ISSN 0971-930X
Journal of Biological Control
Published by Society for Biocontrol Advancement (Copyright 2000 SBA)

Journal of Biological Control, 26 (1): 59-61, 2012

Research Article

Study of fluorescent bacteria antagonistic against *Exserohilum turcicum*

KAUSHAL KUMAR BHATI[*] and RAJESH SINGH[1]

National Agri-food biotechnology Institute, Mohali, India
1. Department of Genetics and Plant Breeding, Banaras Hindu University, Varanasi, Uttar Pradesh, India
Corresponding author E-mail: selvaentomo@yahoo.co.in

ABSTRACT: *Exserohilum 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 rhizospheric fluorescent Pseudomonas having antagonistic property against *E.turcicum*, characterized by biochemical tests and 16S r DNA sequences. Out of total 33 isolates, 6 bacterial strains *viz.,* MBLK1, MBLK3, MBLK6, MBLK15, MBLK17, and MBLK22 were found to be antagonistic against *E.turcicum*. Antagonistic bacteria were analyzed for their plant growth promotion ability on maize variety HUZ-M60.

KEY WORDS: Antagonism; 16s r DNA; PGPRs, *Exserohilum turcicum*, maize

(Article chronicle: Received:19-12-2011 Revised:22-12-2011 Accepted: 24-2-2012)

INTRODUCTION

Turcicum leaf blight is a major constraint to maize production in many maize growing regions worldwide, caused by the fungi *Exserohilum turcicum* (syn. *Helminthosporium turcicum*). The growing season is characterized by high humidity and moderate temperatures (17 to 27°C). Yield losses as high as 70% have been attributed to turcicum leaf blight. Biocontrol of pathogens using natural means is an indispensable approach to minimize yield losses besides environment friendly. *In-vitro* biocontrol of *E.turcicum* using plant extracts has been reported previously (Bhati *et al.,* 2011) and the fluorescent *Pseudomonas* could be potent candidates since, their antagonistic properties to biocontrol pathogens are well characterized (Palumbo *et al.,* 2010; Vidhyasekaran *et al.,* 1997). Antagonistic properties of *Pseudomonas* isolated from rhizospheric soil of tea were found most effective against plant fungal pathogen as compared to bacterial pathogens (Saikia *et al.,* 2011). Plant growth promoting activities of *Pseudomonas* strain reported recently from rice rhizosporic soil (Naganandini *et al.*, 2011) add advantages to use fluorescent bacteria as biocontrol agents. Therefore, the present investigation was aimed to identify fluorescent *Pseudomonas* bacterial isolates antagonistic against *E.turcicum*.

MATERIAL AND METHODS

Isolation of fluorescent bacteria

Soil samples were collected from rhizospheric soil of maize fields at Banaras Hindu University, Varanasi. Ten 10 gm. of each soil samples was serially diluted, streaked on King's B agar (KB) and incubated at 29° C for 48h for the isolation of fluorescent *Pseudomonas* spp. Fluorescent colonies were picked randomly under UV light. These isolates were named as MBLK. The purified isolates were maintained in glycerol stock at -20° C. The fungal pathogen isolated from infected leaf part was identified as *E. turcicum* based on morphological parameters.

Dual culture and *in-vitro* antagonistic assays

The bacterial isolates were screened for *in-vitro* inhibition of *E.turcicum* using dual plate assay. Fresh culture of *E. turcicum* was grown in PDA medium (Potato infusion-200; dextrose- 20; agar-18 (g l^{-1}). A plug from the growing edges (5 mm diameter) of the *E. turcicum* colony was placed in the centre of a fresh plate. The bacterial isolates were grown overnight, spotted along the periphery of 90 mm diameter in a petridish equidistant from the mycelia plug. These plates (3 replicates) along with the uninoculated control were incubated at 30°C for about 72 h or until the leading edge of the fungus reached the edge of the plate. Antibiosis was assessed by measuring the diameter of the mycelial growth of *E.turcicum* alone and in the presence of the bacterial isolate. Zones of inhibition were measured after 5 days using following formula-

$$\text{Percent growth inhibition} = r^{\circ}\text{-}r^{1}/r^{\circ} \times 100$$

where,

r° = colony growth in control plate

r^{1} = colony growth in intersecting plate

Plant growth promotion analysis

Plant growth promotion activity was measured by inoculating seed with bacteria (Zahir *et al*., 2004). Seeds of maize variety HUZ-M60 were surface sterilized with sodium hypochlorite and 0.1% mercuric chloride for 30 sec. and washed with distilled water. The soil bags were prepared with sterilized soil and 5ml sucrose solution was added into each bag along with the maize seeds. The respective bacterial strain was inoculated in the soil bags, mixed properly and kept for 5-6 hrs in an incubator for proper growth. Each maize seed inoculated with bacterial strains were sown in the sterilized soil kept in pots placed inside a plant growth chamber at 25°C, 60% humidity and 12 hr day/night cycle with proper irrigation. After 21 days, the plants were uprooted and the length of root and shoot was measured.

Methyl Red (MR), Voges Proscauer (VP) test

The inoculation tubes were prepared each with 5 ml. Broth media (Peptone – 7.0g, dextrose – 5.0g, potassium phosphate – 5.0g, distilled water –1000.00 ml., pH – 6.9) and autoclaved. For MR and VP test, tubes were inoculated with bacteria and incubated at 35°C for 48 hrs with a control tube. The methyl red indicator in the pH range of 4, remain red inferred positive MR test, while, yellow negative. The development of a crimson- to-ruby pink (red) colour showed positive for VP test and no colour change showed negative.

Indole acetic acid (IAA) production

Bacterial isolates were propagated in DF salts minimal medium with L-tryptophan (1mg, 3mg, 6mg/ml) and incubated at 28±2°C for 48h and later removed by centrifugation at 4,000 rpm for 20 min at 4ºC. One ml of the supernatant was mixed with 4 ml of Salkowski's reagent in the ratio of 1:4 and incubated at room temperature for 20 min. Development of a pink colour indicated the presence of IAA. The absorbance of supernatant mixture (supernatant+ Salkowski's reagent) was measured at 535 nm and the quantity of IAA was determined by comparison with a standard curve.

16Sr DNA amplification and sequencing

DNA extraction from soil microbial isolates was performed using Genomic DNA Mini Kit, GB100 (Geneaid Biotech Ltd.) as per 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). The amplified DNA product was visualized on an agarose gel. The amplified 16s r DNA sequences were sequenced and compared with those available in the NCBI Database (http://ncbi.nlm.nih..gov/blast) using BLAST and the most similar sequences with maximum score were aligned using CLUSTAL W and phylogeny analysis with the neighbour joining method.

RESULTS AND DISCUSSION

Occurrence of plant growth promotion ability attributes may reinforce their effectiveness as microbial inoculants (Lucy, 2004). Around hundred soil samples were analyzed for bacterial strains and 33 strains were screened for fluorescent *Pseudomonas*. All of these isolates were gram-negative, indole-positive, produced fluorescence under UV light.

As evidenced, the technique of dual culture (Yoshida *et al*., 2001) analysis on agar plates is an easy assay to select antagonistic bacteria from a random group of bacterial isolates and to compare these selected strains for their fungal growth inhibition capabilities. Thirty three fluorescent bacterial strains isolated from soil samples were tested for their antagonistic activity against *E. turcicum* and 6 fluorescent bacteria strains were found to have antagonism. The strain MBLK17 was found to have maximum percentage inhibition followed by MBLK1 (Table 1). The antagonistic strains were further checked for their plant growth promotion abilities. Strains MBLK1 had profound effect on both root and shoot growth followed by MNLK22. (Table 1).

Table 1. Effect of isolates of *Pseudomonas aeruginosa* on *Exserohilum turcicum* and on plant growth parameters

Isolate	Percentage inhibition	Root length in cm	Shoot length in cm
MBL–K1	77.7	24.16	36.00
MBL–K3	77.3	17.83	23.66
MBL–K6	74.0	19.83	29.16
MBL–K15	66.9	18.5	26.66
MBL–K17	81.8	21.16	24.16
MBL–K22	68.4	16.33	31.16
CD (*P*=0.01)	2.55	1.65	10.75

These plant growth promotion abilities attribute to the production of IAA like substances and solubilization of phosphate. These antagonist isolates also produced significant level of IAA like substances in culture filtrate of broth media supplemented with tryptophan. Level of IAA ranged from of 14.8–18.9 µg/ml culture filtrates (Table 2). Earlier studies reported level of IAA in vitro to vary from 2.0–21.6 ppm in rice rhizosphere (Thakuria *et al.,* 2004).

The 16s rDNA sequence is a potential tool frequently used for molecular characterization of soil bacterium (Pisa

et al., 2011). The amplified genomic DNA for 16s rDNA sequence was analyzed by agarose gel electrophoresis with 100bp ladder (Figure 1). BLAST hits for all six antagonist

Table 2. Indole production test

S.No.	Strain	1mg/ml tryptophan	3mg/ml tryptophan	6mg/ml tryptophan
1	MBLK1	18.10	54.5	108.90
2	MBLK3	18.90	54.00	104.40
3	MBLK6	14.80	42.60	80.60
4	MBLK15	16.30	48.50	100.00
5	MBLK17	17.40	51.45	106.00
6	MBLK22	16.20	48.10	79.20

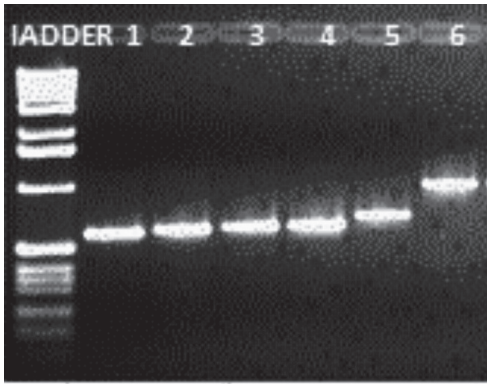


Fig. 1. PCR Amplified product of DNA isolated from antagonistic strains by Polymerase chain reaction using 16S27F and 16S111R primers. 1 = MBLK1, 2 = MBLK3, 3 = MBLK6, 4 = MBLK15, 5 = MBLK17, 6 = MBLK22

Table 3. BLASTN hits for sequences

S. No.	Strain	Highest similarity found	Identity	e– value	Accession no.
1	MBL–K1	*Pseudomonas putida*	97%	0.0	HQ259593.1
2	MBL–K3	*Pseudomonas mosselii*	98%	0.0	DQ095881.1
3	MBL–K6	*Pseudomonas fluorescens*	97%	0.0	HQ236548.1
4	MBL–K15	*Pseudomonas entomophila*	94%	0.0	EF178450.1
5	MBL–K17	*Pseudomonas aeruginosa*	95%	0.0	GU325690.1
6	MBL–K22	*Pseudomonas montelli*	98%	0.0	AF181576.1

isolates showed maximum similarity *Pseudomonas* spp. (Table 3). *Pseudomonas aeruginosa* was found to have maximum *in vitro* inhibitory effect on *E.turcicum*, simultaneously, showing significant increase in shoot length of maize. These antagonistic and plant growth promoting abilities were found as per recent literatures (Palumbo *et al*., 2010, Naganandini *et al.,* 2011).

These finding encourages evaluating the fluorescence *Pseudomonas* spp. for their biocontrol and plant growth promoting abilities.

REFERENCECS

Bhati KK, Prasad R, Singh R. 2011. Screening of certain Ayurvedic plants extracts against *E. turcicum.* Nature Proceedings <http://hdl.handle.net/10101/npre.2011.6199.1>

Lucy M, Reed E, Glick B R. 2004. Application of free living plant growth-promoting rhizobacteria. *Int J Gen Mol Microbiol*. **86**: 1–25.

Naganandini S, Balachandar D, Kumar K, 2011. Diversity analysis of pseudomonads in rice rhizosphere for multifaceted plant growth promotion; *Acta Microbiol Immunol Hung*. **58**: 247–258.

Palumbo JD, O'Keeffe TL, Kattan A, Abbas HK, Johnson BJ. 2010. Inhibition of *Aspergillus flavus* in soil by antagonistic Pseudomonas strains reduces the potential for airborne spore dispersal. *Phytopathology.* **100**: 532–538.

Pisa G, Magnani GS, Weber H, Souza LEM, Faoro LH, Monteiro RA, Daros E, Baural V, Bespalhok JP, Pedrosa FO, Cruz LM. 2011. Diversity of 16S rRNA genes from bacteria of sugarcane rhizosphere soil. *Preto* **44**: 1215–1221.

Saikia R, Sarma RK, Yadav A, Bora TC. 2011. Genetic and functional diversity among the antagonistic potential *fluorescent pseudomonads* isolated from tea rhizosphere, *Curr Microbiol.* **62**: 434–444.

Thakuria D, Talukdar NC, Goswami C, Hazarika S, Boro RC, Khan M R. 2004. Characterization and screening of bacteria from rhizosphere of rice grown in acidic soils of Assam. *Curr. Sci.* **86**: 978–985.

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 Pathol.* **46**: 291–297.

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. *Phytopathol.* **91**: 181–187.

Zahir ZA, Arshad M, Frankenberger WT. 2004. Plant growth promoting rhizobacteria: applications and perspectives in agriculture. *Adv Agron.* **81**: 97–168.