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Co-inoculation of phosphate solubilizing bacteria and rhizobia in the presence of L-tryptophan for the promotion of mash bean (*Vigna mungo* L.)

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Abstract

The development of symbiotic relationship between rhizobia and leguminous plants results in active nitrogen fixation and plays a marvelous role in agriculture systems. L-tryptophan (L-TRP) is an amino acid that acts as physiological precursor of auxins and is responsible for biosynthesis of auxins in the rhizosphere. Pot experiment was conducted at the Soil Bacteriology Section Faisalabad to assess the co-inoculation effect of N_2-fixing (Rhizobium) and P-solubilizing (Bacillus sp) in the presence of L-TRP. Results revealed that co-inoculation improved the pod and straw yield considerably but the effect was more pronounced with L-TRP. Co-inoculation increased the root length, root mass, number of nodule and mass as compared to control with L-TRP. Co-inoculation with L-TRP produced 30.87 pod and 32.73 g pot^{-1} straw yield followed by 30.47 and 31.10 g pot^{-1} with rhizobial inoculation, respectively. Co-inoculation produced higher root mass (33.5 g), root length (36.0 cm), nodule number (34), nodule mass (0.131 g) and these values were further enhanced with L-TRP (40.5 g, 49 cm, 48 and 0.145 g). Co-inoculation enhanced the nutrient concentration in mash plant, grains and improved the nodulation as compared to the separate bacterial inoculations. Co-inoculation with L-TRP produced higher soil N and available P in post harvest soil samples as compared to control. Study demonstrated that co-inoculation of Rhizobium and Bacillus species influenced the yield components positively than their separate inoculation and this effect could be more assenting with L-TRP. However, the approach of precursor-inocula interaction should be studied more comprehensively in different ecological zones to sustain the crop yield.

Key words: co-inoculation, *Rhizobium*, *Bacillus*. L-TRP, mash bean

Introduction

Plants opt different means for better uptake of nutrients including expanded root surface area, organic acid synthesis /exudation, phosphate solubilization, phytohormone production and nitrogen fixation. Legumes display all the possible means for better uptake and to conserve the nutrients (Dobbelaere *et al.*, 2003).

Co-inoculation of plant growth promoting rhizobacteria (PGPR) having the phosphate solubilizing potential with *Rhizobium* species stimulated plant growth more profoundly than their alone inoculation (Zaidi *et al.,* 2003). Studies revealed that the cumulative interaction of N_2-fixing i.e. *Rhizobium* and P-solubilizing i.e. *Bacillus* species exerted significant increase in yield of legumes by providing balanced plant nutrition (Toro *et al*., 1998). Barea *et al.* (2005) suggested that phosphate solubilizers in addition to enhancing P-concentration in plant tissues were also responsible to improve nodulation and N_2-Fixation.

Plant growth promoting substances produced by soil microorganisms like auxins, gibberellins and cytokinins alter the plant physiology quite amazingly. Plants and microbes are the sole sources to produce growth regulators especially auxins. Studies revealed that plants can respond to exogenously applied auxins. Literature revealed that microorganisms produced these hormones in pure culture and in soil (Zahir *et al*., 2004). Plants produce these organic stimulants in particular cells and transfer to other parts where at lower concentration they influence the plant growth and development (Zahir *et al*., 1997).

Use of physiological precursors is an efficient approach than the application of synthetic hormones. Interaction of rhizosphere microbes and plant roots in the presence of precursors provides a continuous source of growth hormones for plant uptake (Arshad and Frankenberger, 1998). Microbial biosynthesis of growth hormones can be increased several folds due to the introduction of precursors in the rhizosphere (Frankenberger and Arshad, 1995; Zahir *et al*., 1997). L-TRP, the physiological precursor of auxins, has been found responsible for biosynthesis of auxins in the rhizosphere. Although, it was observed that microbes can produce auxins in the absence of L-TRP in minute amount yet it was also reported that microbes produced much

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higher quantities in its presence (Davies, 1995; Frankenberger and Arshad, 1995; Zahir *et al*., 2004; Khalid *et al*., 2006).

Studies revealed that plants respond to exogenous application of auxins and their precursors. L-tryptophan application to soils stimulates synthesis of auxins and triggers the plant growth (Sarwar *et al.,* 1992; Frankenberger and Arshad, 1995; Khalid *et al*., 2006). Application of precursors at low concentration is more beneficial than their respective hormones because they are inexpensive, water soluble, lack photosensitivity and provide continuous supply of hormones (Arshad and Frankenberger, 1991).

A little work has been done with precursor inoculum interaction in legumes and use of precursor with co-inoculation of N_2-fixing and P-solubilizing microbes is almost negligible. Hence the present study was conducted to study the influence of L-TRP with co-inoculation of *Rhizobium* and *Bacillus* species on the productivity of mash bean (*Vigna mungo* L.).

Materials and Methods

Isolation of Rhizobium and P-solubilizer

Root samples of mash bean (*Vigna mungo* L.) were collected from the research area of Pulses Research Institute, AARI, Faisalabad. After washing the roots, nodules were separated, the pinkish colored nodules were selected and placed in Petri plates. The surface sterilization of nodules was carried out by dipping in 95% ethanol subsequently rinsed with sterilized distilled water and then by dipping in acidified 0.2% $HgCl_2$ for 3-5 minutes and again rinsing with sterilized distilled water (Russell *et al.*, 1982). Surface sterilized nodules were crushed with the help of sterilized forceps in few drops of sterilized distilled water to obtain a suspension. The Congo red yeast extract mannitol agar medium was inoculated with the using an inoculating needle (Vincent, 1970). Petri plates were incubated at 28 ± 2 ^oC for three days. The colonies that did not attain the color of Congo red were picked and purified. The purified rhizobial cultures were stored at 28 ± 2 ^oC on slants for further screening.

P-solubilizer was isolated by using the dilution plate technique. The serial dilutions of rhizosphere soil samples of mash bean were prepared and subjected to heat shock at 80 ^oC for 30 minutes in an oven as reported by Claus (1964) and on cooling the selective medium (Nautiyal, 1999) was inoculated. The inoculated Petri plates were incubated at 28 ± 2 ^oC for seven days. The growth was purified and screened to get a pure culture. The purified isolates were tested for their solubilization on the Pikovskaya's medium containing (g L^{-1}): glucose, 10; $Ca_3 (PO_4)_2$, 5.0; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4$. $7H_2O$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4.H_2O$, 0.002; $FeSO_4$. $7H_2O$, 0.002; agar, 17 and the pH was adjusted to 7.0 (Pikovskaya, 1948). Isolates forming halos on the Pikovskaya's medium were termed as P-solubilizers and were maintained to assess the extent of solubilization. Isolates of *Rhizobium* sp were further screened out for biochemical tests [Gram (-), Catalase (+), urea hydrolysis (+) and citrate utilization (+)] and P-solubilizer [Gram (+), Catalase (+), urea hydrolysis (-) and citrate utilization (+)], respectively (Krieg and Holt, 1984).

Determination of Auxin biosynthesis

The purified isolates of *Rhizobium* and *Bacillus* (three of each) named as (MR_1, MR_2 and MR_3) and (B_1, B_2 and B_3) were screened for their auxin biosynthesis potential in the absence of L-TRP. Isolates of *Rhizobium* were maintained on the yeast extract mannitol (YEM) for 48-72 hours and *Bacillus* species on Pikovskaya's broth culture. Auxin biosynthesis potential was determined as IAA equivalents using Salkowski's reagent (2 mL of 0.5M $FeCl_3$ +98 mL of 35% $HClO_4$) as reported by Sarwar *et al.* (1992). Isolates having the highest auxin biosynthesis potential were selected for further experimentation.

Determination of phosphate solubilization

Isolates of *Bacillus* (B_1, B_2 and B_3) were tested for the phosphate solubilization on the Pikovskaya's medium (Pikovskaya, 1948). Isolates which formed the halos on the Pikovskaya's medium, were considered P-solubilizers. The growth and solubilization diameter were determined after incubation at 28 ± 2 ^oC for seven days. Solubilization efficiency and solubilization index were determined on the bases of diameter of clearing halo zones (Gaur, 1990; Nguyen *et al.*, 1992; Vazquez *et al.*, 2000) by using the following formula.

$$SE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

Where as solubilization index was determined by the following formula,

$$SI = \frac{\text{colony diameter + halozone diameter}}{\text{Colony diameter}}$$

Auxin biosynthesis potential (as IAA equivalents) of *Rhizobium* varied from 8.49-9.25 µg mL^{-1} while *Bacillus* had 1.82-2.42 µg mL^{-1}. MR_3 and B_3 isolates having the highest auxin biosynthesis potential and phosphate solubilization (Table 1) were selected for experimentation.

Inocula preparation

Broth of YEM and selective medium was prepared for *Rhizobium* and *Bacillus*, respectively and sterilized at 121 ^oC and 15 psi pressure for ½ hour. Both the media were inoculated in 500 mL conical flask containing 200 mL medium and incubated at 28 ± 2 ^{o}C under shaking at 125 rpm for 4 days to have an optical density of 0.5. Leaf mould collected from the Changa Manga forest after composting was used as carrier. Carrier was sterilized at 121 ^{o}C and 15 psi pressure for one hour and inoculated with broth cultures of *Rhizobium* and *Bacillus* species (100 mL per kg of peat). Carrier based inoculum was prepared by adding 10% sugar solution and incubated at 28 ± 2 ^{o}C to increase the population of respective bacteria up to 10^8 CFU mL^{-1}. Inocula of *Rhizobium* and *Bacillus* species at 10^8 MPN bacterial cells per gram of carrier were applied to mash bean as seed coating.

Table 1: Some important characteristics of isolates used in the study

Isolate	IAA equivalent (µg mL^{-1})	Solubilization efficiency (SE)	Solubilization index (SI)
MR_1	8.49	-	-
MR_2	9.09	-	-
MR_3	9.25	-	-
B_1	2.31	240	3.4
B_2	1.82	260	3.7
B_3	2.42	267	3.8

Pot Experiment

Pot experiment was conducted to assess the co-inoculation effect of *Rhizobium* and *Bacillus* sp. on the yield of mash bean with and without L-TRP following the completely randomized design (CRD) with five replication. Seeds of mash bean were soaked in sterilized distilled water (as control) and L-TRP at 10^{-5} M for three hour. The pre-sowing soil samples were collected, air dried, sieved and analyzed for various physico-chemical characteristics. The soil was medium textured having pH, 8.11; EC, 1.42 dS m^{-1}; soil N, 0.038% and available P, 9.52 mg kg^{-1} at research area of Soil Bacteriology Section, Ayub Agricultural Research Institute, Faisalabad. Recommended fertilizer (25 kg N ha^{-1} and 60 kg P ha^{-1}) was applied as basal dose in the form of urea and single super phosphate to all the treatments.

When the crop was at flowering stage, pots of two repeats were excessively irrigated to uproot plants for recording root parameters like number of nodules, nodule mass, root length and mass (three plants in each pot). Data regarding pod, straw yield, N and P concentration in plant and grains and post harvest soil N and available P were determined from the rest of three repeats. Nitrogen was determined according to Kjeldhal method (Bremner and Mulvany, 1982) while phosphorus by modified Olsen method (Olsen and Sommers, 1982). Data were subjected to statistical analysis by following CRD as described by Steel et al., (1997). The differences among treatment means were compared by applying the Duncan’s multiple range tests (Duncan, 1955).

Results

Results showed that microbial inoculations and exogenous application of L-TRP enhanced the yield components of mash bean. It was clearly demonstrated that microbial inoculation supplemented with L-TRP boosted the yield components marvelously.

Co-inoculation significantly influenced the yield parameters (Table 2) as compared to separate inoculation of *Rhizobium* and *Bacillus* sp. Inoculation with either bacterial species or co-inoculation when supplemented with L-TRP proved to be more effective and improved the yield than that without L-TRP. Co-inoculation with L-TRP significantly enhanced the pod yield (30.87 g pot^{-1}) followed by co-inoculation without L-TRP (29.33 g pot^{-1}).

Co-inoculation without L-TRP produced 55.19% more pod yield followed by rhizobial inoculation (41.96%) while increase in pod yield with co-inoculation when supplemented with L-TRP was 24.97% followed by rhizobial inoculation (23.36%) as compared to their respective controls. Similarly, the percent increase in straw yield by co-inoculation without L-TRP was 23.02% and by rhizobial inoculation 13.12% than un-inoculated control. With L-TRP, increase in straw yield was 20.02% and by rhizobial inoculation it was 14.04%. Increase in pod and straw yield with co-inoculation supplemented with L-TRP was 63.33% and 31.29% than the control without L-TRP, respectively.

Data regarding plant N and P content (Table 3) revealed that bacterial inoculation either applied singly or co-inoculation with / without L-TRP significantly enhanced the NP content of mash bean plant matter than control. Co-inoculation with L-TRP demonstrated maximum plant N (1.185%) followed by rhizobial inoculation (1.174%) as compared to un-inoculated control while maximum plant P was observed with co-inoculation (0.257%) followed by *Bacillus* inoculation (0.253%). Co-inoculation without L-TRP gave 11.54% more plant N while increase with L-TRP was 6.56% as compared to their respective controls. Similarly, co-inoculation without L-TRP produced 25.76% more plant P and with L-TRP, increase in plant P was 20.66%. There was significant increase in N and P-content with the application of L-TRP.

Data regarding grain N and P content (Table 4) depicted that microbial inoculations with and without L-TRP significantly enhanced the grain NP-content than un-inoculated control. Results clearly demonstrated that the application of L-TRP increased the NP-content in grains with bacterial inoculations. Co-inoculation with L-TRP produced maximum grain N (2.404%) followed by rhizobial inoculation (2.370%) as compared to un-inoculated control while maximum plant P was observed with co-inoculation (0.330%) followed by co-inoculation without L-TRP (0.320%). Co-inoculation without L-TRP produced 4.57% more plant N while increase with L-TRP was 4.70% as compared to their respective controls. Like wise, co-inoculation without L-TRP produced 25% more plant P and with L-TRP it was 20.88%.

Table 2: Co-inoculation effect on pod and straw yield with and without L-TRP

Treatment	Pod Yield (g pot^{-1})		Straw Yield (g pot^{-1})	
	L-TRP [-]*	L-TRP [+]*	L-TRP [-]	L-TRP [+]
Control	18.90 e**	24.70 d	24.93 g	27.27 e
Rhizobial Inoculation	26.83 c	30.47 ab	28.20 d	31.10 b
Bacillus Inoculation	25.17 d	26.90 c	26.60 f	29.50 c
Co-Inoculation	29.33 b	30.87 a	30.67 b	32.73 a
LSD	1.151		0.592	

*L-TRP [-]: Without L-TRP; L-TRP [+]: With L-TRP

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

Table 3: Co-inoculation effect on plant N and P-content with and without L-TRP

Treatment	Plant N (%)		Plant P (%)	
	L-TRP [-]*	L-TRP [+]*	L-TRP [-]	L-TRP [+]
Control	1.040 g**	1.112 e	0.198 g	0.213 f
Rhizobial inoculation	1.115 e	1.174 b	0.222 e	0.239 d
Bacillus inoculation	1.101 f	1.124 d	0.245 c	0.253 ab
Co-inoculation	1.160 c	1.185 a	0.249 bc	0.257 a
LSD	0.0054		0.0055	

*L-TRP [-]: Without L-TRP; L-TRP [+]: With L-TRP

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

Table 4: Co-inoculation effect on grain N and P-content with and without L-TRP

Treatment	Grain N (%)		Grain P (%)	
	L-TRP [-]*	L-TRP [+]*	L-TRP [-]	L-TRP [+]
Control	2.255 h**	2.296 f	0.256 g	0.273 f
Rhizobial inoculation	2.317 e	2.370 b	0.286 e	0.309 c
Bacillus inoculation	2.276 g	2.328 d	0.295 d	0.309 c
Co-inoculation	2.358 c	2.404 a	0.320 b	0.330 a
LSD	0.0055		0.0077	

*L-TRP [-]: Without L-TRP; L-TRP [+]: With L-TRP

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

Data regarding post harvest soil N and available P (Table 5) revealed that precursor (L-TRP) inocula (co-inoculation) influenced the soil N and available P significantly. Inoculation either applied alone (*Rhizobium / Bacillus*) or in combination yielded higher soil N and available P with and without L-TRP and the effect was more pronounced with the application of L-TRP. Co-inoculation of *Rhizobium* and *Bacillus* species with L-TRP resulted in maximum available P and soil N (12.88 mg kg^{-1}; 0.0497%) followed by (12.46 mg kg^{-1}; 0.0470%) without L-TRP. *Bacillus* inoculation exhibited high available P with / without L-TRP than rhizobial inoculation while rhizobial inoculation resulted in higher soil N than *Bacillus* inoculation. Percent increase in available P and soil N by co-inoculation without L-TRP was (31.02 and 16.63%) and with L-TRP (24.44 and 16.39%) as compared to their respective controls.

Inoculation effects on root parameters (mean of two repeats) with and without L-TRP are in (Figures 1, 2, 3 and 4). Co-inoculation of *Rhizobium* and *Bacillus* affected the root parameters definitely than the separate inoculation and values were further enhanced with the application of L-TRP. Co-inoculation along with L-TRP produced maximum number of nodules pot^{-1} (48), nodule mass (0.145 g pot^{-1}), root length (49 cm) and root mass (40.5 g pot^{-1}) followed by rhizobial inoculation alone (46; 0.144 g pot^{-1}; 44 cm and 38.5 g pot^{-1}), respectively.

Table 5: Co-inoculation effect on soil N and available P with and without L-TRP

Treatment	Available P (mg kg^{-1})		Soil N (%)	
	L-TRP [-]*	L-TRP [+]*	L-TRP [-]	L-TRP [+]
Control	9.51 f**	10.35 ef	0.0403 e	0.0427 d
Rhizobial inoculation	10.77 de	11.20 cde	0.0457 bc	0.0463 b
Bacillus inoculation	11.62 bcd	12.04 abc	0.0430 d	0.0443 cd
Co-inoculation	12.46 ab	12.88 a	0.0470 b	0.0497 a
LSD	0.99		0.0017	

*L-TRP [-]: Without L-TRP; L-TRP [+]: With L-TRP

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

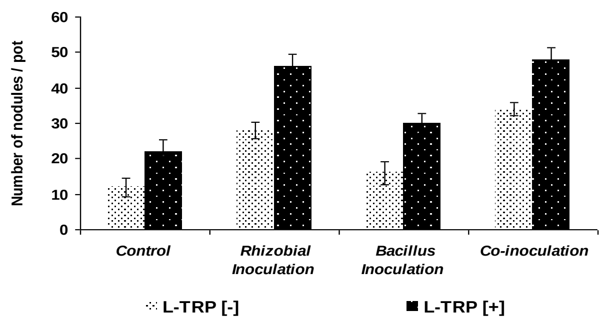

Figure 1: Co-inoculation effect on number of nodules per pot of mash bean with / without L-TRP

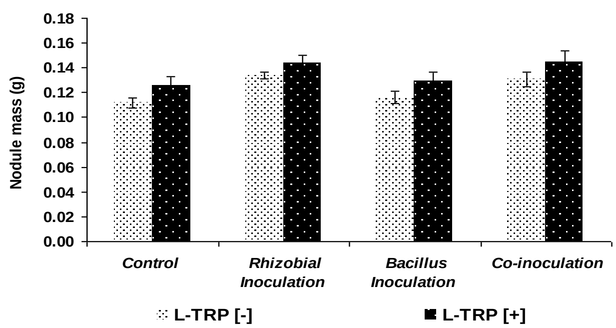

Figure 2: Co-inoculation effect on nodule mass of mash bean with / without L-TRP

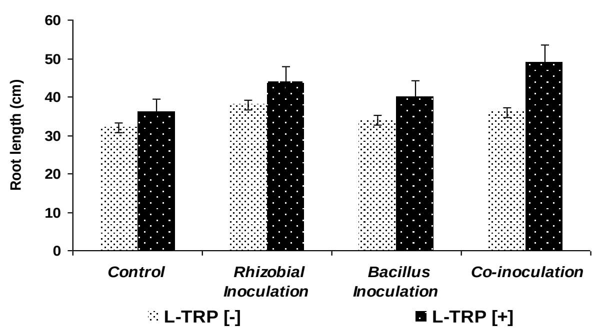

Figure 3: Co-inoculation effect on root length of mash bean with / without L-TRP

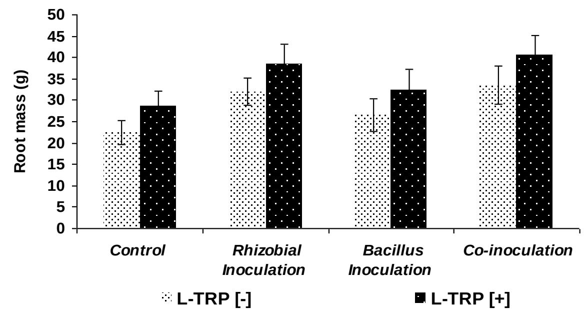

Figure 4: Co-inoculation effect on root mass of mash bean with / without L-TRP

Discussion

Three isolates each of *Rhizobium* and *Bacillus* species were screened for their phosphate solubilization and auxin production potential. All isolates of *Rhizobium* and *Bacillus* species produced IAA equivalents with variable degree. Isolates of *Rhizobium* and *Bacillus* species having highest auxin production and phosphate solubilization extent were used in this study (Table 1). Many researchers have also reported the microbial biosynthesis of plant hormones with and without L-TRP and solubilization of insoluble phosphates (Sarwar *et al.*, 1992; Zahir *et al.*, 2004).

In our experiment, *Rhizobium* and *Bacillus* species were evaluated for mash bean growth promotion in the presence and absence of L-TRP. Results revealed that co-inoculation enhanced the yield and root parameters significantly compared to un-inoculated control and this enhancement was further improved with the supplementation of L-TRP.

Inoculations of *Rhizobium* and *Bacillus* species either applied separately or in combined form enhanced the yield components and NP contents in mash bean. However, *Rhizobium* inoculation proved to be more effective than *Bacillus* inoculation alone (Mirza *et al.*, 2007). L-TRP at 10^{-5} M has influenced the plant growth positively which might be ascribed to provide a continuous source of growth hormones for plant uptake, modification in rhizosphere colonization, and microbial biosynthesis of growth hormones resulting better plant growth as reported by many workers (Zahir *et al*., 2005; Khalid *et al*., 2006).

Rhizobium inoculations has been reported to improve the germination, seedling growth, nodulation and root/shoot mass of crops. *Rhizobium* species besides N_2-fixation, synthesize growth hormones that has been considered the most probable mean to promote plant growth (Zahran, 2001; Zahir *et al*., 2004). *Rhizobium* and *Bacillus* species can synthesize hormones in the absence of precursor and exogenous induction of precursors can enhance the auxin production potential (Khalid *et al.,* 2006; Ali *et al.,* 2009).

Similar to our findings, co-inoculation of *Rhizobium* and *Bacillus* species influenced the yield parameters and nodulation as reported by other workers (Dashti *et al*., 1998; Gupta *et al.,* 2003; Garcia *et al*., 2004). *Rhizobium* and *Bacillus* species inoculation demonstrated higher N and P contents in plant and grains might be due to increase nutrient contents in the root zones. Inoculation of growth hormone producing *Rhizobium* and *Bacillus* species (having high SE and SI) might be responsible for expansion of root surface area and enhanced plant-microbe interaction resulting in more nutrient uptake (Yuming *et al*., 2003). PGPR having the phosphate solubilizing capacity when introduced in the legume rhizosphere produced more available P by production of organic acids and favored more soil N (Zaidi *et al.,* 2004; Khan *et al*.*,* 2006).

In present study, co-inoculation effect of *Rhizobium* and *Bacillus* species was more pronounced and this effect further enhanced the yield and root parameters with application of L-TRP. Precursor-inoculum / inocula interaction enhanced the pod, straw yield and nutrient content in plant and grains; might be due to the increase in root length and mass, thus offering more sites for nodulation. Induction of L-TRP might provide a continuous source of auxins thus causing more root proliferation, nutrient uptake and ultimately growth of the plant (Sarwar and Kremer, 1995). Microbial activities due to the presence of L-TRP provided continuous source of auxins that might alter the endogenous hormonal level. This alteration in the endogenous hormonal level might affect the plant's own synthesis of auxins / modified the translocation of these hormones to other parts. These growth hormones alter the plant growth quite miraculously. Besides the production of hormones, plant also produce these organic stimulants in particular cells and transfer to other cells and influence physiological functions such as enhanced shoot and root growth, stimulated cell division, metabolism and modification of rhizosphere (Frankenberger and Arshad, 1991; Khalid *et al*., 2006**;** Glick *et al.,* 2007; Idris *et al.,* 2007).

Conclusion

Study validated the concept of co-inoculation that influenced the growth and yield of mash bean positively. Study also confirmed the approach of precursor-inoculum interaction which influenced the yield and nutrient content of mash bean with either microbe or with co-inoculation. However, comprehensive and site specific field studies are required to fortify the approach of precursor-inoculum interaction.

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