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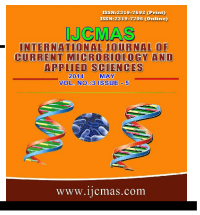

Original Research Article

In vitro antagonistic activity of diverse bacterial isolates against Macrophomina phaseolina (Tassi) Goid

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A B S T R A C T

Macrophomina phaseolina (Tassi) Goid is a fungus that causes charcoal root rot in many plant species and is considered as one of the most important pathogens in forest nurseries. In our studies in vitro antagonistic activity of diverse bacterial isolates against M. phaseolina were carried out. A total of 219 bacterial strains were isolated from the rhizosphere soil samples of some medicinal and aromatic plants viz., Coleus forskohlii, Andrographis paniculata, Withania somnifera, Ocimum sanctum, Aloe vera, Mimosa pudica, Artemisia vulgaris, Acorus calamus and Mentha spicata were collected from different locations in Andhra Pradesh. All the isolates were screened for their antagonistic activity against M. phaseolina. Among the 219 isolates 43 strains were showed antagonistic activity against pathogen but one isolate was showed maximum inhibition (52.22%) against mycelial growth of the pathogen by dual culture plate technique. On the basis of colony morphology and biochemical characterisitics the isolate was identified as Bacillus sp. and further it was characterized through 16S rRNA gene sequencing which led to their identification as Bacillus subtilis (Cf 60). Bacillus species were identified as potential biocontrollers of M. phaseolina which present a background of biological control of diverse plant pathogens. In view of these, the apparent bacterial biocontrol agents could provide a mean for reducing the disease incidence in addition to avoiding the use of fungicides. Such biocontrol approach should be employed as a part of integrated disease management system.

Keywords

Antagonistic activity, medicinal and aromatic plants, *Bacillus sp. M. phaseolina*

Introduction

The *Macrophomina phaseolina* (Tassi) Goid. fungus is the causal agent of charcoal root rot, a worldwide pathology affecting agricultural and forest crops (Shaner *et al*., 1999). Management of soil borne pathogens has become one of the major concerns in agriculture due to great harms caused by chemicals used to control soil-borne pathogens, to environment and focused on searching and selecting antagonist microorganisms on diverse soil pathogens. Among the most used are bacterias like *Bacillus*, *Pseudomonas*, and *Streptomyces*, fungi of the *Trichoderma*, *Penicillium*, *Gliocladium*, *Aspergillus*, *Rhizopus* genera. These microorganisms, natural inhabitants of diverse substrate s, in laboratory tests (*in vitro*) as well as in

the greenhouse and field, have demonstrated antagonistic activity on a wide ranging group of pathogens such as *Sclerotium rolfsii, S. cepivorum*, *Rhizoctonia solani*, *Pythium ultimum*, *Phytophthora parasitica*, and *M. phaseolina* (Bell *et al*., 1982; Balasundaram and Sarbhoy, 1988; Harrison and Stewart 1988; Hussain *et al*., 1990; Adekunle *et al.*, 2001; Singh *et al*., 2008).

In agriculture bacteria, belonging to the genera *Bacillus* has shown effectiveness in the bio-management of different crops. Among the bio-control bacteria, *Bacillus* has become the bacterium of the choice for its versatility and ability to contain a large number of plant pathogens in diverse target environments. Various *Bacillus* isolates are recorded for the control of diseases caused by phytopathogenic fungi (Schisler *et al*., 2004). The application of *Bacillus* reduces incidence of *R. solani*, *Pythium* sp., and other pathogens, as well as stimulating seed germination, plant growth and yield (Kloepper, 1998). They also have the capacity to colonize plant roots, since *Bacillus* is considered as plant growth promoting rhizobacterium (PGPR) (Turner and Backman, 1991). The objectives of this study were to isolate and identify the bacteria which is potential in the control of *M. phaseolina* and molecular characterization at the species level that have potential as biocontroller of the pathogen.

Materials and Methods

Isolation of microorganisms from rhizosphere of medicinal plants

Samples of rhizosphere soils were collected from different medicinal plants grown at the botanical garden, Osmania University, Central Institute of Medicinal and Aromatic Plants (CIMAP) centre, ANGRAU, Hyderabad, India. Intact root system was dug out and the rhizospheric soil samples were carefully taken in plastic bags and stored at 4°C. A total of 25 soil samples were collected from the different medicinal plants located in various regions for the isolation of rhizosphere bacterial isolates.

Rhizobacteria (PGPR) were isolated from the rhizosphere soil samples by serial dilution plate technique (Aneja 2003). Samples were serially diluted with sterile distilled water (10^{-1} to 10^{-7}) and each dilution was used for pouring on nutrient agar plates. After incubation for 48 h at 30°C, colonies were picked from these plates and maintained as pure cultures in nutrient agar slants with periodic transfer to fresh media. The bacterial strains were screened for antifungal activity by using dual culture plate technique.

Antifungal Activity

Macrophomina phaseolina was isolated from diseased plants by using PDA (potato dextrose agar) medium. The pathogen was identified using standard mycological literature. The bacterial isolates were screened for the ability to inhibit *M. phaseolina* by employing dual culture method (Paul *et al.*, 2007) on PDA plates. The bioagent and the pathogen were inoculated side by side on a petri plate containing solidified PDA medium. The width of the inhibition zones between the pathogen and bacteria was categorized as strong, moderate and weak. Three replications were maintained for each isolate with one control by maintaining only pathogen. They were incubated at 28^0 C. Observations were recorded when there was a full growth of pathogen in the control plate (4-7 days). The diameter of

the colony of the pathogen was measured in both directions and average was recorded and the per cent inhibition on growth of the test pathogen was calculated by using the formula given below by Rabindran and and Vidyasekaran (1996).

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent inhibition
C = Radial growth of the pathogen in control
T = Radial growth of the pathogen in treatment

Identification of bacterial isolate

Characterization of selected bacterial isolate by using conventional methods like morphological characters, cultural characteristics on agar plate, growth on broth media was done as described in Bergy's Manual of Systematic Bacteriology (Tein *et al.,* 1979) and the results are presented in Table 2.

Biochemical characterization of bacterial isolate

The biochemical characterization of strain was done by using KB002 Hi Assorted TM Biochemical test kit (HiMedia), the other biochemical tests such as Gram staining, IMViC, catalase tests, gelatin liquefaction, etc. as per the procedures outlined by Aneja (2001) and are listed in Table 3.

16S rRNA Gene sequencing and phylogenetic analysis of *Bacillus* sp

The 16S rRNA gene sequencing was performed by a sequencing service (Macrogen, South Korea). Selected bacterial 16S rRNA was amplified in full length by PCR using two pairs of primers, 518F (CCAg-CAgCCgCggTAATACg) and 800R (TACCAggg-TATCTAATCC) and 27F (AgAgTTTgATCMT-GGCTCAg) and 1492R (TACggYTACCTTgTTA-CgACTT). To evaluate the phylogenetic analysis of 16S rRNA sequences, the resulting sequences were compared with the known sequences using the BLAST function of Gene Bank in the National Center Biotechnology information (http://www.ncbi.nlm.nih. gov). Multiple sequence alignments and consensus sequences were computed using the program CLUSTALW programmed at European Bioinformatics (EBI) site (http://www.ebi.eic.uk/clustalw). The resulted BLAST hits were analyzed for evolutionary significance using tree view programme. Evolution trees for the data sets were inferred from the neighbour-joining methods by using the phylogenetic analysis tool in online. The gene sequence were also submitted to EMBL and accession number was assigned.

Results and Discussion

Medicinal plants support a great diversity of microflora in their rhizosphere including PGPR. The rhizosphere of medicinally and economically important plants was investigated to explore the diversity of plant growth promoting rhizobacteria from different regions of Andhra Pradesh. The rhizosphere soils of medicinal plants i.e. *Coleus forskohlii, Withania somnifera, Ocimum sanctum, Andrographis paniculata, Mentha spicata, Aloe vera, Artemisia vulgaris, Acorus calamus* and *Mimosa pudica* supported a total of 219 rhizobacterial isolates with diversified characteristics suggesting the importance and richness of the niche as a source of plant microbe interactions.

Microbial diversity in soil is considered important for maintaining the sustainability of agriculture production systems. The quantity and activity of microorganisms are a determining factor for the productivity of any kind of soil (Ribeiro, 2011). All the isolated bacterial isolates were screened for their antifungal activity against root rot pathogen i.e. *Macrophomina phaseolina*. Among them 19.6 % (43 strains) showed antagonism against pathogen.

Macrophomina phaseolina (Tassi) Goid. fungus is the causal agent of charcoal root rot, a worldwide pathology affecting agricultural and forest crops (Shaner *et al*., 1999), with more than 500 susceptible hosts (Wyllie *et al.*, 1984). In the last few years, dissemination of the pathogen has been detected from the nurseries to the plantations through asymptomatic plants.

Therefore the antagonistic microorganisms such as bacteria and fungi are an alternative source for controlling these pathogens. *Bacillus* sp. was considered safe biological agents. Different antagonistic studies with *Bacillus* sp. were done (Kim *et al*., 2003; Silo-suh, 1994; Utkhede, 1984). In our studies, 43 isolates were found potential antagonists against *M. phaseolina.* In this screening study, isolates were found potential to antagonize the pathogen at considerable level ranging from 16.66 - 52.22% of inhibition (Table 1).

Antagonistic potential of the isolates was concluded and validated by restriction of the pathogen growth and showed zone of inhibition towards the antagonist as shown in photo-plate of dual culture plate assay (Fig. 1) compared to the control plate. An isolate Cf 60 showed maximum antagonism of 52.22% (Fig. 1) followed by isolate Cf 37 (50.00%), Ap 13 (28.88%), Ac 6, Me 3 showed 25.55% against *M. phaseolina* and remaining isolates showed less antagonism compared to these isolates. Similar type of studies were done by Mallesh *et al*., (2009) and Fravel (1988).

The isolate was motile, rod shaped, Gram positive, produced large, smooth, white colonies with flat edges and elevated centre on nutrient agar. Isolate was positive for utilization of citrate, sorbitol, and negative for lysine ornithine utilization, urease, phenylalanine deamination, nitrate, H_2S production, glucose, adonitol, arabinose, lactose, indole, voges proskaur, gelatinase, methyl red test and positive for catalase activity. These morphological and biochemical activities aided in designating the isolate as *Bacillus* sp. (Table 3). Similar studies were done by Mallesh and Kirankumar (2009, 2007). The isolate was further characterized by 16s rRNA gene sequencing analysis.

Microbial identification by sequencing of 16s rRNA gene is a common identification method of bacterial taxonomists used for a number of years as a measure of DNA similarity between isolates. More recently, 16S rRNA gene amplification and sequence has been used to detect and identify fastidious bacterial pathogens and likely to become an identification tool in clinical laboratory. In the present study the selected strain (Cf 60) was identified as *Bacillus subtilis* from 16S rRNA sequencing. Partial 16S rRNA sequence were submitted to EMBL and accession number was obtained HE659512 (*Bacillus subtilis*).

Table.1 List of the PGPR isolates showing antagonistic activity against *Macrophomiona phaseolina*

S. No.	Isolate	Antifungal activity	Zone of inhibition against M.P (mm)	C (mm)	T (mm)	Zone of Inhibition I (%) (C - T) / C ×100
1	Cf 14	+	2	90	70	22.22
2	Cf 23	++	3	90	65	27.77
3	Cf 24	+	2	90	70	22.22
4	Cf 26	+	2	90	75	16.66
5	Cf 27	+	2	90	70	22.22
6	Cf 31	+	3	90	69	23.33
7	Cf 36	++	3	90	68	24.44
8	Cf 37	+++	28	90	45	50.00
9	Cf 46	+	3	90	70	22.22
10	Cf 60	+++	32	90	43	52.22
11	Oc 1	+	4	90	70	22.22
12	Oc 2	++	7	90	65	27.77
13	Oc 5	+	3	90	69	23.33
14	Ap 2	+	2	90	70	22.22
15	Ap 10	+	2	90	70	22.22
16	Ap 13	++	9	90	64	28.88
17	Ap 14	++	8	90	64	28.88
18	Ws 1	+	2	90	69	23.33
19	Ws 6	+	2	90	70	22.22
20	Ws 8	+	2	90	70	22.22
21	Ws 10	+	1	90	75	16.66
22	Ws 11	+	1	90	75	16.66
23	Ws 24	+	1	90	75	16.66
24	Mp 2	++	7	90	65	27.77
25	Mp 3	+	2	90	70	22.22
26	Mp 8	++	7	90	66	26.66
27	Mp 9	+	1	90	76	15.55
28	Mp 10	+	1	90	75	16.66
29	Mp 18	+	1	90	74	17.77
30	Mp 23	+	2	90	70	22.22
31	Av 9	+	2	90	70	22.22
32	Av 10	+	2	90	70	22.22
33	Ac 3	+	2	90	72	20.00
34	Ac 5	+	2	90	72	20.00
35	Ac 6	++	5	90	67	25.55
36	Ac 11	+	3	90	70	22.22
37	Me 1	+	2	90	70	22.22
38	Me 2	+	2	90	75	16.66
39	Me 3	++	6	90	67	25.55
40	Me 4	+	2	90	70	22.22
41	Me 5	+	1	90	74	17.77
42	Me 6	+	2	90	74	17.77
43	Me 7	+	2	90	73	18.88

I = Per cent inhibition; C = Radial growth of the pathogen in control; T = Radial growth of pathogen in treatment

Table.2 Morphological characteristics of Bacillus sp.

	Morphological characters	**Result**
1	Shape	Long rods
2	Capsules	-
3	Gram stain	Gm +ve
4	Spore stain	+
5	Buds or sheaths	-
6	Motility	Motile
7	**Cultural characteristics on agar plate**	White
8	Colonies	Moderate
9	Growth	Rhizoid
10	Form	Serrate
11	Margins	Flat
12	Elevation	Opaque
	Density	
13	**Growth on broth media**	None
14	Surface growth	Slight
15	Clouding	None
	Sediment	

Table 3. Biochemical characteristics of Bacillus sp.

S. No	**Biochemical test**	**Cf 7**
1	Citrate utilization	+
2	Lysine utilization	-
3	Ornithine utilization	-
4	Urease	-
5	Phenylalanine deamination	-
6	Nitrate	-
7	H_2S production	-
8	Glucose	-
9	Adonitol	-
10	Lactose	-
11	Arabinose	-
12	Sorbitol	+
13	Indole	-
14	Methyl red	-
15	Voges Proskaur	-
16	Catalase	+
17	Gelatinase	-

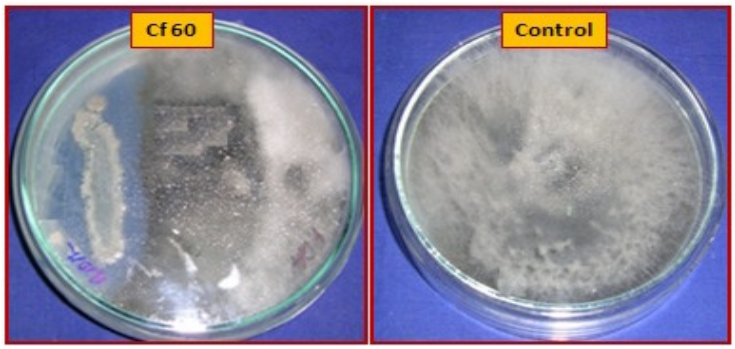

Fig.1 *Bacillus subtilis* (Cf 60) showing zone of inhibition in the dual culture plate assay

It has been determined that *B. subtilis* develops rapidly in culture medium and in nature, produces antibiotics, grows in a wide temperature range, and adapts to various environmental conditions. Furthermore, its metabolites are thermostable and along with stability of the dehydrated antagonist substances are important for its industrialization (Chen and Wu, 1999). It was determined that this species is capable of inhibiting growth of wide range of fungal species (Wilhelm *et al.*, 1998; Li *et al.*, 1998) determining that the biocontrol mechanism is through antibiotic production. The Bacilli are particularly attractive for practical use because they provide endospores which can survive heat and desiccation conditions that may be faced by biocontrol agents (Turner and Beckman, 1991; Lumsden *et al*., 1995; Osburn *et al*., 1995; Sonenshein, 2002). In our studies, among the identified bacteria, *Bacillus* sp. has a background information of being a biological control agent against the pathogen and is considered as a rhizobacterium which promotes plant growth.

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