The impact of Mutants of *Trichoderma* species in modulating salinity stress of beans (*Phaseolus vulgaris* L. CV. Khomein)

Abstract

The occurrence of salinity in Iran's agricultural soils is agrowing problem and can cause a yield loss in the production of many products, including beans. Bio-priming with microorganisms tolerant to salinity stress (such as *Trichoderma*) is one of the biocompatible and effective solutions to solve this problem. The salinity treatment used in this study (NaCl 100 mM) is doubling the tolerance threshold of the bean plant and the effect of biopriming with *Trichoderma* (wild-type and mutant) isolates has been investigated compared to the control plant. In order to increase tolerance to salinity in Iranian *Trichoderma*, mutation induction method using gamma radiation (250 Gy) was used in this research. Growth components, germination, allometric index, seedling tissue water, and vigor were measured at seedling stage. Results from analysis of variance and comparison of the average showed that biological priming of beans with *Trichoderma* (wild type and mutant) fungal species had a significant advantage over control of salinity stress. However, the modulating effect of salt stress was greater in seeds inoculated with mutant species than in their wild type.

Keywords: Biopriming, *Trichoderma* sp., Gamma irradiation, salinity stress.

Introduction

Soil salinity is a condition with high salt concentrations in the soil. These soils' electrical conductivity (EC) equals four desi siemens [1]. This amount of soil salinity significantly reduces the yield of most agricultural products [2]. Presently, a considerable amount of agricultural land is somewhat saline, and the extent of saline land is increasing [3]. For example, one of the reasons for the increasing salinity of agricultural land is improper and excessive irrigation [4]. With the increase in soil salinity, agricultural lands are destroyed, and it is estimated that 50% of these lands will not be usable until the middle of the 47th century [5]. The adverse effects of salinity on plants are divided into three general groups, including the reduction of soil osmotic potential, the destruction of the physical structure of the soil, and the increase in the concentration of sodium (Na+) and chlorine (Cl-) ions [6].

Beans are one of the most important crops, essential for high protein content and use in the diet. This plant is an annual crop cultivated for seed production [7]. Regarding the cultivated area of legumes, beans are the first in Iran [8]. Beans are susceptible to salinity and tolerate soil salinity less than 2 Deci siemens/meter [9].

Many studies showed that increasing salinity in the hydroponic cultivation of beans reduced root dry weight, number of roots, total root length, average root diameter, and total root volume [10]. Also, salinity has adverse effects on bean germination; so, with increasing salinity stress, the germination rate, germination percentage, and root length of this plant decrease [11]. In this regard, from the results of investigating the effects of salinity stress in beans in hydroponic cultivation, it was found that with increasing salinity, fresh stem weight, root fresh and dry weight, and root length of this plant decreased [12].

Seed biopriming is one of the easy and low-cost methods to increase growth quickly, plant efficiency in nutrient absorption, and relative tolerance in the face of harsh environmental conditions such as salinity [13]. Seed biopriming causes an increase in biological and physiological activities in the primed seed and the resulting plant so that these things can be observed in the way of germination, early seedling establishment, early maturity, and quantitative and qualitative increase [14]. Using fungal biological fertilizers in seed inoculation is the most crucial method of using these fertilizers [15]. Trichoderma is one of

the most important members of the rhizosphere microorganism community, and the positive effect of its inoculation on plant growth has been proven [16]. By penetrating the root and establishing an endophytic relationship with the plant root, this fungus changes the gene expression profile of the plant and, in this way, changes the plant's metabolism of the plant [17, 18, 19]. There are reports of increased plant growth due to contact with Trichoderma [20, 21, 22]. The seed responds positively to the treatment with Trichoderma when it is exposed to physiological, biotic, and abiotic stresses. Based on the study that was done on the expression of the most desirable and appropriate biological pretreatment (biopriming) in comparison with the control (no inoculation) when faced with salinity conditions in different concentrations, the salinity of agricultural soils and many reports indicate the improvement of germination behavior and indicators related to It includes average germination time, seed germ, root length, shoot length, germination rate and initial establishment in primed seeds [23, 24, 25].

Studies show the resistance of different plants treated with *Trichoderma* species to salinity stress. Considering the nutritional importance of beans, in this research, wild and mutant species of *Trichoderma* [26] were investigated to study the effect of *Trichoderma* on increasing the resistance of bean plant to salt stress. Investigating the possibility of increasing salinity tolerance in bean plants seedlings by using mutant *Trichoderma* isolates which have a higher salt tolerance than their wild parent isolate has also been the secondary aim of this research.

Material and Methods

Five *Trichoderma* isolates (Table 1) were received from the plant pathology collection of Nuclear Agriculture Research School–Nuclear Science and Technology Institute (NSTRI, and subjected to molecular identification using the ITS-rDNA gene sequencing analysis were used. *Trichoderma* isolate was irradiated in the Nuclear Agriculture Research School (NSTRI, AEOI) using a cobalt-60 γ- irradiator at a dose rate of 0.018 Gy/sec following isolation and identification. The dosimetry procedure was completed using the Fickerefrence standard dosimetry system (Gamma-cell Issledovatel, PX-30). To determine a suitable dose, the specified dose range was 0, 50, 150, 200, 250, 300, 350, 400, and 450 Gy. After dose optimization, irradiation was performed at an optimized dose of 250 Gy (three replicates) on spore suspension (10^6 spores/ml) of *Trichoderma*. Serial diluted (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}) spore suspensions were prepared after irradiation, and 1 ml of each diluted sample was cultured in the PDA medium and incubated at 28 °C for 72 hours 13. To ensure the stability of the mutants, each mutant were sub-cultured for seven times and morphological characteristics were recorded for all mutants. The mutation optimized dose was based on a spore germination inhibitory effect of approximately 40-50 % on a Water Agar (WA) medium [26]. Khomein variety of Pinto bean (Phaseolus vulgaris) was received from the Seed and Plant Improvement Institute (SPII) of Iran. The factorial experiment was conducted as a completely random basic design in three replications at Maragheh University in 2020. The description of the treatments is given in Table 1. Salinity treatment was done by irrigation water with (NaCl 100 mM or 9.1 ds/m), denoted as "S" and "M" are abbreviations of mutant and salt stress, respectively, and compared to the control. In plants, the effect of biopriming with Trichoderma isolates (wild type and mutant) has been investigated to double the tolerance threshold (5 ds/m) of bean plants [27].

Table 1- Treatments used in this research, *Trichoderma* sp. with NCBI code

NCBI Code	NSTRI Code	Treatments	
MW718882	NAS107	*T. harzianum*	*T. harzianum** S
MW719563	NAS114	*T. lixii*	*T. lixii** S
MW719590	NAS108	*T. ghanens*	*T. ghanens** S
MW719876	NAS115	*T. virens*	*T. virens** S
MW719255	NAS112	*T. atroviride*	*T. atroviride** S
MW719591	NAS107 M8	*T. harzianum* mutant	*T. harzianum* mutant * S
MW719564	NAS114 M17	*T. lixii* mutant	*T. lixii* mutant* S
ON545796)	NAS108 M1	*T. ghanens* mutant	*T. ghanens* mutant* S
MW719878	NAS115 M17	*T. virens* mutant	*T. virens* mutant * S
MW719257	NAS112M2	*T. atroviride* mutant	*T. atroviride* mutant* S
		Control	Salt stress (S)

Seeds were superficially disinfected in ethanol 70% for 1 minute and sodium hypochlorite 2% for 40 seconds, and finally, washed 3-4 times with sterile distilled water. Then the seeds were inoculated in the spore suspension with a population of 1 × 108 for 20 minutes on a shaker. Bean seeds were planted in 3-liter pots containing washed perlite, and five seeds were planted in each pot. The pots were kept in the growth chamber with controlled conditions under a lighting period of 8.16 hours (dark/light), a temperature period of 18.28 degrees Celsius (night/day), and relative humidity of 50-60%. It was irrigated with distilled water (Irrigation was twice a week) and Hoagland's nutrient solution (Hogland and Arnon, 1950) [28] with an approximate pH of 7.5±1. The pots were subjected to salt treatment at two concentration levels of zero (control) and 100 mM sodium chloride.

At the end of the experiment, the factors of length, fresh and dry weight of shoots and roots, the relative percentage of leaf water, and emergence were investigated.

The method Cherki et al. [26, 29] was used to measure the percentage of water in fresh leaves. For this purpose, in each replication, a disc with a diameter of one centimeter was prepared from the leaf and then weighed with a scale with an accuracy of 0.0001 gram (FW). The leaf discs were placed in distilled water for 4 hours, and their weight was determined again (TW). These disks were dried in an oven at 70 degrees Celsius, and their dry weight was obtained (DW). The following relationship was used for the seedling tissue water percentage;

Equation 1: $RWC = \frac{FW-DW}{TW-DW} \times 100$

The following equation was used to calculate the vigor index [26];

Equation 2: $Vigor\ Index = \frac{Germination\ Percentage \times Seedling\ dry\ Weight}{100}$

Also, the allometric coefficient was calculated using the following equation [28];

Equation 3: $Alometric\ coefficient = \frac{Stem\ \text{Length}}{Root\ \text{Length}}$

The obtained data were analyzed through the SPSS version 9 statistical program and, Duncan's multi-range test was used to compare the mean at a 5% probability level. Graphs were also drawn using Excel software.

Results and discussion

The analysis of variance showed that the length, wet and dry weight of shoot and roots under the influence of salt stress and biopriming and the mutual effects of stress and biopriming showed a statistically significant difference at the probability level of 1% (Table 2). Also, the allometric index, seedling tissue water percentage, and seed vigor showed statistically significant differences under the effect of salinity stress and biopriming and the mutual effects of stress and biopriming at the probability level of 1 %(Table 2). Germination rate was significant at the probability level of 1% for salinity stress and biopriming with Trichoderma species and was not significant for the interaction effect of salinity stress and biopriming (Table 2).

Table 2- Analysis of variance (mean square) of bean characteristics under salt stress and biopriming treatments.

Source of Variation	df	percentage of Emergence	Length		Fresh Weight		Dry Weight		Alometric Index	Seedling Tissue Water Percentage	Seed Vigor
			Root	Stem	Root	Stem	Root	Stem			
bio-priming	10	321.343**	20.83**	234.59**	5.78**	35.34**	0.84**	0.06**	0.08**	19.64**	84356.4635**
Salt stress	1	6060.325**	30.93**	27.27**	2.43**	2.64**	0.25**	0.005**	0.14**	6.47**	54064.8336**
bio-priming*Salt stress	10	55.36^{ns}	0.14**	0.26**	0.036**	0.007**	0.06**	0.0002**	0.08**	0.52**	435.3597**
Error	10	39.58	0.05	0.05	0.009	0.00003	0.0007	0.00003	0.003	0.03	345.6735
CV.		10.04	2.51	1.06	3.83	1.89	6.94	5.51	12.49	0.12	9.73

ns, ** and *: non-significance, respectively, significant at the probability level of 1 and 5 percent

Germination rate

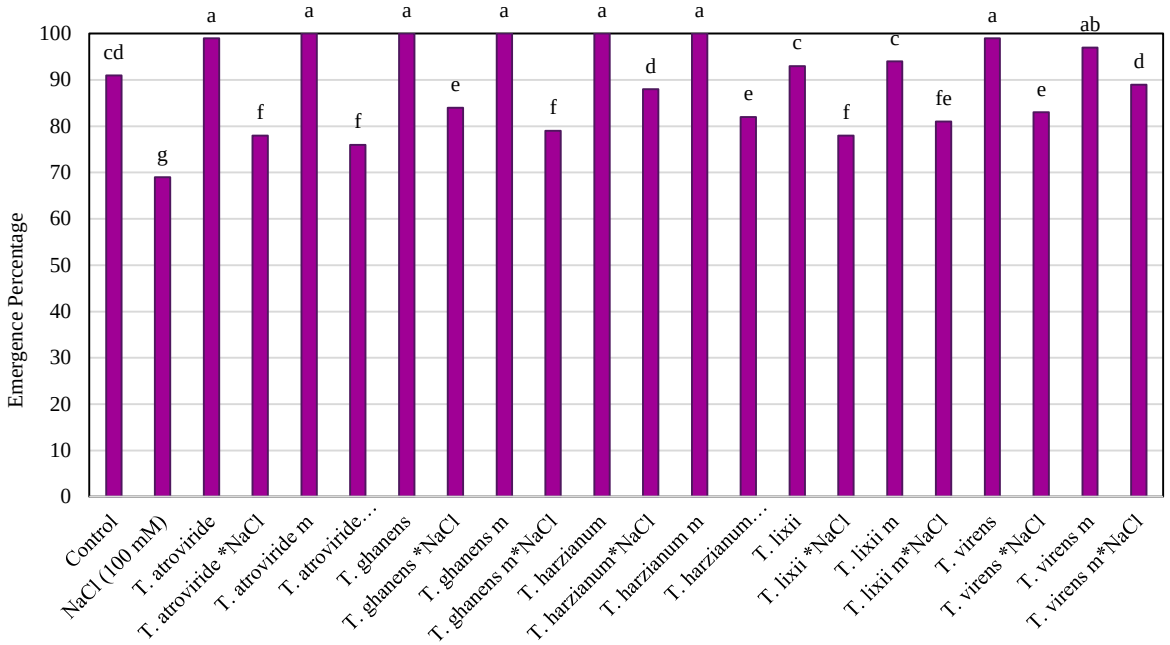
The average comparison between the treatments showed that the 100% germination rate of seeds were observed in *T. atroviride* mutant, *T. ghanens*, *T. ghanens* mutant, *T. harzianum*, *T. harzianum* mutant without stress. The seeds treated with *T. atroviride*, *T. virens*, has no significant difference (99% germination rate). The lowest germination was in the salin stress treatment (69%) which caused a 24.2% decrease in germination compared to the control (91%) (Graph. 1).

In the interaction between salinity and *Trichoderma*, *T. virens* mutant (97%) had the highest salinity stress modulating effect (*T. virens* mutant*S 89%). The effect of salinity stress in *T. virens* mutant*S was 8% less than in *T. virens* mutant (Graph. 1).

The *T. harzianum* *S had the highest salinity stress modulating effect (88%) and was able to reduce the stress effect by 21% compared to S (NaCl 100 mM) (69%) (Graph. 1). Salinity stress in *T. harzianum**S treatment reduced germination by 12% compared to *T. harzianum* (100%) (Graph. 1).

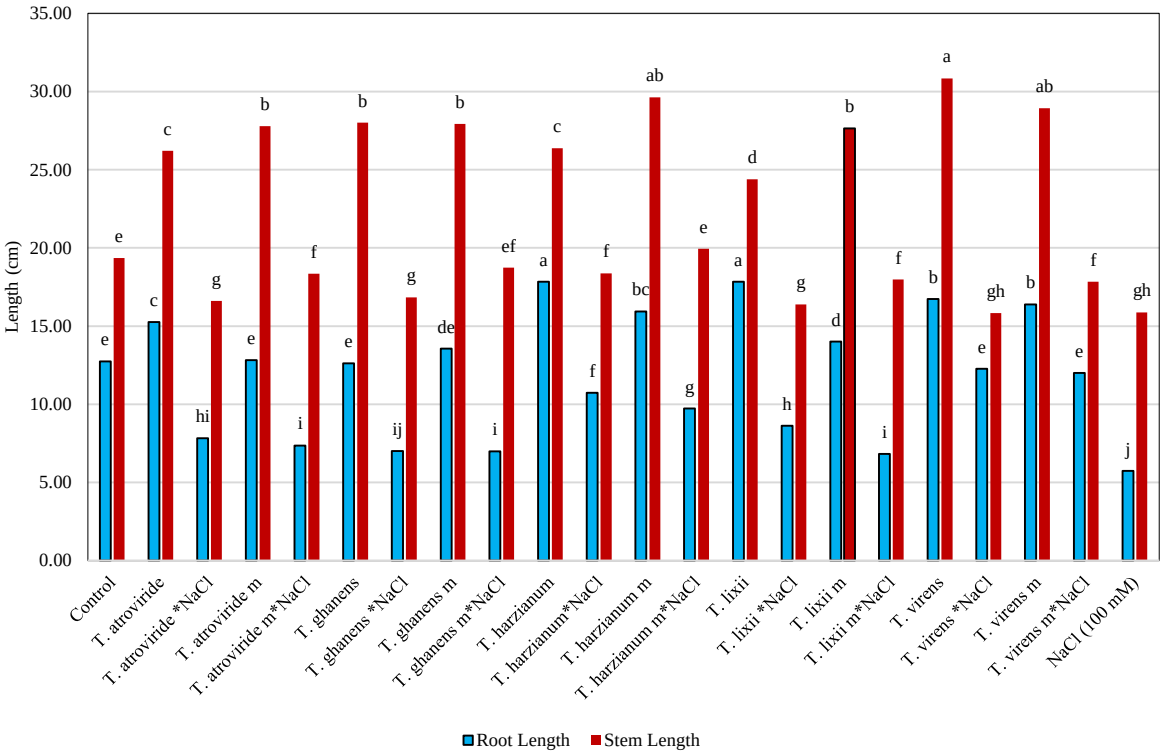
All *Trichoderma* species facilitate the growth and germination of plants in saline soils [30]. Different species of *Trichoderma* fungi affect plant growth and are called growth promoters [31]. Research showed that the inoculation of bean plant seeds with *Trichoderma* fungus could increase the germination rate under salt stress compared to the control [32]. The use of

Trichoderma in bean plants increased the percentage and speed of germination compared to the control [33]. Salinity reduces germination rate by reducing water potential and the effect of absorbed ions on active enzymes and hormones inside the seed [34]. Growth-promoting fungi increase the germination rate by producing indole acetic acid in the growth medium [35]. Ions in irrigation water during germination can stimulate, inhibit or neutralize germination [36]. The lower value of the average germination time means that the seeds have germinated in a shorter period. In the conditions of stress, water absorption by the seed is disturbed or the absorption is slow. In such a case, the metabolic activities of germination are carried out slowly inside the seed, and as a result, the time required for germination increases [37]. It has been reported that the use of biofertilizers and their combination in basil medicinal plants improved germination indicators such as percentage and average duration of germination [38].



Graph. 1 Emergence percentage of bean seeds in salinity stress treatments, biopriming, and interaction effect of salinity and biopriming

Among the treatments, *T. lixii* and *T. harzianum* leads the highest root length of 17.83 cm and 17.84 cm, respectively in seedlings. The stem length in the *T. virens* and *T. harzianum* treatments was 30.83 and 29.62 cm, respectively, which had the longest stem length among the treatments. In S treatment, the length of stem and root was 15.87 cm and 5.73 cm, respectively, and had the highest inhibitory effect on the length of stem and root (Graph. 2). Among the treatments under stress, *T. virens* *S had a root length of 12.26 cm and compared to *T. virens* with a root length of 16.73 cm, it decreased by 26.72%. *T. lixii* mutant *S (6.83 cm) resulted the lowest root length, but this isolate was able to control the stress by 16.11% more than S (Graph. 2). The results of various experiments show that both the length of the root and the stem decreased as a result of salt stress, but the reduction ratio of the length of the stem is more than the length of the root [39]. Many reports indicate the improvement of germination rate, vigor, root length, stem length, and initial establishment in bio-primed seeds with *Trichoderma* spices [40]. The studies of Shafi et al. [41] that investigated the effect of salinity stress (NaCl) on wheat root morphology showed that NaCl reduced root dry weight, root number, total root length, average root diameter, and total root volume.



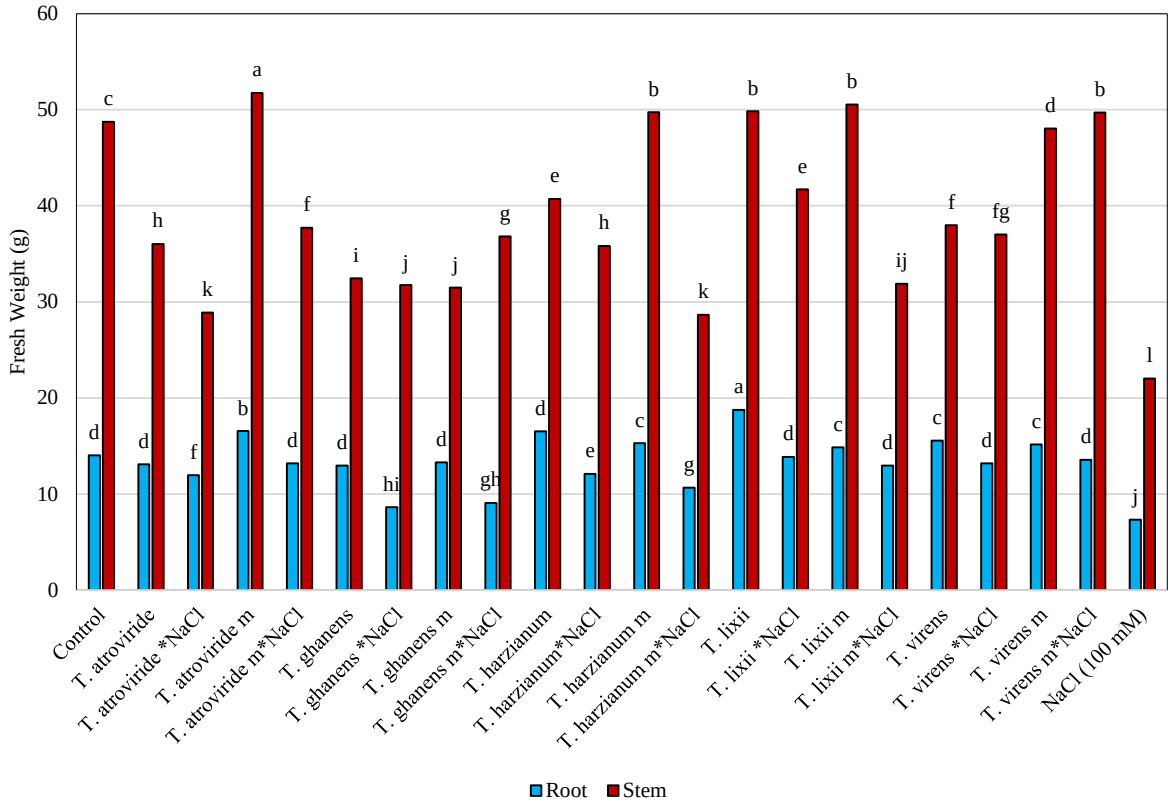
Graph. 2 Length of bean seedling in salinity stress treatments, biopriming, and interaction effect of salinity and biopriming

Fresh Weight

The fresh weight of the root in the seedlings treated with *T. lixii* was 18.74 g, compared to the control (14.03 g), 25.14%, and compared to S (7.34 g), 60.84% increased. *T. virens* mutant *S (13.45 g) had the greatest modulating effect on root fresh weight. This mutant had an increase of 1.86% compared to its wild type (*T. virens*S 13.2 g). S (7.34 g) had the lowest root fresh weight and *T. ghanens* (12.96 g) had the lowest root fresh weight among the wild type. Of course, the effect of gamma radiation in the *T. ghanens* mutant (13.3 g) increased the root fresh weight by 2.94% compared to the wild type of *T. ghanens*. The lowest stress-modulating effect was observed in *T. ghanens* *S (8.63 g) and gamma-ray increased the root fresh weight by 5.09% in *T. ghanens* *S (9.07 g).

The greatest increase in Trichoderma treated seedlings without salinity stress was observed in stem fresh weight of *T. atroviride* mutant (51.73 cm). The lowest stem fresh weight among the treatments was related to S (22.03). Among the treatments under salinity stress, *T. lixii* *S with 41.71 grams of stem fresh weight had the greatest increase and modulating effect of salinity stress and was able to neutralize the effect of salinity by 89.33% compared to S. The lowest stem fresh weight (28.89) was related to *T. atroviride* *S, and the effect of gamma rays in this *T. atroviride* mutant *S species could increase the neutralizing effect of salinity stress by 30.52% (Graph. 3). Sánchez-Montesinos et al. [42] reported that the application of *Trichoderma* fungus leads to an increase in the growth parameters of legumes under salinity stress. Many published studies investigated the effect of some isolates of *Trichoderma* fungus on beans and showed that this fungus improved the growth characteristics of beans in greenhouse conditions [43]. The researchers investigated the response to irrigation water salinity in different stages of root growth and found that the highest root yield and crop yield belonged to the control treatment that received the lowest irrigation water salinity increasing water salinity decreased root yield and crop performance [44]. Also, Zhang et al. [45] studies showed that the effect of sodium chloride salinity stress in a part of the root system on the

yield, quantity, and quality of the fruit was investigated. It showed that by applying salinity to the whole root system, the fruit's fresh and dry weight, the fruit's length, and the fruit's yield decreased significantly.

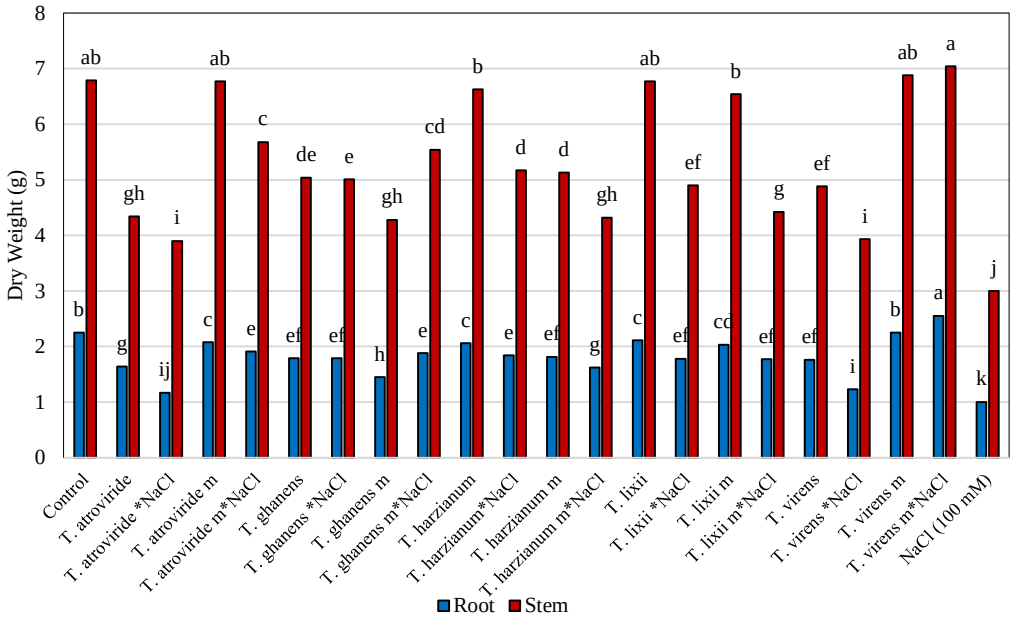


Graph. 3 Fresh Weight of bean seedling in salinity stress treatments, biopriming, and interaction effect of salinity and biopriming

Dry Weight

The highest dry weight of root (2.55 g) of *T. virens* and stem in *T. virens*, *T. virens* mutant, *T. lixii*, *T. atroviride* mutant, and control (respectively: 7.04, 6.88, 6.77, 6.77, 6.79 g) was observed. The lowest dry weight of the root and stem in S was 1.00 and 3.00. Among the non-stressed treatments, the dry weight of the wild *T. virens* species was the highest, and the ratio of the control was 13.33% and 3.68% higher. In the mutants, *T. virens* mutant had the highest dry weight of roots and stems. Among the treatments under the stress of the wild type, *T. harzianum* *S (1.84 g) had the highest salinity modulating effect, which increased root dry weight by 84% compared to S (1.00 g). The dry weight of the stem in *T. harzianum* *S (5.17 g) was higher than other wild species and it was 72.33% higher than S (Graph. 4). *T. atroviride* mutant *S had the highest stem dry weight (5.68 g) among the mutant species. The effect of gamma radiation in this species increased the dry weight of the stem by 45.64% compared to the wild type under the same conditions. The modulating effect of *T. atroviride* mutant *S was 89.33% higher than S (Graph. 4). Rezaloo et al. [20, 22, 26] reported the increasing effect of wet and dry root weight and seedling length in bean, soybean, rapeseed, lettuce, sugar beet, wheat, corn, tomato, and cucumber seeds coated with Trichoderma species (wild type and mutant). The studies of Tabatabaie et al. [46] who studied the effect of uneven distribution of salt on the root and on the ratio of the product to

the amount of water consumed in the hydroponic cultivation of tomato plant showed that in the hydroponic system with mobile branched roots in which the replacement of the root branches between the nutrient solution and Salt water is applied with an interval of 3 days, a significant decrease in the growth of root and aerial organs was observed.



Graph. 4 Dry Weight of bean seedling in salinity stress treatments, biopriming, and interaction effect of salinity and biopriming (abbreviations: m: mutant, s: salt stress, Th: *T. harzianum*, Tl, *T. lixii*, Tg: *T. ghanens*, Tv: *T. virens*, Ta: *T. atroviride*)

Allometric Index

The allometric coefficient of *T. atroviride* *S, *T. atroviride*, and *T. harzianum* mutant *S was higher than in other treatments. *T. ghanens* had the lowest allometric coefficient. (Table 3). In the conditions of not using Trichoderma, the allometric coefficient increased with the increase in salinity, while the use of Trichoderma in saline conditions caused a decrease in the allometric coefficient, which means an increase in the length of the rhizome compared to the stem. This trait may be inducing resistance to salinity or subsequent drought. The use of Trichoderma in inoculation with bean seeds increased root length, stem length, and dry matter and showed characteristics such as organic phosphorus mineralization, auxin production, ACC deaminase activity, ammonia production, and siderophore production [47].

Seedling Tissu Water Percentage

The average water potential of the plant tissue between the treatments varied from 18.92% to 44.55%. The *T. virens* mutant *S had the highest seed tissue water percentage, 135.46% higher than the control at the same level. On the other hand, salinity stress causes a kind of physiological dryness by reducing the osmotic potential of soil water. Such physiological dryness can cause disturbances in photosynthesis and plant life [48]. The decrease in the relative water percentage of leaves is the general response of rice plants exposed to osmotic stress and is a very good indicator of the water status of the plant [49]. It has been stated that the reduction of water stress and during it the reduction of growth is one of the most important effects of high salinity in glycophytes [50]. By using Trichoderma fungi, the relative water content index was improved (table 3). Yeşilyurt et al [51] reported that the

biopriming of the seed with Trichoderma increased the relative water content under salinity stress conditions.

Vigor Index

A comparison of the average between the treatments showed that the highest amount of root index was related to the treatment of seeds with *T. ghanens* mutant without salt stress, which increased by 106.92% compared to the control. S had the lowest vigor index, which decreased by 7.93% compared to the control (table 3). The study of seed treatment with growth-promoting fungi on the germination indices of soybean plants under salt stress by Bakhshandeh et al. [52] showed that seed inoculation with Trichoderma had a positive effect on the germination indices and seed germination. Since Trichoderma can produce siderophore and indole acetic acid, it also has ACC deaminase activity and increases the growth of rice plants [53]. *T. harzianum* was isolated from the rhizosphere of the potato plants and showed the ability to produce a siderophore, dissolve phosphate, and produce indole acetic acid, hydrogen cyanide, ammonia, and exopolysaccharide. This fungus increases the plant's growth activities even in soil contaminated with fungicides [54].

Table 3- Comparison of the average growth components of bean seeds under salinity stress and biopriming treatments and the mutual effect of salinity stress and biopriming.

treatment	Alometric Index	Seedling Tissu Water Percentage	Vigor Index
Control	0.31 cd	42.92 b	441.57 ijk
T. atroviride	0.40 a	27.42 kl	881.82 b
T. atroviride *NaCl	0.41 a	24.63 mn	858.99 bc
T. atroviride m	0.37 b	42.82 b	598.62 g
T. atroviride m*NaCl	0.31 cd	35.90 de	641.46 f
T. ghanens	0.24 g	31.86 i	888.96 b
T. ghanens *NaCl	0.37 b	31.68 i	470.55 ij
T. ghanens m	0.33 c	27.06 l	913.07 a
T. ghanens m*NaCl	0.37 b	35.06 f	738.33 d
T. harzianum	0.33 c	41.96 c	867.40 bc
*T. harzianum**NaCl	0.34 c	32.69 g	879.97 b
T. harzianum m	0.37 b	32.45 gh	694.14 e
T. harzianum m*NaCl	0.41 a	27.30 kl	429.38 jk
T. lixii	0.26 f	42.85 b	494.82 hi
T. lixii *NaCl	0.33 c	30.99 j	576.79 gh
T. lixii m	0.34 c	41.38 cd	538.66 ghi
T. lixii m*NaCl	0.40 a	27.913 k	739.12 d
T. virens	0.37 b	30.83 j	276.09 m
T. virens *NaCl	0.29 e	24.84 m	837.93 c
T. virens m	0.29 e	43.54 ab	508.17 hi
T. virens m*NaCl	0.29 e	44.55 a	496.17 hi
NaCl (100 mM)	0.33 c	18.92 o	409.41 kl

In each column, the averages that have at least one letter in common have no significant difference based on Duncan's test at the 5% probability level.

Fig. 1 Comparison of the mutual effect of Th*S (*Trichoderma harzianum)*, Th m*S *(Trichoderma harzianum* mutant), and S (salinity stress) on bean plant

Fig. 2 Comparison of bean plants treated with *Trichoderma*. The control bean plant is shorter and weighs less compared to Tg (*Trichoderma ghanens)*. The bean plant treated with Tg m (*Trichoderma ghanens* Mutant) has a higher fresh weight and more freshness.

Conclusions

The *Trichoderma* fungus has been effective in improving the growth of bean plants [22]. In addition, it should not be overlooked that plant seed coating with biological agents such as *Trichoderma*, which controls a wide range of fungal and bacterial agents [26], can be proposed as a biotechnological approach to improve growth and resistance to biotic and abiotic stresses. It seems that using the *Trichoderma* fungus has a high ability to improve the strength of the plant to deal with salt stress. As seen in this study, it can be shown that the induction of gamma-ray mutation and the selection of suitable mutants can reduce the initial reactions of some plants to the presence of *Trichoderma* in the ecological root niche and increase the positive effects of this fungus; so, it is recommended as a promising technique. Although the physiological changes of the plant depend on the method of inoculation and different isolates of *Trichoderma* used, it should not be forgotten that before recommending this biological agent, the interactions of different species of this fungus should be investigated independently and then by each plant (and even any varietal variety). It is suggested to investigate the physiological mechanisms of creating this tolerance to salinity in the plant treated with *Trichoderma*, the study of mutated genes in *Trichoderma* mutants that have higher tolerance to salinity, and the interaction studies of the plant under salt stress and treated with this biological agent in the future studies will be done.

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