

IN VITRO PROPAGATION OF PUMMELO (*CITRUS GRANDIS* L. OSBECK)

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SUMMARY

Several experiments were carried out to develop protocols for the *in vitro* propagation of pummelo (*Citrus grandis* L. Osbeck) using shoot-tip explants from seedlings. Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzylaminopurine (BA) and thidiazuron (TDZ), singly or in combination with α -naphthaleneacetic acid (NAA), was used to determine the rate of shoot proliferation. The response of explants to all concentrations of TDZ was very poor. After 6 wk culture, the most adventitious shoots per explant (average 5.2) were obtained on medium supplemented with 1.8 μ M BA. NAA with cytokinin in the medium did not improve the rate of shoot multiplication significantly. Addition of 5.8 μ M gibberellic acid in shoot-proliferation medium during the second subculture improved shoot elongation significantly. Shoot multiplication increased 3.5-fold in each successive subculture. NAA was superior to indolebutyric acid for *in vitro* root induction. Over 75% of the shoots developed roots when transferred to half-strength MS medium with 1.3, 2.7, or 5.4 μ M NAA.

Key words: micropropagation; root induction; shoot tip.

INTRODUCTION

Pummelo or shaddock (*Citrus grandis* L. Osbeck), one of the ancestral species of *Citrus*, is an under-utilized fruit crop with a potential for commercialization in warm, humid climates. Presently it is mainly grown in homestead gardens in most countries of Asia and the Pacific region. The juicy pulp of pummelo is either eaten raw or used for juice. In some countries of South-East Asia, flowers, fruits and seeds are used for medicinal purposes (Aubert, 1990) and flowers are also used for perfume (Niyomdham, 1991). Pummelo fruits are used as 'temple fruits' and auspicious gifts to give at various religious and cultural festivals in Chinese communities (Vinning and Moody, 1997).

Rapid cloning of elite genotypes through *in vitro* adventitious shoot proliferation is extensively employed for many fruit species (Zimmerman, 1986). In a wide range of *Citrus* species, *in vitro* grafting is routinely used to propagate virus-free mother stocks. This technique is not feasible for commercial sapling production, as the success rate is low and saplings are too expensive to produce. The production of *in vitro* plants directly from proliferating shoot tips, nodal stem segments, epicotyl segments and root segments is also reported in sweet orange, citron and lime (Duran-Villa et al., 1989); mandarin (Omura and Hidaka, 1992); and *Citrus mitis* (Sim et al., 1989). Perez et al. (1998) obtained normal plantlets through *in vitro* culture of ovules. In pummelo, morphogenesis and shoot regeneration are obtained through secondary organogenesis of callus derived from immature ovules (Huang et al., 1988; Oh et al., 1991; Song et

al., 1991) and shoots (Zhong et al., 1991), but rooting and establishment in the greenhouse are limited by poor shoot elongation. Although shoot elongation is improved by *in vitro* grafting of the emerging shoots onto Troyer Citrange (hybrid of *Citrus sinensis* and *Poncirus trifoliata*) seedlings, the success rate is low (Huang et al., 1988). Moreover, plants obtained from secondary organogenesis from disorganized tissues such as callus may not be true to type, due to somaclonal variation. Baruha et al. (1995) attempted to propagate pummelo from shoot-tip explants of *in vitro*-grown seedlings. They did not describe the procedure for *in vitro* rooting and the shoot proliferation rate was poor. This paper reports effective methods for propagating complete plants of pummelo using *in vitro* shoot-tip culture.

MATERIALS AND METHODS

Source of tissue and preparation of explants. Six-mo.-old seedlings grown in the glasshouse at $27/15 \pm 2^\circ\text{C}$ day/night temperature were used as a source of explants. Actively growing shoot tips (~1.5 cm long) were soaked in distilled water for 30 min and washed three times with sterile distilled water. Shoot tips were dipped in 70% ethanol for 1 min after washing, then surface sterilized with 9% Domestos bleach (containing ~4.25% w/v available chlorine) for 25 min with gentle stirring. Shoot tips were rinsed three times in sterile distilled water. The young leaves and basal portions of shoots injured by sterilant were removed and 0.8–1.0 cm long shoot-tip explants were cultured.

Culture medium. The basic nutrient medium consisted of MS (Murashige and Skoog, 1962) medium (Imperial, Marlow, UK) with 3% sucrose and vitamins, adjusted to pH 5.7 with 0.1 N NaOH and solidified with 0.8% agar (Sigma, Poole, UK). Each 60 ml jar contained 15 ml medium and was sterilized by autoclaving for 20 min at 121°C , 105 kPa. Cultures were maintained at $25 \pm 2^\circ\text{C}$ under a 16 h photoperiod provided by cool white fluorescent light at $65 \mu\text{E m}^{-2} \text{s}^{-1}$ and subcultured every 3 wk.

Shoot proliferation. In the first experiment, freshly prepared explants

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were cultured on medium containing 6-benzylaminopurine (BA) (0.9, 1.3, 1.8, 2.2, 4.4, 8.9, or 13.3 μM). In the second experiment, similar explants were cultured on thidiazuron (TDZ) (0.05, 0.2, 0.5, 2.3, or 4.5 μM). In both experiments, BA and TDZ were added singly or in combination with α -naphthaleneacetic acid (NAA) (0.5 or 1.1 μM), and one control (without plant growth regulators) was used. In the third experiment, gibberellic acid (GA_3) (5.8 and 14.4 μM) was combined with 1.8 μM BA in the medium to evaluate the effect on shoot proliferation. GA_3 was added to the medium either at the time of primary culture (culture of freshly prepared explants) or during the second subculture of explants (shoots cultured for 42 d in medium supplemented with 1.8 μM BA). Explants from the second subculture contained three to five very short shoots.

Multiplication rate and stability of vigor were determined by recurrent subculture of shoots. The shoot-tip explants were cultured on MS medium supplemented with 1.8 μM BA for 42 d, then transferred to the shoot-elongation medium composed of MS + 1.8 μM BA + 5.8 μM GA_3 . Nodal (two nodes per explant) and shoot-tip explants (~1 cm long) were excised from these cultures at 3 wk intervals and cultured further using the same media. The new shoots arose from the primary explant as well as from shoots removed from original explant, and were cultured on medium containing BA/ GA_3 .

Rooting. Actively growing shoots with four or five nodes from the second subculture produced in medium with 1.8 μM BA + 5.8 μM GA_3 were used for rooting. Initial experiments showed no spontaneous rooting when shoot tips were cultured on MS medium supplemented with no growth regulators. Thus rooting medium consisted of MS macro- and micronutrients at full, half and quarter strength with indolebutyric acid (IBA) at concentrations of 1.2, 2.5, 4.9, 9.8, or 24.6 μM , or NAA at 1.3, 2.6, 5.4, 10.7, or 26.9 μM . Cultures were maintained in a growth room under the conditions described above.

Hardening and establishment of plantlets in the greenhouse. Plantlets were transferred into small plastic pots (7.5 \times 8.0 cm) filled with peat, perlite and sand (1:1:1), and were kept inside the seed propagator of a culture room at 25–30°C, 12 h photoperiod. Each plantlet was fed twice a week with 10 ml 10.7 μM NAA. The plantlets were transferred after 4 wk to a greenhouse at 30–40°C, RH 70%, 12 h photoperiod.

Experimental design and data analysis. All experiments were conducted in a two-factor randomized complete block design with four replicates. Fifteen explants were used for each experimental unit. Data on survival, number and length of shoots were recorded at 3 wk intervals in shoot-proliferation experiments. Rooting experiments were evaluated at 6 wk, and data were recorded on the number of rooted shoots, and the number and length of roots. The data were subjected to ANOVA with a 5% significance level using the statistical software MSTAT. Means were compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

Survival. Table 1 shows the percentage survival of explants for each combination of the plant growth regulators BA and NAA. The survival of shoots decreased when concentrations of BA increased, irrespective of NAA concentration. All shoots in the hormone-free medium (control) survived and resumed normal growth, while only 69% of shoots survived when cultured with 2.2 μM BA. Further significant decrease in survival rate of explants was noted when concentrations of BA exceeded 2.2 μM in the medium.

The shoot tips cultured on media with TDZ showed higher mortality (data not shown). Nearly 30% of explants in the lowest concentration of TDZ (0.05 μM) survived beyond 6 wk initial culture, but their growth and proliferation were very poor. Explants cultured on >0.5 μM TDZ started to die from the second week of culture and all were dead by the end of the fourth week. The shoots cultured on TDZ first developed a brown color on their bases which gradually extended to the upper part of the shoot. The development of browning was followed by yellowing and abscission of leaves, and in severe cases, death of the explants resulted.

Shoot proliferation. The response of shoots to all concentrations

TABLE 1

EFFECT OF 6-BENZYLAMINOPURINE (BA) AND α -NAPHTHALENEACETIC ACID (NAA) ON PERCENTAGE SURVIVAL OF PUMMELO SHOOT-TIP EXPLANTS (DATA RECORDED AFTER 6 WK *IN VITRO* CULTURE)

Percentage survival				
BA concentrations (μM)	NAA concentration (μM)			
	0.0	0.5	1.1	Mean
0.0	100 ^a	—	—	100
0.9	95 ^a	80 ^{bc}	78 ^{bc}	84
1.3	85 ^b	77 ^{bc}	77 ^{bc}	79
1.8	73 ^{bc}	73 ^{bc}	78 ^b	74
2.2	72 ^{cd}	67 ^{cd}	70 ^{cd}	69
4.4	58 ^{de}	50 ^{ef}	48 ^{ef}	52
8.9	53 ^{ef}	38 ^{fg}	42 ^{fg}	44
13.3	24 ^{hi}	43 ^{fg}	31 ^{gh}	32
Mean	70.0	61.1	60.6	

Values followed by a common letter are not significant according to Duncan's multiple range test ($P < 0.05$).

TABLE 2

EFFECT OF 6-BENZYLAMINOPURINE (BA) AND α -NAPHTHALENEACETIC ACID (NAA) ON SHOOT PROLIFERATION RATE OF PUMMELO SHOOTS (DATA RECORDED AFTER 6 WK CULTURE)

Number of shoots per explant				
BA concentrations (μM)	NAA concentrations (μM)			
	0.0	0.5	1.1	Mean
0.0	1.00 ^m	—	—	1.0
0.9	2.6 ^{gh}	2.5 ^{ghij}	2.6 ^{ghi}	2.6
1.3	3.9 ^f	3.7 ^e	4.2 ^{cd}	3.9
1.8	5.2 ^a	4.6 ^{abc}	4.8 ^{ab}	4.8
2.2	4.4 ^{bc}	5.0 ^a	3.9 ^{de}	4.4
4.9	2.3 ^{fg}	2.3 ^{fg}	2.1 ^{kl}	2.5
8.88	2.27 ^{hijk}	2.25 ^{ijk}	2.05 ^{kl}	2.48
13.3	2.0 ^{kl}	2.1 ^{kl}	1.9 ^l	2.0
Mean	3.0	3.3	3.1	

Values followed by a common letter are not significant according to Duncan's multiple range test ($P < 0.05$).

of TDZ was very poor (data not shown), but BA was effective for the induction of multiple shoots (Table 2). The number of shoots per explant increased when BA was included in the medium. The greatest number of shoots per explant (5.2) were produced after 6 wk culture on medium containing 1.8 μM BA. The number of shoots per explant decreased gradually when BA exceeded 2.2 μM . There was no significant difference in shoot proliferation when NAA was included with optimal BA treatments. Baruha et al. (1995) obtained most shoots per explant (3.2) when pummelo shoot tips excised from *in vitro*-grown seedlings were cultured on MS medium supplemented with 3.3 μM BA. The present study differs from those results, as the number of shoots per explant was higher at a lower concentration of BA, and the number of shoots decreased with increased concentration.

Thidiazuron is one of the substituted phenyl ureas, with high cytokinin activity (Capelle et al., 1983), and is reported to be active at lower concentrations than purine-based cytokinins (Mok et al.,

TABLE 3

EFFECT OF GIBBERELIC ACID (GA₃) CONCENTRATIONS AND TIME OF APPLICATION ON *IN VITRO* SHOOT PROLIFERATION OF PUMMELO (DATA RECORDED 6 WK AFTER GA₃ APPLICATION)

Per explant			
Treatment			
GA ₃ concentration (μM)	Application time	Number of shoots (mean ± SE)	Length (mm) of shoots (mean ± SE)
0 (Control)	Primary culture	4.6 ± 0.2 ^c	10.9 ± 1.0 ^c
	Second subculture	4.2 ± 0.1 ^c	10.6 ± 0.6 ^e
5.8	Primary culture	3.2 ± 0.3 ^d	30.7 ± 1.4 ^b
	Second subculture	7.7 ± 0.5 ^a	18.2 ± 0.9 ^d
14.4	Primary culture	2.4 ± 0.2 ^d	34.3 ± 1.7 ^a
	Second subculture	5.8 ± 0.4 ^b	22.2 ± 1.0 ^c

MS medium with 1.8 μM 6-benzylaminopurine.

Values within columns followed by a common letter are not significant according to Duncan's multiple range test (*P* < 0.05).

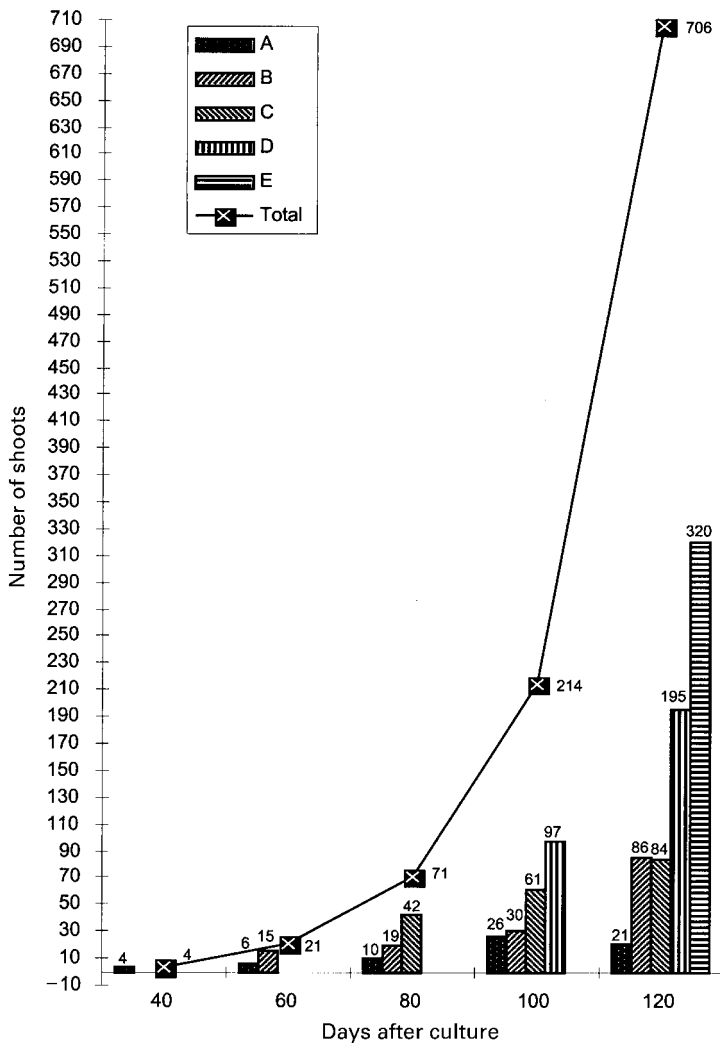


FIG. 1. Shoot multiplication of pummelo over five subcultures on medium MS + 1.8 μM BA + 5.8 μM GA₃. From: A, primary; B, secondary; C, tertiary; D, quaternary; E, pentary explants.

1982). However, the results of the present study indicated that TDZ is unsuitable for *in vitro* propagation of pummelo, as most explants cultured on the medium containing even the lowest concentration (0.05 μM) either died or showed poor proliferation. Pountney and Swietlik (1988) also reported reduced shoot growth of sour orange using TDZ.

Addition of low concentrations of NAA to the medium containing relatively high concentrations of BA did not improve the rate of shoot proliferation in pummelo. This result is in agreement with that of Omura and Hidaka (1992), who found that the addition of NAA is not as critical as that of BA for shoot-tip culture of satsuma mandarin. One reason for shoot proliferation without addition of any

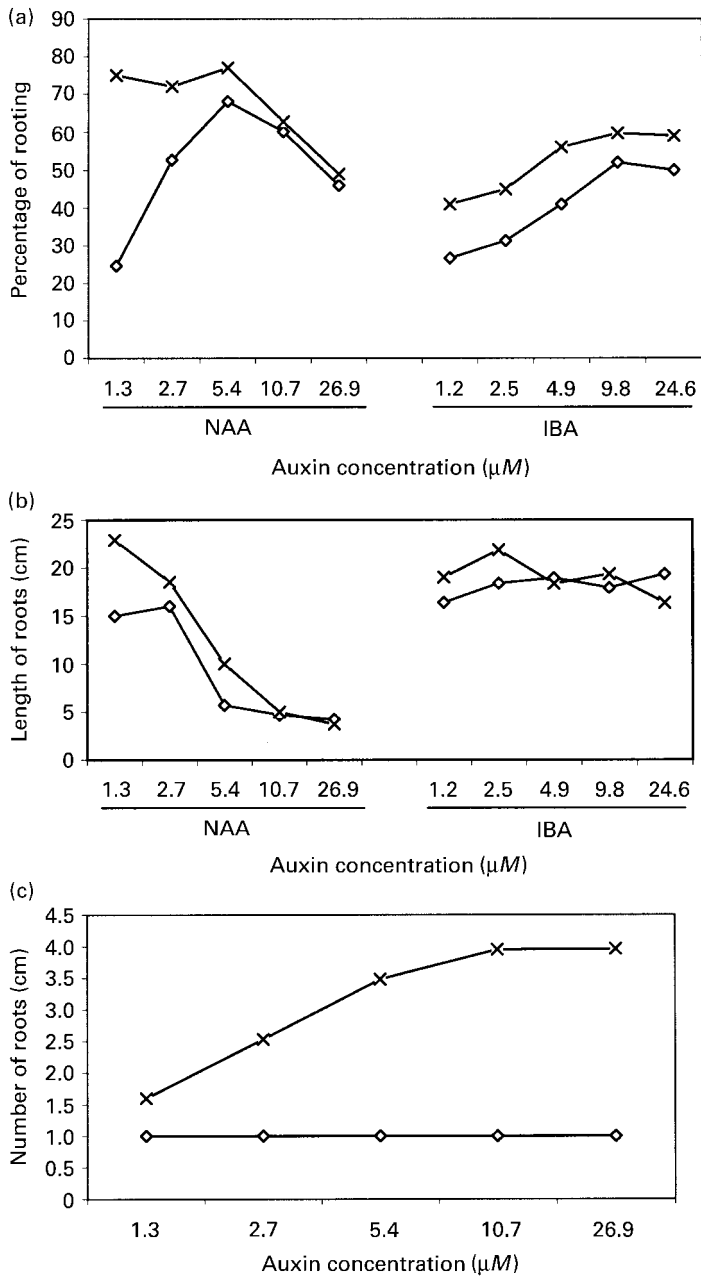


FIG. 2. Effect of auxin concentrations and medium strength on *in vitro* rooting of pummelo. (a, b) Diamond, $1 \times \text{MS}$; cross, $0.5 \times \text{MS}$. (c) Diamond, IBA; cross, NAA.

auxin to the medium may be due to the ability of tissues to synthesize the required amount of auxin endogenously (Smith and Murashige, 1970).

Axillary shoots produced on $1.8 \mu\text{M}$ BA media were short ($10.6 \pm 0.6 \text{ mm}$), and shoot length did not increase with further subculture on the same medium. Individual shoots were difficult to separate due to lack of shoot elongation. Therefore an attempt was made to increase the rate of shoot elongation by adding GA_3 to shoot-proliferation media (MS media containing $1.8 \mu\text{M}$ BA). Concentrations of GA_3 and time of application significantly affected the length of adventitious shoots (Table 3). The length of shoots was found to increase with increasing concentrations of GA_3 . Application of GA_3 in the medium during primary culture produced significantly fewer shoots per explant, and these shoots were very

long, thin, and etiolated. But transfer of shoots to GA_3 -supplemented medium for the second subculture significantly improved shoot proliferation. The most shoots per explant (7.7 ± 0.5) were recorded when shoots were subcultured on medium containing $5.8 \mu\text{M}$ GA_3 . The shoots produced in this treatment were nearly twice the length ($18.2 \pm 0.9 \text{ mm}$) of those produced in GA_3 -free medium ($10.6 \pm 0.6 \text{ mm}$). A significant decrease in number of shoots per explant was noted when the concentration of GA_3 in the medium was increased from 5.8 to $14.4 \mu\text{M}$. Differences in inhibition or promotion of adventitious shoot proliferation by the time of GA_3 application may be due to the fact that this growth regulator inhibits the initiation of meristemoids, but is required for shoot development once meristemoids are formed (Omura and Hidaka, 1992).

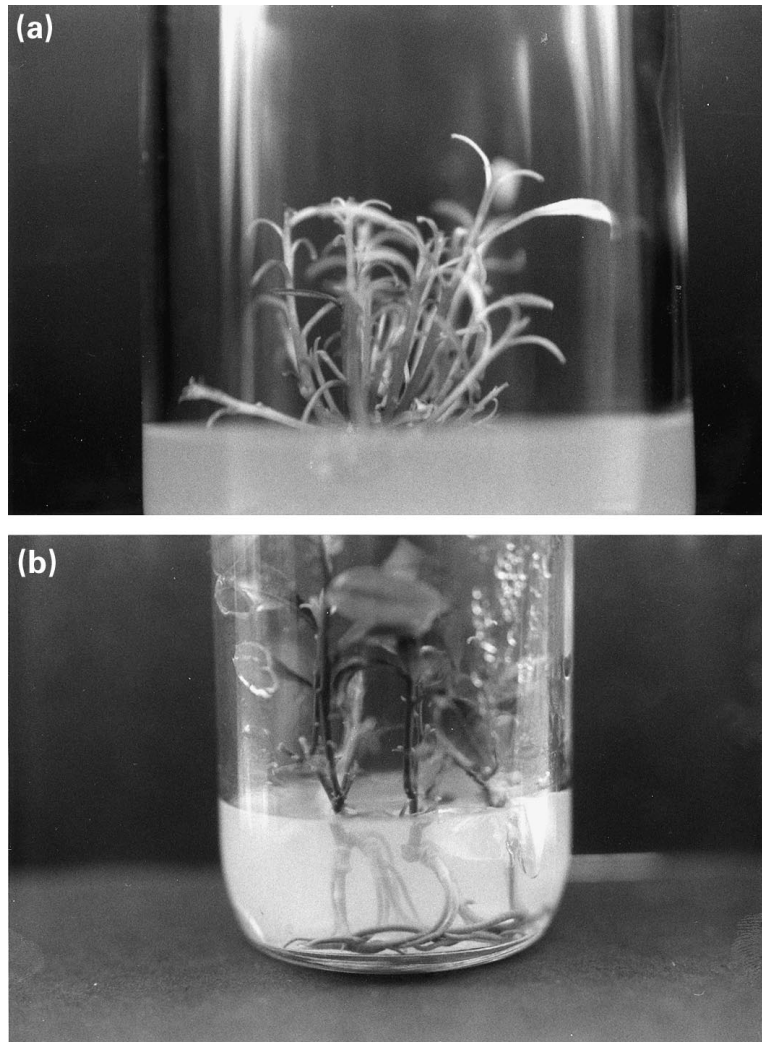


FIG. 3. (a) *In vitro* shoot proliferation on MS medium + 1.8 μM BA + 5.8 μM GA₃. (b) *In vitro* rooting in pummelo on 0.5x-MS medium + 2.7 μM NAA.

A total of 706 shoots were obtained from a single shoot-tip explant after five recurrent subcultures (140 d after the initial culture), with a mean multiplication rate of 3.5-fold (Fig. 1). There was no loss of vigor in the original explant or in regenerated shoots during recurrent subcultures (Fig. 3a).

Rooting. The highest success of rooting ($77 \pm 3.5\%$) was recorded in 0.5x-MS with 5.4 μM NAA (Fig. 2a). 0.5x-MS with 1.3 or 2.7 μM NAA was also effective for rooting of pummelo shoots (Fig. 3b). IBA was less effective for rooting than NAA (Fig. 2a). Rooting success was higher in 0.5x-MS than in full-strength MS (Fig. 2), and no signs of rooting were observed when shoots were cultured on 0.25x-MS (data not shown). The length of roots decreased with higher concentrations of NAA irrespective of medium strength, and the longest roots (19 ± 2 mm) were recorded in 0.5x-MS with 1.3 μM NAA (Fig. 2b). The length of roots (16–22 mm) induced in the IBA-supplemented media did not differ significantly among the concentrations. The shoots cultured with IBA produced only one root per shoot at all concentrations, while those on NAA produced more roots per shoot as the concentration of

NAA increased (Fig. 2c). The number of roots per shoot was not significantly affected by concentrations of macro- and micro-nutrients of rooting media. Variable rooting in response to different types of auxins with mandarin varieties was reported by Omura and Hidaka (1992).

Establishment of plantlets in the greenhouse. The success rate was recorded by the emergence of two or three new leaves. The survival rate was 40%, and these plantlets were slightly yellowish morphologically.

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