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INVESTIGATING THE FUNGICIDAL POTENTIAL OF *ANEURINIBACILLUS MIGULANUS* NR_112214.1 IN THE MANAGEMENT OF PHYTOPATHOGENS INFECTINGCHILLI

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ABSTRACT

Biological control of harmful fungal pathogens in chilli is crucial for ensuring food and environmental safety. Therefore, this study explored the fungicidal potential of various *Bacillus* isolates against *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium oxysporum*, and *Sclerotium rolfsii*, which are known to cause several diseases in chilli plants. A total of 167 bacterial treatments were screened for their antagonistic potential, comprising 161 *Bacillus* isolates (coded B1-B161), and 6 proven bio-control agents. From this initial screening, 25 treatments were chosen for further evaluation using dual plate assays. Among the selected isolates, *Bacillus* isolate B7 demonstrated significant inhibitory effects on the growth of the aforementioned phytopathogens, achieving a reduction of 60-72% in confrontation assay. Pathogenicity tests showed that the soil containing B7 had a notably lower disease incidence (18.7-27.7%) compared to untreated control (60-70%), resulting in an ~ 70% disease reduction over the control. Furthermore, B7 exhibited superior plant growth parameters, including the highest shoot length (8.2-8.8 cm), root length (7.8-9.5 cm), and dry biomass (0.85 g) when challenged with the selected phytopathogens. Additionally, it produced the highest levels of lipase (17 mm), pectinase (25 mm), alongside strong biofilm formation (++++), and hydrogen cyanide activities. B7 has been identified as *Aneurinibacillus migulanus* NR_112214.1. The highest antifungal activity was recorded in the intracellular

crude extract (11.3-14.6 mm), and extracellular crude extract (15-20.6 mm) containing secondary metabolites of *A. migulanus*, both of which surpassed the activity of carbendazim (10-17mm). Furthermore, GC-MSD analysis identified 31 intracellular, and 31 extracellular secondary metabolites. Our research confirms the efficacy of *A. migulanus* in managing diseases in chillies caused by *M. phaseolina*, *R. solani*, *F. oxysporum*, and *S. rolfsii.*

KEYWORDS

Chilli. Phytopathogens. *Aneurinibacillus migulanus*. Antifungal activity. GC-MS

INTRODUCTION

Chili pepper (*Capsicum* spp.) are native to Central and South America, and are now cultivated globally. The two most economically significant species are the bell pepper (*Capsicum annuum*) and the hot pepper (*Capsicum frutescens*) (Hasan et al. 2014; Gowtham et al. 2018). *C. annuum* is known for its mild flavor and large fruit, often consumed either raw or cooked. In contrast, *C. frutescens* produces smaller fruits that are commonly used to add flavor and spice to various cooked dishes. Chilli peppers are rich in nutrients, offering numerous health benefits, including lowering cholesterol levels and strengthening the immune system. These benefits are largely due to their high content of carotene, thiamine, niacin, vitamins A, B, C, E, and K, as well as proteins and fiber (Bashir et al. 2018). Given these health advantages, it is essential to protect chilli peppers from insect pests and diseases while ensuring that harvested produce remain free of pesticide residue.

Bacteria, fungi, viruses, and root-knot nematodes are the primary pathogens in chilli farming, often resulting in significant production losses. Dean et al. (2012) identified *F. oxysporum*, *F. grainearum*, and *Colletotrichum* spp., and *Rhizoctonia solani* as among the top ten important plant-damaging pathogens, with a notable impact on crops like chilli. One of the most significant plant infections contributing to the wilting and reduced production of the chilli is caused by the genus *Fusarium*. *Fusarium* spp. and *F. oxysporum* are the most common fungi associated with wilt in *C. annuum*, including varieties like serrano and jalapeno (Mejia-Bautista et al. 2016; Akash et al. 2022). Martinez et al. (2011) reported that *F. oxysporum*, *F.*

solani, and *F. equiseti* can considerably impact crops, reducing yields in *C. annuum* by up to 20 - 30%.

The Botryosphaeriaceae, *M. phaseolina* causes damping off and charcoal rot (Alizadeh et al. 2025) affecting roots and stems, leading to wilting, stunting, and premature death of chilli plants, resulting yield loss up to 70% when it infests chilli along with *R. solani, Phytophthora capsici* (Anila Younas et al. 2016) *S. rolfsii* is another extremely dangerous fungus that causes southern blight disease in chillies, resulting 16-80% yield loss (Daunde et al. 2018). According to Mahadevara kumar et al. (2018), the disease manifested by yellowing of the leaves and appearance of dark brown lesions in the collar region near the soil line, which eventually leads to wilting of the plant. It produces sclerotia that persist in the soil for many years, complicating management efforts due to its broad geographic host range (Murthy et al. 2018). Thus, *F. oxysporum, R. solani, M. phaseolina,* and *S. rolfsii,* infect chilli crops, leading to significant economic losses in yield. Conventional fungicides are generally advocated for the management of these pathogens.

The excessive use of pesticides to combat phytopathogens affecting chilli plants has negatively impacted soil health, crop quality, and consumer well-being (Shafi et al. 2017; Paddock et al. 2024). Chilli farmers are increasingly turning to biological pest control methods in response to stringent global standards on pesticide residues and a growing awareness of clean food practices (Stenberg et al. 2021). *Bacillus* species have become effective biocontrol agents, offering a sustainable alternative to chemical fungicides. These agents can hamper the growth of the phytopathogens by producing biofilm, hydrogen cyanide, chitin, pectin, siderophore, and synthesis many organic acids and antimicrobial compounds such as lipopeptides, enzymes, and volatile organic compounds (Radhakrishnan et al. 2017; Fira et al. 2018; Miljakovic et al. 2020; Garrido et al. 2020; Poulaki and Tjamos 2023).

Aside from *Trichoderma* and *Pseudomonas*, the majority of bio-fungicides on the market today are derived from *Bacillus* spp. isolates, which are known to produce over 20 structurally diverse antibacterial compounds (Miljakovic et al. 2020). Notable species include *B. pumilus* (Gowtham et al. 2018), *B. subtilis* (Prihatiningsih et al. 2019), *B. amyloliquefaciens* (Abd et al. 2021), and *B. megaterium* (Sharf et al. 2021), all of which have gained recognition as promising bio-fungicides for managing phytopathogenic diseases globally. While commercial formulations of *Bacillus* spp. are available, their effectiveness are often limited to specific fungal phytopathogen(s) (Peter et al. 2021).

We propose that certain *Bacillus* species hold significant potential for effectively managing four key fungal phytopathogens affecting chillies: *M. phaseolina, R. solani, F. oxysporum,* and *S. rolfsii.* This study aims to introduce unique bio-control agent(s) capable of targeting all four phytopathogens. Previously, we successfully evaluated the plant-growth promoting activities of 161 *Bacillus* isolates (Jyothi et al. 2023). In this study, we assessed their bio-control potency against the above-mentioned phytopathogens, comparing them with *B. paramycoides, B. subtilis, B. amyloliquefaciens, P. fluorescens, B. clausii,* and *B. polymyxa*. Our findings demonstrated the antagonistic effects of *Bacillus* species in relation to their pathogenicity and identified *Aneurinibacillus migulanus* (B7) as the most promising biocontrol agent. The objective of the current study is to investigate the bio-efficacy of *A. migulanus* and its mode of action against the targeted fungal pathogens in chilli crop.

MATERIALS AND METHODS

Sampling collection, isolation, and characterization

In India, soil samples were gathered from several agro-climatic zones and isolated in a laboratory (Jyothi et al. 2023). The isolated colonies were streaked individually onto Nutrient Agar (NA) plates and incubated for 48 h at 28±2 °C. A ocular inspection was used to observe the colony's morphological features following incubation (Breakwell et al. 2007).

Culture source

Bacillus paramycoides PP757924, *B. amyloliquefaciens* MTCC610, *B. polymyxa* MTCC9129, *B. subtilis* MTCC1427, *B. clausii* MTCC8326, *P. fluorescens* PE0005, ATCC52761, *R. solani* MTCC4633, *F. oxysporum* MTCC1755, *M. phaseolina* ATCC52761, and *S. rolfsii* ATCC201126 were obtained from M/s Professor TNA Innovation Centre, Varsha Bioscience and Technology India Pvt. Ltd., Greater Hyderabad, Telangana, 508284, India. The bacterial cultures were prepared using nutrient broth (NB). To grow the fungal cultures, potato dextrose broth (PDB) was used.

Screening for antagonistic activity of bacterial treatments

The dual culture method, as outlined by Kim et al. 2008, was employed to screen all the 167 treatments for antagonistic activity and identify possible isolates with antagonistic activity against test pathogens. A 5 mm pathogen disc was placed on one side of the PDA plate (90 × 15 mm). All bacterial cultures were streaked on the opposite side of the medium. Petri plates without bacteria inoculation were served as controls. The dual-inoculated plates were incubated at 28 °C for four days. The level of pathogen growth inhibition in the presence of bacteria, in a dual culture, compared to the control plates, indicated the antagonistic activity of the bacterial isolates. Twenty-five *Bacillus* isolates that exhibited significant antagonism in dual culture assay were further evaluated using the bangle method.

Twenty-five of the most effective treatments, demonstrating significant antagonistic activities against multiple plant pathogens, were cultured in nutrient broth for 24 h and subsequently used in the bangle method assay. A sterile bangle (70 mm in diameter) was immersed in a uniform suspension of the selected isolates/ species (10^8 cfu/ml) for 2 min and then placed on a PDA Petri dish. A 5 mm diameter disc was excised from the periphery of a seven-day old culture of the phytopathogen, and positioned in the centre of the circle of the plate inoculated with bacteria. All treatments were prepared in triplicate and test plates lacking bacterial cultures served as controls. The inoculated plates were sealed with Parafilm and incubated

in an incubator at 28±2 °C for 6 days. After incubation, the radial growth diameter of the fungal pathogen in dual culture was measured. The results were expressed as percentage inhibition of the mycelial growth of the pathogen relative to the control, and calculated using the formula provided by Ramakrishna et al. (2018):

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

where, I = Inhibition of pathogen growth, C= Pathogen growth in control, T= Pathogen growth in treatment.

***In vivo* Bio-efficacy ability of bacterial treatments in sick pot**

To identify the best bio-control agent(s) against the evaluated phytopathogens in chilli, a screen house study was carried out using the sick pot technique. Pathogens were multiplied on a sand-maize medium, which was then mixed with an autoclaved potting mixture comprising soil, sand, and FYM in a ratio of 2: 1: 1 (25g/ kg of potting mixture). The pots, previously disinfected with a 5% copper sulfate solution, were lightly watered and incubated for two weeks within the screen house. Following the incubation, the disinfected pots were filled with sterilized soil and inoculated with pathogen culture grown on the sand-maize medium at a concentration 10^6 cfu/ g. The pots were regularly watered to maintain the sick soil conditions. The efficacy of test bacterial treatments at a concentration of 10^7 cfu/ml, was assessed through soil application to chilli seedlings. Three replications were maintained for each treatment. A water control group was included as a negative control.

A total of three chilli seedlings were planted in each pot containing sick soil and incubated in screen house for 45 days. Observations were made on the number of diseased plants at various intervals and averages from the 3 replicates were calculated. The percentage of disease incidence Gholve et al. (2016), and the percentage disease reduction over control Zeeshan and Kudada (2019), were calculated using the formulas. Additionally, observations on shoot length, root

length, and dry biomass were recorded to assess the growth-promoting ability of the evaluated bacterial treatments in chilli.

$$\% \text{ disease incidence} = \frac{\text{Number of infected plants per units}}{\text{Total no. of plants observed (Healthy and infected)}} \times 100$$

$$\text{Percent disease reduction over control} = \frac{C-T}{C} \times 100$$

Where, C is Percent disease incidence in untreated plants, T is Percent disease incidence in treated plants.

Estimation of Lipase and Pectinase Activities

Hydrolytic activities of lipase and pectinase were estimated following the methods described by Lee et al. (2015) and Mohandas et al. (2018), respectively. To assess lipase activity, test bacterial isolates were spotted on phenol red agar media prepared with 0.1% w/v phenol red, 0.1% v/v olive oil, 0.1% w/v $CaCl_2$, 2% w/v agar, with the pH adjusted to 7.3. The color change from red to yellow indicates lipase production. The diameter of the yellow zone surrounding each spot was measured in mm to quantify lipolytic activity.

For the estimation of pectinolytic activities, all isolates were inoculated as spots on pectin agar media containing 1.0 g $NaNO_3$, 1.0 g KCl, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g yeast extract, 10 g pectin, and 20 g agar, with the pH adjusted to 7.0. Primary screening for pectinase-producing bacteria involved spotting the colonies on pectin agar plates and incubating them at 37 °C for 48 h. Following incubation, the plates were flooded with Gram's iodine solution for 5 min with intermittent gentle shaking. The plates were washed with distilled water and a clear zone around the isolated colony indicated pectinolytic activity

Qualitative Assessment for Biofilm production

The isolates were grown in Trypticase Soy Broth (TSB) with 1% glucose at 28 °C on a shaker at 180 rpm for 24 h. After incubation, the tubes were decanted, washed with phosphate-buffer saline (pH 7.3), dried, and stained with 0.1% crystal violet.

The excess stain was removed with deionized water, and the tubes were held in an inverted position. Biofilm production was confirmed when a visible ring formed on the wall of the test tube, which was weak (+); moderate (++); strong (+++), and highly active (++++) (Hassan et al. 2011).

Estimation of Hydrogen Cyanide (HCN) production

The production of hydrogen cyanide (HCN) was evaluated using both qualitative and quantitative bioassay methods as outlined by El-Rahman et al. (2019).

Quantitative bioassay: To assess the quality of HCN production, selected bacterial isolates were streaked on King's B agar media supplemented with 4.4 g/L glycine. Pre-sterilized Whatman No. 1 filter paper was soaked in a filter sterilized solution of 2% (w/v) sodium carbonate in 0.5% (v/v) picric acid and placed atop the culture plates. The plates were sealed with Parafilm and incubated at 28 °C for 72 h. During incubation period, any color changes of the overlying filter paper from yellow to orange, red, or brown indicated HCN production. The intensity of the color change was noted as low (+), moderate (++), strong (+++), and highly active (++++). No color change was recorded as a negative (−) reaction.

Quantitative bioassay: The quantitative HCN production, 1 ml of bacterial isolate at 10^6cfu/ml was inoculated into 10 ml King's B broth medium supplemented with 4.4 g/l glycine. Each experiment was conducted in triplicate, with non-inoculated tubes serving as controls. Sterile filter paper strips (10×0.5 cm) were soaked in alkaline picrate solution and suspended from the neck of each test tube (one strip per tube). The test tubes were then plugged and sealed with Parafilm. After 4 days of incubation at 28 ± 2 °C with shaking at 140 rpm, the sodium picrate strips color changed to reddish in proportion to the amount of HCN produced. The filter paper strips were then immersed in 10 ml of distilled water, and the absorbance was measured at 625 nm using a UV/Visible Spectrophotometer (Elico make). Prior to measurement, distilled water was used to calibrate the absorbance to zero at 625 nm as described by Reetha et al. (2014).

DNA Extraction and 16r RNA Gene Amplification

DNA was extracted from *Bacillus* isolate B7 culture in the exponential phase using the Qiagen DNA extraction kit (Italy). The quality of the extracted DNA was assessed through 1% agarose gel electrophoresis, revealing a single band of high-molecular weight DNA. The 16S rRNA gene fragment was amplified using 16SrRNA-Forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16SrRNA-Reverse primer 1492R (5′AAGGAGGTGWTCCARCC-3′). The PCR amplification conditions for 16S rRNA were as follows: 30-60 s at 94 °C (denaturation), 30 s at 62 °C (primer annealing), 60 s at 72 °C (primer extension) for 28 cycles, and 240 s at 72 °C (final extension). The resulting discrete PCR amplicon band was purified to remove contaminants. Sequencing of the 16S rRNA was performed using the BDT v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI 3500xl Genetic Analyzer. A consensus sequence of the 16S rRNA gene was generated from forward and reverse sequences using aligner software. The 16S rRNA gene sequence was then subjected to a BLAST search against the 'nr' database of NCBI GenBank. Based on the maximum identity score, the top ten sequences were selected and aligned using the multiple alignment software program, Clustal W. A distance matrix and phylogenetic tree were constructed was constructed using MEGA 10.

Evolutionary Analysis Using the Maximum Likelihood Method

The evolutionary history was inferred using the Maximum Likelihood method, employing the Kimura 2-parameter model (Kimura et al. 1980). The resulting tree with the highest log likelihood (-2110.79) is presented. Initial tree(s) for the heuristic search were generated automatically by applying Neighbor-Join and BioNJ algorithms on a matrix of pairwise distances, which were estimated using the Maximum Composite Likelihood (MCL) approach. The topology with the highest log likelihood value was then selected. The tree is drawn to scale, with branch lengths representing the number of substitutions per site. This analysis involved 11 nucleotide sequences. The codon positions analyzed were the

1st+2nd+3rd+Noncoding regions. There were a total of 1449 positions in the final dataset. All evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Extraction of secondary metabolites

The intra-cellular and extra-cellular secondary metabolites of *A. migulanus* were extracted following the protocol established by Abdel-Nasser et al. (2023). For the intra-cellular metabolites, the centrifuged cell pellet was mixed with an equal volume of ethyl acetate. This mixture was subjected to ultra-sonication and vortexed for 1 h. After phase separation, the organic phase was evaporated using a rotary evaporator at 40 °C. The residual extract was subsequently weighed and re-dissolved in phosphate buffer (pH 7.0). For the extracellular metabolite extraction, 100 mL of bacterial broth supernatant was combined with an equal volume of ethyl acetate in a separatory funnel. The mixture was left undisturbed for 5 min, after which the organic phase was carefully collected.

Evaluation of *in vitro* anti-fungal activity assay

Antifungal activity was assessed using the agar well diffusion method as described by Omar et al. (2006) against four phytopathogens. A PDA plate was inoculated with a 50 µL suspension of fungal pathogen spores at a concentration of 10^6 spores/ mL. In separate wells, 50 µL of intra- and extracellular extracts (as treatment controls), dimethyl sulfoxide (DMSO as a negative control), and 100ppm carbendazim (as a positive control) were added. Plates were sealed and incubated at 28±1 °C for 4 days. After the incubation, the inhibition zones were measured to evaluate antifungal activity using the formula:

$$FI = D - d$$

Where, FI = Fungal inhibition (mm), D= diameter of the inhibition zone appearing on the agar plate (mm), and d= diameter of the agar well (mm).

Identification of Secondary metabolites

The organic phase derived from the intra- and extracellular extracts of *A. migulanus* was analyzed using Gas Chromatography – Mass Spectrometry (GC-MS: Agilent 7890A). A 2µL sample was injected onto a DB-5 MS column (30 cm x 0.25 mm; ID x 0.25 µm thickness). The oven temperature was initially set at 50 °C for 1 min, then increased to 210 °C at 30 °C per min, followed by a further increase to 310 °C at 5 °C/min, where it was held at 310 °C for an additional 3 min. Helium served as the carrier gas a flow rate of 1 mL/min, with the injector and auxiliary heaters maintained at 280 °C. Mass spectrometry (MS) was conducted over a range of 50-550 m/z for compound identification, using the National Institute of Standards and Technology (NIST) -2017 Mass Spectral Library (Rakhmawatie et al. 2023).

Statistical Analysis

A completely randomized design (CRD) was employed for all experimental setups. The significance threshold was set at $p < 0.05$, thus ensuring statistical significance by chance less than 5% of the time. The statistical analysis of the experimental data was performed using Wasp 2.0., a software package designed for advanced statistical computations in research. For the pathogenicity assay, data on multiple measured parameters, including dry biomass, root length and shoot length, which were collected from the sick pot across three replicates were subjected to two-way ANOVA. Following the ANOVA, the means of various treatment groups were compared against one another using Fisher's Least Significance Difference (LSD) test. In this analysis, a more stringent significance level of $P< 0.005$ was adopted to minimize Type 1 errors.

RESULTS AND DISCUSSION

Plant pathogens, particularly pathogenic fungi, pose a significant challenge to successful agriculture production. While chemical pesticides effectively control these pathogens, they are often considered unsafe and harmful to plants, soil, and consumers (Miljakovic et al. 2020). In contrast, biological pesticides and appropriate microbial biocontrol agents offer a more sustainable alternative to

conventional fungicide, promoting overall plant health while effectively managing plant pathogens.

Recent studies have demonstrated the potential of *Bacillus* spp. in mitigating various plant diseases. These beneficial bacteria can effectively reduce instances of wilt disease caused by *R. solani* (Wu et al. 2019), southern blight disease and collar rot by *S. rolfsii* (Sharf et al. 2021), damping off by *F. oxysporum* (Charei et al. 2017), and charcoal rot by *M. phaseolina* (Shahid et al. 2016) in chilli plants. However, it is important to note that none of the *Bacillus* species currently studied exhibit broad-spectrum antagonistic activity against *F. oxysporum, M. phaseolina, R. solani,* and *S. rolfsii.* This limitation highlights the need for the identification of novel *Bacillus* species capable of combating multiple fungal phytopathogens, which could provide more comprehensive protection against various fungal diseases in chilli. Therefore, from 2020-24, a comprehensive investigation was conducted to explore the potential of 161 native *Bacillus* isolates as multifunctional biocontrol against four significant phytopathogens affecting chilli plants: *M. phaseolina, F. oxysporum, R. solani*, and *S. rolfsii*. The efficacy of the isolates was compared to that of six established bio-control agents: *P. fluorescens, B. paramycoides, B. amyloliquefaciens, B. polymyxa, B. clausii*, and *B. subtilis.*

Colony morphology characteristics

The isolated colonies were examined for their morphological characteristics, including size, shape, color, optical properties, and texture. These features offer initial insights into the identity and purity of the isolates. The morphological features of 161 *Bacillus* isolates, as detailed in Fig. 1 include: **Shape**: circle (68), rhizoid (19), spindle (4), irregular (11), and punctiform (59); **Margin**: undulate (75), entire (24), lobate (29), even (6), erose (26), and filamentous (2); **Elevation**: Raised (3), flat (126), umbonate (29), and convex (3); **Size**: Large (37), medium (56), and punctiform(44), small (24); **Texture**: Smooth (67), mucoid (73), and viscid (21); **Appearance**: Creamy-white (2), brown–white (15), yellow (15), white-yellow (3), white (31), yellow-white (77), and pink–white (18); **Optical property**: Opaque (94), translucent (60), and transparent (7).

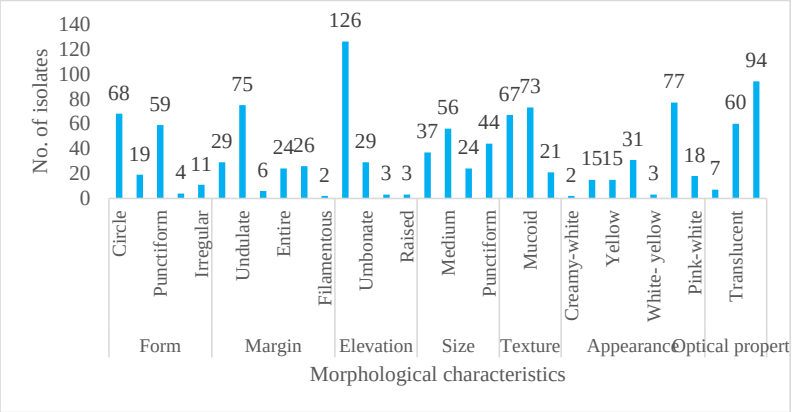

Fig. 1 Morphological characteristics of the 161 bacteial isolates

Screening for *in vitro* antagonistic activity of bacterial treatments

Using the dual culture plate method, we examined the antagonistic effects of 161 bacterial isolates alongside six established biocontrol agents against four fungal phytopathogens. The results revealed that 17, 28, 26, and 15 bacterial isolates exhibited antagonism against *M. phaseolina*, *F. oxysporum*, *S. rolfsii*, and *R. solani* respectively (Table 1; Fig. 2). These findings align with those of Kumar et al. 2014, who conducted a dual plate assay with 120 *Bacillus* isolates reporting antagonistic activity against *Botrytis ricini* (30 isolates), *F. oxysporum* f. sp. *Ricini* (51 isolates), *R. solani* (32 isolates), and *S. rolfsii* (49 isolates), and *M. phaseolina* (49 isolates).

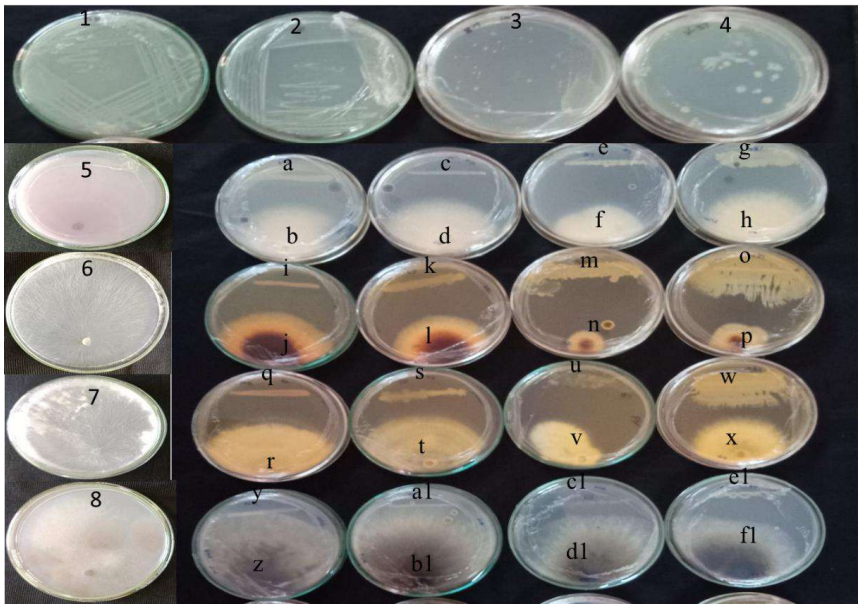

Fig. 2 Primary antagonistic study against fungal phytopathogens. a - *P. fluorescens*, b - *F. oxysporum*, c - *B. subtilis*, d - *F. oxysporum*, e – (B7) *A. migulanus*, f - *F. oxysporum*, g - *B. paramycoides*, h - *F. oxysporum*. i- *P. fluorescens*, j – *R. solani*, k- *B. subtilis*, l - *R. solani*, m - (B7) *A. migulanus*, n - *R. solani*, o - *B. paramycoides*, p - *R. solani*, q - *P. fluorescens*, r- *S. rolfsii*, s - *B. subtilis*, t - *S. rolfsii*, u - (B7) *A. migulanus*, v - *S. rolfsii*, w - *B. paramycoides*, x - *S. rolfsii*, y - *P. fluorescens*, z - *M. phaseolina*, a1 - *B. subtilis*, b1 -*M. phaseolina*, c1 - (B7) *A. migulanus*, d1 - *M. phaseolina*, e1 - *B. paramycoides*, f1- *M. phaseolina*. 1- *P. fluorescens*; 2- *B. subtilis*; 3- (B7) *A. migulanus*; 4- *B. paramycoides*; 5- *F. oxysporum* control; 6- *R. solani* control; 7- *S. rolfsii* control; 8-*M. phaseolina* control.

Table 1 *In vitro* preliminary screening of bacteria against fungal phytopathogens using dual plate assay

Phytopathogens	Name of the bacterial treatments showing positive antagonism
M. phaseolina	B1, B5, B24, B30, B35, B48, B59, B66, B76, B98, B129, B134, B158, *A. migulanus* (B7), *B. paramycoides*, *P. fluorescens*, and *B. subtilis*.
F. oxysporum	B2, B4, B21, B28, B29, B32, B33, B36, B37, B43, B53, B62, B65, B73, B79, B81, B85, B92, B98, B103, B106, B128, B160, *A. migulanus* (B7), *B. paramycoides*, *B. amyloliquefaciens*, *P. fluorescens*, and *B. subtilis*.
S. rolfsii	B4, B21, B22, B28, B32, B35, B36, B37, B43, B73, B81, B85, B88, B91, B98, B101, B119, B130, B154, B161, *A. migulanus* (B7), *B. paramycoides*, *B. amyloliquefaciens*, *P. fluorescens*, *B. subtilis*, and *B. polymyxa*.
R. solani	B21, B22, B53, B62, B65, B79, B88, B111, B125, B142, *A. migulanus* (B7), *B. paramycoides*, *B. amyloliquefaciens*, *P. fluorescens*, and *B. subtilis*.

Of the 167 isolates/ species screened, 25 showed antagonistic effect against minimum two pathogens in a dual culture assay. Subsequently, a confirmatory assay was carried out using the bangle assay method to evaluate the individual antagonistic capabilities of each isolate against the phytopathogen. Among 25 isolates, isolate B28 exhibited the highest antagonism, with a 72.7% inhibition rate against *S. rolfsii*, closely followed by B88 at 72.2%. However, B88 was excluded from further experimentation due to its lack of antagonism against the other three selected pathogens. Isolate B7 demonstrated a 72.0% antagonism against *S. rolfsii* and also showed significant inhibition against *M. phaseolina*, *R. solani*, and *F. oxysporum*, with inhibition rates ranging from 60.7 - 72% compared to the control (Table 2; Fig. 3). Additionally, isolates B21, B98, and *B. amyloliquefaciens* showed antagonism against three fungal phytopathogens. Among the isolates, B4, B21, B22, B28, B32, B35, 36, B37, B73, B85, B88, B7, *B. paramycoides*, *B. subtilis*, and *P. fluorescens* exhibited over 50% inhibition against *S. rolfsii*. For *F. oxysporum*, B7, B28, B65, and B73 demonstrated more than 50% inhibition. Similarly, B7, B21, B65, B98, *B. paramycoides* exceeded 50% inhibition against *R. solani*, while B7, B98, and *B. paramycoides* showed over 50% of inhibition against *M. phaseolina*. Notably, *B. paramycoides* exhibited inhibition rates of 39 - 56.2%, followed by *B. subtilis* with inhibition rates ranging from 25.3 - 50%. *B. clausii* exhibited no antagonism against any of the pathogens (Table 2; Fig. 3).

Kumar et al. 2014 reported that among their 120 *Bacillus* isolates, B77 exhibited a 77.8% inhibition of *S. rolfsii*, while B81 inhibited *M. phaseolina* growth by 58.9%. Isolate B120 demonstrated an impressive 80% inhibition against *R. solani*. Additionally, both B18 and B77 inhibited 66.7% growth of *F. oxysporum* f. sp. *ricini*, with 61.1% inhibition against *B. ricini*. In comparison, the current study found that our isolate B28 achieved a 72.7% inhibition rate against *S. rolfsii*, followed by B88 at 72.2% and B7 at 72.0%. For *M. phaseolina*, B98 demonstrated better inhibition at 64.8%, with B7 following at 63.3%. Additionally, B7 showed over 50% antagonism against both *R. solani*, and *F. oxysporum*. Therefore, B7 was identified as the most effective antagonistic isolate (Table 2; Fig. 3).

Table 2 Growth inhibition of pathogens by antagonistic bacteria recorded in bangle method assay

Strains	% inhibition			
	F. oxysporum	*M. phaseolina*	*R. solani*	*S. rolfsii*
B4	0.0^{m}	0.0^{f}	0.0^{h}	53.6^{efg}
	(0.2)	(0.2)	(0.2)	(47.0)
A. migulanus (B7)	60.7^{a}	63.3^{ab}	68.6^{a}	72.0^{a}
	(51.1)	(52.7)	(55.9)	(58.0)
B21	46.2^{defg}	0.0^{f}	52.6^{de}	63.8^{cd}
	(42.8)	(0.2)	(46.5)	(53.0)
B22	0.0^{m}	0.0^{f}	0.0^{h}	63.8^{cd}
	(0.2)	(0.2)	(0.2)	(53.0)
B28	51.8^{cd}	0.0^{f}	0.0^{h}	72.7^{a}
	(46.0)	(0.2)	(0.2)	(58.5)
B32	36.9^{jk}	0.0^{f}	0.0^{h}	66.2^{bc}
	(37.3)	(0.2)	(0.2)	(54.4)
B35	0.0^{m}	0.0^{f}	0.0^{h}	59.1^{de}
	(0.2)	(0.2)	(0.2)	(50.2)
B36	48.2^{cdef}	0.0^{f}	0.0^{h}	53.6^{efg}
	(43.9)	(0.2)	(0.2)	(47.0)
B37	48.4^{cdef}	0.0^{f}	0.0^{h}	52.2^{fg}
	(44.0)	(0.2)	(0.2)	(46.2)
B43	45.8^{efgh}	0.0^{f}	0.0^{h}	0.0^{k}
	(42.5)	(0.2)	(0.2)	(0.2)
B53	49.8^{cdef}	0.0^{f}	0.0^{h}	0.0^{k}
	(44.8)	(0.2)	(0.2)	(0.2)
B62	32.4^{k}	0.0^{f}	0.0^{h}	0.0^{k}
	(34.6)	(0.2)	(0.2)	(0.2)
B65	51.1^{cde}	0.0^{f}	56.2^{cd}	0.0^{k}
	(45.6)	(0.2)	(48.5)	(0.2)
B73	53.6^{bc}	0.0^{f}	0.0^{h}	54.4^{efg}
	(47.0)	(0.2)	(0.2)	(47.5)

B79	41.1^{ghij}	0.0^{f}	0.0^{h}	0.0^{k}
	(39.8)	(0.2)	(0.2)	(0.2)
B81	38.9^{j}	0.0^{f}	0.0^{h}	40.4^{j}
	(38.5)	(0.2)	(0.2)	(39.4)
B85	39.3^{ij}	0.0^{f}	0.0^{f}	57.8^{ef}
	(38.7)	(0.2)	(0.2)	(49.4)
B88	0.0^{m}	0.0^{f}	0.0^{f}	72.2^{a}
	(0.2)	(0.2)	(0.2)	(58.2)
B98	44.4^{fghi}	64.8a	63.7^{b}	0.0^{k}
	(41.7)	(56.7)	(52.9)	(0.2)
B. paramycoides	39.1^{j}	56.2^{bc}	56.2^{cd}	55.6^{efg}
	(38.6)	(48.5)	(48.5)	(48.1)
B. subtilis	32.0^{k}	25.3^{de}	25.3^{f}	50.0^{g}
	(34.3)	(30.0)	(30.0)	(44.9)
P. fluorescens	38.7^{j}	18.2^{e}	18.2^{g}	64.4^{cd}
	(38.3)	(25.0)	(25.0)	(53.4)
B. amyloliquefaciens	0.0^{m}	49.3^{c}	49.3^{e}	39.3^{j}
	(0.2)	(44.5)	(44.5)	(38.7)
B. clausii	0.0^{m}	0.0^{f}	0.0^{h}	0.0^{k}
	(0.2)	(0.2)	(0.2)	(0.2)
B. polymyxa	20.4^{l}	0.0^{f}	18.4^{g}	0.0^{k}
	(26.6)	(0.2)	(25.1)	(0.2)
CV%	8.415	38.378	13.110	7.873
CD (0.05)	3.426	5.278	2.617	3.499

Average of five replications; figures in the parenthesis indicate angular transformed values; Superscripts a-m – different alphabets indicate significant differences at a 5% ($p < 0.05$) level of significance; CV- Coefficient of Variation; CD- Critical Difference.

Fig. 3 Confrontation study against fungal phytopathogens; **1.** (a – B7, b - *M. phaseolina*, c - B98, d - *M. phaseolina*, e - *M. phaseolina*, f - *B. subtilis*, g - *M. phaseolina)*, **2.** (a-B7, b - *F. oxysporum*, c- B28, d - *F. oxysporum*, e - B37, f - *F. oxysporum*, g - *F. oxysporum*, h - B73, i - *F. oxysporum*, j - B98, k - *F. oxysporum)*, **3.** (a - B98, b - *R. solani*, c –B7, d - *R. solani*, e - *R. solani*, f - B21, g - *R. solani*), and **4.** (a –B7, b - *S. rolfsii*, c - B88, d - *S. rolfsii*, e - B28, f - *S. rolfsii*, g - *S. rolfsii*, h - *P. fluorescens*, i - *S. rolfsii*, j - *B. subtilis*, k - *S. rolfsii*).

***In vivo* Bio-efficacy ability of bacterial treatments in sick pot**

Eleven bacterial isolates exhibiting over 50% antagonism against at least two pathogens were selected for the sick pot assay. The bio-efficacy of these isolates was tested in plastic pots filled with soil infected by phytopathogens+ sand maize media. Seedlings of chilli, treated with the selected bacterial cultures (treatment) and water (control) were planted in the infected soil, and their percentage disease incidence was recorded. Sand maize media was used to grown the phytopathogens (Fig. 4. 1). The water control showed the highest percentage disease incidence, ranging from 61.1- to 70.5% against the four fungal phytopathogens, compared to all other treatments. In contrast, B7 achieved lowest disease incidence in chilli seedlings, ranging from 18.7-27.7% (Fig. 4 A), followed by *P. fluorescens* (25.0-33.3%), and *B. paramycoides* (25.0-38.8%) (Table 3). B7 demonstrated over 70% disease reduction against *M. phaseolina,* and *S. rolfsii*, compared to all other treatments (Fig. 5). Additionally, it showed more than 50% disease reduction against *F. oxysporum*, and *R. solani* with *P. fluorescens* and *B. paramycoides* following closely behind (Fig. 5; Fig. 4 B 1,2). Charcoal rot observed in B65 (Fig. 4 D), B21 (Fig. 4 H), water control (Fig. 4 C), and *B. amyloliquefaciens* (Fig. 4 G) Wilting was observed in B98 (Fig. 4 I), Healthy plant of *P. fluorescens* (Fig. 4 E), and water control (Fig. 4 F).

The chilli plants that survived the pathogenic effects were assessed for shoot length, root length, and dry mass, with results summarized in Table 4; Table 5. The B7 isolate demonstrated the highest performance, achieving shoot length of 8.8 cm against *M. phaseolina,* 8.3 cm for *F. oxysporum,* 8.4 cm for *S. rolfsii,* and 8.2 cm for *R. solani* (Table 4). Similarly, the highest root lengths were noted in B7, measuring 8.4cm against *M. phaseolina,* 9.5cm with *F. oxysporum,* and 7.9 cm, and 7.8 cm with *S. rolfsii,* and *R. solani,* respectively (Table 5). Other isolates showed variable shoot lengths between 6.5-8.5cm, and root lengths from 4.0-9.5 cm across the pathogens, while dry weight ranged from 0.08-0.85g. Our earlier study indicated that application of B7 increased chilli yield (6400 kgs/ha) compared to untreated controls (4850 kgs/ha) (Jyothi et al. 2023). We also demonstrated that *A. migulanus*

moderately improved the activities of amylase, protease, cellulase, and production of ammonia, IAA, and Exopolysaccharide. Similarly, in the present study, B7 consistently exhibited superior growth metrics, affirming its plant growth-promoting capabilities even in the presence of pathogens.

Table 3 Effect of bacterial treatments on disease incidence in chilli

Treatments	% disease incidence			
	M. phaseolina	*F. oxysporum*	*R. solani*	*S. rolfsii*
A. migulanus (B7)	19.6[a]	22.2[a]	27.7[a]	18.7[a]
	(26.20)	(28.0)	(31.7)	(25.4)
B21	60.7[d]	35.1[b]	35.1[abc]	22.2[ab]
	(51.1)	(36.3)	(36.3)	(28.0)
B28	39.2[c]	37.0[bc]	56.4[e]	25.0[abc]
	(38.7)	(37.4)	(48.7)	(29.9)
B65	56.8[d]	38.8[bc]	44.4[cd]	35.4[cd]
	(48.9)	(38.5)	(41.8)	(36.4)
B73	35.2[bc]	44.4[c]	53.7[de]	29.1[abc]
	(36.3)	(41.7)	(47.1)	(32.6)
B. amyloliquefaciens	58.8[d]	35.1[b]	55.5[de]	35.4[cd]
	(50.0)	(36.3)	(48.2)	(36.4)
B98	31.3[bc]	44.4[c]	40.7[bc]	45.8[d]
	(34.0)	(41.7)	(39.6)	(42.6)
B. paramycoides	29.4[b]	38.8[bc]	33.3[abc]	25.0[abc]
	(32.7)	(38.4)	(35.2)	(29.9)
B. subtilis	35.2[bc]	35.1[b]	37.03[abc]	43.7[d]
	(36.3)	(36.3)	(37.4)	(41.4)
P. fluorescens	29.0[b]	33.3[b]	31.4[ab]	25.0[abc]
	(33.0)	(35.1)	(33.9)	(29.4)
Water control	70.5[e]	61.1[d]	62.9[e]	68.7[e]
	(57.2)	(51.42)	(52.6)	(56.0)
CD (0.05)	(5.0)	(4.9)	(6.8)	(7.9)
CV %	7.28	7.79	9.70	13.20

Average of three replications; figures in the parenthesis indicate angular transformed values; Superscripts a-e with different alphabets indicate significant differences at a 5% ($p < 0.05$) level of significance.

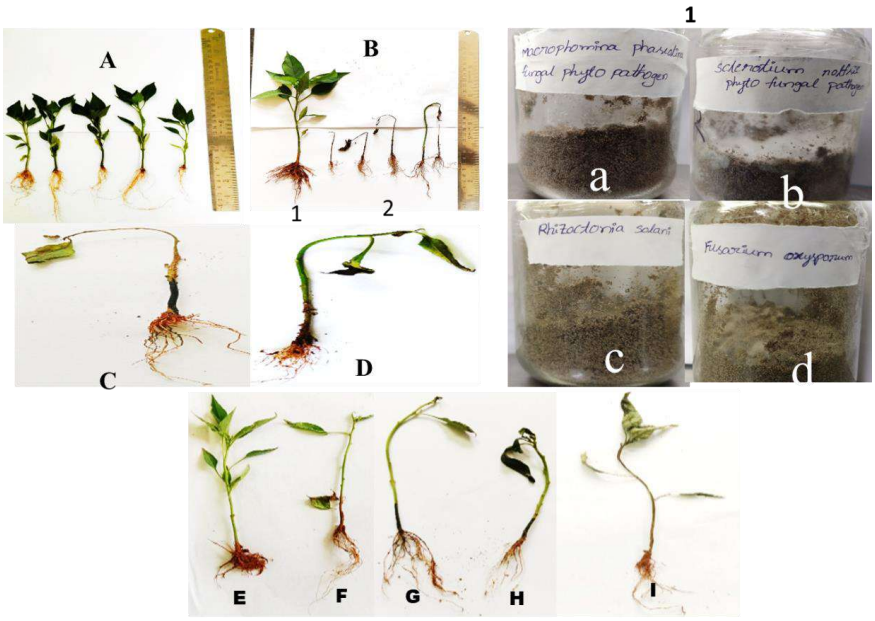

Fig. 4 Pathogenicity test of phytopathogens in sick soil. **1.** four fungal phytopathogens grown in Sand maize media. **A.** Healthy chilli seedlings treated with B7 (*A. migulanus*), **B**. Disease effected chilli seedlings, **1.** Healthy B7 chilli plant **2.** Disease effected water controlled chilli plants, **C.** Charcoal rot observed in water control, **D.** Charcoal rot observed in B65 **E.** *P. fluorescens* control, **F.** Wilting observed in water control, **G.** Charcoal rot observed in *B. amyloliquefaciens*. **H**. Charcoal rot observed in B21, **I.** Wilting observed in B98.

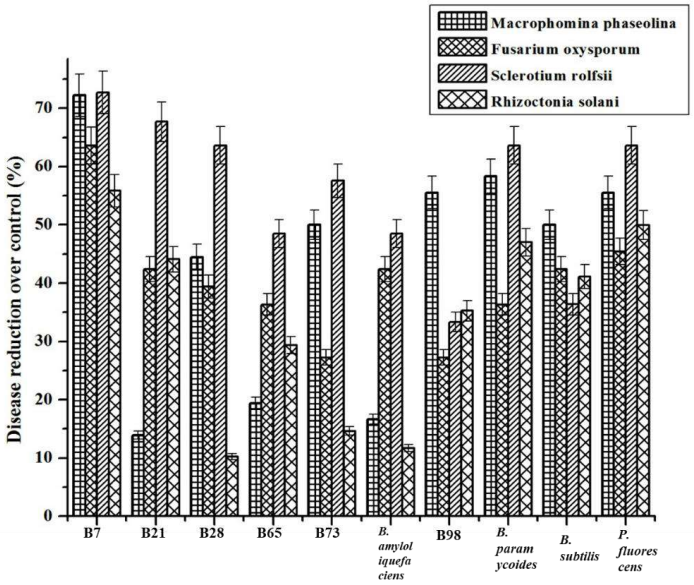

Fig. 5 Percentage Disease Reduction Over Control

Table 4 Pathogenicity study in sick pot using seedlings treated with bacterial treatments on shoot length in chilli against phytopathogens

Treatments	Shoot length (cm)			
	M. phaseolina	*F. oxysporum*	*S. rolfsii*	*R. solani*
B7	8.8[a]	8.3[a]	8.4[a]	8.2[a]
(*A. migulanus)*	(±1.837)	(±0.442)	(±2.1880	(±0.191)
B21	8.2a	6.9[b]	7.9[a]	7.8[a]
	(±1.7.07)	(±0.188)	(±2.054)	(±1.627)
B28	8.5[a]	6.6[b]	6.5[bc]	7.1[ab]
	(±0.384)	(±0.465)	(±1.807)	(±1.514)
B65	8.3[a]	7.4[ab]	7.8[a]	8.1[a]
	(±2.311)	(±1.609)	(±0.580)	(±1.708)
B73	7.6[a]	7.8[a]	7.5[ab]	7.05[ab]
	(±1.555)	(±1.728)	(±1.588)	(±1.841)
B. amyloliquefaciens	7.2[ab]	7.7[a]	7.9[a]	7.6[a]
	(±1.952)	(±1.627)	(±2.093)	(±2.113)
B98	7.4[ab]	7.8[a]	8.1[a]	7.6[a]
	(±1.922)	(±2.067)	(±2.121)	(±1.975)
B. paramycoides	7.2[ab]	8.2[a]	8.08[a]	7.8[a]
	(±0.502)	(±1.688)	(±1.709)	(±0.505)
B. subtilis	7.7[a]	6.7[b]	7.3[b]	6.7[b]
	(±2.002)	(±1.572)	(±2.000)	(±0.235)
P. fluorescens	8.0[a]	7.2[ab]	7.5[ab]	6.5[b]
	(±0.216)	(±1.552)	(±1.576)	(±0.408)
Water	7.2[ab]	7.2[ab]	7.2[b]	7.2[ab]
control	(±1.982)	(±1.982)	(±1.982)	(±1.982)
LSD value	2.4544	2.1177	2.3429	2.2581
CV%	12.77467	6.847967	16.30303	9.077391

Average of three replications; digits in the parenthesis indicate no transformed values; treatments with similar alphabets are not significantly different at 5% level ($p<0.05$).

Table 5 Pathogenicity study in sick pot using seedlings treated with bacterial treatments on root length, and dry biomass in chilli against phytopathogens

Treatments	Root length (cm)				
	M. phaseolina	*F. oxysporum*	*S. rolfsii*	*R. solani*	Dry weight (g)
B7	8.4[a]	0.85[a]	7.9[a]	7.8[a]	0.85[a]
(*A. migulanus)*	(±0.684)	(±0.034)	(±2.139)	(±1.094)	(±0.034)
B21	6.7[ab]	0.55[a]	5[b]	6.5[ab]	0.55[a]
	(±1.744)	(±0.022)	(±1.300)	(±1.125)	(±0.022)
B28	8.2[a]	0.8[a]	6.1[a]	5.8[b]	0.8[a]
	(±2.054)	(±0.032)	(±1.846)	(±1.565)	(±0.032)
B65	7.4[a]	0.36[a]	7.5[a]	6.7[ab]	0.36[a]
	(±2.073)	(±0.023)	(±0.313)	(±2.013)	(±0.023)
B73	6.8[ab]	0.75[a]	6.6[a]	6.4[ab]	0.75[a]
	(±1.426)	(±0.030)	(±1.599)	(±1.447)	(±0.030)
B. amyloliquefaciens	5.4[b]	0.6[a]	5.3[ab]	7.6[a]	0.6[a]
	(±1.417)	(±0.054)	(±1.537)	(±2.559)	(±0.054)
B98	4.02[c]	0.6[a]	6.5[a]	6.8[ab]	0.6[a]
	(±1.056)	(±0.054)	(±1.794)	(±1.862)	(±0.054)
B. paramycoides	5.6[b]	0.8[a]	6.8[a]	6.5[ab]	0.8[a]
	(±0.567)	(±0.032)	(±1.819)	(±1.194)	(±0.032)
B. subtilis	6.3[b]	0.55[a]	6.9[a]	7.2[a]	0.55[a]
	(±1.791)	(±0.022)	(±2.067)	(±1.635)	(±0.022)
P. fluorescens	6.8[ab]	0.48[a]	7.1[a]	5.7[b]	0.48[a]
	(±1.002)	(±0.030)	(±1.577)	(±1.029)	(±0.030)
Water	4.9[bc]	0.08[b]	6.6[a]	6.6[ab]	0.08[b]
control	(±1.324)	(±0.005)	(±1.932)	(±1.932)	(±0.005)
LSD value	2.3372	0.042	2.5769	2.8995	0.042
CV%	11.96649	0.208827	15.25948	9.672663	0.208827

Average of three replications; digits in the parenthesis indicate no transformed values; treatments with similar alphabets are not significantly different at 5% level ($p<0.05$).

Estimation of Lipase and Pectinase Activities

Production of lipase in microbial bio-control agents is an important factor that degrades the lipids of the cell wall membrane of the plant pathogens (Subramoni et al. 2010). The lipase activity of *Bacillus* species is an important criterion for lipid metabolism (Al Mohaini et al. 2022). Lee et al. 2015 demonstrated positive lipase activity in *Bacillus* strain 1-10. However, they did not measure the zone of inhibition. In the current study, the highest lipase production was observed in isolate B7, which exhibited 17 mm zone of inhibition, followed by B37 at 15 mm (Fig. 6; Table 6).

Many plant pathogenic bacteria and fungi are known to produce pectinolytic enzymes that facilitate the invasion of host tissues (Alqahtani et al. 2022), as microbes are known to produce pectinolytic enzymes in response to pectin, which serves as an inducer. Devi and Thakur 2018 reported the pectinolytic activity of bacterial strains, highlighting zones of clearance in their activity diameter (mm) *Viz.*, BTS 14 (6), BTS 16 (15), NTS 11 (2), NTS 78 (12), and NTS 34 (9). In contrast, the current study revealed superior zone of clearance, with B7 exhibiting a diameter of 25 mm, followed by B65 at 24mm (Fig. 6; Table 6). In this study, the highest pectinolytic activity was recorded for B7, with a 25 mm zone of inhibition, followed by B65 (24 mm). Additionally, B7 rendered the highest lipase activity with a 17 mm zone of inhibition, and B37 produced a 15 mm zone of inhibition (Fig. 6; Table 6).

Hydrogen Cyanide (HCN) production

The results presented in Fig. 6 and Table 6 highlight the ability of several bacterial isolates to effectively produce hydrogen cyanide (HCN). Among the tested isolates, only 12 were identified as HCN producers. Notably, isolates B7, B32, and B79 exhibited strong HCN production (+++), while moderate production (++) was observed in B88, B98, and *B. paramycoides*. Weak positive (+) reactions were recorded for B43, B62, B65, B73, *P. fluorescens*, and *B. subtilis*. The use of HCN-producing bacteria as bio-pesticides offer a sustainable and environmentally

friendly approach to agriculture (Sehrawat et al. 2022). Previous studies, such as those by (El-Rahman et al. (2019), quantified HCN production, measuring absorbance at 625 nm *Viz.*, *P. japonica* (0.020), *B. megaterium* (0.002), *Pseudomonas* sp. Strain Gamma -81 (0.005), *P. tolaasii* (0.024), *P. chlororaphis* (0.047), and *P. mosselii* (0.027). In comparison, the current study revealed that isolate B7 had the highest HCN absorbance at 625 nm B7 (0.0546), followed by B32 (0.0343). Thus, isolate B7 stands out as the most prolific HCN producer in this research (Fig. 6; Table 6).

Qualitative Assessment for Biofilm Growth

Biofilm is a complex microbial community comprising living and dead bacterial cells, extracellular polymeric substances (EPS), and other materials secreted by the cells (Flemming and Wingender 2010). This community can grow and interact dynamically with its surrounding environment (Wilson et al. 2018) resulting in various actions, including biological control. In the current study, nine bacterial isolates (*viz.*, B7, B32, B35, B36, B53, B65, B79, B85, and B88) exhibited high levels of biofilm production (++++), while isolates B21, B28, *P. fluorescens*, and *B. amyloliquefaciens* displayed lower levels (+). Notably, some isolates did not produce biofilm at all (Table 6). Several *Bacillus* species are known to be effective biofilm producers on plant roots, offering protection against pathogen invasion while simultaneously promoting plant growth (Pandit et al. 2020; Gao et al. 2015).

Hassan et al. (2011) described a study using a test tube method in which 110 clinical isolates shown biofilm formation. This included 21 high, 33 moderate, and 56 weak or non-biofilm producers. This present research yielded comparable results, categorizing the bacterial isolates into nine with high activity, six with moderate activity, and four with low activity (Fig. 6; Table 6). The isolates also exhibited antagonistic and inhibitory effects against evaluated plant pathogens: *M. phaseolina*, *S. rolfsii*, *F. oxysporum*, and *R. solani*. Notably, *B. clausii*, and *B. polymyxa* did not demonstrate any of the hydrolytic tests, as well as production of HCN, or biofilm. (Fig. 6).

Table 6 Assay of lipase, pectinase, biofilm, and HCN production by selected bacterial isolates

Treatments	Hydrolytic tests (mm)		Plant growth promoting traits		
				HCN	
	Lipase	Pectinase	Biofilm	Qualitative	Quantitative OD @ 625 nm
B4	0	15	0	0	0
A. migulanus(B7)	17	25	++++	++++	0.0546
B21	0	20	+	0	0
B22	0	20	+++	0	0
B28	11	20	+	0	0
B32	0	0	++++	+++	0.0343
B35	0	22	++++	0	0
B36	0	0	++++	0	0
B37	15	0	+++	0	0
B43	0	21	+++	+	0.0123
B53	0	0	++++	0	0
B62	0	20	++	+	0.0143
B65	10	24	++++	+	0.0091
B73	10	15	0	+	0.0102
B79	0	22	++++	+++	0.0302
B81	0	12	++	0	0

B85	0	14	++++	0	0
B88	0	20	++++	++	0.0204
B98	0	18	0	++	0.0202
P. fluorescens	10	15	+	+	0.0307
B. subtilis	0	18	++	+	0.0052
B. paramycoides	0	17	+++	++	0.0032
B. amyloliquefaciens	0	0	+	0	0
B. clausii	0	0	0	0	0
B. polymyxa	0	0	0	0	0

In the table 0, +, ++, +++, and ++++ mentioned to resemble the range of bacterial treatments with lower to higher activity.0- No activity; +- very low activity; ++- low activity; +++ moderate activity; ++++- High activity.

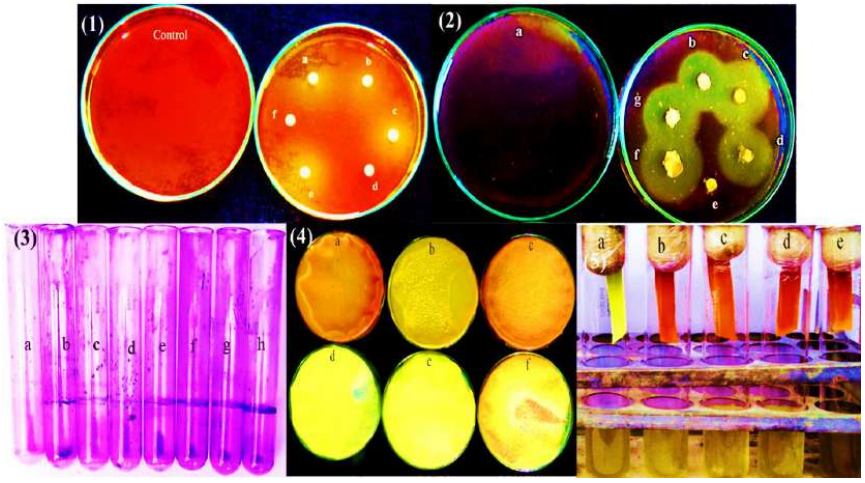

Fig. 6 Biochemical characterization of bacterial isolates. (1) Lipase producing isolate (a - B28, b - *P. fluorescens*, c – (B7) *A. migulanus*, d - *B. paramycoides*, e- B37, f - *B. subtilis*), (2) Pectinase production (a - Control, b - B79, *c* - *A. migulanus* (B7), d - B35, e - B32, f - B65, g - B43); (3) Production of Biofilm (a - Control, b - *B. paramycoides* , c - *P. fluorescens*, d - *B. subtilis*, e - B32, f - B35, g - B36, h - *A. migulanus* (B7), (4) Qualitative estimation of HCN (a - *A. migulanus* (B7), b - B88, c - B32, d - B79, e - Control, f - *B. subtilis*), (5) Quantitative estimation of HCN (a - Control, b - *A. migulanus* (B7), c - B32, d - B79, e - B88).

Genetic identification

The amplified gene was compared with other *Bacillus* spp. Gene sequences at NCBI using the BLAST tool (Fig. 7). A phylogenetic tree was constructed to determine the relationship between bacterial isolate B7 and other *Bacillus* species, or closely related genera submitted at NCBI. B7 was found to be *Aneurinibacillus migulanus*, showed high similarity 99.34% with based on nucleotide homology and phylogenetic analysis.

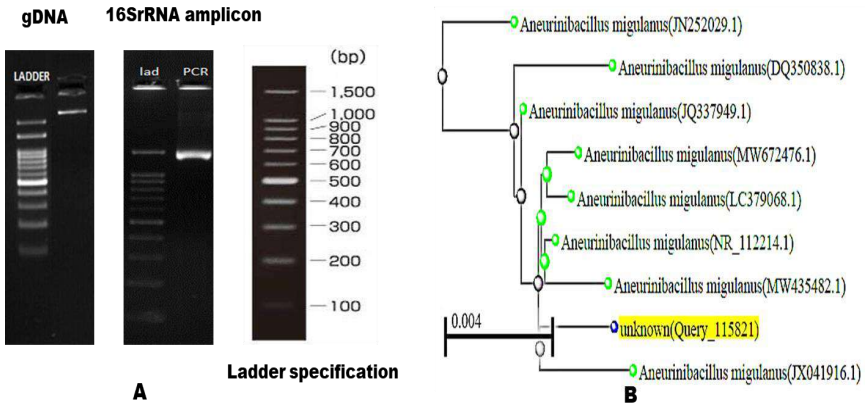

Fig. 7 A. gDNA and 16S Amplicon QC data. **B.** Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationship of the isolate B7 and other members of the genus *Bacillus*

***In vitro* antifungal activity**

The antifungal activities of extra- and intracellular crude extract of *A. migulanus* (B7), and *B. amyloliquefaciens* were evaluated against *M. phaseolina*, *S. rolfsii*, *F. oxysporum*, and *R. solani*. The positive control, carbendazim exhibited inhibition zones of 10 mm against *M. phaseolina*, 15 mm against *S. rolfsii*, 12 mm against *F. oxysporum*, and 17 mm against *R. solani*. The extracellular crude extract of B7 demonstrated superior antifungal activity with an inhibition zones of 19 mm in *M. phaseolina*, 16.6 mm in *S. rolfsii*, 20.6 mm in *F. oxysporum*, and 15 mm in *R. solani*. The intracellular crude extract of *A. migulanus* displayed relatively lower antifungal activity against *S. rolfsii* (13.6 mm), and *R. solani* (11.3 mm) compared to the antifungal activity observed in the extracellular crude extract. *B. amyloliquefaciens* exhibited antifungal activity against *M. phaseolina* with inhibition zones of 10 mm for the intracellular extract and 15 mm for the extracellular extract. The negative control (DMSO) did not inhibit any of the tested phytopathogens (Fig. 8; Fig. 9).

The intra- and extracellular crude extract of *A. migulanus* exhibited the highest antifungal activity against *M. phaseolina*, *S. rolfsii*, *F. oxysporum*, and *R. solani* compared to carbendazim (Fig. 8; Fig. 9). Chu et al. (2022) highlighted *B. velezensis* HN-2's ability to produce antimicrobial metabolites that inhibit *Erysiphe*

quercicola, the fungus responsible for powdery mildew in oak. Similarly, Jan et al. (2023) demonstrated that crude extracts from various *Bacillus* strains inhibited the growth of *F. oxysporum*, *A. niger*, *A. flavus*, and *R. oryzae* by 56-92%. In another study, Soliman et al. (2022) reported significant inhibition against *Alternaria* and *Helmintho sporium* species. Additionally, Bulgasem et al. (2016) found that the supernatant of *L. plantarum* produced a 25 mm inhibition zone against *C. albicans*, while *B. subtilis* showed a 30 mm zone, outperforming the fungicide itraconazole (13.67 mm) (Gharieb et al. 2024).

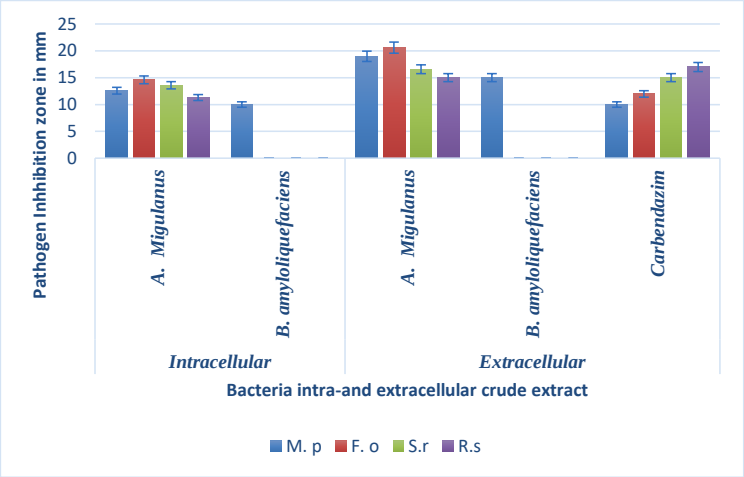

Fig. 8 Bacteria *in vitro* crude extract antifungal activity against phytopathogens

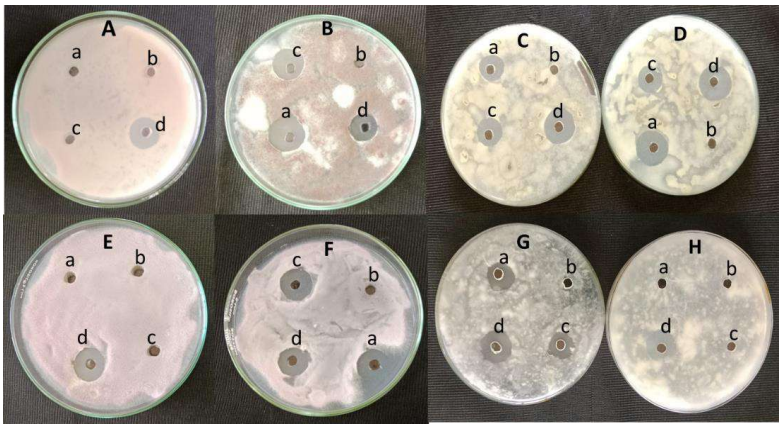

Fig. 9 Bacteria *in vitro* crude extract antifungal activity against phytopathogens. **A –** crude extract of *B. amyloliquefaciens* against *R. solani*, **B –** crude extract of *A. migulanus* (B7) against *R. solani*, **C –** crude extract of *B. amyloliquefaciens* against *M. phaseolina*, **D –** crude extract of *A. migulanus* (B7) against *M. phaseolina*, **E –** crude extract of *B. amyloliquefaciens* against *F. oxysporum*, **F –** crude extract of *A. migulanus* (B7) against *F. oxysporum*, **G –** crude extract of *B. amyloliquefaciens* against *S. rolfsii*, **H –** crude extract of *A. migulanus* (B7) against *S. rolfsii*, a- extracellular crude extract; b- DMSO 10% (Negative control); c- intracellular crude extract; d- carbendazim (positive control).

GC-MS analysis of secondary metabolites emitted by *A. migulanus*

Analysis of the headspace samples from *A. migulanus* revealed a diverse array of secondary metabolites present in both extra- and intracellular extracts following 7 days of incubation. These metabolites encompassed a variety of classes, including organic acids, methyl groups, amine groups, and esters (Fig. 10; Table 7, 8). Gas Chromatography-Mass Spectrometry (GC-MS) analysis facilitated the identification of 31 intracellular (Table 7; Fig. 10) and 31 extracellular (Table 8; Fig. 10) secondary metabolites.

The intracellular extract showcased numerous notable compounds, including benzene, 1,3-dichoro (RT 4.356, 21.1%), benzene nitro (RT 4.809, 24.72%), 1-Dodecanol, methylether (RT 5.237, 27.99%), 2-Cyclopenten-1-one,2,3,4,5-

tetramethyl-(RT4.977, 28.17%), Cyclopentane, undecyl- (RT 7.16, 29.98%), 1-Tetradecanol (RT 6.212, 30.79%), Hexanoic acid, 2- chlorophenylester (RT 5.346, 36.58%), phenol,2,5-bis(1,1-dimethylethyl) (RT 6.789, 53.69%) and heptacosane (RT 19.09, 78.59%). However, in the case of extracellular extract, Benzene nitro (RT 4.804, 35.08%), Hexanoic acid, 3,5-difluorophenylester (RT 5.348, 39.03%), Trichloroacetic acid, hexadecylester (RT 6.219, 62%), phenol,2,5-bis(1,1-dimethylethyl) (6.798, 53.24%), Pentafluoropropionicacid,4-hexadecylester (RT 7.17, 53.21%), 8-Heptadecene,9-octyl-(RT 8.403, 81.25%), 2-Methyl-Z-4-tetradecene (RT 14.88, 88.88%), 1-Hexadecanesulfonyl chloride (RT 17.705, 90.97%) and heptacosane (19.061,85.45%) were identified. The identification of these compounds was performed using the National Institute of Standards and Technology (NIST-2017) Mass Spectral Library. This comprehensive assessment underscores the metabolic diversity of *A. migulanus* and its potential applications in crop protection and biotechnological fields.

Previous studies have suggested that *A. migulanus* could be a superior alternative to conventional fungicides, offering a holistic approach to plant health and pathogen management through the production of various bioactive secondary metabolites. Notably, *A. migulanus* synthesizes the antimicrobial peptide Gramicidin S (Berditsch et al. 2007) and secretes a bio-surfactant that effectively reduces the surface tension of plant leaves, thereby inhibiting pathogen growth (Belbahri et al. 2017). This makes *A. migulanus* as a potential biocontrol agent against several plant diseases including *Botrytis cinerea* (Belbahri et al. 2017), *Dothistroma septosporum* (Alenezi et al. 2016), and *F. oxysporum f. sp. Lycopersici* (Chowdary and Biswas 2018).

In this study, *A. migulanus* was found to be effective against *F. oxysporum, M. phaseolina, R. solani,* and *S. rolfsii,* outperforming other treatments such as *P. fluorescens, B. paramycoides, B. amyloliquefaciens, B. polymyxa, B. clausii,* and *B. subtilis*. The results indicated that *A. migulanus* exhibited lower disease incidence and achieved the highest disease reduction compared to control groups. Additionally, it demonstrated notable antifungal activity, and several secondary

metabolites, along with plant growth, as evidenced by increased dry mass and greater shoot and root lengths. Thus, this is the first report on *A. migulanus* documenting the production of secondary metabolites and anti-fungal compounds, demonstrating significant antagonism against four economically important fungal pathogens affecting chilli.

Table 7 Volatile profile of intracellular crude extract of *A. migulanus.*

RT	Area	Area %	Name	DB Formula
4.356	303540344.5	21.1	Benzene,1,3-dichloro-	$C_6H_4Cl_2$
4.468	202996510.6	14.11	1(4H)-Naphthalenone, 4a,5,8,8a-tetrahydro-4- hydroxy-, (4.alpha.,4a.beta.,8a.beta.)	$C_{10}H_{12}O_2$
4.809	355645597.1	24.72	Benzene,nitro-	$C_6H_5NO_2$
4.977	405151044.3	28.17	2-Cyclopenten-1-one,2,3,4,5- tetramethyl-	$C_9H_{14}O$
5.237	402615202.8	27.99	1-Dodecanol,methylether	$C_{13}H_{28}O$
5.346	526242976.1	36.58	Hexanoic acid, 2- chlorophenylester	$C_{12}H_{15}ClO_2$
5.626	134732363.7	9.37	Hexadecane,1-chloro-	$C_{16}H_{33}Cl$
5.906	187933773.8	13.06	Naphthalene,2-methyl-	$C_{11}H_{10}$
5.998	136421264.9	9.48	Bicyclo[4.4.1]undeca- 1,3,5,7,9-pentaene	$C_{11}H_{10}$
6.102	185149019.6	12.87	Disulfide,di-tert-dodecyl	$C_{24}H_{50}S_2$
6.212	442924693.5	30.79	1-Tetradecanol	$C_{14}H_{30}O$
6.239	124616509.2	8.66	Tetradecane	$C_{14}H_{30}$
6.281	135434419.4	9.42	2-Piperidinone,N-[4-bromo-n- butyl]-	$C_9H_{16}BrNO$
6.556	140931049.8	9.80	1-Hydroxycyclododecanecarbon	$C_{13}H_{23}NO$
6.789	772348235.8	53.69	Phenol,2,5-bis(1,1- dimethylethyl)-	$C_{14}H_{22}O$
6.823	149056154.6	10.36	1-Isopropenylnaphthalene	$C_{13}H_{12}$
6.934	114365536.7	7.95	Disulfide,di-tert-dodecyl	$C_{24}H_{50}S_2$
7.031	204857839.1	14.24	tert-Hexadecanethiol	$C_{16}H_{34}S$
7.16	431235942.1	29.98	Cyclopentane,undecyl-	$C_{16}H_{32}$
7.367	101820094	7.08	8-Dodecen-1-ol,acetate,(Z)-	$C_{14}H_{26}O_2$
7.418	130000610.6	9.04	2,3,3-Trimethyl-2-(4- methylpentanoyl)-cyclopentanone	$C_{14}H_{24}O_2$

7.528	120998198.7	8.41	2-Dodecen-1-yl(-)succinic anhydride	$C_{16}H_{26}O_3$
8.213	105148170	7.31	Trihexadecylborate	$C_{48}H_{99}BO_3$
8.397	1013294467	70.44	8-Heptadecene,9-octyl-	$C_{25}H_{50}$
9.418	252833573.4	17.58	7,9-Di-tert-butyl-1- oxaspiro(4,5)deca-6,9-diene- 2,8-dione	$C_{17}H_{24}O_3$
9.827	111131780.4	7.73	1,2-Benzenedicarboxylicacid, butyl 8-methylnonyl ester	$C_{22}H_{34}O_4$
10.102	310352046	21.58	Acetic acid, 3,7,11,15- tetramethyl-hexadecylester	$C_{22}H_{44}O_2$
14.887	167134789.5	11.62	Cyclohexane,1,2,3,4,5,6- hexaethyl-	$C_{18}H_{36}$
19.09	1130513597	78.59	Heptacosane	$C_{27}H_{56}$
22.44	109380362.1	7.60	Erythro-7,8- Bromochlorodisparlure	$C_{19}H_{38}BrCl$
27.699	113045786.1	7.86	Octa-triacontyl-pentafluoro-propionate	$C_{41}H_{77}F_5O_2$

Table 8 Volatile profile of extracellular crude extract of *A. migulanus*

RT	Area	Area %	Name	DB Formula
4.356	344092605.5	20.77	Benzene,1,3-dichloro-	$C_6H_4Cl_2$
4.471	191055824.2	11.53	Benzeneethanol,beta.-ethenyl-.alpha.-phenyl-	$C_{16}H_{16}O$
4.804	581136659	35.08	Benzene,nitro-	$C_6H_5NO_2$
4.988	467475356	28.22	2-Cyclohexen-1-one, 3,5,5-trimethyl-	$C_9H_{14}O$
5.243	543148512.1	32.78	1-Dodecanol, methylether	$C_{13}H_{28}O$
5.348	646693744.3	39.03	Hexanoic acid, 3,5-difluorophenylester	$C_{12}H_{14}F_2O_2$
5.909	235021627	14.19	Bicyclo[4.4.1]undeca-1,3,5,7,9-pentaene	$C_{11}H_{10}$
6.219	1027254601	62.00	Trichloroaceticacid,hexadecylester	$C_{18}H_{33}Cl_3O_2$
6.507	384582701.8	23.21	Disulfide,di-tert-dodecyl	$C_{24}H_{50}S_2$
6.559	222699397.1	13.44	1-Hydroxycyclododecanecarbon	$C_{13}H_{23}NO$
6.609	146494904.9	8.84	2,5-di-tert-Butyl-1,4-benzoquinone	$C_{14}H_{20}O_2$
6.798	882129914.3	53.24	Phenol, 2,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$
6.823	153504609.4	9.27	1-Isopropenylnaphthalene	$C_{13}H_{12}$
7.17	881540924.2	53.21	Pentafluoropropionicacid,4-hexadecylester	$C_{19}H_{33}F_5O_2$
7.199	165991571.3	10.02	Octadecane,1-chloro-	$C_{18}H_{37}Cl$
7.231	699405758.1	42.22	DiethylPhthalate	$C_{12}H_{14}O_4$
7.36	207794465.8	12.54	8-Dodecen-1-ol,acetate,(Z)-	$C_{14}H_{26}O_2$
7.418	169752689.1	10.25	2-Piperidinone, N-[4-bromo-nbutyl]-	$C_9H_{16}BrNO$
7.64	225688849	13.62	2-Dodecen-1-yl(-)succinicanhydride	$C_9H_{16}BrNO$
8.06	138817438.6	8.38	tert-Hexadecanethio	$C_{16}H_{26}O_3$
8.214	153458039	9.26	1-Decanol,2-hexyl-	$C_{16}H_{34}S$
8.403	1346166610	81.25	8-Heptadecene,9-octyl-	$C_{16}H_{34}O$

9.431	453672513.8	27.38	7, 9-Di-tert-butyl-1- oxaspiro(4,5)deca-6,9-diene2,8-dione	$C_{25}H_50$
9.846	515258114.9	31.10	2-Dodecen-1-yl(-)succinicanhydride	$C_{17}H_{24}O_3$
13.631	225926114.5	13.64	2,3,3-Trimethyl-2-(4- methylpentanoyl)-cyclopentanone	$C_{16}H_{26}O_3$
14.886	1472513054	88.88	2-Methyl-Z-4-tetradecene	$C_{15}H_{30}$
16.341	525030341.4	31.69	Pentacosane	$C_{25}H_{52}$
16.77	536140672.1	32.36	Di-n-octyl phthalate	$C_{24}H_{38}O_4$
17.705	1507129877	90.97	1-Hexadecanesulfonyl chloride	$C_{16}H_{33}ClO_2S$
19.061	1415653227	85.45	Heptacosane	$C_{27}H_{56}$
27.703	119441210.1	7.21	Octatriacontylpentafluoropropionate	$C_{41}H_{77}F_5O_2$

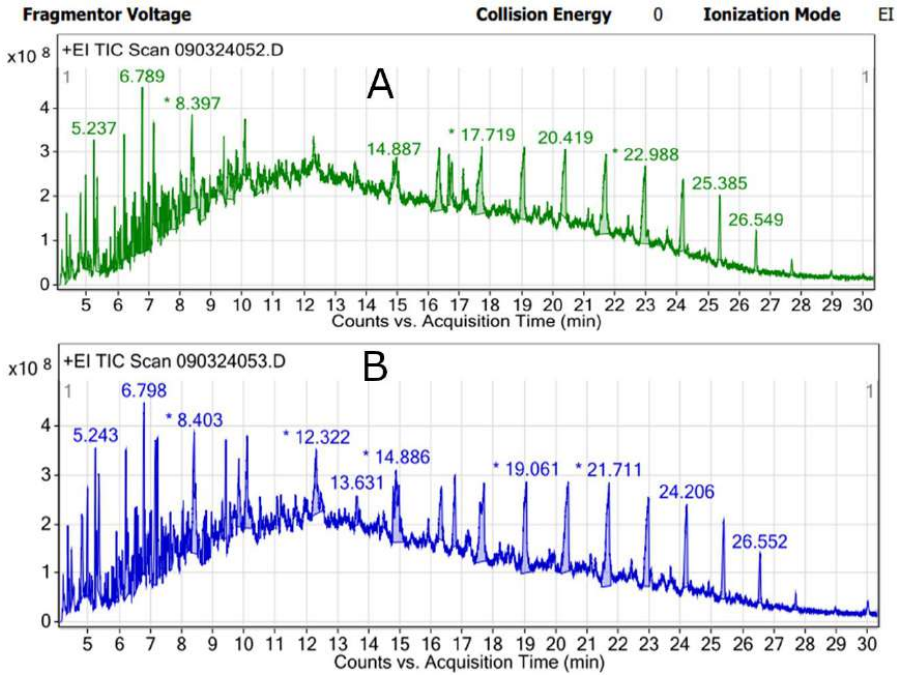

Fig. 10 GC-MS spectrum of secondary metabolites identified in intracellular (**A**) and

extracellular (**B**) extract of *A. migulanus* (B7).

CONCLUSIONS

The study confirmed that *A. migulanus* effectively biocontrol chilli phytopathogens *viz.*, *M. phaseolina*, *R. solani*, *F. oxysporum*, and *S. rolfsii*. GC-MSD analyses indicated that *A. migulanus* secretes bioactive antifungal secondary metabolites crucial for its biocontrol capabilities. It produces various antifungal biochemical substances, such as hydrolytic enzymes, biofilm, hydrogen cyanide, and pectinolytic enzymes, which contribute to its antifungal potential both directly and indirectly. Application of *A. migulanus* to chilli seedlings resulted in the highest percentage of disease reduction compared to control treatments, along with improved shoot, root length, and dry mass, and a decrease in disease incidence. These findings highlight *A. migulanus's* role in promoting plant growth

development more effectively with all the necessary traits for biocontrol. Further research is essential to investigate the specific roles and mechanisms of the various secondary metabolites produced by *A. migulanus* in preventing chilli infestations. Additionally, the synergistic interactions between *A. migulanus* and other biocontrol agents such as *B. paramycoides* and *B. amyloliquefaciens* should be evaluated.

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