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High Chlorogenic and Neochlorogenic Acid Levels in Immature Peaches Reduce *Monilinia laxa* Infection by Interfering with Fungal Melanin Biosynthesis

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ABSTRACT: Chlorogenic acid (CGA) and its isomer, neochlorogenic acid (NCGA), were found to be the major phenolic compounds in the flesh and peel of three peach cultivars. Their concentrations are especially high in immature fruits (CGA, 151−548 mg/kg; NCGA, 85−380 mg/kg), whose resistance to the brown rot fungus, *Monilinia laxa*, is very high. The concentrations of these two phenolic compounds decline in maturing fruits (CGA, 77−181 mg/kg; NCGA, 30−82 mg/kg), and this decline is associated with a concomitant increase in susceptibility to brown rot infection. Other phenolic compounds found in the same HPLC chromatograms at 340 nm from each peach extract at varying sampling dates in each of the three peach cultivars were not correlated with the incidence of brown rot and appeared only in some cultivars. The incidence of brown rot for each cultivar at each sampling date was significantly negatively correlated with the NCGA ($r > -0.85$) and CGA ($r > -0.90$) contents. At concentrations that are similar to those in peach fruit, CGA does not inhibit spore germination or mycelial growth of *M. laxa* in culture but markedly inhibits the production of melanin-like pigments in the mycelia of *M. laxa* in culture (42% melanin reduction). Accordingly, we propose that the high concentrations of CGA and NGA in immature fruits might contribute to their reduced susceptibility or increased resistance to brown rot infection by interfering with fungal melanin production.

KEYWORDS: Chlorogenic acid, neochlorogenic acid, brown rot infection, immature fruit, susceptibility, melanins

INTRODUCTION

Brown rot is a fungal disease of peaches and nectarines, which is mainly caused by *Monilinia laxa* (Aderh. Et Ruhl.) honey in Spain. This disease causes economically important losses, which can be as high as 80% in years when the climatic conditions are favorable for the development of the disease, especially in orchards with late-ripening varieties.[1] The developmental stage of the fruit determines its susceptibility to brown rot infection.[1,2] In general, immature peach fruits are less susceptible to *M. laxa* infection than mature fruits,[1] although fruits at the pit-hardening stage can also be susceptible.[2,3]

Chemical and physical factors are thought to account for the differences in brown rot resistance that have been reported at each of the various stages of fruit development.[2,4] For example, the architecture of epidermal tissue of some peach genotypes, such as the South American cv. Bolinha, which has a high level of brown rot resistance, is distinctly different from that of more susceptible genotypes.[5] In addition to the compactness and arrangement of the epidermal and sub-epidermal cells, Bolinha fruits have fewer trichomes and a thicker cuticle with a higher phenolic content than other fruits of similar maturity from less resistant genotypes.[6]

The potential role of phenols in the resistance to the brown rot fungus has been studied by several groups because their concentrations are particularly high in peach genotypes with a high level of resistance. Specifically, these studies investigated the actions of the major phenolic compounds, namely, catechin, procyanidin B3, chlorogenic acid (CGA; 5-*O*-caffeoylquinic acid), neochlorogenic acid (NCGA), and caffeic acid (CA; 3,4-dihydroxycinnamic acid), on spore germination and mycelial growth of *Monilinia fructicola* in culture.[6−10] Generally, the concentrations of phenol compounds are initially high in immature fruits, then decrease during ripening, and are at their lowest levels at 2 weeks before harvest. However, high amounts of CGA and NCGA (as high as 25 μg/g) persist throughout the maturation stages.[8,9] Lee and Bostock reported that CGA or CA at concentrations that were equal or greater than those in the outermost layer of the skin (exocarp) of an immature resistant peach has no effect on the growth of *M. fructicola* or conidial germination in culture.[2] In another study, Bostock and colleagues[6] demonstrated that some phenolic acids in peaches can suppress cutinase activity in *M. fructicola*. In a follow-up study, the Bostock group showed that CGA and CA, which are naturally occurring antioxidants, caused downregulation of the genes that encoded cutinase and inhibited cutinase production in cutin-induced *M. fructicola* cultures.[11] Because Wang and his colleagues also demonstrated that the antioxidants, glutathione and lipoic acid, could significantly attenuate cutinase production in *M. fructicola*, they concluded that the effect of the phenolic antioxidants is probably a general effect of antioxidants rather than a pharmacological effect of a specific phenolic structural chemistry.[11] Collectively, such findings suggest that phenolic acids may suppress the cellular activities in the fungal pathogen that are crucial for its growth and colonization on a host.

Several lines of evidence support a role for melanin-like pigments in the pathogenicity of several fungal pathogens, such as that of melanized appressoria, which are produced by

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Colletotrichum and *Magnaporthe* spp.[12] We have previously reported on the relationship between melanin and the pathogenicity of *M. laxa*.[13,14] In 1993, we demonstrated that the capacity of *M. laxa* to induce peach twig blight was lost in an albino mutant and in a wild-type strain of the pathogen that was treated with pyroquilon, a melanin biosynthesis inhibitor. We also showed that sporogenesis and the formation of pycnidium-like masses of microconidia of melanized *M. laxa* colonies are greater than those of non-melanized colonies.[14] In a subsequent study, we demonstrated that melanin-like pigments in the hyphal walls of *M. laxa* increase resistance of the fungus to autolysis.[13] Other authors have since reported on the potential importance of melanin for the survival of conidia and the integrity of the stroma of *M. fructicola* against a variety of environmental stresses.[15]

It is against this background that we conducted a study whose aims were to (a) determine the types of the phenolic compounds in peaches and nectarines at various stages of their development, (b) identify and quantify those phenolic compounds that are related to brown rot infection, and then (c) investigate the effect of these phenol compounds on the biosynthesis of melanin-like pigments in *M. laxa* to determine the relationship between fungal melanin biosynthesis and brown rot infection caused by *M. laxa*.

MATERIALS AND METHODS

Chemicals. Analytical-grade CGA (>95% purity) and synthetic melanin were purchased from Sigma-Aldrich, St. Louis, MO, and were used as supplied as reference standards. Methanol [high-performance liquid chromatography (HPLC) grade] was purchased from Labscan, Dublin, Ireland. The water that was used for the mobile phase and in each aqueous solution in HPLC was purified with a Millipore Milli-Q-50 18 mΩ filtering system.

Peach and Nectarine Cultivars. Fruit samples were collected from two fungicide-free peach [*Prunus persica* (L.) Batsch] cultivars, 'Rojo Albesa' and 'Plácido', and one fungicide-free nectarine [*P. persica* (L.) Batsch var. *nucipersica*] cultivar, 'Autumn Free', from three different orchards in 2006 and 2007. In 2006, 'Autumn Free' nectarines were collected at monthly intervals from the start of pit hardening (May 15) until harvest (September 6) (five sampling dates). In 2007, fruits of each peach and nectarine cultivar were collected at weekly intervals for 6 or 7 weeks (six or seven sampling dates) that started from the first appearance of color on the fruit surface (color break) until harvest (September 3, 'Autumn Free'; September 18, 'Rojo Albesa'; October 2, 'Plácido'), with varying sampling dates for each of the three cultivars. A total of 27 fruits of each cultivar were collected at each sampling date to use them to determine the quantification incidence of brown rot infection (the subsequent extraction proportion that displayed signs of phenols from the fruit brown rot) and their phenolic content.

Extraction of Phenolic Compounds. Upon each sampling date, nine fruits of each cultivar were divided into three equal groups and then peeled. The flesh and peel of each group of three fruits were frozen separately in liquid nitrogen and kept at −80 °C until required for analysis of their phenolic contents. Each frozen fruit peel and flesh sample was ground to a fine powder in liquid nitrogen before storing to ensure uniformity. The phenolic compounds were extracted from the fruit powders using a previously described protocol.[10] Briefly, a frozen fruit sample (3.6 g) was homogenized at 6 m s^{-1} for 2 min on silver sand in 7.2 mL of extraction solution [water/methanol (2:4; v/v) that contained 2 mM sodium fluoride] in an ultrahomogenizer (FastPrep-24, Zymo Research Corporation, Irvine, CA) to inactivate the polyphenol oxidases and prevent degradation of the phenolic compounds. The homogenates were then centrifuged at 11 500 rpm for 15 min at 4 °C, and the volume of the supernatant was measured. A 1 mL aliquot of the supernatant was filtered through a 0.45 μm nylon filter (Symta, Madrid, Spain), and an aliquot of the filtrate was analyzed by HPLC within 24 h of preparing each homogenate.

HPLC−Diode Array Detection (DAD) Analysis of the Phenolic Compounds. HPLC−DAD analysis of the phenolic compounds in each filtrate was performed according to a previously described method, with some minor modifications.[10] Aliquots (20 μL) of each filtrate were analyzed using a HPLC system (Agilent 1200 Infinity Series, Agilent Technologies, Palo Alto, CA) that was coupled to a diode array detector, an autosampler, and a fraction collector and operated by Agilent ChemStation software. A reversed-phase C_{18} ACE HPLC column [150 × 4.6 mm (inner diameter); particle size, 5 μm] (Advanced Chromatography Technologies, Aberdeen, Scotland) with a C_{18} guard column was used to separate the phenolic compounds. Formic acid (2%) was added to both water and methanol to increase peak resolution. The mobile phases were 95% water + 5% methanol (A), 90% water + 10% methanol (B), and 20% water + 80% methanol (C). The elution protocol was as follows: 100% A remained isocratic for 5 min, and then a gradient was used to reach 100% B at 10 min and then held isocratic for 3 min. From 13 to 35 min, a linear gradient was used to reach 75% B and 25% C, 50% B and 50% C at 50 min, and 100% C at 52 min and then maintained isocratic until 59 min. The flow rate was 1 mL/min. DAD was performed at wavelengths of 510, 340, and 280 nm. Three samples were analyzed per cultivar and sampling date. The area of the different phenolic compounds in the HPLC chromatograms at 340 nm was determined for each of the three peach cultivars at each sampling date.

Identification and Quantification of Phenolic Compounds. To identify the hydroxycinnamic acid derivatives, mass spectrometry was performed using a HPLC system (series 1100; Agilent Technologies, Palo Alto, CA), which was coupled to a hybrid quadrupole time-of-flight (QTOF) mass spectrometer (QStar Pulsar I, Applied Biosystems). The instrumental parameters were set at the following values: mass range, 50−1200 uma; ion spray voltage, 5000 V; ion source gas pressure 1, 65 psi; ion source gas pressure 2, 65 psi; curtain gas pressure, 20 psi; declustering potential 1, 50 V; declustering potential 2, 15 V; and focusing potential, 250 V. In the tandem mass spectrometry (MS/MS) experiments, the collision energy for each selected ion was kept at −20 eV. The flow rate of the mobile phase was 0.7 mL min^{-1}, and the injection volume was 50 μL.

The hydroxycinnamic acid derivatives were identified using the exact mass measurements of the $[M - H]^-$ ions and their product ions. For this purpose, both positive and negative ionization modes were tested, and we found that the ion response and fragmentation were higher when the negative ionization mode was used. The resultant exact masses were determined using the elemental composition calculator tool, which is incorporated in the Analyst Software (QStar Pulsar I, Applied Biosystems). The identity of extracted CGA was confirmed by comparing its TOF−MS/MS spectrum to that of the reference CGA standard. The amount of CGA and its isomer from the three groups of each cultivar and at each sampling date was quantified by HPLC−DAD at a wavelength of 340 nm using CGA as the reference standard.

Determination of the Incidence of Brown Rot Infection. Upon each sampling date, nine fruits of each cultivar were sprayed until runoff with an aqueous suspension of *M. laxa* conidia (1×10^6 conidia mL^{-1}), which were produced according to a previously published protocol.[1] The control treatment comprised nine fruits of each cultivar that were sprayed until runoff with sterile distilled water. The inoculated and control fruits were then kept in the dark for 7 days at 22 °C. To maintain high humidity during the 7 day incubation period, the fruits were placed on a dry dish in a plastic tray that was lined with moist paper and covered with a plastic film. At the end of the incubation, the disease incidence was determined and expressed as the proportion (%) of peaches and nectarines that displayed signs of brown rot caused by *M. laxa*.

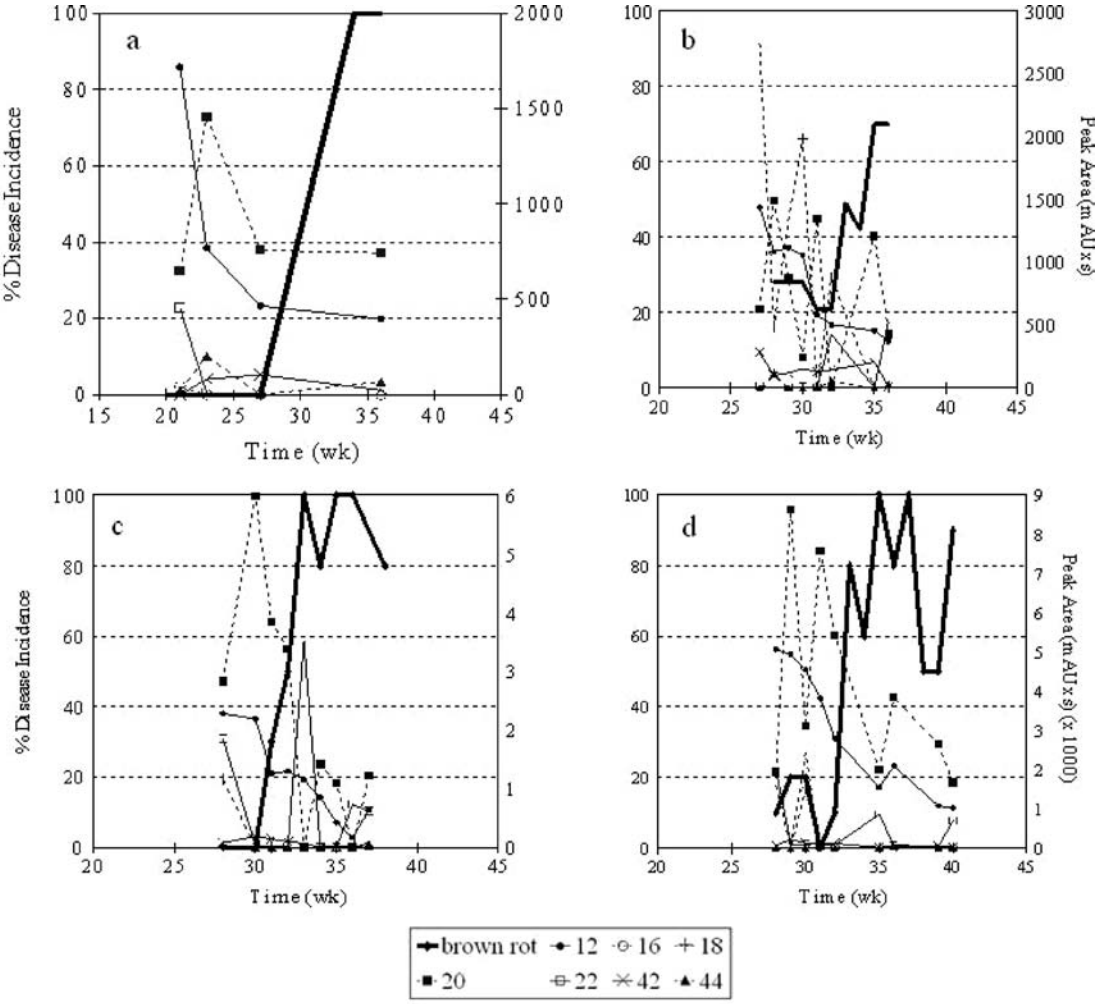

Figure 1. Incidence of brown rot infection (◆) on peach cultivars that were sprayed with a conidial suspension of *M. laxa* (1×10^6 conidia mL^{-1}) and the peak area (absorbance × retention time) of several phenolic compounds in the HPLC chromatograms at a wavelength of 340 nm from each peach extract at the varying sampling dates in (a) 'Autumn Free' nectarines in 2006, (b) 'Autumn Free' nectarines in 2007, (c) 'Rojo Albesa' peaches in 2007, and (d) 'Plácido' peaches in 2007: (●) 12, (○) 16, (+) 18, (■) 20, (□) 22, (×) 42, and (▲) 44 are retention times in minutes.

Effect of CGA on the Melanization and Growth of *M. laxa*. The toxicity of the reference CGA standard on the germination of *M. laxa* conidia was determined using a previously described bioassay.[16] For this purpose, a suspension of *M. laxa* conidia in sterile Czapek broth (Difco, Detroit, MI) (1×10^6 conidia mL^{-1}) and four CGA solutions of different concentrations, namely, 100, 500, 1000, and 2000 ppm, were prepared. An aliquot (30 μL) of the conidial suspension was then mixed with 15 μL of each CGA solution on sterile glass slides and incubated for 16 h at 20−25 °C in the dark. At the end of the incubation, three drops of each suspension were microscopically examined to determine the proportion (%) of germinated spores in each drop. The complete experiment was repeated twice.

Pyroquilon (1,2,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-4-one) (Ciba-Geigy, Greensboro, NC) is a proven inhibitor of melanin biosynthesis in many fungi of the subdivision Ascomycotina,[17] which includes *M. laxa*.[13] Accordingly, we used this inhibitor to investigate the effect of CGA on the extent of melanization and diametrical growth of *M. laxa* colonies on potato dextrose agar (PDA) plates. For this purpose, 750 ppm CGA or 10 ppm pyroquilon were added to the PDA after the medium had been autoclaved for 20 min at 121 °C and 1 atm. Mycelial plugs of *M. laxa* were cut from margins of actively growing 7-day-old *M. laxa* colonies on PDA and then transferred to either unamended PDA plates or PDA plates that were amended with either 750 ppm CGA or 10 ppm pyroquilon. The diameters and extent of melanization of the cultured *M. laxa* colonies were recorded every 7 days for 30 days. The extent of melanization was visually assessed by the absence of brown pigments on the reverse side of the colony according to the following color scale: white, 0; orange, 1; light brown, 2; and dark brown, 3. After 30 days of incubation, the mycelium of *M. laxa* was recovered from each plate to quantify the amount of melanin-like pigments. For this purpose, the mycelia were first homogenized (FastPrep-24) for 10 min and then sonicated 10 times each time for 30 s, before extracting the melanin-like pigments using 1 M KOH at 100 °C for 5 h.[18] The extracted melanin-like pigments were then transferred to a 1 M sodium carbonate solution, and the amount of pigment was determined spectophotometically at OD_{414}, OD_{492}, and OD_{540} in a microplate reader (Multiskan Plus P, version 2.01) using a standard curve that was generated by 1−10 ppm synthetic melanin. The solubility properties of the extracted melanin-like pigments were also determined using previously described assays.[13,17] A total of 10 replicates were used for each compound, and 10 PDA plates without any additives were used as the control. The complete experiment was repeated twice.

Data Analysis. Data were analyzed by a computerized statistical program (Statgraphics Plus for Windows, version 4.1, StatPoint, Inc., Herndon, VA). Infection data (percentage of infected fruit) were arcsine-transformed before analysis of variance for each cultivar and

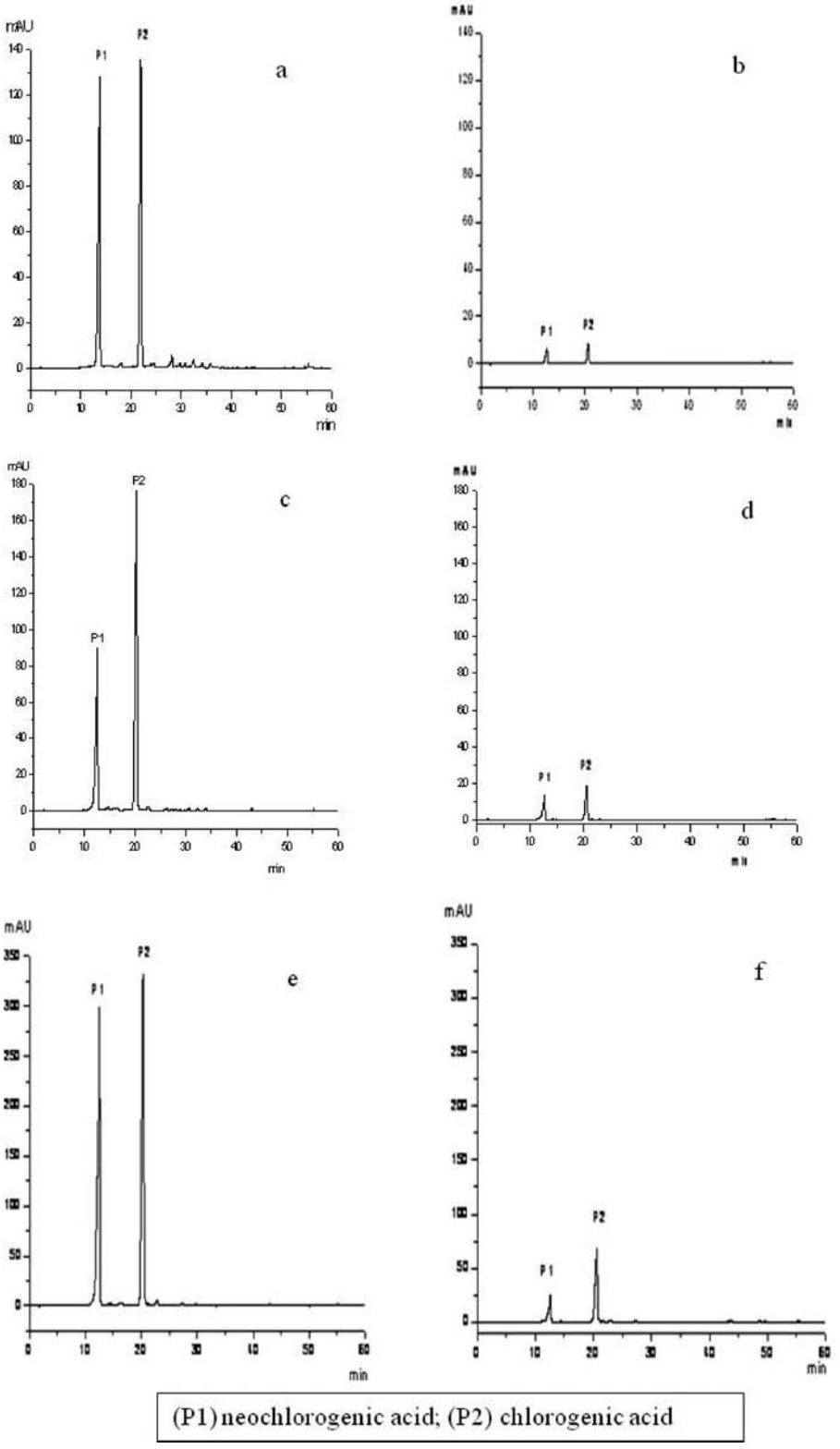

Figure 2. HPLC chromatograms of the phenolic compounds in the different peach cultivars at various stages of fruit development: (a) 'Autumn Free' nectarines that were collected on July 9, 2007 (8 weeks before harvest), (b) 'Autumn Free' nectarines that were collected on September 3, 2007 (9 weeks before harvest), (c) 'Rojo Albesa' peaches that were collected on July 9, 2007 (12 weeks before harvest), (d) 'Rojo Albesa' peaches that were collected on September 10, 2007, (e) 'Plácido' peaches that were collected on July 10, 2007, and (f) 'Plácido' peaches that were sampled on October 2, 2007. The chromatograms were obtained after HPLC analysis of the peach extracts at a wavelength of 340 nm. P1, NCGA; P2, CGA.

sampling date. When the F test was significant at $p = 0.05$, means were compared by the Student−Newman−Keul's multiple range test.[19]

To determine the relationship between the incidence of brown rot and the amount of each phenolic compound in the fruit, correlation and

regression analyses of the area and the incidence of brown rot were performed for each sampling date and cultivar. Each point in each analysis was the average of nine fruit. Data on the incidence of brown rot were arcsine-transformed and the square root (sqrt) of the peak area was calculated before analysis. To determine whether a statistically significant difference exists between the amounts of GGA and its isomer in each cultivar and the stage of development, the CGA peak area and its isomer for each cultivar and sampling date were analyzed by analysis of variance. When the F test was significant at $p = 0.05$, the means were compared by the Student−Newman−Keul's multiple range test.[19]

Data on the effect of the various CGA concentrations on the germination of *M. laxa* conidia were analyzed by regression analysis.[19] For this purpose, the arcsine of the sqrt of the percentage of germinated *M. laxa* conidia was used in the analysis. The results on the effects of CGA and pyroquilon on *M. laxa* growth on PDA plates, the extent of melanization, and the amount of melanin-like pigment in the mycelia of *M. laxa* were analyzed by factorial analysis of variance.[19] When the F test was significant at $p = 0.05$, the means were compared by the Student−Newman−Keul's multiple range test.[19]

RESULTS

Identification and Quantification of Phenolic Compounds. Quantitative differences in the chromatograms of the phenolic compounds were found in the extracts of the analyzed non-inoculated peaches of each cultivar. However, no differences were detected in the chromatograms of the phenolic compounds from the flesh and peel of these fruits (data not shown). Figure 1 shows the peak area of the major phenolic compounds in the HPLC chromatograms at 340 nm from each peach extract at the varying sampling dates in each of the three peach cultivars. The chromatrographic analysis revealed two main peaks in each of the three peach cultivars: a peak with a retention time of 12 min (compound P1) and a peak with a retention time of 20 min (compound P2). There were also differences in the areas of both peaks among the different peach cultivars. The areas of both peaks (compounds P1 and P2) were higher in the immature peaches than those found in the mature peaches (Figure 2). The area of the P1 and P2 peaks in (a) 'Autumn Free' nectarines that were collected on July 9, 2007 (8 weeks before harvest) (Figure 2a), (b) 'Rojo Albesa' peaches that were collected on July 9, 2007 (9 weeks before harvest) (Figure 2c), and (c) 'Plácido' peaches that were collected on July 10, 2007 (12 weeks before harvest) (Figure 2e) were significantly higher than the P1 and P2 peaks in (a) 'Autumn Free' nectarines that were collected on September 3, 2007 (Figure 2b), (b) 'Rojo Albesa' peaches that were collected on September 10, 2007 (Figure 2d), and (c) 'Plácido' peaches that were sampled on October 2, 2007 (Figure 2f).

Liquid chromatography coupled with mass spectrometry was then used to identify P1 and P2. Using the QTOF analyzer, it was possible to obtain highly accurate m/z values and/or isolate different precursor ions to study their MS/MS fragmentation pattern. According to the ultraviolet−visible (UV−vis) absorption spectrum of each compound, both compounds were caffeoylquinic derivatives with a molecular weight of 354 and whose mass spectral fragmentation patterns were very similar.

The accurate mass measurement of the $[M - H]^-$ ion of P1 was 353.0871, and this corresponded to an elemental composition of $C_{16}H_{17}O_9$ (error of 0.2939 mDa). The product-ion mass spectrum of P1 (Figure 3) showed a main fragment at m/z 191.0585 that corresponded to an elemental composition of $C_7H_{11}O_6$ (error of 1.3881 mDa) because of the loss of the caffeoyl moiety. MS/MS fragmentation of this ion at m/z 191 gave a product ion at m/z 173.0459 because of the loss of a water molecule. A second fragment was observed at m/z 179.0365 (error of 0.1468 mDa) and corresponded to the loss of 1,3,4-trihydroxycyclohexanecarboxylic acid. MS/MS fragmentation of this ion at m/z 179 gave a product ion at m/z 135.0460. This product corresponded to an elemental composition of $C_8H_70_2$ (error of 0.1468 mDa) because of the loss of 44 Da and was attributed to the loss of CO_2 from the ester. MS/MS fragmentation of the $[M - H]^-$ ion of P1 also led to the formation of the ion at m/z 161.0232 that corresponded to an elemental composition of $C_9H_5O_3$ because of the loss of quinic acid.

After HPLC analysis of the reference CGA standard, we concluded that the identity of P2 was CGA. According to these results and those of other authors,[8,10] we concluded that P1 was the diastereomer of CGA, NCGA (Figure 4). These two hydroxycinnamic acid derivatives, NCGA and CGA, were then quantified

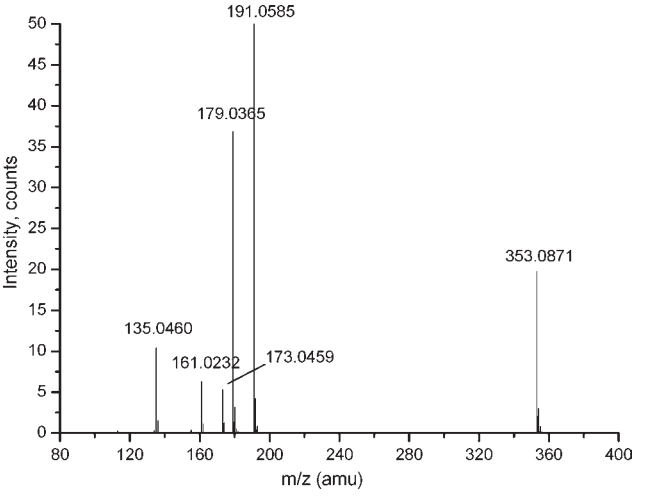

Figure 3. Mass spectrum of NCGA obtained by the HPLC coupled to a hybrid quadrupole time-of-flight mass spectrometer.

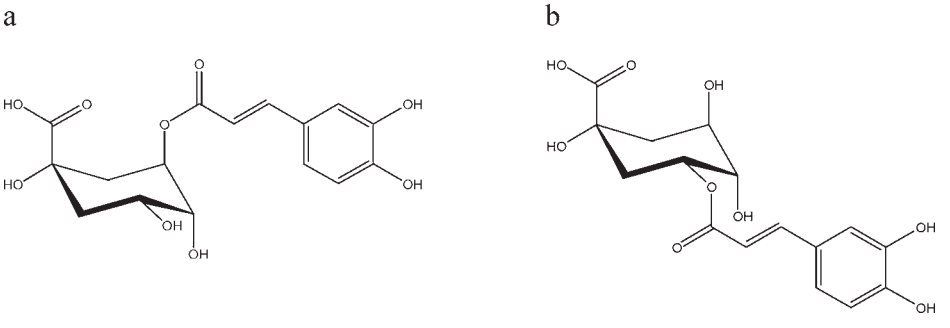

Figure 4. Chemical structures of (a) CGA and (b) NCGA.

Table 1. Quantification of the Phenolic Compounds (NCGA and CGA) in Immature and Mature 'Autumn Free' Nectarines, 'Rojo Albesa' Peaches, and 'Plácido' Peaches in 2007

		NCGA concentration[a]		CGA concentration	
cultivar	date[b]	ppm	mg/kg	ppm	mg/kg
'Autumn Free' 2007	July 9	42.63 ± 18.23	85.27 ± 36.46	75.65 ± 26.08	151.30 ± 52.17
	September 3	15.33 ± 3.17	30.67 ± 6.34	38.55 ± 10.17	77.10 ± 20.34
'Rojo Albesa' 2007	July 9	87.29 ± 4.24	174.58 ± 8.48	216.51 ± 32.99	433.02 ± 65.98
	September 10	26.09 ± 3.84	52.19 ± 7.68	70.57 ± 13.50	141.15 ± 26.99
'Plácido' 2007	July 10	190.13 ± 24.74	380.25 ± 49.48	274.10 ± 30.22	548.20 ± 60.44
	October 2	41.13 ± 4.16	82.27 ± 8.33	90.64 ± 14.74	181.28 ± 29.48

[a] Concentrations are the mean ± standard error of data that were obtained from three groups of each cultivar and at each sampling date expressed as parts per million (ppm) and mg/kg of fresh fruit weight. [b] The dates at which fruits were collected to quantify the amounts of NCGA and CGA in the immature fruits (July) and mature fruits at harvest (September−October) of each peach cultivar using the reference CGA standard.

Table 2. Correlation Coefficients, Incidence of Brown Rot, and the Peak Area of Major Phenolic Compounds with Different Retention Times in the Three Peach Cultivars[a]

	retention time of major phenolic compounds in fruits (min)						
peach cultivar	12	16	18	20	22	42	44
'Autumn Free' 2006	−0.85 (p = 0.0600)	*b*	*b*	−0.37 (p = 0.6316)	−0.39 (p = 0.6081)	*b*	−0.44 (p = 0.4500)
'Autumn Free' 2007	−0.65 (p = 0.1050)	*b*	−0.50 (p = 0.3087)	0.20 (p = 0.7040)	0.26 (p = 0.6110)	−0.34 (p = 0.5094)	*b*
'Rojo Albesa' 2007	−0.85 (p = 0.0076)	*b*	*b*	−0.98 (p = 0.0003)	0.21 (p = 0.6085)	*b*	*b*
'Plácido' 2007	−0.87 (p = 0.0022)	−0.27 (p = 0.4752)	−0.42 (p = 0.2609)	−0.90 (p = 0.0008)	0.04 (p = 0.9109)	*b*	*b*

[a] Data on disease incidence are the mean of three groups of each cultivar on each sampling date (three fruits/group). The peak area of phenolic compounds is the mean of data that were obtained from three groups of each cultivar and sampling date. Data on the incidence of brown rot were arcsine-transformed, and the square roots of the area under each peak were calculated before the correlation analysis. Values are correlation coefficients, and the corresponding p values are displayed in parentheses. The incidence of brown rot infection in each cultivar at the varying sampling dates was determined after the fruits were sprayed until run-off with an aqueous conidial suspension of *M. laxa* (1×10^6 conidia mL^{-1}) and then incubated in trays that were covered with a plastic film for 7 days at 22°C in the dark. The peak area of the different phenolic compounds with different retention times (12, 16, 18, 20, 22, 42, and 44 min) in the three peach cultivars in the HPLC chromatograms was determined at a wavelength of 340 nm. [b] Phenolic compounds not observed.

in each peach cultivar using the reference CGA standard (Table 1). The concentrations of NCGA and CGA were higher in the immature fruits than in the mature fruits (NCGA, 85−380 versus 30−82 mg/kg; CGA, 151−548 versus 77−181 mg/kg).

Incidence of Brown Rot Infection. No brown rot infection was recorded on the fruits at each of the first three sampling dates in 2006 (May, June, and July) for the 'Autumn Free' nectarines that were sprayed with an aqueous conidial suspension of *M. laxa* (Figure 1a). In contrast, the incidence of brown rot infection for these nectarines in the last month before their harvesting in September was 100%. In 2007, the incidence of brown rot for 'Autumn Free' nectarines that were sprayed with the *M. laxa* conidial suspension 3 weeks before harvesting in September was more than 50% (Figure 1b). In 2007, the incidence of brown rot in the 'Rojo Albesa' peaches 6 weeks before their harvesting in September was 50% (Figure 1c), and the incidence of brown rot in the 'Plácido' peaches 7 weeks before their harvesting in October 2007 was 50% (Figure 1d). In 2007, the incidence of brown rot was 100% in 'Rojo Albesa' and 'Plácido' peaches that were sprayed with the pathogen 5 weeks before their harvesting. No brown rot infection was recorded on any fruits that had been sprayed with sterile distilled water (data not shown).

The incidence of brown rot at each sampling date for each cultivar was significantly negatively correlated with P1. This significant negative correlation was not found for P2, except in the case of the 'Rojo Albesa' (r = −0.98) and 'Plácido' (r = −0.90) cultivars (Table 2). In the same chromatogram, the minor peak areas were not correlated with the incidence of brown rot and appeared only in some cultivars (Table 2). The best correlations between the P1 peak and the incidence of brown rot were found for the 'Autumn Free' 2006 (r = −0.85), 'Rojo Albesa' (r = −0.85), and 'Plácido' (r = −0.87) cultivars.

In addition, we found that the incidence of brown rot could be predicted by the NCGA content in the 'Autumn Free' nectarines in 2006 (R^2 = 0.72; p = 0.0600), the 'Rojo Albesa' peaches in 2007 (R^2 = 0.72; p = 0.0076), and the 'Plácido' peaches in 2007 (R^2 = 0.76; p = 0.0020) using the following equations, respectively:

$$\begin{array}{l}\text{arcsin(percent incidence of brown rot)} = \\ \quad \underset{(46.60)}{159.32} - \underset{(1.98)}{5.47}\ \text{sqrt(NCGA in 'Autumn Free')}\end{array} \quad (1)$$

$$\begin{array}{l}\text{arcsin(percent incidence of brown rot)} = \\ \quad \underset{(24.41)}{137.32} - \underset{(0.70)}{2.80}\ \text{sqrt(NCGA in 'Rojo Albesa')}\end{array} \quad (2)$$

$$\begin{array}{l}\text{arcsin(percent incidence of brown rot)} = \\ \quad \underset{(23.75)}{144.38} - \underset{(0.43)}{2.03}\ \text{sqrt(NCGA in 'Plcido')}\end{array} \quad (3)$$

Standard errors of the estimates are shown in parentheses below the parameters for each constant and variable in the equation.

The results of the regression analysis showed that increasing CGA concentrations exerted a significant negative effect ($p < 0.01$) on the germination of *M. laxa* conidia (Table 3). Although the regression coefficient ($R^2 = 0.21$) indicated a relatively weak relationship between CGA and the germination *M. laxa* conidia, the correlation coefficients ($r = -0.46$) indicated that the relationship is strong. The standard deviation of the residuals was 2.69, and this value was used to determine the confidence limits that are displayed in Table 4. Although CGA concentrations higher than 500 ppm could inhibit germination of *M. laxa* conidia in our bioassay, these high concentrations were not found in any of the tested fruits (Table 1).

Effect of CGA on the Melanization and Growth of *M. laxa*. *M. laxa* growth was not affected by the addition of either 750 ppm CGA or 10 ppm pyroquilon to the PDA plates (Table 5). However, both compounds changed the extent of melanization of the *M. laxa* colonies (Table 5). The properties of the melanin-like pigments that were extracted from mycelia of *M. laxa* that was grown on unamended PDA plates and PDA plates that were amended with either 750 ppm CGA or 10 ppm pyroquilon were similar to those of melanin. Specifically, the extracted melanin-like pigments were soluble in hot 0.5 M NaOH and cold 1 M Na_2CO_3, insoluble in water, ethanol, diethyl ether, chloroform, or acetone, and could be precipitated by 7 M HCl.

When the logarithm of absorbance for the reference melanin standard was plotted against the wavelength, the result was a linear curve with a negative slope (−0.0023). When the identical analysis was performed using (a) the melanin-like pigments that were extracted from mycelia of *M. laxa* that was grown on unamended PDA plates, (b) the melanin-like pigments that were extracted from mycelia of *M. laxa* that was grown on PDA plates with 750 ppm CGA, and (c) the melanin-like pigments that were extracted from mycelia of *M. laxa* that was grown on PDA plates with 10 ppm pyroquilon, the slopes of the resultant linear curves were −0.003, −0.003, and −0.0005, respectively. Lastly, the amount of melanin-like pigment that was extracted from mycelia of *M. laxa* that was grown on the unamended PDA plates was 42 and 52% higher ($p = 0.05$) than that extracted from the mycelia of *M. laxa* that was grown on the PDA plates that were amended with either 750 ppm CGA or 10 ppm pyroquilon, respectively (Table 5).

Table 3. Analysis of Variance Table for Simple Regression Analysis on the Effect of the different Concentrations of CGA on the Germination of *M. laxa* Conidia[a]

source	sum of squares	df[b]	mean square	*F* ratio[c]	*p* value[d]
regression	281.03	1	281.03	38.66	0.00001
residual	1024.80	141	7.26		
total	1305.90	142			

[a] Data on the percent germination of *M. laxa* conidia were arcsine-transformed before analysis. [b] df = degrees of freedom. [c] *F* ratio = test statistic that was used to determine whether the between-group variability of the sample means was different from the within-group variability. [d] *p* value = level of statistical significance.

Table 4. Confidence Limits of *M. laxa* Conidia Germination for the Different CGA Concentrations

		95% confidence limits	
CGA concentration (ppm)	percent germination of *M. laxa* conidia[a]	lower	upper
0	66.94	58.8	75.07
100	64.09	56.72	71.46
500	52.70	47.19	58.22
1000	38.47	31.10	45.34
2000	10.01	−6.75	26.77

[a] Data on the percent germination of *M. laxa* conidia were arcsine-transformed before analysis.

DISCUSSION

In this study, we found that susceptibility to brown rot caused by *M. laxa* changes with fruit ripening in peaches; immature fruits are less susceptible than mature fruits to infection to *M. laxa*. Specifically, the susceptibility of the fruits to *M. laxa* infection was greatest between the 32nd and 34th week of growth after the endocarp and pericarp were completely formed. The results of the present study also provide evidence that the amount of phenolic compounds in the fruits could be causally linked to the differences in disease susceptibility of immature and mature fruits. This ripening-dependent difference in susceptibility to brown rot infection caused by *M. fructicola* has been previously described by Gradziel[4] in three clingstone peach genotypes; susceptibility progressively increased from color break to when the color of the epidermis was uniform. Gradziel[4] attributed these differences in disease susceptibility of immature and mature fruits to a wide array of physical and chemical factors, as well as the fruit genotype and other physiological processes.[4]

Phenolic metabolism has been implicated in fungal resistance for a range of horticultural crops.[17] The results of our HPLC−DAD analysis of the phenolic extracts in three peach cultivars showed two main compounds in all cultivars. These compounds were subsequently identified as CGA and its isomer, NCGA, by HPLC−QTOF mass spectrometry, which was a valuable tool for enabling the identification of the isomer for which no standard

Table 5. Effects of CGA and Pyroquilon on *M. laxa* Growth on PDA, the Extent of Melanization of the *M. laxa* Colony, and the Amount of Melanin-like Pigments in the Fungal Mycelia[a]

treatment	*M. laxa* growth (cm^2)	extent of melanization	melanin-like pigment content (ppm)
CGA (750 ppm)	52.46 a	1.64 b (dark orange)	30.70 b
pyroquilon (10 ppm)	50.87 a	1.59 b (dark orange)	24.23 b
control	52.38 a	2.65 a (dark brown)	52.60 a
MSE_{within}[b]	7.45	0.43	345.81

[a] Data are values that were recorded or measured at the end of the 30 day study period and are the mean of 10 replicates per treatment. Means followed by the same letter are not significantly different from each other according to the Student−Newman−Keul's multiple range test ($p = 0.05$). CGA (750 ppm) or pyroquilon (10 ppm) were added to PDA after the medium had been autoclaved. Mycelial plugs were cut from margins of actively growing 7-day-old *M. laxa* colonies and then transferred to either unamended PDA or PDA that was amended with either CGA or pyroquilon. Colony diameters and the extent of melanization of *M. laxa* data were recorded after 30 days of media incubation. [b] MSE_{within} = mean squared error.

reference exists. Concentrations of both compounds, similar to those that we found in this study, have been reported by others in peaches and especially in peach peels.[10,20] However, we did not detect any quantitative differences in the amounts of CGA or NCGA in the flesh and peels of the non-inoculated peaches. We also found quantitative changes in the amounts of CGA and NCGA in the peaches at each sampling date for each cultivar. Specifically, the concentrations of CGA and NCGA were higher in the immature fruits than in the mature fruits. Significant changes in the amounts of volatile compounds, organic acids, and phenolic compounds and cell wall composition occur during development of *Prunus* fruits.[21,22] Some authors[2,6,20] have previously reported that the total phenol content in peaches is higher in immature fruits than in harvested fruits, while other authors did not find a relationship between the phenol content and the stage of maturity in peach peels.[10,23]

We found significant differences between the amounts of NCGA in the 'Autumn Free' nectarines at the various stages in fruit development in 2006 and 2007, as well as large differences in its amounts between the 'Rojo Albesa' and 'Placido' peach cultivars. These seasonal and cultivar differences in total phenolic content are consistent with what has been described for other peach fruits.[8,24] We also found a significant negative correlation between NCGA content and the incidence of brown rot caused by *M. laxa* for each cultivar at the different sampling dates. We also demonstrated that the susceptibility of the each peach cultivar to *M. laxa* infection starts to increase when the amount of NCGA in the fruits begins to decline. A strong correlation between the polyphenolic content and resistance to *Sclerotinia fructigena* has been reported in apples.[25] In addition, a moderate correlation has been reported between the phenolic content of fruits and the activity of the enzymes that are responsible for color breaking on the fruit surface.[2,6,26] Other authors have reported that high levels of CGA and CA in peaches are associated with increased resistance to *M. fructicola*.[2,6]

We found that the germination of *M. laxa* conidia was not affected by CGA at concentrations lower than 500 ppm, which is the concentration that we found in the fruits of the tested cultivars. Bostock and colleagues[6] reported that concentrations of CGA and CA as high as 5 mM did not inhibit spore germination or mycelial growth of cultured *M. fructicola*.[6] Bostock and colleagues[6] also reported that the growth of *M. fructicola* was unaffected by concentrations of CGA or CA that were similar to or greater than those in the exocarp of immature resistant fruits. They attributed these findings to the ability of CGA and its metabolites to chelate iron. In another study, Lee and Bostock[2] found that phenolic compounds, such as CGA, CA, or reduced glutathione, could inhibit the *in vitro* production of cell-wall-degrading enzymes and appressoria of *M. fructicola*. Lee and Bostock[2] also reported that the application of these phenolic compounds to both peach petals and cherry fruits could significantly reduce lesion development caused by the conidia of *M. fructicola*.

We found that *M. laxa* growth in culture was not affected by either CGA or pyroquilon, but both compounds inhibited melanization of the colony, as measured by the reduced extent of melanization of the colony and amount of melanin-like pigments in the mycelia of *M. laxa*. Melanins are naturally occurring stable (biochemically resistant), insoluble, and high-molecular-weight brown−black pigments that are produced by animals, plants, fungi, and bacteria from the oxidative polymerization of phenolic or indolic compounds.[17,27] Melanins have also been reported to enhance the survival and competitive abilities of organisms in certain environments but are not essential for growth and development.[17] The ability of certain microorganisms to produce melanin has been linked to their virulence, and many fungal pathogens, such as *Venturia inaequalis*, *Magnaporthe gray*, *Pyricularia oryzae*, and *Colletotrichum lagenarium*, need melanized appressoria to cause infection and disease development.[28,29] In general, the presence of melanin in the walls of sclerotia, hyphae, or spores of several fungi confer tolerance to environmental stresses, such as UV radiation,[30] microbial lysis,[14] and defense responses of the host plant against fungal infection. We have previously reported that the melanin-like pigments of *M. laxa* have a role in the brown rot infection of peach twigs by the fungal pathogen.[13] We also previously reported that a melanin-deficient mutant strain (albino mutant) and the wild strain of *M. laxa* that was treated with pyroquilon, an inhibitor of melanin biosynthesis in *M. laxa*, could not induce peach twig blight.[14] Collectively, these results led us to conclude that the ability of *M. laxa* to produce melanin is crucial for its pathogenicity.

To summarize, we found that the amounts of NCGA and CGA in immature peach fruits are high and immature peach fruits are very resistant to brown rot infection. We also demonstrated that CGA inhibits melanin biosynthesis in *M. laxa* without any effects on the growth and germination of the fungus. Melanins have been cited as a major determinant for peach infection by *M. laxa*.[13] Therefore, the amounts of CGA or NCGA in harvested fruits could be linked with brown rot infection. If this is indeed the case, applying CGA or NCGA to mature and/or harvested fruits to increase their content might provide a suitable postharvest treatment to complement existing methods that are used to control diseases caused by *M. laxa*.

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