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Composition of phenolic compounds in wild apple with multiple resistance mechanisms against postharvest blue mold decay

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ABSTRACT

Several wild apple accessions (*Malus sieversii*) from Kazakhstan and two (*Malus* × *soulardii*, *Malus sylvestris*) from other parts of the world are highly resistant to blue mold decay caused by *Penicillium expansum*. Previous studies on the wound responses of these apples to infection by this fungus suggest multiple mechanisms of resistance including innate immunity. In this study, the phenolic composition of extracts from mature wild apples resistant (GMAL 4317.f, PI 589391, and PI 369855) and susceptible (GMAL 3623.i) to blue mold, as well as the susceptible cultivar 'Golden Delicious' (*Malus* × *domestica*) were investigated using ultra-high-performance liquid chromatography coupled to diode array detection and high resolution multiple stage mass spectrometry (UHPLC/DAD/HRMS). The metabolomic and quantitative results of this study support the hypothesis of the possible relationship between the phenolic content of wild apples and their resistance to *P. expansum*. Apple accessions resistant to *P. expansum* had higher concentrations of procyanidins, dihydrochalocone, flavonols, and hydroxycinnamic acids.

Findings from this study may lead to development of the physiological markers of resistance that could be used by breeders in evaluating crosses for resistance to blue mold and may be helpful to further define apple defense mechanism(s) against *P. expansum*.

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1. Introduction

Apple production is based on many commercial cultivars with limited resistance to various diseases and pests, and adaptation to environmental stresses. Resistance against *Penicillium expansum*, the most destructive postharvest pathogen causing blue mold decay, declines quickly as the fruit matures and there is practically no resistance at harvest (Ahmadi-Afzadi et al., 2013; Vilanova et al., 2014). This pathogen infects apples through wounds, which must be protected against this fungus in order to prevent decay development (Janisiewicz, 1999). Postharvest treatment of apples with fungicides has been the main weapon against this decay; however, there are various limitations to the use of fungicides and the public increasingly demands produce free of synthetic chemical residues (Caiazzo et al., 2014; Errampalli and Crnko, 2004; Tepper and Yoder, 1982; Vorstermans and Creemers, 2011). Natural disease resistance is the safest and most durable form of disease control. Unfortunately, apple breeders seldom evaluate their crosses for resistance to postharvest diseases because of the general lack of resistance in the cultivated apples and in the progeny resulting from their crosses (Janisiewicz et al., 2008; Spotts et al., 1999).

Recently, resistance to blue mold was identified in wild apple accessions, *Malus sieversii*, the progenitor of the cultivated apple *Malus domestica*, collected from natural wild apple stands in Kazakhstan (Forsline et al., 2003; Janisiewicz et al., 2008; Jurick et al., 2011). The high level of resistance and even immunity in the mature wild apples, suggests a possible constitutive, preformed or/an induced form of resistance (Janisiewicz et al., 2016). Characterization of the wound responses of resistant and susceptible apple genotypes to inoculation with *P. expansum* may be helpful in explaining the underlying mechanisms of resistance. Studies on the histochemical reactions of the wounded area of wild apples resistant and susceptible to *P. expansum* didn't reveal any changes

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that could be clearly associated with the resistance or with resistant responses similar to those observed in immature cultivated apples (Janisiewicz et al., 2016; Lakshiminarayana et al., 1987; Vilanova et al., 2012).

There are a variety of constitutive and induced resistance mechanisms reported to operate in various harvested fruit and phenolic compounds have been often associated with resistance to different pathogens in many fruit including apples (Ahmadi-Afzadi et al., 2015; Ben-Yehoshua et al., 1995; Lattanzio et al., 2001; Petkovšek et al., 2009; Prusky et al., 1992, 2000; Prusky, 2003; Schovánková and Opatová, 2011; Singh et al., 2015; Villarino et al., 2011). One example for the role of preformed substances in fruit resistance is that of diene, one of several preformed antifungal compounds in avocado (Prusky et al., 1992, 2000). This compound is toxic to *Colletotrichum gloeoriosporioides*, the major pathogen causing postharvest decay of avocado. Decline in its concentration during avocado ripening is directly related to increase in susceptibility to the decay. The breakdown of diene is caused by lipoxygenase. Interestingly, epi-catechin is a natural inhibitor of this breakdown as well as pectolytic enzymes produced by the fungus; however, it also declines with fruit maturation. Preformed resorcinols were reported to occur at concentrations fungitoxic to *Alternaria alternata* in the peel of immature mango fruit and were linked to resistance to the fungal decay (Prusky, 1996). This was corroborated by findings that resorcinols concentrations decline to non-toxic levels during ripening of the fruit, decline faster in more susceptible cultivars, the speed of decline was correlated with increase of decay, and the flesh of the fruit, which always contained low concentrations of the compound, was always susceptible to the decay. The role of pre-existing fungitoxic compounds in resistance of banana peel to fungal infection has been extensively studied and reports are contradictory (Abayasekara et al., 1998; Muirhead 1979; Muirhead and Deverall 1984). It appears that resistance is multifaceted and may involve preexisting compounds as well as phytoalexins (Abayasekara et al., 1998).

Accumulation of phenolic compounds, such as hydroxycinnamic acid, flavan-3-ols and flavanols, as well as a higher content of total phenolics in apple leaves and fruit has been reported to occur after infection with *Venturia inaequalis* causing apple scab (Petkovšek et al., 2009).

Also, phenolic acid content was considered an important factor in separating twelve apple cultivars with varying degrees of resistance to this disease (Singh et al., 2015). Thus, considering previous work on the role of preexisting phenolic compounds in resistance of various fruits including apples to fungal invasion, determining the profiles of phenolic compounds in the wild apples is the first logical step to explain the basis of resistance to *P. expansum*.

Liquid chromatography (LC) mass spectrometry (MS) is the most widely used technique for separation and detection of the plant secondary metabolites. There are various targeted assays to quantify specific classes of phytochemicals in apples, such as the quantitation of patulin, sugars, and phenolic compounds using GC–MS or LC–MS techniques (Desmarchelier et al., 2011; Fuzfai et al., 2004; Marks, 2007; Wong et al., 2010; Zhang et al., 2014). In recent years, high-resolution accurate mass MS with multiple-stage fragmentation (HRAM-MS^n) has become increasingly popular and can provide valuable information on the chemical composition and structure, as well as putative identification of phenolic compounds and it becomes the preferred method for the detection of secondary metabolites present in food and botanical materials. Hundreds of secondary metabolites were identified from strawberry, microgreens, apples, red mustard greens, berries and nuts using UHPLC HRAM-MS^n profiling methods (Lin and Harnly, 2007; Lin et al., 2011; Sun et al., 2012, 2013, 2014).

Metabolomics is an emerging field and the ultimate goal of a metabolomics study is capable for profiling and quantitation of all metabolites from the complex mixtures in an untargeted fashion and it is extremely useful to differentiate between species, growing conditions, locations, and/or cultivars. In contrast to traditional targeted food component analysis, which assumes one or several target compounds to be important, metabolomic studies view the whole sample as a complex pool of different secondary metabolites. (Wishart, 2008) However, at current stage, it is more feasible to focus on the identification and quantitation of metabolites differences between groups of samples with different characteristics, in a semi-quantitative manner. In the present study, the phenolic compositions differences of different wild apple accessions, (GMAL 3623.i, GMAL 4317.f, PI 589391, and PI 369855) and the common commercial cultivar 'Golden Delicious' were investigated by a metabolomic approach. The following semi-quantitative analysis on major phenolic compounds profiled was performed to determine the relationships between certain phenolic compounds and the apple's resistance to blue mold.

2. Material and methods

2.1. Fruit

Apple fruit were harvested according to predetermined harvest dates from trees in the germplasm collection located at the USDA-ARS orchard in Geneva, NY. The fruit were placed in cardboard boxes with perforated polyethylene liners and transported to the USDA-ARS AFRS laboratory in Kearneysville, WV. Fruit were stored at 2 °C and were removed from cold storage 1 d prior to start of the experiment to allow for acclimation to room temperature (~22 °C).

2.2. Sampling

For each accession, fruit were randomized and divided into 3 replicates (4–6 fruit each). Tissue samples from each fruit (2–4 plugs, depending on the size of the fruit) were collected with a cork borer (12 mm diameter) and removed, then cut to a depth of approximately 1 cm from the skin. All plugs from each replicate were placed in a 50 mL conical tube, weighed and labeled with the accession, replicate and wet weight. The tubes were placed in liquid nitrogen for 3–5 min to flash freeze the samples and then all tubes for each accession were placed in one Ziploc bag and stored at −80 °C prior to freeze-drying.

2.3. Freeze-drying samples

The samples were moved from the −80 °C freezer and placed in a −20 °C chest freezer with the lid open to prepare tubes for freeze-drying. Glass jars used with the freeze-dryer were also placed in the freezer to cool them before placing samples within. Lids from each of the 50 mL tubes with fruit plugs were removed and replaced with a lid containing 4–5 holes and the 3 tubes (replicates) for each accession were placed in one jar and attached to the Freezemobile 24 freeze-drier (The Virtis Company, Gardiner, NY 12525). Samples were left on the machine for approximately 96 h, then jars were removed and the intact lids of the 50 mL tubes were returned. Tubes were wrapped at the lid-tube juncture with Parafilm and stored at −80 °C until they were transported on ice to USDA-ARS Food Composition and Methods Development Lab in Beltsville, MD.

2.4. Chemicals

Formic acid was purchased from Sigma-Aldrich (St. Louis, MO), Optima™ grade methanol and acetonitrile, were purchased from