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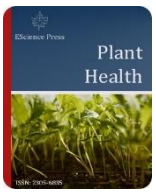

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Isolation and Characterization of Plant Growth Promoting Rhizobacteria from different Soil Layers of Sunflower Crop

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ABSTRACT

Sunflower is one of the four major oilseed crops produced in Pakistan as well as in the globe and it ranks 3rd after peanuts and soybean in contributing to edible oil production across the globe. The Genus Rhizobium is an example of a growth-promoting organisms and is the one of the most widely known group used for growth promotion. Plant growth promoting rhizobacteria (PGPR) has been successfully commercialized with much practical application in agriculture by developing symbiosis with plants. Rhizospheric, rhizoplanic, and endorizoplanic soils were collected from sunflower fields grown at different sites in NARC as they are habitat of PGPR. Ammonia production, HCN production, IAA production, siderophore production, phosphate solubilization, protease production, pectinase production, amylase production, catalase production, and mycelial growth inhibition were recorded during this study. From the samples, 39 strains were tested against all the above-mentioned criterions. All strains exhibited at least one trait amongst the above-mentioned traits. This make these strains as potential plant growth promoting rhizobacteria candidate and their commercial application can be explored for large scale utilization.

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INTRODUCTION

Sunflower (*Helianthus annus L.)* locally known as Surajmukhi belongs to the family Compositeae which is one of the four major oilseed crops globally. It ranks 3rd after peanuts and soybean in contributing to edible oil production across the globe (Jadhao et al., 2023). The seeds of Sunflower contain 40% oil which is high in quality, low in cholesterol, and contains fat-soluble vitamins i.e. A, B, E, and K which is good for cardiac patients (Rahim et al., 2023). Sunflower crops are attacked by numerous diseases that result in growth retardation resulting in poor and low-quality yield (Lukurugu et al., 2023). In Pakistan, Charcoal rot is the most serious disease that attacks sunflower crops (Afshan et al., 2023). (Mirza and Beg, 1983) conducted the first survey of sunflower crops in the central and northern areas of Pakistan during 1982. In the survey, *M. phaseolina* (Charcoal rot) and *Rhizopus spp*. (head rot) was reported as the most destructive on sunflower crops. (Mirza et al., 1984) reported yield loss due to *M. phaseolina* up to 90% in Pakistan on sunflower. The Charcol rot pathogen (*Macrophomina phaseolina*) infects the root or lower stem causing rots that result in

premature loss of plants (Khan and Javaid, 2023 Ghosh et al., 2018). This fungus is a soil born as well as seed fungi. Charcoal rot disease symptoms are the presence of greenish to black discoloration on the plant's stem base which extends upward resulting in hallowing of interior parts. The occurrence of this disease is higher in semi-arid areas with low moisture and higher temperatures. High temperature in soil supports infection and delays the development of roots. The disease was first reported in Pakistan in 1984 (Ahmad *et al*., 1991) and now it has become a major threat to sunflower production.

Plant Growth Promoting Rhizobacteria (PGPR) improve plant growth by closely attaching to plant roots. The promising outcomes of manipulating crop rhizosphere microbial population through inoculation of beneficial bacteria may increase plant growth and excellent results shown in greenhouse studies and laboratories (Timofeeva et al., 2023) whereas the results are variable in the field conditions. The seed dressing with PGPR promotes seedling formation, helps ensure produce, and reduces quality losses which were due to many diseases and pests. Seed treatment helps to control fungi residing over or in the are very effective against pathogens that reside in the soil and results in seed rotting, root rot, and damping off (Das et al., 2024). The present study is designed for disease management of charcoal rot in sunflower crops with the following main objectives: To characterize the plant growth promoting *Rhizobacteria* obtained from sunflower, Assessing the impact of plant growth promoting *Rhizobacteria* strains on Charcoal Rot disease of Sunflower, and testing of selected plant growth promoting Rhizobacteria strains under greenhouse and field conditions to be used as bio-fertilizer for control of charcoal rot disease of sunflower.

MATERIALS AND METHODS

The present study was carried out in the soil Biology and Biochemistry Laboratory at NARC Islamabad.

Collection of Soil Samples

Five soil samples were collected at random from the rhizosphere of sunflowers in three fields of NARC. After harvest, the remaining stubbles were plowed into the soil, sunflower roots were uprooted and one kg rhizosphere soil, the thin layer of soil about 1-2 mm thick surrounding the root, rhizoplane, and endorhizoplane samples were carefully transferred by shaking into sterile plastic bags. They were then transported to the Soil Biology and Biochemistry Laboratory NARC, for isolation of bacteria. Each sample of rhizosphere soil was sieved to remove plant debris before being processed for the isolation of bacteria.

Bacterial Isolation from Soil samples

For the isolation of bacteria from sunflower rhizosphere, rhizoplane, and endorhizoplane, many soil samples were collected. Each soil sample was mixed with one-gram soil and transferred to 9 ml distilled water solution serially diluted up to 10^{-9}. An aliquot of 0.1 ml from four dilutions ($10^{-2,}$ $10^{-4,}$ $10^{-8,}$ and $10^{-9)}$ was spread-plated on Luria Bertani (LB) medium and spread-plate cultures were incubated for 24 hr. at 28ºC. Representative colonies, with different morphological appearances, were selected from the countable plates and re-steaked on a new plate of the same media to obtain pure colonies. A total of 39 isolates were obtained in this manner from sunflowers (Table 1) and maintained on Luria Bertani (LB) agar slants as described in (Aneja, 2002).

Characterization of the Selected Promising Strains

The isolated PGPR strains were characterized based on morphological and biochemical tests as described in Berger's Manual of Determinative Bacteriology. These are detailed as under.

Colony Morphology of the Isolated Strains and Gram's Staining of Bacteria

The isolated strains were examined for their colony morphology, cell shape, form, elevation, margin, and opacity. For this purpose, all the plates were observed using a magnifying glass (Aneja, 2002). Gram's staining was done according to the method of (Vincent et al., 1970). The purple color indicated a gram-positive colony while the pink color indicated the colony as gram-negative.

Biochemical Characterization and Indole Acetic Acid (IAA) production

The biochemical characterization of the bacteria was done as per the procedures outlined by (Beaudoin et al., 2007). Production of indole acetic acid (IAA) was assessed by the method of (Gordon and Paleg, 1957), with pre-sterilized nutrient agar (NA) broth tubes, containing L-tryptophan (5gm/L). The bacterial culture was inoculated and then tubes were incubated for 48 hours at 28ºC, each tube was then added with 10 drops of Kovac's reagent. The production of red color was taken as positive for the indole production.

Table 1. Bacteria isolated from rhizosphere, rhizoplane, and endorhizoplane of sunflower grown in different sites of NARC Islamabad.

Sr.	Strain Code	Source	Location	Sr.	Strain Code	Source	Location
1	NASF-1	Rhizosphere	NARC site 1	21	NASF-21	Endorhizoplane	NARC site 3
2	NASF-2	Rhizosphere	NARC site 1	22	NASF-22	Endorhizoplane	NARC site 3
3	NASF-3	Rhizoplane	NARC site 2	23	NASF-23	Endorhizoplane	NARC site 3
4	NASF-4	Rhizosphere	NARC site 1	24	NASF-24	Rhizoplane	NARC site 4
5	NASF-5	Rhizosphere	NARC site 2	25	NASF-25	Rhizoplane	NARC site 2
6	NASF-6	Rhizoplane	NARC site 2	26	NASF-26	Endorhizoplane	NARC site 1
7	NASF-7	Rhizoplane	NARC site 3	27	NASF-27	Endorhizoplane	NARC site 1
8	NASF-8	Rhizoplane	NARC site 1	28	NASF-28	Endorhizoplane	NARC site 3
9	NASF-9	Rhizoplane	NARC site 3	29	NASF-29	Rhizosphere	NARC site 5
10	NASF-10	Endorhizoplane	NARC site 3	30	NASF-30	Rhizosphere	NARC site 5
11	NASF-11	Rhizosphere	NARC site 3	31	NASF-31	Rhizoplane	NARC site 2
12	NASF-12	Endorhizoplane	NARC site 3	32	NASF-32	Rhizosphere	NARC site 4
13	NASF-13	Endorhizoplane	NARC site 4	33	NASF-33	Endorhizoplane	NARC site 3
14	NASF-14	Rhizoplane	NARC site 1	34	NASF-34	Endorhizoplane	NARC site 3
15	NASF-15	Rhizoplane	NARC site 4	35	NASF-35	Rhizoplane	NARC site 1
16	NASF-16	Rhizosphere	NARC site 5	36	NASF-36	Rhizosphere	NARC site 3
17	NASF-17	Rhizoplane	NARC site 5	37	NASF-37	Rhizosphere	NARC site 2
18	NASF-18	Rhizosphere	NARC site 1	38	NASF-38	Endorhizoplane	NARC site 4
19	NASF-19	Rhizosphere	NARC site 1	39	NASF-39	Rhizosphere	NARC site 5
20	NASF-20	Endorhizoplane	NARC site 3				

Phosphate Solubilization

A pure colony from a fresh culture of each bacterial strain was inoculated on to Pikovskaya media (Pikovskaya, 1948) agar plates, using a sterile needle. Tricalcium phosphate was used as a source of insoluble inorganic phosphate. Phosphate solubilization activity was determined by the development of clear halos surrounding colonies on Pikovskaya agar plates, after incubation for 7 days at 28°C. The halo size produced by the respective bacteria was calculated according to the formula below (Singh *et al.,* 2011).

$$\text{Phosphate solubilization Index} = \frac{\text{Colony Diameter} + \text{Clearing Zone}}{\text{Colony Diameter}}$$

Catalase Activity

For catalase activity, PGPR strains were streaked on LB agar plates. After 24 hrs. of incubation, a single colony of each isolate was picked and put on a glass slide. A single drop of H_2O_2 was added and the formation of bubbles within 10 seconds was observed. Bubble production indicated a positive result, while no bubble indicated a negative result (Montgomerie, 1966; Macfaddin, 2000).

Ammonia and Siderophore Production

All the isolates were tested for the production of ammonia in peptone water, each tube was incubated for 48 h at 30° C. Nessler's reagent (0.5 ml) was added and development of brown to yellow color was a positive test for ammonia production (Joseph *et al.,* 2007; Cappuccino and Sherman, 1992). Siderophore production of rhizospheric isolates was determined with a modified protocol as described by (Schwyn and Neilands, 1987) using blue indicator dye, chrome azurol S (CAS). CAS (60.5 mg) was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3$, $6H_2O$, 10 mM HCL). The CAS and $FeCl_3$ solution were slowly added with constant stirring to hexadecyltrimethyl ammonium bromide (HDTMA) (72.9 mg) dissolved in 40 ml water and then autoclaved. The final mixture of 100 ml was added to 900 ml of autoclaved LB medium with pH 6.8. Bacterial isolates exhibiting a yellowish-orange halo after 5 days of incubation at 28 $\pm$ 2°C were considered positive for the production of siderophores (Lodha et al., 2002; Clark et al., 1994).

Production of Hydrogen Cyanide

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck, (1948). Nutrient broth was amended with 4.4 g glycine/1 and bacteria were streaked on the modified agar plate. A Whatman filter paper No. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36±2°C for 4 days. The development of orange to red color indicated HCN production.

Protease, Amylase and Pectinase Production Activity

For protease activity, the bacterial isolates were grown on skimmed milk agar medium (SKM) by the procedure described by (Kazempour et al., 2004). After incubation at 35°C for 48 hrs. Zone formation was observed around the isolates (Adinarayana *et al.,* 2003). PGPR strains were tested for amylase production on a specific medium. The isolates were streaked on freshly prepared plates and incubated at 35°C. Clear zones were observed after 24 hrs. of incubation (Bertrand et al., 2004; Ashwini *et al.,* 2011). For the pectinase production test, the strains were streaked on a specific medium. Zones were checked after 24 hours of incubation at 35ºC (Stutzenberger et al., 1992; Namasivavam *et al.,* 2011).

RESULTS

Investigations were carried out on the evaluation and potential of biocontrol agent against *M. phaseolina* causing charcoal rot of sunflower. Attempts were also made to elucidate the mechanisms of biocontrol in the potent antagonistic strains of PGPR and their plant growth promotional activity was evaluated.

Isolation of Bacterial Isolates

Overall, 39 bacterial strains were isolated from the rhizosphere, rhizoplane and endorhizoplane of sunflower by serial dilution method from different sites of NARC fields (Table 1).

Colonial Characteristic of PGPR Strains

All the bacterial isolates were characterized on the basis of conventional method like colony morphology viz; shape, elevation, margin, opacity, color. However, eighteen PGPR isolates were selected for further study. The rhizobacteria have been screened for antifungal activity, indole Acetic acid production IAA, Ammonia production, siderophore production, HCN production, other lytic enzymes as catalases, protease, pectinase and amylase production, as described in Bergy's Manual of Systematic Bacteriology. Morphological characteristics of PGPR isolates widely varied. All the isolates produced round shape and raised colonies having smooth shiny surface with smooth margin. They differed in color, but all were odorless and no pigmentation was observed in the colonies of LB agar plates. Diameters of colonies of isolates varied.

Table 2. Colonial characteristics of PGPR bacterial strains isolated from sunflower.

Sr.	Strain Code	Form/ shape	Elevation	Margin	Opacity	Color
1	NASF-1	Circular	Raised	Entire	Opaque	Yellow
2	NASF-2	Circular	Flat	Entire	Translucent	Green
3	NASF-3	Irregular	Convex	Undulate	Opaque	Milky White
4	NASF-4	Irregular	Convex	Undulate	Opaque	Milky White
5	NASF-5	Circular	Flat	Entire	Opaque	Milky White
6	NASF-6	Circular	Flat	Entire	Opaque	Green
7	NASF-7	Irregular	Umbilicate	Undulate	Opaque	White
8	NASF-8	Circular	Umbilicate	Entire	Opaque	Green
9	NASF-9	Irregular	Convex	Undulate	Opaque	Milky White
10	NASF-10	Irregular	Flat	Erose	Translucent	Greenish
11	NASF-11	Circular	Flat	Entire	Opaque	Milky White
12	NASF-12	Irregular	Flat	Erose	Translucent	Milky White
13	NASF-13	Irregular	Umbilicate	Erose	Translucent	Milky White
14	NASF-14	Circular	Flat	Erose	Translucent	Milky White
15	NASF-15	Rhizoid	Convex	Filamentous	Opaque	Yellow
16	NASF-16	Circular	Convex	Entire	Opaque	Milky White

17	NASF-17	Filamentous	Raised	Lobate	Translucent	Green
18	NASF-18	Irregular	Flat	Undulate	Translucent	Green
19	NASF-19	Spindle	Flat	Entire	Opaque	Yellow
20	NASF-20	Filamentous	Flat	Erose	Opaque	Milky White
21	NASF-21	Circular	Flat	Entire	Opaque	Milky White
22	NASF-22	Irregular	Flat	Erose	Translucent	Milky White
23	NASF-23	Filamentous	Flat	Lobate	Translucent	Green
24	NASF-24	Circular	Flat	Entire	Translucent	Yellow
25	NASF-25	Rhizoid	Convex	Erose	Opaque	Milky White
26	NASF-26	Irregular	Flat	Undulate	Opaque	Green
27	NASF-27	Circular	Flat	Entire	Opaque	Orange
28	NASF-28	Filamentous	Flat	Entire	Translucent	White
29	NASF-29	Circular	Raised	Entire	Opaque	Yellow
30	NASF-30	Rhizoid	Convex	Erose	Opaque	Milky White
31	NASF-31	Filamentous	Flat	Erose	Translucent	Green
32	NASF-32	Circular	Flat	Undulate	Translucent	Yellow
33	NASF-33	Circular	Convex	Entire	Opaque	Milky White
34	NASF-34	Circular	Flat	Entire	Opaque	Orange
35	NASF-35	Rhizoid	Convex	Erose	Opaque	Milky White
36	NASF-36	Filamentous	Raised	Lobate	Translucent	Green
37	NASF-37	Circular	Flat	Entire	Opaque	Orange
38	NASF-38	Circular	Raised	Entire	Opaque	Yellow
39	NASF-39	Circular	Flat	Entire	Opaque	Milky White

Isolates which were isolated from sunflower have flat elevation, while NASF3, NASF4, NASF9, NASF16, NASF25, NASF30, NASF33 and NASF35 were convex, NASF1, NASF17, NASF29, NASF36 and NASF38 were raised, NASF7, NASF8 and NASF13 were umbilicate (Table 2, Figure 1). Colony margins were observed from entire to erose; whereas seven isolates NASF3, NASF4, NASF7, NASF9, NASF18, NASF26 and NASF32 have undulated margins. Most of the isolates have opaque to translucent opacity except one isolate NASF18 which has transparent opacity. The colony shape/form in most of the cases was circular to irregular whereas, isolates NASF17, NASF20, NASF28, NASF31, NASF23 and NASF36 were filamentous and one isolate NASF19 was observed as in spindle shape and three isolates NASF15, NASF30, NASF35 were rhizoid. The colony color of isolates varied from yellow to milky white; whereas fifteen isolates NASF3, NASF4, NASF5, NASF9, NASF11, NASF12, NASF13, NASF14, NASF20, NASF21, NASF25, NASF30, NASF33, NASF35, NASF39 were milky white, only one isolate NASF7 was white in color, nine were green. Some of the colonies like NASF1, NASF15, NASF19, NASF24, NASF29, NASF32 and NASF38 were yellow in color.

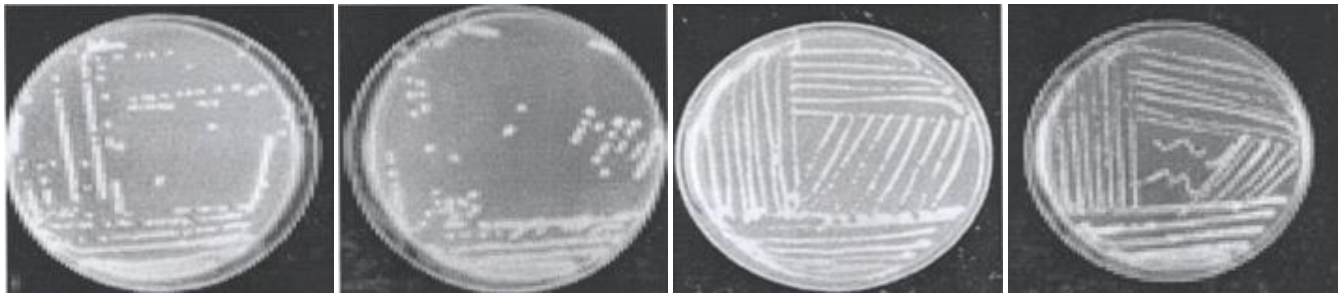

Figure 1. Colonial characteristics of PGPR bacterial strains isolated from sunflower.

Microscopic Observation of PGPR Isolates

All the isolated PGPR strains were found gram +ve and showed purple color under microscope. *Bacillus* species (8 in numbers) were rod shaped, while *Streptococcus* species (13 in numbers) were round. *Micrococcus* species (9 in numbers) were Spherical shaped. Details are given in Table 3 and Figure 2.

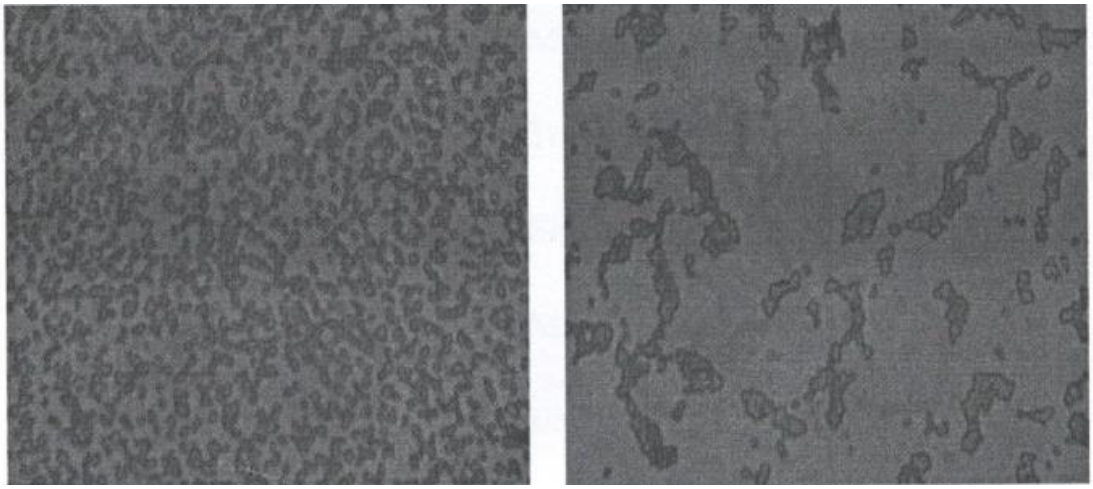

Figure 1. Cell morphology of PGPR bacterial strains.

Table 3. Cell Morphology of and growth promotion traits of PGPR bacterial strains isolated from sunflower.

Sr.	Strain ID.	Gram staining	Shape	Group	Ammonia	HCN	IAA	Catalase	Antagonistic Activity
1	NASF1	+	Round	*Streptococcus*	+	+	+	-	+
2	NASF2	+	Rod	*Bacillus*	-	+	-	+	-
3	NASF3	+	Rod	*Bacillus*	-	-	-	+	-
4	NASF4	+	Spherical	*Micrococcus*	-	-	-	+	-
5	NASF5	+	Round	*Streptococcus*	+	+	+	+	+
6	NASF6	+	Spherical	*Micrococcus*	-	-	-	+	-
7	NASF7	+	Round	*Staphylococcus*	+	+	+	+	+
8	NASF8	+	Spherical	*Micrococcus*	+	+	+	+	+
9	NASF9	+	Round	*Streptococcus*	+	+	+	+	+
10	NASF10	+	Rod	*Bacillus*	+	+	+	+	+
11	NASF11	+	Spherical	*Micrococcus*	+	+	+	+	+
12	NASF12	+	Round	*Staphylococcus*	+	+	+	+	+
13	NASF13	+	Round	*Staphylococcus*	+	+	+	+	+
14	NASF14	+	Spherical	*Micrococcus*	+	-	+	+	+
15	NASF15	+	Rod	*Bacillus*	+	+	-	+	+
16	NASF16	+	Round	*Staphylococcus*	+	+	+	+	+
17	NASF17	+	Round	*Streptococcus*	-	-	-	+	-
18	NASF18	+	Round	*Staphylococcus*	+	+	+	+	+
19	NASF19	+	Round	*Staphylococcus*	-	-	-	+	-
20	NASF20	+	Round	*Streptococcus*	+	+	+	+	+
21	NASF21	+	Round	*Staphylococcus*	-	-	-	+	-
22	NASF22	+	Spherical	*Micrococcus*	+	+	+	+	+
23	NASF23	+	Round	*Streptococcus*	+	+	+	+	+
24	NASF24	+	Spherical	*Micrococcus*	+	+	+	+	+

25	NASF25	+	Round	*Staphylococcus*	-	-	-	+	
26	NASF26	+	Rod	*Bacillus*	-	-	-	+	
27	NASF27	+	Rod	*Bacillus*	-	-	-	+	-
28	NASF28	+	Round	*Streptococcus*	-	-	-	+	-
29	NASF29	+	Round	*Streptococcus*	-	-	-	+	-
30	NASF30	+	Round	*Streptococcus*	-	-	-	+	-
31	NASF31	+	Round	*Streptococcus*	-	-	-	+	-
32	NASF32	+	Round	*Streptococcus*	+	+	+	+	+
33	NASF33	+	Round	*Streptococcus*	-	-	-	+	-
34	NASF34	+	Round	*Streptococcus*	-	-	-	+	-
35	NASF35	+	Spherical	*Micrococcus*	-	-	-	+	-
36	NASF36	+	Rod	*Bacillus*	-	-	-	+	-
37	NASF37	+	Round	*Streptococcus*	+	+	+	+	+
38	NASF38	+	Spherical	*Micrococcus*	-	-	-	+	-
39	NASF39	+	Rod	*Bacillus*	-	-	-	+	-

Screening of PGPR by analyzing their Plant Growth Promoting Traits

Ammonia Production

All the 39 bacterial isolates were then checked for their PGPR activities. First of all, they were checked for ammonia production activity, all the selected rhizobacteria isolated from the rhizosphere of sunflower. Among them 19 isolates NASF1, NASF5, NASF7, NASF8, NASF9, NASF10, NASF11, NASF12, NASF13, NASF14, NASF15, NASF16, NASF18, NASF19, NASF20, NASF22, NASF23, NASF24, NASF32, NASF37 showed positive result for Ammonia production (Table 3, Figure 3).

HCN Production

The ability of all the 39 antagonistic isolates to produce HCN was determined by the picric acid assay. 22 out of 39 isolates produced HCN (Table 3, Figure 4). Isolates NASF1, NASF2, NASF5, NASF7, NASF8, NASF9, NASF10, NASF11, NASF12, NASF13, NASF14, NASF15, NASF16, NASF18, NASF19, NASF20, NASF22, NASF23, NASF24, NASF32, NASF37 showed the change in color of filter paper strip placed on the upper of petri plate indicated the positive production of HCN.

Indole Acetic Acid Production

All of 39 bacterial isolates were examined for the production of IAA. The data presented in table 3 indicated that isolates produced IAA. Isolates were assessed for production of IAA on pre- sterilized nutrient agar (NA) broth tubes, containing L-tryptophan (5 g/L.). Out of 39 isolates, 19 isolates NASF1, NASF5, NASF7, NASF8, NASF9, NASF10, NASF11, NASF12, NASF13, NASF14, NASF16, NASF18, NASF19, NASF20, NASF22, NASF23, NASF24, NASF32, NASF37 showed cherry (deep) red color in the top layer of tube bacteria was found positive test (Table 3, Figure 5).

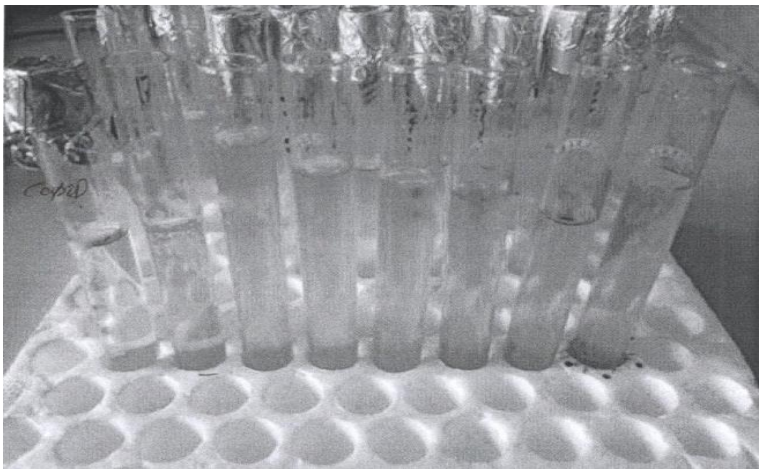

Figure 2. Ammonia production by antagonistic bacterial isolates

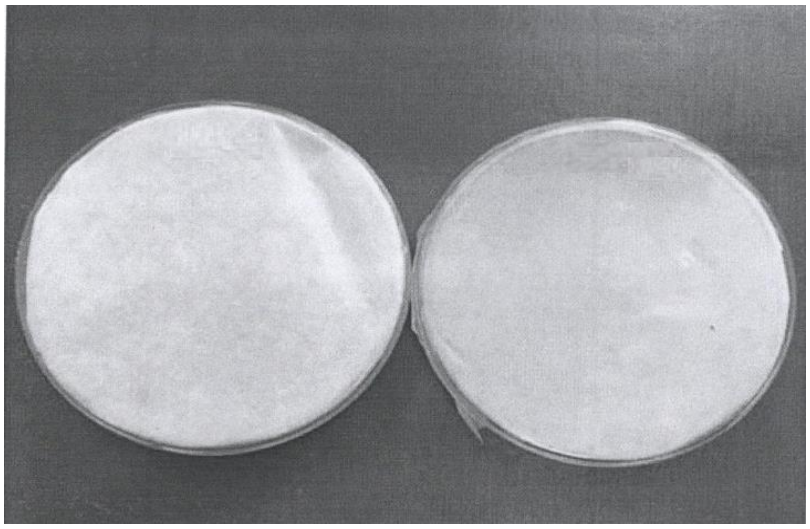

Figure 3. HCN production by strain NASF7.

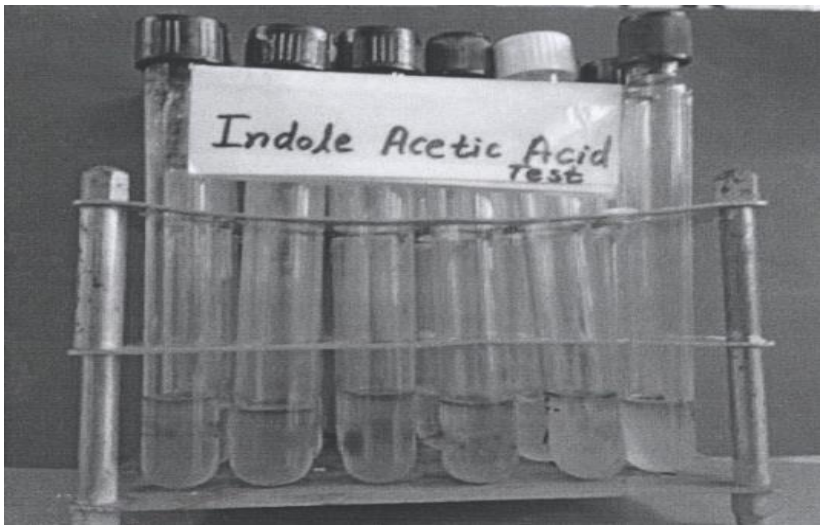

Figure 4. IAA production by PGPR isolated strains.

Catalase Production

The catalase activity of PGPR strains was evaluated by LB agar plates which were used to streak the PGPR strains. After 24 hours of incubation a single colony of each isolate was picked and put on a glass slide. On each colony a single drop of H_2O_2 was added and formation of bubbles within 10 seconds was observed. Among isolates NASF2, NASF3, NASF4, NASF4, NASF5, NASF6, NASF7, NASF8, NASF9, NASF10, NASF11, NASF12, NASF13, NASF14, NASF15, NASF16, NASF17, NASF18, NASF19, NASF20, NASF21, NASF22, NASF23, NASF24, NASF25, NASF26, NASF27, NASF28, NASF29, NASF31, NASF32, NASF33, NASF34, NASF35, NASF36, NASF37, NASF38, NASF39 produce bubble indicated a positive result, while no bubbles indicated negative (Table 3, Figure 6).

Antagonistic Activity

Antagonistic activities of the bacterial isolates were assessed in terms of inhibition zone diameter as a pointer of the reduction in growth of phytopathogenic fungus *M. phaseolina* whereas isolates NASF1, NASF5, NASF7, NASF9, NASF10, NASF11, NASF12, NASF13, NASF14, NASF15, NASF16, NASF18, NASF20, NASF22, NASF23, NASF24, NASF32, NASF37 showed good antagonistic activity toward *M. Phaseolina* (Table 3). This suggests that the modes of action and types of antifungal metabolites produced vary from a rhizobacteria to another.

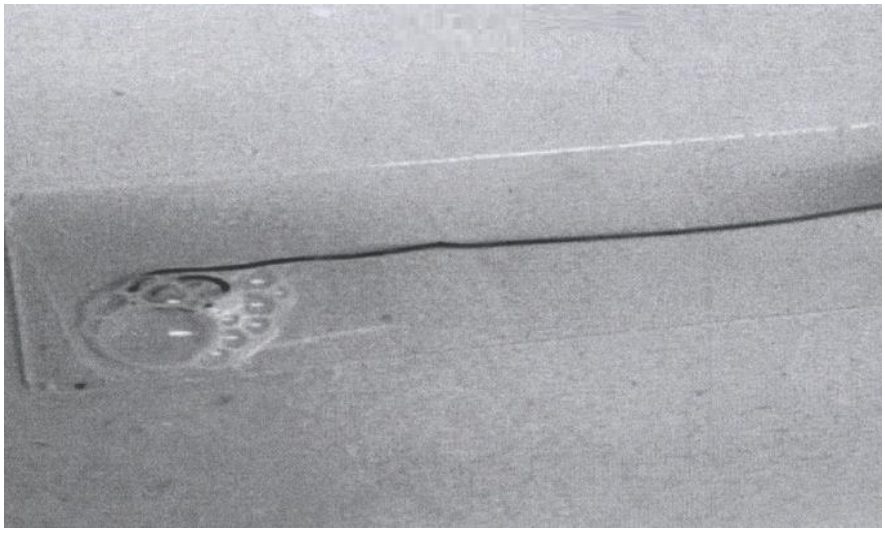

Figure 6. Catalase production by bacterial strain NASF7.

DISCUSSION

Soil is considered as a storehouse of microbial activity although the living microorganisms in soil occupy less than five percent of the total space. These teeming microscopic and macroscopic organisms perform many vital functions including plant growth promoting activities viz; release of mineral elements from organic matter for plant uptake, production of IAA, and phosphorus solubilization etc. (Megha *et al.,* 2007a). The rhizobacteria have unique capability to inhibit, suppress and proliferation of pathogens and to enhance host plant growth by through several different mechanisms (Haas and Defago, 2005). Earlier researchers have found that rhizobacteria obtained from the rhizosphere of plants growing in soil is a favorable habitat for the antagonistic rhizobacteria (Cazorla *et al*., 2006). So, it could be inferred that almost all kinds of agricultural soils induce suppressive effect on various soil borne phytopathogens that is the result of antagonistic activities of these rhizobacteria inhabiting the rhizosphere (Weller *et al.,* 2002). PGPR to produce ammonia is considered as an important measure which influences indirectly growth of plant (karuppiah and Rajaram, 2011). All the selected rhizobacteria isolated from the rhizosphere of sunflower were checked for this property. Nineteen selected isolated NASF1, NASF5, NASF7, NASF8, NASF9, NASF10, NASF11, NASF12, NASF13, NASF14, NASF15, NASF16, NASF18, NASF19, NASF20, NASF22, NASF23, NASF24, NASF32, NASF37 showed positive results for ammonia production. The results are in close conformity with those reported by (Samuel and Muthukkaruppan, 2011) and (Joseph *et al.,* 2007), who reported similar activity in 95% of strains isolated from the rhizosphere of rice, mangroves and soils contaminated by effluent. Bacterial strains producing hydrogen cyanide constitute good biocontrol agents as it induces the resistance in plants against phytopathogens (Berg *et al.,* 2002; Ramette *et al*., 2003; Defago *et al*., 1990). Rhizobacteria inhibit the plant pathogenic fungi by the production of volatile HCN (Ahmad *et al.,* 2008; Blumer and Hass, 2000). Out of 39 isolates, 22 produced HCN, which changed the color of filter paper strip placed on the upper of petri plate indicating positive HCN production. On the basis of HCN production, 18 rhizobacteria were selected, all of them exhibited several desirable characteristics which may suppress the fungal pathogens and promote the plant growth directly or indirectly or synergistically. IAA is one of the most important phytohormone which may function as important signal molecule, plant growth promotion, root initiation, and has an indirect role in disease suppression (Salisbury, 1994). Among the tested isoltes, 16 bacterial isolates were positive for IAA production. The results obtained in the present investigation are also comparable to those of earlier workers (Barea *et al*., 1976; Leinhos and Vaseck, 1994; Mahesh Kumar, 1997; Veena, 1999; Geeta, 2001, Suneesh, 2004; Megha *et al*., 2007b. Naik *et al*., 2008) reported the production of IAA by 63 out of 95 fluorescent pseudomonads isolated from banana rhizosphere. In biological control the role of enzymes is usually associated with the mechanisms called parasitism and antibiosis. Specially, the hydrolytic

enzymes including ß-1,3-glucanases, chitinases, amylases, chitinases, proteases, catalases and cellulases are very important. Expression and secretion of enzymes by rhizobacteria result in the suppression of plant pathogen. These bacterial strains contribute significantly to inhibition of fungal phytopathogens with a significant increase in root colonization and plant development (Gray and Smith, 2005). In our study, protease production was detected in twenty-six rhizobacteria. Earlier studies reported that microorganisms secrete the extra cellular enzyme including proteases which inhibit various bacterial (Johansen *et al*., 2002; Priest *et al.,* 1988.) and fungal communities (Girlanda *et al*., 2001). A number of reporters demonstrated that rhizobacteria that exhibit the protease activity help in the biological control of pathogens (Rakh et al., 2011; Bragger *et al*., 1989). Siderophore production is one of the important traits of PGPR and is driving much attention since last few decades due to applications of siderophores in various other fields apart from agriculture. Under aerobic conditions, most iron exists in an insoluble form ((Fe3+) in soil, which is just not easily available to plants or microbes, even though it is required in a number of major physiological processes like N2-fixation, photosynthesis, respiration, etc. (Dudeja *et al*., 1997). A phosphate solubilization, a plant growth promoting activity to isolate the potent rhizobacteria which not only have the capability to suppress the proliferation of fungal pathogens but also promote the growth of plants. The selected PGPR isolated in this study, not only inhibited the fungal growth of *M. phaseolina* but also increase the agronomic parameters of sunflower plants. Many scientists have also reported the efficacy of PGPR in controlling wide spectrum of plant pathogens such as *Sclerotium rolfsii* (Bhatia *et al*., 2005), *Alternaria helianthi* (Prasad *et al.,* 2003), *Pythium aphanidermatum* (Ramesh and Korikanthimath, 2003), *Rhizoctonia solani* (Nandakumar *et al.,* 2001; Zarrin *et al*., 2009) and *Colletotrichum gleosporiodes* (Vivekananthan *et al.,* 2004).

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