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Intergeneric hybridization between *Diplotaxis siettiana* and crop brassicas for the production of alloplasmic lines

P. B. A. Nanda Kumar*, and K. R. Shivanna**

Department of Botany, University of Delhi, Delhi-110 007, India

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Summary. Intergeneric hybrids were produced between *Diplotaxis siettiana* and *Brassica campestris* through embryo rescue. The hybrids were completely pollen sterile and backcrosses with pollen of *B. campestris* did not yield any seeds. Induction of colchiploidy restored pollen fertility and backcross pollinations yielded viable seeds. Cytological details of the hybrid, amphidiploid and backcross progenies were studied. Both pollen-sterile and pollen-fertile plants have been obtained in backcross 2 progeny. This hybrid (*D. siettiana* × *B. campestris*) was used as a bridge cross to transfer the cytoplasm of *D. siettiana* to two other incompatible cultivars of *Brassica* – *B. juncea* and *B. napus*. Pollinations of the amphidiploid (*D. siettiana* × *B. campestris*, 2n = 36) with pollen of *B. juncea*/*B. napus* readily produced seeds without embryo rescue. These hybrids were grown to flowering and their cytological details were studied. Seeds have been produced from backcross pollinations of both these hybrids with the pollen of the respective cultivars. The results clearly show the feasibility of producing alloplasmic lines in all the three oilseed brassicas.

Key words: Intergeneric hybrids – Bridge cross – Alloplasmics – *Diplotaxis* – *Brassica* cultivars

Introduction

In recent years, there has been an increasing interest in the hybridization of cultivated brassicas with their wild relatives (Nanda Kumar et al. 1988, 1989; Delourme et al. 1989; Agnihotri et al. 1980; Batra et al. 1990; Takahata 1990; Takahata and Takeda 1990). Wide hybrids are being used to introgress desirable genes imparting resistance to biotic and abiotic stresses, and to produce alloplasmic lines (combining the cytoplasm of wild species with the nuclear genome of the cultivars), many of which may exhibit cytoplasmic male sterility (CMS). In Brassicas a few CMS lines have been developed through wide hybridization (Rousselle and Renard 1978; Hinata and Konno 1979; Williams and Heyn 1981; Prakash and Chopra 1988, 1990). However, in oilseed brassicas, commercial exploitation of hybrid vigour through utilization of CMS has not yet been possible because of the unsuitability of existing CMS lines or lack of restorer lines. There is a need for broadening the CMS sources particularly to safeguard against diseases associated with some cytoplasms.

Hinata and Konno (1979) induced CMS by combining the cytoplasm of *Diplotaxis muralis* in the nuclear background of *Brassica campestris*. Ringdahl et al. (1987) attempted to hybridize many *Diplotaxis* spp to *B. napus* through conventional methods with an aim to identify *Diplotaxis* cytoplasms which induce male sterility. Most of these crosses were unsuccessful due to strong crossability barriers. In recent years, however, intergeneric hybrids have been reported between *D. erucoides* and *B. napus* (Delourme et al. 1989) and *D. siifolia* and crop brassicas (Batra et al. 1990) through embryo rescue.

This paper reports: (1) the production of intergeneric hybrids between *Diplotaxis siettiana* and *B. campestris* through embryo rescue, (2) details of the F_1 hybrid, amphidiploid and the backcross progeny and (3) the use of this hybrid as a bridge cross to transfer the cytoplasm of *D. siettiana* to two other cultivars of crop brassicas, *B. juncea* and *B. napus*.

Materials and methods

Plants of *D. siettiana* Maire (2n = 16, DiDi), *B. campestris* L. ssp. *oleifera* var. yellow sarson (2n = 20, AA), *B. juncea* (L.) Czern. cv 'Pusa Bold' (2n = 36, AABB), and *B. napus* L. ssp. *oleifera* strain 706 (2n = 38, AACC) were grown under field conditions. Flower buds were emasculated and bagged one day before anthesis, and were pollinated on the day of anthesis with

* *Present address:* AgBiotech Center, Environmental and Natural Sciences Building, Rutgers University, Cook College, PO Box 231, New Brunswick, NJ 08903-0231, USA
Correspondence to: K. R. Shivanna

fresh pollen of the male parent and rebagged. Some of the pollinated pistils were left on the plant until maturity or senescence and some were used for ovary culture. Pollen germination and pollen tube growth in the pistils were studied using the aniline-blue fluorescence method (Linskens and Esser 1957). In-vitro methodologies used for ovary culture, the induction of amphidiploidy, and multiplication of hybrids are described in our earlier papers (Nanda Kumar et al. 1988; Nanda Kumar and Shivanna 1991). For in-vitro culture studies MS basal medium was used.

For DNA analysis, total DNA of the leaf material was extracted following Dellaporta et al. (1984), purified on a $CsCl_2$ density gradient and digested with the restriction endonuclease *Cla*I according to the manufacturer's instructions and those of Maniatis et al. (1982). The digested DNAs were electrophoresed on 0.8% agarose gels and blotted onto a nitrocellulose membrane (Southern 1975). The membrane was hybridized to *Raphanus* rDNA, pRE 12 (Delseny et al. 1984) and labelled with [^{32}P-αdCTP] using a multiprime labelling kit (Amersham). Hybridization and autoradiography were done following Mukhopadhyay et al. (1991).

For cytological studies young anthers were fixed in Carnoy's solution and squashed in 1% acetocarmine.

Results

D. siettiana × *B. campestris*

Over 200 pollinations were carried out in each of the reciprocal combinations between *D. siettiana* and *B. campestris*. None of the pollinated pistils yielded hybrid seeds when they were maintained on the plant. Fluorescence microscopic studies showed that the pistils of *D. siettiana* supported germination and tube growth of *B. campestris* pollen; many pollen tubes were observed in the ovary. In the reciprocal cross, a few pollen grains of *D. siettiana* did germinate on *B. campestris* stigma; however, pollen tubes failed to enter the papillae.

Ovary culture was attempted to realize the hybrids. In the cross *D. siettiana* × *B. campestris*, out of a total of 136 cultured ovaries about 20% developed into fruits and yielded 28 seeds. Most of these seeds germinated when cultured on a nutrient medium and

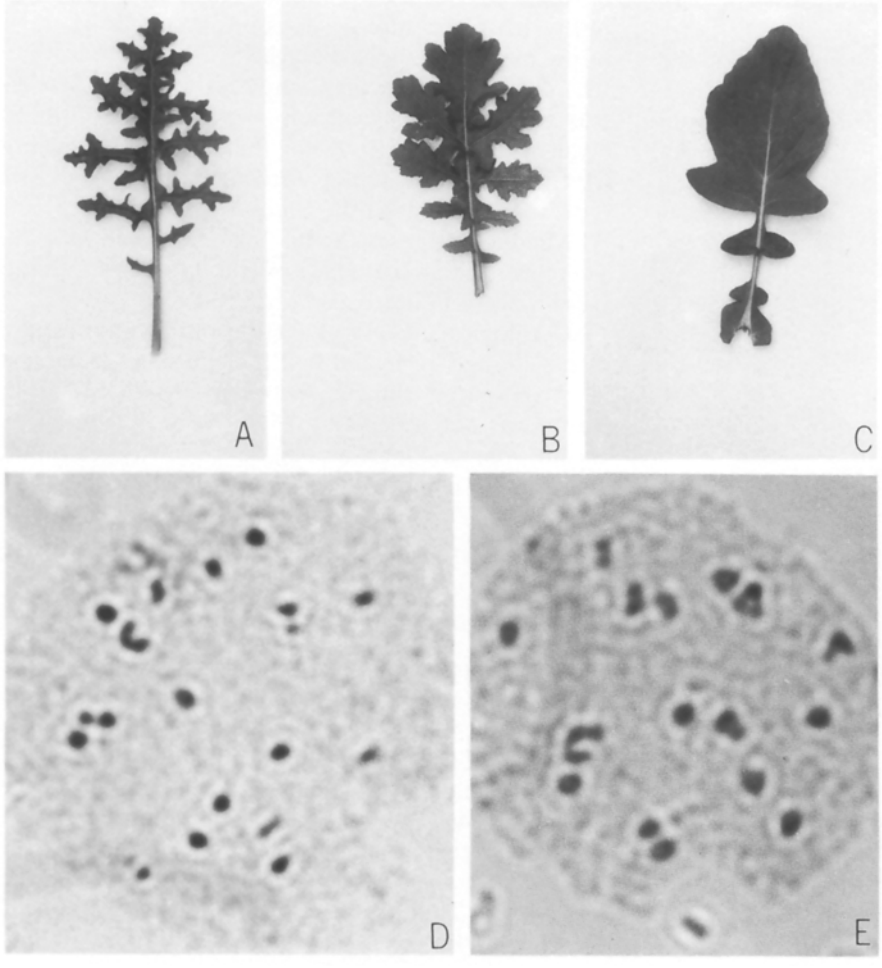

Fig. 1A–C. Leaves of *D. siettiana* (**A**) *D. siettiana* × *B. campestris* F_1 hybrid (**B**) and *B. campestris* (**C**). **D**, **E** Metaphase-I of meiosis showing 14 I + 2 II in F_1 hybrid (**D**) and 15 II + 2 III in amphidiploid (**E**) of *D. siettiana* × *B. campestris*

gave rise to hybrid seedlings. In the reciprocal cross, however, none of the 228 cultured ovaries developed into fruits.

Amphidiploids were obtained through in-vitro colchicine treatment of in-vitro cultured single node segments of the hybrid. The F_1 hybrids and amphidiploids were further multiplied through in-vitro colchicine treatment of in-vitro cultured single node ferred to soil and, in all, 40 F_1 hybrids and 18 amphidiploids were grown to flowering.

F_1 hybrids were intermediate between the parents in general morphological characters such as plant height, lamina shape and dissection (Fig. 1A–C), and flower colour. The anthers were rudimentary in the hybrids and were completely pollen-sterile.

In synthetic amphidiploids, the leaves were more robust with larger stomata (47.7 μm^2 in amphidiploids in contrast to 23.9 μm^2 in F_1 hybrids). The anthers were turgid and contained about 50% fertile pollen grains.

Total DNA from *D. siettiana*, *B. campestris*, the F_1 hybrid and amphidiploid was restricted with *Cla*I and hybridized with the probe pRE12. Hybrids and amphidiploids had two bands (3.5 kb, 4.3 kb) specific to *D. siettiana* and two (6.2 kb, 9.2 kb) for *B. campestris*, indicating their hybrid nature (Fig. 2).

Over 400 backcrosses of the F_1 hybrids using the pollen of *B. campestris* did not result in any seed set. Backcrosses of amphidiploids yielded 138 seeds from 645 pollinations. Most of these seeds germinated when sown in soil and gave rise to backcross-1 (BC_1) progeny. About 25 BC_1 plants were grown to flowering. The BC_1 plants were morphologically uniform and resembled *B. campestris* more in general appearance. Anthers of BC_1 plants, though not as turgid as those in amphidiploid plants, did contain about 3% fertile pollen grains. Further backcrosses of the BC_1 plants with pollen of the cultivar gave rise to BC_2 seeds. Twenty-one BC_2 plants were grown to flowering. They showed morphological variability (Fig. 3A). In nine plants flower buds failed to open and the stigmas protruded. The stamens in these plants were rudimentary and did not contain any pollen. In 11 plants, the flower buds opened normally but were completely pollen-sterile. In one plant flowers opened normally and showed about 26% pollen fertility.

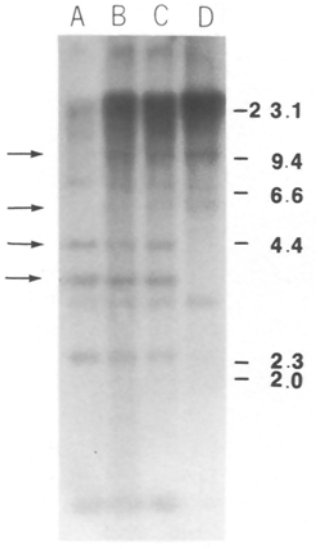

Fig. 2. Five micrograms of total genomic DNA restricted with *Cla*I and probed with pRE12. *D. siettiana* (*lane A*), F_1 hybrid (*lane B*), amphidiploid (*lane C*), *B. campestris* (*lane D*). *Arrows* indicate the parent specific bands in the DNA of F_1 hybrid and amphidiploid. Numbers refer to molecular weight (in kb) of *Eco*RI-*Hin*dIII-digested lambda DNA

Details of meiotic studies on the F_1 hybrid, the amphidiploid and on their progeny are summarized in Table 1. Analysis of F_1 hybrids at diakinesis and metaphase-I (M-I) of meiosis showed the expected chromosome number (2n = 18) with a preponderance of univalents (Fig. 1D) in most of the PMCs examined. However, in four of the 42 PMCs examined, only 4/5 univalents were observed. A low frequency of trivalents were also observed. At anaphase-II, most of the meiocytes showed laggards.

Fertile synthetic amphidiploids showed 2n = 36 and most of the chromosomes were in the form of bivalents (II) (Fig. 1E). A low frequency of univalents (I), trivalents (III) and quadrivalents (IV) were also observed. Anaphase-I disjunction was normal.

Meiotic analysis of the BC_1 plants showed 2n = 28. There was a high frequency of II followed by I. Some III as well as IV were also observed.

Cytological analysis of a fertile and two sterile BC_2 plants showed 10 II and 0–2 I. A clear cut difference in chromosome numbers between fertile and sterile plants was not observed as there were differences in chromosome numbers among the cells within a plant. Surprisingly, eight of 46 PMCs observed each showed only 10 II.

(D. siettiana × B. campestris) × B. juncea and (D. siettiana × B. campestris) × B. napus

Our attempts to raise hybrids of *D. siettiana* with another cultivar, *B. juncea*, through field pollinations were not successful. With the aim of transferring the cytoplasm of *D. siettiana* into the nuclear backgrounds of *B. juncea* and *B. napus*, attempts were made to use the *D. siettiana* × *B. campestris* hybrid as a bridge cross and cross pollinations were carried out with pollen of *B. juncea* and *B. napus*. The results are presented in Table 2. The crosses with F_1 hybrids did not yield any seeds. The crosses with amphidiploids

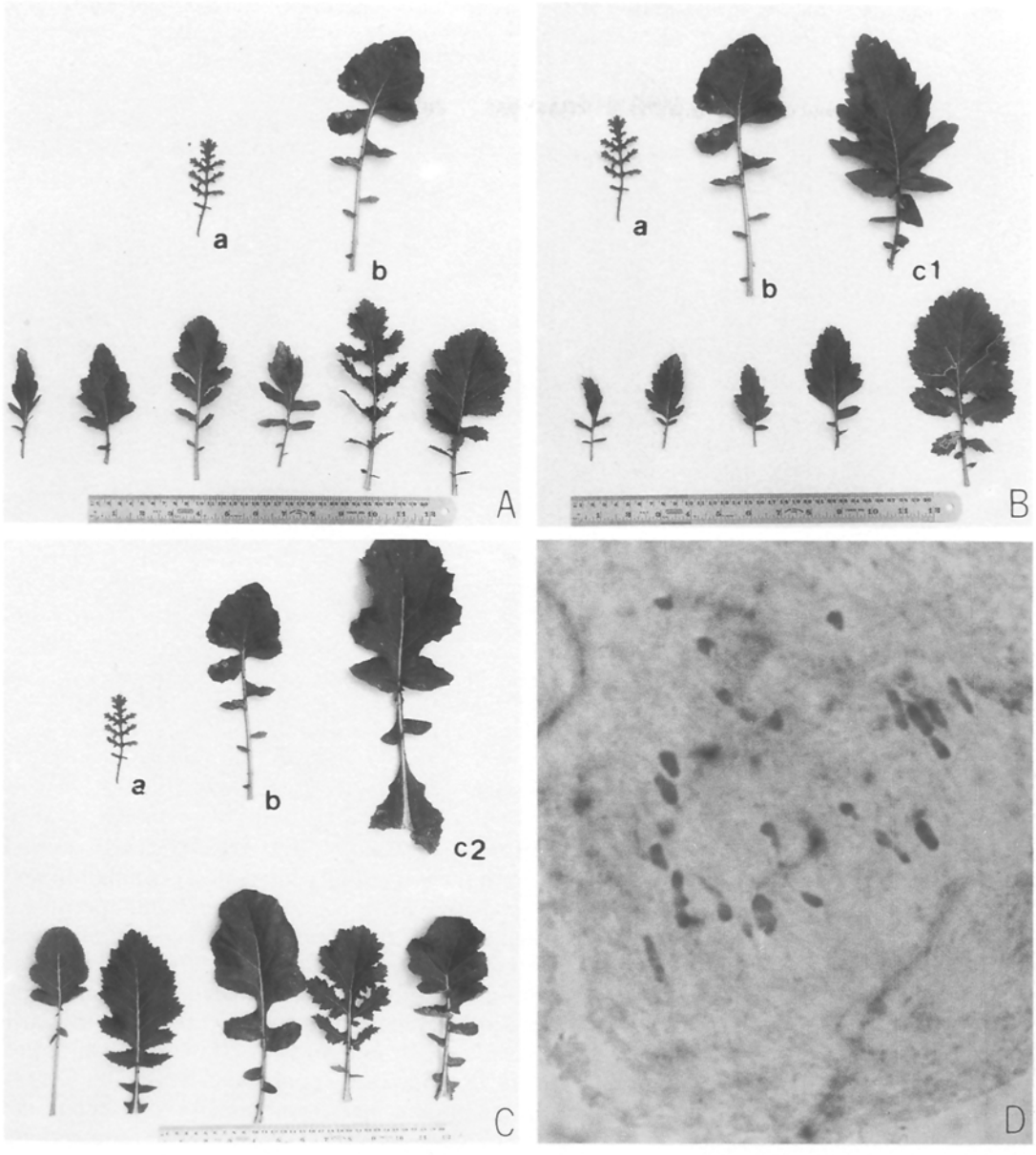

Fig. 3A–C. Morphological variability in leaves of the progeny derived from BC_1 (*D. siettiana* × *B. campestris*) × crop species. Upper rows in each are the leaves of the parents *D. siettiana* (*a*) *B. campestris* (*b*), *B. juncea* (*c1*), *B. napus* (*c2*) and bottom rows of the BC_1-derived progeny. **A** BC_1 × *B. campestris*. **B** BC_1 × *B. juncea*. **C** BC_1 × *B. napus*. **D** Meiotic chromosomes in the BC_1 × *B. juncea* showing 2n = 32 with 14II + 4I

and BC_1 plants gave rise to a good number of seeds particularly with *B. juncea*. Hybrids between amphidiploids (*D. siettiana* × *B. campestris*) (2n = 36) and *B. juncea* (2n = 36)/*B. napus* (2n = 38) were morphologically uniform and showed a low degree of pollen fertility. However, hybrids between BC_1 (*D. siettiana* × *B. campestris*) (2n = 28) and *B. juncea* (2n = 36)/*B. napus* (2n = 38) showed considerable morphological variability (Fig. 3B, C; Table 3). From the backcross populations (BC_1 × *B. juncea* and BC_1 × *B. napus*), plants resembling their respective pollen parents were selected for further backcrossing. Considerable number of seeds were harvested from the backcross pollinations.

The data on the cytology of these hybrids are included in Table 1. Amphidiploid × *B. juncea* and amphidiploid × *B. napus* hybrids, as expected, showed 2n = 36 and 2n = 37 respectively. Analysis at diakinesis and M-I of meiosis revealed that II occurred at a higher frequency followed by I. About 30–60%

Table 1. Details of meiotic studies on F_1 hybrid, of *D. siettiana* and *B. campestris*, their amphidiploid and their progeny[a]

Hybrid	No of meiocytes studied	2n chromosome number	Mean chromosome associations (range) per PMC at diakinesis/M-I			
			I	II	III	IV
F_1 (*D. siettiana* × *B. campestris*)	42	18	9.7 (4–15)	3.5 (0–5)	0.4 (0–2)	0.0 –
Amphidiploid (*D. siettiana* × *B. campestris*)	49	36	1.1 (0–8)	14.1 (8–17)	0.8 (0–3)	1.1 (0–3)
BC_1 (Amphidiploid × *B. campestris*)	52	28	4.9 (1–8)	10.1 (5–13)	0.8 (0–5)	0.1 (0–2)
BC_1 (BC_1 × *B. campestris*)	46	20–22	1.1 (0–2)	10 (10–10)	0.0 –	0.0 –
Amphidiploid (*D. siettiana* × *B. campestris*) × *B. juncea*	51	36	3.9 (1–8)	12.8 (7–17)	0.4 (0–2)	1.4 (0–3)
Amphidiploid (*D. siettiana* × *B. campestris*) × *B. napus*	56	37	4.6 (2–8)	11.9 (9–15)	1.3 (0–4)	1.2 (1–3)
BC_1 (Amphidiploid × *B. campestris*) × *B. juncea*	57	29–32	3.4 (0–7)	11.5 (7–15)	0.4 (0–2)	0.8 (0–2)

[a] BC_1 (Amphidiploid × *B. campestris*) × *B. napus* – microspore mother cells in these plants degenerated at an early prophase stage and details of chromosomes could not be studied

Table 2. Responses of the crosses on F_1 hybrid, amphidiploid and BC_1 plants of *D. siettiana* × *B. campestris* with the pollen of the cultivars

Pistillate parent	Pollen parent	
	B. juncea	*B. napus*
	No. of seeds/no. of pollinations	
F_1 (*D. siettiana* × *B. campestris*)	0/113	0/158
Amphidiploid (*D. siettiana* × *B. campestris*)	232/343	60/270
BC_1 (Amphidiploid × *B. campestris*)	144/242	62/262

of the PMCs showed a low frequency of III and IV in amphidiploid × *B. juncea* hybrids.

Two fertile and two sterile plants of BC_1 × *B. juncea* were examined for meiotic analysis. One of the fertile plants had 2n = 32 chromosomes (Fig. 3D) and another had 2n = 31 chromosomes. One of the sterile plants showed 2n = 29 and the other 2n = 30. These plants also showed a high frequency of II followed by I. Five sterile plants of BC_1 × *B. napus* were analysed to determine chromosome numbers and associations. As these plants showed degeneration of microspore mother cells at an early stage, meiotic chromosomes could not be studied.

Discussion

The cross *D. siettiana* × *B. campestris* clearly showed unilateral incompatibility for pollen germination and tube growth. While *D. siettiana* stigma permitted germination and tube growth of *B. campestris* pollen, the stigma of *B. campestris* strongly inhibited germination and tube entry of *D. siettiana* pollen. Such unilateral incompatibility has been reported in many other wide crosses of *Brassica* (Harberd 1976; Batra et al. 1990: Gundimeda et al. 1992).

In the present investigation, embryo rescue in the form of ovary culture was effective in overcoming post-fertilization barriers in the cross *D. siettiana* × *B. campestris* in which pollen tubes reached the ovary. Ovary culture was not successful in the reciprocal cross in which pollen tubes failed to reach the ovary.

Studies on DNA analysis, morphology and cytology of the plantlets confirmed hybridity. Chromosome pairing in F_1 hybrids was incomplete due to lack of complete homology between the parental genomes. The high frequency of univalents at diakinesis and metaphase-I and chromosome laggards at anaphase lead to irregular meiosis; F_1 hybrids were completely pollen-sterile. Backcross pollinations on F_1 hybrids did not yield any seed. Chromosome pairing with a high frequency of II was observed in amphidiploids. This restored about 50% pollen fertility. Backcrosses on amphidiploids gave rise to a good

Table 3. Details of the progeny realized from pollinations using the hybrid (*D. siettiana* × *B. campestris*) as a bridge cross

Cross	No. of plants grown to flowering	Morphological characters	% Pollen fertility
Amphidiploid (*D. siettiana* × *B. campestris*) × *B. juncea*	52	Uniform, partially pollen fertile	2.3
Amphidiploid (*D. siettiana* × *B. campestris*) × *B. napus*	46	Uniform, partially pollen fertile	12.2
BC_1 (*D. siettiana* × *B. campestris*) × *B. juncea*	37	Highly variable, completely pollen-sterile to partially fertile: 19 plants with abnormal anthesis and pollen-sterile, 16 plants with normal anthesis but pollen-sterile, 2 plants with normal anthesis and partially pollen-fertile	0–30
BC_1 (*D. siettiana* × *B. campestris*) × *B. napus*	44	Highly variable, completely pollen-sterile: 6 plants with abnormal anthesis and pollen-sterile, 38 plants with normal anthesis and pollen-sterile	0–0

number of seeds indicating restoration of partial ovule fertility in addition to pollen fertility.

Many wide crosses in *Brassica* have been reported to produce matromorphic (diploid parthenogenetic) seeds (Eenink 1974; Banga and Labana 1983; Batra et al. 1989; Agnihotri et al. 1990). In the absence of suitable markers expressed at the seedling stage, all the plants have to be grown to flowering before hybrids can be distinguished from matromorphs. This would involve unnecessary time, resources and effort on those plants which would later turn out to be matromorphs. Hence, any method that would enable the identity of the hybrid to be determined at an early stage is very useful in a hybridization programme. DNA analysis is one such a method (Agnihotri et al. 1990). In the present investigation hybrids could be identified unambiguously at the seedling stage through DNA analysis.

A maximum of 5 II and 1 III were found in the F_1 hybrid. This extent of pairing may be due to auto/allosyndesis. Armstrong and Keller (1981) reported that 2 II + 1 III can form autosyndetically in haploids of *B. campestris*. So far there is no report available on the number of possible autosyndetic pairs in *D. siettiana*. However, based on our observations, we presume that *D. siettiana* can form one autosyndetic pair because of the occurrence of a maximum of 3 IV in the amphidiploid. Thus two of the II in the F_1 hybrids seem to be due to allosyndesis.

Backcrosses of the amphidiploid with *B. campestris* increased the frequency of II, although a low frequency of III and IV were also observed in BC_1 plants. In BC_2 plants, higher associations such as III and IV were conspicuously absent probably due to the elimination of *D. siettiana* chromosomes.

The presence of both sterile and fertile plants in BC_2 progeny strongly indicates the possibility of developing CMS and restorer lines in further backcrosses. Moreover, the presence of allosyndetic II and III in F_1, amphidiploid and BC_1 plants indicate partial homology between *D. siettiana* and *B. campestris* genomes and the possibility of introgression of nuclear genes for fertility restoration into alloplasmic *B. campestris*.

D. siettiana shows strong crossability barriers with many other cultivated brassicas. Attempts by Ringdahl et al. (1987) to cross *D. siettiana* with *B. napus* were unsuccessful. Our attempts to cross *D. siettiana* with *B. juncea* through conventional methods were also unsuccessful (data not shown). In many other crop species an intermediate species has been used as a bridge to transfer the genome across incompatible species (Hadley and Openshaw 1980). In the present investigation, we used the amphidiploid of *D. siettiana* × *B. campestris* as a bridge cross to transfer the cytoplasm of *D. siettiana* to *B. juncea* and *B. napus*. A considerable number of seeds were obtained in both these combinations without resorting to embryo rescue. The cytology of these hybrids – (*D. siettiana* × *B. campestris*) × *B. juncea* and (*D. siettiana* × *B. campestris*) × *B. napus* – also indicated allosyndesis to some extent. Both the hybrids readily produced seeds when backcrossed with the pollen of the respective cultivars. Thus the fertile amphidiploid of *D. siettiana* × *B. campestris* serves as a convenient bridge for the transfer of *D. siettiana* cytoplasm and of useful nuclear genes into *B. juncea* and *B. napus* without resorting to the embryo rescue method.

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