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Effect of cytoplasms and genetic backgrounds of parental lines on fertility restoration in *Brassica juncea* hybrids

VINU V[1], NAVEEN SINGH[2], D K YADAVA[3], SUJATA V[4], NAVINDER SAINI[5], S R BHAT[6] and K V PRABHU[7]

ICAR-Indian Agricultural Research Institute, New Delhi 110 012

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ABSTRACT

Cytoplasm diversification was recognized as one of the most important objective in sustainable exploitation of heterosis. Isonuclear alloplasmic cytoplasmic male sterile (CMS) lines with *Moricandia arvensis (mori), Diplotaxis erucoides (eru), Diplotaxis berthautii (ber)* cytoplasms were developed in the six diverse *Brassica juncea* genetic backgrounds (NPJ 112, NPJ 139, LES 1-27, SEJ 8, EC 308575 and Pusa Agarni). Each of these 18 CMS lines were crossed with six locally developed restorers possessing fertility restorer gene from *Moricandia arvensis* to assess the effect of sterile cytoplasms and nuclear backgrounds of parental lines (A and R) on fertility restoration. Comparison of 108 single cross hybrids, 36 hybrids in each cytoplasm, revealed that the hybrids based on *mori* cytoplasm was significantly different from the ones possessing *ber* and *eru* cytoplasms for mean percent pollen fertility. Further, paired comparisons of the mean per cent pollen fertility of hybrids revealed that the per cent pollen fertility in hybrids was influenced by the genetic backgrounds of parents. However, this effect was not consistent for any cytoplasm or nuclear background of parents. Regression analyses involving percent pollen fertility and seed set in the hybrids, both under open and self pollinated conditions, did not observe any significant association. For diversification of *mori* based CMS-FR systems *eru* and *ber* cytoplasms can be used for sustainable exploitation of heterosis in Indian mustard.

Key words: *Brassica juncea*, Cytoplasm diversification, Cytoplasmic genetic male sterility, Fertility restoration, Hybrids, Nuclear background

Deployment of hybrids is most promising strategy for enhancing productivity in *Brassica juncea* [(L.) Czern and Coss)], the third important oilseed crop of the world. For commercially viable hybrid seed production, an efficient pollination control system is one of the important prerequisite. In many field crops such as maize, rice, sorghum, pearl millet, etc. cytoplasmic genetic male sterility (CGMS) systems comprising male sterile (A) line, maintainer (B) line and restorer (R) line have been successfully utilized to produce commercial hybrids. For exploitation of heterosis, set of diverse and improved A and R lines are required. Genes for CGMS is encoded in mitochondrial genome and the effect of this sterility inducing cytoplasm can be counteracted in hybrid by fertility restorer genes present in the nucleus (Chamola *et al.* 2013).

[1]Ph D Scholar (e mail: vinu.kathu@gmail.com), [2]Senior Scientist (e mail: n.singhk@rediffmail.com), [3]Principal Scientist (e mail: dkygenet@gmail.com), Division of Genetics, [4](e mail: sujatavasudev@gmail.com), [5]Senior Scientist (e mail: navin12@gmail.com), [7]Joint Director Research (e mail: kvinodprabhu@rediffmail.com), [6]Principal Scientist (e mail: srbhat22@rediffmail.com), National Research Centre for Plant Biotechnology, Pusa, New Delhi 110 012.

Genetic as well as cytoplasmic diversifications of parental lines are required for sustainable heterosis breeding programmes in any crop. A number of cytosterility sources from *Brassica* coenospecies, viz. *Diplotaxix siifolia* (Rao *et al.* 1994), *Raphanus sativus* (Bannerot *et al.* 1974, Kirti *et al.* 1995a), *B. tournefortii* (Banga *et al.* 1995, Rawat and Anand 1979), *B. oxyrrhina (*Prakash and Chopra 1990), *Trychystoma ballii* (Kirti *et al.* 1995b), *Moricandia arvensis* (Prakash *et al.* 1998), *D. sietiana* (Prakash *et al.* 2001), *D. catholica* (Prakash *et al.* 2001), *Enarthrocarpus lyratus* (Banga *et al.* 2003, Janeja *et al.* (2003b), *Erucastrum canariense* (Prakash *et al.* 2001, Banga *et al.* 2003), Synthetic *B. napus* ISN-706 (Sodhi *et al.* 2006), *D. erucoides* (Bhat *et al.* 2006) and *D. berthautii* (Bhat *et al.* 2008) were identified and used for development of CMS lines and restorers. In *Brassica juncea* large numbers of genetically different sterility inducing cytoplasms have been deployed through intergeneric or interspecific hybridization with wild allied species, for example *ogu* from *Raphanus sativus* (Kirti *et al.* 1995)*, tour* from *B. tournefortii* (Banga *et al.* 1995), *mori* from *Moricandia arvensis* (Prakash *et al.* 1998, Kirti *et al.* 1998), *cath* from *Diplotaxis catholica* (Pathania *et al.* 2003), *lyr* from *Enarthrocarpus lyratus* (Banga *et al.* 2003), *eru* from *Diplotaxis erucoides* (Malik *et al.* 1999, Bhat

et al. 2006), *bar* from *Diplotaxis berthautii* (Malik *et al.* 1999, Bhat *et al.* 2008) etc. Out of these *Raphanus sativus (ogu)* and *Moricandia arvensis (mori)* were widely used for development of commercial Indian mustard hybrids. Among the different sterile cytoplasms rectified *mori* cytoplasm have proved to be stable with almost no adverse effects in *Brassica juncea* nuclear background (Kaur *et al.* 2004) and has been utilized for development of commercial hybrids. On the other hand, commercial application of *D. erucoides* (*eru*) and *D. berthautii* (*ber*) is yet to be demonstrated (Chamola *et al.* 2013).

A major reason for slow progress in heterosis breeding is the absence of fertility-restoring nuclear genes for these synthetic alloplasmic lines in natural populations. Such gene(s) need to be introgressed from the cytoplasm donor species, which is a time and resource consuming tedious job. Common fertility restorer (*Rf*) gene from *mori*, restores fertility in *mori*, *eru* and *ber* cytoplasms (Bhat *et al.* 2005, 2006, 2008) presenting a great opportunity to diversify cytosterility sources without the need of searching for an appropriate restorer gene. The fertility restoration in these three cytoplasms is gametophytic in nature where only *Rf* gene-carrying pollen is functional and F_1 hybrid plants are expected to produce 50% fertile and 50% sterile pollens (Bhat *et al.* 2005).

For diversification of CMS-FR systems it is imperative to study the stability of fertility restoration in hybrids developed from parental lines having different genetic backgrounds. It is also important to check if any variation exists for fertility restoration in isonuclear lines, possessing different cytosterility sources (*mori*, *eru* and *ber*), by a common restorer gene in different nuclear backgrounds. Working out the relationship between per cent pollen fertility and per cent seed set in hybrid plants would further help in providing direction for utilization of restorers having variable pollen fertility in different nucleo-cytoplasmic combinations. To address these issues, alloplasmic CMS lines in six common nuclear backgrounds were developed by continuous backcrossing for seven generations at Indian Agricultural Research Institute, New Delhi. With the availability of alloplasmic isonuclear CMS lines and genetically diverse restorers having *Rf* gene from a common source present study was formulated to (i) examine the effect of genetic background of A and R lines on fertility restoration in hybrids based on *mori*, *eru* and *ber* CMS systems, (ii) investigate the interaction of common fertility restorer gene with three different sterile cytoplasms for restoring the fertility in hybrids and (iii) examine the relationship between pollen fertility and seed set in the hybrids.

MATERIALS AND METHODS

Alloplasmic isonuclear CMS lines in six nuclear, lines viz. NPJ 112, NPJ 139, LES 1-27, SEJ 8, EC 308575 and Pusa Agarni and three cytoplasmic (*mori, eru* and *ber*) backgrounds were developed by six generations of backcrossing to the respective B-lines as given below in Fig 1. The above mentioned nuclear background (maintainers) possesses sufficient genetic diversity among them.

Six above mentioned nuclear backgrounds in each of three sterility inducing cytoplasms (*mori*, *eru* and *ber*) resulted in a set of 18 CMS lines and their respective six common maintainers. To generate single cross hybrids each of these CMS lines were then crossed with locally developed six diverse R lines during 2014 off-season at IARI Regional Station, Wellington, The Nilgiri's, Tamil Nadu, India. The resulting 108 single cross isonuclear and alloplasmic

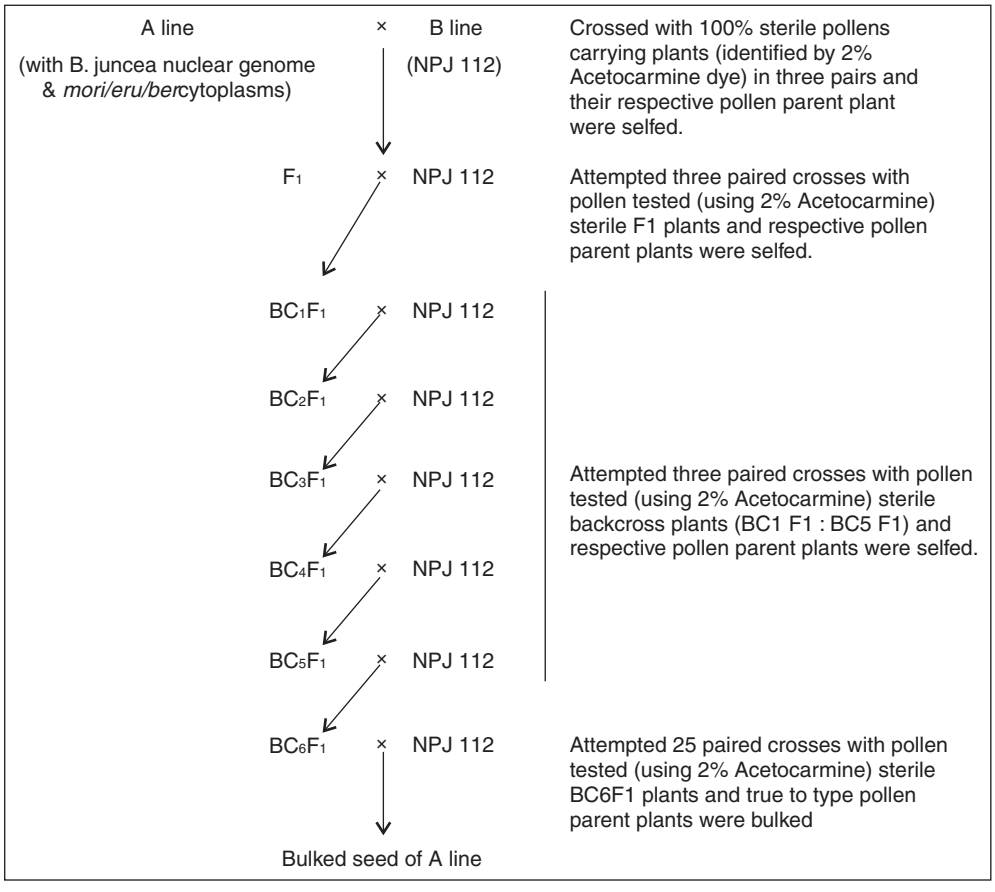

Fig 1 General scheme for development of alloplasmic isonuclear CMS lines carrying *mori, eru and ber* cytoplasms

hybrids along with their respective R-lines were raised during 2014-15 main season at experimental farm, IARI, New Delhi. Standard package of practices were followed to raise a healthy crop.

Fully matured buds from each plant were selected and pollen viability was tested by 2% acetocarmine staining. Under compound microscope, at 10X resolution, the fertile pollens were fully stained, large and round in shape, whereas, the sterile pollens were relatively smaller in size, divided into three lobes and remain unstained. Three microscopic fields per plant were considered to ascertain average and unbiased estimate of pollen fertility in every plants. Per cent pollen fertility was calculated for individual plant as number of fertile pollen grains × 100/ total no. of pollen grains and later averaged. Female fertility was calculated as average seed set per siliqua by taking five siliquae from main shoot of the hybrid plants. Three plants from each hybrid were examined for average seed set under open and self pollinated condition.

Each hybrid was sown in a two row plot of three meter length. Three random plants from each hybrid constituted one replication for examining pollen fertility. Data were recorded in three replications on each hybrid. Factorial RBD analysis was done for the pollen fertility data by using the CPCS1 software to analyze fertility restoration in each cytoplasm and to identify the effect of genetic backgrounds on fertility restoration. Regression analysis was done for per cent pollen fertility and average seed set per siliqua, both under open pollinated and selfed condition, to work out relationship between them.

Table 1 Analysis of variance for per cent pollen fertility on 108 hybrids possessing sterile cytoplasm

Source of variation	DF	Sum of squares	Mean squares	F-calculated
Replication	2	-0.15		
Cytoplasm (C)	2	54.70	27.35	36.64**
Male sterile lines (A)	5	818.01	163.60	219.17**
C × A	10	3177.62	317.76	425.69**
R-lines (R)	5	3773.47	754.69	1011.03**
C × R	10	1460.67	146.07	195.68**
A × R	25	3557.88	142.32	190.65**
C × A × R	50	5319.56	106.39	142.53**
Error	214	159.74	0.75	
Total	323	18321.49		

RESULTS AND DISCUSSION

The analysis of variance indicated significant variation for per cent pollen fertility among hybrids. Influence of cytoplasm, nuclear background of CMS and restorer lines on this trait was also evident (Table 1). The per cent pollen fertility of 108 A × R hybrids ranged from 16.09% to 55.03% with a mean of 42.35%. These hybrids were significantly different from each other for per cent pollen fertility; however, it does not follow any pattern.

Effect of cytoplasm and nuclear background of CMS lines on pollen fertility

Comparison of the mean per cent pollen fertility of hybrids based on CMS lines derived from different cytoplasms revealed that *mori* cytoplasm is statistically significantly different from the *eru* and *ber* cytoplasms (Table 2). The mean pollen fertility in hybrids possessing *mori* cytoplasm was 41.77% while it was 42.64% in hybrids possessing both *eru* and *ber* cytoplasms, a positive change of 0.88% in new cytoplasms. Nuclear background of A- line also influence the pollen fertility in the hybrids and this effect is more prominent than that of cytoplasm as reflected from the range/dispersal (44.60 to 40.58% vs 42.64 to 41.77%) of the trait. This shows that the common fertility restorer gene is interacting differently with different cytoplasm and nuclear background of A-line. The fertility restorer gene was derived from *Moricandia arvensis* genome, however, it imparts better pollen fertility in hybrids possessing *eru* and *ber* cytoplasms indicating better harmony between the nuclear and new cytoplasmic sources. This finding establishes that three cytosterility sources, taken in this study, can be pollinated with restorers having nuclear gene from *Moricandia arvensis* for hybrid seed production and ultimately exploitation of heterosis in Indian mustard. Influence of genetic background of A-lines, carrying A1 cytoplasm, on fertility restoration of hybrids was also

Table 2 Effect of cytoplasm and nuclear background of CMS lines on mean per cent pollen fertility in the set of 108 hybrids (A×R)

Cytoplasm source (C)	Nuclear background of CMS line (A)						
	NPJ112	NPJ139	LES1-27	SEJ 8	EC308575	Pusa Agarni	Mean
Moricandia arvensis	44.61	40.82	43.77	37.22	41.12	43.06	41.77
Diplotaxis erucoides	44.90	46.95	42.68	41.32	35.05	44.92	42.64
Diplotaxis berthautii	43.33	39.66	47.34	44.67	45.56	35.30	42.64
Mean	44.28	42.47	44.60	41.07	40.58	41.10	
CD P=0.05							
Cytoplasm (C)	0.23						
Nuclear background (A)	0.33						
C × A	0.57						

observed in pearl millet (Gupta *et al.* 2010).

Effect of cytoplasm and nuclear background of R-lines on pollen fertility

Mean per cent pollen fertility in hybrids was significantly influenced by the nuclear backgrounds of restorers (Table 3). Maximum and minimum per cent pollen fertility was reported in hybrids derived from the restorers RFP41 (44.98) and RFP26 (36.14), respectively. Such genetic variation existing for pollen fertility in hybrids, imparted by fertility restorers, reveals that improvement in fertility restorers for per cent pollen fertility can be achieved by hybridization among restorers followed by selection. Such effort may also provide opportunity for assembling other favourable alleles leading to improvement in heterosis. Elkonin *et al.* (1998) also reported differences for pollen fertility in hybrids developed from crossing common CMS lines with different male parents in sorghum.

Effect of nuclear backgrounds of A and R lines on per cent pollen fertility

Mean per cent pollen fertility of 36 crosses, pooled over three cytoplasms, possessed significant variation. Averaged over cytoplasms, the mean per cent pollen fertility varied from 40.58 in EC308575 to 44.60 in LES-1-27 nuclear background of A-line (Table 4). The paired comparisons for mean per cent pollen fertility revealed that significant variation existed among different nuclear backgrounds of CMS lines. Similarly for restorer lines, averaged for cytoplasms across A-lines, the mean per cent pollen fertility varied from 36.14 to 44.98 and significantly different from each other (Table 4). The variation in mean pollen fertility per cent is more prominent in restorer lines as compared to A-lines. This may be corrected through the improvement of restorer lines via hybridization followed by selection. These significant variations indicated that the fertility restoration in F_1 generation is influenced by the genetic background of parents. Similar results were also observed in rice (Govinda and Virmani 1998), *Brassica napus* (Pahwa *et al.* 2004) and pearl millet (Gupta *et al.* 2010).

Association between percent pollen fertility and seed set

All possible paired comparisons showed that the fertility restoration in F_1 hybrids is affected by the nuclear backgrounds of the parents, sterility inducing cytoplasms and interaction between them. It is also important to observe the effect of reported variations in pollen fertility on the female fertility of hybrids. For evaluating the female fertility in all the F_1 hybrids, observations on number of seeds per siliqua were recorded under both open and self

Table 3 Effect of cytoplasm of A-line and nuclear background of R-lines on mean per cent pollen fertility in the set of 108 hybrids

Cytoplasm source (C)	Nuclear background of restorer line (R)						
	RFP2	RFP6	RFP19	RFP26	RFP36	RFP41	Mean
Moricandia arvensis	44.76	44.95	36.33	33.78	43.27	47.50	41.76
Diplotaxis erucoides	40.08	40.80	42.03	38.07	44.28	46.57	42.64
Diplotaxis berthautii	44.33	48.10	39.44	36.56	46.55	40.87	42.64
Mean	44.39	44.62	39.27	36.14	44.70	44.98	42.35
CD P=0.05							
Cytoplasm (C)	0.23						
Nuclear background (R)	0.33						
C × R	0.57						

Table 4 Effect of nuclear backgrounds of A and R lines on mean per cent pollen fertility in the set of 108 hybrids of *Brassica juncea*

CMS lines/ Restorer lines	RFP2	RFP6	RFP19	RFP26	RFP36	RFP41	Mean B
NPJ 112	47.24	48.26	46.58	36.55	40.49	46.56	44.28
NPJ 139	48.41	42.35	35.02	32.08	48.64	48.35	42.48
LES 1-27	44.97	43.03	43.13	43.59	46.30	46.56	44.60
SEJ 8	40.79	45.71	38.89	30.74	43.96	46.31	41.07
EC 308575	41.45	46.23	32.19	32.75	44.87	45.96	40.58
Pusa Agarni	43.47	42.13	39.80	41.09	43.94	36.15	41.10
Mean	44.39	44.62	39.27	36.14	44.70	44.98	
CD P=0.05							
A- line (A)	0.33						
R- lines (R)	0.33						
A × R	0.803						

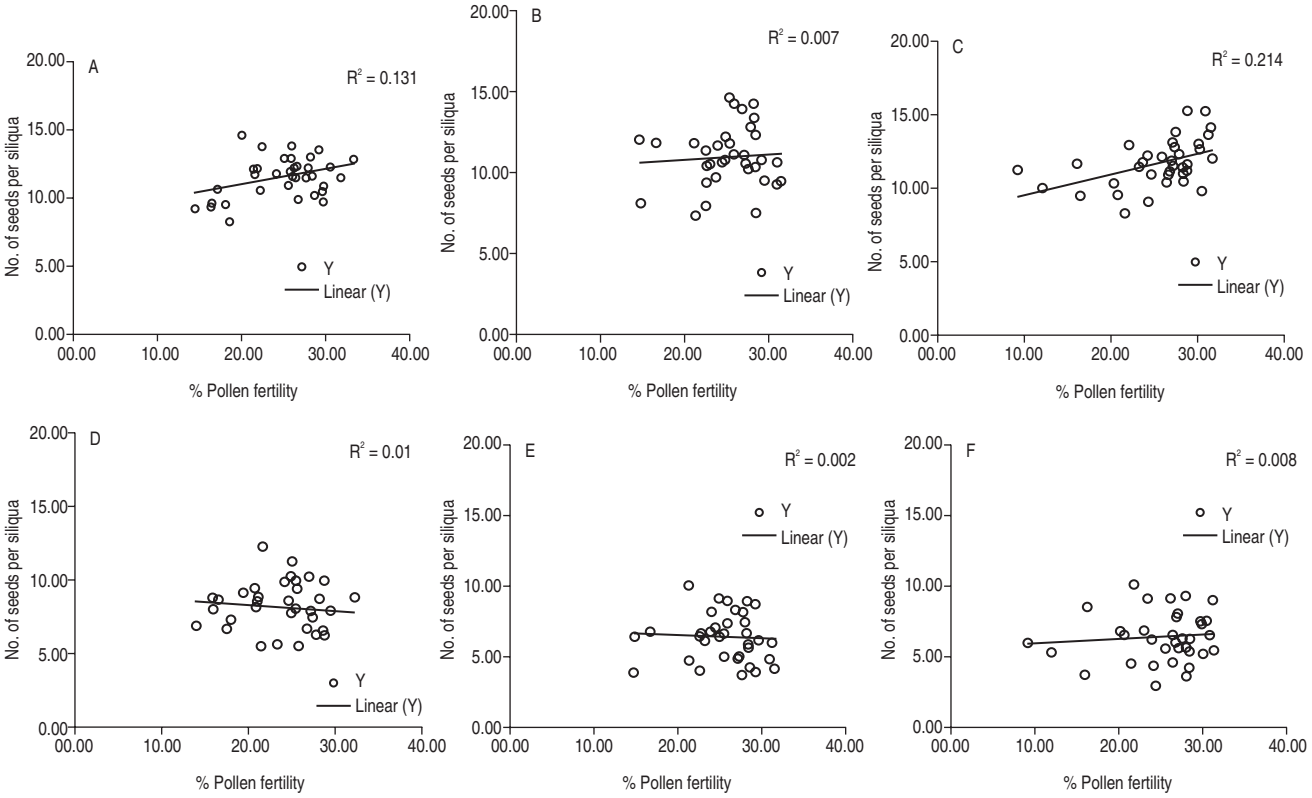

Fig 2 Linear regression curve between per cent pollen fertility and number of seeds per siliqua under A, B, C) open pollinated condition; D, E, F) self pollinated condition in hybrids based on *mori* (A and D), *eru* (B and E) and *ber* (C and F) cytoplasms, respectively

pollinated conditions. The average number of seeds per siliqua varied from 7.47 to 15.20 under open pollinated and 2.93 to 12.4 under self pollinated condition. Even though Indian mustard is a self pollinated species the honeybee population in the field aid considerably in pollination process leading to higher seed set under open pollinated conditions. Consistent lower seed set under self pollinated conditions, as compared to open-pollination, is primarily due to the micro-environmental conditions imposed by selfing bag (Balasubrimanyam 2012) during fertilization and seed development.

The linear regression analysis between per cent pollen fertility and number of seeds per siliqua indicated that there is no significant relationship between them; furthermore, the presence of crossover interactions cannot be ruled out (Fig 2). The R^2 values between these two traits were 0.132, 0.007 and 0.214 under open pollinated condition and 0.010, 0.003 and 0.008 under self pollinated condition for *mori*, *eru* and *ber* cytoplasms, respectively.

The comparative analysis of pollen fertility in 108 hybrids involving three different cytoplasms in six different nuclear backgrounds and six restorer lines revealed significant effect of nuclear backgrounds of parental lines and cytoplasm on per cent pollen fertility, however, these effects were not consistent for any nuclear background or cytoplasm. Furthermore, there is no significant association between the variation in pollen fertility and the seed set in hybrids. It is evident that even with lower per cent pollen fertility, in some hybrids, sufficient number of fertilization events are taking place leading to normal seed set in this prolific pollen producing species. This study demonstrates that the three cytoplasmic male sterility and fertility restoration systems, viz. *mori*, *eru* and *ber* provides great opportunity for genetic and cytoplasmic diversification in parental lines for the development of hybrids and, thus, the sustainable exploitation of heterosis in Indian mustard.

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