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MICROPROPAGATION OF SIX *PRUNUS MUME* CULTIVARS THROUGH AXILLARY SHOOT PROLIFERATION, AND ISSR ANALYSIS OF CLONED PLANTS

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Prunus mume is one of the most popular landscape plants in China and Japan. A successful in vitro propagation system for six cultivars of *Prunus mume* has been developed by in vitro culture of nodal segments from seedling and mature plants. High multiplication rates (from 2.5 to 5.5) were achieved using modified MS media and WPM basic media supplemented with TDZ, BA, IBA, 2,4-D or NAA at concentrations adjusted for each cultivar. All the studied cultivars could be proliferated efficiently on WPM media supplemented with 2.2 μM TDZ, 2.2 μM BA and 2.5 μM IBA. Shoots were rooted on agar-gelled 1/2 MS or WPM basic media containing 2.5 or 5.0 μM IBA, and plantlets were transferred to pots after they had grown more than 3 roots and at least one root was more than 10 mm long. The effects of TDZ, media composition and different genotypes on shoot multiplication and growth were studied in detail. The genetic fidelity of the micropropagated plants from the 'Xuemei' cultivar was examined using PCR-ISSR markers, and the results demonstrated complete genetic stability in the cloned plants.

Key words: *Prunus mume*, micropropagation, genetic stability, ISSR markers, tissue culture.

INTRODUCTION

Prunus mume (Mei flower) is one of the most popular landscape plants in China and Japan. It is well known worldwide for its winter flowering and for the fragrance of its differently colored flowers. Every year, *P. mume* gardens bring commercial benefits by attracting numerous visitors. To date there are 323 cultivars available, thanks to long-term conventional breeding and improvement of cultivars by breeders (Chen, 1997). To satisfy a growing interest in constructing *P. mume* gardens for conservation of their diversity of cultivars, and for commercial reasons, there is an urgent need to raise high-quality seedlings of wild and cultivated *P. mume* varieties. Unfortunately, propagation of *P. mume* through cuttings or through seeds or root suckers is a slow and laborious process. Moreover, the germination frequency of the seeds is very low, as is the survival rate of grafts. Significant progress has been made in the development of tissue, organ and cell culture in different woody plants (Sahoo and Chand, 1998; Birod et al., 2004; Boggetti et al., 1999). Work on the propagation (Webster, 1980; Muna et al., 1999; Duguma and Bell, 2002) and regeneration (James, 1984; Grant and Hammat, 2000) of *Prunus* species has also been reported. In recent years, in vitro culture, a set of techniques that offers a tool for mass multiplication, germplasm conservation and transformation research, has been widely used in *Prunus* species, but very few reports have described micropropagation of *P. mume*.

As in vitro culture is usually associated with somaclonal variation, it is necessary to establish a micropropagation system that produces genetically identical and stable plants, especially in the case of commercial plant species such as *P. mume*. Although RAPD analyses have usually been performed to evaluate genetic variation, ISSR is more effective and reproducible for quick assessment of the genetic stability of plants (Goto et al., 1998; Martins et al., 2004).

To our knowledge, only Harada and Murai (1996) have suggested that it is possible to propagate *P. mume* through in vitro culture, but that work was limited to a few genotypes and had problems in

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enhancing the rooting percentage. In this paper, we describe an optimal protocol for rapid clonal multiplication of six superior cultivars of *P. mume* through high-frequency axillary shoot proliferation from nodal explants, and successful transplanting of those plants to an outdoor environment. In addition, we performed PCR-ISSR analyses to assess the genetic fidelity of the micropropagated plants.

MATERIALS AND METHODS

PLANT MATERIALS

New shoots of 'Jiangnan Zhusha,' 'Gongfen,' 'Xuemei,' 'Lv'e,' 'Bian Lv'e' and 'Fuban Gongfen' cultivars of *P. mume* were collected from mature plants growing in the Wuhan *P. mume* garden.

ESTABLISHMENT AND CONDITIONS OF IN VITRO CULTURE

Fresh shoots, with leaves removed, were washed with running water and cut into ~5 cm lengths and surface-sterilized for ~30 sec immersion in 70% (v/v) ethanol, followed by washing in sterile water, 20 min immersion and agitation (15 min for explants from young seedlings) in 0.1% (w/v) aqueous solution of $HgCl_2$, followed by three washes in sterile water. Node segments 0.5–1 cm in length were then excised from the shoots and placed in shoot-inducing media. The pH of all media, supplemented with 30 g dm^{-3} sucrose, 0.8% (w/v) Agar gel (Sigma) and different hormone combinations, was adjusted to 5.8 with 1 mM NaOH prior to autoclaving, and then sterilized in an autoclave at 121°C for 20–25 min. The explants (4 per flask) were incubated in a 55 75 mm plastic box holding ~30 ml culture media, and sealed with a lid.

For shoot multiplication, three experiments were conducted to determine the most suitable medium for each genotype. In experiment 1, the explants from 6 cultivars were placed on 9 media based on WPM, in which 0.5 mg/L thiamine hydrochloride, 100 mg/L casein and 50 mg/L myoinositol were added again and supplemented with 1 μM 2, 4-D, IBA or NAA, 2.2 μM BA, and different concentrations of TDZ (0, 2.2 or 4.4 μM). The design of experiment 2 was the same except that WPM was replaced by 1/2 MS. In experiment 3, the explants from 6 cultivars were placed on 5 media based on 1/4 MS plus same the organic components as experiment 1, and supplemented with 1.0 μM IBA and different concentrations of BA (0, 4.4, 8.8, 13.2 or 17.6 μM). During culture, if 3 mm long shoots were formed after initiation of the multiplication experiment, the whole culture was transferred to fresh multiplication media or shoot elongation basic media, which were the same as the best shoot multiplication basic media (Tab. 2), except supplemented with 1/5 PGR. Cultures were subcultured at monthly intervals on fresh media of the same composition until the shoots had elongated to 1 cm for further submultiplication or even longer for rooting.

For root induction, shoots (2–4 cm long) with about 5 leaves were harvested and transferred to half-strength MS or full WPM. The medium based on 1/2 MS was supplemented with 0–10 μM IBA. After half a month, rooted shoots were transferred to 1/2 MS or full WPM without plant growth regulators for root elongation.

All the cultures were maintained in a growth chamber at 25±2°C under a 14 h photoperiod, with photosynthetic photon flux (PPF) of 50 μmol $m^{-2}s^{-1}$ provided by 40 W cool white fluorescent tubes. These conditions were also applied for the shoot multiplication and rooting experiments described below.

TABLE 1. Inter-simple sequence repeats (ISSR) primers used to evaluate the stability of 18 plantlets obtained through micropropagation of Xuemei cultivar of *P. mume*. R – A and G residues; Y – C and T residues

Primer	Sequence
N17	GA GA GA GA GA GA GA GA RC
N18	GA GA GA GA GA GA GA GA RG
N19	GA GA GA GA GA GA GA GA RT
N20	GA GA GA GA GA GA GA GA YG
N37	AC AC AC AC AC AC AC AC YC
N40	GACA GACA GACA GACA
N43	GTG GTG GTG GTG GTG
N44	GAC GAC GAC GAC GAC

TRANSFERRING PLANTLETS TO SOIL

Plantlets were removed from the culture flasks and washed thoroughly in running tap water to remove all medium attached to the roots. They were then transplanted to plastic pots containing a mixture of turf soil + garden soil + sand (2:2:1; v/v/v) kept in a growth chamber and maintained at 19°C, and 70% humidity under a 14 h photoperiod. After 2 weeks the pots were transferred to the greenhouse for further growth for 2 weeks and irrigated with tap water every 4 days. Then the plants were transferred to earthen pots containing the same soil mixture.

DNA ISOLATION AND PCR-ISSR ANALYSIS

Genomic DNA from 18 plantlets was extracted from young leaves according to the procedure of Yang et al. (2005). PCR amplifications were performed in a volume of 20 μl containing 10 ng template DNA, 200 nM

TABLE 2. Average number of shoots developing per explant on media in which 0.5 mg/L thiamine.HCL, 100 mg/L casein and 50 mg/L myo-inositol were added besides the components described below for the 6 cultivars after 40 days in culture

Cultivars	Multiplication media that give rise to the best results in this study	No. of shoots (>5mm) developed per node segment
Xuemei	WPM+2.2μM TDZ+2.2μM BA+1.0μM IBA	5.43±1.18
Gongfen	1/4MS+13.2μM BA+1.0μM IBA	4.75±0.25
Lv'e	1/2MS+4.4μM TDZ+1.0μM 2,4-D	3.30±0.80
Jiangnan Zhusha	WPM+4.4μM TDZ+2.2μM BA+1.0μM NAA	2.63±0.65
Bian Lv'e	WPM+2.2μM TDZ+2.2μM BA+1.0μM IBA	2.43±0.20
Fuban Gongfen	1/4MS+13.2μM BA+1.0μM IBA	3.25±0.62

Data were calculated from 36 explants of each cultivar, and only shoots more than 5 mm long. Values represent means ±standard error.

oligodeoxynucleotide primers, 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix and 1 U Taq DNA polymerase. Amplification conditions were one cycle at 94°C for 3 min, 94°C for 30 sec, and 58°C for 45 sec, followed by stepwise reduction of 1°C for the first five cycles and 72°C for 90 sec. In the next 38 cycles, annealing temperature was maintained at 53°C, and final extension at 72°C for 6 min. Forty primers were used for amplification of the template DNA. Eight primers gave clear polymorphic bands, and they were used further in polymerase chain reactions (Tab. 1). The amplification products were resolved by electrophoresis on 2% agarose gel, stained with ethidium bromide, measured with a 100 bp DNA ladder, and finally photographed in a DNA transilluminator.

STATISTICAL ANALYSIS

Four explants were inoculated in three replicates of each treatment. The experiments were done twice for the multiplication stage and three times for the rooting stage. The multiplication rate was calculated by dividing the number of shoots grown after 4 weeks by the initial number of buds. The roots were counted and measured after 5 weeks of culture on rooting media. Data were statistically evaluated by ANOVA, and means were compared with Duncan's test determine the significance of differences. Two-way ANOVA was performed to establish the effect of genotype and basic media.

RESULTS

SHOOT FORMATION AND MULTIPLICATION

The explants showed visible responses on shoot induction medium within 14 days. Regardless of the genotype, direct shoot formation was observed on media with lower PGR concentration; when the concentration of PGR was increased, callus formation took place first, followed by shoot regeneration (Fig. 1a,b). For the three cultivars 'Xuemei,' 'Zhusha' and 'Bian lv'e,' proliferation frequency was higher and shoots grew vigorously on WPM basal media. For the other three cultivars, the best results were achieved on 1/2 MS or 1/4 MS basal media (data not shown). The best media for shoot multiplication of each cultivar are listed in Table 2. From 2 to 6 shoots (>5 mm) were obtained, depending on the cultivar. The multiplication rate varied with the genotype and the TDZ concentration.

On average the shoots elongated to ~1.5 cm three weeks after transfer from the shoot multiplication media to the shoot elongation media. Healthy shoots were obtained in shoot elongation media (Fig. 1c,d).

EFFECT OF THE INTERACTION BETWEEN GENOTYPE AND MEDIA ON SHOOT MULTIPLICATION

To screen for the suitable genotype and basic media combination to get optimal shoot multiplication, we tested the different cultivar explants in WPM, 1/2 MS and 1/4 MS basic media with 1.0 μM IBA, 2.2 μM BA and 2.2 μM TDZ. Analysis of variance for shoot number revealed significant differences between genotypes ($P < 0.01$), while the differences between basic media and their interactions was not significant (Tab. 3).

EFFECT OF GENOTYPE ON SHOOT MULTIPLICATION

The rate of shoot multiplication on the two media was found to depend on the genotype. The fewest plants regenerated in the cultivar 'Bian Lv'e'; 'Xuemei' and 'Gongfeng' produced higher numbers of plants (>4) on both media applied in experiments 1 and 3 (Fig. 2). Most cultures of 'Zhusha,' 'Bian Lv'e' and 'Fuban Gongfeng' turned brown after 2 months of culture in shoot induction media (data not shown). We tested whether ABA (0.1 g dm^{-3}) and different concentrations of activated charcoal would

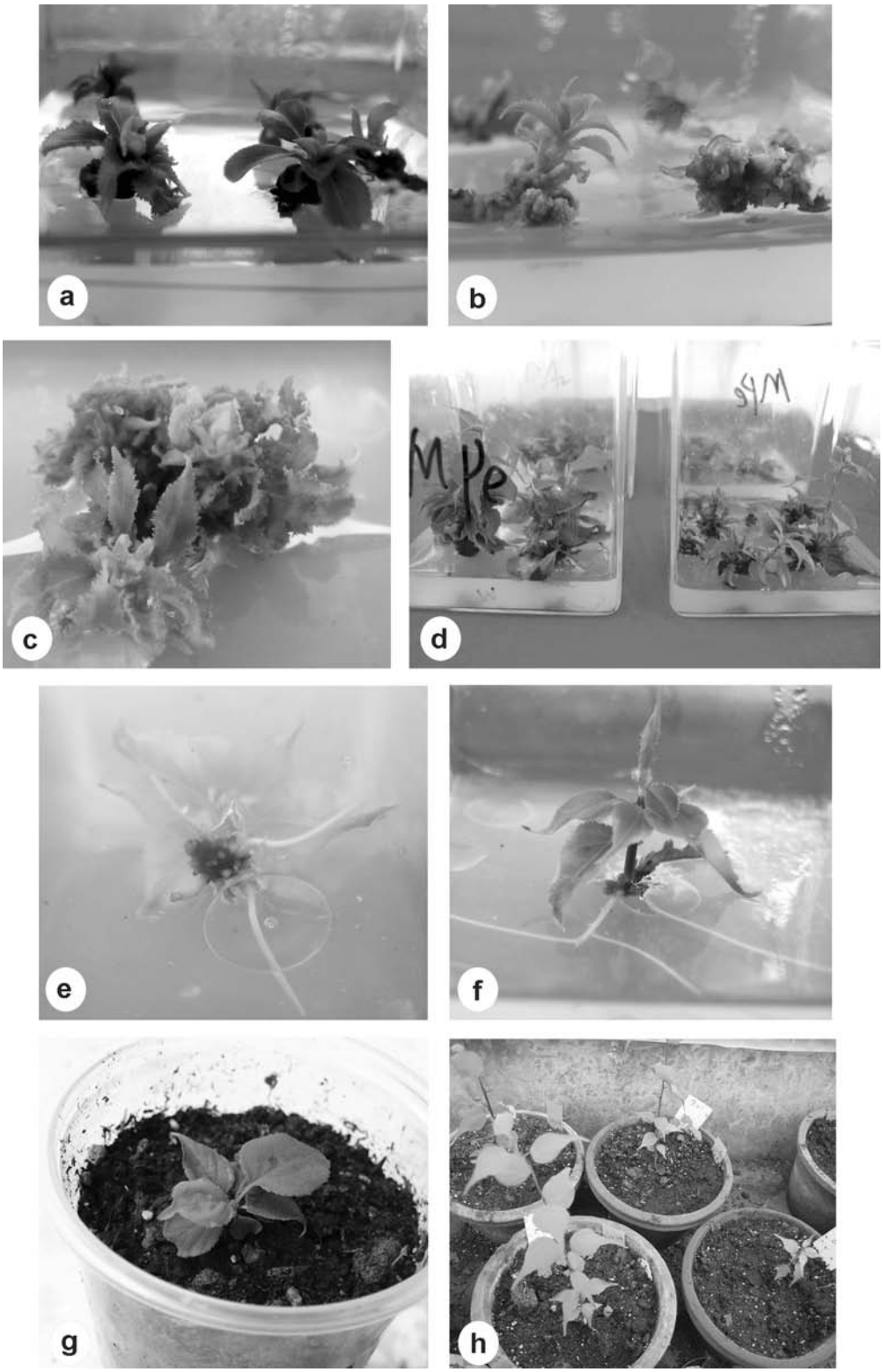

Fig. 1. In vitro culture of *P. mume* cultivars. (**a**) Shoots induced from mature stem; no callus was observed, (**b**) Shoots regenerating from calli induced from seedling, (**c**) Shoot multiplication, (**d**) Shoot elongation, (**e**) Rooting, with large number of small roots; view of bottom of plastic culture box, (**f**) Rooting, with few long roots, (**g**) Hardened micropropagated plantlets, (**h**) Healthy potted plants derived from proliferated shoots after half a year.

TABLE 3. Effects of genotype and media on shoot multiplication. Stem internodes harvested from 'FubanGongFen,' 'Lv'e' and 'Xiemei' were incubated on three basic media: WPM, 1/2MS and 1/4MS, which were supplemented with 2.2 μM BA, 2.2 μM TDZ and 1.0 μM IBA to which were added 0.5 mg/L thiamine.HCl 100 mg/L casein and 50 mg/L myo-inositol, respectively

Source of variation	*df*	Mean squares	*F* value
genotype	5	20.98	12.79**
medium	2	3.87	2.36
genotype*medium	10	0.65	0.4

**Statistically significant at p = 0.01

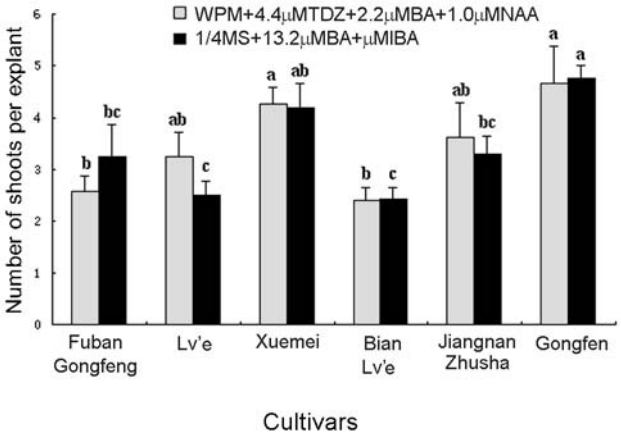

Fig. 2. Effect of genotype on number of shoots developing per explant on two media. Means with the same letter do not significantly differ at p = 0.05 (Duncan's Multiple Range Test). Vertical bar – ± SD.

prevent browning, using stem segments 3 cm long as explants and culturing in the dark in the first 2 weeks. Culturing in the dark proved to be the most effective way to prevent browning and sustain viable growth (data not shown).

EFFECT OF TDZ CONCENTRATION ON SHOOT MULTIPLICATION

Incorporating TDZ in the shoot formation and multiplication media had significant effects on culture of all the cultivars studied. Shoot number for all cultivars was higher when 2.2 and 4.4 μM TDZ was added to media. To examine the effect of TDZ on shoot multiplication in detail, we tested different concentrations of it (0, 2.2, 4.4, 6.6, 8.8 μM) on 'Xuemei.' There were no significant differences ($p < 0.5$) between TDZ concentrations. With the increase of concentration from 0.0 to 4.4 μM the number of shoots developing per explant increased (Fig. 3). Further increasing the concentration from 4.4 to 8.8 μM led to a decrease in shoot number (Fig. 3), and most of the explants formed callus at TDZ concentrations ranging between 6.6 and 8.8 μM.

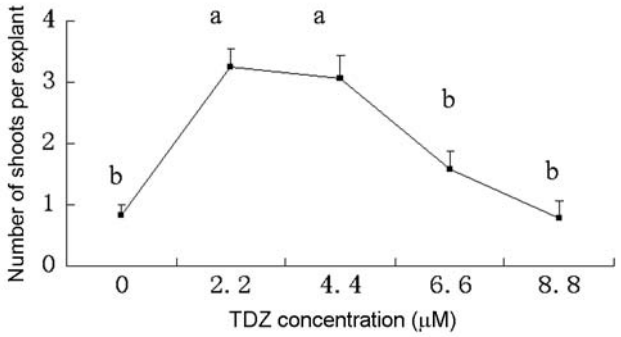

Fig. 3. Effect of TDZ concentration on number of shoots developing per explant of 'Xuemei' cultivar. Means with the same letter do not significantly differ at p = 0.05. Node – ± SD.

ROOTING

Induced shoots longer than 10 mm were excised and transferred to a series of rooting media. It was found that in the absence of plant growth regulators, roots were sparse on shoots growing on 1/2 MS or WPM in all the cultivars used. When the shoots were incubated on 1/2 MS supplemented with 2.5 or 5.0 μM IBA, root initiation was observed at ~8 days. For the 'Xuemei' cultivar there was no significant difference between the basic media with 2.5–5.0 μM IBA for rooting percentage and number of roots per shoot (ANOVA, $p < 0.05$), but a significant difference in shoot length with 1/2 MS supplemented with 5.0 μM IBA (Tab. 4). Moreover, at higher IBA concentrations (>10 μM), calli instead of roots were observed after half a month of culture in root induction media (data not shown). IBA had an obvious effect on rooting in the 'Xuemei' cultivar; more than 80% of the shoots rooted on medium supplemented with 2.5 or 5.0 μM IBA, and all differences between IBA concentrations were significant ($p > 0.05$) (Table 4). Number of roots per shoot and shoot length also significantly differed between the tested IBA concentrations (Tab. 4).

ACCLIMATIZATION AND ESTABLISHMENT OF PLANTS IN SOIL

When the rooted plantlets were transplanted to soil, 100% of the plantlets with more than 3 roots and at least one root longer than 10 mm survived. The survival rate was only 70% for plants with numerous short roots (<10 mm). Under hardening conditions, more than 80% of the plants were success-

TABLE 4. Effect of IBA on rooting of 'Xuemei' cultivar after three weeks of culture. Each value, from 36 explants in each medium, represents means ± SD. Means with the same letter do not significantly differ at p = 0.05 (Duncan's Multiple Range Test)

Rooting medium	IBA (μM)	Rooting (%)	No. of roots per shoot	Root length (mm)
0.5MS	0	10±9c	0.1±0.099c	0.25±0.05b
0.5MS	2.5	86.0±3a	2.89±0.63a	0.39±0.04b
0.5MS	5	85.0±1a	3.14±0.74a	0.99±0.12a
0.5MS	7.5	78±3a	1.09±0.25b	0.98±0.09a
0.5MS	10	35±5b	0.3±0.15c	0.25±0.06b
WPM	5	94.0±6a	5.29±1.17a	0.51±0.15b

fully established in the greenhouse after 4 weeks (Fig. 1g). The plants grew vigorously in the greenhouse or in field conditions after half a year's nursing (Fig. 1h). The appearance and growth of these plantlets were normal.

PCR-ISSR ANALYSIS

At least two independent PCR amplifications were performed for each sample with ISSR primers; only clear bands reproducible from two PCRs were considered for analysis. Each primer generated a unique set of amplification products ranging in size from 200 bp to 1500 bp. The number of bands varied from 1 to 8. The eight primers used in this analysis produced 49 clear bands. The ISSR fingerprints revealed no variation in any of 18 tested micropropagated plantlets. Figure 4 shows representative examples of amplified band patterns produced by primers N37 and N19.

DISCUSSION

To reduce the risk of somaclonal variability during micropropagation of material in vitro, it is advisable to use axillary meristems as explants to obtain uniform planting material on a large scale. Harada and Murai (1996) successfully propagated *P. mume* by in vitro culture, but these studies were genotype-dependent and encountered difficulty in increasing the rooting rate; somaclonal variation was not investigated in that work.

For shoot multiplication, *P. mume* genotype had a significant effect on shoot proliferation frequency in the present study. The number of shoots per explant (2–6) varied significantly between the six studied cultivars. The genetic diversity of these *P. mume* genotypes may be the result of their evolution and of their origin from distantly related ancestors (Bao, 1994).

In our study, TDZ was more effective at lower concentrations. With increased TDZ concentration, shoot number per explant decreased, and finally at 8.8 μM TDZ the shoots were completely replaced by callus formation, indicating that TDZ does not always benefit shoot formation; these results support findings on regeneration of other species (Escalettes, 1993). In addition, high TDZ concentration in the medium markedly inhibited shoot elongation in culture of *P. mume*, consistent with results reported previously (Huetteman, 1993).

Rooting of multiplied *P. mume* shoots was achieved in medium with IBA alone, and the best results were with 1/2 MS or WPM basic medium supplemented with 2.5 or 5.0 μM IBA, respectively. Rooting frequency on 1/2 MS supplemented with more than 7.5 μM IBA was lower than with 2.5 or 5.0 μM IBA, and 10 vM IBA had inhibitory effects (Tab. 4); the same trends were reported in wych elm (Birod et al., 2004). We also found that darkness was more effective than light in promoting root growth (data not shown), as reported by others (e.g., Marin, 1998).

Numerous analyses of somaclonal variation have been done using PCR-based techniques. Genetic stability analyses of micropropagated shoots have been reported in, for example, *Pinus thunbergii* (Goto et al., 1998) chestnut hybrids (Carvalho et al., 2004) and almond (Martins et al., 2004). Our results suggest that the culture conditions applied for axillary bud proliferation are appropriate for clonal propagation of *P. mume*, as the micropropagated plantlets derived from axillary buds were clonally uniform and genetically stable.

Our present work provides a practical protocol for efficient axillary bud multiplication from *P. mume* explants. Compared with previous work (Harada and Murai, 1996), we significantly improved the multiplication frequency, successfully proceeding through root initiation and acclimatization. PCR-ISSR analysis revealed basically no variation in the micropropagated plants obtained by our culture protocol. To the best of our knowledge the literature offers few reports of regeneration of *P. mume*, though there are many descriptions of it in other *Prunus* species (Hammatt and Grant, 1998;

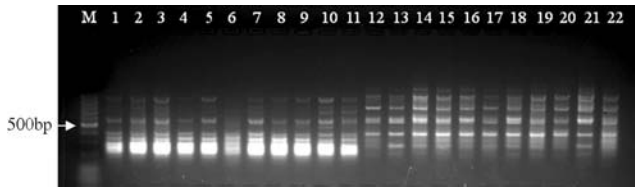

Fig. 4. Agarose gel electrophoresis of ISSR fragments of *P. mume* regenerated shoots. Lanes 1–11 – Monomorphic bands as revealed by gel electrophoresis of ISSR fragments generated with primer N37 (ACACACACACACACA-CYC) in eleven shoots. Lanes 12–22 – primer N19 (GAGA-GAGAGAGAGAGAGART) in eleven shoots (arrow: specific bands in some regenerated shoots). Lane M – 1000 bp molecular markers.

Gentile et al., 2002). Micropropagation of *P. mume* provides an opportunity to maintain virus-free material, conserve important germplasm resources in vitro, and propagate superior cultivars on a large scale.

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