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ANTIFUNGAL ACTIVITY OF THE NATURAL PRODUCT AMBRUTICIN VS4 AGAINST *ALTERNARIA BRASSICICOLA*

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Abstract

Increased concern for health and environmental hazards associated with the use of fungicides has resulted in the need for greater sustainability in agriculture. Naturally occuring molecules represent an important source of antifungal agents that may be used for the synthesis of new compounds.

Myxobacteria are known as excellent and prolific producers of a variety of bioactive secondary metabolites including antibacterial and antifungal compounds. The ambruticin, a natural poliketide originating from the myxobacterium Sorangium cellulosum constitute attractive leads for antifungal drug development.

We have analyzed the effect of ambruticin on different Alternaria brassicicola isolates, an economically important seed-borne fungal pathogen of Brassicaceae species. Several isolates, sensitive and highly resistant to dicarboximides (iprodione), were tested for their susceptibility to ambruticin in vitro.

In vitro assays investigated the responses of Alternaria brassicicola isolates towards ambruticin VS4 by evaluating the potential toxic effects on mycelial growth. Our results show that the polyketide drug ambruticin VS4 exerted antifungal activity against Alternaria brassicicola, mycelial growth being strongly affected.

INTRODUCTION

Naturally occurring molecules represent an important source of antifungal agents that may be used as starting points for the synthesis of new compounds [4]. The ambruticins, another class of natural antifungal polyketides originating from the myxobacterium *Sorangium cellulosum* [4], were reported to have no significant adverse effect on animals [2, 8] and therefore constitute attractive leads for antifungal drug development.

Here we have analyzed the effect of ambruticin VS4, one of the N-methylated forms of ambruticin, on the growth of different genotypes of *Alternaria brassicicola*, an economically important seed-borne fungal pathogen of *Brassicaceae* species. Parallel experiments were also conducted with the model fungus *Neurospora crassa*.

MATERIALS AND METHODS

Fungal isolates. All *A. brassicicola* strains used in this study and listed in table 1 have previously been described [1, 6]. All the strains were purified by monospore isolation and maintained on malt agar medium at 4^0C. All *N. crassa* strains were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City) and grown on agar-solidified Vogel's medium N at 25°C .

Table 1

List of *Alternaria brassicicola* and *Neurospora crassa* strains used in experiments

Strain	Phenotype[a]	Genotype	Source or reference
A. brassicicola			
43	DCF^S	WT	[1, 6]
12 RO	DCF^S	WT	
CM	DCF^S	WT	
40	DCF^R	Ab*NIK1*^{753K}	
43M	DCF^R	Ab*NIK1*^{Q343NS}	
7407	DCF^R	Ab*NIK1*^{W634NS}	
41	DCF^R	Ab*NIK1*$^{\Delta CA}$	
NikΔ3	DCF^R	Ab*NIK1::hph*	
N. crassa			
FGSC 988	DCF^S	WT	Fungal Genetics Stock Center
FGSC 824	DCF^{LR}	*Os1*$^{Q388S-A578V}$	
FGSC 2432	DCF^R	*Os1*$^{G580R-L582M}$	
FGSC 4494	DCF^R	*Os1*^{Q308NS}	
FGSC 4576	DCF^R	*Os5*^{K307FS}	

a. DC, dicarboximides; R, resistant; S, susceptible; LR, low resistance.

Fungicides. The effect of fungicides and ambruticin on mycelial growth and spore germination were tested as described [6]. Antifungal activities of the phenylpyrrole fludioxonil (Syngenta, Agro SAS, Switzerland) and the polyketide ambruticin (Kosan Biosciences, California) on *A. brassicicola* isolates was tested *in vitro*. Mycelium from wild type (WT) isolates and Ab*NIK1* mutants of *A. brassicicola* was exposed to either fludioxonil or ambruticin at various concentrations (0.01 to 100 mg/liter). For each condition, the reduction in radial growth was expressed as a percentage relative to the control (no fungicide or dimethyl sulfoxide). The solvent concentration in both controls and assays never exceeded 1% (v/v). Growth of *A. brassicicola* strains was scored after 4 days of incubation at 25°C. The growth of *N. crassa* isolates was monitored after 24 h of incubation at 25°C on agar-solidified Vogel's medium supplemented with fludioxonil, ambruticin or not supplemented - control (Co).

Results are expressed as the percentage of inhibition in treated samples compared to the control (values are the means of three replicates) and as effective concentration EC50 (the concentration which reduced mycelial growth by 50%).

RESULTS AND DISCUSSIONS

In vitro assays investigated the responses of *Alternaria brassicicola* isolates towards ambruticin VS4 by evaluating the potential toxic effects on mycelial growth and. This growth parameter was strongly affected by ambruticin, with almost complete inhibition at 0.1 mg/liter for all wild-type (WT) strains tested in this study (table 2, figure 1).

Irrespective of the growth parameter studied, the toxicity of fludioxonil to *A. brassicicola* was always lower than that of ambruticin. When conidia from WT strains were germinated in the presence of ambruticin, only short germ tubes with a tendency to swell were observed (data not shown), similar to what was previously observed with DCF^S [1].

For most of the strains, i.e., the Ab*NIK1*-null mutants and the Abra40 substitution mutant, the mycelial growth was not or only slightly affected in the presence of high concentrations (up to 10 mg/ liter) of ambruticin or fludioxonil as compared to control conditions.

We showed here that previously characterized *A. brassicicola* Ab*NIK1*-null mutants expressing high resistance to the dicarboximide iprodione [6] were also highly resistant to the phenylpyrrole fludioxonil as well as to ambruticin. Such cross-resistance was also observed for the *N. crassa os1*- null mutant FGSC4494 (table 3, figure 2).

Table 2

Effects of ambruticin and fludioxonil on *A. brassicicola* isolates

Strain	Phenotype[a]	Genotype	EC50 [mg/l]	
			Ambruticin	Fludioxonil
43	DCF^S	WT	0.006	0.44
12 Ro	DCF^S	WT	<0.01	0.51
CM	DCF^S	WT	<0.01	0.47
40	DCF^R	Ab*NIK1*^{753K}	<0.01	>100
43M	DCF^R	Ab*NIK1*^{Q343NS}	>10	>100
nikΔ3	DCF^R	Ab*NIK1:hph*	>1	>100
7407	DCF^R	Ab*NIK1*^{W634NS}	2.96	>100
41	DCF^R	Ab*NIK1*$^{\Delta CA}$	2.87	>100

Table 3

Effect of ambruticin on *N. crassa* isolates

Strain	Phenotype[a]	Genotype	Fungal growth inhibition [%]			
			Fludioxonil	Ambruticin VS4		
			25 (mg/l)	0.1 (mg/l)	1 (mg/l)	10 (mg/l)
FGSC 988	DCF^{S}	WT	100	88	100	100
FGSC 824	DCF^{LR}	$Os1^{Q388S\text{-}A578V}$	100	72.38	85	100
FGSC 2432	DCF^{R}	$Os1^{G580R\text{-}L582M}$	0	0	0	0
FGSC 4494	DCF^{R}	$Os1^{Q308NS}$	9.52	0	40	88.1
FGSC 4576	DCF^{R}	$Os5^{K307FS}$	0	0	0	0
FGSC 988	DCF^{S}	WT	0	0	0	0

Recently, it has been demonstrated that, like phenylpyrroles, ambruticin interferes with osmoregulation in filamentous fungi, targets group III HK phosphorelay signaling systems in these two filamentous fungi and, at least in *N. crassa*, exhibits fungicidal activity through improper activation of the HOG-related pathway [3].

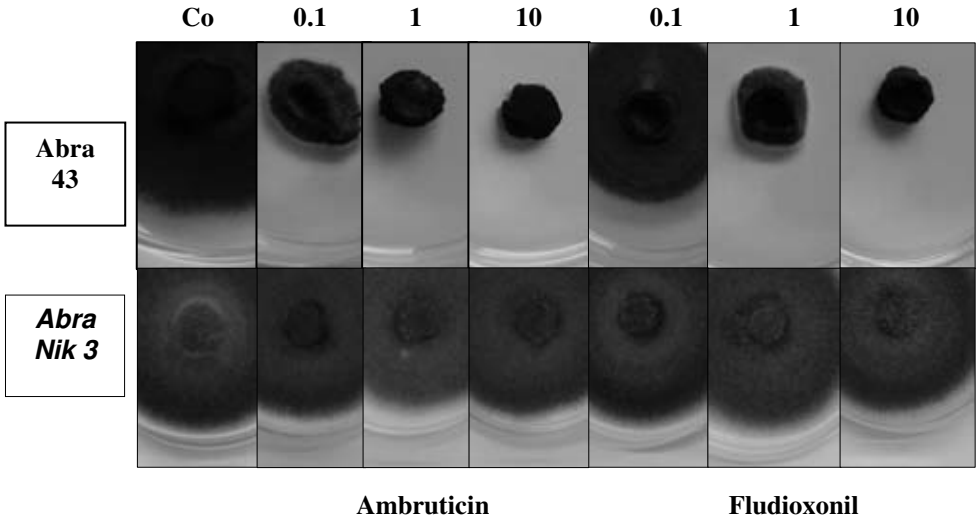

Fig. 1. Effects of ambruticin and fludioxonil [mg/l] on the mycelium radial growth of *A. brassicicola* isolates

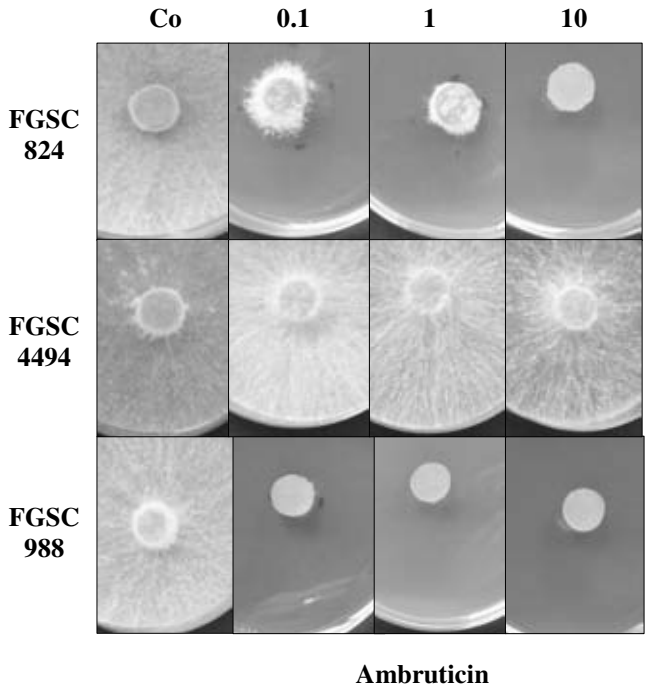

Fig. 2. Effects of ambruticin and fludioxonil [mg/l] on the mycelium radial growth of *N. crassa* isolates

Naturally occurring molecules represent an important source of antifungal agents that may be used as starting points for the synthesis of new compounds. Phenylpyrrole fungicides (fludioxonil) are derived from the natural bacterial antibiotic pyrrolnitrin produced by *Pseudomonas pyrrocinia*. The ambruticins is another class of natural antifungal polyketides originating from the myxobacterium *Sorangium cellulosum*. They were reported to be active against a variety of pathogenic fungi, including *Histoplasma capsulatum, Coccidioides immitis*, and *Blastomyces dermatitides*, as well as the dermatophytic filamentous fungi. Moreover, they are active against *Aspergillus* species that have a high incidence in chronic respiratory infections in humans.

Ambruticins have also been tested successfully *in vitro* against at least one important crop pathogen, i.e., *Botrytis cinerea* [7].

CONCLUSIONS

1. In this study, we demonstrated that the polyketide drug ambruticin exerted antifungal activity against *Alternaria brassicicola*, an economically important seed-borne fungal pathogen of *Brassicaceae* species.

2. The high toxicity of this bacterial metabolite for this fungus was well illustrated by the very low concentrations that were found to significantly inhibit *in vitro* the mycelial growth of several WT isolates.
3. *A. brassicicola* Ab*NIK1*-null mutants expressing high resistance to the DCF iprodione were also highly resistant to the phenylpyrrole fludioxonil as well as to ambruticin.

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