Somatic Embryogenesis in *Hedychium bousigonianum*

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Abstract. An efficient primary somatic embryo (SE) and secondary somatic embryo (SSE) production system was developed for the ornamental ginger *Hedychium bousigonianum* Pierre ex Gagnepain. Addition of two ethylene inhibitors, salicylic acid (SA) and silver nitrate (AgNO₃), to the culture media improved the system. Callus was initiated and proliferated on a medium containing Murashige and Skoog (MS) basal salts supplemented with 9.05 μM 2,4-dichlorophenoxyacetic acid and 4.6 μM kinetin. Friable callus was transferred to a liquid medium containing MS basal salts, B5 vitamins, 0.6 μM thidiazuron, and 8.9 μM 6-benzylaminopurine to induce somatic embryogenesis. The effects of various concentrations of SA (0, 25, 50, 75, 100, 125, 150 μM) and AgNO₃ (0, 10, 20, 30, 40, 50, 60 μM) on callus growth, SE, and SSE development was further evaluated. The rate of callus growth decreased as the concentrations of SA or AgNO₃ increased. AgNO₃ and SA at all concentrations stimulated SE and SSE development better than the control although a decrease in embryo production was observed at higher concentrations of both SA and AgNO₃. The best concentrations for SA were 75 and 100 μM , whereas for AgNO₃, they were 30 to 50 μM for both SE and SSE production.

The genus *Hedychium* belongs to the Zingiberaceae family and consists of ≈ 80 species. They are multipurpose plants (Gao et al., 2008; He, 2000), which are used as ornamentals and as raw material for manufacturing paper and perfumes. They are also used in ethnomedicine as a result of their antibacterial and antifungal activities. Several studies have shown that *Hedychium* species possess antifungal, antibacterial, and insecticidal properties (Bisht et al., 2006; Gopanraj et al., 2005; Medeiros et al., 2003). For transformation and other genetic improvement schemes, an efficient, rapid, and dependable regeneration system is required. Somatic embryogenesis is a regeneration scheme that is generally preferred over other in vitro developmental processes such as organogenesis because it offers more target cells (Ogita et al., 2002) and because somatic embryos (SEs) are believed to be of single cell origin, any regenerated transformed cell would give rise to a transformed plant without unwanted chimeric events often associated with transformed meristems. However, somatic embryogenesis is highly species- or genotype-dependent. Moreover, it is affected

by various factors, including ethylene, a ubiquitous plant hormone with a variety of effects on plant growth and development, which has been reported to inhibit or promote in vitro plant growth and morphogenesis depending on the species (Al-Khayri and Al-Bahrany, 2001; Biddington, 1992; Kumar et al., 1998). Because of this, regulation of ethylene biosynthesis appears to be a promising approach for the development of more efficient tissue culture protocols in plant systems (Kumar et al., 2007). Incorporation of ethylene inhibitors in culture media has resulted in improved in vitro regeneration for several plant species (Adkins et al., 1993; Al-Khayri and Al-Bahrany, 2001; Kumar et al., 1998, 2007). In particular, salicylic acid (SA) has been found to inhibit ethylene biosynthesis (Leslie and Romani, 1986). SA is a hormone-like substance that plays an important role in the regulation of plant growth and development, and it has received much attention in the last two decades because of its involvement in plant defense mechanisms under both biotic and abiotic stresses (Catinot et al., 2008; Métraux et al., 1990). Silver nitrate (AgNO₃), an inhibitor of ethylene signal transduction, has been shown to improve in vitro plant regeneration (Beyer, 1976; Chaudhuri and Kar, 2008; Kumar et al., 2007). Somatic embryogenesis has been achieved in some *Hedychium* species (Sakhanokho et al.,

2008), but there are no reports on somatic embryogenesis in *Hedychium bousigonianum* or on the effect of ethylene inhibitors on somatic embryogenesis response in any *Hedychium* species. Therefore, the objective of this study was to establish high-frequency plant production in *Hedychium bousigonianum* through somatic embryogenesis and assess the influence of the ethylene inhibitors, AgNO₃ and SA, on SE and secondary somatic embryo (SSE) multiplication in this species.

Materials and Methods

Seed sterilization and in vitro germination. Seeds of *Hedychium bousigonianum* were collected from greenhouse-grown plants, scarified in 98% sulfuric acid (H₂SO₄) (Fisher Scientific, Atlanta, GA) for 30 min, rinsed three times for 5 min each time with running tap water to remove acid residues, and then surface-sterilized under a laminar flow hood by dipping them in 100% ethanol for 5 min with gentle shaking. Thereafter, seeds were rinsed with sterile distilled water and transferred to sterilized beakers containing 40% (v/v) sodium hypochlorite solution and one drop of Tween™ 20 (Sigma, St. Louis, MO) and shaken for 20 min at 110 rpm. They were then rinsed three times with sterile distilled water and soaked overnight in sterile distilled water on a shaker at 110 rpm at room temperature (22 °C). The next day, the seeds were rinsed again three to four times with distilled sterile water and transferred to 100 mm $\times$ 15 mm (diameter $\times$ depth) petri dishes (Fisher Scientific) containing Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium supplemented with 20 g·L⁻¹ sucrose, 0.75 g·L⁻¹ MgCl₂, and 2 g·L⁻¹ Gelrite (Sigma). The pH of all media used was adjusted to 5.8 before autoclaving for 15 min at 121 °C. The petri dishes were then placed in an incubator where the temperature was maintained constant at 28 °C with a photoperiod regime of 16-h light (100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 8 h dark. Most seed germinated within 35 to 40 d.

Callus initiation and proliferation. Leaf explants were removed from 5- to 7-d-old in vitro germinated seedlings, cut into 3- to 4-mm segments, and transferred to 100 mm $\times$ 20-mm petri dishes containing the callus initiation and proliferation medium consisting of MS macronutrients and micronutrients (Murashige and Skoog, 1962) and B₅ vitamins (Gamborg et al., 1968) supplemented with 9.05 μM 2,4-dichlorophenoxyacetic acid and 4.6 μM kinetin, 20 g·L⁻¹ sucrose, 0.2 g·L⁻¹ myo-inositol, 1 g·L⁻¹ casein hydrolysate, 1 mg·L⁻¹ thiamine, 0.75 g·L⁻¹ MgCl₂, and 2 g·L⁻¹ Gelrite (Sigma). The explants were incubated at 22 °C and 16-h light (100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)/8-h dark period. Once the cultures were transferred to 100 mm $\times$ 20-mm petri dishes, they were immediately sealed tightly with Parafilm® (Fisher Scientific) to prevent desiccation and microbial contamination. This step was repeated in subsequent cultures throughout the experiment. The drawback of such action,

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however, is the lack of adequate gas exchange leading to excessive ethylene accumulation, which may be detrimental to culture growth. After 4 weeks, induced callus in each petri dish was transferred to the same but freshly prepared callus proliferation medium. This step was performed three to four times every 4 weeks to select and proliferate friable callus.

In vitro liquid culture/primary embryo induction. Approximately 3 g of friable callus was transferred to 125-mL flasks containing liquid media (70 mL/flask). The liquid media consisted of MS basal salts, B5 vitamins, 0.6 μM thidiazuron (TDZ), and 8.9 μM 6-benzylaminopurine (BA), 20 $\text{g}\cdot\text{L}^{-1}$ sucrose, 0.2 $\text{g}\cdot\text{L}^{-1}$ myo-inositol, 1 $\text{g}\cdot\text{L}^{-1}$ casein hydrolysate, and 1 $\text{mg}\cdot\text{L}^{-1}$ thiamine. The liquid media comprised of different treatments consisted of various SA (0, 25, 50, 75, 100, 125, 150 μM) and AgNO_3 (0, 10, 20, 30, 40, 50, 60 μM) concentrations (Sigma). The cultures were placed on an orbital shaker at 22 °C and agitated at 110 rpm for 4 weeks.

Primary somatic embryo development. After the suspension culture step, the embryogenic calli (≈ 0.5 g of embryogenic callus in each plate) were transferred to 100 mm $\times$ 20-mm petri dishes containing SE development media consisting of different treatments of SA and AgNO_3 concentrations. After 4 weeks, somatic embryogenesis response was recorded by counting the number of SEs per gram of callus developed in each plate. The SE development media were similar to the basic liquid media described previously, except that they also contained 0.75 $\text{g}\cdot\text{L}^{-1}$ MgCl_2 and 2 $\text{g}\cdot\text{L}^{-1}$ of Gelrite.

Secondary embryo induction. In a second set of experiments, induction of SSEs was undertaken essentially following a procedure described by Kumar et al. (2007). Briefly, previously developed SEs (100 mg) were subjected to secondary somatic embryogenesis by transferring them to the same SA and AgNO_3 treatments as described previously but containing half-strength MS macronutrients and micronutrients and half-strength B5 vitamins supplemented with 0.6 μM TDZ, 8.9 μM BA, 20 $\text{g}\cdot\text{L}^{-1}$ sucrose, 0.2 $\text{g}\cdot\text{L}^{-1}$ myo-inositol, 1 $\text{g}\cdot\text{L}^{-1}$ casein hydrolysate, 1 $\text{mg}\cdot\text{L}^{-1}$ thiamine, 0.75 $\text{g}\cdot\text{L}^{-1}$ MgCl_2 , and 2 $\text{g}\cdot\text{L}^{-1}$ Gelrite.

Plant regeneration and acclimatization. Plantlets were transferred from the petri dishes to 75-mL (98.5 mm $\times$ 59 mm) culture vessels containing hormone-free MS basal medium supplemented with 20 $\text{g}\cdot\text{L}^{-1}$ sucrose, 0.75 $\text{g}\cdot\text{L}^{-1}$ MgCl_2 , and 2 $\text{g}\cdot\text{L}^{-1}$ Gelrite. The plants were kept in a growth room at 22 °C with a photoperiod of 16 h light (100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 8 h dark. Most of the plants transferred to the vessels produced clumps of rooted multiple shoots. The clumps were divided into separate rooted plants, washed gently with tap water to remove residual callus or gelling agent, planted in premoistened Jiffy-7 peat pellets (Jiffy Products, Ltd., Shippagan, Canada), and covered with clear plastic bags. After 3 d, holes were punched in the plastic bags to allow for gradual acclimatization of the plants before transplanting in the greenhouse after 4 to 6 weeks.

Experimental design and statistical analysis. The experimental design was a completely randomized design comprised of different treatments of various SA (0, 25, 50, 75, 100, 125, 150 μM) and AgNO_3 (0, 10, 20, 30, 40, 50, 60 μM) concentrations. Each plate was weighed before the transfer of embryogenic callus and weighed again 4 weeks later to determine the relative growth rate of the embryogenic callus for each treatment. There were 20 replicates (plates) per treatment, and the experiment was repeated three times. Data collected included the relative growth rate calculated as a percentage of the initial embryogenic callus transferred in a plate and the number of SEs and SSEs per gram after 30 d in culture. The treatment means were calculated using SAS Institute (2003) software.

Results and Discussion

Effect of silver nitrate and salicylic acid on callus growth. Callus formation was observed within 2 to 3 weeks after the transfer of leaf explants to the callus initiation and proliferation medium. SEs (Fig. 1A) could be obtained in ≈ 30 d after transfer of embryogenic callus to the SE development media in all treatments, and plantlet formation could be observed within 2 weeks (Fig. 1A–B). Many plantlets developed roots within this period. Once transferred to culture vessels containing hormone-free MS basal medium, both rooted and nonrooted plantlets developed very quickly and were generally ready for acclimatization in premoistened Jiffy-7 pellets (Fig. 1C). After 4 weeks, callus growth taken after the primary SE development stage reached its maximum (362% increase) after transfer of initiated callus to the control medium (0 μM AgNO_3) before decreasing to only 281% in the medium containing the highest AgNO_3 concentration (60 μM) (Fig. 2A). There seems to be no consensus as to the role or effect of AgNO_3 in callus growth because this effect appears

to be species- or genotype-dependent. For example, AgNO_3 had no effect on callus production of *Zea mays* L. (Vain et al., 1989), whereas it improved callus growth in common wheat (*Triticum aestivum* L.) (Wu et al., 2006). On the other hand, our results are in agreement with those of Tsao and Reed (2002) who reported that increased AgNO_3 concentrations significantly reduced callus production in blackberry (*Rubus* spp.). A similar trend was observed with the addition of SA to the media because higher SA concentrations resulted in reduced callus growth rate, ranging from 358% for the control (0 μM) to 305% for the highest SA concentration (150 μM) (Fig. 2B). As concentration increased, the downward trend for callus growth was more consistent for SA than for AgNO_3 (Fig. 2A–B). There was a zigzag pattern for callus growth for AgNO_3 concentrations between 0 μM (control) and 40 μM (Fig. 2A). The reason for this is unclear, but the downward trend in callus growth became more straightforward steady for AgNO_3 concentrations above 40 μM . Excessive callus proliferation is not generally conducive to somatic embryo initiation and development. The enhanced somatic embryogenesis resulting from the inclusion of SA and AgNO_3 in the media and the subsequent reduced callus growth is not surprising.

Effect of silver nitrate and salicylic acid on primary somatic embryo formation. Addition of AgNO_3 was beneficial to the formation of primary SEs of *H. bousigonianum* (Fig. 3A). This positive effect of AgNO_3 is consistent with previous reports (Beyer, 1976; Kumar et al., 2007). The highest SE production was reached at 40 and 50 μM AgNO_3 with 86 and 88 SEs/g, respectively, compared with 30 SEs/g for the control. The higher concentration of 60 μM AgNO_3 resulted in reduced SE production, but even at this concentration, production of SEs remained higher than that of control SEs (47 ± 0.7 versus 30 ± 0.4) (Fig. 3A). The

Fig. 1. Somatic embryogenesis and plant regeneration in *Hedychium bousigonianum*. (A) (75 μM SA) Development of embryogenic callus and somatic embryos. The basic embryo development medium contained Murashige and Skoog macronutrients and micronutrients (Murashige and Skoog, 1962) and B5 vitamins (Gamborg et al., 1968) supplemented with 0.6 μM thidiazuron, 8.9 μM 6-benzylaminopurine, 20 $\text{g}\cdot\text{L}^{-1}$ sucrose, 0.2 $\text{g}\cdot\text{L}^{-1}$ myo-inositol, 1 $\text{g}\cdot\text{L}^{-1}$ casein hydrolysate, 1 $\text{mg}\cdot\text{L}^{-1}$ thiamine, 0.75 $\text{g}\cdot\text{L}^{-1}$ MgCl_2 , and 2 $\text{g}\cdot\text{L}^{-1}$ Gelrite. (B) (30 μM silver nitrate) Somatic embryo development and subsequent plantlet formation. (C) (30 μM AgNO_3) Plantlets with well-formed roots ready for potting and acclimatization.

Fig. 2. Effect of silver nitrate (A) and salicylic acid (B) on callus growth (percent increase from initial starting callus weight) in *Hedychium bousigonianum*. Each value represents mean $\pm$ SE. Results are from 20 replicates, and the experiment was repeated three times.

addition of SA to the media also had a marked ameliorative effect on SE production (Fig. 3B). The best concentration was 100 μ M SA, which produced 96 ± 0.9 SEs/g of initial callus transferred. This represents a 3.8-fold improvement over the control (25 ± 0.6 SEs/g) (Fig. 3B). On the other hand, reduction in embryo production was observed at the 150 μ M SA level, suggesting an inhibitory effect of high SA concentrations on SE production. However, it is worth mentioning that even at this level, SE pro-

duction was twice that of the control (Fig. 3B). Inhibitory effects of higher SA concentrations have been reported in other crops. For example, Luo et al. (2001) reported a decrease in somatic embryogenesis for concentrations above 200 μ M with *Astragalus adsurgens* Pall. Roustan et al. (1989) found that a 50 μ M concentration of SA was optimal for stimulation of embryo production in carrot (*Daucus carota* L.) suspension cultures but that 250 μ M SA concentration had a toxic effect. In our study, we did not observe

any toxic effect or necrosis using higher concentrations of either SA or AgNO_3 on somatic embryogenesis of *H. bousigonianum* despite a slight reduction in SE production at higher concentrations.

Effect of silver nitrate and salicylic acid on secondary somatic embryo. SSE production followed a similar pattern as that for SE induction, except that significantly more SSEs were produced than SEs with media containing both SA and AgNO_3 (Fig. 3A–B). For AgNO_3 , the best media were those containing 30 and 40 μ M with averages of 212 ± 1 and 225 ± 1.3 SSEs/g, respectively (Fig. 3A). For SA, the 75 and 100 μ M SA concentrations were the best performing media with 285 ± 1.4 and 283 ± 1.7 SSEs/g, respectively. For both SE and SSE production, the SA-containing media generally performed better than AgNO_3 -containing media (Fig. 3A–B). The current study using ethylene inhibitors demonstrated that secondary somatic embryogenesis is an efficient method for multiplication of SEs in *H. bousigonianum*, and plantlets could be obtained within 4 to 8 weeks. Such systems could be very useful for transformation studies or other in vitro genetic manipulations such as polyploidization. The system reported here could be particularly useful for mass propagation of this ornamental species. In addition, SEs may be encapsulated in various gelling agents to form artificial seeds that can be easily stored and transported long distances (Ghosh and Sen, 1994), a feature that can be very useful for a convenient and safe exchange of germplasm. SSEs result from the initiation and formation of new SEs from already formed primary SEs. Advantages of such a regeneration system include a high multiplication rate, independence of an explant source, reproducibility, and maintenance of embryogenicity for long periods of time through repeated cycles of secondary embryogenesis (Karami et al., 2008; Raemakers et al., 1995).

Conclusion

Higher concentrations of the ethylene inhibitors studied reduced callus growth, but there was a marked improvement in SE and SSE production after the addition of SA (75 and 100 μ M) and AgNO_3 (30 to 50 μ M) to the culture media. Overall, more SEs and SSEs were produced using the ethylene inhibitor SA than AgNO_3 . Neither SA nor AgNO_3 is essential for somatic embryo initiation in *H. bousigonianum*. However, the results clearly indicate that significant increases in SE and, notably, SSE production occurred in the presence of SA and AgNO_3 in culture media. In summary, we have established an efficient micropropagation system based on high-frequency SE production that can be useful not only for in vitro genetic manipulations, but also for industrial mass production of *H. bousigonianum*.

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Fig. 3. Effect of silver nitrate (A) and salicylic acid (B) on somatic embryogenesis (number of embryos/g callus) and secondary embryo development (number of secondary embryos/g of primary SE) of *Hedychium bousigonianum*. Each value represents mean $\pm$ SE. Results are from 20 replicates, and the experiment was repeated three times.

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