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Role of MAP Kinase Signaling in Secondary Metabolism and Adaptation to Abiotic/Fungicide Stress in *Fusarium*

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1. Introduction

Phosphorylation by protein kinases including mitogen activated protein kinases (MAPKs) is a major signal transduction mechanism used by eukaryotic cells to regulate different functions, virtually almost all activities that define their phenotypic behavior. Considering their diverse cellular roles, it was not surprising to find that significant portions of eukaryotic genes are devoted to code for protein kinases. For example, the genome of *Saccharomyces cerevisiae,* the budding yeast contains 130 distinct protein kinase encoding genes, representing approximately 2% of the entire yeast genome (Hunter and Plowman, 1997). The human genome contains 518 protein kinase genes comprising 1.7% of the genome (Manning *et al.,* 2002).

The MAPK signal transduction pathways constitute a cascade of phosphorylation events that transmit extracellular signals from membrane-bound receptors to the nucleus. MAPKs are highly selective for phosphorylation of serine/threonine residues lying immediately N-terminal to a proline residue within a peptide substrate (Hanks and Hunter, 1995; Brábek and Hanks, 2004). MAP kinase cascades control almost all aspects of fungal growth, development, sexual and asexual reproduction, metabolism, proliferation and stress tolerance.

Two of the three MAP kinase pathways of filamentous fungi, the HOG1 (*h*igh *o*smolarity *g*lycerol according to yeast nomenclature) MAPK and the CWI (*c*ell *w*all *i*ntegrity) MAPK (homologous to Slt2/Mpk1 in yeast) pathways take part in abiotic stress tolerance including tolerance to salt, osmotic, oxidative, heat, cold, arsenite and citric acid stressors. Recently the HOG MAPK pathway has also been indicated in tunicamycin induced endoplasmic reticulum stress in *S. cerevisiae* (Torres-Quiroz *et al.,* 2010). The third MAPK route, the so-called *p*athogenicity *M*AP *k*inase (PMK) pathway is homologous to the mating/ filamentation Fus3/Kss1 MAPK pathway of the yeast. PMK is required for the infection process including penetration into the host cells and invasive growth. This pathway is also involved in the yeast-to-hyphal transition in dimorphic species. As far as studies on *Fusarium* species are concerned, the Fmk1 and the Gpmk1 MAP kinases of *Fusarium oxysporum* (Di Pietro *et al.,* 2001) and *Fusarium graminearum* (Jenczmionka *et al.,* 2003),

respectively were also found to be essential for pathogenicity. These PMK-type MAP kinases regulates the expression of several genes encoding cell-wall degrading hydrolytic enzymes (Jenczmionka and Schäfer , 2005).

Although PMK-type MAP kinases were generally regarded to have not much role in stress adaptation, recent studies demonstrated that the pathogenicity MAPK pathway controls the oxidative stress response in *Cochliobolus heterostrophus* (Izumitsu *et al.,* 2009). These observations highlight a more complex nature of stress signaling in filamentous fungi as it was anticipated previously (Aguirre *et al.,* 2006).

We present here a functional analysis of Δ*Fvhog1* and Δ*Fvmk2* CWI MAPK mutants of *F. verticillioides* by comparing their sensitivity to different oxidative stressors. *Fusarium verticillioides* (teleomorph: *Gibberella moniliformis*) is a world-wide occurring pathogen of maize that synthesizes a range of secondary metabolites, including fumonisins and carotenoids. To the best of our knowledge this is the first report on the comparison of oxidative stress tolerance of different MAPK mutants of the same filamentous fungus species. We also found that both Δ*Fvhog1* and Δ*Fvmk2* CWI MAPK gene-disruption mutants of *F. verticillioides* resulted in increased sensitivity to methylglyoxal, a toxic glycolytic by-product suggesting a double MAPK regulation of the cellular response to this compound. Secondary metabolite production is also regulated by different MAPK pathways in fungi: we provide here additional information on the recent findings available for fusaria. As the highly conserved fungicide signaling by fludioxonil is not dependent on the histidine kinase-HOG1 MAPK route in all filamentous fungi (Liu *et al.,* 2008) we compared the fludioxonil and hydrogen peroxide sensitivity of three *Fusarium* species. The extreme sensitivity *of F. graminearum* to fludioxonil and hydrogen peroxide was not associated with substantial changes in HOG MAPK mediated osmotic stress tolerance. We also found that Δ*hog1* mutants of two other *Fusarium* species showed fludioxonil tolerant and hydrogen-peroxide sensitive phenotypes, similarly to other filamentous species.

2. The role of HOG1 MAPK signaling in stress and fungicide tolerance of *Fusarium* species

Orthologues of the yeast HOG1 pathway genes have been identified either by functional or *in silico* analysis in several *Fusarium* species, including *F. graminearum, Fusarium proliferatum, F. oxysporum,* and *F. verticillioides* (Di Pietro *et al.,* 2001; Ochiai *et al.,* 2007; Ádám *et al.,* 2008a, b; Rispail *et al.,* 2009; Rispail and Di Pietro, 2010). In *F. proliferatum,* the HOG1 MAPK pathway plays a pivotal role in stress tolerance: this route takes part in salt, osmotic, heat, UV and oxidative (hydrogen peroxide) stress responses, but it is not required for invasive growth, sexual and asexual sporulation (Ádám *et al.,* 2008a, b). Osmotic stress caused a considerably higher rate of cell death in the Δ*Fphog1* MAPK gene disruption mutants as compared to the wild type strain. More importantly, when the fungi were subjected to osmotic (4% NaCl) stress, levels of reactive oxygen species (ROS), mitochondrial membrane permeability transition, nuclear disintegration and DNA fragmentation, four independent markers of programmed cell death (PCD) all showed significant increases in the Δ*Fphog1* mutants in comparison to the wild type strain suggesting that an important role of the functional Hog1 MAPK gene is attenuating apoptotic phenotypes under stress conditions (Ádám *et al.,* 2008a). Fig. 1. shows intense cell death symptoms and accumulation of ROS indicated by blue stained morphologically abnormal cells and green fluorescent cells (indicated by arrow), respectively in a Δ*Fphog1* gene-disruption mutant subjected to salt stress after adding 4%

(w/v) NaCl to the culture medium. Similarly to Δ*hog1* mutants of other fungi, like *Neurospora crassa*, *Aspergillus nidulans*, *C. heterostrophus* and *Colletotrichum lagenarium* the Δ*Fphog1* MAPK mutants of *F. proliferatum* became tolerant to phenylpyrrole and dicarboximide fungicides (Zhang *et al.,* 2002; Noguchi *et al.,* 2004; Yoshimi *et al.,* 2005; Ádám *et al.,* 2008b; Hagiwara *et al.,* 2009). Although the exact mode of action of these compounds is still unclear, the finding that heterologous expression of Hik1, the histidine kinase (HK) gene of *Magnaporthe oryzae* in the yeast, *S. cerevisiae* that contain only one HK gene, Sln1 confers susceptibility in this otherwise fludioxonil-resistant organism, suggests that class III HKs, located upstream of the HOG1 MAPK cascade are possible targets of this fungicide (Motoyama *et al.,* 2005). The class III HKs responsible for elevated osmo-tolerance and increased fludioxonil sensitivity in filamentous fungi are not the orthologues of Sln1 of the yeast (Catlett *et al.,* 2003). Inactivation of Fhk1, a class III HK in *F. oxysporum* resulted in osmo-sensitivity and resistance to phenylpyrrole and dicarboximide fungicides (Rispail and Di Pietro, 2010). The increased tolerance of the Δ*Fphog1* mutants of *F. proliferatum* to fludioxonil and vinclozoline (Ádám *et al.,* 2008b) suggests that functional HK-HOG1 MAPK pathway is required for sensitive response to these fungicides in *Fusarium* species. *In silico* analysis of HKs by reciprocal BLASTP searches in *Fusarium* genome sequences led to the identification of Fhk1 (FOXG_01684) orthologues both in *F. verticillioides* (hypothetical protein FVEG_08048) and *F. graminearum* (hypothetical protein FGSG_07118) (Nagygyörgy and Ádám, unpublished).

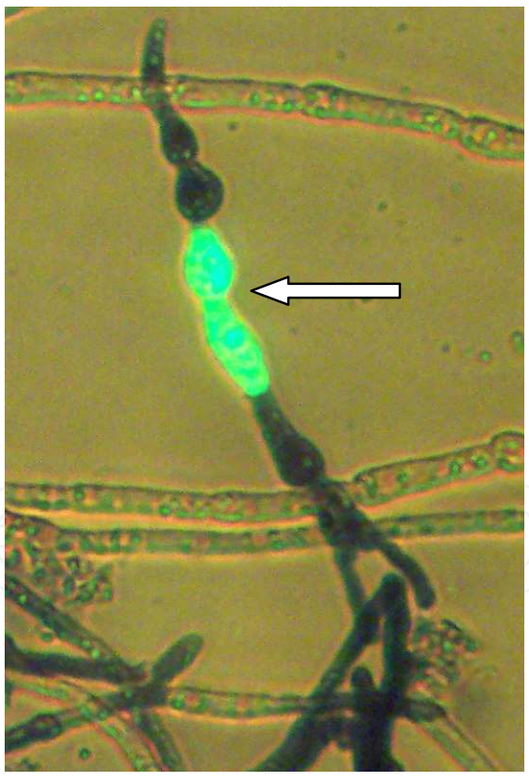

Fig. 1. Double staining of *Fusarium proliferatum* Δ*Fphog1-24* gene-disruption MAP kinase mutant with 2,7-dichlorodihydrofluorescein diacetate (DCHFDA) and Evans blue after NaCl (4% w/v) exposition. Intensive green fluorescence (indicated by arrow) and dark blue discoloration of the cells indicate accumulation of reactive oxygen species (ROS) and cell death, respectively.

HKs have five HAMP (*h*istidine kinase, *a*denylate cyclase, *m*ethyl binding protein and *p*hosphatase) repeats: mutations in these sequences are responsible for the increased osmo-sensitivity and fungicide resistance of *N. crassa*, *C. heterostrophus*, *Alternaria brassicicola* and *Botrytis cinerea* (Ochiai *et al.,* 2001; Yoshimi *et al.,* 2004; Motoyama *et al.,* 2005; Viaud *et al.,* 2006). Recent microarray analyses have further shown that the transcriptional response to fludioxonil depends on a Hog1 orthologue in *A. nidulans*. This response overlaps, in part with the transcriptional response to hyperosmotic stress but depends on factors other than the AtfA transcription factor responsible for conidial stress tolerance (Hagiwara *et al.,* 2009). Thus the identification of transcription factor(s), that are located downstream of Hog1 MAPK and influence gene expression response to fludioxonil requires further studies.

Although fungicide signaling by fludioxonil is highly conserved in filamentous fungi, response to this compound is still not entirely dependent on the HK-HOG1 MAPK route in all species. For example, the Δ*sak1* (Hog1 orthologue) knockout mutants of *B. cinerea* maintained their sensitive phenotype to fludioxionil (Liu *et al.,* 2008) indicating the complex nature of this signaling pathway. On-going research of our laboratory on fludioxonil sensitivity of three *Fusarium* species with available genome sequences (*F. graminearum* PH-1/NRRL 31084, *F. oxysporum* 4287 and *F. verticillioides* FGSC 7600; http: //www.broadinstitute. org/annotation/genome/Fusariumgroup/MultiHome.html) led to somewhat surprising results (Fig. 2).

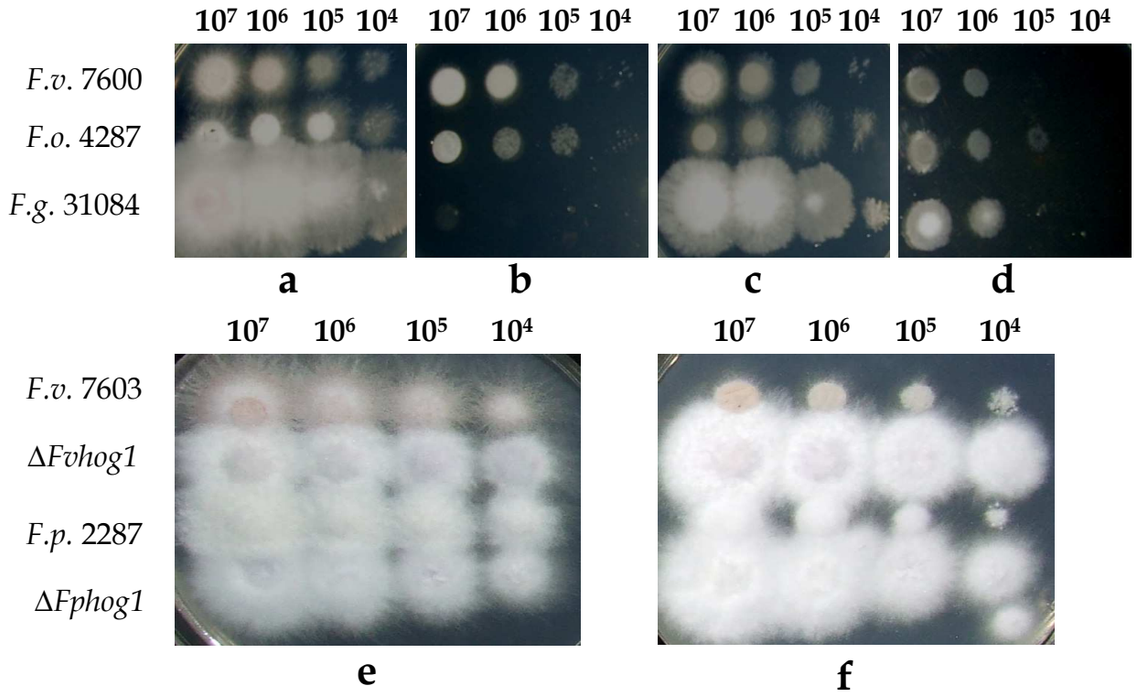

Fig. 2. Growth sensitivity of three *Fusarium* species: *Fusarium oxysporum* 4287, *Fusarium verticillioides* FGSC 7600 and *Fusarium graminearum* PH-1/NRRL 31084 against different stressors (a-d) and sensitivity of Δ*hog1* mutants of *F. verticillioides* FGSC 7603 and *F. proliferatum* FGSC 2287 (Δ*Fvhog1-14* and Δ*Fphog1-24,* respectively*)* against fludioxonil (e-f). Five-five µl of indicated concentrations of conidia (cells/ml) was spotted on (a, e) complex medium (CM) agar plates and CM agar plates supplemented with (b, f) 10 µg/ml fludioxonil, (c) 4% (w/v) NaCl and (d) 2 M sorbitol. Incubation time was 3 days for all plates at 24 °C.

F. graminearum showed increased sensitivity to this fungicide as compared to the other two species (Fig. 2a and b), but fludioxonil sensitivity was not accompanied with substantial changes in osmo-sensitivity of this fungus (Fig. 2c and d). On the other hand, Δ*hog1* mutants of *F. proliferatum* and *F. verticillioides* showed fludioxonil tolerance (Fig. 2e and f), paralleled with elevated osmo-sensitivity. Although these results do not exclude the involvement of the HK-HOG1 MAPK pathway in these phenotypes, further studies are needed to a better understanding of fungicide stress responses in *Fusarium* species.

3. The role of MAPK pathways in secondary metabolism of *Fusarium* species

In addition to its role in stress and fungicide tolerance, the HOG1 MAPK pathway plays also an important role in the regulation of secondary metabolism in different *Fusarium* species (Ochiai *et al.,* 2007; Kohut *et al.,* 2009). Disruption of either Fgos2 (a HOG-type MAPK orthologue) or Fgos4 (encoding a MAPK kinase) or Fgos5 (encoding a MAPK kinase kinase) blocked trichotecene production in *F. graminearum* and substantially reduced expression of the trichotecene gene cluster. On the other hand, amounts of aurofusarin were increased in all three types of mutants (Ochiai *et al.,* 2007). Deoxynivalenol production is controlled by Mgv1 CWI MAPK in *F. graminearum* (Hou *et al.,* 2002). Nitrogen depletion induced the production of fumonisin B1, a polyketide derivative mycotoxin and increased the expression of fuminisin biosynthesis genes in *F. proliferatum*. Under nitrogen starvation (absence of any N-source) conditions deletion of Fphog1, a HOG-type MAP kinase gene resulted in further increases in FUM1 and FUM8 gene expression, as well as fumonisin B1 production suggesting that this response is mediated via the HOG-type MAPK pathway in *F. proliferatum* (Kohut *et al.,* 2009). In a more recent study Fvmk1, a PMK-type MAPK gene was identified as a positive regulator of fumonisin B1 production in *F. verticillioides* (Zhang *et al.,* 2011). On the contrary, fumonisin B1 production was not regulated by cAMP signaling either in *F. proliferatum* (Kohut *et al.,* 2010) or *F. verticillioides* (Choi and Xu, 2010). This signaling route regulates, however negatively regulates the production of bikaverin, another polyketide metabolite in *Fusarium* species (Kohut *et al.,* 2010; Choi and Xu, 2010; García-Martínez *et al.,* 2011). Moreover, the production of another secondary metabolite such as carotenoids is upregulated in *Fusarium* species not only by cAMP signaling (García-Martínez *et al*., 2011) but other regulatory elements related to sexual reproduction (Ádám *et al*., 2011).

4. Complexity of oxidative stress signaling in fungi: Role of the HOG1 and the CWI MAPK pathways

Previous research with different species indicated that, besides the HOG MAPK pathway (Aguirre *et al.,* 2006; Du *et al.,* 2006; Ádám *et al.,* 2008a), the CWI MAPK pathway (Krasley *et al.,* 2006; Valiante *et al.,* 2007) also has a role in oxidative stress tolerance of fungi. To compare the particularities of the two pathways we generated both Δ*hog1* (Δ*Fvhog1*) and CWI MAPK (Δ*Fvmk2*) gene-disruption mutants in a single fungus species, *F. verticillioides* as we have described earlier (Ádám *et al.,* 2008a). These mutants were tested for oxidative stress tolerance in conidial dilution assay using hydrogen peroxide, menadione, diamide and methylglyoxal as stressors. Although all of these compounds elicit finally oxidative stress, the mechanisms they do this are different. Hydrogen peroxide induces lipid peroxidation, protein and DNA damage directly or indirectly and contributes to the formation of hydroxyl radicals (OH·) via

the Fenton reaction (Thön *et al.,* 2007). Menadione is a redox cycling reagent that acts by generating superoxide radicals ($O_2^{\cdot-}$) using NADPH as a cofactor. Diamide causes depletion of the reduced glutathione pool and perturbation of the redox balance of cells; this compound also reacts with sulfhydryl groups of proteins in a reversible way (Pócsi *et al.,* 2005; Thön *et al.,* 2007). Methylglyoxal is a highly toxic natural glycolytic by-product interacting with proteins in a reversible way and, at higher concentrations (8-10 mM) it can deplete the glutathione pool in yeast cells (Aguilera *et al.,* 2005).

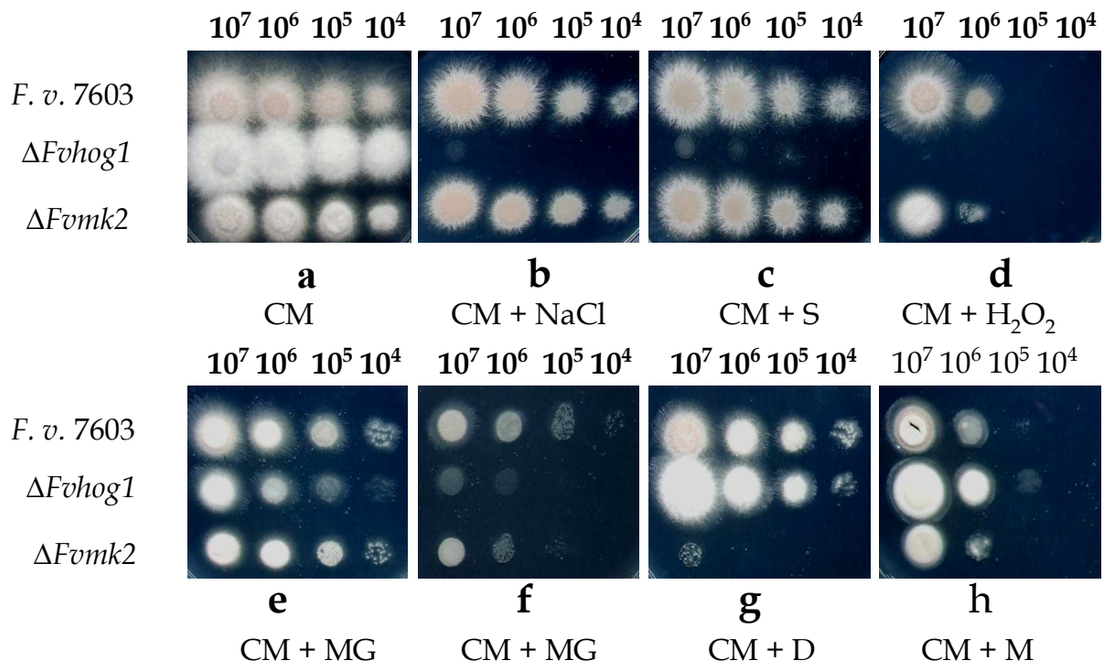

Fig. 3. Differential sensitivity of Δ*Fvhog1-14* and Δ*Fvmk2-16* CWI MAPK mutants of *Fusarium verticillioides* FGSC 7603 against oxidative stressors. Five-five µl of indicated concentrations of conidia (cells/ml) was spotted on (a) complex medium (CM) agar plate and CM agar plates supplemented with (b) 4% (w/v) NaCl, (c) 2 M sorbitol (S), (d) 2 mM hydrogen peroxide (H_2O_2), (e) 5 mM methylglyoxal (MG), (f) 10 mM methylglyoxal (MG), (g) 0,5 mM diamide (D) and (h) 0,03 mM menadione (M). Incubation time was 3 days for all plates at 24 °C.

The Δ*Fvhog1* mutant of *F. verticillioides* was highly sensitive to the osmotic stressors, sodium chloride and sorbitol (Fig 3a, b and c), similar to our former results with *F. proliferatum* Δ*hog1* mutants (Ádám *et al.,* 2008a). On the contrary, the Δ*Fvmk2* mutant showed no elevated osmo-sensitivity (Fig 3a, b and c). The Δ*Fvhog1* mutant was sensitive not only to osmotic stressors but, as well as to hydrogen peroxide and methylglyoxal. However, this mutation caused no change in menadione and diamide sensitivity (Fig. 3a, d, e, f g and h). This is a first report on the involvement of HOG MAPK pathway in methylglyoxal tolerance of a filamentous species. Formerly Aguilera *et al.* (2005) reported on methylglyoxal sensitivity of Δ*hog1* mutants of *S. cerevisiae*. Δ*Hog1* mutants of filamentous species showed different oxidative stress sensitivity. Mutants of *A. fumigatus* lacking the MAP kinase Δ*sakA/*Δ*hog1* were more sensitive to H_2O_2 and menadione compared to wild type strain (Du *et al.,* 2006).

In *F. oxysporum* the *Fhk1* HK mutant, deficient in an upstream element of the HOG1 MAPK pathway is sensitive to menadione induced oxidative stress but not to H_2O_2 (Rispail and Di Pietro, 2010).

Results obtained with the Δ*Fvmk2* CWI MAPK mutants were completely different: they showed no elevated osmo-sensitivity, but their sensitivity to methylglyoxal and diamide increased as compared to the wild type strain (Figs. 3a, b, c, d, e, f, g and h). According to these results, the cellular response to methylglyoxal is regulated by both the HOG1 and CWI MAPK pathway, but the other stressors are signaled separately either by the HOG1 or CWI MAPK pathway. As both methylglyoxal and diamide interact mainly with glutathione metabolism, the possible role of CWI MAPK is to maintain glutathione pools under stress conditions. As regarding the oxidative stress sensitivity of other species, CWI MAPKs played a fluctuating but positive regulatory role in stress tolerance. An exceptional case is *A. fumigatus*: the deletion of MpkA CWI MAPK gene resulted in increased H_2O_2 tolerance and sensitivity to menadione and diamide (Valiante *et al.,* 2007). In the case of *S. cerevisiae* Δ*slt2*/Δ*mpk1* mutants were sensitive to H_2O_2 (Krasley *et al.,* 2006). But in *C. albicans* and *S. pombe*, deletion mutants of Δ*mkc1* and Δ*pmk1*, respectively were sensitive to diamide but not to H_2O_2 and menadione (Navarro-Garcia *et al.,* 2005; Madrid *et al.,* 2006).

5. Sensitivity of different *Fusarium* species to hydrogen peroxide

We compared the hydrogen peroxide sensitivity of three *Fusarium* species, *F. graminearum* PH-1/NRRL 31084, *F. oxysporum* 4287 and *F. verticillioides* FGSC 7600 with available genome sequences (http://www.broadinstitute.org/annotation/genome/Fusariumgroup/ Multi Home.html). *F. gaminearum*, a causal agent of head blight of wheat and stalk/cob rot of maize was the most sensitive to this oxidative stressor both in a decimal conidium dilution assay (Valiante *et al.,* 2007) and in radial growth test (Ádám *et al.,* 2008a). Mycelial growth and conidial germination of this fungus was more strongly inhibited by 5-50 mM and 2 mM H_2O_2 concentrations as compared to *F. oxysporum* and *F. verticillioides* (Fig 4A, 4B), other two plant pathogenic species causing vascular wilt of a wide range of plants and maize cob rot, respectively. In a previous study (Nicolaou *et al.,* 2009), oxidative stress tolerance of 18 fungal species originating from different ecological niches and phylogenetic positions were compared and plant pathogenic species, like *Ustilago maydis*, *F. graminearum* and *M. grisea* were found to be relatively sensitive to oxidative stressors, including hydrogen peroxide. This result was somewhat surprising as both plant and animal pathogens that are exposed to massive oxidative and/or nitrosative stress by the host cells in many host-pathogen interactions (Brown *et al.,* 2009) would have been expected to acquire improved levels of oxidative stress tolerance during their evolution. When the hydrogen peroxide sensitivity tests were extended to other two *Fusarium* species, *F. fujikuroi* MP-C and *F. proliferatum* FGSC 2287, causing the bakane disease of rice and crown and root rot of a wide range of plants, respectively they also showed higher levels of hydrogen peroxide tolerance as compared to that of *F. graminearum.* The increased H_2O_2 sensitivity of *F. graminearum* can be putatively explained by the long saprophytic phase in the life cycle of this species. During the saprophytic phase, *F. graminearum* lives and propagates on dead tissues and in this niche, the fungus is much less exposed to oxidative stress influence than the other species, that spend much of their life cycle inside living plant tissues either as endophytes or vascular colonizers.

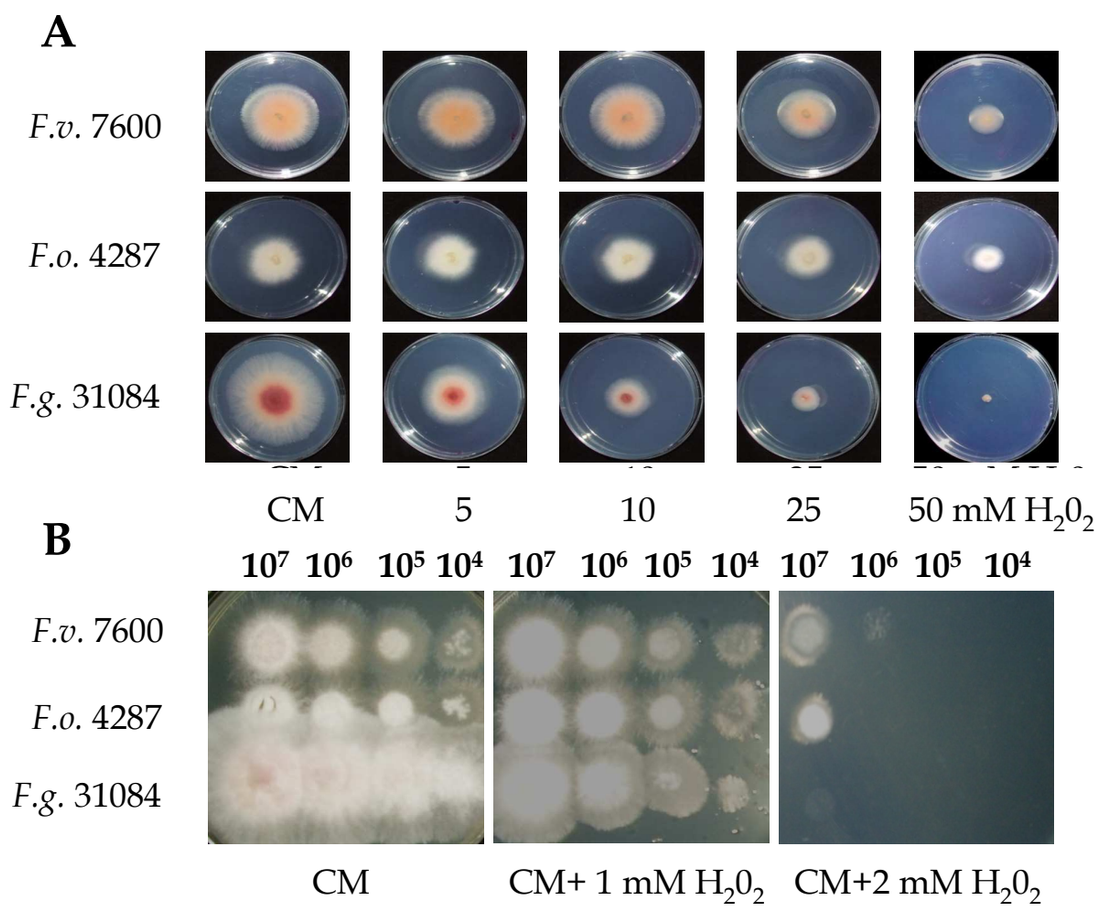

Fig. 4. Growth sensitivity of three *Fusarium* species: *Fusarium verticillioides* FGSC 7600, *Fusarium oxysporum* 4287 and *Fusarium graminearum* PH-1/NRRL 31084 against hydrogen peroxide (H_2O_2) in radial growth test (A) and decimal conidial dilution assay (B). In (B) five-five µl of indicated concentrations of conidia (cells/ml) was spotted on complex medium (CM) agar plate and CM agar plates supplemented with the indicated concentrations of hydrogen peroxide (H_2O_2). Incubation time was 5 days for plates in (A) and 3 days for plates in (B) at 24 ^oC.

In another approach we have studied the role of cAMP signaling in oxidative stress response of different *Fusarium* species. Previous studies in *N. crassa* demonstrated that cAMP signaling and HOG1 MAPK signaling play opposite role in respect to oxidative stress response: disturbances in the cAMP and the HOG1 pathways result in increased and decreased H_2O_2 tolerance, respectively. In *F. proliferatum* and *F. verticillioides,* disruption of Acy1, the adenylyl cyclase gene resulted in enhanced resistance to heat shock and oxidative stress (Kohut *et al.,* 2010; Choi and Xu, 2010). However, in contrast to these data, the *acyA*-mutants of *F. fujikuroi* MP-C were more sensitive to H_2O_2 than the wild type (García-Martínez *et al.,* 2011). This finding suggests the high versatility of the cAMP signaling route even in closely related fungi. These differences are particularly important, if we consider that heat shock and oxidative stress pathways have at least partially overlapping signaling routes and regulated by the same transcription factors in yeast (Ikner and Shiozaki, 2005).

6. Conclusion

Functional analysis of orthologous signal transduction genes in different filamentous fungal species highlighted the complex nature of stress signal transduction. This is especially true for oxidative stress signaling, where all fungal MAPK cascades, the HOG1, CWI and PMK MAPK pathways participate and interact in this regulatory network depending on the fungal species. One of the oxidative agents, mehylglyoxal, a toxic by-product of glycolysis is signaled either by the HOG1 MAPK or CWI MAPK pathway in *F. verticillioides*. All these MAPK cascades are also involved either in positive or negative regulation of secondary metabolite production including mycotoxins in different *Fusarium* species. The high versatility of oxidative stress and secondary metabolite signaling by the above-mentioned MAPK pathways and the cAMP-PKA pathway in different *Fusarium* species denotes that stress signaling is exposed to rapid evolution to tune stress responses in a niche-specific manner, independently of the phylogenetic position of a given species.

7. Acknowledgement

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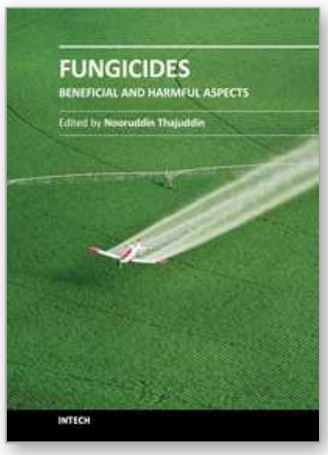

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Fungicides are a class of pesticides used for killing or inhibiting the growth of fungus. They are extensively used in pharmaceutical industry, agriculture, in protection of seed during storage and in preventing the growth of fungi that produce toxins. Hence, fungicides production is constantly increasing as a result of their great importance to agriculture. Some fungicides affect humans and beneficial microorganisms including insects, birds and fish thus public concern about their effects is increasing day by day. In order to enrich the knowledge on beneficial and adverse effects of fungicides this book encompasses various aspects of the fungicides including fungicide resistance, mode of action, management fungal pathogens and defense mechanisms, ill effects of fungicides interfering the endocrine system, combined application of various fungicides and the need of GRAS (generally recognized as safe) fungicides. This volume will be useful source of information on fungicides for post graduate students, researchers, agriculturists, environmentalists and decision makers.

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