ANNALS OF PLANT SCIENCES
ISSN: 2287-688X
OPEN ACCESS
www.annalsofplantsciences.com

ORIGINAL RESEARCH ARTICLE

Isolation and characterization of native *Azospirillium* isolates from normal soils grown rice areas of Chhattisgarh

Neha Kashyap*, S.B. Gupta, Tapas Chowdhary
Department of Microbiology, Indira Gandhi Agricultural university, Raipur-492006, Chattisgarh, India.

Received: January 29, 2016; **Accepted:** March 05, 2016.

Abstract: Sixty soil samples were collected from normal soils of Rice growing areas during year 2014-2015 from various blocks of Raipur district of Chattisgarh for isolation of *Azospirillium*. In this study 60 *Azospirillium* isolates were obtained and were characterized on the basis of Gram's staining, starch hydrolysis, catalase test, urease test, gelatin liquefaction, growth performance in terms of intensity of blue colour produced in N-free malate broth, indole acetic acid (IAA) production and N-fixation capacity. Local *Azospirillium* isolate AZP-R03 was found most effective native Nitrogen fixing bacteria for Rice with a great potential for its use as nitrogen fixing bacterial fertilizer especially for Rice crop under agroclimatic conditions of Chattisgarh.

Key words: *Azospirillium, Characterization, Indole acetic acid (IAA) production, Rice*

Introduction

Rice is an important crop grown in nearly 44 million ha of land in the country with the productivity of 2.2 t/ha which is less than the productivity of many countries. Annual population growth rate of the country is nearly 1.8 %. In Chhattisgarh, rice occupies average of 3.6 million ha. with the productivity of the state ranging between 1.2 to 1.6 t/ha depending upon the rainfall. The state is comprised with three agro-ecological zones i.e. Chhattisgarh plains, Bastar plateau and northern hill region of surguja. These zones have huge variations in terms of soil topography, rainfall intensity and distribution, irrigation and adoption of agricultural production system and thus vary in the productivity of rice in these regions. The rainfall, is quite high in the region and ranges between 1200-1400 mm, during drought years with an irrigated area of nearly 28%. Inspite of that state is well behind the average national productivity. The red and yellow soils are deficient in nitrogen, phosphorus, zinc and boron. Rice is cultivated in all districts of Chhattisgarh; however, none of the district falls in high Productivity (> 2,000-2,500 Kg/ha), and medium Productivity (> 2,500 Kg/ha) group. Traditional practice to achieve maximum yields of rice is associated with the availability of mineral nitrogen and fertilization.

The use of chemical fertilizers, to enhance soil fertility and crop productivity often negatively affect the complex system of the bio-geochemical cycles (Perrott *et al.,* 1992), and has caused leaching and run-off of the nutrients, especially Nitrogen (N_2) and phosphorus (P), leading to the environmental degradation (Gyaneshwar *et al.,* 2002). Among the chemical fertilizers, nitrogenous fertilizers constitute major part of the crop cultivation. Industrial nitrogen based fertilizers are becoming expensive and, to combat with this problem, it is necessary to develop an alternative method of supplying nutrients to plants. These constraints lead to sub optimal productivity of crops raised in acid soils. Enhanced competitive ability in an inoculant strain is a key requirement for successful N_2 fixation (Furseth *et al.,* 2012).

Corresponding Author
Neha Kashyap,
Department of Agricultural Microbiology
College of Agriculture,
Indira Gandhi Agriculture university
Labhandih, Raipur, Chhattisgarh
Pin code- 492006, India.

Bio-fertilization or inoculation with plant growth-promoting bacteria (PGPB) is a sustainable alternative for agro-ecosystems. Microbial inoculants improve plant growth and soil fertility as well. Biofertilizers are cost-effective and ecofriendly compared to the chemical fertilizers. Keeping the above information in view, isolation of native rice loving beneficial bacteria from soils of Raipur district of Chhattisgarh and their characterization could prove useful in order to formulate those isolates for preparation of effective location specific and crop specific Biofertilizers for rice crop.

Materials and Methods

Investigations were conducted in green house of Department of Microbiology, IGKV, Raipur Chhattisgarh (India) for isolation of bacteria from rice grown soil samples collected from different growing areas of Raipur district of Chhattisgarh. To fulfill the above objective a survey was conducted to collect 60 soil samples (Table 1). These soil samples were analyzed for their physiochemical and biological properties such as available N, P, K, soil pH and population of *Azospirillium*. The soil culture was used as inocula to grow rice in green house in disposable cups of 250 gm capacity. At 45 days after sowing the plants were gently uprooted and the rhizosphere soil adhered to the rice roots were collected and used for isolation of *Azospirillium*. The rice roots were used for observing the biomass accumulation. *Azospirillium* isolates were isolated using both rhizosphere soil suspensions and surface sterilized washed root bits. Both the soil suspensions as well as root bits were inoculated in plates containing N free malate medium and incubated for one week at room temperature (25-30 ^{o} C). Change of colour of the medium from green to brilliant blue suggested the presence of *Azospirillum*. The colonies of *Azospirillum* were purified and maintained on nutrient agar slants.

The *Azospirillum* isolates were characterized on the basis of Gram's staining, starch hydrolysis, urease, catalase, gelatin agar test (Table 2), indole-3-acetic acid (IAA) production and N-fixation capacity in N-free malate medium, N- uptake and growth performance in terms of intensity of blue colour produced in N-free malate broth (Table 3). Gram staining was conducted as per standard procedure (Aneja, 2003). The *Azospirillum* isolates were tested for starch hydrolysis on starch agar at 30 ^{o} C for 3 days. After incubation plates were flooded with lugol's iodine solution, allowed to stand for 15-30 min and observed for clear zone around the colony to indicate starch hydrolysis. For catalase test, nutrient slants were inoculated with organisms and were incubated at 30^{o}C for 24 hours and tubes were flooded with hydrogen peroxide and observed for gas bubbles which scored positive for catalase activity (Blazevic and Ederer, 1975). For urease test, urea broth were prepared and inoculated with *Azospirillium* isolates and incubated at 35^{o} C for 48 hours. Development of pink colour in broth is positive reaction for urease test. For gelatin agar, petriplates containing galatin agar were spotted with overnight grown *Azospirillium* isolates and incuabted at 30 ^{o} C for 3 days. Then the plates were flooded with 12 percent mercuric chloride and allowed to stand for 20 minutes and observed for clear zone around the growth of bacterium to indicate gelatin liquefaction (Blazevic and Ederer, 1975). For testing IAA production ability of the *Azospirillium* isolates, cultures were inoculated to sterile czapek's solution (Mahadevan and Sridhar, 1984) supplemented with L-tryptophan @ 5mg ml^{-1} of broth and incubated at 37^{o} C for 7 days in dark. After incubation the cultures were centrifuged at 6000 rpm and the supernatant was collected in a conical flask. 25 ml of supernatant was taken with pH adjusted to 2.8 using one N HCl in a 100 ml conical flask. Equal volume of diethyl ether was added to it and incubated in dark for 4 h. Extraction of IAA was done at 4^{o}C in a separating funnel using diethyl ether. The organic phase was discarded and the solvent phase was pooled and evaporated to dryness. To the dried material, 3 ml of methanol extract was examined using the method of Gordon and Paleg (1957). *Azospirillium* isolates were tested for their growth performance and N-fixation capacity. This study was based on the amount of nitrogen fixed by *Azospirillium* isolates, was estimated by Microkjeldhal method of Jackson (1973). The bacterium was grown in semisolid N free malate medium for seven days which was supplemented with L- glutamic acid @100 mg l^{-1} (okon *et al.,* 1997). Triplicate samples were used for each isolate.

Results and Discussion

Sixty soil samples were collected from different villages of Raipur district of Chhattisgarh (Table 1). The physico-chemical analysis of the soil samples collected showed that the pH of the soils were of normal to alkaline range. The nutrient status was found in medium range. Available N content varied between 156.90 – 178.10 kg ha^{-1}, while available P content ranged between 9.30 – 12.90 kg ha^{-1} and available K content of soils ranged from 286.28 – 498.98 kg ha^{-1}. In representative soil samples, the population of *Azospirillum* was in the range of 4.76 x 10^{3} – 8.81 x10^{4} cfu gm^{-1} soil.

Influence of Beneficial Microbes on Mustard

The collected soil samples were used as inocula to grow rice in green house for 45 days and then gently uprooted and the shoots were dried and weighed to study biomass accumulation. The highest plant height and biomass was obtained in rice grown with soil inoculums numbered 03 followed by 09, 10, 11, 13, 26, 32, 39, 43, 56 etc. (Table 4). This increase in plant biomass may be due to impact of *Azospirillum* on rice plants. Plant growth promoting rhizobacteria use one or more of direct or indirect mechanisms of action to improve plant growth and health. Biological nitrogen fixation, improvement of other plant nutrients uptake and phytohormone production like indole-3-acetic acid are some examples of mechanisms that directly influence plant growth (Glick *et al.,* 1995). Biological control of plant pathogens and deleterious microbes through production of antibiotics, lytic enzymes, hydrogen cyanide and siderophores or through competition for nutrients and space can improve significantly plant health and promote growth as evidenced by increase in seedling emergence, vigor and yield (Antoun and Kloepper, 2001).

Isolation of Crop Beneficial Microbes

The Rhizosphere soils obtained from uprooting of 45 days old rice plants were used for the isolation of Az*ospirillum.* Root bits were also used for the same. *Azospirillium* isolates were obtained from all sixty soil samples.

Characterization of *Azospirillum* isolates

The sixty *Azospirillum* isolates obtained from rice rhizosphere were characterized on the basis of Gram's reaction, starch hydrolysis, urease test, gelatin agar, starch hydrolysis, catalse test, IAA production and N-fixation capacity on N free malate medium. As per Gram's reaction, all of 60

Azospirillum isolates were found Gram negative (Table2). In starch hydrolysis test, 36 isolates were able to hydrolyze starch when grown on starch agar medium while rests of the 24 isolates were not able to do so significantly. The maximum starch hydrolysis was exhibited by AZP-R03 followed by RC AZP-R09.

All the *Azospirillum* isolates when inoculated in malate broth containing BTB indicator turned the initial green colour of broth to brilliant blue after incubation for 3-4 days. Out of sixty *Azospirillum* isolates 42 isolates were able to produce dark blue colour, while rest 18 isolates produced light blue colour. The isolates which produced dark blue colour were AZP-R 03, 09, 10, 11, 13, 26, 32, 39, 43 etc. (Table 3).

All the *Azospirillium* isolates when grown in nutrient agar and tetsed for catalase activity, 36 isolates showed positive results while 24 showed negative results.

All the *Azospirillium* isolates when grown in urea broth and incubated for 24- 48 hours turned the colour to pink showing activity of urease enzyme. However out of sixty isolates, 52 isolates showed dark pink colour while rest 08 showed light pink colour.

All the *Azospirillium* isolates when tested for gelatin agar, showed that 30 isolates showed clearing zone while rest 30 isolates showed negative results.
On the basis of above stated characterization of all sixty *Azospirillium* isolates, top ten isolates along with a standard check were selected for further analysis.

Quantitative analysis of IAA production by *Azospirillum* isolates

Top ten *Azospirillum* isolates were tested for their abilities to produce IAA. The results indicated that all *Azospirillum* isolates were able to produce IAA, however the quantity of IAA produced varied to a greater extent. IAA was produced in the range of 8.86 – 15.8 μg 25 ml^{-1} broth (Table 4). AZP-SC produced highest quantity of IAA i.e. 15.8 μg 25ml^{-1} broth followed by local isolates AZP-R03 and AZP-R09. Many of the rhizosphere organisms are known to produce plant growth promoting substances (Gaur, 1990; Leinhos and Vacek, 1993). In fact, it has been suggested that up to 80 percent of bacteria isolated from rhizosphere can produce IAA (Pattern and Glick, 1994).

Nitrogen fixing efficiency of *Azospirillium* isolates

The result of N fixation capacity of *Azospirillium* isolates are presented in Table 4. The range of nitrogen fixed in the N- free malate medium varied from 8.133- 11.133 mg gm^{-1} of malate in the medium after seven days of incubation. Two strains AZP-R 03 and 09 were best isolates. The standard check that was *A. brasilense* released 11.133 mg N g^{-1} malate after seven days of incubation. Which was followed by local isolates AZP-R02 which relased 10.633 mg N g^{-1} malate following AZP-R09 who released 9.700mg N g^{-1} malate after seven days of incubation. Doberneir and Day (1976) reported *Azospirillium* can fix as much as 115 mg N g^{-1} of malate. Boddey and Doberneir (1982) also reported that the efficiency of nitrogen fixation increased with the age of culture reaching values of 98 mg g^{-1} of glucose and 49 mg g^{-1} of glucose for A. *brasilense* and A. *lipoferum* respectively in early stationary phase. In recent years, great attention has been dedicated to study the role that soil microorganisms play in the dynamics of nitrogen (N), particularly those able to fix nitrogen from atmosphere (Doberneir and Day, 1976).

In soil, physical and chemical environments affect the distribution of microorganisms. Besides these, the tenacy provided by plant roots to alien microorganisms is amazing, because plant roots create a unique habitat for microorganisms due to exudation of photosynthates (Brown, 1975).

Studies on growth, IAA production, biomass production and N fixation in N free malate broth by different *Azospirillium* isolates revelaed that the inoculation with *Azospirillium* isolate AZP-K02 showed best results following standard (*A. brasilense*). From the above study, it was concluded that local *Azospirillium* isolate AZP-K02 is the most effective native nitrogen fixing bacteria for rice under acidic soil conditions and have the tremendous potential to become the specific nitrogen fixing bacterial fertilizer for rice under agro climatic conditions of Chhattisgarh especially for acidic soils. Keeping in view of the above, the isolated and characterized precious microbial cultures were preserved in the department of microbiology, I.G. Agricultural University for future studies.

Table 1: Location from where soil samples were collected

S. No.	Village-Block	District	*Azopsirillium* Isolates	pH range	Ec range
1.	Mandir hasud- Aarang	Raipur	AZP-R01,2,3,4,5,6,7,8,9,10	7.13-7.50	0.05-0.19
2.	Temri- Dharsiva	Raipur	AZP-R11,12,13,14,15,16,17,18,19,20	6.78-8.31	0.06-0.20
3.	Jora- Dharsiva	Raipur	AZP-R21,22,23,24,25,26,27,28,29,30	6.02-6.89	0.11-0.25
4.	Baradera- Dharsiva	Raipur	AZP-R31,32,33,34,35,36,37,38,39,40	6.02-7.80	0.02-0.27
5.	Pirda- Dharsiva	Raipur	AZP-R41,42,43,44,45,46,47,48,49,50	6.75-7.91	0.11-0.24
6.	Darba- Aarang	Raipur	AZP-R51,52,53,54,55,56,57,58,59,60	6.41-7.80	0.12-0.25

Table 2: Grouping of A*zospirillum* isolates on basis of their biochemical characterization

S. No.	Isolate No.	Gram's staining	Intensity of blue colour produced	Starch hydrolysis	Catalase test	Urease test	Gelatin agar
1	AZP-R3	-ve	+++	+++	+++	+++	+++
2	AZP-R9	-ve	++	+++	+++	++	+++
3	AZP-R10	-ve	+++	++	++	+++	+++
4	AZP-R11	-ve	+++	+++	+++	+++	++
5	AZP-R13	-ve	+++	+++	+++	++	+++
6	AZP-R26	-ve	++	+++	++	+++	++
7	AZP-R32	-ve	++	++	++	++	++
8	AZP-R39	-ve	+++	+++	+++	+++	++
9	AZP-R43	-ve	+++	++	+++	++	+++
10	AZP-R56	-ve	+++	+++	+++	+++	+++
Rest 50 isolates		All gram _ve	10+++ 22++ 18+	09+++ 17++ 24+	06+++ 20++ 24 –ve	12+++ 30++ 08+	05+++ 15++ 30 –ve

Table 3: Variation in Biological nitrogen fixing parameter among different *Azospirillum* isolates

S No.	Isolate No.	Plant height (cm)	Shoot (g)	Shoot (g)	Degree of greenness	N-content (%)	N-uptake
			Fresh wt	Dry wt			
1	AZP-R3	19.6	1.394	0.933	Dark	0.672	0.63
2	AZP-R9	18.9	1.389	0.844	Dark	0.654	0.56
3	AZP-R10	19.0	1.387	0.896	Dark	0.671	0.60
4	AZP-R11	18.9	1.381	0.733	Dark	0.627	0.47
5	AZP-R13	18.1	1.375	0.791	Dark	0.619	0.49
6	AZP-R26	18.5	1.364	0.786	Dark	0.603	0.47
7	AZP-R32	18.1	1.283	0.796	Dark	0.601	0.48
8	AZP-R39	17.9	1.256	0.794	Medium	0.592	0.48
9	AZP-R43	18.5	1.253	0.802	Dark	0.572	0.46
10	AZP-R56	18.1	1.276	0.748	Dark	0.589	0.44
Rest 50 isolates		17.8-12.3	1.012-0.762	0.713-0.431	Medium – light blue	0.581-0.327	0.42-0.28

Table 4: IAA production and N-fixation capacity of selected promising *Azospirillum* isolates along with standard check

S.No.	Isolate no.	µg/25ml of Nfb broth`	N-fixation (mg/g malate)
1.	AZP-R SC	15.8	11.133
2.	AZP-R03	13.4	10.633
3.	AZP-R09	11.6	9.700
4.	AZP-R10	11.2	9.633
5.	AZP-R11	10.9	9.867
6.	AZP-R13	10.3	8.833
7.	AZP-R26	10.6	8.500
8.	AZP-R32	9.82	8.500
9.	AZP-R39	9.61	8.400
10.	AZP-R56	8.86	8.133
CD (at 5%)		1.160	1.208

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Cite this article as:
Neha Kashyap, S.B. Gupta, Tapas Chowdhary. Isolation and characterization of native *Azospirillium* isolates from normal soils grown rice areas of Chhattisgarh. *Annals of Plant Sciences* 5.3 (2016): 1284-1288.

Source of support: Nil
Conflict of interest: None Declared