

# プロベナゾール施用が、イネ体中における病害抵抗性関連酵素類の活性に及ぼす影響について

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## Effect of Probenazole on the Activities of Enzymes Related to the Resistant Reaction in Rice Plant\*

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岩田道顕\*\*・鈴木幸雄\*\*・渡辺哲郎\*\*・間瀬定明\*\*\*・関沢泰治\*\*：プロベナゾール施用が、イネ体中における病害抵抗性関連酵素類の活性に及ぼす影響について\*

### Abstract

The activities of enzymes including peroxidase, polyphenoloxidase, phenylalanine ammonia-lyase, tyrosine ammonia-lyase or catechol-O-methyltransferase in rice leaves were surveyed to elucidate the mechanism of action of probenazole in connection with the resistant reaction of host plant against rice blast fungus. The activities of enzymes were increased evidently in the treated-inoculated rice leaves with probenazole and rice blast fungus conidia than in the leaves of treated-noninoculated, nontreated-inoculated or nontreated-noninoculated one. The above results seem to be closely correlated with the facilitated formation of a physical barrier in and around the invaded cell, which was observed microscopically.

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### Introduction

Probenazole (Oryzemat<sup>®</sup>, 3-allyloxy-1, 2-benzisothiazole-1, 1-dioxide) is an effective systemic agricultural chemical for rice blast control. The phytopathological analysis on characteristics of its controlling mechanism was previously given<sup>33,34,35</sup>.

It was found that probenazole was quite effective on the blast disease when it was applied to root system of rice plants<sup>34</sup>. It was also observed that the application of probenazole strongly enhanced the resistance mechanism against the hyphal penetration of rice blast fungus into the leaf cell and against the subsequent extension of the invading mycelium to adjacent cells<sup>35</sup>. It was also noticed that an activity of peroxidase in rice leaves was concomitantly augmented by the treatment with probenazole especially when the conidia of rice blast fungus were inoculated<sup>35</sup>.

Thus, it is evident that both the enhancement of the resistance mechanism of rice plant and the augmentation of the peroxidase activity by the application of probenazole are also closely interrelated in this case, as it is well known that peroxidase plays an indispensable role to form lignoid barrier around the invaded cell preventing the extension of the invading mycelium<sup>1</sup>.

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This paper describes the enzymic surveys upon the changes of apparent activities of peroxidase, polyphenoloxidase, phenylalanine ammonia-lyase, tyrosine ammonia-lyase and catechol-O-methyltransferase in the rice leaves treated with submerged application of probenazole to confirm further the other four enzymic activities, linked with peroxidase activity to form lignoid barrier around the invaded cell.

### Materials and Methods

**Cultivation of rice plant and inoculation of blast fungus.** The cultivation of rice plant and inoculation of blast fungus conidia by spraying were performed according to the methods previously described except otherwise stated<sup>33,34,35</sup>. The inoculation to leaves were also performed on press-injured spots of 2.5 mm diameter made by using a specially designed pressing machine<sup>15,26</sup>. Fifth leaf blades were used for the inoculation. A clot of filter paper powder moistened with spore suspension was placed on the injured spots<sup>15</sup>.

**Application of probenazole.** Eight seedlings were grown in a pot of 6.5 cm in diameter. At 4-leaf stage of the seedlings, each pot was submerged in 200 ml of water in a 500 ml beaker to which 50 ml of solution of probenazole (100 ppm) containing a small amount of acetone and dispersing agent was added. The water level was kept 1 cm above the soil surface during the period of inoculation.

**Extraction of enzymes.** Five days after the application of probenazole, the rice plants were inoculated with blast fungus. The 5th leaf blades of uniform size inoculated by spraying were collected at 2 or 7 days after the inoculation. When the leaf blades were inoculated by the press injury method, the tissues surrounding the inoculation site and those exterior to the surrounding zone were collected separately (see Fig. 1) 2 days after inoculation. The detached fresh leaves or leaf tissues were homogenized at 0-5 C using a polytron (Kinematica, Switzerland) with 40 times volume (w/v) of 0.1 M acetate buffer (pH 5.0) to examine peroxidase or polyphenoloxidase activity, or of 0.05 M sodium borate buffer (pH 8.5) containing 0.5 per cent sodium isoascorbate to examine phenylalanine ammonia-lyase, tyrosine ammonia-lyase or catechol-O-methyltransferase activity. Then the homogenate was centrifuged at  $13,000 \times g$  for 10 min. The supernatant was used as the enzyme source.

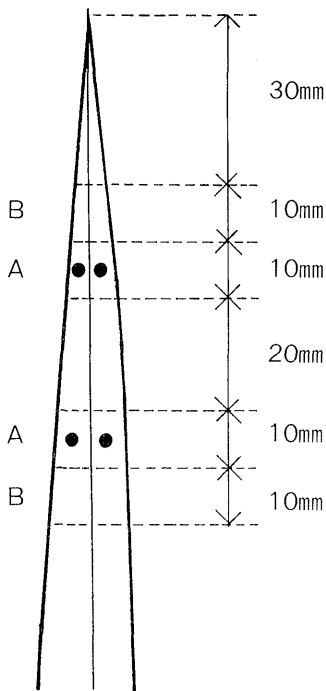


Fig. 1. Inoculated sites by press-injury and collected parts.

A: Inoculated and surrounding zone.  
B: Exterior zone collected for analysis.

**Assays of enzymic activities.** Peroxidase activity was assayed by the modified guaiacol method in which the enzyme activity was determined by the increase of absorption at 420 nm

employing 800 nm as reference light for initial 120 sec at 30 C using a Hitachi Two Wavelength Spectrophotometer Model 156. The reaction mixture consisted of 0.1 ml of 0.1 % guaiacol, 0.1 ml of 0.08 % H<sub>2</sub>O<sub>2</sub>, 0.2 ml of enzyme solution and 0.1 M acetate buffer (pH 5.0) in total volume of 3.0 ml.

Polyphenoloxidase activity was determined by the increase of absorption at 500 nm employing 800 nm as reference light for initial 120 sec<sup>24)</sup> or at 520 nm for 16 hr at 30 C. The reaction mixture consisted of 0.5 ml of 0.01 M chlorogenic acid, 0.5 ml of 0.05 M sodium sulfanilate, 0.01 ml of enzyme solution and 1.0 ml of M/15 phosphate buffer (pH 6.8). By the addition of 0.1 M acetate buffer (pH 5.0) instead of enzyme solution, the enzymic activity was corrected for nonenzymic oxidation of the substrate. In this system, a linear correlation was observed between the concentration of crystalline polyphenoloxidase (mushroom origin, 10,000 units per mg solid, Sigma Chemical) in the extent of 0.001-0.00001 mg per ml reaction mixture and the increase of absorption at 520 nm. The crystalline peroxidase (horse radish origin, 235 purpurogallin units per mg protein, R<sub>z</sub>; 3.14, Sigma Chemical) was used as a reference.

The assay of phenylalanine ammonia-lyase activity was performed determining the amount of cinnamic acid formed<sup>14)</sup>. The reaction mixture consisted of 2.25 ml of 0.01 M phenylalanine dissolved in 0.05 M sodium borate buffer (pH 8.5) and 0.25 ml of enzyme solution. The mixture was incubated for 2 hr at 40 C. The reaction was stopped by the addition of 0.1 ml of 6 N HCl. The acidified mixture was extracted once with 10 ml of ethylether, then the ether phase was concentrated under reduced pressure in a test tube using a centrifugal evaporator (Yamato, Model RC-11) at room temperature. The dryness was dissolved in a small amount of ethylacetate and then subjected to silica gel TLC (E. Merck, Kieselgel 60 F<sub>254</sub>, benzen-ethylacetate-formic acid, 10:5:1). Cinnamic acid separated on the thin layer plate was quantitatively determined by the absorption at 270 nm employing 350 nm as the reference light and using a TLC scanner (Shimadzu, Model CS-900).

The activities of tyrosine ammonia-lyase and catechol-O-methyltransferase were assayed in a similar method to phenylalanine ammonia-lyase. Regarding tyrosine ammonia-lyase, the reaction system consisted of 2.25 ml of 0.01 M tyrosine dissolved in 0.05 M sodium borate buffer (pH 8.5) and 0.25 ml of enzyme solution. The reaction mixture was incubated for 4 hr at 40 C. The ethylether extractable product, that is coumaric acid, was separated by silica gel TLC and quantitatively determined using the scanner by the absorption at 286 nm employing 350 nm as the reference light. Regarding catechol-O-methyltransferase, the reaction system consisted of 0.05 ml of 0.01 M caffeic acid, 0.05 ml of 0.04 M MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.125 ml of 0.002 M S-adenosyl-methionine, 0.125 ml of enzyme solution, 0.1 ml of 1 M tris buffer (pH 8.0) and 0.55 ml of water<sup>19,20)</sup>. The reaction mixture was incubated for 4 hr at 30 C. The amount of ferulic acid separated on silica gel plate was quantitatively determined by the absorption at 300 nm employing 245 nm as the reference light and using the scanner.

## Results

### *Effects of treatment with probenazole on peroxidase and polyphenoloxidase activity*

As previously reported<sup>35)</sup>, peroxidase activity was increased by the treatment of probenazole and/or by the inoculation of rice blast fungus. The activity in the treated-inoculated

leaves was more augmented than that in the treated-noninoculated, nontreated-inoculated and nontreated-noninoculated leaves (Fig. 2).

Peroxidase activity in the rice leaves inoculated by punching was shown in Fig. 3. Higher enzymic activity was observed at the inoculated and its surrounding zone than at the exterior zone, at the inoculated leaves than the noninoculated leaves, and at the treated leaves than the nontreated leaves. The highest level was observed at the inoculated and its surrounding zone on the leaves treated with probenazole. From the above observations, the most augmented activity in treated-inoculated leaves by spraying inoculation (Fig. 2) might be due to the locally increased activity at the invaded sites.

The activity of polyphenoloxidase was assayed spectrophotometrically at 500 nm or 520 nm as shown in Fig. 4 and 5, respectively. Its activity was also more increased in the treated-inoculated leaves than that in the others. Regarding the two oxidases (peroxidase and polyphenoloxidase-like enzyme), a similar tendency as for the shift of respective activity was observed when the events in leaves at 2 days and 7 days after the inoculation were compared. A higher enzymic level observed at 7 days might depend on an age of rice plant<sup>10,13</sup>.

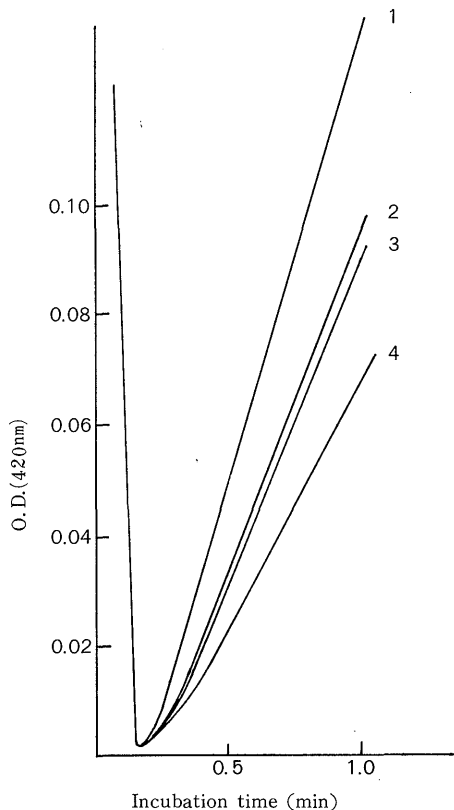


Fig. 2. Peroxidase activity in leaves of rice plant submerged with probenazole.  
1. Treated-inoculated rice leaves.  
2. Treated-noninoculated rice leaves.  
3. Nontreated-inoculated rice leaves.  
4. Nontreated-noninoculated rice leaves.

It was already described that polyphenoloxidase was absent in leaves of aged rice plants<sup>30,32</sup>, and that polyphenols were oxidized by a nonenzymatic system and/or by a peroxidase depending on the increase of  $H_2O_2$  level in the rice tissue<sup>31,32</sup>. Therefore, in order to confirm whether the oxidation of chlorogenic acid by rice leaf homogenate in the present study was due to true polyphenoloxidase, the chlorogenic acid oxidizing activity of rice leaf homogenate was compared with that of the autoclaved rice leaf homogenate and further

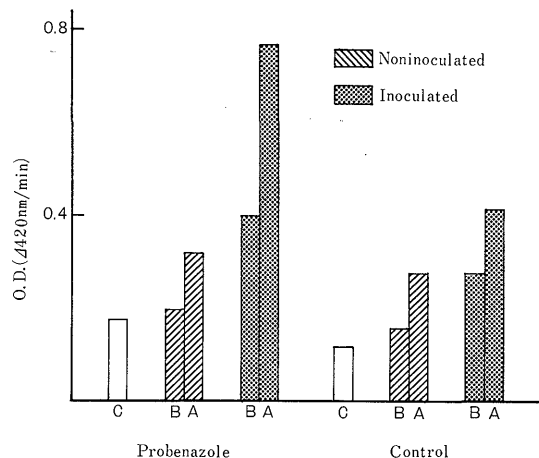


Fig. 3. Peroxidase activity at and around the inoculated site on leaves of plants submerged with probenazole.  
A: Press-injured and its surrounding zone.  
B: Exterior zone.  
C: Noninjured-noninoculated leaf.

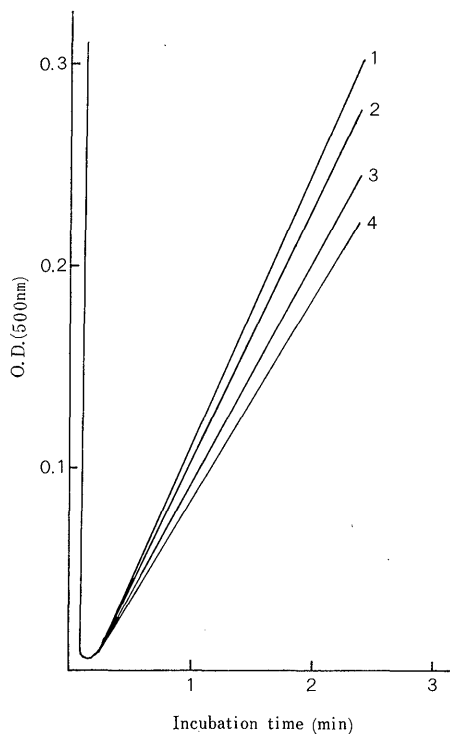


Fig. 4. Polyphenoloxidase-like activity in leaves of rice plant submerged with probenazole.

1. Treated-inoculated rice leaves.
2. Treated-noninoculated rice leaves.
3. Nontreated-inoculated rice leaves.
4. Nontreated-noninoculated rice leaves.

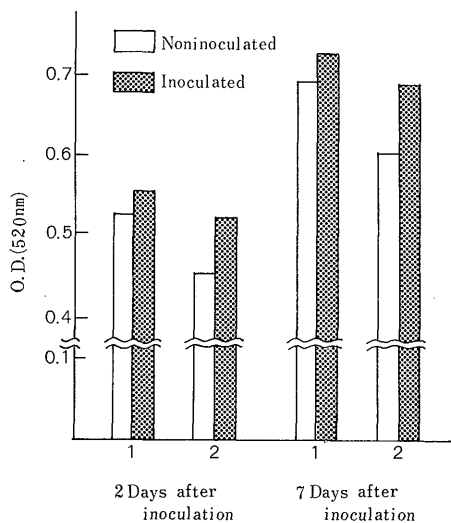


Fig. 5. Polyphenoloxidase-like activity in leaves of rice plant submerged with probenazole.

1. Probenazole.
2. Control.

Table 1. Oxidation of chlorogenic acid by autoclaved or unautoclaved rice leaf homogenate

	Chlorogenic acid oxidizing enzyme in rice leaf homogenates	
	Unautoclaved	Autoclaved
Test 1	1.020 <sup>a)</sup>	0.180 <sup>a)</sup>
Test 2	1.046 <sup>a)</sup>	0.186 <sup>a)</sup>

a) Absorption at 520 nm, corrected for autooxidation of chlorogenic acid in the buffer.

compared with the chlorogenic acid oxidizing activity of the crystalline polyphenoloxidase or that of the crystalline peroxidase. As shown in Table 1, as the chlorogenic acid oxidizing enzymic activity in rice leaf homogenate was practically lost by autoclaving (120 C, 15 min). Thus, the chlorogenic acid in the reaction system shown in Fig. 4 and 5 should be mainly oxidized enzymatically. The minute oxidizing activity in autoclaved rice leaf homogenate might suggest the presence of a weak nonenzymatic system which slightly oxidized chlorogenic acid<sup>32)</sup>.

The Lineweaver-Burk plot on the crystalline polyphenoloxidase, crystalline peroxidase or rice leaf homogenate was respectively shown in Fig. 6 when chlorogenic acid was used as substrate. The Michaelis-Menten constant ( $K_m$ ) for chlorogenic acid on the polyphenoloxidase, peroxidase or rice leaf homogenate was  $5.6 \times 10^{-4}$  M,  $4.8 \times 10^{-3}$  M or  $4.1 \times 10^{-3}$  M respectively. The  $V_{max}$  was  $1.8 \times 10^{-2}$ ,  $1.0 \times 10^{-3}$  or  $2.6 \times 10^{-3}$  as the increase of absorption at 520 nm per minute respectively. From these results, enzymological properties of the rice leaf enzyme, which oxidized chlorogenic acid, were very similar to the peroxidase rather than polyphenoloxidase. Thus, the oxidation of polyphenols in rice tissue might be mainly

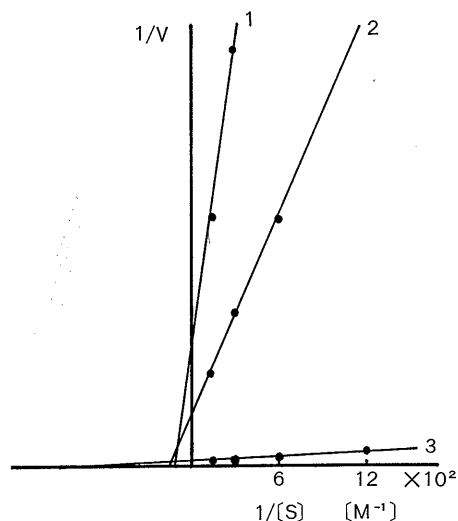


Fig. 6. The Lineweaver-Burk plot on the crystalline polyphenoloxidase, the crystalline peroxidase and the rice homogenate.

1. The crystalline peroxidase.
2. The rice homogenate.
3. The crystalline polyphenoloxidase.

The activity of phenylalanine ammonia-lyase in the rice leaves inoculated by punching method was shown in Fig. 10. Regarding this enzyme, it was also observed that the higher

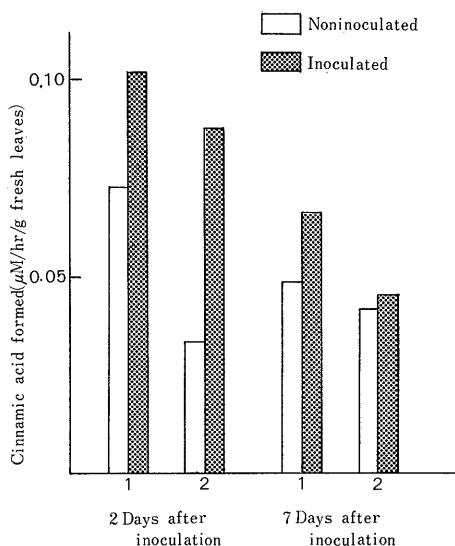


Fig. 7. Phenylalanine ammonia-lyase activity in leaves of rice plant submerged with probenazole. 1. Probenazole. 2. Control.

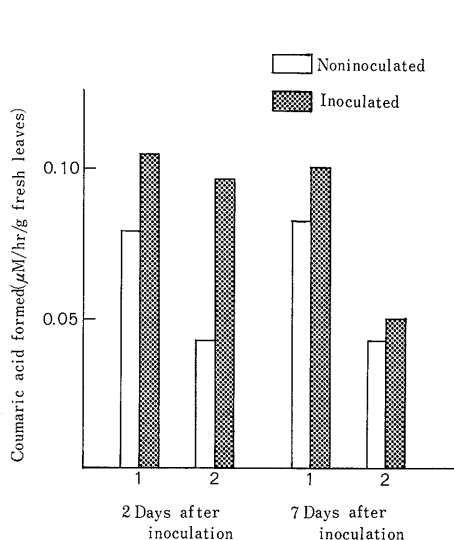


Fig. 8. Tyrosine ammonia-lyase activity in leaves of rice plant submerged with probenazole. 1. Probenazole. 2. Control.

depended on peroxidase as already claimed<sup>32)</sup>.

#### *Effects of treatment with probenazole on phenylalanine ammonia-lyase, tyrosine ammonia-lyase and catechol-O-methyltransferase activity*

The activity of phenylalanine ammonia-lyase, tyrosine ammonia-lyase and catechol-O-methyltransferase was expressed as cinnamic acid, coumaric acid and ferulic acid  $\mu\text{M/hr/g}$  fresh leaves, respectively, when rice leaves were inoculated by spraying method (Fig. 7-9). In these enzymes, the profile of increasing activity of each enzyme was similar to oxidases mentioned above. That is, both at 2 days and 7 days after the inoculation, activity of each enzyme in the treated-inoculated leaves was increased more actively than that in the others. On the contrary, the enzymic level at 7 days after the inoculation was lower than at 2 days. The decrease in deaminases and methyltransferase activities might be due to aging of the plant<sup>13)</sup>.

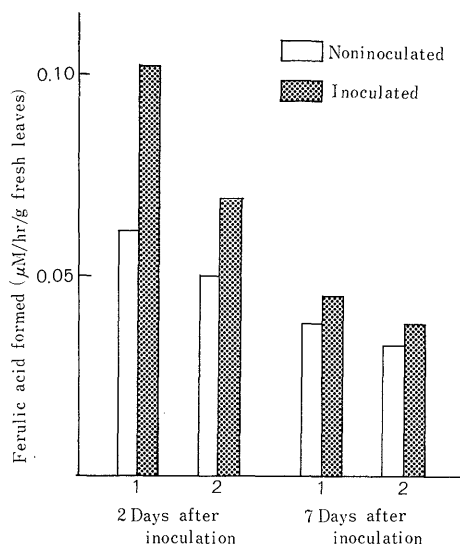


Fig. 9. Catechol-O-methyltransferase activity in leaves of rice plant submerged with probenazole.  
1. Probenazole.  
2. Control.

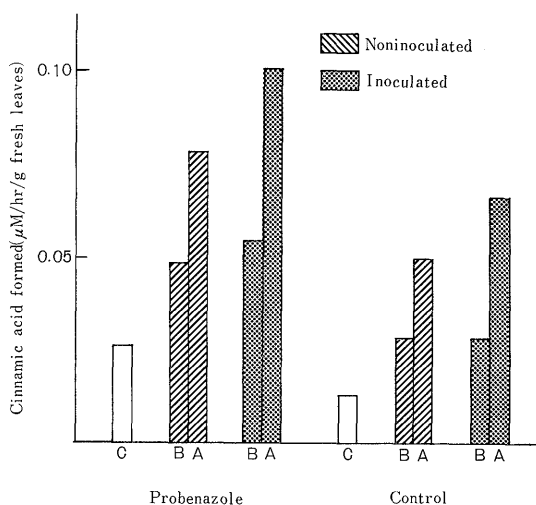


Fig. 10. Phenylalanine ammonia-lyase activity at inoculated site on leaves of rice plants submerged with probenazole.  
A: Press-injured and its surrounding zone.  
B: Exterior zone.  
C: Noninjured-noninoculated leaf.

activity was shown at the inoculated and its surrounding zone on leaves treated with probenazole than the others, reinforcing that the activity was locally increased at the invaded sites.

## Discussion

Five enzymes, which were selected and investigated in this paper, have been described to have indispensable roles in the formation of phenolic substances in plants<sup>2,7,8,9,11,20</sup>. It has been well accepted that these enzymes and phenolic substances formed actually participated in the resistant characteristic of host plants against infection by phytopathogenic microorganisms<sup>16,25,29</sup>.

It was further described that peroxidase and polyphenoloxidase oxidized and polymerized the phenylpropanoids<sup>2,7</sup> and that the oxidized and polymerized products were localized in brownish lesions caused by the infection of pathogens<sup>29</sup>. It was well established that phenylalanine ammonia-lyase and tyrosine ammonia-lyase catalyze respectively a deamination reaction of phenylalanine and tyrosine to form phenylpropanoids<sup>11</sup>. It was also described that the both deaminases were increased markedly in sweet potato roots in response to wounding and infection<sup>14</sup> and that the increases of the enzymic activities were closely paralleled with the accumulation of polyphenols in the tissues<sup>14</sup>. The regulation of lignification or flavonoids synthesis could be effected by the enzymic level of phenylalanine ammonia-lyase<sup>3</sup>. Plant catechol-O-methyltransferase catalyzes the methylation of caffeic acid to form ferulic acid<sup>4,5,9</sup> and also the methylation of 5-hydroxyferulic acid to form sinapic acid<sup>9,20</sup>. It was suggested that catechol-O-methyltransferase might regulate the formation of the lignin-like materials in a resistant potato tuber than phenylalanine ammonia-lyase<sup>6</sup>.

In this paper, it was reported that all of the activities of five enzymes including the



polyphenoloxidase-like enzyme were increased by the inoculation of the blast fungus conidia with the treatment of probenazole. The highest levels of the enzymic activities were observed in treated-inoculated rice leaves, especially at the inoculated and its surrounding zone. The profile of the change of each enzymic activity depending on the treatment or the inoculation showed similar relationship.

During the infection experiments in this study, it was noted that minute brownish lesions, which do not expand further, were often observed on the treated-inoculated rice leaves by spraying inoculation and that microscopic observation upon the section of brownish lesion revealed the facilitated formation of lignoid substance around the invaded cells<sup>12)</sup>. It was also observed that the browning of press injured and inoculated parts on treated rice leaves rapidly occurred even at 1 day after the inoculation. On the contrary, the browning on nontreated rice leaves occurred several days after the inoculation or could not be recognized.

From precedings, it is quite plausible that the application of probenazole induces the activation of a series of reactions related to the formation of lignoid substance around the invaded cells in rice plant. Thus, the rapid accumulation of lignoid substance around invaded cells forms the physical barriers to prevent the extension of blast fungus mycelium to other adjacent cells. It is also plausible from another experiment that the application of probenazole with the subsequent inoculation induces the activation of the formation of antimicrobial substance in host<sup>18, 22)</sup>. Although it was reported that the browning of cell invaded by blast fungus in a highly resistant cultivar did not correlated to the prevention of extension of the fungal hyphae<sup>17, 24)</sup>, the browning or lignoid formation was very often related to resistant reaction in various plants including rice plant<sup>21, 23, 28, 37)</sup>. Therefore, the browning and/or lignoid formation imply the enhancement of the resistance mechanism relating to the defence system of rice plant. A heat-shock experiment previously reported<sup>35)</sup> strongly reinforced the involvement of such a host-mediated reaction induced by the application of probenazole.

As the result from the comparative kinetic study between the chlorogenic acid oxidizing enzyme in rice leaves and crystalline polyphenoloxidase or peroxidase, enzymological properties of the rice leaf enzyme, which oxidized chlorogenic acid, were very similar to the crystalline peroxidase (horse radish) rather than the crystalline polyphenoloxidase (mushroom). Therefore the oxidation of polyphenols in rice leaves should be mainly catalyzed by the peroxidase in the presence of  $H_2O_2$  as already claimed<sup>32)</sup>.

It was reported that some phenolic compounds such as coumaric acid and ferulic acid were accumulated around the blast lesion up to about 5 times as much as the healthy leaves<sup>15)</sup>, as the resultant of increase in activity of tyrosine ammonia-lyase or catechol-O-methyltransferase. And it was suggested that coumaric acid and ferulic acid accumulated might inhibit the expansion of the lesions due to their fungitoxic actions<sup>15)</sup>. The quantitative determination on the increase of coumaric acid or ferulic acid at the invaded sites on rice leaves treated with probenazole was remained for future investigation.

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## 和文摘要

プロベナゾール施用が、イネ体中における病害抵抗性関連酵素類の  
活性に及ぼす影響について

岩田道顕・鈴木幸雄・渡辺哲郎・間瀬定明・関沢泰治

植物の病害抵抗性と関連があるといわれている酵素のうち, peroxidase, polyphenoloxidase, phenylalanine ammonia-lyase, tyrosine ammonia-lyase および catechol-O-methyltransferase について, プロベナゾールを施用した場合のイネ体内における活性変動を調べた。その結果, これらの酵素はいずれもプロベナゾールを施用することによってその活性が増加することが認められた。活性増加は, プロベナゾール施用-いもち病菌接種区で最も大きく, 特にパンチ接種の場合, 接種部位およびその周辺で高く, これに反しプロベナゾール無施用-いもち病菌無接種区の活性は最も低かった。また, プロベナゾール施用-噴霧接種区においては, イネ葉面上に小さな褐点が生じることがしばしば認められた。さらに, プロベナゾール施用-パンチ接種区の場合も, 接種部位が急速に褐変した。従って, いもち病菌の感染を受けた細胞のまわりに, リグニン様物質が蓄積され, それに伴って物理的防御壁が形成されることと, プロベナゾール施用とが, 密接に関連しているものと考えられる。