Effects of Probenazole (Oryzemat[®]) on Rice Plants with Reference to Controlling Rice Blast*

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After treating rice plants by submerged application with probenazole no changes were noted in the siliciferous cells in the leaves, no morphological changes in the leaf tissue and no anti-fungal activity in leaf exudates were detected. When rice plants were treated by submerged application of probenazole and subsequently inoculated with the blast fungus (*Pyricularia oryzae* CAVARA) peroxidase activity (oxidation of phenolic substances) increased and an anti-conidial factor in the rice leaf increased. Also the increase of the factor was observed in rice plants treated by the chemical but not inoculated. A part of the appearance of induced resistant characteristics were thought to be similar to a reaction of resistant rice plant. The induction of resistant characteristics were largely inhibited by a heat shock upon rice plant indicating that the event was host mediated reaction. Probenazole (submerged application) appears to control the blast disease in rice by a combination of the events, such as inhibiting sensitive stages in the life cycle of the blast fungus and also activating the host defence.

INTRODUCTION

Probenazole (Oryzemat[®], 3-allyloxy-1,2-benzisothiazole-1,1-dioxide) was found to be an effective submerging pesticide for rice blast control and its efficacy was investigated.^{1,2)} The tentative conclusion from the earlier reports was that probenazole influenced several stages in the life cycle of the pathogen including a strong inhibitory effect against hyphal penetration into the leaf. These additional effects successfully controlled the disease.²⁾ As reported earlier, although probenazole did not show strong anti-fungal activity *in vitro*, strong inhibitory effects *in vivo* were demonstrated in an experiment on rice plant.²⁾ These data suggested the action of probenazole on

the host plant was to augment its resistant properties. As the next step, the influence of probenazole on the physiological properties of the rice plant was investigated on the bases of the previous findings. This paper describes that the treatment of rice plants with probenazole *via* root system causes increases in an activity of peroxidase and in formation of an unidentified anti-conidial factor.

MATERIALS AND METHODS

1. Cultivation of Rice Plant, Application of the Chemicals and Inoculation of Blast Fungus

These were performed according to the methods previously described except otherwise stated.^{1,2)}

2. Observations on the Siliciferous Motor Cell in the Host Leaf

The rice plants were harvested from the paddy field in an ordinary season with or without the application of the granular formul-

* Studies of the Rice Blast Controlling Agent of Benzisothiazole Analogues (Part 3)

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ation containing 8% of probenazole. The corresponding parts of dried third leaf blades of each rice plant were ignited according to the usual manner respectively, and the number of siliciferous cells was counted microscopically.³⁾

3. Microscopic Observation of the Rice Leaf Tissue

The diseased part on the rice leaf, which was inoculated by the usual procedure with the blast fungus after spraying of probenazole at 400 ppm was sectioned, stained by PAS (periodic acid-schiff) reaction and observed microscopically.⁴⁾

Prior to the observations by electron microscopy, the rice plants were treated with probenazole by submerged application for 5 days. Subsequently the leaf blade inoculated by applying a drop of the conidial suspension on the leaf surface for 1 day was used as the test sample. The ultrathin section of a small leaf piece was observed after ordinary fixation by glutaraldehyde and osmic acid.

4. Examination of Conidial Germination and Appressorial Formation Using an Exudate from the Rice Plant Leaf

The examination was performed according to the method described in the previous paper, with some modification.²⁾ That is, the rice plants of 7th leaf stage were treated for 5 days by submerged application with probenazole and the exudate was then collected from the water pore of the rice leaves. The exudate was mixed with the same volume of a conidial suspension and placed on a slide glass and incubated in a moist chamber at 27°C for 24 hours.

For the appressorial formation test, the cellophane membrane was floated on a small amount of the exudate and inoculated.⁵⁾

5. Extraction of Induced Anti-conidial Factor in Rice Plant

The rice plants treated with 10 ppm probenazole (submerged application for 5 days) were inoculated by spraying the leaves either with conidia of the pathogen or with water (treated control). Rice plants nontreated with probenazole were similarly sprayed with conidia of the pathogen or with water (untreated control). Leaf samples were collected from all

plants 5 days after inoculation, for extraction of the anti-conidial substance.

The extraction was carried out according to a similar method described by Watanabe *et al.*⁶⁾ Ten g of fresh leaves with sheaths from each experiment was cut into small pieces by scissors, suspended in 100 ml of water and homogenized in a whirling blender at 10,000 rpm for 20 minutes. The homogenate was filtered using 2 g of celite as filtering aid. A hundred ml of filtrate was concentrated under reduced pressure to 10 ml. The supernatant of the concentrate was used for anti-conidial germination test.

6. Determination of Conidium-cidal Activity

To examine the influence of the extracts on conidial germination, 30 μ l of sample solution and then 30 μ l of conidial suspension of the pathogen were taken in a hole of slide glass, covered with a cover glass and maintained in a moist chamber at 25°C. After 16 hours incubation, the germination was determined by microscope ($\times 600$). When the length of germ tube was elongated more than 1/3 of the length of longer diameter of conidium, the germination was determined as to be positive.

7. Measurement of Peroxidase Activity of Rice Plant Leaf

The rice plants at about the 4th leaf stage were treated with probenazole under the submerged application for 5 days, and then inoculated at about the 5th leaf stage with the blast fungus. Fifth leaf blades of uniform size were collected at 2 days or 7 days after inoculation and the peroxidase activities were assayed. That is, the fresh rice leaves were homogenized in 10 to 40 volumes (w/v) of M/10 acetate buffer, pH 5.0 at 0–5°C, and the supernatant obtained after centrifugation at 13,000 $\times g$ for 10 minutes was used as the enzyme source. The guaiacol method was employed to assay the peroxidase activity.⁷⁾

8. Determination of Heat-shock Effect on the Induced Resistance

Nine rice seedlings were transplanted apart in 3 groups to each 1/5000 are Wagner pot, which contained 2 kg of fertilized soil, and were cultivated at 25°C in day time and 18°C

in night time in phytotron. At the 5th leaf stage, 16 mg of probenazole in the granular formulation per each pot was given to the 12 pots. Other 12 pots were left as control without the treatment. At 1 day or 1 hour before inoculation, each 3 of the treated and the nontreated pots were heated at $46 \pm 0.5^\circ\text{C}$ for 10 minutes in an air-circulating oven. All pots were inoculated by spraying of conidial suspension of the pathogen and still stood in a moist chamber for 18 hours. And then all the pots were cultured in the phytotron for 1 week. Examination was carried out by counting the developing blast lesions on upper 2 leaves and the number of lesions per leaf was calculated into protective value.¹⁾

RESULTS

1. The Effect of Probenazole on Rice Leaf Tissue

1.1 Siliciferous motor cell formation

The treated rice plants were received two applications at the rate of 320 g of the active ingredient in 10 are of paddy field. No difference in cytological steps between the treated and the nontreated plants were observed; i.e. an average of 3.25 siliciferous cells in a microscopic view ($\times 150$) were observed on the treated plants and 3.50 siliciferous cells in a view were observed on the nontreated ones.

1.2 Microscopic observations

Microscopic observation revealed that the tissue of the diseased leaves changed gradually *via* four cytopathological steps; healthy, toxic, necrotic and degenerative.⁸⁾ No differences between treated and nontreated plants were observed.

1.3 Electron microscopic observations

No differences in the abnormal state on

the cell wall, changes in chloroplast and other contents of the cell were recognized in the leaf tissue of the treated and of the nontreated rice plants.

2. The Influence of Probenazole upon the Conidial Germination in the Exudate from Rice Plant Leaf

The exudate derived from water pores of rice leaves treated with probenazole did not inhibit conidial germination. However, the percentage of appressorial formation in the exudates derived from the rice plants treated by submerged application of probenazole was slightly lower than the control, as shown in Table 1.

3. Induced Formation of an Anti-conidial Factor in the Rice Plant by Probenazole

Inhibition of conidial germination was observed in extracts of leaves derived from plants that had been treated with probenazole with or without subsequent inoculation of the pathogen, as shown in Fig. 1. In another similar experiment, the inhibition was also observed even after washing the conidia, which suggests that the extracts acted as a sterilant on the conidial germination.

4. The Effect of Probenazole upon the Peroxidase Activity

The peroxidase activity was increased with the treatment of probenazole or the inoculation of the pathogen at 2 or 7 days after the inoculation, as shown in Fig. 2, and consequently the activity of the treated-inoculated leaves was more increased 1.68 times or 1.93 times than that of the nontreated-noninoculated leaves at 2 or 7 days after the inoculation,

Table 1 Effect of the exudate derived from the rice plant treated by probenazole upon the germination and the appressorial formation of *Pyricularia oryzae*.

Treatment of the rice plant	No. of conidia examined	Items of observations		
		Not germinated (%)	Germinated without appressorial formation (%)	Germinated with appressorial formation (%)
Submerged application by 10 ppm of probenazole	186	4.3	75.8	19.9
Control	126	3.2	67.4	29.4

Observation was performed after 24 hr incubation.

Fig. 1 The anti-conidial-germination activity of extracts from rice plant treated by submerged application of probenazole. (n=7)

1. Extracts from nontreated-noninoculated rice leaves.
2. Extracts from nontreated-inoculated rice leaves.
3. Extracts from treated-noninoculated rice leaves.
4. Extracts from treated-inoculated rice leaves.
5. Distilled water.

Fig. 2 Peroxidase activity in leaves of rice plant submerged with probenazole.

1. Probenazole,
2. Control

respectively.

5. The Factors relating to the Induced Resistance in Rice Plant

As shown in Table 2, a heat-shock before

Table 2 Determination of heat-shock effect on the induced resistance.

EXP. 1

Probenazole	Heat treatment		
	Non	1 day before inoculation	1 hr before inoculation
Treated	4.4*	14.3	51.5
Nontreated	16.4	20.7	50.7

EXP. 2

Probenazole	Heat treatment		
	Non	1 day before inoculation	1 hr before inoculation
Treated	10.7	18.9	163.7
Nontreated	67.9	112.4	158.3

* Number of developing lesions per leaf.

inoculation greatly reduce the induction of resistant characteristic of rice plant with probenazole.

DISCUSSION

The *in vivo* effects of probenazole upon the blast disease were investigated with reference to the resistance of rice plant, that is, the ratio of silicified cells (especially at motor cell) in the rice leaf blade,⁹⁻¹¹⁾ the morphological change of the leaf tissue examined by using light microscopy and electron microscopy concerning with the first stage of penetration as important phenomena of rice blast infection¹²⁻¹⁵⁾ and the suppression of germination and appressorial formation of the blast conidia by an exudate from rice plants.¹⁶⁻¹⁸⁾ The results in these experiments, however, showed no influence by probenazole. The activity of probenazole should be different from the other fungicides having direct anti-microbial activity,¹⁹⁻²²⁾ and it was assumed that the concentration of probenazole in the exudate might not be enough to show inhibitory activity by *in vitro* assays, because its anti-fungal activity is weak.

By the several experiments, the increase of the small brownish lesions and the decrease of large grayish lesions on diseased rice leaf blades were observed by treatment of probenazole.

Phenolic substances oxidized by enzymes, such as peroxidase, in brownish lesions were thought to play an important role in the resistance of host plants by forming physically obstructing substances against the invasion of the causal fungus in the host leaf tissue.²³⁾ Therefore the involvement of peroxidase in these reactions was expected. As the result at 7 days after the inoculation, the peroxidase activity of the rice plant treated by probenazole (non-inoculated) was increased 1.35 times when compared with that of the nontreated plants (non-inoculated). Besides, the activity of treated-inoculated rice leaves was further increased 1.27 times when compared with that of treated-noninoculated ones. As the reference to the increase of the peroxidase activity by the inoculation in rice plant and potato plant,^{24,25)} it was strongly suggested that the increase of the activity as mentioned above was meaningful as the plant resistance. The details of the changes of the oxidized products by peroxidase, such as quinone-like substances, should be explored in future studies.

It was reported that the suppression of the several diseases was due to the enhancement of resistance in the host plant by the application of several chemical solutions.²⁶⁻²⁸⁾ Anti-fungal factor and phytoalexin in the rice leaves were also reported.²⁹⁻³¹⁾ It was evident that induced formation of an anti-conidial factor by the application of probenazole was phytopathologically demonstrated in this study. Moreover, it was very interesting that conidial germination was inhibited over 99% by a solution of the anti-conidial factor *in vitro*, which concentration was finally half of fresh rice leaves harvested. As already described by Tamari *et al.*,³²⁾ when blast fungus invaded into the rice plant, it produces toxins. And thus toxins may suppress the physiological functions of host plant. In this view, the induced formation of the anti-conidial factor in treated-inoculated host plant might not be more than that of the plant just treated without the inoculation. The heat-shock inhibition on the induced formation of an anti-blast characteristics, which was described in this paper, also implied that the formation of an anti-conidial factor depended strongly on the physiological function of host plant, that is, the

induced formation might be a host-mediated reaction. The induced formation of the factor was also observed in some extent by the inoculation without treatment of probenazole. This might be corresponded with the formation of phytoalexin.^{33,34)} It is quite interesting to consider whether an anti-conidial factor induced by the treatment of probenazole or by the inoculation was chemically same substance. It was also interesting that how long the total resistant characteristics once induced in the host plant by the treatment of probenazole last.

It was stated that peroxidase activity and anti-microbial substance in the host plant are regarded as important factors as for host resistances against pathogens.^{23,34,35)} It must be emphasized that probenazole may participate in the induction of resistance by both increasing the quinone level, which relates to peroxidase activity, and the anti-conidial factor level against the rice blast pathogen in infection process in rice plants.

The characteristics on the action of allyl *o*-sulfamoylbenzoate³⁶⁾ was not examined in this paper, because of the paucity of the isolated sample. Moreover, an accurate quantitative assay method of this intermediate in rice plant was yet unavailable. These experiments including those on a relationship between the dynamic sequence of modified relatives of probenazole *in vivo* and their physiological characteristics in rice plant, which might be related to the durable characteristic of probenazole, remain for future studies.

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要 約

イネいもち病防除におけるプロベナゾールのイネ体に与える影響*

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前報においてプロベナゾール(オリゼメート®)のイネいもち病防除メカニズムに関するいくつかの試験から、薬効の発揮に寄主イネ体の関与が示唆されることを報告した。そこでプロベナゾールの効果をイネ体の変化に重点をおいて研究した。いもち病に対するイネの抵抗性要因として珪質化細胞の形成、イネ葉組織の形態変化およびイネ水孔からの浸出液の抗菌性について試験したが、

* ベンツイソチアゾール系イネいもち病防除剤に関する研究(第3報)

プロベナゾール処理による変化は認められなかった。しかしながらイネ葉中のいもち病菌胞子発芽阻止物質はプロベナゾール処理イネで最も強く発芽阻止を示し、接種のみの区では前者の $1/2$ 以下の発芽阻害にすぎなかった。またフェノール物質の酸化酵素としてのパーオキシダーゼ活性は胞子接種のみの区より本物質の水面施用処

理と接種とを行なった区のほうが増強されている傾向が明らかとなった。したがって前報も含めて考えると、プロベナゾール処理によりイネは強い侵入抵抗とともに拡大抵抗をも示すことになり、これらが総合されて高い防除効果を発揮するものと考えた。