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Original Research Article

Isolation and diversity analysis of Rhizobacteria from sugarcane and its biocontrol potential against *Rhizoctonia solani* – A common plant pathogen

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A B S T R A C T

A total of 298 rhizobacteria were isolated from rhizosphere of sugarcane from three different local agricultural sites of Bardoli area in an attempt to identify their plant growth-promoting rhizobacteria (PGPR) with bio-control potential against *Rhizoctonia solani* bacteria; a common plant pathogen. All isolates were morphologically characterised into different groups on basis of their colony characteristics. Their Gram's sensitive nature and ecological diversity were determined by several diversity indices like Relative Frequency, Similarity Index, Shannon's Diversity Index, Evenness and Richness. The bio-control potential of isolates against *R. solani* was determined by dual culture assay. Out of all the isolates, 3 isolates MP 12, DJ 6 and MP 8 showed maximum inhibition zone of 77%, 74% and 72% against plant pathogen, respectively. The study concluded that these potent strains (MP 12, DJ 6 and MP 8) could be utilized as plant inoculants under greenhouse condition to suppress plant diseases and enhance the production of disease resistance varieties of crop.

Keywords

Bio-control potential, Diversity index, Microbial isolation, Plant pathogen, *Rhizictonia solani*

Introduction

The beneficial plant-microbes interactions in the rhizosphere are determinant of plant health and enhance soil fertility. Plant roots release an enormous amount of root exudates that may represent up to 10–20% of the photosynthethates, leading to a significant stimulation of the microbial density and activity.

In the biogeochemical cycles of both inorganic and organic nutrients in the soil and in the maintenance of soil health and quality, soil micro-organisms are very important (Jeffries *et al.*, 2003). Kloepper and Schroth (1978) have classified bacteria that colonize the rhizosphere of the plant roots and enhance plant growth by any

mechanism as plant growth-promoting rhizobacteria (PGPR). With the increasing global concern for safe food and environmental friendly agricultural input and practices, the use of PGPR as alternative to chemical application for pest and disease control in food and crop production is a novel concept that deserves attention. PGPR have been applied to various crops to enhance growth and crop yield. The use of some PGPR as growth promoter and natural bio-control has been developed and commercialized (Dey *et al.,* 2004; Herman *et al.,* 2008; Minorsky, 2008).

Biological control of plant diseases is gaining attention due to increased pollution concerns because of pesticides use for crop protection and the consequent development of pathogen resistance to such chemicals (Wisniiewski and Wilson, 1992). The use of biological controlled methods in particular the microorganisms have proved useful and fast gaining acceptance for plant-growth promotion and disease control in modern agriculture (Weller, 1988). It is becoming a promising agricultural approach, which plays a vital role in crop protection, growth promotion and biological disease control.

Understanding the diversity of plant-bacterial associations and their role in plant development is necessary if these asso-ciations are to be manipulated to increase crop production, conserve biodiversity and sustain agro-ecosystems. A variety of microbial forms can be found growing in rhizosphere micro-habitats. It is opined that members of any microbial group can develop important functions in the ecosystem (Giri *et al.,* 2005). Biodiversity within a group of organisms may be determined numerically in relation to a variety of parameters (referred to as importance values) including biomass, productivity, and organism count (Odum, 1997). Most estimates of species diversity are determined from species counts, which can be used to generate bio-indices either of species richness, species evenness and or Shannon diversity index.

Rhizoctonia solani, a soil borne plant pathogen which causes root rot, and damages a wide range of host plants by reducing the ability of the plants to take up water and nutrients (Wallwork,1996). The effectiveness of PGPR as biocontrol for soil borne pathogenic organisms has been reported (Jung *et al.,* 2003; Dilantha *et al.,* 2006). However, the beneficial effects of PGPR and its application against soil born pathogen or as biocontrol has not been well documented and reports on its use are scanty in sugarcane cultivation. Sugarcane (*Saccharum* spp), is one of the most important crops in India in particular in the Gujarat region where most folks are involved in its cultivation. Its two main products are crystal sugar and alcohol; a clean biofuel with potential to serve as alternative to fossil fuel. Sugarcane is perhaps the most economically competitive source of ethanol and can effectively contribute to a cleaner environment.

This study therefore attempts to isolate the rhizospheric bacteria from sugarcane and assess the diversity of the isolates by differentiating them on the bases of their morphological features. The study will also evaluate the effectiveness of the isolated bacterial strains as potential biocontrol agent against soil borne pathogen *R. solani*

Materials and Methods

Sample collection, isolation and Gram staining

Soil samples were collected from rhizosphere of sugarcane crop from three different local agricultural sites around

Bardoli area, (Dist Surat), Gujarat, India (Site 1- Vihan village; Site 2- Afva village and Site 3- Ena village). The samples were collected aseptically and immediately transported to lab under cold condition (4^oC) for further process. One (1) g of each soil sample was suspended in 5 ml autoclaved distilled water. After sedimentation of solid particles, the natant (suspension) were serially diluted to 10^{-8}. 0.1 ml of last 3 serial of dilutions were spread by L-shape glass rod on nutrient agar plates supplemented with 70 µg/ml of fungicide (Bavistin) to inhibit the fungal growth and was incubated at 26–28^oC in an incubating chamber. After 3–4days of incubation, morphology and texture of each colony was observed and recorded. On the basis of colony morphology, colonies were selected and further purified by repeated subculturing and maintained on nutrient agar media and stored at 4°C until used. Each strain as obtained from the colonies was characterized by Gram staining method.

Diversity statistics

The diversity of the isolated rhizospheric bacteria was evaluated using Diversity Indices as described by Hajela and Dave (2011).

(i) The Relative Frequency (RF) of occurrence of a bacteria species was determined by:

RF = No. of samples (or) sites in which species is present x100

Total number of samples

Based on value of occurrence, RF was interpreted as 0–25% (just present); 26–50% (common constituents); 51–75% (important) and 76–100%(dominant).

(ii) The Similarity Index (SI) to assess similarities between the crop plants with respect to isolates was determined by the relationship {SI = 2C/(A+B). Where, A and B are the number of varieties obtained from rhizosphere samples between crops and C is the number of varieties common to all the crops.

(iii) Shannon's Diversity Index (H') developed by Shannon Weiner was calculated to assess the diversity of sites for all three crops. The H' values were estimated using the relationship

$$H' = -Epi(lnpi)$$

Where p is the ratio of the number of isolates of i^{th}variety to the sum of isolates of crop plants i.e. (ni/N)

(iv) Evenness (E) and Richness (R); As diversity is composed of two elements (a) evenness (b) richness, the two components were estimated to underscore the diversity that existed among various bacteria from rhizosphere of sugarcane. The two parameters were calculated using the relationships.

Evenness, $\{E_{pielou} = H'/ln(S)\}$ and,
Richness given by $\{R_{menhiniek} = S/ (n)^{1/2}$

Where *H'* is Shannon Weiner Index**,** *S* is number of varieties and *n* is total number of isolates obtained from a crop plant.

***In vitro* Analysis of Biocontrol Potential by dual culture assay**

The *in vitro* inhibitions of mycelium growth of *R. Solani* by the rhizospheric bacterial isolates were tested on potato dextrose agar (PDA) media. A 6 mm agar disc of *R. solani* from fresh PDA culture was placed at the 4 corner of Petri dish containing PDA medium. Each bacterial isolates were placed at centre of Petri plate. A control experiment

was set up by placing a 6 mm agar disc of *R. solani* on PDA medium in absence of any isolates. The plates were incubated at 25 ± 2^{o}C for 6–7 days and the antagonist activity of the isolates was assessed. The growth zone of the fungal pathogen in presence and absence of bacterial colony were recorded. From the data obtained from growth zones, the percentage growth inhibition was calculated as:

$$\% \text{ Inhibition} = [(R_1 - R_2)/R_1 \times 100]$$

Where R_1 = control (growth zone of pathogen) and R_2 = treated (growth zone of pathogen against test bacteria).

Results and Discussion

A total of 298 rhizospheric bacterial isolates were obtained, 178 of the isolates were found in the sample from site1. Based on their colony characteristics, the isolates were classified into 15 different groups. Similarly, a total of 65 isolates were obtained in the sample from site 2 and were classified into 6 types/groups. From samples collected from site 3, a total of 55 isolates falling into six distinctive groups were isolated (Table 1).

These bacterial isolates were predominantly round-shaped while a few of them were slightly curved and amoeboid. The colony colour of isolates varied from white/off-white to bright/dark yellow; with few having green and orange colour (Table 1). The purified isolates from different sites 1 were coded as MP1 to MP15; Site 2 – PM1 to PM6; and Site 3 isolates were – DJ1 to DJ6. Details of colony characteristics are presented in Table 1.

On the basis of Relative Frequency (RF) values obtained, the isolates were distributed into three groups as shown in table (Table 2). Similarity Index (SI) results of three sugarcane plants calculated using Sorensen coefficient index showed that similarity and matrix of the isolates, crop plants from the 3 different sites is in the trend of site 2–3 > site 1–2 > Site 1–3 (Table 3 and 4). Figure 1 showed results of *in vitro* inhibition of mycelium growth of *R. solani* by the isolated strains on PDA media. Among eight isolates which reflected significant inhibition (%> 50), maximum inhibition of *R. solani* mycelia growth was observed in MP12 (77%) as shown in Figure 2. Control plates not treated with the PGPR isolates were completely covered by the phytopathogen showing no inhibition.

In present study, isolated bacterial strains from three different sites were distributed into total of 27 different typology based on their colony characteristics. A further biochemical and molecular characterization may be necessary to identify the species. Results of diversity studies revealed that similarity index (SI) was highest between plants of site 2–3 and the least similarity was between sites 1–3. Consequently, site 1 represented maximum diversity and richness, whereas site 2 had the least. However, with respect to evenness site 3 showed maximum evenness. Thus, site 1 exhibited maximum diversity and richness.

The PGPRs promote plant growth through more than one mechanism that includes secretion of variety of growth stimulating hormones and suppression of plant growth retarding agents that are pathogenic. All isolates were tested for antifungal potential against phytopathogen *R. solani*. The *in vitro* test against *R. solani* revealed that some bacterial isolates were highly inhibitory to *R. solani* growth whereas others were mild or showed no activity at all. This suggests that the mode of action exerted and the type of antifungal metabolites produced by the isolates vary as previously opined by Parke (1991).

Moreover, from the results, eight isolates among all tested, considerably inhibited the mycelial growth of *R. solani in vitro* in the ranking order of MP12>DJ6>MP8> MP1=PM1=DJ1>MP2>MP5. These isolates may further be investigated for their ability to control Rhizoctonia disease in crops. In addition, the isolates may be subjected to further investigation for identifying and understanding the antagonist mechanism, whether it is due to production of diffusible antibiotics, volatile antibiotics and secretion of enzymes involved in bio-antagonism or inhibitory effect of Fe^{3+} in the antagonist. Similar study using *Pseudomonas* spp as a biocontrol against *R. solani* was undertaken and *Pseudomonas aeruginosa* has also been reported to reduce growth of *R. solani* (Podile *et al.* 2005). *Pseudomonas fluorescens* act as both growth promoting and biocontrol against *R. solani* and also *F. oxysporium*. The biocontrol activity of bacterial antagonists such as *P. syringae* and *P. fluorescens* against several important plant diseases have been attributed to the production of antifungal metabolite (Garavel *et al.*, 2005). For example many *P.fluorescens* produce pyrrolnitrin that has strong antifungal activity. Mikani (2008) demonstrated that antagonist activity of *Pseudomonas sp* was due to the production of antibiotic, volatile compounds and siderophore. The present work obtained eight (8) isolates that are beneficial and efficient in the control of *R. solani* from the rhizosphere of sugarcane. There is however the need to investigate the mechanism of antagonism of the isolates against the pathogen.

Table.1 Colony characteristics of isolates form rhizosphere of sugarcane from three different sites

Rhizospheric bacterial isolates from site 1									
Isolate	Total no.	Size	Shape	Edge	Elevation	Pigment	Surface	Opacity	Gram's nature
MP1	6	Medium	Round	Entire	Flat	White	Smooth	Translucent	+ve
MP2	1	Small	Round	Entire	Flat	White	Smooth	Translucent	+ve
MP3	4	Big	Irregular	Wavy	Flat	White	Contoured	Opaque	+ve
MP4	2	Medium	Irregular	Wavy	Flat	yellow	Contoured	Translucent	+ve
MP5	11	Medium	Round	Entire	Convex	Watery	Smooth	Opaque	-ve
MP6	14	Big	Amoeboid	Lobate	Flat	Milky	Corrugated	Opaque	+ve
MP7	1	Verybig	Round	Entire	Flat	White	Smooth	Opaque	+ve
MP8	5	Medium	Round	Entire	Convex	yellow	Smooth	Opaque	+ve
MP9	13	Small	Round	Entire	Convex	Yellow	Smooth	Transparent	+ve
MP10	7	Medium	Round	Entire	Flat	White	Smooth	Translucent	+ve
MP11	18	Medium	Round	Entire	Convex	Milky	Smooth	Translucent	+ve
MP12	35	Medium	Round	Wavy	Convex	Orange	Smooth	Opaque	+ve
MP13	25	Medium	Round	Entire	Convex	Creamish	Smooth	Opaque	+ve
MP14	15	Medium	Round	Entire	Convex	Creamish	Smooth	Opaque	+ve
MP15	21	Big	Round	Entire	Convex	Cream	Smooth	Opaque	+ve

Rhizospheric bacterial isolates from site 2									
Isolates	Total no.	Size	Shape	Edge	Elevation	Pigment	Surface	Opacity	Gram's nature
PM1	6	Medium	Round	Entire	Flat	White	Smooth	Translucent	+ve
PM2	4	Pin point	Round	Entire	Convex	White	Smooth	Translucent	+ve
PM3	25	Big	Amoeboid	Lobate	Flat	Milky	Corrugated	Opaque	+ve
PM4	10	Big	Round	Entire	Convex	Yellow	Smooth	Opaque	+ve
PM5	2	Medium	Round	Entire	Raised	Orange	Smooth	Opaque	+ve
PM6	18	Big	Round	Entire	Convex	Cream	Smooth	Opaque	+ve
Rhizospheric bacterial isolates from site 3									
Isolates	Total no.	Size	Shape	Edge	Elevation	Pigment	Surface	Opacity	Gram's nature
DJ1	3	Medium	Round	Entire	Flat	White	Smooth	Translucent	+ve
DJ2	18	Big	Amoeboid	Lobate	Flat	Milky	Corrugated	Opaque	+ve
DJ3	8	Medium	Round	Entire	Flat	Orange	Smooth	Translucent	+ve
DJ4	8	Big	Round	Entire	Convex	Yellow	Smooth	Opaque	+ve
DJ5	6	Small	Round	Lobate	Flat	White	Corrugated	Translucent	+ve
DJ6	12	Medium	Round	Entire	Convex	Cream	Smooth	Translucent	+ve

Note: Similar coloured on the rows indicate similarities in morphological characteristics among bacterial isolates from different sites.

Table.2 Relative frequency (RF) of bacterial isolates from rhizosphere of sugarcane plant from 3 sites

Isolates			RF value (%)	Grade & Interpretation
Site 1	Site 2	Site 3		
MP1, MP6	PM1,PM3	DJ1,DJ2	100	Dominant
MP11, MP15	PM4,PM6	DJ4,DJ6	66.6	Important
MP2, MP3, MP4, MP5, MP7, MP8, MP9, MP10, MP12, MP13, MP14	PM2,PM5	DJ3,DJ5	33.3	Common Constituent

Group: I: (0-25%) Just present; II: (26-50) common Species; III: (51-75) Important species; IV: (76-100) Dominant Species.

Table.3 Similarity Index between three sugarcane plants

Sites	Site 1	Site 2	Site 3
Site 1	1	0.28	0.19
Site 2		1	0.50
Site 3			1

Note**:** Diversity, Richness and Evenness was calculated by Shannon's Diversity Index (H'); Richness as $R_{Menhinick}$ and Evenness as E_{Pielou} indices

Table.4 Indices representing Diversity, Richness and Evenness of isolates from 3 sites

Sites	Number of Varieties	ShannonWeiner Diversity Index(H')	Richness($R_{Menhinick}$)	Eveness (E_{Pielou})
Site 1	15	**2.36**	**1**	0.87
Site 2	6	1.49	0.74	0.83
Site 3	6	1.64	0.80	**0.91**

Fig.1 Efficiency of inhibition of mycelial growth of *R. solani* by some isolates from rhizosphere of sugarcane collected from three different sites

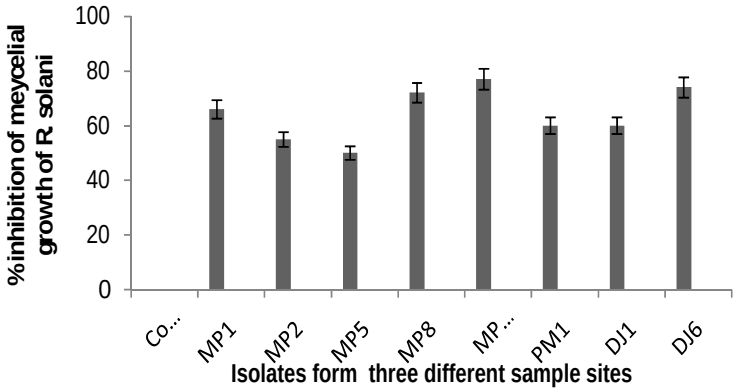
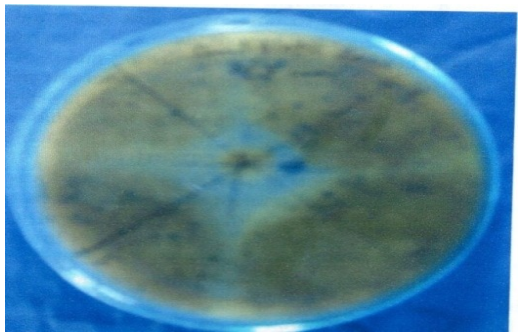


Fig.2 Strain MP12 of isolate from site 1 showing mycelia growth inhibition against *R. solani*



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