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Antioxidative responses of *Echinacea angustifolia* cultured roots to different levels of CO_2 in bioreactor liquid cultures

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Abstract

The effects of CO_2 levels (0.03, 0.5, 1, 2 and 5%) on growth and antioxidant responses in 5 l bioreactor (working volume 4 l) root suspension cultures of *Echinacea angustifolia* were studied. CO_2 caused reduction in growth, malondialdehyde content (MDA), lipoxygenase (LOX, EC 1.13.11.13) activity and superoxide anion (O_2^-) accumulation along with high total phenols accumulation and pH levels. Maximal increases (37%) in total phenols were obtained at 1% CO_2 compared to the control. An increase in total glutathione and total ascorbate (ASC + DHA), accompanied with enhanced ascorbate–glutathione cycle enzymes were observed in CO_2-treated roots, which played an important role for the detoxification of harmful substances. CO_2 also induced an increase in glutathione peroxidase (GPx, EC 1.11.1.9) and glutathione-*S*-transferase (GST, EC 2.5.18) activities. After native polyacrylamide gel electrophoresis (PAGE) analysis, three superoxide dismutase (SOD, EC 1.15.1.1) isoenzymes were detected. Increase in SOD and CAT (EC 1.11.16) activities observed at 0.5% CO_2-treated roots and induced SOD activity seemed to be mainly due to Mn-SOD. However, both SOD and CAT activities were inhibited at 2 and 5% CO_2 but reached similar to control value. Three APX (EC 1.11.1.11) and three guaiacol peroxidase (G-POD, EC 1.11.1.7) isoenzymes were strongly detected, an increase in APX and G-POD activities suggests increased scavenging of ROS, indicating the tolerance to CO_2. These results indicated that limited oxidative damage as shown by lower MDA level, low LOX activity and low O_2^- accumulation may be due to the induced activities of antioxidant enzymes and non-enzymatic antioxidant. It is therefore, suggested that roots cultured in a bioreactor could protect themselves from CO_2 by altering the defense systems.

Keywords: Antioxidant enzymes; CO_2; *Echinacea angustifolia*; Glutathione content; Liquid cultures; Malondialdehyde content

1. Introduction

During the normal processes of growth and development, plants are subjected to different types of biotic and abiotic stress, such as pathogen attack, temperature, ultraviolet light and heavy metal [1]. CO_2 plays an important role in plant growth and development but most plants suffer from both physiological and biochemical changes by exposure to elevated levels of CO_2. These include an increase or decrease in biomass, prevention of fruit ripening, increase in the activity and content of cell wall degrading enzymes [2]. The effect of CO_2 on plants depends on its dosage and environmental conditions such as temperature. Adventitious root and cell cultures techniques have been established in plant and cultivated in small to large-scale bioreactors [3,4]. Growth of the roots and production of secondary metabolites in large-scale bioreactors is influenced by various factors such as shear stress and gas composition. Different bioreactors such as the conventional stirred-tank reactors have been used for the growth of adventitious roots. However, these bioreactors are associated with a high shear stress that reduces the biomass of the plant. Therefore, airlift or bubble column bioreactors are used for providing a more uniform environment for the growth of root. The gas exchange between the gas and liquid phases is another important factor in scale-up of root in bioreactors. Aeration is needed to improve fluid mixing and in association with CO_2 may induce reactive oxygen

Abbreviations: APX, ascorbate peroxidase; C, indicates control roots; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; G-POD, guaiacol peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; LOX, lipoxygenase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; NBT, nitroblue tetrazolium; PMSF, phenylmethylsulfonyl fluoride; PVP, polyvinylpyrrolidone; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid

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species (ROS), which also depends on the composition of the medium [5]. Therefore, it is important to understand growth and physiological changes of plants under elevated CO_2 in bioreactors. Under adverse conditions, ROS are generated and attack membrane lipid, phospholipids causing oxidative damage [1]. To prevent cellular damage caused by ROS, root have developed a protective system involving antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), ascorbate–glutathione pathway enzymes, catalase (CAT), glutathione reductase (GR) and non-enzymatic antioxidant such as ascorbate, glutathione [6–9].

Echinacea angustifolia is a valuable plant (family Asteraceae), and has become a popular medication against colds and flu as well as other viruses and infections, although its true potential for health benefits still remain to be fully understood. Phenolics are the main active components of *E. angustifolia*. Phenolics promote health and longevity by augmenting defense against disease, arresting the ageing process, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental well being [10]. *E. angustifolia* has only recently been regarded as a medicinal herb and has received the global spotlight, mainly due to the geographical confinement of its habitats. To overcome this problem, large-scale cultivation of the plant would be worthwhile. To achieve this, plant root cultures must be optimised to produce high yield of phenolics with improved defense systems. Therefore, root culture would be the best way to produce high value secondary metabolites, which can be used in pharmaceutical and food industries. Scale-up of suspension cultures requires an understanding of bio-chemical parameters for production of biomass and secondary metabolites. To our knowledge little is known about the effects of CO_2 on root culture of *E. angustifolia* for large-scale production in bioreactor. There is a continuous need to know in more detail how an important economic plant as root responds and adapts to such new conditions. The present study was designed to investigate the change in growth parameters, enzymatic and non-enzymatic antioxidant, MDA content, lipoxygenase, O_2^- accumulation and total phenolics in *E. angustifolia* under different levels of CO_2 in bioreactors.

2. Materials and methods

2.1. Plant materials and preparation of explants

Fresh *E. angustifolia* roots were collected from Canada and washed with a detergent solution (AIC Co., Korea) for 10 min and then rinsed with running tap water for 10 min. The root surfaces were sterilized with 70% ethyl alcohol for 30 s, immersed in a 2% NaOCl solution containing two drops of Tween-20 for 30 min and rinsed with sterile distilled water. The sterilized roots were cut into small pieces (1.0 cm × 0.5 cm × 0.3 cm).

2.2. Induction, proliferation of callus and adventitious roots

Root pieces were inoculated on Murashige and Skoog [11] medium supplemented with 1.0 mg l^{-1} indole butyric acid (IBA) and 50 g l^{-1} sucrose (pH 6.0) and cultured at 22 ± 1 °C in the dark for 1 month. After 1 month of culture, the calli were induced and proliferated on the same media under the same culture conditions.

For induction of adventitious root from the callus, proliferated callus were inoculated on 3/4th strength MS medium supplemented with 1.0 mg l^{-1} IBA and 50 mg l^{-1} sucrose (pH 6.0) and cultured at 22 ± 1 °C in the dark for a month. After a month of culture, adventitious roots were induced, which were proliferated further on 3/4th strength MS medium containing 1 mg l^{-1} IBA and 50 mg l^{-1} sucrose by sub-culturing at 4-week intervals and treated as explants for further experiments.

2.3. Treatment procedure and root collection

Four weeks old adventitious roots (1.5–2 cm) were cut in to small pieces (less than 0.5 cm) and then 5 g FW/liter (L) inokula were fed with CO_2 at concentrations of 0.03 (control), 0.5, 1, 2 and 5% containing 4 l 3/4th strength MS liquid medium supplemented with 1 mg l^{-1} IBA, 0.5 mg l^{-1} kinetin and 50 g l^{-1} sucrose. As a control, bioreactor culture was performed in a 5 l bioreactor [4]. The air flow rate was adjusted at 0.1 vvm (400 ml/min) during culture. The compositions of supplied air in control was same as atmospheric air composition (N_2 78%, O_2 20.3%, Ar 0.9% and CO_2 0.03%). Gas was supplied in to each bioreactor at an appropriate flow rate through one of the filters at a total gas flow of 400 ml/min. The percentage of CO_2 concentrations was obtained into each bioreactor by mixing air with different level of pure CO_2 using EYELA gas control unit F140, while the total aeration rate was maintained constant. The gas percentage into each bioreactor was measured by collecting 0.3 ml gas samples from the in-flow tube of each bioreactor weekly and analysed with a gas chromatograph (HP 6890, Hewlett Packard, USA). The air temperature in bioreactor was controlled 22 ± 1 °C using air controller system (Korea). A minimum of three bioreactors from each treatment was harvested at the end of the culture period (4 weeks). The roots were washed with running tap water for 5–7 min and again with nano-pure water. Roots were then blotted dry and weight 1 g and kept in −80 °C for further analysis. The pH of the medium was measured at the end of experiment using pH meter (Tokyo, Japan).

2.4. Determination of growth and total phenolics

The growth of the roots was measured in terms of fresh weight (FW) and dry weights (DW). Roots were placed between the folds of blotting paper to remove excess water and measured as a FW. Dry weight was measured after drying the fresh roots in an oven at 60 ± 1 °C for 48 h. For total phenols, 1 g dry roots were homogenized in 1 ml of 95% ethanol and kept in the freezer for 48–72 h and centrifuged at 19,000 × *g* for 20 min [12]. An aliquot (0.3 ml) of extract solution and 1.5 ml Folin-Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO—10 times dilution of 2N) was mixed and the tube was shaken thoroughly. After 5 min, a 1.6 ml solution of Na_2CO_3 (7.5%, w/v) was added and the mixture was allowed to stand for 30 min in the dark. Absorbance was measured at 715 nm. The phenols content was measured using gallic acid as standard.

2.5. Enzyme extractions and assays buffer

Roots were ground under liquid nitrogen using a specific buffer for each enzyme extraction. The homogenates were squeezed through four layers of cotton cloth, centrifuged at 20,000 × *g* for 20 min, twice and the supernatant was passed through a PVDF syringe filter (Whatman, 13 mm, 0.45 μm) and assayed as described below. All procedures were carried out at 0–4 °C. Protein concentration in the supernatant was estimated according to Bradford [13], using bovine serum albumin (Sigma) as a standard.

2.5.1. Determination of antioxidants enzymes

SOD (EC 1.15.1.1) activity was determined according to Beyer and Fridovich [14]. Roots were homogenized in 100 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 2% insoluble polyvinylpolypyrrolidone (PVPP) and 0.1% (v/v) Triton X-100. SOD activity was determined containing 100 mM potassium phosphate buffer (pH 8), 0.025% Triton X-100, 0.1 mM EDTA, enzyme extract, 12 mM L-methionine, 75 μM nitroblue tetrazolium chloride (NBT) and 2 μM riboflavin. The reaction mixture was kept under fluorescent light for 7 min. One SOD unit (U) was described as the amount of enzyme where the NBT reduction ratio was 50%. NBT reduction ratios were measured at 560 nm.