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Studies on the Leaf Blight of Rice Plant III. A Metabolite of the Pathogenic Bacteria

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Summary

This study is an examination of the relation between the physiological changes of rice leaf infected by bacterial leaf blight pathogen (*Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson) and the metabolites of the pathogen. It became evident that a substance which promoted the water permeability of the cell of rice leaf sheath was contained in the extract of a diseased leaf of rice and in the cultured solution of the pathogen. This substance was identified as phenylacetic acid by searching for a substance having the following three actions; change of permeability of rice cell, destruction of chlorophyll, and inhibition of the growth of rice seedling.

There are many reports indicating that the metabolites (containing enzymes) of the plant pathogen and the substances produced by the host in response to the pathogen are facters causing symptoms. The bacterial leaf blight of rice plant has two characteristics: first the infected area enlarges rapidly, and the color (yellowish to white) of the spot is different from that of other rice diseases. Our previous reports (1, 2) indicated that the diseased leaves became catabolic and the constitutive substances (nitrogenous compounds and carbohydrates) in the leaf were altered. To elucidate relations between the phenomena above described and the metabolite of the pathogenic bacteria (*Xanthomonas oryzae*), effects of the diseased leaf extract and the filtrate of cultured solution of the pathogen on rice plant were examined at first and then the active substance was extracted and identified.

Materials and Methods

Culture of Pathogen: The H-5809 strain of *X. oryzae* was used. The pathogen was shake-cultured at 30 C in potato semi-synthetic medium. After 5 days, the cultured solution was centrifuged (8000 g, 15 min) and the pathogenic bacteria were removed. Then the supernatant was adjusted to pH 6.0 with 1 N HCl and used for experiments.

Inoculation to Rice Leaf: Rice plants (cultivar: Aichi Asahi) were water-cultured in a modified Kasugai's solution and used for inoculation at the shooting stage. The upper three leaves were inoculated with the bacterial suspension by the needle method. Inoculation was done at intervals of 2 cm through the whole length of the half leaf blade. The other half leaf blade was used as a control. It was inoculated with a sterilized physiological salt solution, the same as above.

Extract from Diseased Leaves: After 7 days, the inoculated and the uninoculated half leaf blades were detached separately. Each fresh sample corresponding to one gram of dry weight was ground with quartz sand in mortar. Then a few drops of distilled water were added to the homogenates and they were centrifuged (1000 g, 30 min). Each supernatant was diluted to 10 ml by adding distilled water. One portion of the supernatant and of the cultured solution were boiled for 10 min as a blank test.

Estimation of Cell Permeability: The fourth leaf from the top of the main stem was cut out and the inner epidermis (2—3 cell layers) was removed from the central part of leaf sheath and immersed in the extract of leaf or the supernatant of the cultured solution. After a given time, each piece was transferred to a 0.8 M sucrose solution containing 0.001% neutral red to initiate plasmolysis. After 15 min, these pieces were transferred to a 0.4 M sucrose solution on the slide glass and the time required for deplasmolysis was estimated. It was observed that approximately 50% of the cells displayed deplasmolysis. The estimated value is the mean of three tissue-pieces.

Inhibition of Seedling Growth: Water-presoaked rice seeds were placed in a Petri dish (12 cm in diameter) with two sheets of Toyo filter paper (No. 2) placed on the bottom. Ten ml of the solution to be examined was poured into these Petri dishes. After these dishes were incubated at 30 C, the length of leaf and root were measured.

Effect on Chloroplast: Leaf disks of 0.7 cm in diameter were cut out from the central part of the top, the second and the third leaves of the main stems, respectively and placed for 2 hrs in Petri dishes (9 cm in Diameter) containing distilled water for the purpose of avoiding the effect of cutting. Thereafter, these disks were transferred to a Petri dish containing 10 ml of the solution to be examined and incubated under continuous illumination of 5000 Lux. Then these disks were ground with quartz sand in a mortar and the chlorophyll was extracted by 70% aceton. The homogenate was centrifuged (8000 g, 15 min) and the supernatant was used for chlorophyll estimation by the method of Arnon (3).

Results

1. *Actions of Extract of Diseased Leaf and Cultured Solution*

The effect on the permeability of rice leaf cell was investigated using the extract of diseased leaf and the cultured solution. The results obtained were

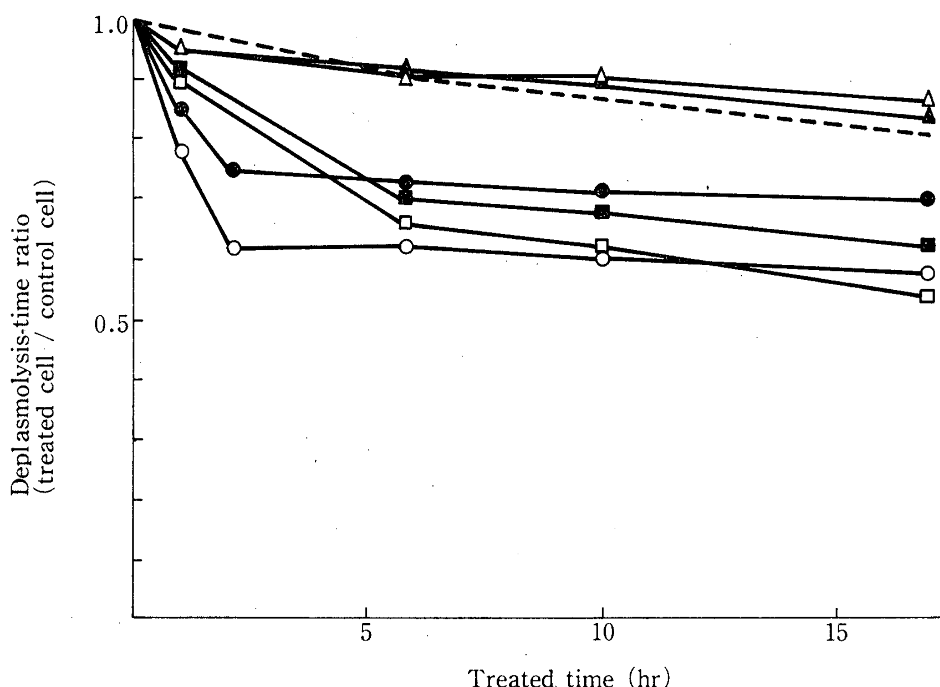
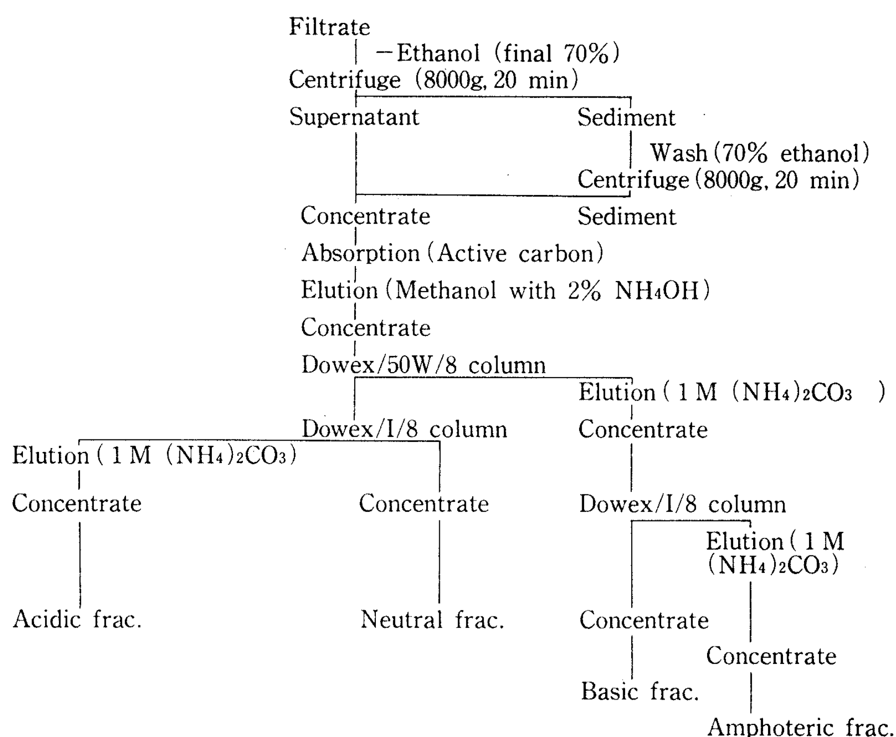


Fig. 1. Effects of the extract of diseased leaf and the cultured solution on permeability of rice leaf cell. ----: uncultured solution, ○—○: cultured solution, ●—●: boiled culture solution, △—△: extract of healthy leaf, ▲—▲: boiled extract of healthy leaf, □—□: extract of diseased leaf, ■—■: boiled extract of diseased leaf

indicated in Fig. 1. The extract of healthy leaf, the boiled extract of healthy leaf and the uncultured solution shortened the time of deplasmolysis slightly, but the difference was very little compared with the control. On the contrary, the extract of diseased leaf and the cultured solution promoted the deplasmolysis and the action was very obvious. By boiling these solutions, their actions were decreased only slightly. These results suggest that some substances in the extract of diseased leaf and the cultured solution may induce a change in the permeability of rice cells. Further treatment of two hours by these solutions induced an abnormal deformation of the protoplast at plasmolysis. Also the bursting of the protoplast was observed often at deplasmolysis by 17 hrs treatment. Such phenomena were never observed in the treatment of the extract of healthy leaf and the uninoculated culture solution. Since the pH values of the extracts of healthy leaf and diseased leaf are 6.0 and 5.8, respectively, it was considered taht the difference of pH value between the extracts did not affect the results.

2. *Extraction of Active Substance*

As it was assumed that the active substance for menbraneous structure of the cell might exist in the extract of diseased leaf and in the cultured solution, extraction of the active substance was attempted. During extraction of metabolic substances of the pathogen, low molecular weight substances in the solution have been extracted generally with organic solvents (ether etc) under acidic conditions.



Culture solution : Polypeptone 5.0gm, $Ca(NO_3)H_2O$ 0.5gm, $Na_2HPO_4 \cdot 7H_2O$ 2.0gm, Sucrose 20gm, Rice green leaf extract 1000ml. pH 7.0. Incubation : 30 C, 7 days.

Fig. 2. Fractionation of metabolites

But in this experiment the extraction with ion-exchange resin was used to increase the yield. Each extract was selected by whether the extract accomplishes the three above described qualifications: the change of permeability of rice cell, the destruction of chlorophyll, and the inhibition of the growth of rice seedling.

a) *Culture of Pathogen*: In order to extract active substance from the culture medium, the pathogenic bacteria were cultured in green rice leaf decoction for vigorous growth of the pathogen. Its composition was as follows: sucrose 15 g, polypeptone 5 g, calcium nitrate 0.5 g, and disodium orthophosphate 2 g in one liter of green rice leaf decoction (200 g/liter). The hydrogen oin concentration is 7.0. The shaking culture was done at 30 C for 7 days.

b) *Extraction and Fractionation*: The process of extraction is indicated in Fig. 2. Each of acidic, neutral, basic and amphoteric fraction was adjusted to a volume to 50 ml by adding distilled water, and their respective actions were examined. The paper-chromatography was done by the ascending method with the solvent (n-butanol: acetic acid: water=4:1:5) by using Toyo Roshi No. 51 paper (40×5 cm). After developing, the chromatogram was detected by UV and brom phenol blue (BPB) spray. UV reactive bands were cut out and the substances in the bands were extracted by ether and concentrated under reduced pressure. The

TABLE 1. *Effect of Each Fraction from the Cultured Solution on the Growth of Rice Seedlings*

Seedling	Water (control)	Fractions			
		acidic	neutral	basic	amphoteric
Shoot(mm)	18.5	17.1	17.9	19.0	19.5
Root(mm)	49.3	2.2	46.8	41.2	43.7

Each reading represents the average for 12 seedlings.

TABLE 2. *Effect of Each Fraction from the Cultured Solution on Plasmolysis of Leaf Cell*

Fractions	Treated time (min)										
	5	10	20	30	40	50	60	90	120	150	180
Water (control)	+	+	+	+	+	+	+	+	+	+	+
Acidic	+	−	−	−	−	−	−	−	−	−	−
Neutral	+	+	+	+	+	+	+	+	+	+	+
Basic	+	+	+	+	+	+	+	+	+	−	−
Amphoteric	+	+	+	+	+	+	+	+	+	+	+

+: Plasmolysis appeared. −: Plasmolysis disappeared.

TABLE 3. *Effects of Acidic and Basic Fractions on Cell Permeability*

Fraction	Experiment			Mean	Ratio
	1	2	3		
Water (control)	191	195	208	198	1.00
Acidic	120	130	123	121	0.61
Basic	201	189	203	197	0.99

The time immersed in each fraction=1 min.
Each reading represents the time (sec.) for deplasmolysis.

residues were dissolved in distilled water.

c) *Actions of Each Fraction*: The effects of each fraction on the growth of rice seedlings are indicated in Table 1. None of the fractions affected the growth of the leaves, but the acidic fraction inhibited the growth of seedling root remarkably. Basic fraction also very slightly inhibited the growth of seedling root. Their effects on the permeability of rice cell are indicated in Table 2. Acidic fraction treatment for 10 min caused rice cells to lose their ability to plasmolyse. Even after treatment for a long time by the basic fraction, the plasmolysis of the rice cell was only slightly affected. The other fractions had no effect. Next, the influence on water permeability was investigated in the acidic and basic fractions, respectively. Table 3 indicates the results. The acidic fraction obviously increased the water permeability of rice cell. The actions of each fraction upon the chloroplast were investigated as to whether the content of total

TABLE 4. *Effect of Each Fraction from the Cultured Solution on the Chlorophyll Content*

Each component of chlorophyll	Water (control)	Fraction			
		Acidic	Neutral	Basic	Amphoteric
Chlorophyll a	135.0	56.4	125.1	118.5	130.7
Chlorophyll b	70.5	44.0	70.0	62.6	68.2
Total chlorophyll	205.5	100.4	195.1	181.1	198.9

Each reading represents contents (μg) contained in 100 mg of rice leaf disks.

chlorophyll decreased or not. The results obtained were indicated in Table 4. The acidic fraction treatment decreased the total chlorophyll content. Especially, the content of chlorophyll a decreased remarkably by the acidic fraction treatment. Chlorophyll b had also the same tendency. The basic fraction had the same action as the acidic fraction, but the action was considerably weaker.

d) *Fractionation of the Acidic Fraction*: As the acidic fraction had strong effects in these three screening tests, this fraction was further fractionated to subfractions. The chromatogram obtained by the paper-chromatography (above described) of the acidic fraction is shown in Fig. 3. Rf-0.97 and -0.39 spots were largest in area among the six spots. The Rf-0.39 spot was not detected with UV, but stained by BPB. Five other spots were detected with UV and BPB. Rf-0.91 and -0.50 spots were positive in their Diazo- and Ferric chloride-reactions. All spots were negative in their Ninhydrin reaction. Each extract from the six spots was examined to elucidate their actions and the results are indicated in Tables 5, 6, and 7. According to the results, the Rf-0.97 fraction alone inhibited the root elongation, affected the plasmolysis and decreased the chlorophyll contents.

3. *Certification of Active Substance*

As one subfraction in the acidic fraction was observed to be capable of causing all three actions, the subfraction was examined further. The Rf-0.97 fraction was analyzed by the thin-layer chromatographical (TLC) method by using Silica gel F (Merk Co.). The TLC plate was developed by the ascending method. The solvent used was a mixture of chloroform: ethylacetate: acetic acid=12:8:1. The two substances detected with UV were extracted by ether and recrystallized with petroleum ether. In this case, the Rf values of the two substances were 0.72 and 0.55, respectively.

The actions of the two substances were examined, but only the Rf-0.55 fraction affected the permeability of rice cell. This fraction was negative for Diazo-, Ferric chloride-, Potassium permanganate-, Ninhydrin- and Brom-reactions. So it was thought that this substance had no free amino groups, phenolic hydroxyl radicals or even double bonds in side chain. This substance has a maximum absorption value of 265 nm and a minimum absorption value of 255 nm in ether

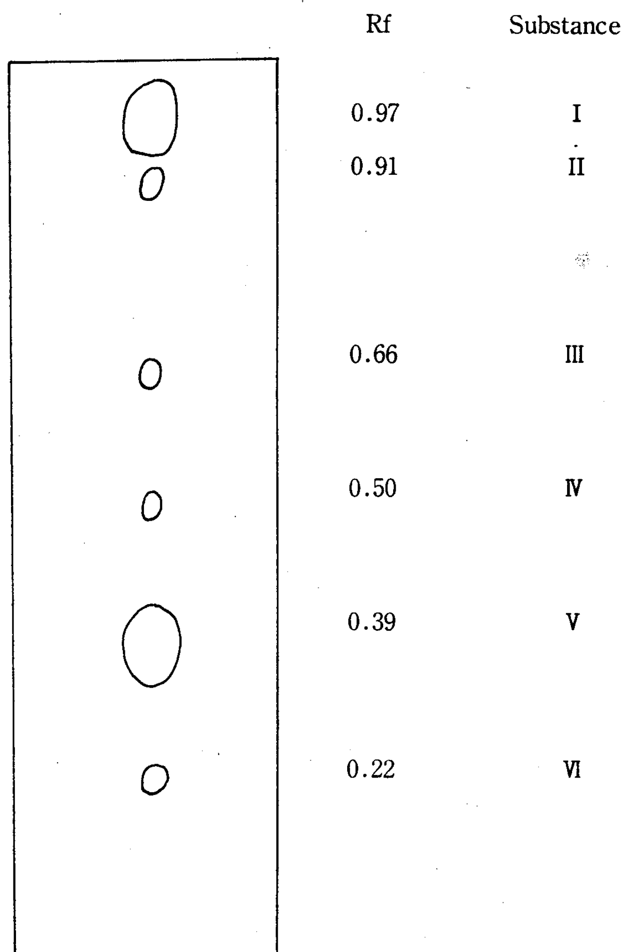


Fig. 3. Chromatography of acidic fraction

TABLE 5. *Effect of Each Substance from Acidic Fraction on the Growth of Rice Seedling*

Seedling	Water (control)	Substances from acidic fraction					
		I	II	III	IV	V	VI
Shoot(mm)	22.6	19.6	21.7	21.5	23.6	20.8	21.9
Root (mm)	41.5	1.5	37.1	40.8	44.5	38.8	40.2

Each reading represents the average for 12 seedlings.

solution. The substance was mixed with potassium bromide and tableted by the usual method and then its infrared absorption curve was estimated to be in the range of 2.5–15.4 μm by an infrared spectrophotometer (Hitachi EPI-S2). The infrared spectrum indicates two peaks (6.2 and 6.6 μm) for the aromatic ring and three peaks (3.7, 5.9 and 7.1 μm) for the carboxyl radical. However, it had no peaks (3.1 and 8.2 μm) for the hydroxyl radical. The melting point of this substance was 76.0 C. Further, this spectrum pattern was consistent with authentic phenylacetic acid. Therefore from the above characters, this substance

TABLE 6. *Effect of Each Substance from Acidic Fraction on Plasmolysis of Rice Leaf Cell*

Substance	Treated time (min.)										
	10	20	30	40	50	60	90	120	150	180	240
I	−	−	−	−	−	−	−	−	−	−	−
II	+	+	+	+	+	+	+	+	+	+	+
III	+	+	+	+	+	+	+	+	+	+	+
IV	+	+	+	+	+	+	+	+	+	+	+
V	+	+	+	+	+	+	+	+	+	−	−
VI	+	+	+	+	+	+	+	+	+	+	+
Water (control)	+	+	+	+	+	+	+	+	+	+	+

+: Plasmolysis appeared. −: Plasmolysis disappeared.

TABLE 7. *Effect of Each Substance from Acidic Fraction on the Chlorophyll Content*

Each component of chlorophyll	Water (control)	Substance					
		I	II	III	IV	V	VI
Chlorophyll a	164.5	48.3	133.1	162.5	170.3	155.5	166.5
Chlorophyll b	75.6	40.6	59.5	75.7	75.8	76.0	77.2
Total chlorophyll	240.1	88.9	192.6	238.2	246.1	231.5	243.7

Each reading represents contents (μg) contained in 100 mg of rice leaf disks.

was identified as phenylacetic acid. This substance also has the characteristic odor of phenylacetic acid. The yield of this substance was approximately 40 mg per five liters of green rice leaf decoction medium in our experiment.

Disccusion

Considering the characteristics of this disease, it was assumed that some substances which affect the physiology of rice plant are contained in rice leaves infected with *X. oryzae* and in the cultured solution of the pathogen. Therefore, the active substances were searched from the standpoints of inhibition of rice growth, alteration of the permeability of rice cell and destruction of chlorophyll. Watanabe et al (4) reported that the substance which increased the water permeability of the inner cells of rice leaf sheath exists in the cell suspension of vigorous strains of *X. oryzae*. They also reported that this substance lost the activity by boiling for 20 min (5). So that substances like enzyme may be involved for inducing change of water permeability of rice cell. But, in our experiment the extract of diseased leaves and the cultured solution of the pathogen lost very slightly their activities by boiling. From this result, we considered that the active substance was tolerant to heat and the substance tolerant to heat was more important for pathogenesis than the substance susceptible to heat. In fact, by boiling the extract of diseased leaves and the cultured solution of the pathogen,

their activities for increasing the permeability of rice cell and altering the protoplasmic menbrane had been lost. On the other hand, the extract of healthy leaves and the uncultured solution had no activities for the phenomena as above.

From these results, we concluded this to be the substance which was synthesized by the pathogenic bacteria. Further, Watanabe and Samejima (5) reported that ^{14}C was remarkably exuded from diseased ^{14}C-labelled-leaves, and that it might be caused by the alteration of the cell permeability. Their presumption is now supported by our experimental results. From the cultured solution of *X. oryzae*, phenylacetic acid was also extracted as the active substance in this experiment. In addition, the active substance in the extract of diseased leaves is presumed to be phenylacetic acid. The evidence of the above presumption will be presented in our next paper. On the other hand, concerning the production of phenylacetic acid by *X. oryzae* in the culture, Egawa et al (6) had already reported it.

In the beginning of our experiment, it was our object to search for other active substances than phenylacetic acid. But *X. oryzae* produces such a large amount of phenylacetic acid that we could not extract any other low molecular weight substances by the method used in this experiment.

References

1) Misawa, T. and Miyazaki, E., *Ann. Phytopath. Soc. Japan*, **38**, 375 (1972) (in Japanese, with English summary)
2) Misawa, T. and Miyazaki, E., *Ann. Phytopath. Soc. Japan*, **39**, 79 (1973) (in Japanese)
3) Arnon, O.I., *Plant Physiol.*, **24**, 1 (1949)
4) Watanabe, M., Konya, K. and Hosokawa, D., *Ann. Phytopath. Soc. Japan*, **36**, 174 (1970) (in Japanese)
5) Watanabe, M. and Samejima, S., *Ann. Phytopath. Soc. Japan*, **38**, 201 (1972) (in Japanese)
6) Egawa, H., Yoshii, K. and Ueyama, A., *Ann. Phytopath. Soc. Japan*, **34**, 46 (1968) (in Japanese, with English summary)