Plantlet regeneration from mature zygotic embryos and embryonic explants of masson pine (*Pinus massoniana* Lamb.)¹

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ABSTRACT

Excised zygotic embryos, cotyledons and hypocotyls of juvenile seedlings of masson pine were grown on DCR medium supplemented with several concentrations of various plant phytohormones. BA (1.0 mg/L) in combination with NAA (0.05 mg/L) in DCR medium was found to increase the formation of adventitious buds from mature zygotic embryos, but most of them were formed at the tips of embryonic cotyledons. Adventitious buds were obtained from cotyledons and hypocotyls from juvenile seedlings when they were cultured on DCR medium containing BA 3-5 mg/L and NAA 0.1-0.2 mg/L. Elongation of buds were observed on hormone-free DCR medium with or without activated charcoal (0.5%). Root initiation was achieved with full or half strength DCR medium supplemented with IBA 1.0 mg/L and NAA 0.25-0.5 mg/L. Approximately 11-20 axillary buds formed on each explant when juvenile seedling explants were treated (3-20 h) with BA 50-100 mg/L, followed by transfer to hormone-free DCR medium. The maximum number of shoots obtained per explant within six months was 33.

Key words: *Pinus massoniana* Lamb., DCR medium, mature zygotic embryos, embryonic explants, plantlet regeneration.

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Abbreviations: BA=N⁶-benzyladenine; DCR=Douglas-fir cotyledon revised medium; GA₃=gibberellin; IBA=indole-3-butyric acid; MS=Murashige-Skoog medium; NAA=α-naphthalene-acetic acid; SH=Schenk and Hildebrandt medium.

INTRODUCTION

The potential value for reforestation offered by plant tissue culture techniques has been widely recognized. For conifer propagation, it is known that the most critical factors are kinds of explants, media components and cytokinin treatment[1,2]. To date, the best results have been obtained using mature zygotic embryos and embryonic explants, and BA alone or in combination with NAA has been shown very effective in promoting bud differentiation in most of the *Pinus species*[2-5].

Masson pine (*Pinus massoniana* Lamb.) is one of the most widespread conifers in the southern part of China, and is highly prized because of its adaptation to both local climate and soil. It is extensively and successfully used in reforestation. Pine moth disease has contributed greatly to its decline in commercial value. Recently, a program has been initiated to obtain transgenic pest resistant masson pine by genetic manipulation in our laboratory. Successful genetic manipulation of conifers through gene transfer and transgenic plant production is dependent upon effective plant regeneration systems. Although *in vitro* plantlet formation has been now achieved with about 28 *Pinus species*[6], no plantlet regeneration, as far as we can aware, has been reported for *Pinus massoniana* Lamb.

The aim of the present study is to develop a method for the induction of adventitious buds on excised whole mature zygotic embryos and embryonic explants of masson pine, and to stimulate the development of these buds into shoots. Whole plantlets were obtained after rooting treatment of elongated shoots.

MATERIALS AND METHODS

1. Seed source and surface sterilization

Seeds of *Pinus massoniana* Lamb. collected from three different regions of China (Fujian-A, Zhejiang-B and Hubei-C, supplied by Nanjing Forestry University, Nanjing) were used in the present study. All seeds were stored at 4°C until used. Seeds were washed under running water for 6 h, surface sterilized with 3.0% (w/v) calcium hypochlorite for 12 min, washed twice with sterile distilled water, and then treated with 0.1% (w/v) HgCl₂ for 2 min. Finally, they were rinsed 3-4 times with sterile distilled water before excision. Zygotic embryos were dissected out of the surrounding endosperms and placed horizontally on the surface of the agar-solidified shoot induction media (Fig 1). Cotyledons and hypocotyls were excised from 7-day-old seedlings.

2. Culture media and shoot production

To compare the bud induction capacity of DCR (Gupta and Durzan, 1985)[7], SH (Schenk and Hildrandt, 1972)[8] and MS (Murashige and Skoog, 1962)[9], the macronutrients, micronutrients and organics of the three media (Tab 1) were tested in a pilot experiment. All media contained 3% (w/v) sucrose and 0.65% Difco purified agar. BA 1.0 mg/L and NAA 0.1 mg/L were added, and the pH was adjusted to 5.9 before autoclaving for 20 min at 121 °C. Each 50-ml Erlenmeyer flask contained 25 ml of the medium. Cultures were incubated at 25 ± 1 °C with 12 h photoperiod of fluorescent light at approximately 50-80 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$. Rates of bud induction were compared at the end of a 4 week incubation period. Since in the pilot experiment DCR medium was shown to be much more effective than SH and MS medium, six BA: NAA combinations (Tab 2) in DCR medium were used to determine the effect of different cytokinin:auxin combination on bud induction

of masson pine zygotic embryos. Twenty to twenty-five embryos were used for each combination at each different concentration, and a control without cytokinins or auxins was used for comparison. Meanwhile, the cotyledons and hypocotyls excised from juvenile seedlings were placed on DCR medium containing BA 3-5 mg/L and NAA 0.1-0.2 mg/L to induce the formation of adventitious buds, and 48 explants were used for each treatment which was repeated at least twice.

Tab 1. Composition of three media used in studies on regeneration of plantlets from mature zygotic embryos of masson pine

Compound\Media	SH	DCR	MS
Macronutrients		mg/L	
KNO ₃	2500	340	1900
Ca(NO ₃) ₂ ·4H ₂ O	-	556	-
NH ₄ H ₂ PO ₄	300	-	-
KH ₂ PO ₄	-	170	170
CaCl ₂ ·2H ₂ O	200	85	440
MgSO ₄ ·7H ₂ O	400	370	370
NH ₄ NO ₃	-	400	1650
Micronutrients			
FeSO ₄ ·7H ₂ O	15.0	27.8	27.8
Na ₂ .EDTA.H ₂ O	20.0	37.3	37.3
H ₃ BO ₃	5.0	6.2	6.2
ZnSO ₄ ·7H ₂ O	1.0	8.6	8.6
MnSO ₄ ·H ₂ O	10.0	22.3	16.9
Na ₂ MoO ₄ ·2H ₂ O	0.1	0.25	0.25
KI	1.0	0.83	0.83
CuSO ₄ ·5H ₂ O	0.2	0.25	0.025
CoCl ₂	0.1	0.025	0.025
NiCl ₂	-	0.025	-
Vitamins & other organic constituents			
Myo-inositol	1000	200	100
Glycine	-	2.0	2.0
Thimine.HCl	5.0	1.0	0.1
Nicotinic acid	5.0	0.5	0.5
Pyridoxine. HCl	0.5	0.5	0.5

Tab 2. Comparison of adventitious shoot production in mature zygotic embryos of masson pine (Fujian-A) as affected by different hormone combination and by different media

Treatment mg/L	No. of embryos tested	No. of embryos with at least 2 shoots	Average no. of shoots per embryo (Mean ± S.E.)
BA/NAA (DCR)			
Control	76	5	1.1 ± 0.2
1.0/0.01	61	11	3.7 ± 1.1
5.0/0.01	65	10	3.4 ± 0.7
1.0/0.05	89	36	13.4 ± 2.0
5.0/0.05	80	20	8.3 ± 1.6
5.0/0.1	57	14	6.5 ± 1.9
10.0/0.1	59	7	3.9 ± 1.3
Media (BA 1.0/NAA 0.1)			
DCR	64	27	11.2 ± 1.4
SH	64	11	5.4 ± 1.2
MS	64	6	2.8 ± 0.7

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In a separate study for pulsing treatment, seedling explants were treated with an aqueous non-buffered solution, pH 5.5, containing 50-100 mg/L BA. The treatment was carried out by immersing the apical buds of seedlings in a 20 ml aliquot of the filter sterilized BA solution in 50 ml glass flasks for 3, 6, 12 and 20 h. After the induction pulse, treated apical buds were blotted on sterile filter paper, and then plated vertically in the agar-solidified DCR medium without hormones.

Four to six weeks after initiation, small shoot buds were transferred to DCR media without hormones for elongation with monthly transfer to a similar medium for 2-3 passages until the shoots reached a minimum height of 10-15 mm. The shoots were then separated individually from the explants and used for rooting. In some cases, activated charcoal (Xi'an, China) 0.5% (w/v) was used in elongation medium.

3. Root induction of adventitious shoots

157 adventitious shoots derived from 16 seeds of masson pine (Fujian-A) were tested. Shoots selected for rooting were distributed for the following two treatments:

a) Shoots were placed on full or half strength macro and microelements of agar-solidified DCR medium containing 1.0 mg/L IBA and 0.25-0.5 mg/L NAA.

b) Shoots were pulse-treated with DCR medium containing NAA 5.0 mg/L for 20 h, blotted on sterile filter paper, and then transferred to the agar-solidified DCR medium without hormones.

Total number of shoots which formed roots were counted after 8 weeks. All experiments were repeated at least twice.

RESULTS AND DISCUSSION

1. Shoot production

The effect of media and BA/NAA concentration on the shoot induction of zygotic embryos is summarized in Tab 2. Among the three media (Tab 1) employed in this study, the promotion of organogenesis was best on DCR medium. This medium is characterized by a low concentration of potassium (4.61 mM) and a high concentration of calcium (3.93 mM). The concentration of ammonium (5.0 mM) is intermediate between that in SH (2.6 mM) and MS (20.5 mM). In fact, this medium has been found to be very useful by other authors [7, 10, 11]. Medium effect has been also reported by Flinn et al. (1986) [12] who found SH medium being superior to MS medium. This may be due partly to the high level of ammonium and high nitrogen concentration in MS medium. According to Perez-Bermudez and Sommer (1987) [13], significantly higher number of shoots was induced when ammonium was omitted.

The embryos grew well in culture and produced normal looking seedlings in hormone-free DCR medium. Adventitious bud formation was apparent in all treatments contained BA and NAA after 4-6 weeks. BA at 1.0 mg/L in combination with NAA at 0.05 mg/L produced the greatest average number of shoots per embryo (13.4 ± 2.0) among the embryos that produced buds. This figure compares favorably with the average shoot production per culture of 15 shoots per embryo reported for *P. strobus* by Schwarz et al. (1988) [4] on MS medium and the 14.7 shoots per cotyledon-hypocotyl explant of *P. strobus* on MS medium achieved by Kaul (1987) [5]. Most of the adventitious buds were formed directly from the cotyledons of masson pine (Fig 2). The pattern of bud initiation at the tips of embryonic

Cotyledons was similar to that observed in *Radiata* pine (Aitiken et al., 1987)[14] and Calabrian pine (Abdullah et al., 1985)[3].

Excised cotyledons and hypocotyls produced numerous adventitious buds at the surface or wound regions of the explants after 4-6 weeks of culture on DCR medium containing BA 3-5 mg/L and NAA 0.1- 0.2 mg/L (Tab 3, Fig 4, 6). The average number of adventitious buds produced per explant varies greatly depending on the different genotypes treated.

Tab 3. Adventitious bud production of cotyledons and hypocotyls of masson pine on DCR medium

Media BA (mg/L)	NAA	Cotyledon	Hypocotyl		
		average No. of buds/explant (Mean $\pm$ S.E.) (Fujian-A)	average No. of buds/explant Fujian-A Zhejiang-B Hubei-C		
3.0	0.1	9.7 $\pm$ 1.3	11.4 $\pm$ 2.3	6.7 $\pm$ 1.5	6.5 $\pm$ 1.4
5.0	0.1	8.4 $\pm$ 1.7	19.3 $\pm$ 2.1	7.5 $\pm$ 1.3	6.2 $\pm$ 1.2
3.0	0.2	12.9 $\pm$ 2.4	nd	nd	nd
5.0	0.2	7.8 $\pm$ 1.9	10.7 $\pm$ 2.7	nd	nd

nd=not done

Buds grown from cotyledonary axils, hypocotyls and those arose on the cotyledon tips, when transferred to hormone-free DCR medium or DCR medium supplemented with IBA 0.5 mg/L, NAA 0.2 mg/L and GA₃ 0.05 mg/L, showed rapid development into normal shoots after 2-4 weeks (Fig 3, 5, 7). However, the best shoot formation occurred with those buds induced at the lower levels of BA (1.0 mg/L) on both DCR and SH. The incorporation of activated charcoal in the medium enhanced the shoot growth slightly (Fig 8).

After pulse treatment with 50-100 mg/L BA and two to three weeks culture on hormone-free DCR medium, 83% of the *P. massoniana* seedling explants had at least one elongated axillary bud. The growth and elongation of axillary buds were more rapid than adventitious buds induced on masson pine hypocotyls and cotyledons. Over 70% of the seedlings formed axillary and adventitious buds after 3-20 h pulse treatment, and the best response was obtained when explants were pulse-treated with BA for 6-9 h (Fig 9). Longer times of pulsing treatment may produce higher average number of buds per explant, but these do not elongate normally.

2. Rooting

Rooting of woody species and especially conifers remains a limiting step for whole plant regeneration in vitro. In general, roots develop either directly from the stem or indirectly via a wound tissue. Some *Pinus* species can spontaneously develop roots directly from the stem, while others need an auxin treatment for rooting. IBA usually has a positive effect on rooting of conifer cuttings. For *P. massoniana*, IBA and NAA are required for direct or indirect root initiation.

The effect of auxins was dependent not only on the concentration but also on the length of treatment. In some cases, regenerated shoots were treated with IBA and

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NAA during the whole rooting period and the percentage of rooting and the number of roots per shoot were increased obviously. The fastest rooting and the highest rooting percentage (40.4%) were obtained with adventitious shoots from Fujian-A treated with DCR supplemented with IBA 1.0 mg/L and NAA 0.25 mg/L for about 6-8 weeks (Tab 4). Treatment with higher NAA concentration (5.0 mg/L) could produce some stronger roots, however, the percentage of rooting and the number of roots per shoot, in this case, were very low.

Tab 4. IBA and NAA stimulation of rooting of Masson pine (Fujian-A) shoots from adventitious buds on DCR medium. Data were taken after 8 weeks.

Treatment (mg/L)	No. of shoots treated	Rooted shoots (%)
DCR+IBA 1.0+NAA 0.25	47	19 (40.4%)
1/2 DCR+IBA 1.0+NAA 0.5	55	12 (22.0%)
1/2 DCR+NAA 5.0	55	3 (5.5%)

Roots were visible to the naked eyes after 35 days. The mean number of roots formed was 1-2.6 per shoot, with the highest 7 roots per shoot. Most roots were about 0.5-1 cm in length, and only a few of them reached 2-4 cm. Numerous lateral roots were present during the whole rooting process (Fig 10, 11).

Fig 1. An initial zygotic embryo, $\times 4.5$

Fig 2. Cotyledon tips with adventitious buds from a zygotic embryo after 8 weeks in culture, $\times 6$

Fig 3. Elongation and the secondary buds from shoot base of an isolated shoot from an embryonic explant on hormone-free DCR medium. $\times 4.5$

Fig 4. Adventitious bud formation from a cotyledon explant after 4 weeks of incubation on DCR medium containing BA 3.0 mg/L and NAA 0.2 mg/L. $\times 4.5$

Fig 5. Adventitious shoot formation of cotyledons on DCR medium supplemented with IBA 0.5 mg/L, NAA 0.2 mg/L and GA_3 0.05 mg/L. $\times 3.5$

Fig 6. Bud formation on the surface of a hypocotyl explant on DCR medium containing BA 5.0 mg/L and NAA 0.1 mg/L. $\times 2.7$

Fig 7. Adventitious shoot formation on hypocotyls on DCR medium supplemented with IBA 0.5 mg/L, NAA 0.2 mg/L and GA_3 0.05 mg/L. $\times 3.5$

Fig 8. Elongation of shoot bud induced on DCR medium containing BA 1.0 mg/L and NAA 0.05 mg/L after 6 weeks on DCR medium with 0.5% activated charcoal. $\times 4.3$

Fig 9. Bud development at the axils of cotyledons of masson pine after 9 h pulse-treated of a 7-day-old seedling explant with 100 mg/L BA. $\times 3.7$

Fig 10, 11. Plantlets with one (10) to four (11) elongated roots on adventitious shoots derived from bud induction DCR medium containing BA and NAA. 10: $\times 0.9$, 11: $\times 2.3$

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