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L-TRYPTOPHAN APPLICATION ENHANCES THE EFFECTIVENESS OF *RHIZOBIUM* INOCULATION FOR IMPROVING GROWTH AND YIELD OF MUNGBEAN (*VIGNA RADIATA* (L.) WILCZEK)

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Abstract

Rhizobium inoculation has successfully been used for improving growth and yield of legume crops in different parts of the world. L-Tryptophan (L-TRP) application may further improve its effectiveness due to substrate-dependent inoculum-derived auxins in the rhizosphere. *Rhizobium phaseoli* strains were isolated using dilution plate technique from the mung bean nodules. Auxin biosynthesis by these rhizobial isolates was determined in the absence and presence of L-TRP, a physiological precursor of auxins. Rhizobial isolates varied widely in auxins biosynthesis capabilities and N42 being the highest auxin producer strain was further evaluated in the presence of different levels of L-TRP (10^{-3}, 10^{-4} and 10^{-5} M) for improving the growth and yield of mung bean. Mung bean seeds were inoculated with peat-based inoculum and sown following randomized complete block design with four replications. Fertilizers, NP were applied at 30-60 kg ha^{-1} as urea and single super phosphate (SSP) in all plots. Results revealed that L-TRP (10^{-4} and 10^{-5} M) and rhizobial inoculation when applied alone significantly increased the growth and yield of mung bean compared to untreated control. However, *Rhizobium* inoculation supplemented with L-TRP (10^{-4} M) gave the most promising results and significantly increased the plant height, number of nodules $plant^{-1}$, nodular mass $plant^{-1}$, number of grains pod^{-1}, number of pods $plant^{-1}$, total plant biomass, grain yield and 1000-grain weight up to 28, 80, 77, 46, 54, 58, 57 and 17%, respectively compared to uninoculated control. Similarly, N concentration in grains also increased significantly at this L-TRP level by the rhizobial inoculation. The results imply that supplementing rhizobial inoculation with L-TRP could be a useful approach for improving growth, nodulation and yield of mung bean.

Introduction

Plant growth regulators (PGRs) play a vital role in controlling plant growth and development. Among the five major classes of PGRs, auxins are one of the important PGRs. Despite the fact that plants are capable to synthesize auxins, yet they respond to exogenously applied auxins during certain growth phases (Frankenberger & Arshad, 1995; Khalid *et al*., 2006).

Soil microorganisms, particularly rhizosphere microflora, are a major natural source of PGRs. Very small quantities of PGRs released by microorganisms can have pronounced effects on plant growth and development. The production of PGRs in pure culture and in soil has been demonstrated by various researchers (Frankenberger & Arshad, 1995; Arshad & Frankenberger, 1998, 2002; Khalid *et al*., 2004; Zahir *et al*., 2004). L-Tryptophan is considered an efficient physiological precursor of auxins in higher plants as well as for microbial biosynthesis of auxins (Davies, 1995; Frankenberger & Arshad, 1995; Khalid *et al*., 2001, 2006). *In vitro* studies have demonstrated that some microorganisms can produce small amounts of auxins in the absence of L-TRP, however, in its presence, the microbiota produce much greater quantities of auxins (Asghar *et al*., 2002; Zahir *et al*., 2004). Exogenous application of L-TRP to soils has also been shown to stimulate synthesis of auxins, influencing plant growth and development positively (Sarwar & Frankenberger, 1994; Frankenberger & Arshad, 1995; Khalid *et al*., 2004).

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Rhizobia are the class of bacteria which frequently nodulate the roots of leguminous plants and fix atmospheric nitrogen (N_2) in the nodules of legumes. In addition to biological nitrogen fixation, they also produce plant growth regulators (auxins, gibberellins, cytokinins, abscisic acid and ethylene) (Frankenberger & Arshad, 1995). Rhizobial inoculants are now widely used in various parts of the world being inexpensive, environment-friendly, easy to use and have no side effects in most of the cases (Burdman *et al*., 1998). They play an important role in improving crop yields (Boonkerd *et al*., 1993).

Auxins biosynthesis by *Rhizobium* spp., in addition to BNF has now been unequivocally demonstrated as a possible mechanism of action (Bartel, 1997; Biswas *et al*., 2000a, b; Zahran, 2001; Zahir *et al*., 2004). *Rhizobium* spp. can synthesize auxins in the absence of TRP but exogenous application of TRP can increase auxin production by several folds (Wheeler *et al*., 1984; Kittell *et al*., 1989; Bartel, 1997; Zahir *et al*., 1997). The effectiveness of TRP-dependent auxin biosynthesis has been tested for improving yields of non-legumes (Bartel, 1997; Zahir *et al*., 2000, 2005; Khalid *et al*., 2006). However, very few studies have been conducted in legumes. Therefore, present study was conducted to evaluate the potential of L-TRP dependent biosynthesis of auxins by *Rhizobium* for increasing growth and yield of mung bean (*Vigna radiata* L.) under field conditions.

Materials and Methods

Isolation of *Rhizobium* and measurement of auxins biosynthesis: Mung bean (*Vigna radiata* L.) root samples were collected from different locations of Faisalabad. Roots were washed gently with tap water to remove the soil and nodules were separated and placed in Petri-plates. The collected nodules were surface-sterilized by momentarily dipping in 95% ethanol solution followed by dipping in 0.2% $HgCl_2$ solution for 3-5 minutes and 6-7 times washings with sterilized water (Russell *et al*., 1982). The surface sterilized nodules were crushed in a minimal volume of sterilized water with the help of a sterilized glass rod to obtain a milky suspension. 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; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.1 g; distilled water, 1000 mL; pH, 6.8] plates and incubated at 28 ± 1 ^{0}C. Well isolated single colonies were picked and restreaked on fresh plates to obtain pure cultures. In this way, 24 fast growing colonies of bacteria were selected, isolated and purified from the mung bean nodules. The purified rhizobial cultures (*Rhizobium phaseoli*) were stored at 4 ± 1 ^{0}C on slants and maintained for further experimentation.

Auxin determination: Sterilized yeast mannitol broth (YMB) was inoculated with rhizobial isolates in the presence and absence of filter (0.2 µm)-sterilized L-TRP in glass tubes and incubated at 28 ± 1^{o}C for 48 hours in shaking incubator at 100 rpm. The contents were filtered through Whatman filter paper No. 2 before measuring auxin production in terms of Indole-3-acetic acid (IAA) equivalents. Auxins biosynthesis was determined colourimetrically by using the method described by Sarwar *et al*., (1992). Rhizobial isolate (N42) giving highest auxin production (Data not given) was selected for field trial.

Preparation of inoculum: Inoculum was prepared in YMB. The *Rhizobium phaseoli* strain (N42) was inoculated in 300 mL conical flask containing 60 mL medium and incubated at 28 ± 1^{o}C under shaking at 100 rpm for three days to give an optical density of 0.5 (recorded at 535 nm wavelengths).

Seed inoculation: For inoculation, mung bean seeds were coated with slurry. Slurry was prepared by mixing 30 mL of 15% sugar solution, 60 mL liquid culture and 200 g of sterilized peat plus clay. Control was treated with sterilized peat plus clay containing sterilized broth and sugar solution. L-Tryptophan solution was applied to the broth at the time of inoculation at different concentrations (10^{-3}, 10^{-4} and 10^{-5} M). Inoculated seeds were placed over night for drying before sowing in plots (5 m x 2 m).

Field experiment: A field experiment was conducted in Research Area, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad to observe the efficacy of rhizobial inoculation along with different levels of L-tryptophan (10^{-3}, 10^{-4} and 10^{-5} M) for improving growth and yield of mung bean (*Vigna radiata* L.) under field conditions. The surface soil was collected from the research area, air dried, thoroughly mixed, passed through 2 mm sieve and analyzed for various physical and chemical characteristics. The soil was sandy clay loam having pH, 7.9; EC, 2.65, dS m^{-1}; CEC, 7.10 C mol(+) kg^{-1}, organic matter, 0.74%; total nitrogen, 0.050%; available phosphorus, 9.1 mg kg^{-1} and extractable potassium, 172 mg kg^{-1}.

Recommended doses of fertilizers NP @ 30-60 kg ha^{-1} were applied as urea and single super phosphate (SSP), respectively in all plots. The whole dose of P fertilizer and half of N was applied at the time of sowing as a basal dose while the second half dose of N was applied after germination with first irrigation. Inoculated/uninoculated seeds were sown by drill. Canal water was used for irrigation. Following treatments were replicated four times in randomized complete block design.

T1 - Control
T2 - L-TRP solution (10^{-3} M)
T3 - L-TRP solution (10^{-4} M)
T4 - L-TRP solution (10^{-5} M)
T5 - *Rhizobium phaseoli* (N_{42})
T6 - *Rhizobium phaseoli* (N_{42}) + L-TRP solution (10^{-3} M)
T7 - *Rhizobium phaseoli* (N_{42}) + L-TRP solution (10^{-4} M)
T8 - *Rhizobium phaseoli* (N_{42}) + L-TRP solution (10^{-5} M)

Following parameters were recorded at maturity and after harvesting, plant height, number and mass of nodules $plant^{-1}$, number of pods $plant^{-1}$, number of grains pod^{-1}, total biomass, grain yield and 1000-grain weight. Grain samples were analyzed to determine the nitrogen concentration (Ryan *et al*., 2001). The data collected were statistically analyzed (Steel *et al*., 1997) and means were compared by Duncan's multiple range test (Duncans, 1955).

Results

This study was conducted to observe the effect of rhizobial inoculation and L-tryptophan (10^{-3}, 10^{-4} and 10^{-5} M) on the growth and yield of mung bean (*Vigna radiata* L.) under field conditions. Results revealed that exogenously applied L-TRP and *Rhizobium* inoculation significantly increased the growth and yield of mung bean when tested in separate treatments. However, *Rhizobium* inoculation when supplemented with L-TRP further improved the crop growth and yield.

Table 1. Synergistic effect of *Rhizobium* inoculation and L-tryptophan (L-TRP) on plant height number of pods plant^{-1}, nodular mass plant^{-1}, number of pods plant^{-1} and number of grains pod+-1 of mung bean (Average of 4 replicates).

Treatment	Plant height (cm)	Number of nodules plant^{-1}	Nodular mass plant^{-1}	No. of pods plant^{-1}	Number of grains pod^{-1}
(T1) Control	55.25 d*	16.92 d	29.45 e	11.4 e	7.0 d
(T2) L-TRP (10^{-3} M)	57.6 cd	19.25 c	30.12 e	12.8 d	7.3 cd
(T3) L-TRP (10^{-4} M)	65.0 b	19.33 c	33.99 d	14.35 c	7.8 c
(T4) L-TRP (10^{-5} M)	67.4 b	20.25 c	38.95 c	14.4 c	8.5 b
(T5) N_{42}	68.2 b	22.55 b	42.35 b	16.1 b	9.7 a
(T6) N_{42}+L-TRP (10^{-3} M)	68.5 b	23.35 b	40.46 b	16.8 ab	8.9 b
(T7) N_{42}+L-TRP (10^{-4} M)	70.8 a	30.54 a	52.15 a	17.6 a	10.2 a
(T8) N_{42}+L-TRP (10^{-5} M)	72.5 a	24.38 b	52.0 a	17.1 a	9.8 a

*Means sharing the same letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test.

Maximum plant height (31.2% higher over untreated control) was recorded with *Rhizobium* inoculation at 10^{-5} M L-TRP that was at par with the treatment where *Rhizobium* and 10^{-4} M L-TRP was applied (Table 1). The *Rhizobium* inoculation significantly increased (23.4% over untreated control) plant height, but it differed non-significantly from *Rhizobium* at 10^{-3} M L-TRP, L-TRP alone at 10^{-4} and 10^{-5} M, respectively.

Data regarding number of nodules plant^{-1} (Table 1) revealed that 80% increase in the nodule number over untreated control was observed with *Rhizobium* inoculation at 10^{-4} M L-TRP. Statistically similar number of nodules was recorded by *Rhizobium* inoculation alone and in the presence of 10^{-3} and 10^{-5} M L-TRP. L-Tryptophan at 10^{-5}, 10^{-4} and 10^{-3} M gave statistically similar results but still significantly higher than untreated control. Similarly, *Rhizobium* inoculation with 10^{-4} M L-TRP produced maximum nodular mass (77% more than control) that differed non-significantly from *Rhizobium* plus 10^{-5} M L-TRP (Table 1). *Rhizobium* inoculation alone produced 44% more nodular mass that was at par with *Rhizobium* in the presence of 10^{-3} M L-TRP solution.

Data in Table 1 showed that *Rhizobium* inoculation alone showed 41% increase in number of pod plant^{-1} over untreated control that was statistically at par with *Rhizobium* plus 10^{-3} M L-TRP solution but significantly different from 10^{-4} and 10^{-5} M L-TRP. However, *Rhizobium* inoculation gave 54% more number of pods than untreated control in the presence of 10^{-4} M L-TRP solution that was statistically at par with *Rhizobium* inoculation at 10^{-5} and 10^{-3} M L-TRP. Likewise, *Rhizobium* inoculation with 10^{-4} M L-TRP gave maximum number of grains pod^{-1} (46% more than untreated control), which was statistically similar with *Rhizobium* inoculation alone and in the presence of 10^{-5} M L-TRP solution (Table 1). Next to these, *Rhizobium* inoculation with 10^{-3} M L-TRP showed 27% increase over control that differed non-significantly from 10^{-5} M L-TRP solution.

Data in Figure 1 showed that *Rhizobium* inoculation showed 41% increase in plant biomass over untreated control that differed non-significantly from *Rhizobium* plus 10^{-3} M L-TRP solution. The total plant biomass with 10^{-5} M L-TRP was 30% more than untreated control and was statistically similar with L-TRP at 10^{-4} M as well as from *Rhizobium* inoculation. However, *Rhizobium* inoculation with 10^{-5} M L-TRP gave 62% increase over untreated control in biomass that differed non-significantly from *Rhizobium* inoculation at 10^{-4} M L-TRP. Regarding grain yield, *Rhizobium* inoculation alone showed 36% increase in grain yield that was at par with *Rhizobium* plus 10^{-3} and 10^{-5} M L-TRP (Fig. 2). *Rhizobium* inoculation at 10^{-4} M L-TRP gave 57% increase in grain yield compared to untreated control that differed non-significantly from *Rhizobium* in the presence of 10^{-5} M L-TRP solution.

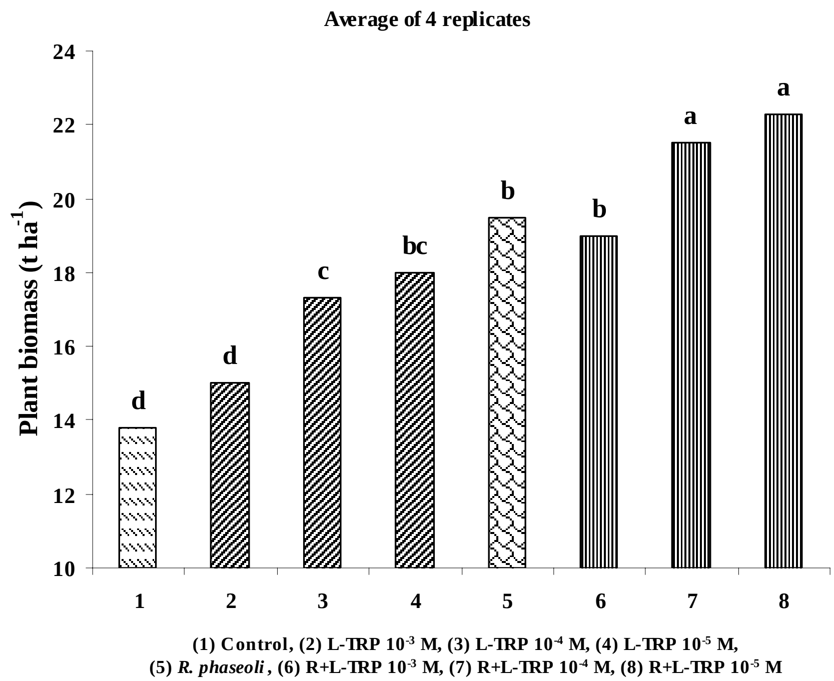

Fig. 1. Integrated effect of *Rhizobium* inoculation and L-TRP on biomass of mung bean.

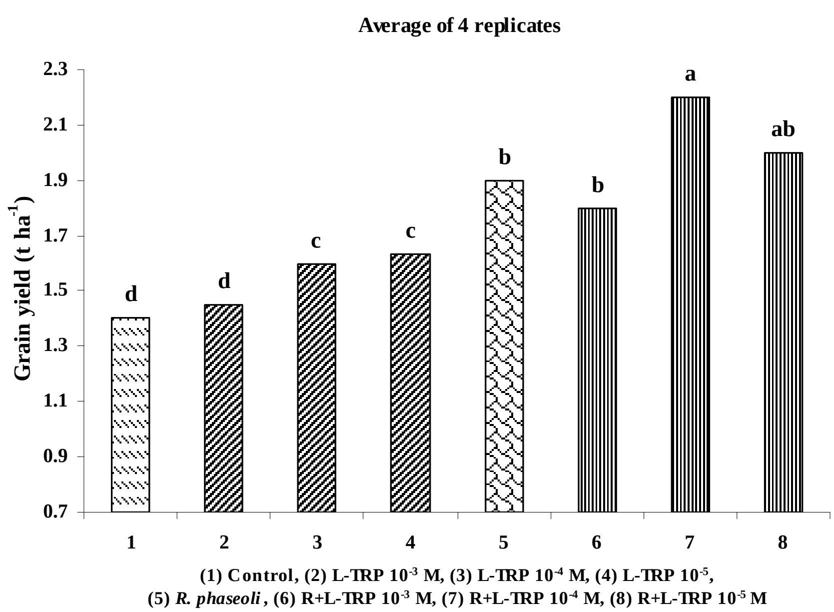

Fig. 2. Integrated effect of *Rhizobium* inoculation and L-TRP on grain yield of mung bean.

The increase in 1000-grain weight with different treatments ranged from 3-17% (Fig. 3), with *Rhizobium* inoculation which produced 12% increase in 1000-grain weight over untreated control. Statistically similar increase in 1000-grain weight was observed by 10^{-5} M L-TRP and *Rhizobium* inoculation when applied separately. However, *Rhizobium* inoculation at 10^{-4} M L-TRP being the most effective treatment produced 17% more 1000-grain weight compared with untreated control.

Figure 4 depicted that *Rhizobium* inoculation gave 71% increase in grain N concentration that differed non-significantly from 10^{-5} M L-TRP and *Rhizobium* inoculation plus 10^{-3} M L-TRP. Rhizobial inoculation at 10^{-4} M L-TRP showed maximum increase in grain N concentration i.e. 86% more than untreated control that differed non-significantly from *Rhizobium* inoculation plus 10^{-3} and 10^{-5} M L-TRP as well as with *Rhizobium* inoculation alone.

Discussion

In addition to BNF, the ability of *Rhizobium* to produce PGRs is considered the most plausible mechanism in controlling plant growth and development in legumes (Dobbelaere *et al*., 2003; Mirza *et al*., 2007). Growth promoting ability of *Rhizobium* may be highly specific to certain plant species, cultivars and genotypes (Mehboob *et al*., 2008).

Rhizobium phaseoli strains were isolated from the nodules of mung bean cultivars. All the strains were characterized for their auxin biosynthesis in the presence and absence of L-tryptophan and the strain N42 (maximum auxins producer) was evaluated by conducting field experiment in the presence of different levels of L-TRP under natural conditions.

In the laboratory study, all the rhizobial isolates produced auxin (expressed as IAA equivalents) in the presence and absence of L-tryptophan but with variable degree of efficacy (data not given). The auxin production by all rhizobial strains increased when the culture medium was supplemented with L-tryptophan. The L-TRP-derived auxins were increased upto 8 fold than that without L-TRP. This increase in auxin production also varied with rhizobial isolates. This contention is supported by many scientists (Leinhos & Vacek, 1994; Sarwar & Kremer, 1995; Biswas *et al*., 2000a, b).

Rhizobium phaseoli strain N42 was evaluated for its growth promoting activity alone and at different levels of L-TRP i.e., 10^{-3}, 10^{-4} and 10^{-5} M under field conditions. The different levels of L-TRP were also tested separately. In most of the cases, a significant increase in crop yield and yield-contributing parameters was obtained when the L-TRP solutions were applied alone except L-TRP level at 10^{-3} M which gave some non-significant results when compared with untreated control. The exogenously applied L-TRP at 10^{-5} M proved to be more effective in improving the growth and yield of mung bean compared to untreated control. The effect in modifying plant growth and development observed by L-TRP in our study was concentration–dependent. The mechanism of action of L-TRP on plant growth may be attributed to direct uptake of these compounds by plant roots, a change in the rhizosphere microflora discouraging root pathogens or by microbial conversion into metabolites resulting in a beneficial rhizosphere for plant growth as reported by other workers (Sarwar & Frankenberger, 1994; Zahir *et al*., 2000, 2005; Khalid *et al*., 2006).

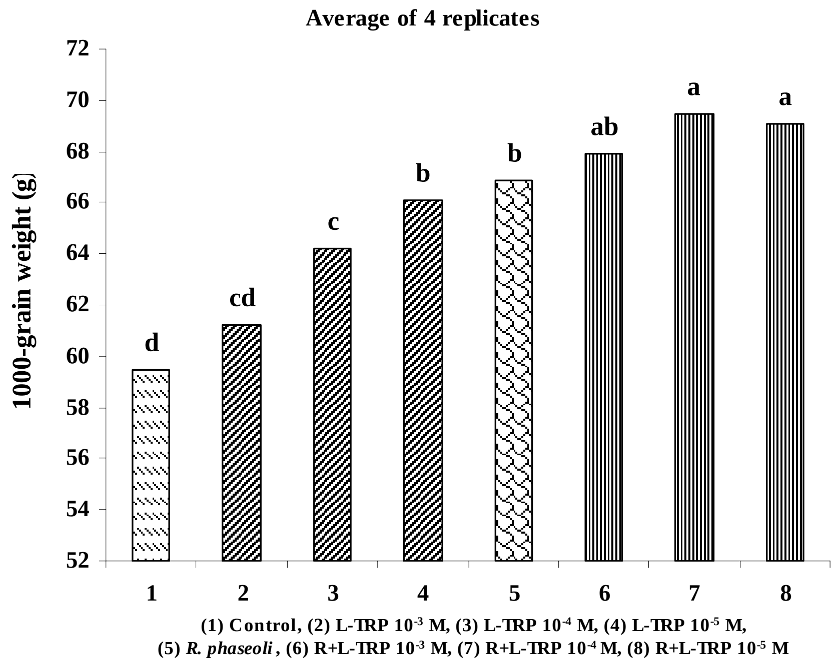

Fig. 3. Integrated effect of *Rhizobium* inoculation and L-TRP on 1000-grain weight of mung bean.

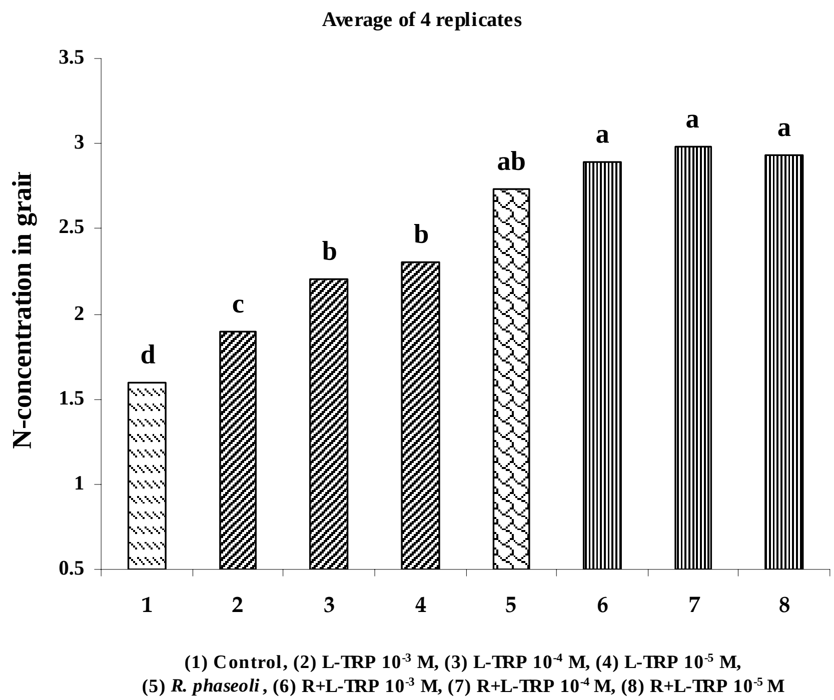

Fig. 4. Integrated effect of *Rhizobium* inoculation and L-TRP on grain N concentration of mung bean.

Inoculation with *Rhizobium* isolate N42 was found to be effective for improving growth and yield of mung bean compared to uninoculated control. The increase in growth and yield parameters i.e. plant height (23.4%), number of nodules plant^{-1} (33%), nodular mass (44%) number of grains pod^{-1} (39%), number of pods plant^{-1} (41%), plant biomass (41%), grain yield (36%) and 1000-grain weight (12%) was recorded compared to uninoculated control. These findings are supported by the work of previous researchers who elucidated the effect of *Rhizobium* inoculation on the growth and development of various leguminous crops (Basu & Bradyopadahyay, 1990; Kumar *et al*., 1993; Bashir, 1994; Roy *et al*., 1995; Provorov *et al*., 1998; Mirza *et al*., 2007).

In the present investigation, besides better nodulation, rhizobial strain also produced considerable increase in yield and yield contributing parameters in mung bean seedlings as compared to control. The reason behind might be the production of phytohormones e.g., auxin (Sevilla *et al*., 2001).

However, *Rhizobium* inoculation in the presence of different levels of L-TRP (10^{-3}, 10^{-4} and 10^{-5} M) further improved the growth and yield of mung bean. Results of field trial revealed that the combined application of *Rhizobium* and L-TRP (10^{-4} M) showed more pronounced effects on mung bean growth, nodulation and yield, however, alone application of L- TRP (10^{-4} M) also gave significantly better results compared to control implying that indigenous microflora was also active in soil in synthesizing auxins from L-TRP under field conditions. Hussain *et al*., (1995) reported the synergistic effect of *Rhizobium* inoculation and L-TRP in lentil in terms of 30.6% more yield than untreated control. The exogenous provision of phytohormones as a result of microbial activity might affect plant's endogenous hormonal levels, either by supplementing the plants own suboptimal levels or by interacting with the synthesis, translocation, or inactivation of existing hormone levels. Plants themselves synthesize a diversity of growth-regulating phytohormones but they also respond to exogenous applications of these PGRs which, after being taken up by the plant, may affect its growth directly as growth stimulants or indirectly by modifying the rhizosphere (Frankenberger *et al*., 1990; Frankenberger & Arshad, 1991a, b; Khalid *et al*., 2006).

In this study, the effectiveness of precursor-inoculum interaction was compared with their separate application for improving the growth and yield of mung bean as a test crop. In most of the cases, combined application proved to be better over separate application. However, further research is needed to unlock several horizons like physiochemical transformation of PGRs in the soil, screening efficient and inexpensive precursors of PGRs and agronomic practices which could enhance the stability and bioavailability of PGRs in plant root zone and to develop an effective approach based upon precursor-inoculum interaction for the prosperity of agriculture industry.

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