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Genetics of the Fertility Restorer *(Rf)* Gene which Restores Fertility in Different Cytoplasmic Male Sterility Systems (*mori, eru and ber*) of *Brassica juncea*

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A B S T R A C T

Hybrid breeding in *Brassica juncea* is suggested as the best strategy to boost rape seed mustard production in India. Diversified male sterile and restorer lines are required for a strong sustainable hybrid breeding programme. Knowledge about the inheritance of male sterile and restorer genes are essential for this. We studied the genetics of fertility restorer gene which can restore the fertility in three different male sterile systems(*mori, eru* and *ber*)in *B.juncea* using nine different BC_1F_1 populations. Monogenic and gametophytic mode of inheritance was observed for all the populations except for the back cross population derived from Pusa Agrani (*ber*). It was observed that few minor genes influence the pollen fertility in all the back cross populations.

Introduction

Indian mustard, *Brassica juncea*, is a major oilseed component in Indian oilseed sector. It contributes more than 80% to the total rape seed mustard production, which is the second most important oilseed crop in India after soybean. *B. juncea* has enormous cultivation potential in semi-arid areas as it is known to be more drought tolerant and shattering resistant than *B. napus* and *B. rapa* (Vinu *et al.,* 2013).Increasing the productivity of this crop can lead to a major breakthrough in the rape seed – mustard production of the country. Indian mustard is a predominantly self-fertilized crop with 5 to 15 per cent cross fertilization (Abraham, 1994); therefore, cultivar improvement has been mostly undertaken by breeding methodologies defined for self-fertilized crops. Significant level of heterosis has been reported in *B. juncea.* In India, different studies reported heterosis over better parent for yield traits to the extent of 136.75 % (Singh *et al.,* 2015),

67.71% (Yadava *et al.,* 2012), 44.80% (Vaghela *et al.,* 2011) and 80.97% (Verma *et al.,* 2011). With highly effective means of hybrid seed production, such as cytoplasmic genetic male sterility and fertility restoration (CMS-FR) system, available level of heterosis in *Brassica* can be exploited commercially. Presently by considering the amenability of Indian mustard for heterosis breeding, hybrid breeding is suggested as a strategy to break the yield barrier in this crop.

The cytoplasmic genetic male sterility and fertility restoration (CMS-FR) system is an efficient pollination control method in hybrid seed production. Cytoplasmic male sterility, leadsto the production of non-functional pollen grains, results from an incompatible nuclear – cytoplasmic (mitochondrial) gene interaction. This maternally inherited male sterility can be restored in the F_1 hybrids by an appropriate fertility restorer gene (Eckardt *et al.,* 2006). These fertility restorer genes may be available in nature or may be introgressed from the wild species from which the CMS was developed. Cytoplasmic genetic male sterility (CGMS) systems comprise male sterile (A) line, maintainer (B) line and restorer (R) line and have been successfully utilized in many crops such as maize, pearl millet, sorghum, rice etc. to produce commercial hybrids.

Large numbers of genetically different CMS-FR systems have been developed in *Brassica juncea* through intergeneric or interspecific hybridization with related wild species. Among these*Raphanus sativus (ogu)* and *Moricandia arvensis (mori)* were used for development of commercial Indian mustard hybrids. Among the different sterile cytoplasms, *Moricandia arvensis* (*mori*) and *Diplotaxis erucoides* (*eru*) cytoplasms are proved to be stable and with almost no adverse effects in *B. juncea* backgrounds (Kaur *et al.,* 2004, Chamola *et al.,* 2013). The *mori* CMS system was developed by Prakash *et al.,* (1998) and subsequently rectified by Kirti *et al.,* (1998). Alloplasmic lines having cytoplasm from *Diplotaxis erucoides*(*eru*)and *Diplotaxis berthautii* (*ber*) were developed by Malik *et al.,* (1999) and later improved by Bhat *et al.,* (2006, 2008).

Development of heterotic restorer lines is an important step in hybrid breeding programmes. The knowledge of the genetics of fertility restorer gene(s) will help the transfer of it from one genetic background to another and thus the development of heterotic restorer lines. Bhat *et al.,* (2005, 2006, 2008) reported that the fertility restorer (Rf) gene from *Moricandia arvensis* can restore the fertility in *ber* and *eru* cytoplasms and the fertility restoration is under monogenetic and gametophytic control. In gametophytic fertility restoration system only *Rf* gene-carrying pollen grains are functional and F_1 hybrid plants produce 50% fertile and 50% sterile pollens (Bhat *et al.,* 2005). In view of the commercial application of *ber* and *eru* cytoplasms, we analysed the genetic behaviour of the common fertility restorer gene for *mori*, *eru* and *ber* cytoplasms using male sterile lines with different *B. juncea*genetic backgrounds.

Materials and Methods

Five genotypes *viz*., NPJ 93, NPJ 112, Pusa Jagannath, SEJ 8 and Pusa Agrani with three different cytoplasms (*mori*, *eru* and *ber*) were selected to study the inheritance of the common restorer gene for these cytoplasms which was derived from *Moricandia arvensis*. The peculiarities of the selected genotypes are mentioned in Table 1. In effect total nine CMS lines such as NPJ 93 and NPJ 112with *mori*, *eru* and *ber* cytoplasms each, SEJ 8 with *mori* cytoplasm and Pusa Agrani and Pusa Jagannath with *ber* cytoplasm were available for this study. These CMS lines derived from

five genotypes in various cytoplasmic backgrounds were developed through 6-7 repeated back crossing with the respective recurrent parents at Genetics Division, IARI, New Delhi.

These nine selected CMS lines were crossed with the Pusa Bold derived restorer line which has the *Rf* gene introgressed from *Moricandia arvensis*. The resulting nine F_1populations were raised during off season 2012-13 at IARI Regional Station, Wellington, Tamil Nadu. The plants in these nineF_1populations were examined for pollen fertility using 2% acetocarmine staining. The F_1 plants produced using these CMS systems will have 50% fertile and 50% sterile pollen grains. The F_1s with 50% pollen fertility in each cross were selected and backcrossed with the respective maintainer lines to generate the nine different BC_1F_1populations. The crossing programme to generate the back cross populations is summarised in figure I. All the nine back cross populations were raised during 2012-13 rabi season at experimental farm, Genetics Division, IARI, New Delhi. Each population was planted in a four-row plot with a spacing of 30 x10 cm (Row x Plant) and standard package of practices were followed to raise a good crop.

Phenotyping of the Back Cross Populations

Every plant in each backcross population was examined for pollen viability. Fully matured buds from each plant were selected and pollen fertility was tested using 2% acetocarmine stain. Three microscopic fields per plant were considered to ascertain average and unbiased estimate of pollen fertility in every plant. Based on this observation, the backcross population was classified into fertile and sterile plants. Because of the gametophytic fertility restoration the heterozygous fertile plants produced both fertile and sterile pollen grains. Per cent pollen fertility of each fertile plant was calculated as number of fertile pollen grains x 100/ total no. of pollen grains and later averaged. Based on the percent pollen fertility the plants in each backcross population were classified as fertile or sterile(Figure II).

Statistical Analysis

To study the mode of inheritance of *Rf* gene, χ^2testof goodness-of - fit against a possible theoretical segregation ratio was done using the formula:$\chi^2 = \sum(O - E)^2 / E$, where O is the observed frequency and E is the expected frequency (Steel and Torrie, 1980).

Results and Discussion

All the nine BC_1F_1populations generated were segregated into male fertile and male sterile progenies. Under compound microscope, at 10X resolution, the fertile pollens were fully stained, large and round in shape, whereas, the sterile pollens were relatively small and trilobular in shape and remained unstained (Figure II).In F_2generation no segregation was observed for the pollen fertility because of the gametophytic nature of the *Rf* gene, thus the BC_1F_1 generation was selected for the inheritance study. The plants with at least 30% pollen fertility were considered as heterozygous male fertile. The per cent pollen fertility of back cross populations ranged from 30.12% to 68.42%. The highest pollen fertility per cent 68.42 was observed in the back cross generation of NPJ 112 with *mori* cytoplasm. The mean and range of per cent pollen fertility of all the BC_1F_1populations are given in table 2. In all the back cross populations few progenies exhibited more than 50% pollen fertility and it was highest (20 progenies out of 40 fertile progenies) with the back cross population from SEJ 8 (*mori*) x Mori Rf. This back cross population had the highest mean pollen fertility per cent with 51.56% but the range was 31.40 – 65.66%. All other

BC_1F_1populations had less than 10% progenies with above 50% pollen fertility. Similarly four BC_1F_1populations had progenies with less than 30% pollen fertility that is partially fertile/ partially sterile plants. The BC_1F_1population derived from NPJ 112 (*mori*) had three progenies with 16.27%, 15.30% and 20.28% pollen fertility respectively. NPJ 93 (*eru*) derived BC_1F_1population had one progeny with 15.68% pollen fertility and the NPJ 112 (*eru*) derived back cross population had three progenies with less than 20% pollen fertility and six progenies with pollen fertility below 15%. This is the back cross population showing highest number of partial fertile/partial sterile plants (nine progenies out of a total 59 progenies). Because of their very low frequency all the partially fertile (16-30% pollen fertility) and partially sterile (1-15% pollen fertility)plants were considered as sterile in this study.

These variations in fertility among the progenies of a cross indicated the presence of minor genes for pollen fertility restoration and the gametophytic inheritance make it more prominent. In case of gametophytic inheritance the expression of a trait in the gamete is determined by the genetic constitution of the gamete rather than the parent. Here the fertile plant has a genotype of *Rfrf* for the pollen fertility restoration loci and during pollen formation two types of pollen grains are produced. The pollen grain with *Rf* allele, the fertile pollen and the pollen with recessive allele *rf*, the sterile ones. Same kind of segregation pattern will occur for the minor genes also. If a pollen grain with *Rf* allele is receiving recessive alleles for the minor genes then its fertility will be less than 50% and vice versa. There is a possibility for the existence of interaction between these minor loci with major locus of fertility restoration also. Apart from this, environmental conditions such as soil fertility, mycorrhizal infection, temperature, stress conditions etc. can affect the production and performance of pollen grains on plants or flowers (Havens *et al.*, 1995; Lau *et al.*, 1995; Lau and Stephenson 1993 &1994, Schlichting, 1986, Jakobsen and Martens, 1994).

Table.1 Characteristics of the *B. juncea* genotypes selected for inheritance study

S.No.	Genotypes	Pedigree/description
1	Pusa Vijay (NPJ 93)	Synthetic *Brassica juncea* / VSL 5
2	Pusa Mustard 25 (NPJ 112)	Short duration genotype of Indian mustard that mature in about 110 days
3	Pusa Jagannath	Varuna / Synthetic *juncea*
4	SEJ 8	Re-synthesized *Brassica juncea*
5	Pusa Agrani	Early maturing *Brassica juncea* / Synthetic amphidiploid (*Brassica campestris* var. toria/ *Brassica nigra*)

Table.2 Mean and range of pollen fertility per cent of back cross (BC_1F_1) populations studied

BC_1F_1 Population	Mean per cent pollen fertility	Range of per cent pollen fertility
[NPJ 93 (*mori*) x Mori Rf] x NPJ 93	46.70 ± 1.25	31.48 - 64.36
[NPJ 112 (*mori*) x Mori Rf] x NPJ 112	51.35 ± 1.93	30.35 - 68.42
[SEJ 8 (*mori*) x Mori Rf] x SEJ 8	51.56 ± 1.24	31.4 - 65.66
[NPJ 93 (*eru*) x Eru Rf] x NPJ 93	43.66 ± 0.99	30.61 - 57.51
[NPJ 112 (*eru*) x Eru Rf] x NPJ 112	41.95 ± 2.03	30.98 - 62.67
[NPJ 93 (*ber*) x Ber Rf] x NPJ 93	47.13 ± 1.11	31.08 - 60.17
[NPJ 112 (*ber*) x Ber Rf] x NPJ 112	47.07 ± 0.89	31.45 - 68.19
[Pusa Jagannath (*ber*) x Ber Rf] x Pusa Jagannath	42.31 ± 0.89	32.06 - 53.31
[Pusa Agrani (*ber*) x Ber Rf] x Pusa Agrani	42.34 ± 1.39	30.12 - 55.26

Table.3 Segregation pattern for pollen fertility restoration in BC_1F_1 progenies

BC1F1 Population	No. of plants		Expected ratio (mf: ms)	$\chi 2$ value	P value
	mf	ms			
[NPJ 93 (*mori*) x Mori Rf] x NPJ 93	50	66	1:1	2.21	0.14
[NPJ 112 (*mori*) x Mori Rf] x NPJ 112	38	40	1:1	0.05	0.82
[SEJ 8 (*mori*) x Mori Rf] x SEJ 8	40	31	1:1	1.14	0.29
[NPJ 93 (*eru*) x Eru Rf] x NPJ 93	48	59	1:1	1.13	0.29
[NPJ 112 (*eru*) x Eru Rf] x NPJ 112	25	34	1:1	1.37	0.24
[NPJ 93 (*ber*) x Ber Rf] x NPJ 93	39	30	1:1	1.17	0.28
[NPJ 112 (*ber*) x Ber Rf] x NPJ 112	70	54	1:1	2.06	0.15
[Pusa Jagannath (*ber*) x Ber Rf] x Pusa Jagannath	37	41	1:1	0.21	0.65
[Pusa Agrani (*ber*) x Ber Rf] x Pusa Agrani	28	75	1:1	21.45	0.00

Fig.1 Crossing scheme for the development of BC_1F_1 populations

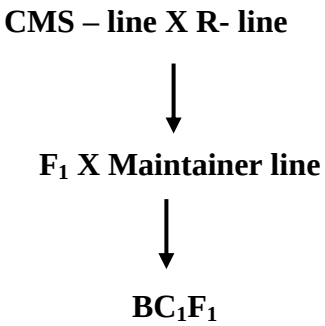
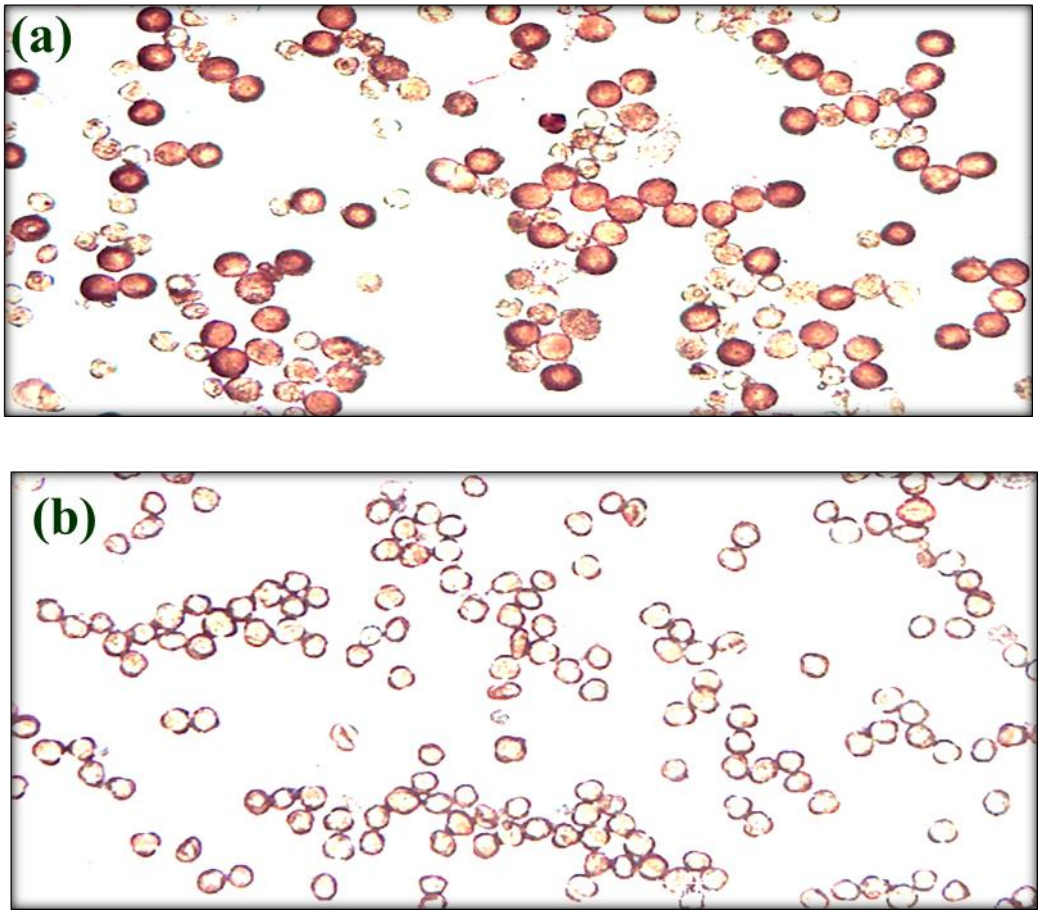


Fig.II Microscopic (10X) image of 2 % acetocarmine stained pollen grains of BC_1F_1 plants derived from cross [NPJ 112 (*mori*) x Mori Rf] x NPJ 112 (a) male fertile plant with large fully stained fertile pollens and small unstained sterile pollens (b) male sterile plant with small unstained sterile pollens



Segregation patterns for pollen fertility of all the nine crosses studied are given in Table 3. The results showed that the fertility restoration is monogenic and gametophytic in nature as reported by Bhat *et al.*, (2005, 2006, 2008) except for the back cross population derived from Pusa Agrani (*ber*). In the back cross generation of Pusa Agrani (ber) x Ber Rf, out of 103 progenies studied only 28 were fertile and the rest 75 were sterile. This segregation pattern, 28 fertile: 75 sterile, is in compliance with 1:3 ratio, the test cross ratio of complimentary gene action (9: 7). In case of complimentary gene action the trait is governed by two major genes and it is expressed when the dominant allele of both the genes are present. Here the 28 fertile progenies may contain the dominant forms of both the genes and the rest of the progenies may have either the dominant form of any one of the gene or recessive forms of both the genes. The pollen fertility of this cross ranged from 30.12% to55.26% with a mean pollen fertility per cent of 42.34%.But for confirmation, extensive study of this cross with more number of progenies testing for pollen fertility status is required.

From this study it is concluded that all the backcross generations studied except the back

cross generation derived from Pusa Agrani (*ber*), the fertility restorer gene for *mori*, *eru* and *ber* cytoplasms has a monogenic and gametophytic inheritance with a major gene and few minor genes influencing the pollen fertility status. The monogenic gametophytic inheritance of the fertility restorer gene derived from *Moricandia arvensis* was first reported by Bhat *et al.,* 2005, 2006, 2008. Even though several CMS-FR systems have been developed in *Brassica juncea* only two systems *ogu* and *mori* were used for the production of commercial hybrids. Chamola *et al.,* (2013) reported that the *erucoides* system has no adverse effect on the agronomic performances of the plants in the *Brassica juncea* background.

In case of *mori*, *eru* and *ber* cytoplasms the per cent pollen fertility in F_1 hybrids was influenced by the genetic backgrounds of the parents but this effect was not consistent for any cytoplasm or genetic background of the parents (Vinu *et al.,* 2017).

This study was conducted as a prior step for the commercial application of *eru* and *ber* cytoplasms. This inheritance study using nine different backcross populations suggested that, *eru* and *ber* male sterile systems along with *Moricandia arvensis* derived *Rf*gene are highly suitable for heterosis breeding in *Brassica juncea*.

The gametophytic inheritance helps to identify the homozygous restorer line by phenotyping itself in the final stage of the restorer line development without going for a test cross. The monogenic and gametophytic nature of restorer gene helps the speedy transfer of *Rf* gene from one background to another and lead to the diversification of restorer lines. The peculiar nature of *Moricandia arvensis* derived *Rf*gene to restore fertility in three different male sterile system (*mori*, *eru* and *ber*) help to broaden the genetic base of male sterile system in *Brassica* hybrid breeding programmes without the search for a new restorer gene.

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