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Auxin production by *Azospirillum*: Role in growth promotion of *Triticum aestivum* L. and *Lens culinaris* Medik

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ABSTRACT

The aim of this study was to evaluate the potential of Azospirillum as a crop inoculant to enhance the growth of Triticum aestivum L. and Lens culinaris Medik. under axenic conditions. Azospirillum was isolated from the rhizosphere of different grasses by using selective and differential medium Congo red agar (CRA). Bacterial auxin production was quantified using colorimetric technique and thin layer chromatography (TLC). Bacterial strain G2 produced the maximum auxin concentration of 47 and 142 µg ml-1 in the absence and presence of L-tryptophan, respectively. Phytostimulatory effect of Azospirillum on T. aestivum and L. culinaris was evaluated under axenic conditions. Inoculations of T. aestivum with G5 improved the fresh weight (30%) and shoot length (31%) over control. However, when soil was amended with L-tryptophan (1000 µg g-I of soil), maximum increase was recorded for dry weight (116%) and number of roots (50%) respectively for strains G2 and G3. In the case of L. culinaris, strain G1 showed maximum increases in shoot length (16%), fresh weight (57%), dry weight (78%), number of leaves (75%) and number of roots (33%). On the other hand, in L-tryptophan amended soil, G3 strain significantly improved the shoot length and number of roots up to 8% and 33%, respectively. Results of this study indicated that auxin production potential of Azospirillum can be used to enhance the growth of agronomic crops.

Keywords: *Azospirillum, Bacterial auxin, Biofertilizers, Lens culinaris, Rhizobacteria, Triticum aestivum.*

INTRODUCTION

Soil environment associated with the plant roots is the area of microbial profusion and action because of the presence of root exudates (Hartmann et al., 20008). Rhizobacteria present in rhizosphere may be having neutral, harmful and beneficial effects on the plant growth and development. Rhizobacteria exert several beneficial effects on host plants through a variety of mechanisms including nutrient solubilization, nitrogen fixation, salt tolerance, production of phytohormones and numerous toxic compounds to eliminate pathogens (Babaloloa, 2010; Lanoue et al., 2010). Such free living soil bacteria are designated as the plant growth promoting rhizobacteria (PGPR) which exhibit the ability to colonize the plant roots (Fischer et al., 2007; Lugtenberg and Kamilova, 2009). Plant growth and soil fertility depends upon the plant-microbe interactions in rhizosphere which can stimulate plant growth by direct or indirect mechanisms (Richardson et al., 2009; Hayat et al., 2010). The indirect mechanism comprises the synthesis of antibiotics and other toxic compounds such as pyrrolnitrin, hydrogen cyanide (HCN), phenazines, and pyoluteorin or fungal cell wall degrading enzymes play very vital role to cope with the deleterious effects of the phytopathogens (Babaloloa, 2010; Hayat et al., 2010). Direct mechanism of plant growth promotion takes into account the function of symbiotic and non-symbiotic PGPR that produce phytohormones such as auxins, cytokinins, gibberellins, ethylene and abscisic acid (Lugtenberg and Kamilova, 2009; Bhattacharyya and Jha, 2012). The bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter* and *Chromobacterium* have been reported beneficial for plant growth by fixing atmospheric nitrogen, solubilization of minerals, improving soil structure by aggregate formation, increasing the amounts of organic matter in the root surroundings (Bashan and de-Bashan, 2010; Hayat et al., 2010). Auxins are known to be most commonly produced by PGPR in comparison with other phytohormones such as cytokinins and gibberellins and to a lesser extent ethylene (Patten and Glick, 2002; Khalid et al., 2004). In case of

Azospirillum-plant interactions, the enhancement of plant growth is attributed mainly to the improved root development due to the production of phytohormones, in particular, auxins rather than biological nitrogen fixation (Hayat et al., 2010). Bacterial auxins can provoke diverse responses like shoot elongation, adventitious shoot induction, callogenesis, callus proliferation and hypertrophy in different media combinations (Ali and Hasnain, 2007). Phytostimulation requires an early contact between *Azospirillum* and germinating seed and the resulting enhanced root development can be short-lived or last till crop harvest (Radwan, 2002; Zemrany et al., 2006).

The main objective of the study was to evaluate the potential of *Azospirillum* as a crop inoculant to enhance the growth of plants. For this purpose, *Azospirillum* was isolated from the rhizosphere of *Oryza sativa*, *Triticum aestivum* and *Cynodon dactylon*, and screened for its potential for auxin production. Pot trials were conducted with *Triticum aestivum* L. and *Lens culinaris* Medik. to evaluate the growth promoting potential of *Azospirillum.*

MATERIALS AND METHODS

Isolation and characterization of Azospirillum

Azospirillum strains were isolated from the roots of *Oryza sativa*, *Triticum aestivum* and *Cynodon dactylon*. In order to remove adherent soil particles , the roots were washed with rapidly flowing tap water for 3-5 min and then rinsed with autoclaved, distilled water for three times. The roots were separated from each other and cut into small pieces of 1.5 cm. The root pieces were macerated with a sterile forcep and then introduced into vials containing 10 ml of semisolid Nitrogen free broth (NFB). After 72 h of incubation at 37˚C, the results were recorded for the presence of the white halo in medium. The bacterial cultures from NFB positive vials then streaked on Congo Red Agar (CRA) plates and incubated at 37˚C for 24 h. The colonies from CRA plates were picked and transferred into semi-solid NFB medium. Pellicle formation in this medium indicated successful isolation of *Azospirillum.* After isolation the strains were characterized morphologically and biochemically (Cappuccino and Sherman, 2002).

Auxin biosynthesis by Azospirillum

The quantification of auxin production was carried out in 100 ml L-broth supplemented with filter sterilized solution of L-tryptophan to a final concentrations of 0, 200, 400, 600, 800, 1000 and 1200 µg ml^{-1}. The flasks were inoculated with 100 µl of bacterial suspension adjusted to 10^7 ml^{-1}. After inoculation, flasks were incubated at 37°C for 72 h in incubated shaker in triplicate at 120 rpm min^{-1}. After incubation, bacterial cultures were centrifuged at 12000 ***g*** for 5 min. One ml of supernatant was taken and mixed with 2 ml of Salkowski reagent and test tubes were left in the dark for 30 min to develop the red color. The intensity of color was measured at 535 nm. Standard curve for IAA was drawn by using different concentrations of authentic auxin to determine auxin biosynthesis in bacterial culture supernatant.

Detection of auxin by thin layer chromatography

For thin layer chromatography (TLC), strains were grown in 100 ml of L-broth containing 1000 µg ml^{-1} of L-tryptophan and incubated at 37°C for 72 h as mentioned above. Twenty ml of bacterial culture supernatant was taken in a separate vial by centrifuging the broth at 1200 ***g*** for 5 min and pH was set up to 2.5. To this supernatant, 20 ml of ethyl acetate was added and left overnight after vigorous mixing. The upper layer of the mixture was collected separately and allowed to complete evaporation at 45°C for 4-5 h. Three ml of methanol was added to dissolve the extract left behind along the walls of beaker after evaporation of ethyl acetate. A small quantity of extract from different strains was spotted on the TLC plate along with standard IAA used as a positive control. When dried completely, the plate was kept in the solvent container with the level of methanol below the line on the plate with spots and left for some time until the solvent reaches the top of the plate. After drying, the chromatogram was observed under the UV light as well as after spraying with Salkowski reagent.

Antibiotic susceptibility of Azospirillum

The antibiotic susceptibility of *Azospirillum* strains was assessed by Kirby-Bauer method (Bauer *et al*. 1966) on Muller Hinton (MH) agar. The susceptibility pattern of the isolates was determined by using antibiotic discs of chloramphenicol (30 µg), ampicillin (10 µg), erythromycin (15 µg), oxytetracycline (30 µg) and carbenicillin (100 µg). The plates of MH agar were heavily inoculated with the respective *Azospirillum* strains to ensure the confluent growth. Five discs, 3 of each antibiotic, were placed with proper distance on the agar surface for each strain. The plates were incubated at 37°C for 24 h and the presence of zones of inhibition (mm) around the antibiotic discs was recorded.

Pot trials with Triticum aestivum and Lens culinaris

Certified seeds of *Triticum aestivum* and *Lens culinaris* were procured from Punjab Seed Corporation, Lahore, Pakistan. Healthy seeds were washed with distilled water and surface sterilized by soaking in 0.1% $HgC1_2$ solution for 10 min, followed by washing 3-4 times with autoclaved distilled water. Sterilized seeds were incubated for 20 min in bacterial suspension

adjusted to 10^7 CFU per ml. Phytostimulatory effect of rhizobacteria on plant growth was evaluated by conducting pot trials in the absence and presence of 1000 µg g^{-1} of soil of L-tryptophan. Water treated seeds were used as control. Pots amended with 50 µg IAA g^{-1} of soil were also kept for comparison to evaluate the effect of standard IAA on plant growth. For each strain and control, eight seeds were inoculated in each pot in triplicate and experiment was repeated twice. Pots were incubated at 25 °C and 12 h photoperiod. After germination, thinning was performed to leave 5 uniform seedlings in each pot. After 3 weeks, different growth parameters such as shoot length, root length, number of roots, shoot fresh weight and dry weight were recorded.

Statistics

Data was subjected to analysis of variance (ANOVA) by using SPSS 16 program. Means of different growth parameters were separated by using Duncan's multiple range test (P = 0.05).

RESULTS AND DISCUSSION

***Results*:**

Characterization of bacterial strains

The vials containing nitrogen free broth (NFB), when incubated with macerated root pieces, showed the white halo formation below the medium surface that indicated the presence of nitrogenase activity. The cultures with positive nitrogenase activity were streaked on CRA plates. The characteristic pink and wrinkled colonies were picked and transferred into semi-solid NFB medium. The white colored pellicle formation in this medium indicated successful isolation of *Azospirillum*. In total 20 bacterial strains with positive nitrogenase activity were isolated. Finally, nine bacterial strains that showed prolific growth and gram negative staining reaction were selected for further studies. The biochemical tests for isolated strains were performed and results were recorded (Table 1). All the isolated strains were found to be positive for cytochrome oxidase, catalase and nitrate reduction tests. However, for urease and methyl red tests, all tested strains were observed to be negative. Variable results were obtained by bacterial strains for indole production, Voges-Proskuer, citrate utilization and starch hydrolysis.

Table 1. Biochemical characterization of *Azospirillum* strains

Biochemical Tests	Strains								
	R1	R2	R3	R4	G1	G2	G3	G4	G5
Indole production	+	-	+	+	-	-	-	+	+
Urease	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	-	-	-	+	+
Cytochrome	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
MR	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	-	-	-	-	+
Nitrate reduction	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	-	-	-	+	-	-	-

Colorimetric analysis of bacterial auxin production

In colorimetric analysis, significant potential for auxin biosynthesis was revealed by bacterial strains upon the provision of varying concentrations of L-tryptophan. In general, more auxin was shown to be produced with the increasing concentrations of L-tryptophan in the media (Figure 1). Maximum auxin production was observed with strain G2 that showed 47 and 142 µg ml^{-1} in the absence and presence of L-tryptophan (800 µg ml^{-1}), respectively. Strains R1, G5, R4 and R3 showed approximately 10, 8, 5 and 5 folds increases, respectively, in amount of auxin produced over their respective non-supplemented controls. The presence of auxin in the ethyl acetate extracts was further confirmed by using TLC. Under UV light, parallel spots of samples and standard IAA were obtained. The plate was stained using Salkowski reagent which gave the pinkish color to the spots. On comparing the R_f of standard IAA and unknown samples, it was observed that the R_f values of unknown were very close to that of the standard IAA. That is indicative of the presence of auxin in our samples.

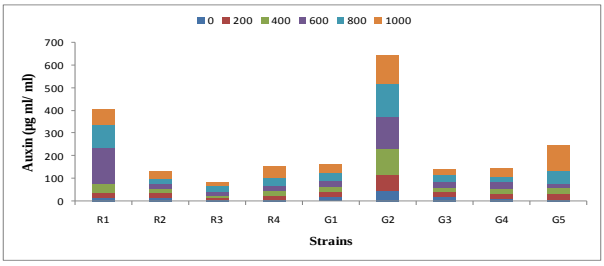


Figure 1. Auxin production by different strains of Azospirillum at increasing concentrations of L-tryptophan (0 to 1000 µg ml^{-1}). Each colored band represents amount of auxin production at specific L-tryptophan concentration

Antibiotic susceptibility of Azospirillum

Bacterial strains showed a variable pattern towards different antibiotics. Strain R1 showed complete resistance against all the antibiotics used. However, Majority of the strains were sensitive for oxytetracycline. All the strains showed resistance against erythromycin except G5 that was resistant only for the carbenicillin. While strains R2, R3 and G1 were resistant to all except oxytetracycline around which measurable zones of inhibition were present. Sensitivity was observed for oxytetracycline and carbenicillin by strains G3 and G4. Similarly R4, G3 and G4 strains showed sensitivity towards carbenicillin (Figure 2).

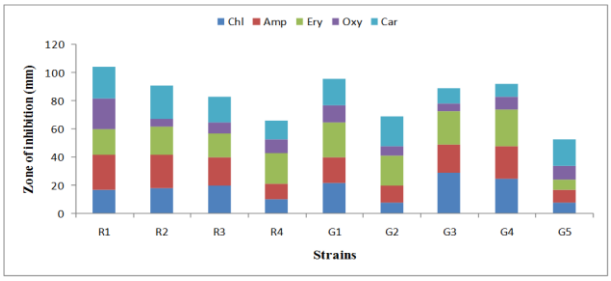


Figure 2. Susceptibility pattern of *Azospirillum* strains against different antibiotics. Each colored band represent zone of inhibition for respective antibiotic

Pot trials with Triticum aestivum

The effect of *Azospirillum* strains on the growth of *T. aestivum* was observed under axenic conditions in the presence and absence of L-tryptophan. The growth parameters considered to study were shoot length, root length, plant fresh weight, dry weight and number of roots. In case of shoot length, 31% and 27% increases were recorded with G5 and R1, respectively, in the absence of L-tryptophan (Table 1). In L-tryptophan amended soil, significant enhancements were observed with G4 (20%) and R2 (19%). Root length was greatly enhanced by G4 (60%) and R3 (56%) in the presence and absence of L-tryptophan, respectively. Moreover, 28% increases in root length was also observed at 50 µg of IAA, over control. In the case of fresh weight, strains R1 and R4 showed significant increases of 32% and 24% respectively, in the absence of L-tryptophan. Whereas, strain R1 increased the fresh weight of the plants up to 160%, over control, when soil was amended with L-tryptophan (Figure 3a). With IAA (50 µg), 16 % increase was observed in the fresh weight. Strains G3 and G1 were shown to be more effective in improving the dry weight up to 40% and 33%, respectively, in the absence of L-tryptophan. Whereas, with L-tryptophan, a significant increase of 116% was observed with the strain G2 (Figure 3b). On the other hand, IAA (50 µg) was also effectual in increasing the dry weight up to 80%. Strains G3 and G1 equally increased the number of roots up to 50% and 75% in the presence and absence of L-tryptophan, respectively. An increase in root number was observed with IAA (50 µg) (Figure 5a).

Table 2. Effect of bacterial inoculations on shoot and root length of *T. aestivum* in the absence and presence of L-tryptophan

Strains	Shoot length (cm)		Root length (cm)	
	L-tryptophan (00 µg g^{-1} of soil)	L-tryptophan (1000 µg g^{-1} of soil)	L-tryptophan (00 µg g^{-1} of soil)	L-tryptophan (1000 µg g^{-1} of soil)
Control	15.5 a	16.0 b	12.5 bc	12.9 b
IAA (50 µg)	15.2 a	17.5 c	16.2 d	15.7 d
R1	20.5 c	17.1 c	11.6 b	11.1 a
R2	16.4 ab	19.4 d	13.4 c	13.1 bc
R3	18.6 b	17.0 c	7.7 a	19.5 f
R4	16.1 ab	18.9 cd	12.9 bc	17.1 e
G1	18.9 b	16.9 bc	15.7 d	14.7 c
G2	15.7 a	19.4 d	15.7 d	17.2 e
G3	15.1 a	14.0 a	13.2 c	12.8 b
G4	15.8 a	19.6 d	20.0 f	13.5 bc
G5	21.0 d	18.5 cd	16.5 e	15.7 d

Mean±S.E. of 30 plants. Different letters within same column indicate significant difference between treatments using Duncan's multiple range test (P = 0.05)

Pot trials with Lens culinaris

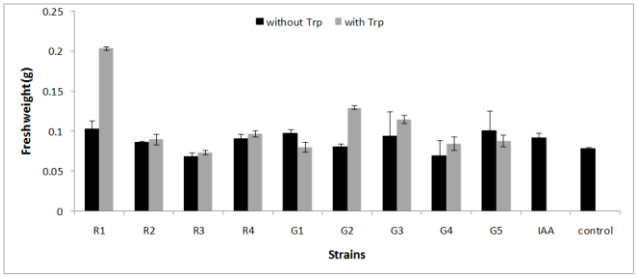
In pot trials conducted with *L. culinaris*, single bacterial cultures and strains in combination with the L-tryptophan exhibited comparable effects on different growth parameters. Shoot length was greatly enhanced by G2 (16%) and G3 (8%) in the presence and absence of L-tryptophan, respectively (Table 3). Strain R1 was equally effective in increasing the root length up to 11% both in the presence and absence of precursor. In the case of fresh weight, 57% and 52% increases were observed with strains G1 and R2, respectively, in the presence of L-tryptophan. While, in its absence strain R1 increased the fresh

weight up to 88% (Figure 4a). Moreover, increase of 52% was also observed with standard IAA (50 µg). Strain G1 was the most effective in increasing the plant dry weight up to 78% and 73% in the presence and absence of L-tryptophan, respectively (Figure 4b). Number of roots was increased with most of the strain in both the conditions. A significant increases in number of roots up to of 33%, in the absence of L-tryptophan, was obtained with strains R3, G1, G2, G3 and G5 (Figure 4b). Similarly, strains R2, R3, R4, G1, G2, G3 and G5 enhanced the number of roots up to 30% in the presence of L-tryptophan. Soil amended with standard IAA also showed increase in root number up to 30%.

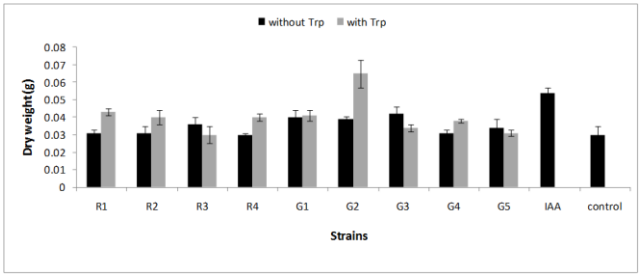
Table 3. Effect of bacterial inoculations on shoot and root length of *L. culinaris* in the absence and presence of L-tryptophan

Strains	Shoot length (cm)		Root length (cm)	
	L-tryptophan (00 µg g^{-1} of soil)	L-tryptophan (1000 µg g^{-1} of soil)	L-tryptophan (00 µg g^{-1} of soil)	L-tryptophan (1000 µg g^{-1} of soil)
Control	17.1 b	16.6 b	10.0 d	15.3 e
IAA (50 µg)	16.5 ab	18.0 c	6.8 a	7.8 a
R1	17.5 b	18.5 c	11.0 d	11 d
R2	19.8 d	17.7 bc	7.5 a	9.5 b
R3	16.9 ab	16.0 b	8.0 b	9.5 b
R4	15.1 a	20.0 d	7.5 a	10.2 c
G1	19.1 d	17.2 bc	8.8 b	10.0 c
G2	20.0 d	17.1 bc	8.2 b	9.5 b
G3	18.7 cd	18.4 c	10.1 d	7.8 a
G4	17.0 b	13.9 a	9.3 bc	7.1 a
G5	19.3 d	17.2 bc	9.6 bc	10.2 c

Mean±S.E. of 30 plants. Different letters within same column indicate significant difference between treatments using Duncan's multiple range test (P = 0.05)

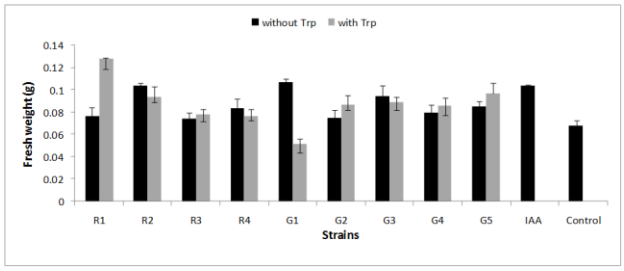


(a)



(b)

Figure 3. Effect of bacterial inoculations on biomass of *T. aestivum*. (a) fresh weight; (b) dry weight (b). Mean±S.E. of 30 plants



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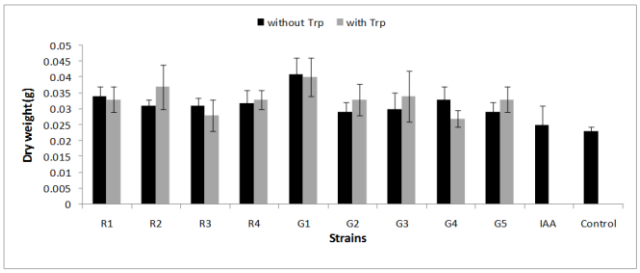
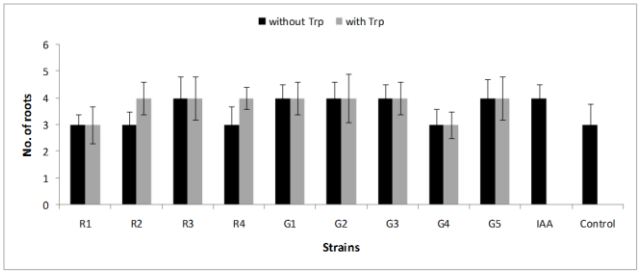


(b)

Figure 4. Effect of bacterial inoculations on biomass of *L. culinaris*. (a) fresh weight; (b) dry weight (b). Mean±S.E. of 30 plants



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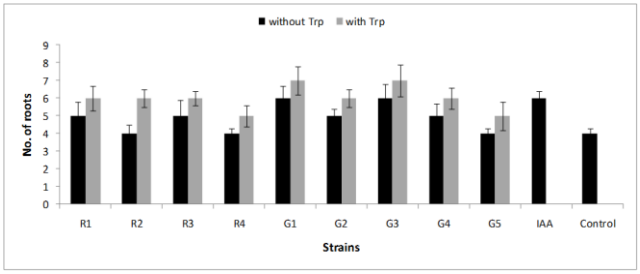

(b)

Figure 5. Effect of bacterial inoculations on number of roots. (a) *T. aestivum*, (b) *L. culinaris*
Mean±S.E. of 30 plants

DISCUSSION

The aim of the study was to evaluate the potential of *Azospirillum* as crop inoculants to enhance the growth of *Triticum aestivum* and *Lens culinaris*. *Azospirillum* strains have been isolated and employed as crop inoculants to lessen the requirements for fertilization and increase the yield of crops such as cereals and grasses. It has been reported that *Azospirillum* induces the plant growth through a variety of mechanisms such as nitrogen fixation and production of plant growth promoting substances including phytohormones, particularly auxins (Bashan and de-Bashan, 2010). Hence, our results are also in agreement with previous findings that showed the growth enhancement effect of *Azospirillum* on plants. In present work, nine strains were selected on the basis of their nitrogenase activity in nitrogen free broth (NFB). The production of auxin in liquid culture was demonstrated in purified strains of *Azospirillum*. Biosynthesis of auxin by bacterial strains showed an increase with the increase in precursor concentration. L-tryptophan is considered as an important physiological precursor of IAA biosynthesis in both plants and bacteria (Spaepen et al., 2007; Sapak et al., 2008). Maximum auxin production was recorded by strain R1 (160 µg) in the presence of L-tryptophan (800 µg ml^{-1}). Similarly, strains G2 and G5 showed auxin production up to 143 µg and 112 µg, respectively, in the presence of 800 µg and 1000 µg of L-tryptophan.

Growth promoting potential of *Azospirillum* strains was evaluated by inoculating the plants of *T. aestivum* and *L. culinaris* under axenic conditions. All of the selected strains possess significant nitrate reductase activity which is indicative of their ability to fix N_2. With *T. aestivum*, bacterized seeds showed a considerable increase in all growth parameters especially root and shoot lengths of seedlings. Under controlled laboratory conditions, bacterial strains G5, G4, R1, G3, and G1 enhanced the shoot length (30%), root length (60%), plant fresh weight (32%), plant dry weight (40%) and number of roots (50%) in the absence of IAA precursor. In a similar study it was reported that wheat seeds when treated with *Azospirillum* spp. formed longer roots (Akbari et al., 2007). With the provision of L-tryptophan, significant increases of 20% in shoot length, 160% in fresh weight, 116% in dry weight and 56% in root length were obtained respectively, with G4, R1, G2 and R3. It was observed that the L-tryptophan application to bacterial treated seeds resulted in increase of root lengths and enhanced fresh and dry weights of plant as compared to treated seeds without L-tryptophan. Different studies revealed that rhizobacteria when provided with L-tryptophan produced high amounts of IAA leading to the enhanced development of plant root system and other growth parameters (Patten and Glick, 2002; Idris et al., 2007).

Bacterization of *L. culinaris* seeds demonstrated results quite comparable in the presence and absence of L-tryptophan. Significant increases in dry weight (78%), fresh weight (52%), shoot length (16%) and number of roots (33%) were observed in the absence of L-tryptophan, respectively, with G1, R2, G2 and G3. The addition of L-tryptophan to the medium significantly enhanced the root growth due to the higher IAA production by bacteria. Root length and number of roots were found to be improved up to 11% and 33% with R1 and G3, respectively. Roots of both *T. aestivum* and *L. culinaris* seedlings acted positively in response to the addition of different concentrations of IAA which was noted as an increase in root length, dry weight and the formation of additional lateral roots. It is well known from recent study that IAA is the dominating phytohormone playing role in elongation of roots and increasing the number of lateral roots in plants (Moghadam et al., 2012).

The findings of this study provided significant evidence that auxin was produced by *Azospirillum* spp. and that it causes significant biological activity in plant roots. The development of techniques for the utilization of plant growth promoting bacteria, such as *Azospirillum*, in order to reduce rates of fertilizer application should be recommended for financial reasons and also to prevent environmental pollution by avoiding excessive applications of industrially produced fertilizers to cultivated fields. *Azospirillum* strains showed maximum production of auxins with increasing concentrations of precursor. Inoculation of *T. aestivum* and *L. culinaris* with *Azospirillum* improved different growth parameters in pot trials. This study suggested that *Azospirillum* along with L-tryptophan amendments can be used as a good biofertilizer to enhance plant growth. This study also encourages the screening of *Azospirillum* as a cost effective biofertilizers on the basis of auxin production for the agronomic crops.

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