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Improved shoot organogenesis of gloxinia (*Sinningia speciosa*) using silver nitrate and putrescine treatment

Eui-Ho Park[1], Hanhong Bae[1], Woo Tae Park[2], Yeon Bok Kim[2], Soo Cheon Chae[3*] and Sang Un Park[2*]

[1]School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Korea
[2]Department of Crop Science, College of Agriculture and Life Sciences, Chungnam National University, 79 Daehangno, Yuseong-gu, Daejeon, 305-764, Korea
[3]Department of Horticultural Science, College of Industrial Sciences, Kongju National University, 1 Daehoe-ri, Yesan-kun, Chungnam, 340-720, Korea

***Corresponding Author: scchae@kongju.ac.kr; supark@cnu.ac.kr**

Abstract

An improved method for shoot organogenesis and plant regeneration in *Sinningia speciosa* was established. Leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with different combinations of benzylaminopurine (BAP) and naphthalene-acetic acid (NAA) for shoot induction. MS media including BAP (2 mg/L) and NAA (0.1 mg/L) resulted in the highest efficiency in shoot regeneration per explant (12.3 ± 0.8) and in the greatest shoot growth (1.2 ± 0.1 cm) after 6 weeks. For improving shoot induction, the ethylene inhibitor silver nitrate and the polyamine putrescine were added to the regeneration medium. The addition of silver nitrate (7 mg/L) increased the shoot number (23.9 ± 1.6) and length (1.7 ± 0.2 cm) after 6 weeks. Similarly, putrescine (50 mg/L) improved the shoot number (19.2 ± 1.6) and growth (1.7 ± 0.2 cm). The rooted plants were hardened and transferred to soil with a 90% survival rate. This method of producing *S. speciosa* regenerated plants could be used as a possible micropropagation and plant transformation protocol.

Keywords: Gloxinia, micropropagation, plant regeneration, shoot organogenesis, *Sinningia speciosa.*

Introduction

Gloxinia (*Sinningia speciosa* Baill) belongs to the family Gesneriaceae and is a commercially important ornamental plant. Gloxinia produces single or double flowers that are available in a variety of colors. This plant can be traditionally propagated by leaf, stem, rhizome, seed, and crown cuttings from a mature plant after blooming. The commercial production of a blooming gloxinia takes approximately 6 to 7 months (Chautems et al. 2000; Zaitlin and Pierce, 2010). Plants show totipotency or the ability to produce a clone from cells in leaves, stems, roots, etc. Plant proliferation by this mechanism is termed plant regeneration. Whole plants can regenerate from excised plant parts by a number of pathways. Two main pathways can be considered, i.e., plant regeneration through shoot organogenesis and somatic embryogenesis (Phillips and Hubstenberger, 1995). Organogenesis is a developmental pathway in which shoots or roots (i.e., organs) can be induced to differentiate from a cell or group of cells. Plant regeneration through organogenesis generally involves induction and development of a shoot from explant tissue, followed by transfer to a different medium for the induction of root formation and development. Research has shown that successful organogenesis in a number of plant species can be achieved by the correct establishment of medium components, selection of a suitable explant, and control of the physical environment (Brown and Thorpe, 1986; Thorpe, 1990). Polyamines are small molecules that have been detected in bacteria, fungi, animals, and higher plants. Although polyamines are not considered plant hormones, they promote cell division and influence morphology (Gaspar et al., 1996; Wallace et al., 2003). Previous studies have shown that exogenously applied polyamines (e.g., putrescine) can result in increased shoot growth, callus formation, and root elongation (Bais et al., 2001; Takeda et al., 2002; Tang and Newton, 2005). Plants growing *in vitro* in closed vessels have been reported to generate ethylene during cell division and that ethylene accumulation occurs in the vessels. Ethylene is a gas hormone that is known to play an important role in plant growth and cell division (Kumar et al. 1998). Ethylene has certain positive effects on callus culture and root growth but largely functions to inhibit the growth of the shoot. Hence, for enhancing shoot production, ethylene inhibitors are added to the plant media to prevent the negative effects of the hormone. $AgNO_3$, amino-ethoxyvinylglycine (AVG), and $CoCl_2$ are known ethylene inhibitors that function to promote shoot growth when exogenously added to the media (Biddington, 1992). In vitro plant regeneration has been reported for gloxinia using leaf explants culture (Scaramuzzi et al., 1999; Nhut et al., 2006; Xu et al., 2009). Direct regeneration of floral buds from sepal segments has also been reported (Pang et al., 2004; Pang et al., 2006). However, the procedures are complicated, show limited efficiency, and are not readily available for use. An improved method for the regeneration of intact plants from tissue culture

is essential for establishing a multiple micropropagation system and a genetic transformation protocol. In this paper, we report the development of an improved method for high-efficiency plant regeneration from leaf explants of *S. speciosa* using benzylaminopurine (BAP) and naphthalene-acetic acid (NAA) in combination with $AgNo_3$ or putrescine.

Results and discussion

Effect of growth regulators on shoot regeneration

An improved and effective method has been developed for the *in vitro* plant regeneration of *S. speciosa*. For establishing a plant regeneration protocol, we investigated the effect of different concentrations of BAP on the efficiency of shoot organogenesis in *S. speciosa* (Table 1). Shoot development from excised leaf explants did not occur in the absence of exogenous BAP. BAP at 4 mg/L in the MS media performed the best for initial shoot regeneration, resulting in the highest number of shoots (6.7 ± 0.6) and in the greatest shoot length (0.95 ± 0.1 cm), followed by BAP at 2 mg/L (shoot number: 5.2 ± 0.4; shoot length: 0.84 ± 0.1 cm). To examine the effect of both auxin and cytokinin, NAA at 0.1 mg/L and various BAP concentrations (1, 2, and 4 mg/L) were used to supplement the media (Table 1). All the combined treatments showed a synergistic effect, with the initial shoot regeneration greater than that of the BAP-treated media. The combination of BAP at 2 mg/L and NAA at 0.1 mg/L was the most efficient for initial shoot regeneration, producing the highest number of shoots and the greatest shoot length (shoot number: 12.3 ± 0.8; shoot length: 1.2 ± 0.1 cm). The shoot number with this treatment was 3.5, 2.4, and 1.8 times more than that with BAP at 1, 2, and 4 mg/L, respectively.BAP has been shown to have a similar influence on shoot induction and regeneration in previous studies on *S. speciosa* (Scaramuzzi et al., 1999; Pang et al., 2006). Scaramuzzi et al. (1999) found that shoot regeneration was affected on MS media supplemented with 5 mg/L of BAP and 5 mg/L of IAA. Moreover, Pang et al. (2006) showed that the combination of GA_3 and BAP were the most affective at inducing the formation of flower buds on sepal segments. When gloxinia leaves were cultured on MS solid media supplemented with 2 mg/L BAP and 0.1 mg/L NAA, the various stages of gloxinia shoot organogenesis were observed. During the initial stage (1–2 weeks of incubation), there was some expansion and proliferation of the cells at the cut surface, but callus growth was limited. At approximately 2 weeks, the cut end of the leaf explant had enlarged, and shoot primordia and small elongated shoots had formed adjacent to the cut surface (Figure 1-A). We observed that the cells of the epidermis proliferated to produce the shoots directly, without an intervening callus phase. The regenerated shoots had developed from the shoot primordia within 4 weeks (Figure 1-B and 1-C). After 6 weeks of culture, fully developed shoots of *S. speciosa* were produced from the leaf explants (Figure 1-D).

Effect of $AgNO_3$ on shoot regeneration

For improving shoot growth, the shoots were cultured on initial shoot regeneration media (MS media supplemented with BAP at 2 mg/L + NAA at 0.1 mg/L) that included $AgNO_3$ (0, 1, 3, 7, 10, 20 mg/L) (Table 2). The addition of $AgNO_3$ significantly improved the initial shoot regeneration. In this study, the highest shoot growth was found when the MS media was supplemented with BAP at 2 mg/L, NAA at 0.1 mg/L, and $AgNO_3$ at 7 mg/L (shoot number: 23.9 ± 1.6; shoot length: 1.7 ± 0.2 cm). This combination resulted in twice the number of initial shoot relative to the control (without $AgNo_3$). Beyond

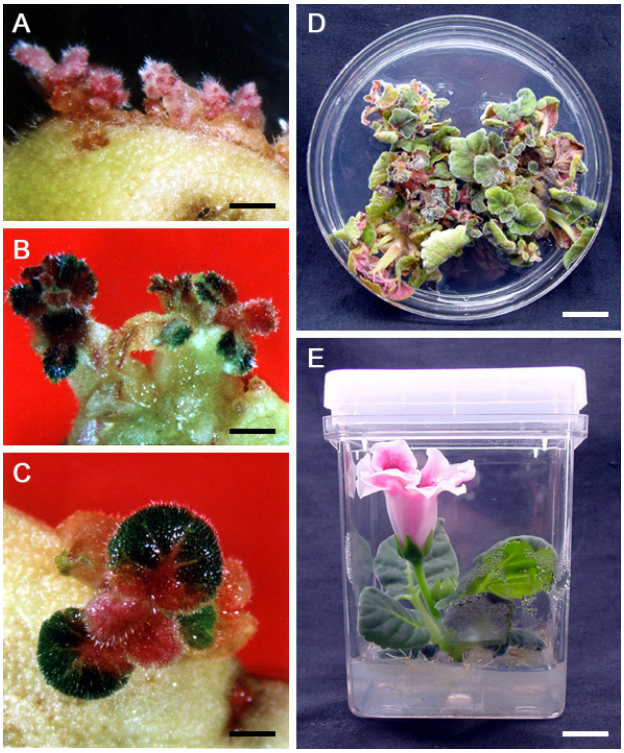

Fig 1. Shoot organogenesis in *Sinningia speciosa*.(A) Shoot primordia emerging from a leaf explant of *S. speciosa* 2 weeks after cultivation on MS solid media supplemented with 2 mg/L BAP and 0.1 mg/L NAA (× 13); (B and C) Shoot induction after 3 and 4 weeks of culture NAA (× 7); (D) After 6 weeks of culture, fully developed shoots had regenerated from the leaf explants (× 0.7); (E) In vitro flowering from a regenerated plant after 8 weeks of culture (× 0.7).

this concentration of $AgNO_3$, the shoot growth declined (Table 2). Ethylene is produced during cell division *in vitro* and is known to act as a growth inhibitor. Ethylene inhibitors such as $AgNO_3$ inhibit the binding of ethylene during cell division (Mohiuddin et al., 1997). Kumar et al. (1998) reviewed the use of silver nitrate in plant regeneration and concluded that this chemical promoted growth. Other species, including cucumber (Mohiuddin et al., 1997), Brassica (Akasaka-Kennedy et al., 2005), and coffee (Kumar et al., 2007) have also been found to be affected by silver nitrate.

Effect of putrescine on shoot regeneration

Putrescine was added exogenously to the initial shoot regeneration media to promote shoot regeneration (Table 3). Shoot growth increased with increasing concentrations of putrescine up to 50 mg/L, but thereafter decreased as the concentrations increased. Putrescine at 50 mg/L produced the highest shoot number (19.2 ± 1.6) and shoot length (1.8 ± 0.2 cm). Both $AgNO_3$ nitrate and putrescine influenced the shoot growth of *S. speciosa*. However, the optimal concentration of $AgNO_3$ (7 mg/L) resulted in a greater number of shoots than the optimal concentration of putrescine (50 mg/L). On the other hand, the 2 treatments were similar in terms of the shoot length. Hence, the $AgNO_3$ resulted in the best overall regeneration.

Table 1. The effect of different concentrations of BAP and NAA on shoot regeneration and growth from leaf cultures of *Sinningia speciosa* after 6 weeks in culture.

Hormones (mg/L)	Number of shoots/explant	Shoot length (cm)
BAP 1	3.5 ± 0.4	0.72 ± 0.2
BAP 2	5.2 ± 0.4	0.84 ± 0.1
BAP 4	6.7 ± 0.6	0.95 ± 0.1
BAP 1 + NAA 0.1	7.8 ± 0.9	0.82 ± 0.1
BAP 2 + NAA 0.1	12.3 ± 0.8	1.19 ± 0.1
BAP 4 + NAA 0.1	11.3 ± 1.3	1.10 ± 0.1

Each value is the mean ± standard error of 3 repeated experiments, with 50 explants used in each treatment.

Table 2. The effect of different concentrations of $AgNO_3$ on shoot regeneration and growth from leaf cultures of *Sinningia speciosa* after 6 weeks in culture on regeneration medium (MS medium with 2.0 mg/L BA and 0.1 mg/L NAA).

$AgNO_3$ (mg/L)	Number of shoots/explant	Shoot growth (cm)
0	12.3 ± 0.8	12.1 ± 0.1
1	13.7 ± 1.2	13.1 ± 0.1
3	19.7 ± 1.1	1.5 ± 0.2
7	23.9 ± 1.6	1.7 ± 0.2
10	16.2 ± 1.3	1.3 ± 0.2
20	13.8 ± 1.2	0.9 ± 0.1

Each value is the mean ± standard error of 3 repeated experiments, with 50 explants used in each treatment.

Table 3. The effect of different concentrations of putrescine on shoot regeneration and growth from leaf cultures of *Sinningia speciosa* after 6 weeks in culture on regeneration medium (MS medium with 2.0 mg/L BA and 0.1 mg/L NAA).

Putrescine (mg/L)	Number of shoots/explant	Shoot growth (cm)
0	12.30 ± 0.8	1.20 ± 0.1
10	12.90 ± 1.3	1.32 ± 0.1
30	16.50 ± 1.7	1.45 ± 0.1
50	19.20 ± 1.6	1.79 ± 0.2
70	17.40 ± 1.2	1.51 ± 0.2
100	13.60 ± 1.1	1.32 ± 0.1

Each value is the mean ± standard error of 3 repeated experiments, with 50 explants used in each treatment.

Rooting and acclimatization of regenerated plants

The regenerated shoots were individually divided and transferred to 1/2 MS without growth hormones to induce root formation. The regenerated shoots were initially rooted after 3 weeks, with more than 95% of the shoots producing roots after 5 weeks. The rooted regenerated plants were washed in tap water to remove the agar and transplanted into pots. The plants were grown in a growth chamber at 25 ± 1 °C with a 16-h photoperiod. They were covered with a plastic bag to maintain high humidity conditions during the 2 weeks. The regenerated plants survived at a rate of 90% and flowered within 3 months. Plant regeneration protocols are an essential part of plant genetic transformation and lead to plant improvement. Currently, shoot organogenesis is the most widely used method of *in vitro* plant regeneration in transformation systems. This regeneration protocol has succeeded for *Sinningia speciosa.* Both $AgNO_3$ and putrescine promoted the shoot regeneration rate of gloxinia. These results will allow the genetic improvement of *Sinningia speciosa* and other flower species.

Materials and methods

Plant material

Seeds of *Sinningia speciosa* were surface-sterilized with 70 % (v/v) ethanol for 1 min and 2 % (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. FIve seeds were placed on 25 ml of agar- solidified culture medium in Petri dishes (100 x 15 mm). The basal medium consisted of salts and vitamins of MS (Murashige and Skoog, 1962) medium and solidified with 0.7 % (w/v) agar. The medium was adjusted to pH 5.8 before adding agar and then sterilized by autoclaving at 121 °C for 20 min. The seeds were germinated in a growth chamber at 25 ± 1 °C under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod.

Shoot organogenesis

Young leaves of *Sinningia speciosa* were taken from in vitro grown plants. Leaves were cut aseptically at the ends, into sections of approximately 7 X 7 mm^2 in size. Explants were placed on the MS medium and solidified with 0.7% (w/v) Phytagar containing various condition media. Seven explants were cultured in each petridish. The pH of medium was adjusted to 5.8 before adding phytagar. The media were sterilised by autoclaving at 1.1 kg cm^{-2} (121 °C) for 20 min. For shoot regeneration from leaf explants, the MS medium was supplemented with 1, 2, 4 mg/L BAP (6-benzylaminopurine) and 0.1 mg/L NAA (1-naphthalene-acetic acid). For improvement of shoot regeneration, the different concentrations of silver nitrate (0, 1, 3, 7, 10, 20 mg/L) and putrescine (0, 10, 30, 50, 70, 100 mg/L) were tested in MS medium supplemented with 2 mg/L BAP and 0.1 mg/L NAA. Cultures were maintained at 25 ± 1 °C in a growth chamber with a 16-h

photoperiod under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}) for 6 weeks.

Rooting and acclimatization of regeneration plants

Regenerated shoots (around 1cm long) were placed in MS medium. The medium was solidified with 3g/l Phytagel and dispensed at 30ml per Magenta box and four shoots were cultured in each box. Regenerated shoots were incubated at 25±1 °C in a growth chamber with a 16-h photoperiod under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}) for 5 weeks. After five weeks, the rooted plants were washed with sterile water to remove Phytagel, transferred to pots containing autoclaved vermiculite, and covered with polyethylene bags for one week to maintain high humidity. The plants were then transferred to soil and maintained in a growth chamber with a 16-h photoperiod, and a night/day temperature of 18/20°C for 2 weeks. These hardened plants then transferred to the greenhouse.

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