

Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericum perforatum* L.

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Abstract In this study, we investigated the influence of initial sucrose concentration on the accumulation of biomass, phenols, flavonoids, chlorogenic acid, and hypericin in adventitious root cultures of *Hypericum perforatum* L. Cultures were initiated in shake flasks by using half-strength Murashige and Skoog (MS) medium, 1.0 mg l⁻¹ indolebutyric acid (IBA), 0.1 mg l⁻¹ kinetin, and different concentrations 0, 1, 3, 5, 7, or 9% in w/v of sucrose and were maintained in darkness. The medium supplemented with 3% (w/v) sucrose resulted in the optimum biomass accumulation, but higher sucrose concentrations (5, 7, and 9%) inhibited biomass accumulation due to the relatively higher osmotic pressure. However, the amount of total phenols, flavonoids, chlorogenic acid, and total hypericin was increased with the roots grown in the medium supplemented with 5, 7, and 9% (w/v) sucrose. The antioxidant potential of methanolic extract [1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid; ABTS) radical scavenging activities] of *H. perforatum* adventitious roots was also assessed and correlated with the metabolite accumulation. Cultures maintained with higher initial sucrose concentration (5, 7, and 9% w/v) showed increased accumulation of phenols,

flavonoids, chlorogenic acid, and total hypericin, and this might be due to the osmotic stress at elevated sucrose concentrations. To verify the effect of osmotic stress on lipid peroxidation, the levels of hydrogen peroxide (H₂O₂), malondialdehyde (MDA), and proline were determined in the adventitious roots and the results revealed a marked increase in the concentrations of these compounds. These results suggest that optimal adventitious root biomass could be achieved in the MS medium with 3% (w/v) sucrose and increased sucrose concentration resulted in osmotic stress and, in turn, induces the accumulation of secondary metabolites.

Keywords Adventitious root cultures · Antioxidant activity · Biomass · Flavonoids · *Hypericum perforatum* · Phenol

Abbreviations

| | |
|-------------------------------|--------------------------------------------------------|
| ABTS | 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) |
| DPPH | 1,1-diphenyl-2-picrylhydrazyl |
| DW | Dry weight |
| FW | Fresh weight |
| H ₂ O ₂ | Hydrogen peroxide |
| IAA | Indole-3-acetic acid |
| IBA | Indolebutyric acid |
| KI | Potassium iodide |
| MDA | Malondialdehyde |
| MS | Murashige and Skoog |
| O ₂ ⁻ | Superoxide radical |
| ·OH | Hydroxyl radical |
| RO· | Alkoxy radical |
| TBA | Thiobarbituric acid |
| TCA | Trichloroacetic acid |

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Introduction

Hypericum perforatum L. (St. John's wort) is an important traditional medicinal plant native to Europe that is grown worldwide for commercial purposes. It has gained international popularity mainly for the treatment of depression and as a dietary supplement in phytomedicine (Miller 1998). The medicinal properties have been related to their phenolic composition and, particularly, hypericins, hyperforins, and flavonoids (Silva et al. 2005; Butterweck 2003). For commercial hypericin production, field-grown plant material has generally been used, but the quality of these products may be greatly affected by different environmental conditions, fungi, bacteria, viruses, and insects, which can result in heavy loss in yield and alter the medicinal content of plants. Further, plants require approximately 2 years to reach maturity and, unfortunately, cultivars that are bred for increased secondary product accumulation are not necessarily disease-resistant (Conceição et al. 2006). Therefore, in vitro cultures may be an alternative choice to manipulate and provide a better control of external factors that can interfere with the metabolic activities.

Cell or organ cultures have emerged as a valuable route for biosynthesizing phytochemicals in a limited time and space (Jeong et al. 2009). For large-scale cultivation, the optimization of physical and chemical conditions, which is varied according to plant species, culture tissues, and culture objectives, is necessitated. For most adventitious root cultures, sucrose is an important carbon and energy source, and its initial concentration can affect such parameters as growth and the yield of secondary metabolites. An increase of sucrose concentration in the medium above the normally used 2–3% level was reported to stimulate the production of *ginseng* saponin and polysaccharides in suspension cultures of *Panax notoginseng* (Zhang et al. 1996). For the adventitious root cultures of *Echinacea angustifolia*, a relatively higher sucrose concentration of 7% (w/v) was found to be more favorable for biomass accumulation, whereas 5% (w/v) sucrose was best for the production of phenols and flavonoids (Wu et al. 2006). In addition to being a major carbon source for in vitro growth, sucrose acts as osmotic agents that may induce osmotic stress above certain concentrations, in which reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), and alkoxyl radical ($RO\cdot$), are produced (Al-Khayri and Al-Bahrany 2002). It is already known that free-radical-induced peroxidation of lipid membranes is a reflection of stress-induced damage at the cellular level. Therefore, the level of malondialdehyde (MDA), produced during the peroxidation of membrane lipids, is often used as an indicator of oxidative damage (Shohael et al. 2006). Fortunately, plants

have developed two antioxidant systems, enzymatic and non-enzymatic scavenging systems, to protect cells by controlling the intra-cellular ROS content. Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of the possible means for overcoming osmotic stress caused by the loss of water (Molinari et al. 2004).

The in vitro propagation of St. John's wort has been reported in gelled medium through shoot regeneration from leaves, hypocotyls, stem cuttings, and stamens, but these techniques are difficult to automate for large-scale production (Zobayed and Saxena 2003). The culturing of adventitious root tissues is an efficient means of biomass production because of fast growth rates and stable metabolite productivity, and adventitious root cultures of several species have been utilized successfully as models to produce root biomass and secondary metabolites (Murthy et al. 2008). However, the adventitious root cultures for *H. perforatum* have not yet been studied. Therefore, in the present study, we have established the adventitious root cultures of *H. perforatum* and examined the effects of initial sucrose concentration on the production of biomass and phenolics in flask cultures. Furthermore, sucrose-induced osmotic stress and antioxidant activity were also determined.

Materials and methods

Induction of adventitious roots and their maintenance

Adventitious roots of *H. perforatum* L. were induced by using leaf explants (0.5×0.5 cm) on full-strength Murashige and Skoog (MS) medium supplemented with B5 (Gamborg et al. 1968) vitamins, 3% (w/v) sucrose, 0.5 mg l^{-1} indole-3-acetic acid (IAA), and 2.3 g l^{-1} gelrite. Adventitious roots were maintained in MS liquid medium supplemented with B5 vitamins, 3% (w/v) sucrose, and 1.0 mg l^{-1} IBA in 250-ml shake flasks (containing 70 ml of medium). Cultures were maintained in darkness at a temperature of $25 \pm 1^\circ\text{C}$ on gyratory shakers at 100 rpm and were subcultured once every 3 weeks.

Optimization of culture conditions: effect of sucrose concentration

Adventitious roots (6 g l^{-1} , 1 cm long) were inoculated in 250-ml shake flasks containing 70 ml half-strength MS medium with B5 vitamins, 1.0 mg l^{-1} IBA, 0.1 mg l^{-1} kinetin, and different concentrations (0, 1, 3, 5, 7, or 9% in w/v) of sucrose. All shake flask cultures were kept in the dark at $25 \pm 1^\circ\text{C}$ and on gyratory shakers at 100 rpm. After 5 weeks of culture, the growth of adventitious roots

was assessed in terms of fresh weight, dry weight, % dry weight, and growth ratio.

Determination of the root weight, % dry weight, and growth ratio

The root fresh weight (FW) and dry weight (DW) were determined as follows. Roots were separated from the medium by passing it through a 1-mm stainless steel sieve. The root FW was then measured after rinsing the roots once with sterile water and then blotting away the surface water. The roots were then dried at 60°C for 1 day, after which the root DW was recorded. The growth ratio and % dry weight were then calculated as follows: growth ratio = [harvested dry weight (g) – inoculated dry weight (g)]/inoculated dry weight (g); and % dry weight = harvested dry weight (g)/harvested fresh weight (g) × 100.

Preparation of root extract

Ground-dried roots (0.5 g) were refluxed with 15 ml of 80% methanol at 80°C for 1 h. After centrifugation (Union 5KR; Hanil, Inchun, Korea) at 4,000×g for 10 min, the supernatant solution was filtered under vacuum into a volumetric flask. The residue was re-extracted in the same way and the final volume of the solution was set at 30 ml.

Determination of total phenol content

The amount of total phenols in our root methanolic extracts was analyzed spectrophotometrically, after modification of a colorimetric method described by Folin and Ciocalteu (1927). The methanolic extracts (100 µl) were mixed with 2.5 ml deionized water, followed by the addition of 0.1 ml (2 N) Folin-Ciocalteu reagent. They were mixed well and allowed to stand for 6 min before 0.5 ml of a 20% (w/v) sodium carbonate solution was added. The color was developed after 30 min at room temperature and the absorbance was detected at 760 nm on a UV visible spectrophotometer (UV-1650 PC; Shimadzu, Japan). These measurements were compared to a standard curve for gallic acid and were expressed as the mg of gallic acid equivalent per gram of dry roots.

Determination of total flavonoid content

The total flavonoid content was determined colorimetrically, according to the method described by Wu et al. (2006). Briefly, 0.25 ml of the methanolic root extract or a (+)-catechin standard solution was mixed with 1.25 ml of distilled water, followed by the addition of 75 µl of a 5% (w/v) sodium nitrite solution. After 6 min, 0.15 ml of a 10% (w/v) aluminum chloride solution was added and the

mixture was allowed to stand for a further 5 min before 0.5 ml of 1 M sodium hydroxide was added. The absorbance was measured immediately at 510 nm on a spectrophotometer (UV-1650 PC; Shimadzu, Japan). The results were expressed as mg of (+)-catechin equivalents per gram of dry roots.

Determination of the content of chlorogenic acid

The analysis of chlorogenic acid was conducted using the method described by Wu et al. (2007). The chlorogenic acid fractions were analyzed using a high-performance liquid chromatography (HPLC) system with a Nova-Pak C 18 column (particle size 4.0 µm, 150 × 3.9 mm). The mobile phase was (A) acetonitrile and (B) 0.1% trifluoroacetic acid. The gradient elution was modified as follows: initial 90% B for 20 min, 60% B for the next 10 min, 50% B for 1 min, after which the mobile phase was returned to its initial conditions for 4 min with a flow rate of 0.4 ml/min. The chlorogenic acid derivatives were detected at 330 nm and then compared to standard chlorogenic acid, which were obtained from ChromaDex (Laguna Hills, CA, USA).

Determination of the content of total hypericin

The total hypericin was determined by a spectrophotometer according to the method described by Pei et al. (2002). The absorbance of methanolic root extract was measured at 590 nm and then compared to standard hypericin, which were obtained from ChromaDex (Laguna Hills, CA, USA).

Determination of residual sugars in the media

Medium samples were collected at the end of the culture period. After filtering (0.45-µm Millipore syringe filter), the medium was diluted 10-fold and 1 ml was used for sugar analysis. The level of sucrose in liquid medium was determined by HPLC (Waters 600S controller, Waters 626 pump; Waters Co., Mildford, MA, USA) with a high-performance carbohydrate column (300 × 7.8 mm; Waters Co., Mildford, MA, USA) and a refractive index detector (Differential Refractometer; Waters Co., Mildford, MA, USA). A mobile phase consisting of 80% acetonitrile (w/v) was used at a flow rate of 1.4 ml/min.

Determination of free radical scavenging activity using DPPH radical

The scavenging activity of the extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured according to the method of Kim et al. (2008), with some modifications. The 0.2 mM DPPH radical solution (0.8 ml) was added to

0.2 ml of the methanolic root extract. After 30 min, the absorbance was measured at 520 nm using a spectrophotometer (UV-1650 PC; Shimadzu, Japan). The antioxidant activity is given as the percentage (%) of DPPH scavenging, calculated as $[(\text{control absorbance} - \text{extract absorbance})/\text{control absorbance}] \times 100$.

Determination of total antioxidant capacity using ABTS radical

The scavenging activity of the extracts on 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation was measured according to Kim et al. (2008). ABTS radical cation was generated by adding 7.4 mM ABTS to 2.6 mM potassium persulfate solution and the mixture was left to stand overnight in darkness at room temperature. The ABTS radical cation solution was diluted with distilled water to obtain an absorbance of 1.4–1.5 at 735 nm (molar extinction coefficient $\epsilon = 3.6 \times 10^4 \text{ mol}^{-1} \text{ cm}^{-1}$). Diluted ABTS radical cation solution (1 ml) was added to 50 μl of extract, ascorbic acid standard solution. After 60 min, the absorbance was measured at 735 nm using a spectrophotometer (UV-1650 PC; Shimadzu, Japan). The ABTS radical cation scavenging activity was expressed as AEAC and defined as the mg of ascorbic acid equivalents per g FW of sample. $\text{AEAC} = (\Delta A_{\text{sample}}/\Delta A_{\text{Aaa}}) \times C_{\text{Aaa}} \times V/W_{\text{sample}}$, where ΔA_{sample} is the change of absorbance in the presence of the sample extracts, ΔA_{Aaa} is the change of absorbance after the addition of ascorbic acid standard solution, C_{Aaa} is the concentration of ascorbic acid standard solution (mg ml^{-1}), V is the volume of sample extracts (ml), and W_{sample} is the weight of sample used for extraction (g).

Determination of hydrogen peroxide, lipid peroxidation, and proline activity

The hydrogen peroxide content of the adventitious roots was measured spectrophotometrically after reaction with potassium iodide (KI) (Sergiev et al. 1997). One gram of adventitious roots was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid in a pre-chilled mortar and pestle by liquid nitrogen. The reaction mixture consisted of 0.5 ml adventitious roots extract supernatant, 0.5 ml of 10 mM K-phosphate buffer (pH 7.0), and 1 ml reagent (1 M KI, w/v in fresh double-distilled water). The blank probe consisted of 10 mM K-phosphate buffer in the absence of adventitious root extract. After 1 h of reaction in darkness, the absorbance was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2 . Lipid peroxidation of adventitious roots was estimated by the level of MDA production using the thiobarbituric acid (TBA)

method as described by Heath and Packer (1968). A quantity of 0.2 g of adventitious roots was homogenized with a mortar and pestle in 4 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $3,000 \times g$ for 10 min. The 1 ml of supernatant was mixed with 0.25 ml of 20% (w/v) TCA, 0.25 ml of 0.01% (w/v) butylated hydroxyl toluene, and 0.5 ml of 0.65% (w/v) TBA and heated at 95°C for 30 min and allowed to cool in an ice bath quickly. The supernatant was centrifuged at $3,000 \times g$ for 10 min and the resulting supernatant was used for the determination of MDA content. Absorbance at 532 nm was recorded and corrected for non-specific absorbance at 600 nm (i.e., $155 \text{ mM}^{-1} \text{ cm}^{-1}$).

Free proline content was measured as described by Bates et al. (1973). Adventitious roots (0.5 g) were weighted, homogenized in 3 ml of 3% (w/v) 5-sulfosalicylic acid in a pre-chilled mortar and pestle in liquid nitrogen, and then centrifuged at $4,000 \times g$ for 10 min; 2 ml of the supernatant were taken for proline estimation by the ninhydrin reagent (0.125 g ninhydrin, 3 ml of glacial acetic acid, 2 ml 6 M H_3PO_4) and incubated for 1 h at 100°C . The reaction was stopped by placing in an ice-cold bath for 15 min. About 4 ml of toluene was used for extraction. The absorbance at 520 nm was determined and the proline concentration estimated from a standard curve.

Statistical analysis

The results shown are the mean values of three independent experiments. One-way analysis of variance (ANOVA) was used to determine if the groups differed significantly. Statistical assessments of the difference between mean values were then assessed by the least significant difference (LSD) test. A P -value of <0.05 was considered to indicate statistical significance and all data were analyzed using the SAS program (SAS Institute, Inc., Cary, NC, USA).

Results and discussion

Effect of sucrose concentration on the accumulation of biomass and metabolites

The results of the accumulation of adventitious root biomass of *H. perforatum* cultured in the medium supplemented with different concentrations of sucrose (0, 1, 3, 5, 7, and 9% in w/v) over 5 weeks is presented in Table 1. The root biomass was optimum at 3% (w/v) sucrose, resulting in maximum fresh weight and dry weight, as well as growth ratio. However, higher sucrose concentrations (5, 7, and 9% in w/v) reduced the dry weight by 31.51, 40.18, and 52.13%, respectively, compared with the response to 3% (w/v) sucrose. In addition, the percentage dry weight

Table 1 Effect of sucrose concentration on the adventitious root growth of *Hypericum perforatum* and amount of phenolics and flavonoids after 5 weeks of culture

| Sucrose conc. % (w/v) | FW (g l ⁻¹) | DW (g l ⁻¹) | % of DW | Growth ratio | Total phenolics (mg g ⁻¹ DW) | Total flavonoids (mg g ⁻¹ DW) |
|--------------------------|-------------------------|-------------------------|---------|-----------------|--------------------------------------------|---------------------------------------------|
| 0 | 3.71 f* | 0.14 f* | 3.85 | – | 0.18 c* | 1.12 c* |
| 1 | 68.14 b | 3.86 e | 5.66 | 6.20 | 44.39 b | 30.05 b |
| 3 | 121.71 a | 13.14 a | 10.80 | 23.53 | 51.42 a | 39.12 a |
| 5 | 58.86 c | 9.00 b | 15.29 | 15.80 | 55.25 a | 42.61 a |
| 7 | 45.29 d | 7.86 c | 17.35 | 13.67 | 51.90 a | 39.54 a |
| 9 | 35.57 e | 6.29 d | 17.67 | 10.73 | 46.80 b | 32.61 b |

* Means with different letters within columns are significantly different at the 5% level of probability using Duncan's multiple range test

was increased with increasing concentration of sucrose; however, the growth ratio was highest with the medium supplemented with 3% (w/v) sucrose. These results indicate that higher sucrose concentrations beyond the 3% (w/v) level inhibited root growth (Table 1, Fig. 1) and this reduced root biomass accumulation might be due to the higher osmotic potential of cultured cells/organs. Similar observations of reduction in biomass with increased osmotic stress are on record with cell suspension cultures of tobacco (Tian and Russell 1998). The accumulation of total phenols and flavonoids were optimum with cultures supplemented with 3, 5, and 7% (w/v) sucrose (Table 1). Similarly, higher levels of chlorogenic acid and total hypericin were accumulated with adventitious roots cultivated in the medium supplemented with higher concentrations (3, 5, and 7% in w/v) of sucrose (Figs. 2 and 3). These findings demonstrate that higher initial sucrose concentration enhanced the accumulation of total phenolics,

flavonoids, chlorogenic acid, and total hypericin. This might be due to elevated levels of osmotic stress (Shohael et al. 2006; Wu et al. 2006).

The analysis of residual sugar levels (sucrose, glucose, and fructose) of the spent medium from 5-week-old cultures revealed that all of the exogenous sucrose had been utilized by the adventitious roots with the cultures supplemented with 1% (w/v) sucrose, whereas less than 1% glucose and fructose remained with the cultures which were initially supplemented with 3% (w/v) sucrose (Fig. 4). However, higher levels of glucose and fructose were present in the cultures which were initially supplemented with higher concentrations of sucrose (5, 7, and 9% in w/v). Thus, the final glucose and fructose content increased by a factor of 5 ~ 18 and 4 ~ 10, respectively, compared with the response to 3% (w/v) sucrose (Fig. 4). These findings indicate that extracellular hydrolysis of the sucrose occurred, which lead to the formation of glucose and fructose.

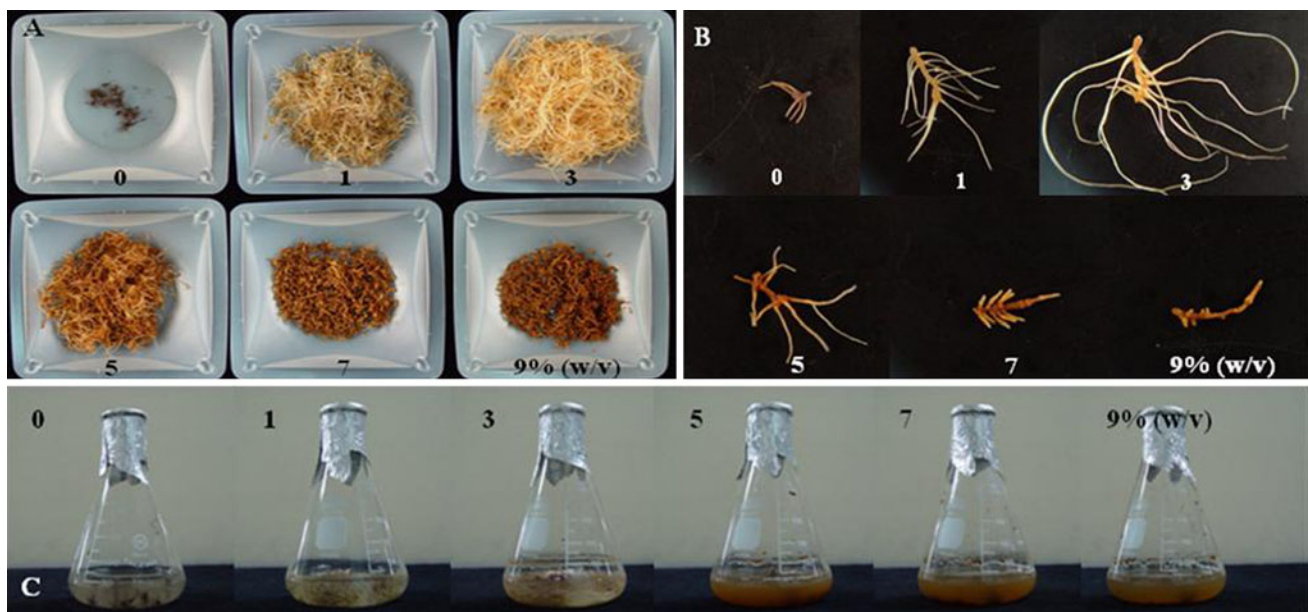


Fig. 1a–c Effect of sucrose concentration on *Hypericum perforatum* adventitious root growth after 5 weeks of culture. Adventitious roots biomass (a), morphology of adventitious roots after 5 weeks (b), and adventitious roots cultures (c)

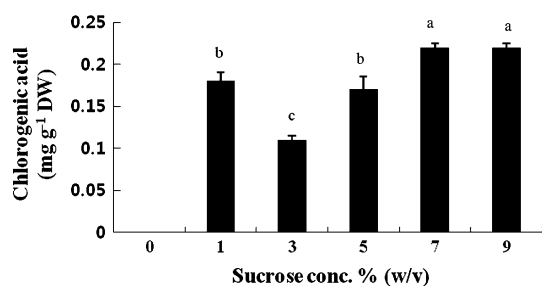


Fig. 2 Effect of sucrose concentration on the production of chlorogenic acid after 5 weeks of culture of *H. perforatum* adventitious root. Means with different letters are significantly different at the 5% level of probability using Duncan's multiple range test. The vertical bars represent the standard error of three replicates

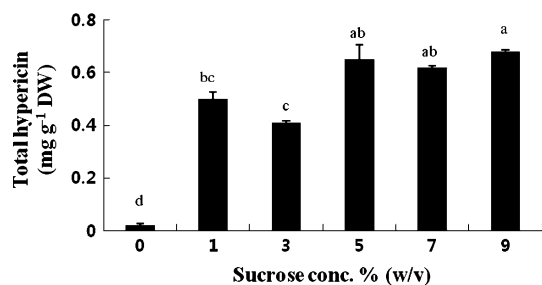


Fig. 3 Effect of sucrose concentration on the production of total hypericin after 5 weeks of culture of *H. perforatum* adventitious root. Means with different letters are significantly different at the 5% level of probability using Duncan's multiple range test. The vertical bars represent the standard error of three replicates

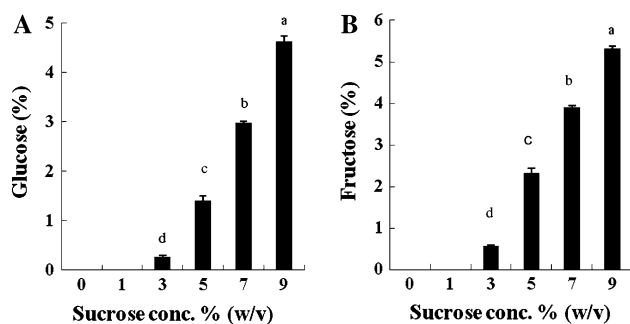


Fig. 4 Glucose (a) and fructose (b) contents in the medium as affected by the initial sucrose concentration after 5 weeks of culture. Means with different letters are significantly different at the 5% level of probability using Duncan's multiple range test. The vertical bars represent the standard error of three replicates

This hydrolysis may have been induced by acid invertase that was secreted from the adventitious root tissue into the medium (Jeong et al. 2009). Thus, the supplementation of cultures with 3% (w/v) sucrose was found to be suitable for the cultivation of adventitious roots of *H. perforatum*. Concentrations of 2–3% (w/v) of sucrose have frequently been reported as optimal for suspension cultures and higher concentrations of sucrose have repressed growth (Tian and

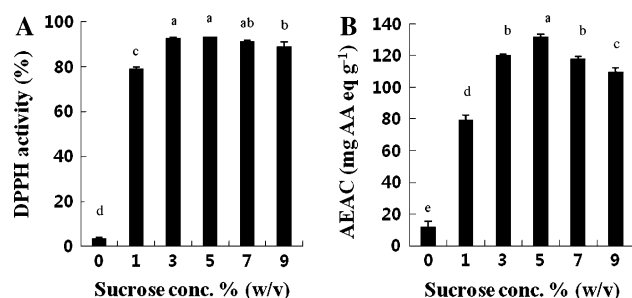


Fig. 5 Effect of sucrose concentration on 1,1-diphenyl-2-picrylhydrazyl (DPPH) activity (a) and total antioxidant activity (AEAC) (b) of *H. perforatum* adventitious root extract after 5 weeks culture. The tested concentration was 1 mg ml⁻¹, respectively. Means with different letters are significantly different at the 5% level of probability using Duncan's multiple range test. The vertical bars represent the standard error of three replicates

Russell 1998). The results of the present study also confirmed the importance of initial sucrose concentration for enhancing adventitious root growth and production of secondary compounds in *H. perforatum*.

Effect of sucrose concentration on stress levels and antioxidant properties of the *H. perforatum* adventitious root extract

Plant cell and organ cultures are usually grown heterotrophically using simple sugars as carbon sources. The level of sucrose has been shown to affect the growth of cells/organs and to affect the productivity of secondary metabolite-accumulating cultures. Sucrose also acts as osmotic agents that may introduce osmotic stress above a certain concentration, in which ROS, such as superoxide radical (O₂⁻), hydroxyl radical (·OH), hydrogen peroxide (H₂O₂), and alkoxy radical (RO·), are produced (Al-Khayri and Al-Bahrany 2002). In the present study, the osmotic stress effect of sucrose was assessed by using free radical scavenging compounds DPPH and ABTS. The results of DPPH scavenging effects of the *H. perforatum* adventitious root extracts which have been produced in cultures supplemented with different concentrations of sucrose (0, 1, 3, 5, 7, and 9% in w/v) is presented in Fig. 5a. The DPPH scavenging activities were 92.63, 93.32, and 91.17% (1 mg ml⁻¹) with root extracts which have been cultivated in the 3, 5, and 7% (w/v) concentrations of sucrose, respectively. The ABTS radical scavenging activities of the extracts also showed similar trends and the highest antioxidant activity was 132.17 mg AEAC per g when the sucrose concentration was 5% (w/v) (Fig. 5b). The roots which were grown in the medium supplemented with higher levels of sucrose accumulated higher amounts of phenolics (Table 1). Such elevated levels of phenolics in the roots grown in the medium with higher levels of

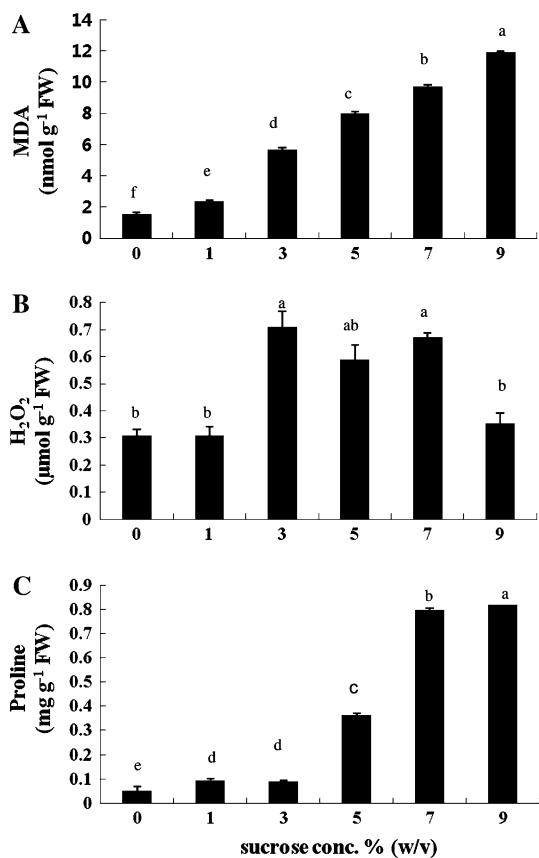


Fig. 6 Effect of sucrose concentration on malondialdehyde (MDA) content (a), H₂O₂ content (b), and proline content (c) in *H. perforatum* adventitious root cultured for 5 weeks. Means with different letters are significantly different at the 5% level of probability using Duncan's multiple range test. The vertical bars represent the standard error of three replicates

sucrose is obvious and are helpful for free radicals scavenging.

The formation of MDA (Fig. 6a) was considered as a measure of lipid peroxidation that was induced by osmotic stress treated roots. MDA, a decomposition product of polyunsaturated fatty acid hydro peroxides, has been utilized very often as a suitable biomarker for oxidative stress. This hypothesis was confirmed by the higher level of MDA in the elevated sucrose-treated adventitious roots (Fig. 6a). Osmotic stress in plant cells produces sub-cellular damage that impairs electron transport systems and leads to the production of ROS (Price et al. 1989). ROS unbalance the cellular redox systems and, in favor of oxidized forms, inactivate enzymes, cause lipid peroxidation, and potentially damage DNA (Smirnov 1993). The production of H₂O₂ (Fig. 6b) was lower in the adventitious roots grown in the medium supplemented with lower levels of sucrose (0 and 1% in w/v), whereas the roots grown in higher concentrations of sucrose (3, 5, and 7% in w/v) showed higher levels of H₂O₂. The production of ROS and

H₂O₂ in plants is known as oxidative stress and also acts as a secondary messenger to signal subsequent defense reactions in plants (Low and Merida 1996). In our study, the total phenolics, chlorogenic acid, and total hypericin accumulation were significantly higher in the roots grown in the medium supplemented with higher levels of sucrose (5, 7, and 9% in w/v) as compared to other treatments (Table 1, Figs. 2 and 3). These results indicate that lipid peroxidation occurred in *H. perforatum* adventitious roots, probably as a consequence of the higher H₂O₂ levels (Fig. 6). H₂O₂ may function as a signal for the induction of defense systems and could enhance secondary metabolite production (Berglund and Ohlsson 1995). Thus, it is speculated that a cascade of events, including lipid peroxidation and accumulation of H₂O₂ contents, may be involved in the induction of secondary metabolite accumulation. In view of this, it appears that osmotic stress is a pre-requisite for secondary metabolite synthesis.

Proline accumulation is one of the adaptations of plants to salinity and water deficit. It has also been widely advocated that proline accumulation is used as a parameter of selection for water deficit stress (Molinari et al. 2004). In this study, the proline content in adventitious roots significantly increased with elevated sucrose concentration, indicating elevated stress levels (Fig. 6c). Such a phenomenon was also claimed with the in vitro culture of *Spathiphyllum cannifolium* shoots and the proline content increased with elevated MS medium salt strength (Dewir et al. 2005). Therefore, when the sucrose concentration was higher than 3% (w/v), the adventitious root of *H. perforatum* was greatly affected by water deficit stress. In this study, H₂O₂ content, MDA content, and proline content increased with elevated sucrose concentration. Therefore, the water deficit stress might be considered to be alleviated or prevented by an increased non-enzymatic scavenging system, such as amino acid proline accumulation.

Conclusion

The results obtained in the present study show that the total phenols and flavonoids were increased considerably in response to high sucrose treatments. Increase in stress levels and antioxidant activities after high sucrose treatment shows evidence of osmotic stress and that the adventitious roots have an enhanced free radical scavenging ability.

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