CHARACTERIZATION OF WINTER SQUASH AGE-RELATED RESISTANCE TO
PHYTOPHTHORA CAPSICI THROUGH FRUIT PEEL TRANSCRIPTOME PROFILING AND
INVESTIGATION OF CELL WALL PROPERTIES

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Breeding, Genetics and Biotechnology-Crop and Soil Sciences-
Doctor of Philosophy

2018

ABSTRACT

CHARACTERIZATION OF WINTER SQUASH AGE-RELATED RESISTANCE TO *PHYTOPHTHORA CAPSICI* THROUGH FRUIT PEEL TRANSCRIPTOME PROFILING AND INVESTIGATION OF CELL WALL PROPERTIES

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Fruit rot of winter squash and pumpkin (*Cucurbita moschata*) caused by the oomycete plant pathogen *Phytophthora capsici* limits factor in the production of these crops. Genetic resistance to fruit rot is not available in commercial cultivars, but age-related resistance (ARR) develops in certain cultivars of *C. moschata* which may benefit management strategies. Earlier ARR studies indicate that the peel provides fruit resistance. The goal of this research was to elucidate the structural, biochemical and genetic basis of ARR of the winter squash fruit peel to *P. capsici*. Five *C. moschata* cultivars were evaluated for *P. capsici* resistance 10, 14, 16, 18, and 21 days post pollination (dpp). The onset of resistance was variable among cultivars. A cultivar with ARR at 14 dpp was selected and the fruit exocarp cell wall examined 7 dpp (susceptible) and 14 dpp, 21 dpp (resistant) using scanning electron microscopy (SEM). An increase in cuticle and epidermal walls thickness as the fruit age increased was observed. According to SEM observations, *P. capsici* caused cell wall degradation/tissue collapse to the 7 dpp fruit within 48- hour post inoculation (hpi) while 14 and 21 dpp fruit remained unaffected suggesting a structural barrier to *P. capsici* in resistant fruit. The contribution of fruit exocarp preformed or induced chemical defense against the pathogen was examined in *C. moschata* cultivars across developmental stages using phytochemical analysis. Results showed a decrease in antifungal activity in non-inoculated fruit peel as the fruit age increased. A significant change in antifungal activity was not observed under induced conditions with inoculation of the fruit peel with *P. capsici*, suggesting that there is not a correlation between preformed or induced chemical defense and winter squash fruit ARR to *P.*

capsici. Transcriptome profiling of fruit peel of two *C. moschata* cultivars at susceptible and resistant developmental stages was performed to uncover the molecular mechanism of ARR. Differential gene expression analysis detected upregulation of multiple genes in the resistant compared to the susceptible stages then functional enrichment analysis detected overrepresentation of these genes in cell wall structures biosynthesis. Pathway enrichment analysis of winter squash orthologous genes detected enrichment in cutin, suberin monomers and phenylpropanoids biosynthetic pathways. Further analysis of genes expression profile in those pathways suggests enrichment in monolignol biosynthesis in the resistant fruit peel.

To my parents;
I cannot ever find enough words to thank you for being a good example in my life.
Love you so much.

ACKNOWLEDGEMENTS

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LITERATURE REVIEW

Cucurbitaceae is a large, diverse family that includes approximately 825 species across 118 genera (Bisognin 2002). The Cucurbitaceae family contains many vegetable crops commonly known as cucurbits. Cucurbits are mostly monoecious, tendril-bearing climbing plants, common in tropical and subtropical areas of Africa, Asia, Australia, and America. They prefer warm temperatures and are frost-sensitive but are found in temperate regions (Crase 2011). Cucurbits include crops grown for food and ornamentation. Some cucurbits have medicinal uses due to the compound Cucurbitacin that has anti-inflammatory properties (Abdelwahab *et al.* 2011). The most important genera of this family are *Cucumis* (includes cucumber and melons), *Citrullus* (includes watermelon), and *Cucurbita* (includes squash, pumpkins, and some gourds) (Whitaker and Davis 1962). Cucumber originated from India and melon and watermelon originated from Africa. Squash was originally discovered in South and Central America (Wehner *et al.* 2003).

The *Cucurbita* genus is one of the most morphological diverse genera of the plant kingdom (Robinson *et al.*, 1976). It includes 22 wild and five cultivated species including *C. pepo* L., *C. maxima Duchesne*, *C. moschata Duchesne*, C. *argyrosperma Huber* and C. *ficifolia Bouché*, with diverse characteristics in color, shape, and size (Bisognin 2002). *Cucurbita pepo* includes pumpkin, gourd, acorn squash, summer squash, and zucchini. *C. moschata* includes winter squash and pumpkin. *C. maxima* produces the largest fruit among the flowering plants. Fruits of these crops are eaten when immature (*C. pepo*) or mature (*C. maxima* and *C. moschata*) (Bisognin 2002). Worldwide, these fruits are known as a rich source of vitamin A that is important for human health (Shrivastava *et al.* 2013). Fruits can be baked as a main dish or served as a dessert, and their seeds toasted providing a source of protein and oil (Wehner *et al.* 2003). Flowers, leaves, and vine tips are also consumed in regions including Latin America (Ferriol and Pico 2008). The cultivated

species *C. argyrosperma* and *C. ficifolia* are not widely distributed and are not economically important.

Squash and pumpkins are grown worldwide for both the fresh market and for processing. China is the top producer of squash followed by India, Russia, and the U.S. (FAO 2012). In the U.S., Michigan leads the country for zucchini, summer and hard squash production for fresh market and processing followed by Florida, California, and New York (USDA 2018). In 2017, squash was grown on 5,800 acres in Michigan generating more than $26 million (USDA 2018). The most popular types of squash grown in Michigan include summer squash (yellow and scallop) and zucchini, and the winter squashes (butternut, buttercup, marrow, and acorn) (Pollack 1996). Michigan ranks 4th for pumpkin production following Illinois, Ohio, and Indiana. In 2017, pumpkins were grown on 5,300 acres in Michigan and valued at more than $8 million (USDA 2018).

In Michigan, summer and zucchini squash are direct seeded in late April through late May with harvest beginning in mid-June. Winter squash planting occurs at the same time and continues through early June with harvest beginning in early September (USDA 2018). Due to their monoecious nature, squash is reliant on bee and insect pollination for complete fertilization and fruit set. Summer squash and zucchini are harvested as immature fruit, and winter squash is harvested when the fruits are fully mature and ripened with a hard rind.

Fruit rot caused by *Phytophthora capsici* Leonian (Leonian 1922) limits the production of cucurbits. This disease also threatens fruiting vegetables including tomato, pepper, and eggplant (Tian and Babadoost 2004; Hausbeck and Lamour, 2004). Cucurbits affected by this pathogen include squash, watermelon, cucumber, pumpkin, cantaloupe, gourd, and honeydew. *P. capsici*

causes symptoms including crown rot, foliar blight, and/or fruit rot (Roberts *et al.*, 2001; Babadoost 2005).

PATHOGEN BIOLOGY

Phytophthora capsici classifies as a eukaryote in kingdom Stramenopila (Cavalier-Smith 1986), family Pythiaceae, order Peronosporales and class Oomycetes. The pathogen is producing oospores and biflagellate zoospore (Erwin and Ribeiro 1996, Hardham 2007, Mrázková *et al.* 2011). The cellulose in its cell wall differentiates it from fungi (Hardham 2007, Erwin and Ribeiro 1996). *P. capsici* is heterothallic, requiring two mating types, A1 and A2, for sexual reproduction (Erwin and Ribeiro, 1996, Lamour and Hausbeck 2000). When both mating types coexist in the same field, the mating types produce specific hormones that stimulate the production of gametangia then outcrossing occur and oospores are produced (Ko 1988). Oospores have a thick-wall containing *β*-glucans and cellulose (Erwin and Ribeiro 1996, Bartnicki-Garcia and Wang 1983), which aids pathogen survival in harsh environmental conditions. Oospores can remain viable in the field for a prolonged period (Erwin and Ribeiro 1996, Lamour and Hausbeck 2000, Hausbeck and Lamour 2004, Babadoost and Pavon 2013). Oospores germinate to produce a germ tube and stimulated by root exudates and natural chemicals produced by the plant (Erwin and Ribeiro 1996, Hord and Ristaino 1991). The germ tube can either elongate to form hypha or develop sporangia (Hord and Ristaino 1991).

During the asexual stage, *P. capsici* produces aseptate hyphae that grow at an optimum temperature of 24 – 33°C and produce papillate sporangia with a long pedicle (Babadoost 2004). *P. capsici* sporangia can be subspherical, ovoid, obovoid, ellipsoid, fusiform, or pyriform (Babadoost 2004) with variation influenced by light or other environmental factors (Erwin and

Ribeiro 1996, Babadoost 2004). Mature sporangia are caducous and depend on water for dissemination (Granke *et al.* 2012). *P. capsici* sporangia can germinate to produce a germ tube and infect directly (Judelson and Blanco 2005), or under saturated conditions, the sporangial cytoplasm differentiates into 20 – 40 biflagellate swimming zoospores (Bernhardt and Grogan 1982, Hausbeck and Lamour 2004, Granke *et al.* 2012). The zoospores are negatively geotropic and are chemotactically attracted to the plant host following the nutrient gradient (Khew and Zentmyer 1973, Erwin and Ribeiro 1996).

INFECTION PROCESS

When zoospores come into contact with a plant's surface, they encyst within 3 hours (Du *et al.* 2013) and produce a germ tube (Hickman 1970). *P. capsici* secretes cell wall degrading enzymes that aid in the penetration of the cuticle (Yoshikawa *et al.* 1977, Jia *et al.* 2009). The mechanism of *P. capsici* hyphal penetration is direct or through natural opening such as a wound or stomata (Judelson and Blanco 2005, Katsura and Miyazaki 1960). However, Du *et al.* (2013) showed no penetration through stomata even when *P. capsici* hypha passed over it. Formation of appressoria was observed in some cases, while not in others (Lamour *et al.* 2012, Du *et al.* 2013). *P. capsici* is a hemibiotroph, the biotroph stage occurs during the 2 to 3 days from infection to sporulation. The necrotrophic stage occurs when clusters of mycelia develop inside the tissue and colonize resulting in damage and maceration of the host tissue (Lamour *et al.* 2012, Du *et al.* 2013). *P. capsici* is a polycyclic pathogen with multiple disease cycles. Thus, even a low amount of inoculum may initiate a significant disease outbreak (Erwin and Ribeiro 1996, Hausbeck and Lamour 2004). When environmental conditions are favorable, the pathogen produces sporangia on the surface of infected tissue (Hausbeck and Lamour 2004, Granke *et al.* 2009). The number

of sporangia produced on a single squash fruit was quantified and estimated to be approximately three billion (Lamour *et al.* 2012).

PATHOGEN HOST RANGE AND SYMPTOMS

Phytophthora capsici was discovered in 1922 by Leon H. Leonian at the New Mexico Agricultural Research Station on chili pepper (*Capsicum annuum* L.) (Leonian 1922). Today, *P. capsici* is known to affect over 50 species, including economically important species in the Solanaceae, Fabaceae and Cucurbitaceae families and some Brassicaceae (Davidson *et al.* 2002, Hausbeck and Lamour, 2004, Gevens and Hausbeck 2005, Krasnow and Hausbeck 2015). Tian and Babadoost (2004) considered cucurbits and pepper to be most susceptible to *P. capsici*. The pathogen can cause root and crown rot, foliar and stem blight, seedling damping off, and fruit rot, depending on the host and environmental conditions (Zitter *et al.* 1996, Holmes *et al.* 2001, Islam *et al.* 2002, Babadoost 2000, 2004, 2005, Hausbeck and Lamour 2004). Generally, when *P. capsici* infects the roots of seedlings, damping off occurs, whereas wilting and plant death may occur in older plants (Lamour *et al.* 2012). In cucurbits, *P. capsici* can infect different parts of the plant including the root, crown, vines, leaves, and fruit. The disease often begins on plants located in the low areas of the field, where the water doesn't drain, and the soil remains saturated for an extended period.

Seedling damping off may occur pre- or post-emergence. Symptoms appear as watery lesions on the hypocotyls followed by wilting and death of the seedlings. Disease on cucurbit vines seems as watersoaked lesions that become dark olive to a brown color as the disease progresses. Disease symptoms on infected leaves are similar to those that appear on vines; lesions are initially watersoaked, become chlorotic, and then turn into necrotic lesions with chlorotic margins in a few days (Babadoost 2004). *P. capsici* may cause fruit rot, which begins with dark watersoaked tissue

followed by pathogen sporulation and then fruit degradation (Bababoost 2000, 2004, Meyer and Hausbeck 2013). Fruit infection often starts on the portion of the fruit that is in direct contact with the infested soil. However, rain splash or overhead irrigation may result in infested soil coming into contact with the upper fruit surface resulting in infection. Two to three days after fruit infection, abundant white sporangia have produced that look like powdered sugar on the fruit surface and result in damage to the whole fruit (Babadoost 2004, Gevens *et al.* 2007, Lamour *et al.* 2012, Meyer and Hausbeck 2013). Infected fruit tissue that remains in the field is a source of inoculum for new infection cycles during the growing season. The pathogen can form oospores when both mating types are present (Lamour and Hausbeck 2000, Hausbeck and Lamour 2004). Symptoms of *P. capsici* infection on less susceptible crops can include plant stunting, stem girdling, and cankers (Quesada and Hausbeck 2010).

Susceptibility of different hosts or different cultivars of the same host is determined by the virulence and pathogenicity of the pathogen population in the field (Ristaino 1990, Kim and Hwang 1992, Foster and Hausbeck 2010a). Quesada *et al.* (2011) studied the population structure of *P. capsici* isolates to investigate the genetic diversity and pathogen virulence. They highlighted the importance of using isolates with diverse genetic backgrounds including isolates collected from temperate and tropical regions (Bowers *et al.* 2007) when screening cultivars for disease resistance in a breeding program.

DISEASE MANAGEMENT STRATEGIES

Disease management strategies include chemical and cultural practices (Ristaino and Johnston 1999, Babadoost 2004, Hausbeck and Lamour, 2004, Kousik *et al.* 2011). However, under conditions favorable for disease, significant yield losses still occur (Granke *et al.,* 2012; Hausbeck

and Lamour, 2004). Several factors make *P. capsici* disease management challenging. The broad range of susceptible hosts and the persistence of *P. capsici* oospores in the soil limit the use of crop rotation (Kousik *et al.* 2015, Quesada *et al.* 2009, Lamour and Hausbeck 2003, Hausbeck and Lamour 2004). In fields infested with *P. capsici*, rotation with non-susceptible hosts for more than 5 years was not adequate to prevent crop loss of *P. capsici* susceptible crops (Lamour and Hausbeck 2003, Hausbeck and Lamour 2004). Cultural control methods include using well-drained fields, since water is a key factor in *P. capsici* dissemination and infection (Ristaino and Johnston 1999). Utilizing raised plant beds and trellises are recommended. However, in Michigan, winter squash and cucumbers grown for processing are mechanically harvested making it difficult to apply these cultural control methods. In areas with frequent rainfall such as the eastern U.S., cultural control methods are not effective when standing water is ≥ 2.5 cm in the field, creating suitable conditions for zoospore release and infection (Hausbeck and Lamour 2004).

In combination with cultural methods and water management, fungicides can provide protection against *P. capsici* (Lamour and Hausbeck 2000, Hausbeck and Lamour 2004). The ability of the pathogen to reproduce sexually increases the likelihood of developing resistance to fungicides, such as cyazofamid and mefenoxam (Hausbeck and Lamour 2004, Jackson *et al.* 2012). Crops with fruits that have long maturation periods, such as winter squash and pumpkins, remain in direct contact with the infested soil for long periods of time increasing the likelihood of fruit rot (Granke *et al.* 2012).

HOST RESISTANCE TO *P. CAPSICI*

Host resistance is an essential element in strategies to control *P. capsici*. Commercial varieties and breeding lines of pepper have been screened for resistance to *P. capsici* (Barksdale *et al.* 1984,

Johnaston *et al.* 2002, Babadoost and Islam 2002, Oelke *et al.* 2003, Foster and Hausbeck 2010a). Foster and Hausbeck (2010a) also identified pepper lines and varieties with crown and root rot resistance to *P. capsici* isolates in Michigan. The fruit of these varieties was susceptible to *P. capsici* infection. The observed variation in susceptibility among pepper tissue types including root, stem, leaves, and fruit suggests that different mechanisms control resistance (Barksdale *et al.* 1984, Oelke *et al.* 2003). Complete resistance has not been detected in cucurbit commercial cultivars (Café-Filho *et al.* 1995). Many cucumber varieties have been screened for fruit resistance against fruit rot; however, only partial resistance has been identified (Gevens *et al.* 2006). In a greenhouse screen for crown rot resistance in a collection of commercial cultivars and germplasm accessions of summer squash, a green zucchini cultivar Spineless Beauty showed less susceptibility to crown rot than the other tested lines (Meyer and Hausbeck 2012). In a laboratory screen of different cucurbit fruits, yellow summer squash was more susceptible to fruit rot than winter squash, cucumber, watermelon, and melon (Ando *et al.* 2009). As complete resistance to *P. capsici* has not been identified in cucurbits, using available resistant cultivars in combination with the fungicides and cultural control may improve the control of *P. capsici* (Meyer and Hausbeck 2012).

Variability in fruit rot susceptibility has been observed in different cucurbit fruits (Ando *et al.* 2009). Young fruits are more susceptible to fruit rot than older fruit (Lamour and Hausbeck 2004, Gevens *et al.* 2006, Ando *et al.* 2009, Meyer and Hausbeck 2013, Krasnow and Hausbeck 2016, Alzohairy *et al.* 2017). This phenomenon is known as age-related resistance (ARR), developmental resistance, or ontogenic resistance (Whalen 2005). Resistance is associated with specific developmental stages of the host (Stermer and Hammerschmidt 1984) and that resistance increases or decreases (Shah *et al.* 2015). Panter and Jones (2002), and Develey-Rivière and Galiana (2007)

described ARR in different plant-pathogen systems. Older hypocotyl tissue in the soybean seedling is resistant to *P. sojae* (Lazarovits *et al.*, 1980). The young soybean hypocotyl tissue developed lesions when inoculated with *P. sojae*, while old tissue was resistant. ARR was correlated with the rapid production of the phytoalexin glyceollin in the older tissue (Ward *et al.* 1980); however, Lazarovits *et al.* (1981) showed that glyceollin was more correlated with necrosis than with resistance, suggesting other mechanisms control ARR.

ARR to *P. capsici* has been described in some cucurbits and pepper (Ando *et al.* 2009, Kim *et al.* 1989, Biles *et al.* 1993). Pepper fruit acquires resistance to *P. capsici* as fruit ripening progresses (Biles *et al.* 1993). Cucumber fruit of a commercial cultivar and other genotypes of plant introductions exhibited ARR two weeks after fruit set. This ARR is likely correlated with the completion of fruit elongation period (Gevens *et al.* 2006). Other cucurbits exhibiting ARR at about 21 days post anthesis include *Cucurbita moschata* and *Cucurbita pepo* (Ando *et al.* 2009, Meyer and Hausbeck 2013, Krasnow and Hausbeck 2016). Some commercial cultivars of *C. moschata* develop resistance 14 days post anthesis (Alzohairy *et al.* 2017). The rates of fruit development are suggested to be responsible for determining the onset of ARR (Gevens *et al.* 2006). Therefore, cultivars/hosts with more days to maturity develop ARR later than those with fewer days to maturity. Selecting cultivars with ARR, especially those exhibiting ARR at earlier stages of fruit development could be beneficial to growers in the timing of fungicide spray and also reducing the number of applications (Ando *et al.* 2009, Hausbeck and Lamour 2004).

The mechanisms behind ARR have been studied in several plants. Meyer and Hausbeck (2013) looked at the physiological and morphological changes as fruit age increased. Soluble solid contents and exocarp firmness were observed to increase with fruit maturing, but they were negatively correlated with the less susceptible phenotype of maturing fruit. Ando *et al.* (2009)

associated a change in fruit color and texture and increased waxiness with ARR in cultivars of *C. pepo* and *C. moschata*. Wounding negates ARR to *P. capsici* in cucumber (Granke and Hausbeck 2010), squash (Krasnow *et al.* 2014), and pepper (Biles *et al.* 1993), suggesting a role of the exocarp in resistance to *P. capsici*. In pepper, cuticle thickness increased with fruit maturity and increased resistance (Biles *et al.* 1993). Similarly, Ando *et al.* (2015) observed an increase in thickness of the cuticle and epidermal wall in cucumber, suggesting the role of a physical barrier. Generally, the plant cuticle is the first barrier that fungi and oomycetes have to penetrate in order to infect. The increase in cuticle thickness is a general protective mechanism against different pathogens such as detected in pepper against *P. capsici* (Biles *et al.* 1993). Also, at approximately three weeks old, bean hypocotyl cuticle thickness increases and becomes resistant to *Rhizoctonia solani* (Stockwell and Hanchey 1983).

Plant production of secondary metabolites with antimicrobial activity is one of the primary defensive mechanisms against microbes (Dixon 2001, González-Lamothe *et al.* 2009). There are two classes of the defensive phytochemicals: low molecular weight preformed compounds that are produced and stored in plant tissues prior to pathogen infection, known as phytoanticipins, and low molecular weight induced compounds that are synthesized *de novo* after infection with pathogen, known as phytoalexins (VanEtten *et al.* 1994, Paxton 1981). The role of phytoanticipins and phytoalexins has been proven in several plant-pathogen systems. The phytoanticipins avenacin is produced by oat roots providing resistance against attack by the take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Osbourn *et al.* 1994). The role of avenacin in defense was demonstrated when a species of *Avena*, *Avena longiglumis*, was found to lack detectable amounts of avenacin and became infected with *Gaeumannomyces graminis* var. *tritici.* Also, oat plants are susceptible to *G. graminis* var. *avenae* that produces avenacinase, which detoxifies

avenacins (Osbourn *et al.* 1994). Also, the phytoanticipins dienes that are produced in unripe avocado fruit peel provide resistance against *Colletotrichum gloeosporioides,* the cause of anthracnose. Ripened fruit become susceptible to anthracnose as the dienes concentration decreases to levels that are not toxic to the pathogen (Prusky *et al.* 1991). A similar mechanism was found in mango fruit that possesses resorcinol in its unripen peel makes it resistant to *Alternaria alternata (*Droby *et al.* 1986, 1987), the levels of resorcinol in ripen fruit is nontoxic to *A. alternata*, and the fruit become susceptible to infection.

The role of phytoalexins in defense has been described in several plant systems. Initially, phytoalexins were first described by Müller and Borger in 1940. Since then, more than 350 phytoalexins have been characterized in plant families, and 130 have been identified in the Leguminoseae family (Joseph 1995). The chemical structure of phytoalexins in the same family is mostly similar. For example, Solanaceae have terpenoid phytoalexins while Leguminoseae have oflavonoid phytoalexins (Singh and Chandrawat 2017). When phytoalexins are produced at the infection site, at the right time and antimicrobial concentrations, they are considered to be involved in defense (Hammerschmidt 1999, Hammerschmidt 2011). One of the best studied phytoalexins to be related to defense is camalexin. Camalexin was first isolated from leaves of *Camelina sativa* infected with *Alternaria brassicae*. It has been further identified in other crucifers (Glawischnig 2007). Camalexin is synthesized from tryptophan, and its production has been induced in *Arabidopsis* after infection by fungi, oomycetes, bacteria, and viruses (González-Lamothe *et al.* 2009). The role of camalexin as a defense mechanism in *Arabidopsis* has been proven. High concentrations of camalexin were detected at the infection site with *A. alternata* and locations near to lesions induced by *Botrytis* sp. (Kliebenstein *et al.* 2005). The accumulation of camalexin was correlated with induction of the precursor tryptophan and camalexin biosynthetic genes

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(Schuhegger *et al.* 2007). Further, the role of camalexin in defense was proven in the mutant line *pad3*, which lacks the accumulation of camalexin. The *pad3* mutant is susceptible to infection with *Alternaria brassicicola* and to *B. cinerea* isolates that were sensitive to camalexin (Kliebenstein *et al.* 2005). The phytoalexin *C*-glycosyl flavonoids, was found to be involved in resistance to powdery mildew in cucumber (McNally *et al.* 2003). When cucumber leaves were elicited with the plant-defense inducer Milsana before infection by the powdery mildew pathogen, the pathogen's conidial chain growth collapsed within 48 hours after infection. Another phytoalexin, *trans-p*-coumaryl aldehyde, a lignin precursor, has been detected in the fruit of *Cucurbita* species including green acorn squash, butternut, and pumpkin (Stange *et al.* 1999).

THE GENETIC BASIS OF RESISTANCE

Early developing cucumber fruit are susceptible to *P. capsici* 8 days post pollination (dpp) and build resistance as the fruit age (Gevens *et al.* 2006). Transcriptomic studies of cucumber fruit across development identified distinct genes characterizing three phases of development. Fruits 0 – 4 dpp are characterized by a group of genes associated with cell division, organization, and biogenesis. Fruit aged 4 – 12 dpp are enriched in genes that function in cell structure and lipid metabolism. Fruit aged 12 – 16 dpp are enriched in genes associated with abiotic and biotic stress and stress-related transcription factors. The change in gene expression associated with fruit growth defense is correlated with the observed shift from young cucumber fruit susceptible to *P. capsici* to maturing fruits that are resistant (Ando et a. 2012). Transcript profiles of cucumber fruit peel of aging resistant fruit exhibit enrichment in genes associated with physical barriers, chemical defense, and molecular pattern-triggered or effector-triggered pathways (Ando *et al.* 2015). Another transcriptomic study of cucumber resistant fruit peel at 16 dpp revealed enrichment in genes encoding for flavonoid and terpenoid biosynthesis (Mansfeld *et al.* 2017). Further

metabolomic studies on the same resistant fruit ages showed enrichment in terpenoid glycosides more so than susceptible young cucumber fruit at 8 dpp, which suggests a correlation between production of terpenoid glycosides in resistant fruit and ARR to *P. capsici* (Mansfeld *et al.* 2017). ARR has been observed in apple leaves and fruit where disease susceptibility to *Venturia inaequalis* decreased with the aging of leaves and fruit. In a transcriptomic study of infected and non-infected leaves with *Venturia inaequalis*, five candidate genes were potentially correlated with ARR. One gene encodes for 'enhanced disease susceptibility one protein' was downregulated in both infected and non-infected leaves. The other four genes encode for metallothionein3-like protein, lipoxygenase, lipid transfer protein, and a peroxidase 3 were upregulated in both infected and non-infected leaves (Gusberti *et al.* 2013). These results suggested a potential mechanism of constitutive gene expression that is correlated with ARR in apple leaves.

CONCLUSION

Winter squash and pumpkin are economically important crops in the U.S. and worldwide. In Michigan and other U.S. states, fruit quality and yield are limited by *P. capsici*. Commercial cultivars are susceptible to fruit rot but ARR has been observed in commercial cultivars of *C. moschata*. Determining the onset of ARR in commercial cultivars and uncovering the mechanism of ARR in winter squash is helpful for integrated management strategies. Using cultivars that exhibit ARR in breeding programs could be used to develop *P. capsici* resistant varieties.

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CHAPTER I: CHANGES IN WINTER SQUASH FRUIT EXOCARP STRUCTURE ASSOCIATED WITH AGE RELATED RESISTANCE TO *PHYTOPHTHORA CAPSICI*

ABSTRACT

Phytophthora capsici is a destructive pathogen of cucurbits causing root, crown, and fruit rot. Winter squash (*Cucurbita sp.*) production is limited by this pathogen in Michigan and other U.S. growing regions. Age-related resistance (ARR) to *P. capsici* occurs in *Cucurbita moschata* fruit but is negated by wounding. The objective of this research was to determine if structural barriers exist in the intact exocarp of maturing fruit exhibiting ARR. Five winter squash (*C. moschata*) cultivars were evaluated for resistance to *P. capsici* 10, 14, 16, 18, and 21 days post pollination (dpp). 'Chieftain' butternut squash fruit expressing ARR 14 dpp were selected for analysis of exocarp cell wall changes and compared to 7 dpp (susceptible) and 21 dpp (resistant) using scanning electron microscopy (SEM). The cuticle, epidermal anticlinal walls, and cortex cell walls were measured and revealed significant increases in the cuticle and epidermal thicknesses as fruit age. *P. capsici* hyphae penetrated susceptible fruit 7 dpp directly from the surface or through wounds before 6- hour post inoculation (hpi) and completely degraded the fruit cell wall within 48 hpi. Resistant fruit, 14 dpp, and 21 dpp remained unaffected. Hyphae did not enter stomata 6 hpi. However, at 24 hpi the pathogen attempted to penetrate through stomata in the maturing resistant fruit. 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.

INTRODUCTION

Phytophthora capsici Leonian, a destructive soilborne oomycete, infects vegetables including crops of cucurbits (Granke *et al.* 2012, Hausbeck and Lamour 2004, Kreutzer *et al.* 1940, Roberts *et al.* 2001, Tian and Babadoost 2004) and pepper (Foster and Hausbeck 2010), snap bean (Gevens and Hausbeck, 2005), and lima bean (Davidson *et al.*, 2002). Under greenhouse conditions, *P. capsici* also infected *Brassica* spp. vegetable and biofumigation cover crops including cabbage, mustard, and radish (Krasnow and Hausbeck 2015). *P. capsici* limits cucurbit production in Michigan (Hausbeck and Lamour 2004, Meyer and Hausbeck 2012) and other U.S. production regions (Miller *et al.* 1994, Roberts *et al.* 2001, Babadoost 2004, Castro-Rocha *et al.* 2017).

Nationally, Michigan ranks number one for production of summer and hard squash *C. moschata* and *C. pepo* repeatidly and fourth for *C. maxima* (pumpkin) production (USDA 2018). Due to the extended maturation period of 80-120 days for hard squash and pumpkin fruits, exposure to *P. capsici*-infested soil occurs for an extended time. Also, the dense plant canopy limits coverage of the fruit by fungicide sprays (Granke *et al.* 2012). Fruit rot can occur in the field (Granke *et al.* 2012) or postharvest (Hausbeck and Lamour 2004). Fruit rot signs and symptoms begin with dark water-soaked tissue followed by pathogen sporulation and fruit degradation (Badaboost 2004). Cucumber (Gevens *et al.* 2006, Ando *et al.* 2009, Hausbeck and Lamour 2004), butternut squash, melon, and watermelon (Ando *et al.* 2009), and pumpkin (Meyer and Hausbeck 2013) fruits are generally susceptible to *P. capsici* infection within 7 days post pollination.

Integrated disease management strategies for *P. capsici* include raised plant beds with plastic mulch, water management, and fungicide application (Hausbeck and Lamour 2004, Ristaino and Johnston 1999). Raised plant beds are not suitable for crops that will be mechanically harvested

such as hard-rind squash and pumpkins used for processing (Granke *et al.* 2012). However, the use of plant beds with plastic mulch was successful in increasing yield and improving quality in many vegetable crops such as cucumbers, tomatoes, pepper, and eggplant (Lamont 2017). Crop rotation offers limited relief since the pathogen produces thick-walled oospores that can survive in the soil for many years in the absence of a host (Babadoost 2005, 2013). *P. capsici* produces zoosporangia and swimming zoospores that are disseminated by rainwater splash or irrigation water (Bowers *et al.* 1990, Granke *et al.* 2012, Babadoost 2004). The pathogen undergoes sexual recombination, increasing the likelihood of developing resistance to fungicides which has occurred for mefenoxam and metalaxyl (Lamour and Hausbeck 2004) and cyazofamid (Kousik and Keinath 2008).

Host resistance along with other disease management strategies can provide long-term management strategy against *P. capsici* (Hausbeck and Lamour 2004, Quesada and Hausbeck 2010, Granke *et al.* 2012). Although complete resistance has not been identified in commercial cultivars of cucurbits (Café-Filho *et al.* 1995), age-related resistance (ARR) has been observed in different cucurbits (Gevens *et al.* 2006, Ando *et al.* 2009, Krasnow *et al.* 2016, Meyer and Hausbeck 2013). ARR or ontogenic resistance is resistance associated with specific host developmental stages (Stermer and Hammerschmidt 1984, Hammerschmidt 2015). ARR has been observed in different host-pathogen interactions (Lazarovits *et al.* 1981, Kim *et al.* 1989, Kus *et al.* 2002, Panter *et al.* 2002). Cucurbits and Solanaceae vegetable crops express ARR to *P. capsici* (Hausbeck and Lamour 2004, Gevens *et al.* 2006, Ando *et al.* 2009, Krasnow *et al.* 2016, Meyer and Hausbeck 2013, Biles *et al.* 1993). Squash (*Cucurbita pepo*, *Cucurbita moschata*) (Ando *et al.* 2009, Krasnow *et al.* 2016), cucumber (Hausbeck and Lamour 2004, Gevens *et al.* 2006, Ando

et al. 2009), and pumpkin (*C. moschata*) (Krasnow *et al.* 2016, Meyer and Hausbeck 2013) develop resistance to fruit rot as they mature.

The time of ARR is variable among cucurbits (Ando *et al.* 2009, Meyer and Hausbeck 2013). It has been observed in cucumber that when the fruit reached its maximum length that overlapped with the resistance against *P. capsici* (Gevens *et al.* 2006, Ando *et al.* 2009, Ando *et al.* 2015). Complete fruit elongation can vary in different cucurbits, wherein cucurbits are producing large fruit such as pumpkins and squash, the fruit size reaches its maximum between 20-24 days-post pollination (dpp). For cucurbits producing small fruit, the fruit maximum size is achieved between 15-20 dpp (Loy 2004). In cucumber, fruit full length is achieved 10-12 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* has been studied in different cucurbits (Mansfeld *et al.* 2017, Meyer and Hausbeck 2013, Krasnow and Hausbeck 2016) and pepper (Biles *et al.* 1993). Wounding resistant fruit negated ARR (Krasnow *et al.* 2014, Biles *et al.* 1993) suggesting the fruit exocarp provides resistance to maturing fruit.

The objectives of this study included determining: 1) the onset of ARR to *P. capsici* during fruit development among five *C. moschata* commercial cultivars and 2) the structural changes in the fruit exocarp cell wall and *P. capsici* hyphal penetration mechanisms in young susceptible and maturing resistant fruit using scanning electron microscopy.

MATERIALS AND METHODS

Five butternut winter squash and processing pumpkin cultivars of *Cucurbita moschata* were selected (Table 1.1). Seeds were planted on 15 June 2015 into 72-cell trays containing soilless peat mixture (Suremix Michigan Grower Products, Inc. Galesburg, MI) and grown for two weeks in the research greenhouse at Michigan State University (MSU) in East Lansing, MI. Thirty seedlings from each cultivar were transplanted on 1 July to a field site, previously planted to pumpkin, at the MSU Plant Pathology Farm in Lansing, MI. 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 days post pollination (dpp) similar to Meyer and Hausbeck (2013) with modification.

Table 1.1.: *Cucurbita moschata* with listed cultivars used in the study.

Cultivar	Cultivar type	Days to maturity	Source
Chieftain	Butternut squash	80	Rupp Seeds Inc., Wauseon, OH
Waltham	Butternut squash	110	Rupp Seeds Inc., Wauseon, OH
Early	Butternut squash	82	Rupp Seeds Inc., Wauseon, OH
Avalon	Butternut squash	90	Rupp Seeds Inc., Wauseon, OH
Dickenson Field	Processing pumpkin	100	Rispens seeds Inc.

Phytophthora 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, then recovered from the infected fruit, and maintained on unclarified V8 agar (143 mL V8 juice, 3g $CaCO_3$, 16 g agar, 850 mL distilled water) (Dhingra and Sinclair 1985, Krasnow *et al.* 2017) under constant fluorescent light at room temperature ($21 \pm 2^{\circ}C$).

Before inoculation, fruits were surface disinfested with 0.4% of 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 mid-point 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 agar plug was used for control fruit. Fruits were incubated in 99 L or 62 L clear plastic bins (Sterilite) lined on the inside edges with water-saturated paper towels to maintain high relative humidity and kept at room temperature ($22\pm2^{\circ}C$) under constant fluorescent light (Meyer and Hausbeck *et al.* 2012, Krasnow *et al.* 2014).

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

'Chieftain' butternut squash fruits were selected for scanning electron microscopy (SEM) studies. 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. Nine nonwounded fruit of 7, 14, and 21 dpp were inoculated with *P. capsici* at three sites as technical replicates as described previously. At 6, 24, and 48 h post inoculation (hpi), cross sections (~2.0 mm x 6-10.0 mm x 2.0 mm; thickness/width/depth) from the three inoculated sites of three fruit/hpi were prepared at the Center for Advanced Microscopy at MSU. Briefly, samples were fixed at 4°C for a minimum of 1 h in 4% glutaraldehyde buffered with 0.1 m sodium phosphate at pH 7.4, then rinsed briefly in the buffer and dehydrated in an ethanol series (25%, 50%, 75%, 95%, 3X 100%) for 1 h. Samples were freeze-dried in an Electron Microscopy Sciences Model EMS750X freeze dryer (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 exam exocarp structural differences at non-wounded and non-inoculated sites. Cross sections (~2.0 mm x 6-10.0 mm x 2.0 mm; thickness/width/depth) of non-wounded and non-inoculated sites of nine fruit/age were prepared as described above. Transverse sections were prepared from nine fruit/age to examine the exocarp surface. After the transverse sections were removed from the fruit, the samples were immediately frozen in liquid N_2, 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 the 6610LV SEM (JEOL Ltd. Tokyo, Japan) and images recorded using software version 3.08 of SEM Control User Interface (JEOL Technics Ltd., Tokyo, Japan).

Image J software, a public domain Java image-processing program (Jensen 1986), was used to visualize the cross-sections images and measure the thickness of different cell wall structures including the cuticle, the top- and mid-point of the epidermal anticlinal walls, and the cortex cell walls of the first four layers below the epidermis. ANOVA ($P = 0.05$) was used to detect significant differences among 7, 14, and 21 dpp fruit ages at the cuticle, the top- and mid-point 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 Tukey's Honest Significant Difference (HSD) test ($P = 0.05$) to determine which age(s) were different. All statistical analyses were conducted using R statistical software (R Development Core Team, 2014).

RESULTS

Fruits of all cultivars harvested 10 dpp developed disease symptoms four days post inoculation and disease incidence across cultivars ranged from 44.4% to 100% (Table 1.2). The onset of ARR was variable among cultivars (Table 1.2, Figure 1.1). 'Early' and 'Chieftain' butternut squash showed resistance (0% disease) beginning 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 18 and 21 dpp were resistant (0% disease). 'Waltham' butternut fruit had 11% disease, 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 (Table 1.2, Figure 1.1). For 'Waltham' and 'Avalon', the highest (1.2 and 1.4) and lowest (0.1 and 0) disease ratings occurred 16 and 18 dpp, respectively (Table 1.2, Figure 1.1). Lesion diameter (LD) decreased as fruit aged in 'Early', 'Chieftain', and 'Dickenson Field', while LD in 'Waltham' and 'Avalon' followed the same pattern as their disease ratings (Table 1.2, Figure 1.1), except that Avalon was not diseased at 14 dpp.

Table 1.2.: Disease rating, lesion diameter and disease incidence four days post inoculation with *P. capsici* for 5 cultivars of *Cucurbita moschata* at fruit developmental stages 10, 14, 16, 18, and 21 days post pollination (dpp).

Fruit age (dpp) Cultivar	Disease rating (0-4 scale)[a]	Lesion diameter (cm)[b]	Disease incidence (%)
10 dpp			
Early	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
14 dpp			
Early	0	0	0
Chieftain	0	0	0
Dickenson Field	2.3	5.2	78
Waltham	0.4	1.1	33
Avalon	0	0	0
16 dpp			
Early	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
18 dpp			
Early	0	0	0
Chieftain	0	0	0
Dickenson Field	0.7	2.4	44
Waltham	0.1	0.6	11
Avalon	0	0	0
21 dpp			
Early	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, b] Disease rating and lesion diameter values represent mean of 9 fruits conducted in three experimental replicates per age and cultivar. Disease scale was 0-4 where 0 is no visible infection, 1 is water soaking, 2 is light pathogen mycelial growth, 3 is moderate pathogen mycelial growth and 4 is extensive pathogen mycelial growth. Rating was done 4 days post inoculation (dpi).
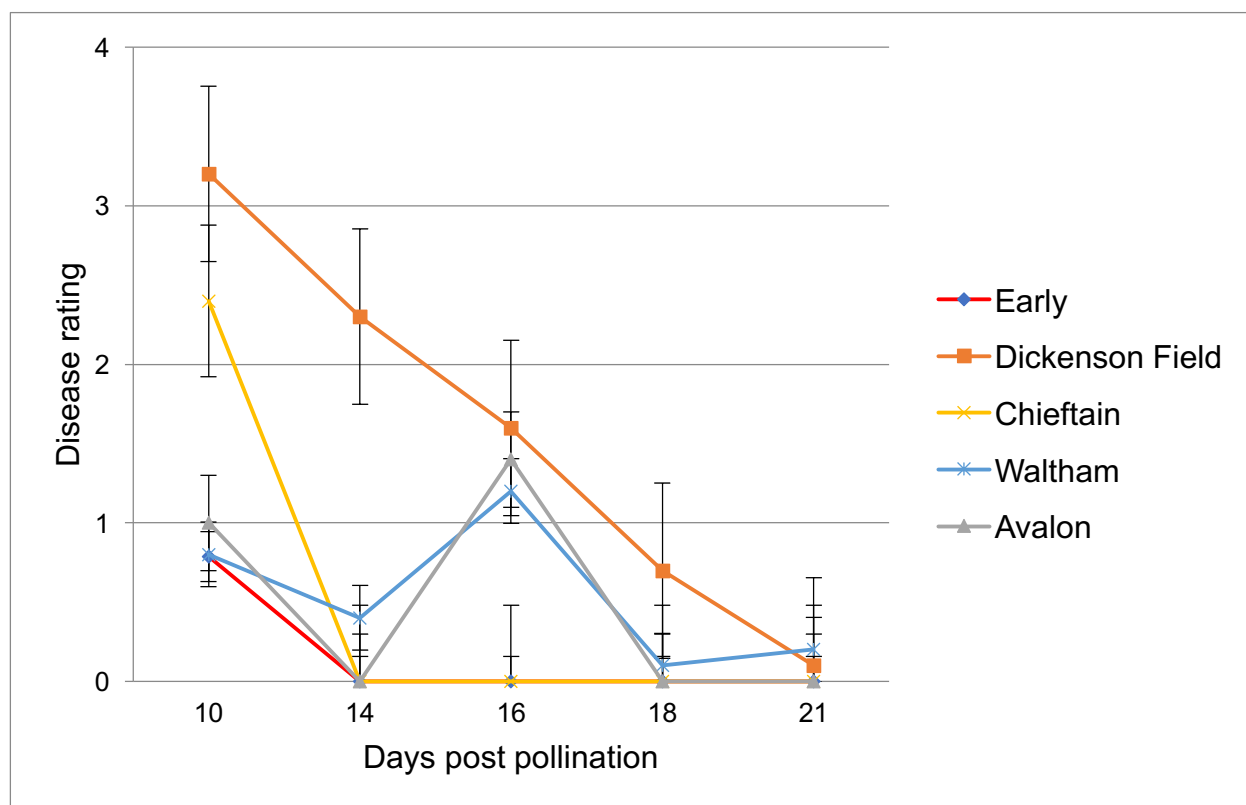
Figure 1.1.: Average disease rating of *Cucurbita moschata* cultivars' fruits at 10, 14, 16, 18, and 21 dpp in response to *Phytophthora capsici* inoculation, four days post inoculation (disease rating 0 = no visible pathogen growth; 1 = water-soaked tissue only; 2 = light visible mycelial growth; 3 = moderate mycelial growth; and 4 = dense mycelial growth).

SEM images revealed increases in the thickness of the cuticle and epidermal anticlinal walls as fruit aged from 7 to 21 dpp (Figure 1.2). The thickness of cuticle and epidermal cell walls increased as the fruit aged but not the cortex cell walls (Table 1.3). 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 (Figure 1.3, Table 1.4). Significant differences between 14 and 21 dpp were detected in the cuticle and top-point of epidermal anticlinal walls thicknesses; differences were not detected in thickness of the mid-point of the epidermal anticlinal walls. Cortex cell wall thickness measurements were not significantly different between 7 and 14 dpp ($P = 0.9$), while 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 (Figure 1.4).
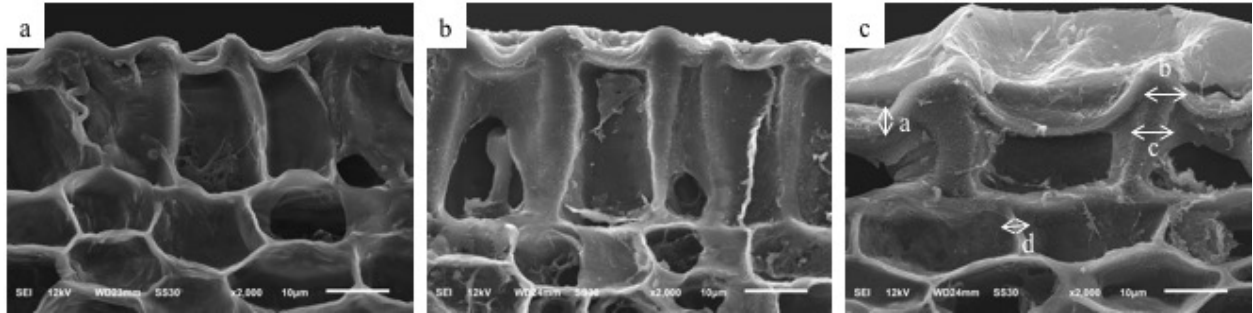
Figure 1.2.: Scanning electron microscopy images of cross sections of non-wounded/non-inoculated fruits of cultivar Chieftain (a) 7 dpp, (b) 14 dpp, and (c) 21 dpp. Arrows for (c) represent the cell wall structures and the measurement direction where a: cuticle, b: epidermal anticlinal wall top-point, c: epidermal anticlinal wall mid-point, and d: cortex cell wall. Arrows heads represent the direction of measurement.

Table 1.3.: Minimum and maximum thickness (µm) of cell wall structures: cuticle, epidermal anticlinal wall top-point, epidermal anticlinal wall mid-point, and cortex cell wall across fruit development at 7, 14, and 21 days post pollination (dpp) in cultivar Chieftain.

Days post pollination (dpp)	Cell wall structures	Thickness (µm) (Min-Max)
7	Cuticle	0.5-0.9
	Epidermal anticlinal wall top-point	1.9-2.5
	Epidermal anticlinal wall mid-point	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 mid-point	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 mid-point	3.6-4.9
	Cortex	0.8-1.0

Table 1.4.: Pairwise comparisons of cell wall structures between fruit developmental stages of cultivar Chieftain.

Days post pollination (dpp)	Cuticle	Epidermal anticlinal wall top-point	Epidermal anticlinal wall mid-point	Cortex
7 vs 14	<0.000001*	5e-07*	<0.00000005*	0.9
7 vs 21	<0.000001*	<0.0000005*	<0.00000001*	0.001*
14 vs 21	0.02*	2e-07*	0.13	0.0004*

*Indicates significant differences between ages in the rows ($P \le 0.05$), HSD $_{0.05}$ (Tukey's Honest Significant Difference test)
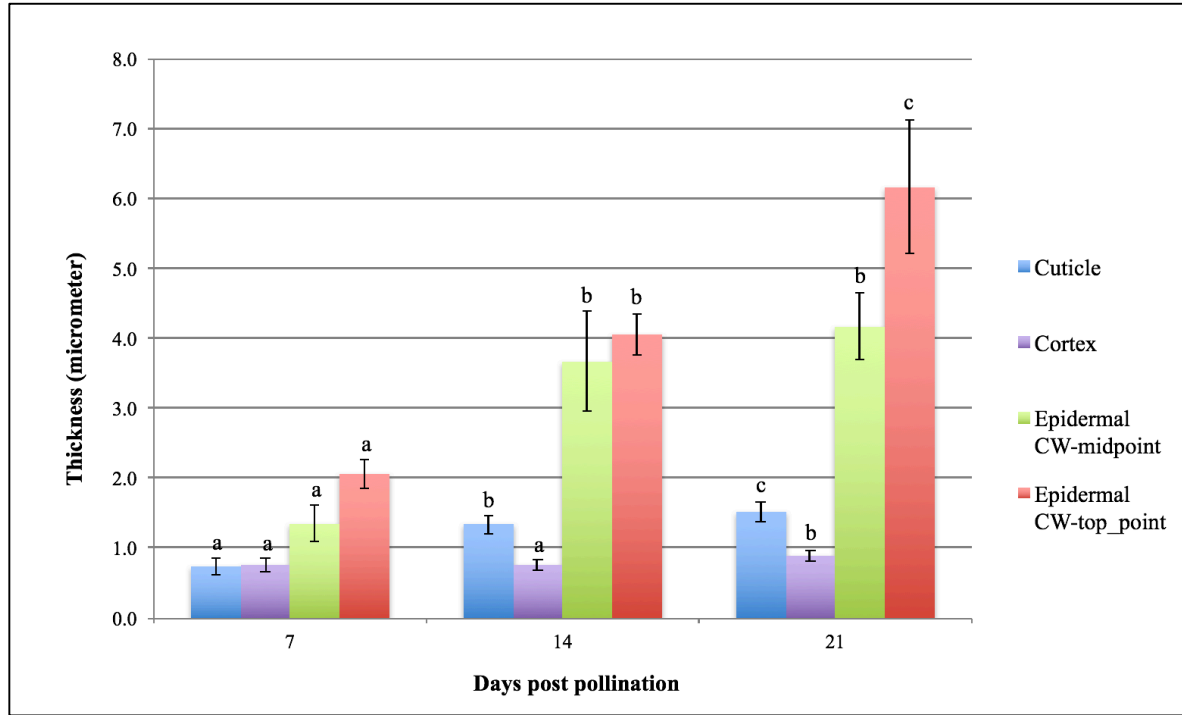
Figure 1.3.: Histogram showing average thickness (µm) of cell wall structures: cuticle, epidermal anticlinal wall top-point, epidermal anticlinal wall mid-point, and cortex cell wall across fruit development at 7, 14, and 21 dpp of cultivar Chieftain. Same color column with different letter indicates significant difference ($P \leq 0.05$) of cell wall structure thickness across ages.
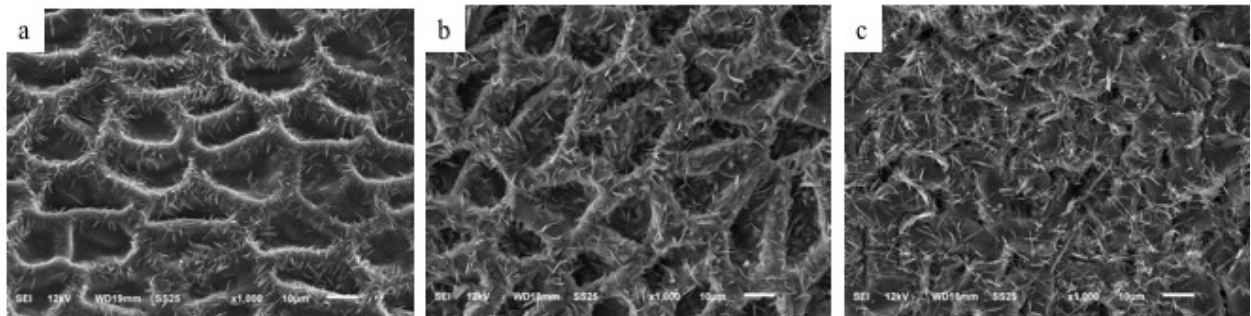
Figure 1.4.: Scanning electron microscopy images of transverse sections at non-wounded/non-inoculated fruits of cultivar Chieftain. (a) 7 dpp, (b) 14 dpp, and (c) 21 dpp. Wax appears as spiny crystals on fruit surface.

When cross sections of 7, 14, and 21 dpp were examined 6 and 24 hpi the cell wall did not appear affected by *P. capsici* (Figure 1.5a-e). While 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 (Figure 1.5g-i). Cross sections of 7 dpp fruit showed that hyphae penetrated

the epidermal layer before 6 hpi and was detected around the vascular bundles at 48 hpi (Figure

1.6a, b). Cross section images at 14 and 21 dpp detected no signs of hyphal penetration of the fruit

tissue among the three-time intervals (Figure 1.6c-h).
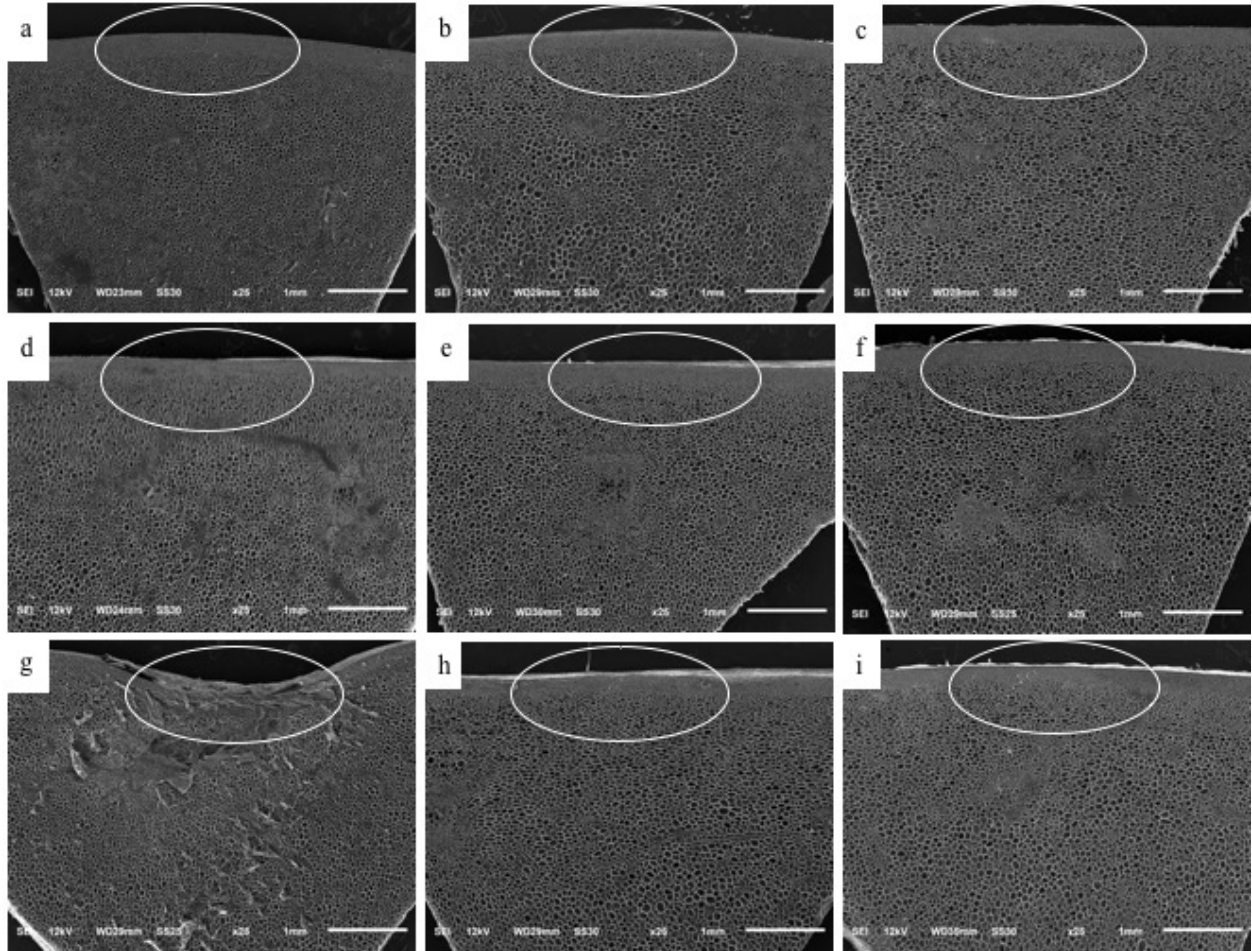
Figure 1.5.: SEM images of cross sections at 6 hour post inoculation (hpi), 24 hpi, and 48 hpi of cultivar Chieftain fruits at (a, d, g) 7 dpp, (b, e, h) 14 dpp, and (c, f, i) 21 dpp, respectively, showing the effect of *Phytophthora capsici* inoculation on cell wall integrity. The white circles point for the inoculation site.
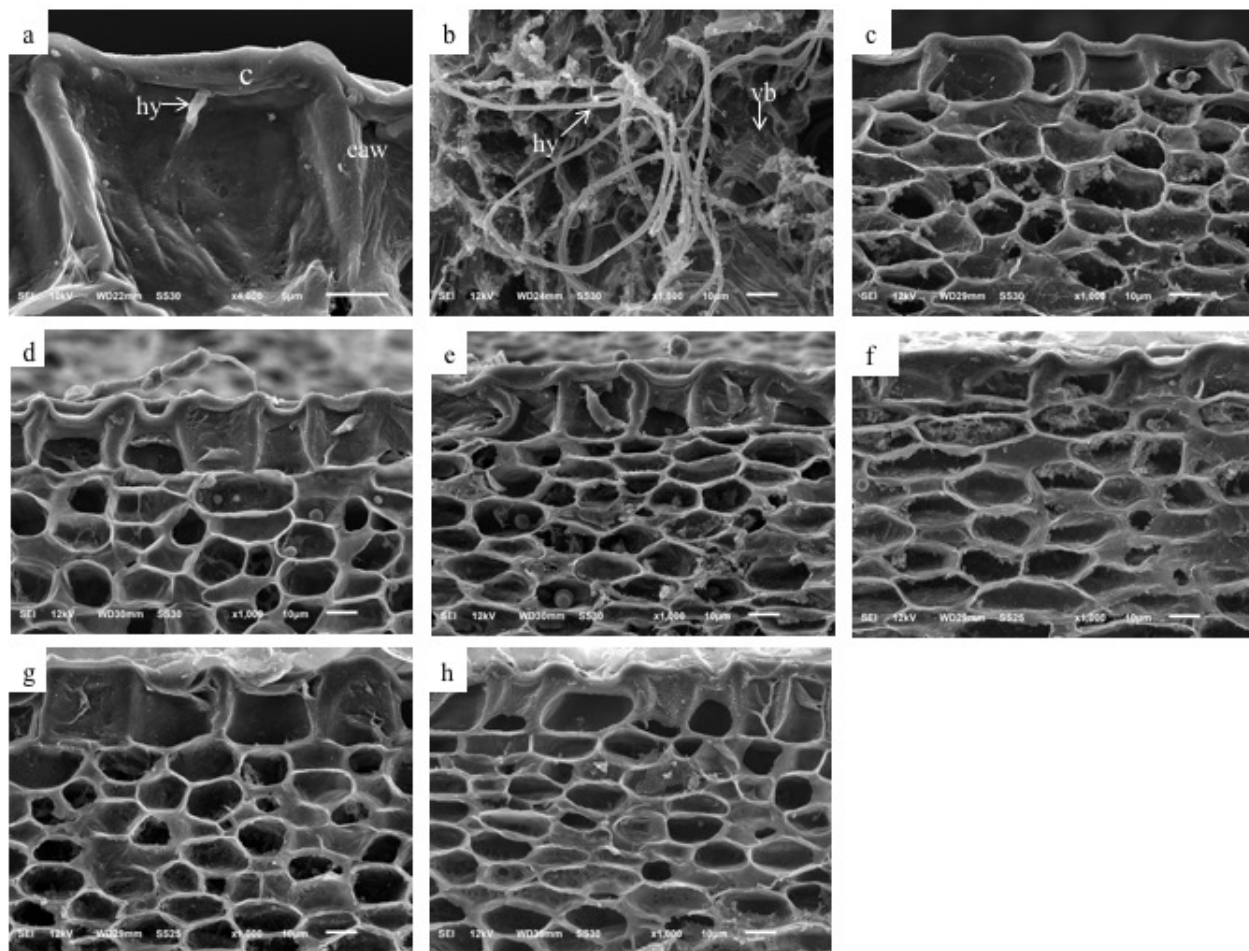
Figure 1.6.: Scanning electron microscopy images of cross sections of cultivar Chieftain fruits of 7 days post pollination (dpp) at (a) 6 hours post inoculation (hpi), (b) 48 hpi; 14 dpp at (c) 6 hpi, (d) 24 hpi, (e) 48 hpi; and 21 dpp at (f) 6 hpi, (g) 24 hpi, and (h) 48 hpi. Hy=hypha; c=cuticle; eaw=epidermal anticlinal wall; vb=vascular bundle.

Top surface views 6 hpi of the same cross-section samples from fruit harvested 7 dpp revealed hyphae directly penetrating the epidermal surface (Figure 1.7a). Hyphae were observed growing over stomata without entering, bypassing multiple stoma cells without penetrating and growing towards a wound (Figure 1.7b, c). At 24 hpi, hyphae were branched and penetrated the epidermal surface directly, and by 48 hpi multiple hyphal penetration points were detected in 7 dpp fruits (Figure 1.7d, 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 (Figure 1.7f, g). However, hyphae were observed penetrating stomata at 24 hpi in both 14 and 21 dpp fruits (Figure 1.7 h, i). At 48 hpi, one appressorium was detected suggesting a direct penetration attempt in 14 dpp fruits (Figure 1.7j). However, visible hyphal penetration was not detected in 21 dpp fruits at 48 hpi.
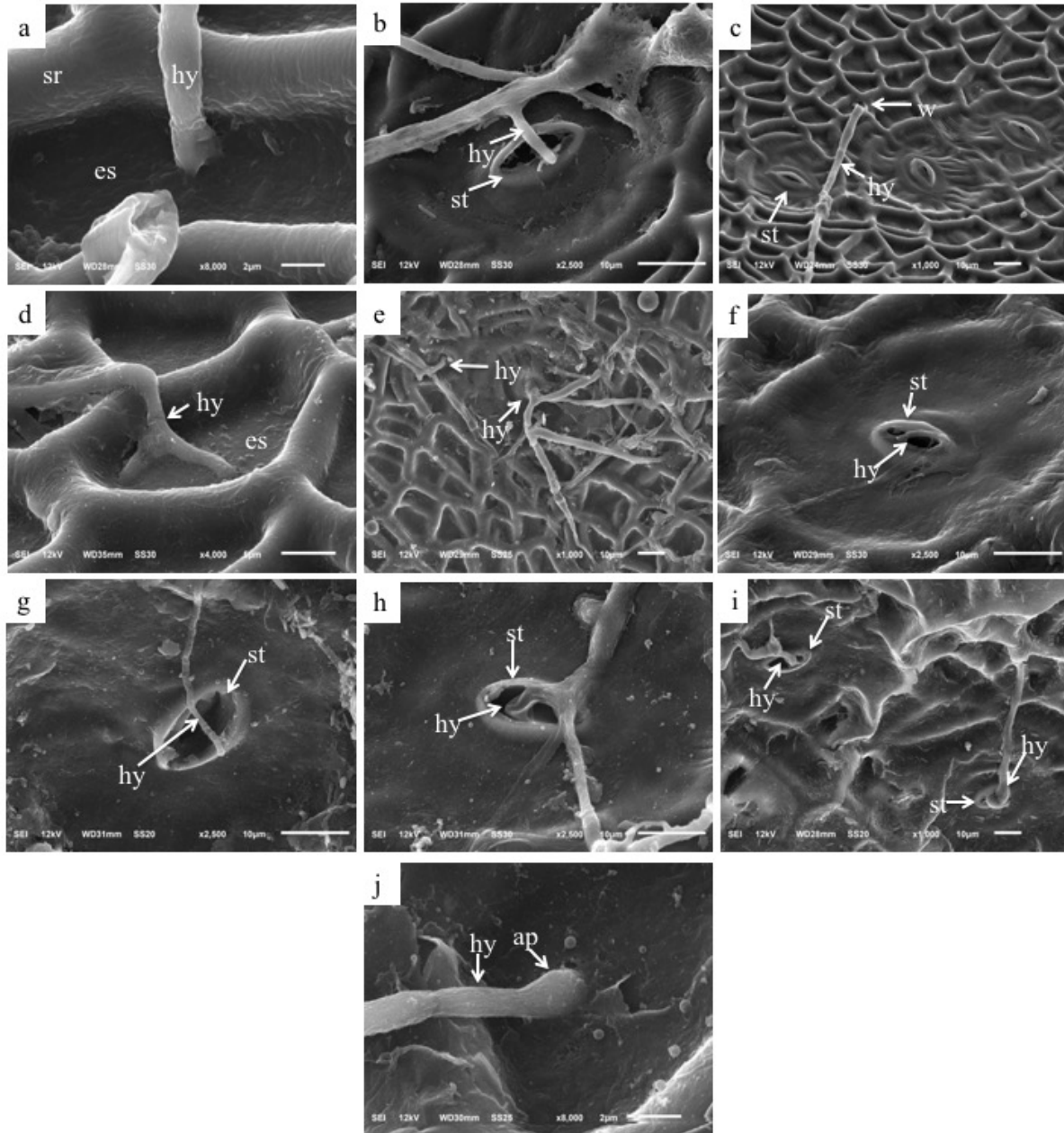
Figure 1.7.: Scanning electron microscopy images of the top surface view of cultivar Chieftain fruits. (a) 7 days post pollination (dpp) at 6 hours post inoculation (hpi), hyphal direct penetration from the epidermal surface (b) 7 dpp at 6 hpi, hypha passing over stomata without entering, (c) 7 dpp at 6 hpi, hypha traveling toward entering through wound and bypassing multiple stomata; (d) 7 dpp at 24 hpi, hypha branching and directly penetrating; (e) 7 dpp at 48 hpi, multiple penetration points; (f, g) 14 dpp and 21 dpp, respectively, at 6 hpi showing hyphae passing over stomata but not entering; (h, i) 14 dpp and 21 dpp, respectively, at 24 hpi showing hyphae entering through stomata; sr=surface ridge; es=epidermal surface; hy=hypha; st=stomata; w=wound; ap= appressorium.

DISCUSSION

In this study, we aimed to study the mechanism of ARR to *P. capsici* in *C. moschcata* cultivars through determining the onset of resistance, studying the fruit exocarp structural changes across development and uncovering the mechanism of hyphal penetration of winter squash fruit. Once a production site becomes infested with *P. capsici*, growers are encouraged to utilize all available tools to manage the disease. Understanding the mechanism of ARR in winter squash is beneficial to growers where choosing a cultivar known to develop ARR can be used as part of an integrated strategy that combines other cultural strategies and effective fungicides. ARR onset differed among the five cultivars of *C. moschata* included in this study. The onset of ARR 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. Results showed a gradual decrease in susceptibility to *P. capsici* as the fruit aged, suggesting changes in fruit development are associated with pathogen resistance. Krasnow and Hausbeck (2016) evaluated several butternut 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 22 dpp. Our results found that at 16 dpp 'Waltham' and 'Avalon' were intermediately resistant and became resistant at 21 and 18 dpp, respectively. 'Early' and 'Chieftain' became completely resistant at 14 dpp and older.

Variable days to maturity among cultivars might be the cause for the observable difference in the onset of ARR. 'Dickenson Field', 'Waltham', and 'Avalon' require more days to mature than 'Early' and 'Chieftain', which may suggest that 'Early' and 'Chieftain' reach full fruit elongation sooner than other cultivars. Fruit transition from a susceptible to resistant state is associated with complete fruit elongation stage (Gevens *et al.* 2006, Ando *et al.* 2009, Ando *et al.* 2015, Krasnow

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and Hausbeck 2016) and maybe 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 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 (Panter & Jones, 2002; Whalen, 2005, Develey-Rivière and Galiana 2007). Structural changes are important factor in plant defense against insects and pathogens (Freeman and Beattie 2008, War *et al.* 2012). The fruit cell wall develops a physical barrier against pathogen attack and provides the first layer of protection (Freeman and Beattie 2008). In the SEM study of 'Chieftain' fruit, cell wall collapse and degradation in 7 dpp fruit were observed within 48 hpi from *P. capsici*, while older (14 and 21 dpp) fruit remained unaffected (Figure 1.5), this suggests a physical barrier prevents hyphal penetration by the pathogen to the inner layer of fruit cell wall.

In plants, epicuticular wax deposition increases as plant age increase (Maiti 2012). Epicuticular wax is a hydrophobic layer which reduces the wet environment needed for pathogen spores to attach and germinate (Maiti 2012). In our study, wax deposition increased with aging fruit that demonstrated resistance to *P. capsici* (Figure 1.4), suggesting the formation of a physical barrier for pathogen hyphal penetration. Wounding negates ARR (Krasnow *et al.* 2014, Biles *et al.* 1993), thus supporting the hypothesis that intact fruit exocarp is essential for resistance. This study and previous studies found nonwounded fruits of different cucurbits exhibit ARR to *P. capsici* as they age (Ando *et al.* 2009, Gevens *et al.* 2006, Meyer and Hausbeck 2013, Krasnow and Hausbeck 2016). However, 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 when compared to susceptible fruit 8 dpp (Ando *et al.* 2015). The resistant phenotype of tomato fruit at the stem end 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 *P. capsici*. In bean, the hypocotyl becomes resistant with age to *Rhizoctonia solani* 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, the cuticle and cell wall thickness increase was observed with maturity (Sutherland and Hallett 1993). Our study showed an increase in the cuticle and epidermal anticlinal wall thickness (Figure 1.2) and wax deposition (Figure 1.4) in aging resistant 'Chieftain' fruit compared to 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 physical 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 to a higher level of significance detected in cuticle and epidermis (Figure 1.3). Thus, the cortex may play a less critical role in resistance since the difference in thickness of cortex cell walls between 7 dpp susceptible fruit and 14 dpp resistant fruit were not significant.

Phytophthora capsici penetrates the plant surface directly or through natural openings such as stomata (Hausbeck and Lamour 2004). On pepper leaves, Du *et al.* (2013) noted that *P. capsici* zoospores encysted within three hours 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 (Figure 1.6a). Appressorium formation was detected 24 hpi on the exocarp surface of the resistant fruit (Figure 1.7j), suggesting an attempt by the pathogen to penetrate the tissue using mechanical force. When fruit were harvested 7 dpp, pathogen hyphae penetrated directly or through wounds by 6 hpi (Figure 1.7a, c). Hyphae were embedded in the epidermal cells of young susceptible fruit (Figure 1.6a). We did not observe penetration through stomata of any of the squash fruit regardless of age 6 hpi (Figure 7b, f). Hyphae were observed growing over the stomata without penetrating (Figure 7b, f), similar to what Du *et al.* (2013) observed in pepper leaves inoculated with *P. capsici*. This suggests that penetration through stomata is not preferred by the pathogen. A similar pattern was observed with *Cercospora henningsii* where the pathogen passed over the stomata without entering (Babu *et al.* 2009). However, a 24 hpi, we detected hyphal penetration through the stomata in squash fruit harvested 14 and 21 dpp was detected (Figure 7h, i), which suggests that when the pathogen encounters obstacles preventing its direct penetration through the surface, it attempts to penetrate through 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 (Figure 1.6c- h). Krasnow and Hausbeck (2016) found that *P. capsici* zoospores accumulated over stomata of a susceptible *C. maxima* cultivar while no accumulation was detected on a *C. moschata* cultivar, which exhibits ARR.

Mycelia colonized the vascular bundles of the susceptible 7 dpp fruit (Figure 1.6b), while colonization was not observed at or near the vascular bundles in the 14 and 21 dpp resistant fruit. Similar results were reported with *Phytophthora 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* colonized the cuticle, epidermis, cortex, and

vascular tissue within 48 hpi (Figure 1.6b). Colonization was not detected in fruit harvested 14 and 21 dpp (Figure 1.6c -h). Fruit harvested 14 dpp or later, exhibited ARR (Figure 1.1). Our results are consistent with a study by Kim and Kim (2009) where *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 due to root structural defenses (Kim and Kim 2009).

In conclusion, our study showed a high correlation between the thickness of cell wall structures including cuticle and epidermis, and ARR (Figure 1.3, Table 1.4)), suggesting the presence of a structural barrier to *P. capsici* invasion of maturing fruit tissue. 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*. Since the early onset of ARR might be related to fewer 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 within the first to the second week of development with fungicides, then a less intensive fungicide program could be used for the duration of the season. Following 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

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CHAPTER II: ANTIFUNGAL ACTIVITY IN WINTER SQUASH FRUIT PEEL IN RELATION TO AGE RELATED RESISTANCE TO *PHYTOPHTHORA CAPSICI*

ABSTRACT

Age-related resistance (ARR), or ontogenic resistance, is associated with host developmental stages. Winter squash fruit (*Cucurbita moschata*) develops resistance to the oomycete plant pathogen *Phytophthora capsici* as they mature. *P. capsici* is soilborne and infects cucurbits, solanaceous crops, lima and snap beans causing damping off, foliar blight, and root, crown rot, and fruit rot. In Michigan and other U.S. growing regions, winter squash production is limited by *P. capsici*. Infection of cucurbit fruit by *P. capsici* appears first as a water-soaked lesion, followed by pathogen sporulation, and fruit rot. ARR in winter squash to *P. capsici* could be exploited to assist growers in limiting crop loss. Previous research suggested the presence of a preformed structural barrier to *P. capsici* in resistant mature fruit exocarp. However, antifungal compounds in the fruit exocarp might also be associated with ARR. The objective of this study to determine whether preformed or induced antifungal activity during fruit development is correlated with ARR. Three winter squash cultivars demonstrating ARR were hand-pollinated and then harvested 10, 14 and 21 days post pollination (dpp). Fruits were peeled with three replicates/dpp/cultivar, and a methanol extract was prepared. A thin-layer chromatography (TLC) bioassay using *Cladosporium cucumerinum* was used to detect antifungal activity in winter squash peel extracts. Results indicated the presence of compounds with antifungal activity in all fruit ages tested, but the antifungal activity decreased with age indicating a lack of correlation between preformed antifungal activity and ARR in winter squash. Induced antifungal activity in winter squash fruit peel from fruits 7, 10, 14 and 21 dpp was examined following inoculation with a *P. capsici* mycelial agar plug at non-wounded sites. Results indicated no significant change in the

antifungal activity among fruit ages regardless of the post inoculation time intervals suggesting no correlation between the induced antifungal activity and ARR in winter squash.

INTRODUCTION

Fruit rot caused by *Phytophthora capsici* is a limiting problem for squash producers in Michigan (Hausbeck and Lamour 2004, Lamour *et al.* 2012, Granke *et al.* 2012) and other states (Miller *et al.* 1994, Ristaino 1999, Robert *et al.* 2005) with entire fields of squash becoming infected (Meyer and Hausbeck 2012). Michigan is ranked number one for summer and hard squash (USDA 2018) where the crops are used for fresh market and processing. Disease symptoms include root and crown rot, fruit rot, and foliar blight (Babadoost 2005). Long-lived oospores in the soil, an expansive host range, and the development of pathogen resistance to fungicide challenges current management strategies (Hausbeck and Lamour 2004, Quesada-Ocampo *et al.* 2009, Lamour and Hausbeck 2000). Commercial cultivars with host resistance are not currently available.

Age-related (ARR) or ontogenic resistance is the development of pathogen resistance during developmental stages of the plant and/or fruit (Stermer and Hammerschmidt, 1984; Hammerschmidt 2015). ARR has been observed in several plant-pathogen systems including *Arabidopsis* and *Pseudomonas syringae* pv. *tomato*, Apple leaves and *Venturia inaequalis*, and soybean seedlings and *Phytophthora sojae* (Panter and Jones 2002, Develey-Rivière *et al.* 2007). ARR to *P. capsici* has been demonstrated with fruits of pepper (Biles *et al.* 1993), cucumber (Ando *et al.* 2009, Gevens *et al.* 2006), and squash (Ando *et al.* 2009, Meyer and Hausbeck 2013, Krasnow *et al.* 2014, Krasnow and Hausbeck 2016, Alzohairy *et al.* 2017). While the onset of ARR is variable among species and cultivars, the early developmental stage of all cucurbit fruits is susceptible to *P. capsici* (Gevens *et al.* 2006, Ando *et al.* 2009, Meyer and Hausbeck 2013,

Alzohairy *et al.* 2017). When resistant squash fruit 21 to 24 days post pollination (dpp) were wounded, they became susceptible to *P. capsici* (Krasnow *et al.* 2014) suggesting that the fruit exocarp forms a structural or chemical barrier to *P. capsici*.

Plants produce secondary metabolites with antimicrobial activity as a chemical defense mechanism against pathogens (Dixon 2001, González-Lamothe *et al.* 2009). Phytoanticipins are low molecular weight antimicrobial phytochemical compounds produced from preexisting constituents that play a role in plant defense (VanEtten *et al.* 1994, González-Lamothe *et al.* 2009, Singh and Chandrawat 2017) and include saponins, glucosinolates, and cyanogenic glycosides (Piasecka *et al.* 2015). Avencains are a saponin produced by oat roots that serve as a chemical barrier against the take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Osbourn *et al.* 1994).

Phytoalexins are low molecular weight secondary metabolites produced in response to pathogen infection (Paxton, 1981). Phytoalexins are antimicrobial compounds that are synthesized via de novo activation of their biosynthetic pathways (González-Lamothe *et al.* 2009). For phytoalexins to serve in defensive response, they must be produced at the site of infection, at the correct time, and at a high enough concentration to inhibit pathogen invasion (Hammerschmidt 1999, Hammerschmidt 2011). Phytoalexins with antimicrobial activity include glyceollin from soybean (Lazarovits *et al.*, 1980), scopoletin from tobacco and camalexin from *Arabidopsis thaliana*, (González-Lamothe *et al.* 2009).

The objective of this study was to determine whether there is chemical components in the fruit peel of maturing, resistant *Cucurbita moschata* fruit that prevent the entry of *P. capsici*. This was achieved by investigating the following: 1) the presence of preformed antimicrobial compounds

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in the fruit peel of three *Cucurbita moschata* cultivars at the developmental stages correlated to fruit resistance; and, 2) the production or accumulation of induced antimicrobial compounds post inoculation in the fruit peel of 'Chieftain' butternut squash fruit, across development stages correlated with ARR using a thin layer chromatography (TLC) bioassay.

MATERIALS AND METHODS

Plant material

Two cultivars of butternut winter squash (*Cucurbita moschata*), 'Chieftain' and 'Early' and a processing pumpkin cultivar, Dickenson Field, (Table 1), previously evaluated for ARR, were used. Seeds were planted on June 20 into 72-cell flats containing a soilless peat mixture (Suremix Michigan Grower Products, Inc. Galesburg, MI) and grown in the research greenhouse on the Michigan State University (MSU) campus in East Lansing, MI, for two weeks at an average temperature of 22°C (±4°C). Plants were watered adequately as needed to maintain soil moisture. On 5 July, fifty seedlings per cultivar were transplanted into a field that was previously planted to a pumpkin at the MSU Plant Pathology Farm in Lansing, MI. The soil type was Capac loam with no known *P. capsici* infestation. Plants were established in raised plant beds covered with plastic mulch and irrigated twice each week via drip emitters. The plant bed length was 30.5 m with 3.7 m between rows. Twenty-five seedlings were planted into each row with two rows per cultivar. Plants were spaced 61 cm apart within the row. At flowering, the stigma of the female flowers was hand-pollinated using stamens from the male flowers and tagged. Fruits were collected 7, 10, 14, and 21 days post pollination (dpp).

Phytophthora capsici inoculum

The virulent *P. capsici* isolate 12889 (mating type A1, insensitive to mefenoxam) from bell pepper (Foster and Hausbeck 2010) was obtained from the long-term stored culture collection of M. Hausbeck's laboratory at MSU, East Lansing, MI. The culture was prepared and maintained on unclarified V8 agar (143ml V8 juice, 3g $CaCO_3$, 16g agar, 850ml distilled water) (Dhingra and Sinclair 1985, Krasnow *et al.* 2017) and grown for 5 to 7 days under constant fluorescent light at room temperature. Before fruit inoculation, pathogen virulence was ensured by inoculating a cucumber fruit, recovering the pathogen, and maintaining it as previously described for use in the experiment.

Non-inoculated fruits for the Thin Layer Chromatography bioassay

'Chieftain', 'Early' and 'Dickenson' field fruits at 10, 14, and 21 dpp were used. Three biological replicates of non-wounded fruits for each age and cultivar were surface sterilized in the laboratory by soaking the fruit in 0.4% of sodium hypochlorite solution for 5 min, rinsing with water for 2 min, and air drying on a paper towel. All fruits were peeled using a vegetable peeler; peels were placed in falcon tubes then stored at -80°C.

Preparation of inoculated fruits for Thin Layer Chromatography bioassay

Three non-wounded biological replicates for 7, 10, 14, and 21 dpp fruits of 'Chieftain' were surface disinfested as described above. Fruits were inoculated with a 7-mm agar plug from a 5- to 7-day-old *P. capsici* culture that was placed colonized side on the fruit at the mid-point between the peduncle and blossom end, then covered with a sterilized screw cap (16.5 mm in diameter) (Axygen Inc., Union City, CA) that was fixed to the fruit using Vaseline. Sterile V8 agar plugs

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were used to inoculate the control fruits. Fruits were incubated in a high relative humidity chamber created by lining wet paper towels on the edges of the 99 L or 62 L plastic transparent bins (Sterilite) (Meyer and Hausbeck 2012, Krasnow *et al.* 2014). Throughout the incubation period, room temperature (22±2°C) and constant light were maintained. Inoculated fruits were peeled 6, 12, and 24 h post inoculation (hpi). Non-inoculated fruits were peeled following 6 h of incubation (6 h control (6hC)). All fruit peels were placed in falcon tubes then stored in the freezer at -80oC.

Thin Layer Chromatography Bioassay Methodology

Cladosporium cucumerinum was obtained for the long-term storage of R. Hammerschmidt's laboratory at MSU, East Lansing, MI. Before the bioassay, cultures were grown on potato dextrose agar (PDA) for 7 to 11 d.

Non-inoculated fruit peels used for analysis of the presence of antifungal compounds associated with ARR were extracted with methanol. Inoculated and non-inoculated control tissues were extracted with absolute ethanol. Ten ml of extraction solvent was used for each gram of tissue extracted. All samples were placed in a glass flask and covered with Aluminum foil. Samples were then boiled for 10 min. The tissues were re-extracted by boiling with the same solvent. All extracts were filtered through Whatman #1 filter paper. Filtrates were combined for each sample then evaporated using a rotary evaporator at 34°C. Evaporated samples were suspended in 1.0 ml of ethanol or methanol per gram fresh weight of tissue. Sonication was used to help dissolve sample extracts. All extracted samples were stored in scintillation vials in the freezer at -20°C until used in the TLC analyses.

Thin Layer Chromatography (TLC) was used to separate compounds in the extracts. Silica gel G or GF TLC plates 20 x 20 cm (Analtech) were used. Samples were divided into two groups for

loading onto the silica plates. Group 1 included non-inoculated samples of 'Chieftain', 'Early' and 'Dickenson Field' 10, 14, and 21 dpp. Group 2 included inoculated 'Chieftain' samples 7, 10, 14, and 21 dpp at 6 hC and 6hpi. Group 3 included inoculated 'Chieftain' samples 7, 10, 14, and 21 dpp at 12 and 24 hpi. Fifty µl of sample was applied to TLC plates prescored to have 2 cm lanes. The plates were developed with CHCl$_3$: methanol (9:1, v/v) to a distance of 10-13 cm. After development, the plates were allowed to dry in the fume hood before placing under vacuum in a desiccator overnight. The plates were observed using UV light (365 nm), and images were recorded using a ChromaDoc-It TLC imaging system with a Digi 105 color camera (12-megapixel), where under the UV light, compounds can be detected and characterized based on their absorbance or emission of fluorescence.

Bioassay

A *Cladosporium cucumerinum* spore suspension was prepared using 50% strength potato dextrose broth (PDB) (1.2 g PDB dehydrates in 100 ml of distilled water). PDB was added to *C. cucumerinum*, and mycelia were scraped to create a spore suspension that was filtered through cheesecloth mycelium and pieces of agar. The developed TLC plates were placed in a fume hood against the wall and sprayed with the prepared spore suspension until wetted thoroughly. Plates were then incubated in a humid chamber for 48 – 72 hrs. After incubation, antifungal activity was determined by the *C. cucumerinum* pigmented mycelia which grew across the plate except for the regions where there were compounds with antifungal activity (Klarman & Sanford, 1968). The TLC bioassay was conducted three times for the three biological replicates for each sample in the study.

RESULTS

To determine the presence of compounds with antifungal activity in the non-inoculated fruits of 'Chieftain', 'Early', and 'Dickenson Field', at 10, 14, and 21 dpp, TLC bioassay was performed. The separation of the methanol extracts from the non-inoculated fruit peel samples of 'Dickenson Field' on cellulose plate and the cultivars Chieftain, Early, and Dickenson Field on silica G plate at 10, 14, and 21 dpp (Figures 1 and 2, respectively) showed presence of non-polar compounds through fluorescence under the UV light. *Cladosporium cucumerinum* grew uniformly and covered the TLC plates except for those areas with antifungal activity (Figures 1 and 2). The *C. cucumerinum* spores and mycelium are darkly pigmented and contrasted with the white areas on the plate representing antifungal activity. In all cultivars, TLC bioassay analyses showed the presence of compounds with antifungal activity 10, 14, and 21 dpp. Zones with antifungal activity decreased as fruit aged (Figures 1 and 2) and was consistent in all cultivars across all replicates. There was no correlation between the presence of constitutively produced compounds with antifungal activity to ARR of winter squash to *P. capsici*.
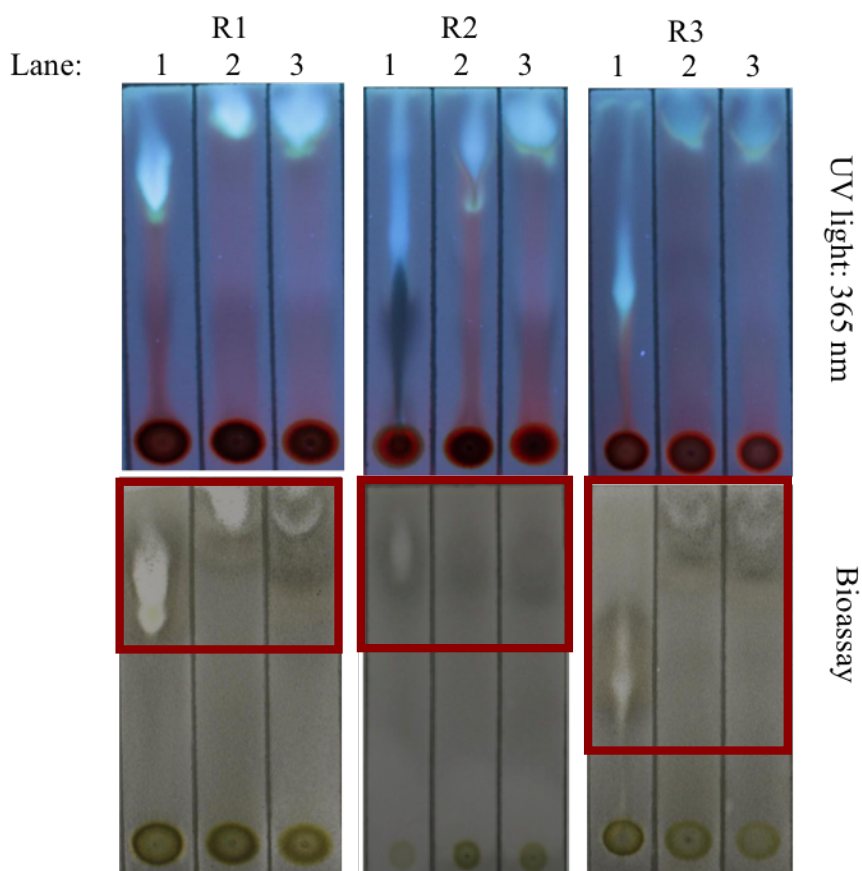
Figure 2.1.: Thin Layer Chromatography (TLC) bioassay of 'Dickenson Field' non-inoculated fruit peel methanol extracts at 10, 14, 21 days post pollination (dpp). Top images are methanol extract of three biological replicates, R1, R2, and R3 of 'Dickenson Field'. The extracts were applied to a cellulose plate and developed in distilled water. Lanes 1, 2, and 3 include 25mg of 10, 14, and 21 dpp respectively. Images were captured under UV light 365nm. Bottom images are the TLC plates sprayed with *Cladosporium cucumerinum* and incubated for 48hrs under humidity. White areas in the red boxes refer to zones of inhibition.
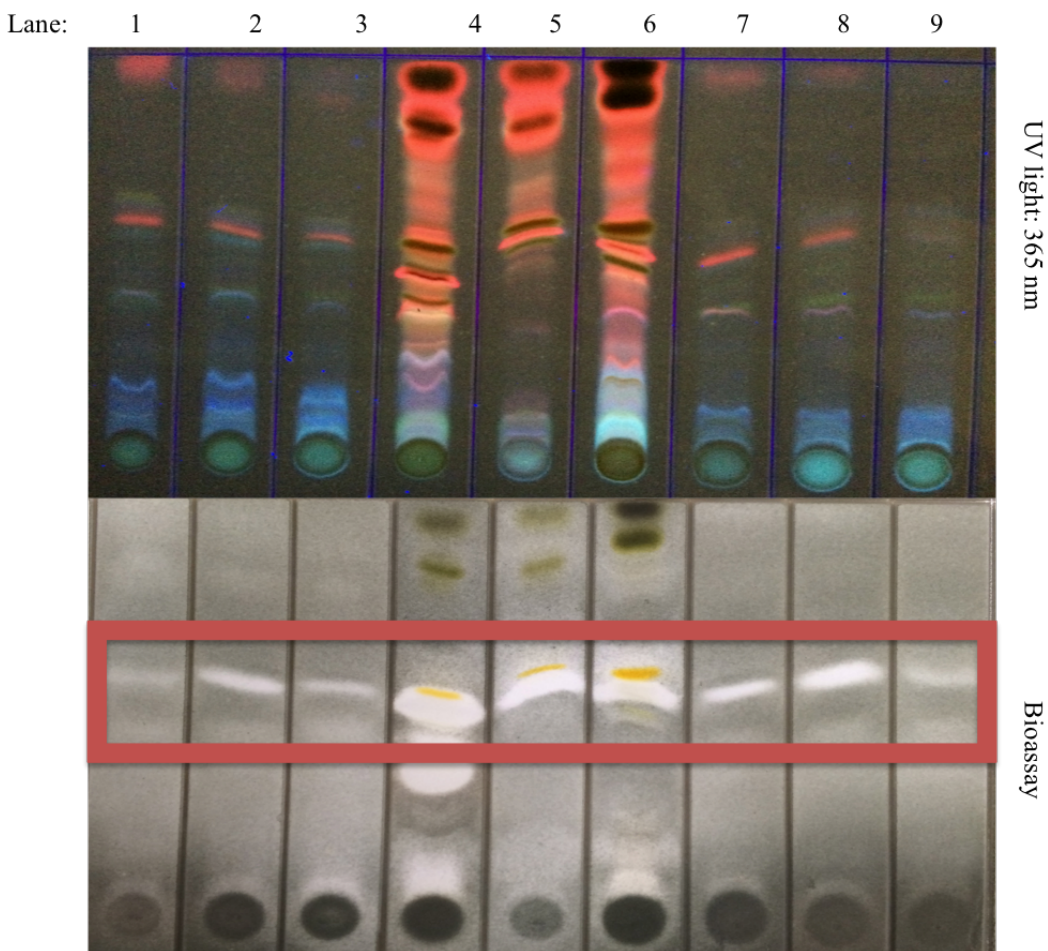
Figure 2.2.: Thin Layer Chromatography (TLC) bioassay of 'Early' (lanes 1 to 3), 'Dickenson Field' (lanes 4 to 6), and 'Chieftain' (lanes 7 to 9) non-inoculated fruit peel methanol extracts at 10, 14, 21 days post pollination (dpp) respectively. Top images are sample methanol extracts applied to a silica-G-gel plate and developed in chloroform-methanol (9:1). Images were captured under UV light 365nm. Bottom images are the TLC plates sprayed with *Cladosporium cucumerinum* and incubated for 48hrs under humidity. White areas in the red box refer to zones of inhibition.

To determine if there is induced production of antifungal compounds, fruits of cultivar Chieftain were harvested at 7, 10, 14 and 21 were inoculated with *P. capsici*. Extracts of inoculated and control tissues were analyzed using TLC bioassay. There was a clear separation of non-polar compounds that were dissolved in ethanol for non-inoculated fruit at 6 hours (control) and inoculated with 6 hpi (Figure 3) and at 12 hpi with 24 hpi (Figure 4) under the UV light as they emitted fluorescence. The bioassay has resulted in a consistent growth of *C. cucumerinum* covering the entire TLC plates except for areas with antifungal compounds. The TLC bioassay revealed areas with zones of inhibition for fungal growth for all fruit ages regardless of the dpp. A significant increase in antifungal activity was not observed in the resistant fruit at 14 and 21 dpp with increased time post inoculation. A correlation between the induced production of compounds with antifungal activity and ARR was not observed.
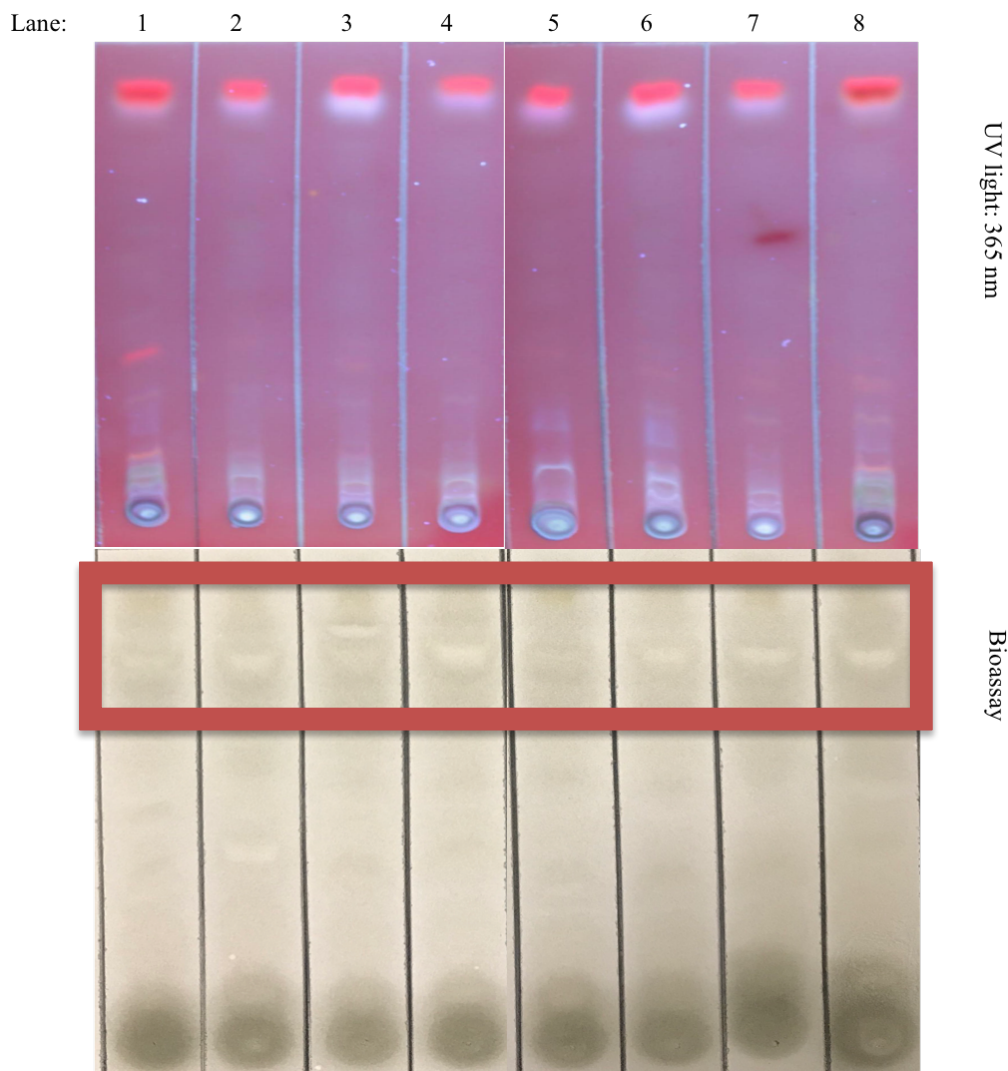
Figure 2.3.: Thin Layer Chromatography (TLC) bioassay of 'Chieftain' inoculated fruit peel at 7, 10, 14, 21 days pos pollination (dpp) with *Phytophthora capsici* at 6 hours control and 6 hours post inoculation (hpi). Top images are samples ethanol extracts applied to a silica-GF-gel plate and developed in chloroform-methanol (9:1). Lanes 1 – 4 include 50mg of 7, 10, 14, and 21 dpp at 6 hours control and lanes 5 – 8 include 50mg of 7, 10, 14, and 21 dpp at 6 hours post inoculation (hpi). Images were captured under UV light 365nm. Bottom images are the TLC plates sprayed with *Cladosporium cucumerinum* and incubated for 48hrs under humidity. White areas in the red box refer to zones of inhibition.
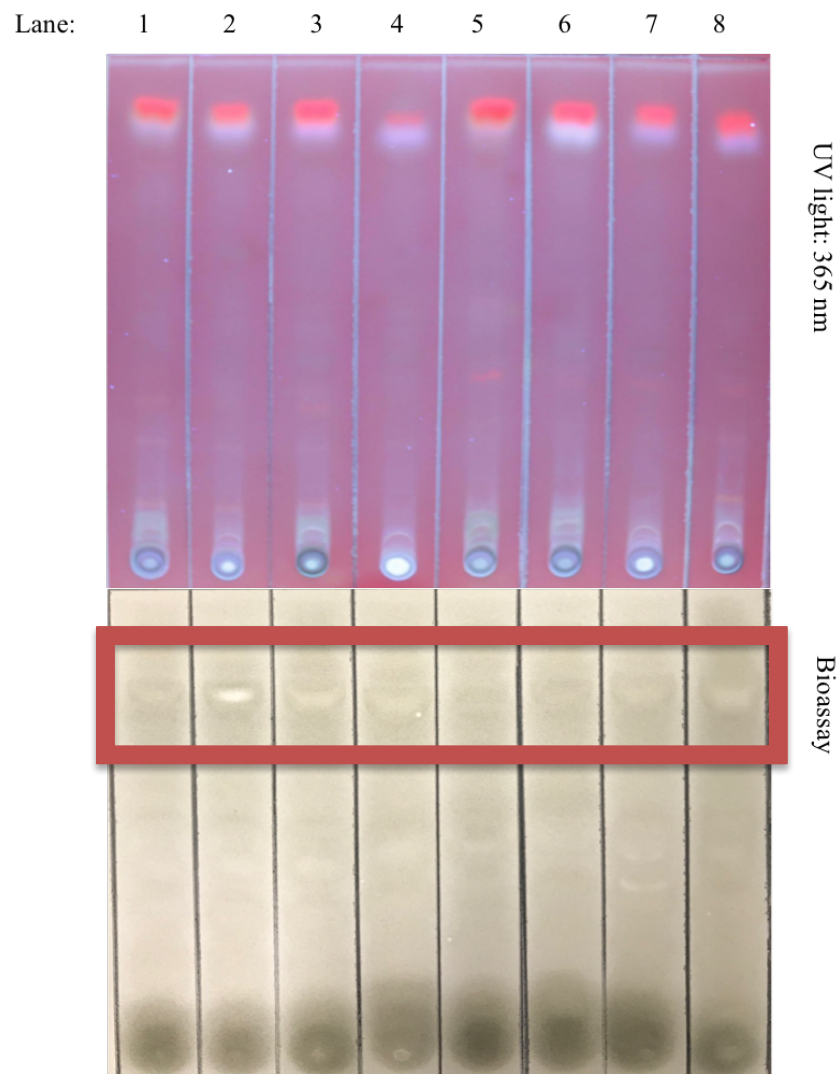
Figure 2.4.: Thin Layer Chromatography (TLC) bioassay of 'Chieftain' inoculated fruit peel at 7, 10, 14, 21 days post pollination (dpp) with *Phytophthora capsici* at 6 hours control and 6 hours post inoculation (hpi). Top images are samples ethanol extracts applied to a silica-GF-gel plate and developed in chloroform-methanol (9:1). Lanes 1 – 4 include 50mg of 7, 10, 14, and 21 dpp at 12 hours post inoculation (hpi) and lanes 5 – 8 include 50mg of 7, 10, 14, and 21 dpp at 24 hours post inoculation (hpi). Images were captured under UV light 365 nm. Bottom images are the TLC plates sprayed with *Cladosporium cucumerinum* and incubated for 48hrs under humidity. White areas in the red box refer to zones of inhibition.

DISCUSSION

ARR to *P. capsici* develops in some cucurbit fruits as they mature. Understanding the mechanisms of this form of resistance may be helpful in integrated management strategy to limit fruit rot disease. In the current study, the possible role of antifungal compounds in ARR to *P. capsici* was examined.

ARR phenomenon has also been observed in several plants against fungi, bacteria, viruses, and oomycetes (González-Lamothe *et al.* 2009). The mechanism of ARR seems to be variable among different plant systems (Panter and Jones 2002). Hypocotyl tissue of cucumber and soybean seedlings become resistant to *Cladosporium cucumerinum* (Panter and Jones 2002, Hammerschmidt 2011) and *Phytophthora sojae* (Lazarovits *et al.* 1980), respectively, as they age. *Arabidopsis* plants develop ARR to *Pseudomonas syringae* pv. *tomato* (Kus *et al.* 2002) and cauliflower mosaic virus (Leisner *et al.* 1993). The mechanism of ARR associated with fruit maturation has been studied in cucumber and pepper (Ando *et al.* 2015, Biles *et al.* 1993).

One of the potential mechanisms of plant defense against pathogens is the production of defensive secondary metabolites with antimicrobial activity (González-Lamothe *et al.* 2009). These defensive phytochemicals can be produced before pathogen infection and stored in plant tissues (i.e., phytoanticipins), or synthesized *de novo* (i.e., phytoalexins) in response to a pathogen attack (VanEtten *et al.* 1994, Paxton 1981). Our results revealed a decrease in constitutively produced antimicrobial activity in the non-inoculated fruit peel as the fruit aged (Figures 2.1, 2.2). A significant change in the antimicrobial activity following *P. capsici* infection was not observed (Figures 2.3, 2.4). Our findings did not support the hypothesis that a chemical barrier to *P. capsici* in resistant maturing fruit is correlated with ARR in *C. moschata* cultivars.

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The role of phytoalexins as defensive antimicrobial compounds has been studied in several plants such as camalexin, from *Arabidopsis* and *Camelina*. Infection of *Arabidopsis* with fungi, bacteria, viruses, and oomycetes induces the production of camalexin (González-Lamothe *et al.* 2009). High concentrations of camalexin were detected at the infection sites of *Arabidopsis* in association with infection by *Alternaria alternate* (Schuhegger *et al.* 2007). In *Cucurbita* fruit, *trans-p-*coumaryl aldehyde, a lignin-like material, was identified in *C. maxima* squash fruit tissue elicited with pectinase. The antifungal activity of this compound was confirmed using TLC-bioassay; *p-*coumaryl aldehyde was also detected in other elicited tissue of butternut, cucumber and pumpkin fruits (Stange *et al.* 1999). This compound was also induced by *C. cucumerinum* in scab-resistant cucumber seedlings (Varbanova *et al.* 2011). Also, *C*-glycosyl flavonoids have shown to function as phytoalexins in cucumber leaves elicited with Milsana and inoculated with powdery mildew fungus.

A role for phytoanticipins as plant defensive secondary metabolites with antimicrobial activity against pathogens have been demonstrated in several plant-pathogen systems (Panter and Jones 2002, González-Lamothe *et al.* 2009). Production of avenacin A-1 in oats roots has been shown to be a factor in resistance of that host to *Gaeumannomyces graminis* var. *tritici* (Osbourn *et al.* 1994). In a cucumber variety with ARR to *P. capsici*, the methanol extract of resistant maturing fruit peel was detected to inhibit *P. capsici* growth in vitro (Ando *et al.* 2015). When this methanol extract was metabolically analyzed, enrichment of terpenoid glycosides was detected (Mansfeld *et al.* 2017), which suggested a potential correlation of the constitutively produced secondary metabolite with the observed ARR in cucumber fruit. Although cucumber is in the same family Cucurbitaceae as squash, it seems that different mechanisms are controlling ARR in the different genera.

In the cucurbit, rockmelon (Cucumis melo L), resistance to Fusarium rot caused by *F. oxysporum* f. sp. *melonis* was associated with immature fruit; susceptibility increased with fruit age (Kumar and McConchie 2010). Kumar and McConchie (2010) detected two preformed antifungal compounds in rockmelon fruit peel, and their antifungal activity decreased with fruit age. In our study, a decrease in antifungal activity was detected and diminished with age. The reduction in antifungal activity in fruit peel correlated with ARR detected in rockmelon but was not detected in *C. moschata* cultivars.

This study did not provide evidence for constitutive or induced antifungal compounds as part of ARR *P. capsici* in winter squash. A study of fruit exocarp structural changes in 'Chieftain' butternut squash across development has shown that cuticle and epidermal walls were significantly thicker in resistant maturing fruit at 14 and 21 dpp than susceptible 7 dpp fruit, which suggests the presence of a structural barrier to *P. capsici* infection of the resistant fruit (Alzohairy *et al.* 2017). Cuticle thickness also increased with fruit age in pepper with ARR to *P. capsici* (Biles *et al.* 1993).

While the mechanism of ARR can be structural or chemical, our study did not show a correlation between the production of either constitutive or induced antifungal activity and ARR to *P. capsici*. These results may rule out a biochemical defense associated with ARR of *C. moschata* fruits and suggests that other structural or genetic factors control ARR. ARR can provide a benefit in managing fruit rot disease. However, its mechanisms in winter squash and pumpkins have not been uncovered.

LITERATURE CITED

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CHAPTER III: TRANSCRIPTOMIC PROFILING OF WINTER SQUASH IMPLICATES MONOLIGNOLS BIOSYNTHESIS AND LIGNIN POLYMERIZATION IN AGE-RELATED RESISTANCE TO THE OOMYCETE *PHYTOPHTHORA CAPSICI*

ABSTRACT

The oomycete plant pathogen, *Phytophthora capsici*, causes root, crown, and fruit rot of winter squash (*Cucurbita* spp.) and limits production. Some *Cucurbita moschata* cultivars develop age-related resistance (ARR) whereby fruit develop resistance to *P. capsici* 21 days post pollination (dpp) due to thickening exocarp, cuticle, and epidermal walls; wounding negates ARR. To determine the molecular mechanisms of ARR two *C. moschata* cultivars that exhibit ARR at different dpp were chosen and the transcriptome of fruit peel at susceptible and resistant time points was sequenced. Upregulated genes in the fruit peel resistant to *P. capsici* in both cultivars tended to play roles in cell wall and lignin biosynthesis and possess peroxidase and cinnamyl alcohol dehydrogenase activities. In addition, cutin, suberin, and phenylpropanoid biosynthetic pathway genes tended to be consistently upregulated when comparing resistant to susceptible time points. Our results provide candidate genes essential for *C. moschata* resistance to *P. capsici*, enabling the development of *C. moschata* varieties with resistance to *P. capsici* for improvement of squash and pumpkin fruit rot management schemes.

INTRODUCTION

Phytophthora capsici is a soilborne oomycete with a host range exceeding 50 plant species (Tian and Babadoost 2004). This polycyclic pathogen is responsible for significant plant losses when environmental conditions are favorable (Erwin and Ribeiro 1996, Hausbeck and Lamour 2004, Granke *et al.* 2009). Economically important crops within the Cucurbitaceae, Solanaceae, and Fabaceae are highly susceptible to *P. capsici* infection (Davidson *et al.* 2002, Hausbeck and Lamour, 2004, Gevens and Hausbeck 2005); cucurbits are considered among the most susceptible (Tian and Babadoost 2004). Symptoms of *P. capsici* infection on cucurbits include root and/or crown rot, foliar blight, and fruit rot (Babadoost 2004). Fruit rot threatens cucurbit crops annually, including squash and pumpkin, in Michigan (Lamour and Hausbeck 2000, Gevens *et al.* 2007, Krasnow and Hausbeck 2016) and other states (Babadoost 2004, Castro-Rocha *et al.* 2017). Michigan is an important producer of summer and hard squash (USDA 2018). The fruits may become rotted while in the field (Meyer and Hausbeck 2013, Granke *et al.* 2012) or postharvest (Hausbeck and Lamour 2004) leading to loss in crop production that may exceed 50% (Babadoost 2000, Meyer and Hausbeck 2013). Protecting squash fruits from *P. capsici* infection is challenging due to a relatively lengthy maturation time and during which the fruits are in direct contact with the soil. While host resistance is critical for long-term management (Quesada and Hausbeck 2010, Granke *et al.* 2012), complete host resistance in commercial cultivars of squash or pumpkin is not available (Café-Filho *et al.* 1995). However, age-related resistance (ARR) to *P. capsici* is expressed in the fruits of specific *C. moschata* cultivars (Meyer and Hausbeck 2013, Krasnow and Hausbeck 2016) and other cucurbit (Gevens *et al.* 2006, Ando *et al.* 2009) and Solanaceae (Biles *et al.* 1993) fruit. ARR is associated with resistance to pathogens that occurs at specific developmental stages (Stermer and Hammerschmidt 1984, Whalen, 2005). Fruits of a number of

cucurbits (Gevens *et al.* 2006, Ando *et al.* 2009, Meyer and Hausbeck 2013, Krasnow and Hausbeck 2016) and pepper (Biles *et al.* 1993) exhibit ARR to *P. capsici* as they mature. However, wounding negates ARR to *P. capsici* (Ando *et al.* 2015, Biles *et al.* 1993, Krasnow *et al.* 2014), suggesting the fruit peel may provide resistance to fruit rot.

The mechanism of ARR has been investigated in different host-pathogen systems (Panter and Jones 2002, Develey-Rivière and Galiana 2007). ARR can be conferred by preformed or induced defenses (Panter and Jones 2002, González-Lamothe *et al.* 2009) where preformed defense is the consequence of structural/physical and/or chemical barriers (Vergne *et al.* 2010). The plant cell wall serves as a physical barrier that forms an obstacle for all pathogens' entry but can be overcome by pathogen-generated cell wall degrading enzymes (CWDE) (Bacete *et al.* 2018, Bellincampi *et al.* 2014). In addition to cell wall, the plant surface is covered by the cuticle made up of cutin polymer that provides another defensive layer against pathogens (Chassot and Métraux 2005). Thickening of bean hypocotyls is correlated with resistance to *Rhizoctonia solani* (Stockwell and Hanchey 1983). Similarly, thickening of the cuticle has been suggested as the mechanism of ARR in pepper fruit (Biles *et al.* 1993) and *C. moschata* cultivars (Alzohairy *et al.* 2017) to *P. capsici*. Thickening of the epidermal walls was also observed in cucumber fruit with ARR to *P. capsici* (Ando *et al.* 2015). Another form of constitutive defense involving strengthened physical barrier is the formation of lignified xylem vessels in bean that leads to resistance against *Colletotrichum lindemuthianum* (Griffey and Leach 1965). In addition, lignin deposition at the cell wall makes it resistant to CWDE as has been observed in resistance of *Camelina sativa* to *Sclerotinia sclerotiorum* (Eynck *et al.* 2012). However, it remains to be determined if increased ligin deposition is important for ARR in cucurbits.

Transcriptomic studies of cucumber fruit with ARR showed an increase in the level of terpenoid glycosides in resistant maturing cucumber fruit compared to the susceptible younger fruit suggesting a role of constitutive chemical defense (Mansfeld *et al.* 2017). When the molecular mechanisms controlling ARR in apple leaves to *Venturia inaequalis* was studied, the constitutive upregulation of genes encoding for metallothionein3-like protein, lipoxygenase, lipid transfer protein, and peroxidase 3, and downregulation of gene encodes for 'enhanced disease susceptibility 1 protein' were highly correlated with the observed ARR of aging apple leaves (Gusberti *et al.* 2013).

Previous studies of fruit ARR in squash and pumpkin against *P. capsici* infection attribute ARR to morphological (Ando *et al.* 2009) or physiological changes (Meyer and Hausbeck 2013). The different developmental time points of ARR onset have been observed in cultivars of *C. moschata* (Alzohairy *et al.* 2017) and other cucurbits (Ando *et al.* 2009, Gevens *et al.* 2006). The difference in ARR onset has been suggested to be related to the difference in rates of fruit development (Gevens *et al.* 2006). However, the genetic mechanism of ARR in winter squash and how the difference in ARR is regulated across cultivars is not known. This study aims to use transcriptomic studies and differential gene expression analysis between resistant and susceptible fruit stages to assess the molecular mechanisms of ARR in two *C. moschata* cultivars with different ARR onset time points.

MATERIALS AND METHODS

Plant material

Two *Cucurbita moschata* commercial cultivars, Chieftain (butternut winter squash, seeds were obtained from Rupp Seeds Inc., Wauseon, OH) and Dickenson Field (processing pumpkin, seeds

were obtained from Rispens seeds Inc.), were previously evaluated for age-related resistance (ARR) (Meyer and Hausbeck 2013, Alzohairy *et al.* 2017). Fruits of these two cultivars were produced in the field according to Meyer and Hausbeck (2013). At anthesis, flowers were hand pollinated then fruits were harvested at 7, 10, 14, and 21 days post pollination (dpp).

RNA extraction

Fruits of 'Chieftain' and 'Dickenson Field' at 7, 10, 14, and 21 dpp were surface disinfested with 70% ethanol then air-dried on paper towel. Sterilized fruits were peeled using the vegetable peeler and immediately frozen in liquid nitrogen. Fruit peels were stored at -80°C until RNA isolation. RNA was extracted from three biological replicate samples for each cultivar and each date using E.Z.N.A. Total RNA kit (OMEGA BIO-TEK) and treated with Turbo DNase (2U/µl, Invitrogen) to remove DNA contamination. RNA concentration was determined using Nanodrop 1000 spectrophotometer (Thermo Scientific). RNA integrity (RIN) was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples had RIN scores ≥8.

RNA sequencing and RNA-seq read processing

RNA library construction and sequencing were done at the Research Technology Support Facility (RTSF) at Michigan State University. RNA libraries were prepared using the Illumina TruSeq Stranded mRNA kit following the Illumina protocols. RNA sequencing was performed using the Illumina HiSeq 4000 platform. Illumina run was in the 2 x 150bp paired-end format. A total of 24 libraries were divided into two pools of 12, and each pool was sequenced on two lanes. Each lane produced an approximate of 30 million reads/sample. Due to a technical error in the sequencing run, two libraries for 'Chieftain' at 14 dpp were resequenced on the same platform, which produced ~180 million read/sample.

An initial quality check of the RNA reads was performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). RNA reads from the two lanes for the same sample were combined in a single fastq file for each R1 and R2. Trimmomatic (Bogler *et al.* 2014) v0.32 was used to filter the RNA reads for adaptors, and low-quality reads. Sliding window method was used to scan the reads with 4-base wide and cut when the base quality is below a threshold of 2. The minimum read length cutoff was 20 bp. Data quality was explored after filtering with FastQC.

STAR v2.5.1b (Spliced Transcripts Alignment to a Reference, Dobin *et al.* 2013) was used to map the RNAseq reads to the *Cucurbita moschata* cv. Rifu reference genome (Sun H *et al.* 2017) from the Cucurbit Genomics Database (http://cucurbitgenomics.org/organism/9). STAR was used with the default settings using the twopassMode Basic option with intron size 21-6000 nt. In all samples, >88% of the RNAseq reads were mapped to the reference genome. The number of sequenced reads, filtered reads, and mapping information are presented in Appendix Table 3. 1.

Differential expression and clustering analysis

The HTseq-count function in HTseq (High-Throughput sequencing) (Anders *et al.* 2015) v0.6.1 was used in the default mode and stranded==reverse for generating read counts. HTseq-count output was fed into DESeq2 (Love and Anders 2014) for differential expression analysis using the standard steps represented in DESeq function (Love *et al.* 2017).

A gene was considered differentially expressed in contrast if the adjusted *p*-value < 0.05 and the log fold change > 1. The *p*-value was adjusted with Benjamini-Hochberg. DEGs from all comparisons among cultivars and were categorized into three main sets including set1: genes that are upregulated or downregulated among all tested comparisons across cultivars; set2: genes that

are commonly upregulated or downregulated in all comparisons per cultivar and set3: genes that are expressed at least in one comparison in both cultivars (Supplementary Table 1).

The clustering of expression data was done using *k*-means clustering with the Complex Heatmap package in R (Gu. *et al.*, 2016) with $k = 16$.

Identification of putative *Arabidopsis* orthologous genes and inference of squash cell well pathway genes

Putative orthologous *Arabidopsis thaliana* genes of *C. moschata* DEGs and non-DEGs were identified using BLASTX (Altschul *et al.* 1990) by comparing the nucleotide sequence of the *C. moschata* DEGs and non-DEGs to the peptide sequence of *A. thaliana* using e-value of 10e-10. *Arabidopsis* pathways related to cell wall structure/composition were downloaded from (https://www.plantcyc.org/). The pathways include cuticular wax biosynthesis, cutin biosynthesis, long-chain fatty acid activation, suberin monomers biosynthesis, esterified suberin biosynthesis, cellulose biosynthesis, homogalacturonan biosynthesis, xylogalacturonan biosynthesis, phenylpropanoid biosynthesis, and xylan biosynthesis.

Functional annotation and pathway enrichment analysis

All DEGs and non-DEGs were functionally annotated through extracting the Gene Ontology (GO) annotations, Interpro, and description from Cucurbit Genomics database (http://cucurbitgenomics.org/organism/9). GO enrichment analysis were done for the different DEGs lists for both cultivars independently. Pathway enrichment analysis were performed using the *C. moschata* genes orthologous to the *Arabidopsis* genes in ten targeted biosynthesis pathways, which are cuticular wax, cutin, long chain fatty acid activation, suberin monomers, esterified

suberin, cellulose, homogalacturonan, xylan, xylogalacturonan, phenylpropanoid. Both GO and pathway enrichment analysis were based on Fisher's exact tests. *p*-values were corrected for multiple testing (Benjamini and Hochberg 1995) and reported as q-values. DEGs were considered significantly overrepresented for a GO-term or pathway when they were positively enriched, and their q-value was <=0.05. Fisher's exact tests were performed using manual python scripts which employ the python package fisher 0.1.5 https://pypi.org/project/fisher/ .

RESULTS

Sequencing and gene expression profile among cultivars

To study ARR in winter squash to *P. capsici*, the fruit peel transcriptomes of two cultivars, Chieftain and Dickenson, at different developmental time points prior to and after the development of ARR were sequenced (Figure 3.1A). *C. moschata* genes were classified into 16 clusters using the transcriptome of both cultivars at different developmental time points to assess the extent to which gene expression patterns correlated with ARR onset (Figure 3.1B). These clusters were grouped into 4 groups based on the similarity in gene expression profile during different time points and between cultivars. We anticipated that ARR-associated genes would have an expression profile that consists of two major patterns. First, groups of genes with a consistent change of expression either by upregulation or downregulation in both cultivars in at least one resistant time point (Figure 3.1B, groups 1 and 2). Second, groups of genes whose expression were upregulated or downregulated in at least one resistant stage in only one cultivar (Figure 3.1B, groups 3 and 4). Groups 3 and 4 represent the opposite expression pattern in both cultivars. For example, in one cultivar, genes may be upregulated in at least one susceptible stage then become downregulated in the resistant stages. This pattern was reversed in the second cultivar in the same cluster. Also,

groups 3 and 4 include genes with irregular expression pattern (e.g. genes can be going up-down-up-down through the four stages of each cultivar). Genes in groups 3 and 4 are less likely to be to be candidates for ARR. Dividing the genes into different clusters facilitated identifying a group of genes that are likely candidates of ARR (Figure 3.1B, groups 1 and 2). Therefore, we performed differential expression analysis to detect potential ARR-associated genes.
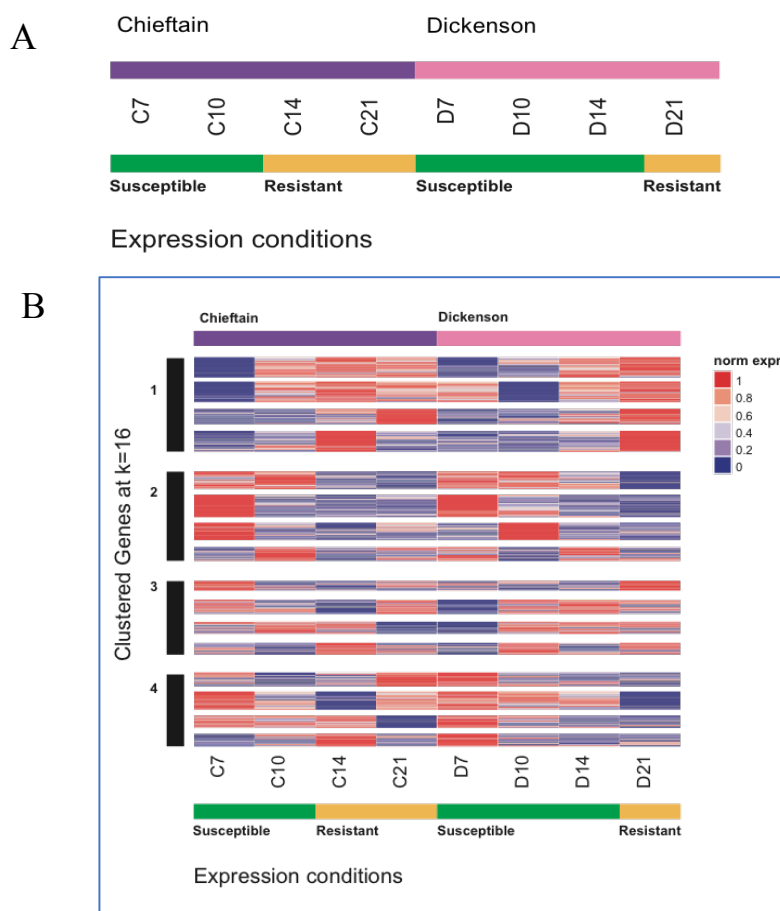
Figure 3.1.: A) Expression conditions of cultivars Chieftain and Dickenson fruit peel at ages 7, 10, 14, and 21 days post pollination. B) Heatmap of K-means clustering of per-gene normalized expression between both 'Chieftain' and 'Dickenson' at 7, 10, 14, 21 dpp. Numbers 1, 2, 3, and 4 are different groups of clusters. Genes were clustered at K=16.

Differential gene expression of 'Chieftain' and 'Dickenson Field'

In 'Chieftain', ARR develops at 14 dpp and continues through 21 dpp, while ARR in 'Dickenson' develops at 21 dpp. To identify candidate genes relevant to resistance development at these ages in 'Chieftain', we contrasted the gene expression of the susceptible fruit peels at 7 and 10 dpp to the resistant ones at 14 and 21 dpp. Similarly, gene expression of the susceptible fruit peels was contrasted at 7, 10, and 14 dpp with the resistant 21 dpp in 'Dickenson'. Candidate genes responsible for resistance against *P. capsici* included genes significantly upregulated or downregulated at 14 and 21 dpp in 'Chieftain' and at 21 dpp in 'Dickenson' when contrasted to their susceptible fruit peel ages (Figure 3.2A). The overlap between the different contrasts per each cultivar for both upregulated (Figure 3.2A, B, C) and downregulated genes (Figure 3.2A, D, E) was used to identify DEGs that were consistently upregulated or downregulated in the resistant stages compared to their susceptible stages. When comparing the resistant stages in 'Chieftain' to the susceptible stages, DEGs tended to be similarly up- or down-regulated. Thus, there are groups of genes with expression patterns that are correlated with the resistant phenotype (Figure 3.2A). In addition, fewer DEGs were detected when comparing 14 and 21 dpp to 10 dpp than to 7 dpp (Figure 3.2B, D) and that difference in DEGs helped to narrow down the list of DEGs that are likely candidate genes for ARR. Similarly, in 'Dickenson', there was an apparent decrease in the number of the determined DEGs as the fruit aged toward the resistance showing also a gradual transition toward resistance that developed at 21 dpp (Figure 3.2C, E). From the differential gene expression analysis, we narrowed down the DEGs to sets 1, 2, and 3 that are potentially included in the ARR-associated genes.
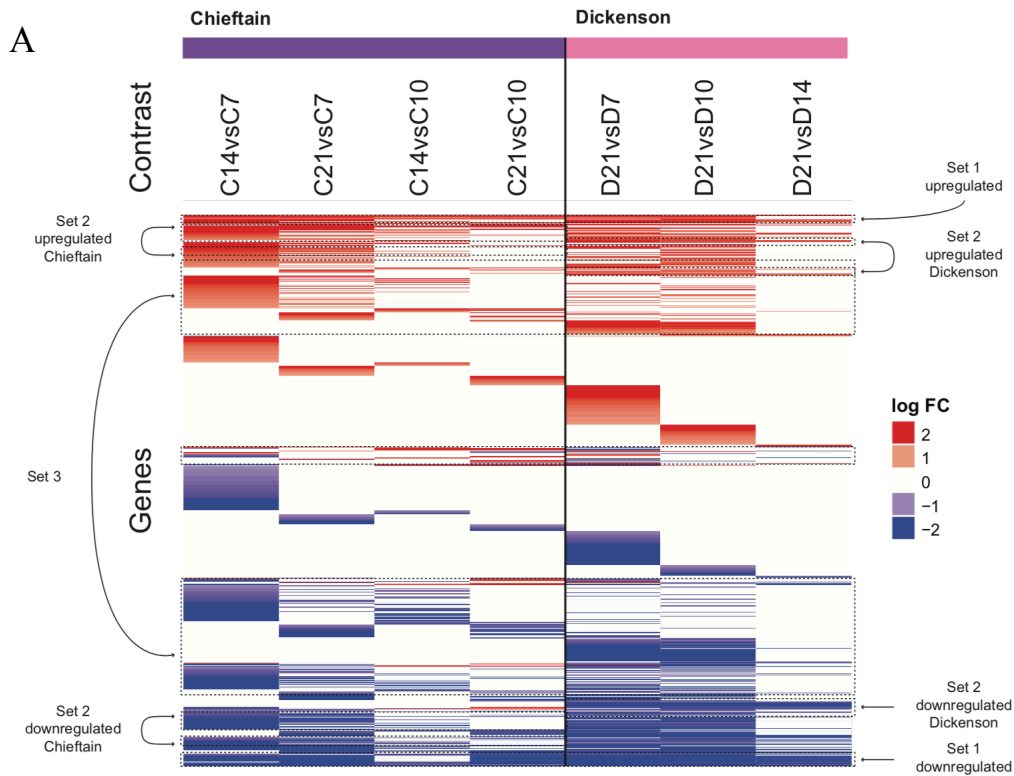
Figure 3.2.: Differential gene expression analysis of 'Chieftain' and 'Dickenson' fruits at 7, 10, 14 and 21 days post pollination (dpp). The onset of age-related resistance (ARR) is at 14 and 21 dpp in 'Chieftain' and 'Dickenson', respectively. A) Heatmap of differentially expressed genes (DEGs) with |log2(FC)|>1, (FC: fold change) and adjusted p-values <0.05. Each column is a contrast between resistant vs susceptible peel of fruit at 7, 10, 14 and 21 dpp. A) and B) Venn diagrams showing upregulated and downregulated genes in all sets of comparisons in 'Chieftain' respectively; C) and D) Venn diagrams showing upregulated and downregulated genes in all sets of comparisons in 'Dickenson', respectively. The letters C and D indicates 'Chieftain' and 'Dickenson', respectively.

Figure 3.2.: (cont'd)

B
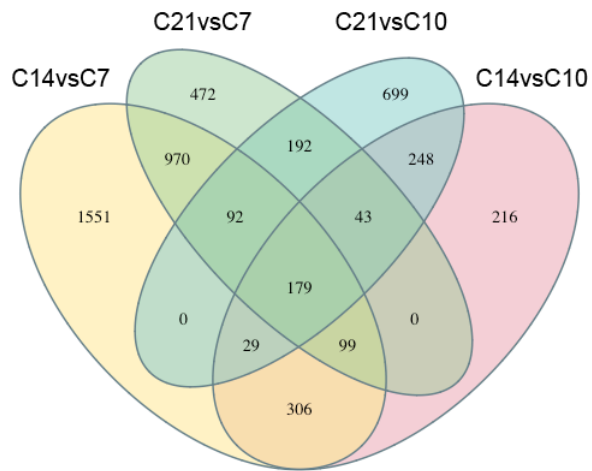
C
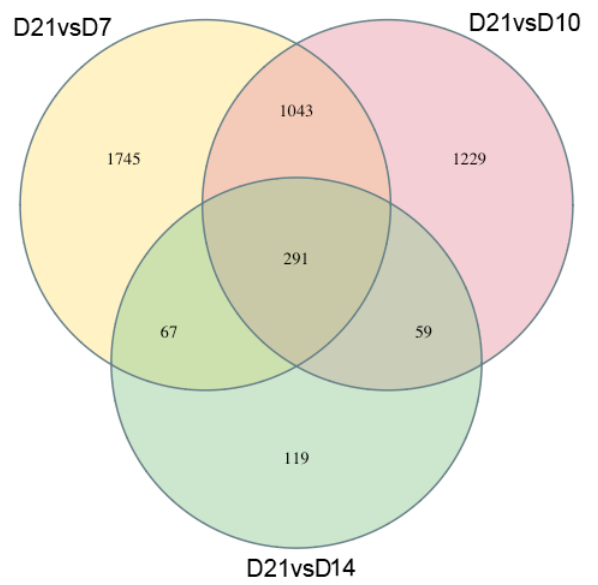
D
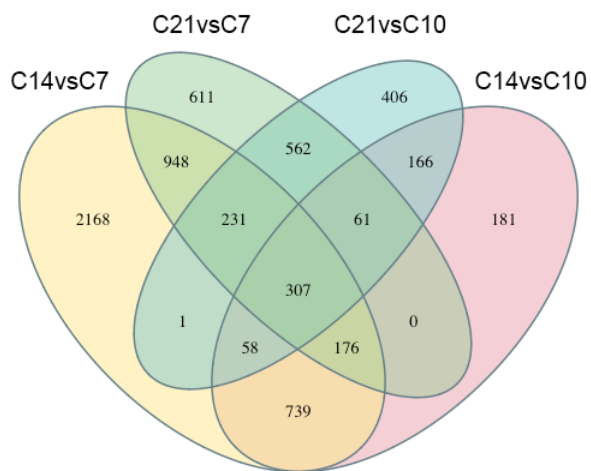
E
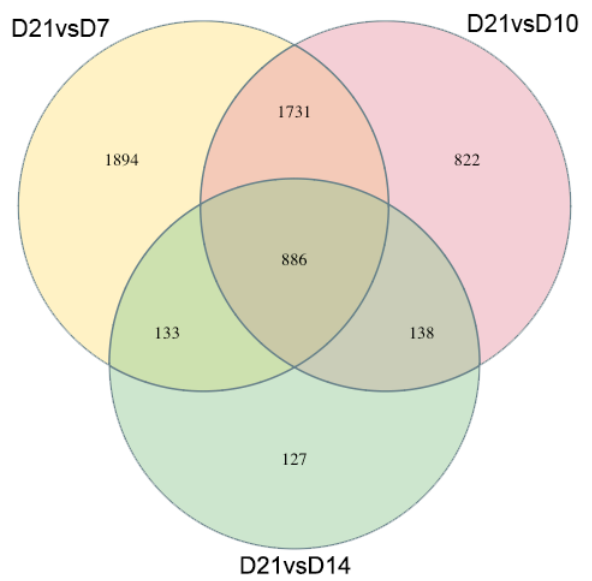
Function of upregulated genes in both cultivars

The function of the upregulated genes in the resistant fruit peel stages when contrasted to their susceptible stages was investigated to determine which genes are candidates for controlling ARR. In 'Chieftain', we included the resistant stages of 14 and 21 dpp to determine if there are stage-specific genes that could be related to ARR versus those that may be constant during both 14 and 21 dpp. Therefore, the Gene Ontology (GO) categories enriched in upregulated genes resulted from individual contrasts were identified. First, the function of the upregulated genes that were detected from the contrast between 14 dpp to 7 and 10 dpp were investigated in order to detect the functions that were consistently present in the resistant stage 14 dpp and related to ARR. Among 3226 and 1120 upregulated genes in 'Chieftain' resulting from the contrast of the early resistant time point (14 dpp) vs. susceptible time points (7 and 10 dpp), they were enriched in 33 and 44 GO-terms, respectively (Appendix Table 3.2). The most significantly overrepresented GO terms during early resistant time point include those relevant to cell wall structures (e.g. lignin biosynthesis process) and phenylpropanoids biosynthesis (e.g. cinnamyl alcohol dehydrogenase activity, sinapyl alcohol dehydrogenase activity), oxidoreductases (e.g. peroxidase activity) and defense (e.g. defense response to bacterium) (Figure 3.3, Appendix Table 3. 2). This result indicates that a group of genes functioning in cell wall structures and phenylpropanoid biosynthesis are likely related to ARR at 14 dpp fruit in 'Chieftain'.
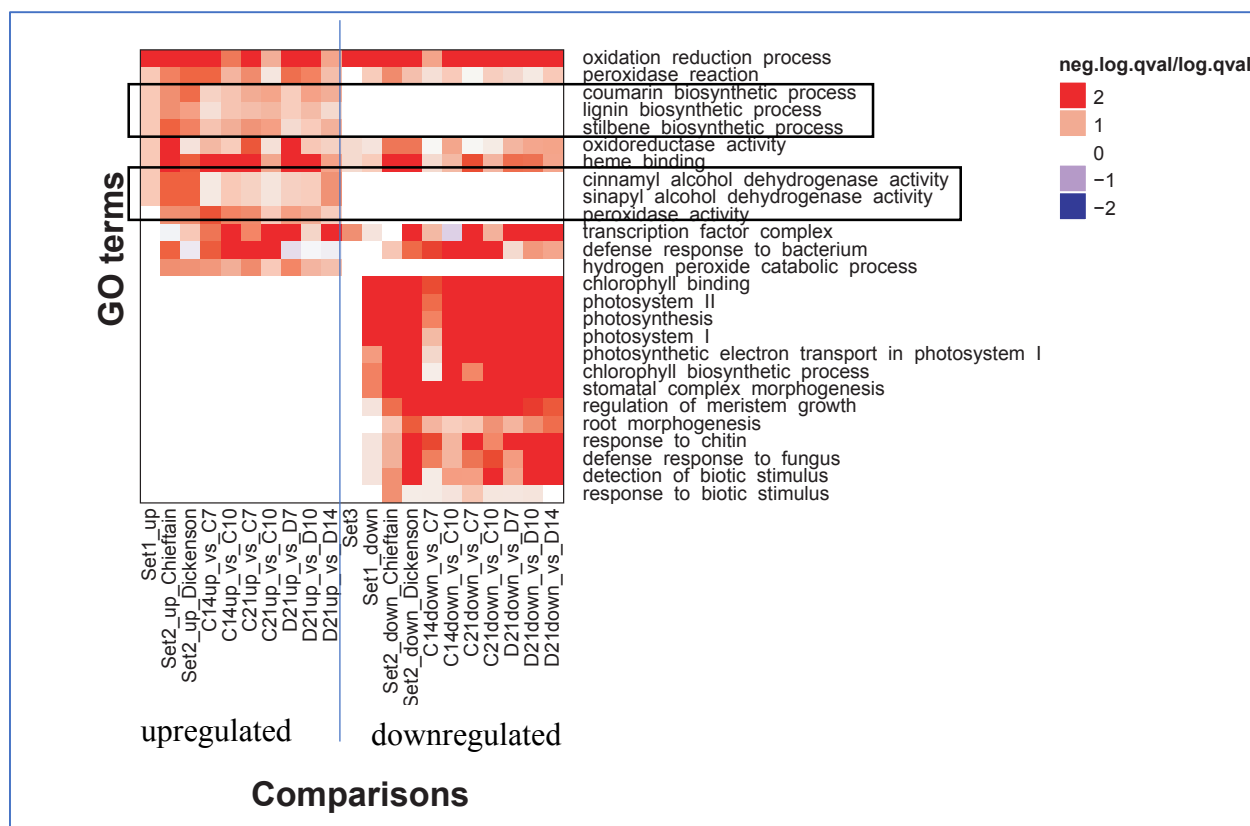
Figure 3.3.: Heatmap showing selected GO terms that are significantly overrepresented in either upregulated genes or downregulated genes in contrasts between resistant vs susceptible peel of fruit at 7, 10, 14 and 21 days post pollination (dpp) in both 'Chieftain' and 'Dickenson'. The onset of age-related resistance (ARR) is at 14 and 21 dpp in 'Chieftain' and 'Dickenson' respectively. The letters C and D indicates for 'Chieftain' and 'Dickenson' respectively. The value range of the heatmap is shown as the result of the Fisher's Exact test. If the GO term was overrepresented, the negative log of the adjusted *p*-value (or q-value) was taken, while if it was underrepresented, the log of the adjusted *p*-value was taken. Therefore, a positive value > or = 1.3 indicates significant overrepresentation while a negative value < or = -1.3 indicates significant underrepresentation. The black rectangles point to functions related to cell wall structures and phenylpropanoid biosynthesis processes that are overrepresented only in the upregulated genes in both cultivars.

Next, we studied the function of the upregulated genes detected from contrast between late resistant stage 21 dpp and susceptible stages (7 and 10 dpp) in 'Chieftain'. By defining the function of the upregulated genes in late resistant stage (21 dpp), the functions that are related to ARR can be detected by filtering the shared functions with early resistant stage (14 dpp). Also, comparing the functions that are detected in both early (14 dpp) and late resistant (21 dpp) stages will define

differences in functions that could be related to each resistant time point. Among 2047 and 1482 'Chieftain' upregulated genes when comparing expression levels during the late stage of resistance (21 dpp) to those during susceptible time points 7 and 10 dpp, they were enriched in 38 and 41 GO terms respectively (Appendix Table 3.2). Similar GO terms were detected between early (14 dpp) and late (21 dpp) resistant stages include cell wall structures (e.g. lignin biosynthesis process) and phenylpropanoids biosynthesis (e.g. cinnamyl alcohol dehydrogenase activity, sinapyl alcohol dehydrogenase activity), oxidoreductases (e.g. peroxidase activity), and defense (e.g. defense response to bacterium) (Figure 3, Appendix Table 3. 2). Few GO terms were different between 14 and 21 dpp when compared to 7 and 10 dpp include GO terms relevant to fruit ripening (e.g. xylem development) and sugar hydrolysis (e.g. beta-galactosidase, glycosaminoglycan catabolic process) (Appendix Table 3. 2). The different GO terms between 14 and 21 dpp did not indicate specific enrichment in functions relevant to resistance (Appendix Table 3. 2), suggesting that the resistance is most likely associated with genes that are consistently upregulated in both 14 and 21 dpp in contrast to the susceptible stage 7 and 10 dpp.

In 'Dickenson' the functions of upregulated genes detected in the contrasts of 21 vs.7 dpp, 21 vs.10 dpp, and 21 vs.14 dpp were enriched in 45, 43, 48 GO terms respectively (Appendix Table 3. 2). Overrepresented GO terms most relevant to the resistance are similar to those detected in 'Chieftain' contrasts included cell wall structures and phenylpropanoids biosynthesis (Figure 3.3). Cultivars distinct GO terms were not related to resistance (Appendix Table 3. 2). Taken together, group of GO terms were identified that were consistently enriched in the upregulated genes detected in the resistant stages in both cultivars when contrasted to their susceptible stages, suggesting that the genes involved in these functions are likely to be ARR-associated genes.

Function of downregulated genes in both cultivars

To identify the genes that are downregulated during fruit development and the transition to a resistant state, the function of the detected downregulated genes in the resistant stages in both cultivars using GO was assessed. In 'Chieftain, among the 4628 and 1688 downregulated genes in 14 vs. 7 dpp and vs. 10 dpp, 127 and 149 GO terms were enriched, respectively. Among the 2896 and 1792 downregulated genes at 21 dpp when compared to 7 and 10 dpp, 152 and 161 GO terms, respectively, resulted (Appendix Table 3.3). These GO terms are highly overrepresented in photosynthesis (e.g. chlorophyll binding, photosystem II, chloroplast thylakoid membrane), cell growth (e.g. regulation of meristem growth, tissue development, regulation of cell size), cell differentiation (e.g. stomatal complex morphogenesis, root morphogenesis) and defense (e.g. defense response to fungi or bacteria, negative regulation of defense) (Figure 3.3, Appendix Table 3.3). This result indicates that in 'Chieftain', ARR associated genes are genes that are upregulated in the resistant stages in comparison to the susceptible stages.

In 'Dickenson', the 4644, 3577, and 1284 downregulated genes in the contrasts of 21 dpp vs. 7, 10, and 14 dpp were enriched for 165, 167, and 170 GO terms respectively (Appendix Table 3. 3). Shared categories of GO terms among the three different contrasts of 'Dickenson' are overrepresented with high significance in photosynthesis (e.g. photosystem I and II, plastid organization, chloroplast thylakoid membrane), and metabolic processes (e.g. sucrose and starch metabolic process), in addition to other GO terms categories such as defense (e.g. systemic acquired resistance, detection to biotic stimulus, defense response to fungus) (Figure 3.3, Appendix Table 3. 3). This result indicates that in 'Dickenson', ARR associated genes are in the upregulated list of genes in the resistant fruit 21 dpp. Our findings from the functional annotation

of both upregulated and downregulated genes detected in the comparisons of resistant vs. susceptible fruit ages in both cultivars indicate that the mechanism of ARR is most likely controlled similarly in both cultivars.

Comparison of ARR mechanism among cultivars

The studied cultivars of *C. moschata* have a different onset of ARR to *P. capsici*, with 'Chieftain' developing resistance at 14 dpp and 'Dickenson' developing resistance at 21 dpp. To determine whether the mechanism of ARR to P. capsici is similar or different across cultivars, DEGs between the two cultivars and among the different developmental stages were categorized into three sets of genes (Figure 3.2A, Supplementary Table 1). The Set1 genes included those that are consistently upregulated or downregulated in both cultivars in the resistant fruit peel ages in comparison to the susceptible fruit peel ages (Figure 3.2A). The Set1 genes are likely to be genes that are associated with resistance in case the mechanism of ARR is similar acoss cultivars. The Set2 genes are commonly upregulated or downregulated in all comparisons within each cultivar. The Set2 genes are likely to be cultivar specific genes, and therefore, we can determine if the mechanism is different between cultivars. Finally, the Set3 genes are those that are either up or down-regulated in at least one contrast between resistant and susceptible stages in both cultivars (Figure 3.2A). The Set3 genes can be related to the resistance mechanism that could be specific to any resistant stage in both cultivars.

Upregulated genes in Set1 were enriched in 38 GO terms (Appendix Table 3. 2) and the overrepresented GO terms included cell wall structures, phenylpropanoid biosynthesis (e.g. lignin biosynthesis process, sinapyl alcohol dehydrogenase activity, cinnamyl alcohol dehydrogenase activity, stilbene biosynthetic process, and coumarin biosynthetic process), and oxidoreductases

(e.g. peroxidase activity). In Set2, upregulated genes in 'Chieftain', were enriched in 40 GO terms. The top ten significantly overrepresented GO terms included the oxidoreductase activity, stilbene biosynthetic process, sinapyl alcohol dehydrogenase activity, and cinnamyl alcohol dehydrogenase activity (Appendix Table 3. 2). However, other overrepresented GO terms for lignin biosynthetic process, peroxidase activity, and coumarin biosynthetic process were still detected (Appendix Table 3. 2).

Upregulated genes of Set2 in 'Dickenson' were enriched in 49 GO terms with the highly scored including sinapyl alcohol dehydrogenase activity, cinnamyl alcohol dehydrogenase activity, lignin biosynthetic process, peroxidase activity, and coumarin biosynthetic process (Appendix Table 3. 2). We also questioned if the ARR phenotype is correlated with genes that are expressed in any comparison in both cultivars, Set3. The function of DEGs in Set3 were enriched in 29 overrepresented GO terms but didn't seem to be relevant to ARR (Appendix Table 3. 2 and 3. 3).

We also investigated the function of downregulated genes detected in all different sets. Downregulated genes in Set1, Set2 in Chieftain, and Set2 in Dickenson were enriched in 114, 154, and 174 GO terms respectively (Appendix Table 3. 3). The highly-scored GO terms for all sets were involved in photosynthesis (e.g., chlorophyll binding, protein chromophore linkage, photosynthesis, chloroplast thylakoid membrane and photosystem I and II) (Figure 3, Appendix Table 3. 3). According to these findings we sought to question which cell wall structures are specifically regulated during development and can be a candidate for the winter squash ARR mechanism against *P. capsici*.

Pathway enrichment of cell wall structure-related genes

In the GO enrichment analysis, we detected that cell wall structures and phenylpropanoid biosynthesis processes are enriched in the upregulated genes in the resistant stages in both cultivars. Based on this finding and also the findings from Alzohairy *et al.* 2017 that cuticle and epidermal walls thickness increase in the resistant fruit ages so we hypothesized that cell wall structures biosynthesis is related to ARR. To define which cell wall structure is related to ARR we studied the enrichment of different cell wall structures biosynthesis pathways in the lists of upregulated and downregulated genes detected in the peel of resistant fruit ages in both cultivars. The winter squash orthologous upregulated and downregulated genes in the resistant stages were examined for enrichment in pathways for cell wall structures biosynthesis. First, the nucleotide sequences of upregulated and downregulated genes in the resistant stages were blasted against *Arabidopsis* peptide database and orthologous squash genes were detected. Then, orthologous squash genes to *Arabidopsis* genes that are involved in ten different cell wall structures biosynthesis pathways were used in the enrichment analysis. Out of ten tested pathways, five were enriched in upregulated and downregulated genes in the resistant fruit stages in both cultivars, which are cutin, phenylpropanoid, suberin monomers, homogalacturonan, and cellulose biosynthesis (Figure 3.4). We detected that cellulose and homogalacturonan biosynthetic pathways were overrepresented in the lists of downregulated genes in resistant stages of both cultivars. While cutin biosynthetic pathway was overrepresented in both lists of up and downregulated genes in both cultivars but more in the downregulated lists of genes in the case of 'Chieftain' (Figure 3.4). Suberin monomers and phenylpropanoid biosynthesis pathways were overrepresented in the upregulated lists of genes for both cultivars more than in the downregulated lists of genes (Figure 3.4).
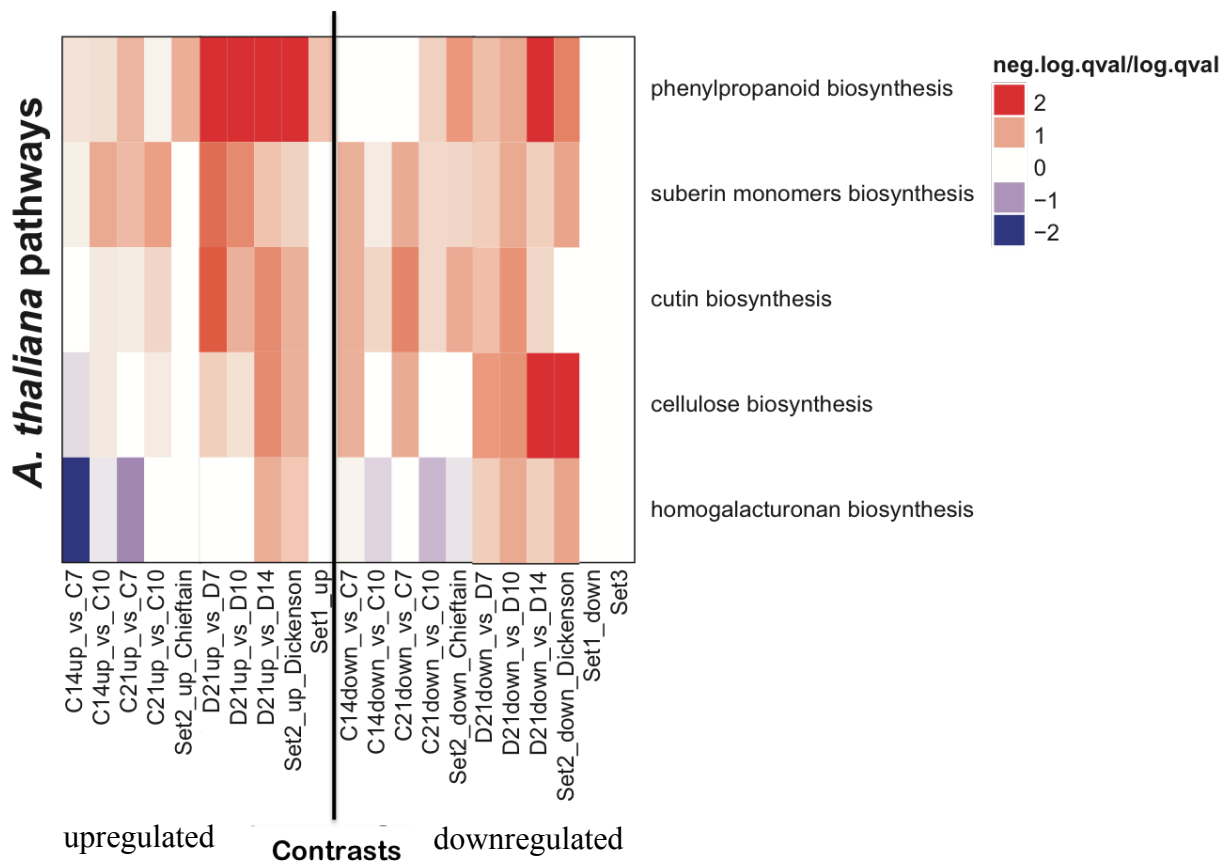
Figure 3.4.: Heatmap showing the pathway enrichment analysis of hard squash of *C. moschata* orthologous genes in *Arabidopsis* cell wall structures biosynthetic pathways. Columns show the contrast between resistant vs susceptible peel of fruit at 7, 10, 14 and 21 days post pollination in both 'Chieftain' and 'Dickenson'. The onset of age-related resistance (ARR) is at 14 and 21 in 'Chieftain' and 'Dickenson' respectively. The letters C and D indicates for 'Chieftain' and 'Dickenson' respectively. The value in the range of the heatmap is shown as the results of the Fisher's Exact test. If the pathway was overrepresented, the negative log of the adjusted *p*-value (or q-value) was taken, while if it was underrepresented, the log of the adjusted *p*-value was taken. Therefore a positive value > or = 1.3 indicates significant overrepresentation while a negative value < or = -1.3 indicates significant underrepresentation.

For a better understanding of the enrichment patterns in upregulated versus downregulated genes, we plotted the expression profile of all orthologous upregulated and downregulated genes in the resistant fruit stages that were detected from the different contrasts in both cultivars and involved in phenylpropanoid, suberin monomers, cutin, cellulose, and homogalacturonan biosynthetic pathways (Figure 3.5A, B, C, D, E). The general expression profile of squash orthologous genes

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showed more upregulated genes associated with the phenylpropanoid, suberin monomers, and cutin biosynthetic processes (Figure 3.5A, B, C) while downregulated genes were more associated with the cellulose and homogalacturonan biosynthetic pathways (Figure 3.5D, E). These results indicate that the phenylpropanoid, suberin monomers, and cutin biosynthetic processes are most likely associated with the observed ARR since they are enriched in the upregulated genes in the resistant stages in both cultivars.
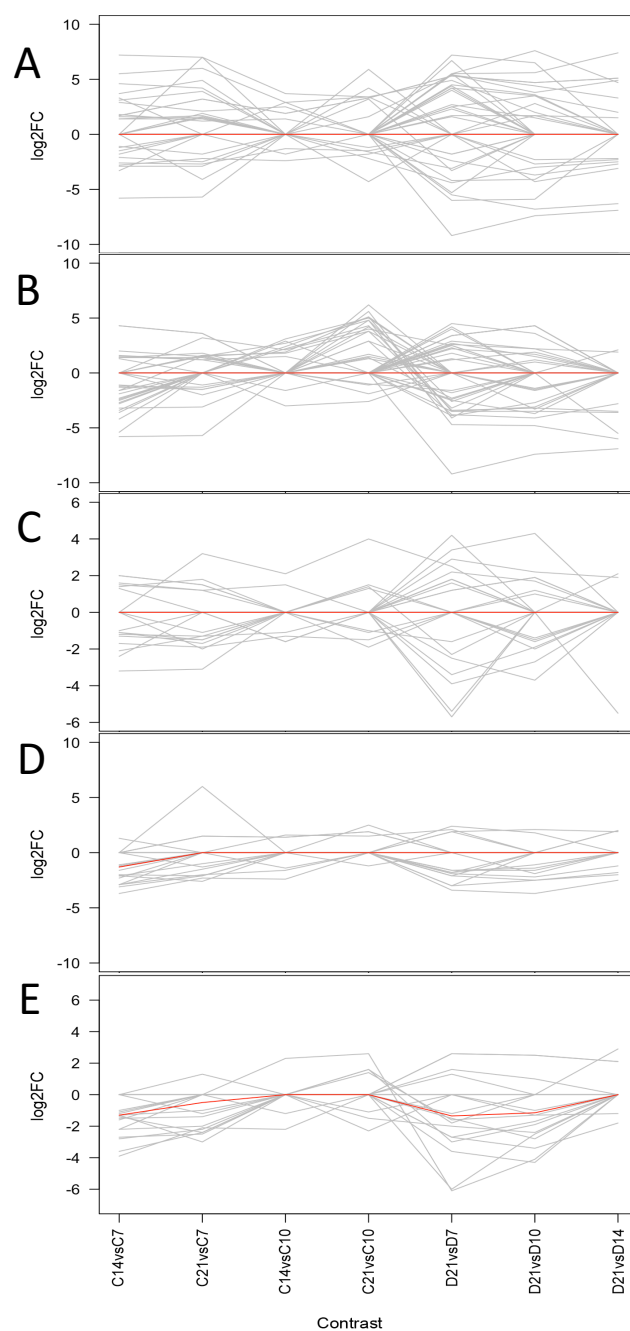
Figure 3.5.: General expression profile of squash orthologous genes that are differentially expressed (*p*<0.05, Log2(FC) >1, FC: Fold Change) in the different contrasts in both cultivars and involved in A) phenylpropanoid, B) suberin monomers, C) cutin, D) cellulose, and E) homogalacturonan biosynthetic pathways.

To determine which genes are highly associated with the ARR of winter squash fruit peel, a few upregulated genes from the three pathways (cutin, suberin monomers and phenylpropanoid) were selected and their expression in both cultivars (Figure 3.6A, B, C) was plotted. Genes encoding for Acyl COA thioesterases and long-chain fatty acid CoA ligase (LACs) involved in cutin biosynthesis pathway were upregulated in both cultivars (Figure 3.6A). Three genes encoding for phenylalanine ammonia-lyase (PAL), caffeoyl-CoA O-methyltransferase (cCoAOMT), and p-coumarate 4-hydroxylase (C4H) involved in suberin monomers biosynthesis pathway (cytochrome P450 protein) were upregulated in the resistant stages in each cultivar (Figure 3.6B). Two genes encoding for cinnamyl alcohol dehydrogenase (CAD) and cinnamoyl-CoA reductase (CCR) (Figure 3.6C) associated with phenylpropanoid biosynthesis pathway were consistently upregulated in all resistant fruit peel ages in both cultivars when contrasted to their susceptible fruit peel ages. An exception occurred with CCR which was not enriched in the contrast of 14 to 10 dpp in 'Chieftain'.
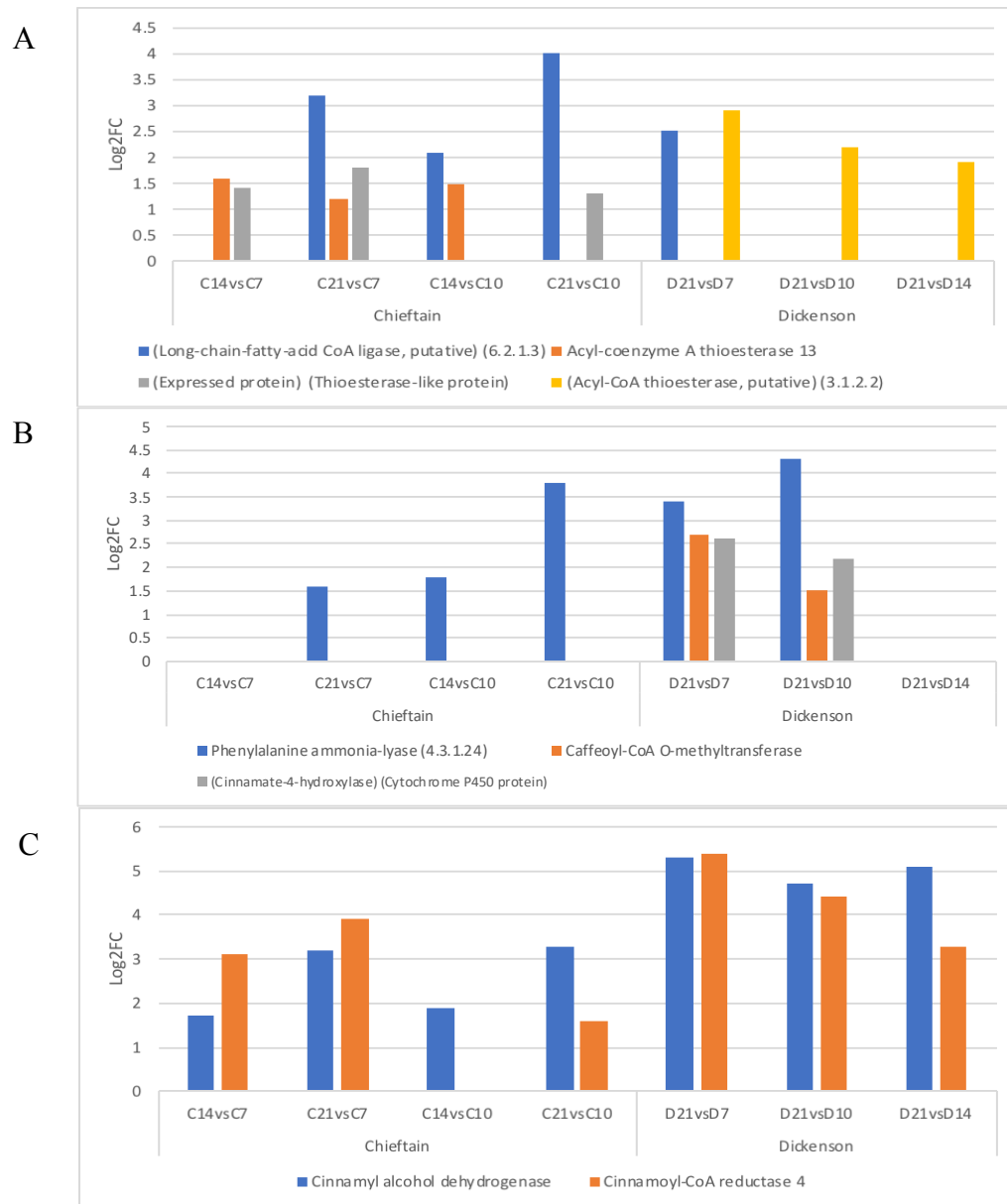
Figure 3.6.: Expression profile of squash orthologous genes that are differentially expressed (*p*<0.05, Log2(FC) >1, FC: Fold Change) in the different contrasts in both cultivars. A, B, C) DEGs involved in cutin, suberin monomers, and phenylpropanoid biosynthetic pathways respectively.

DISCUSSION

We performed transcriptome profiling of the fruit peel of two *C. moschata* cultivars at developmental stages exhibiting susceptible and resistant phenotypes against *P. capsici*. K-means clustering showed two interesting groups of genes that were expected to include age-related resistance (ARR)-associated genes (Figure 3.1B, groups 1 and 2). To identify the candidate genes associated with ARR, we studied the gene expression profile between resistant and susceptible fruit peel ages in both cultivars. Comparison of gene expression profile along the cultivar time points and between cultivars resulted in consistently upregulated genes (Figure 3.2A) with enrichment in function for cell wall structures and phenylpropanoids biosynthesis (Figures 3.3, 3.4). Also, several of the downregulated genes detected in all comparisons between resistant and susceptible fruit peel ages were enriched in photosynthesis and cell growth (Figure 3.3). This was expected as fruit age increases and the fruit color of both cultivars changes from green to beige. Also, by 14 or 21 dpp in 'Chieftain' and 21 dpp in 'Dickenson', the fruits reaches their full expansion, and cell division is not likely to continue. While fruits are approaching complete development, other cellular processes including structural or metabolic may occur similar to cucumber (Ando *et al.* 2015, Mansfeld *et al.* 2017). Following the complete fruit expansion or elongation stage, cell wall structural changes occur with deposition of cell wall materials in the secondary cell wall such as lignin or xylan (Bacete *et al.* 2018). Deposition of primary cell wall polymers such as cellulose, hemicellulose, and pectin is less likely to occur since primary cell wall has been completed (Bacete *et al.* 2018).

In different plant systems, ARR can be regulated by preformed or induced resistance mechanisms and both are regulated by changes in gene expression (Panter and Jones 2002, González-Lamothe

et al. 2009). Preformed defense mechanisms include physical barriers such as cell wall strengthening (Juge 2006) or chemical barriers such as the resistance of oat root against attack by the take-all disease of wheat (Osbourn *et al.* 1994). The plant cell wall constructs the physical barrier that all pathogens have to degrade to be able to infect and colonize the plant (Bellincampi *et al.* 2014, Bacetes *et al.* 2018). Changes in cell wall-related genes either by up or downregulation are significantly affecting disease resistance (Bellincampi *et al.* 2014, Meides *et al.* 2014).

Among the upregulated genes detected in our study in all different comparisons within and between the two studied cultivars (Figure 3.2A, B, C), we identified a candidate group of ARR genes that function in lignin biosynthesis process, sinapyl alcohol dehydrogenase activity, cinnamyl alcohol dehydrogenase activity, stilbene biosynthetic process coumarin biosynthetic process, and peroxidase activity. These genes were upregulated consistently in resistant stages of both cultivars (Figure 3.3, Appendix Table 3. 2), which indicates that metabolic changes in the fruit cell wall are targeting the phenylpropanoids biosynthetic pathway since lignin, coumarin, stilbene, are products of the general phenylpropanoids pathway (Boerjan *et al.* 2003, Vogt 2010, Deng and Lu 2017).

Genes encoding for peroxidase enzymes were expressed in all resistant stages in both cultivars (Figure 3.3, Appendix Table 3. 2). Peroxidases are oxidoreductases, cell wall heme-containing proteins (Vicuna 2005). They are involved in several physiological processes during plant development such as lignin polymerization, fruit ripening, and defense against biotic stress (Passardi *et al.* 2005). The last enzymatic step in lignin biosynthesis is catalyzed by peroxidases, which act to oxidize monolignols utilizing H_2O_2 (Higuchi 1985). Mutation in tomato has proofed the role of peroxidases in lignification (Quiroga *et al.* 2000). In addition to lignification, peroxidases are also involved in cell wall suberization (Quiroga *et al.* 2000). The biosynthesis and

deposition of suberin and lignin polymers in the plant secondary cell wall is developmentally regulated and provide strengthening to the cell wall to perform the physical barrier function against pathogen attack (Miedes *et al.* 2014, Pandey *et al.* 2017).

Sinapyl alcohol dehydrogenase (SAD) and cinnamyl alcohol dehydrogenase (CAD) are enzymes in the phenylpropanoids pathway that catalyze the monolignols biosynthesis from phenylalanine (Vogt 2010, Miedes *et al.* 2014, Deng and Lu 2017). Multiple genes functionally annotated to be involved in lignin biosynthetic process were detected in the resistant stages of both 'Chieftain' and 'Dickenson', however, the highest fold change in the resistant fruit peel ages was for genes encoding for CCR and CAD (Figure 3.6C). In addition, genes encoding for 'MYB transcription factors' which are critical components of the lignin biosynthesis process were found significantly upregulated in several contrasts of both cultivars. A group of MYB proteins are demonstrated as a positive regulator of cell wall structure biosynthesis such as lignin (Zhou *et al.* 2009, Zhong *et al.* 2007, 2008) and cutin and consequently control cuticle and epidermis development (Oshima *et al.* 2013).

Our findings suggest that ARR is potentially regulated in *C. moschata* winter squash cultivars through regulation of cell wall structures biosynthesis. We questioned which cell wall structure is the potential candidate that provides resistance to winter squash fruit against *P. capsici*. The pathway enrichment analysis detected enrichment in cutin, suberin monomers, and phenylpropanoids biosynthesis pathways in at least one resistant stage in both cultivars (Figure 3.4). However, enrichment in phenylpropanoids biosynthesis pathway was consistently detected in upregulated genes in all resistant stages in both cultivars (Figure 3.4). Therefore, we have examined the expression profile of individual genes involved in the three pathways.

In cutin biosynthesis, thioesterases are essential proteins for the release of de novo free fatty acids required for the biosynthesis process (Lowe 2010). The released fatty acids are in turn attached to CoA by the action of long chain acyl–CoA synthetases (LACs) (Schnurr *et al.* 2002). LACs enzymes are plant's long-chain fatty acid, AMP-dependent synthetase and ligase family protein (Li *et al.* 2016). The activation of acyl chains to acyl-CoAs by the LACs is an essential step in the biosynthesis of long chain fatty acids with variable lengths, which are required for cutin and cuticular wax biosynthesis. Cutin and cuticular wax are the components of plant cuticle, and provide a hydrophobic state to the outer plant surface, the cuticle, which acts as a protective barrier against abiotic and biotic stresses (Yeat and Rose 2013). In our results, the upregulated genes in 'Chieftain' and 'Dickenson' were enriched for multiple Acyl COA thioesterases and long-chain fatty acid CoA ligases that are required for the initial steps of cutin biosynthesis (Figure 3.6A). Genes encoding for thioesterase were upregulated in resistant 21 dpp in contrast to susceptible 7, 10, and 14 dpp in 'Dickenson' and in 14 and 21 dpp in contrast to 7 and 10 dpp in 'Chieftain' (Figure 3.6A). LACs gene was only upregulated in 21 dpp in contrast to 7 dpp in 'Dickenson' (Figure 3.6A), and was upregulated in resistant 14 and 21 dpp in 'Chieftain' in contrast to 7 and 10 dpp except the 14 vs.7 dpp was not differentially expressed (Figure 3.6A). LACs perform a key step in cutin and cuticular wax biosynthesis, and absence of their upregulation in 'Dickenson' at 21 dpp, when compared to susceptible 10 and 14 dpp (Figure 3.6A), might indicate that cutin biosynthesis in 'Dickenson' is not the potential mechanism for ARR as the fruit age increases.

Phenylpropanoids are secondary metabolites that include flavonoids, lignin, coumarins, suberin and other phenolic compounds (Mander and Liu 2010). The phenylpropanoids biosynthesis pathway is derived from the shikimate pathway and begins with the amino acid phenylalanine (Mander and Liu 2010, Deng and Lu 2017). By the action of PAL, phenylalanine is converted to

cinnamic acid, then converted by C4H to *p*-coumaric acid then converted by 4CL to *p*-coumaroyl CoA, which is the precursor for several secondary metabolites including monolignols, stilbenes, coumarins, flavonoids and other phenolic compounds (Vogt 2010; Liu *et al.*, 2015, Mander and Liu 2010). The monolignol biosynthesis has two routes, one of them is the conversion of *p*-coumaroyl-CoA to *p*-courmaraldhyde by CCR, then by the action of CAD, *p*-courmaraldhyde is converted to *p*-coumaryl alcohol that is used in building H lignin (Rinaldi *et al.*, 2016). Another route starts by *p*-coumaric acid to produce G lignin, and S lignin utilizes the enzymes C4H and C3H to convert *p*-coumaric acid into caffeic acid then, with the action of another enzyme, it is turned into caffeoyl CoA. Caffeoyl CoA is the substrate for cCoAOMT to produce feruloyl CoA. Feruloyl CoA is then converted to coniferaldhyde by the catalysis of CCR. Coniferaldhyde is the substrate for CAD to produce the coniferyl alcohol, which is the building block for G lignin, or in another route, CAD/SAD convert the coniferaldhyde to sinapyl alcohol to end with building the S lignin (Deng and Lu 2017). The polymerization of the produced monolignols into lignin is catalyzed by peroxidases (Zhao *et al.* 2013, Rinaldi *et al.*, 2016). Lignin is insoluble hydrophobic polymer forms a structural component of the secondary cell wall in plants (Tommerup and Andrews 1997, Miedes *et al.* 2014). Lignin provides structural support to the plant cell wall and has multiple functions including being a structural/physical barrier that defends against wounding and pathogens attack (Buendgen *et al.*, 1990; Bonello *et al.*, 2003, Labeeuw *et al.*, 2015). Cell wall lignification has been known as a disease resistance mechanism in plants (Vance 1980, Nicholson and Hammerschmidt 1992, Sattler and Funnell-Harris 2013). The mechanical strength provided to the plant cell wall by lignin hinders the pathogen penetration using appressoria (Bellincampi *et al.* 2014). Also, the hydrophobic nature of lignin protects against cell wall degradation by the action of the cell wall degrading enzymes produced by plant pathogens (Vance 1980). cCoAOMT and

C4H genes were not upregulated in 'Chieftain' fruit peel of resistant ages 14 and 21 dpp when contrasted to susceptible 7 and 10 dpp (Figure 3.6B). Also, the two genes as well as PAL did not show significant changes in the contrast between 21 and 14 dpp in 'Dickenson' but were significantly upregulated in the resistant stage at 21 dpp vs. the 7 and 10 dpp stages (Figure 3.6B). This might indicate that the observed cell wall thickening in 'Chieftain' resistant fruit cuticle and epidermal walls (Alzohairy *et al.* 2017) that is correlated with ARR might not because of deposition of suberin and that another compound is causing this thickening, which is potentially the primary cause of ARR in hard squash to *P. capsici*.

CAD, and CCR genes were enriched in the phenylpropanoid biosynthesis pathway and their expression was consistently upregulated in all the resistant fruit peel ages in both cultivars when contrasted to their susceptible fruit peel ages. However, CCR that did not change in the contrast of 14 vs.10 dpp in 'Chieftain' (Figure 3.6C). CAD and CCR are the primary two enzymes in the production of the monolignol *p*-coumaryl alcohol that is incorporated in building the H lignin (Rinaldi *et al.* 2016). In a study of the nature of lignin produced in cucumber hypocotyls, it was found that the lignin is derived from *p*-coumaryl alcohol (Robertsen and Svalheim 1990).

The results suggest that CAD and CCR genes are potential candidate genes for controlling ARR through the production of monolignols and then polymerization of lignin at the cell wall by the catalysis of peroxidases. This additional lignin deposited in the peel of the resistant aging squash fruit may provide the thickening to the cuticle and the epidermal walls to resist against pathogens and environmental stresses. Also, the observed increasing of cCoAOMT and C4H expression in the resistant fruit are likely involved in the production of lignin and not suberin.

CONCLUSION

Our results provide evidence that the ARR to *P. capsici* in winter squash is highly correlated with phenylpropanoids production and that lignin is the potential material that is deposited in the cuticle and epidermal cell walls of maturing fruit peel as a constitutive structural defense mechanism against pathogens. Although the ARR onset in the tested cultivars was different, several genes detected in both cultivars were consistently upregulated in the resistant fruit peel and were enriched in phenylpropanoids biosynthesis. This suggests that both *C. moschata* cultivars have similar mechanisms that control ARR to *P. capsici*. The observed difference in the onset of ARR between cultivars is likely due to the difference in their days to maturity as 'Chieftain' matures at 80 days while 'Dickenson' matures at 100 days.

APPENDIX

APPENDIX

Table 3.1.: Number of raw reads, alignment count, and uniquely mapped reads.

Sample No.	Number of sequenced reads	QC-pass and mapping input	Uniquely mapped	Mapping %
C7-1	63739767	55113437	50288035	91.24
C7-2	70456945	66386572	60788885	91.57
C7-3	59026276	56539714	51567392	91.21
C10-1	44371192	44396147	40454480	91.12
C10-2	60930638	51954298	46255164	89.03
C10-3	63003314	59773579	53981968	90.31
C14-1	193,631,816	175601685	162531776	92.56
C14-2	65821663	58883472	53892199	91.52
C14-3	180,842,987	168147746	154877625	92.11
C21-1	67405839	63093646	56908664	90.2
C21-2	70485362	69714639	63134472	90.56
C21-3	57362566	55535461	48999524	88.23
D7-1	48126734	43697048	38574146	88.28
D7-2	42862633	39042901	35207320	90.18
D7-3	45528798	41852230	38426892	91.82
D10-1	44350665	40997570	37564434	91.63
D10-2	58118081	52014876	46467810	89.34
D10-3	54015612	47521845	42776661	90.01
D14-1	59724520	54894218	49579501	90.32
D14-2	49203062	43914637	39735017	90.48
D14-3	54435378	47600991	42138426	88.52
D21-1	56384199	51963759	46334848	89.17
D21-2	55199091	49840126	44627977	89.54
D21-3	49742926	44596852	39845052	89.34

Table 3.2.: Gene Ontology enrichment of upregulated genes in the resistant stages in both cultivars resulted from all contrasts. The values are the negative log of the q-value if the GO term is overrepresented, or the log of the q-value if the GO term is underrepresented. At q = 0.05, a term is significantly overrepresented if the value is > or = to 1.3 and significantly underrepresented if the value is < or = to -1.3.

GO-term	Set 1_up	Set2 _up_ Chieftain	Set2 _up _Dickenson	C14 up_vs_ C7	C14 up_vs_ C10	C21 up_vs_ C7	C21 up_vs_ C10	D21 up_vs_D 7	D21 up_vs_ D10	D21 up_vs_ D14	Set 3
GO:0003677_DNA_binding	3.321	0.972	2.433	0.302	4.37	-0.309	1.076	1.965	0.425	3.864	3.075
GO:0005634_nucleus	3.321	-0.118	0	0	0.529	-0.579	-0.379	-2.004	-0.325	0	3.459
GO:0055114_oxidation-reduction_process	2.637	3.824	2.388	2.247	1.603	3.001	0.956	2.895	2.125	1.084	2.492
GO:0008270_zinc_ion_binding	2.637	0	1.897	-1.373	-0.298	-2.931	-0.943	0.214	-0.545	1.043	1.549
GO:0005515_protein_binding	2.637	-1.458	-0.908	-4.333	-2.198	-3.372	0.276	-4.275	-10.81	-2.123	6.07
GO:0006687_glycosphingolipid_metabolic_process	1.964	2.147	1.788	0.167	0.706	0.571	0.634	0.143	0.158	1.154	0
GO:0046486_glycerolipid_metabolic_process	1.964	1.168	0.879	-0.504	0.247	-0.258	0	-0.255	-0.399	0.413	0
GO:0000785_chromatin	1.964	1.161	2.152	0	0.841	-0.68	-0.497	1.97	0.48	2.75	0.394
GO:0003682_chromatin_binding	1.964	1.091	2.264	-0.228	0.598	-1.068	-0.713	2.023	0.495	2.882	0.402
GO:0046872_metal_ion_binding	1.964	0.283	0.475	0.36	1.564	0.195	0	0.113	0.532	0.406	1.74
GO:0005829_cytosol	1.964	0.151	-0.944	-0.709	-2.871	-0.701	-2.909	-5.346	-2.187	-2.043	1.363
GO:0004565_beta-galactosidase_activity	1.3	1.802	1.361	0	0.648	0.274	0.297	0.184	0	0.924	0
GO:0009341_beta-galactosidase_complex	1.3	1.802	1.361	0	0.648	0.274	0.297	0.184	0	0.924	0
GO:0006027_glycosaminoglycan_catabolic_process	1.3	1.802	1.361	0	0.648	0.274	0.297	0.184	0	0.924	0
GO:0005622_intracellular	1.3	1.284	0.59	-0.143	0.161	-0.154	-0.403	0	0	0.774	0
GO:0006012_galactose_metabolic_process	1.3	1.099	0.159	0.43	1.025	0.471	0.511	-0.386	-1.311	-0.127	0

Table 3. 2 (cont'd)

GO:0030246_carbohydrate_binding	1.3	0.575	0.5	-0.38	0	-0.099	0.588	0.897	-0.617	0.829	0
GO:0009507_chloroplast	1.3	-0.16	-0.506	-1.064	-2.212	-1.615	-3.015	-3.816	-1.399	-0.812	1.74
GO:0005524_ATP_binding	1.3	-0.735	-0.851	-10.67	-3.577	-2.612	0.177	-4.888	-10.91	-0.705	4.635
GO:0005506_iron_ion_binding	0.646	4.785	0.892	2.307	2.36	3.478	1.726	0.446	0.399	0.196	0.211
GO:0020037_heme_binding	0.646	4.197	1.83	3.445	2.293	3.663	1.058	2.994	2.981	1.117	0.411
GO:0016491_oxidoreductase_activity	0.646	2.379	0.312	0.855	0.607	1.856	0.336	2.007	0.663	0.533	0.411
GO:0052747_sinapyl_alcohol_dehydrogenase_activity	0.646	1.802	1.801	0.25	0.648	0.526	0.297	0.54	0.591	1.334	0
GO:0045551_cinnamyl-alcohol_dehydrogenase_activity	0.646	1.802	1.801	0.25	0.648	0.526	0.297	0.54	0.591	1.334	0
GO:0009811_stilbene_biosynthetic_process	0.646	1.802	1.529	0.717	0.972	1.296	1.164	0.406	0.611	0.927	0
GO:0042753_positive_regulation_of_circadian_rhythm	0.646	1.802	0.9	0	0.648	0.274	0.297	0.787	0.297	0.667	0
GO:0005618_cell_wall	0.646	1.56	0	0.86	1.342	1.551	0.884	0.12	-0.137	-0.405	0.211
GO:0006804_peroxidase_reaction	0.646	1.532	1.778	1.778	0.887	1.414	0.278	1.694	1.523	0.771	0
GO:0009805_coumarin_biosynthetic_process	0.646	1.363	1.732	0.524	0.706	0.992	1.092	0.595	1.128	0.971	0
GO:0009809_lignin_biosynthetic_process	0.646	1.363	1.165	0.344	0.706	0.778	0.861	0.595	0.8	0.419	0
GO:0030001_metal_ion_transport	0.646	1.363	0	1.474	3.529	0.571	1.325	0	0.091	0.576	0
GO:0046983_protein_dimerization_activity	0.646	1.1	2.736	0.407	1.295	0.357	0.8	2.235	1.675	2.738	0.402
GO:0019852_L-ascorbic_acid_metabolic_process	0.646	1.099	1.801	0.131	0.172	0.643	0.329	1.573	2.013	1.334	0
GO:0004712_protein_serine/threonine/tyrosine_kinase_activity	0.646	0.664	1.529	-0.791	-0.137	-0.288	-0.319	0.565	0.278	0.927	0
GO:0005887_integral_component_of_plasma_membrane	0.646	0.478	1.361	0	0.172	0	-0.228	0.585	0.637	0.924	0
GO:0008378_galactosyltransferase_activity	0.646	0.478	1.361	-0.202	0	-0.199	0	0.381	0.866	0.924	0
GO:0009117_nucleotide_metabolic_process	0.646	0	1.788	-1.699	-0.291	-0.458	0	0.476	0.707	2.188	0

Table 3. 2 (cont'd)

GO:0006468_protein_phosphorylation	0.646	0	-1.289	-3.683	-0.878	-0.726	1.339	-0.195	-4.867	-0.547	1.723
GO:0042742_defense_response_to_bacterium	0	1.802	-0.212	1.835	2.598	2.038	2.818	-0.316	-0.09	-0.155	0
GO:0006118_electron_transport	0	1.56	0	2.953	0.358	2.392	0.884	-0.152	0.273	-0.553	0.411
GO:0004601_peroxidase_activity	0	1.363	1.403	1.871	1.511	1.413	0.634	1.175	0.975	0.576	0
GO:0042744_hydrogen_peroxide_catabolic_process	0	1.363	1.328	1.261	0.967	1.413	0.634	1.474	0.88	0.756	0
GO:0009055_electron_carrier_activity	0	1.363	0.171	4.099	1.511	3.209	1.547	0.069	0.781	0	0
GO:0010167_response_to_nitrate	0	0.9	1.801	2.03	0.648	2.292	1.164	3.551	3.918	2.671	0
GO:0010089_xylem_development	0	0.9	1.361	0	0.972	0.517	1.446	0	0.2	1.476	0
GO:0015706_nitrate_transport	0	0.478	1.788	0.75	0	1.015	0.511	2.026	2.011	2.188	0
GO:0009723_response_to_ethylene	0	0.478	1.361	0.43	0.365	0.471	0.702	0.999	1.099	0.924	0
GO:0048437_floral_organ_development	0	0	1.801	0	0.217	-0.428	0	0.54	0.297	1.334	0
GO:0000150_recombinase_activity	0	0	1.801	0	0	0	0	0.54	0.297	2.002	0
GO:0045489_pectin_biosynthetic_process	0	0	1.801	-1.629	-0.203	-0.825	0	0.786	0.591	2.002	0
GO:0044237_cellular_metabolic_process	0	0	1.788	0.344	0.706	0.183	1.092	0.143	0.515	1.154	0
GO:0004715_non-membrane_spanning_protein_tyrosine_kinase_activity	0	0	1.732	-0.941	0	-0.458	0.098	0.459	0.226	1.359	0
GO:0043565_sequence-specific_DNA_binding	0	0	1.435	0.991	1.203	1.457	3.023	3.233	1.564	3.193	0.58
GO:0018108_peptidyl-tyrosine_phosphorylation	0	0	1.385	-1.634	-0.093	-1.298	0	0.144	-0.284	0.639	0
GO:0009743_response_to_carbohydrate	0	0	1.361	0	0	0	0	0	0	1.476	0
GO:0045449_regulation_of_transcription	0	-0.119	1.028	1.438	5.889	1.177	6.031	2.891	0.685	3.643	1.363
GO:0005667_transcription_factor_complex	0	-0.119	0.655	1.636	5.133	1.484	5.684	2.089	0.471	2.298	1.358
GO:0003700_sequence-specific_DNA_binding_transcription_factor_activity	0	-0.12	1.049	1.572	5.634	1.413	5.448	2.823	0.625	3.471	1.152

Table 3. 2 (cont'd)

GO:0008152_metabolic_process	0	-0.15	0	-2.603	-1.824	-0.815	-0.811	-0.27	-1.327	0	2.297
GO:0009069_serine_family_amino_acid_metabolic_process	0	-0.191	-0.342	-3.265	-0.591	-1.063	0.657	0.061	-2.811	-0.325	1.549
GO:0003735_structural_constituent_of_ribosome	0	-0.205	-0.119	21.874	3.429	19.275	-0.191	-1.237	20.721	-0.087	1.363
GO:0042254_ribosome_biogenesis	0	-0.205	-0.246	23.576	3.844	20.904	-0.322	-1.268	19.896	-0.43	1.363
GO:0005886_plasma_membrane	0	-0.251	0.177	-0.253	-0.68	-0.438	-0.477	-0.339	-0.323	0.482	2.119
GO:0016021_integral_component_of_membrane	0	-1.012	-0.188	-4.552	-3.844	-1.546	-0.456	-1.013	-1.158	-0.138	4.043
GO:0005739_mitochondrion	0	-1.114	-1.41	0	-1.261	1.004	-0.12	-0.577	0.297	-2.6	1.363

Table 3.3.: Gene Ontology enrichment of downregulated genes in the resistant stages in both cultivars resulted from all contrasts. The values are the negative log of the q-value if the GO term is overrepresented, or the log of the q-value if the GO term is underrepresented. At q = 0.05, a term is significantly overrepresented if the value is > or = to 1.3 and significantly underrepresented if the value is < or = to -1.3.

GO-term	Set1 _down	Set2 _down_ Chieftain	Set2 _down_ Dickenson	C14 down_v s_C7	C14 down_v s_C10	C21 down_v s_C7	C21 down_v s_C10	D21 down_v s_D7	D21 down_v s_D10	D21 down_v s_D14	Set3
GO:0016021_integral_component_of_membrane	10.269	7.323	6.432	2.952	8.416	3.816	4		2.288	2.268	5.553	4.043
GO:0009535_chloroplast_thylakoid_membrane	5.057	7.347	23.401	0.127	6.105	3.198	13.287	10.063	15.531	18.091	0.411
GO:0055114_oxidation-reduction_process	5.057	4.515	5.607	1.102	5.255	2.165	4.698	5.189	3.79	5.234	2.492
GO:0046872_metal_ion_binding	4.727	2.049	1.095	0.389	1.708	0.808	1.632	0.262	0.239	0.479	1.74
GO:0016168_chlorophyll_binding	4.073	9.575	13.202	1.903	5.995	2.949	5.618	4.96	6.463	10.291	0
GO:0019344_cysteine_biosynthetic_process	4.073	6.212	8.826	0.075	2.964	1.319	3.387	1.885	3.076	6.805	0
GO:0006364_rRNA_processing	4.073	5.331	11.469	-0.066	5.285	0.857	5.839	5.059	7.75	8.477	
GO:0005515_protein_binding	3.748	-0.989	-3.47	2.157	-0.793	-0.731	-3.141	-6.642	-3.818	-4.447	6.07
GO:0018298_protein-chromophore_linkage	3.426	7.828	7.154	1.737	4.46	2.051	4.249	1.403	2.3	5.158	0
GO:0009522_photosystem_I	3.426	7.169	14.018	0.842	3.914	2.051	4.487	5.615	7.19	11.156	0
GO:0009941_chloroplast_envelope	3.426	4.961	6.608	0.054	4.413	0.667	6.049	3.61	6.748	5.522	0.212
GO:0009570_chloroplast_stroma	3.426	1.986	2.525	-1.847	1.553	-0.972	1.22	1.11	3.701	1.715	0.394
GO:0005524_ATP_binding	3.426	0.118	-1.507	0.768	-0.126	-0.684	-4.137	-1.17	-0.723	-0.968	4.635
GO:0009765_photosynthesis	3.105	7.503	10.989	1.519	4.323	2.028	3.83	3.398	4.354	8.751	0
GO:0006098_pentose-phosphate_shunt	3.105	6.355	7.148	0.148	6.609	1.647	9.238	1.819	4.147	5.503	0
GO:0009523_photosystem_II	2.787	6.815	13.202	1.728	5.161	2.949	6.415	4.96	6.463	10.291	0
GO:0019288_isopentenyl_diphosphate_biosynthetic_process	2.47	2.852	4.695	-0.365	2.729	0.217	2.78	4.745	7.972	4.028	0

Table 3. 3 (cont'd)

GO term											
GO:0010207_photosystem_II_assembly	2.155	6.414	12.355	1.358	8.144	3.721	10.399	7.339	10.025	10.617	0
GO:0009637_response_to_blue_light	2.155	5.174	9.298	0.557	4.517	2.362	6.335	5.376	6.888	7.979	0
GO:0019252_starch_biosynthetic_process	1.842	4.853	3.162	0.394	3.545	0.663	4.197	0.79	2.296	3.017	0
GO:0010218_response_to_far_red_light	1.842	4.576	7.928	0.842	4.46	2.679	6.383	3.933	5.253	6.362	0
GO:0035304_regulation_of_protein_dephosphorylation	1.842	3.965	5.112	0.55	3.753	2.181	4.489	2.036	2.832	4.043	0
GO:0010155_regulation_of_proton_transport	1.842	3.488	5.154	0.353	2.714	0.964	3.061	0.738	1.814	3.476	0
GO:0010287_plastoglobule	1.842	3.375	6.755	0.128	2.264	0.922	2.702	1.452	2.286	5.323	0
GO:0009507_chloroplast	1.842	0.511	-0.037	-1.85	1.948	-1.962	0.797	0.025	0.521	0.068	1.74
GO:0005634_nucleus	1.842	-2.526	-3.193	-1.164	-3.216	-1.393	-1.316	-1.786	-3.876	-3.303	3.459
GO:0010103_stomatal_complex_morphogenesis	1.531	6.128	4.313	2.967	5.161	4.925	5.892	3.933	5.492	4.228	0
GO:0048046_apoplast	1.531	5.869	6.092	2.962	4.039	4.991	5.105	2.434	3.342	6.883	0
GO:0010114_response_to_red_light	1.531	2.797	7.853	0.265	3.249	1.446	4.937	3.932	5.31	6.457	0
GO:0015995_chlorophyll_biosynthetic_process	1.531	2.584	7.002	0.2	3.139	1.465	5.326	7.138	9.163	5.573	0
GO:0010027_thylakoid_membrane_organization	1.531	2.043	2.905	-0.151	2.729	0.139	2.62	2.872	5.442	1.947	0
GO:0006096_glycolytic_process	1.531	1.251	0.194	-0.074	1.969	0.147	1.062	-1.884	-1.012	0.108	0
GO:0009644_response_to_high_light_intensity	1.531	0.915	5.311	-0.524	0.36	-0.27	0.072	1.478	2.641	5.87	0
GO:0005985_sucrose_metabolic_process	1.531	0.296	10.023	1.536	0.108	1.638	0.419	5.802	6.239	6.546	0.411
GO:0003677_DNA_binding	1.531	0	0.543	0.214	-1.31	1.008	0.428	2.841	0.937	0.577	3.075
GO:0005886_plasma_membrane	1.531	-0.264	0	0.301	0	0.451	-0.332	-0.209	-0.539	-0.029	2.119
GO:0015976_carbon_utilization	1.221	3.941	2.004	0.842	4.187	1.679	4.487	0	0.647	1.848	0
GO:0009773_photosynthetic_electron_transport_in_photosystem_I	1.221	3.393	6.422	0.441	4.323	2.278	5.618	3.518	4.647	6.079	0
GO:0071555_cell_wall_organization	1.221	3.269	2.397	2.304	0.98	3.137	1.881	1.143	0.66	2.353	0

Table 3. 3 (cont'd)

GO:0009744_response_to_sucrose	1.221	2.697	4.368	1.557	3.388	2.48	3.065	0.805	1.408	2.788	0
GO:0019684_photosynthesis	1.221	2.235	1.873	0	1.36	0.131	1.641	0.497	0.917	1.323	0
GO:0051287_NAD_binding	1.221	2.09	2.926	0	1.651	0	0.51	0.302	0.317	2.144	0
GO:0005215_transporter_activity	1.221	1.634	0.18	0	0.79	0.757	1.411	-0.057	-0.21	0.25	0
GO:0006810_transport	1.221	1.386	0.18	-0.064	0.558	0.207	0.617	-0.058	-0.059	0.695	0
GO:0008152_metabolic_process	1.221	0.243	-1.009	0.735	0.412	-0.24	-0.54	-0.727	-0.16	-0.577	2.297
GO:0005982_starch_metabolic_process	1.221	0.188	9.253	1.766	0.11	2.101	0.287	6.672	6.67	6.342	0.411
GO:0009543_chloroplast_thylakoid_lumen	0.913	3.393	4.542	0.675	4.323	1.586	5.382	1.865	3.093	3.481	0
GO:0000023_maltose_metabolic_process	0.913	3.269	2.926	0.088	2.295	0.193	3.001	1.354	2.989	2.668	0
GO:0043085_positive_regulation_of_catalytic_activity	0.913	2.77	4.092	0	2.095	0.193	2.619	1.627	3.198	3.745	0
GO:0070838_divalent_metal_ion_transport	0.913	2.712	5.196	0.65	3.48	2.507	4.892	1.816	2.953	4.577	0
GO:0030003_cellular_cation_homeostasis	0.913	2.712	4.594	0.887	3.48	2.467	5.14	1.452	2.485	3.798	0
GO:0010196_nonphotochemical_quenching	0.913	2.712	2.273	0.226	1.185	0.234	1.058	0.167	0.363	1.714	0
GO:0009902_chloroplast_relocation	0.913	2.29	2.047	0	2.497	0.794	1.881	2.633	4.305	1.431	0
GO:0009657_plastid_organization	0.913	2.092	9.48	0.67	3.641	1.474	4.487	4.706	6.465	8.351	0
GO:0004650_polygalacturonase_activity	0.913	2.092	2.482	0.496	0.648	0.336	0.59	1.847	1.496	1.979	0
GO:0005739_mitochondrion	0.913	-0.301	-2.193	-3.776	-0.48	-4.724	-1.412	-2.761	-1.658	-1.952	1.363
GO:0008270_zinc_ion_binding	0.913	-1.79	-1.773	-0.149	-1.147	-2.754	-1.241	-5.209	-4.152	-1.586	1.549
GO:0005506_iron_ion_binding	0.607	3.447	1.281	2.446	4.211	2.471	3.577	1.152	0.753	1.071	0.211
GO:0020037_heme_binding	0.607	2.887	2.114	0.825	1.895	0.875	1.709	1.673	1.083	1.735	0.411
GO:0006855_drug_transmembrane_transport	0.607	2.092	1.466	1.377	2.877	1.475	1.007	0.968	0.696	0.996	0
GO:0006118_electron_transport	0.607	2.039	0.697	0.69	1.763	2.307	3.954	0.978	0.953	0.899	0.411
GO:0019253_reductive_pentose-phosphate_cycle	0.607	2.032	3.62	0.459	2.343	1.171	2.857	0.902	2.012	3.481	0

Table 3. 3 (cont'd)

GO:0009783_photosystem_II_antenna_complex	0.607	2.032	1.488	0	0.596	0.25	0.546	0.252	0.255	1.187	0
GO:0005576_extracellular_region	0.607	1.658	7.126	1.805	0.926	4.27	2.984	6.516	6.301	5.904	0.211
GO:0009409_response_to_cold	0.607	1.504	1.097	1.391	2.622	2.077	3.519	0.41	1.13	1.651	0
GO:0050661_NADP_binding	0.607	1.504	0.935	0.326	1.615	0	0.384	-0.109	-0.11	0.615	0
GO:0051537_2_iron	0.607	1.504	0.667	0.16	1.124	0.167	2.127	-0.223	-0.098	0.401	0
GO:0006804_peroxidase_reaction	0.607	1.403	0.756	0.352	0.623	0.114	0.566	0.435	0.255	0.641	0
GO:0019852_L-ascorbic_acid_metabolic_process	0.607	1.179	3.976	0.557	0.931	0.426	0.478	2.959	2.688	3.17	0
GO:0019761_glucosinolate_biosynthetic_process	0.607	0.95	3.356	0	1.6	0.511	1.446	0.805	2.105	2.91	0
GO:0050794_regulation_of_cellular_process	0.607	0.95	1.386	0.76	0.136	0.649	0.126	0.335	0.253	1.331	0
GO:0006468_protein_phosphorylation	0.607	-0.208	0.038	0.851	-0.278	0.1	-0.657	0.348	0.589	0.07	1.723
GO:0006633_fatty_acid_biosynthetic_process	0.303	2.77	1.255	1.14	2.703	1.36	1.205	1.182	1.86	2.788	0
GO:0009055_electron_carrier_activity	0.303	2.697	0.477	1.019	2.116	2.886	4.487	1.31	1.395	1.308	0
GO:0010075_regulation_of_meristem_growth	0.303	1.693	2.26	2.384	2.025	4.513	3.142	2.114	1.951	1.848	0
GO:0043481_anthocyanin_accumulation_in_tissues_in_response_to_UV_light	0.303	1.693	0.27	0.711	0.526	1.986	1.224	0.257	0	0	0
GO:0016705_oxidoreductase_activity	0.303	1.62	1.623	0.097	1.071	0.102	0.233	0.872	1.078	1.11	0
GO:0032440_2-alkenal_reductase_[NADP]_activity	0.303	1.427	1.696	1.116	1.312	2.183	1.814	1.863	1.72	1.255	0.402
GO:0009595_detection_of_biotic_stimulus	0.303	1.353	3.62	0.226	1.185	1.171	2.089	1.079	2.213	3.846	0
GO:0043900_regulation_of_multi-organism_process	0.303	1.353	3.025	0.226	1.185	1.171	2.089	0.757	1.742	3.2	0
GO:0010310_regulation_of_hydrogen_peroxide_metabolic_process	0.303	1.353	2.199	0.65	1.185	1.835	2.317	0.872	1.572	2.343	0
GO:0042132_fructose_1	0.303	1.353	1.985	0	1.185	0.234	1.326	0.481	0.982	1.979	0
GO:0008356_asymmetric_cell_division	0.303	1.353	1.488	0.441	0.299	0.94	0.798	1.628	2.209	1.187	0
GO:0019760_glucosinolate_metabolic_process	0.303	1.353	1.488	0	0.596	0.25	0.798	0.252	0.516	1.583	0

Table 3. 3 (cont'd)

GO:0009517_PSII_associated_light-harvesting_complex_II	0.303	1.353	1.488	0	0.596	0	0.546	0.252	0.255	1.187	0
GO:0010277_chlorophyllide_a_oxygenase_[overall]_activity	0.303	1.353	0.992	0	0.596	0	0.798	0.252	0.263	1.187	0
GO:0050278_sedoheptulose-bisphosphatase_activity	0.303	1.353	0.992	0	0.596	0	0.546	0	0.255	1.187	0
GO:0042908_xenobiotic_transport	0.303	1.353	0.618	0.441	0.892	0.234	0.546	0.169	0.363	0.245	0
GO:0008559_xenobiotic-transporting_ATPase_activity	0.303	1.353	0.618	0.441	0.892	0.234	0.546	0.169	0.363	0.245	0
GO:0006006_glucose_metabolic_process	0.303	1.353	0.618	0	0.596	0	0.282	0	0	0.245	0
GO:0016620_oxidoreductase_activity	0.303	1.353	0.618	0	0.596	0	0.282	0	0	0.245	0
GO:0009749_response_to_glucose	0.303	1.353	0.163	0.65	1.185	0.718	0.546	-0.122	0	0	0
GO:0015979_photosynthesis	0.303	1.179	6.315	0	3.501	1.446	5.647	4.184	6.156	5.666	0
GO:0050832_defense_response_to_fungus	0.303	0.942	3.662	1.557	0.883	1.652	1.907	1.215	2.455	3.472	0
GO:0010200_response_to_chitin	0.303	0.942	3.29	1.917	0.883	2.48	1.456	3.146	2.455	4.006	0
GO:0006566_threonine_metabolic_process	0.303	0.942	3.025	0.324	1.865	0.71	0.59	0.747	0.984	2.02	0
GO:0046658_anchored_component_of_plasma_membrane	0.303	0.942	2.716	0.847	0.202	1.871	1.007	1.865	2.663	2.057	0
GO:0019216_regulation_of_lipid_metabolic_process	0.303	0.942	2.482	0	0.42	0	0	1.178	1.736	1.979	0
GO:0006544_glycine_metabolic_process	0.303	0.942	2.199	0.324	2.368	0.703	0.59	0.213	0.222	1.346	0
GO:0010206_photosystem_II_repair	0.303	0.942	1.985	0	0.202	0.167	0.59	0.944	0.982	1.583	0
GO:0009862_systemic_acquired_resistance	0.303	0.942	1.764	0.494	0.648	1.286	1.907	0.661	1.45	2.144	0
GO:0008361_regulation_of_cell_size	0.303	0.942	1.35	0.667	0.42	1.475	1.456	0.872	0.925	0.883	0
GO:0009697_salicylic_acid_biosynthetic_process	0.303	0.705	2.93		0.526	0.431	1.038	1.165	2.095	2.848	0
GO:0010363_regulation_of_plant-type_hypersensitive_response	0.303	0.705	2.117	1.029	0.526	1.991	1.432	1.25	1.193	2.401	0
GO:0009867_jasmonic_acid_mediated_signaling_pathway	0.303	0.705	2.047	0.408	0.337	1.091	1.228	0.797	1.45	2.935	0
GO:0006612_protein_targeting_to_membrane	0.303	0.705	2.004	1.029	0.526	1.991	1.432	0.945	0.878	2.048	0
GO:0009416_response_to_light_stimulus	0.303	0.705	1.985	-0.18	0.16	0	0.304	1.397	1.963	1.979	0
GO:0006636_unsaturated_fatty_acid_biosynthetic_process	0.303	0.705	1.819	0	0.526	0.134	1.228	1.291	2.485	1.75	0

Table 3. 3 (cont'd)

GO:0031348_negative_regulation_of_defense_response	0.303	0.705	1.764	0.402	0.526	0.751	1.224	0.666	1.941	2.144	0
GO:0031408_oxylipin_biosynthetic_process	0.303	0.705	1.426	0	0.16	0	0.307	0.925	0.799	1.451	0
GO:0031969_chloroplast_membrane	0.303	0.424	1.623	-0.106	0.98	-0.104	0.101	0.866	1.572	1.346	0
GO:0003700_sequence-specific_DNA_binding_transcription_factor_activity	0.303	0	3.662	0.868	-0.273	3.041	1.033	2.506	3.54	4.389	1.152
GO:0045449_regulation_of_transcription	0.303	0	3.599	0.925	-0.444	2.975	0.916	2.666	3.606	4.163	1.363
GO:0005667_transcription_factor_complex	0.303	0	3.123	0.846	-0.444	2.735	0.916	2.293	2.946	3.695	1.358
GO:0001053_plastid_sigma_factor_activity	0	2.712	0.496	0.236	0.892	0.718	0.786	0.722	0.768	0.791	0
GO:0015293_symporter_activity	0	2.712	0.224	0.675	1.477	2.028	2.59	1.098	0.627	0	0
GO:0007178_transmembrane_receptor_protein_serine/threonine_kinase_signaling_pathway	0	2.235	0.527	1.177	2.264	1.991	0.66	0.51	0.241	0.599	0
GO:0009825_multidimensional_cell_growth	0	1.693	0.464	1.029	0.725	1.986	1.228	0.385	0.089	0.117	0
GO:0006598_polyamine_catabolic_process	0	1.504	0.992	0.16	0.42	0.703	0.799	1.178	0.768	1.187	0
GO:0042398_cellular_modified_amino_acid_biosynthetic_process	0	1.504	0.992	0	0.42	0.703	0.799	0.94	0.768	1.187	0
GO:0006354_DNA-templated_transcription	0	1.504	0	0	0.42	0	0.59	0.507	0.25	-0.185	0
GO:0006200_obsolete_ATP_catabolic_process	0	1.403	-0.355	0.76	1.184	0.114	0	-0.592	-0.441	-0.596	0
GO:0080167_response_to_karrikin	0	1.353	1.985	0.459	0.596	0.718	0.786	0.94	0.982	1.979	0
GO:0016762_xyloglucan:xyloglucosyl_transferase_activity	0	1.353	1.414	1.096	0.299	1.586	1.326	0.715	0.363	2.407	0
GO:0006073_cellular_glucan_metabolic_process	0	1.353	1.414	1.096	0.299	1.586	1.326	0.715	0.363	2.407	0
GO:0006813_potassium_ion_transport	0	1.353	0.618	0.887	1.477	0	0.798	0.167	0.363	0.51	0
GO:0004707_MAP_kinase_activity	0	1.353	0.618	0.459	1.185	0.25	0.546	0.169	0	0.245	0
GO:0006413_translational_initiation	0	1.353	0.496	0	0.299	0	0.282	0	0	0.395	0
GO:0010264_myo-inositol_hexakisphosphate_biosynthetic_process	0	1.353	0.475	0	3.197	0.234	2.33	0	0	0.643	0

Table 3. 3 (cont'd)

GO:0015299_solute:proton_antiporter_activity	0	1.353	0.27	0.675	1.185	0.25	0.546	0	0	0.245	0
GO:0006865_amino_acid_transport	0	1.353	0.27	0.236	0.596	0.481	0.546	0.525	0	0	0
GO:0000280_nuclear_division	0	1.353	0.27	0.226	0.299	0.718	0.282	0.525	0.56	0	0
GO:0009607_response_to_biotic_stimulus	0	1.353	0.224	0.226	0.299	0.692	0.282	0.279	0.293	0	0
GO:0010311_lateral_root_formation	0	1.353	0	0.887	0.299	0.94	0.546	0	0	-0.285	0
GO:0048829_root_cap_development	0	1.353	0	0.226	0.596	0.481	0.546	0.944	0.263	0	0
GO:0010143_cutin_biosynthetic_process	0	1.353	0	0.226	0.596	0.25	0.282	-0.3	0.178	0	0
GO:0030139_endocytic_vesicle	0	1.353	0	0	0.299	0.25	0.282	0	0.263	0	0
GO:0009888_tissue_development	0	1.353	-0.173	0.441	0.596	0.692	0.282	-0.454	-0.479	-0.149	0
GO:0016747_transferase_activity	0	0.942	1.414	0.16	0.648	0.167	0.384	0.525	0.957	3.119	0
GO:0008289_lipid_binding	0	0.778	1.807	0.095	0.397	0.457	0.111	1.444	0.984	1.735	0
GO:0004497_monooxygenase_activity	0	0.705	2.203	0.408	0.931	0.278	0.478	1.444	1.721	1.451	0
GO:0043086_negative_regulation_of_catalytic_activity	0	0.705	1.819	0.408	0	0.908	0.478	0.659	1.028	1.474	0
GO:0005615_extracellular_space	0	0.705	1.488	-0.18	0	0	0	0.481	1.008	1.583	0
GO:0008447_L-ascorbate_oxidase_activity	0	0.676	2.98	0.65	0.299	1.171	0.546	1.628	1.736	2.376	0
GO:0010015_root_morphogenesis	0	0.676	1.838	0.887	0.596	0.692	1.326	0.902	1.374	1.714	0
GO:0042742_defense_response_to_bacterium	0	0.676	1.749	1.935	3.197	2.064	4.376	0.41	1.258	1.087	0
GO:0006564_L-serine_biosynthetic_process	0	0.676	1.488	0	0.596	0	0	0.252	0.255	1.187	0
GO:0004617_phosphoglycerate_dehydrogenase_activity	0	0.676	1.488	0	0.596	0	0	0.252	0.255	1.187	0
GO:0009767_photosynthetic_electron_transport_chain	0	0.676	1.488	0	0	0.25	0.786	0.484	0.255	1.187	0
GO:0010315_auxin_efflux	0	0.676	1.488	0	0	0.25	0	0.481	0.768	1.187	0
GO:0009768_photosynthesis	0	0.676	1.488	0	0	0	0.282	0.484	0.516	1.187	0
GO:0006629_lipid_metabolic_process	0	0.545	1.426	0.352	0.45	0.649	0.569	1.452	2.138	2.02	0
GO:0004871_signal_transducer_activity	0	0.424	1.426	-0.106	0	0	-0.119	1.098	0.458	0.902	0

Table 3. 3 (cont'd)

GO:0045490_pectin_catabolic_process	0	0.423	1.623	0.667	0	1.286	0.59	0.585	1.078	1.346	0
GO:0051258_protein_polymerization	0	0.423	1.488	0	0.883	0.345	0.59	0.722	0.768	1.187	0
GO:0006782_protoporphyrinogen_IX_biosynthetic_process	0	0.423	1.488	0	0	0	0	1.389	1.244	1.187	0
	0	0.423	1.426	0.16	0.202	0	0	0	0.138	0.902	0
GO:0006563_L-serine_metabolic_process	0	0.423	1.35	0.16	1.865	0.525	0.59	0.102	0.224	0.883	0
GO:0046983_protein_dimerization_activity	0	0.394	2.163	0	0	1.383	0.642	3.667	3.144	2.769	0.402
GO:0005618_cell_wall	0	0.389	2.227	2.311	0.099	3.114	1.27	1.401	2.099	2.199	0.211
GO:0030599_pectinesterase_activity	0	0.294	2.199	0.55	0.526	0.751	1.228	1.019	1.078	1.838	0
GO:0008283_cell_proliferation	0	0.294	1.488	0.265	0	0.922	1.038	6.712	4.354	1.979	0
GO:0004857_enzyme_inhibitor_activity	0	0.294	1.466	0.128	0	0.131	0.304	0.372	0.544	0.996	0
GO:0009505_plant-type_cell_wall	0	0.169	4.428	0.69	0.099	2.155	0.194	2.304	3.367	3.472	0
GO:0009654_photosystem_II_oxygen_evolving_complex	0	0	4.079	0	1.477	0.94	3.369	1.469	2.012	3.119	0
GO:0009538_photosystem_I_reaction_center	0	0	3.976	0	0	0.94	1.326	0.94	1.244	3.17	0
GO:0009664_plant-type_cell_wall_organization	0	0	2.857	0.875	0.228	1.815	0.212	1.739	2.657	2.059	0
GO:0042545_cell_wall_modification	0	0	2.857	0.2	0	0.457	0.111	0.569	0.988	2.059	0
GO:0019898_extrinsic_component_of_membrane	0	0	2.609	0	0.202	0.703	2.127	0.925	1.549	1.735	0
GO:0042973_glucan_endo-1	0	0	2.482	0	-0.371	0.212	-0.128	2.711	2.688	1.979	0
GO:0004190_aspartic-type_endopeptidase_activity	0	0	2.047	0.393	-0.13	0.537	0	0.538	1.448	2.144	0
GO:0042549_photosystem_II_stabilization	0	0	1.985	0	0	0.234	0.798	0.481	0.768	1.583	0
GO:0031072_heat_shock_protein_binding	0	0	1.838	0	0.596	0.25	0.786	0.346	0.756	1.714	0
GO:0045330_aspartyl_esterase_activity	0	0	1.819	1.096	0.299	1.39	1.326	0.373	1.028	1.75	0
GO:0042546_cell_wall_biogenesis	0	0	1.807	0	-0.665	0.278	0	0.424	1.549	1.173	0
GO:0019685_photosynthesis	0	0	1.488	0	0	0.481	0.546	0.944	0.768	1.187	0
GO:0016630_protochlorophyllide_reductase_activity	0	0	1.488	0	0	0.481	0.546	0.944	0.768	1.187	0

Table 3. 3 (cont'd)

GO:0000272_polysaccharide_catabolic_process	0	0	1.488	-0.13	-0.464	0.114	-0.164	1.397	1.496	1.187	0
GO:0042631_cellular_response_to_water_deprivation	0	0	1.488	-0.703	-0.464	0	0	0.94	1.244	1.583	0
GO:0031977_thylakoid_lumen	0	0	1.488	-0.881	0.16	-0.201	0.478	1.389	1.951	1.187	0
GO:0031225_anchored_component_of_membrane	0	0	1.466	0	0	0.239	0	0.812	0.696	0.77	0
GO:0045548_phenylalanine_ammonia-lyase_activity	0	0	1.414	1.096	0	0	0	0.525	0.363	1.378	0
GO:0009800_cinnamic_acid_biosynthetic_process	0	0	1.414	1.096	0	0	0	0.525	0.363	1.378	0
GO:0005199_structural_constituent_of_cell_wall	0	0	1.414	0.441	0	0.692	0.282	0.525	1.168	0.783	0
GO:0009791_post-embryonic_development	0	0	1.414	0.228	0	-0.141	-0.513	1.079	0.957	0.783	0
GO:0016760_cellulose_synthase_UDP-forming_activity	0	0	1.414	0.109	0	0	-0.412	0.902	0.744	0.783	0
GO:0009069_serine_family_amino_acid_metabolic_process	0	-0.067	-0.042	0.318	-0.382	0.334	-1.226	0.26	0.029	0.076	1.549
GO:0043565_sequence-specific_DNA_binding	0	-0.125	1.848	0.25	-0.546	1.202	0.134	1.59	2.289	2.878	0.58
GO:0050662_coenzyme_binding	0	-0.233	1.764	0	-0.721	0.28	-0.353	0.091	0.201	1.217	0
GO:0003735_structural_constituent_of_ribosome	0	-0.323	-0.366	-3.138	-0.895	-1.335	0.072	0.254	0.149	-0.543	1.363
GO:0042254_ribosome_biogenesis	0	-0.323	-0.587	2.37	-0.895	-1.335	0	-0.344	-0.285	-0.769	1.363
GO:0009117_nucleotide_metabolic_process	0	-0.452	1.426	0	-0.497	0	-1.196	1.089	2.534	1.173	0
GO:0005829_cytosol	0	-3.87	-9.04	-4.322	-4.909	-5.425	-5.254	-13.621	-13.221	-7.805	1.363

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FUTURE RESEARCH

In our research, we studied the onset and mechanism of age-related resistance (ARR) to *Phytophthora capsici* in fruits of *C. moschata* commercial cultivars. Two cultivars with early onset of ARR were identified and can be used by growers to limit fruit rot. Incorporating cultivars with ARR may reduce the number of fungicide applications required. Additional screening of squash and pumpkins commercial cultivars for those with early onset of ARR to *P. capsici* is needed. Squash and pumpkins germplasm with resistance to *P. capsici* are needed for breeding programs to develop varieties with fruit rot resistance.

The mechanism of ARR in winter squash to *P. capsici* was investigated. Our results suggest that ARR in winter squash to *P. capsici* is conferred by a physical barrier that is formed by lignin deposition in the fruit exocarp at the cuticle and epidermal walls. Observations from the scanning electron microscope study of fruit exocarp structural defense indicated that at 24 hpi, *P. capsici* attempted to penetrate the resistant fruits through stomatal openings. Cell wall dissociation 24 hpi or at 48hpi suggested that a structural barrier is present at the stomatal opening. Further examination of that structural barrier should be conducted. Lipid stain (ex. sudan IV dye; stains lipids yellow) can be used to examine the suberization at the stomata by comparing young susceptible and mature resistant fruit stages inoculated with *P. capsici* or not inoculated. Therefore, we can detect if suberization occurs prior to *P. capsici* attack (preformed) or induced suberization occurs post inoculation.

Lignin deposition at the fruit exocarp was detected and may confer resistance against *P. capsici*. A disease resistance screening method based on lignin content in the fruit exocarp could be developed. Phloroglucinol stains lignin in red, therefore lignin content can be determined in

proportion to the intensity of the color in the fruit peel. During fruit development, lignin concentration at which resistance occurs can be determined then used in further high-throughput phenotypic screening methods for resistance.

The quantitative expression of lignin biosynthetic genes including CAD, CCR, CCoAOMT and peroxidases in the fruit exocarp could be determined using qPCR and indicate lignin biosynthesis and deposition at the fruit cell wall. If the quantitative expression of these genes can be determined in the resistant fruit stages, then these genes can be used in marker-assisted selection in breeding programs to develop squash or pumpkins varieties with resistance to *P. capsici*.