GESA
GLOCAL ENVIRONMENT & SOCIAL ASSOCIATION

International Journal of Biological Innovations
http://ijbi.org.in | http://www.gesa.org.in/journals.php
https://doi.org/10.46505/IJBI.2026.8202
IJBI 8(2): 156-166 **(2026)**
E-ISSN: 2582-1032

IN VITRO BIO-ASSAY OF THE ANTAGONISTIC POTENTIAL OF *BACILLUS* STRAINS AGAINST *ALTERNARIA* SPP. CAUSING LEAF SPOTS IN MUSTARD

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Article Info:
Research Article
Received
02.02.2026
Reviewed
05.05.2026
Accepted
05.07.2026

Abstract: An efficient technique for screening the potential of antagonistic microbes against plant pathogens in a tripartite environment, host tissues, pathogen, and bio-control agent, is needed. The current research presents a prototype to screen the antagonistic potential of *Bacillus* strains against *Alternaria* spp. causing leaf spots in mustard using the detached leaf assay technique and ImageJ software. During exploration, two methods were used individually to check the pathogenicity or screening for resistance against plant pathogens. However, the use of these two techniques presents a promising and effective method to bio-assay the potential of antagonistic microbes against plant pathogens in a limited time. The findings also promulgate the technique to screen the potent *Bacillus* strains against *Alternaria* spp. efficiently causing a leaf spot in mustard.

Keywords: *Alternaria*, Antagonist, *Bacillus*, Bio-assay, Bio-control, Detached leaf assay, Mustard, Screening.

Cite this article as: Mohit, Puyam A., Jambhulkar P.P., Trivedi S. and Usha (2026). *In vitro* bio-assay of the antagonistic potential of *Bacillus* strains against *Alternaria* spp. causing leaf spots in mustard. *International Journal of Biological Innovations*. 8(2): 156-166. https://doi.org/10.46505/IJBI.2026.8202

INTRODUCTION

Mustard (*Brassica juncea*) ranks as the world's third most significant oilseed crop, following soybean (*Glycine max*) and palm oil (*Elaeis guineensis*). Primarily cultivated in subtropical and tropical regions, it is India's second most vital oilseed crop after groundnut, contributing approximately 27.8 percent to the country's oilseed production (Sang *et al.,* 2014). The primary concern for mustard cultivation is the loss caused by *Alternaria* spp. that inflicts lesions on leaves characterized by necrotic patches and chlorotic borders. This leads to leaf defoliation and hastened senescence due to abscisic acid accumulation, reducing the photosynthetic area. The consequential early defoliation, flower bud abortion, siliquae dehiscence, premature ripening, and seed shriveling collectively result in significant yield losses (Leong, 1886). *Alternaria alternata* alone accounts for 59.4 percent of these losses, while *Alternaria brassicae* and *A. brassicicola*, cause yield reductions ranging from 10 to 70 percent (Sah *et al.,* 2021).

Considering the importance of the disease and its impact on the yield, there is a need to tackle the disease with a sustainable management strategy. In response to the adverse environmental impact associated with chemical fungicides, microbial inoculants have gained widespread usage over the past decade to

combat numerous destructive plant diseases (Chakraborty and Chatterjee, 2008; Akhtar *et al.,* 2010; Shekhawat *et al.,* 2012). Biological disease control stands out as an appealing alternative strategy, aligning well with the objective of sustainable agricultural practices. Microbial biological control agents (BCAs) have garnered increased attention in recent years due to their potential to manage plant diseases sustainably. These considerations have mandated the need for microbial pesticides in the form of bio-control agents to preserve agricultural output, enhance plant growth, and compensate for the reduced use of synthetic pesticides (Rane and Patel, 2021; Chaudhary *et al*., 2024).

The spore-forming PGPR piques significant interest among scientists amidst numerous soil antagonists due to their stable presence in soil (Chakraborty and Chatterjee, 2008; Fousia *et al.,* 2016). Many of these PGPR species serve as robust producers of antibiotics capable of suppressing various phytopathogens (Haas and Defago, 2005; Singh *et al.,* 2013; Hasanah *et al.,* 2023). *Bacillus* spp. in particular, gained recognition as safe biocontrol agent, notably as seed protectants and antifungal agents (Pettitt *et al.,* 2011; Singh *et al.,* 2013). Their spore-forming ability further positions them as advantageous natural formulations compared to other microorganisms (Fousia *et al.,* 2016; Hasanah *et al.,* 2023). *B. subtilis* as a bio-control could be an alternative approach as a biocontrol agent to fungal diseases, improving crop yields and sustainable agriculture. Among microbial BCAs, *Bacillus* strains have been considered one of the efficient antagonistic microorganisms for their ability to produce a variety of metabolites in nature with multiple modes of action, including antimicrobial or growth-stimulating compounds (Moussaid *et al.,* 2025). *Bacillus* spp. are highly effective plant growth-promoting rhizobacteria (PGPR) and antagonists, playing multiple roles in enhancing plant health and resilience. Their effectiveness lies in their ability to improve nutrient availability, suppress plant diseases as biocontrol agents, and enhance soil health through various mechanisms. They employ diverse strategies such as nitrogen fixation, phosphate solubilization, production of phytohormones, induction of systemic resistance, and synthesis of antimicrobial compounds. Additionally, their spore-forming ability and tolerance to environmental stresses contribute to their reliability and persistence across a wide range of agricultural conditions (Cappuccino and Sherman, 1992).

However, screening for an efficient antagonistic microbial is time-consuming as it requires good performance based on dual culture and field data. *In vivo*, screening in natural and greenhouse conditions measured lesion diameter, disease severity, and plant physiological status. *In vitro* assays assessed the antagonistic activity of BCAs against pathogens, focusing on inhibition zones and pathogen growth suppression (Yayis *et al.,* 2018). *In vitro* screening based on inhibition zones and pathogen growth suppression (dual culture or diffusion well assay) gives an idea of the interaction of the pathogen and the antagonist, but there is a thin line that needs to be addressed between their interaction and the involvement of the host. The current research will give a prototype of the tripartite interaction of the host, pathogen, and antagonist under *in vitro* conditions.

Detached leaf assay is being used as a rapid and inexpensive determination of pathogenicity of plant pathogens, all year round like *Pythium* spp. Causing chrysanthemum roots (Nwadili and Aduo, 2021) for screening common beans (*Phaseolus vulgarise* L.) *in vitro* against angular leaf spot, Yam anthracnose disease caused by *Colletotrichum gloeosporioides* and for screening plant pathogen-biological control agents (Liviu *et al.,* 2015). Similarly, ImageJ software is used to study the severity of the disease based on the image captured (Meena *et al.,* 2012). However, using detached leaf assay together with ImageJ software for assessing the potential of an antagonistic microbe is less explored except for a single report (Liviu *et al.,* 2015).

The present research will present a rapid approach to screen bio-control agents against the plant pathogen *Alternaria alternata* causing leaf blight in mustard using detached leaf assay and ImageJ software together for assessing the potential of the antagonist bacterium under *in vitro* condition. It also discusses the characterization of the microbes based on cultural, morphological and biochemical tests associated with plant growth promotion attributes.

MATERIALS AND METHODS

1. Isolation and screening of rhizobacteria

1.1. Sample collection and isolation of bacteria: To isolate bacterial strains during the Rabi 2022-2023, rhizospheric soil samples were collected from various fields in five districts of the Bundelkhand region: Jhansi, Datia, Lalitpur, Tikamgarh, and Nivari. A total of 43 samples were collected from different fields. Till it is used, the soil samples are kept at 4°C in the refrigerator. Root-adhering soil was dislodged and stored in polybags. Bacteria were isolated from the sample following serial dilution with sterile distilled water.

1.2. Primary screening of isolates for antifungal activity: The antifungal activity of all 43 bacterial

isolates was initially evaluated against *Alternaria alternata, A. brassicae* and *A. brassicicola* using the dual-culture method. In this method, four different bacterial isolates were spot-inoculated at equidistant points from the center of Petri plates. Each inoculation comprised of 20 µl bacterial suspension (10^8cfuml^{-1}), placed on the periphery of potato dextrose agar (PDA) plates. Additionally, a single 5 mm plug containing the pathogen, grown for 5 days, was inoculated in the central region of the plates. As a control, one PDA plate was solely spot-inoculated with the pathogen plug. All plates were continuously incubated at 30°C for up to 5 days until pathogen growth reached the edge of the control plate. After this period, fungal growth was assessed by measuring the colony radius, while the inhibition zone of fungal growth around each point of bacterial inoculation was simultaneously measured. The percent inhibition of mycelia growth over control was estimated using the following formula (Vincent, 1947):

$$I = C\text{-}T/C*100$$

where, I = Percent inhibition of mycelium
C = Growth of mycelium in control
T = Growth of mycelium in treatment

All the isolates were grouped based on their performance where, I= ≥ 51 percent, II= 20-50 percent, III= 1-20 percent, and IV=no inhibition (0 percent). Potential bacterial strains, which showed antagonistic activity against the fungal pathogens under study with more percent of inhibition ≥ 51% and at a range of 20-50% in dual culture techniques were selected for further studies.

2. Characterization and identification of antagonists

Antifungal bacterial isolates were morphologically and biochemically characterized following Bergey's manual of determinative bacteriology as per the standard procedures (Asaka and Shoda, 1996; Chaudhary *et al.,* 2024). Different microscopic and cultural parameters like shape and size of cells, colony morphology, elevation, color, surface, and edges were tested in a nutrient agar plate at 28^{0}C for 48-72 h. Gram's reaction, amylase test, catalase, oxidase, motility, and hydrogen sulfide (H_2S) production test, cellulase activity, siderophore production, protease test, phosphate solubilization test were also performed using conventional methods. Another 12 tests *viz*., indole, methyl red, Voges Prokauer's, citrate utilization, glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose and sucrose were performed using KB001 Hi1MViC ™ bio-chemical test kit. Altogether 23 biochemical tests were performed for characterization and assessment of growth-promoting properties.

The full-length 16S rDNA of the putative antagonists was sequenced using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Aneja, 1992; Turner *et al.,* 1999). The accession numbers of the antagonists were obtained after depositing rDNA sequences in the NCBI GenBank database. The nearest strain relationship was established by comparing sequences of the 16S rDNA gene with a database in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTn.

3. Bioassay using detached leaf assay technique

3.1. Preparation of microbial suspension: Bacterial isolates selected for screening potential biocontrol agents were grown overnight in culture tubes (50 mL) with 15-mL nutrient broth medium at 28 ^{0}C on a rotary shaker set at 220 rpm. The tubes were centrifuged for 10 min at 3000 rpm and the pellet cells in sterile distilled water. The bacterial density (OD600) is measured and adjusted to 0.2 by adding autoclaved distilled water. The conidial suspension of *Alternaria alternata* was also prepared by using sterilized distilled water from the 7-day-old culture in a flask, adjusting the same OD i.e., 0.2 at 600 nm. Subsequently, both suspensions were combined, ensuring an optimal blend. The blended suspension of pathogen along with the potent isolate was prepared separately for each treatment. The mixture of the bacterial and conidial suspensions was maintained with a final OD600 of 0.2 and a density of 2 x10^4 spores/ml, respectively.

3.2. Preparation of leaf samples: Older leaves of presumably equal size from the lower side of the fully grown mustard plants will be collected (Fig. 3). Small wounds are made on the abaxial epidermal surfaces of the leaf by gently puncturing with a sterile needle tip under aseptic conditions. Twelve sterilized petri plates of 90mm diameter were taken, and sterilized filter paper moistened with water was spread out to retain the moisture for the leaf. Then, the leaf samples were placed on the prepared moist chamber plates. Additionally, the stalk of the leaves will be plugged with moistened cotton. The setup was prepared for three replications of each treatment. The plates will be sealed with parafilm after inoculation and incubated at 25^{0}C.

3.3. Inoculation of the Microbial suspension: 20 µL droplets of the optimal blend of bacterial and conidial suspension on the injured surface of the leaflets on both sides of the midrib were placed in an aseptic condition under the laminar airflow chamber. Following the inoculation, the samples were allowed to incubate for 10 days at 30°C, providing ample time for the interactions between potent antagonist and *A.*

alternata. A positive control of leaflets inoculated solely with the *Alternaria* spore suspension, and a negative control inoculated only with sterile distilled water were maintained. Ten days after inoculation, the severity of infection is recorded by capturing digital images using camera NIKON D5600. The digital images of the symptoms of the leaf samples in the plates were assessed by image analysis software ImageJ (Pettitt *et al.*, 2011). For processing the image in imageJ, the protocols were followed (Pride *et al.*, 2020). A representation of the ImageJ process image of the control sample is presented in Fig. 4.

Damage diseased area percentage and control efficacy of the artificially inoculated samples were recorded and based on that control efficacy of the potent isolates in different plates was calculated using the formula (Pettitt *et al.*, 2011):

$$\text{Damage Area (\%)} = \frac{\text{Disease leaf area}}{\text{Total leaf area}} \times 100$$

$$\text{Control efficacy (\%)} = \frac{(1 - \text{Damage disease leaf area \% in treated sample})}{\text{Disease Damage leaf area in control}} \times 100$$

RESULTS AND DISCUSSION

All 43 isolates isolated from different rhizospheric soil were put into primary screening for antifungal activity.

1. Primary screening of isolates for antifungal activity

A preliminary screening of 43 isolates was done using dual culture technique and then isolates showing antagonistic activity were subjected to primary screening for antifungal activity. It was found that out of the 43 isolates, seven isolates RB6, 16, 24, 25, 31, 37, and 42 showed visible restriction of the fungal mycelial growth. These seven isolates were seen as potent isolates, showing antagonistic activity against the three *Alternaria* spp. prevailing in the region. Among these seven isolates, only those that showed percent inhibition >50% and at the range of 20-50% *in vitro* evaluation were in the effective range. It was observed that RB6 showed maximum percent inhibition >50% against *Alternaria alternata* and isolates RB16, RB24, RB37, and RB42 showed inhibition at the range of 20-50%. The three isolates RB6, RB16, and 42 showed effective antagonistic activity against all the three *Alternaria* spp. However, isolates RB24 and 37 showed weak antagonistic activity against *A. brassicicola* and *A. brassicae* showing inhibition percentage (IP) in the range of 1-20% as compared to the IP recorded against *Alternaria alternata* (20-50%). Considering these findings, three isolates RB6, RB16, and RB 42 were concluded as promising isolates which can be used for managing the *Alternaria* leaf spot in mustard.

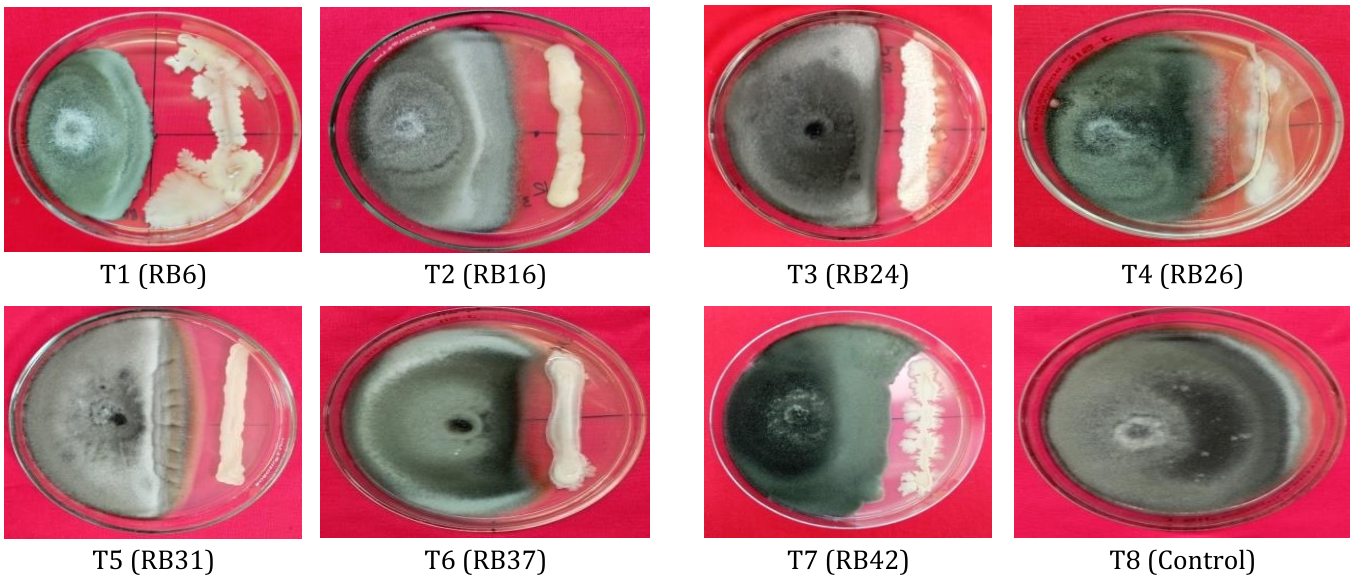

Fig. 1: Dual culture assay of potent bacterial isolates against *Alternaria alternata*.

2. Studies on the growth rate of three *Alternaria* spp. under challenge growth

Growth of *A. brassicae, A. brassicicola,* and *A. alternata,* were recorded for seven days. A post hoc test on the mycelial growth per day and growth rate for 7 days using Duncan pairwise comparisons confirmed that the culture with the treatment RB6 RB16 and RB42 showed the lowest overall colony growth as compared to other treatments. It was also seen that the degree of effectiveness of the three antagonistic bacteria differs according to the species of the pathogen. The growth rate of the fungus in all the treatments per day was less apparent till the 2nd day but on the third day, there was a significant difference among all treatments, and T1 (RB6) showed highly significant as that of control. A clear difference in the growth rate of the fungus for

treatment T1, T2 (RB16), and T7 (RB42) from other treatments on the fourth day was observed, and thereafter, these three treatments showed significantly apparent results from all other treatments.

Considering Fig. 1, on the seventh day, the growth of *A. brassicae* with the treatment T7 (RB42) showed the least growth, followed by T1 (RB6) and T2 (RB16). Post-hoc tests also confirmed the significant difference in T7, followed by T1 and T2, and the remaining treatments T3 (RB24) and T4 (RB26) are at par but significantly different from the control. Based on the growth rate on the seventh day, *A. brassicicola* against the treatment with T1 was the lowest, followed by T2 and T7, and the overall growth rate of the culture under T2 and T7 was also observed to be at par but significantly different from control.\

In the case of *A. alternata,* T1 and T7 showed the lowest mycelial growth radius and are at par but significantly different with other treatments, followed by T2 and others are significantly different. Considering the results, it is seen that T1, T2, and T7 are found to be a potent antagonistic isolate against the pathogen. However, T7 can be considered as the most potent for two *Alternaria* spp. *(A. brassicae* and *A. alternata)* and T1 as the most potent for *A. brassicicola* and also for *A. alternata.* This showed that the effectiveness of the potent isolates may differ according to the differences in the pathogen species, however, it confirms that T1, T2, and T7 were the most effective isolates against the Alternaria leaf spot-causing pathogens.

3. Cultural, morphological, bio-chemical and molecular characterization

3.1. Cultural and morphological characterization: The seven isolates that were found to show antagonistic activity were further taken for morphological and biochemical characterization. The details of the cultural, morphological properties and gram reaction are presented in Table 1. Five of the culture was found to be gram-positive, and the remaining two showed gram-negative, and most were found to be rod-shaped. The three promising isolates T1, T2 and T3 were found to be gram-positive in nature.

Table 1: Cultural, morphological characterization and gram reaction of the potent isolates.

Isolate Name	Colony Shape	Colony Size	Colour	Margin	Gram stain	Cell shape
T1	Irregular	Large	Off white	Serrate	+ve	Rod
T2	Irregular	Medium	Cream	Irregular	+ve	Rod
T3	Irregular	Medium	Off White	Smooth	+ve	Rod

3.2. Characterization of plant growth-promoting attributes based on biochemical properties: A total of 23 biochemical tests were performed (Table 2). All seven isolates showed different bio-chemical test profiles. However, all the isolates showed positive for catalase, citrate utilization, and glucose tests, while for HCN, protease, and cellulase negative. Out of the seven isolates, RB6, which also showed promising results in antagonistic tests, was found to be highly reactive to Voges Proskauer's, citrate utilization, amylase, catalase, and siderophore production tests and positive for the indole test. These may be certain attributes for their antagonistic property under *in vitro* conditions. Similarly, RB42 isolate showed positive reactions for amylase, catalase, and oxidase enzymes, and is highly reactive for siderophore production. Besides, it showed its ability to solubilize different sugar molecules and sugar alcohols (glucose, arabinose, sorbitol, mannitol, sucrose), which indicates its ability to survive under different substrates. However, the antagonistic property of RB 16 is controversial seeing its biochemical profile even though it remains effective in *in vitro* evaluation. Isolates RB24, 26, and 37 showed the same profile in 11 tests. RB6, RB16, and RB42 showed the same reactions in 13 tests. RB 31 showed positive in eight tests and its profile is observed to be different from other isolates. Out of all the isolates, RB 26, 31, and 37 were phosphate solubilizing bacteria, 26 and 37 being highly phosphate solubilizers. Moreover, the two isolates 37 and 31 were efficient siderophore producers. These isolates even though failed to show their antagonistic property in dual culture, they can be explored for these aforesaid beneficial microbial properties. All seven isolates showed positive reactions for catalase activity, glucose, and citrate utilization. Only a single isolate RB 24 showed H2S activity positive.

3.3. Molecular characterization: The BLASTn homology analysis of the amplified 16s rRNA gene sequences of RB6, RB16, and RB42 (Fig. 2) was also carried out. The sequences of these isolate were submitted to NCBI GenBank and obtained the accession no. OR310264, OR304311, and PV550085, listed in Table 3. Comparative 16S rRNA gene sequence analysis has been used for bacterial identification at the genus

Table 2: Response to different biochemical reactions by the potent isolates.

Sl. No.	Tests	RB6	RB16	RB24	RB26	RB31	RB37	Rb42
1.	Indole	+	-	-	-	-	++	-
2.	Methyl Red	+	-	++	-	++	-	-
3.	Voges Proskauer's	++	-	-	+	-	++	-
4.	Citrate utilization	++	+	++	++	++	++	++
5.	Glucose	+	+	++	++	+	++	+
6.	Adonitol	-	-	+	-	-	++	-
7.	Arabinose	-	+	++	++	+	++	+
8.	Lactose	-	-	+	-	-	++	-
9.	Sorbitol	-	-	+	++	-	++	+
10.	Mannitol	-	+	+	++	-	++	+
11.	Rhamnose	-	-	+	++	-	++	-
12.	Sucrose	-	+	++	++	-	++	+
13.	Amylase	++	-	++	-	-	-	+
14.	Phosphate solubilization	-	-	-	++	+	++	-
15.	Catalase	++	+	++	+	++	++	++
16.	Oxidase	+	-	+	-	-	+	+
17.	KOH	-	-	-	++	+	-	-
18.	Motility	-	+	-	+	-	-	-
19.	H2S	-	-	-	+	-	-	-
20.	Siderophore production	++	-	-	-	+	+	++
21.	Cellulase	-	-	-	-	-	-	-
23.	HCN	-	-	-	-	-	-	-
24.	Protease	-	-	-	-	-	-	-

and species levels as well as for inferring phylogenetic relationships among prokaryotic organisms. Comparative sequence analysis of the 16S rRNA gene showed that RB6, RB16, and RB42 showed 96% similarity to the genus *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus safensis*, respectively.

Table 3: NCBI accession number of *Bacillus* spp.

NCBI accession number	*Bacillus* spp.
OR310264	*Bacillus subtilis*
OR304311	*Bacillus pumilus*
Pv550085	*Bacillus safensis*

4. Evaluation of the efficacy of potent isolates against *Alternaria alternata* pathogen

In the detached assay, the three most potent isolates RB6, RB16, and RB42 were used to evaluate their efficacy against *Alternaria alternata*. Damaged diseased area percentage of three different treatments:

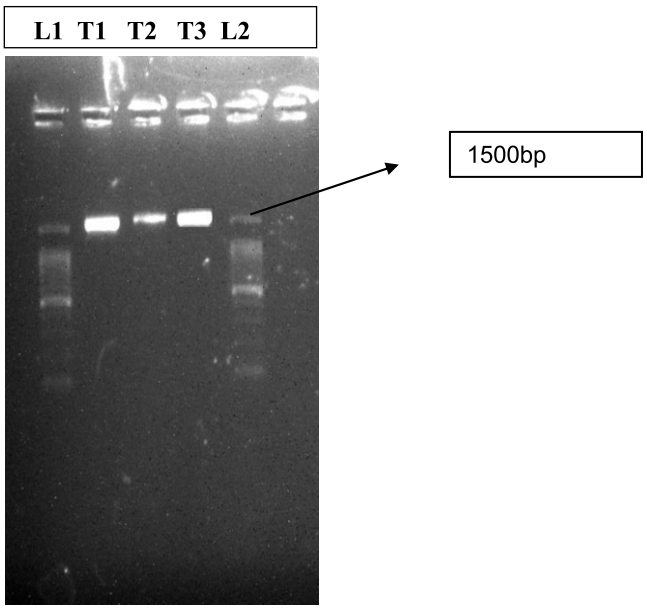

Fig. 2: PCR amplification of DNA of potential bacteria using universal 16S rRNA primers [L1 and L2 are ladder of 100bp, T1 (RB6), T2 (RB16) and T7 (RB42)]

RB6, RB16, and RB42, in the presence of the pathogen inoculum *A. alternata* were recorded against the control. The results showed that the damaged diseased area percent in RB6 treatment was found to be 2.35%, indicating that this particular isolate was able to significantly reduce the spread of the disease compared to the control, in RB16 treatment, it was slightly higher at 2.83% compared to RB6 but still significantly lower than the control. RB42 treatment resulted in a damaged diseased area of 2.46%, which is higher than RB6 and lower than RB16, and remained considerably lower than the disease spread in the control (Table 4).

Table 4: *In vitro* evaluation of potent isolates against *Alternaria alternata* in detached leaf assay.

Treatments	Damaged diseased leaf area (%)	Control efficacy (%)	Mean damaged diseased area (cm)
T1 (RB6)	2.35	96.94	1.71
T2 (RB16)	2.83	96.31	2.11
T7 (RB42)	2.46	96.80	1.78
T8 (Control)	76.80		56.13
CD (5%)			0.17
Sem±			0.07

a) Experimental set up for control plate

b) Experimental set up for treated plate

c) 3 Leaf sample (Pathogen+ T7) seven days after pathogen inoculation

d) 4 Leaf sample (Pathogen+ T1) seven days after pathogen inoculation

e) 5 Leaf sample (Pathogen+ T2) seven days after pathogen inoculation

a) 6 Leaf sample (control) seven days after pathogen inoculation

Fig. 3: Experimental set up for detached leaf assay.

Original leaf sample of control plate

Adjusting color threshold parameters (hue, saturation and brightness) to select the entire leaf (healthy+ disease portion)

Disease or damaged and healthy portion set into Binary form (Black and white image)

Fig. 4: Representation of image (control plate) processing by ImageJ software.

It's important to note that the sample in the control plate displayed a significantly larger damaged diseased area of 76.80%, indicating a severe spread of the disease in the absence of any bacterial antagonist isolate. This highlights the potential of the isolates RB6, RB16, and RB42 to combat the infection and mitigate the disease's impact. Additionally, the control efficacy of each treatment was calculated to be 96.94% for RB6, 96.80% for RB42, and 96.31% for RB16 respectively (Table 4). These high control efficacy percentages demonstrate the effectiveness of these isolates in reducing the disease and protecting the leaves from further development of the pathogen. The control efficacy percentage indicates that the results observed for antagonistic activity in dual culture conjunctions with the results of the screening performed by using the leaf detached assay technique. Conclusively, the three isolates RB6, RB16, and RB42 can be considered to be potent bio-control agents isolated from the Bundelkhand region. Overall, the results indicated that RB6, RB16, and RB42 are promising isolates for disease management and have the potential to be utilized as effective biocontrol agents to combat the spread of the disease. The effects of these same isolates on plant defense have been extensively studied related to mediated induction of histochemical responses, plant defense enzyme in groundnut against early leaf spot (Pride *et al.,* 2020). Further research and experimentation could provide valuable insights into their mode of action and potential applications in plant disease management.

The detached leaf assay (DLA) stands out as a non-destructive technique used to assess the interplay between plants and disease-causing agents, enabling swift evaluation of pathogens' infectivity and plants' resistance. Macan *et al.* (2022) showcased its effectiveness in evaluating potential biological control agents (BCAs), exemplifying its utility in screening BCAs against *Phytophthora cactorum*, the pathogen affecting strawberries (*Fragaria ananassa*) (Liviu *et al.,* 2015). The latest research further underscores its versatility by introducing its application in combating *Alternaria* leaf spot disease in mustard. This study validates the DLA as a reliable and uncomplicated procedure for promptly screening potential bacterial BCAs.

Fousia *et al.* (2016) demonstrated that the application of bacterial antagonists effectively managed disease caused by Pst, reducing the area under disease progress curve (AUDPC) by sevenfold compared to the control treatment and threefold compared to copper hydroxide (Ge *et al.,* 2007). However, they also observed a significant increase in plant height with the application of both bacterial antagonists and copper hydroxide compared to control and mock-inoculated plants. Similarly, the plant growth-promoting traits and antagonistic abilities of phytopathogens and phylogenetic analysis of 16S rRNA sequences of the isolates were identified as new strains of *Pseudomonas stutzeri*, *Bacillus subtilis*, *Stenotrophomonas maltophilia*, and *Bacillus amyloliquefaciens*, which produced high levels of indole-3-acetic acid (Holt *et al.*, 1994).

Plants treated with these strains displayed enhanced germination, seedling vigor, growth, and nitrogen content compared to untreated control plants, while also showing significant suppression of Phytophthora crown rot caused by *Phytophthora capsica* (Holt *et al.,* 1994). Likewise, the plant growth-promoting attributes of eight rhizobacterial isolates obtained from the rhizosphere of various crops (Kandoliya and Vakharia, 2013). These isolates exhibited positive responses for catalase, citrate, oxidase, IAA, siderophore, HCN, and ammonia tests, while displaying negative responses for indole and methyl red, and Voges-Proskauer tests. *In vitro* antagonism tests revealed significant inhibition by *Bacillus subtilis*

and *Pseudomonas fluorescens* against *Fusarium oxysporum* f. sp. *ciceri*, *Rhizoctonia bataticola*, and *Sclerotium rolfsii*.

The study also reaffirmed the antagonistic activity of *Bacillus subtilis* against *Alternaria* leaf spot in mustard, as evidenced by percent inhibition and control efficacy. Additionally, a noticeable reduction in mycelial radial growth of the pathogen was observed when treated with the antagonistic bacterium *Bacillus* spp. Control efficacy (%) of the three isolates, namely T1 (RB6), T2 (RB16), and T7 (RB42), based on leaf detached assay and ImageJ analysis, were reported to be 96.94, 96.31, and 96.80, respectively. These findings underscore the potency of the studied *Bacillus* spp. as potent antagonistic bacteria. Moreover, *in vitro* assays, utilizing dual culture conjuncts, align with the findings of Macan *et al.,* (2022), who developed a screening technique for the biocontrol activity of antagonistic microorganisms based on leaf detached assays and ImageJ analysis (Leong, 1986).

The difference in percent inhibition of mycelial growth indicates the difference in their antagonistic potential for the test pathogen and this may be attributed to diverse antimicrobial secondary metabolites (Siddiqui and Shakeel, 2006; Macan *et al.,* 2022). The antagonistic potential of the potent microbes may positively be correlated with extracellular activities of protease, chitinase, â-1,3-glucanase, and siderophore (Islam *et al.,* 2016), due to the production of siderophores (Leong, 1986; Laha *et al.,* 1992), might be due to volatile antifungal compounds (Abou-Aly *et al.,* 2015), or antibiosis activity (Kandoliya and Vakharia, 2013).

The variance in percent inhibition of mycelial growth signifies discrepancies in the antagonistic capabilities against the test pathogen, potentially attributed to a range of antimicrobial secondary metabolites (Siddiqui and Shakeel, 2006; Macan *et al.,* 2022). The robust antagonistic potential of these microbes may stem from their extracellular activities, including protease, chitinase, β-1,3-glucanase, and siderophore production (Islam *et al.,* 2016). This potency could be linked to siderophore production (Leong, 1986; Laha *et al.,* 1992), the emission of volatile antifungal compounds (Abou-Aly *et al.,* 2015), or antibiosis activity (Kandoliya and Vakharia, 2013).

The present study delves into the utilization of rhizospheric bacteria for the management of the foliar pathogen *Alternaria* leaf spot, which may evoke differing viewpoints. Nonetheless, the concept of priming-mediated induced systemic resistance (ISR) induced by *Pseudomonas azotoformans* GC-B19 and *Paenibacillus elgii* MM-B22 against *Colletotrichum orbiculare* in cucumber has been documented. This approach was observed to inhibit conidial germination and appressorium formation in the fungus, while effectively enhancing defense-related activities such as the rapid elicitation of hypersensitive reaction-like cell death accompanied by hydrogen peroxide generation, and the accumulation of defense-related enzymes including β-1,3-glucanase, chitinase, and peroxidase (Romero *et al.,* 2007).

Similarly, the induction of resistance in groundnut plants against *Botrytis cinerea* infection through the application of the beneficial rhizobacterial strain *Pseudomonas fluorescens* DN16, alongside its association with ThermoSpermine (TSpm) metabolic pathways (Zhu *et al.,* 2021). They emphasized the pivotal role of TSpm catabolism in regulating rhizobacteria-primed defense states by mediating immune responses in groundnut plants following *B. cinerea* infection. Furthermore, a potential increase in ethylene concentration in *P. fluorescens* strains priming plants to produce more ethylene upon pathogen infection thereby enhances their defensive capacity against pathogens (Sah *et al.,* 2021). Given these promising avenues, the future trajectory of the current research aims to assess the impact of potent isolates identified as effective sources of defence-priming microbes against plant pathogens, alongside evaluating the immune response of the host. Bio-agents with no environmental perils are turning out to be the most preferable option for disease suppression in plants (Emmert and Handelsman, 1999).

CONCLUSIONS

Strengthening bio-control production units and promotion of farmer-managed seed hubs need to be stressed on seeing the rising concern about the intensified incidences of diseases in the Bundelkhand region. The identified and characterized novel bacterial strain of the region can be a boon for a region like Bundelkhand, where there is no recommended bacterial biocontrol agent for managing the disease. Furthermore, the ability of these isolates to induce resistance in mustard, against one of the most devastating diseases, *Alternaria* leaf spot can be explored. Using indigenous potent isolate in plant disease management is a crucial aspect of sustainable agriculture with numerous benefits. Their utilization is of paramount importance. Unlike chemical pesticides, which can have detrimental effects on non-target organisms and the environment, indigenous bio-control agents offer a sustainable and eco-friendly alternative. The use of potent bio-control agents

represents a forward-thinking approach to plant disease management that promotes environmental sustainability, biodiversity conservation, and community empowerment. By harnessing the natural mechanisms already present in ecosystems, we can create resilient agricultural systems that benefit both farmers and the environment for generations to come. Besides, the present research promulgated a method for the evaluation of antagonistic microbes using the detached leaf assay technique along with imageJ software for laboratory assessment, the first of its kind, against *Alternaria* leaf spot disease in mustard.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to the content of this article.

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