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Evaluation of endophytes and biocontrol agents for the management of a new leaf spot disease in moringa (*Moringa oleifera* Lam.) in Kerala caused by *Colletotrichum fructicola*

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During 2023, a leaf spot disease causing severe defoliation was observed in moringa from Thrissur district, Kerala. The symptoms initiated as pinpoint papery white spots which later expanded to round to elongated papery spots of varying size ranging from one to ten millimeters.The pathogen appeared with white aerial mycelia upon isolation on PDA and covered the nine cm Petri plate within eight days. It produced hyaline, septate, branched hyphae and hyaline bullet shaped conidia of size measured between 7.13 to 10.46 µm × 1.58 to 2.67 µm, having an oil globule. Molecular characterization carried out by ITS sequencing followed by coupling sequences in*in silico* cap3 contig assembly and further processing through the BLASTn at NCBI database revealed the identity of the isolate as *Colletotrichum fructicola.*To our knowledge, this is the first report of *Colletotrichum fructicola* causing leaf spot of moringa in Kerala. Endophytes were isolated from healthy shoots of moringa devoid of leaf spot disease from Thrissur district. The isolated endophytes, *Cerrena* sp. and *Xylaria feejeensis*, and biocontrol agents such as *Trichoderma asperellum* and *Pseudomonas fluorescens* were tested against the pathogen under *invitro* conditions followed by *in planta* experiment. The results revealed that the endophytes and the biocontrol agents were ineffective in managing the disease in one month old moringa seedlings.

Keywords : *Colletotrichum fructicola,* endophytes, leaf spot, moringa

INTRODUCTION

Moringa (*Moringa oleifera* Lam.) is a multipurpose tree, often called as 'miracle tree', belonging to the family Moringaceae. It is an indigenous crop in India and presently grown all across the world (Jattan *et al*.2021).

It is a fast-growing crop, tolerant to wide range of environmental conditions and is a rich source of nutrients, antioxidants, amino acids and antiaging compounds. The young seed pods and leaves of moringa are used as vegetable while other parts are utilized for medicinal and other purposes. India is the foremost producer of moringa, with an annual production of 2.2 to 2.4 million tonnes of tender fruits from an area of 43600 hectares, with a productivity of around 50 tonnes per ha (Sekhar *et al*. 2017).

The challenges encountered during cultivation of moringa include pests and diseases which account for about 82 per cent rotting of trees when grown on lands vulnerable to flooding (Kumssa *et al*. 2017). Foliar diseases were reported in moringa from different parts of world. *Collectotrichum dematium* and *Fusarium solani* are reported to cause a variety of disease symptoms, including leaf spot, chlorosis, necrosis and wilting, which eventually lead to the death of moringa seedlings (Lezcano *et al*. 2014). The plant has great potential and high demand especially as a cheap source of supplements, but is attacked by microbes, especially fungal pathogens, that causes serious foliar infection resulting in defoliation and crop losses. Hence,

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the study aimed to identify the pathogen causing leaf spot diseases, and to assess the antagonistic efficacy of endophytes isolated from healthy plants and biocontrol agents against the pathogen under *in vitro* and *in planta* conditions.

MATERIALS AND METHODS

Isolation and characterization of pathogen

The study was conducted at Department of Plant Pathology, College of Agriculture, Vellanikkara during 2023-2024. Purposive sampling surveys were conducted in moringa growing homesteads and nurseries of Thrissur district of Kerala. The pathogens associated with diseased specimens were isolated using tissue segmentation method (Rangaswamy, 1958). The collected leaves were washed thoroughly with running water. The leaves were surface sterilized using 2 % sodium hypochlorite solution for 2 min and washed thrice with sterile distilled water. After disinfection the leaves were cut into small pieces of 5 mm and placed over PDA media. After incubation, hyphal tips of the fungal colonies were transferred aseptically to fresh PDA media in Petri plates. The fungal cultures were maintained in PDA slants and stored under refrigeration (4°C) for future studies.The pathogenicity of fungal isolates was tested to prove the Koch postulates. Mycelial bit inoculation method (MBIM) explained by Rocha *et al.*(1998) was followed for inoculation of pathogens to confirm pathogenicity. Healthy leaves on the healthy seedlings were inoculated with mycelial bit of the pathogen taken from seven days old culture and with 1 ml of spore suspension containing 1×10^6 spores/ml. A control was maintained with PDA disc or sterile water spray. The inoculated plants were covered in polypropylene cover and maintained in rain shelter for symptom development. When the symptoms appeared, the pathogen was re-isolated from the symptomatic leaves of the inoculated plants into PDA media. The re-isolated pathogen culture was compared with the original culture to prove Koch's postulates. The morphological identification of the fungus was done on the basis of texture, pigmentation, growth pattern of the colony and characteristics of conidia. Molecular characterization was used to confirm the species of the isolated pathogen. The genomic DNA was extracted, sequences were amplified using ITS primers, ITS-1F (TCCGTAGGTGAACCTGCGG) and ITS-4R (TCCTCCGCTTATTGATATGC) followed by sequencing and coupling using *in silico* cap3 contig assembly and processed through the BLASTn at NCBI database.

Isolation and characterization of endophytes

The endophytic microorganisms were isolated from healthy shoots of moringa from Thrissur district. The samples collected were washed thoroughly under tap water, surface sterilized with 70 % ethanol, and then made into small bits using sterilized blade. The bits were disinfected with 1% sodium hypochlorite for about three minutes and washed with 0.02 M phosphate buffer solution (pH=7.4) for four times followed by three consecutive washes with sterile water. From final wash, 100 µl was taken and streaked over solidified PDA media served as the sterility check. The sterilized bits were dried over sterile bloating paper, transferred and placed over solidified PDA media in Petri plate. The plates are then maintained for about two weeks (Uppala, 2007). Cultural and morphological identification of isolated endophytes were done and were molecularly characterizedusing ITS sequencing and *in silico* analyses.

Evaluation of endophytes and biocontrol agents against leaf spot pathogen

The endophytes and the biocontrol agents (*Trichoderma asperellum* and *Pseudomonas fluorescens*) were tested for antagonistic efficacy against leaf spot pathogen under *in vitro* conditions by dual culture assay. One week old culture of pathogen, endophytes and biocontrol agent were taken for the assay. Mycelial disc of 7 mm size of the pathogen and treatments (endophytes and *T. asperellum*) were transferred onto solidified PDA medium in Petri plate. The mycelial disc was placed 2 cm away from the periphery. For dual culture assay of bacterial biocontrol agent *P. fluorescens*,7 mm mycelial disc of pathogen was placed at center and bacterial antagonist was streaked 2 cm away from the periphery of Petri plate.The dual culture plates were compared with control plates, where pathogen was inoculated alone and

measurements were taken radially. Based on that percent inhibition was calculated using the formula given by Vincent *et al*. (1927) :

$$\text{Percent inhibition of growth (PI)} = \frac{C - T}{C} \times 100$$

Where,
C = Growth of pathogen on control plate (cm)
T = Growth of pathogen in treated plate (cm)

The efficacy of endophytes and biocontrol agents in the management of leaf spot pathogen was studied by pot culture experiment using one month old moringa seedlings.The experiment was conducted during December 2023-January 2024. The experiment was laid out in CRD with six treatments and four replications. The treatments were as given below: T1= *Cerrena*sp. (EM 2), T2= *Xylaria feejeensis* (EM 6), T3= *Trichoderma asperellum* (KAU reference culture), T4= *Pseudomonas fluorescens* (KAU reference culture), T5= pathogen inoculated control (*Colletotrichum fructicola*) and T6= uninoculated control. The fungal endophytes and biocontrol agents were given as seed treatment at the rate of 1 g 100 g^{-1} of seeds and mycelial bit inoculation method or foliar sprayat the rate of 10 g l^{-1}15 days after sowing.

RESULTS AND DISCUSSION

Isolation and characterisation of the pathogen

During 2023, a leaf spot disease causing severe defoliation was observed in moringa from Thrissur district, Kerala. The symptoms of leaf spot initiated as pinpoint white spots which later expanded to round to elongated papery spots of varying size ranging from 1-10 mm. The spots were surrounded by distinct chlorotic halo (Fig. 1). The affected leaves dropped and resulted in completely leafless branches. Many leaf spot diseases in moringa caused by fungal pathogens were reported earlier from different countries. Lezcano *et al*. (2014) described about small round leaf spots with light brown center and dark brown edge. Chuku *et al*.(2015) described about anthracnose disease in moringa characterized by brown lesions in leaves. Ponnuswami (2019) described about leaf spots leading to defoliation of moringa plants. These include scattered brown spots later causing leaf blights and leaf drop, circular spots with whitish-grey center and dark brown margins, and circular to angular dark brown spots with concentric circles. But the leaf spots observed from Thrissur were distinctly white and papery and were surrounded by yellow halo. Though the spots were not covering the entire leaflet, it led to dropping of leaflets.

The disease was observed from Chirakkakode, Thrissur district during February 2023. There the incidence recorded was 50 % and the percent disease severity was 54 %. Cent per cent disease incidence was recorded from Polukkara region of Thrissur district during June 2023. This was followed by Vaniyampara and Kuttanellur regions of Thrissur district where the per cent disease incidence recorded was 60 % and 40 % respectively. Per cent disease severity observed was 74 % from Polukkara, 24 % from Kuttanellur and 18 % from Vaniyampara during June 2023.
The pathogen was isolated on PDA medium from the symptomatic area in the leaf spot samples. It produced white, floccose mycelia.The culture covered entire Petri plate after eight days of incubation with an average growth rate 1.29 cm per day. The pathogen exhibited diurnal zonation with greyish brown colour on the lower surface of the culture plate. The hyphae were hyaline,

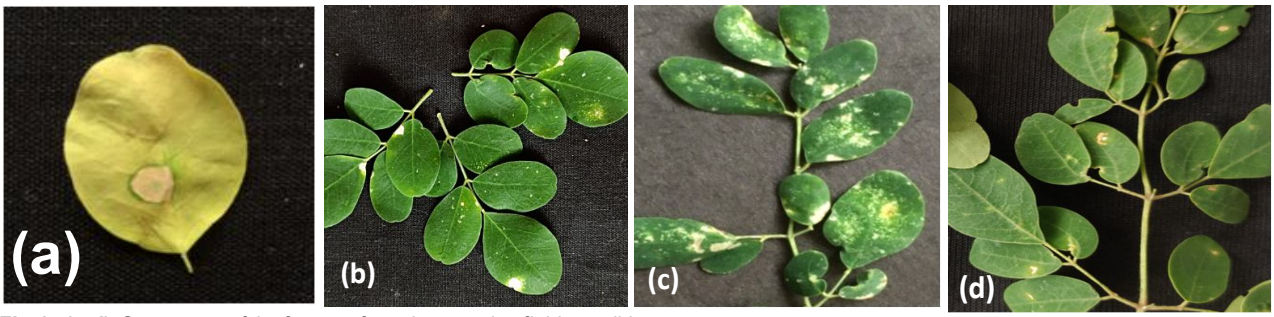

Fig.1: (a-d) Symptoms of leaf spot of moringa under field conditions

septate and branched. The conidia were cylindrical and hyaline with size ranging between 7.13 to 10.46 µm × 1.58 to 2.67 µm (Fig. 2). They were having an oil globule in the center. Based on the cultural and morphological features observed the pathogen was identified as *Colletotrichum* sp. Similar morphological features were documented by Hunupolagama *et al.* (2016) for *Colletotrichum* sp. causing avocado anthracnose in Sri Lanka.

In order to test pathogenicity, mycelial bits were taken from seven days old culture of the pathogen and placed on undersurface of leaflets of healthy plants maintained in rain shelter. The symptoms developed as water-soaked lesions after 24 hrs of inoculation which later turned papery white spots of size ranging from five millimeter to 1.5 cm with darker margins and yellow halo. The entire leaflet turned yellow and finally dropped off (Fig.3**).** Inoculation with spore suspension also produced water-soaked papery white spots surrounded by yellow halo two days after inoculation and the leaflets showing symptoms were fallen down on the following day. The pathogen was re-isolated and compared with the characteristics of original isolateused for inoculation to confirm Koch's postulates. The results revealed that *Collectotrichum* was the causal agent of leaf spot of moringa.

The isolate was molecularly characterized by amplification and sequencing of ITS region using primers ITS-1F/ITS-4R followed by *in silico* analyses. When compared with existing sequences in the NCBI database, the sequence obtained for the pathogen isolate revealed 100 % query cover and 99.66 % similarity with *Colletotrichum fructicola* (accession number MW386819.1). The sequence of the pathogen isolate was deposited in GenBank with accession no. PP830045. The results confirmed the identity of the pathogenic fungus as *C. fructicola.* It has been reported to cause anthracnose disease in various crops such as arecanut, chilli, ivy gourd, ginger, taro, lesser yam, banana, lemon, and mango in India (Chowdappa, 2017). It has also been reported from Kerala, as the cause of leaf spot of culinary melon (*Cucumis melo* var. *acidulus* L.) (Narmadhavathy *et al*. 2016) and rubber (*Hevea brasiliensis*) (Vineeth *et al*. 2024).However, there has been no reports of *C. fructicola* causing leaf spot of moringa in Kerala. According to the available literature, this study can be considered as first report of *C. fructicola* causing leaf spot of moringa in Kerala.

Isolation and characterization of endophytes

The endophytic fungi, EM 2 and EM 6 were isolated from healthy shoots of moringa collected from Thrissur district. The endophytic fungus isolate EM 2 developed cottony white mycelia with entire margin and recorded an average growth rate of 1.87 cm per day (Fig.4 a).The endophytic fungus isolate EM 6 initially produced silky white mycelia with concentric circles and irregular margins. The mycelia later turned to greyish black in colour. The fungus produced stromata on the center of the culture plate after two weeks of incubation (Fig. 4 b). The fungus was slow growing with an average of growth rate of 7.6 mm per day.The endophyte isolates EM 2 and EM 6 were identified through ITS-PCR as *Cerrena* sp.(accession number KX599411.1) and *Xylaria feejeensis* (JQ862606.1) respectively. Endophytic association of *Cerrena* sp. was previously reported in ginger (Anisha and Radhakrishnan, 2017) and cowpea (Divyashree, 2019) from Kerala. Xylariaceous endophytes are generalists and are frequently found in the tropical dicotyledonous woody trees in the forests of Western Ghats, Southern India (Rajulu *et al*., 2013; *et al*. 2018). *Xylaria feejeensis* is also reported to be associated with several plants including orchids (Rajulu *et al*. 2013; Brooks *et al*. 2022; Ma *et al*. 2022).

Evaluation of endophytes and biocontrol agents against leaf spot pathogen

In the dual culture assay, the leaf spot pathogen, *Colletotrichum fructicola* was inhibited by the fungal endophytes *Cerrena*sp. (EM 2) and *Xylaria feejeensis* (EM 6) in the range 64.77 per cent and 65.5 per cent respectively. *Trichoderma asperellum* showed 57.72 per cent inhibition of the pathogen. The bacterial antagonist *Pseudomonas fluorescens* was ineffective in inhibiting the growth of *C. fructicola* (Fig.5, Table1). *X. feejeensis* is reported to have inhibitory activity against plant pathogens such as

Table 1 : *In vitro* evaluation of endophytes and biocontrol agents against *C. fructicola*

Treatments	Per cent inhibition	Antagonistic reaction
Cerrena sp. (EM 2)	64.95 (53.703) [a]	Overgrowth
Xylaria feejeensis (EM 6)	65.62 (54.093) [a]	Overgrowth
Trichoderma asperellum	57.45 (49.286) [b]	Overgrowth
Pseudomonas fluorescens	0.00 (9.594) [c]	Cessation of growth
Colletotrichum fructicola	0.00 (9.594) [c]	Control
CV	1.446	
CD (0.05)	0.768	

*Mean of four replications; in each column figure followed by same letter do not differ significantly according to DMRT; values in parenthesis are angular transformed values

Table 2: Effect of various treatments against *Colletotrichum fructicola*in moringa seedlings at 30 days after sowing

Treatment	Per cent disease incidence (%)	Per cent disease severity (%)	Length of leaves (cm)	Length of root (cm)	No. of leaves	No. of roots	Plant height (cm)	Fresh weight (g)	Dry weight (g)	Root biomass (g) Fresh	Dry
T1	100.00 (10.02)	80.4 (63.790) [c]	9.085 [b]	7.250 [ab]	6.75	32.75	24.625 [bc]	4.883 [a]	0.926	2.320	0.318 [a]
T2	100.00 (10.02)	69.85 (56.721) [d]	8.625 [b]	6.500 [b]	6.50	38.75	24.750[bc]	3.881 [a]	0.731	1.903	0.186 [b]
T3	100.00 (10.02)	85.3 (67.520) [b]	8.625 [b]	7.175 [ab]	6.50	34.25	22.825 [c]	3.916 [a]	0.800	1.730	0.156 [b]
T4	100.00 (10.02)	67.925 (55.514) [d]	8.500 [b]	7.000 [ab]	6.75	50.25	25.750 [bc]	4.240 [a]	0.744	2.006	0.156 [b]
T5	100.00 (10.02)	91.575 (73.266) [a]	9.625 [b]	8.250 [a]	7.00	40.50	28.000 [ab]	2.148 [b]	0.837	1.898	0.187 [b]
T6	0.00 (9.594)	0.00 (9.594) [e]	11.000 [a]	6.175 [b]	7.00	43.50	31.000 [a]	1.565[b]	0.778	1.552	0.202 [b]
CV (0.05) (%)	0.00	3.686	7.499	11.243	8.189	23.928	8.845	32.572	34.123	20.444	33.898
CD	-	2.979	1.03	1.179	-	-	3.437	1.664	-	-	0.101

*Mean of three replications; in each column figure followed by same letter do not differ significantly according to DMRT; values in parenthesis are angular transformed values

Curvularia, *Alternaria* and *Fusarium* (Sopalun *et al*. 2021; Brooks *et al*. 2022). Sopalun *et al*. (2021) reported that *X. feejeensis* isolated from leaf of *Avicennia marina* exhibited highest inhibitory activity against *Curvularia* sp. Crude extract of *X. feejeensis* SRNE2BP isolate obtained from mangrove tree significantly inhibited *Fusarium oxysporum* MFLUCC 19-0157 and *Alternaria solani* MFLUCC 19-0093 growth approximately 60-75 per cent and 56-87 per cent respectively in *in vitro* and *in situ* assays (Brooks *et al*. 2022). The *X. feejeensis* isolate from Cardaba banana showed inhibition of 93.33 per cent against *F. odoratissimum* causing Panama disease in

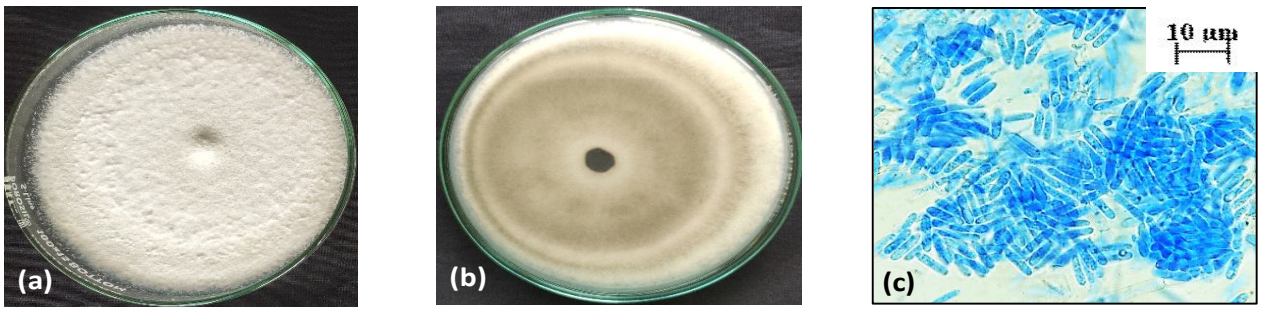

Fig. 2: Cultural and morphological characters of *Colletotrichum fructicola*(a) Front view of colony on PDA (b) Reverse view of colony on PDA (c) Conidia (1000 X)

banana, indicating a very high degree of antagonism (Taping *et al.* 2023).

The endophytes *Cerrena* sp. (EM 2) and *Xylaria feejeensis* (EM 6) and the biocontrol agents *Trichoderma asperellum* and *Pseudomonas fluorescens* were tested in a pot culture experiment on one month old moringa seedlings to evaluate the efficacy in managing leaf spot disease caused by *C.fructicola* (Fig. 6). The results of the experiment are presented in (Table 2). Cent per cent incidence of leaf spot disease was observed after two days of spray inoculation on the following treatments: T1 (*Cerrena* sp. (EM 2)), T2 (*X. feejeensis* (EM 6)), T3 (*T. asperellum*), T4 (*P. fluorescens*) and T5 (leaf spot pathogen *C. fructicola*). No incidence was recorded on the un-inoculated control (T6).The highest disease severity was observed on treatment T5 (leaf spot pathogen *C. fructicola*) (91.5 %) which is the pathogen inoculated control. This was followed by T3 (*T. asperellum*) which showed 85.3 % severity. In comparison to the other treatments, T4 (*P. fluorescens*) and T2 (endophyte isolate *X. feejeensis*(EM 6)) showed the lowest severity of 67.93 % and 69.85 % respectively (Fig. 7). Among the biometric observations, highest plant height (31.0 cm) was noticed in T6 (uninoculated control) and it was on par with T5 (pathogen inoculated control) (28 cm). The lowest plant height of 22.83 cm was recorded for treatment T3, (*T. asperellum*) was on par with plant heights recorded for T1 (*Cerrena* sp. (EM 2)), T2 (*X. feejeensis* (EM 6)) and T4 (*P. fluorescens*).The leaf length of all the treatments except uninoculated control was in the range of 8.5-9.6 cm and were on par.Leaf length was highest (11.0 cm) in uninoculated control plants (T6). But the lowest root length (6.175 cm) was observed in T6 and it was on par with the root length (6.5 cm) observed in T2 (*X. feejeensis* (EM 6)).The fresh weight of the plant was found to be highest (4.883 g/plant) in treatment T1, (*Cerrena* sp. (EM 2)). The lowest fresh weight (1.472 g/plant) was observed in T6 (uninoculated control). The data revealed no significant difference among treatments in case of dry weight of the plant.Fresh and dry root biomass was recorded highest (2.32 g/plant and 0.318 g/plant)in the treatment T1, (*Cerrena* sp. (EM 2)). The fresh root biomass among the treatments were not significantly different. The dry root biomass of the treatments T2, T3, T4, T5 and T6 were on par.The colonization of both the endophytes *Cerrena* sp. (EM 2) and *X. feejeensis* (EM 6), was confirmed by re-isolation from stem taken from T1 and T2. But, the endophytic fungi *viz.*, *Cerrena* sp. (EM 2) and *X. feejeensis* (EM 6) and the biocontrol agents *viz*., *Trichoderma asperellum* and *Pseudomonas fluorescens* were not effective in the management of leaf spot disease in one month old seedlings. No significant growth promotion was also shown by the endophytes and the biocontrol agents.This could be due to the fact that the endophytes isolated from stems may not colonize the leaf tissues or they may take longer to colonize the leaf tissues. Therefore, a long-term study is needed to understand the interaction of these potential antagonistic endophytes*viz*., *Cerrena* sp. (EM 2) and *X. feejeensis* (EM 6), moringa and the pathogen. Though there were some reports of disease reduction by *Pseudomonas*in the case of anthracnose of plum caused by *Colletotrichum fructicola* (Chen *et al*. 2023) under pot culture study, here not much effect was noticed.

CONCLUSION

Leaf spot can be considered as an emerging threat for moringa farmers causing severe defoliation of the plant.The study describes the occurrence and symptoms of leaf spot disease in moringa in Kerala. The pathogen was identified as *Colletotrichum fructicola*. The study also aimed at isolating endophytes from healthy stem of moringa plants which yielded two potential endophytes *Cerrena* sp. (EM 2) and *Xylaria feejeensis* (EM 6). Under *in vitro* conditions, the endophytes and *Trichoderma asperellum* effectively inhibited the pathogen *Colletotrichum fructicola*. But in the *in-planta* experiment, the severity of leaf spot disease didn't reduce much.This may be due to the lower colonization of endophytic fungi and *Trichoderma asperellum* on the leaves of one month old seedlings.

DECLARATION

Conflict of interest**.** The authors declare no conflict of interest.

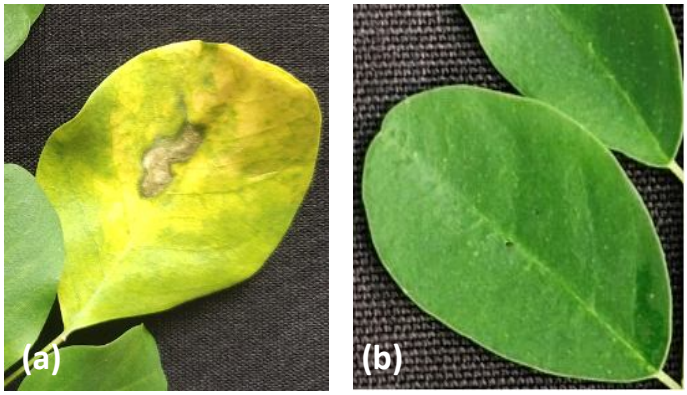

Fig. 3: Symptoms of leaf spot of moringa upon artificial inoculation. (a) Artificially inoculated leaf after 24h (b) Control

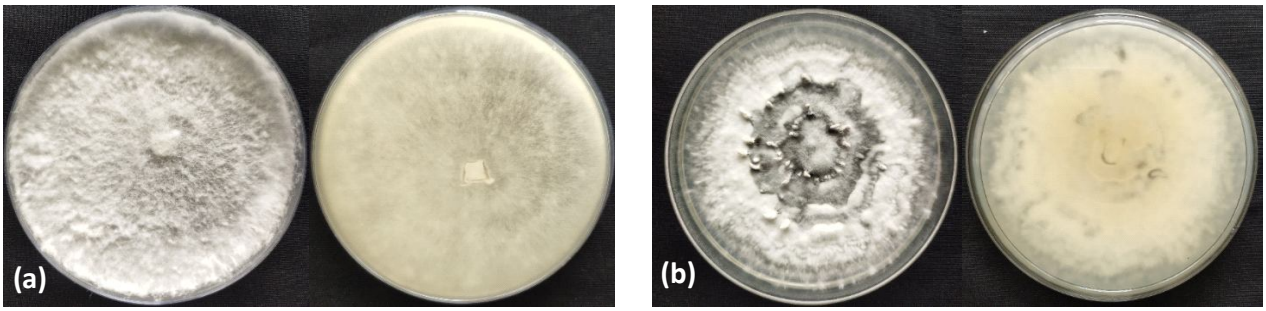

Fig. 4: Endophytes isolated from healthy shoots of moringa. (a) Front and reverse view of *Cerrena* sp. (EM 2) (b) Front and reverse view of*Xylaria feejeensis* (EM 6)

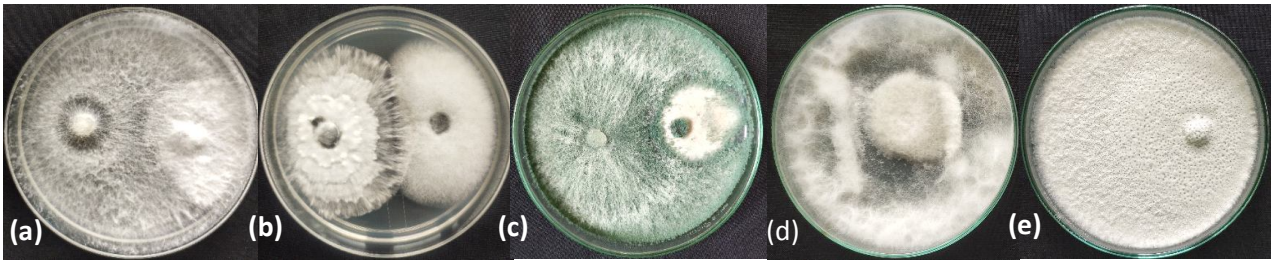

Fig. 5: *Invitro* evaluation of endophytes and biocontrol agents against *C. fructicola.* (a) *Cerrena* sp. (EM 1), (b) *Xylaria feejeensis*(EM 6), (c) *T. asperellum*, (d) *P. fluorescens* and (e) *C. fructicola* (Control)

Fig. 6: General view of experiment –*In planta* evaluation of endophytes and biocontrol agents against leaf spot disease caused by *Colletotrichum fructicola*inmoringa seedlings

Fig. 7: Effect of various treatments on leaf spot disease caused by *C.fructicola*in moringa seedlings

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