Chapter 4

Discussion

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During normal developmental stages of an organism, cells die by a regulated and energy dependent mechanism known as apoptosis or programmed cell death (Halestrap 2005). Programmed cell death in response to external stimuli has been demonstrated in several fungal species (Chen and Dickman 2005; Cheng et al. 2003; Ito et al. 2007a). Most of the currently used antifungal agents kill pathogens through necrosis, which has been reported to induce resistance in pathogens against the antifungal agents (Liu et al. 2010). However, control of pathogens via initiating programmed cell death provides another possible avenue for controlling fungal disease. In fungi, apoptotsis can be either caspase (metacaspase) dependent or independent and is generally mediated by mitochondria (Sharon et al. 2009).

Our study demonstrates that anacardic acid induces inhibition of conidial germination as well as inhibition of mycelial growth in *M. oryzae*. Assays including FM4-64 membrane staining for plasma membrane constriction and Annexin V staining for PS externalization indicate involvement of apoptosis like cell death in *M. oryzae* treated with anacardic acid. Nuclear DNA fragmentation by Ca^{2+} and Mg^{2+} dependent endonucleases is one of the indications of apoptosis. This specific DNA disintegration can be visualized by ethidium bromide staining in gel electrophoresis. Our results from gel electrophoresis of DNA from anacardic acid treated mycelia didn't show a typical laddering pattern as observed during mammalian apoptosis (Tilly et al. 1991). A TUNEL assay of anacardic acid treated mycelia was performed to study DNA disintegration, as a positive TUNEL assay is a strong evidence for apoptosis in yeasts and fungi (Chen and Dickman 2005; Mousavi and Robson 2003). Our results from the TUNEL assay indicate that DNA disintegration takes place in anacardic acid treated *M. oryzae*, which is one of the characteristic features of fungal apoptosis (Liu et al. 2010). Loss of the mitochondrial membrane potential (MMP) during early stages of apoptosis is a well studied process and has been reported in fungal apoptosis as well (Yang et al. 2008).

Our results show a dose dependent loss of mitochondrial membrane potential (MMP) in fungal cells treated with anacardic acid. All these observations suggest that apoptosis may be involved in anacardic acid induced cell death in the rice blast fungus.

Although pancaspase inhibitor Z-VAD-fmk has been reported to reverse the effect of apoptosis inducing factors in some fungi (Liu et al. 2010), our study showed that Z-VAD-fmk was unable to reverse cell death in the rice blast fungus caused by anacardic acid. This indicates that anacardic acid induces caspase independent apoptosis in *M. oryzae*. Our results are consistent with the earlier reports where anacardic acid is reported to cause caspase independent apoptosis in pituitary adenoma cells (Sukumari-Ramesh et al. 2011) and human lung adenocarcinoma cells (Seong et al. 2013).

Earlier, it was widely believed that apoptosis related genes are absent in yeast and filamentous fungi, but more than 50 putative PCD related genes have been reported in *A. fumigatus* (Fedorova et al. 2005). In silico and molecular analysis of fungi suggest that the caspase independent pathway is vastly conserved in yeast and other fungi (Sharon et al. 2009). Homology between fungal and mammalian proteins is not generally very high except for certain specific domains (Sharon et al. 2009). We carried out a bioinformatic analysis to find homologs for mammalian caspases in *M. oryzae* but like other filamentous fungi, no homologs for mammalian caspases are present in the rice blast fungal genome. Instead there are homologs for metacaspases, which represent primitive forms of caspases. It was also found that *M. oryzae* contains a BIR1/ Survivin like protein (Acc. No. MGG_04912), containing two BIR1 domains, which is typical of most filamentous fungi (Sharon et al. 2009). BIR domains are present at the N-terminal part of the protein and are involved in cell survival and anti-apoptotic activity (Widlund et al. 2006).

Apoptosis-inducing factor (AIF) regulates cell death during development and pathological apoptosis in mammals (Wissing et al. 2004). An AIF homologue controls apoptosis in the budding yeast *Saccharomyces cerevisiae* and its action has been shown to be partially caspase dependent.(Wissing et al. 2004). Increased mRNA expression of *aifA,* encoding the apoptosis inducing factor (AIF) like mitochondrial oxidoreductase has been observed in *A. nidulans* during apoptosis (Savoldi et al. 2008). An AIF like protein in *M. oryzae* was identified by bioinformatic analysis and mRNA expression of this protein was found to be upregulated during anacardic acid induced apoptosis. Further characterization of this protein by gene knockout and overexpression studies can reveal its possible role in regulating the fungal apoptosis.

Anacardic acid is a non-specific HAT inhibitor which can inhibit histone acetyltransferases activity of multiple HATs. It has been demonstrated to directly inhibit histone acetyltransferases (HATs) like p300, PCAF and Tip60 (Sun et al. 2006). Tip60 is involved in regulation of DNA repair of double stranded breaks thereby maintaining the genomic integrity of cells (Liu and Sun 2011). Our results show that anacardic acid sensitizes *M. oryzae* to DNA damaging agents. DNA repair proteins similar to Tip60 can be one of the targets of anacardic acid and inhibition of Tip60 may lead to unopposed DNA damage and apoptosis. Blast analysis shows a homolog of Tip60 in *M. oryzae,* that is a histone acetyltransferase with 38% identity to human Tip60. Functional characterization of this protein will be carried out in future in order to know its role in fungal apoptosis.

Accumulation of reactive oxygen species is an immediate and common response during fungal apoptosis (Semighini et al. 2006). However, anacardic acid is known for its preventive antioxidant activity unlike salicylic acid. Anacardic acid prevents generation of superoxide radicals by inhibiting xanthine oxidase without radical scavenging activity (Kubo et al. 2006).

Our results also demonstrate antioxidant activity of anacardic acid rather than accumulation of reactive oxygen species in the cells that normally takes place during apoptosis.

It has been demonstrated that anacardic acid induces intracellular Ca^{2+} mobilization, endoplasmic reticulum stress and autophagy in human lung carcinoma A549 cells (Seong et al. 2014). Also, it has been reported that anacardic acid induced endoplasmic reticulum (ER) stress leads to apoptosis in hepatoma HepG2 and myeloma U266 cells (Huang et al. 2014). Therefore, it will be interesting to study whether ER stress and autophagy precede anacardic acid induced apoptotic cell death in *M. oryzae.*

In conclusion, our study demonstrates that anacardic acid inhibits the mycelial cell growth and conidial germination in *M. oryzae*. It also demonstrates that anacardic acid causes plasma membrane constriction, chromatin condensation followed by DNA disintegration, loss of mitochondrial membrane potential, externalization of phosphotidylserine and other process which are hallmarks of apoptosis.