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In vitro plantlet regeneration from cotyledon, hypocotyl and root explants of hybrid seed geranium

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Abstract

In vitro plantlet regeneration systems for the seed geranium (*Pelargonium x hortorum* Bailey) using cotyledon, hypocotyl and root explants were optimized by studying the influence of seedling age, growth regulators and excision orientation on organogenesis. Indole-3-acetic acid combined with zeatin yielded the highest rate of shoot production on cotyledon explants (0.2–2 shoots per explant). More shoots were produced on explants cut from the most basal region of cotyledons from 2 to 4-day-old seedlings than from older seedlings or more distal cut sites. Hypocotyl explants produced the highest number of shoots, up to 40 shoots per explant, on indole-3-acetic acid (2.8–5.6 mM) + zeatin (4.6 mM) or thidiazuron (4.5 mM). Maximum shoot formation (0.3–1.4 shoots per explant) on root explants occurred when they were cultured on medium containing zeatin. Regenerated shoots rooted best on a basal medium containing no growth regulators. There were substantial differences among cultivars in shoot formation from each of the explant systems.

Abbreviations: BA – 6-benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indole-3-acetic acid; NAA – naphthaleneacetic acid; TDZ – thidiazuron

Introduction

Pelargonium x hortorum L.H. Bailey (geranium) has been grown worldwide for many years as an important floricultural crop. The origins and taxonomy of the Pelargoniums have been reviewed (Mastalerz & Holcomb, 1982). Traditional geranium production methods rely on vegetative propagation, since seed set is low in most cultivars. The difficulty of maintaining near sterile, elite mother-stock plants for the production of cuttings has stimulated the development of the seed propagated geraniums (Mastalerz & Holcomb, 1982). These diploid (2n=18) relatives of the genetically more complex (2n=36) standard commercial geraniums also serve as good model plants for the study of traits such as ethylene response (Evensen *et al.*, 1993) and insect resistance (Grazzini *et al.*, 1995).

A system for producing transgenic geranium plants would make posssible the creation of new cultivars with improved production, postharvest and marketing potential and provide a powerful tool for the study of geranium molecular biology. Such a system requires an efficient DNA delivery system, a method of regeneration, and selection of fertile transgenic plants from plants derived from a single transformed cell. As a first step in the development of such a system, experiments were carried out to optimize the regeneration of seed geraniums from various explant sources, and to compare the efficiencies of the different explants.

Several methods of *in vitro* regeneration of seed geraniums have been reported previously. For example, organogenesis has been induced directly from axillary meristems (Desilets *et al.*, 1993) and germinating seeds (Qureshi & Saxena, 1992), as well as from callus derived from shoot-tips (Debergh & Maene, 1977; Dunbar & Stephens, 1989), hypocotyls (Dunbar & Stephens, 1989), and internode pith (Pillai & Hildebrandt, 1968). Dunbar & Stephens (1989) were unable

to regenerate shoots from cotyledon explants, and we found no reports on regeneration from root explants of geranium.

In this paper, a system is described for regeneration of plantlets from cotyledon, hypocotyl and root explants of several diploid seed geranium cultivars. The cultivars were chosen based on the regeneration potential demonstrated in other research studies (Desilets *et al.*, 1993; Dunbar & Stephens, 1989; Qureshi & Saxena, 1992).

Materials and methods

Plant material

Seeds of the *P. x hortorum* cultivars: 'Pinto Red', 'Pinto Rose', 'Ringo Deep Scarlet', 'Ringo Rose', and 'Ringo Salmon' (Sluis & Groot America, Inc.), and 'Pinto Scarlet', 'Red Elite' and 'Scarlet Orbit' (Goldsmith Seeds, Inc.) were surface sterilized by dipping in 95% ethanol for 60 s and then agitated periodically (for a few seconds each time the seeds sank to the bottom) in 1% (v:v) sodium hypochlorite solution containing one drop of Tween 20 per 100 ml for 30 min. Seeds were rinsed 3 times with autoclaved water, and placed on basal medium in 135 mm dia. Petri dishes sealed with parafilm (see below) for germination. Explants used in shoot regeneration experiments were cotyledons excised from 1- to 5-day-old seedlings and cut in half longitudinally, 1 cm long hypocotyl segments from 1- to 5-day-old seedlings and 2-cm-long root segments excised from plants grown on basal medium. For excision treatment studies, cotyledons from 1- to 5-day-old seedlings of 'Scarlet Orbit' were cut either longitudinally or transversely. For age effect studies, cotyledons were excised and cut longitudinally from seedlings at three different seedling ages: 2 to 4, 6 to 8, and 10 to 12 days after radicles emerged from seed coats. For rooting tests, regenerated shoots (from cotyledons) over 0.5 cm were excised and transferred to root-induction medium in Magenta boxes sealed with parafilm. Each treatment had three replicates with 10 explants each. Explants were arranged in a completely randomized block design on a single shelf in the growth chamber. To determine the efficiency of shoot-induction and root-induction media, the number of regenerated shoots per total explant number and the rooting percentage (shoots with roots formed per total shoots) and root length were recorded after one month. Plants were transferred to a mixture composed of sphagnum moss peat, perlite, and soil (8:4:2), covered with plastic wrap for one week for acclimatization, then uncovered gradually over several days and moved to a greenhouse and grown under partial shade cloth. Analyses of variance were performed on the results of each experiment using an IBM-PC version of Statgraphic and the means were compared using Student's t-test.

Medium preparation and culture conditions

Basal medium consisted of Murashige and Skoog basal salts (Murashige & Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), and 30 g l^{-1} of sucrose. Shoot induction media tested on cotyledon and root explants consisted of basal medium plus IAA (0, 2.8, 5.7, or 11.4 mM) and zeatin (4.6 or 9.1 mM). IAA (2.8 mM), 2,4-D (2.3 mM), or NAA (5.4 and 10.7 mM), each combined with zeatin (4.6 mM) were tested for shoot formation from cotyledons. Basal medium containing BA (4.4 or 8.9 mM) was tested on root explants. Thidiazuron alone (TDZ, 0.5 and 2.3 mM) and IAA (2.8 and 5.7 mM) plus zeatin (4.6 mM) were tested on hypocotyl explants. IAA (0, 5.7 or 11.4 mM) and NAA (5.4 or 10.7 mM) were tested for rooting of regenerated shoots. Growth regulators were added before autoclaving. All media were adjusted to pH 5.7, solidified with 2 g l^{-1} of phytogel (Sigma) and sterilized by autoclaving at 121° C, 15 psi for 30 min. Seeds were germinated in parafilm-sealed Petri dishes on moist Whatman #1 filter paper in the dark at 22–24° C for 10–14 days. Cultures were incubated in Petri dishes or magenta boxes in a growth chamber under an irradiance of 70 or 110 m mol m^{-2} s^{-1} (for Petri dishes or Magenta boxes respectively, measured at the top of the containers) during a 16-h photoperiod provided by a mixture of cool-white Phillips F40CW and Phillips F40 Agro-lite fluorescent lamps at 22–24° C.

Results and discussion

Shoot regeneration from cotyledon explants

Cotyledon explants from 1- to 5-day-old seedlings were cultured for 3 weeks on media containing different combinations of auxins and zeatin. Swelling, cell enlargement, and the growth of a thin layer of light colored callus was observed at the wound site on growth regulator treatments which resulted in regeneration. Shoot regeneration occurred exclusively from

Table 1. The efficiency of shoot formation on cotyledon, hypocotyl, and root explants of six diploid geranium cultivars.

Cultivar	No. shoots per explant		
	Cotyledon explants	Hypocotyl explants	Root explants
Pinto Red	0.6 ± 0.1	ND	2.0 ± 0.3
Pinto Rose	ND [1]	ND	1.0 ± 0.5
Pinto Salmon	0.3 ± 0.1	ND	ND
Pinto Scarlet	ND	ND	2.9 ± 0.6
Red Elite	1.3 ± 0.4	ND	1.1 ± 0.3
Ringo Deep Scarlet	2.1 ± 0.5	30.5 ± 1.7	4.3 ± 2.1
Ringo Salmon	0.3 ± 0.1	ND	0.8 ± 0.5
Scarlet Orbit	2.0 ± 1.0	35.0 ± 1.8	1.9 ± 0.5

[1] ND = not determined

For cotyledon and hypocotyl explants, the growth regulator concentrations were 2.8 mM IAA + 4.6 mM zeatin. For root explants, data is shown for the optimal zeatin concentration, which was 9.1 mM zeatin for 'Scarlet Orbit' and 'Pinto Rose' and 4.6 mM for the other cultivars. Data represent the means of three replications of 10 explants each + standard deviation.

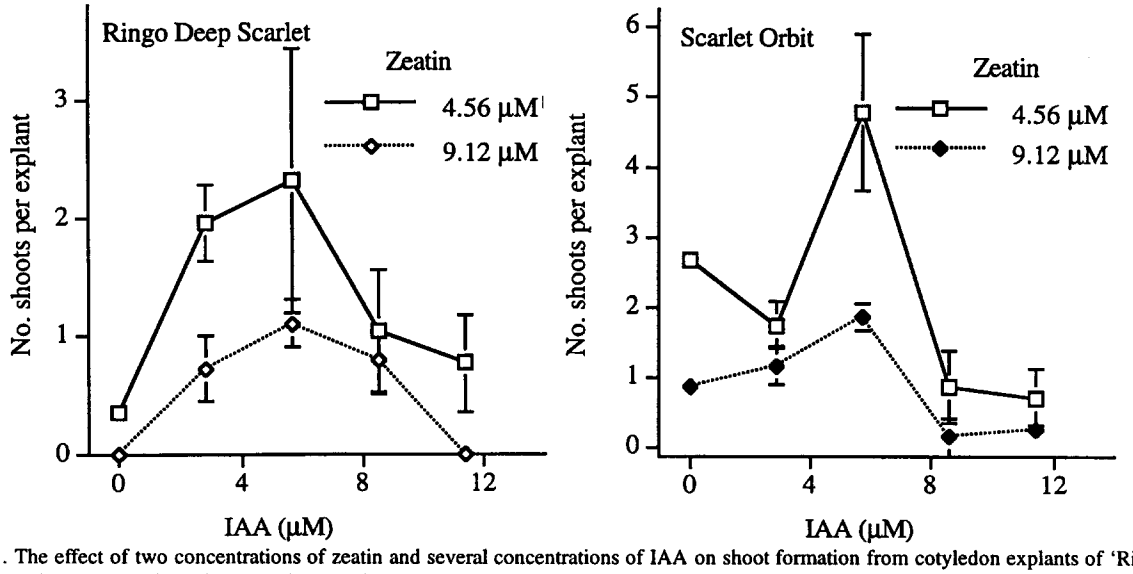

Fig. 1. The effect of two concentrations of zeatin and several concentrations of IAA on shoot formation from cotyledon explants of 'Ringo Deep Scarlet' and 'Scarlet Orbit' geraniums. Values shown are means of 3 replicates of 10 explants each + SE.

cells within or just below this callused area within about two months. There was no shoot formation on 2,4 D (2.7 mM) + zeatin (4.6 mM) and very little on NAA (2.7 mM) + zeatin (4.6 mM) and callus on explants kept on these growth regulator treatments was brown colored (data not shown). All cultivars tested produced shoots from cotyledon explants grown on IAA (2.8 mM) + zeatin (4.6 mM), and the highest rates of shoot production were observed in 'Ringo Deep Scarlet' and 'Scarlet Orbit' (Table 1).

Examination of a range of IAA and zeatin combinations for these two cultivars showed that the optimal growth regulator concentrations were 5.7 mM IAA + 4.6 mM zeatin for both (Fig. 1). Even under these conditions, the rate of shoot production was only 2–4 shoots per regenerated explant, and the proportion of explants forming one or more shoots varied from 9% ('Pinto Salmon') to 50% ('Ringo Deep Scarlet') (data not shown).

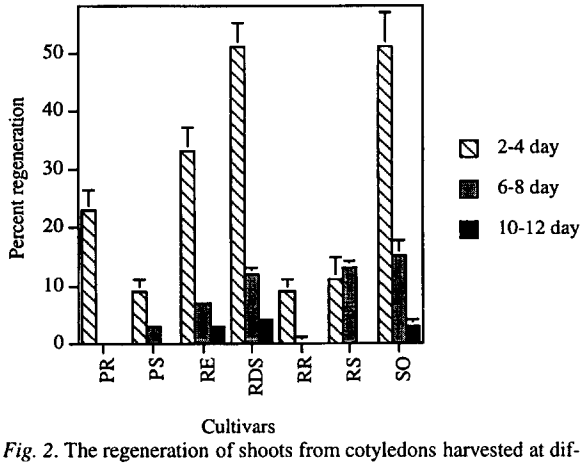

Fig. 2. The regeneration of shoots from cotyledons harvested at different times after germination and cultured on 2.8 mM of IAA and 4.6 mM of zeatin. Cultivars were PR: Pinto Red, PS: Pinto Scarlet, RE: Red Elite, RDS: Ringo Deep Scarlet, RR: Ringo Rose, RS: Ringo Salmon, SO: Scarlet Orbit. Columns represent the mean percent regeneration of shoots from three replications of 10 explants each. No column indicates no regeneration. Bars represent standard deviations. Regeneration of shoots from explants from older cotyledons was significantly less (α=0.05) than from 2–4 day old cotyledon explants for all cultivars except 'Ringo Salmon' (6–8 day old).

Effect of seedling age and position of wound sites on regeneration from cotyledons

It had been previously reported that young cotyledons have a greater regeneration ability than older explants (Chraibi *et al.*, 1992; Power, 1987). Additionally, Dunbar and Stephens (1989) reported that shoots were not produced on cotyledon explants cut from 10- to 14-day-old geranium seedlings.

The effect of seedling age at the time of explant excision on the regeneration efficiency of cotyledon explants was examined. Consistent with the reports cited above, the highest percent of shoot regeneration was found on cotyledons excised from 2- to 4-day-old seedlings on all cultivars tested except 'Ringo Salmon' (Fig. 2).

The frequency of shoot formation on cotyledon explants varied depending on the region of the cotyledon from which the explant was taken. More than 4 shoots per explant were produced at the cut surface of the proximal end of the cotyledon, just beyond the attachment to the petiole, while only 0–0.12 shoots per explant were produced by cutting the cotyledon in half longitudinally or crosswise (data not shown). On explants cut longitudinally, most of the shoots arose from tissue closer to the proximal end. Differential shoot regenerative potentials of proximal and distal parts of cotyledons have also been observed in bell peppers (Arroyo & Revilla, 1991), apples (Kouider *et al.*, 1984) and soybean cotyledons (Mante *et al.*, 1989), suggesting polarity and positional effects on regenerative potential. Practically, this also means that division of cotyledons to increase the number of explants per seed may not be effective.

Shoot formation from hypocotyls

Hypocotyl explants grown on IAA + zeatin produced large numbers of shoots, 30 shoots per explant from 'Ringo Deep Scarlet' and 35 from 'Scarlet Orbit' (Table 1). Explants cultured with TDZ (0.5 or 2.3 mM) or IAA (5.7 mM) + zeatin (4.6 mM) produced 20–35 shoots per explant (data not shown). Over 93% of the explants formed shoots in both cultivars and under all growth regulator treatments (data not shown). Visser *et al.* (1992) reported similar numbers of somatic embryos from hypocotyl sections of 'Scarlet Orbit' using TDZ, but only about 18 embryos per explant with 8 mM BA.

Shoot formation from excised roots

Terminal segments of mature roots were capable of producing shoots, though not with very high efficiency. The number of shoots produced per root segment varied from 0.8 in 'Ringo Salmon' to 4.3 in 'Ringo Deep Scarlet' under optimal growth regulator conditions (Table 1). 'Scarlet Orbit' produced about twice as many shoots on 9.1 mM zeatin as on 4.6 mM, but there was no significant difference in shoot production between the two zeatin concentrations for the other cultivars. No shoots were produced on root explants placed on a medium containing BA (4.4 or 8.9 mM). Under optimal growth regulator conditions, the number of explants producing shoots ranged from 70% ('Ringo Salmon') to 90% ('Pinto Scarlet' and 'Ringo Deep Scarlet') (data not shown).

IAA (2.8–11.4 mM) and NAA (5.4 or 10.7 mM) strongly inhibited shoot formation on excised roots grown on media containing zeatin. 'Pinto Red' and 'Scarlet Orbit' still produced a few shoots in the presence of low auxin concentrations, while the other cultivars produced none (data not shown). The differences among cultivars in auxin inhibition could be the result of endogenous growth regulator balance, differential uptake or degradation, or different tissue sensitivity to auxin.

Table 2. The effect of auxins on root formation from regenerated shoots of 'Scarlet Orbit'.

	IAA (μM)			NAA μM	
	0	5.7	11.4	5.4	10.7
% rooted shoots	100 ± 0	88 ± 12	70 ± 9	71 ± 7	0
Root No. per rooted shoot	29 ± 1	51 ± 7	30 ± 3	18 ± 2	0
Mean root length (cm)	2.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.0	0.4 ±0.1	0

Data represent the means of three replications of 10 explants each + standard deviation. All treatments were significantly different (=0.05) from the controls (no auxin).

Rooting of regenerated shoots and subsequent plant development

In order to produce plants with vigorous root systems capable of growth in a greenhouse, different growth regulator regimes were tested for their effects on rooting of regenerated shoots. Since 'Scarlet Orbit' produced the most uniform growth of shoots from cotyledons, it was used for these studies. The highest percent rooting was obtained on basal, growth regulator-free medium, but the highest number of roots per rooted shoot was obtained when IAA (5.7 mM) was added (Table 2). Since 100% of geranium shoots rooted in media lacking growth regulators, medium with no growth regulator was subsequently used as a rooting medium. Regenerated hypocotyl and root explants also rooted best on medium with no growth regulators (data not shown). Shoots regenerated from root explants took longer to root (about 2 months) than those regenerated from cotyledon or hypocotyl explants (scored after 1 month).

Eleven plants regenerated from cotyledons were grown in the greenhouse to observe phenotypic variation as a result of the culture treatments. After 1 month in the rooting media, plants were removed and planted in soil as described above, acclimated and moved to a greenhouse. Following growth in the greenhouse for 3 months, the morphology (color of vegetative organs and flowers and size of whole plants and flowers) of *in vitro*-regenerated plants was compared to plants germinated from seeds. Approximately 90% of the *in vitro*-regenerated plants appeared normal. The only abnormality observed was lighter colored petals on some of the plants.

Conclusions

The largest number of shoots were regenerated from geranium hypocotyl cultures, which could be easily rooted and grown into normal plants. This regeneration system appears the most promising for transformation, based on the large number of regenerated shoots and the high proportion of explants exhibiting organogenesis (>93% in all cases). Cotyledon and root explants regenerated shoots less efficiently than hypocotyls or shoot tip callus cultures, which produced an average of more than 25 shoots per explant on 'Scarlet Orbit' and 2–25 on other cultivars (Dunbar & Stephens, 1989). Although root explants produced only 1–5 shoots per explant (Table 1), a plant grown from seed in a tissue culture vessel can produce over 30 roots greater than 5 cm in length in one month (data not shown). It would therefore be possible to produce large numbers of regenerated plants from only a few source plants. The proportion of root segments producing regenerated shoots was good (70–90%), although rooting these shoots was slow, usually requiring about 2 months incubation time. Root explants might be particularly useful for sequential multiple transformations, where seed production would add greatly to the time between successive rounds of transformation.

Cotyledon explants do not appear to be well suited for propagating large numbers of plants or for use in transformation studies, since shoot formation was low, and less than half of the explants regenerated at all. Dividing the cotyledons did not increase the number of potential shoot-generating sites. Regeneration of plants from roots and especially hypocotyls might be successfully incorporated into a transformation system.

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