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Effect of Spraying Sulfur and Inoculation Rhizobacteria on Growth and Yield of Canola

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ABSTRACT

Due to the increasing importance of using environmentally friendly methods to increase the yield of crops, two- year experiment carried out in a field located in the Dehgolan region, in the northwest of Iran, to study influence of sulfur spraying and plant growth promoting rhizobacteria inoculation on canola traits. The experiment was arranged as a split-plot factorial arrangement based on randomized complete block design with three replications. The main plots included two levels of sulfur (control and application), and the factorial combinations of strigolactone (control and application) and microorganisms (control, *Funneliformis mosseae*, *Bacillus lentus*, *Pseudomonas fluorescens*, *Thiobacillus* sp.) were allocated to the sub-plots. The results of combined analysis showed that the 1000 seed weights and seed yield increased significantly by sulfur application. At the same time, the 1000 seed weights decreased under the influence of strigolactone. Also, the SPAD number, the number of pods and seeds per plant, the 1000 seed weights and the seed yield increased significantly by influence of microorganisms, especially *Thiobacillus*, compared to control treatment. Based on the results of interaction effects, all traits except the number of SPAD were affected by the interaction effect of sulfur, strigolactone and microorganisms. The application of sulfur along with strigolactone and *Thiobacillus* significantly increased the number of pods per plant (200), the 1000 seed weights (4.53 g) and the seed yield (2552 kg/ha).

INTRODUCTION

Sulfur (S) is very important for achieving maximum yield in canola (Shoja et al., 2018). Therefore, in recent years, various researches have been conducted to determine the optimum amount of sulfur required by this crop. It has been demonstrated that sulfur has a significant effect on the foundation of fatty acid in oilseed crops such as soybean, canola, and groundnuts (Karaaslan et al., 2020). There is greater synergistic influence of sulfur on growth factor, yield and yield attributes, nutrient uptake, protein and oil production in canola (Asadi Rahmani et al., 2018). Sulfur fertilizers have become a main part of the fertilizer mix for canola growers. Sulfur compounds inhibit the growth and activity of mycorrhizal mycelium on the root (Gryndler et al., 1998).

Mycorrhizal fungi are capable of colonizing most of the world's crop plants (95% of the current species of crops belong to families that are mycorrhizal). Many profits accrue to crops from their relationship with arbuscular mycorrhizal fungi (AMF) (Mitra et al., 2019). Mycorrhizal could improve water uptake and nutrient absorption, especially phosphor (Begum et al., 2019). Some plant families, such as *Brassicaceae, Chenopodiaceae* and *Amaranthaceae*are unable to establish symbiosis with mycorrhizal fungi or have partial symbiosis. (Singh et al., 2003). Canola (*Brassica napus* L.) is one of the important oilseed crops in Iran, which is cultivated in rotation with wheat. It ranks tertiary because of world oilseed yield after *Glycine max* and *Arachis hypogaea* (Mohammadi et al., 2012). Poor or no mycorrhizal associations in this family attributed to specific anatomical structures of roots and non-germination of arbuscular mycorrhizal spores due to allelopathic compounds leakage such as glucosinolates, isothiocyanates (Schreiner & Koide, 1993) or defenses, which suppresses activities of pathogenic fungi (Choi et al., 2009). Mukherjee & Ane (2011) found root architecture change and gene expression of symbiotic in crops due to exudates of germinating spores from *Glomus* species.

Supplement effects of arbuscular mycorrhizal fungi well known on the phosphate solubilizing bacteria activity (PSB). Nacoon et al. (2020) showed that beneficial effects of co-inoculation of a PSB strain and AMF, when mixed with addition of rock phosphate, to improve the growth. Some PSB play a role in siderophore production (Hamadali et al., 2008), Secretion of plant hormones includes auxin and gibberellins (Souchie et al., 2007), antibiotics releases (Taurian et al., 2010) and production of secondary metabolites (Yang et al., 2008).

Strigolactones hormones adjust plant growth and development (Yoneyama & Brewer, 2021), enhance seed germination and primary root length and also nodule formation (Mitra et al., 2019). They are derived from carotenoids, which are rich sources of biologically active compounds. Known role of strigolactones is stimulating arbuscular mycorrhizal fungi colonization and promoting of symbiosis with root nodule bacteria (Yoneyama, 2020). Strigolactones help plants confront some kinds of stresses via drought, salinity, heavy metal, nutrient starvation, and heat (Alvi et al., 2022).

However, despite extensive research on the individual effects of sulfur, arbuscular mycorrhizal fungi, phosphate-solubilizing bacteria, and strigolactones on canola growth and yield, the combined impact of these factors remains unclear. Therefore, the present study aims to investigate the synergistic effects of sulfur fertilization, strigolactone application, and AMF inoculation alongside PSB on canola growth, nutrient uptake, and overall yield.

MATERIAL AND METHODS

Experimental Details

The study was conducted in a field located in the Dehgolan region (35.2818° N, 47.4196° E), in the northwest of Iran, over the course of two growing seasons. Figure 1 illustrates the monthly rainfall and temperature data for the years 2020 and 2021 (January to December).

The experimental design followed a split-plot factorial arrangement within a randomized complete block design, with three replications. Sulfur application was assigned to the main plots, while the sub-plot factors included microorganisms (control, *Funneliformis mosseae*, *Bacillus lentus*, *Pseudomonas fluorescens*, and *Thiobacillus* sp.) and strigolactone treatments (seed priming and control). Each sub-plot measured 5 meters in length and consisted of five rows, spaced 1 meter apart. A 2-meter buffer zone was maintained between the main plots to mitigate the effects of lateral water flow and movement during spraying.

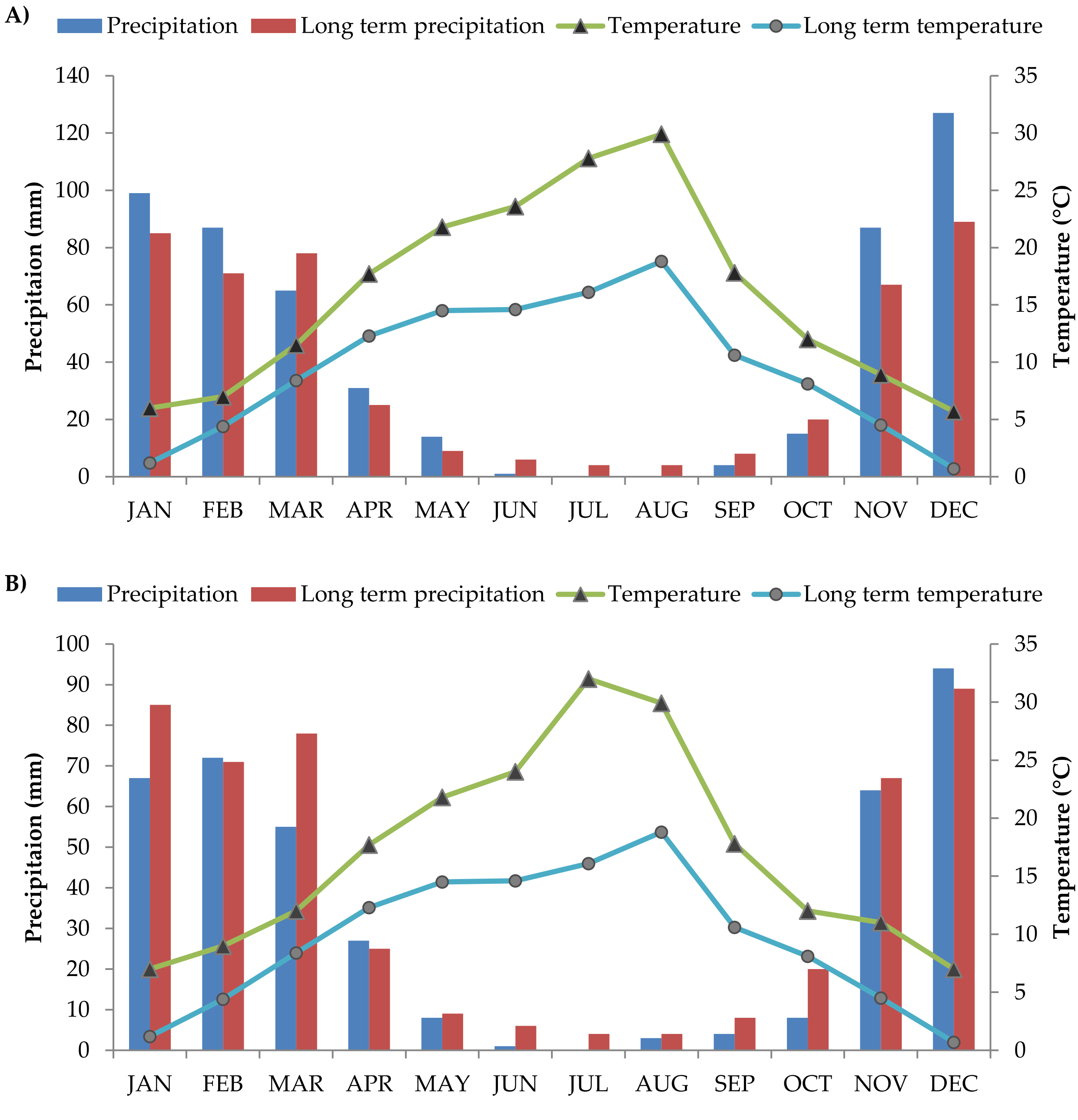

Figure 1. Monthly precipitation and air temperature from January to December for the years 2020 (A) and 2021 (B), along with the long-term average (1980-2020), in Dehgolan, Iran.

The AMF inoculum consisted of soil particles, fungal spores (30,000 spores per 20 ml inoculum), infected root fragments, and hyphae derived from a stock culture of *F. mosseae* with a single subtending hypha. The sporocarps, which contained 2-5 spores enclosed in a peridium, were produced on host plants at the Iranian Soil and Water Research Institute. Mycorrhizal fungal spores were cultured in pots filled with sandy soil (autoclaved at 121°C for 30 minutes, repeated three times) in a greenhouse maintained at 25°C during the day and 16°C at night, with a 16-hour light/8-hour dark photoperiod and a relative humidity of 60-65%. The AMF inoculum was placed 5 cm beneath the canola seeds during sowing.

The phosphate-solubilizing bacteria used in this study were *B. lentus* (P5 strain, lecithinase-negative, gram-positive, peritrichous flagella, ellipsoidal shape, paracentral spore, slightly curved rods) and *P. fluorescens* (S153 strain, gram-negative, rod-shaped, multiple flagella, and obligate aerobes). These strains were isolated and identified at the Soil and Water Research Institute, Iran. Phenotypic characterization was performed using the API system, while phylogenetic identification was conducted through sequence analysis of the 16S rRNA gene. Bacterial strains were extracted and evaluated from soil samples gathered throughout various locations in Kurdistan, Iran. Before inoculation, the bacteria were revived and grown in a nutrient medium. They were grown on a shaker at 37°C and 180 rpm. Afterward, the culture was centrifuged at 10,000 rpm for 15 minutes and washed with 0.9% sodium chloride. The bacterial pellet was then re-suspended in a sterile phosphate buffer (López-Valdez et al., 2011) to achieve a final density of approximately 40×10^6 CFU/cm^3. The selected isolates were subsequently inoculated into the same medium and incubated at 30°C for 10 days. The phosphate-solubilizing bacteria (PSB) were applied 5 cm below the canola seeds during sowing.

To evaluate the effectiveness and persistence of the inoculation, the approach described by Burr (1984) was utilized 30 days following sowing. *Thiobacillus* inoculants were prepared using Postgate culture medium, as outlined by Vishniac & Santer (1975). Once the culture media were ready, the selected bacterial strains were multiplied with the aid of a perlite-based carrier. A total of one kilogram of inoculant was generated and stored in suitable plastic containers, ensuring bacterial viability at temperatures ranging from 4°C to 15°C. All procedures were conducted in shaded areas, away from direct sunlight. The *Thiobacillus* inoculum was placed 5 cm below the canola seeds during planting. Soil samples (0-30 cm depth) were collected before sowing to assess the physicochemical properties (Table 1). In these soil samples, pH was measured in a 1:2.5 (w/v) soil-to-water extract. Total nitrogen (N) was determined using the Kjeldahl method (Bremner & Mulvaney, 1982), while soil Olsen-P content was measured by extracting with 0.5 mol/L $NaHCO_3$ followed by Mo-Sb colorimetry (Olsen & Sommers, 1982).

Available potassium (K) was extracted using varying concentrations of NH_4OAc, including 0.1, 0.25, 0.5, and 1 M. The potassium content in the ammonium acetate extracts was measured using atomic absorption spectrometry (Warncke & Brown, 2011).

Prior to sowing, 100 kg of nitrogen (N)/ha as urea and 75 kg of phosphorus (P_2O_5)/ha as superphosphate were applied during plowing. Additionally, 100 kg of nitrogen (N)/ha was applied as a top dressing during early to mid-flowering. Canola seeds were sown at a density of 80 plants per m^2 on September 16 and 25 for the first and second years, respectively. Pest and weed control measures were implemented throughout the growing season.

Crop Traits Measurement

At the flowering stage, chlorophyll content was measured using a handheld dual-wavelength meter (SPAD-502 Plus). Measurements were taken midway along the uppermost leaf of six randomly selected plants located within the central area of three rows in each plot. At the ripening stage, samples were collected from different plots to measure seed yield and yield components. To avoid potential border effects, three central rows from each plot were sampled. The seeds were manually separated and cleaned. Moisture content was measured using the gravimetric method by drying the samples at 105°C for 24 hours, and the results were standardized to 12% moisture. From the harvested plants, five plants were randomly selected, and the following characteristics were measured: the number of pods per plant, the number of seeds per pod, and the weight of 1000 seeds.

Table 1. Physic-chemical characters of soil analysis in the field of study

Year	Silt %	Sand %	Clay %	Soil Texture	pH	EC (ds/m)	SOC %	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
2020	26	39.8	34.2	Clay loam	7.36	1.21	1.13	0.022	5.3	277
2021	22	42.8	35.2	Clay loam	7.34	1.18	0.68	0.025	6.2	284

Statistical Analyses

After verifying the homogeneity of variances using Bartlett's test, a combined analysis of variance was performed using the SAS 9.4 statistical software. The least significant difference (LSD) test was applied to compare the means of the traits, with a significance level set at $p<0.05$.

RESULTS

The SPAD number, which is an indicator of greenness in plants, became significant under the influence of microorganisms' inoculation at the level of 1% and interaction of microorganism and strigolactone at 5%. In this study microorganism had positive effects on SPAD, as *Thiobacillus* inoculation achieved the maximum SPAD (Table 2).

Also, the pod number per plant significantly affected by year, microorganism, and also some double and triple interaction effects. The effects of microorganisms on the number of pods per plant were effective so that, the maximum number of pods was recorded in the *Thiobacillus* treatment (Table 2). The positive effect of microorganisms in increasing the number of pods per plant was in *Thiobacillus* (4.89%), *Pseudomonas* (3.51%), *Bacillus* (3.1%) and *F. mosseae* (1.96%) compared to control. Triple interaction effect showed that the highest number of pods per plant was recorded by strigolactone application and sulfur foliar spraying and *Thiobacillus* inoculation (Figure 2).

Also seed number per pod affected significantly by microorganism, interaction of strigolactone and microorganism, and also sulfur, strigolactone and microorganism interactions. Numbers of seed per pod reached maximum by no sulfur, no strigolactone and only by *Thiobacillus* inoculation (Figure 3). The number of seeds per pod improved in *Thiobacillus* (5.25%), *Pseudomonas* (0.89%) and *Bacillus* (0.28%) compared to control (Table 2). Also seed weights were affected by main effects and interaction, as seen, the highest seed weight was found by sulfur, strigolactone and *Thiobacillus* inoculation (Figure 4).

Seed weight increased by 12.78% under the influence of sulfur application and decreased by 0.71% under the influence of strigolactone. It seems that the positive effect of strigolactone has led to an increase in the number of pods in the plant. The seed weight enhanced by *Thiobacillus* (1.94%), *Pseudomonas* (0.24%), *Bacillus* (0.97%) and *F. mosseae* (1.94%) compared to control (Table 2).

Based on the two-year results of this experiment, it can be seen that the canola seed yield significantly affected by sulfur and interaction effects of sulfur, strigolactone and microorganism. As shown the seed yield enhanced by 23.22% by sulfur application compared to the control (Table 2). The effect of microorganisms on the canola yield showed that the maximum canola yield achieved by *Thiobacillus* (Table 2). In this study, seed yield enhanced up to 243 kg/ha by microorganisms. The seed yield also improved by *Thiobacillus*, *Pseudomonas*, *Bacillus* and *F. mosseae* as: 12.55, 4.82, 4.44 and 3.96%, as arrangement, compared to control.

Table 2. Influence of sulfur spraying and microorganism's inoculation on canola yield and yield components

Treatment	Method	SPAD number	Pod number per plant	Seed numbers per pod	1000×Seed weight	Seed Yield (kg/ha)
Sulfur spraying	No (Control)	35.86 ^{a}	163.55 ^{a}	28.58 ^{a}	3.91 ^{b}	1829.62 ^{b}
	Spraying	39.36 ^{a}	184.08 ^{a}	27.71 ^{a}	4.41 ^{a}	2254.5 ^{a}
Strigolactone priming	No (Control)	37.51 ^{a}	168.45 ^{a}	28.7 ^{a}	4.18 ^{a}	2019.42 ^{a}
	Priming	37.71 ^{a}	179.18 ^{a}	27.6 ^{a}	4.15 ^{b}	2064.7 ^{a}
Microorganisms	No inoculation (Control)	35.7 ^{c}	169.25 ^{d}	27.79 ^{b}	4.12 ^{b}	1941.91 ^{c}
	Funneliformis mosseae	38 ^{b}	172.58 ^{c}	27.79 ^{b}	4.2 ^{a}	2018.88 ^{b}
	Bacillus lentus	37.25 ^{b}	174.5 ^{b}	27.87 ^{b}	4.16 ^{ab}	2028.2 ^{b}
	Pseudomonas fluorescens	37.58 ^{b}	175.2 ^{b}	28.04 ^{b}	4.13 ^{b}	2035.7 ^{b}
	Thiobacillus sp.	39.54 ^{a}	177.54 ^{a}	29.25 ^{a}	4.2 ^{a}	2185.63 ^{a}

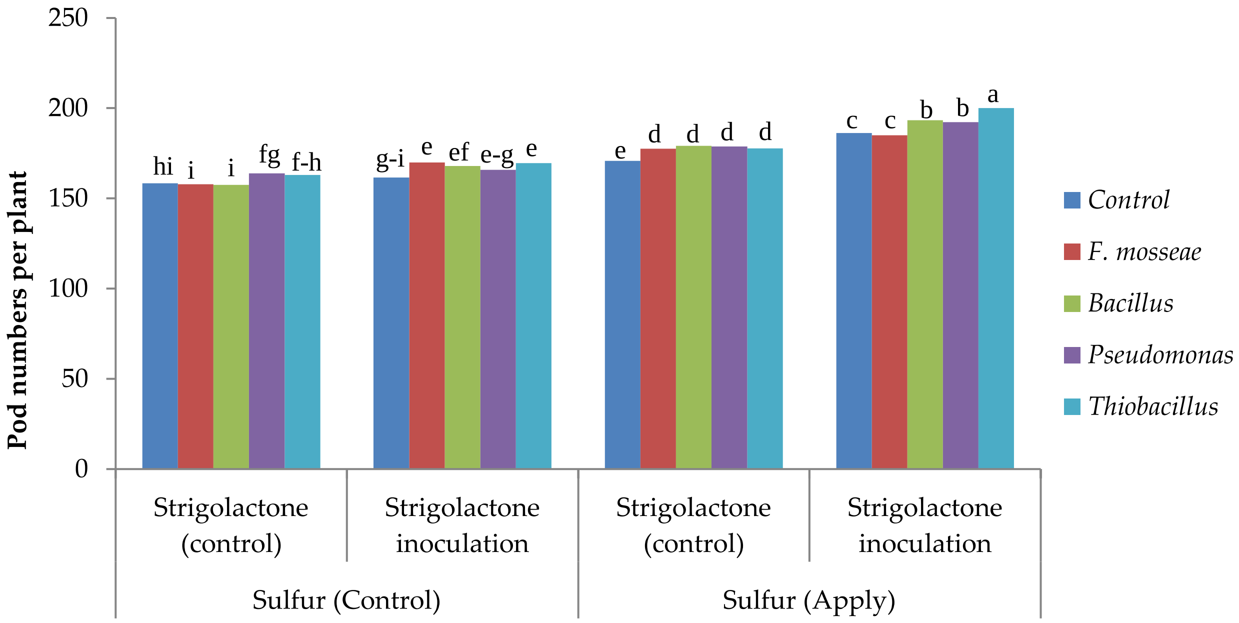

Figure 2. The interaction effect of sulfur spraying and plant growth promoting rhizobacteria inoculation on pod numbers per plant

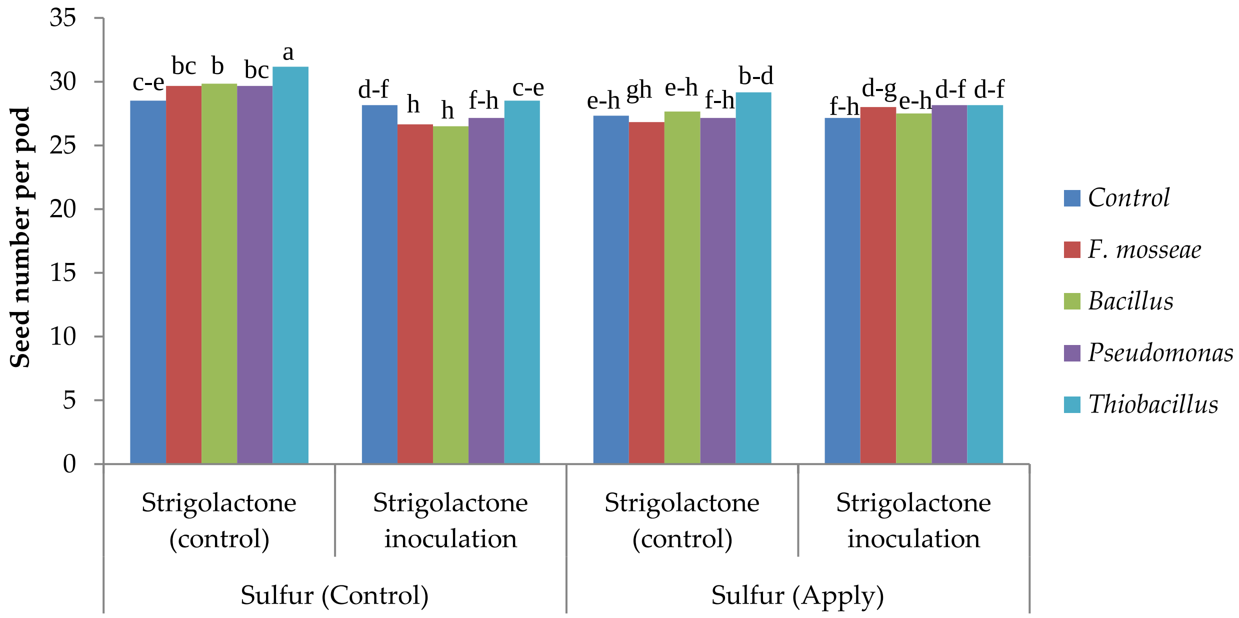

Figure 3. The interaction effect of sulfur spraying and plant growth promoting rhizobacteria inoculation on seed numbers per pod

Also, based on the triple interaction effect of sulfur, microorganisms and strigolactone, a surprising increase of 47% in canola seed yield was observed under the influence of sulfur and strigolactone, and *Thiobacillus* inoculation (Figure 5).

DISCUSSION

Positive but different effects of experimental treatments were observed on greenness index, yield components and canola seed yield as one of the important oilseed plants. Increasing photosynthetic pigments by biofertilizers such as *Bacillus subtilis* and *Pseudomonas fluorescens* inoculation were found by other researchers (Mohamed & Gomaa, 2012). Also, photosynthetic efficiency enhanced by mycorrhizal fungi *F. mosseae* (Begum et al., 2019). Improved leaf chlorophyll index by *Pseudomonas fluorescens* in canola reported by other researchers (Kazemi Oskuei et al., 2023). These researchers attributed the increase in chlorophyll to the improvement of nutrients uptake such as nitrogen, phosphorus, potassium and iron.

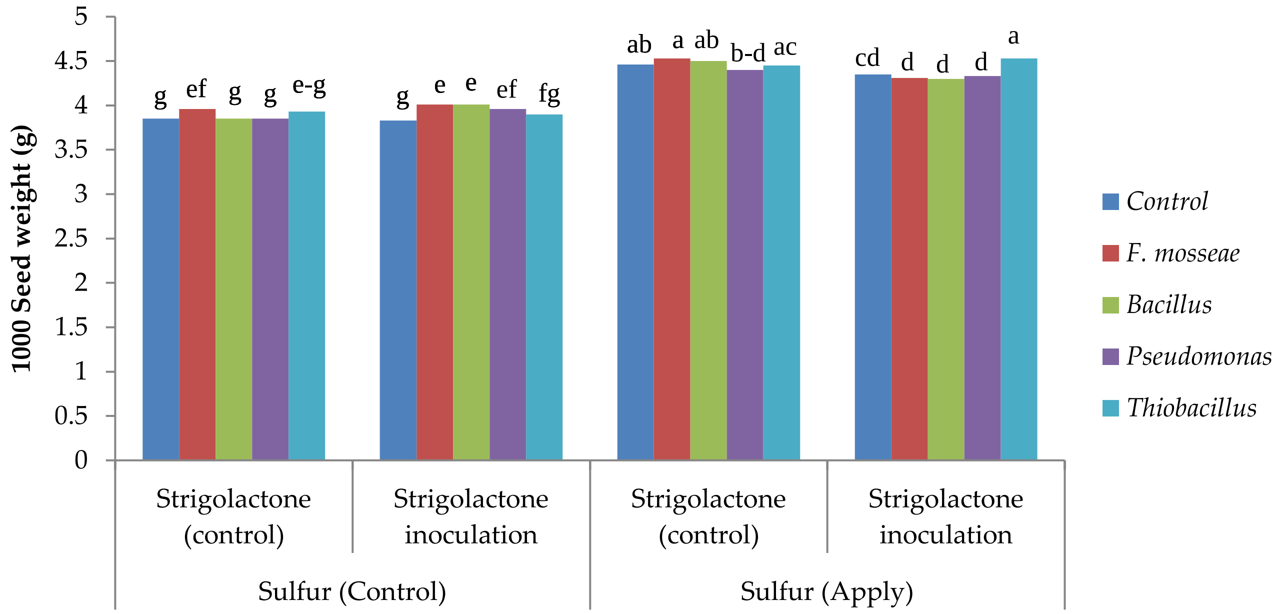

Figure 4. The interaction effect of sulfur spraying and plant growth promoting rhizobacteria inoculation on 1000-seed weight

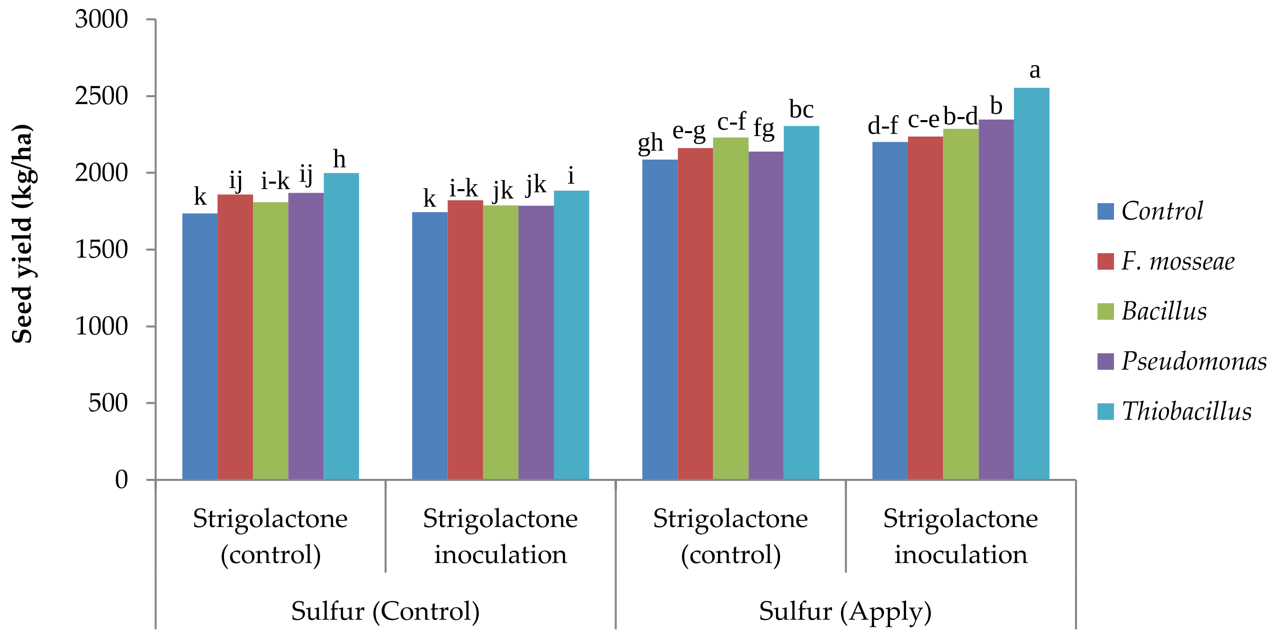

Figure 5. The interaction effect of sulfur spraying and plant growth promoting rhizobacteria inoculation on canola seed yield

The *F. mosseae* significantly enhanced the shoot and root dry matter, plant height, leaf and branch number, photosynthetic efficiency (Mitra et al., 2019). In similar research, the SPAD value increased up to 13% by strigolactone in canola (Ma et al., 2017). They found that strigolactone, improved the growth, leaf chlorophyll, gas exchange characters (net photosynthetic rates, stomatal conductance, concentration of intercellular CO_2, and transpiration rate) and photosystem II quantum yield in oilseed rape.

Considering the increase in the number of pods in the plant under the influence of the treatments in this experiment and based on the negative relationship between the number of pods per plant and the number of seeds per pod, therefore decrease number of seeds per pod seems logical. Ameliorative effect of sulfur on yield attributes such as, pod numbers per plant, seed numbers per pod and 1000-seed weight in canola were found by Ur Rehman et al. (2013) who showed that chlorophyll contents, net assimilation rate, crop growth rate, leaf area index, plant height and number of branches were improved by sulfur. Also, Karaaslan et al. (2020) reported an increasing effect of sulfur on plant height, thousand seed weight, pod per plant, number of primary branches plant and seed per pod and seed yield in canola cultivars. In similar research, sulfur increased the plant height, number of primary branches, pod length and seed attributes such as, number of pods per plant and number of seeds per pod (Poisson et al., 2019). Foliar application of sulfur increased the seed weight of canola by 5.66 and 12.57% (Yari et al., 2022). Also, the numbers of pod per plant increased as 4.33 by sulfur and *Thiobacillus* inoculation (Motamed et al., 2018).

Improving vegetative characters, seed attributes and seed yield of canola varieties by sulfur application were found by Ur Rehman et al. (2013). It seems that in this study, enhanced SPAD number and yield attributes increased seed yield. Yield components were also significantly correlated with canola seed yield (Ma et al., 2015). Enhanced canola seed yield by 7.42% by sulfur related to increasing seed weight (Yari et al., 2022). Rameeh et al. (2019) were found a significant positive correlation between activity of root nitrate reductase and seed yield, they declare that increasing activity of root nitrate reductase in roots by sulfur application, improved the canola seed yield by 17%. Sulfur increased the seed yield via improve of plant height, number of primary branches per plant, number of pods plant, pod length and number of seeds per pod (Tabasi et al., 2017). Also increasing of seed yield and biological yield, in canola proved by sulfur application (Pużyńska et al., 2018; Poisson et al., 2019). In another study, sulfur increased canola yields up to 30% in different places and years (Ma et al., 2015). Sulfur also improved Fe percentage as 15.2% by plant (Baghaie, 2023).

The desirable effects of strigolactones showed on vegetation, biomass and yield under different stress. Exogenous application of strigolactones decrease abscisic acid content whereas enhance auxin and gibberellic acid, after that net photosynthetic rate and the level of carbon dioxide fixation and finally yield increased (Wang et al., 2022). In another study, *Bacillus paralicheniformis* inoculation amplificated the abundance of plants' beneficial microorganisms. In the same ways, *F. mosseae* inoculation intensified uptake and availability of nutrients by plant (Mitra et al., 2019), enhance the root colonization, relative water content, seed yield and yield components (Begum et al., 2019). It has been reported that the significant increase in growth and development, yield components and seed yield under the influence of the use of sulfur and *Thiobacillus* (Motamed et al., 2018).

Asadi Rahmani et al. (2018) attributed the increase in canola yield to enhance uptake of nutrients and also1000- seed weight by Sulfur + *Thiobacillus* inoculation. In similar research, sulfur plus *Thiobacillus* produced the highest number of pods per plant, 1000 grain weight and grain yield of canola (Tabasi et al., 2017). Also, *Pseudomonas fluorescens* enhance activities of antioxidant enzymes hence strengthened the above ground biomass and root length of canola (Kazemi Oskuei et al., 2023).

Neshat et al. (2023) found that inoculated canola by pseudomonas showed higher antioxidant capacity, carotenoids and proline accumulation and total protein under salinity stress. Mohamed & Gomaa (2012) were showed that proline, crude protein contents, IAA and GA3 contents, total free amino acids, and the contents of N, P, K, Ca and Mg enhanced by the inoculation of *Bacillus subtilis* and *Pseudomonas fluorescens*.

CONCLUSION

Canola is one of the valuable oilseed plants that are compatible with large areas with different climatic conditions in Iran. Based on the two-year results of this research, it was observed that the application of treatments increased the seed yield and its components. Among the treatments, sulfur had the greatest effect on increasing the canola seed yield. The effect of strigolactone was positive but minor. Since, based on the research, the usefulness of strigolactone has been reported mostly under stress conditions; it seems that its minor effects were caused by the lack of stress in this experiment. The use of growth-promoting microorganisms was also found to be positive on the studied traits and the most useful in terms of seed yield by *Thiobacillus*.

Compliance with Ethical Standards

Authors' Contributions

SA: Investigation

KM: Methodology, Formal Analysis

BP: Writing – review & editing

AR: Conceptualization, Supervision

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Funding

Not applicable.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AI Disclosure

The authors confirm that no generative AI was used in writing this manuscript or creating images, tables, or graphics.

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