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Research note

Direct organogenesis and plantlet regeneration from mature zygotic embryos of masson pine (*Pinus massoniana* L.)

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Abstract

Mature zygotic embryos of masson pine were cultured as initial explants to investigate the process of direct organogenesis. Adventitious buds were initiated on DCR medium (Douglas-fir cotyledon revised medium) supplemented with 0.5 mg l^{-1} N^6-benzyladenine (BA) and 0.05 mg l^{-1} indolebutyric acid (IBA) or α-naphthaleneacetic acid (NAA). The highest induction frequency of adventitious buds was 99.3%. Subsequent transfer of buds to medium with lower concentrations of plant growth regulators in time was necessary for differentation of high quality adventitious buds. After culturing on elongating medium, in which the proportion of cytokinins to auxins was reduced, shoots higher than 2 cm were transferred for root induction to GD medium with half of the concentration of macro-salts (½ GD) and with 2 mg l^{-1} IBA and 0.05 mg l^{-1} BA. The average root frequency was over 70%. After adventitious roots had appeared, the shoots were transferred to ½ GD medium with a lower concentration of IBA (0.2 mg l^{-1}) for further root development.

Abbreviations: BA-N^6 – benzyladenine; DCR medium – Douglas-fir cotyledon revised medium; GA_3 – gibberellic acid; GD – Gresshoff and Doy; IBA – indolebutyric acid; KT – kinetin; NAA – α-naphthaleneacetic acid; ZT – zeatin

Clonal propagation by organogenesis is a tissue culture preparation method. It allows for *in vitro* regeneration of conifers, and is an efficient tool for mass production of genetically improved material. The plantlet regeneration by tissue culture of conifers was first reported by Sommer et al. (1975) in longleaf pine. Successful plant regeneration has since been reported for a large number of conifer species via organogenesis using various explants (Abdullah and Grace, 1987; Chang et al., 1991; Garcia-Ferriz et al., 1994; Saborio et al., 1997; Mathur and Nadgauda, 1999; Prehn et al., 2003). *In vitro* clonal propagation may bypass the problems associated with irregular seed cone production, long life cycles and vegetative propagation by rooting and cuttings, and can be used to produce plantlets of desirable genotypes, thereby accelerating the selection, breeding and testing cycle (Thorpe et al., 1991).

Pinus massoniana L., the masson pine, is an important tree species for afforestation in South China. The timber is used for construction, railway sleepers, mine timber, furniture, wood pulp, etc., and the trunk as a source of resin and tannin, and for cultivating fungi. It is widely planted in South China and occurs from plains to mountains,

from near sea level to 2000 m elevation (Wu and Raven, 1999). Long-term breeding programs were initiated in China for *Pinus massoniana* L. in the 1980s. So far, *in vitro* regeneration of masson pine has proven to be very difficult. Somatic embryogenesis from mature zygotic embryos of masson pine was reported by Huang et al. (1995), but they only obtained three regenerated plantlets. Kondo and Okamura (1995) reported tissue culture of wakamatsu, the interspecific hybrid between *Pinus thunbergii* and *P. massoniana*. In China, an efficient method for masson pine vegetative propagation would be desirable for forest breeding and afforestation programs. Protocols for genetic transformation in masson pine require improved tissue culture procedures. The present study was undertaken to establish a protocol for direct organogenesis and plantlet regeneration from mature zygotic embryos of masson pine.

Under natural conditions, the production of mature seeds of masson pine takes 24 months from floral bud formation. Mature seeds from open-pollinated *Pinus massoniana* trees were collected from Guangdong and Guangxi provinces (China). Seeds were stored in plastic bags at 4 °C until used as the source of embryos for all experiments. After washing seeds thoroughly under running tap water for 1 h, the seeds were surface sterilized in 70% ethanol (v/v) for 30 s, then treated with 12% sodium hypochlorite solution (v/v) for 12 min, and rinsed five times in sterile, distilled water. Mature zygotic embryos were aseptically excised from megagametophytes and placed horizontally on solidified bud induction medium. Twelve embryos were inoculated per Petri dish (25 ml medium in each 1.5×9 cm dish, sealed with a double layer of Parafilm). All media, except rooting media, were supplemented with 3% sucrose and gelled with 0.65% Difco agar. The pH was adjusted to 5.8 before autoclaving for 20 min at 115 °C. Cultures were incubated at 25 ± 2 °C under a 14-h photoperiod with cool white fluorescent lights (80 μmol m^{-2} s^{-1}).

Mature zygotic embryo explants were cultured on adventitious bud induction medium consisting of different basal media such as DCR (Gupta and Durzan, 1985), GD (Gresshoff and Doy, 1972) and ½ MS (Murashige and Skoog, 1962; half of the concentration of macro-salts), supplemented with 0.3–3 mg l^{-1} benzyladenine (BA), 0.05–0.3 mg l^{-1} α-naphthaleneacetic acid (NAA) or 0.05–0.3 mg l^{-1} indolebutyric acid (IBA). For bud induction, each treatment was replicated at least three times, and each replicate consisted of 50–100 embryo explants. Adventitious buds from the initiation medium were cultured on DCR medium with 0.1 mg l^{-1} BA and 0.2 mg l^{-1} IBA for elongation. After elongation the clusters of adventitious shoots over 1 cm in length were segmented and subcultured on fresh elongation medium every 3–4 weeks. Elongated shoots (2–3 cm in length) were exposed to root initiation medium consisting of ½ GD (half of the concentration of macro-salts) supplemented with 2 mg l^{-1} IBA, 0.05 mg l^{-1} BA and 2% sucrose. Developing roots would appear in 3–5 weeks. The shoots with roots were kept in root induction medium for 1 week. Then they were subcultured on ½ GD medium with 0.2 mg l^{-1} IBA and 0.05 mg l^{-1} BA for further root development (4 weeks). Other auxins and basal media were also tested. For auxin tests, 100 shoots were used for each treatment, for basal media tests 50 shoots were used per treatment. The percentage of shoots forming adventitious roots was scored after 6 weeks on root initiation medium. Rooted plantlets were transferred to pots filled with a mixture of vermiculite and perlite (3:1 v/v). They were covered with a plastic bag to maintain a high relative humidity and placed in the culture room at 25 ± 2 °C under a 14-h photoperiod with cool white fluorescent lights (80 μmol m^{-2} s^{-1}) for 1 week, then the acclimatized plantlets were exposed uncovered to greenhouse conditions.

When mature zygotic embryos were cultured on bud induction medium for 1 week, the cotyledons were splayed and turned green while the radicles dilated and turned red. After 2–3 weeks, adventitious buds developed on the apex of the cotyledons and in the region around the base of the cotyledons (Figure 1a). A large number of buds clustered on the expanded apical dome, most likely because lateral buds elongated consecutively on the apical dome. Among the different basal media used in this study (Table 1), it was obvious that use of DCR basal medium resulted in the greatest frequency of adventitious masson pine bud formation.

BA had a better bud-inducing effect on the mature zygotic embryos than did KT or ZT (Table 2). Most of the mature zygotic embryos treated with ZT showed an initial enlargement and

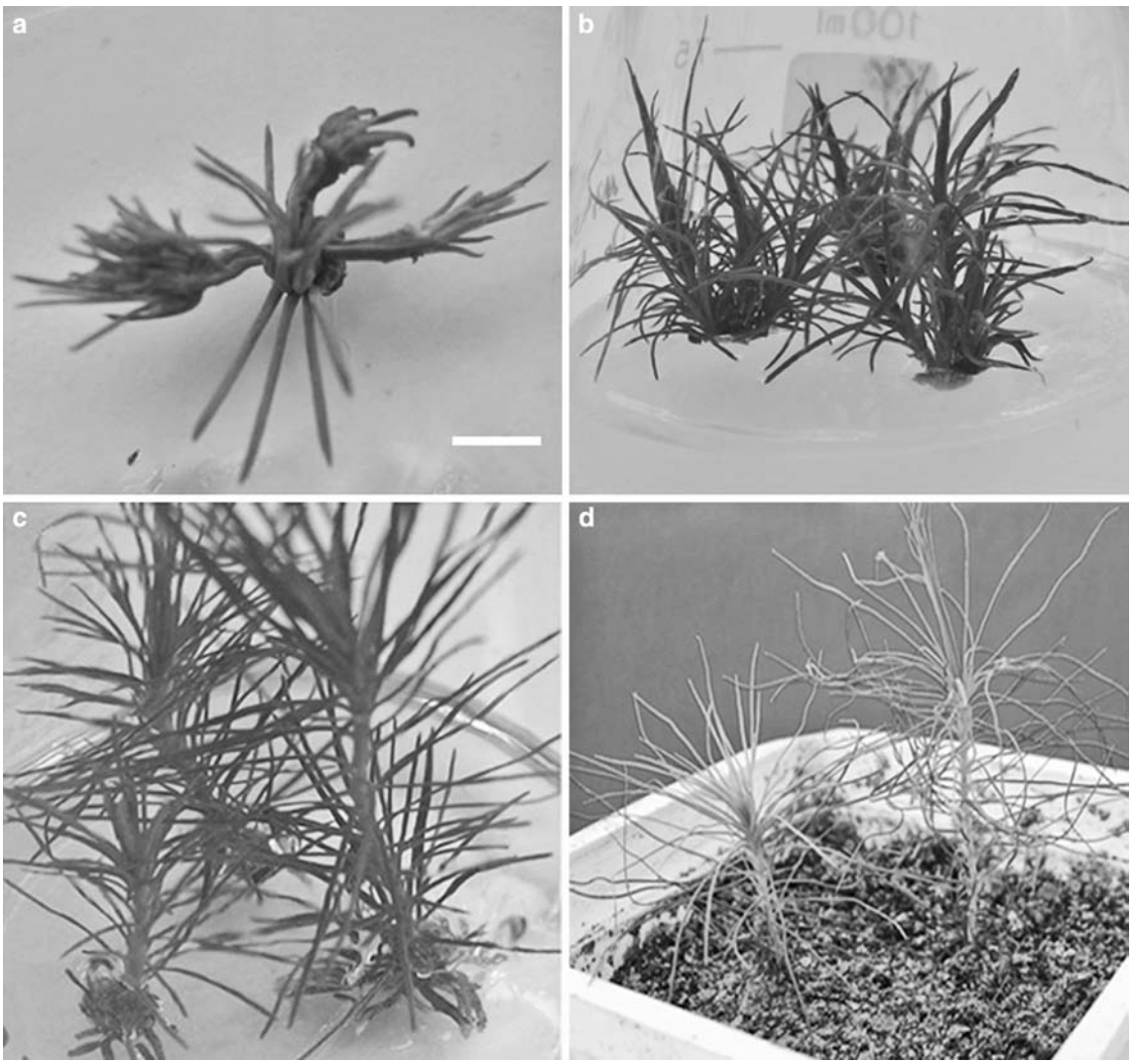

Figure 1. Direct organogenesis and plantlet regeneration from mature zygotic embryos in masson pine. (*a*) Adventitious buds differentiated from a mature zygotic embryo (bar 1 mm); (*b*) adventitious shoots on elongation medium; (*c*) shoots with adventitious roots on rooting medium; (*d*) regenerated plantlets established in a mixture of vermiculite and perlite.

Table 1. Effect of different basal media on adventitious bud induction in *Pinus massoniana* L. mature zygotic embryo

Basal medium	Explant number	Embryos forming buds (%)	Mean number of buds per embryo
DCR	100	99.3 ± 0.6^{a}	9.83 ± 0.72^{a}
GD	100	80.3 ± 1.5^{b}	7.64 ± 0.83^{b}
½ MS	100	31.3 ± 2.5^{c}	3.18 ± 0.29^{c}

All media were supplemented with 0.5 mg l^{-1} BA and 0.05 mg l^{-1} IBA. Values followed by different letters are significantly different at $p < 0.05$ according to LSD multiple range test.

dedifferentiation, but finally turned brown and died. This finding is consistent with that reported for mature zygotic embryo explants of *Pinus wallichiana* (Mathur and Nadgauda, 1999). KT had a mildly stimulating effect on adventitious bud initiation (Table 2). In masson pine, a low concentration of BA (0.3 mg l^{-1}) induced adventitious bud formation. The best adventitious bud induction, 99.3 and 98.7% (Tables 1 and 2), was observed on DCR medium supplemented with 0.5 mg l^{-1} BA and 0.05 mg l^{-1} IBA. Bud induction frequency increased with increasing concentrations

Table 2. Effect of different cytokinins on adventitious bud induction in *Pinus massoniana* L. mature zygotic embryos

Cytokinin	Concentration (mg l^{-1})	Embryos forming buds (%)	Mean number of buds per embryo	Mean number of elongated shoots*
BA	0.3	55.3 ± 3.1^{cd}	2.83 ± 0.31^{de}	1.82 ± 0.03^{ef}
	0.5	98.7 ± 1.2^{a}	10.90 ± 0.95^{a}	4.46 ± 0.12^{a}
	1.0	86.7 ± 3.1^{b}	11.60 ± 0.79^{a}	3.55 ± 0.40^{b}
	2.0	42.0 ± 3.5^{e}	6.53 ± 0.84^{b}	2.25 ± 0.18^{de}
	3.0	18.7 ± 3.1^{fg}	3.30 ± 0.46^{d}	1.39 ± 0.20^{f}
KT	0.5	51.3 ± 1.2^{d}	4.47 ± 0.15^{c}	2.85 ± 0.37^{cd}
	1.0	61.3 ± 3.1^{c}	5.17 ± 0.31^{c}	3.23 ± 0.27^{bc}
	2.0	13.3 ± 3.1^{gh}	2.47 ± 0.21^{de}	Not done
	3.0	7.3 ± 2.3^{h}	2.20 ± 0.17^{de}	
ZT	0.5	15.3 ± 2.31^{g}	2.13 ± 0.15^{e}	
	1.0	24.0 ± 3.5^{f}	2.67 ± 0.25^{de}	
	3.0, 5.0	No bud induction		

*Mean number of elongated shoots (higher than 1.5 cm) per embryo after 2 month cultured on elongation medium.
DCR basal medium contained 0.05 mg l^{-1} IBA. Values followed by superscript letters are significantly different at $p < 0.01$ according to LSD multiple range test. Each treatment contained 50 explants.

of BA until the concentration exceeded 1 mg l^{-1}, at which point the frequency decreased. High BA (exceeding 1 mg l^{-1}) concentration had at least two disadvantages in this experiment:

– it had a negative influence on bud elongation by restricting the elongation of the stem (Table 2) and
– the number of hyperhydrated shoots increased.

Very few hyperhydrated shoots were seen at a BA concentration of less than 1 mg l^{-1}. The development of adventitious buds was asynchronous. There were often many small adventitious, primordial buds surrounding the larger shoots. Elongation of high quality adventitious buds required that they were transferred to the medium with a lower concentration of plant growth regulators in time. Generally, in tissue culture of conifers, the medium needs to be replaced to promote elongation of stem and leaves after bud formation (Okamura and Kondo, 1995). We used DCR medium supplemented with 0.1 mg l^{-1} BA and 0.2 mg l^{-1} IBA as the bud subculture medium for elongation (Figure 1b).

Elongated adventitious shoots, 2–3 cm in height were subjected to a rooting study. NAA and IBA are used widely to induce adventitious roots in plant tissue culture(Saborio et al., 1997; Prehn et al., 2003; Schestibratov et al., 2003). When NAA was used to induce adventitious roots, a mass of callus from which root primordia differentiated was produced on the base of most shoots. Callus production, not only decreased rooting frequency, but also resulted in abnormal roots. Therefore, IBA application was a preferable alternative for the root induction (Figure 2). IBA had a greater effect on promoting root induction and subsequent development of the induced roots proceeded normally. Different basal media were also tested in the experiment (Figure 3). The highest root induction frequency was 78.8% on ½ GD (Figure 3). Root initiation from the base of the excised shoots took 4–6 weeks (Figure 1c). Among 300 adventitious

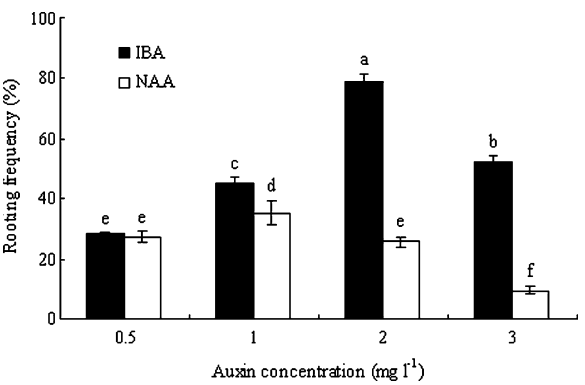

Figure 2. Effect of the auxins IBA and NAA on rooting frequency of adventitious shoots after a 6-week culture on ½ GD basal medium. Values represent the mean of three repeated experiments, each with 100 explants. Columns denoted by different letters are significantly different at $p < 0.05$ according to LSD multiple range test. Vertical lines set in the columns represent the standard error.

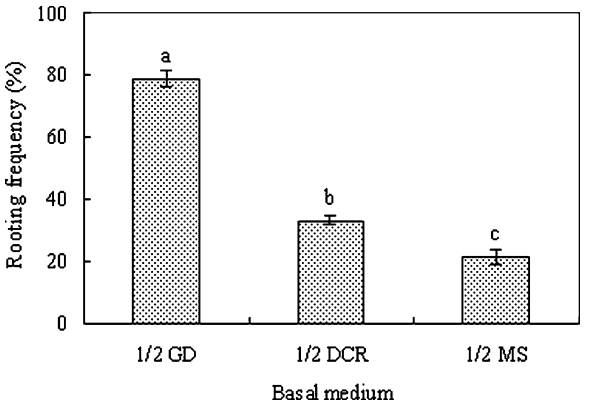

Figure 3. Effect of different basal media on rooting frequency of adventitious shoots after a 6-week culture on medium with 2 mg l^{-1} IBA, 0.05 mg l^{-1} BA and 2% sucrose. Columns denoted by different letters are significantly different at $p < 0.05$ according to LSD multiple range test. Vertical lines set in columns represent standard error.

shoots incubated on ½ GD with 2 mg l^{-1} IBA and 0.05 mg l^{-1} BA, 218 shoots produced initiated roots after 6 weeks. One hundred and sixty-eight plantlets were established in soil in the greenhouse (Figure 1d). Eighty-two of these were survived after 2 months in the greenhouse.

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