RESEARCH ARTICLE

Identification of ploidy levels of in vitro grown purple coneflower (*Echinacea purpurea* L.)

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Abstract: *Echinacea purpurea* L. (Purple coneflower- Asteraceae) is one of the important medicinal herbs. In this study, three morphologically different types, haploid, diploid and tetraploid plantlets, were clearly identified using in *vitro* grown *E. purpurea* plantlets. Although all the rooted plants were almost the same height and possessed of almost the same number of leaves, diploid and tetraploid showed evidently sturdier roots while haploids had slimmer roots. Roots of diploids and tetraploids showed prominent differences; roots of tetraploids were shorter, thicker and darker in colour and with many lateral roots than those of diploids. The difference in the thickness of the roots could be distinguished as early as day 10-15, few days after growing out of adventitious roots. There were no significant differences in flowering time among haploids, diploids and tetraploids grown normally in the field and flowered within 5 months. There were no prominent morphological differences to be identified between *E. purpurea* haploids, diploids and tetraploids plant leaves either under in *vitro* or *in vivo* conditions. Size of stomata on leaves varied even within the same leaf. However, significant differences of the size of stomata could be found among haploids, diploids and tetraploids. The average length of stomata of diploid plants was 122.60 µm and that of tetraploid and haploid plants were 137.59 µm and 72.41 µm, respectively. Overall, tetraploids possessed longer and wider stomata. *E. purpurea* haploid has 11 chromosomes with smaller cells; diploid has 22 chromosomes and tetraploid has 44 chromosomes with comparatively larger cells than haploid and diploid plants.

Keywords: *E. purpurea*, tetraploid, diploid, haploid

Introduction

Plants of *E. purpurea* have been used as a traditional herbal medicine by several groups of Native Americans for hundreds of years (Percival, 2000). Tetraploid plants exhibit significant differences in morphology of flowers, pollens, stomata, shape and size of seeds and fruits compared with diploids (reference). Polyploids usually show different morphology compared with diploids, the amount of differences being very dependent on the plant species, the degree of heterozygosity, the ploidy level, and the mechanism relating to gene interactions, gene dose effects and regulation of specific traits and processes (Leitch and Bennett, 1997). The tetraploid plants are shorter than their diploid counterparts and could possibly be used as dwarfing rootstocks. Tetraploids have been induced from diploid cultivars using *in vitro* technique in number of genera (Cohen and Yao, 1996; Pinheiro *et al.*, 2000, Predieri, 2001; Roy *et al.*, 2001). Tetraploids can also be used to create triploids by crossing with diploid plants (unpublished data Dahanayake *et al.*).

Chromosome size and morphology may help to indicate evolutionary relationships among plant species (Clark and Wall, 1996). Stomata characteristics have been used to estimate ploidy level of the plant. The stomatal size has increased

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with the increasing ploidy level while stomatal density has decreased. The length of stomata guard cell was different among regenerated tetraploid, diploid and haploid plants. This result means that the plant weight or yield of tetraploid plants will increase greatly compared to that of the diploid plants (Gao *et al*., 1996). The aim of the present study was to investigate the morphological differences of tetraploid, diploid and haploid plants of *E. purpurea* for production of superior new varieties which could be used in commercial herbal medicine sector.

Materials and Methods

Preparation of Media

Each bottle was filled with 40 mL medium and covered with an air-tight polycarbonate screwed cap. Shoot regeneration medium for culture of petiole explants comprised of MS salts, 3% sucrose, 0.5 mg/L BA, 0.01 mg/L NAA, and rooting medium of the regenerated shoots comprised of MS salts, 3% sucrose, 0.01 mg/L NAA. All the media used were adjusted to a pH value of with 1 N NaOH or 1 N HCl solution, and gelled with 0.6% agar prior to autoclaving at pressure of $1.4 \, \text{kg} \, \text{cm}^{-2}$ for 20 minutes. When colchicine was used, it was dissolved in distilled water to a concentration of 5 mg/mL, filtered sterilized and then added to warm (about 70°C) autoclaved media.

Plant materials

After 45 days in the shoot elongation and root elongation in the rooting media, healthy and well grown haploid, diploid and tetraploid plantlets of *E. purpurea* were removed from the tissue culture media and gradually acclimatized to outside environment. Acclimatized haploid, diploid and tetraploid plantlets of *E. purpurea* were transplanted to the pots containing soil in the experimental field at the Chinese Medicinal Plant Garden in South China Agricultural University and plants were selected for further identification of agronomic characteristics. Aftercare operations such as watering, fertilizing and applying insecticides were done as necessary.

Stomata analysis

For stomata analysis, a few pieces of epidermal layer were torn from the abaxial side of relatively mature leaves (leaf No. 4 or 5 from the top of the shoot) of haploid, diploid and tetraploid plants separately. These epidermal layers were then mounted on slide glass with one drop of distilled water and a piece of cover glass for measuring sizes. Stomata were observed under a microscope (Leica DLMB2).

Chromosome observation

Actively growing root tips of about 5-10 mm in length were excised from haploid, diploid and tetraploid plants. Cell samples of the root tips were observed for chromosomes under a microscope (Leica DLMB2), and photographs were taken with the associated apparatus.

Data Analysis

All the experiments were arranged in Randomized Complete Block Design (RCBD) and every experiment reported here was with of four replicates. Statistical analysis was carried out using the student Newman-Kuells means separation test of SAS (SAS Institute, Cary, NC, 1995). Significance of differences among means was determined by Duncan's multiple range testes at ≤ 5% level.

Results and Discussion

A plant with all the root tip cells showing 11 chromosomes was determined as haploid, a plant with some cells showing 22 chromosomes was determined as diploid and a plant with all the cells showing 44 chromosomes was determined as tetraploid. Simple methods for early identification of ploidy level in regenerated plants have important application value, especially, when a large amount of regenerated plants are to be identified. Besides, the most frequently used method of counting the visualized chromosome number in metaphase requires fine skill and is
time consuming but it gives the most indubitable results. It was observed that 4x cells visually displayed a greater nuclear area as compared to their diploid counterparts. Generally, chromosome counting in metaphasic cells is considered a reliable method for determining plant cell ploidy levels (Samford, 1983; Hamill *et al*., 1992).
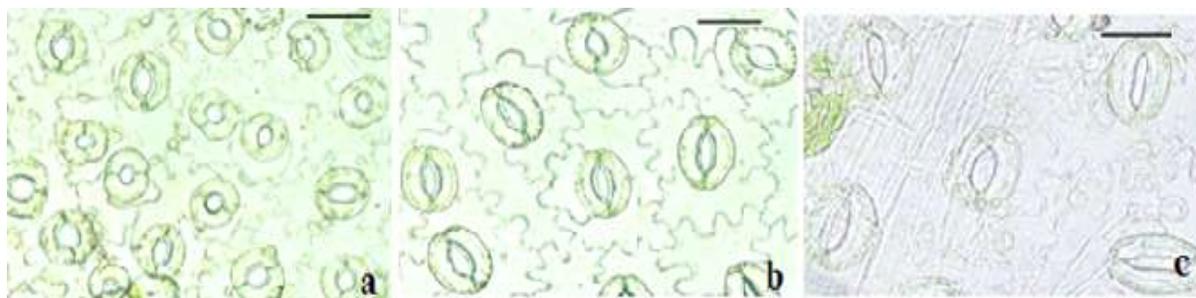
Figure 1. Stomata of *in vitro* grown *E. purpurea* plants
(a: Haploid plant, b: diploid plant, c:tetraploid plant Bar = 40 μm)

There were no prominent morphological differences of plant leaves that could be identified clearly either under *in vitro* or *in vivo* conditions. The sizes of haploid, diploid and tetraploid leaves *in vitro* were not significantly different. However, stomatal sizes were significantly different among the three types. Overall, tetraploids possessed longer and wider stomata (Figure 1). Leaves were taken from *in vitro* grown haploid, diploid and tetraploid plants which were confirmed by chromosome number to observe stomata. It was found that the stomata of the plants were widely open, and sizes of the stomata might vary largely even among closely situated ones for the three plants. Size of stomata on leaves varied largely even among those of the same leaf, however, statistically significant differences could be found among stomata sizes of haploid, diploid and tetraploid.

The average length of the stomata of diploid plants was 122.6 μm, while it was in those tetraploid plants and haploid plants were 137.59 μm and 72.41 μm, respectively (Table 1). Stomata analysis has been proved to be a simple and also a reliable method to determine the poidy level (Borrino and Powell, 1988; Portela de Carvalho *et al*., 2005). In tetraploids, the size of stomata and number of chloroplasts were almost double than those observed in diploids, while the frequency of stomata was lower. The utility of stomatal size in distinguishing plants with different ploidy levels has been used in other plant types (Hamill *et al*., 1992, Sildar and Jolly, 1994, Van Duren *et al*., 1996 and Chakraborti *et al*., 1980). Similar results obtained in this study where stomata on the lower surface of the tetraploid leaves were larger but fewer compared to that of diploids.

Evidently, the bigger mean size of cells observed in tetraploid (44 chromosomes) than that of diploid (22 chromosomes) whereas haploid (11 chromosomes) consisted of smallest cells (Figure 2). These results could be due to doubling of the chromosome number and similar observations have been reported earlier by Dwivedi *et al*., 1986. Increment of the chromosome number and polyploidization are remarkable characteristics in the evolution of plants (Yang, 2001).

Table 1. Comparison of stomata sizes among haploid, diploid and tetraploid plants

Ploidy level of plant	Stomata length (μm)	Stomata width (μm)
Tetraploid	137.59 a	106.66 a
Diploid	122.60 ab	97.59 a
Haploid	72.41 b	75.25 c

* Data in the same column followed by different letters are significantly
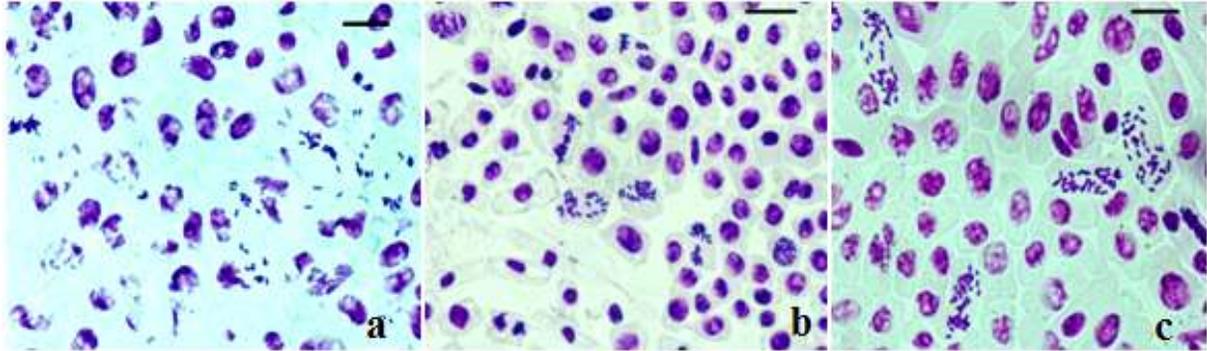different by Duncan's t-test at 95% level

Figure 2. Chromosomes in root tip cells of *E. purpurea* plants
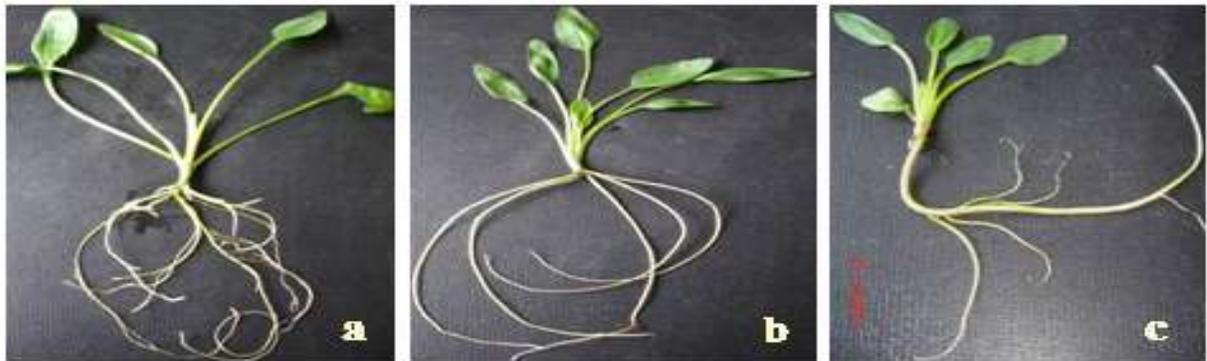a. Haploid (2n=11), b. Diploid (2n=22), c. Tetraploid (2n= 44)

Figure 3. Rooted plants before acclimatization
a. haploid plant, (2n=11); b. diploid plant, (2n=22); c. tetraploid plant, (2n=44)

Figure 4. Field grown five months old diploid (on the left), chimera and tetraploid (on the right) plants with inflorescence

Roots of diploids and tetraploids showed significant differences; roots of tetraploids were shorter, thicker and darker in colour with many lateral roots than those of diploids. Diploid plantlets generally initiated more roots from the base of the shoots, and they were slimmer and had fewer branches. The difference in the thickness of the roots could be distinguished as early as day 10-15, few days after growing out of adventitious roots (Figure 3). The finding of the difference in root morphology between plants of different ploidy levels can serve as a convenient and reliable method for identifying plants of a certain ploidy level in coneflower, and probably be applicable to other plant species as well. Tetraploid plants grew normally in the field and flowered within 5 months. There were no significant differences in flowering time among haploid, diploid and tetraploids (Figure 4).

Conclusion

Tetraploids of *E. purpurea* plant have longer and wider stomata. Clearly, bigger cells were observed in tetraploid (44 chromosomes) than diploid (22 chromosomes) and haploid (11 chromosomes) consisted of the smallest cells in root tip. Roots of tetraploids were shorter, thicker, darker colour and with many lateral roots than those of diploids. Diploid plantlets generally initiated more roots from the base of the shoots, and they were slimmer and had fewer branches while haploids had slimmer roots.

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