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Bypassing prefertilization barriers to hybridization in *Nicotiana* using in vitro pollination and fertilization *

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Summary. In vitro pollination of placenta attached ovules was useful in bypassing unilateral incongruity barriers for several *Nicotiana* interspecific hybrid combinations (*N. tabacum* cv. 'Ky 17' × *N. amplexicaulis*, 'Ky 17' × *N. benthamiana*, and 'Ky 17' × *N. repanda*). By measuring the pollen tube growth over time, prefertilization barriers were determined to be the cause of the incongruity. Seedling necrosis was a problem in the development of the *N. amplexicaulis* hybrid and it prevented maturation of the *N. repanda* hybrid. Callus produced from cotyledons of the *N. amplexicaulis* hybrid eventually resulted in plants that survived to maturity. This procedure was not successful for the *N. repanda* materials. The *N. amplexicaulis* and *N. benthamiana* hybrids were sterile but following chromosome doubling by midrib culture, male and female fertile plants were produced.

Conventional hybridization, fertilized ovule culture, and in vitro pollination were unsuccessful in obtaining hybrids of 'Ky 17' crossed with *N. arentsii* or *N. bonariensis*. Apparently, strong postfertilization barriers prevent the production of viable seed of these hybrids. Each of the *N. repanda* – *N. tabacum* reciprocal hybrids could not be rescued using callus culture; this adds support to the existence of strong sexual postfertilization barriers. A recent report, however, showed that it was possible to obtain this hybrid using the technique of somatic hybridization. Thus, it appears that it may also be possible to obtain asexual hybrids of *N. arentsii* and *N. bonariensis* with *N. tabacum*.

Key words: Wide hybridization – Tissue culture – Ovary culture – Tobacco

Introduction

Sexual barriers preventing interspecific hybridization have been separated into two categories (Stebbins 1950): those occurring prior to fertilization (prefertilization) and those occurring after fertilization (postfertilization). Prefertilization barriers may arise through a number of mechanisms, including the failure of pollen to germinate and penetrate the stigma, the bursting of the pollen tubes in the style, or the failure of the pollen tubes to grow the required distance to effect fertilization (de Nettancourt 1977; Hadley and Openshaw 1980). In *Nicotiana*, interspecific incongruity is frequently unilateral (Swaminathan and Murty 1957; Pandey 1967, 1968; de Nettancourt 1977) suggesting that prezygotic barriers are the factors preventing hybridization.

A number of approaches have been successfully utilized in bypassing stylar barriers. These range from the injection of pollen into the ovary to the application of pollen directly to individual ovules cultured in vitro (in vitro pollination and fertilization) (see reviews by Zenkteler 1980; Collins et al. 1984). These methods have been successful in obtaining both new interspecific and new intergeneric combinations. Circumventing prezygotic barriers, however, does not preclude the existence of postzygotic barriers. For example, with

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Trifolium in vitro pollination of *T. ambiguum* with *T. repens* pollen resulted in hybrid embryos, but these were unable to mature due to endosperm abortion (Richards and Rupert 1980).

The objective of the present study was to evaluate the use of in vitro pollination and fertilization for obtaining unique interspecific hybrid combinations of *N. tabacum* L. with wild *Nicotiana* species. The genus *Nicotiana* contains 66 species some of which are readily available for gene transfer to tobacco as fertile hybrids are easily obtained (Goodspeed 1954). Unfortunately, most other species have been difficult or impossible to hybridize with *N. tabacum* (Mann et al. 1963). The species utilized in the present study belong to the latter group and were chosen as they collectively represent valuable forms of disease, nematode and insect resistance (DeVerna 1984; Stavely 1979; Thurston et al. 1966).

Materials and methods

Plant material

Seed of the *Nicotiana* species *N. amplexicaulis, N. arentsii, N. benthamiana, N. bonariensis,* and *N. repanda* were obtained from L. G. Burk at the USDA Tobacco Research Laboratory, Oxford, N.C. Seed of *N. tabacum* burley cultivar 'Ky 17' were provided by M. T. Nielsen, University of Kentucky. The crosses of interest in this study are listed in Table 1.

Seeds were routinely germinated under aseptic conditions on medium R1/2N (DeVerna and Collins 1984). Plants of each species except *N. bonariensis* were grown in the greenhouse in 25.4 cm plastic pots. *Nicotiana bonariensis* plants, due to their small size, were grown in 11.4 cm pots. All pots contained a mixture of soil, sand, and vermiculite (2:1:1). Plants were fertilized on a weekly basis by watering with a solution of 20:20:20 (N:P:K) commercial fertilizer at a concentration of 3.8 g/l.

In vitro cultures

Fertilized ovule culture (in ovulo embryo culture) was carried out as described by Reed and Collins (1978). The procedures for pollen collection for in vitro pollination, placenta culture and in vitro pollination were carried out as described by DeVerna and Collins (1984) except that four placenta (two ovaries) were cultured per each 60×20 mm Petri dish. Petri dishes containing the ovaries were incubated under continuous light at 25 °C. Six days after in vitro pollination, the placentas were examined for any enlarged ovules. These were subsequently removed and placed on N medium in accordance with the fertilized ovule culture technique of Reed and Collins (1978). Ovules were isolated in this manner in order to bypass post-fertilization barriers that might exist following in vitro fertilization. Cultures were examined on a weekly basis for additional enlarged ovules or ovules that germinated in vitro. Cultures which produced seedlings were transferred to a growth chamber (16 h light/8 h dark). After root tip collection, healthy seedlings were transferred to R1/2N medium and abnormal seedlings to DBI shoot regeneration medium (Linsmaier and Skoog (1965) medium but containing 2 mg/l kinetin and 1 mg/l indole-3-acetic acid).

Table 1. Results from in situ hybridizations of *N. tabacum* burley cultivar 'Ky 17' crossed with five *Nicotiana* species

Cross combination	Total no. crosses	No. crosses setting seed	Hybrid seed germination
N. amplexicaulis × 'Ky 17'	112	7	Yes
N. arentsii × 'Ky 17'	92	0	–
N. benthamiana × 'Ky 17'	46	5	Yes
N. bonariensis × 'Ky 17'	38	0	–
N. repanda × 'Ky 17'	102	15	No
'Ky 17' × *N. amplexicaulis*	27	0	–
'Ky 17' × *N. arentsii*	204	1	No
'Ky 17' × *N. benthamiana*	23	0	–
'Ky 17' × *N. bonariensis*	287	4	No
'Ky 17' × *N. repanda*	50	0	–

Cytology

Root tips were analyzed at mitosis using the procedure of Burns (1982). The viability of hybrid pollen was tested by dusting pollen onto a slide containing one drop of 1% acetocarmine solution followed by examination under a microscope. Pollen stainability was determined using three anthers from each of three flowers from three plants of a particular cross.

The extent of pollen tube growth was evaluated by fluorescent microscopy using the aniline blue staining procedure described by Martin (1959). This was carried out to determine whether stylar barriers were responsible for hybrid incongruity. Flowers were collected from greenhouse-grown plants that were pollinated at the full bloom stage. Styles were collected at periods of 2, 4, 8, 16, 24, 48, and 72 h following pollination. Three replications of each of these treatments were carried out for each cross evaluated.

Chromosome doubling as a means of inducing fertility of sterile F_1 hybrids was done using midrib culture. Midribs were collected from mature greenhouse-grown plants and cultured as described by Kasperbauer and Collins (1972), but modified by placing the midribs onto DBI medium.

Results and discussion

Conventional sexual hybridization of *N. tabacum* burley cultivar 'Ky 17' was attempted via reciprocal crosses with the *Nicotiana* species *N. amplexicaulis, N. arentsii, N. benthamiana, N. bonariensis,* and *N. repanda* (Table 1). Of these ten crosses, only five resulted in pod formation; of these five, only the *N. amplexicaulis* × 'Ky 17' and *N. benthamiana* × 'Ky 17' combinations produced seed capable of germination. Each of these two hybrids matured but were sterile.

Fertilized ovule cultures were initiated with those combinations producing limited seed set (Table 1) but seed which were not capable of developing. Fertilized-ovule culture was successful only in producing seedlings of *N. repanda* × 'Ky 17'. Hybrids from this cross exhibited a seedling lethal necrosis. Numerous attempts to bypass that lethality by the culture of

prenecrotic cotyledons onto a callus induction medium were unsuccessful in producing healthy plants. Similar results with *N. repanda* were previously found by Reed and Collins (1978).

Limitation of pollen tube growth was examined as a possible cause of some of the aforementioned in situ incongruities. Pollen tube growth over time was measured using aniline blue staining for four of the five interspecific crosses involving 'Ky 17' as the pistillate parent. The paternal parents used in this study were: *N. amplexicaulis, N. benthamiana, N. bonariensis, N. repanda,* and 'Ky 17' as control. *Nicotiana arentsii* could not be induced to flower during this aspect of the study and, therefore, was not included. Pollen tube growth data, obtained as percent of total style length traversed by the pollen tubes, are presented in Table 2. The control ('Ky 17'×'Ky 17') had a significantly higher mean than the four other cross combinations. Pollen tubes were observed surrounding the ovules in only the control cross. With *N. benthamiana,* pollen tube growth after 72 h averaged 45.6% of the length of the 'Ky 17' style, and the longest individual entry grew 72.8% (total style length was 33 mm). Pollen tubes of *N. bonariensis* grew vigorously down the style for a distance, but extensive curling of the pollen tubes prevented fertilization. In some instances, pollen tubes of *N. bonariensis* grew close to the end of the style (90% of total style length or about 29 mm) but were never observed within the locule. Occasional production of nonviable seed from in situ hybridization of 'Ky 17' × *N. bonariensis* (Table 1) indicates that fertilization will sometimes occur. For *N. amplexicaulis* and especially *N. repanda,* the percentage of germinating pollen grains was low, and pollen tube growth frequently stopped soon after germination. It is clear then that for the 'Ky 17'×wild species (*N. amplexicaulis, N. benthamiana, N. bonariensis,* and *N. repanda*) crosses the inhibition of pollen tube growth prevented fertilization and, therefore, was at least part of the reason for the failure to in situ hybridize these combinations. Likewise, the inhibition of tube growth also suggests that efforts to obtain progeny from these crosses utilizing fertilized ovule culture would be futile.

The existence of prefertilization barriers led to the evaluation of in vitro fertilization as a means of obtaining hybrids of 'Ky 17' with *N. arentsii* and *N. bonariensis,* and of obtaining novel cytoplasmic-nuclear hybrids of 'Ky 17' with *N. amplexicaulis, N. benthamiana,* and *N. repanda.* In the case of *N. repanda* it was hoped that the seedling lethality apparent in the *N. repanda*×'Ky 17' combination would not exist in the 'Ky 17'×*N. repanda* cross. For all the in vitro crosses, fertilized ovule culture was used in combination with in vitro pollination and fertilization in order to bypass postzygotic barriers that might exist following fertilization.

Pollen applied to the placenta-attached ovules germinated quickly and within 24 h formed a mass of pollen tubes over the surface of the ovules. Pollen germination and pollen tube growth occurred in all crosses; however, pollen tube growth was not evident in every culture. In addition, there appeared to be male

Table 2. Mean percent of total style length traversed by pollen tubes presented as a function of time within each cross involving *N. tabacum* burley cultivar 'Ky 17'

Hours after pollination	% of total style length traversed by pollen tubes[a]				
	'Ky 17'×*N. amplexicaulis*	'Ky 17'×*N. benthamiana*	'Ky 17'×*N. bonariensis*	'Ky 17'×*N. repanda*	'Ky 17'×'Ky 17'
2	0.0*	1.1 a	1.4 a	0.5	1.4 a
4	1.5	2.2 a	6.3 a	0.0	2.5 a
8	4.3	5.3 ab	16.0 ab	1.3	18.3 b
16	6.9	20.9 abc	26.1 b	1.0	27.7 b
24	6.1	27.9 bcd	29.2 b	5.0	49.9 c
48	9.8	30.4 cd	82.3 d	0.4	86.6 d
72	6.6	45.6 d	76.2 d	0.0	99.5 e
LSD (0.05) for hour treatments within genotypes	NS	22.8	15.2	NS	9.6
C.V. (%)	141.4	67.2	25.1	164.4	13.3
Cross means, and LSD (0.05) for between cross means	5.0 a**	19.1 b	33.9 c	1.2 a	40.8 d

*, ** Means within cross columns and across the cross mean row, respectively, which are followed by the same letter do not differ significantly at $P=0.05$ as measured by the Least Significance Difference test

[a] Each value represents the mean of three replicates

Table 3. *Nicotiana* crosses evaluated for their ability to produce hybrid plants via in vitro pollination and fertilization

Cross combination	Total no. cultures	No. enlarged ovules transferred to media N	Total no. plants produced	No. cultures which responded (produced hybrids)	% response[a]
N. arentsii × 'Ky 17'	96	51	0	0	0
N. bonariensis × 'Ky 17'	156	212	0	0	0
'Ky 17' × *N. amplexicaulis*	27	453	124	13	48.1
'Ky 17' × *N. arentsii*	62	105	0	0	0
'Ky 17' × *N. benthamiana*	28	35	16	3	10.7
'Ky 17' × *N. bonariensis*	124	189	0	0	0
'Ky 17' × *N. repanda*	62	199	35	14	21.0
'Ky 17' × 'Ky 17'	47	272	74	16	34.0

[a] Percent response is expressed on a percent basis of the number of cultures which responded divided by the total number of cultures initiated

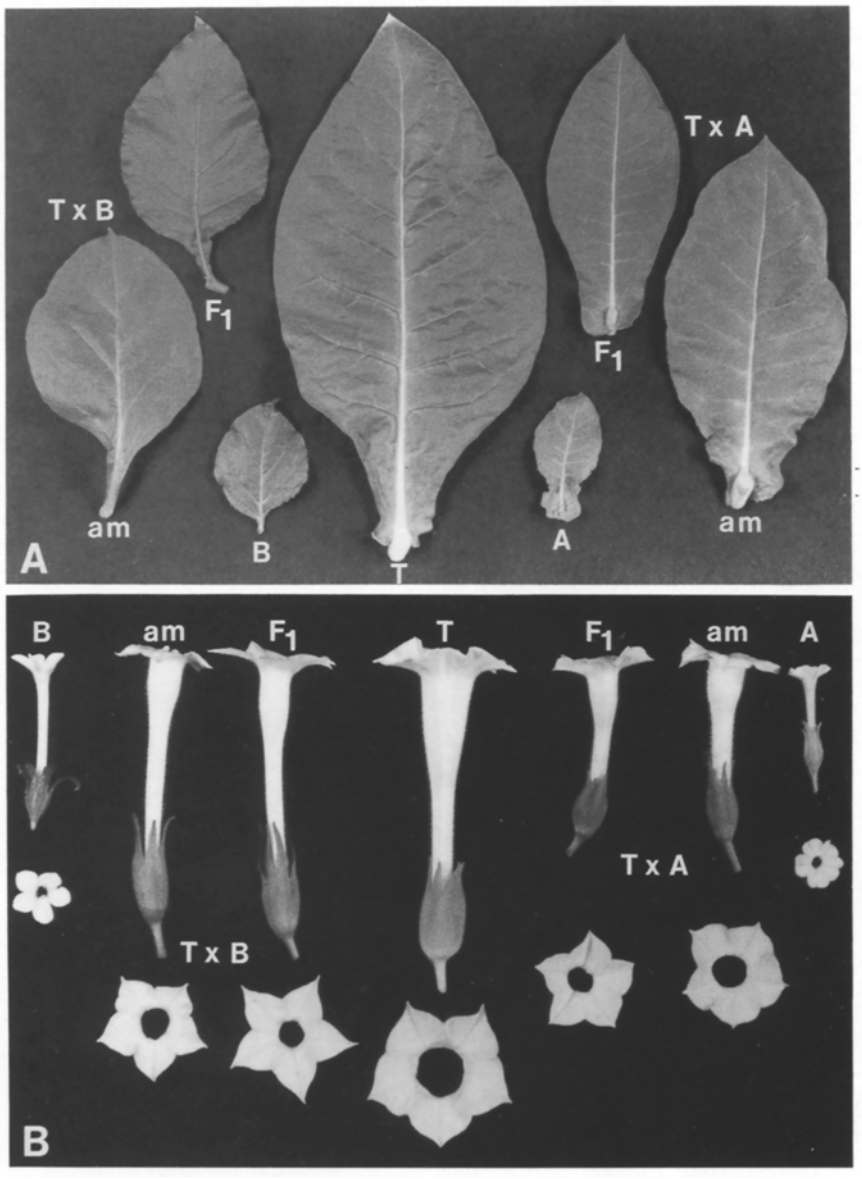


Fig. 1 A, B. Hybrid of *N. tabacum* burley cultivar 'Ky 17' × *N. amplexicaulis* and 'Ky 17' × *N. benthamiana* obtained using in vitro pollination and fertilization: **A** Leaves of 'Ky 17' (T), *N. benthamiana* (B) *N. amplexicaulis* (A), 'Ky 17 × *N. benthamiana* F_1 hybrid (T × B F_1), 'Ky 17' × *N. amplexicaulis* (T × A F_1), 'Ky 17' × *N. benthamiana* amphidiploid (T × B am), and 'Ky 17' × *N. amplexicaulis* amphidiploid (T × A am). **B** Flowers of 'Ky 17' (T), *N. benthamiana* (B) *N. amplexicaulis* (A), 'Ky 17' × *N. benthamiana* F_1 hybrid (T × B F_1), 'Ky 17' × *N. amplexicaulis* F_1 hybrid (T × A F_1), 'Ky 17' × *N. benthamiana* amphidiploid (T × B am), and 'Ky 17' × *N. amplexicaulis* amphidiploid (T × A am)

parent-species dependent differences in the final density of pollen tubes.

Within several days following in vitro pollination, fertilized ovules could readily be distinguished from their unfertilized counterparts by their enlarged size. All cross combinations evaluated produced enlarged – presumably fertilized – ovules (Table 3). The efficiency of in vitro pollination, measured by the number of fertilized ovules transferred to N medium divided by the total number of cultures initiated, was greatest for 'Ky 17' × *N. amplexicaulis*. This cross was approximately three times more efficient than the control ('Ky 17' × 'Ky 17').

Plantlets were produced from ovules which were transferred to N medium six days following in vitro pollination, and from fertilized ovules remaining attached to the placentas. Seedlings were produced only from the 'Ky 17' × *N. amplexicaulis*, 'Ky 17' × *N. benthamiana*, 'Ky 17' × *N. repanda*, and from the control cross (Table 3). The efficiency (percent of cultures producing hybrids) of in vitro pollination and fertilization for the cross 'Ky 17' × *N. amplexicaulis* was greater than that of the control 'Ky 17' × 'Ky 17' (48.1% vs. 34.0%) (Table 3). No hybrid plants were produced in the *N. arentsii* or *N. bonariensis* combinations, although fertilization in vitro was suggested by the presence of enlarged ovules. Apparently strong postfertilization barriers prevented their development. Additional attempts to obtain these hybrids are being carried out using somatic hybridization.

Seedlings produced from the *N. amplexicaulis* and *N. repanda* crosses always exhibited a seedling lethal necrosis. *Nicotiana* interspecific seedling lethality has previously been bypassed by culturing young cotyledons onto a callus induction medium and then recovering 'normal' shoots (Lloyd 1975; Ternovskii et al. 1976). In the present situation, attempts to circumvent the lethality by culturing cotyledons prior to the time of visible necrosis was successful only in the case of 'Ky 17' × *N. amplexicaulis*. Initial shoot regenerates of this cross still exhibited the necrosis but after several subcultures normal-appearing shoots were differentiated. None of the 'Ky 17' × *N. repanda* seedlings survived to maturity. The exhibition of seedling lethality in reciprocal directions for *N. repanda* indicates the existence of strong nuclear or bilateral cytoplasmic-nuclear incongruity.

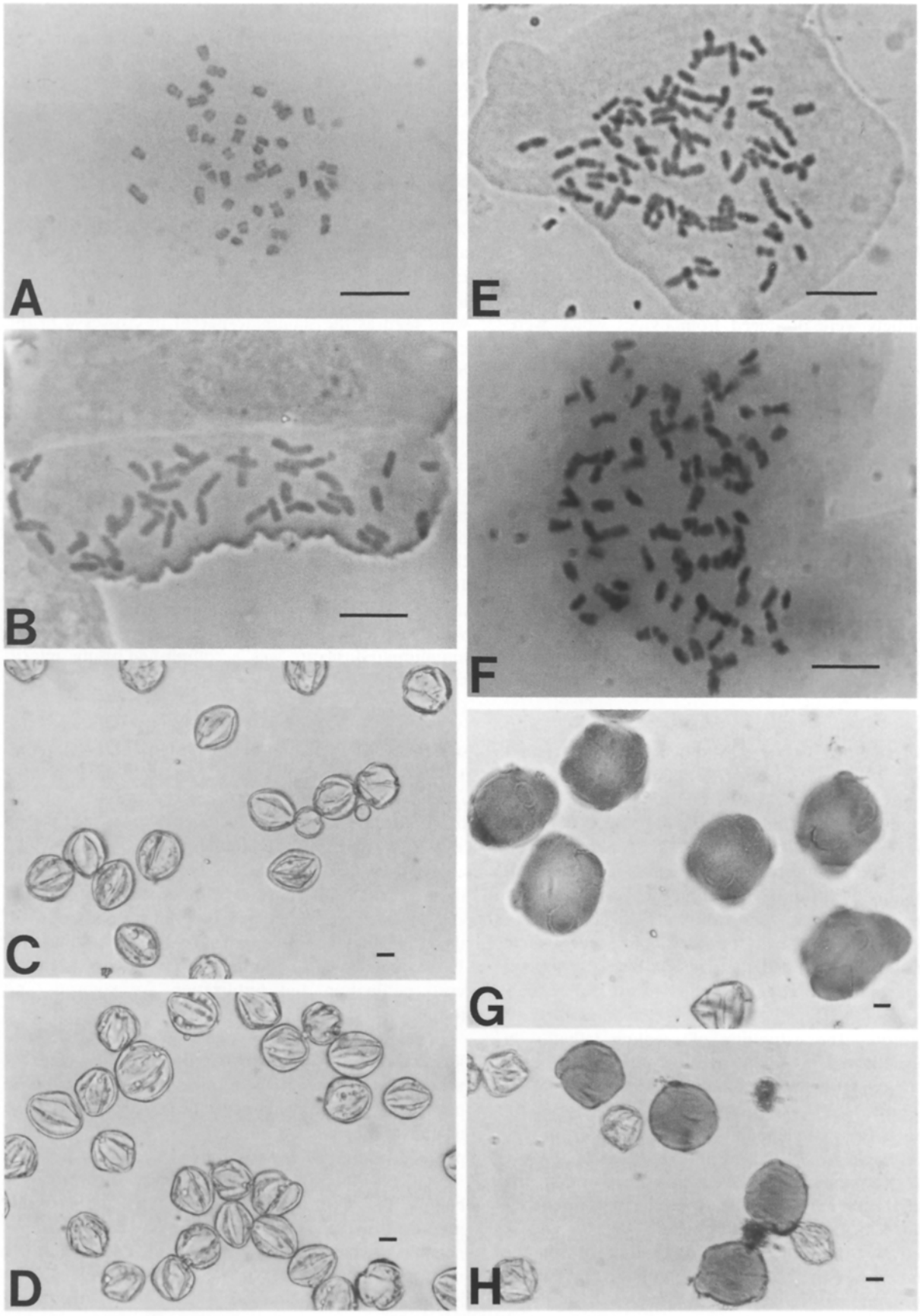
Shoots from cotyledonary-derived callus of the 'Ky 17' × *N. amplexicaulis* hybrid and seedlings of the 'Ky 17' × *N. benthamiana* cross each grew to maturity and flowered. Each hybrid exhibited the characteristic intermediate leaf and flower morphology (Fig. 1 A and B, respectively), and the expected number of somatic chromosomes (Fig. 2 A, 42 chromosomes, and 2 B, 43 chromosomes, respectively).

The 'Ky 17' × *N. amplexicaulis* and 'Ky 17' × *N. benthamiana* amphihaploid hybrids were sterile, each having less than 1% stainable pollen (Fig. 2 C and D, respectively). The midrib culture technique of chromosome doubling was successful for generating amphidiploids of both hybrids. Amphidiploid hybrid plants of 'Ky 17' × *N. amplexicaulis* contained approximately 84 chromosomes and had 69.2 ± 11.6% stainable pollen (Fig. 2 E and G). Amphidiploid hybrid plants of 'Ky 17' × *N. benthamiana* possessed 86 chromosomes and had a pollen stainability of 43.6 ± 29.6% (Fig. 2 F and H). Each amphidiploid is self compatible and female fertile in crosses with 'Ky 17'. When the amphidiploid hybrids were used as the staminate parent in crosses with the cultivated tobacco, however, ovary development began but was followed by floral abortion.

The hybridization of *N. amplexicaulis* and *N. tabacum* has previously been reported (Wark 1970; Lar'kina 1980; Berbec and Doroszewska 1981). From the report of Berbec and Doroszewska (1981) it was apparent that the amphihaploid hybrid plants often were inviable, but a few dwarf sterile plants were eventually produced. Colchicine treatment of these germinating seedlings produced one amphidiploid but it too was sterile. In the present study, the reciprocal hybrid, 'Ky 17' × *N. amplexicaulis*, was vigorous and grew to maturity, but was sterile. The generation of male and female fertile amphidiploid plants was accomplished using midrib culture.

Previous attempts at direct sexual hybridization of *N. benthamiana* with *N. tabacum* have all been unsuccessful (Chaplan and Mann 1961; Ramavarma et al. 1977). In the present study, hybridization of *N. benthamiana* with 'Ky 17' was successful using in situ hybridization with the wild species as the maternal parent and also via in vitro pollination of 'Ky 17' placenta attached ovules. The reasons for the discrepancy of these results with those previously reported are not clear. Genotypic differences or growing conditions of the plants may have been a factor. In any case, the 'Ky 17' × *N. benthamiana* amphidiploids obtained using in vitro pollination and fertilization are vigorous and fertile.

The results from this study indicate that in vitro fertilization in *Nicotiana* is a useful system for bypassing stylar barriers that prevent hybridization in particular *Nicotiana* interspecific combinations. The results of others also support the usefulness of this procedure in *Nicotiana* wide crosses (Berbec and Doroszewska 1981; Lar'kina 1980; Marubashi and Nakajima 1985; Ternovskii et al. 1976). The success of this procedure as reported herein also indicates that bypassing prefertilization barriers will not necessarily result in the production of viable hybrid progeny. This is indicated by the seedling lethality of reciprocal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hybrid progenies of *N. repanda* and by the inability to produce hybrids of *N. tabacum* in either reciprocal direction with *N. arentsii* or *N. bonariensis.*

The recent successes by tobacco researchers with *N. repanda* germplasm introgression suggest that other innovative procedures such as the manipulation of ploidy level (Pittarelli and Stavely 1975), pollen irradiation (Shintaku et al. 1985) or somatic hybridization (Nagao 1982) may be useful in accessing the *N. arentsii* and *N. bonariensis* germplasm.

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Fig. 2A–H. Cytology of interspecific hybrids obtained by in vitro pollination and fertilization between 'Ky 17' × *N. benthamiana* and 'K 17' × *N. amplexicaulis* (bar = 5 μm): **A** Root tip cell of 'Ky 17' × *N. amplexicaulis* F_1 hybrid showing 42 chromosomes. **B** Root tip cell of 'Ky 17' × *N. benthamiana* F_1 hybrid with 43 chromosomes. **C** Pollen stainability of sterile 'Ky 17' × *N. amplexicaulis* F_1 hybrid. **D** Pollen stainability of sterile 'Ky 17' × *N. benthamiana* F_1 hybrid. **E** Root tip cell of 'Ky 17' × *N. amplexicaulis* amphidiploid with 84 chromosomes. **F** Root tip cell of 'Ky 17' × *N. benthamiana* amphidiploid with 86 chromosomes. **G.** Stainable pollen of 'Ky 17' × *N. amplexicaulis* amphidiploid. **H** Stainable pollen of 'Ky 17' × *N. benthamiana* amphidiploid