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Biochemical Characterization and Molecular Identification of Antagonistic Bacteria Identified Against *Exserohilum turcicum* causing Turcicum Leaf Blight in Maize (*Zea mays* L.)

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ABSTRACT

The purpose of this study was to isolate and select the rhizospheric bacteria capable of developing multiple mechanisms of action related to the biocontrol of phytopathogenic fungus *Exserohilum turcicum* affecting maize crops and cause a disease called Turcicum Leaf Blight. The screening procedure consisted of antagonism tests against the fungus, taxonomic identification, biochemical analysis, detection by PCR of several genes related to antifungal activity. Eight isolates, identified and designated as *Pseudomonas* BMLK1, BMLK9, BMLK11 and BMLK18. Phosphate solubilising bacteria desinated as BMLP3 sand BMLP4 and actinomycetes isolates BMLA1 and BMLA11.BMLA1 showed highest percentage of growth inhibition. Biochemical assays were performed for these eight isolates and then after it's DNA extraction, by using universal primers 16S27F and 16S1115R, 16S rRNA genes from these isolates 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. It has been found that BMLP3 and BMLP4 showed similar banding pattern which may emphasize that, they may belong to similar genera of microorganism. BMLK1 and BMLA11 have closely related banding pattern, which may emphasize that they may have closely related antifungal activity.

Keywords: Actinomycetes, Biocontrol agent, Phosphate solubilising bacteria, Pseudomonas in vitro antagonistic,

Introduction

Turcicum Leaf Blight is the cause of maize crop and much loss of it's yields especially in temperate regions. Chemical fungicides are extensively used in contemporary agriculture. However, these products may cause problems such as environmental pollution and affect human health. To reduce the worsening problems in fungicide usage, new methods for plant protection, which are less dependent on chemicals and are more friendly to should be discovered and developed. Microorganisms as biological control agents have high potential to control plant pathogens and no effect on the environment or other non-target organisms. Several soil microorganisms known as phosphate solubilizing bacteria (PSB) have the ability to solubilize insoluble mineral phosphate by producing various organic acids, siderophores, mineral acids, protons, humic substances, CO_2 and H_2S **(**Yadav and Dadarwal, 1997). The precipitated inorganic phosphate is solubilized by the action of mineral and organic acids produced by bacteria and fungus. A large number of PSB have been isolated from the rhizosphere of several crops and these constitute about 20 to 40% of the culturable population of soil microorganisms **(**Ivanova *et al*, 2006). Most actinomycetes and Pseudomonas are found in plant rhizosphere and attention has been paid to the possibility that they can protect roots by inhibiting the development of potential of the fungus *E. turcicum.* The objective of this study were to isolate and identify the three types of bacteria (Phosphate solubilising bacteria, Pseudomonas, and

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Actinomycetes) from rhizospheric soils from eastern regions of Uttar Pradesh. Several isolates were screened for antagonistic activities against the pathogenic fungus *E. turcicum.*

Materials and Method

Isolation of rhizospheric bacteria

Soil samples were obtained from the rhizosphere of Rose, Maize, Brassica, Kamini, Bathua and Wheat plants collected from different regions of Eastern Uttar Pradesh. Actinomycetes strains designated as BMLA1 and BMLA11 were isolated by plating serially diluted samples of the soils on Actinomycetes agar media. Similarly, phosphate solubilising bacteria, BMLP3 and BMLP4 were isolated by using Picovaskaya's agar media and *Pseudomonas* BMLK1, BMLK9, BMLK11 and BMLK18 by King's B media respectively. Isolated colonies were subcultured on nutrient agar media untill pure cultures were obtained.

Antagonistic assay (Dual nature)

All the selected isolates were evaluated by dual culture in vitro assay for their activity towards the fungus *Exserohilum turcicum*. The fungus were maintained on potato dextrose agar (PDA) at optimum temperature (upto 20ºC) and kept in a culture collection at the Department of Genetics and Plant Breeding Laboratory, Banaras Hindu University. Disces (8mm) from 7 days old colonies of the test fungus grown on nutrient agar media were placed at the the center of the Petri plates. Each isolates were streaked on both the sides of the 3 cm away from the fungal disc separately. Plates without the strain served as control. All plates were incubated at 20ºC for 4-5 days and percentage of growth inhibition (GI) was calculated by using the formula of Whipps (1987): R_1-R_2/R_1 X 100. Where R1 is the radial distance (measured in mm) grown by the fungus in direction of antagonist (a control value) and R2 is the radial distance grown by fungus in dual culture.

Biochemical analysis of selected strains

A cascade of biochemical tests have done for evaluation of antagonistic bacteria (according to the reference of Aneja).At first, for Indole Acetic Acid Production test, we prepared nutrient broth for culturing bacteria. In 3 conical flasks took 250ml nutrient broth each. In the first flask added 1mg/ml Tryptophan, in the second 2mg/ml and in the last one 5mg/ml. distributed this media in test tube (5ml per test tube). Then we Autoclaved the test tube after transferring media. The bacteria inoculated (one strain in different concentration of tryptophan) in test tube.After incubation culture centrifuged and taking 2ml supernatants in vial, add 2 drop of orthophasphoric acid and 4ml of Solawaski's regent. Sample gives strong red colour (IAA positive).Yellow colour (IAA negative). After completion,we take O.D. with the help of spectro photometer at 530 nm absorbance. In Methyl- Red & Voges-Proskauer Test**,** prepared of MRVP broth. Then pourd the 5ml broth in each tube and tube and sterilized and inoculated two MRVP tubes with bacteria and keep one tube uninoculated comparative control.

Incubate all the test tubes at 35^0 C for 48 hrs. After the completion of the whole procedure, we have found that in the MR test, the methyl red indicator in the pH range of 4 will remain red and some of them turning to yellow. Similarly, in the VP test, the development of a crimson-to-ruby pink (red) colour, may be most intense of the surface, is indicative of positive VP test while no change in colouration is represent a negative test. For Indole Production Test. at first, preparation of (1%) trypton broth was followed by dissolving 10 g of peptone in one litre of distilled water. Sterilize in the autoclave at 15 psi (121^0C) for 15 minutes. Inoculating one tryptone broth with bacteria and keep third tube as an uninoculated comparative control. Then we incubated the inoculated tubes at 35^0 C for 48 hrs. After 48 hrs of incubation 1ml of Kovac's reagent was added to each tube including control. Shaked the tubes gently after intervals for 10-15 minutes.

Allowng the tubes to stand to permit the reagent come to the top. As the process was maintained, it has been seen that the development of a cherry (deep) red colour in the top layer of the tube in bacteria appear which shows a positive test for Indole production. Absence of red colouration in bacteria represents negative test of Indole production. In antibiotic Assay First of all, stock solutions were made according to different concentrations of dilution as 0.0025, 0.00125, 0.00063, 0.00031, 0.00015 and 0.000075. Two petriplates were taken of each of the bacterial strain. A media was prepared and 300µl of bacterial broth was poured into a petriplate.Then N.A. media was poured into each of the petriplates after autoclaving. Three holes were made in each of the two petriplates of a respective bacterial strain by tips of 1ml. After solidification of N.A. media, stock solutions of different concentrations were poured drop by drop through 1ml tips (stock concentrations of 3,4,5,6,7 and 8 as control). Kept the petriplates in incubator at 28^0C for overnight.After completion of the entire work we got some clear zone of inhibition which inhibit the growth of the antagonistic bacteria. We select the minimum inhibition zone of the bacteria which gave the result of antibiotic assay, i.e. the minimum inhibition zone indicates more resistance against the antibiotics.

Moleculalar Analysis

The comparison of 16s rDNA sequence can show evolutionary relatedness among microorganisms as well as their identification. Similarly in our study we isolated the DNA from antagonistic bacteria against TLB and amplified them by PCR with the help of universal primers (16S 27F and 16S 1115R) and sequenced them for their identification. Bacterial genomic DNA isolation was done by minikit method provided by *Geneaid.* (Vogelstein,B. and Gileespie, D., 1979). In this case, 1.5 ml cultured bacterial were transferred to a microcentrifuge tube, centrifuged for 1 min at full speed and the supernatant was discarded. 200 µl of GT buffer was added. 200 µl of GB Buffer was added to the sample and vortexed for 5 sec. Incubated at 70ºC for 10 min or until the sample lysate was clear. 400µl of W1 buffer added to the GD column, Centrifuged. The flow through was discarded and the GD column placed back in the 2 ml collection tube. 600 µl of wash buffer (ethanol added) was added to the GD column.Through a long procedure, the extraction of genomic DNA of bacterial strains were obtained.

PCR amplification was processed by a cascade of reactions. The DNA template was added as a last component while preparing the PCR reaction mix. Depending upon no. of sample to be amplified it remains better to prepare a master mix depending upon PCR reaction, primer ratio. The amplification was carried out for following reaction conditions/PCR Programming for 30 cycles. Initial temperature 94 ºC for 60 sec .denaturation at 94 ºC for 30 seconds, then annealing, extension was carried out. After completion of reaction the PCR product was taken out and 10µl of aq. Layer was ran out in 1% agarose gel for 1-2 hrs at 100 V. Thereafter, Agarose gel electrophoresis of the PCR products was done. 16S r DNA Sequencing method was used for molecular identification of antagonistic bacteria against TLB**.** In laboratory, we amplified the region of the 16S rDNA gene using Polymerase Chain Reaction (PCR). After PCR we analyse the PCR product by 100 bp Ladder then we confirm that our PCR product is upto the limit for sequencing.

Results and Discussion

Survey for collection of bacteria from rhizospheric soil from different area of eastern Uttar Pradesh

By the extensive survey of different region of Eastern Uttar Pradesh, it became possible to taken up for the collection of soil samples from rhizospheric soil of particular plants. We have collected Hundred soil samples that were mainly

from Maize, Rose, Kamini, Brassica, Wheat and Bathua fields of Varanasi, Ghazipur, Mirzapur and Azamgarh region of Eastern Uttar Pradesh, These samples were stored in the laboratory at -70°C in a deep freezer. The hundred soil samples collected from different locations of Eastern Uttar Predesh was screened for presence of phosphate solublizing bacteria, fluorescent bacteria and soil actinomycetes.

Biochemical analysis of isolated bacteria

In Methyl Red Test, we have found that the methyl red indicator in the pH range of 4 will remain red (throughout the tube) which is indicative of a positive test, while turning of methyl red to yellow gives negative test of this experiment. Thus, we got that the antagonistic strain like BMLP3 and BMLA1showed positive test and the strain BMLK1, BMLK9, BMLK11, BMLK18, BMLP4 and BMLA11 showed negative test. Similarly, in the

VP test, the development of a crimson-to-ruby pink (red) colour, may be most intense of the surface, is indicative of positive VP test while no change in coloration is represent a negative test. Thus, on the basis of this method, it was found that, the antagonistic bacteria which showed antagonistic activity against TLB I.e. BMLK1, BMLA11, BMLK11, BMLK18 and BMLP4 expressed positive test, where as BMLP3 and BMLK9 exhibited negative test.

In an antibiotic assay, streptomycin was used as an antibiotic for detection of MIC (Minimum Inhibition Count) A clear zone appeared in the petri plate. We got minimum inhibition count in most of the the bacterial strain was 0.00015g. The antagonistic isolates BMLP3, BMLA11, BMLK1 and BMLK18 showed MIC 0.00015g whereas, the other antagonistic isolate (Against TLB) like BMLK11 have MIC 0.00031g, BMLA1 and BMLP4 have 0.0025g, BMLK9 showed MIC 0.00063g. Moreover, all isolates were also screened for the Antibiotic Assay.

List of antagonistic bacteria showing antifungal activity.

Isolate	Radial growth (*E. turcicum*)	% of inhibition (mm)
BMLK1	19	78.8
BMLK9	20	77.7
BMLK11	23	74.4
BMLK18	30	66.6
BMLP3	16	82.2
BMLP4	28	68.8
BMLA1	23	85.5
BMLA11	25	72.2

Strain	Methyl Red	Voges Proskauer	Gram Staining	Indole Production
BMLP3	+ve	-ve	+ve	+ve
BMLP4	-ve	+ve	+ve	+ve
BMLA1	+ve	-ve	-ve	+ve
BMLA11	-ve	+ve	+ve	+ve
BMLK1	-ve	+ve	+ve	+ve
BMLK9	-ve	+ve	+ve	+ve
BMLK11	-ve	+ve	-ve	+ve
BMLK18	-ve	+ve	+ve	+ve

Molecular characterization

DNA of the bacteria was isolated for selective amplification of 16S rDNA sequences with the aid of PCR. We have isolated genomic DNA of 11 bacterial isolates in which 8 of them were found to be antagonistic against TLB pathogen. After loading 11 DNA samples (against a 100bp ladder sequences) obtained from bacteria found to be antagonistic against TLB, the samples were run in the Agarose Gel Electrophoretic system for 1.5 hr at 65V. The samples BMLP11and BMLA18 were not visualized clearly. It means the sample may contain inadequate amount of DNA in them, whereas BMLK1, BMLP4, BMLA13, BMLP3, BMLK15, BMLA11, BMLA1, BMLK9, BMLK18 show clear bands. The samples shown clear visualizing bands contain more than 54ng of DNA and are suitable for PCR amplification.

The DNA of antagonistic bacteria (against TLB) was taken for PCR. The characteristic feature of PCR is that it can amplify the DNA even if it is initially taken for a very small quantity. The PCR bands were run in the Agarose Gel Electrophoresis and analyzed under Gel Documentation system. Some bands were found to be clearly visualized and hence contain sufficient amount of DNA required for their sequencing. In the laboratory, we amplified the region of the 16S rDNA gene performing Polymerase Chain Reaction (PCR) using thermal cycler. After PCR we analyze the PCR product by 100 bp Ladder. The bands obtained after gel electrophoresis is shown in Plate 11. Then we confirm that our PCR product is up to the limit for sequencing. Phosphate solubilizer BMLP3 and BMLP4 were found to be show similar banding pattern which may emphasize that, they may belong to similar genera of microorganism. Pseudomonas BMLK1 and actinomycete BMLA11 have closely related banding pattern, which may emphasize that they may have closely related antifungal activity. We have taken the isolates BMLP11, BMLP3, BMLP4, BMLK1, BMLK9, BMLK15, BMLA18, BMLA1, BMLA11, BMLA13, BMLK18 and Ladder respectively. Nonetheless, the selected strains must be subjected to further analysis.

The screening procedure demonstrated to be very effective. Although we did not establish a direct relationship between the described mechanisms and the protection that these strains demonstrate towards turcicum leaf blight in maize, the main goal of this work accomplished and it shows bioactive compounds produced by these strains for the control of the fungus *Exserohilum turcicum* which then might be useful in agriculture practice.

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