

## Article

# Soil-Applied Boron Combined with Boron-Tolerant Bacteria (*Bacillus* sp. MN54) Improve Root Proliferation and Nodulation, Yield and Agronomic Grain Biofortification of Chickpea (*Cicer arietinum* L.)

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**Abstract:** Chickpea is widely cultivated on calcareous sandy soils in arid and semi-arid regions of Pakistan; however, widespread boron (B) deficiencies in these soils significantly decreases its productivity. Soil application of B could improve chickpea yield and grain-B concentration. However, optimizing suitable B level is necessary due to a narrow deficiency and toxicity range of B. Nonetheless, the co-application of B-tolerant bacteria (BTB) and synthetic B fertilizer could be helpful in obtaining higher chickpea yields and grain-B concentration. Therefore, this study optimized the level of soil applied B along with BTB, (i.e., *Bacillus* sp. MN54) to improve growth, yield and grain-B concentrations of chickpea. The B concentrations included in the study were 0.00 (control), 0.25, 0.50, 0.75 and 1.00 mg B kg<sup>-1</sup> soil combined with or without *Bacillus* sp. MN54 inoculation. Soil application of B significantly improved root system, nodulation, yield and grain-B concentration, and *Bacillus* sp. MN54 inoculation further improved these traits. Moreover, B application at a lower dose (0.25 mg B kg<sup>-1</sup> soil) with BTB inoculation recorded the highest improvements in root system (longer roots with more roots' proliferation), growth, nodulation and grain yield. However, the highest grain-B concentration was recorded under a higher B level (0.75 mg B kg<sup>-1</sup> soil) included in the study. Soil application of 0.25 mg B kg<sup>-1</sup> with *Bacillus* sp. MN54 inoculation improved growth and yield-related traits, especially nodule population (81%), number of pods plant<sup>-1</sup> (38%), number of grains plant<sup>-1</sup> (65%) and grain yield (47%) compared with control treatment. However, the grain-B concentration was higher under the highest B level (1.00 mg kg<sup>-1</sup> soil) with *Bacillus* sp. MN54 inoculation. In conclusion, soil application of 0.25 mg B kg<sup>-1</sup> with *Bacillus* sp. MN54 inoculation is a pragmatic option to improve the root system, nodule population, seedling growth, yield and agronomic grain-B biofortification of chickpea.

**Keywords:** agronomic biofortification; plant growth promoting rhizobacteria; grain yield; nodulation

## 1. Introduction

Macro- and micronutrients are essential for optimum plant growth, and their availability has significant impact on crop performance [1]. Boron (B) is a non-metal element and Warington [2] was the pioneer scientist who described it as a micronutrient for the first time during 1923. It plays an important role in human metabolic activities and is highly important for humans [3], animals [4] and unicellular eukaryotes at varying levels [5]. However, different species require varying levels of B for their optimum growth. Boron is involved in brain functioning, psychomotor performance and reactive oxygen and it improves macronutrient metabolism [3]. Plants require B in minute quantities; however, it plays significant role in plant metabolic activities. It helps in stabilizing plant cell walls, membrane integrity, sugar transport and utilization of calcium and nitrogen [6–8]. Moreover, B regulates pod formation during the reproductive phase, which results in a higher yield. Contrastingly, B deficiency at the reproductive stage results in pollen sterility and decreases grain yield to a great extent [9]. Nonetheless, B plays a more critical role in crop performance during reproductive phase than in the vegetative phase, as it helps in seed setting and grain-filling [10]. FSoil application of nutrients is an ancient and economical method, and plants easily uptake soil-applied nutrients. Soil application of B significantly increases stem diameter (~2 cm) at the time of bud emergence [11]. Kanwal et al. [12] reported that a combined application of B and zinc (Zn) enhanced maize (*Zea mays* L.) productivity. Moreover, Panhwar et al. [13] stated that soil application of B and Zn improved the root length, dry weight, plant height, leaf area index and chlorophyll content of maize crop. Soil application of B also improved potassium (K) availability in soil [14]. Boron-deficiency may reduce the dry matter accumulation, photosynthetic activity, plant height and delay fruiting time [15]. Boron availability is extremely important during the reproductive phase to promote the growth and development of pollen tubes and the anther [16]. Yield-related traits of cotton (*Gossypium hirsutum* L.) have been improved by B application [17]. Similarly, soil application of micronutrients improved yield, seed quality and grain Zn concentration in mungbean (*Vigna radiata* L.) [18].

Boron is an important micronutrient, required in a minute quantity; however, its deficiency and toxicity cause serious losses in crop productivity. Cartwright et al. [19] reported that barley (*Hordeum vulgare* L.) production reduced by 17% at higher B concentrations. Pakistani soils are calcareous in nature; therefore, they suffer from micronutrient deficiencies [20]. Boron-deficiency is mostly observed in calcareous soils [21] and poses negative impacts on the productivity of arable crops. Boron-deficiency may reduce chlorophyll contents, disrupt enzyme activities and causes male sterility [22].

Leguminous crops are an integral component of different cropping systems established around the world. Chickpea (*Cicer arietinum* L.) is the third most important crop of these cropping systems after peas (*Pisum sativum* L.) and beans (*Phaseolus vulgaris* L.) [23]. Chickpea is nutritionally rich and can substitute meat for protein intake in humans [24]. It is a rich source of protein (22%) [25] and is consumed as a portion of daily diet in South-Asian region [26]. Luckily, chickpea requires less water and mostly grows in the Thal desert of Punjab province, Pakistan [27]. Chickpea production faces many challenges, including low and high temperature stress, weed infestation, nutrient deficiencies and disease infestation [28–30]. The population of Pakistan is witnessing a rapid increase, which would exert huge pressure on the country's economy and food production. Therefore, rainfed regions of the country must be utilized for food security [31]. Chickpea is grown in sandy soil, while the Thal and Pothohar regions of Pakistan consist of sandy soils that have low organic matter and suffer from B-deficiency, which reduces the crop yield [21]. The sandy soils in arid and semi-arid regions are calcareous and generally deficit in nutrients and soil organic matter [32]. The crops produced in these soils accumulate lower amounts of essential nutrients; thus, human populations fed with the products of these crops face nutrient deficiencies. Several studies have indicated that the application of micronutrients either in the soil or as foliar and exploitation of soil microbes for solubilizing micronutrients have increased crop yield and concentration of micronutrients in harvested plant parts [33,34].

Therefore, chickpeas produced on calcareous soils in arid and semi-arid regions of Pakistan must be enriched with B for higher productivity and essential nutrients' accumulation in grains.

Biofortification is an agronomic and plant breeding technique which enhances nutritional value of edible plant parts [35]. There are many agronomic approaches, which are capable of enhancing nutritive value of plants. These approaches include soil and foliar application of micronutrients to overcome micronutrient scarcity in human body [36,37]. Adequate supply of micronutrients is essential for crops to get better yield and quality [38]. Soil-applied iron (Fe) can potentially mitigate the Fe malnutrition problem of mungbean in Pakistan by improving the grain-Fe concentration and bioavailability [39]. Similarly, recent studies have reported that zinc (Zn) application through various methods have improved the yield and grain biofortification of mungbean [34,40,41]. A recent study has reported that seed coating [42] and seed priming [43] with B combined with B-tolerant bacteria (BTB) have improved chickpea yield under semi-arid conditions of Pakistan. However, the potential of foliar- or soil-applied B combined with BTB has not been explored yet.

Plant growth promoting microorganisms are being exploited in the recent era to avoid the negative impacts of synthetic fertilizers [44]. The ability of these microorganism to improve plant minerals' uptake has been witnessed in the recent decade [45]. Bacteria has ability to survive and work under harsh environmental conditions. Mostly, bacteria are used for phosphorus (P) solubilizing and enhancing P uptake in calcareous soils [44]. However, Samreen et al. [46] reported that *Bacillus* spp. MN-54 strain is boron-tolerant and enhances P uptake. The *Bacillus* spp. MN-54 has been used to improve P uptake and growth of maize under lead [47] and chromium [48] toxicity. Similarly, Khan et al. [44] reported that *Bacillus* spp. MN-54 improved P uptake and enhanced chickpea productivity in calcareous soils. Most of these studies focused on P uptake and ignored B, although the bacteria can tolerate higher levels of B. Several bacteria are known to tolerate higher B levels through high B efflux and exclusions [49]. Two recent studies have indicated that *Bacillus* spp. MN-54 combined with B seed priming [43] and seed inoculation [47] improved the B uptake and productivity of chickpea. Nonetheless, these studies were focused on optimizing B levels due to the narrow deficiency and toxicity range for B. In addition, both studies indicated that *Bacillus* spp. MN-54 inoculation has the potential to improve B uptake and utilization. Seed inoculation is a promising technique, which delivers plant-growth-promoting bacteria in the rhizosphere [50]. The BTB tolerate higher B levels and help plants to uptake B from the soil. The survival of normal bacteria under harsh environmental conditions is a great challenge; therefore, using BTB to improve plants' mineral nutrition, particularly of B, can be a viable option. Application of mineral fertilizer along with plant growth promoting microbes is more adaptable and affordable method to improve crop yield [51,52].

Soil application of B is known to improve crop performance; however, information regarding the interactive effect of soil applied B and *Bacillus* spp. MN-54 in chickpea is missing. Therefore, this study evaluated the effect of soil-applied B and *Bacillus* spp. MN-54 on the growth, productivity and grain biofortification of chickpea. It was hypothesized that *Bacillus* spp. MN-54 and soil B application will enhance chickpea productivity and grain B content. The results will help to improve chickpea productivity on B-deficit calcareous soils in arid and semi-arid regions.

## 2. Materials and Methods

### 2.1. Experimental Material

Seeds of chickpea variety 'NIAB-2016' were procured from Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. *Bacillus* sp. MN54 (accession no. KT375574) isolated bacterial strain was obtained from Soil and Environmental Microbiology Laboratory, Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan. The bacterial strain was identified through molecular approach following Edwards [53] primers. The identified strain has already been used to improve

growth and productivity of several crops [54,55], including chickpea [43,47]. Different concentrations of B were used, i.e., 0, 60, 120 and 180 mM, along with *Bacillus* sp. MN54 in these studies. The lowest growth reduction was observed at higher B concentrations (180 mM), with the presence of strain. The inoculated seeds observed a bacterial count of  $10^8$  per seed [55]. Selected inoculum was prepared in 500-mL Erlenmeyer flask containing 200 mL 10% tryptic soya broth. Inoculated broth was placed in shaking incubator (Firstek, Japan) at 180 rpm for 24 h at  $28 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$  [54]. The optical density of inoculum was adjusted to 0.5 (bacterial population  $10^9$  cfu per mL) before seed inoculation.

## 2.2. Experimental Site

The pot experiments were conducted in the wire house of Department of Agronomy, Bahauddin Zakariya University, Multan, Pakistan during winter, 2018–2019 and 2019–2020 under natural conditions. Before sowing, soil samples were collected and analyzed to evaluate the soil fertility status. The experimental soil was clay-loam with pH of 8.40, 0.65% soil organic matter,  $2.68 \text{ mScm}^{-1}$  EC, 0.021% nitrogen,  $0.62 \text{ mg kg}^{-1}$  boron,  $7.80 \text{ mg kg}^{-1}$  phosphorus and  $240 \text{ mg kg}^{-1}$  available potassium.

## 2.3. Plant Material and Crop Husbandry

Different B concentrations (0.00, 0.25, 0.50, 0.75 and  $1.00 \text{ mg kg}^{-1}$  soil) were applied to the soil before seed sowing in each pot. These pots were divided into two portions. Half of the pots were inoculated with *Bacillus* sp. MN54 ( $10^8$  bacteria per seed), while the remaining half were not inoculated. Boric acid (Merck, Germany product) was used as B source. To fulfill plants nutrients requirements, starter doses of 20 and  $40 \text{ mg kg}^{-1}$  of nitrogen and phosphorus, respectively, were applied to the pots prior to seed sowing. The earthen pots (22-cm diameter and 50-cm height) were filled with 20 kg soil. Initially, 8 seeds were sown in each pot and reduced to 5 per pot after successful seedling establishment. Completely randomized design with factorial arrangement was used in this experiment in both years, and all treatments had three replications. Weeds were controlled manually during experimental years, while irrigation was done daily to exclude the impacts of moisture stress. The experiment was harvested during 18 April 2019, and 23 April 2020 to record data.

## 2.4. Data Collection

### 2.4.1. Nodules Population, Allometric and Roots Related Traits

One plant was uprooted carefully from each pot and number of roots  $\text{plant}^{-1}$  were counted and averaged. Root length and plant height (cm) were measured with the help of a measuring tape. The root samples were kept in an oven at  $70 \text{ }^\circ\text{C}$  for 48 h and dried until constant weight. The weight of the dried samples was measured and averaged to record dry weight. Chlorophyll index was recorded with SPAD meter. The SPAD values were noted from three random leaves from each plant and averaged. The leaves of uprooted plants were separated, and their area was measured with the help of leaf area meter. The first data was collected 55 days after sowing (DAS), while other data was recorded with 40 days interval.

### 2.4.2. Yield-Related Attributes

The crop was harvested at maturity and yield-related attributes were recorded. Total number of branches on each plant were carefully counted and averaged. All pods were harvested from two plants in each pot manually, counted and averaged to record number of pods per plant. To calculate number of grains per pod, total grains were separated from each pod, counted and averaged. Number of grains per plant were calculated according to the Equation (1) as follows:

$$\text{Number of grains plant}^{-1} = \text{Number of pods plant}^{-1} \times \text{Number of grains pod}^{-1} \quad (1)$$

To calculate 100-grain weight, 100 seeds were taken from each of the treatment and their weight was recorded. For biological yield and seed yield per plant, harvested plants from each of the treatment were sun-dried. After drying, their weight was recorded on an electric balance. Afterwards, plants were threshed manually to calculate grain yield per plant. Finally, harvest index of chickpea was recorded by using Equation (2), as follows:

$$\text{Harvest index} = \frac{\text{Grain yield}}{\text{Biological yield}} \times 100 \quad (2)$$

#### 2.4.3. Grain Boron Contents

Seeds were collected and dried in an oven at 70 °C for analyzing grain B contents. In the first step, 1 g seed sample from each treatment was ashed in a muffle furnace at 550 °C [56]. The ashed samples were then extracted by adding 0.36 N H<sub>2</sub>SO<sub>4</sub> 10 mL for 1 h and the solution was filtered through Whatman No. 1 filter paper. The solution volume was raised by adding 50 mL of distilled water and transferred to 50 mL transparent bottle. Buffer was prepared by dissolving 250 mL ammonium acetate and 15 mL ETDA in 400 mL distilled water. Afterwards, 125 mL acetic acid was added in solution slowly. Azomethine solution was prepared which contained 0.45 g of azomethine-H, 1 g of L-ascorbic acid and dissolved in 100 mL distilled water. Finally, 2 mL of buffer solution and 2 mL of azomethine-H solution was dissolved in 1 mL extracted solution and kept for 45 min to develop the required color. B concentration of each sample was determined by using spectrophotometer at 420 nm.

#### 2.5. Statistical Analysis

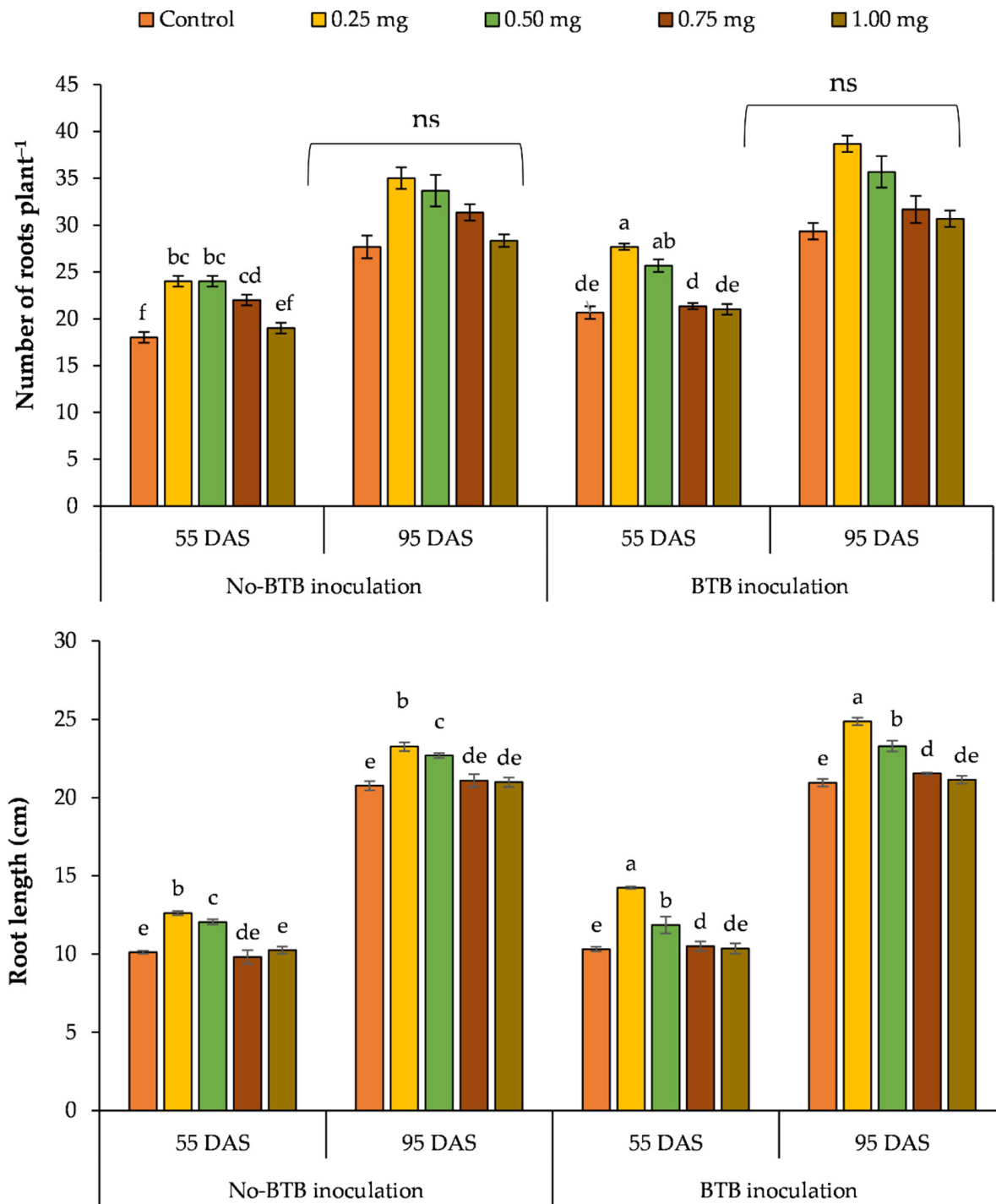
The differences among years were tested by t test, which indicated that year effect was non-significant ( $p > 0.01$ ). Therefore, data of both years were pooled and used in statistical analysis. Two-way analysis of variance (ANOVA) techniques was used to test the significance in the data. Least significant difference (LSD) test at 1% probability was used as post-hoc test to record difference among treatment means [57] where ANOVA indicated significant differences. All statistical computations were done on SPSS statistical software version 20 [58]. Graphical representation of data was done in the Microsoft Excel program. Individual effects of soil-applied B and BTB inoculation were presented where interaction was non-significant. Likewise, individual effects were ignored, and interaction was presented in case of significant effect.

### 3. Results

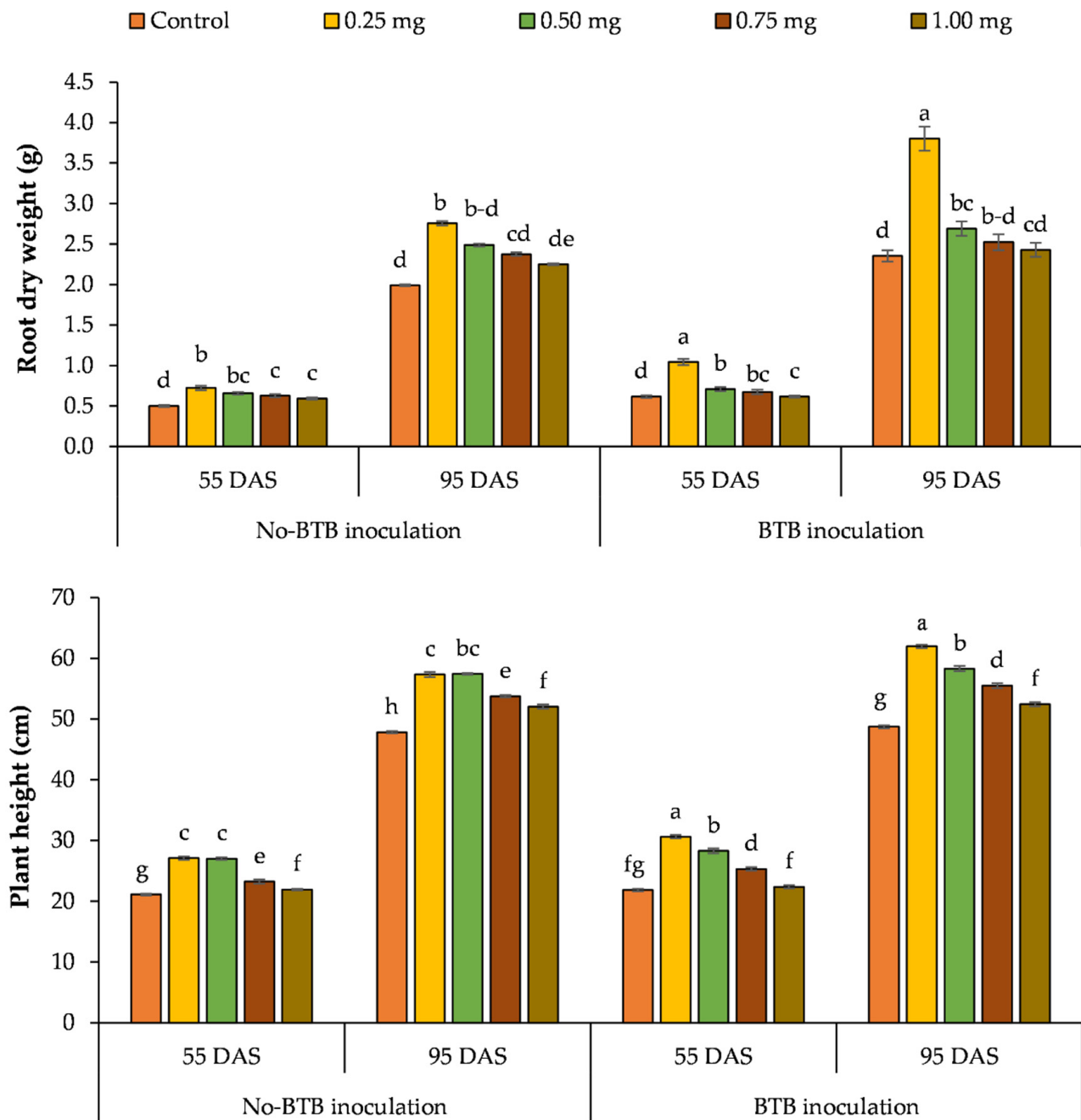
#### 3.1. Root Nodulation, Root System and Growth Parameters

The number of roots, the roots' length and dry weight, and the plant height from 55–95 DAS were significantly altered by B application, BTB inoculation and their interaction, while the B–BTB inoculation interaction had a non-significant effect on the chlorophyll index and leaf area (Table 1).

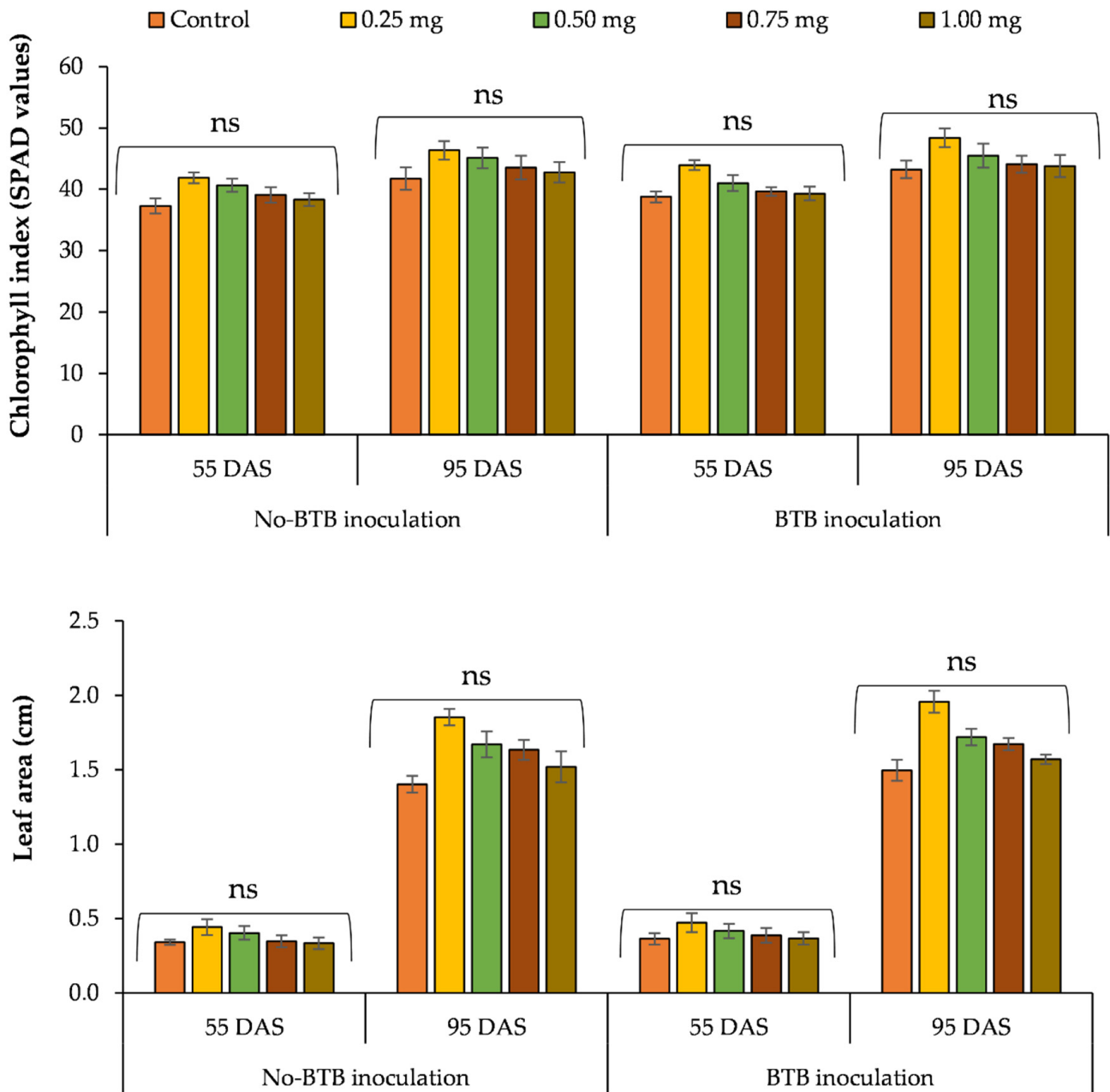
The soil application of B significantly improved the number of roots per plant, the roots' length, the roots' dry weight, the plant height and the leaf area from 55 DAS to 95 DAS (Figures 1–3). The inoculation of BTB improved number of roots per plant, the roots' length and dry weight, the plant height, the chlorophyll index and the leaf area throughout the growing season (Figures 1–3). Regarding interactions, 0.25 mg kg<sup>-1</sup> B application with BTB inoculation recorded the highest number of roots per plant<sup>-1</sup>, root length, root dry weight and plant height compared to the rest of the treatments. The lowest values of these traits were noted for the control treatment without BTB inoculation (Figures 1–3).



**Figure 1.** The influence of different doses of soil-applied boron and inoculation of boron-tolerant bacteria on number of roots per plant<sup>-1</sup> and root length (cm) of chickpea crop ( $\pm$ S.E.). Here, BTB = boron tolerant bacteria, ns = non-significant. Means having different letters are statistically different from each other ( $p < 0.01$ ).



**Figure 2.** The influence of different doses of soil-applied boron and inoculation of boron-tolerant bacteria on root dry weight (g) and plant height (cm) of chickpea crop ( $\pm$ S.E.). Here, BTB = boron tolerant bacteria. Means having different letters are statistically different from each other ( $p < 0.01$ ).



**Figure 3.** The influence of different doses of soil-applied boron and inoculation of boron-tolerant bacteria on chlorophyll content and leaf area of chickpea crop ( $\pm$ S.E.). Here, BTB = boron tolerant bacteria, ns = non-significant. Means having different letters are statistically different from each other ( $p < 0.01$ ).



**Table 1.** Statistical summary (*p*-values) of root proliferation, growth parameters, nodule population, yield-related traits and grain-B concentration of chickpea under soil application of B and BTB inoculation.

Treatment	Soil B Application (B)	BTB Inoculation (BTB)	B × BTB
Number of roots plant <sup>-1</sup> at 55 DAS	0.001	0.001	0.000
Number of roots plant <sup>-1</sup> at 95 DAS	0.003	0.002	NS
Root length at 55 DAS	0.004	0.000	0.000
Root length at 95 DAS	0.002	0.000	0.002
Root dry weight at 55 DAS	0.001	0.000	0.000
Root dry weight at 95 DAS	0.000	0.003	0.000
Plant height at 55 DAS	0.000	0.002	0.000
Plant height at 95 DAS	0.001	0.000	0.001
Chlorophyll index at 55 DAS	0.000	0.001	NS
Chlorophyll index at 95 DAS	0.001	0.000	NS
Leaf area at 55 DAS	0.001	NS	NS
Leaf area 95 DAS	0.002	0.000	NS
Nodules population plant <sup>-1</sup>	0.000	0.001	0.002
Number of branches plant <sup>-1</sup>	0.000	0.001	NS
Number of pods plant <sup>-1</sup>	0.003	0.002	0.001
Number of grains pod <sup>-1</sup>	0.001	0.000	NS
Number of grains plant <sup>-1</sup>	0.001	0.000	0.000
100-grains weight (g)	0.000	0.000	NS
Grain yield (g plant <sup>-1</sup> )	0.002	0.002	0.000
Biological yield (g plant <sup>-1</sup> )	0.001	0.000	0.000
Harvest index (%)	NS	NS	NS
B-grain concentration (%)	0.000	0.000	0.001

NS = Non-significant.

### 3.2. Yield-Related Traits

Soil-applied B and BTB inoculation had significant effect on the number branches, pods and grains per plant<sup>-1</sup>, the number of grains per pod<sup>-1</sup>, the 100-grains weight, the grain yield, the biological yield and the grain B concentration of chickpea (Table 1). The B–BTB interaction had significant effect on number of pods per plant<sup>-1</sup>, the number of grains per plant<sup>-1</sup>, the grain yield, the biological yield and the grain-B concentration (Table 1). Soil application of B at lower levels (<0.75 mg kg<sup>-1</sup> soil) notably improved the yield-related traits, while the higher levels (>0.75 mg kg<sup>-1</sup> soil) caused toxicity and reduced yield (Tables 2–4). Soil-applied B, BTB inoculation and their interaction had a significant effect on the nodule population of plants<sup>-1</sup> (Table 1). Lower B levels (<0.75 mg kg<sup>-1</sup> soil) were more effective in improving nodule population, while the higher levels (>0.75 mg kg<sup>-1</sup> soil) proved toxic and reduced the nodule population (Table 3). Regarding interactions, 0.25 mg kg<sup>-1</sup> B with BTB inoculation had the highest nodule population (81%) compared to the rest of the treatments (Table 3).

Soil application of 0.25 mg kg<sup>-1</sup> soil B improved the yield-related traits, especially the number of branches per plant<sup>-1</sup> (40%), the number of grains per pod<sup>-1</sup> (28%) and the 100-grain weight (25%) as compared to control (Table 2). Similarly, the BTB inoculation significantly improved the number of branches per plant<sup>-1</sup> (18%), the number of grains per pod<sup>-1</sup> (11%) and the 100-grain weight (4%) compared to no inoculation treatment (Table 2).

Regarding B–BTB interaction, 0.25 mg kg<sup>-1</sup> soil B with BTB inoculation recorded better yield-related traits than the rest of the treatments (Tables 3 and 4). Soil application of 0.25 mg B kg<sup>-1</sup> soil with BTB inoculation recorded the highest number of pods per plant<sup>-1</sup> (46%), number of per grains plant<sup>-1</sup> (62%), grain yield (47%) and biological yield (50%) compared to the control treatment (Tables 3 and 4). However, the grain-B concentration was the highest (77%) under higher B levels along with BTB inoculation.

**Table 2.** Effect of soil-applied boron and inoculation of boron-tolerant bacteria on number of nodules per plant and yield-related trait of chickpea.

B Soil Application (mg kg <sup>-1</sup> of Soil)	Number of Branches per Plant <sup>-1</sup>	Number of Grains per Pod <sup>-1</sup>	100-Grains Weight (g)
Boron soil application (B)			
0.00	4.33 ± 0.46 C	1.35 ± 0.06 D	41.31 ± 0.53 C
0.25	7.17 ± 0.46 A	1.87 ± 0.03 A	50.12 ± 0.66 A
0.50	5.83 ± 0.33 B	1.72 ± 0.05 B	48.76 ± 0.50 A
0.75	5.17 ± 0.33 BC	1.58 ± 0.05 C	43.75 ± 0.91 B
1.00	5.17 ± 0.17 BC	1.47 ± 0.05 CD	37.60 ± 0.75 C
LSD at <i>p</i> ≤ 0.01	1.09	0.13	2.20
<i>Bacillus</i> sp. MN54 inoculation (BTB)			
No-BTB inoculation	5.00 ± 0.36 B	1.51 ± 0.04 B	43.96 ± 0.72 B
BTB inoculation	6.07 ± 0.33 A	1.68 ± 0.05 A	45.46 ± 0.62 A
LSD at <i>p</i> ≤ 0.01	0.69	0.08	1.40
B × BTB	NS	NS	NS

Means ± SE (standard error) sharing same case letter, within a column for each trait, did not differ significantly from each other at *p* ≤ 0.01.

**Table 3.** Interactive effect of soil-applied boron and inoculation of boron-tolerant bacteria on number of nodules, pods and grains per plant<sup>-1</sup> of chickpea.

B Soil Application (mg kg <sup>-1</sup> of Soil)	Nodule Population per Plant <sup>-1</sup>		Number of Pods per Plant <sup>-1</sup>		Number of Grains per Plant <sup>-1</sup>	
	No-BTB Inoculation	BTB Inoculation	No-BTB Inoculation	BTB Inoculation	No-BTB Inoculation	BTB Inoculation
0.00	1.67 ± 0.3 e	2.33 ± 0.3 de	12.33 ± 0.3 fg	13.00 ± 0.6 f	14.80 ± 0.8 f	19.53 ± 1.4 de
0.25	5.00 ± 0.6 bc	9.00 ± 0.6 a	17.33 ± 0.3 b	20.00 ± 0.6 a	30.63 ± 1.0 b	39.30 ± 0.7 a
0.50	4.33 ± 0.9 b-d	6.00 ± 0.6 b	15.33 ± 0.3 cd	16.33 ± 0.3 bc	25.03 ± 0.5 c	29.37 ± 0.5 b
0.75	3.33 ± 0.3 c-e	4.00 ± 0.6 b-d	14.67 ± 0.3 de	13.67 ± 0.3 ef	23.00 ± 1.0 c	21.9 ± 1.3 cd
1.00	2.33 ± 0.3 de	1.67 ± 0.3 e	11.00 ± 0.6 gh	10.67 ± 0.3 h	15.37 ± 0.7 f	16.37 ± 0.8 ef
LSD at <i>p</i> ≤ 0.01	2.04		1.59		3.20	

Means ± SE (standard error) sharing same case letter, within a column for each trait, did not differ significantly from each other at *p* ≤ 0.01.

**Table 4.** Interactive effect soil applied boron and inoculation of boron-tolerant bacteria on yield components and grain B concentration of chickpea.

B Soil Application (mg kg <sup>-1</sup> of Soil)	Grain Yield (g plant <sup>-1</sup> )		Biological Yield (g plant <sup>-1</sup> )		Grain B Concentration (mg kg <sup>-1</sup> )	
	No-BTB Inoculation	BTB Inoculation	No-BTB Inoculation	BTB Inoculation	No-BTB Inoculation	BTB Inoculation
0.00	3.47 ± 0.1 e	3.78 ± 0.1 de	9.04 ± 0.2 g	9.52 ± 0.2 fg	18.47 ± 0.6 e	22.8 ± 0.4 e
0.25	4.98 ± 0.2 b	6.58 ± 0.2 a	13.25 ± 0.5 c	18.02 ± 0.4 a	50.8 ± 3.1 d	51.91 ± 2.2 d
0.50	4.79 ± 0.1 bc	5.39 ± 0.1 b	12.96 ± 0.5 c	15.02 ± 0.2 b	65.99 ± 0.8 c	66.73 ± 0.6 c
0.75	4.26 ± 0.1 cd	4.00 ± 0.3 de	11.92 ± 0.5 cd	11.17 ± 0.2 de	67.70 ± 0.7 c	76.26 ± 0.5 b
1.00	4.08 ± 0.2 d	3.82 ± 0.2 de	10.52 ± 0.6 ef	9.95 ± 0.5 e-g	77.9 ± 0.6 ab	82.68 ± 0.6 a
LSD at <i>p</i> ≤ 0.01	0.61		1.35		5.30	

Means ± SE (standard error) sharing same case letter, within a column for each trait, did not differ significantly from each other at *p* ≤ 0.01.

The nodule population, number of pods and yield-related traits had strong positive correlations with grain yield with or without BTB inoculation (Table 5). However, all these traits had non-significant correlations with grain-B concentration with and without BTB inoculation (Table 5).

**Table 5.** Correlation between different growth traits with grain yield and grains B concentrations of chickpea.

Crop Traits	Grain Yield (g Plant <sup>-1</sup> )		Grains B-Concentration (mg kg <sup>-1</sup> )	
	No-BTB Inoculation	BTB Inoculation	No-BTB Inoculation	BTB Inoculation
Number of nodules per plant <sup>-1</sup>	0.98 **	0.97 **	0.33NS	−0.06NS
Number of branches per plant <sup>-1</sup>	0.94 **	0.96 **	0.46NS	0.09NS
Number of pods per plant <sup>-1</sup>	0.82 *	0.86 *	0.00NS	−0.23NS
Number of seeds per pod <sup>-1</sup>	0.98 **	0.99 **	0.48NS	0.03NS
Number of seeds per plant <sup>-1</sup>	0.92 **	0.98 **	0.20NS	−0.15NS
100-grains weight (g)	0.90 **	0.94 **	0.10NS	−0.10NS
Biological yield (g plant <sup>-1</sup> )	0.98 **	0.99 **	0.50NS	0.01NS

\*\* = Significant at  $p \leq 01$ ; \* = Significant at  $p \leq 05$ ; NS = Non-significant.

#### 4. Discussion

The results indicated that soil application of B with BTB inoculation recorded the highest root proliferations, improved morphological parameters and nodule population and increased the yield and grain-B concentrations compared to the control treatment. Soil application of 0.25 mg B kg<sup>-1</sup> soil along with BTB inoculation improved the root traits, growth, nodulation and yield-related traits of chickpea, while higher B levels recorded a greater grain-B concentration (Figures 1–3, Tables 1–5). Boron is involved in sugar translocation from source to sink, carbohydrate metabolism, seed development, pollen fertility, pod setting and grain yield [8]. Boron is most helpful during the reproductive stage rather than the vegetative stage as it is involved in fruit setting and seed development [59].

Soil application of 0.25 mg B kg<sup>-1</sup> soil recorded the highest improvement in root traits and growth parameters, including the number of roots per plant<sup>-1</sup>, the plant height, the root length, the root dry weight and the leaf area. Moreover, seed inoculation with BTB improved root-related traits and growth parameters (Figures 1–3). Plant height was significantly improved by soil application of B and BTB inoculation from 55–95 DAS, which is similar to the findings of Bayrak et al. [60]. Plant height of different chickpea genotypes has been reported to improve by soil application of B [60]. Ceyhan et al. [61] reported that soil application of B (7.5 kg ha<sup>-1</sup>) at sowing significantly improve plant height. Soil application of B increases photosynthesis rate and chlorophyll contents, while B-deficiency reduces chlorophyll and soluble protein contents, which ultimately reduce photosynthesis. Poor growth and the lowest dry matter production were recorded without B application throughout the growing season (Figures 2 and 3). These results are similar to Zhao and Oosterhuis [15] who reported that B-deficiency during the reproductive stage lowers plant height, reduces dry matter production and interrupts photosynthesis. Boron is required by plants in minute quantity; however, metabolic activities are significantly altered by B-deficiency. Boron stabilizes the plant cell walls, membrane integrity and sugar transport and improves the utilization of calcium and nitrogen [6–8]. Nonetheless, pod formation is regulated by B during the reproductive phase, which improves the yield of leguminous plants. Better growth and yield-related traits with 0.25 mg B kg<sup>-1</sup> soil are owed to these physiological and biochemical attributes, which were suppressed under B-deficiency. Boron-deficiency at the reproductive stage results in pollen sterility and decreases the grain yield to great extent [9]. Therefore, the lower yield under no B application can also be explained with pollen sterility.

Soil application with B significantly improved the nodule population, the number of branches and the yield-related traits of chickpea. Boron application at higher levels and no B application recorded a lesser nodule population and reduced numbers of pods and grain sizes. These results are in line with the findings of Agarwala and Sharma [62] and Srivastava et al. [63]. The number of flowers in chickpea were decreased by B-deficiency, which negatively affected photosynthesis, fruit setting, pods formation and yield. Chickpea pods population increased with B application on B-deficient soils [60]. Soil application of 10 kg ha<sup>-1</sup> Zn proved a viable option to improve mung bean productivity along with higher

grain Zn biofortification [64]. However, BTB inoculation improved nodule population and yield-related traits by providing better nutrients and water uptake as compared to non-inoculation. Availability of nutrients (including phosphorous and B) and water uptake is better by soil microbes along with growth stimulating bacteria, which increase growth and production [65]. Seed inoculation in plants involves ion homeostasis, which improves inorganic substances, including B [66]. The *Bacillus* spp. MN-54 improved P uptake and growth of maize under lead [47] and chromium [48] toxicity. Similarly, Khan et al. [44] reported that *Bacillus* spp. MN-54 improved P uptake and enhanced chickpea productivity in calcareous soils. Two recent studies have indicated that *Bacillus* spp. MN-54 combined with B seed priming [43] and seed inoculation [47] improved the B uptake and productivity of chickpea. The current study also reports that *Bacillus* spp. MN-54 can be combined with soil-applied B for improving the growth, yield and grain biofortification of chickpea. The improvement in yield and related traits with the inoculation of *Bacillus* spp. MN-54 could be attributed to higher P and B solubilization and availability to the plants at critical growth stages. Although, *Bacillus* spp. MN-54 tolerates higher B levels, it could not lower B-toxicity at higher levels. Therefore, *Bacillus* spp. MN-54 should be combined with lower B concentration for improving growth, yield and grain B contents. Although a higher B level recorded more grain-B contents, these had toxic effect on plant growth.

Soil application of  $0.25 \text{ mg B kg}^{-1}$  soil significantly improved the grain and biological yields, whereas a higher B level caused toxicity and no B application resulted in B-deficiency. These results agree with the previous study that seed coating of B ( $1.5 \text{ g kg}^{-1}$ ) with bacterial inoculation had positive effects on chickpea growth and production, while higher levels become toxic and reduced production [43]. Chickpea yield has been reported to increase by  $1 \text{ kg ha}^{-1}$  soil application of B [63,67], while its production increased by 54% [68]. These results are in line with our findings that chickpea yield was increased by 47% in response to  $0.25 \text{ mg B kg}^{-1}$  soil BTB inoculation. Moreover, higher levels become toxic and ultimately reduced chickpea yield. These results are similar to the findings of Singh [69] who found that a higher dose of B ( $2 \text{ kg B ha}^{-1}$ ) or B-contaminated irrigation water caused toxicity in plants and suppressed crop growth.

Boron soil application along with BTB inoculation promoted plant growth, nodulation and chickpea production. Different nutrients along with soil microbes enable plants to uptake more essential nutrients, which accelerate their growth and yield [51]. The BTB inoculation significantly improved the number of pods per plant<sup>-1</sup> (5%), the number of grains per plant<sup>-1</sup> (14%) and the grain yield (9%) of chickpea against the non-inoculation treatment. Co-application of BTB along with B seed coating ( $1.5 \text{ g B kg}^{-1}$ ) increased the grain and biological yield by 37 and 40%, respectively [43]. Bacteria are more flexible in moving, transforming and solubilizing the nutrients in plant roots against bulk soil, thus helping plants achieve better nutrient and moisture uptake [70].

## 5. Conclusions

Soil application of B proved to be effective in increasing chickpea growth, yield and grain-B concentration. Similarly, BTB inoculation positively increased chickpea yield compared to the non-inoculation treatment. Soil application of B at a lower dose ( $0.25 \text{ mg B kg}^{-1}$  soil) along with BTB inoculation was the most effective method to increase the nodule population, growth and crop yield. Higher B levels caused toxicity, and no application showed B-deficiency. Therefore,  $0.25 \text{ mg B kg}^{-1}$  soil and BTB inoculation seem to be a viable approach to improving the growth, yield and grain-B concentration of chickpea.

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