Plant Regeneration from Callus Derived from Immature Embryo Cotyledons of *Prunus mume*

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Abstract. A regeneration protocol for *Prunus mume sieb. et Zucc* was developed through indirect organogenesis. Immature cotyledons were excised from the open-pollinated seeds of two cultivars and cultured on a modified MS medium supplemented with various combinations of plant growth regulators. Shoot-organogenic calli were induced on half-strength MS medium supplemented either with combinations of 2.2 μM benzyladenine (BA), 5.4 to 10.8 μM 1-naphthaleneacetic acid (NAA), and 0 to 5.0 μM indolebutyric acid (IBA) or with combinations of 2.2 μM BA, 4.5 to 9.0 μM dichlorophenoxyacetic acid (2,4-D), and 0 to 5.0 μM IBA. High frequencies of shoot regeneration (81.5% and 91.3% in *P. mume* cvs. Lv'e and Xuemei, respectively) were obtained from smooth-white nodular calli cultured on a half-strength MS medium supplemented with 2.2 μM BA, 2.2 μM thidiazuron, and 1.0 μM IBA. A high rate of rooting (90.2% and 88.9% in 'Lv'e' and 'Xuemei', respectively) occurred when shoots were cultured on WPM supplemented with 5.0 μM IBA. Chemical names used: benzyladenine (BA), thidiazuron (TDZ), indolebutyric acid (IBA), dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA).

Prunus mume is a beautiful deciduous tree with fragrant flowers that blooms very early in the spring. It is renowned for its attractive blossoms and longevity throughout China and Japan. In addition, *P. mume* has medical value (Dogasaki et al., 2004) with "pickled plums," made from the unripe fruits of *P. mume*, being used to treat vomiting and fever (Chen, 1962). As a result of its economic importance as an ornamental and medicinal tree, there is significant interest in breeding elite varieties of *P. mume*, and this has primarily focused on using molecular markers to identify desirable phenotypes and characterizing the mechanism of self-incompatibility (Entani et al., 2003; Tao et al., 2000).

During the last 2 decades, efficient in vitro culture systems have been developed for various species of the *Prunus* genus. The majority of these have been achieved by inducing regeneration from either immature tissues (Hashmi et al., 1997; Hokanson and Pooler, 2000; Mante et al., 1989; Pooler and Scorza, 1995; Tang et al., 2000) or mature explants (Andrea and Johannes, 2005; Bhagwat and

David, 2004; Cheong and Pooler, 2004; Declerck and Korban, 1996; Gentile et al., 2002; Hammatt and Grant, 1998; Neil and Neil, 2000; Olaya et al., 2000). For *P. mume*, an in vitro culture system has been reported for the induction of callus, but plant regeneration was not achieved (Liu and Chen, 1999). Although it is possible to conduct traditional breeding programs in *P. mume*, particularly with the assistance of molecular markers, this is a protracted process because of the long intergeneration times. Therefore, to accelerate the genetic improvement of *P. mume*, genetic engineering appears to be an attractive option allowing the introduction of heterologous genes for the enhancement of ornamental or other valued characteristics. Efficient plant regeneration from in vitro cultures is essential for the genetic transformation of *P. mume*. However, to date, there are very few reports of the successful regeneration of plants from cultures of *P. mume*.

This article presents the first report of the successful regeneration of plants of *P. mume* achieved through indirect organogenesis from cotyledons of immature embryos. In the course of this work, we also investigated the effects of the original explant source and the light environment on the rate of shoot organogenesis from callus cultures.

Materials and Methods

Plant material. Open-pollinated, immature seeds (≈ 40 to 50 d after pollination) were collected from two mature trees of *P. mume* cultivars, Xuemei and Lv'e. The trees

had been growing in the Wuhan Mei flower garden (Wuhan, P.R. China) for over 10 years at the time of seed collection during Apr. and May 2005. The seeds were stored at 4 °C until use. After being washed thoroughly with running tap water for 30 min, the immature seeds were disinfected by immersion in 70% (v/v) ethanol for 30 to 60 s followed by one wash in sterile water, then immersion and agitation in a 0.1% (w/v) aqueous solution of HgCl₂ for 20 min, followed by three washes in sterile water. Cotyledons were aseptically excised and placed horizontally on the medium for callus induction. To investigate the effect of the original explant source on callus induction and subsequent plant regeneration, individual cotyledons were divided into proximal or distal sections with the embryonic axis removed.

Culture initiation and plant regeneration. The basal medium consisted of half-strength MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Sigma A1296, Sigma, St. Louis). This was supplemented with various combinations of plant growth regulators (see subsequently). The pH was adjusted to 5.8 with 1 M NaOH before autoclaving at 121 °C for 20 to 25 min. The media were dispensed into 90 mm petri dishes (25 mL of medium per dish), in the case of callus induction, or plastic boxes (250 mL, made in China; 40 mL per box) for shoot induction, shoot elongation, and rooting procedures.

Experiments of callus induction from cotyledon explants were performed on half-strength MS media supplemented with various levels of benzyladenine (BA), dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), and indolebutyric acid (IBA) supplied in 11 combinations (see Table 1). To stimulate adventitious shoot induction, callus outgrowths were transferred to various media supplemented with various levels of either BA and NAA or thidiazuron (TDZ) and IBA (Table 2).

All the cultures (unless stated otherwise) were maintained in a growth chamber at 25 ± 2 °C under a 14-h photoperiod of 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of photosynthetic photon flux (PPF).

Effect of callus source and light on shoot induction. It was found that half-strength MS medium supplemented with 2.2 μM BA, 10.0 μM NAA, and 5.0 μM IBA produced the most vigorous callus growth, and half-strength MS medium supplemented with 2.2 μM BA, 2.2 μM TDZ, and 1.0 μM IBA yielded the highest number of shoots. Therefore, these two media were used in all further experiments designed to test the effects of callus source and light conditions on rates of plant regeneration.

Shoot elongation, rooting, and acclimatization. To promote elongation growth of shoots, the regenerated buds were cultured on WPM medium (Lloyd and McCown, 1980) supplemented with 2.2 μM BA, 0.45 μM TDZ, and 1.0 μM IBA. After incubation for 3 weeks, the most vigorous shoots were cut at the base and transferred

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to a WPM medium supplemented with 2.5 or 5.0 μM IBA and incubated under lights to induce rooting. After 6 weeks, rooted plantlets (defined as having at least one root greater than 10 mm in length) were washed thoroughly with running tap water, to remove the culture medium, and transplanted into plastic pots containing a mixture of 2 peat-moss : 2 garden soil : 1 sand (by volume). These were then kept in a growth chamber set to 70% relative humidity and 19 °C. After 2 weeks, the pots were transferred to a greenhouse and maintained at average 19 °C for further development.

Statistical analysis. For callus induction, seven explants were incubated under each treatment regimen. Experiments of callus initiation and shoot induction were repeated at least three times. The differences between mean values (obtained from at least two independent experiments) were statistically evaluated by analysis of variance (ANOVA) using Duncan's multiple-range test with the least significant difference set to 5% probability.

Results

Callus initiation. No callus was obtained when immature cotyledons of *P. mume* (Fig. 1A) were cultured on a medium that was free from plant growth regulators or contained less than 2.7 μM NAA or 2.2 or less BA. However, calli (Fig. 1B) could be obtained when explants were cultured on half-strength MS supplemented with different concentrations of either BA, NAA, and IBA or BA, 2,4-D, and IBA (Tables 1 and 2). There were significant differences ($P < 0.05$) in the percentage of callus induction resulting from incubation of cotyledon explants on the various media. Across the combinations of plant growth hormones tested and the two cultivars of *P. mume*, the percentage of callus formation varied from 22% to 97% (Table 1). Growth of callus that was induced on various media varied considerably with respect to physical appearance and could be classified into three general types, i.e., 1 = light yellow, loose and globular; 2 = smooth white, loose and nodular (Fig. 1B); or 3 = light yellow, hard and globular.

Shoot regeneration from callus. In the most part, shoots regenerated through organogenesis were derived from the type 2 form of callus (see previously) (Fig. 1B) that had either originated from cotyledons or were from primary callus cultures. These calli maintained their morphogenetic potential for at least two rounds of subculturing. When these calli were cultured on half-strength MS supplemented with 2.7 μM NAA and 13.2 to 22.0 μM BA, small regenerated shoots (Fig. 1C, D) were observed to form on the callus after 4 weeks, but this tended to occur at fairly low frequencies. The further incorporation of TDZ in the regeneration medium significantly stimulated shoot regeneration. Thus, a medium supplemented with 2.2 μM BA and 1.0 μM IBA plus 2.25 μM TDZ

Table 1. Response of callus induction to plant growth regulators in immature *Prunus mume* cotyledons (cultivars Xuemei and Lv'e) on half-strength MS medium.^z

BA	Plant growth regulators (μM)			Percentage of explants forming callus (%)	
	2,4-D	NAA	IBA	Lv'e	Xuemei
2.2	0	0	0	0	0
2.2	0	5.4	0	22.2 $\pm$ 3.1 e	43.0 $\pm$ 4.2 e
2.2	0	5.4	2.5	55.0 $\pm$ 7.1 d	53.0 $\pm$ 4.2 de
2.2	0	5.4	5.0	74.9 $\pm$ 2.2 c	67.5 $\pm$ 10.6 dc
2.2	0	10.8	0	80.0 $\pm$ 1.0 bc	85.0 $\pm$ 1.4 abc
2.2	0	10.8	2.5	97.0 $\pm$ 1.4 a	97.5 $\pm$ 3.5 a
2.2	0	10.8	5.0	95.0 $\pm$ 7.1 a	90.0 $\pm$ 4.2 abc
2.2	4.5	0	2.5	81.6 $\pm$ 2.3 bc	95.5 $\pm$ 6.4 abc
2.2	9.0	0	2.5	91.6 $\pm$ 11.8 ab	85.0 $\pm$ 7.0 abc
2.2	4.5	0	5.0	81.5 $\pm$ 3.0 bc	75.0 $\pm$ 7.1 bc
2.2	9.0	0	5.0	71.5 $\pm$ 2.1 c	50.0 $\pm$ 14 de

^zValues (mean $\pm$ standard error) with the same letter within a column are not significantly different at $P = 0.05$ (Duncan's multiple range test).

BA = benzyladenine; 2,4-D, dichlorophenoxyacetic acid; NAA = 1-naphthaleneacetic acid; IBA = indolebutyric acid.

Table 2. Percentage of regeneration from cotyledon-derived callus in relation to plant growth regulators used with half-strength MS medium for two *Prunus mume* cultivars (Xuemei and Lv'e).^z

BA	Plant growth regulators (μM)			Percentage of calli forming shoots (%)	
	TDZ	NAA	IBA	Lv'e	Xuemei
4.4	0	2.7	0	0	0
8.8	0	2.7	0	0	0
13.2	0	2.7	0	27.8 $\pm$ 7.4 b	50.0 $\pm$ 7.1 cd
17.6	0	2.7	0	36.5 $\pm$ 5.0 b	63.4 $\pm$ 2.4 bc
22.0	0	2.7	0	73.4 $\pm$ 9.4 a	73.4 $\pm$ 4.7 ab
2.2	0.45	0	1.0	32.5 $\pm$ 10.6 b	36.7 $\pm$ 2.4 d
2.2	2.25	0	1.0	81.5 $\pm$ 2.1 a	91.3 $\pm$ 2.7 a
2.2	4.50	0	1.0	74.3 $\pm$ 10.8 a	65.0 $\pm$ 3.5 bc

^zData were collected 6 weeks after transferring the callus to the medium on which they were cultured for the first 2 weeks in the dark and then transferred to light. Values (mean $\pm$ standard error) within a column followed by the different letters are significantly different at $P = 0.05$ (Duncan's multiple range test).

BA = benzyladenine; TDZ = thidiazuron; NAA = 1-naphthaleneacetic acid; IBA = indolebutyric acid.

Fig. 1. Plant regeneration through shoot organogenesis from callus derived from cotyledons of immature embryos of *Prunus mume*: (A) Cotyledons from *P. mume* immature embryos. (B) Calli formed from the cotyledon explants (arrow). (C) Shoots regenerated from smooth white nodular calli of cotyledon explants in darkness. (D) Shoots regenerated from smooth-white nodular calli under light. (E) Shoot elongation. (F) Rooting (view of the bottom of plastic box).

produced the maximal regeneration frequencies of 81.5% and 91.3% in the 'Lv'e' and 'Xuemei' cultivars, respectively (Table 2). No significant difference in regeneration frequency was detected between calli initiated from the proximal or distal sections of

the cotyledons for either cultivar (Table 3). By contrast, the ambient light environment produced different regeneration responses in the two cultivars (Table 4). In 'Lv'e', regeneration frequency did not differ significantly under light compared with dark conditions

as tested by ANOVA analysis ($P < 0.05$). In 'Xuemei'; however, it was apparent that continuous darkness was beneficial for regeneration (Table 4).

Shoot elongation, rooting, and acclimatization. After growth in elongation medium for ≈ 3 weeks, shoots at least 1 cm in length (Fig. 1E) were cut and transferred to rooting medium. Within 4 weeks, these shoots had produced roots from the base of the excision site (Fig. 1F). The number of shoots that produced one or more roots (according to 50 tested shoots) and the number of roots (greater than 5 mm in length) per shoot differed significantly when cultured on WPM media as supplemented with 2.5 or 5.0 μM IBA (Table 5). By contrast, there was no significant difference between the two cultivars at a given concentration of IBA. After 6 weeks of rooting initiation, the plantlets were transferred to soil for "hardening." The survival rate of these regenerated plantlet was greater than 90% under greenhouse conditions.

Discussion

This is the first report, to our knowledge, of the successful in vitro regeneration of *P. mume* through organogenesis from callus derived from immature cotyledons.

In *Prunus* species, somatic embryogenesis has been the most commonly reported regeneration pathway (Cheong and Pooler, 2004; Hashmi et al., 1997; Tang et al., 2000). Directive shoot differentiation had been reported from immature cotyledons of

almond (Phillip et al., 2001; Saafi and Dulal, 2002) and some other woody plant cultures (Tan and Furtek, 2003; Wei et al., 2005). In this study of *Prunus mume*, it was possible to obtain abundant callus from immature embryos cultured on a medium supplemented with specific concentrations of plant growth regulators (Table 1), and shoots could be readily regenerated from the smooth-white nodular form of callus. This result is consistent with that reported for peach (Hammerschlag et al., 1985; Hashmi et al., 1997). During callus induction, increasing the concentration of NAA (in a medium also containing 2.2 μM BA) increased the percentage of callus formation (Table 1) and furthermore eliminated the secretion of phenolic substances from the cut surface of the cotyledons. This observation is consistent with Olaya et al. (2000) who reported that such phenolic substances are particularly liable to be oxidized by auxin oxidase when there is a high concentration of NAA in the culture medium. Callus was also readily obtained on media supplemented with 2.2 μM BA in combination with either of two auxins, i.e., NAA or 2,4-D and IBA. A high level of 2,4-D (9.0 μM) in combination with a low level of BA and IBA (2.2 and 1.0 μM , respectively) led to high callus yields. This was contrary to reports in other plant species (Luo et al., 1999; Nolan et al., 1989). Previous reports of other *Prunus* species have indicated that plantlet regeneration from leaves shows that WPM basic medium is more suitable for the induction of adventitious shoots than is MS basal medium (Andrea and Johannes, 2005;

Bhagwat and David, 2004; Hammatt and Grant, 1998; Neil and Neil, 2000; Tang et al., 2002). However, a high frequency regeneration of *P. mume* was obtained from organogenic callus derived from cotyledons that had been cultured on half-strength MS media supplemented with 2.2 μM BA, 2.25 μM TDZ, and 1.0 μM IBA. There was no difference in regeneration frequency from those calli derived from the distal, as opposed to proximal, parts of the cotyledons (Table 3). This is in contrast to observations from other *Prunus* species (Mante et al., 1989; Tang et al., 2000).

With regard to the frequency of shoot organogenesis from calli derived from the 'Xuemei' cultivar, the effect of a light versus dark incubation was significant (Table 4). In 'Lv'e', there was no significant effect. This result indicates that the importance of the light environment on shoot organogenesis from cotyledon-derived calli is genotype-dependent.

Recently, successful genetic transformation has been reported for a number of plant species through the use of cotyledon explants (Choi et al., 2001; Zaragoz et al., 2004), and transgenic plants have been obtained for some other *Prunus* species (Rosa et al., 2004). Here, we have described the culture conditions that can produce an efficient frequency of plantlet regeneration from immature cotyledon explants of *Prunus mume*. The regeneration method presented here is an essential basis for the introduction of heterologous genes through *Agrobacterium*-mediated transformation for the genetic improvement of this important tree species.

Table 3. Effect of explant source (proximal or distal to the embryonic axis) on adventitious shoot formation of two cultivars (Xuemei and Lv'e) of *Prunus mume*.^z

Cultivar	Source of callus	Percentage of calli forming shoots (%)
Lv'e	Distal section	85.5 $\pm$ 8.0 a
	Proximal section	75.8 $\pm$ 13.0 a
Xuemei	Distal section	81.0 $\pm$ 13.0 a
	Proximal section	87.5 $\pm$ 11.0 a

^zData were collected 5 weeks after transferring callus to shoot formation medium. Values (mean $\pm$ standard error) within a cultivar followed by different letters are significantly different at $P = 0.05$ (Duncan's multiple range test).

Table 4. Effect of light on adventitious shoot formation from cotyledon explants of the Xuemei and Lv'e cultivars of *Prunus mume*.^z

Cultivar	Light condition	Percentage of callus forming shoots (%)
Lv'e	Light	56.3 $\pm$ 14.6 b
	Dark	73.4 $\pm$ 9.4 ab
Xuemei	Light	63.4 $\pm$ 4.7 b
	Dark	91.3 $\pm$ 5.3 a

^zData were collected after 6 weeks after transferring callus to shoot formation medium in different light conditions. Values (mean $\pm$ standard error) followed by different letters within a cultivar are significantly different at $P = 0.05$ (Duncan's multiple range test).

Table 5. Rooting of *Prunus mume* Xuemei and Lv'e after 4 weeks of culture on different media.^z

Rooting media	Cultivar	Rooting percentage (%)	Number of roots per shoot (greater than 5 mm)
WPM + 2.5 μM IBA	Xuemei	81.2 $\pm$ 1.5 b	2.34 $\pm$ 0.1 b
	Lv'e	80.3 $\pm$ 1.2 b	2.16 $\pm$ 0.05 b
WPM + 5.0 μM IBA	Xuemei	88.9 $\pm$ 1.5 a	3.96 $\pm$ 1.0 ab
	Lv'e	90.2 $\pm$ 2.2 a	5.25 $\pm$ 1.7 a

^zValues (mean $\pm$ standard error) followed by different letters within a column are significantly different at $P = 0.05$ (Duncan's multiple range test).

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