THE EFFECT OF MEDIUM COMPOSITIONS AND LIGHT ON THE PRODUCTION OF ASCORBIC ACID, α-TOCOPHEROL AND CAROTENOIDS IN *Centella asiatica* CALLUS

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ABSTRACT

The effect of medium strength, different sucrose and casein hydrolysate (CH) concentrations with varying pH of medium as well as photoperiods and light intensities on the synthesis of ascorbic acid, α-tocopherol and carotenoid content in *Centella asiatica* callus were evaluated. Full strength Murashige and Skoog (MS) medium with 30 to 50 g L^{-1} sucrose, with 0.5 to 1.0 g L^{-1} CH was the most favorable medium composition in enhancing the productions of all antioxidants studied. The pH of MS medium varying from 5.1 to 5.9 did not show any significant effect on the antioxidant activity. Continuous illumination with higher light intensities increased the production of ascorbic acid and α-tocopherol. Carotenoid production was reduced at higher light intensities while light photoperiod has no significant effect on carotenoid content. The above results indicated that the antioxidants produced can be modulated by manipulating the medium composition and physical culture growth conditions in *in vitro* callus of *C. asiatica*.

Keywords: *Centella asiatica*, ascorbic acid, α-tocopherol, carotenoids, antioxidants

INTRODUCTION

Centella asiatica (L.) Urban is a tropical medicinal plant from Apiaceae family which is indigenous to South East Asia, India, Sri Lanka, parts of China, the Western South Sea Islands, South Africa, Madagascar, Mexico, Venezuela, Columbia as well as Eastern South America (Jamil et al. 2007). Locally known as 'pegaga', *C. asiatica* is cultivated due to its medical importance and has a long history of utilization in Ayurvedic and Chinese traditional medicines (Orhan 2012). A wide range of biological activities in *C. asiatica* including antileprotic, antifilarial, antifeedant, antistress, antiviral and antibacterial have been reported by Peiris and Kays (1996). Phytochemical screening of *C. asiatica* has revealed the presence of a vast number of compounds belonging to several chemical classes. Major compounds are triterpenoids including asiaticoside, asiatic acid, madecassoside, madecassic acids and madasiatic acid which are known to be rich in healing properties. Other triterpenes are betulinic acid, thankunic acid and isothankunic acid (Williamson 2002; Pan et al. 2007). The presence of volatile oils, flavonoids and alkaloids were also reported by Peiris and Kays (1996), and Solet et al. (1998).

C. asiatica is a good source of dietary antioxidants including ascorbic acid, α-tocopherol, carotenoids and phenolic compounds. These compounds may contribute as first and second lines of defense against oxidative stress. As a result, they protect cells against oxidative damage and high intake of plant products is associated with a reduced risk of chronic diseases such as cancer, cardiovascular disease, atherosclerosis and diabetes (Temple 2000; Hashimoto et al. 2002; Gundgaard et al. 2003; Gosslau and Chen 2004). α-Tocopherol, ascorbic acid and carotenoids are important antioxidants which protect plants by suppressing oxidative injury. α-Tocopherol, which is located within biological membranes, can quench oxygen radicals, stabilising cell membranes by influencing lipid organization, thus protecting chlorophyll (Hess 1993). Ascorbic acid scavenges many types of free radicals affecting

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many enzyme activities and is also required for regeneration of α-tocopherol (Ahmad et al. 2008). Carotenoid is not only an accessory pigment in the photosynthetic apparatus but also quenches singlet oxygen efficiently (Sharma et al. 2012).

Various strategies using plant *in vitro* systems have been successfully employed to improve the production of valuable compounds and study their biosynthesis and metabolism (Hussain et al. 2012). Resolving the pathways for antioxidant biosynthesis in plants especially ascorbic acid, α-tocopherol and carotenoids, is a major breakthrough that will lead to improved understanding of the physiological and biochemical roles of these antioxidants. Genetic manipulations of these biosynthetic pathways have achieved alteration in composition of ascorbic acid, α-tocopherol and carotenoids. The biosynthetic genes can be isolated and characterised and have the potential to be used in improving the nutritional quality and environmental stress resistance of economically important plants. However, the attempts to increase the concentration of these compounds only gave limited results.

Manipulations of medium compositions, hormonal supplementation and physical growth factors such as temperature, light and atmospheric composition, and treatment with various precursors and elicitors have to be studied in detail to maximise the secondary metabolite production in plant. In addition, manipulating the bioregulators in cell cultures will be useful for breeding and propagation of crop plants and for elucidating primary and secondary metabolic pathways. In this work, manipulation of medium composition namely medium strength, sucrose concentration and CH, and medium pH as well as different light photoperiods (24/0; light/dark, 12/12; light/dark and 0/24; light/dark) and intensities (0 to 103.41 µmol/m^2/s) have been investigated in order to enhance the production of ascorbic acid, α-tocopherol and carotenoids in *C. asiatica* callus.

MATERIALS AND METHODS

Callus initiation and maintenance

Sterilisation of Explants

Young *C. asiatica* leaves (CA03) were washed thoroughly using tap-water for about 15 minutes and then were dipped in 70% (v/v) ethanol for 30 seconds, followed by placing them in 15% Clorox together with a few drops of Tween 20 for 10 minutes. Then, the explants were rinsed 4 to 5 times with sterile distilled water in order to remove detergent residue. Surface-sterilised leaf explants were cut into smaller segments (5 mm x 5 mm).

Culture Medium and Culture Condition

Sterile leaf explants were aseptically cultured on solid MS basal medium (Murashige and Skoog 1962) supplemented with 2.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} Kinetin. Sucrose at 30 g L^{-1} was added as a carbon source and B5 vitamins were used as suggested by Gamborg et al. (1968). Gelrite agar (2.5 g L^{-1}) was used to solidify the culture medium. The pH of the medium was adjusted to 5.7 using 0.1 N HCl or 0.1 N NaOH before autoclaving. The culture media were sterilized at 121°C for 15 minutes. The cultures were maintained by regular subculturing at 8 to 10 days interval on fresh medium. All cultures were incubated in 12 hours/12 hours (light/dark) photoperiod under cool, white fluorescent lamps at 27 ± 2°C. Callus obtained was carefully separated from the leaf explants and was transferred into a fresh medium. Friable callus obtained was used for further studies.

Callus treatments

The control callus was cultured in full MS basal medium supplemented with 2.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} Kinetin, 30 g L^{-1} (w/v) sucrose, B5 vitamins and pH of medium was adjusted to 5.7. About 2.5 g L^{-1} Gelrite agar was used to solidify the medium. The callus was maintained under 12 hours/12 hours (light/dark) photoperiod under cool, white fluorescent lamps at 27 ± 2°C. Each of the studied

parameters (media strength, sucrose and casein hydrolysate concentrations, pH of the medium as well as light photoperiod and intensity) was manipulated separately. Other parameters were maintained in control condition.

Effect of Media

Full (1) and half strength (½) of MS macronutrients from standard formulation of basal medium were used in this experiments and the control consisted of full basal MS medium. Effect of carbon source was studied using different concentrations of sucrose i.e. 0, 10, 20, 30, 40 and 50 g L^{-1} (w/v) with 30 g L^{-1} as a control. Effect of casein hydrolysate (CH) was examined in the range of 0 to 1.0 g L^{-1} with 0.25 g L^{-1} was used as a control. CH was added prior to autoclaving. Effect of initial pH value was examined at the range of 5.1 to 5.9 with 5.7 as the control pH.

Effect of Light

Effect of different light photoperiods i.e. 24/0 (light/dark), 12/12 (light/dark), 0/24 (light/dark) were studied under cool, white fluorescent lamps at 27 ± 2°C. Callus was cultured in different light intensities i.e. 0, 0.42, 1.37, 6.51, 18.85, 31.03 and 103.41 μmol m^{-2} s^{-1} under cool, white fluorescent lamps at 27 ± 2°C .

Antioxidant assays

After 10 days of treatments period, the production of ascorbic acid, α-tocopherol and carotenoids were assayed.

Determination of Ascorbic Acid

Ascorbic acid was extracted according to the procedure of Jagota and Dani (1982). A total of 0.15 g of callus tissue was ground with pre-chilled mortar and pestle in 1.0 mL of 10% trichloroacetic acid (TCA) and clean sand under dim light and in ice-cold conditions. The ground sample was then centrifuged (Eppendorf 5840R) at 10,000 rpm for 10 minutes at 4°C. The supernatant obtained (300 μL) was added into 1700 μL distilled water and 200 μL of 10% Folin reagent. The mixture was gently swirled and left under dim light for 10 minutes. Absorbance of the mixture was then measured at 760 nm. A standard curve was prepared using ascorbic acid at various concentrations (0 to 60 μg mL^{-1}). Ascorbic acid (300 μL) was added into the solution as described above and the amount of ascorbic acid in the sample was calculated based on the standard curve.

Determination of α-Tocopherol

α-Tocopherol was extracted based on the method by Hodges et al. (1996). Under dim light and over ice, 0.15 g of callus tissue was ground with 1.5 mL acetone and clean sand in a mortar and pestle at 0 to 4°C. The mixture was extracted with 0.5 mL hexane followed by vortexing for about 30 seconds. The mixture was then centrifuged at 10,000 rpm (Eppendorf 5840R) for 10 minutes. After the centrifugation, the top layer was removed and the hexane extraction was repeated twice. The assay mixture was prepared as described by Kanno and Yamauchi (1977). A total of 0.5 mL of the hexane-extract was added into 0.4 mL 0.1% (w/v) PDT (3-(2-pyridyl)-5,6-diphenyl-1,2,4 triazine, prepared in ethanol) and 0.4 mL 0.1% (w/v) ferric chloride (prepared in ethanol). The volume was made up to 3.0 mL with absolute ethanol and the mixture was gently swirled and left for 4 minutes for colour development. Following this, 0.2 mL of 0.2 M orthophosphoric acid was added to the mixture and allowed to stand for 30 minutes at room temperature before absorbance of the mixture was measured at 554 nm. The blank was prepared in the same manner except that absolute ethanol was used instead of the hexane-extracts. A standard curve was prepared using α-tocopherol (Sigma, type V) at various concentrations (0 to 1.4 μg mL^{-1}). The amounts of α-tocopherol in the samples were calculated based on the standard curve.

Determination of Carotenoids

Carotenoids content were analyzed according to the method proposed by Lichtenthaler (1987). Callus tissue (0.15 g) was ground up with 3 mL of 80% (v/v) acetone and clean sand in a mortar and pestle. The homogenate was centrifuged at 10,000 rpm (Eppendorf 5840R) for 10 minutes. Supernatant of the samples were measured spectrophotometrically at 663.2, 646.8 and 470 nm, while 80% acetone was used as a blank.

Statistical Analysis

Statistical analysis was performed using the SAS systems Version 6.0 computer statistic program. All experiments were done in five replicates and results obtained were compared by two-way analysis of variance (two-way ANOVA) and tested by Duncan's multiple range test (DMRT) and Least Significant Different (LSD) to determine the differences between treatment means and day of treatments at 95% ($p<0.05$) significance level.

RESULTS

Effects of different culture conditions

Table 1 shows the effects of media strength (full and half strength of MS macro and micronutrients), sucrose concentrations, pH of the medium and different concentrations of CH on the production of α-tocopherol, ascorbic acid and carotenoids in *C. asiatica* callus. Analysis on full and half strength of basal MS media supplemented with B5 vitamins showed that there was no significant difference ($p>0.05$) in the concentration of ascorbic acid and carotenoids in the callus placed on both medium strengths. The results obtained showed that the α-tocopherol concentrations was significantly higher ($p<0.05$) in callus placed in full strength medium (1.24 ± 0.04 μg g^{-1} fwt) compared to half strength medium (0.99 ± 0.11 μg g^{-1} fwt).

Generally, higher concentrations of sucrose (40 and 50 g L^{-1}) produced higher concentration of ascorbic acid (44.68 ± 2.38 and 49.01 ± 4.09 mg g^{-1} fwt, respectively). However, medium without sucrose managed to produce higher amount of ascorbic acid (41.08 ± 3.88 mg g^{-1} fwt) compared to medium with 10 g L^{-1} sucrose (28.11 ± 2.52 mg g^{-1} fwt). Sucrose concentration (0 to 50 g L^{-1}) did not significantly affect ($p>0.05$) the concentration of α-tocopherol. Similarly, callus cultured on medium without sucrose managed to induce higher production of carotenoids compared to those media supplemented with sucrose. The least production of carotenoids was in medium added with 20 g L^{-1} sucrose (0.36 ± 0.01 mg g^{-1} f wt).

The effects of CH were studied in the range of 0 to 1.0 g L^{-1}. CH had significant effect on the production of ascorbic acid. Results obtained indicated that the highest concentration of ascorbic acid was recorded in callus treated with 1.0 g L^{-1} casein (92.25 ± 2.57 mg g^{-1} fwt) while the lowest was observed in 0 g L^{-1} casein (42.16 ± 2.57 mg g^{-1} fwt). As the concentration of casein increased, the production of ascorbic acid also increased. On the other hand, increased concentration of CH did not enhance the production of α-tocopherol. No significant difference ($p>0.05$) was observed in callus without casein and callus treated with 0.5 to 1.0 g L^{-1} casein. Callus treated with 0.25 g L^{-1} casein produced the lowest yield of α-tocopherol (0.29 ± 0.02 μg g^{-1} fwt). No significant difference ($p>0.05$) was observed in the carotenoid content when callus was treated with 0.5, 0.75 and 1.0 g L^{-1} casein (0.56 ± 0.06, 0.51 ± 0.07 and 0.51 ± 0.03 μg g^{-1} fwt, respectively). However, higher concentrations of casein (0.5 to 1.0 g L^{-1}) produced higher yield of carotenoids compared to lower concentrations of casein (0 and 0.25 g L^{-1}).

Table 1 also shows the effects of medium pH on the production of ascorbic acid, α-tocopherol and carotenoids. Varying the pH of the medium (pH 5.1 to 5.9) had no significant effect ($p>0.05$) on the production of ascorbic acid and carotenoids in *C. asiatica* callus cultures. For α-tocopherol concentration, higher pH value (pH 5.9) significantly reduced the production of α-tocopherol

compared to pH 5.1 and 5.5. No significant difference ($p>0.05$) was observed in the α-tocopherol production when pH of the medium was adjusted to 5.1, 5.3, 5.5 and 5.7, respectively.

Effects of Light Photoperiods and Intensities

In *C. asiatica* callus, illumination was found to significantly affect the composition of ascorbic acid and α-tocopherol. Cultures exposed to 24 hours of light illumination significantly stimulated ($p<0.05$) the production of ascorbic acid and α-tocopherol compared to 12/12 (light/dark) and 0/24 (light/dark) photoperiods. The least production was in 0/24 (light/dark) photoperiod. However, different light photoperiods did not lead to a significant difference in carotenoids production of *C. asiatica* callus.

Higher light intensities were found to significantly enhance ($p<0.05$) the production of ascorbic acid and α-tocopherol. Upon elevating the light intensities from 18.85 μmol $m^{-2}s^{-1}$ to 103.41 μmol $m^{-2}s^{-1}$, there was no significant difference ($p>0.05$) observed in the yield of ascorbic acid, whereas the maximum production of α-tocopherol was observed in cultures exposed to 31.03 μmol $m^{-2}s^{-1}$ of light intensity (0.96 ± 0.05 μg g^{-1} fwt). In contrast, higher light intensities significantly ($p<0.05$) inhibited the production of carotenoids (Table 2).

Table 1. Effects of different culture conditions on the production of ascorbic acid, α-tocopherol and carotenoids in *C. asiatica* callus after 10 days of treatment

Treatment		Ascorbic acid (mg g^{-1} fwt)	α-Tocopherol (μg g^{-1} fwt)	Carotenoids (mg g^{-1} fwt)
Medium strength	full	108.83 ± 8.63^{a}	1.24 ± 0.04^{a}	0.19 ± 0.04^{a}
	half	110.63 ± 7.31^{a}	0.99 ± 0.11^{b}	0.25 ± 0.03^{a}
Sucrose (g L^{-1})	0	41.08 ± 3.88^{ab}	0.73 ± 0.10^{a}	0.69 ± 0.02^{a}
	10	28.11 ± 2.52^{c}	0.72 ± 0.07^{a}	0.46 ± 0.06^{ab}
	20	35.67 ± 4.32^{c}	0.66 ± 0.02^{a}	0.36 ± 0.01^{c}
	30	38.39 ± 5.70^{abc}	0.58 ± 0.07^{a}	0.51 ± 0.04^{ab}
	40	44.68 ± 2.38^{ab}	0.66 ± 0.06^{a}	0.61 ± 0.03^{a}
	50	49.01 ± 4.09^{a}	0.56 ± 0.03^{a}	0.61 ± 0.09^{a}
CH (g L^{-1})	0	42.16 ± 2.58^{d}	0.55 ± 0.04^{a}	0.21 ± 0.03^{b}
	0.25	53.33 ± 3.68^{c}	0.28 ± 0.02^{b}	0.27 ± 0.06^{b}
	0.50	57.66 ± 3.65^{c}	0.61 ± 0.04^{a}	0.56 ± 0.07^{a}
	0.75	77.12 ± 6.82^{b}	0.59 ± 0.09^{a}	0.51 ± 0.07^{a}
	1.00	92.25 ± 2.57^{a}	0.58 ± 0.07^{a}	0.51 ± 0.03^{a}
pH	5.1	67.03 ± 6.28^{a}	0.54 ± 0.04^{a}	0.49 ± 0.05^{a}
	5.3	54.78 ± 8.63^{a}	0.48 ± 0.02^{ab}	0.47 ± 0.01^{a}
	5.5	65.59 ± 5.12^{a}	0.51 ± 0.03^{a}	0.46 ± 0.02^{a}
	5.7	60.90 ± 7.61^{a}	0.48 ± 0.04^{ab}	0.39 ± 0.04^{a}
	5.9	74.95 ± 7.35^{a}	0.39 ± 0.04^{b}	0.42 ± 0.04^{a}

Data shown are means ± SE (n=5). Means with the same letters in a column for each treatment are not significantly different at $p>0.05$.

Table 2. Effect of light photoperiods and intensities on the production of ascorbic acid, α-tocopherol and carotenoids in *C. asiatica* callus after 10 days of treatment

Treatment		Ascorbic acid (mg g^{-1} fwt)	α-Tocopherol (μg g^{-1} fwt)	Carotenoids (mg g^{-1} fwt)
Light photoperiod (light/dark)	24/0	114.59 ± 5.54[a]	1.22 ± 0.09[a]	0.17 ± 0.01[a]
	12/12	85.41 ± 11.23b	0.96 ± 0.05[b]	0.19 ± 0.02[a]
	0/24	68.48 ± 2.85[c]	0.72 ± 0.03[c]	0.21 ± 0.02[a]
Light intensity (μmol $m^{-2}s^{-1}$)	0	75.32 ± 2.16[c]	0.71 ± 0.04[bc]	0.26 ± 0.04[a]
	0.42	68.47 ± 4.38[c]	0.74 ± 0.06[abc]	0.13 ± 0.02[bc]
	1.37	63.42 ± 3.75[bc]	0.62 ± 0.05[bc]	0.13 ± 0.003[b]
	6.51	95.08 ± 4.25[ab]	0.58 ± 0.04[c]	0.13 ± 0.02[bc]
	18.85	107.39 ± 11.36[a]	0.65 ± 0.08[bc]	0.06 ± 0.01[d]
	31.03	110.99 ± 11.57[a]	0.96 ± 0.17[a]	0.07 ± 0.01[d]
	103.41	99.46 ± 9.02[a]	0.85 ± 0.08[ab]	0.08 ± 0.01[bcd]

Data shown are means ± SE (n=5). Means with the same letters in a column for each treatment are not significantly different at p>0.05.

DISCUSSION

Numerous strategies have been used to increase the antioxidant production in plant. Manipulation of physical aspects as well as nutritional elements in a culture is perhaps the most fundamental approach for optimization of culture productivity. Manipulation of secondary product formation in medicinal plants is possible by varying the culture conditions, including medium composition and pH, light/photoperiod and growth regulator type and concentration (Collin 2001). Indeed, Aziz et al. (2007) found that the asiaticoside and madecassoside content in leaves of *C. asiatica* varied according to culture medium composition, mainly different concentrations of the growth regulator, 2,4-D. Studies on secondary metabolite production in whole plants are often difficult because the biosynthetic activities may only be expressed in particular cell types within a specific plant organ or at certain time of season. Therefore, plant cell cultures were used in this study as the initiation of cell growth in culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle (Dornenburg and Knorr 1995). Thus, it can provide large scale production of active compounds or secondary metabolites such as alkaloids, terpenes, anthocyanins and anthroquinones (Alfermann and Pertersen 1995; Stockigt et al. 1995; Fu et al. 1999). However, reports on the production of antioxidants particularly on the ascorbic acid, α-tocopherol and carotenoid contents of *C. asiatica* cultures are scarce.

Decreasing the concentration of the macro and micronutrients to half of their usual strength in MS medium significantly lowered the production of α-tocopherol compared with the control while different ionic strengths did not significantly affect the ascorbic acid and carotenoid content in *C. asiatica* cultures. This observation was similar with the results reported by Hilton and Rhodes (1990) where the total amount of other secondary metabolites, hyoscyamine was comparable for cultures of *Datura stramonium* grown on either full or half strength of B5 medium. However, the results for the α-tocopherol level contradicted with the study by Drewes and Staden (1995) on the production of solasodine in *Solanum mauritianum* berries. They found that decreasing the medium strength resulted in a great increase in solasodine production particularly when it was reduced to half strength. Increase in the production may be postulated by the metabolic stress induced by the reduced nutrient in the medium.

Among the nutritional factors, sucrose is of special significance in cell growth, morphology, differentiation and secondary product formation (Selles et al. 1997), serving as the principal energy source and a component for biosynthesis. In addition, sugars could also act as a precursor of metabolic

processes and act as a signalling molecule for promoting gene expressions. Anna (2004) has identified sucrose as the best carbon source for producing the highest yield of triterpenes in callus cultures of *C. asiatica*. A similar sucrose preference rather than glucose or fructose has also been reported in the production of the steviol glycosides in *S. rebaudiana* cultures (Bondarev et al. 2003). Elevating the sucrose concentration in the medium from 0 to 50 g L^{-1} had no significant effect on the production of α-tocopherol, while 30-50 g L^{-1} sucrose in the medium slightly increased the production of ascorbic acid and carotenoid content. These findings seem to be consistent with other research which found a small increase in salidroside content in *Rhodiola sachalinensis* in media containing 40 to 50 g L^{-1} sucrose. Increased sucrose concentrations (40 to 50 g L^{-1}) have also been reported to significantly enhance taxane production in the culture of *Taxus chinensis* (Wang et al. 1999).

In contrast, increase in sucrose concentration (higher than 3%) has been shown to inhibit the production of asiaticoside, madecassoside, asiatic acid and madecassic acid of *C. asiatica* cultures (Anna 2004) and anthocyanin production in cell suspension cultures of *Aralia cordata* (Sakamoto et al. 1993). The different results obtained in this study demonstrated that the effect of carbohydrates on secondary product synthesis *in vitro* are varied, with no general trends being apparent due to the diverse range of products involved. Results also revealed that 30 g L^{-1} sucrose was the optimum concentration in producing the best yield of all antioxidants studied. Similarly, 3% of sucrose induced higher production of digitoxin by cultures of *Digitalis purpurea* (Hagimori et al. 1982) and favoured the anthocyanin accumulation of *A. cordata* (Sakamoto et al. 1993).

In this study, media without sucrose seemed to induce the production of ascorbic acid, α-tocopherol and carotenoid. Previously, Anna (2004) reported that *C. asiatica* callus eventually died when grown in media without sucrose as sucrose is one of the main sources of essential element for any cell growth in tissue culture system. The absence of carbohydrate source initiates changes in physiological and biochemical processes with the goal of sustaining respiration and other essential metabolic processes in plants. Sugar starvation also initiates changes in cellular processes and dramatically changes the pattern of gene expression (Yu 1999). However, the underlying mechanisms used by plant cells to cope with sugar starvation are largely unknown. It was postulated that the cells have evolved mechanisms that allow them to survive under these conditions that lead to the formation of stress-metabolites. It is conceivable that sucrose starvation resembles a stress condition, and the increase in the production of ascorbic acid, α-tocopherol and carotenoid content during this particular time was a stress response. In agreement with this hypothesis, some antioxidant enzymes involved in protection against oxidative stress such as ascorbate peroxidase and catalase, had their highest activity in stationary phase of sunflower cultured cells with a prolonged subculture cycle (Caretto et al. 2004).

CH can act as precursors for the formation of primary and secondary metabolites in plants. As an inducer, CH or other elicitors may bind to certain receptor sites and elicit a phytochemical response from the cell. However, not all receptor binding events will elicit a cellular secondary metabolic response, specific fungal molecules may lead to suppression of the secondary metabolites. Moreover, induction of metabolites by elicitation results from transcriptional activation of genes encoding the enzymes of the particular metabolic pathways, as part of a massive switch in metabolism (Ni et al. 1996).

CH at higher concentrations significantly improved the production of ascorbic acid, α-tocopherol as well as carotenoid content. This study was in accordance with the statement that elicitors, the signalling molecules of plant defense responses can trigger the formation of secondary metabolites in plants under various stresses as a part of defense mechanisms (Ali et al. 2005). Therefore, it can be postulated that treatment of plant with abiotic and biotic elicitors have been an important strategy for inducing secondary metabolites especially ascorbic acid, α-tocopherol and carotenoid content in *C. asiatica* callus. Chong et al. (2005) reported that, other than CH, addition of jasmonic acid in the media could also induce the production of α-tocopherol and carotenoid content in *Morinda elliptica*. However, a reverse trend was observed in the amount of ascorbic acid produced.

Manipulation of the medium pH from 5.1 to 5.9 did not significantly alter the antioxidants studied. Insignificant response to increasing pH values found in this work was identical to that observed by Anna (2004) with the production of asiatic acid and madecassic acid in *C. asiatica* cells at the pH values ranging from 4.0 to 6.0. These results differ with those reported by Merkli et al. (1997) for the production of diosgenin in *Trigonella foenum-graecum* hairy root cultures. They found that increasing the medium pH from 5.0 to 5.9 significantly decrease the production of diosgenin. A change in pH may affect the mineral nutrient uptake particularly NH_4^+ and NO_3^- and affected the activity or metabolism of phytohormones supplied in the medium (Dougall 1980).

Light has important effects on plant growth and secondary metabolite biosynthesis. Plants are sensitive to the radiation and number of hours of light to which they are exposed each day (Zhong et al. 1991). Light exerts an influence on the expression of key enzymes of the phenylpropanoid metabolic pathway such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), at the transcription level and progressively induced the activity of these enzymes as well as secondary product accumulation increases dramatically upon illumination of the cultures (Zhang et al. 2002; Blando et al. 2005).

In this study, higher light intensities significantly enhanced the production of ascorbic acid and α-tocopherol in *C. asiatica* calluses. A photoperiod of 24/0 (light/dark) i.e. continuous illumination was the most favorable condition in inducing both antioxidants. This observation was consistent with the highest production of astaxanthin in green algae, *Haematococcus pluvialis* cultivated in high continuous illumination of 345 μmol photon $m^{-2}s^{-1}$ (Dominguez-Bocanegra et al. 2004). Staba (1980) outlined that plant secondary metabolite can be influenced by light intensity, spectral quality and length of the daily exposure period. However, more effort is needed to understand precisely how light functions in controlling key biochemical regulative mechanisms.

A reverse trend was observed in the production of carotenoid. Calli incubated in dark condition significantly induced the yield of carotenoid. It should be recalled that the production of carotenoid in the callus cultures of *C. asiatica* was significantly repressed compared to intact plant. The outcome was very much in agreement with the study by Wu and Zhong (1999) on ginseng callus and cell suspensions maintained in the dark, where illumination is not required for cell growth and saponin production. According to Furuya (1988), callus cultures under light showed no clear difference in the saponin content, as compared to dark cultures; the effect of light appeared to be dependent on the growth regulators in the medium. Similarly, Butcher and Connoly (1971) showed that the volatile oils (geijerene and pregeijerene) produced by *Andrographis paniculata* cultures exhibited the same amount of volatile oil as those produced in the dark. As for large-scale production of carotenoid, a dark culture may be preferred for the simplicity of equipment and operation as recommended by Wu and Zhong (1999).

CONCLUSIONS

Successful enhancement of ascorbic acid, α-tocopherol and carotenoid content can be achieved by manipulating the medium composition and cultural condition of callus. Full strength MS medium, sucrose feeding at higher concentrations (30 to 50 g L^{-1}) and higher light intensities and a photoperiod of 24/0 (light/dark) increased the production of ascorbic acid and α-tocopherol in CA03 callus. Varying the pH of the medium from 5.1 to 5.9 did not strongly affect the production of all antioxidants studied. CH feeding at lower concentrations could improve the α-tocopherol and carotenoid content whereas increasing the concentrations increased the ascorbic acid content.

A deeper insight into the effects of natural biotic and abiotic elicitors such as yeast extract, chitin, fungal or bacterial lysates or stress response mediators such as salicylate or jasmonic acid/methyl jasmonate and hydrogen peroxide are necessary to successfully stimulate the antioxidant compounds. In addition, feeding of precursors such as coumaric acid, homogentisic acid, phytol, geranylgeraniol, cinnamyl alcohol, naringenin, phenylalanine and a mixture of phenylethanoid glycosides may significantly increase the antioxidants production. Metabolic engineering approach with the

combination of plant cell, tissue and organ culture technologies could be applied to *C. asiatica* as a major breakthrough to improve the production of useful metabolites in this plant.

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