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Somatic embryogenesis of *Asarina erubescens* (A. Gray) Pennel

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Efficient plant regeneration was achieved via direct organogenesis from leaf petiole explants of *Asarina erubescens* (A. Gray) Pennel. Shoots were obtained from explants on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP; 4.40–44.0 μM) alone or in combination with BAP (4.40–44.0 μM) and auxin naphthaleneacetic acid (NAA; 0.54 μM). In MS medium with different BAP concentrations and with BAP plus NAA, calli formed at the ends of explants; however, they were non-embryogenic and plantlets formed directly from epidermal cells. Callus formation was observed after one week of incubation. The age of the leaves also influenced callus formation. The most abundant callus formation took place on leaf petiole explants of intermediate age. The most intensive formation of new plantlets was observed after 2–3 weeks of cultivation. MS medium supplemented with BAP (22.20 μM) was most effective and provided a high shoot-regeneration frequency (100%) associated with a large mean number of shoots per explant (26.8). The presence of NAA in the nutrient medium was not essential for the direct regeneration of plantlets from leaf petiole explants of *Asarina erubescens*.

Key words: plant regeneration, creeping gloxinia, tissue culture
Abbreviations: BAP – 6-benzylaminopurine; MS – Murashige and Skoog; NAA – naphthaleneacetic acid

INTRODUCTION

Tissue culture is a useful method for obtaining many clonal plants. The use of *in vitro* techniques for the propagation of ornamental plants has been widely studied. Ornamental plants have been successfully propagated by shoot morphogenesis or somatic embryogenesis [1–5]. Creeping gloxinia (*Asarina erubescens* (D. Don) Pennell; syn. *Maurandya erubescens*, *Lophospermum erubescens*) belongs to the cosmopolitan family *Scrophulariaceae* and comes from the Mexican mountains. Many members of this family are highly ornamental and are commonly planted in gardens. Several representatives of this family (such as *Mimulus*, *Bacopa*, *Nemesia* and *Diascia*) are now widely grown as ornamental plants. Creeping gloxinia is a rampant climber reaching heights of 4–5 m or more, which produces large pink trumpet-shaped flowers with white throats throughout the summer and autumn. It is relatively rare in Lithuanian gardens. Micropropagation and the *in vitro* growth of some members of the *Scrophulariaceae* have been described previously; for example, *Digitalis lanata* [6–9], *Verbascum thapsus* [10], *Antirrhinum majus* [11], *Torenia* [12] and *Bacopa* [13, 14]. However, there are no reports providing evidence for direct or indirect somatic embryogenesis in *Asarinas*. Plants of this genus have recently been used for planting and other purposes and could potentially be used for breeding, polyploidisation *in vitro,* etc. For creeping gloxinia, which is an ornamental plant propagated by seeds, application of *in vitro* techniques might result in more homogeneous plant material, which could be important in plant breeding and genetic engineering.

The objective of the present study was to develop an *in vitro* method for *A. erubescens* regeneration from leaf petiole explants and to test the potential of 6-benzylaminopurine (BAP) and the auxin naphthaleneacetic acid (NAA) for shoot induction.

MATERIALS AND METHODS

Experiments were carried out in order to evaluate the influence of BAP and NAA on direct shoot formation on leaf petiole explants of *A. erubescens*.

One-year-old maternal plants grown from seeds were stored during winter at a temperature of 10 °C. The following spring they were sprouted and grown in a greenhouse at 15–25 °C under solar irradiance. Leaves were cut from plants when they reached 60 cm in height, avoiding the upper and lower 10 cm sections of the

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shoot. The leaves taken from the middle part of the shoot were divided into the following three groups: leaves from the lowest third were defined as 'old'; leaves from the middle third were defined as of 'intermediate age'; and leaves from the upper third were defined as 'young'. At that time, the leaves were around 7 × 8 cm in size, the young and intermediate petioles were around 0.2 cm thick and the old petioles were around 0.3 cm thick.

Freshly harvested leaf petioles were surface-sterilized in 70% EtOH for 20 s, followed by soaking for 20 min with occasional agitation in a 20% v/v commercial bleach 'Jewel' containing 2.6% active chlorine. They were then rinsed three times for 5 min with sterilized distilled water. After sterilization, the petioles were sliced into 0.5–0.7 cm segments, and the explants were placed onto Murashige and Skoog (MS) agar medium [15]. The induction medium was MS basal medium containing myo-inositol (100 mg l^{-1}), thiamine (0.1 mg l^{-1}), nicotinic acid (0.05 mg l^{-1}) and sucrose (3% w/v). This medium was supplemented with BAP (4.40–44.0 μM) alone or with BAP (4.40–44.0 μM) plus NAA (0.54 μM). The media were solidified with 0.7% agar and the pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl before autoclaving at 121 °C for 20 min. Samples (25 ml) of the molten media were dispensed into 100-ml Erlenmeyer's flasks and capped with aluminum foil. Cultures were incubated at 25 ± 2 °C under a 16-h photoperiod with a cool white fluorescent light. A total of 25 replicates were taken for each treatment and five explants were planted in each flask. Observations were carried out after 1–4 weeks of growth. Differences between the means were scored with Duncan's multiple-range test [16].

RESULTS AND DISCUSSION

The effects of BAP and NAA levels on differentiation were examined in petiole segments (0.5–0.7 cm in length) of fully expanded leaves of *A. erubescens*. The survival rate was 100%.

Figure. Altered shoots

Leaf petiole explants incubated on MS medium with different BAP concentrations or with BAP plus NAA demonstrated callus growth after one week of incubation. The quickest and most abundant callus formation at the cut ends of the petiole explants was observed when the nutrient medium contained 13.32 μM of BAP (100%). Similar results were produced by MS media containing NAA and 22.20–44.0 μM of BAP. Under the influence of other BAP concentrations (4.40; 22.20–44.0 μM) 70–86% of the explants formed calli within one week of growth. Greater numbers of calli formed at the proximal than at the basal end of the petiole at all concentrations. Callus formation was influenced not only by the growth regulators applied but also by the age of the explants. Under the influence of BAP, young explants formed calli at both the distal and basal ends; on the medium with NAA, calli were formed over the entire surface of the explants directed to the medium. This result shows that callus formation in a few cases is affected, among other factors, by the orientation of the explants on the culture medium [17]. Explants of intermediate age formed calli only at the basal end when the medium contained 4.40 μM of BAP or 4.40 μM of BAP plus NAA. In the medium containing 13.32–44.0 μM of BAP, with or without NAA, more calli formed at the basal end. Old explants formed calli at both the distal and basal ends, while in the case of 13.32 μM of BAP calli formed over the entire surface of the petiole directed to the medium. The literature indicates that some other plants in this family form few calli, if any, under *in vitro* conditions. According to Newbury et al. [18], relatively few calli of *Antirrhinum majus* formed when shoot tips cultured on 1 mg l^{-1} BAP were excised from older plants (greenhouse grown plants versus 48-day-old seedlings). Gonzalez-Benito et al. [19] reported no callus growth of *Antirrhinum* on most of the media tested in their study.

After one month of cultivation in the medium with 22.20–44.0 μM of BAP and NAA, the calli started to necrotise. Results of the callus evaluation (given as callus-formation points) after one month of growth in MS medium are presented in Table 1.

Shoot bud formation first started after two weeks of growth on the medium with 4.40 μM of BAP (100%), while under the influence of 13.32–44.0 μM of BAP shoot buds were formed by 85–70% of the explants, respectively.

When the medium contained both the auxin and 4.40–13.32 μM of BAP, growth shoot buds were formed by 57% of the explants after two weeks and by 86% of the explants with 22.20–44.0 μM of BAP. After three weeks of growth in the nutrient medium, all of the petiole explants formed both shoot buds and small shoots, although no shoots were observed on the surface of young petioles under the influence of 44.0 μM of BAP, and few shoots were formed both on young petioles with 22.2 μM of BAP and on intermediate-age petioles with 4.40 μM of BAP plus NAA. The use of intermediate-age petioles resulted in the highest regeneration

Table 1. **Effects of BAP and NAA on callus formation on *A. erubescens* petiole explants after four weeks of cultivation**

Growth regulator (μM)		Callus-formation points*		
		Leaf age		
BAP	NAA	Young	Intermediate	Old
4.40	0	2.6b	2a	3d
13.32	0	2.8bcd	2.8b	3d
22.20	0	2.8bcd	3.4cd	3.6e
44.0	0	2.6b	2.8b	2.4bc
4.40	0.54	3.2d	3.6d	4.8f
13.32	0.54	2.8bcd	3.4cd	2.8cd
22.20	0.54	2.8bcd	2.8b	2.2ab
44.0	0.54	2a	2.2a	1.8a

* Points range from 1 (weak callus) to 5 (abundant callus). Values followed by the same letter within a column are not significantly different according to Duncan's multiple-range test at the 5% level.

levels; after three weeks of growth they formed the largest number of shoot buds and shoots in the medium with 22.0 μM of BAP (Table 2). Direct embryo formation on *Oncidium* leaf explants was retarded by exogenous auxins, but promoted by exogenous cytokinins too [20], and regeneration through direct somatic embryogenesis has been achieved using young leaf explants [21]. Although Asarinas calli formed under all treatments, shoots emerged only from the vegetative tissues and no regeneration occurred from the calli.

Data presented in Table 3 show that the regenerative characteristics of intermediate-age petioles were more strongly expressed than those of young or old petioles. In the medium with 13.32–44.0 μM of BAP, intermediate-age petioles completed the shoot-formation processes both earlier and faster. After four weeks of growth, the largest number of shoots was formed by intermediate-age petioles in the medium with 22.20 μM of BAP (Table 3). By four weeks, young explants formed relatively few shoots (2.0) in the medium with

Table 2. **Effects of different concentrations of BAP and NAA on shoot buds and shoot regeneration from leaf petiole explants of *A. erubescens* after three weeks of cultivation**

Growth regulator (μM)		No. of shoot buds per culture			No. of shoots per culture		
		Leaf age			Leaf age		
BAP	NAA	Young	Intermediate	Old	Young	Intermediate	Old
4.40	0	6.6ab	12.0cd	4.7ab	1.9 bc	4.2bc	2.5a
13.32	0	13.3d	12.7cd	13.5def	3.3 c	4.7bc	8.0bcd
22.20	0	12.7cd	24.2e	10.0cd	0.21 a	17.9e	10.7d
44.0	0	7.3ab	16.5d	16.1f	0 a	3.6b	3.2ab
4.40	0.54	3.7a	3.3a	2.5a	1.1ab	0.2a	1.5a
13.32	0.54	6.1ab	6.5a	6.5bc	6.1d	6.5cd	2.2a
22.20	0.54	6.0ab	6.5ab	6.5bc	3.6c	7.7d	4.7ab
44.0	0.54	8.9bcd	9bc	5.8ab	2.8bc	5.5bcd	3.0a

Values followed by the same letter within a column are not significantly different according to Duncan's multiple-range test at the 5% level.

Table 3. **Effects of different concentrations of BAP and NAA on shoot formation from leaf petiole explants of *A. erubescens* after four weeks of cultivation**

Growth regulator (μM)		No. of shoot buds per culture			No. of shoots per culture		
		Leaf age			Leaf age		
BAP	NAA	Young	Intermediate	Old	Young	Intermediate	Old
4.40	0	9.3ab	20.0e	16.3d	6.0def	4.8ab	6.5cd
13.32	0	19.3d	9.3bc	10.5b	8.1ef	11.7d	13.0g
22.20	0	19.8d	15.3cde	11.5b	8.0f	26.8e	12.8g
44.0	0	11.4b	13.2c	18.5a	2.0ab	7.5bc	9.5e
4.40	0.54	5.7ab	4.1a	3.2b	1.1a	2.3a	1.8a
13.32	0.54	8.6ab	14.0c	5.7a	6.6def	9.0cd	3.2ab
22.20	0.54	8.6ab	4.3a	5.0a	4.6cd	11.5d	9.1de
44.0	0.54	8.3ab	7.1ab	3.7a	3.4bc	12.5d	5.bc

Values followed by the same letter within a column are not significantly different according to Duncan's multiple-range test at the 5% level.

Table 4. **Effects of different concentrations of BAP and NAA on shoot height of *A. erubescens* after four weeks of cultivation**

Growth regulator (μM)		Shoot height (mm)		
		Leaf age		
BAP	NAA	Young	Intermediate	Old
4.40	0	4.5e	3cd	1.3a
13.32	0	2.8bc	4.7e	1.7c
22.20	0	2.7bc	2.7bc	2.5c
44.0	0	3.5cde	3.7d	2.5c
4.40	0.54	1.3a	1.3a	1.7a
13.32	0.54	3.2bc	2ab	1.7a
22.20	0.54	1.2a	2ab	1.7a
44.0	0.54	2.3b	2.7bc	1.7a

Values followed by the same letter within a column are not significantly different according to Duncan's multiple-range test at the 5% level.

44.0 μM of BAP and their basal parts were thickened. Using different BAP concentrations did not help to avoid the growth of calli. However, use of the micropropagation system, which avoids callus formation, is highly desirable to reduce the chances of somaclonal variations during clonal plant production. BAP plus NAA produced the weakest shoot formation by petioles of different ages at the lowest concentration of BAP (4.40 μM) (Table 3). After four weeks of growth in MS medium, the height of the induced shoots varied depending on the age of the explant and the concentrations of the growth regulators. The highest (and least numerous) plantlets were obtained with young and intermediate-age petioles at BAP concentrations of 4.40 and 13.32 μM, respectively, with corresponding heights of 4.5 and 4.7 mm (Table 4). It should be noted that the highest BAP concentration (44.0 μM) in the medium caused the leaves of the shoots to be lighter in color than those formed at other BAP concentrations; moreover, these leaves were irregular in shape and had deformed leaves. However, when the medium contained BAP plus NAA the leaves were of regular appearance.

Root development from shoots was achieved on the MS ½ medium without auxins. Regenerated plants grew into the flowering stage and showed no apparent morphological alterations.

These results showed that the MS medium supplemented with 22.2 μM BAP was most effective, providing shoot regeneration for 100% of the explants with a higher number of shoots per explant. Moreover, petioles of intermediate age were most suitable for the *in vitro* regeneration of *A. erubescens* and for micropropagation of elite ornamental selections.

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ASARINA ERUBESCENS (A. GRAY) PENNEL SOMATINĖ EMBRIOGENEZĖ

Santrauka

Tiesioginė azarinų (*Asarina erubescens* (A. Gray) Pennel) organogenezė indukuota iš lapkočių eksplantų ant Murašigės ir Skugo mitybinės terpės tik su 4,40–44,0 mM citokininu – benzilaminopurinu (BAP) ir, esant tų pačių BAP koncentracijų sąveikai, – su 0,5 mM auksinu – naftilacto rūgštimi (NAR). Ir naudojant citokininą BAP, ir kartu su auksinu lapkočių eksplantų galuose po savaitės formavosi kalius, tačiau jis nebuvo embriogeninis, ūgliai susiformavo tiesiogiai iš epidermio ląstelių. Kaliaus formavimuisi didelę reikšmę turėjo lapkočių amžius – gausiausiai jį formavo vidutinio amžiaus lapkočių eksplantai. Ūgliai gausiausiai formavosi po 2–3 savaičių paveikus 22,20 mM BAP. Tiesioginei augalų regeneracijai iš *Asarina erubescens* lapkočių eksplantų NAR mitybinėje terpėje nebuvo būtinas.