# Reliability of Gentisic Acid, a Fluorescent Marker, for Diagnosis of Citrus Greening Disease

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**ABSTRACT**

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The reliability of gentisic acid (GeA), a fluorescent marker, to predict the presence of citrus greening disease was reevaluated by a modified method for hydrolyzing gentisoyl-β-D glucose to gentisic acid. Bark tissue extracts of random samples of diseased and healthy citrus plants differed significantly in their GeA content. The severity of foliar symptoms was correlated significantly with the amount of GeA in young and old bark tissue. Older tissue had significantly more severe foliar symptoms than younger tissue and yielded more GeA in extracts. However, when dried tissue samples were used, there was a significant difference in GeA content between tender young bark and old bark but no significant difference between mature and old (woody) bark. Although plants infected with several citrus tristeza virus strains contained GeA, greening-affected plants had statistically higher mean levels of GeA. This method is reliable for diagnosing citrus greening disease in citrus when GeA levels are above 300 μg/gm of tissue and when used in combination with other diagnostic criteria under glasshouse conditions.

Citrus greening and greeninglike diseases (likubin, citrus vein phloem degeneration, citrus yellow shoot, and leaf mottle) are confined to the areas surrounding the Indian and Pacific oceans, ranging from South Africa to parts of the Middle East, China, Taiwan, and the Philippines. Greening has recently been described in Japan (17). Attempts have been made to culture and isolate the etiologic agent in vitro, but to date, no confirmed pure culture exists (1,7). The agent is reported to be a fastidious gram-negative bacterium (8–10,18). The known insect vectors, both psyllids, are *Trioza erytreae* (Del Guercio) in Africa (15,16,26) and *Diaphorina citri* (Kuway) in Asia (13).

There has been a great need to develop tests for field diagnosis of citrus greening, because disease symptoms can be confused with other metabolic and/or infectious diseases. While several monoclonal antibody probes have been developed, they have not been universally successful in detecting all serotypes (11,32). Thus far, there are no published reports of successful diagnostic DNA probes. Gentisic acid (GeA), a phenolic compound and plant disease product, is present in low levels in healthy citrus (30) but may become elevated to detectable levels in diseased tissues. Ibrahim and Towers (12) devised a method for two-directional separation by paper chromatography of 21 phenolic acids, including GeA, from several plant species. Feldman and Hanks (4) first reported the presence of hydroxybenzoics, such as GeA, in citrus leaf and

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root tissue. Schwarz (21) discovered a bright blue-violet fluorescent marker in cold ether extracts of greening-affected sweet orange by paper chromatography. This disease-associated phenolic fluorescent marker was identified as the monoglucose ester of GeA, gentisoyl glucose (5). This marker was not detected by observing the fruit albedo of sweet orange under UV light in plants infected with agents that cause exocortis, xyloporosis or psorosis, with tristeza, nor in Zn-, B-, or Cu-deficient plants. However, it was detected in fruit albedo from greening- and stubborn-affected plants (23). The presence of the marker in albedo extracts was consistent in greening-affected sweet orange but was less consistent in greening-affected mandarin, tangelo, lemons, and grapefruit (22–24,27). Detection of the marker in bark extracts was more sensitive for greening diagnosis in sweet orange (23, 25,27), tangelo, and mandarin (23,27) than was indexing based on foliar symptoms. Likewise, GeA fluorescence in fruit albedo of sweet orange was a better predictor of greening disease than fruit symptoms. However, the reliability of GeA in bark tissue of sweet orange is variable because of its uneven distribution, which might be attributable to seasonal variation or bark water content (25,27).

A main problem in the diagnosis of greening based upon the GeA fluorescent marker has been false positives in healthy sweet orange (10–15%) (14). More recently, these inconsistencies have been studied by the comparison of different extraction and chromatographic methods (3,31). Other studies have investigated the relationship among GeA, peroxidase activity, and susceptibility to citrus greening disease (28,29). GeA levels in citrus are positively correlated with greening disease but not with cultivar susceptibility to greening (30). Increased GeA may be caused by or be a reaction to the citrus greening disease pathogen (29).

The purpose of this study is to test whether the presence of elevated levels of GeA, detected as a bright blue fluorescent spot on silica gel thin-layer chromatography (TLC) plates, is a reliable, specific diagnostic marker for citrus greening and greeninglike diseases. Because the detection of GeA in field samples has been inconsistent for diagnosing citrus greening disease, we have evaluated the diagnostic reliability of GeA with an extensive glasshouse collection of a limited number of citrus cultivars infected with several citrus pathogens. We tested two hypotheses: 1) that the presence of GeA reliably predicts the presence of citrus greening disease, and 2) that the level of GeA present is related to severity of foliar symptoms.

**Table 1.** Citrus source plants from Beltsville, Maryland, used in study of gentisic acid in diagnosis of citrus greening disease

| Disease state No. of plants | Greening isolate | Citrus tristeza virus ID no. | Place of origin | Tristeza infection[y] |
|---|---|---|---|---|
| Greening only | | | | |
| 14 | LK5 | ... | Taiwan | − |
| 8 | LK1 | ... | Taiwan | − |
| 1 | B232 | ... | Thailand | − |
| 6 | R1 | ... | Réunion | − |
| 4 | B1b | ... | Réunion | − |
| 2 | SAG | ... | S. Africa | − |
| 2 | ... | ... | India | − |
| Mixed infections | | | | |
| 1 | R1 | ... | Réunion | ND[z] |
| 4 | R1 | ... | Réunion | + |
| 2 | SAG | ... | S. Africa | ND |
| 6 | SAG | ... | S. Africa | + |
| 1 | GF4 | ... | Tanzania | + |
| 1 | GF11 | ... | Tanzania | + |
| 1 | GF13 | ... | Tanzania | + |
| 5 | CYS | ... | People's Republic of China | + |
| 1 | HA | ... | Saudi Arabia | + |
| 1 | B205 | ... | Indonesia | + |
| 1 | ... | B41 | Taiwan | + |
| 1 | ... | B140 | Philippines | + |
| 1 | ... | B143 | Philippines | + |
| 1 | ... | B143sp | Philippines | + |
| 1 | ... | B240 | Brazil | + |
| 1 | ... | B241 | Brazil | + |
| 1 | ... | B241 | Brazil | + |
| 1 | ... | B242 | Brazil | + |
| Psorosis | | | | |
| 1 | ... | B174 | India | − |
| Greening-free CTV | | | | |
| 1 | ... | B14b (new) | Brazil | + |
| 1 | ... | B14 | Brazil | + |
| 1 | ... | B108 | California | + |
| 1 | ... | B107 | California | + |
| 1 | ... | B109 | California | + |
| 1 | ... | B110 | California | + |
| 1 | ... | B156 | Australia | + |
| 1 | ... | B224 | India | + |
| Ringspot | | | | |
| 1 | ... | B104 | Florida | − |

[y] − = Negative, + = positive by enzyme-linked immunosorbent assay.
[z] Not determined.

## MATERIALS AND METHODS

**Source plant material.** All plant material was maintained in a temperature-controlled glasshouse with standard pest control. Citrus greening isolates were maintained in Duncan grapefruit (*Citrus* × *paradisi* Macfady.), rough lemon (*C. jambhiri* Lush.), or sweet orange (*C. sinensis* (L.) Osbeck) cultivars Navel, Valencia, or Madam Vinous. Citrus tristeza virus (CTV) isolates were maintained in sweet orange or *C. excelsa* Wester. Citrus exocortis viroid, citrus variegation virus, citrus leaf rugose virus, and psorosis agent were maintained in citron (*C. medica* L.) and citrus tatterleaf virus in sweet orange. Citrus greening sources were from Africa, South Africa, Taiwan (two different isolates, LK1 and LK2), Peo-

**Table 2.** Virus-infected and healthy citrus source plants from Lake Alfred, Florida, used in study of gentisic acid in diagnosis of citrus greening disease

| Parent plant | Number of plants | Virus[z] |
|---|---|---|
| Citron | 3 | CVV |
| Citron | 3 | CLRV |
| *Citrus excelsa* | 1 | CTV-T26 |
| Sweet orange | 3 | CTLV |
| Grapefruit | 2 | CRSV 4E |
| Grapefruit | 3 | CRSV 6C |
| Citron | 1 | CEV E1 |
| Citron | 1 | CEV E25 |
| Citron | 1 | CEV E8 |
| Citron | 1 | CEV E30 |
| Citron | 10 | CEV |
| Grapefruit | 1 | None (healthy) |
| Sweet orange | 1 | None (healthy) |
| Citron | 1 | None (healthy) |

[z] CVV = citrus variegation virus, CLRV = citrus leaf rugose virus, CTV = citrus tristeza virus, CTLV = citrus tatterleaf virus, CRSV = citrus ringspot virus, and CEV = citrus exocortis viroid.

ple's Republic of China, and Réunion (two different in planta cultures of one isolate, one culture being CTV-free). Double-antibody sandwich enzyme-linked immunosorbent assay (2) was used to confirm CTV infection in the plants used in this study.

**Tissue sampling.** Samples were collected from glasshouse-grown citrus in Beltsville, Maryland (Table 1), and Lake Alfred, Florida (Table 2). These samples consisted of bark from 1) plants affected by greening; 2) plants infected with CTV, psorosis agent, other viruses, or viroids; 3) plants infected with mixtures of CTV and greening; and 4) healthy citrus plants. Where appropriate, young (soft), mature (firm), and old (woody) bark tissue of each plant was sampled. Symptom severity for each plant sampled, based upon typical greening foliar symptoms, was rated 0 (no greening symptoms), 1+ (slight blotchy mottle or yellow-green venation), or 2+ (severe blotchy mottle, woody venation, curled leaves, or small leaves). We used either 0.25 g of fresh tissue (experiments 1 and 2) or 0.05 g of tissue dried overnight at room temperature after being finely chopped (experiments 2 and 3).

**Experiment 1.** We tested the hypothesis that a group of greening-affected plants differed from a group of healthy plants on the basis of the presence or absence of GeA between the groups. A fresh random sample was taken of 41 stock plants, which included both healthy plants and those known to be affected by greening on the basis of foliar symptoms. Plants grafted with greening-affected tissue but not showing obvious foliar symptoms at the time of the experiment were also included. In addition, fresh bark tissue was taken from 32 additional plants, infected with several other viruses, from the collection at the Citrus Research and Education Center in Lake Alfred and tested for the presence of the marker.

**Experiment 2.** We also tested whether foliar symptoms and/or greening source was directly related to the presence of GeA. Fresh and dried tissue from young, mature, and old bark of a sample of 39 healthy or diseased plants was tested. Foliar symptoms and intensities of GeA spots on the TLC plates were rated. The effect of tissue age on GeA intensity also was investigated.

**Experiment 3.** On the basis of prior analyses, several plants infected with other pathogens were positive for GeA. A total of 47 plants were classified according to disease association (CTV only, probable CTV–greening mix, greening only, and healthy). GeA levels among these four plant groups were measured spectrophotometrically after eluting spots from TLC plates. Where possible, young, mature, and old bark tissue was sampled and plants were simultaneously observed for foliar symptoms. Not all plants simultaneously had young, mature, and old bark tissue. However, each plant was sampled only once from the available bark type. Dried bark tissue (0.05 g) was used. TLC results were rated visually and quantitatively by direct spectrophotometric measurements of GeA using a standard curve.

**Analysis of GeA.** We used a modification of the method of Burger et al (3). Bark tissue (0.25 g of fresh or 0.05 g of dried) was finely chopped, added to 0.5 ml of 2 N HCl in a 1.7-ml microcentrifuge tube (PGC Scientifics, Gaithersburg, MD), and boiled for 1 hr. After cooling, 0.5 ml of ethyl acetate was added, and the resulting mixture was vortexed at high speed for 1 min. After centrifugation for 10 min at high speed at 13,000 *g* (BHG Hermle model Z-230-M microcentrifuge, National Labnet Co., Woodbridge, NJ), approximately 200 μl of the organic top layer was removed for analysis. Five-μl samples, in five 1-μl aliquots, were spotted 2 cm from the bottom of 20- × 20-cm TLC plates precoated with silica gel 60 (0.25 mm) without fluorescent indicator (EM Science, Associate of E. Merck, Darmstadt, Germany). Five-μl samples, in five 1-μl aliquots, of GeA (2,5 dihydroxybenzoic acid, free acid; Sigma Chemical Co., St. Louis, MO) (1 μg/ml) were included as internal standards. Ascending chromatography was conducted for approximately 50 min in a sealed glass tank with a water-saturated ethyl acetate–96% ethanol mixture (97:3, v/v) as the irrigant. Plates were air-dried, read over a 302-nm UV transilluminator (model TM-15, UVP, Inc., San Gabriel, CA), and scored. Under our experimental conditions, the GeA standard migrates with an $R_f$ of 0.09. The presence of bright blue fluorescing spots at the appropriate $R_f$ value was visually rated as 0 (absent), 0.5+ (+/−), 1+, 2+, 3+, and 4+, relative to the GeA standard (4+) (Fig. 1).

Ethyl acetate extracts of whole bark tissue and water-eluted extracts of fluorescent spots were further analyzed by high-performance liquid chromatography (HPLC) and by spectrophotometry, respectively. For HPLC, samples were analyzed with a Waters HPLC 600E multisolvent delivery system equipped with a 990 photodiode array detector and a U6K injector. Spectra were taken at the ascending portion, apex, and descending portion of the peak that coelutes with GeA and that appeared at 330 nm. Separations were performed on an 8-mm × 10-cm C18 Radial-Pak column (Waters Associates, Milford, MA) containing 5-μm particles. The mobile phases were 30 mM ammonium dihydrogen phosphate (pH 3.0) containing 50% methanol (mobile phase A) and 10mM ammonium dihydrogen phosphate (mobile phase B). The gradient system began with 30% of the mobile phase A and 70% of the mobile phase B and was changed linearly to 100% mobile phase A after 20 min. After 25 min, the column was reequilibrated to the starting conditions. The retention time of GeA at a flow rate of 1 ml/min is 8.5 min, as determined by coinjection with a GeA standard (Fig. 2).

Spectrophotometric analyses were done with a UV-160A UV-visible recording spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Silica gel spots of the appropriate $R_f$ value and that exhibited the typical blue GeA fluorescence were cut from the TLC plate and suspended in distilled water. The resulting suspensions were clarified by cen-

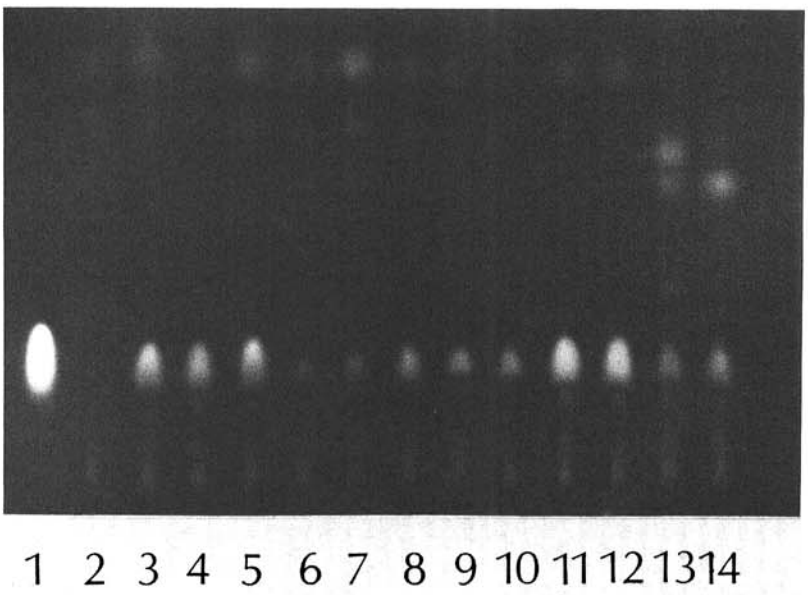


**Fig. 1.** Thin-layer chromatography plate demonstrating a qualitative rating scale on a series of fresh bark tissue extracts spotted in five 1-μl aliquots. Well 1, 4+; wells 3, 4, 5, 11, and 12, 3+; well 8, 2+; wells 6, 7, 9, 10, 13, and 14, 1+; well 2, negative.

trifugation, and the supernatants were scanned from 200 to 800 nm. Under these conditions, the peak absorbance (320 nm) curves were smoothed by instrument software. The absorbance values at this wavelength were converted to concentrations from a standard curve of GeA in water (concentration = 2840.3 × absorbance − 4.3441).

**Statistical analyses.** Data were analyzed parametrically by SAS procedures (19,20), such as analysis of variance (ANOVA) or analysis of covariance. If data did not meet assumptions for parametric analysis, nonparametric procedures (chi-square, Fisher's exact test, Kendall's $\tau$ b correlation, Wilcoxon's signed-rank test, and ANOVA of rank-transformed data) were used. Expected values for chi-square and Fisher's exact tests were computer generated under the null hypothesis of independence.

## RESULTS

Random samples of known healthy and greening-diseased plants differed significantly with regard to the presence of GeA (experiment 1). Of the 15 healthy plants tested, 14 did not have a detectable marker. Conversely, 23 of the 26 greening-affected plants tested had the marker, and three did not ($P < 0.001$, Fisher's exact test) (Table 3). These samples were taken without regard to symptoms. One of the three diseased plants that did not have the marker had severe symptoms; one did not show symptoms; and one apparently healthy plant, which was initially scored positive, appeared negative when the TLC plate was subsequently viewed. The healthy and greening-affected groups differed significantly in their distributions of intensity ratings for GeA ($P < 0.001$, Fisher's exact test) (Table 3); 16 of 26 diseased plants were scored as 3+ or 4+. The one healthy plant was 2+. All the others were 0.

A total of 32 plants infected with other citrus viruses, but not affected by greening, were tested for the presence of GeA to determine if GeA was specific for greening disease. This group differed significantly from random in its distribution of GeA, because only two plants had the marker, and 30 were negative for GeA ($P < 0.001$, Fisher's exact test) (Table 3). The group also differed from random in its distribution of intensity ratings ($X^2 = 37.01$, $P < 0.001$) (Table 3).

Overall, two-factor ANOVA testing the effect of greening isolate (eight levels: SAG, LK1, LK5, B1b, R1, Saudi Arabian, Tanzania, and healthy) and foliar symptoms (three levels: 0, 1, 2) on intensity of GeA was significant for tender young and old bark tissue (young: $F = 8.49$, df = 9, $n = 38$, $P = 0.0001$; old: $F = 8.87$, df = 9, $n = 39$, $P = 0.0001$) (experiment 2). For young and old tissue, degree of foliar symptoms was significantly related to the intensity of GeA

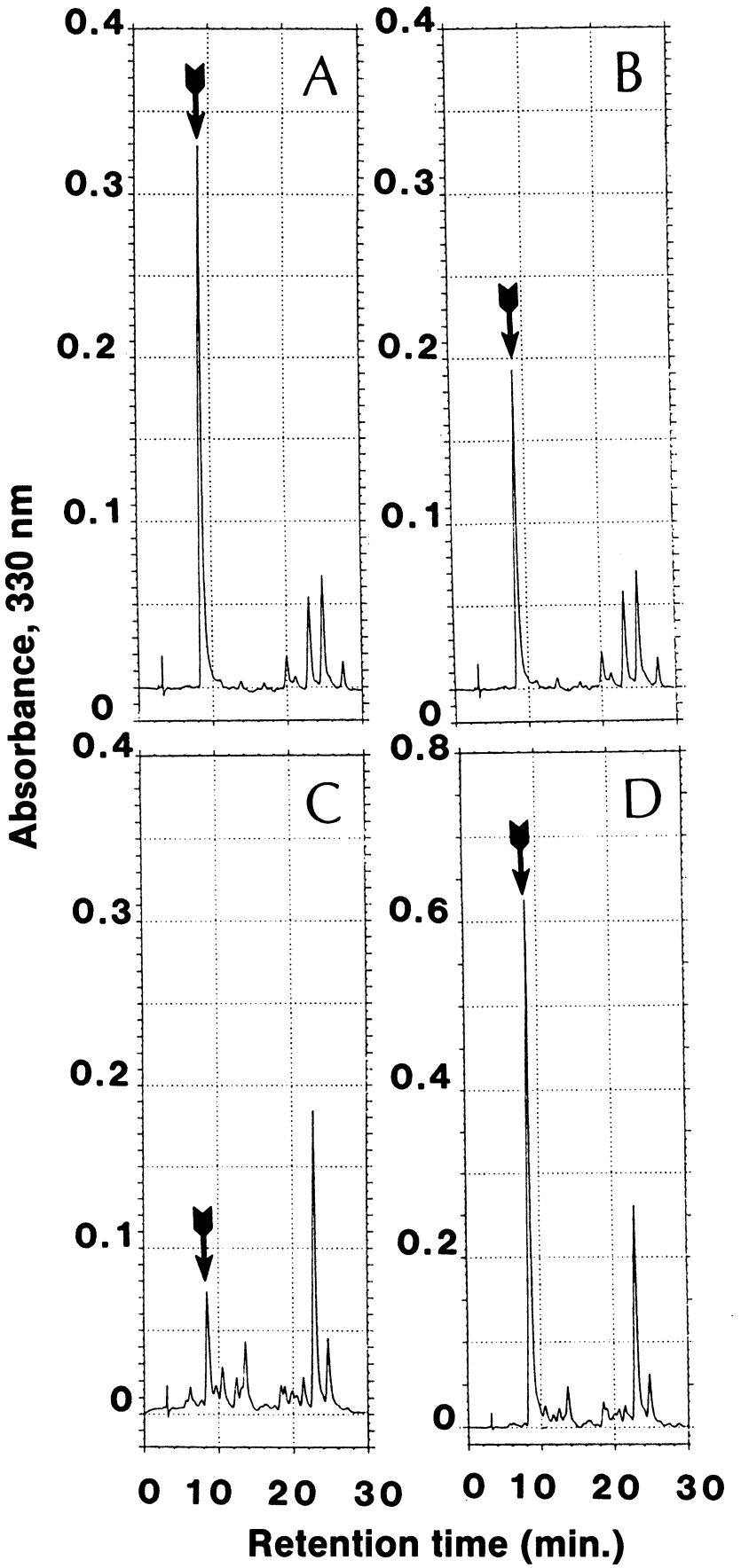


**Fig. 2.** HPLC chromatographs of **(A)** gentisic acid (GeA) standard (3.5 $\mu$g) coinjected with citrus tristeza virus (CTV)-infected tissue extract, and pure tissue extracts of **(B)** CTV-infected, **(C)** CTV- and greening-affected, and **(D)** greening-affected plants at 330 nm. The GeA peak in **(A)** is increased relative to the peak in **(B)** by the addition of standard GeA.

(young: $F = 22.25$, df = 2, $n = 38$, $P = 0.0001$; old: $F = 20.21$, df = 2, $n = 39$, $P = 0.0001$). Greening isolate was not a significant factor. Hence, there were no differences among the intensities of GeA for the individual sources of greening. There were differences in GeA associated with the individual levels of foliar symptoms (no symptoms, slight symptoms, and severe symptoms) in young tissue. All three symptom levels were significantly different from each other in their GeA intensity, with severely symptomatic tissue having the highest GeA level ($P < 0.05$, Scheffé's test). However, in old tissue, only bark extracts from plants with severe symptoms differed significantly from the other two symptom levels in their relationship to GeA intensity ($P < 0.05$, Scheffé's test). The degree of GeA intensity was significantly correlated with degree of foliar symptoms when young and old tissue were considered separately (young: Kendall's $\tau$ [K$\tau$] = 0.7635, $n = 38$, $P = 0.0001$; old: K$\tau$ = 0.7374, $n = 39$, $P = 0.0001$).

GeA intensity (Wilcoxon's signed-rank [WSR] = 126.5, $n = 38$, $P = 0.0001$) and degree of foliar symptoms (WSR = 24.5, $n = 38$, $P = 0.0117$) increased significantly in old tissue compared to young tissue when fresh tissue was immediately extracted for GeA. Mature tissue was not tested. However, when dried samples were tested, there was a significant difference between young and old bark (WSR = 52.5, $n = 16$, $P = 0.0001$) but no difference between the GeA levels in mature and old bark (WSR = 0, $n = 16$, $P = 1.000$).

Because GeA was detected in at least one healthy and two virus-infected plants, additional plants affected by greening and infected by other viruses and a viroid were tested. Ethyl acetate extracts of plants infected by CTV only, affected by greening only, and infected with possible mixtures of pathogens were analyzed by HPLC (experiment 3). Spectra made by diode analysis with a GeA standard coinjected with a CTV-infected tissue extract, CTV-infected plant tissue extract, greening-affected tissue extract, and plants with possible mixed infections are shown in Figure 2. The maximum absorbance at 330 nm from each spectrum was superimposable, and the spectra were considered identical. Likewise, absorption spectra of water extracts of eluted TLC spots from GeA standard, CTV-infected plants, greening-affected plants, and plants with possible mixed infections were indistinguishable spectrophotometrically when scanned from 200 to 800 nm (Fig. 3). Hence, it was concluded that the markers for greening-affected and for the few CTV-infected plants that show the fluorescent marker are chemically identical to GeA. When we measured GeA spectrophotometrically on four groups of plants classified by disease severity, there was no overall detectable difference in young tissue nor in mature bark tissue (Table 4). However, extracts of old woody bark tissue permitted more effective differentiation among the four groups (Table 4). The greening-only group had a significantly higher GeA level than the other three groups, and the CTV and CTV-greening mixed groups were significantly different from healthy and greening-only, but we detected no significant difference between the CTV and the CTV-greening group ($P > 0.0083$, $\alpha$-adjusted for multiple comparisons). On the basis of data from experiment 2, old woody tissue had higher quantitative rating levels of GeA than young tissue.

We then tested whether the distribution of qualitative GeA intensity ratings differed from random among the four groups in mature and old tissue. In young bark tissue, there was no significant association ($X^2 = 6.75$, df = 9, $P = 0.663$). However, there were significant associations in mature and old bark tissue (Table 5). There was a trend toward higher intensity ratings in the greening-only group, especially with extracts from old tissue.

With the exception of extracts of young tissue samples from greening-affected plants, both qualitative (visual) and quantitative (spectrophotometric) ratings of GeA were significantly correlated (mature bark: K$\tau$ = 0.845, $n = 7$, $P = 0.0137$; old bark: K$\tau$ = 0.733, $n = 14$, $P = 0.0013$). This also occurred for the CTV-greening mix plants (young:

**Table 3.** Qualitative scoring of gentisic acid content of healthy, greening-diseased, and virus-infected citrus plants after thin-layer chromatography of bark tissue extracts

| Plant disease state | Source[y] | Number of plants with gentisic acid rating | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1+ | 2+ | 3+ | 4+ |
| Healthy | B | 14 | 0 | 1 | 0 | 0 |
| Greening-diseased | B | 3 | 2 | 5 | 8 | 8 |
| Virus-infected[z] | LA | 30 | 2 | 0 | 0 | 0 |

[y] B = Beltsville, Maryland, collection; LA = Lake Alfred, Florida, collection.
[z] Infected by citrus variegation virus, citrus leaf rugose virus, citrus tristeza virus, citrus tatterleaf virus, citrus ringspot virus, or citrus exocortis viroid.

**Table 4.** Mean gentisic acid concentration in young, mature, and old bark tissue of healthy citrus plants and plants infected by citrus tristeza virus (CTV), affected by greening, or affected by a mixture of CTV and greening

| Plant group | Spectrophotometric measurement of gentisic acid concentration ($\mu$g per gram of tissue) | | |
|---|---|---|---|
| | Bark age | | |
| | Young[w] | Mature[x] | Old[y] |
| CTV only | 42.0 a[z] | 151.6 a | 143.4 a |
| CTV-greening mix | 92.0 a | 200.2 a | 146.3 a |
| Greening only | 140.0 a | 283.7 a | 345.8 b |
| Healthy | 38.0 a | Not done | 24.0 c |

[w] $F = 2.31$, df = 3, $n = 16$, $P = 0.1533$.
[x] $F = 2.40$, df = 2, $n = 22$, $P = 0.1187$.
[y] $F = 12.87$, df = 3, $n = 42$, $P = 0.0001$.
[z] Least-squares means in columns followed by different letters are significantly different at $P = 0.0083$ level, $\alpha$-adjusted for multiple comparisons.

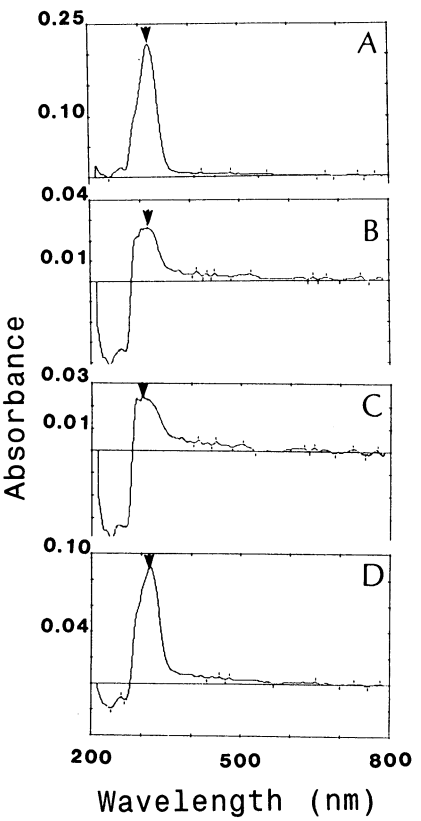


**Fig. 3.** Absorption spectra in the UV-visible range of the gentisic acid peak from **(A)** gentisic acid standard, and **(B)** extracts of citrus tristeza virus (CTV)-infected, **(C)** CTV- and greening-affected, and **(D)** greening-affected tissue.

$K\tau = 1.000$, $n = 4$, $P < 0.0001$; mature: $K\tau = 0.810$, $n = 9$, $P = 0.0064$; old: $K\tau = 0.852$, $n = 13$, $P = 0.0002$). For CTV-infected plants, only old tissue had sufficient sample size for correlation analysis (old: $K\tau = 0.723$, $n = 7$, $P = 0.0365$). Hence, both rating methods for GeA are comparable for determining severity of infection with citrus greening disease.

Under conditions of our assay, a purple fluorescent pigment migrates slightly ahead of the blue GeA marker. This purple pigment was not found in any samples from young tissue. There was no statistically significant association of the purple pigment with the four plant groups in mature tissue ($X^2 = 2.77$, df = 2, $P = 0.250$). However, there was a significant association in old tissue ($X^2 = 15.89$, df = 3, $P = 0.001$). The purple pigment occurred only in bark extracts from plants with greening and doubly infected with CTV, and in greening-only affected plants. Hence, this marker may be specific for greening. Alternatively, its presence may be related to the concentration of GeA present. When the association between this purple pigment and the qualitative rating levels for GeA was tested, there was a significant association in old tissue ($X^2 = 24.85$, df = 4, $P < 0.001$) but not in mature tissue ($X^2 = 8.55$, df = 4, $P = 0.073$). This pigment appears only when GeA is rated 2+ or higher in intensity. The identification and diagnostic significance of this marker remain to be determined.

Because greening-affected plants have a higher GeA concentration than the other groups evaluated, and because degree of foliar symptoms was significantly related to the intensity of GeA, we tested whether the sample of greening plants differed from the sample of CTV-infected plants with regard to severity of foliar symptoms. If the sample of greening-affected plants had a higher frequency of plants with severe symptoms, the GeA level for that group could be biased toward a higher mean than that of the CTV group by virtue of its symptom distribution. Thus, we tested the hypothesis that the distribution of symptom ratings was independent of our classification of plants into the CTV or greening-only group. There was no detectable significant effect of disease group rating (CTV or greening) on symptom rating in young ($F = 0.78$, df = 1, $n = 9$, $P = 0.4071$), mature ($F = 0.61$, df = 1, $n = 12$, $P = 0.4543$), nor old ($F = 0.78$, df = 1, $n = 25$, $P = 0.3895$) bark tissue. Likewise, there were no significant differences in the distribution of symptom ratings between the CTV and greening-affected plants among young, mature, nor old bark tissue (Table 6). These results indicate that the sample was not biased with regard to symptom ratings, and that the severity of symptoms was randomly distributed between the CTV and greening-affected groups. In relation to this, we tested whether CTV-infected and greening-affected plants differed in the length of time that these two groups were diseased. A longer diseased state, as measured from the date of original graft, could affect GeA levels, independent of disease source. Length of disease state, adjusting for disease source, did not significantly affect GeA levels ($F = 1.58$, df = 1, $n = 25$, $P = 0.2287$, analysis of covariance). We conclude that the difference in GeA levels detected was attributable to higher intrinsic GeA levels in greening-affected than in CTV-infected plants.

## DISCUSSION

The citrus plant and disease collection used in this study (from Beltsville and Lake Alfred) is both comprehensive and extensive as a source of CTV and greening isolates. Access to the collection has enabled the testing of a more extensive sample of disease isolates than has been tested previously. A bright blue fluorescent marker, with the same chromatographic and spectrophotometric properties as authentic GeA, was found in bark extracts of the majority (74 of 87) of greening-affected plants. However, we also found, and identified as GeA, a similar marker in several plants presumably infected only with CTV. Both the age of bark tissue and the severity of foliar symptoms affected the fluorescence intensity of the marker, because older woody bark from plants with severe symptoms contained the highest levels of GeA. The sample of greening-affected plants had a higher GeA level than the sample of CTV plants, although both plant groups were rated similarly with regard to disease symptom severity and were diseased for similar time periods.

Although GeA has been considered to be diagnostic for citrus greening disease, its specificity for greening disease has been questioned by investigators who have found similar fluorescent markers in healthy plants or in plants infected with other pathogens (3,21–25,27,30,31). The use of GeA for diagnosis also is limited because GeA has not been consistently associated with greening in all citrus species, including grapefruit (22,23). In more recent studies, including this one, grapefruit that showed prominent greening disease symptoms contained measurably elevated levels of GeA (27,30). Further, in our studies, 85% (74 of 87) of greening-affected plants con-

**Table 5.** Frequency distribution of qualitative ratings of gentisic acid intensity on thin-layer chromatograms among plants grouped by disease and by age

| Plant age / Disease state[w] | Degree of intensity of gentisic acid: 0 | | 0.5+ | | 1+ | | 2+ | | 3+ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | OB[x] | E | OB | E | OB | E | OB | E | OB | E |
| Mature bark[y] | | | | | | | | | | |
| CTV | 0 | 0.2 | 0 | 0.2 | 5 | 2.0 | 0 | 1.8 | 0 | 0.6 |
| CTV–greening | 1 | 0.4 | 1 | 0.4 | 3 | 4.0 | 5 | 3.6 | 0 | 1.3 |
| Greening | 0 | 0.3 | 0 | 0.3 | 1 | 2.8 | 3 | 2.5 | 3 | 0.9 |
| Healthy | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| Old bark[z] | | | | | | | | | | |
| CTV | 1 | 1.4 | 1 | 1.3 | 5 | 2.2 | 1 | 1.3 | 0 | 1.9 |
| CTV–greening | 1 | 2.0 | 4 | 2.3 | 6 | 4.0 | 2 | 2.3 | 1 | 3.3 |
| Greening | 3 | 2.4 | 0 | 2.8 | 1 | 4.8 | 4 | 2.8 | 9 | 4.0 |
| Healthy | 1 | 0.4 | 2 | 0.5 | 0 | 0.8 | 0 | 0.5 | 0 | 0.7 |

[w] Infected by citrus tristeza virus (CTV), affected by greening, affected by a mixture of CTV infection and greening, or healthy tissue. Test not done on mature healthy bark.
[x] OB = observed or E = expected under the null hypothesis of independence.
[y] $X^2 = 17.01$, df = 8, $P = 0.030$.
[z] $X^2 = 29.59$, df = 12, $P = 0.003$.

**Table 6.** Frequency distribution of foliar symptom levels among citrus tristeza virus (CTV)- and greening-diseased plants

| Plant group[x] | Symptom rating: 0 | | 1+ | | 2+ | |
|---|---|---|---|---|---|---|
| | OB[y] | E | OB | E | OB | E |
| Young bark | | | | | | |
| CTV | 0 | 0.4 | 1 | 0.5 | ND[z] | ND |
| Greening | 4 | 3.5 | 4 | 4.4 | ND | ND |
| Mature bark | | | | | | |
| CTV | ND | ND | 4 | 3.3 | 1 | 1.6 |
| Greening | ND | ND | 4 | 4.6 | 3 | 2.3 |
| Old bark | | | | | | |
| CTV | 0 | 0.5 | ND | ND | 4 | 3.40 |
| Greening | 3 | 2.4 | ND | ND | 14 | 14.57 |

[x] Young bark: $P = 1.000$; mature: $P = 0.576$; old: $P = 1.000$ (Fisher's exact test, two-tailed).
[y] OB = observed or E = expected under the null hypothesis of independence.
[z] ND = no data.

tained measurable GeA; the 13 negative plants were asymptomatic.

Reliable detection of GeA can be affected by the different extraction and chromatography methods employed. Acid hydrolysis yields the blue fluorescing GeA (3), whereas water or ether extraction without hydrolysis yields the violet or violet blue fluorescence of the entire moiety, gentisoyl-β-D-glucose (21–23,25,27). Some studies have been complicated by the appearance of so-called interfering blue fluorescing markers in old bark (23) or in healthy plants (3,27,31). Other studies have been difficult to interpret, since markers have been reported as diagnostic because they were elevated in diseased compared to healthy plants (31). Paradoxically, some authors have reported GeA to be diagnostic for greening while simultaneously reporting a similar marker in other diseases, such as stubborn disease in California, which is caused by *Spiroplasma citri* (5,23), or Australian dieback (5), in which the etiologic agent is unknown but may be similar to that of greening disease (6). None of these prior studies has identified these so-called interfering or similar markers. We found that subsequent tests on California *Spiroplasma*-infected tissue were negative for GeA. More work should be done on the purple marker described in this study, and which migrates close to GeA, to determine its identity, its specificity for predicting greening disease, and its relationship to purple markers found in other studies.

By using chromatography (TLC and HPLC) and spectrophotometry, we have demonstrated that elevated GeA is also associated with diseases other than greening. However, on the basis of an extensive sample of diseased citrus, greening-affected plants have higher levels of GeA when compared to plants with similar symptom severity. However, ranges of GeA quantitative values overlapped (for example, with old tissue, CTV: 30.0–268.0 μg per gram of tissue; CTV–greening mix: 32.0–274.0 μg per gram of tissue; greening: 128.0–474.0 μg per gram of tissue; healthy: 10.0–38.0 μg per gram of tissue). Hence, we conclude that GeA is not specific for greening disease but can be used in conjunction with other criteria (such as symptoms and serology), under glasshouse quarantine conditions, in diagnosing citrus greening disease, especially if levels are above 300.0 μg/ml per gram of dried bark tissue. In the absence of an easily cultured pathogen and/or universally diagnostic citrus-greening probes (monoclonal antibodies or DNA probes), we must rely on this plant disease product in conjunction with other criteria in attempts to diagnose the disease in the field and glasshouse.


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