Studies on crossability barriers between cultivated species and wild allies of crop *Brassica*s

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Abstract

Wild species are known to possess reservoirs of genes for resistance to biotic and abiotic stresses. One of the factors responsible for low productivity of oilseed *brassica*s in India is susceptibility of varieties to a number of diseases and pests. But, there is very little information available on crossability behavior of wild relatives and cultivated species of *Brassica*. Such information could be useful to the plant breeders in selecting suitable wild species to incorporate disease and pest resistant genes in improving oilseed *brassica*s through wide hybridization. To understand the extent of reproductive isolation between cultivated species of *Brassica* and some of its wild relatives, pollen-pistil interaction studies were carried out using decolorized aniline blue fluorescence technique. For this study, pistils of all the six important cultivated species of *Brassica* were taken as female parents and pollen from 12 to 20 wild relatives of *Brassica* were used as male parents, depending upon the availability of flowers. Out of the hundred interspecific/intergeneric crosses studied, 73 crosses showed presence of pre-fertilization barriers and 27 crosses showed the presence of post-fertilization barriers. In 49 crosses, pre-fertilization barriers were in the form of inhibition of pollen germination or pollen tube entry into the stigma and 24 crosses due to inhibition of pollen tube entry in the style and ovary. Most of the cultivated species of *Brassica* permitted pollen germination of *Brassica fruticulosa*, *B. maurorum*, *Diplotaxis tenuisiliqua* and *Erucastrum gallicum*. Based on the pollen germination, pollen tube entry into the style and ovary, species have been categorized into those showing pre- and post-fertilization barriers. For those crosses in which pollen tubes entered the ovary, application of embryo rescue technique would be very effective. Thus, studies showed that attempts can be made to develop wide hybrids in *B. nigra* with eight wild species, in *B. rapa* with three wild species, in *B. oleracea* with four wild species, in *B. carinata* with seven wild species, in *B. juncea* with three wild species, and in *B. napus* with two wild species, using embryo rescue techniques. On the basis of results, it can be generalized that amongst wild species, *B. fruticulosa*, *B. maurorum*, *Diplotaxis catholoica*, *Erucastrum gallicum* and *E. cardiminoides* are likely to be more efficient as male parents for developing wide crosses with cultivated *Brassica* species. Thus, the present investigation would be useful to breeders for developing distant hybrids among wild relatives and cultivated *brassica*s.

Key words: Cultivated *Brassica*s, wild relatives, pre- and post fertilization barriers, wide hybridization

Introduction

The genus *Brassica* occupies the second most important position amongst edible oilseeds of India, next only to groundnut. The productivity of oilseed *brassica*s in India is much lower as compare to western countries. This low yield to a large extent is due to the susceptibility of cultivars to many biotic and abiotic stresses. The genus *Brassica* includes a large number of wild relatives with desirable attributes particularly for resistance to diseases. One of the breeding strategies of *Brassica* improvement has been to introgress the desirable genes from the wild species to the cultivars through wide hybridization technique. Several of the wild and weedy relatives of crop *brassica*s form a good source of genes imparting resistance/tolerance to a number of biotic and abiotic stresses. To tap the desirable genes of the wild species, wide hybridization is one of the best approaches used by several workers (Kalloo and Chaudhury, 1992; Shivanna, 2003; Warwick et al., 2001). However, presence of strong pre- and post-fertilization barriers between the cultivars and wild species is the major limitation for such breeding programmes. Present knowledge of reproductive behaviour of the wild species is very limited. For the development of wide hybrids, it is very necessary to study the presence of crossability barriers at morphological, cytological and molecular levels. The information on crossability barriers will be very useful to the plant breeders for developing wide hybrids, genetic enhancement and pre-breeding work for utilization in crop improvement programmes. Present investigations were undertaken with the primary objective of documentation of pollen germination and pollen tube growth in wide crosses between all the six cultivated species of *Brassica* and a number of wild species.

Materials and methods

Different wild relatives (25) and cultivated species (6) of *Brassica*, were used in the present investigation. These were *Brassica maurorum* Dur.,*B. fruticulosa* Cyr., *B. spinescens* Pomel.,*B. cossoneana* Boiss. & Reut.,*B. gravinae* Ten., *Diplotaxis muralis* (L.) DC., *D. siifolia* G. Kunze., *D. siettiana* Maire, *D. berthautii* Br. Bl. and Maire, *D. assurgens* (Del.) Gren., *D. tenuisiliqua* Del., *D. catholica* (L.)DC., *D. tenuifolia* (L.) DC., *D. erucoides* (L.) DC., *Erucastrum gallicum* O.E Schulz., *E. cardaminoides* (A. Rich) O.E. Schulz., *E. abyssinicum* (A. Rich) O.E. Schulz., *Enarthrocarpus lyratus* (Forsk.) DC, *Coincya hispida* (Cav.) Gz. Campo, *Coincya pseuderucastrum* (Brot.)Gz. Campo, *Hutera rupestris* Porta, *Moricandia arvensis* (L.) DC, *Moricandia moricandioides* (Boiss) Heywood, *Rhynchosinapis longirostra* (Boiss)Gz. Campo and *Sinapis* *pubescens* (L.)

To study details of pollen germination and pollen tube growth following wide crosses, several wild and alien species of *Brassica* maintained in pots at National Research Center for Plant Biotechnology (NRCPB) New Delhi were utilized. The plants of cultivated species were grown in the garden of the Department of Botany, University of Delhi. In order to obtain synchronous flowering in the wild and cultivated species and to extend the flowering for as long a period as possible, three consecutive sowings were done at 2-3 week-intervals starting from mid-September to mid-November. Some of the species flowered early during October and others during December and January.

In pollination studies aimed at crossability barriers, interspecific and intergeneric crosses were carried out between wild species and cultivated species. In each inflorescence, 2-4 flower buds, which would open the next day, were emasculated. While emasculating, the sepals and petals were also removed. All the remaining floral buds as well as opened flowers and young fruits were removed from the inflorescence. The emasculated inflorescence was bagged. Anthers from freshly opened flowers were collected from as many flowers as possible and kept in petri-plates covered with muslin cloth under field conditions, for 1-2 h until anthers dehisced. During foggy or cloudy days, however excised anthers were kept in petri-plates under a table lamp in the laboratory until anthers dehisced. On the day of anthesis, the bags from the inflorescences were opened and emasculated flower buds were pollinated by gently rubbing dehisced anthers of the male parent on the stigma; the inflorescences were re-bagged. Flowers were used for the study of pollen germination and pollen tube growth. Pollen germination and pollen tube growth in pollinated pistils were studied using aniline blue fluorescence technique (Shivanna and Rangaswamy, 1992). Pollinated pistils, 2 days after pollination (DAP) were fixed in FAA (formaldehyde: acetic acid: ethanol, 5ml: 5ml: 90ml v/v) for 24 h at laboratory temperature; they were then cleared in 4N NaOH for 18 h at room temperature, washed in water and mounted in decolourized aniline blue (Gurr.) (0.005%) prepared in 0.05M Na_2HPO_4 (pH 8.2). Applying gentle pressure over the cover slip, spread the tissues of the pistils. Whenever observations were made at a later date, a drop of glycerine was also added to the stain before mounting. The preparations were observed under a fluorescence microscope (Nikon) using UV 2A filter combination (excitation filter 330-380 nm, dichromatic mirror 400 nm and barrier filter 420 nm). Pollen germination was scored as pollen grains failed to germinate as nil; $\leqslant$ 10 pollen grains/stigma germinated as poor;11-50 pollen grains/stigma germinated as moderate and > 50 pollen grains/stigma germinated as profuse. Pollen tubes present in the stigma following their entry and those observed in the style/ovary were scored as present (+) or absent (-).

Results

To understand the extent of reproductive isolation between the cultivated species of *Brassica* and some of their wild relatives, details of pollen germination and pollen tube growth were studied following interspecific and intergeneric pollinations. All the six cultivated species of *Brassica* viz., *B. rapa*, *B. oleracea*, *B. nigra, B. juncea*, *B. napus* and *B. carinata,* and 12-20 wild relatives of *Brassica* depending on the availability of flowers, were used in these studies.

Most of the wild species were slow growing. Flowering period also varied considerably in wild species. Many of the wild species such as *Brassica fruticulosa, B. gravinae, B. maurorum, Diplotaxis catholica, D. erucoides, D. siettiana, D. tenuifolia, Erucastrum cardiminoides* and *E. gallicum* flowered early during November and December. Some others such as *Brassica cossoneana, B. spinescens, Diplotaxis berthautii, D. muralis, D. tenuisiliqua, Moricandia arvensis, M. moricandioides and Enarthrocarpus lyratus* flowered late during January-February. Further, the number of flowers available in most of the wild species was limited. Therefore wild species could be used only as male parents in the crosses.

Amongst the cultivated species, *B. nigra, B. juncea* and *B. rapa* were early to flower (November and December) whereas *B. carinata, B. napus and B. oleracea* were late in flowering (January-February). About 10 pistils in each cross combination were pollinated with freshly dehisced pollen of the wild species. The pistils were excised and fixed 24 h after pollination in FAA. Pollen germination and pollen tube growth studies were carried out as per the procedure presented under Materials and Methods, using decolourized aniline-blue fluorescence technique. The results are summarized under each cultivated species.

B. nigra×Wild species

Flowers of *B. nigra* (cv. SRB-98) were crossed with pollen of 17 wild species. Ten species showed moderate to profuse pollen germination, six species showed poor germination and one species *D. tenuifolia* showed no pollen germination. Many species which showed profuse pollen germination, also showed pollen tube entry into style and ovary. Out of 17 crosses, nine crosses showed the presence of pre-fertilization barriers. Eight crosses which showed occurrence of post-fertilization barriers, attempts can be made to develop wide hybrids using embryo rescue techniques. These crosses are: *B. nigra*×*B. maurorum*, *B. nigra*×*B. fruticulosa*, *B. nigra*×*B. cossoneana*, *B. nigra*×*Diplotaxis siettiana*, *B. nigra*×*D. tenuisiliqua*, *B. nigra*×*Erucastrum gallicum*, *B. nigra*×*E. cardiminoides*, and *B. nigra*×*Moricandia arvensis*.

B. campestris×Wild species

Flowers of *B. campestris* (var. brown sarson) were crossed with 12 wild species. In three crosses, there was no pollen germination and non of the cross showed profuse pollen germination. Moderate pollen germination was observed in five crosses and only in three of these crosses, pollen tubes could reach in the ovary. In four crosses, pollen germination was poor and pollen tube growth was inhibited by the development of callose on the tips of stigmatic papillae. Out of 12 crosses, nine crosses showed the presence of pre-fertilization barriers. Three crosses which showed post-fertilization barriers, wide hybrids can be tried through embryo rescue techniques in these crosses-*B. campestris*×*B. fruticulosa*, *B. campestris*×*B. gravinae* and *B.*

campestris×*Erucastrum cardiminoides*.

B. oleracea×Wild species:

Flowers of *B. oleracae* ssp. *italica* (broccoli) were pollinated with pollen of 17 wild species. In most crosses, pollen germination was poor to moderate and failed to germinate in cross-*B. oleracea*×*D. tenuifolia*. Two crosses showed profuse pollen germination and pollen tube growth was observed in style and ovary. Although pollen germination was poor to moderate in another two crosses, but a few pollen tubes could be seen in ovary. Pre-fertilization barriers were recorded in 13 of the seventeen crosses. Four crosses in which presence of post-fertilization barriers was observed, efforts should be made to develop hybrids in these crosses: *B. oleracea*×*B. maurorum*, *B. oleracea*×*Diplotaxis tenuisiliqua*, *B. oleracea*×*D. catholica*, and *B. oleracea*×*Moricandia arvensis*.

B. carinata ×Wild species:

Flowers of *B. carinata* (EC-223405) were crossed with pollen of 20 wild species. Ten crosses showed moderate to profuse pollen germination and seven of them permitted entry of pollen tube into the ovary. In four crosses pollen did not germinate and in three crosses which showed poor pollen germination, pollen tube growth was inhibited by the development of callose plugs on the stigmatic papillae. Out of twenty crosses studied, 13 crosses showed presence of pre-fertilization barriers and seven crosses showed post-fertilization barriers. Thus, attempts could be made to develop wide hybrids in these crosses-*B. carinata*×*B. fruticulosa*, *B. carinata*×*Diplotaxis muralis*, *B. carinata*×*D. siifolia*, *B. carinata*×*D. assurgens*, *B. carinata*×*D. catholica*, *B. carinata*×*Erucastrum gallicum* and *B. carinata*×*Sinapis pubescens.*

B. juncea×Wild species:

Flowers of *B. juncea* (cv. Pusa Bold) were crossed with pollen of 18 wild species and pollen germination was observed in all crosses. Seven crosses showed moderate to profuse pollen germination but only in three crosses, entry of a few pollen tubes were observed in the ovary. Nine crosses in which pollen germination was poor, growth of pollen tubes were inhibited in the stigma or style. Out of 18 crosses, 15 crosses showed the presence of pre-fertilization barriers and three crosses showed the occurrence of post fertilization barriers. Thus, attempts should be made to develop wide hybrids in these crosses i.e. *B. juncea*×*B. fruticulosa*, *B. juncea*×*Diplotaxis erucoides* and *B. juncea*×*Enarthrocarpus lyratus*.

B. napus×Wild species:

Flowers of *B. napus* (ISN-706) were pollinated with pollen of 16 wild species but there was no pollen germination in two crosses. Four crosses in which pollen germination was moderate, entry of a few pollen tubes were recorded in the stigma. Five crosses in which pollen germination was poor, growth of pollen tubes were inhibited by the development of strong callose plugs on tips of the stigmatic papillae. Two crosses (i.e. *B. napus*×*B. fruticulosa* and *B. napus*×*Diplotaxis erucoides*) in which pollen germination was profuse; pollen tube growth was also recorded in the style and the ovary. Attempts should be made to develop wide hybrids in these two crosses. Thus, out of 16 crosses, 14 crosses showed the presence of pre-fertilization barriers and only two crosses showed occurrence of post-fertilization barriers.

Discussion

In the present study, attempt has been made to document crossability barriers in wide crosses of *brassica*s. In recent years, there has been a greater emphasis on the use of wild species in plant breeding programmes. Wild relatives of the crops are often a valuable source of genes for resistance to a range of biotic and abiotic stresses. Several wild species have been effectively used to transfer genes imparting resistance/tolerance to biotic or abiotic stresses to cultivated crop species (Watson, 1970; Harlan, 1976, 1984; Hawkes, 1977; Kalloo, 1992; Prakash et al 2004; Shivanna and Singh, 2000 and Singh 2005). Many wild and weedy members of *Brassica* coenospecies are a rich source of genes conferring resistance/tolerance to biotic and abiotic stresses (Prakash et al, 2004; Warwick *et al*., 2001). However, wide crosses involving wild and cultivated species through conventional hybridization method generally show strong crossability barriers. The stage at which crossability barriers operate depends on the extent of reproductive isolation of the two parental species. In general, the wider the reproductive isolation, the earlier is the stage at which the barriers operate. Thus distantly related species show pre-fertilization barriers, which prevent either pollen germination or pollen tube growth through the stigma and style. While closely related species generally show post-fertilization barriers, often at later stages of embryo development.

The knowledge of wild species, their reproductive behaviour and their crossability barriers with cultivated species are important in pre-breeding and genetic enhancement of crop species; but there is very little information on these aspects. Sporadic efforts have been made to study crossability barriers in crosses involving crop *brassica*s and their wild and weedy relatives. Harberd (1976) reported the studies on crosses between six cultivated species of *brassica*s and 24 wild species. He listed crossing-series among *Brassica* cytodemes and placed species more successful as female parent at the top of the list and species more successful as male parent at the bottom. He placed wild species such as *Erucastrum abyssinicum* and *Diplotaxis erucoides* more successful as female parents at the top; *D. catholica* and *D. tenuifolia* in the middle and *B. fruticulosa* and *B. cossoneana* amongst more successful as male at the bottom.

In the present study, 25 wild species of *Brassica*, *Coincya*, *Diplotaxis*, *Erucastrum*, *Enarthrocarpus*, *Hutera, Moricandia* and *Sinapis* were used to identify the presence of pre-fertilization barriers with all the six cultivated *Brassica*s. Out of hundred interspecific/intergeneric crosses studied, 73 crosses showed the presence of strong pre-fertilization barriers and 27 crosses showed the presence of post-fertilization barriers; in 49 crosses pre-fertilization barriers were in the form of inhibition of pollen germination or pollen tube entry into stigma, and in 24 crosses due to inhibition of pollen tube growth in the style.

Most of the cultivated *Brassica* species permitted germination of pollen of *B. fruticulosa, B. maurorum, Diplotaxis tenuisiliqua* and *Erucastrum gallicum*. *Brassica fruticulosa* showed profuse pollen germination and pollen tube growth in the stigma and style and entry of pollen tubes into the ovary in crosses involving four cultivated *Brassica* species (i.e. in *B. nigra*×*B. fruticulosa*, *B. carinata*×*B. fruticulosa*, *B. juncea*×*B. fruticulosa*, and *B. napus*×*B. fruticulosa*). Thus it is a good male parent for wide crosses with cultivated species. Pollen grains of *D. berthautii, D. tenuifolia*, *D. muralis*, *D. gravinae*, *Enarthrocarpus lyratus*, and *Moricandia moricandioides* were strongly inhibited on the stigmas of cultivated *Brassica* species. Therefore, these species are not suitable as male parents for developing wide crosses with cultivated *Brassica* species.

On the basis of the results obtained in the present studies, it could be generalized that for the development of successful hybrids with wild species, *B. nigra* and *B. carinata* are likely to be more successful as female parents as compared to *B. rapa, B. oleracea, B. juncea* and *B. napus*. Amongst the wild species *B. fruticulosa, B. maurorum, Diplotaxis catholica, Erucastrum gallicum* and *E. cardiminoides* were likely to be more efficient as male parents for developing wide crosses with cultivated *Brassica* species.

Conclusion

The major factor responsible for low yield is susceptibility of cultivars to a range of biotic and abiotic stresses, causing substantial yield losses. One of the breeding strategies of *Brassica* improvement has been to introgress the desirable genes from the wild species to the cultivars through wide hybridization. However, presence of strong pre- and post-fertilization barriers between the cultivars and wild species is the major limitation for such breeding programmes (Shivanna, 1996). All the wild species used for making crosses with the cultivated *Brassica* species, have been categorized into - those showing pre-fertilization barriers at the level of the stigma or style and those in which pollen tubes entred the ovary. To raise hybrids in crosses in which pollen tubes entred the ovary, application of embryo rescue technique wiould be very effective to overcome crossability barriers. Such information will be very useful for the breeders in selecting suitable species to develop wide hybrids. Following are the main conclusion of these investigations:

When *B. nigra* was crossed with pollen of 17 wild species, eight crosses showed occurrence of post-fertilization barriers. These crosses are: *B. nigra*×*B. maurorum*, *B. nigra*×*B. fruticulosa*, *B. nigra*×*B. cossoneana*, *B. nigra*×*Diplotaxis siettiana*, *B. nigra*×*D. tenuisiliqua*, *B. nigra*×*Erucastrum gallicum*, *B. nigra*×*E. cardiminoides*, and *B. nigra*×*Moricandia arvensis*. Attempts can be made to develop wide hybrids using embryo rescue techniques.

B. rapa pistils when crossed with 12 wild species, three of the crosses *B. rapa*×*B. fruticulosa*, *B. rapa*×*B. gravinae* and *B. rapa*×*Erucastrum cardiminoides*, showed post-fertilization barriers; wide hybrids could be tried in these crosses through embryo rescue techniques.

Crosses of *B. oleracea* with 17 wild species, only four crosses: *B. oleracea*×*B. maurorum*, *B. oleracea*×*Diplotaxis tenuisiliqua*, *B. oleracea*×*D. catholica*, and *B. oleracea*×*Moricandia arvensis*, showed the presence of post-fertilization barriers. Efforts could be made to develop wide hybrids.

Pistils of *B. carinata* crossed with pollen of 20 wild species, seven crosses showed post-fertilization barriers. Thus, attempts could be made to develop wide hybrids in crosses: *B. carinata*×*B. fruticulosa*, *B. carinata*×*Diplotaxis muralis*, *B. carinata*×*D. siifolia*, *B. carinata*×*D. assurgens*, *B. carinata*×*D. catholica*, *B. carinata*×*Erucastrum gallicum* and *B. carinata*×*Sinapis pubescens*.

Crosses of *B. juncea* with pollen of 18 wild species, three crosses showed the occurrence of post fertilization barriers. Thus, attempts could be made to develop wide hybrids in these three crosses i.e. *B. juncea*×*B. fruticulosa*, *B. juncea*×*Diplotaxis erucoides* and *B. juncea*×*Enarthrocarpus lyratus*.

Pollinations of *B. napus* with 16 wild species, only two crosses showed occurrence of post-fertilization barriers. Attempts could be made to develop wide hybrids in these two crosses: *B. napus*×*B. fruticulosa* and *B. napus*×*Diplotaxis erucoides*.

Present knowledge on the details of the crossability barriers between *Brassica* cultivars and the wild species is very limited. Documentation of pollen germination and pollen tube growth in wide crosses between all the six cultivated species of *Brassica* and a number of wild species will be very useful for the development of wide hybrids as the knowledge of crossability barriers at morphological, cytological and molecular levels is essential. The information would also help in identifying the techniques required to overcome pre- and/or post-fertilization barriers in various crosses attempted. The information generated through crossability barriers studies, will be useful to the plant breeders for pre-breeding work, developing new wide hybrids, to incorporate genes for biotic and abiotic stresses from wild relatives.

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