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Genetic diversity in seed populations of *Echinacea purpurea* controls the capacity for regeneration, route of morphogenesis and phytochemical composition

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Abstract The production of new varieties and higher quality products from *Echinacea* spp. requires a greater understanding of the regulation of plant growth and the production of specific phytometabolites. The current studies were designed to generate elite varieties of *Echinacea purpurea* based on regeneration efficiency and chemical profile. Clonal propagation of seedling-derived regenerants and screening for antioxidant potential and concentrations of caftaric acid, chlorogenic acid, cichoric acid, cynarin, and echinacoside identified 58 unique germplasm lines. Chemical profiles varied significantly among germplasm lines but were consistent within clones of each line. In temporary immersion bioreactors, exogenous application of the auxin indolebutyric acid significantly increased the cichoric acid and caftaric acid concentration in the root tissues. Together, these demonstrate the potential for selective breeding of elite, highly regenerative, chemically superior, clonally propagated varieties from the naturally occurring genetic variability in the seed populations of *E. purpurea*.

Keywords Caftaric acid · Cichoric acid · *Echinacea purpurea* · Liquid Lab

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Introduction

Echinacea purpurea L. is the most commonly used medicinal plant preparation in USA and many other parts of the world. In a recent survey of the use of complementary and alternative medicines, 62% of respondents reported regularly using alternative medicines and about 40% of adults had used *Echinacea* in the previous 12-month period (Barnes et al. 2004). Retail sales of *Echinacea* products are more than $58 million annually in USA (Blumenthal 2003) and have been estimated at $300 million annually worldwide. A recent NAPRALERT search revealed that there have been at least 86 human clinical trials of *Echinacea preparations* demonstrating efficacy as an immunostimulant, anesthetic, antioxidant, analgesic, and diaphoretic. The same study also identified 216 different medicinally active compounds that have been reported in the literature on *E. purpurea*.

Extensive genetic and chemical diversity has previously been demonstrated in wild and cultivated populations of *Echinacea* (Binns et al. 2002a, b, c). For production of high-quality *Echinacea* for medicinal plant preparations, it is necessary to eliminate the chemical variability, eliminate abiotic and biotic contamination, breed elite plant genotypes and optimize the growing systems (Murch et al. 2000a). *In vitro* regeneration and production offers many advantages for the growth of medicinal plants in general and more specifically *Echinacea* as it offers the capacity to optimize all growth conditions (Murch et al. 2000b). There have been several previous reports of *in vitro* regeneration in the literature from *E. purpurea* (Coker and Camper 1999; Choffe et al. 2000; Koroch et al. 2002; Mechanda et al. 2003; Zobayed and Saxena 2003) and two reports of agrobacterium-mediated transformation in this species (Koroch et al. 2002b; Wang and To 2004) however, a wide range of different responses have been observed in explants cultured on identical culture medium and there has been no systematic study of the chemical diversity among regenerated plants. The objectives of the current study were to compare the responses of explants from

different individual seedlings in two different culture systems, to regenerate clonally propagated germplasm lines derived from the individual seeds and to compare the chemodiversity of the regenerated plants under standardized conditions.

Materials and methods

Development of a seedling-derived germplasm collection

Seedlings of *E. purpurea* L. from three different seed lots (Richter's The Herb Specialists, Goodwood, ON) were used in these studies. Two distinct groups of 50 seeds were surface sterilized by immersion in a solution of 10% PPM (Plant Cell Technology Inc., USA) followed by a 30 s immersion in 70% ethanol, an 18 min immersion in 5.4% sodium hypocholorate containing one drop of Tween 20 per 500 ml and finally three rinses with sterile distilled water as described previously (Choffe et al. 2000). Surface sterilized seeds were germinated *in vitro* on basal medium containing 1/2 strength MS salts and vitamins (Murashige and Skoog 1962), 1.5% sucrose with a pH 5.7 and solidified with gellam gum (Sigma). A third group of 1000 seeds was germinated in 128 plug trays under standard greenhouse conditions and at the 2–3 leaf stage, seedlings were transplanted to baskets containing Hydrotron Leca (Rambridge Wholesale Supply, Calgary, AB) as the growing substrate and fertilized with standard greenhouse fertilizer. Once the *in vitro* or greenhouse grown seedlings reached a height of 2.5–3 in. and had at least three fully unfolded, regular leaves, petioles were assigned a number characteristic of the seedling of origin and harvested for regeneration. Individual petioles from the fully expanded newly emerged leaves of 200 individual plants were separated from leaves and 1 cm segments at the proximal and distal ends of the petiole were excised, flash frozen, and stored at –87°C for antioxidant potential measurements. The mid-region of the petioles was placed in 0.1% PPM solution and stored 2–3 days at 4°C before culturing. The petioles were surface sterilized by immersion for 10 min in 10% solution (5.02%) of sodium hypochlorite (pH 7.0) with a drop of Tween 20 per 500 ml then another 10 min in the solution with the same composition, but without Tween 20. Afterwards the petioles were rinsed three times in sterilized distilled water, cut into 1 cm pieces and cultured onto an induction medium containing MS salts (Murashige and Skoog 1962), Gamborg B5 vitamins (Gamborg et al. 1968), 3% sucrose, and 5 μM benzylaminopurine (BAP; Sigma Chemical Co, St. Louis, MO) at pH 5.6 and solidified with gellan gum (Sigma) added prior to autoclaving. The cultures were incubated in the light (30–50 μmol m^{-2} s^{-1}) with a 16 h photoperiod at 24°C. Regeneration was quantified on day 42 of the culture-induction period as previously determined (Choffe et al. 2000). Regenerants were separated and individually subcultured onto the same medium devoid of growth regulators to develop into whole plants with the identity of the seedling of origin maintained throughout. A total of 28 distinct clonally propagated seedling lines were developed from the first set of 50 *in vitro* germinated seeds, three distinct clonally propagated lines were developed via protoplast isolation and regeneration from *in vitro*-germinated seedlings as described previously (Pan et al. 2004) and 26 distinct clonally propagated seedling lines were derived from the greenhouse-germinated seedlings. The collection of maternal plant materials of the 57 seedling-derived lines were maintained *in vitro* on a culture medium (pH 5.6) consisting of full strength MS salts, Gamborg B5 vitamins, 3% sucrose, 1 μM kinetin and 0.23% gellan gum incubated at 25°C with 16-h photoperiod under cool white light at 40–60 μmol m^{-2} s^{-1} and subcultured at 6–8 week intervals to provide stock plants for the further experiments (Fig. 1a).

Comparison of efficiency of regeneration

For comparison of the efficiency of plant recovery by two different regeneration protocols, petiole explants 2 cm in length were excised from the fully expanded newly emerged leaf of axenic cultures of all seedling lines and cultured on a medium (pH 5.6) consisting of MS salts, B5 vitamins, 3% sucrose, 5 M BAP and 0.23% gellam gum. Leaf explants (1 cm^2) were isolated from the same stock material and cultured onto a medium (pH 5.6) consisting of MS salts, B5 vitamins, 3% sucrose, 5 M BAP and 2.5 M indolebutyric acid and 0.23% gellam gum as described previously (Zobayed and Saxena 2003). The petiole and leaf cultures were randomly divided and incubated at 25°C with either a 16 h photoperiod under cool white light (40–60 mol m^{-2} s^{-1}) or complete darkness for 2 weeks followed by a 16-h photoperiod in the same growth chamber. The experiment consisted of four replicate Petri dishes containing five leaf disks or six petiole explants for each treatment and was replicated twice.

Liquid cultures

Regenerated plantlets were rooted and grown to 3–4 in. height in a Liquid Lab Rocker system (Figs. 1b and c; Southern Sun Biosystems Inc., Hodges, SC). Individual plantlets from each seedling-derived line were gently separated, washed of any solid media residue and subcultured into a Liquid Lab Vessel. Each Liquid Lab Vessel has a total volume of approximately 2.6 l and was cultured with 40 regenerated plantlets from seedling-derived lines. *Echinacea* plantlets were grown in vessels containing 100 ml of liquid medium consisting of MS salts, B5 vitamins, and 3% sucrose at pH 5.7. The media was replenished at 30 day-intervals with a further 100 ml of the same liquid medium. To assess the effects of supplemental auxin on the phytochemistry of *E. purpurea*, replicate vessels of three prolific seedling-derived lines were grown in the same medium supplemented with 0, 5, 10 or 15 μM IBA. Gently rocking the vessels with a 30 s cycle and 1.2 min interval between cycles resulted in an intermittent micorhydrophonic ebb and flow liquid nutrient irrigation thereby creating a

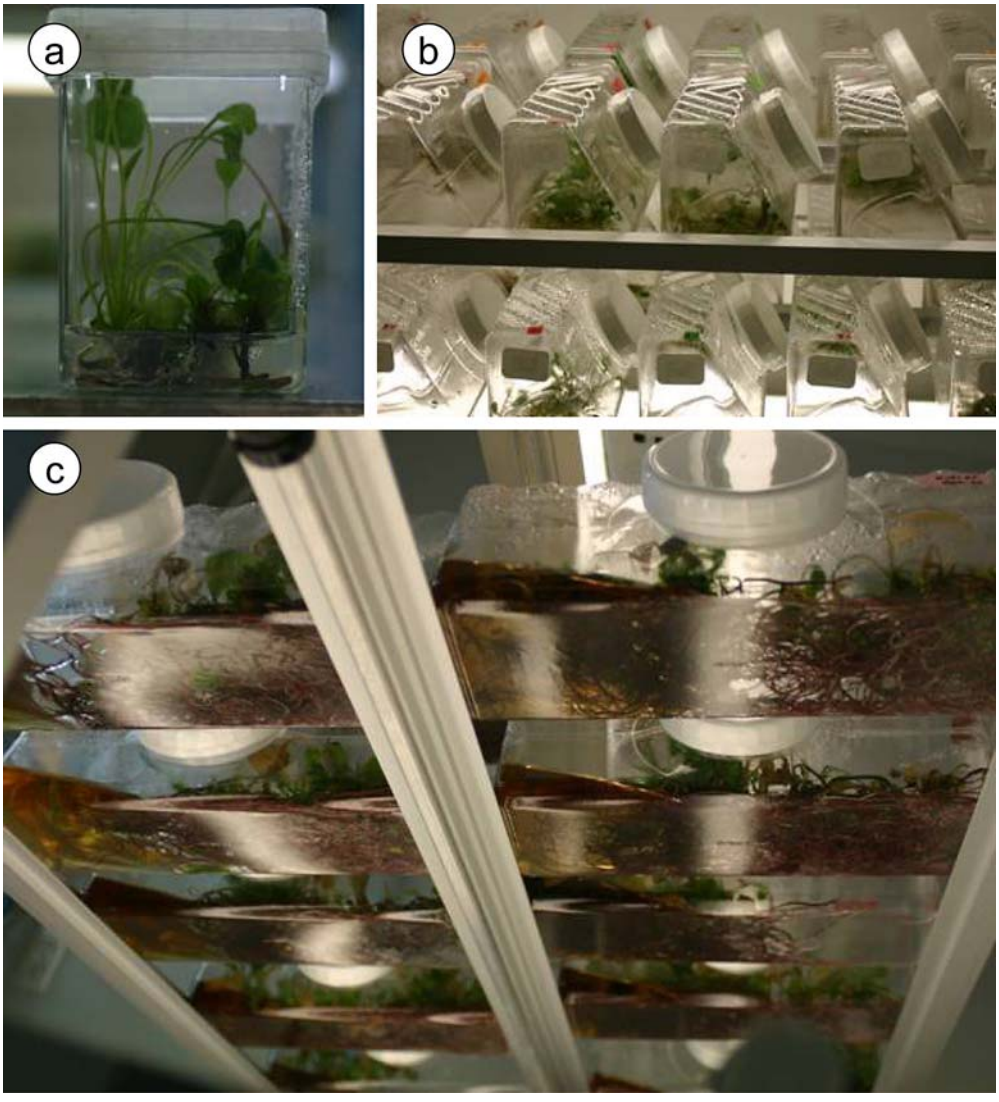

Fig. 1 *In vitro* development of plantlets of *E. purpurea* L. **a** Regenerated plantlet on solid, basal medium (bar = 2.3 cm), **b** Regenerated plantlets in the Liquid Lab Vessels (bar = 10 cm), **c** *E. purpurea* roots grown in the Liquid Lab Vessels produced root biomass at approximately 1 g per Vessel per day (bar = 4.5 cm)

miniature temporary immersion bioreactor by transferring liquid media from side to side.

Antioxidant potential

The antioxidant activity was evaluated according to the method described by Brand-Williams et al. (1995) with some modifications. The high radical generating activity of 1,1 diphenyl-2-picryhydrazyl (DPPH) was determined by absorbance at 520 nm and the capacity of *E. purpurea* extracts to detoxify free radicals were determined relative to a standard curve. Crude plant extracts were prepared by incubating 30 mg DW of plant tissue in 1 ml 80% ethanol overnight at room temperature in the dark. Samples were centrifuged at 13,000 rpm for 3 min to precipitate particulate matter and a dilution series of 0, 2, 4, 10, 20, 30, and 40 µl of the crude extracts was incubated with 125 µl DPPH and allowed to react for 3–5 min. All the samples were loaded in 96 well plate and the absorbance at 520 nm was read using Wallac Victor^2 1420 multilable counter. All germplasm lines were analyzed in triplicate. Data was compared to a standard curve that was prepared with gallic acid at concentrations of 20, 40, 60, 80, 120, 160 and 200 µg ml^{-1}.

Phytochemical analysis

Methods for the isolation and analysis of caftaric acid, chlorogenic acid, cichoric acid, cynarin, and echinacoside were developed by modification of previously described protocols (Perry et al. 2001; Bergeron et al. 2000). Callus, somatic embryos, root and shoot tissues (approximately 0.3 g fresh weight samples) were collected into 2 ml Eppendorf tubes and the tissues was frozen immediately in liquid nitrogen before storage, at -70°C. The frozen plant tissues were freeze-dried for 18 h using a Labconco Freeze Dry System (10×10^{-3} mbar, -40°C) (Caltec Scientific Ltd., Toronto, CA). Immediately after removal from the freeze drier, plant tissues were ground into fine powder using a polyvinyl mortar in the same Eppendorf tube and the

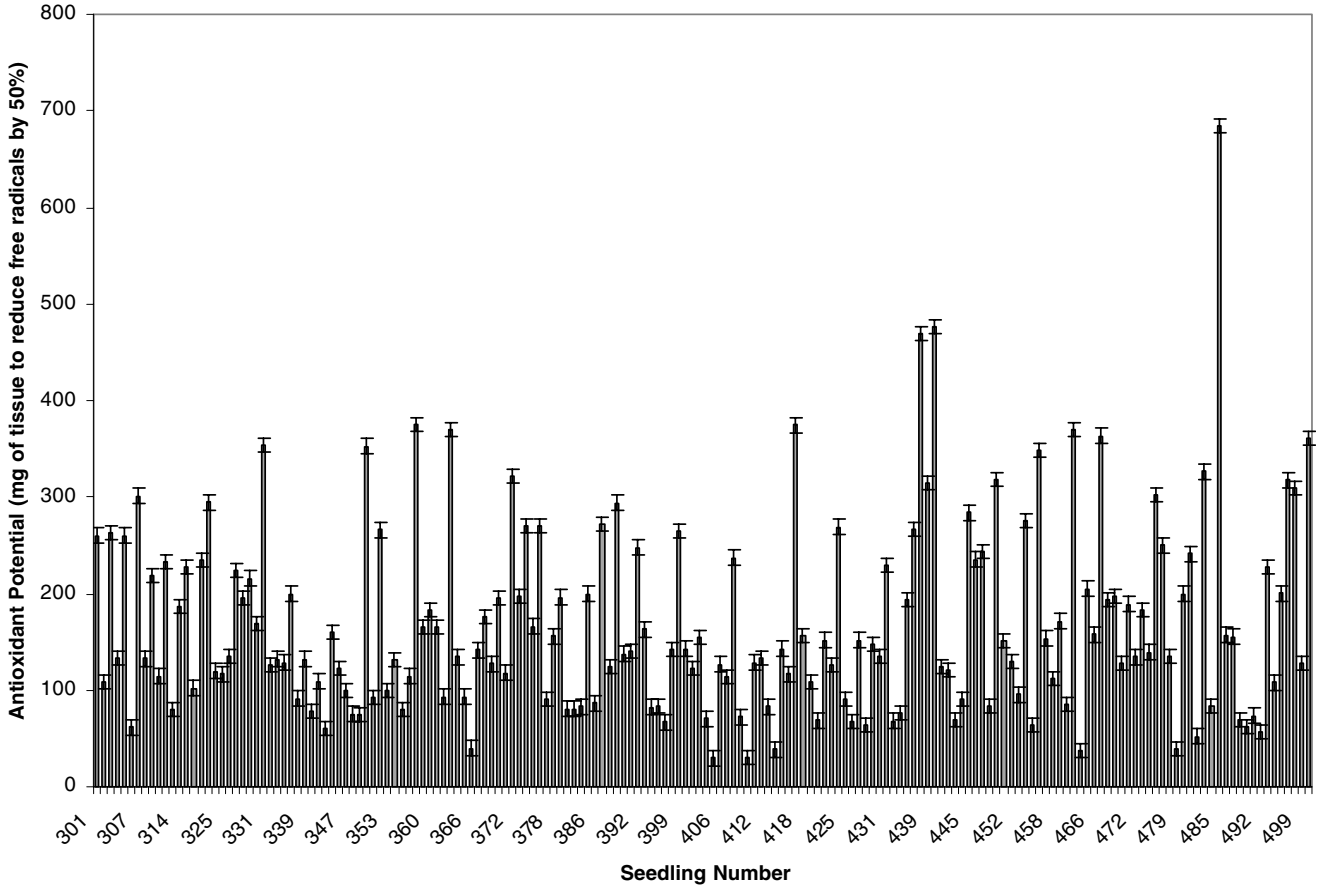

Fig. 2 Diversity of antioxidant potential among seedling of *E. purpurea* L.

finely ground powder was transferred into an 15 ml centrifugal vial. The five bioactive compounds were extracted with 4 ml of 70% methanol for 15 min using an Ultrasonic FS-14 sonicator (Fisher Scientific, Nepean, ON). The vials containing the crude extract were centrifuged at 3000 rpm for 10 min (GS-6 series centrifuge, Beckman Instruments Inc, Palo Alto, CA) to remove the sample residues from the extract. An aliquot (2 ml) of the supernatant was filtered through a 0.2 μm Nylon syringe filter (Waters, Mississauga, ON). After homogenization, 300 μl of the filtered extract from each sample were transferred into a glass HPLC autosampler vial. All the sample preparation steps carried out under the low intensity light conditions at room temperature and once extracted, the samples were injected onto the HPLC within 15 h of extraction.

The HPLC separation was carried out using a Waters 2695 Alliance HPLC system with a Phenomenex Luna C_{18} column (5.0 μm; 4.6 mm × 150 mm) with a C18 guard column (4 mm × 3 mm) (Phenomenex, Torrance, CA). The analytes were separated using a gradient of mobile phase of 0.1% phosphoric acid (A) and acetonitrile (B) at a constant flow rate of 1.3 ml/min flow rate. The gradient separation was obtained by $t = 0$ min, A/B 92/08, $t = 11$ min, A/B 82/18, $t = 15$ min, A/B 60/40, $t = 15.5$ min, A/B 60/40, $t = 16$ min, A/B 92/08, $t = 26$ min, A/B 92/18. Analytes were detected with at 330 nm using a Waters dual λ UV absorbance detector. Reference standards of caftaric acid, chlorogenic acid, cichoric acid, cynarin, and echinacoside were purchased from ChromaDex Inc. (Laguna Hills, CA) and were initially dissolved in 100% methanol to give a concentration of about 2,000 μg/ml and stored at –70°C. Standard curves were obtained as a function of peak area across a dilution series of 5, 25, 50, 100, and 200 μg ml^{-1}. High linearity ($r2 = 0.99$) was obtained for the each calibration curve. A reference sample of greenhouse grown *E. purpurea* roots was repeatedly extracted and analyzed to determine recovery and to ensure consistency throughout the analysis protocols. The quantification of caftaric acid (t_R, retention time of 5.7 min), chlorogenic acid ($t_R = 6.6$), cynarin ($t_R = 10.0$), echinacoside ($t_R = 10.6$), and cichoric acid ($t_R = 14.7$) were determined on the basis of the peak area of UV absorption at 330 nm with comparison to the standard curves of the authentic sample. The content of caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid were adjusted to the corresponding extraction recovery rates as determined.

Data collection

Observations of regeneration were made visually after 42 days. Each shoot and embryo was detected

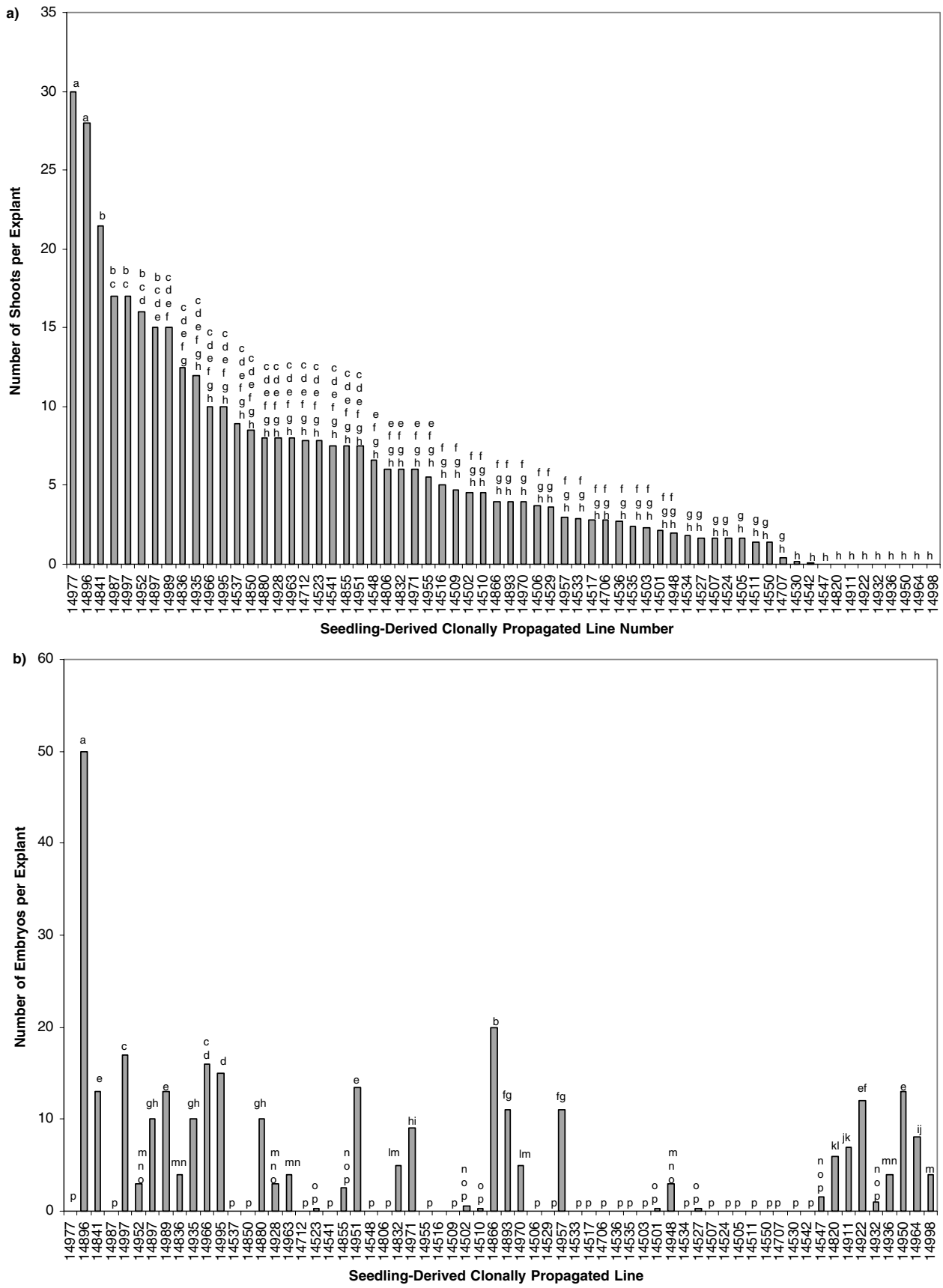

Fig. 3 Competence for morphogenesis of petiole explants cultured on a medium supplemented with cytokinin in the absence of exogenous auxin varied by seedling of origin. **a** Shoot organogenesis and **b** Somatic embryogenesis

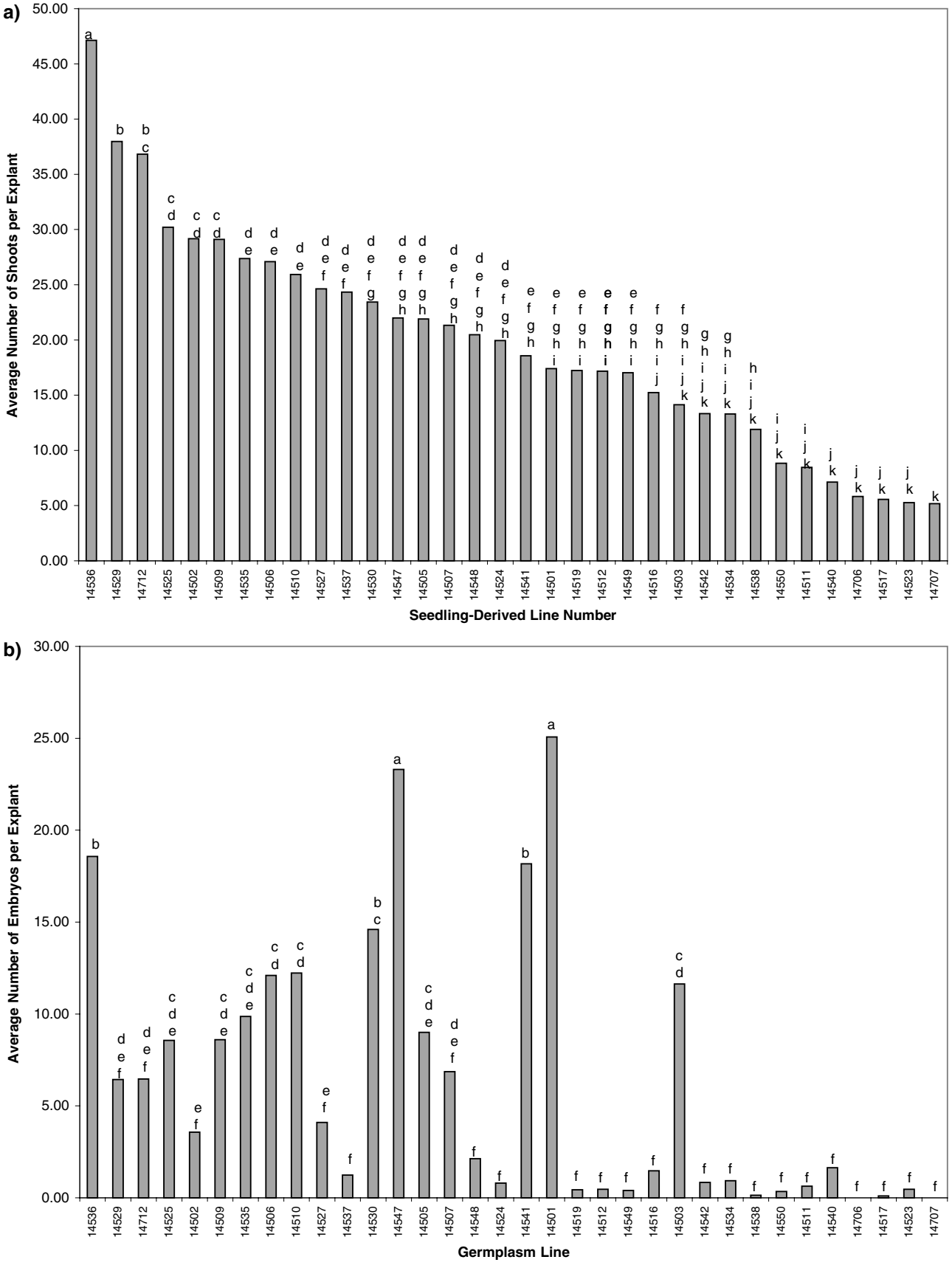

Fig. 4 Competence for morphogenesis of leaf explants cultured on a medium supplemented with both auxin and cytokinin varied by seedling of origin. **a** Shoot organogenesis and **b** Somatic embryogenesis

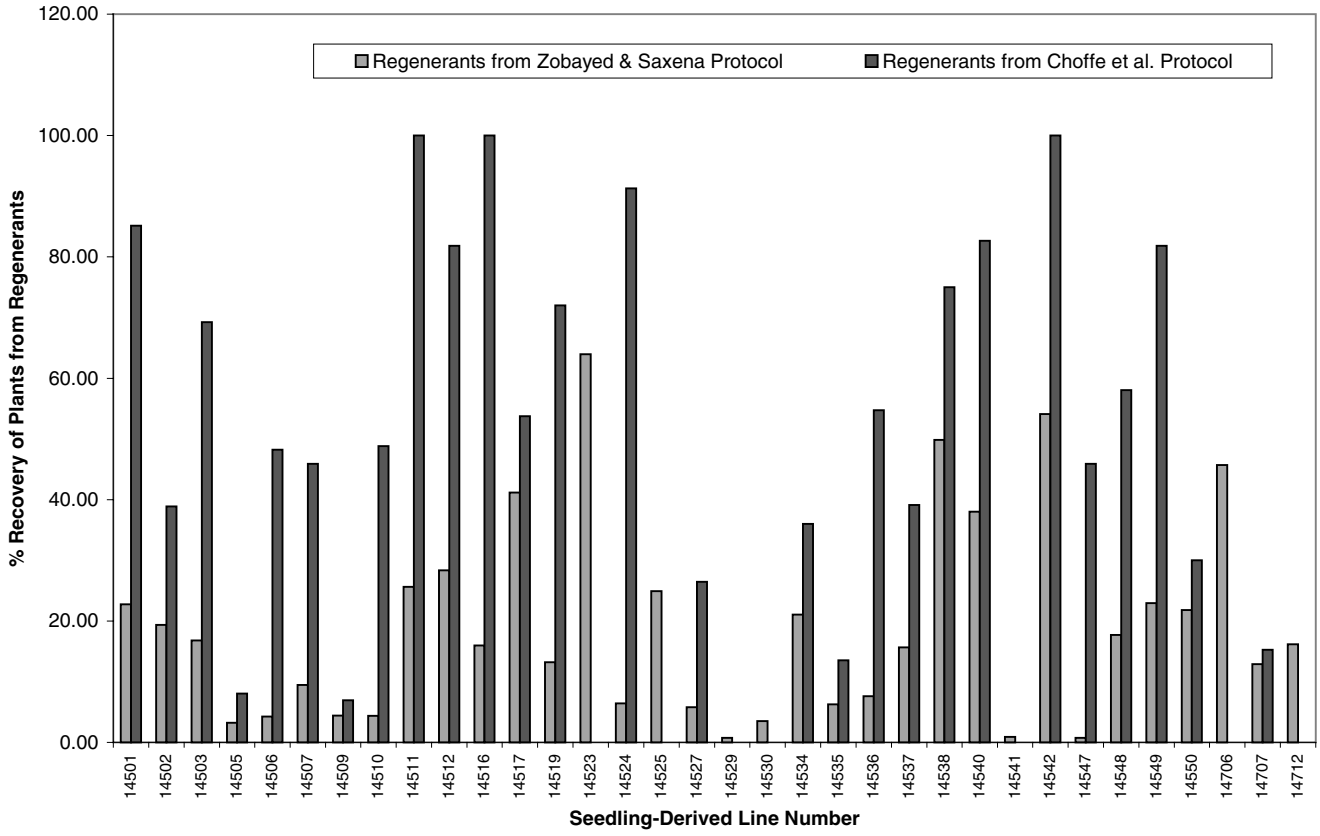

Fig. 5 Transplant efficiency was dependent on the method of induction of morphogenesis

using a phase contrast stereo microscope (Zeiss Stemi 1000/2000/2000C) to distinguish the structure between embryo and shoot. Data were analyzed by Student–Newman–Keuls means comparison test $P \leq 0.05$.

Results

The overall goal of the research project was to assess the physiological differences and morphological potential of a collection of seedling-derived clonally propagated lines of *E. purpurea*. To evaluate different protocols for efficiency of regeneration, petiole and leaf explants were cultured onto the optimal medium for each and incubated as previously described (Choffe et al. 2000; Zobayed and Saxena 2003). There was wide variability in the measurement of the antioxidant potential of the proximal and distal ends of petioles harvested from 200 *E. purpurea* seedlings (Fig. 2), but the capacity to detoxify free radicals was not correlated with the regenerative capacity of the petiole sections. In preliminary experiments, it was shown that the capacity for regeneration is not dependent on the age of the petiole or the origin or orientation of the explant but rather, the critical factor for regeneration competence and the redirection of growth to either embryogenesis or organogenesis is the seedling of origin (unpublished results). In the current investigations, significant differences were observed in the development of shoots or somatic embryos on petiole sections of clonally propagated lines derived from different individual seeds and cultured onto a medium supplemented with only the cytokinin BAP (Figs. 3a and b). There was upto a 30-fold variation in the capacity of individual seedling-derived germplasm to regenerate shoots (Fig. 3a) and greater than 50-fold variation in the competence of cells to undergo somatic embryogenesis (Fig. 3b). In total, only 22% of *Echinacea* seedlings regenerated de novo shoots from petiole explants cultured on the cytokinin medium and 12.8% of the seedlings demonstrated the capacity for somatic embryogenesis. These regenerable lines were then clonally propagated and repeatedly subcultured to produce the seedling-derived lines as stock material for all further experiments. In 54% of the seedling-derived lines, significantly more shoots were regenerated on petiole sections cultured on cytokinin medium and incubated in a 16-h photoperiod than were regenerated on identical petiole sections cultured on the same medium, but incubated in total darkness for 14 days. None of the petiole sections regenerated embryo-like structures when incubated in the dark chamber on cytokinin supplemented medium, but significant differences were observed in the capacity of different seedling-derived lines to regenerate somatic embryos during light incubation. In the second culture system (Zobayed and Saxena 2003), leaf disks of each seedling-derived line were cultured onto the auxin and cytokinin supplemented medium and incubated in a dark chamber for 2 weeks. Both de novo shoots and somatic embryos

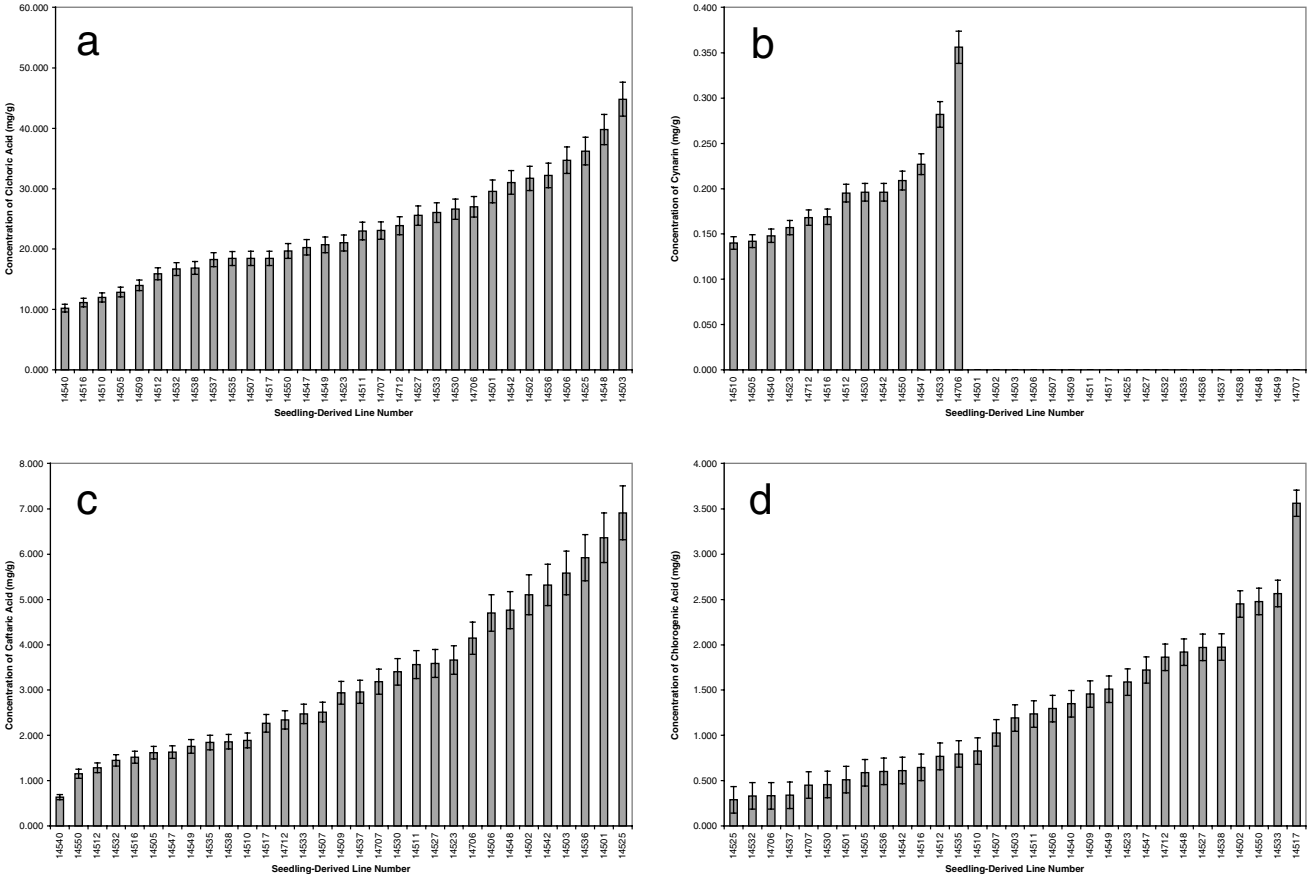

Fig. 6 The phytochemical profile of plantlets depended on the seedling of origin of the regenerants **a** cichoric acid, **b** cynarin, **c** caftaric acid, **d** chlorogenic acid

were formed on leaf disks cultured onto the basal medium supplemented with IBA and BAP at previously optimized concentrations, but the number of regenerants and the relative ratio of the different modes of regeneration were significantly varied with the origin of the seedling-derived lines (Figs. 4a and b). One of the most interesting observations of the experiments to compare the efficiency of the two methods of regeneration of *E. purpurea* was the percentage survival of whole plants after transplanting. While it is possible to recover fully developed plants from regenerants using either protocol (Fig. 1a), the transplant survival rate differed significantly among the seedling-derived lines, potentially as a function of the capacity for root development (Fig. 5). Further, the two regeneration protocols also significantly differed in the capacity for acclimatization and transplant survival. In general, a higher proportion of the plantlets developed from regeneration on the Choffe protocol, viz. petiole sections cultured on cytokinin supplemented medium in light, were developed into whole plants. However, in some of the seedling-derived lines, particularly those that did not respond well to the cytokinin medium, the relationship between induction medium and survival was reversed (Fig. 5). Phytochemical content was determined in callus, shoots, and embryos regenerated from petioles and leaves after 42 days of culture. None of the *in vitro* regenerants were found to have significant concentrations of medicinally active secondary metabolites, regardless of mode of regeneration or seedling of origin (data not shown).

Individual plantlets derived from the clonally-propagated seedling-derived lines were subcultured onto liquid media in the Liquid Lab Rocker system and grown for 60 days to a height of 3–4 in.. Survival of the plantlets in the Liquid Lab was greater than 95% and plantlets from all inductive treatments developed rapidly growing root systems. Major plant losses occurred only when the Liquid Lab Vessels were contaminated. After 30 days in the Liquid Lab Rocker, *E. purpurea* roots were growing at about 1 g per day per vessel. However, there were significant differences in the caftaric acid, chlorogenic acid, cichoric acid, and cynarin concentrations in the tissues of regenerants of the different seedling-derived lines (Fig. 6). Cynarin was present, in detectable, levels in only 42% of seedling-derived lines grown under identical conditions (Fig. 6b) Interestingly, echinacoside was detected in the seedling-derived lines (Fig. 7a) and there were significant differences in the amount of echinacoside quantified in the different regenerated plantlets (Fig. 7b). Supplementation of the basal liquid medium with IBA significantly increased the rate of growth of *E. purpurea* roots in the Liquid Lab (data not shown) and altered phytochemical content. Across all lines, increasing IBA concentration in the medium

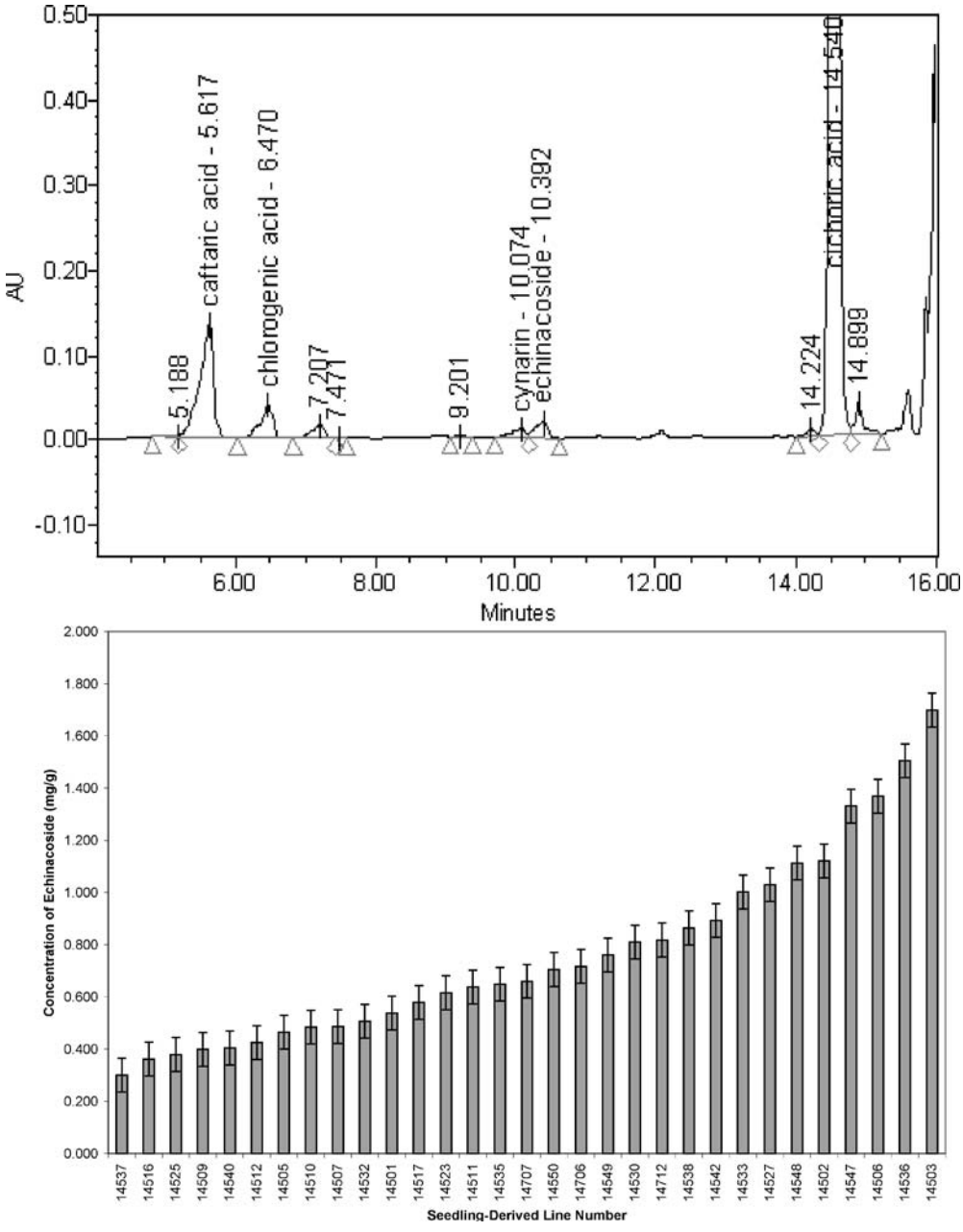

Fig. 7 Echinacoside was found in detectable levels in *in vitro* grown plantlets of *E. purpurea* L. and the concentration was dependent on the seedling of origin

significantly increased the cichoric acid and caftaric acid content of the root tissues of *E. purpurea* (Fig. 8a). As well, there were significant differences in the contents of cichoric acid, cynarin and chlorogenic acid of different seedling-derived lines grown in the medium containing 15 (M IBA (Figs. 8b, c, and d). There was also evidence to indicate that the partitioning of secondary metabolites was different in plantlets derived from different original seedlings. Shoot and root contents of chlorogenic acid were significantly different when the plantlets were grown in the high auxin medium (Fig. 8d). The observation that there was a difference in the chemical profile of plantlets derived from different original seed sources was confirmed with analysis of the whole collection of seedling-derived lines.

Discussion

Ultimately, the development of a high quality *Echinacea* crop requires a detailed understanding of the physiology and metabolism of the species. Yield of the crop must be defined in terms of the production of specific medicinally active metabolites for the medicine to have utility in the treatment of human diseases. The results of this research clearly show that genetic diversity among individuals in the seed population impact the chemical profile of the final products. This chemodiversity also provides the opportunity for the selection of naturally occurring superior individuals and the technology developed facilitates the clonal propagation of these individuals to create novel, consistent plant germplasm. The ultimate result of this research

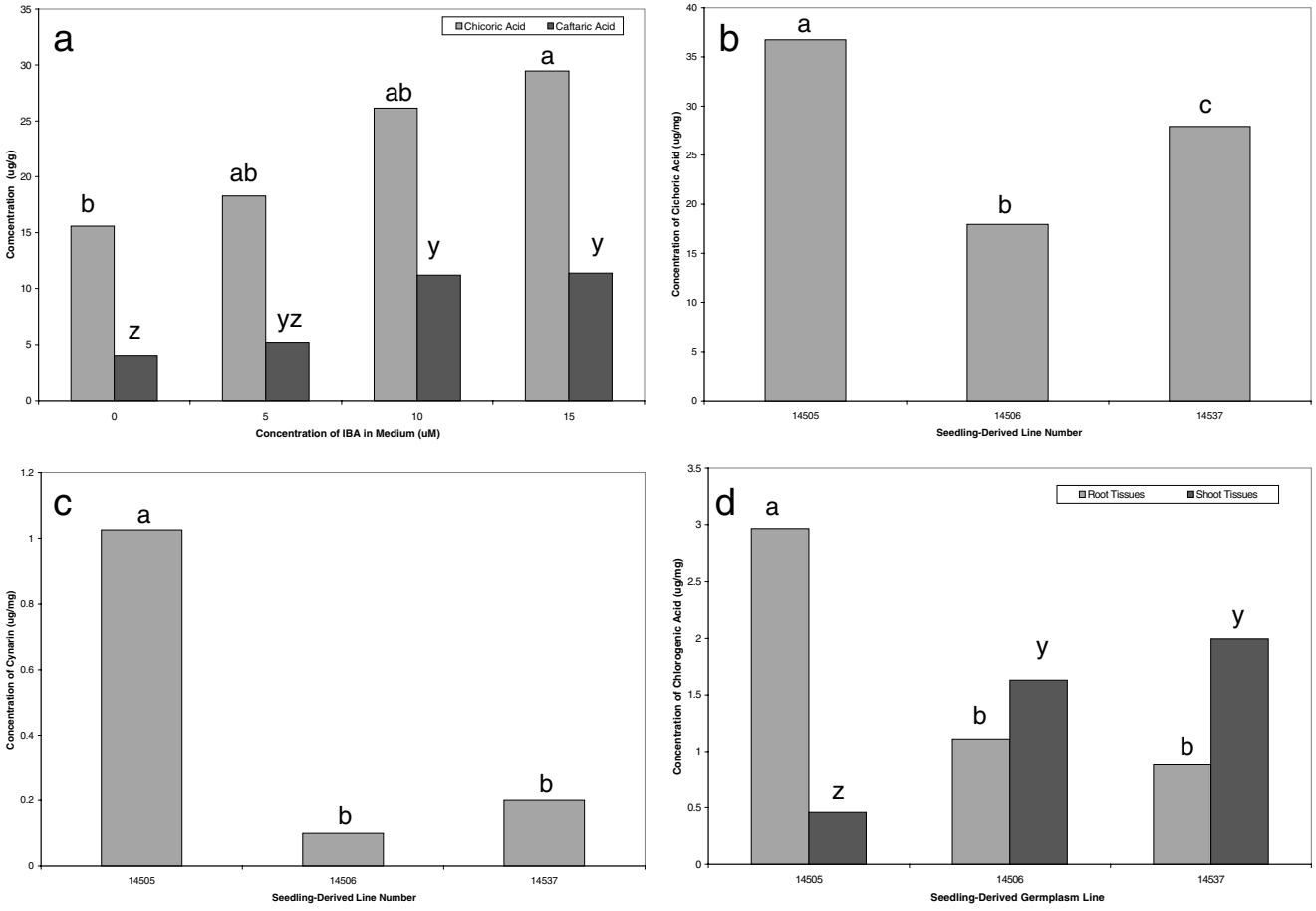

Fig. 8 Phytomedicinal content of in *vitro*-grown plantlets was influenced by supplementation of the medium with indolebutyric acid **a**, and by seedling of origin **b**–**d**

has been the establishment of 57 distinct seedling-derived germplasm lines with different physiological, metabolic and morphogenic potentials. Further evaluation of these lines in model systems will both facilitate the elucidation of the medicinally "active" compounds in *Echinacea* and provide the foundation for superior products and novel applications of specialized chemical profiles.

The medicinally active compounds commonly quantified in *Echinacea* tissues are important antioxidants (Pellati et al. 2005), and root extracts of *Echinacea* have been shown to have a high antioxidant potential (Brand-Williams et al. 1995). Therefore, it was interesting to assess the relationship between medicinally active antioxidants and morphogenesis in *E. purpurea*. In other species, previous researchers have shown that the relative frequency of shoot and embryo production was dependent on the antioxidant enzymes (Kairong et al. 1999; Guptha and Datta 2003) but this relationship was not observed in *E. purpurea* cultures. In preliminary studies, we determined that the morphogenesis response of petioles exposed to cytokinins was dependent on the seedling of origin rather than the age of the petiole, maturity of the associated leaf, petiole color or maturity of the seedling upto about 6 in. height. The data presented here indicates that the capacity for shoot organogenesis or somatic embryogenesis in *E. purpurea* is dependent on the seedling of origin suggesting that the genetic capacity for production of endogenous growth regulators and receptors is essential for the regenerative response and varies within the populations.

Further, the capacity for accumulation of medicinal metabolites was significantly correlated with the seedling of origin and was maintained through multiple clonal propagation cycles indicating stable genetic variations among the original seedling population. Interestingly, the capacity for production of cichoric acid and caftaric acid was enhanced by exogenous auxin application regardless of the seedling of origin. Previous researchers have that high concentrations of chicoric and caftaric acid in all tissues of *E. purpurea* (Cheminat et al. 1988). Our findings suggest that accelerated growth of roots of *E. purpurea* also increases the concentrations of these medicinal caffeoyl conjugates. These findings may be both practical and important since the caffeoyl conjugates have been shown to be effective natural anti-viral compounds (Konig and Dustmann 1985) and may be associated with the medicinal activity of *E. purpurea*. A further interesting finding was the detection and distribution of echinacoside in the *in vitro* grown plantlets of *E. purpurea*. Echinacoside may not be responsible for the immunostimulatory efficacy of *Echinacea* products as it is not commonly found in *E. purpurea* and is used

commercially to distinguish *E. purpurea* from *Echinacea pallida var. angustifolia* (Binns et al. 2002c). Echinacoside has previously been detected in *E. purpurea*, but was present at levels more than 100– fold lower than was observed in *E. pallida var. angustifolia* tissues (Perry et al. 2001). The detectable levels of echinacoside in *E. purpurea* plantlets grown for these experiments may be a reflection of the enhanced metabolism under *in vitro* growing conditions and high sugar content of the growth medium. The current data not only demonstrates the potential for echinacoside production in *E. purpurea* but also reveals a 30-fold difference in the capacity of individuals to produce echinacoside under identical growing conditions.

Production of high-quality *Echinacea* products requires a greater understanding of the factors involved in the production of specific phytometabolites, the growth of tissues and the regeneration of individual clones. The current results provide the foundation for the large-scale production of plants and isolated roots in axenic cultures of *E. purpurea* as plant cell factories for the production of medicinal metabolites. The further scale-up of these protocols will allow for commercial scale production of high-quality plant material free from environmental pollutants, fungi, bacteria, and other contaminants. The further investigation of the regulation of the metabolism of medicinally active compounds will allow for increased medicinal production without the need for increased plant biomass.

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