# PROCEEDINGS OF THE INTERNATIONAL CONFERENCE ON PLANT PHYSIOLOGY 2014

*Enhancing Strategic Plant Physiological Research and Technologies for Sustainable Resources*


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**Malaysian Society of Plant Physiology**

**Indonesian Coffee & Cocoa Research Institute**

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held at Discovery Kartika Plaza Hotel, Bali, Indonesia (26-28 August 2014)

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# CHAPTER 1

# PLANT GROWTH, DEVELOPMENT AND PRODUCTION

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# Vegetative Propagation in Cocoa (*Theobroma cacao*): Effects of Propagation Environment and Rooting Substrates on Rooting Behaviour of Cocoa Stem Cuttings

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## Introduction

Cocoa (*Theobroma cacao* L.) is an important industrial crop with nutritional value. In Malaysia, the cocoa industry is facing an imbalanced growth in upstream and downstream activities due to low cocoa production which need to be overcome in order to sustain cocoa development. The growing demand for cocoa products coupled with increasing number of diseases due to infection has led to the concept of enhancing commercial planting materials which is disease tolerant and is high yielding. Thus, methods have been developed to enhance the development of cocoa planting materials and its deployment through combined approaches of biotechnology, applied horticulture and technology transfer.

Vegetative propagation or macro-propagation is one of the techniques used in propagating superior commercial cocoa clones. Propagation of cuttings can be an alternative method when seedling-supplies are limited by sporadic flowering, low cocoa seed production and poor germination (Pohio et al., 2005, Dickinson et al., 2010, Pijut et al., 2011). However, successful rate of cocoa propagation using cuttings can vary considerably according to their genetic constitution (Nanda et al., 1968). Propagation by stem cuttings especially in recalcitrant species like cocoa needs high technical expertise to achieve an acceptable rooting percentage including specialized facilities that can produce rooted cuttings, thus making it difficult for famers to adopt in a large scale. Non-mist propagation system is a low cost and low-tech system which is suitable to be used by farmers where cuttings are well supplied with water at the cutting base while the leaves are in a shady and moist condition due to evaporation of water from the leaf surface. Non-mist propagators provide good environment for stem cuttings with low vapour pressure deficit (VPD) to minimise water stress. Moreover, in cocoa stem cuttings propagation, environment is the most important aspect that encourages the physiological activity such as photosynthesis and transpiration in the leaf in order to minimize the physiological stress experienced by the stem tissues following harvest (Leakey et al., 1994). A study by Hartmann et al. (1997) indicated that physiological shock arising from the cuttings from its stock plant can be minimized by controlling the propagation environment.

The significant difference in the growing conditions between cuttings grown in other media and those grown in soil is the amount of space available for root development. For example, coconut coir fiber, perlite and vermiculite are suitable for use as growing medium in many crops (Evans et al., 1996). These media improve structure, aeration and moisture capacity when used as growing media. However, there is no research conducted on the utilisation of coconut coir fibre, perlite and vermiculite as rooting substrates in cocoa cuttings. Thus, the objectives of this study were to select cocoa clones suitable to be propagated using stem cuttings and to study the effects of rooting substrates as well as modified atmosphere of propagation chambers on the vegetative propagation of commercial cocoa clones through cuttings.

## Materials and Methods

A series of nursery experiment was conducted in order to investigate the effects of four different rooting substrates (perlite and sand, vermiculite and sand and coconut coir fibre and sand) on five commercial

cocoa clones (KKM22, MCBC1, LKMS1, PBC123 and BR25) using a modified atmosphere of non-mist propagator. Semi-hardwood cuttings of cocoa clones with four internodes were harvested from two-year old trees of different cocoa clones in the fields. The mean diameter of cuttings was 0.4 cm. The base of each cutting was cut with a slant. The leaves were trimmed into half of their original size to reduce transpiration. Cuttings were also disinfected by soaking in a prepared Benex® fungicide containing an active ingredient of 50% w/w benomyl with 5 g in 10 L of distilled water for 15 min. The basal cuttings of 0.5-1.0 cm were dipped with 'Seradix®' (with IBA - indolebutyric acid as active ingredient) hormone powder at 8000 ppm and propagated in a non-mist wooden propagator which acts as a propagation chamber.

The propagator was constructed following the design of Leaky et al. (1990). Wooden propagator (3.0 x 1.0 x 0.8 m) had three compartments. Stones formed as the base material (0.1 m) in each of the compartments followed by gravels (0.1 m thick on top of stones) and lastly the rooting medium (0.2 m thick on top of gravels). Treatments of rooting substrates were Treatment 1 [Perlite and sand (1:2 v/v)], Treatment 2 [Vermiculite and sand (1:2 v/v)] and Treatment 3 [Coconut coir fiber and sand (1:2 v/v)].

Each rooting substrates were sterilized using an autoclave. The non-mist propagation system creates an altered ambient carbon dioxide concentration by enclosing the cuttings in a sealed propagation chamber. Air humidity of the chamber was maintained by the provision of water beneath the rooting medium in the wooden propagator and through spraying of water once a week. The temperature and the relative humidity were recorded every 30 min during rooting period by using a temperature and humidity data logger (Watchdog A-Series Logger-A150, Spectrum Technologies, Inc., United States).

The experiment was conducted for two months using a completely randomized design with four replications. Thereafter, a total of 480 cuttings (3 treatments x 4 replications x 20 samples = 240 samples) were used with rooting allowed in the chamber for 60 days. Assessment of treatment effects were recorded two months after propagation using percentage of survival (percentage of cuttings with roots and shoots divided by the total number of cuttings planted), percentage of cuttings with shoots (total number of cuttings with shoots divided by the total number of cuttings planted) and percentage cuttings with roots (total number of cuttings with roots divided by the total number of cuttings planted). Survived cuttings with successful rooting were transplanted to the polybags. Data were analysed using ANOVA and separation of means was carried out using Duncan's multiple range test.

**Results and Discussion**

Temperature and air humidity of the non-mist propagation chamber were recorded [Figure 1(a)]. The highest and lowest mean temperatures were 29.4 $^0$C and 26.4 $^0$C, respectively [Figure 1(b)]. The figure shows that the non-mist propagator could provide constant high moisture of 99.9% humidity to the cuttings which is important in determining the success of rooted cuttings in the chamber. The results showed that there were significant interaction of rooting substrates and cocoa clones on the shooting, rooting and survival percentages of cocoa cuttings.

Cocoa clone of KKM22 gave the highest percentages on rooting, shooting and survival in the field after transplant following by clone of MCBC1 (Table 1). However, cocoa clones of LKMS1, PBC123 and BR25 did not have pronounced effect even with the application of IBA hormone on rooting, shooting and survivability of cuttings as the percentages achieved were low (2.79-6.43%) (Table 1). Similar result was also found in a previous study on *Intsia bijuga* where treated cuttings with IBA did not show any significant difference in per cent of cuttings survival (Castañeto and Inhumang, 2004).

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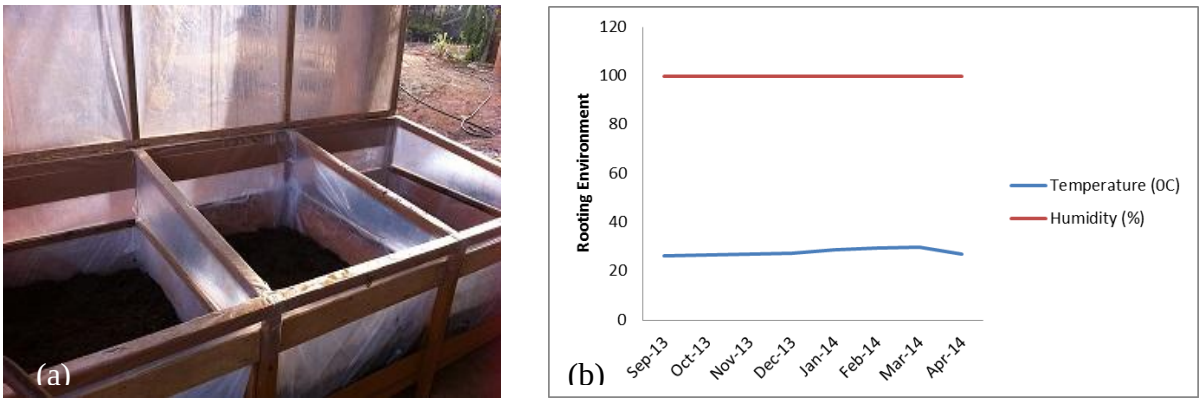


Figure 1. The structure of propagator and the temperature and humidity conditions in the propagator (a) Non-mist propagator (b) Modified environment in the non-mist propagator with temperature and relative humidity recorded during eight month trial of cuttings.

Table 1. Summary of the variance analysis on shooting, rooting and percentage of survival of cocoa cuttings.

| Factor | Percentage (%) | | |
|---|---|---|---|
| | Shooting | Rooting | Survival in field |
| *Rooting substrates (A)* | | | |
| Perlite and sand (P+S) | 22.58 [a] | 21.06 [a] | 8.47 [b] |
| Vermiculite and sand (V+S) | 22.45 [a] | 19.62 [a] | 12.25 [a] |
| Coconut coir fiber and sand (CCF+S) | 14.49 [b] | 12.61 [b] | 7.70 [b] |
| | | | |
| *Clones (B)* | | | |
| KKM22 | 65.42 [a] | 56.43 [a] | 25.76 [a] |
| MCBC1 | 18.75 [b] | 16.93 [b] | 10.08 [b] |
| LKMS1 | 5.93 [c] | 6.43 [c] | 4.96 [c] |
| PBC123 | 5.43 [cd] | 5.53 [cd] | 3.77 [cd] |
| BR25 | 3.68 [d] | 3.49 [d] | 2.79 [d] |
| Interaction A x B | ** | ** | ** |

*Means followed by the same letter in the same column are not significantly different by DMRT at P≤0.05.*

Overall, rooting substrates of vermiculite and perlite amended with soil were suitable to be used as rooting media for all the clones tested compared to coconut coir fibre. KKM22 showed a three-fold higher percentage in shooting compared to other cocoa clones (Figure 2). KKM22 propagated in vermiculite and perlite was successfully shooting at 76.6% and 76.73%, respectively (Figure 2). Figure 3 shows that KKM22 propagated in the non-mist propagation chamber was able to produce rooted cuttings by using vermiculite and perlite mixed with sand with two-fold higher percentage when compared to coconut coir fibre (Figure 3). From the figure, it was observed that the remaining cocoa clones did not show competitive result as compared to KKM22 in producing rooted cuttings under modified environment of non-mist propagator (Figure 3).

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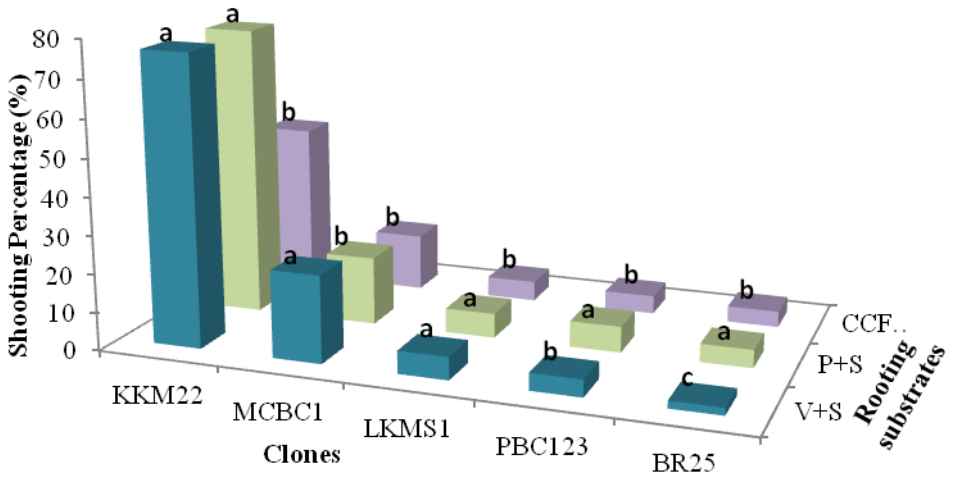


Figure 2. Interactions of rooting substrates and cocoa clones on shooting percentage of cocoa cuttings. Means separations pertaining to each cocoa clones followed the same letter in the same column were not significantly difference by DMRT at P≥0.05.

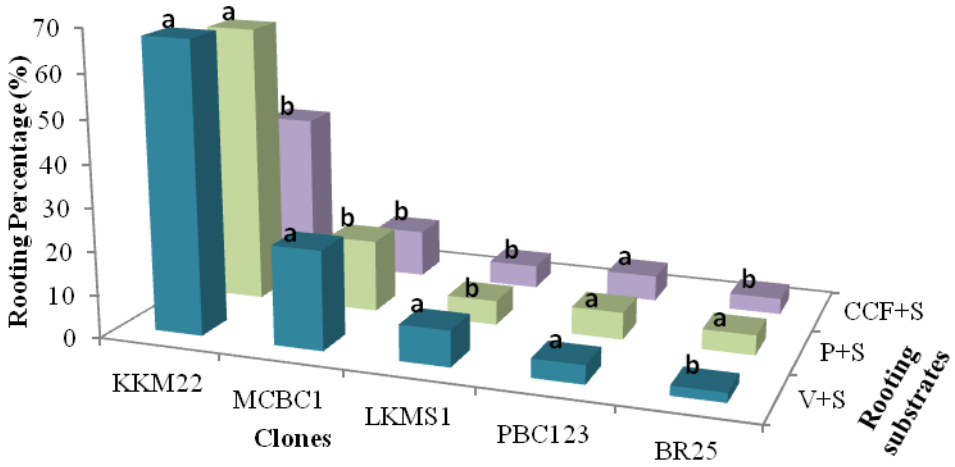


Figure 3. Interactions of rooting substrates and cocoa clones on rooting percentage of cocoa cuttings. Means separations pertaining to each cocoa clones followed the same letter in the same column were not significantly difference by DMRT at P≥0.05.

Next, survivability of KKM22 rooted cuttings following transplantation to the field was the highest when vermiculite (32.6%) was used as rooting media compared to perlite (23.18%) and coconut coir fibre (21.5%) (Figure 4). However, the survival rate for the remaining cocoa clones propagated in the tested rooting media was low after rooted cuttings were transplanted to the field (Figure 4). There was no significant difference observed for cocoa clone PBC123 in survival percentage of all the media tested (Figure 4).

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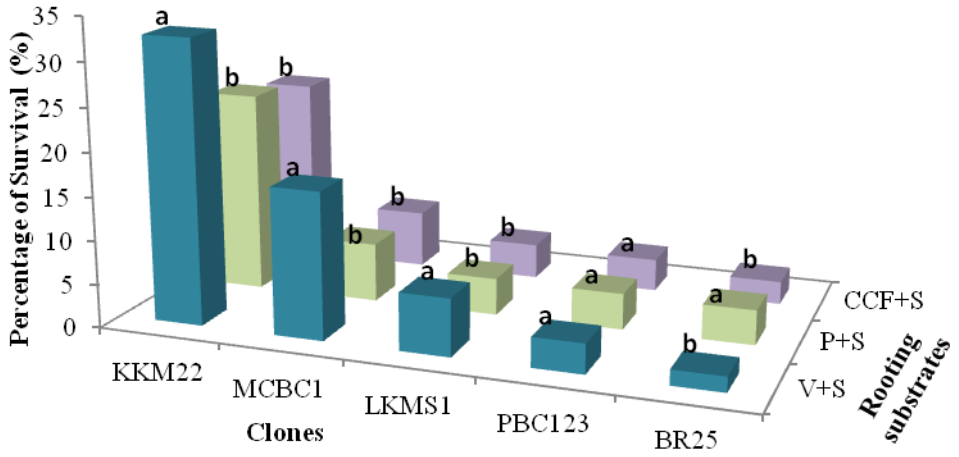


Figure 4. Interactions of rooting substrates and cocoa clones on survival percentage of cocoa cuttings. Mean separations pertaining to each cocoa clones followed the same letter in the same column were not significantly difference by DMRT at P≥0.05.

Newly emerged shoots from the cuttings were initially observed three weeks after planting in the propagator [Figure 5(a)] and during this stage, successful cuttings indicated green leaves and stem. Next, survived cuttings were removed from the propagator after three months when new roots were produced [Figure 5(b)] and the advantage of using coconut coir fibre as a rooting substrate is that the media remained well-shaped and this will make sure that the newly produced roots were undisturbed during transplantation to the polybags [Figure 5(c)]. Successful rooted cuttings were ready to be transplanted to the field after four months in the nursery [Figure 5(d)].

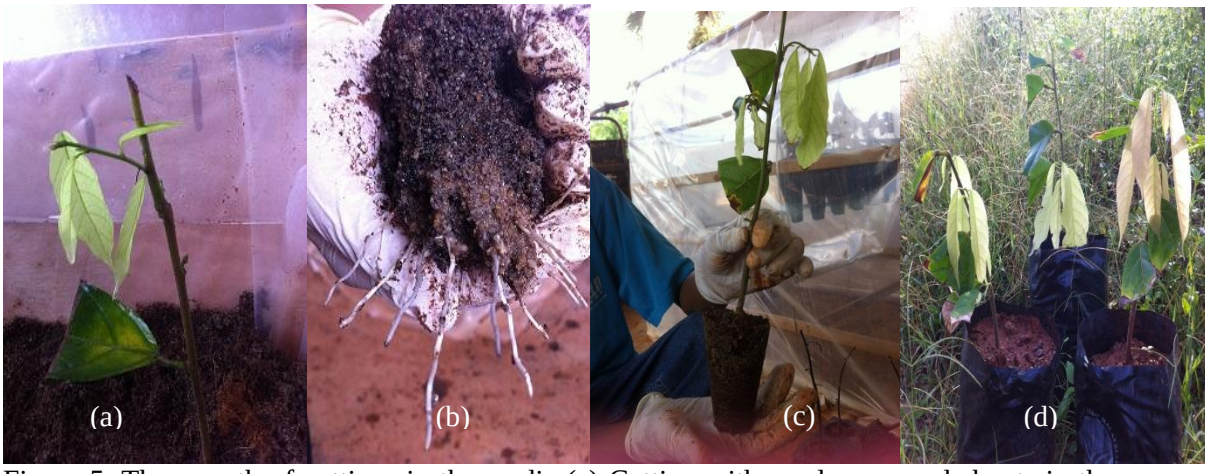


Figure 5. The growth of cuttings in the media (a) Cutting with newly-emerged shoots in the propagator after one month (b) Fresh and new roots growing from the cuttings (c) Rooted cuttings with well-formed coconut coir fibre rooting media (d) Rooted cuttings of 4 month old ready to be transplanted from the polybags to the field.

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## Conclusions

The cuttings of cocoa clone KKM22 can be successfully propagated in the non-mist propagator by applying hormone and all the tested rooting substrates. Non-mist propagator is more economical and affordable to farmers when compared to mist propagator in propagating cocoa through stem cuttings. Furthermore, non-mist propagator can be used in rural areas which have lack of electricity and limited water supply. In addition, the percentages of shooting, rooting and survivability in the field after transplanting can be improved by manipulating the concentration of IBA hormone in order to increase the efficiency of vegetative propagation method by carrying out cuttings in cocoa.

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# Effect of Tuber Seed Size on Vine Growth and Yield of *Dioscorea hispida*

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## Introduction

*Dioscorea hispida* is a long climber plant with thorny vines and fibrous root system. The roots can develop into small oval to round tubers when young and elongate as they grow bigger. The tubers are brown and covered by fibrous rootlets. Locally, it is known as ubi gadong and can be naturally growing mostly along the river banks where the soils are mostly sandy loam. It is neglected due to its poisonous "dioscorine" content. Recently *D. hispida* is becoming popular as the toxins can now be removed and the tuber can then be consumed by both human and as animal feed supplying carbohydrates. Traditionally the toxins are removed by soaking the sliced tuber in running water for about 7 days. Recently rapid toxin removal can be achieved by using an automatic spinning water circulation system (Hudzari et al., 2011).

The plant can easily be propagated using the underground tuber found attached to the main plant. The tuber varies in number and sizes. In this study the different sizes of tuber was grown to determine which size would be optimum to be used for seed tuber. It is important to determine the optimum tuber seed size as this is also the economic part of the plant.

## Materials and Methods

*Dioscorea hispida* tuber were categorised into seven sizes based on tuber weight as 25, 50, 100, 200, 400, 800 and 1600 g. As it was very difficult to obtain the exact tuber size according to the categorised weight, ranges of tuber sizes close to the categorised size were selected. For each category 16 tuber seed were selected and sown in black polyethylene bag of sizes 37 cm x 44 cm. The 7 treatments of different sizes of tuber seed sizes were arranged in randomized complete block design with four replications and four experimental plants per treatments. The categorization and mean tuber seed size was shown in Table 1.

Table 1. Category of seed tuber size of *D. hispida* used in the experiment.

| Treatment Number | Tuber seed size category (g) | Mean of 16 tubers seed size (g) |
|---|---|---|
| 1 | 25 | 25.4 ±1.3 |
| 2 | 50 | 53.0 ± 3.1 |
| 3 | 100 | 103.9 ± 3.8 |
| 4 | 200 | 190.4± 8.6 |
| 5 | 400 | 398.8 ±13.2 |
| 6 | 800 | 819.1± 41.8 |
| 7 | 1600 | 1565.0 ±30.0 |

The rooting media used in the polyethylene bag consisted of mixtures of soil, organic matter and sand in the ratio of 7:3:1. The bag were placed under a constructed trellis 3 m high at a spacing of 0.6 x 1.0 m and the plants were allowed to naturally creep on the trellis. The plants were fertilised every months with 20 g per plant per application which is equivalent to 2200 kg/ha using commercial compound fertilizer NPKMg + trace elements in the ratios of 12:12:17:2. The plants were allowed to grow for eight months after which period the leaves start turn yellow and senesce. The number and diameter of plantlet vines that emerge from each tuber seed, leaf area, number and size of tuber formed, and tuber yield were

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recorded. Harvesting of tubers was done when all the leaves drop and the plant stated to go into dormancy stage.

Data collected were statistically analysed for ANOVA using MINITAB 16 and when significant, comparisons of means were analysed using Tukeys's test at 95% confidence level.

**Results**

*Number and size of vines*

A single tuber seed often produced more than one vine (plantlet) even though when the size was rather small (25-50 g). The number of vines increased from 2.0 to 3.6 when the tuber seed size increased from 25 to 200 g but further increase in tuber seed did not produce more vines (Figure 1a). The diameter of vines significantly increased with tuber seed size. The tuber seed size 1600 g produced significantly bigger vine diameter as compared to other sizes (Figure 1b).

*Leaf area*

The leaf area generally increased with increasing tuber seed size (Figure 2). The tuber seed size 100 and 200 g produced non-significant leaf area compared to the other seed sizes except the biggest tuber size (1600 g). The latter produced significantly ($p<0.05$) the biggest leaves.

*Tuber number and size*

Tuber seed size of 25 and 50 g produced 5 tubers which was significantly ($p<0.05$) lower than those of size 200 g or above which produced 10 or more tubers (Figure 3a). The variations in average tuber size were very high. The effect of tuber seed of sizes 50, 100, 200, 400 and 800 g were not significantly ($p<0.05$) different from each other producing tubers of size ranging from 130-215 g each (Figure 3b). In the wild the tuber size and tuber number was observed to increase with growing seasons.

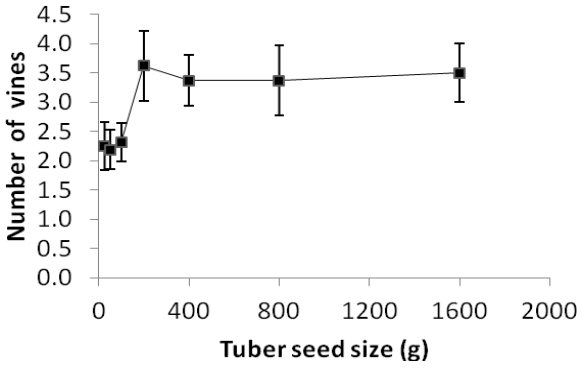


Figure 1a: Effect of tuber seed size on number of vines produced by *D. hispida.* Bars indicate standard error of means.

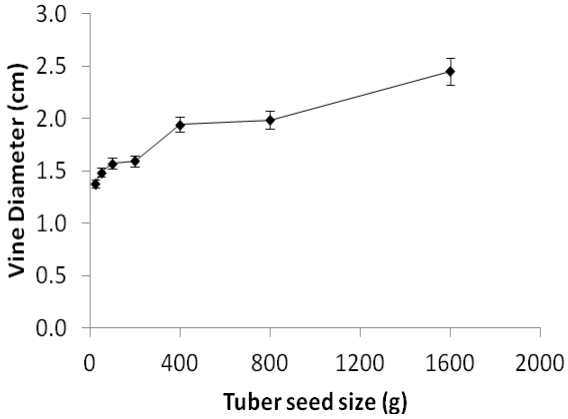


Figure 1b: Effect of tuber seed size on diameter of vines produced by *D. hispida.* Bars indicate standard error of means.

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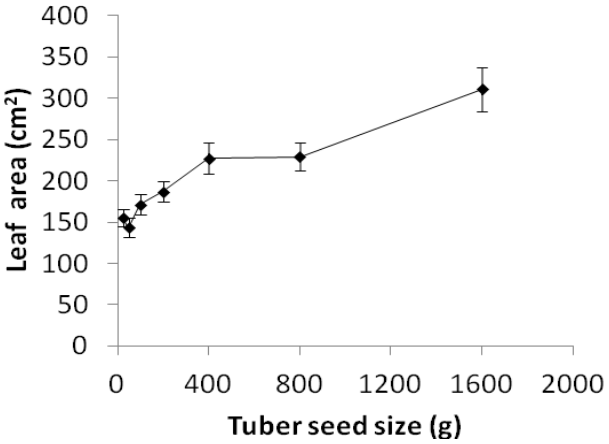


Figure 2: Effect of tuber seed size on leaf area of *D. hispida.* Bars indicate standard error of means.

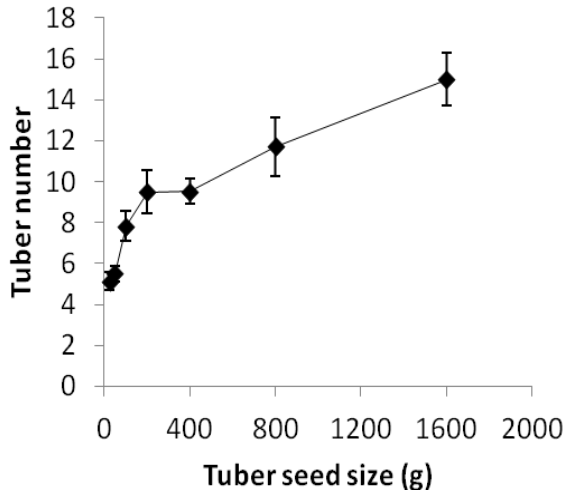


Figure 3a: Effect of tuber seed size on number of tubers produced by *D. hispida.* Bars indicate standard error of means.

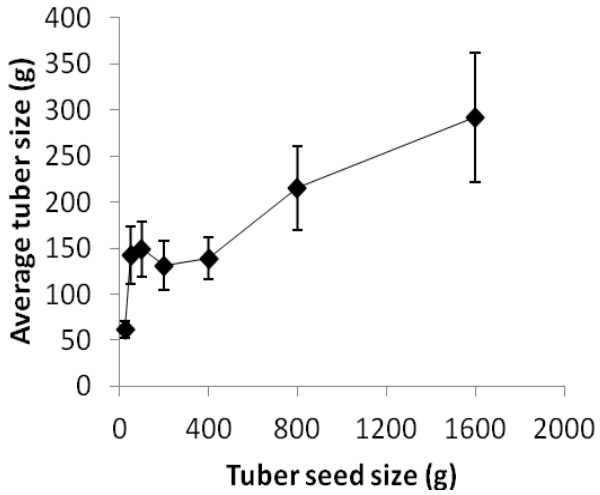


Figure 3b: Effect of tuber seed size on average tubers size produced by *D. hispida.* Bars indicate standard error of means.

*Tuber yield*

Increased in seed size steadily increased the tuber yield (Figure 4). The greatest increase in tuber yield occurred when the tuber seed size was doubled from 25 to 50 g which produced more than double increase in tuber yield. However further increased in tuber seed size did not significantly ($p<0.05$) increase tuber yield. For example increasing tuber seed size from 50 to 400 g produced insignificance increase in the tuber yield. The biggest tuber seed size (1600 g) produced significantly ($p<0.05$) highest tuber yield.

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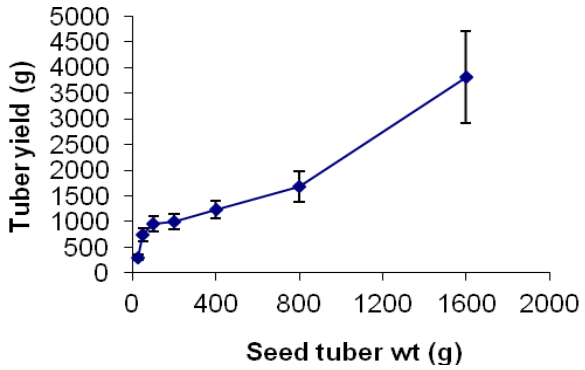


Figure 4: Effect of seed tuber size on tuber yield per seed produced by *D. hispida.* Bars indicate standard error of means.

**Discussions**

The initial food reserve in the tuber seed is important for the subsequent development of a new *D. hispida* plant. Increased in tuber seed size increased all the parameters recorded; number of plantlet, vines diameter, leaf area, number of tubers, tubers size and total tuber yield. The increased in the magnitude of the parameters recorded were significant when the biggest tuber seed size (1600 g) was compared to the smaller sizes.

The growth of *D. hispida* is perennial in nature. New bud sprouted from a tuber when detached from the parent plant or from the existing plant after the monsoon season ended which is in March and continues growing until the monsoon starts again in November. In November the leaves will start turns yellow and finally senesce. The tubers do not normally rot and will remain in the ground until the next growing season. Thus there is a dormant period of about 3 months from November until March before the new bud sprout again. Thus the choice of tuber seed size for planting material will probably depend on how long the plant will be allowed to grow and the availability of the tuber seed.

*Dioscorea hispida* is not normally cultivated but grows naturally in the wild. It reproduces from the underground tubers and probably also from the aerial tuber produced at the leaf axils and once established the plant will grow perennially depending on the availability of other plant to support their twinning vines. From this study it was found that tuber seed size as small as 25 g will be able to produce a healthy new plant but the first year growth will be slow producing small vines, small leaves and small tuber. *Dioscorea hispida* is a perennial plant, the authors observed that in the wild the tuber size progressively increased in size with age. The first year tuber will becomes the following year's tuber seed and due to the big seed tuber size it will subsequently produce a much bigger second year's tuber yield. In the wild the authors have found a single tuber clump weighing up to 25 kg and consisted of up to 70 tubers of various sizes. The villagers in vicinity estimated the age of the clump would have been 5-7 years old. The increase in clump size probably resulted from perennial increase in individual tuber size as well as production of new tubers overlaying the older ones.

For commercial production of *D. hispida* it is suggested that tuber seed size of 100-200 g is used. The tuber yield that can be harvested after a season (a year) of growth will be about slightly more than a

kilogram per clump. However for annual planting system where the tuber is planned to be harvested after one growing season, a bigger tuber seed size of 400 g is recommended, as it will produce higher tuber yield. However further study need to be conducted to determine agronomic requirements especially on planting density, fertilizer requirements and trellis system. With better planting management it is envisaged that higher yield will be obtained.

## Conclusions

Tuber seed size as small as 25 g can be used as planting materials for *D. hispida*. Better growth in the form of more and bigger vines, bigger leaf area, more and bigger tuber sizes and higher yields were obtained by using bigger tuber seed size of 100-200 g. Bigger tuber seed size up to 1600 g produced linearly better plants and higher yield. Tuber seed size up to 400 g is recommended for annual commercial production of *D. hispida*. More studies need to be conducted to determine agronomic requirements especially on planting density, fertilizer requirements and trellis system to produce higher tuber yield.

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# The Effect of Tapping Intensity and Stimulation on Latex Physiological Characters and Incidence of Tapping Panel Dryness

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## Introduction

Rubber tree (*Hevea brasiliensis*) is a main source of the natural rubber which is most widely cultivated. The rubber particles are found in the latex which are synthesized and stored in specific cell called latex vessel. Latex contains 30-45% of the rubber hydrocarbon, 10-20% of lutoid particles, 1-3% of Frey-Wyssling particles, 1-3% of carbohydrates, 1% of proteins, 1.6% of lipids, and other constituents (Nair, 2000). Rubber biosynthesis begins with sucrose as a precursor to the formation of polyisoprene with the cytosolic mevalonate pathway (Keng et al., 2012). Latex was harvested by a system called tapping, done by removing a thin layer of bark so that latex can come out from latex vessel.

An ideal harvest system is to give optimum yield, to reduce tapping cost, and to increase the economical life span, and to maintain plant health. In common harvest system in Indonesian, especially in smallholder, tapping is conducted every day. Although recommended system for smallholder is conventional tapping i.e. half spiral every two days. Intensive tapping can decrease yield and produce low quality latex, indicated by declining dry rubber content. Wimalaratne (1973) reported that increasing intensity of tapping from two days to every day lowered yield by 21.6% in RRIC 52 clone. Latex volume decreased with intensive tapping. Similarly, the dry rubber content decreased by 3.56% with daily tapping. Furthermore, it declined the economical life span, as a result more bark consumption so basal panel only tapped three years. Addition, negative effect of intensive tapping can lead to more incidence of tapping panel dryness (Wimalaratne, 1973; Yeang and Paranjothy, 1982).

On the other hand, the problem in rubber cultivation recently is a shortage labour having good skill in latex harvesting and the severe rising salary of labour. One of the ways to solve this condition is to change of tapping day into low frequency tapping with stimulation. Kuswanhadi and Junaidi (1986) defined that the combination of low frequency tapping and stimulation rose yield. In addition, stimulant application in low frequency tapping resolved the decline of yield due to decreasing of tapping frequency. Further, it can reduce labour requirement, save bark consumption, and maintain the health of trees (Sivakumaran et al., 1982; Abraham, 1984; Kuswanhadi and Junaidi, 1986; Commere and Escbach, 1988). Total production of the low frequency tapping was higher than intensive tapping with equal bark consumption (Sivakumaran et al., 1982).

Stimulant application is the part of a way to increase the production, which has been widely adopted by commercial estates than smallholders. Stimulation can increase yield by extending duration of latex flow as a result increasing of lutoid stability (Wargadipura, 1981; Coupe and Chrestin, 1989; Jacob et al., 1989; Krishnakumar et al., 2011). The using of stimulation in exploitation system must follow by management and discipline in order to get sustainable yield. Incorrect application can lead to more incidence of tapping panel dryness, althought the major factors is still unknown. Many opinions are that the tapping panel dryness is the caused by abiotic factors, biotic factors, variation genotype, nutrient status, and intensity of tapping (van de sype, 1984; Sivakumaran and Zainab, 1996; Senevirathna, 2006). The high metabolism clones are more susceptible than medium and low metabolism clones (Sivakumaran et al., 1988). Both the high intensity of tapping and over stimulation are important factors to cause the

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incidence of tapping panel dryness. The purpose of this study was to evaluate the response of clones having difference in latex metabolism activity to low intensity tapping with stimulation and to study their effects on yield and incidence of tapping panel dryness.

**Materials and Methods**

The experiment was carried out in the experimental field of Sembawa Research Centre since April 2010 to November 2012. The experimental was arranged in a Completely Randomized Block Design with 9 treatments and three replications. The experiment used PB 260, RRIM 600, and PB 217 clones which were planted in 2004. Details of treatments are listed in Table 1.

Table 1. Details of tapping treatments.

| Treatments | Notation | Exploitation system |
|---|---|---|
| A | S/2 d1 | Intensive tapping, without stimulation |
| B | S/2 d1 ET2.5% Ga1 12/y (m) | Intensive tapping, with stimulation 12 times/year |
| C | S/2 d1 ET2.5% Ga1 24/y (2w) | Intensive tapping, with stimulation 24 times/year |
| D | S/2 d2 | Conventional exploitation, without stimulation |
| E | S/2 d2 ET2.5% Ga1 12/y (m) | Conventional exploitation, with stimulation 12 times/year |
| F | S/2 d2 ET2.5% Ga1 24/y (2w) | Conventional exploitation, with stimulation 24 times/year |
| G | S/2 d4 | Low frequency tapping, without stimulation |
| H | S/2 d4 ET2.5% Ga1 12/y (m) | Low frequency tapping, with stimulation 12 times/year |
| I | S/2 d4 ET2.5% Ga1 24/y (2w) | Low frequency tapping, with stimulation 24 times/year |

The production was assessed by weighing the cup lump from each tree. Dry rubber content was assessed by gravimetric method, based on ratio between dry and wet matter from 5 g of latex. Latex drying was conducted at 100 $^0$C until constant weight was reached. The latex physiology parameters i.e. sucrose and inorganic phosphorous concentration in latex were measured every month. The sucrose and the inorganic phosphorous were measured on the clear serum prepared in TCA called TCA serum (Tricloroacetic acid) after latex coagulation, by the anthrone method (Dische, 1962) and the molybdate ammonium method (Taussky dan Shorr, 1953), respectively.

The observation of dry cut length was done by visual estimation of dry cut length percentage which was then converted to value score according to Table 2.

Table 2. Score of tapping panel dryness.

| Score | Tapping panel condition (%) |
|---|---|
| 0 | Healthy cut (no dry cut) |
| 1 | 1-25% dry cut |
| 2 | 26-50% dry cut |
| 3 | 51-75% dry cut |
| 4 | 76-100% dry cut |

**Results and Discussion**

For all of the clones evaluated, the exploitation system with low frequency tapping produced the highest yield in terms of gram dry rubber/tree/tapping (g/t/t), whereas the intensive tapping system produced lower g/t/t than conventional exploitation system. The addition of stimulation in conventional tapping system did not improve yield anymore (Table 3). The total yield during two years on intensive tapping system was the highest (Table 4). It occured on all of the three clones caused by higher number of tapping days in this case. For the mean yield for two years, the highest yield was achieved by intensive tapping

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system and the lowest yield was on treatment low frequency tapping. This condition also seen in every year. The application of intensive tapping system on three clones showed the negative effect on yield and plant health i.e. reduced dry rubber content, reduced g/t/t, and increased the risk of dry tapping incidence (Table 5). Furthermore, the PB 260 clone as a quick starter had a high risk to intensive tapping with stimulation.

Table 3. Mean yield (g/t/t) during two years on different clones and exploitation systems.

| Clones | Treatments | Year of tapping | | Mean |
|---|---|---|---|---|
| | | I | II | |
| PB 260 | A | 11.66 e | 11.63 e | 11.65 e |
| | B | 13.76 e | 14.58 de | 14.17 e |
| | C | 13.76 e | 15.28 de | 14.52 e |
| | D | 18.46 d | 21.39 cd | 19.92 d |
| | E | 22.90 c | 22.25 cd | 22.58 cd |
| | F | 24.52 bc | 26.77 bc | 25.64 bc |
| | G | 28.03 ab | 31.55 ab | 29.79 ab |
| | H | 29.60 a | 34.99 a | 32.29 a |
| | I | 28.22 ab | 33.31 ab | 30.76 ab |
| RRIM 600 | A | 16.02 c | 20.67 a | 18.35 c |
| | B | 17.68 c | 21.24 a | 19.46 c |
| | C | 17.20 c | 22.39 a | 19.80 c |
| | D | 20.63 bc | 19.51 a | 20.07 bc |
| | E | 23.08 abc | 20.37 a | 21.94 bc |
| | F | 21.83 bc | 23.02 a | 22.42 bc |
| | G | 20.42 bc | 18.77 a | 19.60 c |
| | H | 32.30 a | 32.98 a | 32.69 a |
| | I | 29.94 ab | 28.91 a | 29.42 ab |
| PB 217 | A | 10.58 e | 14.14 c | 12.36 e |
| | B | 11.23 e | 18.35 bc | 14.79 de |
| | C | 14.05 de | 18.78 bc | 16.42 cde |
| | D | 15.51 cd | 28.27 ab | 21.89 bc |
| | E | 18.38 bc | 26.41 ab | 22.40 b |
| | F | 19.95 b | 26.72 ab | 23.33 b |
| | G | 17.36 bcd | 19.54 bc | 18.45 bcd |
| | H | 19.74 b | 25.52 ab | 22.63 b |
| | I | 25.10 a | 34.44 a | 29.77 a |

*Values followed by the same letter in the same column are not significantly different at 5%*

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Table 4. Mean of annual yield in kg/ha/year during two years on different clones and exploitation systems.

| Clones | Treatments | Year of tapping | | Total | Mean |
|---|---|---|---|---|---|
| | | I | II | | |
| PB 260 | A | 999.27 abc | 1161.98 a | 2161.25 | 1080.63 bcd |
| | B | 1173.73 a | 1454.13 a | 2627.86 | 1313.93 ab |
| | C | 1179.05 a | 1519.75 a | 2698.79 | 1349.40 a |
| | D | 846.48 c | 1145.62 a | 1992.10 | 996.05 d |
| | E | 1041.74 abc | 1186.78 a | 2228.52 | 1114.26 abcd |
| | F | 1117.45 ab | 1434.23 a | 2551.68 | 1275.84 abc |
| | G | 855.59 c | 1137.28 a | 1992.87 | 996.44 d |
| | H | 897.48 bc | 1253.16 a | 2150.65 | 1075.32 bcd |
| | I | 859.10 c | 1194.97 a | 2054.07 | 1027.04 cd |
| RRIM 600 | A | 1372.34 ab | 1931.38 ab | 3303.72 | 1651.86 abc |
| | B | 1389.19 a | 1973.72 ab | 3362.91 | 1681.45 ab |
| | C | 1111.44 ab | 1948.45 a | 3059.89 | 1529.94 a |
| | D | 868.28 abc | 1076.04 bc | 1944.32 | 972.16 bcd |
| | E | 1051.05 abc | 1018.71 c | 2069.76 | 1034.88 cd |
| | F | 979.27 abc | 1121.12 abc | 2100.39 | 1050.19 abcd |
| | G | 614.29 c | 672.74 c | 1287.03 | 643.51 d |
| | H | 954.17 abc | 908.02 abc | 1862.19 | 931.09 abcd |
| | I | 900.24 bc | 1032.43 bc | 1932.67 | 966.34 cd |
| PB 217 | A | 905.29 b | 1419.11 a | 2324.40 | 1162.20 ab |
| | B | 929.89 b | 1088.83 a | 2018.72 | 1009.36 bc |
| | C | 1202.54 a | 1885.94 a | 3088.48 | 1544.24 a |
| | D | 705.85 cde | 1519.98 a | 2225.83 | 1112.91 ab |
| | E | 837.41 bc | 1414.14 a | 2251.55 | 1125.78 ab |
| | F | 907.48 b | 1428.84 a | 2336.32 | 1168.16 ab |
| | G | 528.63 e | 663.70 a | 1192.34 | 596.17 c |
| | H | 598.74 de | 908.02 a | 1506.76 | 753.38 bc |
| | I | 766.80 bcd | 1234.60 a | 2001.40 | 1000.70 bc |

*Values followed by the same letter in the same column are not significantly different at 5%*

For PB 260 clone, the application of stimulation in conventional and low frequency tapping did not increase g/t/t and the annual yield. This showed that PB 260 clone which had a high metabolic activity have produced a high yield without stimulation application. The latex Inorganic phosporus had reached the maximum limit (without stimulation) so giving stimulation could not improve the energy for latex synthesis anymore. Likewise, latex sucrose content was low in no stimulation treatment. This condition did not support the use of stimulation. Dry rubber content was still normal with different exploitation systems. The Annual yield between d1, d2, and d4 were not significantly different. While the intensity of dry cut lenght in d4 was lower than d2 and d1. The bark consumption with d4 was more scanty than d2 (data not shown). Whereas, the tapping d1 had a higher bark consumption and more trigger of dry cut length.

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Table 5. Latex physiological parameters on different clones and exploitation systems.

| Clones | Treatments | Sucrose (mM) | Inorganic phosporus (mM) | DRC (%) | DCL |
|---|---|---|---|---|---|
| PB 260 | A | 2.34 a | 15.80 ab | 35.45 de | 2 |
| | B | 2.18 a | 19.08 a | 36.42 cd | 4 |
| | C | 2.11 a | 18.79 a | 34.70 e | 4 |
| | D | 2.35 a | 14.50 b | 36.69 bcd | 1 |
| | E | 2.10 a | 15.86 ab | 37.40 abc | 3 |
| | F | 2.09 a | 19.14 a | 36.72 bcd | 2 |
| | G | 1.82 a | 14.26 b | 38.18 ab | 0 |
| | H | 2.30 a | 15.95 ab | 37.75 abc | 2 |
| | I | 2.39 a | 17.54 ab | 38.30 a | 1 |
| RRIM 600 | A | 8.34 a | 11.78 abcd | 34.89 ab | 0 |
| | B | 4.55 cd | 16.04 a | 34.64 ab | 2 |
| | C | 6.88 abc | 14.72 ab | 32.71 c | 2 |
| | D | 7.27 ab | 7.63 d | 35.55 ab | 0 |
| | E | 5.34 bcd | 10.12 bcd | 34.24 b | 1 |
| | F | 5.93 bcd | 11.91 abcd | 34.51 ab | 0 |
| | G | 5.23 bcd | 7.50 d | 34.82 ab | 2 |
| | H | 4.35 d | 9.79 cd | 35.33 ab | 2 |
| | I | 4.88 bcd | 13.49 abc | 35.76 a | 0 |
| PB 217 | A | 7.10 a | 7.35 bcd | 33.98 bc | 2 |
| | B | 4.96 c | 10.48 a | 32.04 d | 2 |
| | C | 4.95 c | 10.85 a | 32.53 d | 1 |
| | D | 6.59 ab | 5.05 d | 36.59 a | 2 |
| | E | 5.15 c | 8.85 abc | 33.94 bc | 2 |
| | F | 4.89 c | 9.65 ab | 33.76 c | 1 |
| | G | 5.87 bc | 6.75 cd | 35.09 b | 0 |
| | H | 5.47 c | 8.16 abc | 35.08 b | 0 |
| | I | 5.37 c | 10.20 a | 34.23 bc | 1 |

*Values followed by the same letter in the same column are not significantly different at 5%*

In contrast, the application of stimulation can increase yield of RRIM 600 and PB 217 by increasing their metabolic activity by increasing (Pi content) and consuming the surose content. It described that the effect of stimulation on latex yield increment was achieved in clones with medium-high sucrose content and low inorganic phosporus content. Stimulation can effect on assimilates circulation i.e. arising of assimilates for latex production.

RRIM 600 and PB 217 clones have a medium and low latex metabolism activity, respectively, had a good response characteristic on stimulation and no negative effect in the long term. For PB 217 clone, in the absence of the problem of the shortage of labour, d2 24/y could be an alternative of exploitation systems. However, if there was a shortage of labour, d4 24/y would be the most suitable. Whereas, treatment d4 12/y in RRIM 600 can produce more latex with no negative impact on plant health.

**Conclusions**

The effect of tapping system on latex physiological characters and incidence of tapping panel dryness was different among the clones with different in latex metabolism activity. An intensive tapping system gave negative effect on physiological parameters and plant health in three clones especially for PB 260 which has high latex metabolism activity, implying high sensitivity towards intensive tapping system. Tapping on d4 frequency without stimulation will be suitable for PB 260. Whereas low frequency tapping with

different frequency of stimulation can increase latex yield without imposing any negative impact on plants health for RRIM 600 and PB 217 which have medium-low latex metabolism activity. d4 12/y and d4 24/y. respectively.

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# Cellular Changes in Cocoa Clones Graft Compatibility

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## Introduction

Clonal planting materials have been widely used in cocoa cultivation in Malaysia. In previous years, many high yielding clones had been released and recommended for planting. However, some of the selected clones were not resistant or at least tolerant to pests and diseases. Among the clones, PBC 130 was considered as a high yielding clone, even though it is highly susceptible to vascular streak dieback disease (VSD) caused by *Ceratobasidium theobromae*. One of the promising methods to control VSD in mature cocoa is through canopy replacement since the fungus penetrates the vascular system of susceptible clones via the shoots. The canopy of the susceptible clones was replaced by the tolerant clones through side grafting on the branches. Initially, the susceptible clone was grafted to hybrid rootstock. Then, it was regrafted using tolerant clones at the stem or branches. However, graft incompatibility was a problem observed in grafted plants, causing dieback. Evaluation of graft compatibility in this experiment was conducted because it involved two stages of grafting since there was no known report on this type of grafting. The first stage involved grafting between hybrid rootstock with first clone (e.g. PBC 130). The second stage involved grafting the first clone (PBC 130) with another clone (e.g. PBC 123). Since there was a possibility of nutrients and water blockage due to the two stages grafting, this study was very important and need to be investigated.

Factors influencing the success of grafting rootstocks on scion include size, growth habit of tree and also fruiting including precocity, bud formation and numbers, fruit set and yield, fruit size, quality and maturity. Using the observed effects of rootstock on vigour to obtain uniform growth in plantings of several cocoa clones requires good knowledge on scions and rootstocks (Phang, 2004). In the present study, PBC 130 clone which is known to be susceptible to VSD disease was grafted with selected VSD tolerant clones. The graft compatibility of the union between PBC 130 and VSD tolerant clones was analyzed through anatomical study. The objective of this study was to observe the development of callus cells and tissues of the graft union during graft union formation.

## Materials and Methods

This study was carried out at the Malaysian Cocoa Board Research and Development Centre, Jengka, Pahang. The treatments were laid out in Randomized Complete Block Design (RCBD) with four replications. Four clones PBC 123, QH 1003, KKM 25 and MCBC 1 were selected for grafting. These clones were considered tolerant to VSD disease based on the previous study.

Cocoa seeds from seed gardens were used to obtain uniform seedlings and to reduce error of experiment. The seeds were sown in polybag of size 30 x 45 cm. There were two stages of graft combinations (Figure 1). After three months, the seedlings were grafted with PBC130 clone using top grafting technique. Three months later, four selected clones were grafted to the PBC130 clone also using top grafting technique. This method was applied to within the natural field conditions of mature cocoa. Graft unions were sampled from each cocoa clones at 10, 20, 30 and 40 days after grafting.

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Figure 1. Cocoa rootstock (a) grafted with PBC 130 (b) and then regrafted with a selected clone (c).

*Fixation*: The graft union portions (approximately 3 cm in length) of the stock/scion combinations at 10, 20, 30 and 40 days after grafting were fixed in formalin acetic acid (FAA) for at least three days to stop physiological processes of the samples before being subjected to further anatomical study. The samples were soaked in 4% ethylenediamine for seven days to soften the woody graft and were subsequently soaked in distilled water at two hours interval in two to three changes to remove ethylenediamine (Carlquist, 1982).

*Dehydration and infiltration processes*: The samples were then dehydrated and infiltrated following the tertiary-butanol alcohol (TBA) series as described by Johansen (1940) with minor modifications.

*Embedding*: Paraplast was liquefied at temperature above 59 $^{0}$C. Liquid paraplast was then poured into a paper mold at half of the mold. The specimens were arranged with the parts to be seen at the bottom surface. When the samples were semi hardened, the specimen was covered again with liquid paraplast for full coverage.

*Sectioning*: Transverse sections of a graft union were cut at 20 µm with a rotary microtome. In order to mount the specimen, a drop of albumin was applied on the slide and was evenly spread. Ribbons containing tissue sections were cut into suitable lengths and placed on the slides that were then placed on a hot plate maintained at 35 $^{0}$C.

*Staining*: The specimens were stained with fuchsin acid and toluidine blue. Slides were prepared and mounted with cover slips and applied with Canada Balsam mounting medium. The stained samples were viewed using CANON digital imaging camera system.

**Results and Discussion**

Cross sections of the cocoa clones graft union after 10 days of grafting were shown in Figure 2. In this study, the graft unions of KKM 25 and QH 1003 clones were successfully processed, while PBC 123 and MCBC 1 samples could not be sectioned. Ten days after grafting, the graft unions of PBC 123 and MCBC 1 did not show any adhesion and were easily separated when the graft union was removed from the combination. Formation of necrotic tissue layers (n) consisting of dead cells were observed along the cut regions of KKM 25 and QH 1003.

It is possible that the necrotic layer was formed because of the dissection of the scion and the rootstock, which compartmentalize the rest of the plant as a defensive mechanism to eliminate invasion of pathogen (Hartmann et al., 2002). Callus bridge was also formed at the interface zone between these two clones from parenchyma tissues adjacent to the necrotic layer. In T-budded citrus, first cell division occurred 24 hours after grafting and the first callus bridge was observed at five days after grafting (www.uky.edu/Ag/HLA/Geneva/Teaching/Graftingandbudding/graftingenvironment.pdf)

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After 20 days of grafting, cell division and enlargement of parenchyma cells along the cut surfaces tend to form an undifferentiated layer (Figure 3). Undifferentiated callus tissue is produced from further division and multiplication of parenchyma cells below the necrotic layer. Callus tissues slowly matured and turned into differentiated cells such as fibres. The differentiation of new vascular tissue was clearly seen for KKM 25, QH 1003 and MCBC 1. There were approximately about five to seven layers of developed parenchyma cells at the graft union. In normal grafting processes for cocoa seedlings, wrapped grafts union were opened 21 days after grafting. At this stage, the stem of PBC 130 and all selected clones were not easily separated. In citrus, differentiation of cambium occurred 10 to 15 days after grafting, followed by first occurrence of xylem tracheids at 15 to 20 days after grafting (www.uky.edu/Ag/HLA/Geneva/Teaching/Graftingandbudding/graftingenvironment.pdf).

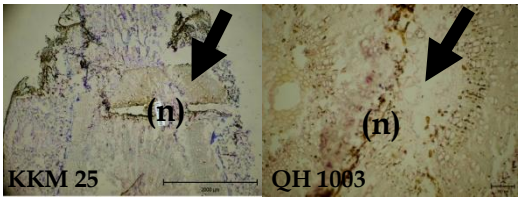


Figure 2. Cross sections of graft union at 10 days after grafting. Necrotic tissue layers were seen on the graft union of KKM 25 (Bars 200 µm) and QH 1003 (Bars 500 µm) clones.

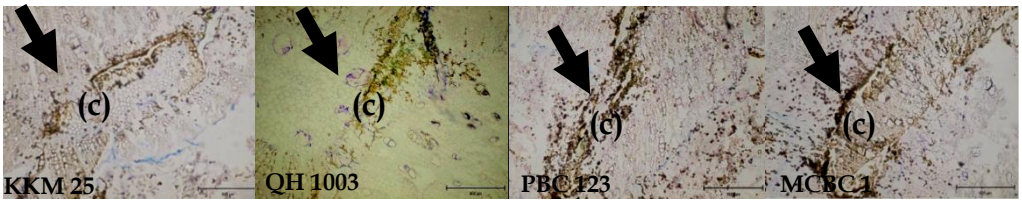


Figure 3. Cross sections of the graft union at 20 days after grafting. Cleared callus (c) cell division and enlargement were seen in KKM 25, QH 1003 and MCBC 1, but uncleared in PBC 123. Bars 500 µm.

New parenchyma cells differentiated into ray parenchyma and produced additional parenchyma cells 30 days after grafting (Figure 4). The additional parenchyma is also known as callus. There were only traces of necrotic tissue layers observed within cortex and vascular tissues, indicating that the graft union was nearly complete. At this time, graft union was considered to be well established. The newly formed callus tissues penetrated the thin necrotic layers of tissue and filled the space between rootstock and scion, interlocking the forming graft union and providing mechanical support where the necrotic layer was broken. Ruiz-Sifre et al. (1997) also observed necrotic layer near the pith area of poinsettia (an ornamental shrub plant) graft union 20 days after grafting. They found that the graft union was still in the formation process. Meanwhile, in citrus, lignification of the callus was completed at 25 to 30 days after grafting in the bark flaps (www.uky.edu/Ag/HLA/Geneva/Teaching/Graftingandbudding/graftingenvironment.pdf).

After 40 days of grafting, traces of the necrotic layer were not evident within the cortex and vascular tissues, demonstrating that the graft union was nearly complete (Figure 5). Although the necrotic layer was probably absorbed, the exact process associated with the elimination of the necrotic layer was not known (Ruiz-Sifre et al., 1997). The true union was achieved only after the xylem and phloem made perfect contact. The eventual differentiation of the elongated cells originated in the cambial area into the new vessels, tracheary elements and phloem sieves through the callus (Estrada-Luna et al., 2002).

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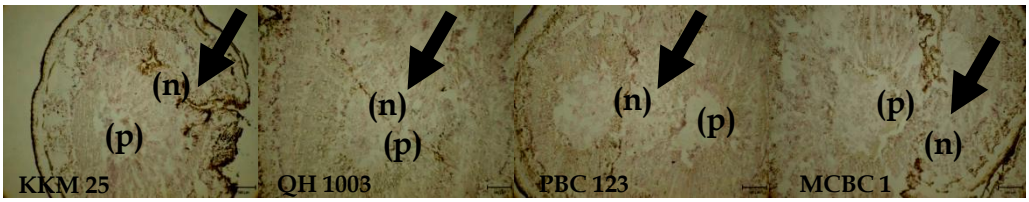


Figure 4. Cross sections of graft union at 30 days after grafting. Traces of necrotic tissue layers (n) were only present near the pith (p) area of the graft in all clones selected for the study. Bars 500 µm.

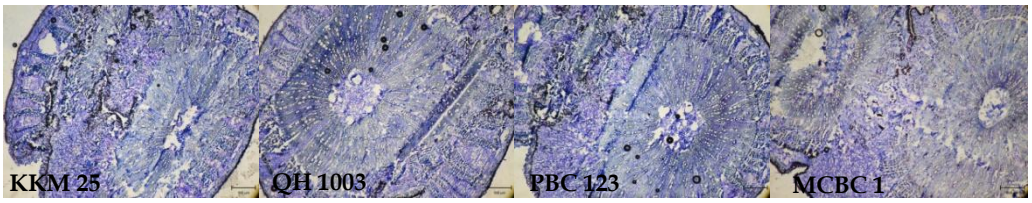


Figure 5. Cross sections of graft union at 40 days after grafting. No traces of necrotic tissue layer were seen within the cortex and vascular tissue.

In cocoa grafting involving woody plants, nodules for the formation of new vascular tissues were formed only after 40 days. Production of new xylem and phloem permitted vascular connection between PBC 130 and selected clones. It is important that production of xylem and phloem from new vascular cambium in callus bridge could be completed before the development of new leaves on scion or else the leaves will wilt and the scion may die.

Estrada-Luna et al. (2002) observed few stages which commonly occur during graft union formation of prickly pear cactus (*Opuntia* spp.). First, it was the development of a necrotic layer and this is followed by proliferation of callus bridge at the graft interface. Third, there will be differentiation of new vascular cambium and fourth, restoration of new vascular tissue. This is followed by restoration of the continuity of the epidermis at the graft union. Similar observation was also seen in the present study.

An observation on anatomical study showed that the four selected clones, KKM 25, QH 1003, PBC 123 and MCBC 1 were compatible to be grafted to PBC 130 although the latter was already grafted to the hybrid rootstock. The possible transmission time requirement for cocoa clones graft union formation was approximately 40 days.

During the early stage of grafting, PBC 123 and MCBC 1 appeared slow to unite when compared to KKM 25 and QH 1003. However, observations 20 days after grafting showed there was no difference in anatomical changes. Callus tissues fill the space between the two components once the graft partners are in contact (Errea et al., 1994). Callus formation was almost complete at 30 days after grafting in all clones suggesting that these clones (PBC 123, KKM 25, QH 1003, MCBC 1) were compatible to the existing cocoa clonal trees (PBC 130). The wound vessels differentiate within the callus at the graft union and were connected into the vascular and cortical parenchyma. The compatibility could be attributed to the structural, physiological and/or biochemical events that may occur in the graft union (Hamdan and Basheer-Salimia, 2010).

Celik (2000) and Hamdan and Basheer-Salimia (2010) stated that the grade of callus formation at the graft union was the main factor for good compatibility between stock and scion. Once the graft partners were in contact, the cambium was capable of meristematic activity producing parenchymatic cells and callus tissues that fill the space between the two components (Errea et al., 1994). When the functional vascular connections were established, translocation of signaling molecules such as polypeptides in the

phloem could be significant for cell recognition and offers good compatibility between graft partners (Hartmann et al., 2002). When several functional phloem and xylem connections cross the graft, a graft union was considered to be successful and complete. Plasmodesmata were important communication pathways in plants. These were channels in the plant cell walls allowing passage of macromolecules and solutes between the neighbouring cells. A recent study demonstrated that insufficient plasmodesmatal coupling at an early stage of development might result in graft incompatibility (Pina et al., 2009).

## Conclusions

KKM 25, PBC 123, QH 1003 and MCBC 1 were compatible with PBC 130 although the latter was already grafted to the hybrid rootstock. Evidence supporting this was the establishment of the vascular tissue continuity observed through histological examination within 40 days after grafting, and also the excellent growth observed 50 days after grafting.

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# Innovation on Pepper (*Piper nigrum* L.) Farming to Ensure High Production of Planting Materials

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## Introduction

Black pepper or scientifically known as *Piper nigrum* L. is a perennial woody vine from the family Piperaceae. The plant is primarily cultivated for its fruit which widely used as spice in culinary preparation, food flavouring, seasoning, perfumery and as a condiment throughout the world (Philip et al., 1992; Bhat et al., 1995; Joseph et al., 1996). It has gained a global recognition as the "King of spice" due to its monetary value and trade in the international spice market (Srinivasan, 2007). Black pepper is an important cash crop with potential for export in Malaysia particularly in Sarawak whereby more than 98% of pepper was produced from the state of Sarawak. According to the International Pepper Community (IPC) 2011, Malaysia has the distinction of being the world's fifth largest pepper producer country with an output of 25,600,000 kg and total export of 14,201,000 kg which worth RM285.27 million in year 2011. In addition to this, domestic consumption of pepper shot up by 11% to 7,828,000 kg compared to 7,069,000 kg in year 2010. Pepper planting was a lucrative endeavor and the demand on food and non-food pepper based products is expected to increase in future. Consequently, a constant supply of planting materials is essential for sustaining and boosting up the pepper productivity of the nation.

A pepper plant has two types of branches. One is the straight, upward growing, orthotropic, monopodial with adventitious roots at each node clinging on the support. This type of branch is referred to as the 'orthotropic branch'. It is also called the "orthotropic stem', 'orthotropic shoot', 'terminal shoot' or simply 'the terminal'. Whilst, the other is the lateral growing, plagiotropic, sympodial branch which bear flower and fruit spikes at the node. This type of branch is known as the 'plagiotropic branch', 'lateral branch' or 'the lateral'.

Pepper cuttings used for planting is sourced from the orthotropic branch (terminal shoot) of a healthy and vigorously growing young vine with varying number of nodes. Traditionally in Sarawak, a five- to seven-node cutting is used for planting. Currently, the five-node cutting is recommended. First round of pruning is normally carried out at six months after planting and subsequent pruning at four-month intervals. Under the traditional practice, a vine normally produces about seven to ten (7-10) five-node cuttings in its first year after planting and another sixteen to twenty-two (16-22) five-node cuttings in its second year of planting (Sim et al., 2011).

The Malaysian Government targets at increasing the pepper cultivated area from the current 14,174 to 15,150 ha by year 2015 and to 15,800 ha by year 2020. This means there will be an increase of 976 ha of pepper cultivated area in the next five years. Based on a planting density of 2,000 vines per hectare, there will be a need for 390,400 cuttings per year in order to achieve this target. However, the traditional planting method is relatively slow and is unable to sustain the constant supply of planting material. Besides low productivity, the occurrence of pests and diseases has restricted pepper cutting production through conventional method. Therefore, this innovative farming method is undoubtedly a novel yet creative way to produce adequate planting material for large-scale pepper cultivation in years to come. Besides, the production of pepper cuttings through this new farming approach would create potential

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extra income for estimated 67,247 of pepper smallholders in Malaysia, who traditionally cultivate pepper only for the peppercorn production.

## Materials and Methods

*Planting materials*

The planting material used in this project is five-node cuttings which obtained from the terminal shoot of young pepper vine with the age of about 1-2 years old. To achieve high survival rate, pepper cuttings were rooted for up to two months at sand bin before transplanting to the field. In order to ensure the homogeneity of the experiment plot, only single pepper variety was used i.e. cv. Semongok Aman.

*Establishment of experimental plot*

An average of 0.1296 ha planting area which is located at Rapak, Sri Aman, Sarawak, Malaysia has been identified as the experimental plot for this invention. Approximately 315 vines of pepper were planted for the assessment on pepper cuttings production. The single pepper vine was trained in a way that three wooden posts of 2 m height were positioned in W-configuration, with one intermediate post set up-righted and the other two lateral posts positioned 45° from the ground. The yield of cuttings production for W-configuration cultivation method was obtained from the cuttings sourced from all of the three posts per vine while the control only taking into account the intermediate up-righted post which comparable to the traditional planting. The production of pepper cutting was statistically analyzed using independent Student-t test at p=0.05 by statistical software, SPSS Statistic Version 16.0 (SPSS Inc., Chicago, IL).

*ROI analysis*

The analysis takes into account for the pepper cuttings production per cycle (2 years) in 1 ha of planting area, with a planting density of 2,000 vines per ha. The parameters of investment cost mainly are made up of non-factor cost which consists of pepper posts (*Eusideroxylon zwageri* post), pepper cuttings, fertilizers, dolomite, weedicide, pesticide and miscellaneous farm implements, whilst, labour cost is considered as factor cost. The gross revenue is solely depending on the cuttings production. All of the statistical data were sourced from Division of Production & Entrepreneurs Farmers Development, Malaysian Pepper Board. The standard market price for fresh cutting or un-rooted cutting is currently RM 3.00 per cutting and the return on investment is calculated based on the formula below:

$$\text{Return on Investment (ROI)} = \frac{\text{(Gain from Investment - Cost of Investment)}}{\text{Cost of Investment}}$$

## Results and Discussion

*Farm structural innovation*

For pepper vine support, Belian (*Eusideroxylon zwageri*) post is recommended by considering the durability and selectivity of pepper adventitious root to cling onto the hardwood support. Thus, the use of Belian wood as support in this new cultivation will be remained as practiced in traditional planting. In W-configuration cultivation method, the suggested size of Belian post is preferably not less than 76.2 mm in diameter and not less than 127 mm in height. W-configuration cultivation method consists of three wooden posts of 2 m height were positioned in W-configuration, with one intermediate post set up-

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righted and the other two lateral posts positioned 45° from the ground as shown in Figure 1. The farm structural design is similar to the traditional planting method whereas both of the distance between row and between vines is recommended at least 2 m (Figures 1C and D).

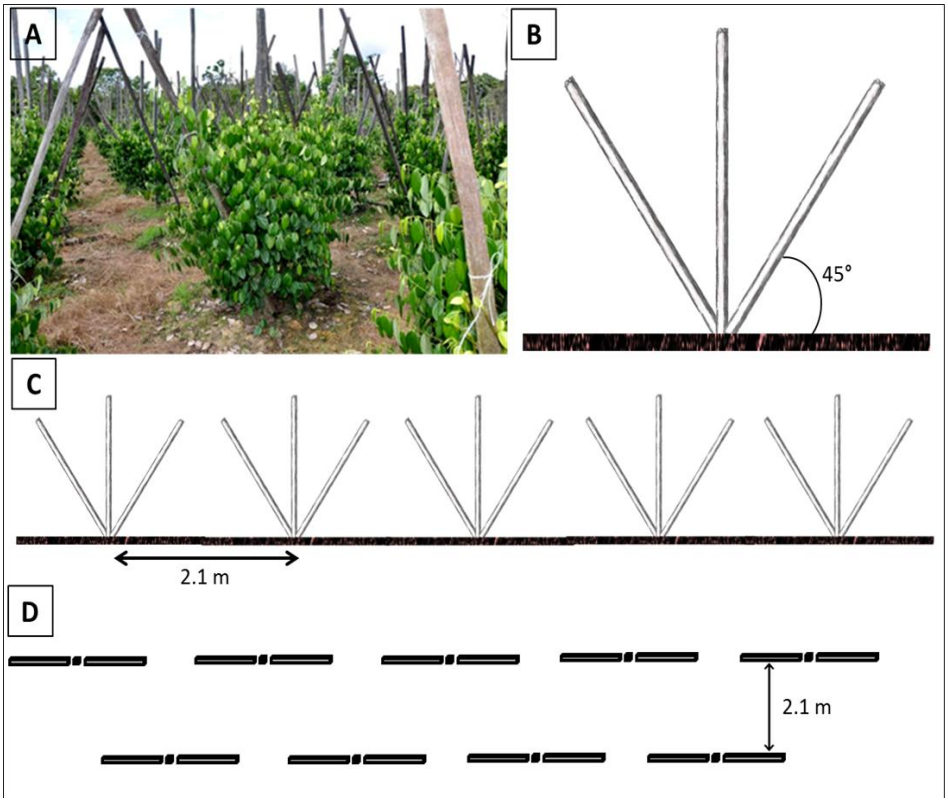


Figure 1. Structural design of pepper vine on Belian post. Experimental plot (A). Post arrangement for W-configuration cultivation method (B). Horizontal view of pepper vine in single row (C). Arial view of two row of pepper vines (D).

*Good agricultural practise (GAP)*

Traditional cultivation practise in Malaysia is to train a five-node cutting onto a post with the three lower nodes are buried in the soil at an angle of 20º to 35° while the fourth node is level with the ground surface. The fifth node is above ground and placed close to the support. All cuttings are advisable to orientate in one direction to ease the fertilizer application later on.

The newly developed terminal shoots are trained on the support by tying at a point just below each node to the support with fiber of plant origin such as raffia or strings removed from a jute sack (gunny sack). To ensure good development of adventitious roots, every node is to be tied properly to the support (Sim et al., 2011). The first harvest of cuttings is normally carried out by the time vine has reached six months old. As a result of cutting harvest, the terminal shoot is pruned back to about 0.5 m from the mound. However, three new terminal shoots which originating from the axillary buds are allowed to develop (Paulus et al., 2006; Sim et al., 2011).

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Unlike the traditional cultivation method, W-configuration cultivation method utilized three wooden posts of 2 m height were positioned in W-configuration, with one intermediate post set up-righted and the other two lateral posts positioned 45° from the ground. The essence of this farming innovation is the use of only one pepper cutting for each planting point with three posts. In this newly developed farming structure, three posts were used per planting point (Figure 1 C). For the up-righted intermediate post, the pepper shoot was trained similar to the traditional planting, by the use of three terminal shoots. Whilst, stolon or locally known as water shoot which emerged from the main vine was trained on the two lateral posts. Before this, stolon (water shoot, hanging shoot or runner shoot) has never been used as planting material in Malaysia due to unpromising growth and it normally retarded before maturity. Therefore, stolon that is hanging or trailing on the ground is normally removed once emerged to reduce fertilizer uptake (Paulus et al., 2006).

The breakthrough of this project is particularly on discovering the potential of stolon (Figure 2a) as planting materials in Malaysia. The stolon was found able to achieve excellence growth performance which is comparable to the terminal shoot with the condition that the nodal part of stolon must buried on the ground with top soil (Figure 2b) prior to be trained on the two lateral posts (Figure 2c). The prerequisites to achieve this are the stolon must emerged from the basal of vigorous growing vine and the nodal region is still viable. This treatment would promote the growth of adventitious root on the buried node and eventually shoot growth. The stolon is ready for cuttings production approximately after 6 months trained on the two lateral posts. In line with the current finding, stolon is extensively used in India for clonal propagation (George et al., 2005).


Figure 2. Runner shoots on the soil (A). Closer view of runner shoot buried with top soil at nodal part (B). Runner was tied and trained on Belian post (C).

*Pepper cuttings production*

The mean number of five-node pepper cutting per vine for the four rounds of pruning is presented in Table 1. Cuttings produced from W-configuration and traditional cultivation method were 12.04 and 9.75 per vine, respectively, indicated that no significant difference between these two cultivation methods for the first round of pruning. This would be attributed to the majority of pepper cuttings were produced from the terminal shoot trained on the main post of the three cultivation methods. While, water shoots which cling onto the side post just about to initiate some new terminal shoots during the first round of pruning.

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The number of five-node pepper cutting per vine varied significantly among the two cultivation methods for the second, third and fourth round of pruning. W-configuration recorded the highest mean number of cuttings produced with 11.19 cutting per vine or equivalent to 59.85% increment as compared to 7.00 cutting per vine produced from traditional cultivation method. This elucidated that the W-configuration planting methods can achieve more promising cutting production rate as compared to traditional planting method.

Table 1. Production of five-node cutting from pepper planted with different cultivation methods.

| Cultivation Method | Number of vine | Number of five-node cutting/vine | | | | |
|---|---|---|---|---|---|---|
| | | P1 | P2 | P3 | P4 | Mean |
| W-configuration | 24 | 12.04[a] | 12.17[a] | 12.38[a] | 11.29 | 11.19[a] |
| Single post (Control) | 24 | 9.75[a] | 6.83[c] | 6.71[b] | 4.71[b] | 7.00[b] |

**Means followed by the same superscript letter within a column are not significantly different at p=0.05 by DMRT*
**Note: P1 = 1st round of pruning; P2 = 2nd round of pruning; P3 = 3rd round of pruning; P4 = 4th round of pruning*

*ROI analysis*

Implementation of W-configuration cultivation method for the production of pepper cutting at stock nursery or plantation is a novelty in of all of the pepper producing countries. A ROI analysis has been carried out to assess the viability of investment into this type of farming approach. The cost estimation for establishment and maintenance of pepper farm mainly for the production of pepper cutting per cycle (1 ha = 2000 vines) was calculated base on the cost of production sourced from the Annual report of Division of Production & Entrepreneurs Farmers Development, Malaysian Pepper Board.

1. ROI on cutting production via W-configuration cultivation method per cycle (2 years) in 1 hectare:

$$\text{Return on Investment (ROI)} = \frac{\text{(Gain from Investment - Cost of Investment)}}{\text{Cost of Investment}}$$

$$= \frac{\text{RM (287,280 - 213,081)}}{\text{RM 213,081}} = 0.35$$

*Gain from Investment = mean number of cutting for each round of pruning X 2,000 vines X price per cutting = [(12.04 x 2,000) + (12.17 x 2,000) + (12.38 x 2,000) + (11.29 x 2,000)] x RM3 = RM287,280.00
*Cost of Investment per cycle (2 years) = RM213,081 (Malaysian Pepper Board 2012).

2. ROI on cutting production via W-configuration cultivation method per cycle (2 years) in 1 ha for the subsequence cycle with excluded cost of Belian support:

$$\text{Return on Investment (ROI)} = \frac{\text{(Gain from Investment - Cost of Investment)}}{\text{Cost of Investment}}$$

$$= \frac{\text{RM (287,280-63,081)}}{\text{RM 63,081}} = 3.55$$

*Cost of Investment per cycle (2 years) after excluded cost of Belian support at the subsequence planting cycle = RM63,081.00 (Malaysian Pepper Board, 2012).

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Based on ROI calculation as in (1), the pepper cutting production plantation via implementation of W-configuration cultivation method can achieved viable index of 0.35 which equivalent to 35% of return from over all of the investment cost. Via the implementation of W-configuration cultivation method, a pepper entrepreneur which invested in a pepper cutting stock nursery with estimated cost of RM213, 081, can generated approximately RM74,578.35 of income in the second year of planting.

In addition, the return would be much more lucrative for the subsequence cycles as the cost of Belian support that has attributed to the greatest amount of investment cost for pepper plantation is reusable. That means the establishment cost for the subsequence planting cycle can be reduced up to 70%. The profit can increased dramatically up to 355% of the investment cost for the subsequence cycles of planting which excluded the cost of Belian support as shown in calculation (2). The ROI analysis revealed that the newly developed farming method for the production and commercialization and of pepper cuttings is very viable for investment.

3. ROI on cutting production via traditional cultivation method per cycle (2 years) in 1 ha:

$$\text{Return on Investment (ROI)} = \frac{\text{(Gain from Investment - Cost of Investment)}}{\text{Cost of Investment}}$$

$$= \frac{\text{RM (168,000-113,081)}}{\text{RM 113,081}} = 0.49$$

*Gain from Investment= mean number of cutting for each round of pruning X 2,000 vines X price per cutting = [(9.75 x 2,000) + (6.83 x 2,000) + (6.71 x 2,000) + (4.71 x 2,000)] x RM3 = RM168,000.00
*Cost of Investment per cycle (2 years) = RM 113,081.00 (Malaysian Pepper Board 2012).

4. ROI on cutting production via traditional cultivation method per cycle (2 years) in 1 ha for the subsequence cycle with excluded cost of Belian support:

$$\text{Return on Investment (ROI)} = \frac{\text{(Gain from Investment - Cost of Investment)}}{\text{Cost of Investment}}$$

$$= \frac{\text{RM (168,000-63,081)}}{\text{RM 63,081}} = 1.66$$

*Cost of Investment per cycle (2 years) after excluded cost of Belian support at the subsequence planting cycle = RM63,081.00 (Malaysian Pepper Board, 2012).

Pepper cuttings production via W-configuration method marked 59.85% greater than the traditional planting methods for the first cycle (2 years) of planting. Inversely, the ROI index on cutting production via traditional cultivation method for the first cycle of planting is 0.49 as compared to 0.35 for W-configuration cultivation method. This could be attributed to the additional cost of two Belian posts for each planting point had increased the cost of investment. The newly developed planting method, even though are less viable as compared to traditional planting method in the first cycle of planting, are able to upsurge the profit as much as 355% when omitted the cost of reusable Belian post during the subsequent

planting cycle. Meanwhile, the traditional method was only capable of achieving ROI index of 1.66 for the subsequence planting cycle. Thus, the W-configuration planting method is more recommended for investment in long run.

## Conclusions

The invention of W-configuration cultivation method is anticipating in overcoming the planting materials shortage problem in Malaysia, at the same time to evaluate the viability of establishing pepper stock nursery or plantation which mainly for cuttings production purpose. The ROI analysis proven the feasibility of this newly developed cultivation method to create potential new income for pepper smallholders in Malaysia.

W-configuration cultivation method is aiming at mass production of planting materials and targeting at the possibility of increasing the yield per hectare in long run. However, the trial is on-going and a conclusive result on yield performance will only revealed at year 2015. This is a breakthrough in pepper farming innovation, particularly in ensuring high production of pepper cutting and at the same time to maximize the land used. Besides, the essence of this method is the utilization of only one five-node pepper cutting for the three posts or per vine. This new farming concept will be disseminating to pepper farmer in Malaysia in years to come as an alternative way to generate income from the selling of pepper cuttings other than peppercorn production.

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# Flowering Improvement of a Landscape Tree, *Xanthostemon chrysanthus* by Using Paclobutrazol and Potassium Nitrate

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## Introduction

Flowering is influenced by endogenous and environmental factors which trigger the change of shoot meristem from generating leaves to the development of reproductive organs. The ability to flower is regarded as an establish state; once maturity is attained, plants will continue to flower provided that the normal-inducing circumstances are imposed (Hackett, 1985). Flower development is manipulated by the climatic conditions during bud differentiation. Dry weather induces flower bud formation and significant precipitation promotes development of vegetative buds (Heller, 1996). Since the climate of the tropical region is uniform throughout the year, the landscapes always look green. Thus, alternative techniques to turn the monotonous landscape into captivating sceneries are crucial. These techniques should be convenient and practical for landscape tree management. Previous study showed that the application of paclobutrazol (PBZ) and potassium nitrate ($KNO_3$) promoted flowering (Ahmad Nazarudin, 2012; Eshghi et al., 2012).

Thus, this study aimed to determine the effects of PBZ and $KNO_3$ on the flowering of *Xanthostemon chrysanthus* (F. Muell.) Benth planted in an urban park in Kuala Lumpur. This species is also known as golden penda (Myrtaceae) and is usually chosen for urban landscapes due to its distinctive yellow inflorescence. It is native to tropical northen Australia, New Guinea, Indonesia and the Philippines (Sosef et al., 1998).

## Materials and Methods

A study plot consisted of 81 existing trees was established in Metropolitan Recreational Batu Park, Kuala Lumpur. These trees aged about six years at the commencement of the study with the average height and average stem diameter at breast height of 6 m and 10 cm, respectively. The experiment was based on a Completely Randomized Design with nine treatments, T1(0 $gl^{-1}$ PBZ + 0 g $KNO_3$), T2(0 $gl^{-1}$ PBZ + 100 g $KNO_3$), T3(0 $gl^{-1}$ PBZ + 200 g $KNO_3$), T4(0.125 $gl^{-1}$ PBZ + 0 g $KNO_3$), T5(0.125 $gl^{-1}$ PBZ + 100 g $KNO_3$), T6(0.125 $gl^{-1}$ PBZ + 200 g $KNO_3$), T7(0.25 $gl^{-1}$ PBZ + 0 g $KNO_3$), T8(0.25 $gl^{-1}$ PBZ + 100 g $KNO_3$) and T9(0.25 $gl^{-1}$ PBZ + 200 g $KNO_3$) and nine replicates. PBZ was applied as soil drench at an application volume of 1 l per tree, while the control plants were applied with 1 l of plain water. Application of PBZ was carried out one time at the start of the study. Meanwhile, $KNO_3$ (13.7:0:38.4) was applied at three months intervals.

Weekly, flower abundance (%) was scored. Inflorescence size (mm) was measured at full bloom by using a digital caliper. Data obtained were subjected to ANOVA and the treatment means were then compared using Duncan's Multiple Range Test.

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## Results and Discussion

Three distinct flowering occurrences were observed, i.e. May 2012, September 2012 and January 2013, irrespective of the treatment (Figure 1). The peak flowering period occurred in January 2013, where trees treated with T8 had flower abundance of about 65.56%. At this stage, the least flower abundance of about 25.56% was measured in T1. Combined effects of PBZ and $KNO_3$ have higher abundance of flowers as compared to single application of PBZ or $KNO_3$. Previous reports stated that PBZ increased the number of flowers in *Lupinus varius* (Karaguzel et al., 2004) and *Hibiscus rosa-sinensis* (Ahmad Nazarudin, 2012).

T4 and T5 gave 10.7 cm and 20.3 cm of inflorescence size at the first measurement, respectively (Table 1). A similar result was also noted with higher dosage of PBZ when T7 and T8 had 10.44 cm and 15.98 cm of inflorescence size. On the other hand, both rates of $KNO_3$ resulted in bigger inflorescence as compared to the control in all measurements. This study proved that, T5-treated tree consistently produced distinctive inflorescence size throughout the study period. PBZ independently increased the number of buds due to the amount of cytokinin formed (Yayat et al., 2013). Cytokinin from the roots was transported to the upper part of plant which further stimulated growth in the axillary buds (Gardner et al., 1991 in Yayat et al., 2013). These lateral buds will transform either as leaf or inflorescence depending on the endogenous and environmental cues. On the other hand, combination of PBZ and $KNO_3$ significantly increased the inflorescence size. As a consequence, it gave a higher abundance of flowers.

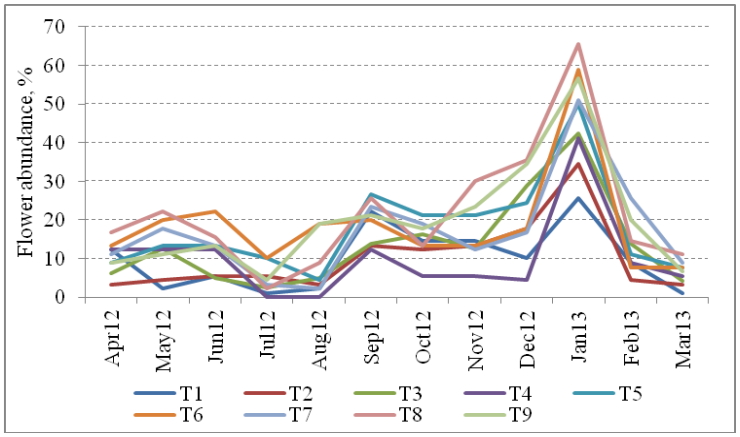

Figure 1. Flower abundance of *X. chrysanthus* after treated with paclobutrazol and potassium nitrate.

Table 1. Inflorescence size of *X. chrysanthus* after treated with paclobutrazol and potassium nitrate.

| Trt | Inflorescence size (cm) | | | | | |
|---|---|---|---|---|---|---|
| | 1st Measurement | 2nd Measurement | 3rd Measurement | 4th Measurement | 5th Measurement | 6th Measurement |
| T1 | 10.00 e | 11.16 f | 10.50 f | 10.40 e | 10.00 f | 9.70 f |
| T2 | 13.94 d | 12.34 e | 12.30 e | 13.10 d | 12.96 e | 13.20 e |
| T3 | 13.96 d | 13.86 d | 14.16 d | 13.44 d | 14.10 d | 13.82 e |
| T4 | 10.70 e | 10.66 f | 10.62 f | 9.94 e | 9.54 fg | 9.78 f |
| T5 | 20.30 a | 17.44 a | 20.68 a | 18.96 a | 20.34 a | 19.70 a |
| T6 | 17.42 b | 16.16 b | 17.80 b | 18.54 ab | 19.50 b | 18.80 b |
| T7 | 10.44 e | 9.98 g | 9.94 f | 8.76 f | 9.00 g | 9.02 f |
| T8 | 15.98 c | 14.64 c | 16.80 c | 17.92 b | 16.46 c | 16.98 c |
| T9 | 14.02 d | 14.34 cd | 14.12 d | 15.68 c | 16.28 c | 14.68 d |

*Means followed by the same letter(s) within column do not differ ($p<0.05$) by DMRT; Trt=treatment*

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## Conclusion

Combined effects of PBZ and $KNO_3$ (T5) increased the flower abundance and inflorescence size. This practice has the potential as a tool to improve the flowering of this species under local climatic conditions.

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# Cadmium Effect on Seed Germination and Seedling Growth of *Amaranthus gangeticus, Cucurbita maxima* and *Brassica alboglaba*

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## Introduction

Heavy metals have been broadly recognized as being very toxic to plants (Li et al., 2005). Plants may be directly affected with heavy metals by air pollutants, and indirectly through contamination of soil and water (Farooqi et al., 2009). As plants are part of the food chain, heavy metal contaminated plants may pose a risk to humans and animals through food contamination. Cadmium is a highly toxic pollutant that affects many metabolic processes of plants. Reduction in the length of roots and shoots in the treatment of cadmium can be caused by depletion of mitotic meristematic zones (Muhammad et al., 2008).

Poultry manure has been used extensively in organic vegetable farming. However, it is an important source of toxic heavy metal contamination introduced to soil as the media for food production. Cadmium is one of the highly toxic heavy metals that are easily found with application of poultry manure on farmlands (Shafiq et al., 2008). This heavy metal is not only affecting seed germination and seedling growth but is also highly dangerous for human health (Das et al., 1997; Munzuroglu and Zengin, 2006; Bavi et al., 2011; Liu et al., 2011; Rehman et al., 2011; Janicka-Russak et al., 2012). In the current paper, several studies were carried out concurrently to determine the seed germination and subsequent early seedling growth of some common vegetables of different families, i.e. *Amaranthus gangeticus*, *Cucurbita maxima* and *Brassica alboglaba*, as affected by cadmium chloride ($CdCl_2$) at varying concentrations.

## Materials and Methods

### *Test material*

Seeds of *A. gangeticus*, *C. maxima* and *B. alboglaba* purchased from a local seed supplier were germinated in three concurrent studies as mentioned.

### *Location of study*

All experimentations were carried out in Laboratory of Plantation and Agrotechnology, Universiti Teknologi MARA, Selangor, Malaysia. The average temperature and relative humidity of the air-conditioned laboratory was 25±2 °C and 55±5%, respectively.

### *Cadmium chloride solution preparation*

$CdCl_2$ solutions of 60, 40 and 20 mg/l were prepared by serial dilution of the stock solution of 80 mg/l $CdCl_2$. Control of 0 mg/l $CdCl_2$ was distilled water.

### *Seed germination and initial seedling growth*

Seeds were germinated in a bright place near the window in the laboratory. Seed germination experimentations were done in enclosed transparent plastic boxes on paper towel moistened with 10 ml

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$CdCl_2$ solution of 0, 20, 40, 60 and 80 mg/l, respectively. $CdCl_2$ solution was dispensed on the paper towel using a syringe.

*Data collection*

Seed germination was recorded daily. Seed was considered to have germinated when visible radical emergence was noted using a magnifying lense. Then, germination index was calculated according to the formula below:

Germination index = $\sum$ (No. of germinated seeds on Gt)/Gt
Where Gt = number of days after germination

The germinated seeds were separated daily into other boxes on paper towel moistened with the same $CdCl_2$ concentration. Then, the length of radicle and plumule of emerged seedling were measured on the next day (for *A. gangeticus*, *C. maxima*) or on the third day (for *B. albograba*) after seed germination. The length of radicle and plumule was measured by using a piece of thread and a ruler.

*Experimental design and statistical analysis*

Studies on the effect of varying concentrations of $CdCl_2$ on germination and early seedling growth of *A. gangeticus*, *C. maxima* and *B. alboglaba* were each based on Completely Randomized Design (CRD). Each treatment was replicated four times. Analysis of variance (ANOVA) was carried out and Tukey's Honestly Significant Difference Test was used for mean comparison. The percentage (%) data were transformed to arc sine value before ANOVA.

**Results and Discussion**

*Amaranthus gangeticus* seed germination was significantly reduced with increasing concentration of cadmium (Table 1). Seed germination was 51.50% with control seeds as there is usually high percentage of non-viable seeds with very small sized seeds of this vegetable. The germination of seed was reduced to 16.75 – 20.25 % with treatment of 20 – 80 mg/l $CdCl_2$. Similarly, the treated seeds also had significantly lower germination index as compared to the control seeds.

The growth of radicles and plumules of *A. gangeticus* was inhibited by this heavy metal (Table 1). The degree of plumule growth inhibition seemed to be greater than that of radicle. Length of plumules was greatly retarded at 80 mg/l $CdCl_2$. There was, however, no significant difference in length of radicles among $CdCl_2$ treatments of 20-80 mg/l.

Table 1. Germination and growth of seedlings of *A. gangeticus* following treatment with $CdCl_2$.

| $CdCl_2$ rate (mg/l) | Germination (%) | Germination index | Length of plumule (cm) | Length of radicle (cm) |
|---|---|---|---|---|
| 0 | 51.50 ± 2.40 [a] | 13.94 ± 0.64 [a] | 1.59 ± 0.04 [a] | 1.26 ± 0.09 [a] |
| 20 | 20.25 ± 3.75 [b] | 5.09 ± 0.86 [b] | 0.80 ± 0.04 [c] | 0.69 ± 0.10 [b] |
| 40 | 17.25 ± 1.10 [b] | 4.50 ± 0.32 [b] | 1.12 ± 0.06 [b] | 0.61 ± 0.05 [b] |
| 60 | 17.00 ± 1.08 [b] | 4.28 ± 0.31 [b] | 1.06 ± 0.09 [b] | 0.54 ± 0.03 [b] |
| 80 | 16.75 ± 2.10 [b] | 4.1 ± 0.49 [b] | 0.59 ± 0.03 [c] | 0.41 ± 0.01 [b] |

*Means with the same letter within the same column are not significantly different at 5% level of significance.*

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Table 2. Germination and growth of seedlings of *C. maxima* following treatment with $CdCl_2$.

| $CdCl_2$ rate (mg/l) | Germination % | Germination index | Length of plumule (cm) | Length of radicle (cm) |
|---|---|---|---|---|
| 0 | 95.00 ± 2.04 [ab] | 4.00 ± 0.13 [a] | 1.25 ± 0.02 [a] | 4.74 ± 0.16 [a] |
| 20 | 96.25 ± 2.39 [a] | 3.91 ± 0.14 [a] | 1.09 ± 0.07 [a] | 4.50 ± 0.18 [a] |
| 40 | 93.75 ± 3.15 [ab] | 3.77 ± 0.07 [ab] | 0.76 ± 0.03 [b] | 3.12 ± 0.20 [bc] |
| 60 | 87.50 ± 3.23 [ab] | 3.26 ± 0.17 [bc] | 0.74 ± 0.03 [b] | 3.69 ± 0.15 [b] |
| 80 | 82.50 ± 2.5 [b] | 3.08 ± 0.77 [c] | 0.70 ± 0.04 [b] | 2.64 ± 0.07 [c] |

*Means with the same letter within the same column are not significantly different at 5% level of significance.*

On the other hand, $CdCl_2$ at concentrations up to 60 mg/l generally did not affect total seed germination of *C. maxima* significantly but those treated with 60 mg/l $CdCl_2$ showed lower germination index than seeds treated with lower concentrations of $CdCl_2$ (Table 2). For subsequent seedling growth performance, $CdCl_2$ at ≥40 mg/l significantly reduced the initial seedling growth in terms of length of plumule and radicle (Table 2). At this level of 40 mg/l $CdCl_2$, soil is generally considered contaminated with cadmium. Growth inhibition of radicle increased significantly and simultaneously with increasing $CdCl_2$ concentration, indicating that radicle of *C. maxima* was more sensitive to this heavy metal than plumule, which is in contrary to that demonstrated by *A. gangeticus* seeds.

Table 3. Germination and growth of seedlings of *B. albograba* following treatment with $CdCl_2$.

| $CdCl_2$ rate (mg/l) | Germination % | Germination index | Length of plumule (cm) | Length of radicle (cm) |
|---|---|---|---|---|
| 0 | 75.00± 2.38 [a] | 9.44±0.12 [a] | 2.00±0.03 [a] | 1.36±0.02 [a] |
| 20 | 76.00± 4.32 [a] | 10.02±0.75 [a] | 1.98±0.05 [a] | 1.23±0.08 [ab] |
| 40 | 69.00± 1.73 [a] | 9.38±0.40 [a] | 1.95±0.03 [a] | 1.04±0.02 [b] |
| 60 | 75.50± 4.57 [a] | 10.57±0.68 [a] | 1.94±0.00 [a] | 0.63±0.09 [c] |
| 80 | 69.00± 3.70 [a] | 9.27±0.56 [a] | 1.71±0.04 [b] | 0.53±0.04 [c] |

*Means with the same letter within the same column are not significantly different at 5% level of significance.*

*Brassica alboglaba* seeds could tolerate $CdCl_2$ up to 80 mg/l (Table 3). These treatments did not result in significant difference in seed germination of this vegetable. However, $CdCl_2$ at concentrations of ≥40 mg/l also resulted in significant inhibition of radicle growth while only the highest concentration of 80 mg/l $CdCl_2$ significantly retarded plumule elongation (Table 3). There was significant and simultaneous greater radical growth inhibition with increasing $CdCl_2$ concentration. This also indicated that radicle of *B. alboglaba* was more sensitive to this heavy metal than the plumule.

The effect of cadmium is dependent on species of vegetables (Raziuddin et al., 2011). Cadmium affects seed germination and development of plumule and radicle of vegetables at certain level. The accumulation of cadmium in leaves and roots was proportional to the concentration of cadmium in the growth solution in the study conducted by Zou et al. (2012). There were studies indicated that the effect of heavy metals on plants is dependent on the amount of toxic substance taken up from a given environment (Farooqi et al., 2009). According to Januskaitine (2012), the roots can easily uptake cadmium and have the heavy metal loaded into the xylem for its transport into leaves (Li et al., 2005; Kiran and Sahin, 2006). Hence, cadmium was more obviously seen as affected development of leaves in this case. According to Yadav (2010), cadmium also caused reduction in photosynthesis, water uptake and nutrient uptake of plants when plants were grown in excessive cadmium.

## Conclusions

$CdCl_2$ at rates of 20-80 mg/l affected seed germination, germination index, growth of plumules and radicles of *A. gangeticus*. The seeds of *C. maxima*, on the other hand, could not tolerate $CdCl_2$ above 40 mg/l. Seed of *B. alboglaba* was more tolerant to $CdCl_2$. They were not affected by $CdCl_2$ of up to 80 mg/l. However, it may tolerate cadmium well at rates up to 60 mg/l $CdCl_2$ only, i.e. 0.33 mM cadmium, as the subsequent elongation of plumule and radicle was significantly affected with cadmium higher than that level. Further studies on the subsequent growth and possibilities of cadmium bioaccumulation in these vegetables are necessary, even with cadmium availability of <40 mg/l $CdCl_2$, for food safety.

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# Yield Analysis of Rice between System of Rice Intensification (SRI) and Conventional Farming in Sabak Bernam District, Selangor, Malaysia – A Case Study

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## Introduction

The production of rice has been started a long time ago since the emergence of the civilization, especially in Asia. Furthermore, it has been considered as one of the world's most sustainable and productive farming systems (Mutert and Fairhurst, 2002). In Malaysia, the production of rice has been highly demanded because of the needs of the Malaysians for it as the staple food. There was an increased production of paddy from 2,375,604 Mt in 2007 to 2,575,988 Mt in 2011 (Jabatan Pertanian Malaysia, 2012). In addition, the high demand for rice leads to the importation of rice (Tey and Radam, 2011). Due to the unstable food security, Malaysian government planned for several new approaches. One of the new approaches in rice production is called System of Rice Intensification (SRI). SRI was developed and started in Madagascar some 30 years ago and started gaining popularity in the other countries. SRI was developed by Fr. Henri de Laulanié after about 20 years of experimentations and observations to help the Madagascar's people in facing the food scarcity and economic unstability (Uphoff, 2006).

In conventional method of rice planting, the recommended age of seedlings used is around 21 – 24 days old (Anon, 2010). In SRI practices, younger seedlings are much required because they can contribute to higher grain yield. The transplanting of younger seedlings avoids additional root and shoot dry matter accumulation. This practice also will not damage the root system during transplanting process. The undamaged root system of the seedlings eventually will reduce the number of unsuccessful growth of the seedlings. The efficiency of young seedlings to utilize the resources can effectively produce a great number of tillers, larger leaf area and much taller plants. The younger seedlings may also promote the microorganism population in rhizosphere and this can favor the growth and yield of paddy.

One or two seedlings transplanting is among the common practices in SRI. One or two seedlings planted per hill in paddy field may reduce the competition in terms of nutrient needs between the root systems (Chapagain and Yamaji, 2010). The increased number of tillers, larger leaf area index and higher dry matter produced from a single seedling are presumed to have contributed to the better yield.

Wider planting spacing in SRI is recommended because it can give higher yield and net income to the farmers (Krishna and Biradarpatil, 2009; Lin et al., 2009; Chapagain and Yamaji, 2010). Wider planting distance per hill produces remarkable longer roots and larger stem diameter. These properties allow the plant to absorb more nutrients and increase the root absorption area in the soil. The wider spacing at reduced plant density also reduces the competition between plants in terms of nutrient and sunlight needs, hence, high yield can be achieved under SRI.

Water management in SRI is a different method as compared to conventional method. In SRI, reduced usage of water by means of intermittent irrigation demonstrated many benefits as compared to continuous flooding in paddy field (Nyamai et al., 2012; Choi et al., 2013). The water requirement in paddy field under SRI was 55.6% less as compared to the conventional practices. However, such irrigation practices in SRI produced less yield as compared to conventional farming in salt – affected soils because of the

salinity stress (Menete et al., 2008). Another advantage in intermittent irrigation is the reduced methane ($CH_4$) emission by 74 – 79.5 % (Setyanto and Kartikawati, 2011; Suryavanshi et al., 2013). The intermittent irrigation also causes unfavorable condition to the pathogen and, therefore, disease severity is reduced (Pathak et al., 2012).

SRI practices recommend the use of organic manures to replace any organic and inorganic fertilizer as far as the cost of production is concerned. Organic manures not only supply the least nutrient for the plant but they are also used as soil amendment. This soil amendment can eventually change the soil chemical and physical properties, affect the microbial activities in the soil to degrade the soil organic matters and help release the nutrients bound to the soil (Rajeshwar and Aariff Khan, 2008; Suryadi et al., 2013).

The Food Price Crisis in 2007 and 2008 influenced the production of food in Malaysia, including rice production. The implementation of SRI to solve the above problem in Malaysia has taken place in several granaries under Department of Agriculture (DoA). As influenced by the conventional practices that have been done for a long time, some farmers, however, may adopt different practices in SRI. Some differences in the SRI practices may lead to differences in yield. These differences can be analyzed against the rice yield under conventional rice farming for improvement purposes.

This study was aimed to analyze the differences in terms of SRI practices adopted by the farmers in Sabak Bernam District, DoA Selangor. It is an important rice granary. The rice yield differences between SRI practices and conventional farming of the main season in 2013 were explained with their agronomic practices.

## Materials and Methods

### *Study location*

The study was conducted in Sabak Bernam District as many farmers there adopted SRI practices. Sabak Bernam is located in the southern west of Peninsular Malaysia. Paddy cultivation is one of the largest food production activities in Sabak Bernam. It is cultivated in irrigated system introduced by British colonial long time ago. Most paddy farmers there planted MR220 paddy variety as recommended by DoA Selangor.

### *Sampling*

There were a total of 104 paddy farmers in Sabak Bernam registered under DoA Selangor. Samples were randomly selected from this area. A total of 50 farmers provided the required information for this study with 28 farmers registered as SRI farmers and the other 22 farmers were still attached to conventional paddy cultivation.

### *Study procedure*

Face to face interviews were used as the tools to collect data from the selected farmers as mentioned. A questionnaire was designed to assist data collection on farmer demography, husbandry practices and yield in main season from the respondents.

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*Data analysis*

Data on demography and husbandry practices were subjected to descriptive analysis. Yield differences, on the other hand, were subjected analysis of variance and treatment means were compared using Duncan's multiple range test.

**Results and Discussion**

From Figure 1, it was found that majority registered SRI farmers were still practicing conventional methods in rice farming. Only 14% of them followed some of the SRI methods in irrigation, nutrient and pest management. This indicates that more intensive extension services or field visits should be considered and implemented by the authorized bodies to promote SRI among farmers, especially the SRI registered farmers.

Figure 1 shows that SRI enabled significantly higher yield as compared to conventional farming. SRI farmers gained mean yield of 9,317 kg/ha. With conventional practices, mean yield was 7,543 kg/ha. Farmers registered for SRI scheme but still practiced conventional farming methods had mean yield of 7,874 kg/ha.

Most SRI farmers were in the classes of 36 – 55 years old (Figure 2). Most conventional farmers, on the other hand, were found in the older age categories, and the oldest farmer of 75 years old was also in the category of conventional farming. Educational background was presumed to affect the perception and adoption of this new rice cultivation technology. Sita Devi and Ponnarasi (2009) suggested that the age differences of farmers were related to the probability of SRI technology adoption. The younger farmers realize that adoption of new practices can increase their yield and productivity.

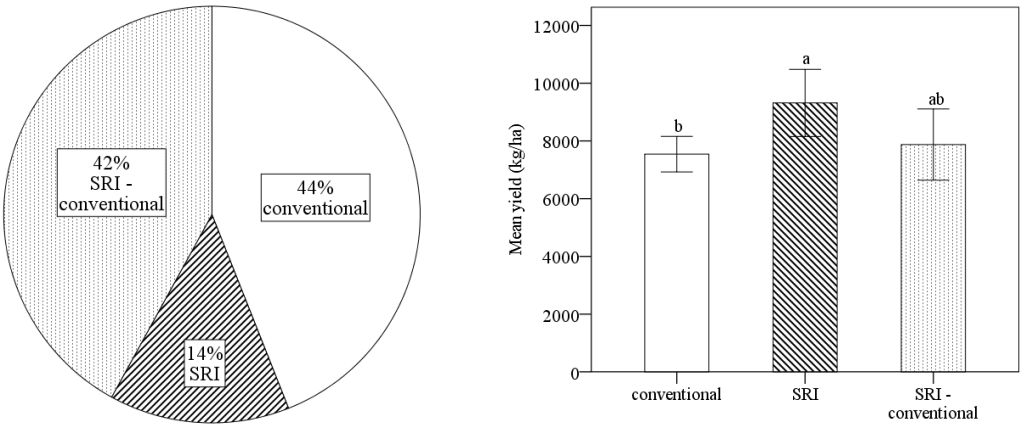


Figure 1. Distribution of farmers according to farming system (left) and yield (right), n=50, bar in the chart on right indicates 95% CI for mean, means having the same letter in the chart on right are not significantly different at 5% level of significance.

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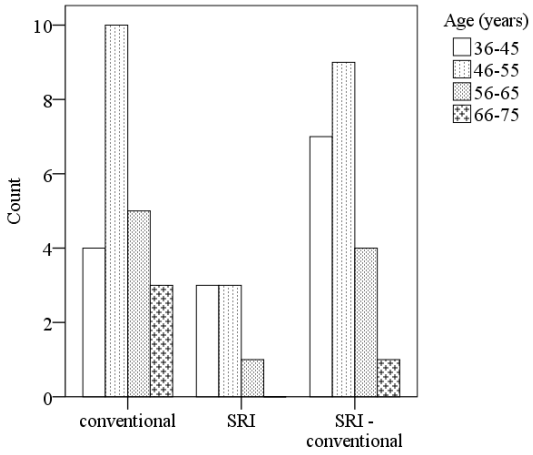


Figure 2. Distribution of farmers according to age.

Most farmers were cultivating paddy in their own land (Figure 3). Only small number of them rented land for living through rice cultivation while other small number of farmers worked on both own and rented land for rice production for living. There was no obvious difference in rice production according to land tenureship in this important rice granary.

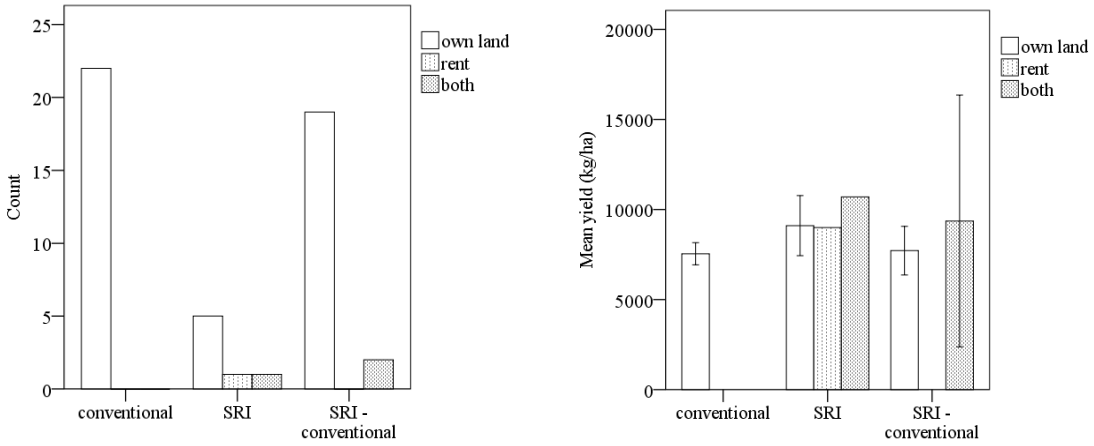


Figure 3. Land ownership (left) and their yield performance (right) according to farming systems, bar in the chart on right indicates 95% CI for mean.

All farmers, including the SRI farmers, practiced direct seeding. Labor shortage was presumed to be related to the failure to adopt reduced seeding rate and plant density practices through transplanting method in SRI. With water management, there were only a few SRI farmers (four persons) that practiced intermittent wet and dry technique, as recommended with SRI, and they obtained high yield of almost 9,000 kg/Ha (Figure 4). With conventional farmers, the best yield achieved was below 8,000 kg/ha.

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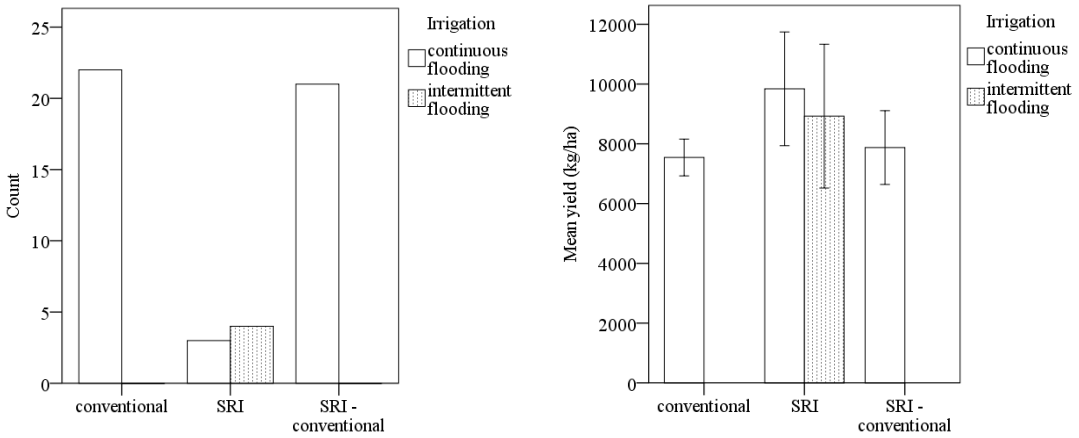


Figure 4. Irrigation practices (left) and their yield performance (right) according to farming systems, bar in the chart on right indicates 95% CI for mean.

In nutrient management, organic fertilizer application was practiced only by small number of SRI farmers while conventional farmers still depended solely on subsidized chemical fertilizers (Figure 5). Organic soil amendment was proved to allow SRI farmers to gain profitable yield of above 9,000 kg/ha while conventional subsidized fertilizers combined with other SRI practices was best in enhancing high yield of paddy, giving yield of almost 10,000 kg/ha. Conventional farmers had yield of below 8,000 kg/ha.

In pest management, most farmers practiced chemical control of insect pests, diseases and weeds with subsidized chemicals (Figure 6). Only a few SRI farmers bought additional effective pesticides, in addition to subsidized pesticides, for the mentioned chemical control of pests and weeds.

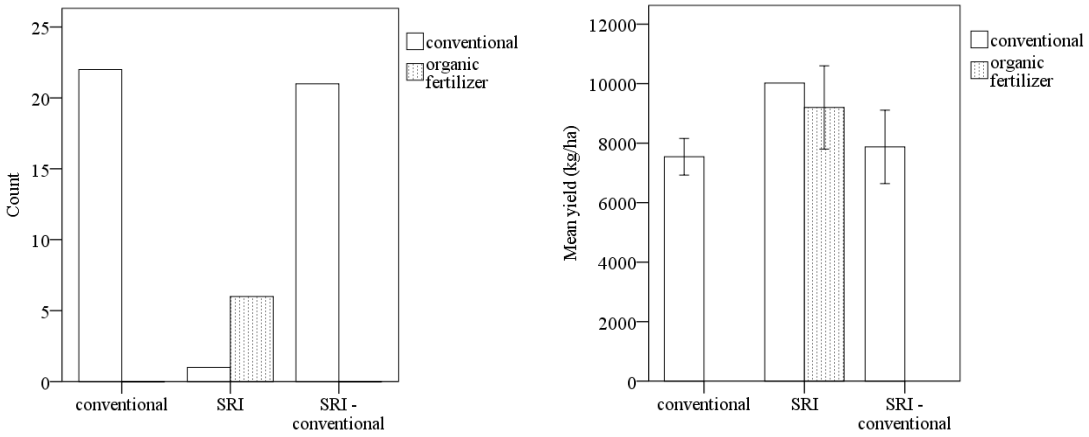


Figure 5. Nutrient management (left) and their yield performance (right) according to farming systems, bar in the chart on right indicates 95% CI for mean.

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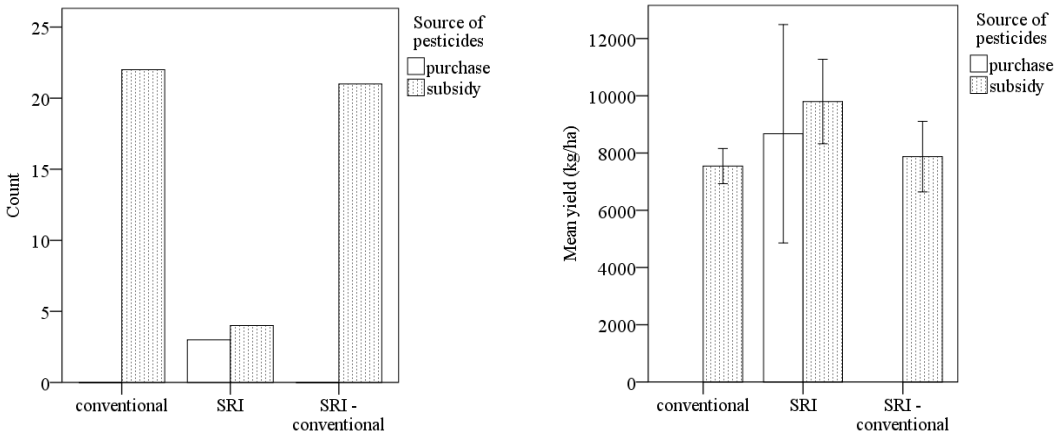


Figure 6. Sources of pesticides (left) and their yield performance (right) according to farming systems, bar in the chart on right indicates 95% CI for mean.

The application of organic soil amendment affects the soil microorganisms. The soil microorganisms such as consortium bacteria can reduce the disease severity, especially in rice (Suryadi et al., 2013). This biological control agent can act as parasite to pathogens and reduce the infection caused by soil borne pathogen. The intermittent irrigation also causes unfavorable condition to the pathogens and the disease severity is therefore reduced. In previous study, there were reductions of disease causing pest populations in SRI plot. Stem borer, leaf folder, cast worm populations were found reduced by 24.2 %, 15.9 % and 11.4 respectively in both CAUR – 1 and Deku variety of rice (Pathak et al., 2012). The population of natural enemies such as dragon fly also reduced as indicator of low population of stem borer, leaf folder and case worm. Moreover, the disease incidence of brown spot, bacterial leaf blight, false smut, bacterial sheath blight and bacterial leaf stripe was also reduced in SRI plots. The study conducted in Chiba Prefecture, Japan, also showed that there was no significant pest or disease incidence during the experiment of SRI (Chapagain et al., 2011). This low incidence of pest and disease was also believed to be related to the lower plant density which reduced the moist microclimate that is favored by the pest and disease causing agents. However, reduced fertilizer and pesticide application advantages were yet to be proven for SRI in Sabak Bernam as the farmers under study still relied greatly on the conventional method and the government intervention in terms of subsidized fertilizers and pesticides. There were, however, positive results with adoption of SRI methods in the under study area as a few SRI farmers achieved higher yield of more than 9,000 kg/ha. Reduced flooding irrigation together with incorporation of organic fertilizer as substitute to chemical fertilizer are believed to bring indirect benefits to maintain healthier environment and ecosystem in the long run (Chapagain and Yamaji, 2010; Nyamai et al., 2012; Choi et al., 2013).

An important concern in SRI practices was the differences of productivity and subsequent income of the farmers. In Tamil Nadu India, a study indicated that the farmers obtained higher income in SRI practices as compared to the conventional farming (Devi and Ponnarasi, 2009). The other study conducted by Barah (2009) also supported that there was an increasing rice production by more than 26% in SRI as compared to the other practices. The increasing rice production combined with the saving of water usage up to as much as 40%, saving on seeds for planting for as much as 42 % and shortened length of rice growing period of approximately 20 days contributed to higher net income to the farmers. The lower pesticide and herbicide usage also contributed to the decreased cost of production. A study conducted in Timor Leste also proved that there was an increase in yield gained by SRI adopters, especially in higher family labor and management requirement as compared to non – SRI adopters (Noltze et al., 2013). This high yield gained was the same for poor and non – poor households in Timor Leste. However, the study conducted in West Bengal, India by Haldar et al. (2012) showed that the labor cost in SRI practices was

75% higher than conventional practices in Kharif season (main season) and Rabi season (off season). Higher labor cost eventually did not affect the net income of the farmers because the higher yield and the reduction of other costs had covered the labor cost as a whole. The increasing rice yield for about 0.3% was corresponding to the additional labor input by 3% (Noltze et al., 2013). It was due to the hiring of the skilled labor in managing SRI practices. The method applied for growing rice in SRI also affects the management of nursery. The lower cost in purchasing seeds, lower irrigation usage, and shorter duration for growing the seedlings in the nursery beds allowed the reduction of nursery cost by 76% as compared to the conventional methods (Haldar et al., 2012). Due to reduction of cost in nursery stages and field stages, the farmers in West Bengal acquired higher income in Kharif and Rabi season of Rs 14,613 and Rs 22,966 per hectare, respectively, as compared to conventional methods.

**Conclusion and Recommendations**

SRI should be encouraged among the rice farmers as there was significant higher yield with such practices as compared to conventional rice farming. Further study should be conducted to represent SRI practiced in other rice granaries in Malaysia. In addition, this study in Sabak Bernam only relied on the production and yield of paddy in main season. Production and yield of paddy in off – season also needs to be studied so that more representing scenario of SRI and conventional farming practices in Sabak Bernam, and in Malaysia as a whole, can be analysed for improvement. The outcome of the study is hoped to be used to strengthen the understanding of SRI among the rice growers in Malaysia. The results on the different SRI practices carried out by the farmers can be a platform for DoA to conduct experimentations and to standardize the practices for the future needs.

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# CHAPTER 2

# ECOPHYSIOLOGY AND STRESS BIOLOGY

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# Comparison of Leaf Wetness in Upper and Lower Citrus Canopy

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## Introduction

Leaf wetness is an important parameter as it influences the infection and growth of many plant diseases. Therefore, many phytopathological models use leaf wetness duration in combination with other factors to assess the infection and pest severity as well as to manage disease control activities in an efficient way. According to Timmer and Zitco (1996) model-based decisions on fungicide applications in citrus resulted in reduced disease, large increases in fruit production and elimination of unnecessary sprays. However, leaf wetness is difficult to measure or estimate because it is driven by both atmospheric conditions and their interaction with the structure, composition and physiology of the crop canopy. Leaves at different positions within a canopy become wet and/or dry at different times, which results in the spatial variation in length of time each leaf is wet. This spatial variability is an important aspect to be considered when measuring leaf wetness duration (LWD) (Santos et al., 2008), since a thorough understanding of the heterogeneity within a crop canopy is critical for implementing a disease-warning system reliant on LWD input data.

Several scientific instruments can monitor leaf wetness, including Campbell Scientific leaf wetness sensor that operates by measuring electrical resistance, and has been used in studies investigating spatial variability of leaf wetness duration in different crop canopies such as apples, coffee, grape, maize, cotton and bananas (Henshall et al., 2005; Sentelhas et al., 2005; Dalla Marta et al., 2008; Santos et al., 2008). However, the sensors may require field calibration. A more recent design, Decagon Devices wetness sensor detects wetness based on electrical capacitance (Gleason et al., 2008) with high resolution and very small amounts of water. This study aimed to compare the upper and lower canopy leaf wetness within a citrus canopy using both these sensors.

## Materials and Methods

### *Description of field site*

The study was conducted at the Welgenvallen Experimental Farm of the University of Stellenbosch (latitude 33.56 S, longitude 18.52 E, altitude 157 m) in Western Cape, South Africa. The citrus orchard, planted in 1994, consists of mature, fruit bearing *Citrus unshiu* Marchovitch citrus trees not irrigated during this field trial experiment. The region is characterized by a Mediterranean climate with cool, wet winters and warm, dry, summers. The mean annual precipitation is 673 mm (about 70% in winter) brought by the dominant westerly and northwesterly moisture laden winds. Mean midday maximum temperatures range from 16 °C in July to 24 $^{o}$C in December and winter night temperature drops to 6.6 °C in July. The 2012 winter season was distinctly wetter and colder, with total rainfall recorded in June, July and August, 2012 about 140 mm more than long-term mean.

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*Leaf wetness sensors*

Flat, printed-circuit wetness sensors, Campbell Scientific sensors (Model 237) (http://www.campbellsci.co.za/237-l) is hard epoxy-fibreglass with interlaced gold-plated copper fingers, measuring 7.5 x 6 cm, with a smaller active surface area of about 30 $cm^2$. Condensation (free water) on the upper surface of the sensor lowers the impedance between fingers (1 mm, grid spacing) and is measured by a data logger. A dry sensor has infinite impedance, recorded as 6999 Ω by a CR10X data logger. Prior to use, the sensors were field calibrated to determine the wet/dry threshold.

Decagon dielectric leaf wetness sensors estimate leaf surface wetness by measuring the ability of the material on the sensor's upper surface to store charge (http://www.decagon.com/products/canopy-atmosphere/water/lws-leaf-wetness-sensor/). The sensor output voltage (mV) is proportional to the dielectric of the measurement zone and proportional to the amount of water on the sensor's surface. It requires an excitation voltage of 2500 mV and data logger reading of less than 274 mV corresponds to a dry sensor. Each of the sensors was individually calibrated in the field.

*Placement of sensors in citrus tree canopy*

Six leaf wetness sensors, (three each type) were installed at two different heights in a citrus tree (about 2.14 m tall). Four sensors, (two Campbell and two Decagon) were mounted in four horizontal positions approximately 30 cm apart along an east-west transect in the top third of the citrus canopy (about 1.63 m height) (Figure 1). Another set of sensors, (one each type) was mounted in the lower one-third of the canopy (64 cm height) surface, with Decagon sensor (D_3) on the eastern side, at a horizontal distance of 70 cm from the Campbell sensor (C_3) on the western side of the canopy. All the sensors were mounted on the end of a PVC pipe, oriented at 45° to the horizontal, facing south. The sensors were checked routinely to avoid leaf contact with their surfaces.

Figure 1. Photo of wetness sensors mounted on the upper citrus canopy at the Welgenvallen Experimental Farm, University of Stellenbosch between July and November, 2012.

Meteorological variables, including relative humidity, air temperature (1.8 m), net radiation (just above the canopy) and wind speed above the canopy as well as leaf temperature were also measured. One thermocouple thermometer, was attached by means of plastic clip to a tagged leaf in upper canopy whilst a second thermocouple to a leaf in the lower eastern canopy. All meteorological sensors were connected to a CR10X – data logger is programmed to scan data from each sensor every five seconds and average it over a 15 min time period. Rainfall data measurement was obtained from a standard weather station located close to the orchard.

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*Visual observations of dew*

The existence of wetness on the leaves was assessed by visual observation, involving visual inspections for wetness on the surface of selected leaves (20 leaves with no visible damage within 30 cm of a wetness sensor) in upper and lower eastern canopy position each tagged and numbered for unique identification. Human visual inspections of dew onset were based on the first appearance of small water droplets on any one of the tagged leaves. A leaf was considered to be "wet" (assigned = 1) when one-tenth (10%) of its upper surface area was visually wet. Leaves with no visual evidence of surface wetness were considered "dry" (flagged as 0). When the leaf upper surface area was completely wet, it was assigned a value of 10 that designated 100 % leaf wetness. These visual observations were done every 15 min starting from 18h00 until complete surface wetness, and again from sunrise until complete dew dissipating in the morning for some selected days from 27 July until 30 November 2012 (total = 62 days) (Kudinha, 2014). A single day (13 Oct, 2012) when dew formed will be presented as an example in this paper.

*Data analysis*

The observed daily LWD data began at 12h15 and ended at 12h00 (24h), by summing all the 15 min time intervals when the wetness was visually observed. The observed days were split into dew days and rainy days, defined as a day with measured rainfall $\geq$ 0.25 mm. T-test at 5% probability was performed to determine if mean LWD were statistically significant from each other during days when wetness resulted from dew, rain or both events.

**Results and Discussion**

*Variation of weather data on a dew day considering the upper canopy*

An analysis of dew days, indicated that dew was mostly deposited on the leaf whenever the leaf temperature reached the dew point temperature (Figure 2). To illustrate the variation of meteorological parameters a typical dew day was analyzed for this paper, 13$^{th}$ October, 2012. The variation of meteorological variables as well as wetness sensors' response was examined from 12h15 on 13$^{th}$ October until 12h00 the next day. On this night, both the air and leaf temperature began to fall sharply after sunset at 18h00. The decrease in air temperature was accompanied by an increase in relative humidity. Dew was observed to form on the night of 13$^{th}$ October 2012 at 19h15 when the leaf temperature reach the dew point temperature at that time (Figure 2) this was also confirmed by the visual observations (Figure 3).

This onset of dew deposition was also recorded by the wetness sensors. In the case of the Campbell sensors, the sensor readings fell from 6999 Ω to below 1000 Ω (Figure 4a), whilst for the Decagon sensors, voltage increased from a baseline voltage of about 270 mV (Figure 4b) as an indication of the leaf wetting process. A sudden change (seen as kink on graph) in one of the Campbell sensors could have been caused by droplets dropping off the sensors, followed by more wetting given the further decrease in temperature (Figure 4a).

The wetness response seemed to mimic the visual observations of canopy wetness because when the canopy was visually observed to be wet, the wetness sensors' outputs also correctly indicated it to be wet in most of the cases. A similar pattern was also observed when the canopy was observed to be dry. Visual observations of wetness indicated that canopy was wet when relative humidity was mainly greater than 82% and above and dry below 45%, which agreed with sensors' response (Figure 5). In the case of vapour pressure deficit, values of vapour pressure deficit less than 1.5 KPa were associated with observed

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wetness when the leaf was evaporating (Figure 6), a trend that the wetness sensors also depicted (Kudinha, 2014).

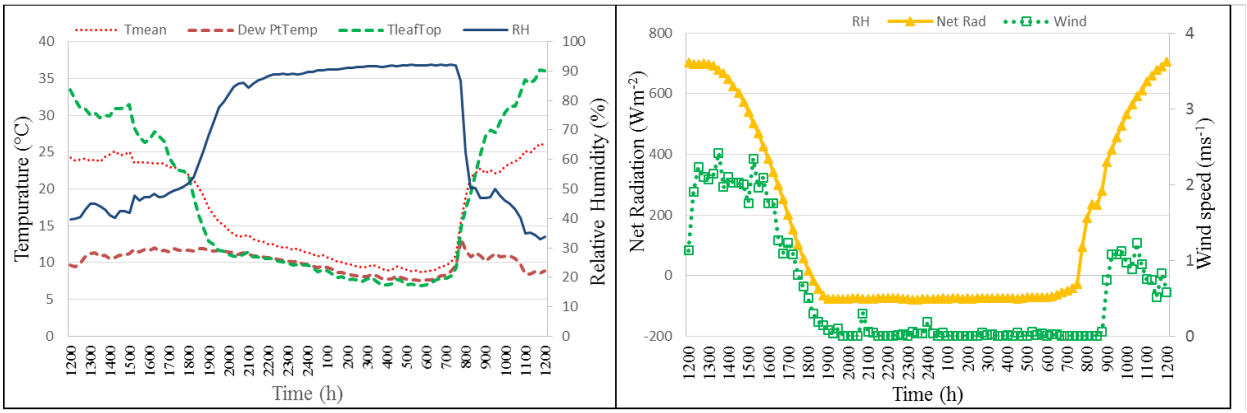


Figure 2. Variation of meteorological parameters at Welgenvallen Experimental Farm of the University of Stellenbosch from 13 October, 2012 until the next day with Tmean = air temperature (°C), RH = air relative Humidity (%), Dew PtTemp = dew point temperature (°C); TleafTop = leaf temperature in upper layer (°C); wind=wind speed above canopy; Net Rad=Net Radiation from the automatic weather station.

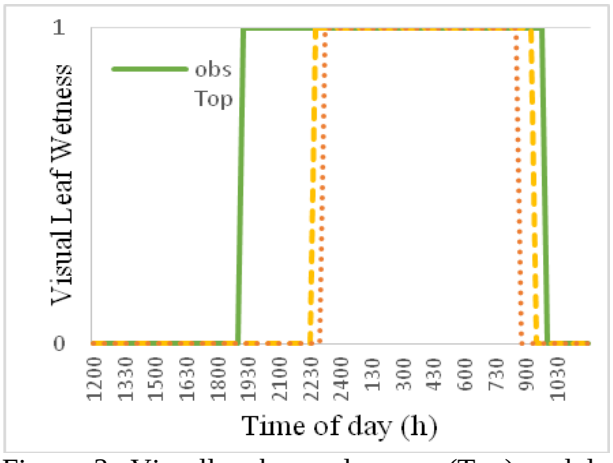


Figure 3. Visually observed upper (Top) and lower (B) canopy wetness within a citrus canopy at Welgenvallen Experimental Farm of the University of Stellenbosch from 13 October, 2012 until the next day. 0= dry, 1=wet.

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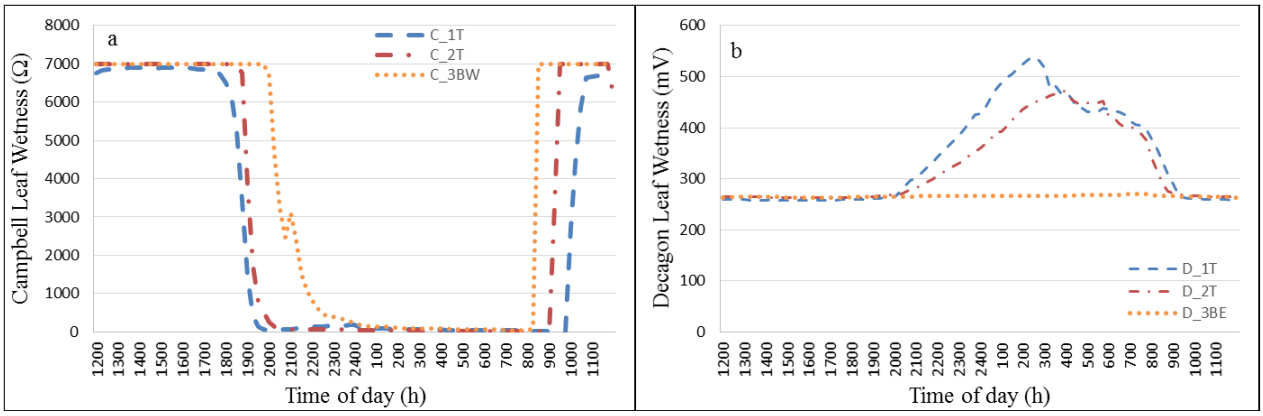


Figure 4. Wetness sensors' response to dew formation at Welgenvallen Experimental Farm of the University of Stellenbosch from 13 October, 2012 until the next day, where T=upper canopy; B=lower canopy; C=Campbell (a), D=Decagon (b), E=East, W=West, #=sensor number.

An analysis of the response of the sensors, showed that the wetting of all the sensors was achieved when the vapour pressure deficit was less than 0.2 KPa, which coincided with the visual onset of dew deposition in the upper canopy level. Evaporation of moisture from the canopy varied according to other conditions and combinations of vapour pressure deficit and air temperatrure. All the sensors exhibited similar patterns, although the two different types showed different sensitivities to the wetting and drying cycle. There was a characteristic hysteresis loop exhibited by the sensor' outputs (Figure 5). The Decagon gave a higher voltage during evaporation part of the cycle (Figure 5b and 5d), thus tending to indicate a wetter value for longer. In contrast, the Campbell sensor went through the whole range of values during both the wetting and evaporation cycles, but gave a high resistance value when dry and near zero value when fully wetted (Figure 5a and 5c). Probably this is why the manufacturers recommend only stating a wet or dry value.

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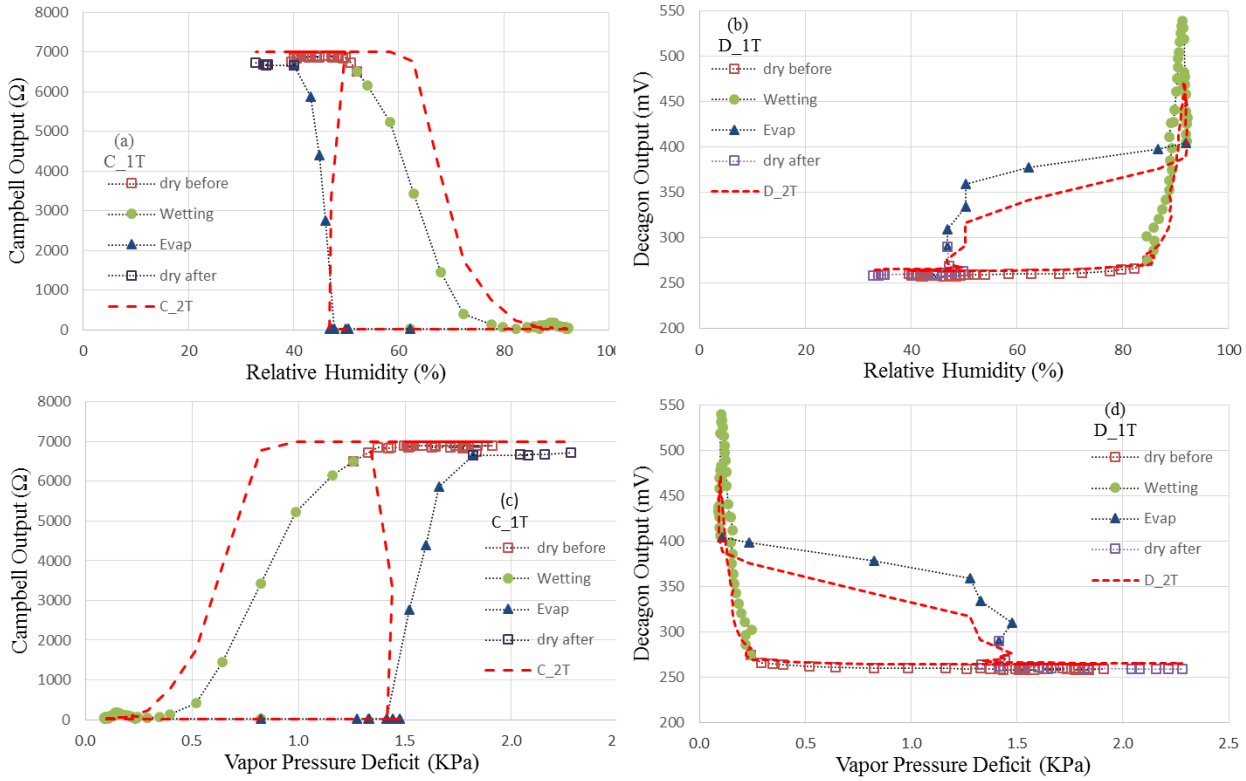


Figure 5. Sensors' response to relative humidity (a and b) and vapour pressure deficit (c and d) at Welgenvallen Experimental Farm of the University of Stellenbosch from 13 October, 2012 till the next day; C=Campbell (a and c), D=Decagon (b and d), #=sensor number, T= upper layer.

*Visual observations of occurrence of leaf wetness*

Although the field trials ran from 27 July 2012 to 31 November 2012, only a total of 62 days were visually observed for leaf wetness throughout the night. Dew formed on the upper canopy more frequently than on the lower canopy position and contributed 47% of all the wetness that occurred during the period of study whilst rainfall accounted for 17 days of wetness in the upper canopy. It was also common to observe that on some days, dew initially formed at night and was followed by rains later during the night or the early part of the next day. The remainder of the days, a total of 10, were dry (no dew having been visually observed). Dew occurred in the lower eastern canopy for a total of 15 days, which was 9 days less than its occurrence of dew in the upper canopy. The number of dry days in the bottom canopy (eastern) was about twice as much as those in the upper canopy (21 days) (for details see Kudinha, 2014).

A comparison of the onset time of LWD observed at both canopy levels indicated different wetness profiles within the citrus canopy. On 9 nights dew formed in the upper canopy level and yet the lower canopy was completely dry. Leaf wetness consistently manifested earlier in the upper canopy layer, hence the leaves in the upper canopy level were usually wetted before dew started to be deposited on the leaves in the lower canopy. On average dew onset on the upper surface occurred 1.5 to 2 h before its dew onset in the lower canopy. There was also a variation of about half an hour between the onsets of wetness on the two sides of the lower canopy. Initial dew onset on the upper canopy was usually detected between 19h15 and 22h00 hours, but only once the first dew occurrence was as late as 1h15 hours. However, dew onset was observed to occur occasionally as late as the midnight in the lower canopy (for details see Kudinha, 2014).

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In the upper, there was a gradual increase of wet leaf area observed as well as the presence of wetness in almost all the leaves. However, in the lower canopy level, the pattern of wetness was that it mainly took place on the outside leaves, followed by some of the leaves in the interior. Wetness was observed in 60% to 100% of the leaves in the upper canopy level, while only a few leaves in the lower canopy wetted (10% to 60%) with exception of two nights when a high incidence was observed in both canopy levels. The two nights were characterized by high relative humidity and lower temperatures which could have resulted in high dew deposition, which could have resulted from rains that fell shortly before that. There was never a single dew day that all the leaves in the lower canopy position became wet (Kudinha, 2014). Rain, especially when intense, caused the leaves in all the canopy positions to wet almost simultaneously, which minimized the differences in LWD between the canopy positions.

*Comparison of visually observed LWD at different canopy positions*

The statistical analysis of the data revealed that LWD was not homogeneous throughout the canopy and varied according to canopy position (height) and type of wetness (Table 1). The vertical distribution of wetness duration in the canopy depended on whether rain or dew was the source of wetness. The differences between mean LWD in the lower eastern and lower western horizontal positions were not as pronounced (not significantly different) as the difference between the upper canopy and any one of the lower canopy positions (significantly different). The greatest differences in mean daily LWD among the canopy positions were observed during days when wetness resulted from dew. These differences in mean LWD between the upper and the two lower canopy positions during dew days were 1.9 h and 1.7 h corresponding to the eastern and western lower canopy positions respectively yet the lower canopy positions differed by less than 30 minutes regardless of the source of wetness. Although the mean daily LWD at the upper canopy and the lower canopy positions were significantly different, the lower canopy positions were not significantly different from each other (Table 1). The same pattern persisted when both rain and dew days were combined. During rainy days, there was no significant difference between all the canopy positions and the mean daily LWD in the upper and lower canopy positions differed by about 1 h.

Table 1. Mean daily leaf wetness duration (LWD) (in hours per day) in three citrus canopy positions, comparing dew only days, rainy only days and days receiving both rain and dew selected from observation period 27 July to 30 November, 2013.

| Type of wetness | Top canopy | Bottom canopy | |
|---|---|---|---|
| | | Eastern | Western |
| Dew | 12.2[a] | 10.3[b] | 10.7[b] |
| Rain | 15.5[a] | 14.3[a] | 14.1[a] |
| Rainy and dew days combined | 14.0[a] | 12.9[b] | 12.6[b] |

*Mean in same row followed by same letter are not significantly different by t–test at 5% probability level*

*Discussion of spatial variations*

The observed total LWD, calculated as the sum of the wet hours during the entire period amounted to 725 h in the upper canopy level compared to 527 h in the lower canopy. This indicates a gap of 198 h between the upper and lower canopy levels and confirms that there are differences between the two canopy positions. These differences can be attributed to the night thermal patterns that occur in the citrus canopy. During the night, the mean air temperature profile at the top of the canopy becomes unstable due to loss of long wave radiation and thus the unstable lower vegetation layer is capped and thereby decoupled from the above the above – canopy region (Jacobs et al., 1995). Air and leaf temperature data show that the leaf temperatures in the upper canopy layer (8.32 ± 3.17 $^{o}$C) were slightly lower than the average air temperature (8.65 ± 2.75 $^{o}$C) compared to higher leaf temperatures in the lower canopy layer (mean 9.12

± 2.97 $^{o}$C). The leaves in the upper canopy were cooler than those in the lower canopy by 0.80 $^{o}$C. A study of leaf wetness spatial variability within grapevine canopy by Dalla Marta et al. (2008) seem to confirm the findings of this study although they found that in the upper canopy layer, leaves were cooler than those in the bottom layer by about 0.6 $^{o}$C. However, the higher leaf temperature difference in the citrus could be due to the fact that the citrus canopy had a very dense canopy. The higher leaf temperatures in this lower canopy layer seemed to have given rise to lower condensation in this canopy layer due the cooling process lasting longer.

The explanation for the early deposition of dew on the top canopy is related to the exposure of the top canopy to the sky. In a mature canopy such as the citrus, the top canopy is directly exposed to the sky, and consequently is generally the first region to exhibit wetness (Santillàn-Nūñez, 2009). The leaves in this layer create a barrier that reduces radiant and convective heat loss from the ground, delaying the cooling of surfaces of the lower canopy, and thereby subsequently delays dew formation (Batzer et al., 2008). The overall result was that, dew deposition occurred earlier in the upper canopy than in the lower canopy. Shorter dew events in the lower canopy level were a result of late dew onset as all the canopy positions dried almost at the same time.

When examining both dew and rainy days, there was a significant effect of canopy position on mean LWD. The differences in LWD variability are associated not only with weather conditions but also the plant structures as well as the planting system, crop age and crop management (Sentelhas et al., 2005). According to Monteith and Unsworth (1990), the LWD inside the crop canopy can be longer or shorter than the top depending on the crop structural characteristics and consequently on its microclimate. For example, in particular the trichomes could act as condensation points preventing dew reaching the leaf surface, which would appear as dry to an observer. In this study, the LWD also showed spatial variability within a citrus canopy. The daily mean LWD between the upper and lower was about 2 h similar to results for apple trees (Sentelhas et al., 2005; Batzer et al., 2008; Wittich, 1995). Generally citrus and apple trees have a similar shape of canopy which is characterized by upper canopy which has unobstructed exposure to the sky and is generally the first to exhibit wetness during both dew and rain events. On the other hand, LWD spatial variability is expected to be different for other canopies. For example, LWD in coffee plants lower was 1.5 h longer than the top (Sentelhas et al., 2005; Santos et al., 2008). The reason for this is that the conical shape of the coffee plants exposes leaves at all the levels to the sky and promotes the beginning of wetness almost immediately.

These findings emphasize it is not only the weather parameters that affect LWD but also by plant structure and height which affect the microclimate. It can therefore be concluded that the citrus canopy in Stellenbosch showed spatial variability in LWD and this variable was not only affected by weather conditions but also by canopy characteristics such as plant height, leaf exposition and leaf area index, whose interaction determines the microclimate These findings point to the importance of taking cognizance of the spatial heterogeneity of LWD in citrus, if measurements are required for disease-warning systems. The understanding of LWD variability is crucial as it can help to improve the performance of the disease warning systems. Since factors such as arrangement of plants, crop age and crop management practices can affect the crop-climate microclimate, it is possible the pattern of spatial variability of LWD in citrus can change as citrus trees grow old and bigger and if crop management practices also change. Griffiths (1978) suggested that during the daylight hours, the added vertical currents of the thermals can cause an almost independent cell (between plants if there is space between them) to develop and at ground level the air may flow at 180$^{o}$ to that of the free air above the trees. This wind flow could promote a drying process that starts in the top canopy followed by the bottom and finally the middle layer. As micromet measurements were not performed between the citrus rows, this cannot be conclusive and would have to be a recommendation for a future micromet study.

Since the daily LWD was longest in the upper citrus canopy, this canopy level should be considered as the as a standard for measurements considering its use in diseases-warning systems in citrus. Moreover, visual observations of wetness in the upper canopy of the citrus can be used as calibration indicators of wetness for the lower canopy. The longer the LWD duration, the more susceptible the citrus would be to diseases that require a longer wetness period to infect a leaf somewhere in the canopy (Hahn, 2009). The mean average LWD was about 10 h, and so the citrus in Stellenbosch could be more susceptible to pathogens that require a minimum of at least 10 h to infect the citrus plants assuming other weather conditions for their development were favourable (Vincent & Garcia-Jimenez, 2008).

**Conclusions**

It can be concluded that both Decagon and unpainted Campbell sensors placed in the upper canopy level proved to be good estimators of leaf wetness duration, although they differ in response to evaporation and drying cycles. However, the sensors placed in the lower canopy provided a poor representation of the leaf wetness, so they should not be used as input into disease warning systems.

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# Antiodixant Defence System in Iron Deficient Groundnut Plants

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## Introduction

Iron is one of the essential micro-nutrients required by the plants. It plays an important role in the synthesis of chlorophyll, carbohydrate production, cell-respiration, chemical reduction of nitrate, sulphate and N assimilation, component of cytochrome oxidase, ferredoxin protein and several enzyme systems. Iron deficiency symptoms appear on younger leaves indicating yellowish inter-veinal areas of leaves (commonly referred to as iron chlorosis). In general, plants are prone to iron deficiency in alkaline, calcareous soils, coarse textured soils, eroded soils, low organic matter soils and cold weathered areas soils except flooded rice field soils. In India more than one-third of the soil is calcareous and spread mostly in the low rainfall areas of the western (Gujarat, Maharashtra, Rajasthan and Karnatka) and central (M.P. U.P.) parts of the country where groundnut is a major crop. Therefore iron chlorosis is causing considerable yield reductions. The crops sensitive to iron chlorosis are groundnut, citrus, field bean, grapes, soybean, vegetables and ornamentals.

Groundnut is grown during kharif (July-October) as well as during rabi-summer (Feb-May) seasons. In calcerous soils of Saurashtra, Gujarat more than 60% groundnut fileds showed chlorosis during rabi-summer season compared with less than 20% during kharif season. During Kharif, groundnut crop is grown as rainfed while during rabi-summer season crop, most of the farmers give frequent irrigation mainly due to the high evapo-transpiration demand of the crop. As a result, majority of the groundnut fields show chlorosis and remain chlorotic throughout the cropping season. Plant Fe deficiency has economic significance because crop quality and yields can be severely compromised and the use of expensive corrective methods is often required (Alvare-Fernandez et al., 2004). Despite the ubiquitous presence of Fe in the earth's crust, the low solubility of Fe compounds in many soils prevents plant Fe uptake and induces development of Fe deficiency symptoms. Crop genotypes differ greatly in their response to iron availability (iron stress) in the soil and have been designated as iron-inefficient (susceptible to iron chlorosis) and iron-efficient cultivars (Brown and Jolley, 1989; Singh and Chaudhari, 1993). Iron efficient genotypes have already been identified for many crop plants, although reports on a limited number of groundnut genotypes are available, an extensive and systematic investigation has not been undertaken for the identification of genotypes tolerant to lime induced iron chlorosis for use in breeding programmes.

A deficiency in mineral nutrients is generally not considered as a stress factor for plants. In this context, the study of Fe deficiency is particularly appealing. First, Fe deficiency is a worldwide problem, enormously detrimental to plant production. Secondly, Fe is a transition metal of pivotal importance in reactions involving active oxygen (Halliwell and Gutteridge, 1989) and a constituent of antioxidant enzymes such as catalase, peroxidases and SOD. On the other hand, there is little information about the relationship between iron-deficiency and the onset of oxidative stress status (Iturbe-Ormaetxe et al., 1995) despite both the world-wide problems represented by scarce iron bioavailability in the soil and the well known double role which iron plays within the cell metabolism. Thus, in this work we have investigated effect of Fe deficiency on oxidative stress in groundnut plants. In view of this, the present research activity was planned to study the correlation between Fe and enzyme activities in groundnut (*Arachis hypogea*) by comparing the antioxidant enzymes of Fe-sufficient (Fe+) and Fe-deficient (Fe-) leaves.

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## Materials and Methods

*Plant materials and treatments*

Eight Fe-efficient and eight Fe-inefficient groundnut genotypes, as listed in Table 1, were sown in twelve nutrient blocks of size 4x5m each in a randomized block design (RBD) with two replicates. In each replicate 60 plants of each genotype were grown in adjacent rows each 5 m long. The row to row and seed to seed spacing were 45 cm and 8 cm respectively with Fe-efficient and inefficient genotypes in separate nutrient blocks. The plants were supplied with iron free nutrient solution at required intervals. At the vegetative stage, 20–25 d after sowing, Fe was added in the form of nutrient solution with $FeSO_4$ through rooting medium. The treatments included $T_1$ as control where no nutrients were applied; $T_2$ was nutrient supply with Fe source (Fe+) and $T_3$ nutrient supply without Fe source (Fe-). The third mature leaf from top was taken for sampling after three days of iron treatments.

Table 1. List of groundnut genotypes.

| Sr No | Fe inefficient | Sr No. | Fe efficient |
|---|---|---|---|
| 1 | NRCG 7472 | 9 | ICGV 86590 |
| 2 | NRCG 162 | 10 | ICGV 86031 |
| 3 | CO2 | 11 | CSMG 9510 |
| 4 | NRCG 7599 | 12 | GG 7 |
| 5 | TIRUPATI 4 | 13 | GIRNAR 2 |
| 6 | MH 1 | 14 | CSMG 84-1 |
| 7 | JL 220 | 15 | ICGV 000348 |
| 8 | VRI 3 | 16 | KADIRI 9 |

*Enzyme extraction:* Fresh leaf samples from control and treated plants were ground with liquid nitrogen, and suspended in 0.1 M phosphate buffer, pH 7.5 containing 0.5 mM EDTA. The Brie was passed through four layers of cheese cloth and the filtrate was centrifuged at 15000 rpm for 20 min at 4°C and resulting supernatant was used for enzyme assays. Then respective enzyme assays were performed according to the following methods:

*Peroxidase* (EC 1.11.1.7): The enzyme activity was estimated by the method of Bergmeyer (1974). To begin with enzyme assay, the reaction mixture containing 0.5 mL enzyme extract, 100 mM phosphate buffer, pH 6.1, 12 mM $H_2O_2$ and 96 mM guaiacol was mixed. Final volume was made up to 3.0 ml with distilled water. Absorbance due to formation of tetra-guaiacol was recorded at 470 nm. The enzyme activity was expressed as units $min^{-1}mg^{-1}$ protein.

*Catalase* (EC 1.11.1.6): Catalase activity was estimated by the UV method of Aebi (1983). The reaction mixture contained 50 µL enzyme extract, 12.5 mM $H_2O_2$ and 50 mM potassium phosphate buffer. Decrease in absorbance was recorded at 240 nm for 30s. The enzyme activity was expressed as µ moles $H_2O_2$ decomposed $min^{-1}mg^{-1}$ protein by using the $H_2O_2$ extinction coefficient 36 $\mu M^{-1}cm^{-1}$.

*Superoxide dismutase* (EC 1.15.1.1): The activity of superoxide dismutase was estimated by the method of Giannopolitis and Ries (1977). The reaction mixture contained 0, 0.2, 0.3, 0.5 and 1.0 mL enzyme extract in different sets. To each set, 200 mM methionine, 2.25 mM nitroblue tetrazolium, 3 mM EDTA and 1.5 M $Na_2CO_3$ were added. Total reaction volume was brought to 3 mL adjusting the pH to 10.2. At the end, 2 µM riboflavin was added. The tubes were shaken and placed 30 cm from the light source consisting of two 15-W fluorescent lamps. The reaction was allowed to run for 15 min and then stopped by switching off the lights. The tubes were immediately covered with black cloth. The absorbance was recorded at 560 nm. A non-irradiated reaction mixture which did not develop a colour, served as control.

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However, in the presence of SOD, the reaction was inhibited and the amount of inhibition was used to quantify the enzyme. $LogA_{560}$ was plotted as a function of volume of enzyme extract used in the reaction mixture. From the resultant graph, the volume of enzyme extract corresponding to 50 % inhibition of the photochemical reaction was obtained and considered as one enzyme unit. The enzyme activity was expressed as units $min^{-1}mg^{-1}$ protein.

*Glutathione reductase* (EC 1.11.1.9): Glutathione reductase activity was estimated by the method of Goldberg and Spooner (1983). To 0.1 mL enzyme extract, 200 mM K-phosphate buffer (pH 7.5), 0.015 mM EDTA and 20 mM oxidised glutathione were added. After 5 min, 2.0 mM NADH was added and mixed thoroughly. The absorbance was recorded at 412 nm at intervals of 5 s. The enzyme activity was expressed as nmole NADH oxidized $min^{-1}mg^{-1}$ protein.

**Results and Discussion**

In leaves supplied with iron source, the catalase and peroxidase activities were higher which decreased by almost 50% with elimination of Fe from nutrients (Fe-), whereas the effect on glutathione reductase was not significant. On the other hand, superoxide dismutase activity was increased by 20-35 % among different genotypes under Fe deficiency in Fe-inefficient genotypes. The genotypes e.g. NRCG 162, MH1, VRI3 etc, which are unable to utilize the available iron efficiently(Fe-inefficient) are having less activities of catalase, peroxidase in the absence of Fe whereas higher activity in presence of Fe. From the results, it is clear that the reactive oxygen scavenging (ROS) enzyme activities are less in the inefficient genotypes showing that the plants are lacking iron and thus showing Fe deficiency symptoms.

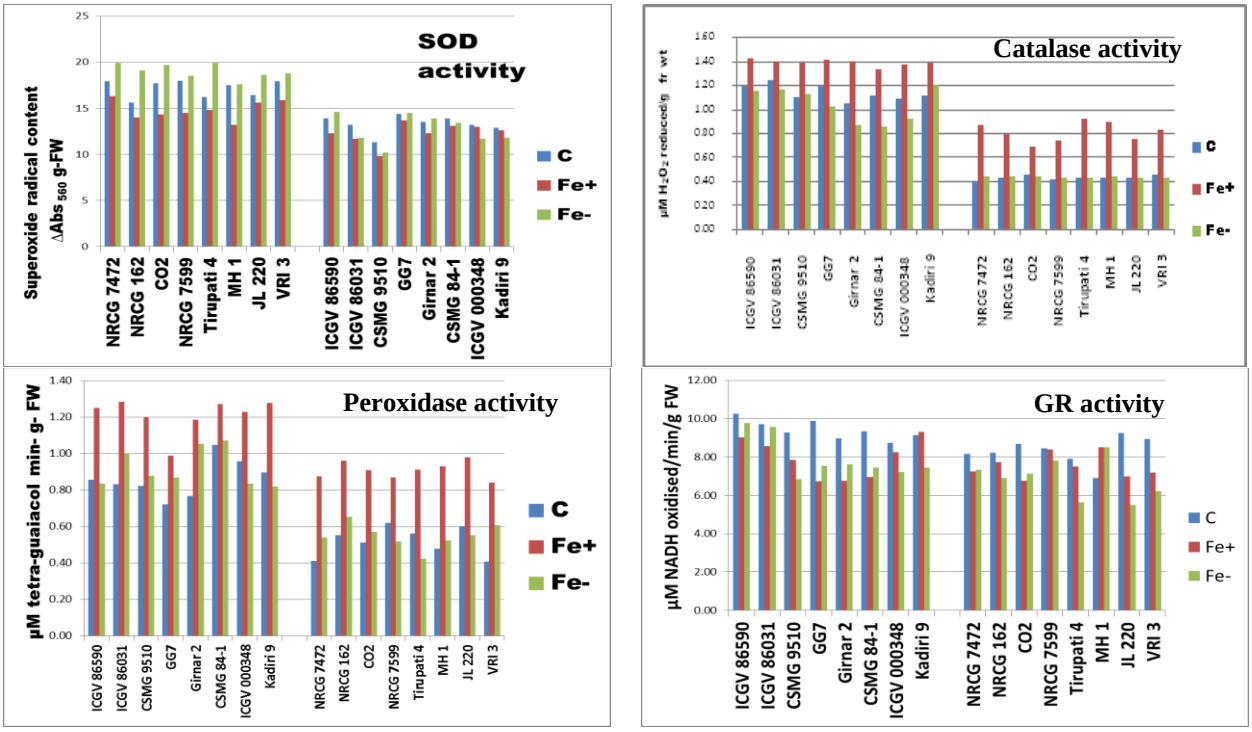


Figure 1. The activities of superoxide dismutase, catalase, peroxidase and glutathione reductase in Fe-efficient and inefficient groundnut (*A. hypogea*) genotypes in presence ($Fe^{+}$) and in absence of ion ($Fe^{-}$).

SOD is a major scavenger of $O^{2-}$ and its enzymatic action results in the formation of $H_2O_2$ and $O_2$. Peroxidase decomposes $H_2O_2$, by oxidation of co-substrates whereas catalase breaks down $H_2O_2$ into water and molecular oxygen (Mittler, 2002). The capacity to scavenge ROS and to reduce their damaging effects on macromolecules appears to represent an important stress tolerance trait. In the present investigation, increased SOD activity following iron deficiency leads to increased $H_2O_2$ production but on the other hand, the decreased capacity to detoxify the enhanced $H_2O_2$ may be due to unsuccessful activation or reduced production of heme containing antioxidant enzymes like peroxidase and catalase. As an abiotic stress for plants, iron deficiency was shown to affect the expression and the activity of certain peroxidase isoenzymes and induces secondary oxidative stress in dicotyledonous species (Ranieri et al., 2001). Zaharieva et al., (2004) found in sugar beet roots that iron deficiency resulted in the decreased activity of APX and increased content of GSH. The activities of several antioxidant enzymes were greater than the controls in Mg-deficient bean leaves (Cakmak and Marschner, 1992) and Mn-deficient needles of Norway spruce trees which may reflect a response of plants to increased free radical production. It has been suggested that plants exposed to iron deficiency may be more sensitive to oxidative stress because iron is a constituent of enzymes associated with the cellular antioxidant system such as APX, CAT, peroxidase, and Fe-SOD (Kumar et al., 2010).

The relationship between decreased iron availability in the nutrient media and the possible onset of oxidative stress is becoming more evident. The decrease in activity of catalase and peroxidase may also be due to the fact that these two are heme containing enzymes and their activities are dependent on Fe. Thus the changes in activities of antioxidant enzymes are due to oxidative stress induced by Fe deficiency suggesting that groundnut plants come under Fe stress affecting their growth.

## Conclusions

The results of the present study clearly showed that there were differential genotypic variations in activities of scavenging enzymes in groundnut cultivars grown under different iron nutrient conditions. The groundnut plants which were moderately tolerant to iron deficiency might have active ROS scavenging system, in addition to other tolerance mechanisms, to cope with stress. Therefore, plants with the ability to scavenge and/or control the level of cellular ROS may be useful in future to withstand nutrient deficiency conditions.

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# The Contribution of Plant Community towards Slope Protection

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## Introduction

Plant diversity might refer to as variety of plant species community existed in an ecosystem. Plant diversity can be measured in different ways, but usually expressed as species richness at a given area (Ihaddaden et. al., 2013). As the slope being cut for urban development due to increasing in population growth, the potential of reducing in variety of plant species was high. Consequently, increased the risk of soil erosion and from time over time might lead to landslide.

Variety of plant community efficiently helps in mitigating the soil erosion in two ways: active and passive protection (Rey et. al., 2004) such as allow soil fixation by the root systems (Gyssels and Poesen, 2003) and increase the quantity of eroded sediments being trapped within the catchment due to the aerial part (Abu-Zreig, 2001) of vegetation, respectively. It is anticipated that plant diversity (Pohl et al., 2009; Genet et. al., 2010) and good pioneer (Normaniza and Barakbah, 2009) were amongst the crucial aspects to enhance slope stability. In addition, higher in plant diversity would also greater the root systems such as root length density (RLD) (Normaniza and Barakbah, 2006) and its distribution, thus, could reduce the soil water content (SWC) on slope and saturation level (STL). Hence, the aim of this study was to observe the influence of different plant coverage with respect to plant diversity.

## Materials and Methods

*Experimental set-up*

The experimental plots of 5 m x 5 m for each were set up with four different coverages; 0% (Treatment A), 10% (Treatment B), 50% (Treatment C) and 100% (Treatment D) in three replications at Guthrie Corridor Expressway, Sungai Buloh, Selangor. The slope coverage had been chosen by visual estimation, and the plant coverage represented the plant diversity as resulted from the preliminary analysis conducted (Figure 1). However, treatment D was not included in the analysis because during the survey conducted, most of 100% coverage slope was dominated by fern and low diversity. Therefore, treatment D was regarded as stable plot as they undergo succession process to reach the climax stage of slope community which slightly different from the forest community. According to Wang et. al. (2006), the plant diversity might be decreasing when the dominance plant species overwhelmed others and might be increasing later when the community developed into its zonal climax.

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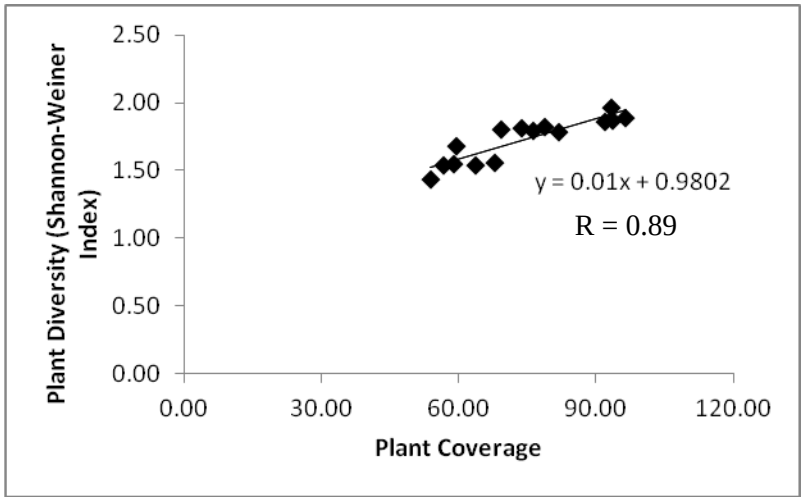


Figure 1. The relationship between plant diversity and plant coverage (treatment D not included).

*Ecological experiment*

The plant species in all treatment plots were identified (Wee, 2005; Ahmad Azly, 1997) and recorded. Plant cover method has been used to measure the relative abundance based on the amount of space covered. This method was practically used for small understory plant such as ferns, shrubs and grass. Instead of counting the number of individual species, each species was assigned to a percent cover class based on a visual estimation of how much they occupied the sample plot. To measure the percent cover, modified Braun-Blanquet cover class scale where >75% cover = 6, 50-75% = 5, 25-50% = 4, 5-25% = 3, 1-5% = 2, and individual cover = 1 (Shono et. al., 2006) has been used. The plant diversity at each treatment, was evaluated by using Shannon-Weiner Index (1). The plant density and plant frequency have been determined in order to measure the Importance Value Index (IVI) of each species (2). All parameters were taken once in three months for 12 months of observation.
Shannon-Weiner Index (1)

$$H' = -\sum Pi \, ln \, Pi$$

Pi = relative importance value of species i (IVi/IV)
IVi = importance value of species i
IV = sum of importance value for all species

Importance Value Index (IVI) (2)

$$IVI = RC + RD + RF$$

RC = Relative coverage
RD = Relative density
RF = Relative frequency

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*Soil hydrological properties*

The soil water profile such as soil water content (SWC), soil field capacity (SFC) and saturation level (SL) were measured. Cylindrical soil cores (11 cm in diameter; 45 cm of soil depth) were sampled by using a soil coring machine (Eijelkamp Agrisearch Equipment, Model Cobra, The Netherlands).

(1) Soil Water Content (SWC)
The soil water content (SWC) was determined by oven-drying the soil sample about 85$^{o}$C in the oven to obtain dry weight (DW). Before that, the fresh weight (FW) of soil sample was taken. The soil water content was calculated by using a formula:

$$[(FW-DW)/FW] \times 100]$$

(2) Soil Field Capacity (SFC)
The soil field capacity was determined by pouring excess water into a container filled with soil so that the soil was supersaturated. The excess water was then drained out through small holes at the bottom of the container. Once the water stop dripping, the saturated soil was weighed (SW) and oven dried at 85$^{o}$C to obtain a constant weight (DW). SFC was calculated by using a formula:

$$[(SW-DW)/SW] \times 100]$$

(3) Soil Saturation level (STL)
Saturation level was the ratio of Soil Water Content (SWC) and Soil Field Capacity (SFC). If the value of SWC is less than SFC, the risk for landslide is low and *vice versa*. This measurement was important to determine the risk of slope failure.

$$[(SWC/SFC)] \times 100]$$

**Results and Discussion**

*Ecological experiment*

Overall, there were 16 plant species found at the study sites. For 12 months of observation, distribution of species showed that *Dicranopteris linearis, Lycopodium sp. and Melastoma malabathricum* were present in all treatments (Table 1). In terms of species richness, treatment A and D recorded the lowest with six species, whilst treatment C was the highest. Species richness of treatment B was nine species. In addition, it has been recorded that the species richness increased from initial observation by 20%, 29%, 25% and 50% increment in treatment A, B, C and D, respectively.

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Table 1. Plant species, species richness (initial and 12 months observation) and species richness increment during 12 months of observation.

| Plot | Plant species (initial) | Number of species (initial - 0 month) | Number of species (12 months) | Increment (%) |
|---|---|---|---|---|
| A | *Dicranopteris linearis* (fern)<br>*Lycopodium* sp. (fern)<br>*Melastoma malabathricum* (shrub)<br>Grass A<br>*Ageratum conyzoides* (weed) | 5 | 6<br>*Lygodium flexuosum* (fern) | 20 |
| B | *Dicranopteris linearis* (fern)<br>*Lycopodium* sp. (fern)<br>*Melastoma malabathricum* (shrub)<br>Grass B<br>*Ageratum conyzoides* (weed)<br>*Acacia mangium* (legumes)<br>*Macaranga* sp. (tree) | 7 | 9<br>*Nephrolepis biserata* (fern)<br>Legume creepers (legumes) | 29 |
| C | *Dicranopteris linearis* (fern)<br>*Lycopodium* sp. (fern)<br>*Stenochlaena palustris* (fern)<br>*Nephrolepis biserata* (fern)<br>*Lygodium flexuosum* (fern)<br>*Melastoma malabathricum* (shrub)<br>*Imperata cylindrica* (grass)<br>*Ageratum conyzoides* (weed) | 8 | 10<br>Legume creepers (legumes)<br>*Asystasia* sp. (weed) | 25 |
| D | *Dicranopteris linearis* (fern)<br>*Lycopodium* sp. (fern)<br>*Melastoma malabathricum* (shrub)<br>*Imperata cylindrica* (grass) | 4 | 6<br>*Nephentes* sp.<br>Species X | 50 |

There was a significant interaction in mean for plant diversity between treatment A, B, C and D, $F(3,16) = 20.89$, $p < .05$ (Table 2). Treatment C was recorded as the most diverse following Shannon-Weiner Index amongst other treatments which was 1.9 followed by 1.7, 1.5, and 1.2 for treatment B, A and D, respectively (Figure 2), indicating higher slope coverage contributed to variety of plant community in the ecosystem. However, treatment D with the highest coverage amongst other treatments had the least diverse in plant diversity. From the observation, treatment D was dominated by fern, *Dicranopteris linearis*. The allelophatic effect (the ability of an organism to produce biochemical that can influence the survival and growth of other species) of this type of fern supress the growth of other plant species. In addition, the mean plant diversity between initial and 12 months of observation was significant, $F(1,16) = 5.22$, $p < .05$ (Table 2). The plant diversity in treatment A, B, C and D has been increased by 5.8%, 8.4%, 14% and 11%, respectively, from the initial observation. This result indicates that the higher the plant diversity, the higher the plant diversity increment.

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Table 2. Two-way ANOVA between treatment and months of observation (0 and 12 months) on plant diversity.

| Tests of Between-Subjects Effects | | | | | | |
|---|---|---|---|---|---|---|
| Dependent Variable: PlantDiversity | | | | | | |
| Source | Type III Sum of Squares | df | Mean Square | F | Sig. | Partial Eta Squared |
| Corrected Model | 1.550[a] | 7 | .221 | 9.827 | .000 | .811 |
| Intercept | 52.333 | 1 | 52.333 | 2322.044 | .000 | .993 |
| Treatment | 1.412 | 3 | .471 | 20.887 | .000 | .797 |
| Month | .118 | 1 | .118 | 5.218 | .036 | .246 |
| Treatment * Month | .021 | 3 | .007 | .303 | .823 | .054 |
| Error | .361 | 16 | .023 | | | |
| Total | 54.244 | 24 | | | | |
| Corrected Total | 1.911 | 23 | | | | |

*a. R Squared = .811 (Adjusted R Squared = .729)*

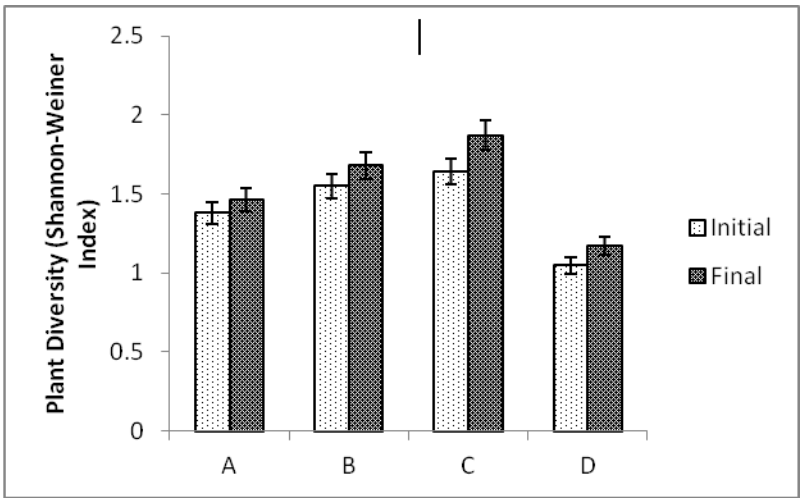


Figure 2. The plant diversity during initial and 12 months of observation.

Figure 3 showed the best three plant species that grow in the treatments based on their importance value index (IVI). *D. linearis* was a dominant plant species that present in all treatments with IVI value 1.20, 0.91, 1.18 and 1.78 in treatment A, B, C and D, respectively. *D. linearis* was a pioneer species which could grow under harsh condition on slope such as lack of water and nutrient availability. Furthermore, as it presents in highway cutslope, *D. linearis* contributed in reducing the risk of soil erosion surface runoff on slope by its rootmat network system (Shono, 2006). The highest IVI value of *D. linearis* in treatment D explained the least diversity at the plot. *D. linearis* commonly forms dense thicket abundantly along the highway, therefore, supress the growth of other plant species, resulting in lower diversity in treatment D.

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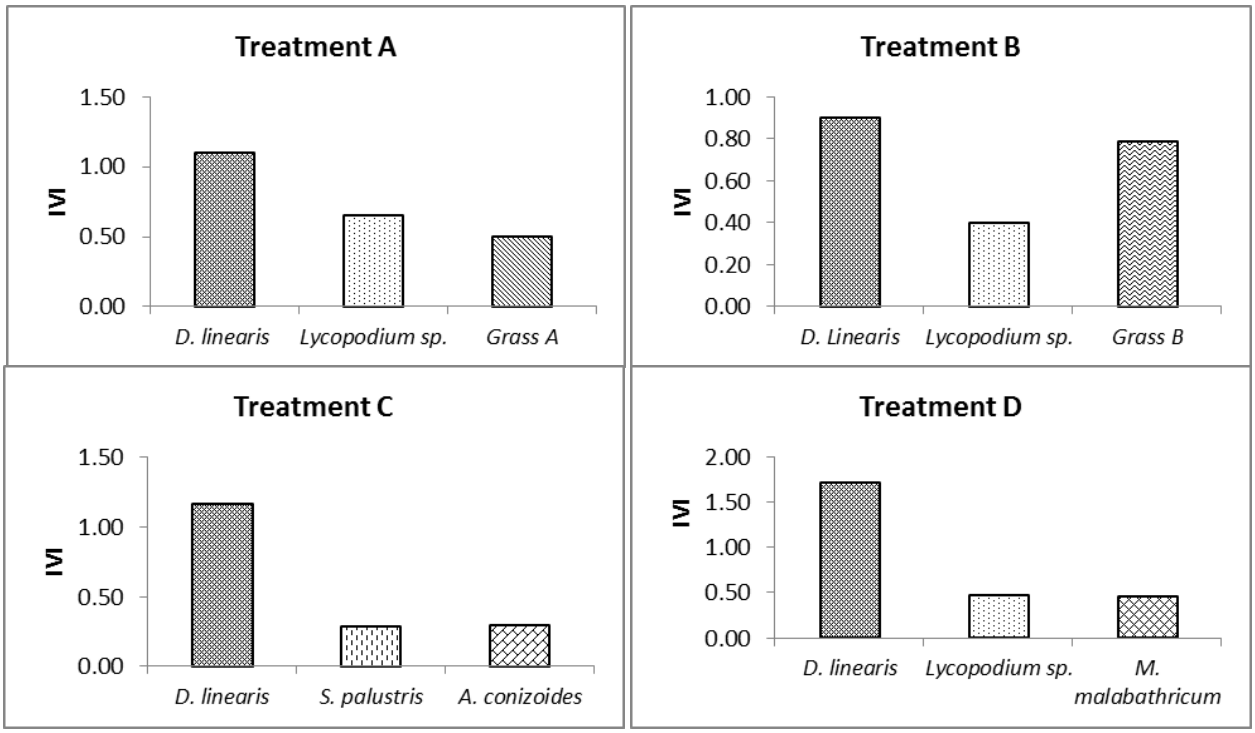


Figure 3. The importance value index (IVI) of the plant species with higher IVI value at treatment A, B, C and D.

*Soil hydrological properties*

Higher in plant diversity would greater the root systems such as root length density (RLD) (Normaniza and Barakbah, 2006) and root distribution, hence the potential of water being absorbed by roots will be higher. The absorbed water transported by the negative pressure gradient to the leaf before transpired out to the atmosphere. This pathway of water from soil to the atmosphere via plants was called soil-plant-atmosphere continuum (SPAC) (Romano et. al., 2012). Throughout 12 months of observation, the soil saturation level (STL) in treatment C showed the lowest value which is 3% (Figure 4). Higher in plant diversity was assisted in reducing the STL of slope. It was observed that the plant diversity in treatment A was much lower compared to treatment B by 18%. The reason behind this was, low diversity in treatment A had caused the evaporation occurred directly from soil, slightly amount of water being absorbed by plant, thus reduced the STL, yet increases the risk of soil erosion. Besides that, the shallow root system of fern in treatment B which has the lower water absorption capacity caused the STL to be higher than treatment A. Even though the STL in treatment D had greatly increased from initial observation, it had been observed that, STL in all treatments were < 50%. Therefore, it indicates that the slope soil in all treatments were at unsaturated level. However, despite of the inconsistent result, interestingly, an inverse relationship between plant diversity and soil saturation level (R = -0.72) approved that higher in plant diversity had reduced the soil saturation level of slope (Figure 5).

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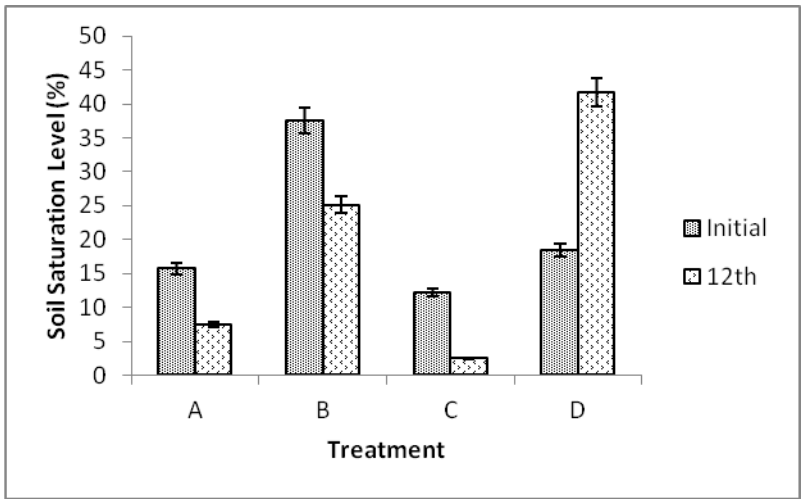


Figure 4. The soil saturation level during initial and 12 months of observation.

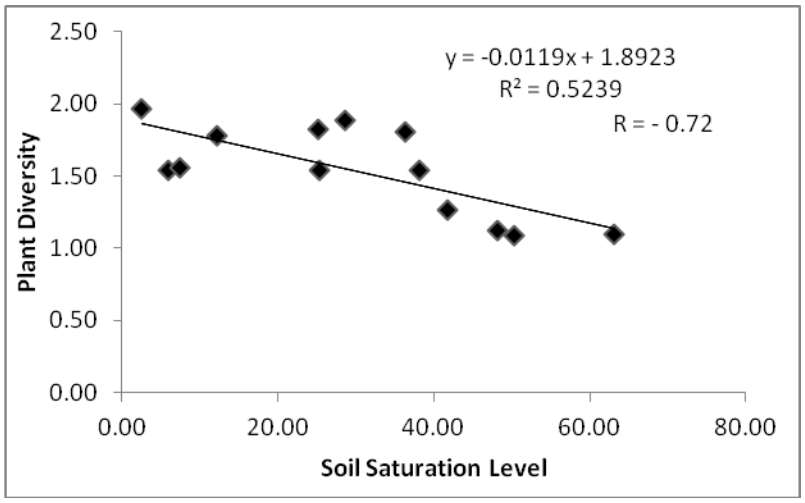


Figure 5. The relationship between plant diversity and soil saturation level.

## Conclusions

In conclusion, treatment C promoted in higher of plant community compared to treatment A, B and D. Even though treatment D was dominated by *D. linearis*, it helps in protecting the slope by its rootmat network system which would reduce the soil erosion and surface runoff. The higher the plant community in the treatment, the lower the soil saturation level, thus enhance the slope stability.

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# Evaluation of Rice Genotypes under Drought and Optimum Environmental Conditions

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## Introduction

Rice production in Indonesia is almost entirely dependent on rice cultivation, and many obstacles occurred such as climate change that causes disruption of water availability. Drought will cause disruption of the metabolic processes of plants such as impaired absorption of nutrients, inhibition of cell division and enlargement, decreased enzyme activity and stomata closure so that the growth and development of plants become constrained (Asmara, 2011). Jumin (2002) reported about the lack of water on the process of photosynthesis as a result of stomata closing. Water has direct and indirect consequences on photosynthesis since crop water deficit will affect all metabolic processes in the plant which resulted in disruption of the growth process (Pugnaire et al., 1999). Drought stress problem can be solved in two ways, either by changing the environment in order to minimize the stress or by improving plant genotypes that are tolerant to drought stress.

Plant breeding is a field of expertise that can be directed to create drought-resistant rice genotypes. Tolerance to drought stress is indicated by its ability to survive and minimize yield lost at low water potential conditions. Drought resistant rice varieties can be obtained by selection of segregated populations derived from crosses involving drought resistant genotypes. This study aimed to understand the phenotypic variability of 10 rice genotypes developed from crosses among two inbred varieties as male parents and five inbred varieties as female parents.

## Materials and Methods

The experiment was conducted from August to November 2012 at IPB Experimental Station Sawah Baru, Bogor. The material consisted of ten genotypes developed from crosses among two inbred varieties, Silugonggo and IPB-3S as male parents (referred to as "tester" thereafter) and five inbred varieties, Mentik Wangi, IR64, Way Apoburu, Jatiluhur and Ciherang as female parents (referred to as "line"). The F1 plants were grown in two irrigation treatments, optimum, where irrigation was applied along the growing season, and sub-optimum, where irrigation was applied until 3 weeks after transplanting and stopped thereafter. A Randomized Complete Block Design with 3 replications was used and the linear model is as follows:

$$Y_{ij} = \mu + \alpha_i + \beta_{k(i)} + \theta_j + (\alpha\theta)_{ij} + \varepsilon_{ijk}$$

where, $Y_{ij}$ is the response variables, $\mu$ is the grand mean, $\alpha_i$ is the effect of environment-i, $\beta_{k(i)}$ is the effect of block-k within environment-i, $\theta_j$ is the effect of genotype-j, $(\alpha\theta)_{ij}$ is the interaction effect of genotype-j and environment-i, and $\varepsilon_{ijk}$ is the intra-block error.

Rice seeds were germinated on petri dish for three days then planted on seedling trays. After three weeks, seeds were transplanted to plots in the screenhouse. Irrigation on a regular basis was applied on the sub-optimum condition for only three weeks after sowing, while sufficient irrigation was applied on the

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optimum environment. Data were collected from a random sample of five plants from each experimental unit and the following characters were measured: plant height, number of tillers, panicle length, flag length, number of filled grains, the number of empty grains, total grain number, grain weight per plant and percentage of filled grains.

**Results and Discussion**

Analysis of variance assumes that the error was distributed normally and had homogenous variance. In our case, this assumption seems to be met for all traits except grain weight, and therefore the log(y+0.5) transformation was used for that trait prior to analysis.

The analysis of variance results showed that the environment factor is highly significant for all traits. The replication within environment factor is also highly significant for all traits except plant height. More importantly, we found that there was very strong evidence that the genotypic (entry) effects were different for all traits except grain weight and percentage of filled grain. The entry factor was explained further by partitioning its sum of squares into three components namely line, tester, and line x tester interaction. The significance of the line effects is in accordance with the genotypic effect whereas the tester effects were only significant for number of tillers, panicle length, number of empty grains and total number of grains (Tables 1 and 2).

The genotype-by-environment interaction was not significant for all traits, except number of filled grains, indicating that genotype ranks did not differ over environments in most cases. The significant entry effects followed by non-significant genotype-by-environment interaction effects, as we found in most of the traits in our study, indicated that there was a genotype that performed well in both environments. Furthermore, the significance of line and tester effect indicated that there were parent genotypes which have superiority to be used in parental crosses for relevant traits.

Performance evaluation of the genotypes showed that the cross combination between Mentik Wangi x IPB-3S had the highest value of plant height, panicle length, flag leaf length, number of total grain and number of empty grains. Jatiluhur x IPB-3S combination had the largest number of filled grains whereas IR64 x Silugonggo had the largest number of tillers. The lowest number of empty grains and the highest grain weight were found in Jatiluhur x Silugonggo genotype (Tables 3 and 4).

Table 1. Summary of the combined analysis of variance for plant height, number of tillers, panicle length, flag length, and grain weight.

| Source | Trait | | | | |
|---|---|---|---|---|---|
| | Plant height (cm) | Number of tillers | Panicle length (cm) | Flag length (cm) | Grain weight (g) |
| Env | 10903.29** | 100.04** | 362.49** | 2901.02** | 41.32** |
| Rep(Env) | 176.89 | 18.06** | 201.09** | 441.65** | 22.46** |
| Entry | 2116.60** | 90.68** | 199.23** | 505.73** | 1.82 |
| Line | 1541.27** | 28.92** | 123.38** | 450.45** | 0.78 |
| Tester | 223.56 | 63.66** | 47.73** | 16.17 | 0.55 |
| Line xTester | 363.32 | 0.13 | 29.90* | 45.70 | 0.62 |
| Entry x Env | 601.06 | 7.80 | 26.51 | 86.63 | 1.14 |

*Note: *=significant at α=5%, ** = significant at α=1%*

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Table 2. Summary of the analysis of variance for number of grains and percentage of filled grains.

| Source | Trait | | | |
|---|---|---|---|---|
| | Number of filled grains | Number of empty grains | Total number of grains | Percentage of filled grains (%) |
| Env | 45212.04** | 7431.04** | 89302.18** | 5898.03** |
| Rep(Env) | 14079.66** | 7172.82** | 41027.84** | 9482.61** |
| Entry | 7987.83** | 22609.54** | 38809.24** | 932.33 |
| Line | 6296.15* | 10250.02** | 18427.32** | 550.83 |
| Tester | 169.52 | 10922.59** | 13813.56** | 358.94 |
| Line xTester | 1359.42 | 1408.47 | 4198.12 | 65.46 |
| Entry x Env | 4803.61* | 4257.31 | 8038.52 | 1062.98 |

*Note: *=significant at α=5%, ** = significant at α=1%*

Table 3. Entry means for plant height, number of tillers, panicle length, flag length, and grain weight.

| Entry | Trait | | | | |
|---|---|---|---|---|---|
| | Plant height (cm) | Number of tillers | Panicle length (cm) | Flag length (cm) | Grain weight (g) |
| Mentik Wangi x Silugonggo | 84.50$^{bcd}$ | 7.43$^{abc}$ | 25.04$^{b}$ | 31.27$^{ab}$ | 1.60$^{ab}$ |
| IR64 x Silugonggo | 80.08$^{cd}$ | 8.52$^{a}$ | 20.36$^{e}$ | 23.58$^{d}$ | 1.06$^{c}$ |
| Way Apoburu x Silugonggo | 75.20$^{d}$ | 7.95$^{abc}$ | 21.60$^{de}$ | 23.60$^{d}$ | 1.13$^{bc}$ |
| Jatiluhur x Silugonggo | 91.93$^{ab}$ | 7.04$^{bcd}$ | 23.92$^{bc}$ | 28.56$^{bc}$ | 1.71$^{a}$ |
| Ciherang x Silugonggo | 78.08$^{d}$ | 8.18$^{ab}$ | 21.37$^{de}$ | 23.29$^{d}$ | 1.49$^{abc}$ |
| Mentik Wangi x IPB-3S | 96.47$^{a}$ | 5.10$^{e}$ | 27.78$^{a}$ | 33.40$^{a}$ | 1.43$^{abc}$ |
| IR64 x IPB-3S | 88.97$^{abc}$ | 6.70$^{cd}$ | 25.27$^{b}$ | 28.19$^{bc}$ | 1.48$^{abc}$ |
| Way Apoburu x IPB-3S | 89.50$^{abc}$ | 7.03$^{bcd}$ | 24.01$^{bc}$ | 28.19$^{bc}$ | 1.49$^{abc}$ |
| Jatiluhur x IPB-3S | 92.90$^{ab}$ | 5.13$^{e}$ | 24.28$^{bc}$ | 27.78$^{bc}$ | 1.41$^{abc}$ |
| Ciherang x IPB-3S | 79.80$^{cd}$ | 5.80$^{de}$ | 22.94$^{cd}$ | 24.67$^{cd}$ | 1.17$^{bc}$ |

*Note: Numbers in the same column followed by the same letter are not significantly different for the DMRT test at α=5%*

Table 4. Entry means for number of grains and percentage of filled grains.

| Entry | Trait | | | |
|---|---|---|---|---|
| | Number of filled grains | Number of empty grains | Total number of grains | Percentage of filled grains (%) |
| Mentik Wangi x Silugonggo | 55.21$^{bc}$ | 72.88$^{bc}$ | 128.08$^{bcd}$ | 44.30$^{a}$ |
| IR64 x Silugonggo | 51.48$^{c}$ | 46.14$^{cd}$ | 97.62$^{d}$ | 40.93$^{a}$ |
| Way Apoburu x Silugonggo | 52.85$^{bc}$ | 56.60$^{bcd}$ | 109.45$^{cd}$ | 41.80$^{a}$ |
| Jatiluhur x Silugonggo | 73.00$^{ab}$ | 34.55$^{d}$ | 107.55$^{d}$ | 46.73$^{a}$ |
| Ciherang x Silugonggo | 47.33$^{c}$ | 45.17$^{cd}$ | 92.50$^{d}$ | 41.05$^{a}$ |
| Mentik Wangi x IPB-3S | 77.08$^{a}$ | 108.54$^{a}$ | 185.62$^{a}$ | 37.15$^{a}$ |
| IR64 x IPB-3S | 62.87$^{abc}$ | 83.23$^{b}$ | 146.10$^{bc}$ | 37.00$^{a}$ |
| Way Apoburu x IPB-3S | 61.53$^{abc}$ | 66.98$^{bc}$ | 128.51$^{bcd}$ | 44.82$^{a}$ |
| Jatiluhur x IPB-3S | 81.68$^{a}$ | 76.92$^{b}$ | 158.59$^{ab}$ | 45.27$^{a}$ |
| Ciherang x IPB-3S | 48.87$^{c}$ | 63.94$^{bc}$ | 112.81$^{cd}$ | 38.81$^{a}$ |

*Note: Numbers in the same column followed by the same letter are not significantly different for the DMRT test at α=5%*

The experiment results from Supriyanto (2013) showed that drought stress significantly affected plant height at 30, 60 and 90 days after planting because plants need sufficient water for its growth and development. Drought stress also significantly affected the number of tillers, percentage of filled grains and grain weight. Water has an important role in the translocation of nutrients from the roots throughout the plant. Insufficiency of water will result in a decrease in the photosynthetic process that resulted in the inhibition of plant growth and development. Tubur et al. (2012) stated that drought stress can reduce plant

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height, number of leaves, number of tillers, panicle length, harvest index as well as increasing the percentage of empty grains.

Genotype performance of line and tester parents was evaluated from the average value of its F1 plants, which also indicated the general combining ability of the respective parent. Genotype Jatiluhur as a parent line had the highest value for plant height and number of filled grains per plant. Genotype IR64 as a parent line had the highest number of tillers whereas Mentik Wangi had the longest panicle and flag leaf, and also the largest total number of grains (Tables 5 and 6).

Table 5. Line and tester means for plant height, number of tillers, panicle length, flag length, and grain weight.

| Entry | Trait | | | | |
|---|---|---|---|---|---|
| | Plant height (cm) | Number of tillers | Panicle length (cm) | Flag length (cm) | Grain weight (g) |
| Line | | | | | |
| Mentik Wangi | 90.48$^{ab}$ | 6.27$^{bc}$ | 26.41$^{a}$ | 32.33$^{a}$ | 1.52 |
| IR64 | 84.93$^{bc}$ | 7.53$^{a}$ | 23.04$^{bc}$ | 26.09$^{bc}$ | 1.29 |
| Way Apoburu | 83.78$^{bc}$ | 7.40$^{a}$ | 23.05$^{bc}$ | 26.36$^{bc}$ | 1.34 |
| Jatiluhur | 92.46$^{a}$ | 6.00$^{c}$ | 24.12$^{b}$ | 28.14$^{b}$ | 1.55 |
| Ciherang | 78.94$^{c}$ | 6.99$^{ab}$ | 22.16$^{c}$ | 23.98$^{c}$ | 1.33 |
| Tester | | | | | |
| Silugonggo | 82.17 | 7.82$^{a}$ | 22.55$^{b}$ | 26.25 | 1.42 |
| IPB-3S | 89.53 | 5.95$^{b}$ | 24.86$^{a}$ | 28.45 | 1.40 |

*Note: Numbers in the same column followed by the same letter are not significantly different for the DMRT test at α=5%*

Table 6. Line and tester means for number of grains and percentage of filled grains

| Entry | Trait | | | |
|---|---|---|---|---|
| | Number of filled grains | Number of empty grains | Total number of grains | Percentage of filled grains (%) |
| Line | | | | |
| Mentik Wangi | 66.14$^{ab}$ | 90.71$^{a}$ | 156.85$^{a}$ | 40.72 |
| IR64 | 57.69$^{bc}$ | 66.37$^{b}$ | 124.06$^{bc}$ | 38.79 |
| Way Apoburu | 58.06$^{bc}$ | 62.83$^{b}$ | 120.89$^{bc}$ | 43.62 |
| Jatiluhur | 77.73$^{a}$ | 57.66$^{b}$ | 135.39$^{ab}$ | 45.93 |
| Ciherang | 48.10$^{c}$ | 54.56$^{b}$ | 102.66$^{c}$ | 39.93 |
| Tester | | | | |
| Silugonggo | 55.73 | 51.47$^{b}$ | 107.20$^{b}$ | 42.99 |
| IPB-3S | 66.40 | 79.92$^{a}$ | 146.33$^{a}$ | 40.61 |

*Note: Numbers in the same column followed by the same letter are not significantly different for the DMRT test at α=5%*

Genotype performance of parent tester IPB-3S had the highest value for panicle length and total number of grains. Genotype Silugonggo as another tester, had the highest value for number of tillers, grain weight per plant and number of empty grains.

**Conclusions**

Genotypic effect was significant for plant height, number of tillers, panicle length, leaf length and number of grains per panicle. In contrast, genotype-by-environment interactions were not significant for all traits except number of filled grains. IPB-3S, Mentik Wangi and Jatiluhur exhibited better estimated

performance than the others in our experiment, indicating that these genotypes could be used as potential parents in subsequent plant breeding programs.

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# Response of $CO_2$ Enrichment on Phytochemical Screening, Fatty Acid Content and Their Antioxidant Activity on *Labisia pumila* var. *alata*

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## Introduction

Plant secondary metabolites are rich sources of bioactive constituents used in pharmaceutical industry, food additives, flavors, and other industrial materials. These substances are also known to play a major role in the adaptation of plants to their environment (Bourgaud et al., 2001). Recent scientific attention has been directed towards the cancer preventive potential for naturally occurring constituents of antioxidant, vitamins, flavonoids and phenolic compounds that have anticarcinogenic or antioxidant potential. Plants products are regarded as potential chemopreventive agents that act to block, reverse or prevent the development of invasive cancers (Reddy et al., 2007).

Elicitation with $CO_2$ enrichment has been shown to increase plant growth, development, and yield of agricultural crops and this response is a function of $CO_2$ concentration and duration as well as the interaction with other environmental factors (Ibrahim et al., 2010). The prospect of improving photosynthesis in crops through $CO_2$ enrichment has created the interest of plant scientists for many years. Enriching plants with high levels of $CO_2$ has been proven to increase plant growth, morphology, development, and yield of many crops, and this response are the functions of $CO_2$ rate and duration of exposure. Crops under enriched $CO_2$ atmosphere acquire positive features with enhanced plant adaptation and growth. The greatest advantages of $CO_2$ enrichment is in the enhancement of leaf gas exchange capacity, particularly under undesirable climatic conditions (Ibrahim, 2012).

*Labisia pumila* (Myrsinaceae), also locally known as Kacip Fatimah has been used by many generations to induce and facilitate childbirth as well as a post-partum medicine by Malay women (Karimi et al., 2013). The exposure of medicinal plants to high level of $CO_2$ may give a positive response in the form of increased of bioactive compounds and their biological activities such as anti-oxidative properties. This study is carried out to evaluate the phytochemical screening, fatty acid and their antioxidant properties of the leaves of *Labisia pumila* var. *alata* under different levels of $CO_2$ enrichment.

## Materials and Methods

### *Plant material*

Three-months old *L. pumila* var *alata* were planted in soil-less medium containing coco-peat, burnt paddy husk and well composted chicken manure in 5:5:1 (v/v) ratio in 25-cm diameter polyethylene bags. Carbon dioxide enrichment treatment started when the seedlings reached four months of age where plants were exposed to 400 and 1,200 μmol $mol^{-1}$ $CO_2$. Healthy and uniform seedlings in terms of leaf numbers were selected, cleaned, separated, freeze dried and stored for further analysis.

### *Plant extraction*

Samples were extracted using methanol solvent and the extraction techniques used were reflux method (Crozier et al., 1997) with slight modifications.

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*Evaluation of phenolic and flavonoid compounds*

The phenolic and flavonoid compounds of samples were quantitatively measured by reversed-phase high performance liquid chromatography (HPLC) technique based on Crozier et al. (1997). The standards for phenolic compounds were ellagic acid, salicylic acid, gallic acid, catechin, epicatechin, caffeic acid, cinnamic acid, and resorcinol. The standard for flavonoid compounds were naringin, apigenin, rutin, quercetin, and myricetin.

*Fatty acid profiles*

The total fatty acids of the leaves were extracted according to the method of Folch et al. (1975). Extracted fatty acids were transmethylated to the fatty acid methyl esters (FAME) using KOH in methanol and boron trifluoride (BF3). A reference standard (C4-C24 methyl esters; Sigma-Aldrich, Inc., St. Louis, Missouri, USA), was used to determine the correction factors for the individual fatty acid composition. The data were expressed as g/100 g of detected total fatty acids.

*Antioxidant activity*

*DPPH free radical scavenging activity*

The 1,1- diphenylpicryl-2-hydrazyl (DPPH) of the extracts were determined by the method of Gulcin et al. (2004). α-tocopherol and vitamin C were utilized as standard antioxidants.

*Nitric oxide scavenging activity*

The nitric oxide (NO) scavenging activity of each plant extract was determined by the method of Tsai et al. (2007). Vitamin C and α-tocopherol were used as controls.

**Results and Discussion**

HPLC and GC analysis revealed a strong influence of increased $CO_2$ concentration on the modification of phenolic and flavonoid profiles and fatty acid contents. HPLC analyses of phenolics and flavonoids indicated the presence of gallic acid, pyrogallol, epicatecin, diadezin, quercetin, naringin and myricetin in the leaves and most of these compounds have increased significantly by increasing $CO_2$ levels from 400 to 1200 μmol $mol^{-1}$ (Table 1). Beside that the proportion of leaf fatty acids such as lauruic acid, pentadecenoic acid, stearic acid, oleic and linoleic acid were quite consistent by increasing $CO_2$ (Table 2). Furthermore, antioxidant activities of leaf extracts of *L. pumila* var. *alata* tested using DPPH free radicals and nitric oxide scavenging activity showed that the antioxidant activities of the extract increased significantly (P= 0.05) by enhancement of the $CO_2$ ( Table 3). These observations are in agreement with that previously reported by Ghasemzadeh et al., (2011) on two varieties of ginger (*Zingiber officinale*). He indicated that total flavonoids, total phenolics and antioxidant activities increased significantly ($P \leq 0.05$) in all parts of the ginger varieties under elevated $CO_2$ (800 μmol $mol^{-1}$). Lavola and Julkunen (1994) reported that phenolic content increased in leaves and stems of *Betula pendula* grown under 700 μmol $mol^{-1}$ $CO_2$.

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Table 1. Concentration of different phenolic and flavonoid compounds in the *L. pumila* var. *alata* grown under different $CO_2$ concentration.

| Phenolic and flavonoid content (µg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Leaf extract (µmol·mol$^{-1}$) | Gallic acid | Pyrogallol | Epicatecin | Catecin | Naringin | Diadezin | Myricetin | Quercetin |
| 400 | 235.1$^{b}$ | 206.2$^{b}$ | 125.2$^{b}$ | ND | 156.4$^{b}$ | 85$^{b}$ | 182.6$^{b}$ | 135.9$^{b}$ |
| 1200 | 619.9$^{a}$ | 395.5$^{a}$ | 181$^{a}$ | 231.2 | 315.2$^{a}$ | 133.1$^{a}$ | 204.7$^{a}$ | 227.5$^{a}$ |

Table 2. Fatty acid composition (%) of *L. pumila* var. *alata* grown under different $CO_2$ concentration.

| Fatty acids | 400 | 1200 |
|---|---|---|
| C12:0 (Lauruic) | 0.76 | 1.08 |
| C14:0 (Myristic) | 1.29 | 1.25 |
| C15:0 (Pentadecanoic) | 1.31 | 1.29 |
| C15:1 (Pentadecenoic) | 1.62 | 2.40 |
| C16:0 (Palmitic) | 24.11 | 21.71 |
| C16:1 (n-9 Plamitoleic) | 0.54 | 0.88 |
| C17:0 (Margaric) | 0.44 | 0.27 |
| C17:1 (Margaroleic) | 1.26 | 0.61 |
| C18:0 (Stearic) | 14.11 | 16.44 |
| C18:1n-9 (Oleic) | 27.01 | 20.81 |
| C18:2n-6 (Linoleic) | 16.11 | 16.54 |
| C18:3n-3 (gamma-Linolenic) | 11.43 | 16.72 |
| TOTAL SATURATED | 42.02 | 42.03 |
| TOTAL MUFA | 30.43 | 24.71 |
| TOTAL N-6PUFA | 16.11 | 16.54 |
| TOTAL N-3 PUFA | 11.43 | 16.72 |

Table 3. DPPH and NO scavenging activities of leaf extraction of *L. pumila* var. *alata* under different $CO_2$ concentration.

| Antioxidant Activity Power (%) | | |
|---|---|---|
| Extract | DPPH | NO Scavenging |
| 400 (µmol·mol$^{-1}$) | 52.35±0.14$^{e}$ | 48.8 ± 1.54$^{e}$ |
| 1200 (µmol·mol$^{-1}$) | 56.42±1.15$^{d}$ | 51.5 ± 0.79$^{d}$ |
| Vitamin C | 92.55 ± 2.09$^{b}$ | 85.41± 3.74$^{a}$ |
| α-tocopherol | 74.29 ± 1.32$^{c}$ | 74.29± 1.51$^{c}$ |

## Conclusions

The manipulation of $CO_2$ may be an effective method to increase secondary metabolites production and enhanced biological activities such as antioxidant properties. This study revealed that the increase in production of plant secondary metabolites in *L. pumila* var. *alata* was followed by enhancement of the antioxidant activity (DPPH and NO scavenging) under exposure of elevated $CO_2$.

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# Assessment of Biochemical Processes on Different Sizes of Air-Layered *Azadirachta excelsa* (Jack) M. Jacobs

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## Introduction

The tree gives response to the changes of the surrounding natural environment. We concern about the factors that actually controlling the growth of trees especially when their cell structures are totally different with other organisms. "Do the trees ageing?" is the question that always keeps reverberating in ecophysiologist minds since the answer still remains unclear. Many theories and hypotheses have derived to explain this issue.

Hazandy et al. (2009) has discussed vastly on some theories and evidences related to net primary productivity of forest. He stated that most of the productivity was triggered by to the existing factors such as nutrient, maturation, reproduction, maintenance respiration, and hydraulic limitation. However, among those factors, hydraulic limitation seems to be the most likely that has caused it. It proposes that as trees get taller, the hydraulic resistance increases and photosynthetic rate decreases, which eventually lead all trees to face hydraulic limitations to growth (Ryan and Yoder, 1997).

The hydraulic limitation hypothesis is proved in reciprocal grafting experiments between seedlings and older trees by Hazandy and Mencuccini (2009). Their studies focused on separating the size effect rather than age, thus it only concludes that size is the dominant factor in controlling tree growth of broadleaved species. Ambrose et al. (2009) has further suggested that tree size correlates more strongly with the observed foliar modifications than tree age and further concluded that the age factor affecting the photosynthetic characteristics is only significant during the early few years of tree development, while the size factor dominates the characteristics afterwards.

Therefore, in this study, we propagated different sizes of branches from similar age donors in order to understand the tree's structures and functions especially involving water relation in trees. It is related to the investigation of soil-plant-atmosphere continuum which is very important in controlling the overall growth of tree and gas exchange. The objectives of this study are to assess the photosynthetic efficiency and capacity of different sizes of air-layered *Azadirachta excelsa* plants affected by different light intensity (A/Q response curves) and carbon dioxide concentrations (A/Ci response curves), respectively and to examine the changes in chlorophyll fluorescence of these plants. These three measurements are the best indicators to determine the internal biochemical changes when plants are vegetatively propagated at different sizes.

## Materials and Methods

The study was conducted in the nursery of Faculty of Forestry, UPM using two donor trees in Ayer Hitam Forest Reserve and Faculty of Forestry, UPM. Thirty air-layered plants from different branches length (30, 40, 50, and 60 cm) and three levels of donor height (3, 4 and 5 m height above ground level) were transferred in polybags and arranged in a complete randomized design (CRD) prior to three months data

collection. The replicates are labelled as L1H1, L1H3, L2H1 and L2H2, all mixed in treatments exclude the donor trees.

Portable Photosynthesis System (LI-6400XT, LI-COR, USA) is used for photosynthetic capacity measurements (maximum carboxylation rate, $Vc_{max}$ and maximum light saturated rate of electron transport, $J_{max}$) and photosynthetic efficiency measurements (maximum photosynthesis rate, $A_{max}$ and Apparent Quantum Efficiency, Φ), while Handy PEA Chlorophyll Fluorometer (Hansatech Ins.) was used to measure chlorophyll fluorescence in order to calculate minimal fluorescence (Fo), maximal fluorescence (Fm), variable fluorescence (Fv=Fm-Fo), and photochemical efficiency (Fv/Fm).

All data were subjected to the repeated measures one-way analysis of variance (ANOVA) with type III test. The means separation was analysed using Bonferroni test to solve the problem of multiple tests of significance. Sigma Plot 10.0 (Systat Software Inc. 2004) was used to plot typical chlorophyll (*Chl a*) polyphasic fluorescence rise by plotting average values of the sum of treatment in amplitude trend lines on a logarithm scale with linear regression analyses (x-axis labels as fluorescence (BITS) or treatment and y-axis labels as log times (μSEC) or parameter).

**Results and Discussion**

In ANOVA, there was no significant difference found in AQE and $A_{max}$ parameters for level (donor height) and length (plant height), so no post-hoc tests are executed. This problem was arisen due to failure in propagating enough branches using air-layering technique. Since there were no significant differences found for photosynthetic efficiency parameters in both levels and length variables, so no size-related trends were observed.

In Figure 1, L1H1 plants recorded the highest mean values for both $A_{max}$ and AQE in March, which were 8.81 μmol $m^{-2}$ $s^{-1}$ and 0.05 μmol $m^{-2}$ $s^{-1}$, respectively. Meanwhile, the lowest mean value for $A_{max}$ and AQE were 3.89 μmol $m^{-2}$ $s^{-1}$ and 0.03 μmol $m^{-2}$ $s^{-1}$, respectively for L1H1 plants in April. Overall, the results showed no size-related trends for every A/Q parameters in every month under study.

Similarly, there was no significant difference for A/Ci parameters. This measurement was more related to the process of generating carbohydrates by uptake of $CO_2$ rather than efficacy in harvesting light. All photosynthetic capacity parameters did not differ significantly between plants propagated at different level and length. Thus, no size-related trends were observed for these parameters again. Mean values for each parameter of A/Ci were plotted and compared (Figure 2), all showed inconsistent trend which did not significant between each other. L2H1 plants in March obtained the highest mean values for $Vc_{max}$ and $J_{max,}$ which were 21.13 μmol $m^{-2}$ $s^{-1}$ and 168.02 μmol $m^{-2}$ $s^{-1}$, respectively. On the other hand, L2H1 plants in April and L1H1 plants in March scored the highest mean values for TPU and Resp, which are 18.93 μmol $m^{-2}$ $s^{-1}$ and 10.52 μmol $m^{-2}$ $s^{-1}$, respectively. In May, L2H2 plants obtained the lowest mean value for $Vc_{max}$ and TPU, which were 7.63 μmol $m^{-2}$ $s^{-1}$ and 2.75 μmol $m^{-2}$ $s^{-1}$, respectively, while L1H1 plants scored the lowest mean value for $J_{max}$ and Resp, which were 31.39 μmol $m^{-2}$ $s^{-1}$ and 1.53 μmol $m^{-2}$ $s^{-1}$, respectively.

Therefore, both photosynthetic capacity and efficiency attributes of air-layered *A. excelsa* plants at different $CO_2$ concentration and light intensity were not able to explain the size-related effect even though they were propagated at different sizes, so the conclusion is not easy to be related to tree growth.

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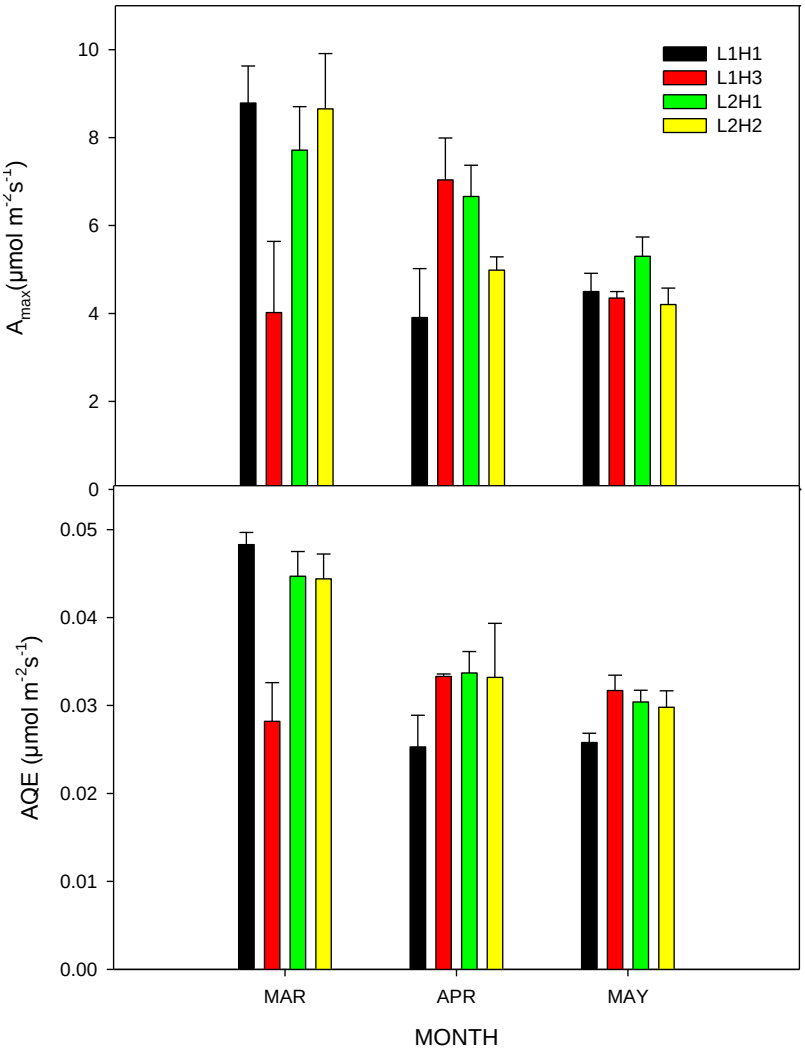


Figure 1. Mean values of $A_{max}$ and AQE for different level and length of branches.

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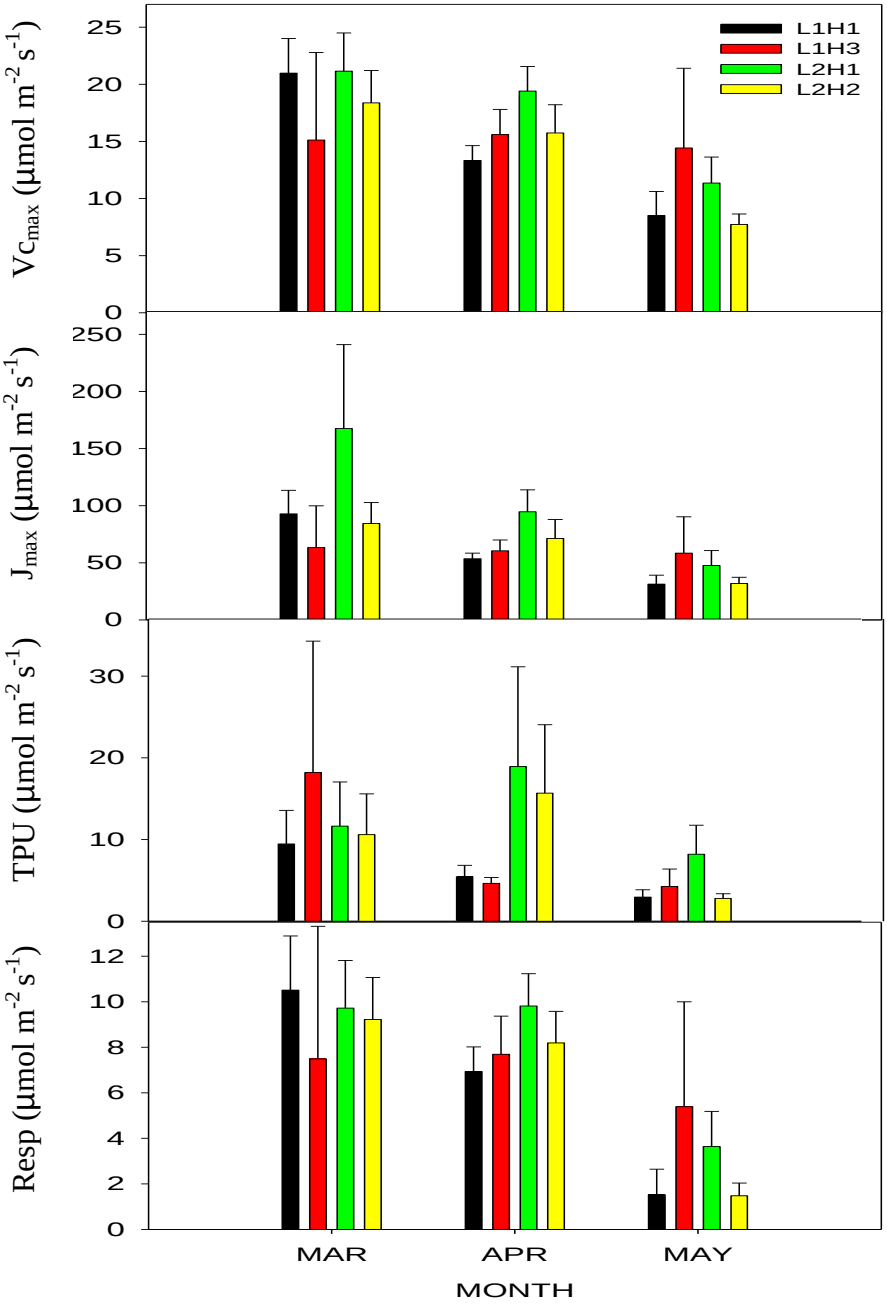


Figure 2. Mean values of $Vc_{max}$, $J_{max,}$ TPU, and Resp for different level and length of branches.

ANOVA also showed that there was no significant difference for chlorophyll fluorescence among plant sizes obtained from different levels of height donor trees. The most interesting parameter in chlorophyll fluorescence attributes is Fv/Fm. This parameter tells us whether a plant or tree growing in a certain condition will have difficulty (stress) or not on their physical and biological environments. The highest peak was shown in L2H1 plants in March, L2H2 plants in April and May, but the amplitude trend showed an ascenders trend with time in sigmoid (Figure 3).

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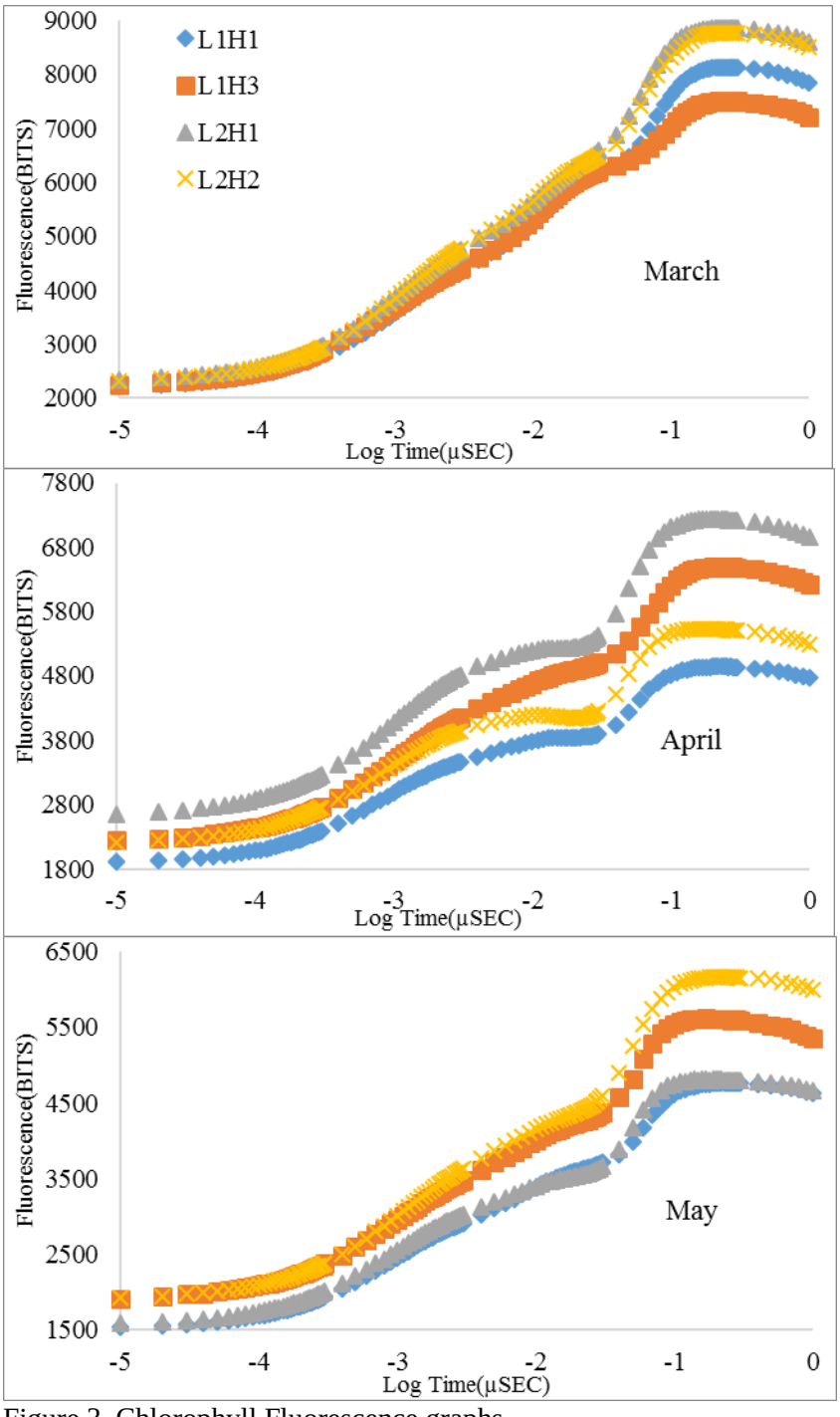


Figure 3. Chlorophyll Fluorescence graphs.

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L2H1 plants scored the highest mean values for Fm, Fv and Fv/Fm parameters in March, which were 8881.14, 6522 and 0.74, respectively (Figure 4). In April, It also scored the highest mean values for Fo parameter, which was 2641.6, but L1H1 plants recorded the lowest mean values for Fv parameter, which was 3042 and L2H2 plants recorded the lowest mean values for Fv/Fm parameter, which was 0.59. In May, L1H1 plants obtained the lowest mean values for Fo and Fm parameters, which were 1541.6 and 4752.71, respectively.

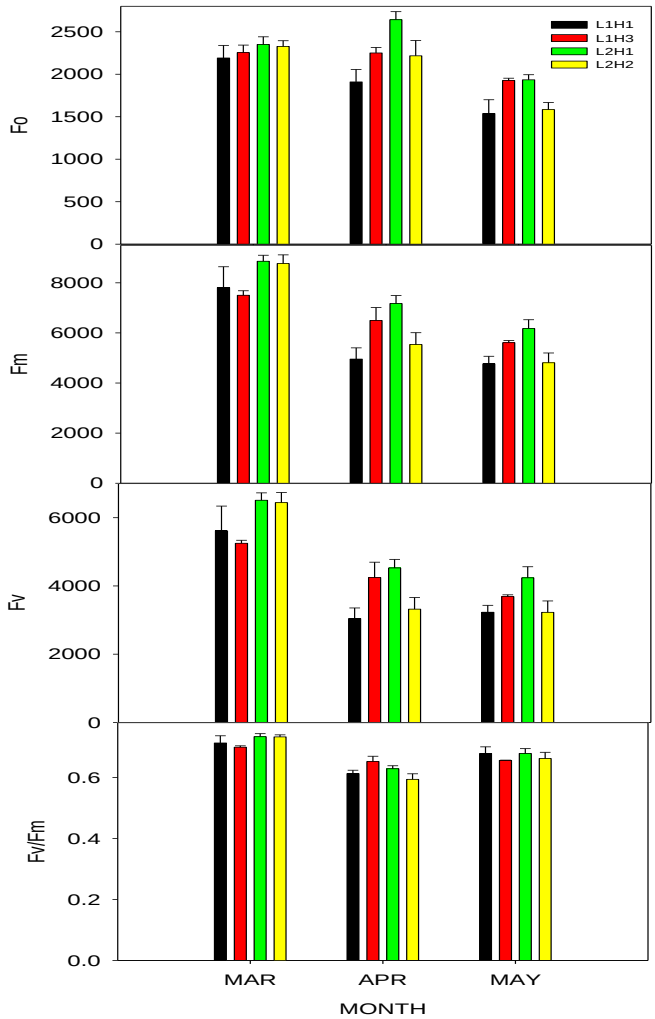


Figure 4. Mean values of Fo, Fm, Fv, and Fv/Fm for different level and length of branches.

At here, the close difference between the branches length does not affect its physiological changes, so chlorophyll fluorescence parameters did not differ significantly between plants propagated at different level and length. The problem might be due to the differences of size were not too significant by just separating the selected lengths at 10 cm apart which eventually does not bring any effects to the difference of the photochemical rates although *A. excelsa* has a high mean annual increment (MAI) of 2.11 in diameter and 2.75 in height (Affendy et. al., 2007). Hadinor (2012) stated that the sizes of length should expand until they are significantly separate apart between one length and others.

## Conclusions

The biochemical processes of air-layered plants of different sizes were observed through measuring the photosynthetic capacity and efficiency. The internal responses of carboxylation and electron transport together with efficiency in harvesting light of every air-layered plant with different sizes did not significantly affected. Overall results showed that the effects of length path of donor trees have been showed through air-layered technique and the selected size differences were found too little to prove that hydraulic constraint affects photosynthesis process as tree increase in height. The air-layering shock was also not found to affect the plants when the mean value of photochemical efficiency was about eighty percent (in a range of 70 to 80% can be considered healthy for tropical plants). Further researches can be carried out by increasing the size of selected lengths, elongating the observation period or comparing the mother tree branches with air-layered plants in a specific time, so that we may observe much to get more comprehensive details on the physiological changes of a stand.

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# Physiological Changes of *Xanthostemon chrysanthus* as Affected by Paclobutrazol and Potassium Nitrate

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## Introduction

Paclobutrazol (PBZ) has been extensively used in controlling excessive growth of plants, improving flower, and increasing drought and salinity tolerance of various plant species. PBZ inhibits gibberellins (GA) biosynthesis in plants, reduces cell elongation and plant growth (Kamountsis and Chronopoulu-Sereli, 1999) but was found highly effective in protecting plants from various environmental stresses that interfere with their normal physiological processes (Fernandez et al., 2006; Sankar et al., 2007).

Urban area is often associated with compacted and infertile soil, lack of nutrients and exposure to heat stress (Lorenz and Lal, 2009; Pickett and Cadenasso 2009), resulting in poor growth quality. Poor growth quality is closely related to soil nutrients. After nitrogen (N) and phosphorus (P), soils are usually most deficient in potassium (K). K plays a significant role in photosynthesis, synthesis of amino acids and proteins, development of lignin and cellulose, resistance against diseases and insect pests and root expansion. Mengel (2007) stated that K has a great regulatory role within plant cells and organs such as activating enzymes, osmosis regulation and photosynthesis, and loading and unloading of sugar in phloem.

Thus, this study was conducted to determine the effects of PBZ and potassium nitrate ($KNO_3$) on the physiological response at three growth stages (flushing, flowering and fruiting) in a landscape tree, *Xanthostemon chrysanthus* (F. Muell.) Benth. This species is native to tropical northern Australia, New Guinea, Indonesia and the Philippines (Sosef et al., 1998) and belongs to the Myrtaceae family.

## Materials and methods

A research plot consisted of 81 trees was established at Metropolitan Batu Park, Kuala Lumpur. These trees aged about six years at the start of the study with the average height and average stem diameter at breast height of 6 m and 10 cm, respectively. The experiment was based on a Completely Randomized Design (CRD) with nine treatments and nine replicates. The treatments were T1(0 $gL^{-1}$ PBZ + 0 g $KNO_3$), T2(0 $gl^{-1}$ PBZ + 100 g $KNO_3$), T3(0 $gl^{-1}$ PBZ + 200 g $KNO_3$), T4(0.125 $gl^{-1}$ PBZ + 0 g $KNO_3$), T5(0.125 $gl^{-1}$ PBZ + 100 g $KNO_3$), T6(0.125 $gl^{-1}$ PBZ + 200 g $KNO_3$), T7(0.25 $gl^{-1}$ PBZ + 0 g $KNO_3$), T8(0.25 $gl^{-1}$ PBZ + 100 g $KNO_3$) and T9(0.25 $gl^{-1}$ PBZ + 200 g $KNO_3$). PBZ was applied as soil drench at an application volume of 1 l per tree, while the control plants were applied with 1 l of tap water. Application of PBZ was carried out one time at the commencement of the study. $KNO_3$ (13.7:0:38.4) was applied at three months intervals.

Randomly, the first three fully expanded leaves from five trees of each treatment were selected for the measurements of photosynthetic rate (A), transpiration rate (E) and stomatal conductance ($g_s$). These measurements were recorded twice, at sixth and twelfth month after treatment by using a portable

photosynthetic system, Li-6400 (LICOR Nebraska, USA). The A and $g_s$ were measured in μmol $m^{-2}s^{-1}$, whereas E was measured in mmol $m^{-2}s^{-1}$. These parameters were recorded with vegetative, flowering and fruiting branches. Data obtained were subjected to ANOVA and the treatment means were then compared using Duncan's Multiple Range Test.

**Results and discussion**

A of *X. chrysanthus* was significantly different between the control and other treatments in all growth stages at sixth and twelfth month after treatment (Table 1). Trees treated with PBZ alone and combination of PBZ and $KNO_3$ gave lower A as compared to the control tree in all growth stages. For vegetative growth stage, the highest A was observed in the control tree, while T7-treated tree had the lowest A at sixth month after treatment. The same pattern was also recorded at twelfth month after treatment. A of the control tree remained the highest whereas T7 resulted in the lowest A in both measurement times. PBZ at this rate consistently resulted in the lowest A even after flowering. These results revealed that A was greatly reduced by PBZ. Greater reduction of A was measured with higher concentration of PBZ. For instance, during flowering, lower A was observed at six months after treatment with T7 (3.04 μmol $m^{-2}s^{-1}$) as compared to T4 (3.57 μmol $m^{-2}s^{-1}$), showing that the concentration of PBZ influenced the physiological capacity of the tree. Similar results of reduced A were also reported in *M. indica* (Shivashankara and Mathai, 2000; Urban et al., 2004). According to Urban et al. (2004), the decrease in A in *M. indica* leaves close to inflorescence was attributed to a decreased in stomatal conductance ($g_s$) and the associated decreased in intercellular partial pressure of $CO_2$. These results suggested that A was also influenced by the growth stages as well as the environmental factors. Gaussoin et al. (1997) also stated that treated plants frequently had a moderate restraining outcome on $CO_2$ exchange rate, thus perhaps minimizing the A.

Meanwhile, E was found highest in all growth stages in the control tree as compared to other treatments (Table 2). Treatment with PBZ alone or in combination of PBZ and $KNO_3$ also reduced the E. In other words, the existence of PBZ affected this physiological performance of the tree. In this study, the cells in the leaf of the treated plants were compactly arranged as compared to the control. As a consequence, the inter-cellular $CO_2$ content in the leaf may be reduced, thus resulting in lower readings of A and E. Significant difference in $g_s$ was only observed between the control and PBZ-treated trees (Table 3). The existence of PBZ consistently reduced $g_s$ in all growth stages. At six month after treatment, T8 and T9 had significantly the lowest $g_s$ as compared to other treatments with fruiting stage. PBZ was presumed to control the stomata aperture which further influences the gas exchange in the leaf. Therefore, PBZ-treated tree had lower rate of biochemical processes as compared to those without PBZ application. The reduction in A would also cause reduction in E as both processes are closely connected with stomata behaviour and LA (Salisbury and Ross, 1992; Abod and Jeng, 1993). The decline in E would subsequently decrease the amount of water lost through stomata. According to Olsen and Andersen (1995), reduction in E would protect the plant against abiotic stress due to water limitation or drought episode.

Plants treated with these triazoles frequently had a moderate restraining effect on $CO_2$ exchange rate, thus reducing A (Gaussoin et al., 1997). In potted *Syzygium myrtifolium*, PBZ reduced A and E but the $g_s$ was not affected (Ahmad Nazarudin et al., 2012). Fletcher et al. (2000) reported that PGRs-treated plants showed reduction in transpiration, used less water and may be able to survive drought better than untreated plants.

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Table 1. Photosynthetic rate in *X. chrysanthus* at 6th and 12th months after treatment.

| Trt | Photosynthetic Rate (μmol $m^{-2}s^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Vegetative | | Flowering | | Fruiting | |
| | 6th month | 12th month | 6th month | 12th month | 6th month | 12th month |
| T1 | 6.56 a | 6.02 a | 5.08 a | 4.67 a | 5.80 a | 5.42 a |
| T2 | 5.28 b | 5.40 b | 4.04 b | 3.99 b | 4.25 bc | 4.25 b |
| T3 | 5.50 b | 5.07 b | 4.07 b | 3.99 b | 4.47 b | 4.43 b |
| T4 | 3.82 cde | 3.53 c | 3.57 cd | 3.37 cd | 3.67 cde | 3.39 c |
| T5 | 4.20 c | 3.58 c | 3.87 bc | 3.41 cd | 3.95 bcd | 3.54 c |
| T6 | 3.92 cd | 3.73 c | 3.63 bcd | 3.52 c | 3.67 cde | 3.54 c |
| T7 | 3.25 e | 3.23 c | 3.04 e | 3.04 d | 3.14 e | 3.2 0c |
| T8 | 3.55 de | 3.31 c | 3.46 cde | 3.24 cd | 3.48 de | 3.29 c |
| T9 | 3.42 de | 3.27 c | 3.26 de | 3.13 cd | 3.37 de | 3.24 c |

*Means followed by the same letter(s) within column do not differ (p<0.05) by DMRT; Trt treatment*

Table 2. Transpiration rate in *X. chrysanthus* at 6th and 12th months after treatment.

| Trt | Transpiration Rate (mmol $m^{-2}s^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Vegetative | | Flowering | | Fruiting | |
| | 6th month | 12th month | 6th month | 12th month | 6th month | 12th month |
| T1 | 3.04 a | 2.72 a | 2.71 a | 2.53 a | 2.89 a | 2.56 a |
| T2 | 2.18 b | 1.90 b | 1.96 b | 1.74 b | 2 .00 b | 1.78 b |
| T3 | 2.01 b | 1.95 b | 1.97 b | 1.82 b | 1.98 b | 1.84 b |
| T4 | 0.78 c | 0.76 c | 0.62 c | 0.65 c | 0.65 c | 0.67 c |
| T5 | 0.84 c | 0.76 c | 0.66 c | 0.64 c | 0.70 c | 0.67 c |
| T6 | 0.77 c | 0.74 c | 0.64 c | 0.64 c | 0.72 c | 0.67 c |
| T7 | 0.76 c | 0.72 c | 0.61 c | 0.63 c | 0.63 c | 0.66 c |
| T8 | 0.76 c | 0.74 c | 0.62 c | 0.61 c | 0.70 c | 0.63 c |
| T9 | 0.75 c | 0.74 c | 0.61 c | 0.60 c | 0.65 c | 0.69 c |

*Means followed by the same letter(s) within column do not differ (p<0.05) by DMRT; Trt treatment*

Table 3. Stomatal conductance in *X. chrysanthus* at 6th and 12th months after treatment.

| Trt | Stomatal Conductance (μmol $m^{-2}s^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Vegetative | | Flowering | | Fruiting | |
| | 6th month | 12th month | 6th month | 12th month | 6th month | 12th month |
| T1 | 0.14 a | 0.14 a | 0.10 a | 0.08 a | 0.11 a | 0.11 a |
| T2 | 0.11 a | 0.12 a | 0.09 a | 0.09 a | 0.10 a | 0.10 a |
| T3 | 0.11 a | 0.11 a | 0.10 a | 0.09 a | 0.10 a | 0.10 a |
| T4 | 0.05 b | 0.05 b | 0.04 b | 0.04 b | 0.04 b | 0.05 b |
| T5 | 0.05 b | 0.03 b | 0.04 b | 0.03 b | 0.04 b | 0.04 b |
| T6 | 0.04 b | 0.03 b | 0.03 b | 0.03 b | 0.04 b | 0.04 b |
| T7 | 0.05 b | 0.04 b | 0.04 b | 0.03 b | 0.04 b | 0.03 b |
| T8 | 0.02 b | 0.03 b | 0.02 b | 0.03 b | 0.02 c | 0.02 b |
| T9 | 0.02 b | 0.03 b | 0.02 b | 0.02 b | 0.02 c | 0.03 b |

*Means followed by the same letter(s) within column do not differ (p<0.05) by DMRT; Trt treatment*

## Conclusions

A, E and $g_s$ were significantly reduced with the existence of PBZ. PBZ may be helpful in controlling water lost from the leaf as it minimizes the stomatal opening. This phenomenon may be useful for *X. chrysanthus* grown in a harsh urban environment.

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# Determining the Influence of Plant Architecture on Light Interception of Virtual Rice Plants on the Simulation Platform GroIMP

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## Introduction

Simulation models make activities of scientific experimental analysis simple and low risk. By analyzing, designing, constructing and parameterizing, the real condition can be precisely simplified and technically shifted into a virtual environment; and it can reduce time, cost, and risk in doing a large number of experiments. One promising approach for simulation in plant sciences is represented by functional-structural plant models (FSPMs). Indeed, FSPMs or virtual plants constitute a new generation of models that can be used to assess plant performance in a virtual world by processing parameters of 3-D structure and development. FSPMs also explicitly reflect the structural aspects of plant architecture, internal functional aspects, and environmental impacts (Renton, 2013). They enable an upscaling from the organ to the individual and plant stand level. One of the pioneering groups was that of de Reffye et al. (1988). In one of their studies (de Reffye et al. 1995), they combined qualitative botanical analysis and quantitative statistical analysis to create a simulation model for 3-D tree architecture with agroforestry applications, with using data that were categorized into growth processes, branching processes, and geometric aspects. Perttunen et al. (1996) designed the model LIGNUM that describes the 3-D structure of a tree crown (segments, branching points, and buds) and defines growth in terms of the carbon metabolism taking place in units which correspond to the organs of the tree. Lindenmayer and Prusinkiewicz used L-systems, i.e., parallel string rewriting systems, for representing the architectural development of plants (Prusinkiewicz and Lindenmayer 1990). This approach from formal language theory was used to simulate ontogenesis in dependence on the light regime in crop plants like maize (Fournier and Andrieu 1999, Pommel et al. 2001) and wheat (Fournier et al. 2003). Cieslak et al. (2008) used an L-system based model of kiwifruit to compare a quasi-Monte Carlo radiation model with a radiosity-based model.

In the field of forest ecology, Umeki et al. (2010) developed an FSPM to calculate annual photosynthetic gains in beech saplings (*Fagus crenata*). The calculation of annual photosynthetic gains was used to determine the influence of foliar phenology and shoot inclination on carbon economy. In a similar spirit, Kennedy (2010) used FSPMs as virtual laboratories to evaluate the effect of alternative local plant architectures on the performance of the whole canopy. He obtained results about the functional significance of foliage parameters for understanding the architectural development of the plant and its foliage. Likewise, Han et al. (2012) used FSPMs for investigating the influence of geometrical traits (internode length, leaf area, branching angle and shoot top diameter) on light interception efficiency of apple trees.

The model GreenLab (Yan et al. 2004), based on the early structural plant model Amap (de Reffye et al. 1988) and its physiologically-enhanced version AmapHydro (de Reffye et al. 1999), calculates the dynamics of organ numbers and organ biomasses of individual plants. It uses a two-scale automaton for morphogenesis and the assumption of constant water-use efficiency to link water conduction and carbon acquisition. Hua and Kang (2013) employed GreenLab to find a shape of a (simplified) tree which maximizes its light interception. Song et al. (2007) used the model GreenLab to assist plant phenotyping on two rice cultivars: Seedlings of the wild type Nippon Bare and one of its T-DNA organogenesis

deficient mutants named Phyllo were investigated to analyze the variability in carbon sinks and sources among genotypes, taking phyllochron and tillering rate into account.

As the rice plant (*Oryza sativa L.*) is a popular staple food in several countries, it is a strong reason to study it intensively. By using an FSPM, we simulated the rice plant under skylight and calculated the total amount of intercepted light at the end of its vegetative growth. In this preliminary study, we used a limited number of combinations of morphological parameters. In a future study, we will use more parameters. It is our objective to use such virtual experiments to identify parameter combinations which are optimal in terms of light interception. In the future, this could guide the breeding of new rice cultivars for improved resource exploitation. Zheng et al. (2008) did also employ a spatial light model to compare the architecture of different cultivars of hybrid rice. However, they did not use virtually-grown plants, but digitized real plants, thus limiting the scope of potential improvements.

**Materials and Methods**

Several previous studies were conceptually adopted to develop our model. We used the model of 3-D virtual rice plants developed by Watanabe et al. (2005) to specify rice plant architecture and to obtain appropriate functions for presenting all growth stages of rice plants, especially for setting the angle between the main stem and tillers, and in modeling the internode length. We also used study results from Gao et al. (1992) for modeling the dynamics of rice development day by day. We used Zhu et al. (2009) to model leaf morphology of rice, especially to determine leaf length, leaf width, leaf number, and to describe the lengths and number of tillers. Furthermore, for the virtual radiation regime of our simulated plants, we employed the Monte-Carlo pathtracer which is implemented as part of the software GroIMP (Kniemeyer et al. 2014) and its configurable sky model which was designed by Hemmerling and Buck-Sorlin (unpubl.) based on concepts from Goudriaan and van Laar (1994).

*Model formalism and language*

Our simulation model is implemented on the software GroIMP, an open-source 3-D modelling platform (Kniemeyer et al. 2014) which uses a graph-based data structure to represent objects and scenes, and which provides a compiler for the programming language XL (eXtended L-system language; Kniemeyer 2008, Kniemeyer and Kurth 2007). XL is an extension of the general-purpose language Java and supports the specification of rule-based developmental models according to the formalism of parallel graph grammars, the latter being a generalization of L-systems from string to graph rewriting. The vertices of the graphs stand for plant organs (leaves, internodes) and for geometrical transformations, the edges stand for relationships among organs (successor, branching, part-of). Among the vertex classes (organ types), a hierarchy with inheritance of properties can be defined, like in Java and other object-oriented languages. XL and the platform GroIMP have already been used for several FSPMs of crop and ornamental plants, including barley (Buck-Sorlin et al. 2005), rose (Buck-Sorlin et al. 2011), cucumber (Chen et al. 2014) and rice (Xu et al. 2011).

*Model structure*

Our model is composed of two submodels, describing the rice plant and the radiation regime (Figure 1). The submodel for the rice plant simulates the 3-D morphology and development of an individual plant and is based on 4 vertex types (classes), named RiceCultivar, Leaf, Stem and InternodeCylinder. In the present study, we restrict ourselves to the vegetative growth stages, but the model could easily be extended to include an inflorescence class as well. The submodel for the skylight is based on 3 classes; SkyLight, Sun and Sky. It describes the movement of the sun and the intensities of direct and diffuse light from different directions, depending on time and date. We parameterized it with a geographical position

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at the equator. GroIMP provides a built-in spectral Monte-Carlo pathtracer (Hemmerling et al. 2008) which sends random rays from the light source (sky) into the virtual scene and allows to estimate the received radiative power for each single object, e.g. for each leaf.

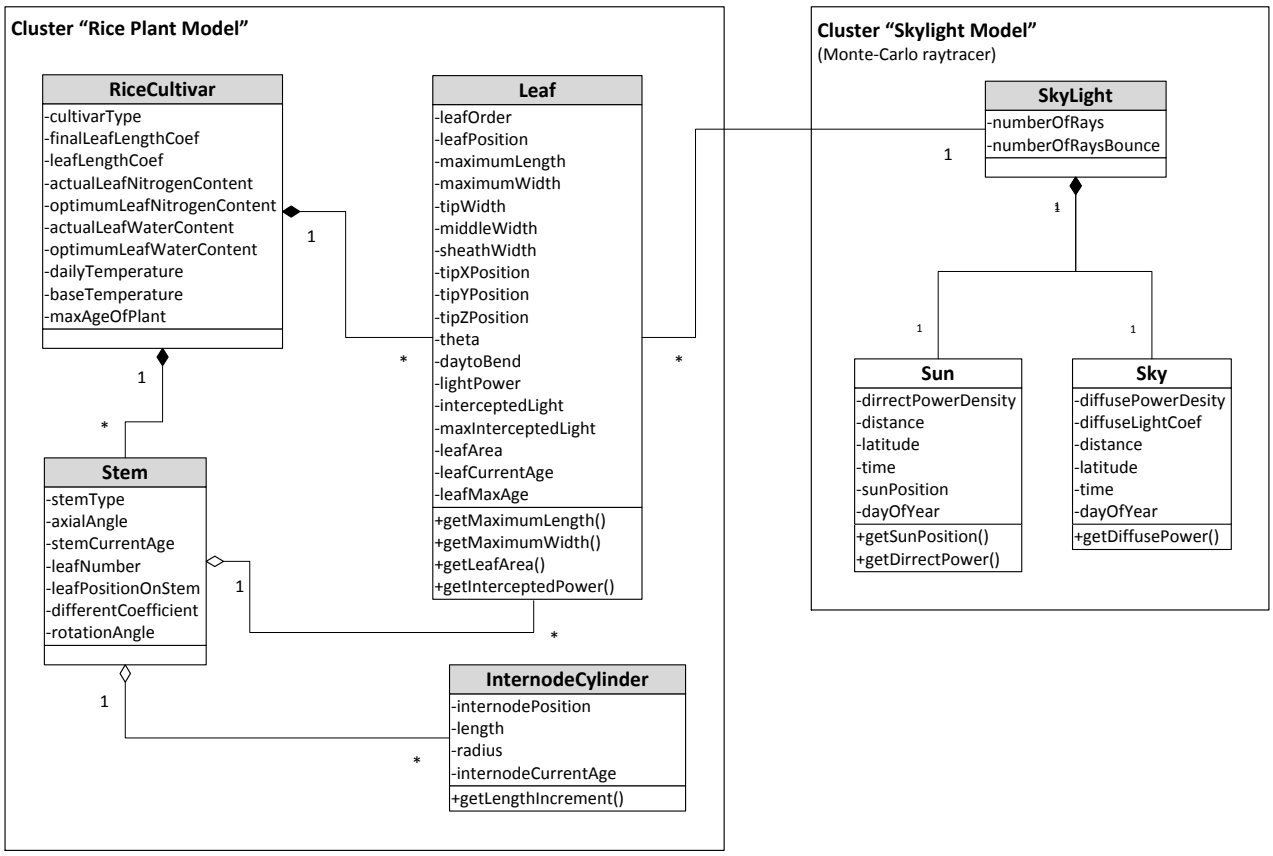

Figure 1. Class diagram of the simulation model.

*Rice plant submodel*

For constructing the simulation model of rice plant growth and architecture, we used several previous studies. For calculating internode lengths, we adopted and simplified an approach from Watanabe et al. (2005), assuming a nonlinear increase of final internode length with the rank of the internode at the axis.

For morphologically modeling the rice plant leaf, we used the model of Zhu et al. (2009), especially for calculating maximum leaf length and width. Many variables are included in the calculation such as the number of growing degree days since leaf emergence (*GDD* ($^o$Cd)), initial number of *GDD* when leaf *n* begins to grow ($IGDD_n$ ($^o$Cd)), number of *GDD* required for completing normal growth of leaf *n* ($\Delta GDD_n$ ($^o$Cd)), factors accounting for nitrogen and water effects (*FN* and *FW*), average daily temperature ($T_i$ ($^o$C)), base temperature ($T_b$ ($^o$C)), leaf age (*LA*), final leaf number (*LN* = 18) and number of elongated internodes on the main stem (*N*). The factors *FN* and *FW* were assumed to be 1 in our simulations, i.e., we assumed non-limiting nitrogen and water supply and a constant temperature of 25.9 $^o$C.

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The rice leaf that is morphologically modeled is illustrated (Figure 2). Here, the virtual leaf is divided into two parts; they are determined by startingPoint, middlePoint and tipPoint. The maximum leaf length $LL_n$ (Zhu et al. 2009) was used to model the fully grown leaf length from startingPoint to tipPoint. The maximum leaf width $LW_n$ (Zhu et al. 2009) was used to model leftCurveOnY and rightCurveOnY.

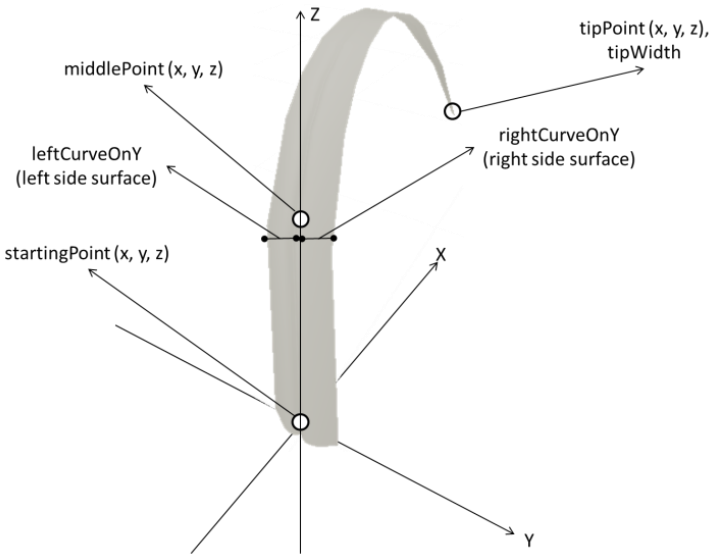


Figure 2. Schematic view of leaf model.

*Skylight model*

Our skylight model utilizes a Monte-Carlo pathtracer which is implemented as part of the software GroIMP (Kniemeyer et al. 2014) and its configurable sky model which was successfully designed by Hemmerling and Buck-Sorlin (unpubl.) based on concepts from Goudriaan and van Laar (1994). We employed the model for the virtual radiation regime of our simulated plants. It can simulate the movement of sun position and the intensities of direct and diffuse light from different directions, depending on time and date. We parameterized it with a geographical position at the equator. To construct the virtual sun that can produce direct light, we used the GroIMP method "lightnode"; and on the other hand, to construct the virtual sky that can produce diffuse light, we initialized the lightnode method for 72 different positions, approximating a continuous sky. The 72 different lightnode positions for modeling the sky dome are visualized in Figure 3.

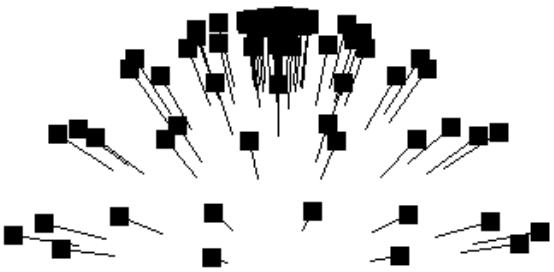

Figure 3. Position of 72 light nodes in our sky model, using GroIMP.

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*Linking light and structure*

To illustrate how light interception of individual leaves is obtained in the simulation program, it can technically be explained by one part of XL code below. We have called the corresponding procedure absorb(). By using several parameters, such as day, hour and leaf order, the procedure can calculate how much light is intercepted by every single leaf. The search pattern 'l:Leaf' in line 3 of the code looks for all leaf objects in the current virtual structure, and assigns to the them the temporary label 'l' used in the following lines. The symbol '::>' indicates an update rule, i.e., a rule which does not modify the topology of the current structure, but only properties of objects – in our case the amount of absorbed light, called 'lightPower', which is technically regarded as a property of each leaf. In this case, the calculation is only done on the main stem, a restriction which is enforced by the condition 'if(l[leafOrder] == 0)'. The leaf order is 0 on the main stem and 1 on tillers. The method call 'lm.getAbsorbedPower3d(l).integrate()' is the command used by our light model to accumulate radiative power over the whole spectrum for leaf l; and 'sum((* l:Leaf, l[leafOrder} == 0 *)[lightPower])' is the command used to sum up all light power that was captured by all leaves on the main stem.

```
protected void absorb (several parameters)
[
        l:Leaf ::>
                if (l[leafOrder] == 0)
                        l[lightPower] = lm.getAbsorbedPower3d(l).integrate();
{
        interceptedLight = sum((* l:Leaf, l[leafOrder] == 0 *)[lightPower]);
        totalInteceptedLight += interceptedLight;
}
]
```

**Results and Discussion**

Two parameters were tentatively chosen to determine the influence of rice plant architecture on light interception. These were the starting day for a leaf to bend (*SDB*) and the inter-segment leaf bending angle (*theta*; see Figure 4). We used 4 values of *SDB* (4, 5, 6 and 7) to modify the day one leaf starts to bend. Indeed, all leaves in our rice plant need 8 days on average to fully grow, except the first leaf that takes 4 days. Here we want to analyze the significance of influence of *SDB* on plant architecture and thereby on accumulated light interception. Regarding *theta* we used 7 values. Then, we can partially determine the influence of plant architecture on light interception by combining these two parameters. In this study, we focused on measuring the accumulation of light intercepted by leaves on the main stem only.

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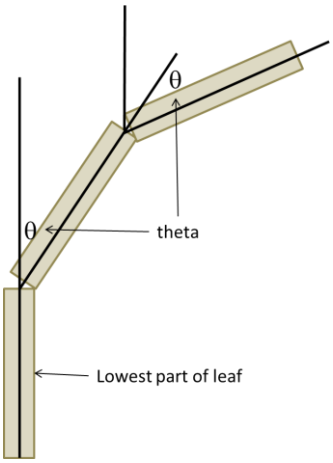


Figure 4. Schematic lateral view of discretized, bent leaf showing the meaning of angle *theta*.

Figure 5 describes one example of data generated from our model. It is a 3-D diagram showing accumulated light interception during the vegetative stage of the modified cultivar Wungxiangjing 14 by combining the two parameters mentioned above. The diagram resulted from two experiments in generating the growth of a virtual rice plant, by rotating the whole virtual plant by 10$^{o}$ for the second experiment. To generate the model, we made several assumptions: 6 types of rice plant cultivar (including 4 artificial cultivars with morphology "between" two existing cultivars, obtained by linear interpolation of parameters); hour by hour sun positions; 4 values of starting day to bend; 7 values of *theta*; 18 tillers, where 12 are primary tillers and 6 are secondary tillers (Watanabe et al. 2005; Zhu et al. 2009); primary tillers have 14 leaves and secondary tillers have 11 leaves (Zhu et al. 2009); the primary tiller axial angle is 32$^{o}$ and the secondary tiller axial angle is 23$^{o}$ (Watanabe et al. 2005); and 65 vegetative stage days. The result from the example above shows that the highest value of accumulated light interception can be reached in combination of *SDB* 4 and *theta* 3.0$^{o}$, we call it the optimum combination. This pattern of the highest accumulated light interception occurred as well on almost all other cultivars (one other existing cultivar and three proposed virtual ones), except the proposed virtual cultivar 3. All results similarly show that the rice plant with optimum combination of *SDB* 4 and *theta* 3.0$^{o}$ experimentally can reach the highest accumulated light interception; except the proposed virtual cultivar 3 which gets the highest value of accumulated light interception for the combination of *SDB* 4 and *theta* 2.8$^{o}$. Figure 6 shows the architecture of the optimal modification of cultivar Wungxiangjing 14 in its vegetative stage.

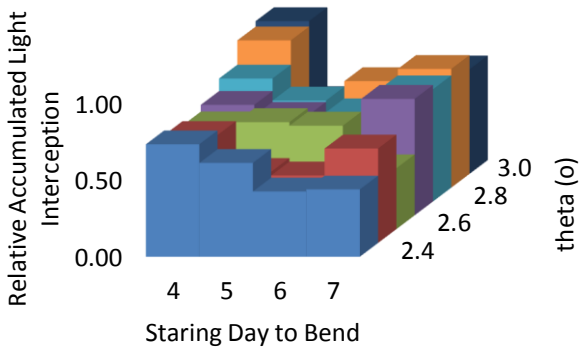


Figure 5. Relative accumulated virtual light interception on cultivar Wungxiangjing 14 with modified architecture.

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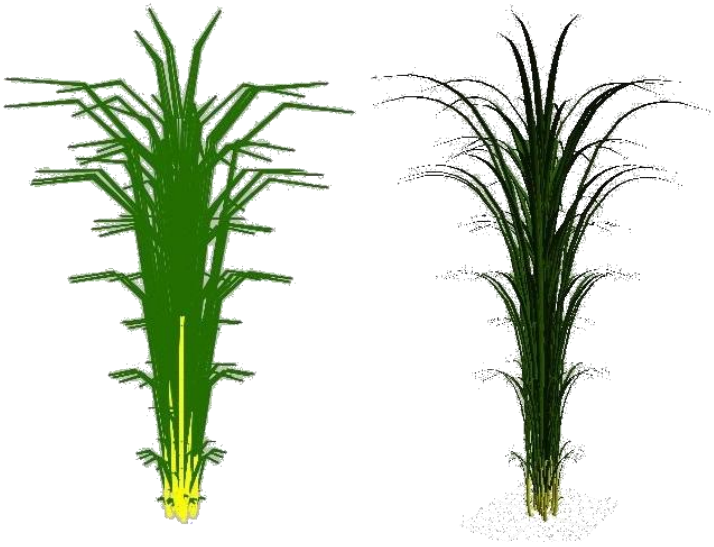

Figure 6. Simplified wireframe (left side) and detailed (right side) view of rice plant 3-D architecture of virtual modified cultivar Wungxiangjing 14. Total height of the plant is 62.33 cm.

Furthermore, figure 7 illustrates the comparison of accumulated light interception based on a fixed value of each cultivar's total leaf area on main stem (*TLA*) for the optimum parameter combination. In this case, we took a *TLA* of approximately 70 $cm^2$. The *TLA* value of 70 $cm^2$ is the highest value of *TLA* that cultivar Wungxiangjing 14 can reach in its vegetative stage. Figure 7 shows that the cultivar Yungdao 6 has the highest value of accumulated light interception for a *TLA* of 70 $cm^2$. Figure 8 shows the temporal development of *TLA* in all 6 virtual cultivars. The accumulated light interception based on *TLA* of each cultivar in their vegetative stage were shown (Figure 9).

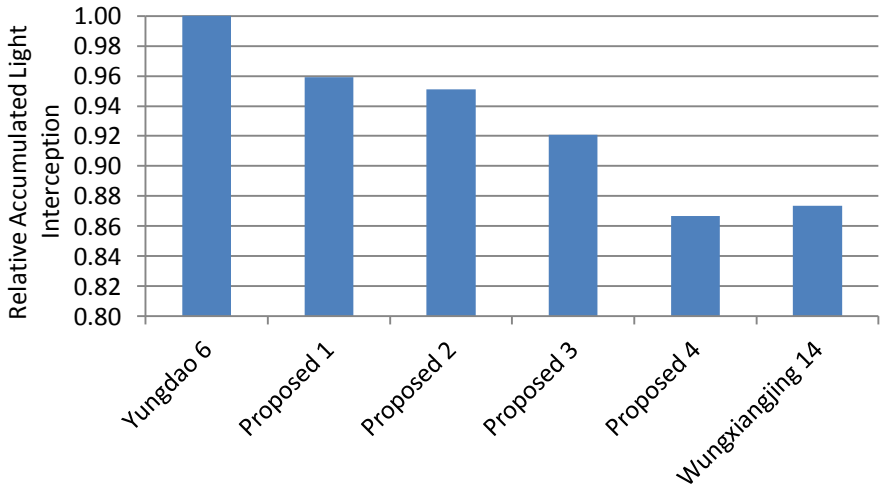


Figure 7. Relative accumulated light interception based on a fixed total leaf area of 70 $cm^2$.

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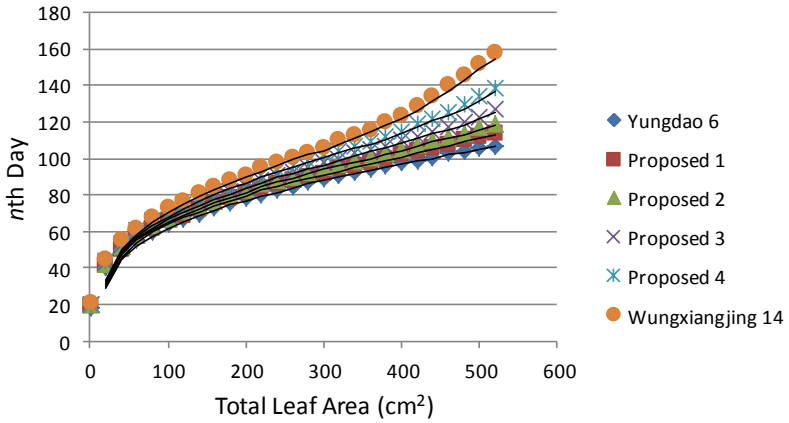


Figure 8. Day-by-day development of total leaf area on main stem in the 6 virtual cultivars.

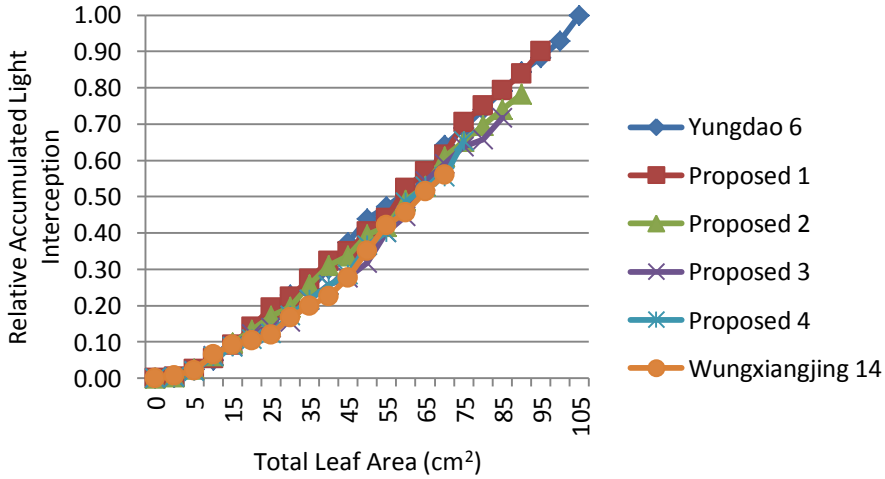


Figure 9. Relative accumulated light interception based on total leaf area in vegetative stage.

## Conclusions

We successfully developed a model that has two submodels: rice plant and skylight model. The rice plant model technically imitates an above-ground real rice plant in its vegetative stage. The skylight model was developed to model the movement of the sun and intensities of diffuse light. From our first results, it can be concluded that the plant architecture and leaf shape influence the quantity of intercepted light, which essentially depends on many parameters, such as leaf-nitrogen content, leaf-water content, daily temperature, maximum plant age, leaf area, starting day for leaves to bend, angle for leaves to bend, number and position of tillers, axial angle of tillers, and other coefficients. Promisingly, future studies can be conducted to study further botanical parameters of the plant by using optimization techniques to propose and recommend a new good performance plant, particularly for rice.

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# Changes of Empty Locul Proportion of Bean Characteristic in Robusta Coffee (*Coffea canephora*) Caused by Drought Stress

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## Introduction

Robusta (*Coffea canephora*) coffee is the second world largest coffee production of the only two important commercial species of 124 members in *Coffea* genus (Davis et al., 2011). Economic value of this commodity has become increasingly important since the contribution to the world coffee production continuously growing up to 42 % in the latest year, compared to Arabica coffee (United States Department of Agriculture, 2014). This species is known sensitive to the drought stress which causing not only loss of production but also can destruct the architectural of plant (Nunes, 1976; da Matta, 2004; Pinheiro et al., 2005). Recently, Brazil as the world biggest coffee producer is suffering due to long dry season that causing loses of 4.2 million of 60 kg-bags compared to the previous year (United States Department of Agriculture, 2014). This stress in the next future is predicted occurred more serious along with the occurrence of global warming.

Composition of bean characteristics in coffee as described by Wormer (1964) is an aspect that also affected by drought stress (Sumirat, 2008). In addition to normal form of bean, there are three forms of abnormal bean which commonly found in Robusta coffee and contributed to the low productivity and low physical quality of coffee bean (Wrigley, 1988, Wintgens, 2004) namely round bean (peaberry or caracole), triage and empty locul. Concerning the empty locul, Sumirat (2008) considered that the origin of the empty locul is actually come from the failure of pollination in one cavity, and also from the failure growth of endosperm. In this study, we would like to report the phenomenon of proportion changes on empty locul caused by drought stress with only discussing the empty locul that originally come from the failure growth of endosperm, including the unusual contrary phenomenon of decreasing proportions of empty locul which possibly play as survival mechanism of coffee tree under drought condition, or even linked to the resistance mechanism.

## Materials and Methods

This study was conducted at Kaliwining experimental garden of Indonesian Coffee and Cocoa Research Institute, East Java, Indonesia. The experimental field is located in the area having climate type C to D (dry climate type) based on Schmidt and Ferguson (1951) classification system on the altitude of 45 m asl. Observation was conducted on 79 progenies of hybrid population of BP 961 x BP 409. The genetic backgrounds of the two parents are Congolese (SG2) group (Crouzillat et al., 2004). BP 409 is a commercial clone having high yield and high tolerance to drought stress, whereas BP 961 is a promising high yielding clone. Each progeny was single tree planted by using 2.5 m x 2.5 m as planting distance under shading tree of Lamtoro (*Leucaena leucocephala*).

Impact of drought stress on the proportion changes of empty locul was studied by comparing the observation on two consecutive harvest years having short two months dry season in the first year and long five months dry season the second year. Observations of proportions of empty locul were done firstly by harvesting 100 random cherries from each progenies, then all type of bean characteristics were

counted to obtain each proportion. Cluster analysis was used to identify the proportion changes of empty locul by using hierarchical clustering method of complete linkage with Euclidean distance.

**Results and Discussion**

*General situation of the site and proportion of empty locul*

Maximum temperature during the longer dry season was 32.6 °C in average, lower than the shorter dry season that was reached 33 °C in average. This condition perhaps due to shorter days light during the longer dry season which only covered 69 % compared to 79 % in average during the shorter dry season. By those conditions, we recorded that numbers of dried branches on this population were 35 % in average during the longer drought season. The coffee flowers were bloomed before the first dry month arrived on shorter dry season, while the flowers in longer dry season were bloomed in the first and second month of dry season.

Observations of proportion of empty locul on two consecutive different length of dry season were found interesting phenomenon of relatively unchanged proportion of empty locul in term of mean, minimum and maximum proportion of population as showed in Figure 1. Minimum proportion was showed decreasing while it is generally known that drought stress is impacted to increase proportion of empty locul as showed in mean and maximum proportion. The unusual phenomenon was also occurred in the histogram that shows decreasing frequency of the member grouped in highest proportion of empty locul, while frequency of the member grouped in lowest proportion was in the contrary. This phenomenon could be the reason for relatively unchanged the mean, minimum and maximum proportion of empty locul in this population. From Figure 1 also, we see that proportion of empty locul could reach more than 30 % which is considering high by Hulupi et al. (1997).

*Changes of empty locul proportion caused by drought stress*

Observations of proportion changes on empty locul due to drought stress were found three groups as showed in Figure 2. According to Figure 2, we then exploring data characteristic from each group to get a better understand of this impact as described in Table 1. Based on Table 1, Group 2 and 3 has the same phenomenon of increasing proportion of empty locul but with different level. In average, Group 3 has an extreme multiplication proportion of empty locul (339.6 %) rather than Group 2 (68.1 %). Although the average proportion of empty locul is seem extremely increasing, the differences proportion of empty locul between short and long drought period were only 12.9 % and 4.2 % in Group 3 and Group 2, respectively. Actually, the most interesting result from this study was the impact of drought stress to 31.6 % of progenies and the parental of BP 961which showed decreasing proportion of empty locul as showed in Group 1. The average proportion of empty locul in this group was multiplied 22.9 % lower after hit by drought condition with the differences only 2.9 % between the short and long dry season. In the contrary, the drought tolerant parental of BP 409 (Nur and Zaenudin, 1992; Erwiyono, 2003) was showed increasing proportion of empty locul, together with Group 2 and 3.

Results of this study showed that most of progenies showing increasing proportions of empty locul when exposed to drought stress as probably inherited from the drought-tolerant-parental BP 409 which showed the same situation. The rest of progenies were showed in the contrary of decreasing proportion of empty locul as probably inherited from the less drought-tolerant-parental BP 961 which also showed the same situation. Increasing proportion of empty locul caused by drought stress is a common phenomenon as like as recently seen in Brazilian coffee. This condition is due to less nutrient uptake and less plant metabolisms (Kumar, 1979) which lead to limited distribution and promote for competition to obtain assimilate products, and finally will be contributed to the increase proportion of empty locul (Sumirat,

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2008; Yahmadi, 1973). However, increasing proportion of empty locul in the high tolerant genotype of BP 409 should be assumed as survival mechanism of coffee plant to drought stress. This assumption was supported by lower changes proportions of empty locul rather than the average value of Group 2 and even for Group 3. These facts suggest that empty locul proportion could be used as selection tool in the breeding to obtain tolerant genotype to drought stress. On the other hand, the unusual phenomenon of decreasing proportion of empty locul was occurred in less drought tolerant genotype of BP 961. Therefore, decreasing proportion of empty locul in Group 1should not be interpreted as a better tolerance to drought stress. We assumed that this situation was due to very low tolerance of the trees to drought stress because actually drought stress will impact to cherries drops, mainly at pinhead stage (Yahmadi, 1973). Longer drought stress will be then impacted to leaves drops and dried branches (Nur and Zaenudin, 1999). High numbers of cherries drops and or dried branches will directly reducing numbers of survival cherries. So when drought condition is finish, the trees will only need to support the growth for less number of cherries and reducing competition on distribution of assimilate products. Due to this condition, beans filling will more supported and finally will reducing proportion of empty locul.

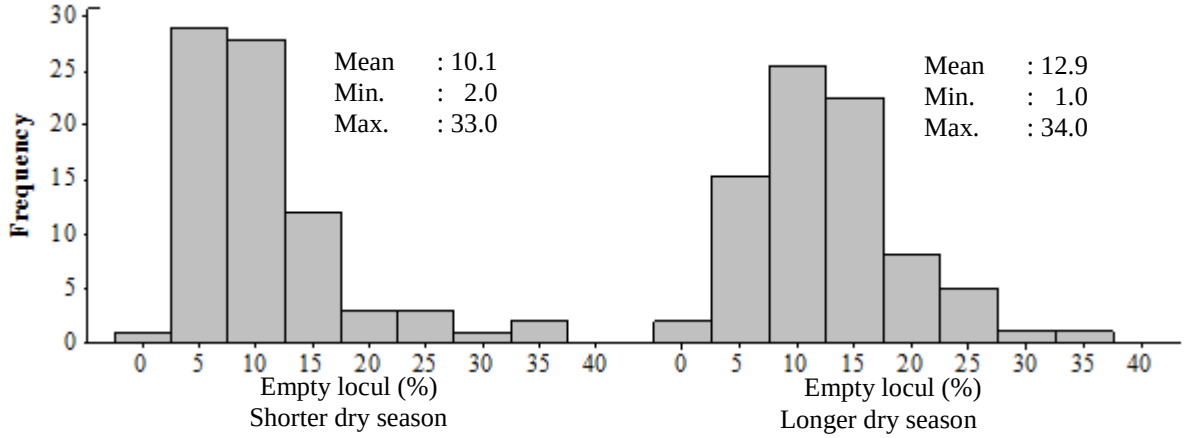


Figure 1. Histogram of proportion of empty locul in shorter and longer dry season.

This result, however, should be verified before it used for selection of drought tolerant coffee, especially to define the tolerance criteria, mainly for selection inside the Group 2 only. One should be considered is how to compromise between the multiplication proportion of empty locul that shows increasing in extreme and the differences proportion between short and long drought period that only reach maximum 20.5 %. For examples, there is a possibility that a genotype shows extreme multiplication proportion of empty locul but with relatively small difference proportion of empty locul between shorter and longer dry season. Referring back to the suggestion of Hulupi et al. (1997) for good commercial variety that contain no more than 10 % for total abnormal bean, proportion of empty locul originally coming from failure growth of endosperm perhaps tolerated to maximum 2 %.

On the other hand, there are also several plant characteristics that have been known linked to the tolerance to drought stress as described by da Matta (2004) and Pinheiro et al. (2005). However, depth of rooting (Pinheiro et al., 2005) is seemed more interesting as plant characteristic for selection in coffee to drought stress especially at seedling phase. No less important, genetic background is also contributed to the level of tolerance to drought stress. From two widest cultivated genetic groups of Robusta coffee in the world, Quillou (SG1) has been known having better tolerance to drought stress rather than Congolese (SG2) (Cramer, 1957).

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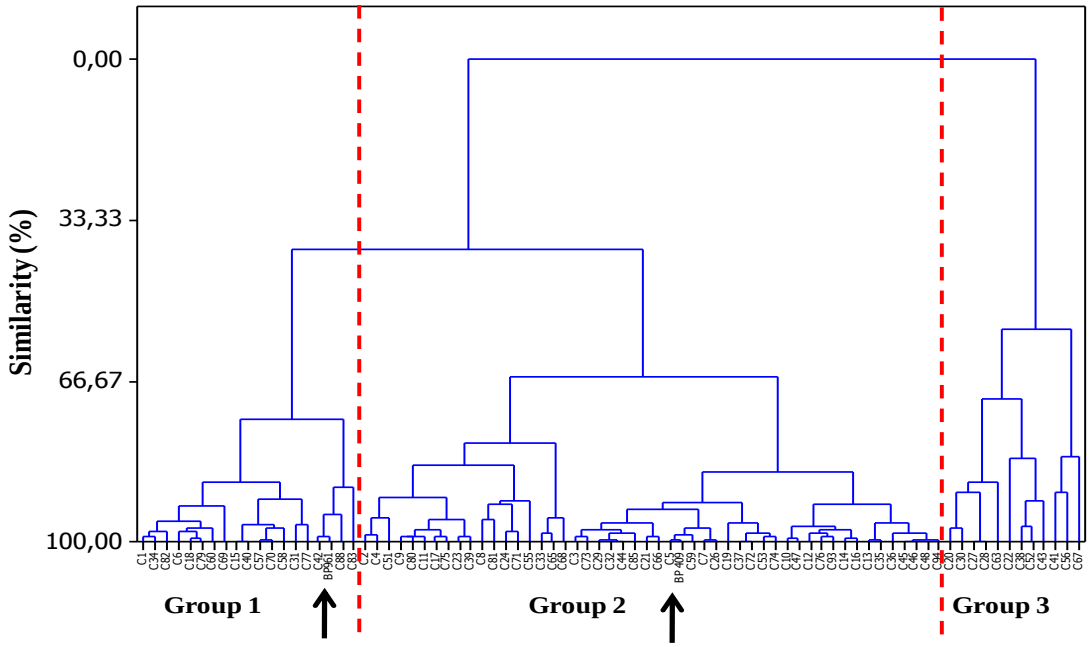


Figure 2. Three groups formed based on changes of empty locul proportion due to drought stress. The arrows show position of the two parental.

Table 1. Description of changes proportion of empty locul from three formed groups

| Group | Members (%) | Differences proportion of empty locul due to drought stress (%) | | | Multiplication proportion of empty locul due to drought stress (%) | | |
|---|---|---|---|---|---|---|---|
| | | Mean | Min. | Max. | Mean | Min. | Max. |
| Group 1 | 31.6 | -2.9 | -10.5 | 0.6 | -22.9 | -77.1 | 4.5 |
| Group 2 | 59.5 | 4.2 | 1.1 | 10.5 | 68.1 | 4.1 | 263.0 |
| Group 3 | 8.9 | 12.9 | 6.0 | 20.5 | 339.6 | 273.3 | 400.0 |
| Parent BP 409 | --- | 3.5 | --- | --- | 38.9 | --- | --- |
| Parent BP 961 | --- | -6.5 | --- | --- | -46.4 | --- | --- |

## Conclusions

According to the results of this research, it could be concluded that:

1. Drought stress is relatively not impacted to the changes proportion of empty locul in term of mean and range value (2-33 % at shorter dry season vs 1-34 % at longer dry season) of studied population.
2. Cluster analysis confirmed three groups correspond to the impact of drought stress to proportion changes of empty locul namely a group that shows increasing proportion in extreme, a group that shows lower increasing proportion, and a group that shows decreasing proportion.
3. Changes proportions of empty locul were shows extreme multiplication, while differences proportion between the shorter and the longer dry season were shows only maximum 20.5 %.
4. The drought tolerant parent was placed in the group that shows lower increasing proportion of empty locul, suggest the increasing proportion of empty locul is a mechanism of resistance to the drought stress.
5. The drought less-tolerant parent was placed in the group that shows decreasing proportion of empty locul, suggest the existence of other survival mechanism under drought stress.

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# Glycosidases Activity in Salinity-Tolerant Rice Grown on Saline Soil under Malaysia Condition

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## Introduction

Rice is well known for carbohydrate source and also one of the major staple food consumed widely by human population all over the world, especially in Asian countries. Demand for the sufficient rice supply increased yearly with increasing human population. However, its production can be influenced by the abiotic stresses, the uncontrollable limitating factors, worldwide. Salinity in the soil is one of the abiotic factors that become the main cause in yield reduction of the crop (Kumar et al., 2010). Salinity lowers the osmotic potential, nutritional disruption and alters various metabolic pathway that lead to morphological and physiological changes (Ashraf and Harris, 2004; Yang et al., 2008). Under salinity stress condition, plants possess various defense strategies, including homeostasis mechanisms of mineral ions (Sairam et al., 2005) and carbohydrate (Gupta et al., 2011). Salinity stress inhibits starch accumulation due to the limited rate of photosynthesis (Danai-Thambale et al., 2011). Under such condition, glycosidase enzyme plays a role in carbohydrate homeostasis by breaking down the polysaccharide into simple sugar which is subsequently utilized as energy in metabolism (Sekhwal et al., 2013). Carbohydrate degradation during growth and development phases may differ between genotypes (Zhen at el., 2012). This paper reports the reducing sugar content and glycosidase enzyme activity in three salinity tolerant rice genotypes grown on saline soil under Malaysian condition.

## Materials and Methods

### *Plant material and treatment*

Three salt-tolerant rice genotypes, namely SS1-40 [IR 66946-3R-178-1-1(FL 478)], SS1-41 [CSR 28] and SS1-42 [IR 55179-3B-11-3] were obtained from International Rice Research Institute (IRRI), Los Banos, and Laguna, Philippines. The experimental design used was Completely Randomized Design and carried out in glass house. Selected seeds were germinated and sown for 15 days in normal soil and subsequently transferred to saline soil for salinity treatment. The soil was taken from Kemasin, Bachok, Kelantan and filled in a planting trough located in the green house at Universiti Malaysia Terengganu. Triplicates were used for each genotype. The salinity level of the soil in the trough was adjusted to EC 8 ds/m with seawater. Plants were observed daily and the water level, pH and salinity were monitored weekly. For enzyme and reducing sugar quantification, one healthy leaf from each genotype was harvested during the vegetative (45 days after seeding), reproductive (70 days after seeding) and ripening (90 days after seeding) stages. The required morphological traits were recorded accordingly.

### *Reducing sugar content*

The total reducing sugar content was measured according to Nelson (1944) and Somogyi (1952). Each sample was ground in ice-cold distilled water at ratio 1:1 (w/v) and centrifuged at $2{,}000_X$ g for 15 min.

Then, 1.0 ml supernatant was added with 2.0 ml sodium phosphate buffer (pH 6.4). Subsequently, 0.5 ml solution was mixed with 0.5 ml distilled water and 1.0 ml Nelson reagent. The mixture was incubated in a water bath at 100 °C for 20 min. The solution was cooled to room temperature, added with 1.0 ml Arsenomolybdate reagent and made up the volume to 10 ml with distilled water. Absorbance was read at 510 nm using spectrophotometer. The reducing sugar content was calculated based on glucose standard curve.

*Glycosidase assay*

At harvest, one gram of fresh leaf was homogenated using a mortar and pestle at 4 °C with 5.0 ml extraction buffer; McIlvaine buffer (pH 5) for β-glucosidase, McIlvainne buffer (pH 3.5) for β-galactosidase and acetate buffer (pH 4.7) for amylase and invertase. Each homogenate was centrifuged at $10{,}000_X$ g for 20 min at 4 °C. The supernatant was discovered and used for the assay or stored at -20 °C. Amylase (EC 3.2.1.1) activity was measured according to Niku-Paavola et al. (1972). One ml of 1% (w/v) starch dissolved in 100 mM acetate buffer (pH 4.7) was added to 250 µl of crude enzyme. The mixture was incubated at 27 °C for 15 min. Subsequently, 2.0 ml dinitrosalicyclic acid (DNS) reagent was added and boiled in water bath for 5 min. The volume was adjusted to 10 ml with 1 ml potassium sodium tartrate and distilled water. Absorbance was measured at 560 nm using spectrophotometer. The absorbance readings were compared against maltose standard.

Invertase (EC 3.2.1.26) activity was measured according to Perumalla et al. (1994). One ml of 2% (w/v) starch dissolved in 20 mM acetate buffer (pH 4.7) was added to 250 µl crude enzyme. The solution was incubated at 27 °C for 30min. Then, 1.0 ml Nelson-Somogyi's solution was added and boiled for 5 min. The mixture was then cooled and 0.5 ml Nelson's Arsenomolybdate solution was added followed by addition of 4 ml distilled water. It was mixed well and absorbance was measured at 650 nm using spectrophotometer and compared against glucose standard.

β-glucosidase and β-galactosidase assay were conducted as the method described by the Alcantara et al. (2006). An amount of 0.2 ml p-nitrophenol glycosidase (pNPG) substrate was added with 10 µl of crude extract in 10 µl McIlvaine buffer of pH 3.2 for β-galactosidase and pH 5 for β-glucosidase. The mixtures were incubated in the water bath at 30 °C for 20 min. The reaction was stopped by the addition of 1.0 ml of 0.2 M $Na_2CO_3$. The amount of p-nitrophenol (pNP) released was measured using spectrophotometer at 420 nm. The specific enzyme activity was quantified by the total activity per total protein. One unit of enzyme released was defined as the amount of enzyme that released 1 µmol pNP per minute from pNP-Gal. Total protein concentration was estimated according to Bradford method (Bradford, 1976), with Bovine Serum Albumin (BSA) as the standard, using spectrophotometer at 595 nm. One unit of enzyme (UE) is defined as the amount of enzyme that released one mol of *p*NP from *p*NPG per ml per min under the assay condition. The specific activity is expressed as UE per mg of protein.

*Statistical analysis*

Data were subjected to normality test and statistically analyzed using multi-variance and the significant differences were identified by post hoc Tukey's honestly significant difference (HSD) test at $p<0.05$ using Statistical Package for Social Sciences (SPSS).

**Results and Discussion**

The salinity stress was found to alter the morphological traits of the three genotypes (Table 1). Salinity stress reduced the length of leaves, panicles and height of plants of SS1-40 and SS1-42, but increased

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these parameters in SS1-41. However, no significant difference was observed on the width of leaves. Salinity stress also affected the leaf angle, type of panicles and leaf senescence, but genotype specific. Interestingly, variety SS1-42 exhibited early and faster leaf senescence than the control plant. Early senescence is a good trait for rice; however, its mechanism remained unclear (Liu et al., 2008).

*Reducing sugar content*

Results showed that reducing sugar content in rice leaf was not significantly different between the control and under salinity stress (Figure 1). The level of reducing sugar in the leaves is dependent on the portioning of sugar and starch accumulation. Rice accumulates starch instead of sugar during salinity stress (Pattanagul and Thitisaksakul, 2008; Danai-Tambhele et al., 2011). In addition, the reducing sugar content was higher during the reproduction stage compared to vegetative and ripening stages.

Table 1. The morphology traits of rice genotypes under control and salinity treatment.

| Morphological traits | SS1-40 | | SS1-41 | | SS1-42 | |
|---|---|---|---|---|---|---|
| | Control | Salinity Treatment | Control | Salinity Treatment | Control | Salinity Treatment |
| Flag-leaf length (cm) | 38.5±0.5 | 25.5±0.5 | 16.3±2.3 | 33.6±1.0 | 35.5±0.5 | 28.6±2.2 |
| Flag-leaf width (cm) | 1.3±0.2 | 1.4±0.2 | 1.1±0.1 | 1.3±0.2 | 1.4±0.2 | 1.3±0.2 |
| Leaf length (cm) | 58.0±2.0 | 25.6±2.2 | 13.5±0.5 | 52.5±0.5 | 37.0±0.8 | 38.9±0.2 |
| Leaf width (cm) | 1.0±0.3 | 0.8±0.1 | 1.0±0.08 | 1.2±0.3 | 1.0±0.3 | 1.0±0.3 |
| Panicle length (cm) | 20.8±1.0 | 17.4±0.6 | 19.3±2.3 | 22.0±2.0 | 18.5±0.5 | 18.1±0.1 |
| Seedling height (cm) | 78.3±2.3 | 65.3±2.3 | 87.8±1.0 | 93.0±3.0 | 84.0±0.8 | 73.2±0.5 |
| Leaf angle | Erect | Erect | Erect | Erect | Erect | Droopy |
| Panicle type | Compact | Compact | Compact | Inter-mediate | Intermediate | Compact |
| Leaf senescence | Slow | Late and slow | Slow | Slow | Intermediate | Early and fast |
| Flag-leaf angle | Erect | droopy | Erect | Erect | Erect | Erect |

*Data are means ± Sd*

*Glycosidase activity*

Amylase activity was influenced by the salinity stress during the vegetative and reproductive stages (Figure 1). Its activity was lower than control plant during the vegetative stage, but higher during the reproductive stage. Under normal condition, both amylase and invertase enzymes were high during the vegetative stage, but gradually reduced during the reproductive to ripening stages. On the other hand, under salinity stress, no significant difference in amylase activity was observed during the vegetative and reproductive stages. However, invertase activity increased during the reproductive stage (Figure 2) and drastically dropped during the ripening stage. This phenomenon suggesting salinity stress triggered the starch and sucrose degrading activity in rice leaves under salinity stress. However, the reducing sugar content in the leaves during these stages was altered by salinity stress. This might be due to active carbohydrate rearrangement and transportation during the reproductive stage.

Glucosidase and galactosidase activities were not significantly different between control and salinity treated plant (Figures 2 and 3). The enzyme activities varied between genotypes and growth stages. Galactosidase activity was highest during the ripening phase (Figure 3). This might be due to its biological functions in fruit ripening, remodeling of carbohydrate for storage (Alcantara et al., 2006) and plant senescence (Tanthanuch et al., 2008). In addition, glycosidase activity is important in hydrolysis of the bond in starch to produce smaller molecules of monosaccharides that are crucial for cereal production (Santosh et al., 1999). Glycoside hydrolases carry out many functions in plants such as starch metabolism, transport, mechanism against biotic and abiotic stress and cell wall remodeling (Sekhwal et al., 2013).

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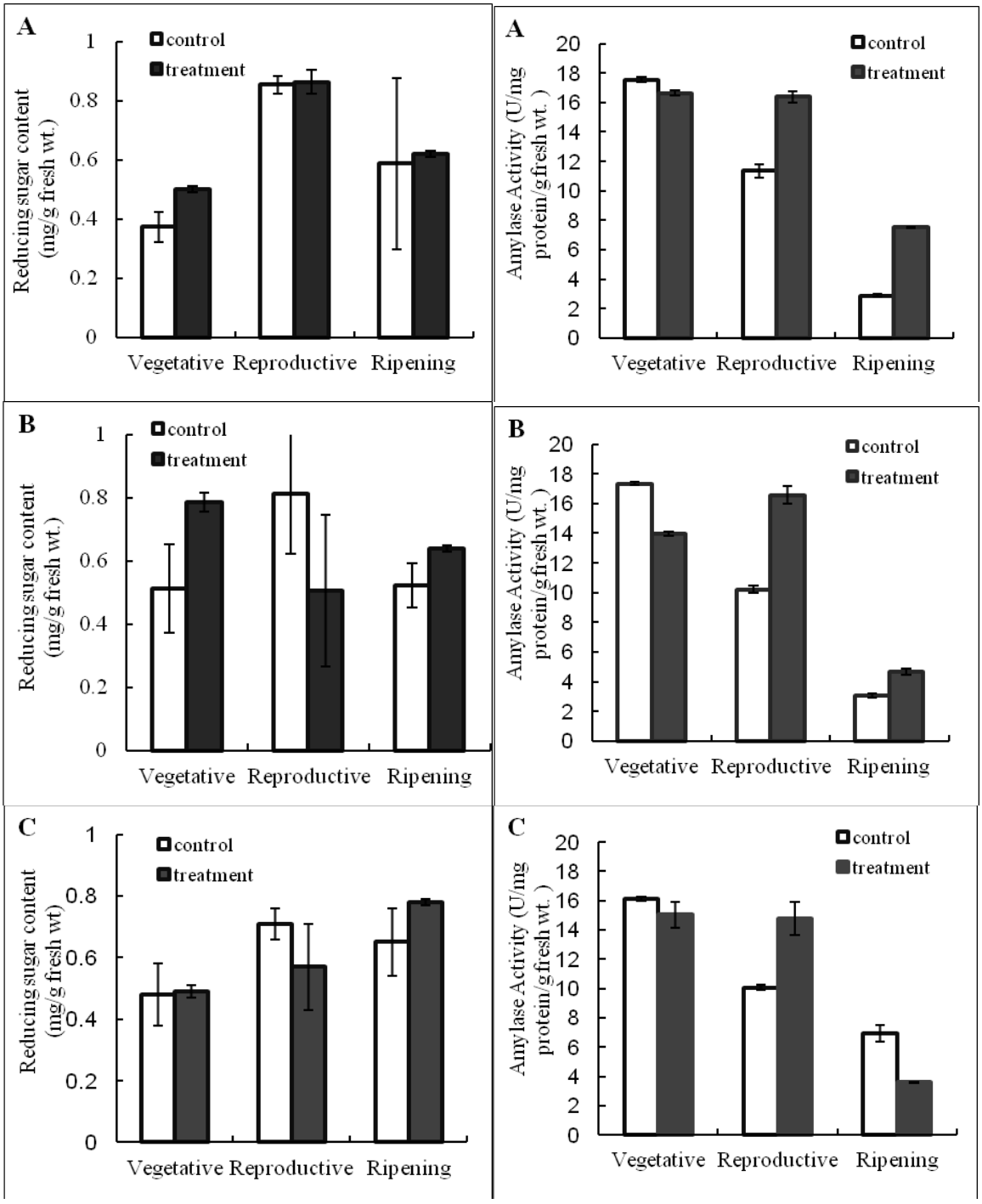


Figure 1. The reducing sugar content (left) and amylase activity (right) in leaves of three rice genotypes, (A) SS1-40, (B) SS1-41 and (C) SS1-42, during the three different growth stages.

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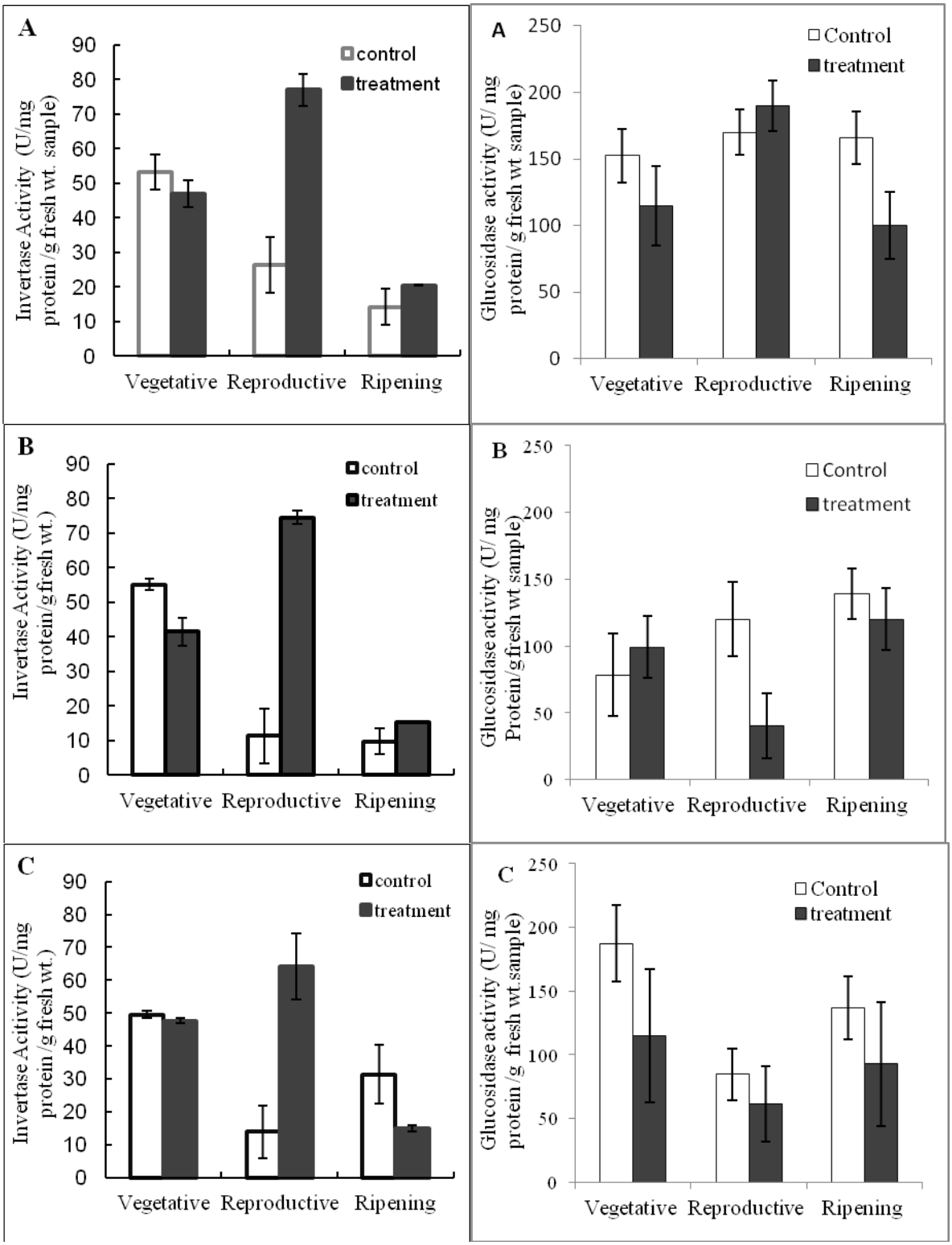


Figure 2. Invertase activity (leaf) and glucosidase activity (right) in leaves of three rice genotypes, (A) SS1-40, (B) SS1-41 and (C) SS1-42, during the three different growth stages.

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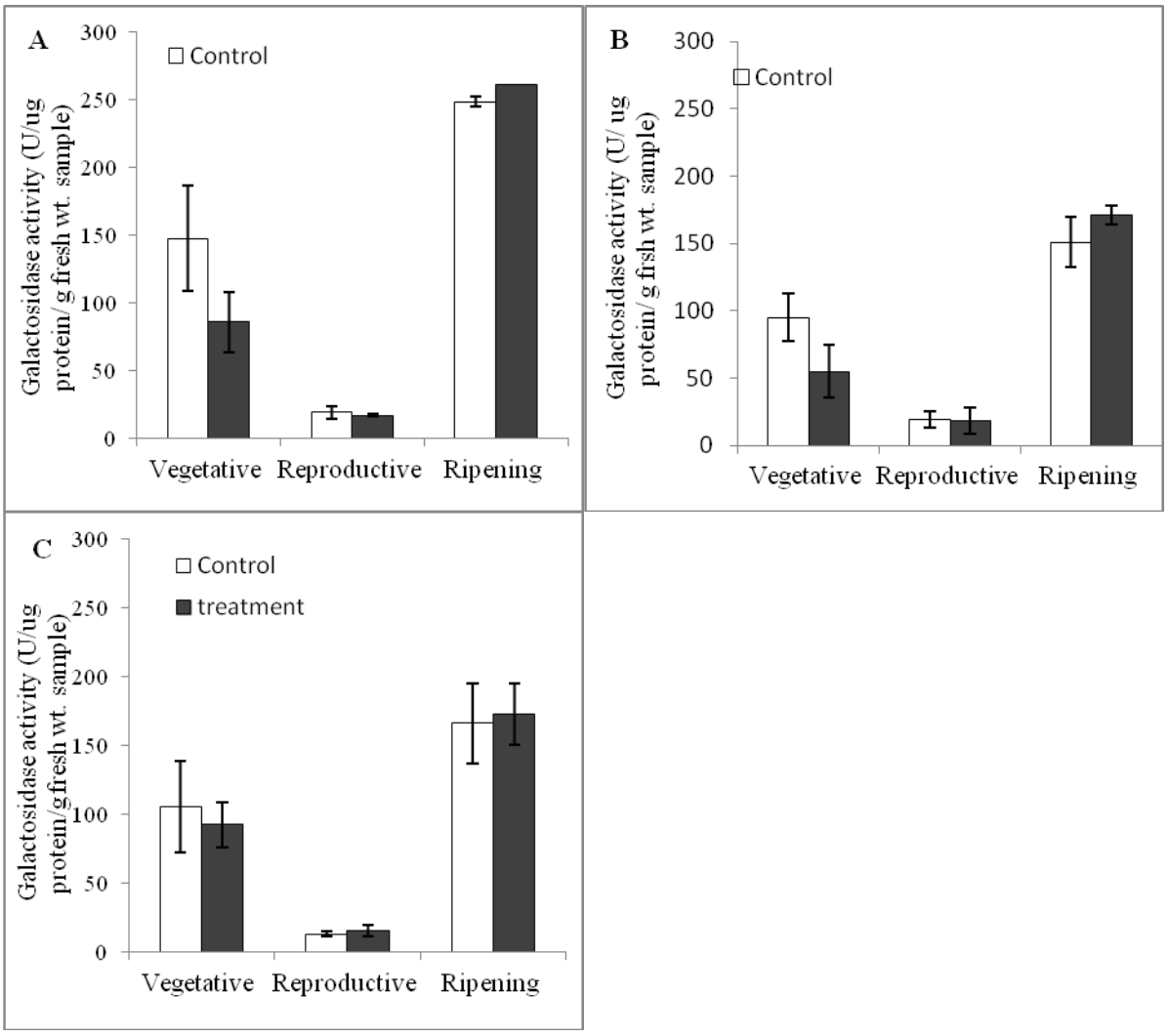


Figure 3. Galactosidase activity in leaves of three rice genotypes, (A) SS1-40, (B) SS1-41 and (C) SS1-42, during the three different growth stages.

## Conclusions

The three rice genotypes used in this study exhibited similar physiological characteristics under normal and salinity stress. Amylase and invertase were two enzymes that were significantly influenced by salinity stress during the reproduction stage.

## Acknowledgements

The authors would like to thank The Director of International Rice Research Institute (IRRI), Los Banos, and Laguna, Philippines, for supplying the rice seeds and Government of Malaysia (KPT-Grant) for research funding under LRGS (Rice-Food Security) Research Grant.

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# Solar UV-B and UV-A/B Exclusion Effects on Intraspecific Variations in Physiological Responses of Wheat Varieties

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## Introduction

Plants are inevitably exposed to solar UV-B radiation, since they are immobile. Owing to natural latitudinal gradient in total atmospheric ozone column thickness, prevailing solar angles, elevation above sea level and optical amplification effect, natural variations in UV-B irradiances at the ground are produced (Ballare et al., 2011). The attenuation of the stratospheric ozone has led to the enhanced UV-B radiation on the surface of land in recent decades (Ballare et al., 2011). The amount of UV-B radiation reaching tropical latitudes is higher than in temperate because the lower solar zenith angle leads to a less atmospheric UV-B absorption in tropics. The significant declining trend in total ozone column (TOC) confirm at numerous stations lying in the northern India suggesting the potential vulnerability of plants to increased UV-B under field conditions (Sahoo et al., 2005). Numerous studies have shown that enhanced and ambient UV-B radiation can affect physiological and biochemical processes of many plant species, including altered plant photosynthesis (Kataria and Guruprasad, 2012a; Kataria et al., 2013), changes in the carbon partitioning from growth pools to secondary metabolic pathways (Bassman, 2004), and thus changes in crop morphology, crop reproductive organ abortion and yield reduction (Mohammed and Tarpley, 2010; Kataria et al., 2013).

Wheat is one of the major world food crops, the main regions of wheat production are tropics and subtropical regions. The effects of enhanced UV-B radiation on intraspecific responses in wheat has been extensively studied on physiology and yield but with the majority of studies considering the effect of enhanced UV-B radiation with lamp supplementation on the physiological and biochemical aspects (Li et al., 2000) in growth chambers and greenhouses where the unnatural spectral balance of radiation can lead to unrealistic conclusions, which may have substantially changed plant sensitivity to UV-B. It is important in experiments to maintain a realistic balance between various spectral regions since both UV-A (315-400 nm) and visible (400-700 nm) radiation can have ameliorating effects on responses of plants to UV-B (Caldwell et al., 1995). Unfortunately, very few studies have been conducted under field conditions to assess the effects of ambient UV-B and UV-A radiation on intraspecific responses in terms of growth and yield of wheat (Kataria and Guruprasad, 2012b) by the exclusion of solar UV components. However, the effects of ambient UV-B and UV-A radiation on intraspecific responses in terms of physiological characteristics like chlorophyll fluorescence, gas exchange parameters and the enzymes involved in carbon and nitrogen metabolism like Carbonic anhydrase, Rubisco and Nitrate Reductase (NRA) in wheat varieties has not been investigated yet.

In this study, we grew wheat varieties in field under ambient and reduced levels of UV-B radiation with the objective to (1) determine, UV-B and UV-A radiation affects wheat physiology under field conditions; and (2) assessment of the intraspecific differences in physiological response of four wheat varieties to UV-B/UV-A radiation in the fields. We hypothesized that exclusion of UV-B radiation will increases specific leaf weight and chlorophyll content, and affects other physiological processes, i.e. carbon fixation by increased PSII efficiency and reducing power will result in the increased activity of CA, Rubisco and nitrogen fixation by increase in Nitrate Reductase activity which will ultimately increased the yield of wheat varieties. These changes will results in intraspecific differences in physiological response under field conditions

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## Materials and Methods

Seeds of Indian wheat (*Triticum aestivum* L.) varieties; Naveen Chandausi (HI 1418), Swarna (HI 1479), Vidisha (DL 788-2) and Purna (HI 1544) were collected from Regional Wheat Research Station, Indian Agriculture Research Institute, Indore. Field experiments under natural sunlight were conducted in the Botanical Garden of School of Life Sciences, Indore (22°44'N), India. The experiments were carried out during October 2012 to January 2013 when the average daily solar UV-B dose was approximately 50% higher than the average daily dose received in the temperate region. Seeds were sown in the field area of 160 cm ×160 cm in 120 cm rows planted 23 cm apart with 5 cm plant spacing within the row under iron cages of dimensions [4 feet L × 4 feet W × 5 feet H]. The iron cages were wrapped with UV cut-off Polyester filters (Garware polyester Ltd., Mumbai) that selectively cut-off UV-B (<315 nm) and UV-A/B (<400 nm) radiation. Two types of controls were taken for the present study; plants were grown either in the cages covered with polythene filter that transmits all the ambient solar radiation (filter control FC) or in open field without any filters, exposed to natural solar radiation (open control OC). Absolute solar irradiance without UV-B or UV-A/B was measured using a radiometer (Solar Light Co. Inc. (PMA 2100), Glenside, PA, U.S.A.).

*Growth data collection and analysis*

Plants were sampled randomly in triplicates from all the treatments at 80 days after the emergence of seedlings (DAE). After 80 DAE the sampled plants in each plot were harvested and taken to the laboratory. The roots of the plants were gently washed in water to remove soil particles, biomass was determined on a top-loading balance. For total biomass accumulation, the above ground parts of the plants and for leaf biomass, leaves were oven-dried at 105°C for 24 h to constant weight. Area of flag leaves was measured using portable laser leaf area meter CID-202 scanning planimeter (CID Inc., USA). Subsequently, plants were dried to a constant weight at 60°C, and the specific leaf weight (SLW; ratio of leaf dry weight to leaf area) of flag leaves was determined.

*Physiological and biochemical measurements*

Photosynthetic pigment, total chlorophyll was extracted from the flag leaves of wheat varieties with 80% acetone at 80 DAE. The amount of total chlorophyll was quantified using the formulae of Wellburn and Lichtenthaler (1984). Accumulation of UV-B absorbing substances (UAS) was determined spectrophotometrically from acidified methanol extract by the method of Mazza et al. (1999).

Chlorophyll *a* (Chl *a*) fluorescence induction kinetics of dark-adapted (30 min) flag leaves of each variety of wheat was measured using a Handy PEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments, King's Lynn, Norfolk, UK). The JIP-test parameters were calculated by using a chlorophyll fluorescence analyzing program (Biolyzer HP 3 software, Bioenergetics Laboratory, University of Geneva, Switzerland). Net photosynthesis (Pn, μmol $CO_2$ $m^{-2}$ $s^{-1}$) and stomatal conductance (gs, mol $H_2O$ $m^{-2}$ $s^{-1}$) was measured by a portable photosynthetic system (Li- 6200, LI-COR Inc., Lincoln, Nebraska, Serial No. PPS 1332 USA) in intact plants grown in normal sunlight or UV excluded sun light under field conditions.

Carbonic anhydrase (CA) activity was determined by the method described by Li et al. (2004). Rubisco activity was measured according to the method of Singh and Singh (2001) by ribulose-1,5-bisphosphate dependent incorporation of $^{14}CO_2$ into acid-stable product which was estimated by liquid scintillation counting in fully opened matured flag leaves at 80 DAE of each variety of wheat. Soluble protein content was estimated by the method of Lowry et al. (1951) using Bovine serum albumin (BSA) as a standard.

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Nitrate reductase activity (NRA) in the flag leaves was determined by the intact tissue assay method of Jaworski (1971).

**Results and Discussion**

To the best of our knowledge, this is the first report to suggest the existence of intraspecific responses to ambient UV radiation under field conditions in terms of photosynthetic characteristics like Chl *a* fluorescence and the activities of enzymes like CA, Rubisco and NRA in wheat varieties. Exclusion of ambient UV radiation from solar spectrum significantly enhanced many components of the growth and biomass allocation of wheat varieties (Kataria and Guruprasad, 2012a). The result indicated that leaf area, specific leaf weight and leaf weight ratio were increased in all the four varieties of wheat grown without UV compared with those grown under ambient UV. Out of the four varieties of wheat, two varieties Purna and Vidisha showed significant enhancement in leaf weight ratio and specific leaf weight (SLW). A maximum of 43% increase in leaf weight ratio was observed after the exclusion of UV-B and UV-A/B respectively in variety Vidisha. SLW was also enhanced 24% by removal of UV-B alone and UV-B along with UV-A in variety Vidisha. It indicates that plant grown in ambient conditions like OC and FC, the leaf weight ratio and SLW was decreased, the decrease in leaf thickness may have increased the UV-B penetration within leaves and it leads to decreased photosynthetic rates and dry weight accumulation in OC and FC plants. Whereas UV exclusion increased the LWR and SLW, indicates larger biomass partition to leaves and increased leaf thickness. Similar results were observed in other UV-B exclusion studies like in *Vaccinium uliginosum* and *Sorghum bicolor* (Albert et al., 2010; Kataria and Guruprasad, 2012a).

Changes in photosynthetic characteristics have often been used as an index to assess crop sensitivity to UV-B radiation. Photosynthetic pigments like Chlorophyll *b* was significantly increased while chlorophyll *a* increased to a lesser extent, leading to a decrease in chlorophyll *a/b* ratio in UV excluded plants in all the four varieties. The total chlorophyll content was also higher after the exclusion of UV radiation. Similarly, Under UV-B exclusion, chlorophyll content of $C_3$ and $C_4$ leaves was higher and the chlorophyll *a/b* ratio was lower compared to ambient controls (Kataria et al., 2013). Polyphasic chlorophyll *a* fluorescence (OJIP) transients from UV excluded plants gave a higher fluorescence yield at I–P phase in wheat variety Purna and Vidisha whereas other two varieties do not show any significant changes (Figure 1). The performance index ($PI_{ABS}$) is a function of three independent functional steps of photosynthesis, the density of reaction centers in the chlorophyll bed (RC/ABS), excitation energy trapping (Φpo) and conversion of excitation energy to electron transport (Ψo). $PI_{ABS}$ was increased in all the four varieties of wheat studied. $PI_{ABS}$ was maximally increased by 75% after exclusion of UV-B and 21% after UV-A/B exclusion as compared to the filter control in wheat variety- Purna.

Net photosynthesis rate in terms of $CO_2$ absorbed and the stomatal conductance was significantly enhanced in the three varieties of wheat by the exclusion of UV-B alone and exclusion of UV-A along with UV-B. But the magnitude of enhancement was more by the exclusion of UV-B as compared to exclusion of UV-A along with UV-B in all the four varieties. UV-B exclusion caused greater enhancement in net rate of photosynthesis in Purna (52%) and Vidisha (48%) than in Swarna (13%) and N. Chandausi (24%) as compared to their filter control. A similar observation of enhancement in the PSII efficiency and net rate of photosynthesis after exclusion of UV-B has been made in many plant species (Albert et al., 2010; Kataria et al., 2013)

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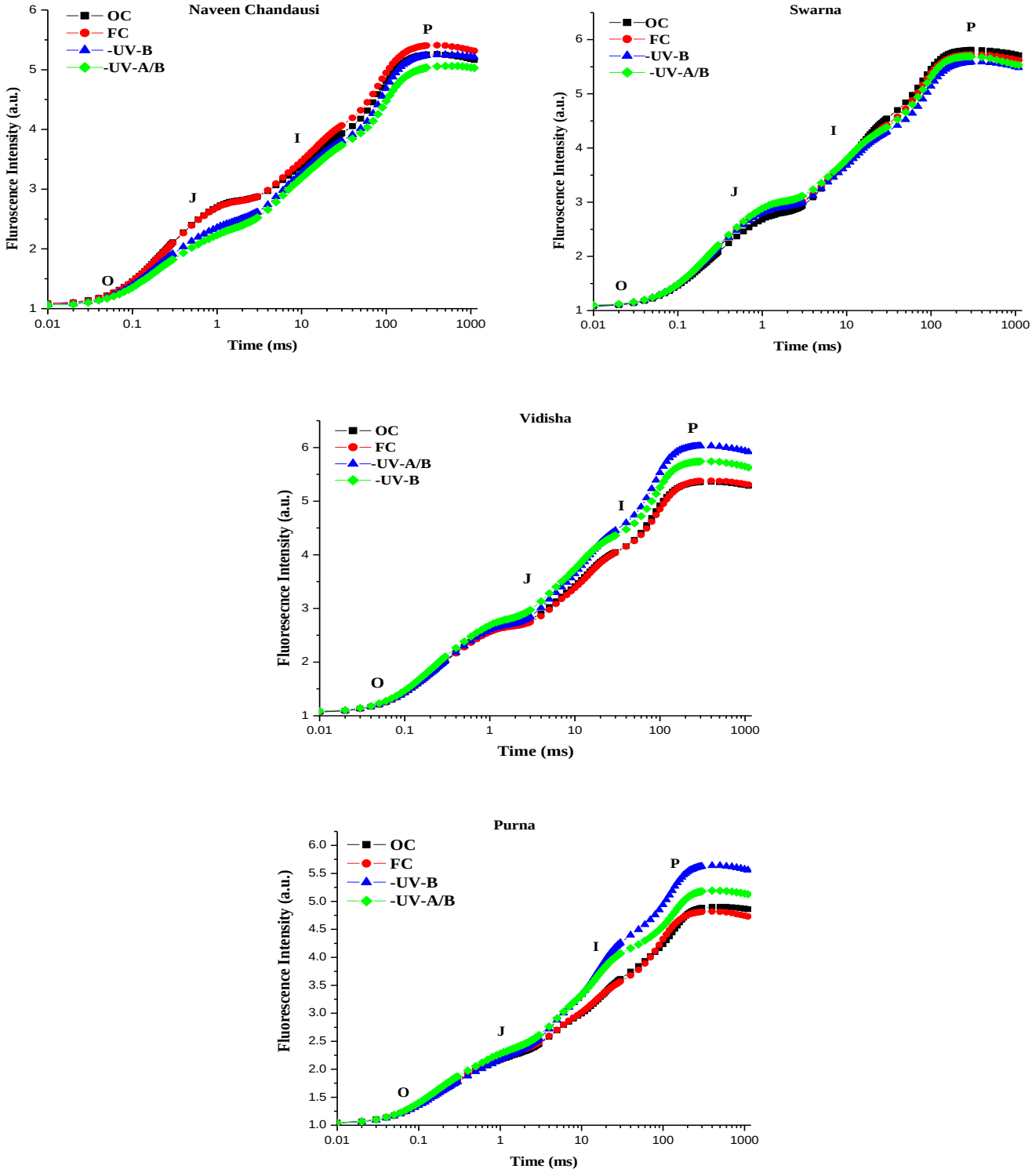


Figure 1. Changes in Polyphasic chlorophyll *a* fluorescence (OJIP) transient curves in four wheat varieties of wheat leaves after UV Exclusion.

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The two enzymes involved in $CO_2$ assimilation and carbon fixation in $C_3$ plants like Wheat, Carbonic anhydrase (CA) and Rubisco were examined for carboxylase activity. CA and Rubisco activity were higher in all the four varieties of wheat by the exclusion of UV. The maximum enhancement in activity of CA was observed in Purna (83%) by exclusion of solar UV-B as compared to FC, which is followed by (39%) in Vidisha, (32%) in Swarna and minimum enhancement was found in N. Chandausi (23%) (Figure 2A). The Rubisco activity was also remarkably enhanced by UV-B exclusion in Purna (88%) which is followed by (49%) in Vidisha, (27%) in Swarna and minimum enhancement was found in N. Chandausi (17%) (Figure 2B). The total soluble protein content was also increased in all the four varieties of wheat.

Nitrate reductase (NRA; EC 1.6.6.4) is a key enzyme of N metabolism, which converts nitrate ($NO_3^-$) into nitrite ($NO_2^-$) on the metabolic pathway leading to the formation of amino acids (Canovas et al., 2007). In this study, exclusion of UV led to an induction in NR activity in flag leaves of all the four varieties of wheat when compared to the filter control treatment. The extent of promotion was greater after exclusion of UV-B as compared to the exclusion of UV-A/B. Maximum enhancement in NR activity was shown by Purna (86%) after exclusion of solar UV-B as compared to FC, which is followed by (57%) in Vidisha, (47%) in Swarna and minimum enhancement was found in N. Chandausi (36%) (Figure 2C). Moreover, enhanced UV-B has been found to inhibit growth and decrease NR activity in dragon spruce (*Picea asperata* Mast.) needles (Yao and Liu, 2007). We have not come across any report of enhanced activity of CA, Rubisco and NR concurrently after exclusion of ambient UV-B and UV-A/B in higher plants as presented here. Although earlier we have reported, the enhancement in Rubisco and CA activity by exclusion of solar UV-B independently in different plant species (Kataria et al., 2013).

Exclusion of UV significantly enhanced photosynthetic capacity as a consequence of increased efficiency of PS II, increased rate of photosynthesis and stomatal conductance along with a remarkable increase in carbonic anhydrase, Rubisco and nitrate reductase activities. Thus UV exclusion increased light utilization efficiency, $CO_2$ fixation and nitrogen fixation and channelized the additional fixation of carbon and nitrogen towards the improvement of yield in wheat varieties. In the present study, increased in grain yield was more pronounced in both the sensitive varieties of wheat (Purna and Vidisha); these results are in agreement with the earlier reports showing the increase in grain yield by the exclusion of UV (Kataria and Guruprasad 2012b; Kataria et al., 2013). Germ et al. (2005) found that exclusion of UV-B from the solar radiation led to more than double the yield of pumpkin fruits. The reason for yield reduction by ambient UV-B may be due to alterations in plant vegetative and reproductive growth, e.g., plant stunting, flower suppression and/or delay of flowering and lower ear/pod set.

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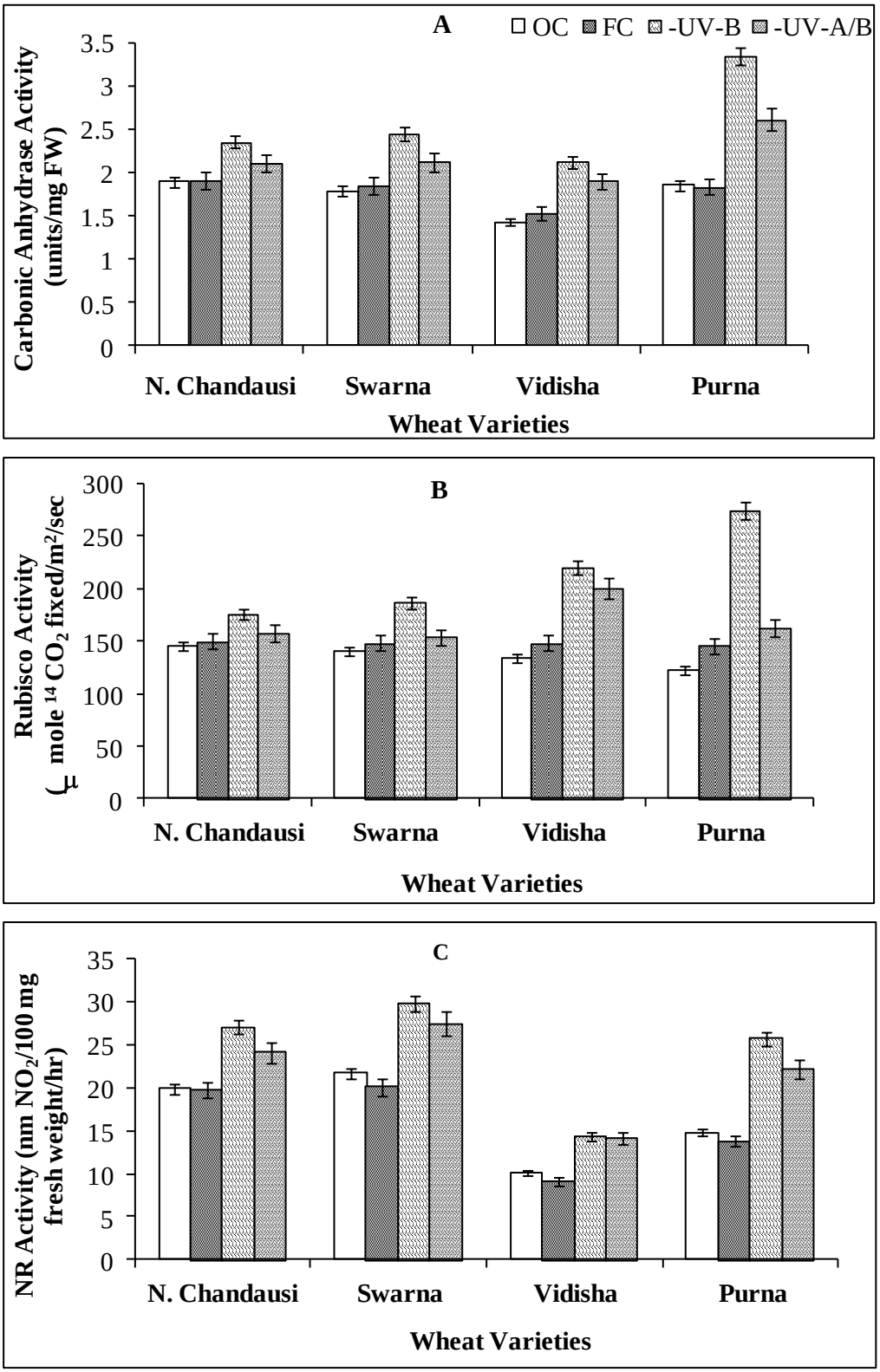


Figure 2. Effect of exclusion of UV-B and UV-A/B on Carbonic anhydrase (A), Rubisco activity (B) and NRA activity (C) in wheat varieties.

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## Conclusions

Photosynthetic characteristics can be used as indicators of the sensitivity of wheat varieties to ambient UV-B radiation under field conditions. In all the four wheat varieties, the responses of each photosynthetic characteristic to ambient UV-B radiation were different. In this study, stomatal conductance, net photosynthetic rate and the activity of CA and Rubisco were significantly enhanced by the exclusion of UV-B radiation. Thus, they might be used as response-indicators for assessing the sensitivity of plant to UV-B radiation. The magnitude of response for UV exclusion for all the measured parameters was higher in two varieties; Vidisha and Purna in terms of photosynthetic $CO_2$ assimilation, PSII photochemistry, pigments, UV-B radiation absorbing ability, nitrogen fixation, grain yield and leaf biomass. On the other hand, not much UV-B damaging effects were observed in the other two varieties; Swarna and N. Chandausi because these varieties have higher levels of photosynthetic $CO_2$ assimilation, reduced damaging effect on photochemistry coupled with higher levels of pigments, and better UV-B screening ability, high nitrogen fixation, grain yield and leaf biomass. Intraspecific differences were found for different measured parameters; apart from variety- Vidisha all the three varieties are more responsive to exclusion of UV-B. Sensitivity of four Indian wheat varieties to ambient level of UV (290–400 nm) radiation at Indore (22˚44ˉN), India had the following sequence; N. Chandausi > Swarna > Vidisha > Purna. Thus Purna was the most sensitive and Naveen Chandausi the least sensitive to current level of solar UV radiation in India. The least sensitive varieties may be used in the development of new varieties tolerant to higher levels of UV-B in future attempts in plant breeding programmes.

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# Role of Salicylic Acid as a Plant Growth Regulator on Some Physiological Features and Antioxidant Enzyme Activity in Ginger (*Zingiber officinale* Roscoe)

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## Introduction

Salicylic acid (SA) is classified as a phenolic compound, a group of substances that can regulate plant growth (Amanullah et al., 2010). SA application influences a wide variety of plant processes, including stomatal closure (Ananieva et al., 2002), plant growth and yield (Khodary 2004) and induction of antioxidant synthesis (Ghasemzadeh and Jaafar, 2012; Yordanova and Popova, 2007). The effect of salicylic acid on plant physiological processes varies depending on species, developmental stage, SA concentration and environmental conditions (Shraiy and Hegazi, 2009). When SA was applied to leaves of *Phaseolus vulgaris* and *Commelina communis*, transpiration rates decreased (Hayat et al, 2005), however, increases in stomatal conductance and transpiration rate were observed in soybean and corn leaves (Khan et al., 2008). In addition, rate of transpiration and internal $CO_2$ increased in soybeans after supplementation of SA (Kumar et al., 2009). Similarly, barley seedlings exposed to SA for one week exhibited increases in $CO_2$ compensation point and stomatal resistance and a decrease in photosynthetic rate (Pancheva and Popova, 1998). In contrast to the above observations, application of SA increased photosynthetic rate in maize and mustard plants (Fariduddin et al., 2011). Reactive oxygen species (ROS), including hydrogen peroxide ($H_2O_2$), superoxide ($O^{2-}$), hydroxyl radical ($HO^-$) and singlet oxygen, can disrupt normal plant metabolism through oxidative damage to proteins, lipids, nucleic acids, photosynthetic enzymes and pigments ( Fu and Huang, 2001). To overcome oxidative stress, plants have developed enzymatic and non-enzymatic antioxidant defence mechanisms to scavenge ROS (Smirnoff, 1993). The most important antioxidant enzymes are peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6) and superoxide dismutases (SOD; EC 1.15.1.1). SODs convert $O^{2-}$ into $H_2O_2$ and $O_2$, with CAT and POD transforming $H_2O_2$ into $H_2O$ (Wang et al., 2009). Ginger (*Zingiber officinale* Roscoe) is a traditional folk medicinal plant extensively used in Malaysian cooking. One of the most widely used herbs, especially in Asia and contains several interesting bioactive constituents and possesses health-promoting properties. In previous studies, we uncovered potent antioxidant and anticancer activities in Malaysian young ginger (halia Bara and halia Bentong) (Ghasemzadeh and Jaafar, 2011). Useful information regarding the effect of SA on growth of Malaysian ginger varieties, with their high levels of beneficial compounds, is still scarce.

The objective of this study were (1) to examine the effect of SA foliar applications on net photosynthetic rate, stomatal conductance, intercellular carbon and biomass production in two varieties of Malaysian ginger, namely, halia Bentong and halia Bara, and (2) to determine the most effective SA concentration and investigated alterations in nitrate reductase, POD, SOD and CAT activities.

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## Materials and Methods

*Plant materials and cultivation*

Rhizomes of ginger varieties halia Bentong and halia Bara were sprouted for two weeks in 10-cm diameter pots filled with peat. Sprouted rhizomes were transferred to polyethylene bags (6 liter) filled with a soilless mixture medium (burnt rice husks:coco peat, 1:1). The plants were raised in specially constructed growth houses at the Glasshouse Complex, Universiti Putra Malaysia. Day and night temperatures were 30±1.0 ℃ and 20±1.5 ℃, respectively, and relative humidity was about 70–80%, 12-h light photoperiod with an average photosynthetic photon flux density of 310 μmol $m^{-2}$ $s^{-1}$. When the ginger seedlings were at the second leaf stage, they were sprayed with two concentrations ($10^{-3}$ and $10^{-5}$ M) of a SA solution containing 2-hydroxybenzoic acid, 100 μL dimethyl sulfoxide, and 0.02% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma). Control plants were sprayed with a similar solution but without SA. Leaves were sprayed at early in the morning, once weeklyfor one month.

*Photosynthetic rate, stomatal conductance and transpiration measurements*

One day after spraying, photosynthetic rate, stomatal conductance and transpiration rate of fully expanded leaves were measured for 6 days using a LICOR-6400 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA).

*Plant biomass*

Plants were harvested 4 weeks after transplanting into the polyethylene bags. Plant parts were separated into leaf, stem and rhizome, dried at 70 °C for 72 h, and weighed on an electronic weighing scale (Mettler-Toledo Model B303-S, Switzerland).

*Nitrate reductase activity*

Leaves from different treatments were collected and cut into small pieces. About 500 mg of leaf pieces from different treatments were incubated separately in a medium containing 1 mL of 1 M potassium nitrate, 2 mL of 0.2 M phosphate buffer (pH 7.5) and 2 mL of 0.5% Triton X-100 for 1 h. One milliliter of reaction mixture was transferred to another test tube and mixed with 1 mL of 1% sulfanilamide in 2 N hydrochloric acid and 1 mL of 0.2% NEEDA (N-1-napthyl ethylenediamine dihydrochloride). Using a mixture of 1 mL NEEDA as a blank, absorbance was recorded at 540 nm in a LKB Biochem Ultra Spectrophotometer. A standard curve was prepared using different concentrations of potassium nitrite. Enzyme activity was expressed as μmol of $NO_2$ liberated per hour per gram FW (Hayat et al., 2009).

*Antioxidant enzyme assays*

The nitroblue tetrazolium (NBT) method was used for determination of SOD activity. One unit of SOD activity was defined as the amount of enzyme required to produce 50% inhibition of NBT reduction at 560 nm. The methods of Fu and Huang (2001) were used to determine POD and CAT activities. For POD, guaiacol oxidation was measured by monitoring increase in absorbance at 470 nm for 1 min. For CAT, $H_2O_2$ decomposition was measured by recording decline in absorbance at 240 nm for 1 min. One unit of CAT or POD activity corresponded to an absorbance change of 0.01 units per min. The activity of each enzyme was expressed on a protein basis. Three replicates per treatment and variety were performed at midday using the youngest fully expanded leaves of different individuals.

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*Statistical analysis*

Data were analyzed using Statistical Analysis System (SAS) version 9.0 (2002). Treatment means were separated using Duncan's Multiple Range Test. Experimental results were expressed as means of three replicates ± standard deviation. A *P*-value ≤ 0.05 was regarded as significant.

**Results and Discussion**

*Effect of foliar application of SA on leaf gas exchange parameters*

Two days after foliar application of $10^{-5}$ M SA, photosynthetic rate increases by 18% and 19.2% in halia Bara and Bentong, respectively. While, spraying with $10^{-3}$ M SA resulted in 9.2% and 10.1% increases in halia Bara and Bentong, respectively within the same time frame (Figure 1a). On day 2 to 5 following either SA treatment, photosynthetic rate decreased significantly in halia Bentong, followed by a slight increase at day 6, whereas photosynthetic rate in halia Bara increased significantly from days 2 to 4 and then decreased. The highest photosynthetic rate in halia Bara was 7.8 µmol/$m^2$/sobserved at day 4, while the highest rate in halia Bentong, was 5.6 µmol/$m^2$/s, at 2 d after spraying. Intercellular $CO_2$ concentration (Ci) was lower in treated plants than in the controls for both varieties (Figure 1c). Stomatal conductance in treated and control plants show similar trend from day 1 to 6 after spray. Plants of both varieties exposed to $10^{-5}$ M exhibited the highest photosynthetic rates. Induction of photosynthetic rate and stomatal conductance by SA has been previously demonstrated (Khodary, 2004). Stomatal regulation and behavior are very important factors controlling photosynthetic rate. While increased photosynthetic rates seen 2 d after SA application were generally accompanied by increased or unchanged stomatal conductance levels and transpiration rates, intercellular $CO_2$ concentrations of treated plants were generally lower than in control plants. If increased stomatal opening was the primary cause of increased photosynthetic activity, increases in internal carbon would be expected. These results thus suggest that increases in photosynthetic rate following SA spray applications were due to increased $CO_2$ uptake at the chloroplast level rather than simple increases in stomatal opening, i.e., reduced resistance to entry of $CO_2$ in the leaves. Increased photosynthetic rates accompanied by increases in stomatal conductance and lowered internal carbon values, as seen in our results, also imply that the photosynthetic rate increase may have been due to enzyme-related activities at the chloroplast level. Stomatal conductance readings, along with decreases in Ci values, strongly suggest that changes in photosynthetic rate were due to changes within the leaf rather than increased stomatal opening. In this study, we used a commercially feasible method of SA application—leaf spraying—to demonstrate how SA might be used to increase photosynthetic and growth rates in a wide range of crop plants. We observed that the lower SA concentration ($10^{-5}$ M) was generally more effective in enhancing photosynthetic rate of the Malaysian young ginger varieties halia Bentong and Bara under greenhouse conditions.

*Effect of SA foliar application on plant height, leaf area and total dry weight*

Foliar application of SA significantly affected ($P \leq 0.05$) plant leaf area and total dry weight for both varieties (Table 1), but not on plant height. Total dry weight and leaf area were greater in $10^{-5}$ M treated plants than in $10^{-3}$ M treated and the controls plants (Table 1). The highest total dry weight (8.68 g per plant) was observed in halia Bentong, and the largest leaf area (719.2 $cm^2$ per plant) was recorded in halia Bara. These results are consistent with published reports. A recent study by Nagasubramaniam et al. (2007) demonstrated that SA ($7.2 \times 10^{-4}$ M) increased plant height, leaf area, crop growth rate and total dry matter production in baby corn, while Jeyakumar et al. (2008) reported that SA ($10^{-4}$ M) enhanced dry matter production in black gram. Plant height and shoot and root dry weight were affected by SA application, but no significant differences were observed between treatments.

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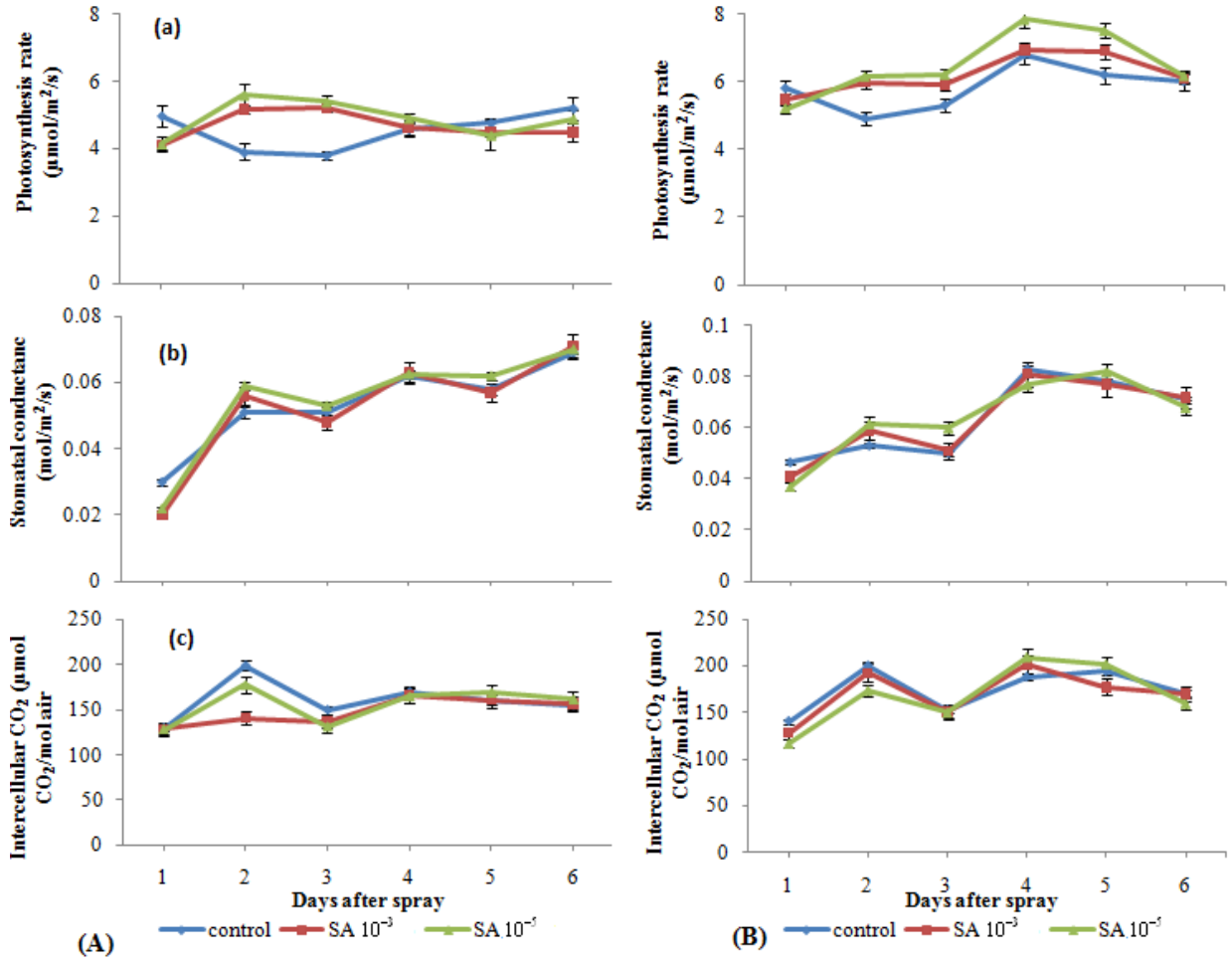


Figure 1. Effect of foliar application of salicylic acid on photosynthetic rate, stomatal conductance and intercellular $CO_2$ (Ci) in ginger varieties (A: Halia Bentong and B: Halia Bara). Error bars indicate means of triplicate measurements ± SEM.

Table 1. Effect of foliar SA application ($10^{-3}$ and $10^{-5}$ M) on plant height, leaf area and dry weight of ginger parts.

| Variety | SA | Plant height | Leaf area | Leaf dry weight | Shoot dry weight | Root dry weight | Rhizome dry weight | Total dry weight |
|---|---|---|---|---|---|---|---|---|
| H.Bentong | Control | 56.66±6.75$^{a}$ | 506.6 ± 15.6$^{c}$ | 1.79±0.53$^{b}$ | 1.86±0.67$^{a}$ | 0.214 ± 0.05$^{a}$ | 3.42±0.66$^{a}$ | 7.28±1.51$^{ab}$ |
| | $10^{-5}$ | 53.26±5.51$^{a}$ | 603.6±16.14$^{abc}$ | 2.31±0.21$^{b}$ | 2.1 ± 0.49$^{a}$ | 0.24 ± 0.06$^{a}$ | 4.03±1.06$^{a}$ | 8.68± 0.71$^{a}$ |
| | $10^{-3}$ | 55.43±4.55$^{a}$ | 541.3 ± 62.02$^{c}$ | 2.55±0.65$^{b}$ | 1.98±0.21$^{a}$ | 0.227±0.035$^{a}$ | 3.56±0.78$^{a}$ | 8.31± 1.09$^{a}$ |
| H.Bara | Control | 49.24 ± 0.9$^{a}$ | 592.4± 16.1$^{bc}$ | 2.64±0.29$^{b}$ | 1.74±0.49$^{a}$ | 0.252±0.068$^{a}$ | 0.74±0.11$^{b}$ | 5.37± 0.68$^{b}$ |
| | $10^{-5}$ | 48.5 ± 3.12$^{a}$ | 719.2 ± 91.5$^{a}$ | 3.72±0.93$^{a}$ | 2.21±0.37$^{a}$ | 0.281 ± 0.03$^{a}$ | 0.99±0.37$^{b}$ | 7.2 ± 1.73$^{ab}$ |
| | $10^{-3}$ | 48.36 ± 8.4$^{a}$ | 673.9 ± 0.9$^{ab}$ | 3.67±0.70$^{a}$ | 2.13± .23$^{a}$ | 0.263 ± 0.06$^{a}$ | 0.95±0.25$^{b}$ | 7.01±1.17$^{ab}$ |

*Data are means of triplicate measurements ± standard deviation. Means not sharing a common single letter were significantly different at $P \leq 0.05$. Plant height is expressed in cm, leaf area in $cm^2$, and dry weight in g per plant.*

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When applied in various quantities, phenolic compounds are well known to rapidly alter plant phenotypes and to influence plant growth from seed germination to senescence, either by enhancing or stimulating the natural growth regulatory system (Amanullah et al., 2010). Our observations that stimulation of photosynthetic rate by SA was due to increases in activity within the leaf generally support previous findings and provide new information regarding mechanisms, levels of application and effective types of phenolic compounds. Our results suggest that increases in photosynthetic rate may have stimulated plant total dry weight production. Application of $10^{-5}$ M SA also resulted in higher net photosynthetic rates, which should lead to increased crop productivity.

*Effect of foliar application of SA on nitrate reductase activity*

Spraying plants with SA, irrespective of concentration, generated significant increases in nitrate reductase activity in rhizomes (Figure 2). Application of $10^{-5}$ M SA was significantly superior to $10^{-3}$ M and no treatment, although lower nitrate reductase activity was observed in control plants. Nitrate reductase activity was higher in Halia Bara than in Halia Bentong for all treatments, including controls. In general, the highest nitrate reductase activity was recorded in Halia Bara sprayed with $10^{-5}$ M SA (621.9 nmol nmol $NO_2h^{-1}g^{-1}$ FW), while the lowest activity was observed in untreated Halia Bentong (300 nmol $NO_2h^{-1}g^{-1}$ FW). This SA concentration-based effect on nitrite reductase activity may indicate that nitrite reductase activity was induced and/or enzyme degradation was prevented. Foliar application of Tween-20 generated a response that was statistically equal to that of the control. This finding was unexpected and suggests that an SA concentration of $10^{-5}$ M might induce nitrite reductase synthesis by mobilizing intracellular $NO_3^-$ and protect against nitrite reductase degradation *in vivo* in the absence of $NO_3^-$ ( Singh, 1993). It can therefore be assumed that SA concentration plays an important role in regulating nitrate reductase activity, with lower concentrations enhancing nitrate reductase protein and higher concentrations decreasing it by affecting the above processes. Increases in nitrate concentration and in turn nitrate reductase activity due to exogenous SA treatment under normal growth conditions have been reported previously ( Hayat et al., 2005) and strongly support our observed results.

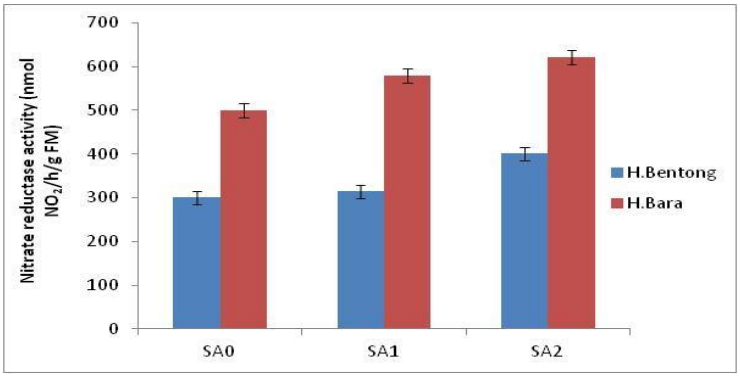


Figure 2. Nitrate reductase activity in Halia Bentong and Halia Bara treated with different concentrations of SA (SA0: control; SA1: $10^{-3}$ M; SA2: $10^{-5}$ M).

*Effect of foliar application of SA on antioxidant enzymes activities*

Exogenous application of SA increased antioxidant enzyme activities, with the maximum response generated in plants sprayed with $10^{-5}$ M SA. Significant increases were observed for POD (45.2%; Figure 3), SOD (44.1%; Figure 4) and CAT (20.1%; Figure 5). Tween-20 application did not generate any significant response. Exogenous application of two different concentrations ($10^{-3}$ and $10^{-5}$ M) of SA resulted in increased growth and physiological responses, with the best response generated from $10^{-5}$ M

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SA. Similar results have been obtained in *B. juncea* (Fariduddin et al., 2003) and wheat (Hayat et al., 2005). Noreen et al., (2009) reported that increase in growth and photosynthetic capacity of sunflower due to exogenously applied SA may have been due to SA-induced increase in activity of peroxidase. After Rubisco, CAT is the most abundant soluble zinc-containing enzyme in $C_3$ plant chloroplasts; it facilitates $CO_2$ diffusion across chloroplast membranes by catalyzing the reversible hydration of dissolved $CO_2$ entering the highly alkaline stromal environment (Majeau and Coleman, 1994) and maintains a constant supply of Rubisco. This enzyme's concentration, and therefore its activity, is finely regulated at transcriptional and/or translational levels (Okabe et al., 1980). In our study, however, neither of these processes was favored by the application of $10^{-5}$ M SA, which increased CAT activity. Exogenous SA is known to increase stomatal conductance (Table 1); by maintaining a constant supply of $CO_2$ for reduction by Rubisco, increased CAT activity in leaves is thus expected to increase photosynthetic efficiency and thereby net photosynthesis (Table 1). The higher SA concentration, however, may have caused permanent changes to cell membrane-level organization. These alterations would injure plant general metabolism and thereby reduce overall growth and photosynthetic attributes. At the lower SA concentration, activities of antioxidant enzymes POD, SOD and CAT were elevated, in accordance with other studies (Hayat et al., 2005). This increased antioxidant enzyme activity might be due to SA's regulatory role at transcriptional and/or translational levels.

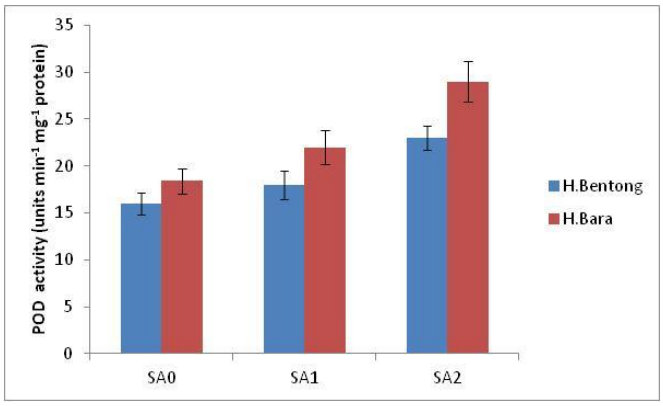


Figure 3. Peroxidase activity in Halia Bentong and Halia Bara leaf extracts treated with different concentrations of SA (SA0: control; SA1: $10^{-3}$ M; SA2: $10^{-5}$ M).

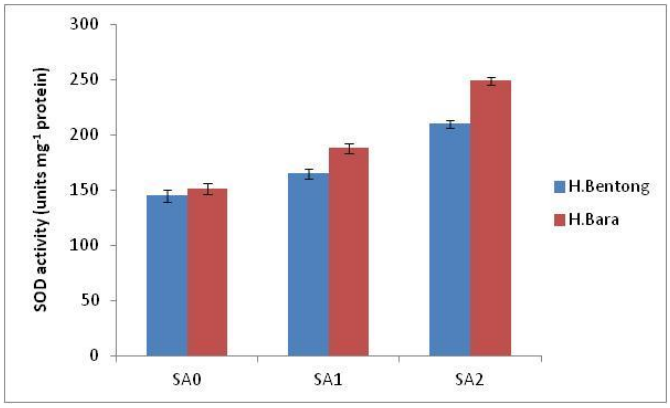


Figure 4. Superoxide dismutase (SOD) activity in Halia Bentong and Halia Bara leaf extracts treated with different concentrations of SA (SA0: control; SA1: $10^{-3}$ M; SA2: $10^{-5}$ M**).**

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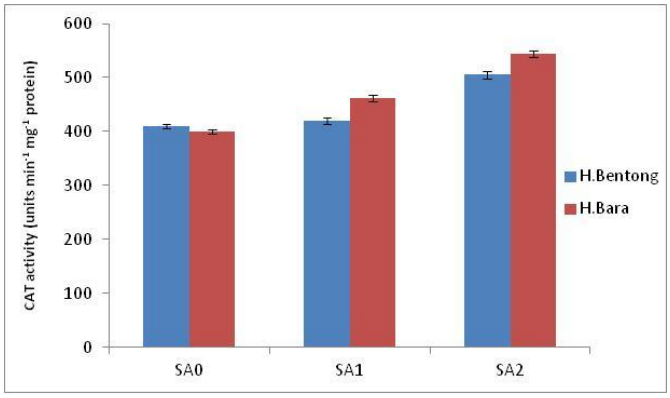


Figure 5. Catalase enzyme activity in Halia Bentong and Halia Bara leaf extracts treated with different concentrations of SA (SA0: control; SA1: $10^{-3}$ M; SA2: $10^{-5}$ M).

## Conclusions

This investigation has highlighted the usefulness of SA in plant growth regulation. One of the more significant findings to emerge from this study is that foliar application of SA can improve plant physiological efficiency, including photosynthetic rate, and can enhance effective partitioning of accumulates from source to sink. Treatment with SA resulted in higher photosynthetic rates and increased plant biomass. Most obviously, antioxidant enzyme activities were enhanced in ginger plants treated with the lower SA concentration ($10^{-5}$ M). In addition, exogenous application of two different concentrations ($10^{-3}$ and $10^{-5}$ M) of SA resulted in increased growth and physiological responses, with the best response generated using $10^{-5}$ M SA. Our results support the idea that low SA concentrations ($10^{-5}$ M) induce nitrite reductase synthesis by mobilizing intracellular $NO^{3-}$ and provide protection to nitrite reductase degradation in the absence of $NO^{3-}$. By boosting photosynthetic rate in response to enhanced antioxidant enzyme activities, it therefore appears that SA can generally be used as a growth regulator to enhance plant growth and yield.

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# Screening of Sorghum (*Sorghum bicolor* L. Moench) Genotypes for Alumunium Stress Tolerance in Nutrient Culture

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## Introduction

Development of agriculture in acid soil (ultisol) in Indonesia is a challenge but opportunity to increase food production towards food sovereignty in Indonesia. Projection of Indonesian food needs in 2025 will reach 38.7 million tons of rice, 19.4 million tons of corn and 2.5 million tons of soybean. Indonesian government policy plays a role in the development of diversification of food other than rice (BPS, 2012). Sorghum, with scientific name *Sorghum bicolor* L. Moench, is a crop that is widely cultivated throughout the world and has the potential as a source of food, feed and industry. Sorghum as food in the world is ranked 5 after wheat, rice, maize and barley (Sirappa, 2003).

Acid dry land in Indonesia suitable for annual crops reached 18.2 million ha (Mulyani et al., 2010). The main limiting factor for production in acid dry land is low soil fertility due to toxicity of aluminum (Al) and deficiency of major nutrients of phosphorus (P), calcium (Ca) and magnesium (Mg) (Marschner, 2012). Al toxicity is a major limiting factor because it can lead to disruption of root growth so that the plant faces problems in water and nutrient uptake (Kochian and Hoekenga, 2004; Kochian et al., 2005).

Impaired root growth begins with the accumulation of Al in the root tip. The genotypes sensitive to Al toxicity will be affected more than tolerant genotypes (Matsumoto and Matoda, 2012). In addition to inhibition of root growth, high Al solubility in the soil can also inhibit nutrient uptake, particularly cations such as P, Mg and Ca (Marchner, 2012).

One of the aspects which indirectly affects the growth and yield of plants is chlorophyll content. Chlorophyll content is an internal factor that greatly affects the efficiency of the plant and the rate of photosynthesis. Plants that have high photosynthetic rates produce high amount of photosynthates. Photosynthate availability is fundamental to plant metabolism (Taiz and Zeiger, 2002). One of the constituent elements of the pigment of chlorophylls is Mg. The low uptake of Mg due to Al toxicity leads to low chlorophyll content in plants (Chen and Ma, 2013).

Each plant has genotype-specific response to aluminum stress. Plant responses to stress can be in the form of morphological, anatomical, and physiological. Changes in physiological responses can be easily observed and can be selection characters to be used to screen and identify tolerant genotypes.

In addition to the characters above used for the selection, identification of genotypes tolerant method also determines the success of the selection method. Generally, tolerant genotype identification is done only on one character or the conclusions are drawn based on the observations of each character. Correlation between the characters and complex tolerance mechanisms are difficult to identify tolerant genotypes based on univariate analysis. Multivariate selection is an alternative approach and aimed to obtain a comprehensive conclusion about the mechanism of tolerance and genotypes that are truly tolerant based on all observed variables. Identification of tolerant genotypes using multivariate approach has been done by several researchers (Natawijaya, 2012) and produced better selection results. This study aimed to

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evaluate the response of sorghum genotypes on Al stress condition, identify Al tolerant genotypes and evaluate and evaluate univariate and multivariate selection methods to identify tolerant genotypes.

**Materials and Methods**

This study was conducted in a greenhouse at Cikabayan University Farm and Laboratory of Spectrophotometry, Faculty of Agriculture, Bogor Agricultural University, Bogor, from March to May, 2014. Randomized complete block design with three replications was used as experimental design. The first factor was the seven genotypes of sorghum. Seven sorghum genotypes used in this study included four sorghum lines of ICRISAT collection, 150-21-A , 5-193-C, 10-90-A and 150-20-A, 1 local sorghum line of Watar Hammu Putih/WHP and 2 national varieties of Numbu and UPCA-S1. The second factor was three levels of Al stress in the nutrient culture, i.e. 0 µM $AlCl_3$ + 0 mM $K_2HPO_4$ (control), 74 µM $AlCl_3$ + 0 mM $K_2HPO_4$ (Al) and 74 µM $AlCl_3$ + 0.1 mM $K_2HPO_4$ (Al+P). Numbu is a known acid soil tolerant variety and was used as positive control.

Seeds were directly sown in the husk. Normal seedlings of one week old with uniform root length were transferred to nutrient culture media. Stems were wrapped and then inserted into the holes of styrofoam and floated in nutrient solution (Ohki, 1987). Oberservation was performed as follows: Plants treated with Al and P for 7 DAP (days after planting) were then harvested to record the length of roots and shoots and the leaves were analysed for chlorophyll *a*, chlorophyll *b* and total chlorophyll content. Plant canopy photographs were captured prior to harvesting.

Data were analyzed using analysis of variance (ANOVA) with a confidence level of 95% to indicate sensitivity index to Al stress. Grouping of genotypes according to tolerance and sensitivity to Al stress was based on the value of the sensitivity index. It was calculated according to Fischer and Maurer (1978) as below:

Sensitivity index (S) = (1-Yp/Y)/(1-Xp/X)

where,

Yp= average value of a genotype that received stress
Y = average value of a genotype that did not receive stress
Xp= average value of all genotype that received stress
X = average value of all genotype that did not receive stress

Tolerant genotypes are those having S<0.5. The moderate genotypes are identified as 0.5<S<1, and sensitive ones are concluded if S>1. Analysis using a multivariate approach was conducted to determine tolerant and sensitive strains, the selection of characters and relationships among strains and character selection based on sensitivity index. The analysis was performed using Minitab software.

**Results and Discussion**

Table 1 indicated that genotype and Al treatment had significant effect on all observed variables. The interaction was significant with contents of chlorophyll *a*, chlorophyll *b* and total chlorophyll, as well as root length. The results were in line with Agustina et al. (2010) where plant tolerance for Al stress was indicated by plant capability to retain potential yield.

Table 1. Recapitulation of the analysis of variance.

| Variable | Chlorophyll *a* | Chlorophyll *b* | Total chlorophyll | Root length |
|---|---|---|---|---|
| Genotype | $0.140^{**}$ | $0.019^{**}$ | $0.273^{**}$ | $17.91^{**}$ |
| Al treatment | $1.09^{**}$ | $0.09^{**}$ | $1.83^{**}$ | $65.3^{**}$ |
| Interaction | $0.310^{**}$ | $0.029^{**}$ | $0.528^{**}$ | $5.031^{**}$ |

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*Selection based on sensitivity index*

Sensitivity index is a measure that describes the deviation of the genotype value from the optimum environmental stress. Greater sensitivity is shown by higher index value and greater reduction in the phenotype. Tolerant genotype is a genotype that has phenotypic deviations lower than the other genotypes.

Identification of tolerant genotypes using univariate analysis was performed by using the sensitivity index values. Higher sensitivity index indicates tolerance to Al stress. Sensitivity index of the physiological character of root length was used to seperate tolerant genotypes (WHP and Numbu) from the sensitive genotypes of 150-21-A, 5-193-C, 150-20-A, UPCA and 10-90-A (Table 2). Root length is a good indicator of growth as a result of the interaction between genetic and environmental factors (Taiz and Zeiger, 2002). In this study, sensitive genotypes (UPCA and 150-20-A) suffered stunted growth due to Al stress. Al stress in acid soils and nutrient deficiency cause stunted growth in plants (Kochian and Hoekenga, 2004).

Table 2. Selection of tolerant genotype based on sensitivity index of root length.

| Genotype | Root length (cm) | | % decrease | Sensitivity index | Criteria |
|---|---|---|---|---|---|
| | Control | Al stress | | | |
| 150-21-A | 20 | 14 | 43 | 1.47 | Sensitive |
| 5-193-C | 20 | 14 | 43 | 1.47 | Sensitive |
| 10-90-A | 20 | 14 | 43 | 1.47 | Sensitive |
| WHP | 20 | 17 | 18 | 0.60 | Tolerant |
| 150-20-A | 20 | 14 | 42 | 1.47 | Sensitive |
| NUMBU | 20 | 20 | 0 | 0.00 | Tolerant |
| UPCA | 17 | 13 | 31 | 1.05 | Sensitive |

Table 3. Chlorophyll *a* content of genotypes as affected by Al stress.

| Genotype | Chlorophyll *a* content (mg/l) | | | % decrease | |
|---|---|---|---|---|---|
| | Control | Al | Al+P | Al | Al+P |
| 150-21-A | 1.96 | 1.60 | 2.29 | 22.50 | -30.13 |
| 5-193-C | 2.05 | 1.28 | 2.01 | 60.16 | -36.32 |
| 10-90-A | 2.05 | 2.00 | 1.38 | 2.50 | 44.93 |
| WHP | 1.51 | 1.67 | 2.02 | -9.58 | -17.33 |
| 150-20-A | 1.66 | 0.67 | 2.22 | 147.76 | -69.82 |
| NUMBU | 1.44 | 1.77 | 1.91 | -18.64 | -7.33 |
| UPCA | 1.46 | 1.02 | 2.08 | 43.14 | -50.96 |

Table 4. Chlorophyll *b* content of genotypes as affected by Al stress.

| Genotype | Chlorophyll *b* content (mg/l) | | | % decrease | |
|---|---|---|---|---|---|
| | Control | Al | Al+P | Al | Al+P |
| 150-21-A | 0.72 | 0.59 | 0.80 | 22.03 | -26.25 |
| 5-193-C | 0.68 | 0.48 | 0.70 | 41.67 | -31.43 |
| 10-90-A | 0.70 | 0.71 | 0.51 | -1.41 | 39.22 |
| WHP | 0.55 | 0.63 | 0.74 | -12.70 | -14.86 |
| 150-20-A | 0.60 | 0.28 | 0.76 | 114.29 | -63.16 |
| NUMBU | 0.51 | 0.64 | 0.68 | -20.31 | -5.88 |
| UPCA | 0.53 | 0.39 | 0.69 | 35.90 | -43.48 |

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The chlorophyll content (chlorophyll *a*, chlorophyll *b* and total chlorophyll) as in Tables 3, 4 and 5 implied that 10-90-A, WHP and Numbu were tolerant genotypes to Al stress. Numbu was known for its tolerance to Al as determined by the sensitivity index (Table 2). The tolerant genotypes were able to maintain chlorophyll *a*, *b* and total chlorophyll content under low Mg condition due to Al stress. The ability to maintain chlorophyll content is closely related to the plant ability to stay green despite stress condition and hence, maintain good photosynthesis. The ability to increase chlorophyll content in culture (Numbu) with Al stress was shown by tolerant genotypes. The same result was shown by Agustina et al. (2010), where the tolerant sorghum genotype of Numbu is capable to produce higher yield of dry matter in acid soil as compared to sensitive genotypes.

Table 5. Total chlorophyll content of genotypes as affected by Al stress.

| Genotype | Total chlorophyll content (mg/l) | | | % decrease | |
|---|---|---|---|---|---|
| | Control | Al | Al+P | Al | Al+P |
| 150-21-A | 2.68 | 2.20 | 3.09 | 21.82 | -28.80 |
| 5-193-C | 2.73 | 1.76 | 2.71 | 55.11 | -35.06 |
| 10-90-A | 2.75 | 2.71 | 2.71 | 1.48 | 0 |
| WHP | 2.05 | 2.30 | 2.76 | -10.87 | -16.67 |
| 150-20-A | 2.26 | 0.95 | 2.98 | 137.89 | -68.12 |
| NUMBU | 1.95 | 2.40 | 2.59 | -18.75 | -7.34 |
| UPCA | 1.99 | 1.42 | 2.77 | 40.14 | -48.74 |

The difference in sensitivity index of the characters in the genotypes may complicate the grouping of genotype tolerance to stress. Multivariate sensitivity analysis based index is an approach that can be used to summarize genotype tolerance in a biplot. The influence of genotype and treatment of Al and P on chlorophyll *a*, chlorophyll *b* and total chlorophyll as in Tables 3, 4 and 5 is only effectively expressed by tolerant sorghum genotypes (10-90-A, WHP and Numbu). Availability of P in the growing media can directly influence the growth of plants tolerant to Al. P deficiency can limit plant growth, and the response will vary depending on the type of plant. According to Agustina et al. (2010), Numbu showed very consistent level of tolerance to Al toxicity and P deficiency in both the field and nutrient culture tests.

*Sensitivity index based multivariate analysis for grouping of tolerant and sensitive genotypes*

Tolerant genotype is a genotype that does not just have a single character that indicates tolerance but many properties. The existence of interdependence between observations and characters makes identification of tolerant genotypes difficult. Principal component analysis is an approach that can be used to identify tolerant genotypes for all the characters at the same time. Multivariate analysis of tolerant character based index and a decrease in the performance index are used in preparing biplots. Genotype having higher index value is the more tolerant genotype. The results of biplot analysis showed that genotypes of Numbu, WHP and 10-90-A had better tolerance to Al stress, based on all the under study characters, as compared to the other genotypes (Figure 1).

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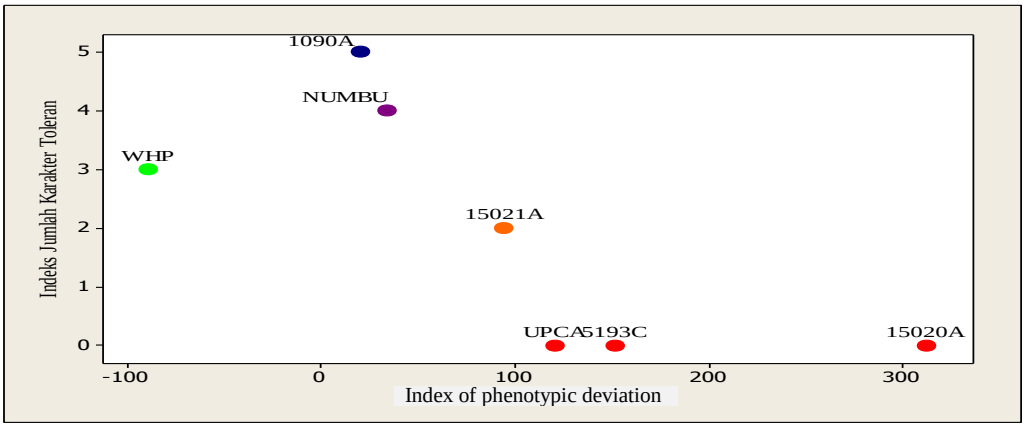


Figure 1. Biplot based on decrease in genotype variability index values; red: most sensitive for all the characters (UPCA, 5-193-C, 150-20-A); orange: relative tolerant (150-21-A); green, purple and blue: tolerant genotypes (10-90-A, WHP, Numbu).

The univariate and multivariate analyses of characters complete the identification of tolerant genotype. Two-dimensional mapping of the index values of characters and a decrease in the performance index showed that WHP had the highest tolerance to Al, followed by 10-90-A and Numbu. Genotypes of 150-20-A, UPCA and 5-193-C had the lowest tolerance, or in other words, were the Al sensitive genotypes.

In general, the results of univariate and multivarate analysis were rather similar in this study on sorghum. However, multivariate analysis provides better results for screening of tolerant genotypes. Based on this analysis, we identified three Al stress tolerant sorghum genotypes.

## Conclusions

The results showed that the tested genotypes had different responses to Al stress. The tolerant genotypes had higher root length, chlorophyl *a*, chlorophyl *b* and total chlorophyll content as compared to sensitive genotypes. In univariate selection, 10-90-A, WHP and Numbu were classified as Al tolerant genotypes while 150-21-A, 5-193-C,150-20-A and UPCA-S1 were classified as Al sensitive genotypes. The tolerant genotypes could be used as potential parents to develop a new Al tolerant sorghum variety.

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# CHAPTER 3

# POSTHARVEST TECHNOLOGY AND QUALITY CONTROL

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# Challenges in Postharvest Handling of Fruits and Vegetables: Reducing Losses and Maintaining Quality

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## Introduction

There are two terms in the postharvest system that is used to define losses which are postharvest losses and food losses. Postharvest losses can be defined as the losses that occur between the completion of harvesting and the moment of consumption by human or consumers (Harris and Lindblad, 1976). De Lucia and Assennato (1994) however, define postharvest losses as a loss in the product during various stages in the postharvest system that can be measured quantitatively and qualitatively. According to Gustavsson et al. (2011), food losses are defined as a decrease in edible food mass during production, postharvest, processing and distribution in value chains that are directed to human consumption. From all the definitions, it can be concluded that postharvest losses are all directed from the point of how producers handle their harvests until it reaches the consumer. These losses have become a major issue in many countries whereby the estimated losses in terms of weight and quality are about 10% to 30% (Hodges et al., 2011). In a study done by the African Postharvest Losses Information System (Rembold et al., 2011), most of the weight loss from the product is due to harvesting or field drying, which is about 4% to 8% fruits and vegetables have the highest rate of wastage as compared to other products at 45% (Anon, 2012). The losses in agricultural production dominate the three industrialized regions whereby the postharvest losses are due to grading caused by quality standards set by retailers which can be seen in Figure 1. In South and Southeast Asia, the losses are due to pre-harvest practices (15%), postharvest practices (10%), processing (18%), distribution (5%) and consumption (5%) (FAO, 2012).

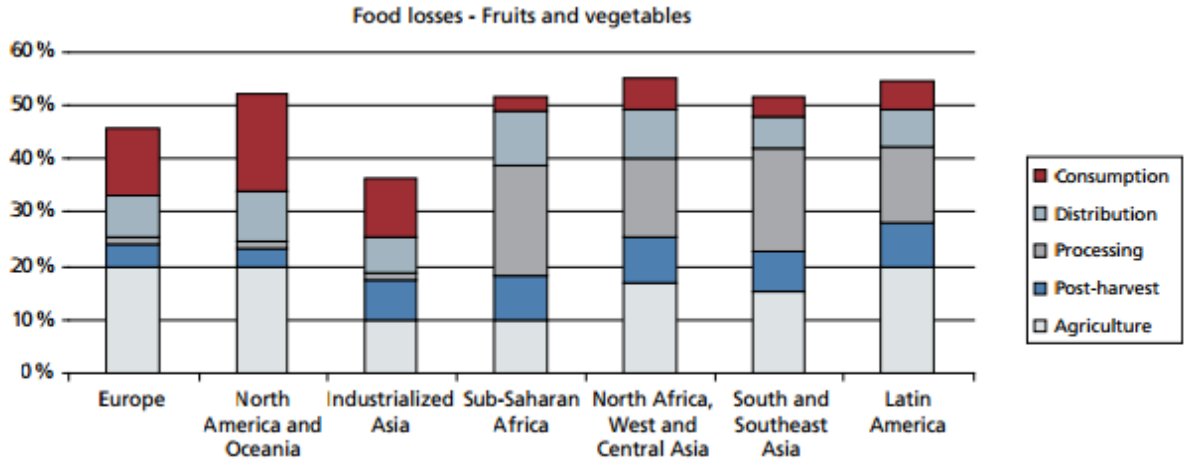


Figure 1. Percentage of postharvest losses for fruits and vegetables in different regions (Gustavsson et al., 2011)

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**Cause of Losses**

The causes of losses are physiological decay, physical damage and water loss or there is a surplus in the market. There are two main causes of losses which are physiological and physical changes. The physiological decay depends on the rates of respiration, transpiration, maturity and senescence, which in turn are influenced by temperature, relative humidity and atmospheric composition. This can be seen in a study done by Ferrante and Maggiore (2007) that a higher storage temperature (10 ºC) will hasten chlorophyll and carotenoid loss as compared to storing at a low temperature (4 ºC). A study done by Banaras et al. (2005) showed that peppers that are stored at 8 ºC only loose their quality after 10 days as compared to peppers that are stored at 20 ºC due to high water loss. This study showed how temperature and relative humidity have an effect on the quality of the fruit during storage. According to a review by Hodges et al. (2011), growing consumer intolerance of substandard food or defect produce has increased the rejection rate of a produce and thus the postharvest losses will also increase. For example, grading of produce according to consumer's preferences has led to the wastage of produce that did not meet the specifications. The amounts of produce being supplied to consumers are mainly influenced by price variation, weather and consumer demand.

Physical changes are related to mechanical damages, water stress, sprouting and rooting, physiological disorders, insect pest and pathological breakdown. These losses can be slight or substantial, leading to a reduction in the quantity, quality and safety of fruits and vegetables. These losses can be seen in a study done by Genova et al. (2006) on postharvest loss of chillies in Vietnam whereby the losses are due to diseases (90%), damage during harvest (10%), damage during transportation (15%) and poor quality fruits (10%). In a survey done in collaboration between the World Vegetable Center (AVRDC) and Department of Agronomy and Agricultural Land Improvement (DAALI) (2005), the main reasons for cucumber postharvest loss at farm level in Cambodia are due to harvesting during hot weather (42%) and diseases (46%). The same trend can be seen for Chinese kale whereby postharvest loss at farm level in Cambodia due to harvesting during hot weather is 81% and due to diseases is 75% (AVRDC and DAALI, 2005). In the two studies, the time of harvesting the produce is also important to reduce the postharvest losses by minimizing the physical change of the produce. High postharvest losses when produce is harvested during hot weather are due to the loss of water from the produce. When there is a high water loss from the produce, the produce will lose its turgidity and this will cause an unpleasant sight for the consumers and they will not purchase the produce.

**Assessment of Postharvest Losses**

It is important to assess the postharvest losses to ensure that the product is being compensated by being able to produce more than the usual standards. One of the methods that can be used to assess the postharvest losses is the Commodity Systems Assessment Method which has 27 components that take into account all steps related to the production, postharvest handling and marketing of any commodity (LaGra, 1990). This process can assist in determining the source of postharvest, the causes of those losses and also the economic value of the losses compared to the costs of current and proposed postharvest practices. The questionnaire that is used in this method can be seen in the manual by LeGra (1990).

The purpose of assessing postharvest losses is to identify at which stage postharvest losses is the highest and also to identify the most suitable strategy to reduce postharvest losses. Besides using a questionnaire to determine the amount of losses that occur at each stage, product sampling at each stage can also be done to determine the optimum maturity of a product. For example, in a research conducted by Fehr and Romao (2001) to measure the losses of fruits and vegetables in Brazil, product sampling was done on growing, marketing, consumption and disposal stages. This research was done to look at the specific reasons of wastage in Brazil and it is found that the marketing stage has the highest wastage at 16.6%

weight. From this study, we can conclude that most of the wastage that occurred in Brazil is identified at the level of producer, wholesaler and retailer. With this information, awareness on how to improved postharvest handling at those stages can be implemented. In another example, Udas et al. (2005) concluded that the highest wastage in the Eastern Hills of Nepal is mainly due to packaging whereby losses as high as 47% for cauliflower from harvest to retail can be seen.

Physico-chemical quality of the produce is also vital in reducing postharvest losses in the world especially in Asia. Physical characteristics that take into consideration are colour, firmness, weight loss, skin browning, decay and defects whereas chemical characteristics such as soluble solids concentration, titratable acidity, pH and ascorbic acid contents need to be evaluated. Biochemical changes in fruits and vegetables during their growth and development is an important factor in fixing the appropriate maturity standards of a particular produce both for local and distant markets. For example, the harvesting 'Kew' pineapple was found to be around 146 to 150 days after flowering which will produce a soluble solids concentration of 18 to 19 ˚Brix, titratable acidity of 0.77 to 0.83% and ascorbic acid of 14.92 mg/100g (Deka et al., 2005). These analyses could also help producers to maximize profit by harvesting at the optimum maturity stage to help maximize consumer satisfaction, add value to the production, reduce wastage from agricultural inputs and choosing the earlier ripening variety. This can be seen in a study done on four accessions of hot pepper whereby each accession has their own harvesting time and storage life ranging from less than one week to more than three weeks (Barrera et al., 2005). In this study, it is also discovered that accession CS219 appeared to be earlier as compared to the other accession. According to Joshi and Roy (1988), soluble solids concentration, starch, sugar, total carotenoid pigments and pH are positively correlated with fruit maturity, whereas moisture content, acidity, ascorbic acid and tannins are negatively correlated. Thus, it is important to harvest the produce at the right stage of maturity to balance out the nutrients in the product. Weight loss in a produce depends on the environment where the produce is kept. It is found that the weight loss of okra showed a positive correlation to the storage duration (Hassan et al., 2010). However, the rates of moisture loss or weight loss are also influenced by the packaging of the product. This can be seen in the study by Hassan et al. (2010), whereby the weight loss in okra is minimal when stored at 10 ˚C with modified atmosphere packaging and the highest weight loss was without packaging and stored at ambient temperature.

**Methods to Reduce Postharvest Losses**

There are various technologies that have been developed to reduce postharvest losses in every stage in the supply chain, but they have not been effectively adopted or implemented. These might due to the lack of training and information exchanges on postharvest handling, inadequate and unavailable tools and equipment to carry out postharvest handling practices, lack of proper transportation, inadequate maintenance of storage facilities and improper marketing systems. Most of these problems occur in countries that are less developed as can be seen in a study carried out in the Eastern Hills of Nepal whereby the main factors responsible for postharvest losses were inappropriate packaging, transportation and grading systems (Udas et al., 2005). This was mainly due to the farmers who are not trained and exposed to proper postharvest handling procedures which can be seen in the selected farm in this study whereby no pre-cooling of produce was adopted both on the farm and collection centre.

Kader (2005) suggested that producers of major commodities should be encouraged to cooperate in order to market their produce. This is because of the small farm size that are scattered around in developing countries. Some of the advantages of marketing cooperatives include being able to provide a central accumulation centre for harvesting commodity, purchasing of agricultural inputs in quantity to reduce costs, providing a place for proper preparation for market and storage when needed, facilitating transportation to the markets, coordinating the marketing program, distributing profits equitable and also distribution of knowledge in the postharvest area. The lack of information on the postharvest handling of

produce in farmers or producers is also one of the main concerns. In order to ensure that any information is readily available, an effective and educational extension program should be carried out by experts that are able to reach the farmers or producers easily.

**Network of Different Agencies to Reduce Losses**

In order to reduce the postharvest losses at every step, it is important to have a network of different agencies to reduce losses. The network of excellence postharvest food losses (NoE) definition process includes relevance, vision, objective, target group, shareholders, stakeholders, network focus and content (Gogh et al., 2013). The relevance of NoE lies in the black spot in which the network will be an additional help to the current practices where PHL is a major issue. This is derived from the lack of knowledge about PHL and the transfer of knowledge to countries with high postharvest losses. The vision of Noah is to recruit an excellent network of experts, sustain local knowledge development, multiformity of involved parties and have projects/investments that have an impact on the local supply chain. The objectives are to have a multidisciplinary approach, transfer and development of knowledge and changes in supply chain systems that are sustainable to a target group which has a direct interest and connection in solving postharvest loss problems such as farmers, retail companies, trades, knowledge institute and others. Next are the shareholders, which play an active role in reducing postharvest losses for the benefits of third parties in developing countries and are selected based on their reputation and track record in their professional field. Stakeholders are those who have a direct interest in the field of operation and activities and may participate based on case by case. Some of the stakeholders are Food and Agriculture Organization (FAO), Asia-Pacific Economic Cooperation (APEC), Multilateral Development Bank, Monetary Financial Institutions and others. The network focuses on products such as fruits and vegetables as they are highly perishable, and they are gaining importance in the local and global supply chains. Besides that, they also focus on the geography of the country whereby food losses in developing countries showed a higher food loss in the upstream activities. A third focus is the content whereby they provide an opportunity for all postharvest experts in order to improve the postharvest performances in the fresh supply chain.

**Conclusions**

Large losses in fruits and vegetables are mainly due to postharvest practices such as improper handling (transport and storage) which will cause the product to be rejected, damaged or reduce in quality. Besides that, consumer awareness of the quality of fruits and vegetables (colour, taste, shelf life, freshness) has caused producers to grade and discard more produce after harvest. This is due to the inability of the producers to maintain their produce quality after harvest to meet the consumer demand. In order to meet consumer demand and also food security, it is important for the producers to be knowledgeable in postharvest handling of fruits and vegetables, in addition to the usage of technologies that have been introduced. Government agencies play an important role in extending service whereby experts in postharvest handling reach out and transfer knowledge to the producers especially in the rural areas. Incentives can also be given to producers that are able to produce good quality fruits or vegetables and at the same time reduce postharvest losses. However, in order to reduce postharvest losses, the main factor is to change the mindset of the producers and consumers so that they will take this matter seriously. In summary, the goal is to ultimately zero down postharvest losses in the future through technology and knowledge.

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# The Growth and Postharvest Performance of Misai Kucing (*Orthosiphon stamineus* Benth) in Relation to Different Soilless Growing Media and Biochar

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## Introduction

Misai kucing or *Orthosiphon stamineus* Benth. is belongs to the family of Lamiaceae. In Malaysia, there are two varieties of Misai Kucing known as MOS1 and MOS2 (Zaharah, 2005). Currently, the production of local herbs mainly Misai Kucing increased year by year indicating the demand is increasing. However, Misai Kucing demand exceed its supply particularly from pharmaceuticals, cosmetics, health care, heal enhancing products, dietary supplements, flavours and fragrances and toiletries and various consumer goods (ECER, 2011). Misai Kucing has become increasingly important worldwide since it has been reported to contain terpenoids, polyphenols, and sterols which have an antiallergic, antihypertensive, antiflammatory and diuretic properties and it is also used as a treatment for anteriosclorosis (capillary and circulatory disorder), ghout, rheutism, kidney stones, diabetis and nephritis (Tezuka et al., 2000). Thus, it is a great challenge to Malaysia government to focus and explore the imperative herbs industry mainly Misai Kucing. In Terengganu, Misai Kucing is not yet exploited due to a large area of Beach Ridges Interspersed with Swales (BRIS) soil which is infertile due to poor physico-chemical characteristics. One of the possible alternatives to explore Misai Kucing cultivation is by using fertigation system. This planting system has been widely used in growing tomato, rockmelon, chilli, and strawberry. However, there is lack of research on growing of Misai Kucing using soilless growing system.

The most common soilless growing media used in fertigation is coco peat (CP) from coconut husk which has the ability to enhance strong and healthy root system, water and nutrient holding capacity. Other than that, new organic media have been widely used to replace soil which is believed to have an excellent moisture holding capacity such as Oil Palm Fruit Bunch (OPFB) and Biochar. OPFB is widely used as mulch for plant cultivation due to highly potential to improve the soil nutrient levels by increased the soil exchangeable K, Ca, Mg, and pH. In the present study, OPFB with the combination of CP were used as newly growth media to determine the growth and postharvest performance of Misai Kucing. Additionally, biochar is an amendment to soilless substrates, which has been reported to improve plant growth and as well as induce systemic resistance to disease (Elad et al., 2010; Graber et al., 2010). However, it is not yet commercially used in herbs plant cultivation. The application these soilless growing media and its combination could be the effective approach to be applied in improving the growth and postharvest performance of Misai Kucing. The information on growth and postharvest performance of Misai Kucing grown on soilless growth media under fertigation system is scarce and not yet extensively studied. Therefore, this study aimed to determine the effects and the best combination of different soilless growing media and biochar on the growth, yield and postharvest performance of Misai Kucing.

## Materials and Methods

The experiment was carried out at the Greenhouse, Universiti Malaysia Terengganu. Misai Kucing plants and coco peat, were purchased from Department of Agriculture, Ajil, Terengganu and Bumi Agro Sdn Bhd, Kuala Terengganu respectively. While, biochar and OPFB were obtained from BERNAS Rice Mill

at Tumpat, Kelantan and Oil Palm Plantation, Terengganu, respectively. Thirty-six of two-node stem cuttings (10-15 cm) of Misai Kucing were used and immediately transfer into seedling tray containing sand. Mature stem cuttings, aged 4-5 weeks, were transplanted into polybags containing different treatments as described below. Each polybags contain 3 kg of growth media either alone or combinations. All Misai Kucing plants were irrigated by using fertigation system. Irrigation has being equipped with 8 L/hr dripper and scheduled for 6-7 min/day.

The experiment was arranged in a Randomized Complete Block (RCBD) with three replications. The treatment were: i) coco peat alone (Cp, 3 kg), ii) coco peat + biochar (Cp, 2.85 kg + Bc, 150 g), iii) coco peat + oil fruit bunch (OPFB) (Cp, 15 kg + Op, 1.5 kg), iv) OPFB alone (3 kg) v) OPFB + biochar (Op, 2.85 kg + BC, 150 g) and vi) Cp + Op + Bc (1:1:1). Two trees represented one experimental unit. Parameters evaluation were pre and postharvest parameters such as stem diameter, number of branches, leaves area, fresh weight (leaves and stem), and dry weight (leave and stem), mineral nutrients in leaf, branch and root [nitrogen (N), pottasium (K), calcium (Ca), and magnesium (Mg)] (Husni et al., 1990). chlorophyll content.

The data were subjected to the analysis of variance (ANOVA) using GLM (General Linear Models) procedures and further separated by LSD for least significance at $P \leq 0.05$ (SAS Institute Inc., 1999).

**Results and Discussion**

The combination of Cp+Bc was significantly higher in number of branches, stem diameter, leaf area, fresh weight, dry weight and cumulative fresh weight (leaves and branches) of Misai Kucing as compared to other media (Figure 1, 2, 3, 4, 5, 6 and 7). However, Cp+Bc had similar values in all parameters as mentioned above with Cp alone. This, reflected to the ability of Cp+Bc media to replace or better than Cp alone based on the comparable values of pre- and postharvest performances evaluated. Wira et al. (2011) claimed that the growth of cherry tomato on Cp+Bc might be associated to the change in soil structure, soil organic content, and also aeration capacity. This was supported by Graber et al. (2010) who claimed that the two possibility of biochar application with other growth media might be stimulated the development of beneficial microorganism which promote plant growth or chemical in biochar directly elicit positive plant responses.

Other than that, the hidden quality attributes evaluated were chlorophyll content, total chlorophylls, macro and micro nutrient content. The chlorophyll content showed the higher value of chlorophyll a and carotenoid which were obtained in plants grown on Cp+Op. While, no apparent effect of chlorophyll b was recorded for all the treatments used (Figure 8). This was in agreement with Erwan et al. (2013), who claimed that the highest value of chlorophyll content in cauliflower were found in plant grown on Cp+Op. Possibly, the plant chlorophyll absorbs sunlight, which converts $CO_2$ and water into glucose (Mansfield et al., 1990; Sims and Gamon, 2003). However, in the present study, the total chlorophyll content was not differ among soilless growing media applied.

Meanwhile for mineral nutrients, the N content in leaf, branch and root was the same among growth media (Figure 9). Contradictly, Erwan et al. (2013) reported that the optimum levels of N were found in the soilless cauliflower grown on Cp+Op. Other macronutrients such as K in Misai Kucing leaf had higher concentration and comparable to Cp alone and Cp+Op (data not included). However, K concentration in branch and root were the same among treatments. In general, the concentration of Ca and Mg in leaf, branch and root showed comparable value among treatments. Regardless of growth media used, the concentration of N was higher in Misai Kucing leaf (ranging from 1.16% to 1.67%), followed by roots (0.01% and 0.43%) and branch (0.04% and 0.21%). In descending order, the K concentration was the highest in leaf and branch (ranged 4.46% to 5.26% and 4.66% to 4.97%, respectively) followed

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by roots (range between 1.94% and 2.50%). Meanwhile, the Ca concentration, leaf > branch > root. Similar pattern was also observed for Mg.

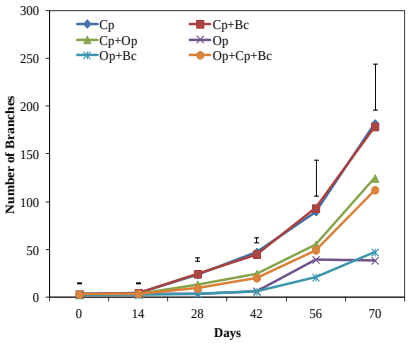


Figure 1. Effects of different soilless growing media and biochar on number of branches of Misai Kucing. Vertical bars represent $LSD_{0.05}$

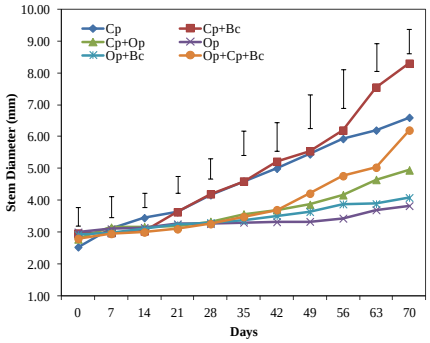


Figure 2. Effects of different soilless growing media and biochar on stem diameter of Misai Kucing. Vertical bars represent $LSD_{0.05}$

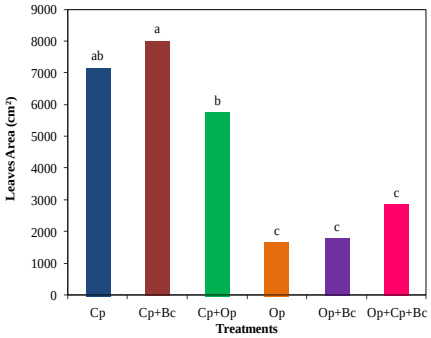


Figure 3. Effects of different soilless growing media and biochar on leaf area of Misai Kucing. Means with different letters are significantly different at the 5% level according to LSD test.

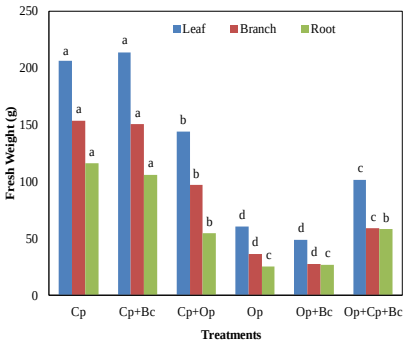


Figure 4. Effects of different soilless growing media and biochar on fresh weight of Misai Kucing. Means with different letters are significantly different at the 5% level according to LSD test.

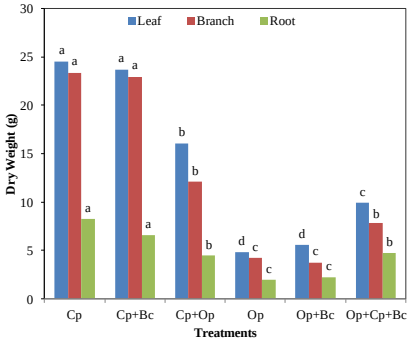


Figure 5. Effects of different soilless growing media and biochar on dry weight of Misai Kucing Means with different letters are significantly different at the 5% level according to LSD test.

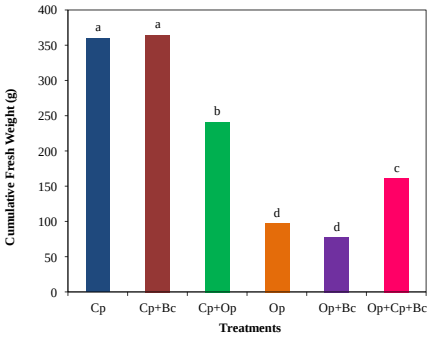


Figure 6. Effects of different soilless growing media and biochar on cumulative fresh weight of Misai Kucing. Means with different letters are significantly different at the 5% level according to LSD test.

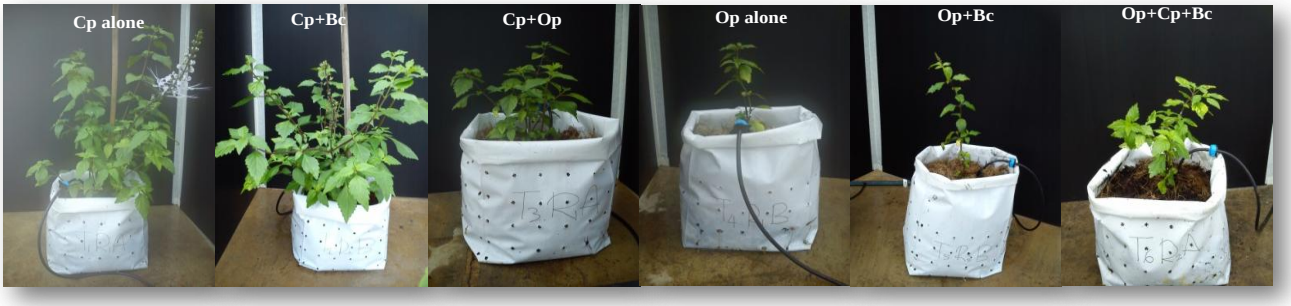


Figure 7. Effect of different soilless growing media and biochar on plant growth of Misai Kucing. Cp alone (coco peat alone), Cp+Bc (coco peat+biochar), Cp+Op (coco peat+Oil palm fruit bunch), Op alone (Oil palm fruit bunch alone), Op+Bc (Oil palm fruit bunch+biochar) and Op+Cp+Bc (Oil palm fruit bunch+coco peat+biochar).

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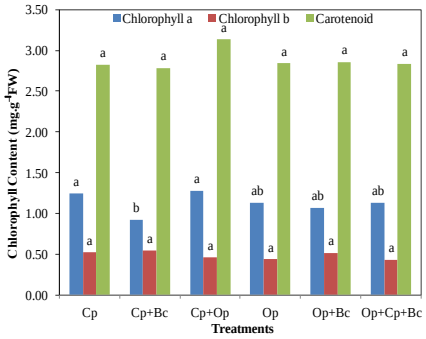


Figure 8. Effects of different soilless growing media and biochar on chlorophyll and carotenoid content of Misai Kucing. Means with different letters are significantly different at the 5% level according to LSD test.

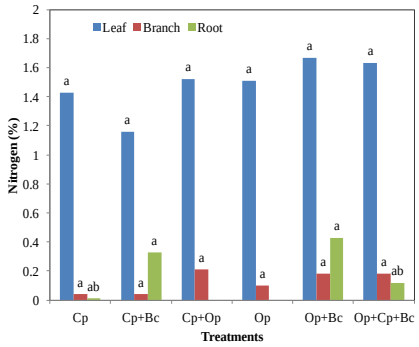


Figure 9. Effects of different soilless growing media and biochar on macronutrients in leaf of Misai Kucing. Means with different letters are significantly different at the 5% level according to LSD test.

## Conclusions

As a conclusion, Cp+Bc growth media had potential to replace commercial growth media (Cp alone) as its exhibit higher and comparable values of yield and growth without significant reduction in postharvest quality attributes. In addition, this newly-developed soilless growth media are light and easy to handle and cost effective. Besides, the usage of fertilizers and environmental pollution could also be reduced by minimizing rice husk waste.

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# Effects of Collecting Systems and Plantation Environment on Debris Accumulation in a Collected Oil Palm Loose Fruits

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## Introduction

The oil palm is one of the key crops that contribute in changing the scenario of Malaysian agriculture as well as the economy. Currently, oil palm plantation sector is a strategic sector of the economy and has contributed the export earnings of about RM61.36 billion for the country from various palm oil based products. The oil palm plantation is also growing steadily to play its role as the main backbone of commodity crop sector in this country. It could be seen from the escalation of total oil palm planted area at about 5.23 million hectares in the year 2013, an increase of 7.27% from 4.85 million hectares recorded in the year 2010 (MPOB, 2014).

The collection of loose fruit is one of the important processes in the oil palm harvesting that need great and serious attention since there is an economic value of the fruits for upstream production of palm oil. According to Turner and Gillbanks (2003), loose fruits are considered as apart of yield, which is derived from oil palm trees, besides a fresh fruit bunch (FFB). Normally in the process of oil palm harvesting, the ripeness of oil palm FFB is determined by the number of fruits that has been detached from the bunch before it cut.

As part of the yield, the plantation management always insists on zero loose fruits left in the estate field in order to minimize the decline of the oil extraction rate due to uncollected loose fruits and at the same time inclines the profits. Seedlink (2008) reported that a loose fruit offers 40% of the oil extraction rate per weight ratio or much higher as compared to 20 to 25% of the oil extraction rate per weight ratio given by a bunch. In fact, a proper collection of only six loose fruits per palm could give an additional RM30 million in net profit to the oil palm plantation company. On the contrary, the loose fruits can give massive lost incomes if loose fruits not well-managed or uncollected

Currently the collection of loose fruits in oil palm plantation is a labor-intensive operation. During the collection process, the worker walks along planting rows while carrying along with him either a sack or wheelbarrow and rake. He stops at the palm tree, collects the scattered loose fruits with rake into one lump before lifting them into the sack or the wheelbarrow until it reaches its full capacity. These activities are repeated at every harvested palm tree until the whole harvested areas are completed.

One of the major problems in the collection of loose fruits is the debris accumulation associated in a collected loose fruits. Debris as defined by Warner (2006) is leaves and anything else that is not part of the fruit. Ahmad et al. (1995) mentioned that debris accumulated in a collected loose fruits can reach up to 60% of its total weight. Shuib and Khalid (2005) stated that the large amount of debris in a collected loose fruits will affect the mill productivity since debris will absorb the oil content of the fruit, hence reducing the oil extraction rate.

It is clear that the loose fruits should be clean before processing it to the mill in order to obtain a higher oil extraction rate. However, it is very difficult for the workers in collecting the loose fruits effectively and without any debris since there are a lot of obstacles faced by the workers such as plantation environment, limitation of collecting system technology used, long working hours, loose fruits condition, increasing the number of loose fruits due to over-ripe bunch and also physical limitation of workers as a human.

The debris accumulation in a collected oil palm loose fruits has made the workers loosing a lot of valuable time. Other than that, the plantation management also has to expense an extra cost to separate the debris from a collected loose fruits or to minimize the percentage of debris in an acceptable level before processing it in the mill. Some palm oil mills even give punishment to the estate management if they keep sending collected loose fruits mixed with debris beyond the acceptable level.

Until today, there is no comprehensive study explored on the effect of collecting systems and plantation environment on debris accumulation in a collected loose fruits. Studies reported by Amirshah and Hoong (2003) and Hitam et al. (1995) were focused on the performance of a rake that is being used as a tool in loose fruits collections in the estate. However, this study did not explore the effects of other conditions such as types of containers used and plantation environment, i.e. level of ground cleanliness of plantation, loose fruits conditions and working experience of the workers to the debris accumulated in a collected loose fruit. All these conditions may influence the debris accumulation in a collected loose fruits.

This study investigates the effects of collecting systems and plantation environment on the percentage of debris accumulation in a collected oil palm loose fruits. Comparisons of debris accumulation in a collected loose fruit as a result of the effect of two above different conditions are presented.

**Materials and Methods**

The comprehensive data collection was carried out in the month of February 2012 at five oil palm estates in Pahang State, Malaysia. The terrain conditions of the estates varied from flat to undulating. A total of 15 plantation workers that have familiarized with the collection of loose fruits were involved in the study. These subjects were randomly selected to sufficiently represent a total of 20 workers population in the estates. Throughout the data collection period, the daily ambient temperature of the estates was in the range of 24 to 35 $^{o}$C. Wind speeds during data collection ranged from 6 to 10 km/h while the relative humidity between 67 to 88%. All the weather data was taken from Viewweather (2012). The collecting systems used were from the two types of the common containers, i.e. sack and wheelbarrow for in field loose fruits collection. Plantation environment refers to the plantation ground cleanliness, working experience and loose fruits conditions. Each subject was assigned to collect loose fruits randomly in his usual manner for three harvesting paths under the same working conditions. Once the container reached its full capacity, the worker recorded the total weight of loose fruits associated with debris using a portable digital weighing machine in the field. Debris accumulated was then separated from a collected loose fruits. Immediately after the separation, he recorded the total weight of debris accumulated only using the same weighing machine. Debris accumulation on a collected loose fruits was calculated by dividing the total weight of accumulated debris with the total loose fruits and accumulated debris and multiplying by 100%.

The T-test and Analysis of Variance of the statistical analysis produced in PC SAS Ver 6.12 software were employed to analyze the collected data. The T-test was used to test the significant effects of two set data of percentage of debris in a collected loose fruits that obtained from sack and wheelbarrow containers used, wet and dry loose fruits condition, < 3 years and > 3 years working experiences. The ANOVA was employed to determine the significant effect of debris accumulation in a collected loose

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fruits under different of ground cleanliness of the estates. Duncan's multiple range test (DMRT) was used to statistically compare means values of the percentage of debris accumulation in the loose fruits.

**Results and Discussion**

There was a significant difference of mean percentage debris accumulation in a collected loose fruits between using a sack and wheelbarrow as shown in Table 1. The mean percentage of debris accumulation in a collected loose fruits using sack was found to be 34.74% of the total weight of collected loose fruits or 11.14% higher than that of the wheel barrow.

Table 1. The mean percentages of debris accumulation in a collected loose fruits using differenttype of containers.

| Type of containers | Mean percentage of debris[1], % | Different,% |
|---|---|---|
| Sack | 37.74[a] | 11.14 |
| Wheelbarrow | 26.60[b] | |

*[1]Means in a given column having suffices with different letter are treated as significantly different at 0.05 probability level*

Several factors have caused the different percentage of debris accumulation in a collected loose fruits between two types containers used. Saving a collected loose fruits into the sack during the collection operation is difficult to be inspected by the estate supervisor. This is because the collected loose fruits were covered by the sack so that it cannot be seen by the supervisor. Normally under this condition, the worker has a tendency to mix a lot of debris along a collected loose fruits into the sack with the purpose of increasing the quantity of sacks that they can be filled with loose fruits in a day. By doing this, the workers can significantly increase their income because usually the payment for the workers was made by the estates management based on the amount of sacks occupied with a collected loose fruits in a day. The situation was different when the worker using a wheelbarrow. The collected loose fruit was easy to be watched by the estate supervisor because the wheelbarrow is an exposed container. The supervisor can easily identify the amount of debris that being mixed together with a collected loose fruit inside wheelbarrow. This situation has reduced the tendency of the workers collect extra debris in a collected loose fruits to obtain additional sacks occupied since the estates send all the loose fruits into a separator to separate the debris from the loose fruits before transporting them to the mill. The management only pays on the basis of the amount of a collected loose fruits weight.

Table 2 shows that the percentage of debris accumulation in a dry collected loose fruits was slightly higher than that of wet collected loose fruits. However, there was no significant difference of the percentages of debris accumulated between two conditions of a collected loose fruits. It was observed that wet and dry conditions did not give any significant effects on percentages debris accumulated in a collected loose fruits. Thus, the collection of loose fruits can be made even after raining day operation to maximize the daily output of the plantation.

Table 2. The mean percentages of debris accumulation in dry-collected loose fruits and wet-collected loose fruits.

| Loose fruit conditions | Mean Percentage of debris[1], % | Different, % |
|---|---|---|
| Dry | 34.60[a] | 4.05 |
| Wet | 30.55[a] | |

*[1]Means in a given column having suffices with the same letter are treated as not significantly different at 0.05 probability level*

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Table 3 shows that there was a significant difference in the percentages of debris accumulation in a collected loose fruits between the workers who has experienced less than 3 years and more than 3 years. The mean percentage of debris accumulation in a collected loose fruits as results of the workers having experience less than 3 years was 39.75% of the total weight of the collected loose fruits or 18.98% higher than that of the workers having experience more than 3 years.

Table 3. The mean percentages of debris accumulation in a collected loose fruits from two different working experiences of the workers.

| Workers experience levels | Mean Percentage of debris[1], % | Different,% |
|---|---|---|
| Less than 3 years | 39.75[a] | 18.98 |
| More than 3 years | 20.77[b] | |

[1]*Means in a given column having suffices with different letter are treated as significantly different at 0.05 probability level*

The workers, having less than 3 years experienced had tendency to collect more debris alongside the loose fruits. They did not yet have enough skill to operate the relevant tools to minimize the amount of debris accumulation in a collected loose fruits. On the contrary, the workers having more than 3 years working experience have skill to minimize the amount of debris in a collected loose fruits. The experienced workers know how to operate rake and container properly. Besides, the workers, having more than 3 years working experience have been classified in the check roll workers or permanent workers while the workers having less than 3 years working experience are still under contract workers. Since the dissimilarity of their status, the management paid the experienced worker on the basis of the weight of the loose fruits itself while contract or inexperienced workers were paid by the numbers of sack collected.

Table 4 shows there was a significant difference in the percentages of debris on the collected loose fruit between estates 1, 2, 3 and estates 4, 5. The mean percentage of debris accumulated in a collected loose fruits from the above estates was in the range of 19.60% to 39.10% of the total weight of the collected loose fruits. The variation of range was created due to the different manner of estates in maintaining their plantation environment. The major factor has been identified to be able to reduce the debris accumulation in a collected loose fruit are cleanliness of plantation environment. The ways of the estate managing the environment in a particularly cleaning the area around the palm base is very crucial. The estates that conduct a regular palm circle spraying at every six month have offered a low debris accumulation in a collected loose fruits as compared to those estates which did not manage to do so. It has been proven that the more clean the field the less debris accumulation in a collected loose fruits. This agree with Orme (2001) stated that a poor palm circle spraying not only results in high debris accumulation but also a significant loss of loose fruit. Management manner of estates in running the collection of loose fruits also affected the amount of debris accumulation in a collected loose fruits. As mentioned earlier, types of container used and payment method also contributes to the debris accumulation in a collected loose fruits. For example, wheelbarrow container has been proven to be able to reduce the amount of debris as compare to the sack. Indirectly payment on the basis loose fruits itself weight has reduced the amount of debris as compared to the payment based on sack.

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Table 4: Percentages of debris accumulated in a collected loose fruits by different estates management style.

| No. of estates | Mean percentage of debris[1], % |
|---|---|
| Estates 1 | 39.10a |
| Estates 2 | 38.70a |
| Estates 3 | 34.70a |
| Estates 4 | 21.50b |
| Estates 5 | 19.60b |

[1]*Means in a given column having suffices with different letter are treated as significantly different at 0.05 probability level*

## Conclusions

Effects of collecting systems and plantation environment with regard to the types of container used, loose fruits conditions, worker experience and estate environment on percentage of debris accumulation in a collected oil palm loose fruits have been presented. Sack provided 37.74% debris accumulation in a collected loose fruits or 11.14% higher as compared to 26.60% of the wheelbarrow. Statistically, there was no significant difference of the percentages of debris accumulated between two conditions of a collected loose fruits. The percentage of debris accumulation in a dry-collected loose fruits was 34.60% or slightly higher as compared to 30.55% debris from wet-collected loose fruits. The workers having experience less than 3 years gave 39.75% debris accumulation in a collected loose fruits or 18.98% higher as compared to the workers with experience more than 3 years.

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# Postharvest Life of Guava Fruit under Selected Postharvest Handling Practices

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## Introduction

Guava (*Psidium guajava* L.) of Myrtaceae family is one of most popular amongst tropical fruit because containing high phytochemical content especially ascorbic acid which measured containing around 60 to 1000 mg/100g. Guava cultivated area in Malaysia showed increasing trend with only around 1,500 ha in 2009 to almost 3,500 ha in 2013 (KPIAT, 2014). Increase in the cultivation area followed by increase production value pattern of only around RM50 million in 2009 into around RM120 million in 2013. The data indicated an increment of 100% on the cultivated area and around 150% on the production value within five years. Guava has a high economic potential with good yield that can produce fruits of around 15 mt/ha/yr (JPS, 2014). The statistics are expected to continue increase in the near future with opening of more production zooning area with basic infrastructures called Permanent Food Production Area (TKPM) throughout the Sabah state of Malaysia.

Like other tropical fruit, guava was also reported rapidly deteriorates in short time after harvest if not properly handled. According to Omayma et al. (2010), guava is highly perishable, susceptible to mechanical damage and chilling injury. Campbell (1994) reported shelf life of this fruit is only 3 to 10 days at room temperature. This fruit reported has a high respiration and ethylene production which lead to a dramatic change on it physical and chemical attributes (Ali and Lazan 1997). Postharvest life or shelf life is simply the time period that a fruit can be expected to maintain predetermined level of quality under specified storage condition. In other word, the period (in days) between initiation or commencement of ripening (i.e. end of green-life) and end of saleable life or edible life (of the fruit) on the shelf. Fruit which have long postharvest life or ripen slowly would facilitate marketing of the fruit and reduce postharvest losses.

Appearance is one of the major factors that a consumer uses to evaluate the quality of fruits and vegetables because quality could be viewed as an absence of defects or degree of excellence (Shewfelt, 1999). Rapid visual assessment can, with experience, be made on the criteria of size, shape, colour, condition (such as freshness), and the presence of defects or blemishes. Therefore, traditionally the appearance, condition and defects qualities are normally done by experts according to his/her senses of sight, touch and smell. However, using traditional methods are not sufficient to define what the consumers want or look for in a certain product (Ismail et al., 2001).

Several postharvest handling have been recommended to extend the postharvest life of horticulture produce. Temperature management has been the dominant main factor to consider longer postharvest life of any harvested produce. For each 10 $^{o}$C rise in temperature, respiration activity is increased by a factor of two or three. Low temperature has been used to extend the shelf life of fruit and vegetable however, a crucial point of this low temperature is that the commodities showed low temperature injury (Paull, 1993). Polysaccharide based coatings can be used to extend the shelf-life of some fruits and vegetables during short term storage by preventing dehydration, oxidative rancidity, and surface browning (Nisperos-Carriedo, 1994). Similar to coating, packaging has been well known to extend shelf life of harvested produce. In addition, proper packaging will give advantages for easier handling and marketing attraction

as buyer buy form their eye judgment. The objective of this write up is to share results of our continuous study to determine effect of several postharvest handling treatments to maintain visual appearances of guava.

**Materials and Methods**

*Plant materials* - Mature-green, uniform sized (350±50 g) guava cv. Kampuchea fruits were harvested from a Farm in Tenom, Sabah. Only well-formed and blemished-free fruits were selected for the experiment.

*Portable hydrocooler* - A portable hydrocooler (PH) was designed and build at the Postharvest Laboratory, Faculty of Sustainable Agriculture Universiti Malaysia Sabah. The principle operation of the hydrocooler was very simple and the material used was considered low cost (Silip et al., 2003).

*Hydrocooling treatments* - Fruits were precooled by immersing them in water (1.5±1 $^{o}$C) containing 300 µg $ml^{-1}$ active benomyl (Benlate®) for 0, 1/8, 1/4 and 1/2 cooling time (CT) in the PH. Fruit for the control treatment was dipped in distilled water (26±1 $^{o}$C) for 1 minute. The storage temperatures (ST) of fruits were 5, 10 and 15 $^{o}$C and storage durations (SD) were 1, 2, 3, 4, 5 and 6 weeks, at relative humidity of 80±15%. Two fruits from each treatment were taken out weekly for the visual quality analysis. The experimental design was a randomized complete block design (RCBD) with a factorial arrangement of treatments (4 CT x 3 ST x 6 SD), with four replications and two fruits per treatment per replication.

*Hydrocooling and packaging study setup* - The study was conducted using 6 x 4 factorial treatments in a completely randomized design (CRD) with 6 hydrocooling and packaging combinations treatments (1. precooling + packing with old new-paper, 2. precooling with plastic, 3. precooling only without packaging, 4. without precooling but packed with old news-paper, 5. without precooling but packed with plastic and 6. without precooling and packaging) with and four storage durations (1, 2, 3 and 4 weeks) with three replications.

*Hot water shocking time study setup* - The study was conducted using 5 x 5 factorial treatments in a CRD with 4 replications for each treatment. There were five dipping durations applied to the guava at temperature of 46±1 $^{o}$C (control, 15, 25, 35 and 45 min) and five storage duration (0, 5, 10, 15 and 20 days).

*Sodium alginate coating study setup* - For this experiment, 4 x 5 factorial treatment arrangements in a CRD were used. Each guava fruit was tested on different concentrations of sodium alginate (1.0, 1.5 and 2.0 % w/v) and with a control (without coating). Each treatment had four replicates. The samples were stored in a cold room 10±2 $^{o}$C for 20 days.

*Visual quality determination (the postharvest life)* - A subjective hedonic score for visual appearances was based on a scale of 9 to 1 where 9=0% defects, 7=up to 25% defects, 5=50% defects, 3=75% defects and 1=100% defects. A score of 6 (37.5% defects) was considered the limit of acceptable visual appearances.

*Statistical analysis* - The differences among the main and interaction effects were subjected to analysis of variance (ANOVA) by using Statistical Packages for Social Science (SPSS). For the subjective visual appearance, the square root transformation was applied before the analysis.

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## Results and Discussion

*Effect of hydrocooling time, storage temperatures and storage duration to guava visual appearances*

Visual appearance was significantly ($P \leq 0.05$) affected by the interaction between storage temperature x storage duration but there were no significant interaction effects of cooling time x storage temperature and cooling time x storage duration on visual appearance. Visual appearance of guava stored at 5 $^{o}$C decreased linearly while those stored at 10 and 15 $^{o}$C decreased quadratically with increased storage duration from 1 to 6 weeks (Figure 1). Fruit stored at 5 $^{o}$C only had acceptable visual appearance when stored up to 1.6 weeks. Guava stored at 10 $^{o}$C had acceptable visual appearance of up to 3.6 weeks of storage. However, the negative quadratic relationship between visual appearance and storage duration of fruit stored at 15 $^{o}$C indicated that the visual appearance decreased rapidly in the first 3 weeks and then the decrease tend to level off until the sixth week of storage. Fruit stored at 15 $^{o}$C had acceptable visual appearance for up to 1.3 weeks. In this study, the acceptable visual appearance was set at 2.44 (square root transformation), which was equivalent to about 37.5% occurrence of defects on fruit. Fruit with visual appearance score of 2.44 could still be sold, although it was of inferior appearance.

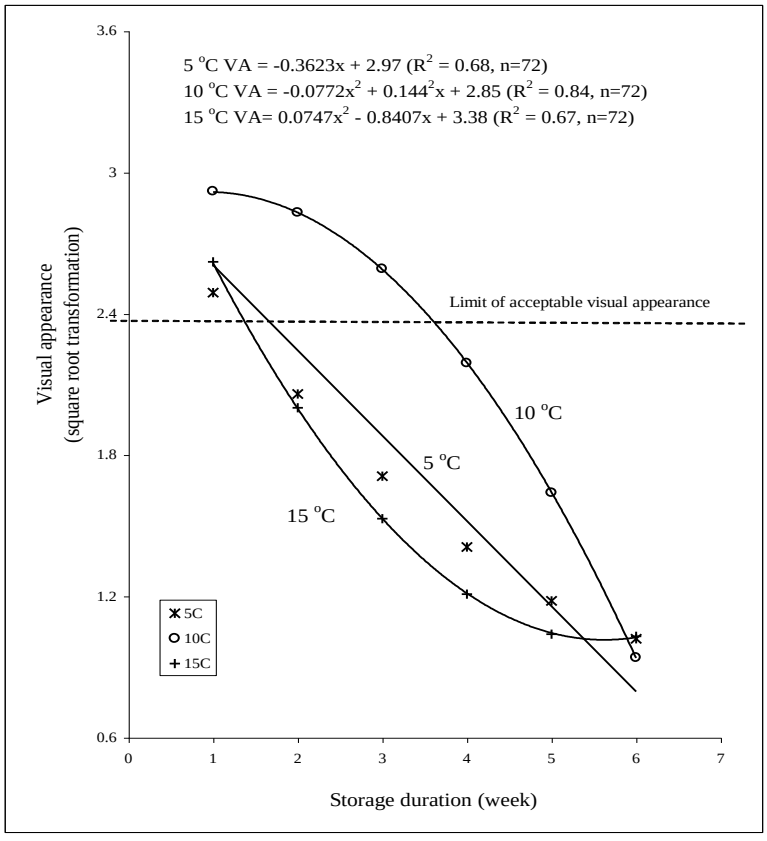


Figure 1. Relationship between visual appearance (VA) and storage duration (week) of guava cv. Kampuchea after storage at 5, 10 and 15 $^{o}$C.

Precooling at 1/8, 1/4 and 1/2 cooling time indicated a reduction of 12.5, 25 and 50% respectively, of temperature differences between product and its surroundings as illustrated by Wills et al. (1998). This study suggested that cooling time of more than 1/2 could be suitable to get better visual appearance of guava. Guillou (1959) reported that the remaining field heat from application of precooling with high

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level of cooling time can be gradually removed with short time and low energy cost at storage. Hence, temperature of products was reduced within a short time, while rate of product metabolic process was expected to be slow while visual appearance in guava could be prolonged. Application of precooling could also ensure greater customer satisfaction is achieved and therefore repeated purchases can be ensured. Freeman (1984) had identified the economic benefits of early precoolings. He also reported that different products are better suited to certain cooling technique so that the cost efficiency could be improved and better results are attained.

In this study, the guava showed different trends of the defects at each storage temperature. The defects in guava stored at 5 $^{o}$C increased linearly while those stored at 10 and 15 $^{o}$C increased quadratically with increased storage duration from 1 to 6 weeks. In this study, increase in defects is referred to as a decrease in the visual appearance. Therefore, this study suggested that the differences could affect the consumer perception to buy this fruit. Dever et al. (1995) reported that even different side of the same fruit could have a different sensory characteristic that effect consumer perception. However, Deliza and MacFie (1996) reported that consumer only reject the products if the disparity was large.

*Effect of hydrocooling, packaging materials & storage duration to guava visual appearances*

Application of hydrocooling followed by any packaging has extended visual appearances of the guava fruits (Figure 2). Precooling followed by plastic give much batter visual appearances compare to other handling in this study. Visual appearances of guava after treatment of precooling and packaging with plastic showed above acceptable visual appearance even after 20 days of storage followed by fruit which previously precooled and packed with newspaper. According to Ahlawat and Jindal, (1980), packaging with plastic will create modified atmosphere that can reduce transpiration, gas exchanges and directly reduce water loss. Appropriate atmosphere condition was expected achieved since the fruit has been precooled earlier. Seymour et al. (1993) confirmed that fruit kept at low temperature has less ethylene production and Lim and Khoo (1985) confirmed less pathogen and rotting in guava stored at low temperature.

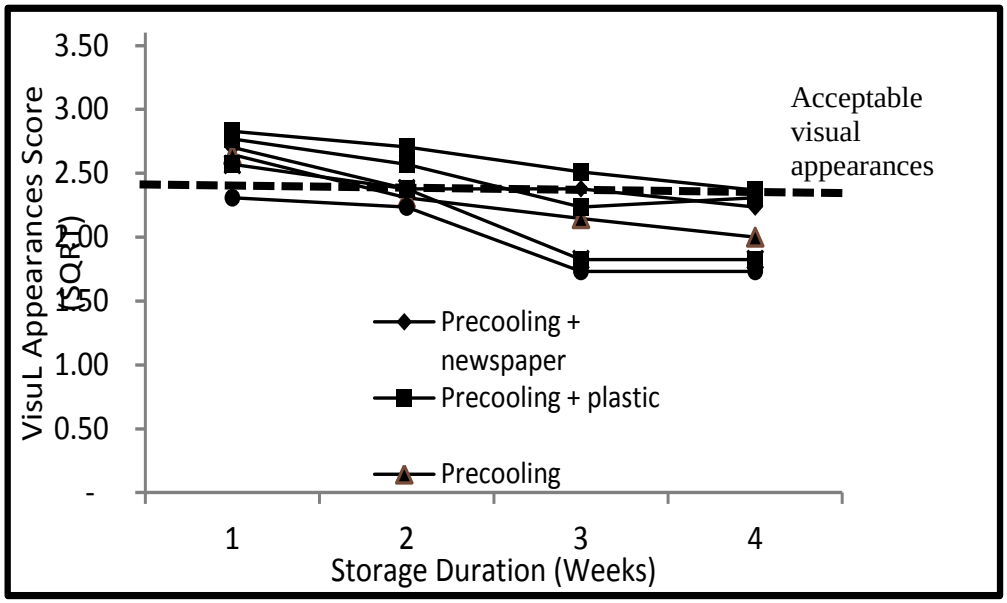


Figure 2. Visual appearance score of guave fruit after subjected to combination treatment with hydrocooling, packaging and storage duration (days).

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*Effect of hot water treatments & storage duration to guava visual appearances*

Dipping duration and storage duration has a significant effect on the visual appearances for guava (Figure 3). The highest visual appearance was always associated with hot water treatment of 15 minutes. Visual appearances are acceptable until the end of observation period compared with other treatments. The lowest visual appearances was always associated with longer dipping duration (35 and 45 minutes). Hot water treatments of 15 min had the highest visual appearances (2.63), while both hot water treatments of 35 and 45 min had the lowest visual appearances (1.98) at the end of 20 days of storage (Figure 3). Yusof and Hashim (1999) pointed out that the incidence of brown scorches especially around the stalk region will be increased with increasing duration of guava pulp exposure to temperature of 46 °C. Mansour et al. (2006) also showed that no blackening occurred on mango dipped in hot water at 45 and 50°C with shorter duration for 5 min, while with raised water temperature and dipping duration at 10 and 15 min, degrees of blackening will increased and severe damage occurred at 50°C and increased gradually as exposure time increased.

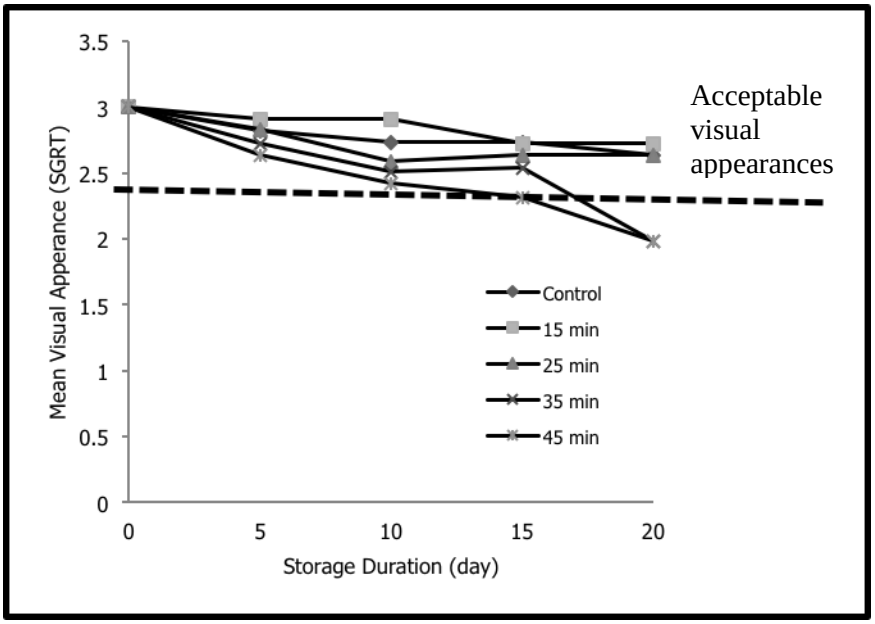


Figure 3. Visual appearance score of guave fruit after subjected to combination treatment hot water treatments and storage duration (days).

Some fruit may be sensitive to heat damage while some are not. According to Woolf and Lay-Yee (1996), avocado fruit are unacceptably damaged by hot water treatments of 3 to 5 min at 50˚C and heat damage symptoms in avocado fruit are usually predominantly in the skin, browning of tissue and hardening when ripe. Hot water treatment with shorter dipping duration can maintain the visual appearances of guava during low temperature storage. However, with increased dipping duration, the level of visual appearance degradation becomes more severe. Therefore, guava should not be treated with longer hot water dipping duration to avoid heat damage.

*Effect of sodium alginate coating concentration and storage durations on guava visual appearances*

Visual appearances was significantly affected by the interaction between the sodium alginate coating and storage duration ($p<0.05$) (Figure 4). The score of visual appearances for each treatment was shown to be decreasing as the storage duration was increased. The result indicated that the fruits coated with 1.5%w/v and 2.0% w/v of sodium alginate showed better visual appearance than control and 1.0%w/v of sodium

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alginate at 15 days of storage. Waxing or coatings of certain fruits and vegetables could reduce their rate of respiration and enhance the gloss product, improving merchandising and marketing (Salunkhe et al., 1991).

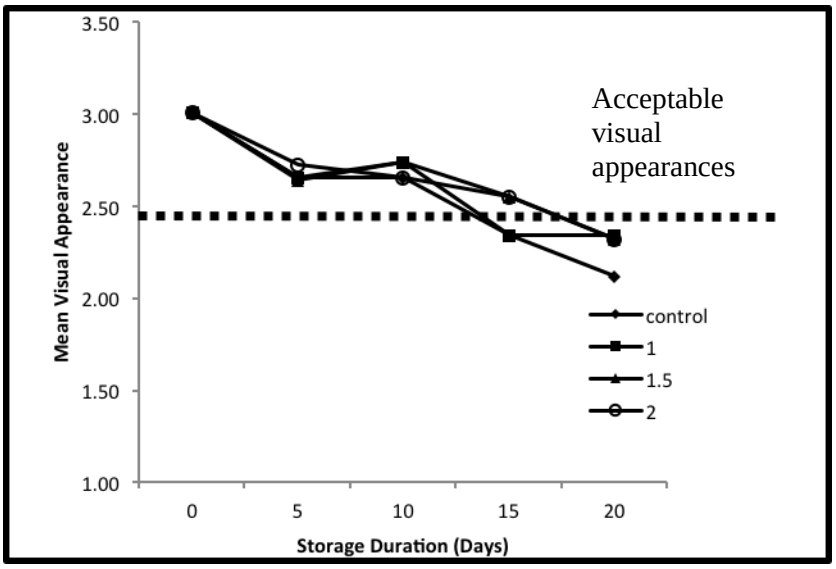


Figure 4. Visual appearance score of guave fruit after subjected to combination treatment of sodium alginate coating concentration and storage duration (days).

**Conclusions and Recommendations**

Temperature management is very important in maintaining the visual appearances of guava fruit. This study recommended precooling guava at minimum of 1/8 cooling time, applying packaging with plastic materials and storing at 10 $^{o}$C. Application of hot water treatments should be carefully supervise to ensure not more than 25 minutes of dipping time with 46 $^{o}$C hot water medium. Producers are recommended to apply coating treatment to get advantages on the extending visual appearances.

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# Sabah Castor (*Ricinus communis* Linn) Maturity Stages Based on Oil Content and Fruit Development Changes

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## Introduction

Castor is an important oilseed crop with indeterminate maturity due to its indeterminate growth habit. The flowering and fruiting of castor in the same raceme do not occurred at the same time (David et al., 2013). In addition, successive generations of racemes continues to sprout before the primary raceme enter full maturity stage resulting in the existence of mature racemes, immature racemes and flowering raceme (Baldanzi et al., 2002; Vallejos et al., 2011; David et al., 2013). The indeterminate maturity of castor causes difficulty in determining whole raceme maturity making it hard to decide the optimum harvest time for castors.

Crop maturity determination is crucial as it affects quality perception and the rate of quality changes during harvesting and postharvest handling (Shewfelt, 2014). The maturation process can be described by crop maturity stages or index which serves as a guide in measuring crop maturity. There is still limited information on castor maturity stages for harvesting and postharvest handling. This study aim to characterize castor maturity stages based on the fruit development changes, rather than seed development due to the fact that fruit changes can easily be observed without destructive sampling. Moreover, the maturity stages also focus on extractable castor oil yield, rather than seed physiological quality, which had been described in many previous study. Thus, the objective of this study is to determine Sabah castor maturity stages based on oil content and fruit development changes.

## Materials and Methods

### *Plant materials*

Wild Sabah castor from Luanti accessions was grown in a plot at UMS Sandakan Campus. Castor seeds were directly sowed at 0.06 m depth in the planting bed size 3.00 m x 1.00 m x 0.15 m. Basal treatments of vermicompost 6 t/ha was applied and left for one week before the sowing process. Five castor plants were planted in each planting bed with the spacing of 0.50 m within row and 1.30 m between rows. Fertilizer, NPK Blue (12-12-17-2) was applied at the rate of 6.65 g at third week and sixth week after planting. Chemical pesticide was used to control pest.

### *Sampling procedures*

Castor tree was tagged after the first days of anthesis to determine sampling time. The sampling began at 21 days after anthesis with seven days intervals for the green stage and was continued until the fruit enter ripe brown stage. Sampling was only conducted on fruits from the first raceme.

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*Determination of castor oil content*

Soxhlet extraction was used for crude castor oil extraction with 95% ethanol as a solvent. The seeds were first oven-dried at 60 $^{o}$C for 7 h before it was crushed. Approximately 10 g of dry ground seeds with 300 mL ethanol were refluxed for 6 hours at 70 $^{o}$C in a Soxhlet extractor. The extracted oil was obtained by using rotary evaporator to filtrate the solvent at 70 $^{o}$C. The extracted oil was heat in the oven at 70 $^{o}$C for 30 min before being weighed. Extracted oil yield was measured by weight basis for oil content determination.

*Determination of castor fruit development changes*

Hundred fruits were randomly selected from each sample for castor fruit colour, size and weight changes determination. The fruit colour was measured by using colour meter, Konica Minolta Japan CR-10. Castor fruit size was determined by measuring the fruit length and width using vernier calliper. Castor fruit moisture content was determined using oven dried method by Zuchi et al. (2009). Fresh fruits were weighed before and after drying at 105 $^{o}$C for 24 h.

*Experimental design and data analysis*

Factorial completely randomized design with maturity stages as a factor was used in the study of fruit development changes with a minimum of five replications. One-way ANOVA and Tukey test using SPSS 21.0 was used to determine if there is significant difference in the data.

**Results and Discussion**

*Castor oil content at different maturity stages*

Castor fruit oil content changes are presented in Table 1. The oil content significantly increased as castor fruits reached the ripened stage. This finding corroborates previous study that reported oil content to increase with fruit maturation (Janick and Paull, 2008; Pinto et al., 2012). There is no significant difference between the oil content of fruits from 21 to 28 DAA green stages. This suggested that castor fruits at 21 and 28 DAA belong in the same maturity stage which is young green stages and castor fruits reached mature green stage at 35 DAA. The oil content is considered as the main indicator for castor maturity stages since it is the main product of castor.

Table 1. Sabah castor oil content changes during castor fruit maturation (N=5).

| Maturity stages | Oil content (%) |
|---|---|
| 21 DAA green | 28.08$^{d}$ |
| 28 DAA green | 29.37$^{d}$ |
| 35 DAA green | 40.21$^{c}$ |
| Dried green | 46.28$^{b}$ |
| Ripe brown | 50.41$^{a}$ |

*Mean followed by the same letter within each column are not significantly different by Tukey test $p<0.05$*

*Castor colour at different maturity stages*

Castor fruit colour changes is shown in Table 2. There is no significant difference in hue, light and chroma between fruit green stages from 21 to 35 DAA. Based on the colour value in Table 2, colour is not a good indicator for maturity determination of castor green stages. The hue value suggested that castor fruits become greener at dried green stages. Castor fruit is a dry fruit with orthodox seeds, thus the fruits

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undergo maturation drying to cracked open after ripened (Black et al., 2006; Schmidt, 2007). The fruits lose its ability to photosynthesise at maturity causing the breakdown of chlorophyll (Rees and Hammond, 2002; Schmidt, 2007; Barry and Roux 2010; Shewfelt, 2014). Therefore, the fruits colour changed from green to brown and the colour value reduced as the fruit colour turned darker as it gradually entered ripening stage.

Table 2. Sabah castor fruit colour ($h^o$, $L^*$ and $C^*$ value) changes during castor fruit maturation (N=100).

| Maturity stages | $L^*$ | $h^o$ | $C^*$ |
|---|---|---|---|
| 21 DAA green | $41.0^{a}$ | $22.7^{b}$ | $114.7^{a}$ |
| 28 DAA green | $41.1^{a}$ | $23.6^{b}$ | $115.3^{ab}$ |
| 35 DAA green | $40.6^{a}$ | $23.8^{b}$ | $128.9^{a}$ |
| Dried green | $37.8^{a}$ | $31.5^{a}$ | $93.1^{bc}$ |
| Ripe brown | $29.0^{b}$ | $14.4^{c}$ | $79.1^{c}$ |

*Mean followed by the same letter within each column are not significantly different by Tukey test p<0.05*

*Castor size and weight at different maturity stages*

Castor fruit size and weight changes are presented in Table 3. The fruit length and width significantly increased from 21 to 28 DAA green stages before significantly decreased from 35 DAA green stages to dried green and increased significantly at the ripe brown stage. The increase in fruit size at brown stage is due to the changes of castor fruit morphology. Castor fruits at ripened stage will split open especially in dehiscent fruit such as Sabah castor to release its seeds, thus it become longer and wider. (Hocking, 1982; Vallejos et al., 2011; Anjani 2012). There are no significant differences between the fruit weight from 21 to 28 DAA green stages and this suggested that the fruits can be categorized in the same maturity stages as young green fruit. The fruits weight starts to significantly decrease from 35 DAA which is similar to the fruit size. Thus, fruit at 35 DAA can be categorized as mature green stage since castor fruits go through drying process at maturity. Although, there is no significant difference in fruit weight between dried green and brown stages, these maturity stages can be differentiated based on its colour changes. Fruit size and weight can be used as a good indicator for castor maturity when combined with the fruit colour. The green stages are hard to be identified only by colour since there is no significant difference between the colour values. However, young green fruits can be differentiated from the mature green by their size. Fruit with bigger size can be considered as mature green, while smaller fruits as young green.

Table 3. Sabah castor fruit size (i.e. length and width) and weight changes during castor fruit maturation (N=100).

| Maturity stages | Length (cm) | Width (cm) | Weight (g) |
|---|---|---|---|
| 21 DAA green | $1.69^{b}$ | $1.63^{b}$ | $2.72^{a}$ |
| 28 DAA green | $1.78^{a}$ | $1.71^{a}$ | $2.83^{a}$ |
| 35 DAA green | $1.62^{c}$ | $1.60^{c}$ | $2.57^{b}$ |
| Dried green | $1.49^{e}$ | $1.51^{e}$ | $1.07^{c}$ |
| Ripe brown | $1.53^{d}$ | $1.55^{d}$ | $0.98^{c}$ |

*Mean followed by the same letter within each column are not significantly different by Tukey test p<0.05*

*Castor fruit moisture content at different maturity stages*

Castor fruit moisture content changes are presented in Table 4. There is no significant difference between fruits from 21 to 28 DAA and fruit from 28 to 35 DAA. The fruit moisture content starts to significantly decrease from 35 DAA as fruit undergoes maturation drying at physiological maturity. Fruit moisture content is not a good indicator for castor young and mature green stages since there is no significant difference. Moreover, fruit moisture content is affected by weather conditions during fruit development and maturation.

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Table 4. Sabah castor fruit moisture content changes during castor fruit maturation (N=5).

| Maturity stages | Fruit moisture content (%) |
|---|---|
| 21 DAA green | 79.30[a] |
| 28 DAA green | 71.78[ab] |
| 35 DAA green | 68.19[b] |
| Dried green | 28.77[c] |
| Ripe brown | 8.62[d] |

*Mean followed by the same letter within each column are not significantly different by Tukey test $p<0.05$*

## Conclusions

The changes in extractable oil content and its fruit physical characters during fruit development and maturation had been observed to produce castor maturity stages. Castor maturity stages can be divided into four indices stages; young green, mature green, dried green and ripe brown. Sabah castor fruits reached its physiological maturity or mature green stage at 35 DAA when the fruit turns to darker green (i.e. 23.8 $h^o$, 40.6 $L^*$, 128.9 $C^*$), measured 1.62 cm in length and 1.60 cm in width, weighed 2.57 g, moisture content reaching 68.19% and produce 40.21% extractable oil yield.

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# CHAPTER 4

# BIOTECHNOLOGY

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# Functional Analysis of *Eucheuma denticulatum* Gene Involved in Phytoremediation

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## Introduction

Phytochelatins (PCs) are short polypeptides which are rich in cysteine residues with the size of 1.5 to 4 kDa (Vestergaard et al., 2008). It acts as thiol-reactive peptides which can chelate heavy metals like cadmium (Cd) (Li et al., 2004). Phytochelatin synthase (PCS; EC 2.3.2.15) is responsible for the synthesis of PCs from glutathione under transpeptidase reaction (Clemens, 2006). The presence of activators like Cd is essential for the synthesis to proceed (Osaki et al., 2008). The PCS gene has been identified in plant, fungi, algae and recently in nematode, *Caenorhabditis elegans* (Vatamaniuk et al., 2002). In this study, the PCS cDNA clone has been isolated from the marine red algae, *Eucheuma denticulatum* and was further used for the expression of recombinant PCS protein in various $Cd^{2+}$ concentrations.

## Materials and Methods

*Isolation and cloning of EdPCS1*

The full-length clone for *E. denticulatum* PCS (EdPCS1) has been isolated using PCR and rapid amplification of cDNA ends (5' RACE-PCR) techniques. Total RNA was extracted from seaweed using the modified method described by Lopez-Gomez and Gomez-Lim (1992). A pair of primers was designed based on the partial sequence of Expressed Sequence Tag (EST) for 5' RACE-PCR, pPCS1 (5'-TTC CTT CGA GTT CAT TGT CGC TGC CT-3') and nPCS1 (5'-CGT TGG TTT GCC GAA TCG CTT CTC G-3'). PCR amplification was performed with the following conditions: 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min (25 cycles). The resulting 1.6kb product was cloned in pTZR/T (Fermentas) and the open reading frame was cloned into pET-32b(+) expression vector (Novagen). The isolated recombinant vector, pET-PCS1 was analysed by PCR, digestion by restriction enzymes and sequencing.

*Expression and purification of recombinant PCS1 in Escherichia coli*

The expression of recombinant PCS1 protein using pET32b(+) was carried out in *E. coli* strain Origami (DE3). The *E. coli* cells transformed with recombinant PCS1 vector were grown in Luria Bertani (LB) medium supplemented with kanamycin (15 µg/ml), tetracycline (12.5 µg/ml) and ampicillin (50 µg/ml). After the cells growth reached about 0.6 at $OD_{600nm}$, the cultures were induced with final concentration of 0.6 mM of isopropyl-ß-D-1-thiogalactopyranoside (IPTG), followed by a further incubation at 16 °C and 150 rpm for 18 h. Cells were harvested and resuspended with lysis buffer, 20 mM Tris-HCl (pH 8). The cells were also treated with lysozyme (1 mg/ml) and β-mercaptoethanol (1 mM). The total protein fractions were collected after sonification and centrifugation. The total protein fractions were analysed by SDS-PAGE and western blot using monoclonal anti-polyhistidine clone His-1. Then, the total soluble protein fractions were purified using HisTrap™ HP column by AKTA-Purifier system. The purified protein fractions dialysed and concentrated using Milipore Vivaspin (10 k MVCO).

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*Growth assay under various concentrations of metals*

Recombinant *E. coli* cells to be used in metal treatment were grown at 37 °C with shaking 250 rpm for overnight. These recombinant cells were spread on the LB agar plates contained 0.6 mM IPTG and various concentrations (0-1000 µM) of different metals ($Cd^{2+}$, $Pb^{2+}$, $Cr^{3+}$, $Cu^{2+}$ and $Mn^{2+}$). The next day, the colony forming unit (CFU) for each plate was collected.

*Phytochelatin synthase enzyme assay*

The PCS activity was measured using the procedure described by Grill et al. (1989) with some modifications. The reaction mixture containing 200 mM TRIS-HCl pH 8, 2 mM 2-mercaptoethanol, 1 mM GSH, and 200 mM $CdCl_2$, respectively, in a total volume of 1 ml. After 5 min preincubation at 35 °C, the reaction was started by adding the 20 µg of enzyme extract. After 30 min incubation, the reaction was terminated by adding 100 µl 10% trifluoroacetic acid and centrifuged at 10,000×g for 10 min. The supernatant was saved and quantitated using a Cl8 RP-HPLC eluted with a 60% acetonitrile and the active fractions were measured at 214 nm. GSH was used as HPLC standards.

*PCS cDNA transformation into Arabidopsis thaliana via Agrobacterium culture*

Primary cloning of full-length PCS1 cDNA clone fused with hexahistidine tag (PCS1h) was carried using pCAMBIA1301 binary vector. PCS1h insert replaced the GUS sequence in pCAMBIA1301. The isolated recombinant vector, pCAM-PCS1h were used as template for secondary cloning of expression cassette consisting of CaMV 35S promoter, PC1S cDNA, hexahistidine tag and NOS poly-A terminator and was cloned into pCAMBIA1301 to form pCAM-gPCS1h. Plant expression vector, pCAM-gPCS1h was transformed into *Agrobacterium tumefaciens* strain GV3101 and verification analyses were performed on pCAM-gPCS1h. *Arabidopsis thaliana* plants were inverted and the inflorescences (flowers) were dipped into the *Agrobacterium* suspension and swirled for about 5 sec. Bacterial suspension were drained from the plants and the plants were laid on their side and covered with plastic wraps to maintain high humidity at low light for overnight. The next day, the plastic wraps were removed and plants were set upright to normal growth. Plants were grown until maturation and seeds were harvested for analysis.

*Transformation of PCS promoter into Arabidopsis thaliana via Agrobacterium culture*

A total of 15 µg of genomic DNA was extracted from 30 g *E. denticulatum* using the method of Kaufman et al. (1995) with modification. The genomic DNA was digested with two restriction enzymes (Dra1 and EcoRV). The amplification the PCS promoter sequence was carried out using Clontech GenomeWalker™ Kit (TaKaRa, USA) according to the manufacturer's recommendations. The primary PCR product with the size of ~1200bp was used for nested PCR. The PCR product of promoter was cloned into pBI121 binary vector producing pB-pPCS construct. Recombinant binary vector, pB-pPCS was transformed into *Agrobacterium* strain GV3101. *A. thaliana* was transformed by floral dip method using *Agrobacterium tumefaciens* strain GV 3101 harbouring PCS promoter construct.

**Results and Discussion**

*Full-length cDNA of E. denticulatum PCS1*

The full-length clone for *E. denticulatum* PCS1 (*Ed*PCS1) has been isolated using PCR and rapid amplification of cDNA ends (RACE) techniques with the size of 1.6kb which contains a single open reading frame encoding a protein containing 218 amino acids. From the BLASTp analysis, the expected

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amino acid sequence of *Ed*PCS1 shared high similarity with PCS1 from *Cyanidioschyzon merolae* (44%) and *Arabidopsis thaliana* (42%). Multiple sequence alignment of *Ed*PCS1 with other PCS sequences showed conserved region at C-terminal (Figure 1).

```
EDPCS          ---MTKP---TNPNPEEPD-----------------------TSPPTHQKSFYRRPL 28
CMPCS          QCPVQTPSSRTQPAPALPENDATVLRTQTPQTITVTTTTTTTRIPSATPPPAVSFYRRPL 120
ATPCS          ---------------------------------------------MAMASLYRRSL 11
OSPCS          --------MASKPSSRAES-----------------------NQAAAAVPSLYGRAL 26
                                                            *:* *.*

EDPCS          PSN-LVDFNSAEGKSRFSSALEAGHAETFFPLISQFQTQSHPALCGLTTLSTILNALQID 87
CMPCS          PAT-CIALDSAEGRALFERSLFSGLAEPFLPLVSQFTTQSEPAFCGLGSLAMVLNALQVD 179
ATPCS          PSPPAIDFSSAEGKLIFNEALQKGTMEGFFRLISYFQTQSEPAYCGLASLSVVLNALSID 71
OSPCS          PSPPAVEFASAEGRRLFAEALQGGTMQGFSSLVSVFQTQSEPAFCGLATLAVVLNALRID 86
               *:   : : ****:  *  :*  *  : *  *:* * ***.** *** :*: :**** :*

EDPCS          PKRVWNHPWRWFAESLLDCCLNMEDMKTEGITMDQLACTAACQGSTVRALRG-------- 139
CMPCS          PGRPWKGPWRWFSEELLDCCLPLHIVAREGITLDEFRCLGQCNGALVETAQPPAPGVAPS 239
ATPCS          PGRKWKGPWRWFDESMLDCCEPLEVVKEKGISFGKVVCLAHCSGAKVEAFRTS------- 124
OSPCS          PGRRWKGPWQWFDESMLDCCEHLDTVRAEGITFGKVACLAHCSGADVRTFRAA------- 139
               * * *: **:** *.:****  :. :  :**::.:. * . *.*: *.: :

EDPCS          --LSAQDARDMIR--DSARGNADGSFEFIVAAYDRQALGQTGTGHFSPIAAYDHASDSVL 195
CMPCS          QHLSLERFRESLQRMCSDRDPRNGS-GFLVLCYAREALQQTGTGHFSPIAAYDEVSDRAL 298
ATPCS          -QSTIDDFRKFVVKCTSSEN------CHMISTYHRGVFKQTGTGHFSPIGGYNAERDMAL 177
OSPCS          -QATLADLRRHLLRCASSQD------CHLVASYHRKLLGQTGTGHFSPIGGYHAGQDMAL 192
                  :    *  :    * ..       .::  * *  : **********..*.   * .*

EDPCS          VLDVARFK---------------------------------------------------- 203
CMPCS          ILDVARFKYPPHWVALPDLYRAMSVLDPATGMPRGLAMVRR------------------- 339
ATPCS          ILDVARFKYPPHWVPLKLLWEAMDSIDQSTGKRRGFMLISRPHREPGLLYTLSCKDESWI 237
OSPCS          ILDVARFKYPPHWIPLPLLWEAMNTIDEATGLLRGFMLISR------------------- 233
               :*******
```

Figure 1. Multiple sequence alignment between *Eucheuma denticulatum* PCS1 (Eucheuma) and amino acid sequences from *Arabidopsis thaliana* (AtPCS1 & AtPCS2), *Oryza sativa* (Oryza) and *Cyanidioschyzon merolae* (Cyanidioschyzon). The conserved region was highlighted in yellow ( ).

*Growth assay under various concentrations of metals*

In the presence of metal, recombinant *E. coli* cells were more tolerant compared to control *E. coli* cells (Figure 2). This shows that recombinant PCS protein was properly expressed and functioned well in *E. coli* cell. The difference in tolerance level of recombinant *E. Coli* and control cell was higher in the metals like $Cd^{2+}$, $Cr^{3+}$ and $Pb^{2+}$ compared to metals like $Cu^{2+}$ and $Mn^{2+}$. This could be because $Cu^{2+}$ and $Mn^{2+}$ are micronutrients that are needed for the growth of organisms (Tangahu et al., 2011). Recombinant *E. coli* cells were tolerant to the concentration of $Cd^{2+}$ up to 600 µM, $Cr^{3+}$ and $Pb^{2+}$ up to 800 µM.

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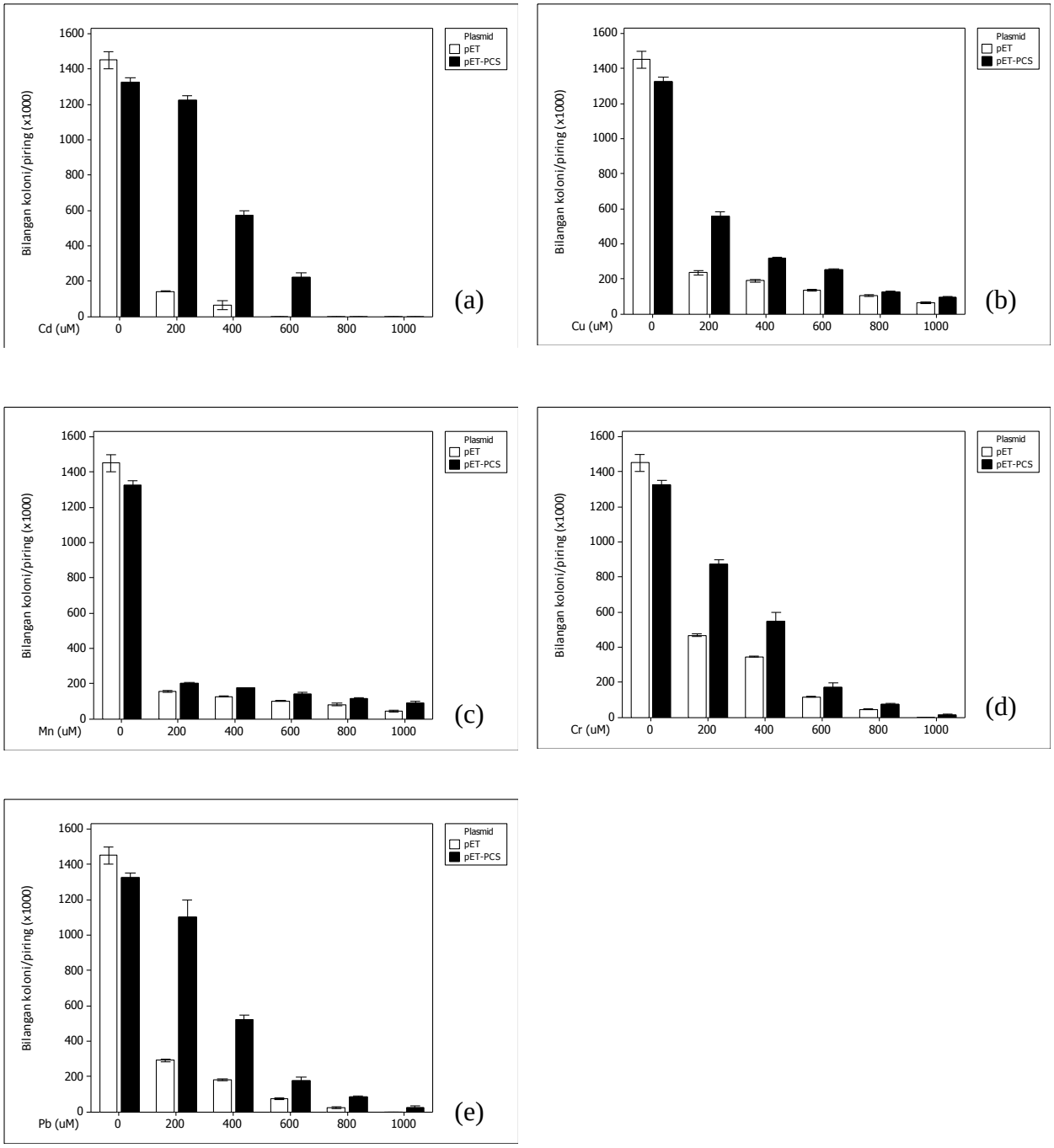


Figure 2. Growth assays of recombinant protein *E. coli* and control cells expressing cells at different metals and at various concentrations (0 - 1000 µM). (a) Growth bar for $Cd^{2+}$. (b) Growth bar for $Cu^{2+}$. (c) Growth bar for $Mn^{2+}$. (d) Growth bar for $Cr^{3+}$. (e) Growth bar for $Pb^{2+}$. Data obtained mean ± standard deviation with $p<0.05$ (n=3).

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*Phytochelatin synthase enzyme assay*

The PCS activity was measured using HPLC method. Enzyme assay mixture was quantitated using a Cl8 RP-HPLC eluted with a 60% acetonitrile as mobile phase and the active fractions were measured at 214 nm. Based on the chromatograms obtained, the reduction of GSH was deduced using the standard curve. The retention time for GSH is about 2 min. The specific activity 2661.3 pmol substrate converted/s/mg (pkat/mg) compared to crude protein at 256.4 3 pkat/mg. The enzyme activity assay showed the Km value for purified recombinant protein was 3.38 mM and $V_{max}$ value of 1610.3 nmol $min^{-1}$ $mg^{-1}$.

*Plant expression vector*

The full-length PCS1 cDNA clone fused with hexahistidine tag (PCS1h) was clone into pCAMBIA1301 binary vector. The isolated recombinant vector, pCAM-PCS1h was analysed by PCR, digestion by restriction enzymes and sequencing. Then, the expression cassette consisting of CaMV 35S promoter, PCS1 cDNA, hexahistidine tag and NOS poly-A terminator (Figure 3) was cloned into pCAMBIA1301's MCS for secondary cloning to form pCAM-gPCS1h.


Figure 3. Expression cassette (gPCS1h) consisting of CaMV 35S promoter, PCS1 cDNA, hexahistidine tag and NOS poly-A terminator.

**Conclusions**

Recombinant PCS1 protein was successfully expressed and purified in *E. coli*. PCS1 protein expressing *E. coli* cells were more tolerant to metals like $Cd^{2+}$, $Cr^{3+}$ and $Pb^{2+}$ compared to control cells. The enzyme activity assay of the purified recombinant protein gave $K_m$ value of 3.38 mM. Functional analysis of PCS1 in transgenic *A. thaliana* may contribute towards further application of this recombinant enzyme in heavy metal remediation.

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# *In vitro* Regeneration of *Jatropha curcas*: Cotyledonary Leaf Induced More Shoots than Other Explants

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## Introduction

Jatropha (*Jatropha curcas* L.) which belongs to the Euphorbiaceae family is a plant that can grow on marginal land. This plant is important for biodiesel production. Besides, the leaves have medicinal properties and the oil seed cakes can be used as fertilisers (Becker and Francis, 2005; Mkoka and Shanahan, 2005). For large scale planting, *in vitro* approaches via multiple shoot induction is needed to mass produce planting materials. Also being a perennial plant, conventional breeding via crossing can be a bottle neck due to its long vegetative growth phase. Thus *in vitro* approaches through adventitious shoot induction coupled with Agrobacterium-mediated transformation can be a very powerful tool in improving the crop and solving the above mentioned problems. In the development of an in vitro regeneration protocol it is important to maximise shoot formation in order to reduce the production cost. Also, the ease in maximizing shoot formation will determine the success of transgenic plant production via Agrobacterium-mediated transformation. Under *in vitro* condition, multiple shoots can be induced either adventitiously or through axillary proliferation depending on the type of explants used. Adventitious shoot can be induced on leaf, root, petiole or hypocotyl explants (Hartmann et al., 2007; He et al, 2007; Wang and Bao, 2007; Tian et al., 2007; Selvaraj et al., 2007; Shang et al., 2006) while axillary budding can be induced from shoot tip and node explants (Rajore and Batra, 2005). There are several factors enhancing multiple shoot induction on an explant. Among them include plant growth regulators, explant type and age, plant genotype and pre-treatment of the mother plant prior to the excision of explant (George et al., 2008). This paper compares the production of adventitious shoots from various explants of *J. curcas* using 6-benzyl amino purine (BAP) at different concentrations.

## Materials and Methods

Fruits of *J. curcas* were collected from mature trees grown in Field 2, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor. The fruits were placed in a 1 L beaker and a few drop of Tween 20 were pipetted on to them followed by washing under running tap water. Then the fruits were brought to the laminar airflow cabinet, the pericarp was peeled off using a sharp sterile blade and the seeds were isolated. The seeds were put in a 1 L beaker, rinsed with 70% ethanol for 20 seconds followed by washing in sterile distilled water. The seeds were then sterilized with 20% Clorox added with a few drops of Tween 20 for 15 min. The seeds were rinsed with sterile distilled water thrice, then transferred into sterile petri dishes containing sterilized dry Whatman filter paper and blotted dry. Using a sharp sterile blade, the seeds were cut into halves, their testa and endosperm removed and the embryos isolated and cultured vertically in vials containing modified MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose, 0.1 $gL^{-1}$ myo-inositol and 4.2 $gL^{-1}$ gelrite. After eight days, cotyledonary leaf, hypocotyl and root explants were excised from the germinated seedling. Hypocotyl and root explants were cultured in vials containing modified Murashige and Skoog (1962) medium supplemented with 3% (w/v) sucrose, 0.1 $gL^{-1}$myo-inositol, 4.2 $gL^{-1}$gelrite and BAP at 0, 1.0, 2.5, 5.0, 10.0 $mgL^{-1}$. Cotyledonary leaf explants were cultured on medium containing 10.0 $mgL^{-1}$ of BAP (the best treatment determined from previous experiment). All media were prepared by adjusting the pH at 5.8 and 10ml of medium was poured into

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each vial before autoclaving at 15 psi and 121 $^{O}$C for 20 min. The cotyledonary leaf explants were cultured with the abaxial surface touching the medium while the hypocotyl and root explants were cultured horizontally with part of the explant submerged in the medium. Ten explants were cultured per treatment per replication. Each treatment was replicated thrice. The experiment was conducted using Completely Randomized Design (CRD). All data were analysed statistically using the analysis of variance (ANOVA) and treatment means were compared using Duncan New Multiple Range Test. Cultures were maintained at $27^{0}$C for a 12 hour photoperiod in the growth room with a daily lighting of 30 µmol $m^{-2}$ $s^{-1}$ intensity using cool white Philips fluorescent tubes.

**Results and Discussion**

Cotyledonary leaf explant cultured on modified MS medium supplemented with 10.0 $mgL^{-1}$ of BAP started to curl at its outer end after a week of culture. At the same time, the injured portion of the veins on the adaxial region of the cotyledonary leaf swelled followed by callus initiation. Adventitious shoots were regenerated from the callus but some were regenerated directly from the explant. Adventitious shoot regeneration from the explant started with the formation of shoot initials, followed by the development of meristem and leaf primordia and then the elongation of shoots, similar to the findings of Cheah and Cheng (1978) on cotyledon explant of Douglas fir. The formation of adventitious shoots on the cotyledonary leaf explant can be seen clearly by the fifth week of culture. By the eighth week, many adventitious shoots were already formed on the cotyledonary leaf explant. Adventitious shoots were initiated from different parts of the cotyledonary leaf explant. Some of the shoots were initiated either directly at the abaxial region or adaxial part of the cotyledonary leaf segment or from callus initiated on that region.

Root explants cultured horizontally on modified MS medium supplemented with BAP started to swell at its proximal end after three days of culture. By the second week, calli were initiated on the wounded end of the root explant. By the fifth week, adventitious shoots were induced from the callus. In the case of hypocotyl, the cut end of the explant began to swell after a few days of culture. By the second week, calli were initiated on the wounded end of the hypocotyl explant. By the third week, greenish spots were observed on the callus. By the fourth week, adventitious shoots could be seen visibly on the callus in the culture vessels.

Table 1 shows the response of cotyledonary leaf, hypocotyl and root explants at different BAP concentrations on percentage and mean number of adventitious shoot formation. Data were taken eight weeks after culturing. It was observed that the cotyledonary leaf explant showed significant difference on percentage of explant regenerating adventitious shoots and mean number of shoots regenerated per explant compared with the hypocotyl explant cultured on most BAP treatments and the root explant in all the BAP treatments. The hypocotyl explant cultured on medium with 1.0 $mgL^{-1}$ BAP did not differ significantly in terms of percentage of explant regenerating shoots compared with the cotyledonary leaf explant. Eighty percent of the cotyledonary leaf explants induced adventitious shoots in the medium supplemented with 10.0 $mgL^{-1}$ BAP with a mean of five shoots per explant. The percentage of hypocotyl explants regenerating shoots ranged from 6.7 to 76.7%, while the root explants exhibited a range of 6.7 to 60.0% in the respective concentrations of BAP tested. The mean number of adventitious shoots regenerated from both the hypocotyl and root explants was very low, within the range of 0.1 to 2.2 shoots only. The success of using cotyledonary leaf explant for in vitro adventitious shoot regeneration has been reported by many researchers (Hartmann et al., 2007; Selvaraj et al., 2007; Lee et al., 2003). Tian et al. (2007) reported high frequency of shoot regeneration from hypocotyl explants of Prunus, while Lee et al. (2003) observed no shoot formation from hypocotyl and root explants of winter squash. In this experiment, cotyledonary leaf explant of *J. Curcas* exhibited prolific shoot regeneration ability compared with the hypocotyl and root explants.

Table1: Adventitious shoot formation from cotyledonary leaf, hypocotyl and root explants of *Jatropha curcas* cultured on medium supplemented with different concentrations of BAP. Data were taken eight weeks after culturing.

| Explant type | BAP ($mgL^{-1}$) | Percentage of explant regenerating shoots | Mean number of shoots per explant |
|---|---|---|---|
| Cotyledonaryleaf | 10.0 | 80.0 a | 5.0 a |
| Hypocotyl | 1.0 | 76.7a | 2.2 b |
| Hypocotyl | 2.5 | 6.7 c | 0.1 c |
| Hypocotyl | 5.0 | 6.7c | 0.1 c |
| Hypocotyl | 10.0 | 0 c | 0 c |
| Root | 1.0 | 50.0 b | 1.7 b |
| Root | 2.5 | 60.0 b | 2.1 b |
| Root | 5.0 | 6.7 c | 0.2 c |
| Root | 10.0 | 6.7 c | 0.1 c |

*Mean followed by the same letter(s) in the same column are not significantly different based on DNMRT at p= 0.05*

## Conclusion

The results revealed that cotyledonary leaf explant regenerated adventitious shoots better than hypocotyl and root explants within the BAP range tested.

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# The Effects of Carbon Dioxide in the *ex vitro* Germination on Coffee Somatic Embryogenesis

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## Introduction

Commercial use of micropropagation by somatic embryogenesis technology is still limited because of its relatively high production cost resulting mainly from high labor costs, low growth rate in vitro and poor survival rate of the planlet during acclimatization. The goal of somatic embriogenesis technology is to mass-produce genetically identical, physiologically uniform, developmentally normal, and pathogen-free planlets which can be acclimatized in a reduced time period and at a lower cost. Development of both automated environmental control system and improved in vitro culture systems are essential for a significant reduction in production cost (Aitken-Christie et al., 1995).

Cultured plant cells require carbohydrate as a source of carbon and energy. Sucrose is the main carbohydrate which supports growth in vitro (Thompson and Thorpe, 1987). Presence of sucrose in the medium induces heterotrophy or mixoheterotrophy in the cultures (Kozai et al., 1992). This and other in vitro condition contribute a culture induce phenotype in planlets (Kozai et. al., 1992), which impedes their normal growth when transferred directly from culture to ambient greenhouse or field conditions and necessitates a period of acclimatization (Deng and Donnelly, 1993). Recent studies have shown that the culture-induced phenotype can be modified towards autotrophy by reducing or completely eliminating sucrose in the medium (Kozai and Iwanami,1988; Nguyen and Kozai, 2001) and increasing $CO_2$ concentration (Desjardins et al., 1988). This concept of photoautotrophic micropropagation has recently been proposed as a mean of reducing production cost, improving planlet growth and survival (Langford and Wainwright, 1987) and reducing the hazards of biological contamination (Hazarika, 2003).

The induction of somatic embryogenesis has been recognized as a technique for producing large numbers of individuals and consequently, there is a great interest in its use for mass propagation (von Arnold et al. 2005; Preil 2005; Takayama and Akita 2005). The application of propagation by somatic embryogenesis required the optimization and control the environment. The influence of environment especially of the concentration of carbon dioxide is a factor that has very little study in the process of somatic embryogenesis from cotyledonary embryo to become planlet. The present research was carried out with the objective to determine the effects of carbon dioxide on cotyledonary embryo in the ex vitro germination.

## Materials and Methods

This research was conducted at Nestle Research and Development Centre, Tours (NR&DC-T), France. This experiment used cotyledonary embryos of the FRT 04 and FRT 65 Robusta genotype. Three systems were tested in germination medium such as rockwoll plugs, Jiffy plugs and paper. Each medium soaked with 50% MS liquid medium and each system were compared with and without a concentration of 3% $CO_2$. Each treatment was replicated three times and observed for four weeks by measuring the percentage of the number of leaves.

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**Results and Discussion**

Micropropagation is being widely used for the clonal propagation of selected horticultural plants, estate crops, and forest trees. In commercial tissue culture labortaories, much concern has been placed on the culture media, optimal culture conditions, and culture techniques for such elite plants. The culture vessels usually used for the shoot development and rooting stages in the micropropagation are still the conventional glass or plasticwares. These vessels, however, may have some disadvantages: they are relatively expensive, less transportable, inflexible in shape and size, and the chance of contamination with microorganisms through their opening is relatively high when using aluminium foil covers or loosely-closed caps. Furthermore, the accumulation of ethylene (Mele et al., 1982) or the decrease in $CO_2$ concentration during light period (Fujiwara et al., 1987) in conventional closed (air tight) culture vessels may have an adverse effect on planlet development.

Plants remove carbon dioxyde from the atmosphere by photosynthesis, also called carbon assimilation, which uses light energy to produce organic compounds (cellulose, lipids, and various proteins) by combining carbon dioxyde and water. Plants can grow up to 50 percent faster in concentrations of 1,000 ppm $CO_2$ when compared with ambient conditions.

The results demonstrate that concentration of 3% $CO_2$ can promote and stimulate the shoot by using plugs of rockwooll plugs, Jiffy plugs and paper. The shoot percentage by using $CO_2$ is higher than that of without $CO_2$ (Figure 1). In FRT 04, the highest percentage was reached by using rockwooll plugs with a concentration of 3% $CO_2$ (Figure 2). Whilst in FRT 65, the highest percentage was reached by using Jiffy plugs with a concentration of 3% $CO_2$ (Figure 3). We conclude that an enrichment $CO_2$ at 3% has strongly improved the percentage of shoot in germination stage of coffee. Whereas, to our understanding, the high $CO_2$ concentrations has never been related to the positive effect on germination. We usually connect with humidity or temperature only. An enrichment of the headspace with exogenous $CO_2$ at 2% strongly promotes the germination when the coffee embryos are grown in a no-emitting media as plugs of rockwool (Ducos et. al., 2009).

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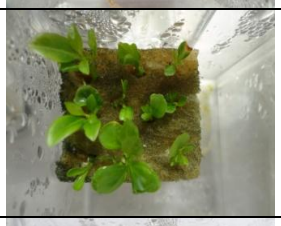
| Treatment | Rockwooll Plugs | Jiffy Plugs | Paper |
|---|---|---|---|
| FRT 04 With $CO_2$ |  |  |  |
| FRT 04 Without $C0_2$ |  |  |  |
| FRT 65 With $CO_2$ |  |  |  |
| FRT 65 Without $CO_2$ |  |  |  |

Figure 1. The performance of leaves after the effect of $CO_2$ released by the media on cotyledonary embryos in FRT 04 and FRT 65.

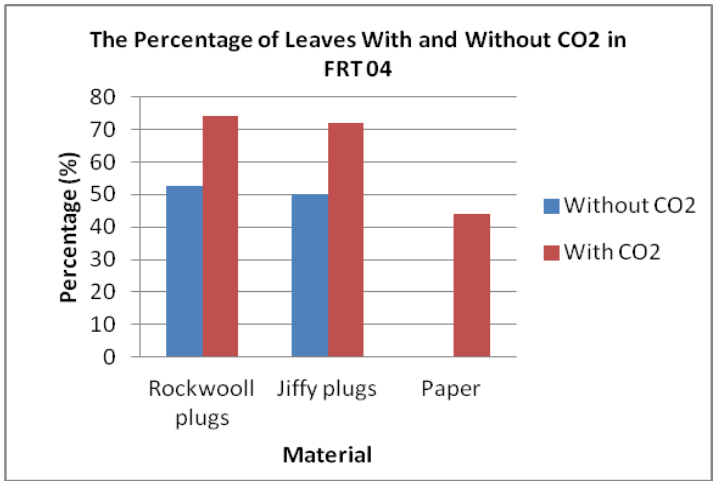



Figure 2. The percentage of leaves in different material on the effect of exogenous $CO_2$ on FRT 04 cotyledonary embryos.

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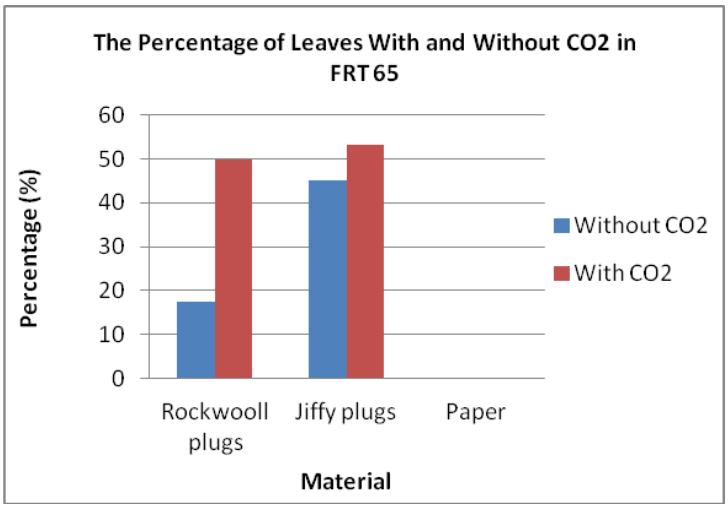


Figure 3. The percentage of leaves in different material on the effect of exogenous $CO_2$ on FRT 65 cotyledonary embryos.

## Conclusions

The concentration of 3% $CO_2$ could directly promote and stimulate the shoot by using plugs of rockwooll plugs, Jiffy plugs and paper in germination stage of coffee. Whereas, to our understanding, the high $CO_2$ concentrations has never been related to the positive effect on germination. We usually connect germination with humidity or temperature only. These results showed the necessity to carry out other studies that allow elucidating the influence of $CO_2$ on cells and tissues cultured in vitro under conditions of non phototrophic.

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# Characterizing Patterns in Developmental Stages of Somatic Embryo of Cocoa (*Theobroma cacao* L.)

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## Introduction

Plant regeneration using tisssue culture was achieved through organogenesis and somatic embryogenesis. Somatic embryogenesis can be described as the process by which haploid or diploid somatic cells develop into structures that resemble zygotic embryos (i.e. bipolar structures without any vascular connection with the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes (Williams and Maheswaran, 1986; Emons, 1994; Raemakers et al., 1995). These methods were also used for plant genetic transformation efficiently (Jimenez et al., 2001).

Somatic embryogenesis process influenced by several factors, such as plant genotype, developmental stage and type of the explant, medium composition, growth regulators, light intensity and physiology of cells that play a crucial role in the induction and maintenance of somatic embryogenesis in many plants (Terzi and Loschiavo, 1990; Ehsanpour, 2002; Jimenez, 2005). The cells which represent an intermediate state between somatic and embryogenic cells are called competent. Cellular competence is associated with the dedifferentiation of somatic cells that allows them to respond to new developmental signals. It is well accepted that embryogenic competent cells can be morphologically recognized as small, rounded cells with rich cytoplasm and small vacuoles (Feher, 2005).

The procedure for plant regeneration of cocoa (*Theobroma cacao* L.) from staminode explants has been developed through somatic embryogenesis technology. In the process of callus induction with staminode explants can be grown rapidly in media containing hormones auxin and cytokinin with a certain balance. Furthermore, the embryo will develop from the callus after sub-cultured on medium without hormones. The concentration of TDZ used in PCG medium significantly affected the rate of callus growth, the frequency of embryogenesis, and the number of somatic embryos produced from each responsive explant. 2,4-dicholorophenoxy acetic acid at 5 μM was the most effective plant growth regulator (PGR) among all PGRs used. A large number of cacao plants have been regenerated from somatic embryos and established in soil in a greenhouse. Plants showed morphological and growth characteristics similar to those of seed-derived plants. The described procedure may allow for the practical use of somatic embryogenesis for clonal propagation of elite cacao clones and other applications that require the production of a large number of plants from limited source materials.

These early studies were very significant, because they confirmed Haberlandt's prediction that embryos can arise from single cells in culture (i.e. cellular totipotency) (Kiyosue et al., 1993; Höxtermann, 1997). The ability to understand the mechanisms involved in the induction and expression of somatic embryogenesis in different species will increase the number of genotypes capable of regeneration by this process.

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## Materials and Mehods

The medium used consisted of MS (Murashige and Skoog, 1962) basal medium supplemented with 3% (w/v) sucrose and 3% gelrite. The pH of media was adjusted to 5.6-5.8 and then autoclaved at 121$^{o}$C for 20 min.

*Plant materials*

Embryogenic callus and somatic embryos were obtained from cocoa flower bud (staminode and petal) induction for five different clones, I03, I04, Sul 1, Sul 2, and Scavina 6. Callus induction were cultivated on induction medium supplemented with 2,4 dichlorophenoxyacetic acid as plant growth regulators. For each condition and cultivar, 50 petridishes with 5 clusters per plate were used. After four weeks, each callus observed their capability to formed embryogenic callus. Embryogenic callus transferred to the expression medium supplemented with cytokinin for four weeks to obtain somatic embryos. Regeneration efficiency and morphological characteristics were observed for each clone. The cultures were subcultured monthly and maintained at 25 $^{o}$C with 16 h photoperiod.

## Results and Discussion

*Developmental stages of cocoa somatic embryo*

During development stages, somatic embryos change in shape and size. Expression pattern influence by gen regulation that controlled morphological and physiological changes. Somatic embryogenesis development broadly divided into two phases, first is competence cell differentiated into embryogenic callus. For the next phase is the expression of embryogenic callus to form somatic embryos. Those two phases can develop independently due to the process influence by different factors such as plant growth regulators. The removal of auxin results in the inactivation of a number of genes so that the embryogenic program can now proceed. The observation that some carrot cell lines are able to develop to the globular stage, but not beyond in the continued presence of auxin, suggests that new gene products are needed for the transition to the heart stage and that these new products are synthesized only when exogenous auxin is removed (Zimmerman, 1993).

*Embryogenic callus induction*

Secondary embryogenic callus was obtained from primary somatic embryos induction. Primary and secondary embryos development exhibits the same morphology during the process (Maximova et al., 2002; Feher et al., 2003). Nevertheless, primary somatic embryos hystologically dominated by multicellular tissue but secondary somatic embryos dominated by unicellular tissue (Alemano et al., 1996).

They can also form indirectly via an intermediary step of callus or suspension culture (in these cases a more complex medium should be used, including additional factors to induce dedifferentiation and reinitiation of cell division of already differentiated cells before they can express embryogenic competence) (Williams and Maheswaran, 1986; Ammirato, 1987). Embryogenic cells are unique: superficially they resemble meristematic cells, though they generally are smaller, more isodiametric in shape, have larger, more densely staining nuclei and nucleoli, and have a denser cytoplasm (Williams and Maheswaran, 1986; Carman, 1990).

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The average of embryogenic callus competence ranged between 11-37% for five clones of cocoa. The highest percentage shown in Scavina 6 clone and ICCRI 04 clone as the lowest. However, after the addition of auxin (2,4 D) then each clone cappability to form embryogenic callus increased to 85% (Figure 1).

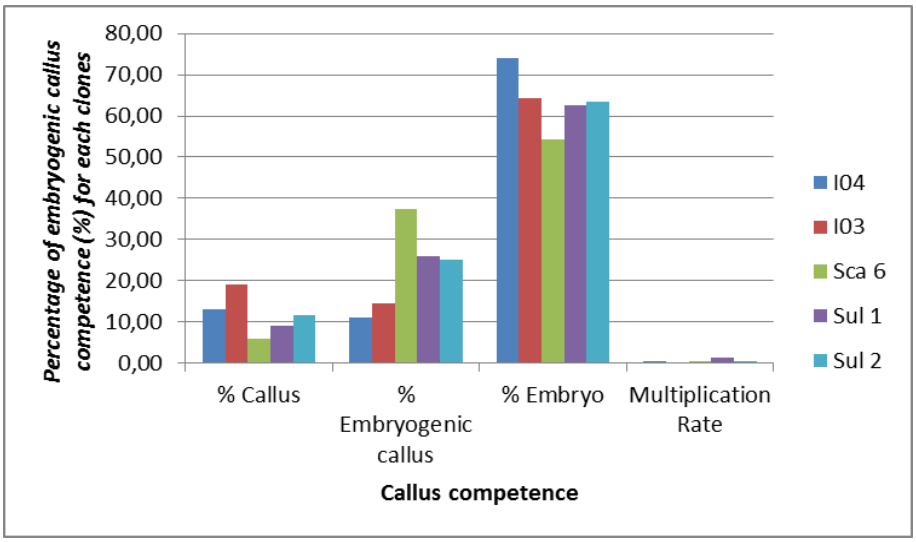


Figure 1. Somatic embryogenesis process of five clones of cocoa on proliferation medium.

Explant (non-embryogenic) cells can be induced to an embryogenic state by a variety of procedures that usually include exposure to plan growth regulators, pH shock, heat shock or treatment with various chemical substances. However, it is still not clear whichchanges asomatic cell must undergo in order to become an embryogenic cell capable of forming an embryo. There appears to be no single, universally applicable signal that renders cells embryogenic (Mordhorst et al., 1998). Moreover, in general only a very limited number of cells in any given explant respond by becoming embryogenic (Toonen and de Vries, 1997). Typically, embryogenic and non-embryogenic callus are distinguishable based on their morphology and color (Von Arnold et al., 2002; Yang et al., 2010). Embryogenic callus presents nodular features and a smooth surface (Yang et al., 2010). They are composed of proembryogenic masses or PEMs (Von Arnold et al., 2002), which can usually be defined as clusters of small cytoplasmic cells (De Jong et al., 1993), i.e. embryogenic cells. Such type of cells form somatic embryos and are generally small and isodiametric in shape (Yang et al., 2010). These types of cells usually appear after re-initiation of cell division and a period of proliferation of the released explant cells in the presence of auxin (De Jong et al., 1993). In contrast, non-embryogenic cells are rough, friable, and translucent (Jimenez and Bangerth, 2001). Callus competences of each clone are shown in the Figure 2.

The capability of explants to generate embryogenics cells is depend on genotype of each clone. However, the addition of suitable plant growth regulators can enhance the competence of clones to proliferate. Auxins are important in somatic embryogenesis for being the primary promoters of growth and differentiation of embryogenic cells through the regulation of changes in cellular gene expression (Gray, 2004).

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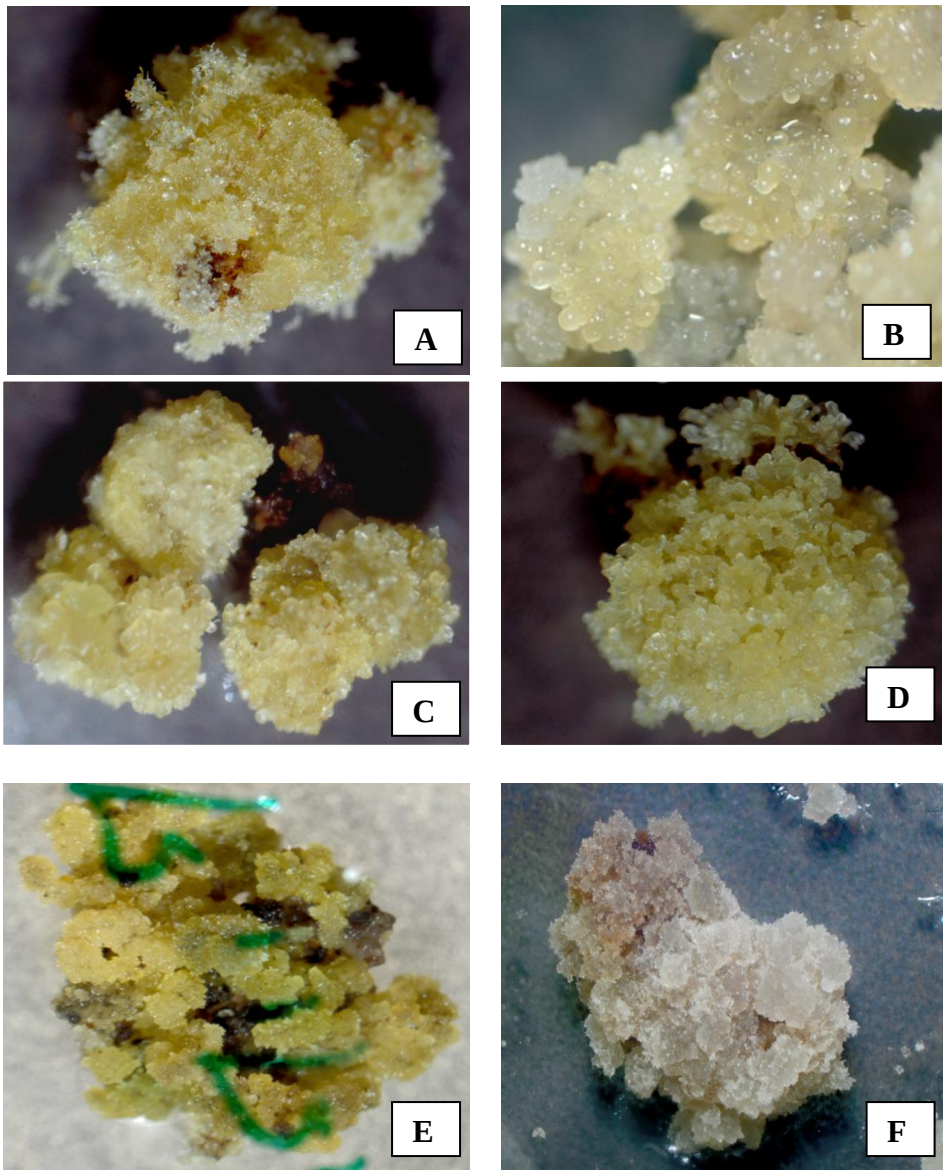


Figure 2. Morphology of callus in different cocoa clones. A) ICCRI 03 clone, B) ICCRI 04 clone, C) Sul 1 clone, D) Sul 2 clone, E) Sca 6 clone and F) Aqueous callus.

*Expression of somatic embryos*

Somatic embryogenesis is defined as a developmental process by which somatic cells induced through the embryogenetic pathway to generate embryogenic cells. These cells then go through a series of morphological and biochemical changes that result in the formation of a somatic embryo making it possible to regenerate new plants in large scale (Schmidt et al., 1997; Komamine et al. 2005). It can be also described in a more simple way as a process by which somatic cells develop into plants through characteristic morphological stages such as globular stage, heart stage, torpedo stages (De Jong et al., 1993). Development through somatic embryogenesis includes a number of characteristic events: dedifferentiation of cells, activation of cell division, and reprogramming of their physiology, metabolism, and gene expression patterns (Yang and Zhang, 2010).

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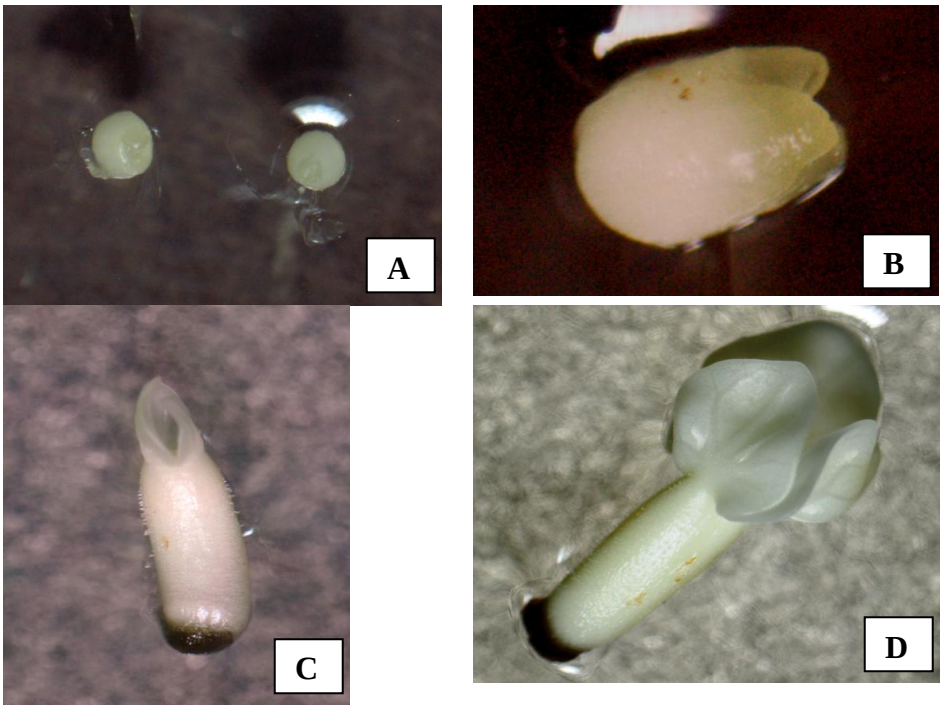


Figure 3. Embryo development phases. A) Globular, B) Heart, C) Torpedo, and D) Cotyledon.

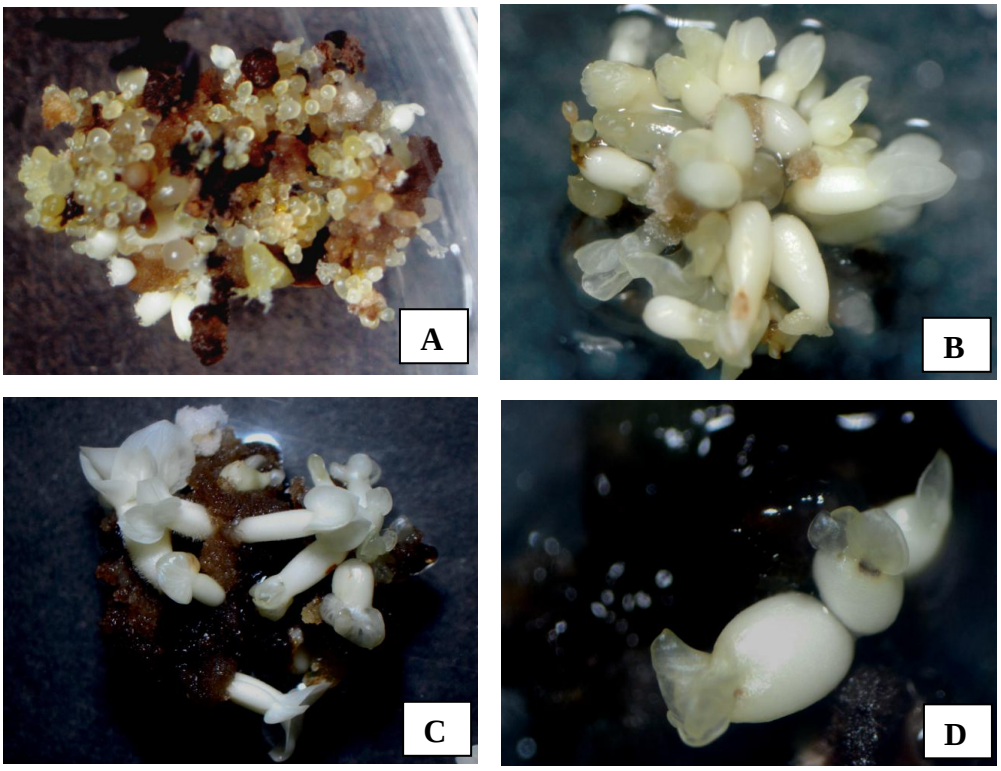


Figure 4. Expression of somatic embryos. A) Proliferation of embryogenic callus, B, C, D) Expression of embryogenic callus becomes somatic embryo.

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Figure 5. Abnormal embryos.

Table 1. Morphological development of somatic embryos on expression medium (Figure 3).

| Embryo phase | Expression medium | | |
|---|---|---|---|
| | Number of embryo | % Abnormal embryo | Embryo size |
| Globular Normal | 3.67 d | 10.67 c | 0.1 c |
| Torpedo Normal | 2.33 d | 13.67 c | 0.4 b |
| Kotiledon Normal | 2.67 d | 6.33 c | 0.7 a |
| Globular Normal | 68.33 a | 7.33 c | 0.1 c |
| Torpedo Normal | 25.67 b | 27.67 b | 0.43 b |
| Kotiledon Normal | 18.67 c | 60.00 a | 0.7 a |

Somatic embryogenesis response was visible within two weeks of explant culturing. Extending incubation for further 4-5 weeks was found optimum for obtaining large numbers of suitable-to-process size somatic embryos. The expressions of embryogenic callus in one cluster are diverse in size and shape, including callus, normal and abnormal embryos (Figure 4). Comparison between the number of normal and abnormal embryos is highly dependent on the composition of the media and plant genotype (Table 1). The embryo will immediately express after hormone auxin was removed.

The Somatic embryogenesis process begins with the induction through cocoa flower induction (petal and staminode). SE development has been divided into two main phases, namely, the one whereby differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells, and the phase whereby the embryogenic cells display their embryogenic competence and differentiate into somatic embryos. Both processes appear to be independent from each other and thus to be influenced by different factors. The term ‘embryogenic cell’ is restricted to those cells that have completed their transition from a somatic (non-embryogenic) state to one in which no further exogenously applied stimuli,

such as the application of growth regulators, are necessary to produce the somatic embryo (Komamine et al., 1992; De Jong et al., 1993). The cells that have reached this transitional state and have already started to become embryogenic, but that still require exogenously applied stimuli, are designated as competent cells (Mordhorst et al., 1997). Although plant growth regulators play a key role in inducing somatic embryogenesis, there are many other factors that have been found which could affect the disposition of a particular tissue to undergo somatic embryogenesis. The range of possible induction treatments suggests that it is unlikely that a single inducing molecule is responsible (Toonen and de Vries, 1996). Examples of these other factors that can direct the transition from somatic cells to cells able to form embryo-like structures are: in Citrus suspension cultures a change in carbon source from sucrose to glycerol (Ben-Hayyim and Neumann, 1983; Gavish et al., 1991; Jiménez and Guevara, 1996); in carrot the $NH4^+$ concentration (Smith and Krikorian, 1989) and pH changes (Smith and Krikorian, 1990, 1992) in the culture medium; in Araujia sericifera the light quality (Torné et al., 2001), in Brassica microspores a temperature shock (Pechan and Keller, 1988); also pre-treatment of donor plants and subculture duration (Mórocz et al., 1990), to name only a few factors.

The process of embryogenesis is influenced by several factors, among others, plant genotype, explant source, the composition of media, growth regulators and physiological state of the cell (Terzi and Loschiavo, 1990; Ehsanpour, 2002). Several previous studies have successfully induced and regenerate plants from a variety of sources via somatic embryogenesis ekspan age. Meristematic tissue is used as a source of explants in meristem culture may be apical meristems or axillary shoot meristem. Propagation of plants through somatic embryogenesis is the formation, growth and development of the embryonic cells of the soma or body cells (Ammirato, 1983). Embryogenesis technique has advantageous for mass vegetative propagation of the species that have high economic value (Blanc et al., 1999). Furthermore, Molina et al. (2002) suggests that somatic embryogenesis can occur either directly or indirectly. Somatic embryogenesis that occurs indirectly preceded by callus formation and embryoid can be generated through callus or cell suspension cultures (Noerhadi, 1974). Embryogenic callus can be generated from the treatment of 2,4-D and or in combination with other plant growth regulators. Somatic embryos have the same clonal characteristics and juvenile as derived from the seed. Propagation of plants through somatic embryogenesis consists of several phases: initiation of embryogenic callus, embryogenic callus multiplication, maturation and germination of somatic embryos (Von Arnold et al., 2002).

## Conclusions

*In vitro* somatic embryogenesis of cocoa is an important prerequisite for the use of many biotechnological tools for genetic improvement, as well as for mass propagation which have limitations of conventional propagation. In this study, a protocol for somatic embryogenesis of cocoa was developed for mass propagation with efficient regeneration. Meanwhile, this protocol offers itself not only as a highly efficient method for mass clonal propagation of this species but also for its conservation. The expression of embryogenic callus in one cluster is diverse in size and shape, including callus, normal and abnormal embryos. Comparison between the number of normal and abnormal embryos is highly dependent on the composition of the media and plant genotype. The embryo will immediately express after auxin was removed.

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# *In vitro* Seeds Germination, Protocorms Proliferation and Shoots Development of Borneo Wild Native Orchid, *Dimorphorchis rossii*

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## Introduction

Most of the orchid could be found in Borneo and particularly, in state of Sabah. Sabah has approximately 150 genera consisting of 1500-2000 orchid species. *Dimorphorchis rossii* belong to the *tribe* Vandeae and *sub tribe* Aeridinae (Beaman and Wood, 2001) and grown as epiphytic and endemic to Sabah. There are only two species in these genera which are *D. rossii* and *D. lowii* (Chan et al., 1994). *D. rossii* is becoming seriously endangered in the wild due to forest clearance for urbanization and agricultural development, and illegal collections (Chan et al., 1994). Therefore, there is an urgent need to conserve this ecstatic species. The conventional propagation methods cannot be applies effectively since this species is monopodial and has a slow growth cycle. In addition, orchid seeds do not have endosperm, so their natural germination is limited and need a symbiotic association with specific mycorrhizal fungus (Arditti and Ernst, 1993). Application of plant tissue culture offered various ways for orchid propagation in large scale by using meristem culture or asymbiotic seeds germination (Arditti and Ernst, 1993). Seeds are able to germinate up to 83% as seen in *Vanda dearei* (Roslina and Gansau, 2007). Thus, the objectives of this present study were to determine optimum growth condition for high percentage of *in vitro* seed germination and protocorm development and established higher number of seedlings/ plantlets through protocorms proliferation study.

## Material and Methods

*Dimorphorchis rossii* grown *ex situ* at Green House of Unit Studies of Orchids, Institute for Tropical Biology and Conservation, University Malaysia Sabah, Malaysia. Flowering orchid was pollinated by transferring pollen to other flower in same plant by using needle. Immature capsules were harvested and cleaned under running tap water for 30 minutes before surface sterilized by soaking in 30% (v/v) sodium hypochlorite solution (Clorox®) with two drops of Tween 20 and agitated for 20 minutes. The capsules were then rinsed five times with sterile distilled water. Seeds from dissected capsule were sprinkled onto the surface of plastic Petri dish containing 25ml media. Series of treatments were studied to optimized nutrient composition such as basal medium [KC (Knudson, 1946), MS (Murashige and Skoog, 1962) and VW (Vacin and Went, 1945)], carbon sources [sucrose, glucose and fructose ,1%, 2%, or 3% (w/v)], complex additives [coconut water (CW) (10-20%, v/v), fresh tomato (FT) (10-20%, w/v), potato homogenate (PH) (10-20%, w/v), yeast extract (YE) and peptone (PE) (0.10-0.3%, w/v)], and light conditions (16 hours light, 24 hours light or 24 hours in the dark). For protocorm proliferation and development, three months old protocorms (1.0 to 2.0mm in size) were cultured on KC, MS, half strength of MS (1/2MS) and VW. Selected basal medium was later supplemented with complex additives such as CW (10-20%, v/v), FT (10-20%, w/v), YE and PE (0.10-0.3%, w/v). All treatments fortified with 2% (w/v) sucrose. The pH of the medium was adjusted to respective pH value of basal medium [KC (pH5.3); MS (pH5.7); VW (pH5.2)] before solidified with 0.9% (w/v) agar (Sigma) and autoclaved at 121 $^{o}$C (15p.s.i) for 20 min. The cultures were grown at 25±2 $^{o}$C and the illumination provided by cool white fluorescent tubes (Philips, Malaysia). All experiments were performed in a Completely Randomized

Design (CRD) and repeated in 5 replicates. Cultures were observed using Dino Lite Digital Microscope. Analysis of variants (ANOVA) was performed on the data and mean values were compared by using Duncan Multiple Range Test (DMRT) at $p<0.05$.

**Results and Discussion**

*In vitro seeds germination*

Seeds of orchids contain small embryos without any associated endosperm storage tissue. During germination process, embryo became swollen and formed small-corm like structure which known as protocorm (George et al., 2008). Now days many studies have been advocate asymbiotic seed germination as suitable propagation technique for conservation of orchid species (Srivastava et al., 2013). Pod that harvested on day 210 showed the highest seed germination compare to other days which indicate $210^{th}$ day was the ideal time to harvest the pod and culture in the laboratory (data not shown). In the basal media studies (Table 1), MS basal media showed the highest seed germination (29.47±5.98%) compare to other media. The response depending on type of media use which related to the nutrient composition in the media. MS medium were highly enriched with macro- and micro-elements compared to KC and VW media (Hossain et al., 2010). Similar result was obtained by Abraham et al. (2012) and Srivastava et al. (2013) where MS medium promoted maximum seed germination of *Vanda coerulea, Coelogyne nervosa* and *Aerides masculum* respectively. Besides that, in carbon source study (Table 2), 2% (w/v) sucrose showed maximum germination (33.33±3.65%) while other treatments especially in high concentration led to necrosis of the seeds. Tokuhara and Mii (2003) had also reported in their study the significant effect of sucrose in callus and protocorm-like-bodies (PLBs) proliferation of *Phalaenopsis* orchids. In complex additives study, 15% (w/v) PH gave the highest percentage of seed germination (95.56%) followed by 20% FT (91.00%) (Table 3). Potato extract alone or potato extract combined with components of conventional culture media has been found to provide a useful medium for the anther culture of wheat and some other cereal plants (Chuang et al., 1978). Addition of potato extract to orchid culture medium was reported by Sagawa and Kunisaki (1982). Potato fresh treatment has also been reported to help cultures of *Doritaenopsis* (Orchidaceae) to recover from hyperhydricity (Zhou, 1995). The seeds also germinated the best when exposed to 16 hour light rather than continuous exposure to light or dark (Table 4).

*Proliferation and shoot development of protocorm*

In protocorm proliferation, MS basal media recorded the highest percentage of protocorm proliferation (33.33±0.49%) as seen in Table 5. For study that involved complex additives (Table 6), 0.2% YE recorded the highest percentage of protocorm proliferation (41.67±0.51%) followed by 20% (v/v) FT and control media (33.33±0.49%) based on. Meanwhile 0.1% (w/v) PE showed no response in protocorm proliferation. YE normally enhances growth in media containing relatively low concentration of nitrogen or where vitamins are lacking and have been shown to have some unusual properties which may relate to its amino acid content (George et al., 2008). Protocorm showed the best growth and development in MS basal media when effect of basal media was studied (Table 5). MS basal media recorded the highest percentage of protocorm formed leaf (41.67±0.51%) and root (16.67±0.39%). Besides that, the highest mean number of new leaf and root was observed in MS basal media as well (4.40±3.82 and 3.50±1.51 respectively). While VW media showed no response in formation of root and most of the protocorm became necrosis (43.75±0.51%). For the effect of complex additives (Table 6), 20% FT recorded the highest percentage of protocorm formed leaf (83.33±0.39%) followed by 10% CW (78.33±0.42%). The lowest percentage of necrosis or dead protocorm was also observed in 10% CW (6.67±0.63%). CW can be beneficial to some orchid plant where it can induce proliferation and development of protocorm into complete seedling (Arditti and Ernst, 1993). Besides that, CW also has been found to be beneficial for

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inducing growth of both callus and suspension culture and for induction of morphogenesis (George et al., 2008).

Table 1. Effect of basal media on seed germination of *Dimorphorchis rossii* under 16h light observed after 140 days of culture.

| Basal medium | Seed germination (%) |
|---|---|
| MS | 29.00±6.98[a] |
| KC | 12.00±5.87[b] |
| VW | 0[c] |

Table 2. Effect of carbon source on seed germination of *Dimorphorchis rossii* on MS medium under 16h light observed after 140 days of culture.

| % Carbon sources (w/v) | | Seed germination (%) |
|---|---|---|
| Control | | 24.00±3.47[b] |
| Sucrose | 1.0 | 22.69±5.35[b] |
| | 2.0 | 33.33±3.65[a] |
| | 3.0 | 31.00±4.65[a] |
| Glucose | 1.0 | 12.10±1.25[c] |
| | 2.0 | 3.10±1.25[d] |
| | 3.0 | 0[e] |
| Fructose | 1.0 | 0[e] |
| | 2.0 | 18.38±3.87[b] |
| | 3.0 | 5.36±2.25[d] |

Table 3. Effect of complex additives on seed germination of *Dimorphorchis rossii* on MS medium supplemented with 2% (w/v) sucrose and grown under 16h light observed after 140 days of culture.

| Complex additives | | Seed germination (%) |
|---|---|---|
| Control | | 10.62±2.56[e] |
| | 10 | 89.44±26.87[ab] |
| Coconut water (CW, v/v) | 15 | 57.53±10.85[d] |
| | 20 | 56.73±10.27[d] |
| | 10 | 88.00±27.94[ab] |
| Potato homogenate (PH, w/v) | 15 | 13.27±5.72[e] |
| | 20 | 91.00±35.41[a] |
| | 10 | 78.44±26.87[c] |
| Fresh tomato (FT, w/v) | 15 | 95.00±38.35[a] |
| | 20 | 74.69±24.87[c] |
| | 0.1 | 0[f] |
| Peptone (PE, w/v) | 0.2 | 44.00±10.00[e] |
| | 0.3 | 0[f] |
| | 0.1 | 57.00±8.00[d] |
| Yeast extract (YE, w/v) | 0.2 | 0[f] |
| | 0.3 | 79.94±26.84[c] |

Table 4. Effect of light conditions on seed germination of *Dimorphorchis rossii* on MS medium supplemented with 2% (w/v) sucrose and 15% (v/v) fresh tomato observed after 140 days of culture.

| Light condition | Seed germination (%) |
|---|---|
| 16 hours light | 96.00±5.94[a] |
| 24 hours light | 88.00±6.87[b] |
| 24 hours dark | 49.26±8.25[c] |

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Table 5. Effect of basal media on protocorm proliferation and growth development under 16h light observed after 130 days of culture.

| Basal media | Percentage of protocorm proliferation (%±SD) | Mean number of new protocorm (±SD) | Percentage number of protocorm with leaf (%±SD) | Number of leaf (±SD) | Length of leaf (mm±SD) | Percentage number of protocorm with root (%±SD) | Number of root (±SD) | Length of root (mm±SD) | Percentage of necrosis (%±SD) |
|---|---|---|---|---|---|---|---|---|---|
| 1/2MS | 16.67±0.58$^{b}$ | 0.67±0.58$^{d}$ | 33.33±0.49$^{b}$ | 2.00±0.98$^{c}$ | 1.27±0.72$^{b}$ | 16.67±0.39$^{a}$ | 1.00±0.39$^{b}$ | 1.11±0.45$^{b}$ | 33.33±0.49$^{c}$ |
| MS | 33.33±0.49$^{a}$ | 2.67±1.60$^{b}$ | 41.67±0.51$^{a}$ | 4.40±3.82$^{a}$ | 2.62±0.59$^{a}$ | 16.67±0.39$^{a}$ | 3.50±1.51$^{a}$ | 4.10±1.69$^{a}$ | 16.67±0.39$^{a}$ |
| KC | 12.50±0.34$^{c}$ | 1.00±0.68$^{c}$ | 25.00±0.25$^{c}$ | 2.00±0.97$^{c}$ | 1.26±0.61$^{b}$ | 6.25±0.25$^{b}$ | 1.00±0.25$^{b}$ | 1.36±0.34$^{b}$ | 18.75±0.40$^{b}$ |
| VW | 12.50±0.34$^{c}$ | 3.75±3.04$^{a}$ | 18.75±0.40$^{d}$ | 2.33±1.03$^{ab}$ | 0.98±0.43$^{C}$ | 0$^{c}$ | 0$^{c}$ | 0$^{c}$ | 43.75±0.51$^{d}$ |

*Note : Significant at P<0.01 level. Means in a column followed by a same letter(s) are not significantly (P<0.05) different according to DMRT.*

Table 6. Effect of complex additives added in MS medium on protocorm proliferation and growth and development observed after 130 days of culture.

| Complex additives | | Percentage of protocorm proliferation (%±SD) | Mean number of new protocorm (±SD) | Percentage number of protocorm with leaf (%±SD) | Mean number of leaf (±SD) | Length of leaf (mm±SD) | Percentage number of protocorm with root (%±SD) | Mean number of root (±SD) | Length of root (mm±SD) | Percentage of necrosis (%±SD) |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | | 33.33±0.49$^{b}$ | 2.67±1.60$^{b}$ | 41.67±0.51$^{f}$ | 4.83±3.82$^{bc}$ | 1.09±0.59$^{c}$ | 16.67±0.39$^{e}$ | 2.33±1.51$^{ab}$ | 0.68±1.69$^{d}$ | 16.67±0.39$^{d}$ |
| CW (v/v) | 10 | 11.67±0.32$^{f}$ | 2.80±2.25$^{b}$ | 78.33±0.42$^{b}$ | 5.12±3.57$^{b}$ | 3.43±2.46$^{a}$ | 66.67±0.48$^{a}$ | 2.27±1.31$^{ab}$ | 2.82±2.93$^{b}$ | 6.67±0.63$^{a}$ |
| | 15 | 16.67±0.39$^{d}$ | 2.33±1.38$^{c}$ | 66.67±0.49$^{c}$ | 6.28±3.90$^{a}$ | 2.48±2.02$^{b}$ | 58.33±0.52$^{b}$ | 2.22±1.50$^{ab}$ | 3.71±3.76$^{a}$ | 16.67±0.39$^{d}$ |
| | 20 | 8.33±0.29$^{g}$ | 1.67±1.44$^{e}$ | 33.33±0.49$^{g}$ | 3.50±3.33$^{e}$ | 0.94±1.49$^{d}$ | 25.00±0.45$^{d}$ | 1.50±1.08$^{c}$ | 1.12±2.25$^{c}$ | 66.67±0.49$^{i}$ |
| TF (w/v) | 10 | 12.50±0.34$^{e}$ | 0.75±0.54$^{g}$ | 56.25±0.51$^{cd}$ | 3.08±1.81$^{e}$ | 0.99±1.15$^{d}$ | 31.25±0.48$^{c}$ | 0.58±0.62$^{f}$ | 0.27±0.82$^{g}$ | 12.50±0.34$^{c}$ |
| | 15 | 12.50±0.34$^{e}$ | 2.00±1.75$^{c}$ | 68.75±0.48$^{c}$ | 2.90±1.72$^{f}$ | 0.93±0.78$^{d}$ | 31.25±0.48$^{c}$ | 2.33±1.56$^{a}$ | 0.54±1.44$^{de}$ | 12.50±0.34$^{c}$ |
| | 20 | 33.33±0.49$^{b}$ | 1.83±1.00$^{e}$ | 83.33±0.39$^{a}$ | 2.22±1.27$^{g}$ | 0.77±0.48$^{e}$ | 16.67±0.39$^{e}$ | 0.67±0.39$^{e}$ | 0.16±0.41$^{h}$ | 8.33±0.29$^{b}$ |
| PE (w/v) | 0.1 | 0.00±0.00$^{h}$ | 0.00±0.00$^{h}$ | 33.33±0.49$^{g}$ | 2.56±2.68$^{f}$ | 1.01±1.68$^{c}$ | 33.33±0.49$^{c}$ | 0.89±0.90$^{d}$ | 0.63±1.43$^{d}$ | 50.00±0.52$^{h}$ |
| | 0.2 | 18.75±0.40$^{c}$ | 2.38±3.16$^{c}$ | 50.00±0.52$^{e}$ | 2.63±1.97$^{f}$ | 0.74±1.06$^{e}$ | 12.50±0.34$^{f}$ | 0.25±0.34$^{g}$ | 0.13±0.39$^{h}$ | 18.75±0.40$^{de}$ |
| | 0.3 | 18.75±0.40$^{c}$ | 1.17±2.22$^{f}$ | 50.00±0.53$^{e}$ | 2.00±1.50$^{g}$ | 0.92±1.26$^{d}$ | 25.00±0.45$^{d}$ | 1.13±0.72$^{c}$ | 0.40±0.72$^{f}$ | 25.00±0.45$^{f}$ |
| YE (w/v) | 0.1 | 12.50±0.34$^{e}$ | 3.00±2.08$^{a}$ | 75.00±0.45$^{b}$ | 2.81±1.76$^{f}$ | 1.27±0.96$^{c}$ | 25.00±0.45$^{d}$ | 0.88±0.60$^{d}$ | 0.24±0.47$^{g}$ | 12.50±0.34$^{c}$ |
| | 0.2 | 41.67±0.51$^{a}$ | 1.94±2.35$^{cd}$ | 50.00±0.52$^{e}$ | 4.11±2.87$^{d}$ | 0.89±0.94$^{e}$ | 25.00±0.45$^{d}$ | 1.83±1.24$^{c}$ | 0.26±0.48$^{g}$ | 16.67±0.40$^{d}$ |
| | 0.3 | 31.25±0.48$^{b}$ | 1.50±1.02$^{e}$ | 56.25±0.51$^{cd}$ | 4.33±2.78$^{d}$ | 0.89±1.06$^{e}$ | 25.00±0.45$^{d}$ | 0.75±0.45$^{e}$ | 0.18±0.32$^{h}$ | 37.50±0.50$^{g}$ |

*Note: CW-coconut water;TF-fresh tomato;PE-peptone;YE-yeast extract. Significant at P<0.01 level. Means in a column followed by a same letter(s) are not significantly (P<0.05) different according to DMRT.*

## Conclusions

The present study has described the efficient method for *in vitro* seed germination and protocorm proliferation and development of *Dimorphorchis rossii*. These findings are important as a reference or guideline to those interested in doing this species as there are few or none paper that had been reported so far. Considering the dwindling population of this orchid, this technique can serve as an alternative to multiply this orchid in large scale for the purpose of conservation.

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# Pregermination of *Coffea canephora* cv Robusta Young Embryo by the Plating Method in Somatic Embryogenesis Propagation

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## Introduction

The capacity to produce morphologically well-formed and normally developed embryos from somatic cells resided uniquely within the plant kingdom (Zimmerman, 1993) up to the present time. In addition, the development of somatic and zygotic embryos is highly similar (Zimmerman, 1993; Dodeman et al., 1997). Therefore, somatic embryogenesis (SE) provides a useful model to study embryo development in plants. In contrast to zygotic embryogenesis, SE can easily be observed, the culture conditions can be controlled, and large quantities of embryos can be easily obtained (Kawahara and Komamine, 1995).

Somatic embryogenesis is the process by which somatic cell develop into plants through characteristic embryological stages without fusion of gamete. Culture tissues of coffee species exhibit a high embryogenic potential with somatic embryogenesis first reported for *Coffea canephora* in 1970 (Staritsky, 1970) and plant formation from *Coffea arabica* embryos in 1977 (Sondhal and Sharp, 1977). Two types of SE are recognized. The term "direct" is applied to explants that undergo a minimum proliferation before forming somatic embryos whereas "indirect" refers to explants that undergo an extensive proliferation before the development of somatic embryos (Sharp et al.,1980). It has been suggested that in direct embryogenesis, embryogenic cells are present and simply require favorable conditions for embryo development whereas indirect embryogenesis requires the redetermination of differentiated cells (Yeung, 1995). However, the terms "direct" and "indirect" are still useful in describing cases in which either very little or a great deal of explant proliferation precedes embryogenesis although not necessarily indicating fundamental differences in the cells involved (Halperin, 1995).

In *Coffea* spp several model systems have been reported for *in vitro* SE induction. SE in *Coffea canephora* was first reported by Staritsky (1970), who described the induction of callus tissue from orthotropic internodes. Subsequently, Herman and Haas (1975) obtained SE for *Coffea arabica* from callus cultures derived from leaf explants. Söndahl and Sharp (1977) developed a two-phase experimental protocol for SE from leaves of *C. arabica*. Dublin (1981) reported SE from leaf explants of Arabusta using a medium with cytokinins (6-Benzylaminopurine [BA] and kinetin) whereas Yasuda et al. (1985) induced embryogenic calli and somatic embryos from *C. arabica* leaf explants using only BA.

From an economical point of view, it is more relevant to achieve somatic embryogenesis in liquid medium. Production of coffee somatic embryos in Erlenmeyer flasks was first reported by Zamarripa et al., (1991) and by Van Boxtel and Berthouly (1996), for *Coffea canephora* and *Coffea arabica* respectively. The yield achieved could be as high as 400,000 torpedo embryos per liter within 7 weeks (Zamarripa et al., 1991).

Somatic embryogenesis process in Indonesian Coffee and Cocoa Research Institute (ICCRI) includes the callus induction, maintenance of the embryogenic callus, multiplication of the embryogenic callus in solid media, expression embryo in solid medium, maturation embryos in liquid medium, shooting and rooting in solid medium. This research aimed to find alternative methods to reduce the numbers of workers in the

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laboratory as reducing the last two steps in maturation embryo, namely shooting and rooting step, to become the pregermination step of young embryo by the plating method.

**Materials and Methods**

This research was conducted at Nestle Research and Development Centre, Tours (NR&DC-T), France. This experiment used embryos of the FRT04 Robusta genotype. The PGR1 medium contained MS medium with cytokinin as the growth hormone and the PGR2 medium contained MS medium free hormone. Embryos were collected using an autoclaved glass system containing a filter (nylon Bluttx 50 µm) fixed by rubber band. Petri dishes were filled with 100 ml of solid medium and boxes containing blue foam with 150 ml of liquid medium. Each recipient was inoculated with 1g of torpedo embryos. All the treatments were replicated twice.

Five protocols were used in this experiment as follows:

- A: Plating of the embryos on PGR1 medium then individual subculture on PGR2 solid medium in large petri dishes (d=140mm) then acclimatization of complete plantlets
- B: Plating of the embryos on PGR1 medium then plating on PGR2 solid medium in large petri dishes (d=140mm) then acclimatization of complete plantlets
- C: Plating of the embryos on PGR1 medium with Whatman paper then plating on PGR2 solid medium with Whatman paper in large petri dishes (d=140mm) then acclimatization of complete plantlets
- D: Plating of the embryos on 100 mm high blue foam soaked wih PGR1 liquid medium then plating on blue foam soaked with PGR2 liquid medium in plastic boxes then acclimatization of cotyledonary embryos
- E: Plating of the embryos on 100 mm high blue foam soaked with PGR1 liquid medium with Whatman paper then plating on blue foam soaked with PGR2 liquid medium with Whatman paper in plastic boxes then acclimatization of cotyledonary embryos.

Development of embryos into cotyledonary embryos were observed at seven days. Observations were made by measuring the percentage of the number of green and brown cotyledonary embryos.

**Results and Discussion**

Stage of embryonic development starts from globular, heart and torpedo shape. In embryonic development, torpedo shape is characterized by the presence of cell differentiation and polarization of growth, in particular the initiation of the formation of cotyledons. Green and open cotyledonary embryo can stimulate apical dominance of shoots (shoot tip of the stem). This phase is the final stage of embryonic development that leads to maturation with marked enlargement of cotyledon size.

The percentage of green and brown embryos would determine the success of the plantlets for acclimatization. The results showed that embryos of FRT04 genotype gave a higher green number of embryos (100%) using protocols A and B after one week starting from 1 g of torpedo. The embryos plated on filter (C) or on blue foam (D, E) turned necrotic (Figure 1). Hence, protocols A and B were better than the other protocols. More than 50% embryos were green using protocol C but more than 70% embryos were brown using protocols D and E (Table 1, Figure 2).

Embryos that had reached physiological maturity usually have better germination capacity, although the conversion of plant from somatic embryos was generally low. The rate ofconversion of somatic embryos into plants averageranged from 0 to 50%. This value was much lower than zygotic embryos of commercial commodity which have capacity for growing an average of over 90%.

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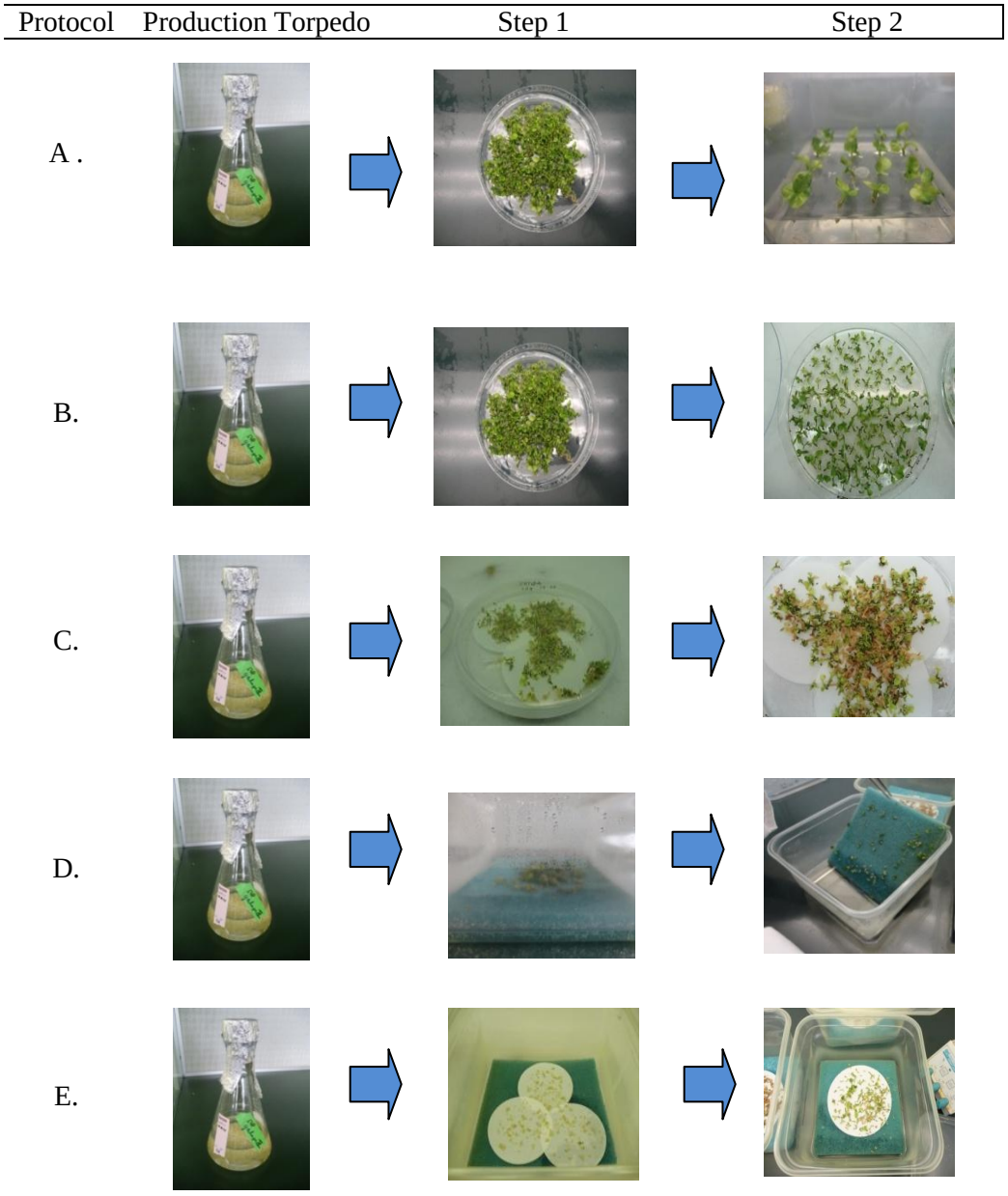


Figure 1. Pre-germination step in various protocols. A: plating DES1 – individual PGR2 with Gelrite, B: plating DES1 – plating PGR2, C: plating Whattman DES1 – plating PGR2 with Whattman, D: plating DES1 – plating PGR2 with Whattman, E: plating DES1 – plating PGR2 without Whattman.

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Table 1. Pre-germination step in various protocols. A: plating DES1 – individual PGR2 with Gelrite, B: plating DES1 – plating PGR2, C: plating Whattman DES1 – plating PGR2 with Whattman, D: plating DES1 – plating PGR2 with Whattman, E: plating DES1 – plating PGR2 without Whattman.

| Protocol | Protocols | % Green embryos | % Brown embryos |
|---|---|---|---|
| A | Plating DES1 – individual PGR2 with Gelrite | 100 | 0 |
| B | Plating DES1 – plating PGR2 | 100 | 0 |
| C | Plating Whattman DES1 – plating PGR2 with Whattman | 54.3 | 45.6 |
| D | Plating DES1 – plating PGR2 with Whattman | 13.8 | 84.8 |
| E | Plating DES1 – plating PGR2 without Whattman | 26.3 | 73.6 |

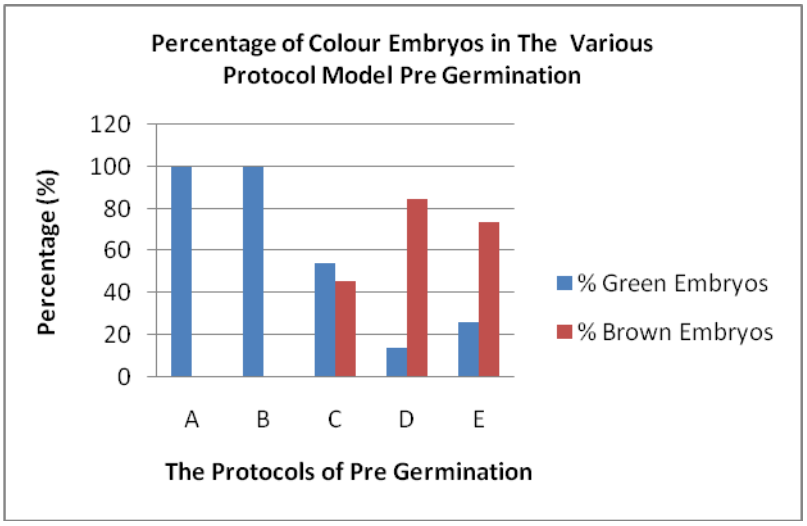


Figure 2. Pre-germination step in various protocols. A: plating DES1 – individual PGR2 with Gelrite, B: plating DES1 – plating PGR2, C: plating Whattman DES1 – plating PGR2 with Whattman, D: plating DES1 – plating PGR2 with Whattman, E: plating DES1 – plating PGR2 without Whattman.

## Conclusions

The percentage of green and brown embryos would determine the success of the plantlets when acclimatized. Protocols A and B described in this work reduced the culture time required, and made it more time- and cost- effective. It will be interesting to validate this protocol with other economically important coffee varieties.

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# Asymbiotic Germination of Borneo Endemic Orchid *Vanda hastifera* Immature Seed

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## Introduction

Orchid seeds are tiny, extremely light and often referred as "dust seeds". The seeds produced in vast numbers and contain very small nutrient reserves (Arditti and Abd Karim, 2000). Seeds may germinate in nature but will not grow unless infected by mycorrhizal fungus, which supplies the young plants with all the sugars and nutrients they need until the plants are old enough to produce food on their own (Rajkumar et al., 2008). In spite of huge number of seeds produce, only few seeds germinated in nature. In natural conditions, the life cycle of orchid is very long, it takes them approximately 4 to 10 years to bloom and produce seeds and this bring the difficulties for wild orchids to re-establish their position in natural habitats (Arditti, 1967). Therefore the application of plant tissue culture technique is proved to be the most efficient approach to conserve orchid species. In the present study, *Vanda hastifera* was selected because of the distribution of this Borneo endemic orchid has been depleted from its natural habitat because of the deforestation activities towards urban and agriculture development. This orchid was originally found mainly in Mt. Kinabalu and Tambunan district of Sabah (Chan et al., 1994). The flower of *V. hastifera* is white-cream with brown spots and it has a sweet scented which remain flowered throughout the year. The purpose of this study is to optimize the best medium for *in vitro* seed germination by determine the effect of basal media, complex additives and carbon sources for nurturing the conservation efforts of this valuable native orchid.

## Materials and Methods

Immature, green *V. hastifera* capsules were harvested at 150 days after pollination from Tenom Orchid Centre in Sabah Agriculture Park, Lagud Sebrang. Capsules of *V. hastifera* were characterized by weight (g), length, diameter (cm) and color. Capsules were cleaned under running tap water for 30 min before surface sterilized by soaking in 30% (v/v) sodium hypochlorite solution (Chlorox™) added with two drops of Tween 20 for 20 min. The capsules were rinsed five times with sterile distilled water, dipped in 95% (v/v) alcohol for five seconds and flamed to evaporate the alcohol. Seeds from dissected capsules were cultured on petri dish containing media treated with different basal media, complex additives and carbon sources. Basal media tested are Knudson C (KC, Knudson, 1946), MS (Murashige and Skoog, 1962), and Vacin and Went (VW, Vacin and Went, 1949). Each basal medium was added with 2% (w/v) of sucrose. Selected basal medium (KC) was then supplemented with various types of complex additives such as 10% (v/v) coconut water, 10% (v/v) tomato juice, 10% (v/v) banana pulp, 10% (v/v) potato homogenate, 0.2% (w/v) peptone and 0.2% (w/v) yeast extract. KC medium without additive served as control. After that, four types of sugars, which are sucrose (Sigma), glucose (Fluka), fructose (Mallinckrodt) and galactose (Sigma) at concentrations of 1 to 4% (w/v) were tested using KC medium supplemented with 10% (v/v) potato homogenate. The medium without sugars served as control. The pH was adjusted to 5.3 and solidified with 0.8% (w/v) of agar (Sigma) prior autoclaving for 20 min at 121 ºC (15 p.s.i). Cultures were incubated at 25±2 ºC under continuous illumination of fluorescent light 800W (Philip, Japan). Cultures were observed weekly for seed germination and protocorm development. The

germination percentage and growth index (GI) were determined as reported by Harrison and Arditti, (1978). All experiments were performed in a Completely Randomized Design with five replicates. Analysis of variants was performed on the data and the mean values were compared using Duncan Multiple Range Test (DMRT) at $p<0.05$.

**Results and Discussion**

*Seed characterization*

The immature capsule of *V. hastifera* harvested after 150 days pollination has 6.88 g of weight, 4.5 cm length, and 2.0 cm diameter (Figure 1b). Seeds of *V. hastifera* were yellowish, very minute and extremely light (Figure 1c). The length of seed ranged from 200 µm to 300 µm. The testa was transparent and the length was double of the embryo, which creates empty space in the seed. This observation is in agreement with Arditti and Abd Karim (2000) for other species of *Vanda*.

*Effect of basal media on seed germination*

After 150 days of culture, it was observed that KC basal medium was found superior to MS and VW media. The germination percentage of *V. hastifera* on KC medium was 77.84±1.14% with growth index (GI) value of 164.72 (Table 1). The seeds started to germinate when the embryo swelled and emerged from testa on this medium after 30 days of culture (Figure 1e). The result also showed that VW basal medium was not suitable to germinate seeds of *V. hastifera*. Knudson C (Knudson, 1946) medium has a simple formulation with few macro and micro salt elements compared to MS basal medium that consists of four main components such as macronutrients, micronutrients, iron and vitamins (Murashige and Skoog, 1962). Meanwhile, VW basal medium is made up only of macronutrients and iron (Vacin and Went, 1949). The variety of macro- and micro-elements in these three types of basal media gave different responses on seed germination of *V. hastifera*. Since *V. hastifera* is an epiphytic orchid, the current result was supported by Arditti (1967) where KC basal medium is commonly used for seed germination of epiphytic tropical orchids. Other differences in KC, MS and VW media are the nitrogen concentration and ratio of ammonium to nitrate. MS medium consists of ammonium nitrate (1650 mg/L) and potassium nitrate (1900 mg/L), KC medium contains ammonium sulfate (500 mg/L) and calcium nitrate (1000 mg/l), while VW medium consists of ammonium sulfate (500 mg/L) and potassium nitrate (525 mg/L). The result showed that nitrogen concentration and the ratio of ammonium to nitrate on KC medium was more suitable for *V. hastifera* seed to germinate. The efficiency of KC as basal medium for seed germination was also reported previously for *V. tessellate* (Roy and Banerjee, 2002), *Vanda* hybrids (Johnson and Kane, 2007) and *Dendrobium lituiflorum* (Vyas et al., 2009).

Table 1. Effect of basal media on seed germination of *Vanda hastifera* after 150 days of culture.

| Treatment | Germination time (days) | Germination (Means±SD)% | Growth Index (GI) |
|---|---|---|---|
| KC | 30 | 77.84±1.14 [a] | 167.72 [a] |
| MS | 30 | 30.75±8.81 [b] | 125.15 [b] |
| VW | 0 | 0 | 0 |

*Mean values within a column followed by the same letters are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test; Treatments were conducted in 5 replicates. SD= Standard Deviation.*

*Effect of complex additives on seed germination*

Addition of 10% (v/v) potato homogenate in KC basal medium has reduced the germination period to 19 days. After 90 days of culture, seed of *V. hastifera* was significantly germinated on KC basal medium containing 10% (v/v) potato homogenate with 95.6±1.67% and GI value of 293.90 (Table 2, and Figure

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1g). From the results, addition of complex additives has significantly increased the germination percentage as compared to control medium which devoid of any complex additives. Previous studies also reported that addition of organic additives such as potato homogenate, banana homogenate and coconut water have a significant contribution on germination of many orchid species (Rosmah et al., 2010; Zeng et al., 2012). Complex additive is natural sources of minerals such as carbohydrates, protein, fat, vitamins, phenolic compounds, growth substances, a lower level of amino acids, and organic acids that can enhance the orchid seeds growth (Islam et al., 2003). In this study, seeds of *V. hastifera* were significantly germinated on medium supplemented with potato homogenate. Potato is a carbohydrate-rich food which contains about 80% water and 20% dry matter (mainly as starch). Potato tubers also contain protein, fat, several vitamins, phenolic compounds and low levels of some amino acids and fatty acids (Islam et al., 2003). The beneficial of potato homogenate was also reported earlier by Rosmah et al. (2010), on seeds germination of *Phalaenopsis gigantea*.

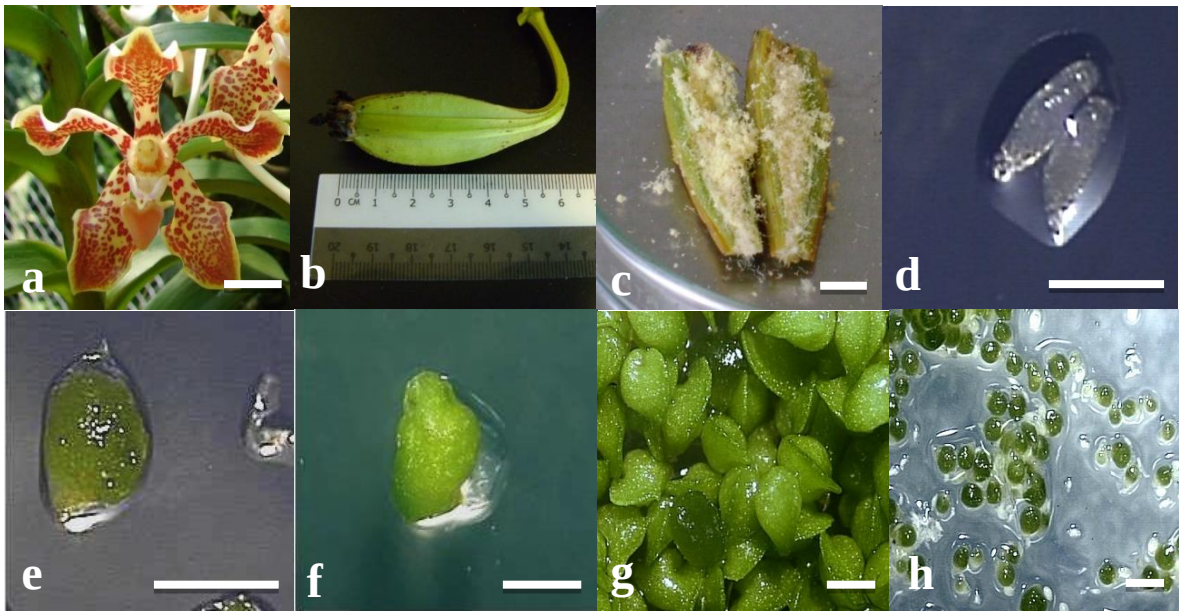


Figure 1. *In vitro* seed germination of *Vanda hastifera*. (a) Flower of *V. hastifera*, (b) Capsule, (c) Seeds of *V. hastifera*, (d) Seeds at Stage 1, (e) Germinated seed (Stage 3), (f) Protocorm, (g) Seeds germinated on KC basal medium supplemented with 10% (v/v) potato homogenate, (h) Germinated seeds treated on medium containing sucrose 1% (w/v), (i) Germination inhibited on medium containing galactose. Bar: (a) and (c) 1cm; (d) and (e) 200µm; (f) and (g) 1mm; (h) 0.5mm.

*Effect of sugars on seed germination*

After 30 days of culture, the germination performance of seeds on Knudson C medium supplemented with 10% (v/v) potato homogenate was greatly influenced by type and concentration of sugar. Culture medium containing 1% and 2% (w/v) of sucrose gave the highest percentage of germinated seeds, that are 86.13±1.90% and 88.39±4.83% and GI value of 281.75±2.95 and 284.20±6.94, respectively (Table 3, Figure 1h). However, higher concentration of sucrose (4%, w/v) reduced the seeds germination percentage. Carbohydrate serves primarily as energy which acts as signaling molecules that involved in the integration and regulation of some important biochemical pathways that influence germination, seed dormancy and seed reserve mobilization (Johnson and Kane, 2007). From this study, sucrose was found superior compared to other source of carbon in germinating the seeds of *V. hastifera*. Sucrose has been widely used as the major carbohydrate source in plant tissue culture because of its efficiency in being

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transported across the plasma membrane (Kumaraswami et al., 2010). Increase osmotic stress at higher concentration of sucrose (4%, w/v) has led to the reduction of seeds germination (Karami et al., 2006). Sucrose at 2% (w/v) in culture medium was also reported beneficial in earlier study for *V. tessellata* (Roy and Banerjee, 2002), *Dendrobium lituiflorum* (Vyas et al., 2009) and *Ansellia africana* (Vasudevan and van Staden, 2010). This study also proof that galactose is not suitable seeds germination of *V. hastifera*. According to Arditti (1967), galactose has long been known to inhibit the growth of orchid seed germination because of orchid's inability to utilize it. George et al. (2008) also reported that galactose is toxic to most plant tissues, which inhibits the growth of orchids and other plants in concentration as low as 0.01% (w/v).

Table 2. Effect of complex additives on seed germination of *Vanda hastifera* after 90 days of culture.

| Treatment | Germination time (Days) | Germination (Means±SD), % | Growth index (GI) |
|---|---|---|---|
| Control | 30 | 15.46±2.58 $^{e}$ | 153.14 $^{e}$ |
| 10% (v/v) coconut water | 28 | 27.45±0.20 $^{d}$ | 205.26 $^{d}$ |
| 10% (v/v) tomato juice | 26 | 81.76±0.46 $^{b}$ | 278.36 $^{b}$ |
| 10% (v/v) banana pulp | 22 | 79.77±5.55 $^{b}$ | 277.23 $^{b}$ |
| 10% (v/v) potato homogenate | 19 | 95.61±1.67 $^{a}$ | 293.90 $^{a}$ |
| 0.2% (w/v) peptone | 29 | 36.91±3.04 $^{c}$ | 221.24 $^{c}$ |
| 0.2% (w/v) yeast extract | 22 | 34.22±2.70 $^{c}$ | 277.24 $^{b}$ |

*Mean values within a column followed by the same letters are not significantly different at p<0.05 according to Duncan's Multiple Range Test; Treatments were conducted in 5 replicates. SD= Standard Deviation*

Table 3. Effect of carbon sources on seed germination of *V. hastifera* cultured on KC medium with 10% (v/v) potato homogenate observed after 30 days of culture.

| Treatment | Concentration (%, w/v) | Germination (Means±SD), % | Growth Index (GI) |
|---|---|---|---|
| Control | | 63.58±13.35 $^{bc}$ | 250.60 $^{c}$ |
| Sucrose | 1 | 86.13±1.90 $^{a}$ | 281.75 $^{a}$ |
| | 2 | 88.39±4.83 $^{a}$ | 284.20 $^{a}$ |
| | 4 | 58.75±12.83 $^{c}$ | 258.62 $^{c}$ |
| Glucose | 1 | 69.30±13.21 $^{bc}$ | 255.60 $^{c}$ |
| | 2 | 76.05±3.00 $^{ab}$ | 270.78 $^{b}$ |
| | 4 | 64.48±12.93 $^{bc}$ | 249.46 $^{c}$ |
| Fructose | 1 | 38.30±10.85 $^{d}$ | 223.33 $^{d}$ |
| | 2 | 32.86±17.66 $^{d}$ | 219.25 $^{d}$ |
| | 4 | 13.07±6.88 $^{e}$ | 200.65 $^{e}$ |
| Galactose | 1 | 0 | 0 |
| | 2 | 0 | 0 |
| | 4 | 0 | 0 |

*Mean values within a column followed by the same letters are not significantly different at p<0.05 according to Duncan's Multiple Range Test; Treatments were conducted in 5 replicates. SD= Standard Deviation*

## Conclusions

From these experiments, this study concluded that the best culture medium for seed germination of *V. hastifera* is KC basal medium supplemented with 10% (v/v) of potato homogenate and 1% (w/v) of sucrose.

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# Formation of Callus in *Hylocereus polyrhizus* Using Immature Flower Parts

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## Introduction

Red pitaya (*Hylocereus polyrhizus*) is a tropical fruit that is well sought after for its nutritive values (Wu et al., 2006), high economic value and potential in the ornamental and fruit production industries (Le Bellec et al., 2006). This red fleshed pitaya is preferred by the farmer for its higher market value compared to the white fleshed *Hylocereus undatus*. Red pitaya had recently been reported as one of the important fruit crops in South East Asia with increasing cultivation land area for each year (Halimi and Satar, 2007). Naturally, *Hylocereus* spp. propagates via stem cuttings and seed germinations but these conventional methods have many weaknesses. Insufficient planting material for propagation had resulted in low multiplication rate; approximately 50 cm long of the cuttings are needed to produce well established plantlets. Seed derived plants have long juvenile period and delay in fruit production even though the seed germination efficiencies were reported to be acceptable. Such drawbacks of propagations are not commercially feasible and were not preferred by the farmers (Le Bellec et al., 2006). Therefore, *in vitro* tissue culture serves as a good alternative for pitahaya vegetative propagation as an unconventional technique for massive plantlet productions (Dhanayake and Ranawake, 2011).

Research on the tissue culture of *Hylocereus* spp has been so far limited to using young stems (Viñas et al., 2012), joints (Mohamed-Yasseen, 2002), seeds (Rodziah et al., 2010) and immature ovules and embryos (Cisneros and Tel-Zur, 2010) as explants. Most of them were concentrating on micropropagation producing multiple shoots. Therefore, the aim of this study was to develop a protocol for *in vitro* establishment and callus formation of *H. polyrhizus* using various parts of flower bud as explants.

## Material and Methods

### *Preparation of explants*

Flower buds of approximately 8 to 10cm in length were obtained from a dragon fruit farm in Lenggeng, Negeri Sembilan. These immature flower buds were kept in a cool box and were transported to the laboratory for immediate use. The flower buds were stripped of all opened sepals and rinsed under running tap water for approximately 5 min. They were then subjected to surface sterilization with 70% ethanol for 1 min followed by 20% commercial bleach (1% (v/v) sodium hypochlorite) for 20 min with constant agitation. The flower buds were then rinsed three times with sterile distilled water before the excision of various flower parts was performed. The sepals, petals, styles and stigmas of the flowers were excised, cut into the size of 0.5x0.5 mm (sepal and petal) or at the length of 0.5 mm (style and stigma). The cylindrical style segments were then cut longitudinally into two. The explants were placed in petri dishes with moistened filter papers for the experiments.

### *Culture conditions*

Explants were cultured horizontally on the medium in petri dishes. The cultures were placed under a photoperiod of 16 h (3,000 lux) at 24 to 25 $^{O}$C.

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*Determination of the best explant for callus induction*

To determine the best explant for callus formation, all four types of explants described earlier were cultured on MS medium supplemented with 2.0 mg/L BAP and 2.0 mg/L NAA. The cultures were put under culture conditions as described for four weeks (28 days).

*Effects of various plant growth hormones on the proliferation of callus using styles as explants*

Various cytokinins and auxins were tested individually to see their effects on *H. polyrhizus* styles for callus production. The cytokinin tested were 6-Benzylaminopurine (BAP), thidiazuron (TDZ) and kinetin (Kn) while the auxin were indole-3-acetic acid (IAA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). These hormones were tested at the concentration of 2.0mg/L.The cultures were put under culture conditions as described for four weeks (28 days).

*Experimental design*

All experiments were conducted using completely randomized block design. Ten explants were used in each treatment and each experiment was repeated three times.

*Statistical analysis*

Non-invasive data was used to prevent contamination. Percentage of explants that produced callus, and the surface area of the callus formed after the designated culture period were evaluated. The data were evaluated by analysis of variance.

**Results and Discussion**

*Determination of the best explant for callus induction*

Physical changes of the explants were observed after a few days of culture. The styles were expanded in size while the sepal curled inward into the medium. The edges of petal and the stigma began to have some sign of browning. Sepal was the first to show signs of callus formation after one week of culture with 16.0% of the sepal explants producing calli (Figure 1). The percentage of callus formation for sepal explants increased slowly to 24.0%, 32.0% and 45.3% after 2, 3 and 4 weeks of culture respectively. Though the petal responded slowly to the medium, high percentage of the explants produced calli (80.0%) after 4 weeks. The style explants started to produce some calli after 2 weeks of culture (12.0%) but the percentage increased quickly to 98.7% after 3 weeks, and all style explants produced calli after 4 weeks. Stigma explants have failed to produce callus and turned brown after 4 weeks (Figure 2). The calli produced by various types of explants differ from one another. The initial calli on sepal explants were loosely packed and light green in colour (Figure 3). The calli then developed into mixture of various shades of green with scarce clumps of whitish callus towards the end of 4 week culture. Calli from the petal explants on the other hand were either whitish or yellowish (Figure 4).

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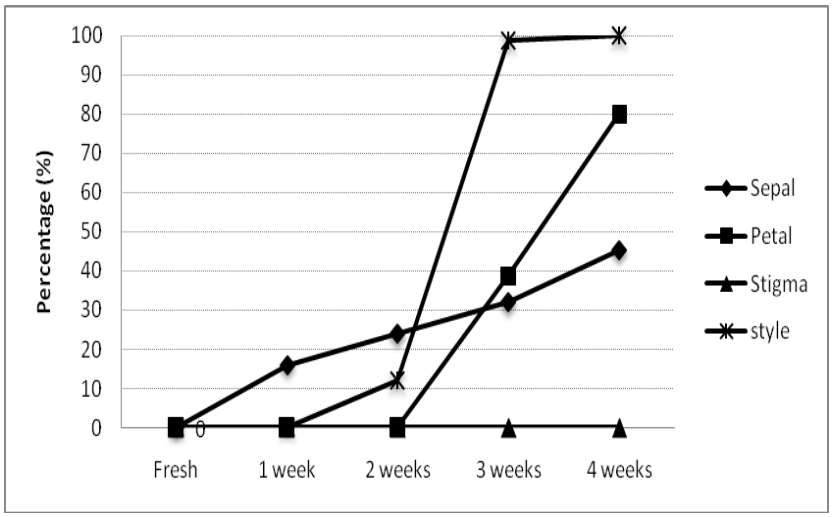


Figure 1. Percentage of various *H. polyrhizus* explants producing calli after 4 weeks of culture. As expected, wounded surfaces of the explants have high tendency to produce callus (Slater, 2008).

Some of these calli changed into compact and light green clumps at the end of 4 weeks. The style explants expanded to approximately three times its original size and produced abundance of calli (Figure 5). Interestingly, the style explants produced some red calli (Figure 6), resembling the peel colour of ripe dragon fruit. The red callus appeared randomly and in mixture with green or whitish calli or sporadic especially the explants that are closer towards the ovary. The red callus was not found on callus formed from sepal or petal.

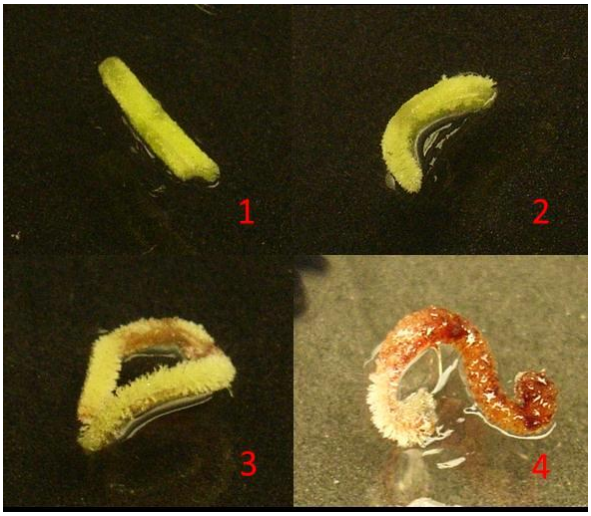


Figure 2. Fresh *H. polyrhizus* stigma explants (1) curved after 1 week (2), slowly turned brown after 2 weeks (3), and became entirely brown after 4 weeks culture (4).

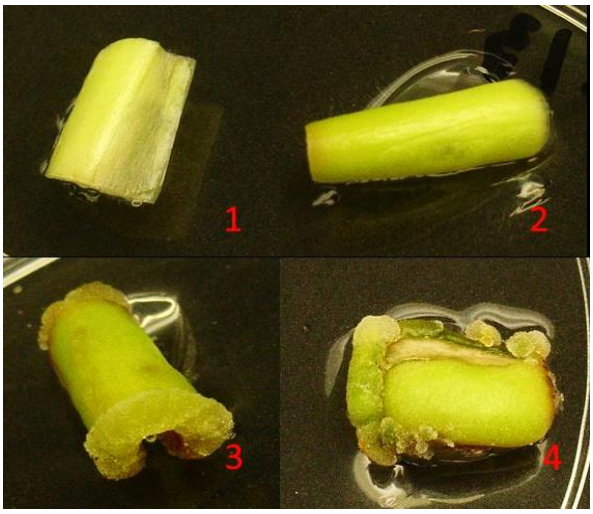


Figure 3. *H. polyrhizus* sepal explants were thin when fresh (1) but thickened after 1 week (2), produced light green callus on the 3rd week (3), and at the end of the 4th week, some calli turned whitish (4).

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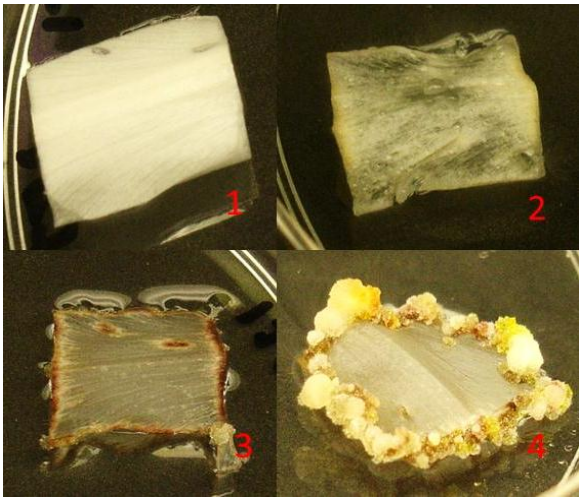


Figure 4. White petal explant of the *H. polyrhizus* (1) turned yellowish and slightly transparent after 1 week (2); the edges turned brown on the 3rd week and some whitish loosely packed calli were produced (3). The calli proliferated on the 4th week (4).

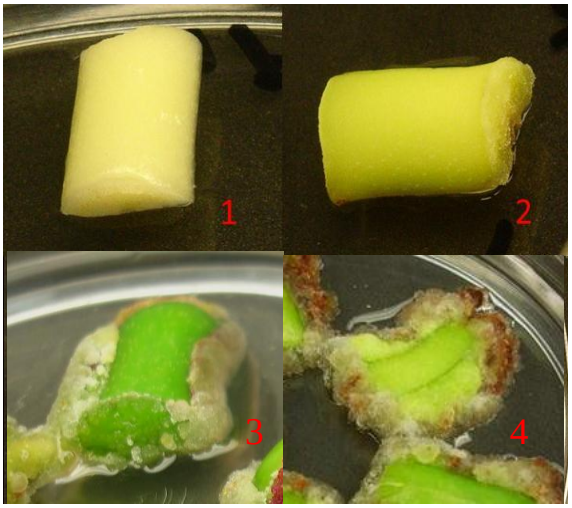


Figure 5. The style explants of *H. polyrhizus* were yellowish when fresh (1), slowly expanded and turned greenish on week 2 (2), the calli were abundant on week 3 (3) and the edges of the explants were fully covered with calli on week 4(4).

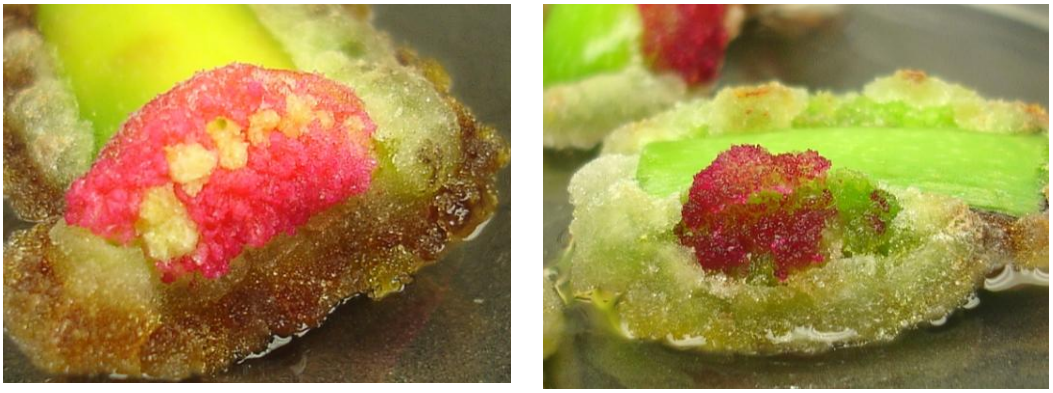

Figure 6: The red calluses produced from the *H. polyrhisuz* style explants were random, either appeared with mixture of other calluses (1) or sporadic (2).

To avoid contamination, non-invasive surface area measurement was used to measure the quantity of calli produced by the various explants. At the end of four weeks, style explants produced highest amount of callus with a mean surface area per explant of 173.3 $mm^2$ (Table 1). Zakaria and colleagues (2010) also found out that the style of *Citrus grandis* was also very responsive to culture media and had demonstrated high capacity of callus production. Undoubtedly, the increase in size of the style during culture had also contributed to the larger wounded surface, thus giving more opportunity to produce calli. Sepal explants came second with a mean surface area of 144$mm^2$. Petal produced significantly lower amount of callus of 82.3 $mm^2$ only.

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Table 1. Mean surface area of calli per explant induced from various parts of *H. polyrhizus* immature flowers after 4 weeks of culture on MS medium supplemented with 2.0mg/L of BAP and NAA each.

| Explant | Mean surface area ($mm^2$) |
|---|---|
| Sepal | $144.0 \pm 16.2_a$ |
| Petal | $82.3 \pm 6.7_b$ |
| Style | $173.3 \pm 18.9_a$ |
| Stigma | $0.0 \pm 0.0_c$ |

*Effects of various plant growth hormones on the proliferation of callus using styles as explants*

The potential of the style explants to produce callus was further investigated using single hormone treatments of cytokinins and auxins at the concentration of 2.0mg/L. It was shown that none of the explant produced callus when cultured on medium with auxin (IAA, NAA and 2,d-D) (Table 2). They all either did not respond to the culture media or turned brown at the end of four weeks culture. The explants seemed to only respond to cytokinins, BAP and TDZ, with 100% of the explants produced callus. Kn had failed to induce any callus. BAP proved to be better and had induced callus formation with significantly higher mean surface area of $60.2 \pm 5.7mm^2$. The calli induced by TDZ were slightly lower at $42.8 \pm 3.4mm^2$. The results showed that culture medium with combination of cytokinin and auxin hormones are superior over medium with single hormone. Similar observation was reported on three types of Cactaceae (Giusti et al., 2002) and *Kigelia pinnata* shoot tips (Thomas and Puthur, 2004).

Table 2. The mean surface area of calli produced from style explants when cultured on various plant growth hormones at the concentration of 2mg/L and cultured for 4 weeks.

| Hormone | Callus (%) | Mean surface area ($mm^2$) |
|---|---|---|
| BAP | $100.0_a$ | $60.2\pm 5.7_a$ |
| TDZ | $100.0_a$ | $42.8\pm 3.4_b$ |
| Kn | $0.0_b$ | $0.0\pm 0.0_b$ |
| IAA | $0.0_b$ | $0.0 \pm 0.0_b$ |
| NAA | $0.0_b$ | $0.0 \pm 0.0_b$ |
| 2,4D | $0.0_b$ | $0.0 \pm 0.0_b$ |

## Conclusions

The style explant of *H. polyrhizus* was found to be very responsive to culture media of MS supplemented with 2.0mg/L of BAP and NAA, producing calli with high mean surface area of $173.3mm^2$. The style explants seemed to be responsive to cytokinin instead of auxin. Further research is required to look into the various combinations of cytokinin and auxin at various concentrations to produce more calli.

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# Effect of *in vitro* Vertical Cutting and Leaf Decapitation on Shoot Multiplication of Janggut Adam (*Tacca integrifolia*)

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## Introduction

In Malaysia, one out of 10 species of bat flowers, the *Tacca integrifolia* or "Janggut Adam" can be found in the tropical rain forest areas and has been specifically used in Malay traditional medicine as well (Nor Aini et al., 2008). In Malaysia, it is also known as Belimbing Tanah dan Keladi Murai (Faridah and Shamsul, 2004). Its values are not only the medicinal properties but also the chemical compounds extracted from rhizome which are able to fight against plant pest (Nuanla and Sruamsiri, 2000). Besides, its inflorescence has an outlandish bract (rabbit-look) and long bracteoles (cat's whiskas-look) with rich dark maroon to purple colour making this plant favourably use as an ornamental purpose.

Indiscriminate collections by native people, habitat destruction and over exploitation for medicinal purposes are the main factors that have threatened the survival of this species. Therefore conservation of *T. integrifolia* is now a matter of universal concern. Normally, the bat flower propagates from seeds and stem budding but plants and seeds are rarely collected from the natural forest. Moreover, its seed germination rate is quite low. Tissue culture technique has been used for the in vitro mass propagation serving the future requirement instead of the conventional propagation method. The micropropagation of *T. chantrieri* has been done using young leaves and leaf stalks of seedling via callus induction (He et al., 2002). There has been a report of using axillary and adventitious shoots of seedling as explants for *in vitro* propagation of *T. chantrieri* (Charoensub et al., 2008). This research is carried out to determine effect of *in vitro* vertical cutting and decapitation on shoot multiplication of *T. integrifolia*.

## Materials and Methods

### *Plant materials*

The plant materials used in this study were collected from local forest in Ayer Hitam Reserve Forest, Puchong, Selangor.

### *Surface sterilization of explants*

The sterile seeds for the sterile seedlings were prepared by removal of sterile seeds from sterilized freshy fruits (berry like). The whole cleaned fruits were dipped in 95% ethanol and directly flamed 2 – 3 times prior to dissection and removal of the sterile seeds. Mature *T. integrifolia* seeds were immersed in 70% ethanol for 1 min prior to surface-sterilization in 10% commercial bleach, Clorox (6%w/w sodium hypochlorite), for 25 – 30 min and rinsed 3 times with sterile water. The sterilized seeds of *T. integrifolia* were grown in culture bottles containing 10 ml of half strength Murashige and Skoog (½ MS). The pH of all culture medium was adjusted to 5.7 with 0.1 N NaOH or HCl before adding 0.75% agar and autoclaving at 1x105 Pa, 121 $^{o}$C for 15 min. The cultures were placed under light conditions (300-350 lux illumination for a 16 h photoperiod) at 27±2 $^{o}$C.

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*In vitro vertical cutting, leaf decapitation and media*

Shoot tips (2–4 cm in length) derived from 16 week-old seedlings were used as explants (Figure 1A). The shoot tips were cut directly by vertical cutting (Figure 1B) and for the decapitation method, 2–3 leaves were removed using a sterile blade (Figure 1C). The explants were cultured on MS hormone-free medium (MS) and MS with 1 mg/L of BAP (MSH). The number of shoot bud, leaves and root were counted after 12 weeks of culture.

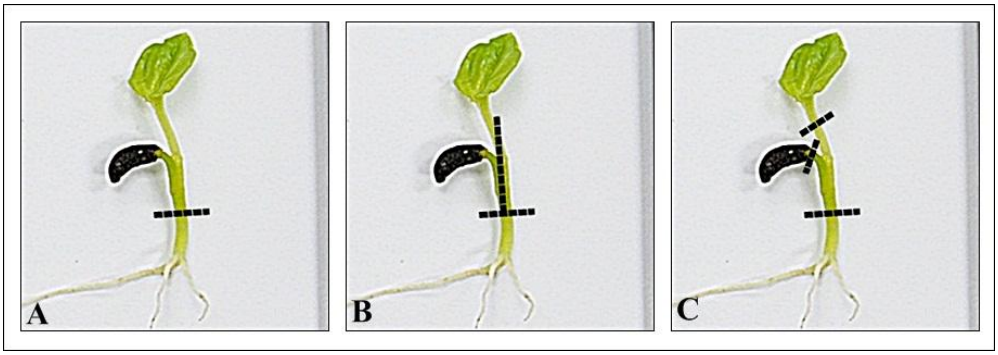


Figure 1. Sixteen weeks-old in vitro seedling were used as explants. (A) intact, (B) vertical cutting and (C) leaf decapitation method for shoot bud induction.

*Experimental design and data analysis*

All experiments were performed in a completely randomized design (CRD). Each experiment included three replicates, with five planlets per treatment. The number of new shoots per explants, number of roots and number of new leaves were determined for each experiment after 12 weeks of culture. Analysis of variance (ANOVA) was performed by using SAS version 9.1 and mean separation with the LSD test. A significance level was set at P=0.05.

**Results and Discussion**

The 16 week-old germinated seedlings maintained in ½ MS medium were used as explants. For each explant, the roots were cut to determine the effect of the growth regulators. The different types of method showed significant differences in shoot multiplication of *T. integrifolia*.

The highest number of shoots was observed when the shoot tips were decapitated as compared to other methods. After 12 weeks in culture, the decapitated explants produced an average of 3.3 new shoots on MS medium supplemented with 1 mg/L BAP, whereas those vertical cutting and intact method showed less shoots (Table 1). The new shoot emerged from tightly grown and around cutting area (Figure 2C). The newly formed young shoots could grow readily on basal MS medium without plant growth regulator for shoot proliferation. Moreover, the original plant of vertical cutting produced callus on MS medium supplemented with 1 BAP, probably due to heavy wounding of the explants (Figure 2B).

There were significant differences between the composition of culture medium on mean number of shoots and roots produced (Table 1). Shoot multiplication was significantly higher on MSH medium than on MS medium. In combination treatments, there were significant differences between different technique of shoot multiplication and composition of culture medium (Table 1). The highest number of shoots was observed on leaf decapitated seedling cultured on MSH medium at 3.33, followed by intact seedling on

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MSH at 1.33 and vertically cut seedling on MSH at 0.67. No development of shoot was observed in the other three combination treatments.

Development of axillary buds following decapitation is a common phenomenon observed in plants. The removal of the apices and whole leaves is used practically in propagation of *Euphorbia pulcherrima* via shoot cuttings (Wilkins, 1988). In this study, the attempts to maximize the production of *T. integrifolia* shoots through vertical cutting and leaf decapitation technique were successful. Leaf decapitated explant induced the highest shoot multiplication using shoot tips as explants placed on MS medium supplemented with 1 BAP. Orlikowska et al. (2000) reported that defoliated shoot tips produced significantly more axillary shoots in comparison to non-defoliated shoot tips on *Codiaeum variegatum* Blume var. *pictum* Muell. Arg. cv. Excellent. This effect was caused by the weakling of shoots (marked by small diameter) due to defoliation.

Table 1. Effect of vertical cutting, leaf decapitation and its combination with media on shoot multiplication and growth of *T. integrifolia* after 12 weeks of culture.

| | | No. of shoot | No. of root | No. of new leaves |
|---|---|---|---|---|
| Method | Intact | 0.67b | 0.00b | 2.67a |
| | Vertical cutting | 0.33b | 0.67a | 0.00c |
| | Decapitation | 1.67a | 0.83a | 1.17b |
| Media | MS | 0.00b | 1.00a | 1.22a |
| | MSH | 1.78a | 0.00b | 1.33a |
| Combination | Intact x MS | 0.00c | 1.33a | 2.00b |
| | Intact x MSH | 1.33b | 0.00b | 3.33a |
| | Vertical x MS | 0.00c | 0.00b | 0.00c |
| | Vertical x MSH | 0.67b | 0.00b | 0.00c |
| | Decapitation x MS | 0.00c | 1.67a | 1.67b |
| | Decapitation x MSH | 3.33a | 0.00b | 0.67c |

*MS= Free-hormone MS medium, MSH= MS medium supplemented with 1 mg/L BAP. Means with the same letter are not significantly different according to Least Significant Different (LSD) test at P=0.05%*

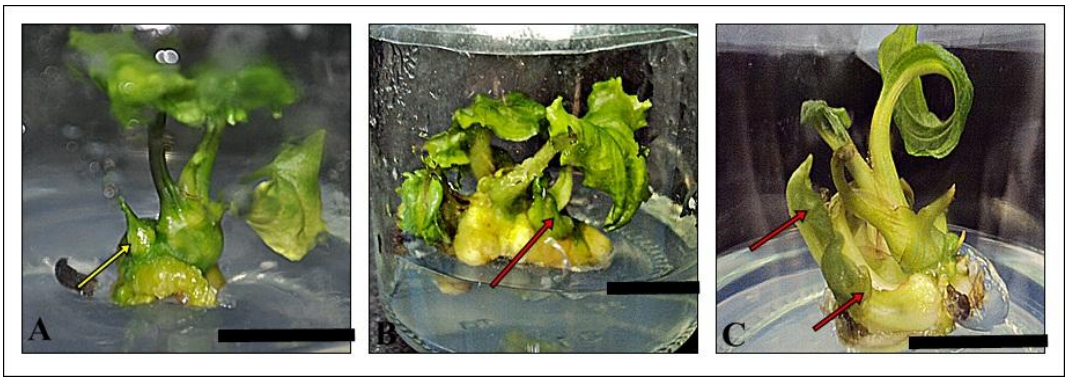


Figure 2. Shoot proliferation by intact (A), vertical cutting (B) and decapitation method (C) for shoot multiplication of *T. integrifolia*. Shoot primordia (yellow arrow) and normal new shoots (red arrow) were obtained when planlets were cultured on MS medium supplemented with 1 mg/L BAP after 12 weeks culture.

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## Conclusions

Micropropagation of *T. integrifolia* can be done by culturing sterile seedlings. Decapitation method is recommended with MS medium supplemented with cytokinin to obtain multiple shoots. Therefore, this technique presents an efficient system of *in vitro* clonal propagation compared to seed propagation for rapid multiplication, production of disease-free plants, non-seasonal production, germoplasm conservation and facilitating their easy exchange.

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# CHAPTER 5

# NUTRITION, PLANT-MICROBE INTERACTION AND INNOVATIVE PRACTICES

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# Mycorrhizal Symbiosis in Managing Phosphorus Efficiency in *Theobroma cacao* L.

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## Introduction

For many tropical countries of Asia, Africa and South and Central America, cocoa (*Theobroma cacao* L.) is an important plantation crop (Hartemink, 2005). Meanwhile soils of most cocoa growing regions are acidic and infertile which caused by nutrient loss by leaching, soil erosion and intensive longterm cultivation (Wessel, 1971). In those highly weathered tropical soils, phosphorus (P) deficiency or unavailability has been generally identified as one of the major limiting factors for cocoa production (Smyth, 1966). As one of the most important nutrients, P plays a main role in development and reproduction for growth of cocoa. To improve the P nutrition of plants, the traditional approach is by applying large amounts of P fertilizers to soils. It has been estimated that around 35 million tons of P-based fertilizers (in terms of $P_2O_5$) are applied worldwide every year (IFIA, 2013). However, the use efficiency of applied P is generally very low, ranging from 10% to 30% in the year applied (McLaughlin et al., 1991). Continuous application of P fertilizers also increases the risk of P loss from soil to water, causing toxic algal blooms in water bodies (Barber, 1984). Improving plant uptake of P from soil is an obvious alternative to the management of low P soils and the enhancement of P efficiency soil P. Phosphorus efficiency is usually known either as P uptake efficiency or as P use efficiency. P uptake efficiency is usually defined as P uptake per plant, or per unit root biomass or root length, while P use efficiency defined as plant biomass produced per unit of available P. Genetic variations in P uptake efficiencies have been widely reported in many crops, such as cocoa (Baon and Winarno, 1998), coffee (Baon et al., 1999), sorghum (Neumann and George, 2004) and millet and cowpea (Bagayoko et al., 2000). Plant traits that can influence P uptake efficiency include rhizosphere acidification, root exudation of organic anions, root morphology, uptake kinetics and symbiotic association with mycorrhizal fungi (Smith et al., 2011). Symbiotic association between plants and arbuscular mycorrhizal fungi (AMF) is widespread and of particular importance in improving plant P uptake efficiency (Smith and Read, 2008). Most plants are engaged in symbiotic relationships with mycorrhizal fungi, and our works have shown that cocoa is a mycorrhizal dependent plant (Baon, 1994). The beneficial effects of AMF are due mainly to the ability of mycorrhizal fungal hyphae to acquire P well beyond the limits of the rhizosphere depletion zone (Li et al., 1991), especially on soils with low available P (Smith et al., 2011). However, the advantages of increased P-uptake and growth over comparative, non-mycorrhizal plants at low P diminish with increasing soil P availability (Rai et al., 2013). As a key component of soil microbiota in rhizosphere, AMF may change several aspects of plant physiology and some nutritional properties of rhizospheric soil. The aim of this review paper was to discuss current developments and future trends concerning the significance of mycorrhizal fungi in contributing P efficiency of cocoa plants.

## Phosphorus Need in Cocoa

Once converted from forest to cocoa farms, tropical soils tend to have structure suitable for crop growth but lack quantities of plant essential nutrients required for long-term agricultural productivity (Lindell et al., 2010). Commonly, productivity of the continuously cultivated tropical soils is limited by insufficient supplies of soil minerals, particularly P (Wood and Lass, 2001). Depletion of soil P pools continues through sequential harvest and removal of cocoa products (Cardoso et al., 2003). Application of inorganic P containing fertilizers to remedy the infertility of these soils is not an option for many subsistence cocoa

farmers in developing countries without money to buy, or means to transport them (Appiah et al. 1997). It is possible that with better management, fertility of these soils can be maintained for a longer period, enhancing their capacity to sustain cocoa productivity (Lindell et al., 2010).

Phosphorus is the second most important macronutrient, second to nitrogen, that is crucial for the stability and continued existence of cocoa life (Wood and Lass, 2001). There are many principal roles of P in the plant physiological processes, such as photosynthesis, utilization of sugar and starch, and energy transfer. Aside from this vital metabolic role, P is an indispensable structural component of numerous molecules, including nucleic acids, which are the building blocks of genes and chromosomes in the cell nucleus and are obligatory for cell division and formation of meristematic tissues (Marschner, 1995). For maximum yield, cocoa plants are in need for a sufficient amount of P from the very early stages of growth. Therefore, direct availability of P determines the crop growth, and limited supply of P results in yield loss (Grant et al., 2005). A regular insufficient early-growth P supply can limit crop yield and thus P fertilizer is commonly applied to ensure that sufficient P is available to optimize crop production.

Removal of nutrients from cocoa ecosystems includes yield (beans and husks), immobilization in stem and branches, and leaching of nutrients below the rooting zone. Most nutrients in cocoa ecosystems are lost by the harvest of beans and husks. About 4 kg P is removed with 1000 kg dry beans, while when the husks are also removed, the amount is increased to about 6 kg P. Nutrients immobilized in the stem and branches of cocoa and shade trees are also considered lost for the system as they are excluded from nutrient cycling. Immobilization of nutrients is particularly important for young cocoa, but is unimportant for mature cocoa (Wessel, 1971).

**Mycorrhizal Dependency of Cocoa**

There is a wide variation in the mycorrhizal dependency of agricultural crop species, even inter clones or hybrids, usually related to root morphology, root hair formation and seed P content (Smith and Read, 2008). Variation between clones of cocoa (Baon and Winarno, 1998) and varieties of coffee (Baon et al., 1999) has also been shown. For cocoa plants, the work showed that there is a tendency for greater reliance on symbiosis in the older cultivated genotypes than in newly cocoa clones. Breeding and choice of cultivar are thus important tools in AM management.

Arbuscular mycorrhizal fungi are associated with improved growth of cocoa crop due to increased nutrient uptake (Widiastuti and Baon, 1994), production of growth promoting substances, tolerance to drought, salinity and transplant shock and synergistic interaction with other beneficial soil microorganisms such as N-fixers and P-solubilizer (Smith and Read, 2008). Symbiotic association of cocoa roots with VA-fungi often result in enhanced growth because of increased acquisition of P and other low mobility mineral nutrients (Baon, 1996). Advantages of AM symbiosis over the non-mycorrhizal state of among cocoa clones are inextricably associated with root architecture, in that plants with extensive, branched root systems, very fine roots and long root hairs (Baon et al., 1994) tend to show relatively low improvement in growth when they are mycorrhizal, even in low P soils (Simpson et al. 2011).

**Factors Affecting Phosphorus Efficiency**

There are two factors controlling the rate of P absorption or P influx into roots (Rai et al., 2013). First, the quantity of various nutrients, available for plant, including C, N, P and others, determine the capability of the root surface area (root morphology) to absorb P from the soil, and controls P absorption by the plant, all else being equal. The more P held by the plant (internal P reserve) the more can be used to produce root surface area in order to acquire P from the soil (Bloom et al. 1985). Therefore, the most critical of

these nutrient elements under the control of the plant is P itself. Second factor is the efficiency with which the internal P reserve is used to absorb P from the soil. There is no immediate negative consequence to inefficient use of a non-limiting resource, simply because it is available in non-limiting amounts. However, immediate benefit is a consequence of more efficient use of plant P, because P is the limiting resource. Koide (1991) defined the efficiency with which the internal P reserve is used to absorb P from the soil as the phosphorus efficiency index (PEI). The PEI is analogous with which determines how efficiently the plant's current dry mass is used to gain more mass. It can often be expressed as the relative growth rate. Similarly, PEI is defined as a measure of how efficiently the plant's internal P reserve is used to gain more P. Thus, phosphorus influx into roots is a function of both plant P content and PEI, and accordingly, growth rate is a function of the PUE, the P content and the PEI.

**Cocoa Growth and Phosphorus Efficiency**

Presence of shade trees in cocoa farm system may delay decomposition rate of organic matter, suggesting slower but sustained release of available nutrients into the topsoil. Isaac et al. (2007) showed that cocoa under artificial shade, both with and without fertilization, exhibited the greatest nutrient responses as compared to unfertilized monoculture cocoa, where P uptake was very stimulated. When fertilizers are undesirable or unavailable, intercropping of cocoa with appropriately selected shade trees will not competitively suppress early growth of cocoa but will improve light regulation and nutritional status of cocoa saplings.

Nutrient efficiency is mainly considered as dry matter produced per unit nutrient element concentration in dry matter (Gourley et al., 1994), which can also be termed as the internal nutrient requirement. Considering yield parameters, efficiency with regard to a specific mineral nutrient, is the capability of any clones or hybrids in producing dry matter, in a soil limiting in that particular nutrient element (Buso and Bliss, 1988). Agronomic efficiency is defined as the total harvestable amount per unit of growth limiting nutrient element applied in the soil (Caradus and Woodfield, 1990). External efficiency is the sum of nutrient content in soil primarily taken up by plants to produce a certain fraction of whole dry matter produced (Fohse et al., 1988). Some researchers have used the term nutrient efficiency ratio, which is calculated as the reciprocal of the nutrient concentration in the whole plant (Gourley et al., 1994). Other workers have used the term nutrient uptake efficiency (Buso and Bliss, 1988). Uptake efficiency is defined in terms of total uptake per plant or specific uptake per unit root length (Marschner, 1995).

Internal P management, like the effects of P stress on yield components and the way the plant distributes the acquired P among shoot organs, varies widely between genotypes (Sattelmacher et al., 1994). However, even greater genetic variation has been found in traits for acquisition of nutrients by roots (Caradus and Woodfield, 1990). Root morphological characters, such as root length, surface area, root radius, and density of root hairs, are regarded as having primary importance for P-uptake efficiency (Sattelmacher et al., 1994). Root morphology is determined not only by genetic but also by environmental factors, including soil P availability (Hajabbasi and Schumacher, 1994). Plasticity of the root system can be exploited for developing P efficient cultivars. Nuruzzaman et al. (2005) hypothesized that P-acquisition efficiency in plant species varies with their rhizosphere volume and chemistry.

The second component of uptake efficiency is root physiological activity such as differing uptake kinetics, i.e. Imax, Km and Cmin, which result in different nutrient uptake rates per unit root and time (Steingrobe and Classen, 2000). Plants differ in P-absorbing capacity of their roots, and even inter-specific variations have been found (Krannitz et al., 1991). The parameter found to differ most among genotypes is the maximum net influx, Imax and plants are considered efficient when Imax is high. However, when soil P availability is low, the rate limiting step in P uptake is its transport to the root surface and a high Imax is useless (Jungk and Claassen, 1997). Other mechanisms or traits that affect

specific uptake efficiency include chemical mobilization of nutrients by root exudates, induced pH changes in the rhizosphere and AM associations (Marschner, 1995).

When a plant is P deficient, two factors control plant growth. One is the rate of P absorption or P influx. The other is the ratio of dry mass increase to P content increase, which can be thought of as the efficiency with which P is used to produce dry matter, the P-use efficiency (PUE) (Koide, 1991). The PUE is expected to vary according to tissue type. Seeds, for example, often have lower PUE than stems, because seeds require more P to produce a given dry mass than do stems. Variation in PUE also occurs among plant species, hybrids and clones (Christie and Moorby 1975), and as a consequence of variation in P availability and mycorrhizal colonization (Haynes et al., 1991).

**Mycorrhizal Role in Phosphorus Transport**

Accordingly, plants have evolved a range of strategies that increase either Pi uptake capacity or availability of Pi in soil. The most widespread of these strategies worldwide is AM symbiosis (Cheng et al., 2011). AM symbioses are widespread in the plant kingdom and contribute significantly to plant P nutrition and growth in natural ecosystems. Association of AM may improve P uptake from soil (Smith et al., 2011). The ability of AM to enhance host-plant uptake of relatively immobile nutrients, in particular P (Smith and Read, 2008), and their requirement for up to 20% of host photosynthate for establishment and maintenance, is well accepted. Plant benefits with this association under nutrient-poor conditions increasing phosphorus uptake by the host plant (Koide, 1991). Miller et al. (1995) reported that disturbance of arable and no-till soil resulted in reduced AM development and subsequently less absorption of P by seedlings of maize in the field. Neumann and George (2004) concluded that mycorrhizal colonization seems to increase P uptake from dry soil. Therefore, mycorrhizal symbiosis may improve the uptake of P by cocoa plants which are commonly grown in dry and hilly area.

P is critical for plant growth and makes up about 0.2% of dry mass, but it is one of the most difficult nutrients for plants to acquire. In soil, it may be present in relatively large amounts, but much of it is poorly available because of the very low solubility of phosphates of iron, aluminium, and calcium, leading to soil solution concentrations of 10 mM or less and very low mobility (Bucher, 2007). In consequence, uptake of orthophosphate (Pi) by root epidermal cells including root hairs leads to lower Pi concentrations in the rhizosphere, as replacement does not keep pace with uptake. Plants and fungi take up P as negatively charged Pi ions ($H_2PO_4^-$), which possess additional problems, because the concentration in cells is about 1000-fold higher than in the soil solution and the cell membrane has an inside-negative electric potential. Pi uptake, therefore, requires metabolic energy and involves high-affinity transporter proteins (Bucher, 2007). Mycorrhizal plants deplete phosphate to lower levels and to a much larger soil volume per unit root length than nonmycorrhizal plants. Influx of P in roots colonized by AM fungi can be three to five times that in nonmycorrhizal roots (Smith and Read, 2008). Jakobsen (1986) reported a two to three times increase in influx of P to roots of pea and clover plants, respectively, due to the effect of mycorrhiza.

Plants grown at high P in the presence of mycorrhizae accumulated only 88% of biomass of plants grown at high P in absence of mycorrhizae, indicating that mycorrhizae can reduce plant growth when not contributing to the symbiosis. Although a beneficial role of mycorrhizal symbiosis has been frequently observed, there have been cases reported where mycorrhizal inoculation has led to a decrease in plant productivity, particularly in AM plants (Schroeder and Janos 2004). Similar result on cocoa was shown by the work of Baon (1996) which showed that in high P content soil, there was no improve in plant growth even decrease in growth of mycorrhizal cocoa.

Plant response to AM inoculation is extremely variable both among and within species. Inoculation by AM has a wide range of effects on different plant species in relation to P nutrition in different situations. The effectiveness of AM fungi in increasing plant growth was observed to be closely correlated with root hair (Baon et al., 1994), soil temperature and light intensity (Baon et al., 1995). Generally, the development of AM inoculation seemed to follow a three phase growth curve i.e. a lag phase, a phase of rapid growth and a phase of constancy or plateau phase with increase age of plants (Saif, 1986). Upon mycorrhizal inoculation, increase in shoot growth is generally lower at the initial stage, and then increases rapidly with age of plants (Bethlenfalvay and Linderman, 1992).

**P Uptake Mechanism by Mycorrhiza**

Leaching losses in cocoa ecosystems are much lower than under annual crops. Van Noordwijk (1989) provide evidence for the "safety net" theory of tree crops such as in cocoa plants whereby nutrients leached to a deeper soil horizon can be taken up by tree roots at great depths. The external hyphae of AM extend from the root surface to the soil beyond the P depletion zone and have access to a greater volume of undepleted soil than the root alone (Lambers et al., 2011). Some hyphae may extend more than 10 cm from root surfaces (Rai et al., 2013) which is further than most root hairs. Also, the small diameter of hyphae (20 to 50 μm) allows access to soil pores that cannot be explored by roots. Therefore, a root system forming a mycorrhizal network will have a greater effective surface area (Richardson et al., 2011) for absorbing nutrients and exploring a greater volume of soil than nonmycorrhizal roots. The volume was calculated to be at least 100 times greater with mycorrhizal association than in its absence. Moreover, mycorrhizal colonization may induce formation of lateral roots or increase root branching (Lynch and Brown, 2001), further increasing the volume of soil explored.

Mycorrhizal plants can absorb more P at lower concentration in the soil solution than nonmycorrhizal plants (Lynch and Brown, 2001). One possible explanation is that mycorrhizal hyphae have a higher affinity (lower Km) for P than roots (Zhu et al., 2010). But this phenomenon is not necessary for explaining improved P uptake by mycorrhizal roots. Barber (1984) explained that there is a very limited concentration gradient around hyphae (i.e., minimal depletion zone) since the radius of hyphae is much smaller than that of roots plus root hairs (0.005 mm versus 0.15 mm). Hence, P concentration in soil solution around hyphae is always higher than in the P depletion zone around roots, and hyphae may absorb more P in low P soil even without having a higher affinity for P.

Organic anion exudation in the rhizospheric environment plays a vital role in the bioavailability of soil P. Mycorrhiza releases organic anions such as citrate, malate and oxalate, which can occupy sorption sites that might otherwize mobilize P, or replace P in the sparingly soluble complexes with aluminium, iron and calcium (Richardson et al., 2011). Thus it can enhance availability of P into soil solution, which might be accountable for the greater release of organic forms of soil P (Wei et al., 2010). Mycorrhiza is also reported to produce carboxylates, which influence ligand exchange, ligand-promoted dissolution of P-bearing minerals such as Fe/Al oxides, and thus having a significant contribution in P mobilization.

Mycorrhiza was shown to induce increased proton efflux or pCO2 activity around the rhizosphere, lowering pH to around 6.3 (Rigou and Mignard, 1994) resulting in greater solubilization of P, predominantly in neutral to calcareous soils. Citric acid and siderophores produced by mycorrhizae can enhance bioavailability of P supply in the soil more specifically in the case of Fe- or Al-bound P in acidic soils (Haselwandter, 1995). Furthermore, mycorrhizae has been reported to produce alkaline phosphatases, which can mobilize P from organic sources, and a low rate of this can have a long-term impact on mobilizing phosphate (Tarafdar and Marschner, 1994). The hydrolysis of organic P by extracellular phosphatases secreted by mycorrhizae, excretion of protons, hydroxyls and organic anions and modifications of the redox potential around mycelium and roots of the mycorrrhizal association might

also hasten the release of P ions from soil to solution. Alteration in rhizospheric pH is also associated with the soil-buffering capacity, microbial activities, and plant genotypes (Hinsinger et al., 2003). These different mechanisms play a significant role, especially in response to phosphorus mobilization (Lambers et al., 2011).

**Management of Mycorrhial Symbiosis**

Several researchers worldwide proposed rhizospheric microbial inoculants as integral and indispensable components of integrated nutrient management systems (Adesemoye and Kloepper, 2009), particularly regarding their ability to increase the availability of P for crops (Smith et al., 2011). Mycorrhizae play a considerable role in alteration of the biochemical environment (Bhadoria et al., 2004) and in the physiological characteristics of the rhizosphere, which can improve P accessibility in the rhizosphere.

Mycorrhizal inoculations can be used in cocoa plantations for stimulating rooting and growth, and thereby transplant survival of cuttings and seedlings rose in sterilized nursery media, which is essential for the establishment of the crop. The inoculation with appropriate AM fungi may give good results in plants (Baon et al., 1994). Successful AMF establishment has been obtained in nursery of cocoa. Mycorrhizas, through their role in increasing the natural resistance of plants to abiotic and biotic stresses and in rendering their underground organs more efficient in exploiting soil resources, have opened interesting possibilities for the production of high-quality micropropagated plants with low inputs (Gianinazzi et al., 1990). AM fungi inoculation has been found to improve growth and nutrient uptake of cocoa plant especially during seedling stage. The ability of the AMF to develop together without negative interactions opens the possibility of creating a given diversified microbial-rich environment around cocoa roots, beneficial for plantlet development and therefore increasing the levels of tolerance of plantlets for a large spectrum of biotic and abiotic stresses, linked to their transplantation into the field.

**Conclusions**

Published works gathered so far in this review indicates that mycorrhiza is likely to have a significant impact on P uptake by cocoa plants followed by its allocation and cycling. There is substantial evidence from the cocoa farm system that mycorrhizal symbiosis facilitate enhancement of phosphorus efficiency. Mechanisms of mycorrhizal mediated advantages have been identified at inside cocoa plant roots and outside cocoa plant roots and new findings continue to highlight the importance of the AMF association in this system. However, the responses of cocoa growth and production to P-limitation can only be understood when the interactions of this limitation with multiple other abiotic resources and biotic interactions are taken into consideration. With rising prices for P fertilizers, the use of mycorrhiza and the breeding of cocoa clones and hybrids with adapted root systems or exudation strategies are possible tools in maintaining or increasing productivity. While not all cocoa plants both as seedlings or plantlets form AMF associations, there is a prospect for development of early association by inoculation either in laboratories or during rising in nursery.

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# Root Nodulation and Nitrogen-Fixation in *Pterocarpus indicus*

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## Introduction

There are a number of commercial timber tree species belonging to the family Leguminosae-subfamily Papilionoideae (Fagaceae) that are N-fixing. These include genera like *Acacia*, *Casuarin*a, *Dalbergia*, *Paraserianthes* and *Pterocarpus*. Despite their flora abundance, diversity and economic importance, few have been studied for their ability to nodulate, fix atmospheric N symbiotically with root nodule bacteria (rhizobia) and soil type preference especially in *Pterocarpus indicus* and *Dalbergia* spp. (Allen and Allen, 1981; de Faria et al., 1989; Sprent, 2001). According to de Faria et al. (1989) and Sprent (1995), there are only 57% and 20% of the genera and species respectively in the tropical legumes that have been examined but poorly studied. *Pterocarpus indicus* or Angsana is of no exception as it is identified as one of the potential 'millenium tree' species for timber forest plantation by FRIM due to its fast growth rates and other desirable characteristics for planting (Appanah and Weinland, 1983; Lok, 1996).

Legumes are often used for managing agricultural ecosystems in order to improve their organic fertility, N economy or farming system flexibility (Brockwell et al., 2005; Sprent, 2001). At times, optimum growth performance of the N-fixing symbiosis depends upon pre-selection of both symbiotic partners for adaptation to the target environment which in turn pose a challenge to root nodulation and survival (Howieson and Ballard, 2004). *P. indicus* are reported to nodulate with *Rhizobium* and *Bradyrhizobium* in the field but little is known on the symbiotic relationships and capacity to fix nitrogen (Lim, 1976; Allen and Allen, 1981; Sprent, 2001). However, inoculation with rhizobia is an advantage where soil populations of compatible microsymbionts are low or absent (Berger, 1993; Ndiaye and Ganry, 1997). Inoculation using effective strains may be useful in commercial forest nurseries so as to increase $N_2$-fixation capability before transplanting to fields (Chee et al., 1989; Perez-Fernandez and Lamont, 2003). Hence, the aim of this study was to determine the ability of *P. indicus* to nodulate with diverse strains of rhizobia for $N_2$-fixation and to identify possible strains with potential for application as inocula in the nursery or field.

## Materials and Methods

A complete randomized block design was used, comprising of 18 strains of rhizobia and two controls (C1 as uninoculated without added inorganic N and C2 as uninoculated plus inorganic N) (Table 1). Four replicates per treatment were set up and carried out at Murdoch University glasshouse. The mean minimum and maximum temperature (±SE) were 18 ± 2 $^{o}$C and 30 ± 3 $^{o}$C respectively.

Seeds and a local strain were collected from the mature *P. indicus* growing in Forest Research Institute Malaysia (FRIM) and surface sterilize before sown in plastic trays containing yellow sand. The sand has a pH ($H_2O$) range from 5.3 to 6.0 and has been used in different plant nutrition and mycorrhizal responses studies (Bougher et al., 1990; Brundrett et al., 1996). After transplanting and inoculation, all pots were given a pulse of 20 ml aqueous solution containing $KNO_3$ and weekly with required nutrient solution (minus N) (Howieson, et al., 1995). The local strain in WSM 3712 was isolated from a nodule of *P. indicus* in a potted seedlings growing in FRIM and extracted according to Vincent, 1970 and transferred

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onto yeast mannitol agar (YMA) (Howieson et al. 1988). All other strains in WSM and TAL were obtained from the Centre for Rhizobium Studies (CRS), Murdoch University, Western Australia.

Table 1. Root nodule bacteria (Rhizobia) used in the study

| Strain No. | Strains | Source |
|---|---|---|
| TAL 643 | *Bradyrhizobium* sp. | RRIM |
| TAL 648 | *Bradyrhizobium* sp. | RRIM |
| TAL 651 | *Bradyrhizobium* sp. | RRIM |
| TAL 656 | *Bradyrhizobium* sp. | RRIM |
| *R 602 | *Rhizobium gallicum* | INRA |
| WSM 2096 | *Bradyrhizobium elkanii* | CRS |
| WSM 2097 | *Bradyrhizobium japonicum* | CRS |
| WSM 2098 | *Bradyrhizobium liaoningense* | CRS |
| WSM 2100 | *Mesorhizobium ciceri* | CRS |
| WSM 2105 | *Rhizobium galegae* | CRS |
| WSM 2106 | *Rhizobium hainanense* | CRS |
| WSM 2108 | *Rhizobium leguminosarum* | CRS |
| WSM 2110 | *Rhizobium tropici* | CRS |
| WSM 2114 | *Sinorhizobium meliloti* | CRS |
| WSM 2115 | *Sinorhizobium saheli* | CRS |
| WSM 2116 | *Sinorhizobium terangae* | CRS |
| WSM 2117 | *Mesorhizobium loti* | CRS |
| WSM 3712 | *Bradyrhizobium* sp. | FRIM |

*Source: CRS: Centre for Rhizobium Studies, INRA: Institute National de la Recherche Agronomique (France), FRIM: Forest Research Institute of Malaysia and RRIM: Rubber Research Institute of Malaysia.*

Sufficient culture strains were then obtained for inoculation using YMA plates and grown at 28 $^{o}$C. A suspension of 10 ml was used to inoculate into each pot for all newly transplanted seedlings to ensure evenly distributed suspension on the soil surface. Plants were harvested after three months and with visible growth effects. Roots were extracted and nodulation was examined based on nodules size, color and position on the roots (Howieson et al., 1995). The shoots were separated and oven dried to constant weight at 70 $^{o}$C. Microscopic studies were fixed overnight in gluteraldhyde (3% v/v) and in phosphate (0.025 M) buffer before being dehydrated and embedded in Spurr's resin (Spurr, 1969). Observations on nodules were made using stereo and compound microscopes. Later, the data were subjected to analysis of variance (ANOVA) using SPSS version 11.5 after three months.

## Results

*Nodulation and shoot dry weight*

Nodules were formed from all four genera of rhizobia: *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Rhizobum* except the two uninoculated controls. Out of eighteen strains used, only ten strains were found to have nodulated. These strains are TAL 643, TAL 651, R602, WSM 2096, WSM 2098, WSM 2100, WSM 2106, WSM 2110, WSM 2114 and WSM 3712. Interestingly, the slow growing strains, *Bradyrhizobium* (WSM 3712, TAL 643, WSM 2096 and TAL 651) formed greater mean number of nodules per seedlings and larger in sizes (Table 2).

*Bradyrhizobium* strains (WSM 3712, WSM 2096 and TAL 643) also formed on the tap and lateral roots while others were only confined to the collar region (Table 2, Figure 1a). The larger nodules formed by WSM 3712 and WSM 2096 were observed to be pink internally (active) whereas all other nodules were whitish (inactive) in color. The nodules of *Pterocarpus indicus* are mainly globose, smooth, internally

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aeschynomenoid in shape, determinate growth in oblate form and containing central infected tissues with few or no uninfected cells (astragaloid in the terminology of Sprent, 2001; Brown and Walsh, 1994).

Nodulated strains in WSM 2096 and WSM 3712 were observed to have dark green shoots as compared to other control uninoculated plants without added N. However, only WSM 2096 had significantly increased shoot dry weight at the time of harvest which was similar to the control with added N (Figure 1b).

Table 2. Mean nodule number, nodulated seedlings, nodule distribution, nodule size and nodule colour in *Pterocarpus indicus* seedlings for ten treatments.

| Strain | Mean number of nodules per nodulated seedling (± S.E.) | Nodulated seedlings (%) | **Nodule distribution[1] (%) | | | #Nodule size[2] (%) | | | Nodule colour |
|---|---|---|---|---|---|---|---|---|---|
| | | | A | B | C | L | M | S | |
| WSM 3712 | 25.3±6.3 | 83 | 93 | 7 | - | - | 18 | 82 | pink |
| TAL 643 | 17.3±5.0 | 83 | 57 | 28 | 15 | - | 19 | 81 | white |
| WSM 2096 | 19.0±9.0 | 42 | 38 | 46 | 16 | 16 | 42 | 42 | pink |
| TAL 651 | 16 | 8 | 100 | - | - | - | - | 100 | white |
| WSM 2106 | 3 | 8 | 100 | - | - | - | 100 | - | white |
| WSM 2114 | 2 | 8 | 100 | - | - | - | 100 | - | white |
| WSM 2100 | 1 | 8 | 100 | - | - | - | - | 100 | white |
| R 602 | 1 | 8 | 100 | - | - | - | - | 100 | white |
| WSM 2098 | 1.0±0.0 | 8 | 100 | - | - | - | - | 100 | white |
| WSM 2110 | 1 | 8 | 100 | - | - | - | - | 100 | white |

*** Nodule distribution (%) at: A = 0-3 cm; B = 3-5 cm and C = >5 cm from the root collar for all four replicates, #Size (%): L = large: > 5mm, M = medium: 3-5 mm and S = small: < 3mm in diameter for all four replicates (Howieson et al., 1995).*

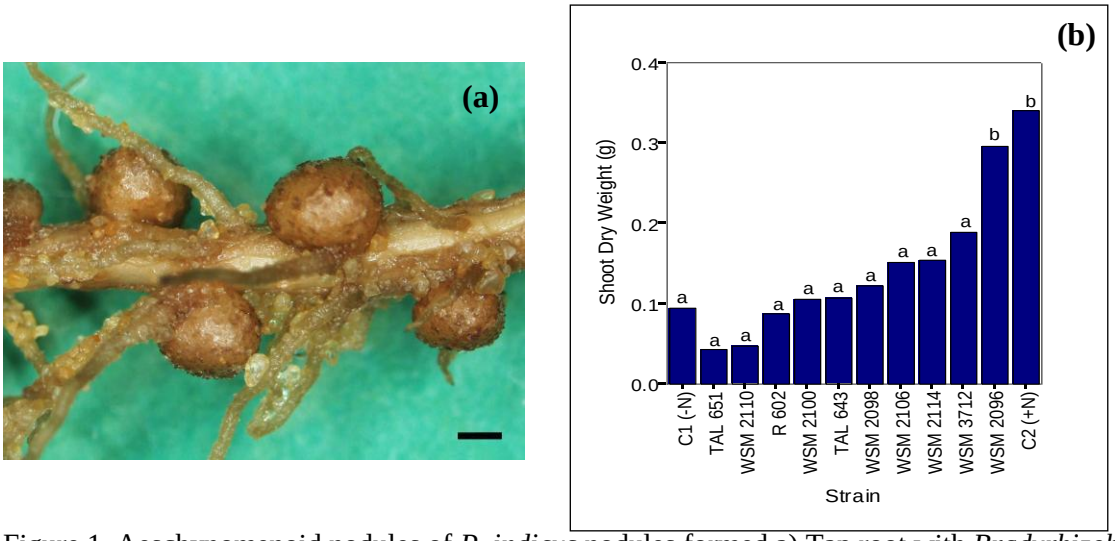


Figure 1. Aeschynomenoid nodules of *P. indicus* nodules formed a) Tap root with *Bradyrhizobium elkanii* (WSM 2096) and b) Mean shoot dry weight obtained from nodulated rhizobia strains. Bars with the same letter are not significantly different at $P \leq 0.05$.

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## Discussion

Knowledge of rhizobia associated with *P. indicus* is lacking. A significant finding from this study shows that *P. indicus* can be nodulated by diverse strains of rhizobia comprising of four genera i.e *Bradyrhizobium, Rhizobium, Sinorhizobium* and *Mesorhizobium*. Nodulation using slow-growing strain such as *Bradyrhizobium*, has previously reported (Lim, 1976) but this is the first study of nodulation on *P. indicus* using diverse strains of rhizobia. This study also provides strong evidence to support the suggestion by Sprent and Parsons (2000) that nodulating species of *Pterocarpus* are promiscuous. Nodulation ability of other tree species has been reported to vary among and within plant species under different locations and environmental conditions (Moreira et al., 1992). However, further work is required to quantify the role for some of these strains and its effectiveness in nitrogen fixation.

## Conclusions

The results showed that *P. indicus* can be nodulated by a diverse range of rhizobia and that it is promiscuous host species. However, this initial study showed that only two strains may be effective. Further inoculation studies with a wide range of strains from taxonomically related species such as in *Dalbergia* are required. Whether woody legumes require inoculation in the nursery depends on the availability of compatible and effective rhizobia in the soils and where they are to be planted in the fields.

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# Interaction of Microbes Application and Vegetation Cover towards Slope Protection

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## Introduction

Most developing countries are now practicing extensive and rapid progress for improvement in terms of infrastructure such as slope cutting for highway development as well as the hillside housing project for a better standard of living. Simultaneously, reduction in vegetation cover has ensued to increase the problems of erosion and landslide. Thus, understanding of both suitable plant species and the role of re-vegetation of slope are vitally needed with a potential towards a systematic and non-destructive methods approach. Microbes are known to play a role in enhancing soil nutrients via its biomass and biological activities (Haslam and Hopkins, 1999) that promotes plant growth. Thus, this study attempts to relate the physiological aspects of selected potential slope plants and soil-microbes application with respect to different vegetation density. It is anticipated that this interaction would enhance the slope plant physiological characteristics, resulting in a more stable and sustainable resources of the slope.

## Materials and Methods

*Experimental set up*

Six experimental plots of three different coverages; bare (control), less dense (50% of plant coverage), and dense (100 % of plant coverage) with the size of 8 m x 8 m for each have been set up on selected slopes at Guthrie Corridor Expressway (GCE), Sg. Buloh, Selangor. Two treatments; microbe (M) and non-microbe (NM) soils were applied on each plant coverage; bare ($B_M$ and $B_{NM}$), less dense ($LD_M$ and $LD_{NM}$), and dense ($D_M$ and $D_{NM}$).

*Plants transplanting on slope and microbe application*

Three species of potential slope plants were chosen and transplanted onto slopes; *Melastoma malabathricum, Lantana camara* and *Bauhinia purpurea.* The transplanting of these species on slope was conducted using a Microclimate Plant Propagation Technique with modified soil depth (Normaniza and Barakbah, 2011). Each seedling was transplanted into holes by using a soil coring machine (Eijelkamp Agrisearch Equipment, Model Cobra, the Netherlands) at 0.6 m of soil depth. Plant supplements such as NPK fertilizer, sphagnum moss and rock-phosphate (15 g each/hole) were applied only in the beginning of treatment in order to initiate the establishment of the roots and other physiological processes. After a week of transplanting, 50 g of commercialized microbe fertilizer was applied at 10 cm of soil depth near the transplanted plants, representing the microbe treated plots.

*Data measurement: isolation of bacteria and fungi, erosion rate, physiological parameters, soil npk and soil saturation level (STL)*

After six months, the soil samples (n=3) for each slope plant species were collected at 10 cm radius from the stem at both treated and non-treated plots using steriled spatula and containers. Bacterial and fungi were isolated on soil extract agar and potato dextrose agar media, respectively. Soil sample (1 g) was ten-

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fold serially diluted quarter strength Ringer's solution and 100 µl of soil suspension spread on to agar plates in triplicates. All the agar plates were incubated for 3 to 7 days at 28 $^{o}$C. Growth of bacteria and fungi were enumerated as colony forming unit per gram of soil (cfu/g).

The eroded soils in the PVC containers that placed below the erosion boxes were collected, air dried and weighed weekly for duration of six months. The erosion rate was manually calculated and determined in g/m$^2$, with three replications. .

Physiological performances of the species studied were observed for six months in triplicates using a portable photosynthesis system (6400XT, LiCOR, USA) in an open system mode, between 0930 and 1200 hours. During early and at the end of experiment, the soil samples within the perimeter of 0.5 m from the root were taken using soil coring machine and the soil nutrient; N, P, and K were analyzed at Department of Geology, Faculty of Science, University of Malaya. N was analysed using Kjeldahl digestion method (Bremmer, 1965) and P was detected by using colorimetric Sharpley et al. (1992). Whilst K was determined by ignition method using atomic absorption spectrophotometry (Fishman and Downs, 1966).

Soil water profiles such as soil water content (SWC), soil field capacity (SFC) and soil saturation level (STL) were determined. The soils were sampled using cylindrical soil cores (11 cm in diameter; 100 cm depth). The measurements of SWC and SFC were taken at the beginning and the end of the experiment, diagonally across the plot with three replications, as follows:

*i)* *Soil water content (SWC)*
The soil samples were oven dried at 85 $^{0}$C to a constant weight. SWC was calculated as [(Fresh weight – dry weight)/ Fresh weight] X 100%.

*ii)* *Soil field capacity (SFC)*
SFC was determined by pouring excess water into a container filled with soil so that the soil becomes supersaturated. The excess water was drained out through small holes at the bottom of the container. Once the water stopped dripping, the saturated soil was weighed (SW) and then dried in the oven at 85 $^{0}$C to obtain a constant weight (DW). SFC was calculated as [(SW – DW)/SW] X 100%.

*iii)* *Soil saturation level (STL)*
The soil saturation level was determined as [SWC / SFC] X 100%.

*Statistical analysis*

Statistical analysis was performed using SPSS version 20. The two way ANOVA was applied to evaluate the significant difference between plant density and microbe application factors. LSD (p=0.05) was calculated using the error mean squares of the analysis of variance.

**Results and Discussion**

*Enumeration of soil microbes*

In general, the counts of bacteria and fungi (both treated and untreated plots) ranged from 5.7 x $10^5$ to 7.1 x $10^6$ cfu/g and 1.0 x $10^3$ to 3.8 x $10^5$ cfu/g, respectively. The result recorded that *L. camara* in treatment $D_M$ exhibited the highest in number of cultured bacteria and fungi (Figure 1), implying that microbe fertilizer has enhanced soil microbial activity of soil. In addition, these results were in accordance with

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finding by Fan et al. (2009) which described that the microbiological properties of arid soil in Southern China had improved in the presence of *L. camara*, thus, it will lead to accelerate the cycles of soil nutrients. Furthermore, bacterial and fungal counts from microbe treated soils of *M. malabathricum* in both LD and D plots showed no significant difference in numbers (Figure 1), indicating that the microbe fertilizer altered the soil microenvironment of the slope plants and the effects varied according to types of plants and density of the plots.

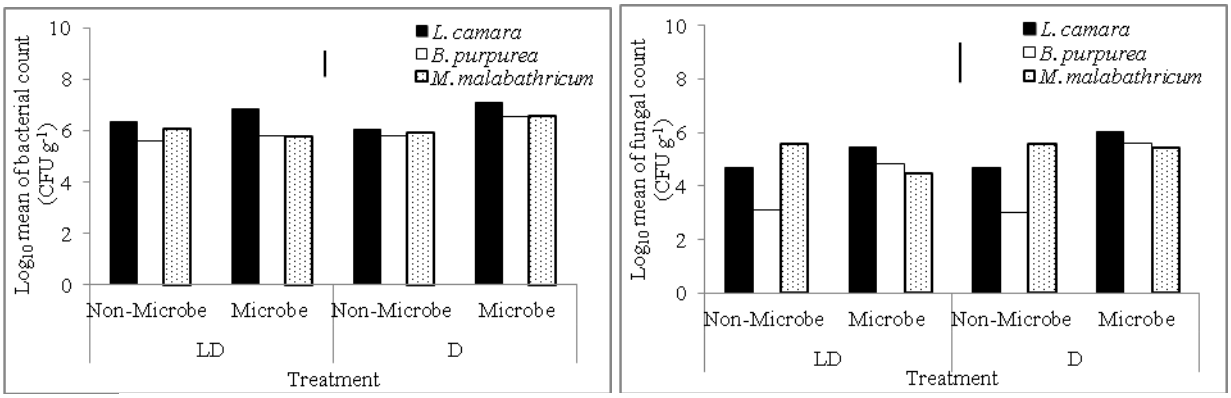


Figure 1: Bacterial and fungal counts in soil collected from the different plant species at six months after microbe application.

*Physiological parameters*

In general, the physiological values of all species were significantly influenced by the interaction between plant density and application of microbe fertilizer. At the end of observation, the stomatal conductance, photosynthetic rate and transpiration rate of *L. camara* of treatment $D_M$ exhibited significantly the highest value amongst species and treatments (Figure 2a-c). Previous study described that this invasive species showed an outstanding performance in growth rate at the harsh environment (Sharma et al., 2005). Thus, this result implies that the sun tolerant feature of *L. camara* as well as having higher number of branching and leaves, resulting in the increased of stomata (Figure 2a). Thus, a higher in stomata opening will subsequently lead to the significant light utilization efficiency of the plant in photosynthesis (Figure 2b). Concurrently, it will contribute in enhancing the transpiration capacity of the plant (Hertwitz et al., 2004) (Figure 2c). Furthermore, dense plant coverage and microbe treatment showed a positive influence on the growth performance of all the species studied. Thus, this will improve the water absorption capacity at the plot areas via evapo-transpiration in order to release excessive water. Subsequently, it may increase the safety aspect of the slope through above ground biomass (Greenwood et al., 2004) and help to reduce the amount of eroded soil.

Interestingly, *M. malabathricum* grown in microbe treated plot has shown a lower assimilation rate as compared to those grown naturally on slopes and in untreated plots (Figure 3). It is known that microbial community structures and ecological functions can have a positive, negative or neutral impact on diversity of plants and soil microbes (Singh et al., 2004). Thus, in this case, the species performance was not influenced by additional microbe fertilizer. This may suggest that plant specific interaction with the microbes is a complex phenomena and this species might be in response to the microbe fertilizer by enhancing growth and development of plant roots rather than the aboveground properties.

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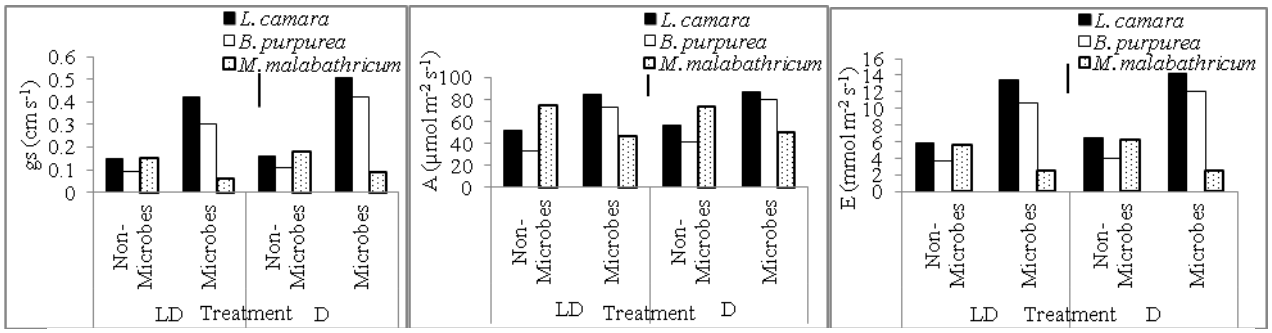


(a) Stomatal conductance (b) Photosynthetic rate (c) Transpiration rate

Figure 2: Physiological performances of species studied grown on the experimental plots.

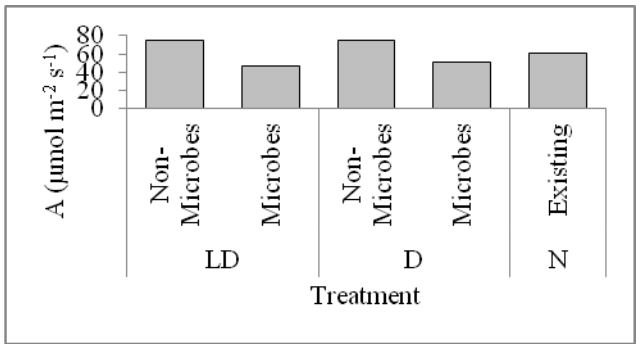


Figure 3: Photosynthetic rate of *M. malabathricum* grown on the experimental and existing plots.

*Soil nutrients (N, P, and K)*

Overall, there was a significant interaction between plant density and application of microbe fertilizer, contributing to the positive changes in soil nutrients. At the end of experiment, the soil NPK values showed the highest in $D_M$ whilst the lowest in $B_{NM}$ (Figure 4), implying the positive contribution of the microbe applied and higher density of the species studied to improve the soil nutrients content. Furthermore, at the end of experiment, the N, P, and K values were higher at the microbe treated plots, indicating that interaction between soil microbe and higher plant density have increased the number of plants associated with the soil microorganisms, thus, helping to obtain nutrients for the plant use (Dobermann and Fairhust, 2000). Consequently, it will enhance the soil fertility as well. Furthermore, a higher number of plants also attributed to the increased of decomposition process of litter, subsequently, releasing the nutrients back into the soil.

In addition, a higher organic matter at the top layer of soil also enhanced the root formation. In aftermath, roots and organic matter physically bind the soil particles and increase the soil aggregation, thus, reducing the soil erosion problem.

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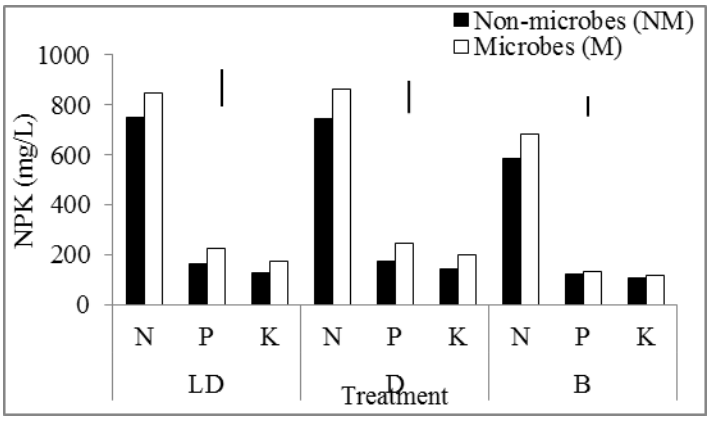


Figure 4: Soil nutrients status at six months of the observation. Vertical lines represent $LSD_{p<0.05}$

*Soil saturation level (STL)*

A significant interaction between plant density and microbes application exhibited the positive effect towards reduction of soil saturation level (STL). The result showed that treatment $D_M$ recorded the lowest value of STL which was by 39.6% (Figure 5). The highest STL was observed in bare treatments, implying that the direct impact of rainfall to the soil surface without interception contributed to the increased amount of water in soil. In addition, it is known that the increment of soil water content will lead to the increasing percentage of saturation level (Bouman and Tuong, 2001). Consequently, it led to the increased soil erosion. In contrast, a higher plant density improved the interception rate of the slope due to the increased leaf area coverage. It indicates that a higher plant density could be considered to be a good indicator as it serves as a good drying agent (Normaniza and Barakbah, 2011) especially during monsoon period, thus, preventing the soil from getting oversaturated. Furthermore, microbe application indirectly reduced the erosion rate of slope soil via increasing the number of bacteria and fungi, thus, enhancing the plant growth performance of the species studied. Therefore, it is anticipated that a higher vegetation coverage would enhance the evapo-transpiration process on slope, hence, alleviating the erosion problem. Moreover, the interaction of roots system amongst species studied tend to increase the surface roughness by adding organic substances to the soil and enhance the creation of soil macropores, thus providing a greater capacity for infiltration (Noguchi et al., 1997).

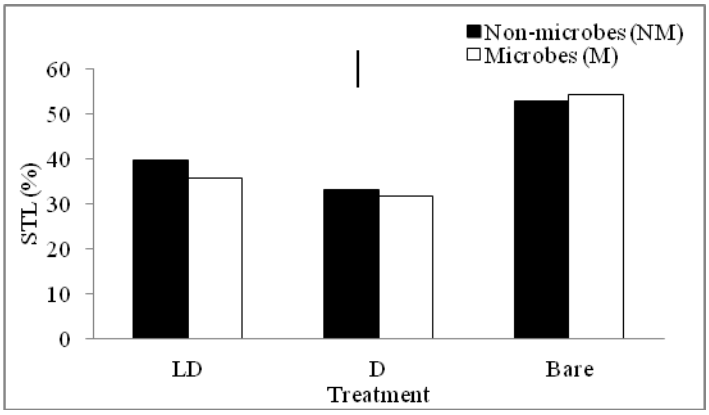


Figure 5: The soil saturation level (STL) at six months of observation. Vertical lines represent $LSD_{p<0.05}$.

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*Erosion rate*

In general, the result showed that the increased of plant density and microbe application had significantly reduced the erosion rate in the plots studied. The treatment $D_M$ recorded the lowest erosion rate by 93.3% at the end of experiment (Figure 6). Furthermore, the higher coverage of plant canopy in treatment $D_M$ resulted in the increased interception rate by foliage and stem, hence, reducing the direct impact of rainfall to the soil surface. In addition, interspecific (mixed species) interaction amongst the species studied as well as microbe application enhanced the canopy sizes and growth performances, thus, anticipating that the evapo-transpiration rate will also increase (Figure 2). Since high amount of water will be extracted out from the soil to the atmosphere, thus, it would prevent the slope soil from oversaturated (Figure 5) and less consolidates, thus, increasing the soil stability.

Despite the effect of plant canopy coverage, the plant litter contributed by reducing the runoff during rainfall events, hence, decreased the amount of soil loss. In addition, a higher plant density (treatment $D_M$), due to their relatively dense plants canopy, giving rise to a thick litter cover at the soil surface, which in turn increased the organic matter at the top layer of soil (Figure 4). Thus, it will enhance the root system and increase the soil-root interaction. Ultimately, it will reduce the soil erosion rate as well as improve the slope stability.

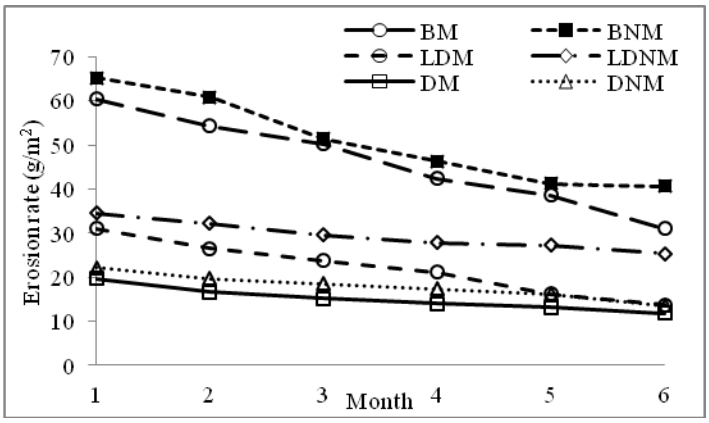

Figure 6: Erosion rate of the experimental plots.

*Correlations amongst the parameters studied*

The STL is positively related to the erosion rate of the slope soil (Figure 7). This indicates that the increased soil saturation level leads to a higher infiltration rate, thus, increased the possibility for the soil to be eroded. In terms of STL, the increasing amount of water in the soil referred to low amount of water extracted through evapo-transpiration process, resulting in oversaturated of the soil and tendency for the soil particles deformation. Therefore, vegetation cover is viewed as an important factor for soil erosion control (Morgan, 2005; Shakesby et al., 2000). The introduction of the suitable species associated with the microbe application on slope will provide the increased in interception of the rainfall (Liao et al., 2001), as well as affected the hydraulic conductivity of the soil by extensive root system activity (Haynes and Naidu, 1998), and accumulation of organic matter to the soil. Ultimately, it will reduce the erosion rate on slope.

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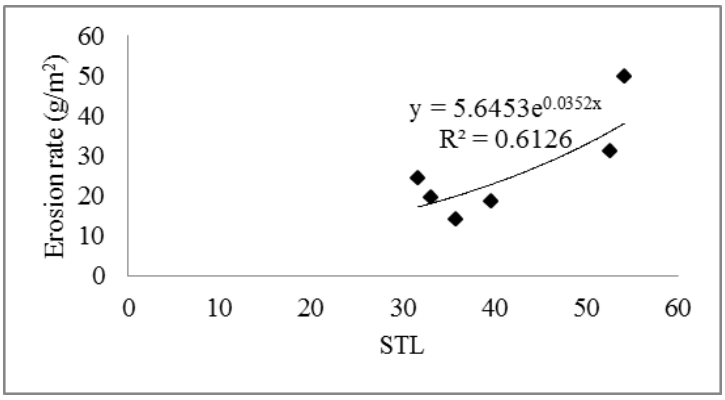


Figure 7: Relationship between soil saturation level (STL) and erosion rate of slope soil.

**Conclusions**

In conclusion, the interaction between microbe and dense coverage have improved the plant growth performance and alleviated the erosion problem. Dense plant coverage contributes to the maximum protection of the soil surface from raindrop impact, reducing the erosion rate of the slope soils. In addition, the performance of species chosen in microbe treated plots was observed higher in terms of physiological performance, consequently, provides a positive influence in reducing the erosion rate of the slope soils. This study has proven that the right planting density and amount of microbe fertilizer application has positively influenced the growth of the species studied and reducing the soil erosion rate of the slope. Thus, become the key factors in bioengineering development towards the slope stabilization.

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# Biomass Production, Physiological Changes and Secondary Metabolites of *Andrographis paniculata* Influenced by EFB Media and Water Stress

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## Introduction

*Andrographis paniculata* commonly known as 'king of bitter' or 'Hempedu bumi' belongs to the family Acanthaceae and it is native to India and Sri Lanka. It is an annual herb that has been commonly grown as a medicinal herb. Recently, the plant has received increased interest due to the valuable pharmaceutical properties that have been used traditionally for the treatments of enormous types of ailment around the world especially in Asia. The active compound of this plant is andrographolide that act as a good source of antioxidants, thereby contributing to treatments of appetite loss, diabetes reduction, liver tonic property, and gallbladder protection (Aziz, 2003). Hempedu bumi' is mainly propagated by seeds and the seedlings are then established in open fields. Soil is mostly used as rooting medium for *Andrographis paniculata* production, which jeopardizes the production of quality seedlings. Thus, cultivation method can be optimized to maximize the organically production of king of bitter by using modern technologies such as soilless media (SM). SM is a system of providing plant with nutrients that results in production of highly qualified crop yields, even in areas with adverse growing circumstance (Gruda, 2012). Waters stress is one of the most essential abiotic stress factors that enhance the biochemical contents of plants. It is known to alter the secondary metabolite production in several varieties of medicinal plants. Therefore, exposure to drought conditions may improve the value of *Andrographis paniculata* as medicinal herb plants which contain higher biochemical contents. However, under certain condition such as water deficit the physical and chemical properties of the growing media influence the biomass production of the plant (Ahmad et al., 2004). Several studies have demonstrated that application of different form of fertilizers either organic, inorganic or bio-fertilizer mitigated the detrimental effect of drought on biomass yield (Karkanis et al., 2007). Microorganism also plays an important role in enhancing the productivity of secondary metabolite in plant. For examples, the content of indole alkaloids ajmalicine, serpentine, and catharanthine has been reported to be increased up to five times when the *Catharanthus roseus* plant treated with fungal cell wall fragments (Namdeo et al., 2002). Little information is available about the effect of drought-stress on biomass production and biochemical content of *Andrographis paniculata* grown under SM warrants further investigation.

Therefore, the objective of this study was to stimulate the accumulation of secondary metabolite in *Andrographis paniculata* and reduce the adverse effect of water stress on biomass production by growing the plant in SM containing EFBC.

## Materials and Methods

### *Plant materials*

The plant material and seed germination of *Andrographis paniculata* was prepared by using a physical scarification method (Talei et al., 2012). After germination, the seedlings with two expanded cotyledons were transferred into tray with equal proportion of EFBC and CCD (1: 1) (v/v) until 6-8 leaf stage germination. Then healthy seedlings were transplanted into plastic pots measuring 12 cm × 17 cm with

different ratio of SM. The plant was established for eight weeks and well watered twice a day then subjected to water stress for 21 days until harvested.

*Experimental site and treatments combinations*

This experiment was also carried out in the glasshouse, Faculty of Agriculture, Universiti Putra Malaysia. It was designed to test two main factors which are soilless media (SM) and water stress (WS) treatment. In this study, the potting media consisted of (i) M1 - solely coconut coir dust (CCD) as control and (ii) M2 - mixture of CCD and EFBC (7:3, v/v) were used. Besides that, the application of indigenous microorganism (IMO) as organic enhancer was added to all treatments a week before transplanting as bio-enhancer and also supplemented with a foliar spray at every 2 weeks. In each application of IMO, the leaves and the surface of the media were sprayed twice to all pots. The treatment combination comprised of three water stress (WS) level, which included well watered (WW), moderate stressed (MS) and severe stressed (SS). Before beginning WS treatment, the field capacity of each pot were measured and all pots were watered to field capacity (FC). When the plants were subjected to WW condition, continuous watering was given to maintain the FC to 100% FC. The FC was determined by weighting the pots every day and at the same time adding the amount of water that equal to the loss in weight from the pots. Under MS, watering was given to 50% FC at which half of the amount of water loss in weight from the pots. For SS treatment, the plants were watered only at 25% FC in which quarter amount of water loss in weight from the pots. The WS treatments were given for 21 days until harvested only after eight weeks of plant establishment. The plants in the pots were received 60 kg N $ha^{-1}$, 15 kg P $ha^{-1}$ and 20 kg K $ha^{-1}$ for each of the plant. Basic fertilizer from urea, muriate of potash (MOP) and Christmas Island rock phosphate (CIRP) as source of N, P and K were used, respectively. The amount of P and K fertilizer were given based on the previous finding (Parvin, 2007). The fertilization was applied by top dressing in three split doses, 50% dose at transplanting, 30% at 15 days after transplanting and 20% before the WS treatment was began. Following the WS treatment, the pots were weighted at 9:00 am in the morning to obtain daily cumulative water transpired. The plant was harvested after 21 days receiving WS treatment. Parameters including plant growth [plant height, number of leaves and total leaf area (TLA)], biomass production and partitioning (fresh and dry weight of shoot), chlorophyll content and proline accumulation were determined once only after 21 days of WS treatment.

*Plant growth, biomass productionand partitioning*

At the end of WS treatment, the plant height and number of leaves were first measured after transplanting using a standard ruler from the media surface to the tip of the main stem while, the number of leaves produced or maintained was also recorded. After harvesting, the leaves were individually separated from the stem and the total leaf area per plant was estimated by using Automatic Leaf Area Meter (LI-3100C Area Meter). Plants were separated into root and shoot (stem, leaves) and the fresh weight of the shoot was measured by using a digital balance (QC 35EDE-S Sartorius, Germany). The plants part were dried individually under sunlight for one day and then oven dried at 40 ℃ for 48 h prior the dry weight was recorded.

*Total chlorophyll content*

Chlorophyll content was determined using chlorophyll meter (Spad-502, Minolta, Japan) and the value was converted to mg $cm^{-2}$ (Coombs et al., 1985).

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*Proline accumulation*

Free proline content in *Andrographis paniculata* leaves was determined by using the methods described by Bates et al. (1973). Proline was extracted by homogenizing 0.5 g young fully expanded fresh leaves in 10 ml of 3% aqueous solution of sulfosalsilic acid at 25 °C. The homogenize was then filtered through Whatman No. 2 filter paper, and 2 ml of the filter reacted with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin in a test tube for one hour in water bath at 95 °C. The reaction mixture was then cooled in an ice bath. Then 4 ml of toluene was added to the reaction mixture and mixed vigorously with a test tube magnetic stirrer for 20 seconds. The toluene layer at the top with pink-red in color was collected with a pipette. The absorbency of the toluene layer was read at 520 nm by using a UV-visible spectrophotometer (UV 3101 PC, Shimadzu Co., Ltd., Japan) and toluene was used as blank. The standard curve was produced with 0 to 30 µg ml-1 of L-proline (Sigma Chemical Co., St. Louis, USA) dissolved in 3% aqueous solution of sulfosalsilic acid. The curve was then used to determine the proline content of the samples on a fresh weight basis as the unit was represent as µmol proline g $FW^{-1}$.

*Statistical analysis*

The statistical analysis was determined by using 1 or 2 ways ANOVA using Statistical Analysis System (SAS) (release 9.3, SAS Institute Inc., Cary, NC, USA). Fisher's Least Significant Differences (LSD) was used for comparison of treatments mean when F values were significant at ($P \leq 0.05$).

**Results and Discussion**

*Plant height, number of leaves and total leaf area*

The plant height of *Andrographis paniculata* was significantly affected by both media and water stress treatments (Figure 1A). Increase in the plant height of *Andrographis paniculata* grown in M2 was noticed compared to plants grown in control media (M1). However, there were no significant different in the plant height of *Andrographis paniculata* grown in M2 media under well watered (WW) and moderate stressed (MS) condition. Plant height under severe stressed (SS) gave minimum value which differed significantly from other WS treatment. The plant grown in M1 and M2 exhibited shorter height under MS (43% and 34.4%) and SS (29% and 11.4%) than under WW condition, respectively. The reduction in the plant height under SS condition in both media is possibly due to the reduction of cell turgor which affect the cell division and expansion (Luvaha et al., 2008). Similar result have been observed by Kharadi et al. (2011) when a minimum plant height was observed indicating that the plant is under deficit water stress. In addition, the sensitivity of the plant cell division, enlargements and differentiations process to water stress depends on the turgor which is responsible for the growth and development of the plant cell. This sensitivity results in the decrease in the cell growth when the plant undergoes water stress condition. These results are in agreement with Tiwari et al. (2013) who found the plant height of each of *Jatropa curcas* and common wheat was adversely affected by subjecting the plants to water stress.

The number of leaves of *Andrographis paniculata* was significantly affected by different combination of SM and WS (Figure 1B). The number of leaves of plant grown in M2 is increased as compared to plants grown in control media (M1). The highest number of leaves was observed in plants grown in M2 media under WW condition. However, there were no significant different in the number of leaves of plant grown in M2 media under MS and SS treatment. Number of leaves is decreased by increasing the water stress level and decreased when exposed to MS (47.03% and 48.2%) and SS (35.3% and 66.1%) compared to WW plant when the plant grown in M1 and M2, respectively. The mean number of leaves of plant grown in M2 media exhibited 66.67% higher than plants grown in M1. The total leaf area (TLA) of

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*Andrographis paniculata* was significantly affected by SM combination and different WS (Figure 1C). The maximum TLA was found in plants grown in M2 compared to that of plants grown in control media (M1) and the highest TLA of plant grown in M2 was registered under WW condition while, there was no significant difference in the TLA of plants grown in same media and water regime treatment. Plant subjected to SS condition reduced the TLA of plant grown in both SM. The percentage of TLA of plant grown in media M2 under SS condition was reduced by 34.4% compared to WW condition. Greater mean of TLA by 31.2% was resulted under M2 media compared to control media (M1) when averaged overall different WS level. There was a significant interaction between the SM and WS in TLA of *Andrographis paniculata*. It has been reported that decreased in the number of leaves and TLA of plants are common occurrences in water stressed plants (Luvaha et al., 2008). Less number of leaves under SS condition attributed to the reduction in the leaf formation and it can be a survival phenomenon by the plant in order to reduce the transpiration rate (Ahmad et al., 2004). The decline in the TLA of the *Andrographis paniculata* under SS might be due to the decrease in the interception of solar radiation which results in the reduction of biomass of most of the crops. These results are in line with the conclusion drawn by Zhang et al. (2004) in soybean,Wullschleger et al. (2005) in populous, and (Hayatu and Mukhtar, (2010) in cowpea in which severe water stress mostly reduced the leaf growth and TLA, implying an important parameter that indicated the level of stress in plant. In addition, the leaf growth and plasticity decreased under drought condition in many plant species, such as peanut and *Oryza sativa* (Demmig-Adams and Adams Iii, 1992).

*Fresh and dry weight of shoot*

The shoot dry weight (SDW) and fresh weight (SFW) of *Andrographis paniculata* was significantly affected by different combination of SM and different WS (Figure 2A and B), respectively. Although, there was a significant difference in the SFW and SDW of plants grown in both media M1 and M2 under different WS, it showed that plant grown in media M2 increased more SFW and SDW as compared to plants grown in control media (M1). However there was no significant difference in the SFW of plants grown in media M1 under MS and SS condition while the SFW of plants grown in M2 were differed significantly under different WS level. In both media WW condition applied exhibited higher SFW and SDW, than plant under MS and SS condition. Under SS condition, SFW and SDW of *Andrographis paniculata* decreased when plant grown in M1 (37.4% and 54.8% ) and M2 (46.1% and 56.7%), respectively, compared to WW condition. Wu et al. (2008) observed that the adverse influence of water stress on growth of *Sophora davidii* could be alleviated with application of appropriate fertilizer. Similarly, application of suitable fertilizers could significantly enhance the shoot biomass production in *Bupleurum chinense* under water stress conditions (Zhu et al., 2009). The mean SFW and SDW of plant grown in M2 was 44.2% and 92.8% higher, respectively, compared to M1. This indicates that the availability of plant nutrient supply in media M2 is more than in control media. Similarly in previous report it has also shown that the EFB compost increased nutrient uptake and yield of palm oil plantation when used as cultivation media (Zaharah and Lim, 2000). Farahzety and Siti Aishah (2013) reported that EFBC has great potential to be used in organic cabbage production. In general, drought stress has adverse effects on the crop production because it inhibits the growth performance of plant, but the application of suitable rate of fertilizers and microorganism can alleviate these effects (Wu et al., 2008; Zhu et al., 2009). There was a significant interaction between SM and different WS in both fertilization applications.

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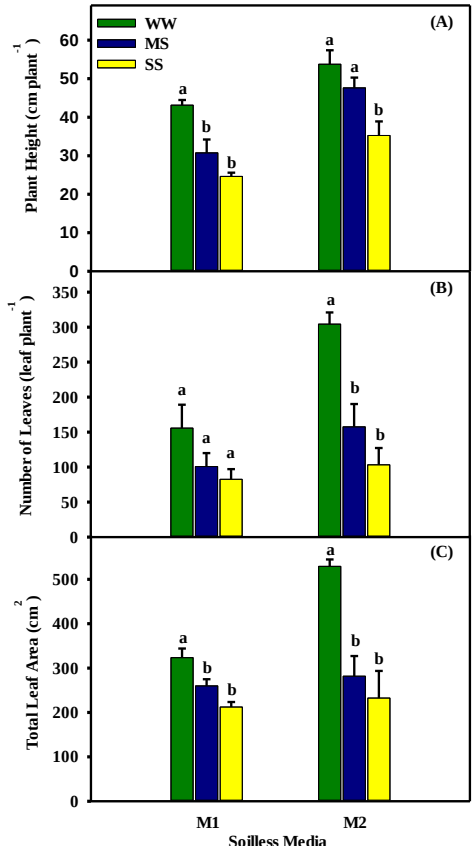


Figure 1: Plant height (A), number of leaves (B) and total leaf area (C) of *Andrographis paniculata* as influenced by soilless media (SM) and different water stress level (WS).Vertical bars represent S.E. of mean followed by the same letter are not significantly different at ($P \leq 0.05$).

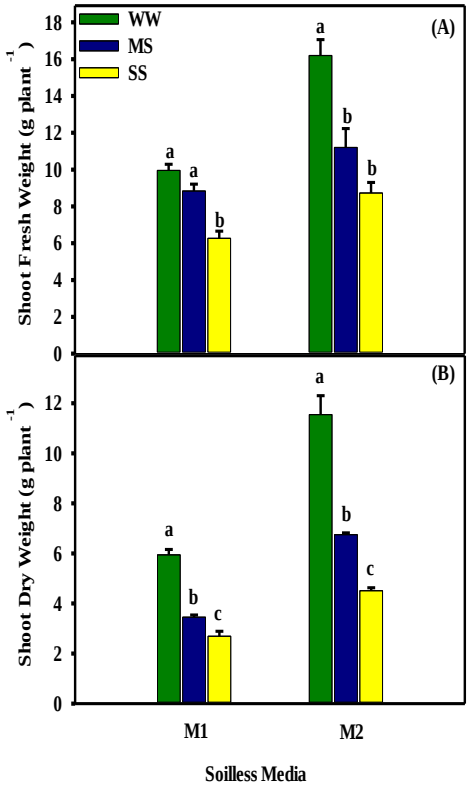


Figure 2. Shoot fresh weight (A) and shoot dry weight (B) of *Andrographis paniculata* as influenced by soilless media (SM) and different water stress level (WS). Vertical bars represent S.E. of mean followed by the same letter are not significantly different at ($P \leq 0.05$).

*Chlorophyll content*

The chlorophyll content in the leaf of *Andrographis paniculata* was significantly affected by growing the plant in different proportion of SM and different WS (Figure 4A). Different WS treatment resulted in higher chlorophyll content of plants grown in media M2 than control media (M1). The maximum chlorophyll content was found in plants grown in M2 under WW condition but the content were not significantly different with subjecting the plant to MS and SS condition. There were a significant different in the chlorophyll content of plants grown in M1 under different WS treatment. Subjecting the plant to SS condition had 44.2% and 51.2% lower chlorophyll content in plants grown in M1 and M2, respectively as compared to the plants under WW condition. These results are in agreement Mafakheri et al. (2010) who described a significant decrease of total chlorophyll content caused by water deficit in six wheat and three chickpea cultivars. A decline of the total chlorophyll due to water regimes treatments reveals a lower capacity for light harvesting. In this study, higher total chlorophyll content of plants grown in M2

media comprising of CCD and EFB (7:3 v/v) regardless of fertilizer application and across different WS was noticed than plant grown in solely CCD medium. Similar result was also obtained in previous studies by Uwumarongie-Ilori et al. (2012) which revealed that additional of EFB as media induced the highest chlorophyll content in palm oil.

*Proline content*

The proline content in fresh leaves of *Andrographis paniculata* was significantly affected by different combination of SM and WS (Figure 4B). Proline content was found significant and highly increased by subjecting the plant to SS condition grown in both media M1 and M2 than plant under MS and WW condition. However, it can be seen clearly that the proline content of plant grown in media M2 under different WS treatment was significantly higher compared to plants grown under control media (M1). However, there were no significant difference in the proline content subjecting to MS and SS condition in plants grown in both media. The proline content of plants grown in M1 and M2 under SS condition was 75.1% and 88.8% respectively higher than under WW condition. Increasing of proline content due to drought stress has been observed in another researches (Safarnejad, 2004).

Increases in proline content under SS condition were in agreement with Wu (2006) who stated that the content of free proline in *Rosmarinus officinalis* L. had significantly increased under water stress. Water deficits induce dramatic increases in the proline content of phloem sap in medicinal and aromatic plants, suggesting that increased deposition of proline at the root apex in water stressed plants could in part occur via phloem transport of proline. A proline transporter gene involved namely *ProT2* was determined strongly induced by water and salt stress in *Arabidopsis thaliana* (Kharadi et al., 2011). Plant grown in M2 increased 11.4% higher proline content compared to M1. Growing the plant in EFBC as a biofertilizer in SM promoted elevated level of proline in the plant leaves. The main reason for this is matric potential of media M2 might be lower during water stress periods. Similar results were also obtained by Sarkar et al. (2008) who found that proline content of tomato plant significantly increased when grown in low matric potential medium.

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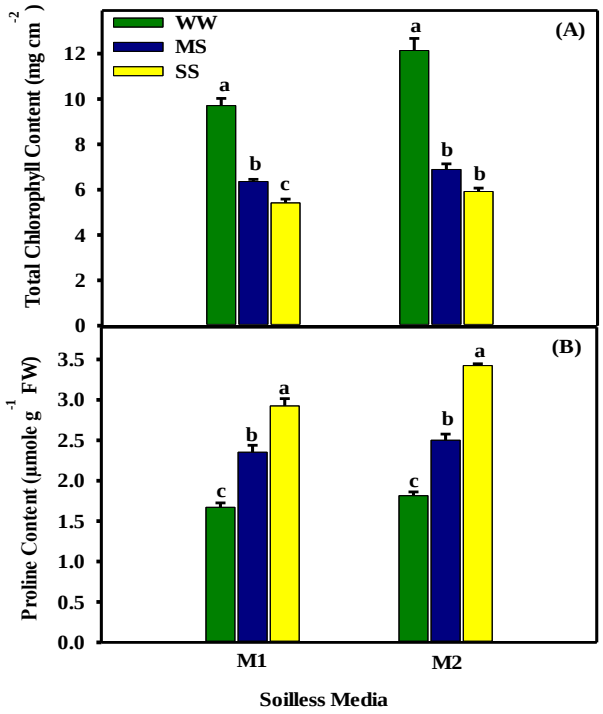


Figure 4.Total chlorophyll (A) and proline content (B) of *Andrographis paniculata* as influenced by soilless media (SM) and different water stress level (WS). Vertical bars represent S.E. of mean followed by the same letter are not significantly different at ($P \leq 0.05$).

## Conclusions

In summary, it can be concluded that different WS and SM have significant effect on the growth, dry herbage yield, plant water status, and proline content of *Andrographis paniculata*. Subjecting the plant to water stress condition adversely affected the plant growth performance. On the contrary the activity of secondary metabolites (proline content) increased in the plant leaf tissue. The results also indicated that application of EFBC (M2) as a growing media and under WW condition for *Andrographis paniculata* can enhance the growth performance while activity of secondary metabolites of the plant was increased in M2 media under SS condition. Growing the plant in optimum proportion of SM significantly alleviated the water stress effect on the plant growth and further increased the proline content of the plant. Proline accumulation was significantly improved with the combined application of EFBC (M2 + SS) as organic amendment to SM showed better effects than solely conventional media (CCD).

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# Approaches Used to Compare Cropping Systems

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## Introduction

Intercropping is growing two or more crop species simultaneously on the same field and is a common practice in most countries in the tropics. Farmers practice different cropping systems in order to increase productivity of their fields and sustainability of their farms (Hauggard-Nielson et. al., 2001). The most common advantage of intercropping is to produce a higher yield from a given piece of land by achieving more efficient use of the available natural resources for crop growth that would otherwise not be utilized by each single crop grown alone (Lithourgidis et. al., 2011). Therefore, in terms of land use efficiency intercropping is regarded as more productive than sole cropping (Andrews and Kassam, 1976). Higher nutrient uptake and better water use efficiency have also been suggested (Dallal, 1974), but probably need to be tested for each different combination of crops used for intercropping systems.

Better use of solar radiation by intercrops was attributed to increased interception of photosynthetically active radiation resulting in a higher radiation use efficiency (RUE). Spatial arrangement of intercrops is an important management practice that can improve radiation interception through a more complete ground cover (Reddy et al., 1989). However, Keating and Carberry (1993) have suggested that increased radiation interception due to better ground cover of intercrops may be due to sub-optimal sole crop population densities used in comparisons.

Doorenbos et al., (1979) defined water use efficiency (WUE) as a measure of the amount of dry matter produced per unit amount of water evapotranspired. When soil water conditions are not limiting, water use efficiency differences between plants are insignificant as long as other conditions are not limiting. However, under water stress conditions crop response to water varies between varieties and at different growth stages. Maize, for example has a relatively higher WUE than sorghum when soil water conditions are not limiting. However, sorghum produces more dry matter than maize when soil water is deficient (Doorenbos et al., 1979). Crop demand for water is generally higher during germination, flowering, pollination and the early grain-filling stage, so these are often called 'critical growth' stages.

The land equivalent ratio (LER) has been developed for use in evaluating the yield advantage of intercropping systems, particularly under a replacement series. LER is defined as the area that a sole crop has to occupy in order to produce the same amount as its component in the intercrop (Mead and Willey, 1980; Baldy and Stigter, 1997). Baldy and Stigter (1997) stated that "it is the only method which permits an effective comparison of different yields from the same surface because each intercrop is compared with its sole stand". The LER compares the yield of each part of the intercrop to the yield of that same species grown alone as a sole crop, thus the advantage of the combination of species or intercrop becomes obvious.

The objective of this paper was to provide an overall view and evaluation of cereal-legume intercropping as compare to sole cropping in terms of productivity; radiation use efficiency (RUE), water use efficiency (WUE) and land equivalent ratios (LER), supported by a number of key examples from the published literature which point out its great value in the context of sustainable agriculture.

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## Materials and Methods

This paper is a review of the literature available and also forms a scoping study in preparation for detailed intercropping trials with underutilised crops, particularly legumes. Searches were performed in the library collection of books, reports, and proceedings of congresses. All efforts have been made to review articles and abstracts.

## Results and Discussion

Tsubo et al., (2001) reported that the radiation intercepted was higher in maize-bean intercropping than the sole crop. Tsubo and Walker (2003) found that intercropped bean and maize had 77 % higher RUE than sole – cropped beans. Keating and Carberry (1993) found that maize – soyabean intercropping has better use of solar radiation over monocrops. Other studies from outside sub-Saharan African region had proven the same results (Reddy et al., 1980; Ennin et al., 2002). For RUE the total system radiation interception can be determined by crop geometry and foliage architecture (Trenbath, 1983). Intercropping combinations of high and low canopy crops is usually to improve radiation interception and hence yields of the shorter crops, but it requires that they be planted between sufficiently wide rows of the taller crop (Seran and Brintha, 2010). Two factors that affects yield in relation to incident radiation in an intercropping system are the total amount of radiation intercepted and the efficiency with which intercepted radiation is converted to dry matter (Keating and Carberry, 1993). Therefore, in an evaluation of an intercropping system, one needs to be sure to measure the total radiation received above and below the canopy, to calculate that intercepted, as well as the biomass accumulation through the season.

When total LER values were higher than one, the advantage of intercropping over sole stands is shown, in regard to the use of environmental resources for plant growth and thus crop production (Mead and Willey, 1980). Similar results, of LER>1, were reported for mix-proportions of pea-barley (Chen et al., 2004), bean-wheat (Hauggaard-Nielsen et al., 2001), and maize-faba bean (Li et al., 1999) showing that the intercropping of a legume with a cereal is advantageous. The LER values from crop mixtures were significantly different with each other, having maximum land indices when two crops were grown in the same rows. It seems profitable on unit area basis to have the two crop combined in the same rows for most economical yields and the best usage of available land. These results provide documentary evidence of the superiority of intercropping over sole cropping by having maximum LER from 1.50 to 1.68 (Zada et al., 1988). Many other scientific articles have reported similar results with various combinations of crops, and under a range of environmental conditions and locations. For example, a LER from 1.2 to 1.5 (Curnard, 1976), 0.9 to 1.4 and 1.2 to 1.4 (Singh et al., 1973) have been reported from intercropping maize with soybean under various combinations.

The availability of water is one of the most important factors determining productivity in cereal- legumes intercropping systems. Improvements of WUE in these type of systems led to an increase in the uses of other natural resources (Hook and Gascho, 1988), and intercropping has been identified to conserve water largely because of early high leaf area index (i.e. initial fast leaf expansion) and higher overall combined leaf area of both crops (Ogindo and Walker, 2005). Garba and Renard (1991) reported that the continuous pearl millet/forage legumes system was the most efficient in terms of production and water use efficiency. Hulugalle and Lal (1986) found that WUE in a maize-cowpea intercrop was higher than in the sole crops, when soil water not limiting. It shows that intercropping results in improved WUE. However, this improvement could not be attributed to improved consumptive use as the values in the monocrops were not significantly different from those of the intercrops, meaning that WUE was not affected by crop interactions arising from intercropping. Rather, WUE improved due to high planting densities in the intercrops which resulted in higher biomass yield for the equivalent amount of water used. The advantage

of intercropping in terms of WUE occurs only when yields are treated on the basis of land equivalents (Trenbath, 1974).

**Conclusions**

Radiation use efficiency (RUE), water use efficiency (WUE) and land equivalent ratios (LER) indices show that intercropping is more efficient that sole cropping. For example, in maize-legume intercropping systems, the legume-based cropping systems were 32–49% more profitable than continuous sole maize under farming conditions in South Africa. These three indices must be calculated from complex measurements, as the biomass outputs from the systems are not all of identical composition, but have different protein and carbohydrate contents, therefore the energy values of each must be used when the combination of crops are compared. This demonstrates that using a combination of physiological, and quantitative approaches can be useful to assess the diversity of any essentially complex system and promote overall benefit to all by improving livelihoods and productivity of the water and land which are the basic natural resources in farming systems.

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# Corm Nursery Technique for Mass Propagation of Banana Seedlings of *Musa acuminata cv. Berangan*

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## Introduction

Banana is the fourth most important fruit crop in the world (Saha et al., 2010) and grown in 132 countries worldwide. It is believed to be originated from the Southeast Asia and it is an important crop in Malaysia. However, disease outbreak had caused the reduction of banana production. The diseases are transmitted from infected banana tree to the banana seedling such as banana suckers and it also can be transferred via pest. Banana suckers are the product of conventional propagation method and through this method it is very difficult to breed banana seedling with resistance to disease (Namuddu et al., 2013). Besides that, naturally regenerated suckers often harbored pest and diseases (Njau et al., 2011). Thus, a simple technique to produce free disease planting materials of banana is required.

Besides, the other constraint in the cultivation of banana is inconsistence supply of quality banana planting materials. Micro propagation technique also known as tissue culture technique is used to propagate disease-free planting materials (Kamaludin et al., 2012) and also as a tool for mass propagation of banana planting materials. However, tissue culture technique required expensive instruments and sophisticated technique, skill and cares to handle (Dayarani et al., 2013). In Malaysia, cultivators of banana are mainly from smallholder. Smallholder always cultivate banana in small scale. Normally, they get the banana seedling either from tissue culture seedling which is sometimes expensive and depends on accessibility.

Since there is no formal standards to regulate banana planting material (Macharia et al., 2010), the growers use conventional method to produce banana planting material as an alternative to reduce the cost of production rather than buying tissue culture banana seedlings. However, these suckers are prone to pest and disease attack. In conventional propagation there are two types of banana sucker being used; sword and water suckers. Sword suckers are recommended because it has better output yield (Robinson and Sauco, 2010). The problem with these conventional seedlings is its availability, where water sucker cannot be utilized because of low quality and sword sucker produced is not as much as tissue culture seedling. Thus, a simple and cheaper technique is needed to produce banana seedling and that is why corm nursery technique of banana was introduced.

Corm nursery technique is a macro propagation method of banana which will not only help the smallholder to cultivate the banana but they also can generate their income from producing banana seedling. Corm nursery technique of banana can provide the smallholder with both availability and accessibility of banana seedling. This technique can be installed in their farm and by performing this technique they can mass propagate banana seedling in faster period. This macro propagation technology can use whole suckers, large pieces of parent corms or sword suckers to produce planting material (Dayarani et al., 2013). In corm nursery technique, the material used is banana corm. This technique consists of several parts; corm preparation (corm-prep), corm seedling in polybag (corm-poly) and stacking of corm-poly (corm-stad). In each technology, selection is made to grade a better quality of banana planting material. In conventional propagation of banana, whole one sucker is used while giving high growth rate but produce less. In corm nursery technique, a whole one sucker's corm is slice to produce more section. Thus, the objectives of this experiment are to evaluate whether corm nursery

technique can mass propagate or not based on seedling emergence percentage and number of seedling produced, and to identify growth performance of new seedling from corm nursery technique based on plant height.

## Materials and Methods

*Study area*

The experiment was taken place at banana plantation of *Musa acuminate cv. Berangan* in Bukit Perawas, Ayer Lanas, Jeli, Kelantan. The plantation was actually integrated farm, where rubber tree is integrated with banana. This farm had been established since 2012 with an area of approximately six acres of land.

*Selection of corm*

The selection of banana corms started from the selection of farm, where banana corms were collected from the non-infected farm. Banana farms are normally infected by diseases such as black and yellow Sigatoka (Castelan et al., 2012) and Panama disease caused by *Fusarium oxysporum* (Borges et al., 2004), where these diseases are very dangerous and need to be avoided in order to produce high quality of banana planting materials. The symptoms of the diseases are clearly can be seen on the tree. Thus, healthy banana corms were selected from healthy banana trees or suckers without the symptoms of those diseases.

*Cleaning and sterilization of corm*

The selected banana corms were sterilized in order to kill microorganism, insect, and nematode. The corms were soaked into sodium hypochlorite (Chikezie, 2012) with 50% concentration for 15 min and were washed with plain water. After the sterilization process, the treated corms were exposed to the sunlight for drying process for one day.

*Corm nursery technique*

Corm Preparation (corm-prep)

Again the treated corms were graded according to size and then it was sliced horizontally from side of the corm. The treated corms then were layout on the bed with the mixture of coco peat and compost with the ratio of 3:1. Coco peat has good physical properties, high total pore space, high water content, low shrinkage, low bulk density and slow biodegradation (Evans et al., 1996; Prasad, 1997; Jadwiga, 2008). The bed with the established treated corms was then covered with polyethylene tarp (PE) which it create the dark environment and retain the humidity and heat of the bed for early bud induction (Robin, 2010).

Corm Seedling in Polybag (corm-poly)

Again banana seedling that grows from banana corm was selected to consider the uniformity. Banana seedlings were then transferred into 22 × 14 cm polybag at the 21 days after layout. The same media was used, the mixture of coco peat and compost were used.

*Propagation banana seedling through conventional method*

The sword sucker and water sucker were excised from the mother plant and the pseudostem was cut left out only corm of the suckers. The materials were then undergoing sanitization process to clean from soil

and pest. Treated corm of sword and water sucker were then layout on the bed same as corm nursery technique. But in the conventional technique of propagating banana, soil is used as a media.

*Maintenance of the corm*

The corms were let to grow for seven weeks and along the weeks the beds were watered evenly with one liter water once every two days and if it is not raining, it was watered every day.

*Experimental design*

There were two factors which are method of propagation (conventional method and corm nursery technique) and types of sucker (sword sucker and water sucker). These two factors were combined to be a factorial design with three replications for each treatment and were layout in a completely randomized design (CRD). Each sample was randomly layout on the bed by using random number table (New York: The Free Press, 1955).

*Data collection*

The plants were let to grow for seven weeks, prior to the emergence or production of new seedlings. Parameters that were taken are seedling emergence percentage, seedling height and number of seedling produced.

*Emergence rate and emergence percentage*

Emergence rate is how fast the new seedlings can emerge and emergence percentage is how many emergences occur out of 80 corms. This data is simple to collect only by counting the emergence of the new seedling for seven weeks.

*Measurement of seedling height*

The height of the new seedling was taken from the point at the above surface up to the point at the last intersection of two new leaves. Plant height was taken to see the growth rate of the plant and to determine the quality of the plant.

*Number of seedling produced*

Number of seedling were count every once a week in each factors or treatment to determine which treatment has the highest number of seedling.

*Data analysis*

Firstly, all of the data taken were test with Kolmogorov-Smirnov Z test to check the normality. The normal data then tested with ANOVA to see the different in each treatment and the significant data then further analyzed with post-hoc analysis. For the abnormal data also called non-parametric data of ANOVA were tested with non-parametric Kruskal-Wallis test (Rosner, 2000).

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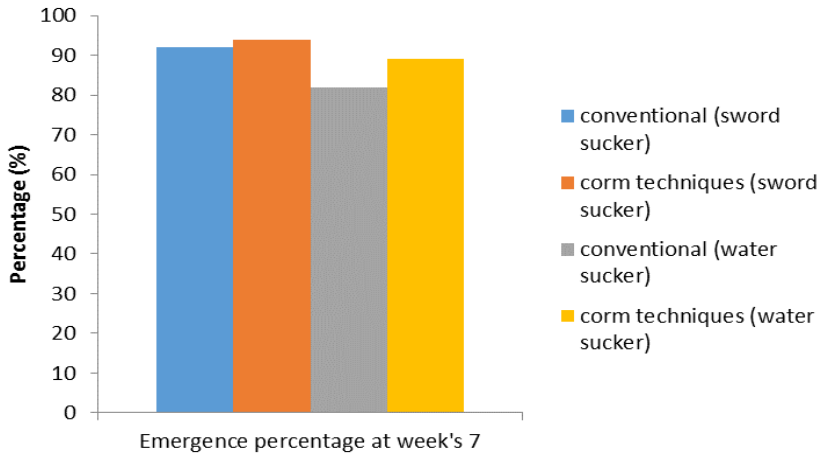
## Results and Discussion

*Percentage of seedling emergence*

Based on Figure 1, there are not much of differences between both methods. Test of normality found that the data were not normally distributed with Kolmogorov-Smirnov Z of 0.00. This suggested that the use of one-way analysis of variance or ANOVA was not appropriate to test the difference of mean emergence percentage among the four groups. Instead the Kruskal Wallis H test, a non-parametric test which does not require normality of the data, was used. The test resulted in a fairly small Chi-Square value of 2.40 which was not significant at 0.05 level of significance (p = 0.49). Therefore, this study concludes that the mean emergence percentage of the different methods of propagation with different sucker's corm type were similar in conventional method (sword sucker), conventional method (water sucker), corm techniques (sword sucker), and corm techniques (water sucker).

Both techniques having similar mean percentage of seedling emergence probably because Bayeri and Aba (2007) said that corm is a nutrient reserve and with the support information from Tropical Permaculture (2007), what can be tells is that corm has growing points and they turn into new suckers with the support from its nutrient reserved. However, there were slight different in types of sucker used, where sword sucker was slightly higher in terms of mean percentage of seedling emergence compared to water sucker based on the preliminary data, Figure 1. Thus, as mentioned by Robinson and Sauco (2010), the growth of sword sucker is better and faster than water sucker because it has strong physical and physiological connection with mother plant.




*Kruskal-Wallis, chi-square=2.40, Significant value=0.49*

Figure 1. The mean percentage of seedling emergence from different methods of propagation with different type of sucker's corms.

*Number of seedling*

Test of normality found that the data were not normally distributed with Kolmogorov-Smirnov Z of 0.00. This suggested that the use of one-way analysis of variance or ANOVA was not appropriate to test the difference of emergence percentage among the four groups. Instead the Kruskal Wallis H test, a non-parametric test which does not require normality of the data, was used. The test resulted in a fairly large Chi-Square value of 9.83 which was significant at 0.05 level of significance (p = 0.02).

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Therefore, this study concludes that the total seedling produced from different methods of propagation with different sucker's corm type were different in conventional method (sword sucker), conventional method (water sucker), corm techniques (sword sucker), corm techniques (water sucker). However, further analysis by using Duncan was used and it discovered that the difference only occurred among method of propagation used and there were no significant different among sucker types in both propagation method (Table 1).

Corm nursery technique has higher number of seedling produced compared to conventional. According to Namuddu et al. (2013), conventional method has low number of seedling is because this method is very difficult to breed banana seedling. Corm nursery technique can produce more seedlings because the corm was sliced the apical dominance of the corm was repressed and this is consistent with finding of Dayarani et al. (2013) about that this technique in which apical dominance is repressed to stimulate lateral bud development and increased suckering rate.

Table 1. The mean rank of seedling number produced in different propagation methods with different sucker types.

| Sources of Mean (Treatment) | Total Seedling Produced | Mean Rank of Seedling Produced |
|---|---|---|
| Conventional (Sword Sucker) | 39 | 48.63[a] |
| Conventional (Water Sucker) | 44 | 53.47[a] |
| Corm Technique (Sword Sucker) | 71 | 71.53[b] |
| Corm Technique (Water Sucker) | 66 | 68.37[b] |

*Kruskal-Wallis, chi-square=9.83, Significant value=0.02, a ≠ b*

*The height of seedling*

Preliminary analysis of the data revealed that an average of seedling height for the different methods of propagation with different sucker types are 19.11 cm, but the average of seedling height in each of the method used seemed to be different. Corm technique with sword sucker had the highest seedling height and conventional method with water sucker had the lowest (Figure 2). Therefore, one of the appropriate hypotheses in his study is that, there is a significant difference in the seedling height for the different methods of propagation with different sucker types. To test this hypothesis, the one-way ANOVA was used. ANOVA yield a significant result with F-ratio 12.97 and p value 0.00 which was significant at the 0.05 level. Therefore, it can be concluded that the different methods of propagation with different sucker types had different average of seedling height.

From the analysis of Post-hoc test by using Duncan test, there were no significant different in seedling height for both type of sucker's corm used in corm technique. However, height of seedlings that emerged in corm technique was varied significantly from the height of seedling grown under conventional method. In the conventional method, seedling height of sword sucker was significantly different from seedlings height of water sucker. Thus, in terms of mean seedling height, corm nursery technique was better than conventional methods. Kasyoka et al. (2010) studies showed that, conventional method of propagation is a slow process and quite often does not yield adequate suckers of the desired varieties, where the results of this experiment also described the process of conventional method in the same manners. Whereas, corm nursery technique has better performance and faster in process this is probably because of the propagation media.

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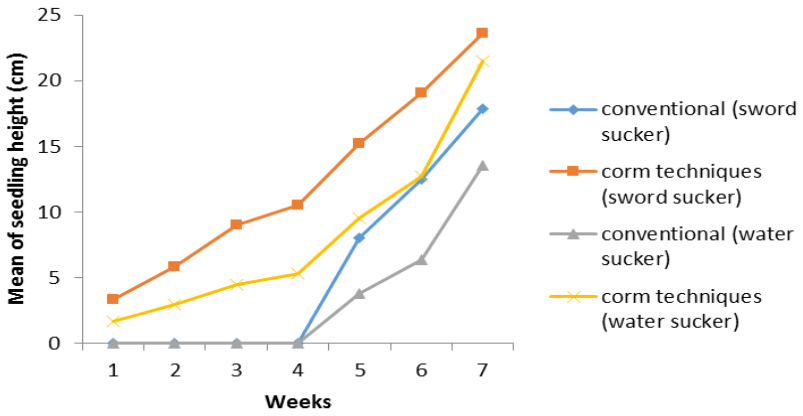


F-value= 12.969, P-value=0.000

Figure 2. The mean of seedling height from different propagation methods and different type of sucker's corm.

The initiation media used in the corm nursery technique was coco peat, as mentioned before by Jadwiga (2008), coco peat has good physical properties, high total pore space, high water content, low shrinkage, low bulk density and slow biodegradation and as reported by Bayeri and Aba (2005), the initiation media which served as anchorage, moisture supply source and helped in proper root aeration. Besides that, further study about this soilless media by Farzad et al. (2011), said that soilless media have proven popular with the majority of producers because of consistency, excellent aeration, reproducibility, and low bulk density. In contrast, the initiation media used in the conventional method was soil which naturally exists in Jeli area and this soil has less aeration for root development and drainage compared to coco peat. Soil is prone to compaction where Beattie and White (1992) and Jadwiga (2008) found that compacted or heavy soils without drainage and less aeration, suppressed the development of root and also increase the chances for the plant to be susceptible to soil borne diseases.

Table 2. The mean of seedling height among propagation methods with different sucker types.

| Sources of Mean (Treatment) | Mean of Seedling Height |
|---|---|
| Conventional (Sword Sucker) | $17.83^{a}$ |
| Conventional (Water Sucker) | $13.55^{b}$ |
| Corm Technique (Sword Sucker) | $23.59^{c}$ |
| Corm Technique (Water Sucker) | $21.45^{c}$ |

*F-value= 12.969, P-value=0.000, $a \neq b \neq c$*

## Conclusions

Corm nursery technique can help in mass propagate banana seedling of *Musa acuminata* cv. *Berangan*. Besides that, the growth of banana seedlings of corm nursery technique was higher than banana seedling from conventional method based on descriptive statistics. Not only banana of *Musa acuminata* cv. *Berangan*, this corm nursery technique can also be implemented in propagating other types of banana and plantain. But further study need to be done to improve the technique, so it can produce better quality of banana planting materials.

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# Effect of Ratios on pH and Selected Nutrient Contents in Compost Tea Produced from Agricultural Waste

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## Introduction

Compost tea or known in agriculture sectors as compost extract is fermented watery extract of compost materials produced from mixing matured composts with water. Compost tea can be prepared using a wide range of composts and nutrients extracted from this process may supply organic matter to the soil, improving soil structure and water holding capacity by improving soil aggregates (Shrestha et al., 2011). Utilization of compost tea improved soil quality because they can alter the chemical and physical properties of soil. Compost tea may improved water holding capacity and organic matter content, microbial population, supply micro and macro nutrient concentration that are necessary in promoting high crop yield. Compost tea can also be used in fertigation system. Cultivation of muskmelon (*Cucumis melo* L.) under fertigation system using compost tea had enhances growth, yield, and quality (Naidu et al., 2013). As an inorganic fertilizer NPK mixed with compost tea at 50:50, the vegetative growth, yield, and antioxidant content in the *Centella asiatica* L. increased (Siddiqui et al., 2011). Compost tea can be used as an alternative source in substituting the chemical fungicides (Siddiqui et al., 2009). Although compost tea has many functions in soil, plant and ecosystem as a whole, limited study can be found in relation to ratios and quality of compost tea (Archana et al., 2012). Thus, a study was conducted in the laboratory to identify the effect of ratio on pH and nutrients content in the compost tea.

## Materials and Methods

Chicken manure was collected from poultry unit, University Agriculture Park at Universiti Putra Malaysia Bintulu Campus (UPMKB) while sawdust was collected from Ling Brothers Sdn. Bhd. at Bintulu City. The dried sawdust and $CaCO_3$ were weighed and mixed thoroughly with the chicken manure slurry in the polystyrene box. The preparation of chicken manure slurry was carried out by dissolving the chicken manure in washing machine wastewater and then it was poured into the polystyrene box. All the materials were mixed thoroughly and the moisture in the compost pile was maintained at the optimum level which was between 60 to 70%. The composting was conducted in polystyrene box size of 38 cm x 36 cm x 32 cm. The composting experiment was conducted at Research Complex in Universiti Putra Malaysia Bintulu Campus. The initial temperature of the compost was monitored 3 times per day (morning, afternoon and evening) until the temperature reached ambient temperature. Mature compost was air-dried to remove the moisture content and pH and nutrients concentration in the compost were analyzed. Compost tea was prepared by mixing washing machine disposal water with mature compost. Washing machine wastewater was firstly collected from the hostel laundry in Sri Rajang College. About 5 g of mature compost was weighed and placed into the tea bag. Then they were placed into the incubation bottle for compost tea extraction purposes. Different amount of washing machine disposal water in different volume was then poured into the incubation bottle and the mixture was leaved for 30 days for incubation. Compost tea was filtered to remove the sediments and nutrient concentrations in compost tea were analyzed for pH, $NH_4^+$, $NO_3$ and P. Total $NH_4^+$ and $NO_3$ were analyzed using distillation method and P concentration was analyzed using Blue Development Method. The pH of compost tea was analyzed by using Mettler Toledo pH meter and other nutrient concentrations were analyzed using Atomic Absorption Spectroscopy (AAS).

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**Results and Discussion**

The characteristics of chicken dung and sawdust compost are presented in Table 1. The produced compost was alkaline in pH (8.45). This indicates that the produced composts are well aerated because well aerated compost pile generally produced high pH compost (Iglesias-Jimenez and Perez-Garcia, 1992). Compost tea prepared from chicken dung and sawdust at 1:1 ratio was significantly affect the pH, $NO_3$ , $NH_4^+$, P, K, Ca, Cu and Zn contents (Table 3 and Figure 1) as different ratios used for the extraction of compost tea (1:10, 1:20, 1:30, 1:40 and 1:50). Ratio of 1:10 was the best ratio to maximize the amount of $NH_4^+$, $NO_3^-$, P, K, Ca, Cu and Zn contents. This might be due to the present of high concentration of humic acid in the compost tea at 1:10 ratio.

The pH of the compost tea ranges from 8.80 to 9.96 (Table 3). The increasing volume of the washing machine disposal water during extracting the compost not significantly affects the pH value. The compost tea showed highest pH at 1:10 ratio (9.66) followed by 1:50 (9.25) (Table 3). This could be due to high amount of compost used during the extraction of compost tea or might be caused by the dissolution of organic acid in the compost. As reported by Stephen (1996), alkaline solution might have neutralization effect on the organic acids present in the compost extract. Alkaline solution and organic acids in compost might react together and produced water and ionic compound which affect the pH value.

The concentration of $NO_3$ , $NH_4^+$, P, K, Ca, and Mg in the compost tea was significantly affected by the volume of the washing machine disposal water added during extraction process. The nutrients present in the washing machine disposal water might contribute to the nutrient concentration in the compost tea. The characteristics of the washing machine disposal water are presented in Table 2. High concentration of Na and surfactants in soap powders used in the laundry has make the compost extract pH to be increased (Dixon et al., 1999). Amount of $NH_4^+$ extracted from the compost was slightly decreased at 1:40 (144.71 mg/L) and 1:50 (147.08 mg/L) ratios as compared to other ratio (Figure 1). In order to maximize the amount of $N0_3$ , P, K, Ca and Zn, the compost tea required 1:10 ratio (Table 3). In contrast, 1:30 was required by the compost to extract the maximum amount of Mg while Na required 1:50 ratio (Table 3). Ratio of 1:40 and 1:50 was required by the compost to extract the maximum amount of Cu (Table 3).

Table 1. Selected characteristics of chicken dung and sawdust compost (1:1 ratio).

| Parameters | Value |
|---|---|
| pH | 8.45 |
| N (%) | 1.26 |
| P (mg/kg) | 3.47 |
| K (mg/kg) | 3783.96 |
| Ca (mg/kg) | 24825.48 |
| Mg (mg/kg) | 1174.08 |
| Na (mg/kg) | 2949.66 |

Table 2. Selected characteristics of washing machine wastewater.

| Parameters | Concentration | Standard Concentration (provided by other researchers) |
|---|---|---|
| pH | 8.78 | 8.3-9.3 |
| Total N (mg/L) | Nd | 14.28 |
| Total P (mg/L) | 99.9 | 51.58 |
| Ca (mg/L) | 385.83 | 18.7-24 |
| Mg (mg/L) | 41.97 | 15.1-60.8 |
| Na (mg/L) | 228633 | 302.1-667 |
| Cu (mg/L) | Nd | 0.0064-0.01 |
| Zn (mg/L) | Nd | 0.14 |

*Source: Dilip and Kunwar (2013); Jamrah et al. (2011); Jamrah et al. (2008); Kotut et al. (2011); Prataphar et al. (2005); Friedler (2004); Jeffersen et al. (2004); Nd- Not determined*

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Table 3. Effect of ratio on pH and selected nutrient concentration in compost tea.

| Ratio | pH | P (mg/L) | K (mg/L) | Ca (mg/L) | Mg (mg/L) | Na (mg/L) | Zn (mg/L) | Cu (mg/L) |
|---|---|---|---|---|---|---|---|---|
| 1:10 | 9.66$^{a}$ | 103$^{a}$ | 295.00$^{a}$ | 374.50$^{a}$ | 49.00$^{ab}$ | 253.50$^{b}$ | 4.08$^{a}$ | 8.27$^{ab}$ |
| 1:20 | 9.11$^{a}$ | 63.5$^{b}$ | 228.50$^{b}$ | 292.00$^{bc}$ | 42.33$^{bc}$ | 250.00$^{b}$ | 3.62$^{ab}$ | 7.93$^{b}$ |
| 1:30 | 8.97$^{a}$ | 53.0$^{d}$ | 277.50$^{a}$ | 317.50$^{b}$ | 52.50$^{a}$ | 237.50$^{b}$ | 3.35$^{ab}$ | 8.53$^{ab}$ |
| 1:40 | 8.80$^{a}$ | 70.0$^{bc}$ | 224.00$^{b}$ | 297.00$^{bc}$ | 37.33$^{bc}$ | 233.50$^{b}$ | 2.90$^{b}$ | 8.97$^{a}$ |
| 1:50 | 9.25$^{a}$ | 71.7$^{b}$ | 240.00$^{b}$ | 282.00$^{c}$ | 32.50$^{c}$ | 303.00$^{a}$ | 3.75$^{a}$ | 8.90$^{a}$ |

*Note: Means with the different letter in column were not significantly different using Tukey Studentized Range Test at p≤0.05*

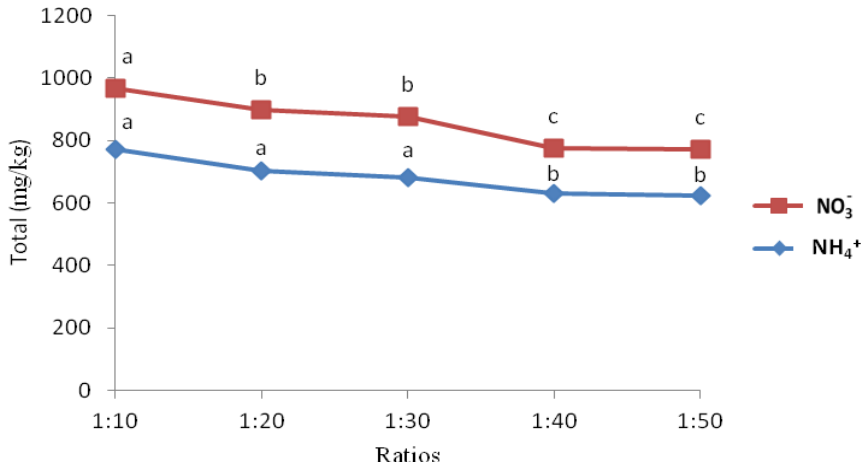


Figure 1 Effect of ratio on $NH_4^+$ and $NO_3^-$ content in compost tea

## Conclusions

Different ratio used during the extraction process affect the characteristics of compost tea. The compost required specific ratio to enhance their pH and nutrients concentration in the compost tea.

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# Effect of Using Leguminous Cover Crop (*Calopogonium mucunoides* Desv.) on Leaf N, Chlorophyll Content and Gas Exchange Rate of Black Pepper (*Piper nigrum* L.)

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## Introduction

*Piper nigrum* Linn.is one of the most common condiments and features prominently in most of the gastronomical cuisines worldwide. Being known as a tropical climbing vine and a member of the family Piperaceae, the genus piper has more than 1000 species but the most economically important species is *P. nigrum* (Shanmugapriya et al., 2012). Pepper cultivation in the Sarawak state of Malaysia dates back to 1856 but more extensive planting started in the 1900s. Overall, the state has produced 10,588 tonnes of pepper valued at RM245 million in the year 2012 thus highlighting the importance of pepper as one of the important cash crops supporting the livelihood of about 67,000 rural dwellers in upland areas of Sarawak (Malaysian Pepper Board, 2013). Currently, Malaysia ranks number five after Vietnam, India, Indonesia, and Brazil in terms of pepper production.

Calopo (*Calopogonium mucunoides* Desv.) is indigenous to tropical America and the West Indies but it is wide-spread in the tropics of Asia and Africa through introduction in the early 1900s. Pizarro (2002) reported that it is the most popular legume amongst Brazilian farmers and it is the legume seed produced in greatest volume in Brazil. As a legume, the plant helps to improve soil fertility with its nitrogen-fixing bacteria found in the root nodules.

Previously, there is no knowledge on how a legume cover crop particularly *C. mucunoides* affects the various physiological characteristics of *P. nigrum*. Furthermore, the documentation on *C. mucunoides* usage in black pepper vineyards in Sarawak is still lacking, which needs attention if cultivation of leguminous cover crops were to be undertaken seriously. Therefore, this study was conducted with the following objectives: (i) to investigate the leaf total N and chlorophyll content of *P. nigrum* grown in a *C. mucunoides* establishment, (ii) to investigate the gas exchange rate of *P. nigrum* grownin a *C. mucunoides* establishment, and (iii) to examine the role of certain soil properties in influencing leaf total N, chlorophyll content and gas exchange rate of *P. nigrum*.

## Materials and Methods

The experiment was a field study and conducted in a 12 m x 12 m plot near Kampung Jagoi, Duyoh, Bau, Sarawak, Malaysia. The soil texture at planting site was sandy clay loam which has a low to moderate permeability status. The crops involved in this study were *P. nigrum* and *C. mucunoides*. *Piper nigrum* var. Kuching cuttings were rooted in a sand bed. After 4 weeks, the pepper cuttings were then selected and transplanted to the planting site.

In order to break the dormancy of the Calopo cover crop seeds, scarification with concentrated sulphuric acid for 30 minutes was done. After scarification, the seeds were germinated on moist cotton for 3 days. The seeds were then transplanted to the planting site by dibbling 3 – 4 seeds into a hole. Calopo seeds were planted in holes placed 20 cm apart within each row. The study was conducted from the month of August 2013 – June 2014.

*Experimental design and treatments*

The experiment was a randomized complete block design (RCBD) with two treatments replicated nine times. Treatments were: (i) control (C0) – *P. nigrum* plot without application of *C. mucunoides* and (ii) Calopo cover crop (C1) –*P. nigrum* plot with application of *C. mucunoides*. Pepper vines were carefully selected to avoid erroneous result due to the nature of most part of the terrain being flat to slightly undulating.

*Selected soil properties determination*

After 10 months, the soil was analyzed for its water content and texture. Soil moisture content was measured by using a soil moisture sensor equipment (WS SMEC 300, Spectrum Technologies, Chicago, Illinois, USA) to obtain the soil volumetric water content (VWC) (% volume).

In order to further understand the fertility status of the soil, samples were collected with an auger at a depth of 0 – 15 cm and analyzed for its total organic carbon (TOC), total nitrogen (N) and available phosphorus (P).The sieved soil samples were analyzed for its chemical properties at Sarawak Plantation Chemistry Laboratory Kuching, Sarawak.

*Leaf total nitrogen, chlorophyll and gas exchange rate measurement of P. nigrum*

At the end of the study, 0.1 g of leaf samples was collected for the purpose of this experiment. The samples were washed clean with distilled water, oven-dried at 60 $^{o}$C and grounded. A kitchen blender (Takada Food Blender, ISB-035) was used to ground the dried leaf samples.The grounded leaf samples were analyzed for total N at Sarawak Plantation Chemistry Laboratory Kuching, Sarawak. The total N for black pepper leaves was determined using the micro-Kjeldahl method (Tan, 1995). Value is recorded as % N for plant tissue on a dry weight basis.

The relative chlorophyll content of leaves was determined by using the SPAD-502 chlorophyll meter (Konica Minolta Co. Ltd., Japan). Readings were recorded when mature fully expanded leaves with the same orientation and the same layer in the crown (middle bottom) are still attached to the tree.

Gas exchange measurement was determined according to the method by DiCristina and Germino (2006), carried out on young fully expanded leaves with the same orientation and the same layer in the crown (middle bottom). Measurements of net photosynthesis on an area basis (*A*) (µmol $CO_2$ $m^{-2}s^{-1}$), leaf stomatal conductance (*g*s) (mol $H_2O$ $m^{-2}s^{-1}$), and transpiration rate (*E*) (mmol $H_2O$ $m^{-2}s^{-1}$) of nine different leaves per treatment were monitored using a photosynthesis system (LI-6400, LICOR, USA) of infrared gas analyzer (IRGA). Light intensity (Photosynthetically active radiation, PAR) within the sampling chamber was set as close to outside PAR at 1500 µmol $m^{-2}s^{-1}$. The $CO_2$ flow into the chamber was maintained at a concentration of 400 µmol $mol^{-1}$. The humidity flow into the chamber was fixed at 500 µmol $s^{-1}$. Statistical assessment was done on gas exchange parameters at between 1100 to 1200 h, which was presumed to be the diurnal period when photosynthetic rates would be maximal.

*Statistical analysis*

Data was analyzed statistically by using an independent and paired t-test to detect the treatment effect. The statistical software used was the SPSS. The relationship between leaf chlorophyll content and leaf total N of *P. nigrum* and photosynthesis and leaf chlorophyll content were correlated using regression of best fit.

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**Results and Discussion**

*Selected soil properties*

Table 1 shows the effect of *C. mucunoides* on soil volumetric water content (VWC). The result indicates that soil VWC in C1 was 40 % higher than that of C0. This result concurs with a report by Hoorman (2009) which mentioned an increased in stored soil moisture was seen in cover crop treatments compared to standard no-till and conventional till treatments after the first major rainfall event after planting. Hoorman (2009) added that deep rooted cover crop such as *C. mucunoides* can improve rooting depth to attain subsoil moisture and water content is conserved by mulching the topsoil as soil compaction decreases and soil quality improves with time. Research by Jasa (2011) has shown that while a cover crop uses some soil moisture as it grows, it tends to use less water than is lost to evaporation from a bare soil surface.

Total organic carbon (TOC) increased significantly in a *C. mucunoides* establishment (Table 1). This may be due to addition of organic matter from cover crop biomass decomposition process. A similar study done by Ngome et al. (2011) observed that cover crop are known to increase soil organic carbon content by expanding biomass production for restoration and maintenance of soil productivity.

The results in Table 1 also show that the usage of *C. mucunoides* has increased both total N and available P in the soil (Table 1). The increased in soil total N indicates that it is most probably due to the capture of atmospheric $N_2$ by nitrogen-fixing bacteria found in the root nodules of *C. mucunoides* into the soil. When a legume cover crop is incorporated into the soil, a substantial amount of nitrogen is usually mineralized, converted from organic to plant-available forms within a few weeks (Ngome et al., 2011). Meanwhile, The increased in soil available P could be partly due to the presence of beneficial fungi known as mycorrhizae housed by the roots of leguminous cover crops which accumulates P. The filaments (hyphae) of these fungi effectively extend the root systems and help the cover crop tap more soil P (Ngome et al., 2011). Parameters such as soil nitrogen (N), total organic carbon (TOC) and available soil phosphorus (P) are major indicators of the productivity and sustainability of an agricultural production system (Kifuko et al., 2007).

Table 1. Effect of *C. mucunoides* on VWC, TOC, Total N and Available P in the soil

| Treatment | VWC (%) | TOC (% oven dry) | Total N (% oven dry) | Available P (ppm oven dry) |
|---|---|---|---|---|
| C0 | $19.81 + 0.90^a$ | $2.57 \pm 0.09^a$ | $0.26 \pm 0.02^a$ | $4.78 \pm 0.67^a$ |
| C1 | $32.89 + 0.73^b$ | $3.82 \pm 0.11^b$ | $0.44 \pm 0.03^b$ | $7.11 \pm 1.05^b$ |

*Note: Means with different alphabets within column indicate significant difference between treatments by independent t-test at $p < 0.05$. Treatments are C0 – control and C1 – Calopo cover crop.*

*Leaf total nitrogen, chlorophyll and gas exchange rate*

Table 2 shows that C1 had a significantly higher leaf total N than those in C0 which could be due to higher availability of N in the soil. Both field and laboratory investigations by Cechin and Fumis (2004) have demonstrated that increasing supply of N availability in soil may result in higher leaf N content. Furthermore, Tucker (2004) hypothesized that total N content in leaves depend on the N content in the soil.

Chlorophyll (SPAD) content increased by 17 % in the *C. mucunoides* treatment when compared to the control which is attributed primarily to higher N concentration and the presence of cytoplasmic fluid at the leaf cellular level (Table 2). Cabrera (2004) and Sulok et al. (2012a) observed that plants with higher nitrogen content and presence of sufficient fluid tend to have darker green leaves.

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Photosynthesis rate (*A*) increased by 27 % in the C1 treatment suggesting that *C. mucunoides* application increased the selected gas exchange rate of *Piper nigrum*. Lawlor (2002) mentioned that the photosynthesis process that leads to increase in reproductive growth and yield is totally dependent upon the adequate supply of nitrogen and soil moisture. Hak et al. (1993) reported that up to 75% of leaf nitrogen is found in the chloroplasts, most of it invested in ribulose-biphosphate carboxylase alone. The process of photosynthesis takes place in the chloroplasts, specifically using chlorophyll, the green pigment involved in photosynthesis.

Table 2 shows that leaf stomatal conductance (*gs*) increased by 44 % in the *C. Mucunoides* treatment when compared to that of the control. The result was parallel to that of Sulok et al. (2012b) where plant subjected to higher soil VWC tends to open its stomata as the soil water potential was at its field capacity.Table 2 shows that transpiration rates (*E*) were higher for black pepper vines subjected to *C. Mucunoides* treatment, increasing by 55 % of the control value. Result for leaf transpiration rates (*E*) concurs to that of Ashizawa et al. (2003) in which they concluded that *E* rate was progressively increased under conditions of sufficient soil moisture. Additionally, Hoorman (2009) reported that due to stored soil moisture in some cover crop treatments, the soil is able to provide adequate supply of water to plants thus negating the effects of lower water availability that inhibits stomatal openings thus led to lower *E* and *A* rates.

Table 2. Effect of *C. mucunoides* on leaf total N, Chlorophyll and selected gas exchange rate of *Piper nigrum*

| Treatment | Leaf Total N (%) | Chlorophyll (SPAD) | Net Photosynthesis Rate (µmol $CO_2$ $m^{-2}s^{-1}$) | Leaf Stomatal Conductance (mol $H_2O$ $m^{-2}s^{-1}$) | Transpiration rate (mmol $H_2O$ $m^{-2}s^{-1}$) |
|---|---|---|---|---|---|
| C0 | $1.63 \pm 0.06^a$ | $33.87 \pm 3.82^a$ | $10.47 \pm 0.65^a$ | $0.09 \pm 0.01^a$ | $1.55 \pm 0.1^a$ |
| C1 | $2.43 \pm 0.12^b$ | $40.60 \pm 2.28^b$ | $14.4 \pm 1.4^b$ | $0.16 \pm 0.01^b$ | $3.41 \pm 0.17^b$ |

*Note: Means with different alphabets within column indicate significant difference between treatments by independent t-test at p< 0.05.Treatments are C0 – control and C1 – Calopo cover crop.*

Figure 1 shows the relationship between chlorophyll (SPAD) and leaf total N of *P. nigrum*. The strong relationship between chlorophyll and leaf total N regardless of treatments shows a polynomial cubic regression line of zero intercept with $r^2 = 0.88$ indicating that higher N content in the leaf increased chlorophyll in plants. Previous report by Tucker (2004) and Daughtry et al. (2000) revealed that because N is a structural element of chlorophyll, thereby it affects formation of chloroplasts and accumulation of chlorophyll in them.

Similarly, the relationship between leaf photosynthetic rate (*A*) and chlorophyll (SPAD) of *P. nigrum* exposed to different treatments were highly correlated, $r^2 = 0.91$ (Figure 2). The relationship between the two regardless of treatments was best described by a polynomial cubic regression line of zero intercept which explains a value of around 91% of the variation in leaf photosynthesis. This consequence showed close relations between the two in which leaf *A*rate increased with increasing chlorophyll (SPAD) content. Ndukwe et al. (2011) revealed that the concentration of chlorophyll affects the rate of photosynthesis as they absorb the light energy without which the reactions cannot proceed. Consequently, higher rates of photosynthesis under conditions of higher nitrogen and soil water availability are often attributed to the formation of chlorophyll photosynthetic pigments and Rubisco activity (Toth et al., 2002).

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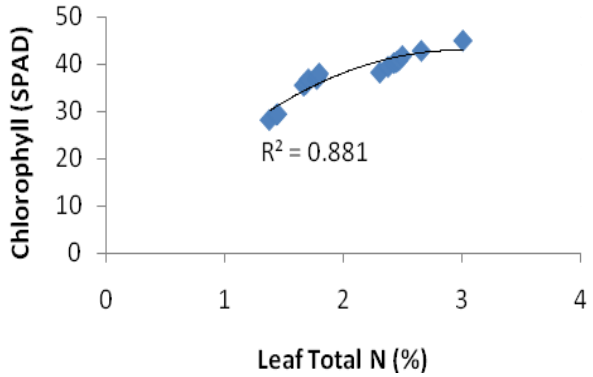


Figure 1: Relationship between chlorophyll (SPAD) and leaf total N of *P. nigrum* subjected to different treatments. Values are means ±s.e. of nine leaves taken from different plants per treatment. The regression line (continuous) is shown. The values of the determination coefficient are included.

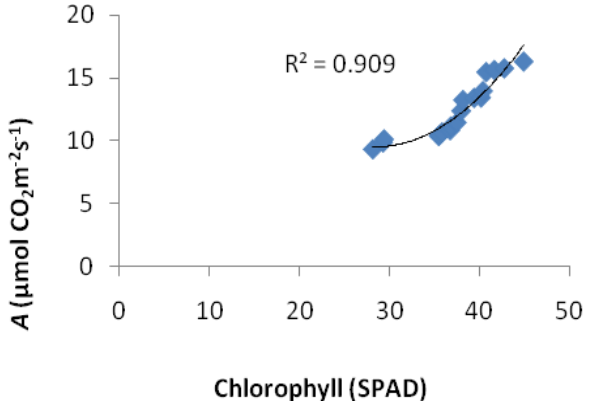


Figure 2: Relationship between leaf *A*rate and chlorophyll (SPAD) of *P. nigrum*subjected to different treatments. Values are means ±s.e. of nine leaves taken from different plants per treatment. The regression line (continuous) is shown. The values of the determination coefficient are included.

## Conclusions

The establishment of the C1 plot using *C. mucunoides* as cover crop responded better in terms of its improvement in soil properties as well as leaf total N, chlorophyll content and gas exchange rate of *P. nigrum*. In the C1 treatment, the application of *C. mucunoides* affects the selected soil properties considerably by showing comparatively highersoil VWC, soil TOC, soil total N and soil available P. Leaf total N, chlorophyll content, photosynthesis rates, stomatal conductance, and transpiration rates of *P. nigrum* grown in the Calopo cover crop treatment were significantly higher than the control. Furthermore, it was found that leaf total N and photosynthesis rate was significantly correlated to chlorophyll content of *P. nigrum*. It can be said that the use of leguminous cover crops such as *C. mucunoides* can bring benefits such as fixing nitrogen, improving soil properties, managing soil moisture and increasing photosynthesis of *P. nigrum.*

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# Improving Rice (*Oryza sativa* L. cv. MR219) Growth, Nutrient Uptake and Recovery Using Clinoptilolite Zeolite

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## Introduction

Nitrogenous fertilizers such as urea in rice fields (under waterlogged condition) are lost through ammonia volatilization. Ammonia loss is higher in waterlogged or anaerobic condition compared to aerobic condition (Zhengping et al., 1991). Besides N fertilizers, phosphorus and potassium fertilizers tend to leach through surface run-off due to high average annual rainfall of 2,500 mm in Malaysia (Huey and Ibrahim, 2012). These problems lead to less availability of nutrients for rice growth and development. Thus, farmers apply fertilizers excessively in rice fields to attain higher yield. However, unbalanced use of fertilizers cause eutrophication, groundwater pollution, acid rain deposition, soil acidification and greenhouse gas emissions (Tang et al., 2008; Adesemoye and Kloepper, 2009; Ju et al., 2009; Moose and Below, 2009).

Ammonia loss and nutrient leaching from fertilizers can be reduced with application of materials which are high in CEC such as Clinoptilolite zeolite (Sommer et al., 2006; Latifah et al., 2011). Clinoptilolite zeolite was mixed with sago waste water and peat water to reduce N loss from urea ( Omar et al., 2010; Latifah et al., 2011). Amending fertilizers with Clinoptilolite zeolite creates a pool of negative charges around nutrients. With the use of Clinoptilolite zeolite, ammonium and potassium ions will also be efficiently retained and released in a timely manner to ensure optimum plant use. Proper retention and release of ammonium ions by Clinoptilolite zeolite during hydrolysis of urea, as an example, may significantly reduce nitrogen loss through ammonia volatilization, hence improving nutrient uptake, nutrient recovery and increase rice plant growth. Amending acid soils and fertilizers with Clinoptilolite zeolite could reduce N loss from urea, nutrient leaching as well as improve rice plants' growth, nutrient uptake, nutrient recovery and selected chemical properties of acid soils. A pot study was carried out to determine the effects of amending an acid soil and fertilizers with Clinoptilolite zeolite on rice plants' growth, nutrient uptake and recovery using Clinoptilolite zeolite.

## Materials and Methods

Typic Paleudults (Bekenu series) soil was sampled at 0 to 25 cm in an undisturbed area of Universiti Putra Malaysia Bintulu Sarawak Campus, Malaysia. The soil was air-dried, crushed, and sieved to pass to a 5 mm sieve. The Clinoptilolite zeolite and soil were analyzed before and at the end of pot study for pH, bulk density, total carbon, total N, available $NO_3^-$, exchangeable $NH_4^+$, exchangeable cations, available P and CEC using standard procedures.

The selected chemical and physical properties of the soil (Table 1) used in this study were typical of Typic Paleudults (Bekenu series) and they were comparable to those reported by Paramananthan (2000), except for CEC. The pH of Clinoptilolite zeolite was higher as expected, but the CEC and total N content of the Clinoptilolite zeolite were lower than expected (Table 2).

The quantity of the soil used in this pot study was calculated based on its bulk density. About 1 kg of air-dried soil was filled in a pot measuring 12.5 cm (top diameter) × 10 cm (bottom diameter) × 9 cm (height). Before planting, rice seeds variety of MR219 were germinated in a plastic ware filled with germination medium (85% sand + 15% Clinoptilolite zeolite). At 7 days after germination, the

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seedlings were transferred into pots (3 seedlings per pot) (Bozorgi et al., 2011) and submerged in tap water. The pot trial was carried out in Completely Randomized Design (CRD) with 4 replications.

Table 1. Selected chemical and physical properties of Typic Paleudults (Bekenu Series) soil before incubation and pot study.

| Property | Soil | Standard Data Range* (0-36 cm) |
|---|---|---|
| $pH_{water}$ | 4.41 | 4.6-4.9 |
| CEC (Cmol(+) $kg^{-1}$) | 11.97 | 3.86-8.46 |
| Total Carbon (%) | 2.43 | 0.57-2.51 |
| Total N (%) | 0.08 | 0.04-0.17 |
| Exchangeable $NH_4^+$ (mg $kg^{-1}$) | 21.02 | Nd |
| Available $NO_3^-$ (mg $kg^{-1}$) | 7.01 | Nd |
| Available P (mg $kg^{-1}$) | 4.85 | Nd |
| Exchangeable $K^+$ (Cmol(+) $kg^{-1}$) | 0.10 | 0.05-0.19 |
| Bulk density (g $cm^{-3}$) | 1.16 | Nd |

*CEC: Cation Exchange Capacity; *Paramananthan (2000)*

Table 2. Selected chemical properties of Clinoptilolite zeolite.

| Property | Clinoptilolite Zeolite |
|---|---|
| $pH_{water}$ | 8.20 |
| CEC (Cmol(+) $kg^{-1}$) | 71.30 |
| Total N (%) | 0.22 |
| Total P (%) | 0.01 |
| Total K (%) | 0.37 |
| Total Ca (%) | 0.67 |
| Total Mg (%) | 0.10 |
| Total Na (%) | 0.76 |

The treatments evaluated were: T1: soil alone, T2: soil + complete fertilization, T3: soil + complete fertilization + 20 g zeolite, T4: soil + complete fertilization + 40 g zeolite and T5: soil + complete fertilization + 60 g zeolite. The complete fertilization was equivalent to 1.31 g urea + 1.39 g ERP + 0.88 g MOP + 0.16 g kieserite + 0.53 g chelated Zncobor per pot. The amount of fertilizers used was a scaled down for plant density of 3 rice plants $hill^{-1}$. The fertilizer rate (151 kg $ha^{-1}$ N, 97.8 kg $ha^{-1}$ $P_2O_5$, 130 kg $ha^{-1}$ $K_2O$, 7.6 kg $ha^{-1}$ MgO) was based on the recommended fertilizer for rice by Muda Agricultural Development Authority, Malaysia, with additional micronutrients (2.3 kg $ha^{-1}$ B, 4 kg $ha^{-1}$ Cu and 4 kg $ha^{-1}$ Zn) (Liew et al., 2010). The amount of zeolite used was deduced from the literature (Kavoosi, 2007; Bernardi et al., 2009; Gevrek et al., 2009; Sepaskhah and Barzegar, 2010), where 5, 10 and 15 tons $ha^{-1}$, equivalent to 20, 40 and 60 g $hill^{-1}$, were used.

Plant height, number of tillers and number of leaves were recorded at harvest (90 days after seeding). The rice plants were oven-dried at 60 °C until a constant weight was obtained. The dried plant samples were ground using a grinding machine, after which they were analysed for total N, P, K, and crude silica using standard procedures. Plant nutrient recovery was calculated using the method of Dobermann (2005).

Analysis of variance (ANOVA) was used to detect significant differences among treatments, whereas Tukey's HSD test was used to compare treatment means using Statistical Analysis System version 9.2 (SAS, 2008).

**Results and Discussion**

The effects of treatments on number of leaves, tillers, plant height, and total biomass production at 90 days after seeding (DAS) are shown in Figure 1. Application of Clinoptilolite zeolite (T4 and T5) significantly increased number of leaves and total dry matter production compared with normal

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fertilization (T2). However, the application of Clinoptilolite zeolite showed no significant effect on number of tillers and plant height.

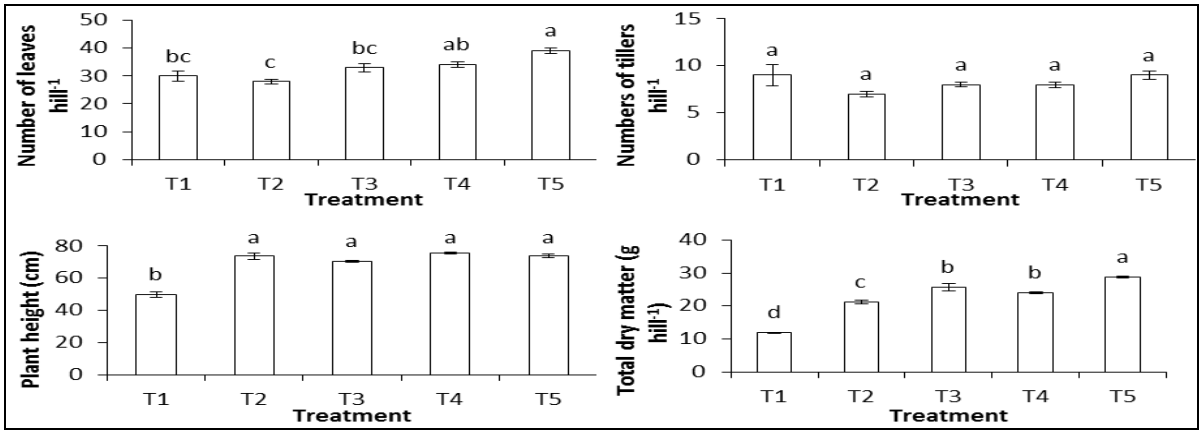


Figure 1. Treatment effects on number of leaves, tillers, plant height and total biomass production of rice plant at 90 DAS. Different alphabets indicate significant difference between means using Tukey's test at $P \leq 0.05$.

The highest amount of Clinoptilolite zeolite (T5) improved nutrient uptake (N, P, K and crude silica) and recovery (N, P and K) compared with the normal fertilization (T2) (Figures 2 and 3). T3 and T4 showed higher N and crude Si uptake and N recovery compared with the normal fertilization. Sepaskhah and Barzegar (2010) also found that nutrient use efficiency and nutrient recovery efficieny in rice plants were increased with increasing rate of zeolite application. The higher cation exchange capacity of Clinoptilolite zeolite enabled nutrients retention from leaching. Thus, the nutrients were timely absorbed to support growth of the rice plants as plant dry matter production and leaf number of rice plant was higher.

T4 and T5 significantly increased soil pH and CEC compared with the normal fertilization (T2) (Figure 4). However, total N, available P and exchangeable K decreased with increasing rate of Clinoptilolite zeolite compared with the normal fertilization. Reduction of nutrients in the soil was because of efficient nutrient uptake in rice plants. The basic nature and higher CEC of the Clinoptilolite zeolite played important role in increasing soil pH and CEC (Kavoosi, 2007).

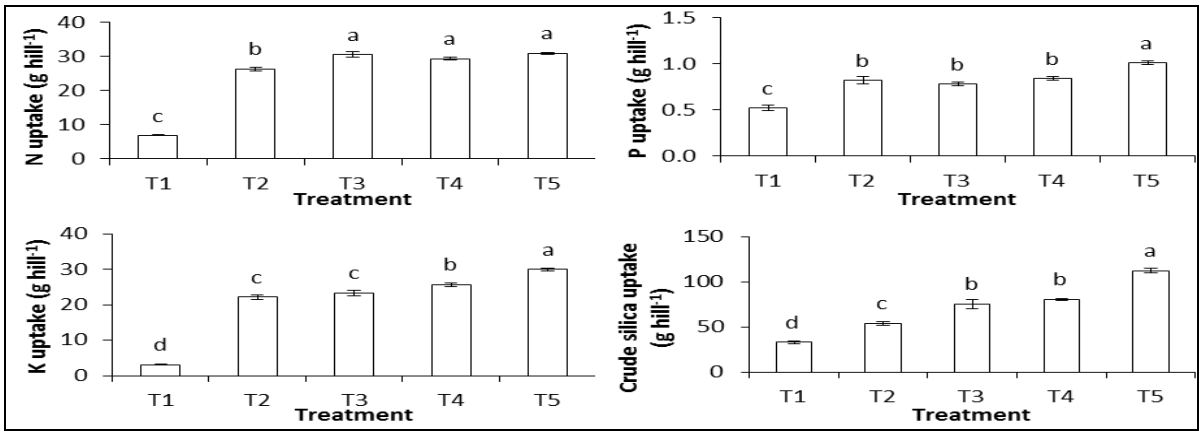


Figure 2. Treatment effects on N, P, K and crude silica uptake of rice plant at 90 DAS. Different alphabets indicate significant difference between means using Tukey's HSD test at $P \leq 0.05$.

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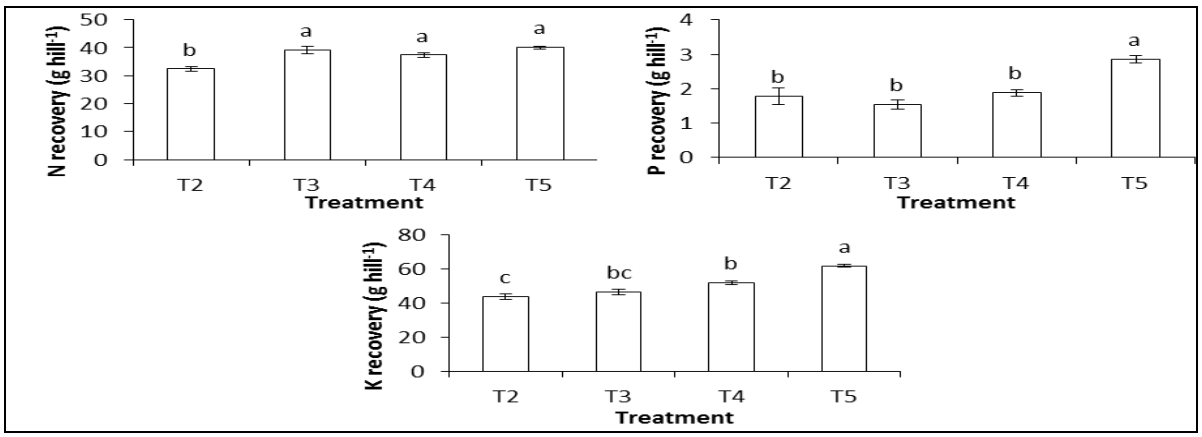


Figure 3. Treatment effects on N, P and K use efficiency of rice plant at 90 DAS. Different alphabets indicate significant difference between means using Tukey's HSD test at $P \leq 0.05$.

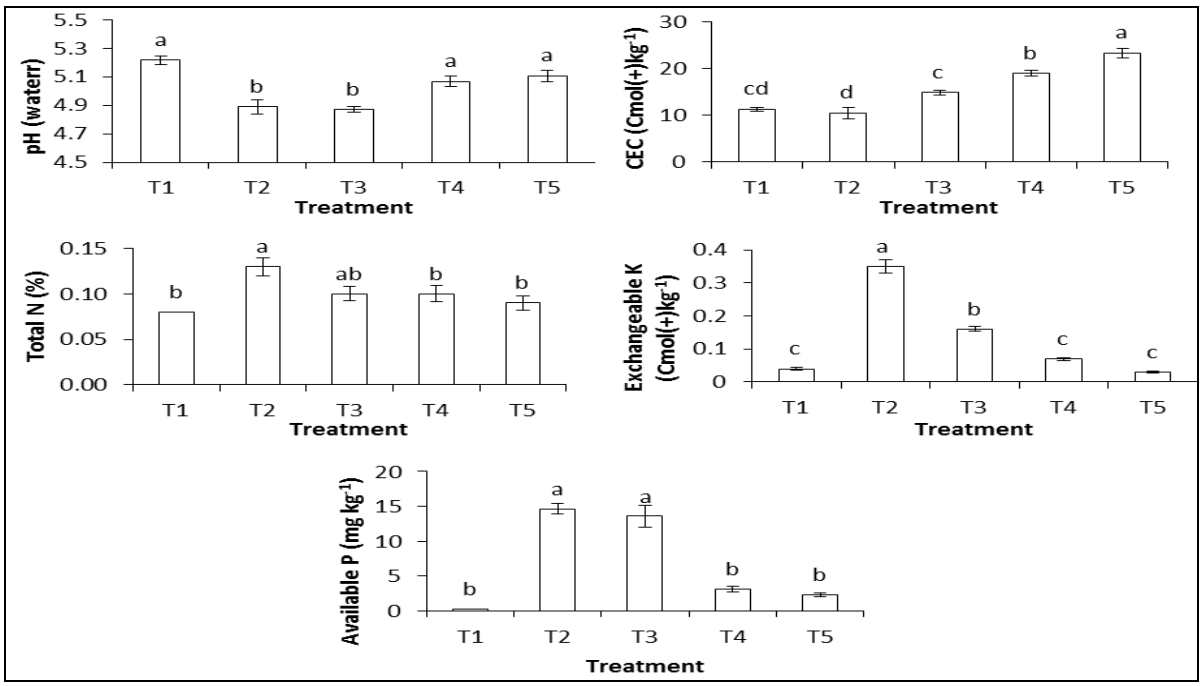


Figure 4. Treatment effects on soil pH, CEC, total N, available P and exchangeable K at 90 DAS. Different alphabets indicate significant difference between means using Tukey's test at $P \leq 0.05$.

## Conclusions

Clinoptilolite zeolite can be used to enhance rice plant growth, nutrient uptake, recovery and improve selected chemical properties of acid soils but field experiment has to be carried out to confirm the findings.

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# CHAPTER 6

# PEST AND DISEASE MANAGEMENT, BEST PRACTICES AND CURRENT TECHNIQUES

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# Association of Copper and Zinc to *Ganoderma* spp. Spatial Distribution in Oil Palm (*Elaeis guineensis*) Plantations on Peat

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## Introduction

*Ganoderma* species are a devastating basidiomycete fungus that has been reported to cause basal stem rot (BSR) and upper stem rot (USR) in oil palm (*Elaeis guineensis*) which threatening the sustainability of the plantations throughout the Southeast Asia region, especially in Malaysia and Indonesia (Hasan et al., 2005; Rees et al., 2012; Rakib et al., 2014a). Oil palm cultivation on peat in Malaysia, especially in the state of Sarawak is becoming more important due shortage of the mineral soil in other regions of Malaysia and the abundance of peat lands in the state (Said et al., 2009).

Although peat soil is suitable for oil palm cultivation through improvements of soil physical and chemical properties (Mutert et al., 1999), oil palms cultivated on peat are more prone to nutrient deficiency as peat soils have very low nutrient availability of the oil palm, especially micronutrients such as copper (Cu) and zinc (Zn) because they are highly adsorbed or fixed in soil organic matter of peat (McGrath et al., 1988; Fageria et al., 2002). Optimum level of essential nutrients in a plant is crucial for normal physiological process, which include the role in the defence mechanisms of the plant against pests and diseases (Marschner, 1995). Therefore, Cu and Zn level in the oil palm may have an important role in the distribution of *Ganoderma* species in an oil palm plantation.

Geographical Information System (GIS) has been adapted for application in plant pathology to quantitatively characterize the spatial distribution pattern of a disease or pathogen. This information is useful as an aid in the design of epidemiological studies, development of more accurate sampling programs, design and analyze experiments more efficiently, monitoring programs, better disease management (Azahar et al., 2011).

Spatial distribution of pathogens and its relation to the nutrient status of its host may provide useful information for disease management through nutrient manipulation. Thus, the objectives of this study were to investigate the association of foliar Cu and Zn of oil palm in relation to spatial distribution of *Ganoderma* species in oil palm plantations on peat.

## Materials and Methods

This study was conducted in two oil palm plantations on peat in Betong (873 ha) and Miri (2801 ha), located in Sarawak, Malaysia, with study areas of 15 ha and 16 ha, respectively. Foliar samples of oil palm were collected from frond 17 (Tohiruddin et al., 2010) based on the geostatistics analysis of spatial distribution of *Ganoderma* species provided in other study (Rakib et al., 2014b), namely, from i) uninfected area (< 15% *Ganoderma* density) and; ii) infected area (15.1-100% *Ganoderma* density). Samples were collected two times (two rounds) from both study sites at one year interval in 2011 and 2012, where a total of 30 samples were collected in each trial (Figure 1).

Total Cu and Zn from the foliar samples were extracted using the single dry ashing method and their concentrations were determined spectrometrically using an atomic absorption spectrometer (AAS)

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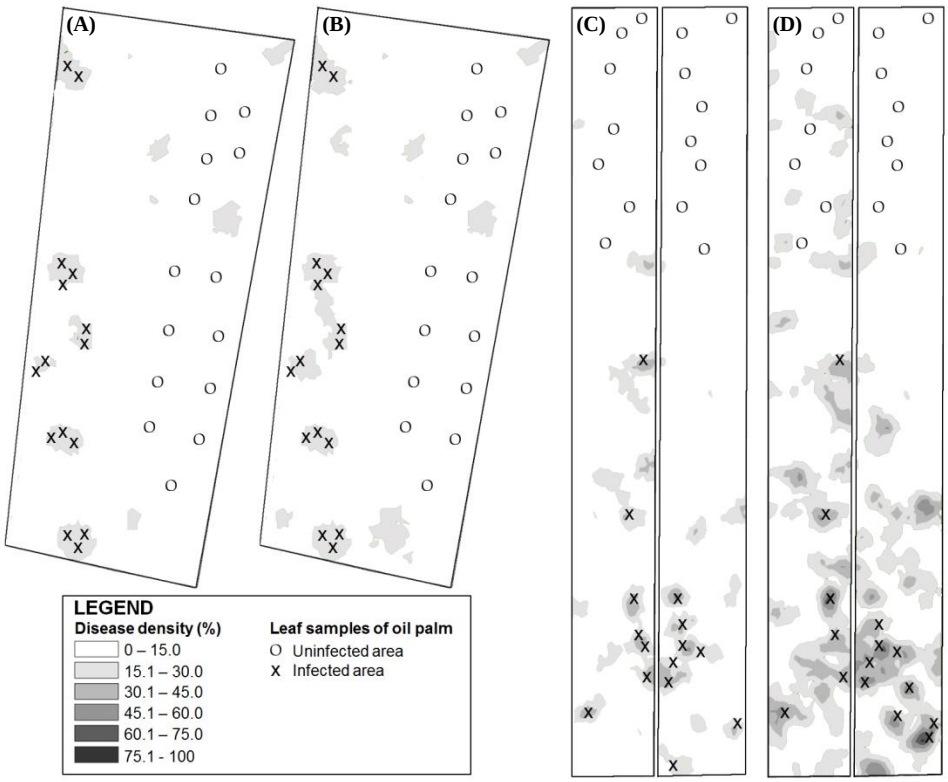
(Korn et al., 2008). A factorial arrangement was adopted in the field design and data were subjected to analysis of variance (ANOVA) and the Duncan's new multiple range test (DNMRT) for mean comparisons.




*Source: Rakib et al. (2014b)*

Figure 1. Foliar samples of oil palm from uninfected and infected area based on the spatial distribution of *Ganoderma* spp. in (A) Betong 2011, (B) Betong 2012, (C) Miri 2011 and (D) Miri 2012.

**Results and Discussion**

Foliar concentration of Cu and Zn in oil palms on infected area was significantly lower compared to an uninfected area (Figure 2). Cu in oil palms on infected area in Betong 2011 and 2012, Miri 2011 and 2012 were 5.41, 6.03, 2.11 and 3.54 $mgkg^{-1}$, respectively, and these were significantly lower compared to uninfected area which was 5.87, 5.67, 4.10 and 6.21 $mgkg^{-1}$, respectively, except inBetong2012. Similarly, Zn in oil palms on infected area in Betong2011 and 2012, and Miri 2011 and 2012 were 15.07, 14.16, 9.58 and 8.91 mg kg-1, respectively, and these were significantly lower compared to an uninfected area which was 16.86, 15.74, 10.61 and 11.43 $mgkg^{-1}$, respectively. Furthermore, oil palms in the infected area in Miri suffered Cu and Zn deficiency, which were below critical level of 3 $mgkg^{-1}$ and 10 $mgkg^{-1}$, respectively (von Uexkull and Fairhurst, 1991; Goh, 2003). Moreover, Miri has higher occurrence and rapid progression of *Ganoderma* species compared to Betong (Rakib et al., 2014b). Comparisons between the two study sites showed that the mean concentration of Cu was significantly lower (3.04 to 4.80 $mgkg^{-1}$) in Miri which has higher *Ganoderma* occurrence, compared to Betong which has significantly higher Cu (5.64 to 5.87 $mgkg^{-1}$) but lower *Ganoderma* occurrence. Similarly, the mean concentration of Zn was significantly lower (10.05 to 10.15 $mgkg^{-1}$) in Miri which has higher *Ganoderma* occurrence, compared to Betong which has significantly higher Zn (15.03 to 15.93 $mgkg^{-1}$) but lower *Ganoderma* occurrence.

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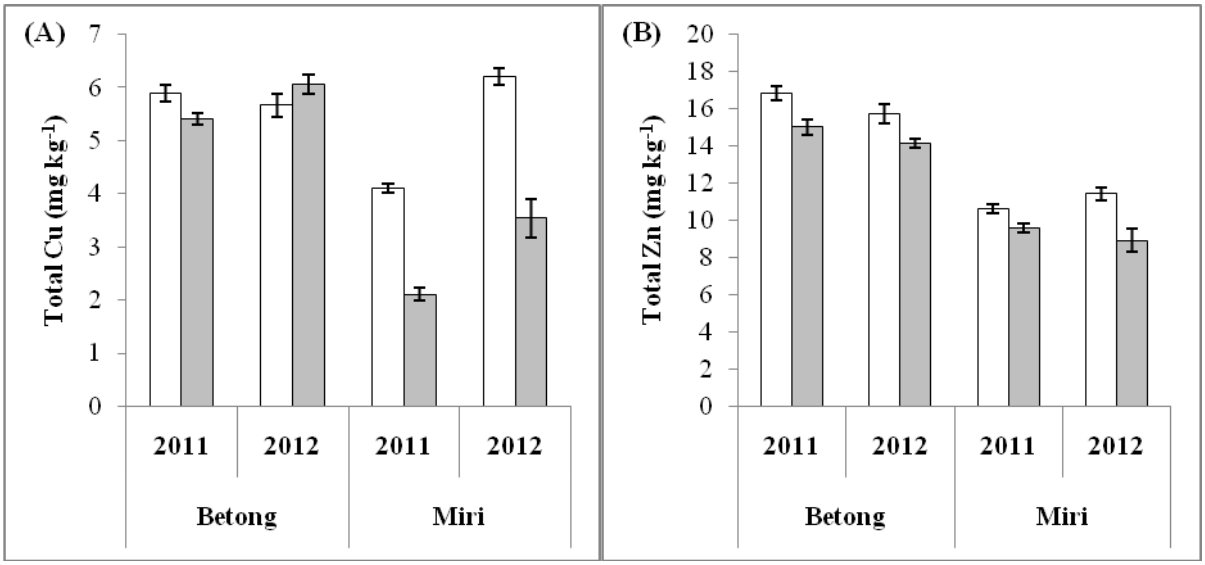


*Note:* □ *= Uninfected area and* ■ *= Infected area*

Figure 2. Foliar (frond 17) nutrient content, (A) total copper and (B) total zinc of oil palms on uninfected and infected area in Betong and Miri in 2011 and 2012.

In other studies, Nur Sabrina et al. (2012) reported reduction in disease progression by *Ganoderma* species in oil palm seedlings supplemented with Cu. Cu also has been reported to reduce verticillium disease by *Verticillium dahlia* in pepper (Chmielowska et al., 2009), leaf blight by *Alternaria palandui* in onion (Karthikeyan et al., 2005) and *Xanthomonasoryzae* in rice (Yuan et al., 2010). Meanwhile, increases in Zn has been related to reduction in *Fusarium graminearum* causing crown rot and (Grewal et al., 1996) *Gaeumannomyces graminis* causing root rot (Graham and Webb, 1991) in wheat.

Both Cu and Zn are essential micronutrients as they are associated with many physiological functions, enzyme systems, antioxidant properties, maintaining cell membrane integrity and required in chlorophyll in a plant. In addition, Cu is required for electron transport in photosynthesis, while Zn is required for protein and starch synthesis, and hormone metabolism (Goh, 2003; Dordas, 2008). The primary function of Cu in plant's defense mechanism against disease is a cofactor for peroxidase and laccase enzymes, which are important in lignification (Diaz et al., 2001; Chen et al., 2002). Lignin provide physical barrier against pathogen penetration into a plant (Rengel et al., 1994; Paterson et al., 2009). Besides that, increase in Cu has also been related to increase in phenolic compounds such as flavonoids as reported in *Digitalis lanata* cell cultures (Bota and Deliu, 2011). This phenolic compounds act as antifungal agent as in wheat (Caruso et al., 2001) and sweet flag (*Acoruscalamus*) (Ghosh, 2006), antioxidant and building blocks for lignin (Lattanzio et al., 2006). Meanwhile, the primary function of Zn in plant's defence mechanism against disease is related to its function in maintaining membrane integrity of a plant. Zn is a cofactor for enzymes involved in protection of membrane against oxidative damage by free radicals (Sakihama et al., 2002; Michalak, 2006) that could cause membrane leakage of high molecular compounds (sugar and amino acids) which favours infection of pathogens (Marschner, 1995; Graham and Webb, 1991). On the other hand, low Zn has been related to accumulation of those sugar and amino acids (Marschner, 1995). Hence, it is assumed that membrane leakage together with increased accumulation of high molecular compounds made the pathogens infection more successful. Furthermore, shortage of Zn also reduces plant's natural antifungal compounds such as phenolic compounds and flavonoids which are important in plant defence mechanism as these compounds are secreted once a plant becomes infected by pathogens (Sakihama et al., 2002). Both Cu and Zn itself are antifungal elements which are toxic to both plants and pathogens, however they are not harmful to plant but pathogen when they are present in optimum level in the plant as the pathogen required much less concentration (Marschner, 1995; Yruela, 2005).

## Conclusions

Low or deficiencies in foliar concentration of Cu and Zn in the oil palm were associated with higher distribution of *Ganoderma* species in oil palm plantations on peat in this study. This information could be useful in management of the disease through nutrients manipulation as in integrated pest management and site-specific disease management.

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# Improving Cocoa Yield and Suppress Pod Rot Disease through Thinning and Pruning Modification

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## Introduction

Indonesia is the third biggest cocoa producer in the world after Ivory Coast and Ghana, by producing about 833,310 tonnes per year. Cocoa farming involves 1.6 million farmers and millions of jobs for the dairy industry (Anonymous, 2011). The cocoa bean productivity is around 564 $kgha^{-1}$ which is higher than Ivory Coast (450 $kgha^{-1}$) and Ghana (500 $kgha^{-1}$). However, this figure is still far lower than the true potential of the clone, which can produce more 2000 $kgha^{-1}$.

There are many causes of this low productivity of cocoa and one of them is due to black pod disease, *Phytophthora palmivora*. This fungus is the main disease in Indonesia and also in the most of other cocoa producers in the world (Semangun, 2000; MacMahon and Purwantara, 2004). It causes the yield loss up to 20-30% (Guest, 2007). In Indonesia, the yield loss was recorded up to 60% in Sulawesi during rainy season (Rosmana et al., 2006) and around 54% in government estate in East Java (ICCRI, 2012). Over-lapped branches due to high humidity and incorrect cocoa pruning have triggered this disease attack. Beside soil, diseased pods left on the tree and stem canker, older trees, high and dense canopy, would complicate sanitation of those sources of inoculation.

The recommended control methods are (a) environmental management by regular pruning of cocoa and shade trees, (b) disease management, by sanitation of infected pods and buried its, (c) pod management by application of protective fungicide. In general, those methods are well versed by farmers but pod rot infection still occurred because those methods were not applied properly. Newest technique in order to control this fungus is by using oil-based formulation of *Trichoderma asperellum*. Tested on detached pods, the formulation was completely found to inhibit the development of the disease. When sprayed in the field on cacao clones that is highly sensitive to *P. megakarya*, the formulation resulted in 90% protection of treated pods after 1 week, and 50% after 3.2 weeks (Mbargaa et al., 2014).

Cocoa farm humidity is influenced by spacing and optimum cocoa spacing is depending on the planting materials, soil fertility and rainfall type. The optimum cocoa spacing vary between countries, i.e. Ghana 2.3 x 2.3 m to 3 x 3m, Ivory Coast 3 x 2 m to 3 x 2.5 m, Trinidad 2.4 x 2.4 m maximum, Brazil 2 x 2 m, Papua New Guinea 3.7 x 3.7 m (Soenaryo and Winaryo, 1988),

Pruning modification to prevent branches over-lapping especially on vigorous farm is able to increase flowering, pod setting and decrease pod rot incidence. Although this method will incur high pruning cost but it is still consider more profitable. Heavier pruning will allow more sunlight to penetrate the canopy and creating a warm condition which lead to induce flowering and pods production (Prawoto, 2008).

Hence, the aim of this research was to determine the effects of cocoa spacing and thinning on clone and hybrid materials as well as the effect of pruning modification on pod and bean yield, incidence of pod rot and mirids attack, and also the net income of farmers.

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## Materials and Methods

*Activity 1: Cocoa spacing and thinning*

a. Young trees

a.1. In spacing treatment, cocoa farms of Pa 191, BL 703 and GC 29 clones planted in 2007 were used. The farms received rainfall under B rainfall type ($0.143 < Q < 0.333$, Q= Ratio between the number of dry months and wet months in a year) according to Schmidt & Fergusson classification (Lakitan, 2002). *Leucaena* sp. planted at 3 x 6 m was used as shade trees. Meanwhile spacing treatments were (a) 3 x 3 m; and (b) 3 x 3 x 6 m. The data were collected from the sampling plot for Pa 191 clone comprising five rows with five trees per row. The observed data was analyzed using descriptive statistic.

a.2. In thinning treatment, hybrid planting materials which was planted in 2006 in C rainfall type ($0.333 < Q < 0.600$). The coconut trees planted at 12 x 9 m was used as shade trees. The initial spacing was 3 x 3 m and some part of them was thinned systematically to 3 x 3 x 6 m (double rows) or one third of the population after 7 years. Therefore, the sampling plots were 3 x 3 m and 3 x 3 x 6 m spacing. The number of replications was 10 rows and 5 plants per row. The observed data was analyzed using descriptive statistic.

The study was conducted in wet (B to A rainfall type) estate on hybrids planting materials planted in 1995. The initial spacing was 3 x 2 m and after 17 years old apart of them thinned selective systematically to 3 x 2 x 4 m (double rows). The sample trees were 5 trees and replicated 10 times (rows).

Variables; for all of treatments the variables were number of harvested pods, pod value, dry bean yield, number of pods infested by pod rot and *Helopeltis antonii*, microclimate indicators of illumination, temperature and relative humidity. Cost components i.e. fertilizer, insecticide, fungicide and their application, cost of cocoa pruning were observed and also gross and net income. Those variables were observed during semester I and II.

*Activity 2: Pruning modification*

This study was conducted in B rainfall type, using Pa 191 clone spacing 3 x 3 m and planted in 2006. The growth was vigorous so at 5 years old the overlapping branches were enormous. The tested pruning type were heavy pruning all of over-lapping branches every two rows of plants and control. Each model was tested on 2 ha. Coconut at 15 x 15 m was the shade trees. The samples were 5 plants and replicated 10 rows. The parameters and analyzed method was similar with the previous study.

## Results and Discussion

*Activity 1: Spacing and thinning*

Wider spacing on young cocoa at 5 years old showed increased pod yield per tree, decrease PPR and Helopeltis (Table 1). But for one area calculation, those increasing pod setting was still lower than the standard spacing 3 x 3 m. This facts lead to lower revenue although cost for pruning, fertilizing, pest and disease controlling one third lower (Table 4). It seen that until 6 years old, cocoa population 730 pph (plant per ha) decrease net income 12.9% to standard population 1100 pph.

Unlike the case with thinning treatment at 7 years old on vigorous hybrid materials, systematic thinning 30% of its population for initial spacing 3 x 3 m (one row for each three rows) increase pod yield per tree about 66%, dry bean 12.9% and net revenue 30.8% (Table 2 and 5). Pod rot and Helopeltis attack in this young plantation was not different to control. Hybrid material is much more vigorous and higher canopy compared to clone materials because of its orthotropic vs plagiotropic

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characters. So, in general it can be said that hybrids planting materials need wider spacing than plagiotropic ones.

The more dynamic impact of cocoa thinning appeared on matured farm. Systematic selective thinning one third population on 17 years old increase yield 36.5% per ha and decrease pod rot attack 11.6%, so this action improve revenue about 11.3%. This study was conducted in wet rainfall type estate (Table 3 and 6). This result was similar with cocoa thinning in Central America that removal of all weak trees (of less than average diameter) on spacing 2x2m and 2x3m increase bean yield from 437 $kg.ha^{-1}$ to 545 $kg.ha^{-1}$ and reduce monilia pod rot (*Moniliophthora roreri*) incidence (Quirós-Conejo et al., 1988).

Table 1. Average per tree of harvested pods, number of pod rot and Helopeltis attack on spacing treatment at 5 years old.

| Spacing (m) | Harvested pods per tree | | | Pod rot per tree | | | Infested pod of Helopeltis |
|---|---|---|---|---|---|---|---|
| | Sem. I | Sem. II | Total | Sem. I | Sem. II | Total | |
| Final 3x3x6 | 18.7± 3.6 | 19.4± 4.3 | 38.1 | 2.8 ±0.6 | 15.4 ±3.2 | 18.2 | 5.2 ±1.2 |
| Initial 3x3 | 12.3 ± 2.7 | 18.6±6.4 | 30.9 | 2.0 ±0.2 | 28.4 ±6.4 | 30.4 | 9.0 ±2.4 |

*Notes: Data ± standard deviation*

Table 2. Average per tree of harvested pods, number of pod rot and Helopeltis attack on thinning treatment at 7 years old.

| Spacing (m) | Harvested pods per tree | | | Pod rot per tree | | | Infested pod of Helopeltis |
|---|---|---|---|---|---|---|---|
| | Sem. I | Sem. II | Total | Sem. I | Sem. II | Total | |
| Final 3x3x6 | 10.4±3.7 | 39.6±12.8 | 50.0 | 5.5±1.2 | 19.0±6.4 | 24.5 | 1.6±0.3 |
| Initial 3x3 | 6.4±2.1 | 23.8±7.4 | 30.2 | 5.2±1.6 | 19.8±7.5 | 24.9 | 1.7±0.5 |

*Notes: Data ± standard deviation*

Table 3. Average per tree of harvested pods, number of pod rot and Helopeltis attack on thinning treatment at 17 years old.

| Spacing (m) | Harvested pods per tree | | | Pod rot per tree | | | Infested pod of Helopeltis |
|---|---|---|---|---|---|---|---|
| | Sem. I | Sem. II | Total | Sem. I | Sem. II | Total | |
| Final 3x2x4 | 11.7 ±4.2 | 23.8 ±9.2 | 35.5 | 17.9 ±4.3 | 13.2 ±4.3 | 31.1 | 2.5 ±0.6 |
| Initial 3x2 | 6.6 ±2.3 | 19.4 ±5.3 | 26.0 | 17.7 ±3.6 | 17.0 ±4.2 | 34.7 | 1.8 ±0.2 |

*Notes: Data ± standard deviation*

Effect of thinning on increasing pod yield physiologically can be explained as follows; to induce cushion to grow flowers, needs warm temperature (Alvim, 1977). This condition can be obtained from pruning or in this case by thinning or by shade tree felling. Air temperature more than 27 °C induced more flower than 23 °C.

Effect of thinning on lowering pod rot disease related with decreasing of relative humidity is showed in Table 7. The moist farm and continued availability of sources of inoculum be the main cause of this disease (Guest, 2007). Cocoa thinning followed by frequent pruning and pod rot sanitation shown effective to suppres its attack. Beside that, in Indonesia RidomillGold fungicide is used for preventive treatment. The use of Trichoderma as biofungicide is still in optimization stage while in Kamerun *Trichoderma asperellum* formulated in oil dispersion has therefore great potential for the control of cacao black pod disease with less recourse to synthetic fungicides (Mbarga et al., 2014). Research in Kamerun showed effect of rainfall on effectiveness method to control *P. megakarya* (Deberdt et al., 2008). The disease rates were 1.73%, 47.1% and 71.23% in the plots treated with fungicide, *T. asperellum* and untreated, respectively, in 2004, and 0.67%, 11.35% and 34.04% in the same plots in 2005.

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As the result of improvement of pod setting and decreament of pod rot attack was appear on improvement of revenue. Cocoa thinning of seedling materials at 5 years old increase net revenue 30.8% and thinning on 17% years old of seedling about 11.3 % (Table 5 and 6).

Optimum population of cocoa which is reflected from the plant spacing treatment tighly correlated with vigority of materials, soil fertility, climate type and plant age. In relation with plant age, this research proved that at the early years of its growth the optimum population more and wider along with the addition of age. Cocoa thinning for the old trees beginning after most of branches overlap, gave positive effect on yield. The problem of overlapping branches actually can be eliminate by frequent pruning, but in this case thinning suspected more effective because wage labor costs become a prominent source in the estate plantation. In addition, crop thinning impact on the lowering need for fertilizers and pesticides and application costs and increase yield and income planters (Table 4, 5 and 6).

There are many research on annual crops about spacing that indicate there was an optimum spacing to obtain maximum yield and income. On *Phazeolus vulgaris*, a results indicate that narrow and equidistant planting has potential to increase bean yield by 30%-70%, when compared to random planting (normal practice) while at the same time suppressing weed growth and is recommended for smallholder farmers in Rwanda and other semi-arid areas in sub-Saharan Africa (Dusabumuremyi et al., 2014). On *Medicago sativa*, Mattera et al. (2013) stated that reducing row spacing to an optimal distance is a practice that allows for more favorable spatial arrangements has a positive impact on forage production.

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Table 4. Economic analysis per ha impact of planting distance at 5 years old.

| Spacing, m | Population, pph | Pod per ha | Dry bean, kg ha$^{-1}$ | Gross income, IDR | Cost, IDR | | | | Total cost, IDR | Net income, IDR | Decreasing, % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Pruning | Fertilizing | Insecticide | Fungicide | | | |
| 3x3x6 | 730 | 27813 | 927 | 27813000 | 250000 | 2344000 | 1840000 | 1800000 | 6234000 | 21579000 | -12.90 |
| 3x3 | 1100 | 33990 | 1133 | 33990000 | 375000 | 3380000 | 2760000 | 2700000 | 9215000 | 24775000 | |

Table 5. Economic analysis per ha impact of cocoa thinning at 7 years old.

| Spacing, m | Population, pph | Pod per ha | Dry bean kg ha$^{-1}$ | Gross income, IDR | Cost of, IDR | | | | Total cost, IDR | Net income, IDR | Increasing, % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Pruning | Fertilizing | Insecticide | Fungicide | | | |
| 3x3x6 | 730 | 36500 | 1304 | 39120000 | 250000 | 1698710 | 1209610 | 1183330 | 4341650 | 34778350 | 19.76 |
| 3x3 | 1100 | 33220 | 1186 | 35580000 | 375000 | 2559700 | 1822700 | 1783100 | 6540500 | 29039500 | |

Table 6. Economic analysis per ha impact of thinning at 17 years old.

| Spacing, m | Population, pph | Pod per tree. year$^{-1}$ | Dry bean kg ha$^{-1}$ | Gross income, IDR | Maintenance cost, IDR | | | | Total cost, IDR | Net income, IDR | Decreasing, % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Pruning | Fertilizing | Insecticide | Fungicide | | | |
| 3x2x4 | 1110 | 35.5 | 1,408 | 42852857 | 333,333 | 2,583,100 | 1,840,000 | 1,800,000 | 6,556,433 | 36296424 | -1.17 |
| 3x2 | 1667 | 26 | 1,393 | 46437857 | 500,000 | 3,750,700 | 2,760,000 | 2,700,000 | 9,710,700 | 36727157 | |

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Table 7. Microclimate condition inside farm plot

| Object | Spacing, m | Temperature, °C | RH, % | Illumination, % direct |
|---|---|---|---|---|
| Seedling, 5 years old | 3x3x6 | 30 | 53 | 2.31 |
| | 3x3 | 28 | 56 | 1.95 |
| Clonally, 7 years old | 3x3x6 | 32 | 56 | 1.98 |
| | 3x3 | 30 | 59 | 1.80 |
| Seedling, 17 years old | 3x2x4 | 29 | 66 | 1.69 |
| | 3x2 | 28 | 69 | 1.45 |

*Activity 2, Pruning modification*

Pruning modification by avoid overlapping branches proved effective to prevent pod rot attack and increase pod yield. Pod yield at 2nd semester increase 26.1% and brings the total pod harvested within one year increased by 19.6% (Table 8). Pod yield during 1st semester was not different to control because pruning treatment was conducted during that semester. Although this pruning modification adds more cost, but this additional was still covered by the increased of yield, the revenue increase about 25.5% (Table 9). As the cause of the fact that due to the pruning modification increase the intensity of radiation, boost air temperature and lower RH inside the canopy.

Table 8. Average of harvested pods, number of pod rot and Helopeltis attack per tree.

| | Harvested pods | | | Pod rot infection, pods | | | Helopeltis attack, pods |
|---|---|---|---|---|---|---|---|
| | Sem. I | Sem. II | Total | Sem. I | Sem. II | Total | |
| Modified trimming | 12.2 ± 4.3 | 34.8 ± 11.6 | 47.0 | 5.4 ± 1.1 | 17.8 ± 4.2 | 23.2 | 13.8 ± 4.7 |
| Control | 11.6 ± 5.2 | 27.6 ± 9.5 | 39.3 | 5.9 ± 2.1 | 39.3 ± 13.6 | 45.1 | 15.5 ± 5.2 |

*Notes: data ± standard deviation*

Table 9. Yield and economic analysis per hectare.

| | Pods | Dry bean, kg | Gross, Rp | Pruning cost | Net | Increase, % |
|---|---|---|---|---|---|---|
| Modified trimming | 54,450 | 1,944,643 | 58,339,286 | 600,000 | 57,739,286 | 25.47 |
| Control | 43,230 | 1,543,929 | 46,317,857 | 300,000 | 46,017,857 | |

Table 10. Microclimate inside tree canopy.

| Treatment | Temperature, °C | RH, % | Illumination, % direct |
|---|---|---|---|
| Modified trimming | 29 | 64 | 2.02 |
| Control | 27 | 68 | 1.85 |

**Conclusions**

a) Wider spacing of 730 pph on young cocoa at 5 years old although increase pod per tree but the revenue still 12.9% lower than control of 1100 pph.

b) Systematic thinning increase pod yield and lower pod rot attack. Thinning cocoa derived from F1 hybrid at 7 and 17 years old, increase pod yield per tree by 66% and 36% and lower PPR 1.7% and 11.6% respectively as compared to the control. Despite the lower cost for fertilizing, pruning, and pest and disease controlling, net income of this methods were 19.8% but -1.2% respectively to the control.
c) Pruning modification increase average of pod yield per tree 19.6%, lower PPR (Phytophthora Pod Rot) incidence 48.6% and Helopeltis attack by 5.1% and net income 25.5% higher.
d) Increasing yield and reduce of PPR are associated with the increase of air temperature and decrease of relative humidity in the farm.

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# Clonal Selection on Cocoa Resistance to Vascular-Streak Dieback

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## Introduction

Vascular-streak dieback (VSD) is the most serious disease on cocoa in Indonesia which results in plant damage. The fungus *Oncobasidium theobromae* of the VSD agent infects cocoa through young emerged-leaves then penetrates to xylem bundle-sheet so the nutrient transport is blocked. Method for controlling VSD has been recommended as integrated management such as regular pruning of the infected branches, application of more organic material to soil and inorganic fertilizer to support plant regrowth followed by spraying of the new emerged-leaves using fungicide of Azocystrobin and Difenoconazole (Sri-Sukamto et al., 2008). These approaches look more effective when applied in case of light and moderate damages but in case of high damages the use of resistant planting material is the most effective method for controlling VSD. Febriantomo (2012) reported the success on reducing VSD incidence from 38% to 9% by implementing those methods in cocoa plantations in Java. As the long term approach for controlling VSD by using resistant planting material, there are some recommended resistant clones and hybrids, namely Sulawesi 01, Sulawesi 02, Sca 6 and ICCRI 06H (Susilo et al., 2013). The genetic potency of cocoa planting material should be improved to contruct polygenic resistance by which the resistant genotypes should also perform resistance to other pests or diseases, besides VSD.

Breeding for VSD resistance on cocoa in Indonesia has been carried out simultaneously by selecting the promising genotypes through hybrid population. Selection was carried out based on individual basis and population basis to develop both clones and hybrids as cocoa planting materials. Clonal selection was carried out in the endemic area of VSD in Kaliwining Experimental Station of ICCRI to select the resistant clones, which are also potentially resistant to other diseases. Results of the selection will be discussed in this paper.

## Materials and Methods

### *Genetic material*

Genetic materials of this trial consisted of the introduced clones from the University of Reading (RU), selected genotypes through hybrid population of multilocation trials (Susilo and Anita-Sari, 2011) and exploratory genotypes at the Sumatra region. A total of 44 clones were collected for testing against both the resistant (Sulawesi 01) and susceptible (NIC 7) (Table 1) controls.

### *Trial design*

Field testing of this trial established in Kaliwining Experimental Station of the VSD endemic were based on randomized complete block design with 3 blocks as replication. Seedlings of the tested clones were propagated by top grafting method in which the scions were derived from plagiotropic branches. The seedlings were planted in 2008 at the spacing distance of 3 x 3 m with 5 trees per plot. Management of the trial was carried out according to the standard of good agriculture practices (GAP) on cocoa.

To perform the resistance record, the symptom of damage due to VSD was noted as scoring of the damage in the tree level using 0-6 scale (Susilo and Anita-Sari, 2011). Resistant evaluations were carried

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out in dry season in the year of 2012-2013. The yield performance of the tree was also recorded by counting the number of pods per tree. The data were then converted to yield potency by multiplying with pod index. The pod index was calculated based on the parameter of the number of beans per pod and weight of a dry bean. Mean of the variables were tabulated to indicate differences among clones.

Table 1. The list of tested clones for selection of the VSD resistant genotype.

| No | KW serried | Selection code/Progeny | Origin |
|---|---|---|---|
| 1. | KW 176 | NIC 7 | Introduction (susceptible) |
| 2. | KW 162 | Sulawesi 01 | Recommended clone (resistant) |
| 3. | KW 255 | JTC 25 | Selected at Jatirono |
| 4. | KW 433 | RU I - SCA 11 | Introduced from RU |
| 5. | KW 443 | RU I - SPA 9 | Introduced from RU |
| 6. | KW 444 | RU I - BORNE 7 A2 | Introduced from RU |
| 7. | KW 454 | RU I - KER 3 | Introduced from RU |
| 8. | KW 467 | RU I -BORNE 7 A6 | Introduced from RU |
| 9. | KW 468 | RU I - EQ X 3360-3 | Introduced from RU |
| 10. | KW 490 | DRC 15 | Selected at Djati Roenggo |
| 11. | KW 514 | ICCRI 07 | Selected at North Sumatra |
| 12. | KW 516 | PABA/VIII/78B/2 | Selected at North Sumatra |
| 13. | KW 535 | ADO 1 | Selected at North Sumatra |
| 14. | KW 562 | PABA/I/90C/2 | Selected at North Sumatra |
| 15. | KW 563 | PABA/I/90C/4 | Selected at North Sumatra |
| 16. | KW 564 | PABA/IX/90O/2 | Selected at North Sumatra |
| 17. | KW 566 | PABA/V/81L/1 | Selected at North Sumatra |
| 18. | KW 567 | PABA/IX/90O/3 | Selected at North Sumatra |
| 19. | KW 570 | Sulawesi 03 | Selected at South Sulawesi |
| 20. | KW 606 | BLITAR 01 | Selected at Blitar |
| 21. | KW 608 | ADO/IV/90B/53/38 | Selected at North Sumatra |
| 22. | KW 609 | ADO/IV/90B/149/13 | Selected at North Sumatra |
| 23. | KW 614 | ADO/IV/91EU/11/33 | Selected at North Sumatra |
| 24. | KW 615 | ADO/IV/91FU/51/32 | Selected at North Sumatra |
| 25. | KW 616 | ADO/IV/91FU/149/96 | Selected at North Sumatra |
| 26. | KW 617 | KWN 2.2.9 (TSH858xKW162) | Selected at Kaliwining, Jember |
| 27. | KW 618 | KWN 3.10.1 (KW162xTSH858) | Selected at Kaliwining, Jember |
| 28. | KW 619 | KPSA - 2.7.16 (KEE2xNIC7) | Selected at Sumber Asin, Malang |
| 29. | KW 621 | KPSA - 3.15.8 (Prop.ICS 60) | Selected at Sumber Asin, Malang |
| 30. | KW 623 | SBRM 01 | Selected at Jembrana, Bali |
| 31. | KW 626 | RANTO JAYA 1 | Selected at Lampung |
| 32. | KW 629 | KALIDADI 1 | Selected at Lampung |
| 33. | KW 630 | GNRG 02 | Selected at Jatirono |
| 34. | KW 631 | KEMBU 01 (TSH 858xPBC 123) | Selected at Kendeng Lembu |
| 35. | KW 635 | KEMBU 05 (TSH 858x BC 123) | Selected at Kendeng Lembu |
| 36. | KW 636 | KEMBU 06 | Selected at Kendeng Lembu |
| 37. | KW 637 | KATE 01 - 1.2.16 (TSH 858xKW 162) | Selected at Kalitelepak |
| 38. | KW 638 | KATE 02 - 2.2.3 (TSH 858xKW 162) | Selected at Kalitelepak |
| 39. | KW 641 | KATE 05 - 2.13.11 (KW163xKEE2) | Selected at Kalitelepak |
| 40. | KW 642 | KATE 06 - 2.3.7 (TSH858xNIC7) | Selected at Kalitelepak |
| 41. | KW 649 | KATE 13 - 3.2.15 (TSH858xKW162) | Selected at Kalitelepak |
| 42. | KW 651 | KATE 15 - 1.4.10 (TSH858xICS13) | Selected at Kalitelepak |
| 43. | KW 684 | RU III - CCN .51 | Introduced from RU |
| 44. | KW 685 | YPSI (MUCHTAR 1) | Selecetd at South Sulawesi |

Other than VSD evaluation, the resistance to pod rot disease was also tested for obtaining the promising clones using laboratory study by inoculating the detached pod using isolate of *Phytophthora palmivora* (Susilo and Anita-Sari, 2014). The tested clones were KW 617 and control of the susceptible clone of TSH 858, the resistant clone of Sca 6, ICCRI 03 and ICCRI 04. Lession size due to *P. palmivora* was measured from 1$^{st}$ to 7$^{th}$ day after inoculation.

*Data analysis*

Means of the scores were clustered by fastclass analysis method using SAS program. The resistance was clasified into 5 groups of resistance, namely resistant, moderate resistant, moderate susceptible, susceptible and very susceptible.

**Results and Discussion**

The results on VSD evaluation during for 2 years (2012-2013) indicate that there was varying performance of VSD resistance among tested clones (Table 2). Mean of the score varied among clones in the range of 1.93 – 4.80. The susceptible clone of NIC 7 had the highest score in contrary with the resistant clone of Sulawesi 01 that had the lowest score. Grouping of the resistance showed that some clones were classified into the group of Sulawesi 01 as the resistant control, namely ICCRI 07, KW 562, Sulawesi 3, KW 606 and KW 617. Of those clones, ICCRI 07 and Sulawesi 3 had been released as the resistant clones to cocoa pod borer (Susilo et al., 2012). This result confirms any potency of polygenic resistant on cocoa to some pests and diseases. For example, Sca 6 had resistance to VSD (Susilo et al., 2009), pod rot (*P. palmivora*) and anthracnose (*Colletotrichum* sp.) (Napitupulu et al., 1991) and witches' broom (Dos Santos et al., 2005). The results also indicated that the introduced clones from international collection of the University of Reading (RU code) also had varying resistance from that of moderate resistance to very susceptible ones. The selected resistant clones were then subjected to selection for the high yielding clones.

Mechanism on VSD resistance has been intensively studied. There were some hypothesis tested to identify the most appropriate mechanism of the resistance that is able for use to develop criteria for selection and control method. Antibiosis mechanism would also control the resistance as reported by Prawoto et al. (2013), in which the resistant clones showed higher concentration of secondary metabolite (terpenoid and polyphenol), which was also supported by structural mechanism as the greater thickness of cuticle, epidermis and palisade tissues in the resistant leaves as compared to susceptible clones. Susilo and Anita-Sari (2014) reported the potency of tolerant mechanism on VSD resistance as the resistant trees exhibited higher rejuvenation process after pruning for controlling VSD. Furthermore, the selected resistant clones can be confirmed for their resistance using those resistant variables in field evaluation of the resistance.

Visual evaluation of the selected clones shows that KW 617 of the selected resistant clone had high potency of yield with higher number of pods per tree (Figure 1). Performance of the clone appeared stable among locations. However, mean pod number for 2 years' evaluation of 2012 - 2013 in Kaliwining was lower than that of Sulawesi 01. KW 617 is of the progeny of Sulawesi 01 with TSH 858 that indicated that there was no hybrid vigor performance for yield component inherited through KW 617 but potential hybrid vigor was found for VSD resistance as the mean score of KW 617 was lower than Sulawesi 01. Pang and Lockwood (2008) suspected that the hybrid vigor was ephygenetic process of the differences in tree performance between parental clones and hybrids. These results also showed that CCN 51 of the recommended clone from Ecuador had lower number of pods as the clone was susceptible to VSD with higher score of 3.21.

Evaluation on bean quality was performed to all the under study clones as whether trees of the susceptible clones are growing well and bearing pods (Table 4). The selected clones of KW 562 and KW 617 gave better quality beans with bean dry weight of 1.0 and 0.97 g respectively (grade A and B of national standard). Their pod indices were up to 21.4 and 23.6 respectively, which are equavalent to that of Sulawesi 01 as the control. The bean dry weight was, however, lower as compared to the highest potency of bean dry weight as cocoa planting material, which was reported on ICS 60 and UIT 1, performing bean

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dry weight of 1.67 and 1.64 g respectively (Iswanto et al., 2001). These two clones were, however, highly susceptible to VSD. Further criteria for selection are not dependent on just a few traits but should refer to some traits which are the attributes of the high quality cocoa planting material.

Table 2. Mean score of VSD-symptom damage of the tested clones in Kaliwining Experimental Station, Jember (2012-2013).

| No | KW serried | Clone | Year | | Mean* |
|---|---|---|---|---|---|
| | | | 2012 | 2013 | |
| 1. | KW 176 | NIC 7 | 5.6 | 4.0 | 4.80 A |
| 2. | KW 162 | Sulawesi 01 | 1.9 | 2.5 | 2.20 E |
| 3. | KW 255 | JTC 25 | 4.7 | 2.3 | 3.46 B |
| 4. | KW 433 | RU I - SCA 11 | 5.3 | 3.5 | 4.42 A |
| 5. | KW 443 | RU I - SPA 9 | 3.5 | 3.3 | 3.43 C |
| 6. | KW 444 | RU I - BORNE 7 A2 | 3.0 | 2.9 | 2.96 D |
| 7. | KW 454 | RU I - KER 3 | 3.5 | 2.8 | 3.15 D |
| 8. | KW 467 | RU I -BORNE 7 A6 | 2.6 | 3.3 | 2.93 D |
| 9. | KW 468 | RU I - EQ X 3360-3 | 4.3 | 2.6 | 3.45 B |
| 10. | KW 490 | DRC 15 | 5.5 | 3.5 | 4.48 A |
| 11. | KW 514 | ICCRI 07 | 2.0 | 2.8 | 2.42 E |
| 12. | KW 516 | PABA/VIII/78B/2 | 2.7 | 3.1 | 2.89 D |
| 13. | KW 535 | ADO 1 | 3.1 | 3.0 | 3.08 D |
| 14. | KW 562 | PABA/I/90C/2 | 2.1 | 2.9 | 2.51 E |
| 15. | KW 563 | PABA/I/90C/4 | 4.9 | 2.8 | 3.83 B |
| 16. | KW 564 | PABA/IX/90O/2 | 3.2 | 3.1 | 3.13 D |
| 17. | KW 566 | PABA/V/81L/1 | 6.0 | 6.0 | 6.00 A |
| 18. | KW 567 | PABA/IX/90O/3 | 2.7 | 2.6 | 2.62 D |
| 19. | KW 570 | Sulawesi 03 | 2.2 | 2.9 | 2.52 E |
| 20. | KW 606 | BLITAR 01 | 2.3 | 2.6 | 2.41 E |
| 21. | KW 608 | ADO/IV/90B/53/38 | 4.7 | 3.2 | 3.95 B |
| 22. | KW 609 | ADO/IV/90B/149/13 | 3.9 | 3.3 | 3.60 D |
| 23. | KW 614 | ADO/IV/91EU/11/33 | 3.0 | 2.8 | 2.89 D |
| 24. | KW 615 | ADO/IV/91FU/51/32 | 4.4 | 3.3 | 3.88 B |
| 25. | KW 616 | ADO/IV/91FU/149/96 | 2.7 | 2.8 | 2.77 D |
| 26. | KW 617 | KWN/II/2/9 (TSH858xKW162) | 1.9 | 2.0 | 1.93 E |
| 27. | KW 618 | KWN/III/10/1 (KW162xTSH858) | 3.6 | 2.1 | 2.86 D |
| 28. | KW 619 | KPSA/II/7/16 (KEE2xNIC7) | 5.1 | 3.0 | 4.07 A |
| 29. | KW 621 | KPSA /III/15/8 (Prop.ICS 60) | 6.0 | 6.0 | 6.00 A |
| 30. | KW 623 | SBRM 01 | 3.4 | 2.7 | 3.04 D |
| 31. | KW 626 | RANTO JAYA 1 | 4.7 | 3.3 | 3.96 B |
| 32. | KW 629 | KALIDADI 1 | 4.7 | 3.0 | 3.83 B |
| 33. | KW 630 | GNRG 02 | 3.2 | 2.4 | 2.78 C |
| 34. | KW 631 | KEMBU 01 (TSH 858xSul01) | 4.7 | 3.0 | 3.87 B |
| 35. | KW 635 | KEMBU 05 (TSH 858x Sul 01) | 5.4 | 3.8 | 4.55 A |
| 36. | KW 636 | KEMBU 06 | 2.8 | 2.5 | 2.64 C |
| 37. | KW 637 | KATE/I/2/16 (TSH 858xKW 162) | 4.3 | 1.8 | 3.05 B |
| 38. | KW 638 | KATE/II/2/3 (TSH 858xKW 162) | 4.6 | 3.0 | 3.80 B |
| 39. | KW 641 | KATE/II/13/11 (KW163xKEE2) | 2.6 | 3.0 | 2.80 D |
| 40. | KW 642 | KATE/II/3/7 (TSH858xNIC7) | 3.1 | 2.6 | 2.84 C |
| 41. | KW 649 | KATE/III/2/15 (TSH858xKW162) | 4.3 | 2.3 | 3.33 B |
| 42. | KW 651 | KATE/I/4/10 (TSH858xICS13) | 4.0 | 3.3 | 3.68 B |
| 43. | KW 684 | RU III - CCN .51 | 3.9 | 2.5 | 3.21 C |
| 44. | KW 685 | YPSI (MUCHTAR 1) | 3.2 | 2.7 | 2.96 D |

*Note: * indicates that means within column with same letter were classified in the same group of VSD resistance based on fastcluss analysis using statistical analysis system (SAS)*

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Figure 1. The performance of KW 617, the selected resistant clones to VSD in three different trial locations; KP Sumber Asin, Malang (left), KP Kaliwing, Jember (centre) and Polman, West Sulawesi (right)

Table 3. Mean of pod number per tree of clones tested in Kaliwining Experimental Station (2012-2013).

| No | KW serried | Clone | Year | | Mean* |
|---|---|---|---|---|---|
| | | | 2012 | 2013 | |
| 1. | KW 176 | NIC 7 | 0 | 18.3 | 9.1 |
| 2. | KW 162 | Sulawesi 01 | 60.8 | 56.0 | 58.4 |
| 3. | KW 255 | JTC 25 | 5.7 | 19.7 | 12.7 |
| 4. | KW 433 | RU I - SCA 11 | 0.5 | 3.5 | 2.0 |
| 5. | KW 443 | RU I - SPA 9 | 13.1 | 15.3 | 14.2 |
| 6. | KW 444 | RU I - BORNE 7 A2 | 27.4 | 12.5 | 19.9 |
| 7. | KW 454 | RU I - KER 3 | 11.5 | 6.0 | 8.7 |
| 8. | KW 467 | RU I -BORNE 7 A6 | 18.1 | 10.1 | 14.1 |
| 9. | KW 468 | RU I - EQ X 3360-3 | 3.9 | 2.4 | 3.1 |
| 10. | KW 490 | DRC 15 | 3.2 | 1.5 | 2.3 |
| 11. | KW 514 | ICCRI 07 | 17.0 | 25.1 | 21.0 |
| 12. | KW 516 | PABA/VIII/78B/2 | 29.9 | 15.2 | 22.5 |
| 13. | KW 535 | ADO 1 | 16.2 | 13.9 | 15.1 |
| 14. | KW 562 | PABA/I/90C/2 | 37.2 | 27.0 | 32.1 |
| 15. | KW 563 | PABA/I/90C/4 | 0 | 0 | 0 |
| 16. | KW 564 | PABA/IX/90O/2 | 19.1 | 21.0 | 20,0 |
| 17. | KW 566 | PABA/V/81L/1 | - | - | - |
| 18. | KW 567 | PABA/IX/90O/3 | 20.0 | 29.7 | 24.8 |
| 19. | KW 570 | Sulawesi 03 | 21.5 | 30.4 | 26.0 |
| 20. | KW 606 | BLITAR 01 | 49.6 | 45.3 | 47.4 |
| 21. | KW 608 | ADO/IV/90B/53/38 | 1.9 | 7.8 | 4.9 |
| 22. | KW 609 | ADO/IV/90B/149/13 | 0.3 | 10.3 | 5.3 |
| 23. | KW 614 | ADO/IV/91EU/11/33 | 16.9 | 15.9 | 16.4 |
| 24. | KW 615 | ADO/IV/91FU/51/32 | 1.9 | 4.9 | 3.4 |
| 25. | KW 616 | ADO/IV/91FU/149/96 | 0.9 | 2.0 | 1.4 |
| 26. | KW 617 | KWN/II/2/9 (TSH858xKW162) | 22.9 | 33.0 | 27.9 |
| 27. | KW 618 | KWN/III/10/1 (KW162xTSH858) | 14.0 | 25.1 | 19.6 |
| 28. | KW 619 | KPSA/II/7/16 (KEE2xNIC7) | 5.3 | 0.8 | 3.0 |
| 29. | KW 621 | KPSA /III/15/8 (Prop.ICS 60) | - | - | - |
| 30. | KW 623 | SBRM 01 | 19.9 | 16.3 | 18.1 |
| 31. | KW 626 | RANTO JAYA 1 | 0.2 | 1.3 | 0.7 |
| 32. | KW 629 | KALIDADI 1 | 0 | 2.4 | 1.2 |
| 33. | KW 630 | GNRG 02 | 13.6 | 30.4 | 22.0 |
| 34. | KW 631 | KEMBU 01 (TSH 858xSul01) | 15.8 | 12.5 | 14.1 |
| 35. | KW 635 | KEMBU 05 (TSH 858x Sul 01) | 0.2 | 7.6 | 3.9 |
| 36. | KW 636 | KEMBU 06 | 9.2 | 8.8 | 9.0 |
| 37. | KW 637 | KATE/I/2/16 (TSH 858xKW 162) | 12.7 | 23.8 | 18.2 |
| 38. | KW 638 | KATE/II/2/3 (TSH 858xKW 162) | 0 | 0.8 | 0.4 |
| 39. | KW 641 | KATE/II/13/11 (KW163xKEE2) | 5.3 | 8.1 | 6.7 |
| 40. | KW 642 | KATE/II/3/7 (TSH858xNIC7) | 7.2 | 15.7 | 11.5 |
| 41. | KW 649 | KATE/III/2/15 (TSH858xKW162) | 19.6 | 24.0 | 21.8 |
| 42. | KW 651 | KATE/I/4/10 (TSH858xICS13) | 3.0 | 12.6 | 7.8 |
| 43. | KW 684 | RU III - CCN .51 | 1.4 | 3.5 | 2.4 |
| 44. | KW 685 | YPSI (MUCHTAR 1) | 24.3 | 27.8 | 26.0 |

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Table 4. Yield component of the clones tested in Kaliwining Experimental Station (2013).

| No | KW serries | Clone | Dry weight bean (g) | Shell content (%) | No of bean per pod | Pod Index |
|---|---|---|---|---|---|---|
| 1. | KW 176 | NIC 7 | - | - | | |
| 2. | KW 162 | Sulawesi 01 | 1.06 | 8.88 | 37.6 | 25.0 |
| 3. | KW 255 | JTC 25 | - | - | | |
| 4. | KW 433 | RU I - SCA 11 | 1.12 | 9.78 | 47.0 | |
| 5. | KW 443 | RU I - SPA 9 | 1.12 | 9.78 | 35.8 | 24.9 |
| 6. | KW 444 | RU I - BORNE 7 A2 | 0.90 | 6.07 | 33.6 | 33.0 |
| 7. | KW 454 | RU I - KER 3 | 0.65 | 8.65 | 34.5 | 44.9 |
| 8. | KW 467 | RU I -BORNE 7 A6 | 0.66 | 15.49 | | |
| 9. | KW 468 | RU I - EQ X 3360-3 | - | - | | |
| 10. | KW 490 | DRC 15 | 1.05 | 9.61 | | |
| 11. | KW 514 | ICCRI 07 | 0.83 | 10.09 | 43.1 | 27.9 |
| 12. | KW 516 | PABA/VIII/78B/2 | 1.04 | 11.37 | 43.0 | 22.4 |
| 13. | KW 535 | ADO 1 | 1.42 | 7.78 | 41.6 | 17.0 |
| 14. | KW 562 | PABA/I/90C/2 | 1.00 | 9.86 | 46.5 | 21.4 |
| 15. | KW 563 | PABA/I/90C/4 | - | - | | |
| 16. | KW 564 | PABA/IX/90O/2 | - | - | | |
| 17. | KW 566 | PABA/V/81L/1 | 0.56 | 14.66 | | |
| 18. | KW 567 | PABA/IX/90O/3 | 1.06 | 7.81 | 39.6 | 23.7 |
| 19. | KW 570 | Sulawesi 03 | 0.75 | 3.95 | 46.5 | 28.6 |
| 20. | KW 606 | BLITAR 01 | 0.67 | 12.74 | 37.7 | 39.5 |
| 21. | KW 608 | ADO/IV/90B/53/38 | - | - | | |
| 22. | KW 609 | ADO/IV/90B/149/13 | - | - | | |
| 23. | KW 614 | ADO/IV/91EU/11/33 | _ | - | 39.6 | |
| 24. | KW 615 | ADO/IV/91FU/51/32 | 1.61 | 6.15 | | |
| 25. | KW 616 | ADO/IV/91FU/149/96 | - | - | | |
| 26. | KW 617 | KWN/II/2/9 (TSH858xKW162) | 0.97 | 14.06 | 43.5 | 23.6 |
| 27. | KW 618 | KWN/III/10/1 (KW162xTSH858) | 1.01 | 12.35 | | |
| 28. | KW 619 | KPSA/II/7/16 (KEE2xNIC7) | - | - | | |
| 29. | KW 621 | KPSA /III/15/8 (Prop.ICS 60) | - | - | | |
| 30. | KW 623 | SBRM 01 | 0.69 | 14.12 | 33.0 | 43.8 |
| 31. | KW 626 | RANTO JAYA 1 | _ | - | | |
| 32. | KW 629 | KALIDADI 1 | - | - | | |
| 33. | KW 630 | GNRG 02 | 0.82 | 8.36 | 41.3 | 29.7 |
| 34. | KW 631 | KEMBU 01 (TSH 858xSul01) | 1.29 | 7.81 | | |
| 35. | KW 635 | KEMBU 05 (TSH 858x Sul 01) | - | - | | |
| 36. | KW 636 | KEMBU 06 | 1.21 | 7.91 | 42.0 | 19.6 |
| 37. | KW 637 | KATE/I/2/16 (TSH 858xKW 162) | 0.77 | 14.13 | 28.0 | 46.4 |
| 38. | KW 638 | KATE/II/2/3 (TSH 858xKW 162) | - | - | | |
| 39. | KW 641 | KATE/II/13/11 (KW163xKEE2) | - | - | | |
| 40. | KW 642 | KATE/II/3/7 (TSH858xNIC7) | 1.62 | 8.32 | 39.3 | 15.7 |
| 41. | KW 649 | KATE/III/2/15 (TSH858xKW162) | 1.03 | 5.70 | 42.0 | 23.2 |
| 42. | KW 651 | KATE/I/4/10 (TSH858xICS13) | 0.78 | 8.68 | | |
| 43. | KW 684 | RU III - CCN .51 | - | - | | |
| 44. | KW 685 | YPSI (MUCHTAR 1) | 0.74 | 17.72 | 40.0 | 33.8 |

Evaluation on the resistance to pod rot (*P. palmivora*) of the promising clone of KW 617 indicated the potency on pod rot resistance. Lesion of KW 617 due to *P. palmivora* infection was smaller than the susceptible clone of TSH 858 and not quite different from the resistant clones of Sca 6, ICCRI 03 and ICCRI 04 (Figure 2). This result confirmed the potency of polygenic resistance in KW 617 to VSD and pod rot.

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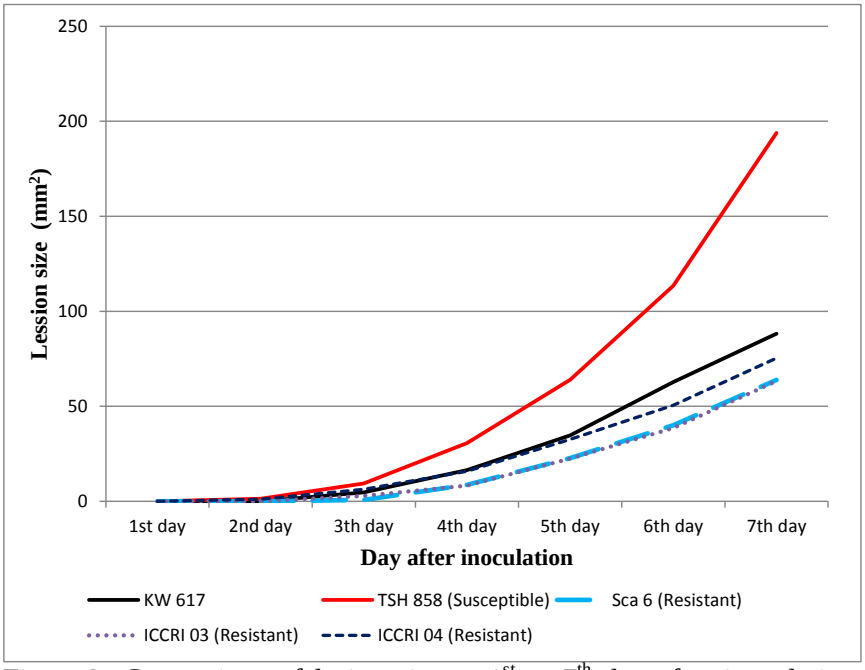


Figure 2. Comparison of lesion size at 1st to 7th day after inoculation of *P. palmivora* in the selected VSD-resistant clone of KW 617 and other susceptible and resistant clones.

## Conclusions

The tested clones had varied VSD resistance in the range of resistant, moderate resistant, moderate susceptible, susceptible, and very susceptible. The resistant group included clones of Sulawesi 01, ICCRI 07, KW 562, Sulawesi 3, KW 606 and KW 617. Among the selected VSD-resistant clones, ICCRI 07 and Sulawesi 03 showed potency on polygenic resistance and were released with cocoa pod borer resistance while KW 617 exhibited smaller pod rot lesion after inoculation by *P. palmivora*. KW 617 was promising VSD resistant clone having yield potency equavalent to Sulawesi 01 as the control for high yielding VSD resistant clone.

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# Bioactive Compounds Profiling and Their Antioxidant Properties in the Leaves of *Curcuma alismatifolia* (Zingiberaceae)

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## Introduction

Bioactive compounds often accumulate in the plant in small quantities and sometimes in specific cells (Finley, 2005). Among them some are known as phenolic, flavonoids, and essential oils, which possess a wide range of biological activities such as antioxidant, anti-inflammatory, anti-aging, anti-bacterial, anti-tumor, and other functions (Karimi et al., 2013).

Antioxidants are compounds known to slow or delay lipid oxidation. Suppressive antioxidants can separate free radicals or single oxygen before any significant oxidant occurrence. However, chain-breaking antioxidants delay or slow the oxidative processes after they start up (Argolo et al., 2004). The genus *Curcuma* from Zingiberaceae family originated from the Indo-Malayan Region (Purseglove, 1968) with a wide-spread distribution in the tropics of Asia to Africa and Australia. *Curcuma alismatifolia* is a monocotyledonous perennial, originating from tropical and subtropical areas of Northern Thailand and Cambodia (Apavatjrut et al., 1999). One of the most challenging pursuits in the realm of pharmaceutical and medical sciences is to investigate the latest and more potent drugs with fewer toxic effects and completely reversible. Much of these features can easily be found from the natural compounds of plants (Lewis, 2001). This research was carried out to analyze the bioactive compounds, such as the phenolic, flavonoid and antioxidant activities using the high performance liquid chromatography (HPLC) in the leaves of *C. alismatifolia* var. *sweet pink*.

## Materials and Methods

### *Plant material*

The plant materials used in this study were the leaves of *C. alismatifolia* var. *sweet pink* which their rhizomes were provided from a *Curcuma* nursery (Ubonrat) in Doisaket District, Chiang Mai 50220, Thailand.

### *Plant extraction*

Samples were extracted using methanol solvent and the extraction techniques used were reflux method (Crozier et al., 1997) with slight modifications.

### *Determination of total phenolic compound*

Total phenolic content of the extract was determined colorimetrically, using the Folin-Ciocalteu method as illustrated by Ismail et al. (2010). The extract was measured using a visible spectrophotometer (Novaspec II Visible Spectrophotometer, Pharmacia Biotech, Cambridge, UK) at absorbance at 765 nm and the result expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (DW).

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*Determination of total flavonoid compound*

Total flavonoid content (TFC) was determined using the method of Ismail et al. (2010) with standard favonoid rutin. The extract was measured using absorbance at 510 nm and the result was expressed as milligrams of rutin equivalents per gram of dry matter.

*Evaluation of phenolic and flavonoid compounds*

The phenolic and flavonoid compounds of samples were quantitatively measured by the reversed-phase HPLC technique based on Crozier et al. (1997). The standards for phenolic compounds were ellagic acid, salicylic acid, gallic acid, catechin, epicatechin, caffeic acid, cinnamic acid and resorcinol. The standard for flavonoid compounds were naringin, apigenin, rutin, quercetin, and myricetin.

*Antioxidant activity*

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

The DPPH of the extracts were determined by the method of Gulcin et al. (2004). Butylated hydroxytoluene BHT, α-tocopherol and vitamin C were utilized as the standard antioxidants.

Ferric reducing antioxidant power (FRAP)

The FRAP property of the extracts was determined using the method as described by Yen and Chen (1995). BHT, α-tocopherol and vitamin C were utilized as the standard antioxidants.

2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ABTS radical cation-scavenging

The ABTS was evaluated by the method of Giao et al. (2007). ABTS radical cation (ABTS*+) was produced by reacting ABTS stock solution with 2.45 mM $K_2S_2O_8$ and allowing the mixture to stand at room temperature (dark place) overnight before utilization.

**Results and Discussion**

*Curcuma* species plants have received much attention, since they produce many beneficial compounds that are useful in the food industry as herbs, flavoring and in the medical industries, as antioxidants and antimicrobial agents. The obtained results showed that the total phenolic value was 2.08±0.02 mg GAE/g DW, and total flavonoid was 1.61±0.17 mg rutin equivalent/g DW. Meanwhile, the HPLC results indicated the presence of phenolic compounds in the leaves of *C. alismatifolia* var. *sweet pink* which were salicylic acid (406.2 µg/g), caffeic acid (125.2 µg/g), catechin (212.9 µg/g), epicatechin (856.2 µg/g), cinnamic acid (10215.4 µg/g), ellagic acid (182.6 µg/g), resorcinol (195.9 µg/g). In addition, flavonoids were rutin (1032.7 µg/g), naringenin (271.5 µg/g), quercetin (964.1 µg/g), and myricetin (166.1 µg/g) respectively. The HPLC chromatogram in Figure 1 shows the phenolic compounds in the leaves of *C. alismatifolia* var. *Sweet Pink*. Furthermore, the antioxidant properties of the methanolic extract at a concentration of 300 µg/ml in all the assays were 53.5%, 51.7% and 54.1%, respectively, but the activities were lower than those of antioxidant standards, such as vitamin C and E. The $IC_{50}$ value of the leaf extracts is presented in Table 1.

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Figure 1. The HPLC chromatogram of phenolic compounds in the leaves of *C. alismatifolia* var. *sweet pink*.

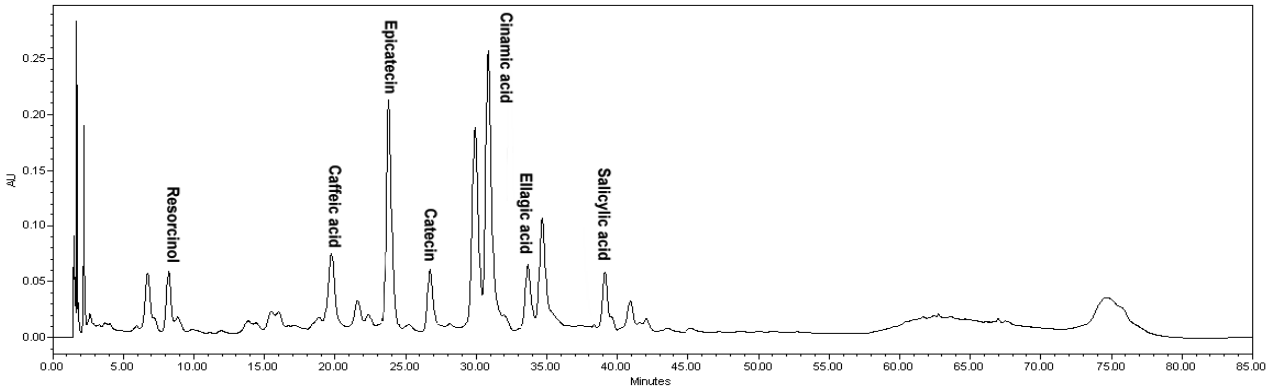


Table 1. The $IC_{50}$ values of extracts and standards in DPPH, FRAP and ABTS scavenging activities (Mean ± SEM; n = 3).

| | $IC_{50}$ (µg/mL) | | |
|---|---|---|---|
| Samples | Free radical Scavenging Activity | Total Antioxidant Activity | ABTS Scavenging Activity |
| Control | 260.7 ± 1.29 | 278.1 ± 1.42 | 288.1 ± 1.35 [a] |
| Vitamin C | 58.1 ± 1.47 | 90.9 ± 2.11 | - |
| Vitamin E | 60.3 ± 3.04 | 61.88 ± 1.86 | - |
| BHT | 89.7 ± 2.43 | 89.7 ± 1.37 | - |
| Trolox | - | - | 174.47 ± 012 [e] |

## Conclusions

Phytochemical compounds like phenolic acids and flavonoids are believed to be responsible for the wide spectrum of pharmacological activities attributed to the herb. It could be concluded that the leaves of *C. alismatifolia* var. *sweet pink* contain variable patterns of phenolic and flavonoid compounds which might play a major role as antioxidants

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# Comparison of Column Performance between Monolithic and Particulate Packing for the Separation of Photosynthetic Pigments

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## Introduction

*Pleomele angustifolia* N. E. Brown was one of the Indonesian local species which has been used as natural colorants. This species provides strong green coloration for food without unhealthy side effects. Accordingly, *P. angustifolia* is considered as one of the potential natural sources for colorants and functional foods. High-performance liquid chromatography (HPLC) method has been employed for analyzing photosynthetic pigments. Ultra-fast liquid chromatography (UFPLC) was one of the newest generations of HPLC which has special advantages in conducting researches with low time consuming and high resolution. It is generally known that in HPLC/UFPLC, separation quality is affected by four main parameters, i.e. mobile phase, flow rate, column temperature and column type. Among them, column had been understood as an important part where pigment separation occurred during analysis. Other separation parameters are depending on the column type used. HPLC/UFPLC columns are distinguished as monolithic and particulate packed type (Unger et al., 2008). Monolithic column is consisting of small-sized skeletons and wide through-pores which can achieve higher separation efficiency than the case with particulate packed columns at a similar pressure drop (Nunez et al., 2008). Thus, this column type has advantages in providing good separation and short time analysis. On the other hand, particulate packed column is also widely used for pigment separation. Two most well-used particulate packed columns are octyl (C8) and octadecyl (C18) types based on silica and they are much used compared to monolithic column in separating photosynthetic pigments (Wright et al., 1991; Jeffrey et al., 1997; Zapata et al., 2000; Garrido et al., 2003). In this report, we compare the column performance between monolithic and particulate packing columns for the separation of photosynthetic pigments extracted from leaves of *P. angustifolia* using a simple isocratic system. Our findings provide important basic information and can help to develop low-cost and simple HPLC technique for separation of plant pigments.

## Materials and Methods

### *Plant materials and pigments extraction*

Leaves of *P. angustifolia* were collected from MRCPP Arboretum and used throughout this study. Sample leaf was ground using a mortar with small amount of sodium ascorbate and $CaCO_3$ to avoid pigment oxidation and acidification. Liquid nitrogen was added to prevent enzymatic reaction which acts on the pigment deterioration. The homogenate (0.2 g wet weight) was extracted with 3 ml of 100% methanol (GR for analysis, MERCK, Darmstadt, Germany). In order to minimize photo-degradation and oxidation of the pigments, the extractions and measurements were carried out under green dimmed light and nitrogen (99%) atmosphere at room temperature. This extraction method was conducted in less than 1 min.

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*UFPLC analysis*

UFPLC analysis was performed using LC-20AD XR (Shimadzu, Kyoto, Japan) equipped with photodiode array detector SPD-20MA and column oven CTO-20AC. Pigments separation was carried out using a simple isocratic mobile phase consisted of acetonitrile (ACN) (HPLC Grade, MERCK) and methanol (MeOH) at a fixed flow rate of 0.5 ml per min. These ACN:MeOH (v/v) compositions were varied for analysis: 20:80 (Sys. 1), 35:65 (Sys. 2), 50:50 (Sys. 3), 65:35 (Sys. 4), and 80:20 (Sys. 5) at temperatures of 30 and 40 °C. Three different types of column were used: i.e. Chromolith (Performance RP-18e, 4.6 mm i.d. x 100 mm, MERCK), Shim-Pack XR-ODS (3 mm i.d. x 100 mm, Shimadzu) and Shim-Pack XR-C8 (3 mm i.d. x 100 mm, Shimadzu). Pigments were detected in the range of absorbance at 190-800 nm. Prior to injection, sample pigment was filtrated through a membrane filter (0.2 µm, Nylon, Whatman, Maidstone, UK). Pigment solution, 20 µL, was injected for analyses through an auto-sampler SIL-20AC XR (Shimadzu).

*Pigment identification and data analysis*

All targeted peaks were isolated for identification. Visible absorption spectra were obtained by UV-Visible Spectrophotometer 1800 (Shimadzu) in the absorbance range of 350-800 nm. Isolated pigments were checked by diluted different solvents. Chlorophyll (chls) groups were measured in acetone, diethyl ether and ethanol, while carotenoid groups in acetone, n-hexane and ethanol. Spectral maximum absorbance was then compared with those of references and standard spectra from plant pigments (Gross, 1991; Britton et al., 1995; Jeffrey et al., 1997; Hegazi et al., 1998). UFPLC data were revealed from original Shimadzu UFPLC operation software, Lab Solution. Plot data and polynomial regression were created by Origin 7.0 (Origin Lab Corp, Northampton, USA). Both numeric and graphic data were representing an average from triplicate.

**Results and Discussion**

Methanol extract of *P. angustifolia* was analyzed using UFPLC in the indicated conditions as mentioned in the text. Six dominant pigment species were separated from all samples using different columns (Figure 1). These pigments were then isolated and subjected to UV-Vis spectroscopy for identification. Pigments were identified as followed: violaxanthin ($1^{st}$ peak), zeaxanthin ($2^{nd}$ peak), chl *b* ($3^{rd}$ peak), chl *a* ($4^{th}$ peak), α-carotene ($5^{th}$ peak) and β-carotene ($6^{th}$ peak) (data not shown). These pigments are found in most of the higher plants as similar to the previous reports (Gross, 1991; Ottander et al., 1995; Schoefs, 2004; Timperio et al., 2007).

Performance of three columns was compared with different column temperatures at 30 and 40 °C under fixed conditions (isocratic, Sys. 3 mobile phase, 0.5 mL/min) (data not shown). Generally, retention times of pigment separation varied depending on the mobile phase compositions used in any column types. In C18 columns, particulate packed column needed longer time analysis than monolithic column. In terms of column temperature, higher temperature (40 °C) shortened analysis times in any column types used. XR-ODS column was superior to other two columns for the separation of non-polar pigments, although it needed longer time of separation and gave lower resolution. XR-C8 column had advantage for fast separation of polar pigments, but it was inferior for separation of non-polar pigments.

Subsequently, several chromatogram parameters were analyzed in order to understand the actions of solvent composition in details. Retention time difference ($\Delta t_R$) and peak retention time ratio ($t_R$ ratio) were important factors which can be applied to analyze separation characteristics of non-polar and polar pigments. Separation of non-polar pigments using a simple isocratic mobile phase based on acetonitrile

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and methanol (Sys. 3, 50:50) was not perfectly sufficient for any columns used and can only be done by changing solvent ratio under the conditions used (0.5 ml/min and 40 °C). We selected retention time of chl *a*, violaxanthin (viol), and β-carotene (β-car), as peak position indicators in calculating $\Delta t_R$ and $t_R$ ratio and also to see time difference between polar (viol) and semi-polar (chl *a*) pigments and between semi-polar (chl *a*) and non-polar (β -car) pigments. Data of $\Delta t_R$ calculated from the results of Chromolith and XR-ODS columns indicated that time of separation for polar pigment increased when acetonitrile concentration increased, but decreased for that of non-polar pigment (Figure 2).

Another parameter, $t_R$ ratio, $t_{R\ \mathbf{chl\_a/viol}}$ and $t_{R\ \mathbf{b\text{-}car/chl\_a}}$ ratios were used as peak indicators. Solvent composition (ACN: MeOH) and column temperature gave the different results of separations. Column temperatures (30 and 40 °C) had little effect on the separation for non-polar pigments (data not shown). These findings were also supported by their polynomial regressions (data not shown). Moreover, $t_{R\ \mathbf{b\text{-}car/chl\_a}}$ polynomial regression ($R^2$=0.99) was obtained at both temperatures in any columns used. These three columns were again proved to be superior in separating polar pigments. The results of $t_{R\ \mathbf{chl\_a/viol}}$ gave slight differences in all conducted analyses (data not shown). It is likely that column temperature at 40 °C and high concentration of methanol decreased time analysis.

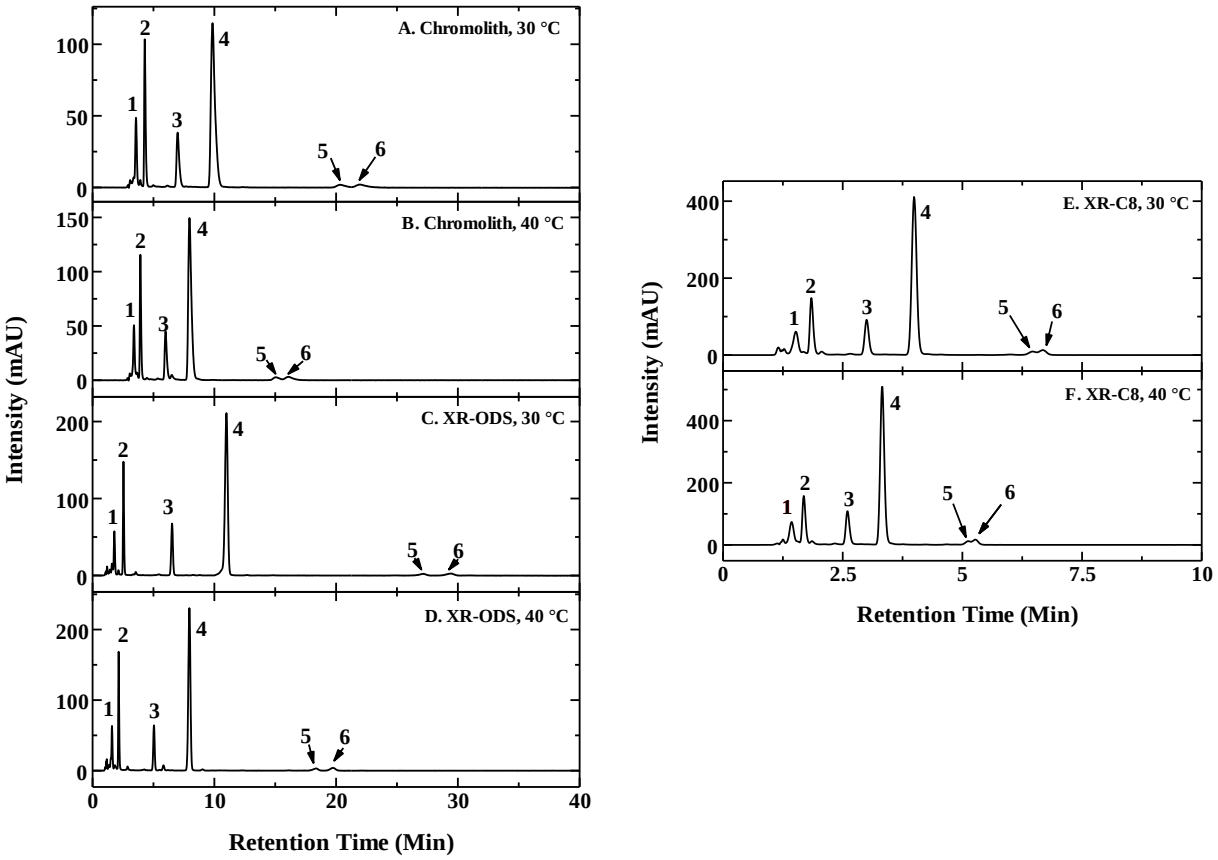


Figure 1. UFPLC chromatograms of photosynthetic pigments from leaves of *P. angustifolia*. UFPLC was carried out on an isocratic mode with ACN:MeOH (50:50, v/v) (Sys. 3) as a mobile phase at a flow rate of 0.5 ml per min. Other conditions are described in the text.

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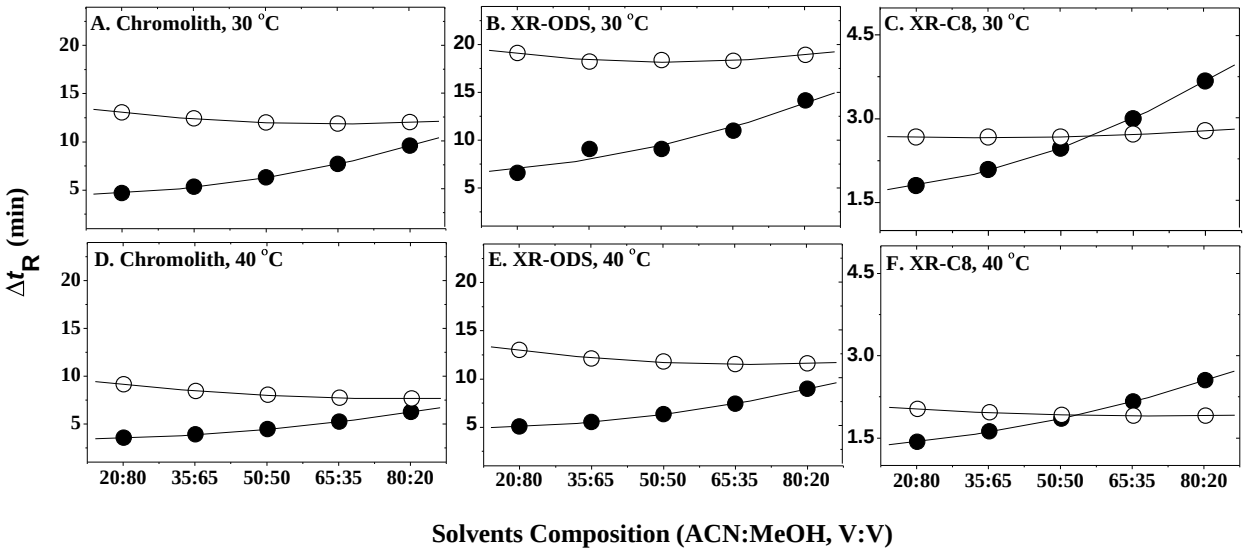


Figure 2. $\Delta t_{R\ \text{chl\_a-viol}}$ (solid circle) and $\Delta t_{R\ \text{b-car-chl\_a}}$ (open circle) were calculated from the results of UFPLC separation of photosynthetic pigments extracted from leaves of *P. angustifolia*. Other conditions are the same as in Figure 1. Data were average of three experiments. SE is less than ±0.5.

To obtain further information to optimize separation, retention time ($t_R$), separation factor ($\alpha$) and resolution ($R_s$) were calculated to chl *a* as a representative pigment using three columns and different solvent systems (Sys. 1 to 5), then the data were analyzed. Separation time was strongly influenced by both high concentration of acetonitrile in methanol mixture and column temperature. The most rapid pigment separation was performed by the use of XR-C8 column (cf. Figure 1). Total analysis was completed within less than 5 min under any solvent systems (Sys. 1 to 5). Highest separation factor was achieved by XR-C8 in Sys. 2 (α 1.3) and Sys 4 (α 1.3) at 30 °C, while the highest resolution was achieved in Sys. 4 at 30 °C ($R_s$ 3.5). High column temperature influenced separation time and only needed 2.8 min in Sys 1. XR-ODS gave the slowest pigment separation, e.g. 6.6 min in Sys 1 at 40 °C to 16.3 min in Sys. 5 at 30 °C. However, highest separation factor was obtained in Sys 3 (α 1.3) at 30 °C as well as highest resolution ($R_s$ 5.3). Chromolith column was likely the most effective column to separate chl *a* among three columns used. At high column temperature (40 °C), it needed minimum 7 min in Sys. 1. High separation factor and resolution were found in Sys. 4 at 30 °C (α 1.7, $R_s$ 5.9).

## Conclusions

In this report, we compared the column performance between monolithic and particulate packing columns for the separation of photosynthetic pigments extracted from leaves of *P. angustifolia* using simple isocratic system. Although each column showed their own features, monolithic column Chromolith provided better resolution and faster analysis among three columns used. These findings can help to optimize pigment separation in rapid time analysis by using simple, safe and low cost mobile phase system.

## Acknowledgements

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# Chlorophyll Values of Local Green Vegetables Common in Malang, East Java

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## Introduction

Healthy human diet is generally associated with an adequate consumption of fresh colored vegetables as source of micronutrient as well as dietary fiber (Jaiswal McEligot et al., 2006). Varieties of vegetable types and quality are normally affected by their cultivation area, particularly related to soil and water quality, as well as temperature and air cleanliness. Malang area is one of the highland areas in Indonesia that has great potential in providing diverse vegetables. The city is surrounded by several active volcanoes and hence providing appropriate soil and environmental condition for vegetable production. In fact, Malang has become the center of urban vegetable production in East Java.

The presence of chlorophylls in the leafy vegetables is a simple and facile indicator of the quality. The consumers usually assume bright green color for fresh and nutritious vegetable. As the major photosynthetic pigments in leaves, chlorophyll is visually observable by human eyes. Two most dominant pigments found in higher plants are chlorophyll *a* and *b* (Quach et al., 2004). These pigments are not only offering us coloration in our daily diet, but also biological activities (Ferruzzi et al., 2002). Having chemical structure which is close to hemoglobin, chlorophylls intake is presumed to play a distinct role in the regeneration of blood cell (Mujoriya et al., 2012).

Several investigations have reported photosynthetic pigment composition which exist in several edible leaves (Lai et al., 1980; de Vogel et al., 2005). However, quantity of chlorophyll *a* in numeric data keeps showing their importance. There is some indication about correlation between leaves color visibility and chlorophyll content, but there are only few reports aimed to prove this phenomenon (Yamamoto et al., 2002; Gitelson and Merzlyak, 2003). Presently, photometric UV-Vis Spectroscopy is enhanced to non-destructive application for conducting simple rapid analysis. Manual color indicators are also replaced by L* (lightness), a* (redness/greenness), b* (yellowness/blueness) based on colorimeter, which is able to reduce error due to human subjectivity (Leon et al., 2006). The development of these instruments offers us a chance to conduct *in vivo* experiments. In this report, we provide comparison data between the chlorophylls content of some Indonesian leafy vegetables and their color value using *in vivo* non-destructive method.

## Materials and Methods

### *Plant materials*

Ten species of edible leafy vegetables (Table 1) were purchased from five different markets in Malang area (East Java, Indonesia). All samples were collected early in the morning to get fresh condition of the product. The samples were directly measured by *in vivo* procedure without any involved sample destruction, and then stored at -20 $^{\circ}$C deep freezer for further analysis. All samples were collected during May until June 2014.

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*Colorimetric assay*

Colorimetric measurement was conducted using Colorflex EZ (Hunterlab, USA). L*, a*, and b* parameters were used to quantify the leaves color. L* value represents color brightness (luminosity; 0: black, 100: white), while a* (positive values: red, negative values: green) and b* (positive values: yellow, negative values: blue) show color dimension.

*Chlorophyll content assay*

The chlorophyll content was determined by using the chlorophyll meter SPAD-502 (Konica Minolta, Japan). Several experiments were accomplished in order to have representative data of each sample. Three leaves per species were used, and then each leaf was spotted in five to fourteen different positions for measurements. As for Kemangi, due to its smaller leaf size, seven leaves were used and two up to three spots were measured per leaf.

*Data analysis*

All data in this report are provided as the average value of multiple experiments. Figures were created from numeric data and plotted using Origin 7 Software (OriginLab).

**Results and Discussion**

Ten leafy vegetables as listed in Table 1 were analyzed using *in-vivo* method. Photometric chlorophyll and color detection were applied in order to evaluate chlorophyll content in its correlation with color appearance. SPAD data provides us representative chlorophyll concentration which is available on the sample (Chang and Robison, 2003). Higher SPAD values signify higher chlorophyll content. The mostly advanced rapid colorimeter assay is according to L*, a*, b* parameter developed by the *Commission Internationale de l'Eclairage* (CIE) of France. L*, a*, b* dimension was built in order to standardize color appearance and decrease subjectivity of human visualization. Lower L* value represents darker appearance, while higher value represents brighter appearance. The chromatic value of a* and b* are the two dimensional color parameter that will provide us color position of the measured samples.

Table 1. The ten species of leafy vegetables collected from five local markets in Malang, East Java, Indonesia.

| Local Name | Abbr. | Common Name | Scientific Name |
|---|---|---|---|
| Kailan | Kai | Chinese kale | *Brassica oleracea* var. *alboglabra* Bailey |
| Chaisim or sawi hijau | Cha | Chinese flowering cabbage | *Brassica rapa* L. var. *parachinensis* (L. H. Bailey) Hanelt |
| Bayam | Bay | Spinach | *Amaranthus spinosus* L. |
| Kangkung | Kan | Water spinach | *Ipomoea aquatica* Forsk |
| Daun Singkong | Sin | Cassava leaf | *Manihot utilissima* Pohl |
| Selada | Sel | Green leaf lettuce | *Lactuca sativa* L. |
| Pakcoy | Pch | Chinese cabbage pak-choi | *Brassica rapa* L. subsp. *chinensis* |
| Kemangi | Kgi | Basil | *Ocimum citriodorum* Vis. |
| Siomak | Sak | Sworn-leaf lettuce | *Lactuca sativa* L. var. *augustana* |
| Kubis | Kbs | Head cabbage | *Brassica oleracea* L. var. *capitata* |

*Abbr. = abbreviation used in the text*

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Figure 1 shows the results of in vivo analysis of green leafy vegetables by means of SPAD-502 Chlorophyll meter as well as the Colorflex. The SPAD, L*, and b* values were significantly varied ($p$<0.01), while the difference of a* values did not show any statistical significance ($p$<0.05) among all the vegetables. Pch was proved to be superior in providing chlorophyll on our daily diet (51.6±2.4 SPAD), while the chlorophyll content of most others species were comparable, i.e. Kai, Cha, Bay, Kan, Sin, and Kgi. Three vegetables which provide less chlorophyll content were Kub, Sel, and Sak (Figure 1A). Further investigation was then carried out by employing colorimetric assays. Kbs exhibited considerably higher L* value (Figure 1B) since its foliage has pale color. This characteristic revealed inverse correlation between lightness value and chlorophyll concentration. The L* value of vegetables rich in chlorophyll were maximum at about forties. On the other hand, according to chromatic values, the parameter of a* includes green to red region, while b* value covers blue to yellow color, from negative to positive denomination, respectively. The SPAD data of chlorophyll contents were found to be positively correlating toward chromatic value. Kbs possessed lowest chlorophyll content as well as lowest green coloration (Figure 2). Therefore, low chlorophyll vegetables, such as Kbs, Sel, and Sak, were easily identified through their higher L* value as well as lower absolute a* value (Figure 1C). Higher b* value indicates yellowish coloration, bearing low chlorophyll content (see b* value of Sel) (Figure 1D). Better interpretation of color space was provided in Figure 2. Compared to L* value, there is no strong correlation between chromatic value and SPAD value. The presence of other colorful plant pigments (e.g. carotenoid, anthocyanin) is suggested to cause greater variety in chromatic value.

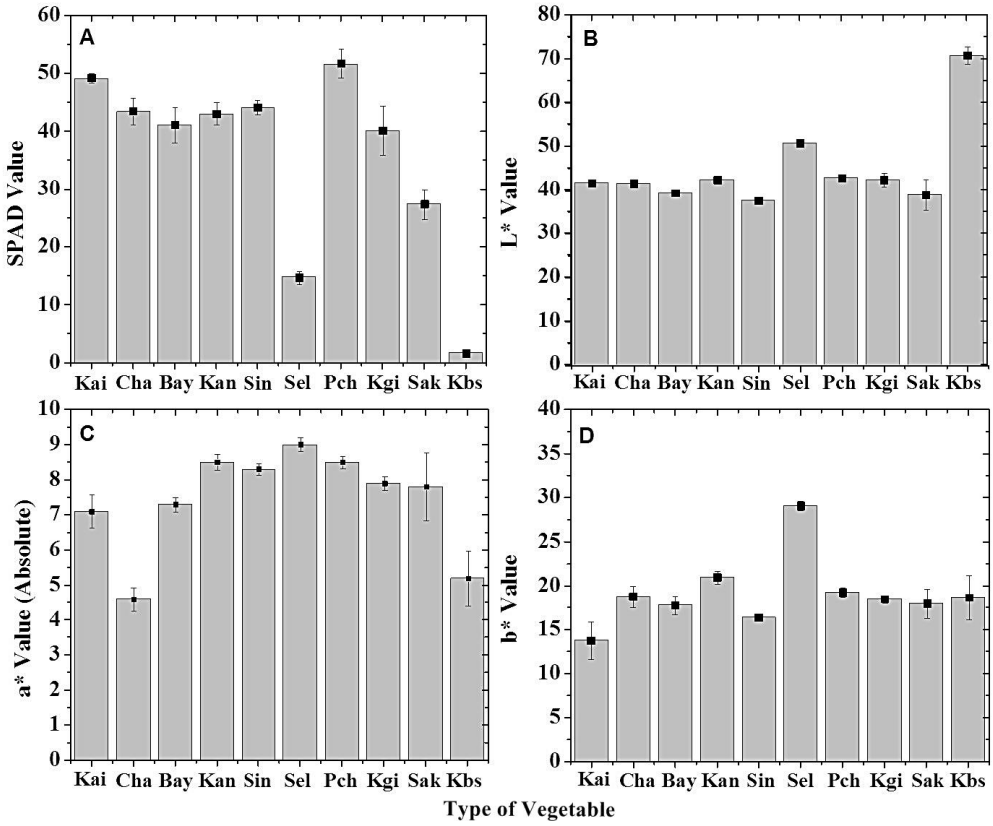


Figure 1. Distribution of chlorophyll content and color parameters of ten leafy vegetables. Abbreviations were defined in Table 1.

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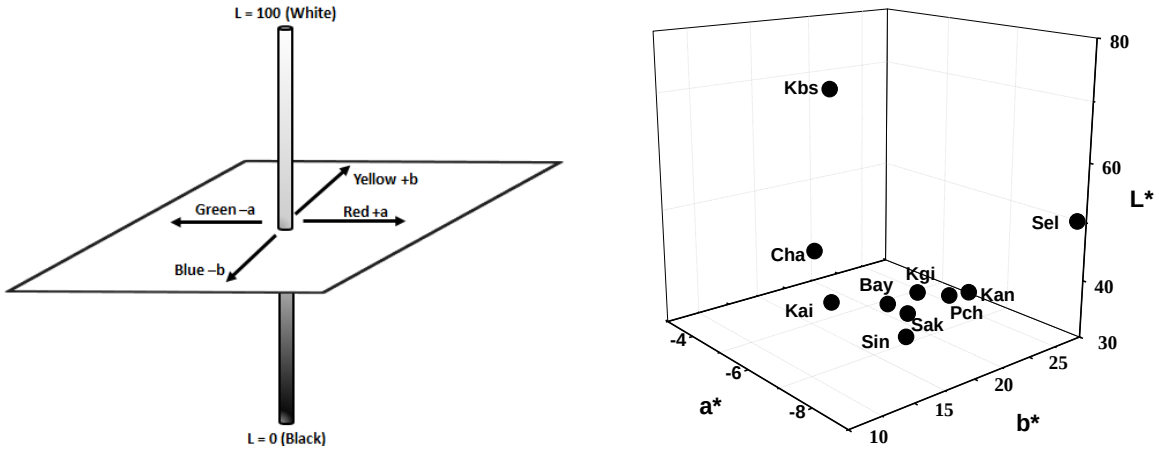


Figure 2. L*, a*, b* color space (left) and 3D Scattergram of colorimetric parameters (right) measured from ten leafy vegetables. Abbreviations were defined in Table 1.

Leafy vegetables, as other higher plant leaves, consist of chlorophylls (green) and carotenoids (yellow to red) to perform its photosynthetic function. Nevertheless, green coloration is generally exhibited in visual appearance due to domination of chlorophylls as the primary photosynthetic pigment, assisted by carotenoids as the accessory pigment (Sarijeva et al., 2007). The a* value could be considered as chlorophyll color space while b* value characterizes the presence of carotenoid group. At the present study, fresh samples were subjected to each measurement in order to provide the best natural condition of the sample. Otherwise, domination of yellowness color space (high b* value) can be interpreted as indication of chlorophyll degradation during senescent or decay process (Tijskens et al., 2001; Schelbert et al., 2009). Overall, colorimetric assay has proven its advantage in providing information which can be used as consideration of chlorophyll content in leafy vegetables. Development opportunity of new and non-destructive method for rapid determination of vegetable quality is still widely opened.

## Conclusions

The correlation between SPAD and L*, a*, b* values were acceptable to evaluate chlorophyll content based on color appearance. Therefore, this information becomes important as the basic information of proposing *in vivo* measurement method to determine vegetable quality as well as chlorophyll analysis based on color values. Among ten different species of leafy vegetables which were obtained from local market in Malang, high chlorophyll content was found in Pch>Kai>Sin>Cha>Kan>Bay>Kgi.

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