JJBS

Jordan Journal of Biological Sciences

Volume 12, Number 2,June 2019
ISSN 1995-6673
Pages 219 - 227

Biological Control of *Macrophomina phaseolina* in *Vigna mungo* L. by Endophytic *Klebsiella pneumoniae* HR1

Sampad Dey, Prajesh Dutta and Sukanta Majumdar*

Microbiology and Microbial Biotechnology Laboratory, Department of Botany, University of Gour Banga, Malda, West Bengal-732 103, India

Received July 18, 2018; Revised August 14, 2018; Accepted August 30, 2018

Abstract

An endophytic isolate HR1 from the root nodules of *Vigna mungo* L. was isolated and identified as *Klebsiella pneumoniae* by 16S rDNA sequencing. The isolate can solubilize phosphate, zinc and produce siderophore, IAA, and HCN. Moreover, the isolate can tolerate heavy metal cadmium and produce hydrolytic enzymes protease, amylase and chitinase. *K. pneumoniae* inhibited the growth of *Macrophomina phaseolina* strongly in a paired culture study. The greenhouse study revealed that HR1 significantly increased the germination percentage, shoot and root length, shoot and root dry weight in the treated plants compared to the healthy control and the *M. phaseolina* infected plant. *K. pneumoniae* reduced the occurrence of *M. phaseolina* which induced the root rot disease in *Vigna*. The lowest percentage of disease incidence (18.2 %) was observed when *K. pneumoniae* was applied in dual-mode (seed bacterization + soil drench application) in the *M. phaseolina*-infested soil. Activities of defense-related enzymes such as, chitinase, phenylalanine ammonia lyase, peroxidase and β-1, 3-glucanase increased significantly following the application of *K. pneumoniae*, and challenged inoculation with *M. phaseolina*. Results clearly indicate that the isolate may be used as biocontrol agent to induce systemic resistance to *M. phaseolina*.

Keywords: Endophytic, *Klebsiella pneumoniae*, Root rot, *Macrophomina phaseolina*, Induced systemic resistance

1. Introduction

Vigna mungo (L.) (Black gram), is an important pulse crop occupying a unique position in Indian agriculture and is a major source of protein (24 %) for humans as well as animals (Satyanandam *et al.*, 2013). Most often, for its nutritional values it has been recommended for diabetes. However, the plant is severely affected by charcoal rot, a devastating root rot disease distributed over arid to tropical regions (Cottingham, 1981; Abawi and Pastor-Corrales, 1990). The pathogenic fungus, *Macrophomina phaseolina* (Tassi) Goid (Dothideomycetes, Botryosphaeriales) is responsible for this devastating root rot disease leading to seedling blight in the initial stage, and brown lesions on roots and stems are found later in the mature stages of the plant (Iqbal *et al.*, 2010b). *M. phaseolina* is a soil and seed-borne fungal pathogen, which produces cushion-like black microsclerotia (Wheeler, 1975). Thus, the management of the root rot disease is essential to meet the enhanced requirements of the crop productivity by an actively growing population. To cope with this problem, farmers generally use chemical fungicides mostly non-judicially. Moreover, the prolonged use of chemical fungicides is environmentally not suited because they could generate resistant pathogen (McMullen and Bergstrom, 1999). So, an alternative method needs to be developed for a sustainable and eco-friendly cultivation of black gram.

In this milieu, plant-associated bacteria can play an important role in the biological control of many plant diseases. Recently, lot of attention has been given to the use of these organisms as biocontrol agents as well as plant-growth promoters to reduce the detrimental effects resulting from using chemical fungicides. Bacterial endophytes can be suitable biocontrol agents as they colonize the same ecological niche, which is inhabited by phytopathogens (Berg *et al.*, 2005). After the colonization in inter tissues, these microorganisms produce some important biochemical compounds, and can alter the plant cell metabolism to enhance host resistance to diseases (Krishnamurthy and Gnanamanickam, 1997; Bloemberg and Lugtenberg, 2001; Lugtenberg and Kamilova, 2009). Several endophytic bacteria such as *Pseudomonas, Bacillus, and Paenibacillus* have been reported for their antagonism against *M. phaseolina* (Atef, 2000; Senthilkumar *et al.*, 2009).

Endophytic *Klebsiella* spp. was reported earlier by authors to possess plant growth- promoting activities (Sharma *et al.*, 2014; Yamina *et al.*, 2014). Reports on heavy-metal resistant strains of *K. pneumoniae, Pseudomonas aeruginosa* and *Pantoea agglomerans* were mentioned earlier by researchers (Nath *et al.*, 2012; Yamina *et al.*, 2014; Bhagat *et al.*, 2016). There have been several reports which emphasized the antagonistic activity of *Klebsiella* sp. and *Pseudomonas* sp. against some pathogenic fungi including *M. phaseolina* (Das *et al.*, 2015; Kumar *et al.*, 2007).

* Corresponding author e-mail: smajumdarwbes@gmail.com.

The current study is conducted to investigate the effectiveness of the endophytic bacteria *Klebsiella pneumoniae* HR1 (Gamma proteobacteria, Entero-bacteriales) in controlling *M. phaseolina* root rot disease in *Vigna*, and to determine the role of the isolate in the induction of plant defence-enzymes in response to the *M. phaseolina* infection.

2. Material and Methods

2.1. Isolation of Endophytic Strain from Root nodule of V. mungo L.

Healthy root nodules of *V. mungo* were collected from different agricultural fields of three districts in North Bengal -Malda, Uttar Dinajpur and Dakshin Dinajpur. The samples were washed with sterile distilled water, and were then surface sterilized using 95 % ethanol for one minute, followed by 0.1 % mercuric chloride ($HgCl_2$) for three minutes. They were finally rinsed six times with sterile distilled water. Then, 1g of nodules was crushed in 5 mL of sterile water resulting in a milky suspension, which was serially diluted up to 10^{-6} dilution. 0.1 mL of each dilution was spread on sterile yeast extract mannitol agar (YEMA) plates containing 0.1 % Congo red. The plates were incubated for forty-eight hours at 28 ± 1°C. The colonies were picked and maintained in YEMA slant at 4°C for further study.

2.2. Characterization and Identification of the Bacterial Strain

The isolates were characterized morphologically on the basis of the colony colour, shape, appearance, diameter, transparency, and gram staining. For the biochemical characterization, oxidase test, production of acid and gas from carbohydrate, citrate utilization, nitrate reduction, gelatin liquefaction, urease, methyl red and Voges Proskauer tests were performed by following the standard protocols (Cappuccino and Sherman, 1992).

For identification, genomic DNA was extracted from the twenty-four-hour-old culture following the method of Stafford *et al.*, (2005). DNA was precipitated from the aqueous phase with chilled ethanol (100 %) and was pelleted by centrifuging at 12000 rpm for fifteen minutes, followed by washing in 70 % ethanol and centrifugation. The pellet was, then, air- dried and suspended in TE buffer pH 8. For ITS-PCR amplification, DNA was amplified by mixing the template DNA (50 ng) with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. The polymerase chain reaction was performed in a total volume of 100 µL containing 78 µL of deionized water, 10 µL 10× Taq polymerase buffer, 1 µL of 1U Taq polymerase, 6 µL 2 mM dNTPs, 1.5 µL of 100 mM reverse and forward primers and 3.5 µL of 50 ng template DNA. The amplification of 16S rRNA gene was carried out by PCR using the forward (27f 5′ GAGTTTGATCACTGGCTCAG 3′) and reverse **(**1492r 5′ TACGGCTACCTTGTTACGACTT 3′) primers (Byers *et al.*, 1998). The PCR was programmed with an initial denaturing at 94 °C for five minutes, followed by thirty cycles of denaturation at 94 °C for thirty seconds, annealing at 61°C for thirty seconds and an extension at 70°C for two minutes and with a final extension at 72°C for seven minutes in a Thermocycler (Applied Biosystems, 2720). The amplified products were resolved by electrophoresis in 0.8 % agarose gel and PCR amplicons were purified. The purified DNA sequenced from Xcelris laboratory, Ahmadabad, India, and the 16S rDNA sequence obtained from PCR products were subjected to BLAST analyses. After complete annotation, the DNA sequences were deposited to NCBI GenBank through BankIt to get accession number. A phylogenetic analysis was conducted in MEGA - 4.0.2 software (Tamura *et al.*, 2007). The evolutionary history was inferred by Neighbor-Joining Method (Saitou and Nei, 1987).

2.3. In vitro Screening for Plant Growth Promoting Activity

2.3.1. Phosphate Solubilization

The phosphate solubilization efficacy of the isolate was determined using both Pikovskya's and National Botanical Research Institute's phosphate (NBRIP) medium (Pikovskaya, 1948; Nautiyal, 1999). The isolate was spot-inoculated at the center of the plate and was incubated for seven days at 28°C. The clear zone around the colony indicated phosphate solubilization activity.

2.3.2. Zinc Solubilizing Activity

A modified Pikovskaya medium supplemented with zinc oxide (Glucose 10g, $MgSO_4.7H_2O$ 0.1g, $(NH_4)_2SO_4$ 1g, KCl 0.2g, K_2HPO_4 2g, yeast extract 5g, ZnO 1g and Agar 20g in 1000 mL dH_2O) was used for determining the zinc-solubilizing activity of the isolate. The plate was streaked and incubated for seven days at 30°C. The clear zone around the colony was considered as a positive result (Pikovskaya, 1948).

2.3.3. Indole Acetic Acid (IAA) Production

IAA production was determined by inoculating the bacterial suspension (100 µL) in a YEM broth supplemented with tryptophan (0.01 %), and was incubated for three days. After centrifugation at 10000 rpm for twenty minutes, the supernatant was collected. A few drops of the orthophosphoric acid and 4 ml of the Salkowski reagent (1 mL of 0.5 M $FeCl_3$ solution in 50 mL of perchloric acid) were added to 2 mL of the supernatant and were kept in the dark for thirty minutes at room temperature. The appearance of the pink colour confirmed the IAA production (Brick *et al.*, 1991).

2.3.4. Production of Ammonia

For ammonia production, a freshly-grown culture was inoculated in 10 mL peptone water and was incubated for forty-eight hours at 28 ± 2°C. Then, Nessler's reagent (0.5 mL) was added. The development of the brown to yellow colour represents positive results for ammonia production (Cappuccino and Sherman, 1992).

2.3.5. Hydrogen Cyanide (HCN) production

The evaluation of hydrogen cyanide (HCN) production was done by inoculating the bacterial isolate on a YEMA medium amended with glycine. A Whatman No. 1 filter paper previously soaked in picric acid solution (0.05% solution in 2% sodium carbonate) was placed at the inner side of the Petri plate. Then the plate was sealed and incubated for forty-eight hours at 30°C. The change in colour of the filter paper from deep yellow to reddish brown was considered as an indication of the HCN production (Bakker and Schippers, 1987).

2.3.6. Siderophore Production

Siderophore production was detected on a Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands, (1987). The isolate (24-hour-old culture) was spotted on a CAS medium, and all plates were incubated at 28±1°C for forty-eight hours. The formation of yellow or orange halos around the colonies was considered as the positive response to the production.

2.3.7. Heavy Metal Tolerance

Heavy-metal tolerance of the isolate was determined using cadmium (Cd) as a heavy metal source. The dilution plate method was used to determine the lowest concentration of Cd that absolutely prevented the growth of the bacterial strain. The YEM agar plate supplemented with 25, 50, 75 and 100 µg/mL $CdCl_2$ was used. The inoculated plates were incubated for seven days at 28°C.

2.4. Production of Extracellular Hydrolytic Enzymes

2.4.1. Protease Production

The assay of protease production was performed on sterile skim milk agar plates. The isolate was spot-inoculated and incubated at 30°C. The zone of clearance around the colony indicated the production of protease (Chaiharn *et al*., 2008).

2.4.2. Amylase Production

For amylase production, the bacterial isolate was spot-inoculated on starch agar medium and was incubated for forty-eight hours at 30°C. After the incubation period, the plate was flooded with Lugol's iodine solution, and was kept for a minute. The colorless zone around the colonies indicated the production of amylase (Shaw *et al*., 1995).

2.4.3. Cellulase Production

The production of cellulase was assessed by following the method of Rangel-Castro *et al*., (2002) with some modifications. The isolate was inoculated on a modified YEMA medium supplemented with CMC (Carboxymethyl cellulose) instead of mannitol. The plate was incubated at 30°C for five days. After incubation, the media was flooded with an aqueous solution of Congo red (1% w/v), and the formation of a clear zone was considered as a positive response in the production of cellulase by the bacterial isolate.

2.4.4. Chitinase Production

Chitinase production efficacy of the isolate was determined by following the method of Roberts and Selitrennikoff, (1988) with some modifications. The chitinase-detection agar (CDA) plate was prepared by replacing mannitol with 1% colloidal chitin in modified YEMA, and the strain was spot-inoculated at the center of the plate. The plate was then incubated at 28°C for seven to ten days. The formation of a clear zone indicated the production of chitinase.

2.5. In-vitro Antagonistic Activity

The *in-vitro* antagonistic activity of the isolate was assessed by a dual culture method against the fungal pathogens: *M. phaseolina*, *Fusarium oxysporum* Smith & Swingle (Sordariomycetes, Hypocreales), *F. semitectum* Berk. and Ravenel (Sordariomycetes, Hypocreales), *Colletotrichum* sp, *Alternaria alternata* (Fr.) Keissl (Dothideomycetes, Pleosporales), and *Aspergillus* sp. in modified PDA supplemented with 2 % sucrose instead of dextrose (Kumar *et al*., 2012). The inoculated plates were incubated at 28 ± 1°C for five days, and the inhibition of the colony growth was measured. The percentage of inhibition was calculated using the following formula –

$$PI = \left(\frac{C - T}{C}\right) \times 100$$

PI = Percentage of inhibition, T = Redial growth of the fungal colony opposite the bacterial colony, and C = radial growth of the pathogen in the control plate.

All the plant pathogenic cultures were obtained from the culture collection of Microbiology and Microbial Biotechnology laboratory at the University of Gour Banga, Malda.

2.6. Assessments of Strain HR1 as a Biocontrol Agent against Root Rot Disease of V. mungo under Greenhouse Conditions

The efficacy of the strain HR1 to control the root rot disease of *V. mungo*, caused by *M. phaseolina* was determined under greenhouse conditions. The seeds of *V. mungo* var. sarada were collected from the 'Pulse and Oil seeds Research Station' Rani Bagan, Behrampore, West Bengal, India. For seed bacterization, the isolate was grown in a YEM broth at 30°C and 140 rpm on a rotary shaker for three days. After centrifugation at 6000 rpm for fifteen minutes, the bacterial suspension was diluted to attain the concentration of 10^8 CFU/mL.

The surface-sterilized seeds were soaked in a bacterial suspension supplemented with 1 % CMC overnight, and were sown (6 seeds/bag) in black polythene bags containing 2 kg of sterilized soil. The bacterium was also applied as soil drench (100 mL/Kg soil, concentration of 10^8 CFU/mL) to the rhizosphere of potted plants after seven days of growth.

The mass inocula of *M. phaseolina* was prepared by inoculating mycelial block in a pre-sterilized 30g moist oat meal medium, and was incubated at 30°C for five days for soil infestation. The inoculum (0.1 % w/w) was mixed thoroughly in the double autoclaved soil.

Growth promotion of *Vigna* plants was measured in terms of increase in the percentage of seed germination, shoot and root length, shoot and root dry weight following the application of the bacterial isolate. The vigor index was calculated as described by Abdul-Baki and Anderson, (1973).

The root rot disease caused by *M. phaseolina* was assessed, after fifteen days of growth by measuring the percentage of disease incidence (PDI) as follows:

$$PDI = \left(\frac{Number\ of diseased\ plants}{Total\ number\ of\ plants}\right) x\ 100$$

The sets of treatments were: I – sterilized soil and unbacterized seed (healthy control), II – *M. phaseolina* inoculated sterilized soil (diseased control), III – bacterized seeds of black gram with *K. pneumoniae* and challenge inoculation with *M. phaseolina* and IV – dual-mode of application (seed bacterization + soil drench) of *K. pneumoniae*, challenge inoculation with *M. phaseolina*. The plants were maintained in a normal daylight condition. Ten replicates were taken for each treatment, and the average of the ten replicate plants were analyzed.

2.7. Biochemical Analysis

Leaves of *V. mungo* were grown in treated or control soil used for all the biochemical analysis. The leaves were collected for assay after fifteen days of germination.

2.8. Enzyme Assay

2.8.1. Peroxidase (EC 1.11.1.7)

The extraction and assay of peroxidase (POX) were conducted by following the method of Chakraborty *et al.*, (1993). Activity was assayed at 465 nm by monitoring the oxidation of O-dianisidine in the presence of H_2O_2. Specific activity was expressed as the increase in ΔA 465/ min/ g tissue.

2.8.2. Chitinase (EC 3.2.1.39)

The extraction of chitinase (CHT) and measurement of its activity were conducted by following the method described by Boller and Mauch, (1998) with modifications. 1g of the leaf samples was crushed in 5 mL of 0.1 M chilled sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 12000 rpm for ten minutes at 4°C. The supernatant was used as the enzyme source.

In the assay mixture, colloidal chitin was used for the detection of chitinase activity, and the activity was determined by measuring the released N-acetyl glucosamine (Glc–Nac). The colloidal chitin was prepared according to the method of Roberts and Selitrennikoff, (1988). The activity was expressed as mg Glc–NAc released/ g tissue /hour.

2.8.3. Phenylalanine Ammonia Lyase (EC 4.3.1.5)

The enzyme phenylalanine ammonia lyase (PAL) was extracted, and its activity was measured by following the method of Chakraborty *et al.*, (1993). PAL activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically at 290 nm. The enzyme activity was expressed as µg cinnamic acid/ min/ g tissue.

2.8.4. β-1, 3-Glucanase (EC 3.2.1.39)

β- 1, 3-glucanase (GLU) was extracted and assayed from the leaf samples following the method of Pan *et al.*, (1991). The reaction mixture consisted of 62.5 µL of the leaf enzyme extract and 62.5 µL of laminarin (4%). The mixture was incubated at 40°C for ten minutes and 375 µL of DNSA (dinitro salicylic acid) was added to the mixture following incubation for five minutes on a boiling water bath. Finally, the coloured solution was diluted with 4.5 mL of water, and the amount of glucose liberated was determined spectrophotometrically using a standard curve. The enzyme activity was expressed as µg glucose released/min/g tissue.

2.9. Statistical Analyses

The results were analysed statistically using one-way ANOVA, SPSS version 22 software. Duncan's Multiple Range Test (DMRT) was used for mean separation wherever appropriate.

3. Results

3.1. Characterization and Identification of HR1

The morphological study revealed that the isolate HR1 is rod-shaped, gram-negative bacterium and showed positive response in the Voges- Proskauer test, acid-gas production test, nitrate reduction test, urease test, as well as the citrate-production test during the biochemical characterization (Table 1).

Table 1. *In vitro* Morphological and Biochemical characterization of the endophytic isolate HR1.

Morphological and Biochemical tests	Bacterial Isolate HR1
Gram stain	-
Shape	Rod
Indole Production	-
Oxidase	-
Citrate	+
Gelatin Hydrolysis	-
Urease	+
Methyl red	-
Voges Proskauer	+
Acid- gas production	+
Nitrate	+

'+' = Activity Present; '-' = Activity Absent.

A continuous stretch of the 1420 16S rRNA gene sequence was obtained from the isolate HR1. The BLAST query of the sequence against GenBank database confirmed the identity of the isolate as *K. pneumoniae* with 99 % similarity with the sequences of other isolates of the same genus deposited in NCBI database. This was confirmed with the phylogenetic analysis, in which the HR1 isolate clustered with other *K. pneumoniae* isolates (Figure 1). The sequence has been deposited in NCBI, GenBank database under the accession No. KY029037.

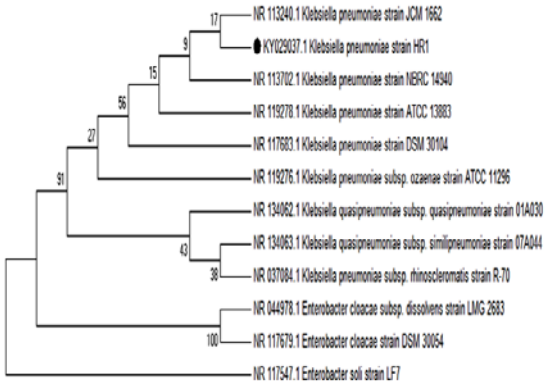

Figure 1. The phylogenetic relationships of the strain *K. pneumoniae* HR1 based on 16S rRNA gene analysis. Evolutionary distances were calculated using the Neighbor – joining method. The level of bootstrap support (1,000 repetitions) is indicated at all nodes

3.2. Evaluation of PGP Activities and Excretion of Extracellular Hydrolytic Enzymes

The isolate was evaluated for *in-vitro* plant growth-promoting (PGP) activities. The phosphate-solubilising activity was assessed both with PKV and NBRIP medium, and the isolate HR1 showed positive results in both cases. The isolate also produced a clear zone on the modified PKV medium containing ZnO, which indicated its efficacy as a zinc-solubilizer. The isolate also produced siderophore, IAA and HCN (Table 2, Figure 2). The *K. pneumoniae* HR1 showed a transparent zone on the chitinase-detection agar plate which clearly indicated the

ability of HR1 to produce chitinase. The halo zones around the bacterial spot on the starch agar and skim milk agar revealed that the strain produced amylase and protease, respectively. However, the isolate could not show positive results on the CMC agar plate (Table 2). The bacterial isolate showed its active growth on the YEM agar plates containing 25, 50, 75 and 100 µg / mL of $CdCl_2$ concentration which shows that the isolate could tolerate cadmium.

Table 2. *In vitro* tests of endophytic isolate HR1 for the determination of PGP activities and production of extracellular hydrolytic enzymes by the isolate.

PGP Activities		Extracellular Hydrolytic Enzymes	
Traits	Response of isolate HR1	Hydrolytic Enzymes	Production by Isolate HR1
Phosphate solubilization	+	Protease	+
Zinc solubilization	+	Amylase	+
IAA production	+	Cellulase	-
Ammonia production	-	Chitinase	+
HCN production	+		
Siderophore production	+		

'+' = Positive activity/ presence of hydrolytic enzymes; '-' = Negative activity / absence of hydrolytic enzymes

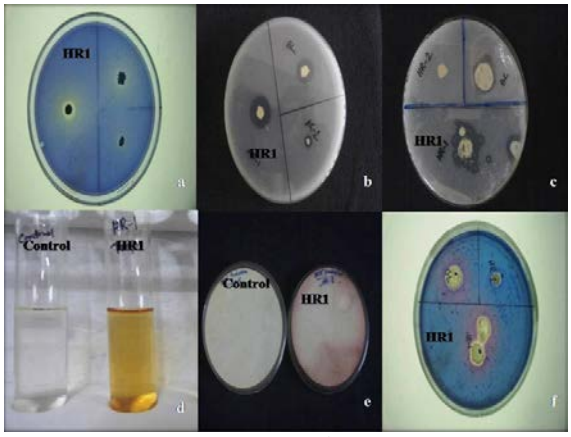

Figure 2. *In vitro* PGP activity of *K. pneumoniae* HR1. a. Phosphate solubilization in NBRIP media, b. Phosphate solubilization in PKV media, c. Zinc solubilization, d. IAA production, e. HCN production, f. Siderophore production

3.3. In vitro Antagonistic Activity of K. pneumoniae against Fungal Pathogens

In vitro antagonism of *K. pneumoniae* was tested against *M. phaseolina, F. oxysporum, F. semitectum, A. alternata, Aspergillus* sp. and *Colletotrichum* sp. The results revealed that *K. pneumoniae* inhibited the tested pathogens significantly (F= 381.662; df= 5; P= 0.05). The highest inhibition observed against *M. phaseolina* was (73.3 %), followed by *Aspergillus* sp. (58.8 %), *F. semitectum* (53.5 %), *A. alternata* (50.8 %) and *Colletotrichum* sp. (42.26 %). However, *K. pneumoniae* showed the lowest percentage of inhibition against *F. oxysporum* (34.12 %) (Figure 3).

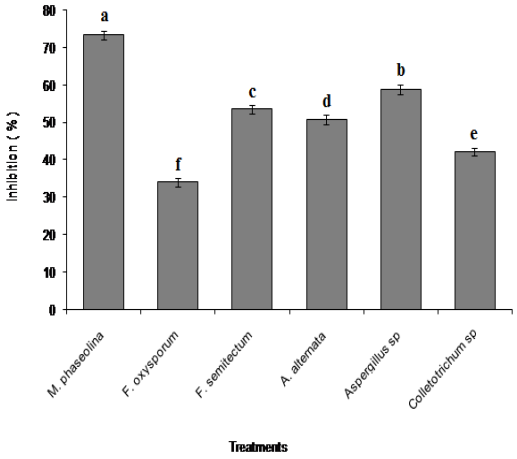

Figure 3. Inhibition of the growth of fungal pathogens by *K. pneumoniae* HR1 expressed in terms of percentage of inhibition. Data represent mean ± SD. Bars not sharing the same letter are different at the *P*< 0.05 level by one-way ANOVA with DMRT

3.4. Assessments of Root Rot Disease of V. mungo

The greenhouse study revealed that the application of *K. pneumoniae* reduced the disease incidence significantly (F= 766.697; df= 2; P= 0.05) in comparison to the control plants in the presence of *M. phaseolina*. It was observed that the percentage of disease incidence was quite high (78.2 %) in the diseased control plants. PDI decreased when the plants were inoculated (seed bacterization) with the isolate; however, the lowest PDI (18.2 %) was achieved when *K. pneumoniae* was applied in a dual-mode (seed bacterization + soil drench application at 10^8 CFU/mL concentration) (Table 3).

Table 3. Assessment of root rot disease of *V. mungo*, caused by *M. phaseolina* in terms of the percentage of disease incidence (PDI) and the percentage of decrease over control (PDOC) following treatment with *K. pneumoniae* HR1 and pathogen challenge.

Days of inoculation	Treatments	Disease assessment	
		PDI	PDOC
15	Control	78.2 ± 3.4^a	-
	T1	27.3 ± 0.5^b	65.02 ± 0.9
	T2	18.2 ± 0.9^c	76.73 ± 0.2

Results represented as mean ± SD, T1 = Seeds of *V. mungo* bacterized with *K. pneumoniae* and challenge inoculation with *M. phaseolina;* T2= Dual-mode of application (seed bacterization + soil drench at 1×10^8 CFU/mL) of *K. pneumoniae*, challenge inoculation with *M. phaseolina*. Values in rows not sharing the same letter are different at the *P*< 0.05 level by one-way ANOVA with DMRT (n=10)

3.5. Effect of K. pneumoniae on the Growth of V. mungo in a M. phaseolina-infested soil

The dual application of *K. pneumoniae* led to a significant increase in the growth of *Vigna* in terms of increase in the germination percentage, shoot and root length, shoot and root dry weight in comparison to the healthy control and the *M. phaseolina*-infected plants (diseased control). A significant decrease in the germination percentage (F= 578.569; df= 4; P= 0.05), shoot length (F= 187.688; df= 4; P= 0.05) and dry weight (F=466.831; df= 4; P= 0.05), root length (F= 112.143; df=

4; P= 0.05), dry weight (F= 969.355; df= 4; P= 0.05) and vigour index (F= 1443.915; df= 4; P= 0.05) were observed in the presence of *M. phaseolina*. As for the percentage of increase over control (PIOC), a statistically-significant increase in seed germination, shoot length, root length and dry weight was observed in the plants in the presence of *K. pneumoniae* alone, in comparison to other treatments (Table 4, figure 4).

Table 4. Effect of *K. pneumoniae* HR1 on the growth of *V. mungo* in a *M. phaseolina*-infested soil.

Growth Parameters	Treatments				
	Untreated Healthy	Untreated Inoculated (*M. phaseolina*)	Treated Healthy (*K. pneumoniae* HR1)	Treated Inoculated [*K. pneumoniae* HR1 (Seed treatment) + *M. phaseolina*]	Treated Inoculated [*K. pneumoniae* HR1 (Seed treatment + Drench Application) + *M. phaseolina*]
Germination (%)	88.27±1.38^{b}	30.4±1.73^{e}	93.3±0.70^{a}	70.2±0.72^{d}	79.1±0.3.20^{c}
Shoot length (cm)	14.3±0.50^{b}	7.2±0.35^{e}	16.1±0.24^{a}	10.3±0.42^{d}	12.6±0.61^{c}
Root length (cm)	3.3±0.17^{b}	1.4±0.11^{e}	3.8±0.19^{a}	2.3±0.14^{d}	2.9±0.13^{c}
Shoot Dry weight (g)	0.23±0.01^{b}	0.09±0.004^{e}	0.29±0.003^{a}	0.18±0.003^{d}	0.21±0.006^{c}
Root Dry weight (g)	0.06±0.002^{b}	0.02±0.001^{e}	0.07±0.001^{a}	0.04±0.0002^{d}	0.05±0.0007^{c}
Vigour Index	1554.9±52.23^{b}	262.0±19.01^{e}	1862.2±16.13^{a}	880.8±21.39^{d}	1225.9±13.66^{c}

Results represented as mean ± = SD, values in rows not sharing the same letter are different at the *P*< 0.05 level by one-way ANOVA with DMRT (n=10)

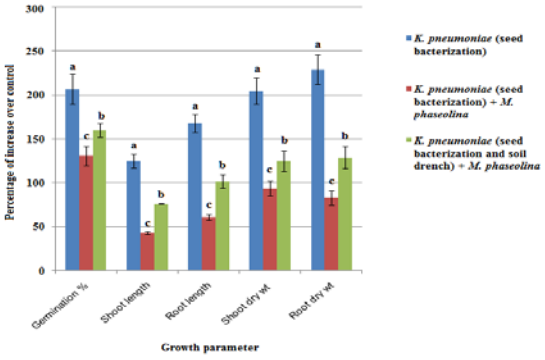

Figure 4. Effect of *K. pneumoniae* HR1 on the growth of *V. mungo*. Control refers to the plants inoculated with *M. phaseolina* only. Data represent mean ± SD. Bars not sharing the same letter are different at the P< 0.05 level by one-way ANOVA with DMRT.

3.6. Activities of Defence Enzymes during Disease Suppression by K. pneumoniae

Activities of defence enzymes were assayed in leaves subjected to various treatments. The activities of defence enzymes increased significantly after the application of *K. pneumoniae* and challenged inoculation with *M. phaseolina*. However, the most significant increase observed was when the isolate was applied in a dual-mode following the challenge inoculation with the pathogen [POX (F= 334.694; df= 3; P= 0.05), PAL (F= 257.825; df= 3; P= 0.05), CHT (F= 3439.517; df= 3; P= 0.05), GLU (F= 262.067; df= 3; P= 0.05)] (Figure 5).

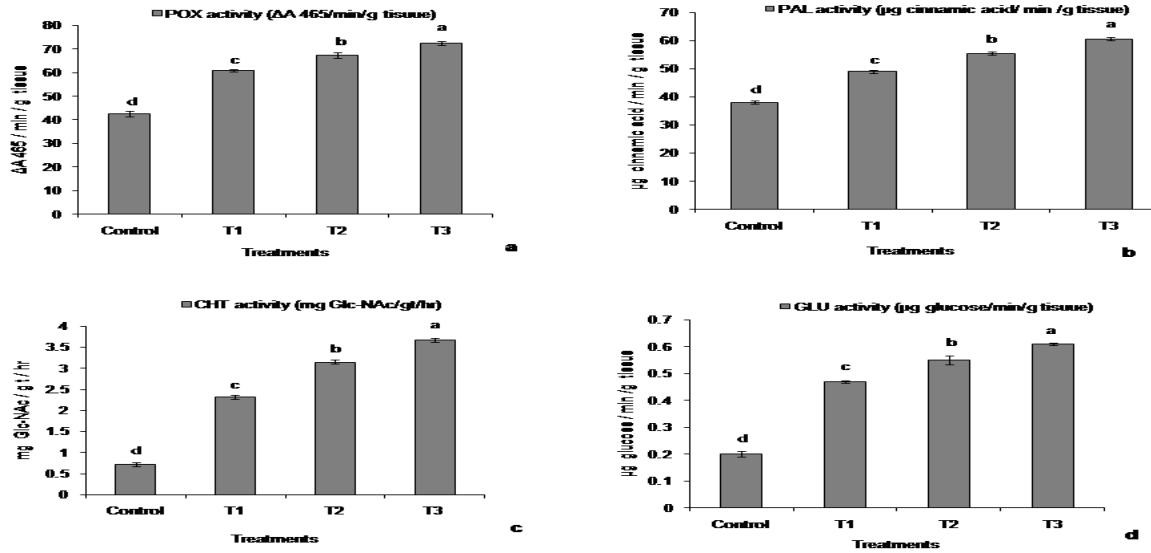

Figure 5. Activities of Peroxidase (a), Phenylalanine ammonia lyase (b), Chitinase (c) and β-1, 3 glucanase (d) in leaves of *V. mungo* after 15 days of growth and challenged inoculation with *M. phaseolina*. T1- *M. phaseolina* inoculated, T2- Bacterized Seeds of black gram with *K. pneumoniae* and challenge inoculation with *M. phaseolina*, T3 – Dual-mode of application (seed bacterization + soil drench) of *K. pneumoniae*, challenge inoculation with *M. phaseolina*. Bars with different letter are significantly different at *P*<0.05 according to DMRT.

4. Discussion

M. phaseolina is the causal agent of charcoal root rot disease. It is a devastating pathogen affecting agricultural and forest crops, with more than five-hundred susceptible host's worldwide (Wyllie *et al.*, 1984). In the present study, the researchers have isolated and characterized one endophytic isolate from *K. pneumoniae* HR1 for its antagonistic and biocontrol efficacy against *M. phaseolina*.

The endophytic gram-negative and rod-shaped bacterium *K. pneumoniae* HR1 was isolated from the root nodules of the leguminous plant, *V. mungo*, on yeast extract mannitol agar medium, and was preliminarily characterized morphologically and biochemically. The HR1 isolate exhibited a phosphate-solubilising activity by producing halos zone on the Pikovskaya's (PKV) and National Botanical Research Institute's phosphate (NBRIP) media. The bacterium could solubilize zinc oxide (insoluble) supplemented in a modified PKV medium. Both phosphate and zinc are essential nutrients and play a crucial role in the promotion of plant growth. Enhancement of growth in *V. radiata* by the zinc-phosphate- solubilizing bacterial isolates was reported previously (Iqbal *et al.*, 2010a). Vaid *et al.*, (2014) also reported that about the Zn-solubilising and IAA-producing *Burkholderia* and *Acinetobacter* strains which increased the uptake of Zn and promoted the growth of rice plant. Plant growth-promoting rhizobacterial strains actively colonize plant roots and exert beneficial effects on the host. Endophytes, residing inside the plants, with plant growth- promoting activities are considered more effective in promoting growth in host plants. In their study, Sharma *et al*., (2014) isolated endophytic *Klebsiella* spp. which could solubilise ZnO. In the present study, it was also observed that the endophytic isolate HR1 solubilized ZnO quite efficiently. Heavy-metal tolerance is considered one of the crucial attributes of PGPRs. Bacterial isolates with this capacity are considered effective to induce systemic tolerance in plants against heavy-metal stress. Several reports suggested that *Klebsiella* sp. has greater cadmium and other heavy-metal tolerance efficiency (Nath *et al.*, 2012; Yamina *et al.*, 2014). In the present study, the isolate *K. pneumoniae* HR1 could tolerate even up to 100 µg/mL of $CdCl_2$. Furthermore, the isolate-HR1 was found to be the producer of IAA, siderophore, hydrogen cyanide, chitinase, amylase and protease. The production of several hydrolytic enzymes such as protease, chitinase, cellulase, lipase and amylase by *Klebsiella* sp. was demonstrated by Mazzucotelli *et al.*, (2013), which showed similar results to the observations of the current study. The production of hydrolytic enzymes, hydrogen cyanide, and siderophore by the isolate HR1 may be the probable mechanisms responsible for the antagonistic activity against phytopathogens. IAA-producing bacteria are believed to influence the endogenous auxin pool of plants, and thus can promote root elongation and plant growth (Patten and Glick, 2002; Ahemad and Khan, 2011a; Ahemad and Khan, 2011b). Jha and Kumar, (2007) also stressed the plant growth promoting traits- phosphate solubilization, IAA production, and nitrogenase activity of *K. oxytoca*. *In vitro* PGP traits such as IAA, siderophore, HCN production, and phosphate solubilization of endophytic PGP bacteria inside the roots of *Brassica napus* were reported by Etesami *et al.*, (2014).

The isolate showed commendable antagonistic activity against phytopathogens used in the present study. The highest inhibition percentage against *M. phaseolina* (73.3 %) and the lowest inhibition against *F. oxysporum* (34.12 %) were observed by *K. pneumoniae* in the paired culture. The inhibition of fungal pathogens by the isolate may be attributed to the production of antifungal enzymes-chitinase, protease, amylase as well as HCN. Simonetti *et al.*, (2015) reported antagonistic activities of eleven bacterial isolates against *M. phaseolina*. Among the isolates *Pseudomonas fluorescens* 9 showed the highest percentage of inhibition (62 %) *in vitro*. Their study is consistent with the results of the current work. Moreover, this study showed that the isolate HR1 exhibited a much more inhibition efficiency against *M. phaseolina*.

In the present study, the application of *K. pneumoniae* both in a single and a dual-mode, in the presence of *M. phaseolina* resulted in a significant increase in growth, measured in terms of increase in shoot and root length, shoot and root dry weight, and vigour index of black gram. However, a significant decrease in the growth parameters was found in the presence of *M. phaseolina* compared to the untreated healthy black gram. During the assessment of the root rot disease, the highest percentage of decrease over control (76.73 %) was observed when *K. pneumoniae* was applied two times (as seed bacterization + soil drench application at 10^8 CFU/mL) in the of *M. phaseolina*. Plants subjected to a dual application with the bacterium in the presence of the pathogen also showed a significantly higher percentage of increase than the control (PIOC) in comparison to the other treatments. It is quite apparent that *K. pneumoniae* not only promoted growth but effectively reduced the root rot disease of *V. mungo* as a potent antagonistic endophytic PGPR strain. Previously, *K. pneumoniae* was reported as disease suppressive and as a plant growth-promoting strain, which reduced 72 % of stem rot severity caused by *Sclerotinia* increasing the plant height by 52–67 % (Abdeljalil *et al.*, 2016).

The role of potential bacterial antagonists in reducing the severity of *M. phaseolina* root rot disease by enhancing the activities of peroxidase, PAL, β-1, 3-glucanase and chitinase was also highlighted by many other authors (Govindappa *et al.*, 2011). Kumar *et al.*, (2007) focused on the control of charcoal rot of chickpeas caused by *M. phaseolina* via the application of a potent antagonistic isolate Pf4-99. Feng *et al.*, (2006) also reported that the rice endophytic strain YS19 promoted growth and increased root biomass. In the current study, it was observed that the activities of defence-related enzymes peroxidase, chitinase, phenylalanine ammonia lyase and β-1, 3-glucanase increased significantly after the application of *K. pneumoniae* and challenge inoculation with *M. phaseolina*. The increased activities of peroxidase (PR9), chitinase (PR3) and β-1, 3-glucanase (PR2) indicate the activation of PR proteins during defence. Since the application of *K. pneumoniae* in the *M. phaseolina*-infested soil increased the accumulation of defence–related enzymes in leaves, it is quite apparent that *K. pneumoniae* HR1 endophytic strain induces a systemic response. Bruce and West, (1989) reported the role of peroxidase in the biosynthesis of lignin which limited the extent of pathogen spread because of antifungal activity. The accumulation of

PR proteins during the induction of resistance in crops against pathogens by biocontrol agents was also reported earlier by some researchers (Bargabus *et al.*, 2004; Bharati *et al.*, 2004). Resistance in *V. mungo* against *M. phaseolina* was induced through the accumulation of phenolics, PR proteins and the induction of defence enzymes by the two potent endophytic strains of *P. fluorescens* – Endo2 and Endo35 (Karthikeyan *et al.*, 2005). Govindappa *et al.*, (2010) reported that higher activities of peroxidase, phenylalanine ammonia lyase, chitinase, polyphenol oxidase and β-1, 3-glucanase involved in phenyl propanoid pathways were observed in the potent antagonistic strain *Bacillus subtilis* which treated safflower plants after challenge inoculation with *M. phaseolina*. The induction of systemic resistance in legumes (*V. mungo*, *V. radiata*) against *F. oxysporum* and *A. alternata* was also highlighted by Rao *et al.*, (2015).

5. Conclusion

The endophytic isolate *K. pneumonia*e HR1 showed strong antagonistic activity and suppression of the root rot disease, caused by *M. phaseolina*. While determining the mechanism of action, it was found to promote plant growth-promotion and induce resistance in the host plants by elevating the synthesis of defence-related enzymes. The results of the current study provide strong evidence that the endophytic *K. pneumoniae* HR1 has a great potential as a candidate for the biocontrol of the soil-borne fungal pathogen, *M. phaseolina*, and as a plant-growth promoter. The use of such beneficial endophytes, which can induce resistance to diseases in the host plants, is a boon to agriculture for a better crop production and a healthy soil.

Acknowledgments

Xcelris laboratory, Ahmadabad and Pulse and Oilseeds Research Station, Rani Bagan, Behrampore, West Bengal, India are thankfully acknowledged for the molecular identification of endophytic strain on the basis of 16S rDNA partial sequencing and for supplying the seeds of *Vigna mungo* var. sarada which helped carry out this research.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abawi GS and Pastor-Corrales MA. 1990. Root rots of beans in Latin America and Africa: Diagnosis, Research Methodologies, and Management Strategies, Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, pp. 114.

Abdeljalil NOB, Vallance J, Gerbore J, Rey P and Daami-Remadi M. 2016. Bio-suppression of *Sclerotinia* stem rot of tomato and biostimulation of plant growth using tomato associated rhizobacteria. *J Plant Pathol Microbiol*, **7**: 331.

Abdul-Baki A and Anderson JD. 1973. Vigor determination in soybean seed by multiple criteria. *Crop Sci*, **13**: 630–633.

Ahemad M and Khan MS. 2011a. Effects of insecticides on plant-growth-promoting activities of phosphate solubilizing rhizobacterium *Klebsiella* sp strain PS19. *Pestic Biochem Physiol.*, **100**: 51–56.

Ahemad M and Khan MS. 2011b. Biotoxic impact of fungicides on plant growth promoting activities of phosphate-solubilizing *Klebsiella* sp isolated from mustard (*Brassica compestris*) rhizosphere. *J Pest Sci.*, **85**: 29–36.

Atef NM. 2000. *In vitro* antagonistic action of eggplant and sweet potato phylloplane bacteria to some parasitic fungi. *Phytopathol Mediter.*, **39**: 366–375.

Bakker AW and Schippers B. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth stimulation. *Soil Biol Biochem.*, **19**: 451–457.

Bargabus RL, Zidack NK, Sherwood JE and Jacobsen BJ. 2004. Screening for the identification of potential biological control agents that induce systemic acquired resistance in sugar beet. *Biol Control*, **30**: 342–350.

Berg G, Eberl L and Hartmann A. 2005. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ Microbiol.*, **7**: 1673–1685.

Bhagat N, Vermani M and Bajwa HS. 2016. Characterization of heavy metal (cadmium and nickel) tolerant Gram negative enteric bacteria from polluted Yamuna river, Delhi. *Afr J Microbiol Res.*, **10**: 127–137.

Bharati R, Vivekananthan R, Harish S, Ramanathan A, Samaiyappan R (2004) Rhizobacteria based bioformulations for the management of fruit rot infection inchillies. *Crop Prot..*, **23**: 835–843.

Bloemberg GV and Lugtenberg BJJ. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr Opin Plant Biol.*, **4(4)**: 343–350.

Boller T and Mauch F. 1998. Colorimetric assay for chitinase. *Meth Enzymol.*, **161**: 430–435.

Brick JM and Bostock RM, Silverstone SE. 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. *Appl Environ Microbiol.*, **57**: 535–538.

Bruce RJ and West CA. 1989. Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiol.*, **91**: 889–897.

Byers HK, Stackebrandt E, Hayward C and Blackall LL. 1998. Molecular investigation of a microbial mat associated with the great artesian basin. *FEMS Mi.crobiol Ecol*., **25**: 391–403.

Cappuccino JC and Sherman N. 1992. **Microbiology a Laboratory Manual**, third ed. Benjamin/Cummings Pub Co, New York, pp. 125–179.

Chaiharn M, Chunhaleuchanon S, Kozo A and Lumyong S. 2008. Screening of rhizobacteria for their plant growth promoting activities. *KMITL Sci Tech J.*, **8**: 18–23.

Chakraborty U, Chakraborty BN and Kapoor M. 1993. Changes in levels of peroxidase and phenylalanine ammonia lyase *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia Microbiol.*, **38**: 491–496.

Cottingham C. 1981. Numbers and distribution of sclerotia of *Macrophomina phaseolina* in soils of South Carolina. *Plant Disease Reporter.*, **65**: 355–356.

Das MP, Devi PV and Yasmine Y. 2015. A study on antagonistic potential of bacteria against phytopathogenic fungi. *Int J Pharm Sci Rev Res.*, **34**: 191–193.

Etesami H, Hosseini M and Alikhani HA. 2014. *In planta* selection of plant growth promoting endophytic bacteria for rice (*Oryza sativa* L.). *J Soil Sci Plant Nutr.*, **14**: 491–503.

Feng Y, Shen D and Song W. 2006. Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects

allocations of host photosynthates. *J Appl Microbiol.*, **100**: 938–945.

Govindappa M, Lokesh S, Rai VR, Naik VR and Raju SG. 2010. Induction of systemic resistance and management of safflower *Macrophomina phaseolina* root rot disease by biocontrol agents. *Arch Phytopathology Plant Protect*, **43**: 26–40.

Govindappa M, Rai VR and Lokesh S. 2011. Screening of *Pseudomonas fluorescens* isolates for biological control of *Macrophomina phaseolina* root-rot of safflower. *Afr J Agric Res.*, **6**: 6256–6266.

Iqbal U, Jamil N, Ali I and Hasnain S. 2010a. Effect of zinc-phosphate solubilizing bacterial isolates on growth of *Vigna radiata*. *Ann Microbiol.*, **60**: 243–248.

Iqbal U, Mukhtar T, Muhammad S, Iqbal, Ul Haque I and Malik SR. 2010b. Host plant resistance in black gram against charcoal rot (*Macrophomina phaseolina* (tassi) goid). *Pak J Phytopathol.*, **22(2)**: 126–129.

Jha PN and Kumar A. 2007. Endophytic colonization of *Typha australis* by a plant growth-promoting bacterium *Klebsiella oxytoca* strain GR-3. *J Appl Microbiol.*, **103**: 1311–1320.

Karthikeyan M, Bhaskaran R, Radhika K, Mathiyazhagan S, Jayakumar V, Sandosskumar R and Velazhahan R. 2005. Endophytic *Pseudomonas fluorescens* Endo2 and Endo35 induce resistance in black gram (*Vigna mungo* L. Hepper) to the pathogen *Macrophomina phaseolina*. *J Plant Interact.*, **1**: 135–143.

Krishnamurthy K and Gnanamanickam SS. 1997. Biological control of sheath blight of rice: induction of systemic resistance in rice by plant-associated *Pseudomonas* spp. *Curr Sci.*, **72**: 331–334.

Kumar P, Dubey RC and Maheshwari DK. 2012. *Bacillus* strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiol Res.*, **167**: 493–499.

Kumar V, Kumar A and Kharwar RN. 2007. Antagonistic potential of fluorescent pseudomonads and control of charcoal rot of chickpea caused by *Macrophomina phaseolina*. *J Environ Biol.*, **28**: 15–20.

Lugtenberg B and Kamilova F. 2009. Plant growth-promoting rhizobacteria. *Annu Rev Microbiol.*, **63**: 541–556.

Mazzucotelli CA, Ponce AG, Kotlar CE and Moreira MR. 2013. Isolation and characterization of bacterial strains with a hydrolytic profile with potential use in bioconversion of agro industrial by-products and waste. *Food Sci Technol.*, **33**: 295–303.

McMullen M and Bergstrom G. 1999. Chemical and biological control of *Fusarium* head blight 1999 projects and progress. In Proceedings of the 1999 National *Fusarium* Head Blight Forum, Sioux Falls, South Dakota, pp. 61–63.

Nath S, Deb B and Sharma I. 2012. Isolation and characterization of cadmium and lead resistant bacteria. *Global Adv Res J Microbiol.*, **1**: 194–198.

Nautiyal CS. 1999. An efficient microbiological growth medium for screening phosphate solubilising microorganisms. *FEMS Microbiol Lett.*, **170**: 65–270.

Pan SQ, Ye XS and Kuc J. 1991. A technique for detection of chitinase, β-1, 3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectric focussing. *Phytopathol.*, **81**: 970–974.

Patten C and Glick CR. 2002. Role of *Pseudomonas putida* indoleacetic acid in development of host plant root system. *Appl Environ Microbiol.*, **68**: 3795–3801.

Pikovskaya RI. 1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Microbiol.*, **17**: 362–370.

Rangel-Castro JI, Levenfors JJ and Danell E. 2002. Physiological and genetic characterization of fluorescent *Pseudomonas* associated with *Cantharellus cibarius*. *Can J Microbiol.*, **48**: 739–748.

Rao GS, Reddy NNR and Surekha C. 2015. Induction of plant systemic resistance in legumes *Cajanus cajan*, *Vigna radiata*, *Vigna mungo* against plant pathogens *Fusarium oxysporum* and *Alternaria alternate*– a *Trichoderma viride* mediated reprogramming of plant defense mechanism. *Int J Recent Sci Res.*, **6**: 4270–4280.

Roberts WK and Selitrennikoff CP. 1988. Plant and bacterial chitinase differ in antifungal activity. *J Gen Microbiol.*, **134**: 169–176.

Saitou N and Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol.*, **4**: 406–425.

Satyanandam T, Babu K, Rosaiah G and Vijayalakshmi M. 2013. Screening of *Rhizobium* strains isolated from *Vigna mungo* native to rice fallows for the production of indole acetic acid. *Int J Pharm Biol Sci.*, **3(4)**: 56–61.

Schwyn B and Neilands J. 1987. Universal chemical assay for the detection and determination siderophores. *Anal Biochem.*, **160**: 47–56.

Senthilkumar M, Swarnalakshmi K, Govindasamy V, Lee YK and Annapurna K. 2009. Biocontrol potential of soybean bacterial endophytes against charcoal rot fungus, *Rhizoctonia bataticola*. *Curr Microbiol.*, **58**: 288-293.

Sharma P, Kumawat KC, Kaur S and Kaur N. 2014. Assessment of zinc solubilization by endophytic bacteria in legume rhizosphere. *Ind J Appl Res.*, **4**: 439–441.

Shaw FJ, Lin PF, Chen CS and Chen CH. 1995. Purification and characterization of an extracellular α-amylase from thermos species. *Bot bull Acad Sin*, **36**: 195–200.

Simonetti E, Viso NP, Montecchia M, Zilli C, Balestrasse K and Carmona M. 2015. Evaluation of native bacteria and manganese phosphite for alternative control of charcoal root rot of soybean. *Microbiol Res.*, **180:** 40–48.

Stafford WHL, Baker GC, Brown SA, Burton SG and Cowan DA. 2005. Bacterial diversity in the rhizosphere of Proteaceae species. *Environ Microbiol.*, **7**: 1755–1768.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol.*, **24**: 1596–1599.

Vaid SK, Kumar B, Sharma A, Shukla AK and Srivastava PC. 2014. Effect of zinc solubilizing bacteria on growth promotion and zinc nutrition of rice. *J Soil Sci Plant Nutr.*, **14**: 889–910.

Vasebi Y, Safaie N and Alizadeh A. 2013. Biological control of soybean charcoal root rot disease using bacterial and fungal antagonists In Vitro and greenhouse condition. *J Crop Prot.*, **2(2)**: 139–150.

Wheeler BEJ. 1969. **An Introduction to Plant Diseases**, John Wiley and Sons Ltd, London.

Wheeler H. 1975. **Plant Pathogenesis**, Academic press, London, New York, pp. 2–3.

Wyllie T, Gangopadhyay S, Teague W and Blanchar R. 1984. Germination and production of *Macrophomina phaseolina* microsclerotia as affected by oxygen and carbon dioxide concentration. *Plant Soil*, **81**: 195-201.

Yamina B, Tahar B, Lila M, Hocine H and Laure FM. 2014. Study on cadmium resistant-bacteria isolated from hospital wastewaters. *Adv Biosci Biotechnol.*, **5**: 718–726.