Chapter 2

Materials and methods

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Fungal strains and growth conditions

M. oryzae B157 strain (MTCC accession number 12236), corresponding to the international race IC9 was used for this study. The fungus was grown and maintained on oatmeal agar (Hi-Media, Mumbai, India) (35 g per liter) at 28°C with a 12 h photo period for conidiation. Complete medium (glucose 1%, peptone 0.5%, yeast extract 0.2%, CAA 0.1%, $NaNO_3$ 0.6 %, KCl 0.05%, $MgSO_4$ 0.05%, KH_2PO_4 1.5%), (pH 6.5) was used for growing culture in broth at 28°C in a shaking incubator.

Extraction of anacardic acid

Extraction of anacardic acid from cashew nut shells was carried out by the procedure reported previously (Omanakuttan et al. 2012). Cashew nut shells were defatted with petroleum ether (PE) using a rotary shaker. The extract was subjected to rotary evaporation below 40˚C to obtain cashew nut shell extract (CNSE). CNSE residue was subjected to thin layer chromatography with a solvent system of PE (70%), ethyl acetate (28%) and formic acid (2%).The bands were visualized by spraying a mixture of aqueous ferric chloride (1%) and potassium ferricyanide (1%; v/v) and followed by methanolic ferric chloride (1%). Anacardic acid was separated from other constituents using SiO_2 column chromatography and eluted with PE containing increasing concentrations of chloroform. The isolated anacardic acid was confirmed by Shimadzu LC-20 HPLC system (Shimadzu Corporation, Kyoto, Japan), equipped with a Phenomenex C18 RP column (Phenomenex, Torrance, CA, USA) and PDA detector using mobile phase of acetonitrile: water: acetic acid (72:18:10) monitored at 245 nm. Anacardic acid showed the presence of triene (56.2%), diene (18.3%) and monoene (24.2%) forms and saturated C15 aliphatic chain (1.3%). Further, HPLC-MS data was generated by an Ultra Performance Liquid Chromatography (UPLC) using Agilent 1290

series (Agilent technologies, Palo Alto, CA, USA) coupled to an Ion Trapped MS (Agilent 6340 series) with electrospray interface. The 3 major molecular peaks showed masses corresponding to 343 m/e (triene), 345 m/e (diene), 347 m/e (monoene) forms for the anacardic acid. The ^{1}H-NMR spectra generated by Bruker AV II 500 spectrometer (Bruker Instruments, Karlsruhe, Germany) was similar to previous reports. 20mg/ml solution of purified anacardic acid mixture and cardol-cardinol extract was prepared using dimethyl sulfoxide (DMSO) and stored at -20˚C. For experimental purposes, anacardic acid from -20˚C stock was diluted with sterile water and working concentration of DMSO was maintained lower than 0.1% in all the experiments.

Spore isolation and infection assay

Vegetative growth and conidiation were measured as described (Liu and Dean 1997) using oatmeal agar plates. Harvesting of spores was done by scrapping mycelia from 7-8 day-old plates and mixing it with 1ml of sterile water and counting the spores using haemocytometer. Infection assays were performed as previously described (Xu and Hamer 1996) .

Conidial germination

M. oryzae wild type strain B157 was cultured on oat meal agar plates at 28°C for conidiation. Conidia from 8-10 day old cultures were harvested in sterile water by filtering it through Miracloth (Calbiochem, La Jolla, CA, USA). The spores were allowed to germinate for 12-14 hours at 28°C in a shaking incubator and observed under light microscope (*Optiphot-2*; *Nikon*, Tokyo, *Japan*). All conidia that could form hyphal growth of any size were considered as germinated conidia. A total of 100 conidia were observed in each of the duplicate samples and each experiment was performed three times. Values were plotted in the graph as mean of three replicates ± Standard deviation (SD).

Mycelial growth inhibition assay

Mycelial cell death assay was performed to evaluate the number of colony forming units in treated and untreated samples (Liu et al. 2010). *M. oryzae* conidia (10^6 conidia/mL) were allowed to germinate in 100 mL flasks with 20 mL complete medium broth (CM) at 28°C in a rotary shaker (200 rpm) for 12 hours. The cultures were exposed to different concentrations of anacardic acid for 2 hours. The germinated conidia were washed with sterile water, diluted to 10^4 conidia/mL and plated on oat meal agar and incubated at 28°C for 3 days. Colony-forming units (CFUs) were counted in each of the three individual experiments performed and values were plotted in the graph as average of three replicates. The data in each sample was expressed as percentage of total number of CFUs observed in untreated or 0.1% DMSO treated control.

TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase fluorescein-12-dUTP nick end labeling) assay was performed by germinating spores of *M. oryzae* in complete medium for 12 h at 28°C. The germinated spores were fixed and double stained with Hoechst 33258 (*Sigma-Aldrich, St Louis, USA)* and TUNEL Dead-End™ Fluorometric TUNEL System (Promega, Madison, USA) by a previously described procedure (Chen and Dickman 2005; Madeo et al. 1997) with some minor modifications. Images were obtained with LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany) using the following lasers: 405 nm for Hoechst 33258 and 488 nm for FITC.

Annexin V staining

Fungal mycelia were washed three times with sterile H_2O and digested using lysing enzyme (*Sigma*, St. Louis, MO, USA) in sorbitol buffer (1 M sorbitol, 10 mM PBS, pH 7.0) at 28°C

for 3-4 h by shaking gently at 100 rpm. The filtered protoplasts were harvested, washed and double stained with propidium iodide (PI) (*Sigma-Aldrich, St Louis, USA)* and FITC-Annexin V using the Annexin V–FITC Apoptosis Detection kit (Invitrogen, Carlsbad, California) using a standard protocol (Chen and Dickman 2005). Images were obtained by Carl Zeiss LSM700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) and numbers of Annexin V positive and PI positive protoplasts were scored. Each experiment was repeated three times and values were plotted in the graph as average of three replicates.

Mitochondrial membrane potential

CMXRos (Mitotracker red) is a highly efficient nontoxic sensitive indicator of relative changes in mitochondrial membrane potential (MMP) (Pendergrass et al. 2004). 0.1 mg/ml of Mitotracker red (Life technologies, Carlsbad, CA USA) was used to evaluate changes in MMP during anacardic acid induced cell death of *M. oryzae*. Fungal spores were allowed to germinate for 12-16 hours at 28°C in complete medium for hyphal growth. After incubating with various concentrations of anacardic acid for 2 hours, the mycelia (both treated and control) were washed with sterile water and stained with 0.1 mg/ml Mitotracker red (Life technologies, Carlsbad, CA USA) in 10 mM PBS (pH 7.0) for 15 mins at room temperature. Staining was followed by fluorescence measurement at Excitation*/Emission* (nm): 590*/*645 using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Each experiment was repeated three times and average values were plotted in the graph.

ROS detection

Intracellular reactive oxygen species (ROS) accumulation was detected with the oxidant-sensitive probe dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma St. Louis,

MD, USA) as described (Machida et al. 1998). Spores of *M. oryzae* were cultured in complete medium for 12 hours and treated with different concentrations of anacardic acid (with untreated and 0.1% DMSO treated as control). The mycelia were washed and resuspended in 10 mM PBS (pH 7.0) and incubated with 40 mM H_2DCFDA (dissolved in dimethyl sulfoxide) for 20 min at room temperature. For fluorescent microscopy, stained mycelia were observed under LSM 700 confocal microscope (Carl Zeiss, Germany) at Excitation*/Emission* 488/530 *nm*. For quantitative estimation of intracellular ROS, mycelia were subjected to fluorescence analysis at Ex/Em (nm): 485/530 using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Each experiment was repeated three times and mean values were plotted in the graph ± SD.

Caspase assay

Fungal spores were harvested and allowed to germinate for 12 hours in complete medium at 28°C. To determine role of metacaspases in anacardic acid mediated apoptosis of *M. oryzae*, 20 mM Z-VAD-fmk (Promega, Co., Madison, WI, USA) was added to germinated spores previously treated with different concentrations of anacardic acid. The germinated spores were allowed to form colonies on oat meal agar plates and the colony forming units (CFUs) were counted after 3 days of incubation at 28°C. Each experiment was repeated three times and values were plotted in the graph as average of three replicates ± SD.

RNA isolation and quantitative Real-time PCR

RNA isolation from fungus was carried out by grinding the mycelia in liquid nitrogen and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA from each treatment was then fractionated in 1.2% w/v agarose gel, stained with ethidium bromide, and then visualized with UV light. First strand cDNA was synthesized from 1 µg of total RNA using Revert Aid First Strand cDNA Kit (*Fermentas*, St. Leon-Rot, *Germany*). AifM

mRNA expression was measured by quantitative real-time RT-PCR in a fluorescent temperature cycler (Lightcycler 2.0; Roche Molecular Biochemicals, Mannheim, Germany). Values were normalized using β-tubulin as the internal reference. The ΔΔ Ct method followed by determination of $2^{-\Delta\Delta Ct}$ was used to determine the fold change in expression. Gene specific primers AIFM-RT-F and AIFM-RT-R used for quantitative RT-PCR expression analysis of AifM are 5'-TGGCGCAAGAGTACAAGTTGA-3' and 5'-AAGACACCCTTGACGAGCG-3' respectively. Expression analysis was repeated three times and values were plotted in the graph as average of three replicates.

Statistics

Statistical analysis of the data was carried out by one way ANOVA followed by *Tukey's HSD* (Honest Significant Differences) test and two way ANOVA followed by Bonferroni's post-hoc test. The results were represented as mean ± S.D using Graph Pad Prism version 5.0 Graph Pad Software, San Diego, USA.