Effects of Antifungal Compounds on Conidial Germination and on the Induction of Appressorium Formation of *Magnaporthe grisea*[§]

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Appressorium formation in germinating conidia of *Magnaporthe grisea* was induced on a hydrophilic (noninductive) surface by antifungal compounds. Respiratory inhibitors or uncoupling agents such as strobilurins, antimycin A, myxothiazol, rotenone, pterulone A, and oligomycin A were particularly effective whereas sodium cyanide had no effect. Cyclosporin A was effective only at high concentrations. These differentiation-inducing effects were only observed at subfungicidal concentrations at which more than 50% of the germinating conidia formed appressoria. Cycloheximide, nystatin, amphotericin B, and papulacandin A did not induce appressoria. Different strains of *M. grisea* displayed the same overall response to the inhibitors, varying merely in the percentage of appressoria formed. A combination of the respiratory inhibitors with 2-phenyl-4H-1-benzopyran-4-one (flavone), diphenyleneiodonium (DPI), or salicylhydroxamic acid (SHAM), compounds which interfere with the cyanide-resistant respiration, resulted in a higher sensitivity of the strains towards the respiratory inhibitors, but had no effect on appressorium formation.

Introduction

The formation of melanized appressoria is a prerequisite for the invasion of host plants by many plant pathogenic fungi such as *Magnaporthe grisea* (Herbert) Barr (anamorph *Pyricularia oryzae* Cavara) or *Colletotrichum* species (Kubo and Furusawa, 1991). In *M. grisea*, the causal agent of rice blast disease, the pre-penetration phase of differentiation from the germinating spores to the appressoria has been subject of many investigations (Talbot *et al.*, 1993; Jelitto *et al.*, 1994; Dean *et al.*, 1996; for review see Dean, 1997).

Differential gene expression has been found to be regulated by a range of signals including contact with a solid surface, the hydrophobicity of the surface, and the presence of plant waxes or cutin monomers (Uchiyama and Okuyama, 1990; Lee and Dean, 1994; Gilbert *et al.*, 1996). Among second messengers, cAMP, 1,2-dioctanoylglycerol, and N-linoleoylphytosphingosine (ceramide IIIa) were reported to exhibit appressorium-inducing activity and at least two signal transduction pathways appear to be involved (Lee and Dean, 1993; Thines *et al.*, 1997a).

During our search for new selective fungicides for the control of rice blast disease, different test systems measuring appressorium formation on inductive and noninductive surfaces were used. This led to the discovery of glisoprenins and several monounsaturated fatty acids as the first inhibitors of the signal transduction pathway activated by the hydrophobicity of the surface (Thines *et al.*, 1997b, 1998; Sterner *et al.*, 1998; Eilbert *et al.*, 1999). In addition, some of these fatty acids inhibit the appressorium formation stimulated by cutin monomers in a competitive manner (Eilbert *et al.*, 1999). In the course of these investigations, culture extracts from several basidiomycetes were found to induce appressorium formation on a noninductive surface. Isolation of the active principle revealed that strobilurins and oudemansins were responsible for this phenomenon. Strobilurins and oudemansins are highly selective antifungal compounds which inhibit respiration (for review see Anke,

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1995, 1997). In order to examine whether the appressorium-inducing activity is connected with the target of the strobilurins, several antifungal compounds with known modes of action were tested for their ability to induce appressorium formation. In the first part of this paper we report that all compounds interfering with eukaryotic respiration, except sodium cyanide, are capable of inducing appressorium formation.

It is known that respiratory inhibitors induce a cyanide-resistant respiration which branches from the cytochrome pathway at ubiquinone and terminates with an alternative oxidase (AOX) that directly reduces oxygen (Minagawa *et al.*, 1992). The signal mediators for transcriptional activation of fungal *AOX* gene by respiratory inhibitors are active oxygen species (Yukioka *et al.*, 1998). Therefore H_2O_2 was tested to elucidate whether it is also involved in appressorium induction.

Flavonoids can block the induction of cyanide-resistant respiration as scavengers of oxygen radicals (Minagawa *et al.*, 1992; Mizutani *et al.*, 1996). Diphenyleneiodonium (DPI) is known as a potent inhibitor of mammalian NADPH oxidase, an enzyme that generates H_2O_2 (O'Donnell *et al.*, 1993). It also inhibits the H_2O_2 production in plant cells as response to pathogen attack (Dwyer *et al.*, 1996). The alternative oxidase itself can be blocked by salicylhydroxamic acid (SHAM) (Moore and Siedow, 1991). In the second part of this paper we report the effects on conidial germination caused by the combination of the respiratory inhibitors with compounds interfering with the induction of the cyanide-resistant respiration or the alternative oxidase.

Materials and Methods

Microorganisms

The strains P1, P3 and P5 of *Magnaporthe grisea* used in this study were obtained from Dr. B. Speakman (BASF AG). They were freshly isolated from infected rice plants. The strains were grown on potato-dextrose agar (Difco) at 23 ± 1 °C. Sporulation was induced by fluorescent light. Conidia were harvested from 10- to 14-day-old cultures and suspended in sterile deionized water. The suspension was filtered through glass wool to remove mycelial debris. Conidia obtained by centrifugation at 2000×*g* for 10 minutes were washed and resuspended in sterile water to give a concentration of 1.25×10^5/ml.

Appressorium formation and germination assays

The development of appressoria in germinating conidia was monitored on a hydrophilic (noninductive) surface in a 24-well microtitre plate as reported before (Thines *et al.*, 1997b, 1998). 40 µl of conidial suspension were placed in each well. After 1 h, sterile water (0.96 ml) was added. Appressorium formation was induced by adding 200 ng of 1,16-hexadecanediol (Aldrich, Steinbronn) or 20 µg of cyclic 8-(4-chlorophenylthio)-adenosine-monophosphate (Roche Diagnostics, Mannheim) per well. Antifungal compounds were dissolved in methanol or ethanol, keeping the final solvent concentration in the assay below 1%. At this level, no effects of the solvent on germination, mycelial growth or appressorium development were observed. The plates were incubated at 24 °C. Appressorium formation was evaluated in all assays after 20 h by light microscopy. Experiments were carried out in triplicates, in each of which three times 100 germinated conidia were counted. The concentrations of test substances were increased until the germination of conidia was inhibited by 90% or until a concentration of 50 µg/ml had been reached. In Fig. 1 and Table II and III, the highest concentrations are not shown.

Effects on spore germination were measured on the hydrophilic surface in a 24 well microtitre plate in the absence of inducing compounds after 12 hours incubation.

Chemicals

Myxothiazol was kindly provided by Dr. G. Höfle, Braunschweig, strobilurin A, and pterulone A by Dr. T. Anke, Kaiserslautern, papulacandin A by Dr. H. Peter, Novartis, Basel, and nikkomycin Z by Dr. H. P. Fiedler, Tübingen. Nystatin was purchased from Serva, Heidelberg, and all other compounds were obtained from Sigma, St. Louis, USA.

Results and Discussion

Strobilurin A was tested for appressorium-inducing activity on a hydrophilic surface with three

Table I. Inhibition of spore germination of *Magnaporthe grisea* strains P1 and P3 by the compounds tested for appressoria inducing activity (SGA_{90}: Concentration at which 90% of the spores did not germinate).

Compound	Site of action	SGA_{90} [µg/ml] *M. grisea* P1	P3	Reference
Inhibition of eukaryotic respiration				
Pterulone A	complex I	5	10	Engler *et al.*, 1997
Rotenone	complex I	>20	>20	Schewe and Lyr, 1995
Antimycin A	complex III	2	2	Schewe and Lyr, 1995
Myxothiazol	complex III	5	10	Gerth *et al.*, 1980
Strobilurin A	complex III	20	>20	Anke, 1995, 1997
Oligomycin A	ATP synthase	1	1	Kobayashi *et al.*, 1987
NaCN	complex IV	>50	>50	Moore and Siedow, 1991
Inhibitors of fungal cell wall or membrane synthesis				
Miconazole	ergosterol biosynthesis	10	10	vanden Bossche *et al.*, 1978
Nikkomycin Z	chitinsynthase	>50	>50	Fiedler *et al.*, 1982
Papulacandin A	glucansynthase	>50	>50	Traxler *et al.*, 1977
Membrane permeability				
Amphotericin B	increase of permeability	1	1	vanden Bossche, 1995
Nystatin	increase of permeability	5	2	vanden Bossche, 1995
Polymyxin B	increase of permeability (bacteria)	>50	50	Matsunga *et al.*, 1991
Miscellaneous				
Cycloheximide	protein synthesis	0.2	0.5	Pestka, 1971
Cyclosporin A	immunosuppressant	>50	>50	Schreier, 1997
Omphalotin A	nematicide	>50	>50	Sterner, 1997

Table II. Induction of appressorium formation in *Magnaporthe grisea* strains P1 and P3 on a noninductive surface by inhibitors of the eukaryotic respiration and by miconazole, cycloheximide and cyclosporin A (A: appressorium formation [%]; B: not germinated spores [%]; n.t.: not tested). The percentage of appressorium formation refers to the number of germinated conidia.

Compound	Concentr. [µg/ml]	Strain P1 A	Strain P1 B	Strain P3 A	Strain P3 B
none		1.6 ± 1.2	1.6 ± 1.3	55.4 ± 5.0	0.8 ± 0.7
Pterulone A	0.5	9.5 ± 1.2	14.5 ± 1.7	72.9 ± 3.4	1.4 ± 1.2
	1	60.1 ± 1.7	64.6 ± 4.0	83.5 ± 2.1	5.0 ± 3.1
	2	62.1 ± 4.5	65.2 ± 4.8	85.2 ± 4.0	10.0 ± 2.1
Rotenone	0.5	3.3 ± 1.6	4.4 ± 2.4	62.5 ± 6.8	1.9 ± 1.9
	5	29.4 ± 2.6	28.3 ± 2.5	90.3 ± 1.9	1.5 ± 0.9
	10	69.1 ± 3.2	66.3 ± 5.5	90.3 ± 1.5	1.1 ± 1.0
Myxothiazol	0.1	6.3 ± 1.2	7.7 ± 1.1	82.3 ± 5.2	1.2 ± 0.8
	0.5	18.5 ± 3.1	19.1 ± 2.5	90.0 ± 3.1	3.3 ± 1.3
	2	69.7 ± 3.9	34.0 ± 2.8	90.4 ± 2.4	8.0 ± 2.2
Oligomycin A	0.05	2.0 ± 1.3	1.7 ± 0.7	67.3 ± 7.7	6.5 ± 2.3
	0.2	6.2 ± 1.6	7.6 ± 2.6	82.3 ± 2.7	4.9 ± 2.8
	0.5	87.5 ± 4.4	65.6 ± 5.0	88.6 ± 5.8	12.7 ± 3.1
Miconazole	0.2	n. t.	n. t.	80.5 ± 3.0	2.3 ± 1.4
	0.5	6.9 ± 3.5	1.2 ± 0.9	88.4 ± 4.3	10.5 ± 1.9
	2	25.6 ± 2.4	1.6 ± 1.1	86.5 ± 4.4	12.0 ± 3.4
Cycloheximide	0.01	3.3 ± 1.5	9.9 ± 3.4	58.9 ± 2.4	1.6 ± 1.4
	0.05	5.0 ± 1.7	31.8 ± 4.0	69.5 ± 3.0	2.3 ± 1.0
	0.1	15.3 ± 1.5	66.0 ± 5.0	60.5 ± 3.3	12.7 ± 1.6
Cyclosporin A	5	14.6 ± 6.4	1.4 ± 0.8	69.7 ± 5.3	2.0 ± 1.8
	20	45.2 ± 9.0	3.8 ± 2.8	83.1 ± 2.3	12.9 ± 2.6
	50	60.4 ± 3.7	5.2 ± 2.0	96.4 ± 3.6	20.3 ± 4.9

strains of *Magnaporthe grisea* (P1, P3 and P5). The strains differed in their sensitivity and their behavior on the hydrophilic surface. In the absence of inducing agents, less than 5% of the germinated conidia of strains P1 and P5 formed appressoria, whereas P3 was partly induced and 50–60% of the germinated conidia formed appressoria. In the presence of 100 ng/ml of 1,16-hexadecanediol or 20 µg/ml of chlorophenylthio-cAMP, all three strains were fully induced and more than 94% of the conidia formed appressoria. At 10 µg/ml of strobilurin A the conidial germination of strains P1 and P5 was inhibited by more than 50%. Strain P3 was less sensitive, the conidial germination was only reduced by 7%. Whether this is due to different expression of ABC transporters or only to different sensitivities of the strains to the antifungal agents, remains to be investigated. ABC transporters are ATP-driven efflux pumps for cytotoxic compounds belonging to the ATP-binding cassette (ABC) superfamily of membrane transporters. Recently, Urban and colleagues reported that an ATP-driven efflux pump is essential for pathogenicity in *M. grisea* (Urban *et al.*, 1999). For further investigations strains P1 and P3 were chosen, because they showed the greatest differences.

The antifungal agents listed in Table I were selected according to their mode of action. Firstly, they were tested for their effects on spore germination. Several of the compounds, e.g. nikkomycin Z and papulacandin A, had no effect on the germination even though they are selective antifungal agents. Sodium cyanide at 50 µg/ml also had no effect on spore germination. All respiratory inhibitors except sodium cyanide induced appressorium

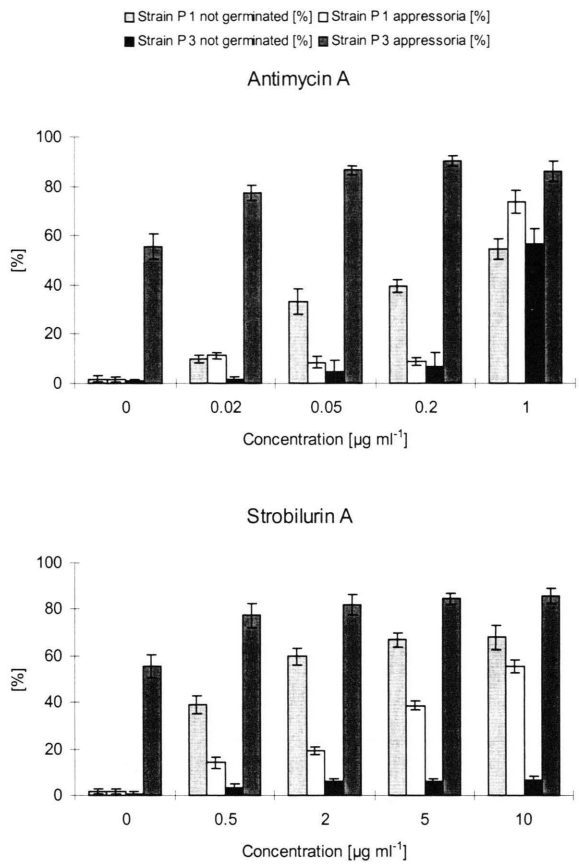

Fig. 1. Effect of antimycin A and strobilurin A on spore germination and appressorium formation in *Magnaporthe grisea* strains P1 and P3 on a noninductive surface. The percentage of appressorium formation refers to the number of germinated conidia.

Table III. Inhibition of spore germination [%] in *Magnaporthe grisea* strain P3 on noninductive surface by respiratory inhibitors in combination with compounds interfering with the induction of cyanide-resistant respiration or the alternative oxidase.

Respiratory inhibitor	none	DPI 10 µM	Flavone 50 µM	SHAM 200 µM
none	0.8 ± 0.7	0.9 ± 0.5	1.2 ± 0.8	1.1 ± 0.7
Pterulone A [1 µg/ml]	5.0 ± 3.1	89.3 ± 8.4	100	100
Rotenone [5 µg/ml]	1.5 ± 0.9	87.2 ± 7.5	100	91.0 ± 5.6
Myxothiazol [2 µg/ml]	8.0 ± 2.2	100	100	100
Antimycin A [0.5 µg/ml]	4.8 ± 4.3	100	100	100
Strobilurin A [5 µg/ml]	6.2 ± 0.8	100	100	100

DPI: diphenyleneiodonium.
Flavone: 2-phenyl-4H-1-benzopyran-4-one.
SHAM: salicylhydroxamic acid.

formation as shown in Fig. 1 and Table II. Generally spore germination in strain P3 was less sensitive to respiration inhibitors as compared to strain P1. Among the other compounds tested only cyclosporin A showed significant activity, whereas the cyclic peptide omphalotin A was ineffective (data not shown). Cyclosporin A had no effect on conidial germination of *M. grisea* strains P1 and P3. This is in agreement with reports that even in sensitive fungi, germination was not affected by cyclosporin A (Dreyfuss *et al.*, 1976). In the presence of nikkomycin Z, papulacandin A, amphotericin B, nystatin, polymyxin B and cycloheximide, less than 15% of the germinating spores of strain P1 formed infection structures. In strain P1 the inducing activity of miconazole was weak, whereas in strain P3 miconazole induced appressorium formation to a similar degree as did respiration inhibitors (Table II). It would be interesting to test additional compounds of this type.

Generally, the effects on appressorium formation in strains P1 and P3 were similar. In strain P1, the percentage of appressorium formation increased from 1.6 to values between 55 and 90, and in strain P3 from 54 to more than 90 in a dose-dependent manner (Fig. 1 and Table II).

When H_2O_2 was tested, no induction of appressorium formation was observed up to a toxic concentration of 400 μM.

A combination of compounds interfering with the induction of cyanide-resistant respiration with respiratory inhibitors resulted in a higher sensitivity of spore germination of both *M. grisea* strains. The results are shown for strain P3 which was less strongly affected by the respiratory inhibitors. As shown in Table III, DPI, flavone and SHAM had no effect on spore germination, but all three greatly enhanced the effects of the respiratory inhibitors. Thus the enhancing effect is independant of the site of action of the compound within the cyanide-resistant respiration. The effect of DPI suggests that in fungi, similar to mammalian systems and plants, a NADPH oxidase is involved in generating H_2O_2.

In our experiments with a range of different antifungal compounds, respiration inhibitors showed the best induction of appressorium formation. Germinating spores develop a vigorous metabolism and have a high energy consumption. This energy is generated by aerobic respiration (Stade and Brambl, 1981). Therefore, inhibition of respiration leads to energy stress that in turn might be an additional signal leading to differentiation such as appressorium formation. The formation of penetration structures on the host leaf surface might be the last chance for the fungus to reach a new energy source. Whether the signal is transferred via the cAMP dependent pathway or the pathway induced by 1,2-dioctanoylglycerol (via protein kinase C) is still under investigation.

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