Studies on Citrus Melanose and Citrus Stem-End Rot by *Diaporthe citri* (Faw.) Wolf. Part 10. 
Hyphal Elongation of *Diaporthe citri* from Citrus Fruit Pedicel to Stem-End and Behavior of Inhibitins

Yasuo Homma*, Hiroharu Takahashi** and Yutaka Arimoto*

Abstract

When satsuma mandarin fruits on trees were inoculated with *Diaporthe citri* to their pedicels, then harvested and stored in the condition of room temperature, stem-end rot was observed on almost all the fruits at the late stage of storage. Despite hyphal penetration and elongation to the pedicel or into pedicel-end core, there was no hyphal existence between the disks and the tissues underneath where a lot of needle-like crystals were distributed. Solutions of the crude crystals at 462~4,620 fold dilutions showed inhibitory activity against the fungus, and higher concentration of the crystals exhibited stronger inhibition to the hyphal elongation. Though the crystals were not observed in pedicel-end of young fruits, many of them were distributed after October when they became matured. Since the crystals did not exist in the stem-end at the end of storage, we assumed that they had disappeared by self-digestion of citrus fruit. A similar inclination on the antifungal activities was already observed in fruit vesicle extract. Similar crystals were also observed in the vascular bundle systems in fruit core and leaves, especially in adult leaves.

(Received July 7, 1988)

Key words: *Diaporthe citri*, stem-end rot, inhibitin, pedicel, hesperetin 7-rhamnogluicoside-like substance.

INTRODUCTION

The authors have clarified that *Citrus unshiu* has some antifungal substances in melanose spots and oil glands on fruit peel1,2,3, vesicles4, as well as around disk, stem button and leaves1,2. It was reported that rain fall more than 10 mm caused *Diaporthe citri* pycnospores to be dispersed and infect new shoots, leaves and fruits on the tree5, but fruits and leaves seldom rotted on the tree6. The authors previously suggested why rot did not occur on these tissues7.

We did not, however, clarify why the fruit which did not rot on the tree started to rot at the last stage of storage. The present study was made to look into the cause of the pathogen development inside the fruit, and the behavior and the effect of one of the inhibitins.

MATERIALS AND METHODS

1. **Inoculation.** As the inoculum, *Diaporthe citri* (Faw.) Wolf was inoculated to pedicels wrapped with absorbent cotton in 1 cm width (about 100 pycnospores under 150× mag-
nification of an optical microscope), at the site 1 cm away from the stem-end of fruits on a tree or picked from a tree. The fruits were incubated in a moist chamber at 25°C for two days or more.

2. **Preparation of materials for the scanning electron microscopy.** Materials were obtained at appropriate time interval after inoculation and the following preparations were made:

   a. pedicel surface
   b. longitudinally and cross-sectionally cut section extending from pedicel to stem-end
   c. inner stem button taken off a fruit

   These materials were attached to specimen stubs with silver-conducting paint, and then observed with or without coating of thin films of carbon and gold under high vacuum according to the method by Homma et al. The samples prepared in this way were observed and photographed with a scanning electron microscope (JSM-U3).

3. **Extract of crystalline product from various parts of citrus tree.** Two hundred g or 100 g each of fresh leaves, stem buttons, the tissues under the disks and the subsequent vascular bundles and the fruit vesicles of satsuma mandarin (*Citrus unshiu* Marc.) were extracted as described in the previous report. The solvent in the fruit tissue extract was completely evaporated by condensation and the weight of crystalline product from each tissue was measured for comparison. The crystalline product dissolved in methanol has UV maximum absorbance at 283 nm and a shoulder at 325 nm. We can distinguish this substance from other by this characteristic absorbance. Therefore, we checked all crystalline products from each tissue by UV absorbance.

4. **Inhibitory effect of crystalline product against Diaporthe citri pycnospore germination.** Ten mg of the residue was extracted from the tissues under the disks by above mentioned method. Another 10 mg of the residue was also taken, but this time crystalline product was excluded. These two of extracts were diluted 10, 10² and 10³ times respectively.

   The final concentration was obtained by mixing the above dilutions with the same volume of *D. citri* pycnospore suspension containing 5% fructose. The suspensions were dropped on a slide glass and incubated at 25°C for 24 or 48 hr as described previously. Germination and germ tube elongation were observed under an optical microscope.

### RESULTS

1. **Infection and translocation of Diaporthe citri from fruit pedicel to stem-end**
   a. **Morphological observation of fruit pedicel and stem-end.** As shown in plate I-1, fruit pedicel had many cracks on the surface. Citrus showed resistance to *Diaporthe citri* in the epidermis of leaf, fruit and fruit pedicel. Plate I-3 shows some parts of pedicel cut longitudinally to the stem-end which consists of a calyx and a stem button. Plate I-2 shows a stem button taken off the fruit.

   In Plate II, pathogens are found on pedicel or in stem-end core. Plate II-1 demonstrates penetration of germ tube into pedicel 48 hr after inoculation on the pedicel near the stem-end. Pathogen seemed to penetrate from the pedicel surface and the existence of hyphae was noted around the inner stem button within a few days of inoculation (Plate II-2).

   Hyphae were observed a month after inoculation to the pedicel surface of a fruit on the tree. Pathogen existed between the bark and parenchyma and around the base of stem button (Plate II-3). However, no hyphae was observed around the base of a disk (Plate II-4) where crystals were distributed.

2. **Existence of crystals around stem-end core**
   a. **Young fruit.** Plate III-1 shows the inner part of stem button of young fruit late in May. Except many holes in the tissues of stem-end, we could find nothing else.

   b. **Fruit in the maturation period.** Plate III-2 shows the inner part of stem button of
fruit in early October. This photograph shows the site around the vascular bundle system of inner stem button. The vascular bundles are covered with many small crystals. Plate III-3 shows the tissues longitudinally cut from stem-end to the tissues under the disk. The upper part shows the disk and the lower part the tissues under the disk. The former has a few small particles in its tissues, while the latter has many.

Plate III-4 shows the cross-sectionally cut tissues under the disk around where small holes are seen. Many of them were filled with small crystals. A photo was taken of the inner part of stem button, after it was dipped in acetone for 5 min (Plate IV-1). The photo shows that none of the crystals existed in the inner stem button after dipping in acetone.

**c. Fruit during the terminal period of storage.** As shown in Plate IV-2, there were none of crystals around the inner part of stem button late in April. The crystals seemed to have disappeared through self-digestion of citrus fruit. By purification in accordance with the usual method, we obtained needle-like crystalline products (Plate IV-3).

3. **Crystal content in various tissues of citrus**

a. **Leaf.** As shown in Table 1, young leaves with or without terminal bud had a few crystals, whereas adult leaves had 10 mg crystalline products per 100 g fresh weight. Crystals seem to have increased as the leaf stage has advanced.

b. **Tissues under stem-end.** Only trace crystals were detected in 200 g fresh weight of disks, whereas 43.3 mg of crystals were found in the same amount of tissues under the disk. Similarly, vascular bundles connected to the tissues under the disks and dorsal vascular bundles had 35.1 and 12.0 mg of crystalline products, respectively. The greatest amount of crystals was found in the tissues under the disks.

<table>
<thead>
<tr>
<th>Age of leaf</th>
<th>Amount of crude crystal (mg)</th>
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<tbody>
<tr>
<td>Young leaf with terminal bud</td>
<td>trace</td>
</tr>
<tr>
<td>Young leaf without terminal bud</td>
<td>trace</td>
</tr>
<tr>
<td>Adult leaf</td>
<td>$10^{10}$</td>
</tr>
</tbody>
</table>

a) Weight per 100 g fresh weight.

<table>
<thead>
<tr>
<th>Tissues used for extraction</th>
<th>Amount of crude crystal (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disk</td>
<td>trace</td>
</tr>
<tr>
<td>Tissues under the disks</td>
<td>43.3</td>
</tr>
<tr>
<td>Vascular bundle connected to tissues under the disks</td>
<td>35.1</td>
</tr>
<tr>
<td>Dorsal vascular bundle</td>
<td>12.0</td>
</tr>
</tbody>
</table>

a) Weight per 200 g fresh weight.

Table 1. Comparison of crude crystal among the growth stages of the satsuma mandarin leaf

<table>
<thead>
<tr>
<th>Period of storage (day)</th>
<th>Date of harvest</th>
<th>Term of extraction</th>
<th>Amount of crude crystal (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>10/10</td>
<td>10/17～11/6</td>
<td>26.6</td>
</tr>
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</table>

a) Weight per 200 g fresh weight.
Table 4. The effect of the extract of the tissues under the disks on the pycnospore germination and germ tube elongation of *D. citri*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Conc. of crude crystalline product</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pycnospore germination</td>
</tr>
<tr>
<td>Extract ×10</td>
<td>× 462</td>
<td>100</td>
</tr>
<tr>
<td>×10²</td>
<td>× 4,620</td>
<td>5</td>
</tr>
<tr>
<td>×10³</td>
<td>×46,200</td>
<td>0</td>
</tr>
<tr>
<td>Extract ×10</td>
<td>× 0</td>
<td>0</td>
</tr>
<tr>
<td>×10²</td>
<td>× 0</td>
<td>0</td>
</tr>
<tr>
<td>×10³</td>
<td>× 0</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Extract excluded crude crystalline product.

c. Vesicle. As shown in Table 3, for short period of storage (7 days) after the harvest, 200 g of fresh weight of vesicle had 26.6 mg crystals, while less crystalline product was found in the same amount of vesicle after long storage (107 days). It seems that the citrus itself has digested these crystalline products. Such outcome is similar to the disappearance of crystals in disk which occurred late in April.

4. Inhibition of *Diaporthe citri* pycnospore germination and germ tube elongation by the crystals extracted from tissues under the disks

Since acetone extract from the tissues under the disks contains crystalline products, it was tested whether the extract had inhibitory effect or not. The results are shown in Table 4. Tenfold dilution of the extract (462-fold dilution in crystalline products) completely inhibited pycnospore germination. One hundred-fold and 1,000 fold dilution did not inhibit pycnospore germination, but suppressed germ tube elongation 63% and 30%, respectively. On the other hand, the same tissue extract from which crystalline products were eliminated did not inhibit any growth of *D. citri* at any concentrations tested. Therefore, it is demonstrated that crystalline products in the extract from the tissues under the disks have inhibitory effect against the mycelial elongation of *D. citri*.

DISCUSSION

*Diaporthe citri* is a pathogenic fungus of citrus melanose and citrus stem-end rot. Brown reported that *D. citri* invaded inside the fruit from the crack between stem-button and the tissue under the disk. By our experiment, this fungus, when inoculated, penetrated from the surface of fruit pedicels and was transmitted to fruit stem-end. It was therefore demonstrated that *D. citri* could enter from fruit pedicel and was transmitted to fruit core.

Citrus seems to have some kind of inhibitins in its different tissues. One of such inhibitins is hesperetin 7-rhamnogluco-side-like substance existing in the stem disk and the tissue under disk where the pathogen did not elongate. This inhibitin was not observed in the disk of young fruit stem-end, but was observed in the same tissues of stem-end of mature fruit at the harvest, and disappeared in the terminal period of storage. The above finding was made by the authors for the first time.

One of the inhibitins was isolated from the fruit stem-ends and inside the fruits, as well as from leaves. Tissues under the disk in the fruit core contained the greatest amount of inhibitin. It was also found in dorsal vascular bundle systems, the vascular bundle connected to the tissues under the disks. A fair amount of the inhibitin was also contained in vesicle. The inhibitin extracted from the tissues under the disks completely suppressed the germ tube elongation at 462-fold dilutions. This inhibitin seems to be one of the factors controlling the fungal development into fruit core.
Literature cited


Explanation of plates

Plate I. Structure of the fruit stem-end of Citrus unshiu. Arrow shows location.
1. The surface of fruit pedicel. c: crack, e: epidermis.
2. The inner part of disk. ab: axial bundle, db: dorsal bundle.
3. The longitudinal section of stem-end. ca: calyx.

Plate II. Penetration of pathogen from fruit pedicel to inner stem-end. Arrow shows location.
1. Penetration of D. citri to fruit pedicel. ps: pycnospore, g: germ tube, pp: penetrated point.
2. Hyphal elongation of D. citri from pedicel to fruit stem-end. eh: elongating hyphae.
3. Hyphal elongation of D. citri at inner stem-end. eh: elongating hyphae, h: hyphae.
4. No hyphae near vascular bundle covered by a lot of crystals. vb: vascular bundle, cr: crystal.

Plate III. Behavior of crystals at inner stem-end. Arrow shows location.
1. Inner disk of young fruit late in May. There is no crystal around inner disk. d: disk end.
2. Inner disk of maturing fruit late in October. A lot of crystals covered inner disk. cr: crystal, d: disk end.
3. Distribution of crystals around the tissues under the disk which is cut longitudinally. There are not many crystals in the upper part while a lot of crystals are observed in the lower part. d: disk end, td: tissue under disk.
4. Distribution of crystals around the tissues under the disk which is cut cross-sectionally. Crystals are filling up cells. cr: crystal.

Plate IV. Behavior of crystals at inner stem-end and feature of crystal. Arrow shows location.
1. Acetone treated inner disk of fruit picked early in October. d: disk end.
2. Inner disk of fruit stored till late in April. Crystal disappeared from inner disk. d: disk end.
3. Needle-like crystals obtained from acetone extract.
Plate I

1. [Image of Plate I with labeled areas: c, e, 200 μm]
2. [Image of Plate II with labeled areas: ab, db, 120 μm]
3. [Image of Plate III with labeled area: ca, 200 μm]
Plate IV

1

2

3

5 μm

5 μm