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Elimination of In vitro Contamination, Shoot Multiplication, and Ex vitro Rooting of *Aglaonema*

W.L. Chen and D.M. Yeh[1]

Department of Horticulture, National Taiwan University, Taipei, Taiwan, Republic of China

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***Abstract*. Elimination of in vitro contamination and shoot multiplication were studied with *Aglaonema* Schott 'White Tip'. Apparently, contamination was reduced, but explants browned when 200 mg·L^{-1} streptomycin was used as either a pretreatment or incorporated into the medium. Reduced occurrence of contamination and browning was achieved in axillary bud explants excised from the stock plants that had not been watered for 2 months. Six shoots per explant elongated normally in Murashige and Skoog (MS) medium containing 30 μM benzylaminopurine (BA). MS medium containing 20 μM thidiazuron (TDZ) also resulted in six shoots per explant, but these shoots failed to extend beyond a rosette. Only microcuttings from 30 μM BA treatment were used for the ex vitro rooting trial, and indole-3-butytric acid (IBA) at 9.8 or 19.7 mM applied to the base of the microcuttings resulted in 100% ex vitro rooting and the longest roots.**

Aglaonema (Araceae) is one of the most popular indoor plant genera due to its attractive foliar variegation and tolerance to drought and low light conditions (Chen et al., 2002). Commercial *Aglaonema* production almost exclusively starts from cuttings. Cutting propagation, however, may transmit pathogens from stock plants to cuttings. Additionally, some *Aglaonema* cultivars may host endogenous pathogens in their vascular tissue (Chase, 1997), which could make cuttings a source for carrying and spreading disease.

Tissue culture is preferable for rapid multiplication of healthy plants. However, endogenous microbial contamination is known to be one of the most serious problems in tissue culture of ornamental aroids, including *Anthurium* Lind. (Kunisaki, 1980), *Dieffenbachia* Schott (Brunner et al., 1995; Voyiatzi and Voyiatzis, 1989), *Philodendron* Schott (Fisse and Pera, 1987), *Spathiphyllum* Schott and *Syngonium* Schott (Kneifel and Leonhardt, 1992), and *Zantedeschia* Spreng. (Kritzinger et al., 1998). Conventional disinfection methods appear to be unsatisfactory because the initial explants are damaged during the long exposure to sodium hypochlorite (NaOCl), which is necessary for the efficient removal of contamination (Kunisaki, 1980). Internal contamination can be minimized or eliminated using antibiotics incorporated into the culture media (Kneifel and Leonhardt, 1992) or with pretreatment of explants before in vitro culture (Kritzinger et al., 1998). Environmentally friendly methods, such as reduced water supply to stock plants, may be an alternative to decrease endogenous pathogens (Debergh and Maene, 1981).

Culture media supplemented with cytokinins are crucial for shoot multiplication in aroids including *Aglaonema* (Hussein, 2004), *Anthurium andreanum* Lind. (Kunisaki, 1980), *Dieffenbachia exotica* Schott 'Marianna' (Voyiatzi and Voyiatzis, 1989), and *Spathiphyllum floribundum* L. (Ramirez-Malagon et al., 2001). Tissue culture has not been particularly successful with *Aglaonema* (Chen et al., 2003), and information in the literature is currently limited. Thus, the objectives of the present work were to develop a procedure for disinfection, to determine the effects of cytokinins on the shoot multiplication, and to evaluate the effects of auxins on ex vitro rooting of microcuttings in *Aglaonema*.

Materials and Methods

Plant material and culture conditions. Stock plants of *Aglaonema* 'White Tip' were grown in a 70% shaded greenhouse with an average noon *PPF* of 360 μmol·m^{-2}·s^{-1} and mean daily temperature of 26 °C. Stock plants were grown in plastic containers containing 2.1 L of a mix of 4 parts sphagnum peat (Fafard No. 1, Conrad Fafard, Agawam, Mass.):1 part perlite:1 part vermiculite (by volume). Stem sections 7–10 cm long, each with 10 axillary buds, were taken from the stock plants, and the buds were excised for in vitro culture. All in vitro media were dispensed as 25-mL aliquots in sterilized 9-cm diameter petri dishes. The environment of in vitro culture was maintained at 25 ± 2 °C under 12-h photoperiod with 45 μmol·m^{-2}·s^{-1} *PPF* provided from cool-white fluorescent tubes.

Expt. 1: Reducing in vitro contamination with streptomycin. Streptomycin was used (Fisse and Pera, 1987) and applied in two ways: stock plant stem section pretreatment and culture medium supplement. For pretreatment, stem sections of stock plants were rinsed with water for 10 min and then transferred to the antibiotic solution containing 0, 25, 50, 100, 200, 300, or 400 mg·L^{-1} streptomycin (Sigma-Aldrich Chemical Co., St. Louis) on rotating drums at 80 rpm for 24 h. Buds were then disinfected with 1% NaOCl (by volume) that contained two drops of Tween-20 per 100 mL of solution for 15 min, followed by three rinses with sterile distilled water. Axillary buds (Fig. 1A) from disinfected stem sections were excised and placed in the culture medium containing half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), 1.34 μM α-naphthaleneacetic acid (NAA), 4 μM thidiazuron (TDZ), 20 g·L^{-1} sucrose, 0.1 g·L^{-1} myoinositol, and 7 g·L^{-1} agar (Sigma-Aldrich Chemical Co.). The pH of the medium was adjusted to 5.6 before autoclaving.

For the culture medium supplement, stem sections were washed with tap water for 10 min and then treated with the NaOCl procedure described above. Axillary buds from NaOCl-disinfected stem sections were then excised and placed in the culture medium to which 0, 25, 50, 100, 200, 300, or 400 mg·L^{-1} streptomycin had been added through minipore sterilization after autoclaving when the temperature of medium was ≈60 °C. For both pretreatment and medium supplement treatments, there were three replicated petri dishes, with 10 explants for each replicate. Visible contamination and explant browning percentage were determined after in vitro culture for 14 d.

Expt. 2: Reducing in vitro contamination with nonirrigation treatments. This experiment was designed to explore the possibility that nonirrigation treatments of *Aglaonema* stock could reduce the subsequent in vitro contamination. Three treatments were used: irrigation once per week (control) and nonirrigation for 1 or 2 months. Six plants were used in each treatment. Water content of the growing medium and water potential of the recently fully developed leaves (leaf 5 from the apex) in each treatment were determined with the W.E.T. sensor kit (Delta-T Device Ltd., Cambridge, U.K.) and Tru Psi (Decagon Devices, Pullman, Wash.). Young, fully developed leaves from all plants in each treatment were sampled to measure the maximal efficiency of photosystem II (PSII) photochemistry (F_v/F_m) values at 25 °C with a modulated-light PAM-210 (Heinz Walz GmbH, Effeltrich, Germany) after the leaves had been dark-adapted for 30 min. Axillary buds were collected and excised from the stem sections, disinfected by the NaOCl disinfection process, and then cultured in the medium as described for Expt. 1. There were three replicated petri dishes, with 10 explants for each replicate. Visible contamination and percentage of explants browning were also determined after in vitro culture for

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[1]To whom reprint requests should be addressed; e-mail: dmyeh@ntu.edu.tw

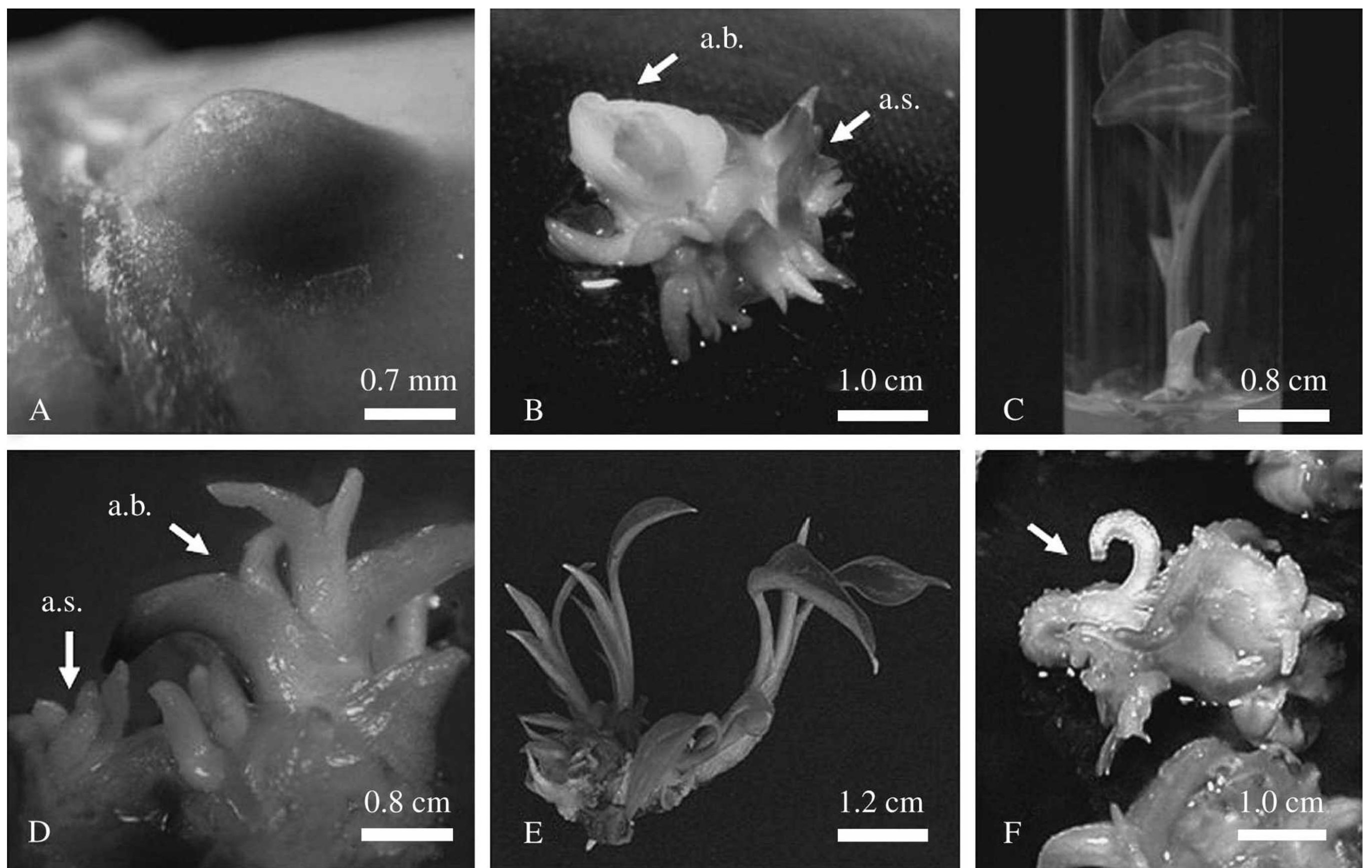

Fig. 1. Shoot multiplication of *Aglaonema* 'White Tip' in vitro culture: (**A**) axillary bud of stem section; (**B**) adventitious shoot formation from axillary bud after culture for 60 d; (**C**) micropropagated plantlet from axillary bud culture after 4 months; (**D**) shoot multiplication from stem section cultured in basal medium with 30 μM BA for 45 d; (**E**) elongated shoots from stem section cultured in basal medium with 30 μM BA; (**F**) rosette shoots with curved leaves (arrow indicated) from stem section cultured in basal medium with 20 μM TDZ. Abbreviations: a.b. = axillary bud; a.s = adventitious shoot.

14 d. In Expts. 1 and 2, surviving explants were subcultured every 2 months. We observed possible recurrence of contamination through four subculture periods.

Expt. 3: Shoot multiplication and ex vitro rooting. Axillary buds were cultured from stock plants that had not been watered for 2 months, as described in Expt. 2. When the micropropagated plantlets developed from axillary buds had 3–4 leaves, the 1.0-cm-long stem sections were cut from the plantlets and used as explants. The basal medium consisted of MS medium with 1.34 μM NAA, 20 $g \cdot L^{-1}$ sucrose, 0.1 $g \cdot L^{-1}$ myoinositol, and 7 $g \cdot L^{-1}$ agar. BA (0, 7.5, 15.0, 22.5, or 30.0 μM) or TDZ (0, 0.4, 2, 4, or 20 μM) was supplemented in the basal medium to determine effects on shoot multiplication. Each treatment included eight replicated explants. Shoot numbers were recorded after 60 d of in vitro culture.

Microcuttings from MS medium supplemented with 30.0 μM BA were distributed in the ex vitro rooting experiment. When the microcuttings had 3–4 visible leaves, they were randomly sampled, transferred to ex vitro, and treated with a basal dip in 0, 2.5, 4.9, 9.8, or 19.7 mM indole-3-butytric acid (IBA; Sigma-Aldrich Chemical Co.) or 0, 3.4, 6.7, 13.4, or 26.8 mM NAA talc, with eight microcuttings per treatment. They were then grown in plastic pots containing a mix of 2 parts sphagnum peat:1 part perlite:1 part vermiculite (by volume) in a growth room at 25 ± 2 °C, 80% to 90% RH, with 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PPF* from cool-white fluorescent tubes for 12 h each day. Percentage of rooting, root number, and root length were recorded after transplanting to the growing mix for 1 month.

Experiment design and data analysis. All experiments were arranged in completely randomized designs. Effects of treatments were determined with analysis of variance followed by Tukey's test at $P \leq 0.05$ or the general linear models procedure. The percentages of contamination and browning were transformed using an arcsine transformation before statistical analysis.

Results

Expt. 1: Reducing in vitro contamination with streptomycin. For both pretreatment and medium supplement treatment, the contamination percentage decreased but the browning percentage increased with increasing streptomycin concentration (Table 1). Explants exhibited browning at ≥50 $mg \cdot L^{-1}$ streptomycin pretreatment or ≥100 $mg \cdot L^{-1}$ streptomycin medium supplement treatment. A high concentration of streptomycin (400 $mg \cdot L^{-1}$) eliminated contamination but caused browning in most explants. Medium supplement treatment caused both higher contamination and browning percentages than pretreatment ($P < 0.001$). Nevertheless, contamination recurred during four subcultures for both treatments.

Expt. 2: Reducing contamination with nonirrigation treatments. Nonirrigation treatments decreased medium water content and leaf water potential but did not significantly affect the leaf F_v/F_m value as compared with the control (Table 2). Nonirrigation treatments reduced the in vitro contamination, with only 6.7% contamination in explants obtained from the stock plants without irrigation for 2 months. Surviving explants did not exhibit browning, and contamination did not recur through four subculture periods.

Expt. 3: Shoot multiplication and ex vitro rooting. Multiple shoots were observed after 2-month axillary bud culture (Fig. 1B). Stem sections were excised from the micropropagated plantlets from 4-month axillary bud culture (Fig. 1C) and cultured on the basal medium with varied concentrations of BA or TDZ. Shoot number increased linearly with increasing BA concentration (Fig. 2). Six shoots formed from each explant and elongated normally in the basal medium containing 30 μM BA (Fig. 1D, E). Regression of shoot number and TDZ concentration showed a curvilinear relationship (Fig. 2). In contrast to the BA treatments, high TDZ

Table 1. Effects of streptomycin pretreatment and medium supplement on in vitro contamination and browning percentages of *Aglaonema* 'White Tip' axillary bud culture.

Streptomycin concn	Pretreatment		Medium supplement	
($mg \cdot L^{-1}$)	Contamination (%)	Browning (%)	Contamination (%)	Browning (%)
0	86.7	0.0	83.3	0.0
25	53.3	0.0	83.3	0.0
50	33.3	3.3	63.3	0.0
100	23.3	23.3	56.7	16.7
200	20.0	33.3	33.3	23.3
300	6.7	46.7	26.7	83.3
400	0.0	66.7	0.0	100.0
Significance	L* Q**	L*** Q**	L*** Q***	L*** Q***

*,**,***Significant at $P \leq 0.05$, 0.01, or 0.001, respectively; L, linear; Q, quadratic.

Table 2. Effects of irrigation treatment on medium water content, leaf water potential and F_v/F_m value of stock plants, and in vitro contamination of axillary bud culture of *Aglaonema* 'White Tip'.

Treatment	Medium water content (%)	Leaf water potential (MPa)	F_v/F_m value	In vitro contamination (%)
Irrigation once per week	32.9 a[z]	–0.49 a	0.81 a	73.3 a
No irrigation for 1 mo.	13.7 b	–0.81 b	0.79 a	53.3 b
No irrigation for 2 mo.	12.8 b	–0.90 b	0.76 a	6.7 c

[z]Means separation within columns by Tukey's test at $P \leq 0.05$.

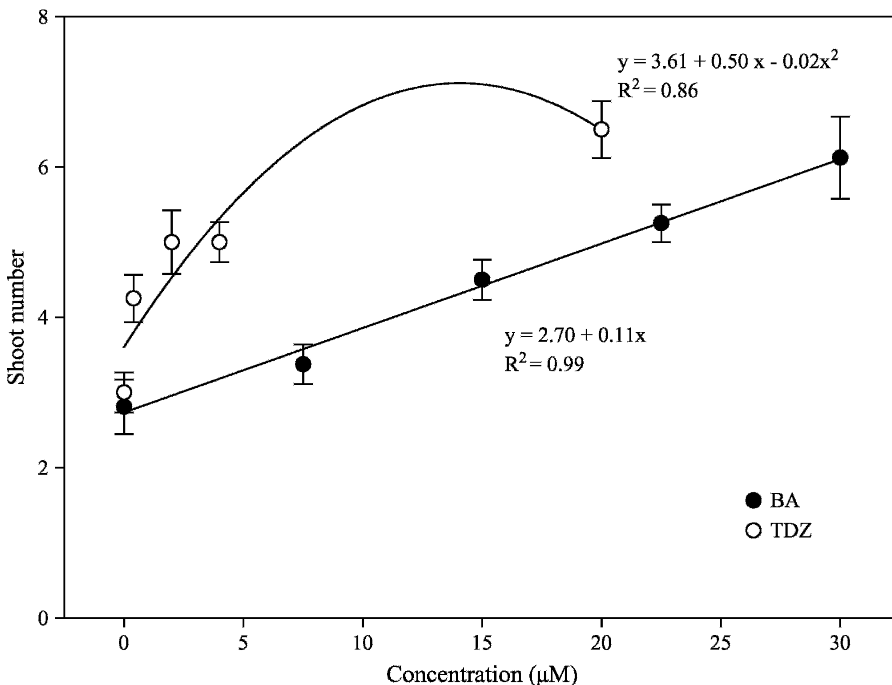

Fig. 2. Effect of BA and TDZ concentration on shoot number of in vitro *Aglaonema* 'White Tip' stem section culture. Bars represent SEM.

concentrations (4 or 20 μM) resulted in rosette (<0.5 cm long) clusters with small and curved leaves (Fig. 1F).

In the ex vitro rooting experiment, all microcuttings from the 30.0 μM BA treatment were successfully acclimatized and rooted when treated with 6.7 or 13.4 mM NAA and 4.9, 9.8, or 19.7 mM IBA after transplanting to the growing mix for 1 month (Table 3). Root number increased when the NAA concentration increased to 13.4 mM and declined when NAA increased to 26.8 mM. Root length was unaffected by NAA concentration. Root number increased with increasing IBA concentration. The 9.8 or 19.7 mM IBA treatments resulted in the longest roots.

Discussion

Serious in vitro contamination problems were shown in *Aglaonema* (Tables 1 and 2), similar to other ornamental aroids (Fisse and Pera, 1987; Brunner et al., 1995; Kritzinger et al., 1998). For *Aglaonema,* in vitro visible contamination could be temporarily reduced using a streptomycin pretreatment (Table 1). Kritzinger et al. (1998) also reported that rhizomes of *Zantedeschia aethiopica* Spreng. pretreated with antibiotic mixtures containing streptomycin before in vitro culture could achieve satisfactory disinfection. When both contamination and phytotoxicity were considered, 200 $mg \cdot L^{-1}$ streptomycin supplemented in the culture medium was more appropriate for *Aglaonema* (Table 1). The best dose for supplementary streptomycin in the culture medium was also 200 $mg \cdot L^{-1}$ for *Philodendron* Schott 'Red Emerald' (Fisse and Pera, 1987). In the present study, pretreatment with streptomycin caused lower contamination percentage than did the medium supplement treatment. This is consistent with the result of Geier (1977), who showed that the inclusion of the antibiotics in sterile distilled water instead of culture medium limited the bacterial growth because no nutrients were involved. However, recurrence of contamination was observed through four subculture periods in both streptomycin pretreatment and culture medium supplement.

Xanthomonas campestris pv. *dieffenbachiae* (Pammel) Dowson, *Fusarium solani* (Sacc.) Mart. emend. Synd. & Hans, and *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey et al. have often been found in vascular tissues or bud axils in *Aglaonema* (Chase, 1997) and other Araceae plants (Debergh and Maene, 1981). We used The Sherlock Microbial Identification System (MIDI, Newark, Del.), a well-established, fully automated gas chromatography analytical system (Cho et al., 2002), in an attempt to identify the microorganisms that caused the contamination. This technique showed that the contamination may have been incited by *Xanthomonas*. However, further investigation is needed to verify the identity of the causal organism.

Water stress can delay or inhibit germination of *Fusarium* (Ramirez et al., 2004) and growth of several fungi (Coleman et al., 1989). In stock plants of *Aglaonema* pretreated with 2 months of nonirrigation, the water potential declined, and thus the pathogens probably could not grow or survive well. This may explain why nonirrigation treatments could minimize in vitro contamination without browning in axillary bud explants (Table 2). Another plausible explanation was that the drought-stressed tissue absorbed bleach solution into deeper layers of explant tissue, resulting in a more effective kill of the microorganisms. Nonirrigation treatments also could prevent infections from soil or water when using overhead watering. These nonirrigation pretreatments did cause the oldest 3–4 leaves to yellow but did not reduce the F_v/F_m value of the fully developed leaves, suggesting that *Aglaonema* is a drought-tolerant plant. Thus, 2-month nonirrigation treatment was considered to be a better alternative than antibiotic treatment for establishment of aseptic cultures of *Aglaonema*.

Increased shoot number with BA have been reported for *Anthurium andreanum* Lind. (Kunisaki, 1980) and *Spathiphyllum*

Table 3. Effects of NAA and IBA concentration on rooting percentage, number of roots, and root length in ex vitro rooting of *Aglaonema* 'White Tip' microcuttings.

Auxin concn (mM)	Rooting (%)	Number of roots	Root length (cm)
NAA			
0	75.0	1.8	0.6
3.4	75.0	2.8	0.6
6.7	100.0	3.0	0.9
13.4	100.0	3.8	0.8
26.8	62.5	1.5	0.8
IBA			
0	75.0	1.8	0.6
2.5	75.0	2.3	0.4
4.9	100.0	3.3	0.4
9.8	100.0	3.5	2.4
19.7	100.0	4.3	2.0
Significance			
NAA concn	Q*	Q**	NS
IBA concn	Q*	Q**	L*
Auxin source	NS	NS	**
Concn × source	NS	*	***

NS,*,**,***Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively; L, linear; Q, quadratic.

floribundum L. (Ramirez-Malagon et al., 2001). Maximum shoot proliferation of *Aglaonema* 'White Tip' was achieved in medium containing 30 μM BA (Fig. 2), and resultant plants continued stable growth through four subcultures. Increasing BA concentration up to 7.5 μM BA resulted in maximum shoot proliferation of *Aglaonema simplex* Blume (Laohavisuti and Mitrnoi, 2005). Inclusion of 7 $mg \cdot L^{-1}$ isopentenyladenine (2ip) in Gamborg (B5) medium resulted in the highest numbers of axillary shoots per explant in *Aglaonema cecilia* 'B.J. Freeman', *A. commutatum* 'Silver Queen', and *A. pictum* 'Tricolor' (Hussein, 2004). It is possible that there are substantial cultivar differences in response to concentration of cytokinins.

TDZ at lower concentrations induced greater shoot multiplication than did BA. However, higher concentrations of TDZ (4 or 20 μM) inhibited shoot elongation (Fig. 2). Similar rosette shoots in other plants incited by high TDZ concentrations have been reviewed by Huetteman and Preece (1993).

Terminal cuttings of *Aglaonema modestum* Schott ex Engler treated with a basal quick-dip in auxins (IBA and NAA) produced more roots than the untreated cuttings (Blythe et al., 2004). The present work also showed that, after the 9.8 or 19.7 mM IBA treatments, all microcuttings rooted and produced maximum root numbers and lengths (Table 3). Thus, ex vitro rooting, compared with in vitro rooting, is of practical value in reducing the time and cost of transplantation.

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