SUMMARY

Group III HHKs (Nik1 orthologs) are important class of sensor kinases ubiquitous in fungi which are involved in osmosensing, virulence, sporulation and hyphal development in fungi. They have been shown to be target of carboximide and phenylpyrrole classes fungicidal agents e.g. fludioxonil, iprodione etc. Fludioxonil belongs to phenylpyrrole group of fungicides which has broad antifungal spectrum and is used to control a variety of important plant-pathogenic fungi (Koch and Leadbeater, 1992). Previous studies have revealed that fludioxonil acts as a fungicide through the inhibition of group III hybrid histidine (Nik1 orthologs) which leads to the improper activation of HOG MAPK pathway in fungal pathogens. However, the cellular processes acting downstream to these events and crucial for toxic effect of fludioxonil remain elusive. Present work is thus a first experimental scrutiny in this regard. Group III hybrid histidine kinases is absent in *S. cerevisiae* and therefore it is naturally resistant to this class of fungicides. Previous work in our lab showed that the heterologous expression of ClNik1 (a Nik1 ortholog from *Candida lusitaniae*) in *S. cerevisiae* confer fludioxonil sensitivity to the host (Randhawa 2013). This model was used in this study to dissect the mechanism of action of fludioxonil.

Present study showed that the fludioxonil acted as fungicide on *S. cerevisiae* cells expressing ClNik1p, as the number viable cells decreased drastically within twelve hours of fludioxonil treatment. These results clearly indicated the suitability of the model. Under hyper osmotic conditions, phosphorylated Hog1p enters the nucleus through the activity of carrier protein NMD5 and regulate the expression of various genes essential for osmoadaptation (Ferrigno *et al.*, 1998). Earlier work in the laboratory monitored the localisation of Hog1p-GFP on fludioxonil treatment and it was suggested that fludioxonil does not induce nuclear translocation of Hog1p (Randhawa 2013). Present study not confirmed this finding but also extended it further. The strain B0119B ($\Delta nmd5$) expressing

pClNik1 was sensitive to fludioxonil. In the absence of Nmd5p, the nuclear translocation of Hog1p was defective in this strain. However, the cells were still sensitive to fludioxonil, indicating that the nuclear translocation of Hog1p was not prerequisite for toxic effect of fludioxonil.

Activation of HOG pathway on fludioxonil treatment led to the elevated level of glycerol in the cell which was thought to be the cause various cellular defects. However cells lacking both *GPD1* and *GPD2*, genes encoding an essential step for glycerol biosynthesis, were found to be sensitive to fludioxonil. Furthermore, the level of intracellular glycerol in Δ*gpd1* strain showed two fold increase on treatment with fludioxonil and this was quite similar to the parental control upon exposure to fludioxonil. In comparison, the cells lacking both the genes (Δ*gpd1*Δ*gpd2*) showed no elevation in the level of glycerol upon fludioxonil treatment. Thus, although fludioxonil hyper-activates the Hog pathway which leads to accumulation of glycerol in the cell but this is not the primary cause of growth inhibition and other cellular defects conferred by fludioxonil.

Aforementioned results showed that Hog1p was phosphorylated and remained in cytoplasm on fludioxonil treatment. Similar pattern of Hog1p activation and intracellular localization was observed on induction of mitophagy recently (Saito *et al.*,2010). Therefore to see whether mitophagy is playing role in the toxic effect of fludioxonil, Om45-GFP processing assay was done with TKYM22 strain expressing pClNik1. There was no degradation of mitochondria in the cells treated with fludioxonil up to 24 hours. These results clearly ruled out involvement of mitophagy in this process. So, next the fludioxonil sensitivity of cells lacking Hsl1p, Sic1p, Rck2p and Ubp3p, important cytoplasmic targets of Hog1p, was checked. Sensitivity to fludioxonil exhibited by the mutants lacking Rck2p and Sic1p was similar to the parental control. Therefore these two proteins are unlikely to be the direct target of fludioxonil. However, the cells lacking Hsl1p were more sensitive as

compared to the wild type BY4741. Fludioxonil induced synthetic growth defect in this strain indicated a role of G2 to M phase regulation by Hog1p in mechanism of action of this compound. In contrast, the deletion of Ubp3p conferred partial resistance to fludioxonil. Therefore, Ubp3p could be one of the direct targets of fludioxonil. Hog1p is known to phosphorylate Ubp3 directly and disruption of *UBP3* gene leads to various defects in the cell such as reduce expression of osmoresponsive genes, impaired ER to golgi transport, hyper-activation of cell wall integrity pathway etc (Cohe *et al.*, 2003; Wang *et al.*, 2008; Sole *et al.*, 2011). It is thus possible that more than one pathway is involve in mode of action of fludioxonil.

Genome wide studies have provided an excellent platform to study impact of various compounds on biological processes and identify their targets. In this study *S. cerevisiae* was used as model to identify and characterize the genes and the pathways involved in the antifungal action of fludioxonil. Firstly, the genome wide screening for drug resistant mutant was carried out by using mTn-LacZ/LEU2 mutagenized library. Four pools of plasmid library DNA was used to transform BY4742 strain expressing pClNik1. Clones which grew on SD (-Leu) media containing 50μg/ml of fludioxonil were selected and considered as fludioxonil resistant mutants. Out of approximately 27,000 transformants, forty one fludioxonil resistant mutants were obtained. Fludioxonil sensitivity exhibited by *S. cerevisiae* cells expressing ClNik1p is through the activation of HOG pathway. The osmosensitivity of the all the drug resistant mutants was checked on 0.7 M NaCl media, to rule out the possibility of the resistance phenotype arising from the mutation in the genes of the HOG pathway. Only one strain was found to be osmosensitive, which was discarded for further analysis.

To classify the remaining forty resistant mutants, diploids were made after mating with wild type strain BY4741 or YPDahl143 (Δ*ssk1*) which carries deletion in *SSK1* gene. Based on the growth pattern of resultant diploid strains on SD agar plates (-His) containing

50 μg/ml fludioxonil, mutants were classified into three groups. Group 1 strains were classified as dominant mutants as the diploids arising out of mating with either wild type BY4741 or YPDahl143 (*ssk1*) remained resistant to fludioxonil. The strains in other two groups were classified as recessive because the diploids arising from mating with wild type BY4741 became sensitive to fludioxonil. Fludioxonil resistance showed by group 3 strains was due to mutation in *SSK1* gene as the diploids from mating with *ssk1* mutant retained resistance to the drug and therefore they were discarded.

Next to identify the mutated genes in the twelve resistant strains in group 2, plasmid rescue method was employed. Site of mTn3 insertions was identified in seven resistant strains, out of which two strains had insertion in *BEM2* gene and rest showed disruption in the coding region of following genes: *ENV11, BNI4, SIR1, ATG1* and *DCG1*. In the remaining five resistant strains, the nucleotide sequencing of the rescued plasmids matched only with vector sequences. Two possible scenarios could be envisaged for this. One is due to cloning artefact. Alternatively, the recovery enzyme EcoR1 might have cut the genomic DNA very close to the LacZ sequence. Use of another recovery enzyme could be a possible solution for the later. Next to confirm that the resistance of the aforementioned seven strains was due to the loss of gene function, the strains Y17304 (Δ*dcg1*), Y11979 (Δ*bni4*), Y14547 (Δ*atg1*), Y14701 (Δ*env11*), Y16017 (Δ*sir1*), Y16152 (Δ*bem2*) having complete deletion in the respective ORF were procured from EUROSCARF. These strains were transformed with plasmid pClNik1 and the fludioxonil sensitivity of the transformants was checked by dilution spotting. Only the strain carrying deletion in *BEM2* showed resistance to fludioxonil. Deletion of *ENV11* also showed partial resistance phenotype. Other deletion strains were sensitive to fludioxonil. Fludioxonil resistance exhibited by these strains could be due to secondary site mutation unlinked to the transposon insertion site. Alternatively, the truncated protein made due to transposon insertion could be responsible for drug resistance phenotype.

To overcome these drawbacks of transposon mutagenesis, screening the library of 5130 haploid deletion strains of *S. cerevisiae* for fludioxonil resistant mutants was done. All the deletion strains were individually transformed in 96 well plate with plasmid pClNik1 and the growth pattern of transformants were checked in presence of 25μg/ml fludioxonil. In primary screening 317 strains were found to be showing two fold better growth than wild type BY4742/pClNik1 in presence of fludioxonil. These strains were subjected to second round of screening and finally sixteen strains were obtained which was consistently more resistant to fludioxonil than the parental control. Out of them, five strains carried deletion of ORF involved in HOG pathway (e.g. *HOG1, SSK1, SSK2, SSK22* and *PBS2*), five strains carried deletion of ORF with unknown function (e.g. YKR004C-A, YPL261C, YJL150W, YPL263C, YLR374C) and the remaining six strain carried deletion in genes involved in various cellular function (e.g. *SSD1, MED1, BEM2, PET494, SET2, BSP1*). Identification of *BEM2* in this screen further supports earlier results. The fludioxonil resistance phenotype of these strains was validated on SD (-His) agar plates. In this assay, Y03652 (Δ*ssd1*), Y05489 (Δ*med1*) and Y05420 (Δ*pet494*) showed complete resistance to fludioxonil. On the other hand the strains Y05586 (Δ*bsp1*) and Y01257 (Δ*set2*) were partially resistant to the drug.

Combining the results from both libraries seven genes (*SSD1, BEM2, ENV11, MED1, BSP1, PET494* and *SET2)* were identified which are involved in the fludioxonil toxicity. The role of Bem2p in this process was further investigated. Bem2p encodes a Rho GTPase activating protein which regulates GTPases to maintain morphogenesis and cell polarity in *S. cerevisiae* (Marquitz *et al.*, 2002). Apart from this, Bem2p is also implicated in various other cellular functions. Bem2p acts as GAP for Rho1 along with other three GAP proteins Sac7p, Lrg1p and Bag7p (Levin, 2011). Deletion of *SAC1*, *LRG1* or *BAG7* did not affect fludioxonil sensitivity of the cell. Thus, Bem2p is the only Rho1 GAP which plays a distinct role in fludioxonil toxicity. Fludioxonil resistance exhibited by Δ*bem2* strain could arise out of a

defect in the activation of the Hog pathway. Therefore, the level of dually phosphorylated Hog1p was measured in Y16152 strain. Activation of Hog1p was observed within fifteen minutes of fludioxonil treatment and it remained activated even after 3 hrs. Thus, Bem2p is acting either downstream of Hog1p or the fludioxonil resistance of Δ*bem2* strain is Hog1p independent phenomenon.

Bem2p is a 2167 amino acid protein, having non catalytic N terminal region which is crucial for its morphogenesis checkpoint function and C terminal region containing pleckstrin homology (PH) and GAP domain which is important for its role in morphogenesis. Interestingly GAP activity of Bem2p is dispensable for its checkpoint role (Marquitz *et al.*,2002). In order to delineate the role of Bem2p in fludioxonil sensitivity, two functional mutants R2003A and Δ2-1749, which completely abolishes the GAP activity and morphogenesis checkpoint role of Bem2p respectively were constructed. The expression of full length *BEM2* restored the fludioxonil sensitivity to Δ*bem2*/ClNik1p strain. However, both the mutant constructs were still resistant to fludioxonil indicating the role of both GAP domain and N terminal region of Bem2p in conferring fludioxonil sensitivity.

BEM2 deletion leads to various cellular defects which includes actin polarity defect, septin assembly defect, cytokinesis defect, hyper activation of cell wall integrity pathway, enlarged vacuoles and morphogenesis checkpoint defect (Wang and Bretscher,1995; Kim *et al.*,1994; Cid *et al.*,1998; Cid *et al.*, 2001; Marquitz *et al.*,2002; Seeley *et al.*, 2002; Atkins *et al.*, 2013). In yeast, actin is an essential protein which participates in majority of cellular processes such as cell polarity, cytokinesis, vacuole fusion and endocytosis (Aghamohammadzadeh and Ayscough, 2010; Eitzen *et al.*, 2002). Exposure to fludioxonil caused actin depolymerisation in 74% of wild type cells (BY4742/pClNik1) and this effect remained even after 4 hours of treatment. This persistent drug-mediated actin depolymerisation is different from that of hyperosmotic shock where depolymerised actin is recovered within one hour

(Yuzyuk *et al.*,2002). In Δ*bem2* strain 34% of cells showed depolarized actin even under normal conditions, which increased to 70% in the presence of the drug. Thus actin disassembly could be one of the general effects of drug treatment. It was not the primary reason for fludioxonil sensitivity, as Δ*bem2* strain, in spite of its actin polarity defect, were resistant.

Bem2 has been implicated to play role in septin assembly (Cid *et al.*, 2001; Caviston *et al.*, 2003). It was thus important to check the effect of fludioxonil on septin organisation. The localization of Shs1-GFP fusion protein was thus examined. Shs1 is a regulator of septin assembly and organisation, and colocalise with other septins at bud neck area (Garcia *et al.*, 2011). Δ*bem2* strain showed altered thickness, shape and localization of septin ring both under normal conditions and on fludioxonil treatment. In contrast, septin organisation was found to be normal on fludioxonil treatment in wild type stain BY4742/ClNik1p. Thus, fludioxonil does not affect septin assembly and organisation.

To understand fludioxonil toxicity, cell cycle analysis was performed and the budding pattern was checked in wild type stain upon fludioxonil exposure. Culture was synchronised in G1 phase and then budding pattern was observed in cells released in normal or drug containing media. It was observed that fludioxonil induced transient cell cycle arrest in G1 phase after release from alpha factor arrest, which was similar to Hog1 mediated G1 arrest observed on osmostress (Escote *et al.*, 2004). Nuclear division analysis revealed that after drug treatment majority of cells were budded with 2 nuclei per cell. Third population of cells also appeared which had two buds and three nuclei per cell. These results indicated that cells exposed to fludioxonil may have cytokinesis defect. To confirm this further, cell cycle analysis was done using flow cytometry. On fludioxonil treatment wild type cells showed altered distribution of different cell cycle phases, there was remarkable decrease in the cell number with G1 and G2 DNA content. At the same time there was increase in population of

polyploid cells. This cell separation defect after fludioxonil treatment indicates that the normal cytokinesis was being affected in the cells. This observation supports the results from deletion sensitivity profiling of fludioxonil in which genes involved in cytokinesis were over represented (Randhawa, 2013). Localisation of cytokinesis protein Hofl, was also found to be altered on fludioxonil treatment in wild type strain (Randhawa, 2013). Thus it is possible that the fludioxonil affects cytokinesis in BY4742/ClNik1p leading to cell separation defect.

It has been recently reported that *bem2-ts* cells show cytokinesis or septum formation defect partly due to inefficient localization of Iqg1 and Inn1 (Atkins *et al.*,2013). Hofl along with Iqg1, Inn1 and cyk3 is involved in actomyosin ring (AMR) and primary septum formation during cytokinesis (Wiloka and Bi, 2012). Since fludioxonil affect localisation of Hofl in wild type stain, it was of interest to see the effect of fludioxonil on localisation of Hofl-GFP in Δ*bem2* strain. It was observed that Hofl was properly localised to bud neck in 52% of budded cells. It was much higher (~3 times) than that reported previously in wild type strain on fludioxonil shock (Randhawa 2013). It may be possible that the cytokinesis defect caused by drug is suppressed by *BEM2* deletion. To investigate this further cell cycle analysis of Δ*bem2*/ClNik1p was carried out. A significant number of cells (13%) with polyploidy was observed in Δ*bem2*/ClNik1p under normal conditions which increased drastically (57%) after four hour of fludioxonil treatment. Thus fludioxonil treatment leads to cytokinesis defect both in wild type and Y16152 (Δ*bem2*) strain. These results indicate the involvement of some other function of Bem2p for drug sensitivity.

S. cerevisiae has six GTPases Rho1-5 and Cdc42 in family of Rho proteins. Since *BEM2* act as GAP for Rho GTPases and its deletion could result higher activation of these GTPases which is responsible for its resistance to fludioxonil. To examine this, Rho1, Rho2, Rho5 and Cdc42 were expressed from plasmid in wild type genetic background. Fludioxonil sensitivity of these strains was quite similar to that of wild type control. Therefore, the

resistance phenotype of *BEM2* deletion strain was not through the over activation of downstream Rho1-GTPases.

At the site of growth Rho1 GTPase activates six major effector proteins such as β 1-3 glucan synthase Fks1/Fks2, formin proteins Bni1 and Bnr1, MAPK kinase Mpk1, Sec23 exocyst component and transcription factor Skn7 (Levin, 2011). Since Bem2 acts upstream of these effector proteins through regulating the activity of Rho1p, therefore the deletion of either of these proteins in Δ*bem2* strain may affect its sensitivity to fludioxonil. It was observed that the deletion of *BNI1, BNR1, CRZ1, FKS2* and *MBP1* in Δ*bem2* had no effect on drug resistance phenotype. However, deletions of genes involve in cell wall integrity pathway and cell cycle regulation Swi6 and Swe1 suppressed the resistance of Δ*bem2* completely, while deletion of Skn7 suppressed the resistance partially. Skn7 is regulated by both Hog pathway and Rho1GTPase and has also role in maintaining cell wall integrity (Levin, 2011). Synthetic lethality of Δ*bem2* strain with *mpk1* and *swi4* also confirms the role cell wall integrity pathway in providing resistance against fludioxonil shock. On environmental stress, Swe1 works in coordination with Hog1p to arrest the cell cycle in G2 phase and has also been implicated in morphogenesis checkpoint delay along with Bem2p (Alexander *et al.*, 2001; Clotet *et al.*, 2006; Marquitz *et al.*, 2002). Deletion strain of *HSL1*, a protein kinase which regulates recruitment and degradation of Swe1p, was found to be slightly more sensitive towards fludioxonil (This study). Together all these observations indicate the role of cell wall integrity and Swe1 mediated cell cycle control in maintaining the cell viability in presence of fludioxonil.

Mpk1 kinase has been implicated in providing resistance against fludioxonil as its deletion leads to drug hypersensitivity (Kojima *et al.*, 2006; Randhawa, 2013). It was found that fludioxonil induced the phosphorylation of Mpk1p in both BY4742 and Δ*bem2* strain. Elevated basal level of phosphorylated Mpk1 was seen even under normal condition in *BEM2*

ɪn strain. Role of Mpk1 in fludioxonil sensitivity was further explored by creating set of ons in Mpk1 including K83A, R196A and K54A which removed the binding of ıs at DB site, SB site and ATP binding respectively. SB site mutant R196A which was ve in transcription through Rlm1 was showing two fold decrease in growth as red to MPK1 suggesting the role of Rlm1 driven transcription of cell wall genes in ing resistance to cell on fludioxonil treatment.

Previous work in our laboratory established that fludioxonil treatment altered the ar morphology to a great extent as most of the budded and unbudded cells were found highly fragmented vacuole. And it was speculated that fludioxonil might cause fusion in the endosomes and vesicles involved in vacuolar protein sorting and vacuole itself ıawa, 2013). To further validate this, vacuolar morphology of Δ*bem2*/ClNik1p was d. Majority of cells had predominant vacuolar morphology of one or two large es even upon fludioxonil exposure. It is already established that Δ*bem2* belongs to the of vacuolar protein sorting (*vps*) mutants, they are characterized by their single large . Class D mutants affect the genes which are involved in anterograde vesicular rt between late golgi to late endosome. It can be speculated that the fission defect of is playing an important role in providing resistance against fludioxonil. If fludioxonil onsible for vacuole fusion defect in the cells then there is a possibility that Bem2 is ing this phenomenon through many ways. Bem2 appears to be crucial for organisation ı cytoskeleton, cell wall integrity, vacuolar functions and cytokinesis, all of these ns are being affected by fludioxonil in one or another way. But its role in vacuole ınd fission pathway seems to be crucial for fludioxonil mechanism of action. However nvestigation will be needed to establish that vacuole fusion defect is the major cause oxonil sensitivity.