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EXPLORING GENETIC DIVERSITY IN *BRASSICA JUNCEA* AND *BRASSICA NAPUS* GENOTYPES FOR LOW ERUCIC ACID AND GLUCOSINOLATE LEVELS: INSIGHTS FROM SSR MARKERS

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Abstract

The development of Chinese mustard (*Brassica juncea*) and canola (*Brassica napus*) cultivars with low erucic acid and glucosinolate content is a primary objective for plant breeders. This study aimed to identify genotypes with reduced levels of erucic acid and glucosinolates by evaluating 20 *Brassica juncea* and 30 *Brassica napus* accessions, for phenotypic traits; oil content, erucic acid percentage, and glucosinolate content. For the molecular analysis, DNA was extracted and amplified using four SSR markers: sS1702, Ra3E05, Na10C01, and sR7178.

High polymorphism was observed reflecting substantial genetic diversity. Among these markers, Ra3E05 demonstrated the highest polymorphism with a PIC value of 0.861. The major allele frequency across the total 50 accessions ranged from 0.52 to 0.79. Notably, genotype BN-003 exhibited the highest major allele frequency of 0.79 but had the lowest PIC value of 0.2743, indicating least polymorphism and genetic diversity in this genotype. Principal Component Analysis was employed to explore the genotype-traits relationship, as well as to assess inter-trait correlations. The biplot revealed a negative correlation between glucosinolate content and erucic acid levels. Additionally, seed oil content showed a negative correlation with erucic acid and glucosinolates. Of all the 50 genotypes, BN-003 recorded least amount of erucic acid 0.05%, whereas, the genotypes “Super canola” and “BN-013” recorded lowest glucosinolates 24 µmol/g with high oil percentage 42.25% and 40.93% respectively. The identified genotypes, particularly Super canola and BN-013, are promising candidates for future breeding programs amid cultivar development contaning low erucic acid and glucosinolates content.

Key words: Brassica; Principle component analysis; Biplot; R software; Erucic acid; Glucosinolates

Introduction

Brassica napus L. also called rapeseed/canola, (AACC, 2n=38) is an important allopolyploid (Lu *et al.,* 2019), developed through natural hybridization between turnip (*Brassica rapa)* and cabbage (*Brassica oleracea)* (Song *et al.,* 2020). Whereas, *Brassica juncea* (AABB, 2n=36) also called Chinese mustard, is developed through natural hybridization between *Brassica rapa* and *Brassica nigra* (Rai *et al.,* 2022). During the last two decades, rapeseed has gained extraordinary importance due to its high quality seed oil (45%) and protein contents (23%) (Matthaus *et al.,* 2016). Its global production has reached 72.3 million tonnes with seed oil 26.3 million tonnes (Anon., 2022) over the cultivated area of 26.02 thousands hectares. *Brassica juncea* has very similar profile to *Brassica napus* due to its desirable fatty acid profile, good stability but contrastingly contain high erucic acid and glucosinolate contents.

The main issue in mustard seed oil is the high contents of erucic acid and glucosinolates (GSL) which needs to be addressed seriously. Mustard seed oil having more than 2% erucic acid is considered as non-nutritious and highly injurious for human health (Gain *et al.,* 2024) due to its cardiotoxic potential (Shyam *et al.,* 2020) and its prolonged intakes can lead to myocardial lipidosis (Galanty *et al.,* 2023). Likewise, the consumption of mustard and canola oil high in glucosinolates (aliphatic, aromatic (phenylalanine-derived) and indole (tryptophan-derived)) (Hasan *et al.,* 2008) through seed meal can result in goitrogen-induced hypertrophy (Galanty *et al.,* 2024).

Now-a-days molecular markers are extensively used by the researchers and scientists for exact estimation of genetic diversity and determining uniqueness of crop genotype for crop improvement in plant breeding. So, in order to develop varieties of *B. napus* and *B. juncea* with seed oil low in erucic acid and glucosinolates content, hundreds of molecular markers have been developed with aim for identification of important seed glucosinolate and erucic acid loci through structure-based allele-trait association studies (He *et al.,* 2018). Among the various types of molecular markers, microsatellite (SSR) markers have been found advantageous for their higher reproducibility, co-dominance nature, easy storability, multiallelic variations and abundant distribution in the whole genome (Verma *et al.,* 2021). Some trait-specific SSR markers have also developed in brassica crop including GSL-associated SSR; Gi31 (Hasan *et al.,* 2008) and erucic acid-associated SSR; rFAE1-5UTR-2F (Karim *et al.,* 2016).

The research aims to screen and evaluate *Brassica napus* and *Brassica juncea* accessions for low erucic acid

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and glucosinolates content. This objective was achieved by employing four candidate-gene SSR markers; sR7178, sS1702, Na10C01 and Ra3E05 previously reported by Bharti *et al.,* (2019) and their validation for double-low trait in both *B. napus* and *B. juncea* accessions. But the tranferability of SSRs across diverse brassica genotypes remains quiet unvalidated and creates a bottleneck in their application in marker-assisted breeding.

Material and Method

Plant material: The characterisation of low erucic acid and glucosinolates content was done using an integrated approach of plant breeding and marker-assisted selection of 30 *Brassica napus* and 20 *Brassica juncea* accessions. Plant materials were collected from the Oilseed Research Institute of Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. Field experiment was performed by growing all the genotypes at single location in the growing season of year 2020 at Ayub Agriculture Research Institute. The chemical plant protection was applied (for disease attack prevention) along with all the agronomic practices for high yield but the weather conditions varied moderately. After harvesting the plants, the biochemical analysis of erucic acid and glucosinolates content in seed oil was done using Hewlett Packard Agilent Technologies 6890N Netwrok GC system.

Phenotyping for the seed oil content (% dry seed weight), erucic acid and glucosinolates (µmoles/g of seeds) was done for the quality seed oil. The seed oil content was measured using the broadband mass NMR spectroscopy.

Marker-assisted selection: Molecular characterization and identification of genes associated with erucic acid and GSLs content were investigated in two brassica species; *Brassica napus* and *Brassica juncea*. Four micro-satellite markers; sS1702, Ra3E05, NA10C01 and sR7178 available in public domain (Bharti *et al.,* 2019) were used to study the genetic diversity and polymorphism for erucic acid and GSLs associated genes among the 50 brassica genotypes (Table 1). The plant DNA extraction was performed by modified Cetyltrimethyl Ammonium Bromide (CTAB) method (Saghai-Maroof *et al.,* 1984). Polymerase Chain Reaction was performed on 25 µl total reaction volume including the DNA template of 5 µl, forward and reverse primer (2µl + 2µl) and 16 µl Green taq master mix, The profile of PCR was consisting of 35 cycles with following temperature details:

Initial temperature 95°C/5 min, denaturation temperature 95°C/1 min, annealing temp 48°C/1 min (for Ra3E05 and sR7178 markers) and 53°C/1 min (for sS1702 and Na10C01 markers), extension 72°C/1 min, final extension 72°C/10 min with store temperature of 4°C.

Statistical analysis

For the data obtained, analysis of variance (ANOVA) and principle component analysis (PCA) were performed using R-Software. Analysis of variance (ANOVA) was used to assess the impact of genotype, environment, and their interaction. Power Marker V3.25 software was used on marker data to obtain the polymorphic information content (PIC) of each primer in the population (Sharma *et al.,* 2022).

Results

Analysis of variance (ANOVA) revealed highly significant variation in oil content (Oil%), erucic acid (EA) and glucosinolates (GSLs) content in *Brassica juncea* (Table 2) and *Brassica napus* (Table 3) genotypes.

In the present study, the EA content in *Brassica juncea* L. varied from 13.26% to 36.25 % while in *Brassica napus* L. the EA% was much less varying from 0.05% to 3.26% minimum and maximum values respectively (Fig. 2.).

The results presented in Figs. 1 and 2 indicate significant findings regarding oil content and erucic acid levels in 30 *Brassica napus* and 20 *Brassica juncea* genotypes. Genotype **BJ-001** exhibited the highest oil content among *Brassica juncea* genotypes at 40.01%, while among the *Brassica napus* genotypes, **Super Canola** showed the highest oil content at 42.25% (Fig. 1). These findings also confirm high oil content in *Brassica napus* than *Brassica juncea* species (Rai *et al.,* 2018). Regarding erucic acid content, among the 50 genotypes evaluated, **BN-003**, **BN-018**, and **BN-004** demonstrated the lowest levels of erucic acid, with values of 0.05%, 0.51%, and 0.58%, respectively (Fig. 2). These findings are consistent with previous studies by Saikia *et al.,* (2018) and Sharafi *et al.,* (2015), which also reported low erucic acid concentrations in Brassica species. Given the growing emphasis in Brassica breeding programs on the development of varieties with **zero erucic acid** and **low glucosinolates** to improve nutritional quality, the genotypes **BN-003**, **BN-018**, and **BN-004** are particularly promising candidates for breeding programs aimed at reducing erucic acid content. These genotypes can contribute significantly to breeding efforts focused on enhancing the nutritional value and health benefits of *Brassica* crops.

Table 1. Primer sequences of SSRs markers and their anealing temperatures.

Sr.	Primer name / Markers	Sequence (Forward/Reverse)	Primer sequence (5'-3')	Annealing Temp. (°C)	Reference
1.	sS1702	F	5` CAGATGAGACAACACAGGAAACA 3`	53°C	(Bharti *et al.,* 2019)
		R	3` ACTCAATACGTTTTTCGCGG 5`		
2.	Ra3E05	F	5` TTCTCATGCTCCAACCACAG 3`	48°C	(Bharti *et al.,* 2019)
		R	3` GTTTCTTCCAAGCCAAGCTG 5`		
3.	Na10C01	F	5` TTTTGTCCCACTGGGTTTTC 3`	53°C	(Bharti *et al.,* 2019)
		R	3` GGAAACTAGGGTTTTCCCTTC 5`		
4.	sR7178	F	5` CTGATGAGGTGACGCAGTGT 3`	48°C	(Bharti *et al.,* 2019)
		R	3`GGCTTGAGTAAAGCGACCTG 5`		

Table 2. Analysis of variance of 20 *Brassica juncea* genotypes.

Source	DF	Oil%	EA	GSLs
Genotypes	19	6.51***	33.56***	637.2***
Blocks	2	11.53***	42.84**	94.0***
Error	38	0.322	7.31	8.7

Table 3. Analysis of variance of 30 *Brassica napus* genotypes

Source	DF	Oil%	EA	GSLs
Genotypes	29	25.85***	2835.0***	2.16***
Blocks	2	26.09***	81.3^{NS}	0.045**
Error	58	0.15	159.0	0.008

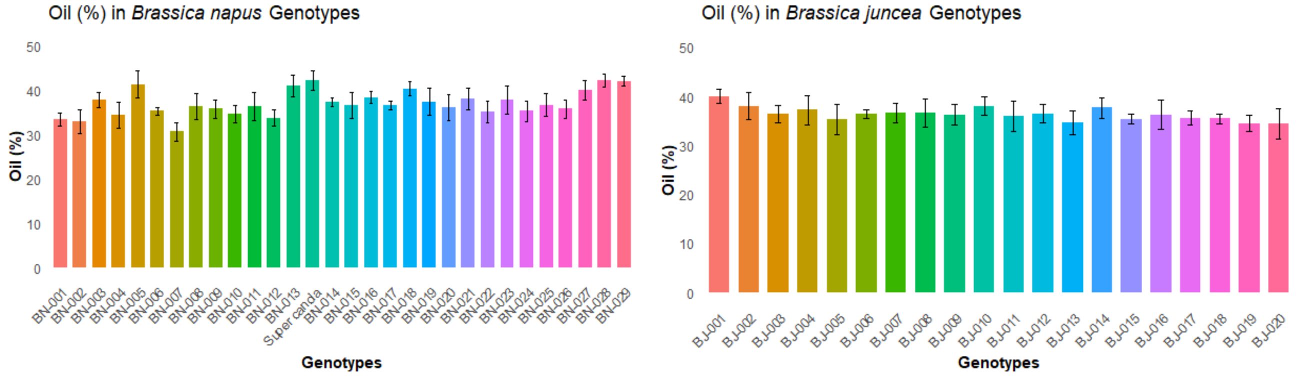

Fig. 1. Oil content (%) in *Brassica napus* L. and *Brassica juncea* L. genotypes.

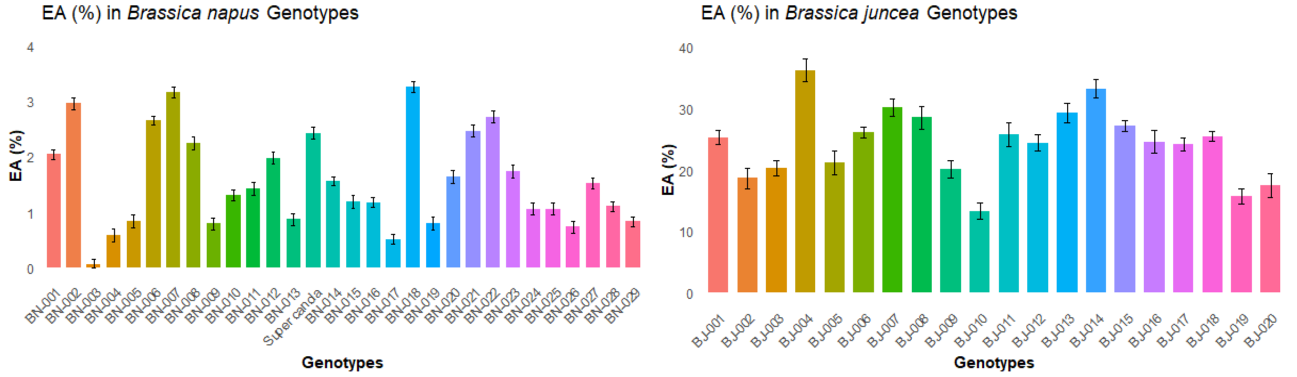

Fig. 2. Erucic acid (%) in *Brassica napus* L. and *Brassica juncea* L. genotypes.

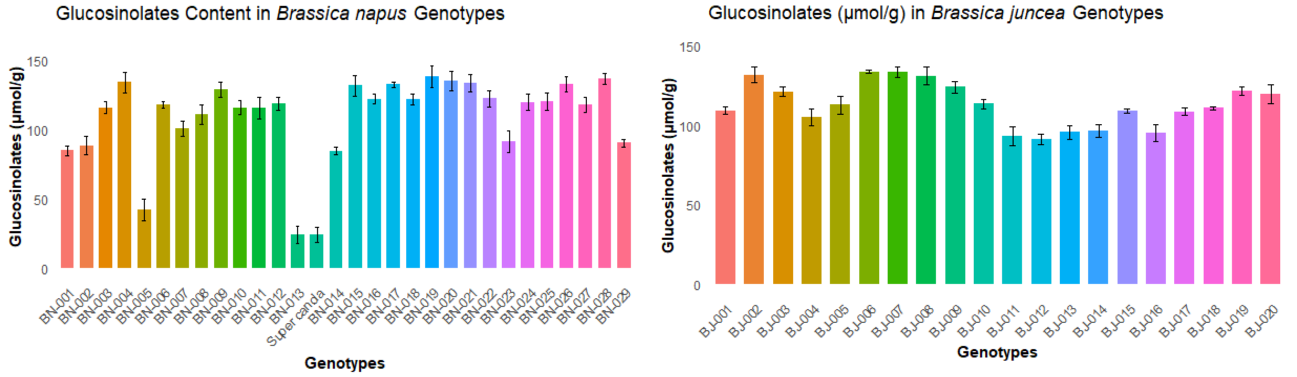

Fig. 3. Glucosinolates content (µmol/g) in *Brassica napus* L. and *Brassica juncea* L. genotypes.

The glucosinolate (GSL) content in ***Brassica juncea*** ranged from **91.41 to 133.84 µmol/g**, whereas in **Brassica napus**, it varied from **24 to 138.6 µmol/g** (Fig. 3). Among the **Brassica napus** genotypes, **"Super Canola"**, **"BN-013"**, and **"BN-005"** had the lowest GSL concentrations, **24 µmol/g**, **24 µmol/g**, and **42 µmol/g**, respectively (Fig. 3), and simultaneously exhibited relatively high oil contents, with **42.25%**, **40.93%**, and **40.37%** oil, respectively (Fig. 1). In contrast, the genotypes **BN-019** and **BN-028** contained the highest GSL levels, **138.6 µmol/g** and **136.78 µmol/g**, respectively (Fig. 3), although their oil content was **37.42%** and **42.16%**, respectively (Fig. 1).

This study demonstrates that certain *Brassica napus* genotypes exhibited the desirable combination of high oil content and low glucosinolate and erucic acid (EA) levels, aligns with the previous findings of Hatzig *et al.,* (2018) and Tiwari *et al.,* (2021). Notably, **Super Canola** was identified as the most promising genotype, showing the highest oil content (**42.25%**) along with low **erucic acid (0.83%)** and **glucosinolate (24 µmol/g)** levels, which makes it a strong candidate for oilseed production. Additionally, **BN-003** emerged as a valuable genotype for future breeding programs targeting **low erucic acid** content, as it exhibited a remarkably low **0.05% erucic acid** (Fig. 2).

The findings suggest that genotypes like **Super Canola** exhibit a favorable combination of high oil content, low erucic acid (0.83%), and low GSL levels (24 µmol/g), which are key factors in improving the suitability of Brassica oilseeds for human consumption and industrial applications, making it an ideal candidate for oilseed production. Additionally, **BN-003** is a valuable genotype for breeding programs focused on reducing erucic acid content, with **0.05%** erucic acid. Overall, these genotypes hold potential for improving both the nutritional quality and oil yield in **Brassica** crops, particularly by reducing undesirable compounds such as glucosinolates and erucic acid, which are important for enhancing the crop's suitability for human consumption and industrial use.

PIC values, genetic diversity and major allele frequency: The polymorphic information content (PIC) gives the genetic diversity analysis among the genotypes (Patil *et al.,* 2021; Sharma *et al.,* 2022). Generally, the PIC values ranging 0.50 - 0.80 for SSRs are considered highly polymorphic and those ranging 0.25 - 0.46 are considered as moderately polymorphic (Patil *et al.,* 2020; Sharma *et al.,* 2022). In the present study, 50 Brassica genotypes were subjected to analysis using four SSR markers, resulting in the detection of 5 to 9 alleles per marker. The Polymorphism Information Content (PIC) values spanned from 0.27 to 0.37, averaging at 0.36 (Table 4), thereby reflecting a moderate polymorphic level of SSRs among 49 genotypes. One genotype, however, demonstrated only a low to moderate level of polymorphism. High polymorphism was evidenced by the PIC values across all four markers; sS1702, Ra3E05, Na10C01, and sR7178 spanned from 0.765 to 0.861 PIC values. Among these, the marker Ra3E05 exhibited the highest polymorphic rate with a PIC value of 0.861 while sR7178 was relatively less polymorphic, with a PIC value of 0.765 (Fig. 4).

Genetic diversity analysis is crucial for identifying superior genotypes in plant breeding (Swarup *et al.,* 2021). Among various molecular markers, microsatellites are particularly effective for assessing genetic variation (Foster *et al.,* 2020). In a study of 50 genotypes, genetic diversity and major allele frequency (MAF) were estimated using Power Marker V3.25 software. The maximum gene diversity among these genotypes was 0.499 with mean value 0.473, indicating a moderate level of genetic variation (Table 4).

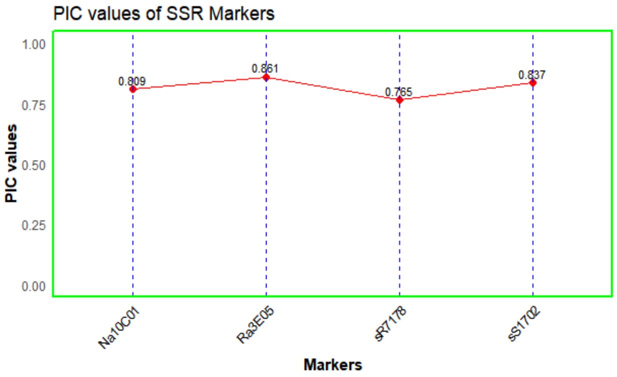

Fig. 4. The polymorphic information content (PIC) values of SSR markers.

Major allele frequency refers to the proportion of the most common allele within a population (Rajpoot *et al.,* 2020). The major allele frequency in the given population of 50 genotypes recorded a range of 0.52 - 0.79 which showed high expression in these genotypes (Table 4). The genotype BN-003 showed highest value for major allele frequency 0.79 with lowest PIC value 0.2743 which showed least polymorphism in this genotype and therefore less genetic diversity (Nefzaoui *et al.,* 2014; Rajpoot *et al.,* 2020). Whereas, the genotypes BN-001, BN-006, BN-009, BN-013, BJ-007, BJ-008, BJ-009, BJ-010, BN-021, BN-022, BN-023, BN-024, BN-025, BN-026, BN-027, BJ-016, BJ-017 and BJ-014 recorded least major allele frequency along with highest PIC value; 0.3747 for the traits of GSLs and EA % (Table 4).

Table 4. The genetic diversity, major allele frequency and PIC values of *Brassica napus* and *Brassica juncea* genotypes.

Genotypes	Genetic diversity	Major allele frequency	PIC values	Genotypes	Genetic diversity	Major allele frequency	PIC values
BN-001	0.499	0.517	0.375	BJ-006	0.495	0.552	0.372
BN-002	0.452	0.655	0.350	BJ-007	0.499	0.517	0.375
BN-003	0.328	0.793	0.274	BJ-008	0.499	0.517	0.375
BN-004	0.471	0.621	0.360	BJ-009	0.499	0.517	0.375
BN-005	0.485	0.586	0.368	BJ-010	0.499	0.517	0.375
BN-006	0.499	0.517	0.375	BN-020	0.495	0.552	0.372
BN-007	0.428	0.690	0.336	BN-021	0.499	0.517	0.375
BN-008	0.495	0.552	0.372	BN-022	0.499	0.517	0.375
BN-009	0.499	0.517	0.375	BN-023	0.499	0.517	0.375
BN-010	0.495	0.552	0.372	BN-024	0.499	0.517	0.375
BN-011	0.495	0.552	0.372	BN-025	0.499	0.517	0.375
BN-012	0.495	0.552	0.372	BN-026	0.499	0.517	0.375
BN-013	0.499	0.517	0.375	BN-027	0.499	0.517	0.375
Super Canola	0.471	0.621	0.360	BN-028	0.495	0.552	0.372
BN-014	0.400	0.724	0.320	BN-029	0.471	0.621	0.360
BN-015	0.452	0.655	0.350	BJ-011	0.471	0.621	0.360
BN-016	0.400	0.724	0.320	BJ-012	0.485	0.586	0.368
BN-017	0.485	0.586	0.368	BJ-013	0.471	0.621	0.360
BN-018	0.400	0.724	0.320	BJ-014	0.499	0.517	0.375
BN-019	0.471	0.621	0.360	BJ-015	0.495	0.552	0.372
BJ-001	0.428	0.690	0.336	BJ-016	0.499	0.517	0.375
BJ-002	0.366	0.759	0.299	BJ-017	0.499	0.517	0.375
BJ-003	0.400	0.724	0.320	BJ-018	0.495	0.552	0.372
BJ-004	0.485	0.586	0.368	BJ-019	0.485	0.586	0.368
BJ-005	0.471	0.621	0.360	BJ-020	0.452	0.655	0.350

Mean Values: Genetic diversity = 0.477, Major allele frequency = 0.587, PIC = 0.361

PCA analysis

Principal Component Analysis (PCA) was conducted to evaluate the correlation between various genotypes and their associated traits, as well as to assess inter-trait relationships. The table 4 shows the numbering of genotypes of *Brassica napus* and *Brassica juncea* displayed in the biplots. The biplot indicated a negative correlation between glucosinolate content and erucic acid levels in both *Brassica napus* and *Brassica juncea* genotypes (Figs. 5, 6; Table 5). In the same way, the seed oil content showed negative correlation with EA but weak positive correlation with GSLs (Fig. 5).

Field data analysis revealed that an increase in seed oil content is associated with a decrease in erucic acid levels, while glucosinolate content either showed no correlation or exhibited a weak positive correlation. The present study is focused on identifying genotypes with a high oil content but low concentrations of erucic acid and glucosinolates. Erucic acid has toxic effect on human health most probably in cardiotoxicity (Galanty *et al.,* 2023) and the GSLs have potential to induce goiter, anemia, hepatic and renal lesions and gastrointestinal irritation) (Bischoff, 2016), that's why the genotypes with low GSLs and EA are desirable. Among all of the genotypes evaluated, **'Super Canola', 'KN279'** and **'RBN18021'** showed exceptional performance, characterized by low glucosinolate and erucic acid accumulation in seed oil, coupled with high oil yield. While the genotype **RBN-18002** was the worst performing with low seed oil yield and high EA% and GSLs (Fig. 5).

Table 5. Labeled accessions numbers of *Brassica napus* and *Brassica juncea* in the respective biplots.

Sr No.	*Brassica napus*	Sr No.	*Brassica juncea*
1	BN-001	1	BJ-001
2	BN-002	2	BJ-002
3	BN-003	3	BJ-003
4	BN-004	4	BJ-004
5	BN-005	5	BJ-005
6	BN-006	6	BJ-006
7	BN-007	7	BJ-007
8	BN-008	8	BJ-008
9	BN-009	9	BJ-009
10	BN-010	10	BJ-010
11	BN-011	11	BJ-011
12	BN-012	12	BJ-012
13	BN-013	13	BJ-013
14	Super Canola	14	BJ-014
15	BN-014	15	BJ-015
16	BN-015	16	BJ-016
17	BN-016	17	BJ-017
18	BN-017	18	BJ-018
19	BN-018	19	BJ-019
20	BN-019	20	BJ-020
21	BN-020	-	-
22	BN-021	-	-
23	BN-022	-	-
24	BN-023	-	-
25	BN-024	-	-
26	BN-025	-	-
27	BN-026	-	-
28	BN-027	-	-
29	BN-028	-	-
30	BN-029	-	-

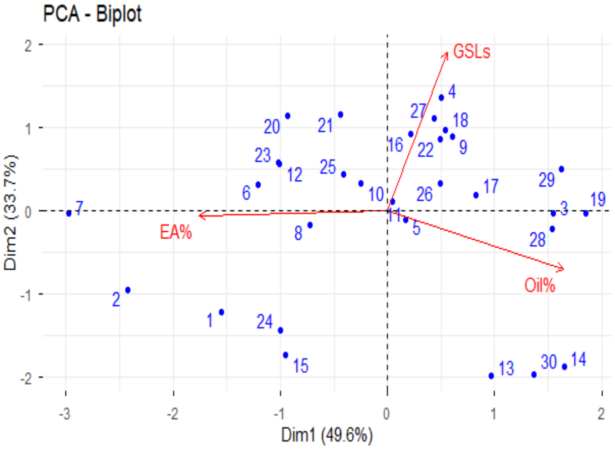

Fig. 5. Principal component analysis of *Brassica napus* genotypes.

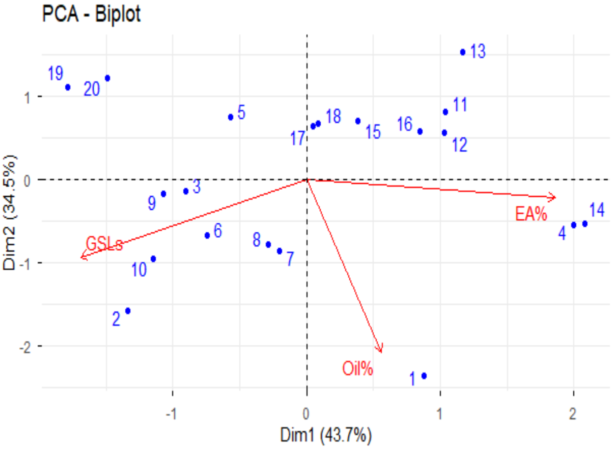

Fig. 6. Principal component analysis of *Brassica juncea* genotypes.

Conclusion

The identification of *Brassica napus* genotypes exhibiting a synergistic combination of oil yield (high oil seed content) and seed quality (low glucosinolates and erucic acid contents) will substantially beneficial in future breeding programs amid developing high value brassica cultivars. Notably, genotype 16CBN007 demonstrated high seed oil content and glucosinolate concentration, with a concomitant reduction in erucic acid content (Fig. 5). In contrast, genotype RBN-18008 displayed a positive correlation between seed oil content and erucic acid levels, while showing an inverse correlation with glucosinolate content (Fig. 5). Moreover, the genotype Super-canola was the best performing in in terms of high oil content and low erucic acid and glucosinolates content (Figs. 1, 2, 3.). These superior genotypes belong to the *B. napus* species and can be used for future breeding programs aimed at developing cultivars with enhanced oil yield and reduced levels of glucosinolates and erucic acid.

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