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Isolation and Characterization of Mustard (*Brassica sp.*) Loving Beneficial Bacteria from the Soils of Raipur District of Chhattisgarh State (India) with Special Reference to *Azospirillum*

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Abstract: Sixty three representative soil samples were collected from mustard growing areas during the year 2007-08 from 3 villages of various blocks of Raipur district of Chhattisgarh (India) for isolation of mustard loving beneficial bacteria and their characterization with special reference to nitrogen fixing *Azosprillum* bacteria. In this study, 59 *Azospirillum* isolates, 51 *Azotobacter* isolates and 31 PSB isolates were obtained. The *Azospirillum* isolates were characterized on the basis of Gram's staining, starch hydrolysis,IAA production and N-Fixation capacity in N-free malate medium and growth performance in terms of intensity of blue colour produced in N-free malate broth. The *Azotobacter* isolates were tested for Gram's staining reaction and for growth performance in terms of turbidity of the broth. The PSB isolates were tested for Gram's staining reaction and their growth performance and phosphate solubilizing efficiency on Pikovskya's medium. This study was based on the clearing zone formed by the different isolates after 48 hours of incubation.

Key words: *Azospirillum* · Azotobacter · PSB · Isolation · Characterization · Indole acetic acid · Mustard

INTRODUCTION

Oilseeds constitute an important group of agricultural crops and are grown in area of about 29mha in India. The crops are generally grown under rainfed areas (80%) and in soils deficient in nutrients in contrast to cereals, which are largely grown under irrigated conditions. Therefore, the yields of oilseed crops are relatively lower and depend much upon the rainfall for successful cultivation. Oil import reached a maximum of some 18.2 lakh tones at a cost of Rs.1061 crores in 2005-06, simultaneously during past years the acreage under oilseed crops has been increasing, but the production and yield has not been increased in that proportion. The reason might be the non-provision of plant nutrients inputs in proportion to that of crop demands. This also proved by the statement given by Subba Rao *et al.* [1] that nitrogen is most limiting plant nutrient threatening sustainability of crop production in the soils. In India *Brassica* crop occupies second largest position after Groundnut with 3.5 million hectares of area and produces about 4.5 million tonnes of seed annually.

This age of increasing prices along with the increasing demand of chemical fertilizers and depleting soil fertility necessitates the integrated use of organic, inorganic and biological sources of nutrients for sustainable crop production and better soil health. Biofertilizers have tremendous potential to provide plant nutrients. Even though we have beneficial microbial strains identified as efficient for production of biofertilizer but there is still scope for improving the efficiency or for identifying a better one. One has to search continuously for more efficient strains. This can be accomplished by isolating and screening numerous wild type strains [2]. There is lack of location specific and crop specific biofertilizers for mustard in Chhattisgarh (India). Hence, isolation of native mustard loving beneficial bacteria from the soils of Raipur district of Chhattisgarh and their characterization with special reference to *Azospirillum* is certainly useful in order to formulate those isolates for the preparation of effective location specific and crop specific biofertilizers for mustard in Chhattisgarh.

MATERIALS AND METHODS

Survey and Soil Sample Collection: A survey was conducted and 63 representative soil samples were collected from important mustard growing regions of Raipur district of Chhattisgarh state of India. These soil samples were analyzed for their physico-

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chemical and biological properties such as available N, P, K, soil pH and population of *Azospirillum, Azotobacter* and PSB.

The soil samples were also used as soil inocula to grow mustard 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 mustard roots were collected and used for the isolation of mustard loving beneficial bacteria. The mustard shoots were used for observing the biomass accumulation.

Isolation of Native *Azotobacter and* Phosphate Solubilizing Bacterial Isolates: For the isolation of phosphate solubilizing bacteria (PSB), appropriate dilutions of the rhizosphere soil were made using sterilized water. The dilutions were plated on Pikovskaya medium [3]. The plates were incubated for three to four days and observed for transparent solubilization zone around the microbial colonies to isolate PSB. Such colonies were purified and maintained on nutrient agar slants.

The same dilutions of soil suspension were used for the isolation of *Azotobacter*. The dilutions were plated on Norris N-free medium with a pH of seven. The colonies of *Azotobacter* were purified and maintained on nutrient agar slants.

Isolation of Native *Azospirillum* Isolates: *Azospirillum* 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 °C). Change of colour of the medium from green to brilliant blue suggests the presence of *Azospirillum*. The colonies of *Azospirillum* were purified and maintained on nutrient agar slants.

Characterization of *Azospirillum* Isolates: The *Azospirillum* isolates were characterized on the basis of Gram's staining, starch hydrolysis, IAA production and N-Fixation capacity in N-free malate medium and growth performance in terms of intensity of blue colour produced in N-free malate broth.

Gram Staining and Starch Hydrolysis Test: Gram staining was conducted as per the standard procedure [4]. The *Azospirillum* isolates were tested for starch hydrolysis inoculating petriplates containing starch agar with test cultures and incubating at 30°C for 3 days. After incubation the plates were flooded with Lugol's iodine solution, allowed to stand for 15-30 minutes and observed for clear zone around the colony to indicate starch hydrolysis [5].

Quantitative Iaa Production Test: For testing of indole-3-acetic acid production ability of the *Azospirillum* isolates, cultures were inoculated to sterile Czapek's solution [6] supplemented with L-tryptophan @ 0.005 gm / ml of broth and incubated at 37°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 hours. Extraction of IAA was done at 4°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 was added, pooled and the IAA present in the methanol extract was examined using the method of Gordon and Paleg [7].

Atomospheric Nitrogen Fixation Capacity Study: The *Azospirillum* isolates were tested for their growth performance and N-fixation capacity. This study was based on the Amount of nitrogen fixed by *Azospirillum* isolates was estimated by Microkjeldhal method of Jackson [8]. The bacterium was grown in semisolid N free malate medium supplied with L-glutamic acid @100 mg/I. Triplicate samples were used for each isolate.

Characterization of *Azotobacter* and Psb Isolates: The *Azotobacter* and PSB isolates were also characterized by Gram staining reaction. The growth performance of *Azotobacter* isolates was tested in terms of turbidity of the broth. Solubilization of insoluble inorganic phosphates by PSB isolates was measured by making appropriate dilutions, which were poured on plates containing media of Pikovskaya's [3]. The plates were then incubated for four to five days and observed for transparent solubilisation zone around the microbial colonies. The zone diameter of solubilization was measured and recorded in mm..

RESULTS AND DISCUSSION

Physico-chemical and Biological Characteristics of Soil Samples: Investigations were conducted at the Department of Microbiology, College of Agriculture, IGKV, Raipur, Chhattisgarh (India) for the isolation of

Table 1: Physico-chemical and biological characteristics of soil samples collected from Raipur district of Chhattisgarh for isolation of beneficial native bacteria

Characteristics of soil samples	Vertisols (40 samples)	Alfisols (11 samples)	Inceptisols (12 samples)
Available N (kg / ha)	178.10	165.94	156.90
Available P (kg / ha)	9.30	11.30	12.90
Available K (kg / ha)	498.98	394.3	286.28
pH	7.89	7.23	6.13
Azotobacter (cfu / gm soil)	9.65×10^4	5.87×10^3	4.53×10^3
Azospirillum (cfu / gm soil)	8.81×10^4	6.54×10^3	4.76×10^3
PSB (cfu / gm soil)	9.26×10^3	6.53×10^3	4.68×10^3

Table 2: Growth performance of green house grown mustard raised with different soil inocula of Raipur district (at 45 DAS)

Name of soil inoculum	Fresh weight (g / plant)	Dry weight (g / plant)
53	4.79	3.39
46	4.76	3.36
43	4.63	3.33
07	4.61	3.31
34	4.58	3.18
21	4.53	3.13
37	4.44	3.04
26	4.37	2.97
52	4.21	2.81
45	4.19	2.79
41	4.14	2.74
40	4.09	2.69
36	3.96	2.56
59	3.93	2.73
15	3.92	2.52
Rest 48 soil inocula	3.36-3.91	2.24-2.51
CD (5%)	0.38	0.84

Table 3: Status of bacterial isolates obtained from soils of Raipur (C.G.)

No. of villages from where soils were collected	No. of soil samples collected	No. of isolates obtained		
		Azotobacter	*Azospirillum*	PSB
3	63	51	59	31

mustard loving beneficial bacteria from soil samples collected from different mustard growing areas of Raipur district of Chhattisgarh. The collected 63 soil samples represented three soil types viz. inceptisols, alfisols and vertisols. The physico-chemical analysis of the soil samples collected showed that the pH of the soils were slightly acidic to alkaline range. The nutrient status of the soils was fairly in a medium range. Available N content varied between 156.90-178.1 kg per ha, while available P content ranged between 9.30-12.90 kg per ha. and available K content of soils ranged from 286.28-498.98 kg per ha. In representative soil samples the population of *Azospirillum* was in the range of 4.76×10^3-8.81×10^4cfu / gm soil where as *Azotobacter* were present in the range of 4.53×10^3-9.65×10^4 cfu / gm soil and PSB were found to be in the range of 4.68×10^3-9.26×10^3 cfu / gm soil (Table 1).

Influence of Beneficial Microbes on Mustard: The collected soil samples were used as inocula to grow mustard in green house for 45 days and then gently uprooted and the shoots were dried and weighed to study biomass accumulation. The highest plant biomass was obtained in mustard grown with soil inoculum numbered 53 followed by 46, 43, 07, 34, 21, etc. (Table 2). This increase in plant biomass may be due to the impact of beneficial microbes like *Azospirillum*, *Azotobacter* and PSB, on mustard plants. Plant growth promoting rhizobacteria use one or more of direct or indirect mechanisms of action to improve plant growth and health. P-solubilization, 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 [9]. Biological control of plant pathogens and deleterious microbes through the 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 increases in seedling emergence, vigor and yield [10].

Isolation of Crop Beneficial Microbes: The rhizosphere soils obtained from uprooting of mustard at 45 DAS were used for the isolation of *Azospirillum, Azotobacter* and PSB isolates. For *Azospirillum* isolation roots were also used. From 63 rhizosphere samples 59 *Azospirillum* isolates, 51 *Azotobacter* isolates and 31 PSB isolates were obtained (Table 3).

Characterization of *Azospirillum* Isolates: The 59 *Azospirillum* isolates obtained from mustard rhizosphere were characterized on the basis of Gram's reaction, starch hydrolysis, IAA production and N-fixation capacity on N free malate medium. As per Gram's reaction, all of 59 *Azospirillum* isolates were found

Table 4: Gram's staining reaction, starch hydrolysis, intensity of blue colour produced and IAA production by the mustard loving isolates of *Azopirillum* obtained from soils of Raipur

Azospirillum isolateNo.	Gram's reaction (+ve or –ve)	Starch hydrolysis	Intensity of blue colour produced	IAA produced(micro g/ 25 ml broth)
Azospirillum brasilense (Standard Check)	Gram-ve	+	+++	25.05
MR AZP 53	Gram-ve	+	+++	25.65
MR AZP 46	Gram-ve	+	+++	21.28
MR AZP 43	Gram-ve	–	+++	23.71
MR AZP 07	Gram-ve	+	+++	23.16
MRAZP 34	Gram-ve	+	++	22.74
MR AZP 21	Gram-ve	+	+++	22.23
MR AZP 37	Gram-ve	–	+++	21.06
MR AZP 26	Gram-ve	+	+++	20.91
MR AZP 51	Gram-ve	+	++	20.04
MR AZP 45	Gram-ve	–	+++	19.89
MR AZP 41	Gram-ve	–	+++	18.46
MR AZP 40	Gram-ve	+	+++	18.13
MR AZP 36	Gram-ve	–	++	16.34
MR AZP 59	Gram-ve	–	++	16.26
MR AZP 15	Gram-ve	–	++	16.03
Rest 44 isolates	All gram–ve	27 +	26+++	9.23-16.01
		16 –	18++	
CD at 5%	-	-	-	1.84

In Gram's reaction, –ve represents Gram–ve bacteria, + indicates growth / reaction and-indicates no growth / no reaction

Table 5: Nitrogen fixation capacity of *Azospirillum* isolates and standard check in the N free medium

Azospirillum isolates	Nitrogen fixation (mg/g malate)
Standard check	15.21
MR AZP 53	15.68
MR AZP 46	15.06
MR AZP 43	14.63
MR AZP 07	13.94
MR AZP 34	13.16
MR AZP 21	13.06
MR AZP 37	12.68
MR AZP 26	11.32
MR AZP 51	9.12
MR AZP 45	10.32
MR AZP 41	9.91
MR AZP 40	9.86
MR AZP 36	8.96
MR AZP59	8.85
MR AZP 15	8.68
Rest isolates	8.09-8.67
CD at 5%	1.89

Gram negative and no were Gram positive (Table 4). In starch hydrolysis test 36 isolates were able to hydrolyze starch when grown on starch agar medium while rests of the 23 isolates were not able to do so significantly. The maximum starch hydrolysis was exhibited by MR AZP 53 followed by isolate 46.

All the *Azospirillum* isolates when inoculated in malate broth containing BTB indicator turned the initial green colour of the broth to brilliant blue after incubation for 3-4 days. Out of 59 *Azospirillum* isolates 36 isolates produced dark blue colour, while the rest 23 isolates produced light blue colour. The isolates, which produced dark blue colour, were MR AZP 53, 46, 07, 43, 34, 41, 52, 21, 45 and 40. (Table 6).

Quantitative Analysis of Iaa Production by Azospirillum Isolates: All the *Azospirillum* isolates were tested for their abilities to produce indole-3 acetic acid. The results indicated that all 59 *Azospirillum* isolates were able to produce IAA however; the quantity of IAA produced varied to a greater extent. The IAA was produced in the range of 9.23-25.65µg / 25 ml broth (Table 4). MR AZP53 produced highest quantity of IAA i.e. 25.65µg. *Azospirillum brasilense,* which was used as standard check produced 25.05 µg/25 ml broth. Many of the rhizosphere organisms are known to produce plant growth promoting substances [8,12]. In fact, it has been suggested that up to 80 percent of bacteria isolated from the rhizosphere can produce IAA [11].

Table 6: Relationship between mustard loving PSB and *Azotobacter* isolates with reference to their growth performance

Name of PSB isolates	Diameter (mm) of the solu. zone	Gram's reaction	Name of *Azotobacter* isolates	Turbidity of growth	Gram'sreaction
MR PSB 46	17.0	-ve	MR AZT 07	+++	-ve
MR PSB 43	14.6	- ve	MR AZT 21	+++	- ve
MR PSB 07	14.2	- ve	MR AZT 23	+	- ve
MR PSB 53	13.5	- ve	MR AZT 26	+++	- ve
MR PSB 21	13.5	- ve	MR AZT 33	++	- ve
MR PSB 34	13.2	- ve	MR AZT 34	+++	- ve
MR PSB 52	12.5	- ve	MR AZT 37	+++	- ve
MR PSB 37	12.3	- ve	MR AZT 40	+++	- ve
MR PSB 26	11.1	- ve	MR AZT 41	+++	- ve
MR PSB 40	10.7	- ve	MR AZT 43	+++	- ve
MR PSB 41	10.4	- ve	MR AZT 46	+++	- ve
MR PSB 09	9.7	- ve	MR AZT 52	++	- ve
MR PSB 03	9.4	- ve	MR AZT 53	+++	- ve
MR PSB 16	8.3	- ve	MR AZT 57	+	- ve
MR PSB 61	7.6	- ve	MR AZT 61	+	- ve
Rest 16 isolates	4-7 mm		Rest 36 isolates	3+++	
				15++	
				18+	

For turbidity, +++, ++ and + mean high, medium and low turbidity respectively

Nitrogen Fixing Efficiency of Azospirillum Isolates: The result of N fixation capacity of *Azospirillum* isolates are presented in Table 5.The range of nitrogen fixed to the N-free malate medium varied from 8.09-15.68 mg/gm of malate in the medium after seven days of incubation. The four strains i.e. MR AZP 53, 46 and 43 were at par with standard check (*A. brasilense*). Among top isolates, isolate number 53 fixed maximum N in the medium that was 15.68 mg/gm malate. The standard check that was *Azospirillum brasilense* released 15.21 mg N /gm malate after seven days of incubation. Dobriener and Day [12] reported *Azospirillum* can fix as much as 115mg N_2 / g of malic acid. Boddey and Dobriener [13] also reported that the efficiency of nitrogen fixation increased with age of culture reaching values of 98mg per gram of glucose and 49 mg per gram of glucose for *Azospirillum brasilense* and *Azospirillum lipoferum* respectively in the 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 [12].

Characterization of *Azotobacter* and Psb Isolates: The *Azotobacter* and PSB isolates were tested for Gram staining reaction and the result of this study showed that all the *Azotobacter* and PSB isolates were Gram negative. The growth performance of the *Azotobacter* isolates was measured in terms of turbidity of the matured broth and the turbidity was classified as high, medium and low turbidity. Out of the 51 *Azotobacter* isolates 13 isolates showed high turbidity, 17 isolates showed medium turbidity while 21 isolates showed low turbidity (Table 6). During the P solubilization zone study of PSB isolates it was found that among the top 10 isolates maximum diameter of solubilizing zone in Pikovskya's medium was recorded as 17 mm for the isolate MR PSB 46. This isolate was followed by isolates MR PSB 43, 07, 53, 21, 34, 52, 37, 26 and 40 (Table 6).

These studies clearly show that the soil samples from which promising *Azospirillum* isolates were isolated also harboured good isolates of *Azotobacter* and PSB which were vigourous in their growth performance and other characteristics. The reason behind this might be the edaphic and micro-climatic factors which influence the composition of the micro flora of that particular soil. Over all a positive correlation was found among the *Azospirillum*, *Azotobacter* and PSB isolates obtained from each particular soil sample in terms of growth performance and other characteristics. In soil, physical and chemical environments affect the distribution of micro-organisms. Besides these, the tenancy provided by plant roots to alien micro-organisms is amazing; because plant roots create a unique habitat for micro-organisms due to exudation of photosynthates [14].

The isolates of *Azospirillum, Azotobacter* and PSB obtained from different mustard growing regions of Raipur district of Chhattisgarh can be used for the development of location and crop specific biofertilizers for mustard in Chhattisgarh. The combined inoculation of *Azospirillum* and PSB on crops like sorghum [15], bajra [16] and cotton have been reported to have given significant increase in dry matter and yield over single inoculation. Hence, crop and locaton specific *Azospirillum* bofertilizer should be developed and its use in mustard either alone or in combination with N-fixing diazotrophs and P solubilizing microbes should be emphasized for getting higher yields besides saving costly fertilizers.

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