Studies on the Bacterial Leaf Blight of Rice Plant V. Effect of Phenylacetic Acid on Rice Leaves

Tohoku Journal of Agricultural Research
Vol. 27, No. 1, August 1976
Printed in Japan

Studies on the Bacterial Leaf Blight of Rice Plant V. Effect of Phenylacetic Acid on Rice Leaves

Eiichiro MIYAZAKI, Susumu YAMANAKA and Tadao MISAWA

Department of Agronomy, Faculty of Agriculture, Tohoku University, Sendai, Japan

(Received, June 7, 1976)

Summary

This experiment was undertaken to determine the effect of phenylacetic acid (PAA) produced by the pathogen of the bacterial leaf blight of rice plant, *Xanthomonas oryzae* (Uyeda *et* Ishiyama) Dowson, on rice leaves.

1) The direct application of PAA to rice leaves resulted in the appearance of yellow spot similar to the initial symptom of this disease,

2) The water permeability of the leaf tissue cells was increased by PAA treatment.

3) At 15 hr after the rice leaves were floated on PAA solution, the electrical conductivity of the solution increased. On the other hand, when the diseased rice leaves were floated on distilled water, a similar result was obtained.

4) The acid phosphatase was liberated from the chloroplast by PAA.

5) The decomposition of chlorophyll was accelerated by protease or PAA, and when both worked at the same time, the decomposition was further accelerated.

6) The fraction-I-protein was remarkably decomposed by the bacterial protease, and the decomposition was more accelerated when PAA also was present.

7) The lesion of a resistant variety "Kidama" which absorbed PAA enlarged more than that without it.

8) From the above results, formation about the lesion characteristic of this disease was discussed.

We have showed in previous reports (1, 2) that *Xanthomonas oryzae* (Uyeda *et* Ishiyama) Dowson, the pathogen of the bacterial leaf blight of rice plant, produced phenylacetic acid (PAA) in both the cultured solution and the diseased tissues of rice leaves. The PAA, possessing a phenolic radical and an acetic radical, seems to penetrate easily into the cell. This fact may suggest that PAA plays an important role in the formation of lesion. Therefore, we conducted the study reported here to determine the effect of PAA on rice leaves.

Materials and Methods

The cultivar of rice plant used in the present study was "Aichi Asahi", which

is susceptible to *X. oryzae*. The rice plants were water-cultured in a greenhouse. The H–5809 strain of *X. oryzae* was used. The pathogen was usually cultured in a semi-synthetic potato decoction medium. The PAA used in this study was a commercial, synthetic chemical. Other materials and methods are described at each section.

Results

1. *Spot Formation due to Applying PAA to Rice Leaves*

If a metabolite (including a toxin) produced by a plant pathogen may be presumed to be at least partly responsible for the appearance of symptom, it will be necessary that at least one of the typical symptoms will be induced by the external supply of the metabolite. In order to clarify the relation between PAA and the symptom appearance of this disease, PAA was applied to the rice leaves. The application of PAA to the leaves was done by punch inoculation at the central part of the upper three leaves. PAA concentrations used in this experiment were 50, 100, 200, 500, and 1,000 ppm, respectively. Each solution was mixed with the powdered filter papers, and the given amount of the mixture was put on the punched portion. In order to prevent the evaporation of PAA, the treated portion was sealed with cellotpae and the plant was kept in a moist chamber at 30°C for 4 days. On the other hand, the control plant was treated similarly with a sterile physiological salt solution instead of PAA solution.

The direct application of PAA to rice leaves resulted in the appearance of yellow spot which developed upward and downward from the punched portion. The length of the spot increased with the rise of PAA concentration. At such a high concentration as 500 or 1,000 ppm, the color of the spot became pale-whitish. These results indicated that the symptom similar to the initial symptom of the disease appeared by the external application of PAA.

2. *Change of Cell Permeability in Rice Sheath Tissues*

We examined the change of cell permeability in the rice sheath tissues caused by PAA treatment. The inner cell layers of the 4th leaf sheath from the top were used in this experiment. The inner cell layers (2–3 cell layers in thickness) were peeled off in the usual way, immersed in PAA solutions with various concentrations for every definite time, and then kept in 0.8 M sucrose solution (containing 0.001% of neutral red) for 15 min. Then, the time for deplasmolysis was estimated in 0.4 M sucrose solution on slide glass. The change of cell permeability was estimated from the difference of the time required for deplasmolysis.

The result is shown in Table 1. The time for deplasmolysis was accelerated by 25 ppm of PAA concentration for 120 min, by 50 ppm for 60 min, and by 100 ppm for 30 min, respectively. These results showed that the water permeability of the

TABLE 1. *Relation between PAA Concentration and Water Permeability*

PAA concentration (ppm)	Immerced time (min)											
	1		5		15		30		60		120	
0	140[a]	1.0[b]	133	1.0	135	1.0	129	1.0	132	1.0	119	1.0
25	142	1.0	145	1.1	138	1.0	124	1.0	125	0.9	94	0.8
50	133	1.0	122	0.9	128	1.0	126	1.0	94	0.7	92	0.8
100	138	1.0	135	1.0	123	0.9	113	0.9	100	0.8	86	0.7
300	121	0.9	108	0.8	92	0.7	75	0.6	—		—	
500	72	0.5	71	0.5	—		—		—		—	
1000	77	0.6	—[c]		—		—		—		—	

[a]Time (sec) for deplasmolysis, average number of 3 tissue-pieces.
[b]Time for deplasmolysis of the treated cell/that of the control cell.
[c]—: No plasmolysis

inner cells of leaf tissue increased with PAA treatment. The loss of water permeability occurred when treated for a shorter time with an increase of PAA concentration. Plasmolysis completely disappeared with 300 ppm of PAA concentration for 60 min, with 500 ppm for 15 min, and with 1,000 ppm for 5 min.

3. *Exudation of Cell Substances from Healthy and Diseased Leaves*

From the above result, it became apparent that the increase of cell permeability was induced by PAA which was produced in the diseased tissues. As it was presumed that the increase of cell permeability in the diseased tissues was accompanied by the increase of exudation from the cells, the following experiment was undertaken.

Leaf disks (0.7 cm in diameter) were cut from the central part of the 2nd leaf of the main stem of rice plant. The leaf disks placed in the Petri dishes were washed well with distilled water to avoid the effect of cutting. After washing, the leaf disks were floated on PAA solutions with various concentrations, and kept at 25°C under 5,000 lux of light intensity. The electrical conductivity of the solution was measured by an electrical conductor (CM-3M type of Towa Denpa Co.). The reducing sugars in various samples were measured by the method of Somogyi-Nelson with glucose as the standard. Total nitrogen was measured by the method of Indo-Phenol after the decomposition by Kjeldahl's method. Nitrogen compound was measured by the Itzhaki and Gills' microbiurett method (3) with bovine serum albumin as the standard.

The results are shown in Fig. 1. At 15 hr after the rice leaves were floated on PAA solution, the electrical conductivity of the solution increased, and at the same time the exudations of soluble sugar and soluble nitrogen compound from the leaf tissues also increased. The amount of exudate increased with the rise of PAA concentration and the period of treatment. The substance which induced the increase of electrical conductivity was not identified, but it would be assumed to be an inorganic substance such as Na^+ or K^+. The nitrogen compound estimated

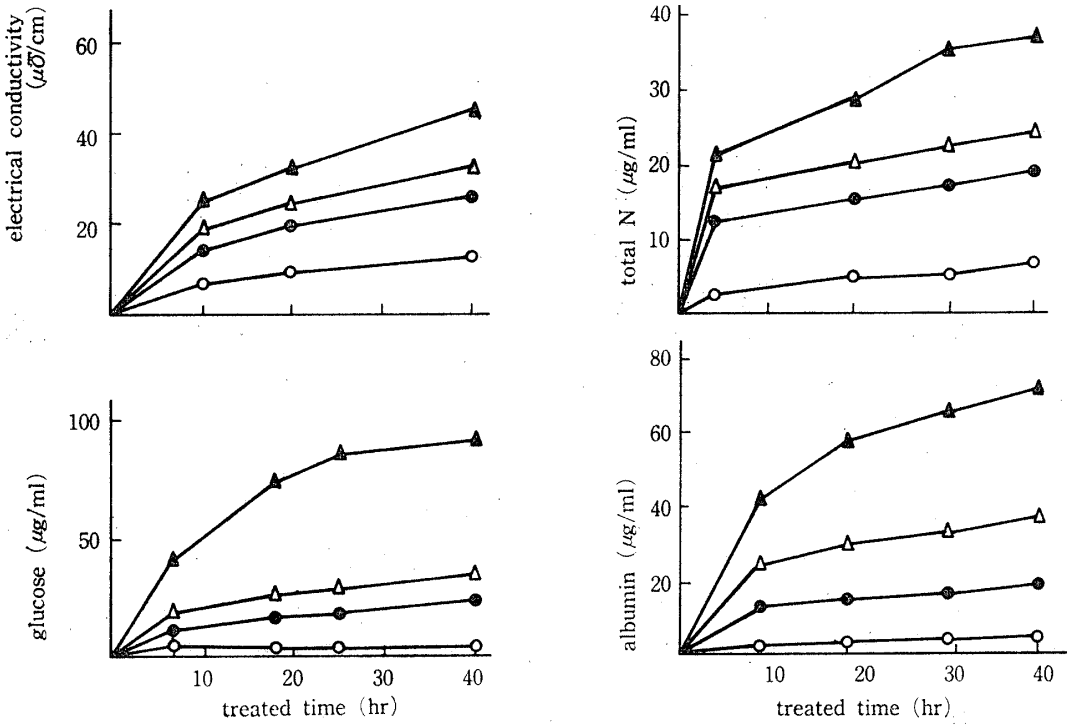

FIG. 1. Relation between PAA concentration and exudates of cell substances from PAA-treated leaf disks
o——o Control (distilled water), •——• 50 ppm of PAA concentration, ▵——▵ 100 ppm of PAA concentration, ▲——▲ 300 ppm of PAA concentration

by the microbiurett method would be a substance with a relatively large molecu la weight such as a polypeptide.

Further, the exudation of cell substances from the diseased tissues was determined. The 2nd leaves of the main stems of rice plants were inoculated with the bacterial suspension by the needle method. The inoculation was done at intervals of 2 cm through the whole length of the half leaf blade. The other half leaf blade was inoculated with a sterilized physiological salt solution as a control. Fifty leaf disks (0.5 cm in diameter) were punched out of both the lesion areas and the

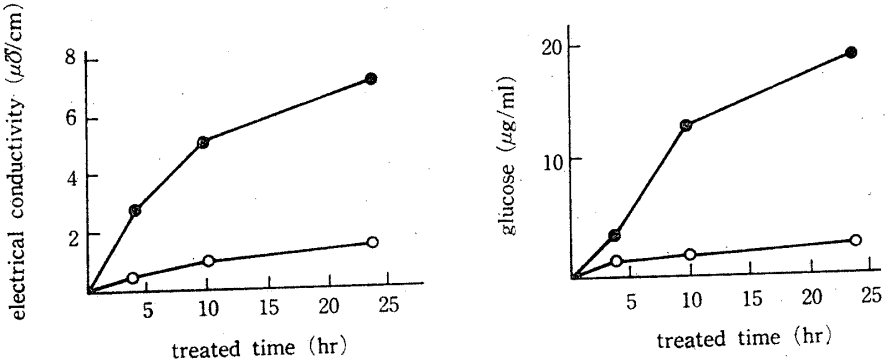

FIG. 2. Exudation of cell substances from the lesion areas
o——o Healthy area, •——• Lesion area

healthy areas by using a cork borer at 5 days after inoculation. The leaf disks were floated on distilled water after washing as described above. The solution was collected at regular times and the bacterial spores were removed by centrifugation at 8,000 g for 20 min. The electrical conductivity and the reducing sugar in the solution were determined.

The results were shown in Fig. 2. The amounts of the salt and the reducing sugar exudated from the lesion areas were about 4 times and 10 times respectively greater than those exudated from the healthy areas. These results closely paralleled those obtained by PAA treatment.

4. *Effect of PAA on Chloroplast*

a) Release of Acid Phosphatase from Chloroplast

It was apparent from the above results that PAA treatment induced both an increase of exudation from the leaf tissues and a decrease of plasmolysis ability of the leaf cells. It was presumed that PAA might denature or destroy the membrane structures of the cell and the cell organelles. On the other hand, the remarkable destruction of chloroplast may have resulted in a release of acid phosphatase from the cell organelles. In this experiment, chloroplast was used to investigate the denaturation or the destruction of the membranes of the cell organelles by PAA. The chloroplast was isolated from the leaf blades of rice plant. Ten g of the leaf blades were gently ground with 10 ml of 0.01 M Tris-HCl buffer solution (contained 0.35 M NaCl, pH 8.5) in a mortar under cooled conditions. The solution was filtrated through a double layer of cheesecloth and the filtrate was centrifuged at 200 g for 90 sec. The supernatant containing chloroplast was further centrifuged at 1,000 g for 7 min, and the chloroplast obtained was suspended in the above buffer solution. In order to purify the chloroplast solution, the same procedure was repeated two times. In addition, the acid phosphatase was isolated from the chloroplast by the method described below. A mixture of the chloroplast suspension and PAA solution with a definite concentration was kept at 30°C. After a given time, the suspension was centrifuged at 10,000 g for 15 min and the acid phosphatase activity of the supernatant was determined by the method of Rogers and Reithel (4). The suspension was allowed to react with p-nitrophenol phosphate-2Na as a substrate at 30°C and pH 5.0 for 5 min. The liberated p-nitrophenol was determined at 420 nm of optical density with a spectrophotometer and its μM were calculated with the working curve.

The result is shown in Fig. 3. The chloroplast not treated with PAA liberated no acid phosphatase, but the chloroplast treated with PAA liberated it in the bathing solution. The activity of acid phosphatase was accelerated in proportion to both the PAA concentration and the treatment period. Thus, the above result indicates that the chloroplast liberated acid phosphatase under PAA treatment.

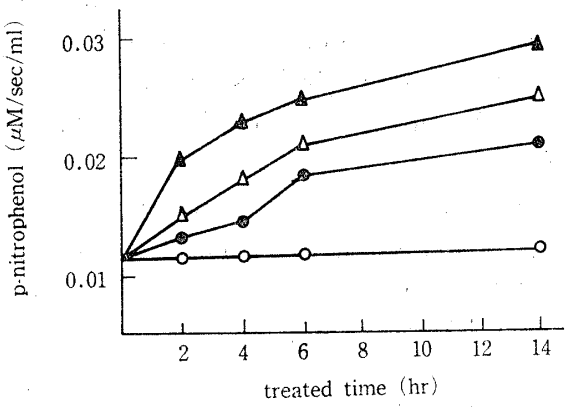

FIG. 3. Acid phosphatase activity liberated from chloroplast by the action of PAA
o——o Control (distilled water), •——• 25 ppm of PAA concentration, ▵——▵ 50 ppm of PAA concentration, ▲——▲ 100 ppm of PAA concentration

b) Decomposition of Chlorophyll-Protein Complex

According to earlier reports, the soluble chlorophyll extracted from the tobacco chloroplast is combined with protein, and the decomposition of protein by protease resulted in a remarkable decrease of chlorophyll. It was previously reported (2,5) that because of the lesion formation in the bacterial leaf blight disease, chlorophyll decreased and the protease activity in the diseased areas was accelerated. In this experiment, the effect of protease on chlorophyll-protein complex extracted from the rice leaf chloroplast was studied for the purpose of observation of the decrease of chlorophyll in the presence of PAA. Chloroplast was extracted from the rice leaves as described above, and the chlorophyll-protein complex was prepared from the chloroplast by the method of Hotta *et al.* (6). Chloroplast was suspended in 0.01 M Tris-HCl buffer solution (pH 8.0) containing 1% Triton X-100, and the solution was stirred for 30 min. After centrifuging at 9,000 g for 60 min, the supernatant was dialyzed overnight against 0.01 M Tris-HCl buffer, and the inner liquid was used as a soluble chlorophyll-protein complex solution. Protease was prepared from the culture filtrate of the pathogen which was shake-cultured in a semi-synthetic potato decoction at 30°C for 5 days. The culture filtrate was centrifuged at 8,000 g for 20 min to exclude the bacterial cells, and the supernatant was added to 75% saturation with ammonium sulfate. The percipitates were collected by centrifuging at 10,000 g for 30 min, dissolved with a small quantity of 0.1 M Tris-HCl buffer, and dialyzed overnight against the same buffer at 4°C. The inner solution was concentrated with carbowax, and the concentrated solution was used as a protease solution. Ten ml of protease solution plus 5 ml of 0.1 M Tris-HCl buffer (pH 8.0) with PAA or without PAA was added to chlorophyll-protein complex solution, and the mixture was allowed to react in the water bath at 30°C. After 15 hr, the absorbance curve in the range of 350–700 nm was estimated. On the other hand, 0.5 ml of the reaction solution was

TABLE 2. *Decomposition of Chlorophyll-Protein Complex by the Treatments with Bacterial Protease and PAA*

Treated time (hr)	Treatments	Chlorophyll A	Chlorophyll B	Total Chlorophyll	Rate of decomposition(%)
0	Control	5.52[a]	1.54	7.06	0
15	Control	4.75	1.35	6.10	13.7
〃	PAA 25 ppm	4.44	1.30	5.74	18.7
〃	PAA 50 ppm	4.29	1.29	5.58	21.0
〃	Protease	4.18	1.29	5.47	22.5
〃	Protease+PAA 25 ppm	4.02	1.27	5.29	25.0
〃	Protease+PAA 50 ppm	3.23	1.09	4.32	38.8

[a]: Chlorophyll contents μg/ml

added to 3 ml of aceton solution. The mixture was shaken and centrifuged at 5,000 g for 15 min. The amount of chlorophyll in the supernatant was estimated by the method of Arnon (7).

The result is shown in Table 2. A decrease of 13.7% in the amount of total chlorophyll occurred in the control with no PAA after 15 hr in this experimental condition. On the other hand, the rate of decrease of chlorophyll was further accelerated with PAA treatment. The decomposition of chlorophyll A was especially remarkable. The decomposition of chlorophyll was more accelerated with protease alone than with PAA alone. When the both worked at the same time, the decomposition was further accelerated.

c) Decomposition of Fraction-I-Protein by Bacterial Protease

As previously reported (8), the soluble protein-N, especially fraction-I-protein remarkably decreased in the leaves affected by this pathogen. It is generally known that fraction-I-protein exists in chloroplast. This bacterium produces an active protease and the activity of this enzyme increases in the diseased tissues. Since these proteases might be presumably associated with the decrease of fraction-I-protein, the action of the bacterial protease on the protein was studied in this experiment.

About 50 g of rice leaves at 5–6 leaf stage was cut finely, and 0.1 M Tris-HCl buffer (pH 8.0) was added to it. They were ground with quartz sand in a mortar cooled with ice and centrifuged at 10,000 g for 20 min after filtrating with cheesecloth. The supernatant fluids were dialyzed overnight against the buffer solution. The dialyzed solution was used as a soluble protein solution. This was treated with Diaflo XM-100 under a cool condition and purified by the repeated sedimental managements with sulfuric ammonium and ethanol. The degree of purification was determined by the electropolyacrylamide gel method. Because only one band appeared at the expected position, it was thought that the protein was considerably purified. The protein was determined by the method of Lowry (9) with bovine serum albumin as the standard. One % of fraction-I-protein was allowed to

TABLE 3. *Decomposition of Fraction-I-Protein by the Bacterial Protease*

Treatments	Reaction time (hr)			
	0	5	10	20
Fraction-I-protein+Protease heated[a]	80[b]	90	111	115
Fraction-I-protein+Protease	80	225	295	362
Fraction-I-protein+Protease+PAA 1000 ppm[c]	80	233	303	375
Fraction-I-protein+Protease+PAA 500 ppm	80	250	313	421

[a]100 C, 10 min. [b]Amounts decomposed μg/ml. [c]Final concentration.

react with protease solution, PAA solution, or the mixture of both solutions in the water bath at 30°C. A given volume of the solution was harvested at regular intervals and the amount of protein decomposed by protease was determined. The result is shown in Table 3.

Fraction-I-protein was remarkably decomposed by the action of bacterial protease. In addition, the degree of decomposition was slightly accelerated when PAA also was present.

5. *Change of Susceptibility of the Rice Plants which absorbed PAA*

From the above results, it became apparent that PAA which was produced by the pathogen was related with the lesion formation. On the other hand, none or only a few small lesions developed in a resistant variety. These might be responsible for the two facts described below: 1) the severe inhibition of multiplication of the pathogen, 2) consequently, minimal or no PAA production by the pathogen. Therefore, when PAA was applied externally, whether or not lesions enlarged in a resistant variety was studied.

In this experiment, a resistant variety "Kidama" at the booting stage was used. The plants were cut at the soil level. The main stems which were further cut in water at the lower portion of the stem were inserted in PAA solutions with various concentrations and were allowed to absorb PAA. The PAA solution was renewed every day till the termination of the treatment. Then, a needle inoculation was made on two upper leaf blades. After inoculation, the inoculated plants were kept at 30°C under high humidity for 48 hr. Afterwards, they were placed in a greenhouse and the lesion areas were measured 7 days after inoculation. The result is shown in Table 4.

With PAA treatment the lesion area enlarged more than in the control. This tendency was more remarkable at the 2nd leaf than at the top leaf. With 50 ppm treatment of PAA solution, the stem portion inserted into the solution became light-brown, and the lower leaves faded slightly. With 30 ppm treatment, the lower leaves became yellow and the upper leaves faded slightly. As the pathogenic bacterial number in the lesions was not estimated, it was not apparent whether the lesion enlargement was caused by the decrease of resistance by PAA treatment or

TABLE 4. *Change of Susceptibility of the Resistant Variety "Kidama" Absorbed PAA*

Leaf position	PAA concentration (ppm)				
	0	50	100	200	300
1st leaf blade	13.0[a]	25.5	29.5	29.0	38.0
2nd leaf blade	19.5	48.0	49.5	67.0	85.0

[a]Mean lesion areas (mm^2).

the multiplication of the pathogen. However, it indicated that the lesion of a resistant variety "Kidama" which absorbed PAA enlarged more than that in the control.

6. *Effect of PAA on the Multiplication of the Pathogen*

This pathogenic bacterium produced PAA as a metabolite in the medium and in the host tissue. Therefore, the effect of PAA on the multiplication of the bacterium was examined. The media used were a semi-synthetic potato decoction medium and a synthetic solution medium. PAA with a given concentration was added to the media and the media were adjusted to pH 7.0. One hundred ml of the medium was placed in a 500 ml of the flask and sterilized at 115°C for 15 min. After the inoculation of the pathogen to the medium, the pathogen was shake-cultured at 30°C. The bacterial cells in the cultured solution were centrifuged at 8,000 g for 20 min at regular intervals. The cells collected were suspended in the sterilized physiological salt solution. The number of the bacterial cells was determined by the measurement of optical density (610 nm). The result is shown in Fig. 4.

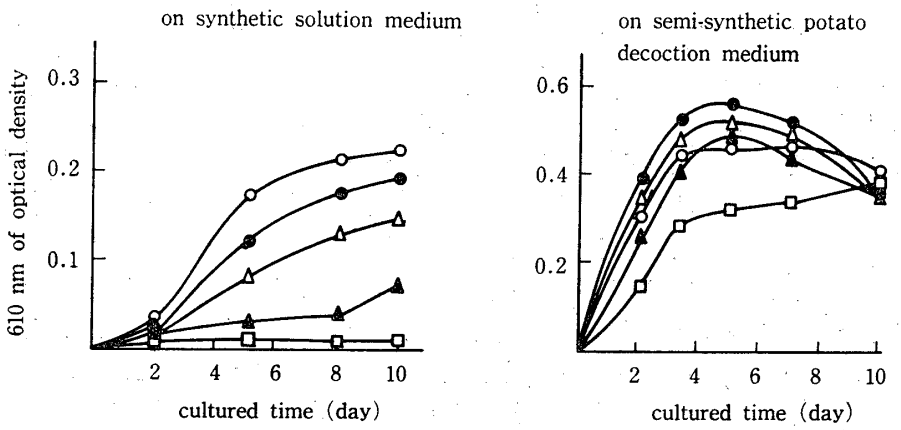

FIG. 4. Effect of PAA on the multiplication of the pathogenic bacterium
o——o Control, •——• 100 ppm of PAA concentration, △——△ 250 ppm of PAA concentration, ▲——▲ 500 ppm of PAA concentration, □——□ 1000 ppm of PAA concentration

The effect of PAA on the multiplication of the bacterium differed between the two kinds of media. At the synthetic medium, the inhibition of multiplication increased with the rise of PAA concentration and no growth of the pathogen occurred in 1,000 ppm of PAA concentration. On the other hand, in a semi-synthetic potato decoction medium, a slight inhibition occurred in 1,000 ppm, but no inhibition in 500 ppm or below. From the above results, it became obvious that the pathogen was remarkably resistant to PAA. The loss of PAA resulted by the sterilization was about 10% in 1,000 ppm of PAA concentration.

Discussion

Previous studies (2) have indicated that the PAA produced in the lesion by this pathogenic bacterium would be an important substance involved in the lesion formation. The high exudation from the lesion suggested the induction of the abnormal permeability of the cell membrane. The treatment of the inner cells of leaf sheath tissues with PAA resulted in the increase of the water permeability and the exudations of inorganic salts, soluble sugars, and nitrogen compounds. It is presumed that these facts are responsbile for the denaturation or the degradation of plasmalemma by PAA. Moreover, PAA would probably affect not only the plasmalemma but also the membrane structures of the cell organellas. Thus, there is an evidence that the acid phosphatase is liberated by the treatment of chloroplast with PAA. As previously reported (8), the hydrolase activity in the lesion area increased remarkably, and it might be associated with the result of PAA action to the tonoplast rather than the production by the pathogen. Thus, the hydrolase transferred to cytoplasm would affect the various organs and the cell substances in cells. When fraction-I-protein and the chlorophyll-protein complex were treated with the bacterial protease, they disintegrated rapidly and the disintegration was further accelerated by the addition of PAA. It resulted in a remarkable decrease of chlorophyll.

The amount of PAA extracted from the diseased areas was calculated as at least 1.5 μg per one lesion at the early stage of the disease development. On the other hand, the number of bacteria in one lesion at the early stage was in the order of 10^4 to 10^5. If the bacterium will produce an equivalent amount of PAA in the host tissues as in the cultured solution, the amount of PAA per one lesion will be presumed to be 1–10 μg. This amount of PAA is approximately consistent with that from the above calculation value. The bacteria do not distribute uniformly in the lesion, but they exist abundantly at the margin of developing lesion. Therefore, it is presumed that the PAA concentration at the margin area is higher than the above calculated value. Judging from the cooperative action between PAA and hydrolase, the PAA might operate at a low concentration in the lesion. The formation of a brownish area around the lesion caused by this bacterium is either very few or not at all. Therefore, some polyphenolic substances or

enzymes referred to as the causal agents of the brownish area formation were examined. According to the result, the accumulation of polyphenolic substances was rarely found, and no activity of polyphenol-oxidase was found. On the other hand, only the peroxidase activity was definitely found. However, as the rise of respiration involved in the source of energy on the accumulation of polyphenolic substances was very slight, the rise of peroxidase activity might be related to the disintegration of the cell contents.

From the above results, the lesion formation characteristic of this disease was presumed as below. The obstruction of the membrane structure of the host cell caused by the pathogen-produced PAA and hydrolase followed by the destruction of chloroplast and the disappearance of chlorophyll. Consequently, the fading of the diseased leaves and the destruction and exudation of the cell substances occur, and at the same time, the cell hydration proceeds. The appearance of the white lesion may be in connection with the above facts. As the amount of PAA is affected by the number of bacterial cells in the tissues, it will be preferable to consider that the small lesion formation in the resistant variety has resulted from other causal agents.

References

1) Miyazaki, E., Yamanaka, S. and Misawa, T., Tohoku J. Agr. Res. **26**, 11 (1975)
2) Miyazaki, E., Yamanaka, S. and Misawa, T., Tohoku J. Agr. Res. **26**, 95 (1975)
3) Itzhaki, R.F. and Gill, D.M., Ann. Biochem. **9**, 401 (1964)
4) Rogers, D. and Reithel, F.J., Arch. Biochem. Biophys. **89**, 97 (1960)
5) Miyazaki, E., Yamanaka, S. and Misawa, T., Ann. Phytopath. Soc. Japan, **42**. 21 (1976)(in Japanese with English summary)
6) Hotta, R., Haraguchi, N. and Shimizu, S., Bot. Mag. (Tokyo) **81**, 347 (1968)
7) Arnon, O.I., Plant Physiology, **24**, 1 (1949)
8) Misawa, T. and Miyazaki, E., Ann. Phytopath. Soc. Japan, **38**, 375 (1972) (in Japanese with English summary)
9) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem., **193**, 265 (1951)