5. DISCUSSION

Sumoylation is a reversible post-translational modification that regulates multiple biological processes including growth, cell cycle, nuclear transport, protein localization, genome integrity, stress resistance, protein-protein interaction and signal transduction (Tanaka *et al.*, 1999; Biggins *et al.*, 2001; Nacerddine *et al.*, 2005; Leach *et al.*, 2011;) by modifying a variety of different substrates in eukaryotic cells. The implications of sumoylation in cellular processes and virulence are yet to be explored extensively. The present study envisages the significance of sumoylation for growth, cell division, appressorial development and pathogenicity of *M. oryzae*. A combination of proteomic and molecular approaches were adopted to identify sumoylation targets and to explore the phenotypes of *sumo* mutant. Although SUMO is characterized in nonpathogenic yeasts like *S. cerevisiae*, *S. pombe* and in human pathogens like *C. albicans* and *A. nidulans*, it has not been studied in plant pathogens. It is also interesting to know the role of SUMO in host-pathogen interaction.

M. oryzae has a single *SUMO* gene just like its homologue *SMT3* in *S. cerevisiae, S. pombe, C. albicans* and *A. nidulans*. *SUMO* genes are highly conserved from yeasts to humans. *In silico* analysis using the GROMO database predicted 6,000 sumoylation sites for 4,494 protein sequences at a high cut-off value in *M. oryzae* (Thakur *et al.*, 2009). MoSUMO contains a single small ubiquitin like modifier

domain; a search for ubiquitin domain containing proteins revealed that *M. oryzae* contains 12 ubiquitin related proteins (Oh *et al.*, 2012). *UEP1* and *UEP3* were previously reported to be expressed abundantly during *M. oryzae* colonization of host tissue but were down regulated by starvation stress whereas *PUB4* was reported to be highly expressed after starvation stress (Talbot *et al.*, 1998; Oh *et al.*, 2012). Further, polyubiquitin was reported to have a role in growth, conidiation, appressorium formation, virulence and sexual development of *M. oryzae* (Oh *et al.*, 2012). The phylogenetic analysis revealed that closest orthologue of MoSUMO was found in *C. fioroniae, F. oxysporum* and *N. crassa.*

Complementation of MoSUMO in *S. cerevisiae* showed that *MoSUMO* is a functional homologue of *S. cerevisiae SMT3*. In *smt3* mutant, sister chromatid separation is abnormal, indicated by inseparable GFP signals, whereas the segregation defect of chromatids was rescued in the complemented strain. An accumulation of large budded cells with abnormal nuclear segregation was observed in *smt3* mutant, while the expression of *MoSUMO* gene in the *smt3* mutant leads to restoration of the wild-type phenotype. The presence of C-terminal diglycine motif is indispensible in SUMO conjugation pathway. To delineate the significance of diglycine motif (GG) of MoSUMO, the GG was replaced with dialanine (AA) and mutated expression construct was transformed in *smt3* mutant strain (SBY331). The positive transformants along with SBY331 strains were shifted to 37^0C for 4hr and

chromosome segregation was analyzed. No restoration of defective phenotype was observed, the abnormal nuclear segregation was like SBY331 strain. This indicates that due to the absence of diglycine motif of MoSUMO in the transformant could not rescue the defective phenotype, showing that conserved diglycine motif plays a pivotal role in sumoylation pathway.

The major aim of the present investigation was to define the role of MoSUMO in the development and pathogenesis of the fungus. The *MoSUMO* knock out strain was generated using split marker approach and various factors critical for pathogenicity like conidiation and appressorium development were checked. Split-Marker Recombination is an efficient method generally used for targeted deletion of fungal genes (Catlett *et al.*, 2002). In this method, substitution of native target *MoSUMO* gene is only feasible when three cross over events occur to generate a functional HPT marker gene. The flanking regions of *MoSUMO* (5' and 3') allow homologous recombination between genomic DNA and the split-marker DNA to substitute the *MoSUMO* gene by the selectable marker. To ensure the phenotype exhibited by the knock out was an effect of MoSUMO, complementation of MoSUMO was carried out using the zeocin selection marker. In addition to reintroducing MoSUMO allele in the *ΔMosumo* mutant strain, an array of tags consisting of three copies of FLAG and six copies of HIS (TAP) were inserted at the N terminus of MoSUMO protein. These tags facilitate the isolation

of endogenously expressed sumoylated conjugates by tandem affinity purification. The transformants were screened on the basis of resistance to zeocin and sensitivity towards hygromycin. The knock-out and complemented transformants were confirmed with Southern blot analysis using several different probes followed by expression study using RT-PCR.

In addition to the molecular analysis, phenotypic characterization was performed which includes assays for growth, conidiation and appressorium development of the fungus. Aerial hyphae and melanin content were unaffected in the *ΔMosumo* mutant. Significant reduction in growth was observed in *ΔMosumo* mutant as compared to wild type. The slow growth of *ΔMosumo* mutant was restored by complementation of MoSUMO. The number of conidia were analyzed statistically using Student t-test ($P<0.05$) and found that conidiation was significantly reduced in the *ΔMosumo* mutant. The slow growth pattern was also reported in yeasts like *S. pombe* and *C. albicans*. (Tanaka *et al*., 1999; Leach *et al*., 2010). In *A. nidulans*, the *sumO* mutant was viable and also had impaired conidiation (Wong *et al*., 2008). The *ΔMosumo* mutant failed to form appressoria from conidia as well as hyphae. All these defective phenotype including reduced growth and conidiation and defective appressorium formation of *ΔMosumo* mutant were rescued in the complemented strain. The deletion of *MoSUMO* leads to loss of pathogenicity in the fungus. The *ΔMosumo* mutant was also unable to form invasive hyphae on leaf

sheath. All phenotypic observations put together, suggest that MoSUMO is not essential protein but it is required for growth, conidiation and it appears to be a key molecule required for the formation of infection structures leading to pathogenicity. Factors associated with this defective phenotype can be demarcated by performing stage specific isolation of MoSUMO conjugates.

The examination of cell division demonstrates that sumoylation is required for mitosis during the execution of cell cycle of *M. oryzae*. Most of the vegetative cells were multinucleate, indicating nuclear segregation defect in *ΔMosumo* mutant. Cell-cycle progression is extensively controlled via complex networks of posttranslational modifications. The *smt3* mutants accumulate at G2/M in the cell cycle with short spindles, unseparated sister chromatids, and undivided nuclei (Li *et al.*, 1999; Johnson *et al.*, 1999; Biggins *et al.*, 2001). Uba2, AOS1 and UBC9, components of SUMO conjugation pathway are dispensable for cell growth, show aberrant mitosis in fission yeast while they are essential in budding yeast (Guntur *et al.*, 1997; Al-Khodairy *et al.*, 1995). In *C. albicans*, many daughter cells remain joined to their mother cell and some are multinucleate suggesting cell cycle defects. Furthermore, growth and cell division related sumoylation targets were identified in *C. albicans* (Leach *et al.*, 2011).The defective phenotype of chromosome segregation in *sumo* mutant seems to be plausible as *SMT3* gene was isolated as a high copy suppressor of mutations in MIF2, which encodes CENP-C

like centromere binding protein in budding yeast (Meluh and Koshland, 1995). In the present study, *ΔMosumo* deletion mutant also displays aberrant chitin deposition during germ tube formation in *M. oryzae*. Since biosynthesis of chitin is catalyzed by seven chitin synthases (CHS) in *M. oryzae*, sumoylation consensus sequences were explored in these CHS. Interestingly, *in silico* analysis of these CHS indicated that three chitin synthases *CHS1p*, *CHS2p* and *CHS7p* have potential sumoylation sites (Table 2) and all these genes were indispensable for development and pathogenicity (Odenbach *et al.*, 2009). The expression level of these three CHS genes was relatively low in vegetative hyphae however; the intensity of CHS1 and CHS7 transcript was highest in conidia and appressoria and lowest expression level was seen in infected rice leaves (Kong *et al.*, 2012). These results suggested that chitin synthases might be the probable targets for sumoylation as chitin deposition was disturbed in *ΔMosumo* mutant.

Moreover, the number of septa was also affected owing to the deletion of *MoSUMO* in *M. oryzae*. Increase in the number of septa was found during conidium germination in *ΔMosumo* mutant as compared to wild type. The sumoylation of septins has been demonstrated in other fungi. Sumoylation of septins occurs abruptly and exclusively on the site of septin collar in budding yeast. Among these, Cdc3, Cdc11 and Shs1 become highly sumoylated during mitosis in budding yeast (Johnson and Blobel, 1999). Inactivation of septin leaded to a branched, highly elongated, multinucleate cellular cluster. These mutants

were unable to organize the bud neck filaments, which have essential roles in cytokinesis. (Cooper *et al.*,1996). Interestingly, when the sumoylation motif of Cdc3, Cdc11, and Shs1 were mutated, septin ring formation was lost, and there was a loss of bud neck-associated SUMO also in budding yeast (Johnson and Blobel, 1999). A family of septins was explored in *M. oryzae* and it was found that out of five septin genes, four showed significant identity to core septins of budding yeast (Cdc3, Cdc10, Cd11, and Cdc12) (Dagdas *et al.*,2012) named as Sep3, Sep4, Sep5 and Sep6 respectively in *M. oryzae*. *in silico* analysis also supported this observations by predicting that Sep3p, Sep10p, Sep11p and Sep12p have potential sumoylation consensus sequence in *M. oryzae* (Table 2).

Localization of MoSUMO during the developmental stages provides some interesting findings. This study revealed that MoSUMO and/or sumoylated proteins were localized in the nucleus and septum of the mycelial cell and it is constitutively expressed throughout all the developmental stages. To track MoSUMO and/or sumoylated proteins during developmental stages, conidia were germinated on a hydrophobic slide and the distribution of sumoylated protein was observed in conidia to appressoria. The time lapse microscopy showed that GFP signal was observed throughout the conidia including nuclei and cytoplasm and was consistent with time progression. Incredibly, as the time progresses, the GFP signal moves towards the pole of the conidia and finally MoSUMO conjugates are

highly accumulated in the appressoria as appressoria showed intense GFP signal. In *A. nidulans*, sumoylated proteins and/or SUMO peptides were localized throughout the nucleus accumulated as punctate spots in chromatin-free sub-nuclear regions in mycelium (Wong *et al*., 2008). These spots were not associated with spindle pole bodies whereas in *S. pombe* Pmt3 fused to GFP is localized in intense signal in the nucleus corresponding to spindle pole bodies (Tanaka *et al*., 1999). Human SUMO-1/2/3 peptides are confined on the nuclear membrane, nuclear bodies and cytoplasm, respectively (Su and Li, 2002). MoSUMO and/or sumoylated proteins were constitutively expressed throughout the life cycle of *M. oryzae* indicating that MoSUMO and its targets play a major role in development of the fungus.

Colocalization of MoSUMO was studied using staining of the GFP::MoSUMO strain with DAPI and CFW which stains nuclei and chitin which is concentrated in the septum of the cell respectively. The result revealed that MoSUMO and/or sumoylated proteins were abundantly localized in the nuclear and at the septal region of hyphae. As mentioned above, defective septation in *ΔMosumo* mutant might be due to the absence of septin sumoylation. The septin sumoylation was reported in yeasts like *S. cerevisiae* and *C. albicans* (Martin and Konopka, 2004, Panse *et al*., 2004, Danison *et al*., 2005), and nuclear proteins were reported as SUMO targets in *A. nidulans* (Wong *et al*., 2008) and the observations in *M. oryzae* also suggesting the role of MoSUMO in septins and nuclei as GFP signal accumulates at the corresponding site of nuclei and septa establishing the link between MoSUMO, septins and nuclear proteins.

Moreover, in the current study, germinating conidia of *ΔMosumo* mutant failed to develop appressoria, forming irregular and bulbous shaped germ tubes, presumably due to disturbed cytoskeleton machinery. As actin is a major a cytoskeletal component and structural unit of microfilaments, it was identified as a bonafide SUMO substrate in human (Vertegaal *et al.*, 2004, Rosas-Acosta *et al.*, 2005, Hofmann *et al.*, 2009,). Consequently, the localization of actin and MoSUMO was observed at the similar sites in conidia, wherein GFP signal and red fluorescence of actin were overlapping, leading to the conclusion that actin could be one of the targets of MoSUMO in *M. oryzae*.

The combination of colocalization and subcellular localization of GFP::SUMO suggests that MoSUMO and/or sumoylated proteins are present at nuclear and septal regions of vegetative hyphae whereas in conidia and appressoria, the distribution was all over the conidia including cell membrane, germ tube and clubbed in appressoria and highly expressed after infection in host tissue. It is noteworthy that highest transcript level of *MoSUMO* was observed maximum during spore germination and it was down regulated during appressorium maturation as previously reported (Oh *et al.*, 2012). On the contrary, the present investigation indicated that expression of MoSUMO occurred during conidial germination and later intensified during appressorial maturation.

The defective phenotype of *ΔMosumo* mutant may be due to the proteins which are necessary for the regulation of different developmental stages like hyphal proliferation, conidiation and appressorium development, and might be absent or unmodified at particular stages due to inactivation of MoSUMO. Above attributes of *ΔMosumo* mutant are in agreement with variety of nuclear and septal targets in other fungi (Johnson and Blobel., 1999; Marin and Konopka, 2004) and with defect in nuclear events due to the inactivation of SUMO. This clearly suggests that the deletion of *MoSUMO* hampered the regulation of MoSUMO targets leading to the defects in development of the fungus.

Sumoylation pathway affects many cellular pathways including a broad spectrum of proteins. Since immunoblotting revealed that a wide range of proteins are targets of MoSUMO, the global protein profiling was performed to analyze the contribution of MoSUMO in differential protein expression using 2DGE of wild type versus *ΔMosumo* mutant. Around 72 spots were absent in *ΔMosumo* mutant while 36 protein spots were significantly up-regulated and 56 spots were down-regulated. As mentioned above, *in silico* analysis predicted 4,494 proteins are the MoSUMO targets (Thakur *et al.*, 2009), however, the present study includes a small number probable MoSUMO targets. Since the database includes the analysis of proteins present throughout the life cycle of the fungus and 2DGE analysis was

the analysis of only single event. Alternatively, some sumoylation targets might present at low level that lie below the sensitivity of analysis. Therefore, it can be presumed that the vast number of proteins affected in *ΔMosumo* mutant might be contributing a major role in the defective phenotype of *ΔMosumo* mutant. The proteins which are absent, up regulated or down regulated, might be the direct or indirect targets of MoSUMO. Among the above category, some proteins might be the legitimate MoSUMO targets while the expression of others could be modulated by set of proteins which might be the direct targets of MoSUMO and which in turn affect the expression of dependent protein.

Randomly, four proteins which were absent in *ΔMosumo* mutant and highly expressed in wild type were selected and identified by peptide sequencing employing MALDI-TOF/TOF and abbreviated as 'S'. The S1 spot was identified as MGG_06958 with 66% coverage score which is HSP like protein. The hsps are a family of ubiquitously expressed heat shock proteins. It is an essential part of the cell's machinery for protein folding and also helps to protect cells from stress. According to *in silico* analysis the above protein is predicted to be localized in the cytosol having three sumoylation sites. The 106th and 525th positions are predicted to be consensus sumoylation sites while peptide 24-28 is found to be the MoSUMO interaction site and it interacts with 27 proteins including MoSUMO. This HSP protein was referred as DK6 by Yi and Lee group (Yi *et al.*, 2009). HSPs were

found to be SUMO targets in *C. albicans* which were constitutively expressed and in response to heat. S2 was identified as MGG_14729 with 51% coverage and found to be a mitochondrial ribosomal protein subunit S18, predicted to be localized in the mitochondria; it interacts with 32 other proteins. It has two sumoylation consensus sites at 183 and 231position. S3, a conserved hypothetical protein (MGG_16530), recently known as MGG_15192 (45% coverage score) is a cytosolic protein having two consensus sumoylation sites at 119 and 244 position. S4 is also a conserved hypothetical protein (MGG_10292) (71% coverage score), predicted to be localized in the cytosol having one SUMO interaction site at 65-69 position and one consensus sumoylation sequence at 69 position. Pfam domain database showed that it has polyketide cyclase/dehydrase and lipid transport domain. The linkage between identified proteins and MoSUMO can be a novel area of research.

As highlighted earlier, a significant number of sumoylated proteins may be expressed below the noticeable limit of 2DGE analysis. Therefore, to isolate such sumoylated proteins, enrichment method was adapted. In this study, a large number of proteins were also detected as MoSUMO targets by immnodetection using anti-SUMO antibody. The identification and validation of these targets can be a future prospective.

Taking all observations together, it is summated that *MoSUMO* is a key molecule of developmental process of the fungus which includes vegetative growth, conidiation and appressorium development, critical for pathogenicity of the rice blast fungus. The bioinformatics, proteomic and localization studies revealed that MoSUMO has various cellular targets, expressed constitutively throughout the life cycle of *M. oryzae*. The identification of more MoSUMO targets with respect to sumoylation will underpin a role of *MoSUMO* in development, pathogenicity and host-pathogen interactions of rice blast fungus.