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Identification and molecular characterization of fluorescent bacteria antagonistic against *Exserohilum turcicum* based on 16S rDNA sequences

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ABSTRACT

Until recently, few studies were carried out in Eastern Uttar Pradesh for identification of bacterial soil communities antagonistic against plant pathogens such as *Exserohilum Turcicum. E. turcicum* is a fungal phytopathogen causing Turcicum Leaf Blight and responsible for a severe loss of yield in Maize. Aiming to characterize the bacterial population in the soil having antagonistic property against *E. Turcicum*, rhizospheric soil was analyzed and 16S rRNA method was employed for their molecular characterization. 100 soil samples were collected from rhizospheric soil of Rose, Kamini, Maize and *Brassica* from different regions of Eastern Uttar Pradesh. Subsequently 33 fluorescent bacteria were isolated on King's B media. Isolates were detected on the basis of antagonistic property and 4 bacteria BMBK1, BMBK6, BMBK11, BMBK18, were found to be antagonistic against TLB. Antgonistic bacteria were then analyzed for their plant growth promotion ability on Maize variety HUZ-M60. Afterwards, all the isolates were tested for gram staining, IMViC test, antibiotic assay and Indole acetic acid production test. IAA production was detected at different concentrations and it was noted that BMBK1 and BMBK6 showing the highest IAA production. Both the isolates were found to be Gram negative, Methyl red positive and Voges-Proskaur positive. The DNA was extracted from both the strains. Using universal primers 16S27F and 16S1115R, 16S rRNA genes from BMBK1 and BMBK6 were amplified by PCR, amplicons were partially sequenced. Finally, the 16S rDNA sequences were compared with those available in the NCBI Database combining a blast search and analysis allowed the identification of BMBK1 as *Pseudomonas entomophila* L-48 and BMBK6 as *Pseudomonas putida* GB-1.

Keywords: Antgonistic bacteria, Exserohilum turcicum, Pseudomonas entomophila, Pseudomonas putida, Turcicum Leaf Blight

Introduction

Since the onset of civilization, plant diseases have had catastrophic effects on crops and the well-being of human population. Infectious plant diseases continue to cause human suffering and enormous economic losses. An increasing human population and decreasing land for agriculture make all approaches of securing the world food supply critical. Protection of crops from diseases can substantially improve agricultural production. Thoughtful application of the plant's own defense mechanisms, combined with understanding of the complex ecology of real-world disease processes, can lead to more effective protection against plant pathogens. The use of microbes to control diseases, which is a form of biological control, is an environment-friendly approach. The microbe is a natural enemy of the pathogen, and if it produces secondary metabolites, it does so only locally, on or near the plant surface. Studies on the control of pathogens by root inhabiting bacteria usually focus on pathogenic microorganisms. It should be noted that some root inhabiting bacteria are also active against weeds and insects. Rhizobacteria are bacteria that colonize plant roots.

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Plant growth promoting rhizobacteria use one or more direct mechanism of action to improve plant growth and health. These mechanisms may be sequentially or active simultaneously at different stages of plant growth are very small portion of rhizobacteria (2–5%) that promote the growth (Antoun and Kloepper, 2001). Biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzyme, hydrogen cyanide and siderophore or through competition for nutrient and space can significantly improve plant health and promote growth by increasing of seedling emergence, vigor and yield (Antoun and Kloepper, 2001). Fluorescent Psuedomonads occur commonly in the rhizosphere of plants and help suppress disease establishment and spread. Members of genus Pseudomonas are rod shaped, Gram negative bacteria characterized by metabolic versality, aerobic respiration, motility owing to several polar flagella, and high G+C content (59.68%) (Holt *et al*, 1994). Fluoresent pseudomonads produce the fluorescent pigment pyoverdin (Pvd) (also known as pseudobactin).This large and heterogenous group comprises most of them notably, *P. putida*,*P. fluorescens* and *P. syringae* (Kumar *et al*, 2005).

Turcicum leaf blight (also known as Northern Corn Leaf Blight) is caused by the Fungi *Exserohilum turcicum* (syn. *Helminthosporium turcicum*) (Pass.) (Leonard and Suggs). It is a major constraint to maize production in many maize growing regions worldwide with a growing season characterized by high humidity and moderate temperatures (17 to 27°C). Yield losses as high as 70% have been attributed to Turcicum leaf blight. Therefore, the expectations from the present investigation was to determine some novel bacterial strains antagonistic against *Exserohilum turcicum* (causing Turcicum leaf blight in Maize) which suppress the deleterious effect of the disease and show Plant growth promotion ability conferred by phosphate solublization activity, biocontrol potential, plant host interaction, soil microflora environment and various other responses. The present study is also aims at understanding the various aspects of disease occurrence and mechanism of phytopathogen suppression by bacterial action. The study was conducted in the Eastern Uttar Pradesh which is still not much explored, expecting the isolation of some new and biologically potential strains of bacteria.

Material and Methods

Isolation and characterization of fluorescent bacteria

The present investigation was carried out in Molecular Plant Breeding Laboratory, Department of Genetics and Plant Breeding, Institute of agricultural sciences, Banaras Hindu University, Varanasi (U.P.). An extensive survey of different region of Eastern Uttar Pradesh was done for the collection of soil samples from rhizospheric soil of particular plants. Hundred soil samples were collected from Maize, Rose, Kamini, Brassica, Wheat and Bathua fields of Varanasi, Ghazipur, Mirzapur and Azamgarh region of Eastern Uttar Pradesh (82° 59’ East, 25° 15’ North and 82° 33’ East, 25° 8’ North). KBM for selective isolation of *Pseudomonas* spp. was prepared by adding 42.23 of King’s medium B base agar (Protease peptone no.3-20.0g, Di-potassium hydrogen phosphate-1.5g, Magnesium sulfate-1.5g, agar-1.5g) in 1000 ml of sterilized distilled water. Upon solidification of media, the plates were incubated in an inverted position at 28ºC for 2-7 days. The fluorescence activity of the strains was detected under the UV rays. 33 isolates were obtained after the screening on King’s B media (KBM). Fluorescent *pseudomonads* were isolated from the soil collected from the rhizosphere of rice with King’s medium B (KMB) (King *et al*, 1954) as described by Vidhyasekaran *et al.* (1997). Therefore a total number of 33 isolates were obtained from 100 samples collected. The bacterial isolates were nominated as BMBK-0, where “B” stands for BHU, Varanasi, “MB” stands for Molecular Breeding Laboratory where the whole investigation was carried out and “K” stands for the King’s B media used for selective isolation of bacteria and “0” stands for any digit based on the sequence in which bacteria were isolated.

Antagonistic assay

Colony interaction between the soil bacteria and the individual species of test pathogen was studied by adopting Dual Culture Experiment. (Haung and Hoes, (1976). Dual culture assay was done by placing the inoculums of test fungi in the centre of petriplates poured with PDA. Then the bacterial inoculants were streaked parallel on opposite sides of fungal inoculums. After inoculation the petriplates were stored at 20±2^{0}C temperature in the incubator for 4-7 days. After few days the cleared zone was observed and diameter is measured. The radial growth of pathogen and bacteria was measured at interval of 24 hrs upto 7 days after incubation. Inhibition of mycelial growth of pathogen fungi by each strain was recorded. Percentage growth inhibition was calculated as per formula (Dennis and Webster, 1971, Arora and Upadhyay, 1978) % growth inhibition = r-r'/r × 100 (Where, r = Colony growth in control plate r' = Colony growth in intersecting plate).

Plant growth promotion analysis

Plant growth promotion activity was detected by inoculating seed with bacteria (Dobbelaere et al., 2001;Vessey, 2003; Lucy et al., 2004; Sahin et al., 2004; Zahir et al., 2004))Seeds of maize variety HUZ-M60 was taken and sterilized properly with Sodium hypochlorite and 0.1% Mercuric chloride for 30 sec, then three times washed with distilled water. Eight sterilized polythene bags were taken and labeled appropriately; 5ml sucrose solution was added in each bag and mixed with maize seed. In each bag respective bacterial strain were inoculated in maize seed and mixed properly and then kept for 5-6 hrs in incubator for proper growth. Each maize seed inoculated with bacterial strains were sown in the soil kept in pots. Pots were transferred into the plant growth chamber and the growth conditions were set at 25^{0}C, 60% humidity and 12 hr day/night cycle appropriately, pots were irrigated properly. After 21 days the grown plants were uprooted and the length of root and shoot were measured.

Biochemical analysis

Gram staining

The bacteria were detected for their gram staining property. Thin smears of bacteria were made on separate glass slides. Smear remains for air drying and heat fixed. Each smear was covered with crystal violet for 30 sec. Each slide was washed with distilled water for few seconds. Each slide was covered with iodine solution for 60 seconds. Iodine solution washed off with 95 % ethyl alcohol. The ethyl alcohol was added drop by drop, until no more colour flowed from smear. The slides were washed with distilled water and drown. The saffranin was applied to the smear. Washed with distilled water and blotted dry with adsorbent paper and be kept for air drying.

Indole production test

1% trypton broth was prepared by dissolving 10 g of peptone in one liter of distilled water and sterilize in the autoclave at 15 psi (121^{0}C) for 15 minutes. Trypton broth was inoculated with bacteria and one of the tube was kept as an uninoculated comparative control. Inoculated tubes incubated at 35^0 C for 48 hrs. After 48 hrs of incubation 1ml of Kovac's reagent was added to each tubes including control. The tubes were shaked gently after intervals for 10-15 minutes. The tubes were allowed to stand, to permit the reagent came to the top. Development of a cherry (deep) red colour in the top layer of the tube in bacteria showed the positive test for Indole production and absence of red colouration was found Indole negative.

Methyl Red, Voges Proscauer test

MRVP broth (Peptone -7.0g, Dextrose-5.0g, Potassium Phasphate-5.0g, Distilled Water -1000.00 ml. pH-6.9) tubes were prepared. 5ml broth medium was poured in each tubes and sterilized by autoclaving at 15 lb pressure for 15 min. MRVP tubes were inoculated with bacteria and one tube kept uninoculated as a comparative control. These tubes were incubated at 35^0 C for 48 hrs. The methyl red indicator in the pH range of 4 will remain red showed

+ve test and yellow –ve. The development of a crimson- to-ruby pink (red) colour showed +ve VP test and no colour change –ve test.

Antibiotic assay

The nutrient broth medium was prepared and bacteria were cultured on a nutrient agar media for antibiotic assay. 100-300µl of nutrient broth culture poured in a sterilized petriplate. After shaking petriplate were poured with nutrient agar medium. Three wells were made per plate and the antibiotic stock solutions of different concentration were poured in the same wells. The plates were incubated for 24 hrs. After incubation period MIC (Minimum inhibition concentration) was evaluated.

Indole acetic acid production test

IAA test was done by method used by Gordon and Weber, 1951. Three conical flasks were taken and poured with 250 ml nutrient broth. In first flask 1mg/ml, in second 3 mg/ml and in the last third 6 mg/ml Tryptophan was added.

The nutrient broth media was added in each test tube (5ml per test tube) and then autoclaved. The bacteria were inoculated (one strain in different concentration of tryptophan) in test tubes. Incubated for 10 days.

After this 2 ml bacterial culture was centrifuged at 10,000 rpm for 10 min. 2 ml supernatant was taken in vial and 2 drops of orthophasphoric acid was added in the supernatant. Then 4ml of Solawaski's regent (50ml, 35% per chloric acid+ 1ml + 0.5 Molar $Fecl_3$) was added.. Samples gave strong red colour were IAA positive whereas Yellow colour confirmed as IAA negative. 10ml standard stock solution was made of different conc. of IAA (40, 60, 80, 100, 150, 200, 250, 300 µg/ml). Optical density (O.D.) was determined with the help of spectrophotometer at a wavelength of 530 nm.

Molecular characterization

DNA extraction, PCR amplification and 16S rDNA sequencing

DNA extraction from soil microbial isolates was performed using Genomic DNA Mini Kit, Catalog no. GB100 (Geneaid Biotech ltd.,) based on method described earlier (Vogelstein and Gileespie, 1979), according to the manufacturers instruction. The isolated DNA was used in PCR reactions for the amplification of 16S rRNA gene, by using universal primers 16S27F (5'AGA GTT TGA TCC TGG CTC AG 3') and 16S1115R (5' AGG GTT GCG CTC GTT G 3') (Geneaid Pvt. ltd). PCR reaction contained PCR 1X buffer (20mM Tris-HCl (pH 8.4), 50mM KCl), 200 µM of each deoxinucleotide, 1.5 mM MgCl2, 50 pmols of each initiator oligonucleotide, 2.5U Taq DNA polymerase (Geneaid pvt ltd.) and 50 ng template DNA, in a final volume of 50 µL. Ten reactions per soil were performed. Samples were then placed in a thermalcycler (Genei pvt ltd.) for amplification of the gene, and the program described by Kuske *et al*., 1997.

Cycling parameters included initial denaturing for 3 min at 95 °C; 40 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C.. To ensure consistency in results, PCR was repeated for each isolate for at least three times. The PCR products (10 µl) were mixed with 6xgel loading buffer (3 µl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5XTAE (Tris-Acetat- EDTA) buffer at 40 V for 200 min. Amplification products separated by gel was stained in ethidium bromide solution (2 µl EtBr/100ml 1xTAE buffer) for 40 min. The amplified DNA product was detected by using the UV gel documentation system (GeneI pvt. Ltd.) and the amplicons were sequenced.

Sequence analysis

The 16S rDNA sequences were compared with those available in the NCBI Database (http://ncbi.nlm.nih..gov/blast). NCBI is a web tool combining a blast research, alignment with CLUSTAL

W and phylogeny analysis with the neighbour joining method (Devulder *et al*, 2003). The combination of theses well-known tools in an automated program facilitates the bacterial identification process (Clarridge, 2004; Raoult *et al*, 2004).We identified the sequence, essentially taking into account the phylogeny, and not simply the percentage similarity and length of the sequence (Fredricks and Relman, 1996; Clarridge, 2004).

Results and Discussion

Identification and characterization of bacteria

The hundred soil samples collected from different locations of Eastern Uttar Pradesh was screened for presence of fluorescent bacteria. Using King's B Media have a total number of 33 fluorescent bacterial colonies were obtained. The total number of hundred soil samples were analysed for bacterial strains and a total of 33 strains were screened as Florescent *Pseudomonas*. The details of the strains isolated have been given in Table 1.

Antagonistic assay As used by several authors (Andrews 1991; Walker *et al*. 1998; Yoshida et al. 2001), the technique of dual culture analysis on agar plates was an easy assay with which to select antagonistic bacteria from a random group of bacterial isolates and to compare these selected strains for their fungal growth inhibition capabilities. In this way Thirty three bacterial cultures isolated from soil samples obtained from different locations were tested for their antagonistic activity against *Exserohilum turcicum*. Only four of the bacterial isolates exhibiting antagonistic property against TLB pathogen. The screening related to antagonism of 33 isolates by dual culture analysis against Turcicum leaf blight has helped in identification of 4 antagonistic bacteria. BMBK-1 has expressed highest percentage (78.5%) of inhibition followed by BMBK-6 (77.3%) and BMBK-11 (74.4%) while BMBK18 has exhibited lowest percentage of inhibition followed by BMBP-30 (61.8%), BMBK-15 (72.2%). Among all the 33 strains, best ones are selected for furture analysis. Pande and Chaube (2003) observed 8.00 mm inhibition zone with *P. fluorescens.* Control at 10 days after application was similar to that at 7 days but at 15 days control levels were lower. Shanmugam *et al.* (2003) pointed out that timely and augmented applications were necessary to aid the establishment or maintenance of antagonists. The details of antagonistic potential, radial growth and percent of inhibition have been presented in Table 2.

Plant growth promotion analysis

In the present investigation we studied the plant growth promotion ability of identified isolates on Maize variety HUZ-M60 for their antagonistic activity as well as their phosphate solublising ability and their biocontrol potential depend upon the various physiological and ecological factors. Among four antagonistic isolates, BMBK1 was found to show maximum plant growth promotion activity, compared to rest of four isolates, determined by measuring the root shoot length of plant. IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral root as well as root length. (Okon and Kapulnik, 1986) The Plant growth promotion analysis of the isolates has been evaluated in Table 3.

Table 1 Details of the isolated bacteria from rhizospheric soil of different plants.

Strain	Source Plant	Location	No. of isolates
BMBK1-BMBK6	Rose	BHU,Varanasi	6
BMBK7-BMBK17	Kamini	Azamgarh	11
BMBK18-BMBK26	Brassica	Mirzapur	9
BMBK27-BMBK33	Maize	Ghazipur	7

Table 2 Radial growth and Percent inhibition of test pathogen (*E. turcicum*) as analyzed by antagonistic assay.

Isolate	Radial growth (*E. turcicum*)	Percent inhibition (mm)
BMBK-1	19	78.5
BMBK-6	20	77.3
BMBK-11	23	74.4
BMBK-18	30	66.5

Table 3 Evaluation of Plant growth promotion ability of antagonistic bacteria on Maize variety HUZ-M60.

Isolate	Root length (cm)			Avg. Root length (cm)	Shoot length (cm)			Avg. Shoot length (cm)
BMBK1	23.5	24.5	24.5	24.16	35	39	34	36
BMBK6	20.5	19	20	19.83	28.5	30	29	29.16
BMBK11	17.5	17	19	17.83	22	25	24	23.66
BMBK18	19	18.5	18	18.5	24	29	27	26.66
CV		3.91		4.21				
SE		0.64		0.99				
CD		1.54		2.38				

Table 4 Biochemical analysis based on Methyl red, Voges-Proskauer, Gram staining and Indole production test.

Strain	Methyl red test	Voges- Proskauer test	Gram staining	Indole production test
BMBK-1	- ve	+ ve	- ve	+ ve
BMBK-6	- ve	+ ve	- ve	+ ve
BMBK-11	- ve	+ ve	- ve	+ ve
BMBK-18	- ve	+ ve	- ve	+ ve

Biochemical analysis

The biochemical analysis was done to determine various properties of strains *viz*. IAA and antibiotic production.

Methyl red, voges- proskauer, gram staining and indole production test

The observations from the Methyl red, voges-proskauer, gram staining and indole production test have been tabulated in the Table4.

Indole actetic acid production test

Different concentrations of tryptophan in µg/ml on 10 days of incubation period were used for the production of Indole acetic acid. BMBK1 strain of bacteria produce 18.10µg/ml of IAA on 1 mg/ml, 54.5 µg/ml of IAA produce on 3mg/ml, 108.40 µg/ml of IAA produce on 6mg/ml tryptophan concentration. BMBK18 strain of bacteria produces 16.30µg/ml, 48.5µg/ml, 100.00µg/ml on 1mg/ml, 3mg/ml, 6mg/ml tryptophan concentration respectively. BMBK6 strain of bacteria 18.90µg/ml, 54.00µg/ml, 104.40µg/ml, on 1mg/ml, 3mg/ml, 6mg/ml tryptophan concentration respectively. BMBK11 strain of bacteria produces 14.80µg/ml, 42.60µg/ml, 80.65µg/ml on 1mg/ml, 3mg/ml, 6mg/ml tryptophan concentrations respectively.

Antibiotic assay

Observations after antibiotic assay of different strains of bacteria were showing different M.I.C (Minimum inhibition concentration). In this observation BMBK-1 was showing the 150µg/ml M.I.C and BMBK6, BMBK11, BMBK18 were showing 630µg/ml, 310µg/ml, 150 µg/ml MIC respectively.

Table 5 Indole production test at three different concentration of tryptophan.

Strain	1mg/ml tryptophan	3mg/ml tryptophan	6mg/ml tryptophan
BMBK-1	18.10	54.5	108.90
BMBK-6	18.90	54.00	104.40
BMBK-11	14.80	42.60	80.60
BMBK-18	16.30	48.50	100.00

Molecular characterization

DNA was isolated from strains which were showing antagonistic activity against *E. turcicum* as well as high Indole production and plant growth promotion activity. After DNA isolation amplified DNA with the help of PCR using Universal bacteria primer Farward-5' AGAGTTTGATCCTGGCTCAG 3' 16S27F Reverse -5' AGGGTTGCGCTCGTTG 3' 16S1115R. 100bp ladder was used in the present investigation. Ten bands were found showing in the figure-1. The two isolates which have shown the highest IAA production i.e. BMBK1 and BMBK6 were showing the bands at 110.33 and 120.66bp respectively. Both the bands were eluted by cutting the gel and partially sequenced. Finally, the 16S rDNA sequences were compared with those available in the NCBI Database combining a blast search and analysis allowed the identification of BMBK1 as *Pseudomonas entomophila* L-48 and BMBK6 as *Pseudomonas putida* GB-1. These species are also reported earlier to have antagonistic activity and growth-promoting effects (Gardner *et al.*, 1984). Analysis of variation of the ribosomal 16S gene allow the inference of the phylogenetic relationships among taxonomically relatively close and distant organisms (Eisen, 1995; Ludwig *et al*., 1998).

Table 5 Antibiotic assay of rhizospheric bacteria showing different Minimum inhibition concentrations.

Strain	Minimum inhibition concentration (MIC)
BMBK-1	150µg/ml
BMBK-6	630µg/ml
BMBK-11	310µg/ml
MBK-18	150µg/ml

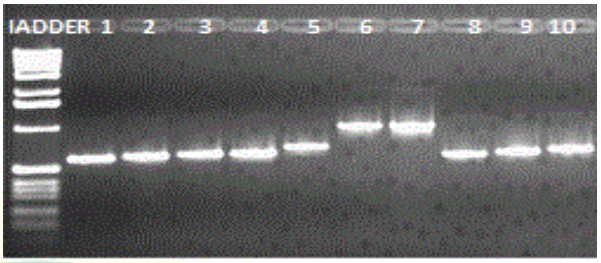

Figure 2 Amplified product of DNA isolated from *Pseudomonas species* by Polymerase chain reaction using 16S27F and 16S111R primers. Lane1 =BMBK1, Lane2 = BMBK2, Lane3 = BMBK3, Lane4 = BMBK4, Lane5 = BMBK5, Lane6 = BMBK5, Lane7 = BMBK7, Lane8 = BMBK9, Lane9 = BMBK11, Lane10 = BMBK18.

References

Andrews JH (1991) Strategies for selecting antagonistic microorganisms from the phylloplane. In: Windels CE, Lindow SE (eds) Biological control in the phylloplane. APS Press, St Paul, Minn., pp 31–44.

Antoun H, Kloepper JW (2001) Plant Growth Promoting Rhizobacteria. Encyclopedia of Genetics. London: Academic Press.

Arora DK, Upadhyay RK (1978) Effect offungal staling growth substances on colony interaction. Plant and Soil 49, 685-690.

Clarridge III JE (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease. Clin. Microbiol. Rev. 17, 840–862.

Dennis C, Webster J (1971) Antagonistic properties of species-group of *Trichoderma*. I. Production of nonvolatile antibiotics. *Transaction of the British Mycological Society*.57: 25-39.

Devulder G, Perriere G, Baty F, Flandrois JP (2003) BIBI, a Bioinformatics Bacterial Identification tool. J. Clin. Microbiol. 41, 1785–1787.

Dobbelaere S, Croonenborghs A, Thys A, *et al.* (2001) Response of agronomically important crops to inoculation with Azospirillum. Australian Journal of Plant Physiology 28, 1–9.

Eisen JA (1995) The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. J. Mol. Evol. 41:1105-1123.

Gardner JM, Chandler JL, Faldman AW (1984) Growth promotion and inhibition by antibiotic producing fluorescent pseudomonads on citrus roots. *Plant Soil* **77**: 103-113.

Gordon SA, Weber RP (1951) Colorimetric estimation of indole acetic acid. Plant physiology. 26: 192-197.

Hoes JA, Huang HC (1975) *Selerotinia selerotiorum:* viability and separation of sclerotia from soil. *Phytopathology* 65, 1431-1432.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) In: *Bergey's manual of Determinative Bacteriology*. Williams and Wilkins Press, Baltimore, U.S.A.

Kumar B, Dubey RC, Maheshwari DK (2005) In: *Microbial diversity: Current perspectives and potential applications* (Eds. Satyanarayana, T. & Johri, B. N.), I.K. International Pvt. Ltd., New Delhi, pp 471-492.

Kuske CR, Barns SM, Busch J (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many Geographic regions. *Appl. Environ. Microbiol*, 63(9), 3614-21.

Leonard KJ, Suggs EG (1974) *Setosphaeria prolata*, the Ascigerous State of *Exserohilum prolatum*, *Mycologia*, Vol. 66, No. 2: 281-297.

Lucy M, Reed E, Glick BR (2004) Application of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology 86, 1–25.

Ludwig W, R Amann, E Martinez-Romero, W Schönhuber, Bauer S, Neef A, Schleifer H (1998) rRNA base identification and detection systems for rhizobia and other bacteria. Plant and Soil 204:1-19.

Okon Y, Kapulnik Y (1986) Development and function of Azospirillum inoculated root. Plant and Soil. 90: 3-16.

Pande VS, Chaube HS (2003) Effect of *Pseudomonas fluorescens* isolates on sclerotial viability of *Rhizoctonia solani. Ann. Pl. Protec. Sci.* **11** : 57-60.

Sahin F, Cakmakci R, Kantar F (2004) Sugar beet and barley yields in relation to inoculation with N2-fixing and phosphate solubilizing bacteria. Plant and Soil 265, 123–129.

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press Cold Spring Harbor, New York.

Shanmugam VT, Raguchander A, Ramanathan, Samiyappan S (2003) Management of groundnut root rot disease caused by *Macrophomina phaseolina* with *Pseudomonas fluorescens. Ann. Pl. Protec. Sci.* **11** :304-308.

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255, 571–586.

Vidhyasekaran P, Rabindran N, Muthamilan M, Nayar K, Rajappan K, Subramanian N, Vasumathi K (1997) Development of a powder formulation of *Pseudomonas fluorescens* for control of rice blast . *Plant Pathology* 46, 291-297.

Walker R, Powell AA, Seddon B (1998) Bacillus isolates from the spermosphere of peas and dwarf French beans with antifungal activity

against Botrytis cinerea and Pythium species. J Appl Microbiol 84:791–801

Webster J, Dennis C (1967) The mechanism of sporangial discharge in *Pythium middletonii*. New Phytologist 66: 307–313.

Zahir ZA, Arshad M, Frankenberger WT (2004) Plant growthpromoting rhizobacteria: applications and perspectives in agriculture. Advances in Agronomy 81, 97–168.

Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A (2001) Antimicrobial activity of culture filtrate of Bacillus amyloliquefaciens RC-2 isolated from mulberry leaves. Phytopathology 91:181–187.