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In vitro Hybridization by Sexual Methods and by Fusion of Somatic Protoplasts

Experiments with *Nicotiana tabacum* × *Petunia hybrida*, *N. tabacum* × *Hyoscyamus niger*, *H. niger* × *P. hybrida*, *Melandrium album* × *P. hybrida*

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Summary. 1. No hybrid plants of *Nicotiana tabacum* + *Petunia hybrida* were regenerated from calluses of fusion experiments with mesophyll protoplasts of *N. tabacum s*, s^2 and *v* and of *P. hybrida* mu_1^2.

2. After in vitro pollination of ovules of *N. tabacum* with pollen of *P. hybrida*, filamentous proembryos and cellular endosperms could be detected. But after 8-10 days, embryos and endosperms degenerated completely, indicating a strong zygotic incompatibility of the two genomes.

3. After in vitro pollination of ovules of *N. tabacum* with pollen of *Hyoscyamus niger*, large globular embryos and well developed cellular endosperms are found, indicating that the method of in vitro pollination was not the reason for the early degeneration of the tobacco × petunia proembryos. The globular embryos of (tobacco × henbane) hybrids developed further on in a culture of ovules in a special medium 'C'. But then they started to produce callus tissue and did not develop normal seeds as (tobacco × tobacco) in vitro self-pollinations were able to do.

4. After in vitro pollination of ovules of *Melandrium album* with pollen of *N. tabacum*, no embryos could be found although several endosperm nuclei could be detected. The hybrid chromosome number was not counted. In *Melandrium album*, ovules pollinated with *P. hybrida* divisions of nuclei of the mother plant and the generative nucleus of the father could be photographed. No further development took place.

Key words: In vitro Hybridization – Fusion of Protoplasts – Gametic and Zygotic Incompatibility – Embryo Development – *Nicotiana tabacum* – *Petunia hybrida* – *Hyoscyamus niger* – *Melandrium album*

Abbreviations
× = sexual cross
\+ = somatic cross
s, *v* = (di)haploid cultivars of tobacco
s^2, v^2 = (amphi)diploid cultivars of tobacco

Introduction

It is now possible by plant protoplast fusion to obtain cell hybrids capable of forming growing callus cultures even when the partners used for fusion are distantly related (the best example at the moment is *Nicotiana glauca* + *Glycine max.*, Kao and Wetter 1977, Kao 1977, Wetter 1977).

However at present, hybrid plants can only be regenerated from hybrid calli when the species involved can also be crossed by usual sexual in vivo methods (Melchers and Labib, 1974, Power et al. 1976, Smith et al. 1976, Melchers 1977, Melchers et al. 1978, Dudits et al. 1978). So far, somatic hybridization by protoplast fusion has led to success in different varieties of *Nicotiana tabacum*, different species of *Nicotiana* and two *Petunia* species (*P. hybrida* and *P. parodii*). Furthermore, a poor growing but morphologically normal sterile sexual hybrid, is known between *N. tabacum* and *P. parodii*. (Pogliaga 1952) Conventional sexual hybrids between *N. tabacum* and *P. hybrida* are not known although it may seem to be only a small step to achieve them through protoplast fusion. During the course of such experiments when a negative result seemed imminent, other experiments involving the method of sexual 'in vitro' hybridization were carried out. The latter method had already many times yielded at least hybrid embryos when normal in vivo hybridization had failed (Zenkteler 1965, 1967, 1971). Positive proof for the significance of the results (should they also prove to be negative) would be indicated if the conditions used were shown to be suitable for successful hybridization of other test plants. Consequently, in the present report, ex-

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periments with *N. tabacum* × *H. niger* and *Melandrium album* × *P. hybrida* are also described.

Material and Methods

Fusion Experiments with Protoplasts

Protoplasts were prepared from two chlorophyll deficient, light sensitive tobacco varieties s^2 (amphidiploid with 48 chromosomes), *s* (dihaploid with 24 chromosomes) and *v* (dihaploid with 24 chromosomes) which had been grown under low light intensity, high temperature and high humidity as described in our previous publication (Melchers and Labib 1974). From *P. hybrida* a yellow-green mutant mu_1^2 was available from J. Straub, Köln-Vogelsang. Unfortunately, the calli from mu_1^2 became normal green under the selection conditions where *v*, v^2, *s* and s^2 calli remained light green or yellow. Consequently we did not have available a selective system to differentiate between *Petunia* and hybrid calluses. Fusion was carried out by the methods of Keller and Melchers (1973) or Kao and Michayluk (1974). From microscopic observations during and after fusion experiments, one can be assured that the fusion products between tobacco and *Petunia* would be in a similar proportion as the products between the tobacco varieties *s* and *v* or *N. tabacum* and *N. silvestris* (Melchers and Labib 1974, Melchers 1977 and Melchers et al. 1978).

In vitro Hybridization by Ovule Pollination

Greenhouse grown plants of *Nicotiana tabacum*, cv. Samsun, *Hyoscyamus niger, Petunia hybrida* and *Melandrium album* were used for the experiments. In the period from April to August 1975, flower buds of *Nicotiana, Hyoscyamus* and *Melandrium* were embryologically analyzed in order to find the exact stage necessary for in vitro pollination of ovules. On the basis of the preliminary observations, flower buds of *Nicotiana* and *Hyoscyamus* were emasculated 48 hours before opening. Flowers of both species as well as of *Melandrium* (buds of that species were not emasculated since it is a dioecious plant) were brought to the laboratory on the day of opening, their pistils were disinfected in 70% ethanol for 45 sec, later immersed in 4% of calcium hypochlorite solution for 10 min and subsequently washed three times with sterile water. Ovules with the placenta were removed and transferred onto the nutrient medium (macroelements according to Nitsch 1951 and microelements according to Rangaswamy 1961). On the same day anthers were excised from flower buds, the pollen grains were scooped out and applied directly onto the ovules. The following crosses were made: *Nicotiana tabacum* × *N. tabacum* (self pollination), *N. tabacum* × *Hyoscyamus niger, N. tabacum* × *Petunia hybrida, H. niger* × *H. niger* (self pollination) *H. niger* × *N. tabacum, H. niger* × *P. hybrida, Melandrium album* × *M. album* (self pollination), *M. album* × *N. tabacum, M. album* × *P. hybrida.*

The cultures were kept in the culture room in darkness at 25-26°C. In order to study pollen germination and pollen tube growth, 12 and 24 hours after pollination the explants were prepared and stained in acetocarmine. For embryological and cytological investigations the ovules were fixed in Carnoy's solution, passed through an alcohol-xylene series and embedded in paraffin. Sections were cut at a thickness of 12-15 μm and stained with crystal violet and orange G.

Ovules of *N. tabacum* containing globular embryos (self-pollination) and those with the hybrid embryos (*N. tabacum* × *H. niger*) were cultured in liquid medium (Murashige and Skoog 1962) supplemented with kinetin, IAA and 2,4-D in various combinations and concentrations. Stock cultures of ovules were maintained in appropriate liquid medium (25 ml in 100 ml Erlenmeyer flasks, each flask containing 15-20 ovules). The flasks were agitated on a rotary shaker at 150 rpm under conditions of total darkness and constant temperature (25°C).

Results

Fusion Experiments with Protoplasts

No plants were obtained from calli, as could be expected in a cross between *N. tabacum* and *P. hybrida*. The plants which were obtained, and in part brought to flowering, were all tobacco or petunia. They sometimes demonstrated unusual characters and abnormal chromosome numbers (polyploidy, aneuploidy). However, the tobacco plants were always chlorophyll deficient under normal

Table 1. Control-experiments. In very many cases the small calluses identified as *v* or *s* by colour, small regenerates as mu_1^2 by the hairs

Number of Protoplasts	Calluses Tested	Calluses not Regenerated by Different Reasons	Calluses Regenerated	Regenerates tested in the Greenhouse
	v			
7 experiments 7 × 10^6 protoplasts	22	20	2	4
	mu_1^2			
20 experiments 2.3 × 10^7 protoplasts	287	284	3	16
	s			
6 experiments 6 × 10^6 protoplasts	45	37	8	33
	s^2			
6 experiments 6 × 10^6 protoplasts	16	14	2	4

Table 2. Fusion experiments with *Petunia hybride* mu_1^2 and tobacco *v*. No mu_1^2 calluses found. Sample tests for chromosome number (exact numbers counted 'diploid'. 'heteroploid' a.s o. estimated by morphology of the plants). In some cases normal 'haploid' *v* regenerates and few shoots with odd chromosome numbers

Experiment	Conditions for Fusion	Number of Protoplasts	Calluses Tested	Calluses not Regenerated by Different Reasons	Regenerated Calluses from *v*	*v*-Regenerates tested in Greenhouse	
6/9/74 $mu_1^2 + v$	pH 9.0 32°, 18′	2×10^5	47	39	8	26	
15/10/74 $mu_1^2 + v$	PEG 1540,12′ pH 9.5 23° 10′ + 37°10′	3×10^6	201	164	37	118	1 callus: 5 regenerates all 49 chromosomes 1 „ 14 „ , 3 w. 52 chromosomes 2 w. 53 chromosomes 1 „ 2 „ , both 52 chromosomes 1 „ 4 „ 1 diploid 1 „ 8 „ all diploid
23/10/74 $mu_1^2 + v$	PEG 1540 8′ pH 9.5,23°,1,5′	$1{,}5 \times 10^6$	160	134	26	91	1 „ 2 „ 1 90-96 chromosomes, 1 haploid
31/10/74 $mu_1^2 + v$I	PEG 1540 23° 10′	4×10^6	43	34	9	30	1 heteroploid
31/10/74 $mu_1^2 + v$II	PEG 1540 23° 10′ PEG replaced by pH 10.5 37° 8′	$1{,}5 \times 10^6$	15	10	5	26	1 callus: 8 regenerates 4 with 46-48 chromosomes 1 callus: 5 regenerates: 1 with 48 chromosomes 1 callus: 4 regenerates: all ca 48 chromosomes 1 callus: 6 regenerates: all 48 chromosomes
12/12/74 mu_1^2 I + *v*	pH 9.0 33° 30′	10^6	22	18	4	10	
9/1/75 $mu_1^2 + v$	pH 9.0 33° 22′	10^6	91	90	1	1	
Σ 7		1.22×10^7	579	489	90	302	

Table 3. Fusion experiments with *Petunia hybrida* mu_1^2 and tobacco *s*. Only a few mu_1^2 = regenerates tested in the greenhouse. Diagnosis of *Petunia* calluses in callus stage. Sample tests like Table 2

Experiment	Conditions for Fusion	Number of Protoplasts	Calluses Tested	Calluses not Regenerated by Different Reasons	*s*-Calluses Regenerated	*s*-regenerated tested in Greenhouse	mu_1^2-callus Regenerated	mu_1^2-regenerates tested in Greenhouse
2/10/74 $mu_1^2 + s$	PEG 1540 30° 20′	1.5×10^6	80	74	6	14	1 callus: 1 regenerates 1 polyploid, 1 „ 2 regenerates, 1 w. 48 Chromosomes	– –
18/2/75 $mu_1^2 + s$	pH 8.5 30° 30′	10^7	69	55	14	67		1 2
25/2/75 $mu_1^2 + s$	pH 9.0 30° 10′	7.5×10^6	3	1	2	9	1 callus: 5 regenerates, 1 hypohaploid.	– –
4/3/75 $mu_1^2 + s$	pH 9.0 30° 12′	10^6	9	8	1	3		– –
18/3/75 $mu_1^2 + s$	pH 9.0 30° 21′	5×10^5	7	4	3	11		– –
20/3/75 $mu_1^2 + s$	pH 9.0 30° 28′	1.25×10^7	49	46	3	16	1 callus: 6 regenerates 1 diploid	– –
Σ 6		3.3×10^7	217	188	29	120		1 2

Table 4. Fusion experiments with *Petunia hybrida* and tobacco s^2. Details like Table 2 and 3

Experiment	Condition for Fusion	Number of Protoplasts	Calluses Tested	Calluses not Regenerated by Different Reasons	s^2-Calluses Regenerates	s^2-Regenerates Tested in Greenhouse	mu_1^2 Calluses Regenerates	mu_1^2 Regenerates Tested in Greenhouse
20/3/75 $mu_1^2 + s^2$	pH 9.0 30° 11′	10^7	105	91	14	58	1	1
23/5/75 $mu_1^2 + s^2$	pH 9.0 30° 15′	7.5×10^6	114	93	21	61	1	1
28/5/75 $mu_1^2 + s^2$	pH 9.0 30° 20′	7.5×10^6	108	97	11	53	1 callus: 4 regenerates 1 heteroploid 1 abnormal Nr. of Chromosomes –	–
12/6/75 $mu_1^2 + s^2$	pH 9.0 30° 15′	5×10^6	276	262	14	78	1 callus: 4 regenerates 1 polyploid 1 heteroploid 6	51 1 callus: 4 regenerates 1 w. 13 chromosomes 2 w. 14 + 28 chromosomes
31/7/75 $mu_1^2 + s^2$	pH 9.0 30° 19′	2×10^6	9	7	2	3	–	–
7/8/75 $mu_1^2 + s^2$	pH 9.0 30° 16′	1.5×10^6	4	4	–	–	–	–
Σ 6		3.35×10^7	616	554	62	253	8	53

greenhouse-light conditions, demonstrating the possession of recessive alleles *v* or *s* in a homozygous state; the petunia regenerates, in contrast to tobacco, could be recognized very early because of the presence of very short stalked glandular hairs.

Table 1 provides a summary of all experiments with unfused protoplasts (controls). It is a fact that here, as well as in all other experiments, there is a large number of infected callus cultures in *Petunia*. The cause is mainly the bacteria which live with the greenhouse grown plants. They are very closely associated with the protoplasts, cells and calli and gradually hinder the growth of the calli. The bacteria survive protoplast preparation and fusion and only later, or not at all, do they break out from the larger calli and cover the medium (In tables 1-4).

Table 2 is a survey of the fusion experiments between dihaploid *v* and diploid mu_1^2. Since at the callus stage *v* is sometimes not so significantly light sensitive as *s*, many calli suspected to be fusion products were tested through plant regeneration.

Table 3 summarizes all experiments with dihaploid *s* and diploid mu_1^2; Table 4, with amphidiploid s^2 and diploid mu_1^2. Random samples taken for chromosome numbers in callus cultures never gave the expected results of 38 for $s +$ or $v + mu_1^2$, or of 62 for $s^2 + mu_1^2$ (chromosome numbers presented in tables). In one culture, which is still being maintained, 41, 42, 83, 84 chromosomes and also 14 (as for *P. hybrida*) could be counted. This means that after prolonged culture there were calli with a chimaeric composition of aneuploid chromosome numbers as well as the normal composition tobacco and petunia. However, no chimaeric plant could be found among the regenerates from each callus. Tobacco plants with markedly abnormal characters (narrow leaves, abnormally developed flowers with chromosome numbers 38, 39, 35-40, 41, 42, 43) show the recessive character yellowish to almost white leaves under greenhouse-light conditions typical for the tobacco variety 'sublethal' and also show no indications of hybrid characters.

Sexual Crossings

Pollination was performed on greenhouse-grown plants. Pollen grains of unrelated plants, when put on stigmas of *Nicotiana tabacum*, *Hyoscyamus niger* and *Melandrium album*, did not germinate, or they germinated only sporadically. Sometimes they produced shorter tubes which later burst. Embryological examination of ovules 3 days after pollination never revealed the entrance of the pollen tube into the embryo sac, nor the presence of endosperm or embryo.

Self Pollination N. tabacum × N. tabacum

N. tabacum pollen grains germinated abundantly on the surface of ovules and formed a dense coating of tubes each several centimetres long. Ten day-old ovules contained heart-shaped embryos surrounded by the cellular endosperm. Six days later, well differentiated dicotyledonous embryos were present in many ovules. Five to six weeks after artificial pollination, seeds started to germinate *in situ* and produced seedlings. About 20-80 seeds developed on each placenta.

Pollination of N. tabacum Ovules with Pollen Grains of Petunia hybrida

Pollen grains of *P. hybrida* germinated profusely and in many cases pollen tubes entered the embryo sacs. Four days after pollination between 2 and 10 ovules on each placenta became enlarged. Inside such ovules filamentous proembryos were discernible (Fig. 1). The development of proembryos concomitantly with the endosperm, or of endosperm only, occured in many examined ovules. It is significant that often the cellular endosperm attained an advanced stage of development (Fig. 2). Eight to ten days after pollination the enlarged ovules became shrivelled and

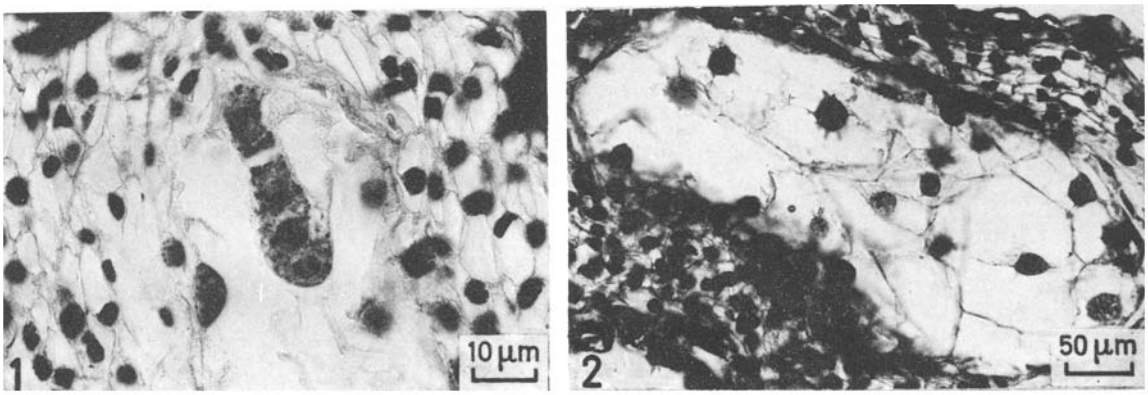


Fig. 1. Linear proembryo in cross *Nicotiana* × *Petunia*, 4 days after pollination in vitro
Fig. 2. Endosperm in the embryo sac of *Nicotiana*, 4 days after pollination in vitro with pollen of *Petunia*

turned brown. Embryological examinations of those ovules revealed a complete degeneration of the linear proembryos.

The number of chromosomes were established only in the metaphase plates of the dividing endosperm nuclei. The mitotic divisions were analyzed in 2 ovules on the 5th day following pollination. The chromosome counts revealed in both cases the presence of 3n = 55 chromosomes. It was not possible to analyze the chromosomes in proembryos as no suitable stages for counting were discernible.

The above finding in the control cross *N. tabacum* × *N. tabacum* which led to new plants, as well as the surprisingly positive results from the cross *N. tabacum* × *H. niger* suggest that the responsibility for the lack of further progress of embryogenesis in the cross *N. tabacum* × *P. hybrida* did not lie with the methods used.

Pollination of N. tabacum Ovules with Pollen Grains of Hyoscyamus niger

Pollen germination was observed 4 to 6 hours after culture and pollen tubes grew luxuriantly among the bare ovules. Ten hours later pollen tubes were observed inside the embryo sacs (Fig. 3). Two days after pollination some ovules showed an incidence of double fertilization. In the next 2 days, between 10 and 15 ovules on each placenta enlarged while the remaining ones started to shrivel, turned brown and stopped growing (Fig. 4). Five to six days after pollination the young developing seeds (fertilized ovules) contained a globular embryo and a cellular endosperm (Figs. 5, 8, 9). In these seeds massive endosperm tissue was also present. In some ovules the globular embryos produced additional outgrowths (like buddings) which presumably had arisen by splitting of the linear proembryo at an early stage of development (Fig. 6, 7).

To prevent the degeneration of globular embryos, whole ovules were excised and transfered to liquid medium (Murashige and Skoog 1962). Three sets of this medium were prepared: A. Kinetin – 2 mg/l, IAA – 1 mg/l and sucrose – 6%; B. Kinetin – 2 mg/l, IAA – 1 mg/l and sucrose – 9%; C. Kinetin – 2 mg/l, 2,4-D – 4 mg/l and sucrose – 6%. The cultures were agitated on a rotary shaker at 150 rpm in the dark for 20 days. Every three days several ovules were taken out from the media and examined microscopically. It was found that only on medium C did the globular embryos develop further. After one week they reached the pre-heart stage (Fig. 10) and during the following 7 days they started to produce a callus. However, medium C did not support the good growth of callus cells and usually the further growth of embryos was arrested (Fig. 11). No indication of embryo development was found in ovules cultured in medium A and B.

Karyological investigations of squashes of the globular embryos revealed the presence of 41 chromosomes (Fig. 8a). Chromosome counts made from permanent preparations of endosperm gave the 3n value as 65 (Fig. 8b).

At the same time, experiments were carried out on ovules of *N. tabacum* (self-pollinated). Ovules, 5 and 6 days after pollination, were transferred to all three

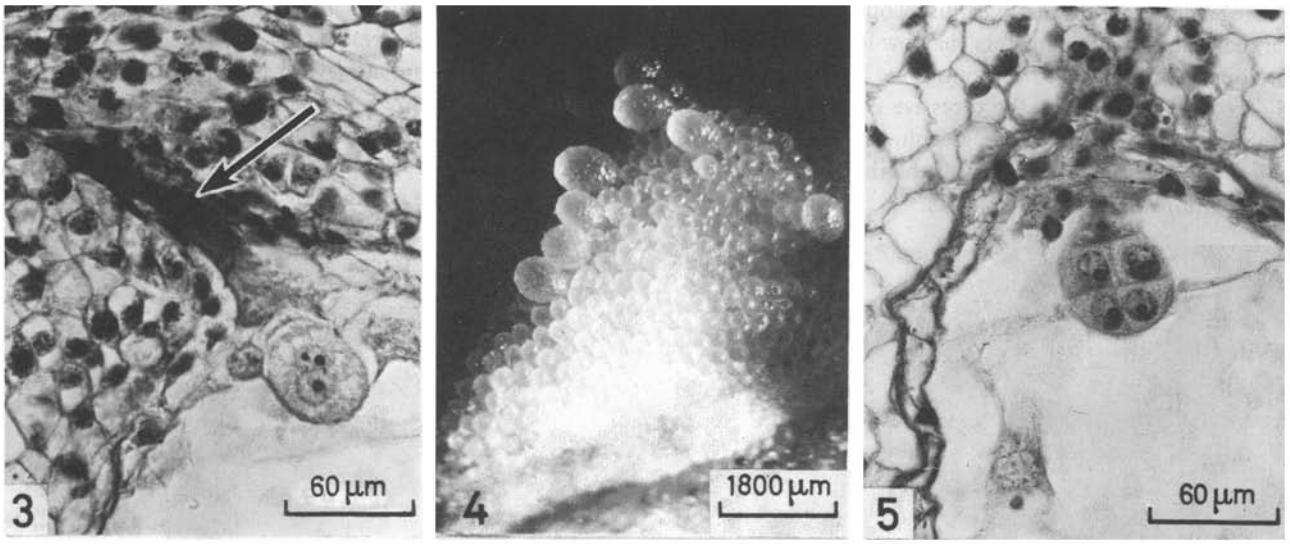
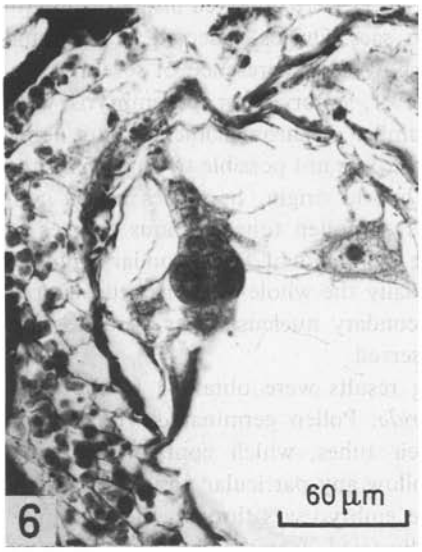


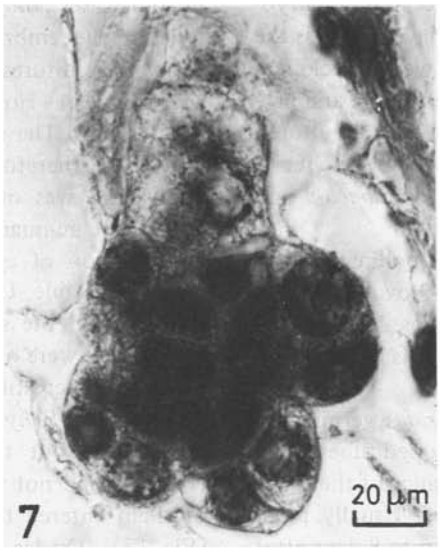
Fig. 3-Fig. 11: *N. tabacum* × *H. niger*

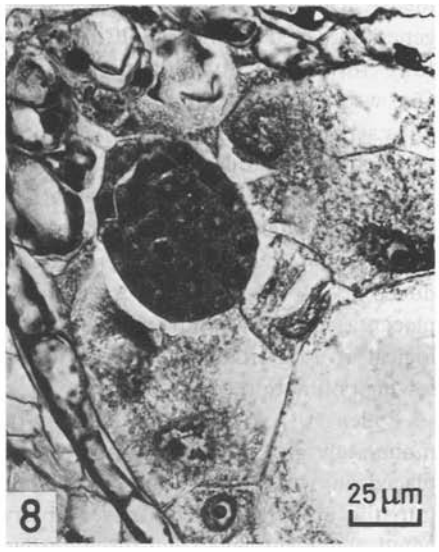
Fig. 3. The entrance of pollen tube (arrow) into the embryo sac

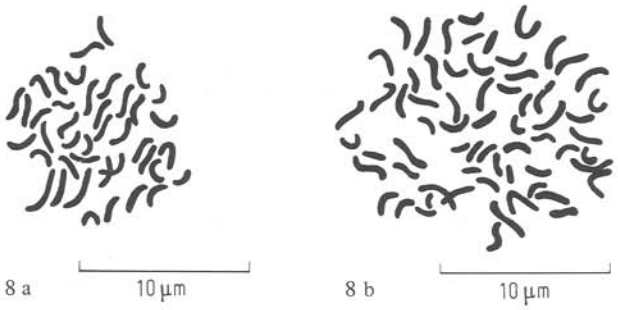
Fig. 4. Enlarged ovules of *N. tabacum* situated on the placenta in the in vitro culture, 4 days after pollination

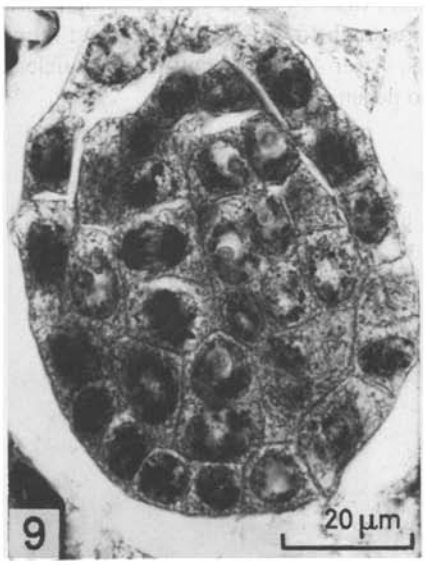
Fig. 5. Hybrid Globular embryos, 5 days after pollination

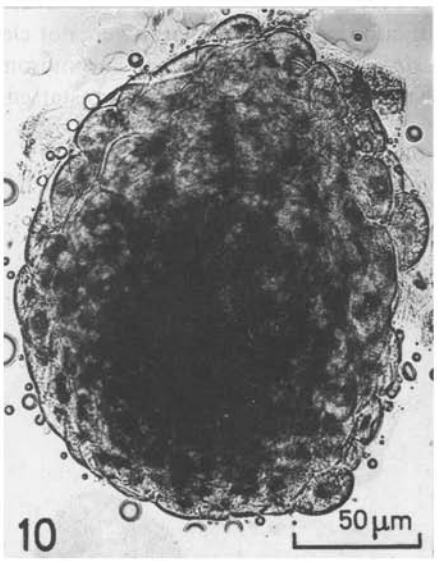


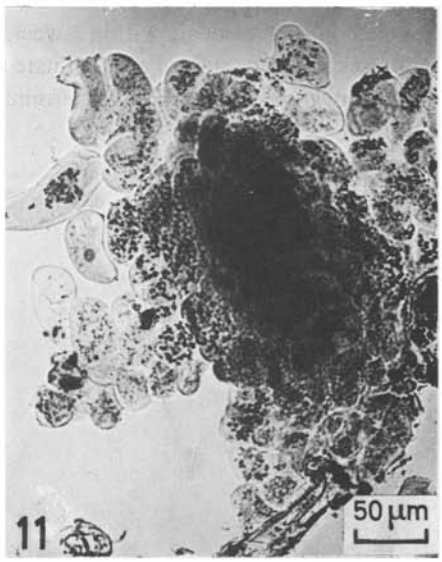












Figs. 6 and 7. Hybrid globular embryo with additional outgrowths (buddings)

Fig. 8. Hybrid globular embryo embedded in endosperm, 8 days after pollination. *a* Chromosome counting (41) of the embryo (s. Fig. 8); *b* chromosome counting (65) of the endosperm (s. Fig. 8)

Fig. 9. Enlarged embryo of Fig. 8

Fig. 10. Hybrid embryo in the pre-heart stage isolated from ovules, 7 days of culture in the liquid medium (15 days following pollination of ovules situated on the placenta)

Fig. 11. Hybrid embryo producing callus after 14 days of culture in liquid medium

media. Only in medium C did the process of embryogenesis proceed normally and in the 20 day old seeds the fully formed dicotyledonous embryos were developed. One week later the embryos started to germinate and produce seedlings. Usually from 70 to 85% of seeds cultured in the liquid medium developed into seedlings. The reciprocal cross with *H. niger* as mother and *N. tabacum* and *P. hybrida* pollen remained unsuccessful.

Ovules of *Hyoscyamus niger*, when self-pollinated, produced in only a very few cases, (usually 1-3 ovules on each placenta) seeds containing mature embryos. No embryological investigations were carried out on this material following pollination.

Pollen of *N. tabacum* and *P. hybrida* germinated moderately and produced normally developed tubes when placed on ovules of *H. niger*, but the entrance of the tubes into the embryo sacs was never observed. Usually pollinated ovules shrivelled and turned brown 6 to 8 days after pollination. Endosperm and embryo formation were never observed.

Pollination of Melandrium album ovules with pollen grains of N. tabacum and P. hybrida

As a result of earlier experience with *Melandrium album* it seemed to be interesting to combine ovules from *M. album* with pollen from *N. tabacum* and *P. hybrida* (Zenkteler 1967, 1971).

When ovules of *M. album* were self-pollinated, fertilization occured normally and about 40 to 70 seeds were developed on each placenta within 3 weeks of culture. In the 4th week embryos started to germinate *in situ*.

Pollen grains of *N. tabacum* germinated profusely and in some cases pollen tubes were observed inside the micropyle of the embryo sacs. Ovules dissected from 2- and 3 day old cultures showed the presence of several endosperm nuclei. However, embryos or proembryos were never found. The number of chromosomes was not determined and therefore it was not possible to ascertain if the endosperm was of hybrid origin. In ovules which contained the remnants of pollen tubes, various degrees of degeneration of egg cells and of the secondary nucleus were observable. Usually the whole egg apparatus deteriorated and in the secondary nucleus scores of nucleoli of various size were observed.

Quite interesting results were obtained in the cross *M. album* × *P. hybrida*. Pollen germinated with varying frequencies but their tubes, which contained 2-nuclei (Fig. 12), did not follow any particular direction. Several of them entered the embryo sacs through the micropyle (Fig. 13). Ovules dissected from 24 hour-old cultures showed the presence of a petunia male gamete (or a generative nucleus) inside the egg cell of *Melandrium* (Fig. 14). In four ovules division of both nuclei inside the egg cell was found. As shown in Figs. 15, 16, 17, one dividing nucleus represents 7 chromosomes of *P. hybrida* and the other one, 12 chromosomes of *M. album*. More advanced stages of development of the hybrid zygote were never observed. Endosperm was represented by several nuclei, however, it was difficult to establish the number of chromosomes. In some cases the dividing nuclei contained ca.. 24 chromosomes (the diploid number of *Melandrium*) and in other ones more than 24 chromosomes. Metaphase plates were not clear enough to distinguish the exact number of chromosomes. After 4 days of culture the nuclear endosperm started to degenerate.

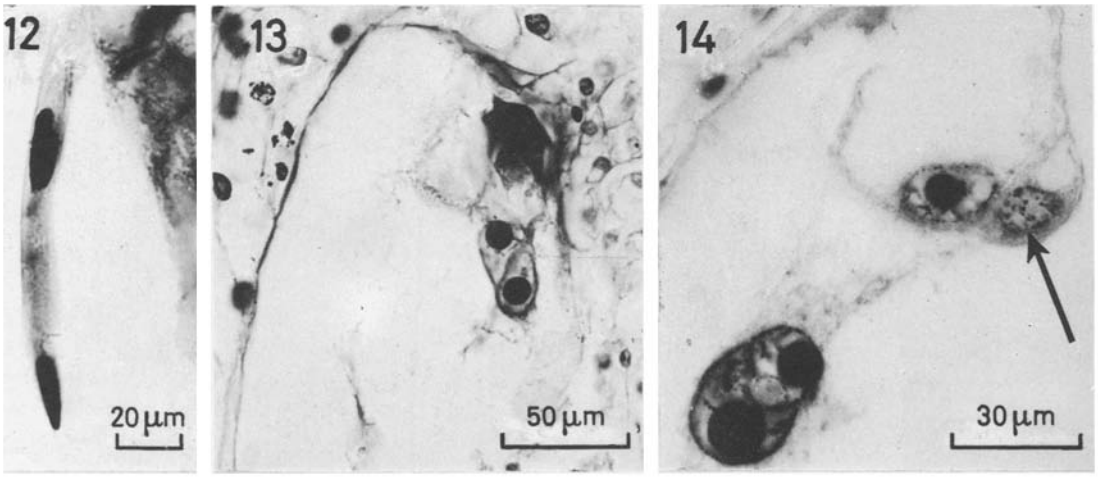


Fig. 12. Pollen tube of *Petunia* germinating on ovules of *Melandrium*, 24 hours after pollination, 2 nuclei visible in the tube

Fig. 13. A discharged pollen tube of *Petunia* at the micropylar end of the embryo sac of *Melandrium*

Fig. 14. The egg cell of *Melandrium* with an additional nucleus – male gamete of *Petunia* (arrow). In the lower part a secondary nucleus with 2 nucleoli

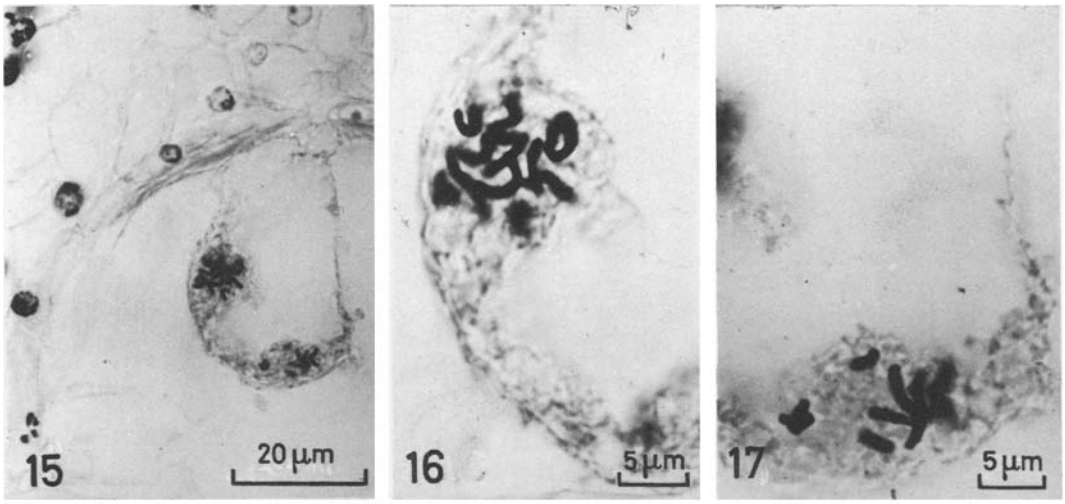


Fig. 15. A mitotic division of the male gamete of *Petunia* and of the egg nucleus of *Melandrium* inside the egg cell of *Melandrium*
Figs. 16 and 17. Enlargements of Fig. 15

Discussion

It has been expected that somatic hybridization by protoplast fusion will supercede the possibilities offered by sexual hybridization. It actually seems that no incompatibility exists at the level of fusion. Further cell hybrids from systematically distantly related partners which divide and make possible studies at the callus culture level have already been described (*N. glauca* + *Glycina max.*, Kao 1977, Wetter 1977, Kao and Wetter 1977). However, at present it is only possible to regenerate hybrid plants from hybrid calli in cases where the hybrids can also arise through sexual hybridization (*N. tabacum* varieties, *Nicotiana* species, *Petunia* species, *Daucus* species). Exceptions are the cases when infertile 'haploids' are somatically crossed (Melchers and Labib 1974) or mixed plastomes are produced by fusion of protoplasts of somatic cells (Gleba 1978). At the moment, the systematic status of the partners of a hybridization is of no special interest. *N. tabacum* and *P. hybrida* were tested not because the hybrid would be an intergeneric hybrid – one conventional sexual hybrid already exists between *N. tabacum* and *P. parodii* – but because in vivo hybridization does not function and because of their close relationship such a fusion would raise hopes of success by other methods. The inability of two partners to cross can have its origin in the gametes. Our experiments have shown that these difficulties can be bypassed through in vitro culture systems. *Petunia hybrida* pollen tubes fertilize *N. tabacum* embryo sacs when the style and stigma of tobacco are removed. Evidently in this case, apart from the gametic incompatibility which can be overcome, there exists a zygotic incompatibility. After a few cell divisions, embryogenesis does not proceed further. It has not yet been tested whether the fusion products of mesophyll protoplasts behave similarly. Such experiments are possible by following individual fusion products by microscopy (Kao 1977). It is established in crosses between *N. tabacum* and *P. hybrida* that zygotic incompatibility exists *in situ*. That does not mean that hybrid cells in an optimal culture medium would not develop further, the zygotic incompatibility could also be partially caused by the surrounding tissue of the mother plant. The culture experiments which have been performed with different media and which were successful only with medium C for ovules containing embryos from the cross *N. tabacum* × *H. niger*, show that there is some hope of success by finding the right medium.

It is at present not possible to predict the future success of distant crosses which are also desired by plant breeders. Inability to cross or the failure to develop fertile products of a hybrid is an important step from the standpoint of natural evolution. Just one gene mutation can be sufficient to isolate a group or an organism from its ancestors. However, for the survival of gametic or somatic fusion products under in vitro conditions and for the division of their nuclei or cells, a highly balanced harmony between the combined genomes does not appear to be a requisite. On the other hand, embryogenesis in vivo and in vitro, or plant regeneration via organogenesis from a callus, obviously requires more balanced gene activities. For many plants the regeneration from callus is not yet possible or the potential for plant regeneration is lost after prolonged maintenance by subculture. General forecasts, positive or negative, are impossible at present. It can only be recommended to try all available methods, in vitro pollination and fusion of protoplasts, especially in combination with embryo culture and plant regeneration from

callus cultures, to test whether a specific cross is possible. This cannot be achieved in vivo and which is a desired breeding aim.

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