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Optimization of Culturing Conditions for the Production of Biomass and Phenolics from Adventitious Roots of *Echinacea angustifolia*

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We investigated different concentrations of auxins (IAA, IBA, NAA), the strength of the MS medium, sucrose and ammonium/nitrate contents, initial medium pH, and inoculum size to determine their effects on biomass increase and the accumulation of total phenols and flavonoids in adventitious roots of *Echinacea angustifolia*. These roots were cultured under darkness in shake flasks for 4 weeks. IBA proved the best auxin for inducing root proliferation. Root growth was inhibited when the initial pH was maintained below 5.0 or above 6.0. Nitrate, rather than ammonium, was more necessary for root growth and phenolics accumulations. Overall, biomass increased and total phenol and flavonoid contents were maximized under the following conditions: half-strength MS medium supplemented with 2 mg L^{-1} IBA, 5% (w/v) sucrose, 5:25 (mM) ammonium/nitrate ratio, pH adjusted to 6.0 before autoclaving, and an inoculum size of 10 g L^{-1} FW. These results indicate that the type of *in vitro* environment strongly affects growth and the accumulation of phenolics from adventitious root cultures of *E. angustifolia*. Such optimization is beneficial to large-scale production of biomass and secondary metabolites in that species.

Keywords: adventitious root culture, *Echinacea angustifolia*, flavonoids, phenols, shake flask culture

Echinacea spp. (family Asteraceae) is a traditional perennial herb that has gained considerable international attention because of its increasing economic value and utility as a medicinal plant. The genus *Echinacea* (purple coneflower) is native to North America (McGregor, 1968). Drugs manufactured from this genus are among the most widely used herbal medicines in Europe, North America, and Australia for the treatment of colds, flu, and chronic respiratory infections (Leung and Foster, 1996). Following extensive qualitative research, its chemical composition is now attracting claims of beneficial pharmacological activity (Hobbs, 1989; Bauer and Wagner, 1991; Pellati et al., 2004). Commercial supplies are obtained primarily from the roots and aerial portions of three species: *Echinacea angustifolia*, *Echinacea purpurea*, and *Echinacea pallida*. Historically, the roots of *E. angustifolia* were the most frequently used medicine among most First Nations groups in the Great Plains region of the United States (Shemluck, 1982; Kindscher, 1989). In the last decade, *Echinacea* species have regained their popularity as the top-selling medicinal-market botanical (Brevoort, 1998). Wild-harvested *E. angustifolia* roots have the highest market value of all *Echinacea* material sold as phytomedicine. Because commercial preparations are commonly made from root tissues, *in vitro* protocols for their culturing could potentially improve this species' commercial availability if manufacturers were able to obtain consistent root material in a shorter time frame than is common with field production.

Plant phenolics constitute one of the major groups of compounds that act as primary antioxidants or free radical terminators (Thumann and Herrmann, 1980; Ramandthan and Das, 1992). The antimutagenic and anticarcinogenic effects of phenolics have also been demonstrated (Newmark, 1987; Deschner et al., 1991), as have their protective roles against cancer, cardiovascular diseases, and cataracts (Hollman et al., 1996). Phenolics possess both antibacterial (Tomas-Lorente et al., 1992) and antifungal effects (Weidenbörger et al., 1990). Flavonoids, some of the most important natural phenolics, are members of a highly diverse and widespread group of compounds (Agrawal, 1989) that also possess a broad spectrum of chemical and biological activities, including radical-scavenging properties. The phenolic phytochemicals present in the roots of *E. angustifolia* can be used as markers to assess its quality in a given product.

The culturing of adventitious root tissues is an efficient means of biomass production because of fast growth rates and stable metabolite productivity (Choi et al., 2000; Kim et al., 2004). Moreover, those roots can serve as a continuous source for obtaining secondary metabolites. Therefore, high product concentrations and efficiency can be achieved by optimizing the *in vitro* culture conditions. Here, we examined the effect of various chemical factors on adventitious root cultures of *E. angustifolia*. Their production of biomass, total phenols, and flavonoids were investigated with the

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aim to establish a protocol suitable for large-scale production of this species.

MATERIALS AND METHODS

Plant Material and Adventitious Root Culture

Ex vitro fresh root explants of *E. angustifolia* were collected from Canada and used for the induction of adventitious roots *in vitro* (Yoo, 2005). The adventitious roots of *Echinacea* were maintained for over two years on a half-strength MS (Murashige and Skoog, 1962) liquid medium supplemented with 5% (w/v) sucrose and 1 mg L^{-1} IBA. The initial medium pH was adjusted to 6.0 before autoclaving (at 121°C and 1.2 kg cm^{-2} pressure for 15 min), and the cultures were kept under darkness at 25°C. These adventitious roots were subcultured monthly.

Optimization of Culture Conditions

Adventitious roots (10 g L^{-1}, 2 cm long) were inoculated in 500-mL shake flasks containing 200 mL half-strength MS medium with 5% (w/v) sucrose and different concentrations (1, 2, 4, or 6 mg L^{-1}) of IAA, IBA, or NAA. The initial concentration of root inoculum was also adjusted to 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5, or 20 g L^{-1} for determining its optimum size for promoting root growth. Various strengths of MS medium (0.25, 0.50, 0.75, 1.0, 1.50, or 2.0), as well as sucrose (0, 1, 3, 5, 7, or 9% in w/v), ammonium/nitrate ratios (0:40, 0:35, 0:30, 5:25, 10:20, 15:15, 20:10, 25:5, or 30:0 in mM), and initial pH of the medium (4, 5, 6, 7, 8, or 9) were applied depending on the objective of the experiment. All shake flask cultures were kept in the dark at 25°C and 100 rpm (Fig. 1A, B). After four weeks of culture, the growth of adventitious roots was assessed in terms of fresh weight, dry weight, % of dry weight, growth ratio, and content of total phenols and flavonoids.

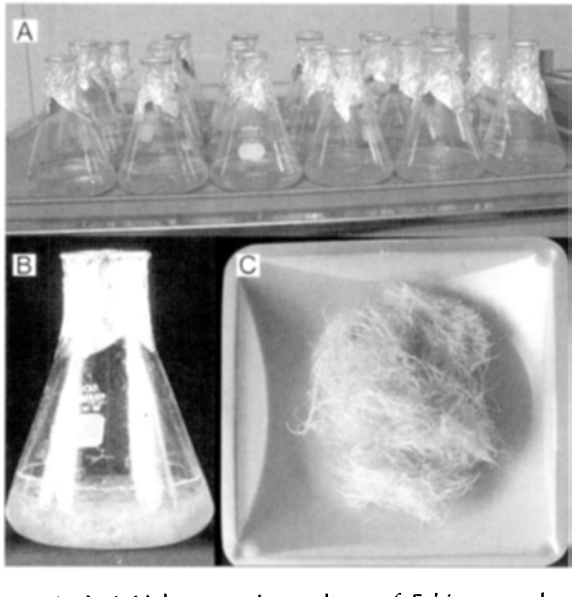

Figure 1. A, Initial suspension culture of *Echinacea* adventitious roots in 250-mL Erlenmeyer shake flasks; **B**, Growth after 4 weeks in culture; **C**, Harvested fresh roots.

Determination of Root Biomass

The roots were separated from the media by passing them through a stainless steel sieve. Their fresh weights were determined after they were rinsed with tap water and the excess surface water blotted away (Fig. 1C). Dry weights were recorded after the roots were dried at 60°C for 2 d to a constant weight. The growth rate (GR) was determined as: GR= {harvested dry weight (g) – inoculated dry weight (g)}/ inoculated dry weight (g).

Preparation of Root Extract

Ground-dried roots (0.2 g) were extracted with 10 mL of 80% methanol at room temperature, using a magnetic stirrer for 15 min. After centrifugation 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 25 mL.

Determination of Total Phenol Contents

The amount of total phenols in our root methanolic extracts was analyzed spectrophotometrically, after modification of a colorometric 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% 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-1650PC; 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 Contents

Total flavonoid content was determined colorimetrically, according to the method described by Zhishen et al. (1999), Dewanto et al. (2002), and Sakanaka et al. (2005). 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% sodium nitrite solution. After 6 min, 0.15 mL of a 10% aluminum chloride solution was added and the mixture was allowed to stand for a fur-

ther 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-1650PC; Shimadzu, Japan). Results were expressed as mg of (+)-catechin equivalents per gram of dry roots.

Experimental Design

Experiments were set up in a completely randomized design, and data were subjected to Duncan's multiple range tests using SAS software (Version 6.12; SAS Institute, USA).

RESULTS AND DISCUSSION

Effect of Auxins on Adventitious Root Growth and Phenolics Content

Adventitious root explants were cultured on half-strength MS media supplemented with various concentrations of auxins (IAA, IBA, or NAA; 1, 2, 4, or 6 mg L^{-1}; Table 1). IBA proved to be the best auxin for adventitious root proliferation. The greatest response in terms of biomass production and contents of phenols and flavonoids was observed on the medium containing 2 mg L^{-1} IBA. However, these responses were markedly suppressed when the medium was supplemented with >2 mg L^{-1} IBA. High auxin levels are often deleterious to secondary metabolite accumulation (Dornenburg and Knorr, 1995; Chan et al., 2005). Our experiments also demonstrated that increasing the NAA concentration had a negative effect on biomass, and on phenol and flavonoid contents. However, the response of adventitious roots to different auxins depends on the plant species. For example, treatment with IBA is more effective than NAA in promoting biomass production from root cultures of *Panax ginseng* (Kim et al., 2003). In contrast, NAA is better at inducing the elongation of tomato lateral roots (Taylor and van Staden, 1998). Finally, naturally occurring auxins (IAA or IBA) show different effects on the induction and elongation of roots compared

Table 1. Effect of type and concentration of auxin on adventitious root growth and contents of phenols and flavonoids from *Echinacea* after 4 weeks in culture.

Auxin (mg L^{-1})		Fresh weight (g)		Dry weight (g)		% of Dry weight	Growth rate	Total phenols (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
IAA	1	3.9	g[z]	0.47	e	12.0	1.4	31.1	D	23.5	de
	2	4.5	fg	0.64	de	14.2	2.2	32.4	D	25.5	d
	4	7.2	cde	1.14	b	15.8	4.7	39.9	C	30.4	b
	6	8.8	b	1.15	b	13.1	4.8	46.9	Ab	34.8	a
IBA	1	8.6	bc	1.31	ab	15.2	5.6	45.6	Ab	34.3	a
	2	11.8	a	1.40	a	11.9	6.0	50.0	A	35.1	a
	4	8.3	bc	1.02	c	12.3	4.1	41.2	B	31.1	b
	6	7.1	cde	0.84	cd	11.8	3.2	37.9	C	29.2	b
NAA	1	7.7	bcd	1.07	bc	13.9	4.4	43.3	B	32.1	b
	2	6.7	de	0.85	cd	12.7	3.2	36.8	Cd	28.8	c
	4	6.1	e	0.67	de	11.0	2.4	36.7	Cd	25.0	d
	6	5.8	ef	0.59	de	10.2	2.0	29.8	E	22.7	e

[z], Means separation within columns by Duncan's multiple range tests at 5% level.

Table 2. Effect of inoculum size on adventitious root growth and contents of phenols and flavonoids from *Echinacea* after 4 weeks in culture.

Inoculum size (g L^{-1} FW)	Fresh weight (g)		Dry weight (g)		% of Dry weight	Growth rate	Total phenols (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
2.5	8.8	d[z]	0.95	d	10.8	18.0	42.4	d	30.0	B
5.0	11.5	c	1.27	c	11.0	11.7	50.9	a	36.7	A
7.5	12.1	b	1.32	b	10.9	8.1	50.2	a	34.2	Ab
10.0	12.5	b	1.45	ab	11.6	6.3	50.3	a	35.2	A
12.5	12.0	b	1.42	ab	11.8	4.7	49.0	b	32.4	B
15.0	12.2	b	1.46	ab	12.0	3.9	45.0	c	29.1	B
17.5	13.5	b	1.56	ab	11.6	3.4	43.9	d	27.8	D
20.0	15.5	a	1.64	a	10.6	3.1	46.1	c	28.1	C

[z], Means separation within columns by Duncan's multiple range tests at 5% level.

with synthetic auxins e.g., NAA (Biondi et al., 1997).

Effect of Inoculum Size on Adventitious Root Growth and Phenolics Content

Inoculum size (2.5 to 20.0 g L^{-1} FW) was investigated for its influence on the root growth from *Echinacea* (Table 2). Final weights for root biomass were low when less dense inoculum sources were used, but development was greatly enhanced with inoculum sizes of 10 to 20 g L^{-1} FW. However, large sizes resulted in low phenol and flavonoid contents. Therefore, the best inoculum size for biomass and secondary metabolites was determined to be 10 g L^{-1} FW. This optimization of size is a known fundamental factor in determining the success of tissue cultures (Ozeki and Komamine, 1985; Su and Lei, 1993; Lee and Shuler, 2000).

Effect of MS Salt Strength on Adventitious Root Growth and Phenolics Content

The strength of the MS medium was tested to determine the optimal salt concentration for promoting root growth in terms of biomass development and the accumulation of phenols and flavonoids (Table 3). The best strengths for the production of phenols and flavonoids were 0.25 and 0.5 MS, whereas only the latter proved better for obtaining both biomass and metabolites. Higher salt strengths inhibited root growth, as manifested in the lowest fresh and dry weights and growth rates, and also resulted in the least accumulations of phenols and flavonoids. This indicates that the adventitious roots of *Echinacea* require only low levels of nutrients because of interactions among the nutritional salts, which enhance the availability of ions to the roots. The optimum nutrient concentration is a critical determinant in controlling the growth of adventitious roots and the accumulation of secondary metabolites. For example, in *Bupleurum falcatum* adventitious root cultures, a full-strength MS medium is sufficient for both root development and saikosaponin production (Yamamoto and Kamura, 1997). In *Panax ginseng* adventitious root cultures, half- and full-strength MS are suitable for biomass production whereas a full-strength medium is optimal for secondary metabolites (Yu et al., 2000).

Effect of Sucrose Concentration on Adventitious Root Growth and Phenolics Content

In testing the range of sucrose concentrations from 0 to 9% (w/v), we found that root biomass was most increased at 5% and 7%, resulting in maximum fresh and dry weight as well as growth rates. However, the highest amount of phenols and flavonoids were accumulated with 5% sucrose (Table 4). This sugar supplement is an important carbon source for adventitious root cultures; its initial concentration can affect several *in vitro* parameters, e.g., growth and the yield of secondary metabolites. Yu (2000) has reported that, for

Table 3. Effects of the salt strength in MS medium on adventitious root growth and contents of phenols and flavonoids from *Echinacea* after 4 weeks in culture.

MS medium strength	Fresh weight (g)		Dry weight (g)		% of Dry weight	Growth rate	Total phenols (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
0.25	9.6	c[z]	1.28	ab	13.3	5.4	48.2	b	33.9	A
0.50	12.8	A	1.41	a	11.0	6.0	50.7	a	35.2	A
0.75	11.4	B	1.16	b	10.2	4.8	20.5	c	15.2	B
1.00	10.0	C	0.80	C	8.0	3.0	11.8	d	7.9	C
1.50	8.4	D	0.76	C	9.0	2.8	11.7	d	7.1	C
2.00	7.4	d	0.80	C	10.8	3.0	11.5	d	7.2	C

[z], Means separation within columns by Duncan's multiple range tests at 5% level.

Table 4. Effects of sucrose concentration on adventitious root growth and contents of phenols and flavonoids from *Echinacea* after 4 weeks in culture.

Sucrose conc. % (w/v)	Fresh weight (g)		Dry weight (g)		% of Dry weight	Growth rate	Total phenols (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
0	1.7	d[z]	0.12	E	7.0	0.0	26.6	D	17.2	D
1	9.2	c	0.95	d	10.3	3.8	28.9	D	19.2	Cd
3	9.8	c	1.15	C	11.7	4.7	33.3	C	23.2	C
5	12.6	b	1.44	b	11.4	6.2	51.0	A	35.1	A
7	13.0	ab	1.56	b	12.0	6.8	51.3	A	35.0	A
9	13.4	a	1.77	a	13.2	7.8	46.6	B	29.8	B

[z], Means separation within columns by Duncan's multiple range tests at 5% level.

Table 5. Effects of ammonium/nitrate ratio on adventitious root growth and contents of phenols and flavonoids from *Echinacea* after 4 weeks in culture.

NH_4^+ : NO_3^- (mM)	Fresh weight (g)		Dry weight (g)		% of Dry weight	Growth rate	Total phenols (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
0:40	9.0	b[z]	1.29	b	14.3	5.4	61.8	a	37.5	A
0:35	9.4	b	1.30	b	13.8	5.5	65.5	a	39.2	A
0:30	9.8	b	1.35	b	13.8	5.8	62.3	a	37.7	A
5:25	11.1	a	1.60	a	14.4	7.0	62.1	a	39.0	A
10:20	11.0	a	1.36	b	12.4	5.8	49.7	b	34.9	B
15:15	6.0	c	0.65	c	10.8	2.2	11.6	C	3.8	C
20:10	5.5	c	0.64	c	11.6	2.2	10.0	C	3.4	C
25:5	3.6	d	0.45	d	12.5	1.2	13.8	C	3.8	C
30:0	1.9	e	0.22	e	11.6	0.1	16.2	C	4.2	C

[z], Means separation within columns by Duncan's multiple range tests at 5% level.

Table 6. Effects of medium pH on adventitious root growth and contents of phenols and flavonoids from *Echinacea* after 4 weeks in culture.

pH	Fresh weight (g)		Dry weight (g)		% of Dry weight	Growth rate	Total phenols (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
4	8.0	c[z]	1.03	C	12.9	4.2	50.8	C	33.2	C
5	10.2	ab	1.23	B	12.1	5.2	59.0	B	37.9	B
6	11.8	a	1.50	A	12.7	6.5	62.0	A	39.1	A
7	9.1	b	1.25	B	13.7	5.2	59.2	B	36.4	B
8	9.1	b	1.21	B	13.3	5.0	58.5	B	36.6	B
9	9.3	b	1.12	C	12.0	4.6	48.7	C	34.7	D

[z], Means separation within columns by Duncan's multiple range tests at 5% level.

adventitious root cultures of *P. ginseng*, a relatively higher sucrose concentration -- 5% (w/v) – is more favorable for biomass development, whereas Zhong et al. (1995) have found that 4.5% (w/v) sucrose is best for the production of anthocyanin. Our experimental data also confirmed the importance of initial sucrose concentration for enhancing adventitious root growth from *E. angustifolia*.

Effect of Ammonium/Nitrate Ratios on Adventitious Root Growth and Phenolics Content

Different ammonium/nitrate ratios were used in half-strength MS media to stimulate *in vitro* root proliferation (Table 5). Here, nitrate rather than ammonium proved more essential for better root growth and the accumulation of secondary metabolites. The highest content of phenols and flavonoids was achieved at a ratio of 0 (mM) ammonium to 35 (mM) nitrate, while a 5:25 ratio (ammonium:nitrate) was optimal for the production of both biomass and metabolites. This ratio has been shown previously to markedly affect plant growth and secondary products. For example, reduced levels of NH_4^+ and increased levels of NO_3 promote the production of shikonin and betacyanins, whereas higher ratios of NH_4^+/NO_3 enhance the production of berberine and ubiquinone (Ikeda et al., 1977; Nakagawa et al., 1984; Bohm and Rink, 1988; Fujita, 1988). Kronzucker et al. (1999) also have reported substantial variability in the adaptation of roots to different NH_4^+ and NO_3^- sources of N. Although the former would seem to be the preferred source because its metabolism requires less energy than that of the latter (Shaul et al., 1999), only a few species actually perform well when NH_4^+ is provided as the N source. However, when both are supplied simultaneously, growth and yield are significantly improved compared with when only one is provided alone. Likewise, our results demonstrate that an optimal ammonium/nitrate ratio is necessary for adventitious root growth and secondary metabolite production.

Effect of Initial Medium pH on Adventitious Root Growth and Phenolics Content

The initial medium pH range of 5.0 to 6.0 was best for promoting the growth of *Echinacea* roots and their accumulation of phenols and flavonoids, but growth was inhibited when the initial pH was maintained either below 5.0 or above 6.0 (Table 6). Although plant tissue cultures generally are effectively maintained when the medium pH is between 5.0 and 6.0, the concentration of hydrogen ions in the medium changes during the development period, decreasing during ammonia assimilation and increasing during nitrate

uptake (McDonald and Jackman, 1989). In ginseng root cultures, root growth and ginsenoside content are greatest in the pH range of 6.0 to 6.5 (Yu, 2000). Moreover, in suspension cultures of red bean (*Vigna angularis*), the onset of phenylalanine ammonia lyase (PLA) activity and the accompanying accumulation of isoflavone glucosides can be accelerated merely by raising the medium pH (Hattori and Ohta, 1985).

Adventitious root culturing is an efficient method for producing useful phytomolecules. In the present study of a shake flask system, we found that *in vitro* conditions strongly affected root growth and the accumulation of secondary metabolites from *Echinacea* tissues. The best performance overall was obtained in a half-strength MS medium supplemented with 2.0 mg L^{-1} IBA, 5% (w/v) sucrose, an ammonium:nitrate ratio of 5:25, media pH adjusted to 6.0 before autoclaving, and an inoculum size of 10 g L^{-1} FW. This particular culture protocol will prove beneficial to the large-scale production of biomass and secondary metabolites in *E. angustifolia*.

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