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Myristica fragrans extract inhibits melanin biosynthesis, hyphal growth and appressorium formation in *Magnaporthe oryzae*

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Abstract

Magnaporthe oryzae, causes rice blast disease, affecting 30% of annual rice production globally. It produces specialized infection structures called appressoria, allowing the pathogen to penetrate the underlying tissue. A dense layer of melanin in the appressorium wall generates turgor pressure to form invasive hyphae. *Myristica fragrans* has potent antimicrobial activity. Therefore, the present study was envisaged to explore the effect of *M. fragrans* extract on inhibition of melanin biosynthesis, hyphal growth and appressorium formation in *M. oryzae*. The results demonstrated that minimum inhibitory concentration (MIC_{100} and MIC_{50}) of *M. fragrans* n-hexane extract (PE-01) were 0.625 mg/mL and 0.312 mg/mL, respectively. Melanin biosynthesis was reduced up to 66.66% at 0.312 mg/mL concentration. Microscopic analysis revealed distorted conidia with no appressorium formation. qRT-PCR showed four-fold upregulation of *alb1/pksP* gene in presence of PE-01 extract as compared to control which may be due to generation of stress. PE-01 treated rice seeds showed 85% suppression of rice blast and 40% enhancement of plant growth. Thus, *M. fragrans* extract has the potential to be developed as a biopesticide against *M. oryzae* infections in rice.

Keywords – DHN-melanin inhibition – *biopesticide* – rice blast – SEM – tricyclazole

Introduction

Various pathogens like *Magnaporthe oryzae*, *Rhizoctonia solani*, *Xanthomonas oryzae* cause significant yield loss of rice crop in South and South-East Asia including India (Sireesha 2013). Of these, rice blast caused by *M. oryzae*, is a serious and recurrent problem in all rice-growing regions of the world (Kapoor & Katoch 2014). The constraints imposed by rice blast include reduced yield, storage rot and fungicide costs.

Magnaporthe oryzae is a heterothallic ascomycete, which produces asexual or sexual spores (ascospores) in structures called asci (Couch & Kohn 2002). It infects rice plants at all developmental stages and can cause blast symptoms in leaf, collar, neck, and panicle (Chandha & Gopalakrishna 2006). It produces a specialized infection structure called appressoria to penetrate the leaves and stems of the rice plant, allowing the pathogen to enter the underlying tissue. *M. oryzae* forms an appressorium on the plant tissue surface by a mechanism involving recognition of

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plant wax components as well as sensing of surface hardness and hydrophobicity (Ryder & Talbot 2015). This sensing triggers the emergence of penetration peg from the appressorium which pierces the host cell wall. As the appressorium matures, a dense layer of melanin is laid down in the appressorium wall, except across a pore at the plant interface. Turgor pressure increases inside the appressorium and a penetration hypha emerges at the pore, which is driven through the plant cuticle into the underlying epidermal cells and subsequently differentiates into invasive hyphae and stops nutrients and water uptake from reaching to the kernels.

There are two classes of fungicides DHN-melanin biosynthesis inhibitors (MBI) and quinine outside inhibitors used for preventing rice blast diseases. MBIs do not interfere with the viability of *M. oryzae* directly but specifically inhibit the pathogen penetration into the host plant (Brent & Hollomon 2007), thereby reducing its growth and virulence (Kunova et al. 2013). Fungicides have been found suitable for controlling rice diseases. However, they prove poisonous to beneficial organisms and become sources of environmental destruction (Morton & Staub 2008). Therefore, an alternative approach needs to be explored to control pathogens which can be approved environment friendly and may also improve the sustainability of rice production.

Plant-based natural extracts have gained importance for managing fungal diseases and controlling pests (Pandey 2018). Plant extracts have been reported to reduce the growth of fungal and bacterial pathogens such as *Botrytis cinerea, Clavibacter michiganensis* and *Fusarium* sp. (Daferera et al. 2003). Natural compounds consisting of essential oils and organic acids have been evaluated for their efficacy against seed-borne pathogens (Groot et al. 2005). The effect of aqueous extracts of *Allium sativum, Aloe vera, Annona muricata, Azadirachta indica, Bidens pilosa, Camellia sinensis, Chrysanthemum coccineum,* processed *Coffae arabica, Datura stramonium, Nicotiana tabacum,* and *Zingiber officinalis* have been previously reported to control rice blast disease caused by *Pyricularia grisea* (Hubert et al. 2015). Previously, Cho et al. (2007) reported that lignans isolated from seed kernels of *Myristica fragrans* inhibit the mycelial growth of most of the plant pathogenic fungi. *M. fragrans*, used as a spice and for medicinal purposes, has potent antimicrobial activity against *Bacillus subtilis, Candida albicans, Staphylococcus aureus* and *Streptococcus durans*. It contains 25–30% fixed oils, 5–15% volatile oils and chemical substances such as macelignan, dihydroguaiaretic acid, elimicin, myristic acid, myristicin and malabaricones (Asgarpanah & Kazemivash 2012).

Thus, the current study investigated the role of various *M. fragrans* extracts on demelanisation as well as inhibition of appressorium formation in rice. Besides, the efficacy of the plant extract against *M. oryzae* was compared with a standard fungicide tricyclazole, used to control rice blast disease.

Materials & Methods

Fungal strains

Magnaporthe oryzae (ITCC 7019) was procured from Indian Agricultural Research Institute, Pusa, New Delhi, India. *M. oryzae* strain was maintained by routine sub-culturing on Potato Dextrose Agar (PDA) slants. The greyish black conidial growth was observed by incubating the cultures at 28 °C for seven days.

Rice seed varieties and standard fungicide

Two varieties of rice seeds- Boro and Black Joha were obtained from Pub Kamrup College, Kamrup, Assam, India. Standard fungicide tricyclazole was procured from Sigma Aldrich (India).

Plant collection and extraction procedure

Myristica fragrans was procured from Spice gardens in Thekaddy, Kerala, India and was deposited and validated by Dr. (Mrs) Sunita Garg (CSIR-Emeritus Scientist, Former Chief Scientist & Head, Raw Materials Herbarium and Museum Delhi (RHMD), CSIR-National Institute of

Science Communication And Information Resources (NISCAIR)). The deposition number of the voucher specimen submitted in RHMD is NISCAIR/RHMD/Consult/2018/3250-51.

The spice *M. fragrans* was cleaned under running tap water and dried. For maceration extraction process, 100 g of spice was crushed to a fine powder using pestle and mortar and sieved using a fine-mesh sieve. The powder was then suspended in 400 ml n-hexane, chloroform, methanol and ethanol sequentially for 72 h with constant shaking (50 rpm) at 25 °C. The solvent was filtered out using a three-layered muslin cloth and the crude extract was completely dried out using rotary evaporator. Once dried, the extract was resuspended in dimethyl sulfoxide (DMSO) to make a stock suspension of 100 µg/µL and stored at 4 ºC. The prepared extracts were coded as PE-01, PE-02, PE-03 and PE-04 for n-hexane, chloroform, methanol and ethanol, respectively.

Phytochemical analysis of extracts

Qualitative phytochemical analysis of the *M. fragrans* extracts was carried out to detect the presence of secondary metabolites like saponins, tannins, terpenoids, carbohydrate, steroids, napthoquinones and flavonoids as per the protocol of Raaman (2006) (Table 1).

Table 1 Protocol for qualitative phytochemical analysis of crude plant extracts.

S.no.	Phytochemical	Protocol	Positive Result
1.	Saponins	1 mL extract + 3 mL distilled water, shaken vigorously for 2 mins	Frothing formation
2.	Tannins	2 mL extract + 1-2 drops of 1% $FeCl_2$ solution	Occurrence of blue-black or blue-green precipitate
3.	Steroids (Salkowaski test)	1 mL extract + 2-3 mL $CHCl_3$ then 2-3 mL conc. H_2SO_4 added carefully along sides to form two different layers	Upper layer turns red and sulphuric acid layer turns yellow-green fluorescence
4.	Flavonoids (Sodium Hydroxide test)	1 mL extract + 2 mL 10% NaOH. Intense yellow colour obtained then 1% HCl added to it	Yellow colour becomes colourless
5.	Terpenoids	1 mL of extract + 2 mL $CHCl_3$ + 2-3 mL of conc. H_2SO_4 along sides of the test tube forming 2 layers	Formation of reddish-violet colour
6.	Napthoquione (Dam-Karrer test)	1 mL extract + 10% KOH (few drops)	Formation of brownish-red colour
7.	Alkaloids (Wagner's reagent)	2 mL extract + 1 mL Wagner's reagent	Formation of brown & reddish-brown colour
8.	Carbohydrates	2 mL extract + 1 mL alc. α- naphthol + 1 mL of conc. H_2SO_4 through sides of test tube	purple or violet colouration

Inoculum preparation

The *M. oryzae* spores were harvested from 7 days old cultures. The final inoculum size was adjusted to a range of 1.0 - 5.0×10^6 spores/mL by microscopic enumeration with a cell-counting haemocytometer (Kunova et al. 2013).

Antifungal activity of *M. fragrans* extracts

Minimum inhibitory concentration (MIC_{100}) of four *M. fragrans* extracts against *M. oryzae* was determined by the Clinical and Laboratory Standards Institute (CLSI) broth micro-dilution method (CLSI 2008). Two-fold serial dilutions of the extracts were made in Potato Dextrose Broth (PDB) to give a final concentration of 5.0 - 0.009 mg/mL. One hundred microliters of *M. oryzae* conidial suspension (2.5×10^4 conidia/mL) was added to each well. The final volume of each well

was 200 µL. Control wells containing conidia mixed with the solvent and chemical pesticide were prepared separately. PDB with conidial suspension was used as a positive control. The experiment was carried out in triplicates. The MICs of the extracts were observed after 7 days of incubation at 28 °C. MIC_{100} was calculated as the lowest concentration of the extract that produced no visible fungal growth (Scorzoni et al. 2007). The extract showing MIC_{100} and MIC_{50} at lowest concentration was taken for further analysis in the study.

Effect of PE-01 (hexane extract) on inhibition of *in vitro* growth of *M. oryzae*

Growth inhibition assays were conducted using pour plate method in triplicates (Kamalakannan & Shanmugam 2005). PDA plates supplemented with PE-01 extract (0.625 mg/mL and 0.312 mg/mL) as well as tricyclazole (12.5 µg/mL) were prepared. Spore suspension (1.0×10^6 spore/mL; 50 µL) was transferred to the centre of each plate and left undisturbed for 15-20 mins. PDA plates inoculated with spores were used as a positive control. Inoculated plates were incubated at 28 °C with 80-90% relative humidity under normal light for 7 days. Colony diameter was measured after 7 days of growth. Growth inhibition was determined as the per cent reduction in colony diameter relative to the positive control (Kamalakannan & Shanmugam 2005).

Extraction, purification and UV-visible spectrophotometric analysis of melanin in solid culture

Melanin estimation in *M. oryzae* conidia has been performed at 0.312 mg/mL of PE-01 using a modified protocol of Kumar et al. (2011). Mycelial plugs (1 cm diameter) were cut from colonies grown on PDA plates and boiled for 5 min in distilled water (5 mL) followed by centrifugation (5000 *g*; 5 min). After washing with distilled water, the pellet was resuspended in 2 mL of 1M NaOH and autoclaved (120 °C; 20 mins) to prevent the formation of melanoidins. The autoclaved suspension was further centrifuged (5000 *g*) for 5 min to recover the supernatant containing the pigment. The alkaline pigmented supernatant was purified by acid hydrolysis (2 mL of 7M HCl) in a sealed glass vial for 2 h at 100 °C. After cooling, the precipitate was recovered by centrifugation (5000 *g*; 10 min). The precipitate was suspended in 100mM borate buffer and UV-visible absorption spectrum was recorded in the wavelength range (250-800 nm) on a UV-visible spectrophotometer. 100mM borate buffer was used as a blank. The experiment was conducted in triplicates.

Chemical analysis of extracted melanin pigment

The chemical analysis of extracted melanin pigment was performed using the modified method of Fava et al. (1993). The solubility of the pigment was assessed in different solvents such as 1N HCl, 1M NaOH, Chloroform, Acetone, DMSO, Ethyl Acetate, Acetic acid and Hexane. Decolourization of pigment was estimated by the oxidizing agents (H_2O_2/$KMnO_4$) and precipitation by 1% $FeCl_3$.

Appressorium formation and penetration test of *M. oryzae* conidia on onion peel epidermis

Onion bulb scales were thoroughly rinsed with distilled water. The inner epidermis of onion bulb scales was peeled off and cut into 1×1 cm^2 strips. Three sections were floated on 4 mL distilled water in separate 60 mm Petri plates. Freshly harvested conidia were washed with sterile water followed by centrifugation at 4500 *g* for 10 min and re-suspended in sterile distilled water to a final concentration of 1×10^5 conidia/mL. The conidial suspension (10 µL) was placed on one strip as positive control. PE-01 extract (0.625 mg/mL) and tricycalzole (12.5 µg/mL) was added with 10 µL of the conidial suspension on the second strip and third strip, respectively in triplicates. The strips were incubated at 28 ºC for 48 h. Conidial suspension with extract was removed after 48 h of inoculation and 30% methanol was applied to prevent further penetration during observation (Chida & Sisler 1987). The strips were stained with lactophenol cotton blue stain and observed under a light microscope (10× and 40× magnification) to observe appressorium formation & penetration.

For scanning electron microscopy (SEM), strips were sputter-coated with gold and observed on Zeiss SEM, MA EVO-18 Special Edition (Liu et al. 2011).

Rice seed infection assay under greenhouse condition

One hundred fifty seeds of boro and black joha rice varieties were washed with distilled water, dried on blotting sheet and then spray-inoculated with 1×10^5 conidia/mL of *M. oryzae* (Namai & Ehara 1986). Inoculated seeds were dried in the laminar flow chamber on single layers of blotting paper in Petri plates for 2-3 h.

Fifteen rice seeds (pre-inoculated with *M. oryzae)* of each variety, were sprayed with approximately 1 mL of PE-01 extract (0.625 mg/mL). Treated seeds were dried for 2 h under sterile conditions and later were planted in pots (15 seeds per pot) containing soilrite mixture and kept under greenhouse conditions (25–30 °C and 85–95% relative humidity) for 14 days. The efficacy of the PE-01 extract in controlling the infection was assessed by comparing root and shoot length of seedlings in treated and untreated samples.

Gene expression analysis of *pksP/ alb1*

Sample preparation

A twelve well plate of PDB media supplemented with PE-01 extract (0.312 mg/mL) and tricyclazole (12.5 µg/mL), in duplicates, was inoculated with *M. oryzae* conidial suspension (1×10^5 conidia/mL). The plate was incubated for 7 days at 28 °C.

Primer designing

The *M. oryzae pksP/ alb1* and *actin* CDS gene sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/pubmed) database for designing the primer for the expression analysis. The primers were designed by Primer 3 software (http://primer3.ut.ee/; Untergasser et al. 2012).

The primer sequences were then analyzed for potential hairpin formation and self-complementarity (http://www.basic.northwestern.edu/biotools/oligocalc.html). The details are given in Table 2.

Table 2 Gene-specific primers used for qRT-PCR.

S.No	Gene Name	Gene Reference IDs	Primer Sequence (5'-3')	Amplicon size (bp)
1	*pksP/ alb1*	XM_003715386.1	F- GATGGCAACTACGTCTTC R- GTAGCTGCGGTACTTCTT	174
2	Actin	XM_003719823.1	F- GACTCTTACGTTGGTGATG R- CTCGTTGTAGAAGGTGTG	129

Total RNA isolation, purification and cDNA synthesis

Total RNA was isolated from *M. oryzae* treated with PE-01 extract, tricyclazole and untreated culture using RNA-Xpress reagent (HiMedia) as per manufacturer's instructions. RNA preparations were subjected to DNaseI treatment according to the defined protocol (Fermentas) to remove genomic DNA contamination. The integrity of total RNA was analyzed through 1.2% agarose gel electrophoresis. The purity of isolated RNA was measured at A260/280 using nanodrop-1000. Two micrograms of total RNA of each sample was used to synthesize the first-strand cDNA by oligo (dT)-18 primer using the Hi-cDNA synthesis Kit (HiMedia). PCR was

performed in a 25 μL reaction volume containing 0.2mM dNTPs, 10 picomoles of each primer, 1.5mM $MgCl_2$, 0.8U Taq DNA polymerase (HiMedia), using 100 ng of cDNA.

Expression profiling of genes by RT-qPCR

Real-time qPCR amplification was carried out by HiMedia LA-1012 RealTime PCR, using a power SYBR-green master mix (HiMedia). The relative quantification of each gene expression was performed using the comparative threshold cycle method. The amplification program used for real time was: 95°C for 3 min, 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The melt curve analysis as per INSTA Q-96 programme quantitative RealTime PCR was at 95°C for 15 s, 60°C for 60 s, 72°C for 30 s and holding time 10 s. The results were analyzed using INSTA-Q96 software and the genes were considered differentially expressed if they were at least two-fold up- or down-regulated.

Gas Chromatography- Mass Spectroscopy (GC-MS) analysis

The chemical constituents of PE-01 extract were analyzed using a gas chromatograph (GC) Varian-450 fitted with a fused silica capillary column TG-5 (30 m × 0.25 mm i.d., 0.25 μm film thickness) under the experimental conditions reported earlier (Joshi 2014, 2016). The oven temperature was programmed from 60°C to 220°C at 3°C/min, using nitrogen as a carrier gas. The injector and the flame ionization detector (FID) temperature were set at 230°C and 240°C, respectively. Gas chromatography–mass spectrometer (GC–MS) analysis employed a Thermo Scientific Trace Ultra GC interfaced with a Thermo Scientific ITQ 1100 mass spectrometer fitted with a ZB-5 fused silica capillary column (30 m × 0.25 mm; 0.25 μm film thickness). The oven temperature range was programmed from 60°C to 220°C at 3°C/min, and helium was used as a carrier gas at 1.0 mL/min for analysis. The injector temperature was set at 230°C, and the injection volume was 0.1 μL in *n*-hexane, with a split ratio of 1:50. MS was taken at 70 eV with a mass range of m/z 40–450 (Joshi 2014, 2015). The relative amounts of individual components were calculated according to GC peak area (FID response) without using a correction factor.

Statistical analysis

The experiments were done in duplicates or triplicates, and the obtained data were expressed as mean ± standard error. All the statistical analysis was performed using Graphpad Prism software version 8.0.2 and Microsoft Excel 2016. $p \leq 0.05$ was considered statistically significant.

Results

Phytochemical analysis of *M. fragrans* extracts

The phytochemical analysis revealed the presence of alkaloids, carbohydrates, steroids, tannins and terpenoids in the *M. fragrans* extracts (Table 3). Flavonoids and saponins were not detected.

Table 3 Phytochemical analysis of *M. fragrans* extracts.

Name of extracts	Alkaloids	Carbohydrate	Flavonoids	Saponins	Steroids	Tannins	Terpenoids
PE-01	+ +	+	-	-	+ + +	+ +	+ +
PE-02	+	+	-	-	+ +	+	+
PE-03	+ +	++	-	-	+ + +	+	+
PE-04	+	+ +	-	-	+	+ +	+

'+,++,+++' showed presence, '–' absence

Antifungal activity of *M. fragrans* extracts

Minimum Inhibitory Concentration-100 (MIC_{100}) and Minimum Inhibitory Concentration-50 (MIC_{50}) of PE-01 (n-hexane extract of *M. fragrans*) was calculated as 0.625 mg/mL and 0.312 mg/mL. However, antifungal activity was not observed in other *M. fragrans* extracts. Therefore, only PE-01 extract was used for further studies. Tricyclazole (TCZ) was used as a positive drug control.

Effect of PE-01 on inhibition of *in vitro* growth of *M. oryzae*

The extract PE-01 (0.312 mg/mL) showed greyish white radial growth (23 mm diameter; Fig. 1b) correspond to 50% growth inhibition as compare to dark grey colour positive control (53 mm diameter; Fig. 1a) after 7 days of incubation. PE-01 extract (0.625 mg/mL) showed 100% growth inhibition of *M. oryzae* (Fig. 1c). Brown colour radial growth (phenotypic change) was observed with tricyclazole (Fig. 1d). Radial mycelial growth was inhibited significantly (p <0.05) at MICs of PE-01 extract.

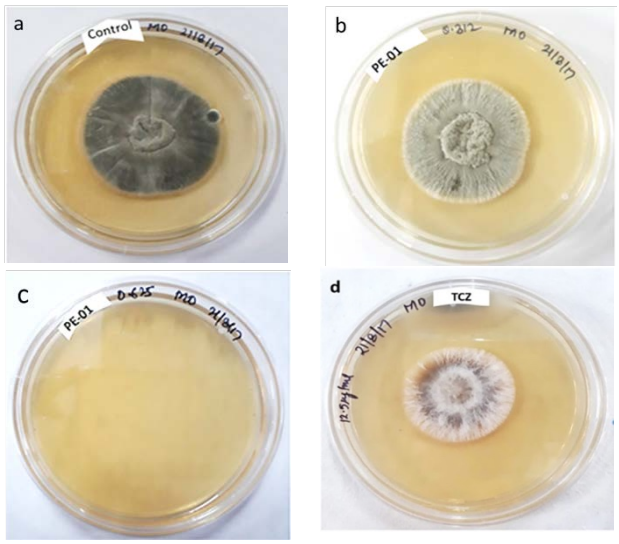

Fig. 1 – Mycelial growth assay of *M. oryzae* in presence of PE-01 extract. a Control plate. b PE-01 extract at MIC_{50}. c PE-01 extract at MIC_{100}. d Tricyclazole (TCZ) treated plate.

Extraction, purification and UV-Visible spectrophotometric analysis of melanin in solid culture

Melanin was extracted from control as well as 0.312 mg/mL PE-01 treated cultures of *M. oryzae*. The results showed a significant reduction in melanin formation in PE-01 treated appressorium and conidial cell wall as compared to control. The absorption spectra showed characteristic absorption peaks in the UV regions ranging from 265-290 nm, but not in the visible region. The overall characteristic absorption peak was shown at 275 nm (Fig. 2). In control and PE-01 treated samples, optical density at 275 nm was 0.93 and 0.62 respectively.

Chemical analysis of extracted melanin pigment

The physico-chemical tests of extracted pigment revealed its solubility in NaOH and KOH. It was insoluble in water or organic solvents like chloroform, ethyl acetate, alcohol, and acetone. The pigment was decolorized by the oxidizing agents H_2O_2 and $KMnO_4$ and precipitated by 1% $FeCl_3$.

Appressorium formation and penetration test of *M. oryzae* conidia on onion peel epidermis

Magnaporthe oryzae conidia were unable to adhere and form appressorium and penetration peg (an initial invasive step of infection) in the presence of PE-01 extract. Only distorted conidia were observed under a light microscope (40× magnification). The microscopic study revealed that the untreated and tricyclazole (chemical pesticide) treated conidia showed germination, hyphal

growth and formed appressorium for penetration as well as invasive hyphae germination on onion epidermis (Fig. 3).

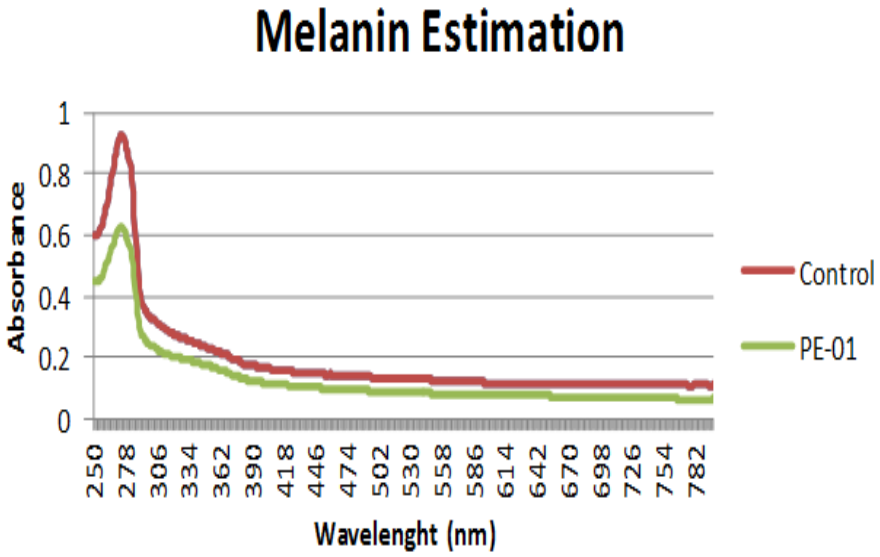

Fig. 2 – Melanin estimation in *M. oryzae* conidia and appressorium after PE-01 treatment at MIC_{50} using UV-Vis spectroscopy.

Scanning electron microscopy

SEM analysis revealed the presence of three-celled stage conidia with proper appressorium and hyphae formation in control whereas PE-01 treated conidia were distorted without septum, no hyphal and appressorium formation (Fig. 4a and 4b, respectively). Tricyclazole treated conidia were present in three-celled stage with a lesser number of appressoria and hyphae (Fig. 4c).

Rice seed infection assay under greenhouse condition

Under *in vivo* conditions, PE-01 extract (0.625 mg/mL) was found to be lethal to *M. oryzae* and reduced the blast incidence in boro and black joha rice seedlings by seed treatment. Besides that, it increased the sprouting of the seeds as compared to the control pots (Pot 1- positive control; Pot 2- negative control; Fig. 5a and 5b).

Shoot and root length of rice seedlings was measured and compared after 14 days of treatment as shown in Table 4. The change in seedlings growth pattern was clearly observed in PE-01 treated Pot 3 as compare to the positive, negative and tricyclazole control pot (Fig. 5c and 5d). There was approximately 1-fold increase in shoot length of both varieties when treated with PE-01 extract in comparison to the untreated. The extract reduced the incidence of rice blast infection significantly ($p<0.05$) when compared favorably with the chemical pesticide tricyclazole.

Expression profiling of *pksP/alb1* gene

The transcript profiling of *pksP/ alb1* gene was performed for *M. oryzae* with and without PE-01 extract and tricyclazole treatment. Differential gene expression was observed. The expression of *pksP/ alb1* gene, a polyketide synthase gene, was found 4 folds up-regulated ($p \leq 0.05$) in PE-01 treated sample and 1.5 folds up-regulated in tricyclazole as compared to untreated (control) sample (Fig. 6). The results indicated that *pksP/ alb1* expression was higher at MIC_{50} of PE-01 as compared to tricyclazole.

GC-MS analysis

The results of GC-MS analysis of PE-01 extract revealed the presence of various biologically active chemical compounds (Table 5).

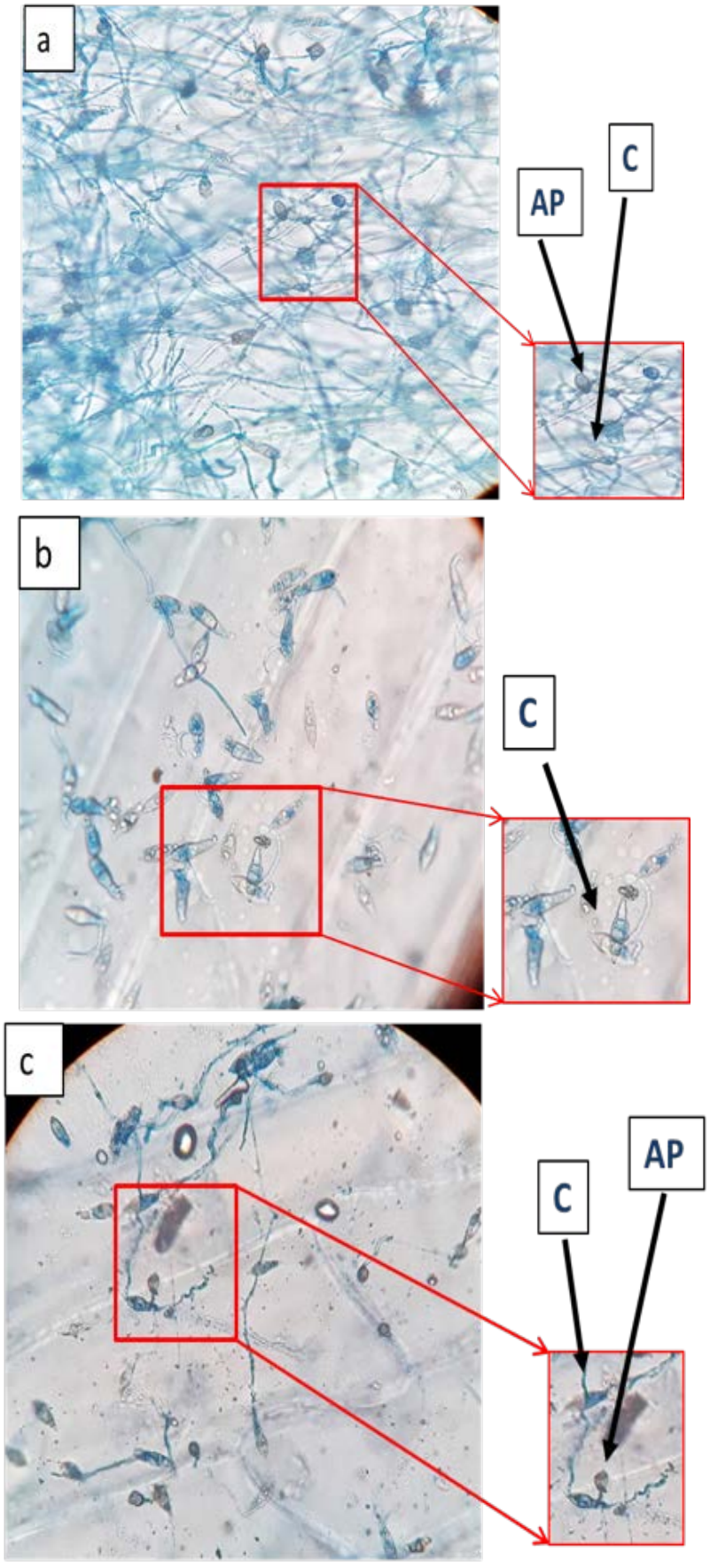

Fig. 3 – Comparison of germination and appressorium formation of *M. oryzae* on onion peel epidermis using the light microscope at 10 × and 40 × magnification; a Control. b PE-01 extract. c Tricyclazole (TCZ). C- conidia, AP- Appressorium.

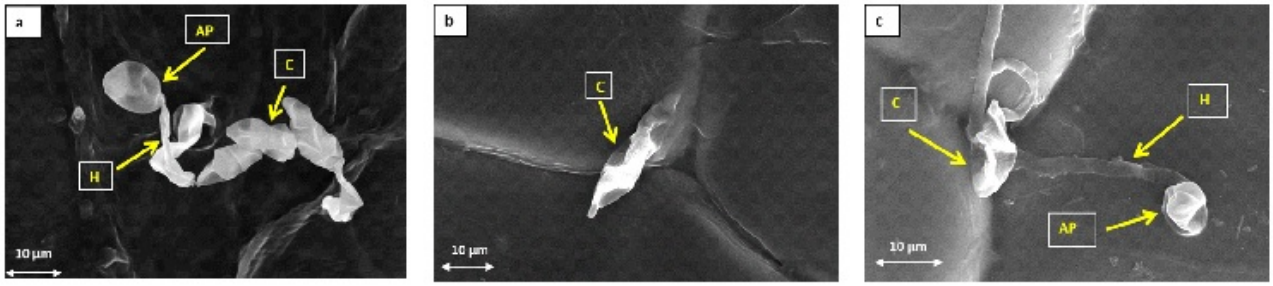

Fig. 4 – SEM analysis of appressorium and hyphae formation of *M. oryzae* on onion peel epidermis a wild type conidia. b PE-01 extract. c Tricyclazole (TCZ) at 4000× magnification. Scale Bars = 10µm. *AP-Appressorium, C- Conidia, H- Hyphae.

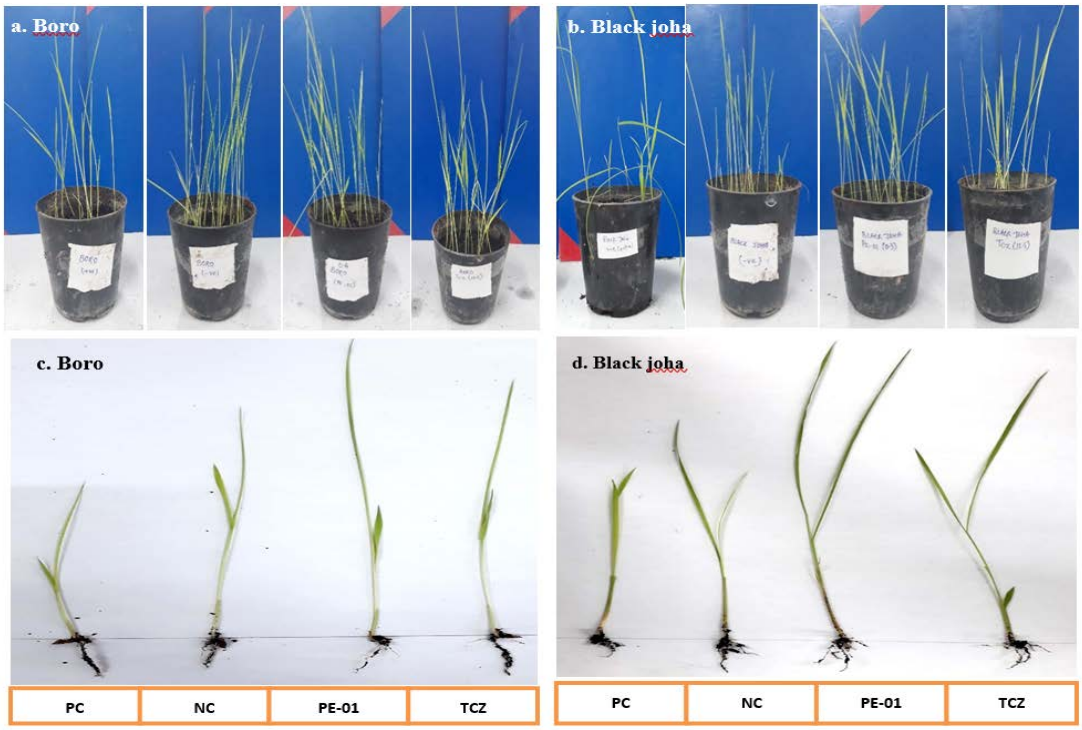

Fig. 5 – Rice seed infection assay a Boro rice variety. b Black joha rice variety grown at MIC_{100} value (0.625 mg/mL) of PE-01 extract in pot 3, positive, negative and Tricyclazole (TCZ) control in pot 1,2 and 4 respectively. c and d Length of shoot and root was measured with the help of scale in cm. PC- positive control, NC- negative control.

Table 4 Effect of PE-01 treatment on growth and development of rice plants by measuring shoot and root length under *in-vitro* conditions.

S.No.	Samples	Boro Variety		Black Joha Variety	
		Means of variables*			
		Shoot length (cm)	Root Length (cm)	Shoot length (cm)	Root Length (cm)
1	PE-01	27.2	3.4	18.9	3.3
2	Positive control (PC)	14.7	3.6	13.4	2.1
3	Negative control (NC)	21.0	2.9	15.0	4.3
4	Tricyclazole (TCZ)	23.7	5.0	17.6	2.9

*Values are means of four replicates. $p \leq 0.05$ was considered statistically significant.

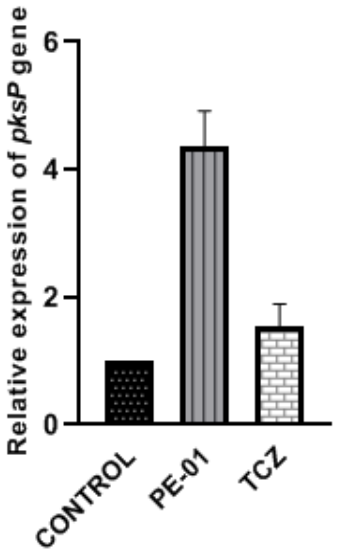

Fig. 6 – Relative quantification of *pksP/ alb1* gene expression (normalised to house-keeping gene, actin) in *M. oryzae* treated with MIC_{50} of PE-01 extract and Tricyclazole (TCZ). Data are means of fold changes with standard deviation from three independent experiments amplified in triplicate. $p \leq 0.05$ was considered statistically significant.

Table 5 Biologically active chemical compounds of n-hexane extract of *M. fragrans.*

S.No.	Chemical Name	Common name	Area %
1	Cyclohexene, 1-methyl-5-(1-methylethenyl)	Sylvestrene	3.13
2	1-Methyl-4-isopropyl-1,4-cyclohexadiene	gamma.-terpinene	1.12
3	Isoeugenol	Isoeugenol	1.33
4	1,3-benzodioxole, 4-methoxy-6-(2-propenyl)	Myristicin	8.62
5	Benzene-1,2,3-trimethoxy-5-(2-propenyl)	Elemicin	12.64
6	L-(+)-ascorbic acid 2,6-dihexadecanoate	Hexadecanoic acid	10.42
7	Cis-9-hexadecenal	Olealdehyde	23.57
8	Pinene	Alpha-pinene	1.42

Discussion

One of the most important plant fungal pathogen causing rice blast is *M. oryzae* (Dean et al. 2012). Chemical fungicides are repeatedly used to controlled plant pathogenic fungi which have disrupted the natural biological system and also lead to increase in antifungal resistance among pathogens. They have showed various detrimental effects on non-target organisms, raising environmental and human health concerns (Yoon et al. 2013). The Economic Survey 2015-2016 in India noted that the use of pesticides without proper guidelines has led to an increase in pesticide residue being found in food products.

Worldwide, biopesticides comprised a value of $3 billion in 2017, which accounts for just 5% of the total crop protection market (Damalas & Koutroubas 2018). There are 175 registered biopesticide active-ingredients and 700 products available in the global market. In the Indian scenario, bio-pesticides represent only 4.2% of the overall pesticide market and are expected to demonstrate a remarkable annual growth rate of about 10% in the coming years (Kumar 2012). The Standing Committee on Agriculture has also recommended the development of an integrated pest-management system, which encourages the use of bio-pesticides (Economic Survey 2015-2016).

Plant oils are important source of antifungal compounds and they may provide alternative source of useful fungicides that can be utilized in antimycotic drugs against fungal pathogens

(Uniyal et al. 2012). In the present study, different organic solvents (hexane, chloroform, methanol, and ethanol) were used for extract preparation based on polarity gradient. It has been reported that the type of solvent used for extract preparation impacts the antimicrobial activity (Witkowska et al. 2013). The phytochemical studies of various *M. fragrans* extracts revealed the presence of steroids, carbohydrates, tannins, alkaloids and terpenoids. However, Asgarpanah & Kazemivash (2012) reported the phytochemical constituents of *M. fragrans* as volatile substances, terpenoids, phenolics, lignins, proteins, mucilage and starch. These variations may be due to various biotic (insects, pathogens) and abiotic factors (climatic conditions, temperature, sources of water, soil salinity etc.).

Myristica fragrans inhibits the hyphal growth and spore formation of various *Aspergillus* sp. which revealed its high antimycotic activity (Uniyal et al. 2012). In the present study, among four extracts, only n-hexane extract of *M. fragrans* (0.625 mg/mL) inhibited the mycelial growth of *M. oryzae* under *in vitro* as well as *in vivo* conditions. The mycelium growth of *M. oryzae* was inhibited with increasing concentrations of PE-01 and showed 50% reduction in growth at 0.312 mg/mL in *in-vitro* studies (Fig. 1). Amadioha (2000) and Kamalakannan et al. (2001) also reported reduced radial growth of *Pyricularia grisea* using neem hexane extract at different concentrations and also incidences and severity of rice blast.

DHN-melanin has been implicated as a virulence factor in various fungal plant diseases. The previous studies have reported that the melanin-deficient mutants or wild-type strain treated with a melanin biosynthesis inhibitor cannot penetrate the host plant (Woloshuk et al. 1983, Chumley & Valent 1990, Howard & Valent 1996). According to Howard et al. (1991), the central role of melanin layer is its permeability to water and solute allowing high turgor pressure in the protoplasm to build up to form specialized penetration peg through the outer plant surface into the plant tissue. In the present study, melanin was extracted and purified from untreated and treated conidia of *M. oryzae*. The result depicted that there was a reduction in melanin formation on PE-01 extract treatment as compared to control (Fig. 2). The absorbance declined as the wavelength increased to the visible region which is the property of aromatic organic compounds. For appressorium formation and infection, conidia require strong adhesion ability and a pentaketide-derived melanin pigment (Howard & Ferrari 1989), but on PE-01 treatment, adhesion ability was disrupted on the conidial surface.

According to Tucker & Talbot (2001), formation of appressorium is a complex process that involves surface attachment and recognition, spore germination, germ tube growth, and cytoskeletal reorganization. PE-01 extract treatment completely inhibited appressorium formation onto the onion surface. Even tricyclazole treated surface showed approximately 50% reduction in appressorium formation, as similarly reported by Chida & Sisler (1987). Cell surface morphology of conidia, hyphae formation, and appressorium formation was analyzed using SEM, which indicated that PE-01 treated conidia were unable to form hyphae and appressorium on the onion epidermis (Fig. 4b). Similar results were reported by Chida & Sisler (1987) where the appressorium of the melanin mutant did not penetrate onion epidermis. Penetration assay of PE-01 treated conidia might speculate that the extract at MIC concentration causes the loss of pathogenicity in *M. oryzae* to penetrate inside the host plant, due to the lack of appressorium pigmentation (Woloshuk et al.1980, Chumley & Valent 1990).

The *in vivo* experiments were also conducted under greenhouse conditions to analyse the efficacy of PE-01 extract against rice blast pathogen. Rice seedlings showed change in growth pattern on PE-01 treatment as compared to control (Fig. 5c, d). These results indicated that the plant extract was not phytotoxic to rice seedlings. No rice blast infection was observed in treated pots. According to Hubert et al. (2015), the shoot length of rice seedlings of cultivar Mwangaza were significantly different when treated with plant extracts and essential oils from *Callistemon, Citrinus and Ocinum gratissimum* in comparison to the control shoot against *Bipolaris oryzae*. It has been reported by Miah et al. (2017) that when infested seeds were sown in the field, the fungus was recovered from different seedling parts, including roots. These results clearly indicated that the

fungus can survive on the grains used for seeding and could serve as primary inoculum (Miah et al. 2017).

The polyketide synthase *pksP/alb1* is the first enzyme of the DHN-melanin biosynthesis pathway. There are three genes (*alb1*, *rsy* and *buf*) associated with three different phenotypical mutants. Mutations at *alb1*, *rsy* and *buf* result in colonies with albino, rosy and buff pigmentation, respectively (Kawamura et al. 1997). Gene expression analysis of *pksP/ alb1* gene in the present study proved that the *pksP/ alb1* gene expression was significantly up-regulated (Fig.6) when *M. oryzae* were treated with PE-01 extract, which may be due to generation of stress. Some previous studies had also suggested that the increased expression of a gene in the presence of an inhibitor could be a feedback mechanism to compensate for its protective response to oxidative damage (Morschhauser 2002, Watamoto et al. 2011). Due to increased expression of *pksP* gene, the current study proposes that the differential expression of proteins involved in melanin biosynthesis pathway need to be evaluated to understand the molecular mechanisms.

The GC-MS analysis of PE-01 extract revealed the presence of sylvestrene, gamma-terpinene, isoeugenol, myristicin, elemicin, hexadecanoic acid, olealdehyde as major constituents. According to Alcazar-Fuoli & Mellado (2012), major constituents in essential oil of *M. fragrans* weremyristicin, safrole, pinene, isoeugenol, 4-terpenol. The quantitative and qualitative divergence of plant constituents may be due to the geographical, climatic, and soil conditions, which in turn may affect the composition and/or synthesize new secondary metabolites from the same plant species (Du et al. 2014). The potential role of these components needs to be further analysed.

Conclusions

The current study concludes that the n-hexane extract of *M. fragrans* is capable of inhibiting the *M. oryzae* conidiation, curbing the initiation of infection at the entry stage of appressorium formation and in inhibiting the formation of melanin in the appressorium. It is restricting the melanin formation pathway via feedback mechanism of *pksP/ alb1* gene overexpression during *M. oryzae* germination. Besides, the extract also enhances the boro and black joha rice seed germination efficiency. Thus, *M. fragrans* extract has the potential to be developed as a bio-pesticide against the rice blast pathogen *M. oryzae.*

Disclosure statement

No potential conflict of interest was reported by the authors.

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