Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(3): 2155-2158
Received: 23-03-2020
Accepted: 24-04-2020

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Isolation and molecular characterization of *Pseudomonas* spp. with antagonistic activity against *Macrophomina phaseolina*

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Abstract
A study was conducted to isolate and characterize bacterial isolates from Rhizosphere soils with potential for suppression of charcoal rot pathogen *Macrophomina phaseolina* causing charcoal rot maize. Out of 10 bacterial isolates collected from forest rhizosphere soils, 2 isolates were found to be antagonistic to *M. phaseolina* in *in vitro* studies. In these best two isolates were molecularly characterized by using 16s rRNA. Data for bacterial antagonists have been deposited under the following accession numbers: MN985505 (*Pseudomonas aeruginosa*) and MN985650 (*Pseudomonas aeruginosa*.).

Keywords: *Machrophomina phaseolina*, *Psuedomonas aeruginosa*, *in vitro*, 16s rRNA

Introduction
M. phaseolina (Tassi) Goid causes charcoal rot in over 500 crop and non-crop plants like cereal crops, maize, sunflower, soybean, cotton, etc. most often in the countries situated in the tropical and subtropical climatic zones. (Piperkova *et al*., 2016 and Indera *et al*., 1986) [13, 7]. The pathogen is also causative agent for charcoal rot, seedling blight, damping off, root rot, basal stem rot in different crops. The characteristic symptoms that appear after flowering are grey black discoloration and shredding of plant tissue at the stem and top of the taproot with getting hollowing of the stem. When the epidermis is removed, minute black microsclerotia may be so numerous as to give a greyish black look to the tissue (Sinclair, 1982; Yang and Owen, 1982; Hoes, 1985; Kolte, 1985) [15, 18, 6, 11]. The pathogen survives primarily in the form of sclerotium free on the soil or as a sclerotium inside diseased plant tissues. The habitat of *M. phaseolina* (Mp) is a soil-borne facultative parasite of low competitive saprophytic ability (CSA), i.e., it is easily suppressed by the activities of competitive, antagonistic and/or hyperparasitic soil microflora (Reis *et al*., 2014) [14]. Hence the *M. phaseolina* is a wide host range pathogen, the present investigation was aimed to characterise the *Pseudomonads* species from different agro-claimatic zones and its antagonistic activity against *M. phaseolina* was carried out to find the potential *pseudomonas* sp to contain the disease incidence.

Material and methods
Soil sample collection
Ten rhizosphere soil samples were collected from the various forest rhizosphere in different parts of Tamil Nadu *viz.* kolli hills, maruthamalai hills, ooty hills, kodaikanal hills, Mangrove forest and Coastal areas

Isolation of *Pseudomonas* from different rhizosphere soils
For isolation of Rhizobacteria, the method proposed by Vlassak *et al.*, (1992) [17] was followed. In this procedure 10 g of soil was taken from each rhizosphere soil sample separately in a conical flask containing 90 ml of sterile distilled water. These samples were agitated for 15 minutes on a vortex and serial dilutions of each soil suspensions were prepared separately. Dilutions prepared from each above soil suspension for *Pseudomonas* spp. is 10^{-3} to 10^{-7}.
0.1ml of respective dilutions was spread on sterilized Petri plates containing specific medium *i.e.* King's B medium for fluorescein (*Pseudomonas*). The Petri plates were incubated at room temperatures (27°C ± 2 °C) for 24-72 h separately. Three replications were maintained for each dilution. The plates were examined daily up to 3 days after inoculation to record the occurrence of bacterial colonies in each Petri plates.

Enumeration of the organism

The plates incubated for a day at 30 ± 1°C were observed for the growth of *Pseudomonas* colonies and the colonies were enumerated manually and recorded separately.

In vitro* evaluation of antagonistic bacterial isolates against *M.phaseolina

The efficacy of twelve bacterial antagonists was evaluated against *M. phaseolina* for radial growth inhibition on the potato dextrose agar medium using dual culture technique under *in vitro* condition. Observation recorded the mycelia growth of fungus up to three days. For each treatment three replications are maintained. Twenty ml of sterilized and cooled potato dextrose agar was poured into sterile Petri plates and allowed to solidify. In case of evaluation of bacterial antagonist, the bacterium was streaked one day earlier at one end of the Petri plate at the middle of the Petri plate and the test fungus placed at the other end. The plates were incubated at 27 ± 1 ºC and inhibition over control was recorded by measuring the clear distance between the margin of the test fungus and antagonistic organism. The colony diameter of pathogen in control plate was also recorded. The per cent inhibition of growth of the pathogen was calculated by using the formula suggested by Vincent (1947).

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

Where,
I = per cent inhibition
C = growth in control
T = growth in treatment

Identification of endophytic bacteria

DNA from each isolate was extracted using the following protocol: 1.5 ml of a 48-hour bacterial culture was centrifuged for 5 minutes at 14000 *g* and resuspended in 1 ml of TE buffer (mM Tris-HCl, 1 mM EDTA, pH 8.0), centrifuged, resuspended in 500 µl of TE buffer and finally adding 0.5 g of glass pearls (0.1 mm in diameter) (Sigma-Aldrich, USA) and 15 µl of 20% SDS. The cells were then homogenized for 30 s in a vortex mixer (AP56 – Phoenix), 500 µl of buffered phenol was added, and the solution was mixed and centrifuged for 5 min at 14000 rpm. The aqueous phase was extracted once with phenol-chloroform (1:1) and once more with chloroform. Following the extraction of the aqueous phase, 20 µl of 5M NaCl was added, the DNA was precipitated with isopropanol (5 min at room temperature) and collected by centrifugation for 10 min at 14000 rpm. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 30 µl of autoclaved, ultrapure water.

16S rRNA gene region was amplified with the universal primers. For setting up PCR, the following reaction mixtures were added into the PCR tube. The reaction mixtures were 5µl of template, Primers: 1 µl of Forward primer- 27F (5' AGAGTTTGATCCTGGCTCAG 3'), 1 µl of Reverse primer-1492R (5' TACCTTGTTACGACTT 3') (Jereny *et al*., 2008) [8], 6 µl of assay buffer, 2 µl of Taq DNA polymerase and 5 µl of Dntp Mix (J.K.Scientific company, New siddhapudhur, Coimbatore, India.). The amplification was carried out in a thermal cycler for 40 cycles using the following reaction conditions, initial denaturation 95 ºC for 5 minute, denaturation of DNA at 95ºC for 1 minute, primer annealing at 60 ºC for 1 minute, extension at 72 ºC at 2 minute and primer final extension at 72 ºC for 10 minute. The amplified PCR product was mixed with 5 µl of gel loading buffer. 1.5% agarose gel was casted. The samples were loaded along with 5 µl of 1kb DNA ladder (HIMEDIA, Mumbai, Maharashtra, India) as a molecular marker. The gel was run and examined on UV transilluminator to visualize the bands.

Results and discussion

12 bacterial isolates were evaluated against *M. phaseolina* by dual culture technique under *in vitro* conditions. Radial growth of pathogen was measured in cm and per cent inhibition was calculated. The data obtained is presented in Table 1 (Fig. 1). The least growth of the pathogen was observed in KHRB -P2 (41.6mm) followed by MMRB – P2 *(*62.3). Maximum inhibition of mycelial growth of *M. phaseolina* was recorded in KHRB -P2 (5370 %), followed by MMRB – P2 (30.7 %). The present study agreement work with of *Adhikari et al*. (2013) reported that among seventy isolates, antagonistic twenty one representing biovars of *P. fluorescens* (biovars I, II, III, and V) were collected from the rhizosphere of okra, chilli, ground nut, brinjal, cabbage and tomato from different agro ecological regions of West Bengal and were subjected to evaluation for their antifungal activity under *in vitro* condition against *Rhizoctonia solani*, the most important soil-borne plant pathogen. Two isolates of *P. fluorescens* PF-8 and PF-7 effectively inhibited the mycelial growth of *Rhizoctonia solani* in dual culture method. Nine strains of bacteria were isolated from plant roots and rhizosphere regions against *A. porri*. Among the isolated strains tested, *B.lentimorbus* AQ1 had higher antagonistic activity against *A. porri* (Kang *et al.*, 2005) [10]. Partial sequence data for the 16S rRNA genes have been deposited in the NCBI nucleotide sequence data libraries. Data for bacterial antagonist have been deposited under the following accession numbers: MN985505 (*Pseudomonas aeruginosa*) and MN985334 (*Pseudomonas aeruginosa*.) (Table 2; Fig. 2). According to Barrey *et al*., 1991 [3], Jensen *et al*., 1993 [9], Gurtler & Stanisich 1996, 16S-23S rRNA intergenic spacer of the ribosomal RNA opearn (RRN) gene region is used for identification of strains and species. Sequence of highly conserved gene region 16S rRNA data helps us for the prediction of correct taxonomy. Of 100 bacterial isolates assessed for antagonistic activity against *P. aphanidermatum*, 20 were found to be antagonistic, all belonging to *P. aeruginosa*. Previous studies reported antagonistic activity of *P. aeruginosa* against some *Pythium* species including *P. splendens*, *P. debaryanum* and *P. myriotylum* (Anjaiah *et al*., 1998; Tambong and Hofte, 2001; Perneel *et al*., 2007) [16] as well as some *Fusarium* species (Anjaiah *et al.*, 1998).

Table 1: Effect of bacterial antogonist on mycelial growth of *M. phaseolina in vitro*

S. No.	bacterial isolates	Isolated from the tree (Rhizosphere)	Mycelial growth of *M. phaseolina* 3DAI*	Percentage of inhibition over control
1	KHRB – PI	Jack	76.3^{e}(60.86)	15.22(22.96)
2	KHRB - P1	Jamun	70.0^{d}(56.78)	22.22(28.12)
3	KHRB -P2	Banyan	41.6^{a}(40.16)	53.7(47.121)
4	MMRB - P1	Peepal tree	64.6^{bc}(53.48)	28.22(32.08)

5	MMRB - P2	Gulmohar	62.3[b](52.12)	30.7(33.64)
6	OYRB-P1	Rivergrasses	79.0[f](62.72)	12.22(20.46)
7	KKRB-P1	Pine	77.6[ef](62.72)	13.77(21.78)
8	MRRB-P1	Avicennia	65.6[c](54.08)	27.11(31.37)
9	THRB-1	Tamarind	90.0[g](71.56)	0.00(0.00)
10	KVRB-1	Pungam	90.0[g](71.56)	0.00(0.00)
11	PF – 1 (Positive control 1) *P. fluorescens*	-	76.3[e](60.86)	15.22(22.96)
12	PK (Positive control 2) *P. koriensis*	-	70.3[d](56.97)	21.8(27.83)
13	Control	-	90.0[g](71.56)	0.00(0.00)

*Values are mean of three replications.
Figures in parentheses represent arcsine transformation.
Means in a column followed by same superscript letters are not significantly different according to DMRT at $P \leq 0.05$.

Table 2: Molecular identification effective bacterial antagonist using 16s rRNA

Isolate	Identified as	Gene bank Accession number
KHRB -P2	*Pseudomonas aeruginosa*	MN985505
MMRB - P2	*Pseudomonas aeruginosa*	MN985650

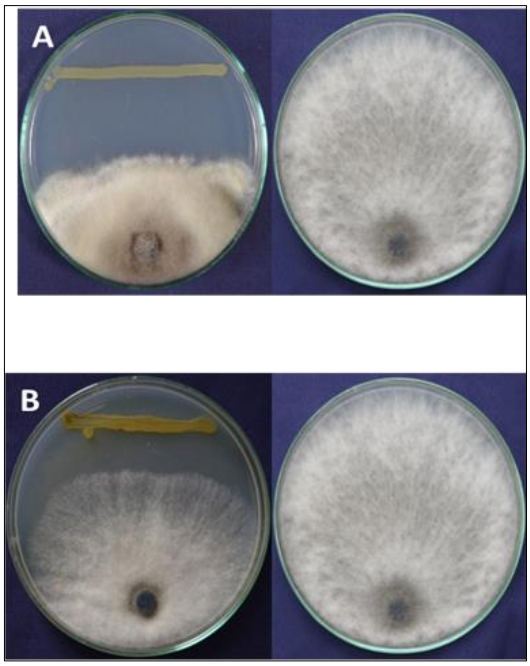

Fig 1.
A. Antagonistic activity of isolate KHRB - P2 against *M. phaseolina* in dual culture on PDA after 3 days of culture at 27 ± 2 °C
B. Antagonistic activity of isolate MMRB - P2 against *M. phaseolina* in dual culture on PDA after 3 days of culture at 27 ± 2 °C

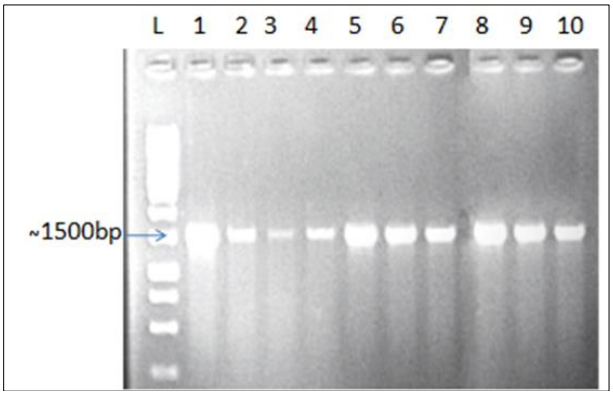

Fig 2: Agarose gel electrophoresis analysis of 16S rRNA genes amplified from two bacterial isolates. PCR amplified products were run on 1.5% agarose gel. Lane L indicates the 1kb ladder (HIMEDIA, Mumbai, Maharashtra, India). Markers with high intensity were indicated by their size. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 indicate the PCR amplified 16S rRNA gene of the KHRB-PI, KHRB-P1, KHRB-P2, MMRB-P1, MMRB-P2, OYRB-P1, KKRB-P1, MRRB-P1, PF-1 and PK of the respective bacterial isolates.

Conclusion

Maximum inhibition of mycelial growth of *M. phaseolina* was recorded in KHRB -P2 (53.70 %), followed by MMRB – P2 (30.7 %). Data for bacterial antagonists have been deposited under the following accession numbers: MN985505 (*Pseudomonas aeruginosa*) and MN985650 (*Pseudomonas aeruginosa*).

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