Optimizing Intercropping Systems of Black Cumin (*Nigella sativa* L.) and Fenugreek (*Trigonella foenum-graecum* L.) through Inoculation with Bacteria and Mycorrhizal Fungi

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This study evaluates the effects of intercropping patterns, plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi (AMF) on seed yield and yield components of black cumin (*Nigella sativa* L.) and fenugreek (*Trigonella foenum-graecum* L.), as well as the essential oil and fatty acid profile of black cumin. A two-year two-factorial field experiment was conducted in 2015 and 2016 to investigate intercropping of black cumin and fenugreek in five ratios and biofertilizer application as AMF and bacteria. Intercropping reveals higher concentrations of nitrogen and phosphorus compared with monocropping, whereas monocropping inoculated with bacteria shows the highest seed yield of both fenugreek (151 g m^{-2}) and black cumin (148 g m^{-2}). Regarding the quality of black cumin, the combination of a black cumin:fenugreek-intercropping pattern of 66:34 with bacteria fertilization is most promising, as it shows i) the maximum essential oil content, oil yield, and fixed oil content, ii) the highest contents of thymol and *p*-cymene, iii) the highest content of linoleic acid, and iv) the maximum land equivalent ratio. Conclusively, bacteria fertilization and black cumin:fenugreek-intercropping pattern of 66:34 helps improving essential oil, fixed oil quality, and quantity of black cumin, thus creating a more sustainable cultivation system for black cumin and fenugreek.

1. Introduction

Intercropping is an important component of sustainable agriculture in which two or more crops are planted simultaneously at the same place of land.[1,2] Well-designed intercropping systems can help to use natural resources more efficiently, increase biodiversity, manage pests and, in many instances, increase crop productivity, quality, and natural soil fertility compared to mono-cropping systems.[2,3] The optimization of intercropping systems therefore follows the call of action of the United Nations to "Diversify species and genetic resources in the agroecosystems over time and space and focus on interactions rather than individual species,"[4] and can contribute in particular to the sustainability development goals 12 (sustainable production), 13 (climate action) and 15 (life on land).[5]

An important approach to optimize intercropping systems could be the use of biological additives such as plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF). Inoculating plant species with PGPR and AMF could facilitate acquisition of nutrients that are heavily required by plants and economically expensive to supply from synthetic fertilizers,[6–10] improve carbon sequestration,[11] and improve seed yield and yield components of black cumin and fenugreek. Biofertilizers such as PGPR are extensively used in small-scale agricultural systems and can play an important role in improving crop productivity through modifying physical

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and chemical properties of soil.[12,13] Plant growth-promoting rhizobacteria refer to a large number of soil bacterial species that live in the rhizosphere. The PGPR benefits plants by producing compounds such as plant hormones (auxin, cytokinin, gibberellin),[14] fixing atmospheric N,[15] increasing the availability of nutrients such as P through organic and inorganic acids, producing siderophores, and increasing iron and manganese availability.[16] The AMFs establish symbiosis with plant root systems and positively affect the growth of host plants by increasing nutrient acquisition, especially P.[17] Previous results have shown intercropping of medicinal plants in low input conditions has increased the quality of essential oils (EOs) of some medicinal plants such as fennel (*Foeniculum vulgare* L. Mill.),[18] fennel and dragonhead (*Dracocephalum moldavica*),[19] and dill (*Anethum graveolens* L.).[20]

A native to India, West Asia, and some parts of Iran, black cumin (*Nigella sativa* L.) is an annual medicinal plant that belongs to the Ranunculaceae family.[21] Black cumin's seeds contain fixed and secondary metabolite such as EO, saponin, and alkaloids.[22] Black cumin's EO contains anethole, *p*-cymene, limonene, carvacrol, thymol, and spathulenol, whereas its fixed oil has important unsaturated fatty acids including linoleic and oleic acids.[20] Black cumin's seeds also contain carminative, which is a menstrual period facilitator, hepatoprotective, anti-hypertensive, nephroprotective, anti-diarrheal, milk enhancer, antibacterial, anti-constipation, and potency enhancing for men.[23]

Fenugreek (*Trigonella foenum-graecum* L.) is an annual plant from the Fabaceae family. Fenugreek was first used in central Asia ≈4000 bc[24] and is known for its medicinal properties.[25,26] The seeds of fenugreek are widely used for their antidiabetic, anticarcinogenic, antioxidant, anticancer, hypocholesterolemic, and immunological properties.[27] Apart from medicinal benefits, fenugreek fixes atmospheric N, decreasing the N fertilizer need in cropping systems,[28] a characteristic of vital importance for low-income farmers around the world.[29] Nitrogen fixing trait of fenugreek also makes it a feasible crop for intercropping with nonlegume crops, a common sustainable practice among small-scale farmers in semiarid regions where plant production is limited by lack of water inputs, water retention, and low organic matter content.[30]

The literature on the interactive effects of intercropping and biofertilizers in black cumin's yield and quality is scant. Therefore, we hypothesized that: i) all intercropping ratios can increase the medicinal properties and the fatty acid profile of black cumin compared with monocropping, ii) application of biofertilizers in intercropping system will improve seed yield of black cumin and fenugreek, and iii) combined application of biofertilizers in intercropping systems will increase N and P uptake and the land equivalent ratio (LER).

2. Experimental Section

2.1. Experimental Site and Weather Condition

A two-year field trial was conducted in 2015 and 2016 growing seasons at the research farm of Naqadeh, West Azerbaijan Province, Iran (36°57″00.0″N, 45°24″00.0″E, 1330 masl). Soil samples were collected from 0 to 30 cm depth, and physical and chemical characteristics were measured. Soil type was silty clay with average pH of 7.9, organic carbon content of 9.5 g kg^{-1}, EC of 0.36 dS m^{-1}, total N of 0.8 g kg^{-1}, available P of 10.45 mg kg^{-1}, and available K of 241.22 mg kg^{-1}. Weather data were obtained from the Iran Meteorological Organization (IRIMO) (**Table 1**).

2.2. Experimental Design and Treatments

The study used a randomized complete block design with factorial arrangement of treatments over three replications (plot size: 4 × 3 m). The treatments included: i) five cropping patterns levels: 50BC:50F (row intercropping) (BC: black cumin, F: fenugreek), 66BC:34F (strip intercropping), 34BC:66F (strip intercropping), 100BC:0F (black cumin monocropping), and

Table 1. Monthly average temperature and monthly total precipitation in 2015 and 2016 growing seasons.

Months	Average temperature [°C]		Precipitation [mm]	
	2015	2016	2015	2016
January	2.9	2.3	24.8	69.3
February	5.5	6.5	25.2	11.4
March	7.3	9.3	51.6	26.4
April	12.7	14.7	26.9	73.1
May	22.5	19.5	21.7	35.3
June	23.8	23.7	0.4	18.0
July	24.5	27.1	0.4	0.4
August	25.5	27.9	0.0	0.0
September	21.2	22.6	3.2	0.0
October	17.0	15.5	44.3	7.0
November	7.8	7.4	27.3	8.0
December	−1.6	−0.4	50.3	74.2
Two-year mean	14.1	14.7	23.0	26.9

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0BC:100F (fenugreek monocropping) where numbers indicate the ratios of BC and F in the intercropping pattern, and ii) the application of AMF (with mix of two AMF species [*Funneliformis mosseae* + *Rhizophagus irregularis*]), bacteria (with mix of phosphate-solubilizing bacteria *Pantoea agglomerans* + *Pseudomonas putida* and N-fixing bacteria *Azotobacter vinelandii*), and an unfertilized control (for each cropping pattern).

2.3. Plant Materials and Cultural Management Practices

Before cultivation, the seeds of both species were inoculated with phosphate-solubilizing bacteria *P. agglomerans* and *P. putida* plus N-fixing bacteria *A. vinelandii* in the form of powder at a rate of 100 g ha^{-1} (Zist Fanavar Sabz Company, Iran). The bacterial population was 5×10^9 colony forming units (CFU) g^{-1} of beneficial bacteria. Bacterial fertilizer powder was mixed with water and uniformly sprayed to cover the seed, and then seeds were air-dried.

The AMF inoculum was obtained from Iran Soil and Water Research Center (Tehran, Iran). At planting, 20 g of inoculum containing ≈4000 spores of the AMF mix of *F. mosseae* and *R. irregularis* were poured into each planting hole. Each gram of inoculum media contained 200 living spores of *F. mosseae* or *R. irregularis*. The origin of mycorrhizal fungi was from the soils of Tabriz plain of Iran.

No chemical fertilizers were used in this study. The seeds of BC and F were each sown at a rate of 33.3 seeds m^{-2} by the furrow method with inter and intrarow spacing of 40 and 7.5 cm, respectively, on March 20, 2014, and March 22, 2015. The rows were 4 m long for both species. Plots and blocks were separated by a buffer space of 1 and 3 m, respectively. The first irrigation was done immediately after planting to facilitate the emergence of the seedlings, and the subsequent irrigations were applied as per the climatic conditions and plant demand every 7 d until the end of the growing season. Weed infestation in this study was manually controlled.

2.4. Crop Sampling and Measurements

2.4.1. Crop Sampling for Determination of Seed Yield and Yield Components

The black cumin and fenugreek plants were hand harvested from a 4.8 m^{-2} central area (i.e., 3 m length of four central rows) at each plot to eliminate the border effect July 5 and August 25, 2015 and July 8 and August 27, 2016, respectively. At harvest, the follicles of the black cumin and the pods of the fenugreek were yellow, corresponding to typical harvesting date for each crop. Harvested seeds were dried at room temperature to reach 14% moisture content.

To determine yield components for each crop, ten plants were randomly selected at each plot. For fenugreek, measurements included plant height, number of pods per plant, number of seeds per pod, and 1000-seed weight. The measured traits for the black cumin were plant height, number of follicles per plant, number of seeds per follicle, and 1000-seed weight.

2.4.2. Plant Nutrient Analysis

Plant material samples were digested following the method proposed by Jones and Case.[31] Following harvest, seed samples of both species were analyzed for macro- and micronutrients content. The Kjeldahl method was used to determine the N content.[32] The concentration of P was determined by the yellow method, in which vanadate–molybdate is used as an indicator.[33] Phosphorus content was measured at 470 nm using a spectrophotometer.

2.4.3. Fixed Oil Isolation and Analysis

The fixed oil content of the black cumin seeds was extracted according to the AOCS (1993) method. The samples were first milled and powdered at 70 °C, and then 10 g subsample was separated after 24 h, and was immersed in Soxhlet with 300 CC of diethyl ether solution. After 6 h, the desired solvent was separated from the oil by rotary. Then, the oil was stored in amber glass bottles to isolate and identify the composition. The fixed oil of black cumin was analyzed using gas chromatography-mass spectrometry (GC-MS) following previously reported methods by Rezaei-Chiyaneh et al.[34]

2.4.4. Essential Oil Extraction and Analysis

The EO extraction was performed by water distillation. To this end, 15 g of BC seed was weighed from each plot and boiled for 3 h in a Clevenger apparatus after briefly milling in 150 mL of water to extract its EO. Then, the content of the EO was calculated by weighing. Following, the EO content and EO yield were calculated as follows[19]

EO yield of black cumin (g m^{-2}) = Seed yield (g m^{-2}) × EO content (%)

Gas chromatography-mass spectrometry analysis was done using an Agilent 7890/5975C (Santa Clara, CA) GC/MSD. For separation of EO components, and HP-5 MS capillary column (5% phenyl methyl polysiloxane, 30 m length, 0.25 mm i.d., 0.25 µm film thickness) was used. The following oven temperature was applied: 3 min at 80 °C, subsequently 8 °C min^{-1} to 180 °C, held for 10 min at 180 °C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The sample was injected (1 µL) in split mode (ratio, 1:50). EI mode was 70 Ev. The mass range was set to be from 40 to 550 m/z. The components were recognized by comparing the calculated Kovats retention indices (RIs), calculated with respect to a mixture of *n*-alkane series (C8-C30, Supelco, Bellefonte, CA), and mass spectra.[35] GC-FID analysis was done by an Agilent 7890 A instrument. The separation was performed in an HP-5 capillary column. The analytical conditions were the same as above. Quantification methods were the same as those reported in previous papers.[18,34,36]

2.4.5. Root Colonization

Root colonization percentage was determined using ten plants from each experimental plot. Plants were carefully uprooted,

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then roots were rinsed with distilled water, cleared in 10% KOH, rinsed with water again, acidified with 1% HCl, and stained in 0.05% Trypan Blue in lacto-glycerol.[37] Mycorrhizal colonization was assessed using the grid-line intersection method described by Giovannetti and Mosse.[38]

2.5. LER

For black cumin and fenugreek intercrops, partial (LERBC and LERF) and total LER (LERT) were calculated according to the following equations[39]

$$LERBC = (YBCI/YBCS) \qquad (1)$$

$$LERF = (YFI/YFS) \qquad (2)$$

$$LER_T = LER_{BC} + LER_F \qquad (3)$$

where YBCI is black cumin seed yield in intercropping; YBCS, black cumin seed yield in the pure stand; YFI, fenugreek seed yield in intercropping; YFS, fenugreek seed yield in pure stand.

2.6. Statistical Analysis

Analysis of variance (ANOVA) was performed using PROC Mixed procedures of SAS version 9.3 (SAS Institute Inc., Cary, NC). The fertilizers application, cropping ratio, and year were considered as fixed effects, whereas blocks were considered random. Mean comparisons for each trait were performed using Duncan's multiple range test at the $P < 0.05$ level.

3. Results

3.1. Plant Performance of Fenugreek

The ANOVA showed that the main effects of cropping patterns and biofertilizers were significant ($P < 0.01$) on all recorded traits (plant height, number of pods per plant, number of seeds per pod, 1000-seed weight, and seed yield) (Table S1, Supporting Information). However, the interaction of cropping patterns and biofertilizer sources was significant for all traits, except for number of seeds per pod and 1000-seed weight (Table S1, Supporting Information).

Means comparison indicated that the highest plant height (54.3 cm) was related to the intercropping pattern of 66BC:F34 inoculated with the bacteria (**Figure 1**A). In addition, highest number of pods per fenugreek plant (19.2) was obtained from bacteria-applied monocropping (Figure 1B). The maximum 1000-seed weight (8.3 g) and seeds per pod (10.5) were obtained from the bacteria fertilizer, respectively. The lowest mentioned attributes were achieved in monocropping without biofertilizer consumption (**Table 2**). On the other hand, the highest seed yield (150 g m⁻²) was produced by a monocropping fertilized with bacteria, whereas that had no significant difference with mycorrhiza-inoculated monocropping system. However, the lowest seed yield (68 g m⁻²) was related to the intercropping

pattern of 66BC:34F without biofertilizer (control) (Figure 1C). Furthermore, the application of AMF and bacteria increased the seed yield by 19.0% and 30.2% compared with control, respectively. In addition, the seed yield in 2016 was 9.6% greater than that in 2015 (Table S3, Supporting Information).

3.2. Black Cumin

3.2.1. Plant Performance

All traits of black cumin (plant height, number of follicles per plant, number of seeds per follicle, 1000-seed weight, seed yield, EOc, EO yield, fixed oil content, and oil yield) were influenced by different planting ratios and biofertilizers (Table S2, Supporting Information). In addition, the interaction of planting pattern and biofertilizers was significant for number of follicles per plant, seed yield, EOc, EO yield, fixed oil content, and oil yield (Table S2, Supporting Information).

Means comparison disclosed that the highest plant height (54.7 cm), seeds per follicle (30.4 cm), and highest 1000-seed weight (2.7 g) were related to the black cumin monocropping, respectively (**Table 3**). In addition, compared to the control conditions, the application of biofertilizer improved the mentioned traits (Table 3). On the other hand, the highest number of follicles per plant (23.2) was obtained from the monocropping of black cumin fertilized with bacteria fertilization (**Figure 2**B).

Besides, the highest seed yield (148 g m⁻²) was recorded from the monocropping fertilized with bacteria biofertilizers, but the latter treatment did not differ significantly from the mycorrhiza-inoculated monocropping system (Figure 2A). Furthermore, inoculation of bacteria fertilization and AMF increased the seed yield by 27.9% and 19.4% compared with control, respectively. Finally, the seed yield of black cumin was 5.8% greater in 2016 compared with 2015, respectively (Table S3, Supporting Information).

3.2.2. Essential Oil Concentration and Yield

The black cumin EO content and EO yield in intercropping were greater than monocropping. It was found that the highest EO content (1.3%) and EO yield (1.7 g m⁻²) were recorded in the intercropping pattern of 66BC:34F fertilized with bacteria fertilization (Figure 2C,D). Moreover, the lowest EO content (0.9%) and EO yield (0.8 g m⁻²) were obtained from a monocropping without fertilizer consumption (Figure 2C,D). In addition, bacteria fertilization and AMF enhanced EO content of black cumin up to 14.7% and 10.8% compared with control, respectively. Furthermore, inoculation of bacteria fertilization and AMF increased the EO yield by 48.4% and 32.6% compared with control, respectively. Also, the EO yield in 2016 was 3.4% higher than that in 2015, respectively (Table S3, Supporting Information).

3.2.3. Essential Oil Compositions

GC-MS analyses showed that 40 components were identified in the black cumin EOs, accounting for 94.7–99.9% of the

(A)

(B)

(C)
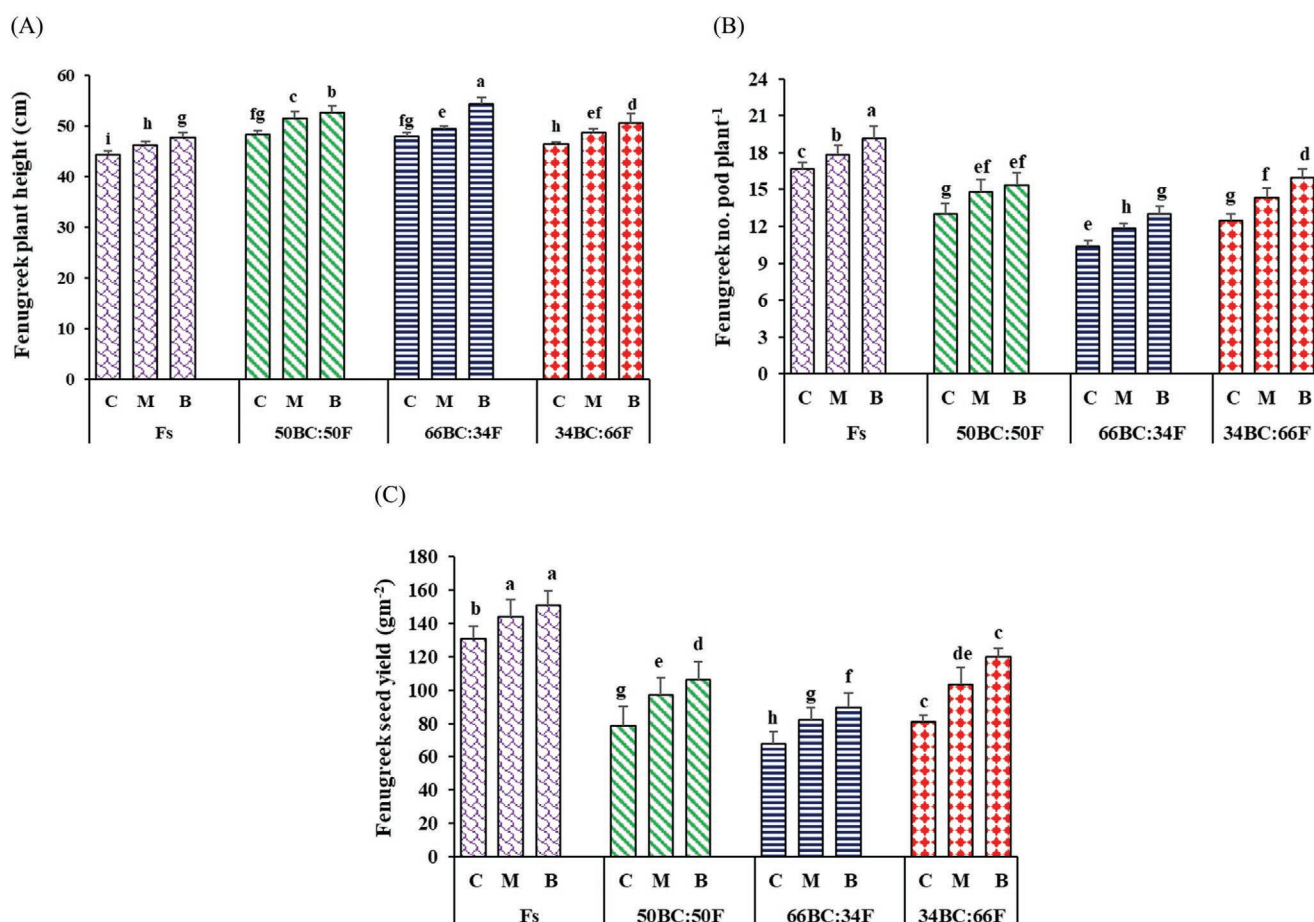
Figure 1. A) Seed yield, B) number of pods per plant, and C) plant height of fenugreek as affected by interaction of different cropping patterns (0BC:100F; 50BC:50F; 66BC:34F; 34BC:66F, where BC and F indicate the ratios of black cumin and fenugreek in intercropping pattern, respectively) and biofertilizers source [C (control); M (mycorrhizal); B (bacterial)]. The error bars indicate standard errors ($n = 3$). The same letters in each shape show nonsignificant difference at $P < 0.05$ by Duncan test.

total compositions. The main EO compounds were thymol (43.63–56.84%), *p*-cymene (13.33–15.59%), geranyl acetate (3.09–5.54%), *trans*-caryophyllene (1.96–4.99%), borneol (1.43–4.23%), and carvacrol (1.14–4.65%), depending on treatments (**Table 4**). The contents of thymol, *p*-cymene, geranyl acetate, *trans*-caryophyllene, and borneol in all intercropping patterns were higher than in black cumin monocropping (Table 4). The highest thymol and *p*-cymene were recorded in the intercropping pattern of 66BC:34F fertilized with bacteria fertilization. In addition, the higher content of geranyl acetate was recorded in the intercropping pattern of 66BC:34F inoculated with AMF. The highest percentages of *trans*-caryophyllene were obtained in the intercropping pattern of 34BC:66F after the use of bacteria fertilization. The highest content of borneol was recorded using the intercropping pattern of 50BC:50F under the use of AMF. In addition, the maximum carvacrol was obtained from the monocropping fertilized with AMF. The relative content of thymol, *p*-cymene, geranyl acetate, *trans*-caryophyllene, and borneol in intercropping patterns enhanced by 12.84%, 2.85%, 17.90%, 22.86%, 38.51%, and 38.51%, respectively, when compared with the monocropping but the average carvacrol in monocropping was 44.47% greater than the intercropping.

Noteworthy, the content of most compounds increased after the use of biofertilizers. Application of bacteria fertilizer and AMF increased the percentages of thymol, *p*-cymene, geranyl acetate, *trans*-caryophyllene, and borneol by16.11–16.68%, 10.87–6.80%, 26.86–42.39%, 34.07–26.54%, and 78.15–46.11% in comparison with control (no fertilizer), respectively (Table 4).

3.2.4. Fixed Oil Content and Oil Yield

The highest fixed oil content (26.7%) was obtained from the intercropping pattern of 66BC:33F fertilized with the bacteria biofertilizer. However, the lowest fixed oil content was observed in the black cumin monocropping without the application of fertilizer, which was 8.3% higher than that of the monocropping under without the application of biofertilizers (**Figure 3**A). Also, the average fixed oil content in intercropping was 3.7% greater than the monocropping. Besides, bacteria fertilization and AMF enhanced fixed oil content of black cumin up to 2.98% and 1.80% compared with control, respectively.

On the other hand, the highest oil yield (52.6 g m^{-2}) was related to the monocropping black cumin fertilized with bacteria fertilization.

Table 2. Number of seeds per pod and 1000-seed weight of fenugreek as influenced by biofertilizer treatments and intercropping patterns.

Treatment	No. of seeds per pod	1000-seed weight [g]
Biofertilizers (B)		
Control	8.66 c	8.03 c
Mycorrhiza	9.83 b	8.20 b
Bacteria	10.45 a	8.30 a
Cropping patterns (C)		
Sole fenugreek	10.94 a	8.37 a
50BC:50F	8.94 c	8.16 b
66BC:34F	8.78 c	7.96 c
34BC:66F	9.94 b	8.18 b
Year (Y)	NS	NS
B	**	**
I	**	**
B × C	NS	NS
Y × B	NS	NS
Y × C	NS	NS
Y × B × C	NS	NS

Notes: 50BC:50F, 66BC:34F, and 34BC:66F (indicate the ratios of black cumin and fenugreek in cropping pattern); *: statistical differences at $P < 0.05$, **: statistical differences at $P < 0.01$, and NS: nonsignificant. The same letters within biofertilizer treatments and intercropping patterns show nonsignificant difference at $P < 0.05$ by Duncan test ($n = 3$).

The lowest (26.4 g m^{-2}) was obtained from the cropping ratio of 33BC:66F without the application of fertilizers (Figure 3B). Furthermore, the application of bacteria fertilization and AMF increased the oil yield by 31.9 and 21.7 compared with control, respectively. In addition, the oil yield in 2016 was 4.6% higher than that in 2015, respectively (Table S3, Supporting Information).

3.2.5. Oil Compositions

The main fatty acids in black cumin oil included unsaturated fatty oleic acid (21.0–22.9%), linoleic acid (47.7–60.9%), and saturated fatty palmitic acid (8.8–15.1%), stearic acid (2.0–3.4%), and behenic acid (2.0–3.4%). According to **Table 5**, the highest content of oleic acid was obtained with the cropping ratio of 33BC:64F with inoculation of AMF and the highest linoleic acid were related to the intercropping pattern of 66BC:34F fertilized with bacteria fertilization, but the highest content of palmitic acid and stearic acid was obtained from the monocropping system that was not fertilized with biofertilizers. In addition, the maximum contents of behenic acid were observed in the intercropping pattern of 33BC:64F inoculated with AMF. Also, the average oleic acid and linoleic acid in intercropping were 3.4% and 8.1% greater than the monocropping. Besides, AMF and bacteria fertilization enhanced oleic acid and linoleic of black cumin up to 2.3–12.1% and 4.6–19.9% compared with control, respectively (Table 5).

3.3. Nutrient Content of Black Cumin

The N and P contents in both plants were influenced by cropping pattern and biofertilizers (Tables S1 and S2, Supporting Information). Also, the interaction of biofertilizer inoculation and intercropping ratio had a significant effect on N and P content

Table 3. Means comparison for the studied traits of black cumin in biofertilizer treatments and intercropping patterns.

Treatment	Plant height [cm]	No. of seeds per follicle	1000-seed weight [g]
Biofertilizers (B)			
Control	43.37 c	24.167 c	2.50 c
Mycorrhiza	47.04 b	26.16 b	2.65 b
Bacteria	50.16 a	27.20 a	2.71 a
Cropping patterns (C)			
Sole black cumin	54.72 a	30.44 a	2.70 a
50BC:50F	43.11 c	24.88 b	2.59 bc
66BC:34F	47.88 b	25.44 b	2.57 c
34BC:66F	41.72 d	22.61 c	2.62 b
Year (Y)	NS	NS	NS
B	**	**	**
I	**	**	**
B × C	NS	NS	NS
Y × B	NS	NS	NS
Y × C	NS	NS	NS
Y × B × C	NS	NS	NS

Notes: 50BC:50F, 66BC:34F, and 34BC:66F (indicate the ratios of black cumin and fenugreek in cropping pattern); *: statistical differences at $P < 0.05$, **: statistical differences at $P < 0.01$, and NS: nonsignificant. The same letters within biofertilizer treatments and intercropping patterns show nonsignificant difference at $P < 0.05$ by Duncan test ($n = 3$).

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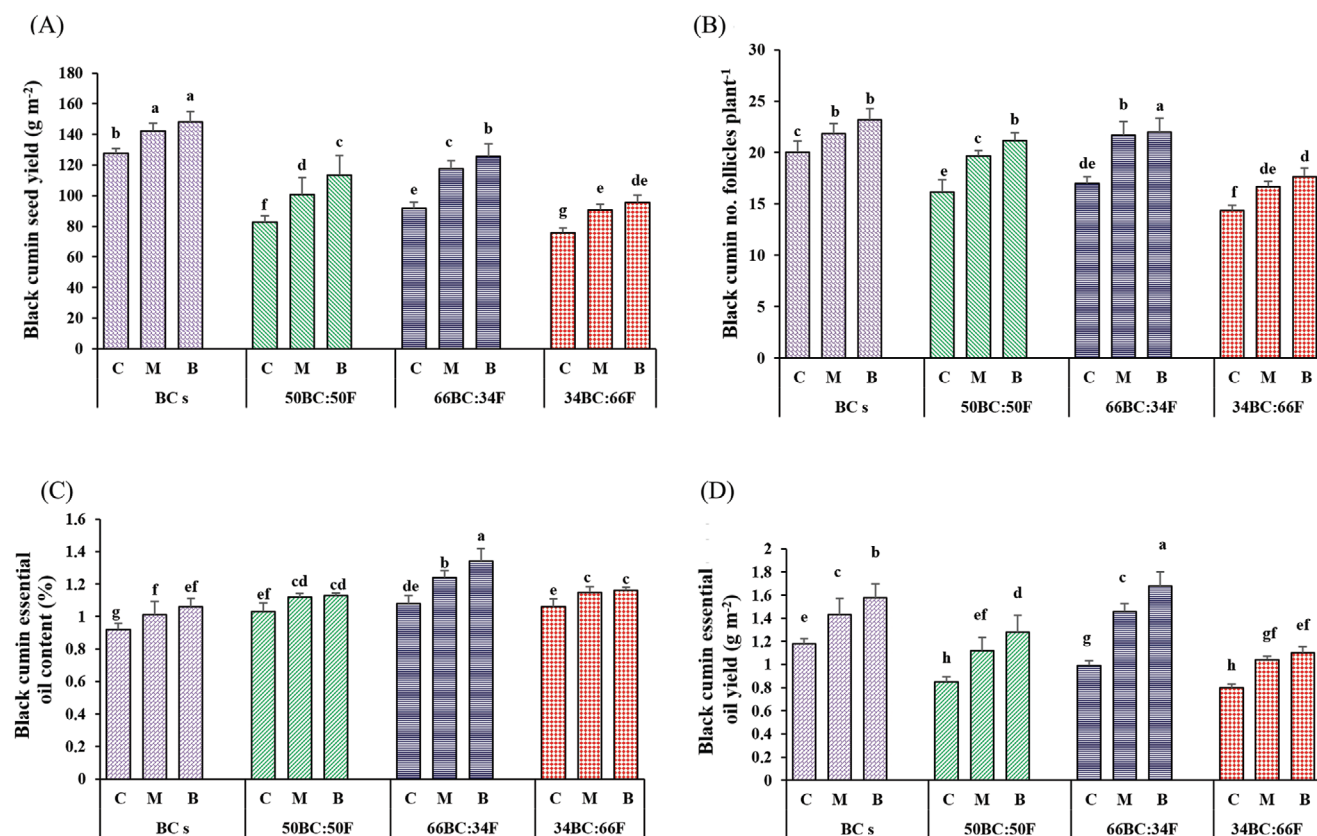
Figure 2. A) Seed yield, B) number of follicles per plant, C) essential oil content, and D) essential oil yield of black cumin as affected by interaction of different cropping patterns (100BC:0F; 0BC:100F; 50BC:50F; 66BC:34F; 34BC:66F, where BC and F indicate the ratios of black cumin and fenugreek in intercropping pattern, respectively) and biofertilizers source [C (control); M (mycorrhizal); B (bacterial)]. The error bars indicate standard errors ($n = 3$). The same letters in each shape show nonsignificant difference at $P < 0.05$ by Duncan test.

(Table S2, Supporting Information). The results indicated that the content of both elements in different intercropping patterns after inoculated with AMF and bacteria fertilization were higher in seeds of both plants than in monocropping without biofertilizer consumption. When compared with AMF, the bacteria application strongly increased the mentioned nutrients content ($p < 0.05$). The highest N and P content of black cumin was observed in the intercropping pattern of 66BC:34F fertilized with bacteria fertilization (**Figure** 4A,C). The lowest contents of N and P in both plants were achieved in monocropping without biofertilizer consumption (Figure 4A,C). Also, the average N and P content in intercropping was 11.0% and 19.2% higher than the monocropping of black cumin, respectively.

3.4. Nutrient Content of Fenugreek

The results indicated that the content of N and P in different intercropping ratio after biofertilization application was higher in seed of fenugreek than in monocropping without biofertilizer consumption. The highest increment level of N and P nutrients was observed in the intercropping pattern of 34BC:66F after use of bacteria (Figure 4B,D). However, there were no significant differences in terms of P content between the intercropping

pattern of 34BC:66F with AMF and bacteria application. The lowest content of N and P was achieved in monocropping without biofertilizer consumption (Figure 4B,D). Furthermore, the average N and P, content of fenugreek in intercropping was 9.48% and 12.70% higher than the monocropping of fenugreek, respectively.

3.5. Root Colonization

In fenugreek, the highest root colonization (75.16%) was obtained from the plants inoculated with AMF in the intercropping pattern of 34BC:66F (**Figure 5**A). Furthermore, the application of AMF and bacteria increased the root colonization by 83.51% and 25.56% compared with control, respectively.

In black cumin, the highest root colonization (60.66%) was recorded in the intercropping pattern of 66BC:34F treated with AMF (Figure 5B). Furthermore, the application of AMF and bacteria fertilization increased the root colonization by 80.99% and 36.95% compared with control, respectively. In addition, the average root colonization in intercropping was 19.78% and 30.95% higher than the monocropping of fenugreek and black cumin, respectively. But the lowest root colonization of both plants was observed in monocropping without the application

Table 4. Proportion of black cumin EO constituents under different planting patterns and biofertilizer treatments (average of two years).

No.	Components	RI[a]	Planting patterns												
			B_S	B_S + M	B_S + B	50BC:50F	50BC:50F+M	50BC:50F+B	66BC:34F	66BC:34F+M	66BC:34F+B	34BC:66F	34BC:66F+M	34BC:66F+B	Average of composition
1	α-Thujene	923	0.48	1.5	1.01	0.16	0.49	1.55	0.3	0.47	0.08	1.87	0.29	1.88	0.84
2	α-Pinene	932	0.48	0.43	–	–	0.5	1.05	–	0.11	0.13	1.19	0.06	0.3	0.47
3	Sabinene	972	0.56	0.55	0.63	1.9	–	1.1	0.36	0.13	0.12	0.85	0.13	–	0.63
4	β-Pinene	976	0.46	0.58	0.68	1.35	–	1.07	0.85	0.17	0.12	1.51	0.15	–	0.69
5	p-Cymene	1022	**13.33**	**13.39**	**14.55**	**13.62**	**13.94**	**14.45**	**14.03**	**15.32**	**15.59**	**13.48**	**14.3**	**14.53**	14.21
6	DL-Limonene	1028	0.58	0.78	0.91	–	0.65	1.59	0.23	0.2	–	1.66	0.29	0.42	0.73
7	1,8-Cineole	1031	0.48	0.25	0.89	–	–	0.52	0.53	0.11	0.18	0.12	0.08	0.24	0.34
8	γ-Terpinene	1057	1.86	2.99	3.19	2.73	2.11	2.79	2.03	2.54	2.73	2.91	2.99	2.3	2.59
9	Cis-Sabinene-hydrate	1066	–	–	–	0.14	0.29		1.34	0.4	0.33	0.11	0.18	0.27	0.38
10	Methyl octanoate	1119	2.28	2.53	3.82	2.59	2.34	3.8	1.38	1.81	3.58	3.93	3.69	2.15	2.82
11	Sabinol, trans-	1146	1.22	0.47	–	0.1	0.68	1	0.96	0.52	0.24	0.18	0.18	0.83	0.58
12	Borneol	1167	**2.06**	**2.31**	**3.48**	**3.86**	**4.23**	**2.17**	**1.51**	**1.58**	**1.43**	**3.19**	**3.95**	**3.68**	2.78
13	4-Terpineol	1178	1.84	0.25	–	0.23	0.58	0.54	1.63	0.9	0.66	0.99	0.68	0.36	0.78
14	p-Cymen-8-ol	1185	0.92	0.41	0.7	0.5	0.89	1	0.55	0.83	0.18	0.68	0.75	0.65	0.67
15	Piperitol, trans-	1204	1.63	0.36	0.99	0.1	0.29	0.93	0.84	0.41	0.15	0.58	0.69	0.25	0.60
16	Sabinene hydrate acetate, cis-	1233	0.71	0.2	0.76	0.55	0.36	–	0.58	0.66	0.42	0.11	0.16	0.87	0.48
17	Carvacrol, methyl ether	1243	0.51	0.58	0.63	0.35	0.77	–	1.41	0.64	0.23	0.12	0.14	0.38	0.52
18	2-Phenylethyl acetate	1251	2.62	2.14	0.45	2.11	2.57	2.1	1.95	2.14	1.16	1.59	2.46	1.28	1.88
19	Geraniol	1253	–	–	–	0.17	–	–	0.4	0.28	0.08	–	–	–	0.23
20	Geranial	1272	0.81	0.83		0.16	0.84	1.1	0.5	0.18	0.2	0.18	0.2	0.46	0.49
21	Thymol	1292	**43.63**	**47.42**	**48.04**	**50.21**	**52.57**	**47.41**	**48.4**	**52.35**	**56.84**	**49.09**	**50.3**	**50.35**	49.71
22	Carvacrol	1300	**4.04**	**4.65**	**4.16**	**3.95**	**2.05**	**2.98**	**3.14**	**2.78**	**1.68**	**1.86**	**3.74**	**1.14**	3.01
23	Thymyl acetate	1352	1.04	0.52	–	–	0.17	–		0.11	0.07	0.16	0.09	0.39	0.31
24	alpha-Longipinene	1354	1.02	0.84	–	–	1.5	1.81	0.67	0.15	0.75	0.65	0.4	–	0.86
25	Carvacrol acetate	1376	1	–	0.55	–	–	0.73	0.21	0.14	0.072	–	0.13	–	0.40
26	Geranyl acetate	1379	**3.09**	**4.05**	**3.61**	**3.12**	**3.23**	**3.24**	**3.63**	**5.49**	**4.59**	**4.18**	**4.86**	**4.27**	3.94
27	β-Bourbonene	1387	0.79	0.44	–	–	0.97	0.56	0.36	0.11	0.18	0.12	0.16	0.04	0.37
28	Longifolene	1409	0.71	0.27	0.58	0.39	0.71	–	0.74	0.83	0.12		1.42	0.92	0.66
29	trans-Caryophyllene	1422	**2.26**	**2.42**	**2.54**	**2.97**	**2.76**	**1.96**	**2.42**	**2.88**	**2.64**	**2.94**	**3.41**	**4.99**	2.84
30	a-Ionone, (E)-	1431	0.08	–	0.08	–	–	0.45	0.26	0.06	–	0.36	0.09	–	0.19
31	α-Humulene	1456	0.09	–	0.09	–	–	0.82	0.42	0.13	0.09	–	0.16	–	0.25
32	β-Acoradiene	1469	0.58	0.24	0.11	0.19	–	0.47	0.71	0.49	0.12	–	0.42	0.52	0.38
33	γ-Himachalene	1478	0.88	0.19	0.21	–	–	0.72	0.69	0.19	0.18	–	0.33	0.4	0.42
34	Germacrene D	1483	–	0.18	0.63	0.16	0.25	–	0.32	0.12	–	0.28	0.06	0.31	0.25
35	α-Selinene	1496	–	–	0.55	–	0.32	–	0.48	0.18	0.09	0.32	0.25	–	0.31
36	a-Bisabolene,	1507	–	–	0.41	–	0.27	0.21	0.34	0.16	–	–	0.16	–	0.25
37	γ-Cadinene	1516	0.59	0.81	0.12	0.61	0.22	0.36	0.95	0.54	0.34	0.09	0.54	0.14	0.44
38	delta-Cadinene	1524	1.12	0.8	–	0.89	0.61	0.29	1.76	0.83	0.68	–	1.21	1.72	0.99

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Table 4. Continued.

No.	Components	RI[a]	Planting patterns												
			B_S	B_S + M	B_S + B	50BC:50F	50BC:50F+M	50BC:50F+B	66BC:34F	66BC:34F+M	66BC:34F+B	34BC:66F	34BC:66F+M	34BC:66F+B	Average of composition
39	Geranyl butanoate	1555	0.48	1.5	–	–	0.34	0.22	0.18	0.09	–	0.87	0.29	–	0.49
40	Caryophyllene oxide	1587	0.48	0.43	0.63	1.05	1.15	0.95	1.91	1.15	1.4	1.19	0.06	0.3	0.89
	Total identified (%)		94.71	95.31	95	94.16	98.65	99.94	98.97	98.18	97.452	97.36	99.45	96.34	99.674

[a]RI, by comparison of retention index with those reported in Adams and NIST 08. M (mycorrhiza), B (bacteria), Bs (black cumin sole cropping), 50BC:50F, 66BC:34F, and 34BC:66F (indicate the ratios of black cumin and fenugreek in cropping pattern); the main components are shown by bold values ($n = 3$).

of biofertilizers (control). In addition, the root colonization of black cumin in 2016 was 8.46% higher than that in 2015, respectively (Table S3, Supporting Information).

3.6. LER

The highest partial LER of the black cumin (0.84) and fenugreek (0.78) was obtained from the intercropping pattern of 66BC:34F and 33BC:64F treated with bacteria fertilization, respectability. The highest (1.44) and the lowest (1.20) total LER was obtained from the intercropping pattern of 66BC:34F inoculated with bacteria fertilization and unfertilized 33BC:64F intercropping ratio, respectively (**Figure 6**).

4. Discussion

4.1. Plant Performance

It seems that the decrease in plant height of fenugreek in intercropping could be because of the competition with black cumin for water, minerals, solar radiation, and space, which reduced the utilization of environmental resources and subsequently reduced its height in intercropping system. On the other hand,

one reason for the increase in the plant height of black cumin in the intercropping probably is because of the light competition with fenugreek, which led to increased light absorption by black cumin, which ultimately increased the plant height and also representing that black cumin has been the dominant crop in the intercropping patterns and was benefited from intercropping compared with fenugreek.[20,40]

Different mechanisms have been proposed for the effect of PGPR and AMF on the growth characteristics of plants. PGPR and AMF affect the uptake of macro- and microelements and enhance the production of plant growth hormones such as gibberellin (effect on longitudinal cell growth, especially on stem internodes), auxin and cytokinin (effect on cell division), which are responsible for increasing plant growth.[41] Numerous reports have pointed out the positive effects of PGPR and AMF on height of different plants.[42,43]

The higher yield and yield components in monocropping could be because of the reduction of interspecific competition in monocropping, which resulted in an increase in the seed yield of both plants compared to other different intercropping ratios.[44] On the other hand, the decrease in the yield components and seed yield of both plants in intercropping system can be attributed to the more excellent synchronization of the black cumin growth with fenugreek, which has resulted in more interspecific competition in different
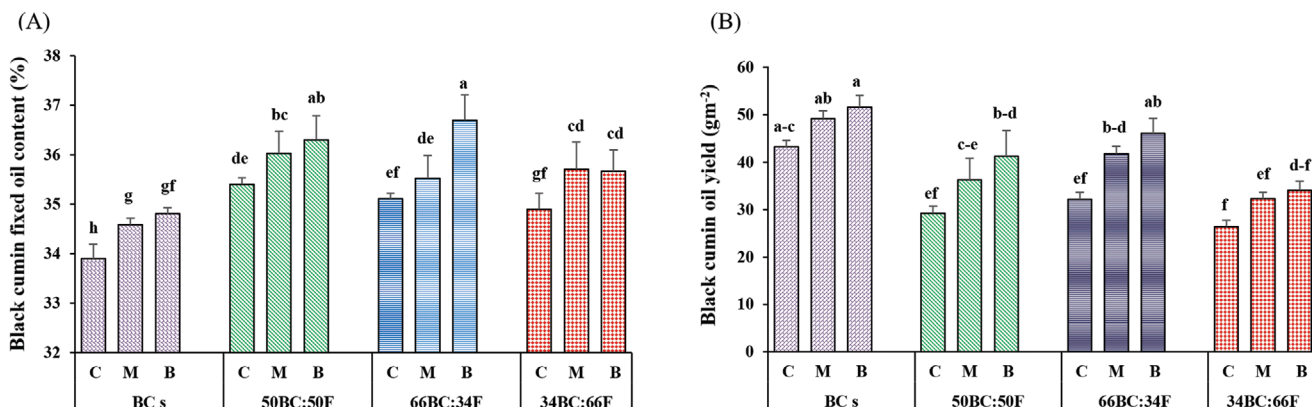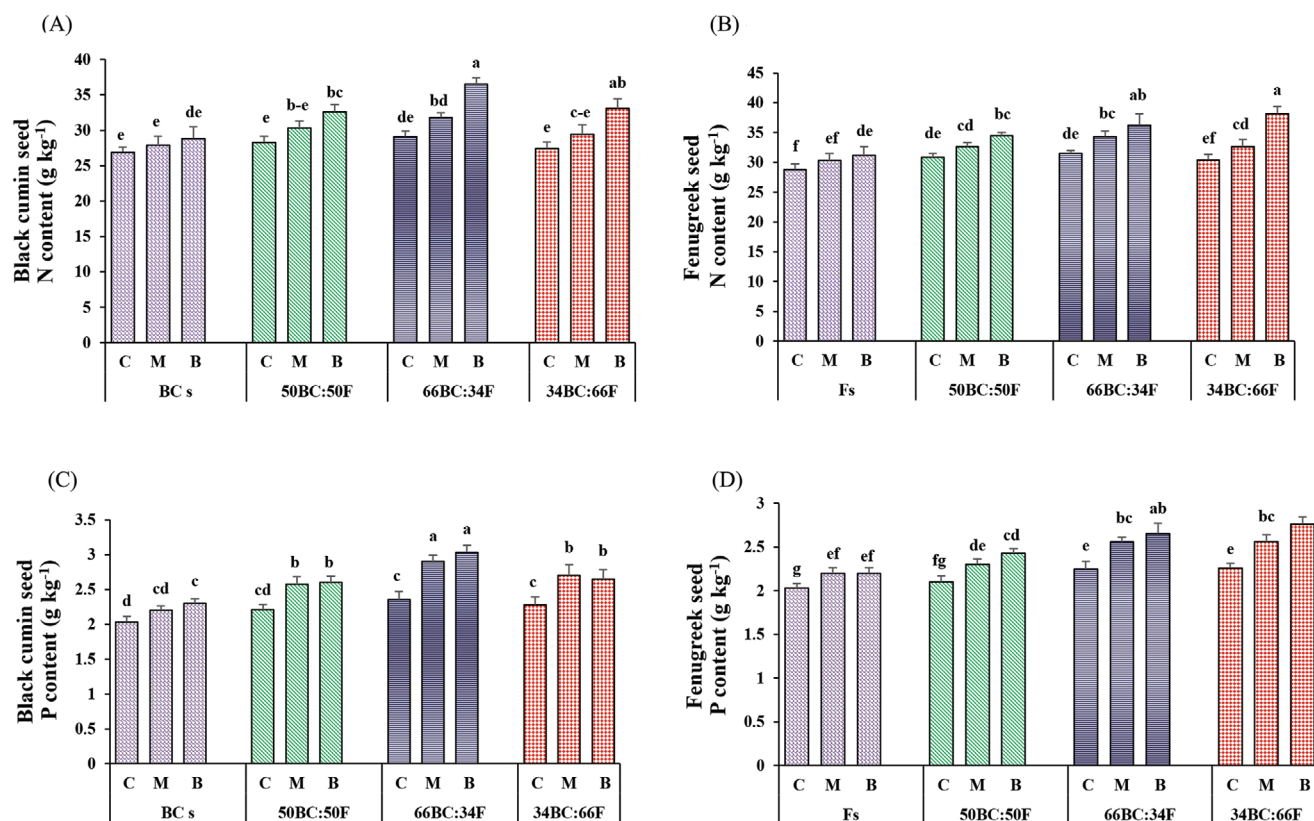

Figure 3. A) Fixed oil content and B) oil yield of black cumin as affected by interaction of different cropping patterns (100BC:0F; 0BC:100F; 50BC:50F; 66BC:34F; 34BC:66F, where BC and F indicate the ratios of black cumin and fenugreek in intercropping pattern, respectively) and biofertilizers source [C (control); M (mycorrhizal); B (bacterial)]. The error bars indicate standard errors ($n = 3$). The same letters in each shape show nonsignificant difference at $P < 0.05$ by Duncan test.

Table 5. Proportion of black cumin oil constituents under different cropping patterns and biofertilizer treatments (average of two years).

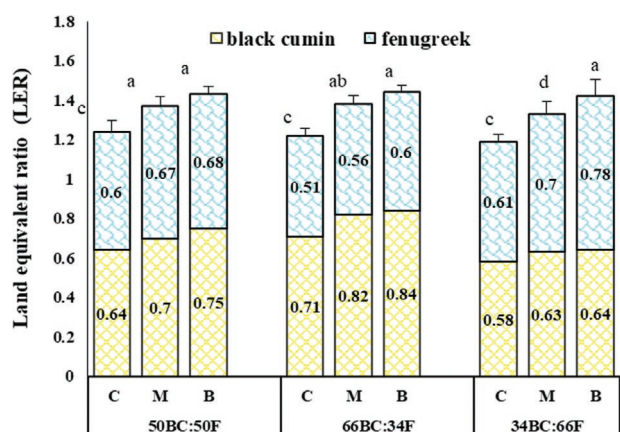| No.	Components	RT	RT	B_S	Planting patterns											Average of composition
					B_S + M	B_S + B	50BC:50F	50BC:50F+M	50BC:50F+B	66BC:34F	66BC:34F+M	66BC:34F+B	34BC:66F	34BC:66F+M	34BC:66F+B	
1	Myristic acid methyl ester (C14:0)	18.78	18.0	0.29	0.26	0.28	0.25	0.27	0.25	0.25	0.22	0.22	0.25	0.24	0.27	0.25
2	Palmitic acid methyl ester (C16:0)	22.20	21.52	**15.14**	**13.76**	**14.92**	**13.46**	**12.27**	**13.01**	**13.23**	**10.61**	**8.77**	**13.25**	**13.59**	**11.79**	12.81
3	Palmitoleic acid methyl ester (C16:1)	23.29	22.57	0.27	0.25	0.27	0.24	0.21	0.21	0.23	0.28	0.19	0.27	0.28	0.29	0.24
4	Stearic acid methyl ester (C18:0)	25.51	24.81	**3.39**	**3.35**	**2.94**	2.91	2.92	3.09	2.91	2.16	2.01	2.49	2.72	2.44	**2.77**
5	*Trans*-9-octadecenoic methyl ester (C18:1n9t)	26.12	25.10	0.12	0.18	0.19	0.16	–	–	–	0.05	0.13	0.08	0.11	0.21	0.13
6	Oleic acid methyl ester (C18:1n9c)	26.43	25.79	**21.14**	**21.79**	**21.86**	**22.17**	**22.49**	**24.12**	**21.00**	**21.45**	**21.86**	**22.42**	**22.94**	**22.62**	22.15
7	Linolelaidic acid methyl ester (C18:2n6t)	27.10	26.77	0.43	0.29	0.26	0.27	–	0.33	0.4	0.22	0.41	0.27	0.23	0.21	0.30
8	Linoleic acid methyl ester (C18:2n6c)	27.80	27.20	**47.65**	**49.08**	**48.15**	**50.82**	**51.13**	**53.79**	**50.79**	**57.58**	**60.92**	**52.88**	**56.31**	**58.51**	53.13
9	Arachidic acid methyl ester (C20:0)	28.63	27.89	0.24	0.25	0.38	0.24	0.24	0.26	0.23	0.23	0.08	0.17	0.21	0.09	0.21
10	Linolenic acid methyl ester (C18:3n3)	29.41	28.62	0.31	0.39	0.59	–	0.48	0.33	0.34	0.46	0.26	0.31	0.33	0.26	0.37
11	*Cis*-11-eicosenoic acid, methyl ester (20:1)	29.58	28.77	0.46	0.48	0.74	0.15	0.39	0.42	0.37	0.52	0.47	0.4	0.42	0.27	0.42
12	Behenic acid methyl ester (C22:0)	30.22	30.07	**4.31**	**4.54**	**6.17**	**4.77**	**3.91**	**4.06**	**3.61**	**5.59**	**2.93**	**3.98**	**1.7**	**2.2**	3.98
	Total identified (%)				93.75	94.62	96.75	95.44	94.31	99.87	93.36	99.37	98.25	96.77	99.08	99.16	96.79

Notes: B (bacteria), M (mycorrhiza), Bs (black cumin sole cropping), 50BC:50F, 66BC:34F, and 34BC:66F (indicate the ratios of black cumin and fenugreek in cropping pattern); the main components are shown by bold values ($n = 3$).

Figure 4. Black cumin and fenugreek seed A,B) N and C,D) P content, respectively, as affected by interaction of different cropping patterns (100BC:0F; 0BC:100F; 50BC: 50F; 66BC:34F; 34BC:66F, where BC and F indicate the ratios of black cumin and fenugreek in intercropping pattern) and biofertilizers source [C (control); M (mycorrhizal); B (bacterial)]. The error bars indicate standard errors ($n = 3$). The same letters in each shape show nonsignificant difference at $P < 0.05$ by Duncan test.
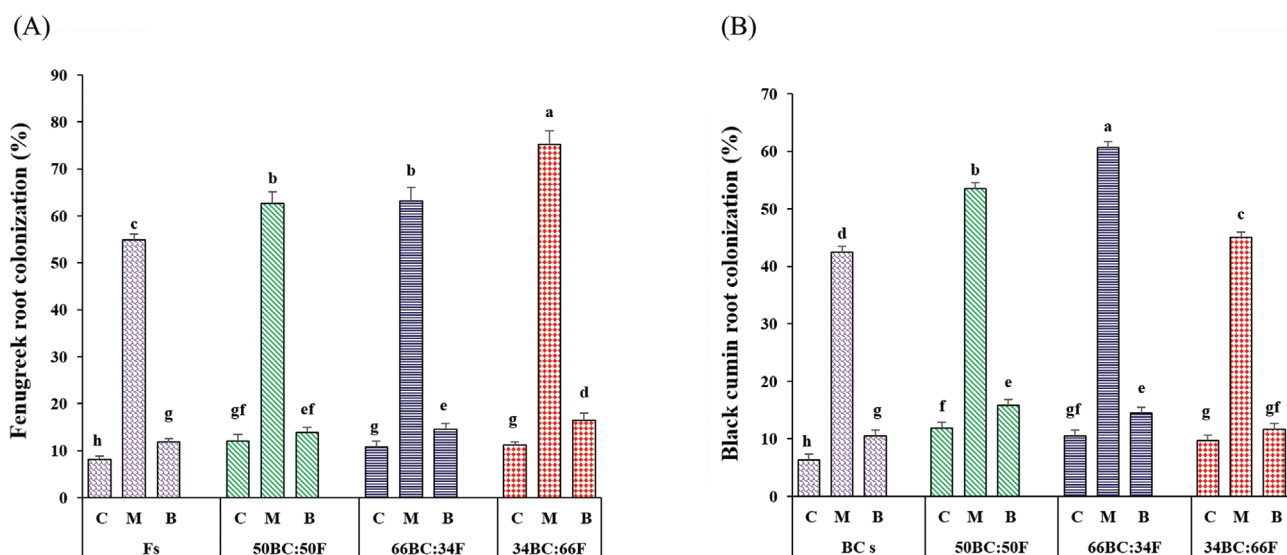
Figure 5. A,B) Black cumin and fenugreek root colonization, respectively, as affected by interaction of different cropping patterns (100BC:0F; 0BC:100F; 50BC:50F; 66BC:34F; 34BC:66F, where BC and F indicate the ratios of black cumin and fenugreek in intercropping pattern, respectively) and biofertilizers source [C (control); M (mycorrhizal); B (bacterial)]. The error bars indicate standard errors ($n = 3$). The same letters in each shape show nonsignificant difference at $P < 0.05$ by Duncan test.

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Figure 6. Land equivalent ratio (LER) of fenugreek and black cumin as affected by interaction of different cropping patterns (50 BC:50F; 66BC:34F; 34BC:66F, where BC and F indicate the ratios of black cumin and fenugreek in intercropping pattern, respectively) and biofertilizers source [C (Control); M (mycorrhizal); B (bacterial)]. The error bars indicate standard errors ($n = 3$). The same letters in each shape show nonsignificant difference at $P < 0.05$ by Duncan test.

intercropping ratio compared to the intraspecific competition in monocropping.[45]

Also, as the plant ratios increased, the yield component decreased because of the reduction of space required for growth and subsequent increase of interspecific competition compared to intraspecific competition between the two species, which resulted in a decrease in seed yield.[19] It also seems that in intercropping with increasing plant ratios, the other plant has less access to environmental factors (light, nutrients, and moisture) and eventually transfers less photosynthate production to the seed, which leads to a decrease in the yield components.[46] Because black cumin is considered as the main plant species in this study (EO yield and quality), the intercropping with higher black cumin proportion (66BC:34F) is considered as the best performing one. However, the higher LER (1.2–1.44) shows that all intercropping systems (50 BC:50F, 66BC:34F, 34BC:66F) allow significantly more efficient agricultural production than monocropping.

The results of this study showed that the yield and yield components significantly enhanced with the application of biofertilizers. It seems that inoculation with these biofertilizers because of enhancing nutrients availability, which is an effective factor in stimulating plant growth and photosynthesis, improves the conditions for growth, and consequently increased yield components and seed yield of both species.[47]

However, it can be concluded that bacterial treatments compared to fungal treatments lead to a positive and significant effect on the yield by bringing about a proper balance between N and P and other microelements.[48] Previous research indicated that the PGPR and AMF increase yield and yield components of plants by increasing root growth and increasing plant access to nutrients and water.[49,50]

4.2. EO Content, EO Yield, and Compositions of Black Cumin

As N is one of the elements that affect the activity of photosynthetic enzymes in plants, any factor that increases N absorption

can eventually lead to an increase in plant's photosynthesis,[51] which can lead to an increase in EO production as well.[52] PGPRs and AMF increase EO of medicinal plants by increasing plant access to important nutrients such as N, P, and micronutrients (iron, zinc, and Cu).[53] Therefore, inoculation with these fertilizers owing to improving availability of nutrients, which is an effective factor in stimulating plant growth and photosynthesis, improves the conditions for growth, photosynthate production, and consequently increased quantitative and qualitative production of the EOs of medicinal plant.[54,55] Vafadar-Yengeje et al.[56] concluded that the Moldavian balm-faba bean (*Vicia faba* L.) intercropping increased the Moldavian balm EO quality by enhancing the amount of geraniol and geranyl acetate compared with the sole cropping system. Rezaei-Chiyaneh et al.[18] reported that PGPR application in the intercropping system improved the EO quality and quantity of fennel.

4.3. Seed Fixed Oil Content, Oil Yield, and Compositions of Black Cumin

It seems that suitable conditions for the growth of black cumin plants such as optimum use of nutrients available in the intercropping pattern of 66BC:33F and better light distribution in the total canopy will improve growth and photosynthesis. Consequently, it leads to an increase in the fixed oil content and oil compositions in intercropping compared to monocropping. Moreover, the use of biofertilizers improved soil microbial activity and root system development and improved access to nutrient absorption and consequently increased fixed oil content.[57] Combined consumption increased biological fixation of N, the solubility of immobilized phosphate, a decrease in soil pH, and the production of various hormones (such as cytokinin, auxin, biotin, and pantothenic acid) because of the synergistic effects of bacteria (azotobacter and pseudomonas). In this way, intercropping stimulates nutrient absorption and improves both quality and quantity of the fixed oil of the black seeds by affecting photosynthetic processes.[58,59] These results agree with the findings of Saeidi et al.[60] in safflower-faba bean intercropping, and Rezaei-Chiyaneh et al.[18] in fennel-common bean intercropping under application of biofertilizer.

4.4. Nutrients

Obtained results demonstrated that the concentrations of nutrients in the intercropping system inoculated with AMF and bacterial biofertilizer were higher than monocropping without application of biofertilizer. Arbuscular mycorrhizal fungi dissolve immobile elements and applicable to the host plant by improving their root uptake, releasing organic acids, and acidifying the rhizosphere environment and biochemical properties of the soil.[61] Besides, AMF have a profound effect on the root physiology of the plant, which activates glutamine synthetase, arginase, and urease enzyme leading to an increase in the content of nutrients concentrations in the plants.[62] On the other hand, the increase in nutrients uptake is related to the improvement of root uptake through the infiltration of the fungal mycelium into the soil, followed by plant access to more nutrients from the soil.[63]

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The flow rate of P into the mycorrhizal plant is 3–6 times higher than in non-mycorrhizal plants.[64] The increasing rate of P uptake by the host plant is because of the presence of mycorrhizal hyphae within the epidermis of the plant, which provides a large surface area for the transfer of the nutrients, especially P to the host plant.[65] Besides, the production and secretion of phosphatase enzyme by hyphae of AMF causes insoluble and stabilized phosphate in the soil to transform to soluble form and be absorbed by the root. Moreover, AMF may increase nodulation and N fixation in legumes by increasing P uptake in these plants.[47] Nitrogen-fixing bacteria can improve N availability to plants by a process of biological N fixation, and phosphate-solubilizing bacteria dissolve insoluble forms of phosphate by releasing some organic acids. As a result, the absorption of nutrients by the plant increases. These results agree with the findings of Weisany et al.[53] and Ingraffia et al.[66] who investigated AMF inoculation in dill (*Anethum graveolens* L.)-common bean and wheat-faba bean intercropping, respectively.

4.5. Root Colonization

Based on the results, the highest root colonization of both plants was obtained from the intercropping systems when they were inoculated with AMF. The high root colonization in the intercropping system compared to monocropping under the use of biological fertilizers might be because of higher greener cover, adequate moisture, and increasing soil biological activities.[67,68] Furthermore, differences in the root system, root depth, and root biomass of two plants, root exudates, and the availability of nutrients provide favorable conditions for root colonization.[69] In addition, inoculation with biofertilizers, especially, induces the creation of a more extensive network of root fungi hyphae and causes root growth along with the increase in root colonization percentage. Hassan et al.[70] reported that the use of plant growth-promoting bacteria plays an important role in root colonization through root exudates such as amino acids, monosaccharides, and organic acids. In agreement with our results, Rezaei Chiyaneh et al.,[71] in the intercropping of isabgol (*Plantago ovata*) and lentil (*Lens culinaris*) and Ingraffia et al.,[66] in the intercropping of wheat/faba bean, reported that the inoculation with AMF increased root colonization.

4.6. LER

Our results indicated that total LER was greater than 1 in all treatments. Therefore, it can be concluded that the intercropping system performed better than monocropping. The higher LER of the intercropping system can be related to the correct arrangement and supplementary use of nutrients, water, and radiation by the components of the intercropping system.[72] Therefore, these conditions improved the growth and yield of both species, and LER increased compared with the plant's monocropping. These results agree with the findings of Fallah et al.[52] in dragonhead–soybean, Koocheki et al.[73] in saffron (*Crocus sativus* L.)–pumpkin (*Cucurbita pepo* L.)–watermelon (*Citrullus lanatus* L.) and Rezaei Chiyaneh et al.[18] in fennel-common bean intercropping.

5. Conclusion

A combination of PGPR and intercropping pattern of 66BC:33F is the recommended treatment to improve LER and nutrient uptake. This is because both seed yield and seed oil quality of intercropped black cumin and fenugreek can be improved through inoculation with PGPR and AMF. This improved overall productivity not only provides economic benefits for growers but also helps to better contribute to SDG 12 (sustainable production) because of the improved LER. Against this backdrop, it can be further assumed that the increased species diversity of intercropped black cumin and fenugreek inoculated with PGPR and AMF also helps to better contribute to SDG 15 (life on land) and 13 (climate action) because of habitat diversification and an improved response diversity. Future research should focus on differences between synthetic fertilizers and biofertilizers in sole and intercropping systems to holistically evaluate the economic and environmental benefits of biofertilization versus synthetic fertilizer application in intercropping black cumin and fenugreek.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

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