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Research Article

Seed oil content, oil yield and fatty acids composition of black mustard [*Brassica nigra* (L.) Koch] in response to fertilization and plant density

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Abstract

The cultivation of black mustard [*Brassica nigra* (L.) Koch] has recently become increasingly popular and there is a raising demand for its oil and seeds from the food, pharmaceutical, and cosmetic industries. A 2-year experiment was conducted in a split-plot design with three replications, two main plots (plant densities: 46 and 76 plants m^{-2}) and four sub-plots (fertilization treatments: control, urea with and without urease and nitrification inhibitors, and compost) to evaluate the fertilization and plant density effect on seed oil content, oil yield, and fatty acids composition of black mustard under Mediterranean environment. The seed yield, oil content and yield were positively influenced by the increase of available nitrogen and negatively by the increase of plant density, with their highest values recorded in the low-density and urea with double inhibitors. In response to the quality characteristics of seed oil, low-density compost application raised the quantities of polyunsaturated fatty acids (PUFA). In conclusion, plant densities higher than 46 plants m^{-2} result in lower seed yield, oil content and yield, while the inorganic fertilization effect, specifically with urea with double inhibitors, was equally important in seed and oil yield; however, when the seed and/or oil are used for their nutraceutical and medicinal value, the use of compost is recommended, resulting in a significant increase in PUFA content.

Keywords: α-linolenic acid; compost; nitrification inhibitor; linoleic acid; oleic acid; polyunsaturated fatty acids (PUFA); seed oil; urease inhibitor

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Introduction

Mustards belong to the Cruciferae or Brassicaceae family. The genus *Brassica* has 150 species of annuals or biennial herbs, many of which are served as one of the most agronomically important oilseeds with a varied range of species that can be used as a variety of oilseed, vegetable, and fodder crops (Sharafi *et al.*, 2015). The main distinguishing feature of *Brassica* species is their high seed oil content, which ranges from 17 to 40% in wild relatives (Kumar and Tsunoda, 1980). The major cultivars of *Brassica* oilseed crops (*B. napus*, *B. juncea*, and *B. rapa*) with average oil content ranging from 45 to 50%, have been released as a result of plant breeding science, and the aforementioned cultivars are currently the world's third most important source of fixed oil (Friedt and Läuhs, 1995; Thomas *et al.*, 2012). The seven principal fatty acids derived from *Brassica* species are palmitic (C16:0), stearic (C18:0), oleic (C18:1 *n-9*), linoleic (C18:2 *n-6*), α-Linolenic (C18:3 *n-3*), eicosanoic (C22:0), and erucic (C22:1) acid (Sharafi *et al.*, 2015). *Brassica* oil contains more genetic variations in its fatty acids profile than other major vegetable oils (Sovero, 1993).

Brassica species seed oil also contains a high concentration of long-chain monounsaturated fatty acids, particularly erucic acid (C22:1), which is not found in any other commercial vegetable oil (Röbbelen and Thies, 1980; Thomas *et al.*, 2012; Sharafi *et al.*, 2015). Although oil with a high erucic acid concentration is anti-nutritional, it is useful for some industrial purposes, such as anti-blocking compounds in polyethylene films and printing adhesives (Vles and Gottenbos, 1989; Uppström, 1995). It could also be employed in the production of cosmetics by synthesising waxes that could be used as a substitute of jojoba oil (Kramer, 2012). Oils having high quantities of arachidic, behenic and erucic fatty acids are required by the oleochemical sector. Oilseed *Brassica* crops have garnered attention in recent decades not only as a source of food oils, but also as a source of bio-fuel and industrial feedstock. These species have regained popularity for application in cosmetics, lubricating emollients, adhesives, and biodegradable plastic products (McVetty *et al.*, 2016; Cartea *et al.*, 2019). Furthermore, the increased emphasis on renewable energy, chemical feedstock, industrial oils, and the bioeconomy will present major growth potential for industrial oils of *Brassica* species (Cartea *et al.*, 2019).

Brassica nigra (L.) Koch, commonly known as black mustard, is an herbaceous annual plant with an unclear native range; however, it is most possibly native to the southern Mediterranean region (Thomas *et al.*, 2012). A black mustard plant has a strong taproot, massive lower leaves, smaller upper leaves, and a finely covered stem with soft hairs. Black mustard is distinguished from commercial *Brassica* species by the absence of a rosette of basal leaves. The seeds of black mustard are globular in shape, 1-1.6 mm in diameter, dark brown to practically black in colour, finely reticulate, and mucilaginous (Bagchi and Srivastava, 2003). The cultivation of black mustard has recently become increasingly popular and has been transferred to nations other than its home continents, such as Australia and America, as a source of edible oil and seeds (Sahay *et al.*, 2015). The present plant species was selected for its capacity to grow in a wide range of agro-ecological conditions, particularly comparatively low temperatures and disturbed soils, making it well suitable for cultivation for domestic and industrial purposes (Angelova and Ivanova, 2009). In addition, due to the rising usage of black mustard oil and seeds in the food, pharmaceutical, and cosmetic industries, black mustard production is expected to increase dramatically in the coming years (Rahman *et al.*, 2018).

Crop environment is referred to be comprised by agronomic practices such as seed rate, plant density, and fertilizer management, which had a significant effect on plant development, productivity, and quality (Dai *et al.*, 2015; Karydogianni *et al.*, 2022). High density is unfavourable because it promotes interplant competition for resources. Prior research found that optimizing plant density has a considerable impact on crop yield, making it an essential component of most cultivation strategies (Hiltbrunner *et al.*, 2007). Moreover, adequate plant density improves the canopy microclimate, promotes photosynthetic capacity, and considerably increases aboveground biomass accumulation, all of which contribute to higher yields (Scheiner *et al.*, 2002; Dai *et al.*, 2015).

The most critical aspect in plant nutrition is fertilization (Naguib *et al.*, 2012). The type, amount, and method of fertilizer application all have a direct impact on plant nutrient availability and an indirect impact on plant physiological and biochemical pathways (Naguib *et al.*, 2012). Chemical fertilizers are commonly used to boost crop yield, but their long-term use affects soil pH, lowers beneficial soil microflora, pollutes water supplies, and disrupts soil biological systems (Bistgani *et al.*, 2018; Fallah *et al.*, 2018). However, due to the health and environmental benefits, demand for organic products, and particularly for pharmaceutical products, has surged in recent years (Fallah *et al.*, 2018).

Nitrogen (N) is among the most critical nutrient for plant growth, development, and quality, as well as the most complex because of the various forms and activities that can occur during its cycle (Montemurro and Diacono, 2016). It is essential for all plant metabolic functions, and the rate of absorption and partitioning is primarily affected by supply and demand throughout the plant's life cycle (Delogu *et al.*, 1998). The most widely utilized inorganic N fertilizers are urea and urea-containing N fertilizers. Urea, a solid fertilizer contained a high rate of N (46%) contributes for over 56% of total global N fertilizer production (Bremner, 1990; Suter *et al.*, 2016). It is easily stored and applied to crops, and can be combined with other N fertilizers in the soil. In addition to good agricultural practices (for instance, proper application procedures and optimum timing), in order to increase the efficiency of N use, the use of N stabilizers and nitrification inhibitors may potentially delay detrimental processes including NH_3 volatilization, nitrate (NO_3^-) leaching, and N_2O emission reduction (Li *et al.*, 2015). A variety of chemical substances that can be added to urea to prolong the transition of N have been found. These slow-release compounds are classified as (a) urease inhibitors and (b) nitrification inhibitors. Urease inhibitors reduce NO_3^- and NH_4^+ generation in soil, slowing urea breakdown. Furthermore, the presence of the urease inhibitor in the soil affects the efficacy of NH_3 loss management (Li *et al.*, 2015; Wang *et al.*, 2020). As for nitrification inhibitors, because N is a component of the chlorophyll structure, the application of these inhibitors enhances the chlorophyll concentration in the leaves, and hence the biomass, seed yield, and crop quality (Wang *et al.*, 2020).

To the best of our knowledge, there is no available information on the influence of fertilization and plant density on seed oil content, yield, and fatty acids composition of black mustard in Mediterranean semi-arid conditions. Furthermore, further research is needed to develop alternative fertilizing solutions for nitrogen fertilization of crops, such as slow-release inorganic nitrogen fertilizers or organic fertilizers such as compost. The overall purpose of this field trial was to investigate the impact of plant density and fertilization (organic with compost and inorganic with urea with and without urease and nitrification inhibitors) on seed oil yield and quality of the black mustard [*Brassica nigra* (L.) Koch cv. 'ISCI20'] crop in order to develop alternatives to fixed oil sources for multiple purposes in the Mediterranean region.

Materials and Methods

Site description and experimental design

A 2-year field experiment was established in the experimental field of the Agricultural University of Athens (AUA) (37°59′ N and 23°42′ E; 30 m altitude) during the 2019-2020 and 2020-2021 cropping seasons. The main physicochemical soil parameters (at 0-30 cm sampling depth) of the experimental site are the following: the soil was a clay loam (29.4% clay, 35.1% silt, and 35.5% sand) with a pH (1:1 H_2O) of 7.39, 0.143% total nitrogen (N), 15,34% calcium carbonate ($CaCO_3$), a sufficient supply of phosphorus (Olsen P: 13.6 mg kg^{-1} soil) and potassium (K: 233 mg kg^{-1} soil), and 1.67% soil organic matter. The weather data (mean monthly air temperature and precipitation) for the experimental site over the growing seasons were gathered from the automatic weather station (Davis Vantage Pro2 Weather Station; Davis Instruments Corporation, Hayward, CA, USA) of the AUA and are presented in Figure 1. Total rainfall in the cropping seasons 2019-

2020 and 2020-2021 (November to May) was 338.4 mm and 204.4 mm, respectively. During the growing seasons, the average air temperature was 14.3°C in 2019-2020 and 14.4°C in 2020-2021.

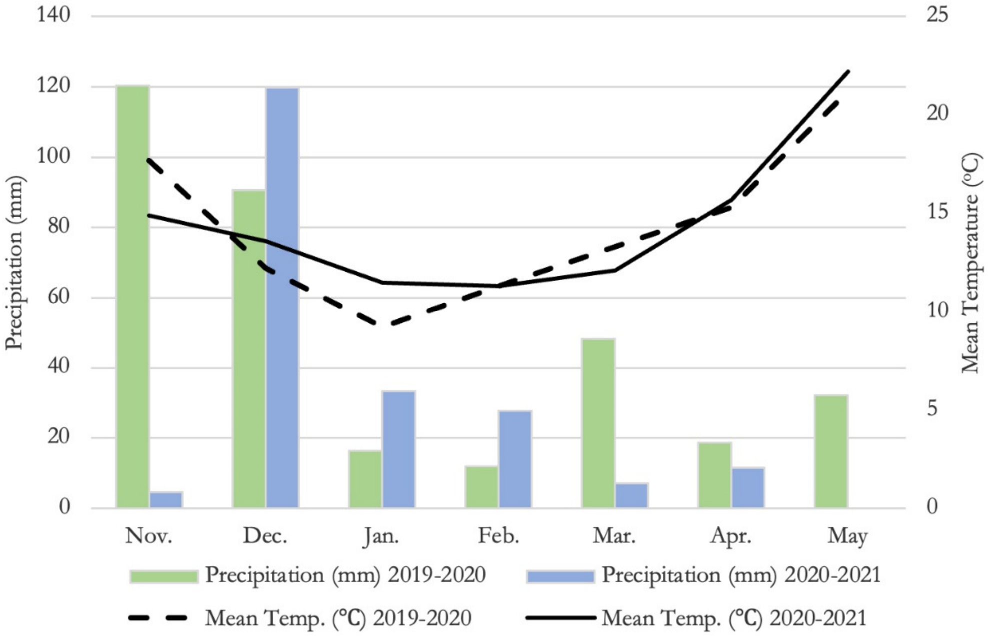


Figure 1. Mean monthly air temperature and precipitation for experimental site during the experimental periods (November-May 2019-2020 and 2020-2021)

The experiment was carried out in an area of 1,015 m^2 using a split-plot design with three replications, in a 2 × 4 factorial scheme. The main plots were plant densities of 46 (PD1) and 76 plants m^{-2} (PD2). Four fertilization treatment sub-plots included untreated (Control), urea (U), urea with Nitrification and Urease Inhibitors (U + NI + UI), and seaweed compost (Compost). The rate of each fertilizer type used in this study is the general recommended dose of the corresponding fertilizer type for black mustard cultivation in clay-loam soils (Thomas *et al*., 2012; Kakabouki *et al.*, 2020). In particular, for urea fertilizers with and without inhibitors, the total applied fertilizer dose was 140 kg N ha^{-1}, whereas the nitrogen application rate for seaweed compost was 50 kg N ha^{-1}. The type of urea fertilizer used was 46-0-0. For the fertilizer with urea and double inhibitors (46-0-0), the nitrification inhibitor was N-((3(5)-methyl-1H-pyrazol-1-yl) methyl) acetamide (MPA; 0.07%) and the urease inhibitor was N-(2-Nitrophenyl) phosphoric triamide (2-NPT; 0.035%). In addition, the nitrogen content of the seaweed compost was 1.98%. The main plot was 140 m^2 (35 m × 4 m) in size, while the sub-plot was 32 m^2 (8 m × 4 m). During each cultivation period, the soil was prepared three days before sowing by mouldboard ploughing to a depth of 25 cm. Fertilizers were distributed by hand as a basal dressing and harrowed into the soil. Seeds of black mustard [*Brassica nigra* (L.) Koch cv. 'ISCI20'] were sown by hand in rows 45 and 30 cm apart for PD1 and PD2, respectively, and 15 cm within each row. For the first and second experimental years, seed sowing took place on November 29th and 24th, respectively. The crop was harvested by hand on June 6, 2020, and May 24, 2021, after the seeds had attained full maturity (seed moisture was about 9%). There were no pests or diseases in the black mustard crop during the cropping periods. Furthermore, weeds were controlled by hand-hoeing as necessary and prior to canopy closure.

Determination of oil content, oil yield and fatty acid composition

For the determination of fixed oil content, seeds weighing a total of 300 g were harvested from each experimental plot. The oil was then extracted using the cold pressing method. Since this method does not use heat or chemicals, it is appreciated by consumers who prefer natural and safe foods. Furthermore, the oil acidity remains low, and the antioxidants and polyphenols are unaffected (Chew, 2020). The oil was extracted at room temperature (25 °C) using a screw expeller SPU 20 (Senta, Serbia) with a capacity of 20-25 kg h^{-1}. The oil samples were stored in plastic bottles (covered in aluminum foil) and kept at room temperature overnight to settle the sediment containing foreign materials. The next day, the samples were centrifuged at 3500 rpm for 15 minutes and filtered through a paper filter (Whatman 41; Whatman International Ltd., UK) in a common glass funnel to remove sediment. The oil samples were then weighed to assess the oil content of the black mustard. The oil content was determined using the following formula:

$$\text{Oil content (\%)} = (m_o * 100)/m_s \qquad (1)$$

Where:
m_o = weight of extracted oil (g),
m_s = sample weight (g)

The fixed oil yield was calculated by multiplying the fixed oil content of the seeds with the seed yield. The fatty acid composition was determined using EU regulation (EC) 2568/1991 (implementing regulation (EC) 2015/1833). Prior to analysis, the fatty acids were converted to fatty acid methyl esters (FAMEs) by stirring a solution of black mustard oil and 3 mL hexane with 0.3 mL of 2N potassium hydroxide methanolic solution for 25 min. A Hewlett-Packard 5890 series II GC chromatograph (Hewlett-Packard Corporation, Palo Alto, California, USA) equipped with a flame ionization detector (FID) and a single capillary column HP-5ms GC (30 m × 0.25 mm i.d. × 0.25 film thickness, Hewlett-Packard Corp.) was utilized to analyse the FAMEs. The carrier gas was helium at a flow rate of 1 mL min^{-1}. The temperatures in the initial and final columns were 170°C and 230 °C, respectively, and the temperature increased at a rate of 4 °C min^{-1}. The temperature of the injector and the detector were both 230 °C. The sample volume was 1 mL, and it was manually introduced into the infusion system using the splitless technique. Data were analysed by Hewlett-Packard 3365 Chemstation data analysis software (Hewlett-Packard Corp.). In the current study, the thirteen major fatty acids (~95%) identified in black mustard oil were studied and expressed as the relative fraction of each individual fatty acid contained in the sample. The thirteen main fatty acids discovered were as follows: pentadecanoic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 *n-9*), vaccenic acid (C18:1 *n*-7), linoleic acid (C18:2 *n*-6), α-linolenic acid (C18:3 *n*-3), arachidic acid (C20:0), cis-11-eicosenoic acid (C20:1), cis-11,14-eicosadienoic acid (C20:2 *n*-6), behenic acid (C22:0), erucic acid (C22:1) and nervonic acid (C24:1). In addition, the following fatty acid combinations were also calculated: omega-3 (*n*-3) fatty acids, omega-6 (*n*-6) fatty acids, total saturated fatty acids (SAFA), total monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), PUFA/SAFA ratio, and *n*-6/*n*-3 ratio.

Statistical analysis

Data analysis procedures were conducted using SigmaPlot statistical software package (ver. 12.0; Systat Software Inc., San Jose, CA, USA). A mixed model was employed for the analysis of variance (ANOVA), with years and replications as random effects and plant density and fertilization as fixed effects. Differences among means were separated by Tukey's honestly significant difference test (Tukey's HSD). In order to estimate the levels of correlation between the studied parameters, Pearson's correlation analysis was performed. For all statistical analyses, comparisons were made at the 5% level of significance.

Results

Regarding the results of the two-year data analysis (Table 1), plant density × fertilization interaction was only significant on seed yield. Moreover, year × plant density interaction was found to be statistically significant for omega-6 (*n*-6) fatty acids. With the exception of monounsaturated fatty acids (MUFA), polyunsaturated to saturated fatty acids ratio (PUFA/SAFA), and omega-6 to omega-3 fatty acids ratio (*n*-6/*n*-3), the main effect of plant density was significant on seed yield, fixed oil content, and yield, as well as on fatty acids composition of black mustard oil and their combinations. As for the fertilization effect, the different regimes had a significant effect on all studied traits. In addition, the main effect of the year had a substantial impact on nervonic acid (C24:1), linoleic acid (C18:2 *n*-6), MUFA, *n*-6, and *n*-6/*n*-3 ratio (Table 1).

Table 1. Combined analysis of variance (*F* values) for the effects of plant density and fertilization on all measured traits of black mustard during the two-year experiment

Source of Variance	**Df**	**Seed Yield**	**Fixed Oil Content**	**Fixed Oil Yield**	**Pentadecanoic acid (C15:0)**	**Palmitic acid (C16:0)**	**Stearic acid (C18:0)**
Year (Y)	1	0.674^{ns}	0.063^{ns}	0.285^{ns}	0.003^{ns}	0.813^{ns}	0.947^{ns}
Plant Density (PD)	1	33.128***	22.588***	32.398***	12.732**	27.427***	12.547**
Fertilization (F)	3	36.537***	26.879***	31.226***	18.305***	19.060***	16.015***
Y × PD	1	0.009^{ns}	0.014^{ns}	0.003^{ns}	0.031^{ns}	0.036^{ns}	0.002^{ns}
Y × F	3	0.005^{ns}	0.066^{ns}	0.009^{ns}	0.106^{ns}	0.018^{ns}	0.004^{ns}
PD × F	3	3.454*	1.433^{ns}	4.098^{ns}	1.124^{ns}	0.228^{ns}	1.014^{ns}
Y × PD × F	3	0.002^{ns}	0.188^{ns}	0.041^{ns}	0.033^{ns}	0.017^{ns}	0.007^{ns}

Source of Variance	**Df**	**Arachidic acid (C20:0)**	**Behenic acid (C22:0)**	**Oleic acid (C18:1 *n-9*)**	**Vaccenic acid (C18:1 *n*-7)**	**cis-11-Eicosenoic acid (C20:1)**	**Erucic acid (C22:1)**
Year (Y)	1	0.952^{ns}	2.131^{ns}	4.529*	0.934^{ns}	1.337^{ns}	2.113^{ns}
Plant Density (PD)	1	12.077**	31.599***	13.108**	27.175***	11.329**	10.959**
Fertilization (F)	3	15.770***	24.558***	7.405***	19.229***	14.919***	15.947***
Y × PD	1	0.001^{ns}	0.055^{ns}	0.921^{ns}	0.022^{ns}	0.050^{ns}	0.119^{ns}
Y × F	3	0.004^{ns}	0.008^{ns}	0.206^{ns}	0.020^{ns}	0.064^{ns}	0.016^{ns}
PD × F	3	1.005^{ns}	0.978^{ns}	0.495^{ns}	0.223^{ns}	0.665^{ns}	0.821^{ns}
Y × PD × F	3	0.001^{ns}	0.061^{ns}	0.220^{ns}	0.012^{ns}	0.061^{ns}	0.026^{ns}

Source of Variance	**Df**	**Nervonic acid (C24:1)**	**Linoleic acid (C18:2 *n*-6)**	**α-Linolenic acid (C18:3 *n*-3)**	**cis-11,14-Eicosadienoic acid (C20:2 *n*-6)**	**Saturated Fatty Acids (SAFA)**	**Monounsaturated Fatty Acids (MUFA)**
Year (Y)	1	9.681**	15.042***	3.927^{ns}	3.252^{ns}	0.225^{ns}	10.343**
Plant Density (PD)	1	17.833***	11.829**	12.102**	37.083***	26.394***	0.721^{ns}
Fertilization (F)	3	5.121**	7.717***	13.561***	31.298***	6.830**	11.254***
Y × PD	1	2.667^{ns}	5.862*	0.029^{ns}	0.034^{ns}	0.012^{ns}	0.226^{ns}
Y × F	3	0.062^{ns}	0.188^{ns}	0.536^{ns}	0.006^{ns}	0.003^{ns}	0.065^{ns}
PD × F	3	0.531^{ns}	1.162^{ns}	0.819^{ns}	1.217^{ns}	0.581^{ns}	0.485^{ns}
Y × PD × F	3	0.015^{ns}	0.020^{ns}	0.137^{ns}	0.012^{ns}	0.003^{ns}	0.096^{ns}

Source of Variance	**Df**	**Polyunsaturated Fatty Acids (PUFA)**	***n*-6**	***n*-3**	**PUFA/SAFA**	***n*-6/*n*-3**
Year (Y)	1	0.444^{ns}	13.790***	3.927^{ns}	0.897^{ns}	7.424*
Plant Density (PD)	1	33.268***	13.446***	12.102**	2.979^{ns}	3.113^{ns}
Fertilization (F)	3	15.336***	6.928**	13.561***	6.693**	11.516***
Y × PD	1	2.123^{ns}	5.683*	0.029^{ns}	0.786^{ns}	0.272^{ns}
Y × F	3	0.809^{ns}	0.187^{ns}	0.536^{ns}	0.375^{ns}	0.418^{ns}
PD × F	3	0.923^{ns}	1.060^{ns}	0.819^{ns}	2.006^{ns}	0.378^{ns}
Y × PD × F	3	0.122^{ns}	0.020^{ns}	0.137^{ns}	0.045^{ns}	0.108^{ns}

F-test ratios are from ANOVA. ns, *, ** and ***: Not-significant and significant at 5%, 1% and 0.1% probability levels, respectively.

Seed yield, oil content and yield of black mustard

The present research demonstrated that plant density and fertilization had a significant impact on the seed yield of the black mustard crop (Table 2). Seed yield was higher in low-density plots (46 plants m^{-2}) than in high-density plots (76 plants m^{-2}) during the two experimental periods, with low-density plants yielding 1817.9 and 1766.0 kg ha^{-1} in 2019-2020 and 2020-2021, respectively. In response to fertilization, the highest yields were found in plots fertilized with inorganic fertilizers. Specifically, the highest values were presented in urea fertilizer with nitrification and urease inhibitors (U + NI + UI) (2011.3 and 1957.4 kg ha^{-1} for the first and second cropping season, respectively) which had no statistically significant differences with urea without inhibitors (1815.9 and 1764.2 kg ha^{-1} for the respective cropping seasons).

Table 2. Seed yield, oil content, and oil yield as influenced by the plant density and fertilization

Treatment	Seed yield (kg ha^{-1})	Oil content (%)	Oil yield (kg ha^{-1})
2019-2020			
Plant Density			
46 plants m^{-2}	1817.9 A	33.27 A	630.7 A
76 plants m^{-2}	1486.1 B	28.39 B	432.9 B
Fertilization			
Control	1205.7 c	24.32 c	297.8 c
Urea	1815.9 ab	33.00 ab	612.9 ab
Urea + NI + UI	2011.3 a	36.94 a	753.7 a
Compost	1575.0 b	29.05 bc	462.8 bc
Source of Variation			
$F_{Plant\ Density}$	15.317**	7.455*	12.772**
$F_{Fertilization}$	16.736***	9.159***	12.549***
$F_{Plant\ Density \times Fertilization}$	1.594^{ns}	0.259^{ns}	1.383^{ns}
2020-2021			
Plant Density			
46 plants m^{-2}	1766.0 A	33.13 A	612.4 A
76 plants m^{-2}	1444.9 B	28.01 B	414.2 B
Fertilization			
Control	1170.3 c	23.49 c	278.4 d
Urea	1764.2 a	33.13 a	596.5 b
Urea + NI + UI	1957.4 a	36.32 a	726.6 a
Compost	1529.9 b	29.32 b	451.5 c
Source of Variation			
$F_{Plant\ Density}$	18.154***	21.201***	22.106***
$F_{Fertilization}$	20.217***	24.574***	20.907***
$F_{Plant\ Density \times Fertilization}$	1.896^{ns}	2.228^{ns}	3.253^{ns}

F-test ratios are from ANOVA. ns, *, ** and ***: Not-significant and significant at 5%, 1% and 0.1% probability levels, respectively. The capital letters compare plant densities within a growing season, and lowercase letters compare fertilization treatments within a growing season, by Tukey's HSD test ($p \leq 0.05$).

Seed oil content was significantly affected by both plant density and fertilization during the two-year study (Table 2). The oil content was substantially higher in the plants of low-density plots, and the values were 33.27% and 33.13% for the first and second experimental year, respectively. In regard to fertilization, the mean values of seed oil content were greatest in urea with nitrification and urease inhibitors (36.94% and 36.32% in 2019-2020 and 2020-2021, respectively) followed by urea without inhibitors (33.13 and 33.00% in 2019-2020 and 2020-2021, respectively), while the lowest values (24.32% and 23.49% for the respective years) were found in the untreated (control) plants.

Seed oil yield was estimated by multiplying the seed yield of the black mustard crop and the oil content of the crop's seeds. According to the combined analysis (Table 1), seed oil yield was influenced by plant density and fertilization. In the low-density plots, the values of oil yield were substantially higher (630.7 and 612.4 kg ha^{-1} in the first and second experimental year, respectively) than those of high-density plots (432.9 and 414.2 kg ha^{-1} in the first and second experimental year, respectively) (Table 2). Averaged over plant densities, the highest values of oil yield were found in plants treated with urea with nitrification and urease inhibitors (753.7 and 726.6 kg ha^{-1} in the first and second experimental year, respectively) followed by urea without inhibitors (612.9 and 596.5 kg ha^{-1} for the respective years).

Fatty acids composition and ratios of seed oil

In the current study, the thirteen main fatty acids detected in black mustard seed oil were studied and expressed as the relative percentage of each individual fatty acid present in the studied oil sample. In particular, the thirteen major fatty acids observed that accounted for about 95% of total fatty acids were pentadecanoic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 *n-9*), vaccenic acid (C18:1 *n*-7), linoleic acid (C18:2 *n*-6), α-linolenic acid (C18:3 *n*-3), arachidic acid (C20:0), cis-11-eicosenoic acid (C20:1), cis-11,14-eicosadienoic acid (C20:2 *n*-6), behenic acid (C22:0), erucic acid (C22:1) and nervonic acid (C24:1) (Table 3).

The combined analysis of variance (Table 1) showed that the contents of saturated (SAFA: pentadecanoic, palmitic, stearic, arachidic and behenic acid), monounsaturated (MUFA: oleic, vaccenic, cis-11-eicosenoic, erucic, and nervonic acid) and polyunsaturated fatty acids (PUFA: linoleic, α-linolenic and cis-11,14-eicosadienoic acid) were significantly affected by fertilization during the two-year experiment. Specifically, averaged over the plant densities, the highest values of SAFA were observed in plots with seaweed compost (6.97% and 7.05% w/w in 2019-2020 and 2020-2021, respectively) followed by urea with nitrification and urease inhibitors (6.77% and 6.86% w/w in 2019-2020 and 2020-2021, respectively) and untreated (6.74% and 6.81% w/w in 2019-2020 and 2020-2021, respectively). Concerning the MUFA, the highest content was observed in the case of untreated plants, with the values being 54.29% and 58.20% w/w in the first and second experimental period, respectively. As for PUFA content, the highest values were achieved in untreated (control: 68.25% and 67.25% w/w in the first and second growing season, respectively) plots (Table 4).

In response to plant density, this had also a significant effect on SAFA and PUFA contents (Table 1). During the two-year experiment, the mean values of SAFA (7.04% and 7.09% w/w in the first and second experimental year, respectively) and PUFA (35.05% and 34.05% w/w in the first and second experimental year, respectively) were greatest in low-density plots (Table 4).

Palmitic acid presented the highest content of SAFA. Specifically, plant density seems to had a statistically significant impact on the values of this trait throughout the two-year experiment and the highest content was found in low-density plots (46 plants m^{-2}), with the values being 3.57% and 3.51% w/w in the first and second growing season, respectively (Table 3). Fertilization had an equally significant effect, with compost (3.64% and 3.59% w/w in 2019-2020 and 2020-2021, respectively) and urea fertilizer with nitrification and urease inhibitors (3.54% and 3.51% w/w in 2019-2020 and 2020-2021, respectively) showing higher values as compared to those of untreated (control: 3.10 and 3.04 % w/w for the respective years).

MUFA consisted of five fatty acids (oleic, vaccenic, cis-11-eicosenoic, erucic, and nervonic acid), and the highest contents were presented by erucic and oleic acid (Table 3). Concerning the erucic acid, the content values were substantially higher in the seed oil of low-density plants, and the values were 20.01% and 20.92% w/w for the first and second experimental year, respectively. In regard to fertilization, this had a significant negative influence on the erucic acid content, with the highest values (22.78% and 23.70% w/w in the first and second experimental year, respectively) obtained in the control plots.

Table 3. Fatty acids composition of seed oil as influenced by the plant density and fertilization

Treatment	*Saturated Fatty Acids (SAFA)*					*Monounsaturated Fatty Acids (MUFA)*					*Polyunsaturated Fatty Acids (PUFA)*		
	Pentadecanoic acid (C15:0) (% w/w)	**Palmitic acid (C16:0) (% w/w)**	**Stearic acid (C18:0) (% w/w)**	**Arachidic acid (C20:0) (% w/w)**	**Behenic acid (C22:0) (% w/w)**	**Oleic acid (C18:1 *n-9*) (% w/w)**	**Vaccenic acid (C18:1 *n*-7) (% w/w)**	**cis-11-Eicosenoic acid (C20:1) (% w/w)**	**Erucic acid (C22:1) (% w/w)**	**Nervonic acid (C24:1) (% w/w)**	**Linoleic acid (C18:2 *n*-6) (% w/w)**	**α-Linolenic acid (C18:3 *n*-3) (% w/w)**	**cis-11,14-Eicosadienoic acid (C20:2 *n*-6) (% w/w)**
2019-2020													
	Plant Density												
46 plants m^{-2}	0.36 A	3.57 A	1.77 A	0.84 A	0.50 A	14.75 B	1.51 A	12.13 A	20.01 A	1.06 B	24.21 A	9.84 A	1.01 A
76 plants m^{-2}	0.31 B	3.26 B	1.51 B	0.72 B	0.44 B	18.77 A	1.38 B	10.36 B	17.04 B	1.49 A	22.36 B	8.37 B	0.90 B
	Fertilization												
Control	0.26 c	3.10 c	2.02 a	0.96 a	0.42 c	15.22 bc	1.31 c	13.81 a	22.78 a	1.19 a	24.23 a	7.09 c	0.83 c
Urea	0.32 bc	3.38 b	1.29 c	0.61 c	0.47 b	19.72 a	1.42 b	8.83 c	14.58 c	1.41 a	22.65 bc	8.93 b	0.94 b
Urea + NI + UI	0.33 b	3.54 ab	1.62 b	0.76 b	0.50 ab	18.39 ab	1.49 ab	11.09 b	18.29 b	1.42 a	22.26 c	9.14 b	1.01 ab
Compost	0.41 a	3.64 a	1.64 b	0.78 b	0.52 a	13.71 c	1.54 a	11.20 b	18.45 b	1.07 a	24.21 ab	11.24 a	1.03 a
	Source of Variation												
$F_{Plant\ Density}$	5.988*	15.567**	6.102*	6.058*	19.917***	7.665*	15.493**	6.407*	6.621*	12.578**	15.207**	6.214*	20.991***
$F_{Fertilization}$	7.947**	9.546***	7.881**	8.036**	14.356***	3.646*	9.901***	8.242**	8.401**	2.007^{ns}	4.279*	8.276**	16.305***
$F_{Plant\ Density\ \times\ Fertilization}$	0.628^{ns}	0.183^{ns}	0.489^{ns}	0.507^{ns}	0.864^{ns}	0.296^{ns}	0.174^{ns}	0.526^{ns}	0.545^{ns}	0.191^{ns}	0.477^{ns}	0.598^{ns}	0.727^{ns}
2020-2021													
	Plant Density												
46 plants m^{-2}	0.35 A	3.51 A	1.84 A	0.87 A	0.51 A	17.46 B	1.48 A	12.59 A	20.92 A	1.40 B	22.23 A	10.79 A	1.03 A
76 plants m^{-2}	0.31 B	3.23 B	1.58 B	0.75 B	0.46 B	19.79 A	1.36 B	11.03 B	18.50 A	1.59 A	21.90 A	9.17 B	0.94 B
	Fertilization												
Control	0.27 c	3.04 c	2.08 a	0.98 a	0.43 c	17.78 ab	1.28 c	14.01 a	23.70 a	1.43 ab	22.69 a	7.97 c	0.86 c
Urea	0.31 bc	3.33 b	1.37 c	0.64 c	0.48 b	20.73 a	1.40 b	9.53 c	15.99 c	1.67 a	21.40 b	9.37 bc	0.97 b
Urea + NI + UI	0.33 b	3.51 ab	1.69 b	0.80 b	0.51 ab	19.76 a	1.48 ab	11.80 b	19.46 b	1.59 a	21.38 b	10.94 ab	1.04 ab
Compost	0.42 a	3.59 a	1.71 b	0.81 b	0.52 a	16.24 b	1.52 a	11.91 ab	19.67 b	1.31 b	22.79 a	11.64 a	1.06 a
	Source of Variation												
$F_{Plant\ Density}$	6.936*	12.085**	6.455*	6.019*	12.593**	5.604*	11.959**	4.964*	4.437^{ns}	5.266*	0.595^{ns}	5.949*	16.412***
$F_{Fertilization}$	10.980***	9.532***	8.142**	7.734**	10.732***	4.151*	9.386***	6.733**	7.554**	3.841*	3.529*	6.084**	15.076***
$F_{Plant\ Density\ \times\ Fertilization}$	0.509^{ns}	0.068^{ns}	0.526^{ns}	0.498^{ns}	0.262^{ns}	0.491^{ns}	0.069^{ns}	0.199^{ns}	0.298^{ns}	0.448^{ns}	0.739^{ns}	0.383^{ns}	0.515^{ns}

F-test ratios are from ANOVA. ns, *, ** and ***: Not-significant and significant at 5%, 1% and 0.1% probability levels, respectively. The capital letters compare plant densities within a growing season, and lowercase letters compare fertilization treatments within a growing season, by Tukey's HSD test ($p \leq 0.05$).

Table 4. Fatty acids ratios of seed oil as influenced by the plant density and fertilization

Treatment	Saturated Fatty Acids (SAFA) (% w/w)	Monounsaturated Fatty Acids (MUFA) (% w/w)	Polyunsaturated Fatty Acids (PUFA) (% w/w)	*n*-6 (% w/w)	*n*-3 (% w/w)	PUFA/SAFA	*n*-6/*n*-3
2019-2020							
Plant Density							
46 plants m^{-2}	7.04 A	49.46 A	35.05 A	25.22 A	9.84 A	5.00 A	2.72 A
76 plants m^{-2}	6.25 B	49.01 A	31.64 B	23.27 B	8.37 B	5.08 A	2.89 A
Fertilization							
Control	6.74 a	54.29 a	32.16 b	25.07 a	7.09 c	4.79 b	3.59 a
Urea	6.08 b	45.96 b	32.53 b	23.60 bc	8.93 b	5.22 a	2.73 b
Urea + NI + UI	6.77 a	50.69 ab	32.41 b	23.27 c	9.14 b	4.81 b	2.64 b
Compost	6.97 a	45.97 b	36.28 a	25.04 ab	11.24 a	5.35 a	2.26 b
Source of Variation							
$F_{Plant\ Density}$	13.807^{**}	0.059^{ns}	18.459^{***}	15.893^{**}	6.214^{*}	0.409^{ns}	0.656^{ns}
$F_{Fertilization}$	3.326^{*}	4.924^{*}	6.087^{**}	3.740^{*}	8.276^{**}	5.085^{*}	6.657^{**}
$F_{Plant\ Density \times Fertilization}$	0.319^{ns}	0.269^{ns}	0.219^{ns}	0.426^{ns}	0.598^{ns}	1.130^{ns}	0.355^{ns}
2020-2021							
Plant Density							
46 plants m^{-2}	7.09 A	53.86 A	34.05 A	23.26 A	10.79 A	4.82 A	2.26 A
76 plants m^{-2}	6.34 B	52.28 A	32.01 B	22.84 A	9.17 B	5.07 A	2.58 A
Fertilization							
Control	6.81 a	58.20 a	31.52 c	23.54 ab	7.97 c	4.67 b	2.99 a
Urea	6.14 b	49.32 c	31.74 c	22.37 b	9.37 bc	5.18 a	2.42 b
Urea + NI + UI	6.86 a	54.10 ab	33.37 b	22.42 b	10.94 ab	4.89 b	2.18 b
Compost	7.05 a	50.64 bc	35.49 a	23.86 a	11.64 a	5.06 ab	2.08 b
Source of Variation							
$F_{Plant\ Density}$	12.603^{**}	1.055^{ns}	15.853^{**}	0.971^{ns}	5.949^{*}	2.996^{ns}	3.180^{ns}
$F_{Fertilization}$	3.507^{*}	6.695^{**}	12.861^{***}	3.309^{*}	6.084^{**}	2.362^{*}	4.977^{*}
$F_{Plant\ Density \times Fertilization}$	0.264^{ns}	0.319^{ns}	1.253^{ns}	0.696^{ns}	0.383^{ns}	0.946^{ns}	0.082^{ns}

F-test ratios are from ANOVA. ns, *, ** and ***: Not-significant and significant at 5%, 1% and 0.1% probability levels, respectively. The capital letters compare plant densities within a growing season, and lowercase letters compare fertilization treatments within a growing season, by Tukey's HSD test ($p \leq 0.05$).

As for the oleic acid, which presented the second higher content among monounsaturated fatty acids, the effects of plant density and fertilization on its content are presented in Table 3. In the high-density plots, the values of oleic acid content were substantially higher (18.77% and 19.49% w/w in 2019-2020 and 2020-2021, respectively) than those of low-density treatment (14.75% and 17.46% w/w for the first and second experimental year, respectively). Furthermore, the mean values of oleic acid content presented good evidence of the fertilization effect. Averaged over plant densities, the highest values were presented in urea fertilizer without inhibitors (19.72% and 20.73% w/w in the first and second cropping season, respectively) followed by urea with nitrification and urease inhibitors (18.39% and 19.76% w/w for the respective years).

In the category of PUFA, the highest content was obtained from linoleic acid, and, in fact, it shown the highest content in relation to all other fatty acids of seed oil (Table 3). The differences among the different plant density plots in terms of linoleic acid content were statistically significant, with the highest values (24.21% and 22.23% w/w in 2019-2020 and 2020-2021, respectively) recorded when plants subjected to low-density. Concerning the fertilization effect, untreated (control: 24.23% and 22.69% w/w in the first and second experimental year, respectively) and compost (24.21% and 22.79% w/w in the first and second experimental year, respectively) presented substantially higher mean values in linoleic acid content.

PUFA are of two types omega 6 (*n-6*) and omega 3 (*n-3*) fatty acids. In the present study, *n-6* fatty acids consisted of linoleic and cis-11,14-eicosadienoic acid. According to the combined analysis of variance (Table 1), *n-6* fatty acids content was affected by both different plant densities and fertilization treatments. *n-6* fatty acids content did not differ among plant densities in 2020-2021; however, significant differences were found in the first experimental year (2019-2020); where the highest value (25.22% w/w) was obtained from the seed oil of low-density plants (Table 4). Concerning the effect of fertilization, the highest values were found in compost (25.04% and 23.86% w/w in the first and second cropping season, respectively) and untreated (control: 25.07% and 23.54% w/w for the respective years) plots.

The results of the present study indicated that omega 3 (*n-3*) fatty acids of black mustard seed oil consisted only of α-linolenic acid (Tables 3 and 4). The differences among plants of different plant densities were statistically significant and the highest values in α-linolenic acid content (9.84% and 10.79% w/w in 2019-2020 and 2020-2021, respectively) were found in plots of low-density (46 plants m^{-2}). In the same manner, fertilization was found to be statistically significant throughout the growing seasons, where compost (11.24% and 11.64% w/w in the first and second experimental year, respectively) presented statistically higher α-linolenic acid content values.

The ratio of PUFA/SAFA was not affected by the plant densities, but it was only influenced by the different fertilization regimes. Specifically, the highest PUFA/SAFA ratio was found in the case of compost (5.35 and 5.06 in 2019-2020 and 2020-2021, respectively) and urea without inhibitors (5.22 and 5.18 in 2019-2020 and 2020-2021, respectively) fertilization, while the lowest values were obtained from urea with nitrification and urease inhibitors (4.81 and 4.89 in the first and second cropping season, respectively) and untreated (control: 4.79 and 4.67 for the respective years) plots (Table 4).

Concerning the ratio of *n-6*/*n-3*, there were no significant differences among the high and low-density plots; however, the plants of high-density treatment (76 plants m^{-2}) presented slightly higher values of this trait (2.89 and 2.58 in 2019-2020 and 2020-2021, respectively) that those of the low-density treatment (46 plants m^{-2}: 2.72 and 2.26 for the respective years) (Table 4). In response to fertilization, this had a significant effect on this trait, and the highest values were found in untreated (control: 3.59 and 2.99 in 2019-2020 and 2020-2021, respectively) plots followed by urea without inhibitors (2.73 and 2.42 in 2019-2020 and 2020-2021, respectively), urea with nitrification and urease inhibitors (2.64 and 2.18 in 2019-2020 and 2020-2021, respectively) and compost (2.26 and 2.08 for the respective years).

Discussion

During the two-year trial, plant density had a substantial effect on the seed yield of the black mustard crop. The two-year average value of seed yield was 22.3% higher with a plant density of 46 plants m^{-2} than at a density of 76 plants m^{-2}. Plant densities of 60-70 plants m^{-2} are generally regarded optimal for oilseed rape (*Brassica napus* L.) hybrids in Europe (Rathke *et al.*, 2006), whereas the usual plant density of hybrid oilseed rape in China is around 30 plants m^{-2} (Momoh and Zhou, 2001; Kuai *et al.*, 2015). In general, the yield of oilseed rape and other *Brassica* species typically demonstrates a quadratic response to plant density, with a relatively close increase along a spectrum of low densities, an incremental decline in yield increase rate, and eventually, a maximum yield at the optimum plant density, which is dependent on crop species, environmental conditions, and cultivation practices (Leach *et al.*, 1999; Kuai *et al.*, 2015). In terms of fertilization, different fertilizations had a significant response on the seed yield of black mustard. The two-year average value of seed yield was statistically considerably higher in plots that had received urea with nitrification and urease inhibitors (1984.4 kg ha^{-1}) followed by urea without inhibitors (1790.1 kg ha^{-1}), and the lowest yield was offered by the control (1188.0 kg ha^{-1}). Several researchers have noticed greater yields in various crops fertilized with inorganic fertilizers since these fertilizers contained soluble inorganic nitrogen with quick availability to cultivated plant species, resulting in enhanced development and greater yields (Mengel and Rehm, 2012; Kakabouki *et al.*, 2019; Zapletalová *et al.*, 2021).

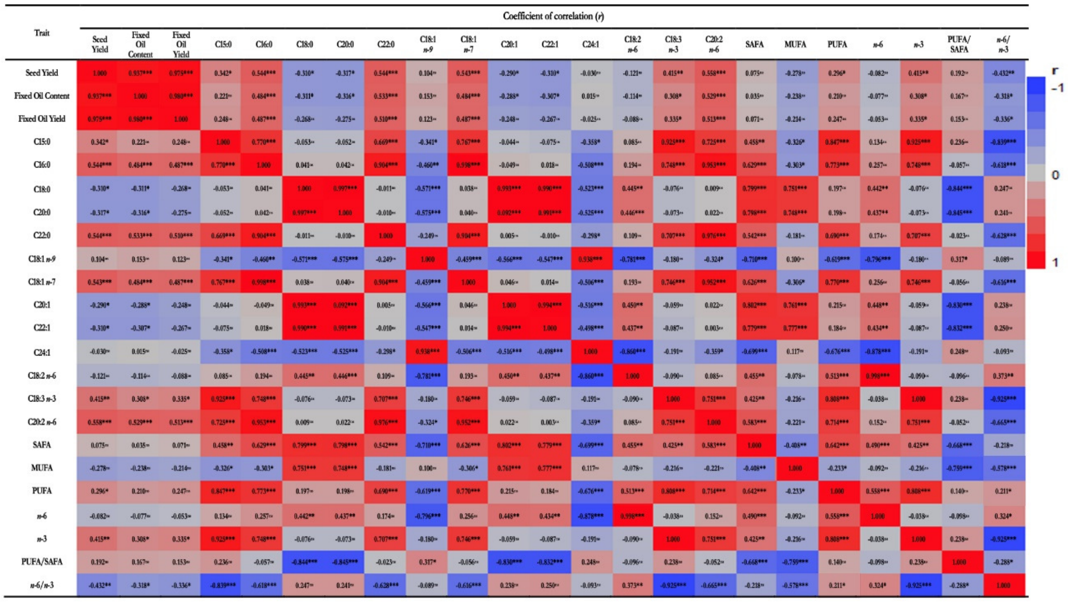
Higher-efficiency fertilizers coated with urease or nitrification inhibitors could be used to prevent nitrogen loss in the soil. Several studies have revealed that utilizing urease or nitrification inhibitors in combination with nitrogen fertilizers is among the most promising new nitrogen loss prevention strategies

(Zaman *et al.*, 2008; Wang *et al.*, 2020). Using inhibitors in combination with nitrogen fertilizers has been found to be a highly effective method for reducing nitrogen fertilizer losses while also improving plant growth and productivity (Drury *et al.*, 2017). A recent research study proved that the use of inhibitors increased the seed yield of maize by 5-7% (Drury *et al.*, 2017).

Regarding the effect of plant density on seed oil content, statistically significant differences were recorded among the different plant densities and the highest content recorded at the plant density of 46 plants m^{-2} with the two-year average being 17.7% higher than the density of 76 plants m^{-2}. These findings are in line with those of Leach *et al.* (1999) and Zhang *et al.* (2012), who discovered that plant densities greater than 50-60 plants m^{-2} significantly reduce seed oil content in oilseed rape crop. In response to the fertilization effect, the two-year average values of the oil content were substantially higher in the plots that had received the urea fertilizer with inhibitors (36.63% w/w) and urea without inhibitors (33.07% w/w), while the lowest value was presented by untreated (23.91% w/w). These results are consistent with the results of Siadat *et al.* (2011), where they found that high doses of nitrogen (from 160 kg N ha^{-1} and over) in rapeseed (*Brassica napus* L. var. *oleifera*) can significantly reduce the seed oil, while lower doses have positive effect on this property. In addition, it is worth underline that according to Sharafi *et al.* (2015), the seed oil content in black mustard crop cultivated under normal cultural practices in Iran ranged from 17 to 24%.

Since the components of the fixed oil yield, namely seed crop yield and seed oil content, showed a comparable trend (Table 2), this trait followed a similar pattern. Low plant density (46 plants m^{-2}) had a two-year average value of 621.6 kg ha^{-1}, which was much greater than of the high-density (76 plants m^{-2}), presenting a two-year average value of 423.6 kg ha^{-1}. In terms of the fertilization effect on this attribute, the highest two-year average values were reported in urea with and without nitrification and urease inhibitors, with values being 61.9% and 32.3% higher than the untreated (control), respectively. The above-mentioned are confirmed by the very strong and positive correlations of fixed oil yield with seed yield and fixed oil content ($r = 0.937, p < 0.001$; $r = 0.975, p < 0.001$, respectively; Table 5).

The seed oil of black mustard contained five saturated fatty acids (SAFA), pentadecanoic, palmitic, stearic, arachidic, and behenic acid, of which the highest content was presented in palmitic acid, with two-year average values ranging from 3.37% to 3.42% w/w. The seed oil also contained five monounsaturated fatty acids (MUFA), namely oleic, vaccenic, cis-11-eicosenoic, erucic, and nervonic acid with the highest contents recorded in erucic and oleic acid, with the two-year average values ranging from 18.53% to 19.71% w/w for erucic acid and from 16.76% to 18.63% w/w for oleic acid, respectively. Finally, its polyunsaturated fatty acids (PUFA), which overall had the highest content of other fatty acids, included three fatty acids, linoleic, α-linolenic, and cis-11,14-eicosadienoic acid. Linoleic acid was the fatty acid with the highest content, and its two-year average values ranged from 22.07% to 23.29% w/w. These findings are in agreement with previous research on black mustard conducted in various geographical locations (Mejia-Garibay *et al.*, 2015; Sharafi *et al.* 2015; Cartea *et al.*, 2019; Kaur *et al.*, 2022).



Trait	Coefficient of correlation (*r*)																					
	Seed Yield	Fixed Oil Content	Fixed Oil Yield	C15:0	C16:0	C18:0	C20:0	C22:0	C18:1 *n-9*	C18:1 *n-7*	C20:1	C22:1	C24:1	C18:2 *n-6*	C18:3 *n-3*	C20:2 *n-6*	SAFA	MUFA	PUFA	*n-6*	*n-3*	PUFA/ SAFA	*n-6*/ *n-3*
Seed Yield	1.000	0.937***	0.975***	0.342*	0.544***	-0.310*	-0.317*	0.544***	0.104ns	0.543***	-0.290*	-0.310*	-0.030ns	-0.121ns	0.415**	0.558***	0.075ns	-0.278ns	0.296*	-0.082ns	0.415**	0.192ns	-0.432**
Fixed Oil Content	0.937***	1.000	0.980***	0.221ns	0.484***	-0.311*	-0.316*	0.533***	0.153ns	0.484***	-0.288*	-0.307*	0.015ns	-0.114ns	0.308*	0.529***	0.035ns	-0.238ns	0.210ns	-0.077ns	0.308*	0.167ns	-0.318*
Fixed Oil Yield	0.975***	0.980***	1.000	0.248ns	0.487***	-0.268ns	-0.275ns	0.510***	0.123ns	0.487***	-0.248ns	-0.267ns	-0.025ns	-0.088ns	0.335*	0.513***	0.071ns	-0.214ns	0.247ns	-0.053ns	0.335*	0.153ns	-0.336*
C15:0	0.342*	0.221ns	0.248ns	1.000	0.770***	-0.053ns	-0.052ns	0.669***	-0.341*	0.767***	-0.044ns	-0.075ns	-0.358*	0.085ns	0.925***	0.725***	0.458**	-0.326*	0.847***	0.134ns	0.925***	0.236ns	-0.839***
C16:0	0.544***	0.484***	0.487***	0.770***	1.000	0.041ns	0.042ns	0.904***	-0.460**	0.998***	-0.049ns	0.018ns	-0.508***	0.194ns	0.748***	0.953***	0.629***	-0.303*	0.773***	0.257ns	0.748***	-0.057ns	-0.618***
C18:0	-0.310*	-0.311*	-0.268ns	-0.053ns	0.041ns	1.000	0.997***	-0.011ns	-0.571***	0.038ns	0.993***	0.990***	-0.523***	0.445**	-0.076ns	0.009ns	0.799***	0.751***	0.197ns	0.442**	-0.076ns	-0.844***	0.247ns
C20:0	-0.317*	-0.316*	-0.275ns	-0.052ns	0.042ns	0.997***	1.000	-0.010ns	-0.575***	0.040ns	0.092***	0.991***	-0.525***	0.446***	-0.073ns	0.022ns	0.798***	0.748***	0.198ns	0.437**	-0.073ns	-0.845***	0.241ns
C22:0	0.544***	0.533***	0.510***	0.669***	0.904***	-0.011ns	-0.010ns	1.000	-0.249ns	0.904***	0.005ns	-0.010ns	-0.298*	0.109ns	0.707***	0.976***	0.542***	-0.181ns	0.690***	0.174ns	0.707***	-0.023ns	-0.628***
C18:1 *n-9*	0.104ns	0.153ns	0.123ns	-0.341*	-0.460**	-0.571***	-0.575***	-0.249ns	1.000	-0.459***	-0.566***	-0.547***	0.938***	-0.781***	-0.180ns	-0.324*	-0.710***	0.100ns	-0.619***	-0.796***	-0.180ns	0.317*	-0.089ns
C18:1 *n-7*	0.543***	0.484***	0.487***	0.767***	0.998***	0.038ns	0.040ns	0.904***	-0.459***	1.000	0.046ns	0.014ns	-0.506***	0.193ns	0.746***	0.952***	0.626***	-0.306*	0.770***	0.256ns	0.746***	-0.056ns	-0.616***
C20:1	-0.290*	-0.288*	-0.248ns	-0.044ns	-0.049ns	0.993***	0.092***	0.005ns	-0.566***	0.046ns	1.000	0.994***	-0.516***	0.450**	-0.059ns	0.022ns	0.802***	0.761***	0.215ns	0.448**	-0.059ns	-0.830***	0.238ns
C22:1	-0.310*	-0.307*	-0.267ns	-0.075ns	0.018ns	0.990***	0.991***	-0.010ns	-0.547***	0.014ns	0.994***	1.000	-0.498***	0.437**	-0.087ns	0.003ns	0.779***	0.777***	0.184ns	0.434**	-0.087ns	-0.832***	0.250ns
C24:1	-0.030ns	0.015ns	-0.025ns	-0.358*	-0.508***	-0.523***	-0.525***	-0.298*	0.938***	-0.506***	-0.516***	-0.498***	1.000	-0.860***	-0.191ns	-0.359*	-0.699***	0.117ns	-0.676***	-0.878***	-0.191ns	0.248ns	-0.093ns
C18:2 *n-6*	-0.121ns	-0.114ns	-0.088ns	0.085ns	0.194ns	0.445**	0.446***	0.109ns	-0.781***	0.193ns	0.450**	0.437**	-0.860***	1.000	-0.090ns	0.085ns	0.455**	-0.078ns	0.513***	0.998***	-0.090ns	-0.096ns	0.373**
C18:3 *n-3*	0.415**	0.308*	0.335*	0.925***	0.748***	-0.076ns	-0.073ns	0.707***	-0.180ns	0.746***	-0.059ns	-0.087ns	-0.191ns	-0.090ns	1.000	0.751***	0.425**	-0.216ns	0.808***	-0.038ns	1.000	0.238ns	-0.925***
C20:2 *n-6*	0.558***	0.529***	0.513***	0.725***	0.953***	0.009ns	0.022ns	0.976***	-0.324*	0.952***	0.022ns	0.003ns	-0.359*	0.085ns	0.751***	1.000	0.583***	-0.221ns	0.714***	0.152ns	0.751***	-0.052ns	-0.665***
SAFA	0.075ns	0.035ns	0.071ns	0.458**	0.629***	0.799***	0.798***	0.542***	-0.710***	0.626***	0.802***	0.779***	-0.699***	0.455**	0.425**	0.583***	1.000	-0.408**	0.642***	0.490***	0.425**	-0.668***	-0.218ns
MUFA	-0.278ns	-0.238ns	-0.214ns	-0.326*	-0.303*	0.751***	0.748***	-0.181ns	0.100ns	-0.306*	0.761***	0.777***	0.117ns	-0.078ns	-0.216ns	-0.221ns	-0.408**	1.000	-0.233*	-0.092ns	-0.216ns	-0.759***	-0.578***
PUFA	0.296*	0.210ns	0.247ns	0.847***	0.773***	0.197ns	0.198ns	0.690***	-0.619***	0.770***	0.215ns	0.184ns	-0.676***	0.513***	0.808***	0.714***	0.642***	-0.233*	1.000	0.558***	0.808***	0.140ns	0.211*
n-6	-0.082ns	-0.077ns	-0.053ns	0.134ns	0.257ns	0.442**	0.437**	0.174ns	-0.796***	0.256ns	0.448**	0.434**	-0.878***	0.998***	-0.038ns	0.152ns	0.490***	-0.092ns	0.558***	1.000	-0.038ns	-0.098ns	0.324*
n-3	0.415**	0.308*	0.335*	0.925***	0.748***	-0.076ns	-0.073ns	0.707***	-0.180ns	0.746***	-0.059ns	-0.087ns	-0.191ns	-0.090ns	1.000	0.751***	0.425**	-0.216ns	0.808***	-0.038ns	1.000	0.238ns	-0.925***
PUFA/SAFA	0.192ns	0.167ns	0.153ns	0.236ns	-0.057ns	-0.844***	-0.845***	-0.023ns	0.317*	-0.056ns	-0.830***	-0.832***	0.248ns	-0.096ns	0.238ns	-0.052ns	-0.668***	-0.759***	0.140ns	-0.098ns	0.238ns	1.000	-0.288*
n-6/*n-3*	-0.432**	-0.318*	-0.336*	-0.839***	-0.618***	0.247ns	0.241ns	-0.628***	-0.089ns	-0.616***	0.238ns	0.250ns	-0.093ns	0.373**	-0.925***	-0.665***	-0.218ns	-0.578***	0.211*	0.324*	-0.925***	-0.288*	1.000

Figure 2. Heatmap of correlation coefficients among evaluated traits

ns, *, ** and ***: Not significant and significant at 5%, 1% and 0.1% probability levels, respectively. C15:0: pentadecanoic acid, C16:0: palmitic acid, C18:0: stearic acid, C18:1 *n-9*: oleic acid, C18:1 *n-7*: vaccenic acid, C18:2 *n-6*: linoleic acid, C18:3 *n-3*: α-linolenic acid, C20:0: arachidic acid, C20:1: cis-11-eicosenoic acid, C20:2 *n-6*: cis-11,14-eicosadienoic acid, C22:0: behenic acid, C22:1: erucic acid, C24:1: nervonic acid, SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, *n-3*: omega-3 fatty acids and *n-6*: omega-6 fatty acids.

Throughout the two-year experiment, the fatty acid composition of the seed oil was substantially affected. With the exception of oleic and nervonic acids, two of the five monounsaturated fatty acids (MUFA), the increase in plant density had a negative impact on all other fatty acids, especially on saturated (SAFA) and polyunsaturated fatty acids (PUFA). These findings are congruent with those recorded by Giridhar *et al.* (2016), who claimed that as seed density increased, the fatty acid content of *Nigella sativa* L. oil changed, with oleic acid increasing and SAFA and PUFA decreasing. The reduced oleic acid content at low sowing density is due to decreased activity of the enzyme Δ9 desaturase, which converts stearic to oleic acid (Ansari Ardali, 2014). In addition, it is worth noting that nervonic acid is synthesized from oleic acid, which occurs naturally in a variety of animal and vegetable fats and oils, after three consecutive chain elongation steps catalysed by elongases (Chimhashu *et al.*, 2018).

Fertilization also had a considerable impact on the fatty acid composition of the black mustard oil. With the exception of stearic and arachidic acid, the remaining saturated fatty acids, namely pentadecanoic, palmitic, and behenic acid were positively influenced by fertilization, exhibiting the highest concentrations after the compost application. In particular, the maximum two-year average values of the oil content in palmitic, pentadecanoic, and behenic acid were 3.62%, 0.42%, and 0.52% w/w, respectively. As for palmitic acid, these findings are comparable to those of Ganjineh *et al.* (2019), who discovered that using compost in sesame production resulted in a higher amount of palmitic acid in the seed oil as compared to the applications of urea and manure. For stearic and arachidic acid, the maximum two-year means were recorded in the untreated (control) with the values being 2.05% and 0.97% w/w, respectively. According to Zapletalová *et al.* (2021), the application of 140 kg N ha^{-1} in oilseed rape crop resulted in a decrease of the stearic acid content of the oil by 1.7% and in an increase of arachidic acid by 5.8%, as compared to the untreated; however, the values among the means were not statistically significant.

Erucic acid is a critical fatty acid that is found most often in the *Brassica* species. This 22-carbon fatty acid belongs to MUFA and is hazardous to human health. In general, high erucic acid oil is significant for industrial applications and is a valuable raw material for the production of industrial products, including plasticizers, detergents, surfactants, as well as polyesters (Coonrod *et al*., 2008; Sharafi *et al.,* 2015). In this study, erucic acid was the main MUFA and the highest contents were presented in untreated plants (22.78% and 23.70% w/w in the first and second growing season, respectively), whereas the lowest values were presented in plots fertilized after the application of urea without inhibitors (14.58% and 15.99% w/w for the respective years) (Table 3). In the same manner, in the research study of Zapletalová *et al.* (2021) on oilseed rape, it was recorded that increasing levels of nitrogen fertilizer up to 140 kg N ha^{-1} resulted in the reduction of erucic acid content by up to 25%.

High oleic acid oils have been proven to exhibit heat stability comparable to saturated fats and are thus a potential substitute for them in commercial food-service applications requiring long-life stability (Sharafi *et al.,* 2015). Furthermore, high oleic acid oil has cholesterol-lowering capabilities, whereas saturated (palmitic and stearic) fatty acids significantly boost blood cholesterol levels (Rakow and Raney, 2003). It is also notable that vegetable oils with a high oleic acid content are gaining popularity for nutritional and industrial purposes. In the current research, oleic acid was the second higher MUFA in content and its highest content was observed after the application of urea with and without nitrification and urease inhibitors with the two-year average values amounting to 20.23% and 19.08% w/w, respectively. The positive effect of increasing levels of inorganic N fertilization on oleic acid and its content has also been observed in other crops, such as sesame (Ganjineh *et al.,* 2019), sunflower (Alzamel *et al*., 2022) and cotton (Sawan, 2018).

Omega 6 (*n-6*) and omega 3 (*n-3*) are fatty acids both types of PUFA. *n-6* fatty acids are distinguished by a double bond six carbons away from the acid's methyl end, whereas n-3 fatty acids share this double bond three carbons away from the acid's methyl end. PUFA are currently sought-after components of specialty oils, which are oils characterized by unique nutritional and functional qualities that are employed as nutraceuticals or cosmeceuticals (Balić *et al*., 2020). Because of a better understanding of their biological and functional properties, as well as their health benefits, PUFA, particularly *n-3* fatty acids, are becoming increasingly important for the health system, not only for disease prevention, but also for the treatment of the most common chronic inflammatory diseases, including inflammatory skin diseases namely atopic dermatitis, psoriasis, and acne (Ferreri and Chatgilialoglu, 2015).

n-6 fatty acids of the black mustard oil consisted of linoleic and cis-11,14-eicosadienoic acid. Linoleic acid and its derivative fatty acids (including cis-11,14-eicosadienoic acid) are necessary fatty acids that cannot be produced by human beings and must thus be received through dietary sources. The high linoleic acid content of oil lowers blood cholesterol levels and aids in the prevention of atherosclerosis (Rakow and Raney, 2003; Balić *et al*., 2020). As a result, edible oil with a high linoleic acid content is considered premium oil. According to the results of the present study, linoleic acid content was highest in the control and compost plots, with two-year average values of 23.46% and 23.50% w/w, respectively. In a previous study of Zapletalová *et al.* (2021), inorganic nitrogen (N) fertilization had a positive effect on oilseed rape crop, and the highest value (18.81% w/w) was recorded in the seed oil of plants fertilized with 140 kg N ha^{-1}. Furthermore, in an experiment conducted by Ahmed *et al.* (2018) in soybean crop, it was discovered that the linoleic acid content in the oil derived from plants that had received compost was statistically significantly higher in comparison to the control and inorganic N fertilization which did not significantly differ among each other. Concerning the oil content of cis-11,14-eicosadienoic acid, the highest contents were found in compost and urea fertilizer with nitrification and urease inhibitors, with the two-year average values differing by 23.7% and 21.3%, respectively, compared to the control that received the lowest value. Previous studies in sunflower and *N. sativa* crop have shown that the content of cis-11,14-eicosadienoic acid, increases with the increasing rates of nitrogen available to the plant (Li *et al*., 2017).

The *n-3* fatty acids in black mustard oil were composed exclusively of α-linolenic acid. Consumption of foods rich in α-linolenic acid has been shown to be advantageous in the prevention of non-communicable diseases including metabolic disorders, inflammatory diseases, and cardiovascular diseases, among others (Ferreri and Chatgilialoglu, 2015). In the current study, the highest content of α-linolenic acid was found in compost with a two-year average of 11.44% w/w. Previous research on oilseed rape proved that nitrogen fertilization in an amount of up to 160 kg N ha^{-1}, had a significant increase in the content of α-linolenic acid with the maximum value amounting to 8.64% w/w (Zapletalová *et al.*, 2021).

Concerning the fatty acid ratios, the PUFA/SAFA was highest in the control and compost plots, with the two-year average values being 5.21 and 5.20, respectively, while, the highest *n-6*/*n-3* ratio was found in the untreated plots with a two-year average value of 3.29. Previously, in the study of Sharafi *et al.* (2015), it was recorded that the mean values of PUFA/SAFA and *n-6*/*n-3* ratios of black mustard fatty acids were 5.90 and 0.91, respectively.

Finally, Figure 2 demonstrates the relationships between the fatty acid attributes evaluated in this study. SAFA had a significant and negative correlation with MUFA (r = -0.408, $p < 0.01$) and a significant and positive association with PUFA (r = 0.642, $p < 0.001$). A significantly negative relationship was also observed among MUFA and PUFA (r = -0.233, $p < 0.05$). In addition, the *n-6*/*n-3* ratio presented a positive correlation with PUFA (r = 0.211, $p < 0.05$) and a negative correlation with MUFA (r = -0.578, $p < 0.001$). These associations appear to be obvious, as an increase in MUFA is frequently accompanied by a reduction in SAFA and PUFA levels. (Li *et al.*, 2017; Ahmed *et al.*; 2018).

As demonstrated in earlier research studies in soybean (Bachlava *et al.*, 2008) and sunflower oil (Li *et al.*, 2017), oleic acid revealed negative relationships with all SAFA and PUFA fatty acids. It is worth mentioning that linoleic and oleic acids have a very significant and negative correlation (r = -0.781, $p < 0.001$; Figure 2). This relationship is because these two fatty acids share the common metabolic pathway in which the enzyme Δ12 desaturase (or FAD2) catalyzes the conversion of oleic acid to linoleic acid, as has been recorded in other crop species (Sharma *et al.*, 2002; Liu *et al.*, 2011; Meru *et al.*, 2018). Moreover, significant correlations of oleic acid with cis-11-eicosenoic acid (r = -0.566, $p < 0.001$), oleic with erucic acid (r = -0.547, $p < 0.001$), and cis-11-eicosenoic acid with erucic acid (r = 0.994, $p < 0.001$) were also found (Figure 2). It is noteworthy that erucic acid biosynthesis is controlled by the expression and the specificity of ß-ketoacyl-CoA synthase (KCS), which constitutes the enzyme responsible for the fatty acid elongation of oleic acid to cis-11-eicosenoic acid and then to erucic acid (Lühs and Friedt, 1997).

Conclusions

The results of the current study demonstrated that the increase in plant density had a negative influence on the seed yield of black mustard, whereas the fertilization presented a beneficial impact, with the greatest values observed in plants of low plant density (46 plants m^{-2}) fertilized with urea fertilizer coated with double (nitrification and urease) inhibitors. In terms of the quantitative characteristics of seed oil, plant density and fertilization also presented a significant effect on oil content with the highest values recorded in plants with low plant density and urease with double inhibitors. The results of the seed oil yield were corresponded to those of the seed yield, with the plants of low-density plots and urea with double inhibitors producing the highest yields. In response to the quality characteristics of seed oil, and specifically with the profile of fatty acids, it was recorded that with the increase of plant density, there was a reduction in saturated (SAFA: myristic, palmitic and stearic acid) and polyunsaturated (PUFA: linoleic, α-linolenic and eicosadenoic acid) fatty acids, while there was an increase in oleic and nervonic acids and a decrease in the rest of the monounsaturated fatty acids (MUFA: vaccenic, cis-11-eicosenoic, and erucic acid). Regarding the fertilization, organic fertilization was the one that contributed positively to the content of the relevant fatty acids. In particular, compost application raised the quantities of polyunsaturated fatty acids (PUFA) that are favorable to human health. Oleic acid showed the highest content after the application of urea with and without inhibitors. In addition, erucic acid was negatively affected by fertilization and the lowest values were presented in plots fertilized after the application of urea without inhibitors. To conclude, plant densities higher than 46 plants m^{-2} result in lower seed yield, oil content, and oil yield, while the inorganic fertilization effect, specifically with urea coated with double inhibitors, was equally important in seed and oil yield; however, when the seed and/or oil are used for their nutraceutical and medicinal value, the use of compost is recommended, resulting in a significant increase in the content of polyunsaturated fatty acids (PUFA).

Authors' Contributions

Conceptualization: S.K. and D.B. (Dimitrios Bilalis); Methodology: S.K., I.R., I.K., A.M., P.S., A.E., N.K., M.G., G.K., D.B. (Dimitrios Beslemes), V.T. and D.B. (Dimitrios Bilalis); Software: S.K., I.R., I.K. and D.B. (Dimitrios Bilalis); Validation: S.K., I.R., I.K. and D.B. (Dimitrios Bilalis); Formal analysis: S.K., I.R., I.K., A.M. and D.B. (Dimitrios Bilalis); Investigation: S.K., I.R., I.K., A.M., P.S., A.E., N.K., M.G., G.K., D.B. (Dimitrios Beslemes), V.T. and D.B. (Dimitrios Bilalis); Resources: S.K., I.R., I.K., A.M. and D.B. (Dimitrios Bilalis); Writing—original draft preparation: S.K., I.R., I.K. and D.B. (Dimitrios Bilalis); Writing—review and editing: S.K., I.R., I.K. and D.B. (Dimitrios Bilalis); Supervision: D.B. (Dimitrios Bilalis). All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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