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Original Article

Production and Biochemical Evaluation of Camelina [*Camelina sativa* (L.) Crantz] Doubled Haploid Lines

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ABSTRACT

The medicinal-oil plant *Camelina sativa* (L.) Crantz belongs to the *Brassicaceae* family. Various experiments have shown that the plant has much fewer water requirements and more resistance to spring cold than other oilseeds, especially Rapeseed. In this study, 136 camelina double haploid lines from anther culture progeny (F1) of crosses of 27 selected parents from different countries were generated. After ensuring that the lines were double haploid, biochemical markers were used to investigate genetic diversity. In the biochemical markers section, grain oil content, grain protein and type of fatty acids were measured. The experiment was carried out based on a randomized complete block design with four replications. To determine fatty acids using chromatography, 18 types of fatty acids were identified in camelina seed oil. Genetic parameters including phenotypic and genotypic variation coefficients, heritability and genetic advance were estimated. In this study, the highest phenotypic and genotypic variation in fatty acids (C14:0-C16:1) were estimated. Also, the highest general heritability for fatty acids (96.49% for C20:0, 98.92% for C20:2 and 98.59% for C20:3) were assessed. In this reserach, two lines with values of 35.81-36.67% linolenic acid and four lines with values between 22.08-23% of linoleic acid were identified. Also, the ratio of omega 6 to omega 3 ranged between 0.479 0 and 0.759.

INTRODUCTION

Approximately one-third of Iran's land is agricultural, but due to poor soil, inadequate water distribution in most areas and poor management, only 12% of them is cultivated [1]. Thus, it appears that the need for new oilseed crops sources that are more compatible than existing plants and more resistant to stresses far exceeds. Oilseeds are the second largest food reserves in the world after cereals [10, 33,34]. The oil-pressing and vegetable oil production industry is one of the strategic industries in most world countries [25]. Also, the cultivation of oilseeds is one of the most essential components of food safety [29].

Camelina is an annual herbaceous plant [9, 13, 16]. Oil is the major component of camelina seeds [38]. Variety of cereal production in rotation with camelina can have many economic and environmental benefits. This can break the cycle of weeds and diseases [7]. Oilseeds are good competitors to compete with weeds early in the growing season because oilseeds have a canopy that encloses the weeds in the early stages [17].

In order to stabilize the crop system, new alternative plants should be introduced to be cultivated alternately with cereals especially in rainfed condition in dryland areas [15]. In recent years, new cultivars in the field of alternative plants, including legumes, fodder and oilseeds have been introduced [30]. Due to the agricultural benefits and the quality of the oil of camelina, it can be a crop with a high potential in meeting the country's need for oil. The introduced camelina must have varieties adapted to different climates. One way to create diversity is crossing and production of haploid and the doubled haploid plants. Production of doubled haploid plants in *Brassicaceae* species is importance as occurs with a low frequency [28].

Understanding the genetic diversity of double haploid lines is possible using molecular techniques

[35]. There are many ways to study diversity, but molecular markers are widely used in the identification and study of plant genetic diversity [27]. Some differences in the DNA sequence between two organisms may manifest as proteins that can be chemically examined, assayed, and recorded. These types of markers are affected by post-translational changes and the quantitative expression of some enzymes and proteins is affected by the growth stage of the plant and the existing environment. The special value of camelina in its oil content is in high contents of unsaturated fatty acids. On the other hand, camelina oil is high in the natural antioxidant tocopherol [9, 20]. The quality characteristics of each edible oil depend on its fatty acid composition [8]. Fatty acid compounds in camelina are affected by cultivars and environmental conditions [21, 23]. Identification of lines with suitable fatty acid profile is necessary for releazing cultivars [4, 9].

In the present study, the camelina doubled haploid lines produced via androgenesis and fatty acid compositions and the ratio between them were investigated. Due to the fact that Iran is facing the problems of drought, high imports of edible oils and the lack of oil plants adapted to the climatic conditions, in this study, diversity was created and the resulting diversity was studied in *C. sativa* (L.) Crantz.

MATERIALS AND METHODS

Plant Materials

The plant materials of this research were hybrids between 27 different *C. sativa* (L.) Crantz cultivars. These cultivars were prepared from Russia, Germany, Denmark, Poland, Kazakhstan, Sweden and the countries independent from the Soviet Union and transferred them to the Campus of Agriculture and Natural Resources of Razi University of Kermanshah, Kermanshah, Iran (Table 1). Parental seeds were planted in pots under greenhouse conditions (16 h light, 8 h dark, 24 °C, 400 μmol m^{-2} s^{-1} light intensity and Relative humidity of around 80%). The potting soil was filled with field soil, rotted manure, peat and perlite in ratios of 2-1-1, respectively. Irrigation period was applied once every two days. For better growth of stock plants, Hoagland [19] liquid fertilizer at the rate of 3.5 per thousand was sprayed on the plants.

Fifteen crosses are shown in Table 2. The progeny (F1 generation) from crosses were harvested separately. The progeny was planted in pots. After growing in greenhouse conditions and at the beginning of the reproductive phase in order to produce double haploid lines by androgenesis method, anther anthers were collected. Unopened buds were collected to collect anthers of F1 generation plants.

In order to make a suitable culture medium in order to separate and not lose water in the pollen grain, the medium containing 40 g / l sucrose was made with pH = 6. The culture medium of embryogenesis used in this study was NLN [26] and 1/4 MS (Murashige and Skoog, 1962). After disinfection, sterile anthers were placed in a 2 ml microtype and the isolation medium was poured into each micro type. The best culture medium for regeneration of the *Brassicaceae* family, especially rapeseed is the B5 medium, so this medium was used for regeneration. After 2 weeks, the embryos that formed the cotyledon structure were removed from the induction culture medium (NLN) and transferred to the regeneration medium (B5) under sterile conditions.

Field Conditions

The morphological and physiological traits was evaluated in field conditions. The lines were planted in the research farm of Razi University Campus of Agriculture and Natural Resources. Agronomic, physiological and morphological traits were measured during plant growth in the field and at the end of the growing season. The field experiment was planted in a randomized complete block design with four replications.

Oil Extraction

Oil was extracted from the grain by cold press. Oil sampling was performed in accordance with Iranian National Standard No. 493. Fatty acid was determined by gas chromatography (Agilent / HP Model 6890). All tests were performed in four replications. The results were analyzed in a completely randomized design using SAS software and graphs were drawn using Excel software.

Data Analysis

The normality of data distribution was checked using SPSS software (version 26). Then, an analysis of variance was performed based on a randomized complete block design with four replications and

mean comparison by LSD method using SAS software (version 9.3). Phenotypic and genotypic variation coefficients (PCV and GCV), general heritability (h^2b) and genetic gain parameters were estimated from the components of variance. genetic advance by selection (GA) was then calculated using these values. Estimates of genetic parameters were estimated using SAS software.

Table 1 Names of parent cultivars and their origin

No.	Cultivar	Origin	No.	Cultivar	Origin
1	Voronezskij 349	Russia	15	Boha	Denmark
2	Omskij Mestnyj	Russia	16	Hoga	Denmark
3	Saratovskij	Russia	17	Kirgizskij 1	Kazakhstan
4	Chulymskij	Russia	18	Blaine Greek	Greece
5	Krupnosemjannyj	Russia	19	Irkutskij Mestnyj	Oblast Irkutsk
6	Borowska	Poland	20	Volyn'askaja	Soviet Union
7	Przybrodzka	Poland	21	Omskij	Soviet Union
8	Brzybrodzka II	Poland	22	Sortandinskij	Soviet Union
9	Czestochowska	Poland	23	Ukrajinskaja	Soviet Union
10	Volynskaja	Poland	24	Voronezh 349	Soviet Union
11	Svalöf	Sweden	25	VNIIMK 17	Soviet Union
12	Calena	Germany	26	Zavolzskij	Soviet Union
13	Lindo	Germany	27	Ukrajinskij	Soviet Union
14	Came	Germany	-	-	-

Parental cross and cultivation of progeny (F1) in greenhouse conditions

Table 2 Parental characteristics and double haploid lines produced by *C. sativa* (L.) Crantz

Number of crosses	Parent (♀) Cultivar	Parent (♂) Cultivar	Naming F1 by number
1	Voronezskij 349	Kirgizskij 1	1,2,5,37,51,62,70,73,77,86,87,93,96
2	Omskij Mestnyj	Irkutskij Mestnyj	9,32,38,43,46,60,71,79,89,113,118,129,3,6
3	Przybrodzka	Hoga	7,14,17,20,22,104
4	Saratouskij	Bronowska	8, 24,40, 96,115,120
5	Chulymskij	Omskij Mestnyj	11,25,29,41,42,58,68,116,121,125,131
6	Krupnosemjanny	Brzybrodzka II	26,36,78,106,110
7	Came	Volynskaja	31,69,76,90,98,119,123
8	Boha	Volynskaja	18,19,23,97
9	Came	Omskij	82,88,91,94,95,100,108,111,112,124,132,4,34,35
10	Svalöf	Ukrajinskij	27,28,50,52,56,103,105,117,130
11	Calena	Blaine Creek	30,36,47,80,83,84,101,114,134,135,136,15
12	Zavolzskij	Sortandinskij	21,39,44,45,59,67,81,99,102,126
13	Vniimk 17	Borowska	48,57,74,75,85,127
14	Voronezh 349	Czestochowska	10,16,49,54,55,61,109,133
15	Lindo	Ukrajinskaja	12,13,33,53,64,65,72,92,107,122,128

Culture medium isolation, embryogenesis and regeneration of plant from anther and embryo formation and their transfer to regeneration medium

RESULTS AND DISCUSSION

Parental Selection

Creating new germplasm and studying genetic diversity in camelina breed is one of the most important priorities of this crop. In the present study, the most successful crossbreeding was observed based on the number of progeny between the cultivars Irkutskij Mestnyj♂ × ♀ Omskij Mestnyj and Omskij♂ × ♀ Came with 14 progenies per crossbreed. In selecting parents, four general

concepts that have been approved by researchers in the field of the race were considered. These concepts include the concept of variety, the concept of adjectives, the concept of gene and the concept of geographical distance. In this study, 27 parents were selected, so selecting a large number of parents in this study also raises the possibility of effective combinations of traits. In this research, the concept of adjective was also observed. For example, Hoga cultivar is mentioned in various sources as a cultivar that contains 30 times more carotenoids [24] and was used in the present study. Based on the concept of genes, Lindo cultivar was selected, which contains 43.6% of oil [32]. Cultivars with geographical distance from each other were also selected. Five cultivars from Russia, five cultivars from Poland, three cultivars from Germany, two cultivars from Denmark, one cultivar each from Sweden, Kazakhstan, Greece and Irkutsk, and eight cultivars from the independent countries of the Soviet Union were selected. In the set, 27 selected parents were placed in 15 crosses and 136 progeny (F1) were obtained.

Results of Variance Analysis of Embryogenesis Medium

In order to investigate the embryogenesis rate in NLN and 1.4 MS media and also to investigate the effect of carbon source content on embryogenesis, a completely randomized design was designed. The treatments of this study were (NLN + 130 gr / lit Sucrose, NLN + 90 gr / lit Sucrose) and (1 / 4MS + 130 gr / lit Sucrose, 1 / 4MS + 90 gr / lit Sucrose).

The highest percentage of embryogenesis due to camelina androgenesis was observed in NLN medium with 130 g / l sucrose at a rate (of 35%). The lowest embryogenesis rate was observed in 4/1MS medium with 90 g / l sucrose. Various factors affect the embryogenesis of microspores.

These factors include genotype, microspore growth stage, pollinating plant growth conditions, medium composition and culture conditions [12]. In varieties of *Brassicaceae* family in a culture medium, we see differences in embryogenesis rates [3]. For example, Ag 45 and Ag46 varieties in *B. Campestris* were reported to be embryogenic in the NLN medium (12.4-13.9%, respectively) [26]. There are limited reports of anther culture for camelina. One of these limited reports is about Ferrie and Bethune in 2011. Since then, no protocol has been reported for anther cultivation.

We observed that embryogenesis in the NLN medium was significantly different from the 1/4 MS medium and was consistent with the results of Ferrie and Keller, 2007 [12] and Ferrie and Bethune, 2011 [11]. In interpreting this, the NLN environment is glutamine-free. The presence of glutamine in camelina embryogenesis has a negative role in embryonic development and the removal of glutamine will increase the embryogenesis rate of undifferentiated cells [11]. Therefore, glutamine deficiency initially increased the embryogenesis rate of camelina pollen seed cells.

Investigation of Biochemical Traits

In total, 18 types of fatty acids [including Lauric acid (C12:0), Myristic acid (C14:0), Palmitic acid (C16:0), Palmitoleic acid (C16:1), Stearic acid (C18:0), Oleic acid (C18:1), Linoleic acid (C18:2), Linolenic acid (C18:3), Eicosanoic acid (C20:0), Eicosenoic acid (C20:1), Eicosadienoic acid (C20:2), Eicosatrienoic acid (C20:3), Behenic acid (C22:0), Erucic acid (C22:1), Docosadienoic acid (C22:2), Docosatrienoic acid (C22:3), Lignoceric acid (C24:0) and Nervonic acid (C24:1)] were identified and measured in the oil extracted from the seeds of camelina double haploid lines.

Analysis of Variance of Fatty Acids in Camelina Seed Oil

Analysis of variance of fatty acid content in camelina seed oil showed that there was a significant difference in the content of fatty acids between the lines studied in this study (Table 3).

The average fatty acids of oil extracted from 137 double haploid lines of camelina showed that there are 18 types of fatty acids in camelina seed oil. Fatty acids are divided into three categories, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids.

Saturated Fatty Acids in Camelina Seed Oil

7 types of saturated fatty acids were observed in the oil composition of camelina seeds. These included lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16: 0), stearic acid (C18: 0), eicosenoic acid (C20: 0), behenic acid (C22: 0) and lignoceric acid (C24: 0).

Table 3 Analysis of variance of the effect of genotype on the amount of different fatty acids in camelina double haploid lines

		Mean Squares					
S.O.V	df	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1
Replication	3	0.00	0.52	14.61	0.47	3.42	119.05
Genotype	135	0.00 **	0.00 **	0.24 **	0.00 **	0.51 **	4.75 **
Error	408	0.00	0.00	0.002	0.00	0.00	0.005
C.V. (%)	-	0.55	0.42	0.22	2.62	0.79	0.64

Continuation of **Table 3**

		Mean Squares					
S.O.V	df	C18:2	C18:3	C20:0	C20:1	C20:2	C20:3
Replication	3	111.59	316.44	0.136	67.97	0.03	0.01
Genotype	135	6.94 **	5.81 **	0.22 **	1.15**	0.01 **	0.05 **
Error	408	0.00	0.00	0.00	0.00	0.00	0.00
C.V. (%)	-	0.36	0.20	0.69	0.20	0.60	0.56

Continuation of **Table 3**

		Mean Squares					
S.O.V	df	C22:0	C22:1	C22:2	C22:3	C24:0	C24:1
Replication	3	0.50	0.69	0.53	0.26	0.46	0.13
Genotype	135	0.00 **	0.23 **	0.00 **	0.01 **	0.00 **	0.00 **
Error	408	0.00	0.00	0.00	0.00	0.00	0.00
C.V. (%)	-	1.88	0.43	0.65	0.69	0.77	0.44

* and **: significant at the 5% and 1% probability levels, respectively.

Monounsaturated Fatty Acids in Camelina Seed Oil

5 types of monounsaturated fatty acids were detected in camelina seed oil, including palmitoleic acid (C16:1), oleic acid (C18:1), eicosenoic acid (C20:1), erucic acid (C22:1) and nervonic acid (C24:1). The ratio of unsaturated fatty acids and the amount of sterols determine the fluidity of membranes in plants. Membrane fluidity in plants is directly related to stability in abiotic stresses [14]. In camelina, finding specific ratios of unsaturated fatty acids, in addition to the specific uses of these fatty acids, is important in improving the plant's resistance to abiotic stresses [6].

Polyunsaturated Fatty Acids in Camelina Seed Oil

Another group of fatty acids detected in camelina seed oil was polyunsaturated fatty acids. PUFAs play a more vital role in the function and structure of living organisms than other fatty acid groups. Since the human and mammalian bodies are unable to make precursors of PUFAs, a sufficient and balanced amount of these fatty acids in the diet is essential (Watts, 2016). Camelina seed oil contains 6 types of fatty acids from PUFAs; linoleic acid (C18: 2), linolenic acid (C18: 3), eicosadienoic acid (C20: 2), eicosatrienoic acid (C20: 3), docosadienoic acid (C22: 2) and docosatrienoic acid (C22: 3), the average value of each of these 6 fatty acids were measured in 137 complete lines.

Linolenic acid (C18: 3) and linoleic acid (C18: 2) are important fatty acids in the PUFA class. The lines studied in this study had different percentages of LA and ALA fatty acids. Omega-6 and omega-3 are fatty acids that the body is unable to make and are therefore essential fatty acids for the body. In this study, the maximum ratio of omega-6 to omega-3 in camelina (0.71 - 0.75) with a frequency of 3 lines and the minimum ratio (0.47 - 0.51) with a frequency of 11 lines.

Erucic Acid in Camelina Seed Oil

Erucic acid (C22: 1) is a 22-mono unsaturated fatty acid (MUFA) that is classified as a very long-chain fatty acid (VLCFAs) [18]. Nutritionally, small amounts of erucic acid are desirable [20]. One of the most important breeding goals of *Brassicaceae* is to reduce the amount of erucic acid [18]. Ward method was used in cluster analysis to group and determine the distance between genotypes. Cluster analysis was performed based on the measured average amount of erucic acid of 137 camelina lines, the results of which were shown as dendrograms (Fig. 1). According to the cut-off point, the studied lines were grouped into four clusters with similar

intragroup and intergroup characteristics. The first cluster (group 1 + group 2) includes 43 lines, the second cluster (group 3) 55 lines, the third cluster (group 4 + group 5) 36 lines, the fourth cluster (group 6) 3 lines. The maximum amount of erucic acid in camelina seed oil was 3.94 and the minimum amount was 2.49%. The fourth cluster (group 6) has three lines with values of 3.94, 3.87 and 3.73 percent of erucic acid, while 134 lines have values less than 3.51 percent. Plants of the genus *Brassicaceae* are divided into two main groups according to the amount of erucic acid. The first category is identified by the abbreviation (HEA- High Erucic Acid). Their oil is more than 5% erucic acid and is not consumed orally. The second category is abbreviated (LEA- Low Erucic Acid). Their oil is consumed orally with less than 5% erucic acid [16]. All camelina double haploid lines in this study contain less than 3.94% erucic acid.

Estimation of Genetic Parameters in Camelina fatty Acids

Genetic and phenotypic variance, phenotypic and genotypic variance coefficient, heritability and genetic progression are evaluated as important parameters in the selection of breeding strategies for different traits [22]. Genetic and phenotypic variance and heritability for different fatty acids are shown in Table 4. Fatty acids (C14: 0) and (C20: 1) had the highest and lowest percentages of phenotypic and genotypic variance, respectively. A high phenotypic variance coefficient for traits indicates that the expression of these traits is greatly affected by the environment [31]. The high coefficients of genotypic and phenotypic variance indicate a wide range of variations for traits. Peripheral variance coefficients in fatty acids (C14:0) and (C20:3) had the highest and lowest percentages, respectively. In general, the proximity of phenotypic and genotypic variance coefficients in some traits indicates that environmental effects on the expression of that trait are negligible. Also, when the phenotypic variance coefficient is much higher than the genotypic variance coefficient, it indicates a high level of environmental effects [22]. This principle was also observed in this study. The small difference between phenotypic and genotypic variance coefficients for fatty acids (C18:3-C18:2-C24:1-C22:1-C20:0-C12:0-C20:2-C20:3<6%ECV) indicates a decrease in environmental variance coefficient and an increase in the effects of genetic factors on the inheritance of these traits. Therefore, parental selection based on these traits is suitable for hybridization for breeding purposes.

Heritability is the most important parameter in genetic studies of quantitative traits [5]. This parameter plays a significant role in decision-making for adjective selection [2]. In this study, the highest phenotypic and genotypic variance was estimated for fatty acids (C14:0-C16:1) and the highest heritability (C20: 2-C20: 3-C20: 0), respectively. Inheritance is considered an indicator of the transmissibility of traits from parents to progeny. The high heritability of traits indicates low environmental effects on the studied traits [2]. The effect of environment on traits that have high heritability is negligible and phenotype-based selection is effective in these traits [22]. High heritability indicates that the selection of optimal genotypes according to phenotype is reliable [31]. But it does not show any indication of the amount of genetic progression to select the best people.

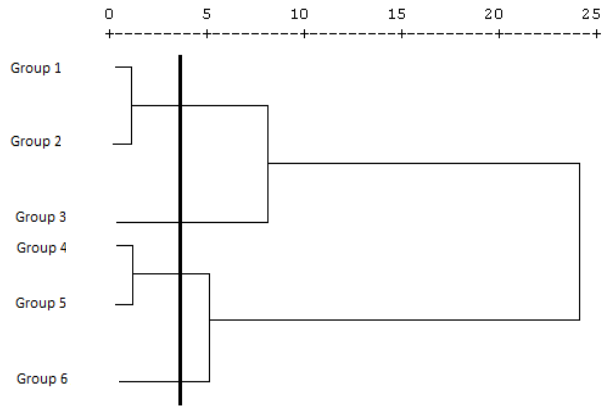

Fig. 1 Analysis of cluster of 137 camelina haploid lines in terms of erucic acid (C22: 1) using the Ward method

To estimate this shortcoming, estimation of genetic advance is used [5]. Combining heritability with genetic advance over heritability alone is more useful for estimating selection effects [22]. It should be noted that high heritability is not always associated with large genetic advances. On the other hand, the continuity of high heritability with low genetic progression for some traits indicates the effects of dominance and epistasis of genes controlling these traits [31]. The results showed that there is the highest rate of heritability and genetic advance for the trait (C12:0-C24:1). Selection for traits that have both high heritability and genetic advance can be successful [5].

Table 4 Genetic parameters for the amount of fatty acids in 137 double haploid camelina lines

Fatty acid	Genotypic Variance Coefficient (%)	Phenotypic Variance Coefficient (%)	Environmental Variance Coefficient (%)	General Heritability (%)	Genetic Advance (%)	Genetic Gain (%)
C12:0	0.89	1.84	1.60	24.7	0.04	90.97
C14:0	41.19	84.08	73.29	24	0.04	0.14
C16:0	1.48	6.63	6.46	5.03	6.82	1.1
C16:1	21.5	44.08	44.33	23.52	0.03	20.88
C18:0	12.59	14.43	7.19	75.16	0.11	4.13
C18:1	5.57	9.24	7.37	36.52	1.06	0.06
C18:2	5.95	8.26	5.73	51.89	1.67	8.68
C18:3	1.70	6.09	5.85	7.83	3.11	9.79
C20:0	12.07	12.28	2.12	96.49	0.47	24.29
C20:1	1.35	6.07	6.07	4.97	9.06	62.21
C20:2	10.59	10.71	1.45	98.92	0.32	21.6
C20:3	9.69	9.7	1.01	98.52	0.24	19.71
C22:0	15.10	21.95	15.93	47.32	0.10	21.25
C22:1	7.81	8.3	2.94	87.57	0.44	14.96
C22:2	20.60	45.75	40.85	20.24	0.03	19.04
C22:3	9.75	14.5	10.72	45.28	0.06	13.44
C24:0	7.51	27.88	26.84	7.27	0.10	41.35
C24:1	13.26	14.17	4.99	87.6	0.19	25.4

CONCLUSIONS AND SUGGESTIONS

The first goal of this study was to address the deficiency of camelina germplasm. With the crosses of selected parents from around the world and culture progeny (F1), 136 double haploid lines were created. Then, the anther culture medium and camelina anther culture protocol were drawn, tested and reported. Optimization of chromosomal observation in camelina was also reported. Finally, diversity at the biochemical level was measured and biometric genetic parameters were estimated for the studied traits. The fatty acids in camelina seed oil were also identified and expressed.

Overall, the results of this study showed that there are 18 types of fatty acids in camelina. Analysis of variance showed that there was a very significant difference ($P \leq 0.01$) between the lines in terms of fatty acid content. Different ratios of fatty acid compounds make it possible to use the lines in terms of nutrition, medicine and industry. Findings from the estimation of genetic parameters of this study, which indicate genetic effects and heritability, make it possible to choose a targeted breeding program for camelina in the future.

It is recommended to create a new germplasm and study the genetic diversity in breeding camelina and improving some of the characteristics and creating ideal types of this plant. It is also recommended to identify and evaluate as many double haploid lines as possible in the continuation of the breeding path of these lines. In camelina, fatty acid compounds, as one of the most important traits, are directly related to cultivars and environmental conditions. Due to this issue, the cultivation of lines in different climatic regions is recommended.

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