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In vitro evaluation of plant growth promoting and antagonistic activities of endophytic bacteria- *Enterobacter cloacae* and *Achromobacter xylosoxidans* isolated from banana

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One of the most widely consumed fruits in the world, is the banana (*Musa* sp.) which suffers from several fungal diseases.The goal of the research was to identify and examine the potential of two endophytic bacteria as antifungal agents against the leaf spot disease and other plant growth promoting (PGP) activities. Endophytic bacteria were collected from the roots of banana, while pathogen was isolated from diseased leaves. Antagonist assays of two endophytic bacteria (MRH -06 and MRH -11) against the fungus pathogen (MLP-01) was carried out using standard methods. For identification, using 16S rDNA and 18s rDNA partial sequencing was done. The two endophytic bacteria which showed antagonistic potentials were identified as *Enterobacter cloacae* (ON955844) and *Achromobacter xylosoxidans* (ON955872), while the fungal pathogen was identified as *Curvularia lunata* (ON246070).

Keywords: Antifungal, endophytic bacteria, leaf spot disease, plant growth promotion

INTRODUCTION

The banana (*Musa* sp) is the most popular tropical fruit and is regularly consumed worldwide (Mendoza and Sikora, 2009). It is high in nutrients content. Besides of its fruit, other parts like leaves, trunk and flower parts are also useful for eating, cooking, vegetables, textiles and many other things. Additionally, the banana plant has religious and cultural importance. The largest herbaceous blooming plant is the banana plant. In many nations, it serves both as a staple food and a source of income for families (Wang *et al.* 2013). They are mostly grown for their fruit and are cultivated in 135 different nations. Almost all edible seedless bananas nowadays come from two wild species of banana *Musa acuminata* and *Musa balbisiana.*

Endophytes are microorganisms that live inside the tissues of plants without affecting their host plant. Nearly every internal part of the host plant, including the stem, leaf, flower, fruit and seed is colonized by endophytes.

Endophytic bacteria are also able to enhance host plant growth directly through mechanisms like phyotohormones (IAA) production or indirectly by suppressing disease. Natural substances from different endophytic bacteria have been reported to inhibit a wide range of pathogenic disease-causing agents like fungi, bacteria, viruses, and protozoans. Biological control, using this bacterial endophytes, have a great promise of providing an alternative to current chemical fungicides for the control of different fungal diseases and it also reduce the health and environmental hazards caused by synthetic fungicides (Wilson *et al*. 2011; Dukare *et al.* 2019; Ons *et al*. 2020; Tariq *et al*. 2020). Like any other plants bananas also have endophytic bacteria which are able to inhibit the growth of pathogenic fungus. Merve and Soylu (2022) reported endophytic bacterial isolates from healthy banana plants which have antagonistic activities against crown rot disease caused by *Fusarium verticillioides*. Leaf spot is one of the most common disease in banana. The present research aimed to isolate endophytic bacteria from the banana plant (*Musa* sp) and their study their role as antifungal agents against leaf spot causing

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pathogen isolated from diseased leaf sample from Raiganj, Uttar Dinajpur.

MATERIAL AND METHODS

Isolation of microorganisms

Root and leaf spot diseased samples were collected from different fields of banana plant (*Musa* sp) of the district Uttar Dinajpur, West Bengal. The root samples were cut into small pieces (1 cm) and transferred into NA (Nutrient Agar slant) after surface sterilization with 0.1% $HgCl_2$ for 2 min, followed by ethanol for 2 min. Two bacteria (MRH -06, MRH-11) were isolated from root pieces onto nutrient agar slant. The diseased leaf sample was also cut into small pieces (1-1.5 cm) and transferred into PDA (Potato Dextrose Agar) medium after surface sterilization by same process. A fungus (MLP -01) was isolated from the infected leaf part onto potato dextrose agar amended with an antibiotic Monocef. The microscopic view was observed under compound light microscope (Fig. 1).

Antifungal assay

The two bacteria MRH-06 and MRH-11 were tested *in vitro* for their antagonistic potential against the fungal pathogen MLP-01. Each bacterium was streaked on the Petridish with medium and an agar disc of the test fungus was placed on the medium 2.5 cm away. There was also a negative control that used solely fungal agar discs without any bacterial culture spots. The inoculated plates were incubated at 28 ± 1°C for five days and the inhibition of the colony growth was measured. The percentage of inhibition was measured by using the following formula – $PI= [(R_1-R_2) / R_1] X 100$ (where PI = Percentage of inhibition, R_2 = Redial growth of the fungal colony opposite the bacterial colony, and R_1 = radial growth of the fungus in the control plate). Scanning electron microscopic images of the bacterial isolates and fungal junction were also taken.

In vitro assay for plant growth promoting (PGP) activities of bacterial isolates

The isolated bacterial strains were examined for plant growth promotion (PGP) traits *in vitro* conditions such as the ability to produce Indole-3-acetic acid (IAA), catalase activity, HCN (hydrocyanic acid) production and the phosphate solubilization.

IAA production

For detection of IAA, the two bacterial isolates were grown for 24 h to 48 h in nutrient broth medium supplemented with Tryptophane (0.1 mM) in the medium to enhance indole acetic acid (IAA) production by the bacteria (Prinsen *et al* 1993). Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaereet al (1999). For the reaction, 1.5 ml of salkowski reagent, consisting of 12 g $FeCl_3$ per litre in 7.9 M H_2SO_4 was added to 1.5 ml of sample supernatant, mixed well and kept in the dark for 25 min at room temperature. The color changes of the supernatant indicated the IAA production.

HCN production

Production of hydrocyanic acid was determined by growing the two bacterial isolates at room temperature (37ºC) on a rotary shaker in nutrient broth (NB) media. Filter paper (Whatman no.1) was cut into uniform strips of 8 cm long and 0.8 cm wide saturated with alkaline picrate solution (0.05% solution in 2% sodium carbonate) and placed inside the conical flasks in a hanging position and incubated at 37ºC for 72 hr. Color changes of the filter paper or the media due to the presence of picrate indicated the hydrocyanic acid production (Bakker and Schippers 1987).

Phosphate solubilisation

Primary phosphate solubilizing activities of the two isolated bacteria were carried out by allowing the bacteria to grow in selective medium i.e., Pikovskaya's agar (Himedia- M520; ingredients- yeast extract-0.50 g/l, dextrose- 10.00 g/l, potassium hloride- 0.20 g/l, magnesium sulphate- 0.10 g/l, manganese sulphate- 0.0001 g/l, calcium phosphate- 5.00 g/l, ammonium sulphate- 0.50 g/ l, ferrous sulphate- 0.0001 g/l and agar- 15.00 g/l) for 7 to 10 days at 37°C (Pikovskaya 1948). The appearance of a transparent halo zone around the

bacterial colony indicated the phosphate solubilizing activity by the bacteria.

Catalase production

Catalase production test was done for the two bacterial isolates. One drop of H_2O_2 was taken in a slide, one loopfullbacteria were added to it. Gas formation had been noticed (Karen Reiner 2010).

Preparation of bacterial sample for Scanning Electron Microscopy (SEM)

The samples were prepared for SEM by using following method as proposed by USIC, University of North Bengal .Samples of bacteria were cultured in NA (Nutrient agar) plate by streaking method and incubated for 24 h. A solution of 0.2% of chilled glutaraldehyde was taken in a 0.5ml Tarson tubes (half of tube was filled). Bacteria were taken from the NA plates by using an inoculation needle and put into the Tarson tubes. The samples were incubated at 4^0C for the entire night. Sterilized distilled water was used to wash the samples at least twice.Then the water was removed by using a micro pipette. Various ethanol grades of 30%, 50%, 70%, 80%, 90% and 100% were used for sample dehydration. The samples were incubated in each ethanol grade for 5 minutes. By placing the bacterial sample on a cut piece of glass slide (10 mm square) by drop fix method, samples of bacteria for SEM were prepared. Scanning Electron microscopy was carried out with a JSM IT-100 model at the USIC, University of North Bengal.

Antibiotic sensitivity test

For the test of antibiotic resistance, the two endophytic bacteria (MRH- 11 and MRH-06) were grown in NA (nutrient agar) plate by using pour plate method and two antibiotic disc (Chloramphenicol 30µg and Ampicillin 10µg) were placed in each plate in slight distance from each other by using a sterile forceps and incubated for 24 hrs. A clear zone around the antibiotic disc represents zone of inhibition or sensitivity to the antibiotic and no zone around the disc represents resistance of the bacteria to the antibiotic.

Identification of endophytic bacterial isolates (MRH-06, MRH-11) based on 16S rRNA Sequence DNA Isolation

DNA Isolation from two bacterial isolates MRH-06 and MRH-11, Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 from Zymo Research was used.

Agarose gel electrophoresis

The amplified DNA was separated by electrophoresis in 0.8% agarose gel run in 1× TAE buffer at 50V for 30 to 45 minute till DNA fragments are migrated well. The gel was photographed on gel documentation system.

DNA quantification

The concentration of each DNA sample was measured by Nanodrop (Biotech instruments, USA). DNA was stored at”80ÚC for further use. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 to 2.0 is generally accepted as “pure” for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

PCR Amplification

The four deoxyribonucleotide triphosphates (dNTPs) (200 M each), the forward and reverse primers (10 pmol each), Taq DNA polymerase (0.5 U), MgCl2 (2.5 mM), and PCR buffer (1x concentration) (Invitrogen, Life Technologies, Brazil) were used to run the PCR in a total volume of 25 ìl . The DNA templates were heated at pre-denaturation temperatures of 95 °C for 5 min to denature them. This was followed by 39 cycles of denaturation at 95 °C for 30 sec, annealing for 45 sec, and elongation for 1 min before a final extension of 7 min at 72 °C. Following that, 1.5% agarose gel with 0.5x tris-acetate-EDTA (TAE) buffer was used to resolve the amplicons.

Sequencing and analyis

DNA was isolated from the bacterial sample. Its quality was evaluated on 1.0 % agarose gel, a

single band of high-molecular weight DNA has been observed. Fragment of 16S rRNA gene was amplified by forward and reverse primer pairs (27F - AGAGTTTGATCCTGGCTCAG) and (1492R - TACGGTTACCTTGTTACGACTT) using BDT v3.1 Cycle sequencing kit in ABI 3730xl Genetic Analyzer. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI GenBank database. Based on maximum identity score sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA7.

Identification of the fungal pathogen (MLP-01) based on 18s partial rRNA sequence

Genomic DNA was isolated using Genei extraction kit using standard procedure. Isolated DNA was amplified with ITS1 and ITS4 universal forward and reverse primers generating a DNA fragment of approximately 958 bp. In a reaction volume of 25 ìL, PCR was carried out using 0.5 pmol of forward and reverse primers, 50-100 ng of genomic DNA, and 1.0 U of Taq DNA polymerase in addition to 1.5 mmolL^{-1} of $MgCl_2$ (Genei, India), 100 L•mol•L-1 of each dNTP, and 1X Taq polymerase buffer (Genei, India). In a thermal cycler, PCR amplifications were carried out (Biorad, USA). The thermal conditions for ITS1 and ITS4 primer pair were [95ÚC for pre-denaturation, 3min(one cycle); 95ÚC for denaturation, 45 sec, annealing 58ÚC, 45 Sec, extension 72ÚC, 1 min (30 cycles); final extension 72ÚC, 10 min (one cycle)]. The amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10 mg/ml) and visualized by gel documentation system. DNA was eluted in 200 ìl preheated elution buffer and stored at -20°C until further analysis. The purified amplicons were sequenced by Sanger method of sequencing. The sequencing results were assembled and compared with NCBI databases.

RESULTS AND DISCUSSION

The scanning electron microscopic images of two bacterial isolates are shown in Fig. 2. These 16S rDNA fragment sequences of strains MRH-06 and MRH-11 were deposited on GenBank with accession numbers ON955844 and ON955872 respectively. By using nucleotide homology and phylogenetic analysis, the two bacterial isolates MRH 06 and MRH 11, were identified as *Enterobacter cloacae* and *Achromobacter xylosoxidans* respectively. Both bacteria showed catalase production , IAA production , phosphate solubilization, and other attributes which are plant growth promoting (PGP) (Table 1; Fig. 3). By forming a clear zone on the Pikovskaya's (PKV) medium, both the bacterial endophytes (*A. xylosoxidans* and *E. cloacae*) indicated phosphate-solubilizing activity. Both isolates produced IAA and catalase *in vitro*, and they were both susceptible to the antibiotic chloramphenicol (30 g) but resistant to ampicillin (10 g) (Fig. 4). Sing *et al*. (2022) described various plant growth-promoting actions by endophytic *E. cloacae* isolated from *Musa acuminata*, including the formation of IAA, phosphate solubilization, nitrogen fixation, siderophore production, and ammonia production. Wheat plants also harbor the endophytic bacterium *A. xylosoxidans*, which aided in the growth of the plants (Jha and Kumar,2009). Vyas *et al*. (2018) reported *A. xylosoxidans*, which was found in the rhizosphere of *Jatropha curcas* L., has certain properties that encourage plant growth. Bhise *et al* (2017) reported plant growth promoting activities of *E. cloacae.* Ramesh *et al*. (2014) reported plant growth-promoting traits in *E. cloacae* subsp. *dissolvens* MDSR9 isolated from soybean rhizosphere which had impact on growth and nutrition of soybean and wheat. *E. cloacae was* also previously reported to have ability of nitrogen fixation (Ji *et al*. 2020), phosphate solubilization, production of phytohormones, acetoin and bioactive compounds (Khalifa *et al*. 2016). Shahid *et al*. (2012) reported that phosphate-solubilizing *Enterobacter* sp. Fs-11 promoted sunflower (*Helianthus annuus* L.) plant's growth. Phosphate solubilization by *E. cloacae* have significant impact on growth

Table 1: *In vitro* plant growth promoting traits of two endophytic bacterial isolates

PGP activities	MRH-06	MRH-11
	(*Enterobacter cloacae)*	(*Achromobacter xylosoxidans)*
IAA production	+	+
HCN production	+	_
Phosphate solubilisation	+	+
Catalase production	+	+

+= activity present; - = activity absent

Table 2: *In vitro* antagonistic tests of *Enterobacter cloacae* (ON955844) and *Achromobacter xylosoxidans* (ON955872) against *Curvularia lunata* (ON246070)

Test fungi	Paired with bacterium	Dia. of fungal growth (cm)	Zone of inhibition (cm)	% of Inhibition
Curvularia	-	8.8±1.2	-	-
lunata	*Enterobacter cloacae*	2.1±0.22	1.7±0.09	75.0±2.91
	Achromobacter xylosoxidans	1.5±0.09	2.0±0.24	83.5±2.65

*After 7 days; ±= SE

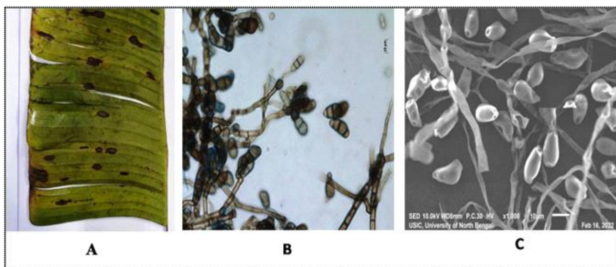

Fig. 1: (A) Leaf spot disease sample, (B) Microscopic view of *Curvularia lunata* spores (40X) (C) Scanning electron microscopic view of *C. lunata* spores

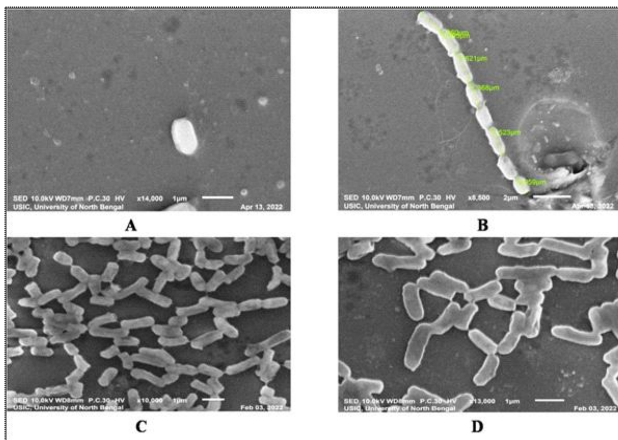

Fig. 2: Scanning electron microscopic views of isolates of endophytic bacteria (A, B) MRH -06 and (C, D) MRH-11

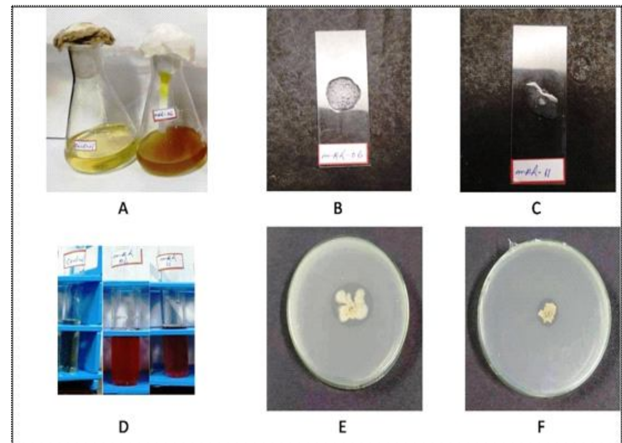

Fig. 3: (A) Colour change of media indicating HCN positive of isolate MRH-06; (B, C) Catalase test positive of MRH- 06 (B) and MRH-11(C) ; (D) IAA test positive of both the bacterial isolates ; (E,F) Halo zones indicating phosphate solubilisation of MRH-06 (E) and MRH-11 (F).

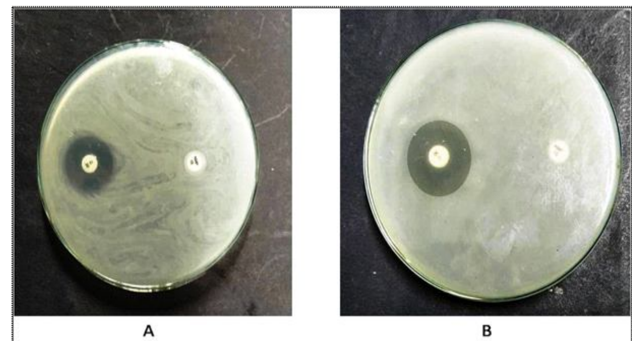

Fig. 4: (A) MRH - 11 showing sensitivity to antibiotic chloramphenicol (30µg) and resistant to ampicillin (10µg); (B) MRH - 06 showing sensitivity to antibiotic chloramphenicol (30µg) and resistant to ampicillin (10µg)

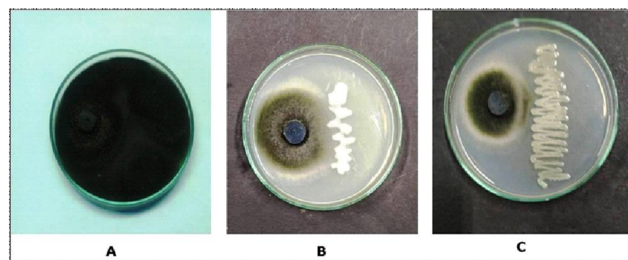

Fig. 5: Inhibition of mycelial growth of *Curvularia lunata* by endophytic bacteria; (A) *C. lunata* growth alone; (B) The antagonistic effect of MRH-06 against *C. lunata* and (C) Antagonistic activity of MRH-11 isolate against *C. lunata*

promotion and boosting yield of wheat plants as reported by Borham *et al.* (2017). IAA-producing bacteria can influence the endogenous auxin pool of host plants and therefore they are able to promote root elongation and plant growth (Patten and Glick 2002). The ability of *A. xylosoxidans* isolate to produce indole acetic acid (IAA) and gibberellins (GA3) was previously reported by

Salem *et al*. (2016). Panigrahi *et al*. (2020) reported that endophytic bacterium *E. cloacae* MG00145, isolated from *Ocimum sanctum* had the ability of Indole Acetic Acid (IAA) production and plant growth promoting capabilities. IAA production, phosphate solubilisation, hydrogen cyanide production by *A. xylosoxidans* were reported by Devi and Mohan (2015).

A fungus (MLP-01) was isolated from the infected leaf part onto potato dextrose agar amended with an antibiotic Monocef. 18S rDNA fragment sequences of strains MLP-01 was deposited on GenBank with accession number ON246070. The fungus was also submitted to NAIMCC (National Agriculturally Important Microbial Culture Collection) with accession number NAIMCC-F-04338 and morphologically identified as *C. lunata* by Agharkar Research Institute, Pune with accession number NFCCI 5361. *E.cloacae* and *A. xylosoxidans*- MRH-06 and MRH-11 showed *in vitro* antagonism against *Curvularia lunata* (Fig.5). The scanning electron microscopic images of antagonist activity of the two endophytic bacterial isolates against *C. lunata* are shown in Fig. 6. *Enterobacter cloacae* inhibited the growth of test pathogens *viz. C. lunata*. *A. xylosoxidans* also checked the growth of the tested pathogen significantly. The tested fungus was inhibited to some degree, the percentage inhibition was 83.5% by *A. xylosoxidans* and 75% by *E. cloacae* (Table 2). According to Ragavi *et al.* (2019), *A. xylosoxidans*, an endophytic banana bacteria, has similar antimicrobial activity against the pathogen *Pectobacterium carotovorum* subsp. *carotovorum*, which causes banana soft rot disease. Devi and Mohan (2015) reported that a bacterial strain of *A. xylosoxidans*, isolated from tannery sludge showed antagonistic activities against various fungi *Alternaria solani*, *Curvularia lunata* and *Fusarium oxysporum*. Endophyte *E. cloacae*, isolated from a banana plant, created a Nutrient-Transfer Symbiosis mechanism with host and also protected the plant from the Black Sigatoka Pathogen Macedo-Raygoza *et al*. (2019).

The Neighbor-Joining approach was used to infer the evolutionary history of all of it .The phylogenetic tree is displayed in Figs. 7 and 8. The tree was built with branch lengths in the same units as the evolutionary distances. The Maximum Composite Likelihood method (Tamura *et al*.2004) was applied to measure evolutionary distances, and the results were summarized in terms of the number of base substitutions per site. All ambiguous locations for each pair of sequences were eliminated using the paired deletion option. 36 nucleotide sequences were used to conduct this analysis for *E. cloacae* MRH-06. the first, second, third, and non-coding coding locations. The final dataset contained 1590 positions in total. The final dataset for analysis of *A. xylosoxidans* MRH-11 comprised a total of 1588 positions and 23 nucleotide sequences . This analysis included a total of 53 nucleotide sequences and 1728 positions for *C. lunata* (MLP-01). By using MEGA 11, these evolutionary studies were carried out (Tamura *et al*. 2021).

CONCLUSION

This study shows that two bacterial isolates- MRH-06 (*Enterobacter cloacae*) and MRH-11 *(Achromobacter xylosoxidans)* have some plant growth promoting characteristics therefore they are beneficial to banana plants and also have leaf spot disease suppression ability against the pathogen *Curvularia lunata*. Utilizing such potent endophytes to restrict or suppress phytopathogenic activity is an alternative method and these have great potential in agricultural in future.

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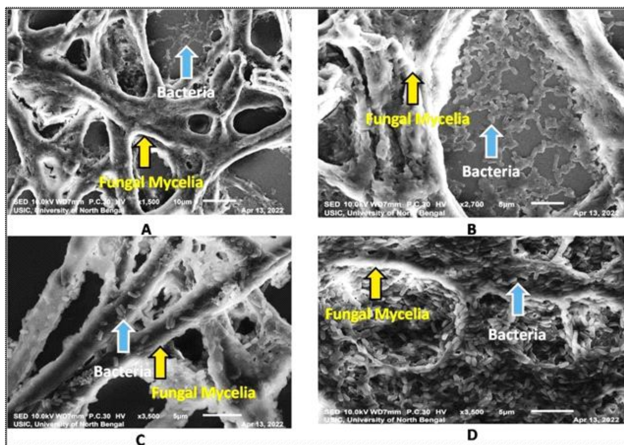

Fig. 6: Scanning electron microscopic view of Junction of MLP-01(*C. lunata*) and (A,B) MRH- 06 and (C,D) MRH-11

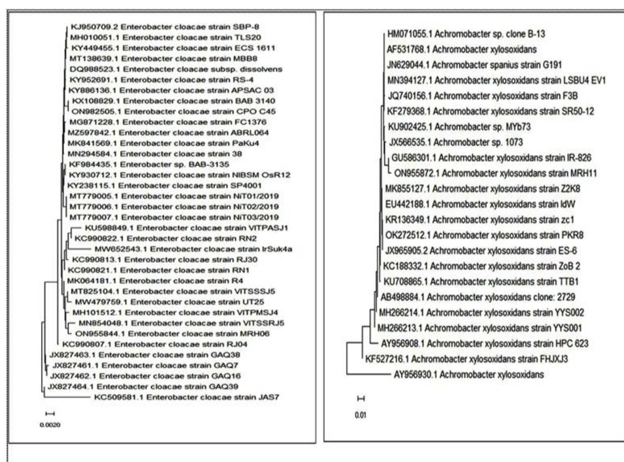

Fig.7: Phylogenetic tree of *Enterobacter cloacae* MRH – 06 (ON955844) which is closely related to strain *Enterobacter cloacae* MN854048 and Phylogenetic tree of *Achromobacter xylosoxidans* MRH – 11 (ON955872) which is closely related to strain *Achromobacter xylosoxidans* GU586301

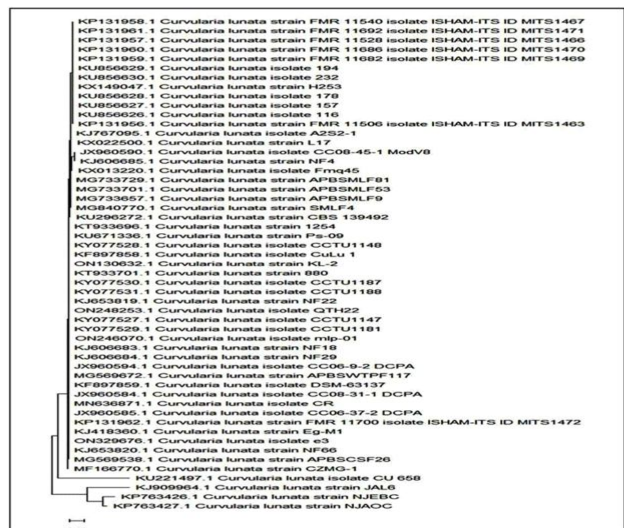

Fig. 8: Phylogenetic analysis of *Curvularia lunata* MLP –O1 (ON246070) which is closely related to strain *Curvularia lunata*KY077529

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