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Research Article

Isolation and Preliminary Characterization of *Fusarium* sp. from Diseased Cardamom and Screening of Bacterial Antagonists

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ABSTRACT

Fusarium species are frequently associated with plant diseases affecting economically important crops, including cardamom (*Amomum subulatum* Roxb.). In this study, a fungal isolate was recovered from diseased cardamom stems collected in Panchthar district, Nepal, and characterized using morphological observations and ITS rDNA sequencing. The isolate was identified as *Fusarium* sp. isolate CS2 showing similarity to members of *Fusarium* species. A wheat seed assay demonstrated fungal colonization, but no visible disease symptoms were observed on wheat seedlings under the tested conditions, likely reflecting that wheat is not the natural host of the isolate. Three bacterial isolates were screened for antagonistic activity against the fungal isolate using dual culture assays. Among them, isolate BGNC-B10, tentatively identified as a putative *Exiguobacterium*-like bacterium based on partial 16S rRNA gene sequencing, showed moderate inhibition of fungal growth, reaching 35.97 ± 2.6% inhibition at Day 6. Inhibition declined to approximately 15% at later incubation times, and the treatment effect was not statistically significant ($p = 0.092$). The isolate also produced extracellular protease on skim milk agar. These findings indicate BGNC-B10 exhibits moderate *in vitro* antagonistic activity against *Fusarium* isolate associated with diseased cardamom. However, pathogenicity toward cardamom was not confirmed.

Keywords: Biological control; Cardamom disease; Exiguobacterium-like bacterium; *Fusarium* sp.; Protease activity

Introduction

Fungal phytopathogens are implicated as major causes of loss of yield in crops across the world, with *Fusarium* being the most destructive pathogen. These pathogens infect crops like cereals, vegetables, fruits, and spice crops. Some of these fungi produce mycotoxins that can pose a threat to food security and international trade (Djemouai et al., 2023). Large cardamom (*Amomum subulatum* Roxb.) is an important high-value spice crop, in Nepal. However, its productivity is threatened by leaf blight and decline diseases associated with members of the *Fusarium graminearum* species complex (FGSC) and several other diseases. Several fungal diseases, including *Fusarium*-associated blight, have been reported as major constraints to cardamom production in Nepal (Belbase et al., 2018). Generally, diseases in cardamom are managed primarily through the use of chemical fungicides. However, their excessive and repeated application has raised concerns regarding the development of resistant pathogen populations, environmental contamination and potential risks to human health, highlighting the need for more sustainable and environmentally friendly management as alternative strategies. Biological control using antagonistic microorganisms represents a sustainable and environmentally friendly strategy for managing diseases caused by *Fusarium* species, and several bacterial genera, including *Bacillus*, *Pseudomonas* and *Streptomyces*, have been extensively studied as potential biocontrol agents (Baffoni et al., 2015). Reported biocontrol mechanisms include antibiotic production, competition for nutrients and space, and the secretion of cell wall–degrading enzymes (Valiz et al., 2017). Despite its metabolic versatility and adaptability to diverse environments, the genus *Exiguobacterium* remains relatively underexplored. Genomic studies indicate that members of this genus can tolerate multiple environmental stresses and produce metabolites with ecological and pharmaceutical importance (Su et al., 2021). Recent studies have demonstrated the antifungal and plant growth–promoting potential of *Exiguobacterium*. For example, *E. acetylicum* SI17 reduced fruit rot severity in litchi (Huang et al., 2024), whereas glacier-derived *Exiguobacterium* sp. KRL4 produced antimicrobial secondary metabolites (Tedesco et al., 2021). Additionally, airborne bacterial communities containing *Exiguobacterium* have shown both biocontrol and plant growth–promoting activities (Guardado-Fierros et al., 2025). Together, these findings suggest that *Exiguobacterium* is a relatively underexplored genus with potential applications in biological control. This study was designed to address this knowledge gap. Specifically, we aimed to: (i) isolate and identify a *Fusarium* species associated with diseased cardamom stems in eastern Nepal; (ii) screen bacterial isolates for antagonistic activity *in vitro*; (iii) characterize the most active bacterial isolate, BGNC-B10, using partial 16S rRNA gene sequencing; and (iv) evaluate enzyme activity that may contribute to its antagonistic potential. To our knowledge, this study represents one of the first reports describing the isolation of a *Fusarium* species associated with diseased cardamom stems and the preliminary evaluation of bacterial antagonists from the cardamom ecosystem in Nepal.

Materials and Methods

Sample collection, pathogen isolation and identification

Cardamom (*Amomum subulatum* Roxb.) stems showing necrotic lesions were collected from plantations in Panchthar District, eastern Nepal (Figure 1). Five symptomatic plants were sampled. Tissue pieces from affected stems were surface-sterilized with 70% ethanol for 30 second, rinsed twice with sterile distilled water, and aseptically placed on potato dextrose agar (PDA; HiMedia, India) (Aneja, 2003). Plates were incubated at 25 ± 2 °C for 5–7 days. Emerging fungal colonies were purified using the hyphal-tip method. Several fungal colonies were obtained from infected tissues, and one representative isolate (CS2) with typical *Fusarium*-like morphology was selected for further characterization. Colony morphology, including pigmentation, texture, and growth rate, was recorded after 7 days of incubation on PDA. Microscopic features were examined using lactophenol cotton blue–stained slides under a compound microscope (40× magnification). Diagnostic characteristics such as hyphal septation, conidiophore structure, arrangement of phialides and conidial morphology were recorded. Identification was performed using standard taxonomic descriptions (Leslie & Summerell, 2006).

Figure 1: Cardamom (*Amomum subulatum* Roxb.), a large cardamom, showing leaf blight and decline symptoms. Field view of affected plants with brown, necrotic and drying leaves in contrast to adjacent healthy foliage (F), Collected samples from symptomatic plants used for laboratory isolation and further study (S).

For molecular identification, genomic DNA was extracted using the phenol–chloroform method (Sambrook & Russell, 2001). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using primers ITS1 and ITS4. PCR products were sequenced at the Nepal Academy of Science and Technology (NAST), Khumaltar, Nepal, and compared with sequences in the NCBI GenBank database using BLAST (Altschul et al., 1990).

Pathogenicity assay

Pathogenicity of the isolate was evaluated using a wheat seed assay. Surface-sterilized wheat seeds were placed on moist sterile tissue in sterilized containers and inoculated with a 5-days-old fungal culture broth containing mycelial fragments. Control seeds received sterile water. Containers were incubated at 25 ± 2 °C, and seed germination and symptom development were recorded over 10 days. In the wheat seed assay, fungal colonization was observed around inoculated seeds, but germination and seedling growth were similar to the control and no disease symptoms were detected after 10 days.

Screening and identification of bioactive biocontrol bacteria against the CS2 phytopathogen

A bacterial isolate, designated BGNC-B10 (NT), originally observed as a contaminant during fungal culturing, was isolated and purified. In addition, two bacillus-like isolates (GR and S3a) were recovered from rhizosphere soil of healthy cardamom plants. All three isolates were screened for antifungal activity against the CS2 phytopathogen using dual culture assays on PDA.

For molecular identification, genomic DNA was extracted from bacterial isolates using the phenol–chloroform method (Sambrook & Russell, 2001). The 16S rRNA gene was amplified by PCR with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). Amplified products were sequenced at the Nepal Academy of Science and Technology (NAST), Khumaltar, Nepal, and compared with sequences in the NCBI GenBank database using BLAST (Altschul et al., 1990).

Dual culture assay

The antagonistic activity of BGNC-B10 was tested using the dual culture method. A 5-mm mycelial disc of *Fusarium* sp. (CS2) was placed on two sides of a PDA plate 5 mm from the edge of a PDA plate, while BGNC-B10 was streaked at center. Control plates contained the fungal disc without bacterial inoculation. Plates were incubated at 28 °C, and radial growth of the fungal colony was measured at 6, 11, and 16 days after inoculation. Colony diameter was determined as the mean of two perpendicular measurements. The percentage inhibition of fungal growth was calculated using the formula described by Pandey et al. (1982). All assays were performed in triplicate:

$$\text{Inhibition (\%)} = ((C - T) / C) \times 100$$

Where C = colony radius in control, T = colony radius in treatment.

Enzyme activity assay

Extracellular protease activity of BGNC-B10 was tested on skim milk agar medium (Hankin & Anagnostakis, 1975). Fresh bacterial cultures were spot-inoculated onto skim milk agar plates (3% skim milk) and incubated at 30 ± 2 °C for 24–48 h. Protease production was indicated by the formation of clear zones around bacterial colonies due to casein hydrolysis. All assays were performed in triplicate. This activity is associated with antifungal mechanisms through degradation of structural proteins in fungal cell walls (Gupta et al., 2002).

Data analysis

All assays were performed in triplicate (n=3). Mean values of fungal growth diameters were used to calculate inhibition percentages. Data were analyzed by two-way ANOVA (additive model) to test the effects of treatment and incubation time. Statistical analyses were carried out in IBM SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Differences were considered significant at $p < 0.05$.

Results and Discussion

Pathogen identification and pathogenicity assessment

Molecular analysis of the fungal isolate recovered from symptomatic cardamom stems showed 96% ITS sequence similarity to *Fusarium graminearum* in BLAST searches. Microscopic examination revealed hyaline, septate, branched hyphae and abundant canoe-shaped macroconidia with three to five septa formed in sporodochial clusters (Figure 2). These morphological characteristics are consistent with the genus *Fusarium*. However, ITS similarity below ~98–99% is generally insufficient for reliable species-level identification within the genus. Therefore, the isolate is conservatively designated here as *Fusarium* sp. isolate CS2, and additional multilocus sequencing (e.g., TEF1-α, RPB1, or RPB2) would be required for definitive species identification.

A wheat seed plate assay was conducted to evaluate the pathogenic potential of the isolate. In the inoculated treatment, visible cottony mycelial growth developed around the seeds, indicating fungal colonization (Figure 3). However, germination and early seedling growth were comparable to the water-treated control, and no visible disease symptoms were observed on germinated seedlings even after 10 days of incubation. These results indicate that although the isolate was able to colonize the seed surface, pathogenicity on wheat seedlings was not confirmed under the assay conditions.

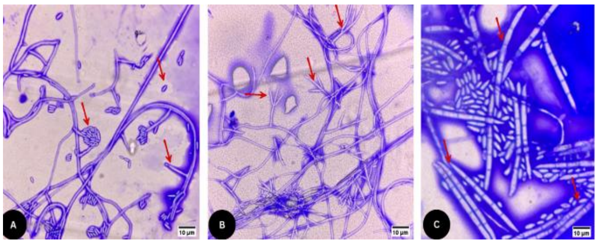

Figure 2: Microscopic characteristics of the *Fusarium* isolate recovered from infected cardamom stem. (A–B) Septate hyphae with branched conidiophores producing phialides. (C) Canoe-shaped, multi-septate macroconidia formed in sporodochial clusters. Stained with lactophenol cotton blue, observed under light microscope at 40× magnification.

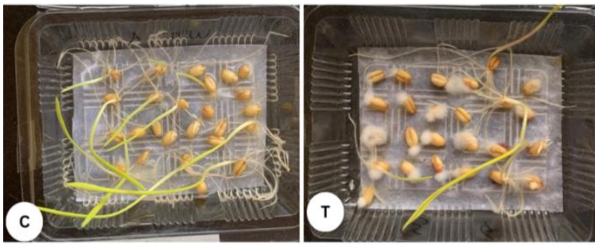

Figure 3: Wheat seed assay showing surface colonization by the Fusarium isolate at day 10. Control seeds treated with sterile water (C), Seeds inoculated with the *Fusarium* isolate (T).

Screening and genetic identification of bioactive biocontrol bacteria against the CS2 phytopathogen

Three bacterial isolates were evaluated for antagonistic activity against CS2, BGNC-B10 (a contaminant isolate) and two bacillus-like isolates (S3a and GR) obtained from rhizosphere soil of healthy cardamom plants. Dual culture assays showed that BGNC-B10 and S3a inhibited fungal growth, whereas GR did not (Figure 4). Both BGNC-B10 and S3a were subjected to molecular characterization. The 16S rRNA gene of BGNC-B10 was amplified successfully (Figure 5) and sequenced in forward and reverse directions. BLAST analysis of the forward sequence showed 95.05% similarity with *Exiguobacterium acetylicum* (38% query coverage), while the reverse sequence showed 92.66% similarity (59% query coverage). Because the sequence similarity (95.05%) and query coverage were relatively low, the isolate is conservatively referred to as a putative *Exiguobacterium*-like bacterium (BGNC-B10) pending further sequencing and taxonomic confirmation. Sequencing of isolate S3a did not yield usable data, and this isolate was excluded from further study. Additional sequencing of both isolates is in progress.

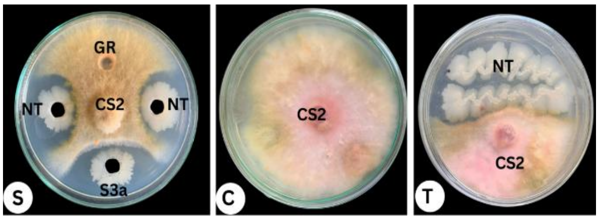

Figure 4: Screening of antifungal activity of bacterial isolates (NT, GR, and S3a) against the phytopathogen CS2. Screening plates showing NT, GR, and S3a (S), Control (CS2 alone) (C); Treated (CS2 challenged with BGNC-B10 (NT) (T).

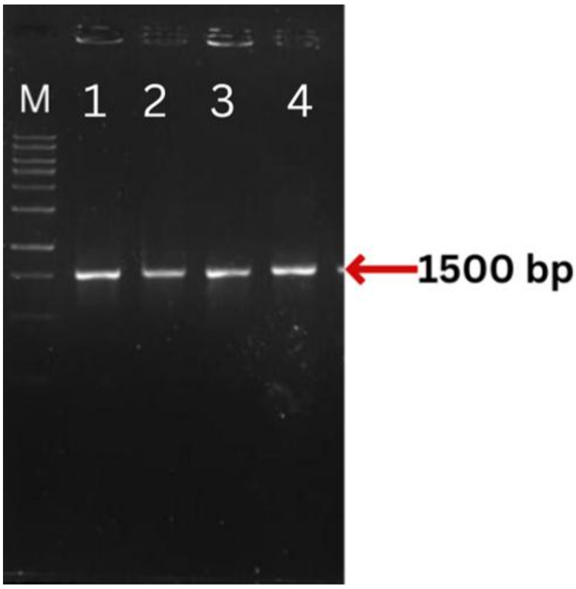

Figure 5: Agarose gel electrophoresis showing PCR amplification of the 16S rRNA gene (~1.5 kb) from bacterial isolates. DNA molecular weight marker (M), BGNC-B10 (lane 1 and 2) and S3a (lane 3 and 4).

Effect of bacterial isolates on the growth of CS2 phytopathogen

Dual culture assays showed partial suppression of fungal growth in the presence of BGNC-B10 compared with the control. Maximum inhibition occurred at Day 6 (35.97 ± 2.6%), followed by a decline to approximately 15% at later time points. Statistical analysis indicated that incubation time had a significant effect on fungal growth, whereas the treatment effect itself was not statistically significant ($p = 0.092$) (Figure 6). At Day-6, average inhibition was 35.97 ± 2.6%. Inhibition decreased to 15.95 ± 7.2% at Day-11 and 15.47 ± 4.5% at Day-16 (Table 1). Statistical analysis using two-way ANOVA (additive model) indicated a significant effect of incubation time ($F = 46.60$, $p = 0.0017$), whereas the treatment effect was not significant ($F = 4.45$, $p = 0.092$).

Table 1: Inhibition of fungal growth by BGNC-B10 in dual culture assays (mean ± standard deviation, n = 3).

Time after inoculation (days)	Inhibition (%) ± SD
6	35.97±2.6
11	15.95±7.2
16	15.47±4.5

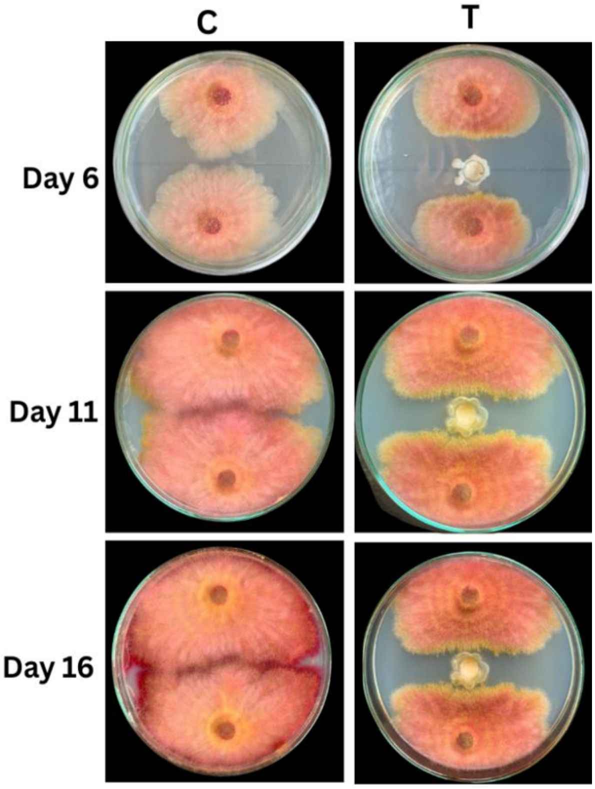

Figure 6: Dual culture assay between the phytopathogen isolate CS2 and the biocontrol agent BGNC-B10. Fungal growth was recorded at 6, 11, and 16 days after inoculation. Control (pathogen alone) (C) and Treated (pathogen with BGNC-B10) (T).

Protease activity of *Exiguobacterium* sp. BGNC-B10

Protease activity of BGNC-B10 was confirmed on skim milk agar. Clear zones were observed around bacterial colonies within 24 to 48 h of incubation, indicating casein hydrolysis (Figure 7).

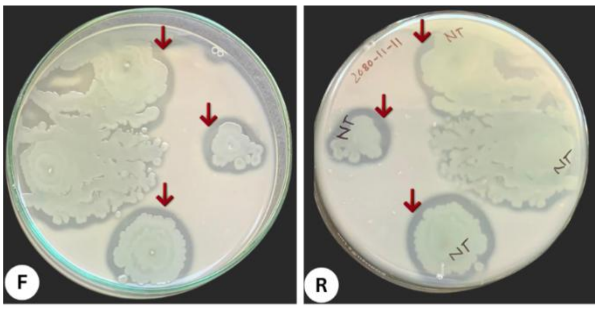

Figure 7: Protease activity of the putative *Exiguobacterium* sp. isolate BGNC-B10 (NT) on skim milk agar. F, front view; R, reverse view of the culture plate. Clear zones surrounding colonies (red arrows) indicate casein hydrolysis and extracellular protease production. NT, isolate BGNC-B10 used in antagonism assays.

The fungal isolate recovered from diseased cardamom stems was identified as *Fusarium* sp. isolate CS2 based on morphological characteristics and ITS rDNA sequencing. Although BLAST analysis showed similarity to members of the *Fusarium graminearum* species complex, the relatively low ITS similarity prevented reliable species-level identification. Furthermore, pathogenicity toward cardamom was not confirmed in this study. A wheat seed assay demonstrated fungal colonization but did not produce disease symptoms. Because wheat is not the natural host of cardamom-associated *Fusarium* isolates, this assay cannot establish pathogenicity according to Koch's postulates. Therefore, the causal relationship between this isolate and cardamom disease remains to be confirmed through inoculation experiments on cardamom plants under controlled conditions.

Dual culture assays indicated that *Exiguobacterium* sp. BGNC-B10 moderately inhibited fungal growth during early incubation. Although BGNC-B10 produced extracellular protease, fungal cell walls primarily consist of chitin and glucans rather than proteins. Therefore, protease activity alone cannot fully explain the observed antagonism. It is possible that additional mechanisms such as competition for nutrients, production of diffusible metabolites, or volatile compounds may contribute to fungal suppression. Similar antifungal activity has been reported for other *Exiguobacterium* isolates (Huang et al., 2024; Tedesco et al., 2021; Guardado-Fierros et al., 2025). Because all experiments were conducted *in vitro,* greenhouse or field studies are needed to evaluate its potential role in disease management. Future studies should investigate additional mechanisms of antagonism including cell-free culture filtrate assays, volatile inhibition assays, and metabolite profiling using LC–MS. Such analyses would provide a more comprehensive understanding of the antifungal activity of BGNC-B10.

Conclusion

A *Fusarium* isolate (CS2) was recovered from diseased cardamom stems in eastern Nepal and identified as Fusarium sp. based on morphological characteristics and ITS rDNA sequencing. A wheat seed assay showed fungal colonization but no disease symptoms under the tested conditions. Pathogenicity toward cardamom was not tested in this study and remains to be confirmed. In dual culture assays, the bacterial isolate BGNC-B10, tentatively identified as *Exiguobacterium* sp., showed moderate inhibition of fungal growth during early incubation. The detection of extracellular protease suggests that enzymatic activity may contribute to the observed antagonism. However, the absence of chitinase and cellulase activity indicates that the antagonistic mechanism may be limited. These results suggest that *Exiguobacterium* sp. BGNC-B10 may have potential as a bacterial antagonist against *Fusarium*-associated fungi under laboratory conditions. Because the present study was conducted in vitro, further work including detailed molecular identification, metabolite analysis, and greenhouse or field experiments is required to evaluate its possible role in disease management of cardamom.

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