Micropropagation and Genetic Fidelity of the Regenerants of *Aglaonema* ‘Valentine’ Using Randomly Amplified Polymorphic DNA

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Abstract. The present study reports a simple protocol for in vitro regeneration of *Aglaonema* ‘Valentine’ using axillary shoot explants for rapid multiplication and production of true-to-type plants. Different concentrations of benzyladenine (BA; 0, 1, 3, 5, and 7 mg·L⁻¹), kinetin (Kin; 0, 1, 3, 5, and 7 mg·L⁻¹), thidiazuron (TDZ; 0, 0.5, 1.0, 1.5, and 2.0 mg·L⁻¹), naphthalene acetic acid (NAA; 0, 0.5, and 1.0 mg·L⁻¹), and indole-3-butyric acid (IBA; 0, 0.5, and 1.0 mg·L⁻¹) were used for shoot regeneration. The highest shoot proliferation (5.0) was obtained on Murashige and Skoog (MS) medium supplemented with 1.5 mg·L⁻¹ TDZ and 1 mg·L⁻¹ NAA. In vitro rooting was easily achieved with 100% at all concentrations of NAA and IBA supplemented to half- or full-strength MS medium. Regenerated plantlets were acclimatized in greenhouse with 100% survival rate. Randomly amplified polymorphic DNA (RAPD) analysis confirmed the genetic fidelity of the regenerated plantlets and mother plant.

Aglaonema, Araceae, is one of the most important ornamental foliage plant genera due to its attractive foliar variegation and tolerance to low light conditions (Chen et al., 2002). The genus has ≈23 species that are herbaceous evergreens native to tropical and subtropical regions of southeast Asia, northeastern India, across southern China, and into Indonesia and New Guinea (Govaerts and Frodin, 2002; Kew Garden, 2015). ‘Valentine’ is a new attractive hybrid from Thailand with beautiful pink and green random blotches. Commercial *Aglaonema* propagation almost exclusively starts from cuttings and by dividing the basal shoots. However, traditional propagation using cuttings sometimes transmits pathogens such as fungal, bacterial and viral diseases from stock plants to cuttings (Chase, 1987). In addition, some *Aglaonema* cultivars may host

endogenous pathogens in their vascular tissue (Chase, 1997), which could make cuttings a source for carrying and spreading diseases. Subsequently, tissue culture is considered the best method to get stock aroids free from endogenous contaminations (Elsheikh et al., 2013; Taylor and Knauss, 1978).

In vitro propagation methods are used for production of ornamental plants to meet the growing demand in both the domestic and the export market. So, the use of tissue culture technique in vegetatively propagated *Aglaonema* is an alternative method to obtain rapid clonal multiplication. However, the difficulty of establishing or maintaining aseptic culture (Chen and Yeh, 2007) and low rate of shoot multiplication (Chen and Yeh, 2007; Zhang et al., 2004) are definite factors in tissue culture of *Aglaonema* plant. The previous studies showed that average shoot number depends on the cultivar and protocol (Chen and Yeh, 2007; Fang et al., 2013; Mariani et al., 2011; Zhang et al., 2004). *Aglaonema* ‘Valentine’ is slow growing in greenhouse and has a low rate of shoot multiplication in tissue culture. Plant growth regulators (PGRs), especially cytokinins, are crucial for shoot multiplication in aroids including *Aglaonema* (Chen and Yeh, 2007), *Dieffenbachia maculata*

(G. Lodd.) and *Dieffenbachia amoena* WB (Elmahrouk et al., 2006), and *Spathiphyllum cannifolium* Engl. (Dewir et al., 2006). In vitro plant propagation methods have been developed for some *Aglaonema* cultivars, including White Tip (Chen and Yeh, 2007), Cochin (Mariani et al., 2011), and Lady Valentine (Fang et al., 2013). However, previous reports on the micropropagation of *Aglaonema* did not determine the homogeneity among the regenerated plantlets.

In recent years, clonal fidelity assessment of the micropropagated plants was carried out using multiple means such as cytology, morphology, protein marker, and DNA-based molecular markers. Therefore, DNA markers have been reported as an important tool to evaluate the genetic homogeneity and true-to-type nature of micropropagated plants (Cheruvathur et al., 2013). Among them, RAPD is a convenient method for analyzing genetic fidelity (Williams et al., 1990). The previous reports have mentioned that RAPD-based detection of genetic polymorphism has been successful in describing somaclonal variability or homogeneity of micropropagated individuals of numerous plant species (Cheruvathur et al., 2013; Haque and Ghosh, 2013; Kumar et al., 2013).

The objective of this study was to investigate the influence of different types and concentrations of cytokinins alone or in combination with auxins on in vitro regeneration of *Aglaonema* ‘Valentine’. RAPD marker was employed to confirm genetic fidelity of the regenerated plantlets.

Materials and Methods

Plant material and surface sterilization.

Stock plants of *Aglaonema* ‘Valentine’ were grown in a shade greenhouse with an average noon photosynthetic photon flux density (PPFD) of 180 μmol·m⁻²·s⁻¹ and mean daily temperature of 25 °C in the nursery of El Kenana Company, Tanta, Egypt. *Aglaonema* shoots (5–10 cm long) were defoliated and washed under running tap water. Then, they were rinsed thrice with distilled water and sectioned to 4- to 5-cm segments containing three to four nodes. They were disinfected for 3 min in 70% ethanol followed by 10 min in a solution of 0.1% (w/v) mercuric chloride containing two to three drops of Tween-20 (Loba Chemical Company, India). After three rinses with sterile distilled water, explants were cultured for 8 weeks on MS medium (Murashige and Skoog, 1962), containing 3% (w/v) sucrose and 2.0 g·L⁻¹ gelrite for induction of axillary shoots.

Shoot multiplication. *Aglaonema* axillary shoots (2.5–3.0 cm in length) were cultured in a cylindrical culture jar (375-mL capacity) containing 60 mL MS basal medium supplemented with 30 g·L⁻¹ sucrose and solidified with 2.0 g·L⁻¹ gelrite. Different concentrations of BA (0, 1, 3, 5, and 7 mg·L⁻¹), Kin (0, 1, 3, 5, and 7 mg·L⁻¹), TDZ (0, 0.5, 1.0, 1.5, and 2.0 mg·L⁻¹), NAA (0, 0.5, and 1.0 mg·L⁻¹), and IBA (0, 0.5, and 1.0 mg·L⁻¹) were added to the media before autoclaving, depending on the objective of the experiment. MS basal medium without PGRs served as control.

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Shoot elongation and in vitro rooting. Shoot clusters of *Aglaonema* were cultured on MS medium without PGRs for their subsequent growth and elongation. The cultures were kept

at 25 °C and 35 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD (16 h/d) for 4 weeks. Shoots (>3 cm long) were separated and used for rooting in different strengths of MS basal medium (full and half strength) supplemented with different concentrations of IBA or NAA at 0, 1, 3, and 5 $\text{mg}\cdot\text{L}^{-1}$.

Culture conditions. The cultures were incubated at 25 $\pm$ 2 °C under a 16-h photoperiod provided by cool-white fluorescent tubes at 35 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD.

Acclimatization. Plantlets at the three to five leaf stage were transplanted into culture pots (5 cm diameter) filled with a mixture of sterilized peatmoss and perlite (1:1). The

plantlets were covered with a clear plastic film during the first 10 d of culture in the growth chamber and watered with a nutrient solution containing half MS salt strength. The environment in the growth chamber was adjusted to 25 $\pm$ 2 °C air temperature, 60% to 70% relative humidity, and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD with a 16-h photoperiod using halide lamps.

Plant DNA extraction and RAPD-polymerase chain reaction conditions. DNA was extracted from fresh leaves (oldest two leaves on the plant) of the mother plant and seven acclimatized plants (two or three plants every treatment) resulted from the best treatments of in vitro shoot multiplication (3 $\text{mg}\cdot\text{L}^{-1}$ BA + 1 $\text{mg}\cdot\text{L}^{-1}$ NAA, 1.5 $\text{mg}\cdot\text{L}^{-1}$ TDZ + 0.5 $\text{mg}\cdot\text{L}^{-1}$ NAA, and 1.5 $\text{mg}\cdot\text{L}^{-1}$ TDZ + 1 $\text{mg}\cdot\text{L}^{-1}$ NAA) by cetyltrimethylammonium bromide according to Doyle and Doyle (1990). Polymerase chain reaction (PCR) was performed and repeated three times using six random decamer primers (Table 1) (Al-Saghir and Abdel-Salam, 2015; Joshi et al., 2009). RAPD-PCR was carried out in presence of 1 $\times$ Taq DNA polymerase buffer (10 mM Tris-HCl of pH 8.3, 50 mM KCl, 1.5 mM MgCl_2), 100 μM dNTPs, 5 pmol single random primers, 25 ng DNA template, and 0.5 unit of Taq DNA polymerase in a total volume of 25 μL . PCR amplification was performed in an automated thermal cycler (MJ Mini; Bio-Rad, Foster City, CA) programmed as follows, 95 °C for 4 min followed by 40 cycles of 1 min for denaturation at 94 °C, 30 s for annealing at 35 °C and 2 min for polymerization at 72 °C, followed by a final extension step at 72 °C for 7 min. The amplification products were resolved by electrophoresis in 1.5% agarose gels in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer and documented on Gel Documentation system (UVITEC CAMBRIDGE Company, Cambridge, UK). This work was conducted at ElKenana Lab. of plant tissue culture, Tanta, Egypt during the years 2013 and 2014.

Experimental Design and Statistical Analysis

Experiments were set up in a completely randomized design and each treatment had three replicates. Each replicate was represented by a culture jar containing four shoots rendering a group of 12 shoots per treatment. Observations on shoot multiplication as well as in vitro rooting were recorded after 8 weeks of culture. Data were subjected to analysis of variance using SAS program (Version 6.12; SAS Institute Inc., Cary, NC). The mean separations were carried out using least significant difference tests and significance was determined at $P \leq 0.05$, 0.01, and 0.001.

Results and Discussion

Effect of cytokinins on in vitro shoot multiplication and growth. Shoot multiplication of *Aglaonema* was significantly influenced by type and concentration of cytokinins (Table 2). The highest shoot number (2.5 and 2.3 shoot per

Table 1. List of the used primers and their nucleotide sequences.

Primer name	Sequence (5'→3')
OPK-11	AATGCCCCAG
OPB-03	CATCCCCCTG
OPA-10	GTGATCGCAG
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPB-01	GTTTCGCTCC

Table 2. Effect of cytokinins on shoot multiplication and growth of *Aglaonema* 'Valentine' after 8 weeks of culturing.

Cytokinin ($\text{mg}\cdot\text{L}^{-1}$)	No. of shoots per explant	Shoot length (cm)	Fresh wt per explant (g)
MS without cytokinin	1.3	7.9	2.16
BA			
1.0	2.1	6.8	2.47
3.0	2.5	6.8	3.06
5.0	2.1	6.6	2.42
7.0	2.0	6.8	2.64
Kin			
1.0	1.3	6.2	1.79
3.0	1.5	7.9	1.90
5.0	1.6	6.9	1.46
7.0	1.3	8.7	2.00
TDZ			
0.5	1.8	6.9	2.30
1.0	2.1	6.9	3.35
1.5	2.3	6.9	3.16
2.0	2.0	6.4	3.13
LSD	0.63	1.54	0.72
Significance ^z			
Cytokinin type (CT)	***	*	***
Cytokinin concentration (CC)	NS	NS	NS
CT $\times$ CC	NS	NS	NS

MS = Murashige and Skoog medium; BA = benzyladenine; Kin = kinetin; TDZ = thidiazuron; LSD = least significant difference.

^zNS, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

Table 3. Effect of different auxins in combination with different cytokinins on shoot multiplication and growth of *Aglaonema* 'Valentine' after 8 weeks of culturing.

Cytokinin ($\text{mg}\cdot\text{L}^{-1}$)	Auxins ($\text{mg}\cdot\text{L}^{-1}$)		No. of shoots per explant	Shoot length (cm)	Fresh wt per explant (g)
	IBA	NAA			
BA 3.0	—	—	2.5	6.9	3.08
	0.5	—	2.0	4.8	0.94
	1.0	—	1.8	4.6	0.85
	—	0.5	3.0	6.3	2.22
	—	1.0	4.0	5.8	2.90
Kin 5.0	—	—	1.6	6.8	1.49
	0.5	—	1.0	4.2	0.55
	1.0	—	1.0	4.7	0.85
	—	0.5	1.5	4.6	1.08
	—	1.0	1.7	4.5	1.47
TDZ 1.5	—	—	2.3	7.3	3.18
	0.5	—	2.7	4.9	3.47
	1.0	—	2.2	4.1	0.81
	—	0.5	4.0	8.5	4.48
	—	1.0	5.0	8.4	4.38
LSD			0.76	0.64	0.61
Significance ^z					
Cytokinin type (CT)	***	***	***		
Auxin type (AT)	***	***	***		
Auxin concentration (AC)	NS	NS	NS		
CT $\times$ AT	NS	***	NS		
AT $\times$ AC	NS	NS	NS		
CT $\times$ AC	NS	NS	*		
CT $\times$ AT $\times$ AC	NS	NS	NS		

IBA = indole-3-butyric acid; NAA = naphthalene acetic acid; BA = benzyladenine; Kin = kinetin; TDZ = thidiazuron; LSD = least significant difference.

^zNS, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

explant) was obtained on MS medium supplemented with 3 mg·L⁻¹ BA or 1.5 mg·L⁻¹ TDZ, respectively. Kin was less effective than BA or TDZ for shoot multiplication of *Aglaonema* in which 1.3 shoot per explant was obtained at 1 and 7 mg·L⁻¹. Many reports demonstrated that BA is superior to other cytokinins for the release of axillary buds from apical dominance in other Araceae members including *Dieffenbachia* (Elmahrouk et al., 2006) and *Spathiphyllum* (Dewir et al., 2006). Chen and Yeh (2007) found that maximum shoot proliferation of *Aglaonema* 'White Tip' was achieved in MS medium containing 6.8 mg·L⁻¹ BA after 60 d of culture. This is contradictory to the study conducted by Fang et al. (2013) who reported that no adventitious shoot formation was observed on *Aglaonema* 'Lady Valentine' stem nodal segments when BA was the sole PGR in the medium. Previous studies showed that different cultivars of *Aglaonema* such as B.J. Freeman, Silver Queen, Tricolor, and White Tip had various responses to different concentrations of cytokinins (Chen and Yeh, 2007). Besides, *Aglaonema* shoots that were cultured on medium with TDZ at 2 mg·L⁻¹ showed swollen shoot base. The abnormal plant growth associated with high TDZ concentrations has been demonstrated by Dewir et al. (2006) and Elmahrouk et al. (2010) in *Spathiphyllum cannifolium* and *Arbutus unedo*, respectively. The highest shoot length (8.7 cm) was obtained by 7 mg·L⁻¹ kin. TDZ at 1 mg·L⁻¹ gave the highest shoot fresh weight (3.35 g), whereas the lowest one (1.46 g) was obtained at 5 mg·L⁻¹ kin. Similar results were mentioned by Chen and Yeh (2007) that higher concentrations of TDZ decreased shoot elongation. TDZ-induced morphogenesis probably depends on the levels of hormones and modulates the endogenous auxin level. The effect depends on the concentration and the duration of its application.

Effect of PGRs on in vitro shoot multiplication and growth. When cytokinins were employed with NAA, the number of shoots per explant increased in comparison with treatments with cytokinins alone (Table 3). Treatments with auxins alone were not included, since preliminary tests had shown that these growth regulators only induced rooting (data not shown). The highest number of shoots, five per explant, was obtained with 1.5 mg·L⁻¹ TDZ combined with 1 mg·L⁻¹ NAA (Fig. 1A). In general, higher shoot number was achieved in all tested levels of cytokinins combined with NAA rather than IBA. Similar results were obtained by Zhang and Chen (2008) on "*Dieffenbachia*." They mentioned that 1.5 mg·L⁻¹ BA + 0.05 mg·L⁻¹ NAA was the appropriate medium for axillary bud development. The highest shoot length (8.5 cm) and fresh weight (4.48 g) per explant were obtained with 1.5 mg·L⁻¹ TDZ combined with 0.5 mg·L⁻¹ NAA. Similar values for shoot length and fresh weight were also obtained when 1.5 mg·L⁻¹ TDZ was combined with 1 mg·L⁻¹ NAA. The use of a cytokinin in combination with an auxin can be effective for increasing shoot multiplication (Dewir et al., 2006; Shen et al., 2007). The present study demonstrated

Fig. 1. In vitro propagation of *Aglaonema* 'Valentine'. (A) Shoot multiplication from axillary shoot on Murashige and Skoog medium supplemented with 1.5 mg·L⁻¹ thidiazuron and 1 mg·L⁻¹ naphthalene acetic acid (bar = 2 cm); (B) plantlets rooted in vitro (bar = 2 cm); (C) acclimatized plantlets in the greenhouse.

the effectiveness of combining NAA with cytokinins for increasing shoot multiplication of *Aglaonema* 'Valentine'. Similar findings on *Aglaonema commutatum* and *Aglaonema* 'Lady Valentine' have been reported by Zhang et al. (2004) and Fang et al. (2013), respectively. The proportion of auxin-cytokinin is a determinant for stem formation and the hormone balance that becomes established between growth regulators determines the type of buds induced (George, 1993). The inherent endogenous auxin and cytokinin levels must have also played part in bud differentiation (Pierik, 1987). These results suggest that one mode of action for auxins could be to downregulate both local cytokinin synthesis and cytokinin export from medium;

this might influence the endogenous cytokinin levels and lead to the activation of buds (Sato and Mori, 2001).

Effect of salt strength and auxin type on in vitro rooting. All concentrations of NAA and IBA supplemented to half- and full-strength MS medium resulted in a 100% rooting of *Aglaonema* 'Valentine' (Table 4; Fig. 1B). Half MS salts was superior to full MS salts for rooting of *Aglaonema*, possibly due to reduced nitrogen content than a reduced osmotic potential (Hyndman et al., 1982). Reducing mineral salts not only increased rooting percentage but also increased number of roots. The highest number of roots (7.4 and 7) was obtained when half MS medium supplemented with 5 mg·L⁻¹ NAA or IBA,

respectively. Several reports demonstrated reducing MS salts for improved in vitro rooting (Dewir et al., 2011; Parveen et al., 2010; Sharma et al., 2014). IBA has been proved superior to other auxins for in vitro rooting (Dewir et al., 2010). However, in the

present study, there were no significant differences between IBA and NAA for in vitro rooting of *Aglaonema*. Microshoots of *Aglaonema* 'Cochin' were rooted and developed on MS medium containing 3 mg·L⁻¹ IBA (Mariani et al., 2011). All rooted plantlets were acclimatized following their transfer to controlled greenhouse condition at 25 °C and 70% relative humidity (Fig. 1C). All plantlets were morphologically identical with the mother plant.

Genetic fidelity test of regenerates by RAPD. RAPD markers were used to investigate genetic fidelity among mother plant and the acclimatized regenerants. The banding profile based on RAPD primers indicated that both mother plant and the randomly tested genotypes have the same banding patterns consequently (Fig. 2). Therefore, using those six primers confirmed that mother plant and in vitro plantlets could not be distinguished. All tested treatments gave plantlets identical to the mother plant. Clonal fidelity is a major consideration in commercial micropropagation (Sharma et al., 2014). Supplementation of plant tissue culture medium with PGRs may increase the occurrence of somaclonal variation (Mujib et al., 2013). Many techniques have been developed to detect and identify genetic variations (Ruibal-Mendieta and Lints, 1998). However, RAPD is a quick and reliable method for distinguishing genetic variation (Dewir et al., 2005; Elbanna et al., 2013; Yadav et al., 2012). Also, RAPD

Table 4. Effect of Murashige and Skoog (MS) salt strength and auxin type and concentration on in vitro rooting of *Aglaonema* 'Valentine' after 8 weeks of culturing.

MS salt strength	Auxins (mg·L ⁻¹)	Rooting (%)	No. of roots per plantlet	Root length (cm)
Full strength	No auxin	87.5	2.1	3.5
	NAA			
	1	100	6.8	4.5
	3	100	6.6	3.4
	5	100	3.8	1.4
	IBA			
	1	100	3.8	2.9
	3	100	5.0	1.4
	5	100	4.0	2.8
	No auxin	100	2.6	4.0
Half strength	NAA			
	1	100	4.0	2.7
	3	100	6.8	2.4
	5	100	7.4	2.3
	IBA			
	1	100	3.8	3.5
	3	100	5.4	3.0
	5	100	7.0	3.0
			1.47	0.82

LSD
Significance^z
Salt strength (SS) *
Auxin type (AT) NS
Auxin concentration (AC) NS
SS × AT NS
SS × AC NS
AT × AC NS
SS × AT × AC NS

NAA = naphthalene acetic acid; IBA = indole-3-butyric-acid; LSD = least significant difference.
^zNS, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

Fig. 2. Randomly amplified polymorphic DNA fingerprint of mother plant (1) and acclimatized regenerants (2–8) through shoot multiplication on Murashige and Skoog (MS) medium supplemented with 3 mg·L⁻¹ benzyladenine + 1 mg·L⁻¹ naphthalene acetic acid (NAA), 1.5 mg·L⁻¹ thidiazuron (TDZ) + 0.5 mg·L⁻¹ NAA, and 1.5 mg·L⁻¹ TDZ + 1 mg·L⁻¹ NAA; (A, B, C, D, E, and F) are OPK-11, OPB-03, OPA-10, OPA-04, OPA-05, and OPB-01 primers, respectively.

analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals (Rani et al., 1995).

The present study reported a simple protocol for in vitro production of *Aglaonema* 'Valentine' plants via axillary shoot explants and the produced plantlets were true to type.

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