Original Article

Comparison of Tetrachlorophthalide and Pentachlorobenzyl Alcohol with Chlobenthiazone and Other Melanin Inhibitors in the Mechanism of Rice Blast Control

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Tetrachlorophthalide and PCBA inhibited melanization of mycelia in *P. oryzae* at higher than 50 μM and 100 μM , respectively. But these concentrations were far above those of chlobenthiazone, tricyclazole, pyroquilon and pp-389 in inhibiting melanin biosynthesis. Moreover, while there was a good correlation between the activities of rice blast control and the capabilities of inhibiting appressorial melanization on leaf surface in chlobenthiazone and tricyclazole, this correlation was not observed in tetrachlorophthalide and PCBA. Microscopic observations showed that some of the appressoria were still melanized on tetrachlorophthalide and PCBA-treated leaves even at concentrations controlling disease, whereas the formation of infection pegs were completely prevented. This indicates that the antipenetrant action of both compounds can not be attributed to the inhibition of melanization of appressoria in *P. oryzae*. Although chlobenthiazone and related compounds were effective in preventing fungal penetration when applied until just before the initiation of appressorial melanization, tetrachlorophthalide and PCBA were only effective when the application was made earlier than this. Both compounds probably interfere with the early process of maturation of appressoria.

INTRODUCTION

There is a group of fungicides which specifically interfere with melanin biosynthesis in rice blast fungus, *Pyricularia oryzae*. The systemic blast fungicides, tricyclazole¹⁾ [5-methyl-1,2,4-triazolo(3,4-b)benzothiazole], pyroquilon²⁾ [1,2,5,6-tetrahydropyrrolo(3,2,1-*i,j*)quinolin-4-one] and pp-389³⁾ [4,5-dihydro-4-methyltetrazolo(1,5-a)quinazolin-5-one] are reported to inhibit melanization of this fungus.^{4–6)} We also showed that the fungicide, chlobenthiazone (S-1901),^{7,8)} 4-chloro-3-methyl-2(3*H*)-benzothiazolone, inhibits melanization in *P. oryzae* cultures⁹⁾ and in the appressoria formed on leaf surfaces.^{9,10)} Furthermore, among a series of compounds analogous to chlobenthiazone, the activities of this inhibition closely correlated to those of disease control.¹¹⁾ It

has been thought therefore that the inhibition of melanin biosynthesis is to be a primary site of action of the compounds and accounts for the controlling effect against blast disease.

More recently, the following fungicides were proposed to be inhibitors of melanin biosynthesis in *P. oryzae*^{12,13)}; tetrachlorophthalide¹⁴⁾ (4,5,6,7-tetrachlorophthalide) and PCBA¹⁵⁾ (2,3,4,5,6-pentachlorobenzyl alcohol). These fungicides have been known to prevent fungal penetration into the epidermis of the rice sheath from appressoria without suppression of other processes of infection,^{16,17)} but the mechanism of antipenetrant action of these compounds had remained unknown. Woloshuk *et al.* reported¹²⁾ however that these compounds inhibit melanin biosynthesis in *P. oryzae* and act on the pathogen as melanin inhibitors by a similar or an identical mech-

anism to protect plants by interfering with appressorial melanization required for epidermal penetration. However, we noticed that there is a considerable diversity in structure between chlobenthiazone and other melanin inhibitors, and both tetrachlorophthalide and PCBA. The present investigation was undertaken to compare the mechanism of antipenetrant action of both types of compounds.

MATERIALS AND METHODS

1. Compounds

Technical grade samples of the test compounds were synthesized according to the following procedures: chlobenthiazone, J. P. 80010589; tricyclazole, D.E.P.2509843 or J. P. 83021916; pyroquilon, U.S.P.3917838; pp-389, J.P.53037695. Respective compounds were purified by silica gel column chromatography followed by thin-layer chromatography (TLC). Tetrachlorophthalide was a gift of Kureha Chemical Industry Co., Ltd., Japan. PCBA was kindly supplied from Sankyo Co., Ltd., Japan. Structures of these compounds are given in Fig. 1.

2. Determination of Mycelial Melanization

P. oryzae Cav. isolates, Ken 60-19, SP-4 and SP-5, were employed as the test organisms. The effect of the compounds on melanization of the isolates was tested by an agar dilution method. Each compound dissolved in methanol containing 1% Tween 80 was added to a potato sucrose agar medium. Rates tested were 0, 0.1, 0.5, 1, 5, 10, 50 and 100 μ M. The final concentration of methanol did not exceed 1%. Inoculation of these isolates was conducted by placing 5 mm ϕ mycelial disks on the agar medium, and the inoculated plates were incubated at 27°C. Minimum inhibitory concentrations (MIC) of melanization against the test isolates were recorded 10 days after the inoculation. Inhibition of melanization was assessed visually by the appearance of red-brown pigments in the medium.

3. Determination of Appressorial Melanization

The test compounds as 5% emulsifiable concentrates were diluted with water to the desired concentrations and sprayed on the potted barley seedlings (*Hordeum vulgare* L., cv.,

Fig 1. Structures of compounds tested.

Kanto No. 6) of the two leaf stage. The spore suspension of *P. oryzae* (Ken 60-19) containing 10^8 spores/ml was then sprayed for inoculation after air-drying, and the plants were placed in the incubation room (28°C, >95% RH). After 4 days disease severities of the leaves were examined and fixed with a carnoy solution. Inhibition of appressorial melanization was observed under an optical microscope.

4. Effect of Application Time on Controlling Activity

The potted rice seedlings (*Oryza sativa* L., cv., Kinki No. 33) of the 2.8th leaf stage were inoculated by spraying a spore suspension (10^5 spores/ml) and placed in the incubation room. After an appropriate incubation period, these inoculated plants were sprayed with a fungicide suspension at a concentration of 2500 μ M, and then returned to the incubation room. Disease severity was determined as the percentage of infected leaf area after 4 days incubation.

The infection process of the fungus at respective application times of the compound was observed under an optical microscope after fixation with a carnoy solution.

RESULTS

1. Antimelanization Activity

Woloshuk *et al.*¹⁸⁾ reported that tetrachloro-

phthalide and PCBA inhibited mycelial melanization in *P. oryzae* (the isolate No. P-2) at concentrations as low as 0.4 and 1.9 μM , respectively. However, at these concentrations, we could not find any influence of these compounds on mycelial melanization in three test isolates of *P. oryzae*. Tetrachlorophthalide and PCBA respectively required more than 50 μM and 100 μM for inhibition (Table 1). On the contrary, chlombenthiazole, tricyclazole, pyroquilon and pp-389 inhibited mycelial melanization at much lower concentrations as compared with tetrachlorophthalide and PCBA (Table 1). Mycelial melanization of the isolates on the agar medium was the most sensitive to tricyclazole among the melanin inhibitors, and was completely inhibited at concentrations of more than 0.5 μM . Inhibition of mycelial melanization by both chlombenthiazole and pyroquilon was attained at concentrations of more than 1.0 μM , and pp-389 required more

than 5 μM for inhibition.

2. Relation between Mycelial Melanization and Disease Control

When the activities of blast control on rice seedlings were compared among melanin inhibitors with those of inhibition of mycelial melanization on an agar medium, tetrachlorophthalide and PCBA differed from chlombenthiazole and others (see Table 1— isolate Ken 60-19 and Table 2). The former compounds inhibited mycelial melanization at concentrations similar to those required for disease control, whereas the latter inhibited melanization at much lower concentrations.

3. Relation between Appressorial Melanization and Disease Control

Woloshuk *et al.*¹⁸⁾ found that tetrachlorophthalide and PCBA inhibited appressorial melanization in *P. oryzae* as did tricyclazole and pyroquilon, and considered that the controlling effect of the former compounds against blast disease must be attributed to the inhibition of melanization of appressoria. Our experiments, however, indicated that while the ratios of numbers of melanized appressoria to unmelanized ones depended on the treated concentrations of tetrachlorophthalide and PCBA, a good correlation between the capabilities of inhibiting appressorial melanization and the activities of disease control was not obtained (Table 3). Even at concentrations which completely controlled disease development on barley seedlings, it was observed that some of the appressoria were still melanized but infection pegs from them were not formed.

Melanization of appressoria on barley leaves treated with chlombenthiazole and tricyclazole was affected at concentrations more than 3 $\mu\text{g/ml}$ and was completely inhibited at concentrations more than 125 $\mu\text{g/ml}$ and 62.5 $\mu\text{g/ml}$, respectively. Moreover, disease control was also shown at the same concentrations in both compounds. Thus the rates of the inhibition of appressorial melanization closely correlated with those of disease control.

4. Effect of Application Time

It was generally accepted that melanin inhibitors are not effective in controlling dis-

Table 1 Effect of blasticides on mycelial melanization in several isolates of *P. oryzae*.

Compound	Isolate		
	SP-4	SP-5	Ken 60-19
Tetrachlorophthalide	10.0 ^{a)}	50.0	50.0
PCBA	50.0	100.0	100.0
Chlombenthiazole	0.5	1.0	1.0
Tricyclazole	0.1	0.5	0.5
Pyroquilon	0.5	1.0	1.0
pp-389	1.0	5.0	5.0

^{a)} Concentration (μM) required for the inhibition of melanization

Table 2 Protective activity of blasticides.

Compound	ED ₉₀ (μM) ^{a)}
Tetrachlorophthalide	45.9
PCBA	94.7
Chlombenthiazole	15.6
Tricyclazole	7.9
Pyroquilon	19.7
pp-389	124.4

^{a)} *P. oryzae* Cav. isolate, Ken 60-19, was employed as the test pathogen. Concentration giving 90% of rice blast control (determined by employing rice seedlings as previously reported¹⁰⁾).

Table 3 Effect of various concentrations of blasticides on appressorial melanization of *P. oryzae* and disease development on barley seedlings.

Concentration ($\mu\text{g/ml}$)	Chlobenthiazone		Tricyclazole		PCBA		Tetrachlorophthalide	
	A	B	A	B	A	B	A	B
1000	0.0	100	0.0	100	35.9	100	45.3	100
500	0.0	100	0.0	100	67.9	100	58.2	100
250	0.0	100	0.0	100	71.1	87	78.0	100
125	0.0	100	0.0	100	76.5	69	74.1	100
62	1.3	100	0.0	100	88.5	55	78.6	98
31	3.2	98	1.8	100	83.1	32	82.1	83
15	9.5	83	7.2	96	88.7	0	83.5	57
7	31.2	52	10.5	85	85.6	0	82.8	19
3	34.3	36	28.2	63	87.3	0	82.0	0

A: % of appressorial melanization.

B: % of disease control (determined by employing barley seedlings as previously reported¹⁰⁾).

Fig. 2 Relation between the time of treatment after inoculation and the rate of disease control of blasticides.

ease development when the application is made after the infection is established. Experiments were therefore made to determine the time when the application became non-effective after inoculation and to compare tetrachlorophthalide and PCBA with chlobenthiazone and related compounds in this respect. As shown in Fig. 2, application of tetrachlorophthalide and PCBA up to 4 hr after the inoculation was highly effective in controlling the disease. Up to 4 hr, most of the spores accomplished germination and initiated to form appressoria in the present experimental conditions. The effectiveness of the application at 6 hr, however, was already incomplete and no control was obtained by the application at 8

hr. Microscopic observations indicated that immature appressoria were formed until 6 hr after the inoculation and grew to full size until 8 hr. A small percentage of the appressoria at 8 hr were in the early stage of melanization.

When the application of chlobenthiazone, tricyclazole and pyroquilone was made up to 6 hr after the inoculation, blast disease was completely controlled. But when these compounds were applied 8 hr or later after the inoculation, only slight or no control of the disease was obtained.

DISCUSSION

Specific characteristics of the action of melanin inhibitors, such as chlobenthiazone,

tricyclazole, pyroquilon and pp-389, in relation to the inhibition of melanization and disease control have been noticed to be; (a) hyphal melanization in *P. oryzae* is inhibited at concentrations well below those that affect mycelial growth,⁴⁾ and there is a good correlation between the activities of rice blast control and the capabilities to inhibit mycelial melanization of the pathogen on a nutrient agar,¹¹⁾ and (b) the rates of inhibition of appressorial melanization closely correlated to those of inhibition of appearance of infection pegs, and of disease development.^{10,11,18)} Although Woloshuk *et al.*¹²⁾ and Yamaguchi *et al.*¹³⁾ suggested that tetrachlorophthalide and PCBA are also included in this group, the present investigations apparently indicated that both the compounds were distinct from chlobenthiazone and other specific inhibitors of melanin biosynthesis in the mode of antipenetrant action. This was supported by the following results; (1) tetrachlorophthalide and PCBA did not have specific characteristics of the action of melanin inhibitors as mentioned above. (2) there was a difference of 2 hr between both tetrachlorophthalide and PCBA, and chlobenthiazone and other compounds in the time when the application became non-effective in controlling the disease after the inoculation.

Microscopic observations showed that the incubation time, when appressoria initiated to reach full size, coincided with that when the application of tetrachlorophthalide and PCBA became ineffective after the inoculation. However, the application of chlobenthiazone, tricyclazole and pyroquilon, were effective in preventing fungal penetration until appressorial melanization was initiated. These experimental results therefore suggest that tetrachlorophthalide and PCBA affect the process of appressorial maturation which occur prior to the inhibition of melanization.

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要 約

テトラクロロフサライド、ペンタクロロベンジルアルコールとクロベンチアゾンその他のメラニン生合成阻害剤のイネいもち病防除機構における比較

井上 悟, 前田清人, 植松多聞, 加藤寿郎
テトラクロロフサライドおよび PCBA は、イネいもち病菌の菌糸のメラニン化を、それぞれ 50 μM および 100 μM の濃度で阻害する。しかし、その阻害濃度は、メラニン生合成阻害剤、クロベンチアゾン、トリシクラゾー

ル、ピロキユロン, pp-389 などと比較し高かった。クロベンチアゾンおよびトリシクラゾールでは、イネいもち病防除効果と、付着器のメラニン化阻害程度間には良い相関が認められるが、テトラクロロフサライドおよびPCBA は穿入糸の形成を抑制するが、発病を阻害する濃度でも付着器のメラニン化を完全には阻害しなかった。以上の結果から、テトラクロロフサライドおよび

PCBA の穿入糸形成阻害は、メラニン生合成阻害によるものではないと考えられた。クロベンチアゾンおよび関連化合物は付着器のメラニン化開始直前までに処理すれば、いもち病菌の侵入を阻止するのに対し、両化合物はメラニン化開始よりさらに早く処理しなければ、効果がなくなることから、付着器のメラニン化以前の成熟過程に何らかの影響を及ぼすものと思われる。