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CELL-FUSION HYBRIDS

HAROLD H. SMITH

Brookhaven National Laboratory
Upton, New York 11973

Abstract. Cell-fusion hybrids were obtained by fusing protoplasts of *Nicotiana glauca* and *N. langsdorffii* in the presence of polyethylene glycol. The hybrid protoplasts were selected out of a mixed population by growing on a culture medium that does not support the growth of parental protoplasts. The cell fusion hybrids had chromosome numbers that were higher (56 to 64) than in the amphiploid (2n = 42). Most of these "hyper-aneuploids" were fertile and their progeny retained the characteristic morphology and approximate chromosome number of their hybrid parent.

The technical advance of being able to remove plant cell walls in order to produce viable wall-less or naked cells (the protoplasts) has opened up new avenues of research in plant propagation and improvement. These fall into two main categories of use: 1) fusion of protoplasts to give hybrid somatic cells, thus by-passing the usual sexual techniques of hybridization by cross-pollination; 2) facilitated entry or more rapid uptake of "genetically informed" particles that are generally excluded by the cell wall; for example, foreign macromolecules (DNA and RNA), chromosomes, nuclei, organelles and viruses. The genetic significance in all these cases is that new additional genetic material migrates into the protoplasts and, in so far as it persists through replication and integration, adds significantly to the genetic variability (8).

The successful production of cell-fusion hybrids has been greatly aided by two additional new techniques for handling protoplasts. These are: 1) the use of polyethylene glycol (PEG) to adhere and fuse protoplasts; and 2) the development of selection methods to recover preferentially regenerated fused hybrid protoplasts from a mixed population of protoplasts.

MATERIALS AND METHODS

The method that we follow to produce fusion hybrids of *Nicotiana* species is outlined below.

1) Pick young leaves from vigorous half-grown plants and surface sterilize them by spraying with 60% ethanol. This, and all subsequent manipulations, are carried out under aseptic conditions in a transfer room or hood.

2) Strip the epidermis from the lower surface of a leaf, slice the epidermis-free areas into strips, and place them into a 1:1 mixture of E1 and M3 solutions. See Kao, *et al.* (4) for preparation of solutions. The E1 solution contains 2% cellulase, 2% hemicellulase and 1% pectinase. The M3 solution is a rich nutrient medium for culturing plant cells. Leave the leaf strips in the solution mixture long enough to give a good yield of protoplasts. This takes 3 or 4 hours at room temperature (about 23°C) with favorable material, as most Solanaceae.

3) Harvest the protoplasts by filtering through two layers of miracloth, which gives an effective mesh of about 50μ, into a centrifuge tube. The two sources of protoplasts, that are eventually to be fused, can be collected into the same centrifuge tube at this time.

4) Wash the protoplast preparation free of enzymes by gradually eluting the solution mixture and replacing it first with pure M3 solution and finally with two washings of solution D of Kao *et al.* (4).

5) Preparatory to fusion, pipette a large drop ($\pm$ 150 μl) of a concentrated suspension of the mixed protoplasts onto a 22 $\times$ 22 mm cover slip that has been adhered to the bottom of a 60 $\times$ 15 mm plastic petri dish, see Smith *et al.* (10). After the protoplasts have settled onto the cover slip (ca 5 min), slowly add 450μl of PEG solution to the protoplast culture. The composition of this solution is PEG 1540 (0.33M), $CaCl_2 \cdot 2H_2O$ (10.5 mM), and $KH_2(PO_4) \cdot H_20$ (0.7mM). Adhesions of membranes begins immediately.

6) Incubate the protoplasts in the PEG solution for about 30 minutes.

7) Gradually elute the PEG from the suspension by repeated washing with M3 solution.

8) Maintain the culture of fused protoplasts under conditions of low light (50 to 100 lux), 23°C and a minimum of vibration. Add more M3 culture medium as cell divisions begin and colonies are formed (in 4 to 5 days).

9) When the colonies reach 2 to 3 mm in diameter (in 4 to 5 weeks) transfer to an agar (0.8 percent) solified medium that has the same sucrose, mineral salt and iron composition as the

Murashige-Skoog formulation (6), the vitamin content of B5 medium (2), and no phytohormones. In our experiemnts with fusing protoplasts of *Nicotiana glauca* and *N. langsdorffii* the absence of phytohormones in the medium selects against the parental species cells and favors the growth of the hybrids, which have less stringent cultural requirements. It is important to initiate some kind of selection system to aid in picking out the hetero-fusion products as early as possible. Others (3,5) have used photosynthesis-deficient complementary markers, to define and eliminate the parental cells and homospecific fusion products.

10) When the calli have reached the size of a small pea, place them on a medium and under conditions that favor differentiation. With our *Nicotiana* material these are a temperature of 25°C, 16 hours of light at ±5000 lux to 8 hours darkness, and a culture medium that contains Murashige-Skoog (6) major and minor salts, 30 g/l sucrose, 27.8 mg/l iron, and no vitamines, glycine, inositol, or hormones. These calli that originated from protoplasts are no different from cultures derived from walled cells or masses of tissue. Extensive reviews and a manual on methods for culturing plant cells and tissues have been compiled recently by Street (11) and by Gamborg and Wetter (2).

Our *N. glauca* + *N. langsdorffii* calli differentiate leaves and shoots more readily than roots. Those without roots were grafted onto plants of either parent as stock (Figure 1). Those with roots were transplated directly into soil in pots and were maintained in a high humidity section of the greenhouse until established. These methods of culture gave mature parasexual hybrids that flowered in about 7 months after protoplast fusion (10).

Figure 1. Teratological scion grown from a differentiated rootless callus of a parasexual hybrid (*Nicotiana glauca* + N. langsdorffii) and grafted onto a stock of *N. langsdorffii*.

All the parasexual hybrids were self-pollinated and most produced seeds. Mature plant progenies have been obtained from these fertile parasexual hybrids (9), which were more frequently derived from rooted calli than from grafted scions.

RESULTS

Cytogenetic analyses of 13 of the parasexual hybrids produced by fusing protoplasts of *Nicotiana glauca* and *N. langsdorffii* are shown in Table 1 along with observations on their progeny.

Table 1. Cytogenetictic analysis of 13 fertile parasexual hybrids (*N. glauca* + *N. langsdorffii*) and their progeny.

Parasexual Hybrids					Progeny		
Callus No.	Propagation	Somat. chrom. No.	No. bivalents	Pollen fert. %	No. pls.	Somat. chrom. No.	Pollen fert. %
7-2	root cal.	62	30	88.6	20	59	90.3
11-5	graft	56	26	85.1	21	54	88.8
23-2	root cal.	60	28	87.7	28	62	83.2
30-8	root cal.	64	32	82.7	23	61	89.0
31-6	root cal.	60	30	86.9	39	59.2	84.9
35-1	root cal.	58	28	86.1	37	59.5	90.4
35-6-1	root cal.	63	30	83.7	40	57	88.6
35-6-2	root cal.	60	27	89.2	2	58	79.3
41-6	root cal.	58	28	80.3	20	60	81.9
46-4-1	root cal.	60	30	90.9	8	60	92.7
46-6	root cal.	58	29	73.5	54	54	65.2
47-1	root cal.	60	30	82.7	29	60	88.4
51-6	root cal.	63	29	93.4	31	62	89.4
Mean		60.2	29.0	85.4	27.1	58.9	85.5

The chromosome numbers in the parasexual hybrids ranged from 56 to 64, averaging 60. The most common number of bivalents found in the first meiotic metaphase was 29, an unexpectedly high frequency. This high degree of meiotic regularity gave a high pollen fertility, averaging 85%. The plants were verified as hybrids based on: 1) requirements for tissue culture; 2) morphological characteristics of the leaf (Figure 2); 3) morphological characteristics of the flower (Figure 3); 4) spontaneous tumor formation on aging (7); and 5) general growth habit (10)

The progeny of the parasexual hybrids (Table 1) ranged in chromosome number from 54 to 62 with an average of 59, which is similar to that of their parental hybrids. The plant progeny of any one parasexual hybrid was relatively uniform in leaf and flower and tended to be similar to its parasexual hybrid parent which, in turn, was similar to the sexually-produced amphiploid between *N. glauca* and *N. langsdorffii*.

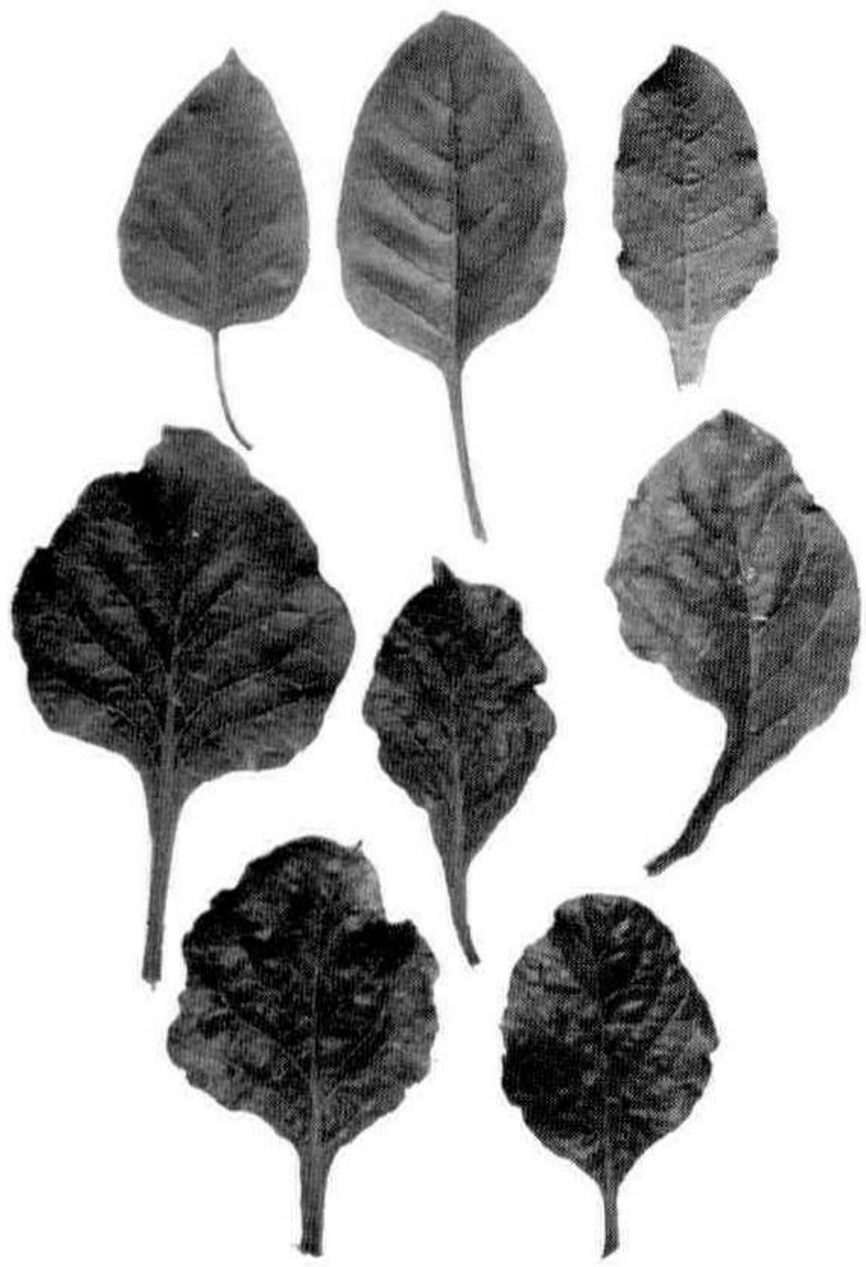

Figure 2. Leaves of *N. glauca*, the amphiploid and *N. langsdorffii* (top row, left to right); and five different parasexual hybrids (below). The cell fusion hybrids show features in common with the amphiploid, but they differ from it and from each other due to their various aneuploid chromosome numbers.

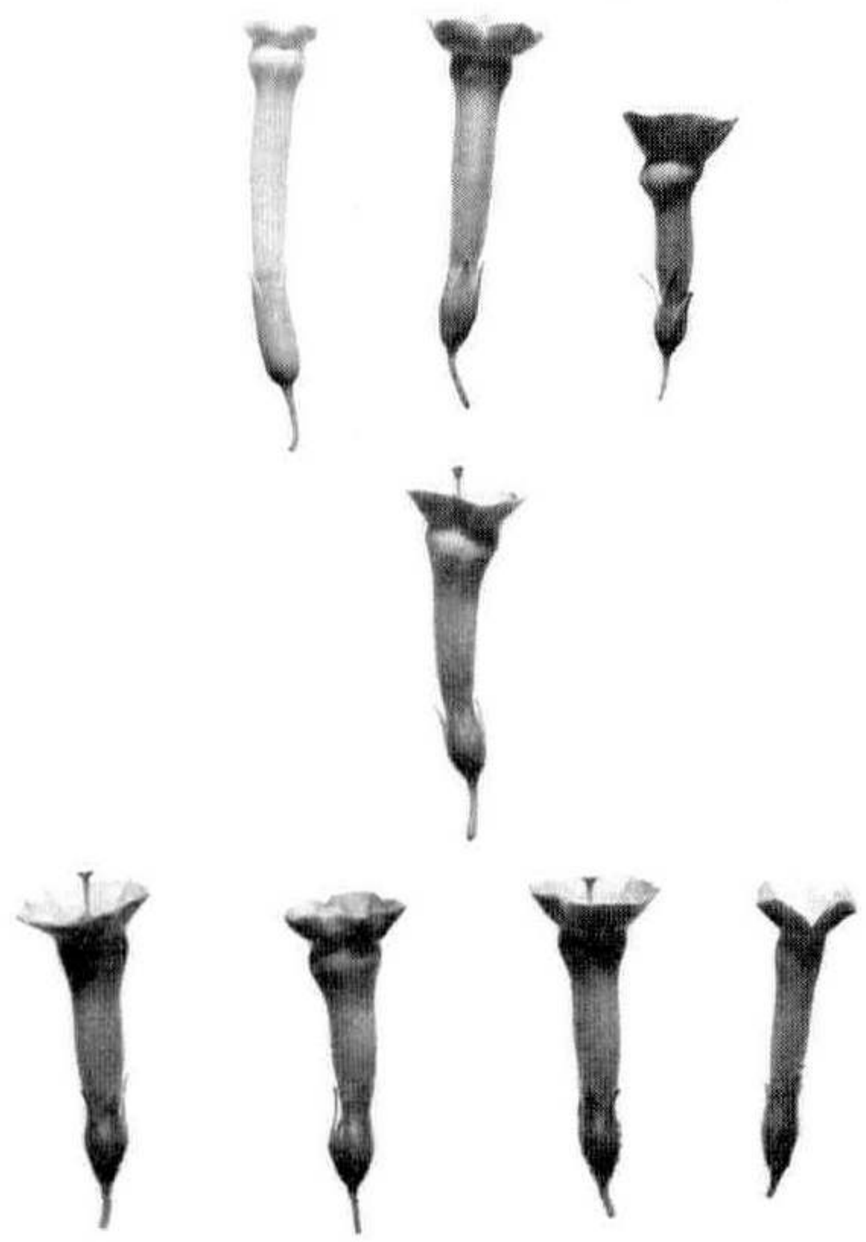

Figure 3. Flowers of *N. glauca*, the amphiploid, and *N. langsdorffii* (top row, left to right); a parasexual hybrid (center) and four progeny of the parasexual hybrid (bottom row).

DISCUSSION

These methods and the hybrids obtained with them have fully confirmed and have extended the results of earlier experiments (1), namely that: 1) parasexual hybrid cells can be produced by fusing somatic protoplasts of *Nicotiana glauca* with *N. langsdorffii*; 2) the hybrid cells can be selected out of a mixed population on the basis of differential growth in specifically defined culture media; and 3) the hybrids can be grown to maturity, are often fertile, and produce abundant progeny.

Although this experimental material serves primarily as a model system to demonstrate parasexual hybridization, its success depends on applying selection based on differential growth of parents and hybrid in a specific culture medium; and an extension of this general principle to other materials may ultimately prove its wider application. For example, the ubiquitous phenomenon of interspecific hybrid vigor, if expressed in more rapid callus growth, might be used to select parasexually produced hybrids from a mixed population containing parental calli.

The chromosome numbers found in the parasexual hybrids were unexpectedly high since a simple addition of the two parental species numbers would give 42; i.e. *N. glauca* = 24 and *N. langsdorffii* = 18. The departures from this number could be attributable to serveral causes e.g.: 1) a different abnormal chromosome number present in source leaf cells of the parental species; 2) multiple protoplast fusion, or 3) unequal mitoses occurring during growth from protoplasts to callus to differentiated plant. The specific range found, i.e. from 56 to 64 chromosomes suggests that triple fusions may have occurred (18 + 24 + 18 = 60, 18 + 24 + 24 = 66), that there was some loss of chromosomes in subsequent mitotic divisions, and that the particular aneuploid types recovered were those that were more successful than others in yielding viable cultures capable of differentiation into mature plants. This phenomenon might find use in genetic and breeding experiments for, instead of producing a single true-breeding amphiploid, a highly variable population of new (mostly fertile) interspecific combinations are found.

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SOME POTENTIALS OF PLANT CELL AND TISSUE CULTURE

AUGUST E. KEHR
National Program Staff-ARS
U.S. Department of Agriculture
Beltsville, Maryland 20705

Plant tissue and cell culture techniques have advanced to the stage where their application to commercial plant propagation is imminent, and in quite a number of cases, is in actual practice. The purpose of this paper is to review some of these techniques and point out their potential practical value for plant propagators and others. These techniques include opportunities for: 1) rapid plant multiplication; 2) eradication of viruses and other tissue-borne pathogens from "old" cultivars; 3) production of homozygous lines; 4) long-time storage of germplasm; 5) transfer of genetic information with isolated DNA, and 6) more efficient plant breeding. Somatic hybridization of protoplasts to develop parasexual plant hybrids has been discussed by Dr. Smith, and the use of shoot-tip culture for plant propagation has been covered by Mark Cunningham.

RAPID MULTIPLICATION OF PLANTS

Techniques are now being used to produce chrysanthemums by the billions, all free of any disease or pest. Four-year-old cultures still produce normal plantlets. In addition to