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Isolation and Characterization of Anti Phytopathogenic Bacteria from Finger Millet Rhizosphere

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ABSTRACT

Phytopathogenic attack is a major constraint in achieving potential yield in finger millet. Rhizobacteria present an eco-friendly and cost-effective alternatives to chemical pesticides. In order to study bacterial diversity having antagonistic potential, a total of 45 rhizobacterial isolates were isolated from 18 soil samples collected from finger millet rhizosphere. Screening for antagonism against phytopathogens revealed that 15 isolates inhibited the mycelial proliferation of Pyricularia grisae and Rhizoctonia solani in the range of 22.22-44.44% and 16.66-33.33% respectively, under dual plate assay. Cultural and morphological characterization of isolates revealed great diversity among them. Most of the isolates appeared as white, creamish white to cream colonies on nutrient agar. Isolate A-4 exhibited characteristic yellow-green pigmentation tentatively indicating its close relation with Pseudomonas sp. Biochemical characterization revealed that all were catalase producers, 13 hydrolyzed starch and showed positive result for citrate utilization. Hydrogen sulphide production was observed with only two isolate while 4 tested positive for TSIA test. Inoculating broth supplemented with different sugars revealed that glucose was utilized by 5 isolates, starch by 4, maltose by 2 and mannitol by 1 while none utilized lactose.

Keywords: *Rhizobacteria, Antagonism, P. grisea, R.solani*

INTRODUCTION

Finger millet (*Eleusine coracana* (L.) Gaertn.) is one of the most important cereal crops sustaining lives of millions of people across the world predominantly in the developing countries[4]. The high nutritional value and the ability to tolerate harsh environmental conditions including high temperature, moisture stress and water stagnation has made this crop an integral part of farmers risk avoidance strategies as well as various health foods[10,14]. However, the crop is mostly traditionally grown in marginal soil conditions and valued as low input crop where it suffers great yield loss. Moreover, the finger millet production is constrained by many abiotic and biotic factors. The biotic factors that seriously compromise the final yields in finger millet include blast, banded blight, smut, rust, foot rot and viral disease.

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Amid these, finger millet blast caused by *Pyricularia grisea* Sacc. (perfect stage = *Magnaporthe grisea* [Hebert]). Barr is considered the most devastating biotic factor resulting in reduction of physiological maturity, biomass and yield of the crop[8]. The pathogen attacks all aerial parts of finger millet plant causing leaf, neck and finger blast and often resulting in >50% yield losses[5]. Besides this, another emerging malady in successful cultivation of finger millet is banded blight incited by *Rhizoctonia solani* (Kuhn.) (Basidial stage: *Thanatephorus cucumeris* (Fr.) Donk). The disease is characterized by oval to irregular light grey to dark brown lesions on the lower leaf sheath. Although chemical control measures for plant diseases have become an integral part of agricultural system, the injudicious use of fungicides is hazardous to human health and ecosystem and not a viable option in organic cultivation systems or for resource-poor farmers. There is therefore an urgent need to identify cost-effective, eco-friendly and farmer friendly practices for management of diseases. Use of soil inhabiting bacterial antagonists as biological control measures presents a viable option to synthetic chemicals. This investigation aimed at exploring the bacterial diversity from finger millet rhizosphere exhibiting antagonism against phytopathogens.

METHODS AND MATERIALS

Fungal pathogens: Pathogens causing blast and banded sheath blight in finger millet i.e. *Pyricularia grisea* causing blast and *Rhizoctonia solani* respectively were procured from our previous study where they were isolated from diseased plant samples. They were maintained on potato dextrose agar slants at 4°C.

Isolation of rhizobacteria

Soil samples were collected from the rhizospheric soil of finger millet grown in different locations of bastar plateau of Chhattisgarh. The rhizosphere samples were collected carefully uprooting the plants using shovel, and the soil loosely attached to the root was collected as rhizosphere soil. The samples were then placed into pre-labeled translucent ziplock bags and maintained at ambient temperature. This collected soil was serially diluted in sterile water and spread on nutrient agar (Beef extract: 3g/l, peptone: 5g/l, NaCl: 5g/l, Agar: 20g/l, pH: 7.0) in petri plates (9 cm dia). The plates were incubated at 30°C for 24 h. The isolated colonies differing in colony morphology were selected and purified by sub-culturing and preserved on nutrient agar slants at 4 to 5°C.

Screening for antagonistic rhizobacteria

Antagonistic activity of the bacterial isolates against *Pyricularia grisae* and *Rhizoctonia solani* was evaluated using dual plate technique. A 5-mm piece of test fungus from a 7 day old culture was placed at the centre of a petri plate containing PDA (Potato infusion: 250g/l, dextrose: 10g/l, Agar: 20g/l, pH: 6.5) and nutrient agar in 1:1 ratio. The rhizobacterial isolates were inoculated at opposite sides and plates were incubated for 96 h at 28ºC. A control plate with fungus alone served as control. Radial growth of the test fungus was measured and percentage growth inhibition was calculated as under:

$$\% \text{ Inhibition} = (R - r)/R \times 100$$

Where, r: Radial growth of fungus in dual plate and R: Radial growth of fungus in control plate.

Characterization of antagonistic rhizobacteria

Cultural Characterization

Initial characterization of all the isolates was done on the basis of colony morphology like colour, pigment, shape, size, diameter, margin, elevation and texture and cell morphology (gram's reaction) as per Bergey's manual of Determinative bacteriology[7].

Biochemical characterization

Catalase production

Catalase production was assessed by direct plate method. A drop of 3% H_2O_2 was taken on to petri plate containing bacterial growth. Rapid and sustained production of gas bubbles or effervescence constituted positive test[2].

Hydrolysis of starch

Sterilized starch agar medium was poured onto petriplates. The log phase cultures were spotted on the plates and incubated at 28°C for 48 hrs. After full growth of cultures, the

petriplates were flooded with Gram's iodine. The hydrolysis of starch was observed as a colorless zone surrounding the colonies against purple background. A blue or purple zone indicated that starch was not hydrolyzed[2].

Methyl red (MR) test

Sterilized glucose –phosphate broth tubes were inoculated with the test culture and inoculated with the test culture and incubated at 28°C for 48 hrs. After incubation five drops of methyl red indicator was added to each tube and gently shaken. Red color production was taken as positive and yellow color production was taken as negative for the test.

Citrate utilization

Isolates were spot inoculated on Simmon's citrate agar plates and incubated at 28°C for 48 hrs. Change in color from green to blue or growth on medium indicated the positive reaction for citrate utilization.

Hydrogen sulphide production

SIM agar medium tubes were stab inoculated by test isolates and incubated fat 28°C for 48-72 hrs[3]. Tubes were observed for the presence or absence of black coloration along the line of inoculation indicating hydrogen sulphide production.

Triple Sugar Iron agar (TSIA) test

Isolates were streaked on TSI agar slants and incubated at 28 ± 2°C for 24-48 h. Change in color of the red slants to yellow indicated positive reaction for the test. Slants were further observed for any change in yellow color of butt and slant and formation of black coloration and gas production.

Carbohydrate utilization test

Screening of pure bacterial isolates for their carbohydrate fermentation abilities was done using different carbohydrates (glucose, lactose, mannitol, maltose and sucrose) in peptone broth medium containing phenol red indicator. Change in color of the broth to yellow from red due to acid production was considered affirmative result for carbohydrate fermentation.

RESULTS AND DISCUSSION

Although finger millet is a hardy crop and can sustain harsh environmental situations, attack by biotic agents is main hurdle in achieving its potential yield. Chemical pesticides no doubt have attained a permanent feature in agriculture, their harmful impact on soil micro and macro-flora and demand for chemical free food necessitates an eco-friendly approach to manage them. In this perspective, this investigation envisaged at exploring the soil micro-flora exhibiting anatgonism against important pathogens so that they can be exploited as potential biocontrol agents. A total of 18 soil samples were collected from different finger millet growing rhizosphere. From these, 45 rhizobacterial isolates showing rapid growth on Nutrient agar medium were isolated and subjected to preliminary screening for antagonism against pathogens, *Pyricularia grisea* and *Rhizoctonia solani*. The *in vitro* dual plate based assay carried out to test inhibitory action of 45 bacterial isolates revealed that bacterial antagonists were the natural resident of chickpea rhizosphere. However, this inhibitory trait was exhibited only by 15 isolates that too in the varying range of 22.22-44.44% against *Pyricularia grisea* and 16.66-33.33% against *Rhizoctonia solani* (Table 1). The bacteria exhibited antagonistic effect on fungal growth was clearly visible by formation of an inhibitory zone preventing the radial proliferation of the pathogens. Antagonist A-9 exhibited maximum growth inhibition (44.44%) against *Pyricularia grisea* followed by A-11 (38.89%) and A-10 (37.77%) while A-6 and A-2 showed highest inhibition (33.33%) against *Rhizoctonia solani* followed by A-7 (31.11%). In a similar report Kumari et al[9] reported that 59 of 174 bacterial isolates from chickpea rhizosphere inhibited the growth of *Rhizoctonia bataticola* causing dry root rot of chickpea. Further corroborating the present finding, Getachew et al[6] reported the antagonistic effects of *P. fluorescens* against *P. grisea* isolates in the range of 48.6-57.2%. The inhibition of mycelial growth of pathogens by antagonistic bacteria has been reported due to production of different secondary metabolites such as siderophores, diffusible metabolites, antibiotics and biocidal volatiles including HCN and ammonia[9].

Characterization of antagonistic rhizobacteria

Soil is a rich depository of microorganisms and screening them for their antagonistic behavior further revealed that there occurs a diverse group of antagonists as natural inhabitant. Our further study envisaged at studying the diversity among these antagonistic bacteria which have immense potential to emerge as biocontrol agent. The antagonists were characterized based on cultural, morphological and biochemical characteristics. Most of the isolates appeared cream, creamish white to white colonies although they differed widely in their colony morphology. The cultural and morphological characteristics of the bacterial antagonists are enlisted in table 2. Isolate A-4 exhibited production of yellowish green pigment which is the characteristic of fluorescent pseudomonds. Likewise, potential of pseudomonads as biocontrol agent against *P. grisea* is well documented[6]. Moreover, gram negative reaction and rod shape further confirmed the identity of bacterial isolate as *Pseudomonas fluorescens*. Nonetheless, the presence of both gram positive and gram negative bacteria was observed. The gram positive bacilli have also been reported to be the biocontrol agents. In their study, Shrestha et al[11] reported that out of 127 rice associated bacteria, 29 exhibited antagonistic behavior against *R. solani* and *Burkholderia glumae.* They further reported the identity of these bacteria by 16S rDNA sequence and found that they were the closest relative of *B. amyloliquefaciens*, *B. methylotrophicus* and *B. subtilis*.

Biochemical characterization of bacterial isolates further revealed diversity among soil inhabiting antagonists. All the tested antagonists were found to be catalase producers while most of them hydrolyzed starch and showed positive result for citrate utilization. When investigated for their ability to produce hydrogen sulphide, only 2 isolate including A-4 (exhibited characteristic yellow green pigmentation of pseudonomads) exhibited positive reaction as indicated by formation of black coloration near the region where cultures were stab inoculated. In their study, Suman et al[13] reported that of 30 *Pseudomonas fluorescence* strains isolated from rice rhizosphere soils of Rangareddy district in Telangana state, 26 confirmed their ability to produce hydrogen sulphide. The carbohydrate utilization test performed by supplementing media with glucose, lactose, maltose, mannitol and maltose sugars revealed that glucose was utilized by 5 isolates, starch by 4, maltose by 2 and mannitol by 1 while none utilized lactose as indicated by change in colour of phenol red indicator to yellow which showed acid production during utilization of sugars. The diversity in sugar utilization pattern indicated the diversity among bacterial antagonist. Fermentation of sugars such as glucose and maltose by gram positive rods could be considered to be the characteristics of bacilli. The affirmative result for TSI test was recorded with isolates A-3, A-4, A-5 and A-9. The fermentation of sugars by the isolate converted the red color of the slant into yellow within 24 h of incubation; however, the yellow color of the slanted portion reverted back to red under aerobic condition while the butt region remain yellow because of anaerobic condition. Moreover, formation of gas was also observed with isolate A-5 as trapped bubbles in the butt region. The observations recorded indicated that it must be the representative of enterobacteriacae[1]. In case of isolate A-3 and A-4, hydrogen sulphide production by reduction of ferrous sulphate of the medium to ferric sulphide was manifested by formation of black precipitate. Furthermore, alkaline butt and slanted region observed with isolate A-4 confirmed its identity as *Pseudomonas*[1]. Bacterial genera including *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia, Bacillus, Paenibacillus* and some members of enterobacteriaceae have been reported to be plant growth promoting rhizobacteria that promote plant growth by either acting as growth stimulators or by preventing attack by detrimental pathogens[12].

Table 1: Antagonistic potential of rhizobacterial isolates against phytopathogens of finger millet

Isolates	*Pyricularia grisea*		*Rhizoctonia solani*	
	Radial growth (cm)*	% inhibition	Radial growth (cm)*	% inhibition
A-1	6.0 ± 0.115	33.33	6.6 ± 0.173	26.67
A-2	5.9 ± 0.173	34.44	6.0 ± 0.115	33.33
A-3	6.3 ± 0.173	30.00	6.5 ± 0.057	27.78
A-4	5.7 ± 0.057	36.67	6.9 ± 0.115	23.33
A-5	6.5 ± 0.115	27.78	7.5 ± 0.11	16.66
A-6	6.0 ± 0.115	33.33	6.0 ± 0.173	33.33
A-7	6.0 ± 0.173	33.33	6.2 ± 0.173	31.11
A-8	6.2 ±0.173	31.11	6.7 ± 0.115	25.55
A-9	5.0 ± 0.115	44.44	6.3 ± 0.057	30.00
A-10	5.6 ± 0.173	37.77	6.7 ± 0.115	25.55
A-11	5.5 ± 0.115	38.89	6.5 ± 0.115	27.78
A-12	5.7 ± 0.173	36.67	6.9 ± 0.057	23.33
A-13	6.3 ± 0.057	30.00	6.3 ± 0.173	30.00
A-15	7.0 ± 0.115	22.22	6.5 ± 0.57	27.78
A-16	6.7 ± 0.057	25.55	7.0 ± 0.173	22.22
Control	9.0 ± 0.00	-	9.0 ± 0.00	-

*Values represent mean ± S.D. of three replications

Table 2: Cultural and morphological characteristics of bacterial antagonists

Isolate	Margin	Texture	Elevation	Consistency	Optical feature	Colony Color	Pigmentation	Gram's reaction	Cell shape	Cell arrangement
A-1	Undulate	Rough	Umborate	Butyrous	Opaque	White	-	Negative	Cocci	Streptococcus
A-2	Undulate	Rough	Raised	Butyrous	Opaque	White	-	Positive	Bacilli	Single
A-3	Undulate	Smooth	Flat	Butyrous	Opaque	Creamish orange	-	Negative	Bacilli	Streptobacilli
A-4	Entire	Smooth	Convex	Butyrous	Transluscent	Glistening	Green	Negative	Bacilli	Single
A-5	Curled	Smooth	Crateriform	Butyrous	Transluscent	Creamish	-	Negative	Bacilli	Single
A-6	Undulate	Rough	Crateriform	Butyrous	Opaque	Creamish white	-	Positive	Cocci	Single
A-7	Undulate	Smooth	Crateriform	Butyrous	Opaque	White	-	Negative	Bacilli	Streptobacilli
A-8	Undulate	Rough	Umborate	Viscous and sticky	Opaque	White	-	Negative	Bacilli	Streptobacilli
A-9	Undulate	Rough	Umborate	Butyrous	Opaque	Creamish white	-	Negative	Bacilli	Single and Streptococcus
A-10	Undulate	Smooth	Flat	Butyrous	Opaque	White	-	Negative	Bacilli	Diplobacilli and Streptobacilli
A-11	Undulate	Rough	Crateriform	Sticky to surface	Opaque	White	-	Negative	Bacilli	Streptobacilli
A-12	Entire	Smooth	Umborate	Butyrous	Transuscent	Creamish white	-	Positive	Bacilli	Streptobacilli
A-13	Undulate	Rough	Umborate	Dry	Opaque	Milky white	-	Negative	Bacilli	Diplobacilli and Streptobacilli
A-15	Entire	Smooth	Crateriform	Butyrous	Opaque	Creamish white	-	Negative	Cocci	Single
A-16	Undulate	Rough	Umborate	Dry	Opaque	White	-	Positive	Bacilli	Single

Table 3: Biochemical characterization of bacterial antagonists

Isolate	Methyl red test	Starch hydrolysis	Catalase production	Hydrogen sulphide production	Citrate utilization	TSIA test	Carbohydrate utilization				
							Glucose	Lactose	Mannitol	Maltose	Sucrose
A-1	-	+	+	-	+	-	-	-	-	-	-
A-2	-	+	+	-	+	-	-	-	-	-	-
A-3	-	+	+	-	+	+	+	-	-	-	+
A-4	-	-	+	+	+	+	-	-	-	-	+
A-5	-	+	+	-	+	+	+	-	+	+	+
A-6	-	+	+	-	-	-	-	-	-	-	-
A-7	-	+	+	-	+	-	-	-	-	-	-
A-8	-	+	+	-	+	-	-	-	-	-	-
A-9	-	+	+	+	+	+	+	-	-	+	+
A-10	-	+	+	-	+	-	-	-	-	-	-
A-11	-	+	+	-	+	-	-	-	-	-	-
A-12	-	+	+	-	+	-	+	-	-	-	-
A-13	-	+	+	-	-	-	-	-	-	-	-
A-15	-	+	+	-	+	-	-	-	-	-	-
A-16	-	+	+	-	+	-	-	-	-	-	-

CONCLUSION

The study concludes that plant rhizosphere is a rich depository of a number of antagonistic bacteria. Exploiting them as potential biocontrol agents would be a step forward towards achieving sustainability in agriculture. Further knowledge on their phenotypic and functional traits will help to determine their fitness for successful biological control.

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