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Isolation and Biochemical Characterization of *Bacillus* spp. from Major Banana growing Areas of Tamil Nadu

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ABSTRACT: *Plant protection through plant growth promoting rhizobacteria (PGPR) has emerged as a sustainable approach for crop health management. In the present study 10 Bacillus strains were isolated from rhizosphere soil of major banana growing areas of Tamil Nadu and subjected to biochemical characterization. Several tests like Malonate, Voges Proskauer's, Citrate, ONPG, Nitrate Reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose, Trehalose, Carbohydrate fermentation, Starch hydrolysis and Citrate utilization were carried out. The isolates BS 30, BS 15 and BS 13 showed positive reaction to all the tests except few isolates. Tests like Gram's staining, Endospore staining, Growth at 45 °C and 4 °C, Growth at 7% NaCl reveals that all the isolates were found to turn positive for Bacillus spp. Further, Bacillus isolates viz, BS 30, BS 15 and BS 13 produced a large orange halo in Chrome Azurol Sulphonate (CAS) agar plate assay and the nature of siderophore was Hydroxymate type. The same strains were found to be positive for hydrogen cyanide production under in vitro. These findings suggest that Bacillus isolates BS 30, BS 15 and BS 13 can be successfully employed as an eco-friendly strategy for the management of diseases in Banana.*

Keywords: *Bacillus spp., Biochemical, Siderophore, Hydrogen cyanide*

INTRODUCTION

The use of plant growth promoting rhizobacteria (PGPR) for biocontrol represents a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops (Jetiyanon and Kloepper, 2002). The strains of *Bacillus* spp. are known to survive both in rhizosphere and phyllosphere. Different mechanisms have been reported for their performance such as production of antibiotics, siderophore, hydrogen cyanide, competition for nutrition and space, inducing resistance, inactivation of pathogen's enzymes and enhancement of root and plant development (Weller, 1988; Intana *et al*., 2008). Among PGPR, the sporulating Gram-positive bacteria like *Bacillus spp*. have also been used successfully for plant disease control (Kloepper *et al*., 2004; Shanmugam and Kanoujia, 2011). The role of plant growth promoting rhizobacteria (PGPR) *viz*., *Bacillus spp.* in biocontrol approaches for managing the pathogen in crop plants are well reported (Manjunath, 2009 and Nagendran, 2011). *Bacillus* spp. isolated from different microhabitats had strong antifungal properties against several foliar pathogens (Duncan *et al*., 2006).

MATERIALS AND METHODS

Isolation of Biocontrol agent – *Bacillus* spp.

Thirty antagonistic *Bacillus sp.* was isolated from healthy banana rhizosphere soil collected from various regions of Tamil Nadu by serial dilution technique and plated onto the Nutrient agar (NA) (g/l; peptone 5, beef extract 2, agar 20, pH 5.0) (Allen, 1953). The isolates of *Bacillus* sp. were identified according to the description given in Bergey's manual for Systematic Bacteriology.

Biochemical Characterization of *Bacillus* spp.

Characterization of *Bacillus* sp. based on primary character and carbon source utilization

Native bacterial isolates were characterized based on standard biochemical tests (Hildebrand *et al*., 1992). Characterization included growth at different temperatures and the ability to utilize different

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substrates as a sole carbon source. Other than this the bacterial biochemical response was tested by using ready biochemical kit for the specific identification of gram positive rods (Rapid biochemical identification test kits-KB002 HiAssorted™ Biochemical test, HiMedia laboratories Pvt. Ltd). Results of these tests were scored either as positive or negative and grouped with the aid of a determinative scheme developed by earlier workers (Ramamoorthy *et al.*, 2002, Ramyabharathi, 2011).

Starch Hydrolysis

Starch was mixed with water until creamy and molten nutrient agar was added, autoclaved for 15 min. and dispensed into steri-lized Petri dishes. The isolate was streaked on starch agar plates and incubated for three days and the plates were flooded with Lugol's iodine solution and observed (Aneja, 1993).

Utilization of Citrate

Stabbed the butt and streaked the surface of a slant of Simmons' citrate agar. Blue color formation due to bacterial growth indicates citrate utilization. Original green color indicates negative results (Schaad, 1992).

Siderophore Production

The production of siderophore by bacterial antagonists was determined by the plate assay method as described by Schwyn and Neilands (1987). The indicator was tertiary complex chromeazural S (CAS) / Fe^{3+} / hexadecyl trimethyl ammonium bromide (HDTMA). Forty eight hour old antagonists was streaked on succinate medium with pH 7.0 amended with indicator dye. To prepare 1 l of the blue agar, 60.5 mg of CAS was dissolved in 50 ml of distilled water and mixed with 10 ml Fe III solution (1mM $FeCl_3$ $6H_2O$) in 10mM HCl). The solution was stirred constantly and slowly added to 72.9 mg of hexadecyl trimethyl ammonium bromide dissolved in 40 ml water. The resultant dark blue liquid was observed for the formation of bright zone with yellowish fluorescent color in the dark blue colored medium. Production of siderophore was scored as none, little, strong and very strong.

HCN Production

The bacterial isolates were streaked into tryptic soya agar (animal peptone – 15.0 g, soyapeptone-5.0 g, sodium chloride-5.0 g, glycine-4.4 g, distilled water-1000 ml). Filter paper disc of 1.5 cm diameter was soaked in picric acid solution (Picric acid, 2.5 g; Na_2CO_3, 12.5 g and distilled water) and placed in the upper lid of each Petri plate (Miller and Higgins, 1970). Plates were sealed with parafilm and incubated for four days. HCN production was assessed by the presence of colored zone around the bacteria and the yellow color of the filter paper turning brown to reddish brown. Reaction were scored as weak (yellow to light brown), moderate (brown) and strong (reddish brown).

Statistical Analysis

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P < 0.05$ and $P < 0.01$) and means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS

Isolation of *Bacillus* spp.

Totally thirty isolates of *Bacillus* sp. were isolated from the rhizosphere soil of healthy banana plants from various locations of Tamil Nadu. *Bacillus* isolates grown on nutrient agar medium were used throughout the study (Table 1).

Table 1
List of *Bacillus* Antagonists Isolated from Rhizosphere Soil of Banana

S.No	*Code.No*	*Place/Village*	*Area*	*Variety/ Cultivar*	*Colony color*
1.	BS1	Sundapalayam	Coimbatore	Nendran	Cream
2.	BS2	Thondamuthur	Coimbatore	Grand Naine	Cream
3.	BS12	Iduganpalayam	Mettupalayam	Grand Naine	Light yellow
4.	BS13	Pulliampatti	Erode	Grand Naine	White
5.	BS15	Aaliyar	Pollachi	Rasathali	White
6.	BS18	Virupatchi	Dindugal	Naatuvazhai	Light yellow
7.	BS19	Chinnamannur	Theni	Rasathali	White
8.	BS28	Kulithalai	Trichy	Poovan	Cream
9.	BS29	Sirugamani	Trichy	NeyPoovan	Light yellow
10.	BS30	Thottiyam	Trichy	Nendran	White

Biochemical Tests for *Bacillus* spp.

The isolates which promoted plant growth and high inhibition to *M. musicola* were characterized by biochemical methods for identification. The different methods *viz*., Malonate, Voges Proskauer's, Citrate, ONPG, Nitrate Reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose, Trehalose, Carbohydrate fermentation, Gram's staining, Endospore staining Growth at 7% NaCl, Starch hydrolysis and Citrate utilization were carried out. All the tests were positive except mannitol for BS 30 and all the tests falls positive except mannitol, sucrose, ONPG and trehalose tests for BS 15 and for BS 13 all the tests falls positive except mannitol, sucrose and trehalose test. The results for other *Bacillus* isolates were shown in Table 2 (Plate 1, 2 and 3).

Siderophore Production

Bacillus isolates exhibited yellow coloured halo around the bacterial streak on dark blue colored agar plates indicating the production of siderophore. The siderophore produced by the isolates BS 30, BS 15 and BS 13 chelated the iron from the chromazurol/Fe^{3+}/ hexadecyl trimethyl ammonium bromide dye and turned dark blue to yellowish fluorescent colour (Plate 4).

HCN Production

The isolates were found to produce hydrogen cyanide (HCN) which acts as an inducer of plant resistance. Among these HCN producing isolates, BS 30, BS 15 and BS 13 medially changed the yellow colour of the filter paper to dark brown when compared to other strains indicating medium level of HCN production whereas in control no change of yellow colour was observed (Plate 5).

DISCUSSION

Biochemical Characterization of *Bacillus* spp.

Various phenotypic methods have been developed and used for characterizing bacterial isolates. In the present study, phenotypic characterization of *Bacillus* isolates were carried out to study the basic characters *viz*., gram reaction, growth at different temperatures and utilization of different carbon sources. BS 13, BS 15 and BS 30 isolates were Gram-positive, spore forming bacteria, able to grow at 45 ^{o}C and in the presence of 7% NaCl. They utilize citrate as sole carbon source. Three *Bacillus* strains were efficiently hydrolyzed starch. The result obtained by analyzing primary character and carbon source utilization of different bacteria revealed that they belong to *Bacillus* sp. The results obtained by analyzing primary character and nutrient source utilization of different bacteria revealed that among the 30 isolates, all 30 were found to turn positive for *Bacillus* sp. This can be supported from the earlier findings as among the various group of *Bacillus* species are the most common bacteria found to colonize plants (Mercado-Blanco and Bakkers, 2007; Ryan *et al.,* 2008; Taghavi *et al.,* 2009). In the present study, 30 strains of *Bacillus* were taken for further studies, since it has great potential in agriculture. Its members are able to produce antimicrobial metabolites to control plant pathogens, to fix nitrogen, to form endospores to resist dessication, heat and UV irradiation and survive in adverse conditions. Preliminary characterization of bacteria showed that approximately equal percentages of Gram positive (41 %) and Gram negative (42 %) bacteria were recovered from the agronomic crop plants (Zinniel *et al*., 2002).

The use of HiBacillus identification kit (KBO13, HIMEDIA) has been shown to give more reliable and reproducible results than classical methods. Ramyabharathi (2011) stated that fifteen *Bacillus* strains were identified by biochemical characterization and confirmed the genus of the *Bacillus* by positive and negative characters. Karthiba (2011) reported that twenty six isolates of *Bacillus* strains were identified by biochemical characterization and confirmed it as *Bacillus* strains. Fifteen isolates of *Bacillus* strains were identified and confirmed by biochemical characterization were reported by Prabhukarthikeyan (2012). However, identification and characterization of isolates are very difficult to confirm based on the morphological and biochemical characteristics up to species level and was opined by Frietas *et al*. (2008).

Siderophore in Biological Control

In the present study, production of yellow halo around the colony in agar plate containing chromazurol S indicated the siderophore production of all the *Bacillus* strains. In most microbes, iron uptake is essential for viability and under iron starvation, these microbes excrete low-molecular weight ferric-iron specific chelators, termed siderophores, to mobilize environmental iron (Neilands, 1995). Subsequently, iron from the ferrisiderophore complexes is recovered via specific uptake mechanisms. Siderophore production under iron stress conditions also confers upon these organisms an added advantage, in inhibiting pathogen growth or metabolic activity (Henry *et al.,* 2008).

Table 2
Biochemical Characterization of *Bacillus* Isolates Collected from Banana Rhizosphere Regions of Tamil Nadu

Isolate/Test	*Malonate*	*Voges Proskauer's*	*Citrate*	*ONPG*	*Nitrate Reduction*	*Catalase*	*Arginine*	*Sucrose*	*Mannitol*	*Glucose*	*Arabinose*	*Trehalose*	*Gram staining*	*Endospore staining*	*KOH test*	*Anaerobic growth*	*Growth at 45°C*	*Growth at 4° C*	*Growth in 7% NaCl*
BS1	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	+	+
BS2	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	-
BS12	+	+	+	-	-	+	+	-	-	-	+	-	+	+	-	+	+	-	-
BS13	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
BS14	+	+	+	+	-	+	+	+	-	+	+	-	+	+	-	+	+	-	-
BS15	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	+
BS18	+	+	+	+	-	+	+	-	-	-	-	-	+	+	-	+	+	-	-
BS19	+	+	+	+	-	+	+	-	-	-	-	-	+	+	-	+	+	+	+
BS28	-	-	+	+	-	+	+	-	-	+	+	-	+	+	-	+	+	-	-
BS29	+	-	+	-	+	+	+	-	-	+	+	-	+	+	-	+	+	+	+
BS30	+	-	+	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

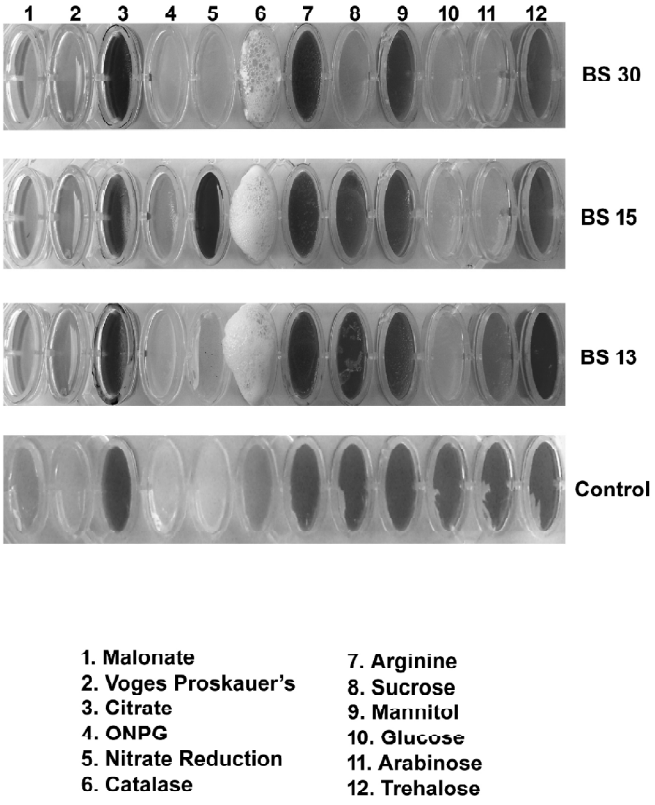

Plate 1: Biochemical characterization of *Bacillus* isolates using standard biochemical kit

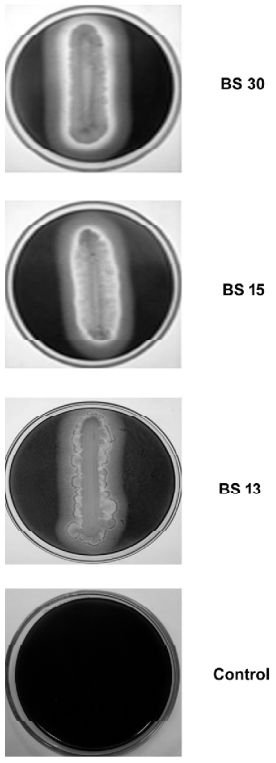

Plate 2: Starch hydrolysis test for *Bacillus* sp.

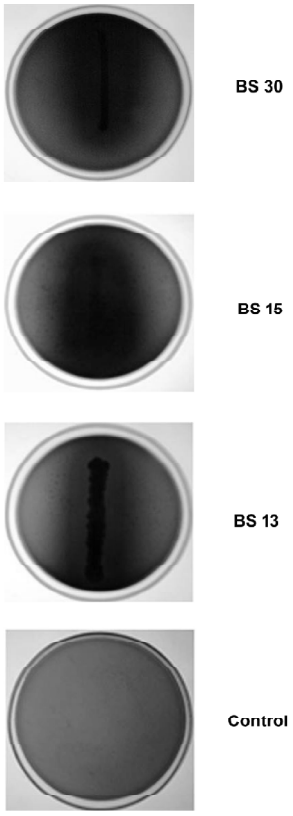

Plate 3: Citrate utilization by *Bacillus* sp.

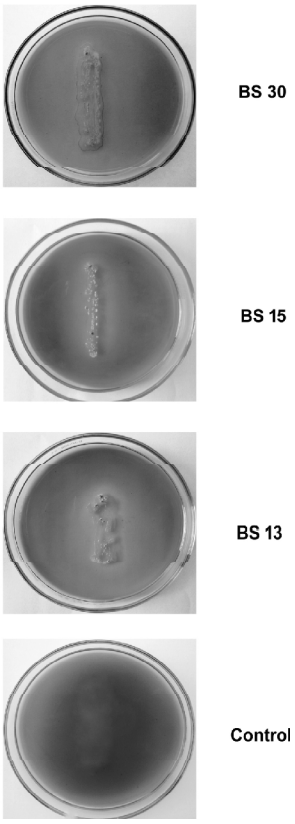

Plate 4: Siderophore production by *Bacillus* sp.

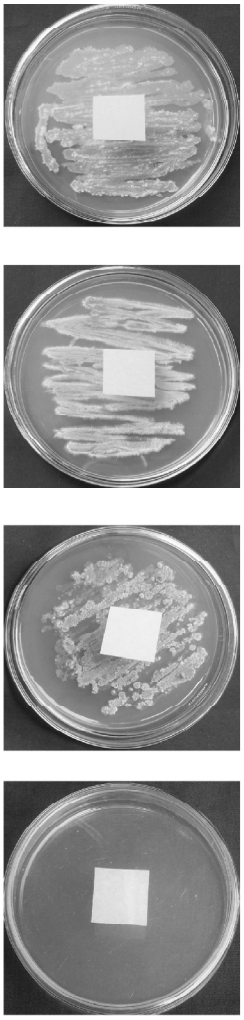

Plate 5: HCN production by *Bacillus* sp.

HCN in Biological Control

The cultures turned yellow color to brown or orange which confirmed HCN. The result was same as like that of siderophore production. The ability to produce HCN and siderophore was associated with their role as growth promoters (Schippers, 1993). HCN is involved in the ability of CHA0 to protect tobacco against black root rot (Voisard *et al*., 1989). Similar finding were reported by Kalpana (2008).

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