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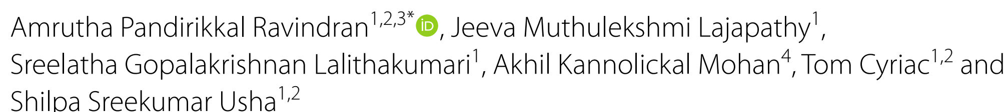
Efficacy of *Bacillus licheniformis*: a biocontrol agent against *Colletotrichum gloeosporioides* Penz. (Penz. & Sacc.) causing anthracnose in greater yam (*Dioscorea alata* L.)



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Abstract

Background Anthracnose, caused by *Colletotrichum gloeosporioides,* is the most dangerous fungal disease of greater yam (*Dioscorea alata* L.), and leads to significant crop loss. Microbial technology in cropping systems is currently focused on biological control agents (BCAs), which include plant endophytes like bacteria, which contribute well to sustainable production. In the present study, the ability of an endophytic bacterium, *Bacillus licheniformis* (CTCRI EB12), isolated from *Aloe vera* was investigated to prevent the disease.

Results The bacterium, CTCRI EB12, was characterized as a member of the *Bacillus* genus by morphological and biochemical tests and confirmed as *B. licheniformis* via 16S ribosomal gene analysis. The effectiveness of the endophytic bacterium was affirmed through cell confrontation assays and scanning electron microscopy. In cell confrontation assay, the isolate inhibited all the three *C. gloeosporioides* isolates used in the study with more than 80% inhibition. The effect of culture filtrate of the isolate was also tested against pathogens that were compliant with the outcome of dual culture technique. The culture and culture filtrate could effectively restrain the spreading of *C. gloeosporioides* in greater yam leaf. The isolate could completely inhibit the growth of the pathogen through an antibiosis study, and the same could be explained through scanning electron microscopy results, in which the endophyte-treated *C. gloeosporioides* hyphae appeared longer and distorted compared to control and conidia possess disfigurement in endophyte-treated sample when compared to control.

Conclusions The study showed that *Bacillus licheniformis* (CTCRI EB12), the endophytic bacterium that has the capacity to enhance the protection of greater yam plants, could be further developed as a microbial pesticide to reduce the effect of anthracnose in greater yam fields. Additional investigation will reveal the endophytic isolate's antifungal capabilities and assessment of their performance in the open field. The present study depicted that it might be a potent biological control agent against *C. gloeosporioides* causing greater yam anthracnose.

Keywords *Colletotrichum gloeosporioides*, Endophytic bacteria, *Bacillus licheniformis*, Antibiosis, Biocontrol, SEM analysis

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Background

Countless infections and pests in fields and storage are hazardous to greater yam production and commercialization. The fungus, *Colletotrichum gloeosporioides*, is the most significant field pathogen which causes foliar anthracnose and poses a serious danger to greater yam farming (Abang et al. 2003). *C. gloeosporioides* was prominent in the tropical and subtropical regions of the world, viz. Sri Lanka, India, China, Brazil, Colombia, Ecuador, Peru, Malaysia, Pakistan, Portugal, Israel, and Turkey (Zakaria 2021). *C. gloeosporioides* causes anthracnose to greater yam plants and is accountable for more than 50% of losses of fresh fruits and vegetables. Other than greater yam, *C. gloeosporioides* causes substantial damage in other significant tropical fruit crops, papaya, mango, and avocado crops.

The application of chemical fungicides reduces fungal infection but comes with significant environmental and public health hazards. As part of integrated disease control programmes, extensive research is being done with a wide range of microbial antagonists against various plant diseases. It was believed that the use of biocontrol agents would be help control the anthracnose disease. Members of the *Bacillus* genus are frequently called as 'microbial factories' because they produce a wide variety of biologically active chemicals that may hinder the growth of phytopathogens. From a technological standpoint, these bacteria are among the finest prospects for creating effective biopesticides due to their capacity to generate endospores (Miljaković et al. 2020). These microorganisms not only protect plants, but also promote growth by producing phytohormones, releasing nutrients from the soil, fixing nitrogen, and decreasing plant stress under various environmental extremes (Ribeiro et al. 2021). *Bacillus methylotrophicus* KE2 produces gibberellins, promotes plant development, and improves the nutritional metabolites and food value of lettuce (Radhakrishnan et al. 2016). Chung et al., (2015) reported that two *Bacillus* species, C7007 and YC7010T, can control rice diseases caused by *Xanthomonas oryzae* pv. *oryzae* and *Burkholderia glumae*. Likewise, *Bacillus licheniformis* (BF3-5 and BF6-6) and *B. pumilus* (BF6-1) strains showed significant antimycotic activity against *Phytophthora capsici*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Pythium ultimum* (Asraful Islam et al. 2010).

In the present study, a potential endophyte, CTCRI EB12, selected against *C. gloeosporioides* causing anthracnose in greater yam through initial screening was tested through cell confrontation assays and SEM analysis, to support the biocontrol efficiency.

Methods

Endophytic bacterial isolate and growth conditions

The endophyte CTCRI EB12 was isolated (Anjum and Chandra 2015) from *Aloe vera* (Indian aloe) leaves from the farm of ICAR-CTCRI. It was cultured in nutrient agar (HIMEDIA) and luria broth (HIMEDIA) in a shaker incubator (JEIO TECH, Korea) at 120 rpm at 35 °C. CTCRI EB12 was stored in 30% glycerol at − 80 °C for long-term sustenance.

Pathogen isolation and culture conditions

The pathogen was isolated from symptomatic leaves of the greater yam, which exhibited necrotic spots, or lesions. Leaves were washed under running tap water to remove any debris or surface contaminants. Infected regions of 5 mm^2 in size, along with a margin of healthy tissue, were excised using a sterile scalpel. The plant tissues were surface sterilized by immersion in a 2.5% sodium hypochlorite (Merck) solution for 2 min, followed by immersion in a 70% ethanol (HIMEDIA) solution for 1 min. The tissues were then rinsed three times with sterile distilled water to remove any traces of the sterilants (Anjum and Chandra 2015). The surface-sterilized tissues were then transferred to sterile Petri dishes containing potato dextrose agar (PDA(HIMEDIA)) containing 10 µl ampicillin (100 mg ml^{-1}, A0166: Sigma-Aldrich) and incubated for 7 days for growth, at 25 ± 2 °C in BOD incubator (Analab Instrument, India) after removing water using sterile filter paper. After 7 days of incubation, a loopful of fungal culture was transferred to a glass slide and examined under a light microscope at 10× magnification (Nikon Eclipse 100 Binocular vision) for the presence of conidia. For morphological study, mycelial and conidial mass colour as well as elevation of the mycelial mat was also observed. Once confirmed, the cultures were subcultured in PDA slants (HIMEDIA) and maintained at 4 °C.

Genomic DNA extraction

The isolates were cultured in potato dextrose broth (PDB) for 3 days at 26 °C, and the mycelia were harvested. The genomic DNA was extracted from the harvested mycelia using the cetyl trimethyl ammonium bromide (CTAB) method as described by Tripathy et al. (2017). DNA purity was assessed by Nano Drop spectrophotometer (DeNovix DS-11) and agarose gel electrophoresis (Bio Rad), then stored at − 20 °C.

PCR amplification

The primer pairs, CgsF1 (GGCGGGTAGGGTCTC CGTGAC) and CgsR1 (TTTGAGGGC CTACAT

CAGC) (Raj et al. 2013), were used in PCR to confirm the pathogen as *C. gloeosporioides*. PCR assays were performed in an automated thermal cycler (Agilent Tech.) using 2 µl of total DNA, 2.5 µl of 10 × KAPA Taq buffer (with 25 mM MgCl2), 0.5 µl of dNTP mixture (10 mM), 1 µl of each primer (10 µM), 0.2 µl of KAPA Taq DNA polymerase (5 U/µl), and 18.8 µl of PCR-grade water in a total volume of 25 µl. The amplification programme consisted of a 2 min initial denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 62 °C, and 40 s at 72 °C, with a final extension at 72 °C for 5 min. Amplified products were resolved on a 1.5% agarose (HIMEDIA) gel stained with 0.5 µg/ml ethidium bromide (Merck) and visualized using a Gel Doc System (Alpha Innotech Corporation, San Leandro, CA).

Identification of the endophytic bacteria

The endophytic isolate, CTCRI EB12, was initially identified through its morphology and with the assistance of biochemical characterization (Mintoo et al. 2019). The genomic DNA of the bacterium was extracted using the CTAB method. The 16S ribosomal gene was amplified (Sure cycler, Agilent Technologies) for phylogenetic analysis using primers, 8F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492R (5′ CGG CTA CCT TGT TAC GAC TT 3′). PCR assays were performed using 2 µl of total DNA (50 ng), 2.5 µl of 10X KAPA Taq buffer (with 25 mM $MgCl_2$), and 0.5 µl of dNTP mixture (10 mM), 1 µl of each primer (10 µM), 0.2 µl of KAPA Taq DNA polymerase (5U/µl) along with 18.8 µl PCR-grade water in a total volume of 25 µl. Amplified products were resolved on a 1.5% agarose gel. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 11 (Tamura et al. 2021) after sequencing.

Antifungal activity of CTCRI EB12 culture against *C. gloeosporioides* via cell confrontation study

To check the antagonistic activity of the isolate, the bacterium was screened against three virulent pathogenic isolates by dual culture technique (Chérif 1990; Salem Elkahoui 2012). In the middle of the Petri dishes, *C. gloeosporioides* agar disc (5 mm) isolated from actively growing cultures was placed, and the bacterial endophyte was streaked on either side of the disc at a distance of 2 cm. The inhibition of fungal growth compared to a cultured pathogen alone served as a measure of the antagonistic activity of the investigated bacterium. Dishes were incubated at 28 ± 2 °C for 7 days, and the fungal growth was observed. The experiment was run twice, with ten replications. The growth of the pathogen towards the endophyte and inhibition zone was measured until control dishes attained complete growth. The growth inhibition of the pathogen was calculated by using the following formulae of Bae et al. (2011).

Percentage inhibition (%) $PI = [(dc - dt)/dc] \times 100$, where dc and dt represent the fungal growth diameter in the control and treated sample, respectively.

Antifungal activity of culture filtrate of bacterium CTCRI EB12 against *C. gloeosporioides*

Endophyte was cultured in nutrient broth at 35 °C for 48 h, and the culture was filtered three times through a mixed cellulose esters membrane syringe filter (0.22 µm pore size) after centrifugation at 8500 × g for 10 min to obtain the culture filtrate (Tian et al. 2021). To determine the antagonistic activities of the culture filtrate, the pathogen was spot inoculated at one end of the Petri dish. To the 8 mm well-made opposite to the point of inoculation of the pathogen, 50 µl culture filtrate was added. As a control group, fungi competed with sterile nutrient broth in well. The tests were run with ten replicates. The growth inhibition of the pathogen was calculated by using the formulae of Bae et al. (2011) shown in abovementioned section.

Antibiosis

For the antibiosis study, the endophytic isolate was spread in Petri dishes amended with PDA (HIMEDIA) and NA (HIMEDIA) in equal proportion and a 5-mm disc from the periphery of actively growing *C. gloeosporioides* culture was inoculated at the centre of the same dish. The control dish was inoculated with *C. gloeosporioides* and asserted without an antagonist. Treatments were kept with ten replications and incubated at 28 ± 2 °C. The growth of the pathogen was observed. The inhibition of the pathogen was calculated by using the formula mentioned by Bae et al. (2011). For microscopic analysis, a small portion immediately around mycelia adsorbed on cello tape was kept on a glass slide provided with a drop of lactophenol cotton blue and observed under a microscope at 10 × magnification (Nikon Eclipse 100 Binocular vision).

Antifungal activity of isolate CTCRI EB12 and culture filtrate against *C. gloeosporioides* on greater yam leaf via detached leaf assay

Detached leaf assay (Pettitt et al. 2011) was carried out using the leaf of susceptible greater yam variety (cv. Orissa Elite) to determine whether endophytic bacterium has a good application potential for the prevention and management of anthracnose. Fresh and healthy greater yam leaves were collected from greater yam plants (until 4–15 leaves were developed) that were maintained in the glass house. The collected leaf samples were washed with tap water, rinsed in 2% sodium hypochlorite for 2 min,

followed by sterile distilled water wash thrice and then air-dried (Tian et al. 2021). The experiment was done as given sets. Set 1: each leaf was challenge inoculated with a 5-mm disc of actively growing pathogen (control). Set 2: each leaf was challenge inoculated with the pathogen and treated with endophytic isolate (1×10^8 CFU ml^{-1}). Set 3: each leaf was challenge inoculated with pathogen as well as treated with the endophytic culture filtrate 10 µl. The experiment was carried out in 200 mm glass Petri dishes at 28 °C and 90% relative humidity and observed daily for lesion development. Assays were performed three times.

Scanning electron microscopy examination (SEM)

SEM was used to discern likely disfigurement in the morphology of *C. gloeosporioides* (most virulent isolate) after treatment with the antagonist. Tissue samples obtained from different greater yam leaves (same as above set 1 and set 2) were collected with a scalpel after incubation for 3 d at 28 °C. The samples were unfixed (Venkatesh Babu et al. 2018). The same was done in sample obtained from Petri dishes of antibiosis study of *C. gloeosporioides* and CTCRI EB12 isolate. Samples were carefully blotted using sterile Whatman No.1 filter paper of 8 mm size, which was transferred into stub and then directly sputter coated in gold using a sputter coater (Quorum SC7620) at 10 mA for 120Sec. The samples were then photographed using a scanning electron microscope (SEM, Carl Zeiss EVO 18 Research).

Statistical analysis

The data were statistically analysed using GraphPad Prism v8.0.2. (GraphPad Software Inc, San Diego, CA, USA). Data were subjected to one-way ANOVA and Tukey's honest significant difference (HSD) tests were carried out. Differences were considered significant if p value ≤ 0.05.

Results

Pathogen isolation and identification

Pathogen was isolated from infected greater yam leaves and cultured on potato dextrose agar successfully. After two days of incubation at 25 ± 2 °C, mycelial growth was observed. The isolate was identified based on its morphological features, including its colony morphology and the microscopic structure of its conidia. The mycelial colour of isolates found to be grey to white. Isolates were named as Cg23, CgS4.7, and Cg12. One of the isolates, Cg23, exhibited elevated mycelial morphology, whereas the other two isolates, Cg S4.7 and Cg12, had flat elevation. The colour of the conidial mass appeared bright orange in the Cg23 isolate compared to the other two, which appeared light orange. Conidia were observed at 10 × magnification under a light microscope (Fig. 1). Conidia appeared one celled and cylindrical with obtuse ends. The morphology and cultural characteristics of the pathogen isolates are given in Table 1.

Molecular confirmation of pathogen

DNA from the pathogen was amplified using PCR with species-specific primers, CgsF1 (GGCGGGTAGGGT CTCCGTGAC) and CgsR1 (TTTGAGGGC CTACAT CAGC). Amplicons of ~ 300 bp were visualized on an agarose gel, confirming the identity of the isolate as *C. gloeosporioides* (Fig. 2).

Table 1 Cultural and morphological characteristics of *Colletotrichum gloeosporioides* used in the study

Sl no	Isolate ID	Mycelial colour	Elevation	Conidial mass colour
1	Cg23	White	Raised	Bright orange
2	CgS4.7	Grey	Flat	Light orange
3	Cg12	Greyish white	Flat	Light orange

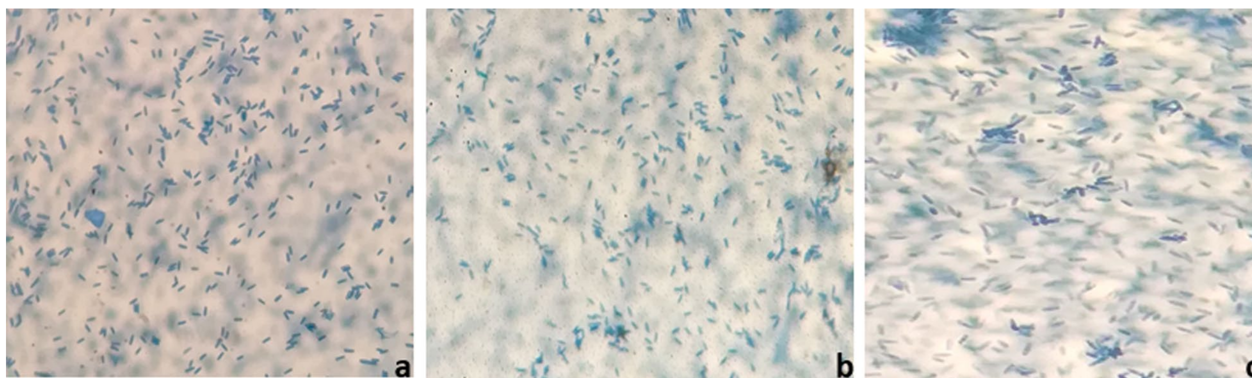


Fig. 1 Light microscope image showing conidia of *Colletotrichum gloeosporioides* under 10 × magnification **a** Cg23; **b** CgS4.7 and **c** Cg12

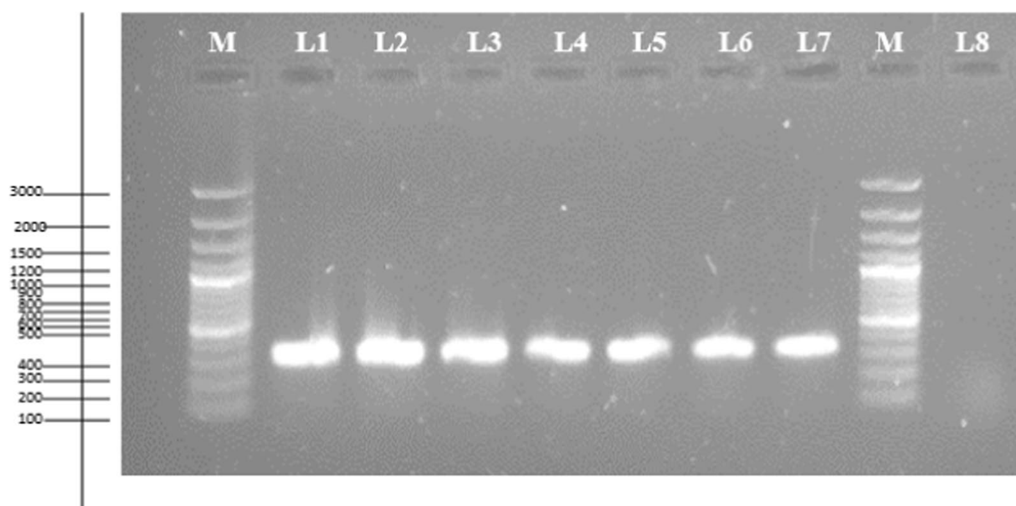


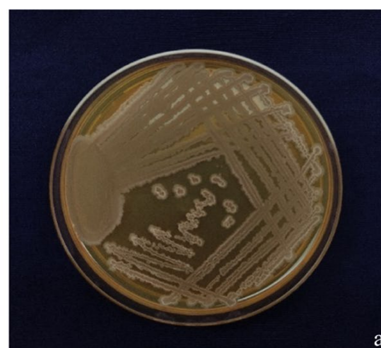
Fig. 2 PCR amplification of *Colletotrichum gloeosporioides* using species-specific primer. *Note*: M: Marker; L1and L2: Cg23; L3 and L4: CgS47; L5 and L6: Cg12; L7: Positive control and L8: Negative control

Identification of CTCRI EB12 culture

Endophyte was maintained in NA 25 °C. The morphological study showed that the bacterium belongs to the genus *Bacillus*. Colony appeared nearly white colour and round in shape with a wrinkled edge of performance on NA (Fig. 3a). It was further confirmed as genus *Bacillus* through biochemical tests. Thus, CTCRI EB12, the bacterium was perceived as *Bacillus* sp. Partial DNA sequencing of the 16S rRNA gene confirmed the isolate as *Bacillus licheniformis*. The 16S ribosomal gene partial sequence was submitted to the GenBank database (www.ncbi.nlm.nih.gov/GenBank) with accession number MK734049. Phylogenetic analysis by MEGA showed that the tested endophyte shared a close relationship with other *B. licheniformis* isolates (Fig. 3b).

Cell–cell confrontation of endophytic bacteria versus *C. gloeosporioides* isolates

The antagonistic effect of endophytic isolate on the three *C. gloeosporioides* isolates was evaluated. The development of pathogens was severely restricted by the bacterial isolate. Compared to the control dish, the endophyte suppressed fungal growth by 70–80% after 7 days of incubation. The highest percentage of inhibition was in Cg23 (84.15 ± 1.8%), followed by CgS4.7 (82.65 ± 1.43%). Compared to the fungus in the control set, the endophyte significantly reduced the density of the hyphal network and caused widespread hyphal thinning (Figs. 4 a, b, c and 5).



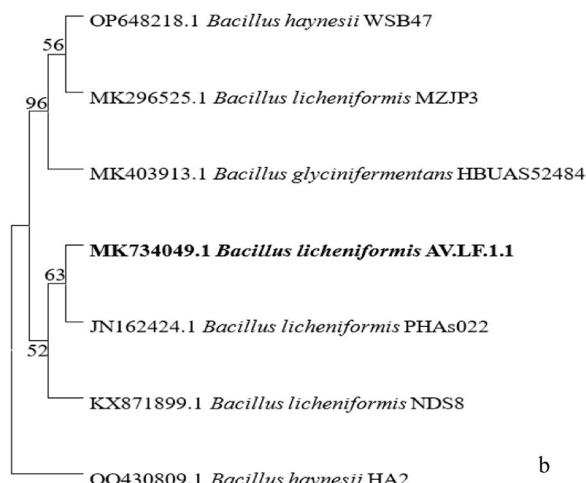


Fig. 3 **a** Pure culture of CTCRI EB12 in NA (2 days old). **b** Neighbour-joining 16SrRNA phylogenetic tree of endophytic bacterium CTCRI EB12 *Bacillus licheniformis*, numbers at nodes indicate bootstrap values (1000 replicates)

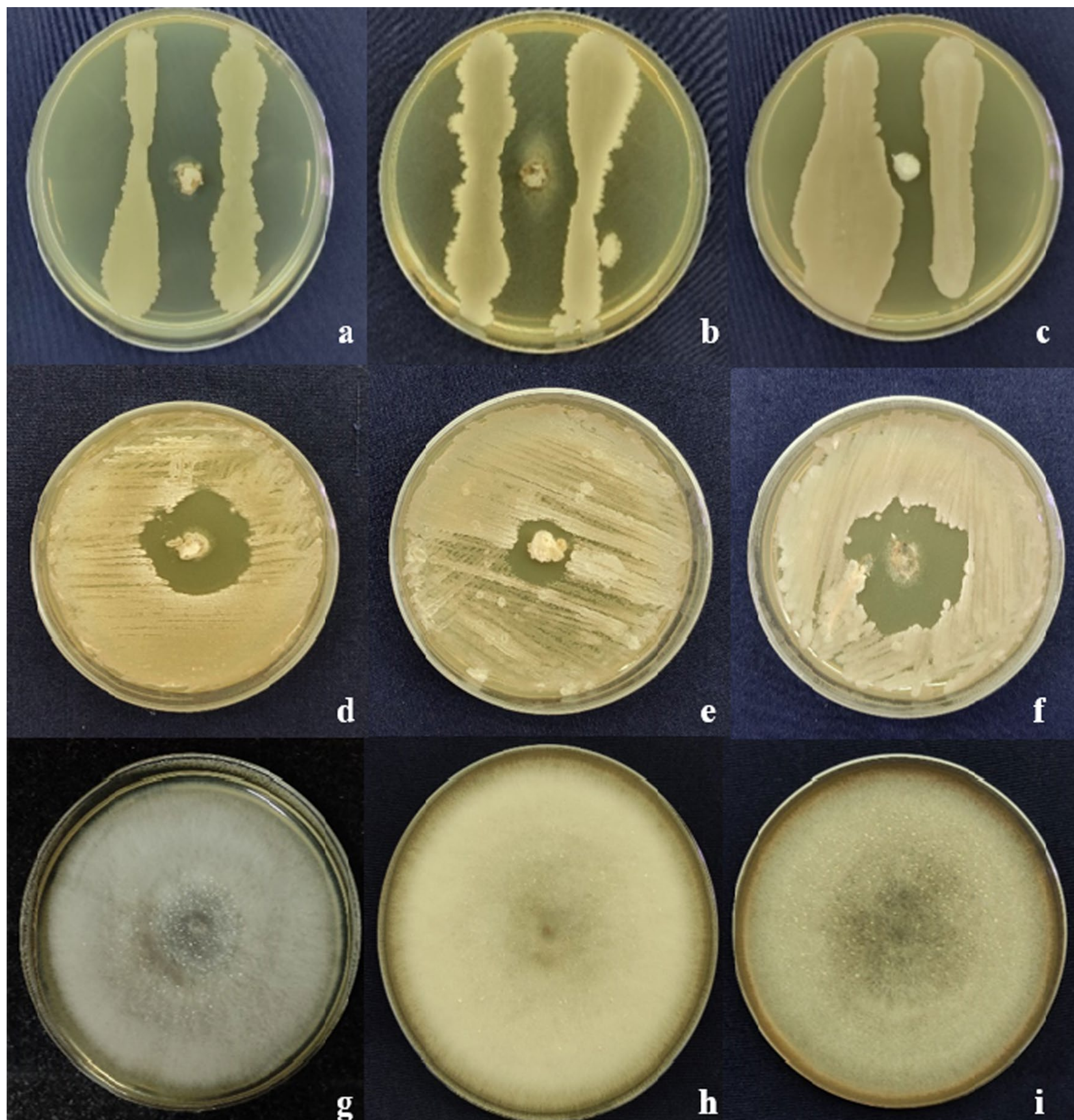


Fig. 4 In vitro, screening of CTCRI EB12 *Bacillus licheniformis* isolate against *Colletotrichum gloeosporioides* isolates **a.** Cg23, **b.** Cg12, **c.** CgS4.7. Antibiosis of CTCRI EB12 and *Colletotrichum gloeosporioides* **d.** Cg23, **e.** Cg12, **f.** CgS4.7, **g.** control Cg23, **h.** control Cg12, **i.** control CgS4.7

Antifungal potential of endophytic bacterial cultural filtrate against *C. gloeosporioides*

The antifungal efficacy of cell-free culture filtrate of the test endophyte against *C. gloeosporioides* isolates was assessed. As shown in Fig. 5, the highest percentage of inhibition was seen against Cg23 (14.2 ± 1.9%), followed by CgS4.7 (13.6 ± 1.6%) and then Cg12 (12.9 ± 1.7%). In addition, similar to the results of the cell confrontation assay, the endophytic isolate caused thinning and reduced fungal mycelial density.

Assay for antibiosis and microscopic examination

In the study of antibiosis, the growth of all three pathogenic isolates was inhibited by the endophyte. The whole mycelia were thin and the mycelial mat also appeared dull, which was the highest (87.2 ± 1.8%) in the Cg23 isolate (Fig. 4d, e, f). The number of acervuli, and the extent of sporulation also appeared extremely low compared to the control plate, where all pathogenic isolates grew splendidly (Table 2). The same samples underwent microscopic examination, and the hyphal morphology

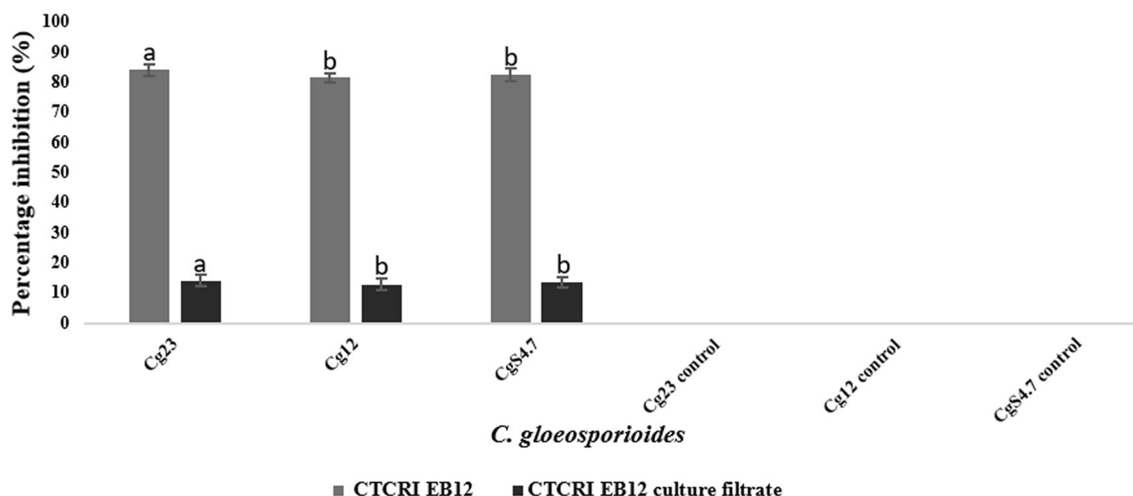


Fig. 5 Mean (± sd) of the ten replicates of percentage inhibition of endophytic isolate and culture filtrate against *Colletotrichum gloeosporioides*. Mean values with the same superscript letter(s) are not significantly different according to Tukey HSD test (p value ≤ 0.05)

Table 2 Antibiosis study of CTCRI EB12 against *Colletotrichum gloeosporioides* isolates

Sl no	Isolate ID	No. of acervuli	No. of conidia	Percentage Inhibition
1	Cg23	+	+ +	87.2 ± 1.8^{a}
2	CgS4.7	+ +	+ +	81.3 ± 1.9^{b}
3	Cg12	+	+ +	81.6 ± 2.5^{b}
4	Cg23 control	+ + +	+ + + +	0
5	CgS4.7 control	+ + + +	+ + +	0
6	Cg12 control	+ + + +	+ + + +	0

Acervuli and conidia of *C. gloeosporioides* isolates represented for their varying number '+ + + +' too many to count, '+ + +' high, '+ +' low, '+' very negligible. Values were recorded based on ten replicates. Means followed by the same superscript letter(s) indicate that they were not significantly different (Tukey HSD test (p value ≤ 0.05)).

around the areas of bacterial and fungal interaction displayed deformities, aberrant swelling, and bulbous formations in mycelia (Fig. 6).

Suppression of anthracnose by CTCRI EB12: detached leaf assay

For the prevention and management of anthracnose in greater yam, antifungal activity of endophytic isolate and culture filtrate against *C. gloeosporioides* on greater yam leaf was assessed using detached leaf assay. Figure 7 depicts inhibition of lesion development on leaves when treated with endophyte with respect to control. Treatment with the culture filtrate of the bacterium considerably ($p \leq 0.05$) prevented the development of anthracnose lesions on leaves. On almost all of the treated leaves, endophytic bacterium inhibited lesion development. Greater yam leaves treated with CTCRI EB12 culture and culture filtrate had smaller lesions than those leaves treated just with *C. gloeosporioides*. Less than 10% leaf degradation and much smaller lesion diameters or altogether no lesion was seen in susceptible greater yam leaves handled by endophytic isolate and its culture filtrate (Fig. 8).

Scanning electron microscopy examination

The cell colonization of *C. gloeosporioides* (Cg23) incubated with CTCRI EB12 on greater yam leaves and the sample from the antibiosis assay were scanned and imaged with SEM. SEM observations of samples from the antibiosis revealed the presence of CTCRI EB12 adhered to the surface of *C. gloeosporioides* (Fig. 9a). The same observation was found in the sample from greater yam leaf co-inoculated with pathogen (Fig. 9d). The mycelia of *C. gloeosporioides* appeared narrow and smooth in all the control samples (Fig. 9c, g), compared to test samples where the same appeared dilated and distorted with an irregular, rough surface, and protrusions that are noticeable through the cell wall that could be the result of internal material leakage (Fig. 9b, e). Abnormal bulging in the hyphae was visible in SEM observations in the most of the test samples. The observation manifests that the typical hyphae were generally uniform in width, whereas those treated with the endophytic bacteria were shrivelled, swelled unevenly at the terminals, and lost cytoplasm. The spores of *C. gloeosporioides* were more disfigured than those of the control group, and they were unusually globose in morphology; some were also constricted to some extent (Fig. 9h, i).

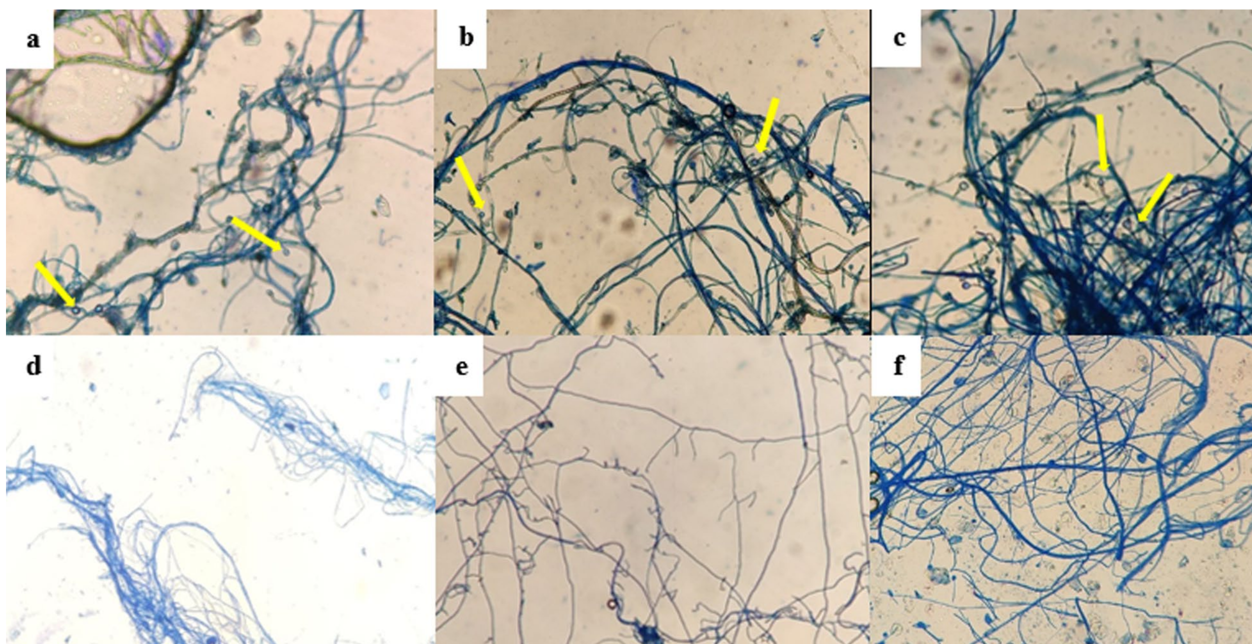


Fig. 6 A photomicrograph showing abnormal hyphae and vacuolar projections in the hyphae of *Colletotrichum gloeosporioides* isolates **a**. Cg23, **b.** CgS4.7, **c.** Cg23, **d.** Cg23 control, **e.** CgS4.7control and **f.** Cg23 control (under 10 × magnification)

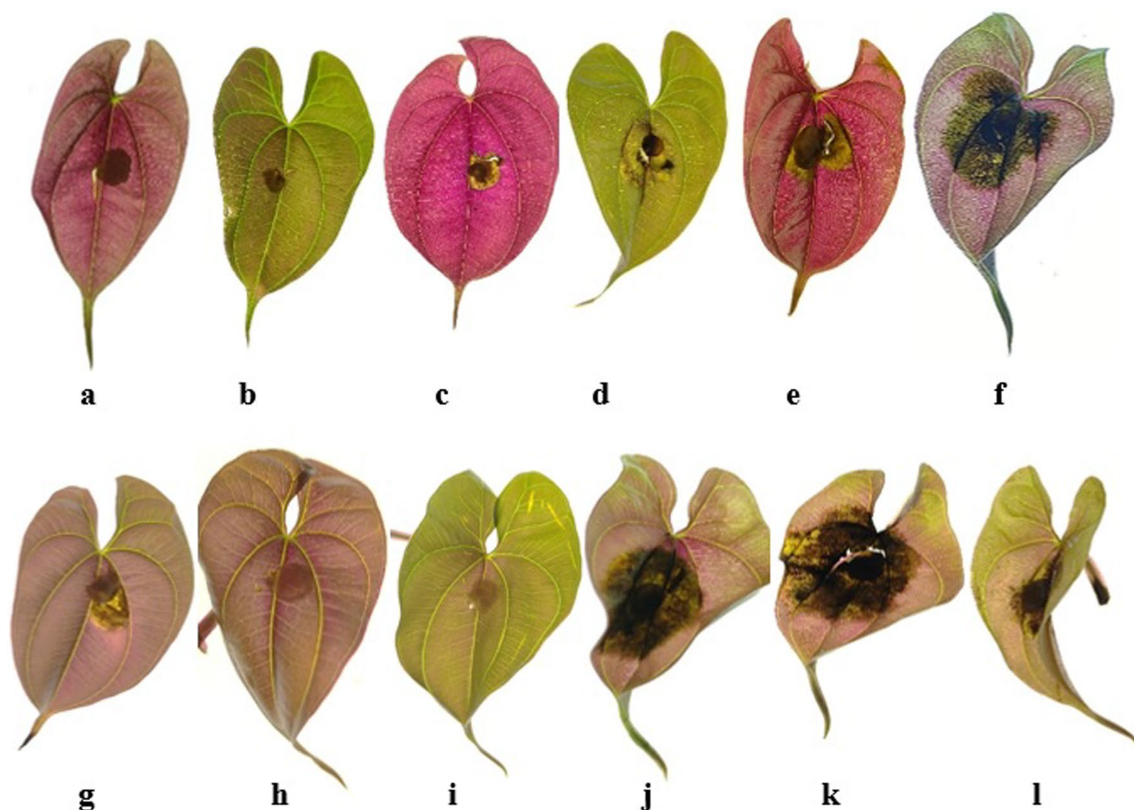


Fig. 7 Effect of endophyte and culture filtrate on the inhibition of *Colletotrichum gloeosporioides* through detached leaf assay. **a–c** *Colletotrichum gloeosporioides* (CgS4.7, Cg12, Cg23) inoculated with CTCRI EB12 on *Dioscorea alata* leaf. **d–f** control (CgS4.7, Cg12, Cg23). **g–i** *Colletotrichum gloeosporioides* (CgS4.7, Cg12, Cg23) inoculated with CTCRI EB12 culture filtrate. **j–l** control (CgS4.7, Cg12, Cg23)

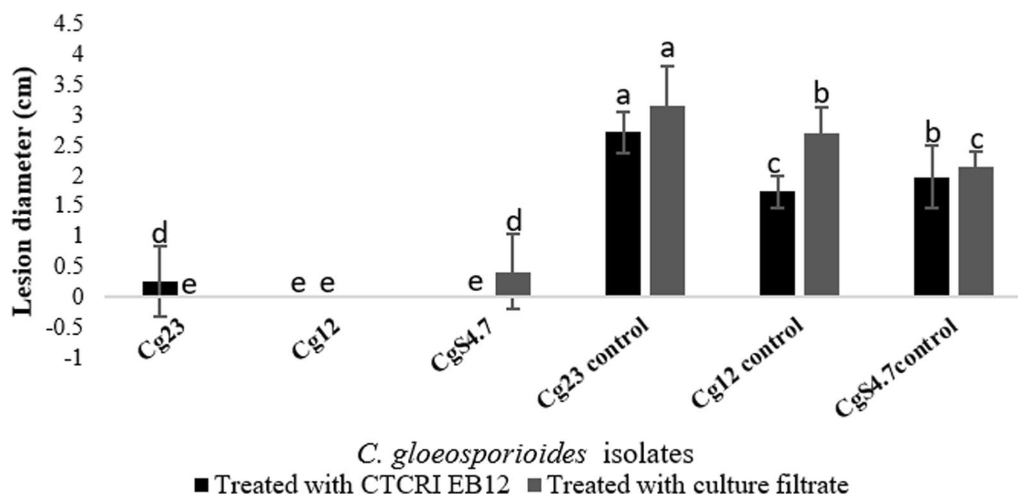


Fig. 8 Effect of endophyte on the inhibition of different *Colletotrichum gloeosporioides* (Cg) isolates through detached leaf assay on greater yam leaves. Mean (± sd) of the three replicates. Mean values with the same superscript letter are not significantly different according to Tukey HSD test (p value ≤ 0.05)

Discussion

Colletotrichum gloeosporioides is a fungal pathogen of greater yam (*Dioscorea alata*), causing the disease anthracnose. It is a major constraint to greater yam production in yam-cultivating regions worldwide, with yield losses of up to 90% as reported by Suresh Veera et al. (2023). It is crucial to make observations on the fundamental morphological and microscopic characteristics of isolated pathogens in order to implement timely disease management strategies for a number of plant diseases. In the present study, all the three *C. gloeosporioides* isolates showed ample mycelia, with bright to light orange conidial mass. Here, conidia appeared single celled and cylindrical in shape. These results are in line with the observations reported by Nuangmek et al. (2005), where distinct morphological culture types of *C. gloeosporioides* isolates were characterized by abundant mycelia with vivid orange conidia masses. These conidial masses were generated in concentric rings on the colonies, along with cottony, white to pale grey mycelium along with single celled and cylindrical conidia.

Molecular diagnosis of the pathogen is indispensable for pathogen identification in addition to morphological characterization. Herein the pathogens were diagnosed by polymerase chain reaction using species-specific primers. Raj et al. 2012 documented the designing of *C. gloeosporioides* specific CgsF1/CgsR1 primers. These primers are found to be specific and more accurate. Here, *C. gloeosporioides* specific CgsF1/CgsR1 primers gave an amplicon of ~ 300 bp in all the three isolates. These outcomes are in corroboration with the observations reported by (Raj et al. 2013), where amplification of *C. gloeosporioides* genomic DNA generated amplicons of ~ 300 bp.

Antibiotic-producing microorganisms are increasingly being addressed for plant disease management due to many advantages over chemical biocides, including simple breakdown and no hazardous residues (Kim et al. 2022). Significant work has been done on the application and development of biological agents as new techniques to control dangerous plant pathogens (Tian et al. 2021). According to Kim et al. (2022), *Bacillus velezensis* CE 100 was shown by a dual culture assay to be a potent antagonist against C. *acutatum, C. coccodes, C. dematium,* and *C. gloeosporioides.* In the present investigation, it was revealed that an endobacterial isolate CTCRI EB12 from *A. vera* was effective in preventing the growth of *C. gloeosporioides*, the causal organism of anthracnose in greater yam. Endophytes of a wide range of plant species frequently contain bacteria of the genus *Bacillus* (Nigris et al. 2018). CTCRI EB12 was isolated and defined as a member of the *Bacillus* species, which could limit the proliferation of *C. gloeosporioides* in vitro. Even though the potent endophytic isolate was identified as *Bacillus licheniformis* via 16SrRNA sequencing, a combination of whole genome sequencing and transcriptome analysis will help to further reveal the exact species-level identification of the endophytic isolate. (Hagiwara et al. 2020).

It is well recognized that unique antimicrobial compounds secreted by *Bacillus* sp. can express expansive antagonistic features, and these antimicrobial elements may be crucial in managing plant diseases. The constituents in the culture filtrate of CTCRI EB12 showed antifungal activity since they were able to inhibit the growth of *C. gloeosporioides* in *vivo*; the same was in compliance

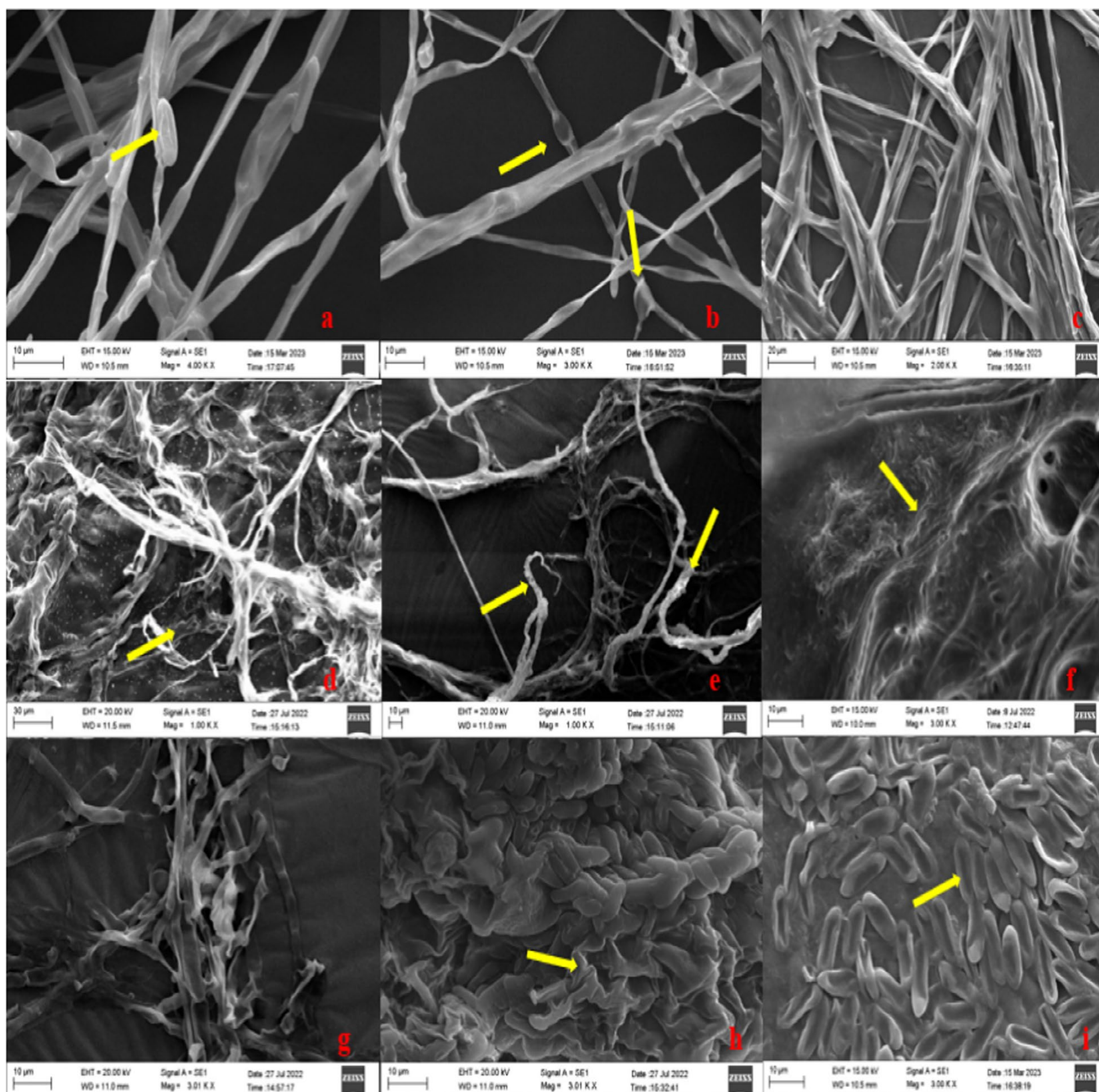


Fig. 9 Scanning electron microscopy of hyphae of *Colletotrichum gloeosporioides* **a**. CTCRI EB12 (yellow arrow) cell on hyphae (antibiosis) **b**. Distortions and bulbous projections in hyphae of *Colletotrichum gloeosporioides* (antibiosis) **c**. Control **d.** CTCRI EB12 (yellow arrow) cell on hyphae (on leaf surface). **e**. Distorted and unhealthy hyphae of *Colletotrichum gloeosporioides* (on leaf surface). **f.** Presence of CTCRI EB12 film on leaf surface **g**. Control (on leaf surface) **h**. Conidia treated with CTCRI EB12 **i.** Control conidia

with the report of Huang et al., (2015). In their study, the endophytic *Bacillus atrophaeus* XW2 culture filtrate was hostile to hyphal development and spore germination of *C. gloeosporioides*. Yoshida et al. (2001) documented that the application of *B. amyloliquefaciens* RC-2 culture filtrate to mulberry leaves prevented the spread of the disease, proving that the antifungal components in the filtrate were responsible for the suppression of *C. dematium*. These findings imply that the antifungal elements in the filtrate can stop the hyphal progression. Antibiosis was profound, about 100%; the mycelia appeared thin and dull when grown together with the endophyte. In Petri dishes, it caused morphological deformity and subcellular abnormalities in *C. gloeosporioides*. It also inhibited the growth and expansion of the fungus, which may be one of its antimicrobial

mechanisms. The number of spores produced and acervuli was less in number than control. Light microscopic examination revealed mycelial distortion and severe impairment due to endophyte. These results were compromising when compared to the results of Srikhong et al. (2018), where microscopic examination showed that an antifungal protein produced by *Bacillus* sp. strain M10 induced anomalous hyphal elongation and conidial swelling and disintegration. In this study, culture filtrate of CTCRI EB12 showed a significant antimycotic effect on morphology, and germination of the conidia of the pathogen.

Pathogenicity can be easily and quickly determined using a detached leaf assay. Young plants and leaves were excised and infected by inserting a mycelium plug into a small puncture in the leaf blade. After incubation, the existence and severity of lesions on leaves were evaluated (Pettitt et al. 2011). In the present study, the endophyte, CTCRI EB12, and its culture filtrate profoundly arrested lesion development when compared to control. In control, lesion development was observed on the second day of inoculation. In contrast, almost complete absence of disease progression was noted when leaves were treated with bacteria and culture filtrate. The outcome manifests that CTCRI EB12 is an active biocontrol agent against *C. gloeosporioides*. The activity of the same was proved via SEM analysis. The SEM observations suggested that the treatment with CTCRI EB12 culminated in fungal hyphae shrinking, cytoplasm leakage, and uneven tip swelling. These showed that the generation of hydrolytic enzymes and antibiosis are the processes responsible for the biocontrol demonstrated by these bacteria. Our results are in accordance with those of Ann et al. (2015). In their study, SEM observations of pepper leaves treated with strains CBF, YCA0098, and YCA 5593 inoculated with *C. gloeosporioides* demonstrate that the treatment of strain CBF cell suspension reduced the hyphal growth on the surface of pepper leaves and slowed down the formation of infection cushions, thereby suppressing infection.

Conclusion

In essence, the present research indicated that *Bacillus licheniformis* CTCRI EB12 had practical biological control effects against *C. gloeosporioides*, and it may one day be used as a powerful tool to combat greater yam anthracnose. The current study may aid in exploiting CTCRI EB12 and its inhibitory activity on *C. gloeosporioides*. This information improved the understanding of the potential inhibitory mechanisms of *Bacillus* species and may offer new approaches for the management of maladies of the greater yam.

Abbreviations

NA	Nutrient agar medium
PDA	Potato dextrose agar medium
PDB	Potato dextrose both
ANOVA	Analysis of variance

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