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RESEARCH PAPER

Isolation, identification of *Azospirillum* and its inoculation effects on maize (*Zea mays* L.)

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Abstract: Among the fifty samples, white undulating pellicle in N free malate semi-solid medium was produced by all the isolates. On the malate agar media with NH_4Cl, typical isolation colonies were circular, small to medium, greenish-white with smooth texture and were opaque. All the isolates were spiral shaped and gram negative. Based on the utilization of carbon sources, thirty isolates were tentatively identified as *Azospirillum brasilense* and twenty isolates were tentatively identified as *Azospirillum amazonense.* Selected isolates were tested for their antifungal activity against *Exserohilum turcicum* under *in vitro* condition and antifungal activity of isolates was determined by the decreased diameter of fungal growth compared to the diameter of the control fungal growth. ACDAZS-35 showed the highest percentage inhibition of 85.56%. The results of pot trials showed that inoculation of maize seeds with *Azospirillum* isolates ACDAZS-09, ACDAZS-30, ACDAZS-35, ACDAZS-47 along with a reference *Azospirillum* spp. ACD-15 at different nitrogen levels showed a significant increase in number of leaves over the uninoculated control. It was found that maximum number of leaves, SPAD values, N content were observed in ACDAZS-9 inoculated with 50 % N application at 30 and 60 DAS. The present study revealed that the selected *Azospirillum* inoculants were capable of increasing the growth of maize plants and can save 25 % of inorganic nitrogen application.

Key words: *Azospirillum*, Maize, Nitrogen, Pellicle

Introduction

Maize (*Zea mays* L.) is one of the major versatile crops grown throughout the tropical as well as the temperate regions of the world. Maize is commonly called the queen of cereals. Globally, maize is grown in an area of 197.28 million hectares with a production of 1120.65 million tonnes and productivity of 5680 kg per hectare. The USA is a leading producer of maize. In India, currently, it is cultivated in an area of 9.70 million hectares with a production of 30.25 million tonnes and with a productivity of 3100 kg per hectare. Maize requires more nitrogen than other essential elements for the development at all growth stages. In view of scarcity and increasing cost of nitrogenous fertilizers, it was felt necessary to find alternate sources to provide major essential nutrients. Biological nitrogen fixation (BNF) is carried out by out by bacteria such as *Rhizobium, Azospirillum*, *Gluconacetobacter, Azotobacter etc. Azospirillum bacteriaare* gram-negative, associative nitrogen fixing rhizobacteria that are found to have a close association with plant roots. They are commonly found in soils and in association with roots of plants namely rice, maize, wheat and legumes (Kanimozhi *et al*., 2011). They can improve plant development and yield in a variety of agronomic crops under a variety of environmental and soil conditions.

Further more, microbial suppression caused by bacteriocins, siderophores phenylacetic acid production (PAA) were reported. Bacteria of the genus *Azospirillum*, which produce siderophores, have been proposed as agents for the biological control of the pathogenic plant fungi *Exserohilum turcicum* (Scavino and Pedraza 2013). The present study is to isolate bacteria of the genus *Azospirillum* associated with cereals and fodder crops to characterise them as biological agents for the control of turcicum leaf blight of maize in *in vitro* condition. These studies indicate that studies on search for efficient nitrogen fixing *Azospirillum* with biocontrol potential appears to be very scarce. Hence, the present investigation was conducted with the intention of reduction of rate of nitrogen fertilizer application.

Material and methods

The present study was carried out in the Department of Agricultural Microbiology, College of Agriculture Dharwad, University of Agricultural Sciences, Dharwad during the year 2019-2020.

The *Azospirillum* strains from samples were isolated by following the enrichment culture technique as adopted by (Dobereiner and Day, 1976) and (Baldani and Dobereiner, 1980). The fresh roots of cereals like maize, wheat, forage grasses *etc*., were collected from the fields of Dharwad district for the isolation of *Azospirillum*. The root bits were aseptically placed in tubes containing sterilized semisolid N-free malate medium (Baldani and Dobereiner, 1980). The tubes were incubated at 30^0C for one week and observed for the growth of *Azospirillum* as subsurface white undulating pellicles. The subculturing was done to confirm the *Azospirillum* isolates *i.e.,* a loopful of culture was streaked on malate agar plates containing 1 per cent NH_4Cl. After a week of incubation, typically small, white dense single colonies were picked and transferred to a culture tube containing semisolid N free malate medium. The isolates forming the characteristic subsurface white undulating pellicle in this medium were tentatively considered as *Azospirillum*. The media

used was NFb medium (g/l): L-malic acid, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.02; trace element solution ($Na_2MoO_4.2H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.235 g; H_3BO_3, 0.28 g; $CuSO_4.5H_2O$, 0.008 g; $ZnSO_4.7H_2O$, 0.024 g; distilled water, 1000 ml), 2.0 ml; bromthymol blue (0.5% aqueous solution [dissolve in 0.2 N KOH]), 2.0 ml; Fe EDTA (1.64% solution), distilled water, 1000 ml), 1.0 ml; KOH, 4.0; pH adjusted to 6.8 with KOH. The *Azospirillum* isolates were characterized morphologically, microscopically by following the standard bacteriological methods (Brenner *et al*., 2005). The Gram staining was done using 24 hours old culture by the modified procedure of Hucker's (Rangaswami and Bagyaraj, 1996). The observations of Gram reactions and cell morphology were recorded.

Tentative identification of native *Azospirillum* isolates

The *Azospirillum* isolates were grown on N free malate broth for 48 hours at 28°C (±2) over a rotary shaker (100 strokes per min) and used for biochemical tests in N free broth amended with different carbon sources (Pridham and Gottlieb, 1948). The isolates were examined for their ability to utilize different carbon sources *viz*., sucrose, glycerol, glucose and mannitol. The carbon sources were added as a sole carbon source individually to the N-free broth medium and were incubated at 30°C for seven days (Brenner *et al*., 2005). The turbidity in the media containing different carbon sources was observed visually and growth was recorded as negative for no growth and positive for turbid growth.

Screening of *Azospirillum* isolates under in vitro conditions

Phosphorous solubilization

Azospirillum isolates were tested for their ability to solubilize phosphorus by spotting 0.1µl of them on Pikovskaya's media (Pikovskaya, 1948). The clear zone of solubilization of tri-calcium phosphate (TCP) around the colony was noted as phosphate solubilizers and no zone of solubilization was recorded as negative. The media composition of pikovskaya media (g/l) was glucose , 10 ; $Ca_3(PO_4)_2$, 5 ; $(NH_4)_2SO_4$, 0.5;NaCl, 0.2;$MgSO_4.7H_2O$, 0.1;KCl, 0.2; Yeast extract, 0.5 ; $MnSO_4.H_2O$, 0.002; $FeSO_4.7H_20$, 0.002; Agar,15; distilled water, 1000 ml;pH.7.

Phosphate solubilization index was calculated by using the following formula

$$\text{Phosphate solubilization index} = \frac{\text{Total diameter (colony + halo zone)}}{\text{Diameter of colony}}$$

Siderophore assay

Siderophore production was detected by following the universal chemical assay using chrome azurol S (CAS) agar (Schwyn and Neilands, 1987). Cultures of *Azospirillum* isolates were grown in nitrogen free broth medium at 30°C for 24 h at 200 rpm on a rotary shaker. 20 µl of bacterial culture filtrate was deposited onto the CAS agar plates. Control plates have sterile broth media without bacteria. The plates were incubated at 37°C for 24, 48 and 72 h and changes in the medium were recorded. *Azospirillum* isolates producing siderophores on CAS agar plates were confirmed by a change in colour of plates from blue to yellow and no colour changes were recorded as negative.

Siderophore producing index was calculated by using the following formula

$$\text{Siderophore producing index} = \frac{\text{Total diameter (yellow zone + colony)}}{\text{Diameter of colony}}$$

***In vitro* assay of *Azospirillum* against *Exserohilum turcicum* causing Turcicum leaf blight of maize**

The assay of antagonism between *Azospirillum* and the fungal pathogen was performed by triplicate on potato infusion agar medium (Somers *et al*., 2005). Six lines of 1.5 cm with 10 µL of the bacterial inoculum each, was extended over the agar; leaving a radius of 3 cm from the center, was incubated at 30°C for 48 hr., after which the fungus was plated, with the pitting at the center of the culture medium. The control was maintained by plating of the fungus without the presence of the *Azospirillum*. The radial growth of the fungus was measured every 24 hr. from 48 hr. to eight days post-incubation. The invitro antagonistic effect was evaluated through the inhibition of radial growth and the application of an analysis of variance and Tukey test (p= 0.05) by using the following formula.(Dennis and webster., 1971). PIS agar: washed, peeled, sliced potatoes, 200 g; l-malic acid, 2.5 g; KOH, 2.0 g; raw cane sugar, 2.6 g; vitamin solution (biotin, 0.01 g; pyridoxin, 0.02 g; distilled water, 1000 ml), 1.0 ml; bromthymol blue (0.5% alcoholic solution), 2 drops; agar, 15.0 g. The potatoes are placed in a gauze bag, boiled in 1 liter of water for 30 min, then filtered through cotton, saving the filtrate. The malic acid is dissolved in 50 ml of water and the bromthymol blue added. KOH is added until the malic solution is green (pH 7.0). This solution, together with the cane sugar, vitamins and agar, is added to the potato filtrate. The final volume is made up to 1 liter with distilled water. The medium is boiled to dissolve the agar, then sterilized by autoclaving.

$$\text{Percentage inhibition} = \frac{\text{Control - Treatment}}{\text{Control}} \times 100$$

Control - Diameter of growth of *Exserohilum turcicum* in control plate.

Treatment - Diameter of growth of *Exserohilum turcicum* in treated plates.

A pot culture experiment was conducted under greenhouse conditions at the Department of Agricultural Microbiology, UAS Dharwad during the year 2020-21, to study the inoculation effect of selected *Azospirillum* isolates on growth of maize and at different nitrogen levels.

Efficient *Azospirillum* strains ACDAZS-9 (A_1), ACDAZS-30 (A_2), ACDAZS-35 (A_3), ACDAZS-47 (A_4) and ACD-15 (reference strain) were grown separately, in a 250 ml flask

containing 100 ml N-free malate broth (Nfb) for 2 days. The grown cultures were homogenizend and then used for inoculation.

Experimental details : The pot culture experiment was conducted by using two factorial complete randomized design with three replications. The experiment consisted of factor A and factor B. Factor A is level of inoculation consisting of four efficient *Azospirillum* strains, one reference strain (ACD-15) and uninoculated control. Factor B is levels of nitrogen fertilization consisting of 100% N, 75% N and 50%N as per recommended dose of fertilizer and without N fertilization. Five seeds were sown in each pots containing field soil. After 20 days, seedlings were thinned to retain 2 seedlings per pot. These seedlings were watered regularly and grown up to 60 days. The reference strain (*Azospirillum* sp. ACD-15) used in the study was obtained from institute of organic farming, Dharwad. Efficient *Azospirillum* isolates ACDAZS-9 (A_1), ACDAZS-30 (A_2), ACDAZS-35 (A_3), ACDAZS-47 (A_4) and ACD-15 (reference strain) was used in pot culture experiment. Efficient isolates, reference strain were grown in nitrogen free broth for seven days. The maize seeds are treated with these isolates with the inoculum count of 10^9 cells/ml. Number of leaves/plant being recorded at 30 and 60 DAS and the mean number of leaves/plant was calculated.The relative chlorophyll content was measured using the SPAD meter at 30 and 60 DAS.

Nitrogen content (%)

Nitrogen content of shoot and root was estimated by modified micro Kjeldahl method (Jackson, 1967) at harvest 60 DAS. The dried shoot sample (0.5 g) and root sample (0.5 g) were powdered and the powdered samples were digested with 5 ml of concentrated H_2SO_4 and 200 mg digestion catalyst (K_2SO_4:$CuSO_4$: Selenium) (100:10:1 ratio) until the contents become clear. After cooling, the volume was made up to 25 ml with distilled water. Then 5 ml of aliquot was transferred to micro Kjeldahl distillation unit. An aliquot of 10 ml of 40 percent sodium hydroxide was added and steam distilled. Ammonia evolved was collected over 2 % boric acid (20 ml) containing 2 drops of double indicator (83.3 mg bromocresol green 16.6 mg methyl red indicator dissolved in 10 ml of 95 % ethanol) and back titrated against 0.05 N H_2SO_4.

Statistical analysis of the data

The data recorded on various parameters in the present study were subjected to Fisher's method of analysis of variance and interpretation of data as given by Gomez and Gomez (1984). The level of significance used in 'F' test and 't' test was P= 0.01 in pot culture experiments. Duncan's multiple range test (DMRT) is used in this study to measure specific differences between pairs of means.

Result and discussion

N free malate semi-solid media was used for the isolation of *Azospirillum* bacteria. Pellicle in the N free malate semi-solid media was produced in all the fifty test tubes inoculated with root system. Different morphological attributes of all the fifty *Azospirillum* isolates were noted and tabulated in Table 1. On the malate agar media with NH_4Cl, typical isolated colonies were circular, small to medium, greenish-white, smooth texture and opaque.The results related to colony morphology were similar to those *Azospirillum* bacteria described in Bergey's Manual of Systematic Bacteriology (Vol.II) (Brenner *et al*., 2005). Similar observations were reported by Vijayalakshmi *et al*., (2019); Senthil and Paneerselvam (2013). All the fifty isolates were observed for their shape in the microscope and gram's reaction. Microscopic observations of all the isolates were noted. All the isolates were spiral shaped and gram negative. These findings were in agreement with the reports of Tarrand *et al*., (1978) .

Utilization of different carbon sources

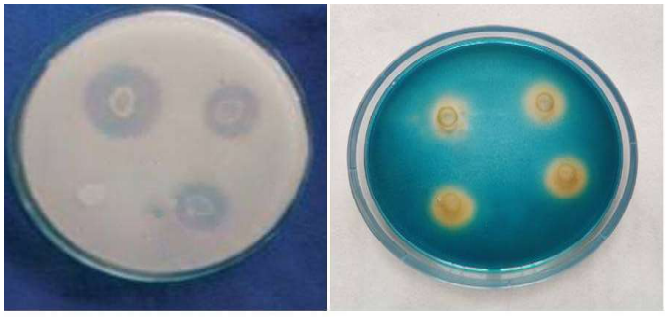
All the fifty isolates were tested for the utilization of different carbon sources. Results are presented in Table 2. Based on the utilization of carbon sources, thirty isolates were tentatively identified as *Azospirillum brasilense* and twenty isolates were tentatively identified as *Azospirillum amazonense*. Classification of the genus *Azospirillum*, according to Bergey's Manual of Systematic Bacteriology (Vol. II) (Brenner *et al*., 2005).

Screening of native *Azospirillum* isolates under *in vitro* conditions

All the fifty isolates were screened for P- solubilization, siderophore assay. Results for functional characterization are presented in Table 3 and figure 1.

P- solubilization

Among fifty isolates, twenty isolates solubilized the phosphorus while thirty isolates did not solubilize the phosphorous. Phosphate solubilization index was ranged from 1.5-3.5. The maximum phosphate solubilization index was found



P-solubilization **Siderophore production**

Fig.1: Functional characterization of *Azospirillum* isolates

in ACDAZS-09 and the minimum phosphate solubilization index was found in ACDAZS-16.These results were similar to those observation of previous studies (Rahman *et al*., 2006 and Gandhimaniyan *et al*., 2020).

Siderophore assay

Among fifty isolates, sixteen isolates were found to produce siderophore, they produced yellow halo zone in CAS media and thirty-four isolates did not produce siderophore. Siderophore producing index was ranged from 1.7-3.4. The

Table 1. Morphological characterization of native *Azospirillum* isolates grown on solid malate agar medium and their gram reaction

Isolate code	Colony shape	Size	Colour	Texture	Elevation	Margin	Opacity	Gram reaction	Cell shape
ACDAZS-1	Circular	Medium	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-2	Circular	Small	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-3	Circular	Small	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-4	Circular	Small	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-5	Circular	Small	Light green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-6	Circular	Medium	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-7	Circular	Medium	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-8	Circular	Medium	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-9	Circular	Medium	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-10	Circular	Medium	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-11	Circular	Small	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-12	Circular	Small	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-13	Circular	Medium	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-14	Circular	Medium	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-15	Circular	Medium	Green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-16	Circular	Medium	Creamy white	Rough	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-17	Circular	Medium	Green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-18	Circular	Medium	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-19	Circular	Medium	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-20	Circular	Small	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-21	Circular	Medium	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-22	Circular	Medium	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-23	Circular	Small	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-24	Circular	Medium	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-25	Circular	Medium	Green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-26	Circular	Small	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-27	Circular	Small	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-28	Circular	Small	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-29	Circular	Small	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-30	Circular	Medium	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-31	Circular	Small	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-32	Circular	Medium	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-33	Circular	Medium	Creamy white	Wrinkle	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-34	Circular	Medium	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-35	Circular	Small	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-36	Circular	Medium	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-37	Circular	Medium	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-38	Circular	Small	Creamy white	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-39	Circular	Medium	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-40	Circular	Small	Creamy white	Rough	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-41	Circular	Medium	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-42	Circular	Small	Light green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-43	Circular	Medium	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-44	Circular	Small	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-45	Circular	Small	Creamy white	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-46	Circular	Small	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-47	Circular	Medium	Light green	Smooth	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-48	Circular	Medium	Dark green	Smooth	Raised	Entire	Opaque	-ve	Spiral
ACDAZS-49	Circular	Small	Dark green	Rough	Convex	Entire	Opaque	-ve	Spiral
ACDAZS-50	Circular	Small	Dark green	Smooth	Convex	Entire	Opaque	-ve	Spiral

(Size: Small - <3 mm, Medium - 3 to 5 mm)

maximum siderophore producing index was found in ACDAZS-36 and the minimum siderophore producing index was found in ACDAZS-31.These results were in confirmitivity with the description of Mall *et al*. (2020) and Sahu *et al*. (2017).

In *vitro* assay of *Azospirillum* against *Exserohilum turcicum* causing Turcicum leaf blight of maize

The four strains of *Azospirillum* and reference strain (*Azospirillum* spp. ACD-15) were selected based on the

Table 2. Tentative identification of native Azospirillum isolates by carbon utilization test

Isolate Code	Carbon Source				Tentative Identification
	Glucose	Sucrose	Glycerol	Mannitol	
ACDAZS-1	-	-	+	-	Azospirillum brasilense
ACDAZS-2	+	+	-	-	Azospirillum amazonense
ACDAZS-3	-	-	+	-	Azospirillum brasilense
ACDAZS-4	-	-	+	-	Azospirillum brasilense
ACDAZS-5	-	-	+	-	Azospirillum brasilense
ACDAZS-6	-	-	+	-	Azospirillum brasilense
ACDAZS-7	-	-	+	-	Azospirillum brasilense
ACDAZS-8	+	+	-	-	Azospirillum amazonense
ACDAZS-9	+	+	-	-	Azospirillum amazonense
ACDAZS-10	-	-	+	-	Azospirillum brasilense
ACDAZS-11	-	-	+	-	Azospirillum brasilense
ACDAZS-12	-	-	+	-	Azospirillum brasilense
ACDAZS-13	-	-	+	-	Azospirillum brasilense
ACDAZS-14	+	+	-	-	Azospirillum amazonense
ACDAZS-15	-	-	+	-	Azospirillum brasilense
ACDAZS-16	+	+	-	-	Azospirillum amazonense
ACDAZS-17	-	-	+	-	Azospirillum brasilense
ACDAZS-18	+	+	-	-	Azospirillum amazonense
ACDAZS-19	+	+	-	-	Azospirillum amazonense
ACDAZS-20	+	+	-	-	Azospirillum amazonense
ACDAZS-21	+	+	-	-	Azospirillum amazonense
ACDAZS-22	+	+	-	-	Azospirillum amazonense
ACDAZS-23	+	+	-	-	Azospirillum amazonense
ACDAZS-24	-	-	+	-	Azospirillum brasilense
ACDAZS-25	+	+	-	-	Azospirillum amazonense
ACDAZS-26	+	+	-	-	Azospirillum amazonense
ACDAZS-27	-	-	+	-	Azospirillum brasilense
ACDAZS-28	-	-	+	-	Azospirillum brasilense
ACDAZS-29	-	-	+	-	Azospirillum brasilense
ACDAZS-30	-	-	+	-	Azospirillum brasilense
ACDAZS-31	-	-	+	-	Azospirillum brasilense
ACDAZS-32	-	-	+	-	Azospirillum brasilense
ACDAZS-33	-	-	+	-	Azospirillum brasilense
ACDAZS-34	-	-	+	-	Azospirillum brasilense
ACDAZS-35	-	-	+	-	Azospirillum brasilense
ACDAZS-36	+	+	-	-	Azospirillum amazonense
ACDAZS-37	-	-	+	-	Azospirillum brasilense
ACDAZS-38	+	+	-	-	Azospirillum amazonense
ACDAZS-39	-	-	+	-	Azospirillum brasilense
ACDAZS-40	+	+	-	-	Azospirillum amazonense
ACDAZS-41	-	-	+	-	Azospirillum brasilense
ACDAZS-42	+	+	-	-	Azospirillum amazonense
ACDAZS-43	-	-	+	-	Azospirillum brasilense
ACDAZS-44	+	+	-	-	Azospirillum amazonense
ACDAZS-45	-	-	+	-	Azospirillum brasilense
ACDAZS-46	+	+	-	-	Azospirillum amazonense
ACDAZS-47	+	+	-	-	Azospirillum amazonense
ACDAZS-48	+	+	-	-	Azospirillum amazonense
ACDAZS-49	+	+	-	-	Azospirillum amazonense
ACDAZS-50	-	-	+	-	Azospirillum brasilense

(+: positive/good growth, -: negative/no growth)

production of siderophore producing index, against fungus *Exserohilum turcicum* causing Turcicum leaf blight of maize. *Azospirillum* isolates inhibited the growth of fungus as revealed by the decreased diameter of fungal growth compared to the diameter of the control fungal and reference strain (ACD-15) growth without the presence of bacteria. The data related to antagonistic test of *Azospirillum* against *Exserohilum turcicum* causing Turcicum leaf blight of maize is tabulated in Table 4 and fig. 2. The radial growth of fungus was measured every 24 hr up to 8 days after incubation. Among the different treatments ACDAZS-35 showed the highest percentage inhibition of 85.56% in all the days. Reference strain showed a

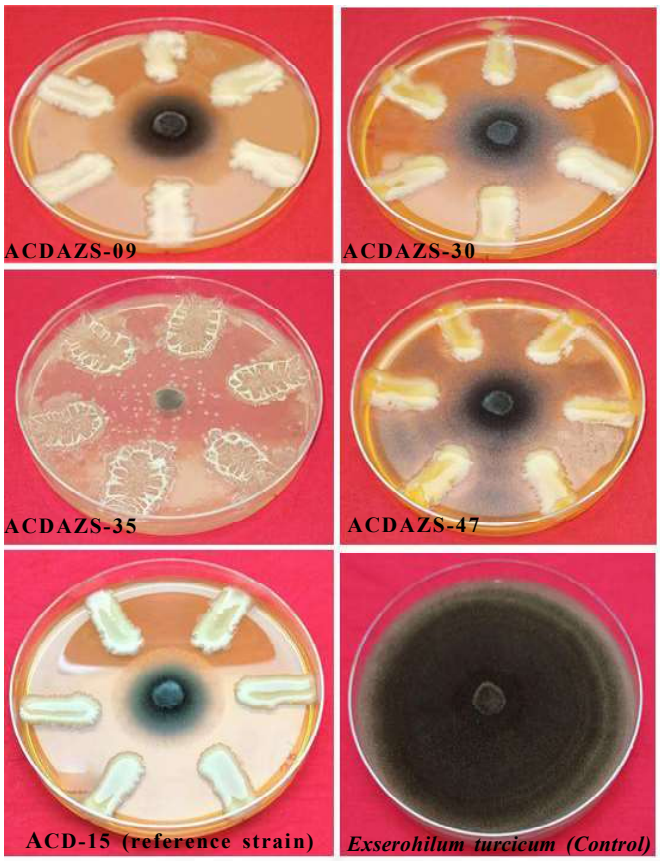


Fig. 2. In vitro *assay of Azospirillum* against *Exserohilum turcicum* causing *Turcicum* leaf blight of maize

percentage inhibition of 38.89%. The lowest percentage inhibition was observed in ACDAZS-47 of 3.33%. The eight-day percent growth inhibition of *Exserohilum turcicum* is presented in plate 5. The inhibition of growth of *Exserohilum turcicum* by *Azospirillum* in in vitro condition could be due to production of siderophores and other unknown metabolites of *Azospirillum* isolates. These results were in agreement with the findings of (Scavino and Pedraza 2013), (Hungria *et al*, 2010), (Zaheer *et al*. 2019).

The production of siderophores by *Azospirillum* may confer a survival advantage on the maize. The *Azospirillum* bacteria fixes atmospheric nitrogen and produces metabolites with growth promoter effects. A.*brasilense* strains had antagonistic effects in in vitro tests against maize phytopathogenic fungi. The production of siderophores is the antagonistic mechanism involved in the inhibition of fungi, e.g., salicyclic acid, with one molecule that can capture the iron precursor being the catechol-type siderophores and which is a process controller for systemic resistance in plants (Scavino and Pedraza 2013).A.*brasilense* can displace pathogenic fungi, which helps to reduce the severity of the disease and promotes plant growth. This can also be attributed to the nitrogen fixation and the production of plant growth promoting substances (Hungria *et al*, 2010). Indirect mechanisms for disease control include competition for nutrients, excluding niche, the induction of systemic resistance and the production of antifungal metabolites such as hydrogen cyanide and phenazines (Zaheer *et al*. 2019).

Table 3. Functional characterization of native Azospirillum isolates

Isolate code	Phosphate solubilization index	Siderophore producing index
ACDAZS-1	1.3	2.0
ACDAZS-2	1.1	1.4
ACDAZS-3	0.0	0.0
ACDAZS-4	1.4	1.2
ACDAZS-5	2.1	1.4
ACDAZS-6	3.0	1.1
ACDAZS-7	1.3	1.4
ACDAZS-8	0.0	2.4
ACDAZS-9	3.5	3.1
ACDAZS-10	2.8	2.0
ACDAZS-11	0.0	2.7
ACDAZS-12	1.9	0.0
ACDAZS-13	0.0	2.3
ACDAZS-14	1.8	2.5
ACDAZS-15	1.6	2.0
ACDAZS-16	1.5	0.0
ACDAZS-17	3.0	1.6
ACDAZS-18	2.1	1.4
ACDAZS-19	0.0	1.5
ACDAZS-20	3.2	0.0
ACDAZS-21	0.0	2.3
ACDAZS-22	3.1	2.6
ACDAZS-23	2.6	0.0
ACDAZS-24	2.4	1.4
ACDAZS-25	0.0	1.5
ACDAZS-26	0.7	0.0
ACDAZS-27	0.9	0.5
ACDAZS-28	0.6	0.0
ACDAZS-29	0.5	3.1
ACDAZS-30	2.5	1.9
ACDAZS-31	1.4	1.7
ACDAZS-32	1.2	2.2
ACDAZS-33	1.5	2.5
ACDAZS-34	1.3	2.5
ACDAZS-35	3.3	2.8
ACDAZS-36	2.9	3.4
ACDAZS-37	1.5	1.4
ACDAZS-38	1.5	1.0
ACDAZS-39	0.0	1.3
ACDAZS-40	2.7	2.1
ACDAZS-41	2.6	0.0
ACDAZS-42	2.5	1.4
ACDAZS-43	0.0	0.0
ACDAZS-44	3.0	2.8
ACDAZS-45	0.0	0.9
ACDAZS-46	2.9	1.2
ACDAZS-47	3.1	2.4
ACDAZS-48	1.3	2.1
ACDAZS-49	1.2	0.0
ACDAZS-50	0.0	1.5
ACD-15 (reference strain)	3.0	2.1
S. Em (±)	0.053	0.061
C.D. (P=0.01)	0.150	0.162

Influence of selected *Azospirillum* isolates on the number of leaves of maize at different nitrogen levels

The effect of inoculating effective *Azospirillum* isolates at varied levels of nitrogen in maize was studied in a pot culture trial with two factors. The experiment consisted of inoculation

Table 4. *In vitro* assay of *Azospirillum* against *Exserohilum turcicum* causing *Turcicum* leaf blight of maize

Treatments (*Azospirillum* isolates)	Percent growth inhibition							
	First day	Second day	Third day	Fourth day	Fifth day	Sixth day	Seventh day	Eight day
ACDAZS-9	35.00	50.00	25.00	34.55	32.43	32.50	24.10	30.00
ACDAZS-30	30.00	17.86	9.09	18.18	29.73	18.75	15.66	32.22
ACDAZS-35	55.00	67.86	77.27	80.00	83.78	83.75	84.34	85.56
ACDAZS-47	25.00	39.29	6.82	18.18	31.08	23.75	10.84	3.33
ACD-15	50.00	60.71	45.45	40.00	52.70	46.25	37.35	38.89
S. Em.(±)	0.983	1.215	1.013	1.072	1.217	1.139	1.050	1.122
C.D.(P=0.01)	3.06	3.78	3.15	3.34	3.79	3.54	3.27	3.49

*Angular transformed value

Table 5. Production of number of leaves of maize as influenced by selected *Azospirillum* isolates at different nitrogen levels

Treatment *Azospirillum* isolates(A)	No. of Leaves/plant									
	30 DAS					60 DAS				
	Nitrogen levels (F)									
	F_1	F_2	F_3	F_4	Mean	F_1	F_2	F_3	F_4	Mean
ACDAZS-9 (A_1)	6.67^{b-f}	8.00^{b}	8.17^{a*}	6.00^{d-g}	7.21	8.67^{c-e}	9.17^{bc}	10.00^{a*}	7.83^{gh}	8.92
ACDAZS-30 (A_2)	7.50^{ab}	6.17^{c-g}	6.00^{d-g}	5.83^{fg}	6.38	8.50^{d-f}	9.33^{b}	8.00^{fg}	7.17^{i}	8.25
ACDAZS-35 (A_3)	6.67^{b-f}	7.00^{bc}	7.00^{bc}	5.67^{g}	6.58	9.00^{b-d}	8.00^{fg}	9.17^{bc}	8.00^{fg}	8.54
ACDAZS-47 (A_4)	7.00^{bc}	7.50^{ab}	6.83^{b-d}	6.17^{c-g}	6.83	8.17^{c-g}	8.33^{c-g}	7.17^{i}	6.33^{j}	7.50
ACD-15 (A_5)	6.83^{b-d}	7.33^{ab}	8.17^{a*}	6.00^{d-g}	7.08	8.17^{c-g}	8.00^{fg}	10.00^{a*}	7.33^{hi}	8.38
UIC (A_6)	6.00^{d-g}	5.87^{fg}	5.83^{gh}	5.17^{h}	5.70	7.83^{gh}	7.33^{hi}	8.00^{fg}	6.33^{j}	7.38
Mean	6.78	6.98	6.99	5.78		8.39	8.36	8.72	7.17	
	S. Em (±)		C.D.(P=0.01)			S. Em (±)		C.D.(P=0.01)		
Isolates (A)	0.14		0.55			0.10		0.37		
Nitrogen fertilizer level (F)	0.12		0.45			0.08		0.30		
Interaction (A X F)	0.29		1.09			0.20		0.74		

Note: - * Means followed by same letters did not differ significantly. ** S. Em; Applicable to Duncan's Multiple Range Test
Level of nitrogen fertilizer 1 (F_1) – 100 per cent N as per RDF
Level of nitrogen fertilizer 2 (F_2) – 75 per cent N as per RDF
Level of nitrogen fertilizer 3 (F_3) – 50 per cent N as per RDF
Level of nitrogen fertilizer 4 (F_4) – 0 per cent N as per RDF

of ACDAZS-9 (A_1), ACDAZS-30 (A_2), ACDAZS-35 (A_3), ACDAZS-47 (A_4) along with a reference strain ACD-15 (A_5) and uninoculated control (A_6) treatments with four different nitrogen levels *i.e.,* 100 percent (F_1), 75 per cent (F_2), 50 per cent (F_3) and 0 per cent (F_4). Number of leaveswere recorded at 30 and 60 DAS. The data on the effects of Nitrogenous fertilizer (F) and *Azospirillum* inoculation (A) either alone or in combination on the number of leaves per plant are presented in Table 5. All the treatments stimulated the number of leaves over the control at different fertilizer dose and selected *Azospirillum* inoculation.

At 30 DAS, with the different levels of N fertilizer application, inoculation of all the selected isolates and *Azospirillum* spp. ACD-15 (Reference strain) resulted in producing higher number of leaves over uninoculated control. In general, plant inoculated with all the selected isolates and reference *Azospirillum* strain produced the maximum number of leaves at 50% N and among the selected isolates of *Azospirillum* and reference strain, inoculation of ACDAZS-9 (A_1) resulted in maximum number of leaves. Inoculation of ACDAZS-9 (A_1) influenced the production of maximum number of leaves at 50% N (F_3) which was 36.71% more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The order of significant increase in the number of leaves due to inoculation of different *Azospirillum* isolates at different dose of N fertilizer was A_1F_3 (8.17 leaves/plant) > A_5F_3 (8.17 leaves/plant) > A_1F_2 (8.00 leaves/plant) > A_4F_2 (7.50 leaves/plant) which was more than number of leaves per plant at 100% N alone without inoculation *i.e.,* A_6F_1 (6.00 leaves/plant). The increase in number of leaves due to inoculation of *Azospirillum* isolate ACDAZS-9 (A_1) at 0% N (F_4) fertilizer was 13.83% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4).

At 60 DAS, with the different levels of N fertilizer application, inoculation of all the selected isolates and *Azospirillum* spp. ACD-15 (Reference strain) resulted in producing higher number of leaves over uninoculated control. In general, plant inoculated with all the selected isolates and reference *Azospirillum* strain produced the maximum number of leaves at 50% N and among the selected isolates of *Azospirillum* and reference strain, inoculation of ACDAZS-9 (A_1) resulted in maximum number of leaves. Inoculation of ACDAZS-9 (A_1) influenced the production of maximum number of leaves at 50%N (F_3) which was 57.97% more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The order of significant increase in the number of leaves due to inoculation of different *Azospirillum* isolates at different dose of N fertilizer was A_1F_3 (10.00 leaves/plant) > A_5F_3 (10.00 leaves/plant) > A_2F_2 (9.33 leaves/plant) > A_1F_2 (9.17 leaves/plant) which was more than number of leaves per plant at 100% N alone without inoculation *i.e.,* A_6F_1 (7.83 leaves/plant). The increase in number of leaves due to

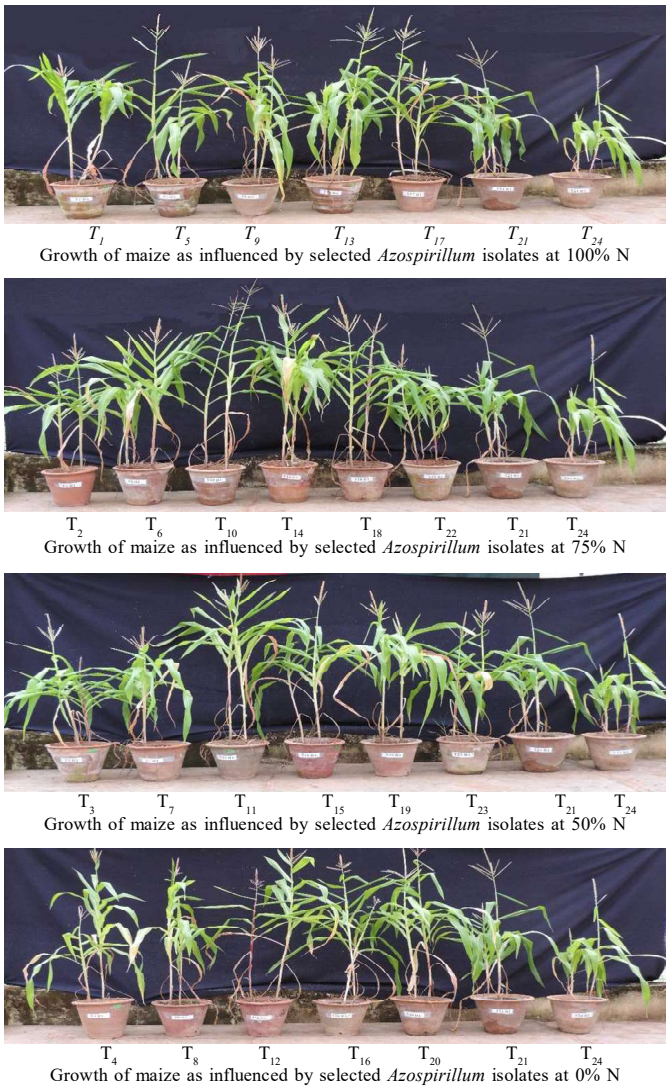


Fig. 3. Growth of maize as influenced by selected *Azospirillum* isolates at 60 DAS at different levels of nitrogen

inoculation of *Azospirillum* isolate ACDAZS-9 (A_1) at 0% N (F_4) fertilizer was 11.76% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4). A pot culture experiment on wheat to evaluate the inoculation of *Azospirillum brasilense* at two levels of nitrogen application (full and half strength) was conducted by El Sayed *et al*. (2015). Shoot length, root dry weight, number of leaves, root length, shoot N uptake, root N uptake and chlorophyll content were all increased after soil was inoculated with *Azospirillum brasilense* HM 1. A. *brasilense* TPS05 strain produced the highest number of tillers, roots, leaves, leaf length and breadth, shoot and root length and grain yield when compared to other isolates. These results indicated that A. brasilense TPS05 has a high potential for getting higher rice production. (Kanimozhi *et al*., 2015)

In the present study, interaction of 75% N and ACDAZS-35 inoculation positively benefitted the plants with enhanced growth with respect to plant height under pot culture trial condition.

Relative chlorophyll content (SPAD values)

SPAD values were recorded at 30 and 60 DAS. The data on the effects of nitrogenous fertilizer (F) and *Azospirillum* inoculation (A) either alone or in combination on the relative chlorophyll content are presented in Table 6. All the treatments stimulated the root lengthover the control at different fertilizer dose and selected *Azospirillum* inoculation.

At 30 DAS, with the different levels of N fertilizer application, inoculation of all the selected isolates and *Azospirillum* spp. ACD-15 (Reference strain) resulted in producing higher relative chlorophyll content over uninoculated control. In general, plant inoculated with all the selected isolates and reference *Azospirillum* strain produced the maximum SPAD values at 75% N and among the selected isolates of *Azospirillum* and reference strain, inoculation of ACD-15 (A_5) resulted in maximum SPAD values. Inoculation of ACD-15 (A_5) influenced the

Table 6. Influence of selected *Azospirillum* isolates on relative chlorophyll content in maize at different nitrogen levels

Treatment	Relative chlorophyll content (SPAD values)									
	30 DAS					60 DAS				
Azospirillum isolates(A)	Nitrogen levels (F)									
	F_1	F_2	F_3	F_4	Mean	F_1	F_2	F_3	F_4	Mean
ACDAZS-9 (A_1)	33.67^{bc}	27.60^{g}	23.23^{ij}	21.53^{jk}	26.26	26.63^{ab}	26.70^{a*}	23.9^{b}	21.53^{e}	24.69
ACDAZS-30 (A_2)	33.87^{bc}	33.47^{bc}	26.90^{f}	24.13^{gh}	30.34	20.27^{fg}	20.10^{fg}	19.27^{h}	19.17^{j}	19.70
ACDAZS-35 (A_3)	35.23^{ab}	31.73^{c-e}	30.23^{e}	29.47^{f}	30.18	23.97^{b}	22.6^{cd}	19.17^{h}	18.23^{ij}	20.99
ACDAZS-47 (A_4)	33.70^{bc}	31.27^{de}	29.70^{e}	26.03^{fg}	32.03	22.20^{de}	20.8^{f}	18.07^{j}	15.70^{k}	19.19
ACD-15 (A_5)	35.83^{a*}	35.73^{a*}	30.97^{de}	29.33^{f}	32.75	24.17^{bc}	22.20^{cd}	21.30^{e}	20.13^{fg}	21.95
UIC (A_6)	29.30^{f}	27.43^{f}	24.43^{gh}	19.30^{j}	25.12	19.10^{j}	18.77^{hi}	17.90^{j}	17.17^{k}	18.23
Mean	34.10	31.54	27.87	25.02		22.72	21.86	19.93	18.66	
		S. Em (±)		C.D.(P=0.01)			S. Em (±)		C.D.(P=0.01)	
Isolates (A)		0.32		1.21			0.09		0.33	
Nitrogen fertilizer level (F)		0.26		0.99			0.07		0.27	
Interaction (A X F)		0.64		2.42			0.18		0.67	

Note: - * Means followed by same letters did not differ significantly. ** S. Em;Applicable to Duncan's Multiple Range Test

Level of nitrogen fertilizer 1 (F_1) – 100 percent N as per RDF

Level of nitrogen fertilizer 2 (F_2) – 75 percent N as per RDF

Level of nitrogen fertilizer 3(F_3) – 50 percent N as per RDF

Level of nitrogen fertilizer 4 (F_4) – 0 percent N as per RDF

production of maximum SPAD values at 75%N (F_2) which was 85.64% more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The order of significant increase in the relative chlorophyll content due to inoculation of different *Azospirillum* isolates at different dose of N fertilizer was A_5F_1 (ACD-15;35.83) > A_5F_2 (ACD-15;35.73) > A_2F_1 (ACDAZS-30;33.87) > A_4F_1 (ACDAZS-47;33.70) > A_2F_2 (ACDAZS-30;33.47) which was more than relative chlorophyll content at 100% N alone without inoculation i.e., A_6F_1 (29.30). The increase in relative chlorophyll content due to inoculation of *Azospirillum* isolate ACD-15 (A_5) at 0% N (F_4) fertilizer was 51.96% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4).

At 60 DAS, with the different levels of N fertilizer application, inoculation of all the selected isolates and *Azospirillum* spp. ACD-15 (Reference strain) resulted in producing higher relative chlorophyll content over uninoculated control. In general, plant inoculated with all the selected isolates and reference *Azospirillum* strain produced the maximum SPAD values at 75% N and among the selected isolates of *Azospirillum* and reference strain, inoculation of ACDAZS-9 (A_1) resulted in maximum SPAD values. Inoculation of ACDAZS-9 (A_1) influenced the production of maximum SPAD values at 75%N (F_2) which was 55.50% more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The order of significant increase in the relative chlorophyll content due to inoculation of different *Azospirillum* isolates at different dose of N fertilizer was A_1F_2 (ACDAZS-9;26.70) > A_1F_1 (ACDAZS-9;26.63) > A_5F_1 (ACD-15;24.17) > A_3F_1 (ACDAZS-35;23.97) which was more than relative chlorophyll content at 100% N alone without inoculation i.e., A_6F_1 (19.10). The increase in relative chlorophyll content due to inoculation of *Azospirillum* isolate ACDAZS-9 (A_1) at 0% N (F_4) fertilizer was 25.39% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4).

The SPAD values in forages was positively influenced by N rates and by inoculation with *Azospirillum*. The increases obtained were attributed to the inclusion of additional nitrogen from both mineral source and biological fixation by bacteria through BNF, since the availability of N to the plant is fundamental for the survival and appearance of new tillers (Benin *et al*., 2012). The improved growth of maize due to inoculation of *Azospirillum* in terms of relative chlorophyll contentin the present study confirmed the findings of inoculating *Azospirillum* by (wheat) El Sayed *et al*. (2015); (maize) Karimi *et al*. (2018); (rice) Primadi *et al*. (2015).

Plant nitrogen concentration (%)

The nitrogen concentration *viz*., shoot and root N concentrationwas recorded at 60 DAS. The data on the effects of nitrogenous fertilizer (F) and Azospirillum inoculation (A) either alone or in combination on the plant dry biomass are presented in Table 7.

Shoot N concentration

At 60 DAS, with the different levels of N fertilizer application, inoculation of all the selected isolates and *Azospirillum* spp. ACD-15 (Reference strain) resulted in producing higher shoot

Table 7. Effect of inoculation of selected *Azospirillum* isolates on shoot, root and total N concentration of maize

Treatments	Nitrogen (%) at 60 DAS														
Azospirillumisolates (A)	Shoot N (%)					Root N (%)					Total N(%)				
	Nitrogen levels (F)														
	F_1	F_2	F_3	F_4	Mean of I	F_1	F_2	F_3	F_4	Mean of I	F_1	F_2	F_3	F_4	Mean of I
ACDAZS-9 (A_1)	3.10^{f}	3.70^{c}	2.40^{ji}	1.90^{l}	2.78	2.40^{f}	3.00^{c}	1.83^{ij}	1.30^{k}	2.13	5.50^{a-e}	6.70^{a-c}	4.23^{b-f}	3.20^{d-f}	4.91
ACDAZS-30 (A_2)	2.93^{fg}	3.53^{cd}	2.20^{k}	1.73^{lm}	2.60	2.30^{fg}	2.70^{e}	1.80^{ij}	1.20^{k}	2.00	5.23^{b-e}	6.23^{a-d}	4.00^{b-f}	2.93^{ef}	4.60
ACDAZS-35 (A_3)	3.63^{c}	4.90^{a*}	2.23^{jk}	2.10^{k}	3.22	3.30^{b}	3.63^{a*}	2.77^{de}	2.10^{gh}	2.88	6.93^{a-c}	8.53^{a*}	5.00^{b-f}	4.20^{b-f}	6.17
ACDAZS-47 (A_4)	2.90^{g}	3.30^{e}	2.60^{h}	1.90^{l}	2.68	3.03^{c}	2.40^{f}	1.63^{j}	1.20^{k}	2.07	5.93^{a-e}	5.70^{a-e}	4.23^{b-f}	3.10^{d-f}	4.74
ACD-15 (A_5)	3.37^{de}	3.90^{b}	2.43^{ij}	2.13^{k}	2.96	2.97^{cd}	3.30^{b}	2.37^{f}	2.07^{gh}	2.53	6.33^{a-d}	7.20^{ab}	4.80^{b-f}	4.20^{b-f}	5.63
UIC (A_6)	2.13^{k}	1.87^{l}	1.69^{lm}	1.00^{m}	1.67	2.20^{fgh}	1.97^{hi}	1.40^{k}	0.82^{l}	1.83	4.33^{b-f}	3.83^{c-f}	3.09^{d-f}	1.82^{f}	3.27
Mean of N	3.01	3.53	2.26	1.79		2.86	2.83	1.97	1.30		5.71	6.37	4.23	3.24	
	S. Em (±) **		C.D. (P = 0.01)			S. Em (±)		C.D. (P = 0.01)			S. Em (±)		C.D. (P = 0.01)		
Inoculation (A)	0.03	0.12				0.04	0.14				0.48	1.81			
Nitrogen fertilizer level (F)	0.03	0.10				0.03	0.12				0.39	1.47			
Interaction (A × F)	0.06	0.24				0.08	0.28				0.95	3.61			

Note: - * Means followed by same letters did not differ significantly. **S. Em; Applicable to Duncan's Multiple Range Test

Level of nitrogen fertilizer 1 (F_1) – 100 percent N as per RDF
Level of nitrogen fertilizer 2 (F_2) – 75 percent N as per RDF
Level of nitrogen fertilizer 3(F_3) – 50 percent N as per RDF
Level of nitrogen fertilizer 4 (F_4) – 0 percent N as per RDF

N concentration over uninoculated control. In general, plant inoculated with all the selected isolates and reference *Azospirillum* strain produced the maximum shoot N concentration at 75% N and among the selected isolates of *Azospirillum* and reference strain, inoculation of ACDAZS-35 (A_3) resulted in maximum shoot N concentration. Inoculation of ACDAZS-35 (A_3) influenced the production of maximum shoot N concentration at 75%N (F_2) which was 79.59% more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The order of significant increase in the shoot N concentration due to inoculation of different *Azospirillum* isolates at different dose of N fertilizer was A_3F_2 (4.90 %) > A_5F_2 (3.90 %) > A_1F_2 (3.70 %) > A_2F_2 (3.53%) which was more than shoot N concentration at 100% N alone without inoculation *i.e.*, A_6F_1 (2.13%). The increase in shoot N concentration due to inoculation of *Azospirillum* isolate ACDAZS-35 (A_3) at 0% N (F_4) fertilizer was 52.38% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The shoot N concentration at only 100%N without inoculation (UIC;2.13 %) is on par with the shoot N concentration with inoculation (A_3F_4;2.10 %) at 0% N.

Root N concentration

At 60 DAS, with the different levels of N fertilizer application, inoculation of all the selected isolates and *Azospirillum* spp. ACD-15 (Reference strain) resulted in producing higher root N concentration over uninoculated control. In general, plant inoculated with all the selected isolates and reference *Azospirillum* strain produced the maximum root N concentration at 75% N and among the selected isolates of *Azospirillum* and reference strain, inoculation of ACDAZS-35 (A_3) resulted in maximum root N concentration. Inoculation of ACDAZS-35 (A_3) influenced the production of maximum root N concentration at 75% N (F_2) which was 77.41% more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The order of significant increase in the root N concentration due to inoculation of different Azospirillum isolates at different dose of N fertilizer was A_3F_2 (3.63 %) > A_3F_1 (3.30 %) > A_4F_1 (3.03 %) > A_5F_1 (2.97 %) which was more than root N concentration at 100% N alone without inoculation *i.e.,* A_6F_1 (2.20 %). The increase in root N concentration due to inoculation of *Azospirillum* isolate ACDAZS-35 (A_3) at 0% N (F_4) fertilizer was 60.95% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The root N concentration at only 100% N without inoculation (UIC;2.20 %) is on par with the root N concentration with inoculation (A_3F_4;2.10 %) at 0% N.

Total N concentration

At 60 DAS, with the different levels of N fertilizer application, inoculation of all the selected isolates and *Azospirillum* spp. ACD-15 (Reference strain) resulted in producing higher total N concentration over uninoculated control. In general, plant inoculated with all the selected isolates and reference *Azospirillum* strain produced the maximum total N concentration at 75% N and among the selected isolates of *Azospirillum* and reference strain, inoculation of ACDAZS-35 (A_3) resulted in maximum total N concentration. Inoculation of ACDAZS-35 (A_3) influenced the production of maximum total N concentration at 75% N (F_2) which was 78.66% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The order of significant increase in the total N concentration due to inoculation of different *Azospirillum* isolates at different dose of N fertilizer was A_3F_1 (8.53 %) > A_3F_1 (6.93 %) > A_1F_2 (6.70 %) > A_5F_1 (6.33%) which was more than total N concentration at 100% N alone without inoculation i.e., A_6F_1 (4.33%). The increase in total N concentration due to inoculation of *Azospirillum* isolate ACDAZS-35 (A_3) at 0% N (F_4) fertilizer was 130% which was more than the uninoculated plants in the absence of N fertilizer (A_6F_4). The total N concentration at only 100%N without inoculation (UIC;4.33%) is on par with the total N concentration with inoculation (A_3F_4;4.20%) at 0%N. The growth of rice plants by inoculation with *Azospirillum* was analysed by Primadi *et al*. (2015). 10 isolates of *Azospirillum* have the highest activity of their N_2 fixation and have highest potential to increase dry weight and N concentration of rice plants. The increased N concentration due to increase in the production of IAA, GA, phosphorous solubilization, inclusion of additional nitrogen from both mineral source and biological fixation by bacteria through BNF.

The increased percent N content in wheat in the present investigation due to inoculation of *Azospirillum* was attributed to the increased population of free living N_2-fixers, which had then probably increased the uptake of nutrients by means of altering the root surface characters involved in nutrient uptake. Isolate A. zea Sp2 that produced higher level of auxins in vitro and also caused higher N content in the grains and straw under pot experiment, also enhanced growth and yield of the wheat under field conditions (Karimi *et al.*, 2018). The improved growth of maize due to inoculation of *Azospirillum* in terms of plant nitrogen contentin the present study could be confirmed by the findings of inoculating *Azospirillum* by (wheat) El Sayed *et al*. (2015); (rice) Primadi *et al*.(2015); (wheat) Vijayalakshmi *et al*. (2019)

Conclusion

The present study revealed that inoculation of maize seeds with *Azospirillum* isolates improved the growth and yield parameters of maize over the uninoculated and inoculation with ACDAZS-09 along with 50 per cent N application, indicating that N application can be reduced by 25 per cent by using *Azospirillum* bio-fertiliser inoculation.

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