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REGENERATION OF PLANTLETS AND TETRAPLOIDY INDUCTION IN *PSEUDOSTELLARIA HETEROPHYLLA*

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This study was aimed at developing an efficient protocol for regeneration of *Pseudostellaria heterophylla* plantlets and induction of polyploidy. Calli of *P. heterophylla* (Miq) from stems, leaves and buds as explants could not differentiate into plantlets. However, young embryo segments used as primary explants produced embryonic calli on MS medium containing 5.0 mg/L 2,4-D and 0.5 mg/L KT. After the embryonic calli with granular protuberances were transferred to MS medium containing 0.5 mg/L BA, they developed shoots and then rooted to form plantlets. Polyploidy was induced when embryonic calli were placed in liquid MS medium containing 0.5% colchicine for 4 days, followed by culturing in solid medium to induce differentiation. Polyploidy was identified by the number of chromosomes and the size of plantlet stomata. The tetraploid plantlets produced larger root tubers than the diploid plantlets.

Key words: Callus, embryonic calli, *Pseudostellaria heterophylla*, regeneration, tetraploidy.

INTRODUCTION

Pseudostellaria heterophylla has been used in traditional Chinese medicine for hundreds of years (Xiao, 2002). The primary medicinal components of the herb include alkaloids, which are located mostly in the root tubers (Wu et al., 2004; Cai et al., 2005). *P. heterophylla* seeds are difficult to germinate because the seed coat is too thick (Wen, 2003). Its root tubers are generally used for propagation in production applications; this results in virus infection, and the tubers degenerate with age as they are used for asexual reproduction (Wu et al., 2004; Zhou et al., 2004).

The use of modern biotechniques to improve *P. heterophylla* propagation has potentially important medical consequences. To date, few reports regarding tissue culture of *P. heterophylla* have been published because it is difficult to get the calli to differentiate (Lin C. et al., 2005; Lin L. et al., 2005; Xie et al., 2006). Here we examine induction of embryonic calli and regeneration of *P. heterophylla* using young embryo segments as explants, to provide a basis for improvement of *P. heterophylla* through biotechniques. Then, having succeeded in that part of the work, we attempt to induce polyploidy with colchicine.

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MATERIALS AND METHODS

CULTURE CONDITIONS

Pseudostellaria heterophylla (YuoSheng No. 1) was grown in Zherong County of Fujian Province in China. We collected two types of explants: stem, leaf and bud segments from sterile plantlets; and segments of young embryos from immature seeds. Thirty explants of each type were placed on MS medium (Murashige and Skoog, 1962) containing 0.5 mg/L KT and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction. Each hormone treatment was made in triplicate. Stem, leaf and bud cultures were kept in the dark for 20 days and then transferred to light (2000 lx) for 12 h each day for 6 weeks. Cultures were maintained in a culture room at 25±1°C. The young

Abbreviations: BA – 6-benzyladenine; 2, 4-D – 2,4-dichlorophenoxyacetic acid; NAA – α-Naphthaleneacetic acid; KT – Kinetin.

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embryo cultures were kept in the dark for 10 days, and then the swollen embryos were cut into small segments and subcultured in MS media supplemented with 3% sucrose and solidified with 0.8% agar. The pH was adjusted to 5.8 prior to autoclaving at 121°C (0.1 Mpa) for 20 min.

CALLUS INDUCTION AND PLANT REGENERATION

The induced calli from young embryo explants were subcultured three times in media supplemented as described above and then transferred to media infused with lower concentrations (1 mg/L) of 2,4-D or without 2,4-D for 60 days. When the embryonic calli reached 3 mm in length they were transferred to medium containing different concentrations of auxins and cytokinins and subcultured 30 days to induce shoots and plantlets. Each hormone treatment was made in triplicate.

POLYPLOIDY INDUCTION

Liquid MS medium containing 0.5% colchicine and 2% DMSO was filtered, sterilized and used for polyploidy induction. The 3–5 mm embryonic calli were placed in the liquid medium and incubated on a shaker (80 r/min) for 2–8 days. Then the calli were washed several times with sterilized water and cultured on MS solid medium to induce differentiation. After 60 days we counted the living and differentiated calli and transferred the shoots to hormone-free solid medium to induce rooting.

We determined polyploidy in the regenerated plantlets by first measuring the size of stoma and then counting the number of chromosomes. The frequency of tetraploid induction was estimated for the different durations of culture.

RESULTS

CALLUS INDUCTION

Calli were produced from stem and leaf explants cultured in media containing 0.25–15 mg/L 2,4-D after 7 days of culture in the dark. After 20 days most explants produced calli which were 2–3 mm long, translucent and very compact. Subcultured calli grew quickly and became buff-colored and less compact (Fig. 1). Explants from buds swelled but produced few calli, and the buds grew to canes when cultured in the dark.

The seeds of *Pseudostellaria heterophylla* were difficult to germinate even when cultured for 90 days. After the immature seeds were peeled out of their seed coats, young embryos (Fig. 2) were placed on media supplemented with different concentrations of 2,4-D in the dark for 10 days and eventually more than 50% swelled (Fig. 3). The swollen embryos were cut into several segments and cultured in the same medium. Some segments of cultured embryos produced calli from their cut end when cultured under light for 15–20 days (Fig. 4). After repeated subculture, these calli became flavescent, with compact structure and vigorous growth (Figs. 5, 6).

The presence of high concentrations of 2,4-D resulted in brown calli which died quickly. Calli grew slowly at low concentrations of 2,4-D and produced many hair-like roots. At a 2,4-D concentration of 5 mg/L, 75% of the explants from stems and leaves and 40% of explants from young embryo segments produced calli (Tab. 1). One reason that fewer calli were induced from embryo segments than from stems and leaves is that roots of embryo died soon after culture. Other factors may also cause embryo calli to grow at a slower rate.

CALLUS DIFFERENTIATION

Calli induced from stems and leaves could not differentiate buds when cultured in media with lower concentrations of 2,4-D and modified concentrations of growth regulators. At low concentrations of 2,4-D the calli continued to grow rapidly but were not differentiated. Calli grew slowly and produced many hair-like roots in medium containing no 2,4-D but with BA and KT, and buds were not produced. Meanwhile, calli from embryo segments grew slowly and became granular and not compact when grown in medium without 2,4-D, and the calli turned from yellow to buff (Figs. 7, 8). When calli were grown further,

Fig. 1. *Pseudostellaria heterophylla* calli induced from stem and leaf explants. ×5. **Fig. 2.** Young embryo dissected from immature seed. ×1. **Fig. 3.** Embryos swollen after culture in dark for 10 days. ×2. **Fig. 4.** Calli induced from embryo segment. ×10. **Figs. 5, 6.** Calli (yellow) after subculture three times. ×5. **Figs. 7, 8.** Calli on differentiated medium (becoming white); note loose structure of callus (Fig. 8). ×10. **Fig. 9.** Callus (green) forming many granular protuberances. ×10. **Fig. 10.** Buds developing from protuberances visible in Fig 9. ×10. **Fig. 11.** Shoots developing from buds visible in Fig. 10. ×2. **Fig. 12.** Plantlets regenerated from shoots. ×2. **Fig. 13.** Metaphase chromosomes (2n = 32) of diploid plantlet. ×1000. **Fig. 14.** Metaphase chromosomes (2n = 64) of tetraploid plantlet. ×1000. **Fig. 15.** Stomata of tetraploid plantlet. ×250. **Fig. 16.** Stomata of diploid plantlet; note size difference as compared with Fig. 15. ×250. **Fig. 17.** Root tubers of tetraploid plantlet. ×2. **Fig. 18.** Root tubers of diploid plantlet; note size difference as compared with tetraploid plantlet in Fig. 17. ×2. **Fig. 19.** Root tubers of tetraploid (right) and diploid (left) plants in field; root tubers of tetraploid plant are bigger than those of diploid. ×1.

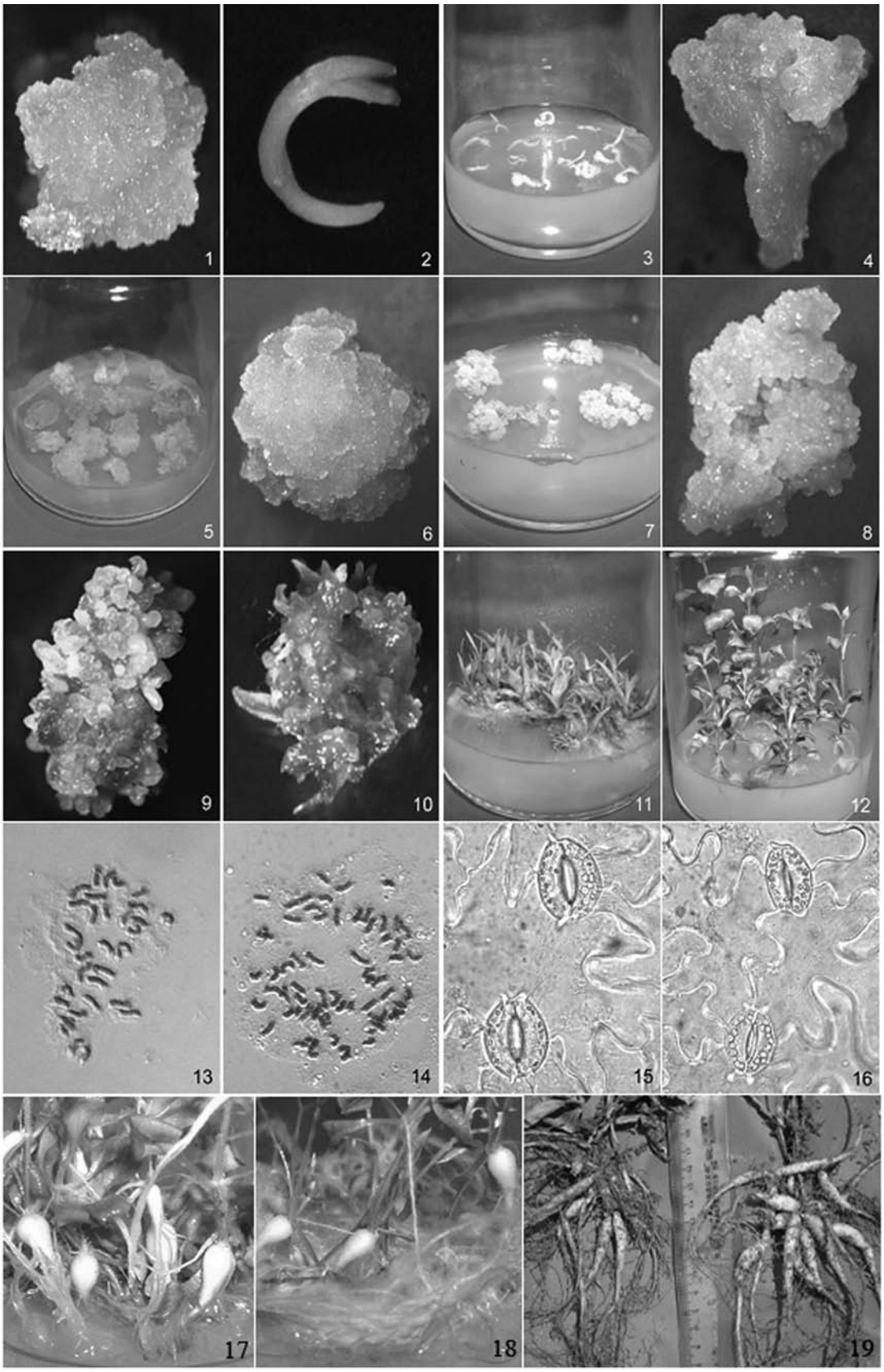
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TABLE 1. Effect of 2,4-D on callus induction from different explants (±SE)

2,4-D concentration (μM)	0	11.31	22.62	31.67	45.25	56.56	67.87
% of calli from stem and leaf	4.4±2.0	55.6±5.1	75.6±5.1	77.8±1.9	78.9±5.1	74.4±2.0	71.1±5.6
% of calli from young embryo	5.6±5.1	24.5±3.9	40.0±3.8	36.7±3.8	35.6±3.9	30.0±5.8	25.6±3.9

TABLE 2. Effect of growth regulators (μM) on callus differentiation. ±SE

Media	% of bud differentiation on calli from young embryo*
MS	55.6±5.1
MS + 2,4-D (4.52)	7.8±1.9
MS + BA (4.44)	22.2±3.9
MS + BA (8.88)	20.0±3.3
MS + KT (9.29)	24.5±2.0
MS + KT (18.58)	23.3±5.8

*No bud differentiation on calli from stem and leaf.

55% changed from buff to green and exhibited small differentiated protuberances on the surface (Fig. 9), developing further into green buds (Fig. 10). The callus differentiation percentage sharply declined when calli were cultured on media containing different concentrations of BA or KT (Tab. 2).

PLANTLET REGENERATION

The green buds were not able to grow further in media without growth regulators, and they gradually died. Growth into shoots and eventually plantlets depended on the presence of growth regulators, especially BA (Tab. 3). When 0.5 mg/L BA was present in the media, 80.0% of the buds formed vigorous shoots after culture for 30 days (Fig. 11). Although 84.43% of the buds grew shoots in the medium with 1 mg/L BA, many of them were gracile and abnormal, and some were vitric. When auxins were present the frequency of bud growth was lower and the shoots grew slowly (Tab. 3). When these shoots were transferred to hormone-free medium, they grew adventitious roots from the stem and node at the sites in contact with the medium (Fig. 12).

POLYPLOIDY INDUCTION

Survivability of calli cultured in the presence of 0.5% colchicine differed between culture times. Calli taken from 2-day culture in medium with 0.5% colchicine suffered no damage and grew vigorously in final culture. Two calli (6.7%) from 4-day culture and 13 (43.3%) from 6-day culture did not survive to the end of final culture. All the calli from 8-day culture failed to survive to final culture, suggesting that 2 days are best for culturing *P. heterophylla* calli in 0.5% colchicine medium (Tab. 4).

Generally, 50% of the surviving calli differentiated into 3 or 4 shoots, which then could be used to induce rooting and produce plantlets. The differentiated shoots easily induced rooting, and all of the shoots formed plantlets in hormone-free medium, meaning there was no effect of colchicine on callus differentiation.

We counted the chromosomes of root and stem tips of 142 regenerated plantlets. Most contained 32 chromosomes (Fig. 13), indicating that they were diploids. Eleven plantlets, healthier and displaying larger stems and leaves, had somatic chromosome number 2n=64, representing tetraploid level (Fig. 14). The stoma of these plantlets were longer (46–57 μm) than in the diploids (35–45 μm) (Figs. 15, 16). The number of plastids in the stomata cells was 20–34 in tetraploids and 12–22 in diploids. After culturing for 120 days the tetraploid plantlets formed root tubers (Fig. 17) that were larger than those of diploid plantlets (Fig. 18). After the plantlets of tetraploids were transplanted in the field, their root tubers grew bigger and 18% heavier than those of diploids (Fig. 19), potentially yielding more value in production of *P. heterophylla*.

DISCUSSION

Over a hundred species produce embryonic calli in plant tissue culture but the induction frequency varies according to the species. Immature embryos have been shown to be a good donor explant for induction of embryonic calli in many plants. Calli were easily induced from vegetative organs in tissue culture of *Pseudostellaria heterophylla*, but differentiation into plantlets was not as easy. There are few papers reporting the use of buds as cultured explants in tissue culture of *P. heterophylla* (Wu et al., 2004; Lin et al. 2005). In the present study, stems and leaves of *P. heterophylla* gave a high frequency of callus induction when grown in medium containing 2,4-D, but the calli were not able to differentiate to form buds. When young embryos were used as explants, embryonic calli easily differentiated into many buds, and calli were even induced in hormone-free medium. Also, plantlets were easily regenerated, suggesting that the use of different donor explants can affect callus differentiation.

TABLE 3. Effect of growth regulator on bud growth. ±SE

Growth regulator (μM)	No. of buds	No. of shoots	% of shoots	State of shoots
MS	90	2	2.2±3.9	Growing slowly
BA (1.1)	90	55	61.1±5.1	Growing vigorously
BA (2.2)	90	72	80.0±3.3	Growing vigorously
BA (4.4)	90	76	84.4±5.1	Growing fast but gracile and vitric
IBA (2.46)	90	26	28.9±3.8	Growing slowly
IBA (1.23) + NAA (1.34)	90	31	34.4±5.1	Growing slowly

TABLE 4. Effect of 0.5% colchicine on callus growth and tetraploid induction

Days of colchicine treatment	No. of calli used	No. of degenerated calli (%)	No. of differentiating surviving calli (%)	No. of regenerated plantlets	No. of tetraploid plantlets
0	30	0	20 (66.7)	96	0
2	30	0	19 (63.3)	72	2
4	30	2 (6.7)	14 (46.7)	45	5
6	30	13 (43.3)	9 (30.0)	25	4
8	30	30 (100.0)	0	0	0

However, the embryonic calli were not able to grow and form shoots in medium without hormones, suggesting that plant regeneration requires the presence of some cytokinins and that plant growth regulators control callus differentiation in late stages. This regeneration system provides a basis for work on genetic manipulation of transgenics and cellular engineering to improve *P. heterophylla*.

The important product of *P. heterophylla* is its root tubers, so increasing their size will improve the plant's economic value. Doubling the number of chromosomes and producing polyploid plants is generally accompanied by an increase in vegetative organ size. Induction of polyploidy has great potential value in plants whose economic products are the vegetative organs, making this a very attractive area of research (Yang et al., 2006; Gu et al., 2005; Jaskani et al., 2005; Urwin et al., 2007). Xie et al. (2006) attempted to produce polyploid *P. heterophylla* plants using apical buds as explants soaked in 0.2% colchicine solution for 12–60 h. They obtained 56.52% survival of buds at 22 h, and those produced 17.39% polyploid plants. At 60 h, however, only 5.56% of the buds survived and colchicine toxicity was evident. Here we used media containing 0.5% colchicine to culture embryonic calli of *P. heterophylla*.

After 2 days of culture in medium with 0.5% colchicine all calli were healthy and grew well in final culture. After 4 days only 6.7% of calli were dead, suggesting short-term colchicine tolerance. When the calli were cultured for 6 days, 43.3% of the calli died, and at 8 days colchicine toxicity was evident and all calli were dead. We infer that the optimal time for calli to be cultured in medium containing 0.5% colchicine is 2–4 days. The percentage of tetraploids induced from calli subjected to colchicine for that period was 7.7% (11/142).

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