

FUNGICIDAL INTERFERENCE DURING INFECTION RELATED DEVELOPMENTAL STAGES IN *MAGNAPORTHE GRISEA*

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ABSTRACT

Rice blast, a serious epidemic disease that limits grain yield worldwide is caused by fungal pathogen *Magnaporthe grisea*. The present investigation was carried out to identify the probable avenues of interference by different fungicides during the critical stages of infection related morphogenesis of *M. grisea*. Effect of six fungicides at different stages of infection related morphogenesis showed variable results like interference in conidial germination, distortion of surface structure of the spores, interference in the germ tube elongation, interference in the transfer of the cell contents from spore to appressorium, deformity in appressorial dome, interference in the melanin deposition. We speculate the critical stages at which these fungicides may interfere. The activity of immunosuppressive drug cyclosporin A (CsA) which is a potential antifungal agent was equated with all the fungicides used. We hypothesize that the exposure of the *M. grisea* spore to the fungicide may lead to the formation of a cyclophilin CYP1-fungicide complex, which inactivates calcineurin and prevents calcium/ calmodulin-dependent protein phosphatase signaling and is therefore one of the target of fungicidal interference. An understanding of how fungal pathogens break the protective barrier that comprise the surface of the host plant as well as precise identification of avenues of fungicidal interference during infection related development in *M. grisea* will lead to novel approach for controlling plant diseases.

Keywords: Appressorium, Fungicides, *Magnaporthe grisea*, Developmental stages, Mode of action, Rice blast.

INTRODUCTION

Magnaporthe grisea is a typical hermaphroditic ascomycete and the causal agent of rice blast, the most disparaging disease of rice. The control measures of this disease have been dependent on breeding resistant cultivars and the application of chemical fungicides. However the frequent appearance of new races has reduced the effectiveness of resistant cultivars in the field (Sonah et al., 2009). Environmental regulations have also restricted the use of chemical fungicides. An understanding of infection mechanism is a prerequisite for the development of new strategies to control this disease. The differentiation and maturation of appressoria are critical steps for successful infection. Environmental cues inducing appressorium formation in *M. grisea* include the hydrophobicity and hardness of the contact surface and chemicals from the plant surface

(Gilbert *et al.*, 1996; Lee and Dean, 1993; Xiao *et al.*, 1994). Intracellular signaling systems involved in appressorium differentiation in this fungus are not fully understood. However, some studies have described role of different signaling molecules in appressorium development (Beckerman and Ebbole, 1996, Choi *et al.*, 1998). It has been observed that the targeted disruption of the catalytic subunit gene (cpkA) of cAMP-dependent protein kinase in *M.grisea* abolished the ability to form normal appressoria. Beckerman and Ebbole (1996) reported that MPG1-distrupted mutants, which are unable to form appressoria but formed appressoria when cAMP was added. Polyamines reduce intracellular cAMP levels in *M. grisea*, leading to the inhibition of appressorium formation (Choi *et al.*, 1998). These experiments suggest that cAMP plays an important role in the regulation of appressorium formation in *M. grisea*. Detailed understanding of both the disease and the developmental biology of *M. grisea* is required for developing novel mechanism to control rice blast.

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Noteworthy progress has been made in identifying the genes required for elaboration of appressoria. In *M. grisea*, appressorium formation and penetration processes have been studied extensively (Dean R. A., 1997; Hamer and Talbot, 1998). Considering the importance of surface interactions to fungi, any interference will reduce the epidemiological potential of the fungus and thus can be a novel and great policy to control blast. Thus in the present investigation different fungicides at different concentrations were used to study their effect on all stages of appressorium development.

MATERIALS AND METHODS

The experimental materials consisted of single spore isolates of *M. grisea* from the infected leaves, which were collected from the naturally infected rice leaves. A highly sporulating isolates were used to check the effect of six fungicides at all stages of appressorium morphogenesis (Table 1). Agar blocks containing embedded fungal growth were induced for sporulation and were used for assaying the infection-related morphogenesis. Developmental stages of *M. grisea* were observed on glass cavity slides which were found equally effective in inducing appressorium formation, and were transparent for viewing with a binocular light/phase contrast microscope. Conidial suspension 200 μ l (containing approx 10³ conidia/ml) was placed on the surface of the

glass cavity slides and left in a humid environment at 24°C. Frequency of appressorium formation on rice leaves was examined by incubating conidia on rice leaves in a humid chamber for 14 hours at 24°C. Six fungicides namely Hexaconazole, Carbendazim, Edifenphos, Isoprothiolane (Diisopropyl(1, 3-dithiolan-2-ylidene) malonate), S-benzyl diisopropyl phosphorothiolate (IBP) and Tridemorph (N-tridecyl-2, 6-dimethylmorpholine) with three different concentrations (0.1%, 0.01%, 0.001%) were used for assaying the effect on infection-related morphogenesis of *M. grisea* (Table1). Approximately 1ml of fungicidal concentration was placed in two cavities of the glass slide and an equal amount of sterilized distilled water in the third cavity, which served as a control. The spores from the sporulating agar blocks were then picked with the help of pointed capillary needle and placed on the glass cavity slides containing the fungicides and left in a humid environment at 24°C. After a brief exposure of the spores, the fungicide was removed with help of sterilized blotting paper and the spores were rinsed several times with sterilized double distilled water and were then incubated in 1ml of sterilized double distilled water. Similarly the pre-germinating conidia on cavity slides were given brief exposure against different fungicides and respective concentrations.

Table 1: Details of fungicides used for the evaluation of their interference in infection related developmental stages in *Magnaporthe grisea*

Trade name	Coined name	Chemical Name	Conc. (a.i.)
Contaf	Hexaconazole	2-(2, 4-dichlorophenyl)-1-(1H-1, 2, 4-triazol-1-yl) hexan-2-ol	90%TC
Bavistin	Carbendazim	methyl benzimidazol-2-yl carbamate	50% WP
Hinosan	Edifenphos	O-ethyl-S,S-diphenylphosphorodithioate	50% EC
Fuji One	Isoprothiolane	diisopropyl-1,3-dithiolan-2-ylidenemalonate	40% EC
Kitazin	IBP	S-benzyl-O,O-di-isopropyl phosphorothioate	48%EC
Calixin	Tridemorph	N-tridecyl-2,6-dimethyl-morpholine	80% EC

All the stages of appressorium formation by *M. grisea* and the fungicidal effects on the infection related morphogenesis were determined by examining under Leica DAS binocular light/phase contrast microscope at 1 hour intervals up to 14 hours and the selected specimens were micro-photographed using KODAK 100 ASA film.

RESULTS AND DISCUSSION

M. grisea is an experimentally tractable fungus, and this has allowed the characterization of a number of

processes related to appressorium development. In present investigation, Hexaconazole (0.01%) was observed as inhibitory to spore germination when exposed for a longer duration, and the cytoplasmic contents were aggregated/ granulated which did not auto-fluoresce under phase contrast imaging. On brief exposures of the spores at 0.001 % Hexaconazole concentrations, the spores were able to germinate but the size of appressoria produced was small and un-melanized (Fig.1A, B, K, Table 2).

Table 2: Effect of Fungicides on developmental stages of infection related morphogenesis at different concentration and time in *M. grisea*.

Effect on different stages of infection related morphogenesis	Exposure time and Concentration				
	24 h	½ h	10 min.		
	0.01		0.1	0.01	0.001
Tridemorph					
Distorted shape of the spores	-	-	++	-	-
Aggregated / granulated cytoplasmic contents in spores	+++	++	-	-	+
A cluster of vacuole intermingled with small lipid droplets	-	-	++	-	-
Increased germ tube length	-	-	++	++	+
Melanization of the elongated germ tube tip	-	-	++		+
Distorted shape of the germ tube	-	-		+++	+
Fragmented cytoplasmic contents transferred	-	-	-	++	-
Abnormal / distorted appressoria	-	-	++		-
Size of the appressoria smaller than the wild type	-	-		++	-
Poorely melanized appressoria	-	-	++	-	-
IBP					
A large central vacuole with lipid bodies	+++	++	-	-	-
Increased germ tube length	-	-	-	+++	++
Distorted shape of the germ tube	-	-	++	-	-
The germ tube did not initiated the appressorial hook	-	-	-	+++	-
Abnormal / distorted appressoria	-	-	-	++	+
Poorely melanized appressoria	-	-	-	+	-
Fuji-One					
Distorted shape of the spores	-	-	-	++	-
Aggregated / granulated cytoplasmic contents in spores	+++	++	++	-	+
A cluster of vacuole intermingled with small lipid droplets	+++	+++	++	-	-
Spores formed chlymadospore like structures	-	-	-	++	-
Increased germ tube length	-	-	++	++	+
Melanization of the elongated germ tube tip	-	-	-	-	++
Distorted shape of the germ tube	-	-	-	++	-
Abnormal / distorted appressoria	-	-	-	-	++
Edifenphos					
The spore s were distorted in shape	++	-	-	-	-
<i>Shrunkn spores</i>	+++	-	++	++	-
Aggregated / granulated cytoplasmic contents in spores	+++	++	+	-	-
A cluster of vacuole intermingled with small lipid droplets	-	-	-	++	-
Increased germ tube length	-	-	-		++
<i>Distorted shape of the germ tube</i>	-	-	-	+++	++
The germ tube did not initiated the appressorial hook	-	-	-	-	++
Abnormal / distorted appressoria	-	-	-	-	++
Carbendazim					
Aggregated / granulated cytoplasmic contents in spores	++	+	-	-	-
Small germ tube	-	-	++	-	-
Abnormal / distorted appressoria	-	-	-	++	-
Size of the appressoria smaller than the wild type	-	-	-	-	++
Poorely melanized appressoria	-	-	-	++	-
Hexaconazole					
Aggregated / granulated cytoplasmic contents in spores	+++	++	++	+	-
Size of the appressoria smaller than the wild type	-	-	-	-	++
Unmelanized appressoria	-	-	-	-	+++

Carbendazim (Bavistin) methyl benzimidazol-2-yl carbamate (MBC) causes loss in the ability of germination. At 0.01% Carbendazim concentration on brief exposure the spores were able to produce short germ tube. After increasing the exposure of spores to this fungicide, the appressoria produced were either abnormal or distorted and the size of the appressorium appeared smaller than the control. The appressorium were poorly melanized at all the concentrations (Fig.1 I-K). Edifenphos (O-ethyl-S, S-phenylphosphorodithioate) showed its effect on different stages of infection related morphogenesis as, either distorted or shrunken spores or the cytoplasmic contents were granulated. In most of the non-germinating spores, cluster of medium sized vacuole intermingled with small lipid droplets were observed. Spores were able to produce germ tube and were very long, distorted and no germ tube hook was

initiated at all the concentrations. Abnormal or distorted appressoria were observed which were poorly melanized at 0.001 % concentration (Fig.1 B-J). Isoprothiolane (diisopropyl-1, 3-dithiolan-2-ylidenemalonate) showed distortion in shape, with granulation of cytoplasmic contents of the spore.

A cluster of medium sized vacuole intermingled with small lipid droplets were observed and in some cases the middle cell of the spores appeared to be transformed in chlamyospore like structures. The spores were able to germinate and produced long germ tube which was distorted at 0.1 to 0.001%. In many cases the melanization of the germ tube tip was observed which resembled the aleurospores. IBP (S-benzyl-O, O-di-isopropyl phosphorothioate) fungicide treated spores were characterized by the presence of a large central vacuole with fewer lipid bodies.

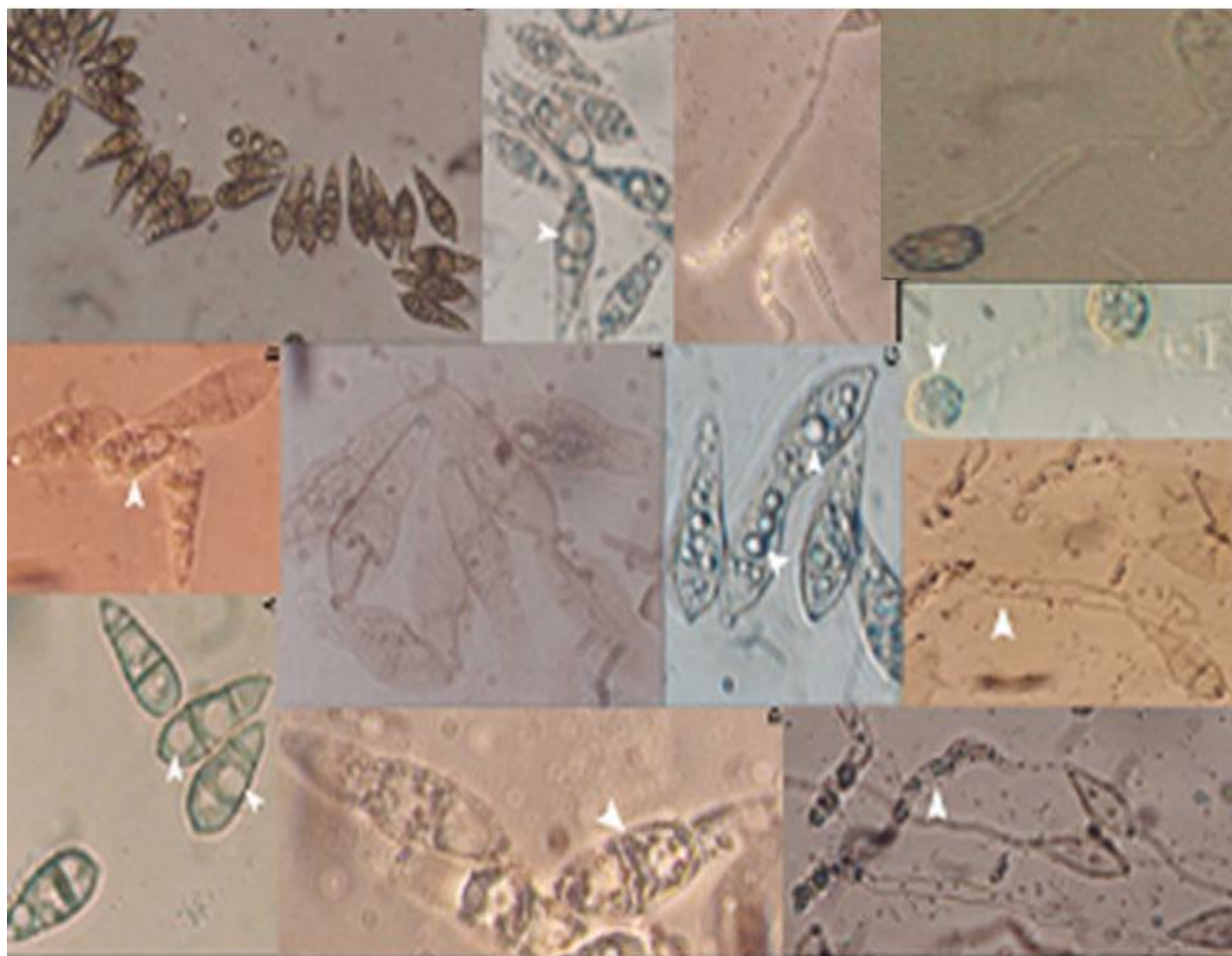


Figure 1: Effect of fungicides on different developmental stages of *M.grisea*. (A) The effect of fungicides were inhibitory to spore germination and cytoplasmic contents were aggregated or granulated. (B, C, D, E) The spores were distorted or shrunken and no germ tube formation, (F, G) The cluster of medium sized vacuole intermingled with small lipid droplets, (H, I, J) Distortion in germ tube elongation. (K, L) Distortion in appressorium formation and poorly melanized. The abnormalities are indicated by arrows.

The spores were able to germinate but the germ tube was either very long or distorted at 0.01 to 0.001%. Abnormal or distorted appressoria were observed or if produced were poorly melanized (Fig.1 E- L).

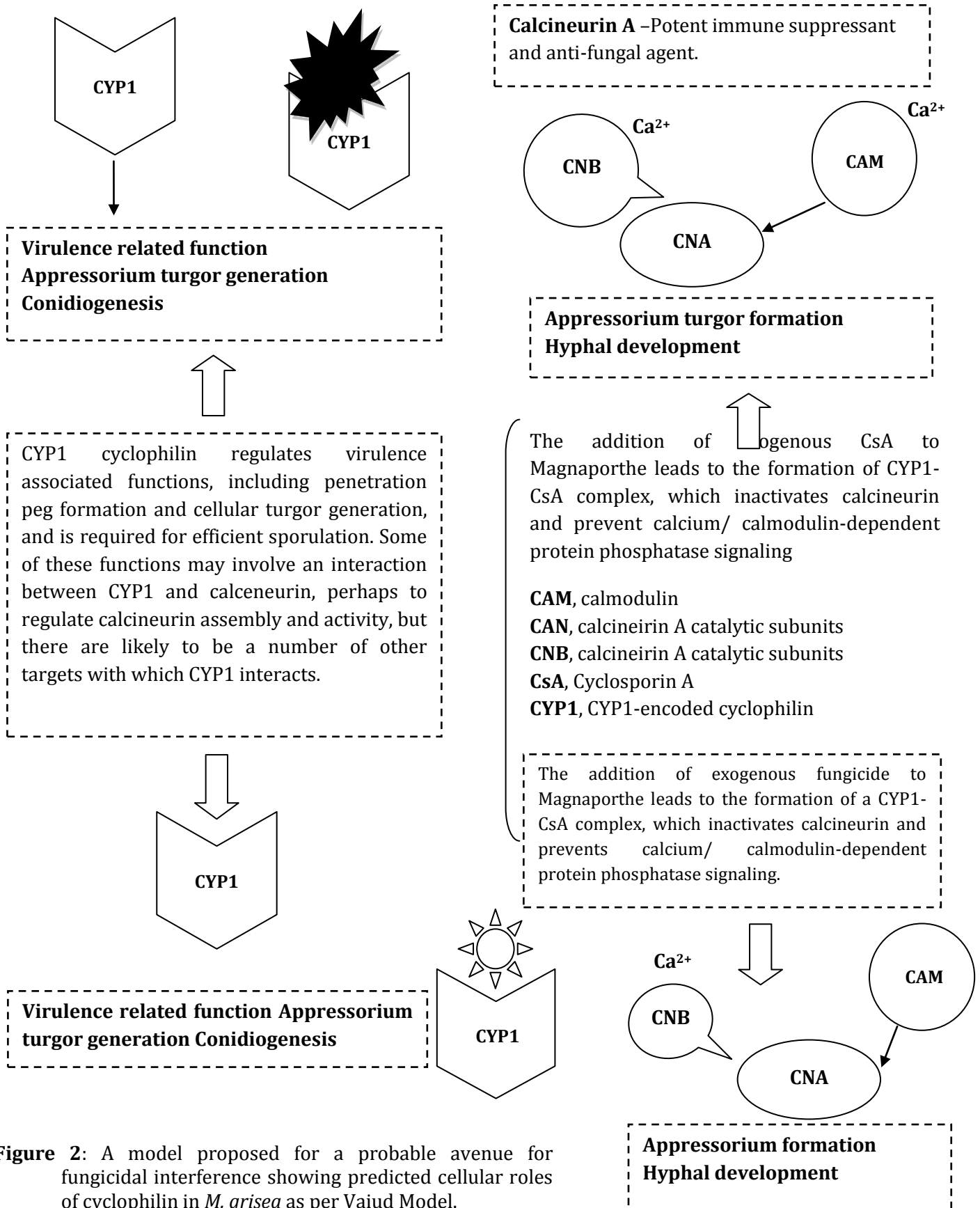
With the Tridemorph (N-tridecyl-2, 6-dimethyl-morpholine), the cytoplasmic contents were granulated and a cluster of medium sized vacuole intermingled with small lipid droplets were observed. The spores were able to germinate, melanization of the elongated germ tube tip was observed, in many cases the shape of the germ tube was distorted, the cytoplasmic contents which were transferred to the germ tube appeared to be fragmented or granulated at 0.1 to 0.001%. Appressoria if produced were either distorted/abnormal in shape or were smaller than the wild type and were poorly melanized (Table 2, Fig.1 G, J-L).

A number of signals have been implicated as triggers for induction of appressorium development in *M. grisea*, including cutin monomers (Gilbert *et al.*, 1996), starvation stress (Jelitto *et al.*, 1994), surface hardness (Xiao *et al.*, 1994) and hydrophobicity of the leaf cuticle (Lee and Dean., 1994). The successful use of anti-penetrant fungicides in blast control provides a useful model for developing future control strategies (Sisler H. D., 1986). The anti-penetrant fungicides are not toxic to the fungus but inhibit melanin synthesis which subsequently prevents penetration of the host by the fungus (Howard and Ferrari, 1989). It has been reported that melanin biosynthesis was blocked by treating with the anti-penetrant tricyclazole and found that appressorium lacked the melanin layer and failed to infect the host plant (Woloshuk *et al.*, 1983). They revealed that this was due to lack of rigidity of the unmelanized appressorial wall. Choi *et al.*, (1998) showed that polyamines, including Putrescine, Spermidine and Spermine, specifically inhibit appressorium formation in *M. grisea* but exogenous addition of cAMP restored appressorium formation inhibited by polyamines. Histo-chemical studies have shown that there is a close correlation between the lignification reaction and inhibition of pathogen development (Thieron *et al.*, 1995). Blockage of lignin biosynthesis in resistant plants leads to uncontrolled spread of the pathogen (Thieron *et al.*, 1995). Carpropamid protects rice plants from the seedling stage upto the panicle formation from damage by rice blast (Kurahashi *et al.*, 1997).

Based on the high resolution light microscopic/ phase

contrast microscopic observations we therefore speculated that the fungicides probably affects the lipid concentration (glycogen and lipid reserves are localized in developing appressoria under the control of the cAMP response pathway, leading to turgor generation and plant infection) in the cells of the spore and these observations were similar to those observed by Vaiud *et al.* (2002). CYP1, which encodes a cyclophilin are the additional group of Magnaporthe genes which were highly expressed during plant infection (Talbot *et al.*, 1993) and were characterized by Vaiud *et al.* 2002.

CsA is also known to be a potent antifungal agent and has fungicidal activity against a wide range of species. Impairment of calcineurin function has been shown to cause defects in the apical growth of hyphae along with gross morphological changes in *N. crassa* (Odom *et al.*, 1997; Prokisch *et al.*, 1997; Wang *et al.*, 2001). It is therefore speculated that calcineurin also is the target of different fungicides used in the present investigation on the spores of *Magnaporthe* and impairment of calcineurin function resulted to cause defects in the gross morphological changes and viability of the spore/ conidia. As per the model proposed by Viaud *et al.*, 2002, CYP1 cyclophilin regulates virulence-associated functions, including penetration peg formation and cellular turgor generation, and is also required for efficient sporulation. Some of these functions may involve an interaction between CYP1 and calcineurin, perhaps to regulate calcineurin assembly and activity, but there are likely to be a number of other targets with which CYP1 interacts. The addition of exogenous CsA to *Magnaporthe* leads to the formation of a CYP1-CsA complex, which inactivates calcineurin and prevents calcium/calmodulin-dependent protein phosphatase signaling. Acute sensitivity to CsA indicates that calcineurin is required for appressorium morphogenesis in addition to regulating hyphal growth and development. We equate the activity of CsA which is a potent immunosuppressant and also is a potential antifungal agent (as per several reports mentioned above) to that with the fungicides used in the present investigation and speculate that the exposure of the *Magnaporthe* spore to the fungicide leads to the formation of a CYP1-fungicide complex, which inactivates calcineurin and prevents calcium/calmodulin-dependent protein phosphatase signaling and is therefore one of the target of fungicidal interference (Fig. 2).



MPG1 encodes a hydrophobin, a new class of hydrophobic proteins that have been described in fungi, which are involved in conferring the hydrophobic character to various fungal structures and are also involved in various developmental processes including pathogenesis (Kershaw and Talbot 1998; Ebbole D. J., 1997; Wessels J. G., 1997, Talbot *et al.*, 1993). Looking to the importance of MPG1, its encoded product the rodlet layers, we speculate that the fungicides seem to have its effects on MPG1, its encoded hydrophobin product or the rodlet layers that lead to a defect in recognizing hydrophobic surfaces thus impairing the ability to make appressorium. The resulting phenotype after the exposure to fungicides is adequately diverse and may have a number of other targets and the possibility for which cannot be overruled.

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