HortScience 23(2):393–395. 1988.

Plantlet Regeneration from Protoplasts of *Petunia alpicola*

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Additional index words. tissue culture

Abstract. **The isolation, culture, and plant regeneration of protoplasts derived from callus or suspension cultures of *Pentunia alpicola* Smith and Downs was established. Protoplasts formed macrocalli, at 85% percent plating efficiency, in Murashige and Skoog (MS) liquid medium containing 2,4–D (1.0 mg·liter^{-1}), NAA (0.5 mg·liter^{-1}), BA (0.5 mg·liter^{-1}), and 20% coconut water. Shoot regeneration from macrocalli occurred at 65% frequency on MS containing zeatin (1.0 mg·liter^{-1}), and shoots were readily rooted on either MS containing NAA (0.01 mg·liter^{-1}) or IBA (1.0 mg·liter^{-1}). However, plantlets failed to grow in artificial planting medium. The results provide the basis for somatic cell genetic studies for a *Petunia* sp. that has distincitive morphology compared to cultivated petunias. Chemical names used: (2,4-dichlorophenoxy)acetic acid (2,4–D), 1-naphthaleneacetic acid (NAA), *N*-(phenylmethyl)-1*H*-purin-6-amine (BA), (*E*)-2-methyl-4-(1*H*-purin-6-ylamino)-2-buten-1-ol (zeatin), and 1*H*-indole-3-butyric acid (IBA).**

Regeneration of plants from protoplasts has been reported for many members of the genus *Petunia* (1, 2, 7). These early studies focused on *Petunia* sp. possessing 2n = 2x = 14 chromosomes, with one exception *P. parviflora* Juss., with 2n = 2x = 18 chromosomes (8). Like *P. parviflora, P. alpicola* also has 2n = 2x = 18 chromosomes and a creeping growth habit, short-petioled succulent leaves, and small magenta flowers (Fig. 1). All these features are in distinct morphological contrast to the 2n = 14 *Petunia* sp. The two 18-chromosome *Petunia* spp. have been recognized by breeders as potential germplasm resources for introgression of desirable genes into cultivated *P. hybrida* Hort., but sexual incompatibility exists (ref. 9 and Ford-Logan, unpublished data). Thus, somatic cell manipulations may be required to realize this potential exploitation. This study was conducted to devise a protocol for the isolation, culture, and regeneration of plants from *P. alpicola* protoplasts.

Plants of *P. alpicola* were obtained from M. Hanson, Cornell Univ., Ithaca, N.Y. Since *P. alpicola* is self-incompatible, a micropropagation system was developed to supply plant material. Excised shoot tips, 1 cm in length, were inserted in Murashige and Skoog (MS) (4) medium + 1.0 mg·liter^{-1} zeatin, and the cultures were placed at 27°C on 16-hr exposure to 17 μmol·s^{-1}·m^{-2} (G.E. F40CW-RS-WM). Every 4 weeks, proliferating shoots were subcultured and some individual shoots were maintained on MS medium for the experiments. Leaves of *P. alpicola* proved to be technically unsuitable for protoplast isolation due to their small size.

Leaf pieces (1 to 2 mm^2) from shoots on MS initiated friable callus when placed on MS + 1.0 mg·liter^{-1} 2,4-D. Primary, dark green callus, 5- to 6-weeks-old, was subcultured, placed at 28°C under 58 μmol·s^{-1}·m^{-2} for 16 hr (G.E. F96-T12-CW), and subcultured thereafter every 21 days. Suspension cultures were established by transfer of 3 to 4 g of callus into liquid MS + 1.0 mg·liter^{-1} 2,4-D (30 ml/flask) and maintained by subculturing every 10 to 14 days. These cultures were maintained in 125-ml flasks on a gyratory shaker at 90 rpm at 27° in diffuse light (16 hr).

Protoplasts were isolated from both callus and cell suspension cultures. Callus (≈3 g and 2 weeks after subculture) was gently separated by passage through a coarse sieve (35 μm) placed in 100 × 15 mm plastic petri dishes and rinsed with a cell protoplast washing (CPW) solution (1) containing 8% (w/v) mannitol (M). Cells in suspension cultures, 7 days after subculture, were collected by transferring the cells to 16 × 125 mm culture tubes and pelleting by centrifugation (80× *g*; 5 min). The growth medium was replaced by CPW 8M and the cell slurry was pipetted onto a coarse sieve (35 μm) and separated in the same manner as the calluses. Callus and suspension cells were subsequently handled in the same manner. They were plasmolyzed in CPW 8M solution for 1 hr at 25°C without agitation. The cell slurry was transferred to screw-capped culture tubes, pelleted by centrifugation (80× *g*; 5 min), and the supernatant was replaced by a filter-sterilized enzyme solution, consisting of 2% Cellulysin and 2% Macerase (Calbiochem), 2% Driselase (Kyowa Hakka Kogyo, Japan), and 8% (w/v) mannitol dissolved in CPW salt solution, pH 5.8. The cells in enzyme solution (≈5 ml packed; 20 ml) were transferred to 100 × 15 mm petri dishes, wrapped with Parafilm, and incubated for 17 to 19 hr in the dark at 27° on a gyratory shaker (35 rpm). Following incubation, the cells were gently teased with a Pasteur pipette to release protoplasts further. The enzyme–protoplast mixture was passed through a fine sieve (61 μm), collected in 100 × 15 ml petri dishes,

Table 1. Media for *P. alpicola* protoplast culture and shoot regeneration.

Compound	Protoplast culture	Shoot regeneration
MS salts and vitamins (mg·liter^{-1})		
2,4-D	1.0	---
NAA	0.5	---
BA	0.5	---
Zeatin	---	1.0
Coconut water (ml)	20	---
Sucrose (mg·liter^{-1})	30,000	30,000
Mannitol (mg·liter^{-1})	130,000	---
Agar (mg·liter^{-1})	---	4.0
pH	5.8	5.8

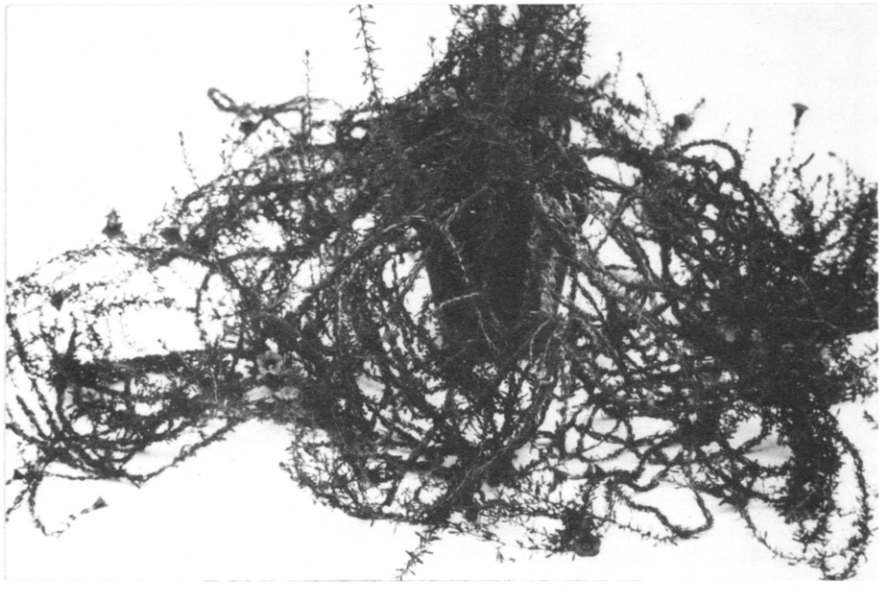

Fig. 1. Flowering plant of *Petunia alpicola.*

Received for publication 21 May 1987. Michigan Agricultural Experiment Station Journal Article no. 12319. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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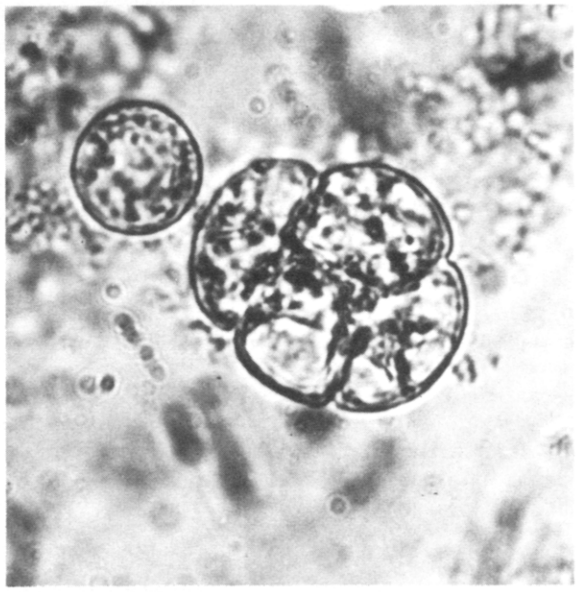

Fig. 2. Second division—four-cell stage of cultured protoplast.

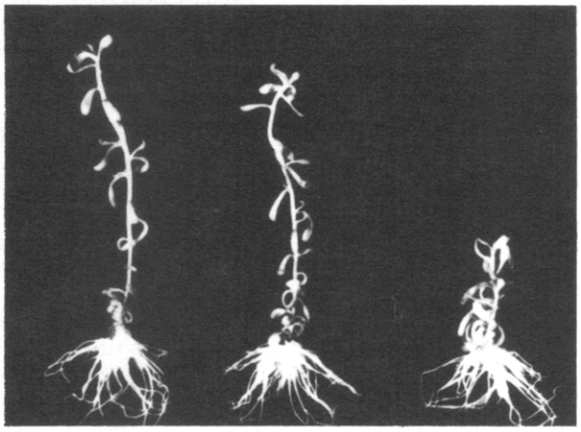

Fig. 3. Regenerated shoots rooted on MS + 1.0 mg·liter^{-1} IBA.

Table 2. Plating efficiencies of *P. alpicola* in modified culture media with protoplasts plated at 1 × 10^5 per ml.

Media component				
2,4-D (mg·liter)	NAA (mg·liter)	BA (mg·liter)	Coconut water (mg·liter^{-1})	Plating efficiency (%)
1.0	0.5	0.5	20	85
1.0	0.5	0.5	---	60
---	0.5	0.5	20	0
1.0	---	0.5	20	0
1.0	0.5	---	20	63
---	0.5	0.5	---	0
1.0	---	0.5	---	0
1.0	0.5	---	---	42

and transferred to culture tubes. The protoplasts were pelleted by centrifugation (100× *g*; 5 min) and the supernatant was removed. Protoplasts were washed free of enzyme by resuspension in CPW 8M and centrifuging (100× *g*; 5 min). The supernatant was replaced by 6 ml of a CPW solution containing 25% sucrose (S) and centrifuged (100× *g*; 15 min). After a final wash in CPW25S, protoplasts at the surface were removed and resuspended in protoplast culture medium (Table 1), counted, and plated in test densities in 60 × 15 mm plastic petri dishes.

Four milliliters of protoplasts were plated in each dish, which were then wrapped with Parafilm and incubated at 25°C with constant illumination of 15 μmol·s^{-1}·m^{-2} (G.E. F20-T12-CW). Viability of protoplasts was monitored by FDA (12).

Dishes were replenished with culture medium, mannitol level held constant, during feeding at 2- and 4-week intervals after plating. Plating efficiency (PE) was determined as the percentage of viable protoplasts 24 hr after isolation that produced cell colonies after 7 days. After ≈6 weeks in culture, the dishes were decanted of used culture medium and the microcalli were placed between two layers of semi-solid agar. This placement was done by adding the microcalli plus 1 ml of fresh MS culture medium to a dish already containing 2 ml of 0.4% agar-solidified medium and layering an additional 1 ml of cooled (45°C) agar culture medium on top and swirling gently. When the cell colonies were of sufficient size, after 2 to 6 weeks of further growth, they were transferred to shoot regeneration media (Table 1).

Protoplast yields were consistently 2 to 3 × 10^6 per g of callus or suspension culture cells. Callus and suspension cultures were readily established and there was no difference in their suitability or response when isolating and culturing protoplasts. Yields were considerably reduced (6 × 10^5) when protoplasts were isolated from cultures nearing the time of subculture. Protoplasts isolated by this method were initially spherical in shape and, after a day in culture, they became oval-shaped, indicating cell wall resynthesis (3) with active cytoplasmic streaming. First division occurred within 48 to 72 hr, and with repeated divisions (Fig. 2) the number of chloroplasts per cell visibly decreased and the cells became more vaculolated, with micro-colony formation observed after 4 days. Pale green, visible colonies (0.3 to 1 mm in diameter) were formed in another 3 to 4 weeks.

Several modifications of the MS-based medium were tested for efficiency of protoplast culture (Table 2). Two tests, media containing BA and NAA, each at 0.5 mg·liter^{-1} with or without coconut water, and lacking 2,4-D, resulted only in budding of protoplasts plated at 1 × 10^5/ml, no first division, and a PE of zero (Table 2). The growth regulator 2,4-D was apparently more important in regulation of cell division than in cell wall regeneration, because in the one test culture medium that lacked only coconut water, protoplasts were observed dividing with a PE of 60% (Table 2). The addition of coconut water to the culture medium containing 2,4-D, NAA, and BA increased the PE to 85%. The 25% increase in PE might be due to the stimulating synergistic effect of coconut water and 2,4-D, as seen by Steward and Caplin (10) with the culture of potato tuber cells. Coconut water generally is believed to contain cytokinin-like substances as well as reduced nitrogen and possesses detoxifying properties, all of which may have value for certain tissue cultures (10, 11).

In initial experiments, the culture dishes were replenished every 2 weeks after plating with 0.5-ml aliquots of the appropriate culture medium containing reduced mannitol levels of 11%, 9%, 6%, 4%, and 0%. This standard dilution procedure resulted in browning and eventual death of all cell colonies. Likewise, transfer of p-calli from liquid culture medium to filter paper laid on agar medium (6) was unsuccessful.

The transfer of microcalli to soft agar was critical in sustaining growth. Plating the p-calli between layers of semi-solid agar allowed further growth and development of the green, discrete colonies. Low concentrations of 0.2% to 0.4% agar apparently allowed a

favorable physical relationship. This method is a modification of that used by Nagata and Takebe (5) for tobacco.

p-Calli transferred to regeneration medium 10 to 12 weeks after plating produced shoots at 65% efficiency. At this stage, the p-calli were moved to a light intensity of 58 $\mu mol \cdot s^{-1} \cdot m^{-2}$ (G.E. F96-T12-CW) for 16 hr at 28°C. Shoot tips 2 cm or longer were dissected and transferred singly to rooting media, either MS with 0.01 $mg \cdot liter^{-1}$ NAA or 1.0 $mg \cdot liter^{-1}$ IBA. MS + NAA gave rise to many short, thick roots (15 to 20 per shoot tip) and concomitant formation of callus tissue at the base of the shoot tips. MS + IBA induced an increased number of roots (20 to 30 per shoot), although they were long and thin (Fig. 3). Root primordia generally emerged between the first and second week, although a few shoots had 1- to 3-mm roots after 6 days. Both auxins produced 100% rooting of shoots. In either medium, 4 weeks in culture were allowed for adequate root development.

Establishment of the plantlets directly into soil or artificial planting medium was unsuccessful. In a mixture of peat, perlite, and vermiculite (V.S.P.-Bay Houston Towing Co.), necrosis of the roots occurred from apparent fungal infection. A survival rate of two out of six regenerates was obtained when the plantlets were transferred to plastic cell pack containing sterilized perlite, but, after limited growth, plantlets were sensitive to moisture stress and susceptible to pathogens. Gradual opening of covering polyethylene bags and increased agar concentration to decrease growth rate and improve hardiness were attempted, but failed.

This study indicated that plantlets can be readily regenerated from p-calli of *P. alpicola* protoplasts and provides an experimental basis for future work in somatic cell genetics with this species.

Literature Cited

1. Frearson, E.M., J.B. Power, and E.C. Cocking. 1972. The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev. Biol. 33:130–137.
2. Hayward, C. and J.B. Power. 1975. Plant production from leaf protoplasts of *Petunia parodii*. Plant Sci. Lett. 4:407–410.
3. Kao, K.N., W.A. Keller, and R.A. Miller. 1970. Cell division in newly formed cells from protoplasts of soybean. Expt. Cell Res. 62:338–340.
4. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497.
5. Nagata. T. and I. Takebe. 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–20.
6. Niedz, R.P., S.M. Rutter, L.W. Handley, and K.C. Sink. 1985. Plant regeneration from leaf protoplasts of seven tomato cultivars. Plant Sci. Lett. 39:199–204.
7. Power, J.B., E.M. Frearson, D. George, P.K. Evans, S.F. Berry, C. Hayward, and E.C. Cocking. 1976. The isolation, culture and regeneration of leaf protoplasts in the genus *Petunia*. Plant Sci. Lett. 7:51–55.
8. Sink, K.C. and J.B. Power. 1977. The isolation, culture and regeneration of leaf protoplasts of *P. parviflora*. Juss. Plant Sci. Lett. 10:335–340.
9. Sink, K.C. and J.B. Power. 1978. Incongruity of interspecific and intergeneric crosses involving *Nicotiana* and *Petunia* species that exhibit potential for somatic hybridization. Euphytica 27:725–730.
10. Steward, F.C. and S.M. Caplin. 1951. A tissue culture from potato tuber: The synergistic action of 2,4-D and of coconut milk. Science 113:518–520.
11. Tulecke, W., L.H. Weinstein, A. Rutner, and H.J. Laurencot, Jr. 1961. The biochemical composition of coconut water (coconut milk) as related to its use in plant tissue culture. Contrib. Boyce Thompson Inst. 21:115–128.
12. Widholm, J.M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47:189–194.