Chapter 3

Results

Results

Anacardic acid inhibits conidial germination.

Evaluation of inhibitory action of anacardic acid on conidial germination of *M. oryzae* was carried out by establishing minimum inhibitory concentration (MIC). 10^5 spores were incubated with different concentrations of anacardic acid ranging from 0-100 µM. After overnight incubation at 28^{o}C, conidial germination was observed under microscope (Fig. S1A: in the Supplementary Material). On an average, 89% ± 4.2 of conidia germinated to form hyphal growth in untreated sample. Strong inhibition of conidial germination was observed in anacardic acid treated samples while no such inhibition was observed in control containing 0.1% DMSO (control). At 75µM anacardic acid, complete inhibition of spore germination was observed (Fig. 1a). Treated spores were then inoculated in complete medium and allowed to grow for 3 days in a shaking incubator at 28°C. It was found that normal mycelial biomass was produced in untreated and 0.1% DMSO treated samples however no biomass was seen in samples treated with or more than 75µM of anacardic acid (Fig. S1B: in the Supplementary Material).

Anacardic acid inhibits mycelial growth

Antifungal potency of anacardic acid on *M. oryzae* was determined by calculating number of colony forming units (CFU) in treated and untreated samples. First, spores were allowed to germinate for 12-14 hours in complete medium to form hyphal growth. Germinated cultures were exposed to various concentrations of anacardic acid ranging from 0-300 µM, with 0.1% DMSO as control. The treated mycelia were allowed to grow on oat meal agar plates for 3 days. Inhibition of mycelial growth was determined by counting number of colony forming units. At 50 µM of anacardic acid around 50 % ± 4 of hyphae survived (control, 100%) and formed colonies. Almost 6% ± 2 colonies survived in samples treated with 300 µM of

anacardic acid (Fig. 1b). No further cell death was observed even at concentrations higher than 300 µM. Our results suggest that mycelial cells are comparatively less sensitive to anacardic acid than conidia.

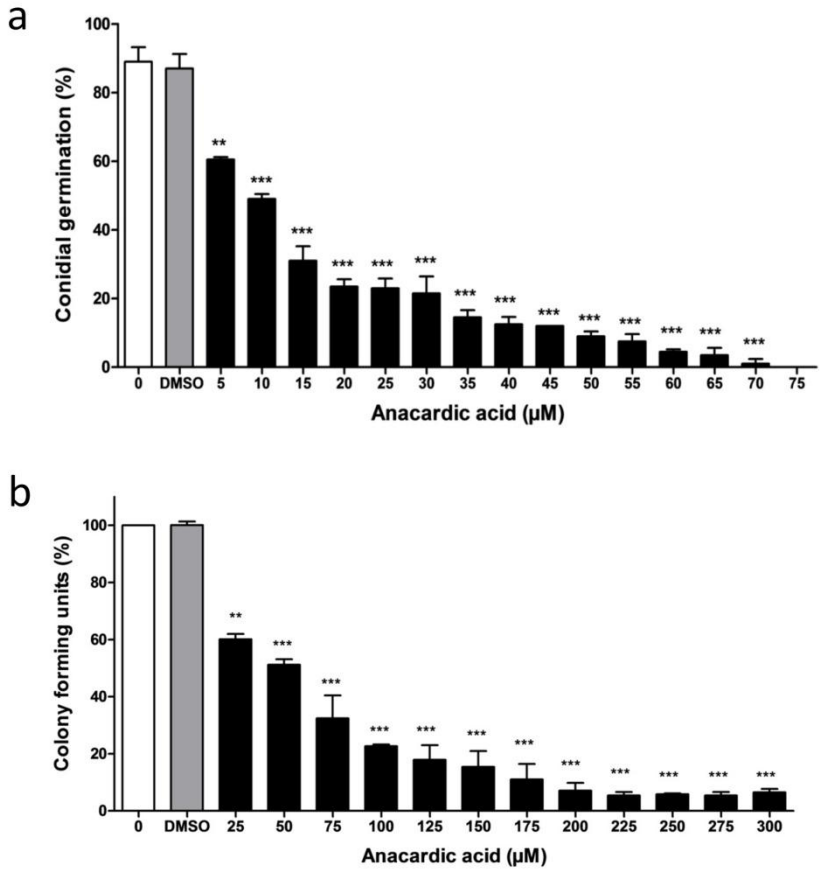

Fig. 1 Effect of anacardic acid on conidial germination (a) and mycelia growth (b). (a) *M. oryzae* conidia were treated with different concentrations of anacardic acid and incubated for 12 hours for germination in complete medium. No inhibition was observed in 0.1% DMSO (control) while complete inhibition was observed at 75µM. (b) Conidia were allowed to germinate for 12-16 hours in complete medium and treated with different concentrations of anacardic acid for 2 hours. Treated germinated spores were allowed to grow on solid agar plates to produce colony forming units (CFU). The number of CFU in untreated samples was taken as 100%. Results indicate the mean and ± SD from three independent experiments where ** = $p < 0.01$, *** = $p < 0.001$ when 0.1% DMSO treated control is compared with anacardic acid treated samples.

Anacardic acid induces apoptosis like phenotype in *M. oryzae*

After investigating inhibitory action of anacardic acid, it was unclear how exactly inhibition of growth is taking place in *M. oryzae*. Since anacardic acid has already been reported to be involved in apoptosis like cell death in mammalian cells, we tried to investigate whether similar kind of cell death is taking place in fungal cells as well. Apoptosis is a sequence of unique morphological events and one of the first visible processes during apoptosis is cell shrinkage. When conidia from *M. oryzae* were treated with 5µM anacardic acid and observed under light microscope, almost 62% ±5 of conidia showed membrane constriction after 2-4 hours post treatment (Fig. 2a). FM4-64 which specifically binds to cell membranes was used to study membrane constriction in mycelia during apoptosis. At 50 µM anacardic acid, almost 72% of anacardic acid treated hyphae showed membrane constriction within 2-4 hours, while no such condition was seen in untreated or 0.1% DMSO control (Fig. 2b). There was a gradual increase in membrane constriction with time which ultimately led to membrane blebbing in anacardic acid treated mycelial cells. To study chromatin condensation and DNA disintegration in anacardic acid treated mycelia, germinated spores were treated with different concentrations of anacardic acid for 2 hours, and chromatin condensation was studied under fluorescent microscope. Hoechst 33258 staining showed an increase in the number of condensed nuclei in anacardic acid treated mycelia (Fig. 2c). 63 % ± 4.1 and 72% ± 5.4 fungal cells showed nuclear condensation at 50 µM and 100 µM of anacardic acid respectively, however only 4% ± 1.4 cells showed nuclear condensation in untreated control (Table. 1).

TUNEL assay: It is commonly used as a marker for apoptosis and is generally detected in situ by the TUNEL assay. Strong green fluorescence was observed in the TUNEL positive hyphae, treated with anacardic acid (Fig. 2d). Almost 61% ± 3.6 hyphal cells showed positive TUNEL staining after treating with 50 µM anacardic acid for 2 hours. In contrast, no

fluorescence was detected in untreated sample or DMSO (0.1%) treated sample. Agarose gel electrophoresis was carried out to detect DNA fragmentation in anacardic acid treated mycelial cells. Unlike classical DNA ladder observed in mammalian apoptosis, a smear without distinct bands was observed (Fig. S2: in the Supplementary Material).

One of the early hallmarks of apoptosis is the externalization of phosphotidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Annexin V conjugated to FITC in combination with PI was used to monitor the extent of flip-flop of PS from the inner to the outer leaflet of plasma membrane. Due to presence of cell wall, fungal cells cannot be directly stained with FITC conjugated Annexin V; therefore protoplasts were generated to investigate externalization of PS. After treatment with anacardic acid, the number of ptotoplasts undergoing apoptosis and necrosis were quantified by Annexin V/Propidium iodide (PI) staining. Green fluorescence was observed in protoplasts treated with anacardic acid whereas very weak or no fluorescence was observed in untreated control or 0.1% DMSO treated protoplasts (Fig. 2e). A total of 61% ± 2.2% protoplasts were found Annexin V positive in sample treated with 50 µM of anacardic acid. 12% of the total protoplasts stained positive with PI (Red fluorescence) indicating necrotic cell death taking place in these cells, however less than 5% of total protoplasts were found to be PI positive cells in untreated protoplasts (control). When protoplasts were incubated with 50 µM anacardic acid for more than 12 hours, almost 83% protoplasts were found to be PI-positive compared to 12 % after 2 hours of treatment. Also, the number of PI positive protoplasts was found to increase with the increase in concentration of anacardic acid. 87% protoplasts stained positive with PI after treating with 300 µM anacardic acid for 2 hours whereas only 6% protoplasts were stained with FITC labeled Annexin V. The percentage of protoplasts stained with FITC labeled Annexin V and PI at 50 µM and 300 µM anacardic acid was represented in a graph (Fig. 2f).

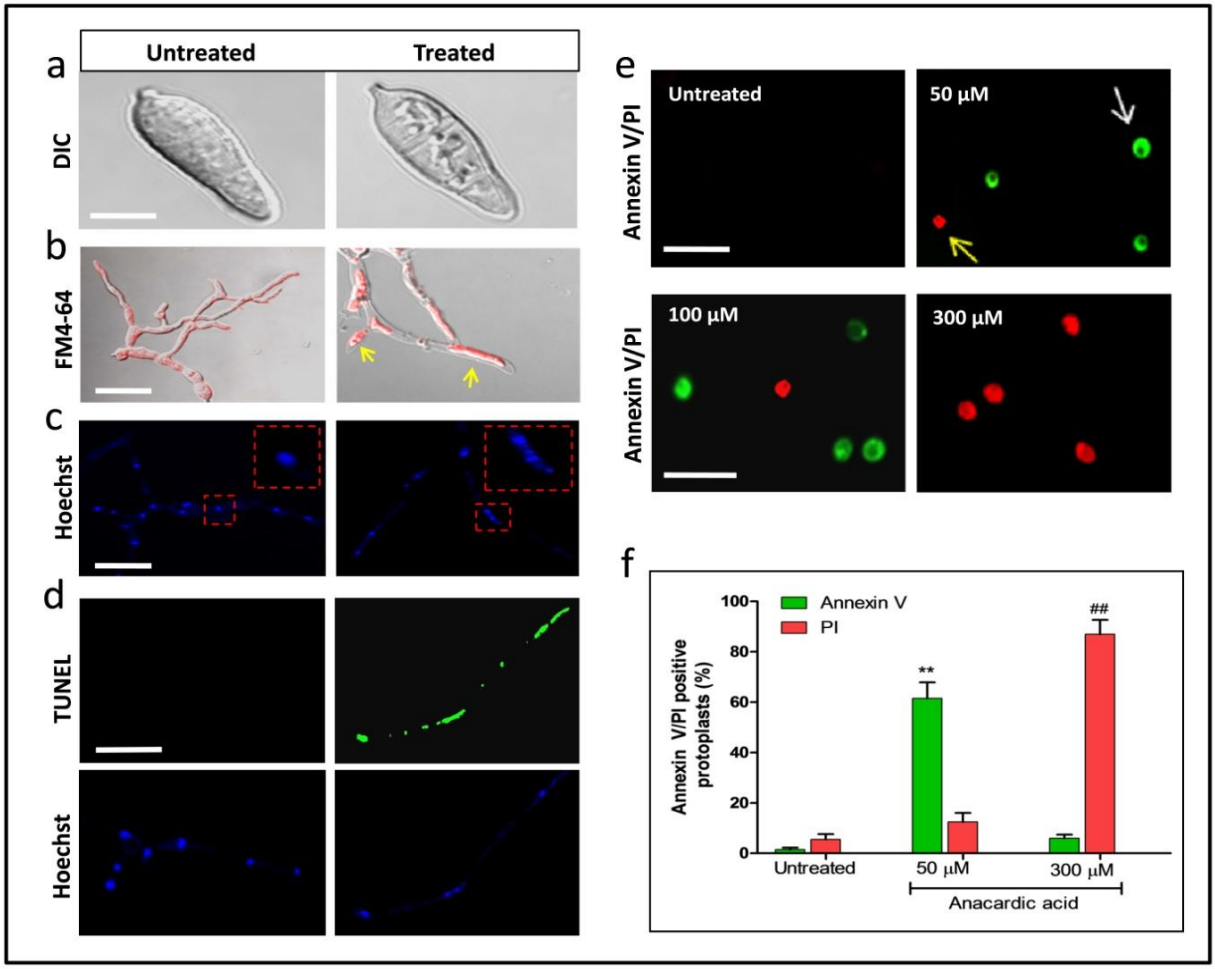

Fig. 2 Anacardic acid induces apoptosis like characteristics in *M. oryzae*. (a) Membrane constriction in conidia. Conidia were isolated and treated with 5 µM anacardic acid for 2 hours and observed under light microscope. Bar = 5 µm. (b) Membrane staining. Germinated spores were treated with 50 µM anacardic acid at 28^{o}C for 2 hours and stained with membrane staining dye FM4-64 to observe membrane constriction in mycelia. Yellow arrows indicate areas of membrane constriction. (c) Nuclear condensation. Germinated spores were treated with 50 µM of anacardic acid and mycelia were stained with Hoechst 33258. Fluorescent microscopy was used to study nuclear condensation and DNA fragmentation. (d) TUNEL staining. Germinated spores were treated with 50 µM anacardic acid for 2 hours and double stained with Hoechst 33258 and TUNEL. (e) Protoplasts were generated and double stained with Annexin-V-FITC and PI. Yellow arrows show PI positive (Red) cells undergoing necrosis and white arrows show Annexin-V-FITC positive cells undergoing apoptosis. Bar = 20 µM. (f) The number of Annexin V and PI positive protoplasts were counted and

represented in the form of a graph as percentage of total number of protoplasts observed. Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$ when the Annexin V positive untreated group was compared with the 50 µM and 300 µM anacardic acid treatments and $^{\#\#} = p < 0.01$ when the PI positive untreated group was compared with 50 µM and 300 µM anacardic acid treatments.

Table. 1 Nuclear condensation in mycelia of *M. oryzae* after treatment with anacardic acid

Anacardic acid (µM)	Control	50	100
Condensed nuclei (%)	4% ± 1.4	63 % ± 4.1	72% ± 5.4

One hundred nuclei from each sample were examined for nuclear condensation. Each value represents the mean ± SD of triplicate determinations from three independent experiments

Anacardic acid induced apoptosis is caspase independent

To evaluate the potential role of caspases (metacaspases) in anacardic acid induced apoptosis, we evaluated whether the broad-spectrum caspase inhibitor Z-VAD-fmk would be able to effectively resist the inhibition of conidial germination and mycelia growth of *M. oryzae*. Although, our results clearly demonstrate apoptosis like cell death in anacardic acid treated *M. oryzae*, Z-VAD-fmk failed to significantly reverse mycelial cell death. As evident from the graph, there is no significant difference between CFU (%) in Z-VAD-fmk treated and untreated groups (Fig. 3a). These results suggest that caspase activation may be dispensable for anacardic acid induced apoptosis.

Anacardic acid leads to loss of mitochondrial potential

The effect of anacardic acid on mitochondrial membrane potential (MMP) of *M. oryzae* was studied using MitoTracker Red (CMXRos). It is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential (Pendergrass et al. 2004). Different concentrations of anacardic acid ranging from 1-80 μM were used in this study and it was found that anacardic acid significantly reduces mitochondrial membrane potential as indicated by diminishing fluorescence of treated mycelia as well as treated fungal spores (Fig. S3: in the Supplementary Material). Quantitative changes in the MMP of treated samples were determined by fluorescence measurement at Excitation*/Emission* (nm): 590*/*645 in 96 well plates with a Synergy H4 Hybrid Multi-Mode Microplate Reader *and it was found that with the increase in concentration of anacardic acid, there was corresponding decrease in MMP . At 50* μM *of anacardic acid, mitochondrial membrane potential was decreased to less than 32% of untreated or DMSO* (0.1%) *treated control* (Fig. 3b).

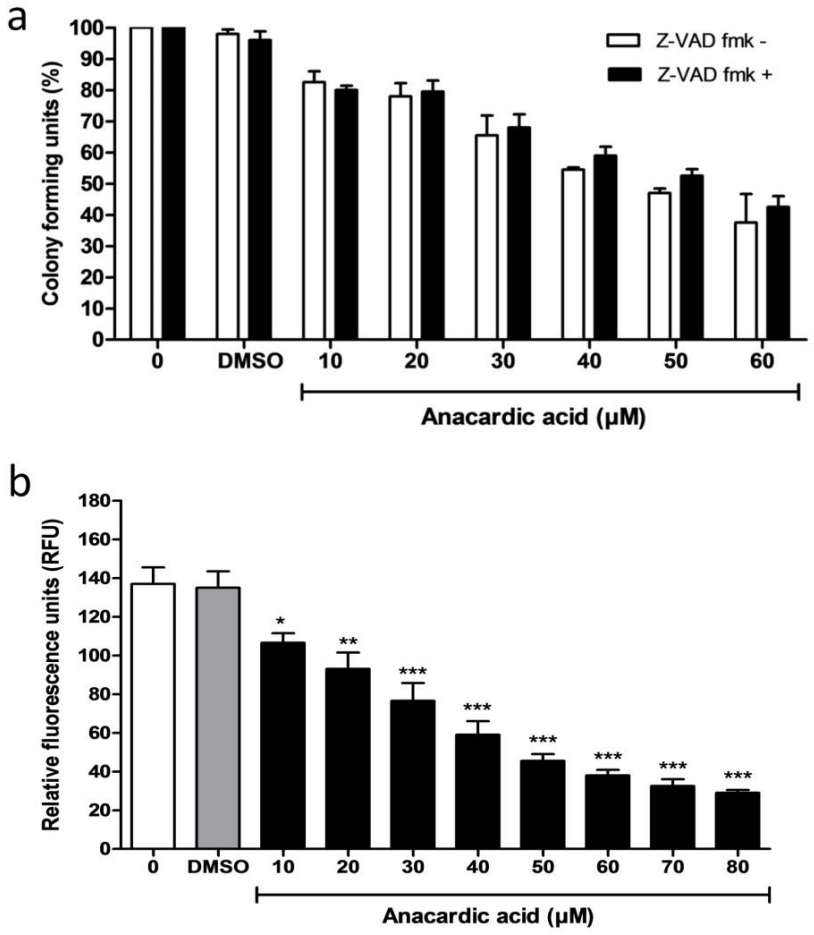

Fig. 3 Determination of caspase involvement (a) and changes in MMP (b) during anacardic acid induced apoptosis. (a) 10^6 spores were germinated in complete medium at 28^0C for 12 h. After treating germinated spores with anacardic acid, cultures were treated with 20 mM Z-VAD-fmk and number of colony forming units (CFU) were calculated in treated as well as untreated samples. (b) Germinated spores were treated with different concentrations of anacardic acid and 0.1% DMSO (control). CMXRos was used as probe to detect changes in mitochondrial membrane potential and fluorescence measurement was carried out at Ex/*Em* (nm): 590/645 using the Multi-Mode Microplate Reader. Values are expressed as ± SD in triplicates where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ when anacardic acid treated groups were compared with the 0.1% DMSO control.

Anacardic acid shows antioxidant property

Accumulation of reactive oxygen species (ROS) is one of the important biochemical responses during apoptosis. In order to monitor ROS accumulation levels in the fungus, fluorescent dye H_2DCFDA was used, which is oxidized to a fluorescent derivative by intracellular ROS. Surprisingly, instead of expected increase in green fluorescence during anacardic acid mediated cell death of *M. oryzae,* there was a significant decrease in fluorescence when visualized under the fluorescent microscope. The relative fluorescence was quantified by a BioTek Microplate Reader and it was found that anacardic acid significantly reduces intracellular ROS (Fig. 4a). These results suggest that anacardic acid prevents generation of ROS and acts as an antioxidant, as has been also demonstrated in earlier studies (Kubo et al. 2006). Hydrogen peroxide is known to induce intracellular ROS production during H_2O_2 induced apoptosis (Ogawa 2003). In order to further confirm antioxidant property of anacardic acid, mycelia were treated with 10 mM H_2O_2. It was found that after H_2O_2 treatment there was a drastic increase of ROS in the cells as expected, but after treating the H_2O_2 treated cells with anacardic acid the level of ROS was reduced again (Fig. 4b). This experiment further confirms potent antioxidant property of anacardic acid. Next, in order to know whether there is any transient accumulation of ROS before antioxidation activity, mycelia treated with 50 µM anacardic acid were stained with H_2DCFDA and relative fluorescence was recorded after every 10 min. It was found that during first 10 min there is a slight increase in intracellular ROS, which starts decreasing again after 20 min. These results suggest that there may be transient but insignificant accumulation of ROS inside the cells before an antioxidation property of anacardic acid comes into play.

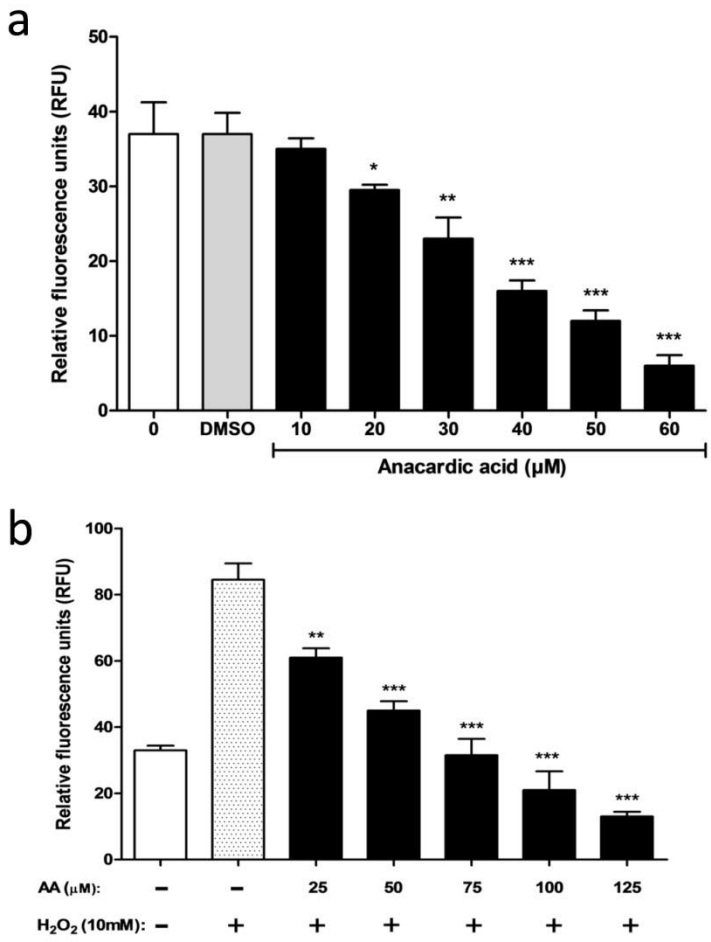

Fig. 4 Antioxidant activity of anacardic acid. (a) H_2DCFDA was used to estimate intracellular ROS in *M. oryzae* treated with various concentrations of anacardic acid and changes in levels of reactive oxygen species were monitored. (b) 10mM H_2O_2 was used to induce production of intracellular ROS in mycelia. Mycelia were then treated with anacardic acid and changes in levels of reactive oxygen species were monitored. Values of RFU are expressed as mean ± SD in triplicates where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ when the anacardic acid treated group was compared with the 0.1% DMSO control.

Apoptosis related proteins in *M. oryzae.*

Apoptosis inducing factor (AIF) is a flavoprotein reported to be involved in initiating the caspase-independent mode of apoptosis by causing chromatin condensation and DNA fragmentation (Cregan et al. 2002). In fungi, apoptosis inducing factor (Aif1) was first characterized in yeast and was described as a mitochondrial protein which translocates from mitochondria to the nucleus during apoptosis (Wissing et al. 2004). In *A. nidulans*, the AIF-like mitochondrial oxidoreductase gene, *aifA* has been found to be involved in apoptosis induced by farnesol and its expression has been found to be upregulated during apoptosis (Savoldi et al. 2008). Using NCBI blast analysis, we found a homolog for *A. nidulans* AifA in *M. oryzae* corresponding to protein accession number MGG_08262, henceforth referred to as AifM. This hypothetical protein shows almost 55% identity with AifA at the protein level. Like *A. nidulans* AifA this protein consists of three major conserved domains including Rieske AIFL N domain, Pyr redox 2 domain and reductase C domain. The AIFL N (apoptosis-inducing factor like) N-terminal Rieske domain family is highly similar to the human AIFL domain. BlastP analysis of AifM showed 25% similarity to the human apoptosis inducing factor and contains similar domains like human AIF including AIFL and pyridine nucleotide-disulphide oxidoreductase domains (Fig. 5a).

Anacardic acid induces upregulation of putative apoptosis inducing factor

As pan-caspase inhibitor Z-VAD-fmk failed to prevent anacardic acid induced mycelial cell death in *M. oryzae*, we wanted to evaluate the possible involvement of apoptosis-inducing factor (AIF) in the apoptotic cell death. It is known that the apoptosis inducing factor is the main mediator of caspase-independent apoptosis-like cell death and its expression is upregulated during the process (Savoldi et al. 2008). Based on the blast analysis, AifM was selected for expression analysis during anacardic acid induced apoptosis. After incubating

mycelia with 25 μM and 50 μM of anacardic acid for 1 hour, *aifM* mRNA levels were upregulated by almost 3.8 ± 0.7 folds and 3.5 ± 0.6 folds respectively. However, no significant changes were observed in untreated or DMSO (0.1%) treated controls (Fig. 5b). It indicates that anacardic acid induced apoptosis might be caspase independent and mediated by apoptosis inducing factor.

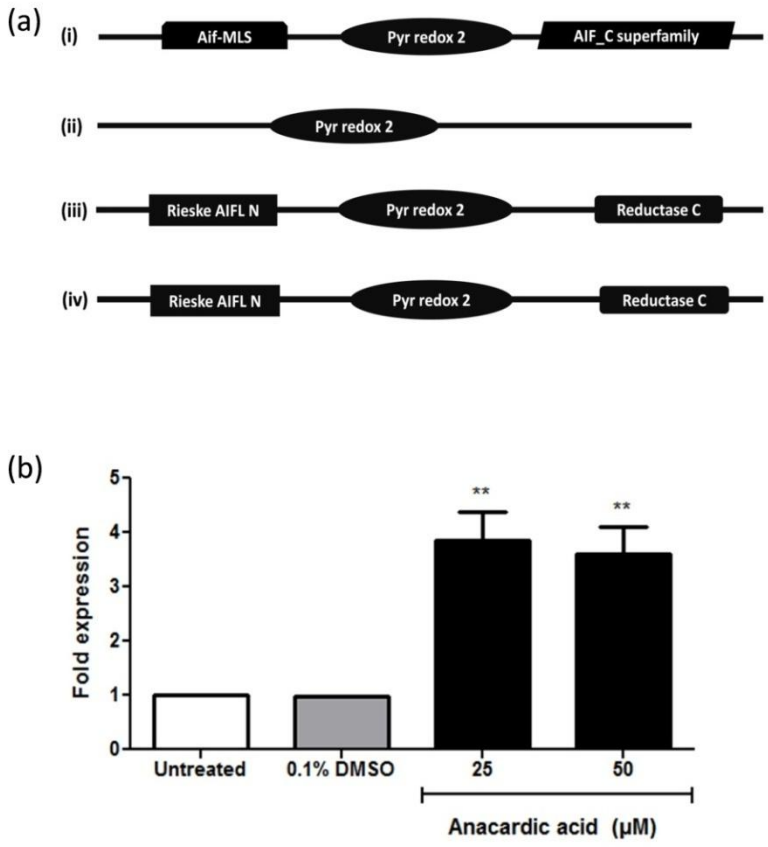

Fig. 5 Domain arrangement (a) and expression analysis of apoptosis inducing factors (AIF) (b). **(**a) NCBI and the Smart domain prediction tool were used to predict possible domains of apoptosis inducing factor in various organisms. (i) *H. sapiens* apoptosis inducing factor (Acc. No. AAD16436) is a 613 amino acids protein with 3 major domains; Aif-MLS (mitochondria localization sequence), Pyr redox 2 (Pyridine nucleotide-disulphide oxidoreductase) and AIF_C (AIF C terminus superfamily) domain. (ii) *S. cerevisiae* AIF1 (Acc. No. YNR074C) is 378 amino acids protein, containing Pyr redox 2 domain. (iii) *A. nidulans*, AifN (Acc. No. AN9103) is a 561 amino acids protein having three major domains including Rieske AIFL N domain, Pyr redox 2 domain and Reductase C domain. (iv) The *M. oryzae* homolog, AifM (Acc. No. MGG_08262) is a 598 amino acids protein that contains similar domains to the *A. nidulans* counterpart. (b) Mycelial cells were treated with 25 µM and 50 µM of anacardic acid and RNA was isolated from treated samples as well as untreated control. After cDNA preparation, quantitative real time PCR was carried out to determine the relative expression analysis of AifM. Values are expressed as mean ± SD in triplicates where ** = $p < 0.01$ when the anacardic acid treated samples were compared with the 0.1% DMSO control.

Anacardic acid inhibits rice blast disease

As already observed, anacardic acid inhibits spore germination completely at concentration of 75 μM. We tried to find out whether this compound can be used to protect rice plants from rice blast infection. For this experiment, rice leaves were sprayed with various concentrations of anacardic acid ranging from 1-75 μM. Then leaves were sprayed with 10^5/ ml conidia and incubated at 28^OC for 7-10 days under optimum humidity. On an average, 10-12 lesions per leaf were observed in leaves without anacardic acid treatment, while as 4-5 lesions were observed in leaves treated with 5μM anacardic acid. It was found that none of the leaves pretreated with more than 10 μM anacardic acid showed any infection lesions, whereas normal disease lesions were found in untreated or DMSO (0.1%) treated controls (Fig. 6a). In order to study effect of anacardic acid on rice, leaves were treated with different concentrations of anacardic acid ranging from 5 -75 μM and stained with fluorescein diacetate (FDA). It was observed that anacardic acid does not induce cell death in rice cells.

Anacardic acid sensitizes cells to DNA damaging agents

In mammals, anacardic acid has been shown to possess anti-HATs (histone acetyltransferase) activity which induces cell death and sensitizes apoptosis-resistance by breaking resistance to DNA damaging agents (Sun et al. 2006). We therefore investigated whether anacardic acid can make fungal cells more susceptible to DNA damaging agents. First, mycelia were treated with various concentrations of anacardic acid for 2 h and then it was followed by treatment with 0.1% ethyl methylsulfonate (EMS) for one hour. After washing thoroughly, the cells were allowed to grow on oat meal agar plates for 3 days till the formation of fungal colonies. It was observed that cells treated with anacardic acid were highly sensitive to EMS. At 50 μM anacardic acid, only 21% ± 2.8 cells formed colonies in sensitized cells in comparison to 47% ± 4.9 recorded in non sensitized cells (Fig. 6b).

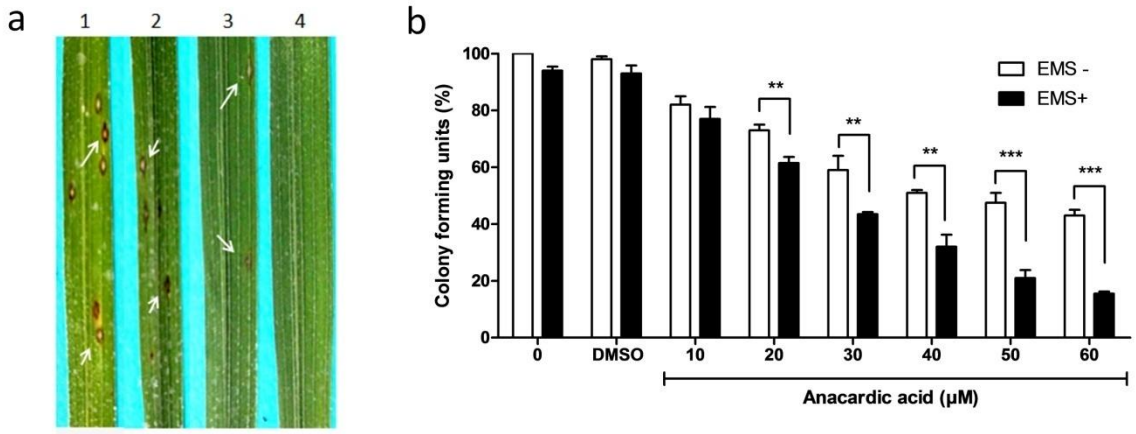

Fig. 6 Infection assays of anacardic acid treated plants. (a) Rice leaves treated with different concentrations of anacardic acid were infected with fungal spores and scored for infection lesions after 8-10 days. 1 Untreated. 2 0.1% DMSO treated. 3 Treated with 5 µM anacardic acid. 4 Treated with 10 µM anacardic acid. (b) Anacardic acid sensitizes *M. oryzae* cells to ethyl methanesulfonate (EMS). Germinated mycelial cells were incubated in DMSO (0.1%) or anacardic acid for 2 h. The cells were then switched to fresh media plates, allowed to grow for 3 days, and surviving cells were assessed for colony formation. Values are expressed as mean ± SD in triplicates where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ when EMS treated samples were compared to the untreated and DMSO treated groups at a particular concentration of anacardic acid.