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Induction of high-frequency somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey cv Ringo Rose) cotyledonary cultures

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Summary. The cv Ringo Rose of hybrid seed geranium (*Pelargonium x hortorum* Bailey), previously shown to be recalcitrant in culture, produced somatic embryos when cotyledonary explants were cultured on regeneration medium containing thidiazuron (TDZ), forchlorfenuron (CPPU), or a combination of indole-3-acetic acid and N^6-benzylaminopurine (IAA+BAP). Amendment of the basal medium with TDZ (0.5 μM) was the most effective treatment. Addition of amino acids to the medium promoted the growth of somatic embryos. Retention of the proximal region of the cotyledon was crucial for regeneration, but the removal of the distal 1/3 to 1/2 cotyledon had no significant effect on somatic embryogenesis. Cotyledonary explants formed somatic embryos in higher frequency and much earlier than hypocotyl explants cultured on the same medium. The somatic embryos induced on cotyledonary explants were germinated on basal medium. More than 70% of the somatic embryos were converted into plants and transferred to soil

Abbreviations: BAP, N^6-benzylaminopurine; CPPU, N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron); IAA, indole-3-acetic acid; TDZ, N-phenyl-N'-1,2,3,-thiadiazol-5ylurea (thidiazuron).

INTRODUCTION

In vitro propagation using somatic embryos has been viewed as a commercially viable alternative for large-scale, rapid multiplication of several ornamental species including geraniums. Development of an efficient somatic embryogenic system to produce high-quality, vigorous somatic embryos with high conversion frequency is prerequisite to artificial seed technology for mass propagation. Methods to obtain regenerants from different explants of geranium (*Pelargonium sp.*) have been developed over the years (Chen and Galston 1967; Pillai and Hildebrandt 1968, 1969; Horst *et al.* 1976; Cassells 1979; Dunbar and Stephens 1989), but the induction of somatic embryogenesis was reported only recently for seed and vegetatively propagated species of *Pelargonium* (Marsolais *et al.* 1991; Visser *et al.* 1992; Gill *et al.* 1993). In an attempt to develop artificial seeds of geranium, Gill *et al.* (1994) encapsulated somatic embryos induced with thidiazuron using sodium alginate. In these studies, a suitable source of plant tissue for inducing embryogenesis was found to be hypocotyl sections obtained from *in vitro* grown etiolated seedlings. However, the regeneration protocols were limited to specific genotypes; the inductive stimuli identified for a particular cultivar could not be successfully exploited for other cultivars of the same species. For example, *Pelargonium X hortorum* cv Ringo Rose was shown to express poor embryogenic potential as compared to another cultivar, Scarlet Orbit Improved (Marsolais *et al.* 1991).

We have recently shown that cotyledons play a regulatory role in the process of somatic embryogenesis of peanut, and the retention of cotyledons on the embryonic axis was essential to induce somatic embryo development (Murthy and Saxena 1994; Murthy *et al.* 1995). Similarly, in several other species belonging to both monocots and dicots, cotyledonary tissue was identified to be highly regenerative (Tepper and Mante 1990). Hence, the objective of the present study was to evaluate regeneration potential of cotyledonary tissue of geranium cv Ringo Rose, and also to test whether there is any specific site of regeneration along the cotyledons. In this report we demonstrate that cotyledons from young seedlings of geranium cv Ringo Rose regenerate rapidly and at a high frequency on a nutrient medium supplemented with the growth regulator thidiazuron.

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MATERIAL AND METHODS

Seeds of diploid Zonal geranium *(Pelargonium X hortorum* Bailey cv Ringo Rose) were obtained from Stokes Seed Co., St. Catharines, Ont., Canada. Seeds were immersed for 30 s in 70% (v/v) ethanol, surface sterilized for 20 min with 1.5% sodium hypochlorite solution with a drop of Tween-20 per 100 ml, and then rinsed 5 times with sterile, distilled water. During the course of the sterilization treatments, the seeds were agitated periodically. Sterilized seeds (10 per plate) were cultured in Petri dishes (100 X 15 mm) containing 25 ml of 0.8% water-agar (Sigma, St. Louis, Mo; purified agar in distilled water). Dishes were incubated in the dark at 24°C after sealing them with Parafilm.

Cotyledon and hypocotyl explants were excised from 6 day-old seedlings. For preparing explants, the cotyledons were separated from the embryonal axis at the attachment point, taking care to avoid the inclusion of cells of the shoot axis and cotyledonary node; thus, only a portion of the petiole and entire cotyledonary lamina were included. In one of the experiments, either the proximal or distal 1/3 to 1/2 of the cotyledon was removed and the explants were tested for their embryogenic potential. Hypocotyl segments, approximately 0.8 cm long, were obtained from the same seedlings for comparative studies. Hypocotyl or cotyledon explants were cultured (6 per plate) in Petri dishes each containing 25 ml of the culture medium. The culture medium consisted of MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968), 30 g L^{-1} sucrose and amended with various growth regulators. The medium was adjusted to pH 5.5 before autoclaving at 1.04 $Kg.cm^{-2}$ for 20 min. Gelrite (Scott Laboratories, Carson, USA) at 3 g L^{-1} was used as gelling agent. The growth regulators added to the basal medium were 1μM IAA+2μM BAP; 1μM IAA+8μM BAP; 0.1, 0.5, 1.0, 2.0 μM TDZ or CPPU. In one of the experiments, the cotyledon explants were also cultured on medium supplemented with amino acids, viz., Glutamine, Proline and Methionine (0.1, 1.0 or 5.0 mM) in the presence of 0.5μM TDZ to study their stimulatory role in morphogenesis.

Cotyledon explants were not oriented in any particular manner with respect to the surface to the medium as there were no differences in the response to differential orientation. However, the surface of the cotyledon explants was always in complete contact with the medium. Petri dishes were sealed with Parafilm and incubated at 24°C under 16 h photoperiod (30-35 μmol $m^{-2}s^{-1}$) provided by cool-white fluorescent tubes (Philips Canada, Scarborough, Ont., Canada). Each treatment consisted of 4 replications (Petri dishes) and all the experiments were repeated at least once. Counts of somatic embryos were made using stereo microscope, 4 weeks after culture initiation. Data were analyzed using analysis of variance (Proc GLM of PC SAS version 6.0) and means comparison was made by Least Significant Difference (LSD) test at 5% level of probability.

For regeneration of plants, the somatic embryos were isolated individually and germinated in Petri dishes containing basal medium. After 2 weeks of growth, the plantlets were transferred to Magenta culture boxes (Magenta Corp., Chicago, Ill., USA) containing 50 ml of the basal medium, prior to acclimatization and planting in the growth chamber.

RESULTS

The cotyledon explants expanded rapidly and began to form callus at the cut end of the petiole within 5 - 7 d when cultured on regeneration medium containing either TDZ, CPPU or IAA+BAP. In all cultures, the cotyledons acquired green colour (originally yellowish) within 24 h when exposed to light. The explants increased in size, 8 - 10 fold, in about 15 d and thereafter the expansion ceased. Growth initially occurred all over the explant, but later was localized in and around the callused area. Cotyledon explants formed somatic embryos much earlier than the hypocotyl explants cultured on the same medium. Somatic embryos first emerged as small protuberances mostly at the cut end of the petiole and they rapidly enlarged into conspicuous structures (Fig. 1). In all cultures, the development of somatic embryos progressed through typical stages of embryo development viz., globular, heart-shaped, torpedo-shaped and cotyledonary stages as perviously documented by Gill *et al.* (1993) for cv. Scarlet Orbit Improved. The somatic embryos were isolated and cultured on basal medium, and more than 70% of the somatic embryos converted into plants.

Somatic embryos originated from the petiole cells immediately adjacent to the callus, as well as a small proportion of them developed from the callus which formed at the cut end. Embryos often occurred in groups or clusters. Somatic embryos were observed only at the cut end of the petiole when the cotyledon was still intact. However, when the petiole was isolated from the cotyledon, and cultured separately, somatic embryos formed at both the ends (Fig. 2) and a few were occasionally seen in the middle region as well. Removal of the proximal region of the cotyledon had a detrimental effect on regeneration potential of the cotyledonary tissue, but removal of the distal 1/3 to 1/2 cotyledon had no significant effect on the frequency of somatic embryogenesis (data not presented). This suggests that there exists a gradient of regeneration potential from the proximal to the distal region in the geranium cotyledons. The apical meristem was excluded from the cotyledonary explants and, hence, was not responsible for somatic embryo production in culture. Explants with intact shoot apex (2-3 mm) and both cotyledons produced callus at the cut end (opposite to the shoot apex) and formed somatic embryos around the callus. However, in peach tissue culture, Mante *et al.* (1989b) observed no regeneration if a portion of the embryonic axis was included.

Cotyledon explants produced somatic embryos in the presence of TDZ at all concentrations tested in the range of 0.1μM to 2.0μM (Fig. 3), however, the frequency varied. The number of somatic embryos developed at 0.5 and 1.0μM TDZ was higher than that observed with either 0.1 or 2.0μM TDZ treatments. Mante *et al.* (1989a, 1989b) found that IBA was necessary along with TDZ to obtain regeneration from cotyledons of soybean and several temperate crops. But in geranium explant cultures, TDZ as the sole inductive signal induced high-frequency regeneration. The response of cotyledon explants on a modified medium with altered levels of auxins and cytokinins (IAA+BAP) or another phenylurea, CPPU, was also examined in an attempt to optimize the conditions for somatic embryogenesis.

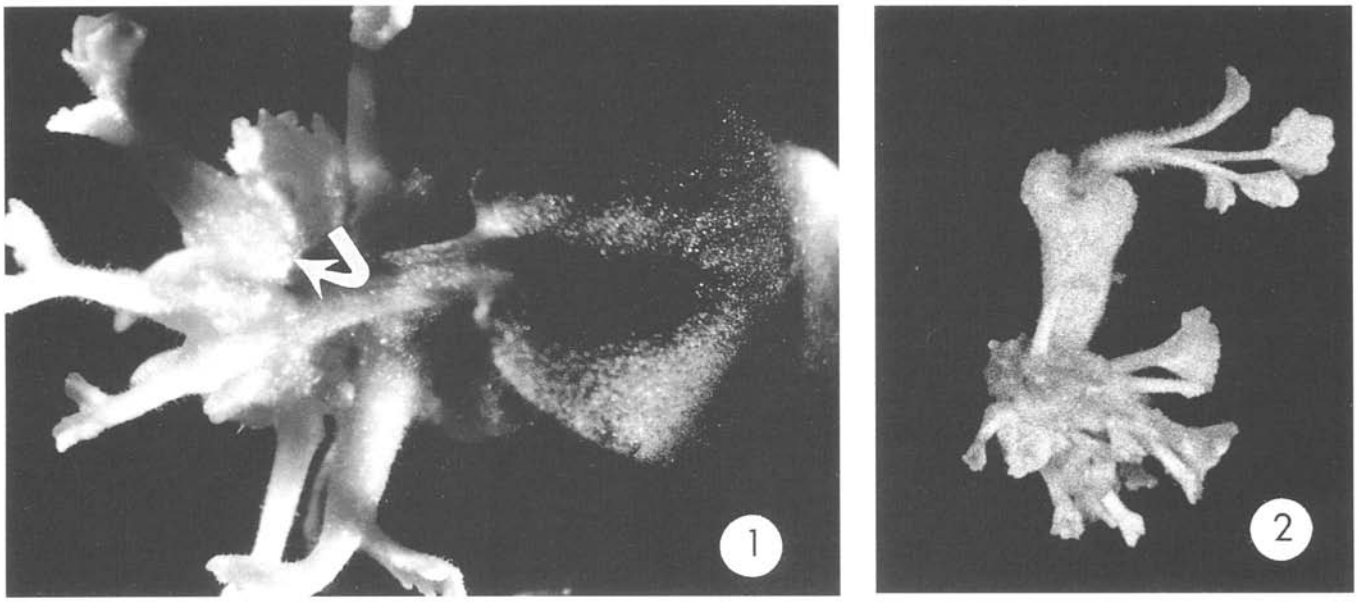

Fig. 1. Somatic embryogenesis in cotyledon cultures of geranium (*Pelargonium x hortorum* Bailey cv Ringo Rose) in response to 0.5 μM TDZ. Note the emergence of somatic embryos (arrow) from the cut end of the petiole. Fig. 2. Emergence of somatic embryos at both cut ends of the petiole explant excised from the cotyledons and cultured for 4 weeks on MS medium supplemented with thidiazuron (0.5 μM).

Although somatic embryos were produced in all these treatments, amendment of the basal medium with TDZ was the most effective treatment. A combination of IAA (1μM) and BAP (2 or 8μM) treatment, previously shown to be a superior treatment for inducing somatic embryogenesis in hypocotyl explants of geranium (Marsolais *et al.* 1991), was least effective. An average of only 1.3 somatic embryos per explant was observed in these treatments. CPPU treatments, although not as effective as TDZ, were better than IAA+BAP treatment. Six to 7 somatic embryos per explant were commonly produced at each of the CPPU concentrations tested.

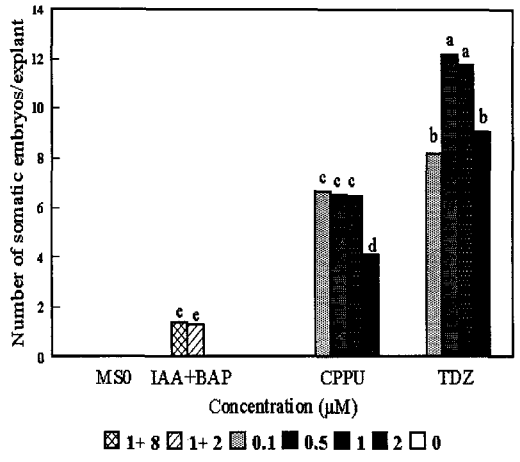

Fig. 3. Mean number of somatic embryos per cotyledon explant of geranium (*Pelargonium x hortorum* Bailey cv Ringo Rose) after 4 weeks of culture on MS medium supplemented with different growth regulators. Bars represented by the same letters are not significantly different from each other at P=0.05.

Hypocotyl explants cultured on medium supplemented with 0.5μM TDZ also enlarged in size and produced somatic embryos. A maximum of only 4 embryos per explant were observed after 4 weeks of culture on TDZ-supplemented medium. 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 differentiated somatic embryos was inconsistent.

Both cotyledon and hypocotyl explants cultured on basal medium in the absence of any exogenous growth regulators never differentiated to produce somatic embryos, but occasionally formed roots. In the case of cotyledon explants roots emerged from the cut end of the cotyledonary petiole, and in hypocotyl explants the rooting was mostly confined to the region next to the cut ends. The failure of explants to develop somatic embryos on basal medium is an indication of the need for the inductive signal, the growth regulators. The explant cells thus have the potential to differentiate into roots or somatic embryos depending on the presence or absence of specific exogenously supplied growth regulators.

None of the amino acids tested viz., glutamine, proline, and methionine, appreciably improved the induction of somatic embryogenesis when compared with the control (TDZ 0.5μM treatment) (Fig. 4). However, with all the amino acid treatments, the growth of the somatic embryos was apparently much better. Small roots were commonly produced from the callused area of the cotyledon explants when the medium was supplemented with proline, and such type of roots were

rarely found on the standard medium (TDZ alone).

The initial growth rate and the frequency of somatic embryo formation was higher in cotyledon explants when compared to hypocotyl sections. The time required to obtain somatic embryos was comparably shorter (2 weeks for cotyledon explants in contrast to 3 weeks for hypocotyl explants), and the embryos of cotyledonary origin grew more synchronously. Hypocotyl segments and cotyledonary petioles, therefore, clearly differed in their response to culture *in vitro*. The reason for an apparent recalcitrance of hypocotyl cells to the same inductive signal which induced high frequency regeneration in cotyledonary tissue is not clear. The morphogenetic potential of explants *in vitro* is influenced by a complex array of factors, both endogenous and exogenous, including the genotype, physiological age of the tissue, size and source of tissue, and their interactions with growth environment. Since the developmental stage of the seedling at the time of excision of the hypocotyl and cotyledonary tissues was the same, the age factor that might contribute for differential response is ruled out in the present case.

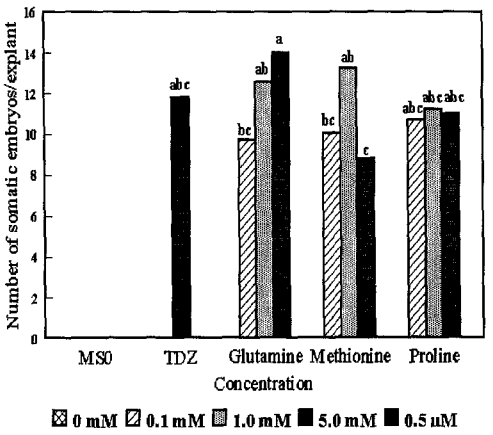

Fig. 4. Mean number of somatic embryos per cotyledon explant of geranium (*Pelargonium x hortorum* Bailey cv Ringo Rose) after 4 weeks of culture on MS medium supplemented with thidiazuron (0.5μM) and different amino acids. Bars represented by the same letters are not significantly different from each other at P=0.05.

The most serious limitation of geranium (cv Ringo Rose) somatic embryogenesis was its poor response in culture. Despite the availability of protocols successfully employed for several *Pelargonium* cultivars, the cv Ringo Rose remained recalcitrant; regeneration response was only improved with the use a growth promotive strain of *Bacillus* (Visser *et al.* 1994). In this report, we demonstrated the potential of cotyledonary tissue to obtain rapid, uniform and high frequency somatic embryogenesis. We have also characterized the response of cotyledons to amino acids commonly known to improve morphogenesis in tissue culture systems. The ability of cotyledonary tissue to produce high frequency somatic embryogenesis in geranium as evidenced in the present study may have a practical value in wide hybridization studies. The protocol could be extended to the cotyledon explants from the seeds (although the seeds themselves may be sterile or non-germinating) to obtain a high degree of regeneration. Seed sterility is common in wide hybridization studies. Furthermore, cotyledon cultures may be potentially valuable for transformation studies and may also serve as a source of somatic embryos for artificial seed technology.

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