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Baseline sensitivity of *Monilinia laxa* and *M. fructigena* to pyraclostrobin and boscalid

Baseline-Sensitivität von *Monilinia laxa* und *M. fructigena* gegenüber Pyraclostrobin und Boscalid

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Summary

Samples infected with *Monilinia laxa* and *Monilinia fructigena* were collected from different European countries and different plant hosts and fungal strains were isolated (102 x *M. laxa*, 37 x *M. fructigena*). In order to determine the baseline sensitivity of these isolates to the newly introduced fungicides pyraclostrobin and boscalid, an appropriate microtiter assay was developed. For both active ingredients, YBA medium was the medium of choice, since it allowed sufficient growth but did not lead to false positive results. The minimum inhibitory concentrations (MIC) were assessed. MIC values ranged for pyraclostrobin and *M. laxa* from 0.01–2.5 ppm and for *M. fructigena* from 0,039–0,625 ppm. For boscalid MIC values were 0.01–2.5 ppm for *M. laxa* and 0.039–2.5 ppm for *M. fructigena*.

Key words: blossom blight, brown rot, microtiter assay, resistance

Zusammenfassung

Aus verschiedenen europäischen Ländern und Wirtspflanzen wurden Proben, befallen mit *Monilinia laxa* und *Monilinia fructigena* gesammelt und zur Gewinnung von Isolaten genutzt (102 x *M. laxa*, 37 x *M. fructigena*). Zur Bestimmung der natürlichen Sensitivität dieser Isolate gegenüber den neu eingeführten fungiziden Wirkstoffen Pyraclostrobin und Boscalid wurde ein Mikrotitertest entwickelt. Für beide Wirkstoffe war YBA-Medium optimal, da das Wachstum in diesem Medium ausreichend war, aber zu keinen falsch-positiven Ergebnissen führte. Mit diesem Test wurde die minimale inhibitorische Konzentration (MIC) bestimmt. Die MIC-Werte lagen für Pyraclostrobin und *M. laxa* von 0,01–2,5 ppm, für *M. fructigena* von 0,039–0,625 ppm. Für Boscalid wurden MIC-Werte von 0,01–2,5 ppm für *M. laxa* und 0,039–2,5 ppm für *M. fructigena* bestimmt.

Stichwörter: Mikrotitertest, Monilia-Fruchtfäule, Resistenz, Spitzendürre

1 Introduction

Monilinia laxa is a serious pathogen on blossoms and fruits of stone fruits worldwide, the special form *M. laxa* f.sp. *mali* can also infect blossoms of pome fruits. *Monilinia fructigena* is an important pathogen on fruits of pome and stone fruits and is mainly distributed in Europe and Asia (Byrde and Willetts 1977). Infections of blossoms are called blossom blight, infections of fruits, brown rot. Depending on host species, variety and disease conditions, up to three fungicide applications from growth stage 59 to 67 are necessary to control blossom blight and up to five sprays are needed to control brown rot during fruit development and ripening. The most important fungicides presently used belong to the group of sterol biosynthesis inhibitors and dicarboximides. Two new fungicides with different modes of action, pyraclostrobin and boscalid, have been launched in a combination product for control of *Monilinia* diseases. Both fungicides show excellent field efficacy against blossom blight and brown rot in stone fruits and pome fruits. Pyraclostrobin is a broad spectrum QoI fungicide, which inhibits the mitochondrial respiration chain by binding to the cytochrome bc1 complex at the Qo site (Sauter et al. 1999; Ypema and Gold 1999; Bartlett et al. 2002). It is registered in many crops against a wide variety of plant pathogens. Boscalid is a new broad-spectrum fungicide belonging to the anilide fungicides and mainly registered in fruits, vegetables and vines. Boscalid inhibits the enzyme succinate dehydrogenase (SDH), which is a functional part of the tricarboxylic cycle and of the mitochondrial electron transport chain.

Resistance to QoI-fungicides is described for different target pathogens. Two amino acid substitutions have been detected in the cytochrome b gene in plant pathogens that govern resistance to QoI fungicides. One mutation leads to a substitution of glycine by alanine at codon 143 and is the main mechanism of resistance to QoIs (Heaney et al. 2000; Gisi et al. 2002). Another mutation at codon 129, which leads to the substitution of phenylalanine by leucine results generally in lower resistance factors (Kim et al. 2003; Pasche et al. 2005). No boscalid-resistant field isolates of the target pathogens have so far been found. Some information on the putative mechanism of resistance in fungal pathogens to carboxin, which is also an anilide fungicide, are published (Bochow et al. 1971; Ben-Yepet et al. 1975; Gunatilleke et al. 1976; Keon et al. 1991; Skinner et al. 1998). It is currently thought that mutations, which lead to amino acid substitutions in the SdhB or SdhC subunit of succinate dehydrogenase, confer resistance to carboxin (Keon et al. 1991; Skinner et al. 1998; Ito et al. 2004).

In order to be able to monitor the sensitivity of *M. laxa* and *M. fructigena* to pyraclostrobin and boscalid after market introduction, it is necessary to determine the baseline sensitivity before product launch. Such baselines reflect the wild-type sensitivity of isolates which have never been exposed to the respective fungicides. Samples included in the studies came from orchards where QoI fungicides were not or rarely used in the past, so that these isolates can be considered to be isolates with wild-type sensitivity. Since there was no registration of an anilide fungicide in pome and stone fruits in the past, all isolates before the market introduction of boscalid can be considered to be isolates with wild-type sensitivity.

To determine the sensitivity of fungal pathogens to pyraclostrobin and boscalid, it is mandatory to use an adequate culture medium. This culture medium must prevent the fungus from gaining energy via alternative pathways as alternative respiration or anaerobic fermentation and ensure the efficacy of the antifungal compounds. Otherwise the *in vitro* sensitivity is reduced and the inhibition of the fungus is not complete, even at high fungicide concentrations, while no loss of efficacy against the same isolates can be detected under *in vivo* conditions (Avila-Adame and Köller 2003; Matheron and

PORCHAS 2004). However, the culture medium should contain enough nutrients to allow sufficient growth of the isolates in the test system. In this study we describe a microtiter assay developed for the determination of the sensitivity of *M. laxa* and *M. fructigena* to pyraclostrobin and boscalid. The baselines for both *Monilinia* species are described.

2 Materials and methods

Samples of *M. laxa* and *M. fructigena* were collected from different plant hosts by transferring conidia from sporulating pustules on the surface of Petri dishes containing 2% malt agar and 200 ppm streptomycin. The Petri dishes were then incubated at 18°C and clean cultures were produced by transferring mycelium of *Monilinia* spp. to new agar plates. For the production of spores, *M. laxa* was cultivated on V8 agar and *M. fructigena* on 2% malt agar. The dishes were incubated at 18°C for 10-12 days under 12 h dark and 12 h white light to promote dense sporulation. The spores were harvested by washing the agar plates with 5 ml double concentrated YBA medium (20 g yeast extract, 20 g Bacto peptone, 40 g sodium acetate in 1 litre sterile deionised water). The resulting suspension was filtered through two layers of cheese cloth and the suspension was adjusted to a spore density of 2 x 10^4 ml^{-1}.

Pure technical active ingredient (a.i.) of pyraclostrobin and boscalid were solved in dimethylsulfoxide and the dilutions were prepared in sterile deionised water immediately before mixing with the spore suspension. Fifty µl fungicide solution and 50 µl spore suspension were mixed in each well of 96-well microtiter plates. The following final concentrations were used in the microtiter assays: 0.002, 0.01, 0.039, 0.156, 0.625, 2.5 and 10 ppm a.i. For each isolate and fungicide concentration two replicate wells were used. The microtiter plates were put into plastic bags to avoid evaporation and incubated at 18°C in darkness. Five days after inoculation the growth was assessed visually in five classes: 0, no growth; 1, less than 50% of control; 2, ~50% of control; 3, > 50% of control; 4, same growth as control. Additionally, the minimum inhibitory concentrations (MIC, lowest concentrations which totally inhibit growth) were determined for each isolate.

3 Results

A total of 102 isolates of *M. laxa* and 37 isolates of *M. fructigena* were tested in microtiter assays for their sensitivity to pyraclostrobin and boscalid. The origins of the collection of the cultures are given in Tables 1 to 4.

Both species caused an intensive browning of the culture medium, which interfered with the photometric measurement of the growth at 405 or 620 nm. This browning could not be circumvented by modifications of the incubation conditions (temperature, light, culture medium, dilutions of medium, addition of different concentrations of antioxidants like ascorbic acid). The interference of this browning with photometric detection led to false values.

Therefore, evaluations were done by microscopic observation of the germination and visual assessment of growth. They showed that pyraclostrobin inhibits the germination completely at the MIC values. In medium containing boscalid the spores were able to germinate even at the highest concentration, but the growth and further development of mycelium was completely stopped (Fig. 1). This could be shown by observations at different time points. Pyraclostrobin inhibited the development of *M. laxa* and *M. fructigena* with the lowest concentration used and provided complete control with 2.5 ppm. Boscalid showed first inhibitory effects with 0.002–0.01 ppm in both species and all isolates were controlled with 2.5 ppm. Tables 3 and 4 show the origin and MIC values of each isolate. There were no significant differences between the origins (plant host, country) of the isolates. Our standard isolate of *M. laxa* (Isolate ID 29) was tested in seven assays and the data indicated that the assay is a valid and reproducible survey of the sensitivity (Table 5). The distributions of the MIC values for pyraclostrobin and boscalid are given in Fig. 2 for *M. laxa* and in Fig. 3 for *M. fructigena*.

4 Discussion

For sensitivity monitoring of pathogens to pyraclostrobin and boscalid the use of the correct methodology is necessary since "rich" media such as malt or V8 medium allow the growth of fungi even at high concentrations of the two fungicides. In such media the fungi are able to use alternative pathways, which are not of relevance in nature. Such false positive data could be misinterpreted as low sensitivity of the pathogens. For *M. laxa* and *M. fructigena* several methods were investigated to measure the sensitivity to pyraclostrobin and boscalid. *In vivo* assays with apple fruits, similar to the method described by FORSTER and MÜLLER (1996) or mycelial growth tests in Petri dishes under different conditions were not appropriate since growth was not completely controlled, the data were not reproducible or the fungal growth was too slow. An unexpected problem in the microtiter assay was the discolouration (browning) of the culture medium. It was not possible to suppress it with different modifications of the test. Since the browning absorbed at the whole spectrum from 405 to 620 nm, it interfered with the photometric detection of the growth at any wavelength chosen for growth detection. Additionally, this browning effect varied between the different fungicide concentrations and depended also on the isolates. Because of this additional absorption a 100% growth inhibition could not be calculated but the total inhibition was clearly visible under the microscope. Therefore we decided to assess the growth visually in five different classes and calculate the frequency distribution with the MIC values by microscopic observation. It is of importance to mention that germination of spores was possible even at high concentrations of boscalid but that the development and further growth was stopped. This was shown by evaluation of the growth at different time points. Frequency analysis gave a relatively broad distribution of the MIC values of boscalid and pyraclostrobin, in particular

Table 1: Origin of *Monilinia laxa* samples

	Apple	Nectarine	Peach	Plum	Sour cherry	Sweet cherry	Sum
Germany	0	1	3	10	32	3	49
France	0	0	3	14	11	0	28
Italy	0	18	0	0	0	0	18
Denmark	0	0	0	0	7	0	7
Sum	0	19	6	24	50	3	102

Table 2: Origin of *Monilinia fructigena* samples

	Apple	Nectarine	Peach	Plum	Sour cherry	Sweet cherry	Sum
Germany	13	0	0	5	1	5	24
France	0	0	11	0	0	0	11
Italy	0	1	0	0	0	0	1
Denmark	0	0	0	1	0	0	1
Sum	13	1	11	6	1	5	37

Table 3: Minimum inhibitory concentration (MIC) values in ppm [mg l^{-1}] active ingredient of isolates of *Monilinia laxa* from different European countries and different plant hosts towards pyraclostrobin and boscalid

Isolate ID	Country	Crop	Year of collection	MIC pyraclostrobin	MIC boscalid
87	DE	Nectarine	2005	0.156	0.156
89	IT	Nectarine	2005	0.01	0.039
113	IT	Nectarine	2005	0.039	0.625
120	IT	Nectarine	2005	0.625	0.156
121	IT	Nectarine	2005	0.039	0.156
122	IT	Nectarine	2005	0.039	0.01
123	IT	Nectarine	2005	0.156	0.156
124	IT	Nectarine	2005	0.039	0.156
125	IT	Nectarine	2005	0.039	0.039
126	IT	Nectarine	2005	0.039	0.039
128	IT	Nectarine	2005	0.039	0.156
129	IT	Nectarine	2005	0.039	0.039
130	IT	Nectarine	2005	0.156	2.5
131	IT	Nectarine	2005	0.156	0.039
132	IT	Nectarine	2005	0.039	0.156
133	IT	Nectarine	2005	0.039	0.625
134	IT	Nectarine	2005	0.156	0.156
135	IT	Nectarine	2005	0.625	0.625
136	IT	Nectarine	2005	0.156	0.625
42	DE	Peach	2005	0.156	0.039
73	FR	Peach	2005	0.156	0.156
82	DE	Peach	2005	0.156	0.039
83	DE	Peach	2005	0.156	0.156
151	FR	Peach	2005	2.5	2.5
155	FR	Peach	2005	0.039	0.039
19	DE	Plum	2005	0.625	0.625
20	DE	Plum	2005	0.625	2.5
23	DE	Plum	2005	2.5	2.5
24	DE	Plum	2005	0.625	0.625
25	DE	Plum	2005	0.156	0.039
26	DE	Plum	2005	2.5	2.5
70	DE	Plum	2005	0.625	0.156
71	DE	Plum	2005	0.625	0.156
72	DE	Plum	2005	0.156	0.156
74	DE	Plum	2005	0.156	0.156
140	FR	Plum	2005	2.5	2.5
142	FR	Plum	2005	0.625	2.5
143	FR	Plum	2005	0.625	0.625
146	FR	Plum	2005	0.625	0.625
159	FR	Plum	2005	0.625	2.5
160	FR	Plum	2005	0.156	0.625
161	FR	Plum	2005	2.5	0.625
162	FR	Plum	2005	0.625	0.156
163	FR	Plum	2005	0.625	0.625
164	FR	Plum	2005	0.156	0.039
165	FR	Plum	2005	0.625	0.625
166	FR	Plum	2005	0.156	0.156
167	FR	Plum	2005	0.625	0.156
168	FR	Plum	2005	0.625	0.039
21	DE	Sour cherry	2005	0.039	0.01
22	DE	Sour cherry	2005	0.156	2.5
27	DE	Sour cherry	2005	0.625	0.156
28	DE	Sour cherry	2005	0.625	0.039

Isolate ID	Country	Crop	Year of collection	MIC pyraclostrobin	MIC boscalid
29	DE	Sour cherry	2005	0.156	0.625
30	DE	Sour cherry	2005	0.156	0.039
31	DE	Sour cherry	2005	0.156	0.039
33	DE	Sour cherry	2005	0.156	0.625
34	DE	Sour cherry	2005	0.625	0.039
35	DE	Sour cherry	2005	0.039	0.625
37	DE	Sour cherry	2005	0.625	0.039
38	DE	Sour cherry	2005	2.5	2.5
39	DE	Sour cherry	2005	0.156	0.039
40	DE	Sour cherry	2005	0.625	2.5
41	DE	Sour cherry	2005	0.156	0.039
43	DE	Sour cherry	2005	0.156	0.039
44	DE	Sour cherry	2005	0.156	0.156
45	DE	Sour cherry	2005	0.039	0.039
47	DE	Sour cherry	2005	0.156	0.156
49	DE	Sour cherry	2005	0.156	0.156
50	DE	Sour cherry	2005	0.625	0.156
51	DE	Sour cherry	2005	0.156	0.039
52	DE	Sour cherry	2005	0.156	2.5
54	DE	Sour cherry	2005	0.156	0.039
55	DE	Sour cherry	2005	0.039	0.156
56	DK	Sour cherry	2005	0.039	0.01
57	DK	Sour cherry	2005	0.039	0.039
58	DK	Sour cherry	2005	0.625	0.156
59	DK	Sour cherry	2005	0.625	0.156
60	DK	Sour cherry	2005	0.156	0.625
61	DK	Sour cherry	2005	0.156	0.625
62	FR	Sour cherry	2005	0.625	0.156
63	FR	Sour cherry	2005	0.625	0.625
64	FR	Sour cherry	2005	0.156	0.156
66	FR	Sour cherry	2005	0.156	0.156
67	FR	Sour cherry	2005	0.156	0.625
68	FR	Sour cherry	2005	0.01	0.039
69	FR	Sour cherry	2005	0.156	0.625
75	FR	Sour cherry	2005	0.625	0.625
76	FR	Sour cherry	2005	0.156	0.625
77	FR	Sour cherry	2005	0.156	0.156
78	DE	Sour cherry	2005	0.156	0.039
79	FR	Sour cherry	2005	0.01	0.01
81	DK	Sour cherry	2005	0.039	0.156
84	DE	Sour cherry	2005	0.039	0.156
85	DE	Sour cherry	2005	0.01	0.039
86	DE	Sour cherry	2005	0.156	0.039
90	DE	Sour cherry	2005	0.156	0.156
169	DE	Sour cherry	1994	0.039	0.156
170	DE	Sour cherry	1994	0.156	0.156
36	DE	Sweet cherry	2005	2.5	0.625
46	DE	Sweet cherry	2005	0.156	0.039
80	DE	Sweet cherry	2005	0.039	0.625
MIC median				0.156	0.156
MIC minimum				0.01	0.01
MIN maximum				2.50	2.50

Table 4: Minimum inhibitory concentration (MIC) values in ppm [mg l^{-1}] a.i. of isolates of *Monilinia fructigena* from different European countries and different plant hosts towards pyraclostrobin and boscalid

Isolate ID	Country	Crop	Year of collection	MIC pyraclostrobin	MIC boscalid
1	DE	Apple	2004	0.625	0.625
2	DE	Apple	2004	0.156	0.625
3	DE	Apple	2004	0.156	2.5
4	DE	Apple	2004	0.156	2.5
5	DE	Apple	2004	0.156	0.156
93	DE	Apple	2005	0.156	0.156
94	DE	Apple	2005	0.156	0.156
95	DE	Apple	2005	0.156	0.625
96	DE	Apple	2005	0.156	0.156
97	DE	Apple	2005	0.156	0.156
105	DE	Apple	2005	0.625	0.156
106	DE	Apple	2005	0.156	0.156
107	DE	Apple	2005	0.156	0.156
88	IT	Nectarine	2005	0.625	0.156
6	FR	Peach	2005	0.156	0.156
7	FR	Peach	2005	0.039	0.156
8	FR	Peach	2005	0.156	0.625
9	FR	Peach	2005	0.156	0.156
10	FR	Peach	2005	0.039	0.039
11	FR	Peach	2005	0.156	0.156
12	FR	Peach	2005	0.156	0.156
13	FR	Peach	2005	0.156	0.156
14	FR	Peach	2005	0.156	0.156
15	FR	Peach	2005	0.156	0.039
16	FR	Peach	2005	0.156	0.625
18	DK	Plum	2005	0.156	0.156
108	DE	Plum	2005	0.156	0.039
109	DE	Plum	2005	0.156	0.156
110	DE	Plum	2005	0.039	0.156
111	DE	Plum	2005	0.625	0.039
112	DE	Plum	2005	0.156	0.039
17	DE	Sour cherry	2005	0.039	0.156
98	DE	Sweet cherry	2005	0.156	0.156
99	DE	Sweet cherry	2005	0.156	0.625
100	DE	Sweet cherry	2005	0.156	0.625
101	DE	Sweet cherry	2005	0.156	0.156
102	DE	Sweet cherry	2005	0.039	0.156
MIC median				0.156	0.156
MIC minimum				0.039	0.039
MIN maximum				0.625	2.5

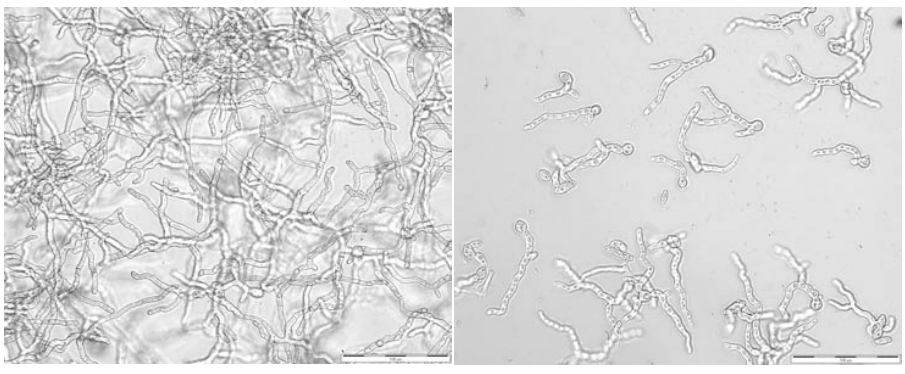

Fig. 1: Growth of *Monilinia laxa* in a microtiterplate without fungicide (left) and 2.5 ppm boscalid (right) after 5 days (bar = 100 µm). Germination was possible in 2.5 ppm boscalid, but further development was stopped.

Table 5: Variability of the sensitivity test. Isolate 29 of *Monilinia laxa* was tested in seven independent tests. The growth was evaluated in 5 classes (0, no growth; 1, less than 50% of control; 2, ~50% of control; 3, > 50% of control; 4, same growth as control). Minimum inhibitory concentration (MIC) values are the lowest concentrations with no growth. MIC values in ppm [mg l^{-1}] active ingredient

Isolate number	Pyraclostrobin concentration								MIC
	Control	0,002	0,01	0,039	0,156	0,625	2,5	10	
29	4	3	2	1	1	0	0	0	0.625
29	4	3	3	2	1	0	0	0	0.625
29	4	2.5	1	1	0	0	0	0	0.156
29	4	3	2	1	0	0	0	0	0.156
29	4	2	2	1	0	0	0	0	0.156
29	4	3	2	1	0	0	0	0	0.156
29	4	3	2	1	1	0	0	0	0.625
Isolate number	Boscalid concentration								MIC
	Control	0.002	0.01	0.039	0.156	0.625	2.5	10	
29	4	4	3	2	1	0	0	0	0.625
29	4	4	3	2	1	1	0	0	2.5
29	4	4	3	2.5	1	1	0	0	2.5
29	4	4	3	2	1	0	0	0	0.625
29	4	4	3	2	1	0	0	0	0.625
29	4	4	3	2	1	0	0	0	0.625
29	4	4	3	2	1	0	0	0	0.625

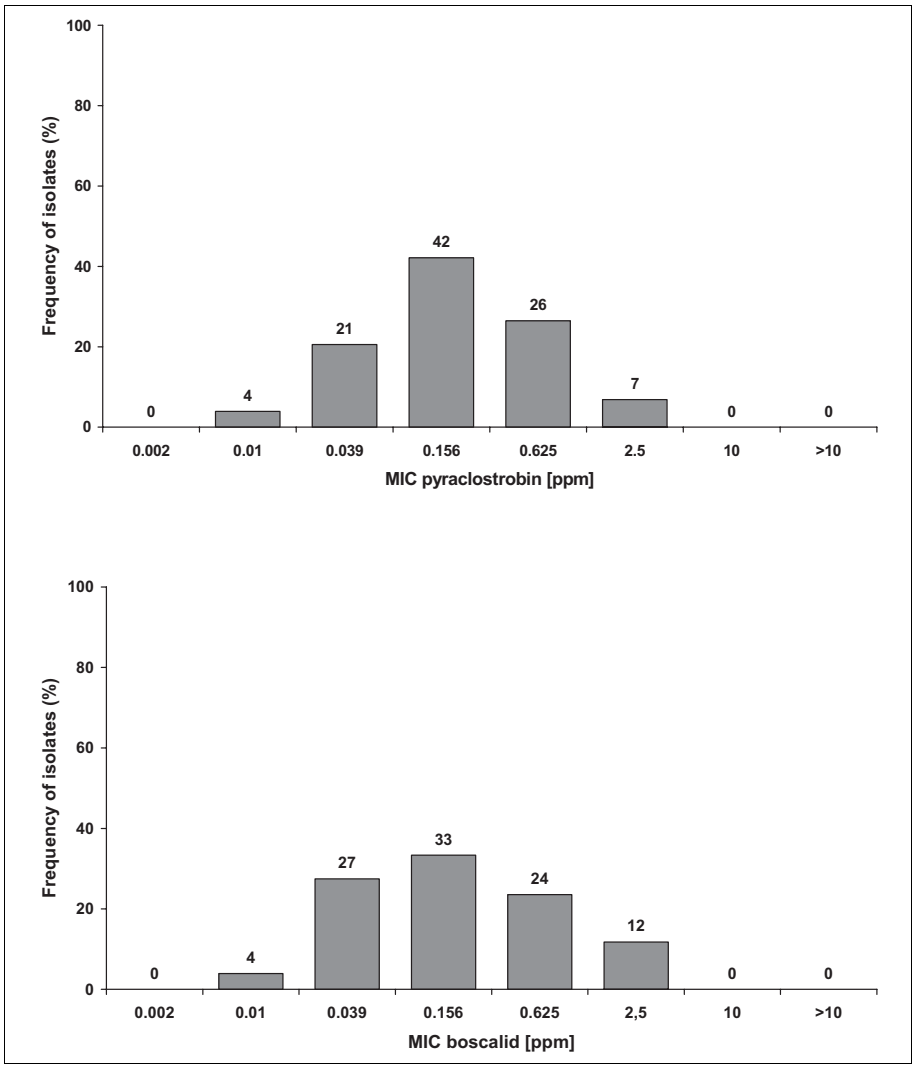

Fig. 2: Baseline sensitivity of *Monilinia laxa* to pyraclostrobin and boscalid. The diagram shows the frequency distribution of the minimum inhibitory concentration (MIC) values of 102 isolates with the described microtiter assay.

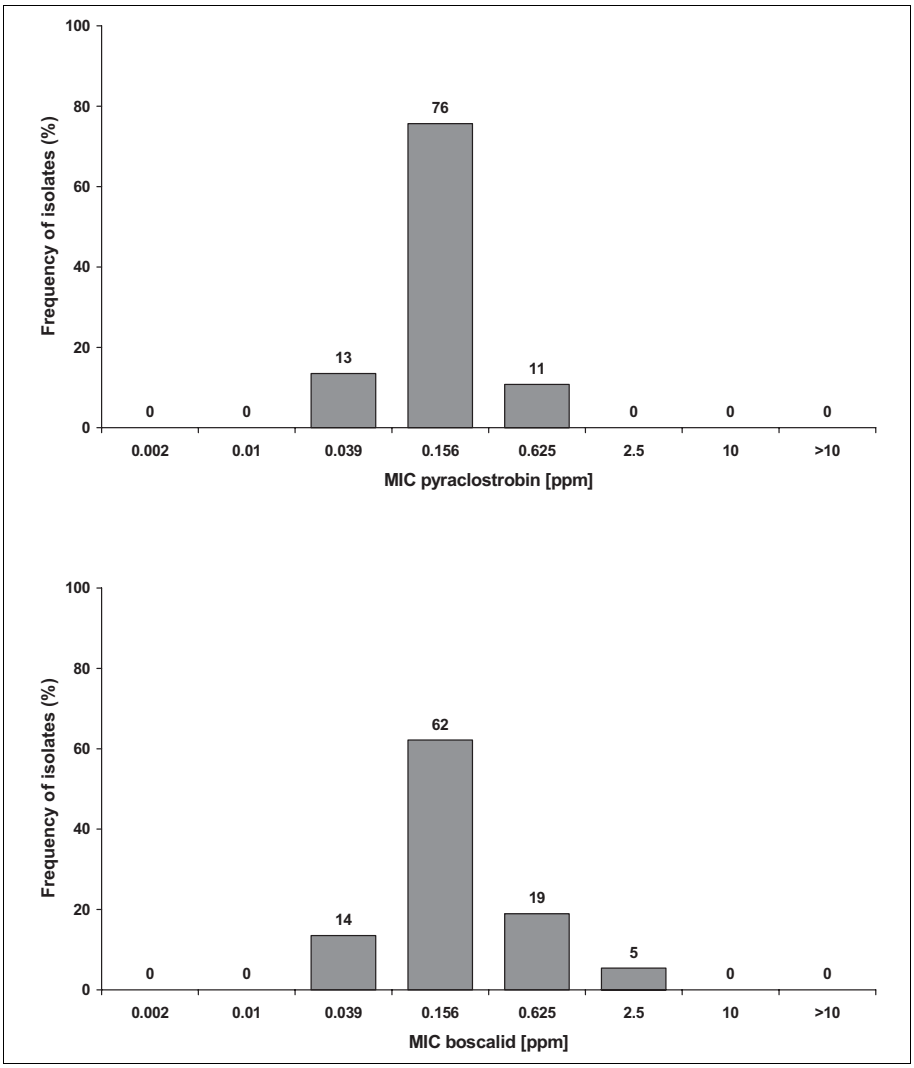

Fig. 3: Baseline sensitivity of *Monilinia fructigena* to pyraclostrobin and boscalid. The diagram shows the frequency distribution of the minimum inhibitory concentration (MIC) values of 37 isolates with the described microtiter assay.

for *M. laxa*. However, at 2.5 ppm the growth of all isolates of *Monilinia* spp. was inhibited. Therefore, this concentration could be used as a discriminatory dose for identification of resistance in *Monilinia* spp. to boscalid or pyraclostrobin.

For pyraclostrobin, an alternative monitoring assay would be a PCR assay for detection of the G143A or F129L mutation in the cytochrome b gene since these two mutations are the only ones which are of relevance for QoI resistance development. For anilides such as boscalid, the mutations, which could be responsible for a target site resistance, are not identified and no resistant strains of the target fungi, like *Monilinia* spp., *Botrytis cinerea*, *Erysiphe necator* or *Venturia inaequalis*, could be identified up to now. Studies with laboratory mutants indicate that different mutations in different fungi could confer resistance to anilides (Skinner et al. 1998; Ito et al. 2004). However, resistance found in laboratory mutants does not always reflect the natural condition, as is the case for dicarboximides, for example (Yamaguchi and Fukimura 2005). Therefore a genetic assay for resistance monitoring of boscalid is not possible at the moment.

The data show that the described method is appropriate to assess the sensitivity of *M. laxa* and *M. fructigena* to pyraclostrobin and boscalid and should be used for sensitivity studies.

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