

Factors affecting plantlet regeneration from *in vitro* cultured immature embryos and cotyledons of *Prunus mume* “Xue mei”

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Abstract Using immature embryos and cotyledons as explants, a successful system to culture immature embryos and induce direct regeneration from cotyledons was established for *Prunus mume* “Xuemai”. For immature embryo culture, a high frequency of plantlet formation (89.5%) from the embryonic axis was obtained using half-strength Murashige and Skoog (1/2 MS) medium supplemented with 13.2 μ M 6-benzyladenine (BA) and 2.7 μ M 1-naphthaleneacetic (NAA). Shoots formed directly from cotyledons with the embryo axis intact when explants were cultured on 1/2 MS medium containing 2.2 μ M BA with different combinations of NAA (2.7, 5.4 μ M) and indole-3-butyric acid (IBA) (0, 2.5, 5.0 μ M). Better results were achieved when the embryonic axis was removed from the cotyledons and cultured on 1/2 MS medium supplement with 13.2 μ M BA, 2.7 μ M NAA or 2.2 μ M BA, 2.2 μ M thidiazuron (TDZ), and 2.7 μ M NAA, respectively. Regenerated shoots were successfully rooted on 1/2 MS or Woody Plant medium (WPM) supplemented with 2.5–5.0 μ M IBA. The effect of the embryonic axis, BA, and TDZ on cotyledon regeneration was investigated in detail. Rooted plantlets were transferred to soil successfully.

Keywords Cotyledons · Direct shoot regeneration · Immature embryos · *Prunus mume* · Organogenesis · Rooting

Introduction

Native to China and Japan, *Prunus mume* has outstanding ornamental features and is one of the longest lived species of the flowering fruit trees. It is planted in the garden as a specimen plant and for cut flowers, and is also generally used for medicinal purposes (Dogasaki et al. 2004). To satisfy a growing interest from the public and commercial growers, there is an urgent need to develop new breeding and improvement methods for this species.

There have been dramatic developments in the tissue culture of *Prunus* species (Lane and Cossio 1986; Declerck and Korban 1996; Hokanson and Pooler 2000; Bhagwat and David 2004), and micropropagation techniques of a few cultivars of *P. mume* have been developed (Harada and Murai 1996). However, little research has been conducted on plant regeneration of *P. mume*, which inhibits the development of breeding and improvement of this species by biotechnology. Moreover, to our knowledge, few researchers have reported on regeneration from immature embryos.

Although there are 323 cultivars of *P. mume* identified (Chen 1997), limited genetic diversity and adaptability restricts the geographical distribution of the species. Recently, interspecific or intergeneric hybridization, polyploidization, and genetic transformation techniques have shown promise in cultivar improvement or introducing useful traits such size control, adaptation to new environments and pest resistance (Katoka 1988; Chen 1997). Successful *in vitro* culture of immature embryos and cotyledons will provide a platform to accelerate the plant breeding and improvement programs. *P. mume* is characterized by abortive hybrid embryos and an efficient and

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reproducible protocol for regeneration has not been developed. To utilize biotechnology in breeding, it is necessary to establish a plant regeneration system for *P. mume*.

In this paper, we detail a successful and efficient protocol for the culture of immature embryos and direct shoot regeneration from the immature embryos and cotyledons of *P. mume*. The response of shoot differentiation to different plant growth regulators and the effect of some factors on shoot formation in regeneration of cotyledons are also discussed.

Materials and Methods

Plant material. Open-pollinated immature fruits were harvested at 50–60 d after flowering from trees of *P. mume* “Xuemei” growing in the Wuhan *P. mume* garden in China, and stored at 4°C in refrigerator until use within a wk. After washing the fruits thoroughly under running tap water for 30 min, the immature fruits were surface-sterilized (for approx 30 s) by immersion in 70% ethanol, rinsing in sterile water, immersion with agitation in a 0.1% (w/v) aqueous solution of HgCl₂ for 20 min, followed by three washes with sterile water.

Explants consisted of either whole immature embryos, intact cotyledons with the embryonic axis removed, or the embryonic axis with approximately 1/4 of the cotyledons, or intact cotyledons. Explants were aseptically removed from the sterile immature fruits using forceps and a scalpel, and placed on the medium for immature embryo culture or shoot induction.

Culture establishment and maintenance. To get suitable media for different explants, explants were tested on 17 media, which were divided into three types: type I: media were supplemented with 2.2 μM 6-benzyladenine (BA) and the factorial combinations of 1-naphthaleneacetic (NAA) (2.7 or 5.4 μM) or indole-3-butyric acid (IBA) (0, 2.2 or 4.4 μM); type II: media were plant growth regulator (PGR)—free or supplemented with BA (2.2, 4.4, 13.2, 22.0, or 30.8 μM) in combination with 2.7 μM NAA; type III: media were supplemented with 2.2 μM BA and 2.7 μM NAA in combination with thidiazuron (TDZ) at a concentration of (0, 0.45, 2.25, 4.50, or 6.75 μM). Once the optimal medium had been defined for each explant type, the experiment was repeated at least once and the data collected was used to investigate the effect of various factors on shoot regeneration of immature embryos or cotyledons. All of the media consisted of 1/2 MS (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar unless otherwise indicated, and were autoclaved for 20 min at 121°C and then 20 ml was dispensed aseptically in Petri dishes. All of the cultures were maintained in a growth chamber at 25±2°C in the dark for the first 3 wk, and then

transferred to light at a photosynthetic photon flux (PPF) of 50 μmol m⁻²s⁻¹ provided by 40 W cool white fluorescent tubes in a 14-h light and 10-h dark cycle.

Elongation and rooting of regenerated shoots from cotyledons. For shoot elongation, the whole clump of immature embryos or cotyledons, comprising regenerated buds, were transferred to WPM (Lloyd and McCown 1980) containing 2.2 μM BA, 0.45 μM TDZ, and 1.0 μM IBA or 1/2 MS supplemented with 13.2 μM BA and 2.7 μM NAA. Elongated shoots were cut from the clump and transferred to 1/2 MS or WPM medium supplemented with 2.5–5.0 μM IBA for rooting when the height was greater than 1.0 cm.

Data analysis. A completely random design with three replicates was performed, each containing at least six explants. Each experiment was repeated at least once. The frequency of germination, shoot regeneration from cotyledons, and the number of shoots formed per regenerating cotyledon were recorded 6 wk after culture initiation. Data were evaluated by analysis of variance (ANOVA) and means were compared using Duncan’s multiple range test to determine significant differences.

Results and Discussion

Culture of immature embryos. When the whole embryo or the embryonic axis with about 1/4 cotyledon attached were cultured on 1/2 MS medium free of growth regulators (Fig. 1a), we found that the embryonic axis could not convert into plantlets (Table 1). In type I medium, most of the adventitious buds appeared on cotyledons after 3 wk of culture, and the embryonic axis did not develop. The best results were obtained when explants were cultured on type II medium, using either the whole embryo or the embryonic axis with about 1/4 cotyledons as explants. A significant difference was observed in growth based on BA concentration, with a high frequency (89.5%) of the whole immature embryo germinating on 1/2 MS medium supplemented with 13.2 μM BA and 2.7 μM NAA (Table 1). There was no significant difference between different explant forms. The embryonic axis formed into a single shoot with a main root after 6 wk of culture (Fig. 1b) on media supplemented various combinations of BA and NAA. A few of the embryonic axis or embryos also formed plantlets on type III medium, but most of those plantlets had usually thick roots or callus instead of roots, and some of the abnormal shoots did not elongate well when transferred to fresh medium (Fig. 1c).

Effect of the presence of the embryonic axis on shoot induction from cotyledons. When cultured on type II or type III media, the cotyledons turned green and expanded when the embryonic axis was not removed (Fig. 1c). When

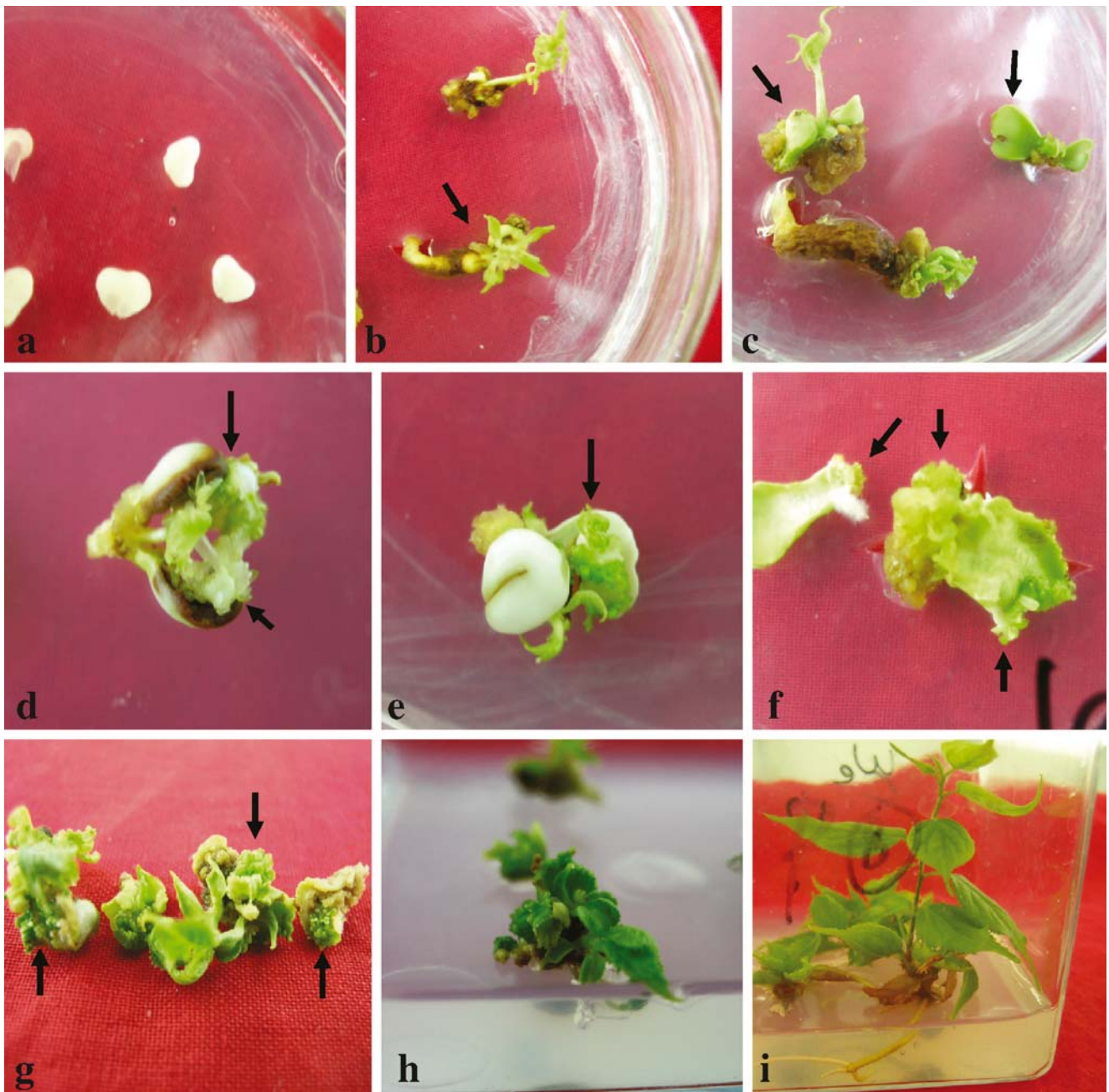


Figure 1. Plantlets forming from immature embryos and shoot regeneration from cotyledons of *P. mume*. (a) Immature embryos or cotyledons inoculated on medium. (b) Plantlets forming from the immature embryos after removing most of the cotyledons (arrow). (c) Plantlets forming from the whole immature embryo without removing cotyledons (arrow). (d) Shoot regenerating from the distal edge of

cotyledon (arrow) with embryonic axis intact. (e) Shoot regenerating from the median section of cotyledons (arrow) with embryonic axis intact. (f) Shoots regenerating from the edges of the cotyledons (arrow) with the embryonic axis removed. (g) Multiple shoot buds (arrow) forming from cotyledons with the embryonic axis removed. (h) Shoots growing in shoot elongation media. (i) Rooting of shoots.

cultured on type I medium (Table 2), after about 20 d of culture, not only did the embryonic axis grow into a plantlet with a few roots, but also multiple clusters of white or green adventitious buds formed on the distal margin and middle of inner surface (Fig. 1*d, e*). These adventitious buds turned into callus or became vitrified if not transferred to new media. However, they grew vigorously when transferred to WPM supplemented with 2.2 μM BA, 0.45 μM TDZ, and

1.0 μM IBA. On 1/2 MS medium containing 2.2 μM BA, the effect of various combinations of NAA and BA on cotyledon regeneration was significant (Table 2). When the embryonic axis was removed from cotyledons and explants were cultured on type II or type III media, direct differentiation of shoot buds and slight callus formation occurred in the distal or proximal region of the cotyledon (Fig. 1*f, g*).

Table 1. Dosage BA in combination with NAA on germination of whole immature embryos of *P. mume*

BA (μM)	NAA (μM)	Germination frequency of Immature embryos (%)
0	0	0
2.2	2.7	22.5 \pm 3.5 ^z d
4.4	2.7	51.0 \pm 5.6 c
13.2	2.7	89.5 \pm 7.8 a
22.0	2.7	66.7 \pm 4.7 b
30.8	2.7	52.5 \pm 3.3 c

Each values represents the mean \pm SE of at least three replicates. Values followed by different letters are significantly different ($P=0.05$).

^zData were collected after 4 wk of culture grown on 1/2 MS basal medium supplemented with different growth regulators.

Effect of BA on shoot regeneration from cotyledons. The addition of BA to 1/2 MS medium containing 2.7 μM NAA significantly increased the shoot regeneration percentage of cotyledons. The highest percentage of shoot occurred on medium containing 13.2 μM BA and 2.7 μM NAA (Table 3). The addition of BA either alone or with NAA was essential in inducing adventitious shoots from the cotyledons. The average number of shoots per cotyledon varied from 1.4 to 5.9, depending on the media composition. A high concentration of BA not only reduced the regeneration frequency, but also the number of shoots per cotyledon (Table 3).

Effect of TDZ on shoot regeneration from cotyledons. There were significant difference among treatments in the percentage of cotyledons that formed shoots and the number of shoots per regenerated cotyledon (Table 4). The highest regeneration frequency was obtained with 4.4 μM TDZ (Table 2), but the number of shoots per regenerated cotyledon was less than that produced by 2.2 μM , which was 5.7 per explant. With increased TDZ concentration, shoot induction was greatly inhibited and the frequency of abnormal shoots increased.

Table 2. Shoot differentiation from immature embryo cotyledons with intact embryonic axis of *P. mume*

BA (μM)	NAA (μM)	IBA (μM)	Immature embryos forming shoots on cotyledons (%)
2.2	2.7	0	30.4 \pm 4.1 ^z d
2.2	2.7	2.5	63.4 \pm 4.7 bc
2.2	2.7	5.0	83.0 \pm 4.2 a
2.2	5.4	0	56.8 \pm 2.6 c
2.2	5.4	2.5	76.0 \pm 5.7 ab
2.2	5.4	5.0	57.5 \pm 10.6 c

Each value represents the mean \pm SE of at least three replicates. Values followed by different letters are significantly different ($P=0.05$).

^zData were collected after 4 wk in culture grown on 1/2 MS basal medium supplemented with different growth regulators and from only those immature embryos in which shoot buds formed from cotyledons.

Table 3. Dosage of BA in combination with NAA on shoot formation from *P. mume* immature cotyledons without intact embryonic axes of *P. mume*

BA (μM)	NAA (μM)	Shoot regeneration from cotyledons (%)	Number of shoots per regenerated explant
0	2.7	2.5 \pm 3.5 ^z c	1.4 \pm 0.22 d
4.4	2.7	14.3 \pm 1.1 c	2.6 \pm 0.32 c
1.3	2.7	72.9 \pm 2.4 a	5.9 \pm 0.43 a
22.0	2.7	58.4 \pm 11.8 ab	4.9 \pm 0.28 b
30.8	2.7	45.9 \pm 5.9 b	4.3 \pm 0.38 b

Each value represents the mean \pm SE of at least three replicates. Value in each column followed by different letters are significantly different ($P=0.05$).

^zData (number of shoots per regenerated explant) were collected after 6 wk of culture grown on 1/2 MS basal medium supplemented with different growth regulators and from only those shoots which were greater than 3-mm long.

Shoot elongation and rooting. Shoots that did not elongate well on shoot induction medium grew vigorously (Fig. 1h) when they were transferred to 1/2 MS supplemented with 13.2 μM BA and 2.7 μM NAA or WPM supplemented 2.2 μM BA, 0.45 μM TDZ, and 1.0 μM IBA. When shoots and embryo-derived plantlets greater than 5 mm long were placed onto 1/2 MS or WPM supplemented 2.5 or 5.0 μM IBA (Table 5), more than 80% formed roots (Fig. 1i) and there was no significant difference in rooting frequency among the medium used. On the contrary, the number of roots per shoot was significantly greater on 1/2 MS medium supplemented with 5.0 μM IBA, where an average of more than five roots formed.

This study demonstrates that successful *in vitro* culture of immature embryos of *P. mume* was not only affected by explant type but also by concentration of plant growth regulators and the ratio of auxin to cytokinin, such as BA

Table 4. Dosage effect of TDZ in combination with BA and NAA shoot induction from *P. mume* immature cotyledons without intact embryonic axis

BA (μM)	TDZ (μM)	NAA (μM)	Shoot regeneration frequency from cotyledons (%)	Number of shoots per regenerated explant
2.2	0	2.7	5 \pm 1.4 d	1.5 \pm 0.32 ^z d
2.2	0.45	2.7	18.8 \pm 5.3 c	2.5 \pm 0.30 c
2.2	2.25	2.7	68.8 \pm 3.3 b	5.7 \pm 0.47 a
2.2	4.50	2.7	84.2 \pm 1.2 a	4.2 \pm 0.43 b
2.2	6.75	2.7	71.3 \pm 1.8 b	3.2 \pm 0.56 c

Each value represents the mean \pm SE of at least three replicates. Value in each column followed by different letters are significantly different ($P=0.05$).

^zData (number of shoots per regenerated explant) were collected after 6 wk of culture grown on 1/2 MS basal medium supplemented with different growth regulators and from only those shoots which were greater than 3-mm long.

Table 5. Effect of basal medium and IBA on rooting response of *P. mume* shoots

Rooting medium	Rooting (%)	Number of roots per rooted shoot
0.5MS+2.5 μ M IBA	82.5 \pm 3.2 ^z a	2.4 \pm 0.46 c
0.5MS+5.0 μ M IBA	83.6 \pm 5.1 a	5.1 \pm 0.54 a
WPM+2.5 μ M IBA	81.5 \pm 4.2 a	3.3 \pm 0.31 bc
WPM+5.0 μ M IBA	90.5 \pm 4.0 a	4.6 \pm 0.57 ab

Each values represents the mean \pm SE of at least three replicates. Values in each column followed by different letters are significantly different ($P=0.05$).

^zData were recorded after 4 wk of culture.

and TDZ. The system described here also opens a new approach for genetic improvement of *P. mume* besides the traditional methods mentioned previously (Katoka 1988; Chen 1997).

The breeding of *P. mume* is hampered occasionally by long seed dormancy and premature embryo abortion, especially as a result of interspecific hybridizations. In our present study, many media types were capable of supporting the conversion young embryos to plantlets. Like studies in other species (Pellegrineschi et al. 1997; Simon et al. 2005; Vilorio et al. 2005), the successful embryo culture reported here suggests the potential of embryo rescue to achieve interspecific hybridization using *P. mume*.

Auxin and cytokinin were both necessary for successful culture of immature embryos of *P. mume*. For *P. mume* “Xuemei”, satisfactory results were obtained on the medium supplemented with 2.7 μ M NAA and 13.2 μ M BA (Table 1), with lower or higher BA concentration having negative effects on embryo development.

Though successful regeneration of immature cotyledons was been achieved in other species (Salajova and Salaj 2001; Singh and Suresh 2003; Han et al. 2004), few reports refer to factor affecting the regeneration of immature cotyledons in *P. mume*. Many reports mentioned that shoot formation on cotyledons from different *Prunus* species depended on the removal of the embryonic axis and the presence of the proximal region of cotyledon (Kouider et al. 1984; Mante et al. 1989). In our study, shoots could regenerate from the distal and median region of cotyledons (Fig. 1d, e), even when the immature embryonic axis was not discarded. This phenomenon occurred only when the whole immature embryo was cultured on type I medium, in which the ratio of cytokinin to auxin was relatively high. With a decrease in the ratio of cytokinin to auxin (Tables 1, 3, and 4), shoots did not form on the cotyledons with the embryonic axis (Fig. 1c). These results indicate that adventitious shoots inducing from cotyledons with the embryonic axis can be altered by exterior PGR. Moreover, it seems that the ratio of cytokinin to auxin plays an

important role in the successful culture of *P. mume* from immature embryos. It is possible that the strongly inhibitory effects on morphogenesis of the cotyledons by the embryonic axes could be weakened by the presence of auxin in the medium.

In contrast to our results with *P. mume*, Antonelli (1991) reported that shoot morphogenesis could not be achieved in the culture of immature almond cotyledons on MS medium with various concentrations of BA and NAA. Furthermore, shoots were able to regenerate from cotyledons with the embryonic axis removed on all media types with BA concentrations ranging from 0 to 30.8 μ M (Table 3). Using 1/2 MS supplemented with 2.2 μ M BA and 2.7 μ M NAA or the addition of TDZ increased the regeneration frequency significantly ($p<0.05$) at all concentrations tested in our study (Table 4). However, TDZ did not always have a positive affect on shoot formation because higher concentration of TDZ inhibited shoot induction. Within a certain concentration range, the inclusion of TDZ in the regeneration medium promotes high levels of bud formation on cotyledons, as reported previously in other species (Escalettes and Dosba 1993; Goffreda et al. 1995; Pooler and Scorza 1995). In agreement with previous reports on rooting of shoots from nodal buds (data not show), there was no difference in the percentage of rooting of shoots when cultured on 1/2 MS or WPM supplemented 2.5–5.0 μ M IBA.

In conclusion, successful immature embryo culture and direct regeneration from immature cotyledons offers unprecedented opportunities for improving the breeding efficiency of *P. mume*. It suggests the potential for interspecific hybridization through embryo rescue, polyploidization, and genetic transformation in the breeding of *P. mume*. In addition, the different regeneration patterns, affected by external sources of PGR, also suggest the potential for studying the distribution and transportation of endogenesis hormones in the immature embryo by *in vitro* culture of *P. mume*.

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