

Antifungal properties exhibited by bacteria isolated from agriculturally cultivable soils and their antagonistic nature towards fungal phytopathogen suppression

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Received: 21-05-2015

Accepted: 28-01-2016

DOI: 10.18805/asd.v35i1.9304

ABSTRACT

Soil samples collected from rhizosphere soil, cultivable field soil, Rhizoplane soil of paddy plants and were used to isolate bacteria by culture methods of which thirty two bacterial cultures were identified with diverse colony characteristics on nutrient agar medium and biochemical characterization. Sixteen bacterial isolates were selected for screening fungal antagonism on PDA and were further tested against *Fusarium* and *Rhizoctonia species*. In this study, two *Pseudomonas strains* (RB15, RB30) and *Bacillus cereus* (RB13) were cyanogen producers. Both strains of *Pseudomonas* RB15 and RB30 and *Bacillus cereus* (RB13) were also siderophore producers. Only three isolates showed chitinolytic activity; *Serratia marcescens* (RB24), *Bacillus cereus* (RB13) and *Enterobacter cancerogenus* (RB17).

Key words: Biocontrol, PDA (Potato dextrose agar), Phytopathogens.

INTRODUCTION

Naturally occurring bacteria have been suggested as a replacement for supplements and chemical pesticides to control plant diseases (Bano and musarrat, 2003). A number of bacterial species have received attention as biocontrol agents due to their catabolic versatility, excellent root colonizing ability, and production of a wide range of antifungal and antibacterial metabolites (Nelson *et al.*, 1983). The beneficial effects of biocontrol PGPB towards various phytopathogens can be explored for sustainable agricultural methods (Cavender *et al.*, 2003).

Selective procedures to differentiate organisms on morphological, biochemical, and growth phenotypes coupled to molecular identification are important to identify “exotic” types of bacteria. Thus identification of bacteria, based on the phenotypic characteristics, can sometimes be achieved by direct comparison of unknown bacteria with known type cultures (Holt *et al.*, 2000).

Some biocontrol PGPB have been found to produce enzymes including β -1,3 glucanase, chitinase, protease, cellulase, amylase that can lyse fungal cells and organic matter (Davis and Henrissat 1995). Chitin is a homopolymer of beta 1-4 linked N-acetyl D-glucosamine residues and can be degraded by microbial chitinases (Davis and Henrissat, 1995). These chitinases act mainly to degrade chitin for utilization by the bacteria as an energy source. In addition some chitinases of chitinolytic bacteria such as *Serratia marcescens* and *Enterobacter agglomerans* are potential

agents for the biological control of plant diseases caused by various phytopathogenic fungi (Zhang, *et al.*, 2001). The enzymes inhibit fungal growth by hydrolyzing the chitin present in the fungal cell wall.

The present work was carried out to isolate bacteria from soil, characterize biochemically and screen for antifungal traits.

MATERIALS and METHODS

Collection of soil samples: Soil samples were collected from agricultural regions of Samalkot, Pithapuram, Rajupalem, Kakinada areas of East Godavari District, Andhra Pradesh, India. Agriculturally cultivable field soils, paddy fields cultivated with pigeon pea, were dug into 60 cms depth for collection of soil samples. The sub surface soils from three different slots were collected and mixed in the sterile black polythene bags. Large and hard soil particles were removed and padded through a 2mm sieve to collect fine soil particles and stored at 4°C for 7-10 days in sterile ziploc polythene bags. Their physico-chemical characteristics such as pH, EC (Electrical conductivity), Nitrogen content, available phosphorus and potassium were analyzed at SIFT (State Institute of Fisheries Technology), Kakinada, Andhra Pradesh.

Enumeration of bacteria and biochemical characterization:

The bacterial colonies obtained in different dilutions were counted using a graduated Quebec colony counter (Dalal). The number of colonies were counted using the Misra and Miles drop count method (1938) on plates containing

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between 30 -300 cfus. Serial dilutions were used to determine the number of bacteria in the original sample and were expressed as colony forming units (cfus) per gram of soil.

$$\text{Colony forming units} = \frac{\text{Number of colonies}}{\text{Dilution}} \times \text{Volume factor}$$

Bacterial enumeration was performed for all soil and compost samples that were diluted and tabulated as the average cfu/gm sample.

Biochemical characterization of bacteria isolated from soil is performed by using KB002 Hi Assorted Biochemical test Kit for gram negative rods and gram positive rods.

This is a standardized colorimetric identification system utilizing seven conventional biochemical tests and five carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation the organisms undergo metabolic changes which are indicated by a color change in the media that can be either interpreted visually or after addition of the reagent.

The organisms are cultured and purified. The purified inoculums in incubated in the test kit for 18-24 hours at the temperature of 37°C. Results are interpreted.

Antifungal traits of bacteria : Qualitative cyanide determination was carried out by Lorck (1948) method modified by Alstrom (1989). Qualitative cyanogen production was determined by inoculating each isolate on Nutrient Agar medium supplemented with glycine (4gms/l). The cultures were spot inoculated on the NA plates. A filter paper after soaking in picrate/ Na_2CO_3 was placed in the petri plate cover lid that was placed over the Petri plate. The Petri dish was sealed with parafilm and incubated at 28°C for 24 and 48 hours. A change of filter paper color from yellow to orange, red, brown reddish brown was recorded as an indication of weak, moderate and strong cyanogenic potential of the bacterial cultures respectively and was recorded. The experiment was repeated 3x for all the sixteen cultures. A qualitative method of screening for the chitinolytic activity by bacteria was performed as described by Cattelan *et al.*, (1999). Bacterial chitinolytic activity was tested by plating bacterial isolates on sterile chitin agar medium. Bacterial test cultures were spot inoculated on colloidal chitin media and incubated at 30°C for 48 hours. Colonies showing zones of clearance against a creamy background were regarded as chitinolytic bacteria producing chitinase. The results were tabulated as positive or negative from the experiment. The experiment was repeated 3x.

RESULTS AND DISCUSSION

Physicochemical characteristics of soils and composts:

Analysis of the physicochemical characteristics of the soil and compost samples used for bacterial isolation is presented in Table 1. All soils and composts tested showed nearly

TABLE 1: Physico-chemical characteristics of Soils & Compost samples

Physico-chemical properties	*RS	*RPS	*CFS
pH	7.1±0.02	6.9±0.03	6.8±0.01
Soil EC mM	0.04±0.01	0.05±0.00	0.03±0.01
Total N%	2.38±0.01	2.87±0.02	1.45±0.03
P205 kg/ha	11.88±0.13	12.14±0.11	11.16±0.11
K kg/ha	148.38±0.04	154.32±0.58	148.8±0.45
Organic C%	13.47±0.18	14.08±0.21	12.48±0.21

*Each value is a mean of triplicate values

RS - Rhizosphere soil

RPS - Rhizoplane soil

CFS - Cultivable field soil

neutral pH, with the exception of RSC which is lowest. Slight variations are recorded for electrical conductivity of composts and soils. Nitrogen content was found more in Rice straw compost (RSC) among the composts and in rhizoplane soil (RPS) among soils lowest was CFS. Available phosphorus levels were found to be slightly more in compost samples than in soils, whereas potassium and organic carbon levels were found to be slightly more in soils compared to composts (Results compared with standard values obtained from Agriculture Research Station, Kakinada). GVC was rich in all the three minerals (N, P, and K) that were tested. Overall, the tested parameters of the samples are not very different from standard values.

Bacterial enumeration of soils and biochemical

characterization: Bacteria were enumerated and evaluated as colony forming units per gram of soil (cfus/gm) and were found to be highest in the Rhizoplane soil compared to Rhizosphere and Cultivable field soils which had moderate bacterial counts (Table 2). The distribution of bacterial genera most numerous in all the composts and soils as recorded in Table 2 *Bacillus* and *Pseudomonas* species were the most numerous in composts and soils were seen. In soils, *Bacillus* sps., were predominant followed by *Pseudomonas* and *Enterobacter*.

TABLE 2: Gram nature and Genera wise distribution of bacterial isolates

Bacteria	Soil samples (cfu/gm)		
	RS	RPS	CFS
*Bacteria	3.5X10 ⁶	4X10 ⁶	2.4X10 ⁶
Gm +ve rods	41±0.33	32±0.58	38±2.31
Gm -ve rods	26±1.75	28±0.23	22±1.76
<i>Bacillus</i>	19.1±0.58	36.1±0.67	31.2±0.9
<i>Enterobacter</i>	17.0±1.88	16.5±1.33	12.1±3.3
<i>Serratia</i>	10.7±3.23	10.0±0.16	8.6±0.11
<i>Klebsiella</i>	18.04±1.12	11.2±2.31	14.0±1.6
<i>Pseudomonas</i>	12.1±0.53	13.3±0.11	16.6±2.3
<i>Azotobacter</i>	4.4±2.53	4.6±0.03	5.9±0.03
<i>Azospirillum</i>	13.0±1.75	6.0±1.33	5.3±0.51
<i>Rhizobium</i>	7.8±2.21	4.6±0.01	8.2±0.03

Fungal antagonistic traits : HCN production by the bacterial isolates was recorded as a change in color of the filter paper from yellow to orange brown. The isolates of genera *Pseudomonas* (RB11, RB15, and RB22) were the best HCN producers compared to the other genera tested. The HCN producers were categorized based on the filter paper color change i.e. rapidly in 24 hours to brown (*Pseudomonas* isolates) or slow change of color from yellow to orange in 48 hours (*Bacillus subtilis* RB1 and *Bacillus cereus* RB13).

Chitinase production was observed via zones of chitin hydrolysis amended medium. Clearing of plates containing colloidal chitin as a sole source of carbon by the bacterium around the colony was used to measure chitin hydrolysis. Only three isolates showed chitinolytic activity; *Serratia marcescens* (RB24), *Bacillus cereus* (RB13) and *Enterobacter cancerogenus* (RB17). The isolates of genera *Bacillus* and *Pseudomonas* show siderophore production.

Higher the value of soil organic carbon matter, the higher the levels of nutrient turnover. In the present study, the rhizoplane soil was reported with the highest potassium levels compared to other soils and composts and this may be due to the increased of cation Exchange Capacity (CEC) of the rhizosphere region which can hold more exchangeable K^+ by mass action as proposed by Bibuthi and Dkhar (2011). Increased N, P, and K concentrations in soils after application of composts was shown in the studies of Hontzas *et al.*, (2004). The mineral contents of composts and soils may vary based on organic matter, carbon substrates and microbial activity (Crecchio *et al.*, 2004).

Biochemical characteristics: The major genera identified in our studies biochemically were *Bacillus*, *Pseudomonas*, *Serratia*, *Enterobacter* and *Klebsiella*. These genera were also found in composts and soils in the studies of Aira *et al.*, (2002). *Klebsiella panticola* was positive for methyl red, while *Klebsiella oxytoca* was positive in urease tests. *Serratia*, *Bacillus* and *Pseudomonas* were positive for enzyme activities including catalase and oxidase. *Bacillus* and *Enterobacter* were positive for amylase production. Thus, the biochemical tests allowed for a robust identification of bacterial genera and often species, in our sample and others.

Antagonistic traits of bacteria : Biological control using microbial antagonists provides an alternative to the use of pesticides for controlling plant diseases. Inhibition of growth of phytopathogens can be due to the production of siderophores, antibiotics, secondary metabolites, and lytic enzymes by microorganisms (Hameeda *et al.*, 2006, Gomathi and Ambikapathy 2011).

In this study, two *Pseudomonas* strains (RB15, RB30) and *Bacillus cereus* (RB13) were cyanogen producers (Table 3a, 3B). Both strains of *Pseudomonas* RB15 and RB30 and *Bacillus cereus* (RB13) were also siderophore producers. According to Annette Wensing *et al.* (2010) *Pseudomonas syringe* produced only siderophores whereas *P. aeruginosa*, *P. fluorescence* produced siderophore and HCN (Table 4). It appears that siderophore production is not directly related to cyanogens production. Voisard *et al.*, (1999) showed iron efficiency is important for HCN production.

In our study, *Pseudomonas* (RB15) produced a color change on filter paper soaked in picric acid via HCN production at early stages of incubation, while *Bacillus cereus* (RB13) produced the change of color at later stages of incubation. Our studies indicate that iron availability had a stimulatory effect on HCN production by both *Pseudomonas* and *Bacillus* bacteria. Voisard *et al.*, (1999) showed that cyanogenesis is minimal during bacterial transition from exponential to stationary phases of growth change and cyanogen production depended on environmental factors such as iron and phosphorus availability.

Bacillus cereus RB13, *Enterobacter cancerogenus* (RB17) were chitinase producers, forming halos of clearance on chitin media i.e. chitinases from these bacteria could utilize the chitin in the media to form clear halos. These organisms were also shown to be gelatin liquefiers enabling their proteolysis. The studies of Wang *et al.*, (2002) showed similar conclusion that chitinolytic bacteria were often also proteolytic.

CONCLUSIONS

Gram positive rods were abundant bacteria in all the soils and composts in our study. *Bacillus* and *Pseudomonas* were found to be the major genera found. The thermophilic and halophilic natures of these bacteria may explain their abundance in the composts and soils used in this study.

Thirty two isolates belonging to eight genera were identified based on colony and microscopic characteristics. The bacteria were further identified to the species level using biochemical tests.

Pseudomonas fluorescens and *Bacillus subtilis* *Bacillus cereus* showed predominant antifungal traits of chitinase and HCN activity.

FUTURE RESEARCH

The bacterial cultures should be further tested for qualitative and quantitative assays for fungal growth inhibition of mycelial mats. The fungal cultures which could be inhibited by bacteria either by the disruption of mycelial filaments or fungal growth biomass should be tested for

TABLE 3a: Biochemical characterization of the bacterial isolates from soils and composts

Name of the test	RB1	RB2	RB3	RB4	RB5	RB6	RB7	RB8	RB9	RB10	RB11	RB12	RB13	RB14	RB15	RB16
Motility	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+
Catalase	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Voges Proskauer	+	+	+	-	+	+	+	+	-	+	D	-	+	D	D	D
Citrate utilization	+	+	+	-	+	+	-	+	-	+	-	-	+	-	-	-
Starch hydrolysis	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Indole	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+
Gelatin hydrolysis	-	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-
Methyl red	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-
Nitrate reduction	-	+	+	-	+	+	-	-	-	+	+	-	-	+	+	+
Ammonification	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+	-
Urease	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	+
Oxidase	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+
H ₂ S	-	+	-	+	+	+	-	-	+	-	-	-	-	+	-	+
Acid production from different carbon substrates																
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
Arabinose	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Mannitol	+	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+
Sucrose	+	-	+	-	+	-	-	+	-	+	-	+	+	-	-	-
Identification of Bacteria	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P

A - *Bacillus subtilis*, B - *Enterobacter aerogenes*, C - *Serratia proteamaculans*, D - *Azospirillum brasilense*, E - *Enterobacter asburiae*, F - *Bacillus licheniformis*, G - *Beijerinckia indica*, H - *Bacillus pumilus*, I - *Enterobacter agglomerans*, J - *Azotobacter chroococcum*, K - *Pseudomonas alkaligenes*, L - *Klebsiella panticola*, M - *Bacillus cereus*, N - *Pseudomonas cepacia*,

TABLE 3 b: Biochemical characterization of the bacterial isolates.

Name of the Test	RB17	RB18	RB19	RB20	RB21	RB22	RB23	RB24	RB25	RB26	RB27	RB28	RB29	RB30	RB31	RB32
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges Proskauer	+	+	+	-	+	+	+	+	-	+	-	-	-	-	-	-
Citrate utilization	+	+	-	-	+	+	-	+	-	+	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	+	+	+	+	+	-	+	+	-	-	+	+	+	-	-
Ammonifiers	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-
Urease	+	-	+	+	-	-	+	+	-	-	+	+	-	-	-	-
Oxidase	-	+	D	+	-	+	+	-	+	+	+	+	+	+	+	+
Acid production from different carbon sources																
Glucose	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Arabinose	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Mannitol	+	-	+	-	+	-	-	+	-	+	-	+	-	+	+	+
Sucrose	+	+	-	+	+	-	+	+	-	+	+	-	-	-	-	-
Identification of Bacteria	A	B	C	D	E	F	G	H	I	J	K	L	M	O	Q	R
A - <i>Enterobacter cancerogenus</i>			B - <i>Serratia marcescens</i>		C - <i>Klebsiella oxytoca</i>					D - <i>Pseudomonas cepacia</i>						
E - <i>Bacillus megaterium</i>			F - <i>Pseudomonas mallei</i>		G - <i>Azospirillum brasilense</i>					H - <i>Serratia marcescens</i>						
I - <i>Sinorhizobium fredii</i>			J - <i>Pseudomonas cepacia</i>		K - <i>Azotobacter beijerinckii</i>					L - <i>Klebsiella oxytoca</i>						

TABLE 4: Siderophore, HCN, Chitinase production by 16 bacterial isolates

BACTERIAL ISOLATES	*Siderophore production	*HCN production	*Chitinase production
<i>Bacillus subtilis</i> (RB1)	-	+	-
<i>Bacillus licheniformis</i> (RB6)	-	-	-
<i>Bacillus pumilis</i> (RB8)	-	-	-
<i>Bacillus cereus</i> (RB13)	+	+	+
<i>Enterobacter agglomerans</i> (RB9)	-	-	-
<i>Enterobacter cancerogenus</i> (RB17)	-	-	+
<i>Klebsiella oxytoca</i> (RB19)	-	-	-
<i>Sinorhizobium meliloti</i> (RB30)	+	-	-
<i>Serratia proteamaculans</i> (RB3)	-	-	-
<i>Serratia marcescens</i> (RB24)	-	-	+
<i>Pseudomonas alkaligenes</i> (RB11)	+	++	-
<i>Pseudomonas fluorescens</i> (RB15)	+	+++	-
<i>Azospirillum lipoferum</i> (RB32)	+	-	-
<i>Azotobacter beijerinckii</i> (RB27)	-	-	-
<i>Azospirillum brasilense</i> (RB23)	-	-	-
<i>Pseudomonas mallei</i> (RB22)	-	+	-

+ positive for presence of trait; - absence of trait*+ weak HCN producer; ++moderate HCN producer; +++strong HCN producer

effective antifungal natures. These cultures once tested quantitatively can be tested *in vitro* and can be used as antifungal agents to suppress fungal diseases caused to pigeon pea and paddy crops.

ACKNOWLEDGEMENTS

Thanks to DST Department of Science and Technology (WOS – B) New Delhi for funding in conduct of this research work.

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