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Research Article

ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY OF RHIZOBACTERIAL MICROFLORA

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Abstract

Rhizosheric microorganisms like bacteria and fungi are renowned entities in enhancing the growth of crop plants and to inhibit other disquieting soil micro-organisms. Current investigations were undertaken to explore such types of rhizobacteria from rice soil. Inhibitory effect of these bacteria was checked against *Fusarium oxysporum* f.sp. *cepae* and *Agrobacterium tumifacians*. Bacterial cultures were isolated by serial dilution, antifungal test was performed by dual culture assay and antibacterial activity was checked by well diffusion method. Research outcomes unveiled that all tested rhizobacterial isolates exerted antagonistic effect against both the tested pathogens. They produced 0.8% to 1.68% inhibitory zone against fungus and 0 to 40 % against bacteria. It can be therefore, assumed that these antagonistic bacterial strains may be employed as biocontrol inoculants against fungal and bacterial diseases of agricultural crops.

Keywords: Rhizobacteria, antagonistic effect, fungi and bacteria.

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1. INTRODUCTION

Bacteria habituating rhizosphere of crop plants are called as rhizobacteria. These bacteria have capability to inhabit the crop roots firming beneficial relationships with plant roots and root hairs (Kennedy, 2005). They multiply, square root their numbers and adhere the roots and rootlets of plants for their nutritional requirements. Beneficial association involves adhering or sticking with rootlets in a feisty soil atmosphere and exercise a useful influence upon the crops (Kloepper and Schroth 1978, Lazarovits and Nowak 1997, Kloepper et al. 1989). Lautenberg and Loemberg, 2001 bifurcated rhizobacteria in to four categories; i) biofertilizer representative (able the plants to take up maximum nutrients). ii) phytostimulators (by releasing phyto-hormones iii) Rhizo-remediator (by changing complex compounds into simplest forms) and iv) biopesticides (controlling diseases by producing antifungal, antibacterial and nematicidal compounds). In current decades bacterial formulations are being used frequently in farmer fields for crop disease management. Moreover, in research findings by Farzana et al., (2009), and Munase and Mulugeta (2014), it has also been confirmed that rhizobacteria can increase the yield of many crop plants like potato sugar beet, reddish and sweet potato effectively. These beneficial bacterial genera include *Flavobacterium Azospirillum*, *Burkholderia*, *Bacillus Alcaligenes*, *Pseudomonas*, *Enterobacter*, *Serratia*, *Acinetobacter*, *Erwinia*, *Arthrobacter*, and *Rhizobium* (Tilak et al., 2005 and Egamberdiyeva, 2005). Due to all

these prospectives these can be applied as an alternative of many pesticides, fertilizers and other supplemental material (Ashrafuzzamanet al., 2009).

As it is evident that plant pathogens can create pandemic conditions at any time with subject to prevalence of necessary disease conditions i.e. wheather, tempreture and humidity and weak host. Which can make a great panic in farming community and agriculture scientists. It has also been reported that about 11 to 20 % yield losses are caused by plant pathogens which can be projected up to 100% (James, 1981 and Serge et al., 2012).

Under these situations It is duty of research scientists to formulate a sound and efficient control measures. Yet use of pesticides is very effective, but their indiscriminate application is considered harmful due to environmental pollution and health hazards, Therefore, alternative to pesticides are use of rhizobacterial inoculants for managing crop diseases. This exercise has been well known as a more safe and responsive practice, (Loon and Bakker, 2003). As the mode of action of biocontrol agents is concerned. They secrete some metabolites i.e. antibiotics, lytic enzymes, hydrogen cyanides etc. which can kill, inhibit or check other pathogenic microorganisms directly and by their competitive ability (Van Loon and Bakker, 2003). These secretions are also safe. These all potentialities of rhizobacteria make them strong biocontrol agents for reducing damages by other phytopathogens. Hence, present research investigations were planned to find such types of rhizobacteria from rice soils, which could have ability to antagonize *Fusarium oxysporum f.sp.alium cepae* and *Agrobacterium tumifacians*. Thus these studies will be helpful for the protection of field crop against diverse fungal and bacterial pathogens.

2. MATERIALS AND METHODS

2.1. Soil sampling, isolation and purification of rhizobacteria:

Soil sampling was done from rice experimental area of Nuclear Institute of Agriculture, (NIA) Tandojam for isolation, purification and characterization of rhizobacteria. Isolation of rhizo-bacteria was made by serial dilution. 200 grams of soil was collected. From this soil, one gram was taken in a conical flask to make soil mixture. Then this solution was vortexed and diluted up to 10^{-8} by serial dilutions. From each dilution 0.1ml was taken and poured on Nutrient Agar (N.A) plates. Then these plates were kept at $28 \pm 3\ ^{0}C$ for 72 hours in an incubator. Selected bacterial colonies were purified by streaking method.

2.2. Characterization of bacterial isolates

Purified single colonies as well as bacterial cells were morphologically characterized on the basis of size, orientation, shape and color. The bacterial cells were also characterized on the basis of their reactions. i.e. negative or positive by gram staining.

2.2.1. Gram staining:

Bacteria were grown in nutrient broth for 24-48 hours. Bacterial smear was made from 1-2 drops of broth on microscopic slide and was heat fixed. Then a solution of reagents was prepared. First of all, 1-2 drops of crystal violet were poured on the fixed smear for 1 minute and then washed with autoclaved distilled water. Then gram's iodine was applied also for 1 minute and then drained by 95% ethonal. The last step was pouring of safranine for thirty (30) seconds and again washing with autoclaved/sterilized water. Then smear was allowed to be dry and viewed using compound microscope with immersion oil. The gram-negative bacteria were seen pink to red while gram positive bacterial cells were found violet.

2.3. Antagonistic test of Rhizobacterial strains

2.3.1. Antagonistic effect of rhizobacterial strains against *Fusarium oxysporum f. sp. cepa*

Antagonistic effect of bacterial strains against *Fusarium oysporum f. sp. cepae* was checked by the procedure as illustrated

by Gupta *et al*., 2001. Fresh cultures of both the test organisms were prepared. Agar piece of 5mm of freshly prepared fungal culture was cut and placed on already prepared PDA (Starch 20gm, dextrose 20gm and Agar Agar 20gm/liter of water) plates. Then on either side of fungal bit, test bacterial cultures were streaked. PDA plates having fungal bit without bacterial streaking were reserved for control treatment. Each treatment was replicated thrice. Test petridishes were placed at 28^0 C for 5 days. Inhibition zone formed by bacterial strains in response to test fungus and control treatment was calculated by the formula as given by Vincent (1947) as follows:

$$I = \frac{(C-T) \times 100}{C}$$

Where,

I= Inhibition percentage of fungal mycelia

C= Mycellial growth in NA plates (served as the control)

T= Mycellial growth in the treatment.

2.3.2. **Antibacterial activity against *Agrobacterium tumifacians***

2.3.2.1. Preparation of cell free supernatant

2% N.A broth was prepared in 4 ml test tubes and inoculated with 48 hours old bacterial culture. This broth was incubated at 28 ^{0}c for 2 days. Broth with reasonable consistency was put in 2ml eppendorf tubes. Then it was centrifuged at 5000 rpm for 15-20 minutes at room temperature. Pellets were discarded and supernatants of each bacterium were separated in separate tubes for use in antibacterial assay.

2.3.2.2. Antagonistic activity

Antagonistic activity of bacterial strains against *Agrobacterium tumefacians* was checked by agar well diffusion method as described by Okeke *et al*., (2001). 24 hours old bacterial culture was used in this protocol. Bacterial strains were inoculated in 24 hr old nutrient broth and were swabbed on media plates amended with nutrient agar. When bacterial growth entirely covers the plates then holes were made on them with the help of 6 mm autoclaved cork borer. Then these holes were filled with 100µl of cell free supernatant in triplicate. Plates were kept in incubator at 37°C for 24 hours. Nutrient broth was considered as negative control treatment. Antibiotic penicillin was used as positive control. After 24h, inhibitory zone was checked in plates and were compared with inhibition zone of positive and negative control.

The relative percentage inhibition of the cell free supernatant was determined by the following formula:

$$\text{Relative \% inhibition of the bacterial isolates} = \frac{(X-Y)}{(Z-Y)} \times 100$$

Where,

X: Total inhibitory zone of cell free supernatant of bacteria,

Y: Total inhibitory zone NB broth,

Z: Total inhibitory zone of penicillin.

2.4. Statistical Analysis

The data generated by these studies was subjected to analysis of variance (ANOVA) for a Completely Randomized Design and the means were compared using post-hoc Tukey's HSD test with $P < 0.05$ being accepted as significance.

3. RESULTS

3.1. Isolation, purification and characterization of rizobacterial isolates.

Isolated bacterial strains were morphologically characterized on the basis of cell morphology, motility and gram staining by light microscopy. The results indicated that they belong to genera *Enterococci, Xyllella, Pseudomonas, Streptococcus* and *Micrococcus* as shown in Table-1

3.2. Antagonistic response between rhizobacteria and fungus (*Fusarium oxysporum f. sp. Cepae)*

Inhibitory effect of rhizobacterial isolates appeared in different levels. All 6 strains significantly retarded fungal growth. Percent inhibition from 0.82 to 1.68% was recorded. Isolate BRS10, BRS11 and BRS12 were observed potential

bioantagonists in vitro and exhibited inhibition of *Fusarium oxysporum. f. sp. cepae* up to 1.65 to 1.68%. The greatest inhibition zone effect was produced by BR12 with 1.68% inhibition and the lowest 0.82 % by BRS 08. The plates served as control were found completely covered by fungal mycelia showing no growth inhibition. Mean mycellial inhibition of the efficient rhizobacterial strain showed that growth inhibition was highly significant at ($p < 0.05$) as presented in (Table-2).

3.3. Antagonistic test between rhizobacterial isolates and *Agrobacterium tumefacians*

All 6 rhizobacterial strains significantly inhibited growth of *Agrobacterium tumefacians* where inhibitory zone varied from 0 to 40 %. Isolate BRS9, BRS10 were found most effective in in-vitro studies and exhibited inhibitory effect against *Agrobacterium tumefactions.* The highest inhibitory effect was recorded in BRS9 with 40% inhibition and the lowest 0% inhibition by BRS 8 and BRS 12. The plates reserved for control treatment were found completely covered by bacterial growth. Mean mycelial retardation of the efficient rhizobacterial strain showed that growth inhibition was highly significant at ($p < 0.05$) as presented in (Table-3).

4. DISCUSSIONS

Rhizobacteria constitute important agents for bio- control of soil-borne disease and for plant growth pro-motion (Rajkumar *et al.*, 2005). In this study 6 rhizobacterial strains were characterized, morphologically identified and checked for their antifungal and antibacterial activities. Almost all the strains showed their antagonistic potential against. *Fusarium oxysporum. f. sp. cepae* and *Agrobacterium*. BR-12 tentatively identified as *Micrococcus* found most effective against fungus inhibiting mycellial growth up to 1.68% as compared to control and BR-09 tentatively identified as *Pseudomonas* showed 40% inhibition against *Agrobacterium tumefactions*. Our results coincides with the findings of Anamika et al 2021 and Rhuoma et al 2008. In the antagonistic assays of Anamanika, 2021 *Micrococuss* dislayed 98% inhibition against *Fusarium oxysorum,* whereas in the finding of Rhouma, 2008, *Pseudomonas* showed best antagonistic potential against *Agrobacterium tumefactions*. Our results are also in accordance with the findings of Adhakari and Manug, 2010, Amarison 2015, Sea 2016, Chung 2016, Hend 2016 and Tariq 2019.

Several modes of action of rhizobacteria are known with which these bacteria inhibit or retard the growth of other phytopathogens (Blanco *et al.,* 2004; Ran *et al.,* 2005). Siderophore is a common metabolite that is released by *Pseudomonas* and *Micrococcus* biocontrol strains, which has ability to inhibit the multiplication and reproduction of pathogenic microflora and onset & development of plant diseases (Duiijff, 1993). Moreover, surfactant rhamnolipid produced by *Pseudomonas aeruginosa* has good fungus-inhibiting activity and it could be used in the petroleum and pharmaceutical industries also.

The application prospect of *Pseudomonads* is precious in the agriculture, medicine, and cosmetics industries. *Pseudomonas aeruginosa* K2187 can retard the mycelial growth of 36 fungal pathogens. Analysis of its components revealed that it can produce chitinase and lysozyme (Wang et al 1999). Studies have also shown that *Pseudomonas aeruginosa* can produce many types of enzymes, such as proteases, dehydrogenases, and lipases to which it uses for biocontrol activity against other pathogens (Peng et al 2010).

Anamika Dubey 2021 highlights his finding as: After testing bio antagonistic potential of various biocontrol bacterial strains, only *Micrococcus luteus* strain AKAD 3-5 exhibited 98% inhibition against mycelia growth of *Fusarium oxysporum* (ITCC 2389) in dual plate culture assay. It was found that it produces cell wall degrading enzymes (chitinase and cellulase) and HCN. Related findings were noted by other research scientists also. Patel *et al.* 2021,

Table-1. Morphological characteristics of antagonistic bacteria

Cell Morphology			Gram reaction	Tentative Identification
Strain No.	**Shape**	**Motile Y/N**		
BRS 7	Cocci	N	+	*Enterococci*
BRS 8	Rod	N	-	*Xyllella*
BRS 9	Cocci	N	-	*Pseudomonas*
BRS 10	Cocci	N	+	*Streptococci*
BRS 11	Cocci	N	-	*Enterococcus*
BRS 12	Cocci	N	+	*Micrococus*

Table-2. Antagonistic activity of rhizobacterial isolates against *Fussarium oxysporum*

Strain No.	*Fussarium oxysporum*	
	Mycelial growth (mm)	**Inhibition over control (%)**
BRS 7	40.34 ± 0.34^{A}	1.10 ± 0.28^{F}
BRS 8	40.00 ± 0.00^{A}	0.82 ± 0.00^{F}
BRS 9	40.34 ± 0.34^{A}	1.10 ± 0.28^{F}
BRS 10	39.67 ± 0.34^{A}	1.65 ± 0.83^{F}
BRS 11	39.67 ± 0.34^{A}	1.65 ± 0.83^{F}
BRS 12	39.67 ± 0.34^{A}	1.68 ± 0.83^{F}

Means sharing similar letters in a column are not significantly different at P< 0.05

Table-3. Antagonistic activity of bacterial isolates against *Agrobacterium tumifacians*

Strain No.	*Agrobacterium tumifacians*	
	Bacterial Zone (mm)	Inhibition over control (%)
BRS 7	1.0 ± 0.00^{ab}	20.00 ± 0.00^{ab}
BRS 8	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
BRS 9	2.0 ± 0.00^{a}	40.00 ± 11.55^{a}
BRS 10	1.67 ± 0.34^{a}	33.34 ± 6.67^{a}
BRS 11	1.34 ± 0.34^{ab}	26.67 ± 6.67^{ab}
BRS 12	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}

Means sharing similar letters in a column are not significantly different at P< 0.05

reported plant-growth-promotion and antagonistic potential of marine bacteria *M. luteus.* and tested their efficacy on vegetative parameters. The results from this investigation revealed that *M. luteus* can protect crops from Fusarium wilts as it has strong antagonistic potential against fungal pathogens and assumed as strong plant growth-promoting bacterial strain to enhance overall plant growth in crops. Most of the studies conducted by different scientists have shown the positive plant-growth-promoting (Patel *et al.* 2021 Matsuura, 2013) desiccation-tolerant

(Ramegowda) 2015 and biocontrol properties of *M. luteus*.

So, studies on detailed characterization and molecular identification of our rhizobacterial strains along with their application in greenhouse and field is needed in 2nd phase of experiment, if their performance will be found satisfactory then can be exploited as effective biocontrol agents to control any plant pathogenic creature.

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