Core Rot Development in Red Delicious Apples Is Affected by Susceptibility of the Seed Locule to *Alternaria alternata* Colonization

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ABSTRACT

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Alternaria alternata is the causal organism of core rot decay symptoms in susceptible cv. Red Delicious but not in resistant cv. Golden Delicious. The two cultivars did not differ in natural colonization of the style and ovary during the first week after full bloom; colonization of the ovary in the susceptible cultivar subsequently decreased with increasing distance from the calycine tube. By 30 days after full bloom, *Alternaria* recovery from ovary 1, adjacent to the end of the calycine tube, was 100 and 40% in the susceptible and resistant cultivars, respectively. In the susceptible cultivar, *Alternaria* recovery decreased from 75 to 20% in ovaries 2, 3, and 4, while there was only minor incidence in the resistant cultivar. In-

oculation of the mesoderms of the two cultivars induced similar decay symptoms, but inoculated locules of Red Delicious were more susceptible than those of Golden Delicious. Increased inoculum concentration or isolate virulence enhanced the difference in locule susceptibility between the cultivars. Inoculation on isolated seed locules or on media amended with susceptible locule tissue as a carbon source induced greater transcript levels of several genes than the inoculation on resistant tissue. Endo- and exoglucanase activity levels were higher at pH 4.8 than at 4.2, conditions typical of the mesoderm adjacent to the seed locules of the susceptible and resistant cultivars, respectively. Current results suggest that susceptibility of Red Delicious apples to core rot decay is dependent on the sensitivity to locule colonization and on mesoderm pH, a factor that enhances fungal virulence.

Additional keywords: apple diseases, *Malus sylvestris*, moldy-core disease.

Core rot symptoms on apple fruits (Fig. 1) are commonly associated with *Pleospora herbarum* and species of *Stemphylium*, *Cladosporium*, *Ulocladium*, *Epicoccum*, *Coniothyrium*, *Penicillium*, *Pezizula*, *Mucor*, *Fusarium*, and *Alternaria*. The most commonly isolated causal agent, however, is *Alternaria alternata* (Fr.) Keissler (1-5,9,27,32). The disease is characterized by dark brown tissue that appears dry and corky within the seed locules and contains air pockets when it penetrates into the fruit mesoderm. The rot may be either a slow, dry rot confined to the mesoderm adjacent to the core or a wet rot that progresses more rapidly and deeply into the mesoderm tissue. External symptoms on the fruit are quite subtle, and typically, the disease goes unnoticed until the fruit is cut open. In some cases, external symptoms of infection may include premature ripening and fruit drop (2,9,27,30).

It has been reported that with warm and moist conditions, *A. alternata* colonized senescing flower parts during and shortly after bloom, and later spread through the open calycine tube into the receptacle or core region of the fruit (20,29). Pathogen spores entered the calyx and colonized the seed locule cavity during the growth season and storage, resulting in core rot symptoms.

Conditions of high relative humidity, mild temperatures, and cultivar susceptibility are important factors in the natural incidence of infection in orchards. However, the mode of infection by the fungus is not fully understood and the conditions for its continued growth inside the fruit have not yet been determined (7,25,28,31,33). Many apple cultivars are affected by *A. alternata*,

but it is particularly evident in those cultivars with an open calyx. The most susceptible is Red Delicious (32), which is the most commonly grown cultivar in Israel, where it constitutes one-third of the entire apple crop. Although there is great variation in the extent of disease development around the core tissue in susceptible cultivars, in cultivars such as Golden Delicious, which do not usually have an open calyx tube, core rot disease is not expressed and symptoms are not observed (Fig. 1).

The natural differences between Golden Delicious and Red Delicious in their susceptibility to core rot development have raised the question of whether the differing susceptibilities to *A. alternata* development may result from: (i) a difference in flower colonization and the presence of the calycine tube, which enables fungal development to extend to the seed locule; (ii) differing colonization in the mesoderms; or (iii) differing capability of *A. alternata* to colonize the respective seed locule tissues, as a first step toward breaching into the mesoderm. The objectives of the current study were to discover the mechanism used by *A. alternata* to penetrate and colonize the seed locule of susceptible cultivars and to identify host factors that might modulate fungal virulence. Current results suggest that the differing susceptibilities of Red Delicious and Golden Delicious are not determined by the initial colonization of the blossom by the pathogen, as reported in the past, but rather by the capability of the pathogen to colonize the host seed locule of the susceptible fruits as a basis for its further development into the mesoderm.

MATERIALS AND METHODS

Fungal cultures, growth conditions, fruit inoculation, and evaluation of decay incidence. *A. alternata* isolates were obtained from fruit of the susceptible *Malus sylvestris* (L.) Mill. var.

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domestica (Borkh.) Mansf. cv. Red Delicious, strain Top Red, showing symptoms of core rot infection in the mesoderm. The fruit were disinfected by washing with 90% ethanol for 5 s, dried with tissue, cut in half longitudinally, and small pieces (1 to 2 mm) of the mesoderm tissue—external to the core region that showed brown *Alternaria* decay symptoms—were removed and incubated on potato dextrose agar (PDA) (Difco, Becton, Dickinson, Sparks, MD) in sterile petri dishes at 25°C. Single-spore cultures were maintained on PDA in petri dishes, and spore suspensions were prepared by adding a small amount of sterile distilled water to 10-day-old cultures of *Alternaria* spores. The spore concentration was quantified with a hemacytometer and adjusted to be between 5×10^5 and 10^6 spores/ml, depending on the experiment.

Isolates of differing virulence had been selected previously by Reuveni et al. (26). Isolates inoculated on Golden Delicious apples that showed a decay diameter of ≤ 3 to 7 mm at 7 days after inoculation were designated as low virulence, in contrast to the high-virulence isolates that showed a decay diameter of >15 mm. Two isolates were used: the high-virulence SH-42 and low-virulence RM-44. Differential pathogenicity was confirmed by measuring the β -1,4-glucanase activity level when isolates were grown on cell walls from Red Delicious apple mesoderm as the main carbon source (10). The β -1,4-glucanase activity level of the high-virulence isolate in culture media 3 days after inoculation was 70 times higher than that induced by the low-virulence isolate (0.072 ± 0.002 nmol min⁻¹ mg protein⁻¹ compared with 0.001 ± 0.0002 nmol min⁻¹ mg protein⁻¹). If not specified otherwise, the isolate used throughout the current study was SH-42.

Inoculation into the mesoderm of the harvested fruit was carried out by wounding, after the apple surface had been disinfected by washing with 90% ethanol as described above. The fruit was wounded with a sterile pipette tip (1 mm wide) that penetrated 2 mm into the mesoderm, followed by inoculation with 10 μ l of a suspension containing 10^6 spores/ml, and were kept at a relative humidity (RH) of approximately 95%. For inoculation of the seed locule, apples were cut longitudinally through the center and the seed locule was dissected from most of the mesocarp. Healthy, half locules showing no dark symptoms were inoculated by placing 20 μ l of spore suspension in the middle of the cavity and kept in a petri dish over a humid filter paper. The inoculated fruits and seed locule tissue were incubated in a growth chamber at 20 to 22°C under an illumination of 100 to 120 μ E·m⁻²·s⁻¹ for 16 h per day.

Disease development in artificially inoculated apples was assessed by daily measurements of the lesion diameter (millimeters) of the developing dark brown symptoms around the inoculated site. The average diameter of decay in six fruits with four inoculation sites per fruit (24 observations) 3 days after inoculation was recorded. The infected surface area of the locule was assessed by

comparing the black-brown sign of infection with drawings of the locule that showed various extents of surface colonization by *A. alternata*. The drawings were adapted from a previous study (22). Colonization by the fungal mycelia 4 days after inoculation was recorded as the average infected locule surface area, as determined from measurements in each of two locules in each of five different fruits (10 observations). The experiments were carried out three times in each of two consecutive harvesting seasons. One representative experiment is reported.

Colonization by *A. alternata* at different stages of fruit development. Fruits and flowers of apple cvs. Red Delicious, strain Top Red, and Golden Delicious were sampled from an orchard in northern Israel, which had shown a consistent natural level of *A. alternata* infection with core rot development during the previous 3 years. Twenty trees of each cultivar that were arranged in parallel rows, separated by 4 to 5 m, were used in the experiment. Seven flowers and/or fruits were randomly sampled from each of three trees (replicates) located three trees apart. The incidence of *A. alternata* on naturally infected flowers and fruits was determined by isolation of the apple tissue to a medium for selective detection of *A. alternata* colonization. Flowers, fruitlets, and fruits were harvested from the orchard on different numbers of days after full bloom (DAFB) and were evaluated in the laboratory. The apple tissues were surface sterilized with 1% NaOCl for 2 min and rinsed twice with sterile distilled water prior to incubation in PDA plates amended with thiabendazole (TBZ) at 20 μ g ml⁻¹. *Alternaria* isolations were identified by the olive-brown color of the culture and confirmed with direct microscopic observation of the spores. At 0 and 7 DAFB, the style and ovary were separated and incubated individually in PDA plates. At 14 DAFB, the cylindrical regions next to the ovaries of fruitlets were each sliced into three regions ('ovaries 1', '2', and '3'), each 3 to 5 mm thick, according to closeness to the end of the calyx tube. Starting at 30 DAFB, fruits of different sizes were evaluated. From each fruit, four sections were sampled and designated as follows, in succession from the end of the calyx tube to the pedicel: ovary 1 (section adjacent to the calyx tube); and ovary 2, 3, and 4 (section nearest to the pedicel). Sections from each fruit were placed in one TBZ-amended PDA plate and labeled according to their specific locations in the ovary. Incidence of *A. alternata* colonization was quantified according to the percentage of tissues infected ([number of tissues colonized/total number of tissues] $\times$ 100) after 5 days of incubation at 25°C.

Evaluation of decay incidence caused by natural infection was based on examination of 300 fruits (100 from each replicate per tree) that were cut transversely through the core. Those fruits in which *Alternaria* had developed into the mesoderm were considered to exhibit core rot symptoms.

In vitro growth condition inducing pectolytic and cellulolytic genes of *A. alternata*. For in vitro studies, the fungus was grown in 40 ml of the medium described by Mandels and Weber (19) ("primary medium"), which contained (per liter of distilled water) 10 g of glucose, 0.5 g of proteose peptone (Difco, Detroit, MI), 200 μ l of Tween 80, 0.3 g of urea, 2.0 g of KH₂PO₄, 1.4 g of (NH₄)₂SO₄, 0.3 g of MgSO₄·7H₂O, 0.3 g of CaCl₂, and 0.01 g of FeCl₃. Forty milliliters of the autoclaved medium in a 125-ml Erlenmeyer flask were inoculated with 1 ml of a suspension containing 10^6 spores/ml. After 96 h of incubation on an orbital shaker running at 150 rpm at 25°C, the cultures were harvested by filtration and washing with 40 ml of sterile water over a Buchner funnel fitted with a 55-mm No. 1 filter paper (Whatman, Maidstone, England) under vacuum. To determine the effect of the carbon source on the activation of gene expression in the fungus, the washed mycelia were resuspended in 40 ml of fresh medium ("secondary medium") containing the following reagents (per liter): 4 g of K₂HPO₄, 2 g of MgSO₄·7H₂O, 5 g of KNO₃, 0.3 g of CaCl₂·2H₂O, 10 mg of FeCl₃, and 1% of seed locule tissue as the carbon source. The collected washed mycelia were resus-

Fig. 1. A, Red Delicious apple showing core rot symptoms caused by *Alternaria alternata* compared with B, symptom-free fruit.

pended for 16 h in 40 ml of secondary medium containing chopped seed locule slices of the resistant (Golden Delicious) and susceptible (Red Delicious) cultivars as the carbon source, instead of glucose, and the mycelia were collected as described above.

To analyze the transcript expression of *A. alternata* in infected locules, isolated dissected seed locules from Red Delicious and Golden Delicious fruits were inoculated with 20 µl of suspension containing 10⁶ spores/ml and incubated at 20°C for 4 days under aseptic conditions, after which the whole locule, whether infected or not, was frozen and ground for RNA extraction.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Transcript expression analysis of the pectolytic and cellulolytic genes of *A. alternata* was done in vitro and in vivo. Lyophilized mycelia (0.1 g) or frozen inoculated locules (0.4 g) from each of the two cultivars were homogenized by grinding with a mortar and pestle under liquid nitrogen. The RNA was extracted with an RNeasy Plant Mini kit (QIAGEN Sciences, Hilden, Germany) and further purified by treatment with TORBO DNA free (Ambion, Austin, TX). The reverse-transcription reaction was performed on 2 µg of total RNA with the Reverse-It First-Strand Synthesis kit (ABgene, Surrey, UK). Samples of cDNA were diluted 1:10 to the final template concentration for real-time PCR. Real-time quantitative PCR was performed with the RotorGene 3000 system (Corbett Research, Sydney, Australia). PCR amplification was performed with 3.5 µl of cDNA template in 10 µl of a reaction mixture containing 5 µl of Syber-Green Amplification kit (ABgene) and primers. The primer sequences, at a final concentration of 300 nM, were as follows, forward and reverse, respectively: for *PG* gene, 5' GAATCTGGCATGTTGCTTGGT and 5' TTACAGCGCAGCACAAAGGAA; for *AaK1* gene, 5' GGTGTCTACATCACACCAGATCTCA and 5' TTGCCGATCAAAGGCTCACT; for *AaCl* gene, 5' TTGCACTGCGAAGGGTAGCT and 5' TCGATGACAATCTTGCCATTCTT; for *AaFl* gene, 5' CCTACGCCGCAAGGTCTA and 5' CCTACGCCGGCAAGGTCTA; for *AaMLG1* gene, 5' AACGGCCAATTCGAGATGAC and 5' CTTGATGAAACGTTCTCGTCTGTA; and for 28S rRNA gene, 5' GGAACGGGACGTCATAGAGG and 5' AGAGCTGCATTCCCAAACAACT. The primers were supplied by Integrated DNA Technologies Pharmacia Bio-Tech (Cambridge, UK).

PCR conditions were as follows: initial denaturation for 15 min at 94°C; 40 denaturation cycles of 10 s at 94°C; annealing at 60°C for 15 s; extension at 72°C for 20 s; and melting at 72 to 95°C. Samples were subjected to melting curve analysis with the RotorGene program. All samples were normalized to 18S rRNA levels, and relative quantification values are expressed as the change in the increase/decrease of the levels relative to a calibration sample as determined by the Delta-Delta Ct ($\Delta\Delta C_t$) method according to Livak and Schmittgen (18). In the $\Delta\Delta C_t$ method, data are expressed as the factor by which gene expression changes, normalized to an endogenous reference gene, and relative to the calibration sample. Normalizing to an endogenous reference provides a means for correcting for differences in the amounts of input RNA (18). The experiment was repeated three times with similar results, of which one set is presented. The variation between the quantification levels of three replicates for each treatment is reported.

Endo- and exoglucanase production assay and pH measurements. Following its growth on a primary medium, the fungus was transferred to a secondary medium similar to the one described above, buffered with phthalate buffered to pH 4.3 to 5.3 but containing 1% of apple cell wall as the carbon source (11,12). Ninety-six hours after inoculation, the culture medium containing the fungus was centrifuged at 5,000 rpm and the collected supernatant was frozen and lyophilized. The dried supernatant was dissolved in 1 ml of water and dialyzed overnight against 500 ml of water at 4°C. Specific activities were expressed as units per milligram of protein, as measured by means of a dye-binding

assay with the Protein Assay kit (Bio-Rad, Hercules, CA), with crystalline bovine serum albumin used as the reference standard.

After incubation under the previously determined optimum conditions of 1 h at 47°C (10), the endoglucanase (EG) activity level was assayed in 50 mM sodium acetate buffer, pH 5.5, containing 0.5% sodium CMC (Sigma) and 10 µl of the sample that contained 1 µg of total protein. The amount of reducing sugar product was analyzed by measuring the OD at 660 nm. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 0.5% CMC to produce an amount of reducing sugar equivalent to 1 µmol of D-glucose per minute under standard assay conditions.

The exoglucanase (EXG) activity level was assayed in 50 mM sodium acetate buffer, pH 5.5, containing 0.5 ml of a solution of *p*-nitrophenyl β-D-cellobioside at 1 mg ml⁻¹ (pNPC, Sigma), D-glucono-1,5-δ-lactone at 0.5 mg ml⁻¹ (Sigma), and 10 µl of the sample that contained 1 µg total protein. The reaction mixture was incubated at 50°C for 1 h and the amount of *p*-nitrophenol produced after the addition of 0.5 ml of 2% Na₂CO₃ was measured spectrophotometrically. The amount of *p*-nitrophenol released was calculated on the basis of a molar extinction coefficient of 18.5 ml µmol⁻¹ cm⁻¹ for *p*-nitrophenol at 410 nm (8). D-Glucono-1,5-δ-lactone is a powerful inhibitor of β-glucosidases, but a poor inhibitor of EXGs (24). Mixtures of the same components incubated at 4°C were used as blanks and showed no activity.

The pH was measured with a micro-combination pH electrode, Model 9810BN (Orion, Beverly, MA). The mesocarp pH close to the seed locule tissue was determined by placing the micro pH electrode directly against the exposed tissue. All measurements were repeated on three different seed locule tissue samples from each of 12 fruits (at least 36 measurements) (21).

Data analysis. Statistical analyses of the data were performed with the JMP-IN software, version 5 for Windows (SAS Institute Cary, NC). For the natural infection colonization experiments, Tukey's HSD test was used to compare means at the *P* < 0.05 level. For the artificial inoculation experiments, Student's *t* test was used to apply pairwise comparison of means at the *P* < 0.05 level. Standard errors of the means were reported in other cases.

RESULTS

Colonization at different development stages of Red Delicious and Golden Delicious apples after natural infection by *A. alternata*. The incidence of *A. alternata* colonization of the styles and ovaries of flowers during flowering, i.e., at 0 DAFB, was similar in the susceptible Red Delicious and in the resistant Golden Delicious apples: both cultivars showed 70 to 80% colonization in the style and almost undetectable levels of colonization in the ovary (Fig. 2A). Seven days after full bloom (Fig. 2B), 100% of the flower styles of both cultivars were colonized. Colonization levels in the ovary close to the end of the calyx tube ('ovary 1') were also similar (as much as 50%) in both cultivars; samples of ovary 2 and 3, far from the calyx tube, showed lower levels of colonization and did not differ between the cultivars. Differences in the level of *A. alternata* colonization between the resistant and susceptible cultivars were first observed at 30 DAFB (Fig. 3). At this stage, the average percentage of infection of ovary 1 was 95% in Red Delicious compared with only 40% in Golden Delicious. The subsequent layers of ovary sections, ovary 2, 3, and 4, showed 75, 40, and 22% infection, respectively, in the Red Delicious, compared with only 5 to 7% in the corresponding sections of the resistant cultivar. Similar behavior was observed after evaluation periods of 45, 60, and 90 days after full bloom, with slight increases in the colonization levels in all the Red Delicious tissue samples, to approximately 85, 65, and 32% in the second, third, and fourth ovary slices, respectively (Fig. 3).

Analysis of the decay severity in naturally infected apples, revealed by slicing 300 fruits, showed that only 8% of the Red Deli-

cious apples exhibited visible core rot symptoms, whereas no symptoms were observed in Golden Delicious fruits.

A. *alternata* colonization of the mesoderm and seed locule.

To determine whether the mesoderm of the fruit affected the differing levels of *A. alternata* decay development in the mesoderms of the resistant and susceptible cultivars, the effects of inoculating external wounds in freshly harvested fruits of both cultivars were compared. Seven days after inoculation, the decay diameters in the susceptible and resistant cultivars were 12.5 ± 2.3 and 16 ± 3.1 mm, respectively, suggesting that mesoderm colonization is not the factor that determines the susceptibility of cv. Red Delicious.

Fig. 2. Colonization of cvs. Red Delicious and Golden Delicious apple flowers and fruitlets by *Alternaria alternata* at **A**, full bloom and **B**, 7 days after full bloom. Flower analysis included isolations of the styles and whole ovary. Fruitlet analysis included isolations from the remnant styles and the ovary region. The cylindrical region adjacent to the ovary was sliced into three sections designated as: ovary 1 = section adjacent to the calycine tube; ovary 2 = section next to ovary 1; and ovary 3 = section nearest to the pedicel and furthest from the calycine tube. Different letters indicate significant ($P < 0.05\%$) differences between the averages according to Tukey's HSD test.

To determine whether the sensitivity of the seed locule was the determining factor in the difference between the cultivars in colonization of the locule and development into the mesoderm, the inner parts of halved seed locules were inoculated with 20 μ l of a suspension containing 10^6 spores/ml. Two days after inoculation, Red Delicious showed 10% surface decay, which increased to 45% after two more days (Fig. 4). Similar inoculation of the seed locule of Golden Delicious resulted in minor surface infection 2 days after inoculation and 4% after two more days (Fig. 4). Differing susceptibility to *Alternaria* colonization of the locule was also observed following inoculation with increasing concentrations of *Alternaria* spores from 10^5 to as much as 10^7 spores per ml (Fig. 5). As the concentration of *Alternaria* spores increased, so did the colonized area of the locule of the susceptible cultivar. At all the concentrations used, the locule of the susceptible cultivar showed significantly greater incidence of infection than that of the resistant one (Fig. 5).

The seed locule of Red Delicious also showed greater susceptibility to colonization than that of Golden Delicious when inoculated with the aggressive SH-42 isolate of *A. alternata*. The percentage of the surface of the seed locule covered by SH-42 colonization was six to seven times greater than that by RM-44 colonization in both the susceptible and the resistant cultivars

Fig. 4. Surface colonization of isolated seed locule tissue of cvs. Red Delicious and Golden Delicious apples following inoculation with *Alternaria alternata* spores. Values of colonization are expressed as the percentage of the surface covered by dark rot symptoms as observed 5 days after inoculation with 20 μ l of *A. alternata* spore suspension containing 10^6 spores/ml and incubation at 20 to 22°C. Different letters indicates significant ($P < 0.05\%$) differences between the averages according to Student's *t*-test.

Fig. 3. Colonization of growing fruits of **A**, Red Delicious and **B**, Golden Delicious apples by *Alternaria alternata* from 14 days until 110 days after full bloom. The cylindrical region adjacent to the ovary was sliced into four sections designated as: ovary 1 = section adjacent to the calycine tube; ovary 2 = section next to ovary 1; ovary 3 = section next to ovary 2; and ovary 4 = section nearest to the pedicel and furthest from the calycine tube. Different letters indicate significant ($P < 0.05\%$) differences between the averages according to Tukey's HSD test.

(Fig. 6). This finding suggests that the differential surface colonization by the two isolates was expressed in both the resistant and susceptible cultivars.

In the susceptible cultivar, *A. alternata* not only colonized the seed locule it also breached the locule and penetrated into the mesoderm tissue, where it caused visible brown symptoms.

Fig. 5. Surface colonization of isolated seed locule tissue of cvs. Red Delicious and Golden Delicious apples following inoculation with increasing concentrations of *Alternaria alternata* spores. Colonization levels are expressed as the percentages of the surface covered with dark rot symptoms as observed 5 days after inoculation with 20 μ l of *A. alternata* spore suspensions containing 10⁵ to 10⁷ spores per ml and incubation at 20 to 22°C. Different letters indicate significant ($P < 0.05\%$) differences between the averages according to Student's *t*-test.

Fig. 6. Surface colonization of isolated seed locule tissue of cvs. Red Delicious and Golden Delicious apples following inoculation with the highly virulent SH-42 and the reduced virulence RM-44 isolates of *Alternaria alternata*. A 20- μ l drop of a 10⁶ spores/ml suspension of each isolate of *A. alternata* was placed on the middle of locule tissues that had been dissected from fully mature fruits, harvested 110 to 120 days after full bloom. Different letters indicate significant ($P < 0.05\%$) differences between the averages according to Student's *t*-test.

Symptoms of mesoderm colonization were observed in 76% of the cases in which the seed locules of Red Delicious were inoculated with 5 μ l of an *A. alternata* suspension containing 10⁶ spores per ml compared with only 12% in Golden Delicious.

Cellulolytic and pectolytic gene expression by *A. alternata* on inoculated seed locules and on medium with seed locule used as the carbon source. Relative expression levels of *Aapg*, encoding for polygalacturonase, *AaK1* encoding for endoglucanase, *AaC1* encoding for exoglucanase, *AaF1* encoding for endoxylanase, and of *AaMLG1* encoding for a mixed-link glucanase were all greater when *A. alternata* was grown in secondary medium in the presence of locule tissue from the susceptible Red Delicious than of tissue from the resistant Golden Delicious apples (Table 1). The difference in relative gene expression ranged from threefold in the case of *AaF1* to approximately 39-fold for *Aapg* (Table 1). Also, when the fungal RNA was extracted from infected locule tissue, the relative expression levels of all tested genes were higher when the tissue was extracted from the Red Delicious locule than when it came from Golden Delicious (Table 1). The transcript levels of *AaMLG1* and *AaK1* showed 12-fold increases in gene expression, that of *Aapg* a sevenfold increase, and that of *AaF1* only a 2.29-fold increase. When uninoculated Golden Delicious locule tissue was used as the template, no amplification was detected. This verified that the RNA obtained by this method was entirely of fungal origin.

Mechanism modulating the differential colonization of the mesoderm by *A. alternata*. The pH of the mesocarp of the resistant cultivar close to the seed locule was 4.2 ± 0.2 compared with 4.8 ± 0.3 in the susceptible cultivar. Growth of the fungus in the presence of secondary inducing medium with apple cell wall used as the carbon source, at an initial pH value of 5.3, induced 12- and 19-fold increases in endo- and exoglucanase activity levels, respectively, compared with the activity levels at pH 4.3 (Table 2). The final pH in the decayed mesocarp tissue of Red Delicious was 5.8.

Relative expression levels of *AaK1* encoding for EG and of *AaC1* encoding for EXG increased 49- and 90-fold, respectively, when *A. alternata* was grown in the presence of liquid medium containing cell wall prepared from mesoderm tissue of cv. Red

TABLE 2. Effect of pH on the activity level of β -1,4 endoglucanase (EG) and exoglucanase (EXG) secreted by *Alternaria alternata*

	Enzymatic activity level (U/mg of protein) ^a		
	4.3 pH	4.8 pH	5.3 pH
EG	0.04 $\pm$ 0.01	0.2 $\pm$ 0.03	0.5 $\pm$ 0.04
EXG	0.20 $\pm$ 0.10	0.5 $\pm$ 0.01	3.9 $\pm$ 0.4

^a Following growth on a primary medium, the fungus was transferred for 16 h to a secondary medium containing 1% of apple cell wall as the carbon source fitted to the corresponding pH. For enzyme activity and unit determination details refer to methods described in Materials and Methods section. The average enzymatic activities of three independent replications $\pm$ standard deviations are presented.

TABLE 1. Relative levels of *Aapg*, *AaK1*, *AaC1*, *AaF1*, and *AaMLG1* expressed by *Alternaria alternata* on locule inducing media or directly seed locule of Golden Delicious (Golden) and Red Delicious (Red) apples

Genes	Relative transcript expression ^a			
	Medium with locule tissue		Seed locule tissue	
	Golden	Red	Golden	Red
<i>Aapg</i>	1.01 $\pm$ 0.25	38.71 $\pm$ 1.71	1.00 $\pm$ 0.0	7.06 $\pm$ 0.14
<i>AaK1</i>	1.00 $\pm$ 0.17	15.30 $\pm$ 0.90	1.07 $\pm$ 0.55	12.00 $\pm$ 7.12
<i>AaC1</i>	1.00 $\pm$ 0.09	27.74 $\pm$ 1.18	1.00 $\pm$ 0.04	2.90 $\pm$ 0.39
<i>AaF1</i>	1.00 $\pm$ 0.06	3.28 $\pm$ 0.65	1.04 $\pm$ 0.39	2.29 $\pm$ 0.26
<i>AaMLG1</i>	1.00 $\pm$ 0.05	11.99 $\pm$ 2.33	1.28 $\pm$ 1.14	12.10 $\pm$ 2.65

^a The relative transcript expression levels in the inoculated media were analyzed 16 h after transfer from the primary media to the inducing secondary media containing 1% of ground apple locule as a sole carbon source, buffered with phthalate buffer at pH 6.0 or 5 days after inoculation of the seed locule. The relative expression levels are the averages of three replications $\pm$ standard deviations of the treatment.

Delicious buffered to pH 4.8 compared with their levels when the medium was buffered to pH 4.2.

DISCUSSION

Fungal spores of *A. alternata* that penetrate the fruit seed locule and cause core rot symptoms may penetrate the fruit via the calyx sinus at any stage of fruit development or postharvest life (17,32). Susceptibility to fungal infection in senescing blossoms may be enhanced by wet weather, which can provide conditions favorable for fungal growth and sporulation (3,6). However, the current results indicate that the core rot-susceptible cv. Red Delicious and the resistant cv. Golden Delicious did not differ in the incidence of *A. alternata* colonization of the style and ovary of the flower during the first 14 DAFB. However, from the 14th until the 110th DAFB, a significant increase in the incidence of *A. alternata* colonization of the core was observed in the susceptible Red Delicious apples. The highest level of colonization of the Red Delicious core was located in the tissue at the end of the calyx sinus, designated as 'ovary 1', which showed almost 100% fungal colonization starting from 30 and continuing up to 110 DAFB, whereas in tissue sampled successively further away from the calyx tube, ovaries 2, 3, and 4, the incidence of colonization declined from approximately 80 to 25%. In the resistant cultivar, however, the incidence of colonization increased from 38 to 82% in the tissue of ovary 1 from 30 until 110 DAFB, but other ovary parts showed low levels of colonization (0 to 15%). The presence of *Alternaria* hyphae on specific tissues of the resistant cv. Golden Delicious suggests that infection of the apple core may occur also in cultivars that have no calyx tube (31). These results raise questions concerning the importance of fungal colonization of senescing blossoms and the importance of the calyx sinus for penetration to the apple core (31).

The finding that several parts of the ovary showed close to 100% colonization by the fungus, although at harvest there were only relative low percent (8%) of core rot symptoms, led us to look for other factors that might affect mesoderm colonization in the resistant and susceptible cultivars, following the colonization of the core (20). Two hypotheses were tested regarding the basis of the differential susceptibility of Red and Golden Delicious: (i) that the differences between cultivars are affected by the differential susceptibility of the mesoderms to fungal colonization; and/or (ii) that the difference between the cultivars resulted from the differing susceptibility of their seed locule walls. No difference was observed between the levels of mesoderm colonization of the susceptible and resistant cultivars by *A. alternata* when the fruits were wound inoculated. However, inoculation of *A. alternata* into the seed locule wall of the susceptible cultivar revealed greater susceptibility to surface colonization than that of the resistant one. Several findings suggest that the Red Delicious locule is more sensitive to *Alternaria* penetration than that of Golden Delicious: (i) the proportion of inoculations of the seed locule that breached to the mesocarp was greater in the susceptible than in the resistant cultivar; (ii) inoculation of the seed locule with successively greater spore concentrations increased the infected surface of the locule of the susceptible cultivar but not that of the resistant one; (iii) inoculation of the seed locule with more virulent *A. alternata* isolates caused greater increases in the locule surface colonization in the susceptible cultivar than in the resistant one; (iv) analysis of transcript levels of *A. alternata* cellulolytic and pectolytic genes following inoculation on the apple seed locule of Red Delicious revealed 2- to 12-fold greater transcript levels than those following inoculation on the Golden Delicious locule. The genes were *Aapg* encoding for polygalacturonase, *AaK1* encoding for EG, *AaC1* encoding for exoglucanase, *AaF1* encoding for endoxylanase, and *AaMLG1* encoding for a mixed-link glucanase; (v) analysis of the transcript levels of the same genes when *A. alternata* was grown in culture medium with Red Delicious seed lo-

cules as the carbon source showed 3- to 39-fold greater transcript levels than when the fungus was grown in the presence of the Golden Delicious locule; and (vi) the pH of the mesoderm tissue adjacent to the seed locule of the Red Delicious was higher than that of the Golden Delicious locule and, therefore, enhanced the activity level of cellulolytic enzymes. All these results suggest that cultivar susceptibility to core rot is strongly affected by the initial colonization of the locule by the fungus and its progress into the mesoderm. However, the incidence of *A. alternata* colonization in Golden Delicious, an apple cultivar without a calyx tube, suggests that the infection of senescing blossoms and the presence of a calyx tube may affect the incidence of colonization but do not determine its development into the mesoderm. The comparison between resistant and susceptible apple cultivars that differ in their susceptibility to core rot has indicated that seed locule colonization is a key factor in modulating the colonization of the mesoderm. Inoculation of seed locules resulted in colonization of the mesoderm only in the susceptible cv. Red Delicious. It is still not known what the basis for locule resistance to fungal attack is. Resistance to the development of wound-infecting pathogenic fungi in apples has been related to the tenderness/toughness of the epidermal layer and the cortical tissue (32). Golden Delicious has a more tender skin and is more susceptible to *Penicillium expansum* than Granny Smith, which has a tough epidermal layer and cortical tissue (32). Concentrations of the phenol-like procyanidine in the peel of some apple cultivars were also hypothesized to be a factor for resistance against *Venturia inaequalis* (34). Although cultivars such as Granny Smith, which have a higher level of polyphenols (more than 2,400 mg/kg), were more resistant to scab than Golden Delicious with 1,200 mg/kg (14), nothing is known about the phenols content in the core tissue. It is also possible that differential core resistance results from lack of activation of fungal enzymes. Epicatechin, a phenol commonly present in apple fruit skin (34), was found to inhibit the activity of pectate lyase secreted by *Colletotrichum gloeosporioides* in avocado peel (35). Another possible cause of the differential attack could be related to the acid content of the fruit and its resulting pH. De Kock et al. (17) suggested that the susceptibility of cv. Starking apples to core rot was due to low malic and fumaric acid contents during the growth and development period. This could account for the current findings that the pH of the susceptible cultivar was almost half a pH unit higher than that of the resistant cultivar (pH 4.8 compared with 4.3), and that endo- and exoglucanase activity levels were enhanced at the pH of the mesocarp of the susceptible cultivar. This suggests that the differing pH levels close to the core could influence the initiation of fungal colonization by activation of specific gene expression, as observed during the activity of alkalinizing or acidifying pathogens (10–12,13,15,16,23,26). The capability of *A. alternata* to alkalinize the host tissue after penetration also accounts for the increase of pH, from 4.8 in healthy mesoderm tissue to 5.8 in the decayed mesoderm tissue close to the core of cv. Red Delicious. In summary, colonization of the seed locule is an important factor in determining cultivar susceptibility, and the understanding of the mechanism of the resistance of this tissue will provide an important tool for future studies on the pathogenicity of *A. alternata*-induced core rot.

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