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POTENTIAL OF *RHIZOBIUM* SPECIES TO ENHANCE GROWTH AND FODDER YIELD OF MAIZE IN THE PRESENCE AND ABSENCE OF L-TRYPTOPHAN

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ABSTRACT

Legumes are very well known for their symbiotic relationships with *Rhizobium*, responsible for specific structure i.e. nodules on 90% of family Fabaceae and displayed all possible means for improving the fertility of soils. *Rhizobium* due to its great colonizing ability may be used in non-legumes for improving crop growth. The physiological precursors are cost effective, water soluble and provide hormones continuously. Present study was planned to assess the *Rhizobium* species of (chickpea, berseem and lentil) with and without L-Tryptophan (L-TRP) on yield parameters of maize. On the basis of auxin biosynthesis potential, isolates of *Rhizobium* sp (Cp_3, Br_3 and Lt_2) were selected for experimentation. Results revealed that *Rhizobium* isolates (Cp_3, Br_3 and Lt_2) improved the growth and fodder yield of maize over control and impact was further prominent with the application of L-TRP. Interaction of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2) increased the fresh fodder and dry matter yield than their separate application. Increase in fresh fodder and dry matter yield (28.02, 37.89 and 26.88%) and (28.82, 39.36 and 25.73%) was observed with interaction of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2), respectively. Study clearly demonstrated that interaction of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2) improved the plant NP content, chlorophyll (a and b) content, photosynthetic, transpiration and photo active radiation and plant physical parameters. Results showed that precursor-inoculum interaction is an efficient approach and may be tested in different ecologies.

Keywords: Precursor-inoculum interaction, L-TRP, *Rhizobium* species, maize.

INTRODUCTION

Maize (*Zea mays* L.) is an important crop grown in summer / spring throughout the Pakistan for both grain and green fodder purposes. It needs substantial amounts of nutrients for rapid growth and provides rich source of nutrients and healthy diet to mammals. Soil microorganisms are considered as indicators of soil health because they mediate many beneficial processes like nitrogen fixation, nutrient mobilization, improve soil structure and involve in biodegradation of agro-chemicals (Lupwayi *et al.,* 2011). The ever-increasing demand of fertilizers to grow crops for huge populations has converted the interest to the use of biofertilizers for the sake of crop growth and soil health. Biofertilizers are eco-friendly, inexpensive and produce systemic resistance to the plants (Hardoim *et al.,* 2008; Mehboob *et al.,* 2008). The beneficial effect of *Rhizobium* in legumes is well known. Recently researchers reported its positive effects on non-legumes and can act as plant growth promoting rhizobacteria (PGPR) (Biswas *et al.,* 2000 a, b). *Rhizobium* is a very good root colonizer of non-legumes like other rhizobacteria and produces plant hormones, siderophores, solubilized phosphorus and exhibited adverse effects to plant pathogens (Antoun and Prevost, 2005; Mehboob *et al.,* 2008). These marvelous roles of *Rhizobium* in non-legumes converted the intentions of Soil Microbiologists for its use and testing in various crops. Recently, literature proved that *Rhizobium* have also been involved in the disease suppression (Elbadry *et al.,* 2006; Huang *et al.,* 2007; Huang and Erickson, 2007; Siddiqui *et al.,* 2007; Mia and Shamsuddin, 2010).

Some PGPR's able to enhance plant growth and development by interfering in the concentration of plant hormones and influenced the physiological processes of plant at very low concentrations (Zahir *et al.,* 2010a). It is very well demonstrated that normal plant growth and development throughout ontogeny is controlled by these compounds produced by the plant itself (Davies, 2004).

L-tryptophan (L-TRP), the physiological precursor of auxins is involved in microbial biosynthesis of auxins in soil and solutions. Khalid *et al.* (2004, 2006) reported that L-TRP is considered an efficient physiological precursor of auxins in higher plants and in microbial derived auxins. Application of L-TRP may increase the growth of plants by its conversion to auxin by soil microorganisms (Khalid *et al.,* 2004; Zahir *et al.,* 2010a). Microbes produced minute amount of auxins in the absence of L-TRP, but in its presence produced much higher values of auxins. (Asghar *et al.,* 2002; Zahir *et al.,* 2004). L-TRP applied exogenously to plants produced higher values of auxins and exerted positive effects on plants (Khalid *et al.,* 2004; Rao *et al.,* 2012). Etesami *et al.* (2009) reported that the different treatments of rhizobial strains and L-TRP levels increased root / stem parameters to many times compared to control.

However, plants may not have the capacity to synthesize sufficient endogenous plant hormones for optimal growth and development under suboptimal growth and environmental conditions. Exogenously applied plant hormones affected plant growth by changing the balance of endogenous levels of hormones, modified the growth and development of plants to the desired extent (Khalid *et al.,* 2004; Zahir *et al.,* 2010a, b). Zahir *et al.* (1999) observed significant effect of indole acetamide and its precursor L-TRP on growth and yield of rice. Zahir *et al.* (2010b) reported that *Rhizobium* inoculation supplemented with L-TRP (10^{-4} M) resulted in significant increase in various physical parameters up to 17-80%, compared to control. *Rhizobium* sp can synthesize auxins in the absence of L-TRP but exogenously applied of L-TRP can increase auxin production to several folds (Asghar *et al.,* 2002; Khalid *et al.,* 2004). Husssain *et al.* (2009) reported that different species of *Rhizobium* effect positively on the rice yield. Present study was designed to assess the effect of interactive effect of different *Rhizobium* species and L-TRP on growth and fodder yield of maize.

MATERIALS AND METHODS

Isolations and Purification of *Rhizobium* Species: *Rhizobium* species were isolated from the specific host plants viz. (chickpea, berseem and lentil) on yeast extract mannitol agar medium (YMA) medium having composition per liter (5% K_2HPO_4:10 ml; 2% $MgSO_4.7H_2O$: 10 ml; 1% NaCl: 10ml; mannitol: 10 g; yeast extract: 1 g; agar: 15.0 g) (Vincent, 1970). The prepared medium was sterilized by autoclaving for 15-30 minutes at 15-20 lb inch^{-2} pressure and 121^{o}C temperature. The sterilized medium was poured on Petri dishes aseptically in laminar air flow cabinets. Then this medium was further sterilized by placing these plates in laminar flow cabinet under UV light. Plants of (chickpea, berseem and lentil) were uprooted at flowering stage. The roots were washed with tap water and nodules were removed and surface sterilized using (95% ethanol and 0.1% $HgCl_2$) and then washed 4-5 times with sterilized distilled water as reported by Russell *et al.* (1982). Nodules were crushed by sterilized forceps and streaked on solidified medium with the help of inoculating needle. Then these plates were incubated at 25^{o}C in the incubator for 48 hours. The prolific single colonies of *Rhizobium sp* (chickpea, berseem and lentil) were picked and purified on fresh prepared plates and purified cultures were stored at 5± 1^{o}C on slants and maintained for further experimentation.

Determination of Auxin Biosynthesis: Isolates of *Rhizobium* species of chickpea (Cp_1, Cp_2, and Cp3), berseem (Br_1, Br_2 and Br_3) and lentil (Lt_1, Lt_2 and Lt_3) were screened for their auxin biosynthesis potential in the presence and absence of L-TRP colormetrically. For this purpose, the isolates were cultured on the sterilized GPM broth in test tubes. The test tubes were incubated at 28 ± 2 °C for one week and un-inoculated control was also kept for comparison. After incubation the contents were centrifuged @1000 rpm for 10 minutes and filtered through whatman filter paper No.2. The auxin biosynthesis potential was determined as IAA equivalents by spectrophotometer at 535 nm using Salkowski's reagent (2 mL of 0.5M $FeCl_3$ + 98 mL of 35 % $HClO_4$) as reported by Sarwar *et al*. (1992). Different biochemical tests like congo red, bromothymol blue (BTB) and gram reaction were performed for these isolates. Isolates showing the highest auxin biosynthesis (CP_3, Br_3 and L_2) with and without L-TRP were selected for the study (Table 1).

Preparation of Inoculum: The yeast extract mannitol broth (YMB) with composition per liter (Mannite: 10 g; Yeast extract: 1g; K_2HPO_4 (5%):10mL; $MgSO_4.7H_2O$ (2%):10mL; NaCl (1%):10 mL) is prepared and autoclaved at 121^{o}C temperature and 15-20 lb inch^{-2} pressure. This autoclaved broth is inoculated with purified and selected isolates of *Rhizobium* sp (Cp_3, Br_3 and Lt_2) separately and incubated at 25 ^{o}C for 3 days.

Pot Experiment: Pot study was conducted in spring having medium textured soil (12 kg) with pH 7.82, EC 1.36 dS m^{-1}, N 0.032 % and available P 7.40 mg kg^{-1} at Soil Bacteriology Section, AARI, Faisalabad. Uniform dose of fertilizer @ 100-60 kg NP ha^{-1}was applied. All phosphorus and half nitrogen were applied as basal and half nitrogen was applied at the time of 3rd irrigation. The experiment was laid out in completely randomized design (CRD) with three replications. Maize seeds (cv. Neelam) were soaked in L-TRP @ 10^{-5} M and respective cultures for three hours. The treatments were viz. T_1: control, T_2: L-TRP @ 10^{-5} M, T_3: *Rhizobium* sp (Cp_3), T_4: *Rhizobium* sp (Br_3), T_5: *Rhizobium* sp (Lt_2), T_6: L-TRP + *Rhizobium* sp (Cp_3), T_7: L-TRP + *Rhizobium* sp (Br_3) and T_8: L-TRP+ *Rhizobium* sp (Lt_2).

The photosynthetic rate, transpiration rate and photoactive radiation were determined by using IRGA (CI-340) after 30 days of germination. Chlorophyll content (a and b) of leaf was determined colorimetrically by crushing the leaves in acetone and then run on spectrophotometer @ 645 nm and 663 nm after centrifuging @ 1000 rpm for 10 minutes (Arnon, 1949). Maize crop was harvested after 45 days of germination and various physical observations were recorded like fresh fodder, dry matter yield, plant height, flag leaf length / width. After one week of sun drying these plants were placed in oven at 70 ^{o}C for 30 minutes for determining the dry matter. The post harvest soil samples were analyzed for extractable P (Olsen and Sommers,

1982) and soil N (Bremnar and Mulvaney, 1982). The recorded data were subjected to statistical analysis by following completely randomized design (CRD) (Steel *et al.*, 1997). The differences among the means were compared by the Duncan's multiple range tests (Duncan, 1955).

RESULTS

Results revealed that bacterial inoculation and application of L-TRP enhanced the maize fodder yield significantly. Interaction of *Rhizobium* sp and L-TRP exerted more assenting effect than their separate application of L-TRP and *Rhizobium* sp. Thus precursor-inoculum interaction enhanced the growth and yield of maize fodder quite miraculously.

Results showed that three isolates of each host produced IAA equivalents in the absence of L-TRP and the effect was more pronounced in the presence of L-TRP (Table 1). Biochemical tests like congo red, bromothymol blue (organic acid production test) and Gram reaction of isolates were carried out. Highest auxin biosynthesis potential as IAA equivalents in the absence of L-TRP was observed by Cp_3, Br_3 and Lt_2 i.e. 7.56, 5.15 and 4.40 µg mL^{-1} and value was increased to 8.61, 5.60 and 4.92 µg mL^{-1} with the application of L-TRP, respectively.

Results regarding fresh fodder, dry matter yield, plant N and P content (Table 2) clearly demonstrated the approach of precursor-inoculum interaction significantly. L-TRP enhanced the fresh fodder and dry matter yield (468.3, 126.7 g pot^{-1}) as compared to control (440.0, 110.0 g pot^{-1}), respectively. Increase in fresh fodder and dry matter yield (23.11, 25.39, and 2.27%) and (24.27, 27.27 and 9.09%) due to separate application of *Rhizobium* species i.e. Cp_3, Br_3 and Lt_2 was observed, respectively. Interaction of precursor and *Rhizobium* species (Cp_3, Br_3 and Lt_2) demonstrated comprehensive increase in fresh fodder and dry matter yield over separate *Rhizobium* sp inoculation and L-TRP. Percent increase fresh fodder and dry matter yield (28.02, 37.89 and 26.88%) and (28.82, 39.36 and 25.73%) was observed with interaction of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2), respectively. *Rhizobium* sp (Br_3) produced the maximum fresh fodder and dry matter yield than the rest of *Rhizobium* species with L-TRP i.e. 606.7 and 153.3 g pot^{-1}, respectively. Interaction of precursor and *Rhizobium* species (Cp_3, Br_3 and Lt_2) produced the maximum plant N and P content than their separate application and control. Interaction of *Rhizobium* sp (Br_3) and L-TRP exhibited the highest plant N and P content i.e. 1.24 and 0.279%, respectively.

Results regarding physical parameters i.e. plant height, flag leaf width and length (Table 3) showed that interaction of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2) significantly enhanced these parameters. The maximum plant height i.e. 104.0, 102.0 and 101.0 cm was observed with L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2), respectively. Flag leaf width and length was maximum with 6.9 and 76.0 cm with L-TRP and *Rhizobium* sp (Br_3) than the rest of treatments, respectively.

Results regarding parameters taken with Infra Red Gas Analyzer (IRGA) showed that photosynthetic, transpiration and Photo Active Radiation (PAR) values (Table 4) were enhanced with the alone application of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2) and further improved with their interaction. The maximum photosynthetic, transpiration and PAR values were observed with L-TRP and *Rhizobium* sp (Br_3) i.e. 87.3 µmole m^{-2} s^{-1}, 8.3 mmole m^{-2} s^{-1} and 902.3 µmole m^{-2} s^{-1}, respectively than their separate application and control.

Results regarding chlorophyll values (a, b) and post harvest soil N and available P (Table 5) presented that the approach of precursor-inoculum interaction had significant effect on chlorophyll content of maize and soil status at harvest. The maximum chlorophyll (a & b) values were observed with L-TRP and *Rhizobium* sp (Br_3) i.e. 0.98 and 0.40 µg mL^{-1}as compared to control i.e. 0.94 and 0.33, respectively. Soil N and available P was also improved with L-TRP and *Rhizobium* sp (Br_3) i.e. 0.039% and 11.76 mg kg^{-1}as compared to other isolates in interaction i.e. 0.038% and 10.27 mg kg^{-1} with *Rhizobium* sp (Cp_3) and their separate application without precursor. It has also been observed that L-TRP and isolates of *Rhizobium* sp produced higher values than control yet their interaction produced much higher values.

DISCUSSION

Rhizobium species affected the maize growth and development significantly and variable response was observed with the isolates and the effect was more pronounced with the exogenous application of L-TRP. Auxin biosynthesis of isolates in the presence and absence of L-TRP affected the growth and fodder yield of maize. Auxin biosynthesis potential of *Rhizobium* sp in the presence and absence of L-TRP was observed by many researchers (Hussain *et al.,* 2013; Zahir *et al*., 2010a, b).

Three isolates of *Rhizobium* sp (chickpea, berseem and lentil) were screened with and without L-TRP and characterized for different biochemical tests. Three isolates of *Rhizobium* sp (chickpea, berseem and lentil) produced IAA equivalents in the absence of L-TRP and increased the value of IAA equivalents in the presence of L-TRP. Isolates selected on the basis of auxin biosynthesis potential with and without L-TRP were Cp_3, Br_3 and Lt_2.

Results clearly demonstrated improved growth and yield components, plant NP content, biochemical rates such as photosynthetic, transpiration, PAR and chlorophyll a, b content due to the precursor-inoculum interaction might be attributed to auxin biosynthesis potential, better root colonization resulted in improved root system architecture and thus growth and yield of maize (Zahir *et al.*, 2004; 2005; 2010). Auxin biosynthesis potential as IAA equivalents confirmed these findings (Khalid *et al.*, 2001).

Separate application of *Rhizobium* species and L-TRP enhanced the yield components of maize fodder and dry matter and plant NP content compared to control. (Pandey and Maheshwari, 2007; Mehboob *et al.*, 2011). Separate application of L-TRP improved the growth and yield might be attributed to biosynthesis of IAA and altered the endogenous hormonal balance and suppressed the ethylene level. Separate application of *Rhizobium* species improved the growth and yield of maize might be attributed to better root colonization, production of plant hormones, siderophores and organic acids, improved nutrient uptake and inducing systemic resistance (Hmissi *et al.*, 2011; Mehboob *et al.*, 2011; Akhtar *et al.*, 2013).

Combined application of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2) promoted the fodder yield by enhancing biosynthesis of auxins in the rhizosphere might be the reason for enhanced growth through precursor-inoculum interaction (Zahir *et al.*, 2005; 2010).

Higher values of plant NP content in maize with L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2) / precursor-inoculum interaction might be attributed to better root system and more prolific root growth owing to presence of growth hormone in the rhizosphere (Hussain *et al.*, 2009). Precursor-inoculum interaction resulted in enhanced soil N and available P might be attributed to the production of organic acids and thus solubilization of phosphates, better root system for acquisition of nutrients and more microbial activities due to presence of precursor (Fatima *et al.*, 2006; Mehboob *et al.*, 2011). It was also observed that application of *Rhizobium* species and L-TRP enhanced the chlorophyll content (a & b), photosynthetic, transpiration rates and PAR values which might be attributed to the production of siderophores, nutrient uptake due to better root system and presence of growth hormones (Huang and Erickson, 2007; Hussain *et al.*, 2009). Increased chlorophyll content (a & b) might be attributed to enhanced photosynthetic leaf area of plant by PGPR inoculation compared to control having less flag leaf width and length (Han and Lee, 2005).

Table 1. Some different traits of microbes under study.

Host Legumes	**IAA equivalents (µg mL^{-1})**			**Congo- red test**	**Bromothymol Blue test**	**Gram Reaction**
	Isolates	**L-TRP (-)**	**L-TRP (+)**			
Chickpea	Cp_1	4.47	5.08	+ve	+ve	-ve
	Cp_2	4.77	5.30			
	Cp_3	7.56	8.61			
Berseem	Br_1	4.02	4.70	+ve	+ve	-ve
	Br_2	4.70	5.15			
	Br_3	5.15	5.60			
Lentil	Lt_1	3.72	4.02	+ve	+ve	-ve
	Lt_2	4.40	4.92			
	Lt_3	3.87	4.62			

Table 2. Influence of treatments on maize yield and NP content.

Treatments	**Fresh Fodder Yield (g pot^{-1})**	**Dry matter Yield (g pot^{-1})**	**Plant N-content (%)**	**Plant P-content (%)**
1. Control	440.0 e*	110.0 d	1.14 e	0.225 e
2. L-TRP @ 10^{-5} M	468.3 d	126.7 c	1.18 d	0.232 e
3. *Rhizobium* sp (Cp_3)	541.7 c	136.7 b	1.20 bcd	0.254 cd
4. *Rhizobium* sp (Br_3)	551.7 bc	140.0 b	1.22 abc	0.263 b
5. *Rhizobium* sp (Lt_2)	450.0 e	120.0 c	1.18 d	0.248 d
6. L-TRP + *Rhizobium* sp (Cp_3)	563.3 b	141.7 b	1.23 ab	0.265 b
7. L-TRP + *Rhizobium* sp (Br_3)	606.7 a	153.3 a	1.24 a	0.279 a
8. L-TRP + *Rhizobium* sp (Lt_2)	558.3 bc	138.3 b	1.19 cd	0.261 bc
LSD	18.28	9.83	0.0355	0.008

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

Precursor-inoculum interaction resulted in an increase photosynthetic, transpiration rates and PAR might be due to enhance root mass, root respiration, better root system architecture thus more nutrient acquisition and more nutrients in the shoots. Interaction of L-TRP and *Rhizobium* species (Cp_3, Br_3 and Lt_2) improved the plant height, flag leaf width and length, photosynthetic, transpiration rates and PAR values might be attributed to improve root mass, increase photosynthetic / transpiration rate, stomatal conductance and flag leaf area known for the highest photosynthetic activity (Feng *et al.*, 2005).

Study concluded that *Rhizobium* sp can be successfully used in non-legumes after screening. Precursor-inoculum interaction is proficient approach to improve the maize fodder yield and plant physiology can be better regulated with this approach. Comprehensive studies in different ecologies are required to confirm this approach.

Table 3. Some physical parameters recorded from maize study at the time of harvest.

Treatments	Plant Height (cm)	Flag leaf width (cm)	Flag leaf length (cm)
1. Control	82.3 f*	5.5 e	52.3 g
2. L-TRP @ 10^{-5} M	87.3 e	5.7 de	64.7 e
3. *Rhizobium* sp (Cp_3)	99.5 c	5.8 d	71.8 d
4. *Rhizobium* sp (Br_3)	99.3 c	6.7 ab	74.2 b
5. *Rhizobium* sp (Lt_2)	90.8 d	5.7 de	59.7 f
6. L-TRP + *Rhizobium* sp (Cp_3)	104.0 a	6.5 b	73.0 c
7. L-TRP + *Rhizobium* sp (Br_3)	102.0 ab	6.9 a	76.0 a
8. L-TRP + *Rhizobium* sp (Lt_2)	101.0 bc	6.1 c	64.7 e
LSD	2.43	0.252	1.127

*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. Parameters taken from the maize study by the IRGA (CI-340).

Treatments	Photosynthetic rate (µmole m^{-2} s^{-1})	Transpiration rate (mmole m^{-2} s^{-1})	Photo Active Radiation (µmole m^{-2} s^{-1})
1. Control	70.3 f*	6.6 e	850.7 e
2. L-TRP @ 10^{-5} M	76.7 e	7.1 de	800.0 f
3. *Rhizobium* sp (Cp_3)	83.0 c	7.7 bc	889.3 bc
4. *Rhizobium* sp (Br_3)	85.0 b	8.1 ab	887.0 c
5. *Rhizobium* sp (Lt_2)	81.7 d	7.5 cd	874.0 d
6. L-TRP + *Rhizobium* sp (Cp_3)	85.0 b	8.3 a	900.3 a
7. L-TRP + *Rhizobium* sp (Br_3)	87.3 a	8.6 a	902.3 a
8. L-TRP + *Rhizobium* sp (Lt_2)	84.7 b	8.3 a	896.3 ab
LSD	1.049	0.580	8.54

*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 5. Chlorophyll determination by the spectrophotometer and Post harvest soil analysis of maize study.

Treatments	Chlorophyll a (µg mL^{-1})	Chlorophyll b (µg mL^{-1})	Soil N (%)	Avail. P (mg kg^{-1})
1. Control	0.94 d*	0.33 c	0.033 d	7.29 g
2. L-TRP @ 10^{-5} M	0.96 bc	0.34 bc	0.035 cd	8.78 e
3. *Rhizobium* sp (Cp_3)	0.96 bc	0.37 abc	0.037 abc	9.52 d
4. *Rhizobium* sp (Br_3)	0.97 ab	0.38 ab	0.038 ab	11.01 b
5. *Rhizobium* sp (Lt_2)	0.95 cd	0.36 abc	0.036 bc	8.04 f
6. L-TRP + *Rhizobium* sp (Cp_3)	0.98 a	0.39 a	0.038 ab	10.27 c
7. L-TRP + *Rhizobium* sp (Br_3)	0.98 a	0.40 a	0.039 a	11.76 a
8. L-TRP + *Rhizobium* sp (Lt_2)	0.96 bc	0.36 abc	0.037 abc	8.78 e
LSD	0.015	0.044	0.008	0.502

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

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