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In Vitro Tetraploid Induction from Leaf and Petiole Explants of Hybrid Sweetgum (*Liquidambar styraciflua* × *Liquidambar formosana*)

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Abstract: *Liquidambar* is an important forestry species used to generate many commercial wood products, such as plywood. Inducing artificial polyploidy is an effective method to encourage genetic enhancements in forestry breeding. This report presents the first in vitro protocol for the induction of genus *Liquidambar* tetraploids based on the established in vitro regeneration system of hybrid sweetgum (*Liquidambar styraciflua* × *Liquidambar formosana*). The leaves and petioles from three genotypes were pre-cultured in woody plant medium (WPM) supplemented with 0.1 mg/L thidiazuron (TDZ), 0.8 mg/L benzyladenine (BA), and 0.1 mg/L α-naphthalene acetic acid (NAA) for a variable number of days (4, 6 or 8 days), and exposed to varying concentrations of colchicine (120, 160, 200 mg/L) for 3, 4 or 5 days; the four factors were investigated using an orthogonal experimental design. Adventitious shoots were rooted in 1/2 WPM medium supplemented with 2.0 mg/L indole butyric acid (IBA) and 0.1 mg/L NAA. The ploidy level was assessed using flow cytometry and chromosome counting. Four tetraploids and nine mixoploids were obtained from the leaves. Pre-treatment of the leaves for 8 days and exposure to 200 mg/L colchicine for 3 days led to the most efficient tetraploid induction. Producing 11 tetraploids and five mixoploids from petioles, the best tetraploid induction treatment for petioles was almost the same as that with the leaves, except that pre-culturing was required for only 6 days. In total, 15 tetraploids were obtained with these treatments. This study described a technique for the induction of tetraploid sweetgum from the leaves or petioles of parental material. Based on the success of polyploid breeding in other tree species, the production of hybrid sweetgum allotetraploids constitutes a promising strategy for the promotion of future forestry breeding.

Keywords: chromosome doubling; sweetgum; allotetraploid

1. Introduction

Liquidambar styraciflua, belonging to the genus *Liquidambar*, is found widely in the southern regions of the United States. *L. styraciflua* has become one of the most important commercial hardwoods in the United States [1,2], because it has a fast growing rate and provides many useful materials such as wood, plywood, pulp and paper production [2]. *Liquidambar formosana*, mainly distributed in East Asia, is an important tree in China owing to its fast-growing properties and use in timber and medicinal production and landscaping [3]. Interestingly, *L. styraciflua* can be interfertile with *L. formosana* [4,5]. Due to the potential of heterosis from interspecies hybridisation, hybrid sweetgum can show robust growth [5].

Allopolyploids are the products of merging two or more genomes by intraspecific or interspecific hybridisation [6,7]. Allopolyploid breeding has been applied successfully in forestry due to its advantages of high biomass and fitness. In vitro regeneration using the reproductive organs of woody plants has been successful in many species. Induction of tetraploidy has been successful in a number of species, including *Populus* [8,9], *Paulownia tomentosa* [10], and Citrus [11]. Unlike meiotic (sexual) chromosome doubling, in vitro asexual polyploidy breeding is not limited by season, and it benefits from a low mixoploids-inducing rate; it has been applied widely in forestry breeding.

Successful induction of tetraploids has been achieved using various chemical reagents, such as colchicine [12], oryzalin [13], and trifluralin [14]. Colchicine is the most widely applied chemical for in vitro tetraploid induction. Moreover, many explant types have been used as materials for in vitro polyploidy induction, such as leaves [8], petioles [15], calluses [12], shoots [16], hypocotyl segments, and cotyledonary nodes [17]. Although leaves and petioles are not used commonly as explants for tetraploid induction, they exhibit a high tetraploid-inducing rate and low number of mixoploids, indicating that they can be used for efficient polyploidy breeding [9,18]. We have improved the media that were applied to the in vitro regeneration of *L. styraciflua* [19,20] and *L. formosana* [21], and the modified medium was suitable for establishing an efficient regeneration system from leaves and petioles of hybrid sweetgum (*L. styraciflua* × *L. formosana*). Therefore, it is possible to acquire in vitro colchicine-induced tetraploid sweetgum.

This study describes an efficient method for the in vitro induction of tetraploids. First, we established multiple hybrid sweetgum genotypes. Then, we investigated the effects of explant type, explant genotype, time of pre-incubation, time of colchicine treatment and concentration of colchicine using an orthogonal experimental design. The tetraploids were determined by flow cytometry and chromosome counting. We hope that the biomass, resistance and ornamental value of *Liquidambar* will be improved by chromosome doubling after in vitro colchicine treatment.

2. Materials and Methods

2.1. Plant Materials

Floral branches of the male parent (*L. formosana*) were collected from five genotypes at Shanghai Chen Shan Botanical Garden (Songjiang District, Shanghai, China); pollen was collected; the same volume of pollen was measured and they were mixed together. In April, the male inflorescences of *L. styraciflua* were removed, and controlled pollination was applied to acquire hybrid seeds by pollination with the pollen of *L. formosana*. *L. styraciflua* was grown at the Shanghai Chen Shan Botanical Garden (Songjiang District, Shanghai, China). The fruits were collected in mid-September 2015, and the seeds were stored at 4 °C.

2.2. Establishment of Aseptic Seedlings and In Vitro Multiplication

Plump seeds were selected and sterilised in 75% ethanol (v/v) for 45 s, rinsed once with sterile distilled water followed by 2% sodium hypochlorite for 8 min, and then washed three times with sterile distilled water. The seeds were added to basal woody plant medium (WPM) supplemented with 4 g/L agar and 2 g/L polygel, and 30 g/L sucrose (pH 5.8–5.9) in a 9-mm culture dish; no phytohormone

was added; in this experiment, all media were semi-solidified. After 20 days, the germinated seedlings were placed in magenta boxes with 50 mL basal WPM [22]. A total of 150 plant genotypes were sub-cultured in rooting medium containing half-strength WPM medium supplemented with 2.0 mg/L indole butyric acid (IBA) and 0.1 mg/L naphthalene acetic acid (NAA). Regeneration medium for leaves and petioles consisted of WPM medium with 0.1 mg/L TDZ, 0.8 mg/L benzyladenine (BA) and 0.1 mg/L NAA supplemented with 2 g/L agar and 4 g/L double coagulation, and 30 g/L sucrose. After 40 days, the explants were transferred into elongation medium supplemented with WPM basal salts, 0.4 mg/L BA and 0.1 mg/L NAA; the concentrations of agar and double coagulation were changed to 2 g/L agar and 2 g/L, respectively.

Three hybrid plant genotypes (named Z1, Z2, and Z3) were selected for tetraploid induction (Table 1). The shoot-inducing rate of all leaves and petioles reached 85%, and they displayed similar morphological characteristics during development.

Shoot-inducing rate: Number of adventitious shoots ($\geq$1 cm)/number of explants $\times$ 100%

Table 1. Influencing factors and level values.

Levels	Factors			
	A	**B**	**C**	**D**
	Genotype	**Colchicine Concentration (mg/L)**	**Pre-Culture Duration (day)**	**Exposure Time (day)**
1	Z1	120	4	3
2	Z2	160	6	4
3	Z3	200	8	5

2.3. Colchicine Application

After sub-culturing for 60 days, the second and third leaves and petioles of Z1, Z2, and Z3 were selected as the materials for colchicine treatment. The leaf samples were proximal halves cut twice through the main vein, and the petioles were cut into 0.8–1.0 cm pieces; both sample types were pre-cultured in WPM supplemented with 0.1 mg/L TDZ, 0.8 mg/L BA and 0.1 mg/L NAA for 4, 6, or 8 days, and then treated for 3, 4 or 5 days with various concentrations of colchicine (120, 160 or 200 mg/L). The genotype, number of days of pre-incubation, colchicine exposure time, and concentration of colchicine were included as variables in the $L(9_3)^4$ orthogonal experimental design (Table 1); two explant types, leaf and petiole were applied to the same design, respectively. Cultures with colchicine treatment were performed in 100 mL flasks and incubated at 25 $\pm$ 2 °C, under dark conditions. Adventitious shoots longer than 1.5 cm were harvested and placed into the rooting medium.

Explants survival rate: Number of survival explants (leaf or petiole)/all explants $\times$ 100% (n = 3)

2.4. Flow Cytometric Analysis of Ploidy Level

We collected leaves that were growing vigorously in vitro, and the ploidy level was analysed by Cyflow Ploidy Analyser (Partec, Görlitz, Germany). The leaves were chopped with a sharp razor blade (Gillette, Boston, MA, USA) in a plastic dish containing 1.25 mL of modified Galbraith's buffer (9.15 g/L $MgCl_2 \cdot 6H_2O$, 4.19 g/L 3-(*N*-morpholino) propansulfonic acid (MOPS), 8.82 g/L sodium citrate, 0.1% polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100), pH 7.0). The crude nuclei solution was filtered through a 50-µm nylon filter. Subsequently, the leachate was stained with 100 µL 4′,6-diamidino-2-phenylindole (DAPI, 10 µg/mL) for 10 s to detect the ploidy level. Leaves from the diploid full-sib family were used as a control to adjust the number of DAPI channels to 50. Sub-culturing was performed twice over a 5-month period, and the ploidy level was examined

twice in this period. Finally, tetraploids, mixoploids, and diploids were placed vertically into Jiffy Mix (Shippagan, NB, Canada). After 2 months, the plants were transferred into plastic pots (height: 12 cm, top width: 10 cm, bottom width: 9.25 cm) containing a 2:1:1 sterilised mixture of peat, vermiculite, and perlite (autoclaved at 121 °C for 30 min). After 2 months, the final ploidy level was determined.

2.5. Chromosome Counting

Shoot growth occurred in rooting medium for nearly 2 weeks, and 5–10 mm of the root tips were examined. The root tips were rinsed and fixed in Carnoy's solution [23] for 24 h at 4 °C. After washing three times, the root tips were hydrolysed in 1 N HCl for 15 min at 60 °C, then the root tips were washed three times for 5 min. The treated root tips were cut into ~1.5 mm sections, stained with 1 drop of carbol fuchsin [24] solution for 15 min, and observed with a microscope using a 100× oil immersion lens (Olympus, Tokyo, Japan).

2.6. Statistical Analysis

Range analysis was used to evaluate the importance of each factor. The value of Range (R = k·(max) − k (mix);) and k_x were positively related to the importance of the factors and levels, respectively. The significance of differences among the treatments were evaluated using the homogeneity test and analysis of variance (ANOVA) using SPSS statistical software (ver. 18.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate statistical significance. Arcsine transformation ($\theta = \sin^{-1}\sqrt{P}$ θ: angle, P: percentage) to determine percentages before ANOVA was performed using Microsoft Excel 2010 software (Microsoft Corp., Washington, DC, USA).

3. Results

3.1. Morphologic Observations in the Initial Stage of Regeneration

Less than 10% of the cultures were contaminated, and 150 genotypes of hybrid sweetgums were established in vitro and sub-cultured. Three genotypes were selected for colchicine treatment. The leaves of the hybrid sweetgum were trisected during the early stage. After incubation for 4 days, no nodules were observed on the wound of the main vein of the leaves and petioles. From 4 to 6 days, nodules appeared and expanded rapidly, and growth continued until 8 days. The nodules in the leaves near the petioles were larger than those found on other wounds (Figure 1). The first bud appeared nearly 20 days after the shoot explants were placed in medium.

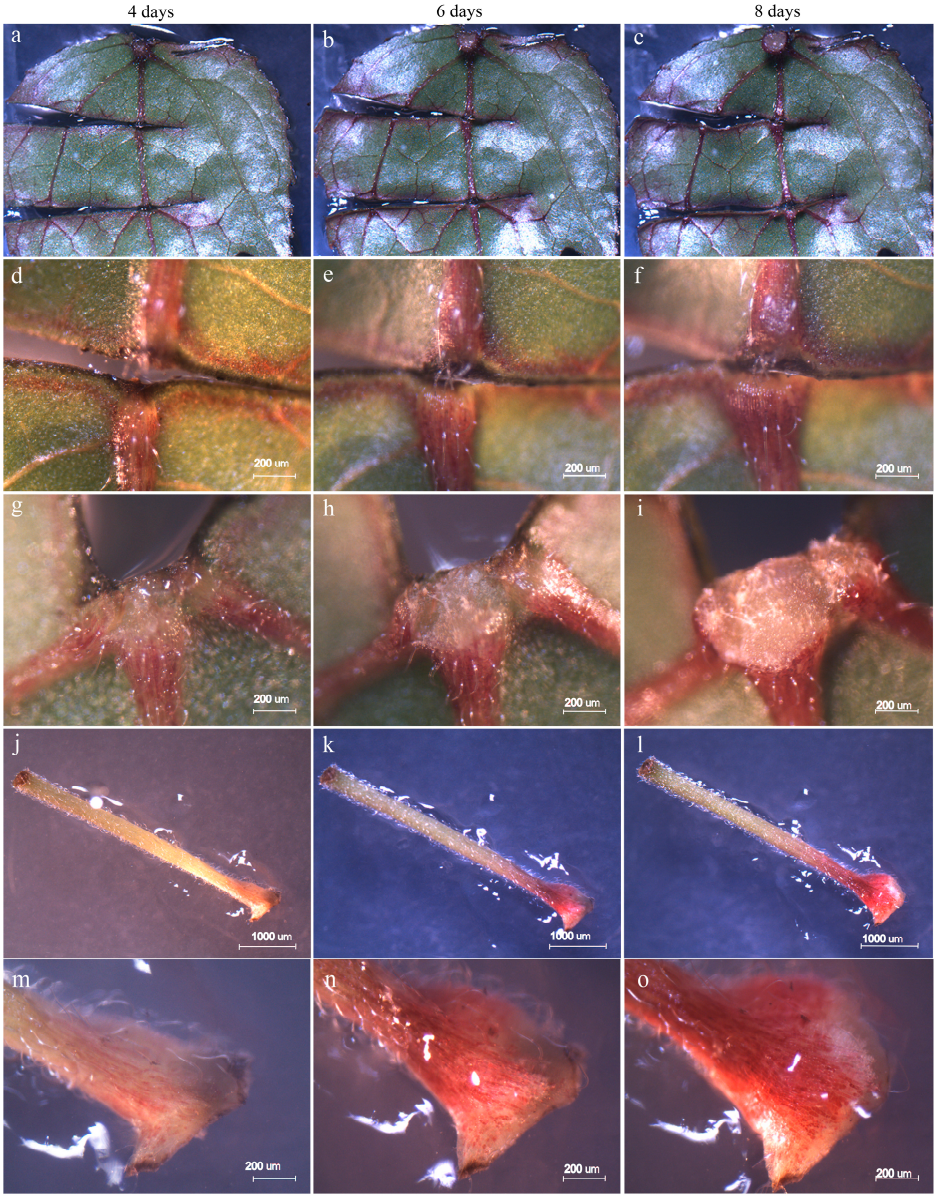

Figure 1. Morphology of leaves and petioles cultured in regeneration medium after 4, 6, and 8 days. (**a**–**c**) morphology of leaves cultured for 4, 6, and 8 days, respectively; (**d**-**f**) morphology of the second wound in the leaf main vein at 4, 6, and 8 days respectively; (**g**-**I**) morphology of the first wound in the leaf main vein at 4, 6, and 8 days, respectively; (**j**-**l**) morphology of the petiole cultured for 4, 6, and 8 days, respectively; (**m**-**o**) morphology of one end of the petiole cultured for 4, 6, and 8 days, respectively.

3.2. Survival Rate and Regeneration of Colchicine-Treated Explants

Under treatment 4, the survival rates were <10% (Tables 2 and 3). Table 4 shows that the concentration of colchicine (120–200 mg/L) had no significant effect on the survival rate of leaf explants. In contrast, genotype, pre-culture time and exposure time had significant effects on the survival rate in leaves (Table 4) and in petioles (Table 5). Exposure time exerted the most significant effect on survival rate for both leaves and petioles (Table 6).

Table 2. Design of orthogonal table $L_9(3)^4$ for leaves.

Treatment	Factors				Number of Shoots Examined	Survival Rate %	No. of Tetraploid	No. of Mixoploid	Tetraploid Induction %
	A	B	C	D					
c1	1	1	1	1	50	95.00	1	1	2.00
c2	1	2	2	2	50	75.00	0	1	0.00
c3	1	3	3	3	30	41.67	1	2	3.33
c4	2	1	2	3	15	8.33	0	0	0.00
c5	2	2	3	1	50	81.67	1	1	2.00
c6	2	3	1	2	50	90.00	0	1	0.00
c7	3	1	3	2	50	75.00	0	1	0.00
c8	3	2	1	3	50	70.00	0	0	0.00
c9	3	3	2	1	50	85.00	1	2	2.00

Table 3. Design of orthogonal table $L_9(3)^4$ for petioles.

Treatment	Factors				Number of Shoots Examined	Survival Rate %	No. of Tetraploid	No. of Mixoploid	Tetraploid Induction %
	A	B	C	D					
c1	1	1	1	1	50	85.00	1	0	2.00
c2	1	2	2	2	50	56.67	2	1	4.00
c3	1	3	3	3	30	28.33	1	0	3.33
c4	2	1	2	3	15	10.00	0	0	0.00
c5	2	2	3	1	50	56.67	3	2	6.00
c6	2	3	1	2	50	75.00	0	0	0.00
c7	3	1	3	2	50	48.33	0	0	0.00
c8	3	2	1	3	50	51.67	0	0	0.00
c9	3	3	2	1	50	70.00	4	2	8.00

Table 4. The variation analyses of survival rates for different genotype leaves (*Liquidambar styraciflua* × *L. formosana*), concentration, pre-culture duration and exposure time.

Variation Source	*df*	MS	*F*	Sig.
Genotype	2	294.147	4.876	0.020 *
Concentration	2	193.362	3.205	0.064
Pre-culture duration	2	1087.422	18.027	0.000 *
Exposure time	2	2769.584	45.913	0.000 *
Error	18	60.322		
Total	27			

df: degrees of freedom; MS: mean square; Sig.: significance; * Represents a significant difference at $p < 0.05$.

Table 5. The variation analyses of survival rates for different genotype petioles (*Liquidambar styraciflua* × *L. formosana*), concentration, pre-culture duration and exposure time.

Variation Source	*df*	MS	*F*	Sig.
Genotype	2	138.765	6.238	0.009 *
Concentration	2	97.631	4.389	0.028 *
Pre-culture duration	2	811.981	36.502	0.000 *
Exposure time	2	1612.270	72.478	0.000 *
Error	18	22.245		
Total	27			

df: degrees of freedom; MS: mean square; Sig.: significance;* Represents a significant difference at $p < 0.05$.

Table 6. The range analysis of the hybrid sweetgum survival rate by orthogonal test.

Explant Type		A	B	C	D
Leaf	K_1	211.67	178.33	255.00	261.67
	K_2	180.00	226.67	168.33	240.00
	K_3	230.00	216.67	198.33	120.00
	k_1	70.56	59.44	85.00	87.22
	k_2	60.00	75.56	56.11	80.00
	k_3	76.67	72.22	66.11	40.00
	R	16.67	16.11	28.89	47.22
Petiole	K_1	170.00	143.33	211.67	211.67
	K_2	141.67	165.00	136.67	180.00
	K_3	170.00	173.33	133.33	90.00
	k_1	56.67	47.78	70.56	70.56
	k_2	47.22	55.00	45.56	60.00
	k_3	56.67	57.78	44.44	30.00
	R	9.44	10.00	26.11	40.56

R: Range. Range = k·(max) − k (mix); $K_{1A} = X_{A1} + X_{A2} + X_{A3}$, $K_{2A} = X_{A4} + X_{A5} + vX_{A6}$, $K_{3A} = X_{A7} + X_{A8} + vX_{A9}$ . . . ; $k_x = K_x$/number of level.

3.3. Analysis by Flow Cytometry and Polyploid Determination

The ploidy levels were determined by three flow cytometry tests (Figure 2). The first test was administered in shoots that were rooted in medium for 1 month, the second test was administered after sub-culturing for 2 months, and the third test was administered after transplantation in the soil for 2 months. Therefore, the chromosomes were counted over a total of 6 months. The results showed that tetraploids and mixoploids were induced in the three genotypes of hybrid sweetgum (Tables 2 and 3). The most effective treatment for inducing polyploidy in the petioles was treatment 9, which consisted of pre-culturing genotype Z3 for 6 days, followed by 200 mg/L colchicine treatment for 3 days. This treatment resulted in 8% tetraploid and 4% mixoploid induction rates (Table 3). For the leaves, one tetraploid was acquired in c1, c3, c5, and c9 (Table 2). The chromosome number of tetraploid hybrid sweetgum was $2n = 4x = 52$ (Figure 3a), and the chromosome number of diploids was $2n = 2x = 26$ (Figure 3b).

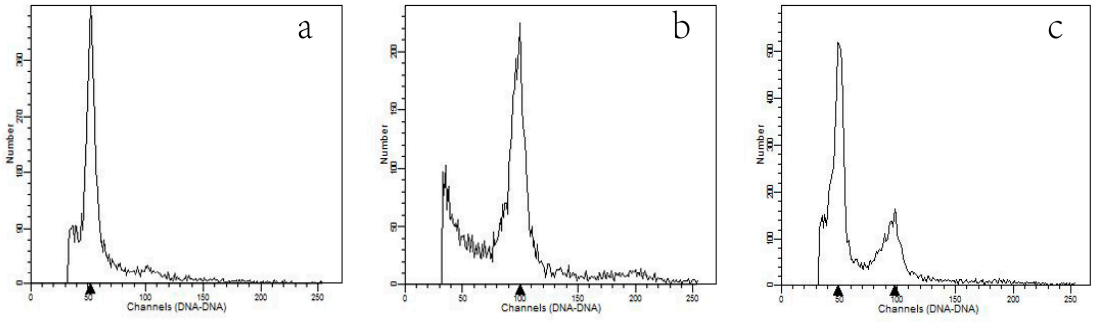

Figure 2. Histograms of flow cytometric analysis of *Liquidambar styraciflua* × *Liquidambar formosana* (**a**) diploid plant (control); (**b**) tetraploid plant; (**c**) diploid + tetraploid plant.

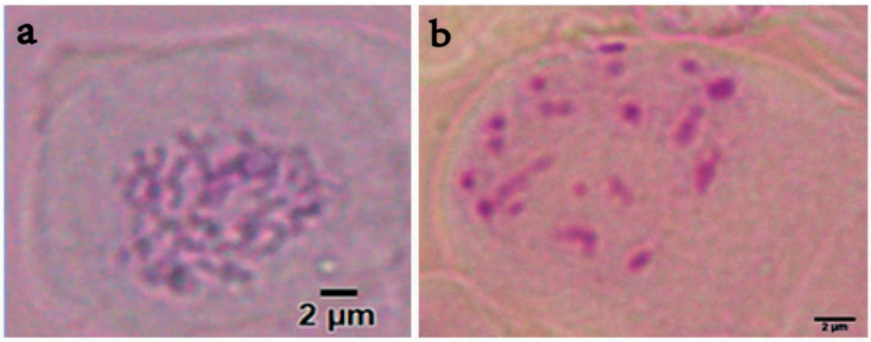

Figure 3. Chromosome counting of regenerated *Liquidambar styraciflua* × *L. formosana* plant (**a**) Chromosomes of a tetraploid plant. (**b**) Chromosomes of a diploid plant.

Range analysis of the results showed that the best concentration of colchicine and exposure time was 200 mg/L and 3 days, respectively, for both leaf and petiole explants. The number of tetraploids was lower than for those exposed to colchicine for 5 days (Table 7). Range analysis also showed that the exposure time was the most important influencing factor in leaf and petiole tetraploid numbers. Moreover, the morphology of the adventitious shoots of leaves were similar to that of petioles (Figure 4a,b), and tetraploid plantlets was significantly different from that found in the diploid plantlets, showing deeper green leaf colour and shorter root length and internodal distance (Figure 4c).

Table 7. The range analysis of the hybrid sweetgum tetraploid-inducing rate by orthogonal test.

Explant Type		A	B	C	D
Leaf	K_1	5.33	2.00	2.00	6.00
	K_2	2.00	2.00	2.00	0.00
	K_3	2.00	5.33	5.33	3.33
	k_1	1.78	0.67	0.67	2.00
	k_2	0.67	0.67	0.67	0.00
	k_3	0.67	1.78	1.78	1.11
	R	1.11	1.11	1.11	2.00
Petiole	K_1	9.33	2.00	2.00	16.00
	K_2	6.00	10.00	12.00	4.00
	K_3	8.00	11.33	9.33	3.33
	k_1	3.11	0.67	0.67	5.33
	k_2	2.00	3.33	4.00	1.33
	k_3	2.67	3.78	3.11	1.11
	R	1.11	3.11	3.33	4.22

R: Range. Range = k (max) − k (mix); $K_{1A} = X_{A1} + X_{A2} + X_{A3}$, $K_{2A} = X_{A4} + X_{A5} + X_{A6}$, $K_{3A} = X_{A7} + X_{A8} + X_{A9}$ . . . ; $k_x = K_x$/number of level.

Figure 4. Morphology of shoot regeneration in Z3 leaves and petioles after 60 days. (**a**) leaf; (**b**) petiole; (**c**) tetraploid; (left) and diploid (right).

4. Discussion

This study describes a method of in vitro tetraploid induction from leaf explants of hybrid sweetgum (*L. styraciflua* × *L. formosana*). Morphological mutation has been found in tetraploid plantlets. Interestingly, this phenomenon has appeared in herbs, fruit and timber trees [9,11,18]. In this paper, we investigated the effects of explant type, genotype, pre-incubation time, exposure time in colchicine and concentration of colchicine. The exposure time had the greatest effect on the tetraploid-inducing rate, this may be due to the lower survival rate of explants after over exposure to colchicine.

All three genotypes acquired tetraploids, with similar results to those obtained using *Actinidia chinensis* and *Populus* [8,15]. Few studies have considered both leaves and petioles together, perhaps due to limited efficiency of regeneration, such as in *Actinidia chinensis* [15]. Therefore, previous studies have provided no evidence as a basis for comparing the induction rates of leaves and petioles. In this study, the ability to induce tetraploidy was higher in petioles than in leaves. It is possible that the nine treatments were more suited to petioles, or the chromosomes and petiole cells were more responsive to colchicine treatment. This method has potential for successful acquisition of polyploids using a high-efficiency regeneration system for leaves and petioles of some plant species. In this study, we found lower induction rates for leaves and petioles pre-cultured for 4 days versus those pre-cultured for 6 or 8 days. Interestingly, we observed that the wounds in the main vein expanded slowly (Figure 1). The relationship between wound expansion and induction rate requires further confirmation in future studies.

The survival rate of leaves was higher than that of petioles subjected to nine treatments. For petioles, the pre-culture duration, colchicine concentration and exposure time significantly affected the survival rate. Similar conclusions were reported by [25]. However, colchicine concentration was not a significant factor for leaf survival. Interestingly, increased pre-treatment time correlated roughly with decreased survival rates, perhaps because inactivation of in vitro explant cells occurred slowly. The duration of colchicine treatment was the most important factor influencing the survival rate, and leaves exposed to colchicine for 5 days acquired only two tetraploids.

Unsynchronised cell divisions may lead to the induction of mixoploids. Mixoploid induction has been reported in *Ranunculus asiaticus* [14], *Echinacea purpurea* [18], and *Pyrus pyrifolia* N. cv. Hosui [26]. Mixoploid induction always accompanies tetraploid induction, and the in vitro regeneration rate of mixoploids was markedly lower than that obtained directly from treated seeds and shoot tips. Although a higher rate of mixoploid induction occurs during direct regeneration from organs versus from somatic embryos, somatic embryogenesis is often limited by its high threshold of regeneration. We hypothesise that increasing the concentration of cytokinin, and selecting younger and more robust colchicine-treated explants, might improve the efficiency of tetraploid induction. Sivolapov and Blagodarova reported that stable mixoploids in poplar showed growth advantages in field observations [27].

In this study, we explored conditions that were conducive for sweetgum polyploidy breeding. Future studies will be aimed at optimising the treatment conditions and regeneration system for other genotypes. In addition, as various regeneration systems have been successfully established in *Liquidambar* [5,20,28], our future studies will incorporate other regeneration systems besides the leaf and petiole to induce polyploidy sweetgum.

5. Conclusions

To the best of our knowledge, this is the first report of in vitro tetraploid induction from leaf and petiole explants of hybrid sweetgum (*L. styraciflua* × *L. formosana*). This method is effective, not limited by flowering period and easy to operate. In the future, this method could be used for tetraploid induction in multiple genotypes, and these tetraploid plants will be observed and measured continuously with the aim of selecting fast growing, high biomass, strong resistance, superior sweetgum with peculiar ornamental value. Furthermore, hybrid tetraploid sweetgum could be

a potential source for the promotion of sweetgum breeding and producing triploids by crossing with diploids.

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Conflicts of Interest: The authors declare no conflict of interest.

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