Australian Journal of Crop Science AJCS
AJCS 7(11):1606-1616 (2013) ISSN:1835-2707

CO_2-enriched microenvironment induces biosynthesis of anthraquinones, phenolics and flavonoids in bioreactor cell suspension cultures of *Morinda citrifolia* (L.): the role of antioxidants and enzymes

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Abstract

In this study, the effects of carbon dioxide (CO_2) levels within the range from 0.03 to 5% on growth and secondary metabolites production, e.g. anthraquinones (AQ), total phenolics and flavonoids in cell suspension cultures of *Morinda citrifolia* were investigated in a 3L balloon-type bubble bioreactor. Besides, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, oxidative stress levels, antioxidative responses and enzymatic activities were also estimated. Results revealed that 0.5% and 1% CO_2 supply enhanced accumulation of cell biomass, whereas high CO_2 levels of 2.5% and 5% uplifted biosynthesis of secondary metabolites in expense of cell growth. In spite of high cell growth at 0.5% and 1% CO_2 supply, the maximum yield of AQ (117.24 mg l^{-1} dry weight), phenolics (147.84 mg l^{-1} dry weight) and flavonoids (68.20 mg l^{-1} dry weight) were achieved at 2.5% CO_2-treated culture. This might be due to upregulated activities of shikimate dehydrogenase (SKDH, E.C. 1.1.1.25), phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5) and cinnamyl alcohol dehydrogenase (CAD, E.C. 1.1.1.195) that stimulated biosynthesis of those metabolites. Cell suspension culture grown with high CO_2 supply progressively stimulated the activities of superoxide dismutase (SOD). Although, 45% and 11% induction of catalase (CAT), 50% and 17% induction of guaiacol peroxidase (G-POD) activities were detected at 2.5% and 5% CO_2 treated cultures, respectively compared to the relative control (0.03%). However, high accumulation of hydrogen peroxide (H_2O_2) and peroxidation of lipids (MDA) were measured at high CO_2 treated cultures (2.5% and 5%). These results suggest that the observed high activities of CAT and G-POD at high CO_2 treated cultures were not sufficient enough to cope with toxic H_2O_2 accumulation, but played a prominent role in reducing stress severity and thereby allowing cells to grow at elevated levels of CO_2.

Keywords: antioxidant enzyme, carbon dioxide, cell suspension culture, *Morinda citrifolia*, oxidative stress, phenylalanine ammonia lyase, secondary metabolite.
Abbreviations: AQ_Anthraquinones, BTBB_Balloon type bubble bioreactor, CAD_Cinnamyl alcohol dehydrogenase, CAT_ Catalase, DPPH_1, 1-diphenyl-2-picrylhydrazyl, G-POD_Guaiacol peroxidase, H_2O_2_Hydrogen peroxide, ICS_Iso chorismate synthase, MDA_Malondialdehyde, NBT_*p*-Nitroblue tetrazolium, PAL_Phenylalanine ammonia lyase, ROS_Reactive oxygen species, SKDH_Shikimate dehydrogenase, SOD_Superoxide dismutase, TBA_Thiobarbituric acid.

Introduction

Cell growth and accumulation of secondary metabolites in large-scale bioreactor are influenced by various physical and chemical factors, including gaseous composition. The gaseous composition in plant cell and tissue culture is an important factor affecting the plant physiology, but gas exchange between the gas and liquid phase is especially important in the context of scale-up of plant cell cultures (Thanh et al., 2006). In bioreactor, forced aeration is needed to supply oxygen and to improve fluid mixing. However, this approach has several limitations, such as high power consumption, cell damage due to the mechanical shear stress, and potential reduction of productivity due to removal of some known (e.g. CO_2, ethylene) or unknown gaseous components (Jeong et al., 2006; Thanh et al., 2006). CO_2 is often considered as an essential component for culturing plant cells (Ducos et al., 1988), because some of the control mechanisms for the photosynthetic dark reactions would be regulated by CO_2 concentrations. This could affect both cell growth and, indirectly, production of bioactive compounds (Mirjalili and Linden, 1995).

The enrichment of CO_2 supply has been shown to significantly affect secondary metabolites formation including phenolics and flavonoids in various plant or cell cultures (Huang and Chou, 2000; Han and Zhong, 2003; Ali et al., 2005. 2006a, 2008; Jeong et al., 2006; Thanh et al., 2006; Ibrahim and Jaafar, 2011; Ali and Jaafar, 2011). However, the elevated CO_2 levels in association with normal air supply in bioreactor may induce reactive oxygen species (ROS), which also depends on the composition of the medium (Vesela and Wilhelm, 2002). It has been reported that the accumulation of CO_2 and its dissociation into bicarbonate (HCO_3^-) and H^+ ions, in cell culture medium and in the cell cytoplasm, may induce oxidative stress, decrease intracellular pH and alter medium osmolality. The role of elevated levels of CO_2 in oxidative stress is primarily due to the formation of carbonate anion (CO_3^-) and peroxymonocarbonate anion (HCO_4^-), which occurs mainly in the presence of strong oxidizing agents like H_2O_2 (Vesela and Wilhelm, 2002; deZengotita et al., 2002). Upon stress, ROS are generated and attack membrane lipid, phospholipids causing oxidative damage (Apel and Hirt, 2004). To prevent cellular damage caused by ROS, plant cells developed various enzymatic and non-enzymatic protective systems such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbic acid, tocopherol and phenolic compounds etc. (Nobuhiro and Mittler, 2006).

It is well-documented that CO_2 is fundamentally important to plants because of photosynthesis. The response of plant to CO_2 enrichment usually leads to increase rates of net photosynthesis due to enhanced activity of Rubisco enzyme and can alter plant growth and partitioning to secondary metabolites (Mattson et al., 2005). Most plant cell suspension cultures are grown heterotrophically in the presence of sugars, whereas a limited number of photoautotrophic cultures have been established which are able to grow with CO_2 as sole carbon source (Roitsch and Sinha, 2002). The majority of photoautotrophic cells require elevated levels of CO_2 (1-5%). Although, the effects of CO_2 on photosynthetic response of heterotrophic cells are scarce, however, in photoautotrophic cell suspension cultures of low CO_2-requiring cotton and high CO_2- requiring soybean, it has been demonstrated that most of the CO_2 was fixed by ribose-1,5-bisphosphate (RuBP) carboxylase. The magnitude of O_2 inhibition of CO_2 fixation was observed similar to that seen in leaves of C_3 plants (Roeske et al., 1989). On the contrary, decrease in Rubisco activity of photoautotrophic calli grown under high CO_2 (5%) supply was observed due to down regulation of photosynthetic capacity (Rey et al., 1990). These results suggest that exogenous supply of CO_2 in cell suspension culture is a critical determinant for enhancing growth and secondary metabolism, and therefore, should be optimized according to plant species. *Morinda citrifolia* (L.), most popularly known as Noni, belongs to the Rubiaceae family has been used for traditional food and folk medicine in Polynesia over 2,000 years (Wang et al., 2002). About 160 valuable pharmaceutically active compounds have been identified in this plant, and the major compounds are polyphenolics, organic acid and alkaloids (Wang and Su, 2001). Among the phenolic compounds, the mostly reported ones are anthraquinones (AQ) that exhibits various therapeutic effects such as hepatoprotective, antitumor, antiviral, antibacterial, antiplasmodial and anticancer activities (Wang et al., 2002; Rao et al., 2006; Osman et al., 2010). Considering potential health benefit and vast repository of those valuable phytomolecules, the cell suspension culture of *M. citrifolia* was first initiated by Zenk et al. (1975) for AQ production. During the last few years, extensive research efforts have been paid for efficient induction of AQ in cell suspension cultures of *M. citrifolia* and other species by attaining exogenous application of various physical and chemical elicitors (Chong et al., 2005a, 2005b; Komaraiah et al., 2005; Ahmed et al., 2008; Baque et al., 2012a). To the best of our knowledge, the effects of CO_2 on cell growth and secondary metabolites production by suspension cultures of *M. citrifolia* are unprecedented. It is already well-known that enrichment of CO_2 supply has significant effect on plant growth and metabolites production. Therefore, in this study we have investigated the effect of elevated levels of CO_2 on cell growth and accumulation of AQ, total phenolics and flavonoids. At the same time, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, oxidative stress levels (hydrogen peroxide, H_2O_2; malondialdehyde, MDA), antioxidant systems (SOD, CAT, G-POD), and enzymatic activities (shikimate dehydrogenase, SKDH; phenylalanine ammonia lyase, PAL; cinnamyl alcohol dehydrogenase, CAD) were also estimated under elevated levels of CO_2.

Results and discussion

Effect of CO_2 on cell growth and bioactive compound production

The differential responses of cell growth of *M. citrifolia* grown in balloon type bubble bioreactor (BTBB) at various levels of CO_2 supply are presented in Table 1 and supplementary Fig. S2 A-B. Cell fresh and dry biomass increased at 0.5% and 1% CO_2 treated cultures compared to control (0.03%). High levels of CO_2 supply (2.5 to 5%) inhibited accumulation of cell fresh and dry biomass. The maximum cell FW (247.80 g l^{-1}), DW (11.11 g l^{-1}) and GR (9.57) were achieved at 0.5% CO_2 supply, as evidence by the increases of 15% and 11% of cell FW and DW, respectively compared to control bioreactor culture (Table 1). Although, CO_2 supply from 2.5 to 5% significantly reduced cell growth but stimulated biosynthesis of those secondary metabolites. As shown in Table 2, accumulation of AQ and flavonoids profusely increased with increasing CO_2 levels from 0.5 to 2.5%, whereas phenolics accumulation increased gradually with CO_2 supply (Table 2). The highest concentrations of AQ (12.98 mg g^{-1} DW) and flavonoids (7.55 mg g^{-1} DW) were achieved when cells were grown with 2.5% CO_2, and phenolics (16.37 mg g^{-1} DW) with 5% CO_2 supply. In terms of productivity, a 2.5% CO_2 supply was found to be an optimal concentration for upswing yield of AQ (117.24 mg l^{-1} DW), and flavonoids (68.20 mg l^{-1} DW) and 1% CO_2 supply for phenolics (161.80 mg l^{-1} DW). The gaseous composition, especially CO_2 in plant cell and tissue culture is an important factor affecting the plant physiology, and thereby has profound effect on cell growth and secondary metabolite production (Jeong et al., 2006). It is reasonable to suspect that some of the control mechanisms for the photosynthetic dark reactions would be regulated by CO_2 concentration. This could affect both cell growth and indirectly production of bioactive compounds (Mirjalili and Linden, 1995). Myriad researches in plant biotechnology have suggested that the effect of CO_2 on cell growth and metabolite production is concentration dependent manner and varied with species to species and types of explants used. For instance, in cell and root suspension cultures of *Panax ginseng*, 1% CO_2 supply is found to be beneficial for biomass accumulation, whereas high levels of CO_2 supply (2.5 and 5%) showed negative effect on biomass and metabolites (ginsenoside, saponin) accumulation (Thanh et al., 2006;

Table 1. Effects of CO_2 on cell growth of *Morinda citrifolia* after 3 weeks cultured in bioreactors.

CO_2 concentration (%)	Fresh weight (g l^{-1})	Dry weight (g l^{-1})	Growth ratio
Control (0.03)	244.33ab	10.01ab	8.63ab
0.5	247.80a	11.11a	9.57a
1.0	215.24b	10.69a	9.22a
2.5	155.51c	9.03bc	7.78bc
5.0	140.51c	7.90c	6.81c

The same letter within a set of values indicates no significant differences by Duncan's multiple range test at 5% level.

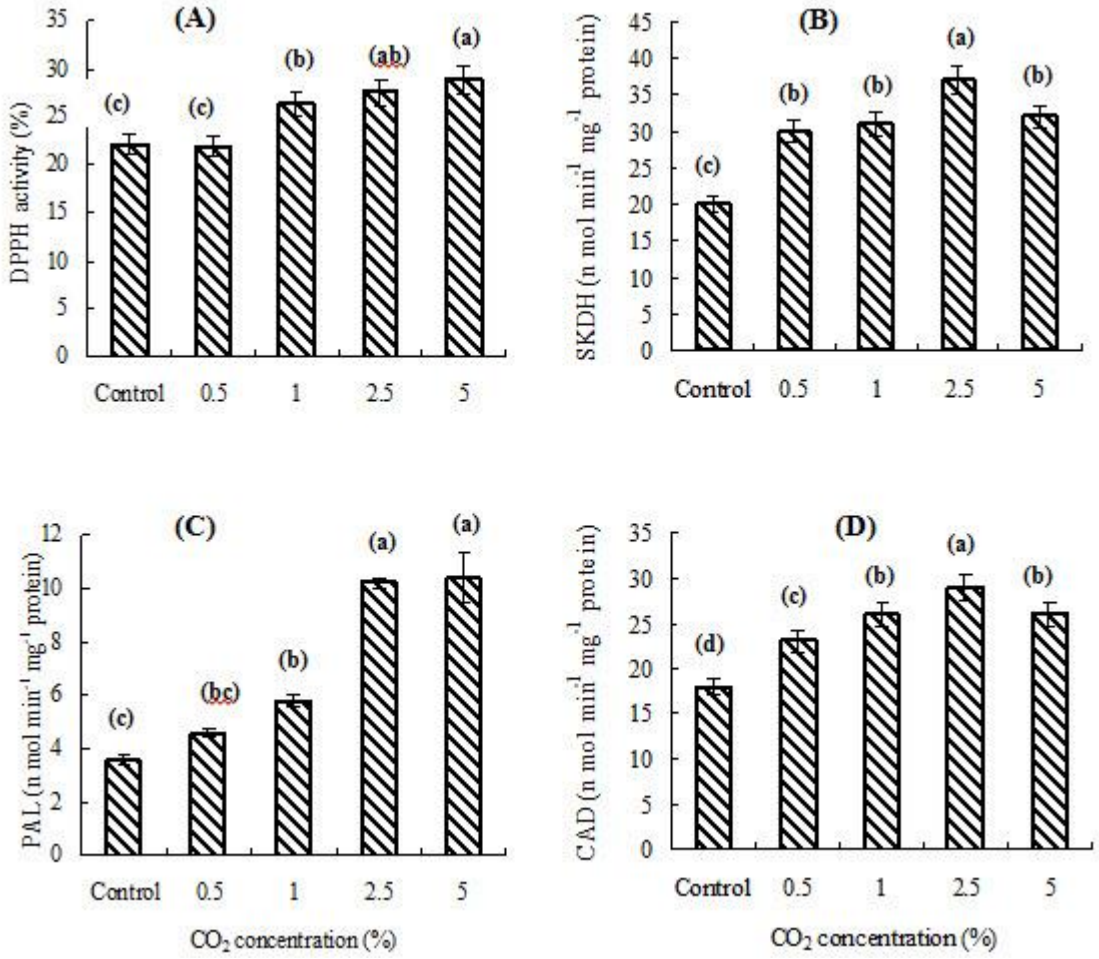

Fig 1. Activities of DPPH (A) and enzymes: SKDH (B), PAL (C) and CAD (D) in cells of *Morinda citrifolia* as affected by different concentrations of CO_2 after 3 weeks cultured in bioreactors. Bars represent mean ±SE (n=3).

Jeong et al., 2006; Ali et al., 2008). In batch cultures of *Acremonium chrysogenum*, high levels of CO_2 (15 to 20%) led to reduction of cephalosporin C production (El-Sabbagh et al., 2008). In contrast, reduced CO_2 concentration had no effect on cell growth of *P. notoginseng* (Han and Zhong, 2003). Using an airlift fermenter, Fowler (1983) observed that supplying CO_2 to a tobacco cell culture increased biomass growth. On the contrary, the beneficial effect of CO_2 supply on desired product yield has been reported by several authors: total phenolics and flavonoids accumulation in bioreactor root suspension cultures of *P. ginseng* and *Echinacea angustifolia* (Ali et al., 2005, 2006a), taxol production in *Taxus cuspidate* (Mirjalili and Linden, 1995), L-DOPA content in cell suspension cultures of *Stizolobium hassjoo* (Huang and Chou, 2000), primary and secondary metabolites synthesis in ginger (Ali and Jaafar, 2011). In this present study, the definite positive effect of CO_2 supply was also observed on the accumulation of cell dry biomass at 0.5% and 1%, AQ and flavonoids at 0.5% to 2.5%, and total phenolics at 0.5% to 5% CO_2 compared to control. High levels of CO_2 supply (2.5 and 5%) significantly decreased the cell growth. This finding indicates that low concentrations of CO_2 (0.5 and 1%) are required for growth and high concentrations (>2.5%) for metabolites synthesis of cells suspension cultures of *M. citrifolia*. These results are in consistent with earlier reported results that CO_2 supply (1%) is beneficial for cell and root growth of *P. ginseng* ((Thanh et al., 2006; Jeong et al., 2006; Ali et al., 2008), total phenolics and flavonoids production (up to 2.5%) in bioreactor root suspension cultures of *P. ginseng* and *Echinacea angustifolia* (Ali et al., 2005, 2006a), berberin production in cell suspension cultures of *Thalictrum rugosum* (Kim et al., 1991). The further increase in CO_2 concentrations (2.5 and 5%) showed negative effect on biomass accumulation (Thanh et al., 2006). The growth promoting effect of additional CO_2 supply could be explained by a stimulation of non-autotrophic CO_2 fixation, involving an enhanced substrate level for pyruvate carboxylase. This effect might be assigned to the enhanced operation of Krebs cycle as a result of more rapid replenishment of intermediates (Schnabl and Mayer,

1976), or linked to marked changes in levels of enzymes of primary carbohydrate metabolism during growth (Ducos et al., 1988). It is well-known that CO_2 is fundamentally important for carbon fixation. Most plant cells are heterotrophic, some are photomixotrophic and a limited number of plant cells are phototrophic, and use chemical energy for their growth. However, bioreactor suspension cultures of *M. citrifolia* exposed to elevated levels of CO_2 (2.5 and 5%) resulted in reduced cell growth. The reduced cell biomass in our study at elevated levels of CO_2 may be associated with the inactivation of certain enzymes particularly involved in carboxylation/decarboxylation process during primary growth metabolism (Dixon and Kell, 1989). Rogers et al. (1987) observed in photoautotrophic soybean suspensions grown at 5% CO_2 a seven-fold lower total Rubisco activity than in leaves. This decrease in Rubisco activity accounts probably for their reduced photosynthetic capacity at high CO_2 levels. In the same way, Rey et al. (1990) have observed a noticeable decrease in the total Rubisco activity of calli grown at high CO_2. They also observed that high CO_2 grown calli accumulated large amounts of sucrose. Such an accumulation which happens under high CO_2, is known to provoke a feedback inhibition of photosynthesis by reducing the levels of orthophosphate. Therefore, it is reasonable to suspect that a feedback inhibition of photosynthesis probably occurs in high CO_2 grown cultures of *M. citrifolia* that may accounts for the reduced cell growth. Another possible mechanism for inhibition of cell growth and stimulation of secondary metabolites biosynthesis at elevated levels of CO_2 is through disruption of intracellular pH regulation. Because, activities of several cellular metabolic enzymes, e.g., phosphofructokinase, are pH sensitive. Changes in intracellular pH affect cell metabolism, ion conductivities, protein synthesis and cell cycle (Madshus, 1988). In this case, the toxic component would be the dissolved CO_2 which can freely diffuse across the cell membrane. As non-polar CO_2 passes across the cell membrane into the cytosol and mitochondrial compartment, the CO_2 would equilibrate to HCO_3^- and H^+, thereby reducing the intracellular pH and placing a burden on the cell to readjust the internal pH by increasing the rate of the Na^+/H^+, HCO_3^-/Cl^- and other similar antiporters, the internal pH may not return to its original value (Madshus, 1988; Ganz et al., 1989; Gray et al., 1996). Moreover, intracellular pH modifications in response to variation in *p*CO2 levels induced perturbation of intracellular pH that may induce several enzyme activities including plasma membrane associated transport systems (Stuhfauth et al., 1987). It has been demonstrated that the increase in plant secondary metabolites production under elevated levels of CO_2 supply might be due to diversion of phenylalanine for protein synthesis to production of secondary metabolites including phenolic compounds (Ibrahim and Jaafar, 2011). It has also been reported that the stimulatory effect of certain level of CO_2 (2.5%) on the accumulation of phenolic compounds may be attributed to changes in pH in the culture media (Huang and Chou, 2000; deZengotita et al., 2002; Ali et al., 2006a). In our study, the enhanced induction of AQ and flavonoids at 2.5% CO_2 supply and phenolics at 5% CO_2 supply were also observed. Although, pH was not measured in this study, however, lending from those results it is tempting to speculate that elevated CO_2 supply might be reduced pH in the culture media that could be linked to the marked changes in the levels of enzymes of phenolics metabolism. Phenolic compounds are known to be involved in protecting plants from stress conditions including CO_2 (Ali et al., 2006a). Because accumulation of phenolics and flavonoids upon stressful conditions function as reducing agents, free radical scavengers and quenchers of singlet oxygen formation (Atoui et al., 2005). Moreover, phenolics are also involved in strengthening the plant cell walls during growth by polymerization into lignins (Ali et al., 2005). Therefore, synthesis of phenolics and flavonoids in cell suspension cultures of *M. citrifolia* might be a part of the defense response against high levels of CO_2.

Effect of CO_2 on activities of DPPH radical scavenging, SKDH, PAL and CAD

The measurement of DPPH as a free radical indicates the radical scavenging capacity of cells, and can be considered as a useful method of investigating the free radical scavenging activities of phenolic compounds (Ali et al., 2006a). As shown in Fig. 1A, DPPH radical scavenging activity elevated with increasing CO_2 levels. More than 30% increase in DPPH radical scavenging activity was noted in cells when exposed to 5% CO_2 supply compared to the relative control. In contrast, when CO_2 levels were increased, accumulation of AQ, phenolics and flavonoids were well-correlated with DPPH activity. A strong positive correlation was observed among DPPH activity and accumulation of AQ (R^2 = 0.914), phenolics (R^2 = 0.926) and flavonoids (R^2 = 0.895), indicating that CO_2 plays an important role in the accumulation of bioactive compounds. Results depicted in Fig. 1B-D show that CO_2 supply significantly induced shikimate dehydrogenase (SKDH), phenylalanine ammonia lyase (PAL) and cinnamyle alcohol dehydrogenase (CAD) activities in cells of *M. citrifolia* after 3 weeks of bioreactor culture. The increases of SKDH (Fig. 1B) and CAD (Fig. 1D) activities were observed with increasing CO_2 levels up to 2.5%, thereafter decreased at 5% CO_2-treated culture. Whereas, PAL activity profusely increased with increasing CO_2 supply, showing almost similar activity when cells were exposed to 2.5 and 5% CO_2 supply (Fig. 1C). A 1.85-, 2.85- and 1.61-fold induction of SKDH, PAL and CAD activities, respectively were detected at 2.5% CO_2 treated cultures compared to the relative control. The induction of SKDH, PAL and CAD activity by CO_2 enrichment was observed coincided with the AQ, phenols and flavonoids accumulation (Table 2), indicating that phenols were likely mobilized by the activities of shikimate, and phenylpropanoid pathway enzymes. SKDH is the first enzyme of shikimate pathway that provides chorismate for the starting product of AQ biosynthesis. Chorismate is not only a precursor for AQ biosynthesis but also a large variety of metabolites such as aromatic amino acid (Hermann et al., 1999; Perassolo et al., 2007). Isochorismate synthase (ICS) could be a regulatory enzyme for channeling of chorismate into AQ biosynthesis in the rubiaceae (Perassolo et al., 2007). The induction of SKDH activity to compensate CO_2 inhibition could partially explain by the activities of ICS. However, it is necessary to point out that the induction of SKDH activity in cells exposed to elevated levels of CO_2 is accompanied by the accumulation of phenolic compounds (Ali et al., 2005). Because, chorismate mutase (CM) converts chorismate to phenylalanine, which is the starting material of the phenylpropanoid pathway (Perassolo et al., 2007). Phenylalanine is required for the synthesis of various polyphenolic compounds such as phenolics, flavonoids, rosamaric acid, lignins and anthocyanins (Herrmann et al., 1999; Perassolo et al., 2007). It has been reported that in AQ producing cell cultures of *Rubia tinctorum*, the increase in AQ biosynthesis is preceded by a proportional increase in isochorismate synthase (ICS) activity and its transcript level

Table 2. Effects of CO_2 on secondary metabolites production from cell suspension cultures of *Morinda citrifolia* after 3 weeks cultured in bioreactors.

CO_2 concentration (%)	AQ		Total phenolics		Total flavonoids	
	Content (mg g^{-1} DW)	Yield (mg l^{-1} DW)	Content (mg g^{-1} DW)	Yield (mg l^{-1} DW)	Content (mg g^{-1} DW)	Yield (mg l^{-1} DW)
Control (0.03)	6.09e	60.98d	13.57c	135.81bc	2.67d	26.74d
0.5	6.89d	76.52c	14.12bc	156.91a	4.07c	45.24c
1.0	9.38c	100.26b	15.14ab	161.80a	5.97b	63.84ab
2.5	12.98a	117.24a	16.37a	147.84ab	7.55a	68.20a
5.0	12.72b	100.45b	16.39a	129.49c	7.18a	56.75b

The same letter within a set of values indicates no significant differences by Duncan's multiple range test at 5% level.
Yield = content (mg g^{-1} DW)* Cell dry weight (g l^{-1}).

(Van Tegelen et al., 1999). Although ICS activity was not measured in this current study, however, it is tempting to speculate that the induction of shikimate dehydrogenase (SKDH) activity by CO_2 enrichment might be induced ICS activity by supplying chorismate, the precursor for AQ biosynthesis. Therefore, further studies should be needed to confirm the relationship between CO_2-induced ICS activity and AQ synthesis. PAL is considered the key regulatory enzyme in phenol biosynthesis since PAL catalyses the first reaction in the general pathway of phenylpropanoid biosynthesis, which includes the formation of flavonoids and hydroxycinnamic acids. These results obtained from this study show that CO_2 supply induces activity of PAL (Fig. 1C), and concomitantly biosynthesis of phenolics and flavonoids (Table 2). It was plausible that most natural phenolic compounds in plants are derived from *trans*-cinnamic acid formed by deamination of L-phenylalanine by PAL (Boudet, 2007). In contrast, PAL plays a pivotal role in phenol synthesis and many reports emphasized the correlation between increase in the corresponding PAL gene expression/activity and increase in phenolic compounds biosynthesis in response to different stress stimuli (Boudet, 2007; Ibrahim and Jaafar, 2011). In our current study, correlation analysis showed that PAL had a strong positive relationship with phenolics (R^2 = 0.954) and flavonoids (R^2 = 0.872) biosynthesis, indicating that over 95% variations in phenolics and 87% flavonoids biosynthesis could be explained from the variations in CO_2-induced PAL activity. The higher induction of PAL activity in CO_2 treated cells indicated that PAL enhancement may be due to ROS generation, which occurs as primary reaction in response to stress, plays a major role in controlling the flux into total phenolics (Ali et al., 2005). The increase in PAL activity in response to various kind of stresses including CO_2, and concomitantly phenolics and flavonoids biosynthesis has also been reported in *spergula avensis* (Hartley et al., 2000), *P. ginseng* (Ali et al., 2005, 2006b), tobacco (Matros et al., 2006), *M. citrifolia* (Baque et al., 2010) and *Labisia pumila* (Ibrahim and Jaafar, 2011). This is basically due to the fact that PAL is a precursor to total phenolics and flavonoids biosynthesis. It has been proposed that cinnamyle alcohol dehydrogenase (CAD) is involved in lignification process, which results in decrease cell wall plasticity and overall cell growth, and therefore represents a mechanism for adaptation to stress. During lignification, upregulation of CAD activity leads to conversion of phenolics to lignin formation to adopt against stress (Chen et al., 2002; Ali et al., 2005). Moreover, induction of CAD activity under stresses is not only closely associated with lignin synthesis but also plays an important role in phenolic compounds synthesis (Cabane et al., 2004; Ali et al., 2005). In this study, upregulation of CAD activity was observed up to 2.5% CO_2-treated cultures (Fig. 1D). Contrary, cultures exposed to high level of CO_2 (5%) supply decreased CAD activity. This may due to inhibition of pH

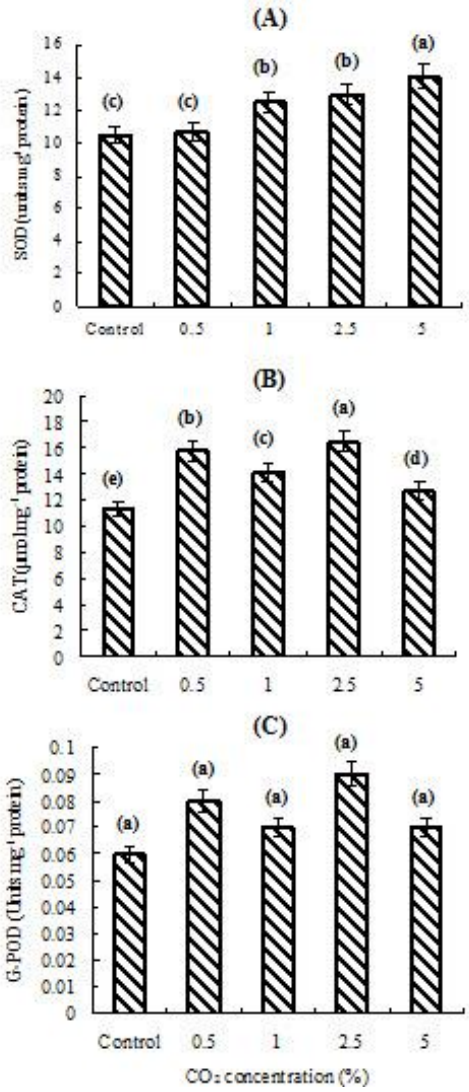

Fig 2. Activities of antioxidant enzymes: SOD (A), CAT (B) and G-POD (C) in cells of *Morinda citrifolia* as affected by different concentrations of CO_2 after 3 weeks cultured in bioreactors. Bars represent mean ±SE (n=3)

level in the culture media exposed to high level of CO_2 supply. In consistent with the present study it has been demonstrated that high CO_2 supply (5%) lowered pH levels of the culture media along with inhibition of enzyme activity (Huang and Chou, 2000; Ali et al., 2006a). Although lignin content was not measured in our study, however, it can be conjectured that induction of CAD activity in high CO_2-treated cells might be involved in lignification process and/or phenolic compounds synthesis. In contrast, inhibition of CAD activity at 5% CO_2 supply (Fig. 1D) might be inhibited

the rate of conversion of phenolics to lignin formation that leads to accumulation of phenolics compared to AQ and flavonoids (Table 2). These results suggest that stimulation of bioactive compound synthesis in expense of cell growth under elevated levels of CO_2 probably accounts for upregulated activities of SKDH, PAL and CAD that induce defense response in cells of *M. citrifolia*.

Effect of CO_2 on activities of antioxidant enzymes (SOD, CAT and G-POD) and stress levels (H_2O_2 and MDA)

Results depicted in Fig. 2A-C show the activities of SOD, CAT and G-POD in cells of *M. citrifolia* in response to CO_2 supply. SOD activity profusely increased with increasing CO_2 supply (Fig. 2A). The maximum induction of SOD activity (34%) was observed when cells were exposed to 5% CO_2 supply compared to control. Contrary, a concentration dependent induction of CAT and G-POD activity was observed after 3 weeks of culture. The maximum induction of CAT (45%) and G-POD (50%) activity was detected at 2.5% CO_2 followed by 0.5% CO_2 (39 and 33%, respectively) treated cultures compared to the relative control (Fig. 2B-C). A significant inhibition of CAT and G-POD activity was observed when cells were exposed to 5% CO_2, indicating that enrichment of CO_2 concentration over 2.5% is deleterious on the activities of CAT and G-POD in cell suspension cultures of *M. citrifolia*. In general, formation of reactive oxygen species (ROS) under different stress stimuli depends on types of species, stress period and intensity, and age of the plant (Navari-Izzo et al., 1996). There is increasing evidence that breakdown of membrane under various stress stimuli is related to a greater production of highly toxic ROS (Mittler et al., 2004). In our study, extent of CO_2-induced oxidative damage was assessed by measuring the content of H_2O_2 and peroxidation of lipids (MDA). As shown in Fig. 3A-B, accumulation of H_2O_2 and formation of MDA in cells increased progressively (except 0.5% CO_2) with increasing CO_2 concentrations. A 6.12- and 1.73-fold increase in H_2O_2 and MDA contents, respectively were measured in cells exposed to 5% CO_2 compared to the relative control. Whereas, a significantly lower H_2O_2 and MDA contents were detected in cells when exposed to 0.5% CO_2. Stress induces formation of ROS such as O_2^-, H_2O_2 and OH^- in plants which create a condition called oxidative stress and can damage cellular components (Apel and Hirt, 2004). Upon stress, plants have developed several antioxidation strategies to demolish the harmful effects of ROS. Several enzymatic and nonenzymatic antioxidative systems such as SOD, CAT, POD, APX, ascorbic acid, tocopherol and phenolic compounds, etc., are responsible for combating ROS-mediated damage (Nobuhiro and Mittler, 2006). Enhancement of antioxidative defense response in plants can thus increase tolerance to different stress factors. Therefore, connections between activities of antioxidant enzymes (SOD, CAT and G-POD) and stress levels (H_2O_2 and MDA) were studied in this study. It was plausible that when CO_2-enriched air transfer to the bioreactor through a sparging tube, CO_2 gas dissolved in water, the dynamics of dissolved inorganic carbon that is, typically, in the form of aqueous dissolved CO_2 or HCO_3^- ion. The capacity of the particular organism to utilize preferably either dissolved CO_2 or HCO_3^- ion is species dependent (Gray et al., 1996; Nedbal et al., 2010). The dissociation of CO_2 into HCO_3^- and H^+ ions in cell culture medium and in the cell cytoplasm may induce oxidative stress due to the formation of CO_3^- and peroxymonocarbonate anion (HCO_4^-), which occurs mainly in the presence of strong oxidizing agents like H_2O_2. The

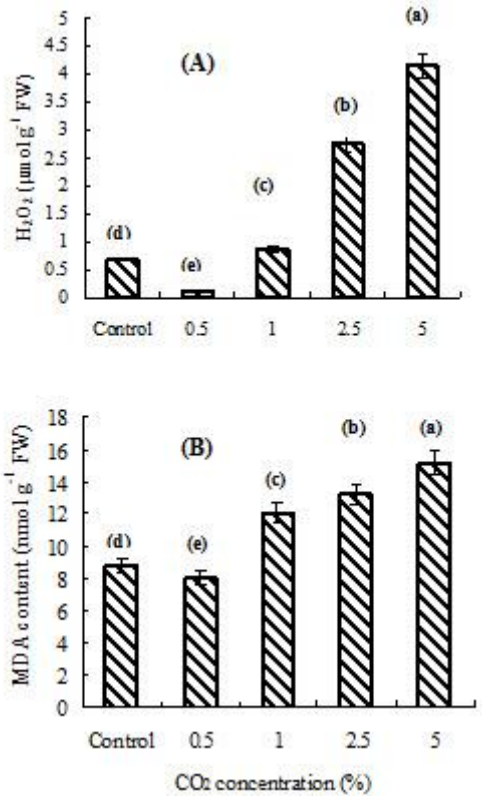

Fig 3. Accumulation of H_2O_2 (A) and MDA (B) in cells of *Morinda citrifolia* as affected by different concentrations of CO_2 after 3 weeks cultured in bioreactors. Bars represent mean ±SE (n=3).

formation of CO_3^- and HCO_4^- can damage proteins and lipids, thereby altering cell metabolism (Vesela and Wilhelm, 2002; deZengotita et al., 2002). The metalloenzyme SOD (Mn-SOD/Cu-SOD) converts O_2^- to H_2O_2, however other different processes also generated H_2O_2 which further scavenged by CAT, APX, POD and various peroxidases (Wang et al., 2001; Neill et al., 2002). It has been reported that upon stress Cu-SOD reacts with H_2O_2 and produce hydroxyl radical that oxidizes HCO_3^- (the dissociated product of CO_2) to produce CO_3^- ion. Additionally, H_2O_2 reacts with HCO_3^- ion to produce HCO_4^- ion (Wang et al., 2001; Vesela and Wilhelm, 2002; deZengotita et al., 2002). Therefore, the combined action of CAT and G-POD is critical to arrest the toxic accumulation of H_2O_2 by converting it to nontoxic H_2O, and thereby mitigating the harmful effects of oxidative stress.

These results obtained from our study show that the enrichment of CO_2 concentrations induced SOD activity (Fig. 2A), which may inhibit superoxide anion (O_2^-) accumulation in cells resulting elevation of H_2O_2 accumulation (Fig. 3A). The induction of SOD activity at elevated CO_2 supply may be due to the presence of Cu/Mn-SOD isoforms. Because, upregulation of SOD activity in CO_2-treated roots of *Echinacea angustifolia* was observed mainly due to the presence of two isoforms (Cu-SOD and Mn-SOD). Moreover, activities of G-POD and APX, which increase with elevated levels of CO_2 supply also revealed the presence of their isoforms (Ali et al., 2006a). Similar to our present study, upregulation of SOD activity under various stresses including CO_2 also reported by several authors (Shalata et al., 2001; Harinasut et al., 2003; Ali et al., 2008; Baque et al., 2010).

CAT and G-POD activities were induced at 0.5% and 2.5% CO_2 treated cultures compared to other treatments (Fig. 2B-C). Although, relatively higher CAT and G-POD activities were observed at 2.5% CO_2 compared to 0.5% CO_2 treated culture, however, higher concentrations of H_2O_2 and MDA formations were detected (Fig. 3A-B). These results suggest that the combined action of CAT and G-POD is not sufficient enough to cope with CO_2-induced H_2O_2 accumulation when cells were exposed to elevated levels of CO_2 (≥1%), therefore peroxidation of lipids occurred. Contrary, the joint function of CAT and G-POD at 0.5% CO_2 treated culture efficiently mitigated toxic H_2O_2 accumulation and consequently less membrane lipid peroxidation was observed compared to other CO_2 treated cultures. This may be the fact that why cell growth was enhanced when cultures were treated with 0.5% CO_2. In root suspension cultures of *P. ginseng* and *E. angustifolia*, it was observed that CO_2 treatments even at 5% did not induce membrane lipid peroxidation, which probably comes from an increased capacity for ROS scavenging by defense enzymes (Ali et al., 2006a, 2008). A relatively lower lipid peroxidation under different stresses has also been reported by several authors (Shalata et al., 2001; Ashraf and Harris, 2004). Contrary, a relatively higher lipid peroxidation was observed when adventitious roots of *Morinda citrifolia* were treated with high salt strength (≥ 1 MS) and high concentrations of sucrose (Baque et al., 2010; 2012b). Consequently, strong inhibition of root growth and higher concentrations of secondary metabolites were detected in the residual media. Therefore, it is reasonable to suspect that H_2O_2-mediated induction of membrane lipid peroxidation under elevated levels of CO_2 (2.5% and 5%) in this study might be caused membrane damage that inhibited cell growth. H_2O_2 burst induced upon stresses plays an important role in inducing secondary metabolism such as activation of PAL and phenolic compounds biosynthesis (Han and Yuan, 2004). Indeed, CO_2-induced H_2O_2 formation was found to be well-correlated with induction of PAL activity and corresponding phenolics biosynthesis. That might be played an important role to combat ROS-mediated damage and thereby allowing cells to grow under elevated levels of CO_2. These results suggest that enrichment of CO_2 concentrations not only stimulated phenolic compounds biosynthesis but also the defense response of *M. citrifolia* cells.

Materials and methods

Induction and proliferation of calli

Calli were induced from *in vitro* grown plantlets of *M. citrifolia*. Leaves from the apical buds were cut into 1x1 cm pieces, and inoculated in Petri dishes containing 25 ml of solid Murashige and Skoog (MS) medium supplemented with 2 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 1 mg l^{-1} kinetin, 30 g l^{-1} sucrose, and 2.3 g l^{-1} gelrite. To induce calli, cultures were kept in the dark at 25±2°C for 4 weeks. For proliferation, the induced calli were transferred to 400 ml flasks containing 100 ml MS liquid medium supplemented with 3.0 mg l^{-1} naphthalene acetic acid (NAA), 0.1 mg l^{-1} kinetin, and 30 g l^{-1} sucrose. The medium pH was adjusted to 5.8 before autoclaving. The cultures were kept on a rotary shaker at 100 rpm at 25±2°C under a 16 h photoperiod and a photosynthetic photon flux (PPF) of 20 µmol m^{-2} s^{-1} and were maintained by subculturing into fresh medium (Ahmed et al., 2008).

Bioreactor cultures

Cell cultures were established as previously described by Ahmed et al. (2008) in a 3 l capacity balloon type bubble bioreactor (BTBB) containing 1.5 l of MS medium supplemented with 3.0 mg l^{-1} NAA, 0.1 mg l^{-1} kinetin, and 30 g l^{-1} sucrose. Thirty grams of 12-mon-old fresh cell per liter was used as inoculum size. The airflow rate was maintained at 0.3 vvm using a flow meter (Dwyer Inc., IN, USA). The cultures were maintained at 25±2°C under a 16 h photoperiod and a photosynthetic photon flux (PPF) of 20 µmol m^{-2} s^{-1} for 3 weeks (Ahmed et al., 2008). To investigate the effects of various levels of CO_2, the inlet air was mixed with CO_2 to yield CO_2 concentrations of 0.03% (control), 0.5%, 1.0%, 2.5% and 5.0% by using a KOFLOC flow meter. Those CO_2 concentrations were maintained during the whole 3 weeks of culture period contentiously supplied from a CO_2 reserve tank. After 3 weeks, cells were harvested for sampling to evaluate the effect of elevated CO_2 supply based on cell growth, metabolites production, stress levels, associated enzymatic activities and antioxidative response. A schematic diagram of the experimental system is shown in supplementary Fig. S1. Each experiment was repeated twice, and included three replications per treatment.

Determination of cell fresh weight, dry weight and growth ratio

After 3 weeks, the harvested cell suspensions were filtered (0.45 µm whatman micro filter) by using a vacuum gauge and the filtered cell fresh weight (FW) was measured. The fresh cells were dried at 60 °C for several days to a constant weight, and then cell dry weight (DW) was determined. The growth ratio (GR) was calculated as: GR = [harvested DW (g) - inoculated DW (g)] / [inoculated DW (g)].

Extraction and determination of total AQ content in cells

The dried cell samples (0.1 g) were digested in 40 ml of 80% ethanol using Cod Oil Bath digestion system (LS-2050-S10, Tech, Korea) for 2 h at 80°C and filtered through filter paper (Advantic, 110 mm, Japan). The procedure was repeated until final extractant was colorless and adjusted to 50 ml by adding rest amount of 80% ethanol. The ethanolic fractions were pooled and filtered by using 0.45 µmWhatman micro filter. Total AQ was determined by a spectrophotometer (UV-1650PC, Shimadzu, Japan) at 434 nm using alizarin (Sigma, Germany) as standard (Zenk et al., 1975).

Determination of total phenolics and flavonoids content in cells

The contents of total phenolics and flavonoids were analyzed spectrophotometrically from the same aforementioned ethanolic cell extracts according to Wu et al. (2006). The measurements of phenolics were compared to a standard curve for gallic acid and were expressed as the mg of gallic acid equivalent per gram of cell DW. The measurements of flavonoids were expressed as mg of catechin equivalents per gram of cell DW.

Determination of DPPH radical scavenging activity in cells

The antioxidant activity of the aforementioned each ethanolic extract was determined by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Hatano et al. (1998) with some modifications. A 0.625 ml of DPPH

(200 µM DPPH radical solution in 99.9% ethanol) solution was mixed with 0.375 ml of extract, vortexed and then incubated for 10 min at room temperature. The control was made by mixing 0.625 ml of DPPH with 0.375 ml 40% ethanol and the absorbance was read by spectrophotometrically against a blank at 517 nm.

Determination of hydrogen peroxide (H_2O_2)

The content of H_2O_2 was determined using a UV-visible spectrophotometer according to the method described by Sergiev et al. (1997).

Determination of lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) reacted with thiobarbituric acid (TBA) (Sigma, USA) to form TBA-MDA complex. Crude extract was prepared as described by Heath and Packer (1968) and final absorbance was measured using a UV-visible spectrophotometer at 532 and 600 nm. Total MDA equivalents were calculated according to Heath and Packer (1968) as the following:
Total MDA (nmol g^{-1} FW) = Amount of extraction buffer (ml) x amount of supernatant (ml) x [Abs 532 – Abs 600/155] x 1000/ Amount of sample (g)
Where 532 nm represents maximum absorbance of the TBA-MDA complex; 600 nm is the correction for non-specific turbidity and 155 mM^{-1} cm^{-1} is the molar extinction coefficient for MDA.

Assay of antioxidants and enzymes activity
Collection of sample for enzyme assay

For enzyme assay, fresh cells were collected immediately after harvest. For each enzymes assay one gram cells weighted and put into liquid nitrogen and stored in -80^{o}C till further analysis.

Enzyme extraction and protein estimation

To measure SOD, CAT and G-POD activity, fresh filtered cells (1 g) were powdered in liquid nitrogen with pre-chilled pestle and mortar then homogenized in 2.0 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2% (w/v) insoluble polyvinyl poly-pyrrolidone (PVPP), 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM EDTA. The homogenate was filtered through two layers of muslin and centrifuged at 10000 rpm (Hanil Science Industrial Co., Ltd., micro 17R, Korea) at 2^{O} C for 10 min. To determine PAL activity, 1 g of fresh cell was powdered and homogenized with 0.1 M sodium borate buffer (pH 7.0) solution. The homogenate was centrifuged at 10000 rpm (Hanil Science Industrial Co., Ltd., micro 17R, Korea) for 20 min. For SKDH and CAD, fresh cell samples (1.0 g) were ground in a cold mortar and pastel under liquid nitrogen and homogenized in 0.1 M K-phosphate buffer (pH 7.4) containing 0.5 mM dithiotreitol (DTT), 2 mM L-cysteine, 2 mM ethylene diamine tetra-acetic acid (EDTA), 8 mM β-mercaptoethanol and 0.5 g of polyvinyl poly-pyrrolidone (PVPP). The homogenate was centrifuged at 17000 rpm (Hanil Science Industrial Co., Ltd., micro 17R, Korea) for 20 min at 4^{0} C and repeated at least twice. The soluble protein contents from the aforementioned prepared samples for the respective enzymes were measured spectrophotometrically at 595 nm according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Assay of phenylalanine ammonia lyase (PAL) activity

PAL activity was measured according to the method described by Dickerson et al. (1984). A calibration curve (r^2 = 0.9998) was prepared for the standard solution *trans*-cinnamic acid following the same procedure. PAL activity is defined as the amount of enzyme forming 1 nmol of *trans*-cinnamic acid from the substrate phenylalanine min^{-1} at 30 ^{0}C.

Assay of SKDH and CAD enzyme activity

The activity of SKDH (EC 1.1.1.25) was assayed in a reaction mixture containing shikimic acid (2 mM) and NADP (0.5 mM) in 0.1 M Tris-HCl buffer (pH 9.0) (Diaz et al., 1997). The reaction was initiated by the addition of enzyme extract and increase in absorbance was monitored after every 60 s for 3 min spectrophotometrically at 340 nm (ε, 6.22 mM^{-1} cm^{-1}) following the NADP reduction. The CAD (EC 1.1.1.195) assay was performed in a reaction mixture (1 ml) containing 0.1 M Tris-HCl, 0.5 mM coniferyl alcohol, 1 mM NADP and enzyme extract (Mitchell et al., 1994). The increase in absorbance was recorded after every 60 s for 3 min spectrophotometrically at 400 nm (ε, 21 mM^{-1} cm^{-1}) following coniferyl alcohol oxidation.

Assay of antioxidant enzyme activity (SOD, CAT and G-POD)

Superoxide dismutase, SOD (EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT at 560 nm. The reaction mixture (5 ml) contained 0.2 M Na-phosphate buffer (pH 7.8), 250 µM NBT, 10 µM riboflavin, enzyme extract and 10 µl TMEDA (modified from Beauchamp and Fridovich, 1971). Reaction mixture was exposed to light at room temperature for 15 min. The activity is expressed as unit's min^{-1} mg^{-1} protein. Enzyme extract corresponding to 50% inhibition of reaction was considered as one enzyme unit. The activity of CAT was determined in a reaction mixture containing 100 mM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.0) and enzyme extract. The disappearance of H_2O_2 after 10 min reaction was measured by reading absorbance at 240 nm of both blank and sample (modified after Bisht et al., 1989). For blank, 1 ml 2N H_2SO_4 was added in the reaction mixture prior to the addition of enzyme extract. G-POD activity was measured by monitoring the formation of tetraguaiacol (extinction coefficient of 6.39 mM^{-1} cm^{-1}) after every 60 s for 3 min at 436 nm according to the method of Putter (1974).

Statistical analysis

The experiment was conducted in a completely randomized design with three replicates. Data were subjected to an analysis of variance (ANOVA). To determine the significant difference, Duncan's multiple range test (DMRT) was performed using SAS software (Version 6.12; SAS Institute, USA).

Conclusion

The results obtained from this study indicate that leaf-originated cells of *M. citrifolia*, tolerated to elevated levels of CO_2 are a good source for the production of phenolic compounds, e.g. AQ, phenols and flavonoids after 3 weeks, a process in which shikimate and phenylpropanoid enzymes play a prominent role. In addition, enrichment of CO_2 upregulated activities of SOD, CAT and G-POD, along with

high concentrations of phenolic compounds in cells, combating the potential danger of H_2O_2. In spite of coordinated increase in these components, a significant reduction in cell growth was observed when suspension cultures were exposed to over 1% CO_2 supply compared to 0.5% CO_2 supply or even control. However, considering total productivity, 2.5% CO_2 supply was found to be an optimal concentration in which 1.53-fold increase in AQ production was achieved compared to 0.5% CO_2 supply. It is widely accepted that other gaseous components such as oxygen and ethylene also have profound effect in controlling growth and regulation of plant secondary metabolism. Therefore, further studies regarding the role of oxygen and ethylene on cell growth and biosynthesis of AQ and other phenolic compounds would be more beneficial for bioreactor suspension culture of *M. citrifolia* as well as for other species.

Acknowledgements

This study was performed with World Class 300 R&D support project (Kolmar Korea, Development of functional herbal cosmetics using native plants for bulk purification technology, No. 10043192) from Ministry of Knowledge Economy, and also partially supported by the Bio-industry Technology Development Programme (Project No.111060-02-1-SB010), Republic of Korea.

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