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CHARACTERIZATION OF STRAINS
OF *BOTRYTIS CINEREA* RESISTANT
TO ANILINOPYRIMIDINE FUNGICIDES

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INTRODUCTION

Pyrimethanil is a new fungicide available for the control of *Botrytis cinerea* in French vineyards. It belongs to the anilinopyrimidine family which also includes cyprodinil and mepanipyrim. These fungicides are able to affect the biosynthesis of some aminoacids (especially methionine) and to inhibit the secretion of enzymes such as pectinases, cellulases, proteinases or laccases. Even their primary target site and the relationship between these two biochemical effects remain unknown, it appears that these anilinopyrimidines have a novel mode of action (Leroux, 1995).

To evaluate the resistance risk to these new botryticides, since 1993, a monitoring was conducted in French vineyards. Additionally we tried to select laboratory resistant strains by exposing mycelium to fungicide amended plates. In both types of experiments, resistant strains were detected and some of their characteristics will be presented here.

MATERIALS AND METHODS

Fungal strains

Most strains of *Botrytis cinerea* were isolated in 1994 from diseased berries collected at the harvest in various French vineyards treated or not with pyrimethanil. Their sensitivity or resistance towards dicarboximides (Sd/Rd), benzimidazoles (Sb/Rb) and phenylcarbamates (Sp/Rp) was tested by conventional methods.

Mycelial growth assay

A synthetic medium containing 10 g glucose, 2 g KH₂PO₄, 1.5g K₂HPO₄, 1 g (NH₄)₂SO₄, 0.5 Mg SO₄.7H₂O, and 12.5 g agar per litre was used to evaluate the fungitoxicity of technical cyprodinil, pyrimethanil and also fludioxinil (a member of the phenylpyrrole group) towards mycelial growth. From the colony diameters measured after an incubation time of 3-5 days (18°C, dark), the EC 50 values were determined.

In vitro spore tests

The effects of fungicides towards conidia of *B. cinerea*, inoculated onto agar medium were evaluated in two manners :

- Lengths of germ tubes after 24 h (under the microscope) ---> EC 50 values.
- Hyphal growth after 4-5 days (macroscopic observation) ---> MIC values.

In vivo assay

Slices of cucumber fruits were dipped (2 min) into water solution of formulated pyrimethanil (Scala) or fludioxinil (Geoxe). After drying, they were inoculated by a spore suspension and then placed in a humid chamber. After 7 days (20°C, daylight), the extension of *B. cinerea* was estimated using a scale from 0 (absence of fungus) to 5 (all the surface invaded).

RESULTS OF IN VITRO ASSAYS

According to the *in vitro* effects of cyprodinil and pyrimethanil towards conidia of *Botrytis cinerea*, the strains from French vineyards could be classified into 3 groups (Table 1) :

□ **Sa** [L, 4.240, 4.300] : strains susceptible to anilinopyrimidines and also to fludioxinil.

□ **Ra1** [3.103, 4.33, 4.128] : strains weakly resistant to anilinopyrimidines (RL or resistance levels : 5 to 10) and also to fludioxinil (RL : 10 to 20) ; they were sensitive or resistant to dicarboximides.

□ **Ra2** [4.271 A and B] : strains highly resistant to anilinopyrimidines [RL : 30 to 70] but sensitive to fludioxinil.

When the tests were realized on mycelial growth, **Sa** and **Ra1** strains appeared equally susceptible to cyprodinil, pyrimethanil and fludioxinil, whereas **Ra2** ones were highly resistant to anilinopyrimidines (RL : 50 to 200) (Table 2).

Among the strains adapted to pyrimethanil in the laboratory, some of them exhibited EC 50 values similar to those of **Ra2** strains [128-Py], whereas others were more resistant to anilinopyrimidines [240-Py, 300-Py] (Tables 1 and 2).

Table 1 : *In vitro* effects of fungicides on conidia of *Botrytis cinerea*

Strains	Phenotypes *	EC 50 germ tubes in mg/l			MIC in mg/l		
		Cyprodinil	Pyrimethanil	Fludioxinil	Cyprodinil	Pyrimethanil	Fludioxinil
L 4.240 4.300	Sa Sd,Rb,Sp Rd,Rb,Rp Sd,Sb,Rp	0.03	0.05	0.01	0.05	0.30	0.05
		0.02	0.05	0.03	0.10	0.50	0.10
		0.02	0.05	0.01	0.05	0.30	0.05
3.103 4.33 4.128	Ra1 Rd,Rb,Rp Rd,Rb,Sp Sd,Rb,Sp	0.10	0.20	0.20	0.50	2.0	0.50
		0.30	0.25	0.20	2.0	3.0	2.0
		0.20	0.30	0.15	1.0	2.0	1.0
4.271 A 4.271 B	Ra2 Rd,Sb,Rp Rd,Sb,Rp	1.0	3.5	0.02	>10	>20	0.05
		1.0	3.5	0.02	>10	>20	0.05
128-Py 240-Py 300-Py	Lab Sd,Rb,Sp Rd,Rb,Rp Sd,Sb,Rp	2.0	4.0	0.10	>10	>20	1
		>10	>10	0.02	>10	>20	0.1
		>10	>10	0.02	>10	>20	0.05

* Sd/Rd : sensitive or resistant to dicarboximides
Sb/Rb : sensitive or resistant to benzimidazoles
Sp/Rp : sensitive or resistant to phenylcarbamates (diethofencarb)

Table 2 : *In vitro* effects of fungicides on mycelial growth of *Botrytis cinerea*

Strains	Phenotypes *	EC 50 mycelium in mg/l		
		Cyprodinil	Pyrimethanil	Fludioxonil
L 4.240 4.300	Sa Sd.Rb.Sp Rd.Rb.Rp Sd.Sb.Rp	0.01 0.02 0.01	0.06 0.05 0.05	0.004 0.005 0.005
3.103 4.33 4.128	Ra1 Rd.Rb.Rp Rd.Rb.Sp Sd.Rb.Sp	0.02 0.07 0.02	0.10 0.10 0.05	0.007 0.02 0.008
4.271 A 4.271 B	Ra2 Rd.Sb.Rp Rd.Sb.Rp	1.5 2.0	4.0 3.0	0.004 0.005
128-Py 240-Py 300-Py	Lab Sd.Rb.Sp Rd.Rb.Rp Sd.Sb.Rp	2.0 >10 >10	5.0 >20 >20	- - -

* see foot note Table1

RESULTS OF *IN VIVO* ASSAYS

When inoculated to cucumber fruits, all the strains from French vineyards and only few laboratory mutants were highly pathogenic.

The **Sa** and **Ra1** strains were completely inhibited by 20mg/l pyrimethanil (or 40mg/l for 4.33). The **Ra2** strains and the laboratory mutants required more than 200mg/l pyrimethanil ; at 800mg/l fungal development was only observed with laboratory resistant strains. (Table 3).

The control of most **Sa**, **Ra1**, and **Ra2** strains was achieved with 10mg/l fludioxonil; only 4.33 required 30mg/l. (Table 3).

Table 3 : Development of *Botrytis cinerea* on slices of cucumber fruits

Strains	Type	Intensity of grey mould after one week *											
		Control	Pyrimethanil (mg/l)							Fludioxonil (mg/l)			
			4	16	40	200	400	800	1	3	10	30	
4.240 4.300	S	5 5	5 3	0 0	0 0	- -	- -	- -	5 5	1 2	0 0	0 0	
3.103 4.33 4.128	Ra1	5 5 5	4 4 3	0 3 0	0 0 0	- - -	- - -	- - -	5 5 5	4 4 5	0 1.5 0	0 0 0	
4.271 A 4.271 B	Ra2	5 5	- -	- -	5 5	2 3	1 2	0 0	5 5	1 1.5	0 0	0 0	
240-Py 300-Py	Lab.	5 5	- -	- -	5 5	5 5	4 5	3 4	- -	- -	- -	- -	

* Scale : 0 absence → 5 : more than 75% of the surface covered by grey mould

CONCLUSION - DISCUSSION

From the survey conducted in French vineyards between 1993 and 1995, we were able to distinguish two types of anilinopyrimidines-resistant strains (Leroux and Gredt, 1995) :

□ **Ra1** : detected in 5% of the monitored locations, they exhibited cross resistance *in vitro* to phenylpyrroles (but not to dicarboximides, benzimidazoles or phenylcarbamates). Resistance was not expressed when inoculated to cucumber fruits.

□ **Ra2** : detected once only in 1994 (Bordeaux area), their resistance *in vitro* and *in vivo* was restricted to anilinopyrimidines. These strains are probably similar to those recently described by Forster et al (1996) or Hilber and Schuepp (1996).

In preliminary biochemical studies we have shown that all the tested field strains produced laccase in liquid culture. However the amount of excreted enzyme was extremely variable and did not seem to be related to anilinopyrimidine resistance. Moreover, in the presence of pyrimethanil (0.3 mg/l), the incorporation of 35 Sulfate into methionine was reduced by 70 % in a sensitive strain whereas it was not affected in a resistance strain (Ra2-type). Additional experiments are required to elucidate the mechanism(s) of resistance to anilinopyrimidines in *B. cinerea*.

Under practical aspects, the biological characterization of anilinopyrimidine-resistant strains allowed the development of suitable methods for monitoring anilinopyrimidine-resistance in *B. cinerea* (Leroux and Gredt, 1996).

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