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SCREENING OF RHIZOBACTERIA FOR PLANT GROWTH PROMOTING TRAITS AND ANTIFUNGAL ACTIVITY AGAINST CHARCOAL ROT PATHOGEN *MACROPHOMINA PHASEOLINA*

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ABSTRACT

Plant growth promoting rhizobacteria stimulate plant growth and suppress the colonization of phytopathogens by involving direct and indirect mechanisms. In our investigation ten predominant rhizobacterial isolates belonging to six genera were isolated and characterized for their multiple growth promoting attitudes and antagonistic activity. The results revealed that 70% of the isolates produced phytohormone IAA, 80% of isolates produced ammonia and HCN. Among the ten isolates, four (40%) exhibited phosphate solubilizing and siderophore producing ability. The cell wall degrading enzymes such as cellulase, protease, chitinase activities of isolates were also recorded. They were in the range of 50%, 10% and 60 %, respectively. The antifungal activity of the isolates were tested against *Macrophomina phaseolina*, among the ten bacterial isolates four (40%) effectively inhibited mycelial growth of plant pathogen. The bacterial strain which showed better results for all the test was identified as *Bacillus amyloliquefacience* by 16SrRNA sequencing.

KEY WORDS: Rhizobacteria, IAA, Siderophores, HCN, Cell wall degrading enzymes, *Macrophomina phaseolina*.

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INTRODUCTION

Sunflower is mainly grown in Rabi season as a rain fed crop mostly by marginal farmers. Charcoal rot, is a major potential threat for crops such as sunflower and soybean. Charcoal rot epidemics are common under severe conditions such as water scarcity and other biotic and abiotic stresses[1]. The pathogen *Macrophomina phaseolina* (Tassi) Goid is an anamorphic ascomycete of the family Botryosphaeriaceae and causes the disease charcoal rot on a broad range of plants in many areas of the world. Its infection on sunflower was first reported from India in 1973. The fungus is reported to be soil, seed and stubble borne. The evidence suggests that it is primarily a root inhibiting fungus and produces a tuber or cushion shaped 1-8 mm diameter black sclerotia. These sclerotia serve as a primary means of survival[2]. Some chemical fungicides are used to control the charcoal rot pathogen. However these chemicals are not ecofriendly, hence an alternative method is needed to manage this disease. Soil-borne diseases have been controlled more recently by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants. The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large and metabolically active groups of bacteria known as plant growth promoting rhizobacteria (PGPR) [3]. PGPR rapidly colonize the rhizosphere and suppress the pathogenic microorganisms at the root surface[4]. PGPR use one (or) more direct mechanism of action to improve plant growth and health. Improvement of plant uptake by phosphate solubilization (or) nitrogen fixation and phytohormone production like indole -3- acetic acid are examples of mechanism of direct influence on plant growth. Biological control of plant pathogens and deleterious microbes, through production of antibiotics, lytic enzymes, hydrogen cyanide and sederophore (or) through competition for nutrient and space can significantly improve plant health and promote growth by increasing of seedling emergence, and yield. Recent studies have demonstrated the importance of strains of non-pathogenic PGPR in enhancing plant resistance[5]. There fore an investigation was carried out to reveal the plant growth promoting activities such as IAA production, Ammonia production, HCN production, Phosphate solubilization, siderophore production, cell wall degrading enzyme activities and antifungal activity against *Macrophomina phaseolina* of rhizobacterial isolates.

MATERIALS AND METHODS

Isolation of Bacteria from Rhizosphere soil

The Rhizosphere soil samples of sunflower plant were collected in polyethylene bags from Pudukkottai districts, Tamil Nadu and transported to the laboratory and kept in refrigerator (4^0C) for further process. The Rhizobacteria were isolated on nutrient agar medium and they were purified by streaking on the same medium. The purified colonies were maintained as pure culture with periodic transfer to fresh media and stocked for further use. All the isolates were identified at genus level based on colony morphology, Gram's staining, motility test and biochemical characteristics.

Production of Indole Acetic Acid (IAA)

Indole acetic acid production was detected as described by Bric *et al*.,[6]. Bacterial isolates were inoculated in nutrient agar amended with L-Tryptophen and incubated at37^0C for 48hrs. Fully grown cultures were centrifuged at 3000rpm for 30 minutes, the supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowaski reagent (50 ml 35% of perchloric acid, 1ml of 0.5m$FeCl_3$ solution). Development of pink colour was indicative of IAA production.

Ability for Phosphates solubilization

Phosphate solublizing ability of the isolate was checked on Pikovskaya (PVK) medium[7], incorporated with tricalcium phosphate ($Ca_3(PO4)_2$). The isolates were spot inoculated on PVK medium. Formation of transparent halo

zone around the developing colonies indicated phosphate solublizing ability of the isolates.

Assay for NH3 production

The rhizobacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated into 10 ml peptone water and incubated at 30^0C for 48 hrs. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production[8].

Hydrogen Cyanide production (HCN)

Production of HCN was determined using the modified procedure of Millar and Higgins[9]. All the bacterial strains were grown on Trypticase Soy Agar (TSA) plates. Sterilized Whatman No. 1 filter paper strips were soaked in picric acid solution (2.5 gm of picric acid, 12.5gm of Na_2CO_3, in 1000ml of distilled water) and were placed in the lid of each Petri dish. Dishes were sealed with parafilm and incubated at $28^0 \pm 1^0$C for 48 hrs. A change in colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong production of HCN by each strain, respectively.

Screening for siderophore production

Production of siderophores by bacterial strains were performed by plate assay according to Schwyn and Neilands[10]. The tertiary complex, Chrome azural S (CAS) / Fe $^{3+}$ / hexadecyl trimethyl ammonium bromide served as an indicator. Forty eight hour old culture of the strains was streaked onto the Succinate medium amended with indicator dye. The formation of bright zone with yellowish fluorescent colour by the culture in the dark colored medium indicated siderophore production. The result was scored either positive or negative to this test, based on the colour change of the medium from blue to fluorescent yellow while no colour change indicated the absence of siderophore production.

Cell wall degrading enzyme production

Cellulase activity

A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on CMC (Carboxy Methyl Cellulose) agar containing (g L^{-1}) KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, $NH_4NO_3$0.3, CMC 10.0, Agar 12.0. The pH was adjusted to 7.0 with 1 M NaOH. The CMC agar plates were incubated at 37°C for 5 days to allow for the secretion of cellulase. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. Clear zone around the colony was indicative of cellulose enzyme production[11].

Chitinase activity

A minimal salt medium containing colloidal chitin as sole carbon and energy source was used. The medium consisted of Na_2HPO_4, 6g; KH_2PO_4, 3.0g; NH_4Cl, 1g; NaCl, 0.5g; yeast extract, 0.05g, colloidal chitin 1.0% (w/v) agar, 15g and distilled water, 1000 ml and incubated at 30ºC. Colloidal chitin was prepared by the method of Hsu and Lockwood[12] from crab shell chitin (Sigma). Colonies showing zones of clearance against the creamy background were recorded as chitinase-producing PGPR.

Protease activity

Protease activity was determined by casein degradation in skimmed milk agar medium[13]. An ability to clear the skimmed milk suspension in the agar plates was taken as evidence for the secretion of protease.

In vitro Antagonistic activity

The bacterial isolates were screened for the ability to inhibit *M. phaseolina* by employing dual culture method[14]on PDA plates.

Individually a loopful of 2 days old bacterial cultures grown in nutrient agar media was streaked on one side leaving 1 cm from the margin, and then 6mm disc of fungal pathogen culture was placed at the other side. Plates

without antagonist served as control. The plates were incubated at 25±2°C for 4-5 days. Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria. The percentage of inhibition was calculated using the following formula: % inhibition = (R - r) / R ×100 Where 'r' is radial growth of the fungal colony opposite the bacterial colony and, R is the radial growth of the pathogen in control plate.

Molecular characterization of isolates

The bacterial isolate which gave promising results in the preliminary test was grown in Luria broth for 24 hrs at 37^0Cfor extraction of genomic DNA through enzymatic lysis[15]. The integrity and concentration of purified DNA was determined by agarose gel electrophoresis. The total genomic DNA extracted was dissolved in water (protease, nuclease free) and stored at 4^0C. PCR amplification was done by using 16S universal primer PA (5^1 - AGAGTTTGATCCTGGCTCAG3^1) and PH (5^1 AAGGAGGTGATCCAGCCGCA -3^1). The PCR (Peliter Thermal Cycler, BIO-RAD) reaction was performed in 100µl volume (10 µl of 10x PCR buffer, 2.5 µl of each primer, 1 µl of Taq DNA polymerase) with 3 µl of DNA Template. The amplication was performed with following program: 5 minutes initial denaturation at 95^0C, followed by 30 cycles of 1minute denaturation at 95^0C, 1 minute annealing at 55^0C, 1 minute extension at 72^0C, and a final extension step of 5 minutes at 72^0C. The amplified product was resolved in 1.2% agarose gel and visualized on gel documentation system (BIO- RAD, USA). The purified 16S rRNA gene was performed using as a template in cycle sequencing reaction with fluorescent dye- labeled terminators (Big dye, Applied Biosystems) of isolate each cluster with same primer and run in 3130 XL ABI prism automated DNA sequence. The sequence was compared with 16S rRNA gene sequence available in the NCBI GeneBank database using BLASTn program.

RESULTS AND DISCUSSION

Isolation and Identification

An investigation was carried out to explore the multiple plant growth promoting activity and antagonistic activity of Rhizobacteria of sunflower crop. Totally ten bacterial species belonging to six genera were isolated and identified based on morphological and biochemical characteristics (Table-1). Similarly Raval and Desai[16] isolated thirty bacterial species from rhizosphere soil of sunflower and also reported that these bacterial isolates increased the seed germination, root length and shoot length of Sunflower (*Helianthus annuus* L.) and other plants in pot culture experiments. The sequence of CCPS1 was compared with previously published sequence on the NCBI database and revealed high similarity to *Bacillus amyloliquefaciens* FZB42 (99% similarity), hence the isolate CCPS1was identified as *Bacillus amyloliquefaciens*. This result supports the previous report of Mubarik *et al*.,[17] they have identified two potential chitinolytic strains namely *Bacillus cereus* and *Bacillus* sp using 16SrRNA sequence.

IAA production

Out of the ten isolates, 70% of isolates produced IAA in broth medium containing Tryptophan. Among the producers, it was noted that the *Bacillus amyloliquefacience* was the strong producer of phytohormone IAA, while the remaining 60% of the test isolates were recorded as weak producer of IAA. The potential for auxin biosynthesis by rhizobacteria can be used as a tool for the screening of effective PGPR strains[18]. Sajani Samuuel and Muthukkaruppan[19] studied the PGPR for their multiple growth promoting traits and reported that all the test isolates were able to produce IAA. Sasirekha Bakthavatchalu *et al*., [20] isolated 51 *Pseudomonas* spp from rhizosphere soils of different crops such as cabbage, wheat, potato, tomato, paddy and garlic. They reported that out of 51 isolates *Pseudomonas aeruginosa* exhibited promising results for IAA production, they also observed that the inoculation of cowpea seeds with this bacteria significantly

increased the seed germination, seedling vigor index, plant height, fresh and dry weight in comparison with the control.

Siderophore production

Siderophores are low molecular weight, iron chelating ligands synthesized by microbes. Siderophores help the microorganisms to compete against fungal pathogens for available iron and the role of siderophores in control of diseases has been well documented[21]. It has been suggested that siderophore producing bacteria could increase the germination power of agricultural plants.[22] In this investigation 40% of isolates screened were positive for siderophore production. *Pseudomonas* sp2, *B. amyloliquefaciens*, *Pseudomonas* sp1, and *Micrococcus* sp1 produced clear yellow zone around the colony. Purified sederophore of *Pseudomonas aeruginosa* showed antifungal activity against *Aspergillus niger*, *A. flavus, A. oryzae, Fusarium oxysporum, Sclerotium rolfsii* [23].

Phosphates solubilization

Phosphorus (P) is a major essential macronutrient for biological growth and development. Microorganisms offer a biological rescue system capable of solubilising the insoluble inorganic P of soil and make it available to the plants. The ability of some microorganisms to convert insoluble phosphorus (P) to an accessible form, like orthophosphate, is an important trait in a PGPB for increasing plant yields[24]. High proportion of phosphate-solubilizing microbes is concentrated in the rhizosphere, and they are metabolically more active than other sources[25]. In our experiment all bacterial isolates were screened for phosphate solubilising capability. The results for phosphate solubilization showed that 40% of the bacterial strains were able to solubilize the tricalcium phosphate, as evidenced by hallow zone around the colony. Among the solubilizer *B. amyloliquefaciens* showed moderate solubilization, *Pseudomonas* and *Staphylococcus* showed weak solubilization. Efficient phosphate solubilizing bacterial species have been identified from the rhizosphere soil of onion, maize, jasmine, and tomato by Ranjan *et al*., [26]. Zarrin Fatima[27] reported that the three strains of bacteria belonging to the genus *Azotobacter* isolated from Wheat rhizosphere possessed phosphorous solubilization and IAA producing characteristics. They concluded that these bacterial strains increased the seed germination, biomass and root, shoot length by inhibiting *Rhizactonia solani* growth when tested in pot culture experiments. The phosphate solubilizing bacterial application was able to mobilize P efficiently in the sunflower and improved seed quality and oil yield. It also enhanced the head diameter, 1000 seed weight, kernel ratio and oil content and led to seed and oil yield increases of 15.0 and 24.7% over no application, respectively[28]. Manivannan *et al*.,[29] isolated ten plant growth promoting bacteria from rhizosphere of rice and reported that among the ten isolates only one was found to be a phosphate solubilizer, also exhibited moderate (30%-40%) antifungal activity against *Fusarium oxysporum*, *Rhizoctonia solani* and failed to inhibit *Sclerotium rolfsii.*

Ammonia production

Production of volatile ammonia has been implicated as a possible mechanism to control soil borne pathogens[30]. In the present study 80% of the isolates were identified as ammonia producers, However *Pseudomonas* sp1 and *Bacillus amyloliquefaciens* were recorded as moderate producer. While the others, *Bacillus* sp1, *Bacillus* sp2, *Pseudomonas* sp2, *Neisseria, Micrococcus* sp1 *Micrococcus* sp2, were recorded as weak producers. The efficacy of ammonia producing PGPR has been well exploited by several previous studies [31]

HCN production

HCN is produced by rhizobateria and has been postulated to play a role in biological control of pathogens[32]. In the present study, we have observed that eight of our isolates produced HCN and among them two were strong producers (+++), one was moderate producer (++) and five were weak producers (+). This report supports the previous reports by Akhtar

and Siddiqui[33] and Ruchi *et al*.,[34]. The role of HCN in bicontrol activity of PGPR has been previously reported. *Pseudomonas aeruginosa* strain NJ-15 has been characterized as potential HCN producer which significantly inhibited the growth of phytopathogenic fungi in the order of *Fusarium oxysporum* > *Trichoderm herizum*> *Alternaria alternata*> *Macrophomina Phaseolina*[35].

Cell wall degrading enzyme production

In order to obtain carbon nutrition several biocontrol agents are able to hydrolyse chitin of fugal cell wall. Several studies have demonstrated the role of cell wall degrading enzyme on the control of pathogenic fungi. Kamil *et al*., [36] have isolated effective chitinolytic bacteria belonging to the genus *Bacillus* namely *Bacillus lichiniformis* and *B.thuringiensis* from the rhizosphere soil of maize, wheat and rice. Yildiz *et al*., [37] screened and selected rhizobacteria against *Fusarium oxysporum* f.sp.melongenae from root zone of eggplant. Out of 261 isolates eight of the isolates showed protease activity and no isolates showed chitinase and cellulose activities. Chaiharn *et al*., [38] observed that 6% of rhizosphere isolates possessed cellulase and chitinase activity and 5% of rhizobacteria showed protease activity. Similarly five of our isolates were screened for cellolytic activity, of which the strong producer was found to be *Bacillus amyloliquefaciens*. The same isolate showed moderate and strong activity of protease and chitinase respectively. 60% of rhizobacterial isolates exerted chitinolytic activity but only two species namely *B. amyloliquefaciens*, *Bacillus* sp1 showed very clear zone on colloidal chitin agar medium (Table - 2).

Antifungal activity against Macrophomina phaseolina

Neetu Singh *et al*.,[39] reported that *Bacillus subtilis* BN1 exhibited strong antagonistic activity against *Macrophomina phaseolina*, and other phytopathogens including *Fusarium oxysporum* and *Rhizoctonia solani*. The antagonist possessed the multiple plant growth promoting activity such as IAA production, phosphate solubilization, siderophore production. Similarly we have observed that the *Bacillus amyloliquefaciens* (35%) was the potent inhibitor of *M. phaseolina*, and also a good PGPR as it possessed the above stated characteristics. *Pseudomonas* sp1 (27%), *Bacillus* sp1 (30%) and *Micrococcus* sp1 (15%) also inhibited the mycelial growth but the rest of the isolates failed to inhibit the growth of pathogenic fungi. These results are in line with report of Usha and Padmavathi[40].

Table - 1
Morphological and Biochemical Characteristics of Rhzobacteria

Isolates	Gram Reaction	Shape of Isolates	Colony Colour	Motility	Indole	M.R.Test	V.P.Test	Citrate	Catalase	Bacterial Isolates
CCPS1	+	Rod	Pale white	Motile	-	+	_	+	+	*Bacillus amyloliquefaciens*
CCPS2	+	Rod	Pale white	Motile	-	+	_	+	+	*Bacillus* sp1
CCPS3	+	Rod	Pale white	Motile	-	+	_	+	+	*Bacillus* sp2
CCPS4	-	Rod	Greenish yellow	Motile	-	+	_	+	+	*Pseudomonas* sp1
CCPS5	-	Rod	Greenish yellow	Motile	-	+	_	+		*Pseudomonas* sp2
CCPS6	-	Rod	Pink	Motile	-	_	+	+	+	*Serratia* sp
CCPS7	+	Cocci	White	Non Motile	-	+	+	_	+	*Staphylococcus* sp
CCPS8	-	Cocci	Yellow	Non Motile	-	_	+	_	+	*Neisseria* sp
CCPS9	+	Cocci	Orange yellow	Non Motile	-	_	+	_	+	*Micrococcus* sp1
CCPS10	+	Cocci	Orange yellow	Motile	-	_	+	+	+	*Micrococcus* sp2

+, Positive, -, negative, MR-Methyl Red, VP, Voges Proskauer

Table - 2
Plant Growth Promoting and traits and antifungal activity

Bacterial Isolates	IAA production	NH_3 production	HCN production	Siderophore production	Phosphate solubilization	Cellulase activity	Protease activity	Chitinase activity	Antifungal activity. Zone of inhibition (%)
Bacillus amyloliquefaciens	+++	++	+++	++	++	+++	++	+++	35
Bacillus sp1	+	+	+	_	_	_	_	+++	30
Bacillus sp2	+	+	+	_	_	_	_	+	-
Pseudomonas sp1	+	++	++	++	+	+	_	+	27
Pseudomonas sp2	+	+	+++	+++	+	+	_	+	-
Serratia sp	+	_	_	_	_	_	_	+	-
Staphylococcus sp	_	_	_	_	+	+	_	_	-
Neisseria	_	+	+	_	_	_	_	_	-
Micrococcus sp1	+	+	+	+	_	+	_	_	15
Micrococcus sp2	_	+	+	_	_	_	_	_	-

- ***Negative, + Weakly Produced, ++ Moderately produced, +++ Strongly Produced***

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