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Evaluation of phosphate solubilizing bacteria isolated from rhizospheric soil of parthenium plant

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ABSTRACT

The export oriented agricultural and horticultural crops depends on the export of residue free produce and has created a great potential and demand for the incorporation of biopesticides in crop protection. There is several fungicides which are causing harm to soil, plant and environment. So, we focus on isolation of such bacteria which having antagonistic property as well as phosphate solubilizing ability for growth promotion. We isolated approximately 54 isolates which having phosphate solubilizing ability from different regions of eastern and western regions of Uttar Pradesh from rhizospheric soil of *parthenium plant*. Among these bacteria we screened for antagonism test against TLB, and 4 bacteria were found which were showing strong antagonism against TLB. Biochemical test were also performed of these bacteria, for Indole acetic production. Among them G1, G7, G13 and G17 produce Indole acetic acid 180µg/ml, 200µg/ml, 220µg/ml and 330µg/ml respectively. These strains have been selected for further studies towards application as BCAs against TLB as well as good plant growth promoting activity. So in this way we can use this strain in future for development of antifumgal chemical which used as a antifungal against TLB and ecofriendly.

Key Words: *Antagonism, Biopesticides, BCA, Fungicide, Phosphate bacteria, Parthenium, Rhizospheric, TLB*

INTRODUCTION

Maize (*Zea mays* L., 2n=20) belongs to tribe Maydeae of the grass family Gramineae (Poaceae). It is the third most important crop worldwide, after rice and wheat, and is grown mainly as a high-energy feed for human and animal consumption, besides diversified end-uses for industrial and pharmaceutical purposes. Maize is the crop with the highest productivity grown all over the world. With a change in food pattern and shift in cropping systems, maize also assumes a significant role in Indian agriculture. There has been a continuous increase in area under maize cultivation. Turcicum leaf blight (also known as northern corn leaf blight) is caused by the Fungi *Exserohilum turcicum.* 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°C to 27°C). Yield losses as high as 70% have been attributed to Turcicum leaf blight. Symptoms of Turcicum leaf blight are easily recognized. Early symptoms are oval, water-soaked spots on leaves (Elliots and Jetkins 1946). The biological control of plant pathogens by antagonistic microorganisms offers an attractive alternative to existing pest management tactics. The antagonistic organisms release antibiotics and other metabolites that are harmful to the turcicum leaf blight pathogen and inhibit their growth. The word antagonism was introduced by Baker 1987. Since then many antibiotics 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 microbes to

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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 i.e., the site where it should act.

The term biocontrol is used not only to control diseases in living plants but also to control diseases occurring during the storage of fruits (also called postharvest control). Studies on the control of pathogens by rhizobacteria usually focus on pathogenic microorganisms. It should be noted that some rhizobacteria are also active against weeds and insects. 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 (Yana and Dadarwal 1997). This results in acidification of the surrounding soil, releasing soluble orthophosphate ions ($H_2PO_4^{-1}$, HPO_3^{-2} and PO_4^{-3}) which can be readily taken up by plants. 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, Bojinova and Nedialkova 2006).The important genera of PSB include Achromobacter, Aerobacter, Alkaligenes, Bacillus, Pseudomonas, Serratia and Xanthomonas.

MATERIALS AND METHODS

The experimental methods applied and procedures adopted during the research work were as follows:

Collection of rhizospheric soil and isolation of bacteria from different regions of eastern Uttar Pradesh.

Experimental site: 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.)

Collection of rhizospheric soil from different region of eastern Uttar Pradesh: The soil samples were collected up to 15cm from the surface of the soil and stored in sterile polythene bags from the experimental fields of Banaras Hindu University, Varanasi, Mirzapur, Bhadohi, Azamgarh, Noida, Jaunpur, Lucknow, ganga ghat Varanasi district. Total number of-samples we have collected from these different regions.

Isolation of rhizospheric bacteria by Serial dilution technique

Pikovaskya agar: Tricalcium phosphate (TCP) is regarded as a model compound for measuring the potential or relative rates of microbial solubilization of insoluble inorganic phosphate compounds. Microorganisms on precipated calcium phosphate agar produces clear zones around their colonies if they are capable of solubilizing calcium phosphate. Suitable dilutions (10:4) of serially diluted rhizosphere soil suspension were poured plated on Pikovskaya's Phosphate solubilization is indicated by the formation of a solubilization or a clear zone around the bacterial colonies. 54 isolates were obtained after the sereening on pikovaskya agar. Single bacterial colonies having clear solubilization zones were isolated separately on to fresh Pikovskaya's agar plates, incubated at 30±5 ^{0}C.

Methods of obtaining pure cultures of microorganisms: The method we used for obtaining pure culture of microorganisms was Streak-plate method.

Method for maintaining pure culture of microorganisms:

1. Around 8ml of nutrient agar was added in the properly sterilized and autoclaved test tubes and the tubes were allowed to solidify in slant position
2. The bacterial inoculums were transferred from streaked plates to the solidified slants under laminar air flow.

3. The slants were then stored in incubator and when the growth was once obtained it was stored in refrigerator.
(Ref.: Aneja KR (2003) Experiments in Microbiology, Plant pathology and Biotechnology).

Collection and isolation of test fungi (Helminthosporium turcicum): The test organisms, the pathogens against which the soil bacteria are to be screened for their antagonistic potentialities, can be either obtained (procured) or isolated from the infected plant tissues after surface sterilized with chemicals such as $HgCl_2$ (0.01%) solution or NaOCl solution. Sample with clear visible symptoms washed thoroughly with distilled water. A small portion of diseased tissues along with a portion of adjacent healthy tissue was cut into small pieces around 1mm in length. Then surface sterilized with chemicals such as $HgCl_2$ (0.01%) solution or NaOCl solution for 2-3 min.

Step	Temperature (ºC)	Time (sec)
Initial temperature	94	60
Denaturation	94	30
Annealing	48	30
Extension	72	60
Final extension	72	120

The pieces were then rinsed thrice with sterilized distilled water and inoculated aseptically on sterilized PDA plates. The inoculated petriplates were incubated at 20ºC-25ºC. When the fungal colony developed, a small cut of single mycelium transferred to another petriplate containing PDA medium to obtain the pure culture. The pure culture of pathogen was maintained in PDA slants and after full growth the slants were stored in refrigerator at 4ºC for further studies.

Screening of antagonistic bacteria gainst *Helminthosporium turcicum*

Antagonistic assay by dual culture technique: *Helminthosporium turcicum* isolated from leaf sample were evaluated against Phosphate solubilizing bacteria in laboratory by dual culture technique to screen out the most efficacious one. 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 ambient temperature in the incubator for 4-7 days. After few days the cleared zone was observed and diameter is measured. Finally after performing the antagonistic assay we have identified 4 isolates out of total 54 bacterial strains.

Molecular characterization of selected isolates showing antagonism against TLB

Bacterial genomic DNA isolation by minikit method: **(**Ref.: Vogelstein B and Gileespie D (1979) Proc Natl Acad Sci USA 76, protocol by GENEAID, www.geneaid.com**)**

PCR amplification of isolated DNA from bacteria
Procedure: PCR mix (50 µl) was prepared by adding the following reagents to the PCR tube. 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.

a) The content mixed gently and reaction mixture was layered with 50 µl of mineral oil. (Optional, as per the specification of PCR machine). The amplification was carried out for following reaction conditions for 30 cycles.

The annealing temperature depends upon the length of the primers and Tm of the primers.

10x assay Taq pol buffer = 15 mM $MgCl_2$	5 µl	(1x)
dNTP mix	1 µl	(200 µm each)
Template DNA (200ng/ µl)	2 µl	(200 ng)
Forward primer (250ng/µl)	2 µl	(250 ng)
Reverse primer (250ng/ µl)	2 µl	(250 ng)
Taq DNA polymerase (3u/ µl)	1 µl	(1.5µ)
Sterile water	37 µl	

b) After completion of reaction the reaction mix was taken out and 10µl of aq. Layer was ran out in 1% agarose gel for 1-2 hrs at 100 V.

c) Stained with EtBr and visualized under UV light for 0.8 kb DNA fragment.

(Ref.: Kumar A, Pandey D (2008) Laboratory Manual Techniques in Molecular Biology).

RESULTS

Results of isolation of Phosphate Solublizing Bacteria: A total of 54 isolates of Phosphate Solublizing Bacteria were isolated from different soil sample on the basis of different parameters of colony characteristic like colony shape, size, surface, margin and colour.

Table 1 Phosphate Solublizing Bacteria isolates obtained from the rhizospheric soil of different plant sample from different location of Eastern U.P.

Rhizospheric soil sample	Location	Number of isolates
Parthenium	B.H.U	10
Parthenium	Ganga ghat varanasi	8
Parthenium	Bhadohi	4
Parthenium	Jaunpur	6
Parthenium	Barailley	8
Parthenium	Mirzapur	10
Parthenium	Noida	4
Parthenium	Azamgarh	4

Result of dual culture: Only 4 phosphate solubilizing bacteria isolates were shown the antagonistic property against the fungal pathogen TLB.

Table 2 Antagonistic potential of selected bacterial isolates against fungal pathogen TLB by using dual culture method.

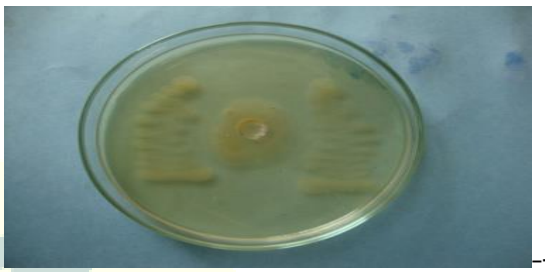
Isolates	% of inhibition	Pathogen
G1	66.6%	TLB
G7	73.7%	TLB
G13	82.6%	TLB
G17	88.8%	TLB

Result of Biochemical Test: 4 isolates shows the +ve result for indole production.

Table 3 Biochemical test for selected isolates.

Isolates	Gram Staining
G1	-ve
G7	-ve
G13	-ve
K17	-ve

Antagonistic result PIC



---- G1

----G7

---G17

----G13

DISCUSSION

Phosphorous deficiency is limiting crop production worldwide (Hamdali *et al* 2008) as most soil phosphates in soil are found bound as insoluble

phosphates which can be released by microorganisms (Arcand and Schneider 2006). P-solubilizing microbes (PSMs) belong to Bacillus, Psuedomonas, Micrococcus and Actinomycetes etc. (Mba 1997, Rudresh *et al* 2005). PSMs are of special interest as they can survive in a variety of soils and produce a plethora of bioactive compounds that may benefit the plant (Hamdali *et al* 2008, Pathom-Aree *et al* 2006, Ikeda 2003, Jain and Jain 2007). Several potential biocontrol agents used for plant disease management behave as opportunistic human pathogens. Though *P. aeruginosa* is a potential biocontrol agent of gray leaf spot on turf, & *P. cepacia*, which is used for the management of pea root rot. Bacillus cereus, being a potential candidate for the management of damping-off and root rot of soybean. Keeping of view in mind we isolate 54 Phosphate solubilizing bacteria on Pikovaskya media from Parthenium rhizospheric soil. Further we screened for antagonistic against TLB and among them 4 isolates G1, G7, G13 and G17 were found to be antagonistic against TLB. Among the above 4 antagonistic, G17 gave the strongest inhibitory effect. All the four isolates gave a positive Indole production test. G17 was the strongest indole producing bacteria produced 300µg/ml followed by G13 i.e. 200µg/ml, G7 i.e. 200µg/ml and G1 i.e. 180µg/ml. So in this way in future we suggest that these 4 isolates use as a strong antagonistic as well as very good plant growth promoter which is no causing any pollution to environment. In other aspect we can say that chemical isolated from these isolates and use as a broadspectrum biofungicide which is cost effective as well as ecofriendly.

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