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ORIGINAL RESEARCH

Antifungal Activity of Rhizobacteria Isolated from Rice Rhizosphere Soil Against Rice Blast Fungus *Pyricularia oryzae*

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ABSTRACT

Rice blast caused by *Pyricularia oryzae* is the major disease affecting the rice production. Application of beneficial bacteria as seed, seedling root dip or foliar spray to protect this disease may be an alternative strategies to chemical control. The objectives of the present study was to isolate and screen the antifungal activity of rhizobacterial strains against rice blast causing fungal pathogen *Pyricularia oryzae* by *in vitro* techniques. Ten rhizobacterial strains and fungal pathogen *Pyricularia oryzae* was isolated from rice rhizosphere soil and blast infected rice leaves respectively. Among 10 isolates, strain RB04 gave significantly higher inhibition of mycelial growth of *Pyricularia oryzae* in dual culture assay and therefore appears to be a potential candidate for control of rice blast disease. The potential candidate RB04 was selected and identified using 16S rRNA sequencing as *Pseudomonas fluorescens* (AUPF25). It possesses a variety of promising physical properties such as protease, IAA and siderophore production, which make it a better biocontrol agent. The result indicated that the rhizobacterial strain *Pseudomonas fluorescens* presented a significant value against *Pyricularia oryzae*.

Key words: Rhizobacteria, *Pseudomonas fluorescens*, Antifungal activity, *Pyricularia oryzae* and Rhizosphere soil.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the three major food crops of the world. Being grown worldwide, it is the staple food for more than one and a half of the world's population. India is the largest rice growing country accounting for about one-third of the world acreage under the crop. The Natural Resources Institute (NRI) London gave first rank to rice blast disease in its study of pre-harvest pests and diseases affecting in south Asia[1]. Rice blast caused by *Pyricularia oryzae* is the major disease affecting rice cultivation[2]. Though, this disease being managed through fungicides, their adverse effects on environment and beneficial soil microorganisms are quite evident. Biocontrol approach for managing these diseases is considered to be a practical and economical alternative[3].

Many microorganisms from the rhizosphere can positively influence plant growth and plant health and are referred to as PGPR. These microbes can act as biocontrol agents in several ways, including niche exclusion, bioantagonism and induction of induced systemic resistance (ISR) against infection by fungal, bacterial and viral pathogens in different plant species[4]. Since biocontrol is a key component of integrated disease management, it is important to search for rhizobacteria active against blast and to evaluate the antagonists for application in field conditions.

The antifungal activity in dual culture screens is due to the production of either antibiotics, toxic metabolites or siderophores as mechanisms for biological control[5]. Siderophore-producing bacteria are able to out-compete other soil microbes, providing one mechanism for their bioantagonistic properties[6]. In addition, siderophores have been identified as molecules able to activate ISR in several plant species, providing a second mechanism for protection of plants against disease by siderophore-producing PGPR[7,8,9]. Recently, the siderophore pseudobactin was found to be an important determinant of ISR against blast disease in rice [10].

In view of this, we have isolated several strains of rhizobacteria from rice rhizosphere and evaluated

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their Antifungal ability against *P. oryzae* by *in vitro* technique. Furthermore, for a better characterization of the selected antagonistic isolate, its potential production of hydrolytic enzymes and secondary metabolites were studied.

2. MATERIALS AND METHODS

Isolation of Pathogenic fungi *M.grisea*

Pyricularia grisea was isolated in the laboratory from infected rice leaves [11]. Fungal cultures were grown and maintained on YEG medium (glucose 10 g l^{-1}, yeast extract 2 g l^{-1}) or oat meal agar. Fungal conidia were harvested by scraping the biomass grown on oat meal agar plates with a sterile surgical blade, resuspended in sterile water and purified by passing through glass wool column. Then the grown culture was overlaid in several sterilized filter paper sections on PDA plates at 26°C. After 14 days the filter paper sections transferred from agar surface to sterile envelope, dry for 3 days at room temperature and stored at -20°C as described by Valent *et al.*[12].

Isolation of Rhizobacteria from Rhizosphere soil

One gram of rice rhizosphere soil sample was suspended in 99 ml of sterile distilled water. Samples were serially diluted and 0.1 ml of sample was spreaded on nutrient agar plates and incubated at 37°C for 24 hrs. All isolates were purified twice and then stored at -80°C in Nutrient broth containing 15% glycerol for further use.

Screening of Antifungal Activity of isolated rhizobacteria by Dual culture method

The isolated rhizobacterial strains were screened for their *in-vitro* antagonistic ability against rice blast pathogen, *P.oryzae* by dual culture technique [13]. Bacterial isolates was streaked at one side of petridish (1 cm away from the edge) containing PDA. Five mm mycelial plug from seven-day-old PDA cultures of rice pathogen *P.oryzae* was placed at the opposite side of petridishes perpendicular to the bacterial streak. Petri dishes were then incubated at 28±2ºC for 5 days. Petri dishes inoculated with fungal discs alone were served as control. There were three replications for each isolate against pathogen. Observations on width of inhibition zone and mycelial growth of test pathogens were recorded and percent inhibition of pathogen growth was calculated.

I =100(C-T)/C

where, **I**= percentage inhibition of mycelial growth, **C**= growth of pathogen in the control plate (mm) and **T**= growth of pathogen in dual cultures (mm). The most effective strain that showed the highest antifungal activity in dual culture was selected and tested for further studies.

Morphological, cultural and biochemical characterization

The selected effective bacteria was confirmed by morphological (staining and motility), cultural (Nutrient agar, King's B agar), biochemical tests (IMViC test, oxidase test, catalase test, triple sugar iron test, nitrate reduction test, gelatin liquefaction test and starch hydrolysis test). The chosen strain was examined for its colony morphology, cell shape and Gram reaction as per the standard procedures[14,15]. The biochemical characterization was done as per the procedures outlined by Cappucino and Sharman[16].

Physiological characterization

P-solublization and IAA production

P-solublization was assessed on Pikovskaya's medium containing tricalcium phosphate[17] and Indole acetic acid production (IAA) production by the selected strain was estimated as described by Hameed *et al.*[18].

Siderophore and Hydrogen cyanide Production

Siderophore production was detected using the universal chrome azurol 'S' (CAS) assay[19]. CAS plate was streaked with overnight grown culture and incubated at 28+2°C for 72 hrs [20]. Production of HCN was determined by modified method of Millar and Higgins[21]. Bacterial culture (48 hrs) was streaked on LB amended with glycine (4.4 g L^{-1}) and a filter paper stoked in 0.5% (w/v) picric acid in 1% Na_2CO_3 placed in the upper lid of the petriplate. After incubation at 28±2°C, changes in colour was examined.

Protease and chitinase production

Bacterial isolate was checked for production of proteases by growing them on skimmed milk agar (SKM) as described by Kazempour[22]. An ability to clear the skimmed milk suspension in the agar was taken as the evidence of the secretion of proteases. To assay the production of chitinases, the bacteria were grown on medium containing chitin as the sole carbon source [23].

Culture identified

The strain RB04 was identified as *Pseudomonas fluorescens* (AUPF25) and the nucleotide sequence (16S rRNA) of this isolate was deposited to Gen Bank under the accession number JQ638587.

3. RESULTS

In the present study, soil samples were plated on nutrient agar. From soil samples, 10 rhizobacterial strains were isolated and designated as RB01 to RB10. The antifungal activity of these

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rhizobacterial strains were screened against plant fungal pathogen *P.oryzae*. Bacterial strain RB04 remained antagonistic at a level of 100% even after 40 days of assay. No physical contact or an inhibitory halo was observed between some of the bacteria tested and *P.oryzae*. With the bacterial strain RB04 production of spores around the fungus causing complete fungal lysis was observed. The experiment was done in triplicate and inhibition zones (expressed in mm) were measured as observed in Dual culture assay method. The data are summarized in (**Table 1**). Among these ten strains, one potential bacterial strain RB04 was selected based on showing highest Antifungal activity. The selected strain identified as *Pseudomonas fluorescens* based on morphological, cultural and biochemical characteristics. The results are shown in (**Table 2**). The strain RB04 exhibited positive for the production of siderophore, P-solubilization, protease and chitinase production. The data are summarized in Table 2. These results showed that these productions by *Pseudomonas fluorescens* play a critical role in Antifungal activity. The phylogenetic tree of the selected isolate RB04 identified as *Pseudomonas fluorescens* (AUPF25) is shown in (**Figure 1**).

4. DISCUSSION

A variety of bio antagonistic bacteria, including members of the genera *Azospirillum, Azotobacter, Pseudomonas, Bacillus* and *Enterobacter*, are known to colonize the rhizosphere of most of the cereals and act as biocontrol agents[24]. In the present study, ten rhizobacterial strains were isolated from rhizosphere soil and tested for its Antifungal activity. The strain RB04 was selected from this study seemed to be highly potential in controlling plant pathogen *P. oryzae* and it was identified as *Pseudomonas fluorescens.*

Rice disease suppression by biocontrol agents is governed by a multitude of factors. The influence of these factors varies with the type of biocontrol agent, plant cultivar and the nature of the pathogen targeted for control[25]. There are different modes of action of biocontrol bacteria, such as inhibition of the pathogen by antimicrobial compounds, competition for iron through production of siderophores[6], parasitism that may involve production of extracellular enzymes, (for example, chitinases and proteases that can lyse pathogen cell walls), and induction of plant resistance mechanisms[26].

Among the strain tested, *Pseudomonas fluorescens* (RB04) show direct bioantagonistic activity against the rice blast fungus *P. oryzae*, which correlates with its ability to produce siderophores and protease. Several of the strains also produce hydrolytic enzymes, which are also likely to play a role in direct antagonism[27,28]. In rice, several bacterial strains have previously been identified that improve rice blast resistance[10,29,30,31]. The results obtained here pointed out the possible use of *Pseudomonas fluorescens* strain as the biocontrol agent of rice blast. However, further research is needed in this strain to control rice blast in the field experiment and to elucidate the mechanism of action of this strain in detail.

Table 1: The effects of rhizosphere isolates on *M. grisea* fungal growth *in vitro* dual culture assay.

	Pyricularia oryzae	
Strain	**Mycelial growth**	**Inhibition over control**
RB01	60	14
RB02	32	54
RB03	56	20
RB04	12	82
RB05	52	25
RB06	39	44
RB07	40	42
RB08	25	64
RB09	46	34
RB10	58	17
Control	70	-

Table 2: Morphological, cultural, biochemical and physical characteristics of *Pseudomonas fluorescens*

Microbial isolate	Variables	Characteristics
Colony and Cell Morphology	Colony size	Large
	Surface	Irregular
	Opacity	Opaque
	Color	Yellow green
	Motility	Motile
	Cell shape	Rod
	Cell size	Small
	Gram's staining	Gram negative
Biochemical characteristics	Citrate	Positive
	Indole	Negative
	MR	Negative
	VP	Negative
	Oxidase	Positive
	Catalase	Positive
	TSI	Positive
	Nitrate reduction	Positive
	Gelatin liquefaction	Positive
	Starch hydrolysis	Negative
Physiological Charactertistics	P-solubilization	Positive
	IAA production	Positive
	Siderophore production	Positive
	HCN production	Negative
	Protease production	Positive
	Chitinase production	Negative

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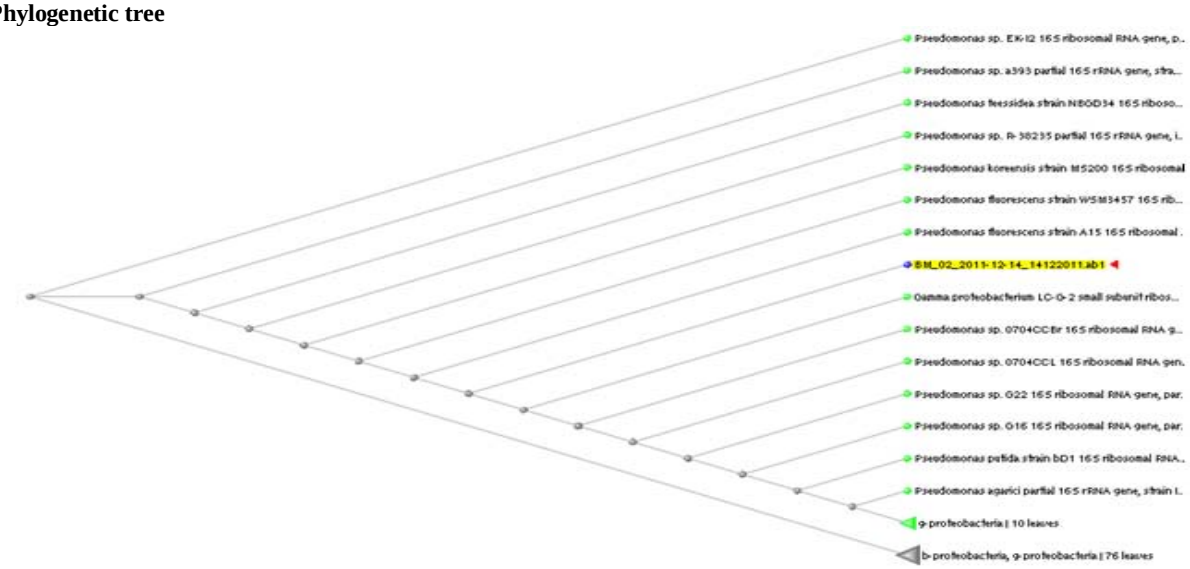
Fig 1: Phylogenetic tree



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