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Adventitious shoot regeneration from leaf explants of *Gypsophila paniculata* L.

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Abstract Adventitious shoots were successfully regenerated from leaf explants of *Gypsophila paniculata* L. The efficiency of shoot regeneration for cv. Arbel was tested on 18 media based on Murashige and Skoog basal medium containing different concentrations of thidiazuron or 6-benzylaminopurine in combination with naphthaleneacetic acid. Both explant age and that of the cuttings used as leaf donors affected the regeneration efficiency. The highest efficiency of adventitious shoot regeneration was obtained with the oldest leaves originating from the youngest cutting analyzed; on thidiazuron-containing medium, shoots regenerated on average from 67% of the leaves, with an average of seven shoots per explant. This regeneration procedure was suitable for all six commercial cultivars studied. Regenerated shoots elongated, rooted and successfully acclimatized to the greenhouse where they were grown to flowering.

Key words Adventitious shoots · Gypsophila · Leaf explants · Regeneration

Abbreviations *BAP* 6-benzylaminopurine · *NAA* Naphthaleneacetic acid · *TDZ* Thidiazuron

Introduction

Gypsophila (*Gypsophila paniculata* L.), also known as baby's breath, is an important cut flower in commercial floriculture. In 1994 it was ranked 11th among all cut flowers exported from Dutch export auctions (Van Vliet 1995). Very little variation exists among the gypsophila varieties used fresh and dried as fillers for bouquets. All these gypsophila genotypes have white flowers, with one exception: cv. Flamingo, which has pale-pink flowers. Gypsophila is therefore an important target for classical breeding. However, breeding programs are severely restricted because these varieties are mainly unisexual and do not produce seeds (Shillo 1985).

Genetic engineering techniques could be useful in the creation of new breeding approaches to producing plant varieties with novel characteristics (Mol et al. 1995). However, adventitious shoot or embryo regeneration systems are a prerequisite for these techniques and, to the best of our knowledge, no such systems have been reported for gypsophila. Tissue culture techniques in gypsophila are currently being used mainly for micropropagation and disease elimination, through shoot tip culture (Henry 1993).

In the present study, two cytokinins in combination with an auxin were tested for their ability to induce adventitious shoot regeneration from leaves at different stages of development. The system developed for adventitious shoot regeneration was found to be both efficient and suitable for all six commercial cultivars studied, and is therefore expected to pave the way for the molecular-biology-based breeding of gypsophila.

Materials and methods

Plant material

Unrooted cuttings of gypsophila (*G. paniculata* L.) cultivars Arbel, Golan, Gilboa, Tavor, Perfecta, and Flamingo were obtained from Danziger "DAN" Flower Farm (Moshav Mishmar Hashiva, Israel). Plants were grown under standard greenhouse conditions and cuttings were harvested and stored for up to 2 weeks at 4°C.

Tissue culture

Cuttings were rinsed with 70% ethanol, then sterilized for 10 min in 1.5% (wt/vol) sodium hypochlorite and rinsed three times for 10 min

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each in sterile water. The five youngest fully expanded leaf pairs below the first pair of leaves covering the apical meristem were excised from each cutting. Leaves were numbered I (youngest) through V (oldest), and removed from the stem in two steps: the fused leaf bases were sliced and then a forceps was used to carefully peel off the leaf. To prepare leaf explants, each leaf was cut twice: longitudinally to divide the leaf into two halves and then across to shorten the explant to about 1 cm. Explants were cultured with their adaxial side in the medium, and after 7 days in culture they were shortened again to about 0.5 cm in length. Murashige and Skoog (MS) (1962) basal medium, supplemented with 30 g/l sucrose and 0.5 g/l tryptone, and solidified with 8 g/l agar oxoid (hereafter IMS medium) was used in all adventitious shoot regeneration experiments. Prior to autoclaving (121°C for 20 min), growth hormones were added to the IMS medium and the pH was adjusted to 5.8. Three concentrations of the cytokinins thidiazuron (TDZ) or 6-benzylaminopurine (BAP) (1.0, 3.0, 6.0 mg/l), in combination with 0, 0.3, or 1.0 mg/l of the auxin naphthaleneacetic acid (NAA), were tested. After 30 days of culture, the plantlets were transferred to SE medium, consisting of MS basal medium supplemented with 30 g/l sucrose, 0.1 mg/l NAA, and 0.1 mg/l gibberellic acid, and solidified with 8 g/l agar oxoid, pH 5.8. Shoot regeneration from leaves of in-vitro-cultured plantlets was obtained as described for greenhouse-grown plants.

All cultures were maintained in a growth room at 25±1°C under a 16-h photoperiod using cool white light (60 µmol m^{-2} s^{-1}). The medium was replaced every 2 weeks. Leaves from at least ten cuttings were cultured on each medium and shoot regeneration efficiency was assessed after 42 days in culture. Shoots developing from axillary buds, occasionally taken together with the leaf explants, could be easily recognized after 1 week in culture and such explants were excluded from further analysis. All experiments were repeated three times and, unless otherwise stated, were performed with standard commercial cuttings used for plant propagation collected 28 days after pruning.

Transfer to soil

Elongated shoots (about 2 cm in length) were rooted in glass jars with vented caps (Osmotek, Rehovot, Israel), on half-strength MS basal medium containing 0.1 mg/l gibberellic acid and 0.1 mg/l NAA, solidified with 8 g/l Difco Bacto agar. The pH was adjusted to 5.8 prior to autoclaving. After about 35 days in culture, roots were cleaned of agar and regenerated plants were transferred to pots containing peat and pumice (Solit potting soil, Soli, Kiryat Malachi, Israel). After 1 week in an aeroponic fogger (Shira Aeroponics, Rehovot, Israel), plants were transferred to the greenhouse and kept under periodic mist (20 s every half hour). Following 2 weeks of misting, plants were moved to a commercial greenhouse for further growth.

Results and discussion

Shoot regeneration

Shoot regeneration from cv. Arbel leaves was tested on the 18 media described in Materials and methods. After 1 week of culture, the explants became swollen, turned dark green and more than doubled in length on all media tested. Callus began to accumulate on both adaxial and abaxial sides and after 12–15 days of culture, shoot formation was visible by stereomicroscope (Fig. 1). On all media, the new primordia always regenerated directly from the transition zone between leaf and stem tissue and never from callus, the accumulation of which was observed mainly on media

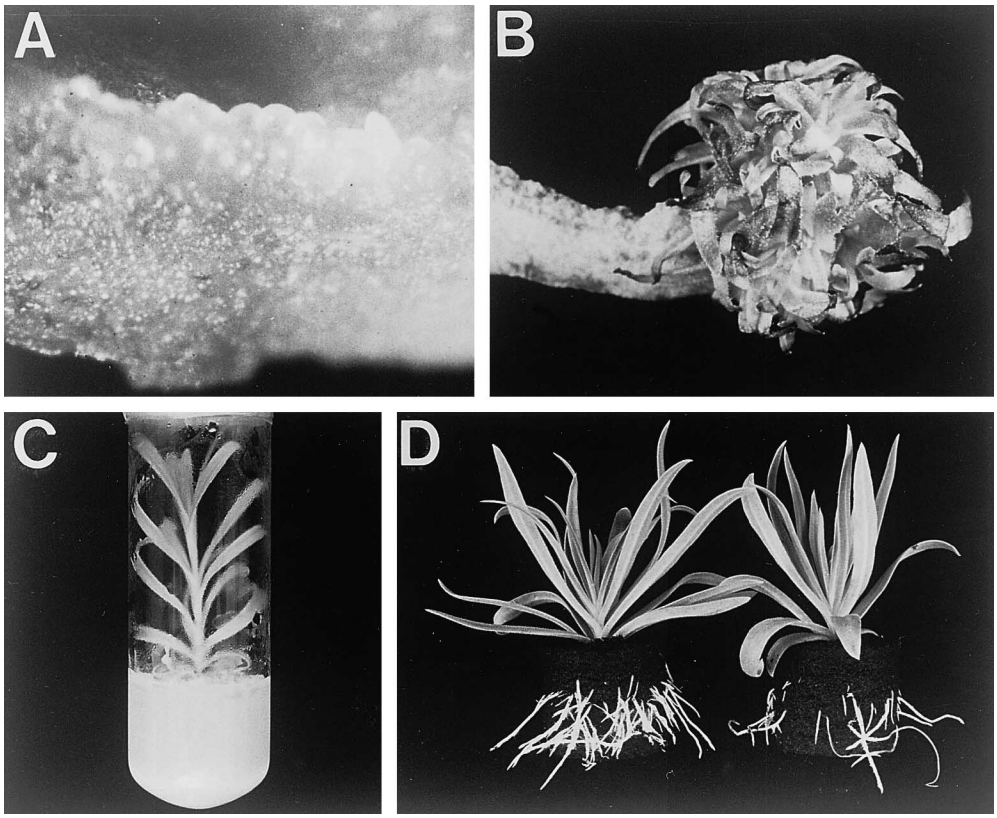

Fig. 1A–D Adventitious shoot regeneration from cv. Arbel leaf explant. **A** Adventitious shoot proliferation from the basal zone of the leaf after 12–15 days in culture. **B** Multiple shoot formation on the explants after 35 days in culture. **C** Rooting of an elongating adventitious shoot. **D** Regenerated plants established in soil

Table 1 Percentage of leaves forming shoots (*LFS*) and average number of shoots per leaf (*AS*) produced from cv. Arbel. Five pairs of leaves from ten cuttings were cultured on media containing TDZ or BAP in combination with NAA. Overall efficiency (*OE*) of shoot regeneration was calculated as the number of shoots per leaf multiplied by the percentage of leaves forming shoots. Regeneration efficiencies were evaluated following a total of 42 days in culture medium. The average number of shoots is given ±SE

Cytokinin/auxin (mg/l)	TDZ/NAA			BAP/NAA		
	LFS (%)	AS (n)	OE (n)	LFS (n)	AS (n)	OE (n)
1/0	35	3±1	105	18	2±1	36
1/0.3	35	3±1	105	20	4±1	80
1/1	30	4±1	120	18	4±1	72
3/0	46	5±1	230	23	3±1	69
3/0.3	47	5±1	235	16	3±1	48
3/1	46	6±1	276	24	3±1	72
6/0	40	3±3	240	29	4±1	116
6/0.3	45	5±2	225	24	3±1	72
6/1	42	6±2	252	24	3±1	72
Average for all media	41	5±1	199	22	3±1	71

containing a high concentration of NAA. Shoot regeneration occurred only from the base of the explants. Fine dissection of the leaf explant prior to its culture did not yield shoot regeneration from other parts of the leaf, and shoot formation remained restricted to the basal zone of the leaf (not shown). This is in agreement with Van Altvorst et al. (1992), who reported the initiation of adventitious shoots from the transition tissues of carnation (*Dianthus caryophyllus* L.) leaf explants.

Irrespective of the media used, some gypsophila shoots (5%) were slightly vitrified. However about 99% of the regenerated plants were successfully rooted and following transfer to the greenhouse, plant vitrification was no longer observed (Fig. 1).

Both cytokinins (TDZ and BAP) induced shoot regeneration with no addition of auxin to the medium (Table 1). Shoot formation efficiency was much higher on TDZ-containing media than on those containing BAP. The average percentage of leaves forming shoots on all tested TDZ-containing media was about twice that on BAP-containing media, and the average overall regeneration efficiency on the former was almost three times that on the latter (Table 1). The ability of TDZ to enhance the shoot regeneration frequency has been reported for various species (Hutteman and Preece 1993). Moreover, in carnation which, like gypsophila, is a member of the Caryophyllaceae family, comparative studies have shown TDZ to be the most effective cytokinin for adventitious shoot regeneration from stem explants (Nugent et al. 1991).

Van Altvorst et al. (1994) reported that adventitious shoot regeneration from leaves obtained from greenhouse-grown plants is more efficient than from leaves of in-vitro-grown plants. In gypsophila we found no such difference. Shoots regenerated from about 50% of the leaves from in-vivo-grown gypsophila plants, with an average number of 4±1 shoots per leaf.

Hormone concentrations of 3 mg/l TDZ and 0.3 mg/l NAA were chosen for subsequent experiments. The low concentration of NAA was mainly selected to minimize callus production.

Effect of tissue age on regeneration efficiency

The regeneration capacity of leaves from the youngest cuttings was much higher than that from the oldest cuttings (Table 2). Improved regeneration results were obtained with respect to both the percentage of leaves forming shoots and the number of shoots per leaf. Overall, per cutting, about 2.5 times more shoots were obtained from 18-day-old cuttings than from 35-day-old cuttings. Moreover, leaf position, especially in the youngest cuttings, strongly affected regeneration efficiency (Table 2). For 18-day-old cuttings, about 2.5 times more shoots were produced from the oldest leaf (V) than from any of the first three leaves (I–III), and about 1.4 times more than from the fourth leaf (IV). Decreasing tissue regeneration potential with increasing donor plant age has been shown for a number of plant systems. Using stem segment explants, Zuker et al. (1995) reported that, similar to gypsophila, the youngest carnation cuttings show the highest shoot regeneration efficiency. However, unlike gypsophila, where the regeneration potential from the youngest cutting increased with leaf age, the youngest carnation leaves were shown to possess the highest regeneration capacity (Van Altvorst et al. 1992). This difference may be because in gypsophila, much more stem tissue remained associated with the older leaves, which are bigger and easier to handle, than with the young leaves. Stem tissue removed with leaves has been strongly implicated in the stimulation of shoot regeneration in carnation (Van Altvorst et al. 1994).

Table 2 Effect of cutting and leaf age on the percentage of leaves forming shoots (*LFS*) and the average number of shoots per leaf (*AS*) produced from cv. Arbel. Overall efficiency (*OE*) of shoot regeneration from leaves was calculated as described in Table 1. Regeneration efficiencies were evaluated following a total of 42 days in culture on medium containing 3 mg/l TDZ and 0.3 mg/l NAA. The average number of shoots is given ±SE

Leaf position	Cutting age								
	18 days			28 days			35 days		
	LFS (%)	AS (n)	OE (n)	LFS (%)	AS (n)	OE (n)	LFS (%)	AS (n)	OE (n)
I	48	4±1	192	40	4±1	160	36	3±1	108
II	48	4±1	192	44	4±1	176	41	3±1	123
III	49	4±1	196	44	4±1	176	35	3±1	105
IV	49	7±1	343	46	5±1	230	35	3±1	105
V	67	7±1	469	60	6±1	360	36	4±1	144
Average for all leaves	52	5±1	278	47	5±1	220	37	3±1	117

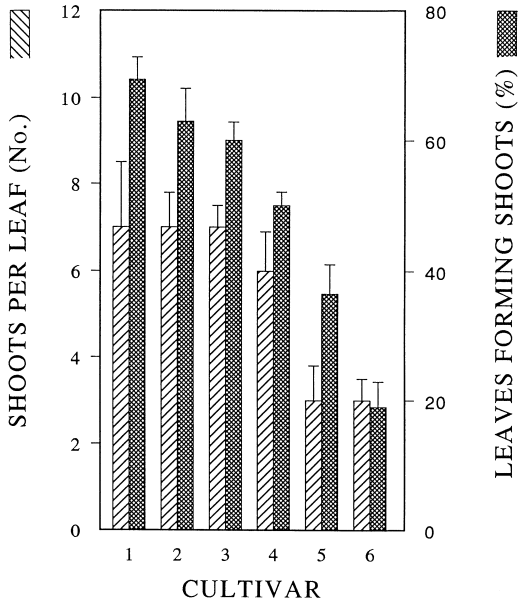

Fig. 2 Percentage of leaves forming shoots and average number of shoots per leaf for various cultivars of G*ypsophila paniculata* L. Explants originating from the fourth and fifth leaf pairs only were cultured in the presence of 3 mg/l TDZ and 0.3 mg/l NAA. Regeneration efficiencies were evaluated following a total of 42 days in culture (*1* Arbel, *2* Gilboa, *3* Golan, *4* Tavor, *5* Flamingo, *6* Perfecta)

Regeneration of various cultivars

The applicability of the regeneration procedure to various gypsophila genotypes was analyzed (Fig. 2), using only the fourth and fifth leaf pairs derived from 18- to 24-day-old cuttings. Regeneration was obtained from all genotypes studied. Cvs. Arbel, Gilboa, and Golan regenerated an average of over six shoots per leaf from more than 50% of the leaves. Cv. Perfecta was the least responsive genotype, and may require fine-tuning of the media components to optimize its regeneration efficiency. It is worth noting that differences in regeneration efficiencies among varieties are a well-established phenomenon in various species (De Jong et al. 1993, Zuker et al. 1995).

In summary, a new procedure for adventitious shoot regeneration from gypsophila leaves has been described. In the future, using biotechnological approaches, the suitability of this system for a variety of cultivars should enable the introduction of useful traits into this important cut flower.

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References

De Jong J, Redmaker W, Wordragen MF (1993) Restoring adventitious shoot formation on chrysanthmum leaf explants following cocultivation with *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult 33:105–119

Henry M (1993) *Gypsophila paniculata* L. (Baby's Breath): in vitro culture and the production of gypsogenin saponins. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 21. Springer, Berlin Heidelberg New York, pp 187–206

Hutteman CA, Preece EJ (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult 33: 105–119

Mol JNM, Holton TA, Koes RE (1995) Floriculture: genetic engineering of commercial traits. Trends Biotechnol 13:350–355

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473–497

Nugent G, Richardson T, Lu CY (1991) Plant regeneration from stem and petal of carnation. Plant Cell Rep 10:477–480

Shillo R (1985) *Gypsophila paniculata*. In: Halevy AH (ed) Handbook of flowering, vol 3. CRC, Boca Raton Florida, Fla, pp 83–87

Van Altvorst AC, Koehorst HJJ, Bruinsma T, Jansen J, Custers J, De Jong J, Dons JJM (1992) Adventitious shoot formation from in vitro leaf explants of carnation (*Dianthus caryophyllus* L). Sci Hort 51:223–235

Van Altvorst AC, Koehorst HJJ, Bruinsma T, Dons JJM (1994) Improvement of adventitious shoot formation from carnation leaf explants. Plant Cell Tiss Org Cult 37:87–90

Van Vliet C (1995) The proportion of imported out of total auction flowers increases from 15.9% to 17.7% (in Dutch). Bloweniste-rij 20:66–67

Zuker A, Chang LPF, Ahroni A, Cheah K, Woodson WR, Bressan RA, Watad AA, Hasegawa PM, Vainstein A (1995) Transformation of carnation by microprojectile bombardment. Sci Hort 64:177–185