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Production of wide hybrids and backcross progenies between *Diplotaxis erucoides* and crop brassicas

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Abstract Intergeneric hybrids were produced between *D. erucoides* (♀), a wild species, and four cultivated species of *Brassica*, *B. campestris*, *B. juncea*, *B. napus* and *B. oleracea*, through embryo rescue. The hybrid nature of these plants was confirmed through morphological and cytological studies. Backcross pollinations with the pollen of the respective cultivars yielded BC progenies in the hybrids *D. erucoides* × *B. juncea* and *D. erucoides* × *B. napus* but not in *D. erucoides* × *B. campestris* and *D. erucoides* × *B. oleracea*. The hybrid *D. erucoides* × *B. campestris* was also used as a bridge species and crossed with *B. juncea* to raise the hybrid and backcross progenies. F_2 progenies were more amenable than F_1 hybrids for raising backcross progenies. Although *D. erucoides* is considered to be a close relative of *B. campestris* and *B. oleracea*, incompatibility barriers of this species with different cultivars do not reflect this relationship.

Key words *Diplotaxis erucoides* · Brassica spp. Intergeneric hybrids

Introduction

Wide hybridization is considered to be an important approach for the improvement of many of the crop species (Kalloo and Chowdhury 1992). The tribe Brassiceae, which includes all of the crop species of *Brassica*, comprises many wild genera that form a good source of genes imparting resistance/tolerance to a gamut of biotic and abiotic stresses (Warwick 1993). Wild species are also a good source of cytoplasm for developing new cytoplasmic male sterile (CMS) systems through the production of alloplasmic lines (Banga 1992). However, strong crossability barriers between the cultivars and the wild species are the major constraints for effective utilization of nuclear and cytoplasmic genes from the wild species.

In recent years there have been many reports of intergeneric hybridization in crop *Brassica* (Warwick and Black 1993). Some of the *Diplotaxis* spp. have been used for intergeneric hybridization with different cultivars (Harberd and McArthur 1980; Ringdahl et al. 1987; Quiros et al. 1988; Delourme et al. 1989; Batra et al. 1990; Nandakumar and Shivanna 1993), and two CMS systems have been developed in the background of *Diplotaxis* cytoplasm (Hinata and Konno 1979; Rao et al. 1994).

On the basis of results obtained from molecular biology studies of nuclear and chioroplast DNA, it has been suggested that crop brassicas have evolved from the *Diplotaxis-Erucastrum* complex (Gomez-Campo and Tortosa 1974) and that *D. erucoides* appears to be the closest ancestor involved in the origin of *B. campestris* and *B. oleracea* (Warwick and Black 1991; Pradhan et al. 1993).

In this paper we report the production of wide hybrids using *D. erucoides* as the female parent and cultivated species of *Brassica* as male parents through embryo rescue, the morphological and cytological details of the F_1 hybrids, and the production of F_2 and backcross progenies.

Materials and methods

Brassica campestris L. ssp. *oleifera* var 'brown sarson' (2n = 20, AA), *B. oleracea* var 'alboglabra' (2n = 18, CC), *B. juncea* (L.) Czern. cv 'Pusa Bold' (2n = 36, AABB), *B. napus* L. strain 706 (2n = 38, AACC) and *Diplotaxis erucoides* (L.) DC (2n = 14, D^e D^e) grown under field conditions were used in the present investigation. Flowers of the wild species which were emasculated and bagged 1 day prior to anthesis were pollinated with freshly collected pollen from cultivated species

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and rebagged. Pollen germination and pollen tube growth were studied using aniline blue fluorescence (Linskens and Esser 1957).

For ovary culture, pistils were excised 4–6 days after pollination (DAP), surface-sterilized with 0.01% mercuric chloride and cultured on White's basal medium supplemented with 500 mg/l casein hydrolysate. The fruits were harvested 40 days after culture, and the seed set was recorded. In another set (sequential culture), cultured ovaries were dissected 5–7 days after culture and ovules, if any, were excised and cultured on Murashige and Skoog's (MS) medium. The cultures were maintained at 25° ± 2 °C.

The seedlings obtained from cultured ovaries/ovules were multiplied in vitro through the culture of shoot tips and nodal segments on MS + 0.2 mg/l BAP medium. The shoots were rooted on MS + 0.1 mg/l NAA medium. The seedlings were hardened and transferred to pots containing garden soil and grown under field conditions. Colchicine (0.1%) was applied in vitro to axillary buds of single node explants for 48 h to induce amphidiploidy. The plants thus developed were subsequently transferred to the field. For cytological studies, buds were fixed in Carnoy's solution, and anthers were squashed in 2% acetocarmine. Pollen fertility was studied using acetocarmine staining.

F_1 hybrids and amphidiploids were allowed to open-pollinate to produce F_2 progenies or backcrossed with their respective male parents to raise backcross (BC) progenies; they were also crossed with other crop species to produce bridge-cross hybrids. As very few of these pollinations resulted in seed set, embryo rescue techniques were also applied to raise the BC progeny and bridge-cross hybrids.

Results

Production of hybrids

Aniline blue fluorescence studies revealed that *D. erucoides* is self-incompatible. Pollen grains of all of the cultivars (*B. campestris*, *B. juncea*, *B. napus* and *B. oleracea*) showed poor to moderate germination on the stigma of *D. erucoides*, but pollen tube entry into the stigmatic papillae was not observed. Field pollinations did not yield seeds in any of the crosses attempted despite initial enlargement of the ovaries.

Ovary culture was successful in only one combination, *D. erucoides* × *B. campestris* (Table 1) in which two seeds germinated within the fruit; these were cultured on a fresh medium, and hybrid seedlings were raised. However, sequential culture was effective in producing hybrids in all four crosses attempted (Table 1).

The seedlings of all the crosses were multiplied through the culture of shoot tips and nodal segments, hardened and transferred to the soil. In vitro colchicine treatment of single node explants was effective in inducing amphidiploidy only in the hybrid *D. erucoides* × *B. campestris*. The seedlings of amphidiploids were also multiplied and transferred to the field.

Morphology and cytology of hybrids

Morphologically F_1 hybrids exhibited many characters intermediate between the two parents (Table 2). However, a few characters were distinctly characteristic of either the male or the female parent. The amphidiploids of *D. erucoides* × *B. campestris* were similar to the F_1 but were more robust. Flowering in amphidiploid was delayed by about 10 days.

Meiotic studies showed the expected chromosome number in two of the hybrids *D. erucoides* × *B. campestris* and *D. erucoides* × *B. juncea* ($2n = 17$ and $2n = 25$, respectively). However, in the other two hybrids, *D. erucoides* × *B. oleracea* and *D. erucoides* × *B. napus*, an additional 9 ($2n = 25$) and 19 ($2n = 45$) chromosomes were observed, respectively. Univalents were more prevalent in all of the hybrids, although a few bivalents and higher associations were also recorded (Table 3). The amphidiploid of *D. erucoides* × *B. campestris* showed, as expected, 17 regular bivalents.

Production of backcross progeny

Backcross pollinations were carried out on F_1 and A_1 plants with their respective male parents (cultivars). The hybrids *D. erucoides* × *B. juncea* and *D. erucoides* × *B. napus* yielded BC_1 progenies (Table 4). In the former, embryo rescue was essential, while in the latter field pollinations also resulted in seed set. The other two hybrids, *D. erucoides* × *B. campestris* (F_1 as well as A_1) and *D. erucoides* × *B. oleracea* failed to produce BC_1 seeds despite embryo rescue.

The BC_1 plants of *D. erucoides* × *B. juncea* and *D. erucoides* × *B. napus* were further backcrossed with their respective male parents to produce BC_2 progeny. The former yielded a few BC_2 seeds through field pollinations, while backcrossing on the latter did not yield any seeds (Table 4).

Use of bridge-cross and F_2 generations

The successful hybrids either failed to produce BC seeds or only produced them at low levels despite the applica-

Table 1 Results of ovary and sequential cultures

Cross	Ovary culture		Sequential culture			
	Number of pistils cultured	Number of hybrids established	Number of pistils cultured	Number of ovules recultured[a]	Number of ovules germinated	Number of hybrids established
D. erucoides						
× *B. campestris*	78	2	405	172 + 31	10	5
× *B. juncea*	40	0	500	201 + 57	69	31
× *B. oleracea*	10	0	95	17 + 18	9	9
× *B. napus*	53	0	225	82 + 5	40	33

[a] Number of shrivelled + healthy ovules

Table 2 Comparison of morphological characters of F_1 hybrids and their parents

Feature	♀	F_1	♂
D. erucoides × *B. campestris*			
Plant height	55.0 ± 2.4	72.8 ± 12.4	125.1 ± 3.1
Leaf	Dark green, hairy, pinnatisect, amplexicaul	Green, glaucescent, pinnatisect, sessile but not amplexicaul	Green, glaucescent, pinnatifid, amplexicaul
Petal colour	White	Pale yellow	Intense yellow
Anther	Introrse	Rudimentary	Extrorse
Pollen stainability (%)	94.0	0	93.2
D. erucoides × *B. juncea*			
Plant height	55.0 ± 2.4	80.0 ± 8.3	158.2 ± 7.4
Leaf	Dark green, hairy, pinnatisect, amplexicaul	Dark green, glabrous, pinnatifid, petiolate	Green, glaucescent, pinnatisect, petiolate
Petal colour	White	White	Intense yellow
Anther	Introrse	Rudimentary	Introrse
Pollen stainability (%)	94.0	1.86	95.2
D. erucoides × *B. oleracea*			
Plant height	55.0 ± 2.4	90.47 ± 24.0	105.2 ± 3.2
Leaf	Dark green, hairy, pinnatisect, amplexicaul	Green with sometimes purple colouration, pinnatisect petiolate	Bluish green, pinnatifid, petiolate
Petal colour	White	Yellow	White
Pollen stainability (%)	94.0	0–5.0	92.0
D. erucoides × *B. napus*			
Plant height	55.0 ± 2.4	100.1 ± 25.8	149.1 ± 8.9
Leaf	Dark green, hairy, pinnatisect, arranged in lax basal rosette	Green, glaucescent, pinnatifed, arranged in lax basal rosette	Green, glaucescent, pinnatifed, alternate
Petal colour	White	Pale yellow	Intense yellow
Anther	Yellow	Yellow with mauve tip	Yellow with mauve tip
Pollen stainability (%)	94.0	0–15.2	92.0

Table 3 Details of meiotic studies of F_1 hybrids

Hybrids	Number of meiocytes studied	Genome	2n	Mean chromosome associations per cell at diakinesis and metaphase I (range in parenthesis)			
				IV	III	II	I
D. erucoides × *B. campestris*	52	D^eA	17	–	0.15	1.37	13.76
				–	(0–1)	(0–3)	(11–17)
D. erucoides × *B. juncea*	35	D^eAB	25	0.14	–	3.57	17.6
				(0–1)	–	(1–6)	(13–23)
D. erucoides × *B. oleracea*	36	D^eCC	25	–	–	2.77	19.44
				–	–	(0–7)	(11–25)
D. erucoides × *B. napus*	38	D^eAACC	45	–	0.10	17.39	9.89
				–	(0–1)	(14–19)	(7–14)

tion of embryo rescue. To improve the production of BC progeny, attempts were made to produce bridge-cross and F_2 generations and use them for backcross pollinations. The amphidiploid of the hybrid *D. erucoides* × *B. campestris* was crossed with *B. juncea*, and two F_1 bridge-cross hybrids were raised through sequential culture. This bridge-cross hybrid (*D. erucoides* × *B. campestris*) × *B. juncea* was pollen sterile and had leaves similar to those of *B. juncea*. These hybrids were further backcrossed with *B. juncea*, and 10 BC_1 seeds were obtained through field pollinations (Table 4).

F_2 progenies of this bridge-cross hybrid (*D. erucoides* × *B. campestris*) × *B. juncea* were also raised. F_2 plants were similar to their F_1 plants in general mor-

Table 4 Details of backcross pollinations on F_1, A_1 and bridge-cross hybrid

Pistillate parent		Pollen parent	Number of seeds[a]/ no. of pollinations through field pollinations	Number of seeds/ no. of pollinations through sequential culture
D. erucoides × *B. campestris*	F_1	*B. campestris*	0/50	0/387
	A_1	*B. campestris*	0/107	0/271
D. erucoides × *B. juncea*	F_1	*B. juncea*	–	2/43
	BC_1	*B. juncea*	4 + 41/150	–
D. erucoides × *B. oleracea*	F_1	*B. oleracea*	–	0/501
D. erucoides × *B. napus*	F_1	*B. napus*	11/48	4/223
	BC_1	*B. napus*	0/192	0/56
	F_2	*B. napus*	1 + 23/192	–
(*D. erucoides* × *B. campestris*) × *B. juncea*	F_1	*B. juncea*	10/410	0/668
	F_2	*B. juncea*	186 + 249/253	–

[a] Number of healthy + shrivelled seed,-not done

phology. Most of these were pollen sterile, but 2 of them showed up to 4% fertility. Backcrossing on F_2 plants yielded a larger number of healthy seeds through field pollinations.

F_2 plants of the hybrid *D. erucoides* × *B. napus* were also produced. These were also similar to their F_1 progeny, but pollen fertility ranged from 0–50% in different plants. Backcrossing on these F_2 plants yielded some seeds through field pollinations. F_2 plants could not be produced in crosses *D. erucoides* × *B. campestris* and *D. erucoides* × *B. oleracea.*

Discussion

The present investigation clearly demonstrates the operation of strong pre- and post-fertilization barriers between *D. erucoides* and the four species of cultivated brassicas. Although some pollen grains did germinate in all of the crosses attempted, pollen tube entry into the stigmatic papillae was inhibited; fluorescent microscopic studies did not reveal pollen tubes in the ovary in any of the crosses. The fact that some hybrids were produced in all of the crosses through embryo rescue indicates that a few pollen tubes do enter the papillae, grow through the style and effect fertilization. This may be in response to variations in the environmental conditions, the physiological status and the genotype of the plants. Studies on pollen germination and pollen tube growth in a limited number of pistils are likely to miss such pistils in which a few pollen tubes enter the ovary. Earlier studies on wide hybridization in *Brassica* have also reported the production of a few hybrids through embryo rescue even in crosses in which pollen tube entry was not observed (Gundimeda et al. 1992).

Ovary culture was successful only in the cross *D. erucoides* × *B. campestris* in which 2 hybrids were obtained. In other crosses, hybrids were raised only through sequential culture. This is in agreement with the earlier reports on the superiority of sequential culture over ovary culture (Nandakumar et al. 1988; Agnihotri et al. 1990; Gundimeda et al. 1992).

Morphological and cytological studies confirmed the hybrid nature of the F_1 plants in all of the crosses. Chromosome numbers in 2 of the hybrids, *D. erucoides* × *B. campestris* and *D. erucoides* × *B. juncea*, showed the full chromosome complements of both parents. However, the other 2 hybrids, *D. erucoides* × *B. napus* and *D. erucoides* × *B. oleracea*, exhibited 2n chromosome numbers of 25 and 45, respectively, while the expected numbers were 16 and 26, respectively. This appears to be due to the fusion of a normal female gamete ($n = 7$) with unreduced male gametes ($n = 2x = 18$ and $n = 2x = 38$). The occurrence of unreduced male as well as female gametes is quite common in Brassiceae (Heyn 1977; Delourme and Renard 1987; Ripley and Arnison 1990).

Chromosome behaviour in the hybrids was characteristic of wide hybrids. Meiosis was irregular and univalents were more prevalent. However, a few higher associations were recorded in all of the hybrids. Haploids of *D. erucoides* have not been studied. However, since the archetype of subtribe Brassiceae is believed to have $x = 6$ (Prakash and Hinata 1980), *D. erucoides* is likely to form at least one autosyndetic bivalent. The hybrids *D. erucoides* × *B. juncea* and *D. erucoides* × *B. campestris* exhibited one quadrivalent and one trivalent, respectively. The occurrence of a quadrivalent in the former hybrid can be ascribed to allosyndesis between the *D. erucoides* and *Brassica* genomes. Although *B. campestris* is theoretically expected to form one trivalent and two bivalents through autosyndetic pairing (Armstrong and Keller 1981), Mizushima (1980) is of the opinion that *B. campestris* forms only one autopair. In that case the trivalent observed in the hybrid *D. erucoides* × *B. campestris* can also be of allosyndetic origin. The other two hybrids, *D. erucoides* × *B. oleracea* and *D. erucoides* × *B. napus*, exhibited less pairing than expected. This may be due to the suppressing effect of the *D. erucoides* genome on both homologous and homoeologous pairing.

In spite of a large number of pollinations carried out and the application of embryo rescue technique, the production of backcross progenies was difficult in most

of the hybrids (see Table 4). There are may reports of the effective use of bridge-crosses to produce incompatibile hybrids and their backcross progenies (Khush and Brar 1992). For example, the hybrid *D. siettiana* × *B. campestris* has been used as a bridge-cross to raise hybrids between *D. siettiana* × *B. juncea* and *D. siettiana* × *B. napus* and their respective backcross progenies without the use of embryo rescue (Nandakumar and Shivanna 1993). In the present investigation also, attempts were made to use the hybrid *D. erucoides* × *B. campestris* as a bridge species to produce (*D. erucoides* × *B. campestris*) × *B. juncea*. However, embryo rescue was necessary to raise the hybrid. Thus, the bridge-cross was not superior to the direct cross *D. erucoides* × *B. juncea*, which also yielded hybrids through embryo rescue. However, bridge-cross hybrid as well as F_2 were more responsive for producing backcross progeny. F_2 progeny (which could be raised without embryo rescue) also showed pollen fertility. As F_1 hybrids are generally pollen sterile, F_2 plants can also be used for the effective application of anther culture technique (for producing haploids) in the breeding programme.

As pointed out earlier, many recent studies have shown that *D. erucoides* is a close ancestor of *B. campestris* and *B. oleracea*. However, present investigations clearly show that both pre- and post-fertilization barriers are equally strong with all the four species of *Brassica*. On the basis of the production of BC progeny, the barriers were stronger with *B. campestris* and *B. oleracea* in which no F_2 or BC progeny were produced in spite of a large number of pollinations (see Table 4). Even the amphidiploid of *D. erucoides* × *B. campestris* failed to produce BC seeds. Thus, incompatibility barriers between *D. erucoides* and different cultivars do not reflect the closer relationship of *D. erucoides* with *B. campestris* or *B. oleracea*.

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