Int.J.Curr.Microbiol.App.Sci (2017) *6*(9): 3457-3463

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 9 (2017) pp. 3457-3463**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.609.424

Screening and Characterization of Rhizospheric Chitinolytic Bacteria for Evaluation of Their Potential as Biocontrol Agent against Phytopathogenic Fungi

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Keywords

Chitinolytic bacteria, Antagonistic activity, Phytopathogenic fungi, *Rhizoctonia solani*, *Bacillus* sp.

Article Info

Accepted:
10 July 2017
Available Online:
10 September 2017

A B S T R A C T

The present study evaluated the antagonistic effect of the rhizospheric chitinolytic bacterial isolates of rice and green gram soil samples against three phytopathogenic fungi, i.e. *Pythium aphanidermatum* (ITCC No.-5488), *Fusarium oxysporum* (ITCC No.-4998) *and Rhizoctonia solani* (ITCC No.-2060). A total of 25 bacterial isolates were obtained based upon their distinct colony characteristics on colloidal chitin agar medium. Only 6 isolates (G1, G2, R3, R4, R9 and R10) showed visible antagonistic activity against the test fungi by dual culture method on potato dextrose agar (PDA) during primary screening. During further screening by well diffusion method, all the 6 showed broad-spectrum antibacterial activity by inhibiting most of the test fungal phytopathogens. The six effective isolates were also able to reduce the fungal biomass of all the three test pathogens by mycelial reduction test. Though R10 inhibited all the three fungal pathogens but maximum reduction was observed against *Rhizoctonia solani*. Physiological & biochemical characterization of the effective isolates indicated that the antagonistic chitinolytic bacterial belong to *Bacillus* sp.

Introduction

Plant diseases cause considerable losses in crop production and storage. Now-a-days growers still rely heavily on chemical pesticides to prevent or control these diseases, which can result in environmental contamination. The presence of pesticide residues on food also cause social and economic problem that heavily affects the economy of the country. Fungi (*Fusarium* sp., *Rhizoctonia* sp., *Alternaria* sp., *Pythium* sp. etc.) are parasitic pathogens which attack crop plants and are very dangerous. Fungal phytopathogens cause serious problems worldwide causing diseases like rusts, smuts, rots and wilt; which damages the crop (Goma *et al.*, 2011). Biological control, using microorganisms to suppress plant disease offers powerful and alternative tool against the use of synthetic chemicals.

Chitinolytic bacteria such as *Aeromonas hydrophila*, *Aeromonas caviae*, *Pseudomonas maltophilia*, *Bacillus licheniformis*, *Bacillus circulans*, *Vibrio furnissii*, *Xanthomonas* sp. and *Serratia marcescens* have been reported as important biocontrol agents. Biological control using microorganism has been studied intensively, since not many alternatives to

control plant diseases are available (Duffy *et al.*, 1995).

Detection of chitin degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antifungal or other novel compounds. There is a higher correlation between chitinolytic and production of bioactive compounds (Hostel *et al.*, 2005). Micro-organisms, which secrete a complex of mycolytic enzymes, are considered to be possible biological control agents of plant diseases against fungal pathogen (Helisto *et al.*, 2001 and Chang *et al.*, 2003). The objective of the present study is to isolate the native chitinolytic bacteria from the rhizosphere soil of rice and green gram as well as from the fish dump waste to study their potential in biocontrol of phytopathogenic fungi.

Materials and Methods

Collection of sample

Rice and green gram rhizospheric soil samples were collected from O.U.A.T farm separately by digging the soil 3-4cm deep after removing the surface soil with the help of sterile spatula carefully from four different regions of each field and brought to the laboratory for microbiological analysis.

Phytopathogens used

The three phytopathogens i.e., *Pythium aphanidermatum* (ITCC No.-5488), *Rhizoctonia solani* (ITCC No.-2060), *Fusarium oxysporum* (ITCC No.-4998) were used in the present study.

Preparation of colloidal chitin

For colloidal chitin preparation, chitin (10g) was added to 170mL of conc. HCl and kept under vigorous stirring for 2 h at room temperature until the chitin was dissolved completely. The above suspension was precipitated by slowly adding ice cold ethanol. The pH of the suspension was adjusted to 7.2 - 7.3 by adding 10 N sodium hydroxide. The above suspension was centrifuged at 8000 rpm for 10 min. The precipitate was collected and used as medium substrate (Faramarzi *et al.*, 2009).

Isolation of chitinolytic bacteria

Isolation of the chitinolytic bacteria was done following tenfold serial dilution of the soil samples on LB agar medium under aerobic condition.

Then selective isolates was done on colloidal chitin agar medium, where chitin was the sole source of carbon and energy. Colonies showing zones of clearance against the creamy background were regarded as chitinase-producer and restreaked until pure cultures were obtained. The selected chitinolytic bacteria were considered for further studies (Kamil *et al.*, 2007).

In vitro antibiosis study of the bacterial isolates against fungal phytopathogens

Only the 6 isolates showing the maximum zone of clearance in the colloidal agar medium were screened against the three fungal phytopathogens to study their antagonistic activity. The preliminary screening was done by dual culture method and the secondary screening test by well diffusion method and mycelial reduction test. All the tests were done by triplicate (Table 4).

Dual culture method

The fungal growth inhibition capacity of the isolates was determined by dual culture method (Balaz *et al.*, 2000). From the fresh fungal culture plates, 6 mm diameter agar

discs of each pathogen were placed at the centre of PDA plates. The chitinolytic bacterial isolates were streaked around the fungal agar discs in rectangular form. The plates were incubated for 72 h at 30ºC and colony diameter of the fungal pathogens were measured and compared with the control (Fig. 2, Table 2).

Well diffusion method

Freshly prepared PDA plates were used to study the antifungal activity of the isolates against fungal phytopathogens.

Two wells were punched on the plates at an equal distance from the centre using a cork borer and 0.1ml of 24 h bacterial broth suspension of the isolates were added to the wells.

The punched test fungal pathogens i.e. *R. solani, F. oxysporum, P. aphanidermatum* from the revived plates were inoculated at the centre of the PDA plate, followed by incubation at 30^{o}C for 5 days in order to study the antibiosis.

Mycelial reduction test in liquid medium

Mycelial reduction test was performed using Potato Dextrose broth (PDB). 20ml of the PDB were taken in screw capped vials. 4 agar discs of 6mm diameter was punched out from fungal culture plate and dispensed in the fungus control vial.

Similarly 4 discs of fungal colony of 6mm diameter were dispensed in the treatments (T_1 & T_2). One loopful of fresh broth culture of chitinolytic bacteria was inoculated in the treatments simultaneously. All the tubes were incubated at 30ºC for 3, 5 and 7 days. After incubation, the fungal mycelia were harvested separately from each flask and dried to constant weight in an oven at 100ºC. The antagonistic effect of the isolates was checked by measuring the % of inhibition of the fungal mass following the formula (Reddy and Hynes, 1993).

% = Control-treatment/control ×100

Morphological characterization

All the bacterial isolates were examined for their cell morphology (cell shape, margin, and cell arrangement) by Gram's Stain technique.

Physiological and biochemical characterization of the chitinolytic bacterial isolates

Physiological and Biochemical characteristics of the antagonistic isolates were checked following the standard methods of identification.

Overnight culture of the purified isolates was used for the entire biochemical tests. All the test isolates were identified using ABIS online software (Table 5).

Results and Discussion

A total of 25 bacteria were selected based on different colony morphology. Out of 25, 10 isolates were found positive for chitinolytic activity using colloidal chitin agar (Table 1, Fig. 1).

Detection of chitin degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antifungal or other novel compounds. There is a higher co-relation between chitinolytic and production of bioactive compounds (Hostel *et al.,* 2005).

Chitinase is known as one of the antifungal protein (Gohel *et al.,* 2006). Chitinase produced by the isolates should be considered as enzyme responsible in lysing chitin polymer of the fungal hyphae (Shanmugaiah *et al.,* 2008).

Table.1 Effective chitinolytic bacterial on their zone of clearance on Colloidal chitin agar medium

Isolates code	Zone of clearance (in mm)
G_1	72.0
G_2	70.0
G_6	65.0
R_3	80.0
R_4	87.0
R_6	77.0
R_7	65.0
R_8	70.0
R_9	72.5
R_{10}	97.0

Table.2 Antifungal activity of the bacterial isolates by dual culture method

Isolates Code	**Zone of inhibition (in mm)**											
	R. solani				***F. oxysporum***				***P. aphanidermatum***			
Days	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Control	--	38	40	40	--	21	28	28	--	12	18	18
G1	--	5	6	6	--	7	8	8	--	8	9	9
G2	--	6	7	7	--	9	9	9	--	6	6.5	6.5
R3	--	7	11	11	--	6	8	8	--	7	8	8
R4	--	7	12	12	--	7	9	9	--	6	6	6
R9	--	8	11	11	--	8	8	8	--	7	8	8
R10	--	9	11	11	--	10	11	11	--	9	9.5	9.5

--: No growth, G: Green gram soil, R: Rice field soil

Table.3 Anti-fungal activity (growth inhibition) of the bacterial isolates by well diffusion method

Bacterial Isolates	**Zone of inhibition (in mm)**		
	R. solani	***F.oxysporum***	***P. aphanidermatum***
G_1	14.0	--	--
G_2	--	8.0	8.0
R_3	6.0	--	--
R_4	6.0	--	--
R_9	--	9.0	--
R_{10}	18.0	6.0	6.0

+: Inhibition, --: No growth

Table.4 Secondary screening of the bacterial isolates by mycelial reduction test

Bacteria	% of reduction								
	F. oxysporoum			*R. solani*			*P. aphanidermatum*		
	Days			Days			Days		
	3	5	7	3	5	7	3	5	7
G_1	50	60	60	40	50	50	56	60	60
G_2	60	65	65	55	60	60	50	55	55
R_3	45	54	54	50	50	50	60	65	65
R_4	55	60	60	60	62	62	65	70	70
R_9	58	60	60	45	52	52	54	58	58
R_{10}	64	70	70	60	78	78	55	67	67

Table.5 Biochemical characterization of the effective chitinolytic bacterial isolates

Strains	In	MR	VP	Ci	NR	Es	Ur	Mo	TSI	Amy	Gel	Oxd
G1	-	+	-	+	+	+	+	+	-	+	+	+
G2	-	+	-	+	-	+	+	+	+	+	+	+
R3	-	-	-	+	+	-	+	+	+	+	+	+
R4	-	+	-	+	-	-	+	+	+	+	+	+
R9	+	-	-	+	+	-	+	+	+	+	+	+
R10	-	+	-	+	+	-	+	+	+	+	+	+

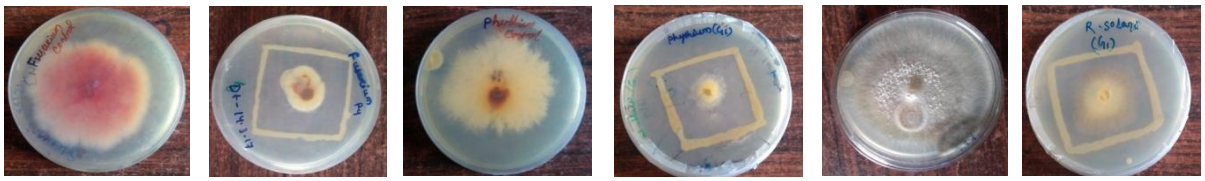
In: Indole test; MR: Methyl red test; VP: Voges Proskauer test; Ci: Citrate utilization; NR: Nitrate reductase; Ur: Urease test; Es: Esculin hydrolysis; Mo: Motility test; TSI: Triple sugar iron test; Amy: Amylase test; Gel: Gelatinase test; Oxd: Oxidase test; +: Positive; - : Negative

Table.6 Test for different sugar utilization by the chitinolytic bacterial isolates

Strains	Galactose	Lactose	Dextrose	Fructose	Maltose	Sucrose
G1	+	-	+	+	+	+
G2	+	+	+	+	+	-
R3	+	+	+	+	-	-
R4	+	+	+	+	+	+
R9	-	-	+	+	-	-
R10	-	-	+	+	+	+

Fig.1 Chitinolytic bacterial isolates on chitin agar medium by spread plate method at Different dilution

Fig.2 Screening of the bacterial isolates by dual culture method



A. *F.oxysporum* (Control & R_4) **B.** *P. aphanidermatum* (Control & G1) **C**. *R. solani* (Control & G_1)

In this study, the ability of the selected chitinolytic strains in suppression of mycelial growth of phytopathogenic fungi was conducted. Following ABIS online software, the isolates were identified as *Bacillus* sp. Bacillus strains have been intensively investigated as biological control agents (Kamil *et al.,* 2007) previously. They all have reported that members of the genus *Bacillus* are well known for their potential to secrete a member of degradative enzymes such as chitinase (Zhang and Zuen *et al.,* 2000; Zhang *et al.,* 2001; Schallmey *et al.,* 2004).

To test the ability of the selected chitinolytic isolates in suppression of mycelial growth of phytopathogenic fungi including *Rhizoctonia solani in vitro,* antifungal assay was performed. It was found that all the 6 chitinolytic isolates antagonized the tested phytopathogen but to a varying degree (Table 3).

All were inhibiting *R. solani* having different inhibition zone size. They have reported a modification of the fungal mycelium appearance due to antifungal secondary metabolite production. Our results are in agreement with those obtained by (Sid Ahmed *et al.,* 2003), who reported that chitinolytic bacteria were active against the fungal pathogens of pepper, sorghum and mango. Protection of plants from disease produced by phytopathogenic fungi is one of the most important challenges in agriculture. Therefore, finding a biological agent that could be used for biocontrol of fungal diseases is very much important in agriculture. The study showed that soil from rhizosphere is the best source for chitinolytic bacteria.

Acknowledgement

The authors are thankful to Dr. B.B. Mishra, HOD, Microbiology Department, O.U.A.T for providing laboratory facilities during the period of study. The authors have no conflict of interst to declare.

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How to cite this article:

Abhilipsa Parhi and Sukantibala Mohapatra. 2017. Screening and Characterization of Rhizospheric Chitinolytic Bacteria for Evaluation of Their Potential as Biocontrol Agent against Phytopathogenic Fungi. *Int.J.Curr.Microbiol.App.Sci.* 6(9): 3457-3463.
doi: https://doi.org/10.20546/ijcmas.2017.609.424