Influence of carbon sources on shoot organogenesis in *Echinacea angustifolia* DC

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Abstract: To achieve better shoot organogenesis and plant regeneration in *Echinacea angustifolia*, we established an efficient tissue culture cycle by adjusting carbon sources and their concentrations. Leaf explants were cultured on initial shoot-regeneration media with 5 different carbon sources (fructose, glucose, lactose, maltose, and sucrose). Then, the best carbon source for shoot regeneration was investigated at 5 different concentrations (10, 20, 30, 40, and 50 mg/L). The best type of carbohydrate for shoot regeneration and growth was sucrose. Shoot regeneration was 1.6-fold higher and shoots were 1.3-fold longer in the explants treated with sucrose than in those treated with the lowest carbon-producing source, lactose. Lactose treatment resulted in the lowest efficiency for shoot regeneration and growth in *E. angustifolia.* The highest shoot regeneration (82%), number of shoots (4.2), and shoot length (15.2 mm) were obtained using sucrose at 40 g/L. This study suggests that carbon sources, particularly sucrose, could be used in the micropropagation and plant transformation protocols for regeneration in *E. angustifolia*.
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1. Introduction

The genus *Echinacea* (family, Asteraceae) is composed of the most popular medicinal plants in North America and Europe (Pepping, 1999; Percival, 2000). In particular, *Echinacea angustifolia* DC has been most commonly used by the Native Americans 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 et al., 2005).

Organogenesis is a developmental process in which shoots or roots are induced to differentiate from a single cell or a group of cells. Regeneration of plants through organogenesis typically involves the induction and development of shoots from explant tissue, followed by transfer to a different medium to induce root formation and plant development (Boudaoud, 2010; Fleming, 2006). Successful organogenesis in many plant species can be achieved by using the appropriate medium components, selecting a suitable explant, and controlling the physical environment (Brown and Thorpe, 1986; Thorpe, 1990).

In living plants, carbohydrates are necessary as a source of energy and a carbon substrate for biosynthesis. In vitro plant cell, tissue, and organ cultures are not fully autotrophic, thereby establishing a need for carbohydrates in the culture media to maintain the osmotic potential, as well as to serve as energy and carbon sources for high-energy developmental processes, such as shoot proliferation, root induction and emission, embryogenesis, and organogenesis (Paiva and Otoni, 2003; Yaseen et al., 2013).

A continuous supply of carbohydrates to plants cultured in vitro is essential because the photosynthetic activity of in vitro-grown tissues is usually low. These compounds are also necessary as osmotic agents in the culture media. Hence, sugars have a potential effect on the physiology, growth, and differentiation of cells (Gibson, 2000). Therefore, the optimal carbon source for callus induction and plant regeneration needs to be considered (Mendoza and Kaeppler, 2002). Here, we report the establishment of an improved method for plant regeneration from stem explants of *E. angustifolia.* To the best of our knowledge, this is the first report on the influence of carbon sources on shoot organogenesis in *E. angustifolia*.

2. Material and Methods

2.1. Plant materials and culture media

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 and then rinsed 3 times in sterilized water. Ten seeds were placed in each petri dish (100 × 15 mm) containing 25 mL of agar-solidified culture medium. The basal medium consisted of salts and Murashige and Skoog (MS) vitamins (Murashige and Skoog, 1962) and 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 μmol·s^{-1}·m^{-2} and a 16-h photoperiod.

2.2. In vitro shoot organogenesis

Stems of *E. angustifolia* were obtained from plants grown in vitro, and they were cut aseptically at the ends into sections of approximately 0.7 cm. Explants were placed in petri dishes (100 × 25 mm) containing approximately 25 mL of basal medium supplemented with 30 g/L of sucrose, 7 g/L of Phytagar, and 2 mg/L of 6-benzylaminopurine (BAP). Seven explants were cultured in each petri dish. The pH of the medium was adjusted according to that required for the germination medium, and the medium was sterilized using the same procedure mentioned above. For improvement in shoot regeneration, the medium was optimized by testing the effect of 30 g/L of each carbon source (fructose, glucose, lactose, maltose, and sucrose) and different concentrations (10, 20, 30, 40, and 50 g/L) of sucrose. Cultures were maintained at 25 ± 1°C in a growth chamber with a 16-h photoperiod under standard, cool-white fluorescent tubes (35 μmol·s^{-1}·m^{-2}) for 6 weeks.

2.3. Rooting of regenerated shoots

Regenerated shoots (~1.5 cm in length) were transferred to 1/2 MS medium in a Magenta box. The medium was solidified using 8 g/L of plant agar, and 50 mL of the medium was placed in each culture vessel. Four shoots were cultured in each vessel. Regenerated shoots were incubated at 25 ± 1°C in a growth chamber with a 16-h photoperiod under standard, cool-white fluorescent tubes (35 μmol·s^{-1}·m^{-2}) for 5 weeks. After 5 weeks, the rooted plants were washed with water to remove the agar, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for 1 week to maintain high humidity. The plants were then transferred to the soil and maintained in a growth chamber with a 16-h photoperiod and a day/night temperature of 18/20°C for 2 weeks. These hardened plants were then transferred to the greenhouse.

2.4. Statistical analysis

Data for the 50 tested leaf explants are expressed as mean ± standard deviation.

3. Results and Discussion

A protocol was established for in vitro shoot organogenesis in *E. angustifolia* (Kim et al., 2010), but the efficiency of shoot regeneration by using this protocol was not satisfactory. For better shoot regeneration, we used different carbon sources (fructose, glucose, lactose, maltose, and sucrose) to investigate the efficiency of shoot organogenesis in *E. angustifolia.* The type of carbohydrate has been found to play an important role in the regeneration of *E. angustifolia*. Among the carbon sources used in this study, sucrose induced the highest shoot regeneration and growth. The highest shoot growth was observed when the generation medium (MS medium with BAP at 2 mg/L) was supplemented with 30 g/L of sucrose, resulting in 72% regeneration frequency with the largest number of shoots (3.7) and the longest shoots (14.1 mm) in each explant (Table 1). Treatment with lactose showed the lowest efficiency for shoot regeneration and growth in *E. angustifolia.* The use of sucrose resulted in 1.6-fold higher shoots per explant and 1.3-fold longer shoots than the use of the lowest carbon-producing source, lactose.

The generation medium (MS medium with BAP at 2 mg/L) was supplemented with different concentrations of sucrose (10, 20, 30, 40, and 50 g/L) to determine an optimum concentration for better regeneration in *E. angustifolia*. Increased shoot regeneration and growth was observed when the concentration of sucrose was increased; however, shoot regeneration and growth declined when the concentration of sucrose was increased beyond 40 g/L (Table 2). The highest shoot regeneration (82%), number of shoots (4.2), and shoot length (15.2 mm) were obtained using sucrose at 40 g/L.

Our results demonstrated that the addition of carbon sources (carbohydrates) was essential, since callus induction and regeneration in the embryo cultures of *E. angustifolia* were affected by the type of carbohydrate and its concentration in the culture medium (Tables 1 and 2). We found that the best type of carbohydrate for regeneration and shoot growth was sucrose at a concentration of 40 g/L. Similar results were obtained by Baskaran and Jayabalan (2005), who reported that sucrose proved to be better than fructose and glucose for promoting shoot regeneration in *Eclipta alba*. Amutha et al. (2003) reported that the use of glucose, fructose, and maltose resulted in very poor shoot differentiation. They observed that sucrose evoked maximum regeneration frequency and increased the number of regenerating shoots. Glucose was the most effective carbon source for both axillary branching and adventitious shoot regeneration in in vitro beech cultures (Cuenca and Vieitez, 2000). In our study, lactose did not result in adequate shoot regeneration and growth. However, Amiri and Kazemitabar (2011) reported that for the embryo explants of *Datura stramonium* L., the best sugar source for callus induction was 2% lactose, and the medium containing 3% glucose showed the

highest regeneration efficiency. Jain et al. (1997) observed that media containing lactose, mannitol, or sorbitol supported limited growth or failed to induce any measurable increase in the fresh weight of callus in *indica* and *japonica* rice varieties. Nowak et al. (2004) suggested that sucrose was a better carbon source than glucose for organogenesis in *Prunus domestica*; however, at lower concentrations, the efficiency of the sugars was comparable.

An efficient protocol for plant regeneration is essential for establishing genetic engineering techniques for plant improvement. In this study, we developed an improved method for the regeneration of *E. angustifolia* by adjusting the carbon sources and their concentrations in the culture media. These results will facilitate research on the genetic enhancement of *E. angustifolia*.

Table 1. Effect of carbon sources on shoot regeneration and growth in *Echinacea angustifolia* explants after 6 weeks of culture on a regeneration medium (Murashige and Skoog medium with 2.0 mg/L BAP).

Carbon sources (30g/L)	Regeneration %*	No. of shoots per explant*	Shoot length* (mm)
Fructose	59	2.6 ± 0.3	11.5 ± 0.8
Glucose	70	3.5 ± 0.4	13.8 ± 0.7
Lactose	54	2.3 ± 0.3	11.0 ± 0.8
Maltose	62	2.8 ± 0.3	12.4 ± 0.6
Sucrose	72	3.7 ± 0.3	14.1 ± 0.8

Regeneration frequency (%) = (No. of explants with shoot differentiation/all explants) × 100
* Values represent the mean ± standard deviation of 50 shoots

Table 2. Effect of sucrose concentration on shoot regeneration and growth in *Echinacea angustifolia* explants after 6 weeks of culture on a regeneration medium (Murashige and Skoog medium with 2.0 mg/L BAP).

Sucrose (g/L)	Regeneration %*	No. of shoots per explant*	Shoot length* (mm)
10	54	2.7 ± 0.3	12.8 ± 0.4
20	67	3.2 ± 0.3	13.4 ± 0.7
30	71	3.5 ± 0.4	13.8 ± 0.6
40	82	4.2 ± 0.4	15.2 ± 0.7
50	80	4.1 ± 0.5	14.9 ± 0.6

Regeneration frequency (%) = (No. of explants with shoot differentiation/all explants) × 100
* Values represent the mean ± standard deviation of 50 shoots

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