Changes in Winter Squash Fruit Exocarp Structure Associated with Age-Related Resistance to *Phytophthora capsici*

Safa A. Alzohairy, Raymond Hammerschmidt, and Mary K. Hausbeck†

Department of Plant, Soil, and Microbial Sciences, Michigan State University, East Lansing, MI 48824
Accepted for publication 24 August 2019.

ABSTRACT

Phytophthora capsici is a destructive pathogen of cucurbits that causes root, crown, and fruit rot. Winter squash (*Cucurbita* spp.) production is limited by this pathogen in Michigan and other U.S. growing regions. Age-related resistance (ARR) to *P. capsici* occurs in *C. moschata* fruit but is negated by wounding. This study aimed to determine whether structural barriers to infection exist in the intact exocarp of maturing fruit exhibiting ARR. Five *C. moschata* cultivars were evaluated for resistance to *P. capsici* 10, 14, 16, 18, and 21 days postpollination (dpp). Scanning electron microscopy imaging of Chieftain butternut fruit exocarp of susceptible fruit at 7 dpp and resistant fruit at 14 and 21 dpp revealed

significant increases in cuticle and epidermal thicknesses as fruit aged. *P. capsici* hyphae penetrated susceptible fruit at 7 dpp directly from the surface or through wounds before 6 h postinoculation (hpi) and completely degraded the fruit cell wall within 48 hpi. Resistant fruit remained unaffected at 14 and 21 dpp. The high correlation between the formation of a thickened cuticle and epidermis in maturing winter squash fruit and resistance to *P. capsici* indicates the presence of a structural barrier to *P. capsici* as the fruit matures.

Keywords: genetics and resistance

Plants are naturally resistant to most pathogenic microbes; however, each species has members that can be infected by one or more microbial pathogens (Lipka et al. 2008). Depending on the plant–pathogen interaction, disease resistance can be divided into two types: nonhost resistance and host resistance (Gill et al. 2015). Nonhost resistance is a form of resistance against all pathogens that are not adapted to cause disease on a particular plant species (Heath 1991, 2000; Senthil-Kumar and Mysore 2013). Nonhost resistance would be useful to deploy by breeding efforts, owing to its durability against multiple pathogens (Niks and Marcel 2009). In host resistance, a cultivar or accession is resistant to a particular pathogen race or isolate that can infect other cultivars (Heath 1991, 2000; Staskawicz et al. 1995). Host resistance can be observed in several different forms, including vertical resistance in which a single resistance gene is responsible for protecting the plants and resists against a specific pathogen race or isolate (gene-for-gene resistance); horizontal resistance in which a group of genes act against a wide range of pathogen races or isolates (Hammerschmidt 2015); or age-related resistance (ARR) in which plants or plant parts acquire resistance with development, which is also known as ontogenic resistance (Whalen 2005).

ARR has been documented in a number of host–pathogen systems (Develey-Rivière and Galiana 2007; Kim et al. 1989; Kus et al. 2002; Lazarovits et al. 1981; Panter et al. 2002). For example, as apple leaves mature, they become more resistant to infection by *Venturia inaequalis* (Gusberty et al. 2013), and hypocotyl tissue in the soybean seedling becomes resistant to *Phytophthora sojae* as the tissue ages (Lazarovits et al. 1981). ARR has also been observed in different cucurbits (Ando et al. 2009; Gevens et al. 2006; Krasnow and Hausbeck 2016; Meyer and Hausbeck 2013) and

Solanaceae crops (Biles et al. 1993; Simonds and Kreutzer 1944). Cucurbits and solanaceous vegetable crops express ARR to *P. capsici* Leonian (Ando et al. 2009; Biles et al. 1993; Gevens et al. 2006; Hausbeck and Lamour 2004; Krasnow and Hausbeck 2016; Meyer and Hausbeck 2013). In the case of squash (*Cucurbita pepo*, *C. moschata*) (Ando et al. 2009; Krasnow and Hausbeck 2016), cucumber (Ando et al. 2009; Gevens et al. 2006; Hausbeck and Lamour 2004), and pumpkin (*C. moschata*) (Krasnow and Hausbeck 2016; Meyer and Hausbeck 2013), the fruit is the pathogen's main target but the fruit develops resistance to infection as it matures.

The timing of ARR development in fruit is variable among cucurbits (Ando et al. 2009; Meyer and Hausbeck 2013). It has been observed in cucumber that the fruit developed resistance to *P. capsici* when the fruit reached its maximum length (Ando et al. 2009, 2015; Gevens et al. 2006). Completion of fruit elongation can vary in different cucurbits. For cucurbits that produce large fruit (e.g. pumpkins and squash), fruit size reaches its maximum between 20 and 24 days postpollination (dpp). For cucurbits that produce small fruit, maximum fruit size is achieved between 15 and 20 dpp (Loy 2004). In cucumber, full length of the fruit is achieved 10 to 12 dpp and corresponds to fruit resistance (Ando et al. 2009). Processing pumpkins *C. maxima* ‘Golden Delicious’ and *C. moschata* ‘Dickenson Field’ have a relatively long maturation period. Dickenson Field develops resistance to *P. capsici* 21 dpp, whereas Golden Delicious remains susceptible (Meyer and Hausbeck 2013).

The mechanisms controlling ARR to *P. capsici* have been studied in different cucurbits (Krasnow and Hausbeck 2016; Mansfeld et al. 2017; Meyer and Hausbeck 2013) and pepper (Biles et al. 1993). However, the mechanism of resistance in squash fruit is unknown. Because wounding resistant fruit negated ARR (Biles et al. 1993; Krasnow et al. 2014), it is hypothesized that changes in the fruit exocarp provide resistance to maturing fruit. Evidence of the presence of antifungal activity has been detected in intact resistant cucumber fruit exocarp (Mansfeld et al. 2017). However, wounding the resistant cucumber demonstrated that the underlying fruit tissue was still susceptible (Mansfeld et al. 2017).

This study aimed to determine the following: (i) the onset of ARR to *P. capsici* during fruit development among five *C. moschata* commercial cultivars, (ii) the cytological change in the fruit exocarp cell wall related to ARR, and (iii) the cytological change in the fruit exocarp cell wall related to ARR under the effect of *P. capsici*

†Corresponding author: M. K. Hausbeck; hausbec1@msu.edu

Funding: This research was supported by Michigan State University (Project GREEN GR16-066), the U.S. Department of Agriculture National Institute of Food and Agriculture (award 2015-51181-24285), Michigan AgBioResearch, and the Michigan Vegetable Council.

The author(s) declare no conflict of interest.

inoculation and detection of hyphal penetration mechanisms in young susceptible and maturing resistant fruit using scanning electron microscopy (SEM).

MATERIALS AND METHODS

Five *C. moschata* cultivars were selected: Chieftain, Early, Waltham, Avalon (butternut squash; Rupp Seeds Inc., Wauseon, OH), and Dickenson Field (processing pumpkin; Rispens Seeds Inc.). Seeds were planted on 15 June 2015 in 72-cell trays containing soilless peat mixture (Suremix Michigan Grower Products Inc., Galesburg, MI) and grown for 2 weeks in the research greenhouse at Michigan State University (MSU) in East Lansing, Michigan. Thirty seedlings from each cultivar were transplanted on 1 July 2015 to a field site, previously planted to pumpkin, at the MSU Plant Pathology Farm in Lansing, Michigan. The soil type was Capac loam with no known *P. capsici* infestation. Plants were grown on raised plant beds covered with plastic mulch and irrigated twice each week via drip emitters. Plant rows were 30.5 m long with 3.7 m between rows and 61 cm between plants. At anthesis, female flowers were hand-pollinated and tagged with the date. Fruits were harvested 10, 14, 16, 18, and 21 dpp similar to Meyer and Hausbeck (2013) with modifications.

P. capsici isolate 12889 (mating type A1, insensitive to mefenoxam) from bell pepper (Foster and Hausbeck 2010) was selected from the long-term collection of M. K. Hausbeck at MSU. To confirm pathogen virulence prior to inoculation, the isolate was used to inoculate cucumber fruit and was then recovered from the infected fruit and maintained on unclarified V8 agar (143 ml of V8 juice, 3 g of CaCO₃, 16 g of agar, and 850 ml of distilled water) (Krasnow et al. 2017; Miller 1955) under constant fluorescent light at room temperature (21 ± 2°C).

Before inoculation, fruits were surface disinfested with 0.4% sodium hypochlorite solution for 5 min, rinsed with water for 2 min, and allowed to air dry. A 7-mm V8 agar plug, removed from an actively growing 7- to 9-day-old *P. capsici* culture using a cork borer, was used to inoculate the fruit. The agar plug was placed mycelial side down on the fruit at the midpoint between the peduncle and blossom end and covered with a sterilized screw cap (16.5 mm in diameter; Axygen Inc., Union City, CA) that was fixed to the fruit with petroleum jelly. A sterile, uncolonized V8 agar plug was used for control fruit. Fruits were incubated in 99-liter or 62-liter clear plastic bins (Sterilite) lined on the inside edges with water-saturated paper towels to maintain high relative humidity and kept at room temperature (22 ± 2°C) under constant fluorescent light (Krasnow et al. 2014; Meyer and Hausbeck 2012).

Disease severity was assessed at 4 days postinoculation (dpi) by measuring the diameter of the lesion and pathogen growth. A 0 to 4 rating scale was used to visually assess the pathogen growth density, where 0 indicated no visible pathogen growth, 1 indicated water-soaked tissue only, 2 indicated light visible mycelial growth, 3 indicated moderate mycelial growth, and 4 indicated dense mycelial growth (Krasnow and Hausbeck 2016; Krasnow et al. 2014; Meyer and Hausbeck 2013). According to Krasnow and Hausbeck (2016), fruits with a mean disease rating <0.5 were considered resistant and those with a mean disease rating >0.5 and <1.5 were considered intermediately resistant. Disease incidence was calculated as a percentage of infected fruits (Krasnow and Hausbeck 2016). After the assessment, a small (1- to 2-mm) tissue segment at the leading edge of the symptomatic tissue was removed and placed onto V8 agar with ampicillin, rifampicin, pentachloronitrobenzene, and benomyl. The recovered isolate was confirmed as *P. capsici* using morphological characteristics (Waterhouse 1963). The entire field experiment was repeated in 2016.

Chieftain butternut squash fruits were selected for SEM studies for two reasons. First, the resistance of Chieftain fruits develops

at 14 dpp. Second, this cultivar has high productivity value in the field. In mid-May 2016, seeds were planted and grown as previously described. Fruits were harvested 7, 14, and 21 dpp and disinfested as previously described. At 7, 14, and 21 dpp, nine nonwounded fruit were inoculated with *P. capsici* at three sites as technical replicates as described previously. At 6, 24, and 48 h postinoculation (hpi), cross-sections (~2.0 mm thickness × 6 to 10.0 mm width × 2.0 mm depth) from the three inoculated sites of three fruit per hour postinoculation (nine cross-sections per age per hour postinoculation) were prepared at the MSU Center for Advanced Microscopy. Briefly, samples were fixed at 4°C for a minimum of 1 h in 4% glutaraldehyde buffered with 0.1 M of sodium phosphate at pH 7.4, then rinsed briefly in the buffer and dehydrated in an ethanol series (25, 50, 75, 95, and 3× 100%) for 1 h. Samples were freeze-dried in a freeze dryer (model EMS750X; Electron Microscopy Sciences, Hatfield, PA) and then mounted onto aluminum stubs using adhesive tabs (M. E. Taylor Engineering, Brookville, MD). Samples were coated with osmium (~10.0 nm thickness) in a NEOC-AT osmium coater (Meiwafosis Co. Ltd., Osaka, Japan). Images for the cross-sections and top surface of each sample's inoculation sites were examined using SEM.

These fruits were also used to examine exocarp structural differences at nonwounded and noninoculated sites. Cross-sections (~2.0 mm thickness × 6 to 10.0 mm width × 2.0 mm depth) of nonwounded and noninoculated sites of nine fruit per age were prepared as described above. Transverse sections were prepared from nine fruit per age to examine the exocarp surface. After the transverse sections were removed from the fruit, the samples were immediately frozen in liquid N₂, placed in an aluminum freeze-drier basket, and allowed to slowly warm to room temperature on aluminum stubs in a desiccator. Both cross- and transverse sections were examined under a 6610LV scanning electron microscope (JEOL Technics Ltd., Tokyo, Japan) and images were recorded using SEM Control User Interface software version 3.08 (JEOL Technics Ltd.).

ImageJ software, a public domain Java image-processing program (Schneider et al. 2012), was used to visualize the cross-section images and measure the thickness at four different points per image of different cell wall structures, including the cuticle, the top point and midpoint of the epidermal anticlinal walls, and the cortex cell walls of the first four layers below the epidermis. A total of 36 measurements per cell wall structure per age were recorded. Analysis of variance (ANOVA) ($P = 0.05$) was used to detect significant differences among 7, 14, and 21 dpp fruit ages at the cuticle, the top point and midpoint epidermal anticlinal walls, and the cortex cell wall. When a significant difference was found in cell wall structures among ages, pairwise comparisons were achieved using the Tukey honest significant difference test ($P = 0.05$) to determine which age(s) were different. All statistical analyses were conducted using R statistical software (R Development Core Team 2017).

RESULTS

Onset of ARR in *C. moschata* cultivars. Fruits of all cultivars harvested 10 dpp developed disease symptoms 4 dpi and disease incidence across cultivars ranged from 44.4 to 100% (Table 1). Onset of ARR was variable among cultivars (Fig. 1; Table 1). Early and Chieftain butternut squash showed resistance (0% disease) beginning at 14 dpp and remained resistant at 16, 18, and 21 dpp. Dickenson Field fruit exhibited ARR 21 dpp with 11% disease. Avalon fruit were resistant 14 dpp but not at 16 dpp. Avalon fruit were resistant at 18 and 21 dpp (0% disease). Waltham butternut fruit had 11% disease at 18 and 21 dpp. Average disease severity ratings from 10 to 21 dpp for cultivars Early, Chieftain, and Dickenson Field ranged from 0.8 to 0, 2.4 to 0, and 3.2 to 0.1, respectively (Fig. 1; Table 1). For Waltham and Avalon, the highest (2 and 4) and lowest (0.1 and 0) disease ratings occurred 16 and 18 dpp, respectively (Fig. 1; Table 1). Lesion diameter (LD)

decreased as fruit aged in Early, Chieftain, and Dickenson Field, whereas LD in Waltham and Avalon followed the same pattern as their disease ratings (Fig. 1; Table 1), except that Avalon was not diseased at 14 dpp.

Changes in noninoculated fruit exocarp structure across development. SEM images revealed increases in the thickness of the cuticle and epidermal anticlinal walls as fruit aged from 7 to 21 dpp (Fig. 2). The thickness of cuticle and epidermal cell walls, but not the cortex cell walls, increased as the fruit aged (Table 2). Significant differences in the thickness of the cell wall structure among ages were detected (ANOVA $P \leq 0.05$). Pairwise comparisons between ages indicated significant differences at the cuticle and both points of epidermal anticlinal walls between 7 and 14 dpp and 7 and 21 dpp (Fig. 3; Table 3). Significant differences between 14 and 21 dpp were detected in the cuticle and top point of epidermal anticlinal wall thicknesses; differences were not detected in the thickness of the midpoint of the epidermal anticlinal walls. Cortex cell wall thickness measurements were not significantly different between 7 and 14 dpp ($P = 0.9$), whereas differences between means of 7 and 21 dpp ($P = 0.001$) and 14 and 21 dpp ($P = 0.0004$) were significant. SEM images of transverse sections of 7, 14, and 21 dpp showed an increase in the wax deposition on the fruit surface as the fruit aged (Fig. 4).

Effect of *P. capsici* infection on fruit exocarp across development and *P. capsici* hyphal penetration mechanism. When cross-sections of 7, 14, and 21 dpp were examined 6 and 24 hpi, the cell wall did not appear to be affected by *P. capsici* (Fig. 5A to F). Although complete cell wall collapse and fruit tissue degradation were detected 48 hpi for 7 dpp fruits, the tissue of fruit harvested 14 and 21 dpp remained unaffected (Fig. 5G to I). Cross-sections of 7 dpp fruit showed that hyphae penetrated the epidermal layer before 6 hpi and were detected around the vascular bundles at 48 hpi (Fig. 6A and G). No hyphae were detected in cross-sections of 7 dpp fruits at 24 hpi (Fig. 6D). However, complete colonization of 7 dpp fruit by 48 hpi (Fig. 6G) and fruit cell wall degradation (Fig. 5G) confirmed that 7 dpp fruit cell wall penetration occurred before 48 hpi. Cross-section images at 14 and 21 dpp detected no signs of hyphal penetration of

the fruit tissue among the three time intervals (Fig. 6B, E, and H compared with C, F, and I, respectively).

Top surface views 6 hpi of the same cross-section samples from fruit harvested 7 dpp revealed hyphae directly penetrating the

Fig. 1. Average disease rating of *Cucurbita moschata* cultivar fruits at 10, 14, 16, 18, and 21 days postpollination in response to *Phytophthora capsici* inoculation, 4 days postinoculation. The disease rating scale is as follows: 0 = no visible pathogen growth, 1 = water-soaked tissue only, 2 = light visible mycelial growth, 3 = moderate mycelial growth, and 4 = dense mycelial growth.

TABLE 1. Disease rating, lesion diameter, and disease incidence 4 days postinoculation (dpi) with *Phytophthora capsici* for five cultivars of *Cucurbita moschata* at fruit developmental stages 10, 14, 16, 18, and 21 days postpollination (dpp)

Cultivar	Fruit age (dpp)	Disease rating (0 to 4 scale) ^a	Lesion diameter (cm)	Disease incidence (%)
Early	10	0.8	0.7	44
Chieftain		2.4	3.2	78
Dickenson Field		3.2	6.2	100
Waltham		0.8	1.7	44
Avalon		1	1.9	44
Early	14	0	0	0
Chieftain		0	0	0
Dickenson Field		2.3	5.2	78
Waltham		0.4	1.1	33
Avalon		0	0	0
Early	16	0	0	0
Chieftain		0	0	0
Dickenson Field		1.6	3.2	56
Waltham		1.2	2.5	33
Avalon		1.4	2.3	44
Early	18	0	0	0
Chieftain		0	0	0
Dickenson Field		0.7	2.4	44
Waltham		0.1	0.6	11
Avalon		0	0	0
Early	21	0	0	0
Chieftain		0	0	0
Dickenson Field		0.1	0.5	11
Waltham		0.2	0.7	11
Avalon		0	0	0

^a Disease rating and lesion diameter values represent the mean of nine fruits conducted in three experimental replicates per age and cultivar. The disease scale ranged from 0 to 4, where 0 indicates no visible infection, 1 indicates water soaking, 2 indicates light pathogen mycelial growth, 3 indicates moderate pathogen mycelial growth, and 4 indicates extensive pathogen mycelial growth. Rating was done 4 dpi.

epidermal surface (Fig. 7A). Hyphae were observed growing over stomata without entering, bypassing multiple stoma cells without penetrating, and growing toward a wound (Fig. 7B and C). At 24 hpi, hyphae were branched and penetrated the epidermal surface directly; by 48 hpi, multiple hyphal penetration points were detected in 7 dpp fruits (Fig. 7D and E). Top surface images of 14 and 21 dpp detected no direct hyphal penetration across the three time intervals, and hyphae did not enter through stomata (Fig. 7F and G). However, hyphae were observed penetrating stomata at 24 hpi in both 14 and 21 dpp fruits (Fig. 7H and I). At 48 hpi, appressorium was detected, suggesting a direct penetration attempt in 14 dpp fruits (Fig. 7J). However, visible hyphal penetration was not detected in 21 dpp fruits at 48 hpi.

DISCUSSION

Once a production site becomes infested with *P. capsici*, growers must use a variety of tools to manage the disease. Having resistance in the fruit throughout all developmental stages would be very useful in the management of fruit rot disease. In this study, we aimed to examine the mechanism of ARR to *P. capsici* in *C. moschata* cultivars through determining the time of resistance onset, studying the fruit exocarp structural changes across development, and uncovering the mechanism of *P. capsici* hyphal penetration of winter squash fruit. Knowing the time of ARR onset in winter squash is beneficial to growers when choosing a cultivar to be grown where the disease is a risk factor. This form of resistance can be used as part of an integrated management strategy that combines other cultural strategies such as raised beds and effective fungicides. Understanding the ARR mechanism in relation to fruit development would generate some new approaches to help in the development of varieties with better fruit resistance and would provide practical information needed to manage winter squash fruit rot caused by *P. capsici*. In the different years of this study, we observed that ARR is a consistent phenomenon that occurs in winter squash fruit during development and that the effect of the environment or the change in weather such as temperature is less likely to affect the development of fruit ARR. This observation is validated by different studies, such as those of Krasnow and Hausbeck (2016) and Meyer and Hausbeck (2013), that examined winter squash ARR over several years. The onset of resistance was consistently observed in the same time of fruit development across the different cultivars in this study and previous studies. However, the environmental effect may be minor because of the consistent results obtained over several growing seasons.

ARR has been used as part of integrated crop management and part of cultural practices to manage *Botrytis cinerea* on strawberry vines (Cooley et al. 1996) and berries against powdery mildew (Ficke et al. 2002). The combination of ARR with cultural practices can reduce the number of fungicide application times and determine

the schedule of the spray; in addition, it can predict the optimum time of the fungicide application when fruit susceptibility is high in the field. Relying on fungicide use solely in managing fruit rot increases the risk of the pathogen developing fungicide resistance; however, when fruit ARR becomes a consideration in disease management, this risk will be reduced.

ARR onset differed among the five cultivars of *C. moschata* included in this study. ARR onset in Dickenson Field, a processing pumpkin, occurred 21 dpp with <11% diseased fruit, consistent with previous findings (Meyer and Hausbeck 2013). This cultivar was used as a control for our study and included fruit aged 16 and 18 dpp that were not tested previously. The results showed a gradual decrease in susceptibility to *P. capsici* as the fruit aged, suggesting that changes in fruit development are associated with pathogen resistance. Krasnow and Hausbeck (2016) evaluated several butter-nut squash cultivars, including Waltham and Avalon, at 7, 14, 22, and 56 dpp and found them to be intermediately resistant to *P. capsici* at 14 dpp and completely resistant at 22 dpp. Our results showed that Waltham and Avalon were intermediately resistant at 16 dpp and became completely resistant at 21 and 18 dpp, respectively. Early and Chieftain became completely resistant at 14 dpp and later.

Variability in fruit maturity among cultivars might be the reason for the observable difference in ARR onset. Dickenson Field, Waltham, and Avalon require more days to mature than Early and Chieftain, which suggests that Early and Chieftain reach full fruit elongation sooner than other cultivars. Fruit transition from a

TABLE 2. Minimum and maximum thickness of cell wall structures: cuticle, epidermal anticlinal wall top point, epidermal anticlinal wall midpoint, and cortex cell wall across fruit development at 7, 14, and 21 days postpollination in cultivar Chieftain

Days postpollination	Cell wall structure	Thickness, minimum to maximum (μm)
7	Cuticle	0.5–0.9
	Epidermal anticlinal wall top point	1.9–2.5
	Epidermal anticlinal wall midpoint	0.9–1.7
	Cortex	0.7–0.9
14	Cuticle	1.1–1.5
	Epidermal anticlinal wall top point	3.8–4.6
	Epidermal anticlinal wall midpoint	2.7–4.7
	Cortex	0.7–0.8
21	Cuticle	1.3–1.7
	Epidermal anticlinal wall top point	5.0–7.8
	Epidermal anticlinal wall midpoint	3.6–4.9
	Cortex	0.8–1.0

Fig. 2. Scanning electron microscopy images of cross-sections of nonwounded/noninoculated fruits of cultivar Chieftain at A, 7 days postpollination (dpp), B, 14 dpp, and C, 21 dpp. Arrows in C represent the cell wall structures. a = cuticle, b = epidermal anticlinal wall top point, c = epidermal anticlinal wall midpoint, and d = cortex cell wall. Bars represent the direction of measurement.

susceptible to resistant state is associated with complete fruit elongation stage (Ando et al. 2009, 2015; Gevens et al. 2006; Krasnow and Hausbeck 2016) and may be the reason we detected an earlier onset of ARR at 14 dpp in Chieftain and Early than in other cultivars.

The underlying mechanisms of ARR to *P. capsici* have been investigated in other plant systems, including cucumber (Ando et al. 2015) and pepper (Biles et al. 1993); however, different plant species and even different cultivars of the same species can have different mechanisms (Develey-Rivière and Galiana 2007; Panter

and Jones 2002; Whalen 2005). Structural changes are important factors in plant defense against insects and pathogens (Freeman and Beattie 2008; War et al. 2012). The plant cell wall provides a physical barrier against pathogen attack and forms the first layer of protection (Freeman and Beattie 2008). In our SEM study of Chieftain fruit, cell wall collapse and degradation in 7 dpp fruit were observed within 48 hpi from *P. capsici*, whereas older fruit (14 and 21 dpp) remained unaffected (Fig. 5). These results suggest that a physical and/or chemical barrier prevents hyphal penetration by the pathogen to the inner layer of the fruit cell wall.

Epicuticular wax deposition increases as plant age increases (Maiti et al. 2012). Epicuticular wax is a hydrophobic layer that reduces the surface wetness needed for pathogen spores to attach and germinate (Maiti et al. 2012). In our study, wax deposition increased with aging fruit that demonstrated resistance to *P. capsici* (Fig. 4), suggesting the formation of a physical barrier for pathogen hyphal penetration. Wax accumulation may also change the surface by creating a hydrophobic texture that reduces pathogen spore attachment. In addition, wax may contain antifungal compounds, as shown in other plants (Cruickshank et al. 1977; Yin et al. 2011).

Wounding negates ARR (Biles et al. 1993; Krasnow et al. 2014), thus supporting the hypothesis that intact fruit exocarp is essential for resistance. This study and previous studies showed that nonwounded fruits of different cucurbits exhibit ARR to *P. capsici* (Ando et al. 2009; Gevens et al. 2006; Krasnow and Hausbeck 2016; Meyer and Hausbeck 2013). A study with pepper demonstrated resistance to *P. capsici* associated with fruit maturity and the ripening process as the fruit changes from green to red (Biles et al. 1993). An increase in cuticle thickness with fruit maturity was detected in pepper, suggesting a role of the cuticle as a physical barrier to *P. capsici* infection (Biles et al. 1993). A similar phenotype was detected in cucumber fruit; cuticle and epidermal cell wall thickness were increased in resistant fruit 16 dpp compared with susceptible fruit 8 dpp (Ando et al. 2015). The resistant phenotype of the stem end of tomato fruit to *P. capsici* is correlated with its thick cuticle and epidermal cell walls (Simonds and Kreutzer 1944). ARR and the role of cuticle thickness in defense are not unique to the *Cucurbita*–*P. capsici* interaction. In bean

Fig. 3. Histogram showing average thickness (in micrometers) of cell wall structures (cuticle, epidermal anticlinal wall top point, epidermal anticlinal wall midpoint, and cortex cell wall) across fruit development at 7, 14, and 21 days postpollination of cultivar Chieftain. Columns with the same shading but different letters indicate a significant difference ($P \leq 0.05$) in cell wall structure thickness across ages.

TABLE 3. Pairwise comparisons of cell wall structures between fruit developmental stages of cultivar Chieftain^a

Days postpollination	Cuticle	Epidermal anticlinal wall		Cortex
		Top point	Midpoint	
7 versus 14	<0.000001*	5e-07*	<0.00000005*	0.9
7 versus 21	<0.000001*	<0.0000005*	<0.00000001*	0.001*
14 versus 21	0.02*	2e-07*	0.13	0.0004*

^a Asterisks indicate significant differences between ages in the rows ($P \leq 0.05$; Tukey honest significant difference test).

Fig. 4. Scanning electron microscopy images of transverse sections at nonwounded/noninoculated fruits of cultivar Chieftain at **A**, 7 days postpollination (dpp), **B**, 14 dpp, and **C**, 21 dpp. Wax appears as spiny crystals on the fruit surface.

(*Phaseolus vulgaris*), the hypocotyl becomes resistant to *Rhizoctonia solani* with age as its cuticle thickness increases (Stockwell and Hanchey 1983). Peach genotypes resistant to *Monilinia fructicola* have thicker cuticles than the susceptible genotypes (Adaskaveg et al. 1989). When the anatomy of *C. maxima* buttercup squash fruit across developmental stages was studied, an increase in cuticle and cell wall thickness was observed with maturity (Sutherland and Hallett 1993). Our study showed an increase in cuticle and epidermal anticlinal wall thickness (Fig. 2) and wax deposition (Fig. 4) in aging resistant Chieftain fruit compared with a reduced thickness of the same structures and less wax deposition detected in young, susceptible fruit. Our findings suggest that the cuticle and epidermis act as a barrier to pathogen penetration and provide resistance to maturing fruit. A lower level of significance was detected between the thickness of cortex cell walls of young (7 dpp) susceptible and older (21 dpp) resistant fruit compared with a higher level of significance detected in the cuticle and epidermis (Fig. 3). Thus, the cortex may play a less critical role in resistance, since the difference in the thickness of cortex cell walls between 7 dpp susceptible fruit and 14 dpp resistant fruit was not significant and wounding that allows direct access to the cortex overcomes ARR.

P. capsici penetrates the plant surface directly or through natural openings such as the stomata (Hausbeck and Lamour 2004). On

pepper leaves, Du et al. (2013) noted that *P. capsici* zoospores encysted within 3 h and then produced two germ tubes that penetrated the surface directly; an appressorium was not detected. In our study, *P. capsici* hyphae penetrated susceptible fruit tissue before 6 hpi (Fig. 6A). Appressorium formation was detected 48 hpi on the exocarp surface of the resistant fruit (Fig. 7J), suggesting an attempt by the pathogen to penetrate the tissue. Appressoria may secrete enzymes to facilitate penetration (Ryder and Talbot 2015).

When fruit were harvested 7 dpp, pathogen hyphae penetrated directly or through wounds by 6 hpi (Fig. 7A and C). Hyphae were embedded in the epidermal cells of young susceptible fruit by 6 hpi (Fig. 6A). We did not observe penetration through stomata of any of the squash fruit 6 hpi regardless of age (Fig. 7B, F, and G). Hyphae were observed growing over the stomata without penetration (Fig. 7B, F, and G), similar to what Du et al. (2013) observed in pepper leaves inoculated with *P. capsici*. This suggests that penetration through the stomata is not preferred by the pathogen at early hours of infection. A similar pattern was observed with *Cercospora henningsii* where the pathogen passed over the stomata without entering (Babu et al. 2009). However, at 24 hpi, hyphal penetration through the stomata in squash fruit harvested at 14 and 21 dpp was detected (Fig. 7H and I); these findings suggest that when the pathogen encounters obstacles preventing its direct penetration through the surface, it attempts to penetrate through

Fig. 5. Scanning electron microscopy images of cross-sections at 6 h postinoculation (hpi), 24 hpi, and 48 hpi of cultivar Chieftain fruits at **A, D, and G**, 7 days postpollination (dpp), **B, E, and H**, 14 dpp, and **C, F, and I**, 21 dpp, respectively, showing the effect of *Phytophthora capsici* inoculation on cell wall integrity. The white circles point to the inoculation site.

the stomata. Barriers to hyphal penetration might also be present in cells in the substomatal cavity, since hyphae were not detected inside the resistant fruit tissue (Fig. 6B, E, and H and C, F, and I, respectively). Krasnow and Hausbeck (2016) found that *P. capsici* zoospores accumulated over the stomata of a susceptible *C. maxima* cultivar, whereas no accumulation was detected on a *C. moschata* cultivar that exhibits ARR.

Mycelia colonized the vascular bundles of the susceptible 7 dpp fruit by 48 hpi (Fig. 6G), whereas colonization was not observed at or near the vascular bundles in the 14 and 21 dpp resistant fruit where colonization was restricted to the surface. Similar results were reported with *P. sojae* on soybean where the pathogen colonized the vascular tissue of the roots of a susceptible cultivar but not of a resistant cultivar (Enkerli et al. 1997). When fruit were harvested 7 dpp, *P. capsici* penetrated the cuticle and colonized the epidermis, cortex, and vascular tissues within 48 hpi (Fig. 6G). Colonization was not detected in fruit harvested at 14 and 21 dpp (Fig. 6B, E, and H and C, F, and I, respectively). Fruit harvested at 14 dpp or later exhibited ARR (Fig. 1). Our results are consistent with a study by Kim and Kim (2009), in which *P. capsici* colonized the epidermis, cortex, and vascular tissue of pepper roots in a susceptible cultivar but did not colonize the vascular tissue of a resistant cultivar. The pathogen was not able to infect or colonize the roots of the resistant cultivar roots owing to root structural defenses (Kim and Kim 2009).

In conclusion, our study showed a strong correlation between the thickness of cell wall structures including the cuticle and epidermis and the onset of ARR in Chieftain winter squash fruits (Fig. 3; Table 3). These results suggest that the mechanism of ARR is attributable to the presence of a structural barrier to *P. capsici*. The results, however, do not explain how the thickened cell walls prevent infection. It is possible that the thickened walls provide a physical barrier that does not allow penetration of *P. capsici* through the cell walls or even between cells. It is also possible that the cell walls have become resistant to the types of cell wall-degrading enzymes that *P. capsici* uses in the colonization of host tissues. It is likely that both changes in physical properties and resistance to degradation play a role in the expression of ARR, but this will require further chemical and physical analyses of the cell walls. Structural defenses, such as cell wall lignification, may play a role in resistance against plant pathogens. However, in our study, we have not determined the chemical composition of the winter squash fruit cell wall and it is important to consider this in future investigations.

Selection of germplasm with resistance to *P. capsici* is of interest to plant breeders and growers; however, complete plant resistance has not been identified. ARR could be a valuable phenotype to integrate into breeding programs. The incorporation of cultivars expressing ARR early (i.e., 14 dpp) could benefit management of *P. capsici*. Because the early onset of ARR might be related to fewer

Fig. 6. Scanning electron microscopy images of cross-sections at 6 h postinoculation (hpi), 24 hpi, and 48 hpi of cultivar Chieftain fruits at **A, D, and G**, 7 days postpollination (dpp), **B, E, and H**, 14 dpp, and **C, F, and I**, 21 dpp, respectively. hy = hypha, c = cuticle, eaw = epidermal anticlinal wall, and vb = vascular bundle. No hyphae were detected in tested cross-sections of 7 dpp at 24 hpi (D).

days to maturity, growers could select cultivars with desired horticultural characteristics that express ARR with fewer days to maturity. Growers should consider protecting the early developing fruit with fungicides within the first to second week of development, and then a less intensive fungicide program could be used for the duration of the season. After harvest, mature fruit of winter squash or pumpkin with ARR could be at a reduced risk of infection by *P. capsici*, provided the fruits are handled carefully to prevent wounding and not exposed to inoculum from infested water washes

or adjacent infected fruit. ARR is an interesting heritable trait for breeders (Panter and Jones 2002). Breeders can select cultivars with early onset of ARR for incorporation in breeding squash cultivars resistant to *P. capsici*. However, ARR-associated genes are not yet identified in winter squash of *C. moschata*; it is essential to identify and utilize ARR-associated genes in screening methods for squash cultivars with resistance to *P. capsici* during fruit development, which may accelerate the process of selection for resistant varieties to *P. capsici*.

Fig. 7. Scanning electron microscopy images of the top surface view of cultivar Chieftain fruits. **A**, Fruit 7 days postpollination (dpp) at 6 h postinoculation (hpi), with hyphal direct penetration from the epidermal surface. **B**, Fruit 7 dpp at 6 hpi, with hypha passing over stomata without entering. **C**, Fruit 7 dpp at 6 hpi, with hypha traveling toward entering through the wound and bypassing multiple stomata. **D**, Fruit 7 dpp at 24 hpi, with hypha branching and directly penetrating. **E**, Fruit 7 dpp at 48 hpi, with multiple penetration points. **F and G**, Fruit 14 and 21 dpp, respectively, at 6 hpi, showing hyphae passing over the stomata but not entering. **H and I**, Fruit 14 and 21 dpp, respectively, at 24 hpi, showing hyphae entering through the stomata. **J**, Fruit 14 dpp at 48 hpi, showing appressorium. sr = surface ridge, es = epidermal surface, hy = hypha, st = stomata, w = wound, and ap = appressorium.

ACKNOWLEDGMENTS

We thank Charles S. Krasnow for timely assistance in some of our experiments.

LITERATURE CITED

- Adaskaveg, J. E., Feliciano, A. J., and Ogawa, J. M. 1989. Comparative studies of resistance in peach genotypes to *Monilinia fructicola*. *Phytopathology* 79:1183-1184.
- Ando, K., Carr, K. M., Colle, M., Mansfeld, B. N., and Grumet, R. 2015. Exocarp properties and transcriptomic analysis of cucumber (*Cucumis sativus*) fruit expressing age-related resistance to *Phytophthora capsici*. *PLoS One* 10:e0142133.
- Ando, K., Hammar, S., and Grumet, R. 2009. Age-related resistance of diverse cucurbit fruit to infection by *Phytophthora capsici*. *J. Am. Soc. Hortic. Sci.* 134:176-182.
- Babu, A. M., Philip, T., Kariappa, B. K., and Kamble, C. K. 2009. Scanning electron microscopy of the infection process of *Cercospora henningsii* on cassava leaves. *J. Phytopathol.* 157:57-62.
- Biles, C. L., Wall, M. M., Waugh, M., and Palmer, H. 1993. Relationship of *Phytophthora* fruit rot to fruit maturation and cuticle thickness of New Mexican-type peppers. *Phytopathology* 83:607-611.
- Cooley, D. R., Wilcox, W. F., Kovach, J., and Schloemann, S. G. 1996. Integrated pest management programs for strawberries in the northeastern United States. *Plant Dis.* 80:228-237.
- Cruickshank, I., Perrin, D., and Mandryk, M. 1977. Fungitoxicity of duvatrienediols associated with the cuticular wax of tobacco leaves. *J. Phytopathol.* 90:243-249.
- Develey-Rivière, M. P., and Galiana, E. 2007. Resistance to pathogens and host developmental stage: A multifaceted relationship within the plant kingdom. *New Phytol.* 175:405-416.
- Du, Y., Gong, Z. H., Liu, G. Z., and Zhao, Y. 2013. Scanning electron microscopic study of the infection process of *Phytophthora capsici*. *Pak. J. Bot.* 45:1807-1811.
- Enkerli, K., Mims, C. W., and Hahn, M. G. 1997. Ultrastructure of compatible and incompatible interactions of soybean roots infected with the plant pathogenic oomycete *Phytophthora sojae*. *Can. J. Bot.* 75:1493-1508.
- Freeman, B. C., and Beattie, G. A. 2008. An overview of plant defenses against pathogens and herbivores. *Plant Health Instr.* doi:10.1094/PHI-I-2008-0226-01
- Ficke, A., Gadoury, D. M., and Seem, R. C. 2002. Ontogenic resistance and plant disease management: A case study of grape powdery mildew. *Phytopathology* 92:671-675.
- Foster, J. M., and Hausbeck, M. K. 2010. Resistance of pepper to *Phytophthora* crown, root, and fruit rot is affected by isolate virulence. *Plant Dis.* 94: 24-30.
- Gevens, A. J., Ando, K., Lamour, K. H., Grumet, R., and Hausbeck, M. K. 2006. A detached cucumber fruit method to screen for resistance to *Phytophthora capsici* and effect of fruit age on susceptibility to infection. *Plant Dis.* 90:1276-1282.
- Gill, U. S., Lee, S., and Mysore, K. S. 2015. Host versus nonhost resistance: Distinct wars with similar arsenals. *Phytopathology* 105:580-587.
- Gusberti, M., Gessler, C., and Broggini, G. A. 2013. RNA-Seq analysis reveals candidate genes for ontogenic resistance in *Malus-Venturia* pathosystem. *PLoS One* 8:e78457.
- Hammerschmidt, R. 2015. Being an adult plant sometimes has its advantages. *Physiol. Mol. Plant Pathol.* 89:v-vi.
- Hausbeck, M. K., and Lamour, K. H. 2004. *Phytophthora capsici* on vegetable crops: Research progress and management challenges. *Plant Dis.* 88:1292-1303.
- Heath, M. C. 1991. Evolution of resistance to fungal parasitism in natural ecosystems. *New Phytol.* 119:331-343.
- Heath, M. C. 2000. Nonhost resistance and nonspecific plant defenses. *Curr. Opin. Plant Biol.* 3:315-319.
- Kim, S. G., and Kim, Y. H. 2009. Histological and cytological changes associated with susceptible and resistant responses of chili pepper root and stem to *Phytophthora capsici* infection. *Plant Pathol. J.* 25:113-120.
- Kim, Y. J., Hwang, B. K., and Park, K. W. 1989. Expression of age-related resistance in pepper plants infected with *Phytophthora capsici*. *Plant Dis.* 73:745-747.
- Kovach, J., and Schloemann, S. G. 1996. Integrated pest management programs for strawberries in the northeastern United States. *Plant Dis.* 80: 228-237.
- Krasnow, C. S., Hammerschmidt, R., and Hausbeck, M. K. 2017. Characteristics of resistance to *Phytophthora* root and crown rot in *Cucurbita pepo*. *Plant Dis.* 101:659-665.
- Krasnow, C. S., and Hausbeck, M. K. 2016. Evaluation of winter squash and pumpkin cultivars for age-related resistance to *Phytophthora capsici* fruit rot. *HortScience* 51:1251-1255.
- Krasnow, C. S., Naegele, R. P., and Hausbeck, M. K. 2014. Evaluation of fruit rot resistance in *Cucurbita* germplasm resistant to *Phytophthora capsici* crown rot. *HortScience* 49:285-288.
- Kus, J. V., Zaton, K., Sarkar, R., and Cameron, R. K. 2002. Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. *Plant Cell* 14:479-490.
- Lazarovits, G., Stössel, R., and Ward, E. W. B. 1981. Age-related changes in specificity and glyceollin production in the hypocotyl reaction of soybeans to *Phytophthora megasperma* var. *sojae*. *Phytopathology* 71:94-97.
- Lipka, U., Fuchs, R., and Lipka, V. 2008. *Arabidopsis* non-host resistance to powdery mildews. *Curr. Opin. Plant Biol.* 11:404-411.
- Loy, J. B. 2004. Morpho-physiological aspects of productivity and quality in squash and pumpkins *Cucurbita* spp. *Crit. Rev. Plant Sci.* 23:337-363.
- Maiti, R., Satya, P., Rajkumar, D., and Ramaswamy, A., eds. 2012. *Crop Plant Anatomy*. CABI, Cambridge, UK.
- Mansfeld, B. N., Colle, M., Kang, Y., Jones, A. D., and Grumet, R. 2017. Transcriptomic and metabolomic analyses of cucumber fruit peels reveal a developmental increase in terpenoid glycosides associated with age-related resistance to *Phytophthora capsici*. *Hortic. Res.* 4:17022.
- Meyer, M. D., and Hausbeck, M. K. 2012. Using cultural practices and cultivar resistance to manage *Phytophthora* crown rot on summer squash. *HortScience* 47:1080-1084.
- Meyer, M. D., and Hausbeck, M. K. 2013. Age-related resistance to *Phytophthora* fruit rot in 'Dickenson Field' processing pumpkin and 'Golden Delicious' winter squash fruit. *Plant Dis.* 97:446-452.
- Miller, P. M. 1955. V-8 juice agar as a general purpose medium for fungi and bacteria. *Phytopathology* 45:461-462.
- Niks, R. E., and Marcel, T. C. 2009. Nonhost and basal resistance: How to explain specificity? *New Phytol.* 182:817-828.
- Panter, S. N., Hammond-Kosack, K. E., Harrison, K., Jones, J. D., and Jones, D. A. 2002. Developmental control of promoter activity is not responsible for mature onset of Cf-9B-mediated resistance to leaf mold in tomato. *Mol. Plant-Microbe Interact.* 15:1099-1107.
- Panter, S. N., and Jones, D. A. 2002. Age-related resistance to plant pathogens. *Adv. Bot. Res.* 38:252-281.
- R Development Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Ryder, L. S., and Talbot, N. J. 2015. Regulation of appressorium development in pathogenic fungi. *Curr. Opin. Plant Biol.* 26:8-13.
- Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods.* 9:671-675.
- Senthil-Kumar, M., and Mysore, K. S. 2013. Nonhost resistance against bacterial pathogens: Retrospectives and prospects. *Annu. Rev. Phytopathol.* 51:407-427.
- Simonds, A. O., and Kreutzer, W. A. 1944. Infection phenomena in tomato-fruit rot caused by *Phytophthora capsici*. *Phytopathology* 34:813-817.
- Staskawicz, B., Ausubel, F., Baker, B., Ellis, J., and Jones, J. 1995. Molecular genetics of plant disease resistance. *Science* 268:661-667.
- Stockwell, V., and Hanchey, P. 1983. The role of the cuticle in resistance of beans to *Rhizoctonia solani*. *Phytopathology* 73:1640-1642.
- Sutherland, P. W., and Hallett, I. C. 1993. Anatomy of fruit of buttercup squash *Cucurbita maxima* D. surface, cuticle, and epidermis. *N. Z. J. Crop Hortic. Sci.* 21:67-72.
- War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., and Sharma, H. C. 2012. Mechanisms of plant defense against insect herbivores. *Plant Signal. Behav.* 7:1306-1320.
- Waterhouse, G. M. 1963. Key to the species of *Phytophthora* de Bary. Commonwealth Mycological Institute, Kew, UK, *Mycol. Papers* 92:22.
- Whalen, M. C. 2005. Host defence in a developmental context. *Mol. Plant Pathol.* 6:347-360.
- Yin, Y., Bi, Y., Chen, S., Li, Y., Wang, Y., Ge, Y., Ding, B., Li, Y., and Zhang, Z. 2011. Chemical composition and antifungal activity of cuticular wax isolated from Asian pear fruit cv. Pingguoli. *Sci. Hortic.* 129: 577-582.