J. Microbiol. Biotechnol. (2010), **20**(9), 1288–1294
doi: 10.4014/jmb.1002.02010
First published online 29 July 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Substrate-Dependent Auxin Production by *Rhizobium phaseoli* Improves the Growth and Yield of *Vigna radiata* L. Under Salt Stress Conditions

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Received: February 7, 2010 / Accepted: April 13, 2010

Rhizobium phaseoli **strains were isolated from the mung bean (*Vigna radiata* L.) nodules, and the most salt tolerant and high auxin producing rhizobial isolate N20 was evaluated in the presence and absence of L-tryptophan (L-TRP) for improving the growth and yield of mung bean under saline conditions in a pot experiment. Mung bean seeds were inoculated with peat-based inoculum and NP fertilizers were applied at 30–60 kg/ha, respectively. Results revealed that imposition of salinity reduced the growth and yield of mung bean. On the contrary, the separate application of L-TRP and *Rhizobium* appeared to mitigate the adverse effects of salt stress. However, their combined application produced more pronounced effects and increased the plant height (28.2%), number of nodules per plant (71.4%), plant biomass (61.2%), grain yield (65.3%), and grain nitrogen concentration (22.4%) compared with untreated control. The growth promotion effect might be due to higher auxin production in the rhizosphere and improved mineral uptake that reduced the adverse effects of salinity. The results imply that supplementing *Rhizobium* inoculation with L-TRP could be a useful approach for improving the growth and yield of mung bean under salt stress conditions.**

Keywords: L-Tryptophan, *Rhizobium phaseoli*, inoculation, salt stress, mung bean

The low economic yield of agricultural crops can be attributed to the crop's susceptibility to a number of biotic and abiotic stresses. Salt stress is of alarming concern among these. Every year, more and more land becomes nonproductive owing to salt accumulation [4], and it has been estimated that more than 50% of arable land will be salt affected by the year 2050 [30]. In the arid and semiarid regions of the world, soil salinity is more severe as a result of low rainfall and high temperature, and has become an important limiting factor for agricultural crops [21].

The *Rhizobium*–legume symbiosis is more sensitive to salinity than free-living bacteria [32]. The legume response to salt stress includes an array of changes at the molecular, biochemical, and physiological levels [17], which mainly depend upon environmental conditions, soil properties, and growth stage [33]. Because of these changes, plants may not have the capacity to synthesize sufficient endogenous plant hormones for optimal growth and development. It is also well accepted at present that the protective response of plants to biotic and abiotic stresses is primarily regulated by phytohormones [22]. However, plants respond to exogenous application of phytohormones, and these exogenously applied phytohormones can mitigate the adverse effects of salinity [27]. It has been evaluated that salinity has adverse effects on wheat growth and yield, but seed treatment with indole-3-acetic acid (IAA) alleviated salt stress effects as apparent from seedling dry mass [9]. Recently, it has been also investigated that IAA is able to enhance protection of *Escherichia coli* cells against different abiotic stresses such as high salt concentration [6]. Thus, exogenous application of phytohormones and their precursors provides an attractive approach to counter the salt stress conditions by changing the balance of endogenous levels of hormones, allowing a modification of growth and development in the desired direction and to the desired extent [20].

Unlike legumes, rhizobia can tolerate high salt concentration and show great variation in their tolerance to salt [10]. The growth of some strains is retarded by 100 mM NaCl, whereas some can tolerate more than 300 mM. This rhizobial salt tolerance involves many physiological and biochemical changes and the activity of different enzymes [10]. Hence, the use of a rhizobial isolate that is highly tolerant to salt stress can increase the growth and yield of legumes under salt stress conditions. Rhizobium is also well reputed to improve the growth and yield of legumes by producing various hormones, besides biological nitrogen fixation [11]. The growth of sorghum increased as a result of production

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of IAA by a recombinant strain of *Azospirillum* [16]. Rhizobial production of these phytohormones can be increased several folds by providing their suitable precursors [1]. L-Tryptophan is considered an efficient physiological precursor of auxins in higher plants as well as for microbial biosynthesis of auxins [2]. Datta and Basu [8] reported that *Rhizobium* isolated from the root nodules of *Cajanus cajan* was found to produce high amounts (99.7 μg/ml) of IAA during growth in basal medium supplemented with L-tryptophan.

Although reports are available that confirm the potential of this approach to improve crop performance under normal conditions [14, 31], to the best of our knowledge, no investigation has been made to verify their potential to improve the growth and yield of legumes under salt stress conditions. Thus, the present study was conducted by assuming that a salt-tolerant *Rhizobium* isolate (also capable of producing IAA) supplemented with L-TRP (precursor of IAA) will improve the growth and yield of legumes under salinity stress by optimizing the plant endogenous level of auxins.

Materials and Methods

Isolation of *Rhizobium*

Root samples of mung bean (*Vigna radiata* L.) were collected from different salt affected locations and were washed gently with tap water to remove the soil. Nodules were separated, placed in Petri-plates, and surface-sterilized by momentarily dipping in 95% ethanol solution, followed by dipping in 0.2% $HgCl_2$ solution for 4–6 min and 5 times washings with sterilized water [24]. A milky suspension was obtained by crushing the surface-sterilized nodules with the help of a sterilized glass rod in a minimal volume of sterilized water. A loopful of the suspension was streaked out on yeast extract mannitol (YEM) agar medium (Yeast, 0.5 g; mannitol, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; distilled water, 1,000 ml; pH, 6.8) plates and incubated at 28±1°C. Single colonies were restreaked on clean plates to obtain pure cultures. In this way, 25 fast growing colonies of bacteria were selected, isolated, and purified from the mung bean nodules. The purified rhizobial isolates (*Rhizobium phaseoli*) were stored at 4±1°C on slants and maintained for further experimentation.

Auxin Determination

Sterilized modified mannitol broth (25 ml) placed in glass tubes was inoculated with rhizobial isolates in the presence and absence of filter (pore size, 0.2 μm)-sterilized L-TRP. The tubes were incubated at 28±1°C for 48 h at 100 rpm in an orbital shaking incubator. The contents of the tubes were filtered through Whatman filter paper No. 2 before measuring auxin production (IAA equivalents) colorimetrically by using the method described by Sarwar *et al*. [26]. The rhizobial isolates were also tested for their ability to survive against salt stress (osmoadaptation).

Osmoadaptation Assay

The salt tolerance of 10 rhizobial isolates was studied at three different salinity levels; that is, original and 5 and 10 dS/m. Petri-plates were prepared with the same nutrients as used in isolation but with different salt concentrations to maintain the EC levels at original, 5 and 10 dS/m. The nutrient agar, with different salt concentrations, was autoclaved before being plated and incubated. A bacterial suspension [10^3 colony forming unit (CFU)/ml] of rhizobial isolates was prepared and 1 ml of that suspension was inoculated on Petri-plates at different salinity levels. The same procedure of salinity development and inoculation was repeated in broth tubes and the bacterial population was adjusted to 10^3 CFU/ml. Both the Petri-plates and tubes were incubated at room temperature. The Petri-plates were simply observed for heavy, medium, and light growth after 2 days of incubation, whereas the broth tubes were checked for absorbance using a spectrophotometer at a wavelength of 540 nm after 6 days of incubation. The population count in the broth tubes was determined using the methods of Vincint [29].

Seed Inoculation

The selected *Rhizobium phaseoli* strain (N20) was used for inoculation of a 250-ml conical flask containing 100 ml of YEM medium and incubated at 28±1°C in a shaking incubator at 100 rpm for 3 days. Mung bean seeds were inoculated by the slurry method. Slurry was prepared by mixing 10 ml of 15% sterilized sugar solution, 25 ml of liquid culture (10^8 CFU/ml), and 50 g of sterilized peat plus clay. Control was treated with sterilized peat plus clay-containing sterilized broth and sugar solution. L-Tryptophan (10^{-5} M) was applied to the broth at the time of inoculation.

Pot Experiment

A pot trial was conducted in the wire house, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan, to study the effect of *Rhizobium* inoculation with and without L-tryptophan application on the growth and yield of mung bean under salt stress conditions. In this experiment, sieved, air dried, well-mixed, and chemically analyzed soil (pH, 7.8; ECe, 2 dS/m; CEC, 7.15 C mol(+)/kg, organic matter, 0.80%; total nitrogen, 0.05%; available phosphorus, 8.2 mg/kg; and extractable potassium, 164 mg/kg) were used. Recommended doses of NP fertilizers (30–60 kg/ha) were applied as urea and single super phosphate (SSP), respectively, to all pots. The whole dose of P and half of N were applied at the time of sowing as a basal dose, whereas the second half dose of N was applied after germination with first irrigation. Three salinity levels (*i.e*., original, 4 and 6 dS/m) were developed by adding a calculated amount of NaCl salt in each pot and mixing it with a mechanical mixer. The actual salinity developed in pots was 2, 3.83, and 5.89 dS/m. Similarly, three pots (original, 4 and 6 dS/m) were maintained as reference pots to check the effect of irrigation water on salinity levels, and the ECe was monitored regularly in the reference pots and from the experimental pots at the end. There was no significant change in the salinity levels at the end. Four seeds were sown in each pot containing 12 kg soil, which was thinned to one plant, 15 days after germination. Treatments were repeated six times by using completely randomized design (CRD). Canal water was used for irrigation when needed. Out of six replications, three were harvested at the flowering stage to collect the data regarding nodule number and the remaining three were harvested at maturity, and data regarding plant height, root length, number of pods/plant, pods dry weight, plant biomass, grain yield, and 100-grain weight were recorded, and grain samples were analyzed for N concentration [25]. The data collected were subjected to the analysis of variance

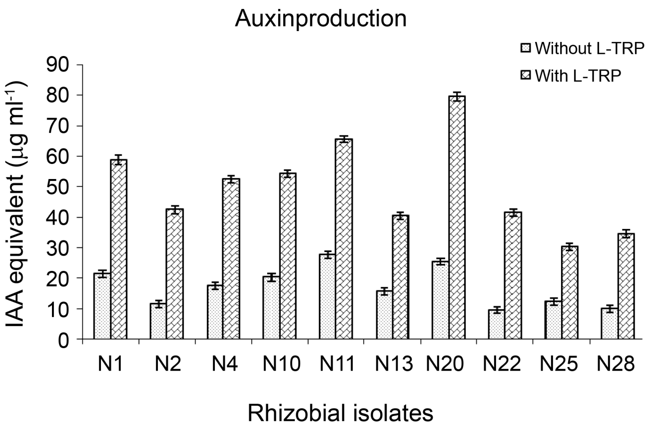

Fig. 1. Auxins biosynthesis by rhizobia in the absence and presence of L-tryptophan.

(ANOVA) technique, and the least significant difference (LSD) test was applied at 5% probability to compare treatment means [28].

RESULTS

Laboratory Study

It is evident from the data (Fig. 1) that although rhizobial isolates produced sufficient quantities of auxins (IAA equivalents) without L-TRP addition, when the medium was supplemented with L-TRP, IAA equivalents increased many folds. The rhizobial isolate N20 produced maximum IAA equivalents (81.25±2.34 µg/ml) when supplemented with L-TRP. The minimum IAA equivalents were recorded with the isolate N25.

Data (Table 1) regarding the type of growth on nutrient agar plates under salt stress conditions indicated that salinity had adverse effects on the growth of the rhizobial isolates. The rhizobial growth, in general, was active at low salt concentration, whereas growth at high salt concentration was poor. Table 2 shows the effects of salinity on the growth and population count of rhizobial isolates in broth tubes and reveals that optical density (shown as mean absorbance) decreased by increasing salt concentration. Moreover, the population count was also affected adversely by high salt concentration. Rhizobial isolate N20 (used further in pot experiment) showed maximum visual growth, optical density (3.10), and population count (30.19×10^3 CFU/ml) at high salt concentration, followed by N11, whereas rhizobial isolate N25 was the most sensitive to salt stress. This growth pattern indicated that rhizobia preferred low salt concentration for their optimal growth.

Pot Study

Data in Table 3 show that salinity affected plant height adversely, but the separate/combined application of L-TRP and *Rhizobium* increased the plant height significantly at all salinity levels, compared with respective uninoculated control. At original and medium salinities (2 and 4 dS/m), combined application (L-TRP and *Rhizobium*) showed significant increase of plant height by 13% and 18%, respectively, over uninoculated control, and differed nonsignificantly from *Rhizobium* inoculation alone. At high salinity (6 dS/m), L-TRP application enhanced the effectiveness of *Rhizobium* and gave the maximum increase (28% higher than respective uninoculated control) in plant height compared with their separate application and uninoculted control.

Data regarding root length of mung bean (Table 3) indicated that the integrated application of L-TRP and *Rhizobium* has more pronounced effects than their separate application, and showed maximum increase in root length that was up to 71% more than untreated control at all salinity levels. *Rhizobium* inoculation was the next effective treatment that was at par with L-TRP application at 6 dS/m.

Separate application of L-TRP and *Rhizobium* showed significant increase in number of nodules/plant compared with respective uninoculated control at all salinity levels (Table 3), except at 6 dS/m where L-TRP application produced similar effects to control. However, their combined

Table 1. Type of growth of different *Rhizobium* isolates on agar plates under salt stress conditions.

Rhizobium isolate	Type of growth on Petri-plates		
	Control	5 dS/m	10 dS/m
N_1	Very heavy growth	Heavy growth	Medium growth
N_2	Heavy growth	Medium growth	Light growth
N_4	Light growth	Light growth	Very light growth
N_{10}	Medium growth	Light growth	Very light growth
N_{11}	Very heavy growth	Very heavy growth	Heavy growth
N_{13}	Light growth	Very light growth	Very light growth
N_{20}	Very heavy growth	Very heavy growth	Very heavy growth
N_{22}	Light growth	Light growth	Very light growth
N_{25}	Very light growth	Very light growth	Very light growth
N_{28}	Light growth	Very light growth	Very light growth

Table 2. Growth of different *Rhizobium* isolates in broth tubes after 6 days under salt stress conditions.

Rhizobium isolates	Absorbance at 540 nm			Population count (CFU/ml×10^3)		
	Control	5 dS/m	10 dS/m	Control	5 dS/m	10 dS/m
N_1	3.96b*	3.00c	2.69c	29.96b	26.88c	23.69c
N_2	3.54c	2.63d	2.38d	26.85c	23.19d	19.53d
N_4	2.68f	1.72g	1.55f	22.64cd	19.34e	16.48e
N_{10}	2.96e	1.90f	1.69e	17.05e	14.86h	12.50g
N_{11}	4.03b	3.17b	2.83b	32.83b	29.89b	26.19b
N_{13}	3.18d	2.19e	1.39hi	21.35d	18.22f	15.59ef
N_{20}	4.15a	3.29a	3.10a	36.11a	33.06a	30.19a
N_{22}	2.69f	1.70g	1.49g	20.08d	17.09g	15.19f
N_{25}	2.48g	1.60h	1.36i	16.98e	13.80i	12.30g
N_{28}	2.61f	1.66gh	1.41h	21.25d	16.23g	14.99f

*Means sharing similar letter(s) in a column do not differ significantly at $p>0.05\%$ according to the LSD test.

application (L-TRP and *Rhizobium*) showed more pronounced effects than their separate application, and showed maximum increase in number of nodules/plant that ranged from 47% to 72% higher than respective uninoculated control.

Data regarding number of pods/plant revealed that integrated application of L-TRP and *Rhizobium* performed significantly better than their separate application at all salinity levels, except at original salinity (2 dS/m) where combined application was statistically similar to *Rhizobium* inoculation alone (Table 3). However, L-TRP application with *Rhizobium* gave maximum increase (up to 67% over uninoculated control) in number of pods/plant at all salinity levels, followed by *Rhizobium* inoculation alone.

Rhizobium inoculation of mung bean supplemented with L-TRP increased pods dry weight at all salinity levels that ranged from 68% to 72%, compared with uninoculated control (Table 4). *Rhizobium* inoculation was the next effective treatment that significantly increased pods dry weight up to 49%, compared with uninoculated control and was at par with L-TRP application at 6 dS/m.

Data regarding plant dry biomass (Table 4) revealed that at original salinity, integrated application of L-TRP and *Rhizobium* showed significant increase of 52% in plant biomass compared with uninoculated control. Similarly, combined application (L-TRP and *Rhizobium*) also showed maximum increase in plant biomass at medium and high salinities (4 and 6 dS/m) that was up to 62% higher than uninoculated control, respectively.

Inoculation of mung bean with *Rhizobium phaseoli* supplemented with L-TRP increased the grain yield up to 66% higher compared with uninoculated control (Fig. 2). At original salinity, combined application (L-TRP and *Rhizobium*) significantly increased the grain yield by 60% compared with uninoculated control. Likewise, integrated application of L-TRP and *Rhizobium* was also an effective treatment at medium and high salinity levels compared

Table 3. Effects of inoculation with *Rhizobium* supplemented with L-TRP on plant height, root length, number of nodules/plant, and number of pods/plant of mung bean at different salinity levels. (Average of 3 repeats)

Treatments	Plant height (cm)			Root length (cm)		
	Electrical conductivity (dS/m)			Electrical conductivity (dS/m)		
	2	4	6	2	4	6
Control	25.47b	21.92d	18.20f	17.23e	14.30g	9.99i
Rhizobium	28.38a	25.63b	20.75e	20.11cd	20.47c	11.85h
Tryptophan	28.19a	23.78c	20.65e	16.68f	19.83d	11.52h
Combination	28.70a	25.88b	23.32c	25.14a	24.05b	17.04ef
	Number of nodules/plant			Number of pods/plant		
Control	15de	11g	7i	13c	8f	6g
Rhizobium	21a	16cd	9h	17a	10e	8f
Tryptophan	17bc	14ef	7i	15b	8f	7f
Combination	22a	18b	12fg	17a	11d	10de

*Values sharing similar letter(s) do not differ significantly at $P<0.05$, least significant difference.

Table 4. Effects of inoculation with *Rhizobium* supplemented with L-TRP on pods dry weight, plant biomass, 100-grain weight, and grain nitrogen of mung bean at different salinity levels. (Average of 3 repeats)

Treatment	Pods dry weight (g)			Plant biomass (g)		
	Electrical conductivity (dS/m)			Electrical conductivity (dS/m)		
	2	4	6	2	4	6
Control	3.38e	1.93h	1.20j	22.83d	16.54f	13.11g
Rhizobium	5.02b	2.87f	1.74i	31.29b	22.58d	16.23f
Tryptophan	4.30c	2.33g	1.65i	26.90c	19.28e	15.98f
Combination	5.65a	3.29d	2.06h	34.65a	26.72c	21.13e
	100-grain weight (g)			Grain nitrogen (%)		
Control	3.99d	3.06f	2.03i	2.92e	2.84f	2.50h
Rhizobium	5.15b	4.71c	2.80g	3.12bc	3.04d	2.85ef
Tryptophan	4.83c	4.09d	2.37h	3.01d	2.90ef	2.57g
Combination	6.20a	5.25b	3.61e	3.24a	3.18ab	3.06cd

*Values sharing similar letter(s) do not differ significantly at $P<0.05$, least significant difference.

with their separate application, which increased grain yield by 62% and 66% over corresponding uninoculated control, respectively.

Combined application (L-TRP and *Rhizobium*) performed significantly better and increased 100-grain weight more than their separate application, ranging from 55% to 78% compared with uninoculated control at all salinity levels (Table 4). Maximum percent increase in 100-grain weight (78% higher than uninoculated control) was observed at 6 dS/m. *Rhizobium* inoculation alone was the next effective treatment that significantly increased the100-grain weight up to 38% compared with uninoculated control.

Data regarding grain nitrogen concentration of mung bean under salinity stress (Table 4) depicted that separate application of L-TRP and *Rhizobium* significantly increased grain nitrogen concentration at all salinity levels except at 4 dS/m. However, *Rhizobium* inoculation along with L-TRP showed maximum increase (up to 23% compared with uninoculated control) in grain nitrogen concentration at all salinity levels. *Rhizobium* inoculation alone showed an increase up to 14% compared with respective uninoculated control and differed significantly from L-TRP application alone at all salinity levels.

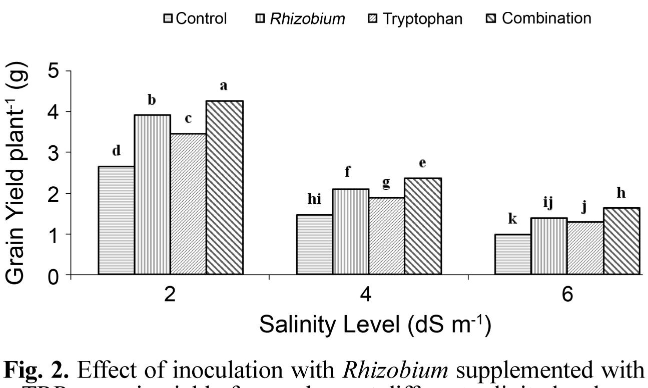

Fig. 2. Effect of inoculation with *Rhizobium* supplemented with L-TRP on grain yield of mung bean at different salinity levels.

DISCUSSION

Salinity can limit plant growth by causing both hyperionic and hyperosmotic stress effects, depressing symbiotic performance, and this can reduce plant yields up to 20% [3]. *Rhizobium* inoculation improves the growth and yield of legumes through multifarious mechanisms in addition to biological nitrogen fixation, and the production of plant growth regulators is considered the most plausible mechanism in controlling plant growth and development [19]. L-Tryptophan is considered an efficient physiological precursor of auxins [2] and its application mitigates the adverse effects of salinity [27]. The present study was conducted to evaluate the effectiveness of precursor (L-tryptophan)–inoculum (*Rhizobium*) interaction for inducing salt tolerance in mung bean, which ultimately improved the growth and yield under salt stress conditions.

During laboratory study, it was observed that all rhizobial isolates were capable of producing auxins (28.32±1.98 μg/ml) and this auxin production increased (81.25±2.34 μg/ml) many folds by supplementing the culture medium with L-TRP. Auxin production by rhizobial isolates has also been evaluated by many research workers [7, 8, 14, 19]. Results from the osmoadaptation assay revealed that high salt concentration has adverse effects on the growth of *Rhizobium*. Tables 1 and 2 indicated that rhizobia preferred low salt concentration for their optimal growth, and it also provided information regarding the isolation of salt-tolerant and salt-sensitive *Rhizobium* isolates, as these isolates had significant differences in the mean values of absorbance and colony count. Previous studies reported by Lloret *et al.* [15] have shown the isolation of some salt-tolerant *Rhizobium* strains that can grow at NaCl concentration up to 0.5 mM. This argument of capability of certain *Rhizobium* strains to tolerate high salt concentration is also supported by Mensah *et al*. [18].

Pot experiment revealed that application of L-TRP and *R. phaseoli*, whether applied separately or in combination, had positive effects on the growth and yield of mung bean. However, their combined application had more prominent effects than their separate application under salt stress conditions. The combined application, in general, was more effective at higher salinity. *Rhizobium* inoculation has proven its worth for improving the growth- and yield-contributing parameters of legumes under salt stress conditions [5]. In our study, *Rhizobium* inoculation significantly increased the growth and yield of mung bean at all salinity levels, compared with respective uninoculated control. This improvement may very likely be attributed to *Rhizobium*'s capability to affect plant growth and development by multifarious mechanisms such as N_2 fixation, production of plant growth regulators (PGRs), and suppression of plant diseases. These findings are also supported by Hafeez *et al.* [12], who studied the effect of salinity and inoculation on growth, nitrogen fixation, and nutrient uptake of *Vigna radiata*, and reported that most of the *Rhizobium* strains were salt tolerant and performed better for plant growth and development under salt stress conditions. These results are also in line with the results of Rao and Sharma [23].

Tryptophan-dependent IAA biosynthesis in soil dramatically affects plant growth [26]. In the present study, results regarding L-TRP application revealed that growth- and yield-contributing parameters were improved with the L-TRP application at all salinity levels. The possible mechanism of action behind this improved growth and yield may be either direct uptake of L-TRP by plant roots or its microbial conversion into auxin metabolites, which may make the plant much healthier to cope with salinity. Our results are in line with the results of Datta *et al*. [9], who conducted an experiment to study the effect of exogenous application of phytohormones on the growth and yield of wheat under salt stress conditions. From their experiment, they suggested that salinity had adverse effects on wheat growth and yield, but pre-sowing seed soaking treatments with indole-3-acetic acid alleviated salt stress effects, as apparent from the seedling dry mass. Similar findings were also reported by Bianco *et al*. [6].

Although separate application of *Rhizobium* and L-TRP showed significant increase in the growth and yield of mung bean, their combined application was found to be more effective than their separate application and showed maximum percent increase for all growth and yield parameters under salinity stress. This was also supported by the work of Hussain *et al*. [14], who conducted a pot experiment on lentil in normal soil conditions and reported that combined application of *Rhizobium* and L-TRP significantly increased grain yield by 30.6% compared with uninoculated control. Our results are also in accordance with the results of Zahir *et al*. [31], who reported that combined application of precursor and inoculum was more effective than their separate application. This improvement in growth and yield parameters under salt stress might be due to either phytohormones production in the rhizosphere, which might optimize the endogenous suboptimal plant hormone level, or improve mineral uptake by plant roots that might reduce the problem of ionic imbalance in the plant body, created by salt stress.

Overall, this study implies that precursor–inoculum interaction is an attractive approach to improving the growth and yield of mung bean under salt stress conditions. However, further field investigations are needed to confirm its potential under various environmental stresses.

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