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Original Article

Effects of Chlobenthiazone on the Infection Process by *Pyricularia oryzae**

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During the infection process of blast fungus, *Pyricularia oryzae*, chlobenthiazone (S-1901), 4-chloro-3-methyl-2(3*H*)-benzothiazolone, inhibited most effectively the appearance of infection pegs from the appressoria at concentrations higher than 10 μM. Melanization of the appressoria was also inhibited by the fungicide at the same concentrations. The rates of this inhibition at respective test concentrations closely correlated with those of inhibition of appearance of infection pegs from the appressoria and to those of control of disease development. On the other hand, when the chemical application was conducted later than 10 hr after the inoculation or the inoculation was made with injury (punch inoculation), the fungicide showed no efficacy in controlling rice blast even at a concentration as high as 2500 μM. These observations indicate that the protective activity of chlobenthiazone against rice blast can be attributed to the inhibition of appearance of infection pegs possibly in consequence of the inhibition of melanization of appressoria.

INTRODUCTION

Chlobenthiazone (S-1901), 4-chloro-3-methyl-2(3*H*)-benzothiazolone, is a new fungicide for the control of rice blast caused by *Pyricularia oryzae*.[1] Disease control can be accomplished by foliar or submerged application.[2] When the foliar application was made before the inoculation, chlobenthiazone showed a protective activity at concentrations higher than 1.5 μg/ml and completely controlled the disease at concentrations higher than 6.2 μg/ml.[2] However, when the application was conducted 16 hr after the inoculation, no effectiveness was obtained even at a concentration of 500 μg/ml.[2] Such a large difference in the effectiveness between preventive and curative application of chlobenthiazone suggests that the compound selectively interferes with an early stage during the infection process of *P. oryzae*.

Chlobenthiazone inhibits normal melanization of blast fungus at lower concentrations as compared with those inhibiting mycelial growth *in vitro*.[3] Melanization in the culture is extremely sensitive to the fungicide and inhibited at a concentration as low as 0.1 μM.[3] Furthermore, when several analogues of benzothiazolone were compared, it was recognized that there was a good correlation between the protective activities of rice blast control and the capabilities to inhibit mycelial melanization of *P. oryzae* on a nutrient agar.[4,5] The purpose of this research is to elucidate the effects of chlobenthiazone during the process of infection of *P. oryzae* and to understand how the inhibition of melanization relates to the process interrupted by chlobenthiazone.

MATERIALS AND METHODS

1. *Fungicide*

A 10% emulsifiable concentrate of chlobenthiazone was employed throughout the experiments.

* Mechanism of Rice Blast Control of Chlobenthiazone (Part 2)

2. Determination of Protective Activity

The potted rice seedlings (*Oryza sativa* L., var. Kinki No. 33) of the 2.8th leaf stage were inoculated with *P. oryzae* (Ken 60–19) by spraying a spore suspension containing 10^6 spores/ml. The inoculated plants were placed in an air-conditioned room maintained at 28°C under a relative humidity of more than 95% (described as the incubation room in the following). After an appropriate incubation period, these inoculated plants were sprayed with the fungicide suspension at a concentration of 2500 μM, and then returned to the incubation room. Disease severity was determined by the percentage of infected leaf area after 4 days incubation.

The infection process of the fungus at respective application times of the fungicide was observed under an optical microscope after fixation with carnoy solution (ethanol : chloroform : acetic acid, 6:3:1, v/v).

3. Observation of the Effects on the Infection Process

The potted barley seedlings (*Hordeum vulgare* L., var. Kanto No. 6) of the two leaf stage were used to determine the effects of chlobenthiazone on the infection process of *P. oryzae*.

The fungicide suspensions were sprayed on the plants. After air-drying, the plants were inoculated with the spore suspension containing 10^3 spores/ml by spraying, and then the plants were placed in the incubation room. After 4 days the leaves were fixed with carnoy solution for microscopic observations. A microscopical study was made on spore germination, elongation of germ tube, appressorial formation, appressorial melanization and formation of infection peg during the process of infection on barley epidermis. Formation of infection peg was determined by observation of developing hyphae emerging from the underside of appressorium or by carefully focusing down to a plane below the appressorium.

4. Punch Inoculation

The potted rice plants at the four leaf stage were treated with the fungicide suspensions. Inoculation of the pathogen was carried out by placing a droplet of the spore suspension containing 10^7 spores/ml onto the leaves with or without injury by using a punch instrument, and then those treated and inoculated plants were put in the incubation room for 1 day and then transferred to a greenhouse. Seven days after the inoculation, disease severity was determined by rating the infected area.

RESULTS

1. Effect of Application Time

The relation between the time of treatment after the inoculation and blast disease control with chlobenthiazone was shown in Fig. 1. Application of the fungicide up to 6 hr after the inoculation was highly effective in controlling the disease. Up to 6 hr, most of the spores accomplished germination and appressoria with a full size were formed on the tips of germ tubes. But, the appressoria did not have a characteristic dark brown color and fungal penetration was not yet observed. When the fungicide was applied 8 hr after the inoculation, only a little control of the disease was obtained. At this time, a few of the appressoria had a dark brown color but the others were not yet melanized. Penetration of infection pegs was observed only from melanized ones. Number of melanized appressoria increased with time, and penetration and invasion into adjacent epidermal cells were found to occur successively.

2. Infection Process

Experiments were made to determine the stage most sensitive to chlobenthiazone during

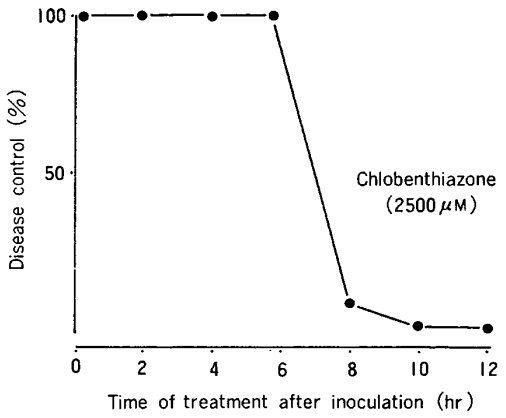

Fig. 1 Relation between the time of treatment after inoculation and blast disease control of chlobenthiazone.

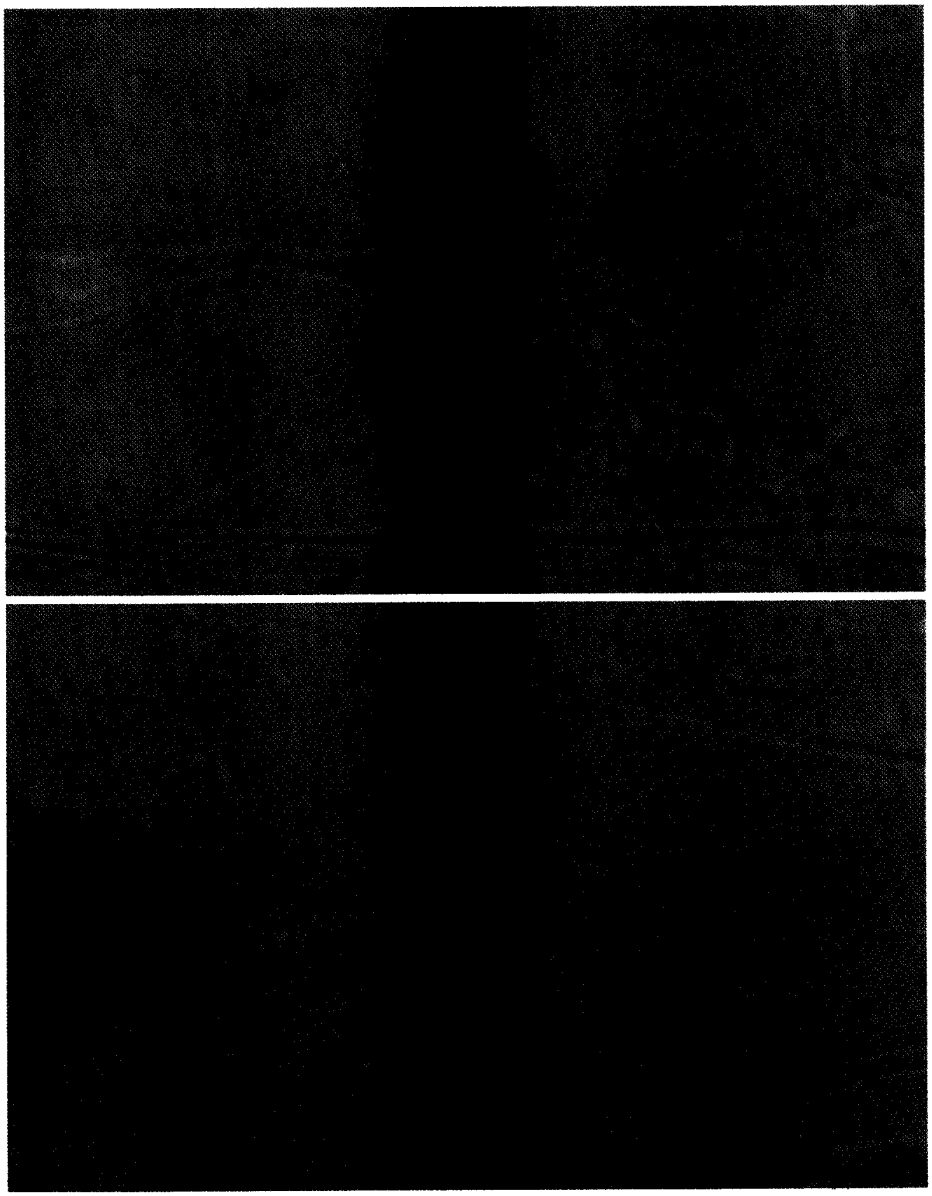

Fig. 2 The effect of chlobenthiazone on the process of infection of *P. oryzae* on barley leaf.
Arrows indicate a melanized appressorium (control, a) and unmelanized one (treated, b).
SP: spore, GT: germ tube, AP: appressorium, DH: developing hypha.

the infection process by using barley plants which have the advantage of ease of observation. Formation of infection pegs was most effectively inhibited by the fungicide and affected at concentrations more than 10 μM. Moreover, disease control was also shown at the same concentrations. In addition, melanization of appressoria was inhibited at concentrations of more than 10 μM. The ratio of number of melanized appressoria (see Fig. 2a) to unmelanized ones (see Fig. 2b) depended on the treated concentrations of the fungicide, and the rates of this inhibition closely correlated with those of control of disease development and with those of inhibition of appearance of infection pegs from appressoria. On the other hand, conidial germination, elongation of germ tubes and appressorial formation were hardly inhibited with the exception of treatment at the highest concentration (Table 1).

3. Punch Inoculation

The penetration of blast fungus into intact host cells requires appressoria and is accomplished by infection pegs, while in injured host

Table 1 Effects of chlobenthiazone on the infection process of blast fungus.

Chlobenthiazone[a] (μM)	Infection process[b]				Appressorial[c] melanization (%)	Disease control (%)
	Conidial germination (%)	Elongation of germ tube (%)	Appressorial formation (%)	Formation of infection peg (%)		
5000	2.3	1.2	0.2	0.0	0.0	100
1000	96.2	93.5	22.3	0.0	0.0	100
500	87.3	86.0	68.2	0.0	0.0	100
100	91.2	87.6	64.3	0.0	0.3	100
50	93.5	90.3	76.1	0.6	1.5	98
10	95.2	88.7	73.5	7.5	12.3	47
5	89.6	86.3	68.3	38.1	89.3	0
1	92.4	89.6	75.7	43.2	98.1	0
0	93.3	90.5	73.1	39.3	97.3	0

a) Aqueous suspensions of chlobenthiazone were sprayed on the leaves of barley at 60 ml/pot.
b) A number of germinated conidia, elongated germ tubes, appressoria or infection pegs was divided by a number of observed conidia, and the percentage was calculated.
c) Appressorial melanization was determined visually, and the percentage was calculated as follows; a number of melanized appressoria was divided by a number of observed appressoria.

cells, the fungus is able to penetrate by single hyphae which originates from germ tubes. Tests were made by injuring host plant cells to determine whether chlobenthiazone acts specifically on appressoria. As shown in Fig. 3, the protective effect of the fungicide against blast disease was not observed even at a concentration of 2500 μM by inoculation with injury (Fig. 3b), while the fungicide controlled the disease at concentrations more than 20 μM when the inoculation was made without injury (Fig. 3a).

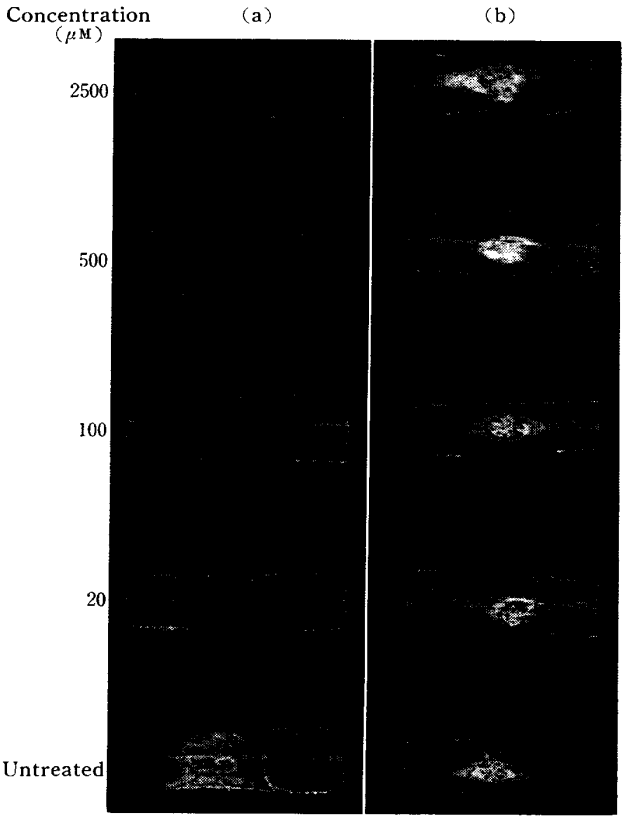

Fig. 3 The effect of inoculation with injury on the controlling activities of blast disease of chlobenthiazone.
(a) indicates the inoculation without injury and (b) indicates the inoculation with injury.

DISCUSSION

Non-fungitoxic blast protectants such as WL-28325 (2,2-dichloro-3,3-dimethylcyclopropane carboxylic acid),[6] probenazole (3-allyloxy-1,2-benzisothiazole 1,1-dioxide),[7] PCBA (2,3,4,5,6-pentachlorobenzyl alcohol),[8] tetrachlorophthalide (4,5,6,7-tetrachlorophthalide),[9] tricyclazole [5-methyl-1,2,4-triazolo-(3,4-b)-benzothiazole],[10] pyroquilon [1,2,5,6-tetrahydropyrrolo-(3,2,1-*i*,*j*)quinolin-4-one][11] and pp-389 [4,5-dihydro-4-methyltetrazolo-(1,5-a)quinazolin-5-one],[12] which do not inhibit major metabolic processes that are essential to fungal growth, are classified into two groups. The first group includes the compounds, PCBA,[13] tetrachlorophthalide,[14] tricyclazole,[15,16] pyroquilon[15] and pp-389,[15] which are known to

inhibit appearance of infection pegs from appressoria without the suppression of other processes. The second includes the compounds, WL-28325[17] and probenazole,[18–21] which are reported to induce resistance in the host plant by either eliciting fungitoxic components such as momilactons A and B, α-linolenic acid, 13-hydroxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid, or accentuating several enzyme activities such as peroxidase, phenylalanine ammonia lyase and catechol-*o*-methyltransferase, contributing to the formation of lignoid barriers around the infection site. Although the blast fungus has the ability to penetrate into host tissues in the presence of the second group of compounds, subsequent spreading of the developing hyphae to adjacent epidermal cells is halted.

The present investigation indicated that chlobenthiazone apparently belongs to the first group. The appressoria treated with the fungicide lost the ability to form infection pegs for penetration into the cuticle and epidermal cells of the host plant. Protective activity of the fungicide can be attributed to this effect. This was supported by the following observations; (a) chlobenthiazone had a high protective activity when the application was conducted up to 6 hr after the inoculation, but showed little or no controlling activity by the application at 8 hr or later, (b) the fungicide protected the intact plants but not the injured ones from the infection of *P. oryzae*.

On the other hand, chlobenthiazone inhibited normal melanization of *P. oryzae* at lower concentrations as compared with those inhibiting mycelial growth, and red-brown pigments were excreted into the medium. Melanization of this fungus was extremely sensitive to the fungicide and inhibited at a concentration as low as 0.1 μM.[3] Furthermore, based on the observation on barley leaves, it was apparent that the rates of inhibition of appressorial melanization closely correlated with those of the inhibition of appearance of infection pegs from the appressoria. Good correlation was also obtained between control of disease development and inhibition of appressorial melanization. Microscopic observation indicated that most of the appressoria reached full size until 8 hr after the inoculation and this was followed by appressorial melanization. The incubation time, when appressorial melanization was initiated, coincided with that when the application of chlobenthiazone became ineffective after the inoculation. These observations indicate that chlobenthiazone acts on the appressorial melanization prior to the inhibition of appearance of infection pegs, and the protective activity of the compound against blast disease can be possibly attributed to the inhibition of appressorial melanization.

Similar observations had been made with tricyclazole, pyroquilon and pp-389 on *Colletotrichum lindemuthianum*,[22] and with tricyclazole and pyroquilon on *P. oryzae*.[15,16] Woloshuk *et al.* showed that the former three compounds inhibited appressorial melanization of *C. lindemuthianum*[22] and prevented penetration with infection pegs into the epidermis of *Bryophyllum* leaf and bean cotyledon, and a similar inhibition of penetration of *P. oryzae* into *Bryophyllum* leaf epidermis was observed with tricyclazole and pyroquilon.[15] Tokousbalides *et al.*[23] showed further that these compounds inhibited hyphal melanization at concentrations well below those that affected mycelial growth as observed by chlobenthiazone. These similarities indicate that chlobenthiazone and these compounds act on the pathogen by a similar or identical mechanism.

Evidence for significance of appressorial melanization is obtained in albino mutants of *P. oryzae* and *C. lagenarium* respectively. Woloshuk *et al.*[24] observed that melanin-deficient mutants of *P. oryzae* which phenotypically resemble the tricyclazole-treated wild-type were nonpathogenic or rarely infected rice plants, and the mutants studied were genetically defective in the melanin biosynthetic pathway at the site blocked by tricyclazole in the wild-type. In *C. lagenarium*, Kubo *et al.*[25] showed that more than 90% of the albino mutant appressoria germinated laterally and formed secondary appressoria, and consequently less than 10% produced the infection pegs to penetrate nitrocellulose membranes, although appressoria of the parent strain rarely germinated laterally and more than 70% produced infection pegs to penetrate the

membranes. Furthermore they[26] also showed that the appressoria formed in the presence of tricyclazole did not produce infection pegs to penetrate cucumber cell wall and nitrocellulose membranes as found in the albino mutant. These observations supported the idea that melanization of appressoria plays an essential role in the appearance of infection pegs during the process of infection. However it is not fully understood how the effect of appressorial melanization relates to the inhibition of appearance of infection pegs. Further studies will be made along these lines in order to understand the detailed mechanism of antipenetrant action of chlobenthiazone.

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要　　約

Chlobenthioazoneのいもち病菌感染過程に及ぼす影響*

井上　悟，植松多聞，加藤寿郎

イネいもち病防除剤 Chlobenthiazone (S-1901) の本病原菌感染過程への影響をオオムギ幼苗を用いて検討した．本薬剤は低濃度で付着器からの穿入糸形成過程を阻害するとともに，付着器の褐変化（メラニン化）過程も同じように低濃度で阻害した．また，それらの阻害程度は，防除効果の程度ともよく相関した．しかし，胞子発芽，発芽管伸長および付着器形成の各過程は，高濃度で

* Chlobenthiazone のイネいもち病防除機構（第2報）

も阻害されなかった．一方，本薬剤は，メラニン化した付着器のいもち病菌に処理をした場合および植物体への侵入に付着器を必要としない傷接種の場合には，高濃度でも防除効果を発揮しなかった．以上の実験結果から，本薬剤のイネいもち病菌感染過程における作用機構は，付着器のメラニン化阻害による穿入糸の形成阻害であると考えられる．