Improved shoot organogenesis of *Echinacea angustifolia* DC treated with ethylene inhibitors

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Abstract: With the goal of achieving better shoot organogenesis and plant regeneration in *Echinacea angustifolia*, we conducted an experiment to investigate the effect of ethylene inhibitors, including silver nitrate ($AgNO_3$), aminoethoxyvinylglycine (AVG), and cobalt chloride ($CoCl_2$). Leaf explants were cultured in initial shoot-regeneration media supplemented with different concentrations of $AgNO_3$, AVG, and $CoCl_2$. The addition of ethylene inhibitors improved regeneration frequency, giving a greater number of shoots per explant, and longer shoots. Shoot growth increased with increasing concentrations of ethylene inhibitors, except for $CoCl_2$. The best shoot growth was found when $AgNO_3$ (10 mg/L) was incorporated in the medium. AVG (10 mg/L) produced the second greatest number of shoots. Treatment with $CoCl_2$ did not result in good shoot organogenesis in *E. angustifolia*. This study suggests that ethylene inhibitors, particularly $AgNO_3$, could be used in a micropropagation and plant transformation protocol for regeneration of *E. angustifolia*.
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1. Introduction

Echinacea species, members of the Asteraceae family, are among the most popular medicinal plants in North America and Europe (Pepping, 1999; Percival, 2000). *E. angustifolia* DC has been used by 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). Plant regeneration through successful shoot organogenesis generally requires correct establishment of media components, selection of a suitable explant, and control of the physical environment (Brown and Thorpe, 1986; Thorpe, 1990).

An important factor in the physical environment of plant tissue culture is ethylene. Ethylene (C_2H_4), a gaseous plant hormone, plays an important role in plant growth and development (Yang and Hoffman 1984). In plant tissue culture, ethylene can affect callus growth, shoot organogenesis, and somatic embryogenesis (Biddington, 1992; Huang et al. 2001; Jha et al., 2007; Chatfield and Raizada, 2008). While ethylene has certain positive effects on callus culture and root growth, this hormone largely functions to inhibit growth of the shoot.

Hence, for enhancing shoot regeneration, ethylene inhibitors are added to plant media to prevent the negative effects of the hormone (Chae et al., 2012; Park et al., 2012). The effects of ethylene inhibitors, including aminoethoxyvinylglycine (AVG), cobalt chloride ($CoCl_2$), benzyl isothiocyanate (BITC), aminocarboxy propionic acid, 1-methylcyclopropene (1-MCP), polyamines, silver nitrate ($AgNO_3$), 3,4,5-trichlorophenol, and salicylic acid (2-hydroxy benzoic acid), on promoting shoot organogenesis in several plant species has been reviewed by Kumar et al. (1998). Regeneration of *E. angustifolia* via organogenesis or somatic embryogenesis from different explants has been previously reported (Harbage, 2001; Lakshmanan et al., 2002; Lucchesini et al., 2009).

In this paper, we report the establishment of an improved method for plant regeneration from stem explants of *E. angustifolia.* We examined for the first time the influence of ethylene inhibitors ($AgNO_3$, AVG, and $CoCl_2$) on shoot organogenesis of *E. angustifolia*.

2. Material and Methods

Plant material and culture medium

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 × 15 mm). The basal medium consisted of salts and Murashige and Skoog (MS) vitamins (Murashige and Skoog, 1962), 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 μmol s^{-1} m^{-2} and a 16-h photoperiod.

In vitro 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. Explants were placed on the medium in Petri dishes (100 × 25 mm). Each Petri dish contained approximately 25 mL of basal medium enhanced with 30 g/L sucrose, 7 g/L Phytagar, and 2 mg/L 6-benzylaminopurine (BAP), and seven explants were cultured in each dish. The pH of the medium was adjusted as for the germination medium, and the medium was sterilized using the same procedure. For improvement of shoot regeneration, the medium was optimized by testing the effect of different concentrations (0, 1, 5, 10, and 20 mg/L) of each ethylene inhibitor . 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.

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 with 8 g/L plant agar and 50 mL was placed in each culture vessel. Four shoots were cultured in each culture 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 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 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.

3. Results and Discussion

Previously, a protocol was established for in vitro shoot organogenesis of *E. angustifolia* (Kim et al., 2010). However, shoot regeneration efficiency using this protocol was not satisfactory. For better shoot regeneration, we investigated the effect of ethylene inhibitors on the efficiency of shoot organogenesis in *E. angustifolia.* Application of $AgNO_3$, AVG, and $CoCl_2$ improved regeneration frequency, giving higher numbers of shoots per explant and greater shoot length (Table 1). Shoot growth increased with increasing concentrations of both $AgNO_3$ and AVG up to 10 mg/L, and declined with concentrations greater than 10 mg/L. The opposite phenomenon was observed in the application of $CoCl_2$, where growth was inhibited with increased concentration. The greatest shoot growth was found when the generation medium (MS media with BAP at 2 mg/L) was supplemented with 10 mg/L $AgNO_3$, achieving 89% regeneration frequency with the largest number of shoots (4.5) and the longest shoots (1.9 cm) in each explant (Table 1). Treatment with $AgNO_3$ produced 45% more shoots per explant, and 58% longer shoots compared to the control. Regeneration frequency was also 17% higher using $AgNO_3$ at 10 mg/L compared to the control. AVG (10 mg/L) produced the second greatest number of shoots, resulting in 39% more and 50% longer shoots compared to the control. Treatment with $CoCl_2$ did not result in good shoot organogenesis in *E. angustifolia.*

Ethylene is a gaseous plant hormone that influences plant growth and development (Yang and Hoffman, 1984). The silver ion (Ag^+) inhibits ethylene action by substituting for Cu^+ at the active site of the ethylene receptor (Beyer, 1979), and cobaltous ions (Co^{2+}) are known to inhibit ethylene synthesis (Lau and Yang, 1976).

Addition of $AgNO_3$ and AVG to the culture media has been shown to greatly improve in vitro plant regeneration of both dicot and monocot species. The use of silver thiosulphate (STS) and AVG increased the percentage of adventitious shoot regeneration from apricot leaf explants (Burgos and Alburquerque, 2003). Addition of $AgNO_3$ and AVG to culture media markedly enhanced the regeneration frequency and number of shoots obtained per explant in pomegranate (*Punica granatum* L.) (Naik and Chand, 2003). Addition of $AgNO_3$ to a callus induction medium was highly effective for shoot regeneration in three genotypes of rapeseed (*Brassica napus* L.) (Akasaka-Kennedy et al., 2005).

An efficient protocol for plant regeneration is essential to the practice of genetic engineering for plant improvement. In this study, we developed an improved method for regeneration of *E. angustifolia* using the ethylene inhibitors $AgNO_3$, AVG, and $CoCl_2$. These results will facilitate research on the genetic enhancement of *E. angustifolia*.

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Table 1. Effect of different concentrations of ethylene inhibitors on shoot regeneration in *Echinacea angustifolia* after 6 weeks of culture on regeneration medium (Murashige and Skoog medium with 2.0 mg/L BAP).

Ethylene inhibitors*	(mg L^{-1})	Regeneration frequency** (%)	Number of shoots per explant**	Shoot length[a] (cm)
Control	0	72	3.1 ± 0.2	1.2 ± 0.1
$AgNO_3$	1	75	3.3 ± 0.2	1.5 ± 0.1
	5	81	3.9 ± 0.2	1.7 ± 0.2
	10	89	4.5 ± 0.3	1.9 ± 0.2
	20	63	2.6 ± 0.1	1.0 ± 0.0
AVG	1	74	3.5 ± 0.3	1.3 ± 0.1
	5	79	3.8 ± 0.3	1.6 ± 0.2
	10	87	4.3 ± 0.4	1.8 ± 0.2
	20	72	2.9 ± 0.1	1.1 ± 0.1
$CoCl_2$	1	74	3.4 ± 0.2	1.3 ± 0.2
	5	72	3.0 ± 0.2	1.3 ± 0.1
	10	65	2.7 ± 0.2	1.0 ± 0.0
	20	58	1.9 ± 0.1	0.7 ± 0.0

* Basal medium consisted of Murashige and Skoog salts and vitamins, 30 g/L 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

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