14. PATHOLOGICAL RESEARCH OF *HELMINTHOSPORIUM* LEAF SPOT

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Introduction

About 20 per cent of the area of Japanese paddy fields originate from degraded soils, which are usually called "akiochi" soils. Rice plants grown in these soils show normal growth in the early stages of development, but they begin to decline gradually at later stages. Moreover, *Helminthosporium* leaf spot often occurs on the rice plants grown in such soils, so that the yield of the crop may be greatly decreased at harvest time. This phenomenon is called "akiochi" in Japanese, which means the "autumn-decline". Although the main cause of the "akiochi" phenomenon is undoubtedly due in part to physical and chemical abnormalities of the soils, the occurrence of *Helminthosporium* leaf spot may also promote the "akiochi" phenomenon.

The causes of the various types of necrotic lesions of *Helminthosporium* leaf spot in rice plants are as follows:

(1) Difference of susceptibility due to the different species or varieties of rice.

(2) Difference of susceptibility due to the different enviromental conditions around the plant.

(3) Difference of pathogenicity due to the different races or isolates of the pathogenic fungus.

(4) Difference of pathogenicity due to the different pre-growing conditions of the pathogence fungus.

Among these causes, differences in the enviromental conditions around the susceptible plant may correlate with the appearance of the largest necrotic spots. Since the present disorder is generally thought of as an enviromental disease, research of disease susceptibility based on the differences in the enviroment is an important approach.

This report deals with the investigation of why the "akiochi" rice plant is more susceptible than the normal rice plant to the causal fungus of *Helminthosporium* leaf spot, *Cochliobolus miyabeanus*.[1]

In the course of the research on disease susceptiblity of plants, properties of both susceptible plant and pathogen should be investigated. At first, the writer wishes to montion on the analyses of susceptibility from the standpoint of the fungus.

Extracellular Enzymes of the Fungus and Their Perthophytic Action

The present fungus grows in the intercellular spaces of cells in rice plants. According to Bonner, middle lamella of Gramineae plants consists mostly of pectic substance, and the cell wall is composed of hemicellulose, lignin and xylan. In order to study the decomposition of middle lamella and the cell wall, the writer investigated the presence of pectic enzyme, cellulase and xylanase in the culture filtrates of the fungus and the changes in their activities during the culture period. Since the duration of the enlargement of the necrotic spots is

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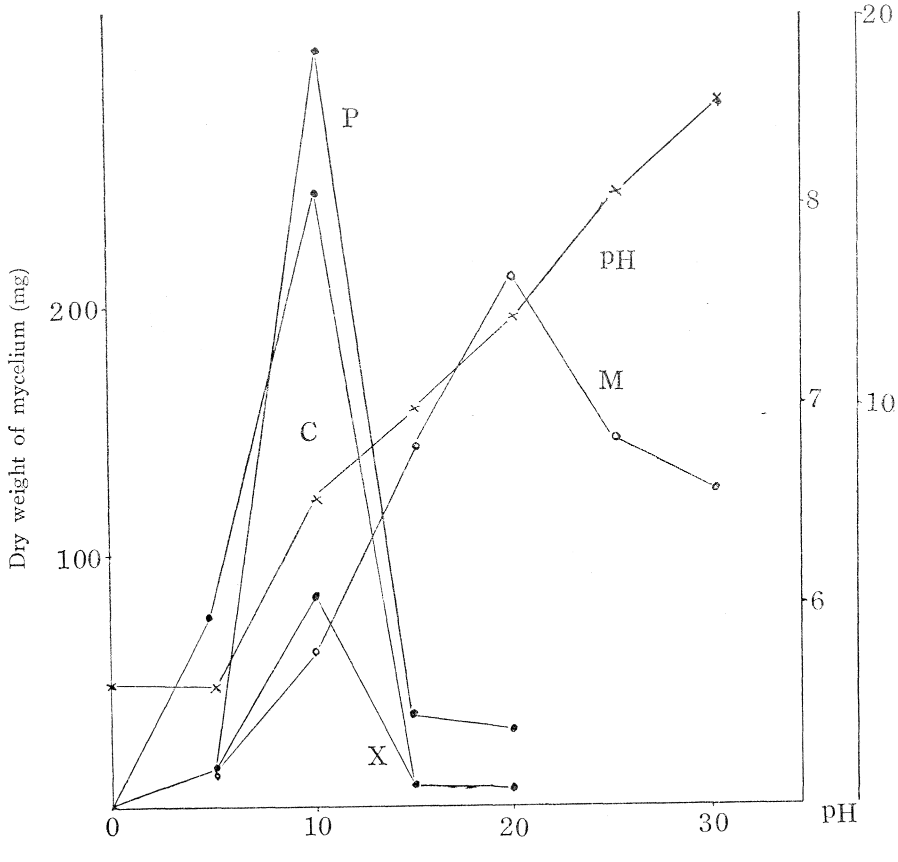
only about a week, it is necessary to investigate whether the above-mentioned enzymes are present in the culture filtrates during early stages of the culture. As shown in Fig. 1, it is



C: Cellulase activity X: Xylanase activity P: Pectic enzyme activity

Enzyme activity is shown by the reducing power of glucose.

Fig. 1. Changes in the mycelial weight, activity of cellulase, xylananse, pectic enzym in the culture filtrate during the cultivation of *Cochliobolus miyabeanus*.

recognized that these enzymes are present and their activities are higher at the logarithmic phase of growth than at the maximum growth phase of the fungus. The amount of these enzymes' secretion seems to be sufficient for the destruction of the susceptible tissues even if the invaded hyphae are few in number. The optimum pH of these enzymes, moreover, is about 6, a value close to that of the rice leaves. From these considerations, the perthopytic action of the fungus to rice leaves may be deduced as follows:

The hyphal invasion is chiefly through the moter cells of the leaves. The hyphae reach the intercellular spaces ofthe parenchyma, and they begin to absord the nutrients from the susceptible cells by the enzymatic decomposition of the middle lamella and the cell wall. The motor cells, penetrated by the hyphae, will die and surrounding cells may also degenerate.

However, the processes, namely, penetration of the hyphae into susceptible cells → death of the susceptible cells → development of the hyphae and secretion of the perthophytic enzymes → death of the susceptible cells, are not actually continuous. Even the enlargement of the necrotic spot in the "akiochi" rice leaves ceases about a week after the fungal in fection, and the defense reaction of the susceptible plant has already occurred. Therefore, it is necessary, to analyze suscept-pathogen interaction.

Preparation of the Artificial "Akiochi" Rice Plant Used in This Study

In the experimental investigations of plants, cultivating conditions are kept at a constant state at each developmental stage. The solution culture method usually satisfies this requirement. An "akiochi" phenomenon, however, is very much complicated, so the use of degraded soil may appear to cause different experimental results because of the variability of the used soil itself. It was our intention to find some methods of treatment to make "akiochi" rice plants similar to the ones grown in nature. The "akiochi" phenomenon seems to require the following conditions.

(1) Keeping the roots in an oxygen deficient state.

(2) Changes in quantitative or qualitative fertilizer elements, especially potassium, nitrogen manganese and ferrous iron.

(3) Addition of hydrogen sulfide or soluble starch.

From combinations of these factors, the writer obtained an artificial "akiochi" rice plant through the following treatment of the culture solution. Non-renewal replacement of culture solution was treated 10 days before the fungal inoculation, and soluble starch was added to the solution at the rate of about 0.1 per cent. The normal plants used in this study were cultivated at all stages of their development in Kasugai's culture solution ($(NH_4)_2SO_4$ 4-40, $Na_2HPO_4 \cdot 12H_2O$ 2-20, KCI 3-30, $CaCI_2 \cdot 5H_20$ 0.4-4, $MgCI_2 \cdot 6H_20$ 0.6-6, $FeCI_3 \cdot 6H_2O$ 0.1-5 (mg), Tap water 1 l.). The "akiochi" plants resulting from this special treatment were almost the same in appearance as those found in nature. Large necrotic spots and rotting of roots or lower leaves could be recognized.

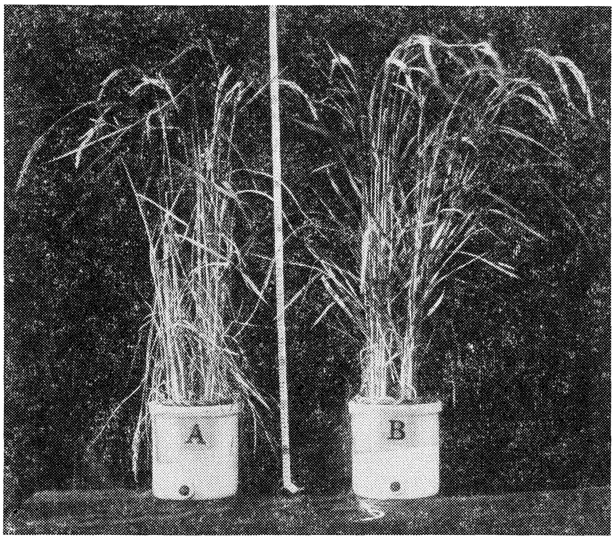
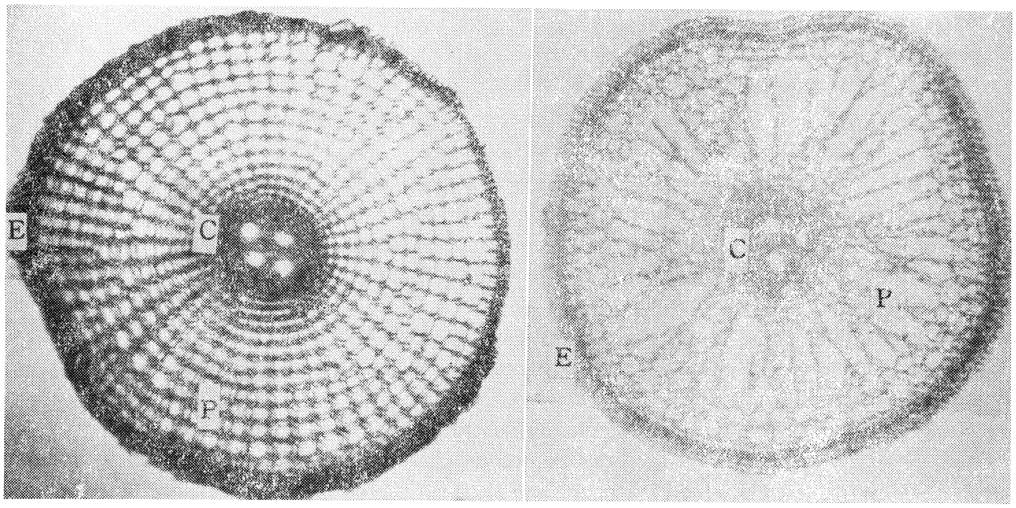


Fig. 2. Artificially induced "akiochi" rice plant (A) and normal rice plant (B).

Fig. 2 shows these two kinds of rice plants and Fig. 3 shows the cross-sections of the roots of both the plants. The "akiochi" plants used in this investigation came from this treatment, the plants grown in Kasugai's culture solution (renewal replacement of the solution is 3-4 days interval) were used as controls.



Root of normal rice plant   Root of "akiochi" rice plant
E: Epidermis   C: Central cylinder

Fig. 3. Cross-sections of the roots of rice plants showing healthy and degraded parenchyma (P).

Existence of Hyphae in the Necrotic Spot

It is well known that some antifungal substances, for example "phytoalexins", appear in infected tissues of plants in response to metabolites produced by a pathogenic fungus. These substances may affect pathogens and the necrotic areas may be kept from enlarging because of the death of infecting hyphae. Nevertheless, the present fungus was isolated even from the border between healthy and necrotic parts of the leaves. This means that even if an antifungal substance is present, the substance does not kill the fungus. The fungus in the necrotic spot remains alive even after the cessation of the enlargement of the spotted area. From these results, the following three hypotheses may be proposed concerning the final stage of the healing.

(1) Secretion of the toxic substance by the fungus may cease, though the fungus still remains alive in the necrotic tissues.

(2) Activity of the toxic substance may be inhibited by the reaction of the susceptible cells, even if the fungus continues to secrete the toxic substance in the necrotic tissues.

(3) Substrates of the substance in the susceptible tissues may be changed, though the fungus continues to secrete the toxic substance in the necrotic tissues.

Therefore, energy is needed for the resistance by infected leaves.

Changes in Respiration and Oxidative Phosphorylation in Rice Leaves due to the Infection of the Fungus

The significance of respiration for energy output consists in the production of energy rich phosphate compounds, such as adenosine triphosphate (ATP). In the normal leaves, oxidative phosphorylation takes place efficiently. However, in the "akiochi" leaves, substrates for respiration are consumed without the production of energy rich phosphate compounds (Table 1).

Table 1. Changes in oxidative phosphorylation in the normal and the "akiochi" rice leaves due to the infection of *Cochliobolus miyabeanus*.

Host plants	Normal rice plants				"Akiochi" rice plants			
Days after inoculation	0	2	4	6	0	2	4	6
P decreased (γ)	7.0	13.7	24.9	10.6	5.6	6.0	5.8	8.6
O_2 uptake (ml)	14.0	14.2	19.2	19.8	14.0	14.8	18.0	18.0
Ratio	0.5	1.0	1.3	0.6	0.4	0.4	0.4	0.5

The uncoupling in the "akiochi" leaves is likely to occur either by a low concentration of adenosine diphospate itself in the leaves, or by hydrogen sulfide originated from the roots. Since the reconstitution of ATP does not take place, energy for the defense reaction must be compensated by ATP which was present before the infection. The disappearance of metabolic activity may permit the enlargement of a necrotic area. This fact seems to be one of the factors which results in the larger necrotic spots in the "akiochi" leaves as compared to the normal ones.

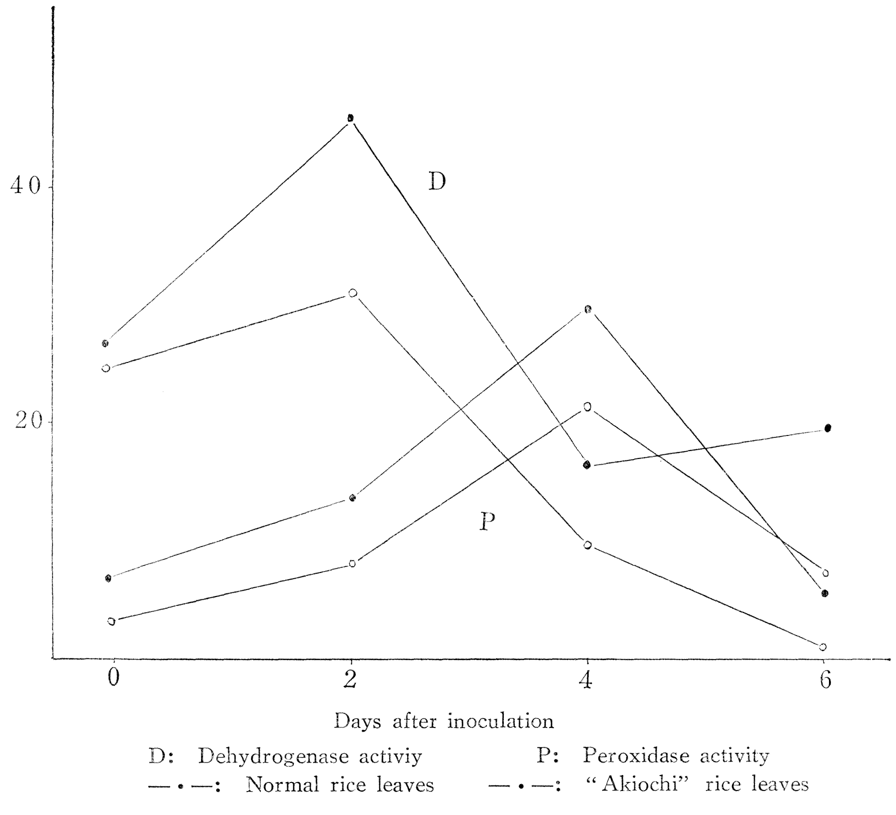
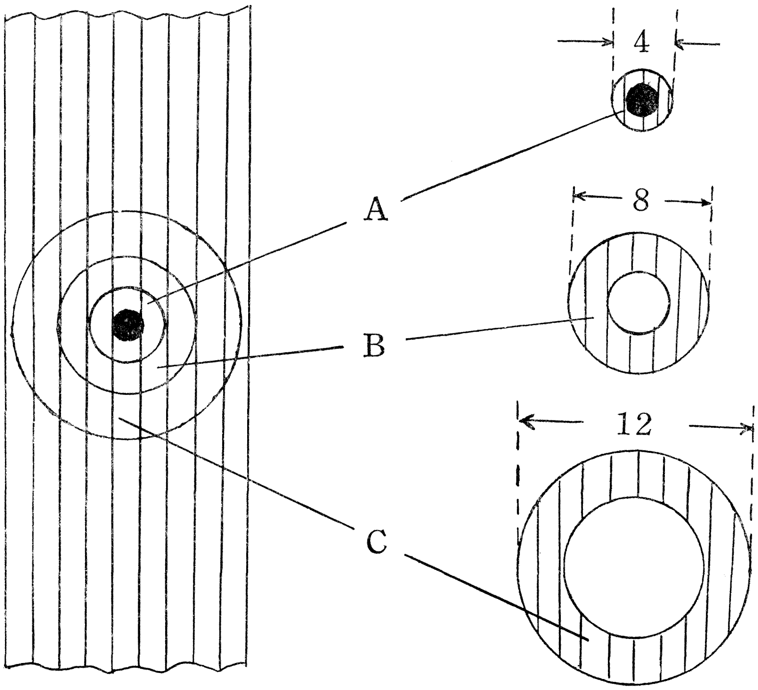


Fig. 4. Changes in the activity of dehydrogenase and peroxidase in the normal and the "akiochi" rice leaves due to the infection of *Cochliobolus miyabeanus*.

Changes in the Activity of Oxidizing Enzymes in Rice Leaves due to the Infection of the Fungus

As previously mentioned, a respiratory enzyme system become active in the infected leaves. Consequently, the writer investigated dehydrogenase activity in the infected rice leaves.

As shown in Fig. 4, dehydrogenase activity in the normal leaves increased 2 days after inoculation followed by a decrease 4 days after inoculation. After 6 days, however, the activity again increased. In the "akiochi" leaves, on the other hand, activity increased 2 days after inoculation followed by a continued decrease in activity. In every experimental stage which was examined, the enzyme activity in the normal leaves was higher than that in the "akiochi" leaves. That is to say, while the susceptible cell is resisting the pathogenic fungus, dehydrogenase activity is generally incresing in proportion to the resistance. In the normal leaves, the spread of the necrotic area is vigorous 4 days after inoculation, and after 6 days the spreading stops. The necrotic area in the "akiochi" leaves is enlarged even 6 days after inoculation and dehydrogenase activity at this time is much diminished. These facts seems to show that the activity of dehydrogenase correlates with the cessation of the necrotic area. Peroxidase activity in both the normal and the "akiochi" leaves is increased 4 days after inoculation, and the activity returns almost to the initial activity after 6 days. Peroxidase activity is also higher in the infected normal leaves than in the infected "akiochi" leaves. It can be shown that many substrates of peroxidase has been produced near the necrotic spot, although no function of peroxidase in the respiratory system is known at present. The writer has not investigated cytochrome oxidase and polyphenol oxidase systems. Also, little is known of the terminal oxidase system in rice leaves.



(unit, mm) Ratio of A: B: C=13: 38: 62 (mm²)=1: 3: 5

Fig. 5. Preparing method of surrounding lesions of necrotic spot

Changes in the Amount of Nitrogenous Compounds at the Surrounding Lesions of the Necrotic Spot

Increase of enzyme activity should be accompanied by an increase in the amount of

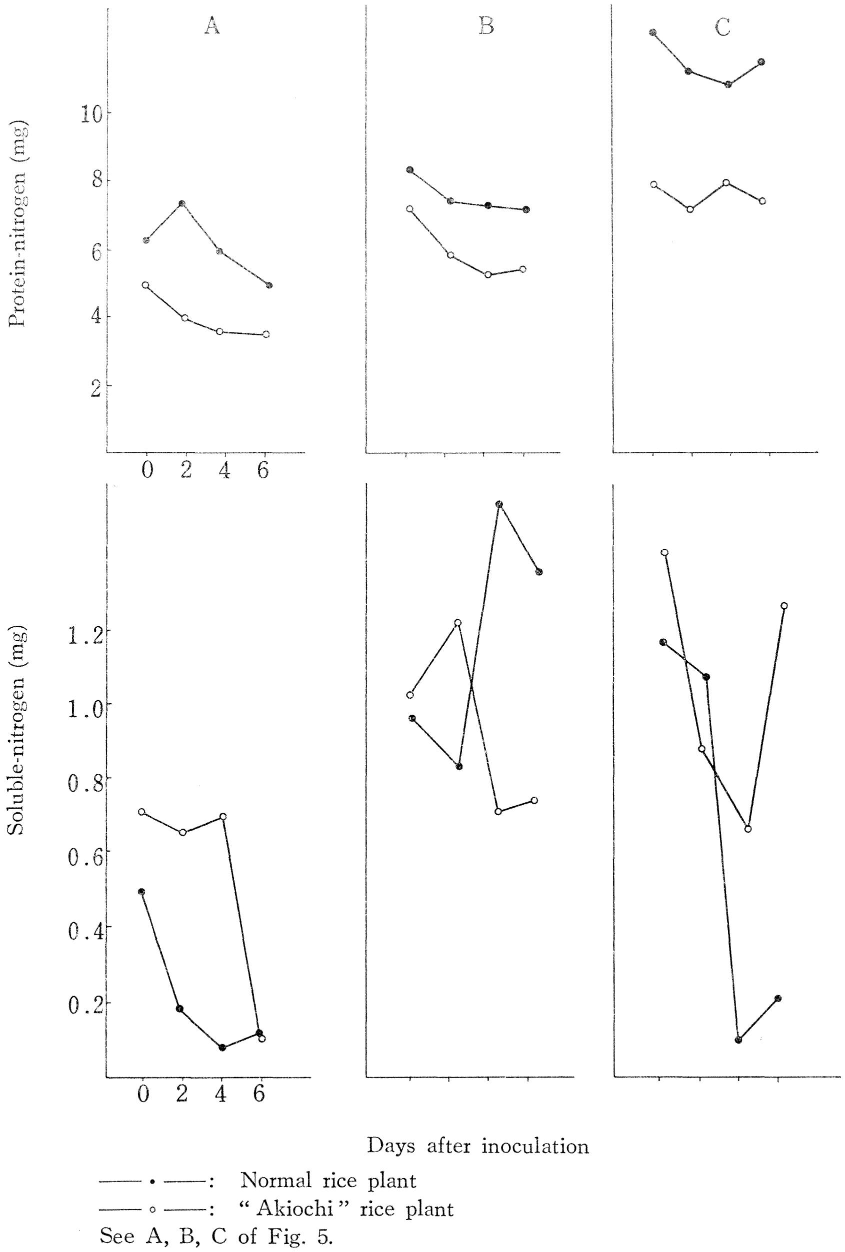


Fig. 6. Changes in the amount of nitrogenous compounds in the surrounding lesions of necrotic spot both in the normal and the "akiochi" rice leaves due to the infection of *Cochliobolus miyabeanus*.

protein. Therefore, the writer investigated the changes in the amount of nitrogenous compound surrounding the necrotic spot.

Fig. 5 shows the method of preparing the material. Every 2, 4, and 6 days after inoculation, the infected and healthy parts of both the normal and the "akiochi" leaves were concentrically cut off at, 4, 8 and 12 mm in diameter. The amount of protein and soluble nitrogen in each part was determined by Micro-kjeldahl.

Fig. 6 shows the changes in the amount of nitrogenous compounds in the lesions surrounds in the lesions surrding the necrotic spot both in the normal and the "akiochi" leaves due to the infection of the fungus. In the normal leaves, a small increase of proteinnitrogen was observed at the early stage of the infection, while a decrease was observed to some extent in the "akiochi" leaves. According to the method of paper electrophoresis, 3 peaks of optical density (α, β and γ) were generally observed in the direction of cathode. In the normal leaves, these 3 protein peaks were prsent even 6 days after inoculation. However, for the "akiochi" leaves the β peak disappeard 2 days after inoculation. This β protein fraction has also peroxidase activity and it has the ultraviolet ab sorption at 270-280 mμ.

Effect of Potassium Permanganate upon the Occurence of *Helminthosporium* Leaf Spot in Rice Plants

Degradation of potassium permanganate in soils may be supposed as follows:

$$2\ KMnO_4 \longrightarrow K_2O + 2\ MnO + 5\ O$$

K, Mn and oxygen are the elements which are lacking in the "akiochi" soils, and application of these elements seems to cause the increase of metabolic activity and exhibition of resistance against the infecting fungus in the "akiochi" plants. The addition of the chemical at the full tillering stage (100-200 g/3.3m^2) of rice plants inhibited the disease occurrence and produced about 10 per cent increases of the harvest grains (Table 2).

Table 2. Effect of potassium permanganate upon the natural outbreak of *Helminthosporium* leaf spot and the yield of rice plants (1957)

Amount of $KMnO_4$ added per 3.3m^2 (g)	Plant height (cm)	Number of effective tillering	Number of spots per 10 cm flag-leaf length	Number of grains per panicle	Percentage of immature ear (%)	Weight of spikelets per 3.3m^2 (g)	Weight of 1000 grains (g)	Weight of grains per 1.8 (g)	Amount of grains L per 10 a (kg)
0	107	17.8	6.1	97.0	4.5	2175	25.7	1449	398.7
I* 100	109	21.6	3.8	77.7	6.4	2428	25.6	1490	449.4
200	109	20.3	2.8	84.4	4.2	2381	25.4	1527	417.4
0	110	16.8	5.9	86.7	5.2	2062	25.9	1416	390.3
II* 100	111	20.6	5.9	75.4	5.9	1846	25.6	1443	343.8
200	115	22.3	3.7	83.0	4.3	2231	25.0	1421	429.0

Preactical application, however, may be difficult because of the high cost.

In summary, the writer have mentioned about his investigations concering the susceptibility of the "akiochi" rice plants to *Helminthosporium* leaf spot. A lower metabolic activity of "akiochi" rice plants is correlated with disease suscceptibility. This lower activity may be due to uncoupl- ing of oxidative phosphorylalion caused by respiration. The uncoupling may be caused by the dacay of roots of the "akiochi" rice plants.

Discussion

D. V. W. Abeygunawardena, Ceylon: High pectinase activity according to your graph is around pH 8. Do you think this is close to the pH of the cell sap? Also I would like to know how you measure pectinase activity in vitro?

Answer: In my exeriment, optimum pH of pectic enzyme activity in the culture filtrate of *Cochliobolus miyabeanus* was 7. The pH of the cell sap in healthy leaves was about 6, and the value was changed into about 7 by the infection. Therefore, I think such high value of the cell sap may permit an effective reaction of pectic enzyme of the infected fungus. The activity was measured by the reducing power. There are three kinds of pectic enzymes. One is polygalacturonase, and the others are pectin esterase and polymethyl galacturonase. Pectic enzyme which I have mentioned here seems to be polygalacturonase.

D.N. Srivastava, India: How did the naturally affected "akiochi" plants compare with the normal plants in regard to anatomy?

Answer: I am sorry, but I have not yet investigated this problem. I have done the root anatomy. The parenchyma of the root of the "akiodhi" plant showed some degradation. No degradation was observed from the normal plant.

K. Goto, Japan: Is there any increase of respiration in the infected plants caused by uncoupled respiration, rather than the increase of coupled respiration?

Answer: Yes, oxidative phosphorylation of the normal leaves takes place efficiently when they are infected with the fungus. However, in the "akiochi" leaves, it is not so effective and substrates for respiration are consumed without the production of energy rich phosphate compounds.

N. Murata, Japan: It seems that peroxidase activity in the normal and the "akiochi" plants shows a little difference even before inoculation. Is the difference significant?

Answer: Yes, high peroxidase activity was always observed in the normal plant without inoculation.

H, Oka, Japan: Could you suggest us a method to evaluate general metabolic activity in relation to the "akiochi" condition?

Answer: I think a metabolic activity of the "akiochi" rice plant is correlated with uncoupling of oxidative phosphorylation caused by respiration when it has been infected with the causal fungus.

H.Asuyama, Japan: How do you think about the role of ophiobolin, a substance which was found by Dr. Nakamura *et al.*, in the pathogenesis of leaf spot?

Answer: Some toxin may play a role in perthopytic patho-genesis. However, the isolate which I used, ophiobolin production has not been observed. The hyphae of leaf spot were always found in the boundary between healthy and infected ones. Therefore, I think opiobolin does not always play a role in the pathogenesis of the leaf spot.

References for paper 14

Asada, Y. 1962. Studies on the susceptibility of "akiechi" (autumn-declined) rice plants to *Helminthosporium* blight (in Japanese: English summary) Mem. Ehime Univ. Sect. VI. 8:1-103.