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α-Tocopherol, Ascorbic Acid and Carotenoid Content in *Centella asiatica* Leaf Tissues and Callus Cultures

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ABSTRACT

Green leafy vegetables constitute a major part of balanced diet and are good sources of minerals and vitamins. These beneficial effects are attributed to the presence of antioxidants. Antioxidants also contribute to the defence mechanisms against oxidative stress. *Centella asiatica*, which is locally known as 'pegaga,' is claimed to be rich in natural antioxidative compounds. This study was conducted to determine the amount of ascorbic acid, α-tocopherol and carotenoid content in twelve accessions of *C. asiatica* (CA01 to CA12-comprises of 'pegaga nyonya', 'pegaga kampung' and 'pegaga salad') leaf tissues and callus cultures. The antioxidative constituents of *C. asiatica* in the leaf tissues and cultures were found to vary significantly between the accessions. In particular, CA03 leaves ('pegaga salad') exhibited the highest concentrations of ascorbic acid (95.86 ± 12.60 mg/g.fwt), whereas CA10 ('pegaga nyonya') produced the highest concentration of α-tocopherol (0.233 ± 0.029 µg/g.fwt) and carotenoids (36.55 ± 0.06 mg/g.fwt). The antioxidants studied were also successfully detected in the cultures of *C. asiatica*, with CA08 callus ('pegaga kampung') being dominant in ascorbic acid (167.21 ± 5.30 mg/g.fwt) and α-tocopherol (5.72 ± 0.29 µg/g.fwt), whereas CA12 callus ('pegaga kampung') had the highest carotenoid content (1.04 ± 0.07 mg/g.fwt). Meanwhile, the amount of non-enzymatic antioxidants (except for carotenoid content) was significantly higher in the cell cultures compared to the leaf tissues. The results indicated that CA03 and CA10 leaf tissues, as well as CA08 and CA12 calli were good sources of natural antioxidants compared to other accessions.

Keywords: *Centella asiatica*, leaf tissues, callus cultures, antioxidants, oxidative stress

INTRODUCTION

Antioxidants are secondary constituents and can be defined as anything that can inhibit or prevent oxidation of a susceptible substrate. Plants produce a wide array of antioxidant compounds which include carotenoids, ascorbic acid, tocopherols, and tocotrienols (Hollman, 2001). These plant-based dietary antioxidants are believed to have important role in the maintenance of human health due to its ability to provide protection or defence mechanism against constant and unavoidable challenges of reactive oxygen species (ROS) (Fridovich,

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1998). The formation of ROS in plants creates a condition called oxidative stress that can damage cellular components, and thus, plants have protective mechanisms to prevent or in defence from oxidative damages (Apel & Hirt, 2004). Meanwhile, the toxic effects of ROS are counteracted by enzymatic and non-enzymatic antioxidative systems, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid, tocopherol, glutathione, and phenolic compounds (Ahmad *et al.*, 2008).

Centella asiatica, which is locally known as 'pegaga', belongs to the family Apiaceae; it is widely found in the tropical and subtropical regions. In addition, *C. asiatica* is commonly consumed as vegetable ('ulam/salad') among the Malays, as a cooling drink by the Chinese and as a brain tonic by the Indians. Moreover, pegaga is claimed to possess a wide range of beneficial effects, and it is treated as a valuable medicinal plant in the Chinese traditional medicine and classical Indian Ayurvedic medicine (Peiris & Kays, 1996). It is also used in the treatment of various skin diseases (Patra *et al.*, 1998), as healing properties (Suguna *et al.*, 1996), anticancer property (Babu *et al.*, 1995), antioxidant property (Zainol *et al.*, 2003) and antileprotic property (Sahu *et al.*, 1989).

To date, relevant research has focused on the production of triterpenoids and phenolic content, as well as the antioxidative properties of the plant. However, studies on the production of antioxidants as defence strategies, particularly vitamin and enzymatic antioxidants from this plant, is still very limited. Therefore, this study was carried out to evaluate the production of ascorbic acid, α-tocopherol, and carotenoid content of the leaf tissues and leaves-derived callus of *C. asiatica*.

MATERIALS AND METHODS

Plant Materials

Twelve accessions of *C. asiatica* (CA01 to CA12, leaves) were obtained from Malaysian Agriculture Research and Development Institute (MARDI), Serdang Selangor.

Callus Initiation and Maintenance

Sterile leaf explants were aseptically cultured on solid Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962), supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l Kinetin, and 30 g/l sucrose was added as a carbon source and B5 vitamins (Gamborg *et al*., 1968). Meanwhile, Gelrite agar (2.5 g/l) was used to solidify the culture medium. The cultures were maintained by regular sub-culturing at 10 days interval on fresh medium. All the cultures were incubated in 12h/12h (light/dark) photoperiod under cool, white fluorescent lamps at 27 ± 2°C. Friable calli obtained were used for the antioxidant assays.

Antioxidant Assays

α-Tocopherol was extracted based on the method by Hodges *et al*. (1996) and the assay mixture was prepared as described by Kanno & Yamauchi (1977). A standard curve was prepared using α-tocopherol (Sigma, type V) at various concentrations (0-1.4 µg/ml). The amount of α-tocopherol in the leaf samples was calculated based on the standard curve. Ascorbate was extracted according to the procedure of Jagota and Dani (1982). Absorbance of the mixture was measured at 760 nm. A standard curve was prepared using ascorbic acid at various concentrations (0-60 µg/ml), and the amount of ascorbic acid was calculated based on the standard curve. Carotenoid content was analyzed according to the method proposed by Lichtenthaler (1987). Supernatant of the leaf samples was measured spectrophotometrically at 663.2, 646.8 and 470nm, while 80% acetone was used as a blank.

RESULTS AND DISCUSSION

The ascorbic acid content in the leaf tissues varied from 27.35 ± 2.33 to 95.86 ± 12.60 mg/g. fwt (*Fig. 1A*). The high productions of ascorbic acid in this study were paralleled with the previous report that *C. asiatica* leaves are rich in carotenoids, vitamin B and C (Paramageetham *et al*., 2004). However, Chanwitheesuk *et al.* (2005) reported much lower content of ascorbic

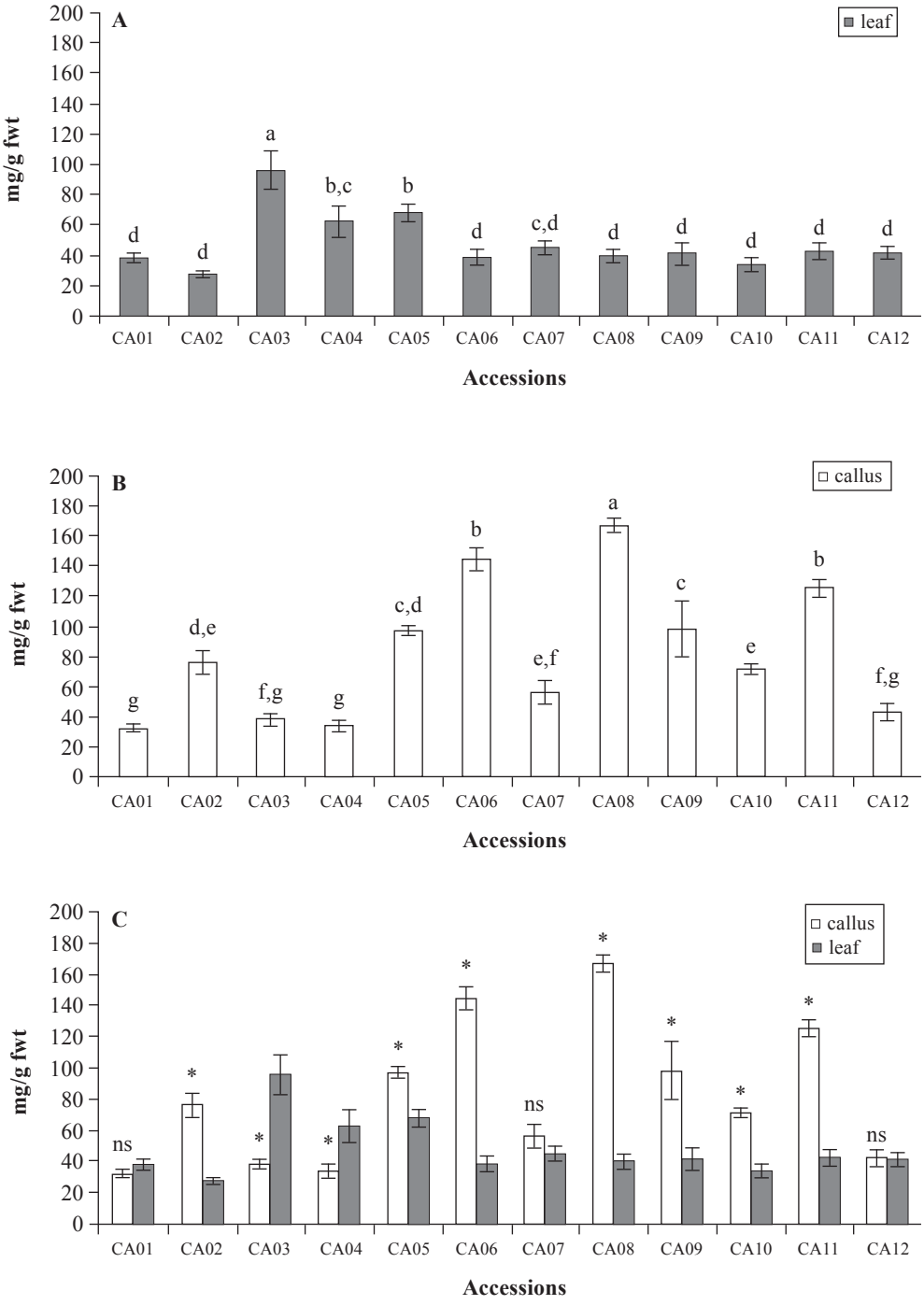

Fig. 1: Ascorbic acid concentrations of C. asiatica*: (A) leaf tissues (B) callus cultures and (C) comparison of leaf tissues and callus cultures. Data shown are means ± SE (n=5). Means with the same letters are not significantly different. * = significantly different, and ns = not significantly different at p<0.05*

acid for *C. asiatica* was cultivated in Thailand, i.e. 6.56 ± 0.10 mg/g dry weight. The variation in the ascorbic acid in this study might be related to the differences in the accessions of *C. asiatica* used. Meanwhile, the variability in ascorbic acid content was also observed in 50 different accessions of broccoli (*Brassica oleracea*), ranging from 0.54 to 1.19 mg/g. fwt. This diversity indicates that potential health benefits depend greatly on the genotype consumed (Kurilich *et al.*, 1999; Vallejo *et al*., 2002). Environmental conditions might also contribute to the alteration of the ascorbic acid concentrations, as reported by Howard *et al*. (1999) in Brasicca vegetables. They observed that the ascorbic acid in plants harvested in the raining season differed than those harvested during high air temperatures. Another factor that might influence the ascorbate pool is light intensities as higher ascorbate was found in the leaves of *Vinca major* and *Schefflera arboricola* grown at high light intensities of 1200 µmol photons $m^{-2}s^{-1}$ (Grace & Logan, 1996; Smirnoff & Pallanca, 1996) and decreases in *Arabidopsis thaliana* and barley leaves during the dark periods (Conklin *et al.*, 1997).

This study revealed that highest concentration of ascorbic acid was detected in CA03 leaf (95.86 ± 12.60 mg/g.fwt). This might be due to the unique characteristics possessed by CA03. Morphological studies by Anna (2004) revealed that the leaf of CA03 is lighter green in colour compared to deep green for all other accessions; the only accession that possesses lobed and crispate leaf margin and also represents the furthest relationship compared to the entire accessions based on the dendogram of genetic distance. Wong (2003) also found that CA03 represented the furthest relationship among the accessions, with 15% of polymorphism with the Amplified Fragment Length Polymorphism (AFLP) analysis.

According to Dietary Reference Intakes and the recommended Dietary Allowances (2001), the current RDA for vitamin C is 15-120 mg/ day depending on the gender and age. Taking into account the quantities of vitamin C found in *C. asiatica*, it becomes obvious that by taking only a portion of *C. asiatica* leaves, one can adequately cover the recommended amount. Large variability of ascorbic acid content (ranging between 32.43 ± 3.09 to 167.21 ± 5.30 mg/g. fwt as shown in *Fig. 1B*) in this study was also observed in the calli. The observation in this study is in agreement with that of Federici *et al*. (2003) who found that the variability observed within the strain collection cultivated under the same culture conditions might be due to the somaclonal variation induced during the initiation of cultures. Successful production of ascorbic acid has also been reported in the two cell lines of *Helianthus annuus* L (Caretto *et al.*, 2002) and in root cultures of *Panax ginseng* and *P. quinquefolium* (Ali *et al.*, 2005).

The α-tocopherol concentrations in *C. asiatica* varied from 0.065 ± 0.001 to 0.233 ± 0.029 µg/g.fwt. The highest amount was found in CA10 leaf. A high content of α-tocopherol was also present in CA01, CA09 and CA02, while other accessions showed lower α-tocopherol concentration (*Fig. 2A*). A higher concentration of α-tocopherol (31 ± 5 µg/g.fwt) was reported in the same plant originated from Thailand (Chanwitheesuk *et al.*, 2005). The concentration of α-tocopherol in this study was also significantly lower compared to the content of α-tocopherol reported by Ching & Suhaila (2001) in *Hydrocotyle asiatica* (29.8 ± 2.2 µg/g edible portion). They also reported that the α-tocopherol content in 62 edible tropical plants, including 3 commonly eaten leafy vegetables or *'ulam'* per 100g fresh weight were 42.68 ± 0.12 mg (*Sauropus androgynus;* cekur manis), 14.68 ± 0.51 mg (*Oenanthe javanica*; selom) and 5.97 ± 0.21 (*Piper sarmentosum*; kaduk). The values of α-tocopherol content reported were significantly higher than those found in this study. The variation of α-tocopherol observed in this study is in agreement with the statement by many researchers that the level of tocopherol derivatives may differs quantitatively and qualitatively among different plant species and varieties, and even within a given plant species or within organs. Strong variations in α-tocopherol contents were also found to depend on the developmental stage of the leaves

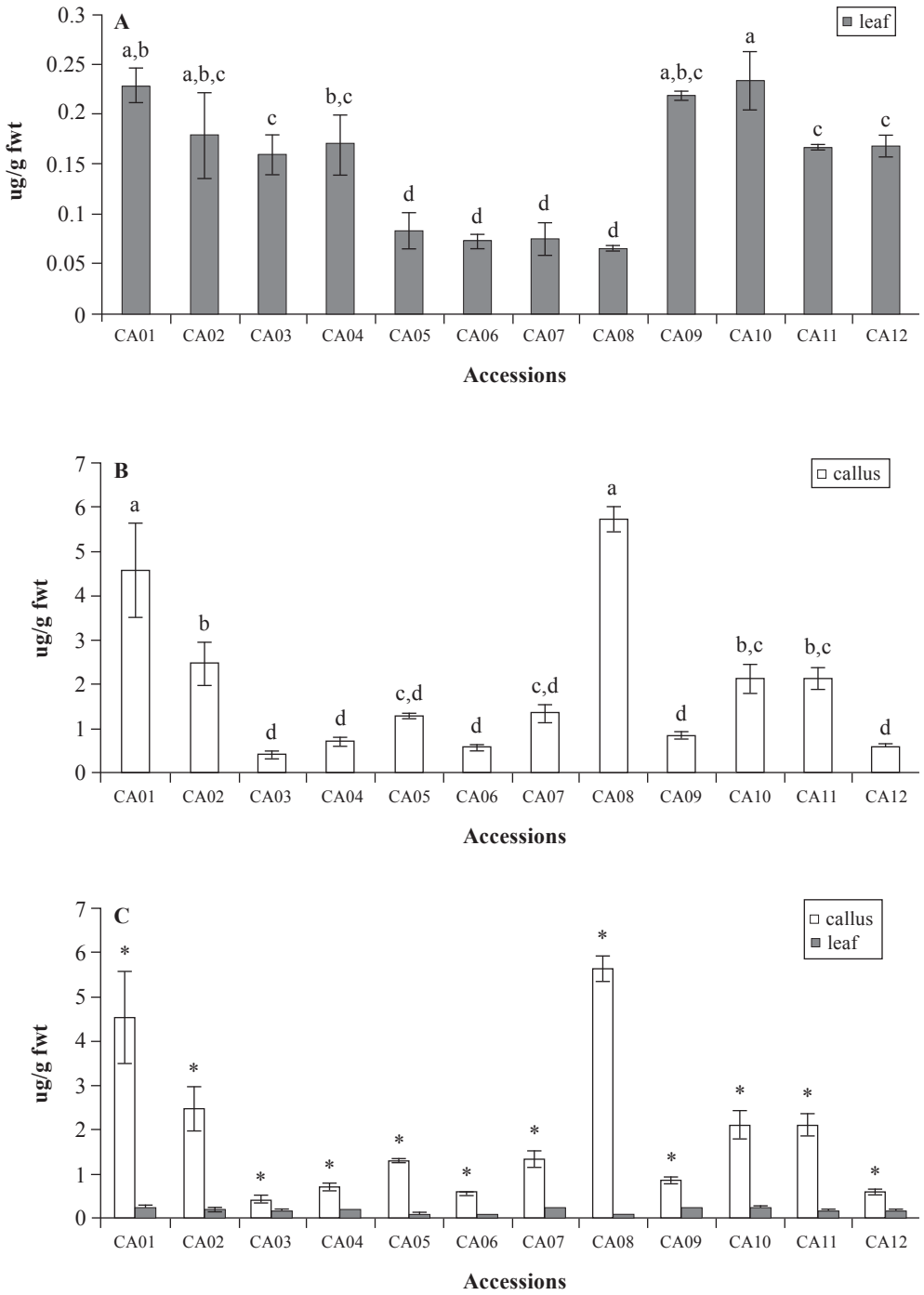

Fig. 2: α-Tocopherol concentrations of C. asiatica*: (A) leaf tissues (B) callus cultures and (C) comparison of leaf tissues and callus cultures. Data shown are means ± SE (n=5). Means with the same letters are not significantly different. * = significantly different and ns = not significantly different at p<0.05*

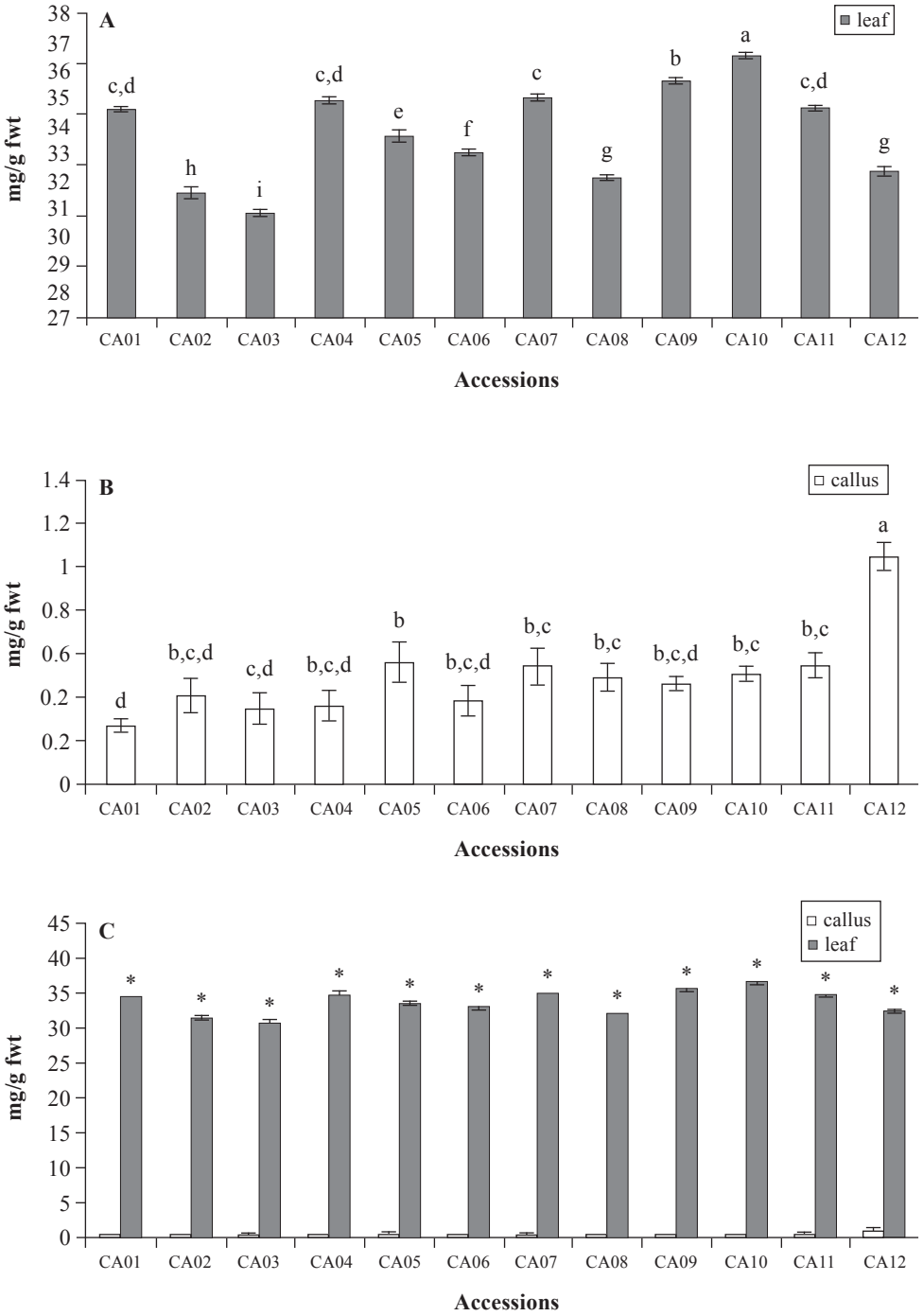

Fig. 3: Carotenoids concentrations of C. asiatica*: (A) leaf tissues, (B) callus cultures, and (C) comparison of the leaf tissues and callus cultures. Data shown are means ± SE (n=5). Means with the same letters are not significantly different. * = significantly different and ns = not significantly different at p<0.05*

and the environmental conditions, such as light intensity, temperature, drought and pollutants (Munne-Bosch & Alegre, 2000). Similarly, *C. asiatica* calli were also identified for their differing capability to produce tocopherols (*Fig. 2B*). α-Tocopherol concentration was dominant in CA08 (5.72 ± 0.29 µg/g. fwt), followed by CA01. Lower concentrations of α-tocopherol were observed in other accessions. The production of α-tocopherol in this study was significantly lower (24 µg/g. fwt) than the value reported in sunflower cell cultures (Caretto *et al.*, 2004) and of 5-13 mg/100 g dry weight in the cell culture of *Carthamus tinctorius* (Furuya *et al*., 1987).

The amount of carotenoid concentration detected in *C. asiatica* was higher than 22 species of vegetables studied by Muller (1997), while Podsedek (2007) reported lower concentrations of carotenoid (0.26 to 6.1 mg/100 g) in Brussels sprouts, broccoli, red cabbage and white cabbage. Several physical factors, including environmental differences (such as temperature, soil and solar intensity) can affect the total nutrient values in plants. Calli of *C. asiatica* managed to produce low amount of carotenoid content except in CA12 which contained almost 2-folds higher of the total carotenoid compared to other accessions (*Fig. 3*). The carotenoid content of *C. asiatica* cultures was in the range of 0.27 ± 0.03 to 1.04 ± 0.07 mg/g fwt and was significantly lower compared to 5.7 to 13.3 mg/g fwt in the *Morinda elliptica* cell cultures in various medium strategies (Chong *et al*., 2004).

It is interesting to note that the analyses conducted in the present work revealed that the greatest accumulation of almost all antioxidant studied occurs in the callus cultures compared to the leaf tissues (*Figs. 1C* and *2C*), except for carotenoids (*Fig. 3C*), while the concentration of the antioxidant studied varied between the accessions.

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