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*Full Length Research Paper*

# Improved shoot organogenesis and plant regeneration of *Echinacea angustifolia* DC

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**We established an improved method for shoot organogenesis and plant regeneration from stem cultures of *Echinacea angustifolia* DC. The regenerated shoots obtained from stem cultures on solid MS medium containing different concentrations of BAP and kinetin. The highest number of shoots per explant (3.2) and shoot length (1.3 cm) was obtained on MS medium containing 2 mg/l BAP. The addition of auxins in MS medium containing 2 mg/l BAP substantially improved the shoot regeneration of *E. angustifolia* and at the optimal concentration of 0.5 mg/l IBA was the most suitable auxin for the highest number of shoots per explant (4.6) and shoot growth (1.5 cm). Plant regeneration was found to be more efficient when Phytagel was used as the gelling agent. The number of shoots produced per leaf explant was 12% higher, and the growth of shoots was 11% greater, on 3 g/l Phytagel compared to 7 g/l Phytagar. The rooted plants were hardened and transferred to soil with an 80% survival rate. The production of *E. angustifolia* regenerated plants could be used as a possible transformation protocol.**

**Key words:** *Echinacea angustifolia* DC, plant regeneration, shoot organogenesis.

## INTRODUCTION

The *Echinacea* species, also known as purple coneflower, belongs to a member of the Asteraceae family and is native to North America. *Echinacea* have a long history of medicinal use for a variety of conditions in North American and currently *Echinacea* products are among the best-selling herbal medicines in several developed countries (Pepping, 1999; Percival, 2000). The *Echinacea* species have been shown to have a variety of beneficial effects and pharmacological actions. Modern interest in *Echinacea* is focused on its immunomodulatory effects, particularly in the prevention and treatment of upper respiratory tract infections (Barrett, 2003; Hostettmann, 2003; Charrois et al., 2006).

The three species of *Echinacea*, *Echinacea* angustifolia, *Echinacea* pallida, and *Echinacea purpurea* are most widely used herbal medicines. *E. angustifolia*, commonly known as Narrow-leaved purple coneflower and black-samson *Echinacea*, is an herbaceous medicinal plant used by all Indians of the Great Plains to treat a wide range of ailments, from venomous bites and stings, to infectious or inflammatory conditions such as cold and flu, toothaches, cough, sore eyes, and rheumatism (Kindscher, 1989; Barnes, 2005).

Plant regeneration through shoot organogenesis generally involves induction and development of a shoot from explant tissue, followed by transfer to a different medium for the induction of root formation and development. Research has demonstrated that successful organogenesis in many plant species can be achieved by the correct establishment of medium components, selection of a suitable explant, and control of the physical environment (Brown et al., 1986; Thorpe, 1990). The plant regeneration of *E. angustifolia* via organogenesis or somatic

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embryogenesis from different explants has been previously reported (Harbage, 2001; Lakshmanan et al., 2002; Lucchesini et al., 2009). The procedure and efficacy of *E. angustifolia* plant regeneration was complicated and even not easily available to the scientists yet. However, an improved method for the regeneration of *in vitro* plants from tissue culture is essential to establish a genetic transformation protocol for E. angustifolia. In this paper, we report the development of a simple method for high-efficiency plant regeneration from the stem explants of *E. angustifolia* and our study on the effect of cytokinins, auxins, and gelling agent, as well as of their interaction, on the induction of shoot organogenesis in *E. angustifolia*.

## MATERIALS AND METHODS

### Seed sterilization and germination

Seeds of E. angustifolia were purchased from Otto Richter and Sons Limited (*Goodwood*, Canada) and stored at 4°C. The seeds were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Ten seeds were placed on 25 ml of agar-solidified culture medium in Petri dishes (100 x 15 mm). The basal medium consisted of salts and vitamins of MS (Murashige and Skoog, 1962) medium and solidified with 0.7% (w/v) agar. The medium was adjusted to pH 5.8 before adding agar, and then sterilized by autoclaving at 121°C for 20 min. The seeds were germinated in a growth chamber at 25°C under standard cool white fluorescent tubes with a flux rate of 35 $\mu$mol $s^{-1}$ $m^{-2}$ and a 16 h photoperiod.

### Shoot organogenesis

Stems of *E. angustifolia* were taken from plants grown *in vitro* and were cut aseptically at the ends, into sections of approximately 0.7 cm in size. Explants were placed on the medium in the Petridish (100 x 25 mm). Petridish contained approximately 25 ml of culture medium. Seven explants were cultured in each Petridish. The basal medium consisted of salts and vitamins of Murashige and Skoog (MS) medium and solidified with 0.7% (w/v) Phytagar. The pH of medium was adjusted to 5.8 before adding Phytagar. The media were sterilised by autoclaving at 1.1 kg $cm^{-2}$ (121°C) for 20 min. For shoot regeneration from leaf explants, the MS medium was supplemented with 0, 1, 2, 4 mg/l kinetin and BAP (6-benzylamino purine).

For improvement of shoot regeneration, the medium was optimized by testing the effect of different concentrations of auxins (0.1 and 0.5 mg/l IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), and NAA (1-naphthalene-acetic acid)) and gelling agents (6, 7, 8, and 9 g/l Phytagar (Gibco-BRL Life Technologies, Rockville, MD, USA) and 2, 3, 4, and 5 g/l phytagel (Sigma Chemical Co, St. Louis, MO, USA)) on shoot formation and growth. Cultures were maintained at 25 ± 1°C in a growth chamber with a 16 h photoperiod under standard cool white fluorescent tubes (35 $\mu$mol $s^{-1}$ $m^{-2}$) for 6 weeks.

### Rooting of regenerated shoots

Regenerated shoots (around 1 cm long) were placed in MS medium. The medium was solidified with 3 g/l Phytagel and dispensed at 30ml per Magenta box and four shoots were cultured in each box. Regenerated shoots were incubated at 25 ± 1°C in a growth chamber with a 16 h photoperiod under standard cool white fluorescent tubes (35 $\mu$mol $s^{-1}$ $m^{-2}$) for 5 weeks. After five weeks, the rooted plants were washed with sterile water to remove Phytagel, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for one week to maintain high humidity. The plants were then transferred to soil and maintained in a growth chamber with a 16 h photoperiod, and a night/day temperature of 18/20°C for 2 weeks. These hardened plants then transferred to the greenhouse.

## RESULTS

An improved and effective method has been developed for the in vitro plant regeneration of E. angustifolia. For establishing a plant regeneration protocol, we investigated the effect of different concentrations of cytokinins (BAP and kinetin) on the efficiency of shoot organogenesis in of *E. angustifolia*. Shoot development from excised stem explants did not occur in the absence of exogenous BAP and kinetin. The treatment with BAP significantly promoted shoot regeneration from stem explants of E. angustifolia. Kinetin also induced shoot regeneration of *E. angustifolia* but its effect was much lower than that of BAP. The highest number of shoots per explant (3.2) and shoot length (1.3 cm) was obtained on MS medium containing 2.0 mg/l BAP (Table 1).

When *E. angustifolia* stems were cultured on MS solid media supplemented with 2 mg/l BAP, various stages of the *E. angustifolia* shoot organogenesis process were observed. During the initial stage (1 - 2 weeks of incubation), there was some expansion and proliferation of cells at the cut surface but callus growth was limited. After the cut end of the stem explant enlarged within 3 weeks shoot primordia and small elongated shoots had formed adjacent to the cut surface (Figure 1A). We observed that cells of the epidermis proliferated to produce shoots directly, without an intervening callus phase. The regenerated shoots were developed from shoot primordia within 4 - 5 weeks. After six weeks of culture, an average of 3 fully developed shoots of at least 1.3 cm in length were produced supposedly from stem explants (Figure 1B).

To study the effects of different auxins on shoot regeneration and growth from excised stem cultures of *E. angustifolia*, stem explants were grown for 6 weeks in basal medium (MS salts and vitamins, 30 g/l sucrose, and 2 mg/l BAP, solidified with 7 g/l Phytagar) supplemented with various concentrations of different auxins. Our results revealed that all tested auxin treatments in basal medium marginally increased the shoot regeneration and growth rates of *E. angustifolia* stem culture (Table 2). The concentration of 0.5 mg/l IBA produced the highest number of shoots (4.6) and the shoot length (1.5 cm).

The effect of different concentration of gelling agents (Phytagar and Phytagel) on the shoot organogenesis of *E. angustifolia* was investigated. Stem explants were grown for 6 weeks in basal medium (MS salts and

**Table 1.** Effect of different concentrations of BAP and kinetin on shoot regeneration and growth from stem cultures of Echinacea angustifolia after six weeks in culture.

| Cytokinin* (mg/l) | Regeneration rate** (%) | No. of shoots /explants** | Shoot length[a] (cm) |
|---|---|---|---|
| Control | | | |
| 0.0 | -- | -- | -- |
| BAP | | | |
| 1.0 | 57 | 1.9 ± 0.1 | 0.9 ± 0.1 |
| 2.0 | 72 | 3.2 ± 0.2 | 1.3 ± 0.1 |
| 4.0 | 70 | 3.0 ± 0.2 | 1.2 ± 0.1 |
| Kinetin | | | |
| 1.0 | 42 | 1.2 ± 0.1 | 0.7 ± 0.1 |
| 2.0 | 63 | 2.8 ± 0.2 | 0.9 ± 0.1 |
| +4.0 | 71 | 3.0 ± 0.2 | 1.1 ± 0.1 |

-- No response
* Basal medium consisted of MS salts and vitamins, and 30 g $L^{-1}$ sucrose, solidified with 7 g/l Phytagar.
** From 100 leaf explants tested.
[a] Values represent the mean ± standard deviation of 50 shoots

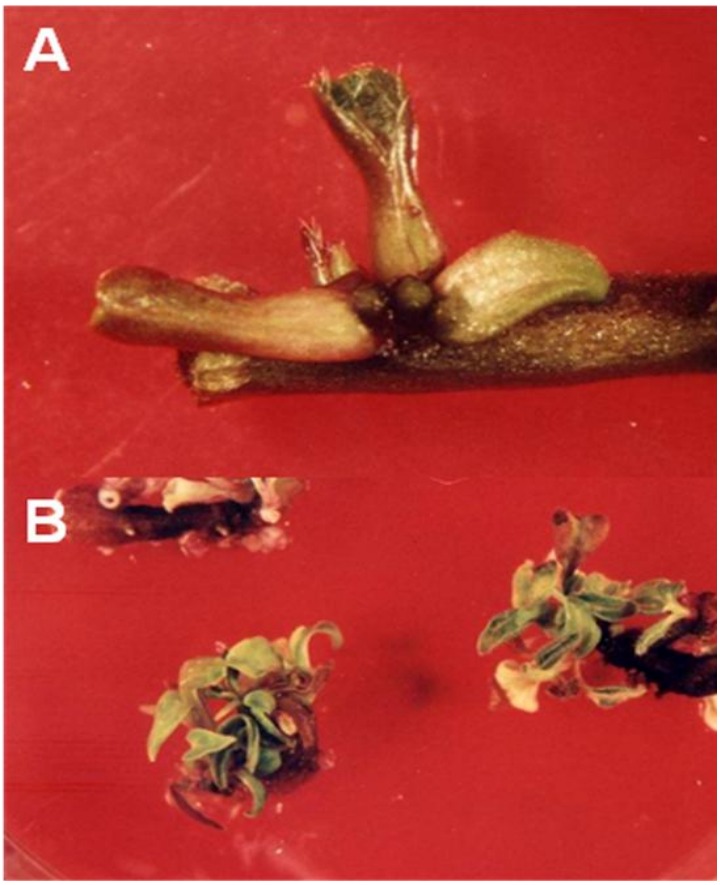


**Figure 1.** Shoot organogenesis in Echinacea angustifolia. A) Shoot primordia emerging from a stem explant of F. esculentum three weeks after the cultivation on MS solid media supplemented with 2mg/l BAP (x 13). B) After six weeks of culture, fully developed shoots were produced from stem explants (x 1.5).

vitamins, 30 g/l sucrose, and 2 mg/l BAP with 0.5 mg/l IBA) supplemented with various concentrations of Phytagar and Phytagel (Table 3). Although these initial experiments were performed on medium solidified with Phytagar, shoot organogenesis was found to be more efficient when Phytagel was used as the gelling agent. The number of shoots produced per leaf explant was 12% higher, and the growth of shoots was 11% greater, on 3 g/l Phytagel compared to 7 g/l Phytagar. Our optimized shoot regeneration medium consisted of MS salts and vitamins, 30 g $L^{-1}$ sucrose, 2 mg/l BAP, 0.5 mg/l IBA and 3 g/l Phytagel.

Regenerated shoots (around 1 cm long) were transferred to MS medium without any exogenous plant hormone. The medium was solidified with 3 g/l Phytagel and dispensed at 30ml per Magenta box. After five weeks, the regenerated shoots induced roots. The rooted plants were washed with sterile water to remove Phytagel, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for one week to maintain high humidity. The regenerated plants were hardened and transferred to soil with an 80% survival rate where they grew normally in greenhouse.

## DISCUSSION

In 1957, Skoog and Miller performed classic experiments demonstrating that shoot and root initiation in callus cultures of tobacco could be regulated by manipulation of the ratio of auxin and cytokinin present in the growth medium. Generally in organogenesis protocols, high cytokinin to auxin ratios induce shoots, high auxin to cytokinin ratios produce roots, and more equal concen-

**Table 2.** Effect of different concentrations of auxins on shoot regeneration and growth from stem cultures of Echinacea angustifolia after six weeks in culture.

| | Auxins (mg/l) | Regeneration rate** (%) | No. of shoots/explants** | Shoot length[a] (cm) |
|---|---|---|---|---|
| Control | 0.0 | 72 | 3.2 ± 0.2 | 1.3 ± 0.1 |
| IAA | 0.1 | 74 | 3.3 ± 0.2 | 1.3 ± 0.1 |
| | 0.5 | 75 | 3.5 ± 0.2 | 1.4 ± 0.1 |
| IBA | 0.1 | 75 | 3.7 ± 0.3 | 1.5 ± 0.2 |
| | 0.5 | 84 | 4.6 ± 0.3 | 1.5 ± 0.1 |
| NAA | 0.1 | 81 | 4.2 ± 0.4 | 1.4 ± 0.1 |
| | 0.5 | 73 | 3.3 ± 0.4 | 1.3 ± 0.1 |

-- No response
*Basal medium consisted of MS salts and vitamins, 30 g L-1 sucrose, and 2 mg/l BAP, solidified with 7 g/l Phytagar.
**From 100 leaf explants tested.
[a]Values represent the mean ± standard deviation of 50 shoots.

**Table 3**. Effect of different concentrations of gelling agents on shoot regeneration and growth from stem cultures of Echinacea angustifolia after six weeks in culture.

| Gelling agent* (mg/l) | Regeneration rate** (%) | No. of shoots/explants** | Shoot length[a] (cm) |
|---|---|---|---|
| Phytagar | | | |
| 6.0 | 85 | 4.3 ± 0.4 | 1.5 ± 0.2 |
| 7.0 | 84 | 4.6 ± 0.3 | 1.5 ± 0.1 |
| 8.0 | 79 | 4.2 ± 0.3 | 1.4 ± 0.1 |
| 9.0 | 61 | 3.2 ± 0.3 | 1.0 ± 0.1 |
| Phytagel | | | |
| 2.0 | 85 | 4.1 ± 0.3 | 1.6 ± 0.2 |
| 3.0 | 92 | 5.5 ± 0.5 | 1.7 ± 0.1 |
| 4.0 | 87 | 5.1 ± 0.5 | 1.7 ± 0.2 |
| 5.0 | 81 | 3.8 ± 0.4 | 1.5 ± 0.1 |

- No response
* Basal medium consisted of MS salts and vitamins, 30 g $L^{-1}$ sucrose, and 2 mg/l BAP with 0.5 mg/l IBA.
** From 100 leaf explants tested.
a Values represent the mean ± standard deviation of 50 shoots.

trations of theses phytohormones are found to cause callus proliferation. Currently, organogenesis is the most widely used method of *in vitro* plant regeneration in transformation systems.

The two gelling agents (*Phytagar* and *Phytagel*) have most commonly been used to solidify the plant tissue culture medium. Agar, commonly known as Phytagar, extracted from red algae such as Gracilaria, Gelidium or Chondrus, and gellan gum, also known as Gelrite or Phytagel, an extracellular polysaccharide produced by the bacterium (Veramendi et al., 1997; Abidine et al., 2008). In this study, shoot organogenesis of E. angustifolia was found to be more efficient when Gelrite *(Phytagel)* was used as the gelling agent. The superiority of Gelrite over agar for the purposes of shoot regeneration has also been reported for apple (Saito and Suzuki, 1999) and *Bacopa monnieri* (L.) Pennell (Shrivastava and Rajani, 1999).

Plant regeneration protocols are an essential part of plant genetic transformation leading to plant improvement. Currently, shoot organogenesis is the most widely used method of in vitro plant regeneration in transformation systems. From our a little effort to establish an efficient protocol for shoot organogenesis and plant regeneration from the stem cultures of *E. angustifolia*, the production of *E. angustifolia* regenerated

plants could be used in a possible transformation protocol which might create new opportunities to study the molecular and metabolic regulation of producing useful secondary metabolites in *E. angustifolia.*

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