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Chemical Characterization of *Citrus sinensis* Grafted on *C. limonia* and the Effect of Some Isolated Compounds on the Growth of *Xylella fastidiosa*

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Citrus sinensis grafted on *C. limonia* produces a considerable number of compounds that are not common in both plants developed from germination of seeds. The chemical profile of scion and rootstock differ notably for absence in the form of flavonoids and coumarins containing C_5 prenyl groups attached to the carbon atoms of aromatic and heterocyclic systems or to oxygen. Only linear pyranocoumarins xanthyletin and xanthoxyletin were found in scion. This observation indicates that the prenylated compounds once biosynthesized in the roots could have been translocated to other organs. *Xylella fastidiosa* colonizes the xylem of plants causing diseases on several economically important crops such as citrus variegated chlorosis (CVC). A number of flavonoids, coumarins, alkaloids, dihydrocinnamic acid derivative, anacardic acid, triterpenes, and limonoids were tested for *in vitro* activity on the growth of *Xylella fastidiosa*. Azadirachtin A was the most active. Hesperidin, which occurs in great amounts in cells of the mesophyll of the affected leaves with CVC, showed a moderate activity suggesting that it can act as a good barrier for small-size colonies from *X. fastidiosa*.

KEYWORDS: *Citrus sinensis*; *C. limonia*; grafting; phytochemical studies; *Xylella fastidiosa*; citrus variegated chlorosis; CVC

INTRODUCTION

Xylella fastidiosa a Gram-negative bacterium is transmitted by xylem-feeding leafhoppers (Homoptera, Cicadellidae) and colonizes the xylem of plants causing diseases on several economically important crops such as citrus variegated chlorosis (CVC) in sweet orange, Pierce's disease (PD) of grapevine, and coffee leaf scorch (CLS) (*1*). The generally accepted cause of the symptoms induced by *X. fastidiosa* is the occurrence of vascular occlusion inside the vessel leading to water stress. It was previously demonstrated that the bacterium is able to grow as a Biofilm, which could be an important factor for pathogenicity (*1*).

CVC has been observed in all commercial sweet orange varieties, with transmission occurring mainly by xylem-feeding insects but also by graft propagation (*2, 3*). Symptoms include leaf chlorosis, stunting, canopy dieback, and fruits that are small and useless for the juicing industry (*4*). To reduce losses and prevent dissemination of the pathogen, pruning, insecticide application, and healthy nursery trees have been used, but effective control has not been reported. CVC has affected up to 36% of commercial sweet orange trees, causing great economic damage to the Brazilian citrus industry, which produces most of the orange juice in the international market (*4*).

Rangpur lime (*Citrus limonia* Osbeck) is the key rootstock for citriculture in Brazil, on which more than 80% of citrus trees are grafted. *C. limonia* trees, developed from the germination of seeds, may be grown in orchards in the presence of high disease and insect pressure, and they do not show foliar symptoms of the disease. However, it does not confer resistance to susceptible sweet orange varieties grafted on to it (*2, 3*). This information stimulated us to undertake phytochemical studies of the *Citrus sinensis* L. Osbeck grafted on *C. limonia*. Thus, roots, stems, and leaves of this combination were examined in order to determine if secondary metabolites present in the rootstock could be translocated into *C. sinensis*. The roots of *C. limonia* were also studied to gain a better understanding of

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whether the compounds obtained from the rootstock in graft occur also in this organ in plants developed from the germination of seeds.

The occurrence in great amounts of hesperidin in the cells of the mesophyll of the affected leaves with CVC and xanthyletin, a linear pyranocoumarin, in the roots of *C. limonia*, observed with this work, has stimulated us to test hesperidin and some roots coumarins on the growth of *X. fastidiosa*. The results could clarify the basis of its resistance to *X. fastidiosa*. However, as part of our efforts to find potential lead compounds as bactericides against this economically important phytopathogenic bacterium, coumarins, flavonoids, alkaloids, dihydrocinnamic acid derivative, anacardic acid, triterpenes, and limonoids isolated from Rutaceae, Meliaceae, and Anacardiaceae plants were also assayed against *X. fastidiosa*.

MATERIALS AND METHODS

Phytochemical Analysis. *General.* Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX 400 and ARX 200, with tetramethylsilane as internal standard. High performance liquid chromatography (HPLC) was performed on a model Shimadzu SCL-10A; detection (Shimadzu SCL-10A). Gas chromatography−mass spectrometry (GC-MS), low resolution, was performed on a HP-2576 instrument. Electrospray tandem mass spectrometry (ESI-MSMS) was performed on a Micromass Quattro LC instrument, equipped with a "Z-spray" ion source.

Plant Material. Nursery trees of *Citrus limonia* developed from the germination of seeds and *C. sinensis* grafted on *C. limonia*, both 10 months old, were produced in the greenhouse of the Centro APTA Citros Sylvio Moreira, Cordeirópolis, SP-Brazil.

Isolation of Compounds. Ground leaves (273 g), stem (625 g), rootstock stem (442 g), and roots (502 g) from two grafts of *C. sinensis* on *C. limonia* were extracted with hexane, then CH_2Cl_2, and finally with MeOH at room temperature 3 times. The concentrated hexane extract from the leaves (6.7 g) was subjected to column chromatography over silica gel (70−230 mesh) under vacuum. Elution with hexane, CH_2Cl_2, EtOAc, and MeOH afforded 8 fractions. Fraction 4 was flash rechromatographed on silica gel eluting with CH_2Cl_2/hexane 3:1, and then by preparative TLC (silica gel; CH_2Cl_2/hexane 7:2) yielding compound **7** (17 mg, 0.25%) and an amorphous solid characterized by low resolution GC-MS as a mixture of (8 mg, 0.12%) sitosterol, campesterol, and stigmasterol. Fraction 5 was rechromatographed as above using hexane/CH_2Cl_2/EtOAc (1:4:1) and CH_2Cl_2/EtOAc (4:1) for preparative TLC (silica gel) to give compound **5** (2 mg, 0.03%). Fraction 6 was rechromatographed as above (column chromatography: CH_2Cl_2/EtOAc 3:1; preparative TLC, CH_2Cl_2/EtOAc/acetone 6:1:0.5) affording compounds **4** (2 mg, 0.03%) and **3** (1 mg, 0.01%). Fraction 7 was flash rechromatographed on silica gel eluting with CH_2Cl_2/EtOAc/acetone (4:1:1) yielding **2** (2.5 mg, 0.04%) and **1** (7.2 mg, 0.11%). During the concentration of fraction 8, a crystalline material separated, which was collected and crystallized from MeOH to give **6** (128 mg, 1.9%). The dichloromethane extract from leaves (4.3 g) was subjected to column chromatography over silica gel (70−230 mesh) under vacuum. Elution with hexane, CH_2Cl_2, EtOAc, and MeOH afforded 10 fractions. Fraction 1−3 was flash rechromatographed on silica gel eluting with a hexane/CH_2Cl_2/acetone/MeOH gradient and then by preparative TLC (silica gel; CH_2Cl_2/acetone 7:2) yielding compounds **11** (4.8 mg, 0.11%) and **8** (23 mg, 0.53%). Fraction 6−7 was twice rechromatographed as above (CH_2Cl_2/acetone 5:1, then hexane/CH_2Cl_2/EtOAc 16:3:1 gradient) affording compound **12** (13 mg, 0.30%). Fraction 8 was purified by preparative TLC (silica gel; CH_2Cl_2/acetone 5:0.5) to give 2 new fractions. Both fractions were repurified by preparative TLC (silica gel) using CH_2Cl_2/acetone (5:0.5) to give compound **9** (23 mg, 0.53%), and CH_2Cl_2/acetone (1:1) to yield **10** (2 mg, 0.05%), respectively. During the concentration of the MeOH extract from leaves (6.3 g) a crystalline material separated, which was collected and crystallized from MeOH to give **6** (812 mg, 1.3%).

The concentrated hexane extract from the stems of scion *C. sinensis* (4.1 g) was subjected to column chromatography over silica gel (70−230 mesh) under vacuum. Elution with hexane, CH_2Cl_2, EtOAc, and MeOH afforded 10 fractions. Fraction 4 was flash rechromatographed on silica gel eluting with a hexane/CH_2Cl_2/EtOAc/MeOH gradient to give **13** (14 mg, 0.34%). Fraction 7−8 was twice rechromatographed as above (CH_2Cl_2/EtOAc 1:2, then acetone/CH_2Cl_2/EtOAc 1:2:0.5) affording compound **11** (1.6 mg, 0.04%). During the concentration of fraction 9−10, a crystalline material separated, which was collected and crystallized from MeOH to give **6** (97 mg, 2.4%). The dichloromethane extract from the stem of scion (4.3 g) was subjected to column chromatography over silica gel (70−230 mesh) under vacuum. Elution with hexane, CH_2Cl_2, EtOAc, and MeOH afforded 8 fractions. Fraction 5 was flash rechromatographed on silica gel eluting with a hexane/CH_2Cl_2/EtOAc gradient to give compound **13** (10 mg, 0.23%) and 3 new fractions. Fraction 5.3 was twice purified by preparative TLC (silica gel; CH_2Cl_2/EtOAc 1:1) to give **4** (4 mg, 0.09%). Fraction 7−8 yielded 3β-*O*-β-D-glucopyranosylsitosterol (112 mg, 2.6%), after crystallization in DMSO. During the concentration of the MeOH extract from the stem of scion (11.7 g), a crystalline material separated, which was collected and crystallized from MeOH to give **6** (560 mg, 4.8%).

The hexane (3 g) and dichloromethane (3.12 g) extracts from the rootstock stem were combined in only one on the basis of analytical TLC, which was subjected to column chromatography on gel permeation Sephadex LH 20 using CH_2Cl_2/MeOH (1:1) to afford 4 fractions. Fraction 2 was flash rechromatographed on silica gel eluting with a hexane/EtOAc gradient affording 4 new fractions. Fraction 2−1 was rechromatographed as described above affording new fractions. Fraction 2−1−2 was subjected to column chromatography on gel permeation Sephadex LH 20 using CH_2Cl_2/MeOH (1:1) to afford a new fraction, which was purified by preparative TLC (silica gel; hexane/EtOAc 3:2) yielding **19** (1.4 mg, 0.02%) and **20** (1.2 mg, 0.02%). Fraction 2−2 was flash rechromatographed on silica gel eluting with hexane/EtOAc (3:1) and then by preparative TLC (silica gel; hexane/EtOAc 3:1) yielding compound **15** (4.5 mg, 0.07%). Fraction 2−3 was flash rechromatographed on silica gel eluting with hexane/EtOAc gradient affording 2 new fractions. Fraction 2−3−1 was purified by preparative TLC (silica gel; hexane/EtOAc 3:2) yielding **7** (28.7 mg, 0.47%) and **14** (7 mg, 0.11%). Fraction 2−3−2 was subjected to column chromatography on gel permeation Sephadex LH 20 using CH_2Cl_2/MeOH (1:1) to afford **21** (2.6 mg, 0.04%) and a new fraction, which was purified by preparative TLC (silica gel; CH_2Cl_2) yielding **16** (5 mg, 0.08%). Fraction 2−4 was purified by preparative TLC (silica gel; CH_2Cl_2) yielding **17** (4.5 mg, 0.07%). Fraction 3 was subjected to column chromatography on gel permeation Sephadex LH 20 using CH_2Cl_2/MeOH (1:1) to give new fractions. Fraction 3−2 was flash rechromatographed on silica gel eluting with hexane/EtOAc gradient affording a new fraction, which was purified by HPLC separation using a 250 mm × 4.6 mm i.d., 5 μm Luna ODS C-18 column (Phenomenex, São Paulo, SP, Brazil), eluted with MeOH-H_2O (3:1) at a flow rate of 1 mL/min and UV/vis detection at 280 nm to yield compounds **18** (2.3 mg, 0.04%) and **21** (2 mg, 0.03%).

The hexane (4.3 g) and dichloromethane (14 g) extracts from the roots (502 g) of the graft were combined in only one on the basis of analytical TLC, and this was subjected to column chromatography over silica gel (70−230 mesh) under vacuum. Elution with hexane, CH_2Cl_2, EtOAc, and MeOH afforded 5 fractions. Fraction 2 yielded compound **7** (110 mg, 0.60%), after crystallization in CH_2Cl_2. Fraction 4 was rechromatographed as described above affording 6 new fractions. Fraction 4−2 was rechromatographed as above affording **11** (10 mg, 0.05%), **15** (5 mg, 0.03%), **16** (3 mg, 0.02%), **22** (3 mg, 0.02%), **23** (2 mg, 0.01%) and **24** (1.5 mg, 0.01%). Fraction 4−3 was rechromatographed as described above affording **26** (2.5 mg, 0.01%) and a new fraction, which was purified by preparative TLC (silica gel; hexane/EtOAc 3:2) yielding **25** (3 mg, 0.02%). Fraction 5 was rechromatographed as described above affording 2 new fractions. Both fractions were repurified by preparative TLC (silica gel) using CH_2Cl_2/acetone (5:0.5) for fraction (Fr) 5−1 to give compounds **19** (23 mg, 0.13%) and **21** (5 mg, 0.03%) and CH_2Cl_2/EtOAc (3:1) for Fr5−2 to yield **12** (8 mg, 0.04%) and **27** (3 mg, 0.02%).

The roots of *C. limonia* developed from the germination of seeds (715 g) were extracted with hexane, then CH_2Cl_2, and finally with

MeOH at room temperature 3 times. During the concentration of the hexane extract (8 g) a crystalline material (68 mg) separated, which was collected and flash chromatographed on silica gel eluting with hexane/EtOAc gradient to give compounds **7** (157 mg, 1.9%), **14** (4 mg, 0.04%), and an amorphous solid characterized by low resolution GC-MS as a mixture of (3.5 mg, 0.04%) sitosterol, campesterol, and stigmasterol. The residual hexane (7.9 g) and dichloromethane (5.7 g) extracts were combined in only one on the basis of analytical TLC, and this was subjected to column chromatography on gel permeation Sephadex LH 20 using CH_2Cl_2/MeOH (1:1) to give 4 fractions. Fraction 2 was flash rechromatographed on silica gel eluting with a hexane/acetone/MeOH gradient affording 2 new fractions. Fraction 2−1 was purified by HPLC using a 250 mm × 6 mm i.d., 10 μm, Phenyl Hexyl column (Phenomenex, São Paulo, SP, Brazil), eluted with MeOH/H_2O (2:1) at a flow rate of 2 mL/min and UV/vis detection at 280 nm to yield compound **18** (2 mg, 0.01%). Fraction 3 was flash rechromatographed on silica gel eluting with a hexane/CH_2Cl_2/MeOH gradient affording compounds **20** (8.1 mg, 0.06%), **7** (35 mg, 0.26%), **14** (30 mg, 0.22%), **31** (3.6 mg, 0.03%), **32** (4.3 mg, 0.03%), and a new fraction. Fraction 3−1 was rechromatographed as described above affording **28** (3.7 mg, 0.03%). Fraction 4 was subjected to column chromatography on gel permeation Sephadex LH 20 using CH_2Cl_2/MeOH (1:1) to give 2 fractions. Fraction 4−1 was flash rechromatographed on silica gel eluting with a hexane/EtOAc/MeOH gradient affording **33** (3.3 mg, 0.02%). Fraction 4−2 was purified by HPLC separation using a 250 mm × 6 mm i.d., 10 μm, Phenyl Hexyl column (Phenomenex, São Paulo, SP, Brazil), eluted with MeOH/H_2O (2:1) at a flow rate of 2 mL/min and UV/vis detection at 254 nm to yield compounds **25** (5 mg, 0.04%) and **11** (6 mg, 0.04%).

The MeOH extract (13.7 g) was subjected to column chromatography on gel permeation Sephadex LH 20 using MeOH, CH_2Cl_2/MeOH (1:1), and CH_2Cl_2 to give 4 fractions. Fraction 1 was flash rechromatographed on silica gel eluting with a hexane/EtOAc/MeOH gradient affording **29** (30 mg, 0.22%). Fraction 2 was purified by preparative TLC (silica gel; hexane/EtOAc 9:1) yielding **7** (20 mg, 0.15%). Fraction 3 was twice flash rechromatographed on silica gel (hexane/EtOAc/MeOH gradient) yielding **30** (2 mg, 0.02%), **32** (2 mg, 0.02%), and **34** (2 mg, 0.02%).

Biological Assays. *Growth Conditions.* To prepare fresh isolated bacteria, *Citrus sinensis* plants were previously inoculated with the 9a5c strain of *X. fastidiosa* and when CVC symptoms were visible; petioles and stems were collected and aseptically ground in PBS (phosphate-buffered saline). The suspension was distributed on plates containing liquid periwinkle wilt medium (PW) (*5*).

Liquid PW Medium. Into a flask containing 500 mL of distilled H_2O was added 4.0 g of phytone peptone (Sigma, São Paulo, SP, Brazil); 1.0 g of trypticase peptone (Sigma, São Paulo, SP, Brazil); 1.2 g of K_2HPO_4; 1.0 g of KH_2PO_4; and 0.4 g of $MgSO_4 \cdot H_2O$; by mechanic agitation was added 10 mL of hemin chloride 0.1% [0.1 g of hemin Cl (Sigma, São Paulo, SP, Brazil), 0.2 g NaOH, and 100 mL H_2O]; and 10 mL of phenol red 0.2% (0.2 g of phenol red, 10 drops of NaOH 20%, and 100 mL H_2O); and distilled water was added to complete 800 mL. Then this solution was transferred into 10 Erlenmeyer flasks, in a total volume of 80 mL in each, and they were autoclaved at 120 °C for 20 min. After autoclaving, 10 mL of bovine serum albumin (BSA) 10% [6 g of BSA fraction V (Sigma, São Paulo, SP, Brazil), 100 mL of autoclaved water, and filtered through a 0.22-μm Millipore membrane] and glutamine 4% [4 g of glutamine (Sigma, São Paulo, SP, Brazil), 100 mL of autoclaved water, and filtered through a 0.22-μm Millipore membrane] were added in each Erlenmeyer flask (*5*).

Solid PW Medium. For the solid medium, 12 g of bacteriological agar was added before adding distilled water to complete 800 mL.

Inoculum Preparation. Flasks containing 30 mL of liquid PW medium were inoculated with 1.0 mL of freshly prepared suspensions of *X. fastidiosa* 9a5c culture grown in solid medium. After 5 days (exponential phase; OD_{600nm} = absorbance 0.080) or 10 days (stationary phase; OD_{600nm} = absorbance 0.281) of growth at 28 °C in a rotary shaker at 140 rpm, these cultures were used for inoculation of each medium used in the growth assays. A mix of the Biofilm (with growth attached to the glass at the medium−air interface) and planktonic (not attached) cells were used in minimal inhibitory concentration experiments (MIC).

Antibacterial Activity. Stock solutions of the compounds were prepared at a concentration of 5 mg/mL in DMSO (Sigma, São Paulo, SP, Brazil)/H_2O (1:1) and diluted to appropriate concentration prior to assays. In Eppendorf tubes containing 100 μL of bacterial culture in liquid PW medium (exponential phase or stationary phase) were added 0.5, 0.6, 1.0, or 2.0 mg/mL of each tested compound and kept in biological oxygen demand for 24 h at 28 °C, and 100 μL was then transferred to a 96-well microplate containing solid PW medium. The microplates were incubated for 10 days in biological oxygen demand at 28 °C. Growth was monitored by the number of bacterial colonies and by the change in color from yellow to red because phenol red produced a red−pink color (alkalized) after cell growth resulting in the consumption of glucose. The MIC was defined as the lowest test compound concentration, which prevented the visible growth of bacteria. This procedure was carried out in triplicate, and in each case, a blank experiment was performed with DMSO/H_2O (1:1) alone (100 μL of bacterial culture, 33.5 μL of DMSO and 33.5 μL of H_2O) in the well and was used as a positive control. The growth of *X. fastidiosa* 9a5c was not significantly affected by 40% DMSO/H_2O (1:1) alone. The positive control well was prepared without the test compound and only with bacterial culture.

Purity Standard. All experiments were carried out with cells verified as *X. fastidiosa* by PCR with specific primers (*6, 7*).

RESULTS AND DISCUSSION

The Centro APTA Citros Sylvio Moreira in Brazil has a citrus sanitation program that cleans citrus material from CVC and other diseases by the use of shoot-tip grafting. There are a limited number of accredited institutions delivering certified disease-free budwoods *Citrus*. The Centro APTA has produced certified budwoods *Citrus* under favorable phytosanitary conditions in a screen house. This work was undertaken on *Citrus sinensis* grafted on *C. limonia* produced at this Centre. Roots, stems, and leaves of this graft were studied separately in order to compare their chemical profile (**Figures 1–3**). The structures of isolated compounds were identified on the basis of spectral data, particularly ^1H and ^{13}C NMR, HSQC, HMBC, and ESI-MSMS, and by comparison of the ^{13}C NMR spectrum with those from the literature.

Compounds Isolated from Leaves of *C. sinensis* Grafted on *C. limonia*. The hexane extract from leaves after successive chromatographic separations afforded the flavonoids tetra-*O*-methylscutellarein (4′,5,6,7-tetramethoxyflavone) (**1**), tangeretin (4′,5,6,7,8-pentamethoxyflavone) (**2**) sinensetin (3′,4′,5,6,7-pentamethoxyflavone) (**3**), nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone) (**4**), and 3,3′,4′,5,6,7,8-heptamethoxyflavonol (**5**); flavanone hesperidin (**6**); the linear pyranocoumarin xanthyletin (**7**); and the steroids sitosterol, stigmasterol, and campesterol. The dichloromethane extract from leaves was purified by repeated column chromatography on silica gel and preparative TLC to give the flavonoids 5-hydroxy-3′,4′,6,7-tetramethoxyflavone (**8**), 5-demethoxynobiletin (3′,4′,6,7,8-pentamethoxyflavone) (**9**), 5-hydroxy-3′,4′,7,8-tetramethoxyflavone (**10**), coumarin xanthoxyletin (**11**), and limonoid limonin (**12**). The methanol extract from leaves afforded only flavanone hesperidin (**6**).

Flavonoids **1**−**4** were previously isolated from leaves (*8, 9*), **1**−**5** from peel (*10*), and hesperidin (**6**) from leaves, stem, and root (*11*) of sweet orange (*C. sinensis*). Flavone **8** has been described from sweet orange peel in the inaccessible patent literature (*12*), **9** from seedless *C. depressa* Hayata (*13*), and **10** from bergamot oil from *C. aurantium* subsp. *Bergamia* (*14*). This appears to be the first record of **5** and **8** from leaves of *C. sinensis* and **9** and **10** from this species.

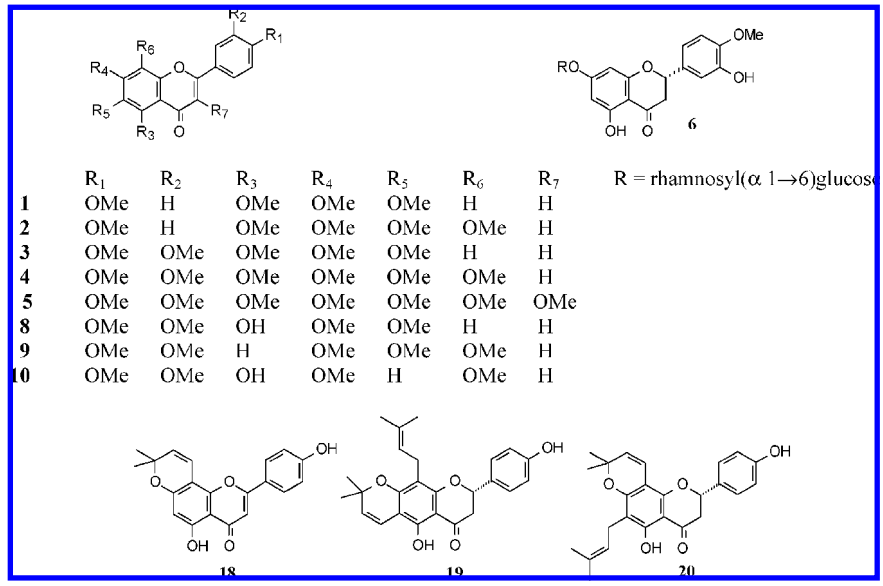

Figure 1. Structure of flavonoids isolated.

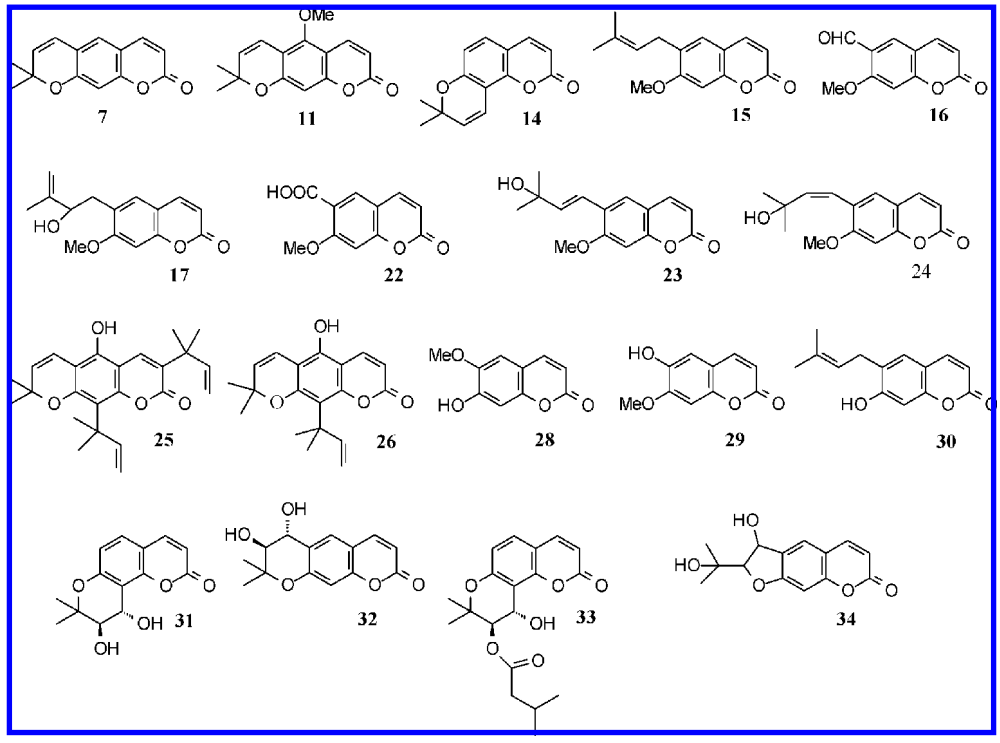

Figure 2. Structure of coumarins isolated.

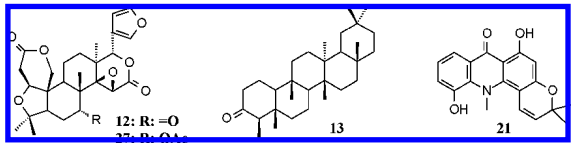

Figure 3. Structure of limonoids, triterpene, and alkaloid isolated.

Coumarins xanthyletin (**7**) and xanthoxyletin (**11**) were previously isolated from the root of *C. sinensis* but not so far in the leaves of this species (*15*).

The limonoid limonin has been found in fruits (*16*), roots (*17*), and shoots (*18*) of *C. sinensis*. In the later reference, the authors observed that limonin concentration in shoots decreased during the growing period in the cultivars. However, in this study limonin was found in the leaves of 10 month old *C. sinensis* plants.

Compounds Isolated from the Stems of *C. sinensis* Grafted on *C. limonia.* The hexane extract from *C. sinensis* stems of scion gave the triterpene friedelin (**13**), coumarin xanthoxyletin (**11**), and flavanone hesperedin (**6**). The dichloromethane extract from stems of scion afforded friedelin (**13**), flavone nobiletin (**4**), and 3β-*O*-β-D-glucopyranosylsitosterol. The methanol extract from scion stems yielded only flavanone hesperidin (**6**).

Nobiletin (**4**) was previously obtained from the dried bark of *C. sinensis* (*19*); however, here the stem and bark were studied together. Xanthoxyletin (**11**) is reported here for the first time from the stem of *C. sinensis*. The pentacyclic triterpene friedelin (**13**) was previously isolated from the leaves (*20*) and peel (*21*) of *C. sinensis*; thus, it is reported here for the first time from the stem.

Compounds Isolated from the Stem of the Rootstock of *C. sinensis* Grafted on *C. limonia.* When the scion and

rootstock are joined, they form a calloused area. It was removed, and only the stem of the *C. limonia* (rootstock) was studied. The hexane and dichloromethane extracts from this stem were combined and after successive chromatographic separations afforded the coumarins xanthyletin (**7**), seselin (**14**), suberosin (**15**), crenulatin (**16**), and tamarin (**17**); the flavonoids limonianin (**18**), lupinifolin (**19**), and erythrisenegalone (**20**); and the acridone alkaloid 5-hydroxynoracronycine (atalaphyllidine, **21**).

Plants of *Citrus sinensis* and *C. limonia* have been widely investigated, but to date, there are no records of studies of their graft working separately with scion and rootstock. The unique investigation of the stem of *C. limonia* revealed the presence of coumarins imperatorin, xanthotoxin, bergapten, isopimpinellin, limettin, scopoletin, umbelliferone, xanthotoxol, and aesculetin, and the steroids stigmasterol and sitosterol (*22*). Thus, all compounds obtained here from the stem of the rootstock of *C. limonia* have not been found in the stem of the plant developed from the germination of seeds. This observation suggests that these constituents could have been translocated from the roots where they have been isolated (*23*). The literature did not mention tamarin **17** from *C. sinensis* and *C. limonia*, but from the roots of hybrid *C. paradise* Macf. X *C. tangerina* Hort. Ex Tanaka (*24*). Thus, the finding of **17** from the rootstock stem in this study suggested that it might have been biosynthesized from suberosin **15** in this part of the graft since it was not found in the roots. The results suggest that the rootstock stem does not biosynthesize metabolites *de novo*; however, it is clear that much further work is need to establish whether the metabolic changes are associated with the graft.

Compounds Isolated from the Roots of the Rootstock of *C. sinensis* Grafted on *C. limonia*. The hexane and dichloromethane extracts from the roots of *C. limonia* rootstock were combined and purified by repeated column chromatography on silica gel and preparative TLC to give the coumarins xanthyletin (**7**), xanthoxyletin (**11**), suberosin (**15**), crenulatin (**16**), buntasin (**22**), (*E*)-suberenol (**23**), (*Z*)-suberenol (**24**), clausarin (**25**), and nordentatin (**26**); the flavanone lupinifolin (**19**), the limonoids limonin (**12**), and limonyl acetate (**27**); and the acridone alkaloid 5-hydroxynoracronycine (atalaphyllidine, **21**).

Compounds **12**, **22**−**24**, and **27** were not previously found from the roots of the plant *C. limonia* developed from the germination of seeds. Limonyl acetate (**27**) has been found in fruits of the *Citrus*−*Poncirus* hybrid (*25*). The coumarins buntasin (**22**) and (*E*)-suberenol (**23**) are cited from the roots of hybrid *C. paradise* Macf. X *C. tangerina* Hort. Ex Tanaka (*24*) and (*Z*)-suberenol (**24**) from *C. funadoko* (*26*). The coumarins xanthyletin (**7**), xanthoxyletin (**11**), suberosin (**15**), crenulatin (**16**), and nordentatin (**26**) were previously isolated from the roots of *C. limonia* (*23*).

Compounds Isolated from the Roots of *C. limonia* Developed from the Germination of Seeds. The roots of *C. limonia* were also studied to gain a better understanding of whether the compounds obtained from the rootstock in grafts also occur in this organ of trees developed from the germination of seeds. During the concentration of the hexane extract, a crystalline material separated, which was collected and chromatographed on silica gel to afford xanthyletin (**7**), seselin (**14**), and the steroids sitosterol, stigmasterol, and campesterol. The hexane and dichloromethane extracts were combined and purified by repeated column chromatography yielding again xanthyletin (**7**), seselin (**14**), and xanthoxyletin (**11**), clausarin (**25**), scopoletin (**28**), *trans*-kellactone (**31**), *trans*-decursidinol (**32**), and junosmarin (**33**); and the flavonoids limonianin (**18**) and erythrisenegalone (**20**). The methanol extract afforded xanthyletin (**7**), isoscopoletin (**29**), 7-demethylsuberosin (**30**), *trans*-decursidinol (**32**), and xanthoarnol (**34**).

Flavona **18** and the coumarin 7-demethylsuberosin (**30**) were previously isolated from the roots of *C. limonia* (*23*), while seselin (**14**), clausarin (**25**), scopoletin (**28**), isoscopoletin (**29**), *trans*-kellactone (**31**), *trans*-decursidinol (**32**), junosmarin (**33**), and xanthoarnol (**34**), and flavanone erythrisenegalone (**20**) are reported here for the first time from this organ. The coumarins **14**, **25**, **28**, **29**, and **31**−**33** have been found from the roots of the hybrid *C. paradise* Macf. X *C. tangerina* Hort. Ex Tanaka (*24*), and flavanone erythrisenegalone (**20**) from several hybrid seedlings resulting from a cross of *C. tamurana* Hort. Ex. Takahashi and *C. kinokuni* Hort Ex. Tanaka (*27*). This appears to be the first record of xanthoarnol (**34**) in *Citrus*, which occurs in rutaceous genus *Zanthoxylum arnottianum* (*28*).

Comparison between the Chemical Profiles of Roots, Stems, and Leaves of *C. sinensis* Grafted on *C. limonia*. Roots, stems, and leaves of *C. sinensis* grafted on *C. limonia* produce a considerable number of compounds that are not common in both plants developed from the germination of seeds. These differences in the chemical profile could be attributed to the influence of the graft on the biosyntheses of these compounds in all organs. However, different investigators seldom publish the developmental stage of the plant, making the above comparisons invalid. Thus, it is premature to draw any conclusions about the role of the graft on the absence or presence of new compounds in roots, stems, and leaves of *C sinensis* grafted on *C. limonia*, until plants developed from the germination of seeds with the same developmental stage can be evaluated. However, little is known about the chemistry of grafts to clarify the translocation of metabolites from the rootstock to the scion or the reasons why some compounds are only present in grafted plant. Our own investigations of the stem of *Cedrela odorata* grafted on *Toona ciliata* (Meliaceae) revealed the presence of some limonoids, which are common in the former developed from the germination of seeds and many others, which were not previously found from that (*29*).

The results above, which suggest that the rootstock stem does not biosynthesize metabolites *de novo*, remain obscure. The chemical profile of the scion and rootstock differ notably in absence of flavonoids and coumarins containing C_5 prenyl groups attached to the carbon atoms of aromatic and heterocyclic systems or to oxygen, in the former. Only xanthyletin (**7**) and xanthoxyletin (**11**) were found in scion. This observation indicates that the prenylated compounds once biosynthesized in the roots could have been translocated to other organs. Xanthyletin (**7**) and xanthoxyletin (**11**) appear to be translocated to scion stems and leaves, while other prenylated coumarins and flavonoids only to rootstock stem. Further experiments on the enzymatic formation of xanthyletin (**7**) and seselin (**14**) from dimethylallyl diphosphate using cell-free extract from leaves, roots, and stems in order to determine if secondary metabolites present in the roots of rootstock could be translocated into rootstock stem and to graft *C. sinensis* are in progress. Preliminary results showed that in the roots of *C sinensis* grafted on *C. limonia* the biosynthesis of xanthyletin (**7**) and seselin (**14**) is time-regulated and that prenyltransferase activity depends upon circadian variation. The cell-free extracts from scion leaves and stems were also similarly incubated with dimethylallyl diphosphate but did not give xanthyletin (**7**) and seselin (**14**), suggesting that the enzyme is not present in these organs.

Effect of Some Sapindale Compounds on the Growth of *Xylella fastidiosa*. A number of flavonoids, coumarins, and limonoids reported here, and alkaloids, dihydrocinnamic acid

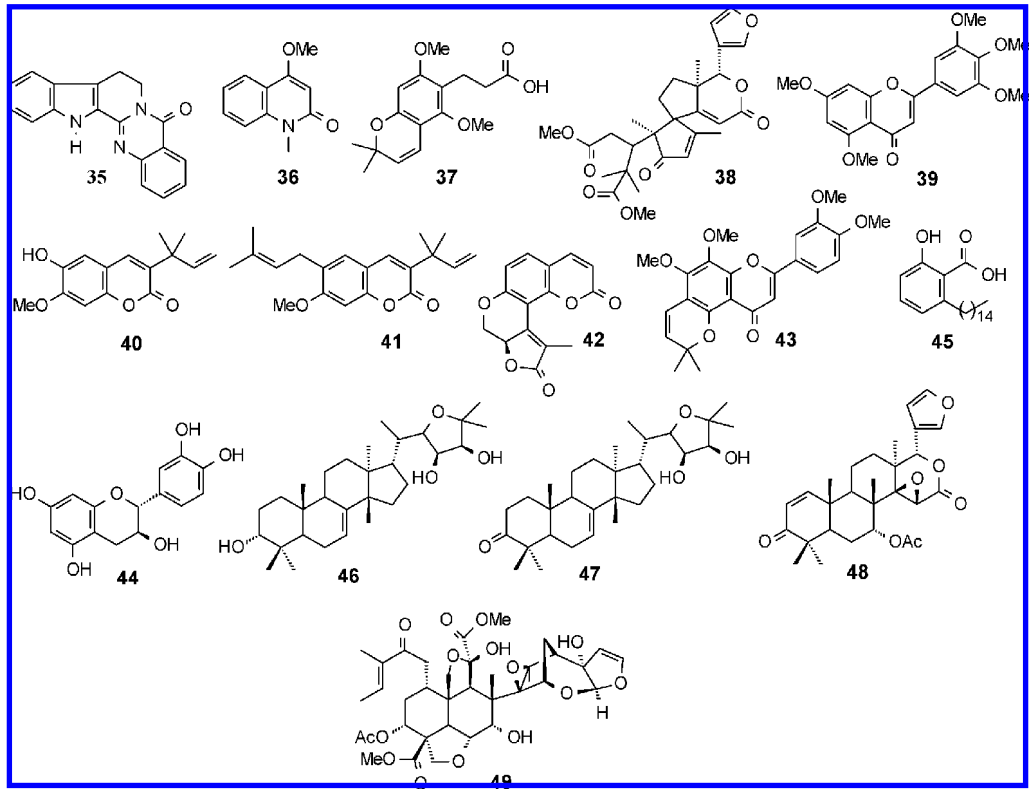

Figure 4. Compounds tested for *in vitro* activity on the growth of *X. fastidiosa*, which were not obtained in this work.

derivative, anacardic acid, triterpenes, and limonoids (**Figure 4**) isolated from other Rutaceae, Meliaceae, and Anacardiaceae (Sapindales) were tested for *in vitro* activity on the growth of *X. fastidiosa*. The tested compounds rutaecarpin (**35**), *N*-methyl-4-methoxyquinolin-2-one (**36**), 5,7-dimethoxy-2,2-dimethyl-2*H*-1-benzopyran-6-propanoic acid (**37**), and guyanin (**38**) were isolated from *Hortia brasiliana* and *H. oreadica*; 3′,4′,5′,5,7-pentamethoxyflavanone (**39**), 3′,4′,7,8-tetramethoxy-5,6-(2″,2″-dimethylpyrano)-flavone (**43**) from *Neoraputia magnifica*; 3-(1,1-dimethylallyl)-isoscopoletin (**40**), raunin (**42**), and gravelliferone methyl ether (**41**) from *Rauia resinosa* (Rutaceae); odoratol (**46**), odoratone (**47**), gedunin (**48**), and catechin (**44**) from *Cedrela odorata* grafted on *Toona ciliata* (*29*); azadirachtin A (**49**) from *Azadirachta indica* (Meliaceae); and anacardic acid (**45**) from Anacardiaceae. All compounds above were isolated in our own investigations of plants from Sapindales.

The 9a5c strain was obtained from the petioles and stems of CVC-affected sweet orange (*Citrus sinensis*) plants (4 months) maintained in a protected greenhouse at Centro APTA Citros Sylvio Moreira, Instituto Agronômico, Cordeirópolis, SP, Brazil. The 9a5c strains evaluated were PCR positive for specific *X. fastidiosa* primers.

The fact that stationary phase bacteria are more resistant to antibiotics than logarithmically growing cells has been known for some time (*30*). Thus, preliminary assays were performed on stationary phase cells of *X. fastidiosa* and with compounds obtained in great amount from *Hortia brasiliana* and *H. oreadica* (Rutaceae). Concentration of rutaecarpin (**35**) at 1.00 mg/mL or higher effectively inhibited *X. fastidiosa* cell proliferation, while *N*-methyl-4-methoxyquinolin-2-one (**36**), 5,7-dimethoxy-2,2-dimethyl-2*H*-1-benzopyran-6-propanoic acid (**37**), and the limonoid guyanin (**38**) showed antibacterial activity only at 1.50 mg/mL. Then, the MICs were determined in dilutions from 1.60 to 0.70 mg/mL on stationary phase. The MICs were, in order of their decreasing activity, 2.9×10^3 μM for **38** and 4.2×10^3 μM for **35**, 4.8×10^3 μM for **37**, and 7.9×10^3 μM **36** (**Table 1**).

Table 1. Inhibitory Effect of Compounds **7**, **12**, **14**, and **35**−**39** on *X. fastidiosa* 9a5c Strain in Stationary Phase and Compounds **6** and **40**−**49** on Exponential Phase

compounds	stationary phase MIC mg/mL (10^3 μM)	exponential phase MIC mg/mL (10^3 μM)
7	1.00 (4.4)	
12	1.60 (3.4)	
14	1.40 (6.1)	
35	1.00 (4.2)	
36	1.50 (7.9)	
37	1.40 (4.8)	
38	1.50 (2.9)	
39	1.40 (3.7)	
6		2.00 (3.3)
40		>2.00 (7.7)
41		>2.00 (6.4)
42		1.50 (5.5)
43		1.30 (3.1)
44		1.00 (3.4)
45		1.70 (4.9)
46		1.80 (3.8)
47		1.10 (2.3)
48		1.30 (2.7)
49		>1.60 (2.2)

The assays with *X. fastidiosa* after 5 days (exponential phase) and 10 days (stationary phase) of growth have been developed in order to compare *in vitro* activity of some test compounds on both phases. Only xanthyletin (**7**), seselin (**14**), limonin (**12**), and 3′,4′,5′,5,7-pentamethoxyflavanone (**39**) were assayed on exponential and stationary phases, and the growth inhibition was very similar for both. This study demonstrated that *X. fastidiosa* was affected by tested compounds **7**, **14**, **12**, and **39** at concentrations ranging from 1.00 to 2.00 mg/mL. In the case of coumarins **7** and **14**, logarithmically growing cells (2.00 mg/mL, complete growth inhibition) were found to be more resistant

than stationary phase cells (1.00 mg/mL, complete growth inhibition). One possible explanation for this resistance is that logarithmic phase bacteria have a more efficient system for the detoxification of foreign compounds within their cells. However, a more systematic analysis is necessary to clarify this resistance. In a recent study, Teitzel and Parsek (*31*) examined the relative effects of heavy metal copper and lead on stationary and logarithmic phase cells of *Pseudomanas aeruginosa* and also found that the latter were more resistant to metals.

Moreover, the exponential phase may be helpful in the estimation of MICs, and it can reduce the time needed for bacterial growth; thus, the other compounds were assayed with *X. fastidiosa* after 5 days of growth. The results for exponential phase are summarized in **Table 1**.

Triterpene derivatives were the most active, odoratone (**47**), gedunin (**48**), and azadiractin A (**49**) showed MICs of 2.3×10^3 μM, 2.7×10^3 μM, and $>2.2 \times 10^3$ μM, respectively. The activities of limonoid limonin (**12**, 3.4×10^3 μM) and triterpene odoratol (**46**, 3.8×10^3 μM) were comparable to those of flavonoids 3′,4′,7,8-tetramethoxy-5,6-(2″,2″-dimethylpyrano)-flavone (**43**, 3.1×10^3 μM), catechin (**44**, 3.4×10^3 μM), and 3′,4′,5′,5,7-pentamethoxyflavanone (**39**, 3.7×10^3 μM).

The cells of the mesophyll of the affected leaves with CVC also present a reduced number of chloroplasts associated with a larger concentration of hesperidin (**6**) crystals (*32*). Then, hesperidin (**6**), 7-rutinosyl-4′-methoxy-3′,5-dihydroxyflavanone, was assayed; however, it showed a moderate activity with MIC at 3.3×10^3 μM. This result suggests that hesperidin (**6**) acts as a good barrier for the growth of *X. fastidiosa* in small-size colonies.

The coumarins xanthyletin (**7**, 4.4×10^3 μM), raunin (**42**, 5.5×10^3 μM), seselin (**14**, 6.1×10^3 μM), gravelliferone methyl ether (**41**, $> 6.4 \times 10^3$ μM), 3-(1,1-dimethylallyl)-isoscopoletin (**40**, $> 7.7 \times 10^3$ μM), and anacardic acid (**45**, 4.9×10^3 μM) showed no appreciable activity against *X. fastidiosa* (**Table 1**).

Comparison of the Antimicrobial Activities of the Sapindale Compounds with Those from the Literature. The biological assay results indicated that the compounds tested *in vitro* against the *X. fastidiosa* 9a5c strain were active at concentrations in the 1.00 mg/mL range, while the antibiotics ampicillin (10 μg/mL), kanamycin (2 μg/mL), neomycin (5 μg/mL), penicillin (50 μg/mL), streptomycin (10 μg/mL), and tetracycline (1 μg/mL) were active in the concentrations lower than 50 μg/mL under the agar disk diffusion and MIC methods (*33*). However, this work did not evaluate the bacterium in Biofilm or in higher density growth; moreover, antibiotics are not utilized in agriculture to treat plants with diseases because of the environmental and economic impact. A second class of organic compounds evaluated for their antimicrobial activity against *X. fastidiosa* (PD00-2 isolated from grapevine with Pierce's disease) was lytic peptides. Peptides cecropin A and B totally inhibited the cell growth of *X. fastidiosa* at a concentration of 0.5 μM (*34*). However, other studies determined cecropin B activity against different sized aggregation of *X. fastidiosa* and demonstrated that this peptide at 10 μM was not efficient to kill large-size colonies from this bacterium. The authors suggested that aggregation of *X. fastidiosa* cells may serve as a mechanism of protection against cecropin B (*35*). In the present work, the tested compounds showed weak activity ranging from 2.2×10^3 μM to 7.9×10^3 μM; however, we utilized cells in higher density and in aggregated form (Biofilm cells), which could reflect what occurs in plants with CVC, and the aggregation of bacteria may serve as a mechanism of protection against bactericides. Thus, if it is assumed that the MICs obtained in this condition suggest continued investigation, clearly the limonoid azadirachtin A (**49**, MIC 2.2×10^3 μM) and the flavanone hesperidin (**6**, 3.3×10^3 μM) need further investigation as potential bactericidal agents. Hesperidin (**6**) is most probably involved as a natural defense or in resistance mechanisms against *X. fastidiosa* in sweet orange varieties (*32*). Thus, it can be efficient to kill small-size colonies from *X. fastidiosa* and could be used as a preventive of disease. It has long been recognized that orange peel represents a promising source of hesperidin. A million metric tons of peel residues are generated as result of fruit processing, and thus, an extract of this residue could be considered for practical use to prevent and control CVC.

Azadirachtin A (**49**) occurs only in the Neem tree *Azadirachta indica* A. Juss. Oil from the seeds of *A. indica* contain a mixture of seven isomeric compounds labeled azadirachtin-A to azadirachtin-G, with the former being present in the highest quantity. This cocktail of compounds significantly reduces the chances of tolerance or resistance developing in any of the affected organisms. In addition, they have significant effect on pests without harming beneficial organisms. Toxicology studies have indicated them to be quite safe to mammals (*36*). Emulsified neem oil (5%) was bactericidal against green pepper bacterial leaf spot incited by economically important phytopathogenic *Xanthomonas campestris* pv *vesicatoria* (*36*). These data stimulated an investigation of neem oil as a *X. fastidiosa* inhibitor. Experiments on the dose response of *X. fastidiosa* Biofilm to hesperidin and neem oil *in vitro* and *in plant* are in progress.

ABBREVIATIONS USED

CVC, citrus variegated chlorosis; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond correlation; GC-MS, gas chromatography−mass spectrometry; ESI-MSMS, electrospray tandem mass spectrometry; HPLC, high performance liquid chromatography; UV, ultraviolet; EtOAc, ethyl acetate; MeOH, methanol; PW, periwinkle wilt; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

Supporting Information Available: References for the compounds tested for *in vitro* activity on the growth of *X. fastidiosa*, which were not obtained from *Citrus sinensis* grafted on *C. limonia*, and the ^{13}C NMR data of isolated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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