African violets' organogenesis responding to hormonal interaction

Maria Missaghi[1], Fataneh Yari[2]*, Amir Mousavi[3], Younes Mostofi[4], and Hamideh Ofoghi[5]

1. Department of Horticultural Sciences and Agronomy, Science and Research Branch, Islamic Azad University, P.O. Box 14515/775, Tehran, Iran
2. Department of Agriculture, Iranian Research Organization for Science and Technology, P.O. Box: 33535111, Tehran, Iran.
3. Department of Plant Molecular Biotechnology, National Institute of Genetic Engineering and Biotechnology, P.O. Box 14155-6343, Tehran, Iran.
4.Department of Horticultural Sciences, Faculty of Agricultural Science and Engineering, College of Agriculture and Natural Resources. University of Tehran, P.O. Box4111, Karaj, Iran.
5. Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), P.O. Box 33535111, Tehran, Iran

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Abstract

In this study, the possibility of organogenesis of leaves and petioles of two African violet cultivars were evaluated using MS media containing various concentrations of plant growth regulators, namely (IAA) (1,2 mg/l), (BA) (0.05, 0.1, 0.2, 0.5 mg/l), (NAA) (1, 2 mg/l), and Thidiazuron (TDZ) 0.01 mg/l)). All experiments were arranged in a completely randomized design. The data were analyzed by one-way ANOVA, and means were separated using Duncan's new multiple range test ($P \leq 0.01$). Direct and indirect organogenesis from the whole leaf with petiole bases were observed in both cultivars. However, the separated leaf blade and petiole were unable to produce any organs. The organogenesis potential of cultivars was significantly different, although the medium containing BA (0.1 mg/l) + IAA (1 mg/l) showed the highest indirect organogenesis (90 %< in both cultivars). In contrast, the media containing BA 0.05 mg/l + TDZ 0.01 mg/l + IAA 2 mg/l and BA 0.05 mg/l + TDZ 0.01 mg/l + IAA 1 mg /l were more successful on the induction of direct organogenesis. Therefore, the level of direct and indirect organogenesis appears to be dependent on the interaction between the plant growth regulators with the explant type having a direct correlation with the genotype.

Keywords: genotype, PGRs, regeneration, *Saintpaulia ionantha*, tissue culture

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Introduction

Saintpaulia is known worldwide as African violet, belonging to the Gesneriaceae family, with shade tolerance, diverse colors, and ever-blooming characteristics that have made it a popular houseplant. The economic value of the genus *Saintpaulia* was known after 1893 (Teixeira et al., 2016). These plants are often propagated by leaf cuttings, which cause simple plantlets from a chimera mother plant, while *in vitro* methods are widely adopted for the commercial propagation of

* *Corresponding Author*
E-mail Address: fataneh.yari@gmail.com
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chimera plants during a short time. On the other hand, micropropagation techniques are mostly employed to induce variation through genetic engineering with commercial values (Silva et al., 2017).

Extensive literature has confirmed that African violet has considerable potential for organogenesis via *in vitro* regeneration or embryogenesis using various explants such as leaf (Sarai et al., 2017), flower bud (Teixeira da Silva et al., 2017), sub-epidermis , anther and protoplasts. Explant type, physiological stage of the mother plant, owner explant, interaction, and concentration of the plant growth regulators (PGRs) in the media are other crucial factors that might be considered in successful organogenesis (Teixeira da Silva et al., 2017).

African violet petioles do not have sufficient *in vitro* organogenesis potential (Teixeira da Silva et al., 2017). There are also studies suggesting that petiole organogenesis possibility is higher than the other part of the leaf, so it has more potential to be used as a micropropagation explant in African violet (Teixeira da Silva et al., 2017).

The results of investigations on the role of PGRs under *in vitro* culture indicates that cytokinins are one of the critical growth regulators for organogenesis. Thidiazuron (TDZ), a phenylurea group that is found in many ornamental plant cultures, has been used as an organogenesis agent. However, its application as the embryonic substance is commonly reported in most species, and this causes somaclonal variation (Dinani et al., 2018). This growth regulator has been widely used alone or in combination with other substances for organogenesis or embryogenesis. Most reports indicated that in shoot regeneration, TDZ was more effective than other cytokinins and results were indicative that the amount of less than 2.5 µM/l leads to organogenesis while higher levels could cause embryogenesis (Teixeira da Silva et al., 2017).

A fast and precise *in vitro* direct-organogenesis protocol could be a critical success for breeders via genetic transformation. Then, the primary goal of this study was to investigate the effect of TDZ on the direct organogenesis of African violets.

Materials and Methods

Establishment of aseptic culture

Initially, healthy fully-expanded young leaves of *Saintpaulia ionantha* cv. Grinia and *Saintpaulia ionantha* cv. Heart Break Kid were washed for 20 minutes under tap water; then, they were surface-sterilized in 30% (v/v) sodium hypochlorite solution (0.15% available chlorine) for 10 min. Next, the leaves were rinsed three times with sterile distilled water, and their margins were cut and removed. Finally, 1.5 cm^2 pieces of leaves were considered as the explant, and were placed on the surface of the media culture. In the case of petiole explants, the same steps were taken, but they were put inside the media. The MS media containing 2 mg/l (IAA) and 0.8 mg/l (BA) were considered for the establishment of initial explants.

Table 1
Organogenesis induction media for African violets

No.	Media	Organogenesis Treatment; PGRs concentration
1	O1	0.1 mg/l BA + 2 mg/l IAA
2	O2	0.05 mg/l BA + 0.01 mg/l TDZ + 2 mg/l IAA
3	O3	0.1 mg/l BA + 1 mg/l IAA
4	O4	0.05 mg/l BA + 0.01 mg/l TDZ + 1 mg/l IAA
5	O5	0.2 mg/l BA + 1 mg/l NAA
6	O6	0.2 mg/l BA + 2 mg/l NAA
7	O7	0.5 mg/l BA + 1 mg/l IAA

Shoot proliferation

Shoot multiplication of explants was monitored on the medium supplemented with 0.12 mg/l BA, 3.0 mg/l IAA and 0.1 mg/l Thiamine-HCl. All multiplication media contained 30 g/l sucrose and 5.6 g/l agar. The pH value was adjusted to 5.7 before autoclaving at 121 ℃, 150 kPa for 20 min. Shoots were sub-cultured three times at a constant three-week subculture interval. Shoot cultures were grown in 150 ml culture vessels containing 30 ml of proliferation medium, at 25 ± 2 ℃ and 16 h-photoperiod (light intensity, 8.85 W/m^2).

Organogenesis

Leaves and petioles from the proliferation part were then used as explants. Three different

types of explants, petioles, leaves segment, and whole leaves with petioles were inoculated onto MS basal media supplemented with different concentrations of IAA, BA, (NAA), TDZ individually (Table 1). The pH of all culture media was adjusted to 5.7 and autoclaved at psi 15 for 20 minutes. After the culture, the dishes were stored at 25 ± 2 ℃ and 8.85 W/m^2 of light for 16 hours of light and 8 hours of darkness. The onset of organogenesis was observed after four weeks of culture, and the number of adventitious buds was counted. After that, the clumps with adventitious buds were divided into smaller clusters with 3-5 buds again before they were transferred to the same culture media for shoot formation and seedling elongation for another four weeks.

The initial establishment, shoot proliferation, and organogenesis data collections were repeated three times with five observations. Data were analyzed by one-way ANOVA using the SAS 9.1, and means were separated using Duncan's new multiple range test (P≤0.01). Charts were plotted using EXCEL software. Percentage of data were square root (each data plus one) transformed before performing ANOVA.

Results

Plant organogenesis from different explant sources

Various explants for their organogenesis potential were initially screened. Leaf, petiole, and whole leaf with petiole bases explants were cultured on MS basal medium containing PGRs (Table 1). There were differences in shoots regeneration among the sources of explants. The whole leaf explants regenerated shoots more effectively than leaf blades or petioles. Just whole leaf explants showed green shoot primordia after 2-3 weeks of culture. These primordia developed into adventitious shoots after 5-6 weeks. In contrast, most leaf blades or petioles explants became pale and necrotic over 4-6 weeks. The whole leaf with petiole bases explants appeared to be superior to the other two explants, and thus, these findings were used in this study.

Analysis variance of data indicated that the genotype affected organogenesis either direct or indirect at 99% confidence level. With the cultivars and PGRs incorporated in the media, adventitious shoots were formed after three weeks. Comparison of shoot regeneration from whole leaf explants on the BA- and IAA/NAA-containing media showed that the number of regenerated shoots per explant was dependent on the genotype and growth regulators, and IAA was more effective than NAA (Table 2). Decreasing IAA and BA enhanced the number of shoot regeneration. The highest number of regenerated shoots was found in the media containing 0.1 mg/l BA plus 1 mg/l IAA. The explants produced no callus on the basal media with TDZ. Meanwhile, NAA-containing media (1-2 mg/l) encouraged root formation with poor shoot regeneration (Fig. I).

Table 2
Comparison of total shoot regeneration efficiency from whole leaf explants in two African violet cultivars; regenerated shoots were scored after six weeks of culture on MS basal medium containing PGRs.

Media No.	Grinia		Heart Break kid	
	Shoot regeneration (%)	Number of shoots per explant* ($\bar{X}$± SD)	Shoot regeneration (%)	Number of shoots per explant* ($\bar{X}$± SD)
O1	50	7.98^{d} ± 1.03	83.33	29.65^{c} ± 1.23
O2	100	34.65^{b} ± 0.93	40	4.32^{e} ± 1.23
O3	100	49.20^{a} ± 0.79	100	39.85^{b} ± 0.85
O4	20.83	3.60^{e} ± 1.23	48.33	6.38^{d} ± 1.21
O5	51.67	8.70^{d} ± 1.01	0	0^{f}±0
O6	0	0^{f}±0	0	0^{f}±0
O7	70	26.40^{c} ± 1.35	31.67	3.98^{e} ± 1.23

* Each value represents the mean number + standard deviation. Means followed by different letters are significantly different at the 99% confidence level; (O1: 0.1 mg/l BA + 2 mg/l IAA; O2: 0.05 mg/l BA + 0.01 mg/l TDZ + 2 mg/l IAA; O3: 0.1 mg/l BA + 1 mg/l IAA; O4: 0.05 mg/l BA + 0.01 mg/l TDZ + 1 mg/l IAA; O5: 0.2 mg/l BA + 1 mg/l NAA; O6: 0.2 mg/l BA + 2 mg/l NAA; O7: 0.5 mg/l BA + 1 mg/l IAA)

Indirect organogenesis

and did not show any sign of vitrification.

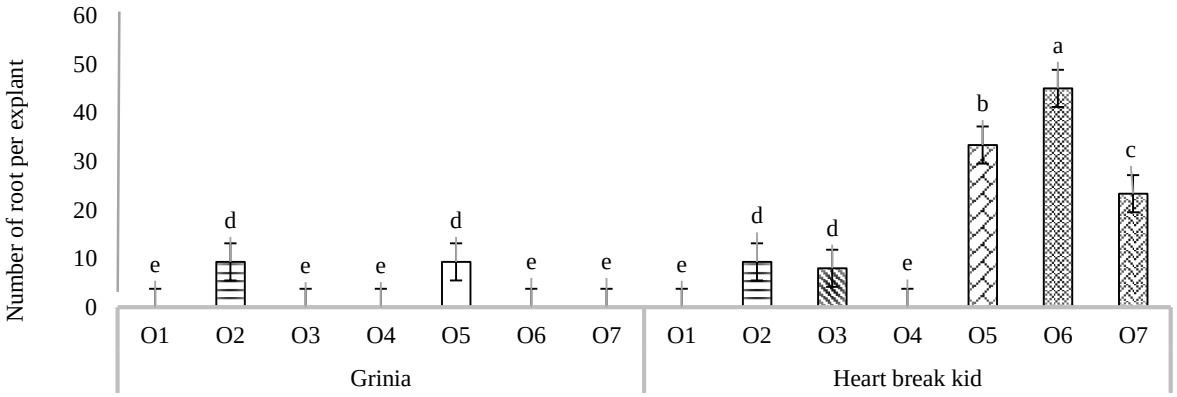

Fig. I. The number of root regeneration from the whole leaf explant on organogenesis induction media, eight weeks after the initial culture

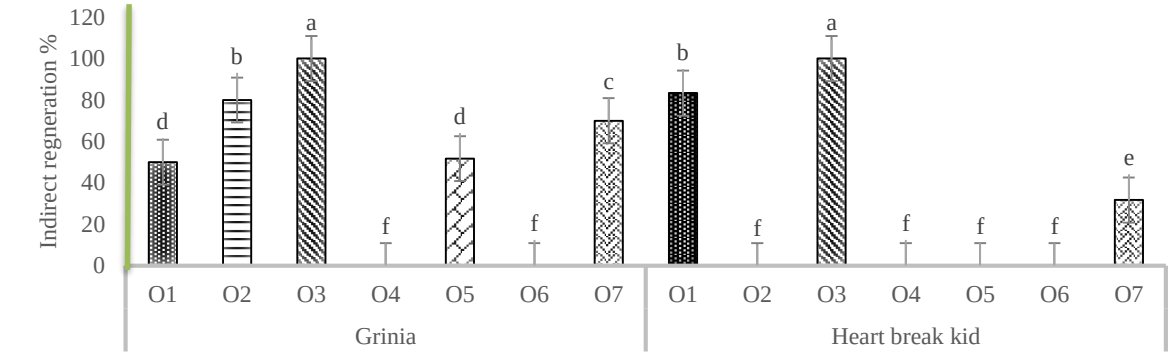

Fig. II. Indirect shoot regeneration from the whole leaf explant on organogenesis induction media, eight weeks after the initial culture

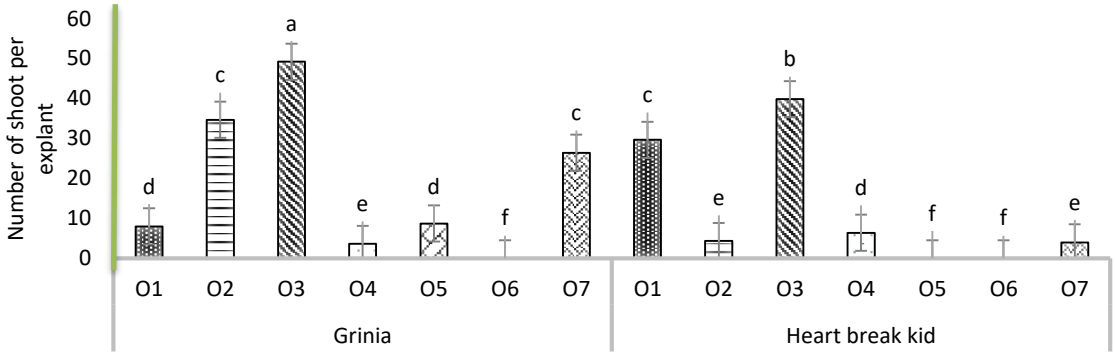

Fig. III. The number of shoot regeneration from the whole leaf explant on organogenesis induction media, eight weeks after the initial culture

Indirect organogenesis in both cultivars was initially affected by the PGRs and not the genotypes (R^2: 0.9982; Adjusted R^2: 0.9977; Predicted R^2: 0.9969). The highest percentage of indirect shoot regeneration was found in the media supplemented with 0.1 mg/l BA and 1 mg/l IAA (O3). Therefore, this medium could be considered as suitable for shoot multiplication goals (Fig. II). Shoot growth continued while still attached to the mother explants. The leaves of the regenerated shoots were healthy with green color

Increasing NAA concentrations reduced shoot organogenesis and at the same time increased root formation just in one cultivar (Heartbreak kid). No shoot formation was evident in this media (O6) in both genotypes (Fig. III). Regeneration capacity and the number of regeneration per explant varied depending on the genotype, and the highest number of shoot per plant was produced by Grinia (Table 2, Figs. III and IV).

Direct organogenesis

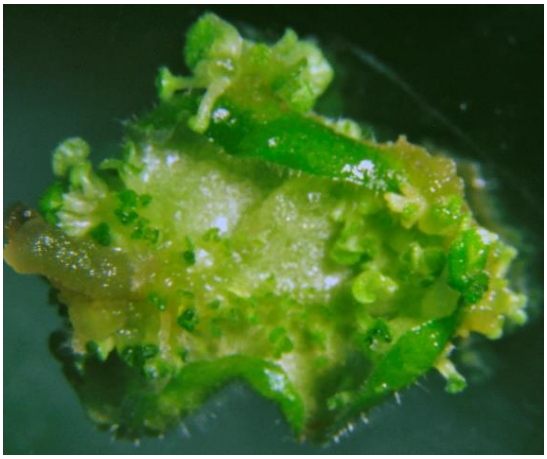

Fig. IV. Indirect shoot induction from whole leaf explant of African violet cv. Grinia on MS media amended with 0.1 mg/l BA + 1 mg/l IAA after 8 weeks

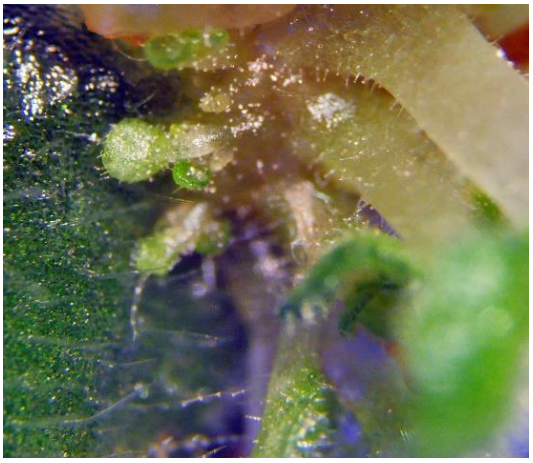

Fig. V. Direct shoot induction from whole leaf explant of African violet cv. Heartbreak kid on MS media amended with 0.05 mg/l BA + 0.01 mg/l TDZ + 1 mg/l IAA after 8 weeks (100X)

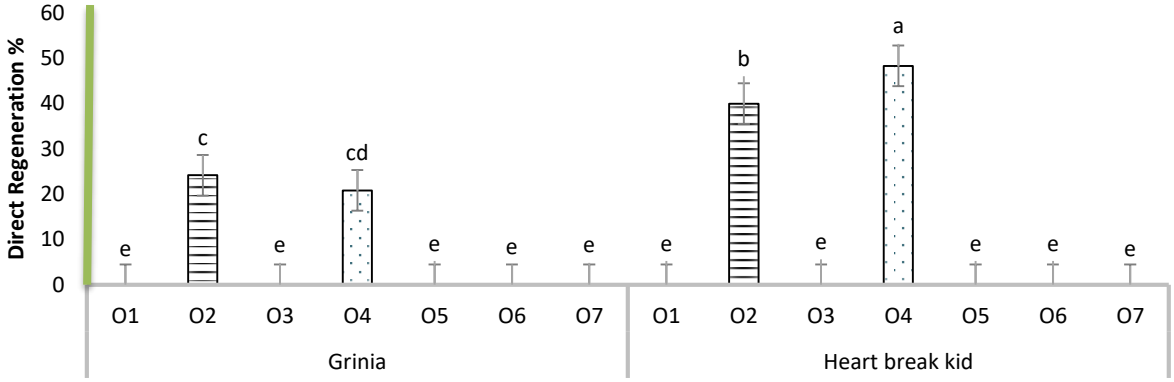

Fig. VI. Direct shoot regeneration from the whole leaf explant on organogenesis induction media, eight weeks after the initial culture.

Subsequent treatments were initiated to assess the effectiveness of whole leaf explants through IAA-BA-TDZ ratios for achieving direct organogenesis. The direct organogenesis of the whole leaf with petiole bases explant (Fig. V) was affected significantly by PGRs and genotype (R^2: 0.9887; Adjusted R^2: 0.9851; Predicted R^2: 0.9799) in both cultivars. The effect of TDZ on direct organogenesis was not consistent and varied with the genotype type and IAA concentration. The highest amount of direct organogenesis was observed in the media containing BA (0.05 mg/l) + TDZ (0.01 mg/l) + IAA (1 mg/l) in Heartbreak kid cultivar (Fig. VI). It seems that the cultivar potential was more effective to this, and the rate of direct organogenesis in Heartbreak kid (48.33%) was higher than Grinia (24.17%).

Discussion

Major factors affecting *in vitro* explant response are genotype (Neumann et al., 2020), physiological stage of donor plant, explant age (Mekala et al., 2016), explant type (Zeng et al., 2016), and explant size (Bakhshaie et al., 2016). The explant sources commonly used are leaves or leaves with petioles, petioles, internodes, intact ovaries, floral buds, sepals, petals, anthers, sub-epidermal tissue, and protoplasts derived from petiole-derived callus, as well as leaves, or young shoots from leaf cultures. Such explants have the potential for inducing direct or indirect organs and embryos (Teixeira da Silva et al., 2016).

Leaf and petiole organogenesis capacity are varied. In some studies, petioles explants failed to produce any tissue or organ (Teixeira da Silva et al., 2017). However, in other reports, the highest organogenesis potential belonged to petioles

explants. Young leaves are one of the primary sources of auxin production in plants (Ćosić et al., 2021). Most *in vitro* leave explants driven from proliferation reveal vegetative phase and are probably abundant in auxin. This may suggest a lack of organogenesis in the petioles that would also be affected by genotype. Genotype could be a critical determining factor of explants capacity for organogenesis. Considering our data and results, it seems that the potential of different tissues as explant in African violet is highly genotype-dependent. Otherwise, the positive interaction of internal tissue phytohormones with the media PGRs is the crucial factor in organogenesis.

Furthermore, BA and NAA/IAA were not essential for shoot direct regeneration via leaf explant, individually. Thidiazuron, which can stimulate callus and shoot formation in woody plants at concentrations higher than 1 µM, was much more active than BA for stimulating regeneration (Mekala et al., 2016; Ramakrishna et al., 2016). In contrast to the results of the current study, the promoting effects of BA to produce adventitious shoots was reported in petiole explants of Saintpaulia (Teixeira da Silva et al., 2017), as it did in other species (Bhatla, 2018; Vinoth and Ravindhran, 2018).

It follows that the leaf blade is necessary for direct shoot regeneration, whereas the petiole enhanced direct and indirect regeneration. The base of the leaf (near the petiole) seem to indicate higher regenerability than the tip region or end of the petiole (Mladenović et al., 2016; Teixeira da Silva et al., 2017). The promoting effects of the petiole on adventitious bud regeneration was evident in many studies (Teixeira da Silva et al., 2017), whereby regeneration was completely inhibited when the petiole was absent (Lee and Pijut, 2017). BA increased shoot regeneration, at high concentration (data was not shown), similar to 0.01 mg/l TDZ but not in the midrib and margins of leaves. Generally, TDZ played an enhancing role in regeneration, whereas PGRs' balance was needed for the induction of regeneration and further development into plantlets. The auxin/cytokinin balance is one of the main factors to determine the morphogenesis pattern of petiole explants. Their interactions resulted in altered morphogenetic responses. Hence when TDZ was used with BA and little amount of IAA, shoot bud regeneration increased markedly. Many reports indicated that TDZ is more effective in shoot regeneration than BA (Mekala et al., 2016; Ramakrishna et al., 2016). TDZ has been used to induce shoot organogenesis in callus culture of *Vigna radiata* (Mekala et al., 2016) and woody species (Vinoth and Ravindhran, 2018). High activities of TDZ in plant regeneration were also reported in other ornamentals such as Campanula (Gehl et al., 2020), orchid (Mose et al., 2017), and roses (Nguyen and Van Le, 2020). In this respect, it has been demonstrated that removal of the PGRs has stopped the regenerative properties of any explants in the African violet (Keutgen et al., 2016; Teixeira da Silva et al., 2017).

Conclusion

It is believed that not all parts or segments of the leaf (leaf blade, petiole and midrib) are able to regenerate shoots. As was reported here, whole-leaf explant showed organogenesis. Although Teixeira da Silva et al. (2017) demonstrated that African violet petiole had a high regenerative capacity, in our investigation BA and NAA/IAA combination had no positive effect on petiole regeneration. In fact, explant regeneration in this study was not possible in the absence of the petiole while shoot regeneration initiation started from the basal of the lamina.

It seems that most competent cells to differentiate shoots are located in the boundary zone between lamina and petiole, and to express their totipotency, they are dependent on some factor such as the hormonal balance of the explants.

In our experiments, the possible role of TDZ on indirect shoot induction was shown conclusively. All cytokinins used in the current study were efficient in enhancing regeneration. We demonstrated that TDZ was efficient in direct-regeneration while other growth regulators including BA were not. In contrast, BA was found necessary for indirect regeneration and was equally effective at different concentrations in regeneration. Furthermore, increasing the concentration of BA resulted in well-developed shoots regeneration. Although it was relatively

low, direct regeneration in the present study was inhibited on a TDZ-free medium, which indicates that the level of endogenous cytokinin in the explants has been sufficient to induce indirect shoot formation. This may be attributed to the presence of regions of meristematic nature. In conclusion, this study demonstrated that direct plant regeneration of African violet through the culture of the whole leaf could be obtained in high frequency. The direct organogenesis protocol described here would allow African violet plantlets' organogenesis in a short time (3 weeks), could be useful for large scale regeneration, and would provide sufficient background to modern breeding via genetic engineering which is precise and fast. This opens the possibility of regenerating plantlets from genetically transferred tissue or looking for somaclonal variations.

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