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Metabolism of cymoxanil and analogs in strains of the fungus *Botrytis cinerea* using high-performance liquid chromatography and ion-pair high-performance thin-layer chromatography

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Abstract

The metabolism of cyano-oxime fungicide 1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea (cymoxanil) and analogs was studied on several strains of the fungus *Botrytis cinerea* owing to their difference in sensitivity towards cymoxanil. Chromatographic analysis of the unextracted culture medium was simpler and more accurate, particularly for ionizable metabolites because it avoids problems associated with extraction. Reversed-phase high-performance liquid chromatography was applied to compare the decrease of cymoxanil and analogs caused by different strains of *B. cinerea*, by periodic injections of incubated culture medium aliquots, directly on a C_4 wide-pore column. Furthermore, a thin-layer chromatographic monitoring on C_{18} bonded silica gel with ion-pairing allowed the monitoring of the ionizable metabolites for substrates that were demonstrated to decompose most rapidly. These complementary analyses showed that the sensitivity of the highly sensitive strain towards cymoxanil was related to the disappearance of cyano-oximes studied from culture medium, namely to the ability of the strain *B. cinerea* to metabolize them.

Keywords: *Botrytis cinerea*; Cymoxanil

1. Introduction

Cymoxanil [1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea] **Ia** (Fig. 1) is an agricultural fungicide, effective against grape downy mildew. Although this compound is not used against the grey mould, it shows in vitro, a significant activity towards strains

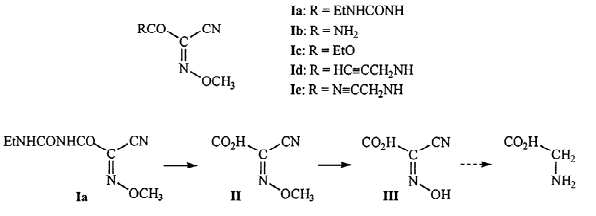

Fig. 1. Cymoxanil **Ia** and analogous cyano-oximes **Ib–e**. Metabolism of cymoxanil **Ia** in plant and animals according to Refs. [7,8].

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of *Botrytis cinerea* [1,2], notably against the sensitive strain L (here named Cya S_1) which is able to decompose this fungicide in less than 6 h [3,4].

For the past few years, several laboratories have been interested in the mode of action of **Ia** [1,5,6]. Although not yet been fully resolved, it seems that **Ia** undergoes a biotransformation triggered by the fungus. This metabolism which results in fungitoxic metabolite(s) would correspond to an activation process, **Ia** behaving as a profungicide [5]. The metabolism of **Ia** has also been studied in plants and animals using radiolabelled material [7,8]. It was found that glycine is the major and ultimate metabolite formed via the intermediates 2-cyano-2-methoxyiminoacetic acid **II** and 2-cyano-2-hydroxyiminoacetic acid **III** (Fig. 1).

The metabolites **II** and **III** were tested in vitro against the sensitive strain Cya S_1 of *B. cinerea*, the hypothesis being that they could be the active principles resulting from the activation of **Ia** in this fungus [9]. However, by contrast to **Ia**, they did not display any fungitoxic activity. The apparent inactivity of **II** and **III** can be explained by their higher polarity which prevents their uptake by the fungus while **Ia**, a less polar molecule, can penetrate into the mycelium where hydrolases trigger its activation.

Recently, during a study dealing with potential fungicides, cyano-oximes analogs to **Ia** were synthesized and other strains than Cya S_1 were tested. The strains were chosen as belonging to phenotypes differing in their behaviour towards **Ia**. Thus a resistant, a highly sensitive and a moderately sensitive strain were chosen: Cya R, Cya S_1 and Cya S_2, respectively. A preliminary screening based on in vitro bioassays concerning mycelial growth and germ tube elongation permitted the selection (for their high fungitoxicity) of the four cyano-oximes **Ib–e** besides **Ia** itself (Fig. 1).

The objectives of this work were threefold. Firstly we aimed to compare the decomposition of fungicidal cyano-oximes: cymoxanil **Ia** and analogs **Ib–e** in the strain Cya S_1 of *B. cinerea* known for its high sensitivity towards **Ia**. Secondly, using cyano-oximes that were demonstrated to decompose most rapidly and the strains Cya R, Cya S_1 and Cya S_2 of *B. cinerea*, we wanted to investigate a possible correlation between the decomposition of cyano-oximes and the strain sensitivity. Finally, we aimed also to study further the mode of action of the previously selected cyano-oximes by focusing on the activation step triggered by the most sensitive strains of *B. cinerea*.

As studies dealing with mycelium are difficult to implement [3,4], we decided to observe the transformations, disappearance of **I** and formation of metabolites in the culture medium, considering that they may reflect what happens in the mycelium.

Residue analysis of **Ia** in grapes has been performed by a multidimensional multicolumn high-performance liquid chromatography (HPLC) technique with complex HPLC instrumentation [10] and also by gas–liquid chromatography with preliminary sample clean-up [11]. In our case, to avoid the multicolumn approach and any sample pretreatment other than simple filtration, we decided to use direct chromatographic monitoring in the presence of the culture medium. This analytical approach was also suggested by our objective to monitor ionic metabolites which may be difficult to extract [9,12]. Previously we used particular HPLC columns that allow direct injection of biological samples containing proteins: ISRP (internal surface reversed phase) [13], and wide-pore packings. In fact, Shihabi and coworkers have successfully used large pore material (300 Å) for the determination of drugs and metabolites by direct serum injection [14,15]. It can be considered that the lower number of plates resulting from the larger pores can be compensated, to some extent by smaller particle size. The advantage of the wide-pore packing is that it provides lower retention due to lower surface area which enables the use of lower concentrations of organic solvents and prevents serum proteins precipitation. This is particularly true when a short alkyl chain (e.g., C_1 column) is used in place of the traditional C_{18} column. Another advantage of this material is that it has an initial low backpressure that is stable after a large number of injections of biofluids. In our laboratory we have also used C_1 and C_4 wide-pore columns (300 Å) to monitor the metabolism of the bioinsecticides destruxins in insect tissues [12,16–18]. Moreover, taking into account that fungi produce and release surface and extracellular enzymes [19], we applied the same approach to study the dynamics of the production of destruxins by the fungus *Metarhizium anisopliae* [20], and the disappearance of cymoxanil **Ia** in two strains of *B. cinerea* directly in

their culture medium using either ISRP or C_1 wide-pore columns [3,17]. Thin-layer chromatography (TLC) may also constitute a direct chromatographic method that we recently implemented in locust biological fluids for monitoring the metabolism of potential proinsecticides of carboxylic acids [21].

In this paper, we present our results concerning the behaviour of cyano-oximes **Ia–e** in a nutritive culture medium of the selected phenotypes Cya R, Cya S_1 and Cya S_2 of the fungus *B. cinerea*. To proceed to a rapid selection of cyano-oximes **I** and strains from the decomposition standpoint, aliquots of the culture medium were firstly analyzed by reversed-phase HPLC as a function of time. Direct injection onto a C_4 wide-pore HPLC column and isocratic elution were used to follow the disappearance of substrates **I**. Then, for the selected strains and substrates the formation of ionic metabolites was monitored by direct application on C_{18} high-performance (HP) TLC chromatoplates impregnated with ion-pairing reagent.

2. Experimental

2.1. Reagents and chemicals

Cymoxanil **Ia** and its precursor **Ib**, and analogs **Id–e** were prepared according to the literature references [7] and [22], respectively.

2-Cyano-2-methoxyiminoacetic acid (metabolite **II**) (Fig. 1), was synthesized from ethyl-2-cyano-2-methoxyiminoacetate **Ic** [22]. However, the method which implies a saponification/acidification sequence [23] was modified in order to increase the yield by isolating the intermediate salt. Thus 1.16 ml (2.9 mmol) of 2.5 *M* aqueous solution of KOH was added to 3 ml of EtOH solution of ester **Ic** (0.47 g, 3 mmol). The mixture was stirred for 90 min at 40 °C then the solvent was evaporated under vacuum at 40 °C. The resulting potassium salt was successively washed with EtOH and Et_2O, then dried by pumping under vacuum, giving a white solid (yield=50%). An aqueous solution of this salt [0.1 g (0.6 mmol) dissolved in 1 ml water] was acidified to pH 1 with concentrated hydrochloric acid. The mixture was extracted with AcOEt, and decanted. Then the organic layers were dried over $MgSO_4$ and concentrated under vacuum. Crude metabolite **II** obtained as a solid was washed with hexane and dried by pumping, yield=90%, Melting point (m.p.)=158–159 °C, identical to the literature [7].

2-Cyano-2-hydroxyiminoacetic acid (metabolite **III**) was prepared from commercial 2-cyano-2-hydroxyiminoacetate [7].

UV–visible spectra were recorded in MeOH solution (Kontron Uvikon 933), λ_{max} (nm) (ϵ_{max}, M^{-1} cm^{-1}): **Ia**, 240.5 (7524); **Ib**, 237.0 (8763); **Ic**, 242.0 (9672); **Id**, 236.0 (4290); **Ie**, 237.5 (8632); **II**, 239 (7375); **III**, 227 (8525).

2.1.1. Chemicals for chromatography

Methanol was of HPLC gradient grade (SDS, Solvent Documentation Synthèse, Vitry, France). Deionized water (18 MΩ) obtained with a Milli-Q apparatus (Millipore, Saint-Quentin en Yvelines, France) was used for the preparation of mobile phases and phosphate buffers by mixing appropriate volumes of dissolved monobasic and dibasic potassium phosphates which were supplied by Acros (Noisy le Grand, France). Tetrabutylammonium bromide (TBAB) and other ion-pair (IP) reagents: cetyltrimetylammonium bromide (CTMAB), tetraoctylammonium bromide (TOAB) and trioctylmetylammonium bromide (TOMAB) were supplied by Acros. Organic solvents and deionized water were both filtered through GHP filters (0.45 μm; Pall, Ann Arbor, MI, USA).

2.2. Preparation of B. cinerea samples

Three strains of *B. cinerea* were used in this investigation: the highly sensitive to **Ia** Cya S_1, the moderately sensitive Cya S_2 and the resistant Cya R.

The strains of *B. cinerea* were maintained on potato dextrose agar. The use of a culture medium rather than a simple buffer system was necessary since *B. cinerea* doubles its mycelial growth over the time in which these studies were conducted (particularly the 6 h studies). Therefore, after 15 days under light, mycelial pellets of *B. cinerea* were prepared by seeding about $10 \cdot 10^5$ conidia per ml in a medium containing 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract for 1 l [24]. The mixture was incubated

at 23 °C on a rotary shaker at 150 rpm for 24 h. Then, 3 ml of these cultures was used to inoculate 150 ml of yeast extract medium in 300-ml Erlenmeyer flasks and incubated again for 24 h as described above to obtain the mycelium used in the experiments. In order to be in a position to compare the biological activities of the different strains, it was necessary to work with the same weight of their respective mycelium, thus the number of inoculated Erlenmeyers were 6, 8 and 9 for Cya S_1, Cya S_2 and Cya R, respectively. The mycelium was harvested by filtering through a gauze (250 μm) and resuspended in six Erlenmeyers with 150 ml volume of yeast extract medium each. Three of these Erlenmeyers were incubated at ''time zero'' with 0.75 ml of an ethanolic solution of 3 mg of every cyano-oxime **Ia–e** (20 μg/ml) and three others were used as blanks (with a simple ethanol addition).

After 0, 2 and 6 h, each inoculated mycelium was separated by filtration from its culture medium which was then frozen, lyophilized, ground and stored at −30 °C until analysis. Aliquots of lyophilized culture medium, 75 mg, were dissolved in 1 ml of phosphate buffer (pH 6.0, 0.1 *M*)–MeOH (or CH_3CN in the case of **Ic**) (75:25, v/v). The samples were successively filtered through 0.45 and 0.2 μm membranes (regenerated cellulose) before HPLC injection or HPTLC application. Table 1 summarizes the preparation of the different samples.

2.3. HPLC instrumentation

For qualitative analysis and quantification of cyano-oximes (**Ia–e**) a system consisting of a Waters LC Module I plus (Milford, MA, USA) operating with Millenium 2010 v2.15.01 chromatography manager software, was used. The separations were carried out with a 5 μm, C_4 Uptisphere wide-pore analytical column (300 Å) (50×4.6 mm I.D.) and a 5 μm, C_4 Uptisphere wide-pore guard column (300 Å) (10×4 mm I.D.) supplied by Interchim (Montluçon, France).

Table 2
Linear calibration parameters for the quantitative HPLC analysis of **Ia** and analogous cyano-oximes **Ib–e**

	$a_0 \pm$SD	$a_1 \pm$SD	r^2	**[Ix]** range, *M*, $\cdot 10^4$	*N*
Ia	6437.83±4418.9	2450.54±17.32	0.9996	0.2–5	5
Ib	8970.09±4219.8	2824.42±9.34	0.9998	0.2–7.5	6
Ic	4306.23±2362.2	1775.08±5.02	0.9999	0.2–5	6
Id	253.04±1864.8	2603.40±7.3	0.9999	0.2–5	5
Ie	3313.77±1861.4	2576.70±7.3	0.9999	0.2–5	5

N = Number of standards.

2.4. HPLC monitoring

Standards were prepared by adding known amounts of **Ia–e** into a mixture composed of 75 mg of untreated sample of the studied strain (0 $CyaS_1$ 0, 0 $CyaS_2$ 0, or 0 CyaR 0) dissolved in 1 ml of phosphate buffer (0.1 *M*, pH 6.0)–MeOH (or CH_3CN for **Ic**) solution (75:25, v/v). See Table 2 for the concentration range.

Injections were of 5 μl for each of **Ia–e** standards or inoculated biological samples. Three replicates of standards at five or six concentration level were analysed, see Table 2.

Elution: isocratic separations were achieved with phosphate buffer (pH 6.0, 0.01 *M*)–MeOH (90:10, v/v) as eluent at a flow-rate of 0.8 ml/min. The capacity factors, *k*, were calculated by the classical relation between the retention time t_R and the hold-up time t_0: $k=(t_R-t_0)/t_0$, t_0 being determined with a diluted $NaNO_3$ solution.

The detection wavelength was set 238 nm for all cyano-oximes **Ia–e**.

Quantification was performed by the external standard method from triplicate injections of stan-

Table 1
Preparation and numbering of the various samples of incubated, or non-incubated strains of *Botrytis cinerea* by cyano-oximes **Ix**

Incubation time (h)	Strain	Cyano-oxime **Ix (Ia–e)** **[Ix]**, μg/ml
0, 2, 6	Cya S_1, Cya S_2, Cya R	0, 20

For example 0.Cya S_1.20.Ia signify: the sample at ''time zero'' of the strain Cya S_1 incubated with 20 μg/ml of **Ia**.

dards or samples. The calibration curves for cyano-oximes **Ia–e** obtained by plotting their concentration y (M) against their peak area x (μV s) are linear within the ranges of concentration which are listed in Table 2 with the corresponding parameters of the linear regressions $y = a_0 + a_1x$. The cyano-oximes **Ia–e** were directly quantified from chromatograms of the various samples (Table 1) by using their peak area and the calibration curves.

2.5. HPTLC instrumentation

HPTLC plates were 0.2 mm thick, 10×10 cm, RP 18WF$_{254s}$ (Merck, Darmstadt, Germany, catalog No. 1.13124). Prior to chromatography the plates were impregnated by vertical dipping for 5 min in methanolic solution of ion-pairing reagent: CTMAB, TBAB, TOAB and TOMAB, then dried at 80 °C for 5 min on a Camag TLC Plate Heater III (Muttenz, Switzerland).

Application of samples was achieved with a Camag ATS III Automatic TLC Sampler 3 and eluted in a Camag horizontal development chamber (HDC) 20×10 cm. The scanning densitometry was performed using a computer-controlled TLC scanner: Camag TLC Scanner operated with CATS 4.06 software residing on a Pentium III personal computer.

2.6. HPTLC monitoring

Standards for calibration, composite standards were prepared by adding known amounts of the mixtures [**II**+**III**+**Ia** or **Ib**] to untreated samples of the strain Cya S$_1$ (75 mg of 0CyaS$_1$0) dissolved in 1 ml of phosphate buffer (0.1 *M*, pH 6.0)–MeOH (75:25, v/v) in order to provide wide ranges of concentration (Table 3).

A 2-μl volume of each standard solution or time zero biological sample and 4 μl of the other biological samples were sprayed as bands of 4 mm length at a band velocity of 10 mm/s onto HPTLC plates, at 8 mm intervals and 8 mm from the lower edge. All values were obtained from duplicate samples, see Table 3 for the range in quantities (nmol).

The plates were eluted in a HDC over a given migration distance MD with a solution of phosphate buffer (0.01 *M*, pH 6.0)–MeOH as eluent. Quantitative measurements were carried out by densitometry (Table 3). The scanning was performed in the UV-reflectance mode (deuterium lamp, scanning speed of 5 mm s^{-1}, 20 nm bandwith, slit dimensions of 5×0.3 mm) with wavelength detection set after optimisation at 243 nm.

3. Results and discussion

To compare the behaviour of the cyano-oximes **Ia–e** towards a given strain, it was necessary to use the same culture medium for the subsequent incubations by **Ia–e**. Moreover, to avoid further metabolism of the samples presented in Table 1, though they were stored at −30 °C, we had to proceed rapidly with their chromatographic analysis.

To provide rapid analyses, the cyano-oximes were resolved from endogenous components by reversed-phase HPLC, whilst HPTLC was used (with an IP

Table 3
Calibration parameters for the quantitative HPTLC analysis of **Ia,b** and metabolites **II** and **III**

	Calibration[a]	a_0	a_1	a_2	Sdv[b]	r^2	Range (nmol)	N
Ia	L	3.74±1.70	0.100±0.003		9.5	0.993	0.05–1	5
II	M	88.19	5065.09	$4.41 \cdot 10^3$	3.2		0.1–1	5
III	M	1.13	417.36	$6.86 \cdot 10^3$	7.5		0.15–1	5
Ib	M	335.	9548.48	$4.58 \cdot 10^3$	12.3		0.05–1.5	5
II	L	61.4±22.1	1.70±0.09		4.5	0.988	0.1–1	5
III	L	−79.56	1.61		6.2	0.987	0.15–1	5

N = Number of standards.

[a] L = Linear calibration: $y = a_0 + a_1x$ with y = quantity expressed in nmol and x = area [a.u. mm (a.u.: arbitrary unit)].
M = Michaelis–Menten correlation with the same signification for y and x: $y = a_0 + [a_1x/(a_2 + x)]$.

[b] Sdv = $100 \cdot [\text{var}(y)^{1/2}/\text{mean } y]$.

reagent to increase retention) to monitor the ionic metabolites.

3.1. HPLC monitoring of the cyano-oximes *Ia–e*

Taking into account literature data [25] and our own observations, it appeared that a slightly acidic medium is generally preferable to maintain the cyano-oximes stability. Thus, eluent optimisation was performed using a phosphate buffer (0.01 *M*, pH 6.0) as the primary solvent and MeOH as a cosolvent.

The feasibility of such an isocratic HPLC analysis performed directly on the culture medium is illustrated in Fig. 2 which represents the chromatographic profiles for the time zero of the less and the more polar cyano-oximes, **Ia** and **Ib**, respectively. It can be seen that phosphate buffer–MeOH (90:10, v/v) represents a good solution for elution, as in such conditions the cyano-oximes **Ia** and **Ib** are well-separated from endogenous components with reasonable *k*: 8.6 and 0.63, respectively. Consequently, cyano-oximes **Ic–e** of intermediate polarity were also analysed with the same eluent, giving as expected intermediate *k*: 7.43, 2.51 and 1.24, respectively. As cyano-oxime **Ic** decomposes slowly in

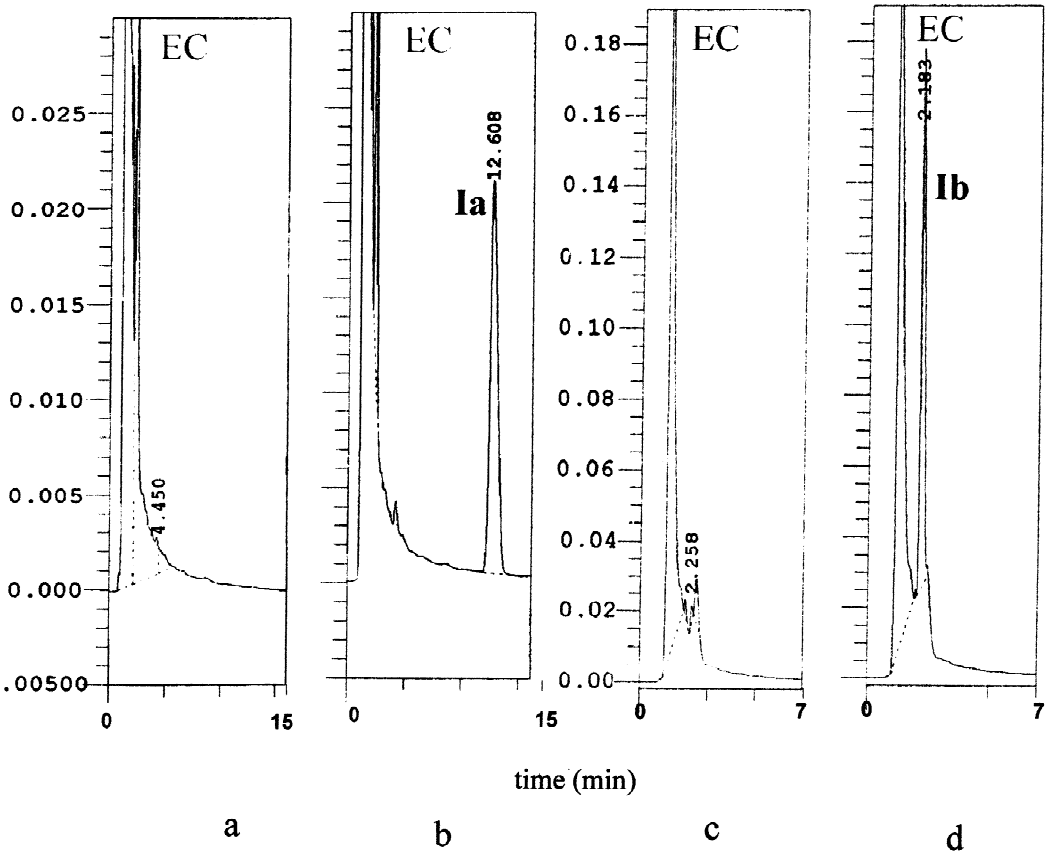

Fig. 2. Chromatograms showing analysis of cymoxanil **Ia** and analogous cyano-oxime **Ib** in the culture medium of Cya S_1. Chromatographic conditions: analytical column 5 μm, C_4 Uptisphere wide-pore (300 Å) (50×4.6 mm I.D.) and a wide-pore guard-column 5 μm, C_4 Uptisphere (300 Å) (10×4 mm I.D.). Isocratic elution with phosphate buffer (pH 6.0, 0.01 *M*)–MeOH (90:10, v/v) at a flow-rate of 0.8 ml/min. UV detection was set at 238 nm. (a) Blank. Endogenous components (ECs) of the biological medium are almost completely eluted in about 2 min. (b) Cymoxanil **Ia**: $k=8.6$. Under these conditions k for cyano-oximes **Ic–e** were 7.43, 2.51 and 1.24, respectively, and metabolites **II** and **III** were eluted beyond the endogenous peaks with $k \leq 0.4$. (c) Blank at a different *x* scale. (d) Cyano-oxime **Ib**: $k=0.63$.

methanolic solutions, the corresponding samples were prepared in acetonitrile solutions. After determination of the stability of cyano-oximes **Ia–e** in the culture media without mycelium, we proceeded to their in situ calibration in the corresponding culture media 0Cya S_1, 0Cya S_2 and 0Cya R for subsequent quantifications (Table 4). The limits of detection (LODs) were about $1.5 \cdot 10^{-5}$ *M* (for $S/N = 3$).

*3.2. Behaviour of cyano-oximes **Ia–e** in B. cinerea strains*

The disappearance of the cyano-oximes **Ia–e** was quantified in the different strains by analysing the different strains of incubated *B. cinerea* Cya S_1, Cya S_2 and Cya R (Table 1) after simple filtration through a membrane.

At first, we compared the behaviour of **Ia–e** in the culture medium of the Cya S_1 mycelium, i.e., that corresponding to the more sensitive strain. Results shown in Table 4 indicate the rapid disappearance of **Ia** (97% decrease within 2 h, **Ia** being undetectable after 6 h). **Ic** shows almost the same metabolism as **Ia**, whereas **Ib** undergoes a slower decrease with time (30 and 80% decrease after 2 and 6 h, respectively). The cyano-oximes **Id–e** are even more slowly metabolized (with about 40% decrease after 6 h). The lack of transformation observed for these cyano-oximes in the controls shows that their disappearance during the above-assays is caused by the mycelium of the Cya S_1 strain.

In a second step, we studied by the same HPLC procedure possible phenotype effects on the metabolism of the cyano-oximes **Ia–c** previously revealed as easily metabolized, using the supplementary Cya S_2 and Cya R strains. With the resistant strain Cya R, as expected, we have not observed after 6 h significant transformation for **Ia** (Table 5). By contrast we have noticed a significant decrease (of about 70%) in the case of **Ib** and **Ic** (Table 5). Their decrease is however less than that observed with the Cya S_1 strain. The same trends were observed with the Cya S_2 strain of intermediate sensitivity.

The results obtained with Cya S_1 and Cya R strains, show that the sensitivity of Cya S_1 strain towards the fungicidal **Ia–c** may be related to the disappearance of these compounds from the culture medium, namely to the ability of the strain to metabolize the cyano-oximes. These results confirm those obtained with ^{14}C cymoxanil [5] which sug-

Table 4
HPLC quantification of cyano-oximes **Ia–e** in presence of the Cya S_1 strain at different incubation times

	C_{HPLC} [a], $M \cdot 10^6$	Q [b], mol·10^6		C_{HPLC} [a], $M \cdot 10^6$	Q [b], mol·10^6
0.CyaS1.20.Ia		15.2[c]	0.CyaS1.20.Ib		23.6[c]
0.CyaS1.20.Ia	374.8	11.8	0.CyaS1.20.Ib	554.7[d]	18.8
2.CyaS1.20.Ia	12.5	0.4	2.CyaS1.20.Ib	384.5[d]	12.1
6.CyaS1.20.Ia	0.0	0.0	6.CyaS1.20.Ib	100.3[d]	2.9
0.CyaS1.20.Ic		19.2[c]	0.CyaS1.20.Id		18.2[c]
0.CyaS1.20.Ic	353.1	12.1	0.CyaS1.20.Id	398.9	13.9
2.CyaS1.20.Ic	54.8	1.8	2.CyaS1.20.Id	306.0	9.8
6.CyaS1.20.Ic	0.0	0.0	6.CyaS1.20.Id	256.7	7.8
0.CyaS1.20.Ie		18.1[c]			
0.CyaS1.20.Ie	564.4	18.1			
2.CyaS1.20.Ie	473.6	14.1			
6.CyaS1.20.Ie	320.2	8.9			

[a] C_{HPLC} = **Ix** concentration determined from HPLC calibration for the considered aliquot of 75 mg in 1 ml of buffer (see Table 2).
[b] Q = Quantity of **Ix** in the whole lyophilised medium.
[c] Theoretical initial quantity of **Ix** in the whole medium.
[d] Due to the presence of an endogenous component (EC) peak in the Cya S_1 blank with similar retention than **Ib**, for the quantification of **Ib** it was necessary to proceed to the subtraction of the area of this peak.

Table 5
HPLC quantification of cyano-oximes **Ia–c** in presence of the Cya S_2 and Cya R strains of *Botrytis cinerea* at different incubation times

	C^a_{HPLC} $M\times10^6$	Q^b $mol\times10^6$		C^a_{HPLC} $M\times10^6$	Q^b $mol\times10^6$
0.CyaR.20.Ia	391.7	12.8	0.CyaS2.20.Ia	449.2	15.0
2.CyaR.20.Ia	385.9	12.5	2.CyaS2.20.Ia	429.8	14.0
6.CyaR.20.Ia	378.4	11.4	6.CyaS2.20.Ia	350.2	11.1
0.CyaR.20.Ib	688.1	22.5	0.CyaS2.20.Ib	566.4	18.8
2.CyaR.20.Ib	517.7	16.8	2.CyaS2.20.Ib	518.2	16.9
6.CyaR.20.Ib	217.1	6.8	6.CyaS2.20.Ib	150.0	4.7
0.CyaR.20.Ic	460.5	14.9	0.CyaS2.20.Ic	326.3	11.2
2.CyaR.20.Ic	313.9	9.9	2.CyaS2.20.Ic	288.0	9.2
6.CyaR.20.Ic	172.2	5.3	6.CyaS2.20.Ic	155.2	4.8

[a] C_{HPLC} = **Ia–c** concentration determined by HPLC calibration for the considered aliquot of 75 mg in 1 ml of buffer (see Table 2).
[b] Q = Quantity of **Ix** in the whole lyophilised medium.

gested that a mechanism of activation is involved. The behaviour of Cya S_2 strain is not so obvious. The variations in sensitivity of this strain may be caused by differences in the uptake of the cyano-oximes (in the permeability of the cell envelope) or differences in the target site.

*3.3. IP-HPTLC monitoring of the cyano-oximes **Ia–b** and their metabolites **II** and **III***

The HPLC method was suitable for the monitoring of cyano-oximes **I** but not for their metabolites. Concerning ionized metabolites, the control of the solvent pH to mask ionic groups is not a good strategy for cyano-oximes analysis [9,25], as also observed for some other acids [26]. By contrast, ion-pairing with IP reagents appears as a good solution for increasing the retention of acidic entities. To avoid the lengthly of IP-HPLC checking-up and the use of several columns, we chose to perform analyses by IP-TLC. This method was optimised using simultaneously several plates for testing different IP reagents.

IP-HPTLC is very useful for the separation of different ionic compounds, particularly acids [26–30]. There are several possible means of introducing the ion-pairing reagent into the chromatographic system: addition to the mobile phase, use of the reagent in both layer and eluent and impregnation of the layer [29,31]. This last method was applied in our laboratory to follow in the Cya S_1 strain the metabolism of **Ia** and **Ib** that have previously been observed as the most rapidly metabolized by this strain. Unfortunately, the metabolism of **Ic** was impossible to follow by this method, due to its instability on IP pretreated plates. Among several procedures, and four IP reagents tested for impregnation, we found the dipping of a C_{18} plate into a solution of TBAB in MeOH to be the best (this was previously found to give good chromatographic results with carboxylic acids and C_2 or C_{18} layers [26]). The TBAB concentration used for pretreating the plates was optimized at 70 m*M* for **Ia, II** and **III** and 50 m*M* for **Ib**. These values represent higher IP concentrations than one generally required in HPLC [26]. With regard to elution, the best results were achieved when two successive migrations were performed, using for the first a phosphate buffer–MeOH (50:50, v/v) mixture over 10 mm. For the second migration, both the migration distance MD and eluent composition were adjusted to each case **Ia** and **Ib**.

Qualitative analysis of the behaviour of **Ia** and **Ib** consisted of the identification of the substrates and their metabolites in the extracts based on both the MD values and the in situ UV-reflectance spectra compared to that of standards prepared in the presence of culture media. From the quantification standpoint the lowest detectable quantities were of about 50, 100 and 150 pmol for **Ia** and **Ib**, **II** and **III**, respectively ($S/N=3$).

The metabolism of **Ia** was studied using only one plate that was eluted with a phosphate buffer–MeOH (60:40, v/v) mixture over 50 mm. Thus both the

disappearance of **Ia** (MD = 21.5 mm), and the formation of two polar metabolites identified as **II**, (MD = 24 mm) and **III** (MD = 28 mm) were quantified (Fig. 3). These results indicate that the ion-pairing was effective since the MD of the metabolites was considerably reduced compared to assays performed without IP, though these MD remained greater than that of **Ia**.

The metabolism of **Ib** was studied using only 35% MeOH in eluent to follow its disappearance**,** due to the greater polarity of this compared to that of **Ia**. Thus two plates were necessary in this case. The first one, eluted with a buffer–MeOH (65:35, v/v) mixture over 70 mm (MD = 40.7 mm for **Ib**) was dedicated to follow the disappearance of **Ib** and the second [eluted with buffer–MeOH (60:40, v/v) up to 60 mm] to the appearance of **II** (MD = 27 mm) and **III** (MD = 32 mm), Fig. 4.

This HPTLC method allowed us to quantify the decrease of the substrate **Ia** (or **Ib**) and the formation

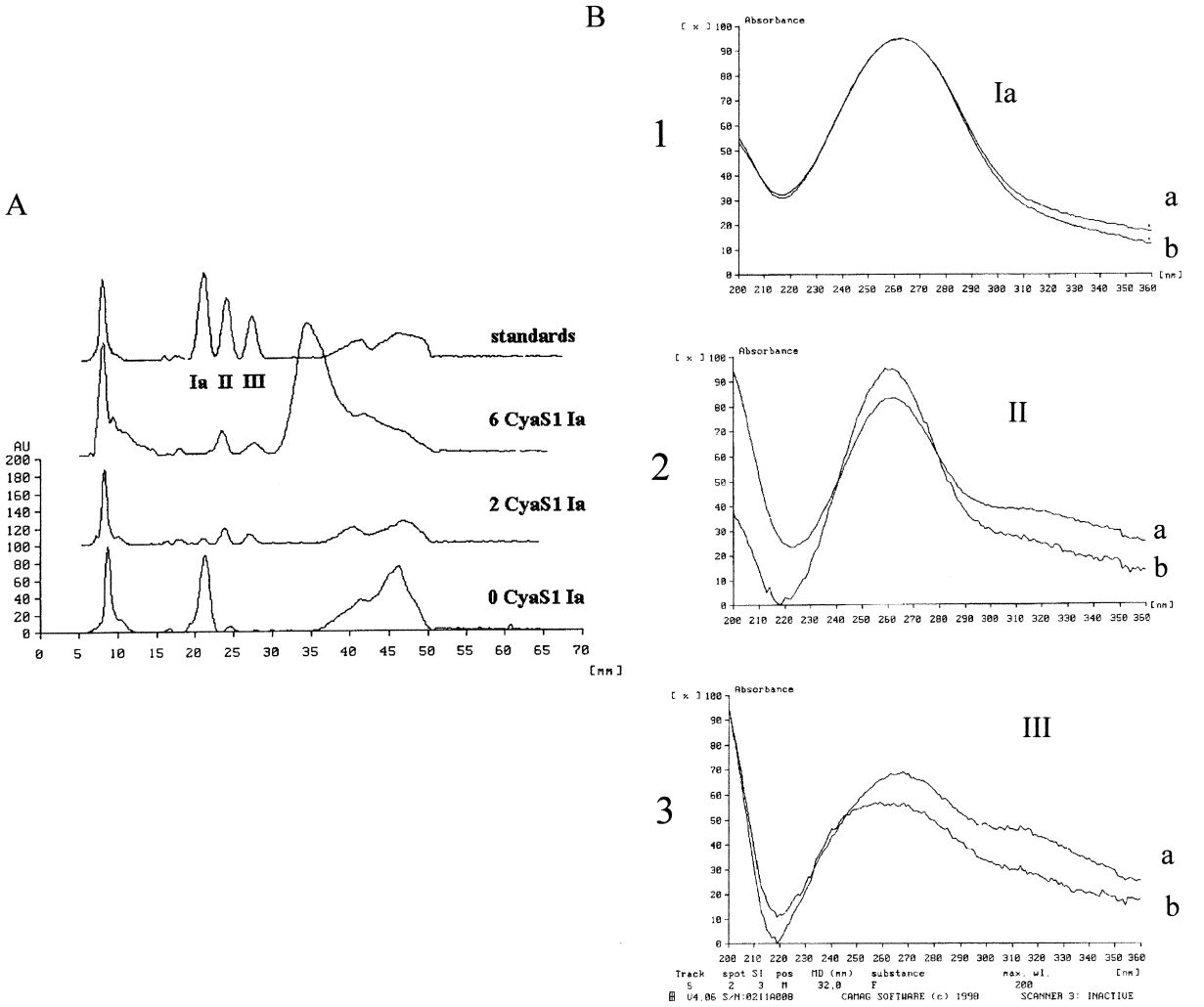

Fig. 3. Direct HPTLC monitoring of cymoxanil **Ia** in the presence of the Cya S_1 strain of *Botrytis cinerea*. (A) Densitograms of a C_{18} chromatoplate impregnated with 70 m*M* TBAB. First migration with phosphate buffer–MeOH (50:50, v/v) over 10 mm. Second elution with phosphate buffer–MeOH (60:40, v/v) over 50 mm. (B) In situ UV-reflectance spectra. (1) 0Cya S_1 Ia (a), standard **Ia** (b); (2) metabolite **II** in 6Cya S_1 Ia (a), standard **II** (b); (3) metabolite **III** in 6Cya S_1 Ia (a), standard **III** (b).

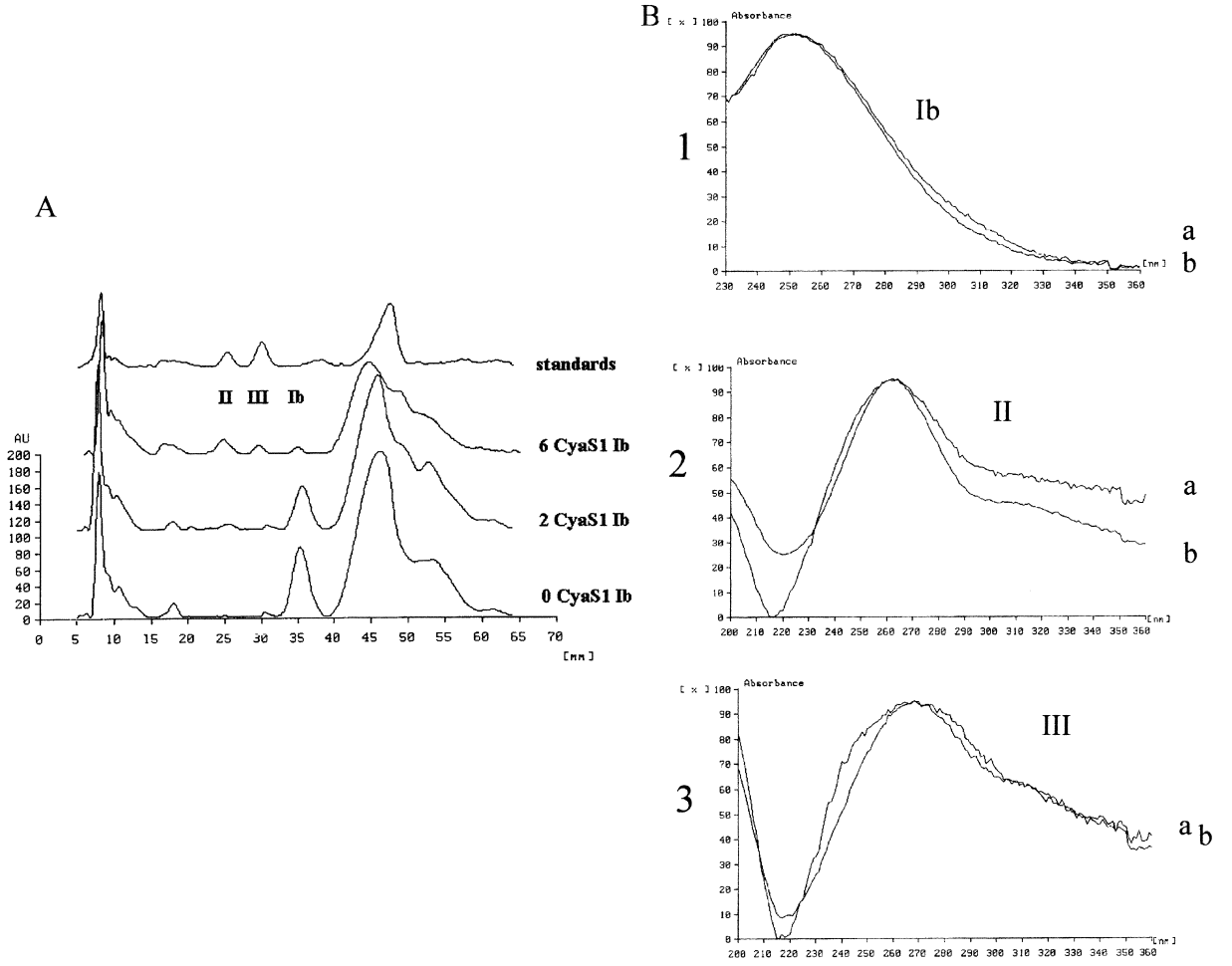

Fig. 4. Direct HPTLC monitoring of **II** and **III** metabolites from the cyano-oxime **Ib** in the presence of the Cya S_1 strain of *Botrytis cinerea*. (A) Densitograms. C_{18} chromatoplate impregnated with 70 m*M* TBAB. First migration with phosphate buffer–MeOH (50:50, v/v) over 10 mm. Second elution with phosphate buffer–MeOH (60:40, v/v) over 60 mm. (B) In situ UV-reflectance spectra. (1) 0Cya S_1 Ib (a), standard **Ib** (b); (2) metabolite **II** in 6Cya S_1 Ib (a), standard **II** (b); (3) metabolite **III** in 6Cya S_1 Ib (a), standard **III** (b).

of the metabolites **II** and **III** in the culture medium. In each case, the amount of metabolites **II** and **III** was shown to increase up to about 10% of the starting material after 6 h (Table 6), leaving most of the initial substrates unaccounted for. This loss can be explained by considering that only a part of these metabolites formed in the mycelium by the cyano-oximes activation is released in the culture medium. Such hypothesis is supported by the fact that attempts to extract **II** and **III** from mycelium have failed.

4. Conclusion

Direct HPLC using wide-pore packing has allowed a rapid and easy comparison between the decrease of cymoxanil **Ia** and other cyano-oximes **Ib–e** in the culture media of different *B. cinerea* phenotypes. We have found that the rate of disappearance for the cyano-oxime follows the order **Ia**~**Ic**>**Ib** in the case of the sensitive Cya S_1 strain. By contrast with the resistant strain Cya R, **Ia** appears to be very stable while **Ib** and **Ic** were extensively transformed.

Table 6
HPTLC quantification of cyano-oximes **Ia–b** and their metabolites **II** and **III** in presence of the Cya S_1 strain of *Botrytis cinerea* at different incubation times

	Q_{HPTLC} [a], mol·10^{12}	Q [b], mol·10^{6}	Q_{HPTLC} [a], mol·10^{12}	Q [b], mol·10^{6}	Q_{HPTLC} [a], mol·10^{12}	Q [b], mol·10^{6}
Cymoxanil	**Ia**		**II**		**III**	
0.CyaS1.20.Ia	1816.2	14.3	0	0	0	0
2.CyaS1.20.Ia	49.1	0.4	86.2	0.7	73.8	0.6
6.CyaS1.20.Ia	0	0	208.3	1.5	182.6	1.3
Cyano-oxime Ib	**Ib**		**II**		**III**	
0.CyaS1.20.Ib	2376.4	20.1	0	0	0	0
2.CyaS1.20.Ib	1123.2	8.8	83.4	0.65	0	0
6.CyaS1.20.Ib	186.0	1.3	207.1	1.5	190.8	1.4

The theoretical quantities for **Ix** in 0.Cya S_1.20.Ia and 0.Cya S_1.20.Ib were 15.2 and 23.6 μmol, respectively.
[a] Q_{HPTLC} = quantity (in pmol corresponding to 4 μl deposited) of compounds **Ia**, **Ib**, **II** and **III** determined from the HPTLC calibration (Table 3) for each aliquot of 75 mg in 1 ml of buffer (see Experimental for the applied volume).
[b] Q = Quantity of **Ix** in the whole lyophilised medium.

Concerning the formation of metabolites, IP-HPTLC was demonstrated to be an appropriate method to follow such ionisable entities, even in very low amounts. The two acidic metabolites **II** and **III** resulting from the activation of both **Ia** and **Ib** were thus identified and quantified. Their presence in the culture medium suggests that the metabolism of cymoxanil **Ia** (and cyano-oxime **Ib**) triggered by *B. cinerea* may be similar to that previously described by other organisms [7,8]. This method which has also confirmed the previous HPLC observations concerning the disappearance of cyano-oximes in Cya S_1 adds to the few examples of systems that have been directly investigated by TLC [21,30–36] without pretreatment.

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