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Micropropagation of Chinese Plum (*Prunus salicina* Lindl.) Using Mature Stem Segments

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Abstract

An effective *in vitro* culture system for mature stem segments of Chinese plum (*Prunus salicina* Lindl. cv. 'Gulf-ruby') was established. The nodal segments of 1 cm were cut from young shoots in open field, sterilized and established *in vitro*. The successfully induced culture was achieved on woody plant medium (WPM) supplemented with 0.05-0.1 mg/l IBA, 0.5-1.0 mg/l BA, 30 g/l glucose, 5 g/l agar and 1.0 g/l Vc. The successful shoot multiplication was achieved on WPM with 0.05-0.1 mg/l IBA, 0.2 mg/l BA, 0.3 mg/l KT and 1.0 g/l casein hydrolysate. Elongation of the shoot *in vitro* was facilitated on WPM with 0.05 mg/l IBA, 0.3 mg/l KT/BA and 1.0 g/l casein hydrolysate. The shoots from microcuttings were rooted in 1/2 MS (half strength MS) supplemented with 0.2-0.5 mg/l IBA, 15 g/l sucrose and 20-40 mg/l phloroglucinol (PG). Regenerated shoots successfully acclimatized to greenhouse conditions and grew vigorously with no apparent phenotypic aberrations.

Keywords: Chinese plum, tissue culture, clonal propagation, phloroglucinol, rooting

Introduction

Plum (*Prunus salicina*) is one of the important fruit trees in China. However, plum trees possess not only longer juvenility but also higher pistil sterile and heterozygosity (Mante *et al*., 1991). In open field, Chinese plum trees are often infected by several viruses including plum pox virus and plum necrotic ringspot virus (Sun, 2004), which severely inhibit plum development in China.

Tissue culture provides opportunity to produce the seedings free from the viruses. The procedures for the production of plants from mature seeds of both *P. domestica* and *P. salicina* and from cotyledons of *P. persica*, *P. domestica* and *P. cerasus* have been described (Mante *et al*., 1989; Tian and Sibbald, 2007). The application of *in vitro* methods in the production of own-rooted planting material of plum significantly increased the production capacities and contributed to the improvement of its quality and health status (Popov and Kornova, 2009). For micropropagation it is often preferable to use explants from mature plants where superior characteristics are evident (Jain and Babbar, 2003). However, it is difficult to culture *in vitro* mature trees including plum due to the higher incidence of microbial contamination, vitrification, difficulty in root induction and high levels of polyphenol exudation (Zimmerman, 1986). Studies on regeneration *in vitro* from mature European plum (*P. domestica*) and ornamental *Prunus* species have been succeeded (Barbara *et al*., 2004; Kalinina and Brown, 2007), but there are less reports on *in vitro* regeneration of Chinese plum species. Herein, an efficient protocol was established for micropropagation of 'Gulf-ruby' Chinese plum (*P. salicina* Lindl. cv. 'Gulf-ruby') using mature stem segments as the explants.

Materials and methods

Plant materials

The explants were harvested from the mature *P. salicina* Lindl. cv. 'Gulf-ruby' trees (3-year-old) grown in the Fruit Garden of Huazhong Agricultural University, Wuhan, China. After being washed with detergent for 30 min, the explants were cut into 1 cm stem segments with an axillary bud. Then the segments were surface-sterilized in 70% ethanol for 30 s, followed by washing one time with sterile water, shaken in 1% NaClO with two drops Tween 20 for 15 min, and finally washed again four times (2 min/time) as described above. These sterile explants were transplanted on the initial medium supplemented with 30 g/l sucrose and 5 g/l agar. The pH was adjusted to 5.5-5.8 prior to autoclaving. These explants were cultured in darkness for 7 days before transferring to white light (Zou *et al*., 2006). The explants were incubated in a growth chamber at 25±1°C, a 15:9-h photoperiod and 3000 Lx illumination intensity.

Initial culture

To select an efficient combination of both IBA and BA, the explants were cultured on the woody plant medium (WPM) supplemented with different concentrations of IBA (0.05, 0.1 and 0.2 mg/l) and BA (0.5, 1.0 and 2.0 mg/l), 30 g/l glucose, 1.0 g/l Vc and 5 g/l agar. Number of regenerated shoots were recorded after culture of 30 days and 60 days.

Multiplicated culture

The cluster buds from the initial culture were cut and cultured on WPM supplemented with 1.0 g/l Vc, 30 g/l glucose, 0.05 mg/l IBA and 1.0 mg/l hydrolyze casein (CH). Three different combinations of BA and KT containing 0.5 mg/l BA+0 mg/l KT, 0 mg/l BA+0.5 mg/l KT, and 0.2 mg/l BA+0.3 mg/l KT were added to the multiplicated medium, resepctively. To obtain the elongation of *in vitro* shoots for the preparation of rooting, the freshly initiated shoots were subcultured on WPM medium supplemented with 1.0 g/l Vc, 30 g/l glucose, 1.0 mg/l CH, 0.05 mg/l IBA, 0-0.3 mg/l BA and 0-0.3 mg/l KT. Each (n=30) of the three treatments was replicated three times, leading to a total of 90 explants.

Rooting

Approximately 15 mm long developed shoots from multiplicated culture were rooted *in vitro* on half strength MS medium (1/2 MS) supplemented with 15 g/l sucrose, 5 g/l agar, and different concentrations of IBA (0.2, 0.5, 0.8 and 1.0 mg/l). Three replicates per treatment were carried out, and each replicate was at least 10 explants.

Due to the rooting difficulty, different concentrations (20, 40, 80 and 160 mg/l) of phloroglucinol (1,3,5-trihydroxybenzene, PG) were added to 1/2 MS additionally containing 0.2 mg/l IBA, 5 g/l agar and 15 g/l sucrose. Three replicates per treatment were carried out, and each replicate was at least 10 explants.

Plantlet acclimatization

The rooted shoots were removed from the culture tubes, washed with tap water and transferred to plastic pots with the mixture of garden soil, perlite and vermiculite (1:1:1, v/v/v). The plantlets were placed in a glass greenhouse without any temperature controlling equipments.

Statistical analysis

Experimental data were tested for significance by analysis of variance (ANOVA) using SAS soft (edition 8.1). Fisher's Protected Least Significant Differences (LSD) were used to compare the differences of means at 5% level.

Results and discussion

After 30 days of initial culture, the combinations of BA and IBA had the profound effects on the multiplication and growth of shoot explants. In the nine combinations, 0.1 mg/l IBA+0.5 mg/l BA treatment exhibited the highest multiplication, quick growth and normal morphology of leaves (Tab. 1). A significant difference was observed between 0.1 mg/l IBA+0.5 mg/l BA treatment and 0.2 mg/l IBA+1.0-2.0 mg/l BA treatment, while the multiplication of explants treated by 0.1 mg/l IBA+0.5 mg/l BA was similar in that of 0.05 mg/l IBA+0.5-1.0 mg/l BA or 0.1 mg/l IBA+0.5-1.0 mg/l BA. The shoot explants treated by the combination of 0.2 mg/l IBA and 2.0 mg/l BA showed slower growth, and also induced vetrification and severe abnormality. Similar results were also observed in 60 days of initial culture. In view of the shoot growth status, the combination of 0.05-0.1 mg/l IBA+0.5-1.0 mg/l BA benefited multiplication and growth of shoot explants in initial culture (Fig. 1 A).

For shoots multiplication, the buds of the recovered shoots from the initial culture were inoculated with different combinations of BA and KT with 0.05 mg/l IBA (Tab. 2). The combination of 0.2 mg/l BA and 0.3 mg/l KT significantly increased the shoot multiplication but reduced the proportion of >1 cm tender shoots after 60 days of culture, compared with the treatment of BA and KT solely. Therefore, different combinations of BA and KT in multiplicated culture would be selected according to the experimental need. For example, the conbination of 0.2 mg/l BA and 0.3 mg/l KT provided more shoot multiplication (Fig. 1 B).

Cytokinins can stimulate growth of lateral buds and thus suppress apical dominance (Te-chato *et al*., 2008). For shoots elongation, the combinations with low concentrations of BA and KT were tested based on the results of Tab. 2. As shown in Tab. 3, the maximum average number

Tab. 1. Effects of different combinations of IBA and BA on explants multiplication and growth of Chinese plum (*Prunus salicina* Lindl. cv. 'Gulf-ruby')

IBA (mg/l)	BA (mg/l)	Number of recovered shoots		Growth status of recovered shoots (30 days)
		30 days	60 days	
0.05	0.5	2.27ab	3.37ab	slow, basically normal
0.05	1.0	2.23ab	3.43ab	slow, basically normal
0.05	2.0	2.50ab	3.07abc	slow, part of leaf presented abnormality
0.1	0.5	2.53a	3.97a	relatively rapid, basically normal
0.1	1.0	2.37ab	3.57ab	relatively rapid, basically normal, very few vitrification
0.1	2.0	1.93abc	2.67bc	relatively slow, part vitrification and abnormality
0.2	0.5	2.17ab	2.60bc	relatively slow, most vitrification and abnormality
0.2	1.0	1.87bc	2.70bc	lower, severe vitrification and abnormality
0.2	2.0	1.40c	2.17c	lower, no elongate, severe vitrification and abnormality

Note: Different letters in the same line denote significant differences (LSD test, $p < 0.05$)

Fig. 1. *In vitro* regeneration from nodal shoot explants of mature Chinese plum (*Prunus salicina* Lindl. cv. 'Gulf-ruby') trees. (A) Nodal shoot explants developed on WPM with 0.1 mg/l IBA and 0.5 mg/l BA in initial culture. (B) *In vitro* shoots multiplied on WPM supplemented with 1.0 g/l Vc, 30 g/l glucose, 0.05 mg/l IBA, 0.2 mg/l BA, 0.3 mg/l KT and 1.0 mg/l hydrolyze casein. (C) *In vitro* shoots elongated on WPM supplemented with 1.0 g/l Vc, 30 g/l glucose, 0.05 mg/l IBA, 0.3 mg/l KT and 1.0 mg/l hydrolyze casein. (D) *In vitro* shoots rooted on 1/2 MS additionally containing 40 mg/l phloroglucinol, 0.2 mg/l IBA, 5 g/l agar and 15 g/l sucrose. (E) Four-month-old plants in pots during greenhouse

of shoots was found in 0.1 mg/l BA+0.2 mg/l KT treatment, which also induced the lowest effects on elongation of the shoots during subculture.

However, 0.3 mg/l BA only or 0.3 mg/l KT only produced both the minimum average number of shoots and elongation of the shoots (Fig.1 C). Therefore, 0.3 mg/l BA only or 0.3 mg/l KT only was considered to be optimal for shoot elongation in Chinese plum.

The multiplicated shoots were induced to regenerate roots in 1/2 MS medium supplemented with different

Tab. 2. Effects of the combinations of BA and KT on shoot multiplication and growth of Chinese plum (*Prunus salicina* Lindl. cv. 'Gulf-ruby') during subculture

BA (mg/l)	KT (mg/l)	Number of recovered shoots (60 d)	>1 cm tender stem after 60 of culture (%)
0.5	0	4.27b	39.1a
0	0.5	4.43b	37.9a
0.2	0.3	5.67a	25.9b

Different letters in the same line denote significant differences (LSD test, $p < 0.05$)

Tab. 3. Effect of the combinations of BA and KT on shoot multiplication and growth Chinese plum (*Prunus salicina* Lindl. cv. Gulf-ruby) during subculture

BA (mg/l)	KT (mg/l)	Number of recovered shoots (60 d)	>1 cm tender stem after 60 of culture (%)
0.3	0	2.63b	53.6a
0	0.3	2.90b	57.2a
0.1	0.2	3.37a	38.6b

Different letters in the same line denote significant differences (LSD test, $p < 0.05$)

Tab. 4. Effect of IBA on shoot rooting of Chinese plum (*Prunus salicina* Lindl. cv. 'Gulf-ruby')

IBA (mg/l)	Rooting percent (%)	Rooting number per plantlet
0	0b	0b
0.2	10.42a	2.0a
0.5	8.33a	1.3ab
0.8	0b	0b
1.0	0b	0b

Different letters in the same line denote significant differences (LSD test, $p<0.05$)

Tab. 5. Effect of phloroglucinol (PG) on shoot rooting of Chinese plum (*Prunus salicina* Lindl. cv. 'Gulf-ruby')

PG (mg/l)	Rooting percent (%)	Rooting number per plantlet
0	10.42c	2.0b
20	48.23b	3.1ab
40	79.76a	4.1a
80	71.98a	3.6a
160	68.11a	4.4a

Different letters in the same line denote significant differences (LSD test, $p<0.05$)

concentrations of IBA (0, 0.2, 0.5, 0.8 and 1.0 mg/l). 0 mg/l IBA resulted in the death of tender shoot, and rooting and callus was invisible (Tab. 4). Lower levels (0.2 and 0.5 mg/l) of IBA supplied on rooting medium facilitated rooting and growth of shoots, and little callus was observed the base of the shoots. In contrast, higher levels (0.8 and 1.0 mg/l) IBA did not induce rooting of shoots but depressed the growth of shoots, which gradually died in late culture. Though 0.2 and 0.5 mg/l IBA could induce rooting, the rooting percent was rather low (<10.50%) and the rooting number per plantlet low (≤2.0). Similarly to other *Prunus* species such as cherry (Pruski *et al*., 2005), *in vitro* produced shoot reosettes of *P. salicina* Lindl. cv. 'Gulf-ruby' rooted difficultly *ex vitro* when treated with IBA.

PG (phloroglucinol), a product of the degradation phloridzin, has been confirmed to enhance growth and rate of axillary shoot proliferation from shoot tip cultures of several woody plants or to act as an auxin synergist during the auxin-sensitive phase of root initiation (Sarkar and Naik, 2000). Preconditioning of shoot cultures by PG has been reported to initiate adventitious roots in different wood species *in vitro*, such as adult wild cherry (*P. avium*) (Hammatt, 1994), almond (*P. dulcis*) (Ainsley *et al*., 2001), and apple (Gur *et al*., 1988). The results obtained (Tab. 5) indicated that incorporation of 20 to 160 mg/l PG into the rooting medium notably stimulated rooting of adult Chinese plum. 40 mg/l PG treatment presented the highest rooting percentage and higher rooting number per plantlet (Fig. 1 D). Moreover, *in vitro* shoot growth treated by 40 mg/l PG was better than other PG treatments. The result was in agreement with the finding of Anirudh and Kanwar (2008), who reported that the highest rooting (88.80%) of wild pear (*Pyrus pyrifolia*) was attained with 40 mg/l PG. However, 20 mg/l PG treatment showed less rooting percentage and rooting number per plantlet than 40, 80 and 160 mg/l PG. Exogenous PG acts as alternative substrates for oxidative enzymes, and may protect auxin from oxidative breakdown, thus inducing rooting (George *et al*., 2008). Based on the above results, 40 mg/l PG applied to rooting medium, which induces more rooting of adult shoot segments of Chinese plum.

A total of 75 rooting shoots were transplanted into greenhouse conditions. Of these, 50 plantlets survived through the initial acclimatization after 120 days of transplant and grew vigorously without morphological abnormalities (Fig. 1 E).

Conclusions

In short, the present results indicated that the effective *in vitro* regeneration of mature shoots of Chinese plum (*P. salicina* Lindl. cv. 'Gulf-ruby') had been established. Based on the above results, the successfully induced culture was achieved on WPM supplemented with 0.05-0.1 mg/l IBA, 0.5-1.0 mg/l BA, 30 g/l glucose, 5 g/l agar and 1.0 g/ L Vc. The successful shoot multiplication was achieved on WPM with 0.05-0.1 mg / L IBA, 0.2 mg/ L BA, 0.3 mg/ L KT and 1.0 g/l casein hydrolysate. Elongation of the shoot *in vitro* was facilitated on WPM with 0.05 mg/l IBA, 0.3 mg/l KT/BA and 1.0 g/l casein hydrolysate. The shoots from microcuttings were rooted in 1/2 MS supplemented with 0.2-0.5 mg/l IBA, 15 g/l sucrose and 20-40 mg/l PG.

Approximately 67% of the plants were successfully transferred to greenhouse conditions.

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