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In vitro antagonistic effects of *Bacillus* species against phytopathogenic fungi

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Abstract

Fungal diseases in plants are a key threat to global food security, causing huge crop losses. Bacterial biofungicides antagonize several fungal phytopathogens, controlling plant diseases. Despite several bacterial species reported as biofungicides, indigenous *Bacillus* species from Nepal have yet to be explored for their antifungal activity, so that they could be used as efficient biofungicides to enhance agricultural production. In this study, we identified 65 isolates of *Bacillus* based on cultural, morphological, and biochemical characteristics from soil, phylloplane, and dead insects. Preliminary antifungal activity against three fungi, *Fusarium* spp. *Curvularia* spp. and *Aspergillus* spp. by dual culture method showed that 16 (26.67%), 3 (5.00%), and 22 (36.67%) isolates of *Bacillus* species exhibited inhibition against *Fusarium* spp., *Curvularia* spp., and *Aspergillus* spp., respectively. Subsequent leaf disk assays on cucumber plants demonstrated 34 (56.67%) isolates of *Bacillus* species exhibiting significant suppression of powdery mildew fungi (*Erysiphe cichoracearum*). Notably, two isolates (NS116, NS114) exhibited the maximum antifungal activity and were characterized by 16S rRNA gene sequencing as *Bacillus cereus* strains. *B. cereus* demonstrated a higher ability to control fungal diseases with a preventive effect exceeding 90%, compared to 3% Anvil (4.8% w/w Hexaconazole), which displayed only a 67.81% preventive effect. Since Anvil induces ecological impact due to its toxicity to aquatic invertebrates and fishes, effective bacterial pesticides like *Bacillus* species could be a better choice. These findings highlight the potential of *Bacillus* spp. as potent biocontrol agents, due to their ability to produce antifungal compounds and manage fungal pathogens.

Keywords: *Bacillus cereus*, powdery mildew fungi, dual plate, leaf disk assay, bio-fungicides.

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Introduction

Pathogenic microbes cause approximately $220 billion loss globally in crop production [1]. The wide group of pathogenic fungi includes *Fusarium oxysporum* (Wilt disease), *Rhizoctonia solani* (Seed rot disease), *and Aspergillus* spp. (Rot-disease), *Erysiphe* spp (Powdery mildew disease) contributes major damage to cucumber production. In general, chemical fungicides are applied to control fungal diseases, resulting the negative effects on the environment and 25 human health, and developing the emergence of resistance strains [2]. Moreover, fungicides are costly, rendering them inadequate for sustainable disease management practices [3].

Cucumber (*Cucumis sativus* L.) is a widely cultivated vegetable in Nepal. According to the Ministry of Agriculture and Local Development, the cucumber farming area covers 160,107 hectares, with a total production of 15.22 metric tons and a yield of 1,289 metric tons [4]. This indicates that cucumber cultivation plays a significant role in Nepal's vegetable production sector. Powdery mildew is a serious fungal disease that primarily targets the young, moist leaves of vegetables and fruits [5]. This disease can cause substantial 30-50% yield losses, especially under high humidity conditions in cucurbits such as cucumbers and pumpkins [6]. Although chemical fungicides are commonly applied, the resistant fungal strains have prompted alternative solutions [7]. Foliar spray of *B. licheniformis* and *B. aerius* enhanced the resistance of cucumber plants affected by *Podosphaera xanthii* [8]. Many microbial biopesticides show lower efficacy and are costly to produce [9]. However, integrating the *Bacillus* species not only helps in controlling powdery mildew fungi (PMF) but also reduces the consumption of chemical fungicides, offering

a healthier and sustainable alternative for agricultural practices.

Microbial strains have been sourced from various environments, including soil, dead insects, leaves, stored grains, and aquatic habitats [10,11,12]. Beneficial microbes protect plants from harmful invaders by releasing chemicals, siderophores, and growth hormones [13]. *Trichoderma* spp, *Penicillium fellutanum*, *Bacillus megaterium, B. subtilis,* and *B. licheniformis* have antagonistic activity against the fungi. Various metabolites such as auxin, gibberellins, cytokinin, antibiotics, siderophore, and hydrocyanide (HCN) act as antimicrobial, plant growth-promoting, and systemic resistance-inducing compounds [14,15].

Among diverse beneficial antagonistic organisms, *Bacillus* species have garnered the attention of researchers because lipopeptides produced by *Bacillus* spp. function as antifungal, antimicrobial, antitumor, and biosurfactant activities [16]. The lipopeptides like fenfycin, surfactin, and iturin inhibit the soil-borne phytopathogens, mainly the fungi, thus resulting in suppression of the plant disease, inhibiting spore germination, and disrupting fungal cell membranes [17,18]. Furthermore, *Bacillus* spp. produces different types of lytic enzymes like cellulases, chitinases, proteases, glucanases, laminaninase that lyse the cells of fungal pathogens in plants. Another key factor of *Bacillus* spp. is siderophore production, which also contributes to fungal inhibition along with the availability of iron for plants [15,19,20].

Although the antagonism of *Bacillus* species isolated from rhizosphere and different soils from Nepal has been studied against bacteria, the antifungal activity of *Bacillus* species from Nepal's soil is rarely investigated [21,22,23]. In addition, indigenous *Bacillus* species from Nepal have not been evaluated against powdery mildew fungi. Various beneficial microbes, such as *Bacillus* and *Pseudomonas*, produce different antagonistic substances that enhance the combat of fungal pathogens. Identifying novel pathogenic strains creates the foundation for developing alternative approaches to control plant diseases. These strains are relatively less harmful to humans and are environment-friendly. The findings of this study explore the opportunities to formulate and develop bacterial biofungicides using Nepalese indigenous *Bacillus* species, which could protect from the huge crop loss due to fungal infections and contribute to fungal disease management practices in Nepal.

Materials and methods

Sample collection and *Bacillus* species isolation, and identification

Altogether, 84 samples comprising soil (n =10), cucumber leaves (n = 30), and dead insects (n = 44) were collected from the vegetable cropland of Kathmandu valley and nearby places like Dhading and Kavrepalanchok.

The *Bacillus* spp. was isolated from soil, cucumber leaves, and dead insects as described by the literature [24,25,26] on Nutrient Agar (Himedia, India). The bacterial isolates showing gram-positive rods in gram staining in microscopy were evaluated for endospore by spore staining and biochemical testing including catalase, oxidase, citrate utilization, triple sugar iron (TSI), Methyl Red–Voges Proskauers, SIM (sulfate, indole, motility), nitrate reduction test, sugar utilization (glucose, starch and mannitol), salt tolerance (2, 5, 7, 10%) and temperature tolerance (20, 30, 40, 50, 60 °C) [27].

Molecular characterization of *Bacillus* spp. by 16S rRNA gene sequencing

The identification and the phylogeny of the two most potent isolates of *Bacillus* were confirmed by sequencing of the 16S rRNA gene. For this, bacterial DNA was extracted by the boiling method. Briefly, a pure-culture colony was transferred and mixed into 100 µL of 50 mM sodium hydroxide. It was incubated at 95 °C for 1 min, subsequently cooled to 4 °C, and then neutralized with 16 µL of 1 M Tris-HCl (pH 8.0). After centrifugation for 2 min at high speed, the obtained pellets (DNA) were resuspended in ultra-pure water [28]. The concentrations and quality of the DNA sample were evaluated by a Nanodrop Spectrophotometer (Thermo, USA). The phylogenetic marker 16S rRNA gene was amplified by PCR using forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1492 R (5'-AGA GCT ACC TTG TTA CGA CTT-3'). Each 25 µL volume contained 12.5 µL of PCR master mix (Promega, USA), 2 µL of template DNA (~ 20 ng) of *Bacillus* strains, 0.5 µL of each forward and reverse primers (10 μmol^{-1} each), and nuclease-free water to make up the volume. The PCR amplifications were accomplished using the thermal cycler (BioRad, USA) with an initial denaturation at 92 °C 1 min, followed by 30 cycles of 45 s at 92 °C, 45 s at 58 °C and 1 min at 72 °C and a final extension of 10 min at 72 °C [29]. Thus obtained PCR product was sequenced at Macrogen, South Korea with Sanger sequencing methods. The raw sequence was edited using Bioedit software for quality control and aligned by using Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11) [30]. The aligned sequences were compared

via Blastn BLAST with the National Center for Biotechnology (NCBI) database. The closest type strain sequences were searched and used for the construction of a phylogenetic tree. The phylogenetic tree was constructed by the Neighbor-Joining method [31], and taxa were clustered together (bootstrap 1000).

Test fungal species

Three fungal cultures, viz. *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp. were obtained from the Plant Pathology Laboratory, Nepal Agricultural Research Council (NARC), Lalitpur, Nepal. Fungi were recovered by culturing on Potato Dextrose Agar (PDA) and reconfirmed based on growth characteristics and microscopic appearance of mycelia, conidia, and spores with Lactophenol cotton blue staining, followed by an antifungal activity test [32]. The fungal cultures were subcultured onto fresh PDA media as well as in PDA agar slant at 4 °C for future use [33].

Another phytopathogenic fungus, powdery mildew fungi was collected from the infected leaves of the cucumber on a farm at the National Horticulture Research Center of the Nepal Agricultural Research Council and identified based on microscopic features of conidia and conidiophore at the Research Centre for Applied Science and Technology (RECAST) in Tribhuvan University. The conidia collected from the infected leaf were stored in distilled water and transferred to healthy cucumber leaves, then the plants were covered with plastic bags and maintained in a growth chamber [34].

Identification of powdery mildew fungi

The morphological characteristics include the morphology of the conidia, the dimensions of conidia and conidiophore, and the presence of fibrosin bodies in the interior of the conidia through the microscope. The powdery mildew fungi were brushed and kept in sterile distilled water in a sterile glass container. One drop was transferred to the clean glass slide and observed in the microscope. The conidial numbers (15 conidia per sample) were counted, and the length and width of the conidia were measured. The conidia structure, the conidia, and the conidiophores were stained with lactophenol cotton blue and observed using a microscope [35].

Antifungal activity of *Bacillus* spp. against *Fusarium* spp., Aspergillus spp., and *Curvularia* spp.

Antifungal activities of *Bacillus* spp. were evaluated by dual (co-)culture technique against *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp. Briefly, a 6 mm disk of freshly grown pure culture of the pathogenic fungal culture on potato dextrose agar (PDA) was transferred into a well made at the center of another PDA. Then, a single straight line of bacteria was streaked on the side of the fungi. After that, the plate was incubated for one week at 25 °C, and inhibition was noted for 7 days [36]. The experiment was done for three replications to confirm the antifungal activity.

Leaf disk antagonism bioassay against the powdery mildew fungi

Adapting a previous protocol [34, 37] with some modification, the leaves of cucumber plants (2nd to 5th node) were detached, and leaf disks of 1 cm in diameter were made with the help of a cork borer. We used 15 leaf disks for each *Bacillus* spp. such that 5 disks were in each of the 3 Petri dishes. The disks were soaked in LB broth with bacterial suspension. The OD_{600} was adjusted to 0.3–0.5 for 1 minute, shaking carefully. Next, 5 disks were relocated into each 6-cm diameter petri dish containing a 5.5 cm Whatman No.1 damp filter paper with the help of sterilized forceps. These disks were placed on the glass slide containing 1% agar media and covered with a 5.5 cm Whatman No.1 damp filter paper [38]. The leaf disks were placed such that the adaxial sides were up. The petri plates were incubated in the dark at a temperature of 30 °C for a period of 24 h. After complete incubation, 10 µL of the freshly prepared suspension of the pathogen's conidia (maintained to 1 X 10^5 CFU/mL, in sterilized ddH_2O) was kept at the center point of each leaf disk as the inoculum. The Petri dishes were then sealed with paper tape (breathable), and incubated under the same conditions as for the pathogens, in a growth chamber set at 24/18 °C for a day/night (16/8 h). A commercially available fungicide, Anvil (4.8% Hexaconazole w/v) was used as a positive control. Anvil acts effectively against powdery mildew fungi, so commonly used as a positive control. Sterile double-distilled water containing powdery mildew was used as a control. The experiment was carried out for five replications. The observation was performed on day 7, when initial symptoms and signs of infection disease occurred. Similarly, data was recorded on day 14, where the full extent of disease development and the long-term efficacy of treatments in controlling disease progression took place.

The occurrence of powdery mildew fungi was assessed visually on each of the leaf disks. The presence of the fungi was ranked as a percentage of the area showing infection with a rating (r) of 0, 1, 3, 5, 7, or 9. This denoted the percentage of the infection on the complete area of a leaf, which was categorized as 0%, <1%, 2–5%, 6–20%, 21–

40%, and >40%, respectively [39]. The disease index (DI %) was estimated with the equation given below:

$$\text{Disease index (\%)} = \frac{\Sigma(\text{number of diseased leaves at each disease scoring value x the corresponding disease scoring value})}{\text{Total number of investigated leaves x the highest disease scoring scale value}} \times 100$$

The preventive effect (PE) % was estimated as the disease index using the undermentioned equation:

$$\text{Preventive effect (\%)} = \frac{DC-DT}{DC} \times 100,$$

Where DT represented the disease index of the treatment, and DC was the disease index of the control.

Statistical analysis

The two-sample t-test was applied to compare the means of preventive effect (PE) % (measured on the 14th day) of each *Bacillus* species with that of Anvil, a positive control chemical fungicide. The null hypothesis set was the mean PE of Anvil – PE of *Bacillus* spp. = 0. The difference was considered significant if $p > 0.05$.

Results

Culture-based identification of *Bacillus* spp. in soil, cucumber leaves, and dead insect

In total, 195 bacterial isolates were recovered from soil, cucumber leaf, and dead insects. Out of 195, 65 bacterial isolates were identified as gram-positive, rod-shaped through Gram staining. The detailed identification of bacterial isolates from different samples was described in **Figure 1**. Further biochemical tests confirmed those bacteria as *Bacillus* spp.

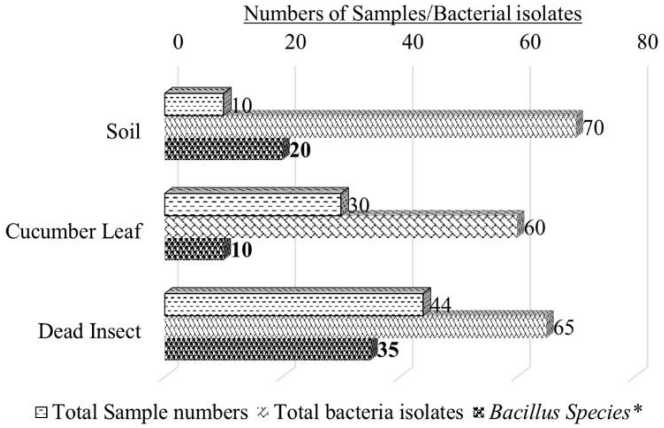

Figure 1. The horizontal bars showed the numbers of the different environmental samples, recovered bacterial isolates, and the identified *Bacillus* species. *The *Bacillus* species were identified based on cultural, morphological (Gram staining), and biochemical testing. The highest numbers of *Bacillus* species were obtained from dead insect samples.

Molecular identification of *Bacillus* spp. by 16S rRNA gene sequencing

The 16S rRNA gene sequence of two selected *Bacillus* spp. isolates - the NS114 and NS116 were confirmed as *Bacillus cereus*. The phylogenetic tree created with the neighboring joining analysis of the sequence is given in **Figure 2**.

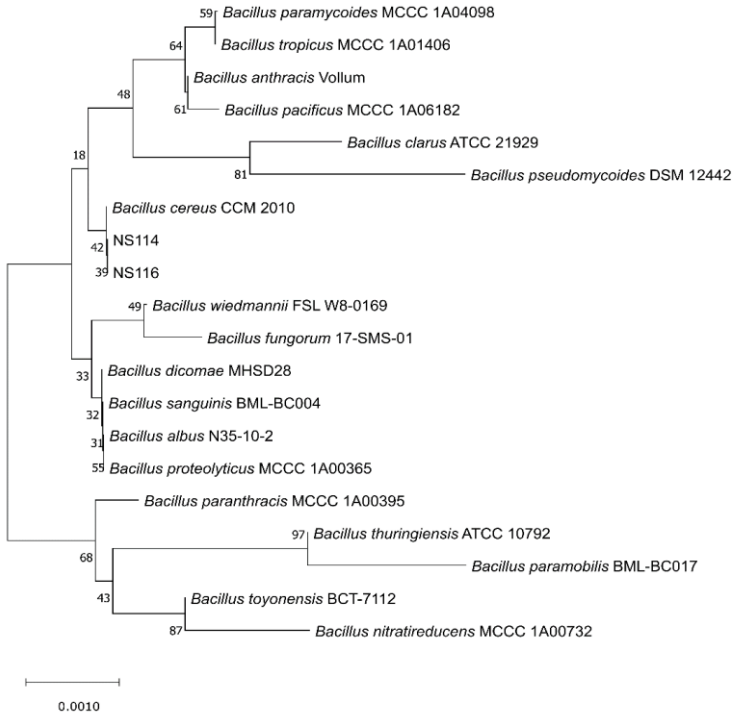

Figure 2. The phylogenetic tree of *Bacillus cereus* (NS114 & NS116) based on 16S rRNA gene sequencing. The neighbor-joining method was used to draw the phylogenetic tree using MEGA V.11. The clustering of related taxa together with the bootstrap test considering 1000 replicates are presented adjacent to the tree branches. The Maximum Composite Likelihood method estimated the evolutionary distances that represent the units of the frequencies of base substitutions per site.

Antifungal activity of *Bacillus* spp. against *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp.

Three fungi sub-cultured in the PDA were morphologically identified by observing the physical appearance of the plate and microscopically mycelia, conidia, and conidiophores. Those fungi cultures were confirmed as *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp.

In primary screening, a total of 65 *Bacillus* spp. was tested for antifungal activity qualitatively against *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp. using dual (co)culture bioassay **(Figure 3)**. The diameter of inhibition zones shown by the bacteria was measured for 7 days after the incubation period. Out of 65 *Bacillus* spp., 16/65 (24.62%) and 22/65 (33.85%) inhibited *Fusarium* spp., *Aspergillus* spp., where only 7 *Bacillus* spp. showed inhibition against both fungi.

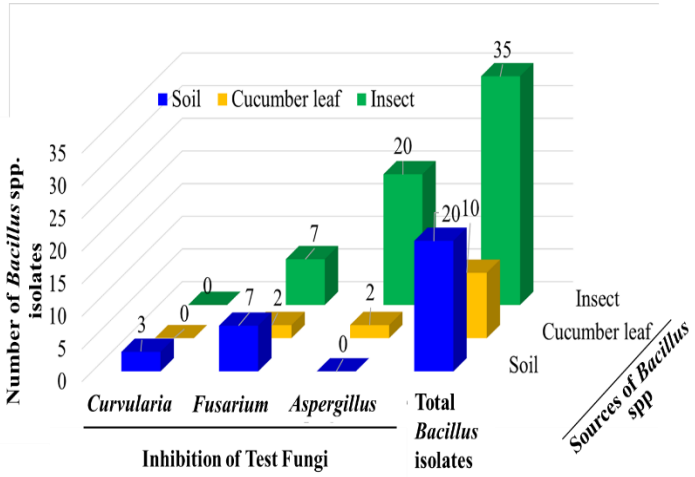

Figure 3. The vertical bars show the number of isolates of *Bacillus* spp. recovered from different sources (soil, cucumber leaf, and insects), and the numbers of those isolates of *Bacillus* spp. which showed antifungal activity against *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp. in dual (co)culture bioassay. The majority of *Bacillus* spp. (n=20) isolated from dead insects were antagonistic to *Aspergillus* spp., while those from soil samples (n=7) were active against *Fusarium* spp. Only a few (n=3) isolates of soil *Bacillus* spp. inhibited *Curvularia* spp.

Only 3/65 (4.62%) *Bacillus* spp. showed a good inhibitory effect against *Curvularia* spp. isolated from the soil samples (**Figure 4**). The results revealed that thirty-four isolates of *Bacillus* spp. demonstrated antifungal activity against three fungal strains as shown in the supplementary information (**Table 1**).

These most promising bacterial isolates were selected for antifungal activity against powdery mildew fungi.

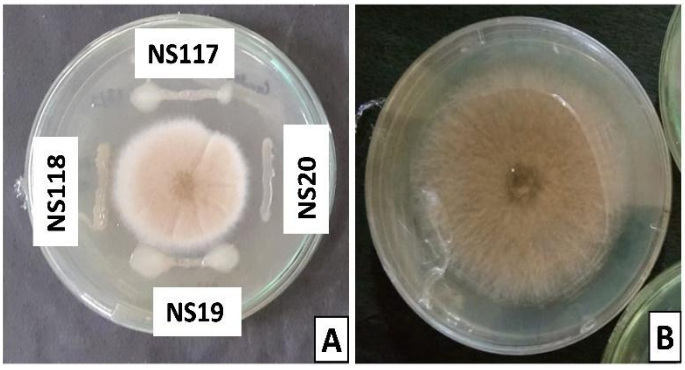

Figure 4: The photographs showing the qualitative assessment of antifungal activity of selected *Bacillus* spp. by dual (co)culture assay. (A) *Bacillus* spp. (NS117, NS118, and NS20) inhibited the growth of *Curvularia* spp., a pathogenic fungus (B) Control plate of *Curvularia* spp. without any treatment.

Bioassay of powdery mildew fungi

The cucumber leaves showing the powdery mildew infected were taken from a cucumber farm. The morphological characteristics were observed by light microscopy and identified as *Erysiphe cichoracearum* [35]**.** The morphology of the powdery mildew in cucumber contained ovoid conidia with fibrosin bodies. The conidial mean length ranges from 25.78 to 28.74 (µm), and the mean width ranges from 18.63 to 20.45 (µm) **(Figure 5**). Mycelium is hyaline septate **(Table 1**).

Table 1. Microscopic morphological characteristics of conidia and mycelium of three powdery mildew fungi collected from infected leaves of cucumbers.

Cucumber leaf Sample	Mean conidia length (µm)	Mean conidia width (µm)	Mycelium
15a	28.74	20.08	Hyaline septate
15b	27.55	20.45	Hyaline septate
17	25.78	18.63	Hyaline septate

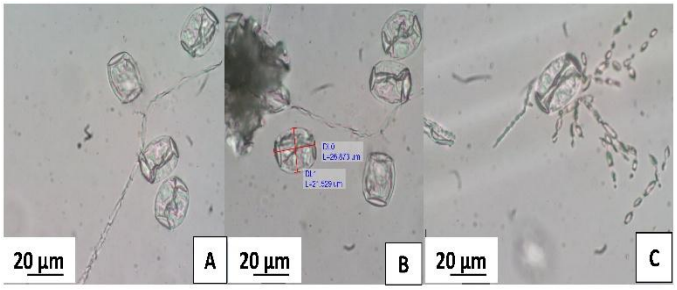

Figure 5. Photograph showing the morphology of conidia of *Erysiphe cichoracearum*, a powdery mildew fungus collected from a cucumber leaf. (A) Ovoid shape conidia, (B) length and breadth measurement of conidia, and (C) bursting of conidia releasing of spores.

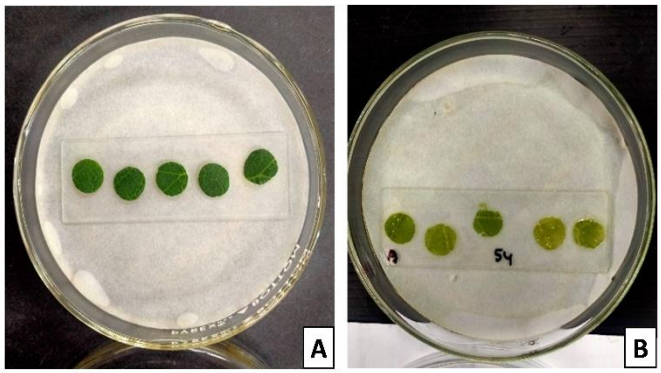

Figure 6. Photograph showing leaf-disk assay for *Bacillus* spp. against powdery mildew fungi. (A) On 1st day, fresh green colored 5 leaf disks were contaminated with powdery mildew fungi and inoculated with *Bacillus* spp. (B) After the incubation on the 14th day color of the leaf disks changed variably from green to brown color indicating the level of infection caused powdery mildew fungi. The color index was used to estimate disease index (DI %) and preventive effect (PE %).

Thirty-four *Bacillus* spp. showing noticeable antifungal activities were further subjected to the antagonistic activity against PMF. Since the PMF is an obligate parasite, the leaf disk bioassay was performed in the laboratory. The percentage of preventive effect (PE%) and disease index (DI%) were estimated for all tested *Bacillus* spp. (**Table 2**).

Table 2. Disease Index (DI) % and preventive effect (PE) % of the *Bacillus* spp. against *E. cichoracearum*, a powdery mildew fungus on 7 days and 14 days. The mean PE % on the 14th day of *Bacillus* spp. was compared with that of Anvil using a two-sample t-test and p>0.05 was considered a significant difference between treatments.

Sample	On 7 days		On 14 days		t-test (*p*-value)
	Mean DI %	Mean PE %	Mean DI %	Mean PE %	
3% Anvil Fungicide (Control)	30.67	60.36	18.22	67.81	
Bacillus spp. NS101	13.33	74.16	11.56	85.77*	0.031
Bacillus spp. NS102	28.89	61.38	5.78	90.05*	0.004
Bacillus spp. NS103	12.00	82.19	9.78	84.56*	0.011
Bacillus spp. NS104	21.34	71.69	12.00	78.44	0.092
Bacillus spp. NS108	19.11	64.65	22.67	71.18	0.558
Bacillus spp. NS110	10.67	78.11	18.67	73.99	0.376
Bacillus spp. NS111	25.78	63.84	6.67	87.4*	0.034
Bacillus spp. NS114	18.22	77.87	4.00	91.30*	0.008
Bacillus spp. NS115	13.78	73.61	14.67	79.92	0.116
Bacillus spp. NS116	16.45	69.44	4.44	93.17*	0.004
Bacillus spp. NS119	18.22	75.08	7.11	89.32*	0.030
Bacillus spp. NS120	18.22	64.29	12.89	83.57*	0.006
Bacillus spp. NS122	22.67	56.35	17.33	76.51	0.286
Bacillus spp. NS137	34.67	36.25	24.47	68.39	0.922
Bacillus spp. NS138	23.56	67.35	11.11	80.67*	0.034
Bacillus spp. NS139	31.56	45.83	36.44	49.66	0.339
Bacillus spp. NS140	34.22	38.36	28.44	60.39	0.339
Bacillus spp. NS141	17.33	76.45	11.11	82.49*	0.033
Bacillus spp. NS142	20.00	72.79	5.78	87.90*	0.025
Bacillus spp. NS143	29.78	60.02	19.56	63.01	0.683
Bacillus spp. NS144	32.00	55.75	24.89	57.42	0.156
Bacillus spp. NS145	19.11	75.39	11.56	82.08*	0.032
Bacillus spp. NS146	30.67	60.88	7.11	89.28*	0.033
Bacillus spp. NS147	32.44	58.46	15.56	75.65	0.375
Bacillus spp. NS148	28.44	60.02	8.00	87.95*	0.034
Bacillus spp. NS149	21.33	71.63	15.11	73.16	0.501
Bacillus spp. NS150	40.89	46.32	16.00	70.79	0.717
Bacillus spp. NS151	31.56	57.55	12.00	75.50	0.597
Bacillus spp. NS152	16.89	75.85	11.55	78.32	0.597
Bacillus spp. NS153	16.00	77.11	5.78	89.35*	0.031
Bacillus spp. NS154	35.11	53.85	13.33	74.81	0.369
Bacillus spp. NS158	24.45	66.60	12.00	80.04*	0.028
Bacillus spp. NS159	35.11	52.15	15.56	70.62	0.669
Bacillus spp. NS160	31.11	58.92	18.67	66.06	0.889

* The significant mean PE difference from that Anvil.

In total, 33/34 (97.06%) showed higher preventive effects on *E. cichoracearum* compared to the control Anvil fungicides. However, nearly half (16/34) isolates of *Bacillus* spp. demonstrated significantly high (t-test, p>0.05) preventive effect (PE %) compared to anvil (**Table 2**). Among them, three potential isolates (*Bacillus* spp. NS116, NS114, and NS102) showed high levels of disease control with Mean PE % values exceeding 90%. Based on 16S rRNA, NS116 and NS114 were confirmed as *B. cereus* with important antifungal activity against PMF. These treatments were promising as they significantly reduced disease severity, followed by three bacterial isolates (NS119, NS146, NS153), resulting in 89% PE reduction. Similarly, NS139, NS140, and NS144 showed lower levels

of disease control with Mean PE values below 70%. Most strains displayed a more preventive effect than the control fungicides applied to control PMF.

Discussion

In the present study, the antifungal activity of all the isolated *Bacillus* spp. was tested against *Fusarium* spp., *Aspergillus* spp., and *Curvularia* spp. Among the sixty-five *Bacillus* spp., it was found that thirty-four *Bacillus* spp. showed antagonistic activity against the three fungi. Our results corroborated with a study by Caulier et al. (2018) that reported high antagonistic activity exhibited by *Bacillus* spp. isolated from soil samples against potato fungal pathogens such as *Phytophthora infestans* (CRA-W10022), *Alternaria solani, Pectobacterium carotovorum* (ATCC 15713), *Fusarium solani* (BCCM-MUCL 5492), and *Rhizoctonia solani* (BCCM-MUCL 51929). The antagonism against *F. solani* is most likely due to competition for surface colonization. Bacilysin, synthesized by *Bacillus pumilus* and *Bacillus subtilis,* contributes antifungal activity through fungal mannoprotein inhibition and chitin biosynthesis [20]. Ezrari and colleagues reported that *Bacillus subtilis* K4-4 and GH3-8 can be promising bio-fungicides to inhibit the *Fusarium* species that cause citrus dry root rot disease because these *B. subtilis* strains produce bacillomycin, surfactin, iturin, fengycin, and subtilosin A responsible for antagonism [40].

Similarly, *B. amyloliquefaciens* that produce ituric lipopeptide, bacillomycin L, and bacillomycin D displayed significant antifungal activity for *R. solani* [41,42]. Iturin A, a lipopeptide produced by *Bacillus* BH072, exhibited high antifungal activity against a wide range of phytopathogens such as *Fusarium graminearum*, *F. oxysporum*, *Pythium irregulare, R. solani*, and *Botrytis cinerea* [43]. Lipopeptide extracts of *Bacillus* spp. showed powerful inhibitory action against *Fusarium solani*, *F. moniliforme, F. oxysporum*, and *Trichoderma atroviride* [44]. *Bacillus* species produced siderophores, which are high-affinity iron chelating compound. These molecules are capable of outcompeting pathogenic fungi for the scarce iron resources in the environment. By binding iron more effectively, siderophores deprive fungi of this essential nutrient, thereby inhibiting their growth and proliferation [45]. Iron-chelating siderophores are also responsible for showing inhibitory effects due to competition for shared iron uptake, limiting the iron availability for pathogens. *Bacillus subtilis* CAS15, a siderophore-producing strain consisting of bacillibactin, reduced the incidence of *Fusarium* wilt [46]. *B. subtilis* producing siderophore showed antagonistic activities against *Cephalosporium maydis,* causing late wilt disease in maize plants. The siderophore accumulates the Fe, suppressing the mycelial growth of *C. maydis* [47]. This may be the reason showing the fungicidal activity when we cocultured *Bacillus* species with the powdery mildew fungi.

Chitinase is an antifungal protein that acts as a biological control agent against phytopathogenic fungi, as this enzyme hydrolyzes chitin available in fungal cell walls, resulting in the inhibition of fungal growth. A rhizospheric bacterium, *Bacillus cereus* sensu lato B25, a rhizospheric bacterium, effectively antagonizes *Fusarium verticillioides,* as the bacterium produces two chitinases, ChiA and ChiB. ChiB exhibits dual substrate activity, functioning as both an exochitinase and endochitinase [48]. Specific strains of *B. subtilis* serve as biocontrol agents in managing the major fungal disease caused in cucumbers through antibiotic production [49]. *Bacillus cereus* produced antifungal activity, showing volatile organic compounds, and polyphenols that morphologically change the fungal conidiophores and conidiospores of *Aspergillus flavus* [50].

Powdery mildew fungi cause serious effects on cucurbits, leading the low production and a significant loss [8]. *Erysiphe cichoracearum* (*Syn. Golovinomyces cichoracearum*) and *Sphaerotheca fuliginea (Syn. Podosphaera xanthii)* are the two major agents that cause powdery mildew on cultivated cucurbits [35]. The morphological study of conidia and conidiophores resembles *E. cichoracearum,* which corroborates with the findings of previous studies [35,51]. In our study, 33 out of a total of 34 isolates displayed a higher preventive effect (>67.81%) compared to the positive control (3% Anvil). Our study revealed that the *B. cereus* identified from nature was highly effective against commercial fungicides, Anvil containing 4.8% hexaconazole, which showed only 67.81% preventive effect. It is reported that hexaconazole exhibited 78.99% in controlling disease caused by *Podosphaera pannosa* in rose powdery mildew [52]. 0.05% hexaconazole was the most effective inhibit spore germination of *Erysiphe pisi,* causing powdery mildew in pea compared with other chemical fungicides [53]. NS116, NS114, and NS102 showed a lower disease index and a higher preventive effect, indicating they can reduce the severity of the disease significantly. Samples NS139, NS140, and NS144 are the least effective, with high DI and low PE indicating they were less effective in reducing the burden of the fungal pathogens. Similar results were found by Sarhan et al. 2020 showing that the higher reduction rate ranging from 91.17% to 76.06% by *Bacillus subtilis, Serratia marcescens, Trichoderma harzianum, T.*

viride, and Paenibacillus polymyxa [54]. *Bacillus thuringiensis* and *B. subtilis* produced antifungal and antibacterial compounds having the ability to inhibit conidial germination of the *Podosphaera fusca*, a cucurbit powdery mildew. It is assumed that antibiosis serves as the primary mechanism for their biocontrol effects [55,56]. Similar lipopeptides bio-surfactants, such as fengycin and iturin, produced by *Bacillus amyloliquefaciens* and *B. subtilis* have strong antifungal properties [16,57]. The mechanism takes place as these lipopeptides are involved in pore formation in the plasmic membrane. Further lipopeptides are responsible for triggering plant defense mechanisms [17]. *B. amyloliquefaciens* reduces the spore germination of *Erysiphe cichoracearum* in tobacco powdery mildew due to the presence of fengycin and bacillomycin D [58]. Fungicides applied to control powdery mildew affect the health and environment, creating disease resistance. Studies on controlling powdery mildew fungi in agriculture often focus on the use of biological control agents. *B. subtilis* combined with the nano molecules treatment decreased disease symptoms and severity, and this combined treatment can be a promising alternative fungicide [59]. Such results correlate to previous studies that also reported the ability of the various biocontrol agents to suppress conidial spore germination [8,60,61]. Therefore, *Bacillus cereus* achieving over 90% disease control is noteworthy and surpasses commercial fungicides against powdery mildew fungi. As many fungal pathogens have emerged as resistant to many chemical fungicides, the application of the biocontrol approach can reduce the burden of fungicide resistance. Increased agricultural productivity can achieve food security by utilizing these natural biocontrol agents. Applying chemical fungicides hampers aquatic invertebrates and fishes, disturbing the ecosystems and disease resistance. However, biocontrol using native *Bacillus* strains is environmentally friendly, and organic farming enhances the value of agricultural products. It can protect the ecosystem minimizing the environmental risks associated with synthetic pesticides. This approach promotes a more resilient and sustainable agricultural system.

The outcomes of this study were obtained in controlled laboratory conditions, which may not be exactly replicated in field conditions. This is the caveat of our study. Although our findings demonstrate clear antagonism between *Bacillus* spp. and test fungal pathogens, future studies with field trials are necessary to validate the practical applications and effectiveness of the isolates in controlling fungal diseases in actual field conditions. Furthermore, identifying the key antifungal compounds and investigating the associated mechanism of antagonism will help to confirm the spectrum of activity.

Conclusion

Over half of the *Bacillus* species bacteria isolated from soil, dead insects, and cucumber leaves in Nepal demonstrated fungicidal potency against test phytopathogenic fungi *Fusarium* spp., *Aspergillus* spp., *Curvularia* spp., and a powdery mildew fungus. Two *Bacillus cereus* isolates exhibited higher preventive effects (>90%) against powdery mildew disease when compared to Anvil (commercial fungicides) and further field trials can confirm their potential application as an effective alternative to conventional chemical pesticides. Although further verifications are warranted to confirm the efficacy and safety of these *Bacillus* spp. under different environmental and climatic conditions, *B. cereus* formulation can be incorporated in integrated pest management practices in Nepal. Since *Bacillus cereus* isolates outperformed Anvil, a chemical fungicide, bacterial biofungicides not only reduce the fungal disease burden and increase agricultural production but also contribute to maintaining soil and aquatic ecosystem health.

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Author's Contribution

EM and DRJ conceptualized the study. EM carried out the laboratory experiments, analyzed the data, and drafted the original manuscript. DJ and MYW provided supervision, resources, and funding for the research. DRJ and RA supervised and oversaw the research, revised, and edited the manuscript.

Conflicts of Interest

There are no conflicts of interest to declare for this research study.

Data Availability

All the data used to support the findings presented in this article are included within the article itself.

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