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Somatic embryogenesis in mature zygotic embryos of *Picea likiangensis*

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Abstract: Somatic embryogenesis (SE) was successfully induced from mature zygotic embryos of seven families of *Picea likiangensis* (Franch.) Pritz after 20 weeks culture on initiation medium. Three basal media (one-half strength LM medium, one-half strength LP medium and improved LP medium) with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (6-BA) were tested but only one-half strength LM medium supplemented with 2,4-D and 6-BA was successful for the embryogenic cultures (EC) initiation. The initiation frequencies of EC varied greatly from different families when culturing on the same initiation medium. The highest frequency (41.3%) was induced from one of the families on one-half strength LM medium supplemented with 3 mg L^{-1} 2,4-D and 1.5 mg L^{-1} 6-BA and 16.83% on average for seven families. EC were subcultured and proliferated on the same medium as the initiation one every 10 days. 3 lines of EC induced from the same family were applied in maturation experiment. Cotyledonary somatic embryos were observed after EC were transferred to maturation media of one-half strength LM medium containing 20–80 mg L^{-1} abscisic acid and 7.5% polyethylene glycol (PEG-4000). However, one-half strength LM medium supplemented with 40 mg L^{-1} or 60 mg L^{-1} ABA and 7.5% PEG gave the best maturation and the 3 lines showed different ability in maturation. Over 80% cotyledonary somatic embryos germinated normally on DCR medium containing 0.2% activated carbon. The success on SE induction of the species has provided an effective clonal propagation method for this important tree's genetic improvement.

Key words: *Picea likiangensis* (Franch.) Pritz; somatic embryogenesis; embryogenic cultures; embryo suspensor masses; plant growth regulators

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 6-BA, 6-benzyladenine; ABA, abscisic acid; PEG, polyethylene glycol; AC, activated carbon; SE, somatic embryogenesis; EC, embryogenic cultures; ESMs, embryo suspensor masses; PGRs, plant growth regulators

Introduction

Distributed widely in Yunnan Province of China, *Picea likiangensis* (Franch.) Pritz is the only tree species in genus of *Picea* growing in the Province. Although it is one of the most important conifers used as timber and garden trees, little progress on its genetic improvement has been made because of its slow growth and low rate of seed maturation in nature. Sexual propagation has been the normal reproduction for this species, which leads to a great variation in commercially interested traits. Due to the long flowering time and difficulty in controlled pollination, it is difficult to produce sufficient seeds for genetic improvement. If clonal propagation is possible, however, we can capture the benefits of genetic variation or genetic engineering programs to improve this species in many commercially interested traits, including growth, wood quality and uniformity.

SE is one of the most practical ways in large scale vegetative propagation. In some cases, SE is favoured over other methods of vegetative propagation because of the possibility to scale up the propagation by using bioreactors. In addition, the somatic embryos or the embryogenic cultures can be cryopreserved, which makes it possible to establish gene banks (von Arnold et al. 2002). Embryogenic cultures are also an attractive target for genetic modification. Although more and more conifer species have been successful in SE induction (Stasolla et al. 2002a, 2003) , it still remains limited in practical application because of low regeneration frequency, EC initiation genotype-dependent and explant limited (always mature or immature zygotic embryo), especially for some conifer tree species.

Conifer SE has been demonstrated for many genera (Park 2002; Sutton 2002; Tautorus et al. 1991). It is a multistage process, which proceeds through initiation, proliferation, maturation and germination. The first reports on conifer SE from seed explants were published for *Picea abies* in 1985 (Chalupa 1985; Hakman et al. 1985). To date, in the family Pinaceae, close to

41 species and hybrids belonging to five genera (*Abies*, *Larix*, *Picea*, *Pinus*, and *Pseudotsuga*) have been reported to undergo SE (Klimaszewska & Cyr 2002). However, most of them have been successful in SE from immature zygotic embryos, especially the trees of *Pinus* and *Larix*. Only a few conifer trees out of *Picea* are successful from mature zygotic embryos, which include *Pinus radiata* (Chandler et al. 1990), *Pinus sylvestris* L. (Hohtola 1995), *Abies alba* (Hristoforglu et al. 1995), *Cunninghamia lanceolata* (Xi & Shi 2006). Compared to other conifers, SE of spruce species has been the most successful and most advanced commercially. Most of them can be induced from immature or mature zygotic embryos. Up to now, more than 10 spruce species have been reported to undergo SE process. These species are: *Picea abies* (Gupta & Durzan 1986a; Egertsdotter & von Arnold 1998), *Picea glauca* (Tremblay 1990; Park et al. 1998; Lamhamedi et al. 2000), *Picea mariana* (Attree S. M. et al. 1990; Adams et al. 1994), *Picea sitchensis* (von Arnold & Woodward 1988; Cyr et al. 2001), *Picea willsonii* (Li & Guo 1990) and *Picea meyeri* (Yang et al. 1997). However, besides *Picea willsonii* and *Picea meyeri*, SE has not been done in any other *Picea* species in China and only a few conifers beyond *Picea* have been reported to do SE. They are *Larix principis-rupprechtii* (Qi et al. 2004), *Larix kaempferi* (Lü et al. 2005), *Pinus massoniana* Lamb. (Huang et al. 1995), *Pinus elliottii* (Tang et al. 1997). The study was the first in China to establish protocols for somatic embryogenesis in *Picea likiangensis* (Franch.) Pritz.

Material and methods

Plant material

Mature seeds were collected from open-pollinated cones of seven *Picea likiangensis* (Franch.) Pritz families in October 2005 in Lijiang Prefecture of Yunnan. The seeds from different families were stored in plastic bags separately at 4 °C before they were used. Mature zygotic embryos dissected randomly from mixed seeds of seven families were used as explants.

Seeds were disinfested by immersion in 70% v/v ethanol for 30 s and in 0.1% mercuric chloride for 30 min, rinsed 3 times in sterile, distilled water and soaked overnight in sterile water. Mature zygotic embryos were aseptically removed from disinfested seeds and placed on solidified induction medium. Each treatment consisted of about 150 explants, and the experiment was repeated twice.

Induction of embryogenic cultures (EC) – explants, media and culture conditions

Two series of experiment were performed for initiation of EC. The first series were to explore effects of basal media and plant growth regulators (PGRs) combinations on EC initiation. Three basal media were used in the experiments and they were one-half strength LM medium (1/2 LM) (Litvay et al. 1985), one-half strength LP medium (1/2 LP) (von Arnold & Eriksson 1981) and improved LP medium (decreasing concentration of NH_4NO_3 from 1200 mg L^{-1} to 600 mg L^{-1}), containing various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) concentration in each basal media (Table 1). In the experiments of this series, explants were mature zygotic embryos picked randomly from mixed seeds of seven families.

Table 1. EC induction from *Picea likiangensis* (Franch.) Pritz mature zygotic embryos on different basal media and PGRs combinations. Explants: mature zygotic embryos from mixed seeds of seven families.

Basal media	PGRs combinations		EC induction (%)
	2,4-D (mg L^{-1})	BA (mg L^{-1})	
1/2 LM	1	0.5	0.74 e
		1.0	2.20 d
		1.5	1.48 d
	2	0.5	8.14 b
		1.0	3.33 c
		1.5	4.76 c
	3	0.5	7.50 b
		1.0	9.60 b
		1.5	15.37 a
			Average for all above **5.90**
1/2 LP	Same as above		0
Improved LP	Same as above		0

1/2 LM – one-half strength LM medium; 1/2 LP – one-half strength LP medium; Improved LP – decreasing concentration of NH_4NO_3 from 1200 mg L^{-1} to 600 mg L^{-1}; 2,4-D – 2,4-dichlorophenoxyacetic acid; BA – 6-benzyladenine; EC – embryogenic cultures; PGRs – plant growth regulators. Percentages followed with the same letter are not significantly different at the 0.05 level of confidence.

Based on the first series of experiment, another series of experiment were set to investigate EC initiation from different families. In these experiments, mature zygotic embryos of seven families were put respectively on two kinds of media selected from the first series experiments (Table 2).

For all media, the concentration of sucrose was 20 g L^{-1}, glutamine was 500 mg L^{-1}, casein hydrolysate was1 g L^{-1}, inositol was 100 mg L^{-1} and agar was 7 g L^{-1} respectively. The pH was adjusted to 5.8 before phytagel was added. The media were then sterilized by autoclaving. Stock solutions of glutamine and casein hydrolysate were filter-sterilized and added to the media after they cooled to about 50 °C. 15 excised mature zygotic embryos were laid on culture media in each petri dish, which were cultivated in darkness at 21 °C. The EC initiation frequency was assessed after 12 weeks culture.

EC proliferation, somatic embryos mature and germination

EC was subcultured on proliferation medium that was same as initiation medium. Proliferated callus were subcultured on the proliferation medium at 10-day intervals in darkness. After 4 times of subculture, EC of 3 lines induced from family DBS-98 were transferred to maturation media. They were one-half strength LM medium containing 20–80 mg L^{-1} abscisic acid (ABA) and 7.5% polyethylene glycol (PEG-4000), which were selected based on previous experiments. EC clumps of growing vigorously and almost the same size (about 1cm^3 in volume) were selected for maturation. 5 Petri dishes each with 5 clumps were prepared for each embryogenic lines and the cultures were maintained in darkness at 21 °C for about 5 weeks when cotyledonary somatic embryos formed. Then, the number of cotyledonary

Table 2. EC induction from seven families of *Picea likiangensis* (Franch.) Pritz on two basal media and PGRs combinations. Explants: mature zygotic embryos from seeds of seven families respectively.

Families / EC induction (%)	Media & PGRs combinations		Media & PGRs average
	1/2 LM + 2,4-D_3 + $BA_{1.5}$	1/2 LM + 2,4-D_2 + BA_1	
AYL-226	16.3 c	2.7 b	**9.5**
BYL-632	2.9 e	0 d	**1.45**
CYL-1102	7.4 d	2.5 b	**4.95**
DBS-98	41.3 a	0.6 c	**20.95**
EBS-690	20.0 c	10.0 a	**15**
FBS-802	0.7 e	0.2 c	**0.45**
GBS-1406	29.2 b	0.6 c	**14.9**
Family average	**16.83**	**2.38**	**9.6**

1/2 LM – one-half strength LM medium; 2,4-D – 2,4-dichlorophenoxyacetic acid; BA – 6-benzyladenine; 2,4-D_3 – the concentration of 2,4-D is 3 mg L^{-1}; BA_1 - the concentration of BA is 1 mg L^{-1}; EC – embryogenic cultures; PGRs – plant growth regulators. Percentages followed with the same letter are not significantly different at the 0.05 level of confidence.

somatic embryos closely resembling their zygotic counterparts on each clumps was recorded. For somatic embryos converting into germinated somatic embryos, 30 randomly selected mature somatic embryos were used. Somatic embryos were transferred to the germination medium and cultured under light. The medium for germination was DCR medium (Gupta & Durzan 1985), containing 0.2% activated carbon (AC).

Results and discussion

The basal media and PGRs affected EC initiation of *Picea likiangensis* (Franch.) Pritz greatly. The typical structure of 10∼12 weeks of EC initiation culture, namely embryonal suspensor masses (ESMs) were induced (Fig. 1c). In conifer trees, ESMs consist of early stages of embryos at varying developmental stages which contained an embryonal head and suspensor system (Jain et al. 1989; Gupta & Durzan 1987). However, ESMs were induced only from the basal media of 1/2 LM with combinations of 2,4-D and BA. None were induced from 1/2 LP and improved LP. Further more, the initiation frequency was different from the different 2,4-D and BA concentrations (Table 1). Most ECs emerged firstly from the part of hypocotyl of zygotic embryos (Figs 1a, 1b). The Fig. 1c shows somatic embryos in early developmental stage. The cultures on 1/2 LM with 3 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BA gave the highest initiation frequency of 15.37% and 1/2 LM with 1 mg Ll^{-1} 2,4-D and 0.5 mg L^{-1} BA gave the lowest frequency of only 0.74%. The average frequency for 3 group PGR combinations on the basal medium of 1/2 LM is 5.9%.

Initiation of embryogenic cultures is very important for the application of somatic embrygenesis in conifers (Li et al. 1998). There were more than 10 species reported to undergo SE process out of all about 40 species in *Picea* spp. to date. The most frequently used basal media for their EC initiation were LP medium, LM medium and MS medium. Among them, LP or 1/2 LP medium was optimal selected. However, our studies showed that 1/2 LM medium was suitable for EC initiation of *Picea likiangensis* (Franch.) Pritz. A relatively high concentration of auxins (0.4–2 mg L^{-1}) and low concentration of cytokinins (0.1–1 mg L^{-1}) were always needed for initiation and proliferation of spruce EC (Yang et al. 1999). In our studies, 3 mg L^{-1} 2, 4-D combined with 1.5 mg L^{-1} BA gave the highest frequency of EC initiation (15.37% for seven families on average, and 41.3% for family DBS-98). Generally, EC initiation frequencies in *Picea likiangensis* (Franch.) Pritz were lower than those reported from mature embryos of *Picea abies* (50%) (von Arnold 1987) and *Picea glauca* (10%-50%) (Tremblay 1990).

EC initiation frequencies of mature zygotic embryos in *Picea likiangensis* (Franch.) Pritz varied greatly among seven families (Table 2). On 1/2 LM media supplemented with 3 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BA, the frequency was from 0.7% (Family FBS-802) to 41.3% (Family DBS-98) and the average of seven families was 16.83%. While on the media of 1/2LM supplemented with 2 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BA, the induction frequency was from 0% (Family BYL-632) to 10% (Family EBS-690) and the average value was only 2.38%. The results suggested that EC initiation in *Picea likiangensis* (Franch.) Pritz was influenced greatly by genotypes. It is still unknown the reasons for the differences in EC induction frequency existed among *Picea likiangensis* (Franch.) Pritz genotypes. Possibly these differences are related to genetic characteristic of genotypes and due to the complex interaction of genes controlling adaptability in genotypes and the culture protocols.

The capacity for somatic embryogenesis is genetically determined. There are major genotypes differences for this trait (von Arnold et al. 2002). The pattern of developmental response of cultured tissue is epigenetically determined and is influenced by the stage of development of the plant, the nature of the explant etc. (Litz & Gray 1995). It has been agreed generally that genotype is one of the internal factor affecting somatic embryogenesis. Many reports show the results that the SE induction varies greatly among different genotypes under completely same culture protocols (Cheliak & Klimaszewska 1991; Jain et al. 1995; Tang et al. 2001). Somatic embryogenesis can proba-

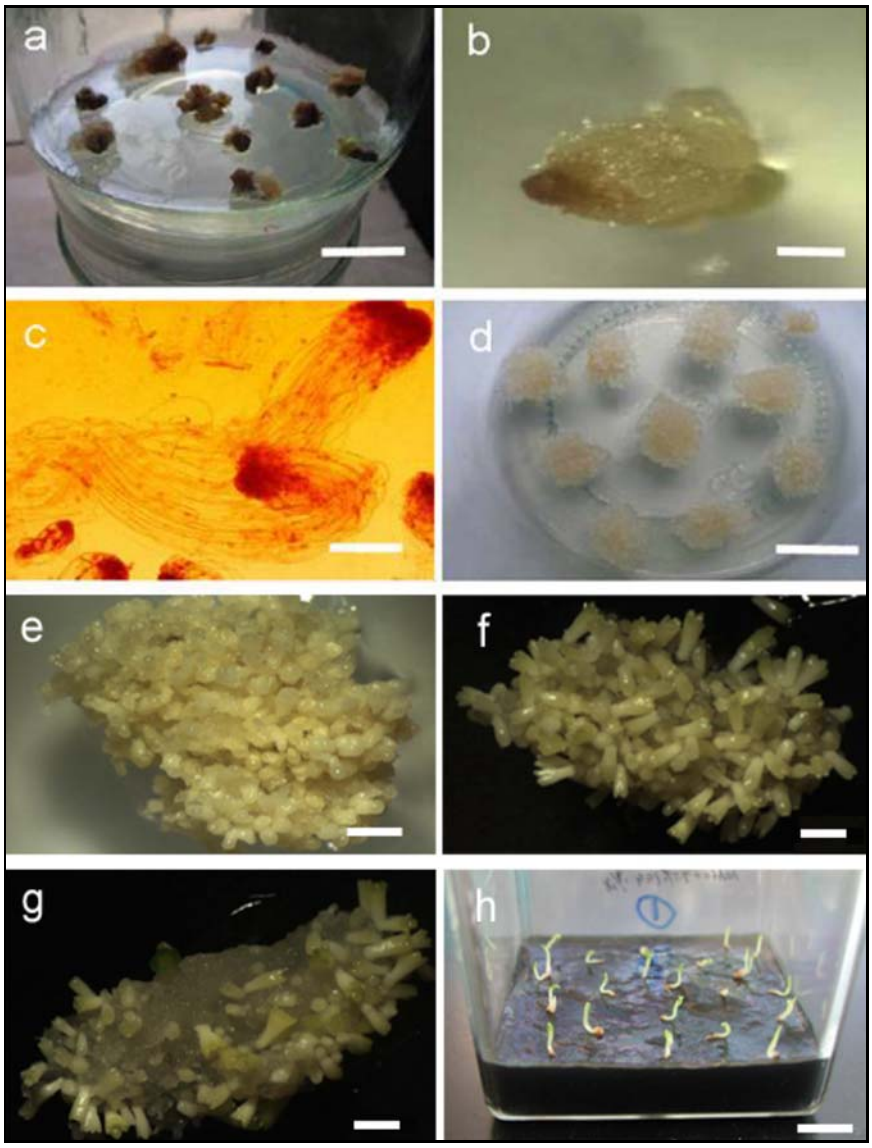

Fig. 1. Somatic embrygenesis from mature zygotic embryos of*Picea likiangensis*. a – initiation of embryogenic callus from mature zygotic embryos (bar = 5 mm); b – embryogenic tissue extruded from radicle part of mature zygotic embryos (bar = 1 mm); c – embryo suspensor masses (bar = 150 μm); d – proliferation of embryogenic callus (bar = 10 mm); e, f g – maturation of somatic embryos (bar = 2 mm); h – germination of somatic embryos (bar = 6 mm).

bly be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed (von Arnold et al. 2002). On this point, it is possible to get SE or relatively high induction frequency for specific genotype of all plant species, especially those of elite genotypes of great commercial value tree species. In our study, the EC initiation protocols are not appropriate for some families, such as family BYL-632, CYL-1102 and FBS-802. So, for them, it is necessary to make further explores on explant, culture media and environmental conditions for EC initiation.

EC of *Picea likiangensis* (Franch.) Pritz was proliferated on the same medium as the EC initiation medium with a better initiation, that was 1/2 LM with 3 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BA . They were subcultured for interval of 10 days and also could be cryopreserved. Fig. 1d shows vigorously growing ECs that have been subcultured for more than half a year and they still have capacity of embryo maturation.

There are several factors involved in maturation and germination of somatic embryos such as osmotic potential of the media, ABA, carbohydrates and plant growth regulators. Gupta & Grob (1995) suggested that osmotic potential of the culture medium and 0.1–25 mg L^{-1} ABA are essential for the development and maturation of conifer somatic embryos. Polyethylene glycol (PEG 4000–8000) was used as an osmoticum together with ABA in the culture medium for conifer somatic embryo maturation (Attree et al. 1991; Gupta & Pullman 1991). It appears that for normal growth of somatic embryos, ABA must be supplied exogenously and its concentration must be carefully optimized depending on species or genotype. As reviewed by Stasolla et al. (2002a), although ABA is commonly utilized to promote somatic embryo development in many coniferous

Table 3. Somatic embryos maturation of *Picea likiangensis* (Franch.) Pritz. EC for maturation: 3 lines induced from the same family.

Media	Cotyledonary somatic embryos					
	Line 1		Line 2		Line 3	
	Number of SE per clump	Total number of SE[a]	Number of SE per clump	Total number of SE[a]	Number of SE per clump	Total number of SE[a]
1/2 LM+ABA20+PEG7.5	38 ± 6	946	45 ± 6	1120	24 ± 2	598
1/2 LM+ABA40+PEG7.5	82 ± 11	2042	95 ± 12	2371	35 ± 2	876
1/2 LM+ABA60+PEG7.5	80 ± 10	1996	90 ± 12	2242	38 ± 3	947
1/2 LM+ABA80+PEG7.5	46 ± 6	1147	48 ± 8	1193	20 ± 2	498

[a] Values are mean ± SE of results; 5 Petri dishes each with 5 EC clumps for each embryogenic line

genera, including *Picea*, *Latix*, and *Pinus*, responsiveness of the tissue to ABA varies widely. For example, relatively high levels of exogenous ABA (40 mM) are needed to allow normal development and inhibit precocious germination in red spruce (Harry & Thorpe 1991). As for *Picea glauca* and *Picea mariana*, embryo growth can be stimulated with lower (12 mM) ABA levels (Attree et al. 1990). PEGs proven to be effective in stimulating water stress in culture and promoting embryo development can inhibit precocious germination and result in a 3-fold increase in the number of cotyledonary somatic embryos produced in white spruce culture (Attree et al. 1991). These embryos had a superior appearance to those matured in the presence of ABA alone (Attree et al. 1995).

In our study, 3 lines of EC induced from family DBS-98 were used for maturation. 4 media of 1/2 LM containing 20 mg L^{-1}, 40 mg L^{-1}, 60 mg L^{-1}, 80 mg L^{-1} ABA and each with 7.5% PEG were applied in the experiment. The EC developed to cotyledonary somatic embryos after about 5 weeks of culture on the media. For all 3 lines, the media of 1/2 LM containing 40 mg L^{-1} or 60 mg L^{-1} ABA and 7.5% PEG gave a higher efficient formation of mature somatic embryos (Fig. 1e, f). The results also showed that the 3 lines performed different embryo maturation, even though they were induced from the same family. As showed in Table 3, line 1 and line 2 gave the highest maturation efficiency on the medium of 1/2 LM containing 40 mg L^{-1} ABA and 7.5% PEG. (82 and 95 somatic embryos per clump EC on average) while line 3 produced only 38 somatic embryos for the most on 1/2 LM containing 60 mg L^{-1} ABA and 7.5% PEG (Fig. 1g). For line 1 and line 2, the number of somatic embryos was less than that reported on *Picea meyeri* Rehd. (109 somatic embryos per callus of $1cm^3$) under the optimum conditions (Yang et al. 1997). When transferred to the germination medium, over 80% somatic embryos germinated normally (Fig. 1h). As we know, variations in the number and quality of somatic embryos exist among species and often among genotypes within the same species. Therefore, optimum maturation media selection for more genotypes of the species, corresponding germinated somatic embryos conversion and plantlet regeneration are under further study.

We can conclude that somatic embryogenesis has the potential for eventual mass propagation of superior genotypes of forest trees, and for genetically engineering genotypes in both conifers and hardwood species, for most trees, especially conifers, limitations due to low initiation frequency and the genetic specificity of explants are important problems associated with embryogenesis. The present work has successfully established protocol for induction of somatic embryogenesis in *Picea likiangensis* (Franch.) Pritz. Further studies are needed to improve frequency of EC initiation and efficiency of somatic embryogenesis maturation and germination, especially for those of the specific elite genotypes, before this species can be propagated in large scales and be of commercially practical.

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