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Production and characterization of interspecific hybrids between *Brassica maurorum* and crop brassicas

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Abstract Seven interspecific hybrids were produced between *Brassica maurorum* (♀), a wild species resistant to Alternaria blight and white rust, and all the monogenomic (*B. campestris*, *B. nigra* and *B. oleracea*) and digenomic (*B. juncea*, *B. napus* and *B. oleracea*) crop brassicas (♂) through embryo rescue. The hybrids were confirmed by means of morphological and cytological studies. All the hybrids were pollen-sterile. Amphidiploids were induced in three of the hybrids: *B. maurorum* × *B. napus*, *B. maurorum* × *B. carinata*, *B. maurorum* × *B. nigra*. The hybrids were also confirmed through DNA analyses for nuclear and organelle genomes using RAPD and RFLP techniques.

Key words *Brassica maurorum* · Crop brassicas · Embryo rescue · Wide hybrids · Amphidiploids

Introduction

Narrow genetic variability in crop brassicas is a major limitation in their breeding programmes. Many wild and weedy relatives of crop brassicas belonging to *Brassica* coenospecies are a rich source of genes conferring resistance/tolerance to biotic and abiotic stresses (Warwick 1993; Chopra et al. 1996). Further, the cytoplasm of many wild relatives induces cytoplasmic male sterility in crop brassicas (Banga 1996). Wide hybridization between wild and cultivated species is an important approach that is used to tap desirable nuclear and cytoplasmic traits (Warwick 1993; Chopra et al. 1996). However, wide hybridization in Crucifers is difficult because of the presence of pre- and post-fertilization barriers (Inomata 1993; Shivanna 1996). In recent years, many biotechnological approaches have been used to overcome these barriers, and a number of wide hybrids have subsequently been produced (Inomata 1992; Shivanna 1996). Nevertheless, there is ample scope for the production of new wide hybrids as a lot of wild germplasm remains unexploited.

Brassica maurorum is endemic to North Africa (Tsunoda 1980). This species shows considerable resistance to white rust and Alternaria blight (Shyam Prakash, personal communication). We have been growing this species during the last 4 years amidst cultivated brassicas. Although the cultivated species, particularly *B. campestris* and *B. juncea*, got severely infected with white rust and Alternaria blight every year, plants of *B. maurorum* never got infected with these diseases. The present paper reports the production of seven interspecific hybrids between *B. maurorum* (♀) and all the mono- and digenomic species of crop brassicas (♂) through embryo rescue and their characterization using morphological and cytological characters, and by DNA analyses.

Materials and methods

The seeds of all the species used in the present investigation were obtained from Dr. Shyam Prakash, National Research Centre for Plant Biotechnology, IARI, New Delhi. The species used are: *Brassica maurorum* Durien (2n = 16, B^mB^m), *B. campestris* L. ssp. oleifera var 'brown sarson' (2n = 20, AA), *B. nigra* L. Koch strain 257 (2n = 16, BB), *B. oleracea* L. Bailey (two varieties: 'alboglabra' and 'botrytis') (2n = 18, CC), *B. juncea* (L.) Czern. cv 'pusa bold'

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(2n = 36, AABB), *B. napus* L. strain 706 (2n = 38, AACC) and *B. carinata* Braun (2n = 34, BBCC). The plants of all these species were grown in the botanical garden of the Department of Botany, University of Delhi.

Flower buds of the female parents were emasculated 1 day before anthesis and bagged. They were pollinated the next day with freshly collected pollen from the male parents and rebagged. Some of the pollinated pistils were used for pollen germination and pollen-tube growth studies using the aniline blue fluorescence method (Linskens and Esser 1957). Some pollinated flowers were left on the plants until their drying out or fruit maturity. The remaining pollinated pistils were used for embryo rescue in the form of ovary, ovule and sequential culture using Murashige and Skoog's (MS) (1962) medium enriched with casein hydrolysate (500 mg/l). For ovary culture, the pistils were excised 4–6 days after pollination, surface-sterilized with 0.1% mercuric chloride and cultured. For ovule culture, pistils 10 days after pollination were surface-sterilized and the ovules were dissected and cultured. For sequential culture, the cultured ovaries were removed from the culture medium 10 days after the initiation of culture; enlarged ovules were then dissected out and recultured on fresh medium.

The seedlings recovered through embryo rescue were multiplied through culture of shoot tips and single node segments on MS + 6-benzylaminopurine (BAP) (0.2 mg/l) + kinetin (Kn) (0.1 mg/l) medium. New shoots produced on this medium were rooted on MS + 3-indolebutyric acid (IBA) or 1-naphthylacetic acid (NAA) (0.2 mg/l) medium. In vitro-raised plantlets were hardened and transplanted to the field. To induce amphidiploidy in the hybrids, we rubbed cotton swabs dipped in a colchicine solution (0.1%) onto axillary buds of in vitro and field-grown plants for 48 h. Alternatively, the in vitro-raised plantlets were immersed in colchicine (0.1%) solution containing 1% dimethyl sulphoxide (DMSO) for 4–6 h before hardening and transplantation (Rao et al. 1996).

For meiotic studies, flower buds were fixed in Carnoy's fixative (ethanol : chloroform : acetic acid, 6 : 3 : 1) for 24 h. Anthers were squashed in 1% acetocarmine. Pollen fertility was determined by staining in 1% acetocarmine.

For DNA analysis, total DNA was extracted from the leaves of parents and hybrids according to Dellaporta et al. (1984). Three restriction endonucleases, *Eco*RI, *Bam*HI and *Hin*dIII, were used for digesting total DNA according to the manufacturer's instructions. The digested DNA was electrophoresed on 1% agarose gel and blotted onto nitrocellulose membranes (Amersham). The membranes were hybridized to radioactive mitochondrial (mt) probes, pCos 61 and pCos 88 (Pradhan et al. 1992), and chloroplast (cp) probe psb D (Alt et al. 1984). Autoradiography was carried out at −70°C. For random amplified polymorphic DNA (RAPD) analysis, primers were obtained from Operon Technologies, Almeda, USA. Amplifications were performed in Perkin-Elmer thermal cycler. Electrophoresis was carried out at 60 V for 5 h on a 1.8% agarose gel, and the bands were visualized after staining with ethidium bromide (0.5 μg/ml).

Results

Production of hybrids and amphidiploids

Aniline blue fluorescence studies of pollinated pistils showed that *Brassica maurorum* is self-incompatible (SI), although this self-incompatibility is weak and results in a few seeds from self-pollinations. Studies on interspecific pollinated pistils revealed the absence of pre-fertilization barriers between *B. maurorum* (♀) and all the crop species (♂); pollen grains germinated and pollen-tubes reached the ovary in all of the crosses. However, reciprocal crosses showed pre-fertilization barriers at the level of pollen germination or pollen-tube entry into the stigma or pollen-tube growth through the style. Field pollinations between *B. maurorum* (♀) and the six cultivated species resulted in a few seeds in only three of the crosses (Table 1).

Table 1 Hybrids obtained through field pollinations

Cross	Number of pollinations	Number of seeds formed	Number of hybrid seedlings established
B. maurorum × *B. nigra*	92	5	5
B. maurorum × *B. oleracea* var botrytis	80	4	2
B. maurorum × *B. napus*	71	7	2

Table 2 Results of embryo rescue methods in realizing interspecific hybrids

Cross	Ovary culture		Ovule culture		Sequential culture			Total no. of hybrid plants established
	Number of ovaries cultured	Number of seeds formed	Number of ovules cultured	Number of seedlings formed	Number of pistils cultured	Number of ovules excised and recultured	Number of seedlings formed	
B. maurorum × *B. campestris*	93	0	45	0	173	60	4	4
B. maurorum × *B. nigra*	170	3	27	1	105	38	16	20
B. maurorum × *B. oleracea* var alboglabra	101	0	18	0	197	30	4	4
B. maurorum × *B. oleracea* var botrytis	201	2	48	0	76	60	4	6
B. maurorum × *B. juncea*	110	0	68	0	147	72	5	5
B. maurorum × *B. napus*	87	4	43	1	83	97	4	9
B. maurorum × *B. carinata*	61	0	23	0	160	51	4	4

Results of embryo rescue methods are presented in Table 2. Ovary culture yielded hybrids in only three of the crosses, which also yielded seeds through field pollination. Ovule culture yielded hybrids in only two of the crosses, *B. maurorum* × *B. nigra* and *B. maurorum* × *B. napus*. Sequential culture was successful in realizing hybrids in all the seven of the crosses attempted (Table 2).

All of the hybrids were multiplied in vitro through the culture of shoot tips and nodal segments. Of the two media, MS + IBA and MS + NAA, tested for the rooting of in vitro-multiplied shoots, the former was better; roots were long with sparsely distributed root hairs, which facilitated the easy transfer of plantlets to the field. One hundred percent transplantation efficiency was obtained when plantlets rooted on MS + IBA were transferred during late November and December, when the ambient temperature was low.

Among the various methods of colchicine treatment attempted, only immersion of plantlets in the colchicine solution was successful in inducing amphidiploidy in three hybrids: *B. maurorum* × *B. nigra*, *B. maurorum* × *B. napus* and *B. maurorum* × *B. carinata*. No amphidiploids were realized in the other hybrids.

Morphology, cytology and DNA analyses

F_1 hybrids were grown to flowering. They were healthy and intermediate in most of their quantitative characters with respect to their parents except for *B. maurorum* × *B. oleracea*, *B. maurorum* × *B. napus* and *B. maurorum* × *B. carinata*, in which the hybrids were smaller than both the parents. Amphidiploids were robust compared to their F_1 hybrids. Flowering in the amphidiploids was delayed by about 15 days. The amphidiploids of *B. maurorum* × *B. nigra* were pollen-fertile with 64% fertility, whereas those of *B. maurorum* × *B. napus* and *B. maurorum* × *B. carinata* were pollen-sterile.

Meiotic analyses of F_1 hybrids and amphidiploids showed the expected chromosome numbers (Table 3). Univalents were prevalent in F_1 hybrids (Fig. 1A, C, D) and bivalents in amphidiploids (Fig. 1B). A quadrivalent and a pentavalent were also observed in F_1 hybrids of *B. maurorum* × *B. juncea* and amphidiploids of *B. maurorum* × *B. nigra*. No higher associations were observed in the amphidiploids *B. maurorum* × *B. napus* and *B. maurorum* × *B. carinata*. Cytological analysis of the remaining three F_1 hybrids (*B. maurorum* × *B. campestris*, *B. maurorum* × *B. oleracea* and *B. maurorum* × *B. carinata*) could not be carried out due to the drying of their flower buds before initiation of meiosis or an unavailability of sufficient number of flower buds. RAPD analyses of parents and hybrids was carried out using 11 single arbitrary primers. The primers OPG 2 and OPD 3 generated polymorphic bands with all the parents. All the F_1 hybrids showed consistently one female parent-specific band at 0.4 kb (Fig. 2A, arrowhead) and two male parent specific-bands at 0.5 kb and 0.43 kb (Fig. 2A, asterisks), thereby confirming their hybridity.

Chloroplast DNA analyses of the parents and hybrids using cpDNA probe psb D gave a female parent-specific band at 5.0 kb which was present in all the hybrids (Fig. 2B). The two male parent-specific bands at 7.0 kb or 8.2 kb were absent in all the F_1 hybrids. Both mtDNA probes, pCos 61 and pCos 88, gave polymorphism with all the parents. With pCos 88 *B. maurorum* gave three female parent-specific bands at 2.7 kb, 1.8 kb and 1.1 kb (Fig. 2C). These female parent-specific bands were present in all the hybrids. None of the hybrids showed any male parent-specific bands. Thus, analyses of the organelle genome confirmed the presence of the cytoplasm of the wild species in all the hybrids.

Some of the F_1 hybrids and all the amphidiploids set seeds on backcross pollinations with pollen of the respective cultivars. These could be used to advance backcross generations to achieve cytoplasmic substitution.

Table 3 Meiotic analysis of F_1 hybrids and amphidiploids

Cross	Genome	Chromosome number 2n	Number of PMCs studied	Mean chromosome associations (range in parenthesis)				
				I	II	III	IV	V
B. maurorum × *B. nigra*-F_1	BmB	16	27	13.3 (8–16)	1.3 (0–4)	0	0	0
B. maurorum × *B. nigra*-amphidiploid	BmBmBB	32	22	0.28 (0–2)	14.7 12–16)	0	0.28 (0–1)	0.14 (0–1)
B. maurorum × *B. Juncea*-F_1	BmAB	26	26	16.7 (11–20)	3.5 (3–4)	0	0.25 (0–1)	0.25 (0–1)
B. maurorum × *B. napus*-F_1	BmAC	27	24	17.5 (9–23)	4.75 (2–9)	0	0	0
B. maurorum × *B. napus*-amphidiploid	BmBmAACC	54	26	1.3 (0–4)	26.3 (25–27)	0	0	0
B. maurorum × *B. carinata*-amphidiploid	BmBmBBCC	50	18	1.0 (0–2)	24.5 (24–25)	0	0	0

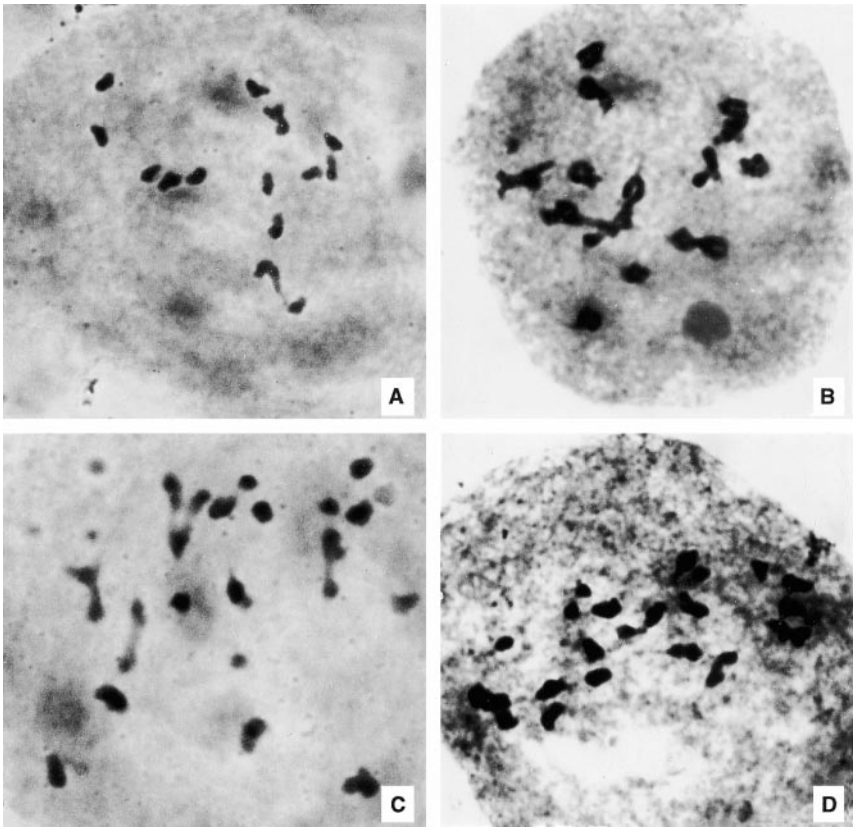

Fig. 1A–D Cytology of F_1 hybrids and amphidiploids. **A, B** *B. maurorum* × *B. nigra* metaphase I of F_1 hybrid showing 16 I **(A)** and diakinesis of amphidiploid showing 16 II **(B)**. **C, D** Metaphase I of F_1 hybrid *B. maurorum* × *B. napus* showing 9 I, 9 II **(C)**, and *B. maurorum* × *B. juncea* 18 I, 4 II **(D)**

Discussion

Studies on pollen germination and pollen-tube growth in crosses between *B. maurorum* and cultivated species showed unilateral incompatibility. Pollen grains of cultivated species germinated on the stigma of *B. maurorum*, and pollen tubes reached the ovary. However, in reciprocal crosses pollen grains or pollen tubes were inhibited. Such unilateral incompatibility is of a common occurrence in wide crosses of *Brassica* (Röbbelen 1960; Shivanna 1996).

Of the three embryo rescue techniques used to overcome post-fertilization barriers, sequential culture was the most successful and yielded hybrids in all seven of the crosses attempted. Superiority of sequential culture over ovary and ovule cultures has also been reported by many earlier workers on wide hybridization in brassicas (Shivanna 1996). To our knowledge, *B. maurorum* is the only wild species which has been successfully crossed with all the six of the cultivated species of *Brassica*. Of these, only the hybrid *B. maurorum* × *B. nigra* has been reported earlier (Truco and Quiros 1991).

Induction of amphidiploidy has been a major constraint in wide hybridization programmes of brassicas (Gundimeda et al. 1992; Vyas et al. 1995). Amphidiploids could be induced in the present study in only three of the seven hybrids produced. For a more successful induction of amphidiploidy, other diplodizing agents such as oryzalin and trifluralin (microtubule depolymerizing herbicides), which have been shown to have a tenfold stronger affinity to plant microtubules than colchicine (Åstrom et al. 1995), may be more effective. These herbicides have been reported to be effective in inducing chromosome doubling in cultured microspores and microspore-derived callus at 100 times lower concentrations than colchicine (Wan et al. 1991; Hansen and Andersen 1996).

Only the amphidiploids of *B. maurorum* × *B. nigra* were pollen-fertile, whereas those of *B. maurorum* × *B. napus* and *B. maurorum* × *B. carinata* were pollen-sterile. Sterility of amphidiploid plants is not uncommon in wide hybrids of brassicas (Mizushima 1968). McNaughton (1973) attributed pollen sterility in amphidiploids to genic imbalance. In the present investigation also, pollen sterility in amphidiploids seems to be the result of genic imbalance since meiosis and anaphase disjunction of chromosomes was normal.

Meiotic analysis of F_1 hybrids and amphidiploids not only confirmed their hybridity but also indicated the extent of genome homology between the parents. F_1 hybrids showed a preponderance of univalents, which is typical, of wide hybrids. Bivalents were common in amphidiploids (Table 3). The basic chromosome number has been suggested to be six in *Brassica*

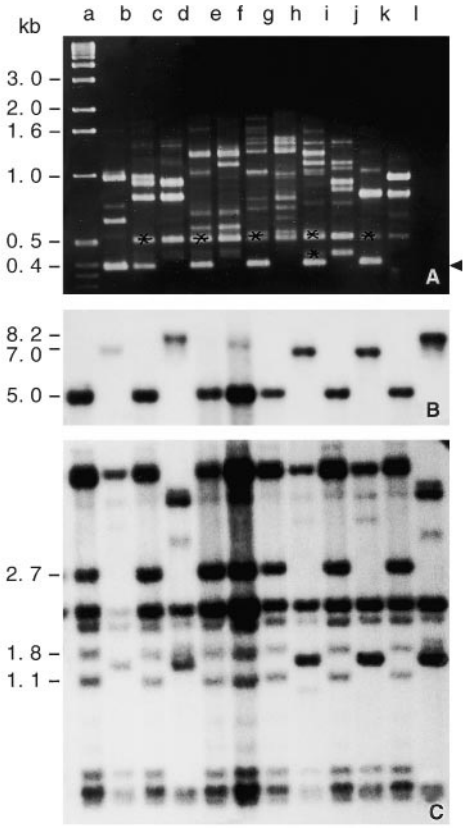

Fig. 2 **A** RAPD analyses of parents and hybrids with the primer OPG 2. *Lane a* 1-kb ladder marker, *lane b Brassica maurorum*, *lane c B. maurorum* × *B. juncea*, *lane d B. juncea*, *lane e B. maurorum* × *B. napus*, *lane f B. napus*, *lane g B. maurorum* × *B. carinata*, *lane h B. carinata*, *lane i B. maurorum* × *B. campestris*, *lane j B. campestris*, *lane k B. maurorum* × *B. nigra*, *lane l B. nigra*. The 0.4-kb female parent-specific band (*lane b*, *arrowhead*) is present in all the hybrids. Many male parent-specific bands are also present in all the hybrids (*asterisks*). **B** Southern hybridization pattern of *Hin*dIII-digested DNA of parents and hybrids probed with cpDNA probe psb D. *Lane a B. maurorum*, *lane b B. campestris*, *lane c B. maurorum* × *B. campestris*, *lane d B. nigra*, *lane e B. maurorum* × *B. nigra*, *lanes f, g* F_1 and BC_1 *B. maurorum* × *B. juncea*, *lane h B. juncea*, *lane i B. maurorum* × *B. napus*, *lane j B. napus*, *lane k B. maurorum* × *B. carinata*, *lane l B. carinata*. The 5.0-kb female parent-specific band (*lane a*) is present in all the hybrids. Male parent-specific bands at 7 kb and 8.2 kb were absent in the hybrids. **C** *Hin*dIII-digested DNA probed with mtDNA probe pCos 88. The lanes are as in **B**. The restriction pattern of the mtDNA of the female parent (*lane a*), with specific bands at 2.7 kb, 1.8 kb and 1.1 kb, and that of the hybrids (*lanes c, e, f, g, i, k*) is the same

species. (Röbbelen 1960; Prakash 1973). On the basis of this basic number, all of the bivalents observed in all of the hybrids can be explained on the basis of autosyndesis within parent genomes. However, the presence of higher associations in the F_1 hybrid of *B. maurorum* × *B. juncea* and amphidiploid of *B. maurorum* × *B. nigra* (Table 3) can be attributed to allosyndesis between the two parental genomes. Thus, it appears that *B. maurorum* has some homology with the B genome (*B. nigra*) and not with the C genome (*B. oleracea*), indicating the possibility of gene introgression from *B. maurorum* to *B. juncea*.

Production of wide hybrids through embryo rescue is cumbersome and time-consuming. Hybrids are generally confirmed through morphological and cytological studies. Confirmation of hybridity at the seedling stage itself is advantageous as the contaminants can be discarded very early, consequently saving the time and effort that would otherwise be spent on such contaminants. In the present investigation, all of the hybrids could be confirmed at the seedling stage through RAPD analyses and the source of the cytoplasm determined through analyses of the organelle genome. This approach has been used earlier in a number of hybrids and alloplasmics of *Brassica* (Erickson et al. 1986; Arumugam et al. 1996; Rao et al. 1996).

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References

Alt J, Morris J, Westhoff P, Herrmann RG (1984) Nucleotide sequence of the clustered genes for the 44 kd chlorophyll apoprotein and the '32 kd' like protein of the photosystem II reaction centre in the spinach plastid chromosome. Curr Genet 8: 597–606

Arumugam N, Mukhopadhyay A, Gupta V, Pental D, Pradhan AK (1996) Synthesis of hexaploid (AABBCC) somatic hybrids: a bridging material for transfer of 'tour' cytoplasmic male sterility to different *Brassica* species. Theor Appl Genet 92: 762–768

Åstrom H, Sorri O, Raudaskoski M (1995) Role of microtubules in the movement of the vegetative nucleus and generative cell in tobacco pollen tubes. Sex Plant Reprod 8: 61–69

Banga SS (1996) Genetics and breeding. In: Chopra VL, Prakash S (eds) Oilseed and vegetable brassicas: Indian perspective. Oxford & IBH Publ, New Delhi Calcutta, pp 50–76

Chopra VL, Kirti PB, Prakash S (1996) Accessing and exploiting genes of breeding value of distant relatives of crop brassicas. Genetica 97: 305–312

Dellaporta SL, Wood J, Hicks JB (1984) Molecular biology of plants: a laboratory course manual, Cold Spring Harbour Laboratory Press, New York

Erickson L, Grant I, Beversedorf W (1986) Cytoplasmic male sterility in rapeseed (*Brassica napus* L.). 1. Restriction patterns of chloroplast and mitochondrial DNA. Theor Appl Genet 72: 145–150

Gundimeda HR, Prakash S, Shivanna KR (1992) Intergeneric hybridization between *Enarthrocarpus lyratus*, a wild species and crop brassicas. Theor Appl Genet 83: 655–662

Hanson NJP, Andersen SB (1996) In vitro chromosome doubling potential of colchicine, oryzalin, trifluralin and APM in *B. napus* microspore culture. Euphytica 88: 159–164

Inomata N (1992) Embryo rescue techniques for wide hybridization. In: Labana KS, Banga SS, Banga SK (eds) Breeding oilseed brassicas. Narosa Publ House, New Delhi, India, pp 94–107

Inomata N (1993) Crossability and cytology of hybrid progenies in the cross between *Brassica campestris* and three wild relatives of *B. oleracea*, *B. bourgeaui*, *B. cretica* and *B. montana*. Euphytica 69: 7–17

Linskens HF, Esser K (1957) Über eine spezifische Anfärbung der Pollenschläuche in Griffel and die Zahl der Kallosepropfen nach Selbstung und Fremdung. Naturwissenschaften 44 : 16

McNaughton IH (1973) Synthesis and sterility of *Raphanobrassica*. Euphytica 22 : 70–88

Mizushima U (1980) Genome analysis in *Brassica* and allied genera. In: Tsunoda S, Hinata K, Gomez-Campo C (eds) *Brassica* crops and wild allies. Japan Sci Soc Press, Tokyo, pp 89–106

Murashige T, Skoog F (1962) Revised media for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15 : 473–497

Pradhan AK, Prakash S, Mukhopadhyay A, Pental D (1992) Phylogeny of *Brassica* and allied genera based on variation in chloroplast and mitochondrial DNA patterns: molecular and taxonomic classifications are incongruous. Theor Appl Genet 85 : 331–340

Prakash S (1973) Haploidy in *Brassica nigra* Koch. Euphytica 22 : 613–614

Rao GU, Lakshmikumaran M, Shivanna KR (1996) Production of intergeneric hybrids, amphiploids and backcross progeny between a cold tolerant wild species, *Erucastrum abyssinicum* and crop brassicas. Theor Appl Genet 92 : 786–790

Röbbelen G (1960) Beitrage zur Analyse des *Brassica*-Genomes. Chromosoma 11 : 205–228

Shivanna KR (1996) Incompatibility and wide hybridization. In: Chopra VL, Prakash S (eds) Oilseed and vegetable brassicas: Indian perspective. Oxford & IBH Publ, New Delhi Calcutta, pp 77–102

Tsunoda S (1980) Eco-physiology of wild and cultivated forms in *Brassica* and allied genera. In: Tsumoda S, Hinata K, Gomez-Campo C (eds) *Brassica* crops and wild allies. Japan Sci Soc Press, Tokyo, pp 109–120

Truco MJ, Quiros CF (1991) Evolutionary study on *Brassica nigra* and related species. GCIRC, Saskatoon, Canada, pp 318–323

Vyas P, Prakash S, Shivanna KR (1995) Production of wide hybrids and backcross progenies between *Diplotaxis erucoides* and crop brassicas. Theor Appl Genet 90 : 549–553

Wan Y, Duncan DR, Rayburn AL, Petolino JF, Widholm JM (1991) The use of antimicrotubule herbicides for the production of doubled haploid plants from anther derived maize callus. Theor Appl Genet 81 : 205–221

Warwick SI (1993) Guide to the wild germplasm of *Brassica* and allied crops. IV. Wild species in the tribe brassiceae (Cruciferae) as sources of agronomic traits. Agriculture Canada Research Branch Tech Bull 17E