Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.7829

ISSN: 2582 – 2845

Ind. J. Pure App. Biosci. (2019) *7*(5), 205-213

Research Article

Indian Journal of Pure & Applied Biosciences

Studying Microbial Diversity Having Biofertilizing and Bioantagonistic Traits from Finger Millet Rhizosphere of Bastar Plateau of Chhattisgarh

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Received: 4.09.2019 | Revised: 11.10.2019 | Accepted: 16.10.2019

ABSTRACT

A total of 111 rhizobacterial isolates were isolated from 24 rhizospheric soil samples collected from different finger millet growing locations of Bastar plateau of Chhattisgarh. Of these 111 isolates, 40 were found to antagonize Pyricularia grisea in the range of 22.22-44.44 %, 38 to Rhizoctonia solani ranging 22.22-38.89 % and 30 to both. Of 30, 24 isolates reduced growth of Pyricularia grisea by producing volatile biocidal compounds while 14 inhibited growth of Rhizoctonia solani. Three bacterial isolates produced HCN while all of them produced ammonia (90.33- 4.38 µmol/ml). Twenty three of 30 isolates produced siderophore in the range of 1.1-3.82. Eighteen bacterial isolates produced β,1-4 glucanases (1.22-2.36) and amylase (1.35-2.33) while 17 were positive for protease (1.38-2.61). Screening of 30 antagonistic bacterial isolates for P-solubilization ability revealed that 19 could solubilize tricalcium phosphate in the range of 1.58-2.55. All the isolates tested positive for IAA production, however, presence of tryptophan greatly affected the IAA production pattern of isolates. In the absence of tryptophan, IAA production ranged from 1.74-4.86 µg/ml in contrast to 3.48-38.08µg/ml in presence of tryptophan.

Keywords: *Antagonism, Biocidal volatiles, Siderophore, P-solubilization, IAA.*

INTRODUCTION

Finger millet (*Eleusine coracana* (L.) Gaertn.) is one of the most important cereal crops sustaining lives of millions of people across the globe predominantly in the developing world (Dida et al., 2007). 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 (Shastri 1989, Taylor & Emmambux 2008). 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.

Cite this article: Kumari, P., Netam, R.S., & Kumar, P. (2019). Studying Microbial Diversity Having Biofertilizing and Bioantagonistic Traits from Finger Millet Rhizosphere of Bastar Plateau of Chhattisgarh, *Ind. J. Pure App. Biosci. 7*(5), 205-213. doi: http://dx.doi.org/10.18782/2320-7051.7829

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 (Lenne et al., 2007). The pathogen attacks all aerial parts of finger millet plant causing leaf, neck and finger blast and often resulting in >50% yield losses (Esele, 2002). 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. Identifying the native microbial flora having both growth promoting and antagonistic activity presents a viable option to synthetic chemicals. This investigation aimed at exploring the rhizobacterial diversity having growth promoting traits and antagonistic behavior to phytopathogens of finger millet.

METHODS AND MATERIALS

Collection of soil samples

A total of 24 soil samples were collected from different finger millet growing rhizosphere of Bastar region of Chhattisgarh. The geographical specifications of the collected soil samples are enlisted in table 1. 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.

Isolation of rhizobacteria

The isolation of rhizobacteria was done using Nutrient agar (Beef extract: 3g/l, peptone: 5g/l, NaCl: 5g/l, Agar: 20g/l, pH: 7.0) media following the method of serial dilution. Colonies which appeared to be morphologically different were isolated and subcultured.

Isolation of fungal pathogens: Leave samples of the aerial part of the plant with more than three blast and sheath blight lesions, were randomly collected from finger millet crop. After washing the tissues thoroughly in sterile water, the causal fungi were isolated from plant tissues exhibiting clear symptoms. The infected tissues along with adjacent small unaffected tissue were cut into small pieces (2–5 mm squares) and by using flame-sterilized forceps, they were transferred to sterile petridishes containing 0.1% mercuric chloride solution used for surface sterilization of plant tissues for a period of 30–60 s. The sterilized pieces were aseptically transferred to petridishes containing potato dextrose agar (PDA) supplemented with streptomycin sulfate, at the rate of three to five pieces of tissues per petriplate and incubated at room temperatures (25–27°C). A portion of mycelium developing on the nutrient medium was transferred to the agar slants for purification and storage for further examination.

Screening for antagonistic rhizobacteria

Antagonistic activity of the rhizobacterial 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 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 rhizobacteria

Initial characterization of all the isolates was

done on the basis of colony morphology and gram's staining. Biochemical characterization of bacterial isolates was done on the basis of catalase production, citrate utilization, starch hydrolysis and fermentation of different sugars. These were conducted as per the standard methods (Cappuccino & Sherman, 1992)

Assessment of functionality traits of antagonistic rhizobacteria

Production of volatile antifungal compounds

The production of volatile antifungal compounds by the isolates was assayed by a sealed plate method as described by Fiddman and Rossal (1993). From a 72 hours broth culture of isolates, 200 µl were spread on nutrient agar medium in a petri dish. After incubation at 37°C for 24 hours, a second petri dish containing PDA was inoculated with a 6-mm plug of the test fungus and placed over the bacterial culture. The two plates were sealed together with parafilm and further incubated at 25°C. This ensured that both organisms were growing in the same atmosphere though physically separated. As a control, a petri plate containing agar medium without bacteria was placed over the PDA medium inoculated with the fungal pathogen, radial growth of the test fungus was measured over 24 h intervals for a period of 5 days.

Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Overnight grown cultures were inoculated in 10 ml peptone water and incubated for 48-72 hours at 30°C. Nessler's reagent (0.5ml) was added in each test tube. Development of brown to yellow color was a positive test for ammonia production (Cappuccino & Sherman 1992). The absorbance was measured at 450 nm with three replicates and ammonia production was quantified by a standard curve prepared using ammonium sulphate.

Production of HCN

Cyanide production was detected as described by Bakker and Schippers (1987). Petri plates containing 10% Trypticase soya agar supplemented with 4.4 g of glycine per litre were inoculated with the bacteria and inverted with a lid containing filter paper, impregnated with 0.5% picric acid and 2% sodium carbonate, over each petri plate. The plates were incubated at 28°C for 3 to 5 days. A change in color from yellow to orange-brown on the filter paper indicated cyanide production.

Production of siderophore

Siderophore production by bacterial isolates was detected by the universal method of Schwyn and Neilands (1987) using blue agar plates containing the dye chrome azurol S (CAS). Production of a halo of orange around the colony indicated production of siderophore. Siderophore production unit was calculated using formula

Siderophore Index = Colony diameter + Halo zone diameter/ Colony diameter

Detection of β- 1, 4 glucanase

Plates containing minimum salts medium with (1 % w/v) carboxy methyl cellulose (CMC) were spot inoculated with the rhizobacterial isolates at the centre. After incubating for 48 h at 30 ºC, the agar medium was flooded with an iodine solution for 15 min, washed and visualized for zones of hydrolysis detecting β-1,4 glucanase.

Detection of protease

The rhizobacteria were spot inoculated on plates with minimum salts medium containing (1 % w/v) casein. After an incubation period of 48 h at 30 ºC, plates were visualized for zones of hydrolysis indicative of protease production.

Detection of amylase:

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.

Production of Indole acetic acid

Characterization of isolates for the production of IAA was done by method given by Gordon and Weber (1951). 10 ml of culture grown in

Luria broth was taken in eppendorfs and centrifuged at 10,000 rpm for 15 minutes. 2 drops of orthophosphoric acid were added to the supernatant (2 ml) if alkaline and then 4 ml of the Salkowski reagent (1 ml of 0.4 M $FeCl_3$ in 50 ml of 35% perchloric acid) and incubated for 25 minutes at room temperature for development of pink colour. Absorbance was taken at 530 nm.

P-solubilization

The cultures were screened for their in-vitro phosphate solubilizing potential in NBRIP medium. Development of yellow halo zone around the colonies was observed and recorded up to 15 days. The Phosphate Solubilization Index (PSI) was identified by measuring the total halo zone of the colony and the colony diameter.

PSI = Colony diameter + Halo zone diameter/ Colony diameter

ACC deaminase production

The qualitative estimation for ACC deaminase production was done by the method prescribed by Govindasamy et al. (2009). Bacterial isolates were grown in 5 ml of LB medium for 24 hours at 150 rpm at 28°C. Cell pellet collected by centrifugation at 8000 rpm for 5 minutes was washed with sterile distilled water and resuspended in 1 ml of sterile water and spot inoculated on Petri plates containing DF salt minimal medium supplemented with 3 mM ACC. Plates containing DF minimal medium without ACC served as a negative control and with $(NH4)_2SO_4$ as N-source served as a positive control. The plates were incubated for 3-4 days at 28°C. Growth of isolates on ACC supplemented plates was compared to positive and negative control plates. Isolates grown well on ACC plates were selected for quantitative assay.

RESULTS AND DISCUSSION

Isolation of antagonistic rhizobacteria, phytopathogens and their characterization

A total of 111 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*.

Pyricularia grisea isolated from diseased plant sample exhibited a wide variation in morphology during its growth stage. It was observed that fungus growth started as grayish white mycelium which later turned blackish gray which could be associated with formation of spores. Geshaw et al. (2014) also reported black color mycelium on PDA media while grey colored mycelium on oat meal agar with smooth colony margin and raised mycelia growth. The identity of pathogen was further confirmed by the shape of the conidia which was typically pyriform with rounded base. Geshaw et al. (2014) also reported pyriform conidia of *P. grisea* with base rounded, apex narrowed, 2-3 septate, 2-4 celled, and broader middle cells.

The *Rhizoctonia solani* exhibited grey color radial colony morphology with very fast growth on PDA plate which covered the entire plate within 72h. Production of aerial mycelium touching the cover of petri plate was also evident. Microscopic study of the fungal mycelium revealed the typical branching at right angle with characteristic constriction at the point of branching. It was an obvious observation for the mycelia branching at right angles as a known feature of *R. solani* (Sneh et al., 1991). Sclerotia production in scattered pattern was observed after 96 h of inoculation with high intensity. The shape of sclerotia was oblong and dark brown in color. In corroboration with the present finding, Sharma et al. (2010) in their study on diversity of *Rhizoctonia bataticola* isolated from chickpea plant reported light black to black and light grey to grey colony color of the pathogen however with very less aerial mycelium with radial to irregular growth of colony which covered the plate within 96 h. Different pattern of sclerocial development i.e. central/peripheral/scattered have been reported by Lal and Kandhari (2009) in *Rhizoctonia bataticola* isolated from rice plant which took 3-5 days for its initiation.

Screening of 111 bacterial isolates isolated from finger millet rhizosphere for antagonism showed that 38 exhibited inhibitory behavior against *Rhizoctonia solani*

while 40 were antagonistic to *Pyricularia grisea.* A total of 30 bacterial isolates exhibiting antagonism against both *Pyricularia grisea* and *Rhizoctonia solani* were selected for further investigation. The inhibitory behavior of antagonistic rhizobacteria varied in the range of The bacteria exhibited antagonistic effect on fungal growth which was clearly visible by formation 22.22-44.44 % against *Pyricularia grisea* and 22.22-38.89 % against *Rhizoctonia solani*. of an inhibitory zone preventing the radial proliferation of the pathogens. In a similar study, Kumari and Khanna (2016) reported that of 174 bacterial isolates isolated from chickpea rhizosphere, 50 exhibited antagonism against *Fusarium oxysporum* and *Rhizoctonia bataticola*.

Characterization of rhizobacterial antagonists

The bacterial antagonists from previous experiment were subjected to cultural and morphological characterization as given in Bergey's manual of systematic bacteriology. While all the 30 bacterial antagonists were bacilli, 27 showed gram positive reaction and 25 of them were observed to produce endospores (Table 2). Cultivation of finger millet under poor neglected soil and harse condition may be associated with the dominance of gram positive bacilli with ability to produce endospores which is the main characteristics of genus bacillus. When tested for hydrolysis of starch, 18 of 30 isolates were found positive for production of amylase while 22 of them were able to utilize citrate and 24 showed production of catalase. Fermentation of different sugars to produce acids is associated with many bacilli and in our study it was observed that 15 of them were able to ferment glucose while 7 fermented sucrose and none mannitol (Table 2). Bacteria representing genera bacillus, pseudomonas and rhizobium which are associated with plant rhizosphere have been reported as potential biocontrol agents. Sivaramaiah et al. (2007) found that out of 124 rhizobacterial isolates obtained from the rhizosphere of field-grown healthy chickpea plants, 45 were gram-positive, sporulating rods.

Functional characterization of rhizobacteria

It has been demonstrated that volatile organic compounds produced by soil bacteria can influence growth of fungi (Blom et al., 2011). Corroborating the same finding in our study, it was observed that 26 of 30 antagonistic bacteria inhibited the growth of *Pyricularia grisea* in the range of 8.23-77.64% and 14 reduced of *Rhizoctonia solani* ranging from 33.33-86.67% due to volatile antimetabolites. Three bacterial isolates produced HCN while all of them produced ammonia (90.33- 4.38 µmol/ml). Fernando et al. (2005) reported the antifungal nature of organic volatiles like HCN and inorganic volatiles such as ammonia. They also reported that production of volatile antifungal compounds by rhizobacteria alter the physiological activities of pathogenic fungi, inhibit sclerotial activity and limit ascospore production thereby managing disease incidence.

The production of siderophores under iron limiting conditions by the biocontrol agents in quantities sufficient to limit Fe^{3+} availability to the pathogen, may lead to induction of host resistance against the pathogen. In the present investigation, 23 of 30 isolates produced siderophore in the range of 1.1-3.82. In a similar study, Kumari and Khaana (2017) reported that of 16 bacillus isolates isolated from chickpea rhizosphere, 6 produced siderophore, highest siderophore index being recorded with *Bacillus* isolate B-I (2.1) followed by B20d (1.9). Several bacteria produce enzymes able to hydrolyze chitin, proteins, cellulose, and hemicellulose, thus contributing to direct suppression of plant pathogens. Finding the similar results in our study, we observed that Eighteen bacterial isolates produced β,1-4 glucanases (1.22-2.36) and amylase 1.35-2.33) while 17 were positive for protease (1.38-2.61) as indicated by halo zone around the colonies (Table 3). It is also known that different types and concentrations of hydrolytic enzymes produced by pseudomonads play an active role in antagonism against different root pathogens such as *Phytophthora capsici* and *Rhizoctonia solani* (Moataza, 2006)

Biofertilizing traits of rhizobacteria

Phosphate solubilizing microorganisms play an important role in utilization of unavailable native phosphates as well as added phosphates. Screening of 30 antagonistic bacterial isolates for P-solubilization ability revealed that 19 of these could solubilize tricalcium phosphate, however, the P-solubilizing potential varied range of 1.58-2.55 as evidenced by the size of orange halo on NBRIP medium. Chen et al (2006) reported that bacterial genera *Bacillus, Rhodococcus, Arthrobacter* and *Serratia* were powerful P- solubilizers.

The production of phytohormones such as auxins by PGPR is one of the most important mechanisms by which most of the rhizobacteria benifit plant health. In many cases these phytohormones are supposed to change assimilate partitioning patterns in plants and affect growth patterns in roots to result in bigger, more branched roots, and/or roots with greater surface area. In present investigation , it was observed that all the rhizobacteria produced IAA, however, presence of tryptophan greatly affected the IAA production pattern of isolates; although it is also thought to be strain dependent (Ahmad et al., 2008). In the absence of tryptophan, IAA production ranged from 1.74-4.86 µg/ml in contrast to 3.48-38.08 µg/ml in presence of 3mM tryptophan after 5 days of incubation. Similar findings have been highlighted by Kumari and Khanna (2016) where IAA production by all the rhizobacterial isolates from chickpea rhizosphere was recorded ranging from 2.4-15.5 µg/ml in the absence of tryptophan and 9.2-48.7 µg/ml in presence of 3mM tryptophan.

The concept of plant growth promoting bacteria (PGPB) containing ACC-deaminase for promotion of plant growth under environmental stress conditions has gained importance (Berg, 2009). The relation between ethylene and rhizobacterial mediated stress alleviation is provided by the enzyme ACC-deaminase which metabolizes ACC in the root of developing plants, thereby reducing the level of ethylene in stressed plant (Selvakumar et al., 2012). Plate assay carried out for screening the ACC deaminase containing rhizobacteria showed that 19 isolates were able to grow on DF medium supplemented with 3 µM ACC as a sole source of nitrogen compared to DF plate where no growth wasobserved signifying the secretion of ACC-deaminase A number of rhizobacteria belonging to genera *Bacillus, Achromobacter, Burkholderia, Enterobacter,* and *Pseudomonas* have been reported to exhibit variable ACC deaminase activity (Chookietwattana & Maneewan, 2012).

Table 1: Geographical location of different soil samples

Blocks/Tehsils	Village	No. of samples	Stage of crop
Bastar	Bhond	1	Flowering
	Lamker	2	Reproductive
	Bade chakwa	1	Flowering
	Parali	1	Flowering
	Narayanpal	2	Flowering
	Seoni	1	Flowering
	Jhartarai	1	Flowering
	Madhota	1	Flowering
	Padarguda	1	Flowering
Lohandiguda	Chhindbahar	1	Reproductive
	Taragaon	2	Reproductive
	Gadhiya	1	Flowering
Tokapal	Kalepal	1	Flowering
	Ghatdhanora	1	Flowering
Jagdalpur	Kumhrawand	4	Maturity
Bastanar	Bastanar	1	Reproductive
	Kodenar	1	Reproductive
Darbha	Tirathgarh	1	Flowering
Total		24	

Table 2: Cultural and biochemical characteristics of rhizobacteria

Morphological characters	
Colony color	Creamish to Creamish white to white
Cell shape	All bacilli
Cell arrangement	Single to diplo to strepto
Biochemical/ cultural characters	**Isolates exhibiting the trait**
Gram's positive reaction	27
Endospore formation	25
Starch hydrolysis	18
Citrate utilization	22
Catalase production	24
Fermentation of Glucose	15
Fermentation of Sucrose	7
Fermentation of Mannitol	0

Table 3: Biofertilizing and antagonistic traits of rhizobacteria

Parameter	**Number of isolates positive for the trait**	**Range**
Antagonistic traits		
Volatiles against *Pyricularia grisea*	26	8.23-77.64 %
Volatiles against *Rhizoctonia solani*	14	33.33-86.67%
HCN	3	-
Ammonia (µmol/ml)	30	0.33-4.38
Siderophore production index	23	1.1-3.85
Glucanase production index	18	1.22-2.36
Amylase productionindex	18	1.35-2.33
Protease production index	17	1.38-2.61
Biofertilizing traits		
IAA with tryptophan (µg/ml)	30	3.48-38.08
IAA without tryptophan (µg/ml)	30	1.74-4.86
P-solubilization Index	19	1.58-2.55
ACC-deaminase	19	-

CONCLUSION

The present investigation clearly indicates the existence of many bacteria in finger millet rhizosphere which have immense potential to act both as biofertililzer and biopesticide. Unraveling these microflora could present a sustainable approach in enhancing finger millet production.

Acknowledgment

Authors are thankful to Department of Science and Technology for possible funding under KIRAN scheme (WOS-A) which made this work a success.

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