For submission to Postharvest Biology and Technology

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*.

Keywords

Penicillium expansum, *Malus sieversii*, apple germplasm, UHPLC-HRMSⁿ

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 have 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 et al., 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 that could be clearly associated with the resistance or with resistant responses similar to those observed in immature cultivated apples (Janisiewicz et al., 2016; Lakshminarayana 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, 2015; Ben –Yehosuha et al., 1995; Lattanzio et al., 2001; Petkovšek et al., 2009; Prusky et al., 1992, 2000, 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 1992; Prusky 2000). This compound is toxic to *Colletotrichum gloeosporioides*, 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 1998; Muirhead 1979; Muirhead and Deverall 1984). It appears that resistance is multifaceted and may involve preexisting compounds as well as phytoalexins (Abayasekara 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 (Shing 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 (Desmarcheli et al., 2011; Fuzfai et al. 2004; Marks, 2007; Wong et al., 2010; and Zhang et al., 2014). In recent years, high-resolution accurate mass MS with multiple-stage fragmentation (HRAM-MSⁿ) 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-MSⁿ 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 hours, 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 Fisher-Scientific (Waltham, MA). De-ionized water was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA). Rutin (quercetin-3-*O*-rutinoside), epi-catechin, catechin, procyanidin B1, procyanidin B2, procyanidin A1, procyanidin C1 and phloretin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Two hundred milligrams of each lyophilized fruit sample was extracted with methanol-water (70:30) for 1 h. The homogenates were then centrifuged at $2,795 \times g$ for 10 min (Fisher Scientific Centrifuge, Waltham, MA). The supernatant was filtered through a $0.22 \mu\text{m}$ PTFE filter, transferred into a 2 mL HPLC vial, and $2 \mu\text{L}$ was injected for UHPLC-HRAMⁿ analysis.

2.5. UHPLC-PDA-ESI/HRAM/MSⁿ conditions

The UHPLC-HRAM analysis was performed on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with an Agilent 1290 Infinity liquid chromatography with G4220A binary pump, G4212 Diode Array Detector, G1316C thermostatted column compartment, and G4226 autosampler with thermostat (Agilent, Palo Alto, CA). An Agilent RRHD Eclipseplus C₁₈ 2.1 mm $\times$ 150 mm, $1.8 \mu\text{m}$ (Agilent, Palo Alto, CA) with an UltraShield pre-column filter (Analytical Scientific Instruments, Richmond, CA) was used for the HPLC separation at a flow rate of $6.67 \mu\text{L s}^{-1}$. Mobile phase consisted of a combination of solution A (0.1 % formic acid in water) and solution B (0.1 % formic acid in acetonitrile). A linear elution gradient was used as followed: 0-10 min increased from 2 % to 10 % B, to 50 % B at 25 min, to 90 % B at 30 min, and the post-run time for re-equilibration was 5 min. The UV-vis spectra were recorded at the range of 200–700 nm for the entire run. The ESI-MS parameters were optimized using a standard solution of quercetin-3-*O*-rutinoside (100 mg L^{-1}) by Tee-in with the initial mobile phase at a flow rate of $6.67 \mu\text{L s}^{-1}$. The MS conditions were optimized as follows: ESI spray voltage at 4.5 kV, capillary temperature at 325°C , capillary voltage at 15 V, and tube lens offset voltage at 150 V. The mass range was recorded from m/z 100 to m/z 2, 000 with a resolution of 30,000, FTMS automatic gain control AGC was set at 5×10^5 , MS/MS AGC was set at 10^4 , the ion isolation width was set 1.5 amu, and max ion injection time of 0.5 s. Data-dependent acquisitions on the

most intense ion were used for MS²-MS⁴ scan and the normalization collision energy was set at 35 %.

2.6. LC–MS data pre-treatment and handling

The UHPLC-HRAM MSⁿ raw files were converted into “.mzXML” universal data format using Proteowizard 3.0. 3569 (<http://proteowizard.sourceforge.net/>) and then processed with XCMS (Tautenhahn, et al., 2012) for peak detection, noise filtering, and peak alignment. A 2D data table was generated including variable index (paired *m/z*-retention time), sample names and peak intensities. Principle component analysis (PCA) was performed on the output table from XCMS using Sigma 13 (Umetrics, Umeå, Sweden).

2.7. Quantification

Stock solutions of chlorogenic acid, phloretin, procyanidin B1, procyanidin B2, procyanidin C1, (–)-epicatechin, sinipic acid, and quercetin-3-*O*-rutinoside (100 mg L⁻¹) prepared in methanol were serially diluted in methanol for developing standard curves (Table S1) for each polyphenol and used for quantification. Compounds were identified by comparison of retention time, UV-spectra and MS, as well as MS/MS information using reference compounds and method was validated with linearity ranges, limit of detection (LOD), and limit of quantitation (LOQ). The results are express as g kg⁻¹ based on the dry weight.

3. Results

3.1. Identification of the major phenolic compounds

The phenolic profile of each apple accession/cultivar was initially examined at UV 280 nm. Under the experimental conditions, 41 chromatographic peaks were observed (Figure 1). Flavan-

197 3-ols (e.g. catechin, epi-catechin), dihydrochalcone glycosides (e.g., phloridzin), flavonols (e.g.,
198 quercetin), procyanidins and phenolic acids (e.g., chlorogenic acid) were found to be the major
199 phenolic compounds in apples from these 5 accessions (Table 1). Metabolite assignments were
200 made by comparing retention time, UV/VIS spectra and MS data (accurate masses, isotopic
201 distribution, and MS²⁻⁴ fragmentation behaviors in negative ion mode) of the compounds
202 detected with those reported for apples in the literature and databases. Identifications were
203 confirmed with standard compounds when available. Among the 41 peaks, Peak 3, 11, 14, 15, 19,
204 21, 24, 31 and 39 were identified unambiguously as malic acid, procyanidin B2, catechin,
205 chlorogenic acid, procyanidin B1, epi-catechin, procyanin C1, quercetin 3-*O*-glucoside and
206 phlorizin by comparing with the reference standards.

207 B-type proanthocyanidin oligomers with degrees of polymerizations from 2 to 5 were the major
208 proanthocyanins identified. The main fragmentation patterns corresponded to heterocyclic ring
209 fission (HRF, loss of D-ring), retro Diels-Alder (RDA, loss of B or E-ring) and cleavage of the
210 interflavan linkage were also used for the confirmation of proanthocyanidins (Gu et al., 2003).
211 One A-type proanthocyanin was identified as procyanidin A2. All the MS² spectra of peaks 31-
212 35 exhibited the same product ion (m/z 301), it was further fragmented into m/z 179 and 151 in
213 the MS³ spectra, which are typical for the quercetin aglycone. Peaks 31 and 32 were thus
214 identified as quercetin 3-*O*-glucoside and quercetin 3-*O*-galactoside respectively. Likewise,
215 peaks 33, 34, and 35 were assigned as quercetin-*O*-pentoside.

216 Peaks 8 and 10 were identified as oxidative coupling products between procyanidin B2 and
217 caffeoylquinic acid according to the similar fragmentation reported in the literature (Poupard, et
218 al., 2011). Peaks 36 and 39 were two major dihydrochalcones found in these five
219 cultivars/accessions. Peak 36 was identified as phloretin-xylosyl-glucoside (m/z 567.1675, -4.45

mmu) and Peak 39 was identified as phloridzin (m/z 435.1278, -1.89 mmu). These results are in agreement with the previous studies (Le Deun et al., 2015; Montero et al., 2013; Ramirez-Ambrosi et al., 2013; Vrhovsek et al., 2012). These results provided a preliminary foundation for further metabolomics study.

3.2. Metabolomic analysis of the UHPLC–HRAM MSⁿ data

Different metabolite patterns could be observed by simple visual inspection of the UHPLC–UV chromatogram at 280 nm for the different apple cultivars/accessions. An in-depth metabolomic study was carried out to explore the differences among these 5 cultivars/accessions. The original UHPLC–HRMS raw data led to the extraction of 318 ion features (m/z , retention time) from the 75 samples analyzed. Principal component analysis (PCA) based on the relative intensities of these ion features revealed the metabolomic differences between these cultivars after pareto scaling (van der Berg et al., 2006) (Figure 2A). The first two components accounted for 73.7% of the total variance (R²), with the prediction goodness parameter Q² of 70.4%. The PC1/PC2 score plot shows that each cultivar was clustered together. ‘Golden Delicious’ and GMAL 3623.i clustered closely, where the other three, PI 589391, PI 369855 and GMAL 4317.f, were separated from each other at the right side of the plot. GMAL 3623.i and ‘Golden Delicious’ are both two susceptible, while the other three are immune accessions. This suggests that the chemical profiles of the apple accessions played an important role on their resistance to fungus. The loading plots revealed ion features that have the largest effect on the respective principle components (Figure 2B). They were m/z 577.1325/6.30, m/z 865.1968/7.59, m/z 191.0547/0.78, m/z 289.0704/6.89, m/z 567.1681/11.07, m/z 353.0862/5.49, and m/z 191.0547, 0.78 for PC1; and m/z 567.1681/11.07 and m/z 583.1635/9.69, for PC2. These features were

identified as procyanidin B2, procyanidin C1, quinic acid, epi-catechin phloretin xylosyl-glucose, and m/z 583.1635 remains unidentified despite our best efforts.

3.3. Quantitation of the major marker phenolic compounds

Under the chromatographic conditions used it was possible to quantify all the major marker compounds present in the extract as all major peaks were well resolved. The epi-catechin, chlorogenic acid, procyanidin B1 procyanidin B2, procyanidin C1, phlorizin and quinic acid were quantified with the respective reference standards, quercetin 3-*O*-glucoside was quantified with rutin as the reference standard and phloretin-xylosyl-glucoside was quantified with phloridzin (Table S1).

Accession PI 369855 had the highest concentration of epi-catechin and procyanidin B1, while the presence of quinic acid was the lowest throughout all apple cultivar/accessions (Figure 3, Table 2) Among monomers and dimers, the most important compounds are procyanidin B2 (0.063-0.396 g kg⁻¹) and epi-catechin (0.128-1.986 g kg⁻¹). The values obtained showed the great difference across these five accessions/cultivars. The resistant accessions, in particular for PI 369855, had a noticeably higher concentration of procyanidin B1. GMAL 4317.f had the highest concentration of procyanidin B2 and procyanidin C1 and quinic acid. The two major dihydrochalcone compounds in this group were phloridzin and phloretin-xylosyl-glucoside. The highest relative and also absolute amounts of phloretin-xylosyl-glucoside were found in PI 589391 (1.457 g kg⁻¹) while the cultivar ‘Golden Delicious’ had the lowest concentration (0.014 g kg⁻¹). Also, PI 589391 had the highest concentration of phloridzin (0.724 g kg⁻¹). Quercetin 3-*O*-glucoside was an important marker compound which also showed the greatest difference between susceptible and resistant apples. It was highest in PI 589391 and PI 369855 followed by GMAL 4317.f and the lowest in ‘Golden Delicious’ and GMAL 3623.i. Chlorogenic acid was

the major hydroxycinnamic acid and was at the highest concentration in PI 369855 followed by PI 589391 then GMAL 4317.f and lastly in GMAL 3623.i and ‘Golden Delicious’. However, it was the main phenolic detected in susceptible GMAL 3623.i and ‘Golden Delicious’ accounting for 27 % and 31 % of total polyphenolics content, respectively, and in resistant GMAL 4317.f, PI 369855, and PI 589391 constituted only 7 %, 8 %, 24 %, of the total phenolic content, respectively (Figure 3 and Table 2).

4. Discussion

The metabolomic and quantitative results of this study support the hypothesis of the existence of a relationship between the content of phenolic compounds in apple and its resistance to *P. expansum*. Apple accessions GMAL 4317.f, PI 589391, and PI 369855 that are resistant to *P. expansum* and didn’t develop any lesions after wound inoculation with a high concentration of the conidial suspension (10^5 conidia/ml) after seven days incubation at 24°C had higher concentrations of procyanidins, dihydrochalcone, flavonols and hydroxycinnamic acids than susceptible accession GMAL 3623.i and ‘Golden Delicious’, which developed lesions 33.0 mm and 31.8 mm in diameter under the same conditions, respectively (Janisiewicz et al., 2016; Janisiewicz, unpublished). The relationship is particularly clear in the case of epi-catechin and procyanidin B1. The concentration of phenolic compounds have relationship with the defense mechanisms against pathogens (Cushnie and Lamb, 2005; Ferreyra et al., 2012). Many flavones and flavanones have been shown to be active against fungal pathogens commonly found during the storage of fruit and vegetables (Cushnie and Lamb, 2005). The efficacy of quercetin in reducing growth of *P. expansum* in vitro and blue mold incidence and severity has already been reported for ‘Golden Delicious’ (*Malus × domestica*) (Sanzani et al., 2012; Mierziak et al., 2014). Phenolic compounds were shown to accumulate in ‘Idared’ apples in response to infection by *P.*

expansum, *Monilinia fructigena* and *Gloeosporium* spp. (Schováňková and Opatová, 2011), and flavonols and procyanidin B2, as well as total phenols, had significant impacts on blue mold development on various commercial apple cultivars with partial resistance to this disease (Ahmadi-Afzadi, et al., 2015). Overall, the much higher concentrations of phenolic compounds in resistant accessions than in susceptible one and ‘Golden Delicious’ increase a likelihood that they play a significant role in the defense mechanism. Considering this, the observed differences in concentrations of the phenolic compounds among resistant accessions may not have a significant effect on their basal level of resistance to *P. expansum*.

The phenolic compounds identified in our study, being in agreement with the previous studies, (Le Deun, et al., 2015; Montero et al., 2013; Poupard, et al., 2011; Ramirez-Ambrosi et al., 2013; Vrhovsek et al., 20120) have provided a foundation for further metabolomics study. The apple metabolome and subsequent quantitative study provided a useful evaluation tool for further understanding of the potential metabolic markers that may be useful for chemical phenotyping and for the development of new apple cultivars with blue mold resistance.

A clear separation of resistant accessions from the susceptible wild apple accession GMAL 3623.i and ‘Golden Delicious’ in the PCA score plot indicates that phenolic compounds are important in the resistance of these apples to *P. expansum*. Among the resistant accessions, the wild apple GMAL 4317.f, had highest concentration of procyanidin B1 and epi-catechin, thus further study should focus on *in vitro* inhibitory effects of these possible marker compounds to *P. expansum*.

Phenolic compounds can affect fruit quality in different ways including fruit color, taste, resistance to pathogens, and shelf-life, in addition they can have significant influence on human health (Hamauzu, 2006). Recent advances in analytical methodologies have allowed for a better

understanding of the role of phenolic compounds in fruit. Understanding the compositions of phenolic compounds in *Malus* species can be very useful for breeding programs and underscores the importance of germplasm conservation. As Francini and Sebastiani, (2013) signified, introduction of highly specific polyphenolic traits to elite apple genotypes will contribute greatly to genetic improvement and may be soon realized due to the revolution in genomics and its biotechnological applications.

5. Conclusion

In conclusion, 41 phenolic compounds were identified from five apple accessions/cultivar. Among them, nine major phenolic compounds showing major differences among the wild apple accessions and the commercial cultivar. The results from both metabolomic and quantitative studies 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, dihydrochalcone, flavonols and hydroxycinnamic acids. Findings from this study may lead to the development of physiological markers of resistance that could be used by breeders in evaluating crosses for resistance to blue mold.

Acknowledgements

This research is supported by the Agricultural Research Service of the U.S. Department of Agriculture, an Interagency Agreement with the Office of Dietary Supplements at the National Institutes of Health (Y01 OD001298-01) and USDA-ARS Project Plan 8080-22000-009-00D.

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492 **Figures**

493

494 **Fig. 1.** The HPLC-UV chromatograms (280 nm) of wild and cultivated apples resistant and susceptible to blue mold

495 (A) 'Golden Delicious', (B) GMAL 3623.i, (C) GMAL 4317.f, (D) PI 369855, and (E) PI 589391.

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497 **Fig.2.** The Principal Component Analysis score plot (A), A = ‘Golden Delicious’, B = GMAL 3623.i, C = GMAL 4317.f, D = PI
 498 369855, and E = PI 589391, and loading plot (B) for five apple cultivar/accessions.

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Fig.3. The major phenolic contents (g kg^{-1} , based on the dry weight) in five apple cultivar/accessions. Standard deviation of the mean from three extraction replicates ($n = 3$).

Table 1. The major phenolic compounds identified in five apple cultivars/accessions

Peak No.	Rt.	m/z	Formula [M-H] ⁻	error (mmu)	Tentative identification
1	0.70	181.0709	C6 H13 O6	-0.84	mannitol
2^a	0.79	191.0541	C7 H11 O6	0.50	quinic acid
3	0.79	133.0139	C4 H5 O5	0.75	malic acid
4	1.23	341.1073	C12 H21 O11	-1.67	di-saccharide
5	1.33	341.1075	C12 H21 O11	-1.44	di-saccharide
6	1.46	341.1078	C12 H21 O11	-1.14	di-saccharide
7	1.83	341.1076	C12 H21 O11	-1.36	di-saccharide
8	2.61	575.1176	C30 H23 O12	-1.89	oxidation product of procyanidin B2
9	3.07	315.0708	C13 H15 O9	-1.36	protocatechuic acid glucoside
10	4.51	573.1017	C30 H21 O12	-2.17	oxidation product of procyanidin B2
11	4.91	577.1336	C30 H25 O12	-1.54	procyanidin B2
12	5.06	865.1848	C45 H37 O18	-3.78	B-type Proanthocynidin trimer
13	5.26	337.1127	C13 H21 O10	-1.32	3-(hexopyranosyloxy)propane 1,2 diyl diacetate
14	5.43	289.0709	C15 H13 O6	-0.84	catechin
15	5.61	353.0850	C16 H17 O9	-2.85	chlorogenic acid
16	5.85	863.1786	C45 H35 O18	-4.30	A-type Proanthocynidin trimer
17	5.97	1153.257	C60 H49 O24	-4.61	B-type Proanthocynidin tetramer
18	6.23	577.1312	C30 H25 O12	-4.41	B-type Proanthocynidin dimer
19	6.42	577.1312	C30 H25 O12	-4.41	procyanidin B1
20	6.79	337.0920	C16 H17 O8	-0.88	p-Coumaroyl quinic acid
21	7.01	289.0688	C15 H13 O6	-3.00	epi-catechin

22	7.22	351.1288	C14 H23 O10	-0.89	cyclohexanecarboxylic acid, 3-[(6-deoxy-3-O-methyl-D-galactopyranosyl)oxy]-1,4,5-trihydroxy-, (1S,3R,4R,5R)
23	7.56	865.194	C45 H37 O18	-4.51	B-type Proanthocynidin trimer
24	7.69	865.194	C45 H37 O18	-4.51	procyanidin C1
25	7.99	517.2274	C24 H37 O12	-1.69	vomifoliol 9-[xylosyl-(1->6)-glucoside]
26	8.15	1153.257	C60 H49 O24	-5.46	B-type Proanthocynidin tetramer
27	8.29	1441.323	C75 H61 O30	-4.63	B-type Proanthocynidin pentamer
28	8.41	1441.323	C75 H61 O30	-4.63	B-type Proanthocynidin pentamer
29	8.73	865.1954	C45 H37 O18	-3.62	B-type Proanthocynidin trimer
30	9.45	425.2012	C18 H33 O11	-1.67	unknown
31	9.71	463.0862	C21 H19 O12	-1.96	quercetin 3-O-glucoside
32	9.96	463.0862	C21 H19 O12	-1.96	quercetin 3-O-galactoside
33	10.40	433.0775	C20 H17 O11	-0.19	quercetin pentoside
34	10.60	433.0775	C20 H17 O11	-0.19	quercetin pentoside
35	10.91	433.077	C20 H17 O11	-0.67	quercetin pentoside
36	11.17	567.1675	C26 H31 O14	-4.45	phloretin-xylosyl-glucoside
37	11.3	447.0921	C21 H19 O11	-1.16	quercetin rhamnoside
38	11.93	429.175	C20 H29 O10	-1.66	phenethyl rutinoside
39	12.20	435.1278	C21 H23 O10	-1.89	phloridzin
40	12.49	473.1646	C21 H29 O12	-1.87	6-Feruloylglucose 2,3,4-trihydroxy-3-methylbutylglycoside
41	12.99	429.1754	C20H29O10	-1.24	phenethyl rutinoside

^a Peaks numbers in bold were confirmed with reference standards.

511 **Table 2.** Phenolic compounds concentrations in wild apple accessions and ‘Golden Delicious’ apples.

Compound Name/Apple	‘Golden Delicious’	GMAL 3623.i	GMAL 4317.f	PI 369855	PI 589391
epi-Catechin	0.128±0.073	0.479±0.148	6.651±0.993	19.86±1.375	1.513±0.361
Chlorogenic acid	0.33±0.027	0.505±0.178	1.104±0.048	3.593±0.127	2.707±0.294
Procyanidin B1	0.224±0.025	0.391±0.125	5.156±1.047	17.742±1.982	1.675±0.765
Quercetin 3- <i>O</i> -glucoside	0.154±0.113	0.11±0.115	0.899±0.114	2.377±0.21	2.668±0.819
Phloretin-xylosyl-glucoside	0.014±0.002	0.02±0.004	0.646±0.06	0.146±0.058	1.457±0.172
Procyanidin B2	0.063±0.005	0.097±0.029	0.396±0.042	0.382±0.072	0.136±0.023
Procyanidin C1	0.054±0.006	0.122±0.07	1.402±0.36	1.34±0.17	0.408±0.117
Phloridzin	0.011±0.001	0.025±0.007	0.292±0.039	0.272±0.03	0.724±0.048
Quinic acid	0.097±0.022	0.103±0.016	0.131±0.016	0.087±0.008	0.105±0.004

512 Data were expressed as mean value ± SD (n = 3, g kg⁻¹) based on the dry weight

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Graphic Abstract

P. expansum
susceptible accession

P. expansum
resistant accession

Metabolomic/
Quantitative Analysis

Phenolic compounds