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Research Paper

Secondary metabolite profiling deciphers the phenolic and volatile organic compound diversity within an interspecific *Malus* collection

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ABSTRACT

Secondary metabolites play fundamental roles in apple, influencing the interaction with pollinators and frugivores for the seed dispersal, contributing to fruit quality and promoting human health through their antioxidant property. Domestication and breeding have significantly re-shaped the apple metabolism, altering both aromatic profiles and nutritional properties. This study assessed the secondary metabolite variation in a comprehensive *Malus* spp. collection comprising 163 accessions belonging to 44 species. The profiling of phenolic and volatile organic compounds (VOCs), performed with Ultra-Performance Liquid Chromatography (UPLC) and Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) instruments respectively, in both skin and pulp tissues, uncovered distinct metabolic patterns between wild and domesticated apples. This investigation underlined the higher concentration of these metabolites in the skin tissue and revealed a clear metabolic divergence between the two groups of *Malus* accessions. Wild *Malus* spp. accessions resulted particularly rich in specific polyphenols characterized by antioxidant activity, including catechin and procyanidins. Conversely, apples of *Malus domestica* accessions exhibited a more abundant VOC profile, particularly represented by esters associated with fruity aroma, enhancing sensory appeal. These findings provide a foundation for leveraging wild germplasm in breeding programs, and the identification of accessions with high polyphenolic concentration and desirable aromatic profiles offers valuable opportunities to improve the aromatic and nutraceutical properties of apple.

Keywords: Apple; diversity; secondary metabolites; polyphenols; VOCs; domestication; quality

1. Introduction

Apples (*Malus domestica*) are among the most widely consumed fruits worldwide, valued for both their taste and health-promoting properties. These attributes are largely influenced by secondary metabolites, particularly polyphenols and volatile organic compounds (VOCs). Phenolic compounds represent a large group of molecules synthesized through the shikimate phenylpropanoid pathway, originating from the aromatic amino acids phenylalanine and tyrosine (Vinson et al., 2001; McGhie et al., 2005). In apples, two main categories of phenolic compounds are found: flavonoids and phenolic acids, distinguished by their molecular structure (Vrhovsek et al. 2004; Serrano et al., 2011; Cuthbertson et al., 2012). Phenolic compounds undergo a catabolic process during fruit growth and ripening (Vilanova et al., 2014). These molecules actively contribute to protecting human health

by preventing the risk of chronic diseases (Willett et al., 2002; Aprikian et al., 2003; Halliwell et al., 2005; McGhie et al., 2005) through their antioxidant properties, which enable free radical scavenging counteracting the effects of reactive oxygen species (ROS) (Manach et al., 2004; Visioli et al., 2011). Beyond their functional role in human health, these secondary metabolites are also crucial for enhancing plant resistance to biotic agents, interfering in the plant-pathogen interaction mechanism. High concentration of phenolic compounds, in particular hydroxycinnamic acid and flavan-3-ols, were observed during infection of *Venturia inaequalis,* the fungus responsible for apple scab (Leser and Treutter, 2007; Petkovsck et al., 2009; Singh et al., 2015). Similarly, high concentration of procyanidins, dihydrochalcone, flavonols and hydroxycinnamic acids have been reported to provide resistance to blue mold decay, caused by infection of *Penicillum expansum* (Sun et al., 2017). Despite their beneficial properties, phenolic compounds are often associated with bitterness and astringency. Due to these undesirable traits, polyphenol concentrations have been significantly reduced, or even entirely removed, through domestication and artificial selection, favoring traits like sweetness, texture and storability (Hyson, 2011; Ma et al., 2018; Spengler, 2020; Davies et al., 2022). The metabolic transition from astringent and bitter to a predominant sweetness and firm/crispy has been suggested as an evolutionary adaptation to promote seed dispersal, initially via zoochory and later through human consumption (Shang et al., 2014; Spengler, 2020). To restore these valuable features lost during the domestication process, the employment of crop wild relatives has been recently considered. To this end, wild apple accessions have been used to reintroduce the red flesh trait, enhancing anthocyanin accumulation and boosting antioxidant properties (Angeli et al., 2024). This class of secondary metabolites typically display a tissue-specific accumulation pattern, also supported by a specific Quantitative Trait Loci (QTL) mapping investigation (Khan et al., 2012). Skin tissue, compared to pulp, is in fact known to be generally characterized by a higher concentration of phenolic compounds, as showed by Busatto et al. (2019).

In addition to polyphenols, volatile organic compounds (VOCs) represent another important group of secondary metabolites, contributing to the aromatic profile of plants and fruits (Pérez and Sanz, 2008). In apple, around 300 VOCs have been identified, with aldehydes, alcohols and esters being the key contributors to the fruit aromatic profile (Espino-Diaz et al., 2016). During fruit development and ripening, these compounds undergo a distinctive transition. Aldehydes dominate in unripe fruit but gradually decrease towards the fruit maturation, while alcohols and esters are produced mainly at the onset of ripening (Fellman et al., 2000). The production of VOCs plays a central role in the ecological signaling and plant phenotype (Niederbacher et al., 2015). According to their regulation, VOCs can be classified as either conservative or stress-induced (Arimura et al., 2005; Loreto and Schnitzler, 2010). Although genetically controlled, VOC variability also exhibits high phenotypic plasticity (Ballhorn et al., 2011). Apples from different cultivars can be distinctively grouped based on their predominant VOCs, particularly alcohols and esters (Farneti et al., 2015). Although VOC concentration is a key factor in determining fruit quality and consumer preference, these molecules also play crucial signaling and communication roles. By interacting with the environment, VOCs can modulate various plant defense mechanisms against biotic and abiotic stresses, as well as influence reproduction (Brosset and Blande, 2022). VOCs emitted by flowers attract pollinators essential for species reproduction (Kessler et al., 2008). The VOC blend, which changes during fruit maturation and ripening, initially promotes the protection of fruits by repelling herbivores when the fruit and seeds are still immature, and later attracts frugivores when fully ripened, allowing the dispersion of mature and vital seeds (Rodriguez et al., 2012).

This study aimed to dissect the metabolic diversity of an extensive multi-species *Malus* collection, focusing on polyphenol and VOC concentration patterns in both skin and pulp tissues. This collection was assembled to uniformly represent the taxonomic and genetic diversity of the *Malus* genus. By comparing wild and domesticated apples, we aimed to identify promising accessions that combine desirable aromatic and nutraceutical properties, supporting future breeding strategies. The generation and selection of novel apple accessions through traditional breeding is a very time-consuming and laborious practice, especially for its biology. Despite annual crops, perennial trees are normally distinguished by a long juvenile unproductive phase. Breeding in apple is moreover limited by its gametophytic type of self-incompatibility, which force out-crossing to prevent self-fertilization. To introduce disease resistance or promoting resilience into new accessions, crop wild relatives can represent a valuable reservoir of untapped diversity but they can often lack a detailed phenotypic and metabolic characterization, making breeding and selection even more complicated. This work addresses this large gap, providing new insight in the metabolic characterization of a large set of accessions that could represent potential parents in future breeding activities oriented to improve fruit sustainability and quality.

2. Materials and methods

2.1. Plant materials and sample preparation

This study utilized a diverse apple collection consisting of 163 *Malus* spp. accessions representing 44 different *Malus* species. Apple accessions were located in the Trentino Alto-Adige region, in the northern part of Italy. Wild *Malus* species were collected from the experimental orchard of Fondazione Edmund Mach (Trento, Italy), while the *Malus domestica* accessions were selected from the RefPOP, a reference apple collection representing the genetic diversity existing within *M. domestica* (Jung et al., 2020), located at the Laimburg Research Centre (Bolzano). The two locations are closely located (Lat: 46.19442, long: 11.13635 and lat: 46.38277, long: 11.28804 for the two locations respectively) with similar temperate climatic conditions. Each accession was represented by three plants, and at horticultural maturity, determined based on standard indicators (skin color, starch index and historical data from previous assessments), five uniform fruit per accession were collected and initially assessed for fresh weight and diameter. The skin was accurately separated from the pulp tissue, and both tissues were individually frozen in liquid nitrogen and ground into a fine powder using an IKA analytical mill (Staufen, Germany). The powdered samples were stored at –80 °C for subsequent metabolite analysis. Each sample was then used to profile two metabolite categories: polyphenols and VOCs.

2.2. Polyphenolic analysis

Polyphenols were extracted following the protocol reported by Busatto et al. (2021). Briefly, 100 mg of frozen apple powder was extracted with water/methanol/chloroform (20:40:40) solution for two times. The final extract was dried under nitrogen flow, resuspended with 500 uL of the water/methanol (1:2) mixture and transferred to High-Performance Liquid Chromatography (HPLC) vials. Before injection, samples were stored at –20 °C. The Ultraperformance liquid chromatography (UPLC) was carried out through a Waters Acquity UPLC system (Milford, MA), equipped with a Waters Acquity HSS T3 column 1.8 µm, 100 mm × 2.1 mm (Milford, MA, USA). The UPLC system was coupled with a mass spectrometer, the Water Xevo TQMS (Milford, MA, USA) in ESI ionization mode, as described in Busatto et al. (2018). Capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode and MRM conditions (precursor and product ions, quantifiers and qualifiers, collision energies,

and cone voltages) were optimized for the analysis of the samples as described in Vrhovsek et al. (2012).

2.3. Volatile organic compounds analysis (VOCs)

The VOCs analysis of the two apple tissues (skin and pulp) was performed through a commercial PTR ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria; Mazzucotelli et al., 2022) following the methodology described in Farneti et al. (2015). Each tissue was analyzed in triplicates, and 0.5 g of frozen powder were weighted in a 20 mL glass vial equipped with PTFE/silicone septa (Agilent, Cernusco sul Naviglio, Italy) and mixed with 0.5 mL of deionized water, 200 mg of sodium chloride, 2.5 mg of ascorbic acid, and 2.5 mg of citric acid. Each measurement was conducted automatically after 20 min of sample incubation at 40 °C by using an adapted gas chromatography (GC) autosampler (MPS Multipurpose Sampler, GERSTEL), and 1 min of time between each measurement was applied to prevent the memory effect. The sample headspace was withdrawn with the 2.5 mL syringe (CTC Analytics AG, Zwingen, Switzerland) and injected into the static headspace module (Ionicon Analytik GmbH, Innsbruck, Austria). The flow of zero air inside the static headspace module was 90 $cm^3 \cdot min^{-1}$, and the syringe was injected with the speed 250 $\mu L \cdot s^{-1}$, which provoked a 7 fold dilution of the sample. The injection of the headspace mixture into the PTR-ToF-MS drift tube allowed the measurement of the VOCs. Temperature, pressure and voltage of the drift tube; E/N ratio; ToF acquisition, were set up as described by Di Pierro et al. (2022).

2.4. Data analysis

Polyphenolic data were processed using the Waters MassLynx 4.1 and TargetLynx software (Vrhovsek et al., 2012). VOC spectral data obtained from the PTR-ToF-MS was analyzed following the procedure outlined in Farneti et al. (2017). The array of mass peaks detected with PTR-ToF-MS was refined by applying noise and correlation coefficient thresholds. Initially, mass peaks not significantly different from blank samples were removed. Mass peaks with over 99% correlation were excluded, as these mostly corresponded to isotopes of monoisotopic mass peaks (Farneti et al., 2017). Log transformed data were utilized to perform multivariate statistical analysis through the software R 4.3.2 and its external packages “mixOmics” for Principal Component Analysis (PCA) and “ggplot2” for their visualization. “ComplexHeatmap” and “dendextend” were utilized for the creation and visualization of the heatmaps and relative dendrograms. For the analysis of variance performed to define difference statistically significant a *P*-value threshold of 0.05 was considered.

3. Results

3.1. Polyphenolic variability assessment using Principal Component Analysis

The pulp and skin tissues from 163 apple accessions were analyzed to assess the polyphenolic concentration (Table S1). Targeted analysis identified 19 different compounds, grouped into 7 main classes: dihydrochalcones, flavanols/flavan-3-ols, flavonols, hydroxycinnamic acid, phenolic acid, phenylpropanoid, and stilbenes. Polyphenolic concentration was evaluated using a multivariate statistical approach through principal component analysis, which distributed the samples across a 2D-PCA scatter plot defined by two principal components (PCs), together explaining 61.68% of the total metabolic variability (Fig. 1, A). The first PC (PC1), explaining 35.17% of the total variability, clearly separated the two tissues. Skin samples from both *M. domestica* and wild *Malus* spp. accessions clustered in the positive PC1 area of the plot, while pulp samples from the same accessions were instead positioned in the negative PC1 area. The second principal component (PC2), accounting for 26.51% of the total variability, primarily distinguished between species. Most *M. domestica*

accessions clustered in the positive PC2 area, while wild *Malus* spp. accessions were predominantly located in the negative PC2 area. The distribution of the set of samples illustrated in the PCA, derived by the specific accumulation patterns of each polyphenolic compound, as shown in the correlation circle variable plot (Fig. 1, B). All compounds were located in the positive PC1 area, indicating a general quantitative distribution of polyphenols. In contrast, PC2, explained, instead, a qualitative distinction among the compounds. The two radians of the correlation variable plot indicate which compounds accounted for most of the variability for each principal component. In the positive quadrant of PC2, characterizing the skin tissue of *M. domestica* accessions, were identified compounds including vanillic acid, coniferyl alcohol, kampferol-3-rutinoside, *cis*-piceide, arbutin, rutin, isorhamnetin-3-glc, quercetin-3-rhamnoside, and quercetin-3-glucoside + quercetin-3-galactoside (as quercetin-3-glucoside). In the PC2 negative area of the plot, distinguishing instead skin tissue of *Malus* spp. accessions were projected vanillin, t-piceide, neochlorogenic acid, cryptochlorogenic acid, chlorogenic acid, phlorizin, catechin, epicatechin, procyanidin B1 and procyanidin B2+B4. The compound positions within the two radii further defined their contribution to the metabolite variability, with the first radius explaining 0–50% of the variability, and the second radius explaining 50%–100% (Fig. 1, B). Compounds like quercetin, rutin, arbutin, phlorizin, catechin, epicatechin and procyanidins, were positioned in the high-variability zone of the core-plot, emerging therefore as key contributors of the sample distribution.

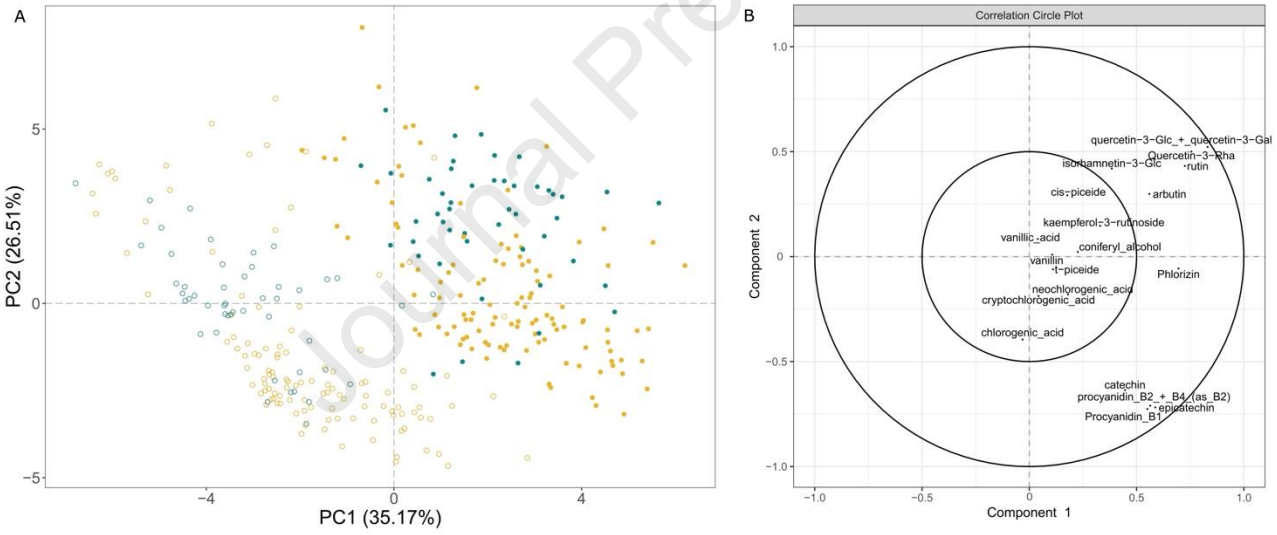

Fig. 1 Analysis of phenolic compound variability by principal component analysis

(A) In the PCA plot is illustrated the distribution of each sample depicted by color (blue: *M. domestica*, yellow: *Malus* spp.) and types of dots (full: skin tissue, empty: pulp tissue). (B) The contribution of each phenolic compound is instead reported in the correlation circle variable plot defined by the two radii.

3.2. Phenolic compound assessment across the Malus genus

The concentration of the 19 polyphenolic compounds detected in the two tissues, pulp and skin, was visualized through hierarchical heatmaps (Fig. 2, A and B) that revealed a substantial variability in compound concentration among the 163 accessions. The heatmaps evidenced an overall higher metabolite accumulation in the skin compared to the pulp. In pulp tissue, the most abundant compounds were chlorogenic acid, procyanidin B2 + B4 (identified as B2) and epicatechin (Fig. 2, A; Fig. S1). Together with phlorizin, these compounds were also abundant in the skin (Fig. 2, B; Fig. S2). Notably, chlorogenic acid exhibited higher concentrations in wild *Malus* spp. compared to the *M. domestica* accessions, with a fold change of 1.80 in pulp and 1.93 in skin. Interestingly,

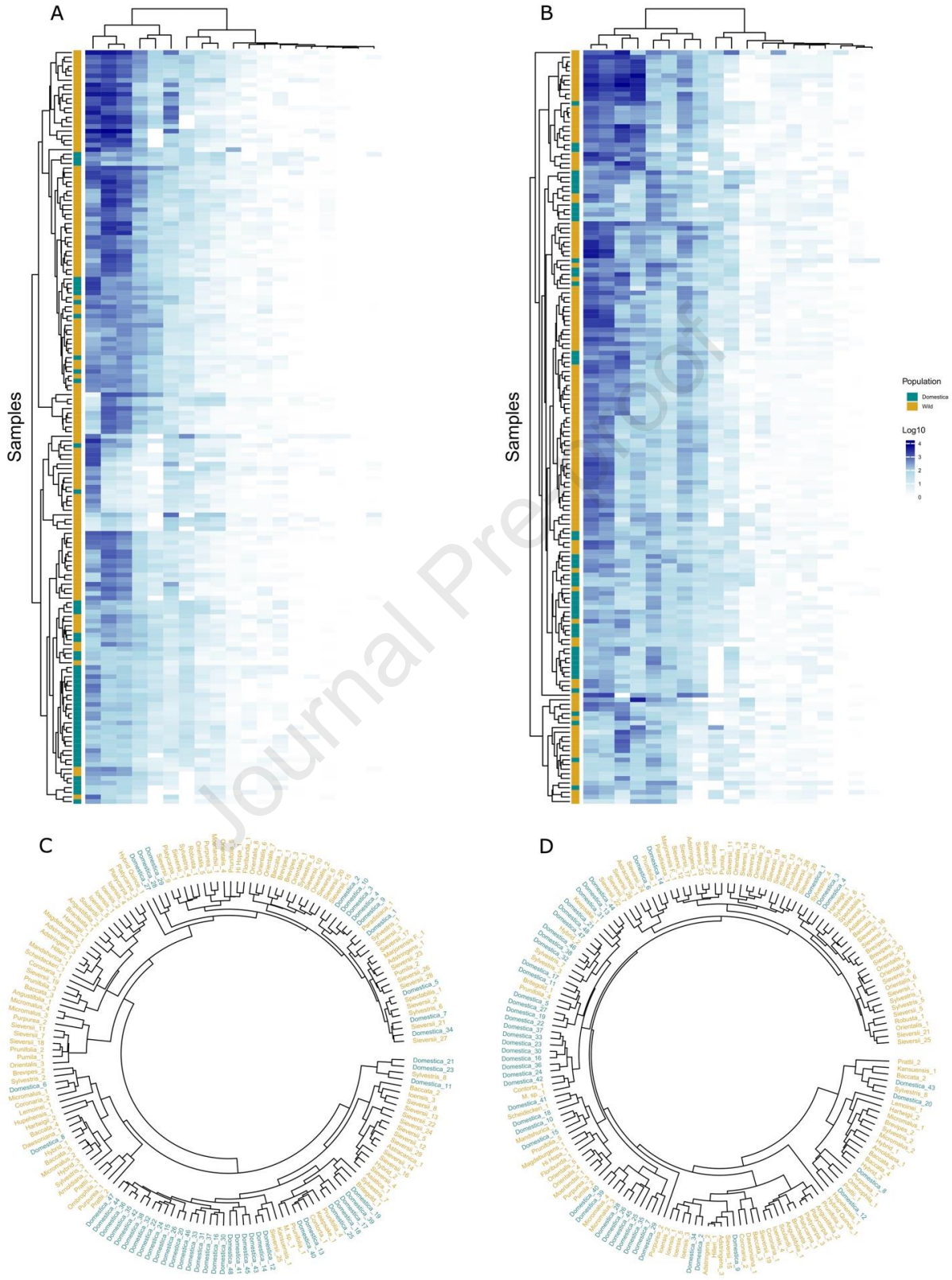

Fig. 2 Heatmap of polyphenols concentration in apple pulp and skin

(A, B) Concentration pattern of phenolic compound visualized by heatmap for pulp (panel A) and skin (panel B). The accumulation pattern is reported in

logarithmic scale. Samples were clustered and indicated by color on the left side of each heatmap, with blue and yellow color for *M. domestica* and *Malus* spp., respectively. (C, D) The clustering of the accessions is also magnified and reported in the circular dendrogram reported in panel C and D for the two types of tissues, respectively. The identity of each accession is reported in Figs. S1–S4.

this polyphenolic compound was more concentrated in the pulp tissue compared to skin, with a fold change of 1.35 and 1.26, for *M. domestica* and *Malus* spp., respectively. Procyanidin B2 + B4 distinctly distinguished wild *Malus* spp. from *M. domestica*, with an average concentration 8.04 times higher in pulp and 4.43 times higher in skin. When comparing tissues within the same species, the fold changes were 1.83 for *M. domestica* and 1.00 for wild *Malus* spp (skin/pulp). Epicatechin followed a similar trend, with fold changes of 4.39 and 2.58 for the pulp and skin tissues, respectively. Tissue-specific differences within each species were smaller, showing a fold change of 1.73 in *M. domestica* and 1.02 in wild *Malus* spp. (skin/pulp). Phlorizin resulted to be a polyphenolic compound particularly associated with the skin tissue of both groups. In the *M. domestica* accessions, phlorizin was 7.84 times more concentrated in the skin than the pulp. Similarly, the skin tissue of the wild *Malus* spp. was concentrated 5.40 times than the pulp tissue. It is worth noting that in both tissues of the wild *Malus* spp. this compound was more concentrated than their *M. domestica* counterpart, with fold changes of 7.02 for the pulp and 4.84 for the skin tissue, respectively. Kaempferol-3-rutinoside, vanillin, and vanillic acid were the least concentrated in both tissues. Despite its low concentration, kaempferol-3-rutinoside showed remarkable tissue-specific differences. *M. domestica* skin had three orders of magnitude more than pulp, while in wild *Malus* spp. the difference between tissues was less extreme but still significant. Vanillin and vanillic acid were characterized by a similar accumulation pattern. In wild *Malus* spp., tissue differences were minimal, with fold changes of 0.85 for vanillin and 0.46 for vanillic acid (skin/pulp). Conversely, *M. domestica* accessions showed a marked accumulation in the skin tissue, with fold changes of 6.60 for vanillin and 14.89 for vanillic acid.

3.3. Similarity relationship based on phenolic concentration within the interspecific apple collection

The metabolic profile of the analyzed polyphenolic compounds was employed to cluster the accessions through a similarity dendrogram based on hierarchical clustering. In both pulp and skin tissues, the dendrograms (Fig. 2, C and D; Figs. S3 and S4) revealed a clear separation between *M. domestica* and wild *Malus* spp. accessions. For pulp tissues, the circular dendrogram identified a specific cluster containing half of the analyzed *M. domestica* accessions (24 out of 48), characterized by a total polyphenol concentration ranging from 14.32 $\mu g \cdot g^{-1}$ to 54.58 $\mu g \cdot g^{-1}$. *M. domestica* accessions with higher averaged polyphenol concentration (from 61.64 $\mu g \cdot g^{-1}$ to 173.98 $\mu g \cdot g^{-1}$) clustered separately from this group and resulted more spread over the dendrogram plot showing concentration of phenolic compounds more similar to *Malus* spp. accessions. The circular dendrogram representing the similarity relationship among the accessions based on the polyphenolic accumulation in the skin showed a less defined grouping compared to the pulp tissue. From the main cluster of 24 *M. domestica* accessions identified in the pulp dendrogram, only seven accessions clustered together also in the skin dendrogram, with a polyphenolic concentration between 35.85 $\mu g \cdot g^{-1}$ and 67.88 $\mu g \cdot g^{-1}$. The ten *M. domestica* accessions with higher polyphenolic concentration, thus metabolically closer to the wild *Malus* spp. accessions, showed concentrations ranging from 118.77 $\mu g \cdot g^{-1}$ to 232.75 $\mu g \cdot g^{-1}$ in the skin tissue and resulted scattered across the dendrogram. Eight of these accessions also exhibited high polyphenolic concentration in the pulp. In the profiles of both tissues, pulp and skin, the three *M. domestica* accessions with the highest phenolic compound concentrations were ‘Ard Cairn Russet’, ‘Biesterfelder Renette’, and ‘Discovery’. On the opposite, the accessions with the lowest phenolic concentration were ‘William Crump’, ‘Prinses Marijke’, and ‘McIntosh’.

Three key polyphenolic compounds, namely phlorizin, catechin and chlorogenic acid, known for their nutraceutical benefits, were specifically analyzed to illustrate their accumulation patterns across the apple collection (Fig. 3, A, B, C; Fig. S5). Phlorizin showed a predominant accumulation in the skin tissue of both *M. domestica* and wild *Malus* spp., with concentrations ranging from 18.07 $\mu g \cdot g^{-1}$ to 6943.47 $\mu g \cdot g^{-1}$ in the wild *Malus* spp. skin tissue. The species with the highest skin phlorizin concentration were 'Soulardii', 'Angustifolia' and 'Bracteata', with 'Soulardii' displaying a concentration more than double compared to the others. 'Contorta', 'Asiatica' and 'Kansuensis' had instead the lowest phlorizin concentration in the skin tissue. *M. domestica* showed a mean skin concentration value of 105.74 $\mu g \cdot g^{-1}$. In the pulp, phlorizin concentration was significantly lower, with wild *Malus* spp. ranging from 1.37 $\mu g \cdot g^{-1}$ to 931.72 $\mu g \cdot g^{-1}$ and *M. domestica* averaging 13.49 $\mu g \cdot g^{-1}$. Catechin exhibited a more balanced distribution between skin and pulp across both species' groups. Wild *Malus* spp. ranged from 0 to 297.70 $\mu g \cdot g^{-1}$ in the skin and from 0 to 203.77 $\mu g \cdot g^{-1}$ in the pulp. 'Pumila' and 'Paradisiaca' were the wild species with the highest catechin concentration, while 'Ombrophila', 'Prattii', 'Hupehensis' and 'Dawsoniana' showed the lowest. In *M. domestica*, mean catechin concentrations were 31.55 $\mu g \cdot g^{-1}$ in the skin and 27.23 $\mu g \cdot g^{-1}$ in the pulp. Chlorogenic acid, unlike the other two compounds, was more accumulated in the pulp than the skin. Wild *Malus* spp. accessions showed a mean concentration of 799.12 $\mu g \cdot g^{-1}$ in the pulp and 635.15 $\mu g \cdot g^{-1}$ in the skin. In *M. domestica* concentrations were instead lower: 444.20 $\mu g \cdot g^{-1}$ in the pulp and 328.73 $\mu g \cdot g^{-1}$ in the skin. Among wild species 'Angustifolia', 'Coronaria' and 'Ioensis' showed the highest pulp concentrations, while 'Ombrophila', 'Prattii' and 'Purpurea' had the lowest. It is also interesting to highlight the peculiar accumulation pattern of a specific set of phenolic compounds observed for a hybrid quince included in this collection. Quince (*Cydonia oblonga*) is another *Rosaceae* species sharing, together with pear, the same chromosomic number of apple (2n = 2x = 34). The pulp of this hybrid showed an exceptionally high concentration of neochlorogenic acid, cryptochlorogenic acid, chlorogenic acid and rutin, with a fold change regarding the rest of the accessions included in the collection, ranging from 2.65 to 186.3. The skin tissue, in addition to these compounds, resulted to be particular rich also in quercetin, querceting-3-glc and kampferol, with a fold change ranging from 3.3 to 355 (Table S1 and Fig. S5).

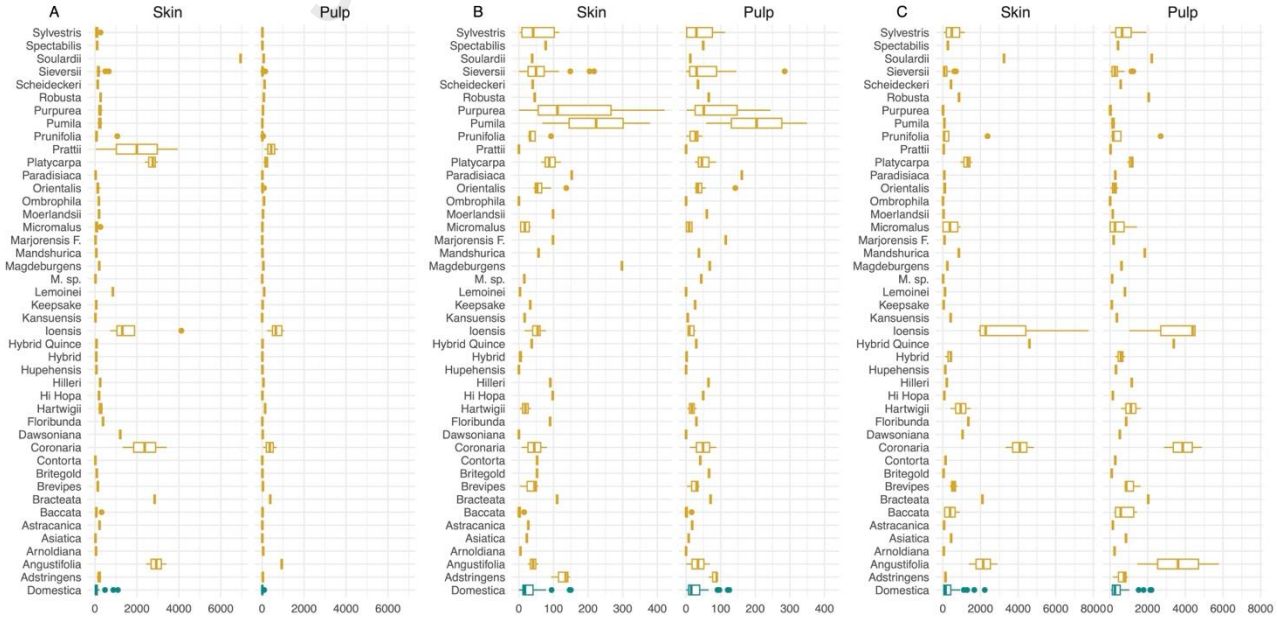

Fig. 3 Box plot indicating the accumulation pattern for each species for phlorizin (A), catechin (B) and chlorogenic acid (C)

Yellow color indicates *Malus* spp., while *Malus domestica* is highlighted in blue color. Data are reported in $\mu g \cdot g^{-1}$.

3.4. VOCs variability assessment using Principal Component Analysis

The whole VOC spectra of each apple accession, assessed in triplicate for each accession by PTR-ToF-MS, were reduced to 170 VOC mass peaks (Tables S2 and S3), applying noise and correlation coefficient thresholds. Tentative identification of each mass peak detected by PTR-ToF-MS relied on an in-house library of chemical standards, and on the list of compounds detected by SPME/GC-MS analysis in previous experiments on apple fruit (Farneti et al., 2015). The multivariate analysis of VOCs in the pulp and skin tissues of the 163 *Malus* accessions was carried out using PCA, like in the assessment of the polyphenolic concentration (Fig. 4, A). The VOC analysis generated a 2D-PCA plot with the first two principal components (PCs) accounting for 66.25% of the total variability. The first PC (PC1), explaining 48.99% of the volatilome variability, clearly separated the two apple groups: wild *Malus* spp. and *M. domestica*. Wild *Malus* spp. accessions, regardless of the analyzed tissue, clustered predominantly in the negative area of PC1, while *M. domestica* accessions were mainly distributed in the positive area of PC1. The second PC (PC2), accounting for 17.26% of the total variability, differentiated the two tissues. Skin tissue samples were mostly located in the positive area of PC2, while pulp tissue samples were primarily positioned in the negative. The projection of each VOC, contributing to the distribution of samples in the PCA, is visualized in the correlation circle variable plot (Fig. 4, B). Most of the VOCs clustered in the quadrant corresponding to the positive area of PC1, aligning with the position of *M. domestica* accessions. It is also worth noting that the highest proportion of the volatilome assessed (140 compounds over 170) clustered into the second radius of the corr plot, explaining from 50% to 100% of the entire variability of the volatile compounds. In the group of *M. domestica* accessions, the skin tissue was represented by a slightly higher number of VOCs with regards to the pulp tissue.

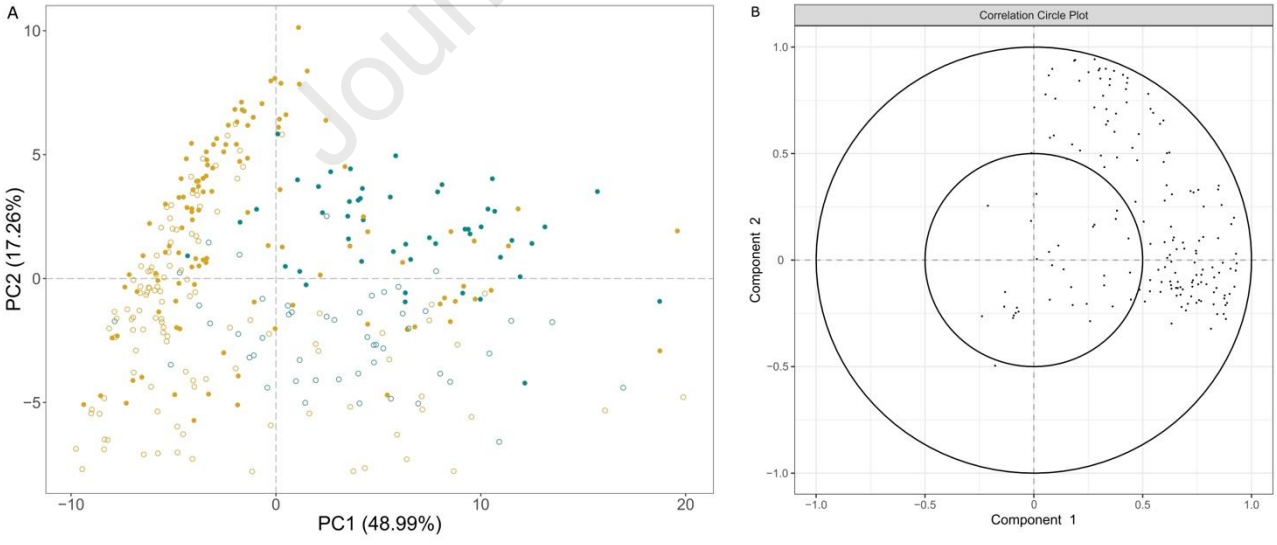

Fig. 4 Analysis of volatile organic compound (VOC) variability by principal component analysis

(A) In the PCA plot is illustrated the distribution of each sample depicted by color (blue: *M. domestica*, yellow: *Malus* spp.) and types of dots (full: skin tissue, empty: pulp tissue). (B) The contribution of each VOC compound is instead reported in the correlation circle variable plot defined by the two radii.

3.5. Heatmap visualization of VOCs variability

The VOC concentration in the two tissues of the 163 samples was visualized using the hierarchical heatmap

profiles (Fig. 5, A, B; Figs. S6 and S7), for pulp and skin tissue, respectively. In both tissues, the most concentrated VOC resulted to be the mass m/z 45.034 (Acetaldehyde), with an average concentration of 2486.06 $\mu g \cdot m^{-3}$ n pulp and 2332.44 $\mu g \cdot m^{-3}$ in skin. The lowest concentrated compound differed between tissues, m/z 189.162 ($C_{14}H_{21}^{+}$), was the lowest in the pulp (0.032 $\mu g \cdot m^{-3}$), while m/z 156.105 (unknown), had the lowest concentration in skin (0.028 $\mu g \cdot m^{-3}$). Despite these differences, the two tissues shared a similar accumulation pattern, with 18 mass peaks highly concentrated in both, including mass peaks m/z 33.0342 (Methanol), 39.0247 (Common fragment), 41.0387 (Common fragment), 43.0183 (Ester fragment), 43.0547(Alcohol fragment), 45.0340 (Acetaldehyde), 47.0500 (Ethanol), 55.0544 (Butenol), 57.0337 (Acrolein), 57.0696 (Butanol), 59.0491 (Acetone), 61.0289 (Acetic acid or ester fragment), 71.0847 (Methyl-butanol and Pentanol), 79.0527 (Benzene), 81.0695 (Monoterpene fragment), 83.0853 (3-Hexen-1-ol), 89.0602 (Ethyl-acetate) and 99.0790 (Hexenal). The pulp was also distinguished by the high concentration of m/z 107.0846 (Ethylbenzene). The listed compounds showed however a different behavior between the two *Malus* groups. Most of the highest accumulated compounds resulted in fact more concentrated in the *M. domestica* accessions compared to *Malus* spp. accessions, exception made for m/z 47.0500 (Ethanol), 79.0527 (Benzene) and 81.0695 (Monoterpene fragment), which were more abundant in wild accessions. The most concentrated VOC, m/z 45.0340 (Acetaldehyde), averaged 3513.96 $\mu g \cdot m^{-3}$ in *M. domestica* pulp and 3107.35 in the skin, with a fold change of 1.13. Interestingly, among the list of the 18 highest accumulated mass peaks, the largest difference in VOC concentration between tissues was found for m/z 43.0183 in wild *Malus* spp., with a 1.62 fold change for the pulp/skin comparison, while *M. domestica* had a more pronounced 3.91 fold change for m/z 81.0695 in the skin/pulp comparison. The similarities among the accessions, based on VOC profiles, were visualized through a hierarchical clustering plot, showing a comparable organization between *M. domestica* and wild *Malus* spp. for both tissues (Fig. 5, C, D; Figs. S8 and S9). The circular dendrogram of the pulp tissue (Fig. 5, C; Fig. S8) separated *M. domestica* into two main groups. While the first group composed by 32 accessions clustered together, the second group of 16 accessions was closer to the wild *Malus* spp. accessions and spread over the dendrogram. The largest cluster within *M. domestica* featured 8 accessions, with a mean VOC concentration ranging from 12108.49 $\mu g \cdot m^{-3}$ to 40634.49 $\mu g \cdot m^{-3}$. The VOC accumulation pattern in the skin tissue, illustrated through circular hierarchical clustering (Fig. 5, D; Fig. S9), showed a distinct grouping of *M. domestica* and wild *Malus* spp. accessions. Only seven *M. domestica* accessions clustered together with the wild *Malus* spp., while the other 41 *M. domestica* accessions were instead clustered together in distinct groups. The major cluster found in this circular dendrogram was composed by 8 *M. domestica* accessions commonly found also in the previously described cluster represented by 12 *M. domestica* accessions for the pulp circular dendrogram. The average minimum value of this cluster was 16839.75 $\mu g \cdot m^{-3}$ to 40954.33 $\mu g \cdot m^{-3}$. The *M. domestica* accessions showing the highest VOC concentration in both pulp and skin tissues were 'Discovery', 'Aroma' and 'Red Ingrid Marie'. The *M. domestica* 'Discovery' was also characterized by the highest concentration of polyphenolic compounds.

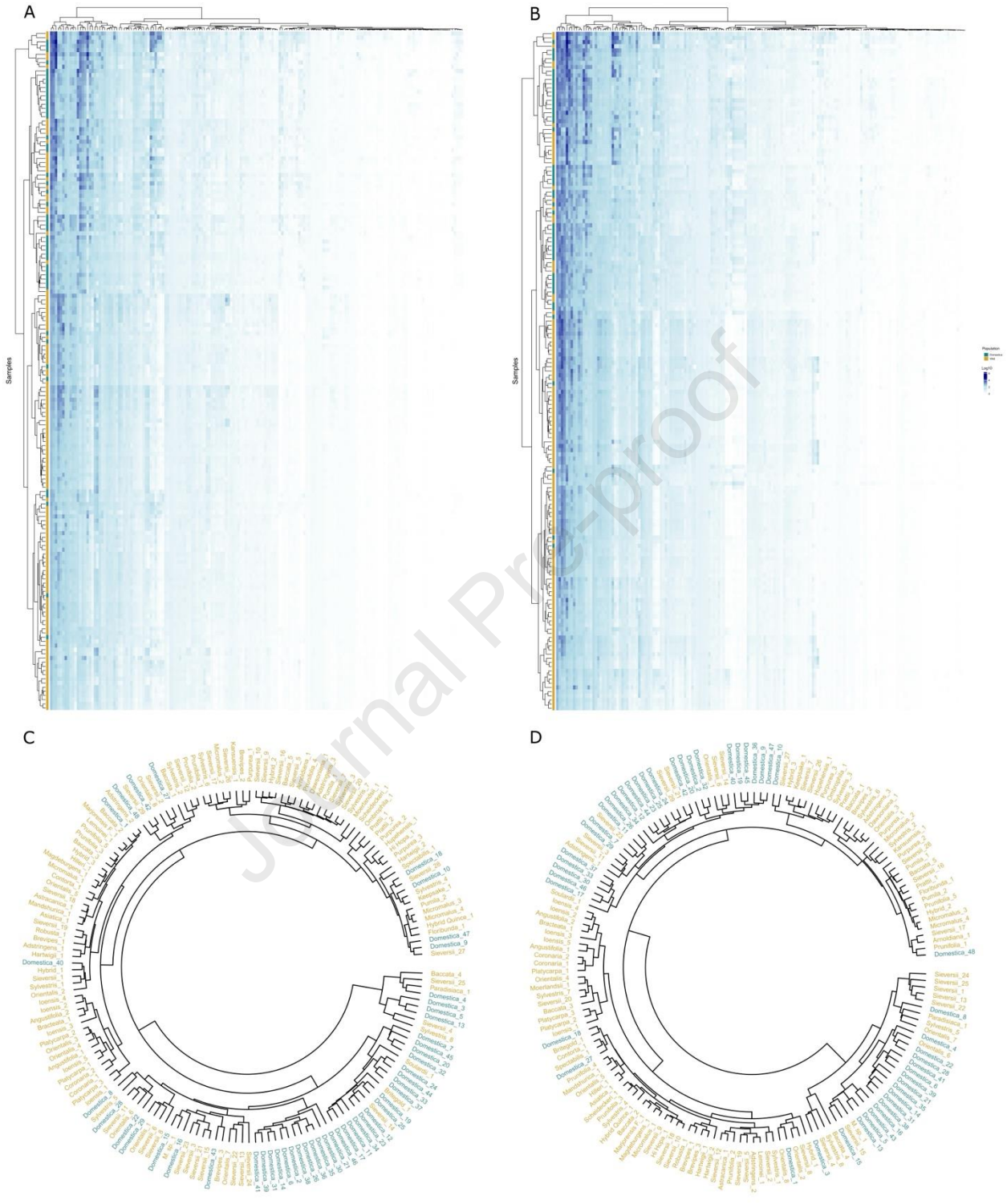

Fig. 5 Heatmap of VOC concentration in pulp and skin

(A, B) Accumulation pattern of volatile organic compounds (VOC) visualized through heatmaps for pulp (A) and skin (B). The accumulation pattern is reported in logarithmic scale. Samples were clustered and indicated by colors on the left side of each heatmap, with blue and yellow color for *Malus domestica* and *Malus* spp., respectively. (C, D) The clustering of the accessions is also magnified and reported in the circular dendrogram reported in panel C and D for the two types of tissues, respectively. The identity of each accession is reported in Figs. S6–S9.

3.6. Pattern of VOC accumulation

The pattern of the entire volatilome, composed of 170 mass peaks assessed across 163 *Malus* accessions was organized into nine distinct accumulation patterns based on which species and tissue showed the highest VOC accumulation (Fig. 6). Among the several profiles, the group of three models indicated whether the highest accumulation was observed in the wild *Malus* spp. (Fig. 6, A, B, C), with an equal concentration between the two groups (Fig. 6, D, E, F) or with a higher accumulation in the *M. domestica* accessions (Fig. 6, G, H, I). Each of these groups was further refined by the type of tissue, showing whether the accumulation was greater in the pulp (Fig. 6, A, D, G), in the skin (Fig. 6 C, F, I) or evenly distributed between the two tissues (Fig. 6, B, E, H). The first model (Fig. 6, A) comprised cases where wild *Malus* spp. displayed a higher concentration in the pulp tissue. This pattern included 8 mass peaks among the 170 analyzed (Table S4). The second model (Fig. 6, B) showed wild *Malus* spp. accessions with consistently higher VOC concentrations across both tissues, though this group was smaller, with only 3 mass peaks. The third model (Fig. 6, C) reflected a more skin-focused accumulation in wild *Malus* spp. represented by just 2 mass peaks. The fourth model, with the pulp tissue being the most concentrated in both groups of *Malus* spp. (Fig. 6, D) was represented by 8 mass peaks, while the fifth model (Fig. 6, E), illustrated a balanced VOC concentration between the two tissues, equally distributed between wild and domesticated accessions, and included another set of 8 mass peaks. The sixth model (Fig. 6, F) was represented by 16 mass peaks, with the skin tissue showing the highest accumulation in both *Malus* groups. Most of the volatile compounds (125 mass peaks over 170) showed instead an accumulation profile distinguished by a higher concentration in the tissues collected from the *M. domestica* accessions. The seventh model (Fig. 6, G), represented by 11 mass peaks, highlighted higher VOC concentrations in the pulp tissue. The eighth model (Fig. 6, H) showed an even distribution between pulp and skin tissues and was notably larger, with 47 mass peaks. Lastly, the ninth model (Fig. 6, I) was the most prominent group, comprising 67 mass peaks, where VOCs were most concentrated in the skin tissue of *M. domestica* accessions.

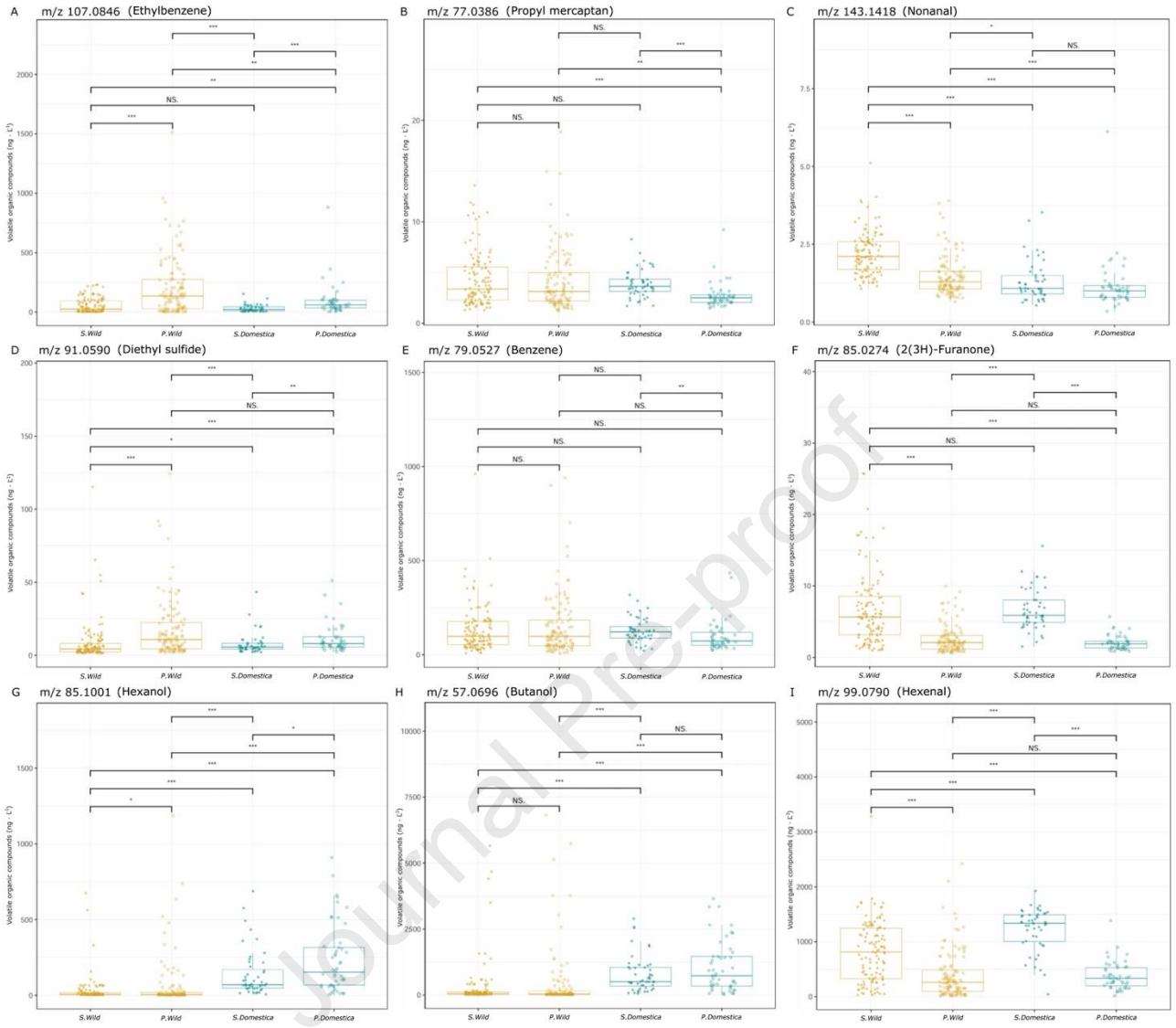

Fig. 6 Volatile organic compounds (VOC) pattern of nine specific mass peaks representing the volatilome accumulation profiles mainly detected within the collection

In each plot yellow color is for wild *Malus* spp., while blue indicated the *M. domestica* accessions. Full and empty dots indicate skin and pulp tissues, respectively. Differences among each compound were defined according to the analysis of *t*-test. NS.: not significant, *: *P*-value < 0.05, **: *P*-value < 0.01, ***: *P*-value < 0.001. The mass peaks included in each model are specified in Table S4.

4. Discussion

4.1. Polyphenolic compounds are more concentrated in the wild Malus spp. accessions than in Malus domestica

The targeted analysis of 19 polyphenolic compounds revealed that wild *Malus* spp. accessions generally contains higher concentration of these metabolites than the *M. domestica* accessions. However, the Principal Component Analysis, revealed as the major distinction emerged between the two tissues singularly assessed in this survey, skin and pulp, rather than between species, as explained by the first principal component (PC1). The distinction between wild and domesticated apples accessions only emerged along the second component (PC2), which accounted for a smaller proportion of the total variance. Beyond quantitative differences, it is also interesting to note as the skin of the two groups of species was also distinguished by a qualitative organization of

different metabolites. The skin tissue of the *M. domestica* accessions was in fact mainly characterized by quercitin, arbutin and rutin, whereas M*alus* spp. exhibited higher concentration of catechin, procyanidin B1 and B2+B4. This pattern aligns with previous studies suggesting a link between water content and polyphenol concentration. Specifically, apple skin tends to have less water (Kaeswurm et al., 2022), and because domesticated apples are typically larger and more hydrated — a result of selective breeding — the water content likely dilutes polyphenol concentrations. The different concentration observed among the accessions included in this large apple collection is also consistent with the observation of Khan et al. (2014) and Busatto et al. (2019). In this survey, a slight correlation between the fruit size and the metabolite concentration was in fact observed for specific phenolic compounds in the two types of tissues. For the skin tissue, compounds like phlorizin, epicatechin, procyanidin B1 and B2+B4 showed correlation values between –0.23 and –0.44. In the pulp, the same compounds, alongside chlorogenic acid and quercitin, also showed negative correlations with values ranging from –0.24 to –0.46. It is worth noting as the number of phenolic compounds negatively correlated with the size are higher in the pulp than skin, and these phenols were also more relevant in the contribution of the functional quality properties in apple. Although the concentration of the phenolic compounds has been heavily reduced through the domestication process because of their astringency, recent breeding programs are seeking to re-increase the concentration of these metabolites due to their already established beneficial role on the human health (Boyer and Liu, 2004). The nutraceutical role of polyphenols as antioxidant, scavenging ROS and free radicals, elevated these molecules to essential determinants for the promotion of the functional quality of fruits, important to prevent the development of chronical diseases (Vauzour et al., 2010). The red-flesh apple represents one of the most important successes in this regard. This property, already present in wild germplasm collection, was for long time selected out from breeding program due to its scarce appreciation. Because the red color is fundamentally dependent on the high concentration of anthocyanins, known for their antioxidant activity (Butkeviciute et al., 2022), wild red-flesh accessions have been recently selected as parental lines in recent breeding programs to reintroduce functional traits. This trend reflects a broader shift in breeding strategies, which now incorporate genetic resources from gene banks, such as landrace and crop wild relatives to recover valuable traits lost during domestication, with the final goal to increase resistance, resilience and nutraceutical properties (McCouch, 2013; Alseek et al., 2021). However, this strategy must carefully balance the benefit of increasing genetic diversity with the potential loss of agronomically desirable traits achieved through decades of selective breeding. The extensive survey carried out on this apple collection enabled the description of the accumulation pattern in a diverse panel of apple accessions and identified *M. domestica* accessions distinguished by an enhanced polyphenolic content, close to the concentration observed in the group of wild accessions. These promising individuals, combining high polyphenolic content with a domesticated genetic background, represent valuable breeding material. Within the collection investigated in this work, three varieties have been identified as the most promising due to their specific polyphenolic concentration, such as ‘Discovery’, ‘Ard Cairn Russet’ and ‘Biesterfelder Renette’. It is worth noting the high polyphenolic concentration in the last two varieties, which are specifically distinguished by a russeted skin. The high concentration of these molecules in cultivars showing this characteristic have been already reported by other authors, validating these findings (Khanizadeh et al., 2007; Marks et al., 2007; Mullen et al., 2007; Busatto et al., 2019). The biosynthesis of suberin in response to an impaired accumulation of cuticle can determine an initial process of water loss for a high permeability of this type of polymer, contributing to a

partial concentration of these molecules with regards to other varieties with a more complete cuticle structure (Hen-Avivi et al., 2014; Lashbrooke et al., 2015, 2016).

4.2. The VOCs are predominantly emitted in Malus domestica accessions

VOCs represent another essential category of secondary metabolites playing a key role in fruit metabolism, contributing significantly to fruit quality. VOCs are responsible for the distinct aromas typical of fruits, and their production and composition shifts considerably through the fruit's maturation and ripening process (Dudareva et al., 2004). In apple, most VOCs originate from fatty acid and amino acids, which are subsequently transformed into aldehydes, alcohols and esters. These classes of compounds are specifically accumulated in distinct phase of the fruit physiology, with aldehydes predominantly accumulated in pre-ripening stage and alcohols and esters more specifically produced towards the full ripening (Espino-Diaz, 2016). This distinct accumulation pattern influences frugivore behavior, attracting animals when seeds are mature and suitable for dispersal (Dudareva et al., 2013; Rodriguez et al., 2013; Brosset and Blande, 2021). In contrast to polyphenols, the overall VOC profile effectively differentiated *M. domestica* from *Malus* spp. (rather than tissue), as shown by PCA results, with PC1 explaining 48.99% of the variance (Fig. 4, A). Examination of the complete VOC profile revealed that 74% of these compounds were more abundant in *M. domestica* tissues, especially esters that contribute to the fruit's pleasant aroma. Only 8% of VOCs showed higher accumulation in wild species. This observation highlights a contrasting evolutionary trend for the two classes of metabolites. While phenolic compounds decreased significantly during domestication, VOCs were fundamentally re-programmed and enhanced. This divergent pattern can be attributed to their role in attracting seed-dispersing organisms. Moreover, it has been revealed that esters can contribute to the sweetness perception by consumers (Aprea et al., 2017). Therefore, increased aroma production in *M. domestica* can be the result of an initial domestication operated initially by animals and thereafter through artificial selection of breeding activities oriented to improve quality features.

4.3. The process of domestication and breeding has reshaped the secondary metabolite background in apple

The domestication process in apple began in the area of Tian Shan, in Central Asia, and progressed westward along the Silk Road towards Europe and North America. The travel of humans and animals facilitated the hybridization amongst several apple species, in particular *M. sieversii* with *M. baccata, M. orientalis* and *M. sylvestris* (Harris et al., 2002; Cornille et al., 2012; Duan et al., 2017), ultimately resulting in the modern *M. domestica*. The human driven domestication and the subsequent breeding shaped therefore the features of the modern apple varieties. During this process, the entire metabolome of apple experienced important changes and modification, especially in the two main categories of metabolites investigated in this work. The domestication process prioritized palatability and yield, reducing phenolic compounds responsible for bitterness and stringency favoring increased sweetness or size (Toivonen et al., 2006; Soares et al., 2013; Sun et al., 2017; Davies et al., 2022). Beside negatively impacting fruit quality, polyphenolic compounds, however, contributed to the resistance mechanisms against several pathogens (Sun et al., 2017), and their reduction in domesticated accessions may underline the increased susceptibility of the *M. domestica* accessions (Drewnoski et al., 2001; Huang and Xu, 2021; Wu et al., 2021). On the contrary, the volatilome experienced an increase in both diversity and concentration. This divergent pattern is evident in the PCA plots. While the plot generated with the phenolic data revealed as the major contribution was associated to the type of tissue (with the wild accession playing a more relevant role), for the VOCs, the major determinant was represented by the *M. domestica* accessions (Fig.

4, A and B). This different pattern was supported by the role that certain VOCs have in attracting frugivores or seed dispersing animals. The aromatic bouquet, together with color, are the two main features developed by fruit to attract frugivores with the final goal to disperse the seeds contributing to the spread of the species over time and space (Valido et al., 2011). Moreover, the initial guided selection that occurred during domestication was performed by mammals using mostly the olfactory sense for the detection of ripe fruit rather than visual cues, due to their chromatic vision (Veilleux et al., 2001; Bicca-Marques and Garber, 2004; Corlett, 2011; Rodriguez et al., 2011). With contemporary climate change and increasing interest in functional foods, previously eliminated traits like high phenolic content are now valued for their antioxidant properties and health benefits. While crop wild relatives offer valuable genetic resources for agronomic improvement, especially considering the high recurrence of a few founders in the pedigrees of modern cultivars (Noiton and Alspach, 1996), breeding with non-domesticated materials may compromise quality traits achieved through domestication and human guided advanced selection. In particular, the loss of the high storability trait, acquired through a century of modern breeding would be especially detrimental to new elite varieties, potentially increasing food waste due to the poor storability typical of wild species. Our investigation identified several *M. domestica* accessions with phenolic profiles comparable to wild *Malus* species, representing promising materials for breeding programs aimed at enhancing these compounds while preserving the domesticated genetic background, potentially facilitating more efficient introgression of agronomically valuable traits.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.hpj.2025.

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Declaration of competing interest

The authors declare that they have no competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.