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IN VITRO PROPAGATION OF *SAINTPAULIA IONANTHA* WENDL. GENOTYPES AND ASSESSMENT OF GENETIC STABILITY OF REGENERATED PLANTS USING CDDP MARKERS

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Abstract

A micropropagation protocol via direct shoot organogenesis from leaf explants of six commercial varieties of Saintpaulia ionantha Wendl was established in this study. The shoot induction was successfully achieved on Murashige and Skoog (MS) media supplemented with 0.2 mg L^{-1} indole-3-acetic acid (IAA) and 0.5 mg L^{-1} benzylaminopurine (BA). In proliferation stage, the effects of two combinations of PGRs (V1-0.2 mg L^{-1} IAA + 0.2 mg L^{-1} BA and V2-0.2 mg L^{-1} NAA +1 mg L^{-1} BA) on shoot number and length were examined for each genotype. The results suggest that PGR's combinations significantly influenced shoot proliferation in all analysed variety and among the treatments 0.2 mg L^{-1} NAA in combination with 1 mg L^{-1} BA was the most effective for in vitro shoot multiplication. The in vitro rooting percentage was 86.86-96.66% and was varieties-dependent. In vitro-raised plants showed a very high rate of survival (82-94 %). The genetic fidelity between the selected vitro-plants and mother plants were confirmed by CDDP markers.

Key words*: in vitro culture, African violets, molecular markers, genetic fidelity.*

INTRODUCTION

Saintpaulia ionantha H. Wendl., commonly known as the African violet (Moore, 1957), is a species that includes varieties showing a wide range of morphological characteristics with ornamental value. This species from the Gesneriaceae family is cultivated worldwide as a houseplant by flower enthusiasts, becoming an important asset to the floriculture industry. Visual appeal, compact size, attractive flowers and leaves, shade tolerance, and the ability to bloom under artificial light are some characteristics that make Saintpaulias popular potted houseplants (Ghimire and Fang, 2023; Grout, 1990). In the last century, the African violets were mainly propagated by seeds and leaf cuttings from the mature plants. These propagation techniques were considered time-consuming and usually, a small number of plants were regenerated from the mother plants (Preece, 2003). In this context, the development of valuable techniques for rapid propagation of African violets in a short time and limited space has been an important goal for scientists and floriculturists (Torres, 1989; Maghami, 2003). The capacity of African violets for easy regeneration has been extensively explored in vitro (da Silva et al., 2016). Thus, organogenesis via in vitro regeneration have been reported from different types of explants such as lamina leaves or leaves with petioles, petioles, internodes, floral buds, anthers, and sub-epidermal tissue (da Silva et al., 2017). It is worth noting that micropropagation has become a reliable approach for the commercial propagation of African violets, with an important objective of producing a large number of new and true-to-type plants in a relatively short time (Sharma and Kathayat, 2021; Sunpui and Kanchanapoom, 2002). However, the environmental conditions of in vitro culture and its duration may induce somaclonal variations in micropropagated plants which can lead to a reduced commercial value (Missaghi et al., 2023; Shukla et al., 2013). During *in vitro* culture, some genetic changes that occur at the molecular level can be expressed at the phenotypic level, but sometimes cannot be identified and evaluated even in plants acclimatized under *ex vitro* conditions (Hârţa et al., 2018). Nevertheless, DNA-based molecular markers can be successfully used for evaluating the genetic stability of in vitro cultivated plants

of Gesneriaceae (Hârţa and Clapa, 2022). Among DNA-based molecular marker systems, Conserved DNA-derived polymorphism (CDDP) marker system is based on PCR amplification using a single primer that acts as reverse and forward, relies mainly on the conserved regions and provides comprehensive genomic information (Char et al., 2023). Genetic homogeneity testing of *in vitro*-grown plants adds value to any technology used for commercial purposes (Saidi et al. 2018). Based on the above-mentioned context, the main objectives of the present research were: to determine the influence of plant growth regulators (PGRs) on the *in vitro* culture of six varieties of African violets, to acclimatize micropropagated plantlets and to evaluate themunder greenhouse conditions, and also to evaluate the genetic fidelity of *in vitro*-raised plants with their mother plants after the 4th successive subculture using CDDP molecular markers system.

MATERIALS AND METHODS

Plant material

In this study, six hybrid varieties of *Saintpaulia ionantha* were used as mother plants. Plant material was provided by a certified nursery in Mijdrecht (Holland) and was purchased from a flower store. Before starting the experiments of the present study, the mother plants were cultivated under greenhouse conditions (22°C; 80% humidity). The varieties of Saintpaulias used as a source of explants for the establishment of *in vitro* culture are shown in Figure 1.

Figure 1. The African violet varieties used from the 'Voilà' plant assortment(original images)

Establishment of in vitro culture

In vitro culture initiation was done following the methodology described by Hârţa and Clapa (2022). After 60 days of *in vitro* culture on the initiation medium, adventitious shoots proliferated from the leaf explants were divided and further multiplied at 21-day intervals with two passages on MS medium supplemented with 0.2 mg L^{-1} IAA and 0.5 mg L^{-1} BA to provide plant stock for subsequent stages of *in vitro* multiplication of each African violet variety used.

Shoot proliferation and organogenesis

At the proliferation stage, adventitious shoots were divided and transferred to 370 mL (v/v) culture jars containing MS medium solidified with Plant agar (0.6% w/v) and supplemented with two variants of PGRs: V1 - 0.2 mg L^{-1} IAA + 0.2 mg L^{-1} BA and V2 - 0.2 mg L^{-1} NAA + 1mg L^{-1} BA. Adventitious shoots were subsequently transferred in aseptic condition to culture jars and cultured (21 days/subculture) at 24 ± 1°C under fluorescent white light (33.6 µmol m^{-2} s^{-1}) conditions with a photoperiod of 16/8 h light and dark cycles. All the chemicals and reagents were purchased from Duchefa, BiochemieBV, Holland. The average number of shoots/explant and the average shoot length were recorded after four repeated subculture in V1 and V2 proliferation culture media.

In vitro rooting and acclimatization

After the 4th successive subculture, the proliferated shoots were separated and then were rooted using MS medium without hormones (V3) and½ MS medium supplemented with 1 mg L^{-1} IAA (V4). Twenty explants (obtained from the shoot induction and multiplication experiment) were inoculated to each formulation for each variety. Data were recorded weekly for four weeks and values presented as means ± standard deviation. The rooted plantlets were thereafter subjected to acclimatization process. Briefly, the plantlets were taken out from the culture jars, rinsed carefully with sterile distilled water, and then grown in transparent plastic containers filled with moistened perlite. After 42 days, the plantlets were transplanted individually into plastic pots (6 cm Ø) filled with a potting

mixture of peat, vermiculite and perlite (2:1:1) and hardened under greenhouse conditions.
The average value of the survival rate for each analysed African violet variety was recorded after two months of growth in the greenhouse.

Genetic stability assessment

To confirm that the micropropagated plantlets were genetically true-to-type to their mother plants, genetic fidelity assessment by CDDP molecular markers was performed. Genomic DNA was isolated from fresh leaves weighing 10 mg. The ex-vitro mother plants and a total of five randomly chosen *in vitro* regenerated and subsequently greenhouse-grown plantlets from each variety analysed were used.
The extraction of total genomic DNA was performed using a Quick-DNA Plant/Seed Miniprep kit (ZymoResearch, USA) following the protocol described by the supplier company. Prior to CDDP analysis, DNA samples were diluted to 50 ng μL^{-1} using sterile double distilled water. Six CDDP primers were used to amplify DNA from each analysed African violets varieties and to confirm the genetic uniformity *of vitro*-plants with their mother plants. The six primers used generated detectable fragments in all samples analysed. To ensure the reproducibility of results, all PCR reactions were repeated twice. PCR was performed using a thermocycler system (SuperCycler Trinity by Kyratec, Australia) in 15 µL of PCR mixture containing 3 µL gDNA, 5.6 µL nuclease free H_2O for the PCR reactions, 2.5 µL GoTaq Flexi Green buffer, 2.5 µL MgCl2, 0.25 µL dNTP mix (Promega, USA), 1 µL CDDP primer (GeneriBiotech, Czechia), and 0.15 µL of GoTaq polymerase (Promega, USA). The PCR amplification process was started at 94°C for 5 min, followed by 35 cycles of 94°C for 60 s of denaturation, 60 s of annealing at 50°C, 120 s of extension at 72°C, and final extension at 72°C for 7 min. Separation of PCR products was performed by electrophoresis on 1.6% agarose gels (Promega, USA) stained with RedSafeTM Nucleic Acid staining solution (iNtRON Biotech, South Korea) in 1X TAE (Tris-acetate-EDTA buffer), at 80V and 88 mA for 2.5-3 hours. The electrophoretic bands profiles were visualized in UVP Biospectrum AC Imaging System (UVP BioImaging Systems, Germany).

Data analysis

The experiment was arranged in a completely randomized design (5 replication x 5 inoculums/each variant). One-way ANOVA was performed to check the differences between the experimental variants. When the null hypothesis was rejected, post-hoc Duncan test ($\alpha < 0.05$) was used to separate and highlight the differences among means. The values shown are means ± S.E. CDDPs gel images were analysed using TotalLab TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to count the number and the range size of the amplified bands.

RESULTS AND DISCUSSIONS

Shoot induction and organogenesis

In this study, the induction of adventitious shoots (rosettes) from leaf explants of six Saintpaulia varieties, grown under *in vitro* conditions, was successfully done using MS medium supplemented with 0.2 mg L^{-1} IAA and 0.5 mg L^{-1} BA (data not shown). These results are consistent with those reported in other studies that revealed the high capacity of African violet leaves for in vitro shoot induction (Cassells and Plunkett, 1984; Shukla et al., 2013). In the present study, two different combinations of PGRs were tested to analyse the influence of phytohormones on the shoot proliferation stage. Thus, the PGRs combinations significantly influenced shoot proliferation in all analysed African violet varieties (Table 1).

Table 1. The influence of PGRs on the multiplication rate of proliferated shoots (number of shoots/inoculum) of the analysed varieties of African violets

Varieties	Multiplication rate	
	V1 MS+0.2 mg L^{-1} IAA+0.2 mg L^{-1} BA	V2 MS + 0.2 mg L^{-1} NAA+1 mg L^{-1} BA
Voilà Light pink	9.80 ± 0.26 D*	16.87 ± 0.24 F
Voilà Dark pink	8.80 ± 0.22 BC	15.45 ± 0.24 E
Voilà Dark blue	8.66 ± 0.23 B	17.09 ± 0.11 FG
Voilà Light blue	9.49 ± 0.20 CD	17.64 ± 0.21 G
Voilà Red	6.97 ± 0.13 A	16.62 ± 0.20 F
Voilà Pink-white	8.35 ± 0.18 B	16.51 ± 0.25 F

*The values shown are means ±SE. Different letters indicate significant differences between the variants according to Duncan's test.

The results show that the addition of cytokinins such as benzylaminopurine (BA) at a concentration of 1 mg L^{-1} and 0.2 mg L^{-1} auxins

(NAA) in MS medium had a stimulatory effect on the multiplication of Saintpaulia's adventitious shoots. As shown in Table 1, the aforementioned PGRs combination (V2) was found to be the best variant for shoot proliferation and significantly improved the number of shoots/inoculum for the blue-flowered cultivars named 'Voilà Light blue' (17.64 ± 0.21) and 'Voilà Dark blue' (17.09 ± 0.11). On the same variant of the culture medium (V2), non-significant differences were recorded between 'Voilà Light pink' (16.87 ± 0.24), 'Voilà red' (16.62 ± 0.20) and 'Voilà Pink-white' (16.87 ± 0.24) varieties. The lowest number of shoots/inoculum was recorded in the variety with dark–pink flowers (15.45 ± 0.24). High rates of direct shoot organogenesis were observed on V2 for all African violets varieties, indicating the genotype dependence of the PGRs combination (Figure 2).

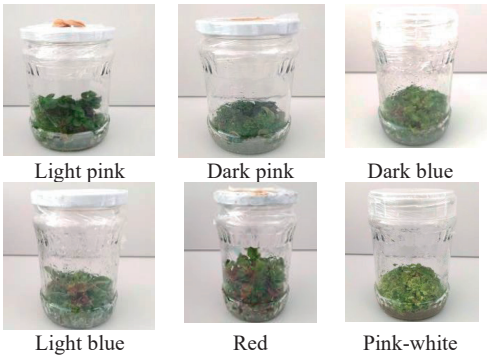


Figure 2. *In vitro* shoot proliferation of six African violet varieties on MS medium supplemented with 0.2 mg/L^{-1} NAA and 1mg/L^{-1} BA on 4th successive subculture

Thus, the results of the present study are in agreement with those reported by other authors (Sunpui et al., 2002; Shukla et al., 2013) who stated that MS medium containing BA in concentrations between 0.2 to 2.0 mg L^{-1} and NAA (0.1-0.2 mg L^{-1}) led to the highest efficiency of shoot proliferation per inoculum. High rates of direct shoot organogenesis were observed for all varieties, indicating that the genotype was dependent on the PGRs combination (Figure 2). Another important parameter of *in vitro* grown plants is the length of shoots. As shown in Table 2 significant differences were observed between the two variants of PGRs used (V1 and V2) in the case of all the analyzed varieties.

Table 2. The influence of PGRs on the length of proliferated shoots for analysed varieties of African violets

Varieties	Shoot length (cm)	
	V1 MS+0.2 mg L^{-1} IAA+0.2 mg L^{-1} BA	V2 MS + 0.2 mg L^{-1} NAA+1 mg L^{-1} BA
Voilà Light pink	1.48 ± 0.1 A[8]	1.97 ± 0.03 B
Voilà Dark pink	1.47 ± 0.12 A	2.01 ± 0.08 B
Voilà Dark blue	1.94 ± 0.08 B	2.18 ± 0.02 BC
Voilà Light blue	1.97 ± 0.05B	2.41 ± 0.04 CD
Voilà Red	1.67 ± 0.04 A	2.15 ± 0.04 B
Voilà Pink-white	2.00 ± 0.07 B	2.53 ± 0.09 D

*The values shown are means ±SE. Different letters indicate significant differences between the variants according to Duncan's test.

In this study, MS + 0.2 mg L^{-1} NAA+1mg L^{-1} BA was the best option for the elongation of Voilà Pink-white cv. shoots (2.53 ± 0.09 cm). The lowest shoot length value was recorded on the Voilà Light pink (1.97 ± 0.03 cm). This finding is consistent with the results reported by us in a previous study regarding the influence of PGRs combination on in vitro shoot growth from Gesneriaceae species (Hârţa and Clapa, 2022). Moreover, Daud and Taha (2008) reported that the proliferation and shoot growth of African violets from floral explants were also stimulated when MS medium was supplemented with 1 mg L^{-1} BA and 2 mg L^{-1} NAA. These results indicated the importance of auxin (NAA) as a plant growth regulator that stimulates both the number and length of African violet shoots grown *in vitro* conditions.

Rooting and acclimatization of plants

In vitro rooting of multiple shoots (rosettes) was recorded on MS medium without hormones (V3) and ½ MS+1mg L^{-1} IAA (V4) for all of six varieties of African violets used in the present study. The results summarized in Table 3 show that ½ MS supplemented with IAA stimulated the rooting of regenerated shoots for all six cultivars of *Saintpaulia ionantha*. Moreover, there were significant differences between cultivars regarding in vitro rooted shoots on the V4 variant except for the two genotypes named: Voilá Light pink and Voilá Dark blue. Data were recorded weekly for four weeks and values were presented as means ± standard deviation (Table 3).

Table 3. The influence of PGRs on the *in vitro* rooting of shoots for analysed varieties of African violets

Varieties	*In vitro* rooting (%)	
	V3 MS without hormones	V4 ½ MS + 1 mg L^{-1} IAA
Voilà Light pink	85.64 ± 0.16 A[8]	87.01 ± 0.01 AB
Voilà Dark pink	88.55 ± 0.11 A	89.48 ± 0.09 C
Voilà Dark blue	86.03 ± 0.29 A	86.94 ± 0.16 A
Voilà Light blue	88.58 ± 0.12 BC	89.44 ± 0.11 C
Voilà Red	93.55 ± 0.11 D	94.44 ± 0.12 D
Voilà Pink-white	94.66 ± 0.16 D	97.66 ± 0.29 E

*The values shown are means ±SE. Different letters indicate significant differences between the variants according to Duncan's test.

It is noted that, for some varieties analysed (Voilà Dark blue and Voilà Red) there were no significant differences between the percentage of root induction on V3 and V4 rooting media (Table 3). Similar response was observed in two previous studies of *Saintpaulia ionantha*. (Shajiee, 2007; Zeljković et al., 2021). These results might be due to the fact that the in vitro-grown plantlets of African violets produced a sufficient amount of auxins endogenously and no exogenous auxins were required for the induction of roots (Khan et al., 2007).

Regarding the acclimatization process, it is noteworthy that more than 82% of in vitro growth plants of the six analysed African violet varieties survived (Table 4).

Table 4. The survival rate of *in vitro* raised plants of the analysed varieties of African violets

Varieties	The survival rate (%)
Voilà Light pink	82.22 ± 0.01 A[8]
Voilà Dark pink	88.66 ± 0.11 B
Voilà Dark blue	86.88 ± 0.29 AB
Voilà Light blue	94.44 ± 0.12 C
Voilà Red	93.44 ± 0.11C
Voilà Pink-white	94.66 ± 0.16 C

*The values shown are means ±SE. Different letters indicate significant differences between the variants according to Duncan's test.

It was observed that although the plantlets were sufficiently rooted, it is nearly mandatory for the plantlets that they should be kept one month covered with the plastic lids, prior to the direct exposure of the plantlets under greenhouse conditions.

After 1 month, raised-plants were successfully transferred in greenhouse, followed by normal plant growth and flower development. In a previous study, also, Khan et al. (2007) observed that although African violet plantlets were sufficiently rooted, it was almost mandatory to be kept in a tunnel covered with a plastic sheet, before direct exposure of the plantlets under greenhouse conditions. The period required to keep the plants in the tunnel varied with the season and suggested that the best time for the transfer of plantlets for the acclimatization stage is the winter season.

Genetic stability assessment

Several strategies were followed to assess the genetic fidelity of the in vitro-raised progenies of ornamental plants since the sustainability of the micropropagation technique depends on ensuring the genetic fidelity of the regenerated plants (Alizadeh et al., 2015).

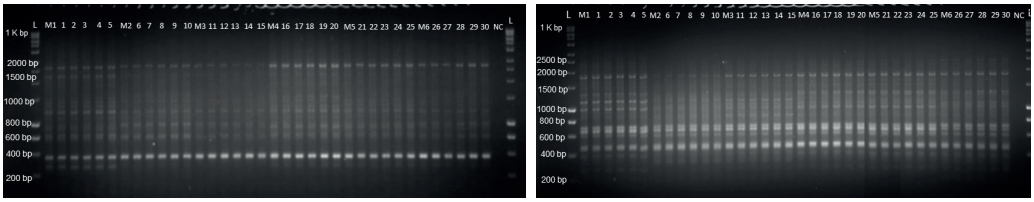
Despite the availability of abundant literature supporting the importance of large-scale multiplication of African violets using in vitro cultures, it is also very important to evaluate the genetic uniformity of acclimatized plants using molecular markers systems (Hârţa and Clapa 2022; Tyagi et al., 2022; Biswas and Kumar, 2023). To confirm the uniformity of in vitro grown plants with their mother plants, CDDP markers were employed to analyse the genetic fidelity of five randomly selected acclimatized plants from each variety. All CDDP primers used generated clear and detectable bands for all samples analysed (Table 5). The genetic analysis with CDDP markers showed a total number of monomorphic bands between 64 (Voilà Dark blue) and 70 (Voilà Light blue) as shown in Table 5. Each CDDP markers generated monomorphic bands and their length ranged between 200 and 2100 bp.

Moreover, no genetic differences were observed in any of the analysed African violets variety.

As shown in Figure 3 *in vitro* raised plants have the same banding patterns as the mother plants, indicating their uniformity at the DNA molecular level.

Table 5. Sequences of CDDP primers and the number of monomorphic bands generated in the analysed Gesneriaceae cultivars

Primer name	Primer sequence 5'-3'	Number of monomorphic bands						Range size of bands (bp)
		Voilà Light pink	Voilà Dark pink	Voilà Dark blue	Voilà Light blue	Voilà Red	Voilà Pink-white	
Knox 1	AAGGGSAAGCTSCCSAAG	9	8	7	10	9	10	300-2100 bp
WRKY-R3B	CCGCTCGTGTGSACG	12	11	11	12	13	9	380-2100 bp
Myb1	GGCAAGGGCTGCCGC	13	13	14	13	12	13	200-1800 bp
Myb2	GGCAAGGGCTGCCGG	12	12	11	12	12	13	200-2000 bp
ERF1	CACTACCCCGGSCTSCG	10	11	11	12	11	11	300-1600 bp
ERF2	GCSGAGATCCGSGACCC	9	10	10	11	12	9	300-1800 bp
Total		65	65	64	70	69	65	



(a) (b)

Figure 3. PCR banding patterns obtained with Knox1 (a) and WRKY-R3B (b) CDDP primers after the PCR amplification of African violets varieties: Voilà Light pink (M1; 1-5); Voilà Dark pink (M2; 5-10); Voilà Dark blue (M3; 11-15); Voilà Light blue (M4; 16-20); Voilà Red (M5; 21-25) and Voilà Pink-white (M6; 26-30). Lane L-1kb DNA Ladder; M-mother plants; NC-negative control

Although genetic fidelity has been assessed by various DNA-based molecular marker systems in African violets (Biswas and Kumar, 2023), to our knowledge, this is the first paper to report the use of the CDDP marker system to evaluate the genetic uniformity of African violet varieties.

CONCLUSIONS

This research demonstrated that direct plant regeneration of *Saintpaulia ionantha* Wendl. through leaf explants was successfully achieved, but is cultivar dependent. The genetic fidelity between the selected vitro-plants and mother plants were confirmed by CDDP markers.

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