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Sensitivity of *Botrytis cinerea* to propamidine: in vitro determination of baseline sensitivity and the risk of resistance

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Abstract The baseline sensitivity of *Botrytis cinerea* to propamidine and assessment of the risk of propamidine resistance in vitro are presented in this article. The baseline sensitivities of 41 wild-type strains were distributed as a unimodal curve with EC_{50} values of mycelial growth ranging from 0.182 to 1.460 μg ml^{-1}, with a mean of 0.79±0.27 μg ml^{-1}. A total of 10 resistant mutants, obtained from one parental strain, were induced by UV irradiation and selected for resistance to propamidine with an average frequency of 1.98×10^{-9} and 0.025 respectively. These mutants were divided into three classes of resistant phenotypes with low (LR), moderate (MR) and high (HR) levels of resistance, determined by the EC_{50} values of 5.0–15.0 μg ml^{-1}, 15.1–75.0 μg ml^{-1} and more than 75.0 μg ml^{-1} respectively. Neither positive cross-resistance nor negative cross-resistance was detected between propamidine and the fungicides, benzimidazole carbendazim, anilino-pyrimidine pyrimethanil, dicarboximide iprodione or procymidone. All 10 propamidine-resistant mutants showed reduced mycelial growth in vitro, sporulation, spore germination and pathogenicity when compared with the parental strain. These studies demonstrated that propamidine possesses a low risk of resistance developing. However, as *B. cinerea* is a high-risk pathogen, appropriate precautions against resistance development should be taken.

Keywords *Botryotinia fuckeliana* · Botryticides · Aromatic diamidines · Fungicide-resistance

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Introduction

Botrytis cinerea (teleopmorph *Botryotinia fuckeliana*), is a ubiquitous fungus which causes grey mould on many important economically crops worldwide (Prins et al. 2000). In addition, grey mould is one of the most serious vegetable diseases in greenhouses in China (Ji et al. 1998). Management of this disease often depends on frequent application of fungicides (Rosslenbroich and Stuebler 2000). Unfortunately, *B. cinerea* is a classical 'high risk pathogen' with regards to resistance management (Brent and Hollomon 1998). The development of resistance to fungicides is a serious problem in controlling grey mould disease. Many botryticides such as benzimidazoles, diethofencarb, dicarboximides, anilinopyrimidines and hydroxyanilide have already been confirmed to face the possibility of resistance development in Europe and China (Beever

et al. 1989; Latorre et al. 1994; Zhou et al. 1994; Pappas 1997; Leroux et al. 1999; Dianez et al. 2002; Baroffio et al. 2003; Zhang et al. 2003; Ji et al. 2003). Moreover, multiple fungicide resistance of *B. cinerea* has appeared in Shandong province in China (Ding et al. 2001). As a consequence, new types of fungicides, which have no cross-resistance with botryticides already in use, are required as the candidates for grey mould managing (O'Neill et al. 2004).

Propamidine, 4,4′-[1,3-propanediylbis(oxy)]bis-benzenecarboximidamide (Fig. 1), is an aromatic diamidine compound. The medicinal activity of propamidine was first reported in 1939 (Lourie and Yorke 1939) and was used for the treatment of *Acanthamoeba* keratitis and other corneal infections (Bailly et al. 1997). Its agricultural activity was first reported in 2005 and was patented as a novel fungicide against grey mould (Chen et al. 2005). It has been registered for control of *B. cinerea* on tomato and cucumber in China. Little is known about the mechanism of propamidine to *B. cinerea* and it is also unclear about cross-resistance with other fungicides such as pyrimethanil, iprodione, procymidone and carbendazim.

In order to study the possibility of development of resistance to propamidine, a series of tests were conducted with the objectives to: (a) establish baseline sensitivity of *B. cinerea* to propamidine; (b) induce resistant-mutants and to determine the mutation frequency and the resistant level of *B. cinerea* to propamidine; (c) elucidate the cross-resistance relationships between propamidine and other fungicides and (d) compare the fitness and pathogenicity between propamidine resistant mutants and their parental strain.

Materials and methods

Isolation of *Botrytis cinerea* and culture conditions

The wild-type strain of *B. cinerea* was isolated from infected green tomatos (*Lycopersicum esculentum*) collected from five commercial tomato fields in the cities of Xi'an, Xianyang, Baoji, Weinan, Yangling in Shaanxi Province in China. All the isolates were grown on potato dextrose agar (PDA) in a climate chamber at 22C with 14 h light produced by fluorescent lamps with a 70% relative humidity. Each isolate was purified by transferring a single conidium to a PDA plate. The purified strains were maintained in glass tubes on PDA at 4°C in dark for long term storage.

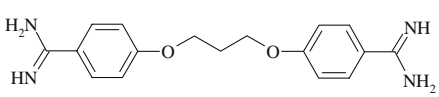

Fig. 1 Chemical structure of propamidine

Fungicides

Propamidine for resistant induction and bioassay tests was technical grade (≥97%) provided by the Research & Development Center of Biorational Pesticide in Northwest Agriculture and Forestry University (China). Technical grade (≥95%) benzimidazole carbendazim, anilino-pyrimidine pyrimethanil, dicarboximide iprodione and procymidone for the cross-resistance tests were provided by Jiangsu Branch of National Pesticide Research & Development South Center (China). Propamidine was dissolved in sterile distilled water to 1 mg ml^{-1} as a stock solution. The other fungicides were dissolved in acetone, except carbendazim, which was dissolved in 0.1 M HCl, as a stock solution. In all cases, the final amount of solvent (except water) was no more than 1% (v/v) for the actual application.

Propamidine baseline-sensitivity assay in vitro

The fungicidal sensitivity of the wild-type strains in vitro was assessed by transferring plugs (5 mm in diameter) from 3-days-old colony margins to PDA in 9-cm Petri dishes amended with 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 or 3.2 μg propamidine ml^{-1}. Each concentration was replicated three times and the experiment was performed twice. The plates were incubated at 22C for 3 days in the dark. Mean colony diameter (minus the diameter of the inoculation plug) was measured for each treatment and expressed as percentage growth inhibition. For each strain, a linear regression of percentage inhibition related to mycelial growth in the control against the $\log_{10}$ transformation for each concentration of propamidine was obtained. The median effective concentration (EC_{50}) value was calculated with the regression equation for each strain.

Induction of resistant mutants

One wild-type sensitive strain, 091, was selected as the parental strain for induction of resistant mutants.

Conidial suspensions (5.6×10^7 conidia ml^{-1}) of the selected parental strain were obtained from 15-days-old PDA plates cultures. The suspension was spread 0.1 ml per dish on PDA medium containing 10 μg ml^{-1} propamidine in 9-cm Petri dishes. PDA plates with no fungicide used as controls to calculate the frequency of mutation. After incubation at 22C for 2 h in darkness, dishes were placed 20 cm from a 254-nm UV lamp (25 w) for 55 s and then incubated in darkness at 22°C for 5 days, to enable resistant colonies to appear. The selected resistant isolates were transferred on to PDA containing 10 μg ml^{-1} propamidine for resistance identification. The number of germinated spores on control plates was quantified after UV irradiation for 55 s and incubation at 22°C for 12 h in order to calculate the frequency of mutation. Frequency of mutation was calculated as: number of resistant mutants / (mean number of germinated spores on each plate × number of propamidine-amended plates used to obtain mutants). The level of fungicide resistance (resistance factor) was equal to the EC_{50} for the resistance phenotype/EC_{50} of the sensitive wild-type parental isolate.

Selection for resistance to propamidine

Four mycelial plugs (5 mm in diameter) from 3-days-old colony margins of the selected parental strain (091) were inoculated inverted on each PDA plate containing 10 μg ml^{-1} propamidine. After incubation at 22C for 10 days, spontaneous and fast growing sectors from inhibited colonies on PDA plates were collected. Each resistant mutant was identified on PDA plates with 10 μg ml^{-1} propamidine. In order to keep the resistant strains selected by propamidine stable, a single conidium culture was used. The frequency of mutation was calculated as: number of resistant mutants / (number of mycelial plugs inoculated on each plate × number of propamidine-amended plates used to obtain mutants). The resistance factor of each resistant strain was calculated as mentioned above.

Determination of fitness parameters

Mycelial growth rate, sporulation and spore germination as the fitness parameters of a resistant strain were tested. To determine fungal growth rate, mycelial plugs of each strain were inoculated in the centre of PDA plates without propamidine. After incubation at 22C for 3 days in dark, colony diameter of each strain was measured. To determine conidial production, a 0.1 ml conidial suspension (6.0×10^6 conidia ml^{-1}) of each strain was sprayed onto PDA plates without propamidine. After incubation at 22C with 14 h day^{-1} illumination and 70% relative humidity for 15 days, the conidia of each strain were washed off with sterile distilled water and filtered through cheesecloth. The volume of the conidial suspension was adjusted to 20 ml with sterile distilled water and the concentration of the conidial suspension was measured with a Neubauer haemocytometer. The concentration was expressed as the number of conidia per square centimeter of the plate culture. Spore germination was determined by calculating the germination percentage on 2% water agar plates (WA). Aliquots of a 30 μl suspension with 1×10^5 conidial ml^{-1} were spread onto 2% WA plates. Five plates for each isolate were used, and the experiment was repeated three times. After 10 h of incubation at 22C, germinating conidia were counted under a microscope and germination percentage was calculated.

Pathogenicity tests on tomato leaves

Pathogenicity of the resistant strains was determined by measuring the diameter of the lesions caused by each strain on tomato leaves. Tomato leaves were collected from the tip of the branch of 30 days old plants and were placed in the 9-cm Petri dishes on a wet filter paper. The petioles of the leaves were wrapped with wet absorbent cotton for moisture retention. The mycelial plugs from a 3-days-old colony margin of each strain was inoculated on the centre of the fresh leaves. After incubation with 90% humidity at 22C in dark for 3 days, the diameter of the lesion on inoculated leaves was measured for statistical analysis as mentioned below.

Cross-resistance

The mycelial plugs (5 mm in diameter) from 3-days-old colony margins of the resistant mutants and their parental strain were inoculated on PDA plates with a series of concentrations of carbenda-

zim, pyrimethanil, iprodione and procymidone. Colony diameters were measured after incubation at 22C for 3 days and inhibition rates and EC_{50} values were calculated as mentioned above. The sensitivity of resistant strains to propamidine and to the other four fungicides was compared and correlation analysis was adopted in analyzing cross-resistance.

Statistical analysis

Analysis was conducted using with the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA). Linear regression analysis was performed using the REG procedure of SAS and EC_{50} values were calculated according to the dose response curves. LSD was performed using ANOVA of SAS in order to assess differences in mycelial growth rate, sporulation, spore germination and pathogenicity.

Results

Baseline sensitivity to propamidine

A total of 41 *B. cinerea* strains isolated from the field near different cities in China were tested for their sensitivity to propamidine. The EC_{50} values for sensitive strains ranged from 0.182 to 1.460 μg ml^{-1} and the frequency distribution represented a unimodal curve (Fig. 2). The mean EC_{50} value was 0.79± 0.27 μg ml^{-1}, representing a range-of-variation factor of 8.0. None of the 41 strains showed resistance to propamidine. The mean EC_{50} could be used as a baseline for observing the sensitivity in *B. cinerea* populations to propamidine.

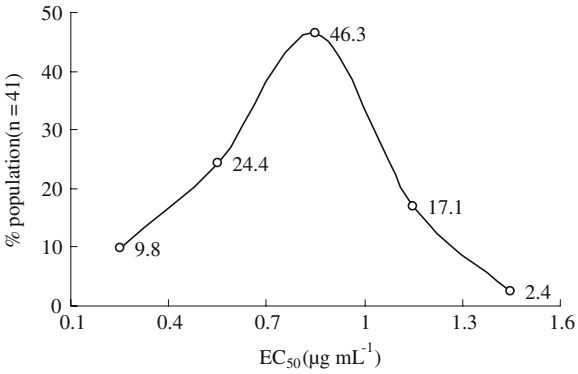

Fig. 2 In vitro sensitivity of 41 field strains of *B. cinerea* to propamidine

Resistant mutants and resistant level

The results of the resistant induction test in vitro showed one mutant was obtained by UV irradiation and nine mutants were selected by fungicide with the frequency 1.98×10^{-9} and 0.025, respectively. Although these mutants were able to grow on PDA plates containing 10 μg ml^{-1} propamidine, while their parental strain could not, changes in sensitivity to propamidine were small, with a resistance factor ranging from 3.9 to 14.8 (Table 1). Based on the standard of FAO (Anon 1982), the resistant mutants were divided into low (LR), moderate (MR) and high (HR) resistant levels, determined by the EC_{50} values of 5.0–15.0 μg ml^{-1}, 15.1–75.0 μg ml^{-1} and more than 75.0 μg ml^{-1}, respectively. Eight mutants belonged to LR, 2 mutants to MR and none of the mutants belonged to HR.

Fitness parameters and pathogenicity of resistant mutants

The growth rates and sporulation of all the mutant strains in vitro were significantly (P=0.05) lower than their sensitive parental strain, except F091-29, which was similar to its parental strain. In addition, spore germination of each propamidine-resistant mutant was substantially lower than their parental strain. The pathogenicity of the resistant mutants, UV091-1 and F091-16, which belongs to the MR level, possessed lower infection ability than their parental strain and LRs (Table 2). These results suggest that the propamidine-resistant mutants had reduced fitness.

Cross-resistance

Comparison of the sensitivities of propamidine-resistant mutants and their wild-type parental strain showed little correlation between sensitivities to propamidine and the other fungicides, benzimidazoles (carbendazim), anilino-pyrimidines (pyrimethanil), dicarboximides (iprodione and procymidone), indicating neither positive cross-resistance nor negative cross-resistance between propamidine and the tested fungicides (Fig. 3).

Table 1 Effect of propamidine on the mycelial growth of wild-type parental strain of *B. cinerea* and propamidine-resistant mutants

Strains	Regression equation $y=bx+a$	Correlation coefficient(r)	EC_{50} value ($\mu g\ mL^{-1}$)[d]	Resistance factor[e]
091[a]	y=1.0368x+7.9398	0.9758	1.46	–
UV091-1[b]	y=5.2633x−2.0279	0.9930	21.64	14.8
F091-14[c]	y=2.7634x+1.8241	0.9736	14.10	9.7
F091-15	y=1.7492x+3.3392	0.9764	8.90	6.1
F091-16	y=6.0842x−2.3547	0.9887	16.17	11.1
F091-17	y=1.9670x+3.4226	0.9914	6.34	4.3
F091-25	y=1.8505x+3.4530	0.9633	6.85	4.7
F091-26	y=2.5392x+2.0470	0.9960	14.55	10.0
F091-27	y=3.0025x+2.1274	0.9969	9.05	6.2
F091-29	y=0.5390x+4.5948	0.9000	5.65	3.9
F091-31	y=2.0777x+2.8199	0.9934	11.20	7.7

[a] Wild-type parental strain

[b] UV indicates that the propamidine-resistant mutant was recovered by UV irradiation

[c] F indicates that the propamidine-resistant strain was inducted by selection for resistance to the fungicide

[d] Concentration to reduce growth by 50%

[e] Resistance factor = EC_{50} resistant mutants/EC_{50} sensitive parent

Table 2 Comparison of growth rate, sporulation, spore germination and pathogenicity between parental wild-type strain of *B. cinerea* and propamidine-resistant mutants

Strains	Sensitivity phenotype	Hyphal growth(mm)[a]	Sporulation ($\times 10^6$)[b]	Spore germination(%)[c]	Lesion diameter(mm)[d]
091[f]	S	63.2 a[e]	14.8 a[e]	82.8 a[e]	19.6 a[e]
UV091-1[g]	MR	44.7 f	0.2 e	22.0 f	7.0 e
F091-14[h]	LR	60.3 b	2.8 c	37.9 e	19.1 a
F091-15	LR	33.0 h	6.3 b	59.0 c	11.1 c
F091-16	MR	26.2 i	1.9 cd	49.0 d	9.3 d
F091-17	LR	53.5 d	7.3 b	71.1 b	18.5 a
F091-25	LR	41.5 g	1.3 d	43.1 e	10.5 c
F091-26	LR	50.7 e	1.7 d	27.2 f	15.1 b
F091-27	LR	27.2 i	1.3 d	69.6 b	10.5 c
F091-29	LR	64.8 a	13.9 a	50.1 d	18.7 a
F091-31	LR	56.5 c	6.8 b	40.1 e	19.4 a

[a] Mean colony diameter measurements after 3 days of incubation (n=3)

[b] Mean number of conidia per cm^2 of colony after 15 days of incubation (n=3)

[c] Proportion of germinated conidia after 10 h incubation (n=3)

[d] Mean lesion diameter after 3 days of incubation (n=6)

[e] Values followed by the same letter with a column were not significantly different in LSD (least significant difference) tests at P=0.05

[f] Wild-type parental strain

[g] UV indicates that the propamidine-resistant mutant was recovered by UV irradiation

[h] F indicates that the propamidine-resistant strain was inducted by selection for resistance to the fungicide

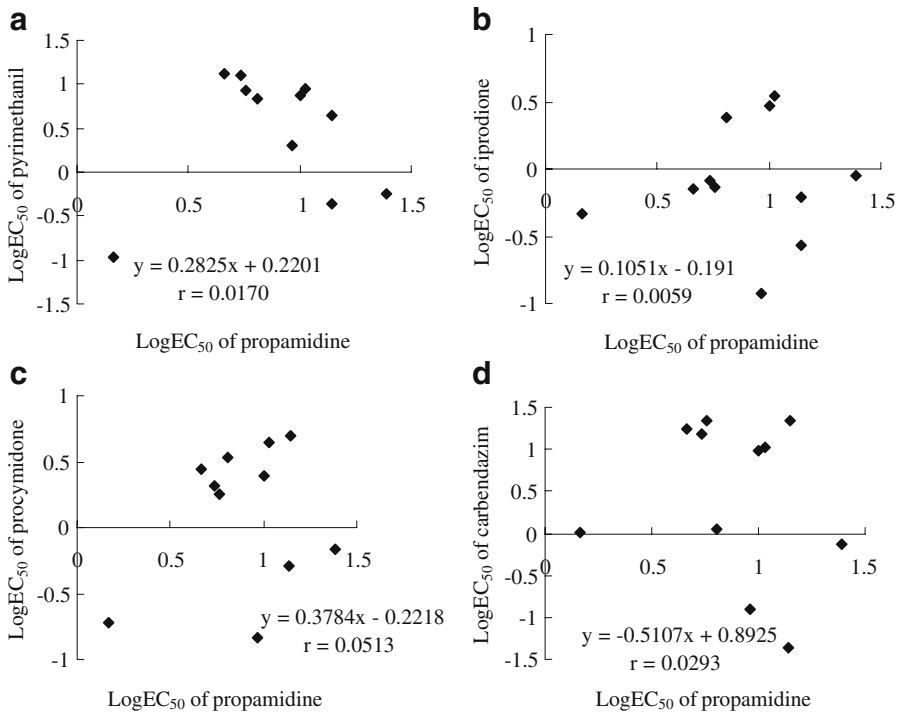

Fig. 3 Correlation between propamidine-resistant mutants and their parental sensitive strain to propamidine and the fungicides pyrimethanil (**a**), iprodione (**b**), procymidone (**c**) and carbendazim (**d**)

Discussion

A total of 41 field isolates of *B. cinerea* were tested for their baseline sensitivities to propamidine in this study. Their EC_{50} values showed a narrow range of distribution from 0.182 to 1.460 μg ml^{-1} for the most- and least-sensitive isolates respectively. Isolates collected from five geographical origins possessed similar sensitivity levels. Therefore, 0.79±0.27 μg ml^{-1} propamidine could be used as baseline for observing the sensitivity in *B. cinerea* populations to propamidine (Russell 2004).

According to this study, there was no cross-resistance between propamidine and other fungicides such as benzimidazoles, anilino-pyrimidines and dicarboximides, indicating that propamidine might be a new class of fungicide with a novel mechanism of action. Meanwhile, propamidine showed high activity in inhibiting both mycelial growth and spore germination (Chen et al. 2005). In a medical study, propamidine bound to the minor groove of DNA resulting in activity against *Acanthamoeba polyphaga* (Bailly et al. 1997). With the fact that more and more botryticides were reported to have resistant problems (Beever et al. 1989; Latorre et al. 1994; Zhou et al. 1994; Pappas 1997; Leroux et al. 1999; Dianez et al. 2002; Baroffio et al. 2003; Zhang et al. 2003; Ji et al. 2003), propamidine, which is able to be easily degraded and presents a favorable toxicological profile and environmental behavior (Chen et al. 2005), is a potentially alternative candidate for the management of grey mould disease.

In this study, propamidine-resistant mutants were obtained at low frequencies and with small resistance factors. Compared with the wild-type parental strain, most resistant mutants showed decreases in mycelial growth, sporulation, spore germination and pathogenicity. The fitness parameters and pathogenicity of MRs were smaller than LRs. The resistance risk of *B. cinerea* to propamidine is currently being assessed and it seems that propamidine possesses a low risk in controlling grey mould disease. According to the results of this study, resistance population(s) may develop slowly following field application. However, as *B. cinerea* is a high-risk pathogen (Brent and Hollomon 1998), fitness of natural populations may be promoted by continued selection. Therefore, appropriate precautions against resistance development should be taken.

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