

## Seed fixed oil content, oil yield, and fatty acids profile of *Nigella sativa* L. in response to fertilization and plant density

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### Abstract

The current study aimed to assess the impacts of fertilization and plant density on fixed oil content, oil yield, and fatty acids profile of *Nigella sativa* L. under Mediterranean environment. The 3-year experiment was set up in a split-plot design with three replications, two main plots (plant densities: 200 and 300 plants m<sup>-2</sup>) and four sub-plots (fertilization treatments: control, seaweed compost, farmyard manure and inorganic fertilizer). The seed yield, fixed oil content, as well as the fixed oil yield were positively affected by the increase of available nitrogen and negatively by the increase of plant density, with their highest values recorded in the low-density and inorganic fertilization. Regarding the composition in fatty acids, it was found that with the increase of plant density there was a decrease in saturated (SAFA: myristic, palmitic and stearic acid) and polyunsaturated (PUFA: linoleic,  $\alpha$ -linolenic and eicosadenoic acid) fatty acids, while there was an increase in oleic acid which was the only monounsaturated fatty acid detected in fixed oil. In terms of fertilization, the organic fertilizers were the ones that contributed positively to the content of the respective fatty acid. As a conclusion, plant densities greater than 200 plants m<sup>-2</sup> result in lower seed yield, fixed oil content and yield, whereas the effect of inorganic fertilization was equally important in seed and fixed oil yield; however, when the seed and/or its fixed oil are utilized for their high medicinal and nutritional value, the application of compost is indicated, resulting in a significant increase in the content of PUFAs, characterized for their beneficial effects on human health.

**Keywords:**  $\alpha$ -linolenic acid; compost; fixed oil; inorganic fertilizer; linoleic acid; oleic acid; polyunsaturated fatty acids (PUFAs)

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## Introduction

*Nigella sativa* L. is an annual herbaceous plant of the Ranunculaceae family, whose growth area extends from the Mediterranean basin's southern and eastern rims to Iran, Pakistan, and India, and is characterized by a wide application for culinary and medicinal purposes. It has been commonly used for its anti-microbial, anti-oxidant, anti-hyperlipidemic, anti-inflammatory, anti-cancer, anti-diabetic, wound healing activities, and also cardiovascular-protective, gastro-protective, nephro-protective, hepato-protective and immuno-protective (Sicak and Erdogan Eliuz, 2019). *N. sativa* is primarily used as a spice in traditional medicine, but it is also used in the pharmaceutical and food industries nowadays. According to several studies, it is a medicinal plant that contains both fixed and essential oils, proteins, alkaloids, and saponins (Darakhshan *et al.*, 2015; Kooti *et al.*, 2016).

*N. sativa* is greatly known because of its high fixed oil content in seeds (30-35% w/w) obtaining a large number of lipids, vitamins, and enzymes (Ashraf *et al.*, 2006). Fatty acids are major components of the lipids in *N. sativa* fixed oil. Specifically, its fixed oil contains around 60% of essential fatty acids (FAs), such as linoleic acid (omega-6) and  $\alpha$ -linolenic acid (omega-3) which are effective and beneficial in reducing the risks of blood pressure, vascular and chronic diseases by balancing and strengthening the immune system (Simopoulos, 2008; Terzi *et al.*, 2010). Currently, it is well understood that humans and many other animals may derive monounsaturated fatty acids from sugars (present in the body); however, the human body is unable to convert oleic acid into linoleic and linolenic acids due to a lack of the  $\Delta$ 12-desaturase enzyme (Lin, 2006). Therefore, humans are forced to append these fatty acids to our diet directly to supply the required polyunsaturated fatty acids. In the human body, polyunsaturated fatty acids are transformed back into important compounds, such as antioxidants which protect the body (Ruxton *et al.*, 2005; Calder and Yaqoob, 2009). Because of these substances and their benefits, seeds and fixed oil have found widespread application in functional foods, nutraceuticals, and pharmaceutical products (Hassanien *et al.*, 2015).

Agronomic practices such as seed rate, plant density, and fertilizer management are referred to comprise crop environment, which affects plant growth, productivity, and quality (Roussis *et al.*, 2019, 2022). High density is unfavourable because it increases competition for resources among interplants. A prior study discovered that optimizing plant density has a significant impact on crop output, making it a critical component of most cultivation practices (Hiltbrunner *et al.*, 2007). Furthermore, sufficient plant density optimizes the canopy microclimate, increases photosynthetic capacity, and significantly increases aboveground biomass accumulation, all of which contribute to increased yields (Dai *et al.*, 2015; Yao *et al.*, 2015).

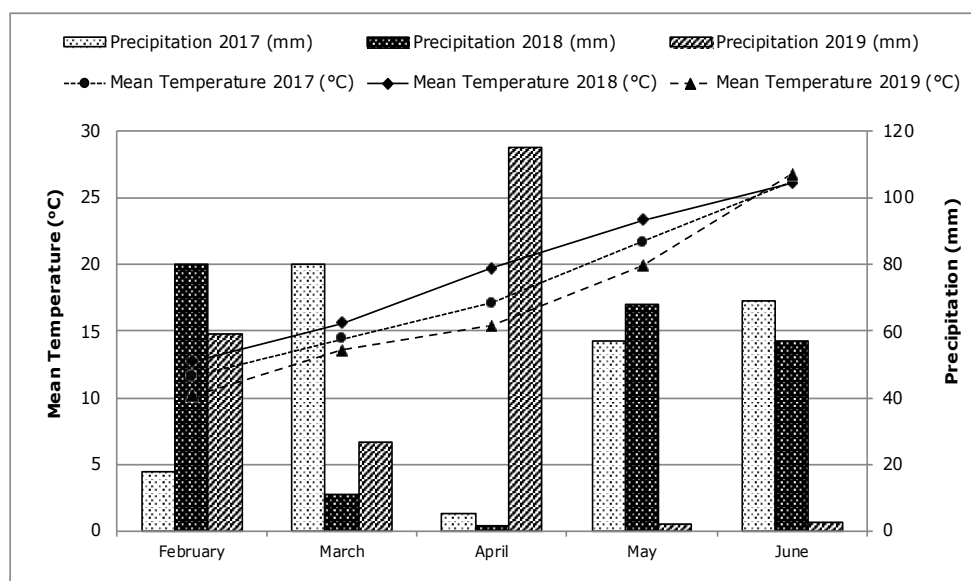
Fertilization is regarded as the most important factor in plant nutrition (Naguib *et al.*, 2012). The type, amount, and application method of fertilizer have a direct impact on plant nutrient availability and have an indirect impact on physiological and biochemical pathways in plants (Naguib *et al.*, 2012). Chemical fertilizers are widely used to increase crop output, but their long-term use alters soil pH, reduces beneficial soil microflora, pollutes water supplies, and disrupts soil biological systems (Fallah *et al.*, 2018; Bistgani *et al.*, 2018). However, demand for organic products has increased in recent years due to their health and environmental benefits, particularly for pharmaceutical products (Fallah *et al.*, 2018). In addition, the tendency for medicinal plants cultivation with the application of organic soil amendments has been increased (Bajeli *et al.*, 2016; Fallah *et al.*, 2018; Roussis *et al.*, 2019).

To our knowledge, limited data are available concerning the effects of fertilization and plant density on seed oil content, oil yield and fatty acids profile of *N. sativa* under Mediterranean semi-arid environment. Therefore, the present study aimed to investigate the influence of organic and inorganic fertilization, as well as plant density, on variation of fixed oil content, yield and fatty acids composition of *N. sativa* crop.

## Materials and Methods

### *Site description and experimental design*

A 3-year field experiment was conducted from 2017 to 2019 in the organic experimental field of the Agricultural University of Athens (Latitude: 37°59'1.70" N, Longitude: 23°42'7.04" E, Altitude: 29 m above sea level). The soil was a clay loam (29.1% clay, 35.3% silt, 35.6% sand) with pH (1:1 H<sub>2</sub>O) 7.43, 15.93% CaCO<sub>3</sub>, 0.121% total nitrogen, a sufficient supply of phosphorus (Olsen P: 13.4 mg kg<sup>-1</sup> soil) and potassium (206 mg kg<sup>-1</sup> soil) and 1.82% organic matter. The site was managed according to norms of European Union (EU) legislation on organic agriculture (EC 834/2007). 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 Agricultural University of Athens and are presented in Figure 1.



**Figure 1.** Meteorological data (mean monthly air temperature and precipitation) for experimental site throughout the duration of the experimental periods (February–June 2017, 2018, and 2019)

The experiment was conducted in an area of 302 m<sup>2</sup> according to a split-plot design with two main plots, four sub-plots, and three replications. The main plots were plant densities of 200 and 300 plants m<sup>-2</sup>. Four fertilization treatment sub-plots included control (untreated), seaweed compost (2000 kg ha<sup>-1</sup> Posidonia by Compost Hellas S.A. Piraeus, Greece; 1.98% N, equivalent to 40 kg N ha<sup>-1</sup>), farmyard manure (2000 kg ha<sup>-1</sup>, solid; 1.52% N, equivalent to 30.5 kg N ha<sup>-1</sup>), and inorganic fertilizer (300 kg ha<sup>-1</sup> Enpeka 15-15-15+5 S by Compo Expert GmbH, Münster, Germany; equivalent to 45 kg N ha<sup>-1</sup>). The amount of each type of fertilizer employed in the present study is the general recommended dose for *N. sativa* cultivation on clay-loam soils (Tuncurk *et al.*, 2012; Kakabouki *et al.*, 2021). The sizes of the main plot and sub-plot were 42.25 m<sup>2</sup> (6.5 m × 6.5 m) and 9 m<sup>2</sup> (3m × 3 m), respectively. Every experimental year, two days before sowing, the soil was prepared by mouldboard ploughing at a depth of 25 cm. Fertilizers were broadcasted by hand as a basal dressing hand and incorporated into the soil by harrowing. *N. sativa* seeds were sown by hand in rows 30 cm apart at a depth of 0.5-1 cm. The sowing rate was 50 kg ha<sup>-1</sup>, and seed sowing was performed on the 1<sup>st</sup> of February in all experimental years. Emergence was done on the 25<sup>th</sup>, 19<sup>th</sup>, and 27<sup>th</sup> of February in the first (2017), second (2018), and third experimental year (2019), respectively. Seedlings were thinned at the four-true leaf stage to the examined plant densities. During the 3-year experiment, there was no incidence of pest or disease on *N. sativa* crop. In addition, weeds were controlled by hand-hoeing when necessary and before canopy closure.

Plants were harvested at full seed maturity (seed moisture content of 12%) on the 3<sup>rd</sup>, 6<sup>th</sup>, and 8<sup>th</sup> of June in 2017, 2018, and 2019, respectively. The seed yield was determined by plants derived from the middle sub-plot area (1 m<sup>2</sup>).

#### *Determination of fixed oil content, oil yield and fatty acid composition*

For the determination of the fixed oil content, seeds with a total weight of 300 g were harvested from each experimental plot. This was followed by the extraction of oil by the cold pressing method. This method does not contain heat and/or chemicals and is therefore preferred by consumers interested in natural and safe foods. In addition, the acidity of the oil remains low, while the antioxidants and polyphenols are not altered (Kiralan *et al.*, 2014). The oil was extracted with a screw expeller SPU 20 (Senta, Serbia) with a capacity of 20-25 kg h<sup>-1</sup> at room temperature (25 °C). The oil samples were stored in plastic bottles (wrapped in aluminum foil), and left overnight at room temperature to allow the sediment containing foreign matter to settle. The next day, the samples were centrifuged at 3500 rpm for 15 min 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 and the oil content of the *N. sativa* seeds was determined. The oil content was calculated by the following formula:

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

Where:

$m_o$  = weight of extracted oil (g),

$m_s$  = sample weight (g)

The fixed oil yield was determined by multiplying the fixed oil content of the seeds with the seed yield. The composition of fatty acids was determined in accordance with EU regulation (EC) 2568/1991 (implementing regulation (EC) 2015/1833). Prior to analysis, the fatty acids were converted to fatty acid methyl esters (FAME) by stirring a solution of *N. sativa* oil and 3 mL of hexane with 0.3 mL of 2N potassium hydroxide methanolic solution for 25 min. The fatty acid methyl esters were analyzed by gas chromatography using a Hewlett-Packard 5890 series II GC chromatograph (Hewlett-Packard Corporation, Palo Alto, California, USA) combined 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.). Helium with a flow rate of 1 mL min<sup>-1</sup> was used as the carrier gas. The initial and final column temperatures were 170 °C and 230 °C, respectively, and the temperature increased at a rate of 4 °C min<sup>-1</sup>. The temperature of the injector and the detector was 230 °C (Gharby *et al.*, 2015). The amount of sample was 1 mL and the introduction into the infusion system was done manually with the splitless technique. Data were analyzed using Hewlett-Packard 3365 Chemstation data analysis software (Hewlett-Packard Corp.). In the present study, the seven major fatty acids (~99%) detected in *N. sativa* oil were examined and expressed as the relative percentage of each individual fatty acid present in the sample. The seven major fatty acids observed were myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 n-9), linoleic acid (18:2 n-6),  $\alpha$ -linolenic acid (18:3 n-3) and eicosadienoic acid (20:2 n-6).

#### *Statistical analysis*

The experimental data were analyzed using the SigmaPlot 12 statistical software (Systat Software Inc., San Jose, CA, USA). The parameter values produced by plant density and fertilization treatments in the three years were assessed by adopting a 3 × 2 × 4 factorial design (three years; two plant density treatments and four fertilization treatments) laid out in a split-plot design with three replications. For the analysis of variance (ANOVA), a mixed model, with years and replications as random effects and plant density and fertilization as fixed effects, was used. 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. All comparisons were made at the 5% level of significance.

## Results

The results of the three-year data analysis (Table 1) indicated that plant density  $\times$  fertilization interaction was significant on fixed oil yield. Moreover, the interaction of year  $\times$  plant density was statistically significant for seed yield and fixed oil yield. The main effects of plant density and fertilization application were significant on seed yield, fixed oil content and yield, as well as, on fatty acid composition of *N. sativa* fixed oil. In addition, the main effect of the year had a significant impact on oleic and linoleic acid (Table 1).

**Table 1.** Combined analysis of variance (*F* values) for the effects of plant density and fertilization on measured properties of *N. sativa* During the 3-year experiment

Source of Variance	Df	Seed Yield	Fixed Oil Content	Fixed Oil Yield	Saturated Fatty Acids (SAFA)		
					Myristic Acid (14:0)	Palmitic Acid (16:0)	Stearic Acid (18:0)
Year (Y)	2	1.0164 <sup>ns</sup>	0.6709 <sup>ns</sup>	0.7571 <sup>ns</sup>	2.4506 <sup>ns</sup>	0.5354 <sup>ns</sup>	0.4808 <sup>ns</sup>
Plant Density (PD)	1	192.62 <sup>***</sup>	25.396 <sup>***</sup>	224.91 <sup>***</sup>	17.648 <sup>***</sup>	30.917 <sup>***</sup>	18.116 <sup>***</sup>
Fertilization (F)	3	86.882 <sup>***</sup>	35.463 <sup>***</sup>	133.94 <sup>***</sup>	21.064 <sup>***</sup>	24.836 <sup>***</sup>	22.672 <sup>***</sup>
Y $\times$ PD	2	11.977 <sup>***</sup>	0.0839 <sup>ns</sup>	9.6089 <sup>***</sup>	0.0509 <sup>ns</sup>	0.0493 <sup>ns</sup>	0.1001 <sup>ns</sup>
Y $\times$ F	6	0.4720 <sup>ns</sup>	0.0242 <sup>ns</sup>	0.2787 <sup>ns</sup>	0.4398 <sup>ns</sup>	0.0386 <sup>ns</sup>	0.0129 <sup>ns</sup>
PD $\times$ F	3	3.4119 <sup>ns</sup>	0.2536 <sup>ns</sup>	7.1494 <sup>***</sup>	1.0389 <sup>ns</sup>	0.1737 <sup>ns</sup>	1.3804 <sup>ns</sup>
Y $\times$ PD $\times$ F	6	0.5430 <sup>ns</sup>	2.1828 <sup>ns</sup>	3.2742 <sup>***</sup>	0.0688 <sup>ns</sup>	0.0245 <sup>ns</sup>	0.0026 <sup>ns</sup>
Source of Variance	Df	Mono Unsaturated Fatty Acids (MUFA)		Polyunsaturated Fatty Acids (PUFA)			
		Oleic Acid (18:1 n-9)	Linoleic Acid (18:2 n-6)	$\alpha$ -Linolenic Acid (18:3 n-3)	Eicosadienoic Acid (20:2 n-6)		
Year (Y)	2	4.5673*	6.2232**	2.3577 <sup>ns</sup>	1.1905 <sup>ns</sup>		
Plant Density (PD)	1	25.434 <sup>***</sup>	16.857 <sup>***</sup>	17.239 <sup>***</sup>	36.158 <sup>***</sup>		
Fertilization (F)	3	7.9496 <sup>***</sup>	11.188 <sup>***</sup>	20.500 <sup>***</sup>	33.510 <sup>***</sup>		
Y $\times$ PD	2	1.2471 <sup>ns</sup>	1.7562 <sup>ns</sup>	0.0603 <sup>ns</sup>	0.0608 <sup>ns</sup>		
Y $\times$ F	6	0.0605 <sup>ns</sup>	0.0757 <sup>ns</sup>	0.4676 <sup>ns</sup>	0.0132 <sup>ns</sup>		
PD $\times$ F	3	0.6186 <sup>ns</sup>	1.0150 <sup>ns</sup>	1.0496 <sup>ns</sup>	1.0949 <sup>ns</sup>		
Y $\times$ PD $\times$ F	6	0.0408 <sup>ns</sup>	0.0587 <sup>ns</sup>	0.0671 <sup>ns</sup>	0.0229 <sup>ns</sup>		

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

### *Seed yield, fixed oil content and yield of N. sativa*

The results of the present study indicated that the seed yield of *N. sativa* crop was affected by both plant density and fertilization (Table 2). Specifically, seed yield was higher in the low-density plants (200 plants m<sup>-2</sup>) than in high-density plots (300 plants m<sup>-2</sup>) during the three experimental periods, with the values of low-density plants being 677.4, 601.9 and 699.5 kg ha<sup>-1</sup> in 2017, 2018, and 2019, respectively. In response to fertilization, the plant seed yield increased up to the inorganic fertilization with the averaged values being 677.7, 703.4, 706.3 kg ha<sup>-1</sup> in the first, second, and third year of the experiment, respectively.

According to the combined analysis of variance (Table 1) and Table 2, fixed oil content was significantly influenced by plant density and fertilization. During the 3-year study, the fixed oil content was significantly higher in the low-density plots (300 plants m<sup>-2</sup>), and values were 30.22, 29.38 and 29.12% for the years 2017,

2018, and 2019, respectively. Concerning the effect of fertilization, averaged over the year and plant densities, the mean values of fixed oil content were greatest in the inorganic treatment (31.79, 31.11 and 30.97% in 2017, 2018, and 2019, respectively) followed by compost (31.30, 30.60 and 30.06% in 2017, 2018, and 2019, respectively), while the lowest values were found in the untreated (control) plants.

Fixed oil yield was estimated by multiplying the seed yield of *N. sativa* crop and the fixed oil content of the crop's seeds. According to the combined analysis (Table 1), fixed oil yield was influenced by the plant density and fertilization. Averaged over fertilization treatments, the highest yields (204.7, 176.8 and 203.7 kg ha<sup>-1</sup> in 2017, 2018, and 2019, respectively) were recorded when plants subjected to low-density (Table 2). During the three-year experiment, the mean values of fixed oil yield were greatest in the inorganic treatment (215.4, 218.8, and 218.7 kg ha<sup>-1</sup> in 2017, 2018, and 2019, respectively) followed by compost (199.2, 189.7 and 199.9 kg ha<sup>-1</sup> in 2017, 2018, and 2019, respectively), while the lowest values (96.8, 87.0, and 92.3 kg ha<sup>-1</sup> for the respective years) were observed in the untreated plants.

**Table 2.** Seed yield, fixed oil content and fixed oil yield as affected by the plant density and fertilization

Treatment	Seed Yield (kg ha <sup>-1</sup> )			Fixed Oil Content (%)			Fixed Oil Yield (kg ha <sup>-1</sup> )		
	2017	2018	2019	2017	2018	2019	2017	2018	2019
Plant density									
200 plants m <sup>-2</sup>	677.4 A	601.9 A	699.5 A	30.22 A	29.38 A	29.12 A	204.7 A	176.8 A	203.7 A
300 plants m <sup>-2</sup>	446.8 B	506.6 B	455.8 B	26.39 B	26.24 B	25.52 B	117.9 B	132.9 B	116.3 B
Fertilization									
Control	429.8 c	386.7 d	425.6 c	22.52 c	22.49 c	21.68 c	96.8 c	87.0 d	92.3 c
Manure	504.5 b	507.0 c	513.6 b	27.62 b	27.02 b	26.57 b	139.3 b	136.9 c	136.5 b
Compost	636.4 a	619.8 b	665.3 a	31.30 a	30.60 ab	30.06 a	199.2 a	189.7 b	199.9 a
Inorganic	677.7 a	703.4 a	706.3 a	31.79 a	31.11 a	30.97 a	215.4 a	218.8 a	218.7 a
Source of variation									
<i>F</i> <sub>Plant Density</sub>	102.22***	14.67**	109.29***	11.31**	5.40*	10.16**	83.68***	30.56***	127.16***
<i>F</i> <sub>Fertilization</sub>	25.45***	30.48***	31.56***	14.19***	8.71**	13.92***	33.46***	52.43***	54.19***
<i>F</i> <sub>Plant Density × Fertilization</sub>	1.13 <sup>ns</sup>	1.11 <sup>ns</sup>	2.31 <sup>ns</sup>	1.12 <sup>ns</sup>	2.23 <sup>ns</sup>	0.97 <sup>ns</sup>	3.60*	3.19 <sup>ns</sup>	7.31**

F-test ratios are from ANOVA. ns, \*, \*\* and \*\*\*: Not-significant and significant at 5%, 1% and 0.1% probability levels, respectively. The different capital letters in the same column denote statistically significant differences according to the Tukey's HSD test ( $p \leq 0.05$ ) under different plant density, and the different lowercase letters in the same column denote statistically significant differences according to the Tukey's HSD test ( $p \leq 0.05$ ) under different fertilization.

#### *Fatty acid composition of fixed oil*

In the present study, the seven major fatty acids detected in fixed oil of *N. sativa* were studied and expressed as the relative percentage of each individual fatty acid present in the studied oil sample. Specifically, the seven major fatty acids observed that accounted for about 99% of total fatty acids were myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 n-9), linoleic acid (18:2 n-6),  $\alpha$ -linolenic acid (18:3 n-3), and eicosadienoic acid (20:2 n-6).

The combined analysis of variance (Table 1) showed that the contents of saturated (SAFA: myristic, palmitic and stearic acid), monounsaturated (MUFA: oleic acid) and polyunsaturated fatty acids (PUFA: linoleic acid,  $\alpha$ -linolenic and eicosadienoic acid) were 14.63-18.49% w/w, 18.08-29.10% w/w and 53.65-63.78% w/w, respectively.

Palmitic acid had the highest content of saturated fatty acids. Specifically, plant density seems to have had a statistically significant effect on palmitic acid content values throughout the three-year experiment and the highest content was found in low sowing density (200 plants m<sup>-2</sup>), with the values being 15.36, 15.10, and 15.32 % w/w in the first, second and third year of the experiment, respectively (Table 3). Fertilization had an equally significant effect, with compost (15.67, 15.49 and 15.78% w/w in 2017, 2018, and 2019, respectively) and inorganic fertilizer (15.28, 15.13 and 15.47% w/w in 2017, 2018, and 2019, respectively) showing higher values as compared to those of manure (14.58, 14.32 and 14.53% w/w in 2017, 2018, and 2019, respectively) and control (13.37, 13.09 and 13.24 % w/w for the respective years) (Table 3).

Monounsaturated fatty acids consisted only of oleic acid. The differences between plants of different plant densities were statistically significant and the highest values in oleic acid (25.83, 27.26 and 25.13% w/w in 2017, 2018, and 2019, respectively) were found in plots of high plant density (300 plants m<sup>-2</sup>). In terms of fertilization treatments, manure (26.64, 28.56 and 22.59% w/w in 2017, 2018, and 2019, respectively) and inorganic fertilizer (24.63, 27.23 and 24.36% w/w in 2017, 2018, and 2019, respectively) showed statistically higher oleic acid content values versus compost (18.64, 22.35 and 18.04% w/w in 2017, 2018, and 2019, respectively) and control (20.71, 24.48 and 20.81% w/w in 2017, 2018, and 2019, respectively). Regarding the growing year, it was found that the average value of oleic acid content during the second growing year (25.65% w/w) was significantly higher ( $F = 4.5673$ ,  $p = 0.0153$ ; Table 1) compared to third (22.20% w/w) and the first year (22.16% w/w).

**Table 3.** Fatty acid composition of fixed oil as affected by the plant density and fertilization

Treatment	Saturated Fatty Acids (SAFA)											
	Myristic Acid (14:0) (% w/w)			Palmitic Acid (16:0) (% w/w)			Stearic Acid (18:0) (% w/w)					
	2017	2018	2019	2017	2018	2019	2017	2018	2019			
Plant density												
200 plants m <sup>-2</sup>	0.43 A	0.43 A	0.47 A	15.36 A	15.10 A	15.32 A	1.73 A	1.80 A	1.75 A			
300 plants m <sup>-2</sup>	0.36 B	0.37 B	0.40 B	14.08 B	13.91 B	14.20 B	1.48 B	1.55 B	1.49 B			
Fertilization												
Control	0.31 c	0.33 c	0.35 c	13.37 c	13.09 c	13.24 c	1.97 a	2.03 a	1.96 a			
Manure	0.39 b	0.38 bc	0.41 bc	14.58 b	14.32 b	14.53 b	1.26 c	1.33 c	1.30 c			
Compost	0.49 a	0.50 a	0.50 a	15.67 a	15.49 a	15.78 a	1.58 b	1.66 b	1.61 b			
Inorganic	0.40 b	0.39 b	0.47 ab	15.28 ab	15.13 ab	15.47 ab	1.60 b	1.65 b	1.62 b			
Source of variation												
$F_{Plant\ Density}$	6.26*	7.04*	5.10*	13.36**	10.60**	7.81*	5.81*	6.04*	6.28*			
$F_{Fertilization}$	8.01**	11.16***	4.86*	8.30**	8.54**	8.11**	7.70**	7.75**	7.22**			
$F_{Plant\ Density \times Fertilization}$	0.56 <sup>ns</sup>	0.57 <sup>ns</sup>	0.18 <sup>ns</sup>	0.16 <sup>ns</sup>	0.06 <sup>ns</sup>	0.02 <sup>ns</sup>	0.47 <sup>ns</sup>	0.48 <sup>ns</sup>	0.42 <sup>ns</sup>			
Treatment	Mono Unsaturated Fatty Acids (MUFA)			Polyunsaturated Fatty Acids (PUFA)								
	Oleic Acid (18:1 n-9) (% w/w)			Linoleic Acid (18:2 n-6) (% w/w)			$\alpha$ -Linolenic Acid (18:3 n-3) (% w/w)			Eicosadienoic Acid (20:2 n-6) (% w/w)		
	2017	2018	2019	2017	2018	2019	2017	2018	2019	2017	2018	2019
Plant density												
200 plants m <sup>-2</sup>	18.49 B	24.05 B	19.27 B	60.82 A	55.34 A	59.96 A	0.55 A	0.61 A	0.59 A	2.02 A	2.07 A	2.05 A
300 plants m <sup>-2</sup>	25.83 A	27.26 A	25.13 A	55.36 B	53.91 A	55.79 B	0.47 B	0.51 B	0.51 B	1.82 B	1.89 B	1.88 B
Fertilization												
Control	20.71 ab	24.48 bc	20.81 ab	60.97 a	57.29 a	60.87 a	0.39 c	0.45 c	0.45 c	1.68 c	1.73 c	1.72 c
Manure	24.64 a	28.56 a	22.59 a	56.13 bc	52.33 b	55.11 b	0.50 bc	0.52 bc	0.52 bc	1.90 b	1.96 b	1.94 b
Compost	18.64 b	22.35 c	18.04 b	60.29 ab	56.62 a	60.67 a	0.63 a	0.65 a	0.69 a	2.07 a	2.13 a	2.12 a
Inorganic	24.63 a	27.23 ab	24.36 ab	54.97 c	52.28 b	54.83 b	0.51 b	0.61 ab	0.54 b	2.04 ab	2.10 ab	2.09 ab
Source of variation												
$F_{Plant\ Density}$	13.60***	5.74*	7.12*	11.54**	1.60 <sup>ns</sup>	5.12*	5.49*	5.33*	7.05*	14.89**	12.25**	9.57**
$F_{Fertilization}$	2.25*	4.33*	2.44*	3.45*	5.73**	3.30*	7.64**	4.69*	11.16***	11.37**	11.48**	10.75**
$F_{Plant\ Density \times Fertilization}$	0.22 <sup>ns</sup>	0.51 <sup>ns</sup>	0.14 <sup>ns</sup>	0.39 <sup>ns</sup>	0.77 <sup>ns</sup>	0.23 <sup>ns</sup>	0.53 <sup>ns</sup>	0.21 <sup>ns</sup>	0.57 <sup>ns</sup>	0.59 <sup>ns</sup>	0.37 <sup>ns</sup>	0.21 <sup>ns</sup>

F-test ratios are from ANOVA. ns, \*, \*\* and \*\*\*: Not-significant and significant at 5%, 1% and 0.1% probability levels, respectively. The different capital letters in the same column denote statistically significant differences according to the Tukey's HSD test ( $p \leq 0.05$ ) under different plant density, and the different lowercase letters in the same column denote statistically significant differences according to the Tukey's HSD test ( $p \leq 0.05$ ) under different fertilization.

In the category of polyunsaturated fatty acids, the highest content was presented by linoleic acid, and, in fact, it presented the highest content in relation to all other fatty acids of fixed oil. The differences between the plots of different sowing density in terms of linoleic acid content were statistically significant, with highest contents (60.82, 55.34 and 59.96% w/w in 2017, 2018, and 2019, respectively) were recorded when plants subjected to low-density. Concerning the effect of fertilization, control (60.97, 57.29 and 60.87% w/w in 2017,

2018, and 2019, respectively) and compost (60.29, 56.62 and 60.67% w/w in 2017, 2018, and 2019, respectively) showed significantly higher mean values in linoleic acid content. Finally, regarding the growing year, it was found that the average values of linoleic acid content during the first (58.09% w/w) and third growing year (57.87% w/w) were significantly higher ( $F = 6.2232, p = 0.0040$ ; Table 1) compared to the second year (54.63% w/w).

## Discussion

Throughout the three-year trial, plant density had a substantial effect on the seed yield of the *N. sativa* crop. The three-year average value of seed yield was 40.4% higher with a plant density of 200 plants  $m^{-2}$  than at a density of 300 plants  $m^{-2}$ . Mollafilabi *et al.* (2010) discovered that the highest seed yield in *N. sativa* was attained at a sowing density of 180  $m^{-2}$  plants (809  $kg\ ha^{-1}$ ) and that increasing the density to 240 plants  $m^{-2}$  lowered the yield by 38%. In terms of fertilization, different fertilizations had a substantial effect on the seed yield of *N. sativa*. The three-year average value of seed yield was statistically considerably higher in plots that had received inorganic fertilizer (695.8  $kg\ ha^{-1}$ ) followed by compost (640.5  $kg\ ha^{-1}$ ), and the lowest yield was offered by the control (414.0  $kg\ ha^{-1}$ ). According to several researchers, higher yields have been reported in various crops fertilized with inorganic fertilizers because these fertilizers contained soluble inorganic nitrogen with rapid availability to the cultivated plant species, resulting in increased growth and higher yields (Mengel and Rehm, 2012; Bilalis *et al.*, 2018; Kakabouki *et al.*, 2018).

Regarding the effect of plant density on fixed oil content in *N. sativa* seeds, statistically significant differences were observed between the different plant densities and the highest oil content found at the plant density of 200  $m^{-2}$  plants with the three-year average being 13.5% higher than the density of 300  $m^{-2}$  plants. These findings are consistent with the findings of Meena *et al.* (2011), Gholinezhad and Abdolrahimi (2014) and Giridhar *et al.* (2016), where they found that with the increase of plant density in *N. sativa* crop, the content of fixed oil in seeds was significantly reduced. In addition, the oil content in the plant density of 200 plants  $m^{-2}$  (29.12-30.22% w/w) is in complete agreement with the results of Hosseini *et al.* (2019), where Iranian local varieties of *N. sativa* were studied at the same plant density (200 plants  $m^{-2}$ ) and the seed oil content ranged from 27.57% to 33.04% depending on the cultivated variety.

In terms of fertilization, there was a significant effect of different fertilization treatments on the fixed oil content. Specifically, the average three-year values of the oil content were statistically significantly higher in the plots that had received the mineral fertilizer (30.29% w/w) and compost (30.65% w/w), while the lowest value was presented by control (22.23% w/w). These results are consistent with the results of Ashraf *et al.* (2006) and Aytac *et al.* (2017), where they found that high doses of nitrogen (from 60  $kg\ N\ ha^{-1}$  and over) can significantly reduce the content of seeds in stable oil, while lower doses (up to 30-40  $kg\ N\ ha^{-1}$ ) have positive effect on oil content. In contrast, Özgüven and Sekeroglu (2007) and Shah (2007) did not find significant differences between the different doses of nitrogen in the fixed oil content of *N. sativa* seed. In addition, many studies in oilseed crops have shown that high and increasing doses of nitrogen reduce the oil content of the seeds (Taylor *et al.*, 1991; Scheiner *et al.*, 2002; Brennan and Bolland, 2007).

As the components of the fixed oil yield of the crop, i.e., the seed crop yield and the oil content of the seeds showed a similar trend (Table 2), this trait also followed an equivalent pattern. Specifically, the low plant density (200  $m^{-2}$  plants) with a three-year average value of 195.1  $kg\ ha^{-1}$ , was significantly higher than that of the 300  $m^{-2}$  plants with a three-year average value of 122.4  $kg\ ha^{-1}$ . Concerning the fertilization effect on this property, the highest three-year average values were recorded in inorganic fertilization with compost following and values being 137.1% and 115.2% higher than the control, respectively. The above is confirmed by the very strong and positive correlations of fixed oil yield with seed yield and fixed oil content ( $r = 0.9489, p < 0.0001$ ;  $r = 0.8696, p < 0.0001$ , respectively; Table 4).

**Table 4.** Correlation coefficients among evaluated properties

Property	Coefficient of correlation ( $r$ )								
	Seed Yield	Fixed Oil Content	Fixed Oil Yield	Myristic Acid	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	$\alpha$ -Linolenic Acid
Fixed Oil Content	0.6899***								
Fixed Oil Yield	0.9489***	0.8696***							
Myristic Acid (14:0)	0.6044***	0.5538***	0.6284***						
Palmitic Acid (16:0)	0.7223***	0.6781***	0.7441***	0.7928***					
Stearic Acid (18:0)	-0.0007 <sup>ns</sup>	-0.0832 <sup>ns</sup>	0.0006 <sup>ns</sup>	-0.0332 <sup>ns</sup>	0.0625 <sup>ns</sup>				
Oleic Acid (18:1 n-9)	-0.3143**	-0.1728 <sup>ns</sup>	-0.3046**	-0.3633**	-0.5183***	-0.5715***			
Linoleic Acid (18:2 n-6)	0.1123 <sup>ns</sup>	-0.0322 <sup>ns</sup>	0.0929 <sup>ns</sup>	0.1360 <sup>ns</sup>	0.2520*	0.5806***	-0.9565***		
$\alpha$ -Linolenic Acid (18:3 n-3)	0.5798***	0.5584***	0.6139***	0.9077***	0.7678***	-0.0244 <sup>ns</sup>	-0.2891*	0.0542 <sup>ns</sup>	
Eicosadienoic Acid (20:2 n-6)	0.7346***	0.6891***	0.7465***	0.7658***	0.9718***	0.0502 <sup>ns</sup>	-0.4379***	0.1655 <sup>ns</sup>	0.7746***

ns, \*, \*\* and \*\*\*: Not-significant and significant at 5%, 1% and 0.1% probability levels, respectively.

The fixed oil of *N. sativa* contained three saturated fatty acids (SAFA), myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0), of which the highest content was presented in palmitic acid, with three-year average values ranging from 13.83% to 16.24% w/w. The fixed oil also contained a mono unsaturated fatty acid (MUFA), namely oleic acid (18:1 n-9) with three-year average ranging from 18.09% to 29.10% w/w. Finally, its polyunsaturated fatty acids (PUFAs), which overall had the highest content of other fatty acids, included three fatty acids, linoleic acid (18:2 n-6),  $\alpha$ -linolenic acid (18:3 n-3) and eicosadienoic acid (20:2 n-6). Linoleic acid was the fatty acid with the highest oil content and had three-year average values ranging from 51.11% to 61.48% w/w. These observations are consistent with previous studies conducted on *N. sativa* in various geographical areas (Nickavar *et al.*, 2003; Ashraf *et al.*, 2006; Cheikh-Rouhou *et al.*, 2007; Aytac *et al.*, 2017).

The composition of the fixed oil in fatty acids was significantly affected throughout the three-year experiment. With the exception of oleic acid, which was the only monounsaturated fatty acid (MUFA), all other fatty acids (SAFA and PUFA) were adversely affected by the increase in plant density. These results are consistent with the findings reported by Giridhar *et al.* (2016), where they argued that with increasing seed density, the fatty acid content changed, with increasing oleic acid and decreasing saturated and polyunsaturated fatty acids. The reduced content of oleic acid at low sowing density lies in the reduced activity of the enzyme  $\Delta 9$  desaturase which converts stearic to oleic acid (Ansari Ardali, 2014). Fertilization also had a significant effect on the composition of the oil in fatty acids. With the exception of stearic acid, the remaining saturated fatty acids, i.e., myristic and palmitic acid, were positively affected by the fertilization, presenting the highest contents after the application of compost. Specifically, the maximum three-year average values of the oil content in myristic and palmitic acid were 0.50% and 15.65% w/w, respectively. These results are similar to the results of Ganjineh *et al.* (2019), where they found that the application of compost in sesame cultivation resulted in a higher content of myristic and palmitic acid in the oil compared to the application of urea and manure. For stearic acid, the maximum three-year mean was found in the control with the value being 1.99% w/w. According to Ashraf *et al.* (2006), applications of 30 and 60 kg N ha<sup>-1</sup> in *N. sativa* crop resulted in a reduction of the stearic acid content of the oil by 15.5% and 48.3% w/w, respectively, compared to the control, where no fertilization was applied.

Regarding oleic acid, the highest content was found after the application of manure with the three-year average value amounting to 26.27% w/w. The positive effect of manure against inorganic fertilization on oleic acid and its content has also been observed in other crops, such as oilseed rape (Gao *et al.*, 2010) and sesame

(Ganjineh *et al.*, 2019). Linoleic acid showed the highest contents in the control and compost plots with average three-year values of 59.71% and 59.19% w/w, respectively. Ashraf *et al.* (2006) found that the application of nitrogen up to 90 kg N ha<sup>-1</sup> had no significant effect on the content of *N. sativa* fixed oil in linoleic acid; however, the highest value was presented in the unfertilized plants. In addition, in an experiment performed by Ahmed *et al.* (2018) in soybean crop, it was observed that in the oil derived from plants that had received compost, the linoleic acid content was statistically significantly higher in relation to the control and nitrogenous inorganic fertilization which did not differ statistically significantly between each other.

As for  $\alpha$ -linolenic acid, the highest content was found in compost with a three-year average of 0.66% w/w. According to Ashraf *et al.* (2006), the application of nitrogen in an amount of up to 30 kg N ha<sup>-1</sup>, had a significant increase in the content of  $\alpha$ -linolenic acid with the maximum value amounting to 0.60% w/w. In contrast, Aytac *et al.* (2017), found that increasing the amount of nitrogen up to 90 kg N ha<sup>-1</sup>, increased the  $\alpha$ -linolenic acid content; however, the differences compared to the control were not significant. Regarding the oil content of eicosadienoic acid, the highest contents were found in compost and inorganic fertilizer, with the three-year average values differing by 23.4% and 21.6% compared to the control that received the lowest value. Previous studies in *N. sativa* have shown that the content of eicosadienoic acid increases with increasing nitrogen available to the plant, but this increase is not significant (Ashraf *et al.*, 2006; Giridhar *et al.*, 2016; Aytac *et al.*, 2017; Li *et al.*, 2017).

Finally, Table 4 shows the correlations between the seed oil content and the individual fatty acid contents. As found in previous studies in soybean oil (Bachlava *et al.*, 2008) and sunflower oil (Li *et al.*, 2017), oleic acid showed negative correlations with all saturated (SAFA) and polyunsaturated (PUFA) fatty acids. It is worth noting that a very strong and negative correlation found between linoleic and oleic acid ( $r = -0.9565$ ,  $p < 0.0001$ ; Table 4). This relationship lies in the fact that the two fatty acids share a common metabolic pathway by which the conversion of oleic acid to linoleic acid is catalyzed by the enzyme  $\Delta 12$  desaturase (or FAD2), as has been shown in other cultures (Sharma *et al.*, 2002; Liu *et al.*, 2011; Meru *et al.*, 2018).

## Conclusions

The results of the present study indicated that the seed yield of *N. sativa* was negatively influenced by the increase in plant density and positively affected by the fertilization with the highest values found in plants with low plant density and those received inorganic fertilizer. In terms of quantitative characteristics of fixed seed oil, seed density and fertilization had a significant effect on fixed oil content with the highest values being found in plants with low plant density (200 m<sup>2</sup> plants) and inorganic fertilizer, with compost following and not significantly differing from those of inorganic. Corresponding to the results of seed yield were those of fixed oil yield, where the highest yield was found in the experimental plots of 200 m<sup>2</sup> plants and inorganic fertilization. Regarding the quality characteristics of the fixed oil, and specifically with the composition in fatty acids, it was found that with the increase of plant density there was a decrease in saturated (SAFA: myristic, palmitic and stearic acid) and polyunsaturated (PUFA: linoleic,  $\alpha$ -linolenic and eicosadienoic acid) fatty acids, while there was an increase in oleic acid which was the only monounsaturated fatty acid detected in *N. sativa* fixed oil. In terms of fertilization, the organic fertilizers were the ones that contributed positively to the content of the respective fatty acid. In particular, the application of compost has increased the levels of both saturated (SAFA) and polyunsaturated fatty acids (PUFA) beneficial to human health. Oleic acid showed the highest content after the application of manure. As a conclusion, plant densities greater than 200 plants m<sup>-2</sup> result in lower seed yield, fixed oil content and fixed oil yield, whereas the effect of inorganic fertilization was equally important in the yield of the crop in seed and fixed oil; however, when the seed and/or its fixed oil are utilized for their high medicinal and nutritional value, the application of compost is indicated, resulting in a significant increase in the content of polyunsaturated fatty acids, characterized for their beneficial effects on human health.

### Authors' Contributions

Conceptualization: I.R. and D.B.; Data curation: I.R., I.K. and D.B.; Formal analysis: I.R., I.K. and D.B.; Investigation: I.R., I.K., A.M., V.T., A.Z., C.K. and D.B.; Methodology: I.R., I.K., A.M., V.T., A.Z., C.K. and D.B.; Project administration: I.R. and D.B.; Resources: I.R., I.K., A.M., V.T., A.Z., C.K. and D.B.; Software: I.R., I.K. and D.B.; Supervision: I.R. and D.B.; Validation: I.R., I.K. and D.B.; Visualization: I.R., I.K. and D.B.; Writing - original draft: I.R., I.K. and D.B.; Writing - review and editing: . I.R., I.K. and D.B. 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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