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MODULATION OF SOMATIC EMBRYOGENESIS IN HYPOCOTYL-DERIVED CULTURES OF GERANIUM (*PELARGONIUM X HORTORUM* BAILEY) CV RINGO ROSE BY A BACTERIUM

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SUMMARY

The Ringo Rose cultivar of zonal geranium (*Pelargonium x hortorum* Bailey) has been shown to be morphogenetically unresponsive. Attempts to improve somatic embryogenesis using various seed stress treatments before germination proved ineffective. However, bacterial contamination of one of the seed-stress treatments led to infected explants that had a significant increase in frequency of high-quality somatic embryos. The co-cultivation of explants with the isolated bacterium (tentatively identified as *Bacillus* sp.) was found to be repeatable, and potentially represents a novel way to improve morphogenesis in geranium and possibly other species.

Key words: geranium; *Pelargonium;* somatic embryogenesis; bacterium; co-cultivation.

INTRODUCTION

Somatic embryogenesis is an efficient tool for rapid propagation of horticultural crops (Redenbaugh et al., 1987). The ability to produce somatic embryos, coupled with the development of synthetic seeds, may offer tremendous benefits to high-value ornamental crops such as geranium (*Pelargonium x hortorum*). Regeneration of plants via somatic embryogenesis in seed-propagated *Pelargonium* has been reported only recently (Marsolais et al., 1991), although stages of embryo development were observed earlier in callus cultures derived from petiole explants (Cassells 1979). Marsolais et al. (1991) found significant variation for somatic embryo production among 30 diploid *P. x hortorum* genotypes in response to auxin and cytokinin-supplemented media. The number of somatic embryos induced ranged from 6.9 embryos/explant for the most responsive variety Scarlet Orbit Improved to 0.2 for the cultivar Ringo Rose, indicating that embryogenic capacity was under genetic control.

A variety of stress treatments applied to the source tissue have resulted in improved morphogenic potential of carrot, perhaps due to the creation of competence (Kiyosue et al., 1990). Our efforts to enhance somatic embryogenesis in the cultivar Ringo Rose using seedlings exposed to wet, dry, and variable temperature conditions met with little success. Interestingly, however, a bacterial contaminant in one of the treatments was associated with the hypocotyl explants that produced an unusually high number of somatic embryos. In this communication we describe the isolation of a growth-promoting bacteria, as well as the modulation of somatic embryogenesis and regeneration of whole plants from cultures of the recalcitrant cv Ringo Rose after inoculation with the isolated bacterium.

MATERIALS AND METHODS

Induction of somatic embryogenesis. Seeds of the diploid zonal geranium *Pelargonium x hortorum* Bailey cv Ringo Rose (Stokes Seed Co., St. Catharines, Ontario) were surface sterilized by dipping in 95% ethanol for 30 s and then immersing them in 30% (vol/vol) Sanitizing Sodium hypochlorite (Lilo Products, Hamilton, Ontario) containing Tween 20 (20 drops per liter). Seeds were agitated in this solution for 20 min and then rinsed 3 times with sterile distilled water. Ten seeds were aseptically cultured in 100 × 15-mm petri dishes containing 30 ml of 0.85% water-agar (Sigma, St. Louis, MO, purified agar in distilled water). The dishes were sealed with Parafilm and incubated in darkness at 24° C. Hypocotyl explants were excised from 6-day-old seedlings by removing shoot and root apices. Nine hypocotyl segments, approximately 8 mm long, were cultured in 100 × 15-mm petri dishes on 30 ml of MS (Murashige and Skoog, 1962) medium amended with various supplements.

Thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea; TDZ; 0.2–2.0 μM) was the sole growth regulator in the initial experiment for culturing the hypocotyl explants. In subsequent experiments to induce somatic embryogenesis, TDZ at 0.4 μM was used in combination with 0, 0.25, 0.5, or 1.0 μM indole-3-acetic acid (IAA). In a separate experiment, 0.4 μM TDZ was used in combination with 0, 0.5, 1.0, 2.5, or 10.0 mM K_2SO_4. Four replications were used per treatment. All hypocotyl cultures were incubated at 25° C with a 16-h photoperiod (30 to 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$; cool white fluorescent tubes).

Seed stress treatments. Seeds were subjected to a number of stress treatments. Sterilized seeds were placed in petri dishes for 1 to 3 days on the following: a) filter paper disks which had been soaked in 20% polyethylene glycol (PEG) solutions; b) filter-paper disks which had been soaked in sterile, distilled water; or c) dry filter-paper disks with subsequent exposure to either warm (37° C), cold (4° C), or room temperature (25° C). The seeds, treated as above, were then placed on 0.85% water agar for germination. After 6 days, hypocotyl explants were prepared as described above and cultured on MS medium amended with 0.4 μM TDZ.

Bacterial media. Bacterium was isolated from the infected explants and cultured on the following two media: a) MS-yeast extract medium, 4.33 $g \cdot liter^{-1}$ MS salts and vitamins, 20.0 $g \cdot liter^{-1}$ sucrose, 0.1 $g \cdot liter^{-1}$ yeast extract. Medium pH was adjusted to 5.5 and 3.0 $g \cdot liter^{-1}$ gelrite was added prior to autoclaving. b) Bacto-peptone medium, 10.0 $g \cdot liter^{-1}$

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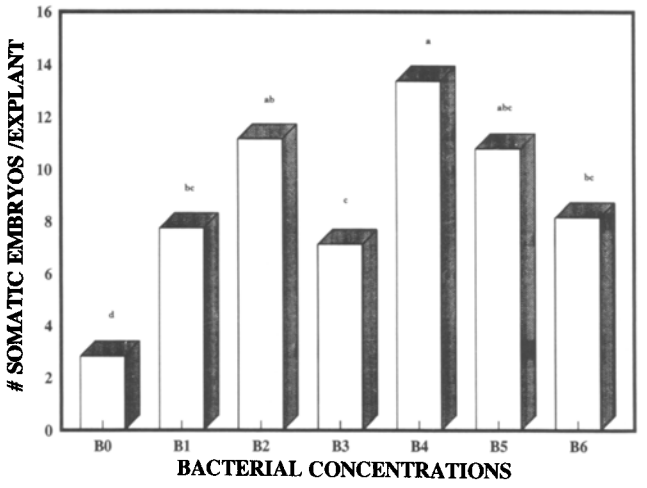

FIG. 1. Somatic embryogenesis in hypocotyl-derived cultures of geranium (*Pelargonium x hortorum* Baily) in response to co-cultivation with various concentrations of bacterial inoculum on TDZ-supplemented medium. *B0*, control (no bacterium); *B1*, 0.47×10^8 CFU/ml; treatments *B2* to *B6*, serial dilutions prepared with bacterial suspension at a density of 0.47×10^8 CFU/ml and sterile distilled water (1:10). *Bars* represented by the same letters are not significantly different from each other at $P = 0.05$.

Bacto-peptone, 10.0 g·liter^{-1} GIBCO-yeast extract, with or without 2.0 g·liter^{-1} sodium chloride with a pH of 6.95. For solid medium, 15.0 g·liter^{-1} phyto-agar was added before autoclaving.

Explant infection with bacterial suspension. A bacterial suspension was prepared by placing a small amount of the bacterium cultured on solid bacto-peptone medium in a 125-ml flask containing liquid bacto-peptone medium and grown at 37° C for 48 h. Explants were prepared and cultured on TDZ (0.4 μM)-amended MS medium as described previously. Different inoculum densities (0.47×10^8 colony forming units (CFU)/ml and serial dilutions) used for explant infection are shown in Fig. 1. Dilution series was prepared by mixing the bacterial suspension and sterile distilled water (1:10). The number of CFU/ml for the treatments B1-B6 were 0.47×10^8 to 0.47×10^3. A volume of 10 μl from the bacterial suspension was distributed over each explant.

Four replicates were prepared for each treatment, with 24 explants per treatment. The experiments were arranged in a completely randomized design. Counts of somatic embryos were determined from an overhead view 14 and 28 days after hypocotyl culture. Any somatic embryos underneath the explant were obstructed from view and were not included in the results. Data were analyzed using SAS GLM and means separated using a protected LSD ($P = 0.05$).

Conversion of somatic embryos to plants. Somatic embryos induced on the explants co-cultivated with bacteria and on control explants were converted to plantlets by culturing them on MS medium lacking growth regulators. The pH of the medium was adjusted to 5.5, and 0.3% gelrite was added and dissolved with heating before being dispensed to magenta boxes and autoclaved. Explants (cultured for 6 to 8 wk on TDZ medium) bearing somatic embryos were cut into 0.5-cm sections and four to six explant sections were placed in each magenta box. Visual observations were recorded after approximately 2 mo., and regenerated plantlets were transferred to soil in the greenhouse.

RESULTS

Response to TDZ. Explants cultured on media supplemented with 0.2 to 0.8 μM TDZ enlarged and produced somatic embryos after 4 wk. Concentrations higher than 1.0 μM were lethal to explant survival and somatic embryo development. A maximum of four embryos per explant were produced after 4 wk of culture with 0.4 μM TDZ compared to none in the absence of TDZ. The embryos were generally light-green, poorly differentiated, and a high percentage (>90%) of them did not develop beyond the globular stage. Whole plant regeneration from these poorly organized somatic embryos was inconsistent. If explants bearing somatic embryos were allowed to remain in culture for more than 8 wk, they became disorganized and developed callus or produced abnormal structures. Supplementation of culture medium with IAA (0.1 to 1 μM) or potassium sulphate (1 to 10 mM) did not improve the embryogenic response (data not shown).

Response to seed stress treatments. Seeds were subjected to a number of stress treatments with the aim of increasing embryo yield by preconditioning the source seedling. Sterilized seeds were exposed to hot, cold, wet, or dry conditions for 1 to 3 days before germination on water agar (*see* Methods). Hypocotyl explants were prepared from the resulting seedlings and cultured on MS medium amended with 0.4 μM TDZ.

Stress treatments given to the seeds proved ineffective for increasing the frequency of embryogenesis. However, one of the treatments (placing seeds for 3 days on filter-paper disks soaked in sterile, distilled water) was found to be contaminated with a bacterium, presumably as a result of improper sterilization of the distilled water. The bacterium grew very slowly on the MS medium, forming a white halo around the explants. Many infected explants were brown, shrivelled, and dying, but some that were less heavily contaminated were relatively green and displayed an increased frequency of well-differentiated embryos over all other treatments.

Response to co-cultivation with isolated bacterium. Bacterium surrounding the explants was isolated and cultured on MS-yeast extract or bacto-peptone medium and used for further experimentation. In the absence of bacterium, hypocotyl sections enlarged and developed globular embryos in 2 to 4 wk on TDZ-supplemented medium (Fig. 2*A*). No signs of growth and differentiation were observed in explants cultured on basal medium (Fig. 2*B, left*). Co-cultivation of explants with bacterial suspension and their culture on a medium lacking TDZ caused swelling and callusing, or root formation or both (Fig. 2*B, right; arrows*). Somatic embryos were induced on explants co-cultivated with bacterium on the medium containing TDZ (Fig. 2*C*). The experiment designed to study the relationship between the density of bacterial suspension and embryogenic response revealed that the highest number of somatic embryos was induced with a bacterial concentration of 0.47×10^5 CFU/ml (Fig. 1*B4*). At lower inoculum densities, the embryogenic response decreased significantly but was still higher than the control (Fig. 1*B6*). It should be noted, however, that the data shown in Fig. 1 was taken after 28 days. Somatic embryo production from bacterium-treated cultures continued even after transfer of explants to basal medium, resulting in higher embryo numbers that were difficult to determine because the embryos germinated precociously and formed shoots (Fig. 2*D*). Regenerated shoots continued to grow further and developed plantlets on MS basal medium (Fig. 2*F*). Plantlet formation from somatic embryos developed on control explants, i.e., without co-cultivation with bacterium was inconsistent and limited to only one or two plantlets per explant (Fig. 2*E*). Bacterial growth was checked without the addition of an antibiotic, and the bacterium did not inhibit growth and differentiation of plantlets.

An attempt was made to identify the organism. The bacterium was analyzed using gas chromatography in an automated identification process by Microbial Identification System (MID), which identifies bacteria on the basis of their unique fatty acid profiles. The

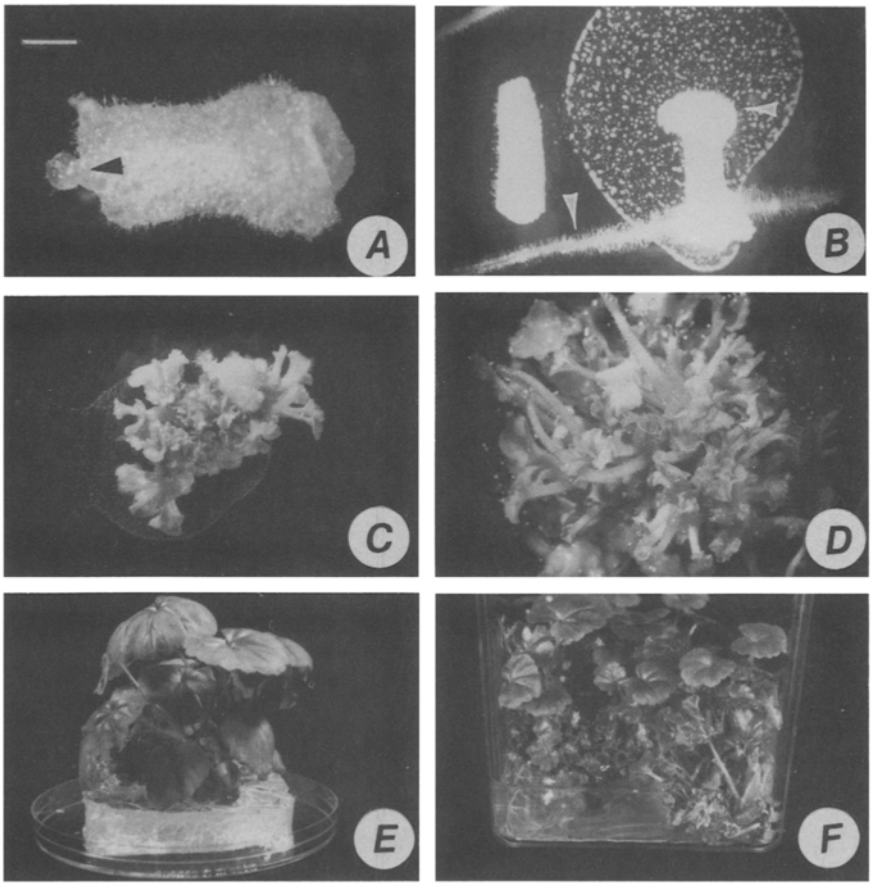

FIG. 2. Somatic embryogenesis in hypocotyl cultures of geranium (*Pelargonium x hortorum* Bailey) in response to bacterial co-cultivation. *A*, control explant cultured on 0.4 μM TDZ (4 wk). *Note* the presence of only a few somatic embryos (*arrow*). *B*, explants cultured on basal MS medium (4 wk). The one on the *right* was inoculated with bacteria (*note* swelling and root formation) whereas the explant on the *left* was not. *C*, somatic embryos induced on infected explant in the presence of 0.4 μM TDZ (4 wk). *Note* the presence of the bacterium around the explant. *D*, further development of somatic embryos on the explant cultured on 0.4 μM TDZ and infected with bacterium (6 wk). *E*, plantlet formation on MS medium from non-infected explant (10 wk). Compare plantlet number with *F*. *F*, multiple plantlets formed from somatic embryos induced on infected explants (10 wk). *Bar* in *A* = 2.0 mm; *B-D* = 4.0 mm; *E* = 17.0 mm; *F* = 12.5 mm.

results of the analysis (Microbial ID, Inc., Newark, DE; University Hospital, London, Ontario) suggested that the organism in question belongs to the genus *Bacillus*. It was not possible to obtain species-level identification.

DISCUSSION

Although somatic embryos can be produced in many plants, it remains difficult to develop embryos at a high frequency with some species (Thorpe 1988). Regeneration in diploid geranium (*Pelargonium x hortorum* cv. Scarlet Orbit Improved) via somatic embryogenesis was first achieved in hypocotyl-derived cultures induced with an auxin (IAA or PAA) and a cytokinin (BAP) (Marsolais et al., 1991; Slimmon et al., 1991). Later, we demonstrated that the substitution of the auxin-cytokinin complement of the induction medium with TDZ significantly improved somatic embryo production (Visser et al., 1992). Somatic embryos developed from the subepidermal layers and various stages of embryonic development (i.e., globular, heart-shaped, and cotyledonary) were observed in hypocotyl cultures as well as those derived from thin cell layer explants (Visser et al., 1992; Gill et al., 1993). Further, the TDZ-induced somatic embryos of geranium could be developed into artificial seed by encapsulating them in alginate hydrogel, stored at 4° C, and later converted to plants (Gill et al., 1994).

However, the induction of somatic embryogenesis from the Ringo Rose cultivar of geranium with TDZ was inefficient: the highest number of embryos per explant was less than four and embryo conversion to plantlets was inconsistent. These results are not surprising when one considers that Ringo Rose was the least responsive of the 30 *P. x hortorum* varieties tested on BAP/IAA-supplemented medium with a mean of 0.2 embryos per explant (Marsolais et al., 1991). It was concluded that Ringo Rose cultivar was genetically restricted in its ability to yield large numbers of embryos. The potential for somatic embryogenesis has been reported to be genotype-dependent in a number of species including alfalfa (Brown, 1988), corn (Hodges et al., 1986), clover (Keyes et al., 1980), soybean (Barwale et al., 1988), and wheat (Ou et al., 1989). In previous reports on *Pelargonium* (Pillai and Hildebrandt, 1968;

Theiler, 1977; Dunbar and Stephens, 1989), differences among genotypes for in vitro shoot regeneration potential and nutritional requirements have been reported.

A growth-promoting bacterium was isolated from infected Ringo Rose explants that produced somatic embryos which could be converted to plantlets. Co-cultivation of explants with isolated bacterium improved the induction of somatic embryos and their conversion to plantlets, and the promotive effect of co-cultivation was reproducible. Enhanced somatic embryogenesis in our experiments by co-cultivation with bacterium (Fig. 1) suggests that Ringo Rose is indeed capable of embryogenesis and that apparent genetic unresponsiveness may be modulated by a favorable cultural environment. Co-cultivation of explants with the bacterium may have increased the embryogenic competence due to the elicitation of a plant stress response by interacting at the cell wall or membrane or by secreting substances such as plant growth regulators, nutrients, vitamins, or enzymes. It is also possible that the bacterium acts by causing changes in medium pH and the uptake of nutrients.

Co-cultivation of explants with bacterium on a medium lacking growth regulators resulted in callusing or root formation or both at the cut ends of the explants, but failed to induce somatic embryos. Obviously, the bacteria alone is not able to elicit an embryogenic response but requires the presence of a growth regulator, in this case TDZ, for manifestation of its activity. The occurrence of callusing and rhizogenesis suggests that auxin(s) may be involved. One could speculate that in combination with an exogenous cytokinin (TDZ), proper hormonal ratios are established which permit the expression of the embryogenic response. Many microorganisms are known to produce or destroy growth regulators, or both. Plant pathogens may produce more of the same growth regulators or inhibitors of the growth regulators as those produced by the plant; they may produce new and different growth regulators or inhibitors of growth regulators; or they may produce substances that stimulate or retard the production of growth regulators or growth inhibitors by the plant (Agrios, 1988). Recently, Wake et al. (1991, 1992) reported the promotion of carrot somatic embryogenesis by hot-water extracts containing low and high molecular weight substances, possibly including plant growth regulators, polyamines, oligosaccharides, or polypeptides, prepared from 21 strains of marine cyanobacteria.

The bacterium was tentatively analyzed as belonging to the genus *Bacillus*, but so far it has not been possible to obtain species-level identification. Research is in progress in our laboratory to further identify the bacterium and to characterize its mode of action in the modulation of somatic embryogenesis. Regardless of the nature and the mechanism of action, co-cultivation of explants with bacteria resulted in a significant and reproducible improvement in TDZ-induced embryogenesis over the control. Thus, the system offers potential for other unresponsive cultivars of *P. x hortorum* and possibly for other recalcitrant plant species.

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