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The Interspecific Hybrid *Petunia parodii* x *P. inflata* and its Relevance to Somatic Hybridization in the Genus *Petunia**

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Summary. Attempts at the reciprocal cross between *Petunia parodii* and *P. inflata* using standard emasculation and pollination techniques failed. Limited pollen tube growth down the style in reciprocal crosses led to reproductive isolation between the self-compatible *P. parodii* and self-incompatible *P. inflata*. The interspecific hybrid was successfully produced by bud-pollination of *P. parodii* with *P. inflata* as the male parent in 22 percent of attempts, but not in the opposite direction. In vitro pollination of *P. parodii* ovaries with *P. inflata* pollen also produced hybrids. The small size of the ovary made it technically impossible to use *P. inflata* as the female parent for in vitro pollination. The interspecific hybrids were intermediate, as compared to the two parents, for six of the seven plant and flower characters measured. Furthermore, the hybrids had high pollen fertility, set abundant seed upon self-pollination, and readily inter-crossed with the parental species. The results are consistent with a high degree of chromosomal homology in the parental species and with minor genetic divergency leading to reproductive isolation that is pre-zygotic in nature. Overcoming the barriers to cross-incompatibility by practical techniques resulted in fertile interspecific hybrids that segregated for parental characters. The potential value of employing the parental species in somatic hybridization experiments is discussed.

Key words: *Petunia* – Unilateral cross-incompatibility – Bud-pollination – In vitro pollination – Somatic hybridization

Introduction

Our studies on the genus *Petunia* have been primarily concerned with the inheritance of monogenic traits (Sink 1973), their action in morphological development (Natarella and Sink 1971) and biochemical expression (Knowlton and Sink 1977) and the regeneration to whole plants of cultured protoplasts (Hayward and Power 1975; Power et. al. 1976b). The crossing behaviour of *P. hybrida* genetic lines with *P. axillaris, P. inflata, P. parodii* and *P. violacea* has also been examined (Sink 1975). During these studies, the intercrosses among these selected *Petunia* species were made not only to determine the origin of the cultivated types, but to identify possible new sources of variation, and to define taxonomic relationships. We observed that all the *Petunia* species employed readily intercrossed among themselves and with selected *P. hybrida* cultivars. An exception proved to be the attempted cross between *P. parodii* and *P. inflata*. We report here our subsequent studies in which we have examined by histological procedures, the stage where failure occurs in the reproductive cycle between these two species and the successful production of the interspecific hybrid by the in vitro pollination method and by bud-pollination. The information gained is important in view of the current aim of plant breeders and geneticists to extend the degree of genetic variation that can be incorporated into economically important crop and horticultural species. The results are discussed in relation to two in vitro approaches that are presently being intensively studied and further developed for their potential in overcoming sexual incongruity at various taxonomic levels. These methods include in vitro pollination (Rangaswamy 1977) and somatic hybridization (Smith 1974).

Materials and Methods

The *Petunia* species *P. inflata* Fries (Fries 1911) 2n = 14, and *P. parodii* W.C.S. (Steere 1930) 2n = 14 were obtained (Natarella and Sink 1975) and verified by Dr. L.B. Smith (Personal Communication 1972). Voucher specimens of these two species and their interspecific hybrid have been deposited in the U.S. National Herbarium. The plants used in this study were grown from seed ob-

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Table 1. Plant and flower character means and standard deviations for the interspecific hybrid *Petunia parodii* by *P. inflata* and the parental species. Measurements are in centimeters

				Corolla						
Species	Veg lv length	Veg lv width	Pedicel length	Total length	Tube length	Lobe diameter	Length fillament attach	Percent fillament attach	Flower color	Percent Pollen Viability
inflata	6.0 ± 0.86	2.9 ± .48	3.5 ± .47	2.7 ± .19	1.5 ± .15	2.8 ± .31	0.4 ± .40	26.6	magenta	95.6
parodii	8.8 ± 1.54	3.1 ± .51	4.0 ± .85	9.1 ± .50	6.9 ± .38	4.2 ± .61	3.8 ± .25	55.0	white	99.0
parodii *inflata*	8.6 ± 1.58	3.5 ± .68	3.8 ± .92	4.7 ± .94	3.0 ± .67	3.5 ± .73	1.1 ± .37	36.7	lt. magenta	93.4

tained by sibcrossing a minimum of 4 plants of each generation and massing the harvested seed. Seed was germinated and the plants grown to flowering under greenhouse conditions. Reciprocal cross pollinations were attempted between the two *Petunia* species by emasculating flowers in the bud stage one day prior to anthesis and pollinating in the standard manner. Bud-pollination between the parental *Petunia* species was achieved by slitting open the corolla tube at various lengths of bud elongation prior to anthesis. Immediately after opening the corolla tube, and following emasculation, the immature stigma was pollinated and the cross tagged. All reciprocal standard and bud-pollinated crossing attempts were recorded relative to success or failure to set seed. Seed counts, on a per capsule basis and for ten capsules, were made for standard self- and bud-pollinations of the parents together with the successful crosses.

Pollen grain germination and tube penetration in the style was observed in reciprocal standard and bud-pollinations between *P. parodii* and *P. inflata*, 24 h after pollination, by use of the analine blue fluorescence technique (Linskens and Esser 1957). In vitro pollination followed the procedure of Rangaswamy and Shivanna (1967) as described for *Petunia*. Hybrid seed and that of the parents was sown on March 23, 1973, on terralite. Germination was at 22° ± 2 in an environmental chamber with a 16 h photoperiod of 10,500 lux. Seedlings were transplanted to 2-1/4" peat pots on April 16, and plantlets into 5" clay pots on May 7, using a pasteurized soil mix (soil, peat moss, and perlite, 1:1:1 v/v/v). The plants were grown to flowering in the greenhouse incorporating standard cultural, disease and insect practices. At flowering, seven plant and flower characters were recorded (Table 1). The phyllotaxy of *Petunia* is such that during vegetative growth the leaves are opposite and when flowering commences, they become alternate with each leaf subtending a solitary flower. Thus, leaf measurements were made on the sixth pair of opposite leaves prior to flowering. The first pair of opposite leaves being designated those subtending the first alternate leaf. Data on the floral characters was obtained from the tertiary flower to bloom on an unbranched stem. At anthesis this flower was detached at the pedicel base by a single cut with a sharp razor blade, placed in a vial of water and the measurements subsequently recorded.

Corolla length was defined as the distance from the base of the corolla tube to the tip of the lobe when the flower was pressed flat. Corolla tube length was measured as a portion of the total length of the corolla after the lobe was removed at the tapered point of the flower throat (Fig. 1). Filament attachment was the length of adnation of the filament to the inside of the corolla tube whilst percent filament attachment was calculated as the length of filament attachment/corolla tube length X 100. The experimental design was a randomized block with four replicates, 8 plants per replicate and duplicate data obtained from each plant by random selection of two unbranched stems.

Results and Discussion

Cross-incompatibility between *P. parodii* and *P. inflata* was confirmed using the standard pollination technique for hybridizing *Petunia*. Approximately 500 pollinations were conducted in each direction. In addition, pollination of mature stigmas throughout different growing seasons failed to produce hybrid seed. The corolla of pollinated flowers of both species wilted and abscissed, the ovary turned brown and shriveled within 10-14 days, but the calyx persisted. Histological observations of pollen grain germination and pollen tube growth in the styles of the reciprocal crosses indicated the cross-incompatibility was pre-zygotic in nature. Twenty-four hours after pollination, the pollen grains of *P. parodii* had germinated readily on the stigmatic surface but the pollen tubes failed to penetrate further than the neck region of the style of *P. inflata*. In the reciprocal cross, *P. inflata* pollen grains ger-

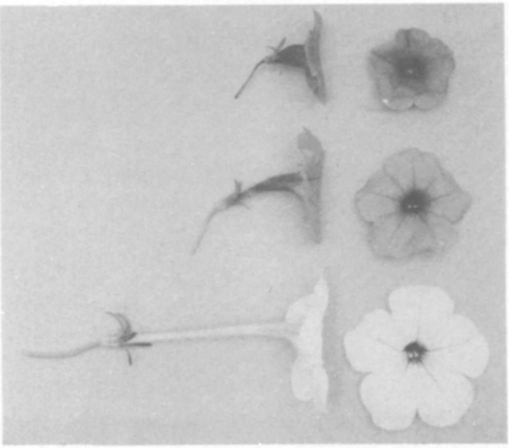

Fig. 1. Flowers of, top to bottom, *Petunia inflata*, interspecific hybrid *P. parodii* X *P. inflata, P. parodii*

minated in high numbers but pollen tube growth only penetrated 1.6 to 1.9 cm down the 5.4 cm long mature style of *P. parodii*. *Petunia parodii* plants set abundant seed upon normal self-pollination but *P. inflata*, a gametophytic self-incompatible species (Brewbaker and Majumder 1961), was inconsistent. Approximately 100 self-pollinations were carried out on 6 seedling *P. inflata* plants. Two seedlings failed to set self-seed and the 4 remaining ones ranged from 4-25 seed capsules per plant; the capsules averaged 8.9 seeds.

Subsequently, hybrids were obtained by bud-pollination or by the in vitro pollination technique. Hybrids were only produced by the bud-pollination method when *P. parodii* was the female parent. Eighteen out of 80 bud-pollinations resulted in hybrid seed set; these were on flower buds in which the style length was 1.1 to 2.3 cm. Thus, bud-pollination of immature *P. parodii* flowers provided a short style length that could be penetrated by *P. inflata* pollen tubes since it had the inherent potential to grow at least 0.8 cm to achieve self-pollination. This was confirmed by histological studies which indicated pollen tube growth to the base of the style of *P. parodii* 24 h after bud-pollination. The bud-pollinated *P. parodii* × *P. inflata* capsules averaged 78 seeds, considerably lower than the average 430 seeds per capsule for the bud-pollinated selfing of *P. parodii*. Bud-pollinating 180 flowers in the reciprocal direction failed to produce hybrids. Twenty-four h after pollination, *P. parodii* pollen tube growth had only extended to the neck region of the stigma-style of *P. inflata*. A similar situation was encountered using the in vitro fertilization method. Ovaries of *P. inflata* proved to be too small to handle for the in vitro pollination method. However, hybrid seeds were obtained by in vitro pollination of *P. parodii* ovules exposed by removal of the ovary wall.

No differences were observed in the mean values obtained for hybrid plants produced by in vitro fertilization (4) compared to those obtained by bud-pollination (32); thus the data were combined. The means for the seven characters measured are shown in Table 1 and the flowers of the parental species and their hybrid are illustrated in Fig. 1. Except for vegetative leaf width (3.5 cm), all characters of the interspecific hybrids were intermediate with respect to those of both parents (Table 1 and Fig. 1). Leaf size in *Petunia* varies considerably depending on container size, soil fertility level, light intensity and plant age, thus, the variance in mean data for leaf characters as reflected in high standard deviations and a vegetative leaf mean of 3.5 cm for the hybrids is not unexpected. Floral characters in *Petunia* are more reliable indices of taxonomic delineation (Fries 1911), and this was confirmed in the interspecific hybrid of *P. parodii* and *P. inflata* when compared to the parents (Table 1).

For example, a character commonly employed in *Petunia* taxonomy is the percent filament attachment. In *Petunia*, the filaments are partially adnate to the corolla tube; *P. parodii* is taxonomically characterized by a greater than 50 percent adnation (Steere 1930) whilst *P. inflata* has less than 50 percent adnation (Fries 1911). Thus, the hybrid of the two species was intermediate with 36.7 percent filament attachment. The observed mean values for the other floral characters of the interspecific hybrid approximated to the calculated means of the two parental species. In the hybrids therefore, the measured morphological characters were expressed as a genetic interaction of the parents and not with *P. parodii* showing dominance over the smaller *P. inflata* species in the hybrids. The flower color of the hybrids was a light magenta, or shades thereof, as compared to the magenta color of *P. inflata* and white for *P. parodii*. Hence, the flower color of the hybrids is in agreement with a previous report that floral pigmentation in *Petunia* is controlled by dominant genes to white (Mather and Edwardes 1943). The hybrids had a high pollen viability (93.4 percent) as measured by lactophenol blue staining and readily set seed upon self-pollination. They also intercrossed in both directions with the parental species to produce viable offspring that segregated for the parental characters documented in Table 1 (unpublished data). Although no cytological studies were conducted on the hybrids, the fact that both *P. parodii* and *P. inflata* have a diploid chromosome number of 2n = 14, vigor in the hybrid plants, together with a high pollen fertility and ease of setting self seed and that they hybridized with the parents can only be interpreted as evidence for the existence of a high degree of chromosomal homology linked with no major abnormalities during meiosis.

As North (1976) stated, failure to produce hybrids may be due to any one or more of three incompatibilities: pre-fertilization, fertilization or postzygotic. Pre-fertilization includes pollen-stigma or pollen-style incompatibilities which prevent fertilization from occurring. The observations made herein on the *P. parodii* × *P. inflata* cross indicated that reproductive isolation occurred by a pre-fertilization type failure. It has been reported that these types of pollen-stigma or pollen-style isolating mechanisms may be controlled by a few genes (Levin 1971). The *P. parodii* by *P. inflata* cross would thus appear to be a system that merits experimental study for the genetics of pre-fertilization type reproductive isolation. Furthermore, the unilateral incompatibility system studied herein between a self-compatible, *P. parodii*, and a self-incompatible, *P. inflata*, species is paralleled in several other pairs of *Solanaceous* species: for example, in the genera *Solanum* (Grun and Aubertin 1966), Nicotiana (Anderson and De Winton 1931), *Lycopersicon* (Hogenboom 1972) and also in *Petunia violacea* × *P. axillaris* (Mather 1943).

In vitro fertilization is primarily a method to overcome pre-fertilization incompatible factors (Rangaswamy 1977). In the genus *Petunia*, Rangaswamy and Shivanna (1967) used the in vitro fertilization technique to overcome a pollen-style type incompatibility in a line of *P. axillaris*. Since then, this technique has been extended to overcome self and cross-incompatibility barriers in several plant species (Rangaswamy 1977). The use of this technique to produce the *P. parodii* by *P. inflata* hybrid represents an alternative approach to bud-pollination in an attempt to overcome an apparent pollen-style cross-incompatibility. If the failure had been due to a strict pollen-stigma barrier operative both in physiologically mature and immature *P. parodii* stigmas, in vitro fertilization may have been the only method to obtain interspecific hybrids.

Somatic hybridization is a recently developed procedure aimed at overcoming sexual incompatibility barriers between plant species by the fusion of isolated protoplasts (Smith 1974). To date, somatic hybrids in higher plants have been produced within the genera *Nicotiana* and *Petunia* (Power et. al. 1976a), and only between sexually compatible species. The technique of plant protoplast fusion and subsequent nuclear fusion during culture to produce somatic hybrids is not a synonymous system to the in vivo steps involved during sexual reproduction in higher plants. Somatic hybridization may, therefore, be used to overcome both pre- and post-zygotic type incompatibility barriers. *Petunia* is one of a few plant species from which numerous protoplasts can be isolated, cultured, and regenerated into plants. To date, leaf protoplasts of cultivars of *P. hybrida* (Frearson et. al. 1973) and selected *Petunia* species (Power et. al. 1976b), including *P. parodii* and *P. inflata*, have been successfully regenerated into intact plants, and with a high efficiency. Also, Power et. al. (1976a) have described the somatic hybridization of *P. hybrida* and *P. parodii*. The next logical intermediate step leading to somatic hybridization of strictly sexually incompatible species within the genus *Petunia* may be to employ *P. parodii* and *P. inflata*. Provided, a successful selective cultural procedure could be designed to preferentially recover somatic hybrids, pre-zygotic incompatibility may be overcome in this system through somatic hybridization.

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