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Overcoming prefertilization barriers in the cross *Diplotaxis siettiana* × *Brassica juncea* using irradiated mentor pollen

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Abstract

Hybridization between the nearly extinct species *Diplotaxis siettiana* and *Brassica juncea* is prevented because of strong prefertilization barriers. Use of mentor pollen of *D. siettiana* irradiated with 1000 Gy gamma radiation before the incompatible pollination led to fertilization. 5 d after pollination 17 % ovules showed entry of pollen tubes, 10 d after pollination 27 % ovules showed small globular embryos which grew no further. No embryos were found in control pollinations. Thus, use of irradiated mentor pollen brings about fertilization in this difficult cross and hybrids can be obtained if embryos are rescued.

Key words: gamma radiation, incompatibility, pollen germination

Introduction

The genus *Diplotaxis* belongs to the tribe *Brassiceae* and has about 20 species. Of these *D. siettiana* is an important species because it became extinct in nature and was reintroduced from seed material stored in the IBPGR designated Crucifer Seed Bank at Institute Nacional de Investigaciones Agrarias in Madrid, Spain (Gomez-Campo 1990). Different species of *Diplotaxis* have been crossed with brassicas, an important oilseed crop for the development of alien cytoplasm lines (Ringdahl *et al.* 1987). Yet, very few intergeneric hybrids have been reported (Delourme *et al.* 1989, Hinata and Konno 1979, Batra *et al.* 1990). Of these only *D. siifolia* has been successfully crossed with *B. juncea.* Nandakumar and Shivanna (1993) failed to obtain hybrids between another species *D. siettiana* and *B. juncea.* Our studies on crossability of *D. siettiana* with six *Brassica* species showed that prefertilization barriers prevent hybridization in the cross *D. siettiana* × *B. juncea.* Use of irradiated mentor pollen is a relatively simple technique to overcome prefertilization barriers in self and interspecific crosses (Knox *et al.* 1987). However, its application in intergeneric

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Abbreviations: dap - days after pollination.

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crosses has not been demonstrated. This paper reports the use of mentor pollen to overcome prefertilization barriers in the cross *D. siettiana* × *B. juncea* and to obtain embryos.

Materials and methods

Forty seeds of *Diplotaxis siettiana* (obtained from Crucifer Seed Bank, Madrid, Spain) were sown in pots. The seeds are very small, light and very few germinated. Only six plants could be grown to maturity. *Brassica juncea* var. Pusa Bold was grown in beds.

Buds of *D. siettiana* were emasculated 1 d before anthesis in the evening, bagged and pollinated the next morning. Four sets of pistils were pollinated, three controls (C_1, C_2, C_3) and one experimental (E).

C_1 - pistils pollinated with normal *D. siettiana* pollen
C_2 - pistils pollinated with irradiated *D. siettiana* pollen (mentor pollen)
C_3 - pistils pollinated with normal *B. juncea* pollen
E - pistils pollinated with irradiated *D. siettiana* pollen followed by normal *B. juncea* pollen after 30 min.

In each, pollen from freshly dehisced anthers of 5 - 6 plants was used. For the experimental set E and the control C_2, *D. siettiana* pollen was irradiated with 1000 Gy γ-rays from a ^{60}Co source at Genetics Division, IARI. 50 pistils were pollinated in each of the four sets and the inflorescences bagged. Pollen germination and pollen tube growth were studied in 25 pistils each at 1 and 5 d after pollination (dap) in each set.

Pistils were fixed in 3:1 alcohol:acetic acid (v/v) for 24 h and transferred to 70 % alcohol and stored at 10 °C. They were hydrolysed in 8 M NaOH at 60 °C for 5 - 10 min, washed in water and mounted in 0.005 %. Aniline Blue (Merck) dissolved in 1/15 M K_3PO_4 (pH 8.0) and observed under a UV fluorescence microscope. In addition, 10 pistils in each set were fixed at 5 dap and 10 dap and turgid ovules squashed in 2 % acetocarmine to observe the presence of embryos, if any.

Results

Observation on *D. siettiana*: *D. siettiana* plants grow upto 30 cm tall. About 15 - 30 flowers are borne in a very compact inflorescence. The small flowers (petal limb 0.3 cm long, claw 0.5 cm long, 0.5 cm wide) produce few pollen. The siliqua has prominent seedless beak and the seed bearing region below the beak is about 1.2 cm long. On maturity about 60 % siliqua abscise. Thus seed set is very low.

Thirty flowers on three plants each of *D. siettiana* were selfed and crossed with each other to determine compatibility and percent ovules showing pollen tube entry. At 5 dap in the first plant 9 % ovules showed pollen tube entry when selfed and the second plant had 20 % ovules showing pollen tube entry. No pollen tubes entered the

ovules in case of selfing of the third plant. This plant was excluded from the study. On the other hand, when mixed (self + cross) pollen was used 12 - 14 % ovules showed pollen tube entry. In subsequent studies (C_1, C_2 and E) mixed *D. siettiana* pollen was used.

Unpollinated stigmas of *D. siettiana* did not show any fluorescence. The results of pollinated pistils (C_1, C_2, C_3 and E) observed at 1 and 5 dap are reported below. In addition, studies on embryo development observed at 5 and 10 dap are also presented.

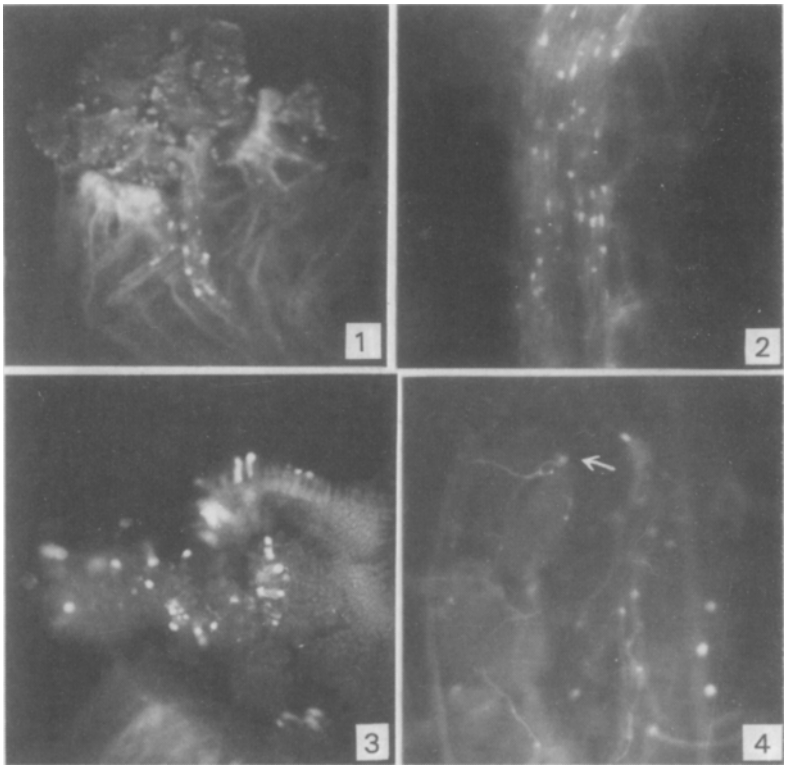

Fig. 1 - 4. Fluorescence micrographs of aniline blue stained pistills of *Diplotaxis siettiana* pollinated with *Brassica juncea* with (Fig. 1, 2, 4) and without (Fig. 3) the use of mentor pollen.
Fig. 1. Pollen germination and pollen tube growth in the stigmatic region 1 d after pollination (× 160).
Fig. 2. Pollen tubes showing callose plugs in the transmitting tissue of the lower stylar region 1 d after pollination (× 400).
Fig. 3. Stigmatic papillae showing fluorescence and few ungerminated pollen grains 1 d after pollination (× 400).
Fig. 4. Pollen tubes in the ovular region 5 d after pollination. Arrow indicates a pollen tube in the micropyle (× 400).

Pistils, 1 d after pollination showed pollen germination and pollen tube growth in E (Fig. 1), C_1 and C_2. Pollen tubes were observed in the style in E (Fig. 2), C_1 and C_2. In C_3, *B. juncea* pollen did not germinate in 14 out of the 15 pistils observed (Fig. 3). One exceptional pistil showed a few germinated pollen which did not enter the stigmatic papillae. The *B. juncea* pollen used was viable as it germinated on a separate *B. juncea* stigma. The *B. juncea* pollen germinated in pistils prepollinated with mentor pollen (E). The pollen of *B. juncea* is spherical (appears round) and that of *D. siettiana* slightly elongated (appears rectangular with rounded ends) and can be distinguished. Pollen tubes emerging from *B. juncea* pollen were traced at least upto the upper stylar region.

Table 1. Effect of irradiated (1 000 Gy) mentor pollen in the cross *Diplotaxis siettiana* × *Brassica juncea* (5 d after pollination).

Crosses	Pistils studied	Extent of pollen germination	Stigmatic fluorescence	Pollen tube growth in ovary	Total number of ovules	Number of ovules showing pollen tube entry	Ovules showing pollen tube entry [%]
C_1	15	+	-	+	224	27	12.05
C_2	15	+	-	+	203	9	4.43
C_3	15	-*	+	-	136	0	0.00
E	10	+	-	+	161	28	17.39

+ present, - absent, * only one pistil showed a few germinated pollen.

Table 2. Range and mean of ovules/pistil, ovules with tubes at 5 dap and embryos at 10 dap in C_1 and E.

Crosses	Days after pollination	Range of ovules/pistil	Range of fertilized ovules/pistil	Mean number of ovules/pistil	Mean number of ovules with tubes/pistil	Mean number of embryos/pistil
C_1	5	11 - 37	0 - 7	14.9	1.8	0.0
	10	0 - 18	0 - 7	7.2	-	0.9
E	5	7 - 36	0 - 7	16.1	2.8	0.0
	10	0 - 10	0 - 6	6.6	-	2.2

15 pistils were examined in C_1 at 5 dap and 10 pistils in all the other cases.

Stigmatic papillae showed high fluorescence only in C_3. In all, 384 ovules (from 21 pistils) were observed in C_1, 306 (from 21 pistils) in C_2. 404 (from 21 pistils) in C_3 and 295 (from 16 pistils) in E and none showed pollen tubes entering the micropyle (1 dap).

Pistils, 5 d after pollination showed more pollen tubes in the style compared to 1 dap and in the ovular region in E (Fig. 4) C_1 and C_2 and even entered the ovules.

The frequency of fertilization varied from 0 - 17 % (Table 1). In C_3, the incompatible cross, no pollen tubes were observed near the ovules even at 5 dap.

Embryos were not observed in ovules at 5 dap in all the four sets of pistils (40). However, at 10 dap, embryos were observed but at a very low frequency and only in C_1 and E. 9 embryos were observed in 72 ovules (12.5 %) of C_1 and 18 embryos in 66 ovules (27.3 %) of E. The embryos in E were at the globular stage (Fig. 5). The frequency of embryos at 10 dap in C_1 and E is compared with pollen tube entry at 5 dap (Table 2). The per cent embryos in C_1 appeared rather low. To check, if this

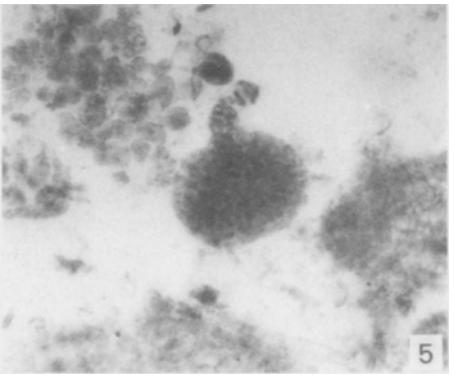

Fig. 5. Acetocarmine stained squashed ovules 10 d after pollination showing globular embryo (with a conspicuous suspensor) in the cross *D. siettiana* × *B. juncea* using mentor pollen (× 1200).

low frequency was due to the mixed pollen used in C_1, 10 flowers of plant 2 were pollinated with self pollen and another 10 flowers on the same plant were pollinated with mixed pollen (used in C_1). These pistils were fixed at 15 dap. The pistils pollinated with self pollen showed 60.7 % embryos (in 89 turgid ovules), while those pollinated with mixed pollen showed 38.5 % embryos (in 65 turgid ovules). The frequency of embryos was 38 % and 28.4 %, respectively, if both the turgid and degenerated ovules were counted as in other cases. In both the cases most of the embryos were heart shaped. No embryos were seen in the ovules of C_2 and C_3, *i.e.* from pistils pollinated with irradiated pollen and the incompatible *B. juncea* pollen, respectively. Embryos have thus been obtained from the cross *D. siettiana* × *B. juncea*. Since they could not be grown to maturity their hybrid nature remains unconfirmed. However, their slow growth and absence in control pollinations (C_2 and C_3) suggests their hybrid nature.

Discussion

It is difficult to obtain *Diplotaxis* × *Brassica* hybrids using conventional crossing techniques (Ringdahl *et al.* 1987). The reasons for the failure of these crosses have not been studied. Studies on pollen germination and pollen tube growth in such

crosses would help in using suitable techniques to obtain hybrids as has been done in the case of *D. siifolia* (Batra *et al.* 1990).

Among the *Diplotaxis species*, *D. siettiana* appears to be the most difficult to cross. Ringdahl *et al.* (1987) failed to obtain *D. siettiana* × *B. napus* hybrid from 276 pollinations. Takahata and Hinata (1983) obtained one hybrid *D. siettiana* × *B. nigra.* They suggested that *D. siettiana* should be placed in another genus because it is difficult to cross even with other *Diplotaxis* species. *D. siettiana* sets very few seeds on selfing flowers. When selfed two plants of *D. siettiana* showed 9 % and 20 % pollen tube entry into the ovules. On the other hand pollen tube entry was in 12 % of the ovules when mixed pollen was used for pollination. To determine the compatibility status of *D. siettiana* a detailed study needs to be done using a large population.

Our results indicate that it is possible to obtain hybrids in the cross *D. siettiana* × *B. juncea* using irradiated mentor pollen. Nandakumar and Shivanna (1993) failed to obtain hybrids from this cross and attributed it to a strong prefertilization barrier. They observed pollen germination but no pollen tube growth in this cross. We also observed that fertilization was prevented. Use of irradiated mentor pollen facilitates fertilization in this cross. At 10 dap 27.3 % ovules had embryos when pollination with irradiated mentor pollen was followed by incompatible pollen (E). However, at 5 dap in the same cross only 17.4 % ovules showed pollen tube entry. This increase in % ovules fertilized at 10 dap is possibly due to slow pollen tube growth and delayed fertilization. No embryos were found when only irradiated mentor pollen was used, thus ruling out occurrence of parthenogenesis. The hybrid nature of the embryos could be confirmed if they could be grown into plantlets using embryo rescue techniques.

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