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An Improved System for Rapid *in vitro* Regeneration of *Saintpaulia ionantha*

Zahra Ghorbanzade* and Mohammad Ahmadabadi

Department of Biotechnology, Faculty of Agriculture, Azarbaijan Shahid Madani University, 35 km Tabriz-Maraqeh Road, Tabriz, Iran

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Abstract

For rapid multiplication and genetic manipulation of African violets (*Saintpaulia ionantha* Wendl.) was aimed at developing a rapid and efficient regeneration and adaptation system from leaf explants. Using RM medium supplemented with different combinations of growth regulators, we developed a highly efficient and time-saving *in vitro* regeneration protocol. The developed system was also successfully applied for plant regeneration from petiole and internode explants of several *Saintpaulia* cultivars at a high efficiency. Regeneration occurred through both somatic embryogenesis and shoot organogenesis at a high frequency. Also, we developed a rapid root formation protocol followed by their efficient adaptation for *in vitro* regenerated plantlets.

Introduction

African violet (*Saintpaulia ionantha* Wendl.) is an economically important ornamental plant with many varieties with diverse colors and shapes. Propagation of African violet through traditional method of leaf cuttings is highly time-consuming. Also, a limited number of plants are produced through this method and therefore, development of a rapid propagation method of this plant species will be of a great economic benefit in ornamental plant industry. One of the best methods for rapid plant propagation is *in vitro* culture technology that enables fast multiplication of plants in a short time and limited space. So far, successful plant regeneration from different parts of the African violets has been reported in leaves (Cooke 1977, Daud et al. 2008, Mithila et al. 2003, Shukla et al. 2013, Smith and Norris 1983, Start and Cumming 1976, Sunpui and Kanchanapoom 2002), anther (Weatherhead et al. 1982), sub-epidermal tissue

*Author for correspondence: <m.ahmadabadi@azaruniv.edu>.

(Bilkey and Cocking 1981), protoplasts (Hoshino et al. 1995, Winkelmann and Grunewaldt 1995), petioles (Mithila et al. 2003, Sunpui and Kanchanapoom 2002) and floral buds (Daud and Taha 2008, Molgaard et al. 1991). However, most of the methods are largely time-consuming and in several cases, regenerated plants lose their flowering competence (Daud and Taha 2008, Daud et al. 2008). Also, in most of the protocols for *in vitro* propagation of African violets, shoot regeneration has been considered as the most important step, however, successful root induction and uninterrupted robust growth are critical for rapid and efficient plant hardening and adaptation. It is, therefore, necessary to optimize *in vitro* regeneration systems for rapid and efficient shoot and root induction with no significant effects on the plant phenotype. Several factors are involved in optimizing a tissue culture system; therefore, that the combination of appropriate concentrations of plant growth regulators is considered as the most important one. In this study, we aimed at developing a rapid shoot regeneration and subsequent root formation system for efficient multiplication of African violets from leaf and petiole segments.

Materials and Methods

Five local cultivars of *Saintpaulia ionantha* Wendl. from Guilan province, Iran were used in this experiment. For surface sterilization, plant materials were washed under running tap water and disinfected with 70% ethanol for 30 sec followed by incubation in 5% sodium hypochlorite for 15 min. The plant segments were then washed with sterile water under aseptic conditions and cultured on MS.

For leaf culture, leaves of young sterile plants were cut in about 3 × 3 mm pieces. Leaf segments were then cultured on RM medium (Linsmaier and Skoog 1965) complemented with 30 g/l sucrose and different combinations of hormones for shoot induction (Table 1). In addition, MS supplemented with different plant growth regulators has been evaluated for shoot elongation as well for root formation and growth from *in vitro* regenerated shoots. Petioles of sterile leaves were cut into about 1 mm pieces followed by culturing with the basal side on desired media. Three mm long root sections of sterile plants were cultured on the optimized medium.

The pH was adjusted to 5.8 and the media were solidified by adding 7 g/l agar-agar (HIMEDIA-India). thidiazuron (TDZ) and GA_3 were filter-sterilized (0.2 μm, Whatman, UK) and added to the medium after autoclaving.

Cultured plates were incubated in growth chamber under a diurnal cycle of 16 hrs light of 25 $\mu E/m^2/s$ at 25°C followed by an 8 hrs dark period at 22°C. After four - six weeks (without subculture) regeneration was evaluated and data were

noted. The adopted experimental design was completely randomized with three replicates. The statistical comparison was performed by one-way ANOVA using SPSS software version 19.0, and means were compared using the Duncan's test ($p < 0.05$).

Results and Discussion

For rapid and efficient shoot regeneration and subsequent root formation a protocol for African violets (*Saintpaulia ionantha* Wendl.) was developed. Shoot induction in *in vitro* culture usually requires the exogenous application of growth regulators such as auxin and cytokinin (Riou-Khamlichi et al. 1999). In African violet, application of zeatin (Daud et al. 2008), BAP (Daud and Taha 2008, Sunpui and Kanchanapoom 2002) and TDZ (Mithila et al. 2003, Shukla et al. 2013) have been reported to play an important role in *in vitro* shoot regeneration. However, most of the experiments have been carried out using MS basal medium. Our initial studies using two basic media including MS and Revised-MS (RM) (Linsmaier and Skoog 1965), fortified with 1 mg/l BAP and 1 mg/l NAA indicated that the RM basic medium is more suitable for African violet regeneration (data not shown). Therefore, all other experiments for shoot regeneration were performed using RM basic medium. To find out the best hormonal combination and concentration for shoot induction from leaf discs, we tested different concentrations of BAP and TDZ in combination with NAA (Table 1) on shoot regeneration from 0.3 cm^2 leaf fragments of African violet in RM basal medium. Results showed that shoot induction on TDZ-containing media starts about two

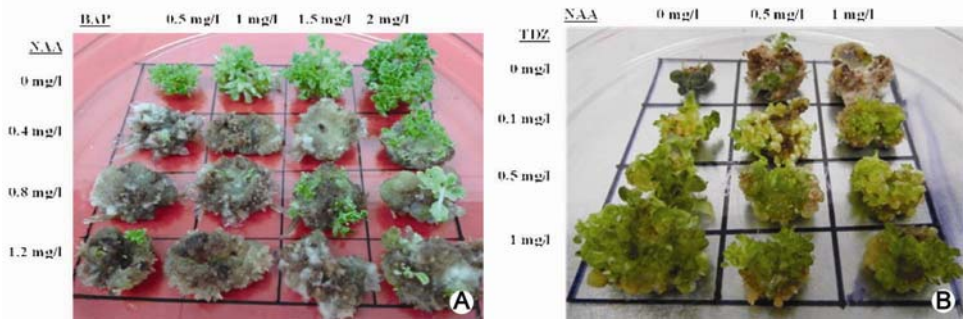

Fig. 1. *In vitro* regeneration response of *S. ionantha* leaf tissues cultured on different media containing various combinations of NAA and BAP (A) or NAA and TDZ (B).

weeks earlier than BAP-containing media. The highest shoot induction rate (100%) was also observed on medium containing 1 mg/l TDZ (Table 1 and Figs 1 and 2A). It should be mentioned that on BAP containing media, regeneration occurred mainly through direct organogenesis, while the addition of TDZ promoted somatic embryogenesis (Fig. 1).

In general, in both experiments, NAA application resulted in a dose-dependent callus induction (Table 1, Fig. 1). The calli on BAP-containing media were largely non-embryogenic with a high tendency to root formation (Fig. 1A), while the calli produced on TDZ-containing media were mainly embryogenic with ability to generate shoots over the time (Fig. 1B).

Taking all important parameters including regeneration rate and frequency into account (Table 1 and Fig. 1), RM medium supplemented with 1 mg/l TDZ has been recognized as the best medium for rapid and efficient shoot induction (Fig. 2A). We have tested this medium for shoot regeneration from petiole, internode and root explants. High efficiency rapid regeneration was obtained from petiole and internode explants. The results were comparable to those obtained from leaf explants (Fig. 2B and data not shown). However, for root explants, plant regeneration occurred through callus induction with lower efficiency (50%) (Data not shown). Also, the same protocol has been successfully used for shoot regeneration from at least five varieties of African violets. Similar high shoot regeneration rates were observed for all varieties, indicating genotype-independency of the developed protocol (Data not shown).

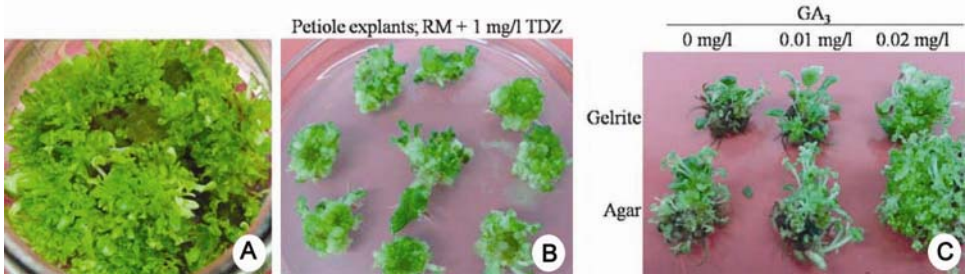

Fig. 2. *In vitro* regeneration response of *S. ionantha* from leaf (A) and petiole (B) explants cultured on RM + 1 mg/l TDZ after six and four weeks, respectively. (C) The effects of added GA_3 in the MS on shoot multiplication and elongation of regenerated plantlets are shown. Two different gelling agents, gelrite and agar, were tested in this experiment.

Application of GA_3 in the culture media has been reported to have different effects on shoot and root induction and elongation. In tobacco, for example, addition of GA_3 has led to irreversible repression of shoot formation (Thorpe and Meier 1973). In buckwheat, GA_3 application has resulted in accelerated root initiation and increased root number (Srejović and Nešković 1985). Positive effects of GA_3 on shoot induction and elongation have also been reported in ginger (Lincy and Sasikumar 2010), *Cocos nucifera* L. (Montero-Cortes et al. 2010), *Tylophora indica* (Sahai et al. 2010) and *Peperomia* (Ahmadabadi and Bock 2010). Therefore, we tested low concentrations of GA_3 for its effects on *in vitro*

regenerated shoots of African violet. The results showed that GA_3 has positive effects on shoot multiplication of African violet cultures; however, shoot elongation as well as root formation have not been affected significantly (Fig. 2C).

Rapid root induction and elongation is one of the most important steps toward fast and successful production of *in vitro* regenerated shoots. However, this issue has not been considered in most of the protocols developed for *in vitro* propagation of African violets. We, therefore, aimed at developing a reliable root induction and an elongation system for *in vitro* regenerated shoots of African violets. Incubation of regenerated shoots on hormone free MS or half strength of MS did not result in efficient root induction (Fig. 3A). As auxins are well known to induce root induction (Verstracten et al. 2013), different concentrations of IAA

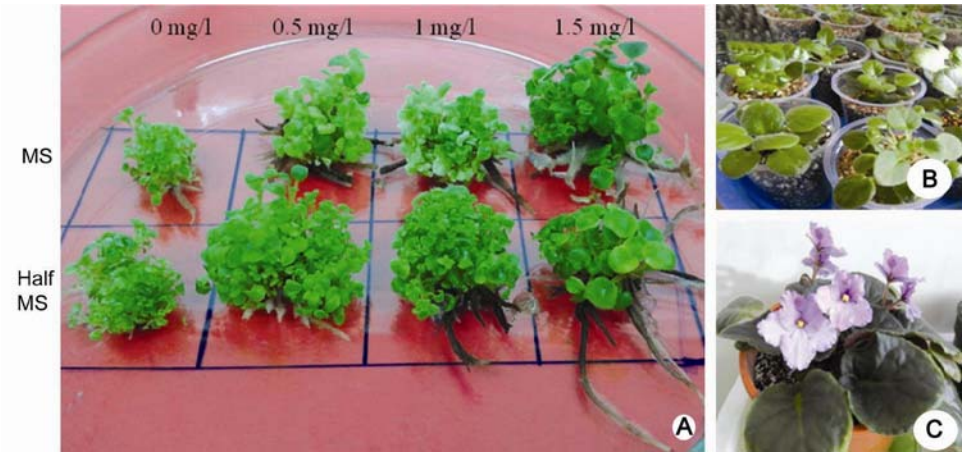

Fig. 3. Effects of four concentrations of IAA on root induction and elongation from *in vitro* regenerated shoots of *Saintpaulia* in MS or half strength of MS after four weeks of culture (A). A number of acclimated plants and one plant in flowering are respectively, shown in Fig. (B) and (C).

were tested for their effects on root formation from regenerated shoots (Table 1). The results showed that highly efficient (100%) root induction and growth occurs on half strength of MS fortifed with 1.5 mg/l IAA (Table 1, Fig. 3A). Note that shoots grown in this medium were more suitable for transfer into the soil for hardening (Figs. 3A, B). Rooted plantlets were successfully transferred to soil at a high efficiency (95%, Fig. 3B) followed by normal plant growth and flower development (Fig. 3C).

Table 1. Medium composition and *in vitro* shoot and root regeneration and elongation response of *Saintpaulia* from leaf tissues. For shoot induction, two experiments using RM medium supplemented with various combinations of NAA and either BAP (First experiment) or TDZ (Second experiment) hormones were carried out. For root induction and elongation response from *in vitro* regenerated shoots, MS and half strength of MS media supplemented with different concentrations of IAA were tested. The data are averages of four replications.

Purpose	Serial No.	BM	IAA	NAA	BAP	TDZ	GA_3	Shoot/ root ind. (%)	No. of shoots/root per exp.	Callus ind. (%)	Av. root length per exp. (mm)
					First experiment						
Shoot induction	1	RM	-	0	0.5	-	-	$65^{b}/0^{g}$	$20^{d}/0^{e}$	15^{d}	-
	2	"	-	0	1.0	-	-	$70^{b}/0^{g}$	$48^{c}/0^{e}$	15^{d}	-
	3	"	-	0	1.5	-	-	$85^{ab}/0^{g}$	$64^{b}/0^{e}$	14^{d}	-
	4	"	-	0	2.0	-	-	$95^{a}/0^{g}$	$76^{a}/0^{e}$	13^{d}	-
	5	"	-	0.4	0.5	-	-	$16^{cd}/40^{de}$	$5^{fgh}/11^{d}$	50^{c}	-
	6	"	-	0.4	1.0	-	-	$9^{d}/60^{b}$	$5^{fgh}/15^{d}$	70^{b}	-
	7	"	-	0.4	1.5	-	-	$9^{d}/90^{a}$	$4^{gh}/12^{d}$	95^{a}	-
	8	"	-	0.4	2.0	-	-	$20^{cd}/90^{a}$	$12^{e}/18.5^{c}$	100^{a}	-
	9	"	-	0.8	0.5	-	-	$8^{d}/55^{bc}$	$5^{fgh}/10^{d}$	100^{a}	-
	10	"	-	0.8	1.0	-	-	$18^{cd}/90^{a}$	$9^{efg}/19^{bc}$	95^{a}	-
	11	"	-	0.8	1.5	-	-	$30^{c}/45^{cd}$	$13^{e}/12^{d}$	90^{a}	-
	12	"	-	0.8	2.0	-	-	$30^{c}/30^{ef}$	$14^{e}/11^{d}$	80^{ab}	-
	13	"	-	1.2	0.5	-	-	$8^{d}/25^{f}$	$2^{h}/11^{d}$	95^{a}	-
	14	"	-	1.2	1.0	-	-	$9^{d}/48^{bcd}$	$3^{h}/14^{d}$	80^{ab}	-
	15	"	-	1.2	1.5	-	-	$8^{d}/58^{bc}$	$6^{fgh}/23^{b}$	95^{a}	-
	16	"	-	1.2	2.0	-	-	$25^{cd}/80^{a}$	$11^{ef}/30^{a}$	95^{a}	-

(contd.)

(Contd)

Second experiment											
	17	RM	-	0	-	0	-	$35^{def}/25^{c}$	$18^{de}/6^{bc}$	4^{f}	-
	18	"	-	0.5	-	0	-	$15^{f}/40^{b}$	$6^{f}/10^{b}$	35^{e}	-
	19	RM	-	1	-	0	-	$13^{f}/63^{a}$	$5^{f}/27^{a}$	65^{bcd}	-
	20	"	-	0	-	0.1	-	$60^{c}/0^{e}$	$64^{c}/0^{e}$	54^{de}	-
	21	"	-	0.5	-	0.1	-	$50^{cd}/0^{e}$	$24^{d}/0^{e}$	60^{cd}	-
	22	"	-	1	-	0.1	-	$35^{def}/10^{b}$	$17^{de}/2^{de}$	74^{bcd}	-
	23	"	-	0	-	0.5	-	$80^{b}/0^{e}$	$73^{b}/0^{e}$	55^{de}	-
	24	"	-	0.5	-	0.5	-	$30^{def}/10^{de}$	$13^{ef}/3^{cde}$	80^{bcd}	-
	25	"	-	1	-	0.5	-	$19^{ef/}10^{de}$	$10^{ef}/3^{cde}$	65^{bcd}	-
	26	"	-	0	-	1	-	$100^{a}/0^{e}$	$102^{a}/0^{e}$	92^{a}	-
	27	"	-	0.5	-	1	-	$38^{de}/25^{c}$	$21^{d}/5^{cd}$	88^{ab}	-
	28	"	-	1	-	1	-	$28^{def}/20^{cd}$	$16^{de}/7^{bc}$	84^{abc}	-
Shoot elongation and growth	29	MS	-	-	-	-	0	-	-	-	-
	30	"	-	-	-	-	0.01	-	-	-	-
	31	"	-	-	-	-	0.02	-	-	-	-
Root induction and growth	32	½ MS	0.0	-	-	-	-	–/36.7^{e}	–/4.0^{f}	-	6.0^{e}
	33	"	0.5	-	-	-	-	–/83.3^{ab}	–/11.7^{cd}	-	14.7^{cd}
	34	"	1.0	-	-	-	-	–/93.3^{a}	–/15.2^{b}	-	19.0^{bc}
	35	"	1.5	-	-	-	-	–/100.0^{a}	–/36.7^{a}	-	24.0^{a}
	36	MS	0.0	-	-	-	-	–/50.0^{cd}	–/8.3^{f}	-	7.3^{d}
	37	"	0.5	-	-	-	-	–/66.7^{bc}	–/11.3^{e}	-	11.0^{d}
	38	"	1.0	-	-	-	-	–/93.3^{a}	–/11.3^{de}	-	12.3^{d}
	39	"	1.5	-	-	-	-	–/96.7^{a}	–/16.7^{bc}	-	16.7^{b}

Data were analyzed using SPSS statistical software, and means were compared using the Duncan's test. Values indicated by different letters (superscript), show significant differences at $p < 0.05$, in comparison to the corresponding column values of each experiment. M No.: Medium number, BM: Basal medium, exp: Explant. The concentrations of growth regulators are expressed in mg/l.

The whole procedure from sterile leaf culture to plant hardening takes around 11 - 12 months. No visible phenotypic difference was observed among regenerated plants during this period. However, longer incubation on regeneration medium and repeated rounds of regeneration resulted in phenotypic variation, especially in flower color (data not shown).

In conclusion, here we report a fast protocol for African violet regeneration from leaves that can provide a reliable tissue culture system for commercial propagation. The protocol is highly efficient, time-saving and genotype-independent, and is applicable to other tissues: leaf petioles, internode segments, and root explants.

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