Plant Cell, Tissue and Organ Culture **77:** 251–255, 2004.

Plant regeneration from mesophyll protoplasts of *Echinacea purpurea*

Z.G. Pan[1], C.Z. Liu[1,2], S.M.A. Zobayed[1] & P.K. Saxena[1,*]
[1]*Department of Plant Agriculture, University of Guelph, Guelph, Ont., Canada N1G 2W1;* [2]*Present address: Institute of Process Engineering, Chinese Academy of Science, Beijing 100080, P.R. China (*request for offprints: Fax: +1-519-7670755; E-mail: psaxena@uoguelph.ca)*

Received 3 April 2003; accepted in revised form 23 September 2003

Key words: Echinacea purpurea L., medicinal plant, plant regeneration, protoplasts

Abstract

An efficient plant regeneration system was developed from isolated protoplasts of *Echinacea purpurea* L. using an alginate block/liquid culture system. Viable protoplasts could be routinely isolated from young leaves of *Echinacea* seedlings in an isolation mixture containing 1.0% cellulase Onozuka R-10, 0.5% pectinase and 0.3 mol l^{-1} mannitol. Purified protoplasts were embedded in 0.6% Na-alginate block at a density of 1×10^5/ml and cultured in a modified MS medium containing 0.3 mol l^{-1} sucrose, 2.5 μmol l^{-1} BA and 5.0 μmol l^{-1} 2,4-D. Cell colonies were observed after 4 weeks of culture, and the protoplast-derived colonies formed calluses when transferred onto 0.25% gellan gum-solidified MS medium supplemented with 1.0 μmol l^{-1} BA and 2.0 μmol l^{-1} IBA. Shoot organogenesis from protoplast-derived callus was induced on MS medium supplemented with 5.0 μmol l^{-1} BA and 2.0 μmol l^{-1} IBA. Complete plantlets were obtained from the regenerated shoots on MS basal medium. The protoplast to plant regeneration protocol developed in this study provides the prerequisite for creating novel genotypes of this valuable medicinal species through genetic manipulation.

Abbreviations: BA – 6-benzylaminopurine; B5 – Gamborg et al. (1968); 2,4-D – 2,4-dichlorophenoxyacetic acid; FDA – fluorescein diacetate; MES – 2-(*N*-morpholino)-ethanesulfonic acid; MS – Murashige and Skoog (1962); NAA – 1-naphthaleneacetic acid

Introduction

Echinacea purpurea L., belongs to the family Asteraceae, and has been used extensively in medicinal preparations for the treatments of many diseases, including colds, toothaches, snake bites, rabies and wound infections (Bauer, 1999). *Echinacea* species are currently one of the best-selling herbs in North America and have gained great attention because of their increasing economic value. However, commercial production of *Echinacea* has been limited by a range of issues including contamination of plant materials by micro-organisms, pollution from the environment, variability of active components and lack of pure, standardized plant material for biochemical analysis. To address these issues, *in vitro* technology for this species has been developed recently for potential use in the production of high quality, consistent plant material as well as for genetic improvement (Choffe et al., 2000a, b; Koroch et al., 2002; Zobayed and Saxena, 2003).

Genetic improvement of *Echinacea* will be an important strategy to develop a superior germplasm and value-added products. In addition to conventional genetic modification, cell manipulation techniques such as somaclonal variation and somatic hybridization using protoplasts also provide useful means of genetic improvement (Nagata and Bajaj, 2001). Somatic hybrids of *Echinacea* species (*E. purpurea*, *E. angustifolia* and *E. pallida*) or those between clones selected for unique phytochemical profiles may generate novel germplasm and provide an excellent system to investigate the biosynthesis of species-specific medicinal compounds. A basic requirement for achieving this goal is the successful regeneration of plants from isolated protoplasts. Therefore, the overall

objective of this research was to develop a protocol for plant regeneration from protoplasts of *E. purpurea.*

Materials and methods

Seeds of *E. purpurea* L. (Richters, The Herb Specialists, Goodwood, Ont., Canada) were surface sterilized by immersing in 70% ethanol solution for 30 s and soaking in a 5.4% sodium hypochloride solution containing one drop of Tween 20 per 500 ml for 20 min, followed by three rinses in sterile distilled water. Surface-sterilized seeds were germinated and maintained on a basal medium (referred to as MSO) containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 30 g l^{-1} sucrose, and 2.5 g l^{-1} gellan gum (Gelrite, Schweitzerhall, South Plainfield, NJ, USA). PPM (0.3% v/v; Preservative for Plant Tissue Culture Media; Plant Cell Technology Inc., USA) was added to the MSO for eliminating fungal contamination prior to autoclaving at 121 °C. All cultures were incubated in a growth cabinet in darkness at 26 °C. After 2 weeks, the germinated seedlings were maintained in a growth room with 16-h photoperiod under cool-white light (50 μmol m^{-2} s^{-1}).

One hundred milligrams of young leaves of 4-week-old seedlings grown in light were sliced into 1–2 mm wide strips and incubated in 5 ml of an isolation mixture containing 1.0% cellulase Onozuka R-10 (Yakult Honsha Co., Japan), 0.5% pectinase (Sigma, USA), 5 mmol l^{-1} MES and 0.3 mol l^{-1} mannitol in CPW medium (Patat-Ochatt et al., 1988) in a Petri dish (60 mm × 15 mm). The pH of the isolation solution was adjusted to 5.6 prior to filter sterilization using 0.2 μm pore diameter filter disc (Millipore, Billerica, MA, USA). The incubation was carried out in darkness for 18 h at room temperature on a shaker (Stovall Life Science Inc., Greensboro, NC, USA) at 30 rpm. After digestion, the enzyme mixtures were screened through a 50 μm nylon mesh, and resulting protoplasts were collected by centrifugation at 120 × *g* for 8 min. The pellet was gently suspended in 6 ml of CPW solution with 0.5 mol l^{-1} sucrose, and 2 ml of CPW solution with 0.3 mol l^{-1} mannitol was gently loaded on top of the sucrose solution. Viable protoplasts formed a band at the interface between sucrose and mannitol solutions following centrifugation at 100 × *g* for 6 min. The protoplasts were collected using a pipette, rinsed once with 8 ml of CPW containing 0.3 mol l^{-1} mannitol, and centrifugated at 120 × *g* for 8 min. Viability of protoplasts was assessed by FDA staining method (Widholm, 1972).

For liquid culture, 2.5 ml of the purified protoplasts at a density of 2×10^5 ml^{-1} in a liquid culture medium were pipetted into Petri dish (35 mm × 10 mm). For alginate block/liquid culture, the purified protoplasts were suspended at a density of 2×10^5 ml^{-1} in 0.3 mol l^{-1} mannitol solution containing 1.2% (w/v) Na-alginate (Sigma, USA). The alginate-protoplast suspension (0.8 ml) was poured onto a medium containing 20 mmol l^{-1} $CaCl_2$, 0.3 mol l^{-1} mannitol and 1.5% (w/v) agar (Fisher, USA), and the resulting solidified protoplast-alginate blocks were transferred into 60 mm × 15 mm Petri dishes containing 4 ml of a liquid culture medium. The culture medium contained MSO medium ingredients as described above with 0.3 mol l^{-1} sucrose, 2.5 μmol l^{-1} BA and 5.0 μmol l^{-1} 2,4-D. Various liquid culture media tested for protoplast culture included:

- MS;
- B5;
- MS salts and B5 vitamins; and
- Modified MS (500 mg l^{-1} KNO_3 and 500 mg l^{-1} NH_4NO_4).

All media were amended with varying levels of sucrose (0.2, 0.3, 0.4, 0.5, 0.6 mol l^{-1}). The pH was adjusted to 5.6 before autoclaving.

All cultures were incubated at 26 °C in the dark and transferred to dim light (5–10 μmol m^{-2} s^{-1}) after 2 weeks of culture. Protoplast-derived colonies (1–3 mm diameter) were gently transferred onto 0.25% gellan gum-solidified MS medium with 1.0 μmol l^{-1} BA and 2.0 μmol l^{-1} IBA and grown in light to induce callus growth. After 4 weeks, the calluses were subcultured on MS medium supplemented with 5.0 μmol l^{-1} BA and 2.0 μmol l^{-1} IBA according to the procedure developed for regenerating leaf tissues (Zobayed and Saxena, 2003) to induce shoot formation. Regenerated shoots were separated and transferred to MS basal medium for plantlet formation.

Table 1. Effect of various liquid culture media on cell division and colony formation of mesophyll protoplasts of *E. purpurea* L.[a]

Medium	Division frequency (%)	Colony formation (%)
MS	12.2 ± 0.7	0.31 ± 0.05
B5	5.1 ± 0.8	0
MS salts + B5 vitamins	9.4 ± 1.2	0.23 ± 0.01
Modified MS	35.7 ± 3.9	1.24 ± 0.22

[a] All media contained 0.3 mol l^{-1} sucrose, 2.5 μmol l^{-1} BA and 5.0 μmol l^{-1} 2,4-D. Modified MS had a reduced concentration of potassium and ammonium nitrate (500 mg l^{-1}).

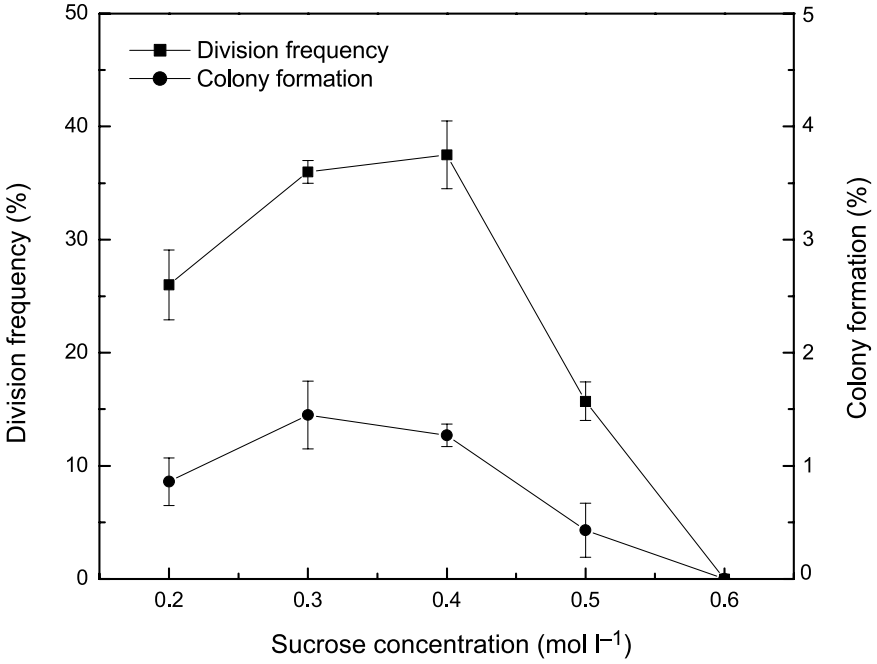

Figure 1. Effect of sucrose in liquid modified MS medium on cell division and colony formation of mesophyll protoplasts of *E. purpurea* L. The culture medium was supplemented with 2.5 μmol l^{-1} BA and 5.0 μmol l^{-1} 2,4-D.

Division frequency (the percentage of protoplasts with at least one cell division) was estimated after 2 weeks of culture, and colony formation (the percentage of protoplasts developing cell colonies of 32–64 cells) was determined after 4 weeks of culture on the basis of total numbers of protoplasts cultured and the cell colonies developed (Saxena et al., 1987). Each treatment had three replicates and all experiments were repeated three times.

Results and discussion

Young leaves of 4-week-old *Echinacea* seedlings grown in the light were used for protoplast isolation. About 3×10^6 protoplasts with a viability of 85% were obtained per gram fresh weight of leaf tissue with optimized isolation conditions. The isolated protoplasts were cultured in MS medium containing 0.3 mol l^{-1} sucrose and a growth regulator combination of 2.5 μmol l^{-1} BA and 5.0 μmol l^{-1} 2,4-D using liquid culture and alginate block/liquid culture. The protoplasts divided with a cell division frequency of 12.7 and 0.38% protoplasts formed colonies in the alginate block/liquid culture. Only a few cell divisions with no cell colony formation were observed in liquid cultures (data not shown). The combination of alginate block with liquid medium has been reported to be beneficial for a number of protoplast culture systems (Patat-Ochatt et al., 1988; Rozwadowski et al., 1990; Schlangstedt et al., 1992; Perales and Schieder, 1993). The efficacy of solid/liquid culture method has been attributed to the neutral and less toxic nature of alginate that protects the protoplasts and cells from chemical and physical damages from the culture microenvironment (Rozwadowski et al., 1990). In addition, the block/liquid cultures also provide an easy way to replace medium for accurately decreasing the osmoticum concentration during culture. Therefore, the alginate block/liquid culture was employed for further experiments with *Echinacea* mesophyll protoplasts.

The effect of medium components on cell division and colony formation of *Echinacea* protoplasts was examined. As shown in Table 1, MS medium was found more effective than B5 medium for improving cell division and colony formation. The maximum cell division (35.7%) and colony formation (1.24%) of *Echinacea* protoplasts was obtained when protoplasts were cultured on a modified MS medium. Nitrogen was an important factor in *Echinacea* protoplast regeneration, the modified MS medium with a reduced ammonium concentration (500 mg l^{-1} NH_4NO_4) improved cell division and colony formation (Table 1). The deleterious effects of ammonium have also been

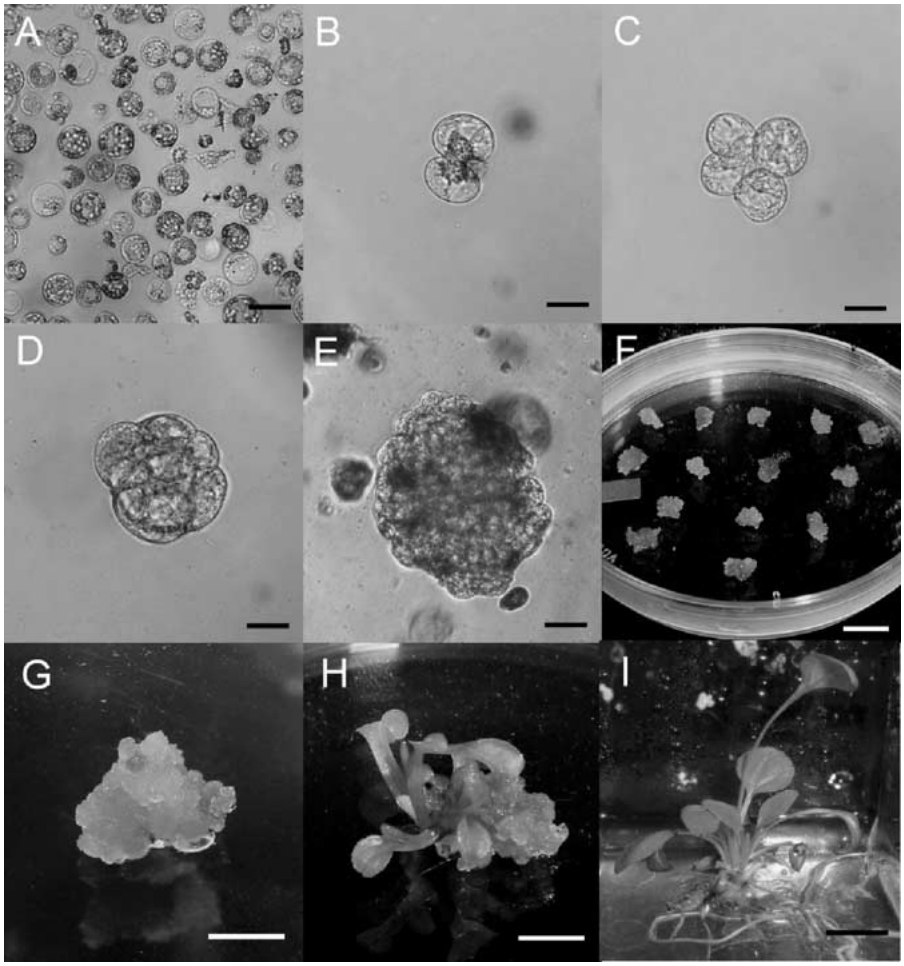

Figure 2. Plant regeneration from mesophyll protoplasts of *E. purpurea* L. (A) Freshly isolated mesophyll protoplasts (bar 50 μm); (B) first division of protoplast after 6 days of culture (bar 25 μm); (C and D) second and third cell division of protoplast-derived cells (bar 25 μm); (E) protoplast-derived cell colony after 6 weeks of culture (bar 100 μm); (F) callus formation from protoplast-derived cell colony (bar 1.0 cm); (G) organogenesis from protoplast-derived callus (bar 1.0 cm); (H) regenerated shoots from protoplast-derived callus (bar 1.0 cm); (I) complete plantlet regenerated from protoplast-derived shoots of *E. purpurea* (bar 1.0 cm).

reported in other protoplast culture studies (Xia et al., 1992; Wang et al., 1995). Sugar in liquid culture medium also influenced the cell division and colony formation (Figure 1). Sucrose appeared to be the more effective for cell division and colony formation from protoplasts than glucose, fructose, mannitol and their combinations (data not shown). The best colony formation was observed at a sucrose concentration of $0.3\,\mathrm{mol\,l^{-1}}$, and the number of colonies decreased as sucrose concentration increased beyond $0.4\,\mathrm{mol\,l^{-1}}$ (Figure 1).

The freshly isolated mesophyll protoplasts were green in color with an average diameter of 35 μm (Figure 2A). The first divisions of protoplasts occurred after 6 days (Figure 2B). Sustained divisions and colony formation was observed after 4 weeks (Figure 2C, D and E). Calluses were visible after 4 weeks of transfer of protoplast-derived colonies onto solidified MSO medium with $1.0\,\mu\mathrm{mol\,l^{-1}}$ BA and $2.0\,\mu\mathrm{mol\,l^{-1}}$ IBA (Figure 2F). Shoot regeneration was initiated from the calluses on MS medium with $5.0\,\mu\mathrm{mol\,l^{-1}}$ BA and $2.0\,\mu\mathrm{mol\,l^{-1}}$ IBA (Figure 2G), and regenerated shoots further developed on the same medium after 4 weeks (Figure 2H). Complete plantlets were obtained from protoplast-derived shoots on MS basal medium (Figure 2I). About 75% of protoplast-derived calluses formed shoots and 56% of these regenerated shoots formed roots. Protoplast-derived plants appeared morphologically normal and were successfully transplanted to greenhouse.

This study describes the development of a protocol for plant regeneration from protoplasts of *E. purpurea*, one of top 10 best-selling medicinal crops in North American and Germany. To our knowledge, this is the first report of protoplast culture and plant regeneration in *E. purpurea*. Although further experiments are needed to increase the percentage of colony formation, the protocol provides the prerequisite for using protoplasts as a tool to create novel medicinal germplasm. Protoplast fusion allows transfer of bulk or limited DNA between closely and distantly related species compared to transfer of one or two genes by current methods of genetic transformation. Therefore, the somatic hybrid cells have tremendous potential for investigating the biosynthesis of secondary metabolites regulated by multiple genes (Murch and Saxena, 2001).

Acknowledgements

The financial support of the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Food Agriculture is gratefully acknowledged.

References

Bauer R (1999) Chemistry, analysis and immunological investigations of *Echinacea* phytopharmaceuticals. In: Wagner H (ed) Immunomodulatory Agents from Plant (pp. 41–48). Birkhauser-Verlag, Basel, Boston, Berlin

Choffe KL, Victor JMR, Murch SJ & Saxena PK (2000a) *In vitro* regeneration of *Echinacea purpurea* L.: direct somatic embryogenesis and indirect shoot organogenesis in petiole culture. *In Vitro* Cell. Dev. Biol. Plant 36: 30–36

Choffe KL, Murch SJ & Saxena PK (2000b) Regeneration of *Echinacea purpurea*: induction of root organogenesis from hypocotyl and cotyledon explants. Plant Cell Tiss. Org. Cult. 62: 227–234

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158

Koroch A, Juliani HR, Kapteyn J & Simon JE (2002) *In vitro* regeneration of *Echinacea purpurea* from leaf explants. Plant Cell Tiss. Org. Cult. 69: 79–83

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Murch SJ & Saxena PK (2001) Somatic cell fusion: relevance to medicinal plants. In: Saxena PK (ed) Development of Plant-Based Medicines: Conservation, Efficacy and Safety (pp. 167–182). Kluwer Academic Publishers, Dordrecht, The Netherlands

Nagata T & Bajaj YPS (2001) Biotechnology in Agriculture and Forestry. Vol. 49: Somatic Hybridization in Crop Improvement II. Springer-Verlag, Heidelberg, New York

Patat-Ochatt EM, Ochatt SJ & Power JB (1988) Plant regeneration from protoplasts of apple rootstocks and scion varieties. J. Plant Physiol. 133: 460–465

Perales EH & Schieder O (1993) Plant regeneration from leaf protoplasts of apple. Plant Cell Tiss. Org. Cult. 34: 71–76

Rozwadowski KL, Saxena PK & King J (1990) Isolation and culture of *Lens culinaris* Medik. cv. Eston epicotyl protoplasts to calli. Plant Cell Tiss. Org. Cult. 20: 75–79

Saxena PK, Gill R & Rashid A (1987) Optimal conditions for plant regeneration from mesophyll protoplasts of eggplant (*Solanum melongena* L.). Sci. Hort. 31: 185–194

Schlangstedt M, Hermans B, Zoglauer K & Schieder O (1992) Culture of sugar beet (*Beta vulgaris* L.) protoplasts in alginate-callus formation and root organogenesis. J. Plant Physiol. 140: 39–344

Wang Y, Huang MR, Wei ZM, Sun YR, Chen DM, Xu ZH, Zhang LM & Nu N (1995) Regeneration of simon poplar (*Populus simonii*) from protoplast culture. Plant Cell Rep. 14: 442–445

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47: 189–194

Xia GM, Li ZY, Guo GQ & Chen HM (1992) Direct somatic embryogenesis and plant regeneration from protoplasts of *Bupleurum scorzonerifolium* Wild. Plant Cell Rep. 11: 155–158

Zobayed SMA & Saxena PK (2003) Auxin and a dark incubation increase the frequency of somatic embryogenesis in *Echinacea purpurea* L. *In Vitro* Cell. Dev. Biol.-Plant (in press)