

## Article

# Salinity Stress and the Influence of Bioinoculants on the Morphological and Biochemical Characteristics of Faba Bean (*Vicia faba* L.)

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**Abstract:** Faba bean (*Vicia faba* L.) is an economically important crop cultivated globally for fulfilling human requirements. However, the productivity of the faba bean has declined due to poor management of soil, particularly under salt stress. Salt stress is a major constraint to crop productivity worldwide. Therefore, the objective of the present investigation is to check the behavior of faba bean genotypes on the basis of morphological and biochemical traits in response to salinity. In this study, we studied seven different treatments (including control) applied to faba bean under salt stress. Bioinoculants such as *Trichoderma viride*, *Pseudomonas fluorescens*, *Glomus mosseae*, and *Gigaspora gigantea*, each separately and in combination, were tested for their efficacy under salinity stress. Data recorded on days to flowering ( $48.92 \pm 1.15$ ), days to maturity ( $144.56 \pm 1.95$ ), plant height ( $141.93 \pm 4.81$  cm), number of branches per plant ( $4.87 \pm 0.09$ ), number of clusters per plant ( $18.88 \pm 0.24$ ), number of pods per plant ( $48.33 \pm 1.06$ ), pod length ( $5.31 \pm 0.02$  cm), catalase ( $222.10 \pm 2.76$  mg), hydrogen peroxide ( $24 \pm 4.58$  mol/g), malondialdehyde ( $45 \pm 1.00$  mol/g), electrolyte leakage ( $54.67 \pm 5.03$ ), chlorophyll ( $51.67 \pm 3.06$  mg/g), proline content ( $2.96 \pm 0.12$  mg/g), and on other parameters indicated the combined inoculation of all the species (consortium) was taken to be highly effective even under salt stress. Overall, the consortium treatment comprising all of the bioinoculants was observed to be the most efficient treatment in improving all the morphological and biochemical traits of faba bean under salt stress. Although, other treatments also demonstrated considerable effects on faba bean as compared to one without bioinoculants under salt stress.

**Keywords:** bioinoculants; faba bean; NaCl; salinity; stress



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## 1. Introduction

Faba bean (also known as broad bean, horse bean, and filed bean) cultivation dates back to the global region. It is one of the oldest crops that belong to the Fabaceae family [1] and is salt-sensitive, mainly grown in the rabi season [2,3]. Studies show that the chromosome number of faba bean is diploid  $2n = 2x = 14$ , and progress has been made in developing an excellent genetic understanding for better variety development. The genetic architecture of this crop explains that the chromosome size of faba bean is large and is considered to be the largest genome in the grain legume family [4]. Faba bean is a good source of protein for human beings, may be utilized as fodder for animals, and has an excellent ability to fix nitrogen [5]. There is increasing demand for faba bean due to its consumption globally, but the yield has been declining due to abiotic and biotic stresses, which

creates a need to develop tolerance and resistance in varieties [6]. Plants suffer different environmental conditions, such as soil salinity which limits the growth and productivity of the crop. The excessive level of salt stress in agriculture fields is a major concern of global issue and has a harmful impact on crops [7].

Salinity is a major problem in agriculture, especially for field crops where rainfall is insufficient to leach salts from the root zone [7]. Additionally, salinity stresses are known to be detrimental to plants which may reduce their yield [8]. Due to reduced nutrient utilization, hormonal imbalance, ROS generation, ionic toxicity, and osmotic stress, salinity-stressed plants develop poorly. Moreover, salt stress reduces osmotic equilibrium, nutrient balance, stomatal activity, hydraulic conductance, and photosynthesis [9]. Salinity can also alter the physicochemical properties of soil and reduce microbial diversity and health. In the future, the growth of the population will increase, and more grains will be needed to feed the population [10]. Existing farming techniques and salt-tolerant crops only will not be sufficient for future needs [8]. In this regard, biological control, particularly biocontrol agents, could be the most effective method of crop management under salt stress [11]. Sustainable agriculture can be applied through the utilization of these microbes, which do not leave any harmful effects when applied to soil and seed. These beneficial microorganisms improve seed germination, root and shoot growth, and abiotic and biotic stress tolerance [12].

The biological control for salt stress has been utilized by several researchers, and satisfactory results have been obtained. The *Trichoderma viride* is a biological control agent that can reduce the effect of salt stress. The genus *Trichoderma* shows a wide range of capabilities among its different strains as multi-functional fungi found in different ecosystems [13]. *Trichoderma*, a genus of fungi in the *Hypocreaceae* family, is one such rhizospheric inhabitant whose application in microbial inoculants draws researchers' attention to other plus points of *Trichoderma* spp. [14]. Moreover, the interactions of *Trichoderma* spp. with plants effectively regulate the yield by increasing other subcomponents [15]. Some other bioinoculants, such as *Pseudomonas fluorescens*, *Glomus mosseae*, and *Gigaspora gigantea*, also have a beneficial interaction with plants [16,17].

*Pseudomonas fluorescens* is rendered an essential bacterium in the rhizosphere for optimal growth and development of a plant. *Pseudomonas fluorescens* is a plant-growth rhizobacterium in the plant rhizosphere. It has acquired wide attention globally and has the capacity to produce a wide range of enzymes and metabolites that help to withstand abiotic stresses. The application of *pseudomonas fluorescens* inoculant in combination with microbial fertilizer plays an effective role in stimulating the yield and growth traits of crops. Field trials of *pseudomonas* strain lead to a great increase in yield in plants, and it also works with other microorganisms such as *Glomus mosseae* and *Gigaspora gigantea* [18].

Under salt stress, faba bean suffers from  $\text{Na}^+$  toxicity, and accumulations of  $\text{Na}^+$  toxicity correspond to  $\text{K}^+$  toxicity resulting in cellular organs' damage and declined efficiency of photosynthesis in faba bean [19,20]. Additionally, these below-ground microbes adapt to hostile environmental conditions and arouse the cyclization of minerals and other soil nutrients [21]. The mechanism involved, however, remains unresolved. So far, studies on salt stress tolerance in plants have suggested that treated plants grow better due to improved mineral nutrition and physiological processes like photosynthesis or water use efficiency, the production of osmoregulators, higher  $\text{K}^+/\text{Na}^+$  ratios, and the compartmentalization of sodium within some plant tissues [22]. The microorganisms can survive in saline soils and help plants to alleviate salinity stress by enhancing nutrient uptake, photosynthetic activity, water-use efficiency, and the accumulation of compatible solutes and enzymatic antioxidants [22]. Therefore, the purpose of this study was to investigate the effects of bioinoculants on various characteristics of faba bean in order to determine whether or not they have the potential to be used to control the impact of salinity stress on faba bean.

## 2. Material and Methods

### 2.1. Experimental Location

The experiment was performed at Ranchi University, Ranchi, India (29.94° N and 76.89° E) in a Randomized Complete Block Design with three replications during the year 2019–2020. The average temp recorded was 25.5 °C ± 6.0 with relative humidity of 50–68%. The crop was recommended for cultivation with 80 kg nitrogen, 60 kg phosphorus, and 80 kg potash (per acre) [23].

### 2.2. Soil Characteristics and Treatments

The characteristics of the soil included 70.5% of sand, 24.8% of silt, 4.7% of clay, 0.048% of nitrogen, 0.020% of available phosphorus, 0.05% of organic carbon, and pH 6.5 to 9.0. The method of pH determination included CaCl<sub>2</sub> solution –0.01 M. Dissolve 14.7 g CaCl<sub>2</sub> 2H<sub>2</sub>O in 10 L H<sub>2</sub>O. Based on requirement, pH was adjusted with pH of Ca(OH)<sub>2</sub> or HCl. Salts like carbonates of calcium, magnesium, and sodium also give a preponderance of OH ions over H ions in the soil solution. When salts of strong base such as sodium carbonate go into soil solution and hydrolyze, they give rise to alkalinity. Salt stress was given by applying NaCl with irrigation water with an electrical conductivity (EC<sub>w</sub>) of 1.0 (low), 2.0 (medium), and 3.0 dS/m (high) in addition to distilled water with an electrical conductivity of 0.05 dS/m. The water requirement for faba bean was 231–297 mm. In the present study, seven treatments were studied, which were control (T1), salt stress (T2), *Trichoderma viride* + salt stress (T3), *Pseudomonas fluorescens* + salt stress (T4), *Glomus mosseae* + salt stress (T5), *Gigaspora gigantean* + salt stress (T6), and *Trichoderma viride* + *Glomus mosseae* + *Gigaspora gigantean* + salt stress (T7). Hence, there were two controls T1 control under normal conditions and T2 control under salinity stress, respectively.

### 2.3. Experimental Design

Faba bean seeds were surface sterilized with 2% sodium hypochlorite solution for 6 min then washed 3 times with deionized sterilized water. The sterilized seeds of faba bean were applied with *Pseudomonas fluorescens* (1 mL) with a concentration of ~10<sup>6</sup> cfu/mL inoculum in 100 mL of solution (IMTECH, Mohali, India) on a rotary-shaker for 5 h at 37 °C. Then the treated seeds were air-dried and subsequently analyzed in the experiment. Nutrient broth medium was made in purified water, kept at 32 °C for incubation for 48 h and used to prepare inoculum of *P. fluorescens*. The seedlings that were found healthy were transplanted in plots having size of 3.0 m × 2.7 m × 45 cm and spacing of 45 cm between every row. The *Trichoderma* inoculum was taken from Sri Ram fertilizers, Kota, India. The soil-dilution-plate method was then utilized to produce the required inoculum on potato-dextrose-agar medium. The incubation of plates was performed at 30 °C for 4 days. Wheat bran, sawdust, and water in a 3:1:1 ratio were added to the inoculum. The inoculum of *Glomus mosseae*, with colonization of 80–86% and AM spores of 780–800 w/w, was taken from Botany Department, Kurukshetra University, Kurukshetra, India, and the inoculum of *Gigaspora gigantea*, with colonization of 75–79% and AM spores of 870–890 w/w, was obtained from the Forest Pathology Discipline, FRI, Dehradun, India. Moreover, one plot without inoculation and salt stress was left for control (with normal water), and one plot was left for plants with salt stress.

### 2.4. Morphological Characterization

Morphological characters studied included days to 50% flowering (calculated as mean of five flowers per plant for each replication), days to Maturity and Leaf area in cm<sup>2</sup> (measured at maturity), Plant height (cm), Number of branches per plant, and Number of clusters per plant (recorded after plants emergence). Number of pods per plant and Pod length (cm) were taken as five readings for every plant. Seeds per pod, seed weight (gm), Grain yield (gm), Shoot length (cm), Root length (cm), Root dry mass (mg/plant), Shoot dry mass (mg/plant), Internode length were calculated as the mean of five plants after harvesting, for each replication.

### 2.5. Biochemical Characterization

Biochemical characters included proline conc. (mg/g), chlorophyll (mg/g), Electrolyte leakage, malondialdehyde level (mol/g), hydrogen peroxide (mol/g), catalase activity (mg), peroxidase (g), superoxide dismutase (mg), SPAD, Stomatal conductance (mmol), and leaf relative water (%) were measured at the time of processing as mean of five plants, for each replication. Procedures for biochemical characterization are given below.

For estimation of proline concentration, method suggested by Bates et al. [24] was used, which involved homogenization of 0.5 g of plant sample in 10 mL 3% aq. sulfosalicylic acid and filtration of homogenate through 'Whatman filter paper 2'. Two ml filtrate was treated with acid ninhydrin (2 mL) and glacial-acetic acid (2 mL) at 100 °C for about 1 h in ice bath. After that, toluene (4 mL) was used for the reaction mixture extraction, mixed vigorously for about 15–20 s. The chromophores having toluene were then warmed to room temp. After aspiration from aqueous phase, the absorption was measured at 520 nm for a blank with toluene [24].

Chlorophyll content was determined by Ignat et al. method [25]. For this, 0.7 g of sample was absorbed in 80% ethanol solution and kept at temp. of −18 °C then used for analysis. The frozen tissue sample was then soaked with absolute ethanol in a mortar until only white tissues were left. After this, the soaked tissue was allowed to vacuum-filter through a fiberglass disk filter (Whatman, GE Healthcare, Buckinghamshire, UK). Finally, the leftovers on the filter disk were carefully rinsed using ethanol (absolute) and again passed through the filter, then the last filtrate volume was recorded. The spectrometric analysis of the sample was performed using a Gensys Spectrophotometer (Thermo-Electron Scientific Instruments LLC, Madison, WI, USA) in a cuvette made of quartz [25].

For electrolyte leakage determination, 100 mg of fresh sample of leaves were cut into pieces of 5 mm length and kept in test tubes with 10 mL distilled (deionized) water. The tubes were then covered properly and kept in a water bath at 32 °C. After 2 h, electrical conductivity initially,  $EC_1$ , was recorded with an electrical conductivity meter (CM-115, Kyoto Electronics, Kyoto, Japan). Afterward, the sample was autoclaved for 20 min at temp. of 121 °C to completely damage the tissues and liberate the electrolytic solutions. The sample was then allowed to cool down to 25 °C, and electrical conductivity,  $EC_2$ , was again recorded. Finally, the electrolytic leakage was calculated with the formula  $EC_1/EC_2 \times 100$  [26].

The malondialdehyde content was estimated using the Heath and Packer method [27]. The method involves maceration of 1 g of sample in 5 mL 0.1% trichloroacetic acid followed by centrifugation of the homogenate at  $10,000 \times g$  for 5 min. After that, 0.5% thio-barbituric acid (4 mL) in 20% trichloroacetic acid was mixed with the supernatant. The resulting mixture was then kept for heating for 30 min at 95 °C, followed by cooling in ice bath immediately. The mixture obtained was centrifuged at  $10,000 \times g$  for 15 min, and finally, the absorbance at 532 nm was measured. The malondialdehyde concentration was determined using extinction coefficient of  $155 \text{ mM}^{-1}\text{cm}^{-1}$  [27].

Hydrogen peroxide was estimated according to Sergiev et al. method [28]. About 500 mg of leaf-tissues were allowed to homogenize in ice bath with 5 ml of 0.1% trichloroacetic acid. Then centrifugation of homogenate was obtained, followed at  $12,000 \times g$  for about 15 min and 0.5 ml super-natant mixed with 0.5 ml of 10 mM potassium phosphate buffer (pH = 7.0) and 1 ml of 1 M potassium iodide. Absorption of the resulting supernatant was determined at 390 nm, and the amount of  $\text{H}_2\text{O}_2$  was obtained on a standard curve [28].

For enzyme activity analysis, the sample was imbibed at 30 °C for 24 h in a beaker. Then 200 mg of this sample was crushed in a pestle-mortar containing 10 mL of phosphate buffer (pH = 7.8). The homogenate (10 mL) was centrifuged at 12,000 rpm for 20 min at 4 °C, and recentrifuged at 15,000 rpm for about 10 min. This supernatant was, further, used for estimation of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) activity. The POD activity was measured by observing the tetraguaiacol formation from guaiacol ( $\epsilon = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$ ). Reaction mixture containing 0.5 mM of phosphate buffer (pH = 6.1); 16 mM of guaiacol; 2 mM of  $\text{H}_2\text{O}_2$ ; and 20  $\mu\text{L}$  of enzyme extract was made, and the variations in absorption at 470 nm were recorded after 30 s. The CAT activity was measured according to the  $\text{H}_2\text{O}_2$  disappearance

( $\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Reaction-mixture comprising 50 mM sodium phosphate buffer (pH = 7.0) and 10 mM  $\text{H}_2\text{O}_2$  along with enzyme extract was made and the absorption at 240 nm was measured with spectrophotometer (Specord-205, Analytik Jena AG, Jena, Germany). The SOD activity was measured based on the decrease in formazone optical density, prepared by superoxide radical with nitro-blue tetrazolium dye by the enzymes, and the absorbance at 560 nm was measured with a spectrophotometer [29].

Stomatal conductance was determined with a leaf porometer (SC-1, Decagon Devices, Inc., Pullman, WA, USA). Calibration of the instrument was performed prior to each measurement set according to the guidelines of manufacturer [30]. Relative water content (RWC) was estimated from leaf discs of 100 mg fresh weight immersed in distilled water until saturation was attained. The leaf discs were removed after 6 hrs. The water on the surface of the discs was then blotted-off without applying any pressure, and the discs were subsequently weighed for their saturated weight. Lastly, their dry weight was determined after air-drying the leaf discs at 70 °C for 72 h [31].

### 2.6. Data Analysis

ANOVA was directed toward the detection of variations among mean values of every treatment using the software package STAR (Statistical tool for agricultural research). Every mean value underwent two-way ANOVA, which observed the effects of each studied treatment. The outcomes of the experiment were analyzed for examining different parameters in each treatment, and the least significant differences (LSD) were used to calculate significance of differences,  $p < 0.05$ . In addition, the results obtained were expressed as the mean value  $\pm$  standard deviation, calculated, and scrutinized statistically. The statistical significance is marked at  $p < 0.05$  unless stated otherwise.

## 3. Results

### 3.1. Analysis of Variance

The variance analysis for morphological and biochemical traits is represented in Tables 1 and 2, respectively. It showed the highly significant mean sum of squares of genotypes for each and every character. Significant mean showed the existence of considerable variability in the material studied for the improvement of various morphological traits (Table 1) and biochemical traits (Table 2).

**Table 1.** Variance analysis of the effects of different treatments on morphology of faba bean.

Traits	Treatments	Replication
DF	6	2
Days to 50% flowering	117.14	95.69
F	1.46	1.19
P	0.27	0.33
Days to maturity	292.42	0.78
F	20.15	0.05
P	0.00	0.94
Plant height	1894.33	3.05
F	302.43	0.49
P	0.00	0.62
Number of branches per plant	3.42	0.11
F	78.71	2.71
P	0.00	0.10
Number of clusters per plant	157.25	0.57
F	365.08	1.35
P	0.00	0.29

**Table 1.** *Cont.*

Traits	Treatments	Replication
Number of pods per plant	432.74	6.85
F	573.33	9.08
P	0.00	0.00
Pod length (cm)	3.80	0.00
F	176.78	0.33
P	0.00	0.72
Seeds per pod	0.71	0.03
F	16.63	0.81
P	0.00	0.46
Seed weight (gm)	177.44	1.47
F	149.81	1.24
P	0.00	0.32
Grain yield (gm)	313.25	1.76
F	483.86	2.72
P	0.00	0.10
Shoot length (cm)	156.03	0.53
F	303.09	0.34
P	0.00	0.32
Root length (cm)	173.89	1.30
F	343.56	0.34
P	0.00	0.65
Root dry mass (mg/plant)	145.43	1.23
F	278.34	1.67
P	0.00	0.21
Shoot dry mass (mg/plant)	59.23	1.65
F	233.45	0.34
P	0.00	0.23
Internode length	324.56	1.45
F	525.54	2.34
P	0.00	0.54
Leaf area	178.04	1.35
F	231.32	0.67
P	0.00	0.23

**Table 2.** Variance analysis of the effects of different treatments on biochemical traits of faba bean.

Traits	Treatments	Replication
Stomatal conductance (mmol)	233.21	1.42
F	456.43	0.76
P	0.00	0.00
SPAD	122.45	5.76
F	56.45	1.23
P	0.00	0.23
Superoxide dismutase (mg)	90.31	6.04
F	22.01	1.47
P	0.00	0.26

Table 2. Cont.

Traits	Treatments	Replication
Peroxidase (g)	68.38	15.42
F	38.81	8.76
P	0.00	0.00
Catalase activity (mg)	2248.49	87.76
F	51.25	2.00
P	0.00	0.17
Hydrogen peroxide concentration (mol/g)	112.66	16.04
F	12.45	1.77
P	0.00	0.21
Malondialdehyde level (mol/g)	367.38	2.33
F	68.88	0.44
P	0.00	0.65
Electrolyte leakage	391.15	0.90
F	53.22	0.12
P	0.00	0.88
Chlorophyll (mg/g)	289.3	1.33
F	50.07	0.23
P	0.00	0.79
Proline content (mg/g)	1.12	0.01
F	75.38	0.90
P	0.00	0.43
RWC	735.09	4.00
F	98.01	0.53
P	0.00	0.59

### 3.2. Effects on Morphological Traits

Table 3 suggests that plants treated with bioinoculants *Trichoderma viride*, *Pseudomonas fluorescens*, *Glomus mosseae*, and *Gigaspora gigantean* exhibited significant improvement in each treatment as in comparison to salt stress. The treatment with C (T1) and TV + GM + GG + SS (T7) was found to be the most efficient treatments on the morphological characters. However, other treatments such as TV + SS (T3), PF + SS (T4), GM + SS (T5), and GG + SS (T6) also demonstrated considerable effects on Faba bean. Data on days to flowering showed that minimum duration was observed with T1 ( $47.24 \pm 1.25$ ), followed by T7 ( $48.92 \pm 1.15$ ), T5 ( $50.67 \pm 23.18$ ), T6 ( $51.33 \pm 2.52$ ), T4 ( $57.19 \pm 0.41$ ), and T3 ( $59.76 \pm 0.63$ ) treated plants which was far better than salt stress ( $64.08 \pm 5.39$ ) (Table 3). Similarly, the decrement of days to maturity in the same treatment, T1 ( $140.56 \pm 5.34$ ), was observed, followed by T7 ( $144.56 \pm 1.95$ ), T5 ( $155.85 \pm 2.08$ ), T6 ( $158.22 \pm 1.07$ ), T4 ( $159.37 \pm 5.78$ ), and T3 ( $160.78 \pm 0.51$ ), as compared to T2 ( $169.33 \pm 4.05$ ). The plant height (cm) was observed to be maximum with T7 ( $141.93 \pm 4.81$ ), followed by T1 ( $134.19 \pm 0.41$ ), T6 ( $122 \pm 1.09$ ), T5 ( $115.96 \pm 1.15$ ), T4 ( $110.46 \pm 0.49$ ), T3 ( $106.44 \pm 0.61$ ), as compared to T2 ( $64.36 \pm 3.77$ ) (Table 3).

**Table 3.** Effects of different treatments on morphological traits of faba bean.

Traits	Control (T1)	Salt Stress (T2)	<i>Trichoderma viride</i> + Salt Stress (T3)	<i>Pseudomonas fluorescens</i> + Salt Stress (T4)	<i>Glomus mosseae</i> + Salt Stress (T5)	<i>Gigaspora gigantea</i> + Salt Stress (T6)	Stress + TV + GM + GG (T7)
Days to 50% flowering	47.24 ± 1.25 bc *	64.08 ± 5.39 g	59.76 ± 0.63 ac	57.19 ± 0.41 d	50.67 ± 23.18 ab	51.33 ± 2.52 g	48.92 ± 1.15 f
Days to maturity	140.56 ± 5.34 c	169.33 ± 4.05 a	160.78 ± 0.51 ab	159.37 ± 5.78 ab	155.85 ± 2.08 b	158.22 ± 1.07 b	144.56 ± 1.95 c
Plant height	134.19 ± 0.41 b	64.36 ± 3.77 f	106.44 ± 0.61 e	110.46 ± 0.49 de	115.96 ± 1.15 cd	122 ± 1.09 c	141.93 ± 4.81 a
Number of branches per plant	5.28 ± 0.26 a	2.17 ± 0.55 e	3.38 ± 0.03 cd	3.04 ± 0.06 d	3.95 ± 0.04 bc	4.09 ± 0.01 b	4.87 ± 0.09 a
Number of clusters per plant	25.31 ± 1.17 a	3.72 ± 0.59 f	9.51 ± 0.96 e	15.71 ± 0.35 d	17.16 ± 0.19 cd	18.88 ± 0.24 c	21.02 ± 0.56 d
Number of pods per plant	51.06 ± 1.32 a	18.29 ± 0.88 g	26.21 ± 2.51 f	34.16 ± 0.20 d	30.31 ± 1.11 e	42.44 ± 0.40 c	48.33 ± 1.06 b
Pod length (cm)	5.74 ± 0.05 a	2.48 ± 0.36 e	3.4 ± 0.02 d	4.14 ± 0.01 c	4.2 ± 0.01 c	4.91 ± 0.08 b	5.31 ± 0.02 b
Seeds per pod	3.43 ± 0.02 a	1.96 ± 0.42 c	2.81 ± 0.06 b	2.92 ± 0.06 ab	3 ± 0.00 ab	2.83 ± 0.29 b	3.39 ± 0.15 ab
Seed weight (gm)	34.27 ± 2.55 a	10.98 ± 0.78 f	19.39 ± 0.99 e	22.52 ± 0.54 d	24.05 ± 0.10 cd	27 ± 0.06 c	30.9 ± 0.38 d
Grain yield (gm)	37.17 ± 0.81 a	10.44 ± 1.38 g	13.94 ± 0.28 f	17.82 ± 0.42 e	25.5 ± 1.45 d	30.54 ± 0.49 c	33.21 ± 0.71 b
Shoot length (cm)	42 ± 0.56 b	14 ± 0.21 a	19.65 ± 0.06 e	21.67 ± 0.43 d	23.67 ± 0.34 c	31.65 ± 0.52 ac	34.76 ± 0.23 ab
Root length (cm)	48.5 ± 1.01 c	9.89 ± 1.10 f	12.65 ± 0.06 g	14.76 ± 0.10 e	18.78 ± 0.52 d	29.23 ± 0.32 bc	35.78 ± 0.78 c
Root dry mass (mg/plant)	1.1 ± 0.04 ac	0.12 ± 0.06 b	0.32 ± 0.01 f	0.42 ± 0.02 bc	0.54 ± 0.03 c	0.62 ± 0.05 d	0.76 ± 0.01 cd
Shoot dry mass (mg/plant)	2.9 ± 0.02 ab	0.54 ± 0.03 c	0.79 ± 0.02 e	0.91 ± 0.10 d	1.1 ± 0.06 f	1.35 ± 0.01 c	1.79 ± 0.01 ab
Internode length	1.76 ± 0.02 bc	0.34 ± 0.01 g	0.56 ± 0.02 e	0.68 ± 0.02 ab	0.91 ± 0.13 bc	1.05 ± 0.02 cd	1.2 ± 0.03 b
Leaf area	14.69 ± 1.03 c	3.73 ± 0.61 a	7.45 ± 0.76 b	7.89 ± 0.64 f	8.34 ± 0.54 ac	9.07 ± 0.32 ab	12.36 ± 0.67 g

\* Values in a column followed by the same letter are not significantly different,  $p \leq 0.05$ , LSD.

The number of branches per plant was highest with T1 ( $5.28 \pm 0.26$ ), followed by T7 ( $4.87 \pm 0.09$ ), T6 ( $4.09 \pm 0.01$ ), T5 ( $3.95 \pm 0.04$ ), T3 ( $3.38 \pm 0.03$ ), and T4 ( $3.04 \pm 0.06$ ), as compared with T2 ( $2.17 \pm 0.55$ ). Similarly, the maximum number of clusters per plant was observed with T1 ( $25.31 \pm 1.17$ ), followed by T7 ( $18.88 \pm 0.24$ ), T6 ( $18.88 \pm 0.24$ ), T5 ( $17.16 \pm 0.19$ ), T4 ( $15.71 \pm 0.35$ ), and T3 ( $9.51 \pm 0.96$ ) (Table 3). Further, the highest number of pods per plant was recorded with T1 ( $51.06 \pm 1.32$ ), followed by T7 ( $48.33 \pm 1.06$ ), T6 ( $42.44 \pm 0.40$ ), T4 ( $34.16 \pm 0.20$ ), T5 ( $30.31 \pm 1.11$ ), and T3 ( $26.21 \pm 2.51$ ). The results with pod length (cm) were the same when treated with T1 ( $5.74 \pm 0.05$ ), followed by T7 ( $5.31 \pm 0.02$ ), T6 ( $4.91 \pm 0.08$ ), T5 ( $4.2 \pm 0.01$ ), T4 ( $4.14 \pm 0.01$ ), and T3 ( $3.4 \pm 0.02$ ), as compared to T2 ( $2.48 \pm 0.36$ ). Furthermore, the maximum increment in seeds per pod was also recorded with T1 ( $3.43 \pm 0.02$ ), followed by T7 ( $3.39 \pm 0.15$ ), T5 ( $3 \pm 0.00$ ), T4 ( $2.92 \pm 0.06$ ), T6 ( $2.83 \pm 0.29$ ), and T3 ( $2.81 \pm 0.06$ ). Similarly, seed weight (gm) was maximum recorded with T1 ( $34.27 \pm 2.55$ ) followed by T7 ( $30.9 \pm 0.38$ ), T6 ( $27 \pm 0.06$ ), T5 ( $24.05 \pm 0.10$ ), T4 ( $22.52 \pm 0.54$ ), and T3 ( $19.39 \pm 0.99$ ) (Table 3). Grain yield (gm) was the highest recorded with T1 ( $37.17 \pm 0.81$ ),



followed by T7 ( $33.21 \pm 0.71$ ), T6 ( $30.54 \pm 0.49$ ), T5 ( $25.5 \pm 1.45$ ), T4 ( $17.82 \pm 0.42$ ), and T3 ( $13.94 \pm 0.28$ ), which again proved the efficiency of combined application of bioinoculants over separate inoculations (Table 3).

The treatments showed different results in RWC with an application of T1 ( $65 \pm 5.00$ ), T7 ( $70 \pm 1.00$ ), T6 ( $58 \pm 2.00$ ), T4 ( $57.67 \pm 2.52$ ), T5 ( $52 \pm 2.00$ ), and T3 ( $44.67 \pm 1.53$ ); here it is indicated that T7 showed better results than rest of the treatments (Table 3). The maximum shoot length (cm) ( $42 \pm 0.56$ ) of faba bean was found in T1-treated plants, followed by T7 ( $34.76 \pm 0.23$ ), T6 ( $31.65 \pm 0.52$ ), T5 ( $23.67 \pm 0.34$ ), T4 ( $21.67 \pm 0.43$ ), and T3 ( $19.65 \pm 0.06$ ) (Table 3). The same result was found in root length (cm), and the maximum root length was recorded with the application of T1 ( $48.5 \pm 1.01$ ), followed by T7 ( $35.78 \pm 0.78$ ), T6 ( $29.23 \pm 0.32$ ), T5 ( $18.78 \pm 0.52$ ), T4 ( $14.76 \pm 0.10$ ), and T3 ( $12.65 \pm 0.06$ ) (Table 3).

The maximum root dry mass (mg/plant) was observed with the application of T1 ( $1.1 \pm 0.04$ ), followed by T7 ( $0.76 \pm 0.01$ ), T6 ( $0.62 \pm 0.05$ ), T5 ( $0.54 \pm 0.03$ ), T4 ( $0.42 \pm 0.02$ ), and T3 ( $0.32 \pm 0.01$ ). Similarly, shoot dry mass (mg/plant) was highest recorded with T1 ( $2.9 \pm 0.02$ ), followed by T7 ( $1.79 \pm 0.01$ ), T6 ( $1.35 \pm 0.01$ ), T5 ( $1.1 \pm 0.06$ ), T4 ( $0.91 \pm 0.10$ ), and T3 ( $0.79 \pm 0.02$ ) (Table 3). The highest internode length was recorded in plants treated with T1 ( $1.76 \pm 0.02$ ), followed by T7 ( $1.2 \pm 0.03$ ), T6 ( $1.05 \pm 0.02$ ), T5 ( $0.91 \pm 0.13$ ), T4 ( $0.68 \pm 0.02$ ), and T3 ( $0.56 \pm 0.02$ ). Similarly, Leaf area ( $\text{cm}^2$ ) was the highest when applied with T1 ( $14.69 \pm 3$ ), followed by T7 ( $12.36 \pm 0.67$ ), T6 ( $9.07 \pm 0.32$ ), T5 ( $8.34 \pm 0.54$ ), T4 ( $7.89 \pm 0.64$ ), and T3 ( $7.45 \pm 0.76$ ) (Table 3).

### 3.3. Effects on Biochemical Traits

Further, Table 4 also demonstrated the beneficial effects of bioinoculants used for biochemical characters in each treatment in comparison to salt stress. Data on stomatal conductance (mmol) showed the highest value with T1 ( $367.34 \pm 86.41$ ), followed by T7 ( $145.78 \pm 1.32$ ), T6 ( $120.67 \pm 2.89$ ), T5 ( $110.45 \pm 2.54$ ), T4 ( $91.45 \pm 1.25$ ), and T3 ( $76.32 \pm 2.65$ ). Similarly, SPAD was highest recorded with T1 ( $32.02 \pm 4.32$ ), followed by T7 ( $29.78 \pm 1.23$ ), T6 ( $27.67 \pm 1.32$ ), T5 ( $22.89 \pm 0.32$ ), T4 ( $22.03 \pm 0.54$ ), and T3 ( $21.45 \pm 1.21$ ), as compared to T2 ( $13.45 \pm 1.04$ ) (Table 4). The maximum superoxide dismutase (mg) was recorded in plants treated with T1 ( $30.67 \pm 1.53$ ), followed by T7 ( $25.67 \pm 1.53$ ), T6 ( $24.67 \pm 1.53$ ), T5 ( $23.33 \pm 3.06$ ), T3 ( $21 \pm 1.00$ ), and T4 ( $18.33 \pm 3.06$ ). Similarly, peroxidase (g) was highest recorded with treatment T7 ( $27.33 \pm 1.53$ ), T6 ( $24.67 \pm 1.53$ ), T4 ( $24.33 \pm 3.06$ ), T3 ( $22.33 \pm 1.53$ ), and T5 ( $21.67 \pm 1.53$ ) (Table 4). The highest catalase activity (mg) was observed with T7 ( $235 \pm 10.15$ ), followed by T6 ( $205.67 \pm 4.16$ ), T5 ( $194 \pm 1.73$ ), T4 ( $193 \pm 6.08$ ), and T3 ( $184.33 \pm 11.37$ ), as compared to T2 ( $184 \pm 7.55$ ) (Table 4).

The minimum hydrogen peroxide (mol/g) was recorded with T1 ( $18.33 \pm 3.06$ ), followed by T7 ( $24 \pm 4.58$ ), T6 ( $26.33 \pm 2.52$ ), T5 ( $31 \pm 1.00$ ), T4 ( $32.67 \pm 4.73$ ), T3 ( $32.87 \pm 2.00$ ), and T2 ( $36.33 \pm 2.52$ ). Based on this investigation, it can be concluded that T1 and T7 have much more constructive effects than other treatments. The minimum malondialdehyde level (mol/g) was recorded with T1 ( $32 \pm 2.00$ ), followed by T7 ( $45 \pm 1.00$ ), T5 ( $51.33 \pm 2.52$ ), T6 ( $52.67 \pm 2.52$ ), T4 ( $58.67 \pm 3.21$ ) and T3 ( $58.84 \pm 1.53$ ), and T2 ( $66 \pm 2.00$ ). The minimum electrolyte leakage was recorded with the treatment of T1 ( $40.67 \pm 1.53$ ), followed by T7 ( $54.67 \pm 5.03$ ), T6 ( $61.67 \pm 2.08$ ), T5 ( $63.67 \pm 1.53$ ), T4 ( $65.31 \pm 2.00$ ), and T3 ( $66.33 \pm 1.53$ ). The chlorophyll (mg/g) with the application of T7 ( $51.67 \pm 3.06$ ) was the highest, followed by T6 ( $41 \pm 1.00$ ), T1 ( $39.33 \pm 2.08$ ), T5 ( $35.33 \pm 1.53$ ), T4 ( $32.67 \pm 3.51$ ), and T3 ( $31.67 \pm 2.08$ ). The proline content (mg/g), however, showed no significant differences when treated with T1 ( $1.08 \pm 0.03$ ), T7 ( $2.96 \pm 0.12$ ), T6 ( $2.72 \pm 0.15$ ), T5 ( $2.63 \pm 0.21$ ), T4 ( $2.4 \pm 0.11$ ), and T3 ( $2.06 \pm 0.04$ ) (Table 4).

**Table 4.** Effects of different treatments on biochemical traits of Faba bean.

Traits	Control (T1)	Salt Stress (T2)	<i>Trichoderma viride</i> + Salt Stress (T3)	<i>Pseudomonas fluorescens</i> + Salt Stress (T4)	<i>Glomus mosseae</i> + Salt Stress (T5)	<i>Gigaspora gigantea</i> + Salt Stress (T6)	Stress + TV + GM + GG (T7)
Stomatal conductance (mmol)	367.34 ± 86.41 b *	56.76 ± 3.45 ab	76.32 ± 2.65 d	91.45 ± 1.25 bc	110.45 ± 2.54 f	120.67 ± 2.89 a	145.78 ± 1.32 ac
SPAD	32.02 ± 4.32 a	13.45 ± 1.04 ac	21.45 ± 1.21 b	22.03 ± 0.54 d	22.89 ± 0.32 g	27.67 ± 1.32 e	29.78 ± 1.23 f
Superoxide dismutase (mg)	10.67 ± 1.53 f	15 ± 2.00 a	21 ± 1.00 b	18.33 ± 3.06 bc	23.33 ± 3.06 ac	24.67 ± 1.53 ab	25.67 ± 1.53 g
Peroxidase (g)	12.67 ± 2.52 e	19 ± 1.00 ab	22.33 ± 1.53 bc	24.33 ± 3.06 e	21.67 ± 1.53 f	24.67 ± 1.53 b	27.33 ± 1.53 ac
Catalase activity (mg)	143.67 ± 2.08 b	184 ± 7.55 ac	184.33 ± 11.37 c	193 ± 6.08 f	194 ± 1.73 cd	205.67 ± 4.16 f	235 ± 10.15 g
Hydrogen peroxide concentration (mol/g)	18.33 ± 3.06 b	36.33 ± 2.52 bc	32 ± 2.00 c	32.67 ± 4.73 cd	31 ± 1.00 e	26.33 ± 2.52 d	24 ± 4.58 f
Malondialdehyde level (mol/g)	32 ± 2.00 g	66 ± 2.00 f	58.67 ± 1.53 ab	58.67 ± 3.21 ac	51.33 ± 2.52 d	52.67 ± 2.52 ad	45 ± 1.00 a
Electrolyte leakage	40.67 ± 1.53 ab	77.33 ± 2.08 ac	66.33 ± 1.53 a	60 ± 2.00 f	66.67 ± 1.53 ad	61.67 ± 2.08 a	54.67 ± 5.03 ac
Chlorophyll (mg/g)	39.33 ± 2.08 ab	19.67 ± 1.53 ad	31.67 ± 2.08 a	32.67 ± 3.51 ac	35.33 ± 1.53 b	41 ± 1.00 f	51.67 ± 3.06 ac
Proline content (mg/g)	1.08 ± 0.03 ac	1.8 ± 0.10 a	2.06 ± 0.04 ac	2.4 ± 0.11 f	2.63 ± 0.21 g	2.72 ± 0.15 bc	2.96 ± 0.12 d
RWC	65 ± 5.00 ab	22.67 ± 2.52 e	44.67 ± 1.53 d	57.67 ± 2.52 bc	52 ± 2.00 cd	58 ± 2.00 bc	70 ± 1.00 a

\* Values in a column followed by the same letter are not significantly different,  $p \leq 0.05$ , LSD.

#### 4. Discussion

Excessive salt stress in soil exhibits a negative impact on the growth and development of plants, resulting in a loss in productivity and quality of crops. Salinity stress conditions affect the physiological features of a plant by the accumulation of salt in the soil through excessive ions such as  $\text{Na}^+$  and produce reactive oxygen species, impairing enzymes and cell organelles, thereby disturbing photosynthesis, protein synthesis, and respiration in plants. Soil microbes possess different substances or compounds that may enhance the morphological as well as biochemical characteristics of plants. The present study was conducted to determine the effect of different microbes on the morphological and biochemical traits of faba bean under salinity stress. It was observed that faba bean grown in adverse conditions shows negative effects on both the morphological and biochemical traits. Similar results were found among different treatments under salinity stress, as studied by Afzal et al. [32]. However, a significant effect on faba bean cultivated under salinity stress in field conditions utilizing microbes and their interaction has their effectiveness.

Filipovic et al. [33] found the same result and suggested that an increment of water salinity reduced the morphological and biochemical characteristics [33]. The symbiotic interactions in different treatments on faba bean showed different results. However, we noted the significant effects of these interactions with the microbes *Trichoderma viride*, *Pseudomonas fluorescens*, *Glomus mosseae*, *Gigaspora gigantea* on faba bean. These microorganisms help to recover soil biodiversity by improving unfavorable conditions of the soil. Previous findings on legume crops also established the positive effects of these microbes on plant growth and yield [34,35]. The interaction of these microbes assists the host by improving the uptake of water and minerals. It is clear from the study that microbes used in plants show better results in different treatments than salt stress [36]. Inoculation with

these microbes has displayed a better capacity to tolerate and resist a large number of environmental stresses, especially abiotic stresses [37]. The interaction among treatments may be related to the plant metabolism of the bacterial population in nodules, as suggested by Mhadhbi et al. [38]. These results showed that the improvement in legumes was not only due to the selective genotypes but also the assessment of the most effective association between genotypes and bacteria [39].

The significance of *Trichoderma viride*, *Pseudomonas fluorescens*, *Glomus mosseae*, and *Gigaspora gigantea* on faba bean under salinity stress in field condition has been proven through numerous other studies [36,40]. Moreover, the interaction of these microbes can be beneficial for each other and helps to grow plants and maintain yield and biomass production as well [41,42]. It has earlier been proved that these interactions can be beneficial for further use in salinity stress on the development of faba bean [43,44]. Bio-inoculation in plants led to considerable uptake of water and nutrients, allowing better plant growth and development under salt stress compared to non-inoculated plants [45]. The salt stress disrupts the physiological process, especially photosynthesis, resulting in a decrease in plant growth and productivity. Oxidative stress could stem from a decreased stomatal conductance in response to osmotic imbalance and reduced leaf-water potential [46], ultimately leading to a decrease in photosynthesis. Moreover, the accumulation of intracellular sodium ions at salt stress changes the ratio of K:Na [47], which seems to affect the bioenergetic processes of photosynthesis. Moreover, the vapor-loss regulation through stomata is very subtle to salinity stress [48]. Closure of leaf pores is considered the most adaptive mechanism for the prevention of cell turgor loss due to an inadequate supply of water.

The salt stress results in reduced conductance through stomata; however, the photosynthesis rate per unit area of the leaf may sometimes remain unaffected [47]. However, the application of TV + GM + GG + SS enhanced the photosynthetic activity significantly in faba bean. It might be due to the higher interaction of these microbes improving plant nutrition [49,50]. However, in addition to inhibition of photosynthesis, plants of faba bean treated with the interaction of these microbes TV + GM + GG + SS were able to maintain a constant K<sup>+</sup> content independently of Na<sup>+</sup> and Cl<sup>-</sup> accumulation. These results suggest that K<sup>+</sup> maintenance might be a common mechanism of protection against salt damage [51]. Photosynthetic pigments were affected due to salt stress concentration, which reduced the yield, but the combination of these microorganisms provides sufficient scope for improvement; it may be an effect of photosynthesis. Many studies have shown shoot length and dry mass to be affected either negatively or positively by changes in salt concentrations, type of salt, and plant genotype present. The increment of the shoot dry mass may be due to the ability of plants to increase the size of their vacuole, which allows for storing a good amount of water, and this, in turn, dissolves the salt. Moreover, enhancement of shoot length might be associated with enhanced photosynthesis, as suggested by an increase in Chlorophyll which was treated plants with TV + GM + GG + SS [52]. The increment in leaf area could be attributed to the decreasing concentration of NaCl which has a positive effect on photosynthesis and leads to improvement in plant growth, development, and yield.

It was verified that continuous use of saline water during the flowering stage reduced maturity, the number of branches, the number of clusters per plant, the number of pods per plant, seeds per pod, pod length, seeds weight, and grain yield. This investigation on faba beans demonstrated that plants treated with TV + GM + GG + SS exhibited a greater loss in ROS concentration and restricted cellular oxidative damage [19]. The uncontrolled rise in lipid peroxidation leads to a decline in the functional and structural integrity of the cellular membrane. The regular metabolism of plant cells is disturbed by ROS due to oxidative damage caused to nucleic acid, photosynthetic pigments, and lipids. Numerous studies have revealed that microbial interaction may intensify the anti-oxidative defense system of plants via increased activity of antioxidants of bio-inoculated plants hastening superoxide dismutations to H<sub>2</sub>O<sub>2</sub>, obstructing H<sub>2</sub>O<sub>2</sub> build up by increased activities of enzymes CAT, POD, and SOD. Some novel genetic tools, along with physiological activities, may help in the recognition of *Trichoderma* spp. associated with secondary metabolite production [53].

It is well known that plants subjected to abiotic stresses suffer cell damage due to oxidative stress, yet in many cases, plants are able to avoid this fate by activating their inbuilt antioxidant mechanisms. Numerous studies have shown that one method these biocontrol agents can protect from the harmful effects of abiotic stresses, particularly salt stress, is by enhancing the capacity of their antioxidant machinery [54,55]. The results obtained in this study presented the increased antioxidant activity of enzymes, also established in the study conducted by other researchers [37], due to amplified SOD, CAT, and POD synthesis. It was also found that vegetable crops, after colonization, possess higher anti-oxidants capable of scavenging H<sub>2</sub>O<sub>2</sub> directly [56]. Further, the reduced electrolytic leakage, as indicated in TV + GM + GG + SS mediated plants, may be accredited to enhanced nutrient uptake, osmotic homeostasis, and the reduced toxicity of ions [57,58]. Some probable processes by which biocontrol agents improve plant tolerance to salinity stress have been proposed, but their precise nature is still largely unknown. The possible mechanisms include the release of hormones like abscisic acid, gibberellic acid, cytokinins, and auxin; the release of ACC (1-aminocyclopropane-1-carboxylic acid) deaminase, which decreases ethylene levels in the developing plant roots; and bacterial-induced systemic tolerance [59]. Researchers have also linked plant salt tolerance to the accumulation of certain metabolites like amino acids, amides, amino acids, proteins, glycine betaines, and polyamines [60].

## 5. Conclusions

*Trichoderma viride*, *Pseudomonas fluorescens*, *Glomus mosseae*, and *Gigaspora gigantea* have emerged as capable bio-inoculants for faba bean for nutrient-procurement and maintenance. Faba bean cultivation is popular because of its exceptional nutritional qualities and is recognized as a valued food product. Faba bean cultivation requires extensive maintenance and eco-friendliness. They are well-known for supplying plants with necessary quantities of nutrients, which are essential for the plants' continued growth and development. In this regard, the TV + GM + GG + SS interaction seems appropriate for improving plant traits and the quality and quantity of the crop in the instance of faba bean. However, in order for the morphological and biochemical characteristics of the plants to be relevant, the plants also need an appropriate quantity of regular water. In general, the findings of this study suggest that the interaction between *Trichoderma viride*, *Pseudomonas fluorescens*, *Glomus mosseae*, and *Gigaspora gigantea* has a substantial impact on the yield and growth development of faba bean.

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