CHAPTER 2

MICROPROPAGATION VIA ORGANOGENESIS IN SLASH PINE

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1. INTRODUCTION

Highly efficient and reproducible *in vitro* regeneration systems via somatic embryogenesis or organogenesis are a prerequisite for clonal propagation of elite genotypes of specific plant species and for production of transgenic plants (Becwar et al., 1990; Attree & Fowke, 1993; Tang & Newton, 2003). Although plant regeneration via somatic embryogenesis has been reported in a number of coniferous species, plant regeneration via organogenesis from callus cultures has been obtained in only a few conifers (Hakman & Fowke, 1987; Nørgaard & Krogstrup, 1991; Tang et al., 2004). Routine methods of transformation are still hampered by the lack of readily available, highly efficient, and long-term regenerable cell and tissue culture systems in conifers (Handley et al., 1995; Tang & Newton, 2004).

Currently, a variety of explants have been successfully used for obtaining morphogenesis *in vitro* in conifers (Nagmani & Bonga, 1985; Gladfelter & Phillips, 1987; Tremblay, 1990; Guevin & Kirby, 1997; Salajova et al., 1999; Zhang et al., 1999), of which the most common are immature and mature embryos (Attree & Fowke, 1993; Find et al., 2002; Vookova & Kormutak, 2002). However, developmental progression has been limited to cultures capable of somatic embryogenesis and plant regeneration directly from the explant or via a callus phase using immature embryos (Krogstrup, 1990; Harry & Thorpe, 1991; Jalonen & von Arnold, 1991; Nørgaard, 1997; Klimaszewska et al., 2000). The successful regeneration of somatic embryos and plantlets is achieved using immature embryos (Campbell et al., 1992; Attree & Fowke, 1993; Guevin et al., 1994) as the target tissues in Fraser fir and Nordmann fir. Nevertheless, these explants require that their collection be limited to a special season of the year. In addition, there is a strong genotype dependency involved in tissue culture and efficient regeneration with embryogenesis. Furthermore,

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regeneration efficiency is still low, especially in commercial cultivars, due to various factors affecting the frequency of plant regeneration after transformation and selection (Find et al., 2002; Vookova & Kormutak, 2002). Therefore, a highly efficient regeneration system is needed for the genetic transformation of conifers.

Because of its rapid growth rate, slash pine (*Pinus elliottii* Engelm.) is a valuable southern pine for reforestation projects and timber plantations throughout the south eastern United States. Slash pine is also widely planted in the tropical and subtropical regions over the world. Slash pine is naturally found in wet flatwoods, swampy areas, and shallow pond edges. It can occur in the low sandy soils that are poor in nutrients. Millions of acres of slash pine have been planted and grown in the south eastern United States, where younger trees are harvested for pulpwood. Plant regeneration via somatic embryogenesis from embryogenic callus initiated from immature embryo explants of different slash pine genotypes has been reported (Jain et al., 1989; Newton et al., 1995). However, the development of a significantly improved plant regeneration system through multiple shoot differentiation from callus cultures derived from mature embryos would be valuable to clonal propagation and to genetic transformation in slash pine. In this study, we report the establishment of an efficient plant regeneration system via organogenesis from callus cultures in slash pine. The method presented here will be most useful for future slash pine clonal propagation and genetic transformation programs.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

Mature seeds of genotypes 1177, 1178, 7524, 7556 of slash pine (*Pinus elliottii* Engelm.) are provided by Penny Sieling and Tom Byram (Texas Forest Service Forest Science Laboratory, Texas A&M University, College Station, TX 77843-2585, USA). All seeds are stored in plastic bags at 4°C before they are used for callus induction. Seeds are washed in tap water for 20 min, then disinfected by immersion in 70% w/w ethanol alcohol for 30 s and in 75% house breach for 15 min, followed by five rinses in sterile distilled water. Mature zygotic embryos are aseptically removed from the megagametophytes and placed horizontally on a solidified callus induction medium in 15 × 100 mm Petri dishes (Fisher Scientific) with 20 ml medium. Make sure the whole embryos are touching the medium. Plates with embryos are incubated in the dark at 23°C.

2.2. Culture Medium

Basal media used in this investigation included BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004) media (Table 1). Plant growth regulators (Table 2) used in callus induction medium include α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and

Table 1. *The basal media used in tissue culture of slash pine. The basal media used for callus induction, adventitious shoot formation, shoot elongation, and rooting included BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004) medium.*

Chemical formula	BMS	DCR	LP	MS	SH	TE
$Ca(NO_3)_2 \cdot 4H_2O$	0	556	0	0	0	556
KNO_3	2,500	340	1,900	1,900	2,500	340
$CaCl_2 \cdot 2H_2O$	200	85	1,760	440	200	85
NH_4NO_3	0	400	1,200	1,650	0	400
$MgSO_4 \cdot 7H_2O$	400	370	370	3,70	400	720
KCl	0	0	0	0	0	1,900
KH_2PO_4	0	170	340	170	0	170
$NH_4 H_2PO_4$	300	0	0	0	300	0
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	0	8.6	1.0	25.8
$MnSO_4 \cdot H_2O$	16.9	22.3	2.23	16.9	10.0	25.35
H_3BO_3	6.2	6.2	0.63	6.2	5.0	6.2
KI	0.83	0.83	0.75	0.83	1.0	0.83
$Na_2MoO_4 \cdot H_2O$	0.25	0.25	0.025	0.25	0.1	0.25
$CoCl_2 \cdot 6H2O$	0.025	0.025	0.025	0.025	0.1	0.025
$CuSO_4 \cdot 7H_2O$	0. 025	0.025	0.025	0.025	0.2	0.025
$FeSO_4 \cdot 7H_2O$	27.8	27.8	13.9	27.8	15.0	27.8
NaEDTA	37.3	37.3	0	37.3	20.0	37.3
Myo-inositol	1,000	1,000	1,000	1,000	1,000	1,000
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5	0.5	0.5	0.5
Thiamine HCl	0.1	0.1	0.1	0.1	0.1	0.1
Glycine	0.1	0.1	0.1	0.1	0.1	0.1
Sucrose	30,000	30,000	30,000	30,000	30,000	30,000
Glutamine	0	0	0	0	0	500
Casein hydrolyzate	0	0	0	0	0	500
Gelrite	0	0	0	0	0	3,000
pH	5.7	5.7	5.7	5.7	5.7	5.7

2-isopentenyladenine (2iP). The pH is adjusted to 5.8 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121°C for 20 min. All media are adjusted to pH 5.8 prior to autoclaving for 20 min at 121°C. All tissues are cultured at 23°C. Adventitious shoot induction is conducted in the dark, and adventitious shoot differentiation and proliferation and rooting are conducted at 23°C under a 16-h photoperiod with cool fluorescent light (100 μmol m^{-2} s^{-1}). Each experiment is replicated three times, and each replicate consisted of 50–200 embryos for callus induction, 30–50 pieces of calli (0.5 × 0.5 cm in size) for adventitious shoot formation, and 30–45 elongated shoots for rooting. For shoot proliferation and maintenance, the multiplied shoots of

each clump are cultured in the same shoot formation medium for 6 additional weeks. All cultures are subcultured every 3 weeks.

Table 2. *Procedure for plantlet regeneration in slash pine. The basal media used for callus induction, adventitious shoot formation, shoot elongation, and rooting include BMS (Boulay et al., 1988), DCR (Gupta & Durzan, 1985), LP (von Arnold & Eriksson, 1979), MS (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1972), and TE (Tang et al., 2004).*

Plant growth regulators	Stage of plantlet regeneration			
	Induction	Differentiation	Elongation	Rooting
α-Naphthaleneacetic acid (NAA)	12 μM	0	0	0
Indole-3-acetic acid (IAA)	0	0	2 μM	0.01 μM
Indole-3-butyric acid (IBA)	0	2 μM	0	0.01 μM
2,4-Dichloroxyacetic acid (2,4-D)	15 μM	0	0	0
6-Benzyladenine (BA)	0	3 μM	1 μM	0
Thidiazuron (TDZ)	0	9 μM	0	0
2-Isopentenyladenine (2iP)	6 μM	0 μM	0	0
L-Glutamine	500 mg/l	500 mg/l	400 mg/l	400 mg/l
Myo-Inositol	500 mg/l	500 mg/l	250 mg/l	250 mg/l
Sucrose	30,000 mg/l	30,000 mg/l	20,000 mg/l	10,000 mg/l
Phytagel	4,500 mg/l	4,500 mg/l	5,000 mg/l	5,000 mg/l
PH	5.8	5.8	5.8	5.8
Culture time	6 weeks	6–12 weeks	6 weeks	6 weeks

2.3. Shoot Regeneration and Maintenance

The procedure of plant regeneration involving callus induction, adventitious shoot formation, shoot elongation, and rooting is shown in Table 2. Basal media used for callus induction include DCR, BMS, LP, MS, SH, and TE media. The frequency of callus formation is determined 6 weeks after culture. After calli are transferred onto adventitious shoot regeneration medium consisting of DCR, BMS, LP, MS, SH, and TE media for 6 weeks (Table 1), differentiation is evaluated by the percentage of calli forming adventitious shoots on the medium for a 6-week period.

1. Subculture calli every 3 weeks before the induction of shoot formation.
2. Transfer calli onto shoot formation medium supplemented with IBA, BA, and TDZ for 2–3 subcultures. If more calli are needed, subculture calli 4–6 times.
3. Make sure the whole calli are touching the medium.
4. Culture calli at 23°C under a 16-h photoperiod with cool fluorescent light (100 μmol m^{-2} s^{-1}).
5. Subculture calli with adventitious buds in LifeGuard plant growth vessels (Sigma) every 3 weeks on fresh shoot formation medium.
6. Determine the frequency of calli forming shoots, 6 weeks after calli are transferred onto shoot formation medium.

Among 6 basal media (BMS, DCR, LP, MS, SH, and TE) used in this study, higher frequency (34%–46%) of callus induction is obtained on BMS, SH, and TE, compared to DCR, LP, MSG, and MS. Similar callus induction frequency is obtained in four genotypes of slash pine. The frequency of callus formation increased during 4–6 weeks on fresh callus induction medium supplemented with NAA, 2,4-D, and 2iP. The highest frequency of callus formation is obtained on TE medium. After callus cultures (Figure 1A) are transferred onto shoot formation medium for 6 weeks, frequency of calli forming adventitious shoots is evaluated. Adventitious shoots (Figure 1B, C) are regenerated from callus cultures of four slash pine genotypes on BMS, SH, and TE media, with higher frequency (26%–35%) on SH and TE media and lower frequency (6%–9%) on BMS medium. The frequency of adventitious shoot formation increased during 6–12 weeks on fresh shoot formation medium supplemented with IBA, BA, and TDZ. The highest frequency of callus forming shoots is obtained on TE medium.

2.4. Rooting

Elongated, well-developed individual shoots with more than 8 needles are separated from the mother clumps and transferred onto rooting medium for 6 weeks. After elongated shoots are transferred onto rooting medium, rooting (Figure 1D, E) is evaluated by the percentage of shoots forming roots on the test medium for 6 weeks. Higher rooting frequency (26%–35%) is obtained in four genotypes on SH and TE media, compared to BMS medium (7%–9%).

1. Transfer shoots onto shoot elongation medium supplemented with IBA and BA.
2. Subculture shoots every 3 weeks.
3. Culture shoots at 23°C under a 16-h photoperiod with cool fluorescent light (100 μmol m^{-2} s^{-1}).
4. Subculture shoots every 3 weeks on fresh shoot elongation medium for 6 weeks.
5. Transfer elongated shoots 3–5 cm in height onto rooting medium supplemented with IAA and IBA.
6. Culture the elongated shoots for 6 weeks.
7. Rooting is conducted at 23°C under a 16-h photoperiod with cool fluorescent light (100 μmol m^{-2} s^{-1}).
8. Determine the frequency of shoots forming roots, 6 weeks after shoots are transferred onto rooting medium.
9. Plantlets with roots 2–5 cm in length can then be hardened.

2.5. Hardening

After rooting of adventitious shoots, regenerated plantlets from organogenic calli are treated at 4℃ for 1 week. Regenerated plantlets are then transferred from culture in 125 ml Erlenmeyer flasks into a perlite:peatmoss:vermiculite (1:1:1 v/v/v) soil mixture. For acclimatization, plantlets are covered with glass beakers for 1 week. After acclimatization by decreasing relative humidity to ambient condition over a period of 1 week, plantlets are exposed to greenhouse conditions (Figure 1F).

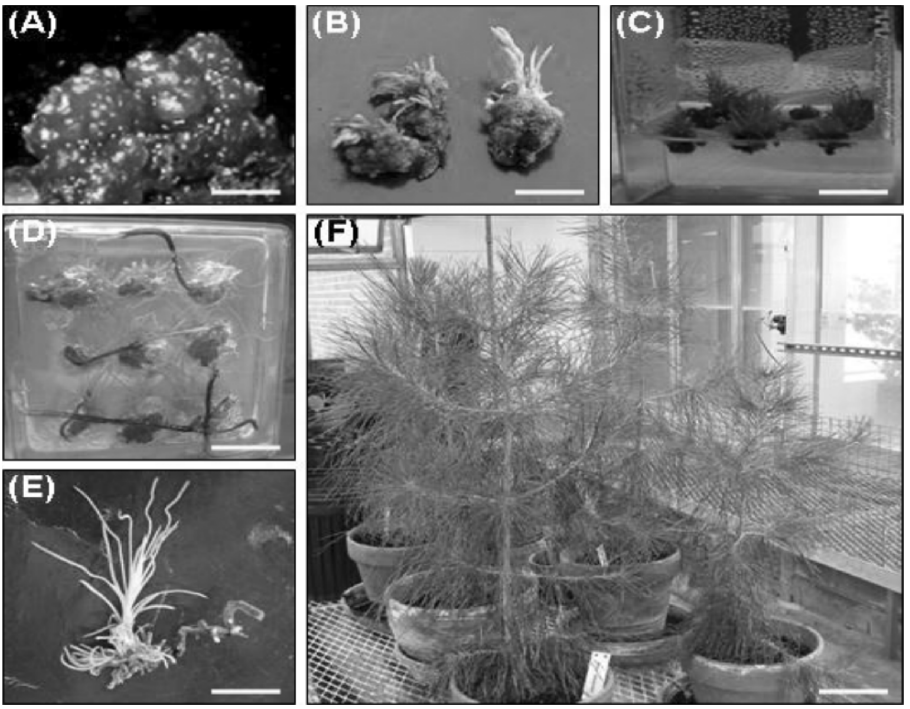

Figure 1. *Plantlet regeneration via organogenesis from callus cultures in slash pine. A) Callus cultures induced from mature embryos cultured for 3 weeks on callus induction medium. B) Clusters of adventitious shoots 6 weeks after callus cultures are transferred into shoot formation medium. C) Clusters of adventitious shoots 9 weeks after callus cultures are transferred onto shoot formation medium. D) Rooting of elongated shoots on rooting medium for 6 weeks. E) Plantlets before transferring into potting soil. F) Regenerated plants established in potting soil in greenhouse for 18 months. (A, bar = 0.5 cm; B, bar = 0.8 cm; C and D, bars = 1.1 cm; E, bar = 2 cm; F, bar = 8 cm.)*

2.6. Field Testing

After acclimatization, plantlets are taken out from the LifeGuard plant growth vessels (Sigma) and washed completely in tap water to remove the medium. The washing takes about 30 min. Plantlets are then planted into potting soil. In the first week, plantlets are watered two times a day. After that, they are watered once a day. Survival rate of regenerated plantlets is evaluated 6 weeks after their transfer to soil. More than 90% of the acclimatized plantlets survived in greenhouse.

3. CONCLUSION

The protocol established here is highly reproducible for the production of plantlets via organogenesis in four genotypes of slash pine. Plant growth regulators and the physiological activity of the explants are very important for successfully inducing plant regeneration via organogenesis in pine species. Mature zygotic embryos are good explants for the establishment of highly regenerable multiple shoot cultures of slash pine. The procedure presented here has several advantages over previously published reports of successful embryogenic callus induction from immature embryos. First, seeds of slash pine can be easily provided at any time throughout the year, but immature embryos are only available at the specific season of the year. Second, the process from callus to plant regeneration takes only a few months (8–10 months) which is less than plant regeneration via somatic embryogenesis. Third, plant regeneration from organogenic calli is a simple and highly efficient short-term in vitro regeneration system. There is no difference in the survival rate regenerated plantlets among different genotypes (genotypes 1177, 1178, 7524, 7556) used in this study. Regenerated plantlets produced from six basal media (DCR, BMS, LP, MS, SH, and TE) have very similar survival rates. The plant regeneration protocol established in this investigation may facilitate future research in genetic transformation in slash pine and other conifers.

4. REFERENCES

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