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Isolation and physiological characterization of effective antagonistic rhizobacteria from plant rhizospheric soil of *Helianthus annuus*

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Abstract

Diverse chemical pesticides/fungicides are used in particular doses for plant development purpose in agriculture. But for numerous side effects of chemical pesticides there is a better concern on the mutual activities between plants and the rhizospheric microorganisms. So, recently the Plant Growth Promoting Rhizobacteria (PGPR) application opening a new opportunity to resolve these issues. The rhizobacteria not only can decrease the disease incidence, but also increase the plant growth. In this article we are going to focus on collection of soil samples, isolation of bacteria, colony morphology, cell morphology and Gram's reaction of bacterial isolates, isolated from the rhizosphere, rhizoplane and endorhizoplane of sunflower. Mainly isolates of sunflower were flat elevation, colony margins were observed from entire to erose. Most of the isolates showed opaque to translucent opacity, but one isolate NASF18 was transparent opacity. The colony color of isolates varied from yellow to milky white. From isolated PGPR strains twenty six were gram -ve bacteria and showed pink color during gram staining reaction, whereas isolates NASF2, NASF6, NASF8, NASF9, NASF17, NASF20, NASF22, NASF24, NASF26, NASF26, NASF35, NASF38 were gram +ve and showed purple color under microscope. All isolates were examined microscopically and different cell shapes were observed from *Bacilli* to *Coccus* and cell grouping of the bacterial isolates varied from *streptococcus* to *bacillus*.

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Introduction

From previous three decades with green revolution, production of agriculture has increased due to indiscriminate use of inorganic fertilizers and high yielding crop varieties. Although the unnecessary use of chemical fertilizers and crop defensive chemicals resulted in degradation of flora and fauna of the ecosystem. Hence it is essential to evolve and adapt a strategy for sustainable crop production by using judicious combinations of chemical pesticides and organic biocontrol agents (Tank and Saraf, 2003). A huge variety of microorganisms such as, fungi and bacteria are found in the rhizosphere of plants. Rhizosphere is nutrient rich area that may exhibit beneficial, neutral and perilous effects on the plants (Berendsen *et al*., 2012).

Rhizobacteria can increase plant growth and reproductive development (Bloemberg and Lugtenberg, 2001) through various actions such as, by synthesizing of numerous compounds (siderophores, hydrolytic enzymes, antibiotics, phytohormones, organic acids) and through Plant Growth Regulators (PGRs) or synthesizing of different biological active compounds (Arshad and Frankenberger, 1998). It is seen that the PGPR show more flexibility in mobilization, transformation and solubulization of various nutrients from bulk soils (Hayat *et al*., 2010). Rhizobacteria are naturally occurring free-living bacteria and they have the capability to assault the different plants and can produce indicative disease infections (Sturz and Nowak, 2000). These beneficial microbes are coated on seeds and they can reduce the harmful effects of soil borne pathogens and also promote the growth of plant (Rangarajan *et al.,* 2003).

A lot of studies have shown that rhizobacteria had beneficial effects on vegetables (Kurabachew and Wydra, 2013), cereals (Shaharoona *et al*., 2006), flowers (An *et al*., 2010), spices like black pepper (Diby and Sarma, 2006) and fruits (Kavino *et al*., 2010). Soil microorganisms (PGPR) have been found to increase the effectiveness of manures and fertilizers, consequently it decreased the recommended rates of fertilizers and manures (Adesemoye *et al*., 2009).

PGPR have also been constitute to increase the biological worth of soils by increasing enzymatic and microbial actions (Dinesh *et al*., 2013). In this study the preliminary objectives were to isolate bacteria from the rhizosphere, rhizoplane and endorhizoplane of sunflower plants, then to characterize their colony morphology, Gram's staining and cell morphology of isolated strains.

Materials and methods

Collection of soil samples

Five soil samples were randomly collected from the rhizosphere of sunflower in three fields at NARC Islamabad. Islamabad is located at 73.04°E longitude and 33.43°N latitude with the elevation of 540 meters of Pothohar Plateau having screne beauty of Capital Territory at Margalla Hills. These sites were selected because the fields were under sunflower cultivation for a long time. After harvesting the crop at maturity, sunflower stubbles were removed, and samples collecting sites were marked. The samples consisted of 1 kg soils collected at 1-2 cm depth and stored in sterile plastic bags, refrigerated at 4°C until processed. Plant debris were removed after sieving the rhizosphere soil samples and further processed for isolation of bacteria.

Bacterial isolation from soil samples

One gram soil sample was added to nine ml distilled water and then serially diluted from 1×10^{-1} to 1×10^{-12}. An aliquot of 0.1ml from four dilutions (1×10^{-2}, 1×10^{-4}, 1×10^{-8} and 1×10^{-9}) was taken out and spread on plates containing Luria Bertani (LB) medium (L3926, Sigma) (Majeed *et al*., 2015). The plate were placed in incubator at 28°C for 24 hrs and observed under a steromicerscope for the growth of PGPR colonies. To obtain pure culture of bacteria, the dissimilar representative colonies were selected and re-streaked on new plates of the same media. A total of thirty nine bacterial strains retrieved by this method were preserved on Luria Bertani (LB) agar slants (Aneja, 2002).

Characterization of the selected promising strains

By following Berger's Manual of Determinative Bacteriology, the above mentioned thirty nine isolates of PGPR were characterized.

Morphological characterization

The isolated strains initially were examined for their colony morphology (form, elevation, margin, opacity, color) cell morphology (cell shape, cell grouping) and Gram's reaction (Aneja, 2002).

Gram's staining of bacteria

Gram's staining of bacterial isolates was done as reported by Vincent (1970). A loop of bacteria culture was picked, a drop of distilled water was placed on a glass slide and the contents were mixed. Smear was initial air dried, fixed by heat and then stained for 1 minute with crystal violet. The slides were rinsed with distilled water after thirty seconds, solution of iodine was put on the smear and pursue by washing in distilled water, the smear was decolorized with 75 percent alcohol for 1 to 2 minutes.

The bacterial smear was washed and then stained with safranin. Glass slides were washed with water and cover slips were placed on top. Then slides were observed under steromicroscope at 100x magnification. Gram positive colonies appeared as purple and gram negative colonies indicated pink color.

Results and discussion

Isolation of bacterial isolates

During the present investigation, over all 39 bacterial strains were isolated from the rhizosphere, rhizoplane and endorhizoplane of sunflower by serial dilution method from different sites of National Agricultural Research Centre (NARC) fields (Table 1).

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

Strain code	Source	Location
NASF1	Rhizosphere	NARC site 1
NASF2	Rhizosphere	NARC site 1
NASF3	Rhizoplane	NARC site 2
NASF4	Rhizosphere	NARC site1
NASF5	Rhizosphere	NARC site2
NASF6	Rhizoplane	NARC site2
NASF7	Rhizoplane	NARC site3
NASF8	Rhizoplane	NARC site1
NASF9	Rhizoplane	NARC site3
NASF10	Endorhizoplane	NARC site3
NASF11	Rhizosphere	NARC site3
NASF12	Endorhizoplane	NARC site3
NASF13	Endorhizoplane	NARC site4
NASF14	Rhizoplane	NARC site1
NASF15	Rhizoplane	NARC site4
NASF16	Rhizosphere	NARC site5
NASF17	Rhizoplane	NARC site5
NASF18	Rhizosphere	NARC site1
NASF19	Rhizosphere	NARC site1
NASF20	Endorhizoplane	NARC site3
NASF21	Endorhizoplane	NARC site3
NASF22	Endorhizoplane	NARC site3
NASF23	Endorhizoplane	NARC site3
NASF24	Rhizoplane	NARC site4
NASF25	Rhizoplane	NARC site2
NASF26	Endorhizoplane	NARC site1

NASF27	Endorhizoplane	NARC site1
NASF28	Endorhizoplane	NARC site3
NASF29	Rhizosphere	NARC site5
NASF30	Rhizosphere	NARC site5
NASF31	Rhizoplane	NARC site2
NASF32	Rhizosphere	NARC site4
NASF33	Endorhizoplane	NARC site3
NASF34	Endorhizoplane	NARC site3
NASF35	Rhizoplane	NARC site1
NASF36	Rhizosphere	NARC site3
NASF37	Rhizosphere	NARC site2
NASF38	Endorhizoplane	NARC site4
NASF39	Rhizosphere	NARC site5

Earlier researchers have found that rhizobacteria obtained from the rhizoplane and rhizosphere of crops growing in soil constitutes a favourable habitat for the antagonistic rhizobacteria (Cazorla *et al.,* 2006). It could be thus inferred that most of agricultural soils induce some suppressive effect on the soil borne phytopathogens which may be the result of antagonistic activities of these rhizobacteria inhabiting in rhizosphere. Generally a fertile soil is prevailed by many beneficial microbes which devise a number of siderophores, fungicidal compounds and antibiotics, enabling them to compete with other microorganisms and induced systemic resistance against plant pathogenic organisms (Weller *et al*., 2002).

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

Sr. No	Strain-code	Form/Shape	Elevation	Margin	Opacity	Color
1	NASF1	Circular	Raised	Entire	Opaque	Yellow
2	NASF2	Circular	Flat	Entire	Translucent	Green
3	NASF3	Irregular	Convex	Undulate	Opaque	Milky white
4	NASF4	Irregular	Convex	Undulate	Opaque	Milky white
5	NASF5	Circular	Flat	Entire	Opaque	Milky white
6	NASF6	Circular	Flat	Entire	Opaque	Green
7	NASF7	Irregular	Umbilicate	Undulate	Opaque	White
8	NASF8	Circular	Umbilicate	Entire	Opaque	Green
9	NASF9	Irregular	Convex	Undulate	Opaque	Milky white
10	NASF10	Irregular	Flat	Erose	Translucent	Greenish
11	NASF11	Circular	Flat	Entire	Opaque	Milky white
12	NASF12	Irregular	Flat	Erose	Translucent	Milky white
13	NASF13	Irregular	Umbilicate	Erose	Translucent	Milky white
14	NASF14	Circular	Flat	Erose	Translucent	Milky white
15	NASF15	Rhizoid	Convex	Filamentous	Opaque	Yellow
16	NASF16	Circular	Convex	Entire	Opaque	Milky white
17	NASF17	Filamentous	Raised	Lobate	Translucent	Green
18	NASF18	Irregular	Flat	Undulate	Transparent	Green
19	NASF19	Spindle	Flat	Entire	Opaque	Yellow
20	NASF20	Filamentous	Flat	Erose	Opaque	Milky white
21	NASF21	Circular	Flat	Entire	Opaque	Milky white

22	NASF22	Irregular	Flat	Erose	Translucent	Milky white
23	NASF23	Filamentous	Flat	Lobate	Translucent	Green
24	NASF24	Circular	Flat	Entire	Translucent	Yellow
25	NASF25	Rhizoid	Convex	Erose	Opaque	Milky white
26	NASF26	Irregular	Flat	Undulate	Opaque	Green
27	NASF27	Circular	Flat	Entire	Opaque	Orange

Colonial characteristic of PGPR strains

Characterization of all the PGPR isolates was done on the basis of conventional method like colony morphology namely; shape, opacity, color, margin and elevation as described in Bergy's Manual of Systematic Bacteriology. All the bacterial strains were found to be, dissimilar in their morphological characteristics from each other (Tein *et al.*, 1979).

Table 3. Cell morphology of PGPR strains isolated from sunflower.

Sr. No.	Strain Code.	Shape	Group	Color	Gram reaction
1	NASF1	Coccus	Streptococcus	Pink	Negative
2	NASF2	Bacillus	Bacillus	Purple	Positive
3	NASF3	Spirochete	Bacillus	Pink	Negative
4	NASF4	Bacillus	Micrococcus	Pink	Negative
5	NASF5	Coccus	Streptococcus	Pink	Negative
6	NASF6	Coccus	Micrococcus	Purple	Positive
7	NASF7	Coccus	Staphylococcus	Pink	Negative
8	NASF8	Coccus	Micrococcus	Purple	Positive
9	NASF9	Coccus	Streptococcus	Purple	Positive
10	NASF10	Coccus	Bacillus	Pink	Negative
11	NASF11	Coccus	Micrococcus	Pink	Negative
12	NASF12	Coccus	Tetrad	Pink	Negative
13	NASF13	Coccus	Staphylococcus	Pink	Negative
14	NASF14	Bacillus	Micrococcus	Pink	Negative
15	NASF15	Bacillus	Bacillus	Pink	Negative
16	NASF16	Coccus	Tetrad	Pink	Negative
17	NASF17	Coccus	Streptococcus	Purple	Positive
18	NASF18	Coccus	Staphylococcus	Pink	Negative
19	NASF19	Coccus	Staphylococcus	Pink	Negative
20	NASF20	Coccus	Streptococcus	Purple	Positive
21	NASF21	Coccus	Staphylococcus	Pink	Negative
22	NASF22	Bacillus	Micrococcus	Purple	Positive
23	NASF23	Bacillus	Streptococcus	Pink	Negative
24	NASF24	Bacillus	Micrococcus	Purple	Positive
25	NASF25	Bacillus	Staphylococcus	Pink	Negative
26	NASF26	Bacillus	Bacillus	Purple	Positive
27	NASF27	Coccus	Bacillus	Pink	Negative
28	NASF28	Coccus	Streptococcus	Pink	Negative
29	NASF29	Coccus	Streptococcus	Pink	Negative
30	NASF30	Coccus	Streptococcus	Pink	Negative

31	NASF31	Coccus	Streptococcus	Pink	Negative
32	NASF32	Coccus	Streptococcus	Purple	Positive
33	NASF33	Coccus	Streptococcus	Pink	Negative
34	NASF34	Coccus	Streptococcus	Pink	Negative
35	NASF35	Coccus	Micrococcus	Purple	Positive
36	NASF36	Bacillus	Bacillus	Pink	Negative
37	NASF37	Bacillus	Streptococcus	Pink	Negative
38	NASF38	Bacillus	Micrococcus	Purple	Positive
39	NASF39	Bacillus	Bacillus	Pink	Negative

Mainly isolates of sunflower have flat elevation, while NASF3, NASF4, NASF9, NASF16, NASF16, NASF25, NASF30, NASF33 and NASF35 were convex, NASF1, NASF17, NASF29, NASF36 and NASF38 were raised, NASF7, NASF8 and NASF13 were Umbilicate (Table 2). Colony margins were observed from entire to erose; whereas seven isolates NASF3, NASF4, NASF7, NASF9, NASF18, NASF26 and NASF32 had undulate margins. Most of the isolates had opaque to translucent opacity expect one isolate NASF18 which had transparent opacity. Circular to irregular, colony shape was observed in the majority cases whereas PGPR isolates NASF17, NASF20, NASF28, NASF31, NASF23 and NASF36 were filamentous and 1 isolate NASF19 was recognized as spindle shape and three isolate NASF15, NASF30, NASF35 were rhizoid (Fig 1). The difference in phenotypic characteristics explains that bacterial morphology is generally related to the adaptation of bacterial strains to different ecological aspects of particular areas (Bochner, 2009; Mishra *et al.*, 2010; Manivannan *et al.*, 2012).

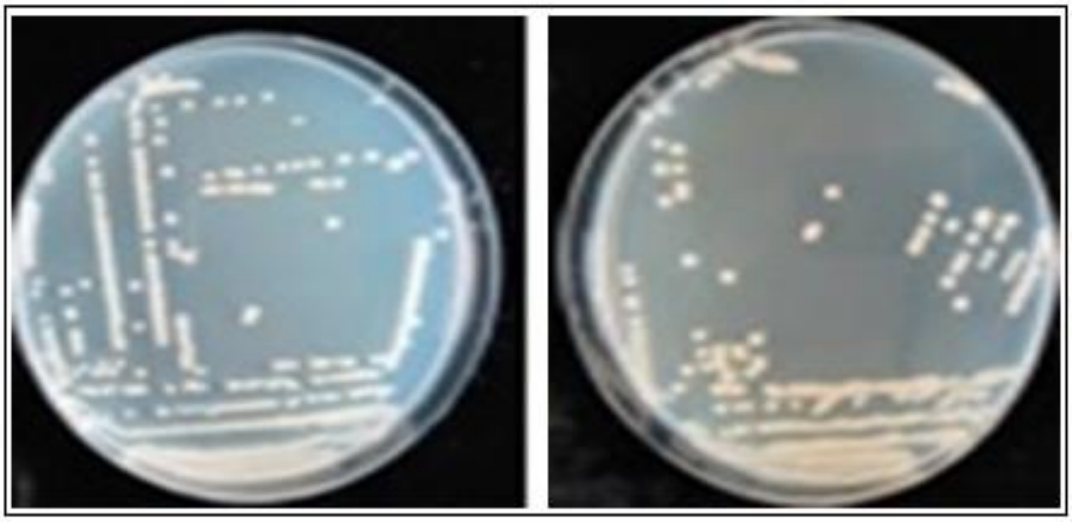

Fig. 1. Colony morphology of PGPR.

Microscopic observation of Rhizobacterial isolates

From isolated bacteria twenty six PGPR strains NASF1, NASF2, NASF4, NASF5, NASF7, NASF11, NASF12, NASF13, NASF14, NASF15, NASF16, NASF18, NASF19, NASF21, NASF23, NASF25, NASF27, NASF28, NASF29, NASF30, NASF31, NASF33, NASF34, NASF36, NASF37 and NASF39 were gram -ve and showed pink color during Gram's staining whereas, isolates NASF2, NASF6, NASF8, NASF9, NASF17, NASF20, NASF22, NASF24, NASF26, NASF26, NASF35, NASF38 were gram +ve and showed purple color under microscope. All isolates were examined microscopically and different cell shapes were observed from bacilli to coccus (Fig 2). Twenty five isolates NASF1, NASF5, NASF6, NASF7, NASF8, NASF9, NASF10, NASF11, NASF12,

NASF13, NASF16, NASF17, NASF18, NASF19, NASF20, NASF21, NASF27, NASF28, NASF29, NASF30, NASF31, NASF32, NASF33, NASF34 and NASF35 were coccus, isolates NASF2, NASF3, NASF4, NASF14, NASF15, NASF22, NASF23, NASF24, NASF25, NASF26, NASF36, NASF37, NASF38, NASF39 were bacillus and one isolate NASF3 was observed as spirochete (Table 2).

The cell grouping of isolates varied from *streptococcus* to *bacillus*; whereas nine isolates NASF4, NASF6, NASF8, NASF11, NASF14, NASF22, NASF24, NASF27, NASF35 were *micrococcus*, isolates NASF7, NASF13, NASF21, NASF25, NASF12, NASF19 were *staphylococcus,* whereas NASF16 and NASF12 were tetrad (Mishra *et al*., 2010).

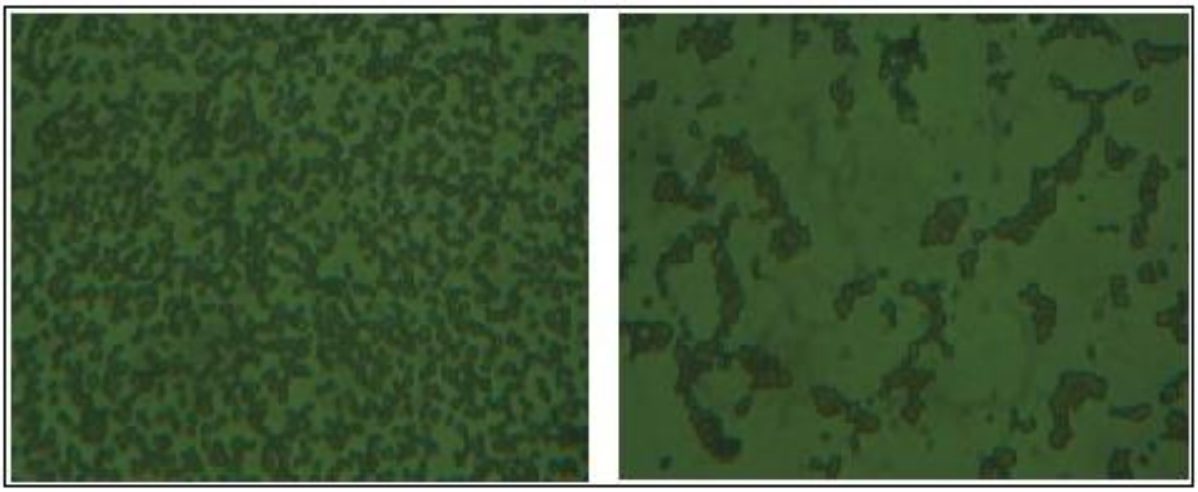

Fig. 2. Cell morphology of bacterial strains NASF31 and NASF13.

Conclusion

This study on the PGPR strains of sunflower plants showed that isolates are growth promoter and disease reducer which play very important role in plant' growth and development. So, the root of plants embellished with these rhizobacteria will probably enhance the soil fertility and substitute of chemical fertilizers/pesticides for sustainable cultivation of sunflower.

In conclusion from isolated PGPR strains twenty six were gram -ve bacteria and showed pink color during gram staining reaction, whereas thirteen isolates were gram +ve and showed purple color under microscope. The cell shapes were observed from bacilli to coccus and cell grouping of the bacterial isolates varied from streptococcus to bacilli.

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