Culture Filtrates of Plant Growth Promoting *Bradyrhizobium* sp. (*Vigna*) Strains VR1 and VR2 Inhibit Growth and Sclerotia Germination of *Macrophomina phaseolina in vitro*

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Abstract: Six isolates (VR1−VR6) of *Bradyrhizobium* sp. from *Vigna mungo* were evaluated for their plant growth promoting (PGP) attributes and antifungal properties *in vitro*. All the isolates produced IAA but none of them produced HCN. Isolates VR1 and VR2 produced siderophore, and enzymes chitinase and ACC deaminase besides phosphate solubilisation and antagonism against *M. phaseolina*. Cell-free culture filtrates of *Bradyrhizobium* strains VR2 resulted in maximum growth inhibition of pathogen in dual culture causing several deformities in hyphae and sclerotia such as fragmentation, shrinkage and lysis of hyphae, cytoplasm vacuolation, loss of mycelial pigment, and inability of sclerotia formation and germination. However, complete inhibition in mycelial dry weight and sclerotia germination of pathogen was recorded at 45% concentration of culture filtrates of strain VR2. Moreover, patterns of sclerotia germination varied with concentration of culture filtrates of VR1 and VR2. The number of hyphae produced per sclerotium was more in control than the culture filtrate-amended plates. The number of sclerotia producing less hyphae got increased with increasing the concentration of culture filtrate of strains VR1 than VR2. These results suggest that the presence of inhibitory properties in culture filtrates of *Bradyrhizobium* strains help to act as potential biocontrol agent for control of *M. phaseolina*.

1. Introduction

Cow pea or black gram [*Vigna mungo* (L.) Hepper] is one of the important pulse crops which is widely grown in different states of India and used in multifarious ways in daily uses as well as during certain rituals and festivals. It undergoes symbiotic association with a commonly slow growing soil bacterium called *Bradyrhizobium* that fixes atmospheric nitrogen. In addition, cow pea also suffers from many fungal diseases that lower growth and yield of the plant. *Macrophomina phaseolina* (Tassi) Goid. is a major pathogen of more than 500 hosts, including cow pea. Its microsclerotia produced during parasitic phase and/or saprophytic phase (Dubey and Upadhyay 2001) serve as the primary source of inoculum and persist in soil for 2–15 years depending on environmental conditions, and association of sclerotia with host residue (Short *et al*. 1980; Baird *et al*. 2003). Sclerotia germinate through mycelia that ramify on root surface, penetrate through wound or cracks and enter inside the root before proper establishment (Ammon *et al*. 1975) resulting in development of charcoal rot disease.

Rhizobia that readily colonise the root surface of legumes and effectively form symbiotic associations have also been reported to reduce the incidence of plant disease. Moreover, they are directly involved in increased uptake of nitrogen, synthesis of phytohormones, solubilization of minerals such as phosphorus and production of siderophores that chelate iron and make it available to the plant root (Lalande *et al*. 1989). Several fungi have shown sensitivity to the presence of rhizobia. Malajczuk *et al*. (1984*)* have reported growth inhibition of *Phytophthora* and *P. cinnamomi* to different degrees by 15 *Rhizobium* isolates *in vitro*. Inhibitory effect of *R. leguminosarum* strains in reducing mycelial dry weights of fungal isolates *in vitro* has been demonstrated by Chao (1990)*.* Kelemu *et al*. (1995) have reported inhibitory effects of *Bradyrhizobium* on mycelial growth, sclerotial formation and germination of *Rhizoctonia solani* AG-1. The antagonistic activity of the cultural filtrates of the three wild rhizobial isolates from wild legumes and their synergistic effects with arbuscular mycorrhizal (AM) fungi for the biocontrol of damping-off and root rot diseases of faba bean caused by *Rhizoctonia solani*, *F. solani* and *Fusarium* spp. have been reported by El-Batanony *et al*. (2007) and Mazen *et al*. (2008).

Antagonistic properties of culture filtrates of *Bradyrhizobium* strains on sclerotia germination of *M. phaseolina* are not known. Hence, the present work reports the deleterious effects of culture filtrates of two plant growth promoting *Bradyrhizobium* sp. (*Vigna*) strains VR1 and VR2 on mycelial yield and

sclerotia germination of *M. phaseolina* that causes charcoal rot of *Vigna mungo*.

2. Material and Methods

2.1. Isolation of *Bradyrhizobium* isolates and fungal pathogen

Bradyrhizobium isolates were screened from root nodules of healthy plants of *V. mungo* from the crop field in Haridwar (India) as described by Vincent (1970). On the basis of preliminary investigation six isolates (VR1 − VR6) were selected and maintained on yeast extract mannitol agar (YEMA) medium at 4°C for further use. Physiological and biochemical characterization of isolates were performed by using HiCarbohydrate™ Kit (Himedia Laboratories Pvt. Ltd., Mumbai, India) following the manufacture's instruction. The cultures were identified following the Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994) and compared against a standard culture of *Bradrhizobium* sp. NAIMCC-B-00262 procured from the National Bureau of Agriculturally Important Microorganisms Culture Collection (NAIMCC), Azamgarh, U.P. (India).

The fungal pathogen *M. phaseolina* was isolated from diseased roots of *V. mungo* on sterilized Capek's Dox agar (CDA) medium grown at 30±1°C for 7 days. Characteristics of *M. phaseolina* were identified following mycological literature available in the laboratory. The fungal culture was maintained by regular subculturing on CDA medium at 4°C for further use.

2.1. 16S rDNA gene sequencing

The genomic DNA was isolated following Sambrook and Russel (2001). Amplification of 16S rRNA gene of VR1 and VR2 was carried out by a Bio-Rad thermal cycler using the primers PA: 5′-AGAGTTTGATCCTGGCTCAG-3′; PH: 5′-AAGGAGGTGATCCAGCCGCA-3′ (Banglore genei) with the Taq buffer (10X), dNTP mix (2.0mM), primers (10 ng/μl) and Taq polymerase (1 U). The PCR product was analyzed on 1.2% agarose in a TAE buffer. A 1 kb ladder DNA (Bangalore genei) was used as size marker. The PCR product was sequenced using ABI 3130 XL Sequencer, and similarity of 16S rRNA gene sequences was aligned using BLAST programme of GenBank database (NCBI).

2.3. Plant growth-promoting properties of *Bradyrhizobium* isolates

Exponentially grown culture of isolates were separately grown in broth medium at 28°C and 150 rpm for 24h. Cultures were separately centrifuged at 10,000 rpm for 10-15 minutes at 4°C. Supernatant was transferred into fresh tubes. 100 μl of 10 mM *O*-phosphoric acid was added to 2 ml of supernatant. Development of pink color confirmed IAA

production (Gupta *et al*. 2002). HCN production was determined following the modified method of Bakker and Schippers (1987). The exponentially grown cultures of bacterial isolates were separately streaked on agar plates supplemented with or without 4.4 g glycine l^{-1}. Filter paper soaked with 0.5% picric acid in 1% Na_2CO_3 was transferred in the upper lids of each plate and sealed with parafilm. The plates were incubated at 28 ±1°C for 72 h along with un-inoculated control. Change in colour from yellow to brown was indicative of HCN production.

Siderophore production was detected on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). Bacterial isolates (24 h old culture) were spotted on CAS medium and all plates were incubated at 28 ±1°C for 48 h; development of orange halo around the colonies confirmed siderophore production.

Bradyrhizobium isolates were separately spotted on Pikovskaya's agar plates for detection of phosphate solubilization. Plates were incubated at 28 ±1°C for 72 h. Formation of clearing zone around the colonies confirmed solubilization of inorganic phosphate by bacterial isolates (Pikovskaya 1948).

Defined medium was used to detect chitinase activity having colloidal chitin as sole carbon source following the method of Renwick *et al*. (1991). Bacterial Isolates were spotted on the plates contained defined medium and incubated at 28±1°C for 5-6 days; thereafter, plates were observed for development of clear zones around bacterial colonies.

2.3.1. Assessment of 1- aminocyclopropane-1-carboxylate (ACC) deaminase activity

The bacterial isolates were separately grown in YEM broth and the cells were hasrvested from their respective log phase cultures. Bacterial culture of each isolate was centrifuged at 9,000 rpm for 10 min to collect cell pellets. The pellets were washed with sterile distilled water and re-suspended in 1 ml of sterile water. Cell suspension was spot inoculated on Petri plates containing minimal medium amended with 3 mM ACC. The minimal medium plates devoid of ACC served as negative control; plates containing minimal medium with $(NH4)_2$ SO_4 as a nitrogen source served as positive control. All inoculated plates were incubated at 28±1°C for 3–4 days (Honma and Shimomura 1978).

2.4. Antagonistic activities of *Bradyrhizobium* strains against *M. phaseolina in vitro*

2.4.1. Colony growth inhibition of *M. phaseolina*

Antagonistic property of *Bradyrhizobium* strains against *M. phaseolina* was tested by using dual culture technique (Skidmore and Dickinson 1976). A 5 day-old mycelial disc (5 mm diam.) cut from actively growing margin of pathogen was placed in the centre of sterilized modified medium containing

YEMA and Capek's Dox agar (CDA) in a ratio 1:1. A loop full exponentially grown culture of each isolate of *Bradyrhizobium* was spotted 2 cm juxtaposed from the fungal disc and incubated at 28±1ºC for 5 days. Growth inhibition was calculated by measuring the distance between the edge of bacterial and fungal colonies as compared to control (without bacterium). The zone of inhibition was recorded and growth inhibition (%) was calculated by using the formula: $100 \times$ C-T/C, where C is radial growth in control and T is radial growth in dual culture.

2.4.2. Scanning electron microscopic study

Mycelia were collected from the zone of interaction between two microorganisms with the help of a sterile needle for sample preparation for scanning electron microscopy (SEM). The mycelia were fixed overnight using 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) at 48°C, and washed thrice in phosphate buffer for 10 min. Then, samples were serially dehydrated through 70, 80, 90 and 100% ethanol (5 min at each stage) and finally three changes in 100% ethanol at room temperature. Ethanol was replaced with liquid CO_2 and air dried following Lopez-Llorca and Valiente (1993). The samples were mounted on stubs followed by coating with gold; the coated specimens were observed at 15 kV in a LEO 485 VP SEM and suitable photomicrographs were taken.

2.4.3. Growth inhibition of *M. phaseolina* by culture filtrates of isolates VR1 and VR2

2.4.3.1. Preparation of Cell-free culture filtrates of isolates VR1 and VR2

Cell-free culture filtrates of antagonistic *Bradyrhizobium* strains VR1, VR2 and the standard culture NAIMCC-B-00262 were prepared separately. Log phase cell suspension (1 ml) of *Bradyrhizobium* strains was separately transferred in 50 ml YEM broth and incubated at 150 rpm for 72 hours at 28±1°C on a rotary shaker. The cell suspension from each tube was centrifuged at 10,000 rpm for 10 minutes, supernatant was collected separately in 50 ml beakers and few drops of chloroform was added to each beaker to kill the bacterial cells if any. Culture filtrate of each isolate was left open aseptically for 1 hour to evaporate the chloroform vapours. The filtrate was used directly for colony growth inhibition of *M. phaseolina*, measuring inhibition of mycelia dry weights, and in preparation of agar plates for sclerotial germination.

2.4.3.2. Colony growth inhibition by culture filtrates

Sterilized Capek's Dox agar (CDA) plates were prepared and wells of 5 mm diam. were was prepared at 2 cm distance from the centre. An agar block (5 mm diam.) containing mycelia mat from actively growing margin of *M. phaseolina* was inoculated in the centre of CDA plates. Aliquot of cell-free culture filtrate (0.5 ml) of a strain was poured in both wells of each CDA plate. Sterilized distilled water was poured in control plates in the same way. All the plates were incubated at 28±1°C for 5 days and colony growth inhibition (%) of the pathogen was recorded as described earlier.

2.4.3.3. Mycelial dry weight measurement

Czapek Dox broth (CDB) was prepared and sterilized at 121±1°C for 20 min. For estimation of mycelial yield cell-free culture filtrate of each *Bradyrhizobium* strain and NAIMCC-B-00262 was added separately in 50 ml pre-sterilized CDB in three replicates so as to get 15, 30 and 45 % concentration (v/v). Sterilized distilled water was added in the same concentration in control sets in triplicate. The flasks were inoculated with 5 agar blocks (each of 5 mm diam.) cut from actively growing margin of *M. phaseolina*. Each culture was filtered through pre-weighed Whatman filter paper No. 1 after incubation for seven days at 30±1°C. The mycelial mat of each treatment was dried at 85°C for 24 h to determine the mycelial yield, and loss (%) in mycelial dry weight was calculated by using the formula : $100 \times$ (C-T)/C, where C = mycelial dry weight in control, T= mycelial dry weight in treatment.

2.4.4. *In vitro* sclerotia germination of *M. phaseolina*

2.4.4.1. Harvesting of sclerotia

Sclerotia of *M. phaseolina* were harvested by cellophane disc method of Ayanru and Green (1974). Cellophane paper discs (90 cm diam.) were cut and boiled in 100 ml distilled water in a beaker for 30 min to remove plasticizers. Then cellophane discs were aseptically removed and spread onto the surface of sterilized and solidified potato dextrose agar (PDA) medium in Petri dishes. An agar block (5 mm diam.) from margin of actively growing margin of *M. phaseolina* was put in the centre of these Petri plates and incubated at 30±1°C for 5 days. Thereafter, each cellophane paper was aseptically removed from each Petri dish and scrubbed with a blade to harvest the mycelial mat containing sclerotia. The mixture of mycelia and sclerotia was dried overnight at 35-40°C onto sterile filter paper, mashed using a sterile pestle and mortar, and passed through a sieve of 50 µm pour size. The sclerotial powder was transferred into a vial and stored at 4°C for further use.

2.4.4.2. Sclerotia germination

Sclerotia germination and hyphal development *in vitro* was studied on sterilized water agar medium (2%) plates. Cell-free culture filtrate of each isolate prepared as above was poured into 20 ml culture tubes each containing 2% water agar medium in three

replicates so as to get 15, 30 and 45% concentration (v/v) of filtrate of each isolate. The control plates were devoid of any filtrate. Sclerotia germination was studied following the standard tube dilution method (Baily and Scott, 1974) modified by Dwivedi and Dubey (1986). A small amount of sclerotia prepared as above were put in 100 ml beaker, washed with sterile phosphate buffer (0.1 M, pH6.5), centrifuged at 1000 rpm for 3 min and decanted to remove mycelia fragments and exogenous inhibitors. Then 50 ml sterile distilled water was poured into the beaker to prepare sclerotial suspension (about 300 sclerotia/ml). One ml suspension was poured separately into each plate amended with different concentrations of culture filtrate in triplicate along with proper control. The Petri plates were incubated at 30±1°C in dark and sclerotia germinating in each plate were counted after 48, 72 and 96 h of incubation.

2.4.4.3. Patterns of sclerotia germination

Effect of cell-free culture filtrate of each *Bradyrhizobium* isolate on development of hypha/mycelia was studied after 48 h of incubation as described by Dubey (1992). In both the cases 400 sclerotia per treatment were counted in three replicates. Germination was defined as the hypha production by a sclerotium to about half of its diameter. The germinating sclerotia were examined under light compound microscope (Olympus BX 51 TRF), and images of mycelial/sclerotial deformities, if any, were captured by using an Image Anlyser (Biovis)

3. Results

3.1. Isolation and identification of *Bradyrhizobium* isolates and *M. phaseolina*

Morphological, physiological and biochemical (including carbon and nitrogen utilization) characteristics of all six isolates showed them as Gram-negative, rod shaped, creamy white, translucent, round, highly gummy and forming convex colonies on YEMA plates. All of them were motile, slow grower, and found positive for catalase, and negative for H_2S production, methyl red and Voges Proskaur reaction. Most of them tolerated temperature up to 40°C, pH 7-8 and salinity by 4 % NaCl concentration. The various phenotypic characters of these isolates were compared with standard strain *Bradyrhizobium* sp. NAIMCC-B-00262. The isolates VR1 was found more identical with *Bradyrhizobium japonicum* and VR2 with *Bradyrhizobium* sp.

Fungal colonies were greyish to black in colour producing cottony mycelia and numerous jet black microsclerotia.

3.2. 16S rRNA gene sequencing

The 16S rRNA gene sequence of VR1 and VR2 comprised of 1374 bp and 1257 bp, respectively (NCBI Gene Bank Accession Numbers VR1 JX001401 and VR2 JX001402). The isolate VR1 showed 100% sequence similarity with *Bradyrhizobium japonicum* EU333382 and *Bradyrhizobium* sp. NR042177, and VR2 showed 100% sequence similarity with *Bradyrhizobium* sp. AB681396 and *Bradyrhizobium elkanii* AB672634.

3.3. Plant growth promoting (PGP) attributes in *Bradyrhizobium* strains

Presence of PGP attributes in different *Bradyrhizobium* strains (VR1−VR6) of *V. mungo* are shown in Table 1. All the isolates produced IAA and solulilised phosphorus but did not produce HCN. Only three isolates VR1, VR2 and the standard culture *Bradyrhizobium* sp. NAIMCC-B-00262 showed production of siderophore. Isolates VR1 and VR2 as well as the standard culture produced ACC deaminase, whereas the other isolated failed to show positive activity.

Table 1: Plant growth promoting (PGP) and antagonistic attributes in different *Bradyrhizobium* strains from *V. mungo*.

Bacterial strains	Plant growth-promoting attributes				Antagonistic attributes		
	IAA[a] production	HCN[b] production	Phosphate solubilization	Siderophore production	Chitinase activity	ACC[c] deaminase	Antagonism against *M. phaseolina*
VR1	+++	−	+++	++	++	+	++
VR2	+++	−	+++	++	++	++	+++
VR3	+	−	+	−	−	−	−
VR4	+	−	+	−	−	−	−
VR5	+	−	+	−	−	−	−
VR6	+	−	+	−	−	−	+
Bradyrhizobium sp. NAIMCC-B-00262	++	−	+++	−	+	+	++

Abbreviation: − = negative; + = positive, + = small halos >0.5 cm wide surrounding colonies; ++ = medium halos; +++ = large halos >1.0 cm wide surrounding colonies.
[a] Indole-3-acetic acid; [b]Hydrogen cyanide; [c]1- aminocyclopropane-1- carboxylate (ACC) deaminase.

3.4. Antagonistic properties in *Bradyrhizobium* isolates against *M. phaseolina*

Isolates VR1, VR2 and VR5 displayed more chitinase activity than strain NAIMCC-B-00262. The other isolates did not show chitinase activity. None of isolates showed ACC deaminase activity except VR1, VR2 and NAIMCC-B-00262. Except VR3, VR4 and VR5, the other strains inhibited colony growth of *M. phaseolina in vitro* (Table 1). In dual culture isolate VR2 resulted in maximum growth inhibition (71.5%) of the pathogen followed by VR1 (50.5).

Cell-free culture filtrates of VR1 and VR2 inhibited colony growth by 37.6% and 49.2%, respectively (Figure 1).

Light microscopic study and scanning electron micrographs (SEM) show post-interaction events in hyphae, mycelia and sclerotia of *M. phaseolina* caused by *Bradyrhizobium* sp. VR2 displaying several deformities besides colony growth inhibition, such as hyphal fragmentation (Figure 2 A), cytoplasm vacuolation and lysis of hyaline mycelia (B), production of hyaline sclerotium due to loss of cell pigments resulting in structural integrity and viability (C), hyphal shrinkage, fragmentation and lysis (D-E).
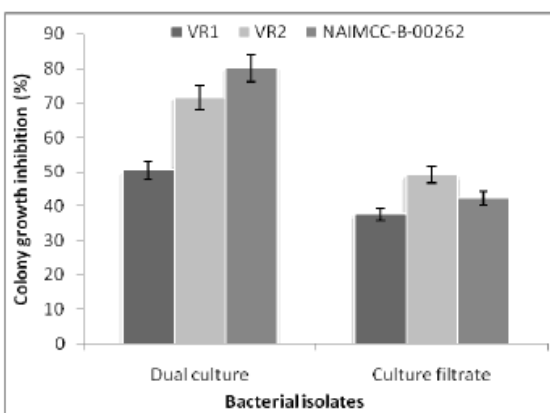

Figure 1: Growth inhibition (%) of *M. phaseolina* in dual culture and by cell free extract of *Bradyrhizobium* isolates at 28±1°C *in vitro*. Values are mean of three replicates ± standard error of mean.

3.5. Growth inhibition of *M. phaseolina* by culture filtrates of *Bradyrhizobium* strains

Cell-free culture filtrate of *Bradyrhizobium* strains VR1 and VR2 caused 37.6% and 62.2% colony growth inhibition respectively. *Bradyrhizobium* sp. NAIMCC-B-00262 caused 56.3% colony growth inhibition of the pathogen (Fig. 1). High concentration of culture filtrates (45 %) of strains VR1 and VR2 significantly (P >0.1) inhibited mycelia yield by 100%, whereas NAIMCC-B-00262 caused 91.3% inhibition in yield. However, inhibitory

effect on mycelia yield was significantly (P >0.1) low at 15% concentration of all culture filtrates. In control sets, increase in dilution of growth medium resulted in a decline in mycelia production (Figure 3).
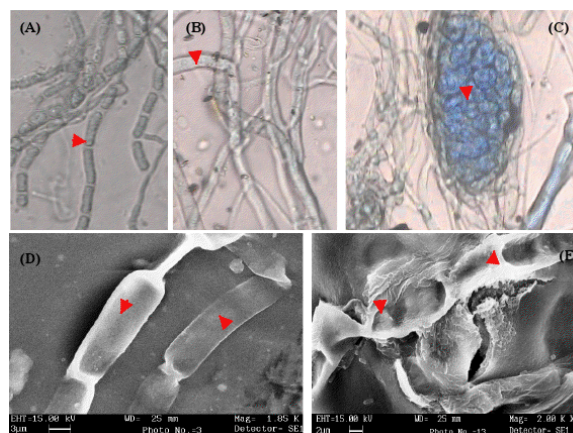

Figure 2: Light and scanning electron micrographs (SEM) showing post-interaction events between *Bradyrhizobium* strain VR2 and *M. phaseolina*, hyphal fragmentation of *M. phaseolina* by VR2 (A), cytoplasm vacuolation and lysis of hyaline mycelia (B), loss of viability of a hyaline sclerotium (C); hyphal shrinkage, lysis of loss of viability (D-E).
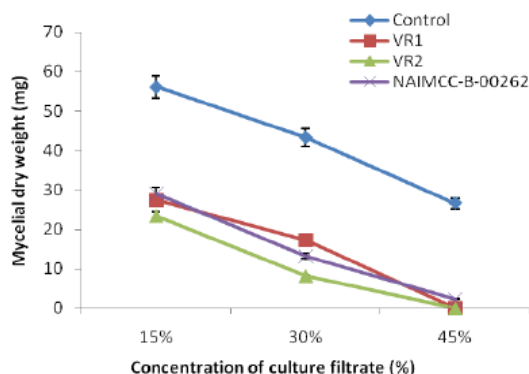

Figure 3: Effect of cell-free culture filtrates of *Bradyrhizobium* isolates on mycelia yield of *M.phaseolina*. Values are mean of triplicate ± standard error of mean. LSD at P>0.1 in comparison with control values.

3.6. Study of sclerotia germination

Sclerotia germination of *M. phaseolina* gradually increased with incubation time. *M. phaseolina* sclerotia showed germination at 15% and 30% concentration of culture filtrates of VR1 and VR2, but inhibitory effect was more pronounced in culture filtrate of VR2 than that of VR1. Moreover, sclerotia germination was completely inhibited at 45% concentration of culture filtrates of VR1 and VR2 strains, whereas 11% and 15% sclerotia germination

was recorded after 76 and 96 hours respectively in media mixed with 45% culture filtrate of *Bradyrhizobium* sp. NAIMCC-B-00262 (Table 2).

Table 2. Effect of cell-free culture filtrates of *Bradyrhizobium* strains on germination of *M. phaseolina* sclerotia.

Bacterial isolates	Concentration (%)	Sclerotia germination (%)*		
		Incubation (hours)		
		48	72	96
Control	-	80±2	86±3	93±3
VR1	15	32±3	42±2	54±1
	30	23±1	26±1	31±2
	45	0	8±3	12±1
VR2	15	28±2	37±2	47±2
	30	8±1	12±1	17±1
	45	0	0	0
Bradyrhizobium sp. NAIMCC-B-00262	15	36±2	47±1	58±1
	30	20±1	25±2	31±3
	45	0	11±1	15±2

*Values are mean of ten replicates ± standard error.

The pattern of sclerotia germination varied at different concentrations of culture filtrates of *Bradyrhizobium* strains VR1 and VR2. The number of sclerotia producing >7 hyphae was more in control (80) than the culture filtrate-amended Petri plates, while the least number of sclerotia (4) produced 1-3 hyphae. Moreover, the number of sclerotia producing less number of hyphae was more in Petri plates amended with 30% culture filtrate of isolate VR2 than the other isolates. The number of sclerotia producing 1-3 hyphae got increased and that of producing >7 hyphae got decreased with increasing the concentration of culture filtrate of both strains (Table 3). Viable sclerotia of *M. phaseolina* showed myceliogenic germination on water agar medium which in turn produced small-sized secondary sclerotia singly or in chain (Figure 4 A). However, sclerotium lost vigour to germinate at higher concentration of culture filtrate of VR2 (B). Hyaline mycelia failed to form sclerotia after treatment of culture filtrate (C).

Table 3. Effect of culture filtrates of *Bradyrhizobium* isolates on hyphal development during sclerotia germination of *M. phaseolina* after 48 h of incubation.

Bacterial isolates	Concentration (%, v/v)	Sclerotia germination (%)*			
		No. of hypha produced /sclerotium			
		1-3	4-6	>7	Total
Control	-	4±1	26±3	50±1	80±2
VR1	15	4±2	10±1	18±2	32±1
	30	21±1	3±2	0	23±3
	45	0	0	0	0
VR2	15	4±2	17±3	7±2	28±2
	30	6±3	2±2	0	8±2
	45	0	0	0	0
Bradyrhizobium sp. NAIMCC-B-00262	15	11±1	18±3	7±2	36±2
	30	3±2	16±4	1±1	20±3
	45	0	0	0	0

* Values are mean of ten replicates ± standard error.
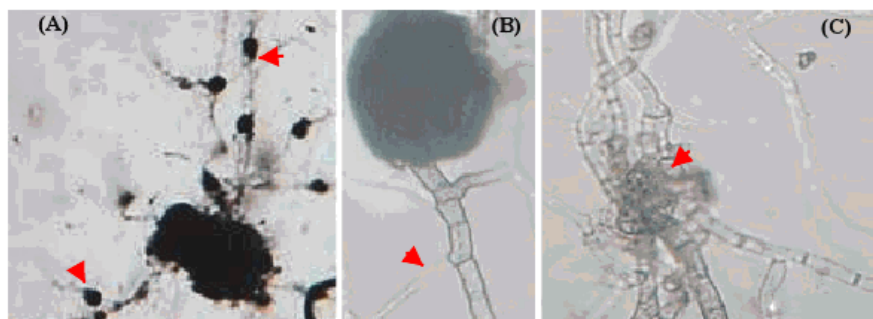

Figure 4: Effect of 30% culture filtrate of strain *Bradyrhizobium* strain VR2 on germination of *M. phaseolina* sclerotia. Myceliogenic germination of a single sclerotium (A), sclerotium lost vigour to germinate after treatment with culture filtrate (B), hyaline mycelia failed to form sclerotia after treatment with culture filtrate (C).

4. Discussion

On the basis of morphological, biochemical, physiological and 16 S rDNA gene sequencing, the bacterial isolates were identified as *Bradyrhizobium japonicum* strain VR1 and *Bradyrhizobium* sp. (*Vigna*) strain VR2. Presence of plant growth promoting attributes and antagonistic characteristics in both strain VR1 and VR2 corroborate other's observations also (Lalande *et al.* 1989, Deshwal *et al.* 2003, Mazen *et al.* 2008).

Fungal colonies were greyish to black in colour producing numerous jet black microsclerotia. Presence of these features confirms the characteristics of *M. phaseolina* as have been

decribed by other workers (Dubey and Upadhyay 2001).

In the present investigation different *Bradyrhizobium* isolates (VR1−VR6) of *V. mungo* showed the presence of PGP attributes. The beneficial effect *of Rhizobium* and *Bradyrhizobium* in legumes in terms of biological nitrogen fixation has been a main focus in the recent past (Deshwal *et al.* 2003). Production of siderophore and IAA, and phosphate solubilization by *Rhizobium* and *Bradyrhizobium* (Antoun *et al.* 1998) and *Bradyrhizobium* strains AHR-2, AHR-5 and AHR-6 (Deshwal *et al.* 2003) have also been reported, besides producing exopolysaccharides and chitinase (Mazen *et al.* 2008). Many species of rhizobia promote plant growth besides inhibiting the growth of certain pathogenic fungi (Lalande *et al.* 1989). Dubey and Upadhyaya (2001) have reviewed the antagonistic properties of many species of rhizobia against several pathogenic fungi, such as *M. phaseolina, Rhizoctonia* solani, *Fusarium oxysporum, Pythium* spp., etc. both in leguminous and non-leguminous plants.

Isolates VR1 and VR2 displayed chitinase and ACC deaminase activities resulting in maximum growth inhibition by71.5% and 50.5%, respectively in dual culture. Many previous workers have reported the production of chitinase, *β*-1,3-glucanase and ACC deaminase by *Rhizobium*, *Bradyrhizobium* and other bacteria (Gupta *et al.* 2006, Kumar *et al.* 2010, Dubey *et al.* 2012). Biological control involves destruction of the propagating units of plant pathogens, prevention of formation of surviving inocula, weakening of pathogen in infested residues, reduction of pathogen's vigor by antagonistic microorganisms. The disease is controlled by various modes of action of antagonism and induced resistance or plant growth promotion. Bacterial antagonism responsible for biological control may operate by antibiosis, competition and/or parasitism. Parasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi (Chet *et al.* 1990).

Metabolites of isolate VR2 secreted in zone of interaction caused several deformities such as fragmentation, lysis, shrinkage, perforation and loss of pigment in hyphae and sclerotia of *M. phaseolina*. Such abnormalities result in loss of fungal viability. Similar post-interaction events in *Sclerotinia sclerotiorum* by *P. aeruginosa* GRC₁ (Gupta *et al.* 2006), in *Cajanus cajan* by *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 (Kumar *et al.* 2010), and in *M. phaseolina* by *Azotobacter chroococcum* AZO2 (Dubey et al. 2012) have also been reported.

In the present investigation mycelia dry weight of *M. phaseolina* was inhibited more by *Bradyrhizobium* strain VR2 than the other strains. The inhibitory effect may be due to presence of toxins and/or cell wall lytic enzymes produced in culture filtrates. Inhibition of myclelial dry weight in control sets may be explained to be due to dilution of nutrient medium that affected dry weight. The presence of toxin(s) in culture filtrate of *Bradyrhizobium* cannot be ruled out (Deshwal *et al.* 2003). The inhibitory properties of rhizobial culture filtrate containing rhizobitoxin have been reported by Chakraborty and Purkayastha (1984). Rhizobitoxin is an important compound involved in symbiosis between rhizobia and legumes that enhances nodulation and competitiveness of *Bradyrhizobium elkanii* on a legume host (Yuhashi *et al.* 2000). Chitinolysis plays an important role in biological control of plant diseases and has been substantiated with increased disease control by chitin-supplemented application of chitinolytic biocontrol agents. Earlier, we have reported the role of chitinase (E.C.3.2.1.14) and *β*-1,3-glucanase (E.C.3.2.1.39) activities produced by fluorescent pseudomonad which inhibited growth of *Fusarium oxysporum, M. phaseolina* and *Sclerotinia sclerotiorum in vitro* (Gupta *et al.* 2002, 2006).

Different concentrations of culture filtrate of *Bradyrhizobium* strains VR1 and VR2 effectively inhibited sclerotia germination of *M. phaseolina* causing complete inhibition at 45% up to 48 h. Inhibition in scelrotia germination may be explained to be due to presence of inhibitory factors in culture filtrate. Microsclerotia are made up of mycelia network the cells of which are tightly cemented. Individual cell acts as a unit and all of them show germination; this is why a sclerotium produces many hyphae emerging from it. Only the viable cells of sclerotium germinate due to the presence of inhibitory factors, and immediately produce secondary sclerotium required for its survival. This is why number of hypha emerging from a sclerotium was more in control than culture filtrate treated cell (Dubey 1992).

Kelemu *et al.* (1995) have reported the inhibitory effects of *Bradyrhizobium* strains or their cell-free culture filtrates on mycelial growth, sclerotial formation, and sclerotial germination of *Rhizoctonia solani* AG-1, a pathogen of tropical forage legumes. Besides, cell-free culture filtrates of three *Bradyrhizobium* strains had inhibitory effects on the growth of the other bacteria such as *Escherichia coli* DH5α and *Xanthomonas campestris* pv. *phaseoli* CIAT 555. Das *et al.* (2008) have found that the cell-free culture filtrates of fluorescent pseudomonads strains at 20% concentration significantly reduced the formation and germination of microsclerotia of *M. phaseolina*. Role of chitinase in control of *Sclerotium*

rolfsii and *Rhizoctonia solani* by *Serratia marcescens* (Chet *et al.* 1990), and *Sclerotinia sclerotiorum* by *Pseudomonas aeruginosa* GRC₁ (Gupta *et al.* 2006) associated with certain plant diseases has been reported. Chet *et al.* (1990) have found that *S. marcescens* releases N-acetyl D-glucosamine from cell walls of *S. rolfsii* due to presence its chitinolytic activity.

Use of antagonistic *Bradyrhizobia* strains has dual advantage compared to other biocontrol agents as the former assimilate atmospheric nitrogen besides killing deleterious phytopathogens. It may be concluded that the presence of inhibitory properties in culture filtrates of *Bradyrhizobium* strains help to act as potential biocontrol agent for control of *M. phaseolina*.

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