Nature of Specific Susceptibility to Alternaria kikuchiana in Nijisseiki Cultivar among Japanese Pears (III)*

Chemical and thermal protection against effect of host-specific toxin

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and Keisuke Kohmoto**

Abstract

Pear leaves susceptible to Alternaria kikuchiana and to its host-specific toxin were treated with various compounds to test for possible protective effects. Disulfide-reducing reagents (dithiothreitol and mercaptoethanol), applied to susceptible leaves before exposure to toxin, gave partial protection against toxin-induced loss of electrolytes. This protection was completely reversed by an oxidizing reagent [5,5'-dithiobis(2-nitrobenzoic acid)]. When an alkylating reagent (N-ethylmaleimide) was applied after application of the reducing reagent, the protective effect was not reversed by the oxidizing reagent. The results suggest that disulfide groups in susceptible cells may be involved in the reaction to toxin. Partial protection by thermal treatments (55°C for 2 sec in water or 35°C for 16 hr in air) was evident when measured by toxin-induced electrolyte losses from susceptible tissues and by a decrease in toxin-induced necrosis. The protective effects of thermal treatments were gradually lost by the tissues. The data suggest the existence of toxin receptor sites or substances that can be saturated.

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Introduction

Alternaria kikuchiana Tanaka produces a metabolite (a host-specific toxin) that is strikingly toxic to the susceptible pear (Pyrus serotina Rehd. cv. Nijisseiki and its derivatives) but has little or no effect on resistant pear cultivars. The toxin has a rapid effect on functions of the plasma membrane of susceptible but not resistant cells. Evidence for such an effect is the almost instantaneous increase in loss of electrolytes from susceptible tissues. The pathogen also causes a drastic increase in electrolyte losses during the infection process. Genetic data have shown that susceptibility to the fungus and to its toxin is controlled by a single dominant gene.

Scheffer et al. have postulated the existence of receptor sites for the toxin of Helminthosporium victoriae in susceptible but not resistant oats. The H. victoriae toxin has a rapid effect on plasma membranes of susceptible cells. It is possible that A. kikuchiana toxin has a mechanism of action similar to that of H. victoriae toxin. We have attempted to find toxin receptor sites, but have not been successful to date. Therefore, an indirect approach was tried.

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toxin molecule may not act if its hypothetical sites are covered or protected in some way. Several chemical treatments and brief exposures to high temperature, prior to toxin exposure, were tested for possible protective action. Brief reports of some of this work have been published.

**Materials and Methods**

Freshly-harvested young leaves of pear cultivars susceptible (cv. Nijisseiki) and resistant (cv. Chojuro) to *A. kikuchiana* and to its toxin were used in all experiments. Partially purified host-specific toxin was prepared from culture filtrates as described previously. Toxin solutions were adjusted with distilled water to a concentration approximately 500 times greater than the minimum required to form characteristic black necrosis on young susceptible leaves.

Compounds of several types were tested for ability to prevent toxin-induced loss of electrolytes from susceptible leaves. Twenty leaf disks, each 1.0 cm in diameter, were vacuum-infiltrated for 30 min with solutions of the test compounds, and incubated in the same solutions for 90 min. When treatment with two or more compounds was required, tissues were treated first with one, then with the other, for 2 hr each. After treatment with test compounds, leaf tissues were rinsed in distilled water and vacuum-infiltrated for 15 min with toxin solution or water. Each sample was then rinsed again in distilled water and incubated on a shaker in 10 ml glass distilled water. Conductance of each ambient solution was measured at specific intervals with a conductivity bridge.

The effect of heat treatment on toxin-sensitivity of pear leaves was tested by holding leaves in water or in air at several different temperatures. Leaf disks, 1.0 cm in diameter, were cut from the heated leaves and wounded slightly in the center with a needle. One drop of toxin solution was then placed on the wound. After 12 or 24 hr at 28°C in a moist chamber, the area of each characteristic black necrotic spot on leaf disks was measured. In other experiments, heated leaf disks were vacuum-infiltrated with toxin solutions for 15 min, and loss of electrolytes from the disks was measured at intervals. Experiments were repeated several times.

**Results**

**Chemical protection of pear tissues against toxin-induced loss of electrolytes**

Prior to toxin-exposure, tissues were treated with dithiothreitol (DTT) or mercaptoethanol (ME), which are disulfide-reducing reagents. DTT (1 mM) gave >50% protection against toxin-induced loss of electrolytes from susceptible leaves, as determined 2 hr after toxin treatment. ME (1 mM) gave about 30% protection during the 2 hr period (Fig. 1). The protective effects of these reducing reagents were lost gradually after exposure to toxin. The decrease in protection might result from oxidation of sulfhydryl groups in pear cells. Therefore, we reasoned that sulfhydryl groups produced by DTT might be stabilized by alkylation with N-ethylmaleimide (NEM; 1 mM) prior to toxin treatment. However, DTT plus NEM did not give better protection against toxin than did DTT alone (Fig. 2).

Tissues were treated with several concentrations of
DTT for 2 hr before exposure to toxin. Results confirmed that DTT at 1 to 5 mM gave some protection against toxin. Possible protection with higher concentrations could not be determined, because DTT at >5 mM caused leaves to leak electrolytes. In other experiments, tissues were treated with DTT (1 mM) plus toxin for 2 hr, or with toxin for 15 min, followed by DTT (1 mM) for 2 hr. Maximum protection of 15 to 20% was obtained by simultaneous treatment with DTT plus toxin; little or no protection was evident when DTT was applied after tissues were exposed to toxin.

Apparent protection induced by reducing reagents might result from a reaction of the test compound with the toxin molecule. Therefore, possible inactivation of toxin by the test compound was tested. DTT (2 mM) or ME (2 mM) was mixed with an equal amount of toxin solution. After incubation for 24 hr, the mixture was diluted serially with distilled water, and the dilution series was tested for ability to induce black spots on leaves. Results indicated that DTT and ME did not modify the biological activity of the toxin molecule.

There were several experiments to determine whether or not reduction is the basis of protection against toxin. Tissues treated with DTT (1 mM) were infiltrated with a second test compound before exposure to toxin. DTT protection was completely eliminated by treatment with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; 1 mM), an oxidizing reagent. When NEM (1 mM), an alkylating reagent, was applied after DTT treatment, DTNB did not eliminate protection by DTT (Fig. 2). All these results suggest that disulfide groups in susceptible tissues may be part of, or associated with, the toxin receptor in susceptible cells.

The following compounds were tested for possible protective effects against toxin-induced losses of electrolytes: iodoacetic acid and p-chloromercuribenzoic acid (sulphydryl-binding reagents); semicarbazide (a carbonyl-binding reagent); O-methyl-isourea (an amino-binding reagent); pronase and nagarse (proteolytic enzymes); hydroxyproline (an amino acid component of cell walls); 2,4-dinitrophenol (an uncoupler); and cycloheximide and blasticidin S (protein synthesis inhibitors). Leaf samples were pre-treated with these compounds for 2 hr before exposure to toxin, except for the case of protein synthesis inhibitors where pre-treatment was given for 12 hr. However, these compounds gave no protection against toxin-induced losses of electrolytes from susceptible leaves.

**Effect of pre-treatment with heat on sensitivity of leaves to toxin-induced necrosis**

Susceptible and resistant pear leaves were held at 40 and 55°C in water for different times, then infiltrated with toxin and incubated at 28°C. Black necrotic areas on the leaves were measured after incubation for 12 and 24 hr. Lesions on susceptible leaves treated for 6

**Fig. 2. Effect of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and N-ethylmaleimide (NEM) on the dithiothreitol (DTT)-protection against toxin-induced loss of electrolytes from susceptible pear leaves. Conductance of each ambient solution was measured 2 hr after toxin treatment and was adjusted by calculating the value of each toxin control to 100 μmhos. Treatments are as follows: A, toxin control; B, water control; C, DTT-toxin; D, DTT control; E, DTT-NEM-toxin; F, DTT-NEM control; G, DTT-DTNB-toxin; H, DTT-DTNB control; I, DTT-NEM-DTNB-toxin; J, DTT-NEM-DTNB control.**
Table 1. Effect of pre-incubation at high temperatures on toxin-induced black lesion formation by susceptible pear leaves

<table>
<thead>
<tr>
<th>Temperature of treatment</th>
<th>Length of treatment (min)</th>
<th>Necrotic area (mm²) at 12 hr(a)</th>
<th>Necrotic area (mm²) at 24 hr(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. None</td>
<td>4</td>
<td>47</td>
<td>52</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
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</tr>
<tr>
<td>55</td>
<td>8</td>
<td>7</td>
<td>48</td>
</tr>
<tr>
<td>2. None</td>
<td>1</td>
<td>65</td>
<td>71</td>
</tr>
<tr>
<td>55</td>
<td>2</td>
<td>39</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
<td>24</td>
</tr>
</tbody>
</table>

a) Size of toxin-induced necrotic area on pear leaf disks (1.0 cm in diameter).
b) Time after toxin treatment that observations were made.

or 8 min at 40°C were smaller than lesions on control leaves, at 12 hr after exposure to toxin (Table 1). Two sec exposure at 55°C also reduced toxin-induced necrosis when observed at 12 hr. The protective effect of a 40°C treatment was soon lost; no decrease in necrosis was evident when observations were made 24 hr after toxin exposure. However, some protective effect of the 55°C treatment was still evident at the 24 hr observation time (Table 1). Toxin caused no necrosis in resistant leaves, regardless of leaf temperatures before toxin exposure.

Susceptible leaves were held at 55°C for 4 sec, then were incubated at 28°C in a moist chamber for various times. After incubation, leaf disks (1.0 cm in diameter) were cut from the leaves and infiltrated with toxin. The resulting necrotic areas on leaves were measured 12 hr after exposure to toxin. Results showed that the heat-induced protective effect was gradually lost; no significant protection remained 12 hr after the heat treatment (Fig. 3).

Leaves were given various thermal treatments in air or in water, then were infiltrated with toxin and incubated for 12 hr at 28°C. Size of necrotic area resulting from each treatment was compared. As air temperature was increased from 33 to 45°C, the treatment time required to give significant protection was decreased from 24 hr to 0.5 hr (Table 2). When leaves were placed in water at temperatures from 40 to 60°C, the treatment times for significant protection against toxin were decreased from 300 to 1 sec (Table 2). The greater efficiency of the water treatment may be related to the fact that transpiring leaves usually are at lower than ambient temperatures.

Susceptible leaf disks (1.0 cm in diameter) were vacuum-infiltrated for 15 min in toxin solution, and were incubated at 28°C in a moist chamber for various times. Tissues were then held in water at 55°C for 4 sec, and incubated again at 28°C. After 8 hr of toxin treatment, intensity of darkness which appeared on the tissues was measured with a spectrophotometer (absorbance at 530 nm). The
Table 2. Comparative effects of thermal treatments in air or water on induced protection against toxin, as measured by size of black lesions on susceptible leaves

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (hr)</th>
<th>Temperature (°C)</th>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Treatment in air</td>
<td>33 24</td>
<td>2. Treatment in water</td>
<td>40 300</td>
</tr>
<tr>
<td></td>
<td>35 16</td>
<td></td>
<td>45 30</td>
</tr>
<tr>
<td></td>
<td>40 2</td>
<td></td>
<td>50 2</td>
</tr>
<tr>
<td></td>
<td>45 0.5</td>
<td></td>
<td>55 2</td>
</tr>
</tbody>
</table>

a) Thermal treatment time required to give 50% or more reduction in size of necrotic areas on susceptible tissues, determined 12 hr after exposure to toxin.

Fig. 4. Effect of thermal treatments following exposure to toxin on development of necrotic lesions. Tissue sections (1.0 cm in diameter) were exposed to toxin, incubated at 28°C for times as indicated, and heated at 55°C for 4 sec. The intensity of lesion development was measured 8 hr after initial toxin treatment, by measuring light absorption in the lesion at 530 nm. Treatments were as follows: — , toxin-treated tissues that were heated; — , toxin-treated tissues that were not heated; — , water-treated tissues that were heated; — , water-treated tissues that were not heated.

heat treatment effectively protected leaves against toxin, provided heat was applied within 1 hr after exposure to toxin. If more than 1 hr elapsed from toxin exposure to heat treatment, there was no protective effect (Fig. 4). We interpret these data to mean that all toxin receptor sites are affected by toxin within 1 hr. After that time, these are no more sites that can be altered by heat.

**Thermal protection of pear tissues against toxin-induced loss of electrolytes**

Pear leaves were given a series of thermal treatments differing in temperature and exposure time. Five leaf disks (1.0 cm in diameter) were cut from the leaves of each treatment type and vacuum-infiltrated for 15 min with toxin solution. The leaf disks were rinsed with distilled water, placed in 20 ml glass distilled water, and incubated on a shaker at 28°C. Conductivity of the ambient water was measured at intervals. Susceptible pear leaves pre-heated at 33 to 55°C had less toxin-induced leakage of electrolytes than did control leaves (Fig. 5, Table 3). Heat treatment in air at 33 to 35°C gave maximum protection against toxin-induced loss of electrolytes from leaves (Fig. 5). Higher temperatures (above 55°C) injured the leaves and caused leakage without toxin exposure. The time required to observe the induced protective effect on the loss of electrolytes was far quicker than that of the tissue necrosis method (Tables 2, 3). Heat-treated resistant leaves were not affected by toxin.
Table 3. Comparative effects of thermal treatments in air or water on induced protection against toxin, as measured by changes in loss of electrolytes

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Timea) (hr)</th>
<th>Temperature (°C)</th>
<th>Timea) (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Treatment in air</td>
<td>33</td>
<td>2. Treatment in water</td>
<td>40</td>
</tr>
<tr>
<td>33</td>
<td>14-24</td>
<td>45</td>
<td>20-30</td>
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<tr>
<td>35</td>
<td>8-24</td>
<td>50</td>
<td>3-4</td>
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<tr>
<td>40</td>
<td>0.5-2</td>
<td>55</td>
<td>1</td>
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<tr>
<td>45</td>
<td>0.25-0.5</td>
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</tbody>
</table>

a) Thermal treatment time required to develop a significant protective effect against toxin-induced loss of electrolytes from pear leaves.

An attempt was made to determine whether or not thermal and chemical protection against toxic effects are based on a common change in susceptible cells. Heat-treated leaf disks were treated with DTNB or DTT, each at 1 mM, for 2 hr. Each sample was rinsed with distilled water and vacuum-infiltrated for 15 min in toxin solution. Leakage of electrolytes from tissues was measured at intervals. The experiment was repeated several times, but the results showed no common relationship between thermal and chemical protections.

**Discussion**

Several lines of evidence show that the host-specific toxin of *A. kikuchiana* is an essential or controlling factor in its pathogenicity. The characteristic necrotic reaction of pear leaves to the toxin occurs only in cultivars that are hosts of the fungus, and fungus isolates that have lost pathogenicity also lose the ability to produce toxin in culture. All known physiological and biochemical changes induced by *A. kikuchiana* infection can also be reproduced in the susceptible plant by the toxin. Susceptibility of the Nijisseiki pear to *A. kikuchiana* and to the toxin are controlled by a single dominant gene. All these characteristics indicate that the toxin is an essential determinant of disease, and that further study of this case could help us to understand...
parasitism in plants.

Present results confirm previous findings that the toxin causes a rapid loss of electrolytes from susceptible but not resistant pear leaves within 5 min of initial treatment\(^{10}\). Drastic permeability increases are induced prior to the appearance of visible lesions during pathogenesis\(^8\). Therefore, toxin-induced loss of electrolytes was selected as a tool to determine the possible location and chemical nature of toxin receptor sites in pear cells. Experiments described above provide indirect evidence concerning the nature of toxin receptors, and show the pliable nature of susceptibility in the Nijisseiki pear. Disulfide-reducing reagents (dithiothreitol and mercaptoethanol) among various other test compounds reduced the sensitivity of pear leaves to toxin, as indicated by changes in losses of electrolytes. These compounds delayed the onset of toxin-induced electrolyte losses, but had little effect on the rate of loss, once the delay period is passed (Fig. 1). The results suggest that a disulfide group may be a part of the sensitive sites in cells. Work with \textit{H. victoriae} toxin showed that sulfhydryl-binding compounds, carbonyl-binding compounds, and cycloheximide gave protection, and that these compounds decreased the rate of electrolyte loss from toxin-treated tissues\(^2\)-\(^{13}\). These compounds gave no protection against \textit{A. kikuchiana} toxin. On the other hand, cycloheximide and phospholipase D gave protection against \textit{Periconia cincta} toxin, whereas the sulfhydryl-binding compounds gave no protection\(^{1}\). Thus the toxin receptors or the mechanisms of action for the three toxins would seem to differ somewhat.

Leaves of susceptible pears were protected in part against \textit{A. kikuchiana} toxin by prior exposure to mild heat. This was evident whether the protection was measured in terms of electrolyte losses, or as a decrease in characteristic black necrosis. One possible explanation is that the hypothetical sites of toxin attack are easily changed by heat treatment. Conceivably, the site could be a protein containing a disulfide group, although the failure to show a relationship between thermal and chemical protection doe not seem to fit this possibility. Our recent experiments have indicated the presence of a toxin-binding protein in only susceptible tissues. The proteinous nature of the substance was evidenced because when susceptible tissues were pre-treated with heat, the toxin remained in a free state (unpublished). All these results support a toxin receptor site hypothesis, first proposed for \textit{H. victoriae} toxin.

Acknowledgement

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Literature cited

和文摘要

ナシ黒斑病菌の宿主特異的作機能 (III)
化学物質ならびに熱処理による毒素効果の減少

尾谷敬・西村正昭・甲元啓介

ナシ黒斑病菌の宿主特異的毒素は、感受性品種のナシ葉ののみに、透過性の異常増大を引き起こし、その結果、ナシ葉は、葉脈に沿う特異的な壊死斑を形成する。このような毒素による透過性の異常増大は、ナシ葉をあらかじめ S-S 結合還元剤処理することによって、ある程度抑制された。さらに、この抑制効果は、SH 結合酸化剤処理によって完全に打消されるが、還元剤処理後にアルカリ化剤処理すると、酸化剤処理の効果はみられなくなった。一方、感受性ナシ葉を、あらかじめ 40℃で 6 分以上、あるいは 55℃で 2 秒以上温湯処理すると、毒素による透過性の異常増大、さらに、壊死斑の形成は極端に抑えられた。この抑制効果は、33〜60℃の範囲の温処理で常に認められた。なお、熱処理葉は、時間の経過とともに徐々に抑制効果を失ない、再びもとの感受性にもどることが認められた。以上の諸結果から、毒素作用の特異性に関与する因子は、感受性品種の側に存在し、抵抗性側には存在しないと推論できた。