https://doi.org/10.17221/74/2026-CJGPB

Deciphering the genetic basis of heterosis for yield and its component traits using *Brassica carinata*-derived *B. juncea* alien introgression lines

Prashant Vasisth[1], *Mohit Sharma*[1], *Omkar Maharudra Limbalkar*[1,2], *Gokulan Dhanasekaran*[1], *Mohan Lal Meena*[1], *Priyanka Jain*[3], *Bhaskar Chandra Sahoo*[1], *Sourav Kumar Moharana*[1], *Mir Asif Iquebal*[3], *Kiran B. Gaikwad*[1], *Anshul Watts*[4], *Naveen Singh*[1]*

[1]*Division of Genetics, Indian Council of Agricultural Research (ICAR)-Indian Agricultural Research Institute, New Delhi, India*
[2]*ICAR-Indian Institute of Agricultural Biotechnology, Ranchi, Jharkhand, India*
[3]*Division of Agricultural Bioinformatics, Indian Council of Agricultural Research -Indian Agricultural Statistics Research Institute, New Delhi, India*
[4]*ICAR-National Institute for Plant Biotechnology, New Delhi, India*

**Corresponding author: ns1.genet@gmail.com*

Citation: Vasisth P., Sharma M., Limbalkar O.M., Dhanasekaran G., Meena M.L., Jain P., Sahoo B.C., Moharana S.K., Iquebal M.A., Gaikwad K.B., Watts A., Singh N. (2026): Deciphering the genetic basis of heterosis for yield and its component traits using *Brassica carinata*-derived *B. juncea* alien introgression lines. Czech J. Genet. Plant Breed., 62: 213–226.

Abstract: The genetic basis of heterosis, which is widely exploited to increase crop yields, remains incompletely understood. Introgression lines created through interspecific hybridisation have been found to be useful for understanding the genetic basis of heterosis for economically important traits. Therefore, we used *B. carinata*-derived *B. juncea* introgression lines (ILs) to study the genetic basis of heterosis and identify genes associated with heterosis in elite hybrids. To assess the contribution of introgressed genomic segments in both homozygous and heterozy-gous states, ILs, introgression line hybrids (ILHs), and the *B. juncea* recipient parents were com-pared. High mid-parent heterosis in the hybrids for yield-related traits highlights the heterotic potential of the ILs. Comparisons among ILs, *B. juncea* parents, and ILHs showed that most ILHs outperformed both parents in seed yield and its component traits. Candidate genes such as *ARR1*, *ARF5*, *TCP22*, and *BKK1* for seed weight; *SASP* and *SBT1.8* for secondary branches and total siliquae; *ATKT2*, *KASIII*, *UGT73B3*, *UGT72E1*, and *SESA1* for oil content; and *FLA3* for siliqua length displayed a heterozygote advantage in ILHs. Therefore, the observed pheno-typic overdominant effect and heterozygote advantage at key candidate genes indicate that overdominance or pseudo-overdominance is a primary genetic basis of yield heterosis in *Brassica*.

Keywords: *Brassica juncea*; genetic hypothesis; heterosis; interspecific hybridization; *B. carinata* derived lines

Supported by the ICAR-IARI in-house Project CRSCIARISIL2021008293 and ICAR funded Consortium Research Project on Hybrid Technology: 12-142D.

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https://doi.org/10.17221/74/2026-CJGPB

The *Brassica* genus is the most economically important genus of the *Brassicaceae* family, which encompasses three diploids, viz., *B. nigra* (*BB*), *B. oleracea* (*CC*), and *B. rapa* (*AA*), and three allopolyploids, viz., *B. juncea* (*AABB*), *B. carinata* (*BBCC*), and *B. napus* (*AACC*) species (Warwick et al. 2006). *B. juncea* (2*n* = 36), also known as Indian mustard, is an amphidiploid species that evolved in nature following hybridisation between diploid species *B. nigra* (2*n* = 16) and *B. rapa* (2*n* = 20). It has been cultivated for edible oil in Eastern Europe and the Indian subcontinent, for vegetables in China, and for condiments in Europe, Canada, and the United States (Gautam et al. 2013; Kumar et al. 2015). In India, *B. juncea* is the predominant oilseed crop, accounting for over 90% of the total acreage under the rapeseed-mustard group of crops (Chauhan et al. 2011).

Rapeseed-mustard ranks second in global oilseed production after soybean, with a contribution of 13.66% in 2023–2024 (Anonymous 2025a). However, it ranks first in oilseed production in India, contributing 13.16 million metric tonnes (33.24%) to the total oilseed (39.59 MMT) production in 2023–2024 (Anonymous 2025b). Despite this, India requires over 25 million metric tonnes of edible oils to achieve self-sufficiency (Jat et al. 2019). To achieve self-sufficiency in edible oil, the productivity of *B. juncea*, a key oilseed crop in India, needs to be enhanced immediately (Sharma et al. 2024). Concerted efforts by various researchers led to the development of high-yielding varieties of Indian mustard; however, the nation is still struggling to meet edible oil demand.

The exploitation of the phenomenon "heterosis" for the development of high-yielding hybrids has emerged as an effective strategy for achieving significant gains in seed yield (Gaikwad et al. 2014; Singh et al. 2015) across various field crops. Despite this, the genetic basis of heterosis remains perplexing. In the rapeseed-mustard group of crops, *B. napus* has been extensively studied to dissect heterotic loci and resolve the genetic mechanism of heterosis. Radoev et al. (2008) and Basunanda et al. (2010) reported the significant epistatic influence in the manifestation of heterosis in *B. napus.* The pseudo-overdominance and synergistic allelic effect on the expression of heterosis for plant weight in Chinese cabbage were also reported (Yue et al. 2022). However, a limited number of studies have been conducted to understand the genetic basis of heterosis in *B. juncea*. Aakanksha et al. (2021) reported that over-dominance is the predominant type of effect in determining the expression of heterotic quantitative trait loci (QTLs) in *B. juncea.* Furthermore, the presence of epistatic interactions in mapping populations, such as F_2, F_{2-3}, recombinant inbred lines, and double haploid lines, makes it challenging to precisely identify individual heterotic loci that contribute to hybrid vigour (Lippman & Zamir 2007). Alien introgression lines (ILs) have been utilised by researchers in different crops to investigate heterosis at the molecular level. Due to their uniform genetic background, ILs are valuable genetic resources used to confine epistasis (Gaikwad et al. 2019; Zhang et al. 2022). To the best of our knowledge, ILs have never been deployed to understand the genetic basis of heterosis in oilseed Brassicas.

B. carinata is endowed with several agronomically desirable traits (Thakur et al. 2019). Interspecific hybridisation between two *B. juncea* cultivars (DRMRIJ 31 and Pusa Mustard 30) and the BC-4 accession of *B. carinata* has led to the development of *B. carinata*-derived *B. juncea* ILs at ICAR-IARI, New Delhi. These ILs have already been characterised, exhibiting a wide range of variability for yield-related traits, and are also expected to express high heterosis for yield and its contributing traits (Limbalkar et al. 2021). The present study was, thus, conducted to (*i*) elucidate the genetic basis of heterosis for seed yield and its contributing traits and (*ii*) identify candidate genes introgressed from *B. carinata* conferring heterosis for yield-related traits in the *B. juncea* background.

MATERIAL AND METHODS

Development of introgression lines. Interspecific hybridisation was employed to introgress genomic segments from *Brassica carinata* accession BC-4 into two elite *Brassica juncea* cultivars, DRMRIJ 31 and Pusa Mustard 30. The cultivars DRMRIJ 31 and Pusa Mustard 30 were developed under the ICAR-All India Coordinated Research Project on Rapeseed and Mustard. DRMRIJ-31 (Giriraj) is a released variety from ICAR – Indian Institute of Rapeseed and Mustard Research, Bharatpur, whereas Pusa Mustard 30 and BC-4 were developed at the ICAR – Indian Agricultural Research Institute, New Delhi. Biparental mating was used in the F_2 generation to break the undesirable linkages and reduce the size of introgressed genomic segments (Limbalkar et al. 2024). In the following filial generations, until homozygosity was achieved, stringent phenotypic selection was employed to select and self-individual plants that morphologically

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resembled *Brassica juncea* (Figure 1). As a result, ILs of *B. juncea* containing genomic segments from *B. carinata* have been developed, demonstrating stable phenotypic and cytological traits. A total of 40 ILs, 27 in DRMRIJ 31 (DRMRIJ31_ILs) and 13 in Pusa Mustard 30 (PM30_ILs) genetic backgrounds, were selected and used in the present study.

Generation of hybrids. Forty ILs were crossed to their respective recipient parents, viz., DRMRIJ 31 and Pusa Mustard 30, to generate a total of 40 introgression line hybrids (ILHs; Figure S1 in Electronic Supplementary Material (ESM)) during the *rabi* 2020–2021 crop season. Emasculation and the hand pollination method, though difficult, was used to generate sufficient seeds of these 40 ILHs to raise a replicated trial.

Phenotypic evaluation and statistical analysis. A set of 40 ILs and 40 ILHs was evaluated along with recipient parents (DRMRIJ 31 and Pusa Mustard 30) in paired rows plots (45 × 15 cm^2) in a randomised complete block design (RCBD) with three replications at ICAR-IARI, New Delhi, during the *rabi* 2021–2022 crop season. Observations were recorded on 17 yield-related traits, viz., plant height (cm), height to the first branch (cm), number of primary and secondary branches per plant, main shoot length (cm), height to first siliqua on main shoot (cm), total siliquae on main shoot, total siliquae per plant, siliqua length (cm), number of seeds per siliqua, days to 50% flowering, 1 000-seed weight (g), seed yield per plant (g), biological yield per plant (g), harvest index, seed yield/ha (t) and percent oil content. The data were recorded on five randomly selected competitive plants in each plot and replication, except for seed yield/ha (t) and days to 50% flowering, which were recorded for the entire plot. The biological yield was recorded at physiological maturity after the harvested plants had dried. Data on plot yield (g) was used to calculate seed yield/ha (t).

An analysis of variance for a RCBD was carried out for all the studied traits using the R software package "Agricole" (R Core Team 2023). The statistical model for RCBD is as follows:

$$Y_{ij} = m + g_i + r_j + e_{ij}$$

where:

Y_{ij} – phenotypic observation of ith genotype in j^{th} replication;

m – general mean;

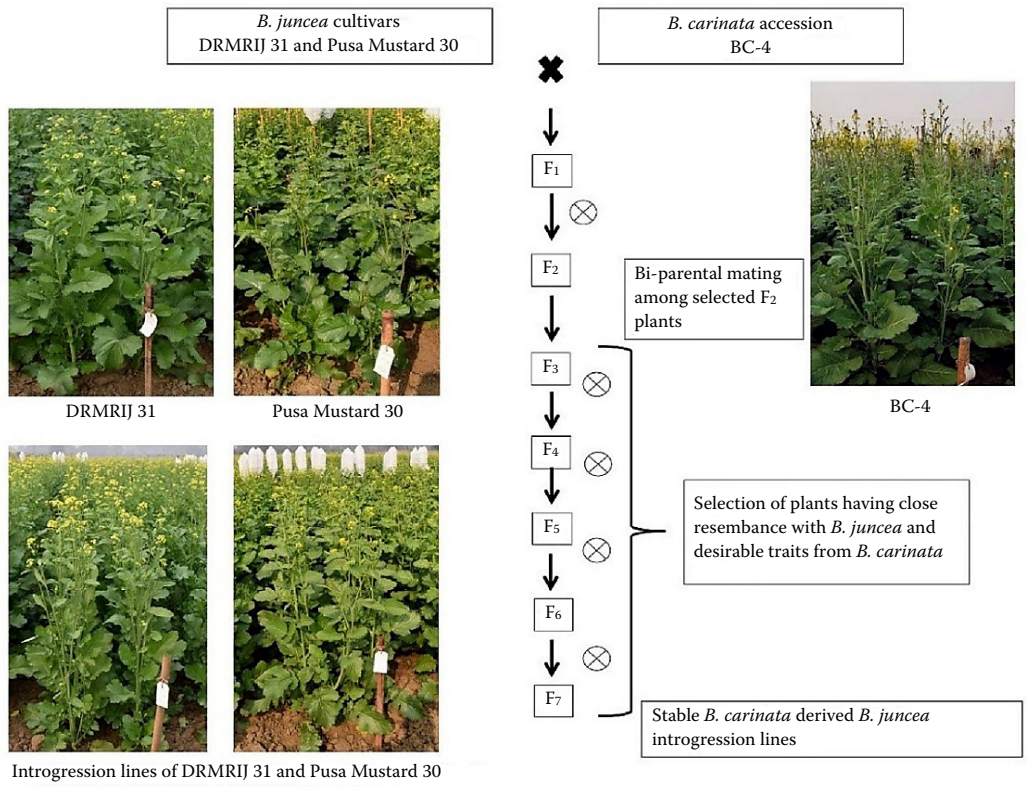

Figure 1. Flow chart depicting the development of *B. carinata*-derived *B. juncea* introgression lines

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g_i – effect of ith genotype;
r_j – effect of jth replication;
e_{ij} – random error.

Mean values of ILs, ILHs, and the recipient parent were used for analysis for variance (ANOVA), followed by Fisher's least significant difference (LSD) test at the 5% level of significance. In addition, correlation analysis was conducted using the "metan" package in R software (Olivoto & Lúcio 2020) to determine the association between the studied traits and seed yield/hectare (tonnes). Mid-parent heterosis (MPH) was calculated using the standard equation:

$$\text{MPH} = \text{heterosis (\%)} = \frac{\overline{F_1} - \overline{MP}}{\overline{MP}} \times 100$$

where:
$\overline{F_1}$ – mean phenotypic value of F_1;
$\overline{MP}$ – mean phenotypic values of parents.

The significance of heterosis was determined at a 5% probability level using the Benjamini–Hochberg false discovery rate (FDR) correction.

Determination of the heterotic effect. Heterotic ILs, their recipient parents, and ILH derived from them were statistically compared at a 5% level of significance for yield and yield-contributing traits. The heterotic effects were determined using the "decision tree" proposed by Semel et al. (2006) in tomato and modified to simplify for this study (Figure S2 in ESM). Heterotic effects were estimated only for ILs whose hybrids showed significant mid-parent heterosis and for which either the IL or the IL-derived hybrid differed significantly from the recipient parent. If significant differences were observed between the mean phenotypic value of any IL and its recipient parent (RP), and the mean phenotypic value of the ILH falls between the phenotypic value of any IL and its RP, then three possible mechanisms of heterosis can be anticipated: (a) if the mean phenotypic value of ILH was significantly different from the RP but not from IL, then the heterotic effect of introgressed segments in ILH was attributed to be dominant; (b) if phenotypic value of ILH, for any trait, was significantly different from IL but not from the RP, effect of introgressed segments was considered to be recessive; and (c) if ILH was either significantly different from both IL and the RP or non-significant from both, this effect was considered to be additive in nature. If the mean phenotypic value of ILH was significantly higher or lower than the values of both parents, the effect of introgressed segments in ILH was considered to be due to overdominance.

Genotyping of introgression lines using the genotype by sequencing approach. Genomic DNA was extracted from all the ILs and their respective parents in this study and outsourced for genotyping by sequencing (GBS). The high-quality filtered sequencing reads were mapped to the *Brassica juncea* cv. reference genome. Varuna (UDSC Var 1.1) (Paritosh et al. 2021) using the Burrows-Wheeler Aligner (BWA) version 0.7.17 (Li & Durbin 2009). Following alignment, duplicate reads were identified, and variant calling (including SNPs and InDels) was performed using the Genome Analysis Toolkit (GATK Ver. 3.6). Polymorphic SNPs were then identified based on their allelic differences relative to the parental lines. A total of 5 157 and 4 893 SNPs in the genetic background of DRMRIJ 31 and Pusa Mustard 30, respectively, were used to genotype ILs using GGT2 software (http://www.dpw.wau.nl/pv/pub/ggt/) (Van Berloo 2008). Donor-derived marker introgressions in these ILs were subjected to candidate gene analysis (1 000 bp upstream and downstream of the SNP) using the annotation of the *Arabidopsis* genome and physical positions of SNPs in the reference *B. juncea* genome (Paritosh et al. 2021).

RESULTS

Evaluation of introgression line hybrids, introgression lines and *B. juncea* parent(s)

The data recorded on 40 ILs, their recipient parents, and 40 hybrids generated from them (ILHs) were subjected to ANOVA. The mean sum of squares due to treatment was significant for all traits across both genetic backgrounds (Table S1 in ESM). Furthermore, there were no significant differences in mean performance between ILs and their respective recipient parents for all traits studied (Table 1). The average performance of ILHs for traits like siliqua length, seeds/siliqua, total siliqua on the main shoot, seed yield/plant, biological yield/plant, 1 000-seed weight, and seed yield/ha (t) in the DRMRIJ 31 genetic background, as well as seed yield/plant, oil content, 1 000-seed weight, and seed yield/ha (t) in the Pusa Mustard 30 genetic background, was significantly better than that of the respective ILs or their recipient parents. Furthermore, ILHs observed either lower or comparable trait values to their respective ILs or parents for some of the agro-morphological traits,

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Table 1. Mean performance of recipient parent, introgression lines, and introgression line hybrids for seed yield and its contributing traits

Traits	DRMRIJ 31 genetic background					Pusa Mustard 30 genetic background				
	DRMRIJ 31	ILs	ILHs	*CV* (%)	H^2 (%)	Pusa Mustard 30	ILs	ILHs	*CV* (%)	H^2 (%)
Plant height (cm)	212.27^{a}	$+ 200.86^{a}$	206.92^{a}	4.75	55.4	213.47^{A}	$+ 208.43^{A}$	215.01^{A}	3.99	41.10
Point to first branch (cm)	54.40^{a}	$+ 52.65^{a}$	57.69^{a}	13.17	63.20	76.33^{A}	$+ 54.25^{B}$	64.41^{A}	9.33	73.30
Primary branches/plant	4.03^{ab}	$+ 4.63^{a}$	4.16^{b}	10.23	52.20	3.73^{A}	$+ 4.96^{A}$	4.68^{A}	9.50	64.40
Secondary branches/plant	9.47^{a}	$+ 11.77^{a}$	11.03^{a}	13.97	47.30	8.13^{B}	$+ 13.36^{A}$	11.04^{B}	11.94	67.20
Main shoot length (cm)	84.13^{a}	83.32^{a}	$+ 85.59^{a}$	6.04	59.70	81.47^{A}	87.19^{A}	$+ 88.27^{A}$	5.12	67.70
Point to first siliqua on main shoot (cm)	8.20^{a}	$+ 9.19^{a}$	8.88^{a}	13.16	64.80	8.07^{A}	7.83^{A}	$+ 7.80^{A}$	10.78	52.30
Siliqua length (cm)	3.87^{ab}	3.67^{b}	$+ 4.02^{a}$	6.44	75.50	3.90^{AB}	3.73^{B}	$+ 4.21^{A}$	7.57	65.60
Seeds/siliqua	$+ 15.63^{ab}$	12.70^{b}	14.18^{a}	6.43	70.10	11.17^{A}	$+ 13.47^{A}$	13.25^{A}	5.67	59.30
Total siliquae on main shoot	$+ 56.27^{ab}$	48.16^{b}	51.03^{a}	7.99	43.80	43.87^{A}	$+ 51.78^{A}$	49.53^{A}	6.64	69.00
Total number of siliquae/plant	291.47^{a}	307.20^{a}	$+ 313.25^{a}$	13.72	38.50	233.80^{A}	$+ 357.12^{A}$	333.34^{A}	12.28	56.10
Seed yield per plant (g)	19.10^{ab}	16.48^{b}	$+ 21.50^{a}$	13.55	66.00	12.77^{AB}	17.97^{B}	$+ 21.45^{A}$	13.27	66.60
Biological yield per plant (g)	74.67^{ab}	76.35^{b}	$+ 87.12^{a}$	13.00	56.60	59.73^{A}	78.21^{A}	$+ 87.58^{A}$	12.25	64.40
Oil content (%)	$+ 37.13^{a}$	35.35^{a}	36.37^{a}	3.19	75.50	31.77^{B}	35.11^{AB}	$+ 35.31^{A}$	2.73	65.80
Days to 50% flowering	50.33^{ab}	49.00^{a}	$+ 46.53^{b}$	3.50	79.80	52.00^{A}	47.85^{AB}	$+ 47.26^{B}$	2.38	68.20
1 000-seed weight (g)	4.07^{ab}	4.52^{b}	$+ 4.88^{a}$	8.06	58.20	$+ 5.97^{AB}$	4.81^{B}	5.52^{A}	8.10	72.60
Harvest index (%)	$+ 25.60^{ab}$	21.76^{b}	24.80^{a}	11.86	39.00	21.30^{A}	23.09A	$+ 24.54^{A}$	9.67	27.80
Seed yield/ha (t)	2.73^{ab}	2.05^{b}	$+ 3.07^{a}$	11.91	82.10	2.03^{B}	2.38^{B}	$+ 2.95^{A}$	11.33	65.00

+Denotes the higher trait mean values among introgression line (IL), Introgression line hybrid (ILH), and respective recipient parent (DRMRIJ 31, Pusa Mustard 30) for most of the traits except plant height, point to first branch, point to first siliqua on main shoot (cm), and days to 50% flowering where lower values are considered desirable; means with same letter not significantly different (LSD, $P = 0.05$) and uppercase (A, B) or lowercase (a, b) letters depict Pusa Mustard 30 and DRMRIJ 31 backgrounds, respectively; *CV* – coefficient of variation; H^2 – broad sense heritability

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such as plant height, point to first branch, primary branches/plant, secondary branches/plant, main shoot length, and point to first siliqua on the main shoot in both the genetic backgrounds (Table 1).

Comparison of introgression line hybrids to the mid-parental value. A high mid-parent heterosis was observed for seed yield/ha (t) in ILHs generated for both genetic backgrounds (Tables S2 and S3 in ESM). In the DRMRIJ 31 genetic background, a higher number of ILHs were observed to have significantly high mid-parent heterosis for siliqua length (10), seed yield/plant (12), biological yield/plant (10), 1 000-seed weight (15), seed yield/ha (t) (21) and days to 50% flowering (20) as compared to other traits (Table S4 in ESM). Furthermore, seed yield/plant, biological yield/plant, 1 000-seed weight, and seed yield/ha (t) expressed > 10% mid-parent heterosis in ILHs developed in the DRMRIJ 31 genetic background. Whereas, ILHs in the Pusa Mustard 30 background, on the other hand, expressed significant mid-parent heterosis for the siliqua length (8), seeds/siliqua (5), seed yield/plant (10), biological yield/plant (7), oil content (10), days to 50% flowering (9) and seed yield (11; t/ha) traits. Six traits, viz., siliqua length, total siliquae/plant, seed yield/plant, biological yield/plant, harvest index, and seed yield/ha (t), observed > 10% mean mid-parent heterosis in hybrids generated in the Pusa Mustard 30 genetic background. These results indicated that high mid-parent heterosis was trait-specific in each genetic background rather than across all agro-morphological traits.

Comparison of introgression line hybrids to the recipient parents and introgression lines. Higher heterosis was expressed by the ILHs over DRMRIJ31_ILs, as compared to DRMRIJ 31 for traits including seed yield/ha (t), harvest index, oil content, seed yield/plant, total siliquae on main stem, siliqua length, and seeds/siliqua. This suggests that the homozygosity of the introgressed segments may result in lower performance of ILs. However, an inverse trend was observed for primary branches/plant, secondary branches/plant, and 1 000-seed weight, where ILHs expressed higher heterosis over DRMRIJ 31 than over their derived ILs (Table S2 in ESM). Except for 1 000-seed weight and siliqua length, the ILHs developed in the genetic background of Pusa Mustard 30 exhibited higher heterosis over Pusa Mustard 30 than the PM30_ILs for nearly all studied traits. This implies that for the majority of traits, PM30_ILs performed better than the recipient parent, Pusa Mustard 30 (Table S3 in ESM).

Estimation of heterotic effects for seed yield and its contributing traits in introgression lines

The seed yield/ha (t) of most DRMRIJ31_ILs was inferior to that of their recipient parent; however, their derived ILHs showed significant superiority over both parents (Figure 2A; Table S5 in ESM). This suggests the presence of heterotic genomic segments with a preponderance of overdominant and negative recessive effects. Traits such as seed yield/ha (t), seed yield/plant, biological yield/plant, siliqua length, and 1 000-seed weight predominantly exhibited overdominant heterotic effects in the majority of heterotic ILs. Additionally, a larger number of hybrids have been observed to express significant mid-parent heterosis for these traits. In contrast, negative overdominant and dominant heterotic effects were observed for days to 50% flowering.

Introgression line hybrids in the Pusa Mustard 30 background exhibited significantly higher seed yields (t/ha) compared to their respective parents (Figure 2B; Table S6 in ESM), confirming the overdominant heterotic effect. Further, traits such as oil content, seed yield/plant (g), biological yield/plant (g), seeds/siliqua, main shoot length, and total siliquae/plant exhibited positive overdominant and dominant heterotic effects, with few exceptions, and thus contributed to higher seed yield in the ILHs. A negative overdominant effect was observed for traits such as days to 50% flowering and point to first siliqua on main shoot, whereas point to first branch exhibited a negative dominant heterotic effect. Overall, traits for which a higher number of ILHs exhibited significant mid-parent heterosis were generally governed by a positive overdominant heterotic effect across the genetic backgrounds. Thus, overdominant heterotic effects was identified as the most prominent genetic mechanism for the expression of heterosis in *B. carinata*-derived *B. juncea* ILs.

Dissecting candidate genes conferring heterosis for yield-related traits

Genotyping of DRMRIJ31_ILs and PM30_ILs revealed the presence of introgressed genomic segments from *B. carinata* into the *B. juncea* backgrounds (Figure 3). These introgressed regions were associated with the expression of trait-specific mid-parent heterosis observed in the ILHs. Notably, ILHs developed in the DRMRIJ 31 genetic background, such as ILH 82, ILH 119, ILH 136, and ILH 156, exhibited significantly higher 1 000-seed weight than their respective parental IL. Candidate gene analysis in these

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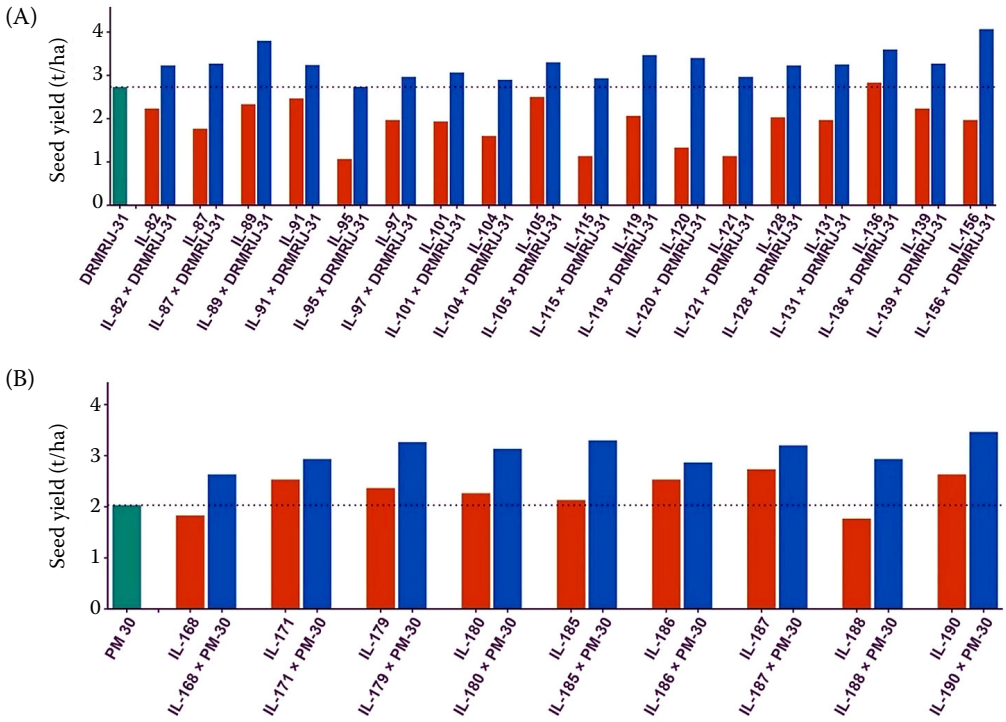

Figure 2. Mean seed yield of introgression lines (ILs), their recipient parents, and introgression line hybrids (ILHs) derived from them in the genetic background of (A) DRMRIJ 31 and (B) Pusa Mustard 30

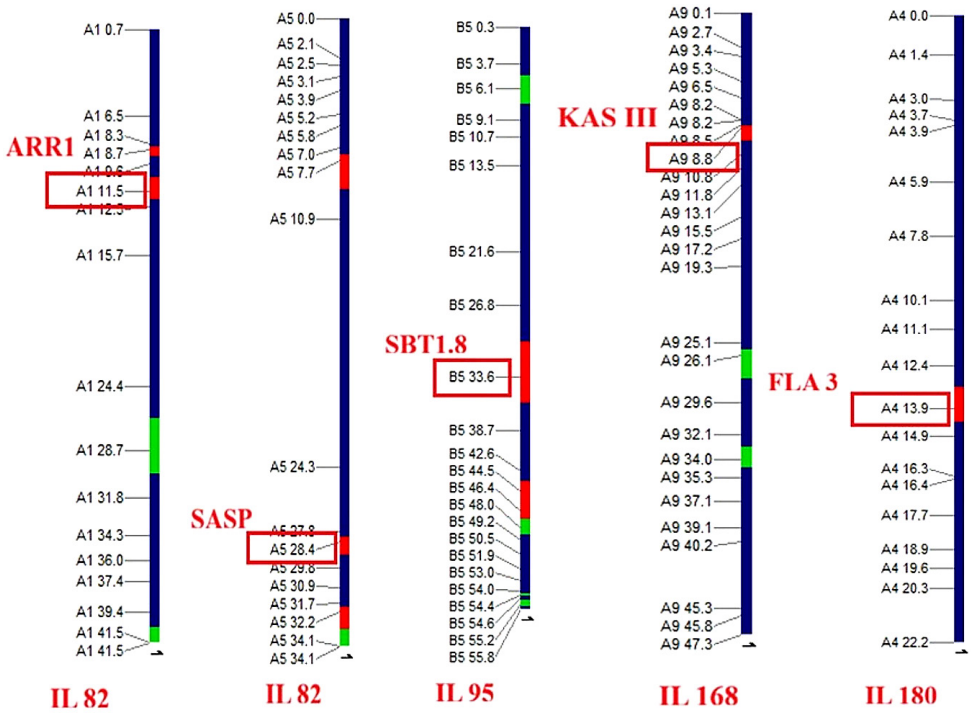

Figure 3. Graphical genotyping of selected introgression lines (ILs) showing candidate genes within introgressed genomic segments

Colour key: blue colour – recipient parent allele; red colour – donor introgression; green colour – missing data; SNP ID format: e.g., A1 11.5 – genome A, chromosome 1, position 11.5 cM; candidate genes are indicated by red boxes

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ILs identified key genes, including *ARR1* (*Response regulator 1*; *AT3G16857*), *ARF5* (*Auxin Response Factor 5*; *AT1G19850*), *TCP22* (*TCP Domain Protein 22*; *AT1G72010*), and *BKK1* (*BRI1- Associated Kinase 7*; *AT2G13790*), all of which are known to regulate different developmental pathways influencing seed weight in various species (Tables S7 in ESM; Figure 3). These results suggest that the superiority of ILHs over ILs in terms of 1 000-seed weight may be primarily due to the overdominance of these genes (Figure 4). Furthermore, ILH 82 and ILH 95 exhibited a significantly higher number of secondary branches and siliquae/plant as compared to their respective parents (Figure 4). These significant effects could be attributed to the heterozygosity of the genes such as *SASP* (*Senescence-associated subtilisin protease*; *AT3G14067*) and *SBT1.8* (*subtilisin-like protease*; *AT2G05920*) in the respective ILHs (Table S7 in ESM).

Most of the ILHs, developed from PM30_ILs and Pusa Mustard 30, exhibited higher oil content than their respective ILs, which can be attributed to the heterozygosity of the introgressed loci. Candidate gene analysis (Table S8 in ESM) revealed the presence of oil biosynthesis-associated genes such as *ATKT2* (*Potassium Transporter 2*; *AT2G40540*), *KASIII* (*3-Ketoacyl-Acyl Carrier Protein Synthase III; AT1G62640*; Figure 3), *UGT73B3* (*UDP-Glucosyl Transferase 73B3*; *AT4G34131*), *UGT72E1* (*UDP-Glucosyl Transferase 72E1*; *AT3G50740*), and *SESA1* (*Seed Storage Albumin 1*; *AT4G27140*). Further, IL 168, IL 181, IL 187, and IL 190 harboured the *FLA3* (*Fasciclin-Like Arabinogalactan Protein 3 Precursor*; *AT2G24450*; Figure 3) gene, and the ILHs derived from these lines showed a significant increase in siliqua length over their respective parental ILs.

Understanding heterosis mechanisms in productive and non-productive traits

An association analysis was performed between seed yield/ha (t) and other agro-morphological traits to determine productive, non-productive, and intermediate productive groups of traits (Figure S3 in ESM). Among lines and hybrids in DRMRIJ 31 background, nine traits viz., seed yield/plant, biological yield/plant, harvest index, siliquae on main shoot, oil content, point to first branch, 1 000-seed weight, plant height and seeds/siliqua were found to be significantly associated with seed yield/ha (t) and, thus, considered productive traits. Secondary branches/plant, primary branches/plant, days to 50% flowering, point to first siliqua on main shoot, and main shoot length, were independent of seed yield/ha (t) and were delineated into the non-productive group. Furthermore, the heterotic effects were compared between productive and non-productive traits, and positive over-dominance and negative recessive effects were found to be major effects in the expression of heterosis for productive traits. Whereas the non-productive group exhibited mostly positive recessive, negative dominant and overdominant heterotic effects (Figure 5A).

Considering the lines and hybrids developed in the Pusa Mustard 30 genetic background, seed yield/plant, biological yield/plant, siliqua length, harvest index and plant height (cm), which had significant correlation with seed yield/ha (t), were grouped

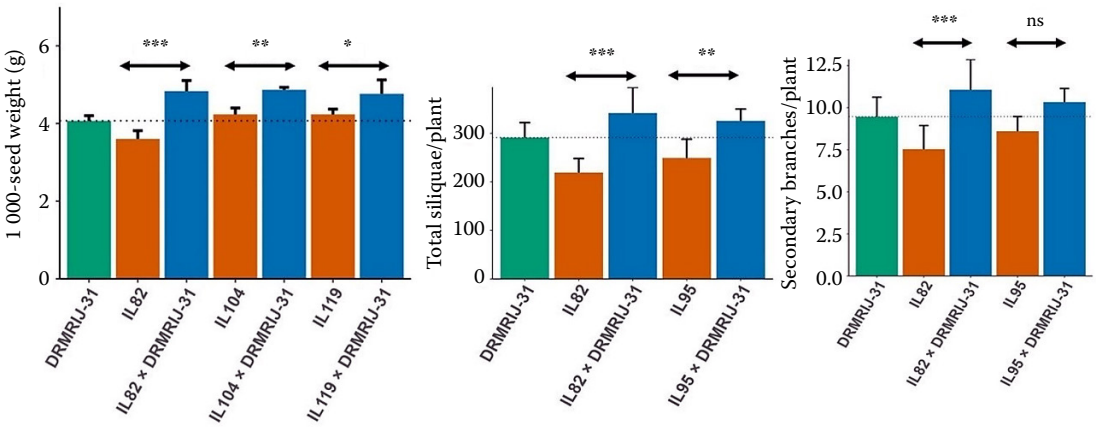

Figure 4. Performance of DRMRIJ 31, introgression lines (ILs) in DRMRIJ 31 genetic background, and introgression line hybrids (ILHs) generated using them for 1 000-seed weight, total number of siliquae, and secondary branches

Error bars represent standard deviation (SD) of three replications; *,**,***significant at P = 0.05, 0.01 and 0.001; ns – non-significant

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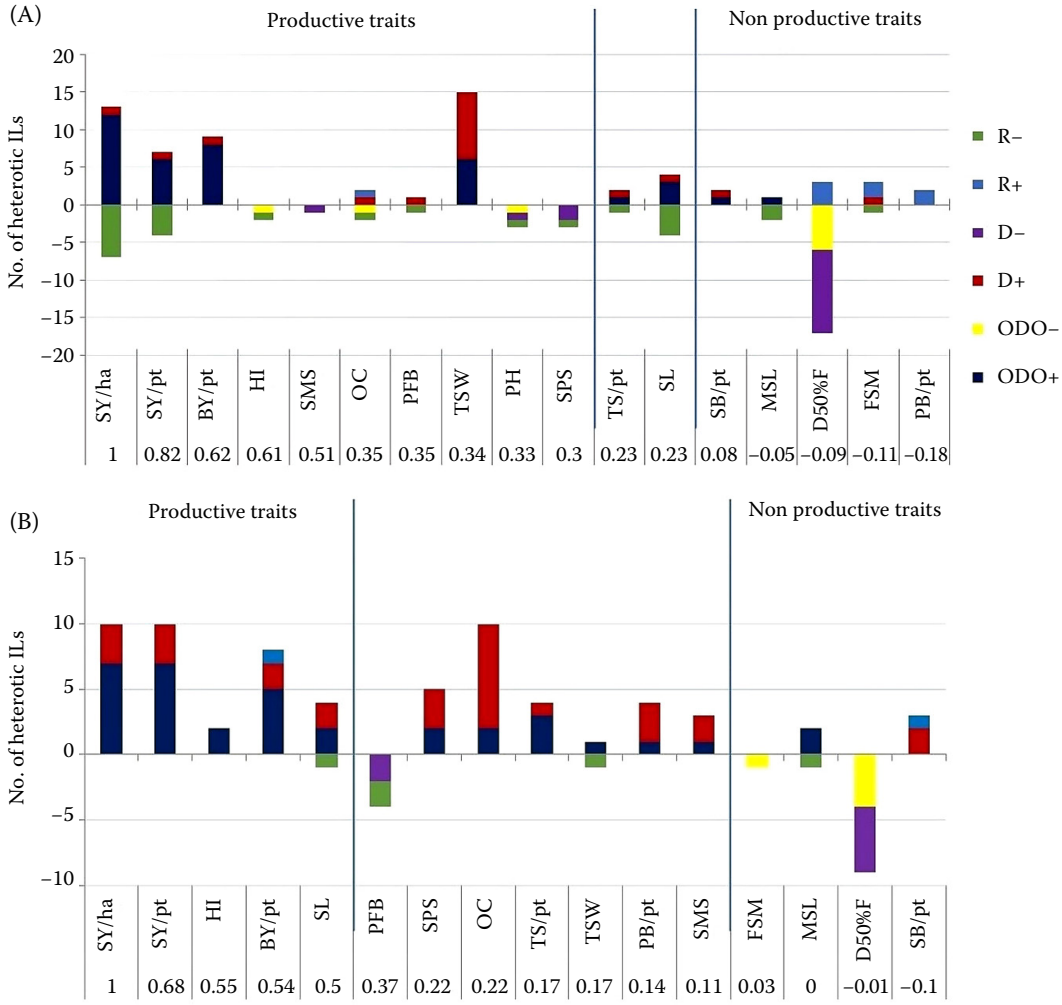

Figure 5. The comparison of the genetic basis of heterosis among productive and non-productive traits in the genetic background of (A) DRMRIJ 31 and (B) Pusa Mustard 30

The number of heterotic introgression lines (ILs) for a specific trait is represented by a vertical bar, the colour of which is based on the mode of inheritance; ILs with increasing heterotic effects are represented by those bars above the zero line, whereas ILs with decreasing effects relative to the recipient *B. juncea* parent are represented by the negative bars; the correlation value for each trait with seed yield/ha (t) is shown at the bottom; PH – plant height (cm); PFB – point to first branch (cm); PB – primary branches/plant; SB – secondary branches/plant; MSL – main shoot length (cm); FSM – point to first siliqua on main shoot (cm); SL – siliqua length (cm); SPS – seeds/siliqua; SMS – total siliqua on main shoot; TS/pt – total number of siliqua/plant; SY/pt – seed yield per plant (g); BY/pt – biological yield per plant (g); OC – oil content (%); D50%F – days to 50% flowering; TSW – 1 000-seed weight (g); HI – harvest index (%); SY/ha – seed yield/ha (t); R–: negative recesive; R+: positive recessive; D–: negative dominant; D+ positive dominant; ODO–: negative overdominant;, ODO+: positive overdominant

as the productive group. On the other hand, four traits, viz., secondary branches/plant, point to first siliqua on main shoot, main shoot length, and days to 50% flowering, were independent of seed yield/ha (t) and thus classified as the non-productive group. Overall, association studies involving plant material from this genetic background revealed that the productive traits predominantly exhibited positive overdominant heterotic effects, while the non-productive traits were mainly associated with negative dominant, overdominant, and recessive heterotic effects (Figure 5B).

DISCUSSION

The exploitation of the phenomenon "heterosis" through hybrid breeding has become a highly effective strategy for significantly increasing seed yield

https://doi.org/10.17221/74/2026-CJGPB

across various field crops (Hallahan 2024). Despite the successful use of heterosis to boost yields, its genetic basis remains puzzling (Liu et al. 2019). A thorough understanding of the mechanism underlying heterosis requires two key elements: higher heterosis expression for yield and related traits, and an ideal genetic system to explain it. In *B. juncea*, the relatively limited expression of heterosis has hindered its effective use in hybrid breeding and the understanding of its genetic basis (Mei et al. 2022; Vasisth et al. 2023). Additionally, researchers have utilised mapping populations, such as F_2, recombinant inbred lines, and doubled haploids, to study heterosis; however, these populations have limitations due to epistatic interactions among segregating loci across the genome (Semel et al. 2006; Tian et al. 2019). Therefore, classifying heterotic effects and the genomic regions controlling them into different modes of inheritance remains a major challenge.

Inter-subspecific hybridisation is useful in increasing diversity and heterosis (Saravanan et al. 2025). The use of *B. carinata* to create new genetic variation in *B. juncea* and to identify QTLs that control yield traits under rainfed conditions has been established (Limbalkar et al. 2023, 2024). The expanded genetic base in the *B. juncea* ILs, achieved through the introgression of genomic segments from *B. carinata*, has also improved heterosis levels. Additionally, ILs were considered the best genetic material for better understanding the heterosis mechanism, as genome-wide epistasis could be avoided in ILs due to the high genome similarity between ILs and recipient parents (Limbalkar et al. 2021). Therefore, the phenotyped ILs, B. juncea parents, and introgression line hybrids derived from them were statistically compared, and the genetic basis of heterosis was identified.

Dissecting the genetic basis of heterosis in introgression line hybrids. Deciphering the genetic basis of heterosis is crucial for developing more targeted and efficient hybrid breeding strategies, particularly for traits such as seed yield, grain quality, and disease resistance (Manlulu et al. 2025). Research across different crop species consistently shows that the genetic basis of heterosis is complex and lacks a clear explanation for its manifestation (Liu et al. 2019). The present study revealed that the overdominant heterotic effect is the primary basis of heterosis, as most ILHs produce higher seed yields (t/ha) than their respective parents. Additionally, most DRMRIJ31_ILs have lower seed yields (t/ha) than DRMRIJ 31, while ILHs have higher yields than both ILs and DRMRIJ 31, indicating positive overdominant and negative recessive effects. Traits such as seed yield/plant and biological yield/plant exhibited overdominant heterotic effects in both genetic backgrounds. However, few hybrids displayed overdominant effects, while others showed dominant heterotic effects for traits such as 1 000-seed weight in the DRMRIJ 31 genetic background and oil content in the Pusa Mustard 30 genetic background. Aakanksha et al. (2021) also reported a range of heterotic effects from dominance to overdominance for 1 000-seed weight and oil content. Overdominant effect has been identified as the primary genetic basis of heterosis by various researchers across different crops like maize (Stuber et al. 1992), rice (Gaikwad et al. 2019), tomato (Krieger et al. 2010), and cotton (Ma et al. 2019). The heterotic effects of the hybrids are consistent in both genetic backgrounds. We evaluated them at a single location using a replicated randomised block design, as producing enough hand-pollinated hybrid seeds for testing at multiple locations always remains challenging. It would be desirable to validate these results in trials across different environments.

Candidate genes expressing overdominance for yield-related traits in heterotic ILs. Genes regulating yield-contributing traits can be used to predict the genetic basis of heterosis (Dan et al. 2019). This study identified potential candidate genes, including *ARR1*, *ARF5*, *TCP22*, *BKK1*, *SASP*, *SBT1.8*, *ATKT2*, *KASIII*, *UGT73B3*, *UGT72E1*, *SESA1*, and *FLA3*, which show overdominance in ILHs.

In the DRMRIJ 31 genetic background, the genes *SASP/SBT1.4* and *SBT1.8* were identified in IL82 and IL95, respectively, and associated with a significant increase in the number of secondary branches and siliques in the ILHs compared to their respective parents. This suggests that heterozygosity at the *SASP/SBT* loci in ILH82 and ILH95 may have resulted in an overdominant effect. Martinez et al. (2015) reported a significant increase in branching and silique numbers as compared to wild-type plants due to a loss-of-function mutation in the *SASP* gene in *Arabidopsis*. Furthermore, heterozygosity at key regulatory genes, including *ARR1*, *ARF5*, *TCP22*, and *BKK1*, was associated with the development of bold seeds in ILHs compared to their respective parents. These genes are already known to regulate seed weight. Specifically, *ARR1* influences cytokinin-mediated growth processes that affect seed development (Argyros et al. 2008; Dhaka et al. 2017); *TCP22* impacts seed size indirectly by regulating floral organ

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development (Danisman 2016); *BKK1* functions within brassinosteroid signalling pathways that are critical for seed maturation (Jiang et al. 2013); and *ARF5* plays a role in the auxin signalling cascade, regulating early embryogenesis and seed formation (Cao et al. 2020).

Despite the 1 kb window size not efficiently capturing distal regulatory elements, and a moderate marker density can only be achieved for an allopolyploid genome through the GBS approach, the genes *ATKT2*, *KASIII*, *UGT73B3*, *UGT72E1*, and *SESA1* were identified in ILs whose respective ILHs exhibited high mid-parent heterosis for per cent oil content in the Pusa Mustard 30 genetic background. Among these, *KASIII* plays a crucial role in fatty acid biosynthesis by catalysing the initial condensation of acetyl-CoA and malonyl-ACP in the fatty acid elongation cycle, directly influencing oil accumulation in *Brassica* species (Zhou et al. 2024). Conversely, *UGT73B3*, *UGT72E1*, *SESA1*, and *ATKT2* have been previously reported to indirectly influence oil content through their roles in metabolic regulation, glycosylation, and nutrient partitioning during seed development (Kumar et al. 2022). Furthermore, heterozygosity of the *FLA3* gene significantly increased siliqua length in ILH 180, ILH 181, ILH 187, and ILH 190, indicating an over-dominance mechanism. Previous studies have also shown that the *FLA3* gene controls siliqua length and seed per siliqua in *Arabidopsis* and Brassicas (Li et al. 2010; Wang et al. 2019). Although the identified candidate genes align with previous studies, they require functional validation before deployment in hybrid breeding programs.

Overdominance for the productive group of traits. Overdominance is the primary genetic basis of yield heterosis in interspecific ILs of *B. juncea*. High trait-specific mid-parent heterosis was observed in ILHs across both genetic backgrounds, consistent with earlier studies in interspecific ILs (Semel et al. 2006). In the DRMRIJ 31 genetic background, a relatively higher number of ILHs exhibited significant mid-parent heterosis for siliqua length, seed yield/plant, biological yield/plant, 1 000-seed weight, and seed yield/ha (t), and these traits exhibited positive overdominant heterotic effects in ILHs. Similarly, more ILHs demonstrated significant mid-parent heterosis for siliqua length, seeds/siliqua, seed yield/plant, biological yield/plant, oil content, and seed yield/ha (t) in the Pusa Mustard 30 genetic background. Among these traits, seed yield/plant (g), biological yield/plant, siliqua length, and seed yield/ha (t) more often showed overdominant heterotic effects in the heterotic ILs.

Correlation analysis grouped traits into productive and non-productive categories. A similar approach was employed in earlier studies on heterosis in various field crops (Tian et al. 2019). Most of the heterotic ILs for productive traits exhibited overdominant heterotic effects, consistent with reports in corn (Wang et al. 2016), tomato (Semel et al. 2006), and rice (Tao et al. 2016). Aakanksha et al. (2021) identified 695 additive QTLs for 14 yield-related traits, and overdominance was reported as the primary effect driving heterotic QTL expression in *B. juncea*.

Overall, limited research has been conducted to understand the genetic basis of heterosis for yield-related traits in *B. juncea*. The present study utilised novel interspecific ILs, which provide greater accuracy and efficiency in determining the genetic basis of heterosis. Though overdominance emerged as the primary genetic mechanism underlying heterosis for seed yield and related traits, the role of pseudo-overdominance could not be ruled out. The lower seed yield of a few ILs compared to the recipient parent is likely due to retained genetic load in the repulsion-phase linkage, particularly in genomic regions with suppressed recombination, thereby making the condition favourable for pseudo-overdominance. This phenomenon has also been observed in rice (Gaikwad et al. 2019) and tomato (Semel et al. 2006). Differentiating true overdominance from pseudo-overdominance will require fine-mapping and haplotype-resolved sequencing of key heterotic genomic regions. Furthermore, functional validation of the candidate genes identified in this study remains an exciting and necessary avenue for unravelling the molecular basis of heterosis and deploying them to improve yield heterosis in *B. juncea.*

CONCLUSION

To elucidate the genetic basis of heterosis, *B. carinata*-derived *B. juncea* ILs and their ILHs were evaluated. The high genomic similarity between ILs, ILHs, and the recipient parent suggests that minimal "epistasis" accounts for the observed heterosis. Statistical comparisons among ILs, ILHs, and the recipient *B. juncea* parents (DRMRIJ 31 and Pusa Mustard 30) revealed a widespread overdominant effect, as most ILHs produced higher seed yields (t/ha) than their respective parents in both genetic backgrounds. Correlation analysis categorised yield-

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related traits into three groups: productive, medium productive, and non-productive. Most heterotic ILs for productive traits exhibited predominantly overdominant heterotic effects compared to non-productive traits in both backgrounds. Candidate genes such as *ARR1*, *ARF5*, *TCP22*, *BKK1*, *SASP*, *SBT1.8*, *ATKT2*, *KASIII*, *UGT73B3*, *UGT72E1*, *SESA1*, and *FLA3* showed heterozygote advantage at these loci in ILHs for yield-related traits. This suggests that the overdominance or pseudo-overdominance model, emphasising phenotypic overdominant effects and heterozygote advantage at genomic loci, is the primary genetic mechanism underlying heterosis for seed yield and its component traits. Overall, the material and information generated in this study have implications for enhancing heterosis in *B. juncea*.

Acknowledgements. Prashant Vasisth is grateful to the Post-Graduate School, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, for granting a Senior Research Fellowship. The authors are grateful to the Division of Genetics, ICAR-IARI, New Delhi, for providing facilities for hybridisation and evaluating parental lines and hybrids, as well as to ICAR-IASRI, New Delhi, for providing facilities for bioinformatic analysis.

Data availability statement. The datasets generated and/or analysed during the current study are available in the NCBI Sequence Read Archive (SRA) repository under BioProject accession number PRJNA1400575 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1400575?reviewer=mg48523jd3qc2q2gl283kltk8 m).

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Received: June 4, 2026
Accepted: July 31, 2026
Published online: August 19, 2026