Theor Appl Genet (1984) 69: 187–192

TAG THEORETICAL AND APPLIED GENETICS

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Maternal haploids of *Petunia axillaris* (Lam.) B.S.P. via culture of placenta attached ovules *

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Received April 5, 1984; Accepted May 4, 1984
Communicated by H. F. Linskens

Summary. Hybridization of *Petunia axillaris* and *P. parodii* with *Nicotiana tabacum* was attempted using the method of in vitro pollination and fertilization. Seedlings were produced when the *Petunia* species and *N. tabacum* were used as the maternal parents; however, most of these had the identical somatic chromosome complement of the maternal parent. With crosses involving *P. axillaris* as the maternal parent, a low frequency of haploids was also produced. Due to the potential of haploids in basic and applied genetic research, additional experiments were carried out to determine whether in vitro pollination was necessary to stimulate haploid production and to more closely define the optimal time for ovule excision and culture. Four treatments were applied to accomplish these objectives. They were: placentas cultured prior to the time of anthesis, with and without pollination, and placentas cultured after the time of anthesis, with and without pollination. In vitro pollination had no effect on the frequency of haploids produced. Placenta attached ovules cultured prior to the time of anthesis produced significantly more haploids than those cultured after anthesis. The preanthesis treatment produced a frequency of 6.5 haploids per 100 ovaries cultured. The culture of placenta attached ovules provides an alternative to anther culture as a means for haploid production.

Key words: In vitro pollination – Ovary culture – Tissue culture – Haploid production – Placenta culture – *Petunia*

Introduction

An efficient means for haploid production in plants is important since haploids are useful in basic and applied genetic research. Haploids have been produced spontaneously and also by a variety of methods and mechanisms including: semigamy, pseudogamy, androgenesis, as a member of a set of twins, chromosome elimination, interploid crosses, the use of alien cytoplasms, anther culture, and ovule culture (San Noeum 1976; Hermsen and Ramanna 1981; Peloquin 1981). Even though such an array of methods exists, the potential of haploidy has not been realized in most crop species due to a lack of an economical means for haploid production.

In the genus *Petunia,* haploids have been produced by anther or microspore culture, and by crossing lines with specific genetic markers and then screening the progeny for their expression (Sangwan and Norreel 1975; Singh and Cornu 1976; Mitchell et al. 1980). Anther culture is most popular; however, the frequency of haploids produced from this method is low, especially in comparison to other members of the Solanaceae (Zenkteler 1971; Engvild et al. 1972; Rashid and Street 1973; Tomes and Collins 1976). For *Petunia* anther culture, typically less than 2% of anthers produce plantlets (Hanson, in press). Maizonnier (in press) states that the most efficient method for producing *Petunia* haploids is through hybridization and subsequent chromosome elimination; however, the frequency for this method is also low (in the range of 10^{-4}) and requires the use of genetic markers.

The culture of ovaries and ovules is a method of producing haploids that has been used successfully in only a few cases but over a wide range of genera. The first report of

* The investigations reported herein were supported by USDA/SEA/CRGO Project 59-2213-1-1-613-0 and the paper (No. 84-3-36) is published with the approval of the Director of the Kentucky Agricultural Experiment Station

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haploid plants being produced by ovary culture was with *Hordeum vulgare* (San Noeum 1976). The generation of haploid plants via ovary or ovule culture has since been reported for *Gerbera jamesonii, Lilium daviddii, Nicotiana tabacum, Oryza sativa, Triticum aestivum,* and *Zea mays* (see Yang and Zhou 1982 for review). The generation of maternally derived haploids by ovary or ovule culture may be advantageous for a number of reasons. It may be the only efficient means of producing haploids, and also may be useful for comparison to paternally derived haploids. This latter point is important since significant differences have been shown between maternally and paternally derived dihaploids in barley (*H. vulgare*) and in some types of tobacco (*N. tabacum*) (San Noeum and Ahmadi 1982; Reed and Wernsman 1983).

The research reported here involved the development and evaluation of a technique to produce maternally derived haploids of *P. axillaris* (Lam.) B.S.P. (2n = 14). The approach was derived from an observation in experiments designed to hybridize *P. axillaris* and *P. parodii* (2n = 14) with *N. tabacum* cv. 'Ky 17' (2n = 48) using in vitro pollination. From this material, a low frequency of *P. axillaris* maternally derived haploids were generated. Additional experiments were carried out to determine whether pollination with 'Ky 17' pollen was necessary for haploid production, to more closely define the optimal time for placenta excision and culture, and to establish the efficiency of haploid production.

Materials and methods

Experiment I

The first experiment was initiated in an attempt to hybridize tobacco burley cv. 'Ky 17' (*N. tabacum*) with *P. axillaris* and *P. parodii.* The cross combinations utilized and the number of cultures that were initiated are indicated in Table 1. Placentas used in this study were all from flowers collected one to three days after the time of anthesis. Four placenta (two ovaries) were inoculated onto each petri dish and pollen was applied to all placentas.

Experiment II

The effects of in vitro pollination and placenta maturity on haploid production were tested using a 2×2 factorial design nested within weeks. In order to compensate for weeks in which a particular treatment did not produce plants, a weighted least squares analysis was done using the SAS system procedure FUNCAT, with log (p/q) as the response function were p is equal to the proportion failing to respond and q is equal to the proportion responding. The first factor (A) was the effect of pollination on haploid production. The two levels of factor A were pollination and no pollination of the cultured placenta attached ovules. The second factor (B) was placenta maturity as measured by the age of flowers in relation to anthesis. The two levels of factor B were placentas cultured from flowers collected prior to anthesis and from flowers collected postanthesis. The approximate size of the flowers collected from each of these maturity groups is illustrated in Fig. 1A. Two hundred replications of each of the four treatments were carried out, with one petri dish containing one ovary (two placentas) constituting a replication. For each day the experiment was performed, ten replications of each treatment were initiated. The cultures were initiated on two days per week over a period of ten weeks and extended from June to August of 1982. The percent response was measured as the number of ovaries which produced plants divided by the total number of ovaries initiated per treatment.

Plant material

Seed of *P. axillaris* and *P. parodii* were supplied by R. Thurston (Univ. KY) and M. Hanson (Univ. VA), respectively. Seed of 'Ky 17' were provided by M. Nielsen (Univ. KY). Seed were germinated under aseptic conditions on the media designated R 1/2 N (modified from Bourgin and Nitsch 1967 by Tomes and Collins 1976). Plants were grown in 25.4 cm plastic pots under greenhouse conditions. All pots contained a mixture of soil, sand, and vermiculite (2 : 1 : 1). Fertilization was on a weekly basis with 20-20-20 (N : P : K) soluble fertilizer at a concentration of 3.8 g/l.

Pollen collection

Pollen was collected from flowers of burley tobacco cultivar 'Ky 17', prior to anthesis, two to three days in advance of use. The anthers were removed under aseptic conditions according to the following procedure. Corollas of the flowers were gently cotton swabbed with 70% ethanol and allowed to dry. Anthers were then carefully removed with forceps and placed into 60×20 mm plastic petri dishes. Petri dishes containing the anthers were wrapped in parafilm and incubated at room temperature overnight.

Placenta culture and in vitro pollination

Flowers were collected in the greenhouse and brought to the laboratory for culture. Following the removal of the calyx and corolla, the pistils with attached receptacle were sterilized by a brief rinse (ca 30 s) in 70% ethanol and then by a ten minute treatment in 0.5% sodium hypochlorite. Rinsing was done sequentially by a 3 min and 2.5 min treatment in sterile deionized, distilled water. Then, using a dissecting scope, the ovary wall was removed aseptically with scapel and forceps. The bicarpellate ovary was split by cutting through the septum. Each placenta, with ovules attached, was placed in 60×20 mm petri dishes containing the media designated NP (modified Nitsch's (1969) with 500 mg/l casein hydrolysate, 0.250 mg/l D-Ca-pantothenate, and sucrose at a final concentration of 40.0 g/l). No hormones were added to the media.

Pollen was applied by holding the anthers with forceps and gently dusting the pollen onto the surface of the ovules. Based on the observations of Balatkova and Tupy (1972), care was taken to apply pollen only to a small region of the placenta rather than dusting over its entire surface.

Petri dishes containing the ovaries were incubated under continuous light at 25 °C. Six days after pollination the placentas were examined for enlarged ovules. These were then removed and placed on media N (Nitsch 1969) according to the technique of Reed and Collins (1978). This was done to bypass postfertilization barriers to hybridization by culturing fertilized ovules prior to the time of endosperm abortion. Cultures which produced seedlings were transferred to a growth chamber having a day/night cycle of 16 h/8 h, respectively. After root tip collection, seedlings were removed and cultured on the media R 1/2 N for additional root formation and growth. Seedlings that were abnormal in morphology were placed on a callus-inducing media from which plants were later regenerated (Budding media, designated as DBI,

Table 1. In vitro pollination and fertilization on crosses involving *N. tabacum* cv. 'Ky 17' with *P. axillaris* and *P. parodii*

Cross	Number			Percent response
	Cultures initiated	Plants produced	Cultures responding	
'Ky 17' × *P. axillaris*	96	5	4	4.2[a]
'Ky 17' × *P. parodii*	54	0	0	0
P. axillaris × 'Ky 17'	152	11	5	3.3[b]
P. parodii × 'Ky 17'	67	2	1	1.5[a]

[a] Plants had the somatic chromosome complement of the maternal parent
[b] Plants were found to be both haploid and diploid

Kasperbauer and Collins 1972). This was based on the technique of Lloyd (1975), who cultured cotyledons of *Nicotiana* interspecific hybrids on a callus inducing media to bypass seedling lethal traits.

Cytology

Root tip mitosis was examined using the procedure of Burns (1982). Root tips were collected three to four hours after the initiation of the daylight period from seedlings that had been acclimated to the growth chamber photoperiod regime.

Results

The technique of in vitro pollination was used in an attempt to produce hybrids of two *Petunia* species with *N. tabacum*. Plantlets appearing to arise from an embryogenic pathway (Fig. 1 B) were observed within a three to twelve week period following culture initiation. Seedlings were produced both from the enlarged ovules that were transferred to media N and also from ovules still attached to the placenta (Figs. 1 C and D, respectively). Of the four cross combinations tested, three produced plants (Table 1). None of the plants were hybrid and, for the most part, they had the identical somatic chromosome complement of the maternal parent. An exception was noted in the case where *P. axillaris* was used as the maternal parent. Here, both diploid and haploid plants were produced. Figure 1 E shows a root tip preparation from one such haploid individual. The maternally derived haploids were smaller in stature and their flowers and leaves were reduced in size in comparison to their diploid counterparts (Fig. 1 f).

A second study was initiated to further characterize this system for its potential in *P. axillaris* haploid production. Four treatments were evaluated to accomplish the objectives and haploids were produced from all of them (Table 2). The modal number of haploid plants generated per culture was one. Only two of the 29 responding cultures produced more than one plantlet; each of these produced two. No significant difference in the percentage of cultures producing haploid plants was found between pollinated and unpollinated treatments (3.0% vs. 4.0%, respectively). In contrast, the stage of floral maturity at the time of placenta culture had a significant effect on the frequency of haploids produced (Table 2). Those flowers cultured prior to anthesis produced significantly more haploids than those cultured after the time of anthesis (6.0% vs. 1.3%, respectively).

In addition to haploid production in this experiment, many cultures produced diploid seedlings (Table 3). Diploids were produced in all four of the treatments; however, a significantly greater number of

Table 2. The effect of placenta maturity and pollen application on the number of ovaries which produced haploid plants. The numbers in parentheses indicate the total number of plants produced per treatment

Floral maturity at time of culture**	Placenta pollination		
	Yes	No	Total
Before anthesis	10 (10)	14 (16)	24 (26)
After anthesis	3 (3)	2 (2)	5 (5)
Total	13 (13)	16 (18)	29 (31)

** Significant differences ($P \leq 0.01$) were detected between the two floral maturity groups

Table 3. The effect of placenta maturity and pollen application on the number of ovaries which produced diploid plants. The numbers in parentheses indicate the total number of plants produced per treatment

Floral maturity at time of culture**	Placenta pollination		
	Yes	No	Total
Before anthesis	2 (3)	2 (2)	4 (5)
After anthesis	10 (77)	13 (46)	23 (123)
Total	12 (80)	15 (48)	27 (128)

** Significant differences ($P \leq 0.01$) were detected between the two floral maturity groups

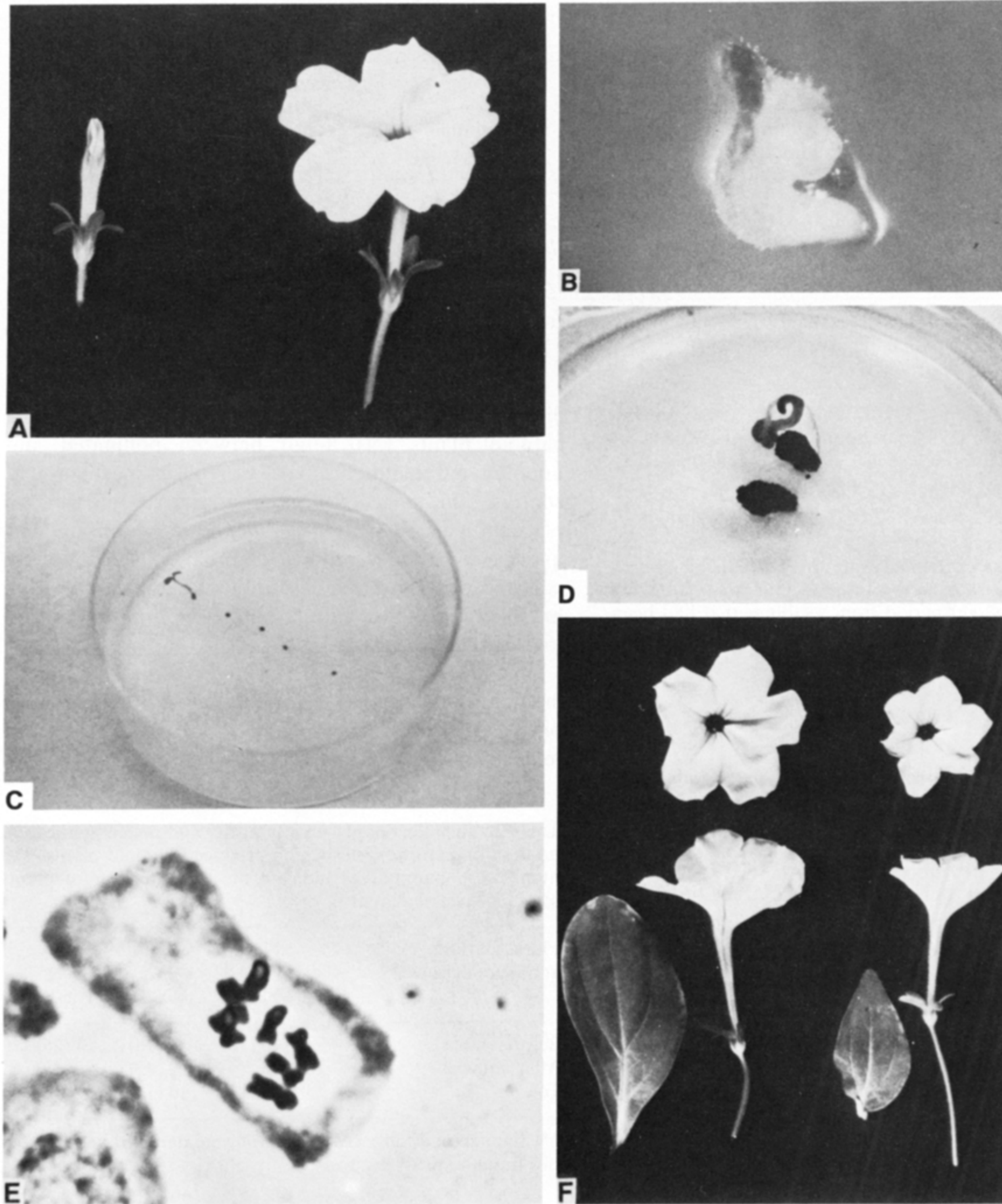

Fig. 1. A Placentas were cultured at two stages of floral development: before anthesis (*left*) and after anthesis (*right*); **B** well formed root and shoot primordia indicate that haploid seedlings arose through an embryogenic mode; **C** haploid seedling germinating from enlarged ovules that were transferred to media N six days after pollination; **D** haploid seedling germinating directly on the placenta; **E** root tip cell of a haploid ($N=7$) individual derived from the culture of placenta attached ovules; **F** leaves and flowers of diploid (*left*) and haploid (*right*) *P. axillaris*

ovaries responded (produced diploid plants) in the postanthesis treatment than in the preanthesis treatment (5.8% vs. 1.0%, respectively). This high frequency of diploids at the onset of the experiment led us to suspect that contaminant pollen may have been a problem. To remedy this, extra precautions were taken for the last 80 replications. In addition to emasculation, the pistils of the flowers from the post anthesis treatment were covered with paper straws to insure against contaminant pollination. This had a dramatic effect, and decreased the number of diploid plants produced by 68%.

Seedlings from the preanthesis treatment which were transferred directly to the rooting media were all found to be haploid. It is important to note that all of the higher ploidy level individuals that were produced in the preanthesis treatment were from explants initially cultured on a callus inducing media. In addition, seedlings from the postanthesis treatments initially cultured on callus inducing media generated plants of various ploidy levels. Up to nine regenerates were evaluated from a single explant source and, depending on the explant, were found to be: all diploid, haploid and diploid, diploid and tetraploid, or haploid, diploid and tetraploid. In two instances, root tips from regenerated individuals were observed to be mixoploid. One individual contained both haploid and diploid cells, and the other contained diploid and tetraploid cells.

Discussion

In vitro pollination has been previously utilized in obtaining a number of hybrid combinations that were not possible by conventional sexual hybridization (see Zenkteler 1980 and Collins et al. 1984 for reviews). The direct application of pollen to the ovules is advantageous in instances where the pollen is not able to traverse the length of the style to effect pollination. However, the bypassing of prezygotic barriers does not insure that postzygotic barriers do not also exist. In the present study, it appeared that postzygotic barriers prevented the recovery of intergeneric hybrid plants.

The method of in vitro pollination was unsuccessful in the attempts to hybridize *N. tabacum* with *P. axillaris* and *P. parodii.* Though hybrid plants were not produced, three of the four cross combinations produced plants that were similar in appearance to the source used as the maternal parent. In two of the three combinations, all of the plants had the same number of somatic chromosomes as the maternal parent. This indicated that either accidental in vivo pollination had occurred or that some other process in the cultured material led to the production of diploids. When *P. axillaris* placenta attached ovules were cultured, both haploid and diploid individuals were produced. Haploids were not produced when *P. parodii* was used as the maternal parent indicating that this method of producing haploids may be species dependent.

Pollination of cultured *P. axillaris* placentas was found to have no effect on the number of haploids which responded. This implies that pollination is not necessary for haploid production and does not affect the frequency of haploids produced. A significant increase in the response was observed when placentas were cultured prior to the time of anthesis. This is an indication that the stage of embryo sac maturity is important for haploid production. For *Petunia* anther culture the stage of microspore development also has been shown to be critical for haploid development (Engvild 1973; Sangwan and Norreel 1975; Mitchell et al. 1980). For the utilization of ovule or ovary culture for haploid production, it is not practical to directly evaluate the effect of embryo sac maturity. A more feasible approach is to correlate embryo sac development with microspore maturity (Yang and Zhou 1982).

A common problem with *Petunia* anther culture has been the high proportion of polyploid plants that are produced (Raquin and Pilet 1972; Wagner and Hess 1974; Sangwan and Norreel 1975; Mitchell et al. 1980). An advantage of culturing placenta attached ovules is the relatively high proportion of haploid plants produced. In fact, all of the preanthesis seedlings that were transferred directly to the rooting media were haploid. Seedlings from both the preanthesis and postanthesis treatment that underwent a callus inducing phase regenerated plants that were haploid, diploid, tetraploid, and mixoploid. It is likely that all of the non-haploid preanthesis derived plants were originally haploid and subsequently doubled through an endomitotic event during the callus culture treatment. A more definitive method for determining the origin of these higher ploidy levels would be the use of genetic markers. It is suggested that, for haploid plant production via the culture of placenta attached ovules, cultures be initiated from flowers collected prior to the time of anthesis. This, in combination with allowing the ovules to develop and germinate directly on the placentas, should save considerable effort in the application of the technique.

Factors influencing the frequency of haploid production from cultured ovaries and ovules were recently reviewed by Yang and Zhou (1982) and can be broken up into three categories: 1) age, genotype, and condition of the donor plant, 2) media modifications, and 3) environmental treatments before and after culture initiation. In the present study, environmental and media modifications were not evaluated for their effect on haploid production. Thus, potential exists to further increase the number of ovaries producing haploids and, also, the number of haploids produced per ovary beyond that reported here. Overall, the frequency of ovaries which produced haploids and the number of haploids produced per ovary was low in this study. This low response is typical for *Petunia* anther culture and has also been shown for ovary culture of rice (Chang and Hong-Yuan 1981). However, the frequency of haploids produced in the preanthesis treatment (6.0%) was relatively high in comparison to the response that was previously reported for anther culture of *P. axillaris* (1.1% by Swamy and Chacko 1973). Therefore, in addition to generating maternally derived haploids, this technique has potential for increasing the efficiency of haploid production.

The conclusions that can be drawn from this procedure follow: 1) The culture of placenta attached ovules has potential for efficient haploid production in *Petunia.* 2) This technique appears to be species depen-

dent as *P. axillaris* produced haploids and *P. parodii* did not. 3) Placenta attached ovules cultured before anthesis produced more haploids than those cultured after anthesis (6% vs. 1%, respecitvely). 4) The transfer of enlarged ovules to media N was not necessary for haploid production. The elimination of this step and the pollination treatment would save considerable time in the application of this technique. 5) Other factors influence haploid production via ovule culture and the evaluation of these has potential in increasing the efficiency of the technique.

Acknowledgements. The authors extend their appreciation to S. Daole, N. Giri, and T. Ragland for their skillful assistance and to W. L. Mesner for his assistance with some of the photography.

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