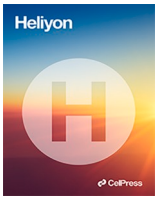
Heliyon 10 (2024) e32310

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Mangrove endophytic fungi: Biocontrol potential against *Rhizoctonia solani* and biofertilizers for fragrant rice cultivation

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ARTICLE INFO

Keywords:
Bioactive compounds
Endophytes
MRQ76
Oryza sativa
Sheath blight disease
Seed inoculation
Soil inoculation

ABSTRACT

The mangrove ecosystem has emerged as a fascinating source for exploring novel bioresources which have multiple applications in modern agriculture. This study evaluates the potential applications of mangrove endophytic fungi (MEF), such as biocontrol agents against *Rhizoctonia solani* and as biofertilizers for improving the yield of fragrant rice variety Malaysian Rice Quality 76 (MRQ76). Through the antagonism assays, it is observed that among the 14 MEF studied, 4 fungal isolates (*Colletotrichum* sp. MEFN02, *Aspergillus* sp. MEFN06, *Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10) exhibited promising antagonistic effect against the pathogen *R. solani* compared to the chemical fungicide (Benomyl). These isolates also revealed significant production of enzymes, phytochemicals, indoleacetic acid (40.96 mg/mL) and ammonia (32.54 mg/mL) and displayed tolerance to salt and temperature stress up to 2000 mM and >40 °C respectively. Furthermore, employing the germination and pathogenicity test, inoculation of these endophytes showed lower percentage of disease severity index (DSI%) against *R. solani*, ranging from (24 %–46 %) in MRQ76 rice seedlings. The *in-vivo* experiments of soil and seed inoculation methods conducted under greenhouse conditions revealed that these endophytes enhanced plant growth (8–15 % increase) and increased crop yield (≥50 %) in comparison to control treatments. The current findings provide valuable insights into eco-friendly, cost-effective and sustainable alternatives for addressing *R. solani* infection and improving the agronomic performance of the fragrant rice cultivar MRQ76, contributing to food security.

1. Introduction

Rice (*Oryza sativa* L.) is a widely cultivated crop which serves as the staple dietary for more than half of the world's population, particularly to Asians and Africans [1]. However, it is prone to both biotic and abiotic stresses whose combined effect can reduce the average crop yield by more than 50 % [2–5]. Sheath blight, triggered by the soil-borne, necrotrophic fungus *Rhizoctonia solani* (*R. solani*) J.G. Kühn, ranks as the second most devasting disease in rice, affecting yield significantly, especially in tropical Asia where it can reduce outputs from 10 to 50 % in major rice-producing areas [6,7]. Brunei, introduced Malaysian Rice Quality 76 (MRQ76) to

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https://doi.org/10.1016/j.heliyon.2024.e32310
Received 10 February 2024; Received in revised form 22 May 2024; Accepted 31 May 2024
Available online 1 June 2024

increase the country's rice production. However, this variety faced challenges with *R. solani* infestation [8,9]. The use of chemical pesticides to control pathogens benefits the crop yield, in turn, reduces the beneficial microbes and increases the salinity of the soil, leading to a decline in soil quality [10]. Therefore, there is a growing need to explore and adopt eco-friendly biocontrol methods and biofertilizers as sustainable alternatives to chemical treatments [11].

Researchers are increasingly focusing their attention on beneficial microbes as a promising biocontrol agent (BCA) and biofertilizer [12,13]. For example, Abbas et al. [14] documented a noteworthy antifungal effect of isolates from *Trichoderma virens*, demonstrating their antagonistic properties against *R. solani* in cotton and zinnia. *Aspergillus terreus* exhibits an antifungal mode of antagonistic effect to suppress *R. solani* in legumes, such as *Phaseolus vulgaris* and Vicia *faba* [15]. Safari Motlagh et al. [7] demonstrated that *Trichoderma virens* and *Aspergillus fumigatus* act as mycoparasite antagonists, effectively controlling rice sheath blight disease.

Furthermore, *Trichoderma* isolates have proven to be effective agents in controlling *Rhizoctonia* root rot of strawberries by producing antifungal volatile metabolites that reduced mycelial growth of *R. solani* [16]. *Bacillus amyloliquefaciens* (3MPE1) and *Pantoea ananatis* (1MSE1) endophytes isolated from *Rhizophora apiculata* have been shown to enhance the growth of rice seedlings [17]. Sreeja et al. [18] identified 23 endophytic fungal species from *Piper nigrum*, with displaying inhibitory effects to *Phytophthora capsici*, a pathogen causing blight and root rot in plants. The colonization of *Aspergillus awamori* Wl1, isolated by Asif Mehmood et al. [19], and *Colletotrichum* sp. SL4, isolated by Roy et al. [20], were found to improve plant growth through the production of IAA, secondary metabolites, ammonia and siderophores.

Mangrove endophyte fungi (MEF) have garnered attention for their resilience to extreme climatic and salinity conditions. These MEF produce secondary metabolites, phytohormones, siderophores and enzymes that enhance plant protection against pathogens and promote growth in biotic and abiotic stress [21–24]. Although extensive research has been conducted on the medicinal properties of MEF, their promising applications in the agricultural sector, particularly in rice cultivation, remains largely unexplored. This study aims to bridge this knowledge gap by examining the potential uses of selected MEF to serve as biocontrol agents against *R. solani* and as biofertilizers to improve MRQ76 rice yield.

2. Materials and methods

2.1. Biological and chemical materials

Fungal cultures were obtained from the microbial culture deposits available at the Faculty of Science, Universiti Brunei Darussalam (UBD), Brunei Darussalam. This includes 14 mangrove endophytic fungi (MEF) Mohamad et al. [25]: 3 species isolated from *Rhizophora apiculata* (Rhizophoraceae), 5 species from *Nypa fruticans* (Arecaceae) and 6 species from *Xylocarpus granatum* (Meliaceae) and the biological data and gene bank accession numbers of the aforesaid 14 MEF are given in Table 1. *Rhizoctonia solani* AG1-IA Kühn (*R. solani*), the causative agent of sheath blight disease in rice (GenBank Acc No. MW876227), was also obtained from UBD [6,26]. A fragrant rice variety MRQ76 seeds and fungicide (Benomyl) was benevolently granted by the Rice Industry Unit, DOAA. Analytical grade organic and inorganic chemicals, fungal growth media and broth were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA.). and Hi-Media Laboratories Pvt. Ltd (Mumbai, India).

2.2. Cultivation

All the fungal isolates were cultured on potato dextrose agar (PDA) and then incubated at 25 °C for a week duration [27]. For stock maintenance, the culture was regenerated every 3 weeks on fresh PDA plates and refrigerated at 4 °C. The cultured fungal isolates were then used to study antagonistic activities.

Table 1
Mangrove endophytic fungi (MEF) with gene bank accession numbers (GenBank Acc. No.) and their host plants [25].

No.	Host plant	MEF Family	MEF species	GenBank Acc No.
1	*Rhizophora apiculata*	Hypoxylaceae	*Annulohypoxylon* sp. MEFR03	0P508381
2		Trichocomaceae	*Penicillium* sp. MEFR09	0P508383
3		Glomerellaceae	*Colletotrichum* sp. MEFR10	0P508384
4	*Nypa fruticans*	Glomerellaceae	*Colletotrichum* sp. MEFN01	0P508391
5		Glomerellaceae	*Colletotrichum* sp. MEFN02	0P508392
6		Glomerellaceae	*Colletotrichum* sp. MEFN03	0P508393
7		Glomerellaceae	*Colletotrichum* sp. MEFN04	0P508394
8		Trichocomaceae	*Aspergillus* sp. MEFN06	0P508395
9	*Xylocarpus granatum*	Hypoxylaceae	*Daldinia* sp. MEFX01	0P508398
10		Hypoxylaceae	*Annulohypoxylon* sp. MEFX02	0P508399
11		Chaetomiaceae	*Chaetomium* sp. MEFX06	0P508401
12		Botryosphaeriaceae	*Lasiodiplodia* sp. MEFX09	0P508404
13		Trichocomaceae	*Aspergillus* sp. MEFX10	0P508405
14		Hypocreaceae	*Trichoderma* sp. MEFX11	0P508406

2.3. Biocontrol agent assay

2.3.1. Fungicide tolerance level

The fungicide tolerance level of *R. solani* was estimated based on the method of Lezama et al. [28], with slight modification. The stock concentration of the fungicide (Benomyl) was prepared by mixing it (50 mg) in distilled water (100 mL), then serially diluted to obtain four different concentrations (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL and 50 mg/mL), which were used as positive controls. Sterile distilled water (SDW) was used as the negative control. The antagonist effect of the endophytic fungal species was estimated by the agar disk diffusion method using the aforementioned concentrations. The concentration of the fungicide that showed an inhibitory effect on the pathogen was further used in antagonistic assays below.

2.3.2. Antagonistic activity against pathogen

Antagonistic activities of the 14 MEF (refer Table 1) against *R. solani* were evaluated through dual culture plate assay, non-volatile compounds assay and volatile compounds inverted assay.

2.3.2.1. Dual culture plate assay. The dual culture technique was conducted based on the method of Sornakili et al. [29]. The antifungal activities of 14 MEF were tested against *R. solani* by co-inoculating the mycelial plugs of both pathogen and endophyte in each half of the PDA plates. The pathogen-inoculated PDA plates that replace MEF with fungicide (disk diffusion) and SDW served as positive control and negative control, respectively. The plates were incubated at 27 °C for a week. Six replicates were carried out to determine their antagonistic activities and the fungal interactions between the pathogen and endophytes [30]. The percentage inhibition (PI) for dual culture plates was computed using the following equation:

$$PI(\%) = 100 * [(C - T) / C] \quad \text{Equation (1)}$$

where PI, C and T represent percent inhibition, radial pathogen growth in the control plate and radial pathogen growth in the treated plate, respectively.

2.3.2.2. Non-volatile compound assay. The antagonistic effects of non-volatile metabolites produced by the 14 MEF were investigated using the techniques described by Hamzah et al. [27]. A MEF (5 mm disc) was inoculated in a conical flask (250 mL) containing 100 mL of potato dextrose broth. The mixture was subsequently placed in an incubator shaker at 27 °C for a week. After incubation, the culture filtrate was filtered using Whatman No.1 filter paper, and then mixed with molten PDA (20 % v/v final concentration). The mix was poured into petri dishes and left to solidify. For negative and positive controls, culture filtrate was replaced by SDW and Benomyl fungicide respectively. Once solidified, the plates were inoculated with *R. solani* (5 mm disc) by placing the disc at the center of a PDA plate. The plates were incubated at 27 °C for 7 days. After the incubation period, the radial growth of the pathogen on the plate was measured and compared with the pathogen growth on the control plates. The percentage inhibition was also calculated using Equation (1).

2.3.2.3. Volatile compounds inverted assay. Antagonistic volatile compounds produced by 14 MEF, which can inhibit the pathogen's growth, were evaluated by the volatile compounds inverted assay, following Sornakili et al. [29]. An antagonist and the pathogen (*R. solani*) mycelial plug (5 mm) were individually inoculated in a PDA and placed overnight at 20 °C. The plates with the pathogen and endophyte were opened, and placed over each other in an inverted position, sealed in such a way that both cultures were facing each other. For negative and positive controls, endophytes was replaced by SDW and Benomyl fungicide respectively The plates were then incubated at 27 °C for 7 days, ensuring that the pathogen was exposed to the volatile compounds liberated by MEF [31,32]. The experiment was replicated six times to determine the antagonist efficacy of MEF's volatile compound. The percentage inhibition was also computed using Equation (1).

Only four of the 14 MEF studied (*Colletotrichum* sp. MEFN02 and *Aspergillus* sp. MEFN06 from *N. fruticans* and *Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10 from *X. granatum*) exhibited promising forms of antagonism (matrix competition and antibiosis) based on the results of the antagonistic assays. As a result, they were further employed in the subsequent experiments below.

2.3.3. Hyperparasitism activities assay

The slide culture test of four antagonistic MEF was conducted using a sterile slide, filter paper and a U-shaped glass rod [29]. Firstly, the filter paper was placed inside the petri dish, followed by placing the slide containing the thin square slice of water agar medium over the U-shaped glass rod. Then, small mycelial plugs of an antagonistic individual MEF and *R. solani* were placed opposite each other on the medium at a distance of 2 cm. The moisture in the petri dish was maintained by adding SDW (2 mL). After 7 days of incubation, the interactions between the hyphae of MEF and the pathogen were observed under a DP20 digital microscope camera (Olympus, Japan) with a 1200-1600 X pixel monitor, and their images were captured.

2.3.4. Hydrolytic enzyme activities assays

The four MEF were evaluated for their extracellular enzyme activities by growing them on specific indicator media containing corresponding substrates following the methods described by Sornakili et al. [31] and Hankin and Anagnostakis [33], for amylase and cellulase, Toghueo et al. [34] for laccase activity, Abe et al. [35] for lipase and protease activity, and Rodríguez and Fraga [36] for phosphatase activity. After incubating the plates at 27 °C for 3–5 days, the area of clearance around each fungal colony, indicating

enzyme activity, was measured in accordance with the method described by Sornakili et al. [29]. However, for laccase enzyme activity, a color change was observed instead, as described by Toghueo et al. [34]. Enzyme Index (EI) of amylase, lipase, cellulase, protease and phosphatase were calculated using the formula as described by Sornakili et al. [29]:

$$EI = R/r \quad \text{Equation (2)}$$

where R is the average diameter of the clear zone and r is the average diameter of the colony.

2.3.5. Phytochemical screening assays

The presence of alkaloids, phenols, tannins, triterpenoids, saponins, and flavonoids was investigated in the fungal crude extract adapting the standard procedure described by Thorati and Mishra [37], Jagadevi Shivaputrappa [38] and Bhardwaj et al. [39]. Distilled water (5 mL) and hydrochloric acid (2 mL) were combined with the extract (2 mg), and subsequently, Dragendorff's reagent (1 mL) was introduced. The emergence of an orange or orange-red precipitate signified the presence of alkaloids. To ascertain the presence of phenols, lead acetate (10 %) was introduced into the extract, leading to the formation of a substantial white coloration. For triterpenoids presence, few drops of 5 % w/v ferric chloride are added to the 2 mL extract resulted in the formation of a green or blue-green precipitate, indicative of the presence of tannins. When concentrated sulfuric acid was carefully introduced into the test solution, the lower layer turning yellow indicated the existence of triterpenoids. To detect saponins, the extract (5 mL) was mixed with a drop of sodium bicarbonate solution and agitated, yielding the formation of a frothy, honeycomb-like structure. For flavonoids presence, the addition of 10 % sodium hydroxide solution to the test solution led to the development of a yellow coloration, which subsequently became colorless upon the introduction of a few drops of diluted acid, confirming the presence of flavonoids.

2.3.6. Temperature and salinity tolerance assays

The four MEF isolates were inoculated on PDA plates and cultured at three different temperatures (25 °C, 30 °C, and 35 °C) for a week. To determine fungal species with greater temperature tolerance, those fungi that demonstrate growth at 35 °C were further subjected to additional testing at 40 °C, 45 °C and 50 °C [40]. All experiments were conducted in triplicates.

For screening the salinity tolerance in MEF, five-day-old cultures were introduced to PDA plates containing five concentrations of sodium chloride, NaCl (200 mM, 400 mM, 600 mM, 800 mM and 1000 mM), the control group was maintained in the absence of NaCl, also in triplicates. Following a one-week duration of incubation at 27 °C, the percentage reduction of mycelial growth compared to the respective controls was calculated by implementing the procedure described by Bekker et al. [41] and Sampangi-Ramaiah et al. [42]. For the specific species that exhibited higher NaCl tolerance, further testing was carried out using elevated NaCl concentrations of 1500 mM and 2000 mM.

2.4. Biofertilizer assay

2.4.1. Ammonia screening assay

The four fungal isolates were screened for ammonia production, as described by Asif Mehmood et al. [19]. The fungal isolates were cultured in 100 mL flasks containing 15 mL Czapek broth medium, composed of glucose (1 %), KCl (0.05 %), peptone (1 %), $FeSO_4.7H_2O$ (0.001 %) and $MgSO_4.7H_2O$ (0.05 %) at pH 7.3 ± 0.2. The cultures were incubated in a shaker at 120 rpm for 7 days at 28 °C, with a control treatment maintained. For qualitative analysis, the cultures were centrifuged at 5000 rpm for 5 min and the supernatant was analysed. The presence of ammonia in the culture filtrate was assessed by observing a brown color change when 0.5 mL of Nessler's reagent was added, and the absorbance was measured at 530 nm using a SpectraMax Plus 384 (Molecular Devices, USA).

2.4.2. Indoleacetic acid (IAA) screening assay

Each of the four isolates was inoculated in Erlenmeyer flasks containing 25 mL of Czapek broth, consisting of glucose (1 %), yeast extract (0.1 %), $FeSO_4$ (0.001 %), $MgSO_4$ (0.05 %), K_2HPO_4 (0.1 %) at pH 7.0. The medium was supplemented with L-tryptophan (0.1 %) as a metabolite precursor and a control was maintained. The flasks were incubated in a shaker at 120 rpm and 28 °C for 7 days following Asif Mehmood et al. [19]. The presence of IAA in the culture filtrate was determined by detecting a red color change after adding 0.5 mL of Salkowski reagent. The absorbance at 530 nm was measured using a SpectraMax Plus 384 microplate reader.

2.4.3. Germination bioassay and pathogenicity tests

This study utilized MRQ76 rice variety which yields low production due to the outbreak of soil borne pathogen *R. solani* [9]. The MRQ76 seeds were sterilized using a 5 % (v/v) Clorox solution and rinsed with SDW. After sterilization, the seeds were treated with a unique mixture of four antagonistic MEFs combined with *R. solani*, while the control group was treated with *R. solani* alone for 4 h using the spore suspension method, following Safari Motlagh et al. [7] and the hyphal fragment suspensions for the *R. solani* isolate was prepared using the method described by Wang et al. [43] with minor adjustments.

The *in-vitro* pathogenicity assay for *Rhizoctonia solani* involves growing fragments of hyphae on artificial media. To produce hyphal fragment suspensions, *R. solani* was cultivated on 2 % PDA in a Petri dish at 25 °C for 7 days. The mycelium was scraped from the agar surface using a sterilized bamboo stick and transferred to 2 % potato dextrose broth (PDB). The mixture was then incubated at 25 °C with shaking at 200 rpm for 3 h. Microscopic examination confirmed the presence of only mycelial fragments. After an additional 2 days of incubation, the mycelial cultures were homogenized at 20,000 rpm for 2 min, yielding 80 % of hyphal fragments, as described by Wang et al. [43]. The fragment length ranges around 100 μm. The fragments from the hyphal suspension were mounted in a drop of

sterile water on glass slides and examined under an Olympus microscope. The concentration of the hyphal fragment suspension and conidia count was measured using a hemocytometer. The suspension concentrations were maintained at 2.5×10^5 conidia/mL for antagonistic MEF and 4×10^5 hyphal fragments/mL for *R. solani* (hyphal fragments were used because fungi do not produce asexual spores and rarely form sexual spores in nature).

Following treatment, approximately 10 seeds were placed on moist Whatman No. 1 filter paper in a petri dish and allowed to germinate for 10 days in an incubator at 27 °C. The experimental design encompassed five treatments comprising of four fungal isolates and a control, each with 10 seeds and 5 replications. On the 10th day, disease development on the hypocotyls of germinated seeds was assessed by evaluating the size of the necrotic area. Scale values were assigned to all seeds in each replicate to determine the diseases severity of each isolate [44]. The percentage of disease severity index (DSI%) was calculated using the Townsend-Heuberger's [45], formula based on scale values obtained from the pathogenicity tests. Germination parameters such as germination percentage (GP%), vigor index1 (VI-1) and vigor index 2 (VI-2) were also determined, following Abdul-Halim et al. [26].

$$\mathrm{DSI\%} = \left[\sum (100 * \mathrm{Score} * \mathrm{Number\ of\ plants\ with\ scores})\right] \Big/ (\mathrm{Total\ number\ of\ plants} * \mathrm{Highest\ Score}) \quad \text{Equation (3)}$$

[45].Where DSI represent disease severity index.

$$GP(\%) = 100 * (Ni / Nt) \quad \text{Equation (4)}$$

[26].Whereby GP, Ni and Nt represents germination percentage, germinated seeds and total number of seeds, respectively.

$$VI - 1 = GP * SL\ (cm) \quad \text{Equation (5)}$$

[26].Whereby VI-1, SL, and GP represents vigor index 1, seedling length, and germination percentage.

$$VI - 2 = GP * SW\ (gm) \quad \text{Equation (6)}$$

[26].Whereby VI-2, SW and GP represents vigor index 2, seedling dry weight and germination percentage.

2.4.4. Greenhouse pot experiment bioassay

2.4.4.1. Plant and soil materials. Healthy and viable MRQ76 seeds were chosen by the seed floating technique [26]. The selected seeds were subjected to surface sterilization by soaking them in SDW overnight, after which they were treated with 75 % ethanol for 30 s, 1 % NaOCl for 10 min and 70 % ethanol for 30 s. The effectiveness of the surface sterilization was confirmed by incubating the seeds on PDA plates supplemented with chloramphenicol [46]. For the pot experiments, we obtained soil from the Wasan agricultural farm, collected in March 2021 with the assistance from the rice Industry Division, DOAA. The soil properties of the Wasan field are given in Table 2.

2.4.4.2. Soil and seed inoculation methods. We employed soil and seed inoculation methods to investigate the effect of MEF on the growth of MRQ76, following the procedures outlined by Wijesooriya and Deshappriya [46]. For soil inoculation, a 100 mL spore suspension (1 x 10^5 spores/mL) of each MEF (4 antagonistic MEF) was added to the sterilized soil surface before planting. In control pots, SDW was added instead of fungal cultures. The non-inoculated seeds were wrapped in moist sterile tissue paper and incubated at room temperature (27 °C) for 5 days or until visible germination occurred. Conversely, for seed inoculation, 25 seeds were immersed in a 30 mL suspension of distinct MEF or SDW (control) for 4 h in a shaker at 180 rpm. The immersed seeds were then dried for 20 min in sterile petri dishes. Similar to the soil inoculation method, the inoculated and non-inoculated seeds were germinated.

For both the soil and seed inoculation methods, pots (28 cm in height and 30 cm in diameter) were filled with 7 kg of autoclaved rice field soil and five 5-day-old healthy seedlings were transplanted into each pot corresponding to each treatment. The pots were

Table 2
Soil properties of Wasan agricultural farm (Crop Protection and Soil Science Section, Rice Industry Division, DOAA, unpublished data).

Soil properties	Mean values
pH (1:2.5, soil:H2O)	3.37
Electrical Conductivity – mS/cm	0.20
Organic Carbon – OC%	5.56
Nitrate (NO_3) – ppm	0.70
Exchangeable potassium – K, meq/100g	0.25
Exchangeable Magnesium – Mg, meq/100g	1.38
Exchangeable Calcium – Ca, meq/100g	1.06
Exchangeable Aluminium – Al, meq/100g	12.53
Available Phosphorus – P, ppm	3.92
Available Zinc – Zn, ppm	1.7
Available Copper – Cu, ppm	2.01
Available Iron – Fe, ppm	659.67
Available Manganese – Mn, ppm	4.15

†Soils were collected in March 2021.

placed in a greenhouse for 120 days, with average day and night temperatures of 30 ± 5 °C and 20 ± 5 °C, respectively. Since the experiments were not rain-fed, the pots were regularly watered, ensuring the water level in each pot remained 5 cm above the soil surface until day 70. After panicle formation, the water level was reduced to 1 cm above the soil surface and left to dry for 7 days prior to harvesting, following the guidance of DOAA. In total, there were 5 treatments, including 4 fungal isolates and a control and 5 replicates per treatment, resulting in 25 pots per soil or seed inoculation method.

The following growth parameters were evaluated as described by Wijesooriya and Deshappriya [46]: a) shoot height per pot, measured at 2-week intervals from 2nd week (vegetative stage) up to 10th week (tiller initiation stage) after planting, b) chlorophyll content per pot of leaves, measured on the 8th week prior to tiller appearance using a chlorophyll content meter (CCM-200 plus, Opti-Sciences, USA), c) tiller count per plant, measured at the panicle initiation stage, d) grain count per plant and 100-grain weight per treatment, measured after final harvesting.

2.5. Statistical analysis

The data collected in this study were subjected to statistical analysis using the GraphPad Prism version 8.0.2 for Windows, GraphPad Software, San Diego, California USA, Home - GraphPad [47]. Collected data were either analysed by one-way or two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests at 5 % significance level. Prior to analysis, all data were checked for normality and homogeneity of variance. All the assays, such as hyperparasitism assay, enzyme assay, phytochemical screening assay, salinity and temperature tolerance assay, and IAA and ammonia screening assays were performed in triplicates. Antagonistic assays, such as dual culture plate, non-volatile and volatile compounds assays were conducted with six replicates. Germination bioassay and greenhouse pot experiments were carried out with five replicates.

3. Results

3.1. Biocontrol agent

3.1.1. Antagonistic activity against pathogen

There are three distinct forms of antagonism: matrix competition, mycoparasitism, and antibiosis. This study attempted to assess the antagonistic effects of 14 MEF (3 isolates were from *R. apiculata,* 5 isolates from *N. fruticans* and 6 isolates from *X. granatum*) against the rice crop pathogen *R. solani*. The investigation revealed that the MEF strains demonstrated antagonistic effects against *R. solani*

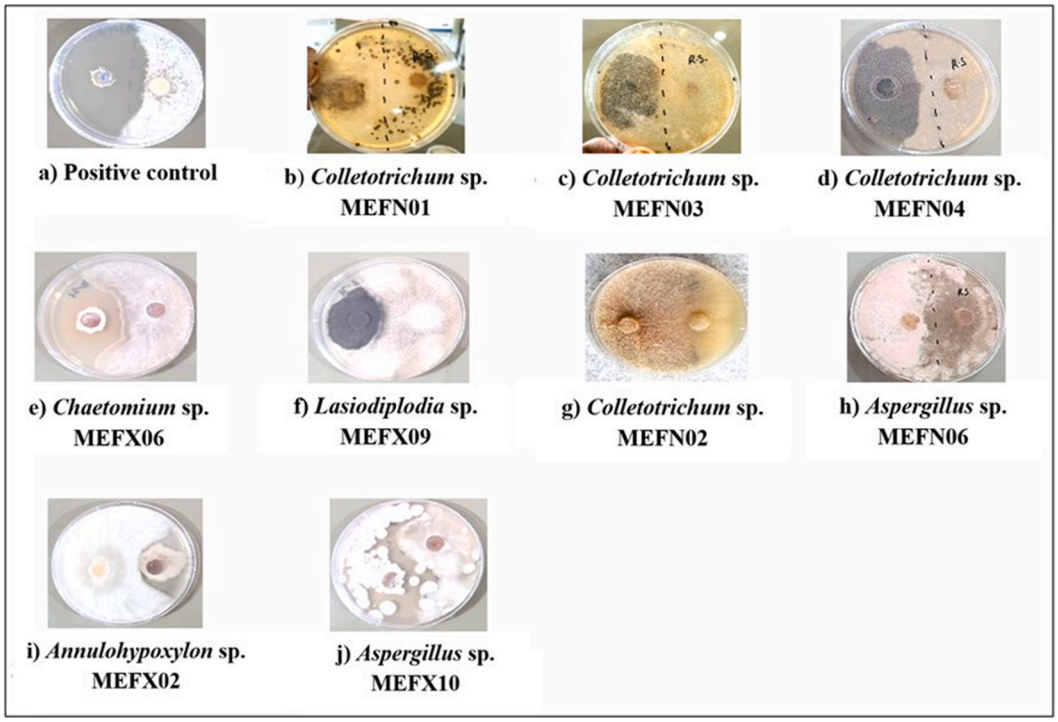

Fig. 1. Antagonistic effect of mangrove endophytic fungi (MEF) against *Rhizoctonia solani* through dual culture plate assay. Dual culturing plate assay demonstrates the antagonistic interactions between *R. solani* (right) and MEF colonies of *Rhizophora apiculata* (MEFR), *Nypa fruticans* (MEFN), *Xylocarpus granatum* (MEFX), and Benomyl fungicide (a positive control) (left). Note: Antagonistic interactions between the MEF and *R. solani* is grouped as follows: (a) Type B interaction, (b) Type C interaction, (c) to (f) Type E interactions, (g) to (j) Type F interactions.

through mechanisms such as matrix competition, mycoparasitism, and antifungal compounds. The dual culture plate assay reveals a scenario of matrix competition, indicating four distinct fungal interactions between MEF and the pathogen *R. solani* as depicted in Fig. 1 *Colletotrichum* sp. MEFN01 displayed a type C interaction, wherein the pathogen colonies penetrate and grew through the MEF colonies. Similarly, *Colletotrichum* spp. MEFN03 and MEFN04, *Chaetomium* sp. MEFX06 and *Lasiodiplodia* sp. MEFX09 exhibited type E interactions, with the MEF inhibitor species grew through the pathogen colonies. *Colletotrichum* sp. MEFN02, *Aspergillus* sp. MEFN06, *Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10 demonstrated type F interactions, as they overgrew and surrounded the pathogen colonies. In contrast, the positive control of 6.25 mg/mL fungicide (the least effective concentration) exhibited a type B interaction, indicating mutual inhibition upon contact.

Table 3 presents the percentage of inhibition of pathogen growth through various antagonistic assays for fourteen mangrove endophytic fungi against the pathogen *Rhizoctonia solani*. It is observed that MEF derived from *N. fruticans*, specifically *Colletotrichum* sp. MEFN02 and *Aspergillus* sp. MEFN06 as well as MEF from *X. granatum*, namely *Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10, exhibited promising antagonistic effects against *R. solani*, with growth inhibition percentages ranging between 52 % and 64 %, then that of both the negative (SDW) and positive (fungicide) controls ($P < 0.05$). One can also note that *Colletotrichum* spp. MEFN03 and MEFN04, *Lasiodiplodia* sp. MEFX09 and *Chaetomium* sp. MEFX06 showed significantly moderately high growth inhibition percentages, which were also significantly different from the positive and negative controls ($P < 0.05$). In addition, *Colletotrichum* sp. MEFN01 exhibited the least growth inhibition against the pathogen, even significantly lower than the fungicide, but significantly higher growth inhibition than the negative control ($P < 0.05$). Conversely, the negative control, as well as all three MEF derived from *R. apiculata* (*Annulohypoxylon* sp. MEFR03, *Penicillium* sp. MEFR09, *Colletotrichum* sp. MEFR10) and two MEF from *X. granatum* (*Daldinia* sp. MEFX01 and *Trichoderma* sp. MEFX11), did not exhibit any antagonistic effects against *R. solani*, showed no growth or competitive inhibition, hence there was no statistically significant differences ($P > 0.05$).

From the results of non-volatile and volatile compounds assays (refer Fig. 2, Fig. 3 and Table 3), it is recorded that out of the fourteen MEFs that were tested, *Colletotrichum* sp. MEFN02, *Aspergillus* sp. MEFN06, *Annulohypoxylon* sp. MEFX02, and *Aspergillus* sp. MEFX10 showed significant antifungal mechanism of antagonistic activity against *R. solani*. These endophytes exhibited the highest growth inhibition, with percentages ranging between 72 % and 87 %, in non-volatile compound assay, whereas, it shows inhibition percentages ranges from 18 % to 25 % in volatile compound assay ($P < 0.05$). In non-volatile assay, it was observed that *Colletotrichum* sp. MEFN04 and *Chaetomium* sp. MEFX06 exhibited a moderate inhibitory effect against the pathogen. However, in the volatile assay, only *Colletotrichum* sp. MEFN04 showed a moderate inhibitory effect, while *Chaetomium* sp. MEFX06 demonstrated the weakest inhibitory effect against the pathogen, with statistical significance ($P < 0.05$). Moreover, for both the assays, the remaining MEFs, including all three from *R. apiculata* (*Annulohypoxylon* sp. MEFR03, *Penicillium* sp. MEFR09, *Colletotrichum* sp. MEFR10), three from *X. granatum* (*Daldinia* sp. MEFX01, *Lasiodiplodia* sp. MEFX09, and *Trichoderma* sp. MEFX11), and two from *N. fruticans* (*Colletotrichum*

Table 3
In-vitro antagonism of fourteen mangrove endophytic fungi against the pathogen *Rhizoctonia solani* using various assays.

Mangrove endophytic fungi	Mean ± SD percentage of growth inhibition of pathogen		
	Dual culture plate assay	Non-volatiles compound assay	Volatile compound inverted assay
	No growth		
Negative control	0.00 ± 0.00^{i}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
Annulohypoxylon sp. MEFR03	0.00 ± 0.00^{i}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
Penicillium sp. MEFR09	0.00 ± 0.00^{i}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
Colletotrichum sp. MEFR10	0.00 ± 0.00^{i}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
Daldinia sp. MEFX01	0.00 ± 0.00^{i}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
Trichoderma sp. MEFX11	0.00 ± 0.00^{i}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
	Type B		
Fungicide (Positive control)	48.38 ± 1.88^{de}	87.15 ± 1.26^{a}	26.67 ± 2.58^{a}
	Type C		
Colletotrichum sp. MEFN01	24.71 ± 1.57^{h}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
	Type E		
Colletotrichum sp. MEFN03	41.13 ± 3.04^{g}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
Colletotrichum sp. MEFN04	45.17 ± 2.05^{ef}	55.53 ± 2.53^{d}	20.55 ± 1.861^{c}
Chaetomium sp. MEFX06	49.58 ± 2.08^{cd}	55.14 ± 1.75^{d}	5.13 ± 1.75^{e}
Lasiodiplodia sp. MEFX09	41.75 ± 2.25^{fg}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
	Type F		
Colletotrichum sp. MEFN02	53.79 ± 2.86^{b}	86.57 ± 0.85^{a}	17.77 ± 1.94^{d}
Aspergillus sp. MEFN06	52.60 ± 1.04^{bc}	86.76 ± 1.16^{a}	23.31 ± 1.55^{b}
Annulohypoxylon sp. MEFX02	63.86 ± 1.39^{a}	71.93 ± 3.01^{c}	20.37 ± 2.32^{cd}
Aspergillus sp. MEFX10	51.97 ± 2.71^{bc}	82.02 ± 2.92^{b}	24.69 ± 1.73^{ab}

†Mangrove endophytic fungi (MEF) colonies of *Rhizophora apiculata* (MEFR), *Nypa fruticans* (MEFN) and *Xylocarpus granatum* (MEFX). The fungal interactions between pathogen and MEF in dual culture assay were classified as no growth, Type B, C, E and F.

‡Data presented are mean ± standard deviation (SD). Six replicates per treatment.

§Data were analysed using one way ANOVA test to analyze statistical differences between MEF and control groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), followed by a Dunnett's multiple comparison post hoc test. Mean values with same letters within a column were not significantly different.

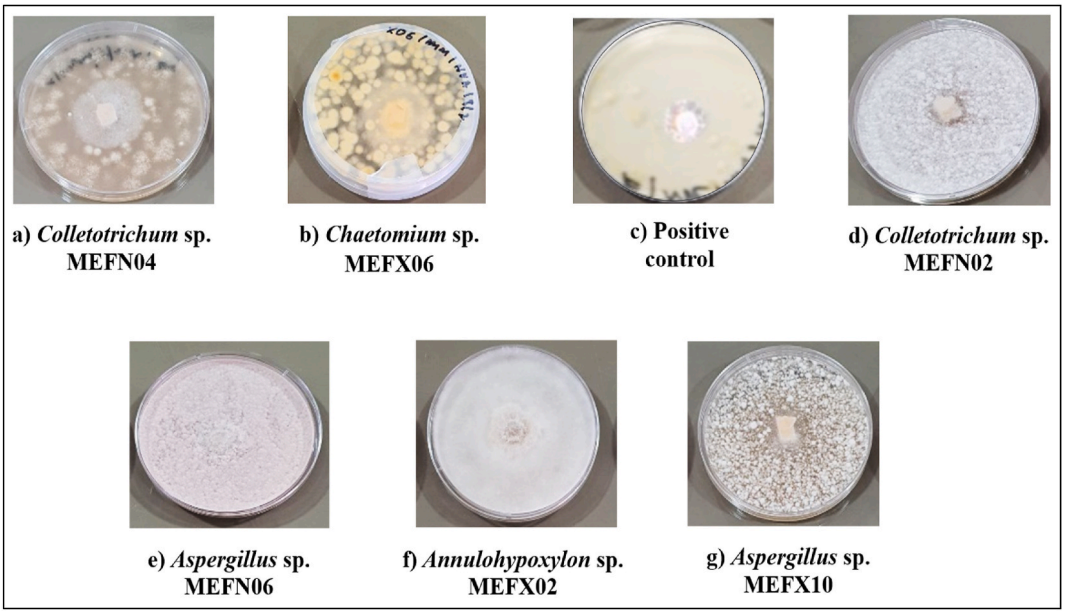

Fig. 2. Antagonistic activities of mangrove endophytic fungi (MEF) against *Rhizoctonia solani* via non-volatile compound assay. Colonies of *R. solani* were inoculated at the center of the PDA media (distinctly mixed with mangrove endophytic fungi colonies from *Rhizophora apiculata* (MEFR), *Nypa fruticans* (MEFN) or *Xylocarpus granatum* (MEFX) and Benomyl fungicide (a positive control), respectively. Note: Antagonistic impact of MEF on *R. solani* can be categorized as follows: (a) and (b) moderate level of antagonism, (c) to (g) high level of antagonism.

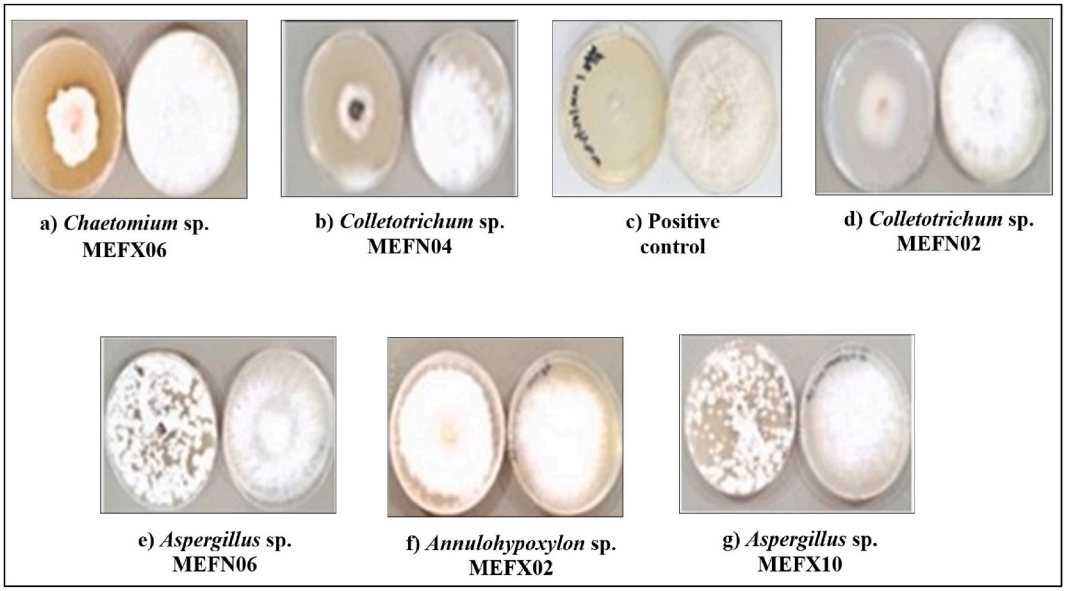

Fig. 3. Antagonistic activities of mangrove endophytic fungi (MEF) against *Rhizoctonia solani* by utilizing volatile compounds inverted assay. Colonies of *R. solani* were inoculated at the right-hand side and the distinct colonies of mangrove endophytic fungi from *Rhizophora apiculata* (MEFR), *Nypa fruticans* (MEFN) or *Xylocarpus granatum* (MEFX) and Benomyl fungicide (a positive control) were at the left-hand side. Note: Antagonistic impact of MEF on *R. solani* can be categorized as follows: (a) low level of antagonism and (b) to (g) moderate level of antagonism.

sp. MEFN01 and *Colletotrichum* sp. MEFN03), as well as the negative control, did not exhibit any significant inhibitory effect against the pathogen control ($P > 0.05$).

3.1.2. Hyperparasitism activities assay

This assay unveils the microscopic analysis on the occurrence of specific mycoparasitism mode of antagonism within the MEF of

N. fruticans and *X. granatum* when subjected to *R. solani*, as depicted in Fig. 4. More precisely, *Annulohypoxylon* sp. MEFX02 exhibited hyphal coiling, while *Aspergillus* sp. MEFX10 displayed hook formation. In contrast, *Colletotrichum* sp. MEFN02 showcased clamp formation, and *Aspergillus* sp. MEFN06 exhibited hyphae thickening.

3.1.3. Hydrolytic enzyme activities assays

As shown in Fig. 5, *Aspergillus* spp. MEFX10 and MEFN06, and *Colletotrichum* sp. MEFN02 exhibited the presence of all enzyme activities, except for lipase in *Aspergillus* sp. MEFN06 and laccase in *Colletotrichum* sp. MEFN02. On the other hand, *Annulohypoxylon* sp. MEFX02 fungi solely exhibited amylase activity, with the absence of all other enzyme activities. From the results given in Table 4, it is noticed that *Colletotrichum* sp. MEFN02 had significantly higher enzyme index (EI) values for amylase, cellulase and phosphatase, whereas *Aspergillus* spp. MEFN06 and MEFX10 displayed significantly greater EI values for protease ($P < 0.05$). Furthermore, both *Colletotrichum* sp. MEFN02 and *Aspergillus* sp. MEFX10 demonstrated a significantly higher EI value for lipase.

3.1.4. Phytochemical screening assays

The qualitative analysis of ethyl acetate crude MEF extract revealed the presence of several secondary metabolites, including alkaloids, phenols, tannins, triterpenoids, saponins and flavonoids (refer Table 5). MEF *Colletotrichum* sp. MEFN02 exhibited the presence of phenols, tannins and triterpenoids, whereas *Aspergillus* sp. MEFN06 displayed the presence of all tested phytochemical compounds except for saponin. Similarly, *Annulohypoxylon* sp. MEFX02 has shown the presence of alkaloids, phenols and triterpenoids, while *Aspergillus* sp. MEFX10 showed the presence of phenols, tannins, triterpenoids and saponins.

3.1.5. Temperature and salinity tolerance assays

Based on the temperature and salinity screening protocols (as given in Tables 6 and 7), two MEF, namely *Aspergillus* spp. MEFN06 and MEFX10 exhibited the ability to withstand the extreme temperatures of 40 °C and 45 °C, but unable to tolerate higher temperatures. Furthermore, these MEF demonstrated promising salt tolerance levels up to 2000 mM, with a significantly lower growth reduction ranging from 0 to 2 % across various salinity concentrations. These species revealed that salt concentrations are not significantly affecting their growth ($P > 0.05$). On the other hand, the MEF *Colletotrichum* sp. MEFN02 and *Annulohypoxylon* sp. MEFX02 displayed moderate temperature tolerance in the range of 25 °C–35 °C and exhibited low salt tolerance ranging from 200 to 1500 mM with a growth rate of about 8–82 % for *Colletotrichum* sp. MEFN02 and 4–84 % for *Annulohypoxylon* sp. MEFX02.

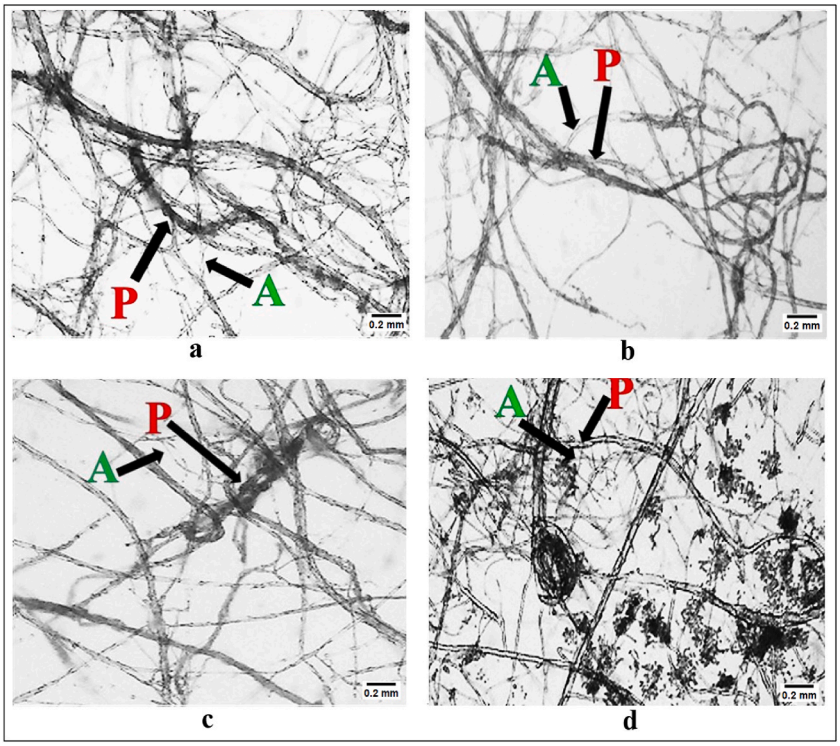

Fig. 4. Microscopic observation of Hyperparasitism effect of mangrove endophytic fungi (MEF) obtained from *Nypa fruticans* and *Xylocarpus granatum* against *Rhizoctonia solan,* (by slide culture method). a) *R. solani* x *Colletotrichum* sp. MEFN02, b) *R. solani* x *Annulohypoxylon* sp. MEFX02, c) *R. solani* x *Aspergillus* sp. MEFN06, d) *R. solani* x *Aspergillus* sp. MEFX10. A and P represent the antagonistic MEF and *R. solani*, respectively.

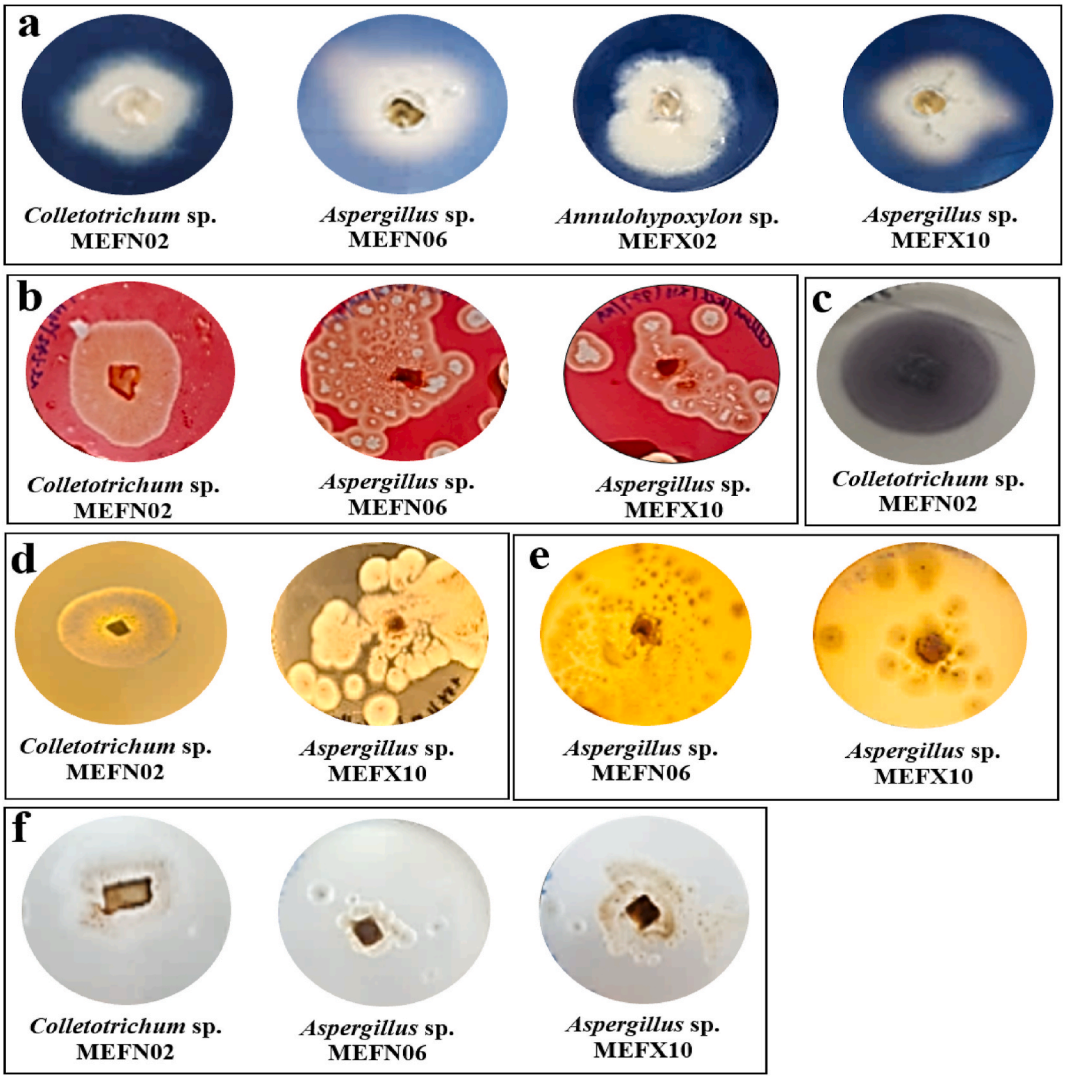

Fig. 5. Hydrolytic enzyme activities in mangrove endophytic fungi (MEF) obtained from *Nypa fruticans* and *Xylocarpus granatum*. a) amylase, b) cellulase, c) laccase, d) lipase, e) protease, and f) phosphatase enzymes.

Table 4
Extracellular hydrolytic enzymes index (EI) present in four selected antagonistic mangrove endophytic fungi (MEF).

Enzymes	*Colletotrichum* sp. MEFN02	*Aspergillus* sp. MEFN06	*Annulohypoxylon* sp. MEFX02	*Aspergillus* sp. MEFX10	P Value
Amylase	1.15 ± 0.014^{a}	1.08 ± 0.013^{b}	1.06 ± 0.004^{b}	1.06 ± 0.003^{b}	***
Lipase	1.06 ± 0.003^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	1.05 ± 0.004^{a}	***
Cellulase	1.07 ± 0.006^{a}	1.04 ± 0.007^{c}	0.00 ± 0.00^{d}	1.06 ± 0.003^{b}	***
Protease	0.00 ± 0.00^{b}	1.07 ± 0.015^{a}	0.00 ± 0.00^{b}	1.06 ± 0.003^{a}	***
Phosphatase	1.15 ± 0.014^{a}	1.07 ± 0.015^{b}	0.00 ± 0.00^{c}	1.06 ± 0.003^{b}	***

†Antagonistic MEF obtained from *Nypa fruticans* (*Colletotrichum* sp. MEFN02 and *Aspergillus* sp. MEFN06) and *Xylocarpus granatum* (*Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10).
‡Data presented are mean ± standard deviation (SD) of Enzyme Index (EI). Experiment was triplicated per treatment.
§Data were analysed using a one-way ANOVA test to analyze statistical differences between MEF (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), followed by a Dunnett's multiple comparison post hoc test. Mean values with the same letters within a row were not significantly different.

3.2. Biofertilizer assay

3.2.1. Indole acetic acid (IAA) and ammonia screening assays

Table 8 illustrates the production levels of indole-3-acetic acid (IAA) and ammonia by four antagonistic microorganisms. Among

Table 5
Phytochemical properties present in four selected antagonistic mangrove endophytic fungi (MEF).

Phytochemical compounds	*Colletotrichum* sp. MEFN02	*Aspergillus* sp. MEFN06	*Annulohypoxylon* sp. MEFX02	*Aspergillus* sp. MEFX10
Alkaloids	–	+	+	–
Phenols	+	+	+	+
Tannins	+	+	–	+
Triterpenoids	+	+	+	+
Saponins	–	–	–	+
Flavonoids	–	+	–	–

†Antagonistic MEF obtained from *Nypa fruticans* and *Xylocarpus granatum*. Presence of phytochemical (+); absence of phytochemical (−).

Table 6
Growth of four selected antagonistic mangrove endophytic fungi (MEF) in various temperature.

Temperature (°C)	*Colletotrichum* sp. MEFN02	*Aspergillus* sp. MEFN06	*Annulohypoxylon* sp. MEFX02	*Aspergillus* sp. MEFX10
25	+	+	+	+
30	+	+	+	+
35	+	+	+	+
40	–	+	–	+
45	–	+	–	+
50	–	–	–	–

†Antagonistic MEF obtained from *Nypa fruticans* (*Colletotrichum* sp. MEFN02 and *Aspergillus* sp. MEFN06) and *Xylocarpus granatum* (*Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10).
‡Temperature tolerance (+) and temperature intolerance (−).

Table 7
Percentage of growth of four selected antagonistic mangrove endophytic fungi (MEF) in various salt concentration.

Salt concentration (mM)	*Colletotrichum* sp. MEFN02	*Aspergillus* sp. MEFN06	*Annulohypoxylon* sp. MEFX02	*Aspergillus* sp. MEFX10	P value (MEF)
Control	0.00 ± 0.00^{aF}	0.00 ± 0.00^{aA}	0.00 ± 0.00^{aF}	0.00 ± 0.00^{aA}	NS
200	8.33 ± 1.91^{aE}	0.00 ± 0.00^{cA}	3.53 ± 1.18^{bF}	0.00 ± 0.00^{cA}	***
400	10.42 ± 1.91^{aE}	0.00 ± 0.00^{bA}	12.55 ± 1.36^{aE}	0.39 ± 0.68^{bA}	***
600	29.58 ± 2.89^{aD}	0.00 ± 0.00^{bA}	25.49 ± 1.80^{aD}	0.39 ± 0.68^{bA}	***
800	35.42 ± 3.61^{bD}	0.78 ± 1.36^{cA}	45.49 ± 2.72^{aC}	0.39 ± 0.68^{cA}	***
1000	46.25 ± 3.30^{bC}	1.57 ± 1.79^{cA}	61.57 ± 2.96^{aB}	0.39 ± 0.68^{cA}	***
1500	59.58 ± 1.44^{aB}	1.57 ± 2.72^{bA}	63.92 ± 1.80^{aB}	0.78 ± 0.68^{bA}	***
2000	81.67 ± 3.82^{aA}	1.96 ± 1.80^{bA}	84.31 ± 1.80^{aA}	1.57 ± 1.80^{bA}	***
P value (salt concentration)	***	NS	***	NS	

†Antagonistic MEF obtained from *Nypa fruticans* and *Xylocarpus granatum*, control - (PDA unsalted).
‡Data presented are mean ± standard deviation (SD) of % growth reduction in various salt concentration. Experiment is triplicated.
§Data were analysed using a two-way ANOVA test to analyze statistical differences between MEF groups or salt concentration treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), followed by a Dunnett's multiple comparison post hoc test. Mean values with the same lowercase letters within a row (salt concentration) or the same uppercase letters within a column (MEF species) were not significantly different.

Table 8
Screening of indole acetic acid (IAA) and ammonia productions in four mangrove endophytic fungi (MEF).

Compound (mg/mL)	*Colletotrichum* sp. MEFN02	*Aspergillus* sp. MEFN06	*Annulohypoxylon* sp. MEFX02	*Aspergillus* sp. MEFX10	P value (MEF)
IAA	15.70 ± 0.006^{d}	40.96 ± 0.006^{a}	19.07 ± 0.015^{c}	33.04 ± 0.058^{b}	***
Ammonia	26.83 ± 0.0012^{c}	32.54 ± 0.006^{a}	29.70 ± 0.006^{b}	24.57 ± 0.006^{d}	***

†Antagonistic MEF obtained from *Nypa fruticans* and *Xylocarpus granatum*.
‡Data presented are mean ± standard deviation (SD). The experiment was triplicated per treatment.
§Data were analysed using a one-way ANOVA test to analyze statistical differences between MEF groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), followed by a Dunnett's multiple comparison post hoc test. Mean values with the same letters within a row were not significantly different.

these, *Aspergillus* sp. MEFN06 demonstrated significantly higher concentrations of both IAA (40.96 mg/mL) and ammonia (32.54 mg/mL) compared to the other endophytes ($P < 0.05$). *Aspergillus* sp. MEFX10 produced 33.04 mg/mL of IAA and 24.57 mg/mL of ammonia. *Annulohypoxylon* sp. MEFX02 yielded approximately 19.07 mg/mL of IAA and 29.70 mg/mL of ammonia. *Colletotrichum* sp. MEFN02 exhibited IAA production at 15.7 mg/mL and ammonia production of 26.83 mg/mL.

3.2.2. Germination bioassay and pathogenicity test

The germination parameters of MRQ76 rice seeds, treated with MEF mixed *R. solani* and *R. solani* (control) are presented in Table 9. When subjected to *R. solani* treatment, the aforesaid four MEF isolates revealed significant enhancement of seed germination (increased by 87 %–134 %) with higher vigor index-1 (VI-1) (increased by 291 %–596 %) and vigor index-2 (VI-2) (increased by 364 %–662 %), along with significantly lower percentage of disease severity index (DSI%) of *R. solani*, ranging from (24 %–46 %), compared to the control ($P < 0.05$). Seeds treated with *Aspergillus* sp. MEFN06:RS displayed notably higher germination percentage (20 %), VI-1 (43.8 %), VI-2 (39 %) as well as least DSI% (24 %) compared to other MEF ($P < 0.001$). Fig. 6 represent the disease severity of the various treatment.

3.2.3. Greenhouse pot experiment assay

Through seed and soil inoculation methods, during the 2nd and 4th week of the vegetative stage, the heights of MRQ76 plants were not significantly affected by the MEF and control treatments (refer Fig. 7, Table 10 and Table 11). However, during the 6th week onwards until panicle initiation stage (10th week), the plants treated with MEF by seed inoculation exhibited a considerable increase in their height (11 %–16 %, $P < 0.001$), whereas, by soil inoculation, the plant height significantly increased in the range 4 %–11 % ($P < 0.001$). Through both inoculation methods, the result of MEF treatments exhibited significant improvements over the result of control plants in terms of chlorophyll levels (17 %–46 %), tiller counts (28 %–93 %), grain counts (43 %–102 %), and 100-grain weights (13 %–41 %), when compared to corresponding results of control plants. *Aspergillus* sp. MEFN06 demonstrated the highest chlorophyll concentrations (41 %–46 %), tiller counts (92 %–93 %), grain counts (93 %–102 %) and 100-grain weights (37 %–41 %).

4. Discussion

Rhizoctonia solani, a soil-borne pathogen, poses significant economic threats to various crops, including rice, as it lacks a completely resistant cultivar [6,7]. Hence, there is an urgent need to explore non-chemical fungicidal measures to protect plants without harming soil biota. Over the past 2 decades, researchers have shown significant interest in endophytes derived from mangroves, owing to their resilience and ability to secrete a diverse range of unique secondary metabolites, enzymes, phytohormones, siderophores, and mineral-solubilizing agents [12,48]. These endophytes play a crucial role in controlling both biotic and abiotic stressors, offering potential applications as agents to manage stress and as eco-friendly alternatives to chemical compounds [21–24]. This study assesses the potential applications of 14 endophytic fungi found in mangroves (MEF), derived from three mangrove plant species. It explores their potential as agents for controlling *Rhizoctonia solani* and as biofertilizers to enhance the productivity of the fragrant rice strain Malaysian Rice Quality 76 (MRQ76).

In this study, through antagonistic assays, only four out of fourteen MEF (*Annulohypoxylon* sp. MEFX02, *Colletotrichum* sp. MEFN02, *Aspergillus* sp. MEFN06, and *Aspergillus* sp. MEFX10) demonstrated efficient control or inhibition of *R. solani* growth compared to the fungicide (Benomyl) as noted in Table 3. Furthermore, it was notable that these four MEF exhibited a significant inhibitory effect (Type F interaction) against the pathogen, surpassing the effectiveness of the fungicide (Type B interaction).

Dual culture examination of the interaction between these MEF and the pathogen revealed evidence of matrix competition, indicating that the endophytes compete with the pathogen for nutrients, leading to its suppression due to their vigorous growth, ultimately restraining the growth of *R. solani*. Moreover, these examination not only revealed the matrix competition between them, but also pointed out that the live fungal antagonists were more efficient in controlling pathogens in an eco-friendly way than the chemical fungicides, since the effectiveness of chemical fungicides depends on their availability and also they were prone to denaturation or degradation over time [11,49–51]. Furthermore, the residues of the fungicides cause lethal or adverse effects on humans, such as damage to the endocrine and nervous systems [52]. Similarly, Vaish and Sinha [53], reported the inhibitory effect of the endophyte *Aspergillus niger* (isolated from rice, rice rhizosphere and soil) over *R. solani* with Type F interaction.

Likewise, both volatile and non-volatile assays of this study reveal that all 4 MEF exhibit significant inhibitory effects on *R. solani*. These compounds have antifungal effect which contribute to curb the pathogen. This is consistent with the results obtained by Rabha et al. [54] whose observations on *Colletotrichum gloeosporioides* endophytic fungus present in *Camellia sinensis* (tea) exhibiting antifungal antagonistic effect on *Pestalotiopsis theae* and *Colletotrichum camelliae*. Sreeja et al. [18] reported that *Annulohypoxylon nitens, Ceriporia lacerate, Daldinia eschscholtzii, Diaporthe* spp., *Fusarium* spp., and *Phomopsis* spp. of *Piper nigrum* showed an antifungal inhibitory effect on *Phytophthora capsica*.

Mycoparasitism, a direct mode of antagonist action, has been observed in several endophytes, particularly in the genus *Trichoderma* [11,55]. The microscopic analysis of the interaction between MEF and *R. solani* clearly revealed the formation of hyphal coiling, hyphal clamping or thickening of the pathogen which indicates the presence of the mycoparasite mode of defense mechanism [56,57]. These MEF outgrow and encircle the pathogen, releasing spores that ultimately constrained the pathogen's hyphal growth. It is interesting to note that the antagonistic effects of MEF emphasized direct mode of defense mechanism such as, matrix competition, non-volatile and volatile antifungal compounds and mycoparasitism, as major contributors to the inhibitory effect on pathogen and these characteristics are in agreement with the findings of [57–60].

From Tables 4 and it is observed that the MEF such as *Colletotrichum* sp. MEFN02, *Aspergillus* spp. MEFN06 and MEFX10 are found to produce most of the hydrolytic enzymes. Furthermore, antagonism and hyperparasitism assays revealed that enzymes produced by these MEF are capable of lysing the cell wall and obtaining nutrition of the pathogenic species, thereby inhibiting the mycelial growth of the *R. solani* [11,61]. Maria et al. [62] reported that endophytic fungi of *Acrostichum aureum* L. (mangrove fern) and *Acanthus ilicifolius* L. (mangrove angiosperm) were capable of producing extracellular enzymes, such as amylase, cellulase, lipase and protease except laccase, tyrosinase or chitinase. Gupta and Das [63] and Hiruma et al. [64] also observed the production of phosphatase

Table 9
Seed germination and seedling growth parameters and percentage reduction of disease symptoms (%RDS) in MRQ76 rice strain bioprimed with an individual mangrove endophytic fungus (MEF) mixed with *Rhizoctonia solani*.

Parameters	Control (RS)	*Colletotrichum* sp. MEFN02: RS	*Aspergillus* sp. MEFN06: RS	*Annulohypoxylon* sp. MEFX02: RS	*Aspergillus* sp. MEFX10: RS	P-Value
GP (%)	32.00 ± 2.74^{d}	65.00 ± 0.00^{b}	75.00 ± 0.00^{a}	60.00 ± 0.00^{c}	67.00 ± 2.74^{b}	***
VI-1	83.1 ± 8.0^{e}	384.8 ± 8.5^{c}	579.0 ± 12.3^{a}	325.2 ± 9.9^{d}	449.0 ± 25.7^{b}	***
VI-2	0.74 ± 0.10^{e}	4.68 ± 0.05^{c}	5.64 ± 0.06^{a}	3.44 ± 0.05^{d}	4.86 ± 0.16^{b}	***
DSI (%)	98 ± 0.84^{d}	34 ± 0.55^{b}	24 ± 0.55^{a}	46 ± 0.55^{c}	36 ± 0.55b^{c}	***

†MRQ76 rice seeds were treated with a mixture of individual MEF and *Rhizoctonia solani* (*Colletotrichum* sp. MEFN02:RS and *Aspergillus* sp. MEFN06: RS obtained from *Nypa fruticans* and *Annulohypoxylon* sp. MEFX02:RS and *Aspergillus* sp. MEFX10:RS from *Xylocarpus granatum*) and *R. solani* alone as the control (RS). Parameters are germination percentage (GP %), vigor index-1 (VI-1), vigor index-2 (VI-2) and percentage disease severity index (DSI %).

‡Data presented are mean ± standard deviation (SD). Five replicates per treatment.

§Data were analysed using a one-way ANOVA test to analyze statistical differences between MEF and control groups (*P < 0.05, **P < 0.01, ***P < 0.001), followed by a Dunnett's multiple comparison post hoc test. Mean values with the same letters within a row were not significantly different.

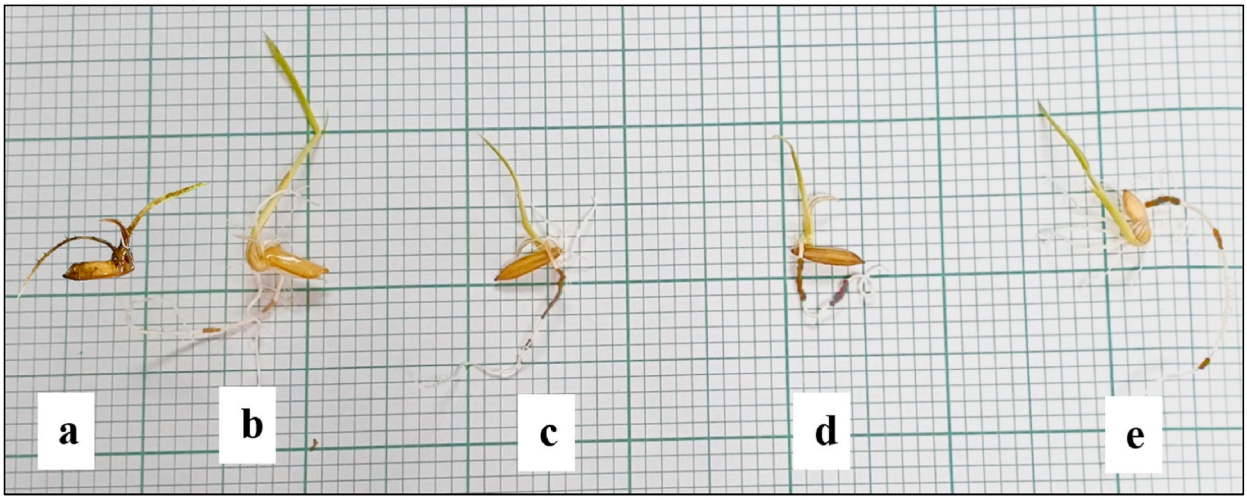

Fig. 6. The disease severity in rice MRQ76 treated with mangrove endophytic fungi (MEF) using in-vitro germination assay. The seeds were treated with a unique mixture of four antagonistic MEFs combined *with R. solani*, while the control group was treated with *R. solani* alone. a) *Rhizoctonia solani*, b) *Aspergillus* sp. MEFN06 with *R. solani*, c) *Aspergillus* sp. MEFX10 with *R. solani* d) *Annulohypoxylon* sp. MEFX02 with *R. solani* and e) *Colletotrichum* sp. MEFN02 with *R. solani*.

enzymes and reported that fungal species, such as *Aspergillus* PF8, *Aspergillus* PF127 and *Colletotrichum tofieldiae*, had the ability to solubilize phosphate, facilitating the transfer of the macronutrient phosphorus to aboveground tissues of *Arabidopsis thaliana*, ultimately enhancing plant fertility under phosphorus-stressed condition. It is of interest to note that the result of our study is in alignment with the aforesaid results by Maria et al. [62], Gupta and Das [63] and Hiruma et al. [64].

Through the results of Tables 5 and it is found that in the present study, various phytochemicals such as phenols, tannins, triterpenoids, alkaloids, saponins and flavonoids are present at different degrees in the MEF. These phytochemical compounds act as antioxidants and antimicrobial agents, thus the MEF could enhance crop protection. Findings of this research are in alignment with similar results obtained by Sopalun et al. [65] and Munshi et al. [66] propounded the presence of phytochemicals in the endophytes derived from the mangrove plants.

Plants have been exposed to abiotic stresses such as temperature and salinity stresses [5]. Temperature and salinity stresses causes various disruptions at the molecular and metabolic levels such as the inhibition of stomatal conductance, reduction of photosynthetic activities, disturbance of osmotic pressure, affect the seed germination, root growth, and crop productivity, particularly in major crops like rice [67–69]. From the results obtained in Tables 6 and 7, it is inferred that all tested MEF were able to withstand temperature and salinity stress which enhance the plant growth under drastic climatic conditions, since these endophytes are derived from mangrove plants that are resilient to extreme temperature and salinity [24,63]. Furthermore, these endophytes contribute to the regulation of the plants' morphological traits and physiological as well as biochemical processes [70,71]. Similarly, Dastogeer et al. [40] reported that endophytic fungi isolated from *Nicotiana* species exhibited higher salt and temperature tolerance.

Endophytic fungi isolated from mangrove plants produce siderophores, ammonia, phosphate and IAA which contribute to plant's protection against pathogens and promote its growth [22,23,72]. From the results of Tables 8 and it is noted that all the four MEF

Fig. 7. Growth of MRQ76 rice plants when treated with mangrove endophytic fungi (MEF) through (A) soil inoculation and (B) seed inoculation methods in greenhouse pot experiments. The MEF treatments are (a) control, (b) *Colletotrichum* sp. MEFN02, (c) *Aspergillus* sp. MEFN06, (d) *Annulohypoxylon* sp. MEFX02 and (e) *Aspergillus* sp. ME.FX10

Table 10
Growth parameters of MRQ76 rice strain treated with mangrove endophytic fungi (MEF) through seed inoculation method.

Parameters	Control (SDW)	*Colletotrichum* sp. MEFN02	*Aspergillus* sp. MEFN06	*Annulohypoxylon* sp. MEFX02	*Aspergillus* sp. MEFX10	P Value
2nd week height (cm)	27.6 ± 0.22^{a}	29.8 ± 1.14^{a}	25.5 ± 5.46^{a}	27.3 ± 2.24^{a}	27.3 ± 2.33^{a}	NS
4th week height (cm)	53.7 ± 3.75^{a}	59.6 ± 0.90^{a}	56.7 ± 3.37^{a}	55.4 ± 1.74^{a}	55.9 ± 5.10^{a}	NS
6th week height (cm)	74.9 ± 1.73^{b}	87.1 ± 1.40^{a}	83.8 ± 2.01^{a}	84.6 ± 0.97^{a}	85.1 ± 3.42^{a}	***
8th week height (cm)	87.9 ± 2.88^{a}	92.8 ± 5.13^{a}	91.2 ± 3.43^{a}	93.9 ± 3.49^{a}	90.0 ± 4.42^{a}	NS
10th week height (cm)	89.7 ± 3.16^{b}	96.4 ± 4.54^{a}	95.9 ± 3.03^{ab}	97.2 ± 3.20^{a}	95.5 ± 2.30^{ab}	*
Chlorophyll	16.02 ± 0.16^{d}	19.96 ± 0.33^{b}	22.6 ± 0.55^{a}	18.8 ± 0.40^{c}	20.4 ± 0.21^{b}	***
Tiller count	3.2 ± 0.45^{d}	4.8 ± 0.45^{bc}	6.2 ± 0.45^{a}	4.2 ± 0.45^{c}	5.4 ± 0.55^{ab}	***
Grain count	32.2 ± 2.17^{d}	51 ± 3.74^{c}	65 ± 3.08^{a}	48.2 ± 1.30^{c}	57.2 ± 3.70^{b}	***
100-Grain weight (g)	1.57 ± 0.02^{d}	1.92 ± 0.06^{b}	2.20 ± 0.03^{a}	1.83 ± 0.06^{c}	1.99 ± 0.01^{b}	***

†MRQ76 rice seeds were inoculated with individual MEF (*Colletotrichum* sp. MEFN02 and *Aspergillus* sp. MEFN06 obtained from *Nypa fruticans* and *Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10 from *Xylocarpus granatum*) and sterile distilled water (SDW) as the control. Parameters include shoot height, which was measured at two-week intervals starting from second week (vegetative stage) to tenth week (tiller initiation stage) after planting, Tiller count per plant (measured at the panicle initiation stage i.e., 10th week after planting), chlorophyll content (measured on the 8th week prior to tiller appearance), and grain count per plant and 100-grain weight (measured after final harvesting).
‡Data presented are mean ± standard deviation (SD). Five replicates per treatment.
§Data were analysed using a one-way ANOVA test to analyze statistical differences between MEF and control groups (NS $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), followed by a Dunnett's multiple comparison post hoc test. Mean values with the same letters within a row were not significantly different.

enhanced plant growth in the presence of IAA and this observation is in agreement with the findings of Asif Mehmood et al. [19] who reported that the colonization of maize roots by *Aspergillus awamo*ri Wl1 strain obtained from *Withenia somnifera* effectively improved the growth of the host plant mainly due to the production of IAA as well as the synthesis of secondary metabolites such as phenols and sugars.

Ammonia production by microorganisms plays a pivotal role in nitrogen fixation, especially in nitrogen-deficient soils [19]. It can also help to restrict pathogens and improve soil fertility and plant growth. From Tables 8 and it is also observed that all four MEF significantly produced ammonia. The ability of production of IAA and ammonia by these MEF facilitates the enhancement of plant growth under drastic climatic conditions. This observation aligns with findings by Khan et al. [73], which reported that the production of both IAA and ammonia by *Penicillium kongii* FETW4 and *Aspergillus oryzae* FETW6, derived from *Taxus wallichiana*, were found to promote the growth and yield of mung bean and fenugreek plants. Likewise, Roy et al. [20] found that the fungal endophyte *Colletotrichum* sp. SL4 isolated from *Plumbago zeylanica* Linn had the ability to enhance plant growth through the production of ammonia, IAA, and siderophore.

Table 9 describes the results of germination bioassay conducted with the four MEF which revealed the improvements in the growth and germination abilities of the MRQ76 seedlings, along with significantly lower percentage of disease severity index (DSI%) of

Table 11
Growth parameters of MRQ76 rice strain treated with mangrove endophytic fungi (MEF) through soil inoculation method.

Parameters	Control (SDW)	*Colletotrichum* sp. MEFN02	*Aspergillus* sp. MEFN06	*Annulohypoxylon* sp. MEFX02	*Aspergillus* sp. MEFX10	P-Value
2nd week height (cm)	49.2 ± 4.85^{a}	47.4 ± 8.32^{a}	45.6 ± 6.45^{a}	48.6 ± 8.45^{a}	44.4 ± 3.01^{a}	NS
4th week height (cm)	69.1 ± 5.07^{a}	67.8 ± 11.6^{a}	68.8 ± 8.92^{a}	66.1 ± 12.2^{a}	66.9 ± 6.82^{a}	NS
6th week height (cm)	77.8 ± 0.71^{b}	84.5 ± 3.13^{a}	81.2 ± 3.14^{ab}	86.3 ± 4.11^{a}	83.0 ± 4.06^{ab}	**
8th week height (cm)	81.5 ± 1.65^{a}	85.9 ± 3.61^{a}	83.9 ± 2.03^{a}	87.9 ± 5.66^{a}	85.2 ± 2.95^{a}	NS
10th week height (cm)	83.1 ± 2.70^{b}	92.4 ± 3.81^{ab}	89.9 ± 8.28^{ab}	95.7 ± 6.73^{a}	91.2 ± 6.16^{ab}	*
Chlorophyll	14.9 ± 0.32^{d}	19.0 ± 0.64^{bc}	21.8 ± 0.44^{a}	18.3 ± 1.19^{c}	20.1 ± 0.44^{b}	***
Tiller count	2.8 ± 0.45^{d}	4.2 ± 0.45^{bc}	5.4 ± 0.55^{a}	3.6 ± 0.55^{cd}	4.6 ± 0.55^{ab}	***
Grain count	30.2 ± 3.27^{d}	48.8 ± 3.63^{bc}	57.8 ± 2.28^{a}	43.4 ± 4.51^{c}	51.2 ± 4.08^{ab}	***
100-Grain weight (g)	1.53 ± 0.02^{d}	1.81 ± 0.06^{c}	2.13 ± 0.00^{a}	1.72 ± 0.07^{c}	1.93 ± 0.02^{b}	***

†MRQ76 rice seedlings were planted in soils inoculated with individual MEF (*Colletotrichum* sp. MEFN02 and *Aspergillus* sp. MEFN06 obtained from *Nypa fruticans* and *Annulohypoxylon* sp. MEFX02 and *Aspergillus* sp. MEFX10 from *Xylocarpus granatum*) and sterile distilled water (SDW) as the control. Parameters include shoot height, which was measured at two-week intervals starting from second week (vegetative stage) to tenth week (tiller initiation stage) after planting, Tiller count per plant (measured at the panicle initiation stage i.e., 10th week after planting), chlorophyll content (measured on the 8th week prior to tiller appearance), and grain count per plant and 100-grain weight (measured after final harvesting).
‡Data presented are mean ± standard deviation (SD). Five replicates per treatment.
§Data were analysed using a one-way ANOVA test to analyze statistical differences between MEF and control groups (NS $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), followed by a Dunnett's multiple comparison post hoc test. Mean values with the same letters within a row were not significantly different.

R. solani, ranging from (24 %–46 %), compared to the control exposed to the pathogen *R. solani*. Among the four tested MEF, *Aspergillus* sp. MEFN06 showed significant increase in the growth of MRQ76 seedlings as well as control against *R. solani*. Thus, enhancement of seedling and control of pathogen were mainly occurred due to their production of enzymes, phytohormones, minerals and phytochemicals. These findings are similar to the result of Abdul-Halim et al. [26], reported on Laila rice variety inoculated with *Trichoderma* sp. showed improved growth of rice plants and also a substantial decrease in *R. solani* disease symptoms.

Tables 10 and 11 spell out the enhancement of plant growth using soil and seed inoculation techniques and revealed that seedlings treated with MEF displayed improved performance in terms of shoot height, chlorophyll content, tiller count, grain count, and grain weight. Notably, among the various MEF investigated, the strain *Aspergillus* sp. MEFN06 exhibited the most effective results. This strain significantly improved both germination and growth of MRQ76, demonstrating its superiority in both soil and seed inoculation methods. These findings align with Wijesooriya and Deshappriya [46] and Priyadarshani et al. [1], which reported that endophyte-treated seedlings with soil and seed inoculation methods demonstrated more effective growth in terms of shoot height, tiller count, grain weight, and root height.

5. Conclusion

This study reveals that among 14 mangrove endophytic fungi (MEF), only four species, namely *Colletotrichum* sp. MEFN02, *Aspergillus* sp. MEFN06, *Annulohypoxylon* sp. MEFX02, and *Aspergillus* sp. MEFX10, demonstrate a promising ability to directly combat *R. solani* through the production of antifungal volatile and nonvolatile substances, phytochemicals and hydrolytic enzymes, as well as engaged in hyperparasitism. These endophytes serve as natural biocontrol agents, mitigating biotic stress induced by pathogens.

Additionally, these endophytes contribute to plant growth promotion in MRQ76 rice plants by producing indole-3-acetic acid (IAA), ammonia, and phosphatase, acting as biofertilizers. Originating from mangrove plants, they possess resilience to extreme temperatures and salinity, further supporting plant growth under abiotic stress conditions. These discoveries underscore the potential role of MEF in enhancing sustainable agriculture and ensuring food security. Further in-depth research is recommended to fully exploit the use of MEF in agriculture, with a focus on understanding the dual benefits of disease prevention in host plants and the promotion of plant growth.

Data availability

All data required to support this study is included in the manuscript.

Funding

This project is supported by Universiti Brunei Darussalam (Grant No. UBD/RSCH/1.4/FICBF(b)/2020/030). MMN is grateful to Universiti Brunei Darussalam for the UBD Bursary Award (UBA) Scholarship.

CRediT authorship contribution statement

Manjula Muthu Narayanan: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Faizah Metali:** Writing – review & editing, Validation, Supervision, Software, Conceptualization. **Pooja Shivanand:** Writing – review & editing, Supervision, Conceptualization. **Norhayati Ahmad:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the Department of Agriculture and Agrifood (DoAA), Ministry of Primary Resources and Tourism for supplying MRQ76 rice seeds and the Environmental and Life Sciences Programme, Faculty of Science, Universiti Brunei Darussalam for providing assistance and chemicals/consumables.

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