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Adventitious shoot induction and somatic embryogenesis with intact seedlings of several hybrid seed geranium (*Pelargonium* × *hortorum* Bailey) varieties

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Summary. Intact seedlings of hybrid seed geranium (*Pelargonium x hortorum* Bailey) were tested for their ability to produce adventitious shoots and somatic embryos by direct culture of mature seeds on Murashige and Skoog (1962) medium (MS) supplemented with growth regulators BAP, BAP + IAA, or thidiazuron (TDZ). Ten varieties were tested in the presence of different BAP concentrations, four with BAP + IAA, and two with TDZ. Varieties used in this study differed in their response to BAP in the medium. Multiple adventitious shoots were produced by seven of the ten varieties tested. Multiple adventitious shoots were induced at all levels of TDZ in the medium. TDZ also induced callusing from roots and direct embryogenesis from intact hypocotyls. Adventitious shoots were separated, rooted and transferred to soil where they grew as normal healthy plants and flowered.

Key Words: Geranium - *Pelargonium* - Micropropagation - Adventitious shoots - Somatic embryogenesis

Abbreviations: BAP = N^6-benzylaminopurine; IAA = indole-3-acetic acid; MS = Murashige and Skoog (1962); TDZ = thidiazuron

Introduction

Micropropagation is perceived to be a potential large-scale propagation method for plant species of ornamental interest. Unfortunately, this method is neither available to most ornamental plant species nor is it always advantageous as a commercial option (Pierik 1988). Nevertheless, micropropagation can be attempted where conventional methods are simply not feasible owing to the technical difficulties, the length of time involved for multiplication, and/or the high production cost.

In the micropropagation industry, cost effectiveness of the process and genomic fidelity of the resulting micropropagules are two very important considerations. In this context, methods relying upon regeneration of plants through a callus phase may not be considered ideal, as genomic alterations in the regenerants are often believed to originate during callusing, and the accumulation of variations in the cultured cells seems to be directly proportional to the duration of culture (Larkin and Scowcroft 1981; Evans and Sharp 1983; Gavazzi et al. 1987). Avoiding an intervening callus phase by inducing direct organogenesis may, on the other hand, produce a multitude of plants without risking clonal fidelity.

Geranium (*Pelargonium x hortorum*) is a popular floricultural crop in Europe and North America. Prior to 1965 seed propagated geraniums were not commercially available, but since then F1 hybrid seed of a number of varieties is available in the market. Because of manual handling and the relatively limited supply of hybrid seed, production costs are high.

Methods of *in vitro* micropropagation of hybrid seed geranium have been described previously. For instance, shoot and root regeneration has been achieved using shoot-tips (Horst et al. 1976; Debergh and Maene 1977), apical meristems (Pillai and Hildebrandt 1968) and other explants (Pillai and Hildebrandt 1969). Callus induction and plant regeneration from cultured stem segments (Hammerschlag and Bottino 1981) and shoot-tips (Dunbar and Stephens 1989) have also been described. Recently, Marsolais et al. (1991) described a method of direct somatic embryogenesis from cultured hypocotyl explants of many hybrid seed geranium varieties. They found that a combination of IAA and BAP induced direct somatic embryogenesis in hypocotyl sections. Using the same procedure but replacing indoleacetic acid with

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phenylacetic acid, direct somatic embryogenesis in hypocotyl explants of seed geraniums has also been reported (Slimmon et al. **1991**).

We here describe a procedure to induce adventitious shoots and somatic embryogenesis to recover normal healthy plantlets using intact geranium seedlings. This procedure is simple, direct, and bypasses explanting and callusing which may be a potential source of variation in the clonal population.

Materials and methods

Seeds of ten hybrid seed geranium varieties (*Pelargonium x hortorum* Bailey) were obtained from Stokes Seeds, St. Catharines, Ont. Seeds were surface sterilized by immersing in 70% ethanol for 30 sec followed by a treatment with 30% (v/v) commercial sodium hypochlorite solution for 20 min. To this solution Tween-80 (2 drops/100 ml solution) was added as a surfactant. During the course of the treatment, seeds were agitated periodically. Following sterilization, the seeds were thoroughly washed with sterile distilled water to remove all traces of the bleach.

Medium preparation. The basal medium used in this study contained MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968) and 30 g/l sucrose. The medium was adjusted to pH 5.5 and was solidified with 3 g/l Gelrite (Scott Laboratories, Carson, USA). The medium was sterilized by autoclaving at 0.122 MPa for 20 min. Depending upon the need, this medium was modified to contain different concentrations of BAP (5 - 80 μM) with or without IAA (1 μM), and TDZ (1 - 10 μM).

Seed culture in the presence of BAP and IAA. Surface-sterilized seeds (5/dish) were placed in Petri dishes containing 30 ml culture medium. Three dishes/treatment were inoculated and sealed with Parafilm. Various treatments were : 0, 5, 10, 20, 40, and 80 μM BAP with and without 1 μM IAA. The seeds were allowed to germinate in the dark for 10 days at 25 °C. After this incubation, the dishes were transferred to an incubator with a 16h day light and 25 °C temperature. The illumination (20-25 $\mu E.m^{-2}.s^{-1}$) was provided by cool white fluorescent tubes. The dishes were placed individually on incubator shelves to allow uniform exposure to light.

Seed culture in the presence of TDZ. Surface-sterilized seeds (5/dish) were germinated in sealed Petri dishes as described above on MS medium without any growth regulators (0MS; 20 dishes) and with 1, 2, 5, or 10 μM TDZ (two sets of 4 dishes/treatment). After ten days of germination in the dark, Petri dishes were moved into the light (16h day, with a light intensity of 20-25 $\mu E.m^{-2}.s^{-1}$, at 25 °C). While maintaining one set of seedlings on 1, 2, 5, or 10 μM TDZ in Petri dishes, the other set was transferred to Magenta boxes (5 seedlings/box, 4 boxes/treatment) containing MS medium with respective TDZ concentrations. At the same time the seedlings which were germinated on 0MS for 10 days were transferred to Magenta boxes (5 seedlings/box, 4 boxes/treatment) containing 1, 2, 5, and 10 μM TDZ. Magenta boxes were sealed with Parafilm.

Fig. 1a-d. **(a)** Control : "Scarlet Orbit Improved" growing on 0MS. **(b)** "Scarlet Orbit Improved" growing on MS medium supplemented with 20 μM BAP exhibiting induction of adventitious shoots. **(c)** Rooted plantlets derived from the adventitious shoots induced on "Scarlet Orbit Improved" in the presence of 20 μM BAP. **(d)** A mature flowering plant derived from an adventitious shoot taken from a "Scarlet Orbit Improved" seedling.

Results and Discussion

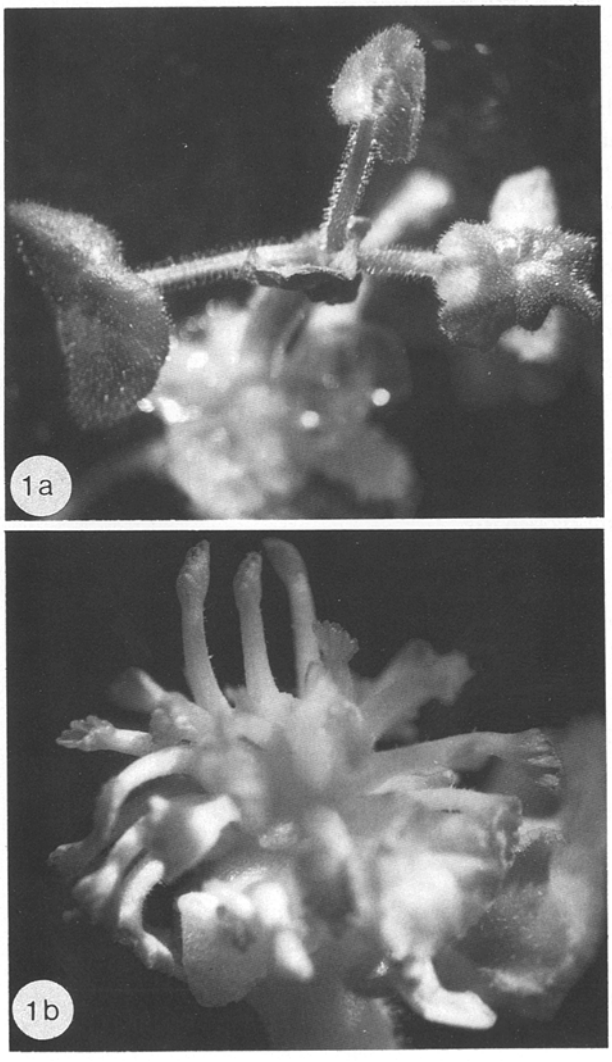
BAP and IAA treatment

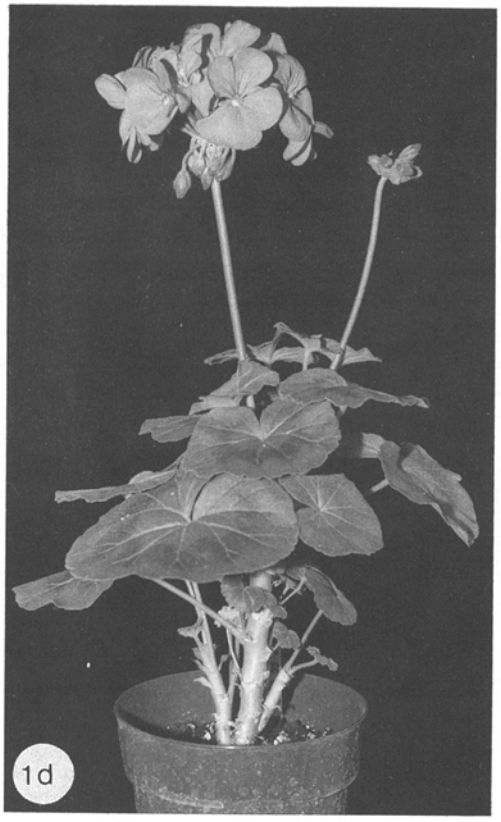
Germination

The presence of BAP in the medium produced a variable response for germination. Out of the 10 cultivars tested, in four BAP had no effect on germination up to a concentration of 20 μM, whereas it had an adverse effect on six others. The germination percentage among these sensitive varieties in the presence of BAP ranged from 13.3 to 100% at different BAP concentrations (Table 1). Due to the failure of germination at 40 and 80 μM BAP in most cases, observations only up to 20 μM BAP are included in the Tables 1 and 2.

Adventitious shoot induction

No adventitious shoot induction occurred, in any variety tested, in the absence of exogenous BAP in the medium (Fig. 1a). In the presence of exogenous BAP in the medium, the varieties tested showed a clear genotypic





difference in response. BAP alone at 5, 10, and 20 μM in the medium favoured multiple adventitious shoot induction in six varieties. Among these six varieties, **Scarlet Orbit Improved** (SOI) showed the best response (Table 1). The optimum BAP concentration for **SOI** was found to be 20 μM (23 shoots/seedling). The other five varieties showed a modest response ranging from a mean of 2.4 to 5.7 shoots/seedling. Within this range, most differences between different BAP concentrations and among different varieties were not significant. Adventitious shoots were produced from the enlarged apical region which in many cases appeared swollen giving rise to a ring of adventitious shoot buds below the apex (Fig. 1b).

Table 1. Adventitious shoot induction in several varieties of hybrid seed geranium. Seeds were germinated in MS medium supplemented with different BAP concentrations. Scoring was done after six weeks of incubation. CR = Callusing roots, HC = Callusing hypocotyl, CC = Callusing cotyledon, AP= Callusing apex, SN = Senescence, SOI = Scarlet Orbit Improved, SPSC = Sprinter Scarlet, SEO = Scarlet Eye Orbit, SSM = Summer Shower Mix, MBL = Multibloom Lavender, MBS = Multibloom Salmon. Standard error of means is shown in parenthesis.

Variety	BA (μM)	%Germination	Callusing	Shoots/Plant
SOI	0	100	-	1
	5	100	CR	8 (1.5)
	10	100	CR	6.4 (0.4)
	20	100	CR	23.4(1.9)
Mustang	0	100	-	1
	5	100	CR	4.2 (2.5)
	10	100	CR	2.4 (0.8)
	20	100	CR	1 SN
Red Elite	0	100	-	1
	5	100	CR	1
	10	100	CR	1
	20	100	CR	1
SPSC	0	100	-	1
	5	100	CR	4.2 (0.8)
	10	100	CR	3.4 (1.2)
	20	100	CR	5.7 (1.8)
SEO	0	100	-	1
	5	73.3	CR,HC	1
	10	20.0	-	1 SN
	20	60.6	-	1 SN
Ringo Rose	0	100	-	1
	5	86.6	-	2.3 (1.3)
	10	33.3	HC	1
	20	60.0	-	1
SSM	0	100	-	1
	5	100	CR	2.5 (1.2)
	10	100	CR	3.2 (0.4)
	20	60.0	-	1 SN
Cherry Orbit	0	100	-	1
	5	73.3	HC	4.7 (3.7)
	10	40.0	HC	1
	20	53.3	-	1 SN
MBL	0	100	-	1
	5	20.0	-	1
	10	13.3	HC,CC	1
	20	20.0	-	1
MBS	0	100	-	1
	5	26.6	AP	1
	10	40.0	AP	1
	20	20.0	HC,CC	1

Four cultivars were tested with BAP (5-80 μM) and IAA (1 μM) together in the medium. All four cultivars showed an overall better response than BAP alone. **Red Elite** which did not produce adventitious shoots with BAP alone, responded at 10 μM BAP together with IAA and produced 10.8 shoots/seedling. Other concentrations (5, 20, 40, and 80 μM BAP) failed to produce any response in this cultivar. Progressively more shoots per seedling were produced by **SOI** with increasing BAP concentration. The optimum BAP concentration for **SOI** and **SPSC** was 20 μM. **Mustang** produced more shoots at 5 μM BAP (Table 2).

Conversion of adventitious shoots to individual plants

In order to test the reliability of this micropropagation system to produce whole plants, a set of twenty plants was raised to maturity using adventitious shoots obtained from **SOI** seedlings. Bunches of adventitious shoots were excised and transferred to Magenta boxes (each containing 50 ml of 0MS) without separating individual shoots for further development. Within 2-3 weeks these shoots grew further and gained vigour. At this point, individual shoots were separated from the bunches and 3 shoots per Magenta box were inserted for rooting on the same medium. Within next 2-3 weeks, individual shoots developed strong root systems (Fig.1c). At this stage, individual plantlets were transferred to soil (Rediearth, 1 plant/6" diameter plastic pot) and were kept covered using inverted Magenta boxes to maintain a humid environment around them. All plants transferred to soil in this manner survived, continued normal growth and flowered (Fig.1d). Morphologically, these plants and the flowers produced on them appeared normal and true-to-type.

TDZ Treatment

This treatment was used with two cultivars, **SOI** and **Mustang** only. For this experiment, seeds were germinated on medium containing different TDZ concentrations (0, 1, 2, 5, and 10 μM). After 10 days of initial incubation in the dark, the dishes were moved to light (16h day at 25°C) for further incubation. At this time, one set of seedlings grown on different TDZ concentrations in Petri dishes was transferred to Magenta boxes containing the same medium. This experiment was designed to study the effect of the kind of culture vessel used on the degree of adventitious shoot induction from geranium seedlings in the presence of TDZ.

Multiple adventitious shoots were induced in both cultivars at all levels of TDZ used (Fig.2a). While growing in boxes the range of **SOI** was between 4-8 shoots/seedling, the optimum being 2 μM. Under the same conditions, the mean range observed in the cultures of **Mustang** was between 4-6 shoots/seedling (Table 3).

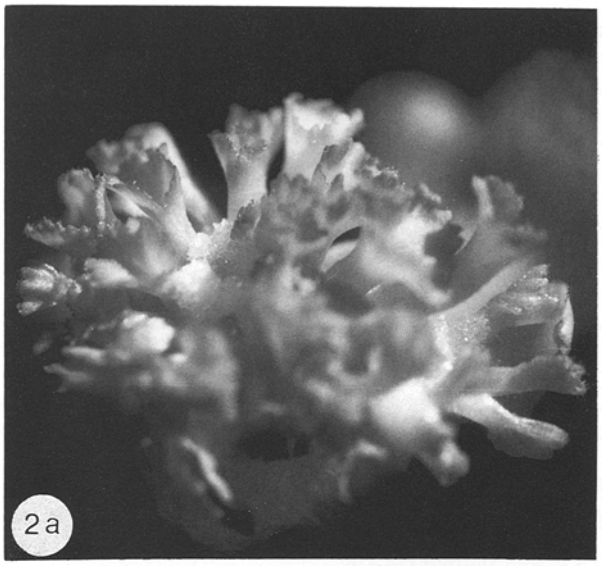
Table 2. Adventitious shoot induction in four hybrid seed geranium varieties under the influence of BAP (5-20 μM) and IAA (1 μM) together in the growth medium. SOI = Scarlet Orbit Improved, SPSC = Sprinter Scarlet, CR = Callusing roots. Scoring was done six weeks after incubation. Germination in all varieties was 100 percent. Standard error of mean is shown in parenthesis.

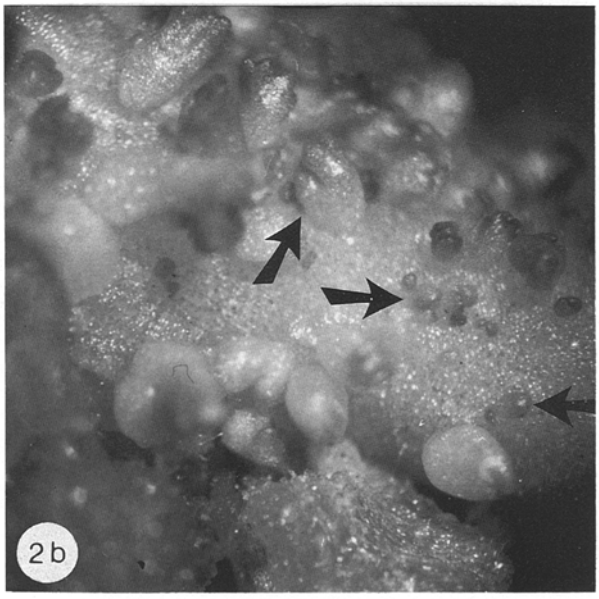
Variety	BA(μM)+IAA	Callusing	Shoots/Plant
SOI	0	-	1
	5	CR	4.9 (0.4)
	10	CR	17.1 (1.1)
	20	CR	20.4 (1.4)
Mustang	0	-	1
	5	CR	7.3 (2.5)
	10	CR	4.0 (0.7)
	20	CR	1
Red Elite	0	-	1
	5	CR	1
	10	CR	10.8 (3.1)
	20	CR	1
SPSC	0	-	1
	5	CR	1
	10	CR	4.1 (1.4)
	20	CR	11.8 (4.3)

Table 3. Adventitious shoot induction in two hybrid seed geranium varieties. Seeds were germinated in Petri dishes on MS medium containing various TDZ concentrations (1, 2, 5, and 10 μM). Seedlings were either transferred after ten days to Magenta boxes containing corresponding TDZ concentration or were left in the dishes where these were germinated. Scoring was done six weeks after incubation. Standard error of means is given in parenthesis.

	Scarlet Orbit Improved			
μM TDZ	# of seedlings		Shoots/plant	
	box	dish	box	dish
0	20	15	1	1
1	20	15	4.1 (0.3)	19.0 (2.2)
2	20	15	8.3 (0.5)	17.6 (2.1)
5	20	15	6.9 (0.5)	17.0 (1.5)
10	20	15	5.7 (0.5)	13.4 (0.9)
	Mustang			
0	20	15	1	1
1	20	15	6.0 (0.4)	17.4 (0.7)
2	20	15	5.8 (0.3)	19.8 (2.1)
5	20	15	4.7 (0.6)	11.8 (0.8)
10	20	15	4.3 (0.5)	NA

The number of shoots produced at 1 and 2 μM TDZ was not significantly different, but was higher than the number produced at 5 and 10 μM TDZ. The difference between the seedlings growing in Magenta boxes and Petri dishes was very clear. The number of shoots/seedling was higher in each treatment when the seedlings remained in Petri dishes. The mean range in this case was 13-19 shoots/seedling for **SOI**, and 11-19





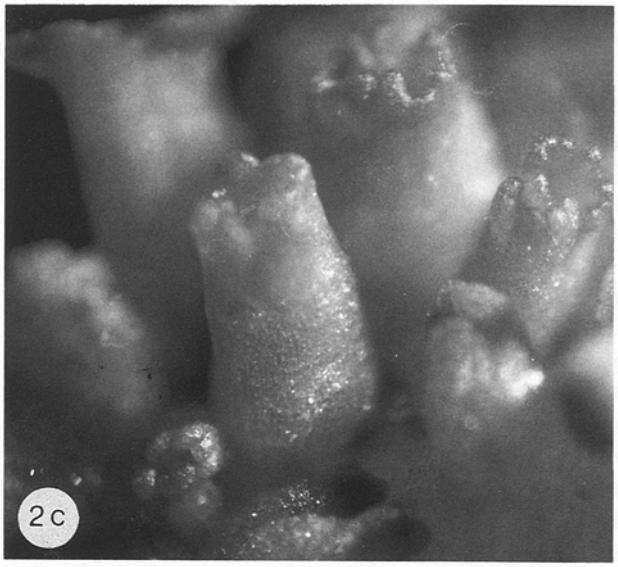


Fig. 2a-c.(a)"Mustang" growing on MS medium supplemented with 1 μM TDZ exhibiting induction of adventitious shoots.**(b)** Direct somatic embryogenesis at the basal end of the intact hypocotyl of "Scarlet Orbit Improved". Arrows indicate somatic embryos and embryoidal structures. **(c)** A magnified view of somatic embryos produced directly on the intact hypocotyl.

shoots/seedling for **Mustang**. The mean number of shoots produced by **SOI** seedlings did not differ between 1, 2 and 5 μM TDZ treatments, whereas at 10 μM TDZ the number was reduced. For **Mustang**, the mean number of shoots produced at 1 and 2 μM TDZ did not differ significantly, but was significantly higher than the number produced at 5 μM TDZ. The cause of this difference in response between Petri dishes and boxes is yet unknown, but a different micro-environment of the culture vessels may be one plausible explanation. Petri dishes being a tight compartment as opposed to Magenta boxes, may provide a TDZ-rich environment where the apical region remains very close to the medium surface.

Actual physical contact with the medium containing TDZ has been seen to induce changes in the plant parts as opposed to the parts which remain away from the medium surface. For example, if one cotyledon remains in contact with the medium containing TDZ and the other on the same seedling remains away, the one which remains in contact with the medium enlarges two to three fold (unpublished observation).

The roots of the cultivar **SOI** produced callus at 5 μM TDZ which was morphogenic. The roots of **Mustang** also produced morphogenic callus at 1, 5, and 10 μM TDZ.

When the seeds of **SOI** and **Mustang** were germinated on OMS for 10 days and were transferred to Magenta boxes containing MS medium with 1, 2, 5, and 10 μM TDZ following observations were made. At 1 μM TDZ in the case of **SOI**, the main shoot senesced, basal region swelled, roots were thickened and turned green. From the swollen basal portion, direct somatic embryogenesis was observed (Figs.2b & 2c) as has previously been reported using geranium hypocotyl sections (Marsolais et al. 1991; Slimmon et al. 1992; Wilson 1990). No adventitious shoots were formed. At higher levels of TDZ (2, 5, and 10 μM), similar observations were made, except that swelling of the basal portion of the stem was lesser and fewer somatic embryos were produced.

Mustang seedlings at 1 μM TDZ showed senescence of the main shoot and swelling of the basal part of the stem. Roots were thickened and turned green. In some seedlings, occasionally adventitious shoots were seen to emerge from the middle portion of the hypocotyl but not from the apical region. This drastic difference between the seedlings germinated and grown on the medium containing TDZ versus seedlings germinated in the absence of TDZ and transferred onto TDZ medium is rather interesting. Perhaps conditioning of the meristem

is required during the germination for later morphogenic expression (Malik and Saxena 1992). At 2 and 5 μM TDZ, a similar pattern of response was found except that more callusing was observed. At 10 μM TDZ, less swelling of the basal region of the stem was observed and fewer somatic embryos were seen. Adventitious shoot primordia appeared from the apical region and from roots.

Thidiazuron, previously used as a cotton defoliant (Arndt et al. 1976) has been shown to possess high cytokinin activity (Mok et al. 1982). In other studies involving tissue morphogenesis *in vitro*, TDZ was found to exert a much greater influence than cytokinins (Thomas and Katterman, 1986 ; Fiola et al. 1990). Our observations concur with these findings. Induction of somatic embryogenesis directly on the intact hypocotyls indicates the influence of this compound over endogenous cytokinins and auxins. Increased level of cytokinins has been found in callus tissue treated with TDZ which may suggest that the activities associated with TDZ may be related to its regulation and/or accumulation of cytokinins (Capelle et al. 1983). Considering our observation of direct somatic embryogenesis induced by TDZ alone, and in view of the generally accepted notion that morphogenic expression relies upon auxin availability and a balance between the amounts of endogenous growth regulators (Skoog and Miller 1957; Ammirato 1983), TDZ may also be involved in conserving and influencing the mobility and direction of the endogenous auxin.

In conclusion, this report describes a procedure for direct adventitious shoot initiation and the successful use of thidiazuron as an alternative and more potent compound expediting micropropagation. The conditions developed in our study may offer a less labour intensive and hence economically viable proposition to geranium hybrid seed industry. This procedure can also be of special value for multiplying certain genotypes carried by only a few hybrid seeds resulting from a difficult cross.

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