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IMPROVEMENT OF *IN VITRO* SHOOT REGENERATION AND FLOWERING FROM PETAL EXPLANTS OF *BEGONIA* X *HIEMALIS* FOTSCH

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ABSTRACT

Begonias hold significant importance due to their use as culinary plants, medicinal remedies, decorative elements, and high-value crops. This research aims to find new explant materials for regeneration shoots and direct flowering with high frequency. In this study, high-frequency shoot regeneration and *in vitro* flowering from petal explants of *Begonia x hiemalis* Fotsch were achieved. Whole petal explants disinfected with an AgNPs solution at 0.5 g/L for 15 min achieved the highest survival rate. The highest shoot regeneration rate reached 86.66%, with 65.33 shoots/explant from ½-petal explants. Direct formation of the flower consists of two different structural forms, including single petal clusters and complete flower buds, with the corresponding flower formation rates of 19.66 petals/explant; 0.53 flower buds/explant, respectively, at the keel of the petals on the MS medium supplemented with 1000 mg/L myo-inositol, 1.0 mg/L BA, 1.0 mg/L NAA, 30 g/L sucrose and 9.0 g/L agar. Interestingly, increasing the concentration of myo-inositol in the culture medium led to the appearance of red-leaf shoots at the highest rate (12.66 shoots/explant), reaching a maximum shoot height of 1.40 cm after 8 weeks of culture. After each subculture, the red color on leaves tended to decrease gradually, and they were *in vitro* flowered after the second subculture at a rate of 15% and the third subculture at a rate of 10% on medium supplemented with 40 mg/L adenine, 1.0 mg/L NAA, 1.0 mg/L BA, 30 g/L sucrose, 9 g/L agar and 1 g/L activated charcoal. The plantlets regenerated from petal cultures bloomed with standard red pigments, similar to those grown in natural growing conditions.

Keywords: *Begonia x hiemalis* Fotsch, *inflorescences*, *myo-inositol*, *petal explants, red-leaf*.

INTRODUCTION

Begonias are popular as one of the ornamental plants used for garden decoration, and indoor plants. *In vitro* quality seedlings play an important role in cultivation, trade, and export (Chebet *et al.,* 2003). Comment explants for direct or indirect Begonia shoot regeneration were leaves, petioles, stems and inflorescences (Espino *et al.,* 2004; Nhut *et al.,* 2005; Awal et al., 2008, 2010; Mendi *et al.,* 2009; Snježana *et al.,* 2011; Kabirnataj *et al.,* 2012; Awal *et al.,* 2013; Lai *et al.,* 2018; Ismaini *et*

al., 2021). Using petals as initial materials for tissue culture has been reported in some crops such as *Gerbera*, *Sedum* and *Chrysanthemum* (Surinder and Jitender, 2006; Jesmin *et al*., 2007; Maria, 2009; Rezvanolsadat *et al*., 2014). In *B. elatior*, *in vitro* callus induction and shoot regeneration from the petal were initially obtained with certain success (Velasco *et al.,* 2018). The main limitation in this study was that the disinfectant caused damage to the epidermis of the petals, leading to necrosis or unresponsiveness regeneration. Therefore, it is very meaningful to use a new disinfectant with less damage and high viability for petal explants. In a recent study, silver nanoparticles (AgNPs) were applied as a new disinfectant with high efficiency in the process of disinfecting explants of on *Saintpaulia ionantha*, *Kappaphycus striatus* and *Fragaria* × *ananassa* in *in vitro* culture (Nhut *et al*., 2018, Tung *et al*., 2018, Mo *et al*., 2020, Tung *et al*., 2021).

In some reports, the role of sucrose and adenine had a significant effect on flowering *in vitro* cultures of Begonia sp. (Zhang *et al*., 2008; Awal *et al.,* 2013). Myo-inositol is a versatile compound that plays a vital role in plant biochemistry and physiology. They participate in many stress-induced processes, such as having a positive role in salinity tolerance, immunity, and programmed cell death; this suggests that they have a role in protecting plants against adverse conditions (Loewus and Murthy, 2000; Tan *et al*., 2013; Hu *et al*., 2018). Besides, myo-inositol is also considered a carbohydrate source, an *in vitro* growth agent with osmotic ability, and an enzyme that catalyzes the biosynthesis of inositol involved in various physiological processes such as embryogenesis, seedling development and resistance to biotic and abiotic stresses (Bellini *et al*., 1990; Sepehr and Ghorbanli, 2002; Mazri *et al*., 2016; Hu *et al*., 2020; Sharma *et al*., 2020). Furthermore, myo-inositol is a type of plant-derived carbocyclic sugar and is commonly used at a concentration of 100 mg/L in a culture medium (Schenk and Hildebrandt, 1972). The change in leaf color has been found to closely relate to the accumulation or destruction of anthocyanin in response to changes in the external environment (Michal, 2009).

In this study, petal explants were used as the initial source of culture material. AgNPs was used as a new disinfectant with better disinfection efficiency than $HgCl_2$ and $Ca(ClO)_2$. The petal explants were cut in half lengthwise for high-frequency regeneration. Myo-inositol has been used as a source of energy, a plant growth regulator, and an enzyme that slows down the decomposition of anthocyanin pigments in flower petals, resulting in the appearance of red leaf buds and flowering directly from petal cultures. This opened up new research directions on physiology and biochemistry related to pigment accumulation and storage in cells, as well as a selection of somatic mutant lines for further studies.

MATERIALS AND METHODS

Medium and culture conditions

The basal MS medium was used in all experiments (Murashige and Skoog, 1962). The explants were cultured at 25 ± 2°C, humidity of 55 - 60%. There was also the use of fluorescent light with an intensity of 40 - 45 $\mu mol.m^{-2}.s^{-1}$ and the photoperiod is 16h/day.

Effects of AgNPs on the surface disinfection of petal explants

Petal explants with a 2.5 - 3.0 cm diameter located on the first and second layers from the outside of *Begonia x hiemalis* Fotsch flower grown in the greenhouse of Tay Nguyen Institute for Scientific Research (Fig. 1a) were washed under a running water tap for 30 min, then soaked in 70% ethanol for 30 s, and washed three times with sterile distilled water. Next, the petal explants were disinfected in 0.1, 0.2 and 0.5 g/L AgNPs solution (< 20 nm and provided by the Institute of Environmental Technology) in 10, 15 and 20 min. The controls were disinfected with 1 g/L $HgCl_2$ and 10 g/L $Ca(ClO)_2$ for 7 and 10 min, respectively (Tung *et al*., 2021; Nhut *et al*., 2018). Finally, the whole petal explants were washed three times with sterile distilled water and transplanted on MS medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 9 g/L agar and a pH = 5.8.

Effect of petal sizes on shoot regeneration

Surviving *Begonia* petal explants from the surface disinfection experiment were used as a source to investigate the effect of a vertical cut line (¼-petals, ½-petals, whole petals) on shoot regeneration on MS medium supplemented with 1.0 mg/L BA, 1.0 mg/L NAA, 30 g/L sucrose, 9.0 g/L agar and pH = 5.8 (Velasco *et al*., 2018).

Effect of myo-inositol on *in vitro* flower formation and regeneration of shoots bearing red leaves

Surviving petal explants obtained from the concentrations and times described in the above experiment were used to cut in half lengthwise (cut from the tip to the keel of the petal). Then, explants were cultured on medium supplemented with (100, 250, 500 and 1000 g/L) myo-inositol, 1.0 mg/L BA, 1.0 mg/L NAA, 30 g/L sucrose, and 9 g/L agar to induce flower formation and regeneration of shoots bearing red leaves (Velasco *et al*., 2018).

Changes in leaf color and *in vitro* flowering

Single shoots bearing red leaves were sub-cultured on medium supplemented with 40 mg/L adenine, 1.0 mg/L NAA, 1.0 mg/L BA, 30 g/L sucrose, 9 g/L agar and 1 g/L activated charcoal to study the change of red color on leaves and *in vitro* flowering after three subcultures (8 weeks/cycle) (Awal *et al*., 2013).

Statistical analysis

Each experiment was repeated three times with fifty vessels/treatment (two explants/vessel) for disinfection and regeneration induction. The change in leaf color was observed in thirty shoots per treatment. All data were processed by Microsoft Excel 2010 software and statistical analysis software SPSS 16.0 using Duncan's test with $p < 0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

AgNPs on the surface disinfection of *Begonia x hiemalis* Fotsch petal explants

The results of surface disinfection of *Begonia x hiemalis* Fotsch petal explants with AgNPs, $HgCl_2$ and $Ca(ClO)_2$ at various concentrations and exposure times are shown in Table 1. Treatment with 0.1 g/L AgNPs in 10 - 20 min resulted in a low survival rate (25.33 - 42.66%) due to high contamination rates (52.33 - 73%). At AgNPs of 0.2 g/L and 20 min disinfection time, the survival rate of petals was significantly increased up to 70.66% and coupled with a 21% decrease in the contamination rate. The highest explant survival rate of 81.33% and the lowest

contamination rate of 10.33% were observed in the treatment with 0.5 g/L AgNPs in 15 min (Table 1). Meanwhile, the explant survival rates with the two common disinfectants $HgCl_2$ and $Ca(ClO)_2$ were much lower than using the optimal AgNPs. Furthermore, under AgNPs treatments, the color of petals was unaltered, while it turned into a lighter color with a brown edge or necrosis after being disinfected with $HgCl_2$ or $Ca(ClO)_2$, respectively, after 1 week of culture. These results demonstrate that AgNPs are more efficient at surface sterilization than the old ones and minimal damage to the petal explants. The healthy petal explants remaining after surface sterilization with AgNPs showed that *Begonia x hiemalis* Fotsch petals could serve as a source of explants for the establishment of new propagation protocols. Begonia petals surface-sterilised with hypochlorite at 4 - 50% had been used as explants in previous studies, in which they became either necrosis (Awal *et al*., 2013) or poor callus induction (Velasco *et al*., 2018). Previous publications have shown that AgNPs are used as alternative disinfectants for the commonly used decontamination substances without causing adverse effects on plant growth and development of the explants. Additionally, AgNPs stimulate the induction of shoot regeneration from the petiole explants of African violet (*Saintpaulia ionantha* H. Wendl.) (Nhut et al., 2018) and strawberry leaves and *Chrysanthemum* flowers (Tung et al., 2021). In this study, AgNPs at a concentration of 0.5 g/L were safe and effective in sterilizing Begonia x hiemalis Fotsch petals, with a survival rate of 81.33%.

Table 1. Effect of AgNPs on the surface disinfection of petal explants of *Begonia x hiemalis* Fotsch after 1 week of culture.

Disinfectant agent	**Concentration (g/L)**	**Exposure time (min)**	**Survival rate (%)**	**Necrosis rate (%)**	**Contamination rate (%)**	**Explant appearance**
AgNPs	0.1	10	25.33^{h*}	1.66^{h}	73.00^{a}	The red color of the petals was preserved
		15	33.00^{g}	3.33^{g}	63.66^{b}	
		20	42.66^{f}	5.00^{f}	52.33^{c}	
	0.2	10	51.00^{e}	3.33^{f}	45.66^{d}	
		15	61.00^{d}	6.66^{e}	32.33^{e}	
		20	70.66^{c}	8.33^{d}	21.00^{f}	
	0.5	10	68.00^{c}	5.00^{f}	27.00^{e}	
		15	81.33^{a}	8.33^{d}	10.33^{g}	
		20	78.00^{ab}	14.00^{b}	8.00^{g}	
$HgCl_2$	1.0	7	76.33^{b}	11.66^{c}	12.00^{g}	Red petals faded to pink; the edges of petals turned brown

$Ca(ClO)_2$	10	10	48.33[e]	28.00[a]	23.66[f]	Most of them are necrotic

*Different letters (a, b, c,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).

Shoot regeneration from petal cuttings of *Begonia x hiemalis* Fotsch after 8 weeks of culture

Regenerated shoots were observed from vertical cut petals after 8 weeks on culture media. The highest shoot regeneration rate and number of shoots per explant (86.66% and 65.33 shoots/explant, respectively) were obtained on ½-petal (Table 2). Clusters of shoots formed at the keel and on the vertical cut lines of the petals (Fig. 1b). In the case of intact petal explants, shoot clusters mainly formed at the keel of the petals or appeared with a certain frequency at wounds created during explanation. The shoot regeneration rate and number of shoots per explant are 80% and 50.33 shoots/explant, respectively. The lowest shoot regeneration rate was obtained from ¼-petal explant treatment, with shoot clusters mainly formed at the keel of the petals. Therein, the petal explants with ¼ wing showed a low induction rate or unresponsiveness of shoot regeneration.

In vitro callus induction and shoot regeneration from petal explants have been reported in several ornamental plants. Velasco *et al.* (2018) obtained callus from the petals of 2 out of 4 cultured *Begonia* varieties at the keel but had poor shoot regeneration. In the study of Surinder and Jitender (2006), shoot regeneration at high frequency was achieved from petal-derived callus of *Gerbera*. The same successes with high frequency callus formation and shoot regeneration from petals were also reported in *Chrysanthemum* (Jesmin *et al*., 2007; Rezvanolsadat *et al*., 2014). *In vitro* shoots of *Sedum* were formed from epidermal and parenchymal cells directly or indirectly via the embryogenesis callus from the epidermal layer of petal explants (Maria, 2009). In this study, *in vitro* direct or indirect shoot regeneration of *Begonia* can be obtained from the keel or the vertical cut of petal explants (Fig. 1b and Fig 2h).

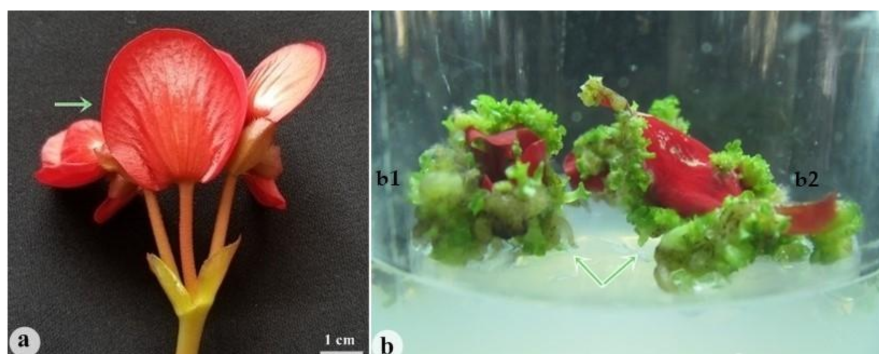

Figure 1. Petal explants were used for in vitro surface disinfection and regeneration experiment.

(a) 3-week-old flower bud which has a diameter of about (2.5 - 3 cm); (b) Regeneration of shoot cluster at the keel of the petal (b1: indirect shoot regeneration; b2: direct shoot).

Table 2. Effect of cutting method petal explants on shoot regeneration after 8 weeks of culture.

Cutting method	Shoot regeneration rate (%)	Number of shoots per explant	Shoot formation sites
Whole petal	80.00[ab*]	50.33[b]	The keel of the petal
½ Petal	86.66[a]	65.33[a]	The keel and vertical cut lines of the petal
¼ Petal	75.00c	44.66[b]	

*Different letters (a, b, c,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).

Myo-inositol on *in vitro* flower formation and regeneration of shoots bearing red leaves

Results of direct flower formation from ½-petal explants of *Begonia x hiemalis* Fotsch after 8 weeks of culture are shown in Table 2. Directly formed flowers consist of two different structural forms, including a single petal cluster and complete flower buds forming mainly at the keel of the petals. When increasing the concentration of myo-inositol in the culture media, the flower formation rate increased accordingly and reached its highest value (19.66 petals/explant; 0.53 flower buds/explant) on the medium supplemented with 1,000 mg/L myo-inositol (Fig. 2a-j).

Meanwhile, the direct shoot clusters were formed mainly from the keel and decreased to the wing of the petals (Fig. 2h). It is interesting to observe that when myo-inositol was added to the culture media, the appearance of shoots with red leaves was observed. These red-leaved shoots were formed with higher numbers on the keel of the petals than on the vertical cut line and the wing. The highest rate of shoot formation with red leaves was 12.66 shoots/explant, with the maximum shoot height was 1.40 cm on medium supplemented with 1000 mg/L myo-inositol (Table 3, Fig. 2g-h). The appearance of red leaves was due to the deposition of pigment in the vacuoles and cytoplasm in the epidermal cell layers of the petals (Erdelska´ and Ovecˇka, 2004). The expression of pigment in leaves was related to the myo-inosytol added to the culture media. Myo-inositol is a plant-derived pentose, which is the precursor and energy provider for anthocyanin synthesis (Vuylsteke, 1989). It was added to MS and B5 media at a concentration of 100 mg/L (Murashige and Skoog, 1962; Gamborg et al., 1968). Subsequently, it was also added to the culture medium at the same concentration in banana tissue culture (Vuylsteke, 1989). In SH medium, they were supplemented with 1000 mg/L (Schenk and Hildebrandt, 1972). Myo-inositol biosynthesis includes three successive steps: (1) phosphorylation of glucose to glucose-6-phosphate by hexokinase; (2) conversion of glucose-6-phosphate to myo-inositol-1-phosphate by myo-inositol-1-phosphate synthase; and (3) dephosphorylation of myo-inositol-1-phosphate to myo-inositol by myo-inositol monophosphatase. Myo-inositol-1-phosphate by myo-inositol-1-phosphate synthase is the rate-limiting enzyme in the biosynthetic pathway of myo-inositol and its derivatives (Hu *et al.*, 2020). Thus, it is considered an enzyme that catalyzes the biosynthesis of inositol which participates in various physiological processes such embryogenesis, plantlet

growth, and resistance to biotic and abiotic stresses (Mazri *et al*., 2016, Hu *et al*., 2020; Sharma *et al*., 2020). In previous studies, different carbohydrate sources (sucrose, glucose and fructose) were reported to have an effect on the enhancement of anthocyanin synthesis and accumulation (Hara *et al*., 2003; Zhang *et al*., 2015). The expression of red pigment in callus with high frequency was induced by petal culture of rose to obtain anthocyanins with a high content of β-pinene and higher antimicrobial activity compared with the original petal tissues (Pınar *et al*., 2017; Tarrahi and Rezanejad, 2013). In this study, the results showed that the expression of red pigment in the leaves is an issue that needs to be studied further in order to serve Begonia's breeding through the culture of petal explants in the future.

Figure 2. Direct-shoot regeneration and *in vitro* flowering from 3-size petal explants.

(a, b, c) The formation of petal clusters and shoots from whole petals, ½-petals; ¼-petals; (d, e, f) Complete flower buds forming were obtained on ½-petal explants; (g) Cluster shoots formation with red leaves; (h) Direct shoot regeneration at the keel of the petals; (i) Direct clusters single petal formation at the keel of the petals; (j) Petal layering of the complete flower bud.

Table 3. Effect of myo-inositol on *in vitro* formation of flower and regeneration of shoots bearing red leaves after 8 weeks of culture.

Concentration of myo-inositol added (mg/L)	**Flower buds formation (%)**	**Flower formation**		**Shoot regeneration**			
		Number of petals per explant	Number of flower buds per explant	Shoot regeneration rate (%)	Number of shoots with green leaves	Number of shoots with red leaves	Average height of shoots (cm)

					per explant	per explant	
100	0.00[d*]	0.00[c]	0.00[c]	85.00[a]	65.33[a]	0.00[d]	1.06[c]
250	25.00[c]	13.33[b]	0.00[c]	55.00[b]	39.66[b]	5.33[c]	1.20[bc]
500	33.33[b]	18.33[a]	0.26[b]	46.66[c]	20.00[b]	10.33[b]	1.26[ab]
1000	53.33[a]	19.66[a]	0.53[a]	26.66[d]	12.66[c]	12.66[a]	1.40[a]

*Different letters (a, b, c,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).

Effect of the number of subcultures on the change in leaf color of shoots and *in vitro* flowering

The shoots with red leaves were continuously sub-cultured 3 times (8 weeks/time). After the first subculture, the color of new leaves began to appear with half red and half green, while there was a reduction in red color observed on the old leaves. After the second subculture, the shoots continued to grow and formed leaves with 3 different colors (red leaves, mosaic leaves, and green leaves) (Fig. 3b). However, after the second subculture, newly formed flowers (15%) were derived from shoots with red and red mosaic leaves on medium supplemented with 40 mg/L adenine, 1.0 mg/L NAA, 1.0 mg/L BA, 30 g/L sucrose, 9 g/L agar and 1 g/L activated charcoal (Table 4, Fig. 3d). After the third sub-cultured, expression of red pigment was not observed in the leaves of the transplanted shoots, and the newly formed shoots developed normally as they were regenerated from other parts of the plant. However, observation of the flowering process (10%) was continued from shoots with green leaves (Fig. 3c).

The petal pigments are primarily distributed in the upper and lower epidermal layers of the petals. The red color displayed on the leaves of shoots regenerated from petal may be due to pigment deposition in the vacuoles and cytoplasm of the petals. During the process of regeneration and cell division, these pigments were transferred into daughter cells, which led to a decrease in the expression of red color on the leaves after each subculture. The shoots that originate from the epidermal tissues of the petals still retain the typical cellular structures of a petal, including conical and flat structures. The characterization of different types of epidermal cells, such as conical and flat cells, might also have an important impact on the expression level of petal color. The presence of cones can increase the incidence of direct light on epithelial cells, which enhances the light absorption of pigments, thus creating the dark color of the flower. In contrast, cells with a flat cell structure can reflect more incident light, resulting in a lighter flower color (Zhao and Tao, 2015). The translocation of red pigment from petals to the stem organs via phloem was reverse translocated to the maternal storage organs during aging (Erdelska´ and Ovecˇka, 2004). Indirect flowering processes in cultured *Begonia* were also observed in petiole explants (Awal *et al*., 2013). In this study, the results showed that inflorescences bloomed *in vitro* with normal red pigment like those bloomed under natural growing conditions and the expression of red color on leaves regenerated from petal culture tended to decrease after each subculture.

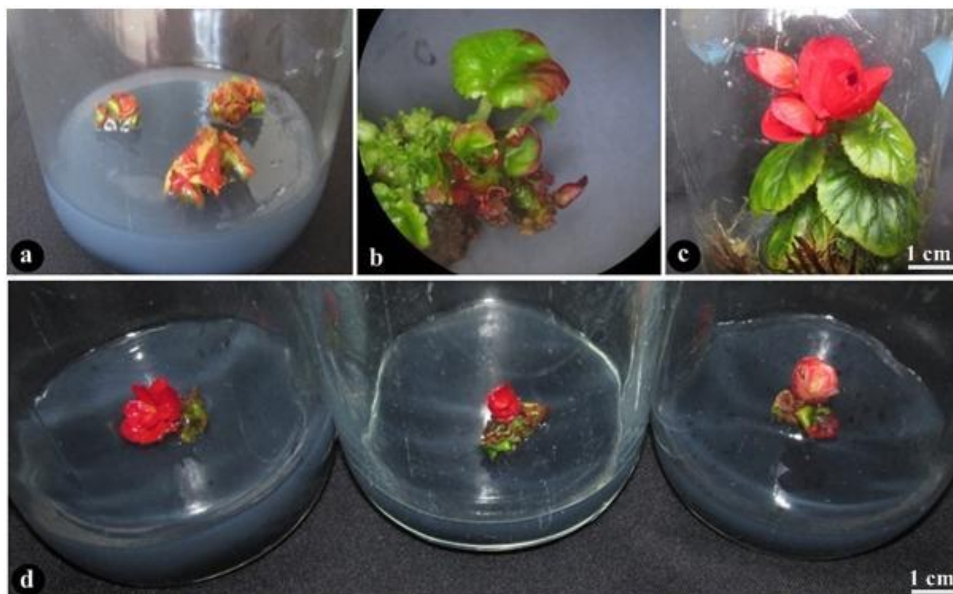

Figure 3. Change of leaf color and *in vitro* flowering after 3 subcultures.

(a) shoots with red leaves; (b) Shoots bearing red and green mosaic leaves; (c) Shoot formation inflorescence with multiple flower buds after third time subculture; (d) Shoots bearing green mosaic and red leaves flowering after the second time subculture.

Table 4. Effect of the number subcultures on the change of leaf color of shoots and *in vitro* flowering.

Number of subcultures	**The colors of the shoots**			***In vitro* flowering rate (%)**	**Notes**
	Shoot with red leaves (%)	Shoot with red and green leaves (%)	Shoot with green leaves (%)		
1st	71.66^{a*}	28.33^{b}	0.00^{c}	0.00^{b}	-
2nd	21.66^{b}	65.00^{a}	13.33^{b}	15.00^{a}	*In vitro* flowering of shoots bearing red and green leaves
3rd	0.00^{c}	0.00^{c}	100.00^{a}	10.00^{a}	*In vitro* flowering of shoots bearing green leaves

* Different letters (a, b, c,…) in the same column represent statistically significant differences at $p < 0.05$ (Duncan's test).

CONCLUSION

The results of this study showed that AgNPs are a new disinfection with a higher survival rate (81.33%) than $HgCl_2$ and $Ca(ClO)_2$. *Begonia* petals are considered a new source of materials in the study of direct shoot regeneration with high frequency (86.66 %; 65.33 shoots per explant). Increasing the concentration of myo-inositol (1,000 mg/L) promoted flower formation directly at the keel from ½-petal explants and the formation of shoots with red leaves. The reduction of red pigment in leaves after each *in vitro* subculture, and the plantlet flowering with normal structure and color have been found to be similar to natural growing conditions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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