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Production of hybrids, amphiploids and backcross progenies between a cold-tolerant wild species, *Erucastrum abyssinicum* and crop brassicas

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Abstract Three intergeneric hybrids were produced between a cold-tolerant wild species, *Erucastrum abyssinicum* and three cultivated species of *Brassica*, *B. juncea*, *B. carinata* and *B. oleracea*, through ovary culture. The hybrids were characterized by morphology, cytology and DNA analysis. Amphiploidy was induced in all the F_1 hybrids through colchicine treatment. Stable amphiploids and backcross progenies were obtained from two of the crosses, *E. abyssinicum* × *B. juncea* and *E. abyssinicum* × *B. carinata*. The amphiploid, *E. abyssinicum* × *B. juncea*, was successfully used as a bridge species to produce hybrids with *B. napus*, *B. campestris* and *B. nigra*. These hybrids and backcross progenies provide useful genetic variability for the improvement of crop brassicas.

Key words *Erucastrum abyssinicum* · *Brassica* spp · Intergeneric hybrids · Bridge crosses · Amphiploidy · Stress resistance

Introduction

Wild species have been effectively used to transfer genes for resistance to a range of biotic and abiotic stresses to cultivated species in many crops (Kalloo 1992). *Brassica* coenospecies, a group of wild and weedy species of crop brassicas, are repositories of genes conferring resistance to biotic and abiotic stresses (Warwick 1993; Cole 1994). Many of the alloplasmics developed by transferring the cytoplasm of the wild species to crop brassicas exhibit cytoplasmic male sterility (CMS) (Banga 1993). Some of the alloplasmics have also been reported to show agronomically valuable characters (Namai 1987; Downey and Rimmer 1993). However, strong crossability barriers limit the introgression of these genes through conventional methods. Recently, through the embryo rescue technique, it has been possible to overcome post-fertilization barriers and to produce many wide hybrids (for reviews see Inomata 1993; Warwick and Black 1993).

Erucastrum abyssinicum, a wild species inhabiting the Ethiopian highlands (> 3000 m), is reported to be cold tolerant (Al-Shehbaz 1985 cited in Warwick 1993). It has been suggested that some genetic homology exists between *Erucastrum* species and crop brassicas (Gomez-Campo and Tortosa 1974), which would allow introgression of genes from the wild species to the cultivars. The present communication reports the production of wide hybrids between *E. abyssinicum* (♀) and all the cultivated species of *Brassica* (♂), through the use of embryo rescue and bridge-cross methods, as well as the morphology, cytology and DNA analysis of the hybrids, and the production of stable amphiploids and backcross progenies.

Materials and methods

Plants of *E. abyssinicum* (Rich.) O.E. Schulz (2n = 32, EaEaEaEa), considered an autotetraploid (Harberd and McArthur 1980), *B. campestris* L. ssp. *oleifera* var. brown sarson, (2n = 20, AA), *B. nigra* L. Koch strain 257 (2n = 16, BB), *B. oleracea* var. alboglabra L.H. Bayley (2n = 18, CC), *B. juncea* (L) Czern. cv pusa bold (2n = 36, AABB), *B. napus* L. strain 706 (2n = 38, AACC) and *B. carinata* Braun (2n = 34, BBCC) were grown under field conditions. Flower buds were emasculated and bagged 1 day before anthesis; they were pollinated on the day of anthesis with fresh pollen and were re-bagged. In vivo pollen germination and pollen-tube growth were studied using the aniline-blue fluorescence method (Linskens and Esser 1957).

For ovary culture, pistils were excised 4–6 days after pollination, surface-sterilized with 0.1% mercuric chloride and cultured on MS (Murashige and Skoog 1962) medium supplemented with casein hydrolysate (500 mg l^{-1}).

The hybrid seedlings obtained from cultured ovaries were multiplied in vitro through the culture of shoot tips and single-node segments (Nanda Kumar and Shivanna 1991) on MS medium supplemented with BAP (0.3 mg l^{-1}). The multiplied shoots were rooted on MS medium supplemented with NAA (0.1 mg l^{-1}) or IBA

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(0.2 mg l^{-1}). The seedlings were hardened and transferred to pots containing garden soil, and grown under field conditions.

To induce amphiploidy, in vitro raised plantlets were treated (before hardening) by immersion of whole plantlets or only the roots for 4 h/48 h, respectively, with 0.1% colchicine (Sigma) dissolved in 0.5% dimethyl sulphoxide. For cytological studies, flower buds were fixed in Carnoy's solution and squashed in 1% acetocarmine. Pollen fertility was assessed through acetocarmine staining.

For DNA analysis, total DNA from the leaf material was extracted following the method of Dellaporta et al. (1984) and digested with a restriction endonuclease, *Sau*3AI, according to the manufacturer's instructions. The digested DNA was electrophoresed on 1.5% agarose gel and blotted onto Hybond N^+ membrane. The membrane was hybridized to a *B. campestris* species-specific DNA tandem repeat probe, pA2-78 (Lakshmikumaran and Ranade 1990), labelled with $\alpha^{32}P$ dCTP. Hybridization and autoradiography were carried out according to Lakshmikumaran et al. (1985). RAPD amplification was carried out according to Jain et al. (1994).

Results

Hybrid seeds were realized through field pollinations in only one cross, *E. abyssinicum* × *B. oleracea*. Two other crosses (*E. abyssinicum* × *B. juncea* and *E. abyssinicum* × *B. carinata*) did not yield hybrid seeds, though there was some fruit development. Fluorescent microscopic studies following intergeneric pollinations revealed profuse pollen germination and pollen-tube growth in all the three crosses. Many pollen tubes were seen entering the ovules.

Ovary culture was successful in all the three crosses attempted. Approximately 50% of the cultured pistils developed into fruits in vitro. From two of the crosses (*E. abyssinicum* × *B. oleracea* and *E. abyssinicum* × *B. carinata*), bold hybrid seeds were recovered after about 50 days. The hybrid seed of the cross *E. abyssinicum* × *B. juncea* germinated in situ and the cotyledons emerged, breaking open the fruit wall.

All the hybrids were multiplied through culture of single-node segments or shoot tips. Though both the methods were effective, the shoots raised from single-node segments were more robust. The shoots were rooted on NAA (0.1 mg l^{-1} or IBA (0.2 mg l^{-1}) medium. Although both the auxins were effective in root induction, the rooting and transplantation of plantlets was better on IBA medium.

Immersion of whole plantlets in colchicine solution induced amphiploidy in two hybrids: *E. abyssinicum* × *B. oleracea* (6.6% of the treated plantlets) and *E. abyssinicum* × *B. juncea* (50% of the treated plantlets). In the former, all the inflorescences bore fertile flowers, whereas in the latter only about 25% of the inflorescences bore fertile flowers. The fertile inflorescences were of a chimeric nature: approximately 60% of the basal flowers were fertile and the rest were sterile.

Immersion of only the roots of in vitro raised plantlets in colchicine was also effective in inducing colchiploidy in the hybrids *E. abyssinicum* × *B. juncea* and *E. abyssinicum* × *B. carinata* (54% and 12.5% of the treated plantlets, respectively). These colchiploids were also chimeric.

Morphology and cytology of hybrids

Though all the hybrids were intermediate to their parents in most of the quantitative characters, morphologically they showed greater resemblance to the female parent (*E. abyssinicum*). The branching was profuse in the F_1 hybrids as compared to either of their respective parents. All the flowers of the female parent were bracteate, while those of the male parents were ebracteate. In all three hybrids, 3–5 basal flowers of each inflorescence were bracteate, while the remaining flowers were ebracteate. The flowers opened normally but were small and pollen sterile. The petals of all the parents were yellow except for those of *B. oleracea*, in which they were white. The petal colour of the hybrid *E. abyssinicum* × *B. oleracea* was similar to that of the male parent (white). The anthers in all the F_1 hybrids were flaccid and did not dehisce.

All the synthetic amphiploids largely resembled their respective F_1 hybrids. The leaves present at the time of colchicine treatment turned brittle and the new leaves that emerged after treatment were normal. The number of inflorescences in the amphiploids was greatly reduced as compared to their respective F_1 hybrids. The inflorescences were highly condensed and the number of flowers reduced to almost half. In two of the amphiploids (*E. abyssinicum* × *B. juncea* and *E. abyssinicum* × *B. carinata*) the size of the flowers was comparable to that of the corresponding male parent. In the amphiploid of *E. abyssinicum* × *B. oleracea*, though the flower was larger than that of F_1 it was smaller than that of *B. oleracea*.

All the hybrids showed the expected chromosome numbers. There was a preponderance of univalents in all F_1 hybrids though a significant number of bivalents was observed. Also, occasional trivalents were seen in all the F_1 hybrids.

The amphiploid of *E. abyssinicum* × *B. oleracea* showed 25 bivalents in 5 of the 44 meiocytes studied. Quadrivalents (0–4) were observed in a majority of meiocytes. There were occasional uni-, tri- and pentavalents. A majority of the meiocytes of *E. abyssinicum* × *B. juncea* showed 34 bivalents. Quadrivalents were also common. There were also a few tri- and pentavalents.

DNA analysis

The hybrids were characterized by RFLP and RAPD analysis. A DNA tandem repeat, (pA2-78) of unit size 177 bp, from *B. campestris* (Lakshmikumaran and Ranade 1990) was used for RFLP analysis. Under high-stringency hybridization and washing conditions this probe hybridizes only to DNA having an A or a C genome. As all the three male parents used possess either the A or the C genome, this probe was effective in detecting the presence of the male parent genome in the hybrid. The DNA of the wild species did not give any

signal with the probe (Fig. 1A, lane a). The DNA of F_1 hybrids and their corresponding male parents (Fig. 1A, lanes b–g) gave multimeric bands of 177 bp (177, 354, 531 etc.). The results, thus confirm the presence of the male parent genome in the hybrids.

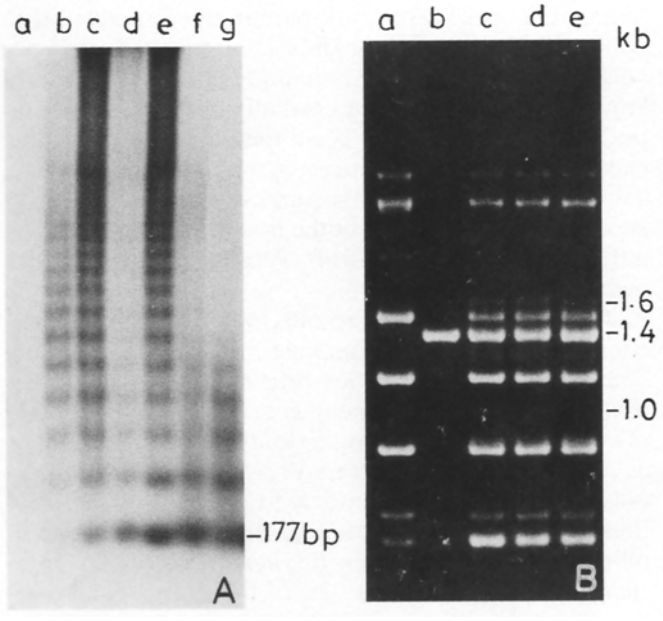

Fig. 1 A Southern-blot hybridization pattern of radio-labelled *B. campestris* species-specific probe pA2-78 with *Sau*3AI-restricted total DNAs of parents and F_1 hybrids: *E. abyssinicum* (*lane a*), *E. abyssinicum* × *B. juncea* (*lane b*), *B. juncea* (*lane c*), *E. abyssinicum* × *B. carinata* (*lane d*), *B. carinata* (*lane e*), *E. abyssinicum* × *B. oleracea* (*lane f*) and *B. oleracea* (*lane g*). The probe does not hybridize with the female parent DNA (*lane a*) but hybridized with the DNAs of all the hybrids and their male parents giving multimeric bands of 177 bp, confirming the presence of male-parent DNA in the hybrids. **B** Ethidium bromide-stained gel of RAPD of the F_1 hybrid *E. abyssinicum* × *B. juncea*, and its parents: *E. abyssinicum* (*lane a*), *B. juncea* (*lane b*) and F_1 hybrid (*lanes c–e*). The species-specific bands amplified in both the parents are represented in the F_1 hybrid, confirming its hybrid nature

RAPD analysis was confined to a single hybrid *E. abyssinicum* × *B. juncea*. Of the 16 primers (OPC 01-OPC 16, Operon technologies) used, some generated conclusive polymorphism. The bands specific to both the parents were represented in the hybrid confirming its hybrid nature (Fig. 1B). However, some amplification products from the male parent were missing in the hybrid. Also, some novel bands were seen in the hybrid.

Male fertility and seed-set in the F_1 hybrids and amphiploids

All the F_1 hybrids were pollen sterile and there was no seed-set on backcrossing (Table 1). Embryo rescue was also not effective in realizing backcross progeny.

Though the amphiploid *E. abyssinicum* × *B. oleracea* showed about 95% pollen fertility, very few seeds were obtained on selfing, as well as on backcrossing to the male parent (Table 1). The seeds obtained from selfing failed to germinate in the soil. Though one seedling could be raised from backcross seeds, it failed to establish itself in the field.

In the remaining two amphiploids (*E. abyssinicum* × *B. juncea* and *E. abyssinicum* × *B. carinata*) also pollen fertility was above 95%. In both the amphiploids seed-set was obtained both on selfing as well as on backcrossing (Table 1). Apart from bold seeds, considerable numbers of shrivelled and in situ germinated seeds were also harvested. The seed-set was better on backcrossing compared to selfing in both the amphiploids. Bold seeds from selfing and backcrossing readily germinated in the soil.

A_2 and BC_1 progeny

A_2 progeny of *E. abyssinicum* × *B. juncea* and *E. abyssinicum* × *B. carinata* largely resembled their respective male parents. The plants of A_2 progeny were taller and

Table 1 Results of field pollinations of F_1 hybrids and amphiploids

Hybrid	Male parent	No. of pollinations	No. of fruits formed	No. of bold seeds developed	No. of seeds per pod
E. abyssinicum × *B. carinata*					
F_1 hybrid	*B. carinata*	213	18	0	0.00
Amphiploid(A_1)	Selfing	42	41	21	0.51
	B. carinata	22	21	51	2.42
E. abyssinicum × *B. oleracea*					
F_1 hybrid	*B. oleracea*	178	0	0	0.00
Amphiploid(A_1)	Selfing	112	109	5	0.04
	B. oleracea	75	69	3	0.04
E. abyssinicum × *B. juncea*					
F_1 hybrid	*B. juncea*	452	5	0	0.00
Amphiploid(A_1)	Selfing	375	370	392	1.07
	B. juncea	228	220	467	2.12
Amphiploid(A_2)	*B. napus*[a]	52	48	672	14.00
	B. campestris[a]	62	51	18	0.35
	B. nigra[a]	48	39	5	0.12

[a] Bridge cross pollinations

more robust as compared to their respective A_1 progeny. There was no difference in the morphology of flowers and fruits or in the pollen fertility between A_1 and A_2 plants. However, the seed-set on selfing improved (2.52 and 1.2 seeds/pod respectively) in the A_2 progeny.

BC_1 plants of *E. abyssinicum* × *B. juncea* were more close to their male parent, (*B. juncea*) than to A_2. Some of the BC_1 plants possessed more primary and secondary branches as compared to *B. juncea*. Also the length of the main raceme and the number of pods on the main raceme were significantly greater. The pollen fertility was about 98% and the seed-set was 4.5 seeds/pod on backcrossing. Two male-sterile plants were also isolated from the 62 BC_1 plants raised.

The BC_1 progeny of *E. abyssinicum* × *B. carinata* resembled *B. carinata* except for their smaller size. However, they were taller than A_1 and A_2 progeny. All the BC_1 progeny were male sterile and female fertility (3.5 seeds/pod) was better as compared to A_1/A_2 progeny.

Bridge-cross hybrids

The amphiploid *E. abyssinicum* × *B. juncea* (A_2) was used as a bridge species to produce hybrids with *B. napus*, *B. campestris* and *B. nigra*. Hybrid seeds were realized in all the crosses, without resorting to embryo rescue (Table 1). The seed-set with *B. napus* was markedly higher when compared to the other crosses.

Discussion

It has been reported that crosses between tetraploids (♀) and diploids (♂) are more successful for wide hybridization (see Nishiyama et al. 1991). In the present investigation too *E. abyssinicum* (♀, autotetraploid) × *B. oleracea* (♂, diploid) yielded hybrid seeds through field pollination while the other two crosses, in which male parents were allopolyploids, were not successful.

As the number of hybrids realized was small, they were multiplied in vitro through culture of shoot tips and single-node segments. This has been very useful in raising a large number of hybrid plants for cytological studies, for colchicine treatment, and to carry out backcross pollinations (Nanda Kumar and Shivanna 1991). Colchicine treatment of the roots or the whole plantlets was effective in inducing colchiploidy. However, the chimeric nature of amphiploids observed in two of the hybrids, in which only the basal flowers in some of the inflorescences showed pollen fertility, is difficult to explain on the basis of the available literature.

Matromorphy has been reported in several wide crosses in brassicas (see Namai 1987). To distinguish hybrids from matromorphs through conventional methods, it is necessary to grow the plants up to flowering. This would involve unnecessary time, input and effort on those plants which would turn out to be matromorphs. Hence, any technique to evaluate the hybrids at an early stage is very useful in the hybridization programme, particularly when this involves the use of laborious and time-consuming methods of embryo rescue, in vitro multiplication, and the induction of colchiploidy. One such approach to characterize the hybrids is the use of the RFLP technique employing repetitive DNAs as probes (Saul and Potrykus 1984; Schweizer et al. 1988). Another approach is RAPD analysis of DNA (Heun and Helentjaris 1993; Mukhopadhyay et al. 1994). In the present study both these methods were effective in confirming hybrids unambiguously at the seedling stage.

Meiosis in the F_1 hybrids was characteristic of wide hybrids, with a preponderance of univalents. A high incidence of bivalents in the F_1 hybrids seems to be largely as a result of autosyndetic pairing due to autotetraploidy in *E. abyssinicum* (Harberd and McArthur 1980). However, higher (than expected) bivalent associations observed in some meiocytes (> 8) and the presence of trivalents and higher associations in the hybrids suggest allosyndetic pairing between different parental genomes, indicating the possibility of gene introgression from wild species to crop brassicas.

All the F_1 hybrids were pollen sterile and failed to set seed on backcrossing. Although the pollen fertility was $> 95\%$ in the amphiploids, the seed-set as reported in other studies was low. However, there was an improvement in seed-set in the A_2 and BC_1 progeny. The low seed-set in the amphiploids has been held to be due to meiotic disturbances and disharmonious interaction between parental genomes (Olsson 1986). According to Dolstra (1982) residual factors of incongruity between parental genomes operate between newly established amphiploids and may cause sterility.

The production of stable amphiploids is important for successful gene transfer, and for reliable evaluation of the genetic value of the alien genes in the genetic background of the cultivar. To obtain a complete set of addition lines also, it is essential to produce stable amphiploids, as the F_1 hybrids exhibit strong male and female sterility (Jiang et al. 1994). Of the three amphiploids synthesized in the present study two were stable with good pollen fertility and moderate female fertility. The female fertility is likely to improve in subsequent generations.

In wide hybridization of *Diplotaxis siettiana* (Nanda Kumar and Shivanna 1993) and *D. erucoides* (Vyas et al. 1995) with different crop brassica species, bridge crosses have been more effective than the embryo rescue technique. In the present study also, a considerable number of hybrid seeds were realized in crosses between *E. abyssinicum* × *B. juncea* amphiploid and the cultivars, *B. napus*, *B. campestris* and *B. nigra*.

Earlier reports on hybrid production between *E. abyssinicum* and crop brassicas (*B. campestris*, *B. oleracea*, *B. juncea*) are confined to meiotic analyses (Harberd and McArthur 1980; Sarmah and Sarla 1994). To our knowledge, ours is the first report both on the

production of hybrids with *B. carinata*, *B. napus* and *B. nigra* and on the amphiploids and backcross progenies obtained from many of the hybrids. The amphiploids and backcross progenies produced in the present investigation provide useful genetic variability in crop brassicas for their improvement through breeding, and for developing new alloplasmics.

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