Tropical Agricultural Research & Extension 13(1): 2010

SEPARATION OF TETRAPLOID AND DIPLOID PLANTS FROM CHIMERAS IN *IN-VITRO* CULTURES OF PURPLE CONEFLOWER (*ECHINACEA PURPUREA* L)

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Accepted: *09th February 2010*

ABSTRACT

Doubling the chromosome number of diploid and haploid *Echinacea purpurea* plants have high applicable values for genetic improvements of the crop. Field experiments have shown that the tetraploid plants grow vigorously as compared to diploid plants. Tissue culture methodology provides a useful way to separate plant chimeras into their component genotypes. In general, mutated cells are difficult to monitor but mutations which result in a change in genome chromosome number are an exception, because chromosome number mutation can be identified by chromosome counting. In the present study, chimeric materials were used as explant source, and higher percentages of tetraploid shoots were induced from explants with higher ratio of tetraploid cells to diploid cells; explants possessing 26% tetraploid cells regenerated 10% tetraploid plants, explants possessing 15% tetraploid cells regenerated 4% tetraploid plants, and explants possessing 11% tetraploid cells regenerated 2% tetraploid plants. The reliability of the tetraploid nature of the regenerated plants, directly from colchicine treated culture and from chimeric materials was confirmed by regenerating buds again from explants of these plants, and amongst the six plants tested, five were confirmed to be true tetraploids that regenerated 100% tetraploid plants, and the rest one to be a chimera which regenerated 93% tetraploid plants. Results of the experiments indicate that *in vitro* culture method could provide a useful way to separate chimeras into individuals with one of the component cell genome numbers, and by this it could produce 100% pure tetraploids from chimera plants for further genetic studies of *Echinacea purpurea* L and for direct agricultural application.

Key words: Breeding, Chimera, Chromosome, Purple coneflower, Regeneration, Tissue culture

INTRODUCTION

Echinacea purpurea (Asteracea) has a deserved reputation for enhancing the human immune system (Barnes et al. 2005). Chimeras may arise when cells undergo mutations. These mutations could occur spontaneously or as a result of induced irradiation or treatment with chemical mutagens. Many ornamental plants with variegated leaves are chimeras, with the outer layer lacking chlorophyll. Separation of the chimera into component genotypes has been observed in *Ananas comosus* (Jones et al. 1974) and *Dracaena marginata* "Tricolor" (Chua et al. 1981).

This paper describes the production of tetraploid and diploid *E. purpurea* using mixoploid plants as starting material and the reliability of tetraploids which are directly produced by colchicine treatment or regenerated from explants of chimera materials.

MATERIALS AND METHODS

Establishment of aseptic seedlings

Surface sterilized seeds were cultured in a medium comprised of half-strength MS (Murashige and Skoog 1962) salts, 1% sucrose and 500mg/l lactalbumin hydrolysis. This medium was solidified with 0.2% phytagel prior to autoclaving at 1.4 kgcm^{-2} for 20minutes. Petioles of 2-month-old seedlings cut into 7-10mm long segments were used as explants.

Preparation of media

Shoot regeneration medium for culture of petiole explants comprised of MS salts, 3% sucrose, 0.3 mg/l BA, 0.01mg/l NAA. The media were adjusted to pH6.0 with 1N NaOH or 1N HCl solution, and gelled with 0.6%agar prior to autoclaving. Colchicine was dissolved in distilled water to a concentration of 5mg/l, filtered and sterilized before adding to warm (about 70 ^{0}C) autoclaved media.

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Paper presented at the 2nd National Symposium, Faculty of Agriculture, University of Ruhuna

Maintenance of cultures
Cultures were kept in a 12h photoperiod regime under cool-white light (about 50μmolm^{-2}s^{-1}). All cultures were kept in a same room at 25 - 27 ^{0}C.

Investigation of regenerated plantlets from chimera materials
From our earlier experiments six aseptic chimeric plants (3 plants each with chimeric percentage <4% and >10%) were selected as materials for investigating chromosome levels of plants regenerated from chimeras. Chimera petiole of explants were cut into approximately 5mm lengths and inoculated on to regeneration medium containing 0.3mg/l BA and 0.01mg/l NAA. After 40days the regenerated buds were transferred to the root induction medium. Actively growing root tips were excised and prepared for chromosome counting. According to the chromosome counts, plantlets were separated into diploid, tetraploid and chimera and the percentages of the newly produced diploid, tetraploid and chimeric plants were calculated for the mother chimeric plants.

Comparision of the reliability of tetraploid plants
Petiole explants obtained from the six *in vitro E. purpurea* tetraploid plants,(three of which were directly produced from colchicine treatments and the other three were regenerated from chimera plants), were cultured on MS medium containing 0.3mg/l BA and 0.01mg/l NAA for the regeneration of adventitious buds. Roots of plantlets recovered from these buds were sampled and the number of chromosomes of the root tip cells was counted for comparison of the reliability of the selected tetraploid plants.

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 diploid, tetraploid, and chimeric plants separately and mounted on glass slides with a drop of distilled water for measuring sizes and photography (microscope Leica DLMB2).

Data analysis
Statistical analysis was carried out using the student Newman-Kuells means separation test of SAS (SAS Institute 1995). Significant differences among means were determined by Duncan's Multiple Range testes at P≤0.05.

RESULTS AND ANALYSIS

Initiation of shoots from explants
As a number of mixoploid plants were identified from tetraploid and diploid plants regenerated after colchicines treatment, it could be possible to obtain tetraploids from these mixoploids by regenerating shoots from tetraploid cells of mixoploid explants. The medium used with a combination of 0.3mg/l BA and 0.01mg/l NAA induced the highest response (100%) of shoot regeneration. Explants inoculated on the medium formed noticeable callus at the cut surface in two weeks and the callus produced multiple shoots in another three weeks.

Chromosome level of regenerated plantlets from different chimeric plants
The regenerated shoots were induced to root, and the number of chromosome in the root tip cells were analyzed for clarifying the ploidy conditions of the regenerated plants from the chimeric materials (Plate 1). Tetraploid, diploid and again chimeric shoots were obtained from all mixoploids with different percentages of tetraploid and diploid cells. The highest percentage (10%) of tetraploid induction occurred in the chimera plant which possessed 25.9% tetraplois cells. Chimeric plants with 10.9% and 15.0% tetraploid cells generated lower percentages of tetraploids, 2% and 4%, respectively where were no tetraploid plants observed from chimeric plant materials which possessed lower percentages of tetraploid cells of no more than 4%. However, all chosen six chimeric plants again produced chimeric plantlets, displaying different tetraploid cell percentages (Table 1).

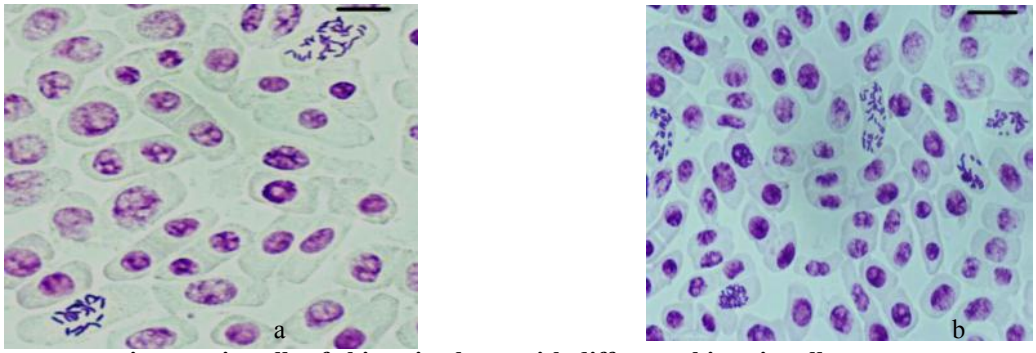

Plate 1. Chromosome in root tip cells of chimeric plants with different chimeric cell percentages
a, chimera with 2x 98% and 4x 2% cells; b, chimera with 2x 90% and 4x 10%

Table 1 Comparison on chromosome level of plants regenerated from materials with different chimeric levels

Chimeric level (% tetraploid cell)	Number of plants sampled	Number of root tip sampled	Number of cells observed	Number of cells with 2x chromosomes	Number of cells with 4x chromosomes	Number of each kind of plants on the base of chromosome counting		
						2x plants	4x plants	Chimeric plants
1.48	50	96	4034	4033	1	49	0	1(1/115/2)*
2.40	50	88	3343	3342	1	49	0	1(1/83/2)
3.37	50	92	3747	3743	4	48	0	2(4/196/4)
10.90	50	77	4276	4200	76	44	1 (59/59/1)	5(17/433/9)
15.00	50	92	3415	3289	126	41	2(109/109/4)	7(17/748/19)
25.90	50	88	3628	3335	294	35	5(249/249/10)	10(45/1026/22)

*No. of 4x cells/ No. of cells observed/ No. of root tips sampled, in all the plants examined

Table 2 Haploid, diploid and tetraploid cell ratio of chimera plants regenerated from haploid explants treated with different colchicines concentrations

Colchicine conc. (mg/l)	Treatment duration (days)	No. of chimera plants	In the regenerated chimera plants				
			No. of root tips sampled	No. of cells observed	% 1x cells	% 2x cells	% 4x cells
30	3	8	12	484	33.88	66.12	0
	9	11	15	423	11.35	66.43	22.22
	18	9	16	396	9.6	54.55	35.85
60	3	9	12	352	17.62	56.53	25.85
	9	15	28	769	10.14	71.00	18.86
	18	14	27	707	11.18	58.27	30.55
120	3	11	16	386	15.55	62.69	21.76
	9	14	21	460	23.7	47.82	28.48
	18	10	13	293	3.4	42	54.6

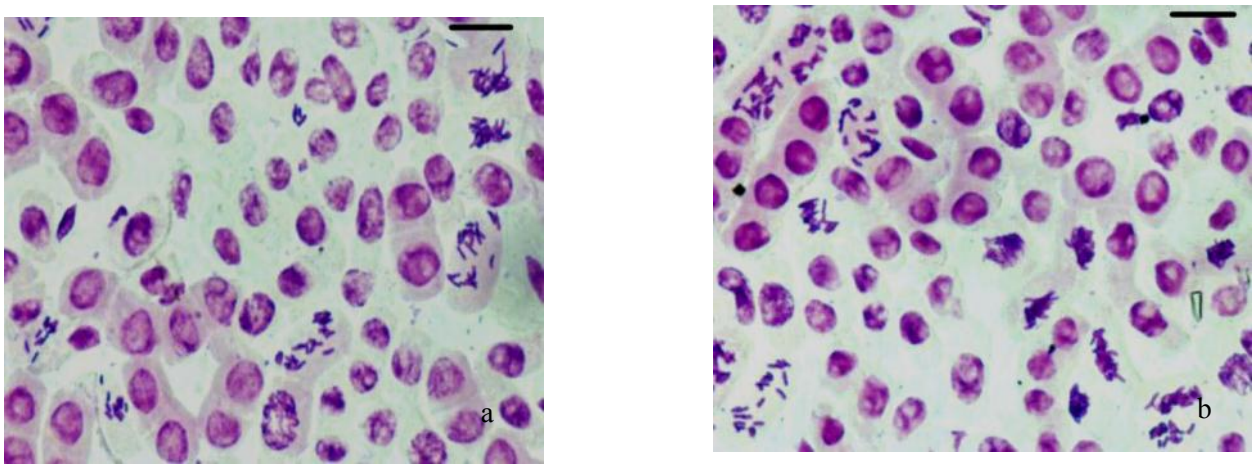

Plate 2. Chromosomes in root tip cells of *E. purpurea* chimeric plants: a- chimera with cells of 1x and 2x; b- chimera with cells of 1x, 2x and 4x

Chimera plants from haploid explants treated with different colchicine concentrations

Data in Table 2 indicate three kinds of chimeric plants; cells with x + 2x chromosomes, 2x + 4x chromosomes and x + 2x + 4x chromosome numbers. The ratio of x cells to 2x cells or 2x cells to 4x cells was generally high. Cells of different chromosome levels in a single root tip of two chimeric plants were shown in Plate 2.

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Stomata analysis

Samples were taken from *in vitro* grown haploid, putative diploid, putative tetraploid and the three different types of chimera plants which were confirmed by chromosome counting for observing stomata. Although deviation in the sizes of the stomata was evident even in the true haploid, differences in size among all types of non chimeric plants could still be detected through statistical analysis (Table 3, Plate 3). However, significant

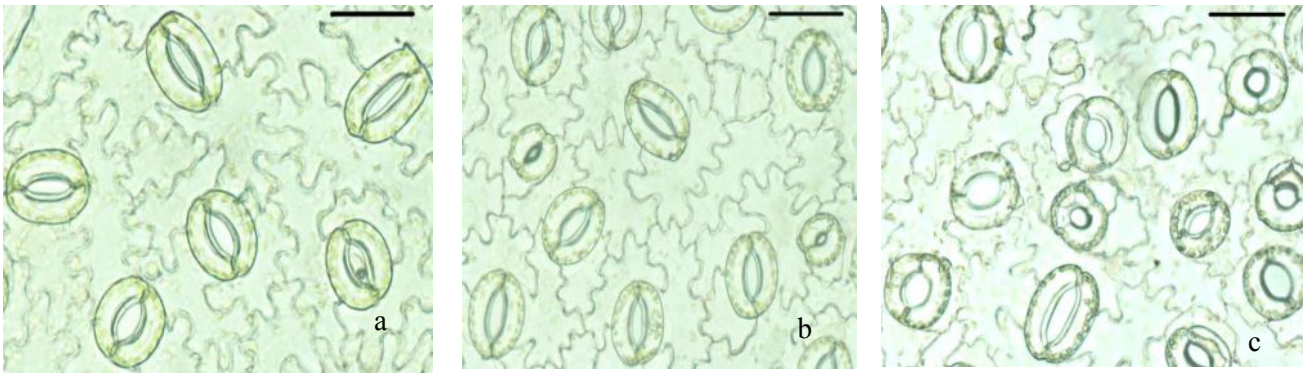

Plate 3 Stomata of *in vitro* grown *E. purpurea* chimeric plants regenerated from colchicines treated haploid materials a- chimera 2n=2x and 4x; b- chimera 2n=1x and 2x; c- chimera 2n=1x and 2x and 4x

variance in length and width of stomata of the three different types of chimeras were not detected.

Comparison of the reliability of tetraploid plants regenerated from different explant sources

Upon culture, explants taken from tetraploids which were produced from chimera 1, chimera 2 and chimera 3 regenerated only tetraploid buds, and explants taken from three tetraploid plants produced directly from colchicines treated diploid materials regenerated not only tetraploid but also 3.3% diploid plants and 3.3% chimeric plants. Results of this experiment indicated that it was almost certain that within the six plants selected, five of them were pure tetraploid and the other one was a chimera, however, the ratio of 4x to 2x cells in this chimera was high (Table 4).

DISCUSSION

Mixoploidy is a major problem in the recovery of tetraploids using *ex vitro* conditions, and the presence of mixoploids could confuse the situation in some cases. In comparison, *in vitro* methods may minimize the frequency of mixoploids by treating the plant materials under more favorable conditions, especially for longer duration to ensure the chromosomes in more cells to be doubled.

Table 3 Comparison of stomata size among haploid, diploid, tetraploid and chimera plants with various chimeric levels

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 d	75.25 c
Chimera (x:2x = 7:23)	99.35 c	83.03 bc
Chimera (2x:4x =25:6)	113.60 bc	105.52 a
Chimera (x:2x:4x =12:15:2)	106.70 bc	94.55 ab

* Data in a column followed by different letters are significantly different by Duncan's Test at ≤5% level.

In our study with lower (30mg/l) or higher (240mg/l) concentrations of colchicine for 30day treatment and 120mg/l for 14day treatment, substantial percentage of chimeras with various combinations of 2x and 4x cells were regenerated. Because the cell cycle for 2x and 4x cells is different, the ratio of 2x cells to 4x cells will change along with the growth of the plant, the stability of genomic chimeric plants is poor.

As a number of mixoploids had been identified in our early experiments, we considered it possible to obtain pure tetraploids from these mixoploids by regenerating shoots from tetraploid cells of chimeric petiole explants. Results of the experiments confirmed our hypothesis. It is a common phenomenon and also a problem that a high frequency of chimeras is often associated with colchicine treatment (Ackerman and Derman 1972; Schifino and Fernandes 1987). The present study indicates that chimeras can be separated by regenerating buds from chimeric explants, providing a big opportunity to obtain pure tetraploids from chimeras with 2x and 4x cells.

Tetraploid shoots were obtained from all mixoploids of 2x and 4x cells with an average tetraploid percentage of more than 10.9%, and the reliability of these plants to be of pure tetraploid nature were confirmed by regenerating buds from explants taken from these plants.

The leaf appearance of ornamental *Alocasia* plants produced from colchicine and oryzalin treatments was very variable, including deformed or asymmetric leaves (Nguyen *et al*. 2003). This may be due to the fact that, in chimeric plants, some portion of the leaf may be tetraploid and other parts diploid (Nguyen *et al*. 2003). The variability of the ploidy level of regenerated plants within the same treatment condition could be due to the stage of the cells that were responsible for the initiation of new

Table 4 Comparison of the reliability of tetraploid plants regenerated from chimeric explants and from colchicines treated diploid explants

Source of tetra-ploid material	Number of plants sampled	Number of root tip sampled	Number of cells observed	Number of cells with 2x chromo-somes	Number of cells with 4x chromo-somes	Number of plants with the indicated chromosome number		
						2x plants	4x plants	Chimeric plants
Chimera 1	30	54	1981	0	1981	0	30	0
Chimera 2	30	53	1806	0	1806	0	30	0
Chimera 3	30	55	1926	0	1926	0	30	0
Diploid 1	30	56	1915	0	1915	0	30	0
Diploid 2	30	55	1787	84	1703	1(41/41/2)*	28	1(5/48/2)
Diploid 3	30	55	1849	0	1849	0	30	0

shoots. Although a number of cells in meristem tissue may become polyploidized, many others may be unaffected and remain diploid. Thus, we can find the areas where both normal and polyploidized cells are intermixed (Dermen and Henry 1944; Pryor and Frazier 1968; Wan *et al*. 1989).

ACKNOWLEDGEMENT

This research was funded by a grant (No. 2008B090500250) from a special program for Enterprise, University and Research Institute Cooperation of Guangdong Province and the Ministry of Education of China.

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