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Study of fluorescent bacteria antagonistic against *Exserohilum turcicum*

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ABSTRACT

E. Turcicum is a fungal phytopathogen causing Turcicum Leaf Blight and responsible for a severe loss of yield in Maize. This work was aimed 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 Banaras Hindu University. Subsequently 33 fluorescent bacteria were isolated on King's B media. Isolates were detected on the basis of antagonistic property and 6 bacteria MBLK1, MBLK3, MBLK6, MBLK15, MBLK17, and MBLK22 were found to be antagonistic against TLB. Antagonistic bacteria were then analyzed for their plant growth promotion ability on Maize variety HUZ-M60. Afterwards, all of these antagonistic strains were tested on biochemical tests. The genomic DNA was isolated and amplified for 16s rRNA gene & these amplified segments were sequenced and compared for similar sequence with those available on database for public domain for identification of antagonistic fluorescent strains.

Keywords: Antoun and Kloepper, Fluoresent pseudomonds

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. 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 *Psuedomonas* occur commonly in the rhizosphere of plants and help suppress disease establishment and spread. Members of genus Pseudomonas are rod

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shaped, Gram negative bacteria characterized by metabolic versatility, aerobic respiration, motility owing to several polar flagella, and high G+C content (59.68%) (Holt *et al*, 1994). *Fluoresent pseudomonds* 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.

Material and methods

Isolation and characterization of fluorescent bacteria

Soil samples were collected from rhizospheric soil of particular plants and hundred soil samples were collected from Maize, Rose, Kamini, Brassica, Wheat and Bathua fields at Banaras Hindu University Varanasi,. King's B media 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. Fluorescent *pseudomonades* were isolated from the soil collected from the rhizosphere of rice with King's medium B as described by Vidhyasekaran *et al*. (1997). A total number of 33 isolates were obtained from 100 samples collected. The bacterial isolates were nominated as MBLK0, where "MBL" 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.

Dual culture & Antagonistic assays

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 test fungi in the centre of petriplates poured with PDA. Then the bacterial inoculants were streaked parallel on opposite sides of fungal inoculums & were stored at 20 ± 2^0C temperature in the incubator for 4-7 days. After few days the cleared zone was observed and diameter measured. 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 surface sterilized with Sodium hypochlorite and 0.1% Mercuric chloride for 30 sec. & 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 25^0C, 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

Bacterial colonies with anatagonistic properties were examined by various biochemical tests, including Gram staining for morphological characterization, and few of major such tests as given below-

Indole production test

1% trypton broth was prepared by dissolving 10 g of peptone in one liter of distilled water and sterilize in the autoclaved. Pepton broth was inoculated with bacteria and one of the tube was kept as an uninoculated comparative control & incubated at 35^0C for 48 hrs. After 48 hrs of incubation 1ml of Kovac's reagent was added to each tubes including control & gently shake 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

The Broth (Peptone -7.0g, Dextrose-5.0g, Potassium Phasphate-5.0g, Distilled Water -1000.00 ml. , pH-6.9) tubes were prepared. Each tubes was poured with 5ml broth medium in and autoclaved. 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.

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 orthophosphoric acid was added in the supernatant. Then 4ml of Solawaski's regent (50ml,35% perchloric acid $HClO_4$+ 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, 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 p.moles 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 thermal cycler (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 examine on agarose (1.5% w/v) gel electrophoresis in 0.5XTAE (Tris-Acetet-EDTA) buffer at 40 V for 200 min. Amplification products separated by gel was visualized 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 amplified 16s rDNA sequences were sequenced by Automated Sanger Di-deoxy termination method sequenced & Sequence data generated by both ends (forward and reverse) using BDTv3.1 or DTCS using ABI 3730XL at Xcelris™ Labs Ltd, Ahmedabad-Gujarat, India. The 16S rDNA sequences were compared with those available in the NCBI Database (http://ncbi.nlm.nih..gov/blast) using BLAST n (Altschul *et al*.,1997) and the most similar sequences with maximum score were alignment with CLUSTAL W (Thompson *et al*.1994) 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 Banaras Hindu University were screened for presence of fluorescent.

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*.

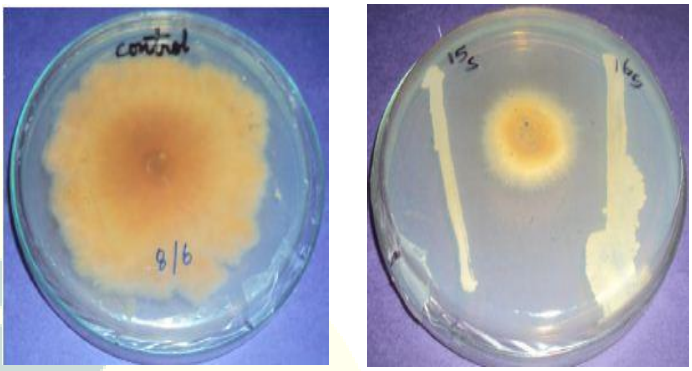
The screening related to antagonism of 33 isolates by dual culture analysis against Turcicum leaf blight has helped in identification of 6 antagonistic bacteria. These antagonistic details are summarized in table-2 and demonstrated in figure 1. bacteria. Using King's B media have a total number of 33 fluorescent bacterial colonies were obtained. The total of hundred soil samples was analyzed for bacterial strains and a total of 33 strains were screened as Florescent *Pseudomonas.* The strains isolated have been given in Table 1.

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

Strain	Source Plant	Location	No. of Isolates
MBLK1-MBLK6	Rose	BHU, Varanasi	6
MBLK7-MBLK17	Kamini	Azamgarh	11
MBLK18-MBLK26	Brassica	Mirzapur	9
MBLK27-MBLK33	Maize	Ghazipur	7

Table 2 Percent Inhibition on TLB Pathogen

Isolates	Pathogen	% of Inhibition
MBL-K1	TLB	78.8
MBL-K3	TLB	77.7
MBL-K6	TLB	74.4
MBL-K15	TLB	66.6
MBL-K17	TLB	82.2
MBL-K22	TLB	68.8



Control Dual plate culture

Figure 1 showing effect of isolated strains on Root & shoot growth.

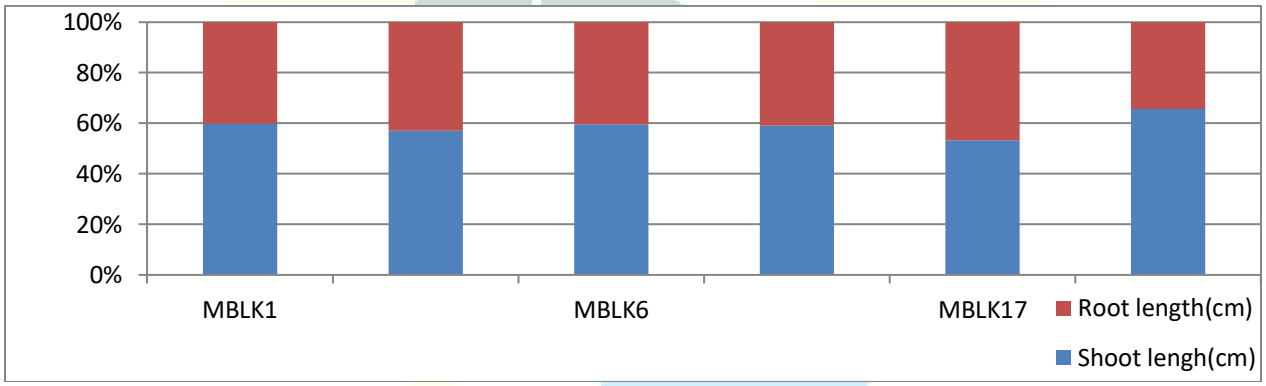


Figure 2 (A and B) showing the percentage inhibition.

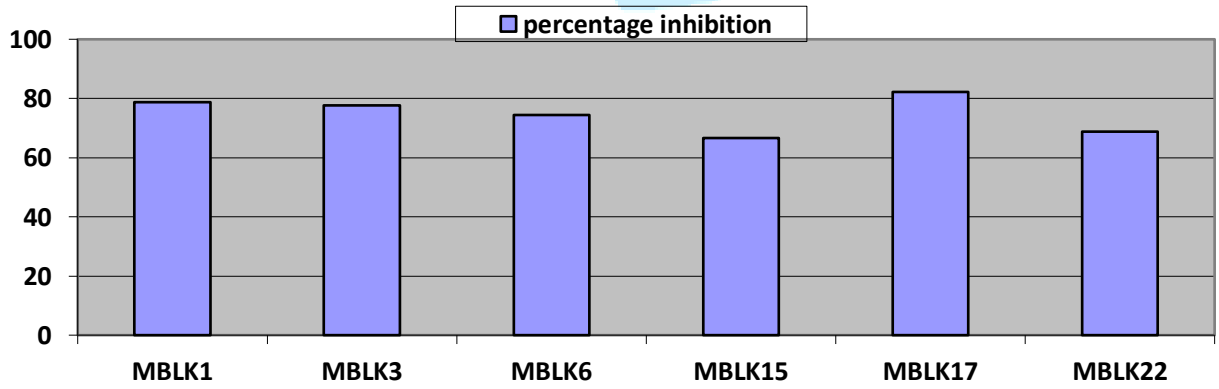


Table 3 Evaluation of Plant growth promotion ability of antagonistic bacteria on Maize variety HUZ-M60**.**

Isolates	Maize variety	Root length in cm			Avg.	Shoot length in cm			Avg.
MBLK1	HUZ -M60	23.5	24.5	24.5	24.16	35	39	34	36
MBLK3	HUZ-M60	17.5	17	19	17.83	22	25	24	23.66
MBLK6	HUZ-M60	20.5	19	20	19.83	28.5	30	29	29.16
MBLK15	HUZ-M60	19	18.5	18	18.5	24	29	27	26.66
MBLK17	HUZ-M60	20	21	22.5	21.16	24	24.5	24	24.16
MBLK22	HUZ-M60	17	16.5	15.5	16.33	31	33	29.5	31.16

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
MBLK1	- ve	+ ve	- ve	+ ve
MBLK3	- ve	+ ve	- ve	+ ve
MBLK6	- ve	+ ve	- ve	+ ve
MBLK15	- ve	+ ve	- ve	+ ve
MBLK17	- ve	+ ve	- ve	+ ve
MBLK22	- ve	+ ve	- ve	+ ve

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

Strain	1mg/ml Tryptophan	3mg/ml Tryptophan	6mg/ml Tryptophan
MBLK1	18.10	54.5	108.90
MBLK3	18.90	54.00	104.40
MBLK6	14.80	42.60	80.60
MBLK15	16.30	48.50	100.00
MBLK17	17.40	51.45	106.00
MBLK22	16.20	48.10	79.20

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 their bio-control potential depends upon the various on *E. turcicum* by different isolates physiological and ecological factors. Among four antagonistic isolates, these results are given in table-3 and evaluated in figure-2 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).

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, and Gram staining and indole production test have been tabulated in the Table.4

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. These production results are summarized in table-5. The maximum IAA production given by MBLK1 on 6mg/ml tryptophan.

Molecular characterization

The amplified genomic DNA for 16s rDNA sequence was analyzed by Agarose gel electrophoresis.100bp ladder was used in the present investigation. Ten

bands were found showing in the figure-3. 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 by running a BLASTN search and these sequences were aligned on CLASTALW, this alignment is given in Figure 4. The BLAST results with highest similarity are compiled in table 6.

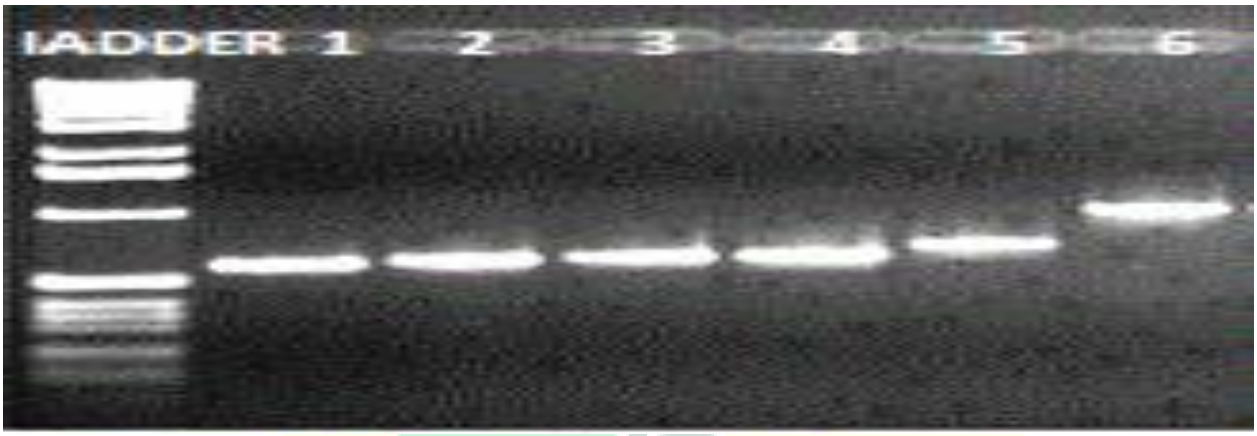
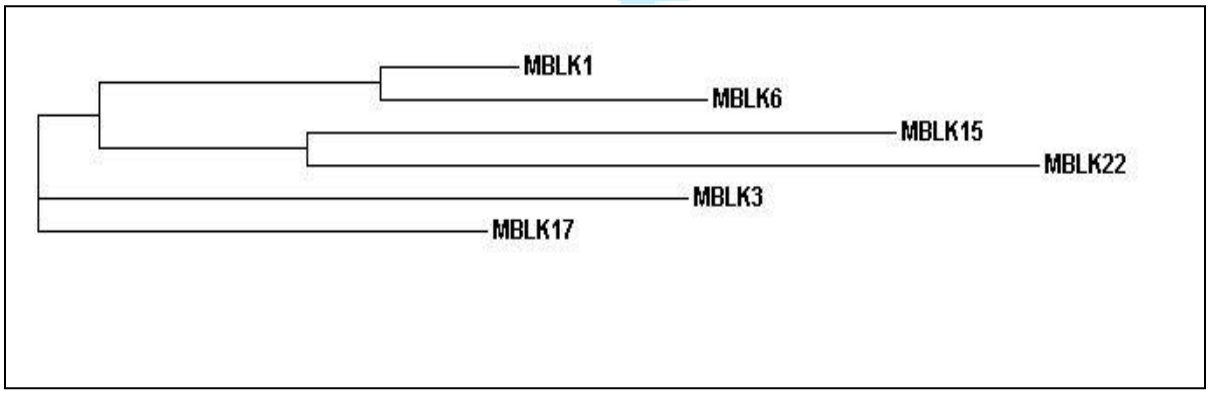


Figure 3 Amplified product of DNA isolated from *Pseudomonas species* by Polymerase chain reaction using 16S27F and 16S111R primers. Lane1 =MBLK1, Lane2 = MBLK3, Lane3 = MBLK6, Lane4 = MBLK15, Lane5 = MBLK17, Lane6= MBLK22.

Table 6 Showing BLASTN results for sequences.

Strain	Highest Similarity Found	Identity	E- value	Accession No.
MBL-K1	Pseudomonas putida	97%	0.0	HQ259593.1
MBL-K3	Pseudomonas mosselii	98%	0.0	DQ095881.1
MBL-K6	Pseudomonas fluorescens	97%	0.0	HQ236548.1
MBL-K15	Pseudomonas entomophila	94%	0.0	EF178450.1
MBL-K17	Pseudomonas spp.	95%	0.0	GU325690.1
MBL-K22	Pseudomonas montelli	98%	0.0	AF181576.1

Figure 7 showing Phylogenetic tree for isolated antagonistic strains based on 16s rDNA alignment by CLASTALW.



Discussion

The biological control of plant pathogens by antagonistic microorganisms offers an attractive alternative to existing pest management. The word antagonism was introduced by Baker, (1987). Since then many antibiotics & fungal cell wall degrading enzymes have been isolated and characterized from both actinomycetes and bacterial biocontrol agents. A group of root-associated bacteria, plant growth promoting rhizobacteria (PGPR), intimately interact with the plant roots and consequently influence plant health and soil fertility. The use of Pseudomonades for biocontrol of *E. turcicum* has been clearly proved in this experiment as well as their plant growth promoting abilities reveled. The maximum percentage inhibition shown by MBL17 and most effective PGPR activity is observed on treatment of MBLK1.hteir molecular and chemical characterization proved their identities as different strains of flurescent bacteria specifically belongs to genus Pseudomonas.

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 allows the inference of the phylogenetic relationships among taxonomically relatively close and distant organisms (Eisen, 1995; Ludwig *et al*., 1998).

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