Tropical Agricultural Research & Extension 19 (1): 2016

INDUCED POLYPOIDY UNDER *IN VITRO* CONDITIONS AND HIGHLY EFFICACIOUS SCREENING OF HEXAPLOIDS IN PURPLE CONEFLOWER (*Echinacea purpurea* (L.) Moench)

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Accepted: 15th June 2017

ABSTRACT

Leaf, petiole, and root explants from *in vitro* maintained triploid purple coneflower plantlets were treated with120 mg·L^{-1}colchicine for the induction of hexaploid plants. Best result for induction of hexaploid was obtained by treating the triploid root explants with 120 mg·L^{-1}colchicine for 25 days. Regeneration of adventitious buds from the triploid root explants on Murashige and Skoog (MS) medium with 6-Benzyladenine (BA) and Naphthalene acetic acid (NAA) after the colchicine treatment persisted for a time longer than from those untreated. Only slow-growing plantlets in a same genotype-and-treated population were selected and detected to increase the screening efficiency as well as save time and efforts. Chromosome counting confirmed that most early-regenerating and fast-growing buds were triploid, while most late-regenerating and slow-growing buds were hexaploid, screening only those slow-growing regenerated plantlets could increase effectively the hexaploid. In the present study, screening efficiency increased from 21% (detected all plantlets) to 53% (detected only the slow-growing plantlets). On the other hand, hexaploid plants had much larger stomata and more stomatal guard cell chloroplasts. The stomatal guard cell chloroplast number in hexaploid plants has a good linear relationship with those in the diploid, triploid, and the tetraploid progenitors. Results indicate that the colchicine-induced hexaploid could be induced and screened out with high efficiency, making this process worth further exploring in other species as well.

***Key words*:** chloroplast, *Echinacea purpurea*, hexaploid, polyploidy, screening, stomata

INTRODUCTION

As an internationally recognized immunoregulatory herb, purple coneflower (*Echinacea purpurea* (L.) Moench, has gained much attention throughout the world in the past decades (Barrett 2003; Bauer 1999).In China, the purple coneflower plant, its powder and the oral solution were certified by China's Ministry of Agriculture as the first class of new veterinary drugs in China (Certification Nos.: New Veterinary Drugs 2012- 23th, -24th, and -25th) (2012). Later, the purple coneflower root and root powder were certificated as the second class new veterinary drugs (Certificate Nos.: New Veterinary Drugs 2014-44th, and -45th) (2014). Plants are the resources of these purple coneflower products, hence the promulgations promoted demand of plants to increase rapidly and often this demand cannot be met. Growing well-selected clonal populations may help to enhance productivity because the pur-

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ple coneflower is cross-pollinated and plant grown from normal seeds are different and a large number of individuals may contain low concentrations of functional components. The differences between individuals could be large, for example, variation on *in vitro* regeneration abilities of explants from plants with different genotypes (Li, et al. 2013). Some efforts have been made on enhancing the *in vitro* regeneration and the rooting ability (Chen, et al. 2013). However, as at present there are no published reports on breeding of new varieties with improved medicinal value in purple coneflower till now.

Polyploidization is always an important force promoting evolution in plants (Liu, et al. 2014). It has been considered to stimulate the appearance of many unique characteristics, for example, the existence of greater vigor than that of the diploid progenitor (Comai 2005). Doubling the chromosome number may increase the contents of certain secondary metabolites in medicinal plants. To enhance productivity of the functional compounds in medicinal plants, polyploidization has been reported as a feasible strategy (Abdoli, et al. 2013; Berkov and Philipov 2002; Dhawan and Lavania 1996). As for purple coneflower, the normal chromosome number of purple coneflower is twenty-two (2N=2X=22) (Qu, et al. 2004). In previous studies, we have succeeded in obtaining haploid (2N=1X=11) (Zhao, et al. 2006), tetraploid (2N=4X=44) (Chen, et al. 2015; Nilanthi, et al. 2009), octaploid (2N=8X=88) (Nilanthi and Yang 2013), and triploid (2N=3X=33) (Chen, et al. 2014)plants through anther culture, colchicine treatment, and/or hybridization. Since the plants of purple coneflower with one, two, three, four, and eight gene dosages showed significantly different characteristics (Chen, et al. 2012; Chen, et al. 2015; Chen, et al. 2014; Chen, et al. 2014; Nilanthi, et al. 2009; Xu, et al. 2014),the triploid and tetraploid plants have been proved to contain higher concentrations of certain functional compounds, such as chicoric acid, and have higher economic value for cultivation than the diploid did (Abdoli, et al. 2013; Chen, et al. 2014; Xu, et al. 2014). As octaploid coneflower plantlets cannot survive in the natural environment easily (Nilanthi and Yang 2013), would the pentaploid and hexaploid ones be more viable? To answer this question, the creation of a hexaploid should be the first step, because the pentaploid can be obtained through a cross between tetraploid and hexaploid progenitors (Figure 1). Creating plants with more gene dosages, such as pentaploid and hexaploid, might help to reveal more relationships between gene dosage and some epigenetic characteristics.

Colchicine treatment is an efficient method to induce polyploidy. A certain concentration of colchicine could inhibit chromosome segregation during meiosis because of its action as a microtubule toxin. This chemical substance has successfully been used on *E. purpurea* to create tetraploid and octaploid plants (Abdoli, et al. 2013; Nilanthi, et al. 2009; Nilanthi and Yang 2013). Meanwhile, it could be poisonous to the cells and could kill the *in vitro* cultured explant if the concentration is too high or the treatment lasts too long. In earlier experiments, we compared the doubling effects of different concentrations of colchicine, and the results showed that 120 $mg \cdot L^{-1}$was a suitable concentration for doubling the chromosome number of purple coneflower (Nilanthi, et al. 2009; Nilanthi and Yang 2013).

In the present paper, details of obtaining hexaploid plants by treating *in vitro* derived triploid explants with colchicine was described. For example, efforts of selecting hexaploid could be significantly saved by only surveying the late-regenerating and slow-growing buds.

In addition, some interesting features of the hexaploid were discovered by comparing the hexaploid with the diploid, triploid, and tetraploid plants. Stomatal length and the chloroplast number in stomatal guard cells, for instance, could be predicted through calculation with equations once we knew the ploidy

level. By the same equations, the ploidy of a purple coneflower bud can be preliminarily estimated by measuring and analyzing its stomatal length or counting and analyzing the chloroplast number in stomatal guard cells. These discoveries may help the purple coneflower breeding and are worth being validated in other species.

MATERIALS AND METHODS

Materials

Originally diploid purple coneflower plants were grown in the field from seeds provided by the Company of Plantation Products (Norton, MA, USA). The diploid seeds were sterilized and then cultured under aseptic conditions in bottles with 40 ml MS medium to provide diploid seedlings. Diploid plants used in present study were obtained by *in vitro* propagation of one diploid seedling (Chen, et al. 2012). Tetraploid plants were obtained by *in vitro* treatment of diploid explants with colchicine, and plant regeneration was induced from these colchicine-treated explants (Nilanthi, et al. 2009). Triploid plants were generated by crossing diploid and tetraploid plants and growing the resulting seeds. The ploidy levels of these plants were confirmed by counting the chromosome numbers of root tip cells. Plantlets of diploid, tetraploid, triploid, and hexaploid genotypes were cloned by stimulating axillary bud proliferation (Chen, et al. 2012) from one individual plantlet, and the produced plants were used in the following experiments.

Preparation of explants

Leaves, petioles, and roots were isolated from *in vitro* maintained clones of intact diploid, triploid, and tetraploid plants. All explants were prepared by cutting: leaf explants were cut to about 0.6 cm^2, while petiole and root explants were cut to about 8 mm in length.

Preparation of medium

Each jar was filled with 40mL of medium and covered with a polycarbonate screw cap. The medium for pre-culture (Chen, et al. 2015) was Murashige and Skoog (MS) basal elements, 3% sucrose, 0.5 $mg \cdot L^{-1}$ BA, and 0.01 $mg \cdot L^{-1}$ NAA. The medium for colchicine treatment consisted of MS basal elements, 3% sucrose, 0.5 $mg \cdot L^{-1}$ BA, 0.01 $mg \cdot L^{-1}$ NAA, and 120 $mg \cdot L^{-1}$ colchicine (Nilanthi, et al. 2009; Nilanthi and Yang 2013).The medium for inducing adventitious bud formation from explants contained MS basal elements, 3% sucrose, 0.3, 0.7, 1.4$mg \cdot L^{-1}$ BA, and 0.01$mg \cdot L^{-1}$ NAA. The essential components of the medium for culture of the regenerated buds included MS basal elements, 3% sucrose, and 0.05$mg \cdot L^{-1}$ NAA. All media were gelled with 0.45% agar and sterilized by autoclaving at 1.4 $kg \cdot cm^{-2}$ for 20min.

Maintenance of cultures

All cultures were stored at 25°C–27°C. Cultures used for seed germination were first kept in darkness for 10 days and subsequently under a photoperiod of 12h light (about 50$\mu mol \cdot m^{-2} \cdot s^{-1}$)/12h darkness. Cultures for explant pre-culture, regeneration of adventitious buds, and rooting of the isolated buds were kept under a photoperiod of 12h light (about 50$\mu mol \cdot m^{-2} \cdot s^{-1}$)/12h darkness, where as those for the treatment with colchicine were not exposed to light. When the cultures lasted more than 50 days, they were sub-cultured and inoculated on to a newly made medium with the same components.

Confirmation of diploid, triploid, and hexaploid

Only plantlets without hyperhydricity (Chen, et al. 2014) were detected. The ploidy levels were confirmed by counting the chromosome numbers of root tips (Qu, et al. 2004). Three root tips in each plantlet were cut for detection. Ten days later, another three root tips from the same plantlet were cut for a second check. Detection was repeated after another ten days for samples in which observations on chromosome numbers were not clear, Suspected chimeras (Nilanthi, et al. 2010) were

not counted.

Data collection and analysis

Experiments were repeated at least twice with consistent results and in *in vitro* cultures at least 15 explants were inoculated. Data for adventitious bud formation were recorded 50 days later through one-time subculture to a fresh medium of the same composition. Cultures kept for more than 50 days were then subcultured every other month until the culture ended. Buds of at least 1 cm in height, without hyperhydricity (Chen, et al. 2014) and at least two visual leaves were considered as valid.

Survival rates: explants were initially cultured in the colchicine-containing agar-solidified medium for 19 and 25days and then were cultured for bud regeneration for 22 days before living explants were counted and survival rates were calculated. Regeneration rate: in the bud regeneration cultures, number of regenerated buds from each explants was counted. Data were collected 50 days after initiation of the bud regeneration cultures. Regeneration rate was calculated by the number of buds per explant.

Values of survival, regeneration, and hexaploid production rates, as well as those of stomatal length and chloroplast numbers in stomatal guard cells were expressed as means ± SE.

Statistical analysis of the data was carried out using the SPSS 19.0 software, and the significant differences among the means were determined by Duncan's Multiple Range Tests for more than two data sets, or by the independent sample *t*-test for two sets of data. Tests for more than two data sets, the differences were considered significant when values were less than 0.05.

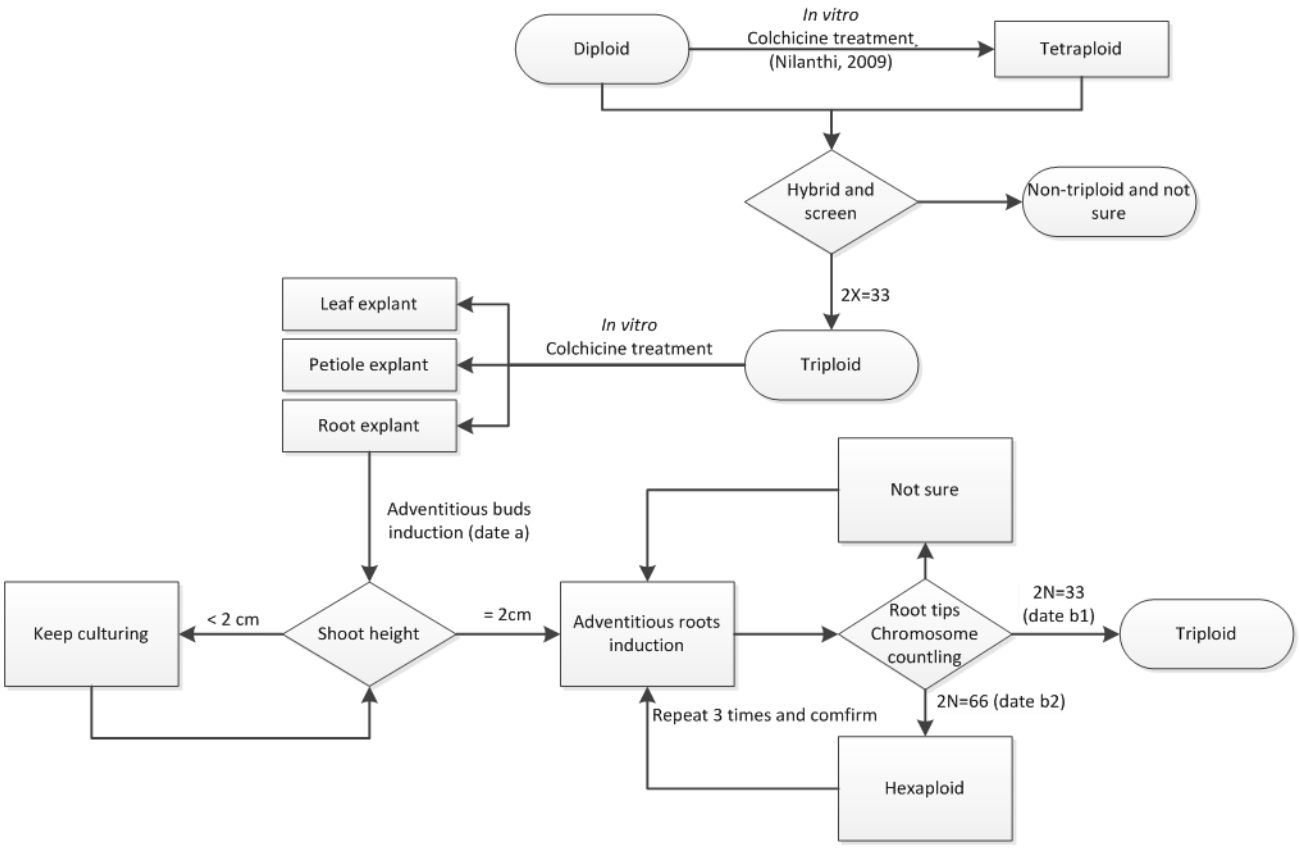

Figure 1. Technology road map of induction of hexaploid

RESULTS AND DISCUSSION

Survival status of triploid explants after treatment with colchicine

After the treatment with colchicine, the triploid root and petiole explants had a better chance of survival than did the leaf explants. No shoots were regenerated from either of these explants after the application of 120 $mg \cdot L^{-1}$ colchicine for 19 or 25 days, and the subsequent culture on MS medium with 0.4 $mg \cdot L^{-1}$ BA and 0.01 $mg \cdot L^{-1}$ NAA for 22 days. Approximately 20% leaf explants turned yellow and died, while almost all petiole and root explants survived (Table 1).

Regeneration rates of explants from different root parts treated with colchicine

Root sections from different parts of healthy roots were cut into pieces of about 8 mm in length. All root sections were initially treated with 120 $mg \cdot L^{-1}$colchicine for 25 days and then were cultured on a MS medium with 0.7 $mg \cdot L^{-1}$ BA and 0.01 $mg \cdot L^{-1}$ NAA. Regeneration rates were calculated 50 days after culture.

Compared with root explants obtained from the proximity of root tips (including the root tip itself) and the ones isolated near the rhizome, explants from the intermediate section of roots regenerated a considerably higher number of shoots (Table 2). However, there were no significant differences among them (as determined by Duncan's multiple range test at $P<0.05$ level).

Confirmation of diploid, triploid, and hexaploid purpurea

The ploidy levels were confirmed by counting the chromosome numbers of root tips. As seen in Figure 2A, the diploid had 22 chromosomes, and the triploid and the hexaploid contained 33 and 66 chromosomes, respectively.

Table 1. Survival status of triploid explants after colchicine treatment

Explants	On colchicine-free medium		On medium with 120 $mg \cdot L^{-1}$ colchicine			
	For 25 days		For 19 days		For 25 days	
	Explants No.	Survival rates (%)	Explants No.	Survival rates (%)	Explants No.	Survival rates (%)
Leaf	15	100.0	15	80.0±10.70b*	15	73.33±11.82b
Petiole	15	100.0	15	100.0a	15	93.33±6.67ab
Root	15	100.0	15	100.0a	15	100.0a

*Values of survival rate calculated 22 days after the treatment are expressed as the mean ± SE. Data in the same column followed by different letters are significantly different, as determined by the Duncan's multiple range test at $P<0.05$ level. (n =15)

Table 2. Regeneration rate of root explants from different position treated with 120 $mg \cdot L^{-}$

Root sections	Explants No.	Total shoot No.	Shoots No. per explant
Proximity of root tip (including the root tip)	21	3	0.14±0.078a*
Intermediate section	20	8	0.40±0.11a
Near rhizome	20	6	0.30±0.11a

*Values of No. of shoots per explants are expressed as the mean ± SE. Data in the same column followed by same letter are not significantly different, as determined by Duncan's multiple range test at $P<0.05$ level.

Effect of BA on the induction of shoots in root explants of E. purpurea after colchicine treatment

Table 3 shows that the largest amount of regenerated shoots, 0.38 shoots per colchicine-treated root explant, were obtained when the explants were inoculated on MS medium with 0.7 mg·L^{-1} BA and 0.01 mg·L^{-1} NAA. Almost no buds regenerated when the colchicine-treated triploid explants were cultured on MS medium supplemented with 0.3 mg·L^{-1} BA and 0.01 mg·L^{-1} NAA. Similar results were achieved when the concentration of BA was 1.4 mg·L^{-1}.

Effect of the duration of colchicine treatment at a concentration of 120 mg·L^{-1} on the hexaploid ratio of E. purpurea

Data in Table 4indicate that root explants treated with 120 mg·L^{-1} colchicine for 19days manifested a hexaploid ratio of 6.78%.The hexaploid ratewas4 times higher(34.38%)and significantly different ($P<0.05$) when explants were treated for 25 days.

Relation between growth speed and hexaploid selecting ratio of E. purpurea

Once the 25-days-long colchicine-treatment was finished, all root explants were trans-

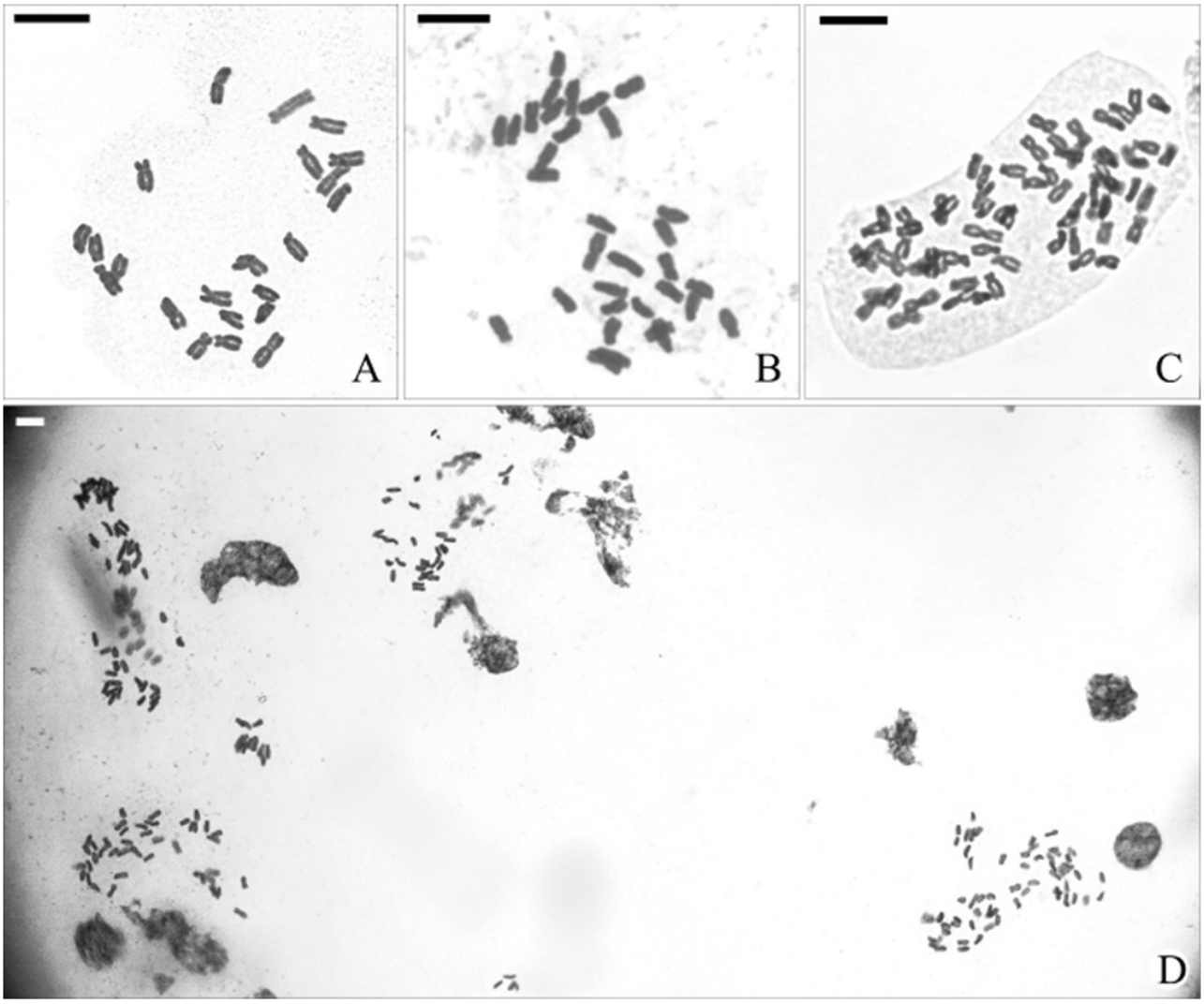

Figure 2A. Chromosome number counting of root tip cells for confirmation of the ploidy level of diploid, triploid and hexaploid *E.purpurea* plantlets. A: Diploid; B: Triploid; C and D: Hexaploid; Bar=10 µm.

ferred onto the MS medium with 0.7mg·L^{-1} BA and 0.01 mg·L^{-1} NAA to induce bud regeneration. When shoots were regenerated and grew to1 cm in height, they were shifted to an MS medium with 0.05mg·L^{-1} NAA for induction of roots. After roots reached 2 cm in length, their root tips were cut and chromosome numbers in the root tip cells were counted to identify ploidy of the plantlets.

Figure 2B shows the accumulation of triploid and hexaploid plantlets as number of days in culture increased. The slope of data fitting of the triploid plantlets (y_1 in Figure 2A) was higher than the one of the hexaploid plantlets (y_2 in Figure 2A). In other words, hexaploid plantlets accumulated more slowly than triploid plantlets (Figure 2A). However, the proportion of the hexaploid plantlets increased

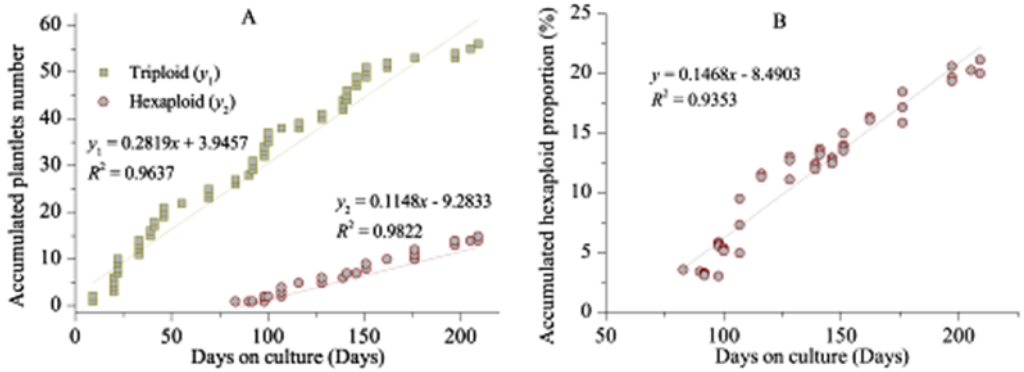

Figure 2B. Data fitting of the accumulation of triploid and hexaploid plantlets. A: Total number of triploid and hexaploid plantlets; B: Proportion of hexaploid plantlets.

Table 3. Effect of BA on the induction shoots in root explants of *E. purpurea* after colchicine treatment. [1]colchicine.

BA concentration (mg·L^{-1})	Explants No.	Shoots No.	Shoots No. per explant
0.3	31	0	0.00±0.00b*
0.7	34	13	0.38±0.10a
1.4	27	4	0.15±0.07b

*The values of shoot Nos. per explants are expressed as the mean ± SE. Data in the same column followed by different letters are significantly different, as determined by Duncan's multiple range test at P<0.05 level.

Table 4. Effect of the duration of colchicine treatment (120 mg·L^{-1}) on the hexaploid ratio of *E. purpurea.*

Duration (days)	Plantlets No.	Triploid plantlets No.	Hexaploid plantlets No.	Hexaploid rate (%)
19	59	55	4	6.78±3.30b*
25	32	17	11	34.38±8.53a

*Values of the hexaploid ratio are expressed as the mean ± SE. Data in the same column followed by different letters are significantly different, as determined by the independent sample t-test at P<0.05 level.

(Figure 2B). At the end of the period of culture, hexaploid plantlets accounted for 21.13% of all surveyed plantlets (Figure 2B, Table 5).

Table 5 indicates that almost all of the fast growing plantlets were triploid. Their root(s) grew to 2 cm in length within 50 days. On the other hand, 53.33% of the slow-rooting plantlets were hexaploid. They took more than 150 days to complete the same process.

Morphological observation of hexaploid. purpurea

The hexaploid purple coneflower plants were obviously different from the diploid, triploid, and tetraploid ones. It formed small, fragile, and thick leaves and remained in the vegetative growing stage for at least one year. The whole plant was short, small, and developed slowly (Figure 3).

Stomatal observation of hexaploid

The shapes of stomata of the hexaploids were similar to those of the diploid, triploid, and tetraploid. The stomatal frequency of hexaploid was $42mm^{-2}$ and significantly lower than that of diploid, triploid, and tetraploid (Table 6, Figure 4A, 5);furthermore stomatal frequency of diploid, triploid, tetraploid, and hexaploid plants highly conformed to a curve fitting, described by the formula $y=14.868x^2-$

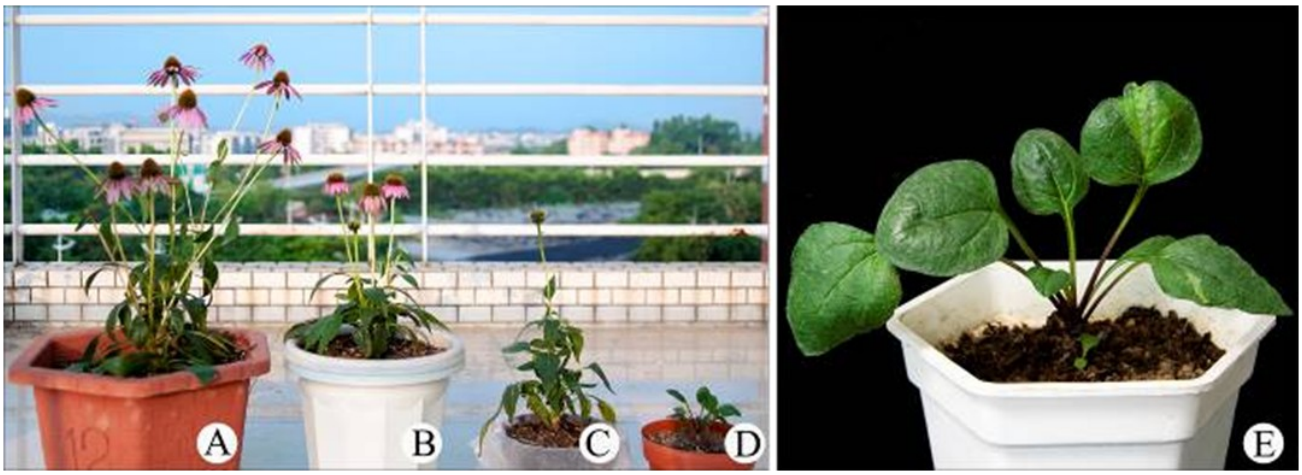

Figure 3. Morphological observation of six-month-old hexaploid *E. purpurea* plants. A-C: diploid, triploid, tetraploid, and D and E, hexaploid during early summer

Table 5. Relation between growth period and hexaploid ratio *of E. purpurea*

Days on culture	Surveyed plantlets No.	Triploid plantlets No.	Hexaploid plantlets No.	Hexaploid rate (%)
0–50	21	21	0	0.00±0.00c
51–100	18	16	2	11.11±7.62bc
101–150	17	12	5	29.41±11.39ab
151–210	15	7	8	53.33±13.33a
Total (0–210)	71	56	15	21.13±4.88bc

*Values of the hexaploid ratio are expressed as mean ± SE. Data in the same column followed by different letters are significantly different, as determined by Duncan's multiple range test at $P<0.05$

157.44x+ 452.6, R^2=0.9997 (Figure 4A). Meanwhile, stomata of hexaploid were significantly longer than those of the diploid, triploid, and tetraploid plants (Table 7, Figs. 4B, 5).Stomatal length of diploid, triploid, tetraploid, and hexaploid plants also highly conformed to a curve fitting, described by the formula y=1.144x^2–3.69x + 47.4, R^2=0.9986 (Fig.4B). Under the same culture conditions (temperature, humidity, and light intensity) and at the same time point, the stomatal aperture of the hexaploid was obviously narrower than those of diploid, triploid, and tetraploid plants (Figure 5).

Chloroplast numbers in stomatal guard cells

The hexaploid contained approximately 27 chloroplasts in each guard cell of the stomata, which was significantly more than did the diploid, triploid, and tetraploid. One layer of chloroplasts was present in the diploid guard cells, while one and a half layers were available in those of the triploid. In the guard cells of the tetraploid, two chloroplast layers were detected, whereas there were three or more in those of the hexaploid (Table 8, Figure 6). The chloroplasts number in each guard cell of stomata of the diploid, triploid, tetraploid, and hexaploid highly conformed to a linear fitting, represented by the formula y=3.86912x + 4.28273, R^2=0.9994 (Figure 7).

The present results evidenced that root explants may be a good material for chromosome doubling because of their high survival rate (100%) after treatment with colchicine at a certain concentration and duration of exposure. In our former experiments, we realized that explants from roots could be suitable for

Table 6. Stomatal frequency of diploid, triploid, tetraploid, and hexaploid *E. purpurea*

Ploidy	Area No.	Total area (μm^2)	Total stomata No.	Stomatal frequency (mm^{-2})
Diploid	5	5761341	1067	197.76±33.17a*
Triploid	5	3146428	343	112.57±7.31b
Tetraploid	5	9523832	561	61.86±12.91c
Hexaploid	10	10833644	459	43.01±1.84c

*Values of stomatal frequency are expressed as the mean ± SE. Data in the same column followed by different letters are significantly different, as determined by Duncan's multiple range test at P<0.05 level. (for diploid, triploid, and tetraploid, n =5; for hexaploid, as the stomatal frequency was low, we surveyed more areas, n =10)

Table 7. Stomatal length of diploid, triploid, tetraploid, and hexaploid *E. purpurea*

Ploidy	Surveyed stomata No.	Stomatal length (μm)
Diploid	230	44.59±0.16d
Triploid	230	46.06±0.36c
Tetraploid	230	50.94±0.11b
Hexaploid	230	66.37±0.26a

*Values of stomatal length are expressed as mean ± SE. Data in the same column followed by different letters are significantly different, as determined by Duncan's multiple range test at P<0.05 level. (n =230)

micro propagation due to their excellent regeneration ability, and the achieved value of 100% regeneration rate and 1.75 shoots per explants (Nilanthi, et al. 2009). Considering the combination of a great survival rate and strong regeneration ability, we surmise that the root could be the best explant source for inducing chromosome doubling.

As there were no significant differences among the regeneration rates of root explants, including that of root tip, middle part, or near rhizome, sections from any part of healthy roots could be used as explants for duplication of the chromosome number.

Colchicine was utilized at 120 mg·L^{-1}in the chromosome doubling treatment, because this concentration had been proven suitable for the treatment of explants of tetraploid and octaploid purple coneflower (Nilanthi, et al. 2009; Nilanthi and Yang 2013).The longer treatment duration (25 d) resulted in a significantly higher hexaploid rate (64%), while the shorter one (18 d) led to achieving a lower chromosome doubling rate (7%).
After the treatment with 120 mg·L^{-1} colchicine, the explants were transferred to a medium containing 0.7 mg·L^{-1} BA. Either the lower concentration of 0.3 mg·L^{-1}or the higher concentration of 1.4 mg·L^{-1}of BA caused a reduction in rate of induction of adventitious bud regeneration.

When hexaploids were screened out among all regenerated plantlets by root-tip chromo-

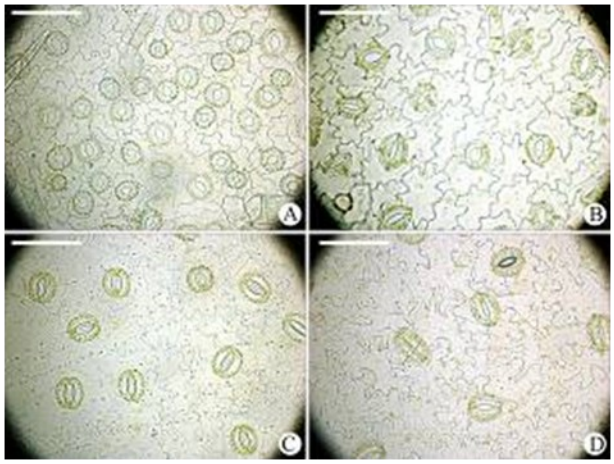

Figure 5. Stomata of hexaploid *E. purpurea*. A: Diploid; B: Triploid; C: Tetraploid; D: Hexaploid; Bar=100 μm.

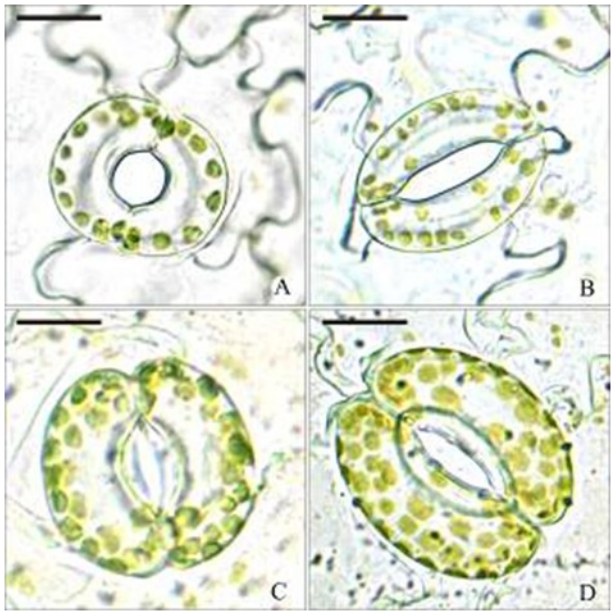

Figure 6. Chloroplasts in stomatal guard cells of *E. purpurea*(40×). A: Diploid; B: Triploid; C:Tetraploid; D:Hexaploid; Bar=20 μm.

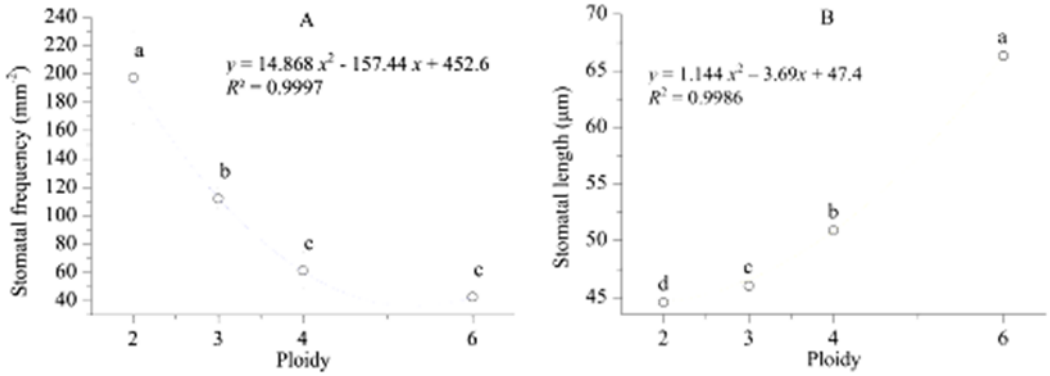

Figure 4. Data fitting of stomatal frequency (A) and stomatal length (B) of *E. purpurea*.

some counting, it was found that the slow-growing shoots, which needed more than 150 days to reach1 cm of height and had 2-cm-long root(s), were more likely to be hexaploid (53.33% of the slow-growing shoots were hexaploid). On the other hand, the most fast-growing shoots, which gained1 cm of height and developed 2-cm-long root(s) within 50 days, were triploid (almost 100% of the fast-growing shoots were triploid). Counting the chromosome number of all regenerated plantlets without differentiating them into fast- and slow-growing groups, leads to a waste of time for screening, since hexaploids may not at all might regenerated during the first 50 days. The triploid plantlets always increased faster than the hexaploids, but the proportion of the hexaploid plantlets still increased with time. Making good use of the fact that hexaploids grow more slowly than their mother triploid plants, the hexaploid individuals could be selected more easily, and a lot of time could be saved (Figure 8).

Morphological and phonological observation of hexaploid plants in present study, showed that they may exhibited a reduced growth speed in comparison with those their diploid and triploid relatives. According to our previous observations, the volume of pollens of the diploid, triploid, and tetraploid highly conformed to a linear fitting (Chen, et al. 2014). The volume of pollens of the hexaploid can be predicted to amount 28261.79 μm^3on the average. Although an octaploid plant was produced with the highest gene dosage level of purple coneflower ever obtained, it could hardly grow under normal conditions (Nilanthi and Yang 2013).It is possible that the hexaploid plant can reach a level of maturity enabling it to flower, because the tetraploid can grow quite normally (Abdoli, et al. 2013; Chen, et al. 2014; Chen, et al. 2014; Xu, et al. 2014). Hexaploidy, as a polyploidy level, may contribute to the accumulation of medical chemicals in concentrations higher than those in the diploid, triploid, and tetraploid (Xu, et al. 2014).

In our earlier studies, the triploid and tetraploid flowers similar to the diploid, but

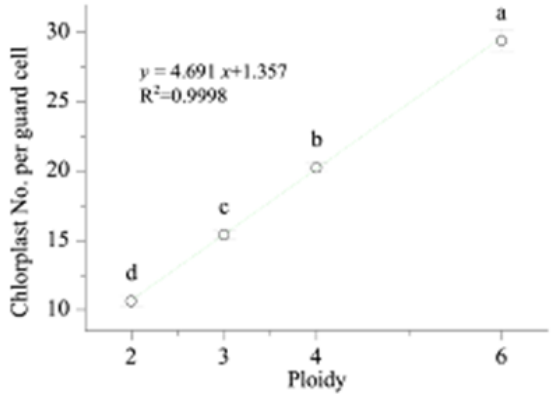

Figure 7. Data fitting of chloroplasts in stomatal guard cells of *E. purpurea*. Chloroplast numbers in stomatal guard cells

Table 8. Chloroplasts in stomatal guard cell of hexaploid *E. purpurea.*

Ploidy	Stomatal guard cell No.	Total chloroplast No.	Chloroplast No. per guard cell
Diploid	30	320	10.67±0.44d
Triploid	30	463	15.43±0.32c
Tetraploid	30	608	20.27±0.34b
Hexaploid	30	883	29.43±0.77a

*Values of the chloroplast No. per guard cell are expressed as mean ± SE. Data in the same column followed by different letters are significantly different, as determined by Duncan's multiple range test at $P<0.05$ level. (n=30)

they had a certain extent of abnormal meiosis of the pollen mother cells, and a small but stable percentage of very tiny pollens were formed (Chen, et al. 2014). What would happen in the meiosis of hexaploid pollen mother cells? May more aneuploidy pollens be yielded? It may also be interesting to investigate the performance of the hexaploid plants when crossed with the tetraploid purple coneflower plants and/or with other ploidy levels– providing that the hexaploid plants can grow to the flowering stage.

From the results obtained in the present investigation, it is also noteworthy that the stomatal frequency, stomatal length, and number of chloroplasts in the guard cells of stomata of hexaploids could be easily predicted, because those data followed certain simple rules. The volume of pollens, stomatal length, and chloroplast number in stomatal guard cells usually varied among the different levels of polyploidy in one spices. The pollen volumes of the diploid, triploid, and tetraploid purple coneflower highly conformed to a linear fitting (Chen, et al. 2014), and so did the stomatal length and of the chloroplast number in the stomatal guard cells of the diploid, triploid, tetraploid, and hexaploid plants. On one hand, the stomatal frequency complied with a curve fitting model associated with the ploidy level. We noticed that the fitting curve may be inaccurate if it is used to predict the stomatal frequency of pentaploids, a ploidy that has not yet been achieved. For obtaining a pentaploid plant, the most direct way might be crossing a hexaploid with a tetraploid. Considering that a hexaploid still could not be grown to bloom,

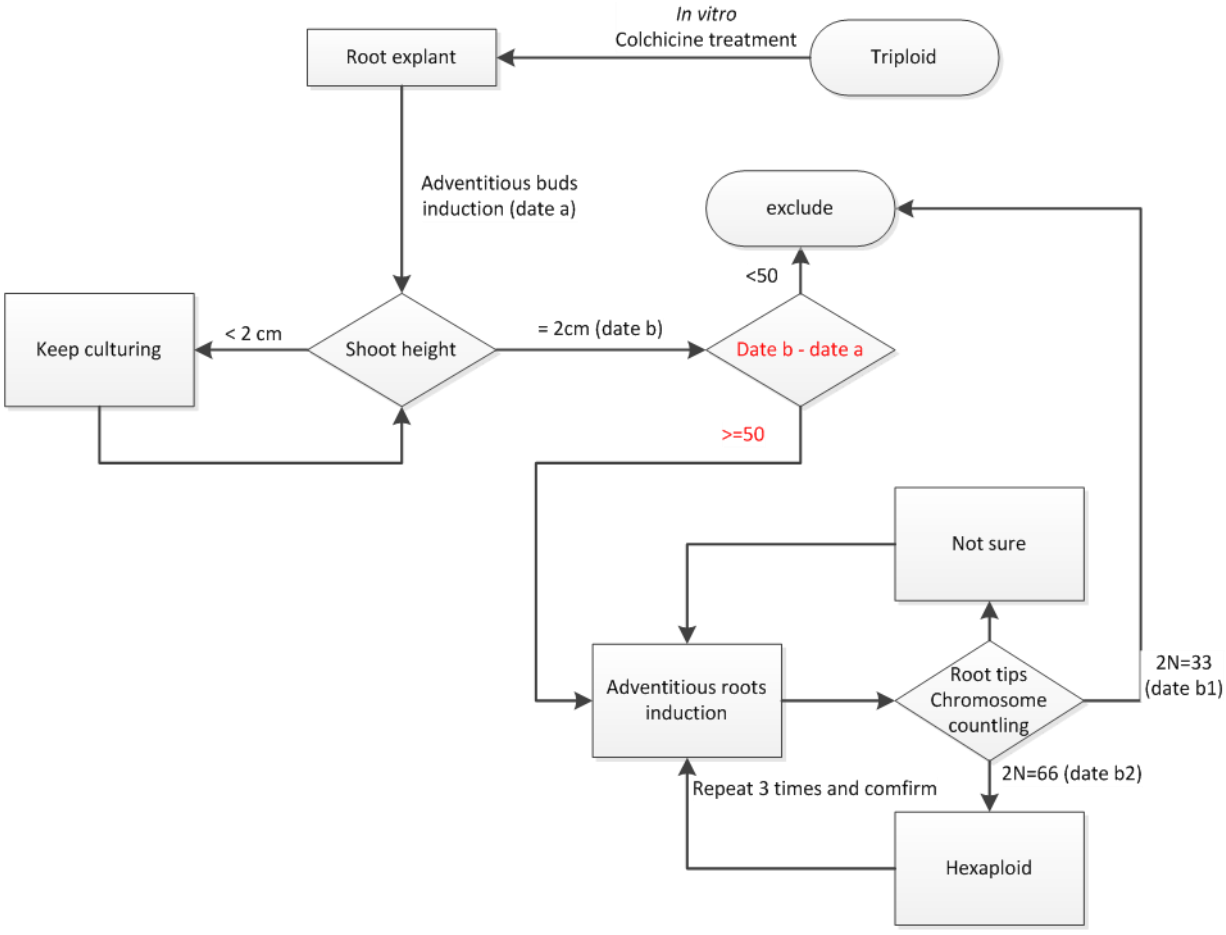

Figure 8. Protocol of screening hexaploid with higher efficiency

obtaining a pentaploidis difficult. On the other hand, because the stomatal length and chloroplast number in stomatal guard cells conformed to line fittings with the ploidy, the operation of the longer, lower-populated, and less opened stomata may result in a diminished gas exchange capability of the hexaploid leaves. The function of the guard cell chloroplast in stomatal responses has been considered to be related with stomata regulation and stomatal opening; however, this is still a subject of debate. The above results might help to understand the effects of polyploidy on photosynthesis (Warner and Edwards 1993).

To sum up, although the hexaploid purple coneflower may have had a poor economic value of cultivation until now, it could be valuable for completing the germplasm pool and exploring gene-dosage effects. The present study may help to accelerate the process of obtaining more hexaploid purple coneflower germplasm. As germplasm accumulated, some hexaploid genotypes perform better, such as to grow faster and to be able to bloom might be discovered. In addition, results of this examination may offer some tips for chromosome doubling of other ploidy levels and/or in other plant species and may be useful in producing plants with special water use efficiencies, capable of resisting the abnormal climatic changes (Lawson 2009).

CONCLUSION

Root explants of triploid *Echinacea purpurea* (L.) Moench is the best explant to produce hexaploid plants.120 $mg \cdot L^{-1}$ colchicine for 25 days resulted in a significantly higher hexaploid rate (64%), while the shorter one (18 d) led to achieving a lower chromosome doubling rate (7%). Hexaploids grow more slowly than their diploid and triploid relatives. Hexaploid plants had much larger stomata and more stomatal guard cell chloroplasts compare to mother triploid plants.

ACKNOWLEDGEMENT

This work was supported by, the Science and technology cooperation projects of Hainan Province, China (KJHZ2015-15), the Natural Science Fund of China (81603245), and the Science and Technology Planning Project of Guangdong Province, China (2016A020210075).

AUTHOR CONTRIBUTIONS

XLC: Experiments, interpretation of data, wrote the manuscript andreferences management. DLL: Experiments,interpretation of data,and modify the manuscript. JJZ,Rong Chen,andQing-Ling Li:Experiments. DN: Revising the manuscript critically for important intellectual content.YSY: Conceived and designed the experiments, revising the manuscript critically for important intellectual content, and overall responsibility. HW: Conceived the experiments; provided purple coneflower seeds and greenhouse used in the experiments.

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