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In vitro regeneration of *Echinacea purpurea* from leaf explants

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Abstract

Efficient plant regeneration was achieved via organogenesis from callus cultures derived from leaf tissue of *Echinacea purpurea.* Proliferating shoot cultures were obtained by placing leaf explants on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) combinations. MS medium supplemented with BAP (4.44 μM) and NAA (0.054 μM) was the most effective, providing high shoot regeneration frequencies (100%) associated with a high number of shoots per explant (7.7 shoots/explant). Plantlets were rooted on MS medium alone or in combination with different concentrations of indole-3-butyric acid (IBA), and high rooting and survival was achieved using MS media without plant growth regulators (PGR). All plantlets survived acclimatization producing healthy plants in the greenhouse. This study demonstrated that adventitious shoot regeneration of *E. purpurea* from leaf explants can be a useful method for the multiplication of this important medicinal plant.

Abbreviations: BAP – 6-benzylaminopurine; IBA – indolyl-3-butyric acid; NAA – naphthaleneacetic acid; MS – Murashige and Skoog (1962); PGR – plant growth regulator

Introduction

Echinacea has gained considerable attention because of its increasing economic value and use as a medicinal plant. The genus *Echinacea* (purple coneflower) is represented by 11 taxa found in the United States and in south central Canada. *Echinacea purpurea* is the most widespread species (McGregor, 1968) and the most widely cultivated medicinal species of the genus (McKeown, 1999). *Echinacea* species have long been recognized as important medicinal plants used by Native Americans for the treatment of many diseases, including colds, toothaches, snake bites, rabies and wound infections (Bauer and Wagner, 1991).

Recently, *in vitro* shoot regeneration of *E. purpurea* from petiole explants (Choffe et al., 2000a) and root organogenesis from hypocotyl and cotyledon explants (Choffe et al., 2000b) have been reported. However, there are no reports of regeneration using leaf tissue as an explant source for this species.

In vitro techniques can facilitate molecular genetic manipulations. However, the successful application of *in vitro* methods is greatly dependent on a reliable regeneration system. Skoog and Miller (1957) first demonstrated that one of the key variables in the chemical regulation of *in vitro* organogenesis was the ratio of auxin – cytokinin present in the medium. Since then, protocols for regeneration have been elucidated for many plant genera.

The objective of this work was to develop an *in vitro* regeneration method for *E. purpurea* from leaf explants. The use of leaf tissue will facilitate the application of plant tissue culture and genetic engineering in *Echinacea purpurea.*

Materials and methods

Plant material

Young and not fully expanded leaves of *E. purpurea* were collected from 4 month-old plants grown at the Rutgers University, Cook College, Department of Plant Biology greenhouse. Freshly harvested leaves were surface sterilized by soaking for 17 min with occasional agitation in a 1.05% (w/v) sodium hypochlorite solution (20% v/v commercial bleach) containing 0.1% Tween 20. Leaves were subsequently washed three times with sterile water in a laminar flow hood.

Callus and shoot initiation

The leaf margins were removed along with the tip and basal portions. Leaf sections (0.7 × 0.7 mm) were placed on callus and shoot induction media with the adaxial surface toward the media. The callus and shoot induction media was composed of MS (Murashige and Skoog, 1962) basal medium (4.32 g l^{-1}) containing myo-inositol (100 mg l^{-1}), thiamine (0.4 mg l^{-1}, and sucrose (2% w/v); this media was supplemented with different concentrations of 6-benzylaminopurine (BAP) alone or in combination with naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.8 with KOH before adding agar (7 g l^{-1}). Medium without plant growth regulators was used as a control. Cultures were maintained in dark conditions at 28 °C. Each treatment consisted of 12 explants per dish (100 × 15 mm) and was replicated 10 times, representing a total of 120 observations per treatment. The rate of callus formation and the number of shoots/explant was determined after 4 weeks. After incubation in darkness, all treatments were moved to lighted conditions with a 16-h photoperiod of 25 µmol $m^{-2}s^{-1}$ at 25 °C for 1 week.

Table 1. Effect of different combinations of NAA and BAP on shoot regeneration from leaf explants of *E. purpurea* after 4 weeks of culture

Growth regulator (µM)		% explants producing shoots	No. shoots per explant ± SE*	% explants producing callus
NAA	BAP			
0	0	0	0	23
	0.44	17	0.2 ± 0.05^{j}	48
	2.22	97	4.2 ± 0.2^{cd}	100
	4.44	97	2.3 ± 0.1^{efg}	100
	8.88	59	1.9 ± 0.2^{de}	100
	17.76	0	0	65
	31.08	0	0	44
0.054	0	0	0	87
	0.44	65	1.3 ± 0.1^{ghij}	100
	2.22	75	3.3 ± 0.2^{de}	100
	4.44	100	7.7 ± 0.5^{a}	100
	8.88	0	0	98
	17.76	0	0	39
	31.08	0	0	16
0.54	0	0	0	95
	0.44	63	1.2 ± 0.1^{ghij}	100
	2.22	89	1.8 ± 0.1^{fgh}	100
	4.44	78	2.1 ± 0.1^{efg}	100
	8.88	73	2.7 ± 0.2^{ef}	100
	17.76	32	0.6 ± 0.1^{hij}	100
	31.08	0	0	12
2.69	0	0	0	100
	0.44	30	0.5 ± 0.1^{ij}	100
	2.22	70	1.3 ± 0.1^{ghij}	100
	4.44	91	5.7 ± 0.4^{b}	100
	8.88	82	$1.9 \pm ^{fgh}$	100
	17.76	96	4.7 ± 0.4^{bc}	100
	31.08	0	0	0

* Values are means ± standard error. Means followed by the same letter do not differ statistically at $p \leq 0.001$ different according to Tukey test.

Root initiation of regenerated shoots

For rooting induction isolated shoots (1.5 cm or longer) were transferred to basal medium alone or in combination with indolebutyric acid (IBA), pH 5.8. Shoots of this size were obtained 5 weeks after culture initiation. Each rooting treatment consisted of 10 explants per vessel (Phytacon, 108 mm) replicated 5 times, representing a total of 50 observations per treatment. The frequency of rooting and average number and length of roots per shoot was determined and recorded after 4 weeks of culture. Rooted plants were removed from culture, rinsed in water to remove media, and transferred to potting medium (Pro-Mix BX, Premier Company, PA, USA) in a mist chamber in the greenhouse. After two weeks, plants were transferred to the greenhouse supplemented with a 16-h photoperiod of 85 µmol m^{-2} s^{-1}. The percent survival of regenerated plantlets was recorded after a total of 4 weeks in potting medium and greenhouse conditions

The experimental design was fully randomized. Data were analyzed statistically by analysis of vari-

ance (ANOVA) followed by the Tukey test, with the level of significance set at 5%.

Results and discussion

Leaf explants incubated on basal medium with different combinations of auxin /cytokinin demonstrated callus formation after 4 weeks of incubation (Table 1). The rate of callus induction varied depending on the combination of applied growth regulators. Callus initiation was observed to develop from the margins of the leaf explant. In addition to the differences in the frequencies of callus formation, differences in shoot induction were also observed (Table 1).

BAP alone produced green callus for each concentration tested, however only the lower concentrations (0.44–8.88 μM) showed adventitious shoot formation after 4 weeks. BAP concentrations at (17.76 and 31.08 μM) inhibited shoot formation after 4 weeks. These calli required 7–8 weeks to show shoot induction (data not shown).

Of the combinations, MS medium supplemented with BAP (4.44 μM) and NAA (0.054 μM) was the most effective, providing shoot regeneration for 100% of explants associated with a high number of shoots per explant (7.7 mean shoots per explant). Explants grown in this medium for two weeks formed callus at the cut surface, and after 3 weeks the callus began to produce multiple shoot primordia, which developed into adventitious shoots after 5 weeks (Figure 1). Evidence of hyperhydricity was observed for shoots produced in the presence of more than 2.22 μM BAP, a problem previously observed at high concentrations of this PGR (Ziv, 1991). However, these morphological symptoms rapidly disappeared when the shoots were transferred to MS media without PGR.

Increasing NAA concentration resulted in increased callus production and low shoot initiation (Table 1). NAA alone (0.54 μM, 2.69 μM) induced direct and indirect root formation (data not shown). However, a low concentration of BAP (0.44 μM) added to the medium resulted in a stimulation of shoot induction. The balance of auxin to cytokinin is a determining morphogenic factor. A combination of a high amount of NAA (2.69 μM) and a small amount of BAP (0.44 μM) induced shoot proliferation and some adventitious roots. However, these roots formed independently of the shoots (data not shown).

In contrast, regeneration was slow or absent for explants grown on medium containing high levels of BAP alone or with NAA. The callus observed with higher BAP and NAA concentrations were brown and exhibited excessive necrosis, indicating toxic effects.

In previous reports, plant regeneration from petiole explants of *E. purpurea* was achieved by using only a small amount of BAP (Choffe et al., 2000a), whereas, in the present study, BAP in combination with NAA was most effective in inducing adventitious shoot regeneration from leaf explants. This difference between petiole and leaf explant response to BAP and NAA concentrations in media could be a reflection of probable differences of endogenous growth regulator levels in the explant sources or different tissue sensitivities to these plant growth regulators (Lisowska and Wysonkinska, 2000).

Reports of auxin and cytokinin combinations supporting organogenic differentiation in other species have been well documented for several species (Lisowska and Wysonkinska, 2000; Pereira et al., 2000, Pretto and Santarém, 2000). Results presented here are in agreement with these studies, where it was observed that low concentrations of NAA in combination with BAP were successful for the induction of callus and the subsequent proliferation and differentiation of shoots from such callus.

All shoots longer than 1.5 cm were transferred to rooting media treatments. The highest number of roots per shoot was induced at concentrations of 2.46 and 4.9 μM IBA (Table 2). However, the highest frequency of rooting alone with the highest root length occurred for the control media (0 μM IBA) and 0.49 μM IBA. IBA at concentrations greater than 2.46 μM resulted in callusing at the base of the shoots, and root elongation was clearly suppressed with increasing IBA concentration in the media.

The survival rate of regenerated plantlets transferred to soil was highest following root initiation in tissue culture with 0.49 μM IBA and for the control (0 μM IBA) (Table 2). The three higher IBA concentrations resulted in a significantly lower survival rate after transfer to the greenhouse compared to the above treatments. This low rate of survival could be due to the absence of roots or an anomalous adventitious root system formed by indirect origin via callus.

In contrast, no difference in survival rate (95%) was observed when elongated shoots were placed on MS media without IBA for 30 days and subsequently transferred to rooting media (data not shown). All shoots began rooting on MS media without PGR. Those subsequently transferred to rooting media containing IBA produced an increased number of roots

Figure 1. Shoot organogenesis on echinacea leaf explants on 0.054 μM NAA+ 4.44 μM BAP medium after 5 weeks culture.

Table 2. Effect of IBA concentrations on rooting of *in vitro* regenerated shoots of *E. purpurea* after 4 weeks of rooting treatment and percent survival of regenerated plantlets transferred into potting media and grown for 4 weeks under greenhouse conditions

IBA (μM)	Root number[a]	Root length (cm)[a]	% rooting[b]	% survival[b]
0	2.4 ± 0.2 b	4.9 ± 0.4 a	97[a]	95[a]
0.49	3.1 ± 0.3 ab	4.3 ±0.4 a	92[a]	97[a]
2.46	4.8 ± 0.6 a	2.6 ± 0.2 b	82	86
4.90	4.8 ± 0.7 a	1.4 ± 0.2 c	60	87
9.8	2.9 ± 0.5 ab	0.9 ± 0.1 c	70	75

[a]Means ± standard error. Means followed by the same letter within a column are not significantly different according to Tukey test at 5% level.
[b] Significant at 5% (test for comparing proportions) within a column.

after the rooting treatment. Despite the difference in the number of roots induced by the IBA treatment, there was no difference in the survival rate between the control and IBA treated plantlets.

These results show that IBA treatment is unnecessary for induction of a root system that is sufficient for acclimatization and survival in potting medium and greenhouse conditions. Survival after acclimatization was equally high for the control (no IBA) as for 0.49 μM IBA treatment, although the root number for this IBA treatment was higher than for the control.

These results demonstrate that leaves of *E. purpurea* have a great organogenic potential for shoot formation, however the response is highly sensitive and directly related to the combinations of exogenous growth regulators in the culture medium.

The *in vitro* regeneration of adventitious shoots is an essential component for most methods of genetic transformation (Schwarz and Beaty, 2000). The system described here provides an efficient method for adventitious shoot regeneration from leaf explants of *E. purpurea* that will be useful for both genetic transformation studies and for micropropagation of elite ornamental or chemotype selections of this medicinal plant species.

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