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Interspecific hybridization between *Nicotiana repanda* Willd. and *N. tabacum* L. through the pollen irradiation technique and the egg cell irradiation technique *

Y. Shintaku, K. Yamamoto and T. Nakajima [1]

Laboratory of Plant Breeding, Faculty of Agriculture, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, 113, Japan
[1] Present address; Laboratory of Plant Breeding, Faculty of Agriculture, Tamagawa University, Tamagawa-gakuen 6-1-1, Machida city, Tokyo, 194, Japan

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Summary. Two techniques were useful in overcoming hybrid inviability between *N. repanda* and *N. tabacum*. These techniques combine gamma-ray irradiation to pollen or to egg cells (in ovules) with in vitro culture of fertilized ovules. When in vitro culture of fertilized ovules from in situ hybridization of *N. repanda* × *N. tabacum* was combined without gamma-ray irradiation to pollen or to egg cells (in ovules), all of the resulting seedlings developed chlorosis and died. Furthermore, in the case of in situ hybridization of *N. repanda* × *N. tabacum* with gamma-ray irradiated *N. tabacum* pollen, no viable seeds were obtained. By using both techniques, combining gamma-ray irradiation to *N. tabacum* pollen or to egg cells in (*N. repanda* ovules) with in vitro culture of fertilized ovules, we were successful in obtaining flowering hybrid plants. Thus, it appears that it may be possible to overcome hybrid inviability to a certain extent using both the pollen irradiation technique and the egg cell irradiation technique, i.e., gamma-ray irradiation to pollen or to egg cells (in ovules) before pollination and in vitro culture of fertilized ovules.

Key words: Wide hybridization – Hybrid inviability – Irradiation – Ovule culture – Tobacco

Introduction

Conventional hybridization between distantly related species is usually unsuccessful, with the exception of a few compatible crosses between particular species or genera. The causes of barriers to sexual reproduction can be grouped into four distinct classes of phenomena: (1) inhibition of pollen germination in stigmata and/or pollen tube growth in styles; (2) spontaneous abortion of the ovules after fertilization; (3) inviability of hybrid embryos and/or young hybrid plants; and (4) deformity or sterility of reproductive organs in hybrids or their progenies. Much research has been done to attempt to overcome these barriers to sexual reproduction (Zenkteler 1967, 1980; Zenkteler and Melchers 1978; Kameya and Hinata 1970; Marubashi and Nakajima 1985), but research on hybrid inviability is still relatively new and this problem remains unresolved (Kostoff 1930; Mok et al. 1978; DeVerna et al. 1987).

In the genus *Nicotiana*, ovule culture was successfully used to obtain hybrids in several interspecific combinations, and the typical pattern of ovular death within several days of cross-pollination was prevented (Reed and Collins 1978; Shizukuda and Nakajima 1982; Douglas et al. 1983). However, in the combination between *N. repanda* and *N. tabacum* the hybrid seedlings died in the younger stages of development (Reed and Collins 1978). This phenomenon is a manifestation of hybrid inviability. If a small portion of the male or female genome that causes hybrid inviability could be eliminated, hybrid plants might be obtained. Although the cross-combination between *N. rustica* and *N. tabacum* does not normally show hybrid inviability (Shizukuda and Nakajima 1982), Shizukuda et al. (1983) successfully produced "partial hybrids" in which an incomplete chromosome complement of *N. tabacum* was added to the haploid chromosomes of *N. rustica*. They used a combination of two techniques: (1) pollination after the destruction of chromosomes in pollen grains with ionizing radiations; and (2) in vitro culture of fertilized ovules.

In this report, the combination of techniques used by Shizukuda et al. (1983) was applied to interspecific hybri-

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dization between *N. repanda* ad *N. tabacum* to investigate the possibility of overcoming hybrid inviability. Moreover, irradiation was applied not only to pollen grains but also to egg cells in ovules. Thus, two different techniques were used in this study.

Materials and methods

Plant materials

Nicotiana repanda Willd. and *N. tabacum* L. cv Hicks 2 (supplied by Japan Tobacco Inc.) were used in the experiments.

Irradiation

Cesium 137 gamma-rays were used and radiation treatment was conducted at the Gamma-ray Irradiation Facilities, Research Center for Nuclear Science and Technology, The University of Tokyo.

Pollen irradiation technique

The anthers of *N. tabacum* were collected the day before anthesis and placed in small vials. They were then stored in the desiccator overnight. The following day, the fresh pollen grains of *N. tabacum* were exposed to radiation. Doses ranging from 5 kR to 40 kR were achieved by applying radiation at a constant rate of 2.5 kR per minute. The flowers of *N. repanda* were emasculated the day before anthesis and pollinated with the irradiated pollen at the time of anthesis. On the 4th, 5th and 6th days after cross-pollination, the developing ovules were excised from their placentae and inoculated onto culture medium.

Egg cell irradiation technique

The flowers of *N. repanda* were collected before anthesis. Sepals, anthers and petals were removed under aseptic conditions. The pistils were planted on the surface of the same agar medium that was used in the pollen irradiation technique. The following day, they were exposed to radiation. Doses ranging from 0.5 kR to 4 kR were achieved by applying a constant rate of 0.5 kR per minute. The fresh pollen grains of *N. tabacum* were gathered under aseptic conditions and were dusted onto the stigmata of irradiated pistils. On the 4th, 5th and 6th days after cross-pollination in vitro, the developing irradiated ovules were excised from placentae and inoculated onto the fresh medium of the same composition.

Culture media and condition

The medium used in both techniques contained the mineral solution of Nitsch (1972), vitamins (Maheshwari and Lal 1961) and 7% sucrose. The medium was adjusted to pH 5.8 and 0.8% agar was added.

To promote rooting, hybrid plantlets with 4–6 leaves were transferred to agar (0.7%) medium containing the mineral solution of Nitsch (1972), 1 mg/l indoleacetic acid and 1% sucrose at pH 5.8.

All cultures were maintained under continuous illumination (about 3,000 lux) at 28 °C.

Chromosome analysis

Root tips of the hybrid plants were pretreated with a 0.5% colchicine solution for 90 min or with 0 °C water for 24 h. They were then fixed in a solution of 3 parts absolute alcohol to 1 part glacial acetic acid for 30 min, hydrolyzed in 1 N HCl for 6 min at 60 °C and stained with Feulgen reagent. From each hybrid plant, five roots were extracted and squashed. More than three cells with well spread chromosomes were observed for chromosome counts.

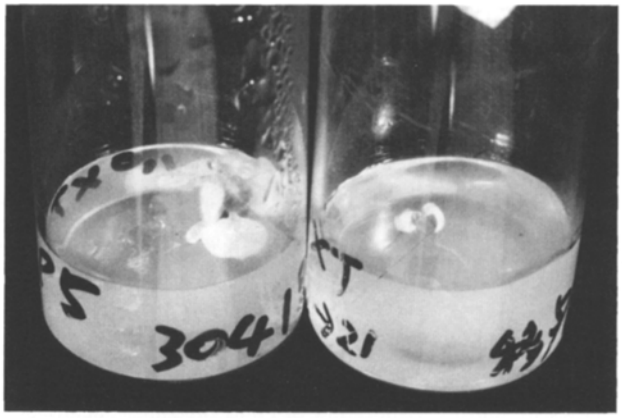

Fig. 1. Hybrid plantlets obtained using ovule culture after in situ hybridization of *N. repanda* × *N. tabacum* without gamma-irradiation; they developed chlorosis and died; they showed hybrid inviability

Table 1. Production of seedlings in situ hybridization of *N. repanda* × *N. tabacum* through ovule culture

Days after pollination	No. of ovaries inoculated	No. of ovules inoculated	No. of seeds germinated	Percentage of seeds germinated
3	11	2 860	0	0
4	6	1 560	1	0.06
5	14	3 640	56	1.54
6	6	1 560	8	0.51
7	7	1 820	6	0.33
8	5	1 300	4	0.31
Total	49	12 740	75	0.59

Results

Hybrid inviability between N. repanda and N. tabacum

When pollen grains of *N. tabacum* were pollinated on stigmata of *N. repanda* in situ, the pollen germinated normally and pollen tubes extended far enough to reach ovules of *N. repanda*. Although most of the ovules started to develop 3 days after pollination, within 9 days all embryos were aborted. No viable seeds from in situ hybridization of *N. repanda* × *N. tabacum* were obtained. The ovules that developed on the 4th, 5th and 6th days after pollination were cultured. After approximately 20 days of ovule culture, a large number of seedlings were obtained (Table 1). The seedlings grew to plantlets bearing 4–6 leaves and slender roots. All of the plantlets obtained by ovule culture eventually developed chlorosis and died (Fig. 1), as reported by Reed and Collins (1978).

Table 2. Production of seeds in situ hybridization of *N. repanda* with irradiated pollen of *N. tabacum*. Significance between every treatment and non-treatment was tested by t-test

	N. repanda self	Dose (kR)					
		0	05	10	20	30	40
No. of pollinated flowers	3	2	3	5	7	2	2
No. of seeds obtained per ovary	329±24	268±71	124.3±41.7	84.8±12.8	139.9±22.3	124.5±84.5	0
t-test	–	–	$0.2<P<0.3$	$P<0.001$	$P<0.001$	$0.4<P<0.5$	–
Percentage of germinated seeds	96.9	0	0	0	0	0	0

Table 3. Effect of gamma-irradiation on survival of hybrid plantlets

Experiment	Dose (kR)	No. of seedlings obtained	No. of plantlets which survived			No. of flowering plants in pots
			Up to 3 months	Up to 6 months	More than 12 months	
Pollen irradiation technique	0	124	–	0	0	0 (0)
	5	71	–	7 (9.9)	0 (0)	0 (0)
	10	33	–	4 (12.1)	0 (0)	0 (0)
	20	20	–	6 (30)	4 (20)	4 (20)
	30	8	–	0 (0)	0 (0)	0 (0)
	40	0	–	0 (0)	0 (0)	0 (0)
Egg cell irradiation technique	0	62	0 (0)	0 (0)	0 (0)	0 (0)
	0.5	71	29 (40.8)	14 (19.7)	10 (14.1)	0 (0)
	1	382	186 (48.7)	119 (31.2)	66 (17.3)	1 (0.26)
	2	120	33 (27.5)	17 (14.2)	5 (4.2)	1 (0.83)
	3	15	3 (20)	2 (13.3)	2 (13.3)	0 (0)
	4	21	5 (23.8)	3 (14.3)	2 (9.5)	0 (0)

Hybridization of N. repanda with irradiated pollen of N. tabacum

As shown in Table 2, most of the seeds obtained aborted and were non-viable. A few of them looked like viable seeds, but when they were sown on moistened filter papers in petri dishes, none of them germinated.

Pollen irradiation technique

Most of the plantlets obtained by the pollen irradiation technique died, as did the plantlets obtained by in vitro culture of the fertilized ovules without gamma-ray irradiation to *N. tabacum* pollen, but several survived. The results of the production of seedlings obtained by the pollen irradiation technique were presented in Shintaku et al. (1985).

The surviving plantlets were subcultured once a month on the root-promoting medium. Some of them continued to grow in culture vessels for more than 6 months. The results in relation to the radiation dosage are shown in Table 3. Though all of the hybrids in 0 kR died, survival rates increased with higher radiation up to a point (9.9% in 5 kR, 12.1% in 10 kR and 30% in 20 kR survived as long as 6 months after germination). At extremely high dosages, however, the rate of viable hybrids declined. In 30 kR, 8 seedlings were obtained, but none survived. In 40 kR, no seedlings at all were obtained. Thus, 20 kR appears to be the most effective dose for obtaining viable hybrids. Four flowering hybrid plants were eventually obtained in 20 kR. Thus, this technique seems to be effective in obtaining viable plantlets.

Egg cell irradiation technique

The results obtained in seedlings produced by the egg cell irradiation technique were similar to those obtained by the pollen irradiation technique, as described in Shintaku et al. (1986).

Moreover, the rates of hybrid survival using the egg cell irradiation technique were similar to the results for the pollen irradiation technique (Table 3). Most of the plantlets obtained died, but several survived. The dose with the highest survival rate was 1 kR. Two flowering hybrid plants were eventually obtained in 1 kR and 2 kR. Thus, this technique also seems to be effective in obtaining viable plantlets.

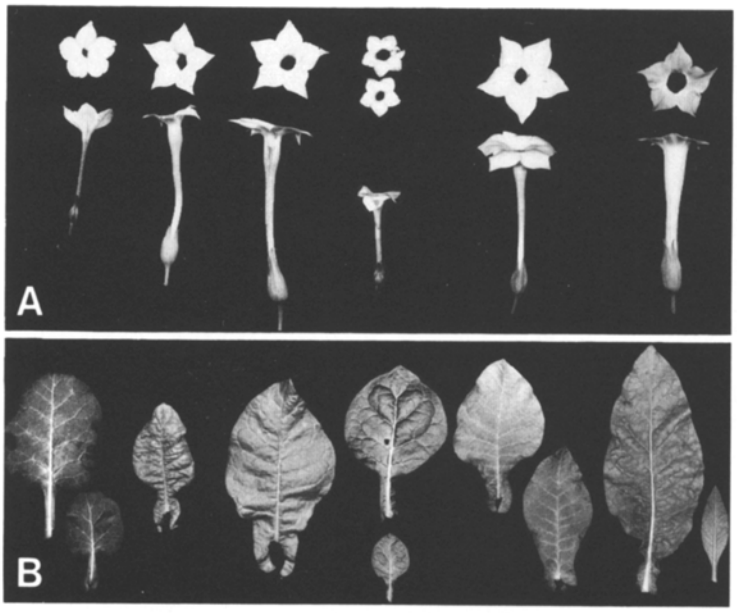

Fig. 2.

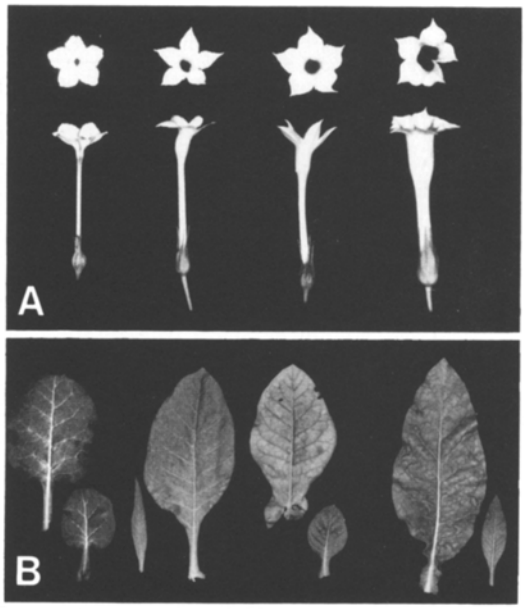

Fig. 3.

Fig. 2 A and B. Hybrids obtained using the pollen irradiation technique, which overcame hybrid inviability; **A** flowers of *N. repanda*, hybrids (nos. 5–8) and *N. tabacum* (*left to right*); **B** leaves of *N. repanda*, hybrids (nos. 5–8) and *N. tabacum* (*left to right*)

Fig. 3 A and B. Hybrids obtained using the egg cell irradiation technique, which overcame hybrid inviability; **A** flowers of *N. repanda*, hybrids (nos. 18 and 19) and *N. tabacum* (*left to right*); **B** leaves of *N. repanda*, hybrids (nos. 18 and 19) and *N. tabacum* (*left to right*)

Table 4. Morphological analysis of flowering hybrid plants obtained by the pollen irradiation technique and by the egg cell irradiation technique

	Species of parents		Code no. of hybrids[a]					
	N. repanda	*N. tabacum*	5	6	7	8	18	19
Dose (kR)	0	0	20	20	20	20	1	2
No. of chromosomes ($2n$)	48	48	47	46	44	48	48	46
Apical dominance	–	+	+	+	–	+	+	+
Flower shape	*N. repanda*	*N. tabacum*	medium	medium	small	medium	medium	medium
Flower color	white	pink	light pink	light pink	light pink	light pink	light pink	light pink
Leaf shape	*N. repanda*	*N. tabacum*	medium	medium	small	medium	medium	medium
Petiole	–	+	–	–	+	–	+	–
Survival duration after germination[b]			−10	−10	28+	−12	18+	12+

[a] Nos. 5, 6, 7, 8 were obtained using the pollen irradiation technique, and nos. 18, 19 were obtained using the egg cell irradiation technique

[b] −10 shows that hybrid was dead in vitro or in the pot up to 10 months after germination, and 12+ shows that hybrid survived in the pot for more than 12 months after germination

Analysis of hybrids

The plantlets that survived were potted in vermiculite and transferred into a greenhouse. Most of the transplanted plantlets displayed poor growth and died within several months. Only six of them were able to reach maturity. Characteristics of the viable hybrid plants are shown in Table 4 and Figs. 2 and 3. Four of them (coded nos. 5–8) were obtained using the pollen irradiation technique with a dose of 20 kR. The rest of them (coded nos. 18–19) were obtained using the egg cell irradiation technique with doses of 1 kR and 2 kR.

The plant type of the flowering hybrid plants (except no. 7) was similar to that of the male parent, *N. tabacum*,

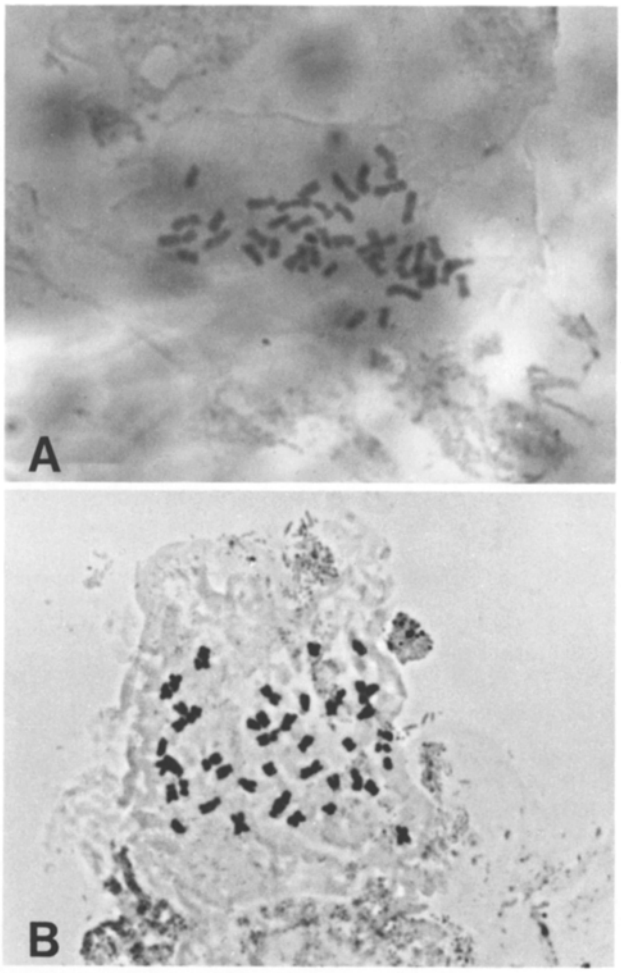

Fig. 4 A and B. Cytology of interspecific hybrids obtained by the pollen irradiation technique and by the egg cell irradiation technique; **A** root tip cell of no. 7 with 44 chromosomes; **B** root tip cell of no. 19 with 46 chromosomes

with regard to plant height and branching habit, rather than lying somewhere in the two parent plants. Flower shapes and leaves were intermediate in appearance between those of the parents, but the flower shapes were more similar to *N. tabacum,* the male parent (Figs. 2 and 3). The flower color of *N. repanda* is white and that of *N. tabacum* is pink. The flower color of the hybrids was light pink. The most variable hybrid plant was no. 7, whose chromosome number was the smallest. The plant height, leaf size and flower size in no. 7 were the smallest of the hybrids obtained. In addition, no. 7 did not show apical dominance (Fig. 2, Table 4).

The chromosome numbers of the flowering hybrid plants are presented in Table 4 and Fig. 4. Nos. 5, 6, 7 and 19 exhibited aneuploidy. As mentioned earlier, no. 7 was the hybrid with the smallest number of chromosomes. Nos. 8 and 18 had the same chromosome numbers ($2n=48$) as an intact hybrid.

Thus, it is shown that mature sexual hybrid plants between *N. repanda* and *N. tabacum* can be obtained, which has never been reported previously. None of the hybrids were able to produce fertile pollen and all were completely self-sterile. Research for the production of amphidiploids of the hybrid plants is currently under way.

Discussion

In this study, the production of aborted seeds from in situ hybridization of *N. repanda* × *N. tabacum* and hybrid inviability in plantlets obtained by the culture of fertilized ovules (Table 1) were found to be the same as in previous reports (Reed and Collins 1978; DeVerna et al. 1987). Thus, it is clear that there are two kinds of barriers to sexual reproduction in the hybridization of *N. repanda* × *N. tabacum*: seed abortion and hybrid inviability. The causes of seed abortion in crosses between *Nicotiana* species have previously been investigated and discussed (Cooper and Brink 1940; Brink and Cooper 1941; Shizukuda and Nakajima 1982). From these studies, it was apparent that the hybrid seeds aborted because of abnormal growth in nucellar tissue. Thus, it was suggested that the cause of seed abortion in the hybridization of *N. repanda* × *N. tabacum* was also abnormal growth in nucellar tissue. Moreover, the causes of hybrid inviability in the hybridization of *N. repanda* × *N. tabacum* have also been previously investigated (DeVerna et al. 1987). DeVerna et al. (1987) reported that the exhibition of hybrid inviability in reciprocal directions for *N. repanda* indicated the existence of strong nuclear or bilateral cytoplasmic-nuclear incongruity.

Seed abortion in other *Nicotiana* interspecific crosses has previously been overcome by pollinating with irradiated pollen and then developing viable hybrid seeds (Tanaka 1961; Pandey 1980, 1983). In the case of in situ hybridization of *N. repanda* with irradiated pollen of *N. tabacum,* no seeds germinated (Table 2). Thus, it is important to combine gamma-ray irradiation to pollen or to egg cells (in ovules) with in vitro culture of fertilized ovules in order to increase the germination rate of hybrid seeds.

The results obtained indicate that it is possible to overcome hybrid inviability to a certain extent by using the pollen irradiation technique or the egg cell irradiation technique. In spite of low doses and dose rate in the egg cell irradiation technique, compared with those in the pollen irradiation technique, it was possible to obtain mature hybrid plants (Table 3). This suggests that sensitivity to irradiation is different between pollen and egg cells. It has already been shown that pollen has a considerable degree of resistance to irradiation (Brewbaker and Emery 1962).

Numerous attempts to overcome hybrid inviability in the cross between *N. repanda* and *N. tabacum* by the culture of prenecrotic cotyledons onto a callus induction medium were ineffective in producing mature hybrid plants (Reed and Collins 1978; Iwai et al. 1985, DeVerna et al. 1987). Similar methods were successful in producing mature hybrid plants between *N. suaveolens* and *N. tabacum* that are normally subject to hybrid inviability (Lloyd 1975). The successful hybrid plants were not aneuploids because of the occurrence of point mutation. In this study, all flowering hybrid plants except nos. 8 and 18 exhibited aneuploidy. Nos. 8 and 18 had the same chromosome number as an intact hybrid (Table 4). It is not clear whether point mutation is likely to have occurred in these two hybrid plants or if a part of a chromosome might be lost. The chromosomal variation in the hybrids obtained will be discussed in detail elsewhere.

Nagao (1982) obtained somatic hybrids between *N. repanda* ad *N. tabacum* using protoplast fusion. Iwai et al. (1985) also obtained a regenerated hybrid between *N. repanda* and *N. tabacum* using callus culture. No mature hybrids have thus far been obtained through sexual reproduction. In this study, it is shown that mature hybrids may also be obtained through sexual reproduction using the pollen irradiation technique or the egg cell irradiation technique.

In conclusion, the results obtained indicate that it may be possible to overcome hybrid inviability to a certain extent by using both the pollen irradiation technique and the egg cell irradiation technique, i.e., pollination after the destruction of inviability causing chromosomes in pollen grains or egg cells (in ovules) with ionizing radiation and in vitro culture of fertilized ovules.

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