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Peroxidase Activity as a Marker in Greening Disease of Citrus for Assessment of Tolerance and Susceptibility

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Abstract

The possible connection between peroxidase activity and citrus species and cultivars was investigated in relation to tolerance and susceptibility to greening disease.

Zusammenfassung

Peroxidaseaktivität als Indikator in Greening Disease des Zitrusgewächses in der Bewertung von Toleranz und Anfälligkeit

Untersucht wurde eine eventuelle Verbindung zwischen Peroxidaseaktivität und Zitrusgewächsarten und -sorten in Zusammenhang mit Toleranz und Anfälligkeit gegen Greening Disease.

Peroxidase (PO) activity was found to be higher in the greening-tolerant Tahiti lime and decreased with increased susceptibility of species and cultivars. In leaves with greening symptoms PO activity was not affected compared with apparently healthy leaves of the same species or cultivar. In leaves from diseased branches PO activity decreased with increased susceptibility of species and cultivars. It appears that PO activity assay of citrus leaves can be used as a reliable marker for greening tolerance and susceptibility as it is not affected by the greening organism.

It has taken over 60 years to elucidate the etiology of greening disease. It was originally ascribed to nutrient deficiencies, until McClean and Oberholzer (1965) transmitted the disease and postulated that it was caused by a virus. After
observing prokaryotes in the phloem LaFLECHE and BOVE (1970) maintained that the disease was caused by a mycoplasma-like organism, but these organisms were later found to possess cell envelopes similar to that of gram-negative bacteria (MOLL and MARTIN 1974, GARNIER and BOVE 1977). It is generally accepted now that greening disease is caused by a type of gram-negative bacteria approximately 3000 nm by 350 nm with a double track cell envelope 20 nm to 30 nm thick.

Greening disease is one of the most serious diseases of citrus trees in the world and has been known in South Africa since 1929. Severity of the disease varied, with peaks during 1932 to 1936 and 1939 to 1946 (OBERHOLZER et al. 1965). Since 1958 disease severity has increased and certain areas have become unsuitable for growing citrus.

Diseased trees can be treated successfully by trunk injections of antibiotics (SCHWARZ and VAN VUUREN 1971, BUITENDAG and BRONKHORST 1983). Re-infection by the insect vector (Triozia erytreae Del. G.), still occurs, even with the use of insecticides (BUITENDAG 1976, VAN VUUREN and DA GRACA 1978). This forces re-application of antibiotics which leads to the development of resistance by the organism. The only effective control measure for greening disease is likely to be the use of tolerant or resistant plant material.

The susceptibility of different cultivars of several citrus species was studied by SCHWARZ (1968) and McCLEAN and SCHWARZ (1970). Lemon (Citrus limon [L.] Burm.), rough lemon (C. jambhiri L.), lime (C. aurantifolia Swing.), and Poncirus trifoliata and its hybrids were found to show milder symptoms. DE LANGE (1976) indicated that Tahiti lime (C. latifolia Tan.) is highly tolerant to greening.

Since the testing of hybrids, bred for greening resistance, is of a long term nature (DE LANGE et al. 1984), a laboratory test to determine greening resistance will be of great value.

Various authors found increased activity of PO to be directly involved in disease association with virus (SIMONS and ROSS 1970, VAN LOON 1976, DA GRACA and VAN LELYVELD 1978, WAGIH and COUTTS 1982) and bacterial infection (LOV-ERIKOVICH et al. 1968, URS and DUNLEAVY 1974 a, 1974 b, 1975) and fungi (FEHRMANN and DIMOND 1967, VAN LELYVELD and BRODRICK 1975, YAMAMOTO et al. 1978, SAINI et al. 1985, ARORA and WAGLE 1985, REUVEN and FERREIRA 1985). The purpose of this study was, therefore, to establish whether PO activity may be involved in citrus greening.

**Materials and Methods**

**Plant material**

Citrus trees of species and cultivars which are known from observations to be susceptible or tolerant to greening disease (SCHWARZ 1968, McCLEAN and SCHWARZ 1970, DE LANGE 1976) were selected to provide leaf material. The analysis of mature leaves for PO in citrus (VAN LELYVELD et al. 1975) and avocado (VAN LELYVELD and BESTER 1978) has been shown to give the most reliable results. Mature leaves, two to three months old, were picked during August and September, 1983 and 1986, from 10 to 20 year old orchards on the Citrus and Subtropical Fruit Research Institute. Leaves from symptomless (apparently healthy) and diseased branches from the same tree as judged by the presence of symptoms (McCLEAN and SCHWARZ 1970), were collected and analysed separately. Each sample consisted of 20 g healthy or diseased leaves for each species or cultivar. The species and cultivars
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chosen for the investigation are listed in Table 1. From cultivars that did not show visual disease infection, only apparently healthy samples were taken. All samples were replicated four times.

After picking, the leaves were placed in a refrigerator (0 to 4°C) and homogenized the same day. All diseased branches were through natural infection. Tolerance and susceptibility were visually judged by observations in the orchard for greening symptoms (McCLean and Schwarz 1970) using a scale of 0 to 3 where 0 = tolerant and 1, 2 and 3 = increasing severity of symptoms.

**Peroxidase extraction and assay**

Acetone powder was prepared by homogenizing 20 g leaves per sample in 100 ml cold (−20°C) acetone for 2 min using an Ultra Turrax.

The homogenate was filtered under suction through a Sartorius glass fibre filter and washed repeatedly with cold acetone to remove chlorophyll. The acetone powder was air dried and kept in glass bottles in a freezer at −20°C until needed.

Each acetone powder sample (0.5 g) was homogenized in 10 ml cold (0 to 4°C) 50 mM phosphate buffer, pH 6 and Polycar AT (0.2 g) with a mortar and pestle for 1 min. The homogenate was filtered through gauze cloth and then centrifuged for 25 min at 15,000 × g and 4°C. The supernatant was used immediately for PO assay.

The protein content of the crude extract was determined by the method of Lowry et al. (1951) as modified by Legget-Bailey (1962). Bovine serum albumin was used as standard.

Peroxidase assay was based on the method described by Van Leuven et al. (1975) by the addition of 20 μl incubation mixture of 10 ml 50 mM phosphate buffer, pH 6.0, 0.5 ml 0.04 M guaiacol and 0.5 ml 0.1 M H₂O₂. The initial rate was measured at 420 nm and 25°C. The units of specific activity for PO are given as number of absorbance units min⁻¹ mg protein⁻¹.

**Table 1**

Peroxidase activity in leaves of greening affected and healthy branches from several citrus species and cultivars

<table>
<thead>
<tr>
<th>Species and cultivars</th>
<th>Tolerance rating**</th>
<th>PO activity* units (Greening negative)</th>
<th>Greening positive (diseased)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. latifolia (Tan) — Tahiti lime</td>
<td>0</td>
<td>21.344</td>
<td></td>
</tr>
<tr>
<td>C. aurantifolia (Christm.) Swing. — West Indian lime</td>
<td>1</td>
<td>12.394</td>
<td></td>
</tr>
<tr>
<td>C. paradisi (Macf.) — Grapefruit</td>
<td>2</td>
<td>11.220</td>
<td></td>
</tr>
<tr>
<td>C. limon (L.) Burm. — Lemon</td>
<td>2</td>
<td>9.109</td>
<td></td>
</tr>
<tr>
<td>C. grandis (L.) Osbeck — Pumelo</td>
<td>2</td>
<td>8.480</td>
<td></td>
</tr>
<tr>
<td>C. jambhiri Lush — Rough lemon</td>
<td>2</td>
<td>3.742</td>
<td></td>
</tr>
<tr>
<td>A. aurantium Linn. — Sour orange</td>
<td>2</td>
<td>1.342</td>
<td></td>
</tr>
<tr>
<td>C. sinensis (L.) Osbeck — Sweet Orange (Valencia)</td>
<td>3</td>
<td>3.157</td>
<td>4.379</td>
</tr>
<tr>
<td>C. sinensis — Sweet Orange (Navel)</td>
<td>3</td>
<td>1.432</td>
<td>1.097</td>
</tr>
<tr>
<td>C. paradisi × C. reticulata — Minneola</td>
<td>3</td>
<td>2.484</td>
<td>2.888</td>
</tr>
<tr>
<td>C. reticulata Blanco-Dancy tangerine</td>
<td>3</td>
<td>1.551</td>
<td>1.456</td>
</tr>
<tr>
<td>L.S.D. P = 0.05</td>
<td></td>
<td>5.23</td>
<td>4.703</td>
</tr>
<tr>
<td>P = 0.01</td>
<td></td>
<td>5.82</td>
<td>5.991</td>
</tr>
</tbody>
</table>

Results from 4 replications (different samples).

* Number of absorbance units min⁻¹ mg protein⁻¹.

** Tolerance rating 0 = tolerant, 1 = slightly susceptible, 2 = susceptible, 3 = highly susceptible.
Results

Peroxidase activity in the tolerant Tahiti lime was significantly (P < 0.01) higher in apparently healthy leaves than in leaves of all other species and cultivars (Table 1).

Statistical examination of the data for PO activity in apparently healthy leaves shows that the different species and cultivars can be grouped as follows:

Group 1 C. latifolia Tan
Group 2 C. aurantifolia (Christm.) Swing., C. paradisi Macf., C. limon (L.) Burm., C. grandis (L.) Osbeck
Group 3 C. jambhiri Lush., C. sinensis (L.) Osbeck (Valencia), C. sinensis (Navel), C. paradisi × C. reticulata, C. reticulata Blanco, C. auran-
tium Linn.

With the exception of C. jambhiri Lush., it is clear that significant differences (P < 0.05) are found between groups but not within groups (Table 1).

In a statistical analysis of data for observed symptom ratings of susceptible and the PO activity in apparently healthy leaves a correlation of \(-0.70451\) was found to be significant at \(P < 0.01\). A linear regression coefficient significant at \(Y = 19.4178 - 5.95598 \times X\) was established between symptom ratings of species and cultivars and the decrease of PO activity in apparently healthy leaves.

There was no significant difference in the PO activities between apparently healthy and affected branches from the same cultivar or species. This indicates that PO activity was not affected by the presence of disease symptoms.

Discussion

A negative correlation, significant at \(P < 0.01\), exists between PO activity, in the leaves of different citrus species and cultivars and their tolerance rating. It appears, therefore, that PO activity in mature leaves can be used as a parameter in the assessment of susceptibility of citrus cultivars to greening disease. Similar results were obtained with avocado leaves and susceptibility to Phytophthora cinnamomi root rot (VAN LELYVELD and BRODRICK 1975).

The possible reasons for this PO reaction may be explained by the findings of various authors who reported an increase in PO activity as a result of infection (OOGUCHI and ASADA 1975, VANCE et al. 1976, HEALE and SHARMAN 1977, HAMMERSCHMIDT et al. 1982, THORPE and HALL 1984). HAMMERSCHMIDT et al. (1982) have shown that acquired resistance can be developed in cucumber as a result of increased PO activity after infection. In the present investigation, however, PO activity in leaves of the least tolerant cultivar and species, with greening symptoms was not significantly different in healthy trees of the same four cultivars or species (Table 1). It would appear, therefore, that resistance, as measured by PO activity, to greening in citrus is species and cultivar bound and not acquired. URS and DUNLEAVY (1974 a, b) suggested that PO is bactericidal in combination with other cellular constituents. URS and DUNLEAVY (1974 a, b) found that the PO in the presence of certain phenols can be bactericidal probably because of the formation of toxic quinones.
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In the present investigation, therefore, it is possible that the high natural PO activity of Tahiti lime results in larger quantities of quinone being produced in its leaves than are produced in leaves of more susceptible species and cultivars. This aspect is now being investigated.

We wish to thank Dr. J. N. Moll for his suggestions and valuable discussions, and Mr. A. Toerien for statistical analysis.

Literature


— —, and — —, 1974b: Bacterial activity of horseradish peroxidase on Xanthomonas phaseoli var. sojae. Phytopathology 64, 542—545.


