Increases in Peroxidase-and Polyphenoloxidase-Activities and Production of Antifungal Substances in Mulberry Shoots following Wounding or Infection by Pathogenic Fungi

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ペルオキシダーゼおよびポリフェノールオキシダーゼの増加ならびに抗菌性物質の産生

Abstract

Activity of peroxidase in cut surface tissue of wound made in the bark of mulberry shoots increased almost immediately (within 1 min) after cutting, accompanied by a decrease in activity in tissues next to the cut surface. This sudden change in peroxidase activity was found to be due to peroxidase contained in latex which flowed out from laticifers in the tissue next to the cut surface. Later increase in peroxidase activity in the tissue neighbouring wound, however, was found to be due to *de novo* synthesis of protein since blastcidin S (Bc S), an inhibitor of protein synthesis, inhibited the increase. Browning of the bark segments cut from the shoots was also inhibited by treatment with Bc S. The pattern of increase in peroxidase activity in the tissue neighbouring wound of shoots was altered when they were infected with *Stigmata mori* and *Diaporthe nomurai*. The highest peaks of peroxidase- and polyphenoloxidase-activities were then formed in the border part of the disease lesion. Four bands A-1, A-2, A-3 and A-4 were found by the gel isoelectric focusing on peroxidase activities of extracts obtained from mulberry shoots infected by *Fusarium solani* and *D. nomurai*. With latex secreted from wound, the bands A-2, A-3 and A-4 were found. In the case of the healthy tissue two bands A-2 and A-3 were distinct. Phytoalexin like substances were found in the bark tissue infected by *S. mori*, *D. nomurai* and *F. solani*.

(Received December 9, 1977)

Introduction

Shoot rearing technique, harvesting mulberry leaves with shoot, has been used in sericulture in Japan for the sake of saving labor. However, this technique often causes browning and death of shoots in mulberry fields since the wounds provide openings for invasion by pathogens. It is important to clarify defense mechanisms of mulberry shoots.

It is well known that plant tissues when wounded or infected with a pathogen often turn brown and the activities of peroxidase and polyphenoloxidase increase^{3,7,15,18,19}. These changes are associated with general metabolic alterations induced by mechanical injury or infection by pathogens and often considered to be involved in the mechanisms of resistance^{9,21,22}. In many plant diseases, phytoalexins are

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known to accumulate in the brown infections, and to play an important role in disease resistance^{1,8,10}).

The experiments reported here were carried out to clarify the qualitative and quantitative changes in activities of peroxidase and polyphenoloxidase and the production of phytoalexin-like substances in the mulberry-shoot cortex which were induced by injury or infection by pathogens.

Materials and Methods

Materials. The basal parts of shoots about 1.5 cm in diameter taken from one-year-old mulberry of cultivar, Ichinose, were cut 20 cm long and coated at the both cut surfaces with paraffin, enclosed in polyethylene bags, then stored at 5 C until used. *Stigmina mori* (Nomura) Shirata et Takahashi, *Diaporthe nomurai* Hara and *Fusarium solani* (Maltius) Appel et Wollenweber f. sp. *mori* Sakurai et Matuo were used as pathogens.

Inoculation. A center part of shoot was girdled in a zone 1 mm in width with a razor. The girdled zones were filled with mycelia that had been grown on potato dextrose agar medium (PDA), and then the zones were sealed with cellophane-tape, and then the shoot was kept in a moist chamber.

Enzyme preparation. Pieces of shoot cortex 5 mm in length were sampled in succession starting from the infection zone toward the distal end. The sample (0.2 g) was ground in a mortar with 10 volumes of 0.05 M phosphate buffer, pH 7.0, containing 0.01 M $MgCl_2$, 0.04 M NaCl and 8.0 mM β -mercaptoethanol. The homogenate was diluted with distilled water to 10 times the original volume and centrifuged at $10^4 \times g$ for 10 min. An aliquot of supernatant was used for gel isoelectric focusing. The rest of the supernatant was dialyzed overnight by using Viskig Dialysis Tubing. All procedures were performed in a cold room at 5 C. The dialyzed solution was used for measurements of enzyme activities and determination of protein content.

Determination of protein content. The concentration of protein in the dialyzed samples was measured by the method of Lowry *et al*¹³) using Bovine serum albumin (Armour Pharmaceutical company) as the standard protein solution.

Measurement of enzyme activity. Activities of peroxidase and polyphenoloxidase were measured by the method of Maxwell and Bateman¹⁶) with some modifications. The activity of peroxidase was assayed spectrophotometrically. The reaction mixture, 3 ml in total volume, consisted of 1.5 ml of 0.1 M, pH 6.5, sodium phosphate buffer, 0.5 ml of 0.1 M *p*-phenylenediamine, 0.5 ml of 0.03% hydrogen peroxide and enzyme solution and water. Polyphenoloxidase was assayed spectrophotometrically using a reaction mixture, 4 ml in total volume, consisting of 1.0 ml of 0.1 M catechol and enzyme solution and water. In the assay of both enzymes, measurements were done throughout the time period in which the reaction followed a straight line. Peroxidase and polyphenoloxidase activities were expressed as the change in optical density at 485 nm and 415 nm respectively within one minute. The enzyme activity was calculated on protein weight basis.

Gel isoelectric focusing. Gel isoelectric focusing was carried out by the method of Hirano⁶). Acrylamide solution (7.5%) which contained 1.5% aniline (pH range 3.5–10), 0.2% N, N-methylene-bis-acrylamide and 0.00075% riboflavin was made as thin layer gel (14×10×0.2 cm). After the gel was exposed to daylight for 30 min, 10 μ l of the sample was poured into the ditch (0.1×0.1 cm) which was made at a distance of 4 cm from the anode. Electrophoresis was run under a constant

voltage of 40 V/cm for 4 hours by immersing a platinum anode in 1% phosphoric acid solution and a platinum cathode in 2% N,N,N',N'-tetramethylethylenediamine solution. These solutions were connected to gel with filter paper bridges. To stain peroxidase, Endo's method⁴⁾ was applied with slight modification as follows: 49 mg of o-dianisidine and 29 mg of β -naphthol were dissolved in 20 ml of acetone and added with 10 ml of 0.1 M tris-acetic acid buffer solution (pH 4.5), 1 ml of 0.3% hydrogen peroxide solution and an adequate amount of deionized water to make 100 ml of solution. The gel was soaked in the solution for 15 min at room temperature.

Measurement of latex secretion. The cortex of a mulberry shoot was wounded crosswise with a razor. Secreted latex from the wound was wiped at intervals of 2 seconds with a small piece of filter paper which had been weighed. The weight of the filter paper was measured immediately after each wipe. The difference between the weight of the filter paper before and after wiping gave the weight of secreted latex.

Measurement of tissue browning. The pieces (10×5 mm) of mulberry cortex were put on a filter paper in a line and held between two sheets of glasses. The color was measured in a Shimadzu chromatoscanner (CS-90) at 545 nm and the degree of browning was expressed as OD₅₄₅ measured by adjusting the OD of intact cortex to zero point of the scanner.

Determination of antifungal activity. A segment of cortex tissue cut out from the infected mulberry shoot (0.1g) was immersed in 1 ml of acetone and kept overnight at room temperature. The cortex was discarded and the acetone extract was used for germination experiments. One drop of Na-phosphate buffer (0.1 M, pH 4.5) and one drop of the acetone extract were put on a depression slide and one drop of spore suspension was added. After the slide was incubated in a moist chamber at 30 C for 4 hr, germination of spores was observed under a microscope. Spores of *Cochlioborus miyabeanus* were used in these assays.

Results

Browning of cortex in cut and inoculated pieces of bark

Pieces of bark cut out from the mulberry shoots and kept on wet filter paper in a Petri dish at 25 C turned brown ununiformly if they were not washed. When they were washed with running water for 1 hr immediately after cutting and then treated with distilled water for 1 min, they turned uniformly but less brown than those not washed. If the washed pieces of bark were treated with 1 ppm of blastidicin S (Bc S) for 30 min, their discoloration was greatly inhibited (Fig. 1 and 2). When the pieces were treated with latex which was collected from wounds of mulberry shoots, discoloration of the cortex occurred a little bit more rapidly than those treated with water (Fig. 1). Treatment with Bc S inhibited also the increase in discoloration of the pieces caused by treatment with latex (Fig. 1).

The pieces of bark which had been treated with water or 1 ppm of Bc S for 30 min, were dipped in spore suspension (1.1×10^4 /ml) of *D. nomurai* for several minutes. Browning of the pieces, which were treated with Bc S but not inoculated with the spores, was greatly inhibited and marked browning did not occur for 3 days. However, in the case of Bc S-treated and spore-inoculated pieces, browning was completely inhibited for 1 day but later the pieces became brown (Fig. 2).

If the cut surface of a shoot was inoculated with *D. nomurai*, browning of cortex extended about 10 mm from the inoculated girdle-end at 15 and 25 C 4 days after inoculation. If the shoot was inoculated with *S. mori*, browning of cortex extended

Fig. 1. Effect of latex and blasticidin S on browning of excised cortex and phloem tissue. The bark segments of shoots were dipped in 1 ppm blasticidin S or water for 30 min and then dipped in 10% latex solution or water for several seconds. They were incubated in moist petri dishes at 25 C. Each point represents the mean of three determinations.

—●— : non-treated,
○..... : latex,
□..... : Bc S and latex,
 —■— : Bc S.

This lowering is assumed to be due to movement of latex from the inner tissue to the cut surface.

When the girdle-end of shoots were inoculated by *D. nomurai*, the high peroxidase activities in the cut surface were not observed a day after inoculation. However,

Fig. 2. Effect of inoculation with spores of *D. nomurai* and blasticidin S on browning of cortex and phloem tissue. The bark segments of shoots were dipped in 1 ppm of blasticidin S or water for 30 min and then dipped in spore suspension (1×10^4 /ml) for several seconds. They were incubated in moist petridishes at 25 C. Each point represents the mean of three determinations.

—●— : non-treated,
○..... : spores,
□..... : spores and Bc S,
 —■— : Bc S.

about 20 mm from the inoculated girdle-end at 15 C, but 25 C, the browning covered only the inoculated surface and did not extend toward distant part of the tissue 4 days after inoculation. At 5 C both *D. nomurai* and *S. mori* caused only slight yellowing at the girdle-end surface.

Changes in peroxidase activity caused by infection.

Peroxidase activities in girdle-end tissue of the shoots non-inoculated or inoculated with mycelia of *D. nomurai* and *S. mori* were measured at various temperatures (5, 15 and 25 C), and the results are summarized in Fig. 3.

In the case of non-infected girdle-end tissue, the highest activities of peroxidase were found in the tissue 2 mm in length from the girdle-end at all incubation temperatures. One day after girdling, peroxidase activities were found to be lower in samples from about 2 mm to 15 mm from the cut end as compared with the tissues above and below this zone.

Fig. 3. Peroxidase activities in cortex and phloem-tissue of mulberry shoots in inoculated girdled tissues and tissues distal to the girdle. Sampling for determinations of peroxidase activities were made 1 and 4 days after girdling and inoculation. Each point represents the mean of two determinations.

1 min) after cutting (Fig. 4). The results indicated that the pattern of peroxidase activity curve had been already established about 1 min after cutting; that is, activity was highest in the cut surface tissue, lower in the middle tissue and again high in the most distant tissue. Activities calculated on a protein basis and on a fresh weight basis both gave similar results (Fig. 4).

Peroxidase activity in various tissues taken from mulberry shoots is shown in Table 1. Latex was found to contain an unusually large quantity of peroxidase.

Results from experiments to determine the rate of latex secretion showed that most of the latex was secreted within a few seconds (Fig. 5). If the shoot was wounded with a knife, and then cut after a few minutes at a place 3-4 cm distant from the first wound, no latex was secreted from the new wound. This fact and Fig. 4 suggested that the latex flowed out from the laticifers extending a few cm distant from the wound.

Degree of browning, protein contents, peroxidase- and polyphenoloxidase-activities

4 days after inoculation, the peroxidase activity in the inoculated surface tissue was higher than that of the non-inoculated shoot when incubated at 25 C.

When the shoots were inoculated with *S. mori* at 5 C and 25 C, slight invasion by the latter occurred and the pattern of peroxidase-activity was similar to that of non-infected tissue. However, the activity of peroxidase in the cut-surface tissue was greatly reduced by the infection in the shoots incubated at 15 C, the temperature a little higher than optimum temperature for disease development. In this case, the highest activity of peroxidase was found in the tissue about 15 mm distant from the inoculated girdle-end, suggesting that peroxidase activity may be, in some way, associated with the host response to invasion by the parasite. Polyphenoloxidase activity had the similar pattern as peroxidase activity in all cases with regard to the activity curve constructed with the enzyme activity as ordinate and the distance from the girdle-end as abscissa, and also time course of activity change. The patterns of activity curves of both enzymes were similar whether calculated on a protein basis or a fresh weight basis.

Peroxidase activity in samples from the girdle-end of non-infected shoot and from tissues distal to the girdle-end was measured immediately (about

Fig. 4.

Peroxidase activities and protein contents in the bark segments of mulberry shoots in the cut surface of the girdled zone and in tissues distal to the girdle. Sampling for determination of peroxidase activities were made within 1 min after girdling. Each point represent the mean of two determinations.

electrolytes coincided with degree of browning. However, activities of both peroxidase and polyphenoloxidase were found to be highest in the tissue zone neighboring the brown zone. Protein content, on the contrary, was higher in the tissue distant from the infected tissue than in the brown infected tissue.

To learn whether or not the increase in peroxidase activity in the tissue neighbouring girdle-end of the shoots requires *de novo* protein synthesis, the following ex-

Fig. 5. Weight (mg) of latex secreted from the wounds in mulberry shoots made by a razor. Determinations were made at intervals of 2 seconds. Each point represents the mean of three determinations.

and leakage of electrolytes were determined in tissue segments cut out in succession from the girdle-end of the shoot infected by *S. mori* toward the distal tissue 12 days after inoculation (Fig. 6). The inoculated shoots were kept at 15 C. The rate of leakage of

Table 1. Protein content and peroxidase activity in the various tissues of mulberry shoot. Each figure is the mean of three determinations

Tissues ^{a)}	Protein, fresh weight basis ^{b)}	Peroxidase activity	
		Fresh weight basis ^{c)}	Protein basis ^{d)}
Epidermis, periderm	7.8	35	4.5
Cortex	9.7	94	9.7
Phloem	5.4	89	16.5
Xylem	2.4	21	8.8
Latex	13.5	1693	125.4

a) Samples were taken from intact shoots.

b) mg/g Fresh weight

c) $\Delta O.D./min/g$ Fresh weight

d) $\Delta O.D./min/mg$ Protein

Fig. 6. Data from the cut surface of the girdled zone toward distal intact tissue for degree of browning, protein content, leakage of electrolytes, peroxidase- and polyphenoloxidase-activities in cortex and phloem tissues of mulberry shoots inoculated with *S. mori*. Sampling was made 12 days after inoculation. The inoculated shoots were incubated at 15 C. Each point represents the mean of two determinations.

Fig. 7. Effect of Bc S and incubation temperature on the increase in peroxidase activities in the bark segments cut from mulberry shoots. The segments were dipped in 1ppm of Bc S or water for 30 min, and incubated on wet filter paper in petri dishes.

—●— : non-treated at 15 C,
 —■— : Bc S at 15 C,
○..... : non-treated at 5 C,
 Each point represents the mean of three determinations.

periments were carried out. The cortex pieces of shoots were washed with water to eliminate latex, and then treated with 1 ppm of Bc S for 30 min (Fig. 7). The enzyme activity increased greatly in non-treated tissue at 15 C 4 days after cutting, but the increase at 15 C was greatly inhibited by treatment with Bc S, and also the activity of non-treated tissue did not increase at 5 C.

Electrophoregram of peroxidase isozymes

Electrophoregram of peroxidase isozymes in extracts from mulberry shoot is shown in Fig. 8. Isozymes in cathodic and anodic sides were designated C-1, A-1, A-2, A-3 and A-4, respectively. The three peroxidase isozymes, C-1, A-2 and A-3 were found in the extract from healthy cortex. Isozymes A-1, A-2, A-3 and A-4 were found in the extract from infected tissue, and the color intensities of A-2 and A-3 were slightly greater than those of healthy tissue. The electrophoregram in the extract from cut-injured tissue was similar to that of brown infected tissue except that color intensities of A-1 and A-4 were lower. Three isozymes, A-2, A-3 and A-4, were found in latex.

Antifungal activity in acetone extract of infected shoot

Acetone extracts from various parts of the infected brown zone and the healthy part of mulberry shoots were added to an appropriate amount of water, and the acetone was then evaporated. The solutions obtained were used for the assay of toxicity (Fig. 9). Spores of *C. miyabeanus* germinated in the extract from the

healthy part which had been concentrated 8 folds above the original concentration. Extracts from the infected brown parts, on the contrary, inhibited germination of the

Fig. 8. Electrophoregrams of peroxidase activities of extracts obtained from mulberry shoots inoculated with *F. solani* and *D. nomurai*, and that of peroxidase contained in latex. Sampling were made 12 days after inoculation.

- 1: Healthy part of the intact cortex and phloem tissue,
- 2: Healthy tissue adjacent to the girdled zone 12 days after cutting,
- 3: Browned tissue infected by *F. solani*,
- 4: Browned tissue infected by *D. nomurai*,
- 5: Latex secreted from wound made on the intact shoot.

spores. In each of brown lesions of the shoots infected by *S. mori*, *D. nomurai* and *F. solani* the toxic principles were contained in the highest concentration in the tissues bordering lesion (Fig. 9). The toxic principles inhibited spore germination of *D. nomuri*, *S. mori*, *F. solani* and *F. lateritium* which are pathogens of mulberry shoot.

Discussion

When mulberry shoots were girdled, the peroxidase activity was the highest in the cut-surface tissue of the girdle-end, the lowest in next zone to the cut-surface tissue and again relatively high in the distant tissue 1 and 4 days after cutting at

Fig. 9. Diagrams of infected mulberry shoots showing type and location of discoloration in relation to antifungal activity of cortex- and phloem-tissue's extracts. Antifungal activities of the extracts from bark segments, removed epidermis, cut out from various parts of lesions of shoots infected by *S. mori*, *D. nomurai*, and *F. solani*. The shoots inoculated with *S. mori* were incubated at 10 C for 5 days followed by on additional 20 days at 25 C. The shoots inoculated with *D. nomurai* and *F. solani* were incubated at 25 C for 25 days. Cortex- and phloem-tissue, each 0.1 g were extracted with 1 ml of acetone for 5 hr at room temperature. The figures show the minimum dilution point for the inhibition of conidial germination of *C. miyabeanus*.

■ : black, ▨ : brown,
 ▩ : light-brown, ▤ : deep-brown.

5, 15 and 25 C. These curves of peroxidase activity were generally reduced by efficient infection by *S. mori* and *D. nomurai* at favourable temperatures (Fig. 3). In the tissue infected by *S. mori* at 15 C, the highest peak of peroxidase activity was found in the borders of brown disease lesions 4 days after inoculation, suggesting that an increase in peroxidase activity was involved in the response of shoots to infection. The similar curve as that of peroxidase activity distributed from the cut surface to distal tissue observed a day after cutting was also found immediately (within about 1 min) after cutting (Fig. 4). The rapid increase of activity in the cut surface tissue of girdle-end accompanied the reduction of the activity in the distal tissue. These results suggested that secretion of latex from the cut surface may be the cause of the rapid response. The experiments showed that latex seemed to be secreted within a few seconds after cutting from the laticifers located in tissue a few cm from the cut-end (Fig. 4 & 5). These results clearly indicated that the initial increase of peroxidase activity in the cut surface tissue was due to secretion of latex from the inner tissue. We suspect that latex may play some roles in protecting the cut surface from microbes or insects. Further investigation is necessary to make clear the role of latex in the disease resistance of mulberry shoots. Later increase in peroxidase activity in the tissue segments cut from the shoots was shown to be caused by *de novo* protein synthesis, since the increase was inhibited by treatment with Bc S or by placing them at 5 C. It is, however, unclear whether the increase in peroxidase activity was due to the synthesis in laticifers or in cortex- or phloem-cells. The cortex and phloem segment cut from the shoots and washed with water were browned within a few days. This browning was also inhibited by treatment with Bc S.

A great amount of work have been reported on the relationship between oxidative enzymes and disease resistance in plants. It is without doubt that oxidative enzymes such as polyphenoloxidase and peroxidase are partly involved in the entire sequence of plant response to wound or infection. However, it is difficult to evaluate the role of peroxidase in plant resistance⁵⁾. In some disease, high activity of peroxidase seemed to be a result of resistance but not the cause²⁾. In some disease, on the contrary, peroxidase seemed to play an important role in disease resistance^{11, 12, 14)}. Further investigation is necessary to evaluate the role of peroxidase in the disease resistance of mulberry shoot.

Antifungal activity was found in acetone extracts from browned tissue of shoot cortex infected by pathogenic fungi. This may have been due to some kinds of phytoalexins. Under our experimental conditions the rate of progress of brown symptoms toward distal tissue from inoculation point was slowest in the tissue inoculated with *F. solani*, and extract from this brown tissue showed the highest antifungal activity. This result suggested that the antifungal substance was closely related to the resistance of mulberry shoots to infection by pathogens. Isolation and characterization of antifungal substances are now in progress.

Authors are grateful to Mr. H. Hirano for his benevolent guidance and help in gel isoelectric focusing, and also to Dr. W. R. Bushell for his help in preparation of the manuscript.

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和文摘要

付傷または感染クワ枝皮層部組織におけるペルオキシダーゼおよび
ポリフェノールオキシダーゼの増加ならびに抗菌性物質の産生

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クワの枝条を環状剥離すると、切口付近でペルオキシダーゼおよびポリフェノールオキシダーゼの活性が異常に高まるが、この主な原因は両酵素を多量に含む乳液の移動によるものであった。流水で乳液を除去した皮層部組織切片を湿室に保つと、切片は数日で褐変し両酵素活性の増加がみられた。しかし、低温に保つか、蛋白合成阻害剤のブラストサイジンSで処理すると、切片は褐変せず両酵素活性の増加がみられなかった。

病原菌を接種し病斑が拡大しつつあるクワの枝条の皮層部を調べると、褐変し始めた部位で蛋白含量の減少、両酵素活性の増加および電気伝導度でみた膜透過性の増大がみられた。さらに病徴が進み変色が激しい部位では蛋白含量および酵素活性の減少がみられ、付傷の場合とは様相を異にした。

ペルオキシダーゼのアイソザイムを調べたところ、健全のクワ枝条皮層部組織からはC-1, A-2, A-3の3つのバンドが得られた。罹病褐変組織からは新しくA-1, A-4の2つのバンドが増加したが、C-1は消失した。また健全クワ枝から採取した乳液にはA-2, A-3, A-4の3つのバンドがみられ、C-1はみられなかった。

健全クワ枝条の表皮を除去した皮層部組織のアセトン抽出液には抗菌作用は認められなかった。しかし、罹病褐変皮層部組織からの抽出液には強い抗菌作用が認められ、クワ枝条と病原菌との相互作用の結果、ファイトアレキシン様物質が産生蓄積されたものと考えられた。