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Research Article

New report of root endophyte - *Enterobacter cloacae* having *in vitro* growth promoting attributes and biocontrol potential against *Curvularia lunata* causing leaf spot disease of banana

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Abstract

The Banana plant (*Musa paradisiaca*) worldwide cultivated crop harbors many endophytic bacteria. Endophytic bacteria are those that live inside plant tissues without producing visible symptoms of infection or having an adverse effect on their hosts. Root samples were collected from different fields of banana (*Musa paradisiaca*) of the District Uttar Dinajpur. GPS locations of the banana fields were also taken by using mobile GPS location application.Bacterium-MRH-6 was isolated from root pieces. The scanning electron microscopic image of bacterial isolate (MRH 6) was taken. 16S rDNA fragment sequence was found to be *Enterobacter cloacae* and was deposited to GenBank with accession number ON955844. *E. cloacae* showed plant growth promoting (PGP) traits like IAA production, phosphate solubilization. The bacterium showed phosphate-solubilising activity by producing a clear zone on the Pikovskaya's (PKV) medium. *E. cloacae* was positive in IAA production and catalase production. For quantification, HPLC analysis of IAA from culture filtrate of *E. cloacae* was done by injecting 10µl of the filtered extract onto a (C18, 5µm 25×0.46 cm, Make: Agilent, Model: Infinity 1260) in a chromatograph equipped with a differential ultraviolet detector absorbing at 254 nm. Mobile phase was acetonitrile 60% and Water 40% and flow rate 1.0ml/min. Retention times for peaks were compared to IAA standard and quantified. *E. cloacae* recorded IAA production of (3.509 × 20)-70.18 ng/µl. The bacterium showed sensitivity to antibiotic chloramphenicol (30µg) and resistant to ampicillin (10µg). The leaf spot disease in banana from Sarai Raiganj was first reported by Chowhan and Chakraborty (2022) and Koch's postulate was successfully established for the pathogen causing leaf spot disease in banana. 18S rDNA fragment sequence of strain MLP-01 was deposited on GenBank with accession number ON246070. The fungus was morphologically identified as *Curvularia lunata* by Agharkar Research Institute, Pune, India with Accession number NFCCI 5361. E. cloacae showed in vitro antagonism against *C. lunata* (ON246070). *E. cloacae* also checked the growth of the tested pathogen significantly. The tested fungus was inhibited to some degree, the percentage inhibition was 75% by *E. cloacae*.

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Introduction

The widely grown banana plant (*Musa paradisiaca*) is host to a large number of endophytic bacteria. as defined by Kandasamy *et al*. (2023), Tiwari *et al.* (2023) and Ayilara *et al.* (2023), endophyte bacteria are bacteria that reside without causing damage to their hosts inside plant tissues or exhibiting outward signs of infection. Endophytic bacteria, then, are microbes that reside in plant tissues without appearing to harm the host plant. These bacteria develop symbiotic connections with their hosts by colonising the intracellular, vascular, and intercellular regions of plants (Dutta *et al.* 2024). Roots, stems, leaves, seeds every parts of plants are all home to endophytic bacteria (Bharadwaj et al.

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2024, Shukuru *et al.* 2014). Numerous advantages can be bestowed upon their host plants by endophytic bacteria. It is possible for some endophytic bacteria to fix atmospheric nitrogen and release it for use by the host plant. According to Nath *et al.* (2024), this can promote the plant's growth and development, particularly in soils low in nitrogen. Antimicrobial substances produced by some endophytic bacteria can shield the host plant from parasites, bacteria, and other diseases. Additionally, they may strengthen the plant's defenses against disease by stimulating several plant resistance mechanisms (Salim *et al*. 2024). Auxins, cytokinins and GAs are examples of phytohormones produced by some endophytic bacteria that can enhance plant growth and development, increasing biomass output and enhancing agricultural productivity (Mamarasulov *et al.* 2024). Keeping these in mind, the present study has been undertaken to evaluate the role of *Enterobacter cloacae* as plant growth promoter and biocontrol agent to suppress the leaf spot disease of banana, caused by *Curvularia lunata*.

Material and methods

Sample collection and isolation

In the District of Uttar Dinajpur, banana (*Musa paradisiaca*) fields were selected for the collection of root samples. Moreover, mobile GPS location applications were used to obtain the GPS positions of the banana fields (Fig. 1). Bacterium-MRH-6 was isolated (surface sterilization was carried out by using 0.1 % mercuric chloride and 70 % ethanol) (Babu *et al.* 2022, Çağlar *et al.* 2023, Chowhan *et al.* 2023) from root pieces in a NA (Nutrient Agar) slant, similarly the leaf spot disease causing fungal pathogen of banana was isolated in a PDA (Potato Dextrose Agar) plate (Lv *et al.* 2023, Huang *et al.* 2023, Chowhan *et al.* 2023).

IAA production

The bacterial isolate was cultured for 24 hours in nutritional broth medium supplemented with 0.1 mM tryptophane to increase the formation of indole acetic acid by the bacteria in order to detect IAA (Prinsen *et al.* 1993). According to Dobbelaere *et al.* (1999), The amount of IAA generated in the culture supernatant was measured using the Pillet-Chollet method. For the reaction, 2 ml of sample supernatant and 2 ml Salkowski reagent (12 g $FeCl_3$ per litre in 7.9 M H_2SO_4) were combined, thoroughly mixed, and allowed to stand at 28C in the dark for 30 minutes. IAA generation was determined by colour changes in the supernatant (Chowhan *et al.* 2023).

Phosphate solubilization

The phosphate solubilizing properties of bacterium MRH-06 were investigated by growing the bacteria for seven to ten days at 37°C on a selective medium, namely Pikovskaya's agar (PKV) (Himedia- M520) (Pikovskaya 1948). The creation of a halo zone surrounding the bacterial colony demonstrated its capacity to solubilize phosphate.

Catalase production

For the bacterial isolate MRH-06, a catalase production assay was conducted. One loopful of bacteria was placed to a slide containing one drop of H_2O_2. There had been indications of gas development (Karen Reiner 2010).

Protease production

Protease activity was carried out on 3% powdered skim milk-agar plates according to Walsh *et al.* (1995). The creation of a transparent halo zone surrounding the bacterial colony demonstrated its protease activity.

HCN production

Bacteria MRH-06 was grown in nutritional broth (NB) media at 35ºC to detect the production of hydrocyanic acid. After that small strips of filter paper dipped in picrate solution (0.05% solution in 2% sodium carbonate), was placed inside conical flasks in a hanging position and incubated for another 72 hours at 35ºC. The reaction with picrate solution caused colour changes in the medium, which indicated the generation of hydrocyanic acid (Bakker and Schippers 1987).

Quantification of IAA through HPLC

In order to do an HPLC evaluation of IAA from the culture filtrate of *E. cloacae*, 10µl of the filtered extract was injected onto a (C18,5µm 25×0.46 cm, Make: Agilent, Model: Infinity 1260) in a chromategraph that had a differential ultraviolet detector that absorbed light at a wavelength of 254 nm. Mobile phase was acetonitrile 60% and Water 40% and flow rate 1.0ml/min.

Antibiotic sensitivity test

Using the pour plate method, the bacterium MRH-06 was grown in nutrient agar (NA) plates to test for antibiotic resistance. Two antibiotic discs, 30µg of chloramphenicol and 10µg of ampicillin, were placed in the plate slightly apart using sterile forceps, and the plate was incubated for 24 hours. A clear zone encircling the antibiotic disc suggests a zone of sensitivity to the antibiotic, whereas no zone

surrounding the antibiotic disc denotes bacterial resistance.

Antifungal assay

The antagonistic capacity of the isolate MRH-06 against the fungal pathogen MLP-01 was evaluated *in vitro*. An agar disc containing the fungus was placed 2.5 cm in the petriplates away from Bacterium streaked on medium on it. Additionally, a negative control consisting only of fungal agar discs without any bacterial inoculation spots was employed. The colony growth inhibition was assessed after four days of incubation at 30 ± 1°C on the infected plates. The following formula was used to calculate the percentage of inhibition: PI= [(R1-R2) / R1] X 100. (where PI = Percentage of fungal inhibition, R2 = Redial mycelium growth of the fungas opposite the bacterial colony, and R1 = radial mycelium growth of fungus in the control plate) (Dey *et al.* 2019).

Results and discussion

By sequencing the 16S rDNA fragment of the bacterial isolate MRH-06, which was uploaded to GenBank under the accession number ON955844, *Enterobacter cloacae* was identified. Mahlangu *et al*. (2022) reported *E. cloacae* as an endophyte bacterium in *Centella asiatica*. *E. cloacae*, an endophytic bacterium that promotes plant growth, was isolated from ground nuts and described by Ramakrishnan *et al.* (2023). In addition to forming a halo zone on the PKV medium, *E. cloacae* isolate MRH-06 was found to be positive for both IAA, catalase, HCN, protease production (Fig. 2) shown in Table 1. Fajingbesi et al. (2018), Fasiku et al. (2020) reported protease production by *E. cloacae.* It also demonstrated phosphate-solubilizing activity. Borham et al. (2017) reported phosphate-solubilizing activity of *E. cloacae* which help in increase the productivity of wheat. According to Chen et al. (2022), *Enterobacter cloacae* strain NG-33 that could solubilize phosphate and promote maize growth. The genes of *E. cloacae* linked to plant growth promotion were reported by Wang *et al.* (2023). Scanning electron microscopic (SEM) image of bacterial isolate (MRH 6) was captured by using JSM IT 100 (Fig. 3).

Table 1. *In vitro* plant growth promoting traits of *Enterobacter cloacae* (ON955844)

Plant growth promoting traits	MRH -06
IAA production	+++
Catalase production	+++
Phosphate solubilization	+++
Protease production	+++
HCN production	+++

+=positive response and -= negative response

Retention times for peaks were compared to IAA standard and quantified. *E. cloacae* recorded IAA production of (3.509 × 20)-70.18 ng/µl (Fig. 4) much higher as compared to control IAA 30 p.p.m. which is 30.051 ng/µl (Fig. 5). IAA production from endophytic bacteria was previously reported by many researchers (Lata *et al.* 2006, Pantoja-Guerra *et al.* 2023). Di *et al.* (2023) reported synthesis of IAA from an endophytic bacterium- *Bacillus subtilis*, isolated from Sugarcane. Semwal *et al.* (2023) reported production of IAA from endophyte bacteria belonging to *Bacillus* genera isolated from a medicinal plant *Gloriosa superba* L. Boonmahome *et al.* (2023) reported IAA production from an endophytic bacterium- *Micrococcus luteus.* The bacterium exhibited resistance to ampicillin (10µg) and sensitivity to the antibiotic chloramphenicol (30µg) (Fig. 2).

Chowhan and Chakraborty (2022) were the first to report the leaf spot disease in bananas from Sarai Raiganj, and they effectively established Koch's postulate for the pathogen causing the disease. The strain MLP-01 18S rDNA fragment sequence has been uploaded into GenBank under the accession number ON246070. Agharkar Research Institute, Pune, India identified the fungus morphologically as *Curvularia lunata*, with Accession number NFCCI 5361 (Fig. 6). *In vitro*, *E. cloacae* displayed antagonistic behaviour towards *C. lunata* (ON246070). Additionally, *E. cloacae* considerably inhibited the tested pathogen's growth. *E. cloacae* inhibited the tested fungus to a certain extent; the percentage of inhibition was 75% (Fig. 7) shown in Table 2.

Table 2. *In vitro* antagonistic tests of *Enterobacter cloacae* (ON955844) against *Curvularia lunata* (ON246070)

Test fungi	Paired with bacterium	Dia. Of fungal growth (cm)	Zone of inhibition (cm)	% of Inhibition
Curvularia lunata	-	8.8±1.2	-	-
	Enterobacter cloacae	2.1±0.22	1.7±0.09	75.0±2.91

*After 7 days; ±= SE

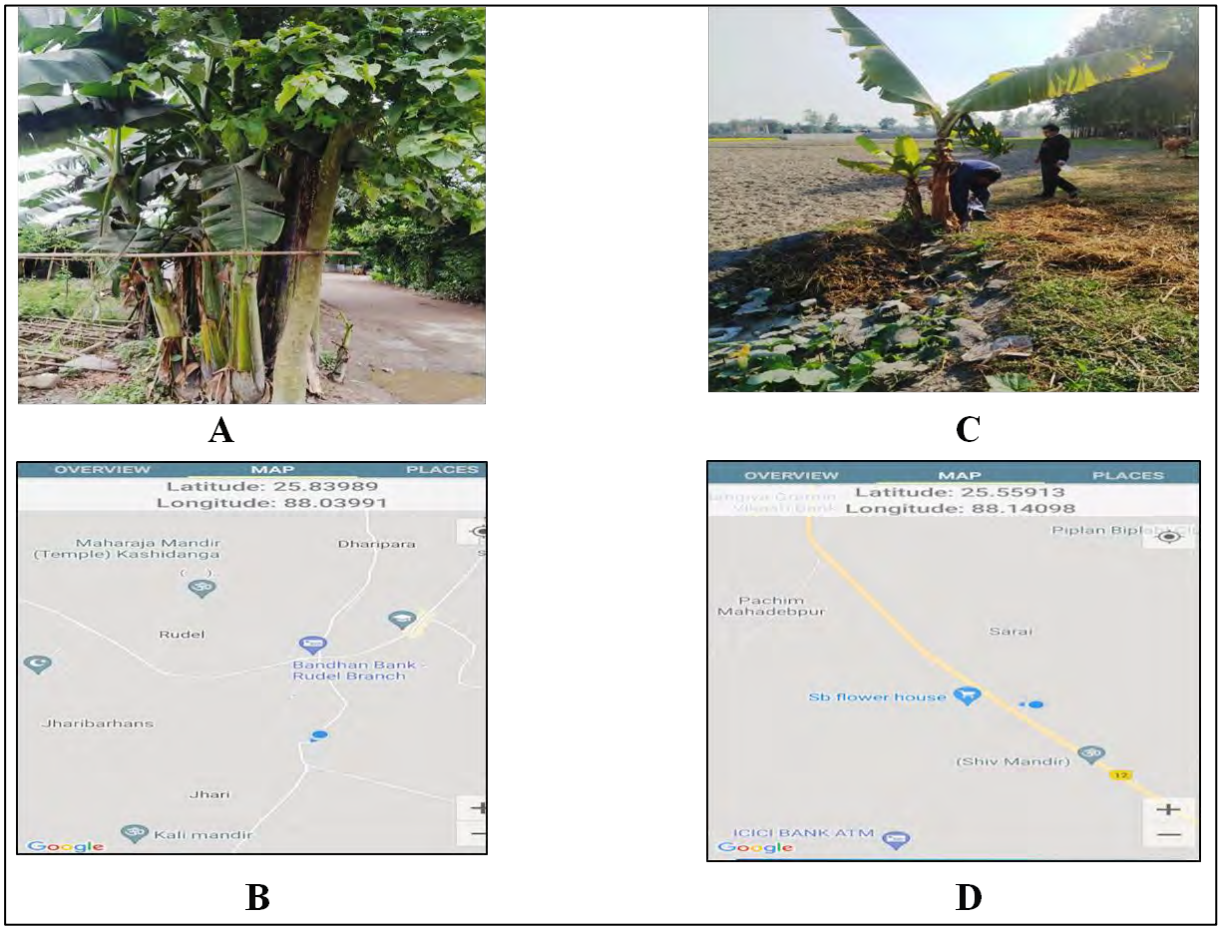

Fig. 1. Collection of root sample and GPS location of MRH 06 (A, B); Collection of leaf spot disease sample and GPS location of MLP-01 (C, D)

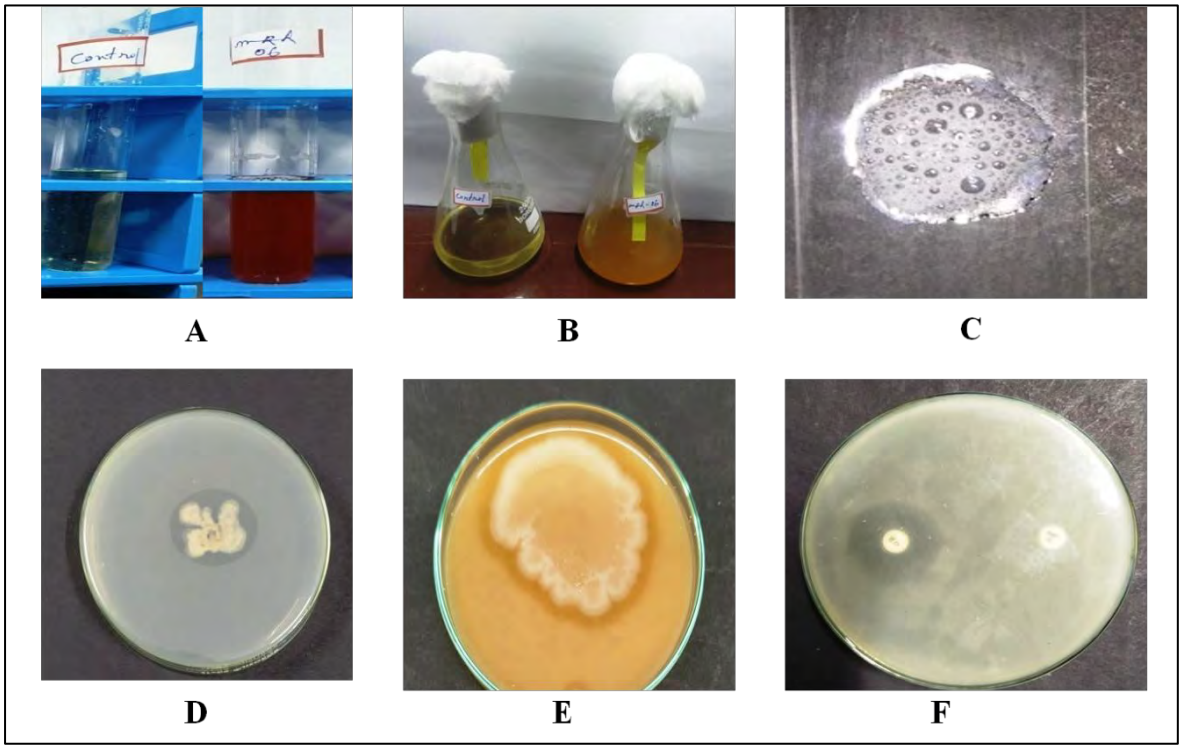

Fig. 2. IAA test positive (A), HCN test positive (B), Catalase test positive (C), Phosphate solubilization test positive (D), Protease test positive (E) of bacterial isolate- *Enterobacter cloacae*- MRH-06; *E. cloacae* was

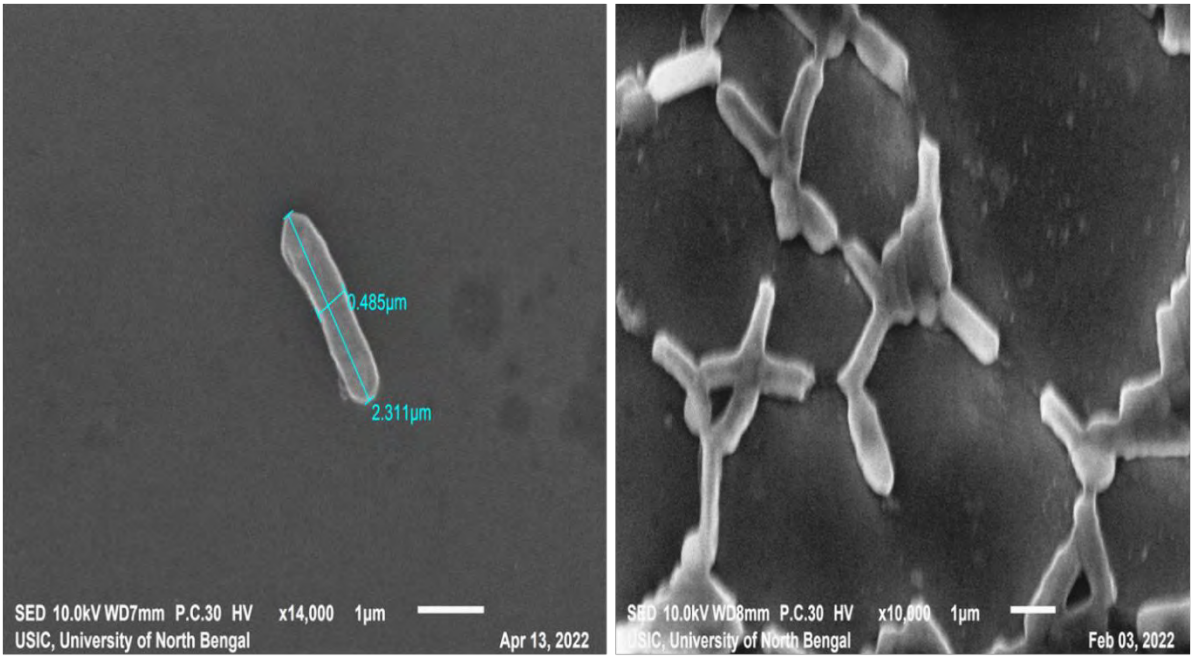

Fig. 3. Scanning Electron Microscopic view of *Enterobacter cloacae*- MRH-06

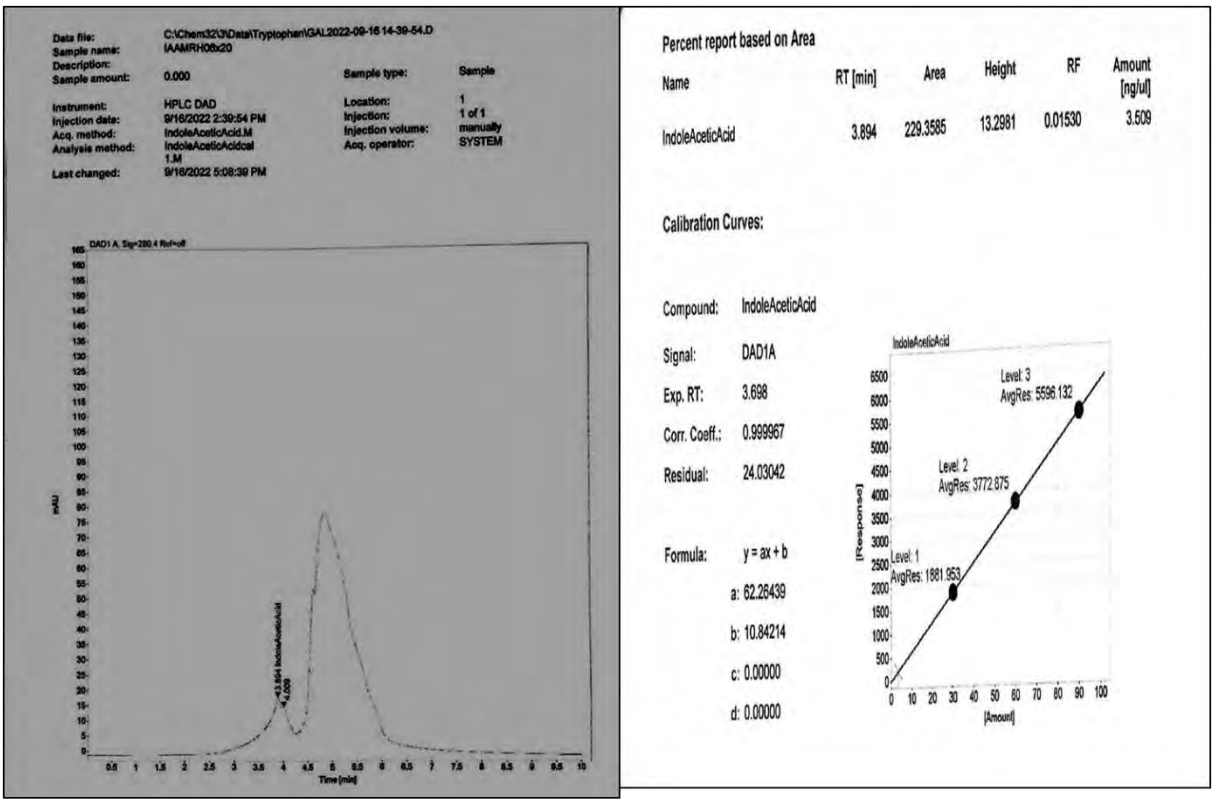

Fig. 4. IAA production of *E. cloacae* isolate which is 70.18 ng/µl much higher as compared to control IAA-30.051 ng/µl

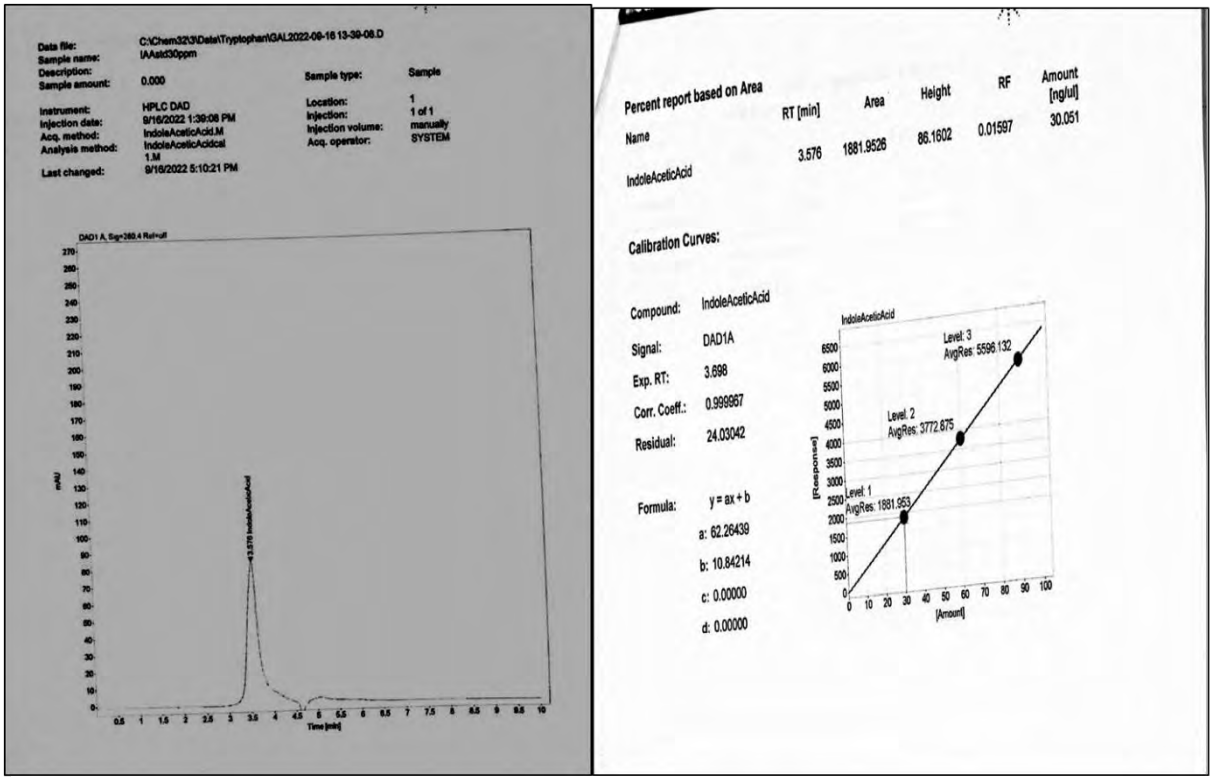

Fig. 5. HPLC analysis of control IAA at 30 p.p.m. which is 30.051 ng/µl

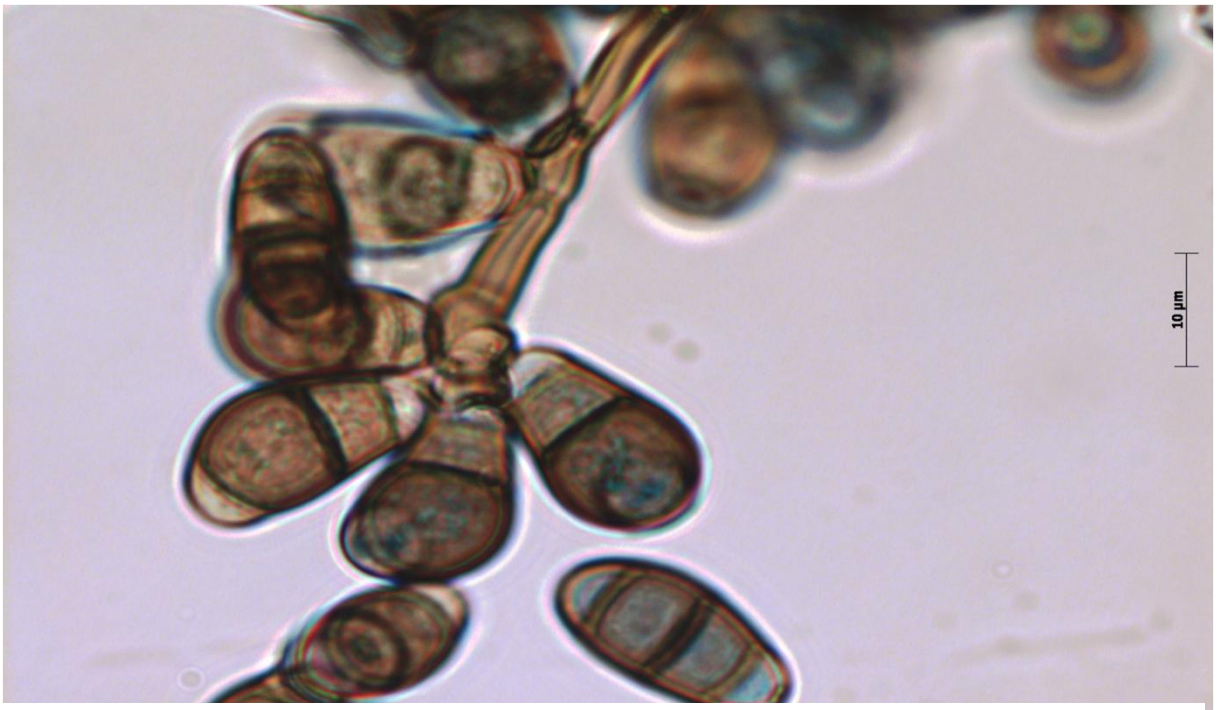

Fig. 6. Light Microscopic view of MLP-01 fungal pathogen- *Curvularia lunata*

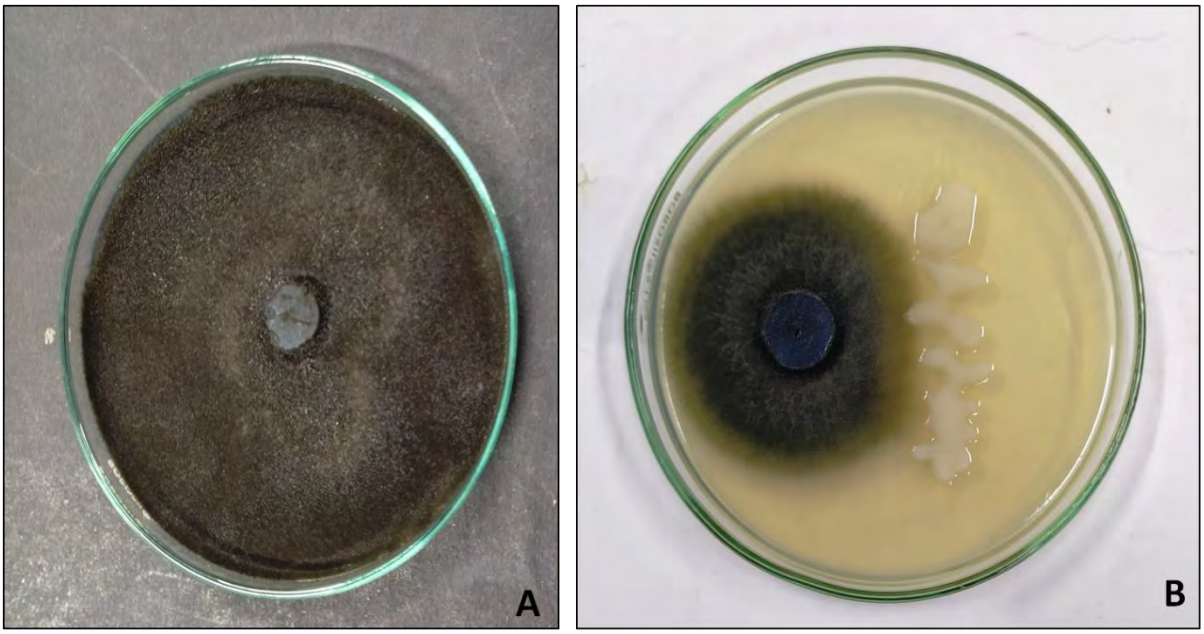

Fig. 7. *Enterobacter cloacae* inhibited the mycelia growth of *Curvularia lunata. C. lunata* growth alone (A); antagonistic effect of *E. cloacae* against *C. lunata* (B)

During the *in vitro* examination, the scanning electron microscopic picture of the transition zone between the cells of *E. cloacae* and mycelia of *C. lunata* was examined (Fig. 8). In 2023, Hamane *et al*. confirmed the plant growth-enhancing and biocontrol properties of *Enterobacter* sp., an endophyte isolated from *Sulla flexuosa* L. root nodules, against a variety of fungal diseases, including *Fusarium* sp. and *Botrytis* sp. Chowhan *et al.* (2023) earlier reported on the plant growth-promoting and antagonistic potential of another endophyte bacterium, *Achromobacter xylosoxidans*, isolated from bananas against the fungal disease *Curvularia lunata*.

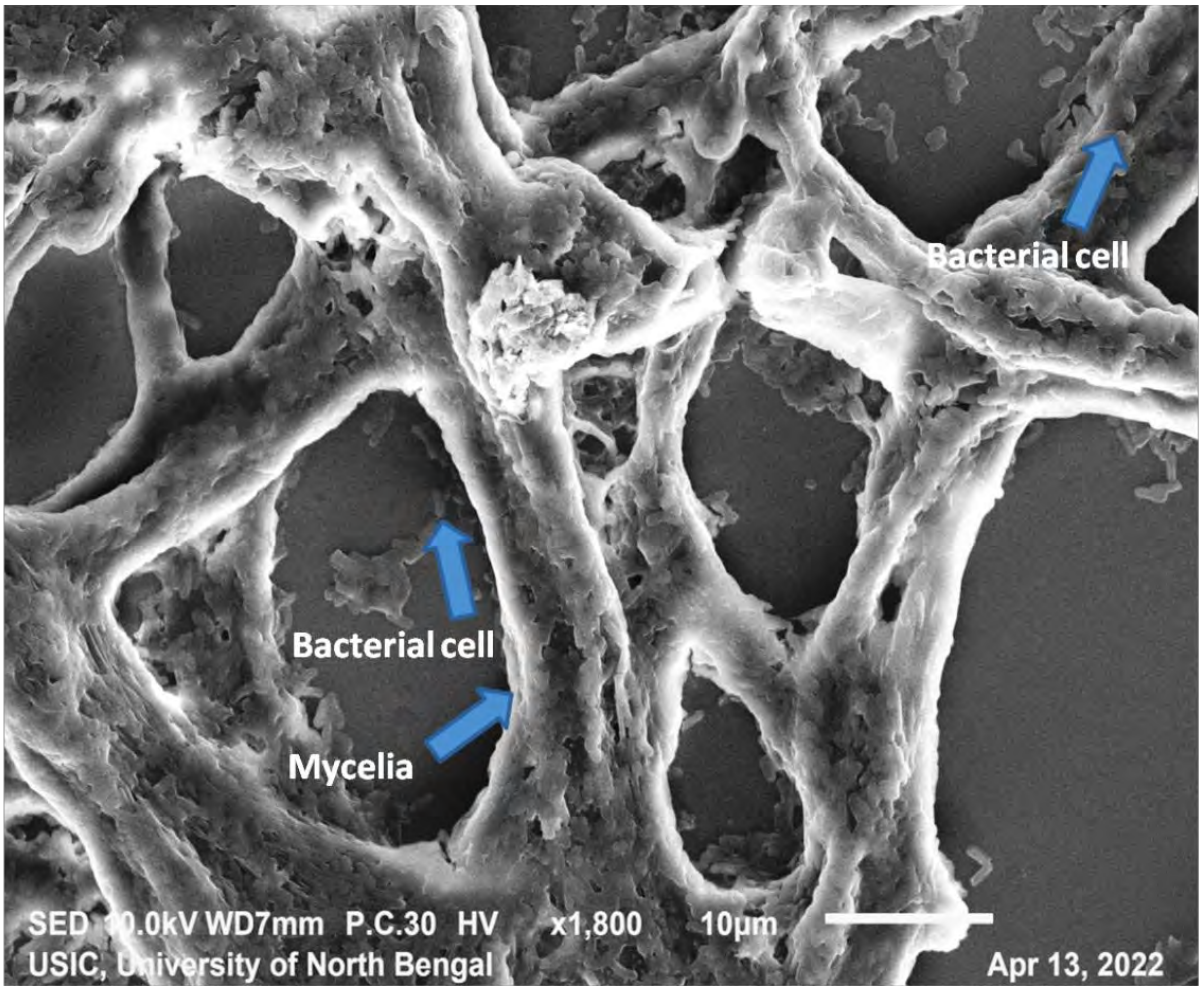

Fig. 8. Scanning Electron Microscopic view of junction between *Curvularia lunata* and *Enterobacter cloacae*

Conclusion

Overall, the study provides insight into the plant growth promotion activities like IAA production and interaction between endophytic bacterium- *E. cloacae* and pathogenic fungi like *C. lunata* in banana plants, highlighting potential biocontrol applications for managing plant diseases. Research on endophytic bacteria is ongoing and scientists are exploring their potential applications in agriculture, bioremediation and phytoremediation. By harnessing the beneficial effects of endophytic bacteria, it may be possible to develop sustainable agricultural practices and improve crop productivity while reducing the need for chemical fertilizers and pesticides.

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Data availability

No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest

The authors declare that they have no conflicts of interest.

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