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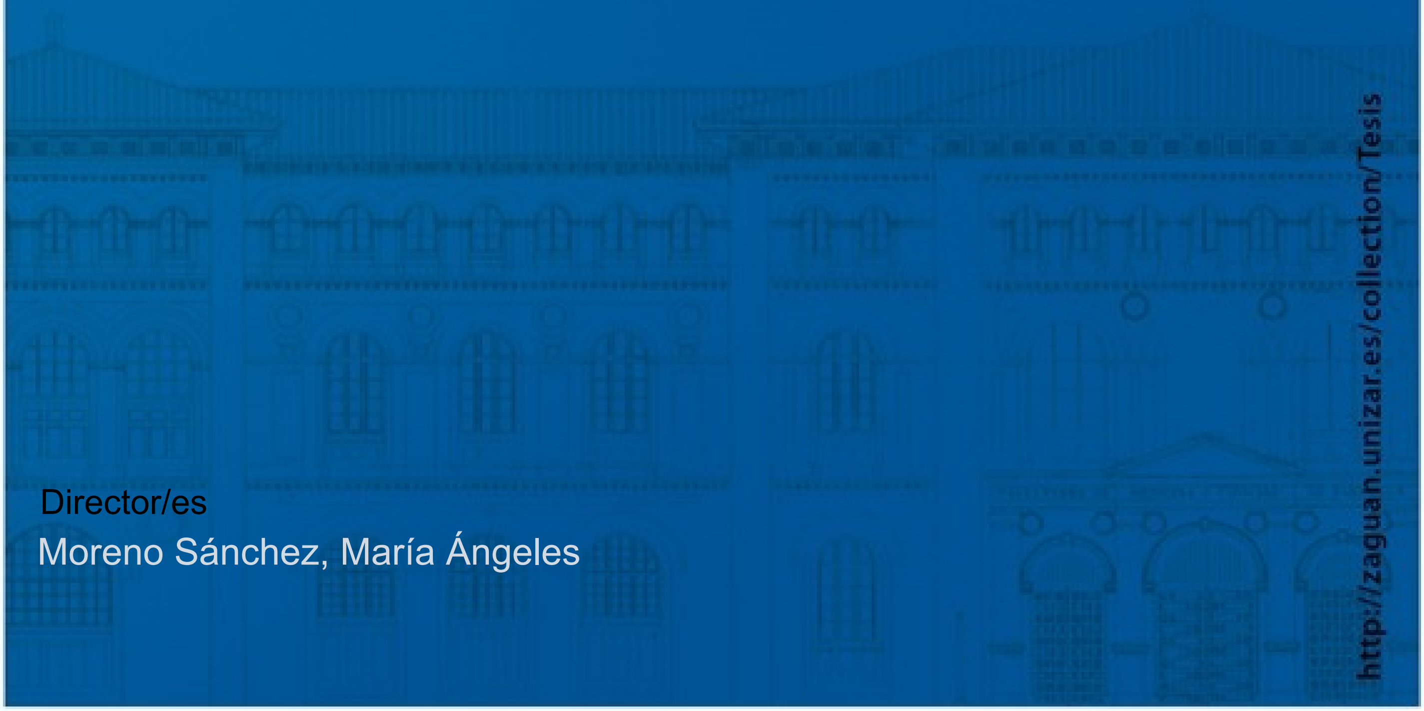
Pierre Mignard

Análisis genómico y fenómico de los caracteres de calidad del fruto en un banco de germoplasma de manzano en Aragón



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Tesis Doctoral

ANÁLISIS GENÓMICO Y FENÓMICO DE LOS CARACTERES DE CALIDAD DEL FRUTO EN UN BANCO DE GERMOPLASMA DE MANZANO EN ARAGÓN

Autor

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UNIVERSIDAD DE ZARAGOZA
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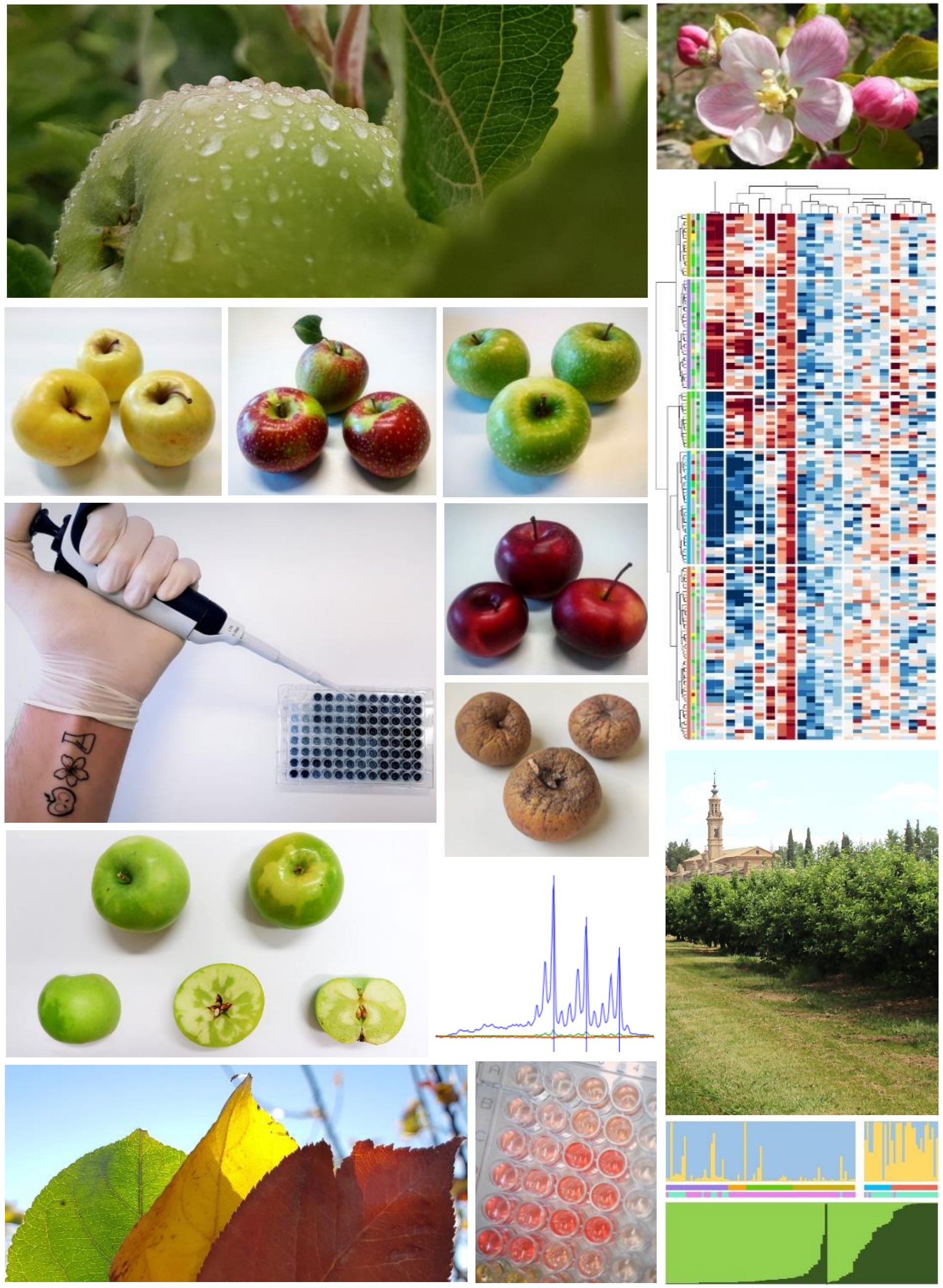

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Análisis genómico y fenómico de la calidad del fruto en un Banco de Germoplasma de manzano de Aragón – **Pierre MIGNARD**

2022

Tesis Doctoral

Análisis genómico y fenómico de los caracteres de calidad del fruto en un Banco de Germoplasma de manzano de Aragón

Pierre MIGNARD

Zaragoza, 2022

Le Fils de l'Homme, René Magritte, 1964

Estación Experimental de Aula Dei, Zaragoza
Consejo Superior de Investigaciones Científicas (CSIC)
Departamento de Pomología

Tesis Doctoral

Análisis genómico y fenómico de los caracteres de calidad del fruto en un Banco de Germoplasma de manzano de Aragón

Memoria presentada por Pierre MIGNARD, para optar al grado de Doctor por la Universidad de Zaragoza en el programa de doctorado: Ciencias Agrarias y del Medio Natural (CAMN).

Directora/Tutora:

Mª Ángeles Moreno
Dra. Ingeniero Agrónomo

Zaragoza, 2022

Dña. Mª ÁNGELES MORENO SÁNCHEZ, Investigadora Científica del Consejo Superior de Investigaciones Científicas (CSIC)

CERTIFICA

Que la Tesis Doctoral titulada **“Análisis genómico y fenómico de los caracteres de calidad del fruto en un Banco de Germoplasma de manzano de Aragón”**, ha sido realizada por el biólogo **Pierre Mignard**, en el Departamento de Pomología de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su dirección y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor.

Zaragoza, 2022

Fdo. Mª Ángeles Moreno Sánchez

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Et à moi tiens… Merci à moi aussi!

Pourquoi? Parce que!

&·

Pour ma Mère, mes sœurs,

Et la famille que je porte dans mon cœur

Resumen

La manzana (*Malus x domestica* Borkh) tiene una gran importancia en el mercado mundial gracias al hecho de que se encuentra disponible todo el año. Es una de las frutas más consumidas en el mundo y de las más producidas en climas templados. Es muy apreciada por su sabor, características organolépticas, compuestos nutricionales y sus efectos beneficiosos para la salud.

Como objetivo general, esta tesis doctoral pretende caracterizar a nivel pomológico, bioquímico y genético, las accesiones de manzano conservadas en la colección de germoplasma de la Estación Experimental de Aula Dei (EEAD – CSIC). Se busca la progresión en el conocimiento de los factores asociados al control genético de la calidad organoléptica del fruto, así como de los factores climáticos (radiación solar, precipitación, temperaturas), de una fisiopatía (vitrescencia) y de la conservación en cámara frigorífica.

Por todo ello, se evaluó el comportamiento agronómico y la calidad organoléptica y nutricional del fruto de 155 accesiones de manzano (99 accesiones autóctonas españolas y 56 cultivares extranjeros). Este estudio mostró que las variedades españolas presentaban mayores valores para algunos caracteres de calidad básica del fruto (sólidos solubles, acidez valorable e índice de madurez) y compuestos bioquímicos (compuestos antioxidantes, azúcares individuales y ácidos orgánicos mayoritarios), demostrando el interés de los recursos fitogenéticos autóctonos españoles. Además, este estudio demostró la influencia significativa de los factores climáticos en los perfiles de los diferentes metabolitos estudiados. Los fenoles y flavonoides totales tendieron a disminuir, en general, con temperaturas más altas, y aumentar con mayor precipitación y radiación solar. El contenido en azúcares, disminuyó con la precipitación y aumentó con la temperatura y la radiación solar, demostrando la importancia de la región geográfica del cultivo.

Los estudios de estructura de población, mediante 23 marcadores moleculares del tipo microsatélites (SSRs), mostraron dos subpoblaciones para las accesiones diploides y cuatro para las triploides. Además, el análisis de agrupamiento según la distancia genética mostró que las accesiones tendían a agruparse de acuerdo con su origen (español/no-español). El estudio genético por mapeo de asociación para 118 accesiones evaluadas con 21 caracteres agronómicos y bioquímicos reveló un total de 126 asociaciones significativas. Las asociaciones con parámetros tales como fecha de

cosecha, color de la piel, calidad básica del fruto, contenido en antioxidantes, en azúcares y en ácidos orgánicos, muestran el potencial interés de los marcadores utilizados (SSRs) para la selección asistida en los programas de mejora genética.

Asimismo, el estudio de ‘vitrescencia’ en tres variedades sensibles a esta fisiopatía, mostró que los frutos de manzana afectados tenían mayor densidad y mayor contenido en sorbitol y compuestos antioxidantes. Además, la expresión relativa de los genes de los transportadores de sorbitol *MdSOT1*, *MdSOT2*, *MdSOT2.2* y *MdSOT5.3* fue menor en los frutos con vitrescencia, mientras que la expresión de *MdSOT5.2* fue aumentando con la madurez del fruto y mayor en comparación con las manzanas sanas. Por otra parte, el mayor contenido de antioxidantes en las manzanas con vitrescencia se vio respaldado por una mayor expresión general de genes relacionados con los antioxidantes (*MdPAL*, *MdPPO* y *MdPOX*).

El estudio de 17 accesiones de manzana en postcosecha mostró que tras seis meses de conservación en cámara frigorífica, dos accesiones locales (‘Reneta’ y ‘Solafuente’) no presentaron ningún síntoma de daños por frío y conservaron su calidad nutricional. Por el contrario, las accesiones triploides con presencia de ‘russeting’ (‘Bossost’, ‘Reineta Blanca del Canadá’ y ‘Reineta Gris’), presentaron más daños de pardeamiento y deshidratación, posiblemente debido a sus mayores contenidos en fenoles y características de su piel.

Los factores climatológicos, de conservación en postcosecha o de fisiopatías típicas de algunas variedades, influyeron significativamente en las características de la calidad del fruto. Los estudios genéticos mostraron que la estructura genética de accesiones autóctonas españolas podría representar una fuente inexplorada de recursos fitogenéticos dadas las asociaciones genéticas encontradas. Esta tesis doctoral potenciará las variedades locales españolas mejor adaptadas en el área de cultivo para los trabajos de selección y mejora genética del manzano.

Abstract

Apple (*Malus x domestica* Borkh) has a great importance in the world thanks to be one of the most produced fruit in temperate climates. It is one of the most consumed fresh fruits worldwide and it is available in the market all year long. It is highly appreciated for its taste, organoleptic characteristics, nutritional compounds and its beneficial influence on human health.

The present Doctoral Thesis aims to characterize at the pomological, biochemical and genetic levels, apple accessions preserved at the germplasm collection of the Aula Dei Experimental Station (EEAD - CSIC). It searches the progression in the knowledge of the factors associated with the genetic control of the organoleptic fruit quality, as well as on the climatic factors (solar radiation, precipitation, temperatures), on a physiological disorder (watercore) and cold storage of apples.

Indeed, the agronomic behavior, and the organoleptic and nutritional fruit quality of 155 apple accessions (99 Spanish native accessions and 56 foreign cultivars) were assessed. This study showed that the Spanish accessions exhibited higher values for some basic fruit quality traits (soluble solids content, titratable acidity and maturity index) and biochemical compounds (antioxidants, individual sugars and organic acids), demonstrating the interest of these autochthone Spanish genetic resources. In addition, this study demonstrated the significant influence of climatic factors on the profiles of the different metabolites studied. Thus, total phenols and flavonoids tended to decrease, in general, with higher temperatures and increase with greater precipitation and solar radiation. In turn, the sugar content decreased with precipitation and increased with temperature and solar radiation, indicating the importance of the geographical region of the growing conditions.

The population structure studies, using 23 microsatellite molecular markers (SSRs), determined two subpopulations for the diploid accessions and four, according to triploids. In addition, the genetic distance analysis showed that accessions tended to be grouped according to their origin (Spanish/non-Spanish). The genetic study by association mapping for 118 accessions evaluated with 21 agronomic and biochemical characters revealed a total of 126 significant associations. Associations with parameters such as harvest date, skin color, basic fruit quality, antioxidants content, sugars and organic acids, show the potential interest of the markers used (SSRs) for assisted selection in breeding programs.

Furthermore, the evaluation of the watercore disorder for three sensitive varieties, showed that watercored apple fruits had higher density and contents of sorbitol and antioxidant compounds. In addition, the differential gene expression for the sorbitol transporters genes expression, *MdSOT1*, *MdSOT2*, *MdSOT2.2* and *MdSOT5.3* was lower in the watercore affected fruits, while the *MdSOT5.2* gene expression increased along with the ripeness of the fruit and showed higher expression compared to healthy apples. On the other hand, the higher antioxidants content in watercored apples was supported by a higher overall expression of related genes to antioxidants (*MdPAL*, *MdPPO* and *MdPOX*).

The study of 17 apple accessions in postharvest showed that after six months of cold storage, two local accessions (‘Reneta’ and ‘Solafuente’) did not present any symptoms of chilling injury and maintained their nutritional quality. In contrast, triploid accessions with the presence of russeting (‘Bossost’, ‘Reineta Blanca del Canadá’ and ‘Reineta Gris’) showed higher damages from browning and dehydration, possibly due to their higher polyphenols content and skin characteristics.

Climatological factors, conservation in post-harvest or typical physiological disorders for some varieties, significantly influenced the organoleptic and nutritional quality of the fruit. Genetic studies showed that the genetic structure of native Spanish accessions could represent unexplored phytogenetic resources, given the genetic associations found in the present work. This doctoral thesis will enhance the local Spanish varieties best adapted to the growing conditions for selection and breeding of apple trees.

Résumé

La pomme (*Malus x domestica* Borkh) a une grande importance sur le marché mondial grâce au fait qu'elle est disponible pour sa consommation durant toute l'année. C'est l'un des fruits les plus consommés dans le monde et les plus produits dans les climats tempérés. Elle est très appréciée pour sa saveur, ses caractéristiques organoleptiques, ses composés nutritionnels et ses effets bénéfiques sur la santé.

Comme objectif général, cette thèse de doctorat vise à caractériser au niveau pomologique, biochimique et génétique, les accessions de pommier conservées dans la collection de germoplasme de la Station Expérimentale de Aula Dei (EEAD - CSIC). Elle cherche à permettre de progresser dans la connaissance des facteurs associés au contrôle génétique de la qualité organoleptique du fruit, ainsi que des facteurs climatiques (rayonnement solaire, précipitation, températures), d'un déséquilibre physiologique (vitrescence) et de la conservation en chambre froide.

Pour toutes ces raisons, le comportement agronomique et la qualité organoleptique et nutritionnelle du fruit de 155 accessions de pommier (99 accessions autochtones espagnoles et 56 cultivars étrangers) ont été évalués. Cette étude a montré que les variétés espagnoles présentaient des valeurs plus élevées pour certains caractères de la qualité du fruit (solides solubles, acidité et indice de maturité) et des composés biochimiques (composés antioxydants, sucres individuels et acides organiques majoritaires), démontrant l'intérêt des ressources phytogénétiques locales espagnoles. En outre, cette étude a démontré l'influence significative des facteurs climatiques sur les profils des différents métabolites étudiés. Les phénols et les flavonoïdes totaux ont eu tendance à diminuer, en général, avec des températures plus élevées, et à augmenter avec plus de précipitations et de rayonnement solaire. La teneur en sucres a diminué avec les précipitations et a augmenté avec la température et le rayonnement solaire, démontrant l'importance de la région géographique de la plantation.

Les études de structure de population, utilisant 23 marqueurs moléculaires de type microsatellite (SSRs), ont montré deux sous-populations pour les accessions diploïdes et quatre pour les triploïdes. En plus, l'analyse de regroupement selon la distance génétique a montré que les accessions avaient tendance à se regrouper selon leur origine (espagnole/non-espagnole). L'étude génétique par cartographie d'association pour 118 accessions évaluées avec 21 caractères agronomiques et biochimiques a révélé un total de 126 associations significatives. Les associations avec

des paramètres tels que la date de récolte, la couleur de la peau, la qualité du fruit, la teneur en antioxydants, en sucres et en acides organiques montrent l'intérêt potentiel des marqueurs utilisés (SSRs) pour la sélection assistée dans les programmes d'amélioration génétique.

De même, l'étude de la vitrescence sur trois variétés sensibles à ce déséquilibre physiologique a montré que les fruits de pomme affectés avaient une densité plus élevée et une plus haute teneur en sorbitol et en composés antioxydants. En outre, l'expression relative des gènes de différents transporteurs de sorbitol *MdSOT1*, *MdSOT2*, *MdSOT2.2* et *MdSOT5.3* a été plus faible dans les fruits vitrescents, tandis que l'expression de *MdSOT5.2* a augmenté avec la maturité du fruit et était plus élevée par rapport aux pommes saines. D'autre part, la plus grande teneur en antioxydants des pommes vitrescentes a été soutenue par une plus grande expression générale des gènes liés aux composés antioxydants (*MdPAL*, *MdPPO* et *MdPOX*).

L'étude de 17 accessions de pomme post-récolte a montré qu'après six mois de conservation en chambre froide, deux accessions locales ('Reneta' et 'Solafuente') ne présentaient aucun symptôme de dommages causés par le froid et de plus, conservaient leur qualité nutritionnelle. En revanche, les accessions triploïdes avec présence de 'russeting' ('Bossost', 'Reinette Blanche du Canada' et 'Reinette Grise') présentaient davantage de dommages de brunissement et de déshydratation, peut-être en raison de leurs teneurs plus élevées en phénols et caractéristiques de leur peau.

Des facteurs climatiques, de conservation post-récolte ou de déséquilibre physiologique, typiques de certaines variétés, ont eu une influence significative sur les caractéristiques de qualité du fruit. Les études génétiques ont montré que la structure génétique des accessions locales espagnoles pourrait représenter une source inexplorée de ressources phytogénétiques étant donné les associations génétiques trouvées. Cette thèse de doctorat renforcera les variétés autochtones espagnoles les mieux adaptées dans la zone de culture pour les travaux de sélection et d'amélioration génétique du pommier.

Índice General

Abreviaturas

ANOVA	Análisis de varianza
DL	Desequilibrio de ligamiento
EEAD	Estación Experimental de Aula Dei
GL	Grupo de ligamiento
ha	Hectárea
N	Newton
ns	No significativo

Abbreviations

AsA	Ascorbic acid
DNA	Desoxirribonucleic Acid
DPPH	2,2-bipyridyl-1,1-diphenyl-2-picrylhydrazyl
FW	Fresh weight
GAE	Gallic acid equivalents
HPLC	High performance liquid chromatography
LD	Linkage disequilibrium
LG	Linkage Group
MAS	Marker assisted selection
N	Newton
ns	Not significant
PCA	Principal component analysis
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAC	Relative antioxidant capacity
RI	Ripening index
SE	Standard error
SSC	Soluble solids content
SSR	Simple sequence repeats
SNP	Single nucleotide polymorphism
SP	Spain
TA	Titratable acidity
TFC	Total flavonoids content
TPC	Total phenolics content
UPGMA	Unweighted pair group method average

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Capítulo 1

Introducción General

La manzana (*Malus x domestica* Borkh) tiene una gran importancia en el mercado mundial gracias al hecho de que se encuentra disponible durante todo el año para su consumo. Es una de las frutas más consumidas en el mundo y de las más producidas en climas templados. La manzana es muy apreciada por su sabor, por sus compuestos nutricionales y también por sus efectos beneficiosos para la salud. En 2020, la producción mundial de manzanas alcanzó más de 86,4 millones de toneladas, por detrás del plátano (119,8 millones de toneladas) y de la sandía (101,6 millones de toneladas) (FAOSTAT, 2022). En España, se produjeron más de 617 mil toneladas en 2021. Aragón representó un 13,1% del total nacional, por detrás de Cataluña (48,4%) y Galicia (17%) (MAPAMA, 2022).

Actualmente, existen más de 10.000 cultivares de manzana documentados en el mundo (Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2016). Entre ellos, se encuentran las variedades comerciales y mundialmente conocidas, pertenecientes a los grupos ‘Fuji’, ‘Gala’, ‘Golden’, ‘Granny Smith’ o ‘Red Delicious’, así como accesiones autóctonas de los diferentes países productores de manzana (Urrestarazu et al., 2016). No obstante, en España, como en otros países productores, los fruticultores basan la producción del manzano en variedades no autóctonas, principalmente de los diferentes grupos antes citados, o que son resultado de mutaciones y/o cruzamientos entre ellas. Los agricultores se centran en estas variedades debido a su rápida entrada en producción y su mayor rendimiento económico (Iglesias et al., 2016; 2018; Urrestarazu et al., 2016).

Sin embargo, los problemas de calidad del fruto, mala adaptación en condiciones limitantes del cultivo y menor aceptación del consumidor en las últimas décadas por algunas variedades autóctonas, han llevado al arranque de plantaciones y, por ello, a una creciente dependencia de las importaciones en España (Iglesias et al., 2016). Este hecho está llevando a una pérdida dramática de la diversidad genética y podría obstaculizar los programas de mejora genética, ya que se requiere variación genética en dichos programas. Como consecuencia, las variedades autóctonas, mejor adaptadas a las zonas de origen, en cuanto a resistencia frente a plagas, enfermedades y características específicas de calidad de fruto, han ido desapareciendo del mercado (Mignard et al., 2021; Reig et al., 2015). Para evitar su extinción y la pérdida de recursos fitogenéticos, muchas de las variedades españolas se han conservado en bancos de germoplasma. Por lo tanto, las colecciones locales de manzano desempeñan un papel crucial en la preservación de la diversidad genética y el suministro de material a potenciales interesados (Pereira-Lorenzo et al., 2017; Reig et al., 2015; Swarup et al., 2020). Esta diversidad genética es necesaria para la posible mejora de las variedades actuales, debido a factores relacionados con el cambio climático, como el incremento de las temperaturas y la mayor incidencia de plagas y enfermedades, así como la aparición de nuevas especies de patógenos (Muranty et al., 2020; Stewart et al., 2020; Swarup et al., 2021).

Además, la manzana es conocida por sus propiedades beneficiosas para la salud. Su consumo está asociado a una dieta más sana, con un menor riesgo en el desarrollo de enfermedades crónicas y relacionadas con la edad (Boeing et al., 2012; Garcia-Oliveira et al., 2021; Qanash et al., 2022), gracias a los diversos compuestos que contiene (Lattanzio, 2013; Preti y Tarola, 2021). En efecto, la manzana forma parte de las frutas que presentan las principales fuentes de sustancias biológicas activas beneficiosas para la salud humana. La manzana contiene pectinas, fibras, vitaminas, oligosacáridos, compuestos fenólicos (fenoles y flavonoides específicos, ácido ascórbico - vitamina C), azúcares individuales (sacarosa, fructosa, glucosa y sorbitol) y ácidos orgánicos (ácido málico, oxálico, tartárico, cítrico, quínico, succínico y shikímico) (Boeing et al., 2012; Castel et al., 2020; Lattanzio, 2013; Mignard et al., 2021; 2022; Zhang et al., 2016). El contenido en estos compuestos varía considerablemente en función del genotipo de las diferentes variedades, de las condiciones ambientales, prácticas de cultivo, fechas de maduración o de las condiciones de conservación en postcosecha (Alhaj Alali et al., 2020; Mignard et al., 2021; 2022; Wu et al., 2012). Además, el perfil de azúcares y de ácidos orgánicos, así como la concentración de los azúcares solubles y ácidos orgánicos individuales confiere propiedades organolépticas y nutricionales específicas a las variedades (Aprea et al., 2017; Font i Forcada et al., 2014, 2019; Mignard et al., 2022).

La caracterización de dichos compuestos es primordial para su uso en los programas de mejora genética y para conocer la adaptación de las nuevas variedades a distintas áreas de cultivo y condiciones climáticas (Fernández-Cancelo et al., 2021; Parajuli et al., 2019; Stewart y Ahmed, 2020; Swarup et al., 2021; Tyagi et al., 2017). Sin embargo, muy pocos trabajos han evaluado los parámetros mencionados en las condiciones edafoclimáticas del Valle del Ebro (Castel et al., 2020). Por otra parte, el aumento de las temperaturas, observado en las últimas décadas, ya ha tenido un impacto visible en el crecimiento y desarrollo de las plantas (Fujisawa y Kobayashi, 2011; Li et al., 2020; Parry, 2019). Además, existe el riesgo de que los rangos de temperatura actuales puedan ampliarse debido a cambios climáticos futuros (Li et al., 2020; Swarup y Singh Kushwaha, 2022). A menos que se establezcan políticas drásticas para reducir los gases de efecto invernadero en los próximos años, estos cambios afectarán a todos los agroecosistemas, y se deberán desarrollar estrategias de adaptación (Beguería et al., 2003; Iglesias et al., 2018).

El objetivo general de esta tesis consiste en la caracterización bioquímica, pomológica y molecular de accesiones de manzana (*Malus x domestica* Borkh) de la colección establecida en la Estación Experimental de Aula Dei del Consejo Superior de investigaciones Cientificas (EEAD-CSIC) (Cambra, 1975; Herrero et al., 1964) en condiciones típicas del Valle Medio del Ebro (Figura 1.1). Este área está considerada como una región vulnerable para el cultivo del manzano debido a las condiciones edafoclimáticas actuales y al cambio climático (Cantín y Gracia, 2022; Gracia y Cantín, 2022; Iglesias et al., 2016; 2018; Reig et al., 2015).

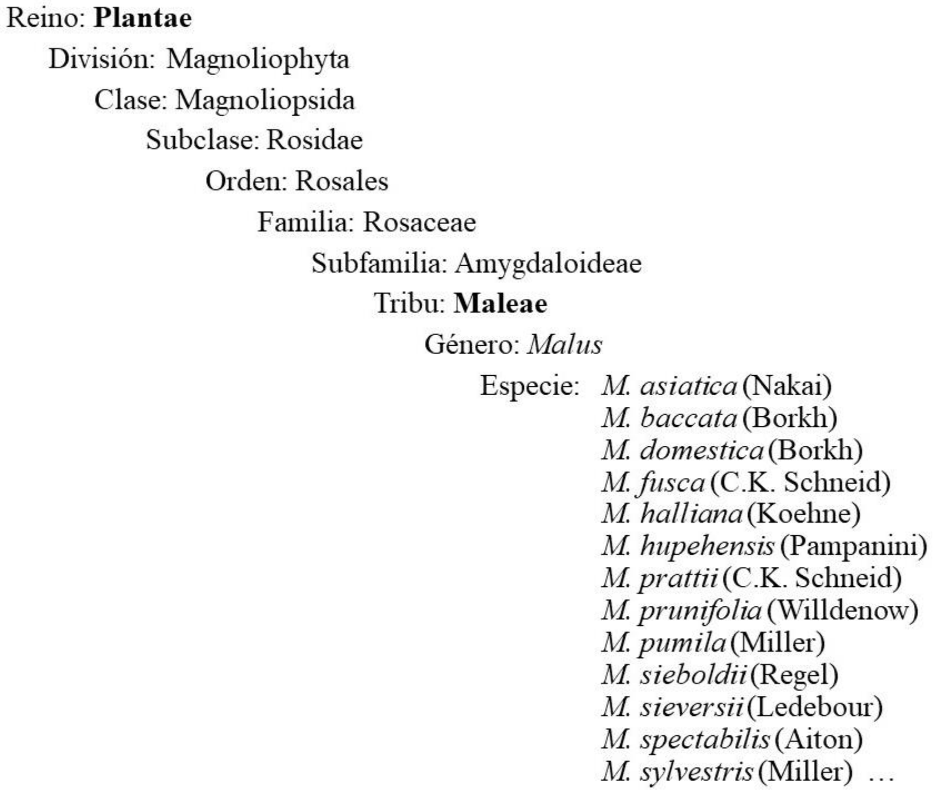
Figura 1.1. Colección del Banco de Germoplasma de manzano establecido en la Estación Experimental de Aula Dei (EEAD-CSIC), Zaragoza.

Este trabajo prioriza el estudio de las características de calidad del fruto (antioxidantes, perfiles de azúcares y ácidos orgánicos, entre otros) así como la estructura genética de variedades autóctonas españolas y accesiones extranjeras de referencia conservadas en dicho Banco de Germoplasma, mediante la utilización de técnicas de espectrofotometría, cromatografía líquida de alta eficacia o *High Performance Liquid Chromatograph*y (HPLC) y marcadores moleculares del tipo microsatélites (SSRs).

Los programas de mejora genética tienen como objetivo la obtención de variedades más productivas, mejor adaptadas a las condiciones de cultivo y con la mejor calidad organoléptica y sensorial del fruto. Por ello, en esta tesis se pretende profundizar en el conocimiento de los factores asociados con el control genético de la calidad organoléptica del fruto y la influencia de los factores climatológicos, para poner en relación la calidad del fruto con el clima de la zona de cultivo. Este trabajo potenciará las variedades locales mejor adaptadas en el área de cultivo para futuros trabajos de selección y mejora genética de manzano.

1.1. TAXONOMÍA, CLASIFICACIÓN BOTÁNICA Y DESCRIPCIÓN DEL MANZANO

La manzana es el fruto comestible del manzano común, un híbrido inter-específico que generalmente se designa como *Malus x domestica* Borkh. Es un miembro de la subfamilia *Maloideae*, de la familia de las Rosaceae y pertenece al género *Malus*. Dicho género comprende de 25 a 30 especies diferentes procedentes de todo el mundo (Robinson et al., 2001; Volk et al., 2021) (Figura 1.2).



Reino: **Plantae**
División: Magnoliophyta
Clase: Magnoliopsida
Subclase: Rosidae
Orden: Rosales
Familia: Rosaceae
Subfamilia: Amygdaloideae
Tribu: **Maleae**
Género: *Malus*
Especie: *M. asiatica* (Nakai)
M. baccata (Borkh)
M. domestica (Borkh)
M. fusca (C.K. Schneid)
M. halliana (Koehne)
M. hupehensis (Pampanini)
M. prattii (C.K. Schneid)
M. prunifolia (Willdenow)
M. pumila (Miller)
M. sieboldii (Regel)
M. sieversii (Ledebour)
M. spectabilis (Aiton)
M. sylvestris (Miller) …

Figura 1.2. Clasificación botánica del genero *Malus* según Volk et al. (2021).

El manzano tiene un número básico de cromosomas igual a 17. La mayoría de las variedades cultivadas son diploides (2n = 2x = 34). Sin embargo, también existen variedades triploides (2n = 3x = 51) y tetraploides (2n = 4x = 68). El manzano común se cultiva en todo el mundo y la especie *Malus x domestica* Borkh es la más utilizada del género *Malus*. Pudo originarse en Asia Central, donde su ancestro silvestre, *Malus* *sieversii*, todavía existe, además de ser el área donde se encuentra la mayor diversidad dentro del género *Malus* (Gao et al., 2015; Vavilov, 1930). En efecto, estudios recientes (Brite, 2021; Davies et al., 2022) demuestran la hipótesis de Vavilov. El manzano se ha cultivado durante cientos de años en Asia y Europa y fue llevado a América del Norte y del Sur por los colonizadores europeos. Actualmente, la zona de cultivo del manzano abarca muchas partes del mundo, con amplias variaciones de clima y terrenos muy diferentes (Jung et al., 2020; Howard et al., 2019; Lassois et al., 2016).

Los árboles de manzano son muy vigorosos y longevos si se cultivan a partir de semillas y no entran en fructificación antes del sexto o séptimo año (Del Bo, 2011). En la actualidad, los cultivares de manzano se propagan por injerto sobre patrones seleccionados (Reig et al., 2020a), controlando el tamaño y el vigor del árbol y adelantando la entrada en fructificación. Existen dos tipos básicos de patrones que se clasifican como patrones francos y clonales. Mientras que los primeros proceden de semillas de la misma especie que la variedad injertada (Howard, 1987), los clonales proceden de su propagación vegetativa. La propagación clonal asegura la uniformidad en el desarrollo vegetativo y en la producción del material utilizado (Felipe, 1989). Existen diversos patrones que buscan controlar el vigor y aumentar la productividad del árbol (Kim et al., 2020; Reig et al., 2020a; Wang et al., 2019). En Europa, los más usados, en general, son las selecciones M9 y M26 (Kim et al., 2020; Wang et al., 2019). Estos dos patrones tienden a mejorar la producción, tamaño de fruto, calidad del fruto y resistencia a plagas y enfermedades (Wang et al., 2019). En la Estación Experimental de Aula Dei (CSIC), la mayoría de las accesiones están injertadas sobre el patrón semi-vigoroso y resistente a algunas plagas MM106 (Wang et al., 2019). No obstante, es un patrón muy susceptible a la podredumbre de cuello de raíz (*Phytophtora* sp.) (Verma et al., 2021).

El manzano es un árbol caducifolio que puede tener una vida de 60 a 80 años. Puede alcanzar los 10 m de altura en estado silvestre, aunque normalmente no sobrepasa los 2,5-3 m. Su copa es globosa y se dispone de forma espontánea en vaso (Del Bo, 2011). Su tronco es bastante recto, con la corteza escamosa cubierta de lenticelas. Además, en las zonas viejas del árbol esta corteza se encuentra con un color gris pardo mientras que en las ramas más jóvenes el color es más verdoso (Coque, 1996). Las hojas son ovaladas, verde oscuro en la parte superior y más claras en la parte inferior. Las flores se hallan reunidas en corimbos y, en general, tienen una corola con cinco pétalos de color blanco rosado (Figura 1.3). Dado que la pulpa del fruto deriva de un engrosamiento del receptáculo, se dice que el fruto es falso. El verdadero fruto es el corazón de la manzana, que contiene de cinco a diez semillas (Del Bo, 2011). La forma, tamaño o el color de las manzanas dependen mucho del cultivar (Davies et al., 2022; Reig et al., 2015; Urrestarazu et al., 2016).

Figura 1.3. Representación botánica de una variedad de manzana: a, flor; b, corte longitudinal de una flor de manzano; c, hoja; d, fruto o manzana; e, semilla. Illustración de Jacob Sturm (1771-1848) botánico e ilustrador naturalista alemán.

Se estima que existen más de 10.000 cultivares conocidos de manzano con diferentes características (Jung et al., 2020; Marconi et al., 2018; Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2016), que son usados para consumo en fresco (fruta de mesa), zumos, cocinar, producción de sidra y otras bebidas alcohólicas.

1.2. IMPORTANCIA ECONÓMICA DEL CULTIVO

1.2.1. Interés económico del manzano

La manzana es posiblemente una de las frutas más comercializadas en el mundo por su interés y por sus condiciones más fáciles de conservación y de transporte (Acerete, 1949). La producción ha pasado de unos 18 millones de toneladas en 1961 hasta más de 86 millones de toneladas, 60 años después (Figura 1.4), casíicinco veces más (FAOSTAT, 2022).

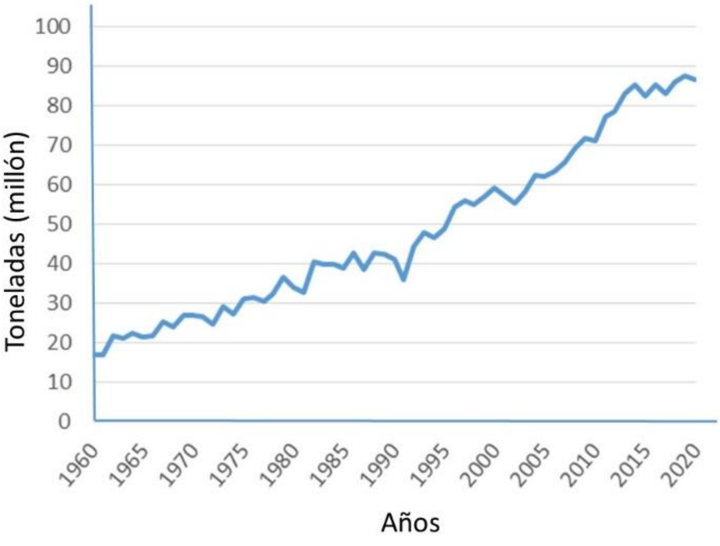


Figura 1.4. Evolución de la producción mundial de manzana desde 1960 hasta 2020 en millones de toneladas (FAOSTAT, 2022).

La superficie total mundial cultivada de manzano ha pasado de 1,7 millones de hectáreas en 1961 a 4,6 millones de ha en 2020 (FAOSTAT, 2022). Tanto para la producción como para la superficie de manzano cultivada, Asia representa más del 60 %, con una producción de 55,7 millones de toneladas, mientras que Europa representa un 20 %, con una producción de 17,5 millones de toneladas (Figura 1.5). China representa más del 72 % del total asiático en términos de producción y más del 60 % para la superficie cultivada. La manzana se produce sobre todo en Asia (60%), Europa (20%) y América (10%) (Wang et al., 2019).

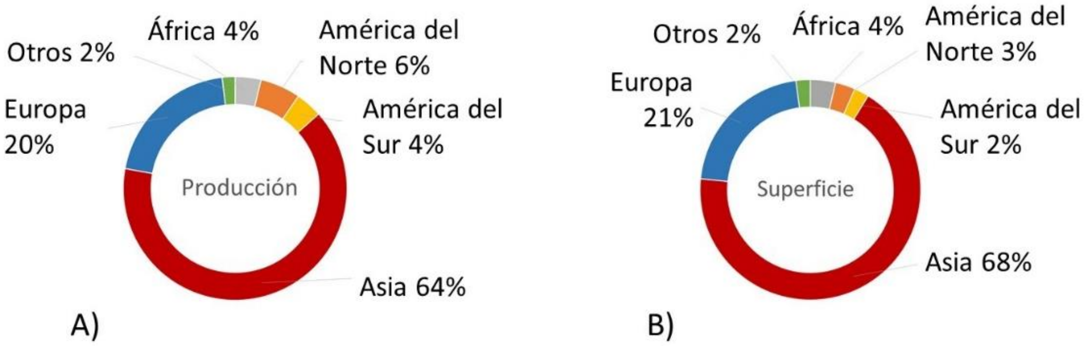


Figura 1.5. Distribución (%) de la producción mundial (A) y de la superficie mundial cultivada (B) de manzano, en 2020 (FAOSTAT, 2022).

1.2.2. Producción y superficie cultivada en España

En España, la producción de manzana con unas 617 mil toneladas producidas en 2021, va muy por detrás de la del melocotón y de la nectarina (1206 miles de toneladas) aunque por delante de la de pera (317 miles de toneladas). Cataluña es la principal región productora de manzana de mesa, seguida por Aragón, Galicia y la comunidad de Castilla-León. Estas cuatro comunidades autónomas produjeron en 2021 el 80% de la producción de manzana de mesa. En cuanto a la manzana de sidra, tiene su mayor importancia en Galicia, como principal región productora para la elaboración de sidra (Pereira-Lorenzo et al., 2003; 2007), seguida por Asturias y País Vasco. No obstante, los volúmenes de producción media de estas dos últimas comunidades autónomas son muy inferiores respecto a Galicia (MAPAMA, 2022) (Figura 1.6). En cuanto a las variedades mayoritarias producidas en España, como ocurre en la mayoría de países, están las de los grupos antes citados (‘Fuji’, ‘Gala’, ‘Golden’, ‘Granny Smith’, ‘Red Delicious’ o ‘Starking’). Se cultiva también alguna variedad autóctona como la ‘Verde Doncella’ en

Aragón, con su característico color amarillo blanquecino y su tendencia a la vitrescencia, aunque su impacto en el mercado no sea de gran importancia (Herremans et al., 2014; Melado-Herreros et al., 2013).

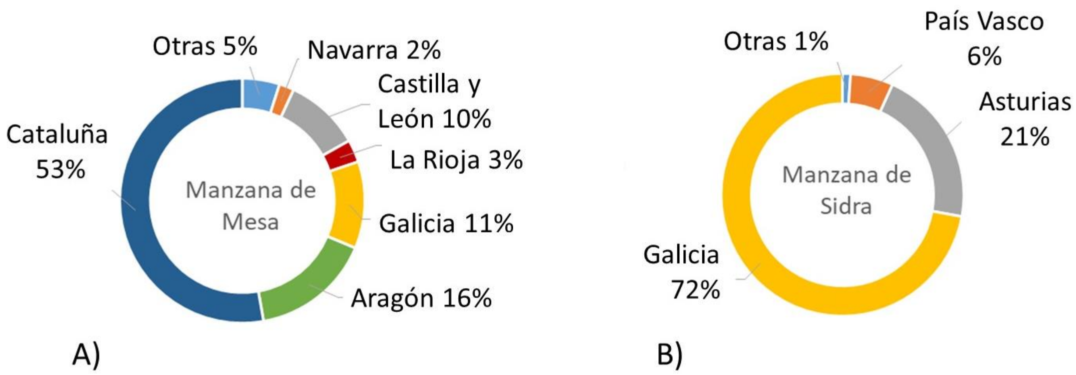


Figura 1.6. Distribución de la producción de manzana de mesa (A) y de sidra (B) en España, en 2021 (MAPAMA, 2022).

1.3. UNA MANERA DE CONSERVAR LOS RECURSOS FITOGENÉTICOS: LOS BANCOS DE GERMOPLASMA

En 1996, en la "Declaración de la Conferencia de las Partes del Convenio sobre la Diversidad Biológica a la Conferencia Técnica Internacional sobre la Conservación y Utilización de Recursos Fitogenéticos" (FAO, 1996), se citó: *"Se ven amenazados muchos recursos fitogenéticos que pueden ser vitales para el desarrollo agrícola y la seguridad alimentaria en el futuro. En los informes de los países se indica que las pérdidas recientes de diversidad han sido elevadas y que continúa el proceso de "erosión". Preocupa de manera particular la pérdida irreversible de genes, unidad funcional básica de la herencia y fuente primordial de la variación del aspecto, las características y el comportamiento de las plantas."*.

Además, más de cincuenta años antes, en Francia, Auguste Chevalier mencionó: "*Il est très urgent d'organiser l'expérimentation scientifique en pomologie en constituant plusieurs stations regionales, des fermes expérimentales et un Institut central d'études fruitières. Toutefois, il est nécessaire de former au préalable des chercheurs qualifiés.*" (Chevalier, 1942). Lo cual, se podría traducir por: "*Es muy urgente organizar la experimentación científica en pomología constituyendo varias estaciones regionales, granjas experimentales y un Instituto central de estudios frutales. No obstante, es necesario formar de antemano a investigadores cualificados."*

Con esta misma idea y dada la necesidad de evitar la pérdida de genotipos autóctonos, la Estación Experimental de Aula Dei del CSIC (EEAD-CSIC) acometió en los años cincuenta, un programa de prospección, caracterización y conservación de recursos genéticos (Cambra, 1975; Herrero et al., 1964). En efecto, los programas de investigación en fruticultura requieren un gran esfuerzo en la caracterización, evaluación, selección y mejora de estos valiosos recursos fitogenéticos. Permiten revalorizarlos y poner a disposición de los agricultores, mejoradores o investigadores, un material vegetal de alto valor agronómico y tecnológico.

Actualmente, en España, existen varios bancos de germoplasma de manzana, en distintas regiones, como el de la Estación Experimental de Aula Dei (CSIC) y la del Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), las dos en Zaragoza, y el del Banco Nacional de Germoplasma de la Estación de Villaviciosa de Asturias, del Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA). Estos bancos conservan variedades antiguas que se han cultivado tradicionalmente en sus respectivas regiones, pero también incluyen otros cultivares comerciales o extranjeros (Cambra, 1975; Dapena, 1996; Pereira-Lorenzo et al., 2017).

La conservación de la diversidad genética es importante para todas las especies cultivadas, mediante la evaluación y propagación de cultivares locales o antiguos, así como de especies silvestres próximas, para evitar la pérdida de recursos fitogenéticos y genes que podrían ser importantes en la adaptación agronómica (Reig et al., 2015). Hoy en día, el banco de germoplasma de la EEAD - CSIC dispone de más de 180 accesiones (Figura 1.7) con una diversidad significativa respecto al color, tamaño, sabor, uso y perfiles bioquímicos de los frutos de dichas accesiones (Reig et al., 2015). Existen numerosos trabajos que han caracterizado bancos de germoplasma alrededor del mundo, como los realizados en Suiza (Bühlmann et al., 2015), Noruega (Meland et al., 2022), Portugal (Ferreira et al., 2016), Italia (Marconi et al., 2018), Francia (Lassois et al., 2016), Turquía (Bakır et al., 2022), Estados Unidos (Howard et al., 2018), China (Gao et al., 2015) y España (Castel et al., 2020; Pereira-Loreno et al., 2017; Urrestarazu et al., 2012), entre otros.

Aparte del trabajo de evaluación y caracterización en los diferentes bancos de germoplasma, otro gran aspecto para evitar la pérdida de cultivares y de recursos genéticos valiosísimos, es la financiación pública y privada y los trabajos de divulgación. En efecto, es muy importante la tarea que consiste en dar a conocer lo que se hace en los centros de investigación mediante actividades de divulgación con el objetivo de acercar a los potenciales usuarios lass variedades disponibles para su preservación en condiciones reales de cultivo (fruticultores) y fomentar la demanda por el consumidor (Reig et al., 2018).

Figura 1.7. Ejemplo de diversidad fenotípica de la colección de la EEAD – CSIC.

1.4. CALIDAD DEL FRUTO EN MANZANO

1.4.1. Noción de calidad

La palabra “*calidad*” proviene del latín *qualitas*, que significa “*atributo”*, “*propiedad”* o “*naturaleza básica”* de un objeto. Según el Diccionario de la Real Academia Española de la Lengua, la *“calidad”* es la propiedad o conjunto de propiedades inherentes a algo, que permiten juzgar su valor. No obstante, otra definición que se puede citar, habla de la buena calidad como algo superior o excelente (RAE, 2022). Asimismo, se puede decir que un producto es de mejor calidad cuando es *"superior"* en uno o varios parámetros valorados (Font i Forcada, 2012). No obstante, entendemos de manera general, la calidad, como las diferentes características de un producto que determinarán su grado de aceptabilidad por parte del consumidor (Cantín y Gracia; 2022; Kramer y Twigg, 1966; Song et al., 2022).

En las últimas decadas, ha aumentado el interés de los productores, comercializadores y consumidores hacia la búsqueda de una mayor calidad en los diferentes productos agrícolas, y especialmente en las frutas. Para facilitar su estudio, la calidad de un producto vegetal puede dividirse en cuatro bloques principales: la calidad organoléptica o sensorial, la calidad nutricional, la calidad postcosecha y por último, la calidad sanitaria (Musacchi y Serra, 2018; Tyagi et al., 2017).

En esta tesis, no se ha abordado la calidad sanitaria del fruto. Tampoco se ha considerado la evaluación sensorial pero si aspectos organolépticos, nutricionales y postcosecha (Bowen y Grygorczyk, 2021; Cantín et al., 2022). Los aspectos de tamaño, color, forma, firmeza, aroma, sabor, acidez o sólidos solubles, formarían parte de la calidad organoléptica, y los componentes bioquímicos como los azúcares solubles, vitaminas, fenoles, flavonoides, antocianinas o la capacidad antioxidante, se incluirían en la calidad nutricional (Boeing et al., 2012; Font i Forcada, 2012; Lattanzio, 2013; Preti y Tarola, 2021). La calidad postcosecha recopilaría todos los parámetros anteriores y se centraría en la evolución de estos compuestos a lo largo de su conservación, después de la cosecha (Bui et al., 2019; Corollaro et al., 2014; Davey et al., 2007; Vallarino y Osorio, 2019; Zhu et al., 2022a).

1.4.2. Calidad organoléptica

El tamaño y la forma son dos características muy importantes de la calidad organoléptica de la manzana. Se pueden evaluar de forma sencilla con un calibre del tipo pie de rey (Figura 1.8). Estos parámetros se definen durante el desarrollo del fruto, mientras que la coloración y otros parámetros de calidad básica del fruto se alcanzan durante la maduración. El color es uno de los principales parámetros para estimar el grado de madurez de un fruto, ya que es el aspecto externo más fácil de valorar por el consumidor y facilita la aceptación del mismo (Pathare et al., 2013). Por eso, los productores utilizan el color para la conveniente comercialización de la fruta. Para la medición del color, el método CIELAB es uno de los más usados en los trabajos experimentales y se puede determinar mediante un colorímetro (Seymour, 2022) (Figura 1.8).

Otra manera de expresar el nivel de madurez de la fruta es el índice de madurez, (IM) resultante del cociente entre el contenido en sólidos solubles (CSS) y la acidez valorable (AV). El contenido en sólidos solubles se evalúa con un refractómetro (ºBrix) (Figura 1.8) y la acidez valorable se mide mediante valoración acido/base (Milošević et al., 2022; Vuković et al., 2020).

También se puede determinar el nivel de madurez según el índice de almidón (Peirs et al., 2002; Shewa et al., 2022; Smith et al., 1979). Esta prueba determina la cantidad de almidón en la pulpa del fruto por medio de una solución de yodo. Este índice es especialmente adecuado para los frutos de las pomáceas. El almidón se acumula en las manzanas durante el crecimiento del fruto y se hidroliza en azúcar según madura (Shewa et al., 2022).

Finalmente, la firmeza de la pulpa es otro de los indicadores más utilizados para determinar el grado de maduración adecuado para que los frutos sean cosechados (Cárdenas-Pérez et al., 2017). La firmeza se mide generalmente utilizando un penetrómetro (Figura 1.8). Sin embargo, existen otros métodos no destructivos para medir la firmeza, como los durómetros o la espectroscopia de reflectancia en el infrarrojo cercano, que estiman la firmeza de una fruta sin dañarla (Bureau et al., 2009; Sudebi y Walsh, 2009).

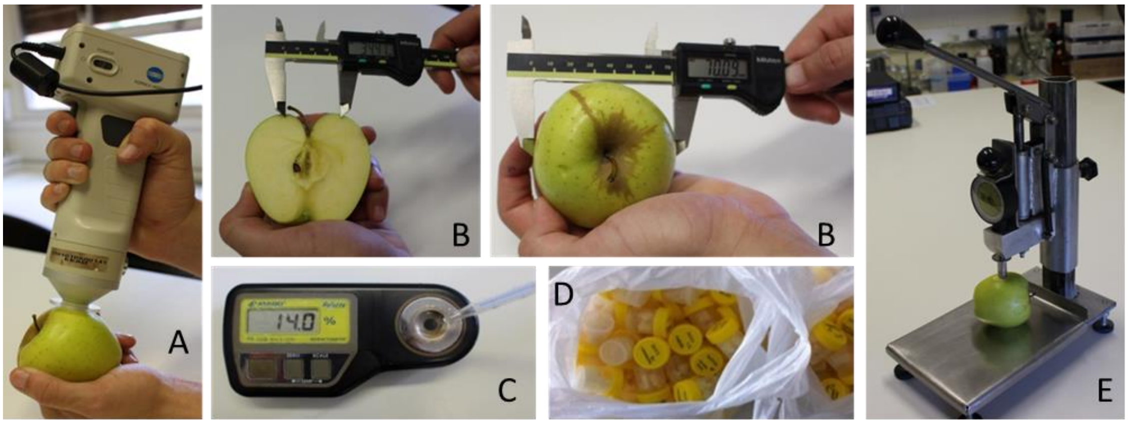


Figura 1.8. Aparatos utilizados para medir la calidad organoléptica de la manzana: A: colorímetro (Konica Minolta, CR-400, Osaka, Japón); B: Calibre digital del tipo Pie de Rey; C: Refractometro digital (Atago PR-101, Tokyo, Japan); D: muestras procesadas; E: Penetrómetro (FT-32).

1.4.3. Calidad nutricional

La calidad nutricional se refiere a la capacidad de los alimentos para proporcionar elementos nutritivos que favorezcan un buen estado de salud y prevengan la aparición de enfermedades en los consumidores (Boeing et al., 2012; Michalska & Łysiak, 2015). Dentro de la calidad nutricional, la calidad nutraceútica se centra en la presencia de sustancias antioxidantes que actúan frente a los radicales libres y podrían ayudar a prevenir enfermedades como el cáncer, la diabetes y enfermedades cardiovasculares según estudios realizados *in-vitro,* en animales y en humanos (Boeing et al., 2012; Gibney et al., 2019; Ho et al., 2020; Michalska & Łysiak, 2015; Zhang et al., 2016).

Asimismo, la calidad nutritiva depende de la cantidad y calidad tanto de los macronutrientes (proteínas, carbohidratos, ácidos y lípidos) como de los micronutrientes (vitaminas y elementos minerales) que entran en su estructura. La figura 1.9 muestra algunas biomoléculas que forman parte de los compuestos fenólicos, azúcares y ácidos orgánicos. Entre los compuestos bioquímicos más importantes en la manzana, se pueden destacar los azúcares, los ácidos orgánicos, la vitamina C, los compuestos fenólicos y los flavonoides. También se puede determinar la capacidad antioxidante relativa (RAC). Estos compuestos se pueden estimar de una forma sencilla mediante técnicas de espectrofotometría (Figura 1.10) aunque los métodos más precisos incluyen técnicas de cromatografia líquida de alta eficacia (HPLC) (Font i Forcada et al., 2019; Kschonsek et al., 2018; Vasile et al., 2021). Sin embargo, la gran diversidad de compuestos fenólicos y la mayor complejidad en su detección hacen los analisis por HPLC más costosos.

Además y con el fin de conservar las muestras, se deben congelar a -20ºC / -80ºC y/o liofilizar (Figura 1.11) para hacer los análisis necesarios posteriormente. En el segundo caso, las determinaciones pueden hacerse en peso seco en vez de peso fresco (Amri et al., 2021; Mignard et al., 2021).

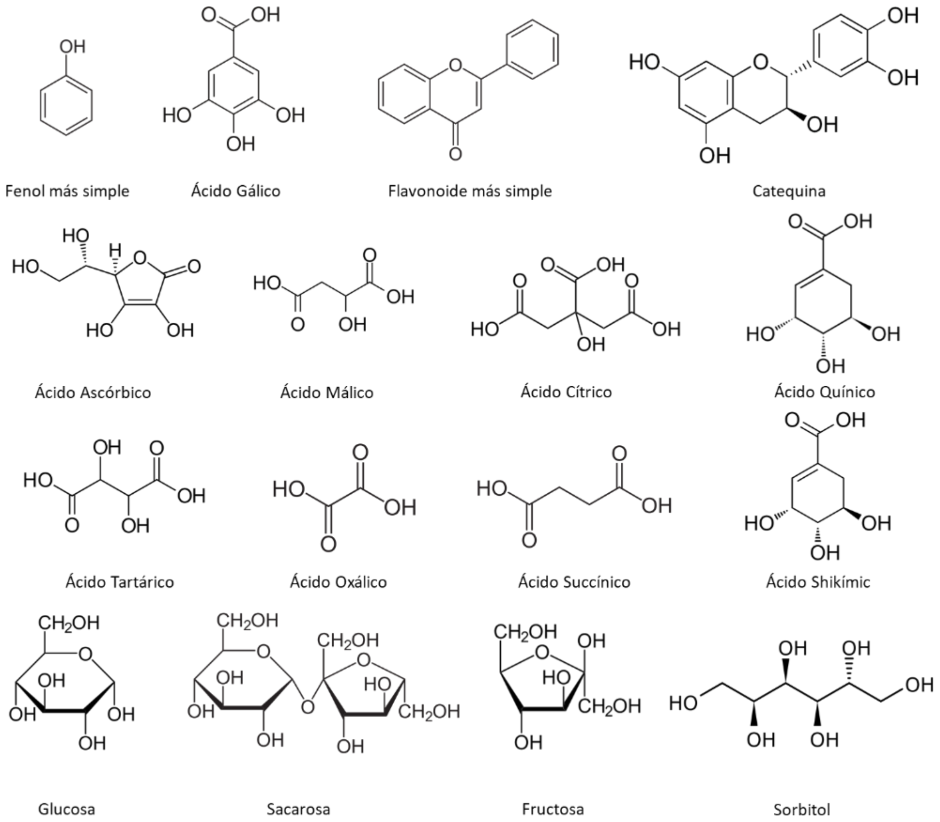


Figura 1.9. Diferentes bio-moleculas que forman parte de los compuestos bioquímicos presentes en el fruto de la manzana (azúcares, ácidos orgánicos y compuestos antioxidantes).

Figura 1.10. Aparatos utilizados para la preparación de muestras y estimación de parámetros bioquímicos y antioxidantes. A: Polytron (T25D Ultra-Turrax, IKA Works Inc., Wilmington, USA); B: Micropipeta y microplaca de 96 pocillos tipo placa ELISA; C: Espectrofotómetro de placas (Asys UVM 340 microplate reader; Biochrom, Cambridge, UK); D: Placa ELISA; E: Micropipeta tipo multicanal; F: Agitador de placas (Thermo-Shaker PST-60HL, Biosan).

Figura 1.11. Liofilizador en la EEAD – CSIC, modelo M301 mill (Retsch, Dusseldorf, Alemania, GmbH).

Los carbohidratos y los ácidos orgánicos juegan un papel importante en la calidad del fruto e influyen en la textura del fruto y en su sabor (Aprea et al., 2017; Font i Forcada et al., 2019; Liu et al., 2013; Vallarino et al., 2019; Yang et al., 2021). Conforme el fruto madura, el contenido en azúcares totales aumenta mientras que el contenido total en ácidos orgánicos disminuye. Sin embargo, la proporción de los diferentes compuestos también varía según el estado de madurez de la fruta (Grammen et al., 2019; Yang et al., 2021). En general, en manzana, el azúcar predominante es la fructosa, seguido por la sacarosa, la glucosa y finalmente el sorbitol (Figuras 1.12 y 1.13) (Liu et al.. 2013; Mignard et al., 2022; Yang et al., 2021). La fructosa tiene mayor capacidad edulcorante que la glucosa y la sacarosa (Doty, 1976; Zhang et al., 2020). Además, el sorbitol es más beneficioso que otros azúcares para la salud humana (Forni et al., 1992). De hecho, el sorbitol se puede utilizar como sustituto de la glucosa en las dietas para la diabetes, y es un edulcorante natural alternativo a la sacarosa. A través de la vía poliol, también llamada vía sorbitol-aldosa reductasa, la glucosa se reduce a sorbitol y posteriormente se oxida a fructosa (Lu et al., 2018).

Figura 1.12. Análisis por cromatografía líquida de alta eficacia (HPLC) en la Estación Experimental de Aula Dei (CSIC) [de izquierda a derecha: bomba 515 HPLC Pump, inyector, detector UV-Visible (Waters 2489) y detector índice de refracción (IR) (Waters 2410), Milford, USA].

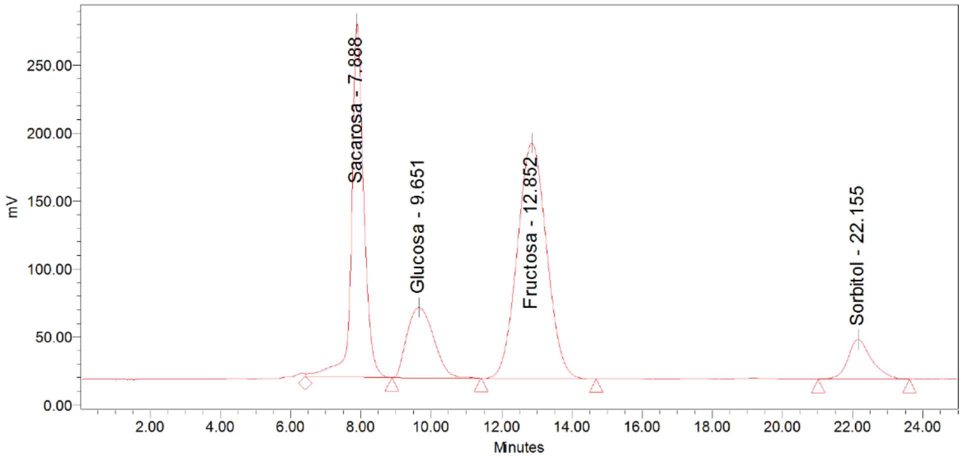


Figura 1.13. Ejemplo de cromatograma de un análisis por HPLC de los diferentes azúcares individuales de una variedad de manzana. Analisis mediante columna Aminex HPX-87C (300 x 7.8 mm), Biorad, y detector IR (Waters, 2410).

La acidez del fruto viene determinada por el contenido total e individual de los diferentes ácidos orgánicos (Font i Forcada et al., 2019). Junto a los azúcares individuales, intervienen en la percepción del dulzor y del aroma de la manzana (Iwanami et al., 2012; Mignard et al., 2022; Verma et al., 2019). El ácido orgánico mayoritario en la manzana es el ácido málico. No obstante, también el ácido cítrico, quínico, shikímico oxálico, tartárico y succínico (Figura 1.14) pueden estar presentes (Aprea et al., 2017; Vallarino et al., 2019; Zhu et al., 2022a). La acidez alcanza un máximo durante el desarrollo inicial de la manzana y luego disminuye con la maduración, tanto en el momento de la cosecha como en la postcosecha (Liu et al., 2013).

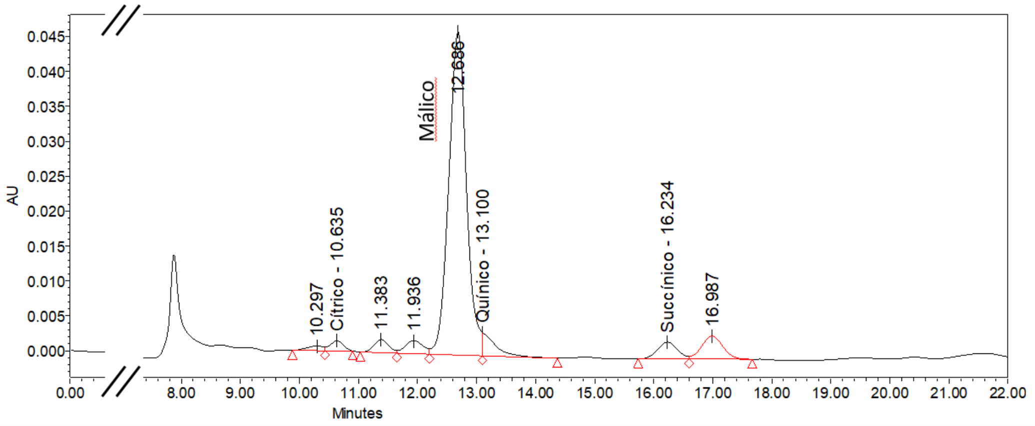


Figura 1.14. Ejemplo de cromatograma de algunos ácidos orgánicos de una variedad de manzana. Análisis de HPLC mediante columna Rezex™ ROA-Organic Acid H+ (8%), (300 x 7.8 mm), Phenomenex y detector UV-Visible (Waters, 2489).

1.4.4. Maduración del fruto y calidad postcosecha

Se pueden distinguir dos tipos de maduración en la manzana, la madurez fisiológica y la madurez comercial. La primera es el estado en el cual el fruto ha alcanzado un estado de desarrollo suficiente para que tras la cosecha, su calidad sea la mínima aceptable para el consumidor. La segunda es el momento óptimo de recolección para cada fruto y cultivar, es decir cuando la fruta ha alcanzado su calidad óptima aún estando en el árbol. La madurez comercial es crucial a la hora de conseguir una buena calidad final para el consumidor, ya que influye en la calidad organoléptica, nutricional, postcosecha y en la vida útil del fruto (Davey et al., 2007; Ehsani-Moghaddam et al., 2013; Kader et al., 1999).

En el caso de la manzana como fruta climatérica, se recoge antes de su maduración fisiológica, que debería completarse en un almacén o una cámara frigorífica bajo condiciones controladas de temperatura y humedad. La manzana se cosecha antes de que su estado fisiológico sea el adecuado para el consumidor pero apta para su conservación (Ehsani-Moghaddam et al., 2013; Grammen et al., 2019). Sin embargo, si se recolecta mucho tiempo después de su estado fisiológico adecuado, su vida útil se acortará y será más susceptible a los daños mecánicos (Zhu et al., 2022a). El momento de la cosecha es un paso decisivo en la calidad del producto.

Las técnicas de postcosecha buscan reducir los niveles de pérdida de peso de la fruta una vez cosechada y mantener la calidad organoléptica y uniforme del fruto en su paso del campo (Figura 1.15) a la mesa del consumidor. De ahí la importancia de evaluar la calidad de la fruta y observar la evolución del fruto, la ausencia de daños por frío y evaluar los niveles de diferentes compuestos bioactivos, como antioxidantes, azúcares individuales y ácidos orgánicos mayoritarios, ya que pueden reducir el riesgo de algunos daños y enfermedades postcosecha (Alhaj Alali et al., 2020; Cainelli & Ruperti, 2019; González et al., 2022; Zhang et al., 2016; Zhu et al., 2022a) y aumentar la vida útil de la fruta (Bui et al., 2019; Davey et al., 2007).

El ‘bitter pit’ o depresión amarga es uno de los trastornos fisiológicos más frecuentes que ocurren en postcosecha en la fruta (Jemrić et al., 2016). En efecto, los síntomas se desarrollan durante el almacenamiento, apareciendo varias semanas o meses después de la cosecha. Este trastorno fisiológico fue asociado durante mucho tiempo con la absorción o falta de calcio en la fruta (Bonomelli et al., 2020; Krawitzky et al., 2016). La gravedad del ‘bitter pit’ a menudo se asocia con la concentración de nutrientes (N, K, Mg, Ca) y minerales, las precipitaciones estacionales y las temperaturas, que se sabe que mejoran el desarrollo del trastorno (Al Shoffe et al., 2020; Krawitzky et al., 2016; Reid et al., 2020).

Además, las nuevas técnicas moleculares de asociación genética podrían identificar y localizar genes asociados a los parámetros agronómicos y nutricionales de calidad del fruto y/o de fisiopatías y trastornos relacionados con la postcosecha (Howard et al., 2019; Kenis et al., 2008). Por ello, la caracterización de dichos compuestos podría contribuir a conocer la adaptación de las variedades a su almacenamiento en postcosecha y utilizar esta información en conjunto con los análisis de asociación en los programas de mejora genética (Leja et al., 2003; Wu et al., 2012).

Figura 1.15. Manzanas con problemas de conservación tras varios meses de conservación en cámara frigorífica.

1.5. CARACTERIZACIÓN MORFOLÓGICA, MOLECULAR Y ESTUDIOS DE ASOCIACIÓN EN MANZANO

1.5.1. Caracterización morfológica y bioquímica

La caracterización de las variedades se lleva a cabo determinando sus características morfológicas, pomológicas, agronómicas y/o bioquímicas, siguiendo las directrices de los distintos organismos internacionales como pueden ser el IPGRI (*The International Plant Genetic Resources Institute*), la UPOV (*International Union for the Protection of New Varieties of Plants*) y la oficina comunitaria de la Unión Europea (CPVO - *Community Plant Variety Office*) (Reig et al., 2015). Sus directrices incluyen observaciones sobre el vigor del árbol, la precocidad, la producción, la aptitud a la propagación vegetativa, la susceptibilidad a ciertas plagas y enfermedades, tamaño del fruto, prominencia y/o profundidad de la cavidad pistilar y pedúncular, color de fondo, color de la chapa, firmeza de la pulpa, dulzor y acidez del fruto, fechas de inicio, plena y final de floración, y época de madurez o la determinación de la calidad tanto organoléptica como nutricional (apartados 1.4.II y 1.4.III), así como la caracterización bioquímica (Castel et al., 2020; Fernández-Cancelo et al., 2021; Gibney et al., 2019; Gracia y Cantín, 2022; Melado-Herreros et al., 2013; Mignard et al.,, 2021; 2022; Reig et al., 2015; Yang et al., 2018).

1.5.1.1. Factores geográficos y climáticos que influyen en la calidad

En el contexto actual de cambio climático, es cada vez más relevante el hecho de preservar la variabilidad genética disponible (Parajuli, 2019; Parry, 2019). El aumento de las temperaturas observado en las últimas décadas ya ha tenido un impacto visible en el crecimiento y el desarrollo de las plantas (Fujisawa & Kobayashi, 2011; Li et al., 2020). Por lo tanto, los agricultores y los investigadores están cambiando su comportamiento al impulsar y seleccionar cultivares de manzanas tolerantes frente a estos estreses climáticos (Beguería et al., 2003; Boudichevskaia et al., 2020). Los productores de manzana reconocen el retraso en la madurez de la fruta como un efecto directo del aumento de las temperaturas, y se debe profundizar en las estrategias de adaptación para enfrentar el calentamiento global (Ahmadi et al., 2019; Gitea et al., 2019).

Además de la gestión y manejo agronómico, los parámetros ambientales como la radiación solar, las precipitaciones, las temperaturas, la humedad relativa o la altitud, influyen en el crecimiento y proporcionan una amplia variación en los nutrientes y la acumulación de compuestos bioquímicos en el fruto (Cirilli et al., 2016; Mignard et al., 2021; 2022; Stewart & Ahmed, 2020; Yuri et al., 2009). Por ejemplo, la concentración de azúcar en la fruta suele disminuir en proporción al suministro de agua o de las precipitaciones (Mills et al., 1996). No obstante, la dilución del agua depende de la dinámica del crecimiento del fruto y, por lo tanto, varía según la especie y las condiciones climáticas (Cakpo et al., 2020; Cirilli et al., 2016). La altitud influye en las temperaturas, la intensidad de la luz y el fotoperiodo. Por ello, la altitud juega un papel crucial en el metabolismo de las plantas y el contenido de compuestos bioquímicos en las frutas, destacando la importancia de la región donde se cultivan (Kumar et al., 2019; Li et al., 2009).

Además, las quemaduras o golpes de sol en la piel de las frutas siguen siendo una causa de pérdida económica significativa en la producción de manzanas (Reig et al., 2020b). Existe un consenso en que las quemaduras están relacionadas con la combinación de altas temperaturas y una mayor radiación de solar durante el período de crecimiento de la fruta (Ranjan et al., 2020), unas condiciones que encontramos en nuestra zona de estudio, el valle medio del Ebro. Sin embargo, el conocimiento sobre los aspectos fisiológicos que inducen los cambios en la calidad interna de la fruta (Severino et al., 2020) y la generación de trastornos fisiológicos relacionados con el sol en la fruta son limitados. En efecto, los estudios sobre la influencia del clima en la calidad organoléptica de la manzana son escasos (Ahmadi et al., 2019; Boudichevskaia et al., 2020; Kim et al., 2019). Sin embargo, dado que los expertos en clima han anticipado un aumento de las temperaturas del aire en un rango de 2-5 °C en la cuenca mediterránea (Benlloch-González et al., 2018), es necesario mejorar la comprensión de cómo el clima y sus componentes influyen en las variables organolépticas de la fruta. Para ello, los bancos de germoplasma ofrecen una excelente plataforma para evaluar esta situación (Swarup et al., 2021).

1.5.1.2. Evaluación de compuestos bioquímicos en manzana con vitrescencia

La vitrescencia (Figura 1.16) es un una fisiopatía que ocurre cuando la fruta todavía está en el árbol (Buccheri et al., 2020; Herremans et al., 2014; Melado-Herreros et al., 2013). La vitrescencia se puede observar en el corazón de la manzana, en la pulpa o incluso en la piel, y se debe a que se llenan de líquido los espacios intercelulares del tejido afectado (Kasai & Arakawa, 2010). Como el líquido acumulado entre las células de la fruta reduce la dispersión de la luz que pasa a través del tejido afectado por vitrescencia, la pulpa parece ser translúcida (Itai, 2015; Kasai & Arakawa, 2010). Este líquido es rico en sorbitol, siendo el principal poliol (alcohol polihídrico con varios grupos hidroxilo) transportado en los espacios intercelulares de tejido de manzana (Itai, 2015; Kasai & Arakawa, 2010; Tanaka et al., 2020; Yamada et al., 2006; Yang et al., 2018). Además, debido al menor volumen de espacio aéreo intercelular, los tejidos afectados sufren estrés oxidativo y, por ello, aumenta la formación de especies reactivas de oxígeno (ROS). Por lo tanto, las enzimas antioxidantes son más activas en los tejidos afectados por la vitrescencia debido al estrés oxidativo, y se consumen en mayor medida los sustratos antioxidantes como pueden ser los fenoles o los flavonoides (Buccheri et al., 2020; Zupan et al., 2016).

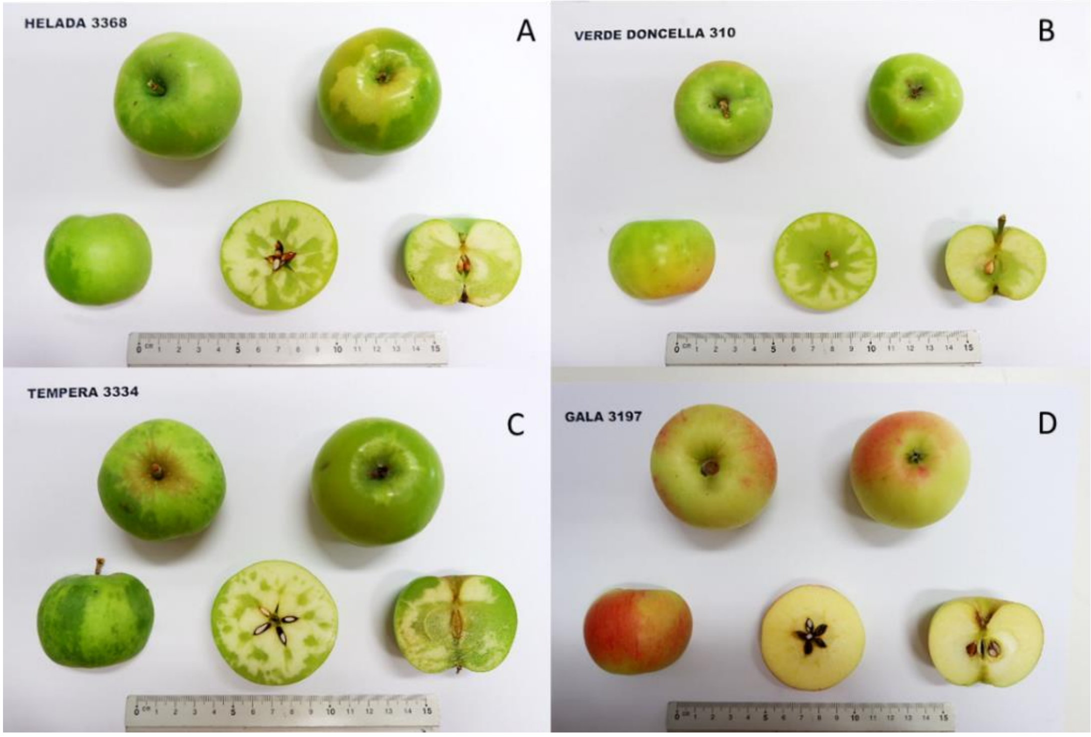


Figura 1.16. Ejemplo de caracteres morfológicos y pomológicos de distintas variedades de manzana. Presencia de vitrescencia (A - Helada, B - Verde Doncella, y C - Tempera) o ausencia de vitrescencia (D - Gala) en la piel y en la pulpa.

Los azúcares y los compuestos antioxidantes juegan un papel crucial en los diferentes procesos metabólicos de las plantas como el crecimiento, la fotosíntesis, la defensa contra patógenos o en respuestas frente a estreses abióticos como la sequía, temperaturas extremas, salinidad e incompatibilidad patrón variedad (Amri et al., 2021; Li et al., 2012; Mignard et al., 2021; 2022; Ruan, 2014; Zhao et al., 2020). De hecho, la protección y la permeabilidad de las membranas celulares de las manzanas pueden ser inducidas por acumulación de sorbitol (Yang et al., 2018).

La vitrescencia en la fruta puede provocar importantes pérdidas económicas para los agricultores, dado que como consecuencia de esta fisiopatía, otros problemas como el pardeamiento o la podredumbre son más frecuentes en postcosecha (Itai, 2015; Kasai y Arakawa, 2010). La vitrescencia en las manzanas está relacionada, en general, con una aceptabilidad negativa por parte de los consumidores debido a estos trastornos indeseables que ocurren en los frutos vitrescentes (Tanaka et al., 2020). Sin embargo, dado que la vitrescencia está relacionado con la madurez de la fruta (Itai, 2015; Yamada, 2004), en algunos países como Japón, las frutas con vitrescencia son verdaderamente apreciadas debido a su sabor y su aroma. También en algunas partes de España, algunas variedades autóctonas susceptibles a la vitrescencia son especialmente apreciadas y valoradas al sufrir esta fisiopatía, como la ‘Esperiega de Ademuz’, la ‘Helada’ o la ‘Verde Doncella’ (Melado-Herreros et al., 2013). Además, como la vitrescencia está directamente relacionada con la madurez de la fruta (Charles et al., 2019), puede ser una prueba de que los frutos han madurado completamente en el árbol (Tanaka et al., 2020). El rasgo vitrescente se ha convertido en un objetivo en algunos programas de mejora genética, como con la variedad ‘Fuji’, procedente del cruzamiento entre ‘Red Delicous’ x ‘Ralls Janet’, en Japón (Kunihisa et al., 2016; Tanaka et al., 2020).

En *Malus*, como en otras *Rosaceas*, el sorbitol es uno de los principales productos fotosintéticos sintetizados y en los frutos existen evidencias de acumulación de sorbitol apoplástica (Pleyerová et al., 2022; Yang et al., 2019; Zhang et al. 2004). El sorbitol viaja en el floema para el transporte de larga distancia, y luego se descarga en los diferentes órganos a través de vías simplásticas o apoplásticas (Patrick et al., 2013). La vía simplástica utiliza el gradiente de concentración para mover los azúcares solubles a través de las membranas. No obstante, el proceso es complejo y requiere transportadores de azúcar especificados y codificados por múltiples familias de genes (Wei et al., 2014; Zhu et al., 2022b).

La expresión de los transportadores de sorbitol en la especie *Malus x domestica* Borkh (MdSOT) es menor en los tejidos de manzana con vitrescencia que en tejido de manzana sana (Gao et al., 2005). De hecho, Li et al. (2012) reportaron que los genes MdSOT1 y MdSOT2 se expresaban en todos los tejidos del fruto pero en menor proporción en frutos vitrescentes. Sin embargo, las expresiones de los genes MdSOT3, MdSOT4 y MdSOT5 fueron significativas en los órganos vegetativos mientras que los frutos muestraban una baja expresión de estos transportadores de sorbitol (Gao et al., 2005; Li et al., 2012). Los transportadores de sorbitol en manzana (MdSOTs) son específicos del sustrato y su afinidad por el sorbitol es muy alta (Pleyerová et al., 2022). Las deficiencias en estos transportadores de sorbitol son directamente responsables de la fisiopatía de la vitrescencia en manzana (Gao et al., 2005).

1.5.2. Caracterización molecular

La caracterización molecular se concentra en el uso de marcadores moleculares, basados en el ADN, el ARN o las proteínas, para determinar las características genéticas de los organismos estudiados. Esta caracterización molecular permite un conocimiento más completo y eficiente de los diferentes recursos fitogenéticos conservados en los bancos de germoplasma y en programas de mejora (Bianco et al., 2014; Chagné et al., 2012; Jung et al., 2021; Pereira-Lorenzo et al., 2017; Muranty et al., 2020; Urrestarazu et al., 2016). La identificación molecular y las caracterizaciones morfológica y agronómica se complementan ya que permiten identificar los caracteres a nivel genético aunque estén ausentes como rasgos fenotípicos (Font i Forcada, 2012).

Los marcadores llamados comúnmente “microsatélites” (*Simple Sequence Repeats – SSRs*) están entre los más utilizados, por su fácil uso y bajo coste (Pereira-Lorenzo et al., 2008; Urrestarazu et al., 2012). Además, su alto nivel de polimorfismo los convierte en una técnica muy útil. Los SSRs son unas secuencias muy abundantes en el genoma, constituidas por unidades cortas (motivos básicos) de 1 a 6 pares de bases, que se repiten en tándem un elevado número de veces. Cada secuencia SSR se define por el tipo de unidad repetida (lo más frecuente mono, di, tri o tetra, aunque también penta o hexa nucleótidos) y por el sitio que ocupan en el genoma (locus) (Pereira-Lorenzo et al., 2017; Pina et al., 2014). La variación se manifiesta normalmente como diferencias en longitud entre los distintos alelos del mismo locus. Estas diferencias en longitud surgen de la existencia de un número diferente de repeticiones del motivo básico en cada genoma. Son altamente polimórficos, multi-alélicos, codominantes y reproducibles entre laboratorios (Miranda et al., 2014; Pereira-Lorenzo et al., 2017; Pina et al., 2014). En los bancos de germoplasma de manzano españoles, ya se han hecho estudios diversos para caracterizar las accesiones conservadas (Arnal et al., 2020; Pereira-Lorenzo et al., 2008; 2017; Pérez Romero et al., 2015; Pina et al., 2014; Urrestarazu et al., 2012; 2016).

Los alelos amplificados pueden separarse mediante electroforesis en geles de agarosa, acrilamida o electroforesis con marcaje fluorescente. Permiten en muchos casos definir si una accesión es diploide o triploide, aunque no siempre puede verse (Figura 1.17). Por ejemplo, en manzano, los marcadores MdSWEET2e y MdSWEET7b no permiten distinguir si la muestra es diploide o triploide.

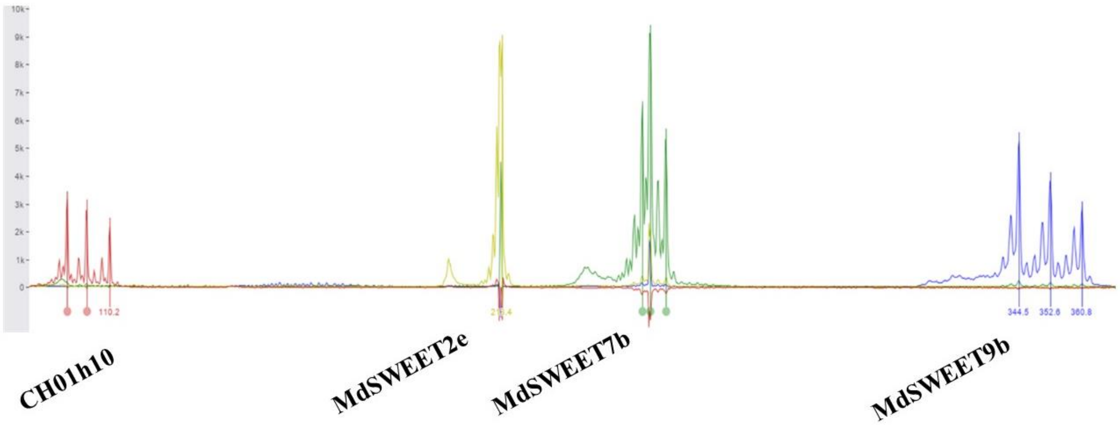


Figura 1.17. Ejemplo de perfil de una accesión triploide (‘Reineta Inesita Asua’), caracterizada con cuatro SSRs y separados mediante electroforesis con marcaje fluorescente.

Por otra parte, los polimorfismos basados en un único nucleótido (*Single Nucleotide Polymorphisms - SNPs*), se han convertido en los más utilizados actualmente, debido a su menor tasa de mutación, abundancia en los genomas vegetales y a la robustez del análisis automatizable (Jung et al., 2020). En efecto, dado que lo que se caracteriza es la sustitución de un solo nucleótido por otro en una región específica del material genético, el análisis está mucho más dirigido (Bianco et al., 2014; Jung et al., 2020). En muchos casos, el polimorfismo puede resultar en un cambio de aminoácido, una cadena polipeptídica con características diferentes a la cadena original, y a su vez, presentará un posible cambio en el fenotipo (Vanderzande et al., 2019). Su utilización es muy valiosa para la construcción de mapas genéticos, la identificación y localización de genes o caracteres de interés y la genética de asociación (Bianco et al., 2014; Chagné et al., 2012; Jung et al., 2020; 2021; Muranty et al., 2020). Sin embargo, en manzano, esta técnica sigue siendo muy costosa. Por ello, en este trabajo se han utilizado los marcadores moleculares de tipo SSRs (23 SSRs), con el fin de estudiar la variabilidad molecular en la colección de germoplasma de manzano de la EEAD – CSIC y analizar las posibles asociaciones genéticas utilizando los datos fenotípicos disponibles. Once de los 23 SSRs fueron recomendados por el grupo de trabajo del ‘*European Cooperative Programme for Plant Genetic Resources* (ECPGR) *Malus*/*Pyrus*’ con una metodología estándar para hacer posible las comparaciones entre diferentes laboratorios (Lateur et al., 2012). Además, otros ocho microsatélites fueron utilizados, dado que fueron especialmente diseñados para amplificar en zonas del genoma en relación con la ruta metabólica de los azúcares en manzano: los ‘SWEET Genes’ (Zhen et al., 2018).

1.5.3. Estudios de asociación genética en manzano

Todos las variedades comerciales de manzana descienden de un complejo híbrido ínter-específico perteneciente a la familia de las rosáceas, y tienen un número cromosómico básico de 17 (Marconi et al., 2018). La mayoría de los cultivares de manzana domesticados son diploides (2n = 34). El manzano moderno es el resultado de un largo proceso evolutivo a lo largo de cientos de años con varias especies que contribuyeron al 'pool' genético encontrado hoy en día (Velasco et al., 2010). De hecho, en el contexto del cambio climático global, la diversidad alélica del manzano debe usarse para enfrentar problemas bióticos y abióticos, tanto existentes como futuros, y relacionados con la producción (Lassois et al., 2016). La diversidad a nivel genético entre las accesiones de manzana refleja una combinación de selecciones históricas y procesos adaptativos que resultan en una variación genética extensa pero en una estructura poblacional limitada (Urrestarazu et al., 2016). Estudios basados en marcadores moleculares han evaluado la diversidad disponible en las accesiones del genero *Malus* en diversos países (Pereira-Lorenzo et al., 2017; Gross et al., 2012; Urrestarazu et al., 2012; Gao et al., 2015; Liang et al., 2015; Lassois et al., 2016; Vanderzande et al., 2017).

Los marcadores utilizados para caracterizaciones genéticas también se han asociado con loci de caracteres cuantitativos (*Quantitative Trait Loci* - QTLs) con un gran interés para variables agronómicas, morfológicas y/o organolépticas. Son utilizados como herramientas moleculares para la selección asistida por marcadores (*Marker Assisted Selection* - MAS) en los programas de mejora (Liu et al., 2016; Marconi et al., 2018). Igualmente, el mapeo de asociación, conocido como mapeo de desequilibrio de ligamiento (*Linkage Disequilibrium* - LD) se basa en la fuerza de asociación entre los marcadores genéticos y el fenotipo. Por lo tanto, la asociación mediante LD es un enfoque que detecta y localiza genes en relación con un mapa existente de marcadores genéticos (Font i Forcada et al., 2015). En consecuencia, este método detecta relaciones entre la variación fenotípica y el polimorfismo génico en el germoplasma existente y en individuos no relacionados. Además, el mapeo de asociación complementa y mejora la información genética anterior de QTLs al incorporar los efectos de la recombinación que ocurren en muchas generaciones pasadas, en un solo análisis. La caracterización genética y asociación con el fenotipo se ha utilizado con éxito para identificar genes implicados en varios caracteres y variables de la manzana. No obstante, en la bibliografía, los estudios respecto a asociaciones mediante marcadores del tipo SSRs respecto a la calidad organoléptica y/o calidad básica del fruto son todavía escasos. Se encuentran, no obstante, varios estudios utilizando QTLs y/o marcadores tipo SNPs (Amyotte et al., 2017; Gutiérrez et al., 2018; Kumar et al., 2022; Larsen et al., 2019; Lee et al., 2017; Liao et al., 2021; McClure et al., 2018; 2019). Con los recientes avances en tecnologías de secuenciación de alto rendimiento, un nuevo enfoque ha abierto nuevos horizontes para el genotipado extensivo gracias al “genotyping-by-sequencing”. Este método permite la selección genética a nivel genómico, llamada selección genómica y que podría facilitar la selección de variables en el sector de la fruticultura en futuros programas de mejora genética (Zahid et al., 2022).

1.6. REFERENCIAS

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Capítulo 2

Objetivos

Como objetivo general, esta tesis doctoral pretende caracterizar tanto a nivel pomológico como bioquímico y genético, diferentes accesiones de manzano (*Malus x domestica* Borkh) del banco de germoplasma de la Estación Experimental de Aula Dei (EEAD – CSIC). Se trata, a lo largo de los diferentes capítulos, de profundizar en el conocimiento de los factores asociados con el control genético de la calidad organoléptica y nutricional del fruto. Además, se aspira a determinar la influencia de otros factores sobre los perfiles de metabolitos y compuestos bioquímicos, como el efecto de los factores climáticos (radiación solar, precipitación, temperaturas,…), la influencia de una fisiopatía (vitrescencia) y la conservación de larga duración en cámara frigorífica.

Los objetivos secundarios son:

1- Evaluación y caracterización en el período de cinco años (2014-2018) de parámetros básicos de calidad del fruto, compuestos antioxidantes, ácidos orgánicos mayoritarios y azúcares individuales de 155 accesiones y sus correspondientes correlaciones con los factores climáticos para los cinco años de cosecha mediante análisis de regresión con modelos de efectos mixtos **(Capítulo 3 y Capítulo 4)**.

2- Estudios de caracterización genética y estructura poblacional en 186 accesiones de manzano (150 diploides y 36 triploides), mediante el uso de 23 marcadores moleculares del tipo microsatélites (SSRs). Además, se pretende hacer un estudio de mapeo por asociación y del desequilibrio de ligamiento de 118 variedades diploides y fenotipadas, con el fin de identificar marcadores moleculares asociados a los parámetros agronómicos, bioquímicos y organolépticos de la calidad del fruto **(Capítulo 5)**.

3- Estudio de una fisiopatía denominada ‘vitrescencia’ tanto a nivel pomológico, mediante caracterización de los compuestos antioxidantes y azúcares individuales, como a nivel genético, mediante expresión diferencial de genes, de cuatro variedades de manzana (tres susceptibles a vitrescencia y una de referencia) para determinar los factores relacionados con esta fisiopatía **(Capítulo 6)**.

4- Evaluación de 17 accesiones de la colección de manzano de los síntomas visuales de daños por frio (deshidratación y/o pardeamiento), de la calidad básica del fruto y de diferentes compuestos bioactivos (antioxidantes, azúcares individuales y ácidos orgánicos mayoritarios), tras seis meses de almacenamiento del fruto en cámara frigorífica a baja temperatura y con humedad controlada **(Capítulo 7)**.

Capítulo 3

Genetic origin and climate determine fruit quality and antioxidant traits on apple (*Malus x domestica* Borkh)

3.1 Abstract

Apples are highly appreciated in terms of flavor, nutritional and health-promoting compounds and are associated with a reduced risk to develop age-related and chronic diseases. In this study, 155 accessions of *Malus x domestica* Borkh from the apple germplasm collection, situated at the Experimental Station of Aula Dei in NE Spain, were analyzed during five consecutive years (2014-2018). Basic fruit quality traits including soluble solids content (SSC), titrable acidity (TA) and the ratio SSC/TA (ripening index - RI) were obtained. In addition, biochemical compounds such as total phenolics content (TPC), flavonoids (TFC), vitamin C (Ascorbic acid - AsA) and relative antioxidant capacity (RAC) were determined. Statistical analysis was used to determine differences in trait values among accessions and years. The well adapted local accessions showed, in general, higher average content of antioxidants and RAC compared with the foreign and commercial ones. A multivariate model was fitted with the accessions and climate features of each year as independent variables. A cluster analysis was then performed on the model coefficients space to classify the 155 accessions within five groups. The cluster analysis showed that foreign cultivars (i.e., those not originating from Spain) were concentrated in two groups while local accessions could not be segregated and had very different profiles. Furthermore, the concentration of bioactive compounds tended to decrease with higher temperatures, while increased with higher solar radiation. Statistical analyses emphasized differences between groups and highlighted accessions and climate as main factors affecting metabolite profiles and fruit characteristics.

Keywords: Flavonoids, meteorogical parameters, mixed-effects model, phenols, plant breeding, vitamin C

DOI: https://doi.org/10.1016/j.scienta.2021.110142**.**

3.2 Introduction

Apple (*Malus x domestica* Borkh, family *Rosaceae*, tribe *Pyreae*) is among the most widely consumed fresh fruits in the world. It has a special importance as one of the major temperate fruit crops cultivated globally. In 2019, more than 87 M tonnes were produced worldwide (FAOSTAT, 2021) and it ranked second in production after peaches and nectarines, and before pears, in Spain with more than 638 thousands of tonnes. Since its consumption is widespread in many countries and it is available on the market for the whole year, apple is, among fruits and vegetables, a major source of nutrients and bioactive compounds for humans (Michalska & Łysiak, 2015). The content of antioxidants present in apples is also important because of their contribution to the sensory quality of fresh fruit and processed apple products (Khanizadeh et al., 2008). Bioactive compounds improve the quality and the shelf life of vegetables and reduce the risk of post-harvest diseases (Bui et al., 2019; Davey et al., 2007). They also provide important health benefits to humans (Boeing et al., 2012; Gibney et al., 2019; Ho et al., 2020). In fact, apples are an important dietary source of potentially healthy biomolecules such as antioxidants. Moreover, there is an increasing evidence of a relationship between the consumption of fruits and vegetables and a reduced risk of human diseases such as cancer, heart coronary, cardiovascular, diabetes, Alzheimer’s diseases, and age-related functional decline (Zhang et al., 2016).

Currently, there are more than 7,000 documented apple cultivars in the world (Urrestarazu et al., 2016; Pereira-Lorenzo et al., 2017). However, the global production is dominated by relatively few well-adapted cultivars (Fuji, Gala, Golden, Granny Smith and Delicious), many of which are closely related (Urrestarazu et al., 2016; Ordidge et al., 2018), to the detriment of the locally well-adapted apple cultivars (Reig et al., 2015). This fact is leading to a dramatic loss of genetic diversity in the orchards and may also hamper future plant breeding while breeders require genetic variation for plant improvement (Swarup et al., 2020). Therefore, apple collections play a crucial role in preserving genetic diversity and providing breeding material (Muranty et al., 2020; Reig et al., 2015). Several apple germplasm banks are presently maintained in Spain, preserving mainly old cultivars which have been grown traditionally in their respective regions of origin (Pereira-Lorenzo et al., 2017), but also other foreign or commercial cultivars. Conservation of genetic diversity is important not only for wild species but also for cultivated plant species (Font i Forcada et al., 2014a; Guajardo et al., 2020; Reig et al., 2015). Monoculturalization has already driven out many local cultivars. Consequently, the collection, conservation and evaluation of local or old cultivars and wild relatives in germplasm banks are urgently required to prevent their extinction. Genetic diversity in local cultivars and wild relatives is thus very important, as it preserves genes that might be relevant from agronomical and fruit quality points of view (Reig et al., 2015; Font i Forcada et al., 2019a; Guajardo et al., 2020).

Preserving genetic variability is especially relevant in the context of climate change (Parajuli, 2019; Parry, 2019). Indeed, the complex quantitative nature of basic fruit quality traits and bioactive compounds can be affected by environmental conditions and agronomical management (Stewart & Ahmed, 2020). Actually, environmental variables such as climate parameters (solar radiation, precipitation, temperature…) influence the tree-growing environment providing wide variations in bioactive compounds accumulation (Cirilli et al., 2016; Yuri et al., 2009). The increasing temperatures observed in recent decades have already had a visible impact on plant growth and development (Fujisawa & Kobayashi, 2011; Li et al., 2020). Farmers and researchers are changing their behaviour by boosting and selecting climate-resilient apple cultivars (Beguería et al., 2019; Boudichevskaia et al., 2020). At this respect, there is a need to improve our understanding on how climate affects relevant fruit traits, and germplasm banks offer a good platform to assess this concern (Swarup et al., 2020). Climate experts have anticipated an increase in air temperature in the range of 2–5 °C (Benlloch-González et al., 2018) in the Mediterranean Basin.

To the best of our knowledge, no study has previously focused on the study of antioxidant compounds of local apple cultivars grown in Spain and conserved in germplasm banks. Nevertheless, most local and traditionally grown Spanish accessions have been characterized with SSR markers (Pereira-Lorenzo et al., 2017) as well as using morphological and phenological parameters (Reig et al., 2015). In other countries, several studies have reported apple antioxidant compounds only for a few number of cultivars and one or two years (McClure et al., 2019; McGhie et al., 2005; Yuri et al., 2009), in comparison with the 155 accessions and the five years considered in the present work. Moreover, studies concerning climate relationship with fruit quality traits are really scarce (Ahmadi et al., 2019; Kim et al., 2019).

Therefore, the goal of this study is to better characterize a higher number of apple accessions, increase the knowledge of nutritional fruit quality in local accessions and commercial cultivars, and search for the role of the climate and its importance onto the apple fruit quality to facilitate breeders improving nutraceutical properties and facing the changes due to global warming. To do that, fruit quality and biochemical compounds of 155 accessions from the first germplasm bank established in Spain will be assessed. In addition, a mixed model will be built searching the relationship between climate parameters and fruit quality traits.

3.3 Materials and methods

3.3.1. Plant material and field trial

The research work was conducted on the apple germplasm bank established at the Experimental Station of Aula Dei (EEAD-CSIC, Zaragoza, NE Spain: 41° 43′ 42.7″ N, 0° 48′ 44.1″ W). A total of 155 apple accessions [*Malus x domestica* Borkh], consisting of 99 local accessions and 56 foreign accessions were studied (Table 3.1). Indeed, most of the foreign accessions are commercial cultivars meanwhile, within the local accessions, autochthone commercial cultivars and traditional landraces are represented. The accessions were classified according to their skin colour as bicolour (95 accessions), red (9), green (34), yellow (14) and brown (3), with brown corresponding to skin completely covered by russeting. Each accession had three-tree replications established in a unique block design in the orchard. Trees were trained to a low density open-vase system (6 m × 5 m). Cultural management practices, such as fertilization and winter pruning, were conducted as in a commercial orchard. Trees were hand-thinned at 40–45 days after full bloom (DAFB), leaving one fruit per cluster. The orchard was flood irrigated every 12 days during the summer.

Table 3.1. Information of the 155 apple accessions used on this study.

Accession	Nº	Code EEAD	Classification	Origin	Skin color	Ploidy
Aciprés	1	3339 AD	Local	Huesca, SP	Bicolor	2
Akane	2	2902 AD	Foreign	Japan	Bicolor	2
Almenar_2 - MRF 46	3	3555 AD	Local	Lérida, SP	Bicolor	2
Ascara_1	4	3423 AD	Local	Huesca, SP	Bicolor	2
Ascara_2	5	3424 AD	Local	Huesca, SP	Bicolor	2
Astrakan Red	6	3378 AD	Foreign	Rusia	Bicolor	2
Audiena de Oroz	7	3375 AD	Local	Navarra, SP	Green	2
Augüenta	8	3335 AD	Local	Lugo, SP	Green	2
Averdal_1	9	882021	Foreign	-	Red	2
Averdal_2	10	892340	Foreign	-	Red	2
Baujade	11	923284 AD	Foreign	France	Green	2
Bellaguarda Lardero - MSV 27	12	3547 AD	Local	La Rioja, SP	Yellow	2
Belleza de Roma	13	638 AD	Foreign	Italy	Bicolor	2
Biscarri_1 - M 107	14	3726 AD	Local	Lérida, SP	Bicolor	2

Blackjon	15	2690 AD	Foreign	Wenatchee, USA	Bicolor	2
Bofla	16	3418 AD	Local	La Rioja, SP	Green	2
Boluaga	17	3340 AD	Local	Guipúzcoa, SP	Bicolor	3
Bossost_1 - MRF 75	18	3626 AD	Local	Lérida, SP	Bicolor	3
Bossost_2 - MRF 76	19	3627 AD	Local	Lérida, SP	Brown	3
Bossost_4 - MRF 78	20	3629 AD	Local	Lérida, SP	Bicolor	2
Bossost_5 - MRF 79	21	3630 AD	Local	Lérida, SP	Bicolor	3
Bost Kantoia	22	3341 AD	Local	Guipúzcoa, SP	Yellow	2
Cabdellà_2 - MRF 49	23	3613 AD	Local	Lérida, SP	Bicolor	2
Cabello de Angel	24	3255 AD	Local	Calatayud, SP	Yellow	2
Calvilla de San Salvador	25	3342 AD	Local	Zaragoza, SP	Bicolor	2
Camosa - MRF 42	26	3553 AD	Local	Lérida, SP	Bicolor	2
Camosa - MRF 60	27	3620 AD	Local	Lérida, SP	Bicolor	2
Camuesa de Daroca	28	3371 AD	Local	Zaragoza, SP	Green	2
Camuesa de Llobregat	29	1342 AD	Local	Barcelona, SP	Green	2
Camuesa Fina de Aragón	30	3372 AD	Local	Huesca, SP	Bicolor	2
Carapanón	31	3634 AD	Local	Asturias, SP	Bicolor	3
Carrió	32	3636 AD	Local	Asturias, SP	Bicolor	3
Cella	33	2512 AD	Local	Teruel, SP	Green	2
Ciri Blanc	34	3402 AD	Local	Gerona, SP	Green	2
Cirio - MRF 52	35	3615 AD	Local	Gerona, SP	Green	2
Cox's Orange Pippin	36	2889 AD	Foreign	England	Bicolor	2
Cripps Pink	37	933540 AD	Foreign	England	Bicolor	2
Cuallarga	38	3467 AD	Local	Gerona, SP	Green	2
Cul de Cirio - MRF 39	39	3551 AD	Local	Lérida, SP	Bicolor	2
De Agosto - MRF 57	40	3619 AD	Local	Lérida, SP	Bicolor	2
De Pera	41	3416 AD	Local	La Rioja, SP	Yellow	2
De Valdés	42	3632 AD	Local	Asturias, SP	Bicolor	2
Delciri	43	3413AD	Local	Baleares, SP	Yellow	2
Delcon	44	2896 AD	Foreign	USA	Bicolor	2
Delgared Infel	45	902708 AD	Foreign	-	Red	2
Deljeni	46	851305 AD	Foreign	Malicorne, France	Yellow	2
Delkistar	47	923273 AD	Foreign	USA	Bicolor	2
Delorgue Festival	48	913044 AD	Foreign	Malicorne, France	Bicolor	2
Elista	49	912883 AD	Foreign	Netherlands	Bicolor	2
Esperiega	50	3420 AD	Local	La Rioja, SP	Yellow	2
Esperiega de Olba - M 106	51	3725 AD	Local	Teruel, SP	Bicolor	2
Eugenia	52	3468 AD	Local	Gerona, SP	Bicolor	2
Evasni - Scarlet Spur	53	933554	Foreign	France	Bicolor	2
Florina	54	3633 AD	Foreign	Angers, France	Bicolor	2
Fuji	55	3488 AD	Foreign	Japan	Bicolor	2
Gala	56	3197 AD	Foreign	New Zeland	Bicolor	2
Galaxy	57	892451 AD	Foreign	New Zeland	Bicolor	2
Golden Delicious_675	58	675 AD	Foreign	-	Yellow	2
Golden Delicious Infel_972	59	2491 AD	Foreign	France	Yellow	2
Golden Paradise	60	3739 AD	Foreign	Spain	Yellow	2
Golden Smoothee	61	3286 AD	Foreign	West Virginia, USA	Yellow	2
Granny Smith_1	62	2614 AD	Foreign	Australia	Green	2
Granny Smith_2	63	3196 AD	Foreign	Australia	Green	2
Guillemes	64	3411 AD	Local	Baleares, SP	Bicolor	2
Hared	65	892232 AD	Foreign	France	Bicolor	2
Helada	66	3368 AD	Local	Baleares, SP	Green	2
Hierro	67	3374 AD	Local	Navarra, SP	Bicolor	2
Idared	68	2484 AD	Foreign	Idaho, USA	Bicolor	2
Irgo_2 - MRF 66	69	3622 AD	Local	Lérida, SP	Bicolor	2
Jonadel	70	2650 AD	Foreign	Iowa, USA	Bicolor	2
Jonagored	71	882001 AD	Foreign	Halen, Belgium	Bicolor	3
Jonathan_1	72	2495 AD	Foreign	New York, USA	Bicolor	2
Jonathan_2	73	3096 AD	Foreign	New York, USA	Bicolor	2
Jubilee	74	851304 AD	Foreign	Middlesex, England	Bicolor	2

Landetxo	75	3343 AD	Local	Navarra, SP	Bicolor	2
Les_1 - MRF 73	76	3624 AD	Local	Lérida, SP	Bicolor	2
Les_2 - MRF 74	77	3625 AD	Local	Lérida, SP	Bicolor	2
Mañaga	78	469 AD	Local	Huesca, SP	Green	2
Mañaga - MRF 43	79	3554 AD	Local	Lérida, SP	Bicolor	2
Marinera	80	3412 AD	Local	Baleares, SP	Bicolor	2
Marquinez	81	3419 AD	Local	La Rioja, SP	Bicolor	3
McIntosh	82	3192 AD	Foreign	Canada	Bicolor	2
Médulas_1 - MSV 38	83	3548 AD	Local	Lérida, SP	Bicolor	2
Melrose	84	2482 AD	Foreign	Ohio, USA	Bicolor	2
Merrigold	85	851307 AD	Foreign	France	Yellow	2
Montcada_1 - MRF 82	86	3631 AD	Local	Lérida, SP	Bicolor	2
Morro de Liebre	87	3256 AD	Local	Zaragoza, SP	Bicolor	2
Nesple	88	3410 AD	Local	Baleares, SP	Bicolor	2
Normanda	89	3252 AD	Local	Zaragoza, SP	Bicolor	3
Nueva Starking	90	1899 AD	Foreign	-	Red	2
Ortell	91	413 AD	Local	Zaragoza, SP	Bicolor	3
Ortell - MSV 24	92	3546 AD	Local	La Rioja, SP	Bicolor	2
Pera_2	93	3417 AD	Local	La Rioja, SP	Yellow	2
Pera de Sangüesa	94	3379 AD	Local	Navarra, SP	Green	3
Peromingan	95	1158 AD	Local	Asturias, SP	Green	2
Pero Pardo	96	3369 AD	Local	Navarra, SP	Green	3
Peruco de Caparroso	97	3373 AD	Local	Navarra, SP	Bicolor	2
Plaona	98	923283 AD	Foreign	-	Green	2
Poma de San Juan - MRF 47	99	3556 AD	Local	Lérida, SP	Bicolor	2
Prau Riu_3	100	3491 AD	Local	Asturias, SP	Bicolor	2
Prau Riu_4	101	3492 AD	Local	Asturias, SP	Bicolor	3
Prau Riu_5	102	3493 AD	Local	Asturias, SP	Green	2
Prima	103	851306 AD	Foreign	Illinois, USA	Red	2
Rebellón	104	3370 AD	Local	Navarra, SP	Bicolor	2
Red Delicious	105	3085 AD	Foreign	USA	Bicolor	2
Red Elstar	106	882002	Foreign	Netherlands	Bicolor	2
Red Rome Beauty	107	2897 AD	Foreign	Ohio, USA	Bicolor	2
Redaphough	108	933411 AD	Foreign	USA	Red	2
Red Chief	109	851308 AD	Foreign	USA	Bicolor	2
Regal Prince_1	110	882022 AD	Foreign	France	Bicolor	2
Regal Prince_2	111	892341 AD	Foreign	France	Bicolor	2
Reguard_1 - MRF 53	112	3616 AD	Local	Lérida, SP	Bicolor	2
Reguard_2 - MRF 54	113	3617 AD	Local	Lérida, SP	Bicolor	2
Reguard_4 - MRF 56	114	3618 AD	Local	Lérida, SP	Bicolor	2
Reina de Reinetas	115	2488 AD	Foreign	Netherlands	Bicolor	3
Reineta Blanca Canada_1	116	308 AD	Local	Zaragoza, SP	Green	3
Reineta Blanca Canada_2	117	3111 AD	Foreign	France	Green	3
Reineta Blanca Canada_3	118	3194 AD	Local	Zaragoza, SP	Green	3
Reineta Encarnada	119	3635 AD	Local	Asturias, SP	Bicolor	2
Reineta Gris	120	2883 AD	Local	Spain	Brown	3
Reineta Inesita Asua	121	2543 AD	Local	Bilbao, SP	Bicolor	3
Reineta Regil	122	3466 AD	Local	Vizcaya, SP	Green	3
Reneta	123	3408 AD	Local	Mallorca, SP	Bicolor	2
Roja Valle Benejama	124	1038 AD	Local	Valencia, SP	Bicolor	2
Roser de la Reula - MRF 40	125	3552 AD	Local	Lérida, SP	Bicolor	2
Royal Red Delicious	126	2363 AD	Foreign	USA	Bicolor	2
Rubinete	127	861526 AD	Foreign	Switzerland	Bicolor	2
Ruixou_1 - MRF 51	128	3614 AD	Local	Lérida, SP	Bicolor	2
San Felipe	129	3376 AD	Local	Navarra, SP	Bicolor	2
San Miguel	130	2579 AD	Local	La Rioja, SP	Bicolor	2
Sandia	131	3336 AD	Local	Lugo, SP	Bicolor	2
Sant Jaume	132	3470 AD	Local	Gerona, SP	Bicolor	3
Sant Joan	133	3409 AD	Local	Mallorca, SP	Bicolor	2
Santa Margarida	134	3401 AD	Local	Gerona, SP	Bicolor	3

Signatillis	135	3403 AD	Local	Gerona, SP	Green	2
Solafuente	136	3559 AD	Local	Cantabria, SP	Bicolor	3
Starking_1	137	2964 AD	Foreign	USA	Bicolor	2
Starking_2	138	632 AD	Foreign	USA	Bicolor	2
Starkrimson_1	139	1904 AD	Foreign	USA	Red	2
Starkrimson_2	140	3195 AD	Foreign	USA	Red	2
Taüll_1 - MRF 67	141	3623 AD	Local	Lérida, SP	Green	2
Telamon	142	3398 AD	Foreign	Kent, England	Bicolor	2
Tempera	143	3334 AD	Local	Lugo, SP	Green	2
Terrera	144	3469 AD	Local	Gerona, SP	Brown	3
Top Red Delicious	145	2651 AD	Foreign	USA	Red	2
Totxa	146	3471 AD	Local	Gerona, SP	Green	2
Transparente	147	3377 AD	Local	Navarra, SP	Green	2
Transparente Blanca	148	3344 AD	Local	Navarra, SP	Yellow	2
Urarte	149	3415 AD	Local	La Rioja, SP	Green	3
Urtebete	150	3345 AD	Local	Navarra, SP	Green	2
Valsaina	151	3558 AD	Local	Cantabria, SP	Bicolor	2
Verde Doncella - MRF 36	152	3549 AD	Local	Teruel, SP	Green	2
Verde Doncella_1	153	2125 AD	Local	Zaragoza, SP	Green	2
Verde Doncella_2	154	310 AD	Local	Zaragoza, SP	Green	2
Vinçada Tardía - MRF 61	155	3621 AD	Local	Lérida, SP	Green	3

AD, Aula Dei; EEAD, Experimetal Station of Aula Dei; SP, Spain; USA, United States of America

3.3.2. Leaf and Fruit sampling

To assess the ploidy level of the different accessions studied, newly expanded mature leaves were collected from each accession and analysed as described in Reig et al. (2015). The accessions were therefore classified into diploids (129 accessions) and triploids (26 accessions).

Regarding to the fruit sampling, a representative sample of 15 fruits (5 fruits × tree × rep.) were harvested when fruit firmness (FF) attained a value around 70–80 N or when they exhibited the ground colour representative. Maturity date ranged from late June to early December, depending on the accession. Fruit traits were measured for each accession at least three years within the period 2014-2018, and means for each season and accession were calculated.

3.3.3. Basic fruit quality traits

Soluble solid content (SSC) and titratable acidity (TA) were determined on flesh juice extracted by an automatic juicer (Philips, HR185890) with three replicates per accession and five fruits per replicate. Soluble solid content of fruit juice was measured with a digital refractometer (Atago PR-101, Tokyo, Japan) and was expressed as ºBrix. Titratable acidity was determined using an automatic titration system (EasyPlus Titrator, Mettler Toledo, US) with 0.1 N NaOH to a pH end point of 8.1. Results were expressed as g malic acid per litre. Ripening index (RI) was calculated based on the SSC/TA ratio.

3.3.4. Phytochemical traits

For the analysis of the total phenolics content (TPC), total flavonoids content (TFC), vitamin C (ascorbic acid – AsA) and the relative antioxidant capacity (RAC), a flesh sample composite of 5 g of five peeled fruits per replicate was frozen in liquid nitrogen and kept at −20°C until further analysis. Three replicates per accession were sampled and prepared. Samples were homogenized in a polytron (T25D Ultra-Turrax, IKA Works Inc., Wilmington, NC, USA) after one night in 10 mL of extraction solution [methanol/Milli-Q water, 80% (v/v) for TPC, TFC and RAC, and metaphosphoric acid, 5% (w/v) for vitamin C - AsA]. Extracts were centrifuged at 20,000 g for 30 min at 4°C, and the supernatant was collected and stored at −20°C. The phytochemical compounds were analysed using a 96-well microplate spectrophotometer photodiode array detector (Asys UVM 340 microplate reader; Biochrom, Cambridge, UK) as described by Font i Forcada et al. (2019a). The standard calibration curves were daily prepared on each microplate and eight different concentrations were used.

TPC was determined using the Folin-Ciocalteau method (Singleton et al., 1965) with modifications. Ninety microliters of diluted extract (1:8) were mixed with 80 µL of Folin-Ciocalteau reagent (0.25 N). The sample was incubated for three minutes before adding 30 µL of sodium carbonate [NaCO3, 11% (w/v)]. Then it was incubated for one hour in the dark at room temperature. Absorbance was measured at 725 nm and the results expressed in mg of GAE per 100 g of FW.

TFC was determined using a colorimetric assay based on the method of Zhishen et al. (1999) with minor modifications. Fifty microliters of the extract were mixed with 100 µL of Milli-Q water, 15 µL of sodium nitrite [$NaNO_2$, 5% (w/v)] and incubated for five minutes before adding 15 µL of aluminium chloride [$AlCl_3$, 10% (w/v)]. The microplate was incubated for three minutes more and finally 100 µL of sodium hydroxide 4% (NaOH) was added. Absorbance was measured at 510 nm. The results were expressed in mg CE per 100 g FW.

The RAC was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method adapted from Brand-Williams et al. (1995) with modifications. Ten microliters of the extract were mixed with 290 µL of DPPH (100µM in methanol, 80:20, v/v) and incubated in the dark at room temperature for 10 min. The absorbance was measured at 515 nm. The results were expressed in mg Trolox per 100 g FW.

Vitamin C – AsA was determined using the method for the spectrophotometric determination of ascorbic acid (AsA) as described by Zaharieva and Abadía (2003) with modifications. The extract (75 µL) was mixed with 75 µL of Milli-Q water, 60 µL of phosphoric acid diluted (H_3PO_4 85%/Milli-Q water 1:1), 60 µL of 2,2-bipyridyl [2,2-bipyridyl, 40:60, (w/v)/ethanol, 70:30, (v/v)] and 30µL of iron (III) chloride $FeCl_3.6H_2O$, 3% (w/v). Then it was incubated in a Memmert TM oven during one hour at 37ºC. Absorbance was measured at 525 nm and the results were expressed in mg AsA per 100 g FW.

3.3.5. Climate data

Climate variables as relative humidity (rh), maximum, minimum and mean temperature (tmx, tmn and tmd), rainfall (pre), and solar radiation (rad), were recorded daily during the study period (1st January 2014 to 31st December 2018). Data were downloaded from the Aula Dei meteorological station, nearby the orchard, which belongs to the official network of the Spanish meteorological service (AEMET). Relative humidity, maximum and minimum temperatures were daily recorded on a Rotronic HC2-S3 thermometer. Mean temperature was calculated as the average between tmx and tmn. Precipitation was recorded using a Pulsos ARG100 automatic raingage. Finally, solar radiation was measured on a Skye SP1110 pyranometer. The data were recorded automatically in a data-logger, and downloaded daily to a central server.

3.3.6. Data analysis

All statistical analyses were carried out using R software (R Development Core Team, 2019). The one-way analysis of variance (ANOVA) was run to determine whether there were any statistically significant differences between the means of the evaluated traits for the five years of study. The year effect, the accession, and their interaction (accession x year) effect were considered. The Pearson's correlations and a Principal Component analysis (PCA) were performed to understand how biochemical traits contribute to variability among accessions. A linear mixed-effects model (Pinheiro et al., 2019) was constructed using the different traits studied as dependent variables, the climate features of each growing season as independent variables or fixed effects, and the cultivar as random effect affecting both the intercept and the fixed effects coefficients. The growing season for the mixed-effect model was defined as the period between the 1st June and harvest. The date 1st of June was chosen in the aim to cover each accession growing period because the first harvest date is late June. On this date, all the accessions studied are on fruit setting and begin to grow regardless the harvest date.

The climate features were the total precipitation (mm), mean, maximum and minimum daily temperatures (ºC), mean daily temperature range (ºC), mean daily relative humidity (%) and mean daily solar radiation (W m^{-2}). Both the dependent and independent variables were centered and scaled to a common range so the model coefficients could be compared. A stepwise procedure based on the Akaike Information Criterion (AIC) statistic was used to select between alternative model configurations with different independent variables. The model coefficients were fitted by the maximum likelihood method, as implemented in the nlme package for R (Pinheiro et al., 2017).

A cluster analysis was applied to the accessions on the model coefficients' space, in order to determine groups of accessions with similar relationship between climate and biochemical traits. The cluster analysis was based on the Ward's D method based on the Euclidean distance (Ward et al., 1963).

3.3.7. Chemicals

All chemicals were of analytical grade. Aluminium chloride ($AlCl_3$), 3,4,5 trihydroxybenzoic acid (gallic acid), sodium carbonate (Na_2CO_3) and sodium hydroxide (NaOH) were purchased from PanReac Quimica SA (Barcelona, Spain). The bipyridyl, cathequin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), folin-ciocalteau's reagent, phosphoric acid (H_3PO_4), iron (III) chloride ($FeCl_3$), metaphosphoric acid (HPO_3), sodium nitrite ($NaNO_2$) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

3.4 Results

3.4.1. Accession and year effect

The statistical analysis was carried out with the 155 accessions presented in the Table 3.1. The ANOVA analysis showed significant differences ($P \leq 0.001$) among the different apple accessions for each year and for all traits evaluated (Figure 3.1). Additionally, statistical significance differences were also obtained for the interaction between accession and year for all traits studied ($P \leq 0.001$) (Supplementary Files 3.1 and 3.2).

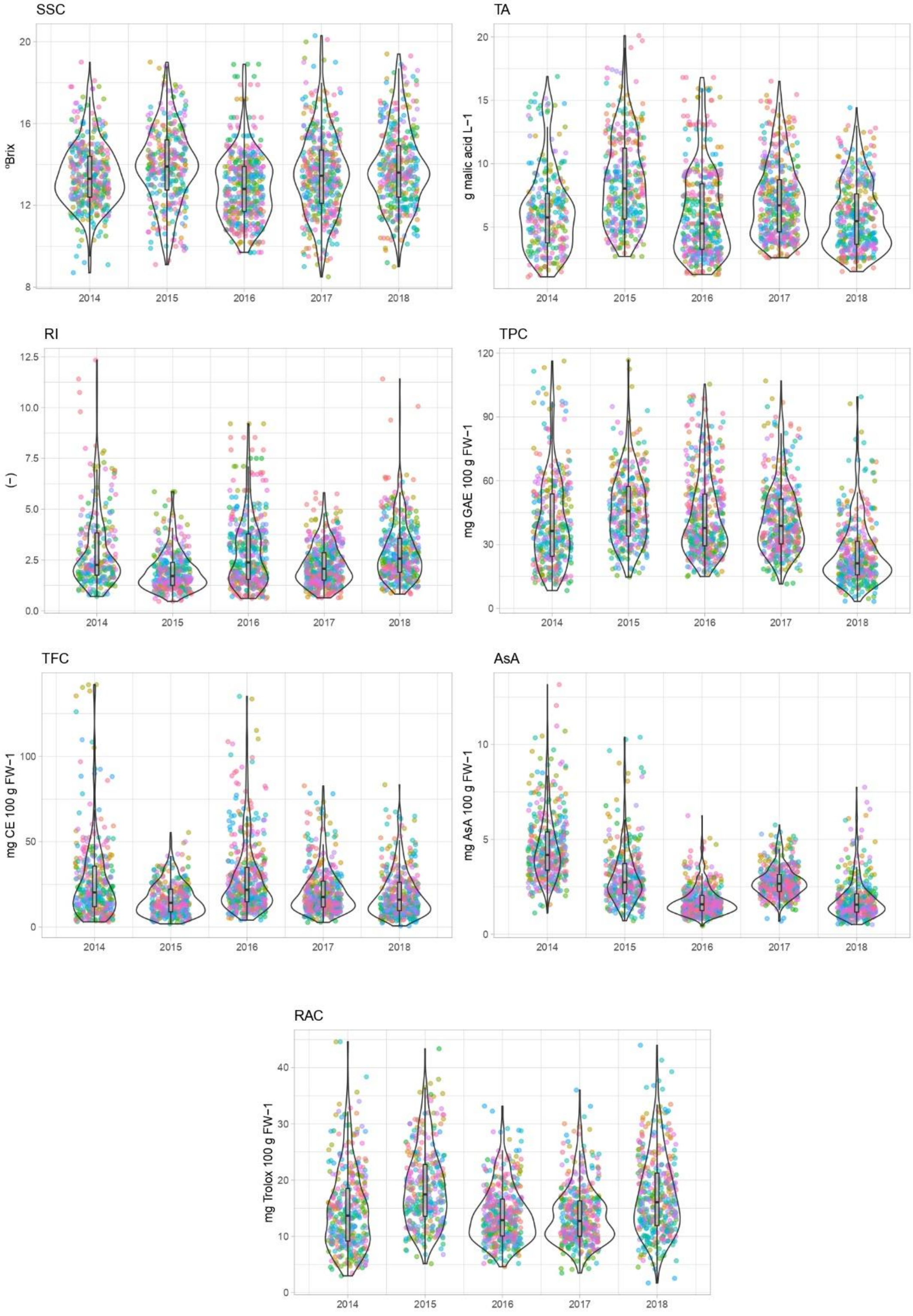


Figure 3.1. Violin plots showing the variability between accessions (dots) and years of fruit quality parameters, computed between June 1st each year and the harvest day. Abbreviations: soluble solids, SSC; titrable acidity, TA; ripening index, RI; total phenolic content, TPC; total flavonoid content, TFC; relative antioxidant content,RAC; ascorbic acid, AsA.

Differences on average daily temperature (tmd), average minimum daily temperature (tmn), average maximum daily temperature (tmx) and mean daily temperature range (trg), as well as solar radiation (rad), mean daily relative humidity (rh) and total precipitation (pre_tot) during the five years of the study were also found (Supplementary File 3.1). The 2018 climatic traits during the fruit growth period for each accession (from 1st June to harvest date) varied less than the other years of the study. Noneless, 2016 was the year with less precipitation and lower levels of relative humidity for the major part of the accessions. Meanwhile, 2017 seemed to be the year with more precipitation and higher relative humidity. Nevertheless, the values for rh in 2018 were even higher compared to the other four years (2014 – 2017). The years 2015 and 2017 were the hottest with higher values for tmd, tmn and tmx, while the 2014 was the coldest. The temperatures (tmd, tmx, tmn and trg) for 2014 were very similar for all the accession growing periods studied. According to the solar radiation, 2014, 2015 and 2017 had similar profiles for the growing periods of the different accessions, while solar radiation values tended to be lower in 2018.

3.4.2. Basic fruit quality traits, biochemical compounds and antioxidant activity

The soluble solids content (SSC) ranged among apple accessions and years from 8.5 (‘De Agosto – MRF 57’, in 2017) to 20.3 (‘Pera_2’, in 2017) ºBrix. Regarding the titrable acidity (TA), values varied greatly ranging from 1.1 (‘Verde Doncella – MRF 36’, in 2014) to 20.1 (‘Urarte’, in 2015) g malic acid per liter. Ripening index (RI=SSC/TA) values ranged from 0.5 (‘Urarte’, in 2015) to 12.3 (‘Verde Doncella – MRF 36’, in 2014). The standard deviation for SSC, TA and RI respectively were fitted at 1.9, 3.3 and 1.5 (Table 3.2). According to the mean of the five years of study (Supplementary file 3.3), SSC ranged from 10.1 (‘Bellaguarda Lardero – MSV 27’) to 17.8 (‘Terrera’) ºBrix, TA varied from 1.8 (‘Verde Doncella – MRF 36’) to 17.3 (‘Reguard_2 – MRF 54’) g malic acid per liter and RI varied from 0.7 (‘Urarte’) to 8.6 (‘Verde Doncella – MRF 36’).

The total phenolics content (TPC) varied greatly among apple accessions and years ranging from 3.3 (‘Poma de San Juan’, in 2018) to 116.7 (‘Camuesa Fina de Aragón’, in 2015) mg gallic acid equivalents (GAE)/100 g FW. For the total flavonoids content (TFC), values ranged from 0.7 (‘Poma de San Juan’, in 2018) to 142.1 (‘Camuesa Fina de Aragón’, in 2014) mg catechin equivalents (CE)/100 g FW. Regarding AsA (ascorbic acid – Vitamin C), values ranged from 0.4 (‘Delgared infel’, in 2016) to 13.2 (‘Transparente’, in 2014) mg AsA/100 g FW. Finally, relative antioxidant capacity (RAC) values ranged from 1.7 (‘Poma de San Juan’, in 2018) to 44.6 (‘Les_1 – MRF 73’, in 2014) mg trolox/100 g FW. The standard deviation for TPC, TFC, AsA and RAC were fitted at 19.4, 18.4, 1.6 and 6.6 respectively (Table 3.2). Regarding the mean of the five years of study (Supplementary File 3.3), TPC varied from 15.2 (‘Biscarri_1 – M 107’) to 98.1 (‘Camuesa Fina de Aragón’) mg gallic acid equivalents (GAE)/100 g FW, TFC ranged from 6.0 (‘Biscarri_1 – M 107’) to 89.0 (‘Camuesa Fina de Aragón’) mg catechin equivalents (CE)/100 g FW. According to AsA, values ranged from 1.4 (‘Delgared infel’) to 5.9 (‘Reguard_1 – MRF 53’) mg AsA/100 g FW. Finally, RAC varied from 5.9 (‘Delgared infel’) to 30.8 (‘Les_1 – MRF 73’).

Table 3.2. Basic statistics of fruit quality traits, biochemical compounds and antioxidant capacity over the accessions and years of the study: units, number of observed accessions (n), minimum, maximum, mean values, and standard deviation (SD).

Trait	Units	n	Minimum	Maximum	Mean	SD
SSC	ºBrix	155	8.5	20.3	13.5	1.9
TA	g malic acid L-1	155	1.1	20.1	6.8	3.3
RI	-	155	0.5	12.3	2.9	1.5
TPC	mg GAE 100 g FW-1	155	3.3	116.7	39.7	19.4
TFC	mg CE 100 g FW-1	155	0.7	142.1	23.1	18.4
AsA	mg AsA 100 g FW-1	155	0.4	13.2	2.8	1.6
RAC	mg Trolox 100 g FW-1	155	1.7	44.6	15.4	6.6

SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; RAC, relative antioxidant content.

Comparison of means for the different basic quality traits, biochemical compounds and antioxidant capacity (Figure 3.2), showed no significant differences between foreign and local accessions for SSC and RI. Nevertheless, several foreign accessions such as ‘Granny Smith_1’, ‘Granny Smith_2’, ‘Reineta Gris’ or ‘Redaphough’ showed lower levels of acidity (TA) than the local ones as the ‘Reguard_2 – MRF 54’, ‘Urarte’, ‘Bossost_2 – MRF 76’ and the ‘Transparente Blanca’ accessions. Regarding the bioactive compounds and the RAC, all traits showed significant differences between foreign and local accessions. Indeed, for all the parameters, foreign cultivars such as ‘Akane’, ‘Deljeni’, ‘Delorgue Festival’, ‘Reineta Gris’, among others, had lower values than local accessions such as ‘Camuesa Fina de Aragón’, ‘Les_1 – MRF 73’, ‘Peruco de Caparroso’, and ‘Prau Riu_5’, among others.

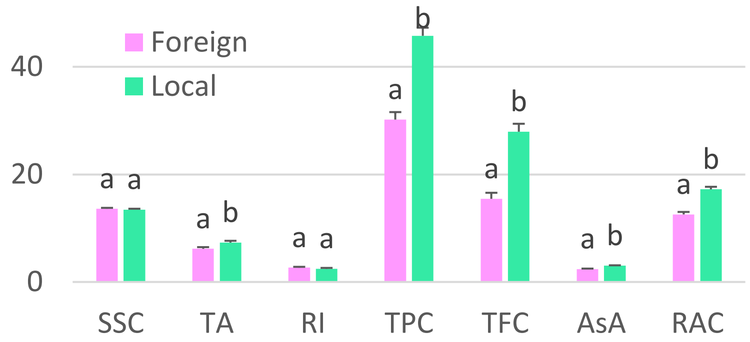


Figure 3.2. Mean for the different basic quality traits and biochemical compounds studied according to foreign or local accession. Vertical bars indicate ±SE. Different letters indicate significant differences ($p \leq 0.05$). Abbreviations: soluble solids, SSC; titrable acidity, TA; ripening index, RI; total phenolic content, TPC; total flavonoid content, TFC; relative antioxidant content,RAC; ascorbic acid, AsA.

3.4.3. Pearson's correlations

Significant ($P \leq 0.01$) bilateral correlations were found between all traits evaluated (Table 3.3). As expected, RAC was highly and positively correlated with TPC (r=0.901) and TFC (r=0.865). TPC was also significantly and highly positively correlated with TFC (r=0.963). In a minor proportion, AsA showed a significant and moderate positive correlation with TPC (r=0.415). In addition, significant moderate positive correlations were found between TA and TFC (r=0.464) and TPC (r=0.450).

Table 3.3. Pearson's correlation coefficients between traits.

	TFC	TPC	AsA	TA	RI
RAC	0.865**	0.901**	0.369**	0.282**	ns
TFC		0.963**	0.386**	0.450**	-0.315**
TPC			0.415**	0.464**	-0.296**
AsA				0.326**	-0.247**
SSC				ns	0.241**
TA					-0.841**

**: Statistical significance at $P \leq 0.01$; ns: not significant; SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; RAC, relative antioxidant content.

Table 3.4 shows the Pearson's correlation between the different climatic traits studied. Thus, tmd was highly and positively correlated with tmx (r=0.979), tmn (r=0.948) and trg (r=0.645). The mean daily temperature range (trg) was also highly correlated with tmx (r=0.780). Solar radiation (rad) was positively correlated with tmx (r=0.678), tmn (r=0.582), tmd (r=0.699) and trg (r=0.541). Relative humidity (rh) showed negative correlation with tmx (r=-0.739), tmd (r=-0.664) and trg (r=-0.822). Solar radiation was also highly correlated but in a negative way with rh (r=-0.777). Finally, the total precipitation showed a positive correlation with the rh (r=0.591) but negative with the solar radiation (r=-0.772). These correlations helped to the choice of the more significant parameters useful for the mixed-effects model and for the two-way hierarchical agglomerative cluster analysis.

Table 3.4. Pearson's correlation coefficients observed between climatological traits.

	pre	**tmx**	**tmn**	**tmd**	**trg**	**rh**	**rad**
pre_tot	0.085	-0.296**	-0.127	-0.281**	-0.399***	0.591***	-0.772***
pre		-0.127	0.007	-0.076	-0.251**	0.408***	-0.030
tmx			0.873***	0.979***	0.780***	-0.739***	0.678***
tmn				0.948***	0.376***	-0.454***	0.582***
tmd					0.645***	-0.664***	0.699***
trg						-0.822***	0.541***
rh							-0.777***

Statistical significance at **: P ≤ 0.01; ***: P ≤ 0.001; Total precipitation, pre_tot; precipitation, pre; average maximum daily temperature, tmx; average minimum daily temperature, tmn; average daily temperature, tmd; mean daily temperature range, trg; mean daily relative humidity, rh; solar radiation, rad.

3.4.4. Principal components analysis

A principal component analysis (PCA) was carried out to understand how traits could segregate the different accessions studied (Figure 3.3). The first two PCs, PC1 and PC2, accounted respectively for 50.5% and 21.3% of the total variability (Supplementary File 3.4). Indeed, a total of 71.8% of the variance could be explained accounting with only the two first PCs. The PC1 mainly contributed to biochemical traits (TPC, TFC, RAC and AsA) (Supplementary File 3.5). Accessions on the positive side of PC1 corresponding mainly to local accessions, induced, in general, higher values of those biochemical compounds (for instance 'Camuesa Fina de Aragón', 'Transparente', and 'Les-1'). In contrast, accessions on the negative side of PC1, corresponding to most of the foreign cultivars (for instance 'Nueva Starking', 'Averdal' and 'Evasni'), showed, in general, lower values for those biochemical compounds. Indeed, only eight foreign accessions were situated on the positive side of the PC1 (‘Akane’, ‘Astrakan Red’, ‘Cox’s Orange Pippin’, ‘Deljeni’, ‘Granny Smith_1’, ‘Granny Smith_2’, ‘Reineta Blanca del Canadá_2’ and ‘Reineta Gris’).

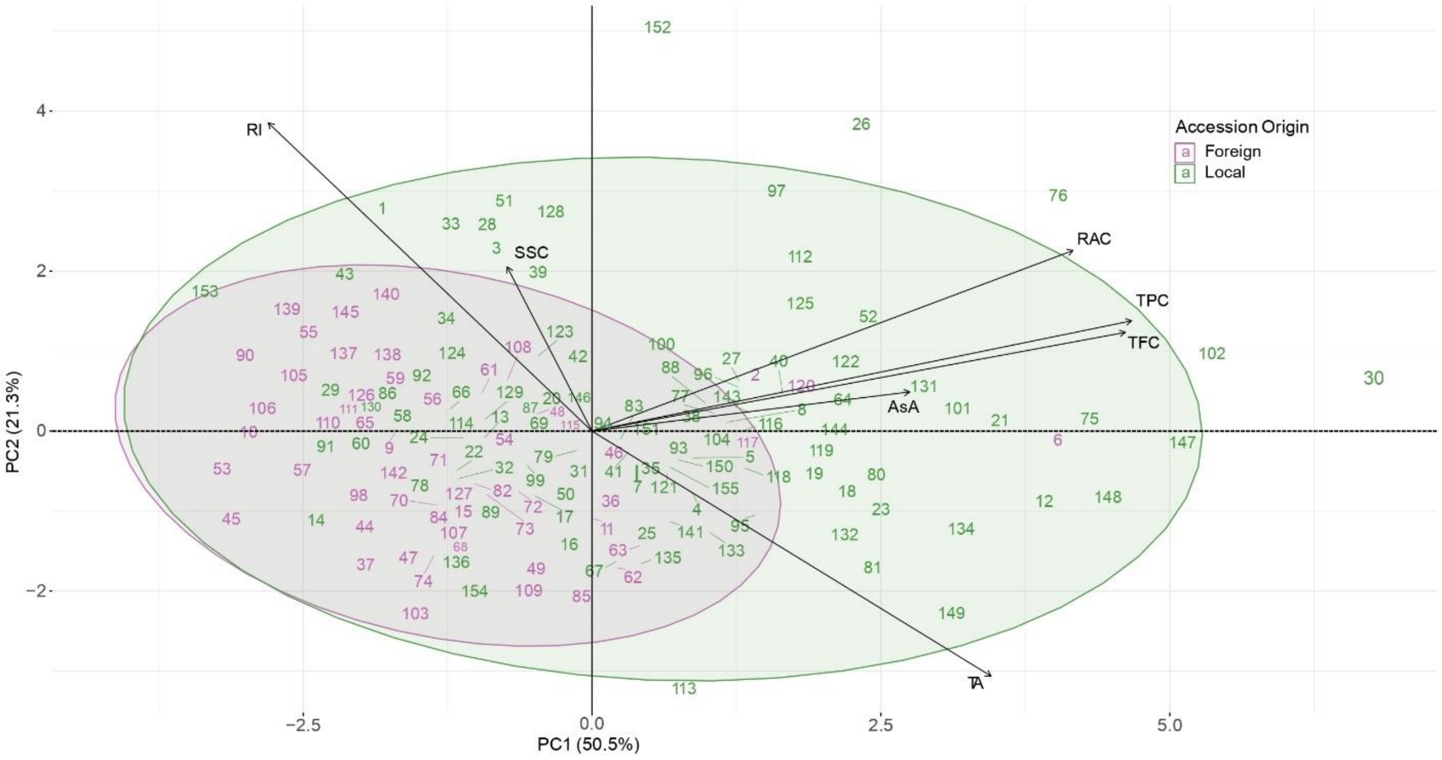


Figure 3.3. Principal components analysis (PCA) for the basic fruit quality and biochemical antioxidant traits evaluated on the 155 apple accessions. Analysis was performed using mean data of the 5 years of study (2014-2018). Abbreviations: SSC, soluble solids content; TA, titrable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; RAC, relative antioxidant content; AsA, ascorbic acid.

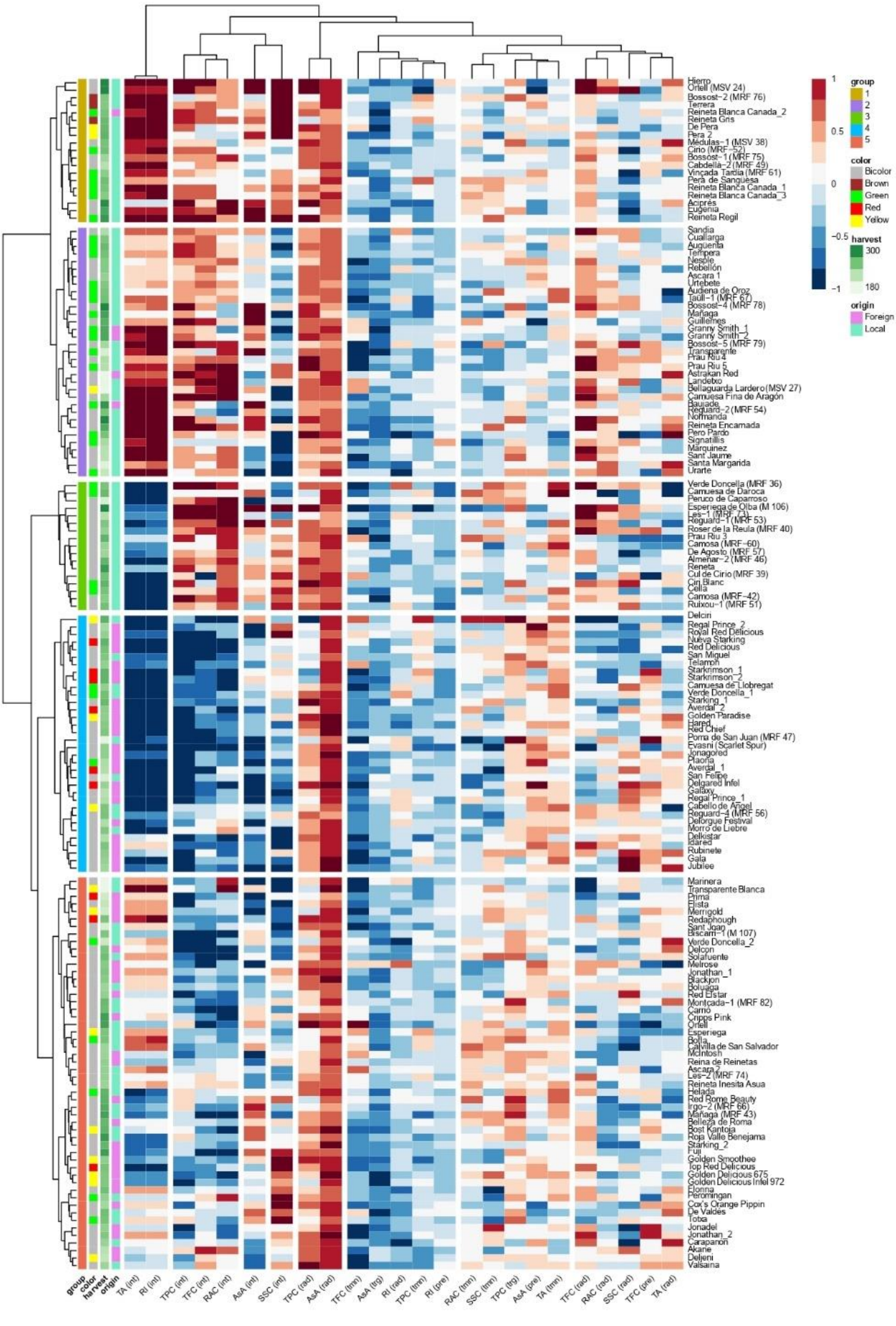
Moreover, the PC2 loadings suggested that separation on this component was mainly due to basic fruit quality parameters (TA, SSC and RI) (Supplementary File 3.5). On the negative side of PC2, accessions showed higher values for TA (for instance ‘Reguard-2’ and ‘Urarte’). A pool of foreign accessions with specific fruit quality values could be also identified through PCA analysis. The ‘Granny Smith’ cultivar seem to be the more acidic foreign accession according to the PCA, as it is commonly known. Nevertheless, the more acidic accession was ‘Reguard-2 - MRF 54’ which is a Spanish local accession. The ‘Granny Smith_1’ is the first most acidic foreign accession but there are 22 local accessions with higher TA values (Supplementary File 3.3).

3.4.5. Mixed-effect model and relationships between accessions, climate and genetic origin

Based on the highest correlations between the climatic traits above mentioned and a stepwise procedure based on the Akaike Information Criterion (AIC) statistic, the best significant model (lowest AIC) for the TPC analysis included the following three variables: tmn, trg and rad. The random effects indicated that there were differences between accessions in both their intercepts (mean TPC values) and climate coefficients. For the TFC analysis, the best significant model (lowest AIC) included pre, rad and tmn. The best significant models for RAC, SSC and TA analysis included rad and tmn. Regarding the AsA analysis, the best significant model included pre, rad and trg. Finally, the RI analysis model included pre and rad. The results of the mixed-effects models were significant for all variables (basic fruit quality traits, antioxidant compounds and relative antioxidant capacity), demonstrating that there is a close relationship between the evaluated fruit traits and the climate characteristics of the growing season.

A two-way hierarchical agglomerative cluster analysis was used to classify the 155 apple accessions studied into groups according to their model coefficients (Figure 3.4). Clusters of accessions would therefore indicate a similar relationship between antioxidant traits and climate, while clusters of variables would group model coefficients that tended to behave similarly across the accessions.

Concerning the effect of climate traits, there were small differences between clusters regarding the effect of solar radiation, with an overall positive effect for TPC and AsA in all cases (shown in red colours in Figure 3.4) and negative effect for RI. Similarly, temperature tended to have a negative effect on TPC, TFC, and AsA, while the results were more mixed for other variables. Precipitation had an overall negative effect on RI, although lower than the other climate variables. The main effect of high temperature was to reduce the phenolics and flavonoids content and RAC, while solar radiation had a positive and strong influence on all of them.


Hierro
Orlell (MSV 24)
Bossost-2 (MRF 76)
Terrera
Reineta Blanca Canada_2
Reineta Gris
De Pera
Pera 2
Médulas-1 (MSV 38)
Cirio (MRF-52)
Bossost-1 (MRF 75)
Cabdellà-2 (MRF 49)
Vinçada Tardia (MRF 61)
Pera de Sangüesa
Reineta Blanca Canada_1
Reineta Blanca Canada_3
Aciprés
Eugenia
Reineta Regil
Sandia
Cuallarga
Agüenta
Tempera
Nesple
Rebellón
Ascara 1
Urtebete
Audiena de Oroz
Taüll-1 (MRF 67)
Bossost-4 (MRF 78)
Mañaga
Guillemes
Granny Smith_1
Granny Smith_2
Bossost-5 (MRF 79)
Transparente
Prau Riu 4
Prau Riu 5
Astrakan Red
Landetxo
Bellaguarda Lardero (MSV 27)
Camuesa Fina de Aragón
Baujade
Reguard-2 (MRF 54)
Normanda
Reineta Encarnada
Pero Pardo
Signatillis
Marquinez
Sant Jaume
Santa Margarida
Urarte
Verde Doncella (MRF 36)
Camuesa de Daroca
Peruco de Caparroso
Esperiega de Olba (M 106)
Les-1 (MRF 73)
Reguard-1 (MRF 53)
Roser de la Reula (MRF 40)
Prau Riu 3
Camosa (MRF-60)
De Agosto (MRF 57)
Almenar-2 (MRF 46)
Reneta
Cul de Cirio (MRF 39)
Ciri Blanc
Cella
Camosa (MRF-42)
Ruixou-1 (MRF 51)
Delciri
Regal Prince_2
Royal Red Delicious
Nueva Starking
Red Delicious
San Miguel
Telamon
Starkrimson_1
Starkrimson_2
Camuesa de Llobregat
Verde Doncella_1
Starking_1
Averdal_2
Golden Paradise
Hared
Red Chief
Poma de San Juan (MRF 47)
Evasni (Scarlet Spur)
Jonagored
Plaona
Averdal_1
San Felipe
Delgared Infel
Galaxy
Regal Prince_1
Cabello de Angel
Reguard-4 (MRF 56)
Delorgue Festival
Morro de Liebre
Delkistar
Idared
Rubinete
Gala
Jubilee
Marinera
Transparente Blanca
Prima
Elista
Merrigold
Redaphough
Sant Joan
Biscarri-1 (M 107)
Verde Doncella_2
Delcon
Solafuente
Melrose
Jonathan_1
Blackjon
Boluaga
Red Elstar
Montçada-1 (MRF 82)
Carrió
Cripps Pink
Orlell
Esperiega
Bolla
Calvilla de San Salvador
McIntosh
Reina de Reinetas
Ascara 2
Les-2 (MRF 74)
Reineta Inesita Asua
Helada
Red Rome Beauty
Irgo-2 (MRF 66)
Mañaga (MRF 43)
Belleza de Roma
Bost Kantoia
Roja Valle Benejama
Starking_2
Fuji
Golden Smoothee
Top Red Delicious
Golden Delicious 675
Golden Delicious Infel 972
Elonna
Peromingan
Cox's Orange Pippin
De Valdés
Totxa
Jonadel
Jonathan_2
Carapanon
Akane
Deljeni
Valsaina
group
color
harvest
origin
TA (int)
RI (int)
TPC (int)
TFC (int)
RAC (int)
AsA (int)
SSC (int)
TPC (rad)
AsA (rad)
TFC (tmn)
AsA (trg)
RI (rad)
TPC (tmn)
RI (pre)
RAC (tmn)
SSC (tmn)
TPC (trg)
AsA (pre)
TA (tmn)
TFC (rad)
RAC (rad)
SSC (rad)
TFC (pre)
TA (rad)
1
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0
-0.5
-1
group
1
2
3
4
5
color
Bicolor
Brown
Green
Red
Yellow
harvest
300
180
origin
Foreign
Local

Figure 3.4. (Page 74) Two-way hierarchical analysis of the 155 accessions based on the Euclidean distance on the model coefficients space. Apple accessions are grouped into five groups according to the dendrogram. Positive and negative coefficients indicate the intercept (int) and the effect of climate variables (minimum temperature, tmn; temperature range, trg; precipitation, pre; radiation, rad) on different biochemical traits (SSC, soluble solids content; TA, titrable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; RAC, relative antioxidant content; AsA, ascorbic acid). Additionally, values of auxiliary variables not used in the cluster analysis are also shown: skin colour, harvest date (in Julian days), and origin.

Table 3.5. Mean values of the mixed-effect model coefficients of the traits studied for each cluster for the 155 accessions.

Clusters	**1**	**2**	**3**	**4**	**5**
AsA (int)	0.492	0.244	0.283	-0.568	-0.056
AsA (pre)	-0.044	-0.060	-0.037	0.396	0.126
AsA (rad)	0.694	0.688	0.780	0.880	0.811
AsA (trg)	-0.561	-0.495	-0.495	-0.325	-0.442
RAC (int)	0.398	0.466	0.932	-0.582	-0.332
RAC (rad)	0.051	0.277	0.129	-0.144	-0.158
RAC (tmn)	0.156	-0.190	0.080	-0.082	0.079
RI (int)	1.133	0.962	-0.953	-0.931	0.102
RI (pre)	-0.064	-0.033	-0.365	-0.276	-0.131
RI (rad)	-0.200	-0.182	-0.024	-0.055	-0.243
SSC (int)	0.943	-0.483	0.346	-0.239	0.031
SSC (rad)	-0.243	0.092	-0.015	0.167	-0.132
SSC (tmn)	0.006	-0.098	-0.002	-0.114	0.082
TA (int)	1.064	0.893	-1.022	-1.000	0.033
TA (rad)	0.156	0.098	-0.025	0.015	0.053
TA (tmn)	-0.017	-0.002	0.439	0.346	0.142
TFC (int)	0.546	0.533	0.508	-0.598	-0.313
TFC (pre)	-0.049	-0.033	-0.094	0.196	0.000
TFC (rad)	0.458	0.600	0.475	-0.098	0.077
TFC (tmn)	-0.236	-0.467	-0.313	-0.505	-0.321
TPC (int)	0.743	0.671	0.790	-0.908	-0.362
TPC (rad)	0.700	0.658	0.671	0.344	0.422
TPC (tmn)	-0.117	-0.285	-0.202	-0.070	-0.151
TPC (trg)	0.218	0.156	0.225	0.304	0.309

Intercept, int; minimum temperature, tmn; temperature range, trg; precipitation, pre; solar radiation, rad; soluble solids, SSC; titrable acidity, TA; ripening index, RI; total phenolic content, TPC; total flavonoid content, TFC; relative antioxidant content,RAC; ascorbic acid, AsA.

Table 3.5 shows the mean for each cluster; meanwhile the Table 3.6 considers whether the accession is a local or a foreign one. It is easily noticed that the foreign accessions showed in general lower values for TPC, TFC, RAC, AsA, SSC, TA and RI. As previously said for the TPC and AsA, solar radiation had a great and positive effect independently of the cluster. However, solar radiation showed different profiles whether the accession was local or foreign for TFC, RAC and SSC (Table 3.6).

Table 3.6. Mean values of the mixed-effect model coefficients of the traits studied according to foreign or local accession.

Clusters	Foreign	Local
TPC (int)	-0.624	0.353
TPC (rad)	0.442	0.558
TPC (tmn)	-0.118	-0.189
TPC (trg)	0.235	0.266
TFC (int)	-0.356	0.201
TFC (pre)	0.133	-0.045
TFC (rad)	0.043	0.352
TFC (tmn)	-0.432	-0.352
RAC (int)	-0.464	0.280
RAC (rad)	-0.170	0.088
RAC (tmn)	≤ 0.001	-0.007
AsA (int)	-0.353	0.200
AsA (pre)	0.260	0.020
AsA (rad)	0.855	0.741
AsA (trg)	-0.403	-0.473
SSC (int)	-0.002	0.015
SSC (rad)	0.017	-0.040
SSC (tmn)	-0.036	-0.008
TA (int)	-0.339	0.191
TA (rad)	0.065	0.017
TA (tmn)	0.229	0.135
RI (int)	-0.270	0.260
RI (pre)	-0.188	-0.143
RI (rad)	-0.159	-0.160

Intercept, int; minimum temperature, tmn; temperature range, trg; precipitation, pre; solar radiation, rad; soluble solids, SSC; titrable acidity, TA; ripening index, RI; total phenolic content, TPC; total flavonoid content, TFC; relative antioxidant content,RAC; ascorbic acid, AsA.

Two big clusters were identified in the accessions axis, and a separation into five smaller clusters was selected as optimum (Figure 3.4). The clusters were numbered according to the height at which they separate from the remaining group. The first three clusters included respectively 19, 33 and 17 accessions; the fourth cluster grouped 34, while the last one included 52 accessions. There seemed to exist a relationship between the clusters and the origin of the accession. Thus, clusters 1, 2 and 3 included mostly local accessions (64 out of 69 accessions). Clusters 4 and 5, included 51 out of the 56 foreign accessions present in the collection. With regard to the ploidy of the accessions studied, more than 75% of the triploid accessions were grouped in the first major cluster which included clusters 1 and 2.

Regarding the independent variables, the hierarchical classification tended to group together the intercepts of the models, related to the mean values of the biochemical traits, and then the climatic variables. Accessions from clusters 1 and 2 had similar profiles for the intercept of basic fruit quality traits (TA and RI) and antioxidant parameters (TPC, TFC, RAC, AsA), which were both very high. Cluster 3 had very high antioxidant profiles as clusters 1 and 2, but instead it had low values of the basic fruit quality traits as the fourth cluster. Cluster 4 had low coefficients for both fruit quality traits and antioxidants, while cluster 5 was characterized by relatively low correlations profiles for antioxidants and more mixed profiles for basic quality traits. The profiles for ascorbic acid were more mixed, although some clusters had consistently low (Cluster 4) or high (clusters 1, 2 and 3) profiles. A similar situation was found for the SSC.

3.5 Discussion

In the present study, basic fruit quality traits (TA, SSC and RI) and biochemical compounds (TPC, TFC, AsA), as well as antioxidant capacity (RAC), were evaluated on 155 apple cultivars for five years (2014-2018). As expected, the levels of the assessed traits differed greatly among accessions and years, as demonstrated in previous studies (Boyer and Liu, 2004; Reig et al., 2015; Van Der Sluis et al., 2001), indicating that both accession and year had consistent effects on the evaluated parameters. Significantly, other studies in peach (Font i Forcada et al., 2014b, 2019a, Iglesias et al., 2019) and apricot (Gómez-Martínez et al., 2021) also reported a year effect due to the climatic parameters that affected fruit quality, biochemical compounds and relative antioxidant capacity. Values were, in general, within the range reported for apple cutivars for the basic fruit quality traits (Guan et al., 2015; Reig et al., 2015; Slatnar et al., 2019; Wu et al., 2007; Zhen et al., 2018), as well as for the antioxidant biochemical compounds (Boyer and Liu, 2004; Slatnar et al., 2019; Yuri et al., 2009). However, in the present work, the range varied in a higher extent due to the extremely different plant material studied, probably because of different genetic background in comparison to commercial cultivars. In fact, this is the first study reporting fruit quality, bioactive compounds levels and relative antioxidant capacity profile of these accessions for a long period of time (five years) and relating these parameters to the climatic conditions. It is interesting to note that the local accessions ‘Camuesa Fina de Aragón’, ‘Les_1 – MRF 73’, ‘Prau Riu_5’ and ‘Transparente’, showed higher values than expected for TPC, TFC and RAC. In fact, the first twenty accessions ranking with higher TPC, TFC and RAC values were local. With regards to the foreign accessions, ‘Akane’, ‘Deljeni’, ‘Delorgue Festival’, ‘Granny Smith_1’, ‘Granny Smith_2’, ‘Red Rome Beauty’, ‘Reineta Blanca Canada_2’ and ‘Reineta Gris’ had the highest content of TPC, TFC and RAC, but, in general, they had 1.5-2 times lower values than those presented by ‘Camuesa Fina de Aragón’, ‘Les_1 – MRF 73’, ‘Prau Riu_5’ and ‘Transparente’.

The PCA analysis of the 155 accessions demonstrated a great variability between accessions. It also showed that foreign accessions were more closely related between them, constituting a foreign genetic pool. The PCA results showed that foreign accessions can be segregated in a single group because they shared similar values for the traits studied. However, local accessions cannot be segregated in a single group, as they have some really different profiles, being diffuse in the whole graph. Pereira-Lorenzo et al. (2017) studied a large set of apple genotypes conserved in different Spanish collections using microsatellite markers. They discriminated an Iberian genepool of apple accesions from a wide set of foreign cultivars. Similarly, in the present work, we discriminated, in the PCA results, two groups in accordance with the genetic diversity of the Spanish core collection proposed by Pereira-Lorenzo et al. (2017). In the two-way hierarchical analysis based on the Euclidean distance we could find these two different groups whether the accession is local or foreign too. Indeed, clusters 1, 2 and 3 included 64 local accessions out of 69 total accessions and clusters 4 and 5, grouped 51 out of the 56 foreign accessions present in the collection.

The health benefits could be one of the main attributes to promote fruit consumption. Many studies have shown that antioxidant compounds are among the most benefic biomolecules ingested in fruit consumption (Boeing et al., 2012; Boyer et al., 2004; Lattanzio, 2013). This study showed lower values for TA for most of the foreign accessions and higher values for SSC and thus for RI. This fact leads us to hypothesize that foreign accessions or commercial cultivars had the acidity (TA) balanced by the sweetness (SSC) in the aim of the acceptance by customers, as it has also been proposed by other studies (Jakobek et al., 2020; Musacchi et al., 2018). Nevertheless, as the acidity was correlated moderately but significantly with the biochemical compounds, this could indicate that taste and the organoleptic properties of pulp were ranked first on the past breeding programs beyond health benefits provided by antioxidants (Gómez-Martínez et al., 2021; Preti & Tarola, 2020).

Interestingly, the two-way cluster analysis showed apparently no relationship between the different clusters and harvest date or skin color. All studied genotypes had similar tree management and were grown under the same climatic and soil conditions. Actually, the climatic conditions for a given year only differed between cultivars due to the different lengths of their growing periods, controlled by the harvest date. It must be noted that the variability range of the climatic conditions was limited to between-years variation, as the experiment was carried out in one single location. There is a much wider range of variability to be expected if the experiment was carried out in a variety of location of contrasting environmental conditions (climate), which could possibly lead to a stronger climatic variability than found here.

The year effect should be explained by the climatic variables (temperatures, precipitation, radiation, etc.), although it is difficult to explain the individual contributions of the various possible environmental factors (Lattanzio, 2013). Actually, in addition to a direct impact on metabolite levels (basic fruit quality traits and antioxidant biosynthesis), environmental conditions will also affect general fruit physiology and development (Davey et al., 2007). Light and temperature affect many fruit quality traits, including sugar nutrient levels, texture, taste and flavour (Davey et al., 2007; Fischer et al., 2016). Apples contain several antioxidants originated from different pathways and accumulated in the fruit as a consequence of sun exposure (Lattanzio, 2013). It is well known that flavonoids are an important group of phenolic compounds in apples and both contribute significantly to the relative antioxidant capacity of fruits (Bui et al., 2019; Lattanzio, 2013; Marks et al., 2007). Previous studies in apples reported similar positive correlations between TPC, TFC, and RAC, as found in this work (Cocci et al., 2006; Preti & Tarola, 2020; Raudone et al., 2017; Vieira et al., 2009; Wang et al., 2015). The total phenolics are the main compounds responsible for keeping fruits from UV/high light damage (Li et al., 2013). This accumulation of phenolics might be related to the photoprotective function of phenolics under high light and UV irradiation (Lattanzio, 2013; Li et al. 2008). Agroclimatic traits also influenced bioactive compounds values (AsA, TFC, TPC and RAC), in general, by increasing their levels, as previously demonstrated in other studies with apple cultivars (Yuri et al., 2009; McGhie et al., 2005). Furthermore, Li et al. (2013) showed that Gallic acid content became higher in the sun-exposed peel as fruit developed. Our work shows a positive effect of solar radiation in the flesh fruit by increasing the biochemical compound levels for AsA, TFC and TPC. In the case of the RAC, solar radiation increased their values for the local accessions, but decreased them for the foreign cultivars. In the present study, solar radiation exhibited also a high contribution to the AsA values in flesh tissue for all the studied accessions. In contrast, previous works demonstrated that solar radiation only influenced the AsA values in the apple peel and not in the apple flesh (Li et al., 2009). In some works, a higher accumulation of AsA was found in the sun-exposed side compared to the shaded side (Bui et al., 2019). However, the exact impact of solar radiation in AsA contents in apple flesh fruit is not clear yet, as the available studies and cultivars evaluated show contradictory results with the present work (Bui et al., 2019; Li et al., 2009). Nevertheless, few cultivars have been studied, and very few multi-year studies exist. The same occurs with the temperature traits. Davey et al. (2007) showed a negative correlation between average preharvest daytime temperature and AsA values in whole fruits, whereas Łata (2007), found a significantly higher total amount of AsA in the warmer year of their study. In the present work, considering five years and 155 accessions, a significant effect between lower temperatures and higher biochemical compound values was obtained. Lattanzio et al. (2001) demonstrated that the low temperature effect involved a cold-induced stimulation of the phenylalanine ammonia-lyase (PAL) activity in apple as well as other enzymes important in the phenolic biosynthetic pathway.

Biochemical compounds play an important role in postharvest prevention of several diseases (Chagné et al., 2019; Davey et al., 2007). Indeed, different links exist between crop antioxidant defence, maintenance of postharvest fruit quality and harvest date (Davey et al., 2007). Piretti et al. (1994) found that the concentration of antioxidant compounds decreased during storage in ‘Granny Smith’ apple cultivar. Bui et al. (2019) showed that higher exposure of apple fruit to sun in the field might lead to an improved tolerance to postharvest fungi infection by increasing the antioxidant superoxide dismutase (SOD) and ascorbate peroxidase (APX) enzyme activity. The general accumulation and increase in TPC, TFC, AsA and higher RAC values due to solar radiation, demonstrated in the present work, highlight the positive effect of radiation on the bioactive compounds. Besides this positive effect, local accessions seem to be more affected than the foreign ones, demonstrating the importance of the local fitogenetic resources and their adaptation to the conditions prevailing in the central Ebro Valley (Reig et al., 2015). In fact, some of the antioxidant compounds might have antifungal activity too, as the flavonoid, quercetin-3-galactoside reported by Bui et al. (2019) Both higher content of phenolic compounds (Li et al., 2013) and higher activities of key enzymes involved in the antioxidant metabolism (Davey et al., 2007, Lattanzio et al., 2001) suggest that the polyphenolic metabolism in apples is upregulated by high irradiance (Lattanzio, 2013; Li et al., 2013).

Finally, statistical analyses highlighted apple accessions, year and climate traits as main factors affecting metabolite profiles and fruit quality characteristics. Our work emphasizes that research could lead to the development and selection of cultivars (Pereira-Lorenzo et al., 2017; Verma et al., 2019) having the additional benefit of improved nutritional value (Font i Forcada et al., 2019a; Yuri et al., 2009) for the consumer. Indeed, accessions that have a higher content of antioxidants can be selected to promote their positive effect on health (Boeing et al., 2012; Boyer et al., 2004), and can be selected to be consumed in different ways (fresh, juice, dry snacks, etc.). Moreover, application of high-throughput phenotyping techniques in the germplasm collection, in combination with SSRs (Pereir-Lorenzo et al., 2017) or high-density SNP genotyping (Font i Forcada et al., 2019b; Vanderzande et al., 2019), could offer additional valuable information in interpreting the genetic control of fruit quality traits in apple.

3.6 Conclusions

This study shows a considerably high biodiversity in the apple germplasm studied for the content of bioactive compounds and basic fruit quality traits. We found a higher average content of antioxidants in the local accessions as compared with the foreign ones, which should increase awareness of the importance of the local phytogenetic resources. Moreover, we found that climatic traits such as precipitation, solar radiation, and temperature strongly influenced the antioxidant and metabolite profiles of the different accessions studied. The bioactive compound values tended to decrease, in general, with higher temperatures, while they increased with precipitation and solar radiation. Albeit the limited range of variation of climatic conditions in our study (as only between-year variation in a single location was considered), this significance of the climatic parameters highlights the importance of the geographic region where the crop is cultivated in the resulting bioactive compounds. Genetic progress could be greatly affected by selecting the right accessions on future breeding programs to improve biochemical characteristics desired of fruits depending on the area of the crop.

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Capítulo 4

Effect of genetics and climate on apple sugars and organic acids profiles

4.1 Abstract

Apple is widely cultivated in temperate regions. The beneficial properties of apple for preventing several illnesses are widely known. Nevertheless, qualitative variables such as sweetness or sourness may influence consumer satisfaction. They are critical factors for fruit consumption and essential in plant breeding. In the present work, 155 apple accessions were assessed during five consecutive years (2014-2018). Four individual sugars and seven organic acids were analysed by HPLC. A mixed-effects model was fitted with accessions and the years' climatic features as independent variables. A cluster analysis was applied on the mixed-effects model coefficients. Four groups were considered as optimum. Genetics seemed to have the strongest effect and showed clear differences between accession groups, while climate effects were strong only for certain compounds and had a more horizontal behaviour affecting equally the different accession groups. In fact, non-Spanish cultivars tended to concentrate while autochthone accessions had a much wider spread. Individual sugars and acids concentrations correlated negatively with precipitation and positively with temperature range and solar radiation in all accession groups. The geographic region where the orchard is grown is thus very important in the resulting metabolites profiles. Moreover, apple genetics would also play a decisive role as highlighted in the cluster analysis.

Keywords: *Malus x domestica* Borkh, fruit quality, temperature, solar radiation, HPLC, fructose, sorbitol, malic acid

DOI: https://doi.org/10.3390/agronomy12040827.

4.2 Introduction

Apple has been cultivated in Europe and Asia since antiquity (Mushtaq et al., 2020), and it is currently the largest fruit crop in temperate regions. Globally, apple production ranked 86.4 million tons worldwide (FAOSTAT, 2021). Therefore, its availability in markets all year around makes apple fruit the most consumed fresh fruit in the world.

Apple is well known for being a source of dietary fiber, micro-nutrients such as vitamin C, sugars, organic acids, minerals, and phytochemical compounds (Kim et al., 2019; Mignard et al., 2021; Verma et al., 2019; Zhen et al., 2018), and its beneficial properties for human health is also well established (Boeing et al., 2012; Gibney et al., 2019; Ho et al., 2020; Zhang et al., 2016). Qualitative traits such as sweetness, sourness, skin colour, fruit firmness, and other organoleptic characteristics strongly influence consumer satisfaction (Font i Forcada et al., 2019; Jakobek et al., 2020; Minas et al., 2018. Among the latter, sourness and sweetness are the main factors influencing fruit palatability and consumption. Therefore, they are among the most relevant traits considered in plant breeding programs (Aprea et al., 2017). A combination of organic and mineral acids such as citric, malic, tartaric, quinic or succinic are responsible for sourness (Briand et al., 2016), but fruit acidity not only affects the overall apple flavour but also influences the perception of other organoleptic traits such as sweetness and aroma (Iwanami et al., 2012; Verma et al., 2019). Apart from individual acids concentration, sourness is usually assessed by the titratable acidity (TA), while the soluble solids content (SSC) is used as a proxy to fruit sweetness (Charles et al., 2018; Yang et al., 2021; Zhang et al., 2010).

Besides playing an important role in fruit consumption, sugars and acids play an essential role in plant development and stress response (Amri et al., 2021; Zhao et al., 2020). Sucrose is the main product of photosynthesis in mature green leaves (Ruan, 2014), and it can be further transformed to fructose and glucose (Yang et al., 2018). Fructose is essential for regulating carbon fluxes in apple sink cells (Li et al., 2012; Yang et al., 2018; Zhao et al., 2020). Sorbitol accumulation, because of its high level of transport in apple trees, protects the cell membranes, decreases membrane permeability, and increases apples' sweetness (Aprea et al., 2017). It is the major sugar alcohol found in *Rosaceae* plants. Organic acids, as malate and citrate in lower extend, are the primary acids involved in fruit acidity. They are synthesized in plants during the oxidation of different photosynthetic products (Vallarino & Osorio, 2019). The organic acids play an essential role in plants by providing redox equilibrium, supporting ionic gradients on membranes, and acidifying the extracellular medium (Igamberdiev et al., 2016; Maeda & Dudareva, 2012). Improving our understanding of the genetic variability of apple acids and sugars is therefore highly desirable, as it could help breeders develop new apple cultivars with target sourness and sweetness levels.

There are more than 7,000 apple cultivars worldwide (Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2016). Nevertheless, a relatively small number of well-adapted and closely-related cultivars dominate the global apple production. This lack of production diversity impedes consumers to access well-adapted local apple accessions from countries where apple is traditionally grown, such as Spain (Mignard et al., 2021; Urrestarazu et al., 2016; Ordidge et al., 2018; Swarup et al., 2020). While breeders require more and more genetic variation for plant improvement, apple monoculturalization is causing a loss of genetic diversity and puts autochthone cultivars at risk of disappearing. Therefore, the collection and conservation of local and ancient accessions and wild relatives are top priority activities. Germplasm banks are crucial tools to preserve genetic diversity, as well as to provide breeding material (Font i Forcada et al., 2014; Guajardo et al., 2020; McClure et al., 2019; Muranty et al., 2020; Swarup et al., 2020; Vanderzande et al., 2019). Spain has very rich apple genetic resources spanning a wide range of agro-climatic conditions, including historical cultivars selected for centuries from wild local populations. This rich heritage is preserved in several apple collections that include traditional cultivars (Castel et al., 2020; Pereira-Lorenzo et al., 2017; Reig et al., 2015). These collections also contain non-Spanish accessions and commercial cultivars, enabling comparison between cultivars of varying origin.

In the increasingly relevant context of climate change, preserving genetic variability is especially important (Parajuli et al., 2019; Parry, 2019). Selection and enhancement of climate-change resilient apple varieties is becoming a priority for both growers and scientists (Beguería et al., 2003; Boudichevskaia et al., 2020). Besides orchard management, environmental parameters such as altitude or climate have an enormous effect in apple's nutrients and biochemical traits (Cirilli et al., 2016; Mignard et al., 2021; Stewart et al., 2020; Yuri et al., 2009). Sugar concentration in fruit usually decreases with water supply due to dilution (Mills et al., 1996), but this process depends on the fruit growth dynamics, and thus it varies between cultivars and in interaction with the environmental conditions (Cakpo et al., 2020; Cirilli et al., 2016). It has also been demonstrated that altitude causes concurrent changes in temperature, light intensity, and photoperiod. These elements play a crucial role in controlling plant metabolism, and therefore affect the final contents of several biochemical compounds in fruits, highlighting the importance of the region where the fruits are grown (Kumar et al., 2019; Li et al., 2009). Several studies have highlighted the impacts of increasing temperatures on plant growth and development (Fujisawa and Kobayashi, 2011; Li et al., 2020), and in most regions farmers have already acknowledged its effect on delaying fruit maturity, leading to the adoption of varied adaptation strategies (Ahmadi et al., 2018; Gitea et al., 2019).

There is an essential need to improve our understanding of the differences between apple cultivars in organoleptic traits and in the concentrations of biochemical compounds, and to better know how climate interacts with genetics in their determination. Germplasm banks offer an excellent platform to undertake this assessment (Swarup et al., 2020), although there's still a relatively small number of studies concerning climate and apple quality traits (Ahmadi et al., 2019; Boudichevskaia et al., 2020; Kim et al., 2019). Regarding specifically the relationships between climate and individual sugars and organic acids in apple cultivars of varying origin, we are not aware of any previous study.

This study evaluates individual sugars and organic acids of 155 accessions during five consecutive years from the first established Spanish apple collection. The main objectives of the present study were: i) to improve our knowledge of nutritional and organoleptic traits according to the cultivar's origin; ii) to assess the role of each years' climate on the apple metabolite profiles and iii) improving our understanding of the genetic variability of apple acids and sugars as it could help breeders develop new apple cultivars with target sourness and sweetness levels.

4.3 Materials and methods

4.3.1. Field trial, plant material and climate data

The location, names and number of the 155 accessions evaluated are described in Supplementary File 1. All the studied plant material was grafted on MM 106 rootstock, except for four accessions ('Delgared Infel', 'Red Elstar', 'Rubinete' and 'Regal Prince_1') which were grafted on the M9 rootstock. Trees were trained to a low density open-vase system (6 m × 5 m). Fertilization and winter pruning were conducted as in a commercial orchard. Trees were hand-thinned at 40–45 days after full bloom (DAFB), leaving one fruit per cluster. The orchard was flood irrigated every 12 days during the summer. The climatic parameters assessed for the years of the study were reported by Mignard et al. (2021).

4.3.2. Fruit sampling, individual sugars, and organic acids analysis

Each year, a representative random sample was harvested for each accession. Fruits were harvested when flesh fruit firmness (FF) attained a value around 70–80 N or when they exhibited the ground color representative. Maturity date ranged from late June to early December, depending on the accession. Then, apple flesh was sampled during the following 1-2 days as described by Mignard et al. (2021). Three biological replicates per accession were prepared. Individual sugars and organic acids content was analysed by HPLC as reported by Font i Forcada et al. (2019). In summary, sugars were analyzed using an Aminex HPX-87C column (300 mm x 7.8 mm, Biorad) with a refractive index detector at 35ºC (Waters 2410, Waters Corporation, Milford, USA) with milliQ water as mobile phase. For the organic acids, a Rezex™ ROA-Organic Acid H+ (8%) column (300 mm x 7.8 mm, Phenomenex) was used with a photodiode array detector (Waters 2489, Waters Corporation, Milford, USA) at 210 nm. A sulphuric acid solution (0.005N) was used as mobile phase in the latter case. Individual sugars (glucose, fructose, sucrose, and sorbitol) and organic acids (citric, malic, oxalic, quinic, succinic, shikimic, and tartaric) concentrations (expressed as g per kg of fresh weight) were determined by their characteristic retention times following standards mentioned in the chemicals paragraph (section 2.4).

4.3.3. Data analysis

All statistical analyses were carried out using the R language (R Development Core Team, 2019), involving the following packages: *ggplot2*, *factoextra*, *FactoMineR*, *ggpubr*, *dplyr*, *Hmisc*, *corrplot* and *nlme* (Pinheiro et al., 2017). Two-way ANOVA was used to examine accession x year effects. Pearson’s correlation was used to study the relationships among traits, and Principal Component Analysis (PCA) was used to simplify the dimensionality of the dataset and find the principal axes of variability.

A mixed-effects model was then constructed using the individual sugars and organic acids traits as dependent variables, the climate characteristics of each growing season as fixed effects, and the accession as the random effect affecting both the intercept and the fixed effects coefficients. All variables were centred at zero and scaled to a common range in order to make the model coefficients comparable. Hierarchical agglomerative cluster analysis based on the Manhattan distance was then used to assess similarities between climatic and biological variables across the accessions.

4.3.4. Chemicals

Chemicals of analytical grade were used in all analyses. Sugar standards (sucrose, glucose, fructose, and sorbitol) and the sulfuric acid were purchased from PanReac Química, S.A. (Barcelona, Spain), while organic acids standards (citric, malic, oxalic, quinic, succinic, shikimic, and tartaric) were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

4.4 Results

4.4.1. Accession and year effects

There were significant differences among the 155 accessions for all the evaluated traits according to ANOVA analyses. In addition, the interaction between accession and year was significant for all traits evaluated ($P \leq 0.001$) (Supplementary Files 2, 3 and 4).

The climatic characteristics during the fruit growth season also showed significant differences between years. A thorough description of the climatic variables during the five years of study was reported by Mignard et al. (2021).

4.4.2. Individual sugars and organic acids profiles

Total sugars values (Sugars) ranged significantly among apple accessions and years from 25.02 (‘Morro de Liebre’, 2015) to 159.79 (‘Baujade’, 2015) g kg^{-1} FW (Table 1). Regarding the individual sugars, sucrose (Suc) values varied considerably, ranging from 3.26 (‘Morro de Liebre’, 2016) to 61.18 (‘Baujade’, 2015) g kg^{-1} FW. Glucose (Glu) values ranged from 2.18 (‘McIntosh’, 2016) to 37.56 (‘Baujade’, 2015) and fructose (Fru) values ranged from 14.63 (‘Morro de Liebre’, 2016) to 87.40 (‘Médulas_1 - MSV 38’, 2015) g kg^{-1} FW. Finally, the alcohol sugar sorbitol (Sor) values varied from 0.48 (‘Red Chief’, 2018) to 26.23 (‘Bossost_2 - MRF 76’, 2015) g kg^{-1} FW.

The Supplementary File 4 shows the mean values for the five years of study. Total sugars ranged from 61.85 (‘Urarte’) to 121.61 (‘Reineta Regil’) g kg^{-1} FW. Sucrose varied from 10.29 (‘Akane’) to 44.47 (‘Reineta Blanca Canada_1’) g kg^{-1} FW, while glucose and fructose ranged from 6.23 (‘Roja Valle Benejama’) to 24.29 g kg^{-1} FW (‘Ruixou_1 – MRF

51') and 31.39 ('Baujade') to 61.41 ('Akane') g kg^{-1} FW, respectively. Lastly, sorbitol varied from 1.20 ('Plaona') to 12.29 ('Terrera') g kg^{-1} FW.

Table 4.1. Average values for individual sugars and organic acids traits over accessions and years: units, minimum, maximum, mean values, and standard deviation (SD).

Trait	Units	Minimum	Maximum	Mean	SD
Sugars	g kg^{-1}	25.02	159.79	88.94	17.80
Sucrose	g kg^{-1}	3.26	61.18	25.75	9.17
Glucose	g kg^{-1}	2.18	37.56	13.16	5.68
Fructose	g kg^{-1}	14.63	87.40	45.38	8.96
Sorbitol	g kg^{-1}	0.48	26.23	4.65	3.41
Acids	g kg^{-1}	0.773	22.832	6.786	2.336
Oxalic	g kg^{-1}	0.010	0.020	0.015	0.001
Citric	g kg^{-1}	0.010	0.312	0.057	0.034
Tartaric	g kg^{-1}	0.017	0.271	0.047	0.021
Malic	g kg^{-1}	0.435	19.843	5.689	2.119
Quinic	g kg^{-1}	0.116	2.035	0.435	0.210
Succinic + Shikimic	g kg^{-1}	0.080	2.284	0.555	0.285

Total acids values (Acids) among accessions and years ranged from 0.77 ('Golden Smoothee', 2016) to 22.83 ('Astrakan Roja', 2017) g kg^{-1} FW (Table 1). Regarding the individual acids, oxalic acid (Oxa) values varied from 0.01 ('Starking_1', 2018) to 0.02 ('Terrera', 2016) g kg^{-1} FW. Citric acid (Cit) values ranged from 0.01 ('Roja Valle Benejama', 2017) to 0.31 ('Transparente', 2016) g kg^{-1} FW and tartaric acid (Tar) values ranged from 0.02 ('Roja Valle Benejama', 2017) to 0.27 ('Transparente', 2016) g kg^{-1} FW. Malic acid (Mal) ranged considerably, compared with the others, from 0.43 ('Golden Smoothee', 2016) to 19.84 ('Astrakan Roja', 2017) g kg^{-1} FW and quinic acid (Qui) varied significantly from 0.12 ('Golden Smoothee', 2016) to 2.03 ('Terrera', 2016) g kg^{-1} FW. Finally, succinic + shikimic acids (Succ+Shi) varied from 0.08 ('Delorgue Festival', 2017) to 2.28 ('Poma de San Juan - MRF 47', 2015) g kg^{-1} FW.

Regarding the five-years mean values (Supplementary File 4), total acids varied among apple accessions from 3.42 ('Verde Doncella_2') to 12.58 ('Urarte') g kg^{-1} FW. Oxalic acid ranged from 0.014 ('Starking_2') to 0.018 ('Prau Riu_5') g kg^{-1} FW, whereas citric and tartaric acid ranged from 0.02 ('Cul de Cirio - MRF 39') to 0.15 ('Transparente') and 0.03 ('McIntosh') to 0.09 ('Camuesa de Llobregat') g kg^{-1} FW, respectively. Malic acid varied notably from 2.68 ('Delciri') to 10.69 ('Urarte') g kg^{-1} FW, while quinic acid ranged from 0.24 ('Verde Doncella_2') to 0.87 ('Urarte') g kg^{-1}

FW. Finally, Succinic + Shikimic acids varied from 0.20 (‘Granny Smith_2’) to 1.68 (‘Poma de San Juan - MRF 47’) g kg^{-1} FW.

The quantitative profiles of the individual sugars and organic acids for the 155 apple accessions for the mean of the five years of study are shown in Figure 4.1. Firstly, it is easily noticed the great variability found in this study for all the traits assessed. Moreover, for most of the variables, the data set could be well modelled by a normal distribution and thus, seemed to be normally distributed. In general, the concentration of the organic acids were lower than the sugars amounts even though malic acid had higher concentrations. According to the two major sugar and organic acid respectively, fructose reached 61.41 g kg^{-1} FW (‘Akane’) while normally was between 40-50 g kg^{-1} FW and malic acid reached the largest concentration in ‘Urarte’ (10.69 g kg^{-1} FW) although usually it was within the range 4-8 g kg^{-1} FW.

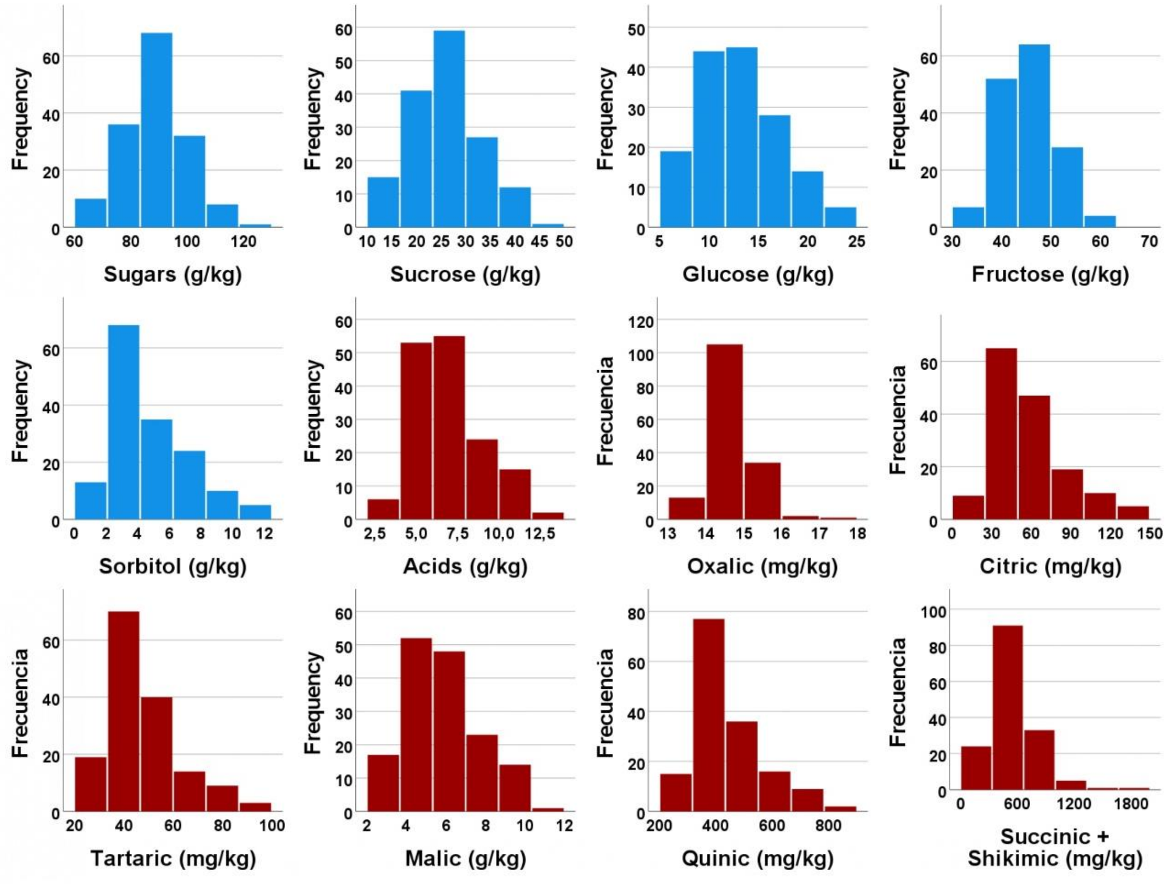


Figure 4.1. Quantitative profiles of the individual sugars and organic acids for the 155 apple accessions during the five years of study (2014-2018).

4.4.3. Pearson correlation coefficients

There were significant bilateral correlations between traits ($P \leq 0.01$) (Figure 4.2 and Supplementary File 5). Total sugars were significantly and positively correlated with the individual sugars (Suc: r=0.639; Fru: r=0.691; Sor: r=0.651 and Glu: r=0.285). Glucose and sucrose were significantly and negatively correlated (r=-0.340). The three major acids (malic, quinic and citric) were significantly and positively correlated between them and with total acids. First, malic acid showed significant and positive correlations with total acids (r=0.990), as well as quinic acid (r=0.803) and citric acid (r=0.856). Quinic acid was significantly and positively correlated with total acids (r=0.853), and citric acid (r=0.724). Moreover, total acids were significantly and positively correlated with citric acid (r=0.861). In general, individuals and total sugars were negatively correlated with the individuals and total organic acids, when significant.

In addition, a hierarchical agglomerative cluster heatmap on the Pearson correlation coefficients was used to split the different traits into two groups of increasing similarity (Supplementary Files 6). All the sugars were grouped in one cluster, while a second cluster grouped all the organic acids traits.

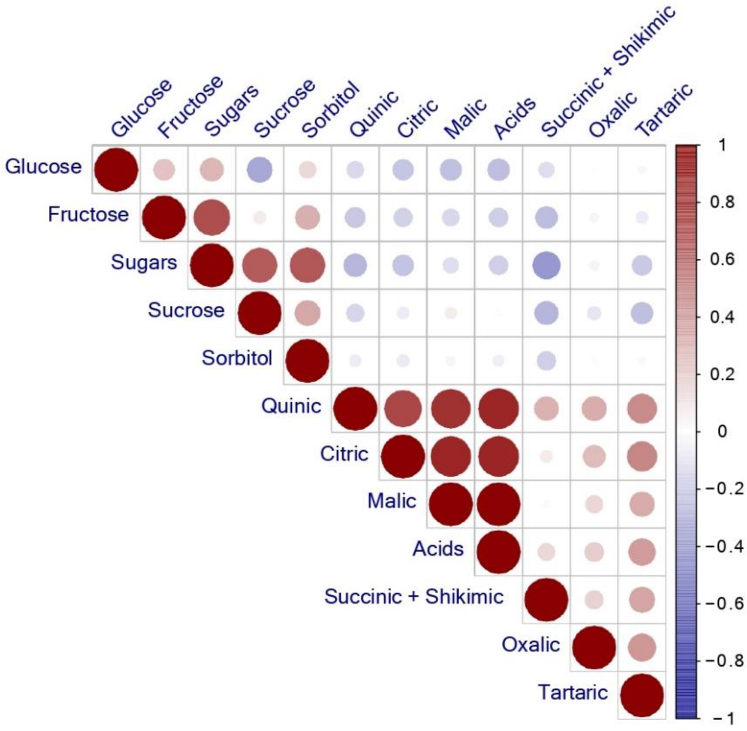


Figure 4.2. Pearson's correlation coefficients for the traits studied for the 155 accessions assessed and the five years of study. Abbreviations: Sugars, total sugars; Acids, total organic acids. The size of the circle for each correlation and the colour depicts the significance and the magnitude of the correlation coefficient, respectively

4.4.4. Principal components analysis

The first two components accounted for 55.5% of the total variance, with PC1 accounting for 35.6% and PC2 accounting for 19.9% (Figure 4.3, Figure 4.4 and Supplementary File 7). Therefore, further analysis was based on these two components.

PC1 correlated mostly with total and individuals organic acids (Figure 4.3 and Supplementary File 8). Total acids and sugars were the two traits with more contribution in the two-dimensional principal component analysis. Malic, citric and quinic acids had a great contribution in the total variance while glucose and oxalic acid had a very low contribution in the two first PCs (Figure 4.3). The Figure 4.4 showed the PCA plot for the 155 accessions included in this study for the 12 variables studied. Accessions with positive loading on PC1, mainly Spanish accessions (‘Bellaguarda Lardero – MSV 27’, ‘Santa Margarida’, ‘Transparente’, ‘Transparente Blanca’, ‘Urarte’, ‘Verde Doncella_MRF 36’) as well as the non-Spanish accession ‘Astrakan Red’ were characterized by higher values of organic acids and other biochemical compounds. On the contrary, accessions with negative loadings (including 44 out of 56 non-Spanish accessions such as ‘Fuji’, ‘Nueva Starking’, ‘Regal Prince_1’, ‘Galaxy’, ‘Redaphough’, ‘Starkrimson_1’, or ‘Evasni’) had lower values for these traits.

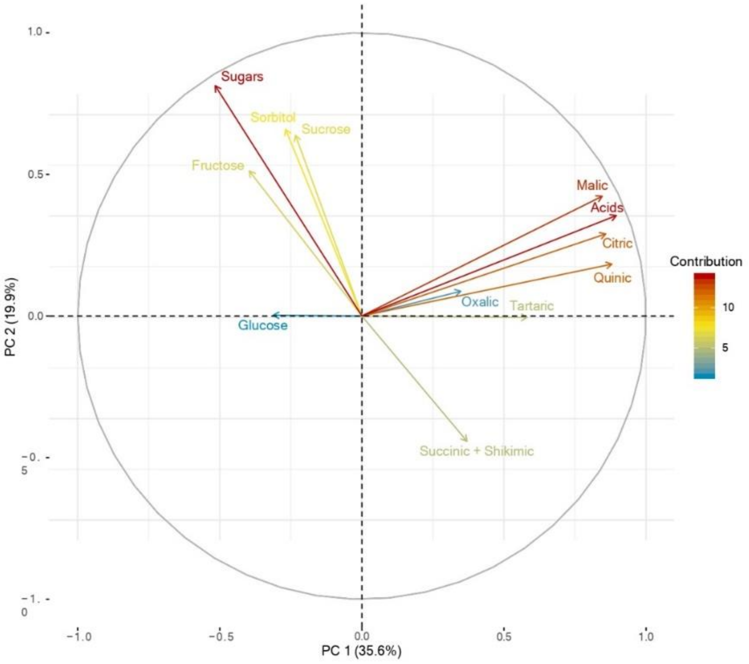


Figure 4.3. Two-dimensional principal component analysis (PCA) plot for all the 12 variables assessed. The colours showed the contributions of variables accounting for the total variability. Abbreviations: Sugars, total sugars; Acids, total organic acids.

Separation on the PC2 component was mainly due to total and individuals sugars (Supplementary File 8). Accessions with positive loadings on PC2 showed higher values for sweetness traits: 'Eugenia', 'Reineta Regil', 'Reineta Blanca Canada_1', 'De Pera' or 'Pera 2'. Moreover, the three brown accessions of the study ('Bossost_2 - MRF 76', 'Reineta Gris' and 'Terrera'), should be highlighted for their higher sugar values. Furthermore, it is noteworthy that the non-Spanish accession 'Fuji' showed an extreme sweetness value compared to other non-Spanish accessions.

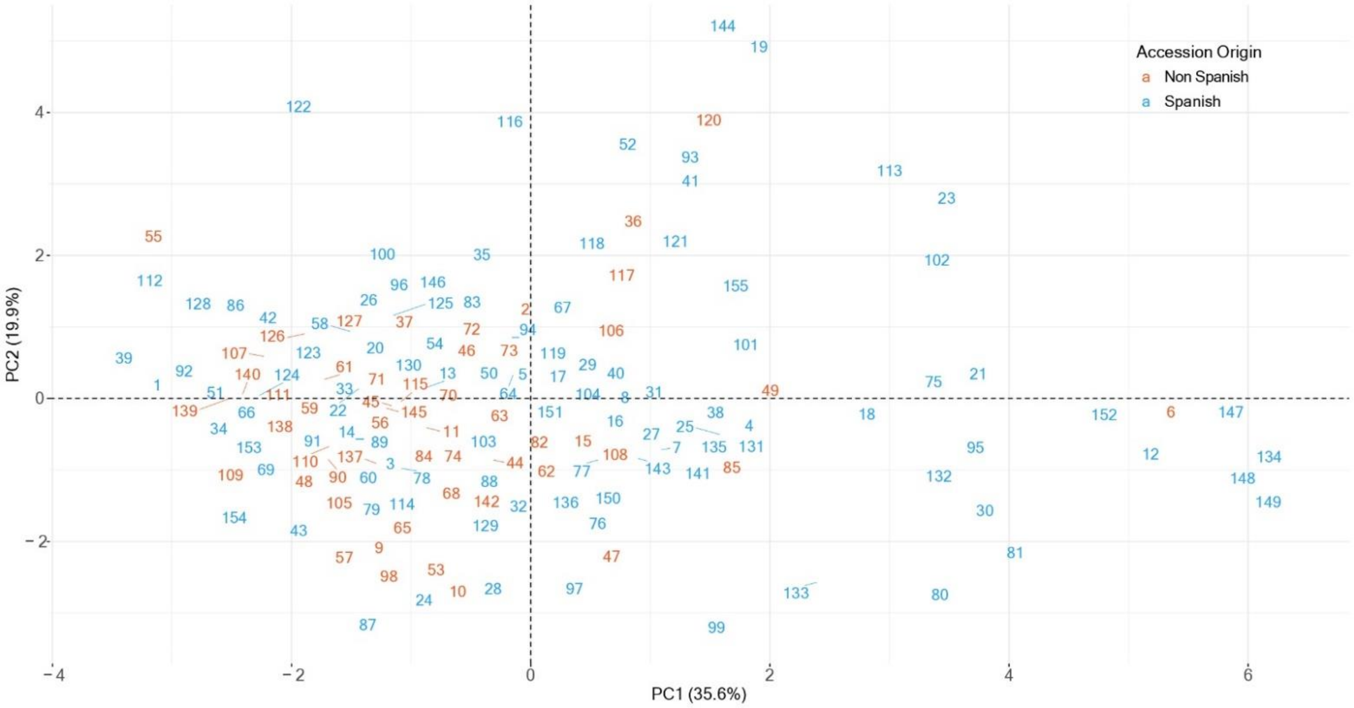


Figure 4.4. PCA plot showing the relation among the 12 evaluated traits on the 155 apple accessions.

4.4.5. Mixed-effect model results: relationships between climate and genetic origin

Figure 4.5 and Supplementary File 9 show correlations between mean values of sugars and organic acids of the 155 accessions and the different climatic traits. Correlations with total precipitation (pre), mean daily temperature range (trg), relative humidity (rh), and solar radiation (rad) tended to attain the highest values. Notwithstanding these results, a stepwise procedure based on the Akaike Information Criterion (AIC) statistic selected the best model (lowest AIC) that included fewer climatic variables than these four. The best model for oxalic acid, for instance, included the following climatic variables: pre and rad. The random-effects coefficients revealed differences between accessions in both intercepts (mean Oxa values) and climate coefficients. For the remaining traits (Suc, Glu, Fru, Sor, Sugars, Cit, Tar, Mal, Qui, Succ + Shi, and Acids), the best model included pre, trg and rad. The model coefficients were significant for all variables (all individual sugars, total sugars, all organic acids, and total acids).

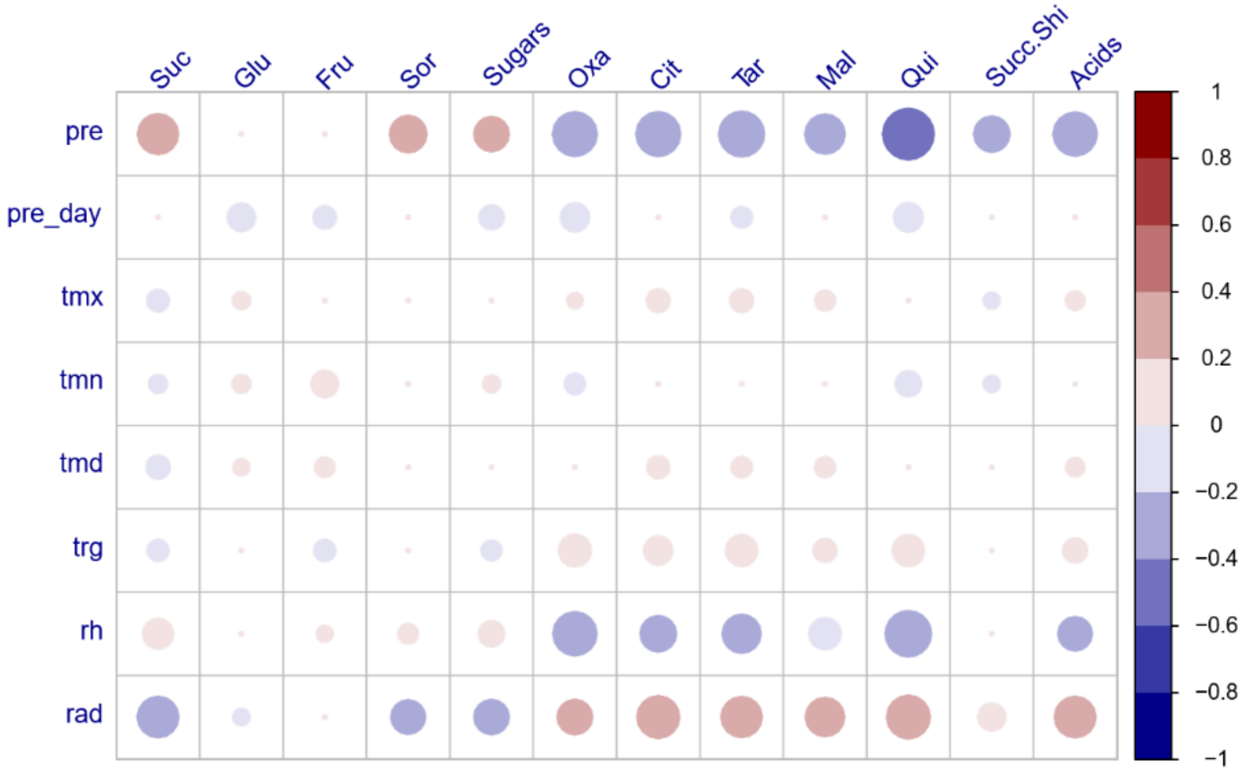


Figure 4.5. Correlations between the different sugars and organic acids of the 155 accessions studied and the climatic traits. Abbreviations: Suc, sucrose; Glu, glucose; Fru, fructose; Sor, sorbitol; Sugars, total sugars; Oxa, oxalic acid; Cit, citric acid; Tar, tartaric acid; Mal, malic acid; Qui, quinic acid; Succ+Shi, succinic + shikimic acid; Acids, total organic acid; pre, total precipitation; pre_day, mean daily precipitation; tmx, mean daily maximum temperature; tmn, mean daily minimum temperature; tmd, mean daily temperature; trg, mean daily temperature range; rh, relative humidity; rad, solar radiation. The size of the circle for each correlation and the colour depicts the significance and the magnitude of the correlation coefficient, respectively.

A two-way cluster analysis based on the Manhattan distance was used to hierarchically classify the 155 apple accessions and the independent variables according to the model coefficients (Figure 4.6). Manhattan distance is the sum of the distances between the accessions and traits assessed. Groups of accessions would indicate similar mean concentrations of biochemical traits and similar climate influence, while clusters of variables would group together model coefficients that tend to behave similarly across the accessions.

The model intercepts (labelled as ‘int’) correspond to mean compound concentrations for average climatic conditions; that is, they represent the genetic component as they only vary per accession (Figure 4.6). The model coefficients (labelled as ‘pre’, ‘rd’, or ‘trg’), on the other hand, capture the climate’s influence, which varies in interaction with the accession. Since the variables were standardized prior to the analysis, their coefficients can be compared among them. Thus, values closer to the limits (-1, 1) indicate a stronger influence than values close to zero.

Reading the heatmap in Figure 4.6 from left to right, the variable clusters are sorted by their decreasing effect in compound’s concentration, with the first cluster having the overall strongest effect. The analysis tended to form groups of either genetic effects (intercepts) or climatic variables, with the former (genetics) tending to have stronger effects than the later. Thus, between the first three groups of variables, two consisted on intercepts while the other contained climate coefficients. The first three groups of variables showed clear patterns across the accession groups, while the latter two did not show distinctive patterns and had, in general, coefficient values closer to zero.

Regarding the genetic variables (model intercepts), there were clear differences between accession groups. The first group of variables, that included the intercepts for citric, malic and total acids, showed contrasting values between accession groups 1 and 2 with predominantly positive values indicating higher than the global mean concentrations and groups 3 and 4 with negative values. Nevertheless, the climate’s effects did not show large differences across accession groups, and tended to have similar values across the dataset instead. Thus, no differences between accession groups were evident in the case of the second group of variables, integrating the effect of precipitation on oxalic and quinic acid. Precipitation for these two variables had a strong negative effect. In fact, precipitation had, in general, a negative effect for all the traits and most of the accessions.

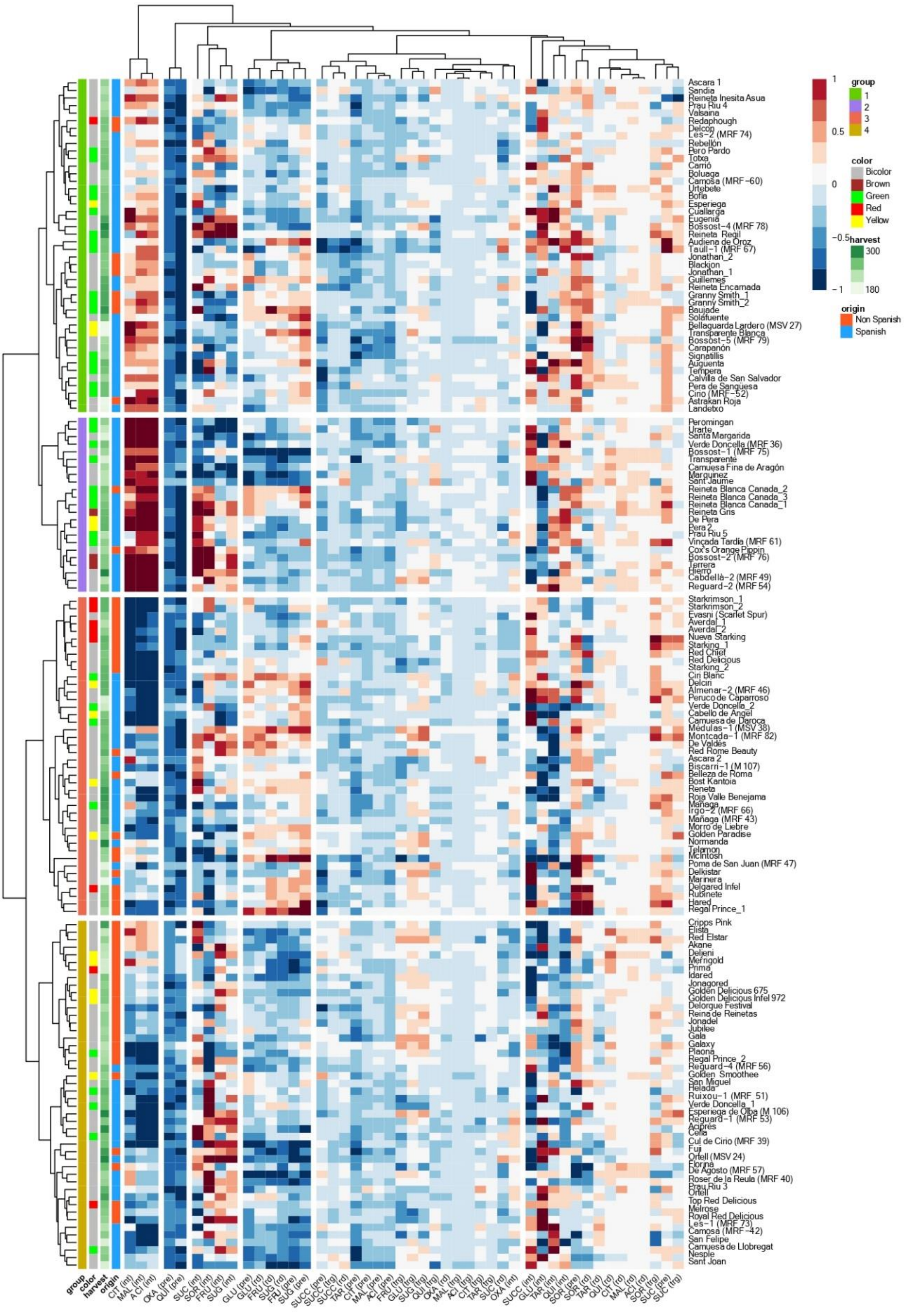
On the other hand, the fourth group of variables included the effect of radiation on glucose, fructose and total sugars, with a marked positive effect indicating that higher radiation was related to higher compound concentration in the third group of accessions (Figure 4.6). Group 4 showed a negative effect of the solar radiation, while the effect for the first two clusters of accessions was less marked and heterogeneous.

The fifth group of variables included the effects of precipitation, temperature range, and radiation on most of the variables studied and the intercept of oxalic acid. The model coefficients for all these effects were markedly negative for all the accession groups, indicating a negative effect. The last group of variables is more difficult to interpret since, as mentioned earlier, the coefficients tended to be closer to zero and did not show as clear patterns as the previous groups.

Reading the plot in Figure 4.6 from top to bottom, two large accession clusters were identified. Segregation into four smaller clusters was deemed as optimum. The first two clusters included 44 and 23 accessions, respectively; the third cluster had 42 accessions, while the last one included 46 accessions. As previously seen, there were clear differences between the accession groups regarding the model intercepts, while the climate effects where more homogeneous across groups.

A relationship between clusters and accession origins was apparent. Indeed, clusters 3 and 4 grouped 45 out of 56 non-Spanish cultivars, while clusters 1 and 2 grouped 56 out of 99 local-Spanish accessions. No relationship between the clustering analysis and the harvest date was detected. Supplementary Files 9 and 10 provide mean values of sugars (Supp. File 9) and organic acids (Supp. File 10) for each accession cluster according to the accession's origin (Spanish or non-Spanish) and skin colour. They show that non-Spanish cultivars had, in general, lower organic acids and sorbitol contents than autochthone accessions. On the other hand, it is also quickly noticed that brown accessions had higher total sugars content due to high sucrose and sorbitol values and higher total acids content. Indeed, the three brown skin accessions are included in the second group of accessions.

Figure 4.6. **(Next page)** Heatmap of the 155 accessions grouped into five groups. Abbreviations: intercept, int; temperature range, trg; total precipitation, pre; radiation, rd; Suc, sucrose; GLU, glucose; FRU, fructose; SOR, sorbitol; SUG, total sugars; OXA, oxalic acid; CIT, citric acid; TAR, tartaric acid; MAL, malic acid; QUI, quinic acid; SUCC, succinic + shikimic acid; ACI, total organic acid.


Ascara 1
Sandia
Reineta Inesita Asua
Prau Riu 4
Valsaina
Redaphough
Delcon
Les−2 (MRF 74)
Rebellón
Pero Pardo
Totxa
Carrió
Boluaga
Camosa (MRF−60)
Urtebete
Bofla
Esperiega
Cuallarga
Eugenia
Bossost−4 (MRF 78)
Reineta Regil
Audiena de Oroz
Taüll−1 (MRF 67)
Jonathan_2
Blackjon
Jonathan_1
Guillemes
Reineta Encarnada
Granny Smith_1
Granny Smith_2
Baujade
Solafuente
Bellaguarda Lardero (MSV 27)
Transparente Blanca
Bossost−5 (MRF 79)
Carapanón
Signatillis
Augüenta
Tempera
Calvilla de San Salvador
Pera de Sangüesa
Cirio (MRF−52)
Astrakan Roja
Landetxo
Peromingan
Urarte
Santa Margarida
Verde Doncella (MRF 36)
Bossost−1 (MRF 75)
Transparente
Camuesa Fina de Aragón
Marquinez
Sant Jaume
Reineta Blanca Canada_2
Reineta Blanca Canada_3
Reineta Blanca Canada_1
Reineta Gris
De Pera
Pera 2
Prau Riu 5
Vinçada Tardía (MRF 61)
Cox's Orange Pippin
Bossost−2 (MRF 76)
Terrera
Hierro
Cabdellà−2 (MRF 49)
Reguard−2 (MRF 54)
Starkrimson_1
Starkrimson_2
Evasni (Scarlet Spur)
Averdal_1
Averdal_2
Nueva Starking
Starking_1
Red Chief
Red Delicious
Starking_2
Ciri Blanc
Delcin
Almenar−2 (MRF 46)
Peruco de Caparroso
Verde Doncella_2
Cabello de Angel
Camuesa de Daroca
Médulas−1 (MSV 38)
Montcada−1 (MRF 82)
De Valdés
Red Rome Beauty
Ascara 2
Biscarri−1 (M 107)
Belleza de Roma
Bost Kantoia
Reneta
Roja Valle Benejama
Mañaga
Irgo−2 (MRF 66)
Mañaga (MRF 43)
Morro de Liebre
Golden Paradise
Normanda
Telamon
McIntosh
Poma de San Juan (MRF 47)
Delkistar
Marinera
Delgared Infel
Rubinete
Hared
Regal Prince_1
Cripps Pink
Elista
Red Elstar
Akane
Deljeni
Merrigold
Prima
Idared
Jonagored
Golden Delicious 675
Golden Delicious Infel 972
Delorgue Festival
Reina de Reinetas
Jonadel
Jubilee
Gala
Galaxy
Plaona
Regal Prince_2
Reguard−4 (MRF 56)
Golden Smoothee
San Miguel
Helada
Ruixou−1 (MRF 51)
Verde Doncella_1
Esperiega de Olba (M 106)
Reguard−1 (MRF 53)
Aciprés
Cella
Cul de Cirio (MRF 39)
Fuji
Ortell (MSV 24)
Florina
De Agosto (MRF 57)
Roser de la Reula (MRF 40)
Prau Riu 3
Ortell
Top Red Delicious
Melrose
Royal Red Delicious
Les−1 (MRF 73)
Camosa (MRF−42)
San Felipe
Camuesa de Llobregat
Nesple
Sant Joan
group
color
harvest
origin
CIT (int)
MAL (int)
ACI (int)
OXA (pre)
QUI (pre)
SUC (int)
SOR (int)
FRU (int)
SUG (int)
GLU (pre)
GLU (rd)
FRU (rd)
SUG (rd)
FRU (pre)
SUG (pre)
SUCC (pre)
SUCC (trg)
SUCC (rd)
TAR (pre)
CIT (pre)
MAL (pre)
ACI (pre)
FRU (trg)
GLU (trg)
SUG (trg)
QUI (trg)
OXA (rd)
MAL (trg)
ACI (trg)
CIT (trg)
TAR (trg)
SUC (rd)
OXA (int)
SUCC (int)
GLU (int)
TAR (int)
QUI (int)
SOR (pre)
SOR (rd)
TAR (rd)
QUI (rd)
CIT (rd)
MAL (rd)
ACI (rd)
SOR (trg)
SUC (pre)
SUC (trg)
1
0.5
0
−0.5
−1
group
1
2
3
4
color
Bicolor
Brown
Green
Red
Yellow
harvest
300
180
origin
Non Spanish
Spanish

4.5 Discussion

One of the objectives of this work was to better characterize a large number of apple cultivars and autochthone accessions. Iglesias et al. (2016) and Urrestarazu et al. (2016) reported that in Spain 87% of apple production is limited to four cultivars: 'Fuji', 'Gala', 'Golden Delicious' (more than 50% of total production) and 'Red Delicious'. These figures highlight the loss of genetic diversity in the Spanish apple fruit industry.

As expected, there was a large variability in sugars and organic acids content among the 155 accessions. Values for the different traits varied significantly among accessions and years, as reported in previous studies (Aprea et al., 2017; Begić-Akagić et al., 2014; Bureau et al., 2012; Donahue et al., 2021; Yang et al., 2021). Since the only source of variability between years was the climatic characteristics during the growing season, these results highlight that both **accession and inter-annual climate variability have a relevant role in the assessed traits**. In other tree crops, year and accession effects were also reported (Font i Forcada et al., 2019a; Iglesias et al., 2019; Gómez-Martínez et al., 2021). In addition to a direct impact on fruit physiology and development, metabolite profiles, sugars, and organic acids could also be significantly affected by the growing seasons' climatic characteristics (Davey et al., 2007).

The measured values for sugars and organic acids were within previously reported ranges (Aprea et al., 2017; Begić-Akagić et al., 2014; Castel et al., 2020; Jing et al., 2020; Oszmiański et al., 2020; Yang et al., 2021). As it was foreseeable in apple, fructose had the highest values among individual sugars, followed by sucrose, glucose and the sugar alcohol, sorbitol, in agreement with Liu et al. (2013) and Yang et al. (2012). Regarding organic acids, malic was the major acid with values ranging from 10 to 100 times higher than the other acids. Previous works detected a smaller number of organic acids than the present study. Moreover, our measured values varied considerably, probably because of the **different genetic background of the large number of accessions studied**, when compared to other studies that used only a few commercial cultivars. Actually, it is the first work reporting sugar and organic acid profiles for a large number of accessions (99 Spain native accessions and 56 non-Spanish cultivars) over several consecutive seasons (five years). Notably, it is also the first one to relate these traits to climatic characteristics.

The cluster analysis did not show association between the clusters and other characteristics such as the harvest date or the skin colour. Indeed, the 155 accessions studied could not be segregated by their harvest date or skin colour. Nevertheless, it is worth noting that the three **brown accessions (fully russeting covered) had higher values for sugars and organic acids** ('Bossost_2 – MRF 76', 'Reineta Gris' and 'Terrera') than the other skin colour groups. Nevertheless, russeting is usually seen as a negative trait and is not appreciated by consumers, with the exception of a few varieties such as 'Reineta Blanca Canada' (which is not fully covered by russeting) or 'Reineta Gris'. As the cuticle and cork coat protect plant tissues against environmental stresses, changing their components in the pericarp may affect the fruit resistance to biotic and abiotic stresses. Nevertheless, most apple accessions exhibiting russeting are triploids (Busattto et al., 2019; Reig et al., 2015; Wang et al., 2014), and thus, less suitable in breeding programs, even though russeted apples could improve sugars and organic acids profiles.

Fructose is sweeter than glucose and sucrose (Zhang et al., 2020). Moreover, sorbitol is more beneficial than other sugars for human health (Forni et al., 1992; Font i Forcada et al., 2019). Indeed, sorbitol can be used as a glucose substitute in diabetes diets, and it is an alternative natural sweetener to sucrose. Through the polyol pathway, also called the sorbitol-aldose reductase pathway, glucose is reduced to sorbitol and subsequently oxidized to fructose (Lu et al., 2018). This process allows explaining the positive and significant correlations between the different individual sugars. Furthermore, sugars were positively and significantly correlated with SSC ($r = 0.87$) according to Mignard et al. (2021), but they did not significantly correlate with TA. Individual organic acids, in turn, were significantly correlated with TA ($r = 0.86$), but they did not significantly correlate with SSC. These **results support the common use of SSC and TA as proxy measures for fruit sweetness and acidity**, respectively. Nevertheless, it is challenging to define fruit sweetness or sourness by only chemical methods without counting on the experience of regular panellists or consumers (Giné-Bordonaba et al., 2016).

Spanish native accessions showed, in general, higher acid levels (e.g.,'Urarte', 'Santa Margarida', 'Pero Pardo', 'Transparente', 'Transparente Blanca' or 'Bellaguarda Lardero – MSV 27'). Previous studies determined that total acids negatively correlated with sensory acceptability, reducing panellist acceptance (Keenan et al., 2012). In general, the equilibrium of sweetness and sourness determine consumer satisfaction for apple flavor. All these compounds influence the organoleptic perception of sweetness, sourness, and aroma (Aprea et al., 2017; Yang et al., 2021) and contribute to the fruit-eating quality and consumers' acceptance. Apple breeding programs should target achieving a good balance between sourness and sweetness in apples. This is not only to achieve good consumer acceptance, as some studies reported an influence of sweetness and sourness characteristics on chemical composition changes during apple ripening (Aprea et al., 2017; Bayarri et al., 2001; Corollaro et al., 2014).

The Central Ebro Valley area is characterized by a semi-arid climate with warm and dry summers, high radiation, and large day-night temperature variation. For both sugars and organic acids, no previous study assessed the relationship with climatic characteristics. Nonetheless, significant correlations with climate have been reported for antioxidants and basic fruit quality traits (Li et al., 2013; McGhie et al., 2005; Mignard et al., 2021; Yuri et al., 2009).

Spanish native accessions exhibited a tendency to show higher values for sorbitol and organic acids than the non-Spanish cultivars. The increment of sugar concentration could respond to the high-energy demand required because of different stress responses and the supply of carbon to the organic and amino acids (Giné-Bordonaba et al., 2019; Li et al., 2012). Moreover, different metabolic pathways could explain the negative correlation between individual sugars and organic acids (Ruan, 2014). Indeed, organic acids degradation can occur through dicarboxylates (malate and other organic acids) decarboxylation. Decarboxylation permits phosphoenolpyruvate (PEP) production which plays a role in gluconeogenesis activation, resulting in increased glucose production (Vallarino & Osorio, 2019).

According to the effect of climate, minor differences between clusters were found except for sorbitol, which was significantly and positively correlated with precipitation for most accessions. Sorbitol is the main photosynthesis product in the *Rosaceae* family and has a prominent role in osmoregulation. As such, it can be accumulated when plants are under drought, cold, salinity or other stresses (Amri et al., 2021; Dietrich et al., 2007; Zhang et al., 2011). Moreover, the effect of radiation on sorbitol could be observed in the obtained two-way hierarchical analysis. When radiation was higher, in general, accessions showed higher sorbitol concentration. These results agree with Li et al. (2013), who showed that the sun-exposed peel of 'Jonamac' apple cultivar had higher sorbitol contents. The sorbitol-6-phosphate dehydrogenase (S6PDH) gene controls sorbitol production, while osmosis regulates its expression (Zhang et al., 2011). **Sorbitol values could be related to the adaptability of plants to different climatic stresses** such as water deficit, excessive cold or salinity throughout osmotic regulation (Dietrich et al., 2007; Gao et al., 2005).

The PCA analysis showed substantial variability between accessions. It identified a pool of non-Spanish cultivars with specific and differentiated biomolecules profiles. **The non-Spanish accessions or commercial cultivars seemed to be sweeter and less acidic**, in general, than autochthone accessions. They also had more similar profiles between them. In contrast, Spanish accessions were more heterogeneous and tended to have, in general, higher sorbitol and organic acids concentration. Nevertheless, we can highlight two non-Spanish cultivars which were outliers from the above grouping tendency because of their extreme values. 'Fuji' (Nº55) had higher sugar levels, while 'Astrakan Red' (Nº6) had very high organic acids values.

Segregation between non-Spanish and local-Spanish accessions was already reported by Mignard et al. (2021) for the same plant material studying antioxidants traits. Similar results were found by Pereira-Lorenzo et al. (2017) using SSRs for a great group of apple genotypes from Spanish germplasm banks. The segregation according to the origin of the accessions was also apparent in the two-way hierarchical analysis on the model coefficients. Consequently, most non-Spanish cultivars were grouped into clusters 3 and 4. This reinforces the similarity between the non-Spanish cultivars and their differences concerning the autochthone accessions.

In a previous study, local-Spanish accessions showed higher antioxidant levels than non-Spanish cultivars (Mignard et al., 2021), probably because of their better adaptation to the stressful and thus less optimum agroclimatic conditions of NE Spain (Reig et al., 2015). Antioxidants are secondary metabolites involved in division and development, and are fundamental in the response to biotic and abiotic stresses (Amri et al., 2021; Li et al., 2012). The secondary metabolism is merged to primary metabolism (soluble sugars and organic acids) because of the different substrates diverted from primary routes and directed into the secondary pathway (Amri et al., 2021). The correlations between individual sugars and organic acids and the different antioxidants (data not shown), showed the linkage between the primary and the secondary metabolisms. Therefore, abiotic and biotic stresses influencing photosynthesis and primary metabolism will thus affect secondary metabolism (Li et al., 2012).

Moreover, the activation of repair mechanisms during the development associated with climate stress, such as high radiation or high temperatures in the ripening period, could lead to a higher need for carbon skeletons, new molecules synthesis or increased antioxidative enzyme activity. As a reply, **the plant generates an antioxidative defensive system composed of enzymes or increased antioxidants and specific sugars**

as sucrose or sorbitol (Amri et al., 2021; Li et al., 2012; Pandey et al., 2017). Many of these processes can be supported by photosynthetic activity (Pandey et al., 2017). The extreme accumulation of reactive oxygen species (ROS), as a consequence of biotic and abiotic stress, could disrupt the regular physiological as well as the cellular effective performance, resulting in cell wall damage and oxidative stress (Pandey et al., 2017).

Lastly, our results emphasize that basic research provides useful insight for selecting and developing new cultivars (Pereira-Lorenzo et al., 2017; Verma et al., 2019) with a particular sensory taste for consumers. Indeed, the present work highlighted **climate traits and apple accessions as the main drivers of organic acids, sugars, and fruit quality characteristics**. Between these two drivers, genetics seemed to have the strongest effect and showed clear differences between accession groups, while climate effects were strong only for certain compounds and had a more horizontal performance affecting equally the different accession groups. Therefore, accessions with a higher content of sugars or organic acids could be selected to promote their acceptability in the market. Furthermore, the evaluation of the germplasm bank assessed in this study with SSRs (Pereira-Lorenzo et al., 2017) or high-density SNP genotyping (Font i Forcada et al., 2019b; Jung et al., 2020; Winfield et al., 2020), may offer supplementary and precious material in understanding the genetics and its control on biochemical traits in apple. Similarly, a selection of the most suitable accessions could be done considering the climatic characteristics of cultivation sites.

4.6 Conclusions

The present work showed high biodiversity in the EEAD-CSIC apple collection concerning sugar and organic acid contents. Higher contents of organic acids were observed in the Spanish accessions, while the sugar profile was more heterogeneous according to the accession's origins. These results strengthen the consciousness of the importance of autochthone phytogenetic resources. Furthermore, the differences shown in sugar and organic acid profiles between accessions stress the relevance of these biomolecules in the global quality of apples. Besides genetics, climatic characteristics during the growing season such as rainfall, solar radiation, and temperature substantially determine metabolite profiles. Organic acids content was, in general, inversely related to precipitation, and positively related to temperature and solar radiation. Sugars were also affected by climate parameters. The climatic parameters emphasize the relevance of the geographic region where the fruit tree is cultivated in the resulting metabolites profiles, even though the genetics seemed to have the strongest effect on them. In future breeding programs, it could be worth considering the climate of the growing area in combination with genetics to further improve the biochemical characteristics of the cultivars and identifying the more resilient genotypes to climate change.

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Capítulo 5

Population structure and association mapping for agronomical and biochemical traits of a large Spanish apple germplasm

5.1. Abstract

A basic knowledge on linkage disequilibrium and population structure is necessary in order to determine the genetic control and identify significant associations with agronomical and phytochemical compounds in apple (*Malus x domestica* Borkh.). In this study, 186 apple accessions (Pop1) representing both Spanish native accessions (94) and non-Spanish cultivars (92) from the EEAD-CSIC apple collection, were assessed using 23 SSRs markers covering the apple genome. Four populations were considered. The Pop1 was divided into 150 diploid (Pop2) and 36 triploid accessions (Pop3) while for the linkage disequilibrium and the association mapping analysis, 118 diploids and all phenotyped accessions were considered as population 4 (Pop4). Thus, average number of alleles per locus and observed heterozygosity for the overall sample set (Pop1) were 15.65 and 0.75 respectively. Genotypes were considered to be duplicated if they matched at all alleles across the markers being in this case eight accessions. Population structure analysis identified two subpopulations in the diploid accessions (Pop2 and Pop4) and four according to the triploids (Pop3). Regarding the Pop4, the population structure with K=2 subpopulations segregation was in agreement with the UPGMA cluster analysis according to the genetic pairwise distances. Moreover, the accessions seemed to be segregated by their origin (Spanish/non-Spanish) in the clustering analysis. One of the two subpopulations encountered was quite-exclusively formed by non-Spanish accessions (30 out of 33). Furthermore, agronomical and basic fruit quality parameters, antioxidant traits, individual sugars and organic acids were assessed for the association mapping analysis. A high biodiversity was exhibited in the phenotypic characterization of Pop4 and a total of 126 significant associations were found between the 23 SSR markers and the 21 phenotypic traits evaluated. This study also identified many new marker-locus trait associations such as in the antioxidant traits (TPC, TFC, AsA, and RAC) or in sugars (sucrose, glucose, and sorbitol) and organic acids (oxalate, quinate, malate, citrate, tartarate, succinate and shikimate), which may be useful for predictions and for a better understanding of the apple genome.

Keywords: Antioxidants, Apple, Genetic association, Linkage disequilibrium, simple sequence repeats - SSRs, SWEETs genes.

5.2. Introduction

Modern cultivated apple (*Malus x domestica* Borkh.) is the third most important fruit crop, after bananas and watermelons, grown in temperate zones worldwide. Apple is the most significant and ancient fruit crop of the Rosaceae in the world as well as in Spain (Urrestarazu et al., 2012). In 2020, more than 86M tonnes of apple were produced in the world (FAOSTAT, 2022). In 2021, an average of 617 thousand tonnes was produced in Spain (MAPAMA, 2022). Indeed, more than 516 thousand tonnes of the national production were dessert apple for fresh consumption and juices, while cider apple production reached only 100 thousand tonnes. Nevertheless, apple production around the world is based on a reduced number of modern bred varieties (Mignard et al., 2021a; 2022; Urrestarazu et al., 2012). In fact, apple production is dominated by some well-adapted modern group of cultivars (‘Fuji’, ‘Gala’, ‘Golden’, ‘Granny Smith’ and ‘Delicious’), many of which are genetically linked (Urrestarazu et al., 2016; Ordidge et al., 2018). Indeed, the ‘Golden Delicious’ cultivar represented more than 46% of the apple Spanish production for fresh consumption with more than 240 thousand tonnes (MAPAMA, 2022). As a consequence, many of the traditional and/or locally well-adapted cultivars have been considered outdated and a dramatic loss of genetic diversity has been noticed in the apple gene pool in the last centuries (Lassois et al., 2016; Gao et al., 2015; Marconi et al., 2018; Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2017; Vanderzande et al., 2017).

All commercial apple cultivars descend from an inter-specific hybrid complex belonging to the Rosaceae family, having a basic chromosome number of 17 (Marconi et al., 2018; Velasco et al., 2010). Most domesticated apple cultivars are diploid ($2n = 2x = 34$), even though triploid ($2n = 3x = 51$) and tetraploid ($2n = 4x = 68$) cultivars can also be found. The modern apple should be the result of a long evolutionary process throughout thousand years with several species contributing to the domesticated apple gene pool encountered nowadays (Cornille et al., 2012).

Despite the high genetic variability of the apple gene pool, the breeding programs, over the years, were mainly based on few organoleptic traits, aesthetic criteria and resistance to specific diseases (Marconi et al., 2018) resulting in few cultivars and causing a loss in diversity. Moreover, the breeding programs have limited and reduced the use of the total number of cultivars, such as ‘Cox’s Orange Pippin’, ‘Golden Delicious’, ‘Jonathan’, ‘Red Delicious’, and ‘McIntosh’, although more than 10,000 different apple accessions have been described globally (Marconi et al., 2018; Noiton and Alspach, 1996; Urrestarazu et al., 2016). Indeed, a great number of new cultivars from breeding programs were obtained during the twentieth century from a reduced number of progenitors and, consequently, share a high degree of parentage (Noiton and Alspach, 1996; Reig et al., 2015; Urrestarazu et al., 2016). For this reason, germplasm evaluation, characterization and conservation of genetic diversity is crucial for the management of genetic properties (Fernández i Martí et al., 2015a). Therefore, there is an increased neediness in studying and collecting old local apple accessions that could provide a better knowledge of the history of the crop and could be used in future breeding programmes aiming to select better adapted cultivars to climate conditions (Fernández i Martí et al., 2015a; Mignard et al., 2021b; 2022). In fact, the apple allelic diversity should be used to face existing and future biotic and abiotic issues with respect to production in the context of global climate change (Lassois et al., 2016). Furthermore, in the last decades, the interest of the genetic and molecular characterization of apple has increased. The diversity found at genetic level between apple accessions reflects a combination of historical selections and adaptive processes resulting in an extensive genetic variation but in a limited population structure (Urrestarazu et al., 2016).

The microsatellite markers or Simple Sequence Repeats (SSRs) have been in apple and other fruit crops, in general, one of the most used markers for molecular characterization together with the Single Nucleotide Polymorphism markers (SNPs). Although SNPs analysis has not been assessed in this study, it would be a good option for further analyses. SSR markers are DNA subdivisions containing short repeated sequences and are very abundant in genome. They are made up of short units of 1 to 6 base pairs, which are repeated in tandem all along the DNA. The genome sequence of the domesticated apple has been released, and accounts for an approximately 750 Mb per haploid (Velasco et al., 2010). The SSRs analysis is a good technique within crop species, due to their abundance, codominance, high polymorphism, easy use with the PCR conditions and low cost (Fernández i Martí et al., 2015b; Urrestarazu et al., 2018). Indeed, many studies based on these molecular markers have assessed the diversity in *Malus* accessions (Pereira-Lorenzo et al., 2017; Gross et al., 2012; Urrestarazu et al., 2012; Gao et al., 2015; Liang et al., 2015; Lassois et al., 2016; Vanderzande et al., 2019). Gross et al. (2012) demonstrated that nine SSRs loci were sufficient to determine potential duplicates and study differences among *Malus* accessions. In the present work, 23 microsatellites markers were used for the molecular characterisation of a large set of accessions from the North and North-eastern of Spain. The European Cooperative Programme for Plant Genetic Resources (ECPGR) has published several lists of recommended markers including SSRs that span most of the apple genome (Lassois et al., 2016; Patocchi et al., 2009), and have been tested on a set of standard *Malus* accessions. Many of these mapped SSRs have been associated with quantitative trait loci (QTL) with a great interest in agronomical, morphological, and/or organoleptic traits and could be used as molecular tools for marker assisted selection (MAS) in future plant breeding programs (Liu et al., 2016; Marconi et al., 2018).

Apart from the recommended SSRs, the present work aims to report the identification of the MdSWEET genes by SSRs markers designed by Zhen et al. (2018). In plants, SWEET transporters function as bidirectional uniporters that mediate influx and efflux of sugars across cell membranes. SWEETs can be divided into four subgroups (Eom et al., 2015; Zhen et al., 2018). Clades I, II, and IV of the SWEETs transport predominantly hexoses, whereas clade III of the SWEETs appear to be sucrose transporters (SUTs) (Zhen et al., 2018). This study will allow a better understanding of the effect of SWEETs on fruit sugar accumulation and it will also be helpful for genetic improvement of fruit sugar accumulation in apple-breeding programs.

Moreover, association mapping, also known as linkage disequilibrium (LD) mapping, relies on the strength of association between genetic markers and phenotype. Therefore, LD mapping is an approach that detects and locates genes relative to an existing map of genetic markers (Font i Forcada et al., 2013; 2015). Consequently, this method detects relationships between phenotypic variation and gene polymorphism in existing germplasm and in unrelated individuals. In addition, association mapping complements and improves previous QTL information by incorporating the effects of recombination occurring in many past generations into a single analysis. Association mapping has been successfully used to identify genes involved in flowering and ripening traits in apple (Urrestarazu et al., 2017), although the bibliography for organoleptic and fruit quality traits is scarce (Amyotte et al., 2017; Gutierrez et al., 2018; Larsen et al., 2019; McClure et al., 2018; 2019).

The present work aims to study the genetic characterization of the *Malus x domestica* Borkh germplasm located at the Experimental Station of Aula Dei (EEAD-CSIC), Spain, to assess the population structure, linkage disequilibrium and association mapping between the local well-adapted accessions compared with the modern and commercial cultivars. This work provides thus molecular tools for genetic improvement of fruit quality in apple breeding programs and a better knowledge of the apple genetic resources available through a common set of 23 SSR markers. Our results will highlight the importance of genetic variation in germplasm collections for the effective conservation of biodiversity in domesticated apple crop.

5.3. Materials and methods

5.3.1. Plant material and field trial

This research counted with 186 accessions (*Malus x domestica* Borkh) from the apple germplasm bank established at the Experimental Station of Aula Dei (EEAD-CSIC, Zaragoza, NE Spain: 41° 43′ 42.7″ N, 0° 48′ 44.1″ W). The 186 genotypes (Population 1) were grown under Mediterranean soil conditions, typical of the Central Ebro Valley area. This geographic area is characterized by a semi-arid climate with warm and dry summers, high radiation, and large day–night temperature variation. The accessions assessed in this work (Table 5.1) consisted in a wide range of geographic origins (94 Spanish accessions and 92 non-Spanish accessions). Indeed, most of the non-Spanish accessions were commercial cultivars meanwhile autochthonous commercial cultivars or traditional landraces represented the local-Spanish accessions. The accessions were categorized according to Mignard et al. (2021b; 2022). In the field, each accession had three-tree replications established in a single block design. Trees were trained to a low-density system (6 m × 5 m) and the cultural management was carried out as in a commercial plantation. The orchard was flood irrigated every 12 days during the summer.

Table 5.1. Basic information of the 186 apple accessions assessed in this study.

Nº	Accession	EEAD Code	Ploidy	Origin
1	Acipres*	3339	2n	Spanish
2	Akane*	2902	2n	non-Spanish
3	Almenar-2*	3555	2n	Spanish
4	Ascara-1	3423	3n	Spanish
5	Ascara-2*	3424	2n	Spanish
6	Astrakan Red*	3378	2n	non-Spanish
7	Audiena de Oroz*	3375	2n	Spanish
8	Augüenta*	3335	2n	Spanish
9	Averdal-1*	882021	2n	non-Spanish
10	Averdal-2	892340	2n	non-Spanish
11	Baujade*	923284	2n	non-Spanish
12	Belgolden	3193	2n	non-Spanish
13	Bellaguarda Lardero*	3547	2n	Spanish

14	Belleza de Roma*	638	2n	non-Spanish
15	Biscarri-1*	3726	2n	Spanish
16	Blackjon*	2690	2n	non-Spanish
17	Blacktayman	2490	3n	non-Spanish
18	Bofla*	3418	2n	Spanish
19	Boluaga	3340	3n	Spanish
20	Boskoop Rouge	2898	3n	non-Spanish
21	Bossost-1	3626	3n	Spanish
22	Bossost-2	3627	3n	Spanish
23	Bossost-4*	3629	2n	Spanish
24	Bost Kantoia*	3341	2n	Spanish
25	Cabdellà-2*	3613	2n	Spanish
26	Cabello de Angel*	3255	2n	Spanish
27	Calvilla de San Salvador*	3342	2n	Spanish
28	Camosa-1*	3553	2n	Spanish
29	Camosa-2*	3620	2n	Spanish
30	Camuesa de Daroca*	3371	2n	Spanish
31	Camuesa de Llobregat*	1342	2n	Spanish
32	Camuesa Fina de Aragón*	3372	2n	Spanish
33	Carapanón	3634	3n	Spanish
34	Carrió	3636	3n	Spanish
35	Cella*	2512	2n	Spanish
36	Cepiland	881967	2n	non-Spanish
37	Charden	303	3n	non-Spanish
38	Ciri Blanc*	3402	2n	Spanish
39	Cirio*	3615	2n	Spanish
40	Cox's Orange Pippin*	2889	2n	non-Spanish
41	Cripps Pink*	933540	2n	non-Spanish
42	Crispin	3080	3n	non-Spanish
43	Cuallarga*	3467	2n	Spanish
44	Cul de Cirio*	3551	2n	Spanish
45	De Agosto*	3619	2n	Spanish
46	De Pera*	3416	2n	Spanish
47	De Valdés*	3632	2n	Spanish
48	Delbar Estivale	3262	2n	non-Spanish
49	Delciri*	3413	2n	Spanish
50	Delcon*	2896	2n	non-Spanish
51	Delgared Infel*	902708	2n	non-Spanish
52	Deljeni*	851305	2n	non-Spanish
53	Delkistar*	923273	2n	non-Spanish
54	Delorgue Festival*	913044	2n	non-Spanish
55	Democrat	2892	3n	non-Spanish
56	Elista*	912883	2n	non-Spanish
57	Esperiega*	3420	2n	Spanish
58	Esperiega de Olba*	3725	2n	Spanish
59	Eugenia*	3468	2n	Spanish
60	Evasni (Scarlet Spur)	933554	2n	non-Spanish
61	Florina*	3633	2n	non-Spanish
62	Fortuna Delicious	2702	2n	non-Spanish
63	Freyberg	2611	2n	non-Spanish

64	Fuji*	3488	2n	non-Spanish
65	Fukutami	2895	2n	non-Spanish
66	Gala*	3197	2n	non-Spanish
67	Galaxy*	892451	2n	non-Spanish
68	Gloster 69	3140	2n	non-Spanish
69	Golden Auvil Spur	2402	2n	non-Spanish
70	Golden Delicious*	675	2n	non-Spanish
71	Golden Delicious Infel*	2491	2n	non-Spanish
72	Golden Paradise*	3739	2n	non-Spanish
73	Golden Smoothee *	3286	2n	non-Spanish
74	Granny Smith-1*	3196	2n	non-Spanish
75	Granny Smith-2*	2614	2n	non-Spanish
76	Gravenstein	3109	3n	non-Spanish
77	Guillemes*	3411	2n	Spanish
78	Hared*	892232	2n	non-Spanish
79	Harrold Red Delicious	2899	2n	non-Spanish
80	Helada*	3368	2n	Spanish
81	Hierro*	3374	2n	Spanish
82	Idared*	2484	2n	non-Spanish
83	Irgo-2*	3622	2n	Spanish
84	Jerseymac	3141	2n	non-Spanish
85	Jonadel*	2650	2n	non-Spanish
86	Jonagored	882001	3n	non-Spanish
87	Jonathan-1*	2495	2n	non-Spanish
88	Jonathan-2*	3096	2n	non-Spanish
89	Jubilee*	851304	2n	non-Spanish
90	Kidd's Orange Red	2888	2n	non-Spanish
91	Kinrei	2900	2n	non-Spanish
92	Lancer	881968	2n	non-Spanish
93	Landetxo*	3343	2n	Spanish
94	Les-1*	3624	2n	Spanish
95	Les-2	3625	3n	Spanish
96	MacIntosh*	3192	2n	non-Spanish
97	Mañaga-1*	469	2n	Spanish
98	Mañaga-2*	3554	2n	Spanish
99	Marinera*	3412	2n	Spanish
100	Marquiñez	3419	3n	Spanish
101	Médulas-1*	3548	2n	Spanish
102	Melrose*	2484	2n	non-Spanish
103	Merrigold*	851307	2n	non-Spanish
104	Montcada-1*	3631	2n	Spanish
105	Morro de Liebre *	3256	2n	Spanish
106	Mutsu	2487	3n	non-Spanish
107	Nesple*	3410	2n	Spanish
108	Normanda	3252	3n	Spanish
109	Nueva Starking*	1899	2n	non-Spanish
110	Ortell-1	413	3n	Spanish
111	Ortell-2*	3546	2n	Spanish
112	Ozark Gold	3175	2n	non-Spanish
113	Pera 2*	3417	2n	Spanish

114	Pera de Sangüesa	3379	3n	Spanish
115	Pero Pardo	3369	3n	Spanish
116	Peromingan*	1158	2n	Spanish
117	Peruco de Caparroso*	3373	2n	Spanish
118	Plaona*	923283	2n	non-Spanish
119	Poma de San Juan*	3556	2n	Spanish
120	Prau Riu-3*	3491	2n	Spanish
121	Prau Riu-4	3492	3n	Spanish
122	Prau Riu-5*	3493	2n	Spanish
123	Prima*	851306	2n	non-Spanish
124	Prime Gold	3198	2n	non-Spanish
125	Rebellon*	3370	2n	Spanish
126	Red Delicious*	3085	2n	non-Spanish
127	Red Elstar	882002	2n	non-Spanish
128	Red King Delicious	2688	2n	non-Spanish
129	Red Rome Beauty*	2897	2n	non-Spanish
130	Redaphough*	933411	2n	non-Spanish
131	RedChief*	851308	2n	non-Spanish
132	Redspur Delicious	3082	2n	non-Spanish
133	Regal Prince-1	882022	2n	non-Spanish
134	Regal Prince-2*	892341	2n	non-Spanish
135	Reguard-2*	3617	2n	Spanish
136	Reguard-4*	3618	2n	Spanish
137	Reina de Reinetas	2488	3n	non-Spanish
138	Reineta Blanca del Canada-1	308	3n	non-Spanish
139	Reineta Blanca del Canada-2	3111	3n	non-Spanish
140	Reineta Blanca del Canada-3	3194	3n	non-Spanish
141	Reineta Encarnada*	3635	2n	Spanish
142	Reineta Gris	2883	3n	non-Spanish
143	Reineta Inesita Asua	2543	3n	Spanish
144	Reineta Regil	3466	3n	Spanish
145	Reneta*	3408	2n	Spanish
146	Richared Delicious	2481	2n	non-Spanish
147	Roja Valle de Benejama*	1038	2n	Spanish
148	Roser de la Reula*	3552	2n	Spanish
149	Royal Red Delicious	2363	2n	non-Spanish
150	Rubinete*	861526	2n	non-Spanish
151	Ruixou-1*	3614	2n	Spanish
152	San Felipe*	3376	2n	Spanish
153	San Miguel*	2579	2n	Spanish
154	Sandía*	3336	2n	Spanish
155	Sant Jaume	3470	3n	Spanish
156	Sant Joan*	3409	2n	Spanish
157	Santa Margarida	3401	3n	Spanish
158	Shelred	2893	2n	non-Spanish
159	Signatillis*	3403	2n	Spanish
160	Solafuente	3559	3n	Spanish
161	Spartan	2483	2n	non-Spanish
162	Starking-1*	2964	2n	non-Spanish
163	Starking-2*	632	2n	non-Spanish

164	Starkrimson-1*	3195	2n	non-Spanish
165	Starkrimson-2	1904	2n	non-Spanish
166	Stayman Waynesap	3110	3n	non-Spanish
167	Taüll-1*	3623	2n	Spanish
168	Telamon*	3398	2n	non-Spanish
169	Tempera*	3334	2n	Spanish
170	Terrera	3469	3n	Spanish
171	Topred Delicious	2651	2n	non-Spanish
172	Totxa*	3471	2n	Spanish
173	Trajan	3396	2n	non-Spanish
174	Transparente*	3377	2n	Spanish
175	Transparente Blanca*	3344	2n	Spanish
176	Turley Winnesap	2884	3n	non-Spanish
177	Tuscan	3397	2n	non-Spanish
178	Urarte	3415	3n	Spanish
179	Urtebete*	3345	2n	Spanish
180	Valsaina*	3558	2n	Spanish
181	Vance Delicious	2647	2n	non-Spanish
182	Verde Doncella-1*	2125	2n	Spanish
183	Verde Doncella-2	310	2n	Spanish
184	Verde Doncella-3*	3549	2n	Spanish
185	Vinçada Tardía	3621	3n	Spanish
186	Wellspur Delicious	3081	2n	non-Spanish

*: Accessions assessed for the association mapping using the TASSEL Software: 118 accessions (Pop4).

5.3.2. Leaf and Fruit sampling

For the evaluation of the ploidy level for the 186 accessions (Population 1) of this work, newly expanded leaves were collected from each accession and analysed as described in Reig et al. (2015). The accessions were consequently classified into diploids (Population 2: 150 accessions) and triploids (Population 3: 36 accessions). According to the fruit sampling, 30 fruits (10 fruits × tree × rep.) were harvested when fruit firmness (FF) attained a value around 70–80 N or when they displayed the peel colour representative of each accession. Accessions were harvested during at least three seasons within the 2014-2018 period, and means for each year and accession were calculated (Mignard et al., 2021b). Maturity date fluctuated from late June to early December.

5.3.3. Phenotypical evaluation of biochemical traits

5.3.3.1 Basic fruit quality

Soluble solids content (SSC) and titratable acidity (TA) were determined on flesh juice as described by Mignard et al. (2021b). Soluble solids content were expressed as ºBrix. Titratable acidity results were expressed as g malic acid per liter and ripening index (RI) was thus calculated based on the SSC/TA ratio.

5.3.3.2. Antioxidant compounds, vitamin C, and relative antioxidant capacity (RAC)

For the analysis of total phenolics content (TPC), total flavonoids content (TFC), vitamin C (ascorbic acid – AsA), and the relative antioxidant capacity (RAC), a flesh sample composite of 5 g of five peeled fruits per replicate was frozen in liquid nitrogen and kept at −20°C until further analysis. The biochemical compounds were analysed using a 96-well microplate as described by Font i Forcada et al. (2019).

TPC was determined using the Folin-Ciocalteau method (Singleton et al., 1965) with modifications, and the results were expressed in mg of gallic acid equivalent (GAE) per 100 g of FW. TFC was determined using a colorimetric assay based on the method of Zhishen et al. (1999) with minor modifications, and the results were expressed in mg catechin equivalent (CE) per 100 g FW. The RAC was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method adapted from Brand-Williams et al. (1995) with modifications. The results were expressed in mg Trolox per 100 g FW. Finally, vitamin C – ascorbic acid (AsA) was determined using the method for the spectrophotometric determination of AsA as described by Zaharieva and Abadía (2003) with modifications. The results were expressed in mg AsA per 100 g FW.

5.3.3.3. Individual sugars and organic acids

Individual sugars and organic acids contents were assessed by HPLC as reported by Mignard et al. (2022). Sugars were analysed using an Aminex HPX-87C column (300 mm x 7.8 mm, Biorad) with a refractive index detector at 35ºC (Waters 2410, Waters Corporation, Milford, USA) and with milliQ water at 85ºC as mobile phase. Organic acids were assessed with a Rezex™ ROA-Organic Acid H+ (8%) column (300 mm x 7.8 mm, Phenomenex) with a photodiode array detector (Waters 2489, Waters Corporation, Milford, USA) at 210 nm and a sulphuric acid solution (0.005N) at room temperature as mobile phase. Individual sugars (glucose, fructose, sucrose), the sugar-alcohol (sorbitol) and main organic acids (citric, malic, oxalic, quinic, succinic, shikimic, and tartaric acids) concentrations were determined by their characteristic retention times following standards and expressed as g per kg of fresh weight.

5.3.4. Microsatellite loci analysis and genotyping

For genomic DNA extraction, young leaves were collected from each accession, frozen instantly in liquid nitrogen, and stored at -20°C until use. DNA was isolated using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA quality was examined by agarose gels (1%) and DNA concentration was determined by spectrophotometry using NanoDrop™ (Waltham, Massachusetts, USA). Twenty-three Simple Sequence Repeats (SSRs) markers previously described in *Malus* were tested in the apple population (Table 5.2). Eleven of these 23 SSRs were recommended by the 'European Cooperative Programme for Plant Genetic Resources (ECPGR) *Malus*/*Pyrus*' working group with a standard methodology proposed to allow comparisons of the same accessions between different laboratories (Lateur et al., 2012). In addition to these eleven SSRs, eight of them were specially designed to amplify in areas of the genome in relation to the metabolic pathway of sugars: the 'Sugar Will Eventually be Exported Transporters – SWEET' genes (Zhen et al., 2018).

Six different multiplexed reactions were used for these 23 SSRs. Forward SSR primers were labelled with 5'-fluorescence dyes including PET, NED, VIC and 6-FAM. The polymerase chain reactions (PCR) for the multiplexed PCRs were performed in a final volume of 10 µL using 10 ng of DNA template, 0.1 µM of each primer (with the exception of some markers as described in Table 5.2), and 1 × PCR Master mix of QIAGEN kit multiplex PCR (Qiagen, Hilden, Germany). Two PCR cycling conditions were used. The first PCR cycling was as follows: pre-incubation for 15 min at 95°C, followed by 5 cycles using a touchdown amplification program with an annealing temperature reduced by 1°C per cycle from 65°C to 60°C. The next step involved 30 cycles, each consisting of 30 s denaturing at 95°C, 60 s annealing at 60°C, and 60 s elongation at 72°C. The last cycle ended with a final 30 min extension at 72°C. The second PCR cycling consisted in: pre-incubation for 15 min at 95°C, followed by 7 cycles with a touchdown amplification program from 65°C to 58°C, followed by 30 cycles, each consisting of 30 s denaturing at 95°C, 60 s annealing at 58°C, and 60 s elongation at 72°C. The last cycle ended with a final 30 min extension at 72°C. Fragment analysis and sizing were carried out using Geneious Prime v.2022.0.1 software (Thermo Fisher Scientific, Waltham, USA). The PCR products were diluted with milliQ water 1:5 (v/v) and mixed with 12 µL Hi-di Formamide (Applied Biosystems) and 0.5 µL size standard Gene Scan™

600 Liz® (Applied Biosystems). The fragment analyses were performed using an Applied Biosystem 3130 DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 5.2. Characteristics of the 23 SSR markers used in this study with indication of the corresponding multiplex and dye.

Locus	N°	LG	Multiplex	Dye	Size Range (bp)	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)	Primer concentration	Reference
CH-Vf1	1	1	MP_5	VIC	130-171	ATCACCACCAGCAGCAAAG	CATACAAATCAAAGCACAACCC	[0.1 µM]	Urrestarazu et al.(2012)
Hi02c07	2	1	MP_3	VIC	98-146	AGAGCTACGGGGATCCAAAT	GTTTAAGCATCCCGATTGAAAGG	[0.1 µM]	Urrestarazu et al. (2012)
CH02c06	3	2	MP_3	PET	201-261	TGACGAAATCCACTACTAATGCA	GATTGCGCGCTTTTTAACAT	[0.4 µM]	Urrestarazu et al. (2012)
GD12	4	3	MP_3	NED	139-189	TTGAGGTGTTTCTCCCATTGGA	CTAACGAAGCCGCCATTTCTTT	[0.1 µM]	Urrestarazu et al. (2012)
MdSWEET2a	5	3	MP_6	VIC	331-357	ATACCGAGGAACTGTAGGACCAAGC	CTCCACACTAAACAACCAGAAAGCA	[0.1 µM]	Zhen et al. (2018)
MdSWEET9b	6	4	MP_4	6-FAM	336-360	GCGCCAATGTAAGACCCTTTACTTT	CTGACCTTGTCCTTCTTGGATGCGTA	[0.1 µM]	Zhen et al. (2018)
CH05f06	7	5	MP_2	NED	161-189	TTAGATCCGGTCACTCTCCACT	TGGAGGAAGACGAAGAAGAAAG	[0.1 µM]	Urrestarazu et al. (2012)
MdSWEET2d	8	5	MP_6	PET	265-289	CATTCAATTTATTCGACCGGACGAC	TGGGTTCATCCCTCACTTTCACTCA	[0.1 µM]	Zhen et al. (2018)
MdSWEET7b	9	6	MP_4	VIC	230-267	GGGTTTTGAGAATCTTGAGGGTAGG	TTTGATGGGTTGGACTGTAACTTGC	[0.1 µM]	Zhen et al. (2018)
CH04e05	10	7	MP_1	PET	163-228	AGGCTAACAGAAATGTGGTTTG	ATGGCTCCTATTGCCATCAT	[0.1 µM]	Urrestarazu et al. (2012)
CH01h10	11	8	MP_4	PET	81-120	TGCAAAGATAGGTAGATATATGCCA	AGGAGGGATTGTTTGTGCAC	[0.1 µM]	Urrestarazu et al. (2012)
CH01f03b	12	9	MP_5	NED	127-177	GAGAAGCAAATGCAAAACCC	CTCCCCGGCTCCTATTCTAC	[0.1 µM]	Urrestarazu et al. (2012)
CH02c11	13	10	MP_2	PET	208-238	TGAAGGCAATCACTCTGTGC	TTCCGAGAATCCTCTTCGAC	[0.15 µM]	Urrestarazu et al. (2012)
MdSWEET2e	14	10	MP_4	NED	205-243	GTGAGCCCACAACTAATCCCAT	CTTGTGCGTAGGAATCCCGATA	[0.1 µM]	Zhen et al. (2018)
CH02d08	15	11	MP_1	VIC	196-256	TCCAAAATGGCGTACCTCTC	GCAGACACTCACTCACTATCTCTC	[0.1 µM]	Urrestarazu et al.(2012)
MdSWEET2b	16	11	MP_5	6-FAM	249-263	TGAGGCAGAAACAATCATAAGGGTC	GAGCACGGAATTTGAAGCTGTAAAA	[0.1 µM]	Zhen et al.(2018)
MdSWEET7a	17	11	MP_5	PET	340-376	TTCTATCTCCCCTTCCCAAACTTCC	GCTAAACAGTGCCACTGCATAAGGT	[0.1 µM]	Zhen et al. (2018)
CH01f02	18	12	MP_1	6-FAM	155-212	ACCACATTAGAGCAGTTGAGG	CTGGTTTGTTTTCCTCCAGC	[0.1 µM]	Urrestarazu et al. (2012)
GD147	19	13	MP_3	PET	124-158	TCCCGCCATTTCTCTGC	GTTTAAACCGCTGCTGCTGAAC	[0.1 µM]	Urrestarazu et al. (2012)
CH04c07	20	14	MP_2	VIC	93-139	GGCCTTCCATGTCTCAGAAG	CCTCATGCCCTCCACTAACA	[0.1 µM]	Urrestarazu et al. (2012)
MdSWEET12a	21	14	MP_6	NED	223-253	ATGACAGGGCAACTTCAGGGT	CGTAATAGTCCTTTGCCCTCC	[0.1 µM]	Zhen et al. (2018)
CH02c09	22	15	MP_2	VIC	203-254	TTATGTACCAACTTTGCTAACCTC	AGAAGCAGCAGAGGAGGATG	[0.1 µM]	Urrestarazu et al. (2012)
CH01h01	23	17	MP_2	6-FAM	92-130	GAAAGACTTGCAGTGGGAGC	GGAGTGGGTTTGAGAAGGTT	[0.1 µM]	Urrestarazu et al. (2012)

5.3.5. Data analysis for the whole dataset

5.3.5.1. Phenotype statistical analyses

All statistical analyses were carried out using R language (R Development Core Team, 2019). The one-way analysis of variance (ANOVA) was run to determine whether there were any statistically significant differences between the means of the evaluated traits. Moreover, the Pearson's bivariate correlations were performed to better determine how biochemical traits contribute to variability among accessions.

5.3.5.2. Diversity and variability assessment

Genetic parameters were carried out for the 23 microsatellites and for the whole dataset (Population 1) corresponding to the 186 accessions from the EEAD – CSIC germplasm bank (186 accessions in total divided in two different pools: 150 diploids, Population 2; and 36 triploids, Population 3). No multiloci SSR marker was detected in this study. The number of observed alleles per locus (N_A), effective number of alleles per locus (N_E) (Kimura and Crow 1964), and rare alleles (N_B: alleles with a frequency below 5%), were determined using the Genodive software (Meirmans, 2020). Observed heterozygosity (H_o: number of heterozygous accessions/number of accessions assessed), expected heterozygosity ($H_e = 1 - \sum \rho i2$, where ρi is the frequency of the ith allele) (Nei, 1973), Wright's fixation index ($F_{is} = 1 - H_o / H_e$), and Shannon's information index (I) (Lewontin, 1972) were calculated using the PopGene 1.32 software (Yeh et al., 1997, http://www.ualberta.ca).

5.3.5.3. Analysis of population structure

STRUCTURE analysis was first performed on the whole dataset. All the accessions were categorized as Spanish or non-Spanish accessions. The program STRUCTURE (version 2.3.4) implements a model-based clustering criterion for inferring population structure using genotypic data from unlinked markers (Pritchard et al., 2000). All kinds of models including both "ancestry" and "allele frequency" models were fitted with the selection of admixture and allele frequency correlated, respectively. Furthermore, we also performed ten independent runs per K value with 10,000 burn-in period and 100,000 MCMC replications starting with K=1 to K=6, under the admixture model. The statistic ΔK was then carried out with K, where K specifies the number of subpopulations or clusters. This analysis was based on the rate of change in the log probability of the data (Evanno et al., 2005) to select the optimum number of K subpopulations.

5.3.6. Data analysis for the association mapping for 118 accessions

Genetic parameters for trait markers association were carried out for the 23 microsatellites and for the Population 4 corresponding to 118 diploids. Indeed, from the Population 2 (150 diploids), only 126 accessions were phenotyped and eight resulted duplicated (126 – 8 = 118 diploids). In fact, from these 126 accessions and using the software Cervus v.3.0.7 (Kalinowski et al., 2007), genetic uniqueness of each accession, and redundancy were eliminated. The multi-locus DNA profile of all the accessions was compared pairwise under the identity analysis, setting the minimum number of matching loci as 23 and 0 mismatch. Indeed, eight duplicated diploid accessions resulted from pairwise comparison of SSR profiles analysed by Cervus software. Although the ‘Averdal-1’, ‘Averdal-2’, ‘Evasni’, ‘Red Elstar’, ‘Royal Red Delicious’, ‘Starkrimson-2’, and ‘Topred Delicious’ cultivars were duplicated among them, the ‘Averdal-1’ was kept in the study. Moreover, the ‘Galaxy’ and the ‘Regal Prince-1’ were duplicated between them and the ‘Verde Doncella-1’ and ‘Verde Doncella-2’ were also duplicated between them. The ‘Galaxy’ and the ‘Verde Doncella-1’ accessions were kept in the study.

5.3.6.1. Linkage disequilibrium

The analysis of linkage disequilibrium (LD) was calculated using the Trait Analysis by Association, Evolution and Linkage (TASSEL) version 3.0.174 software (http://www.maizegenetics.net). Alleles with frequency below 5% were removed (minor allele frequency - MAF). Linkage Disequilibrium between pairs of multiallelic loci was calculated using the r^2 coefficient, separately for loci on the same or on different linkage group (LG). The statistical r^2 gives an indication of both recombination and mutation (Flint-Garcia et al., 2003). The significance level of LD between loci was examined using a permutation test implemented in TASSEL software for multiallelic loci, using the “rapid permutation” option.

5.3.6.2. Association mapping

TASSEL (v.3.0.174) was used with the General Linear Model (GLM) option (Yu and Buckler, 2006) to examine association between the phenotypic traits and the 23 DNA markers (Table 5.2). Structured associations approach could correct for false associations using a Q-matrix of population membership estimates (Yu and Buckler, 2006). Therefore, the population membership estimates obtained from STRUCTURE analyses were fitted as a covariate in a GLM where, phenotype = population structure + marker effect +

residual. In the present study, association mapping was used to identify linked markers for pomological and biochemical traits by using the general linear model (GLM). As permutation methods can provide exact control of false positives and allow the use of non-standard statistics making only weak assumptions about the data, a standard correction for multiple testing consisting of 10,000 permutations were run before carried out the GLM.

5.4. Results

5.4.1. Phenotypic evaluation and Pearson's correlations

The phenotypic evaluation and the statistical analysis were carried out for the Population 4 (118 diploids) out of the 186 apple accessions described in Table 5.1 and more extensively by Mignard et al. (2021b). The ANOVA analysis showed significant differences ($P \leq 0.001$) among the different apple accessions for all traits evaluated (Table 5.3).

The SSC ranged among apple accessions from 10.14 ('Bellaguarda Lardero') to 17.03 ('Eugenia') ºBrix. Regarding the TA, values varied greatly ranging from 1.77 ('Verde Doncella') to 17.29 ('Reguard-2') g malic acid per liter. The RI values ranged from 0.76 ('Reguard-2') to 8.55 ('Verde Doncella'). The standard deviation for SSC, TA and RI were fitted at 1.36, 2.92, and 1.34 respectively (Table 5.3). The TPC varied greatly among accessions ranging from 15.24 ('Biscarri-1') to 98.07 ('Camuesa Fina de Aragón') mg GAE/100 g FW. For the TFC, values ranged from 6.00 ('Biscarri-1') to 88.95 ('Camuesa Fina de Aragón') mg CE/100 g FW. Regarding AsA, values ranged from 1.37 ('Delgared Infel') to 5.30 ('Transparente') mg AsA/100 g FW. Finally, the RAC values ranged from 5.93 ('Delgared Infel') to 30.82 ('Les-1') mg trolox/100 g FW. The standard deviation for TPC, TFC, AsA and RAC respectively, were fitted at 15.76, 14.67, 0.82, and 5.08 (Table 5.3).

Total sugars values (Sugars) ranged significantly among apple accessions and years from 63.69 ('Transparente Blanca') to 115.27 ('Fuji') g kg^{-1} FW. Regarding the main individual sugar, fructose values ranged from 31.39 ('Baujade') to 61.41 ('Akane') g kg^{-1} FW and the alcohol sugar sorbitol values varied from 1.20 ('Plaona') to 11.96 ('Prau Riu-3') g kg^{-1} FW. The standard deviations for total sugars, fructose and sorbitol, were 9.87, 5.17, and 2.43 respectively. Total acids values (Acids) ranged among accessions and years from 3.42 ('Verde Doncella') to 11.39 ('Astrakan Red') g kg^{-1} FW. Regarding the main individual acids, malic acid, ranged considerably compared with the others from 2.68 (‘Delciri’) to 9.81 (‘Reguard-2’) g kg^{-1} FW. The standard deviations for total acids and malate, were fitted at 1.82 and 1.69 g kg^{-1} FW, respectively.

Table 5.3. Basic statistics of phenotypical traits over the Population 4 (118 diploid accessions) during the 2014-2018 period: units, minimum, maximum, mean values, standard deviation, and standard error of the mean.

Trait	Units	Minimum	Maximum	Mean	SD	SE	ANOVA
SSC	ºBrix	10.14	17.03	13.40	1.36	0.47	***
TA	g malic acid L^{-1}	1.77	17.29	6.61	2.92	1.35	***
RI	-	0.76	8.55	2.62	1.34	1.45	***
TPC	mg GAE 100 g FW^{-1}	15.24	98.07	39.54	15.76	0.08	***
TFC	mg CE 100 g FW^{-1}	6.00	88.95	22.84	14.67	0.12	***
AsA	mg AsA 100 g FW^{-1}	1.37	5.30	2.83	0.82	0.27	***
RAC	mg Trolox 100 g FW^{-1}	5.93	30.82	15.44	5.08	0.12	***
Sucrose	g.kg^{-1}	10.29	42.14	25.52	7.01	0.64	***
Glucose	g.kg^{-1}	6.23	24.29	13.17	4.12	0.38	***
Fructose	g.kg^{-1}	31.39	61.41	45.55	5.17	0.48	***
Sorbitol	g.kg^{-1}	1.20	11.96	4.55	2.43	0.22	***
Sugars	g.kg^{-1}	63.69	115.27	88.76	9.87	0.91	***
Oxalic	g.kg^{-1}	0.0136	0.0176	0.0147	0.0006	0.0001	***
Citric	g.kg^{-1}	0.0178	0.1482	0.0556	0.0279	0.0026	***
Tartaric	g.kg^{-1}	0.0260	0.0910	0.0480	0.0146	0.0013	***
Malic	g.kg^{-1}	2.6831	9.8144	5.5182	1.6930	0.1559	***
Quinic	g.kg^{-1}	0.2350	0.8005	0.4231	0.1151	0.0106	***
Succinic+Shikimic	g.kg^{-1}	0.1948	1.6766	0.5570	0.2520	0.0232	***
Acids	g.kg^{-1}	3.4242	11.3889	6.6085	1.8218	0.1677	***

ANOVA: significant differences at $P \leq 0.001$ (***) among the different apple accessions. Abbreviations: SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; RAC, relative antioxidant content; Sugars, sum of individual sugars; Acids, sum of organic acids.

Significant ($P \leq 0.01$) bivariate correlations were found between the different traits evaluated except for the peel color which did not shown any correlation (Figure 5.1). Positive and significant ($P \leq 0.01$) correlations were found between phenolics and flavonoids ($r = 0.96$) and between phenolics and flavonoids with RAC ($r = 0.90$ and $r = 0.87$ respectively). Significant positive correlations were also found between the antioxidant compounds and the organic acids. The individual sugars and the sum of sugars showed significant but low negative correlations with the bioactive compounds (TPC: r = -0.21, TFC: r = -0.26, and RAC: r = -0.25) and the organic acids. Moreover, high and significant correlations were found between harvest date, and concentrations of soluble solids, sorbitol, sugars, and as expected, with the ripening index. Significant and negative correlations were also found between the harvest date and individual/total organic acids, the titratable acidity, the antioxidant compounds (TPC and TFC) and the relative antioxidant capacity (RAC) (Figure 5.1). According to the Pearson's correlations, the non-Spanish accessions seemed to present lower organic acids and antioxidant compounds (TPC, TFC, and RAC) concentrations compared with the Spanish accessions.

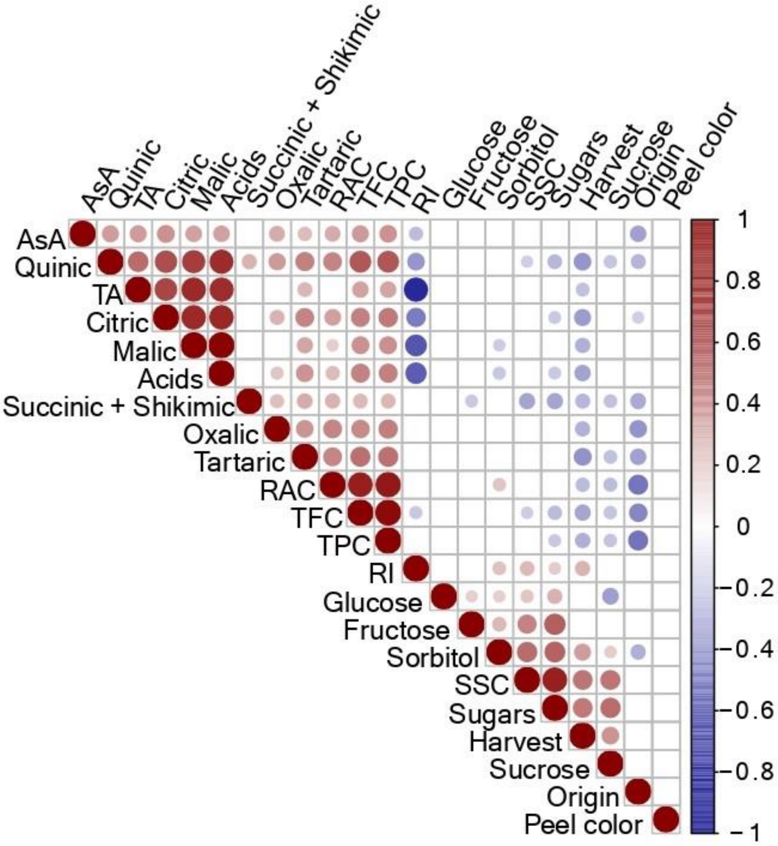

Figure 5.1. Pearson's bivariate correlations among the different traits assessed over the 118 accessions phenotyped. Abbreviations: SSC, soluble solids content; TA, titratable acidity; RI, ripening index; RAC, relative antioxidant content; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; Sugars, sum of individual sugars; Acids, sum of individual organic acids. The size of the circle for each correlation and the colour depicts the significance and the magnitude of the correlation coefficient, respectively.

5.4.2. Genetic Diversity

The mean estimated values for genetic parameters based on the 23 SSRs loci assessed are presented in the Table 5.4. According to the population 1 (186 accessions), the 23 microsatellites were all polymorphic and amplicons could be observed for all of them with a total of 360 alleles. The average number of alleles per locus (N_A) was 15.65, ranging from 4 (MdSWEET2b) to 35 (CH01f02). Nevertheless, the number of effective alleles per locus (N_E) was significantly lower (Table 5.4). Indeed, the average number of effective alleles (N_E) overall the loci was 4.90, ranging from 1.5 (MdSWEET2e) to 8.98 (CH02d08). Moreover, rare alleles (N_B) were found in all loci and the number of them increased with the number of alleles per locus (r=0.96). Alleles with a frequency lower than 5% (N_B) varied from 33.3% at locus CH02c11 (5 out of 15) to 86.7% at locus CH04e05 (26 out of 30). Furthermore, all loci except five (CH04e05, CH01f02, CH01h01, MdSWEET2e, and MdSWEET2d) were not at Hardy-Weinberg Equilibrium ($P \leq 0.05$). In fact, the 186 accessions assessed in this study do not belong to a panmictic population. While mean observed heterozygosity (H_o) was 0.75, ranging from 0.19 (MdSWEET2e) to 0.96 (MdSWEET7b) (Table 5.4), average expected heterozygosity (H_e) was 0.77, varying from 0.41 (MdSWEET2e) to 0.92 (CH05f06). F_{is} values were positive in 10 primers and negative in the remaining 13 SSRs, indicating a high level of heterozygosis in the genotypes assessed.

Among the population 2 (150 diploid accessions), and population 3 (36 triploid accessions), the average number of alleles per locus (N_A) was 14.7 and 10 respectively, and the number of effective alleles per locus (N_E) were fitted at 5.23 (Pop2) and 4.66 (Pop3). The observed heterozygosity and the expected heterozygosity were higher in diploids ($H_o = 0.79$; $H_e = 0.77$) than in triploids ($H_o = 0.71$; $H_e = 0.73$). The F_{is} value was negative for the population 2 while it was positive for the population 3.

Pairwise comparison of multiloci profiles revealed eight duplicated diploid accessions of redundancies leading to the removal of redundant accessions before further analyses (4.3 % of redundancy). Several cases of homonymy (i.e., accessions with the same name but different profiles according to the 23 SSRs) were also found (‘Camosa-1’ and ‘Camosa-2’, ‘Jonathan-1’ and ‘Jonathan-2’, ‘Granny Smith-1’ and ‘Granny Smith-2’, ‘Mañaga-1’ and ‘Mañaga-2’, ‘Starking-1’ and ‘Starking-2’, and ‘Verde Doncella-1’ and ‘Verde Doncella-3’). The final number of unique diploid genotypes further analyzed was therefore 118 (Pop4).

Table 5.4. Mean estimated values for different genetic parameters of the 186 apple accessions based on 23 SSRs loci.

SSR	N_A	N_E	N_B	Ho	He	F_{is}
CH-Vf1	13	3.50	10	0.75	0.73	-0.03
Hi02c07	9	3.40	4	0.68	0.70	0.03
CH02c06	21	8.54	14	0.93	0.90	-0.03
GD12	17	2.85	13	0.71	0.69	-0.03
MdSWEET2a	12	4.28	8	0.83	0.81	-0.02
MdSWEET9b	9	2.53	5	0.66	0.63	-0.05
CH05f06	14	5.79	7	0.86	0.92	0.07
MdSWEET2d	13	4.88	8	0.44	0.81	0.46
MdSWEET7b	15	5.17	9	0.96	0.87	-0.10
CH04e05	30	3.47	26	0.67	0.73	0.08
CH01h10	18	3.23	14	0.78	0.72	-0.08
CH01f03b	15	5.24	11	0.92	0.82	-0.12
CH02c11	15	8.72	5	0.89	0.90	0.01
MdSWEET2e	12	1.50	10	0.19	0.41	0.54
CH02d08	25	8.98	18	0.85	0.90	0.06
MdSWEET2b	4	1.96	2	0.51	0.50	-0.02
MdSWEET7a	12	3.61	8	0.72	0.74	0.03
CH01f02	35	8.60	29	0.81	0.90	0.10
GD147	15	4.29	9	0.83	0.78	-0.06
CH04c07	19	7.06	11	0.92	0.91	-0.01
MdSWEET12a	8	2.42	4	0.60	0.58	-0.03
CH02c09	13	6.96	5	0.93	0.87	-0.07
CH01h01	16	5.80	10	0.75	0.83	0.10
Mean - 186 accessions (Pop1)	15.65	4.90	10.43	0.75	0.77	0.03
Mean diploids - 150 accessions (Pop2)	14.70	5.23	9.22	0.79	0.77	-0.03
Mean triploids - 36 accessions (Pop3)	10.00	4.66	5.26	0.71	0.73	0.03

N_A: observed number of alleles per locus; N_E: effective number of alleles per locus; N_B: number of rare alleles; Ho: observed heterozygosity; He: expected heterozygosity, F_{is}: Wright's fixation index; I: Shannon's information index; Pop: population.

5.4.3. Population structure

Bar plots were obtained according to the values of K assumed number of subpopulations, corresponding to the number of clusters defined by delta K (Evanno et al., 2005) (Figure 5.2). Model-based clustering analyses were used to determine the genetic diversity structure within the 186 (Pop1) assessed *Malus x domestica* (Borkh) genotypes. A pairwise STRUCTURE analyses based on 23 allelic SSR molecular data was carried out and the maximum value of delta K was observed at K = 2 for Pop2 (150 diploids) and Pop4 (118 diploids), suggesting two genetic clusters. Along with the 36 triploids of the study (Pop3), the maximum value of delta K was revealed at K = 4, although at K = 2, an increase of delta K was observed (Figure 5.2). Genotypes were divided into two or four clusters respectively based on their membership coefficients (Q), considering the genotypes as pure when the membership coefficient (qI) was greater than 0.80 and as an admixture or hybrid when the membership coefficient (qI) was lower than 0.80 (Pritchard et al., 2000).

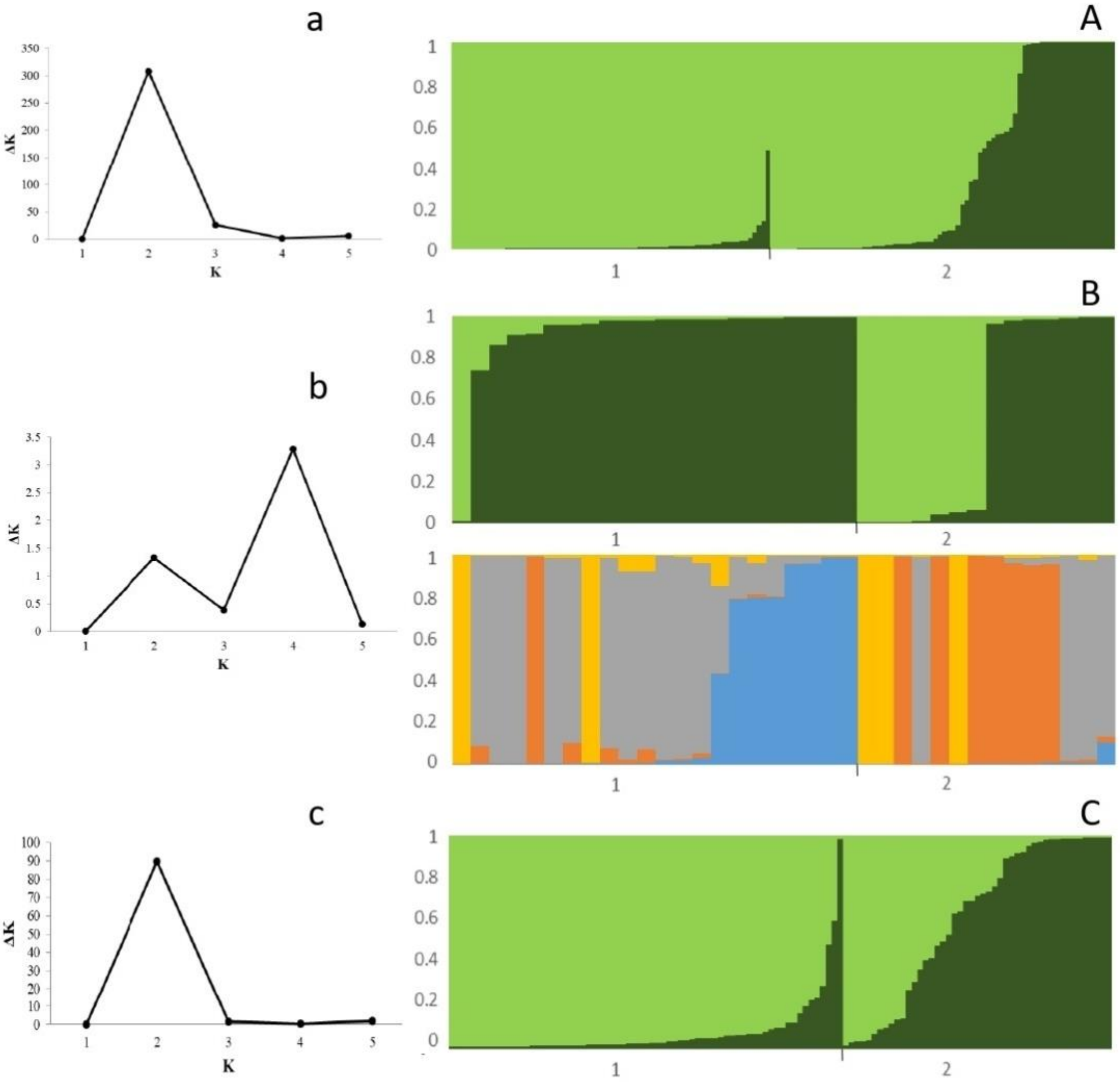

Figure 5.2. Estimation of Malus x domestica Borkh collection using LnP(D) derived Δk for k from 1 to 5 based on: a) Population 2 (150 diploids apple accessions); b) Population 3 (36 triploids); and c) Population 4 (118 diploids) and STRUCTURE bar plots based on: A) Pop2 at K=2; B) Pop3 at K=2 and K=4; and C) Pop4 at K=2, sorting by subpopulation (Spanish/Non-Spanish) and the coefficient of membership (Q).

The population 2 (150 diploids) was represented by 72 Spanish and 78 non-Spanish accessions, Pop3 (36 triploids) by 22 Spanish and 14 non-Spanish and Pop4 (118 diploids) by 72 Spanish and 46 non-Spanish accessions. However, the separation at K = 2 was congruent for all the diploid populations assessed, and admixture in each subpopulation was observed demonstrating an allele sharing (Figure 5.2). For population 4 (118 diploids), the mean values of genetic differentiation (F_{st}) among the two subpopulations obtained with K = 2 were 0.25 and 0.0024 respectively. The F_{st} of the first subpopulation indicated a strong genetic differentiation for the accessions of this group. In addition, the allele frequency divergence among subpopulations were 0.0678 and demonstrated significant genetic differences between subpopulations (>0.05). Furthermore and regardless of the population studied (Pop2 and Pop4), the first subpopulation was represented mostly by Spanish accessions and the second by the non-Spanish reference cultivars.

Moreover, the dendrogram obtained by the unweighted pair group method with arithmetic mean (UPGMA) analysis was compared with the phenotypic data (Figure 5.3) and the two subpopulations of the STRUCTURE results for the population 4 (Figure 5.4). In the dendrogram obtained from the similarity matrix of pairwise analyses for the 23 SSR markers, there is a clear agreement between clusters representing genetic diversity and subpopulations obtained with STRUCTURE at K = 2. Furthermore, the population assessed can clearly be divided into six clusters/groups from the first two major clusters (cluster 1: groups 1, 2, 3, and 4; cluster 2: groups 5 and 6). According to the Spanish/non-Spanish classification, the first cluster of the UPGMA analysis (groups 1, 2, 3, and 4) included only 16 non-Spanish accessions out of 85. The group 1 included 15 non-Spanish and 13 Spanish accessions. The groups 2 and 3 did not show any non-Spanish accession and the group 4 included only one: ‘Jonadel’ accession. The second cluster (groups 5 and 6) was represented by 33 accessions and 30 were non-Spanish. The three Spanish accessions observed in this second cluster were: ‘Biscarri-1’, ‘De Valdés’, and ‘Valsaina’ (Figure 5.3 and 5.4).

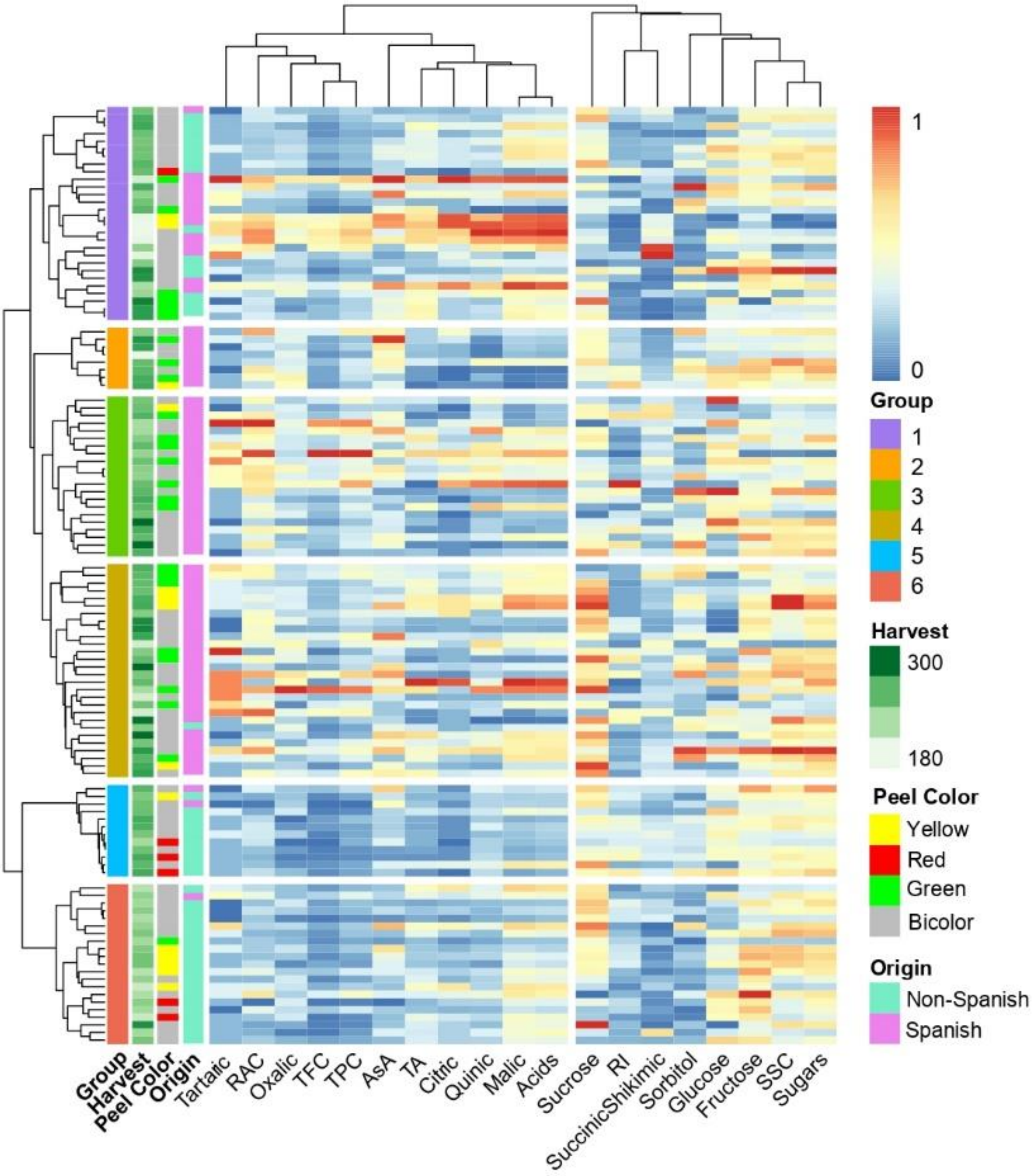

Figure 5.3. Pheatmap of 118 apple accessions based on pairwise genetic distances with 23 SSR markers and fruit biochemical characteristics as basic fruit quality (soluble solids content, SSC; titratable acidity, TA; ripening index, RI), antioxidants (total phenolics content, TPC; total flavonoids content, TFC; vitamin C - AsA), individual sugars (glucose, fructose, sucrose and sorbitol), and organic acids (malate, quinate, citrate, tartarate, oxalate, succinate, and shikimate). Annotations as group (K=6 clusters), harvest date, peel color and origin (Spanish/Non-Spanish) for each accession are shown.

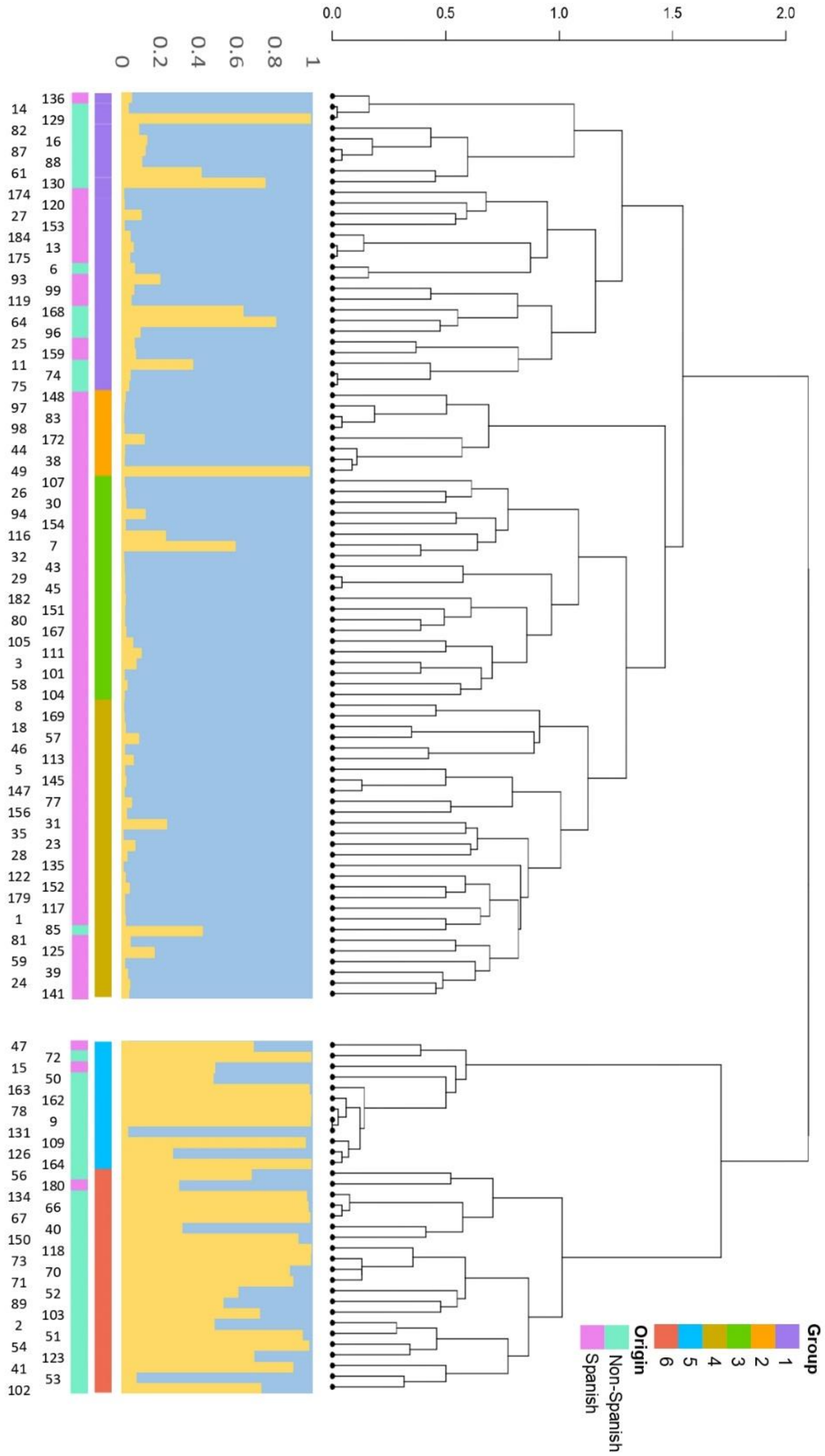

Figure 5.4. Dendrogram of Population 4, corresponding to 118 diploids apple accessions, based on pairwise genetic distances according to 23 SSR markers and STRUCTURE bar plot at K=2, optimum number of K subpopulations for Pop4.Moreover, annotations for the accessions assessed as group/cluster number (n =6 clusters) and origin (Spanish/Non-Spanish) for each accession are shown.

The 19 phenotypic traits were divided into two groups of variables. Firstly, the TA, TPC, TFC, RAC, AsA, tartarate, oxalate, citrate, quinate, malate and the sum of organic acids were clustered. On the other hand, the succinate + shikimate, SSC, RI, sucrose, glucose, fructose, sorbitol, and the sum of individual sugars were grouped. Indeed, clusters would group variables that tended to behave similarly across the accessions. The first group of traits was dominated by the antioxidant traits, the titratable acidity and the individual organic acids (except the sum of shikimate and succinate), while the second corresponded to the individual sugars, the SSC and the RI traits. The cluster 1 (groups 1, 2, 3, and 4) of accessions seemed to present a more acidic profile while groups 5 and 6 showed a sweeter profile. Nevertheless, the more acidic cultivar assessed was non-Spanish ('Astrakan Red') and was in the cluster 1 – group 1. The cluster 1 (groups 1, 2, 3, and 4) showed more antioxidant compounds than the groups 5 and 6. Indeed, the 'Camuesa Fina de Aragón' accession exhibited the most TPC values with 98.1 mg GAE 100 g FW^{-1}, far away from the first non-Spanish accession ('Akane') with 55.59 mg GAE 100 g FW^{-1}. The 'Les-1' accession was the accession with the most relative antioxidant capacity (RAC) found among all the Pop4 data set (30.8 mg Trolox 100 g FW^{-1}) while the 'Delgared Infel' (non-Spanish) was the accession with the less RAC observed (5.9 mg Trolox 100 g FW^{-1}). According to the harvest date, ranging from 170 ('Mañaga-2') to 316 ('Bossost-4') Julian days, the first cluster (groups 1, 2, 3, and 4) seemed to cluster accessions which ripened later than the accessions of the second cluster (groups 5 and 6). The peel color did not seem to influence in clustering the population 4 (Figure 5.3). Indeed, six accessions were categorized as 'Red' and they were in different groups: 'Redaphough' belonged to group number 1; 'Averdal', 'Nueva Starking', and 'Starkrimson' were in group 5, while 'Delgared Infel' and 'Prima' belonged to group 6.

5.4.4. Linkage disequilibrium and association mapping

The linkage disequilibrium (LD) arrays of all the 253 pairwise combinations of the 23 SSRs were assessed using TASSEL (Figure 5.5). The LD r^2 values varied greatly from 0.00 to 0.77, with $r^2 = 1$ when the markers provide exactly the same information. The highest LD value was recorded between CH02c11 and CH02c06 markers. The significance cut-off threshold values from the distribution of LD was assigned at $r^2 = 0.2$. Indeed, 15 pairwise combinations of the 23 SSRs assessed recorded values above the cut-off. Thus, CH01h10 and CH02c11 were considered at linkage disequilibrium as well as CH02c06 with Hi02c07, CH05f06, CH02c09, and CH02c11; Ch02c09 with CH02c11;

CH02c11 with CH05f06; MdSWEET2d with CH02c09, CH02c06, and CH02c11; and finally, MdSWEET7b with CH01h10, CH02c11, CH02c06, and CH02c09 markers.

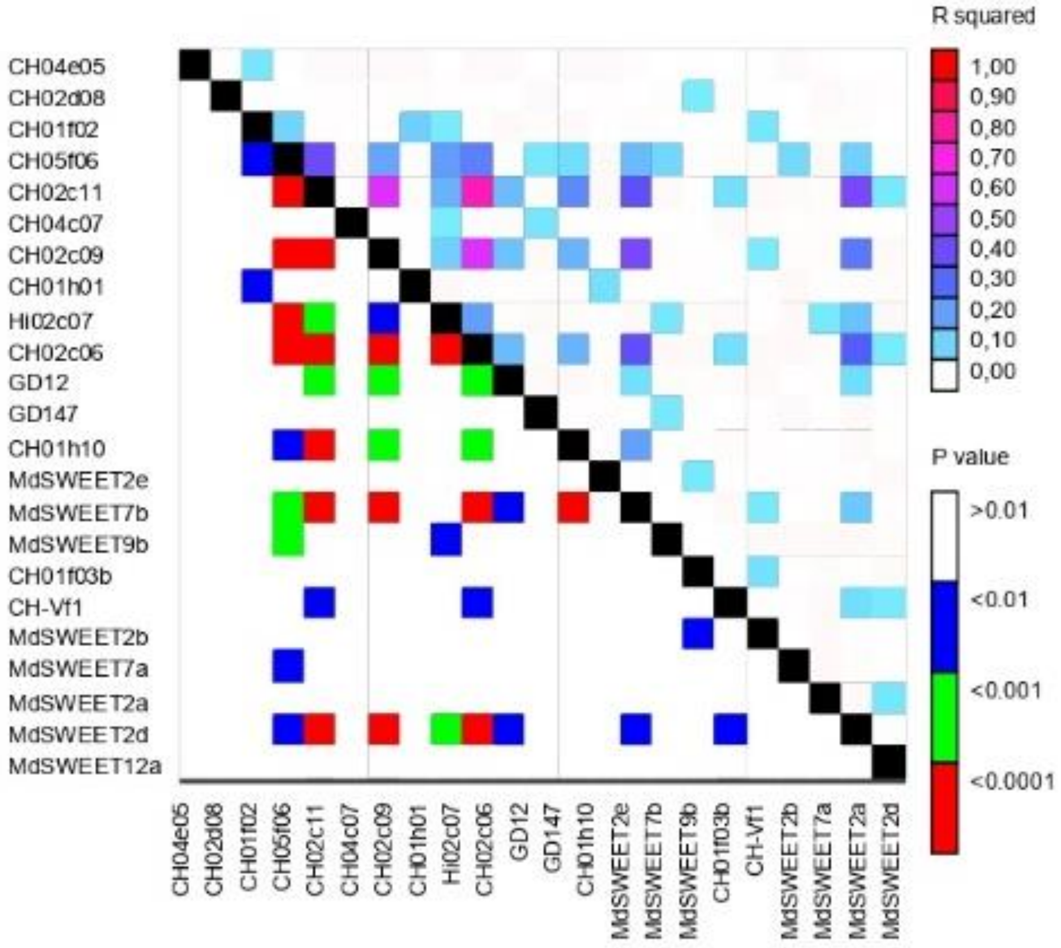

Figure 5.5. Linkage disequilibrium plot based on 23 SSR markers screened among Population 4 (118 apple accessions). The r^2 values are shown in the upper right of the plot and the P-values are represented in lower left of the plot.

The results showed that the number of associations reached were 126 (P ≤ 0.01) using a modeling coefficient of membership (Q) values estimates from STRUCTURE as covariate (Table 5.5). In total, 20 SSRs (out of 23 SSRs) contributing of the phenotypic variation were significantly associated with at least one trait studied. Moreover, only fructose was not significantly associated with any of the 23 SSRs assessed. Furthermore, the SWEETs genes markers MdSWEET2a, MdSWEET2d, MdSWEET7b, MdSWEET2b, MdSWEET7a, and MdSWEET12a markers showed significant associations (P ≤ 0.01) with 17 out of the 21 phenotypic traits assessed (harvest, peel color, RAC, TPC, TFC, SSC, TA, RI, sucrose, sorbitol, sum of sugars, oxalate, citrate, tartarate, malate, quinate, and sum of organic acids). We will focus on the 10 SSR markers which showed 19 significant associations at $P \leq 0.0001$: CH01f02, CH01f03b, CH02c06, CH04c07, CH04e05, CH05f06, CH-Vf1, MdSWEET12a, MdSWEET2a, and MdSWEET7b markers. According to the agronomical parameters, only one significant association was found between the harvest date and the MdSWEET12a marker.

For the antioxidant compounds, five associations were found. Thus, the relative antioxidant capacity (RAC) was associated to the MdSWEET7b marker, the TPC with CH05f06 and MdSWEET7b marker and finally, the TFC was linked with two markers (CH02c06 and CH05f06). For the basic fruit quality parameters, two associations were found. The first was found between the ripening index and the CH04c07 marker while the second was observed between the titratable acidity and the CH01f02 marker. Seven associations between the individual organic acids and markers were observed. The succinate and shikimate were associated with the CH01f03b, CH02c06, and CH04e05 markers. The citric and the quinic acid were associated with the CH05f06 marker while the oxalate was related with the CH02c06 and MdSWEET2a markers. Lastly, according to the sugars compounds, four associations have been observed: two between the sorbitol content and the CH01f02 and CH02c06 markers, another one between the sucrose content and the CH-Vf1 marker, and the last one between the sum of the individual sugars and the MdSWEET12a marker.

Table 5.5. Significance (*P*-value) of association between 23 SSRs polymorphic loci and biochemical traits among 118 apple accessions.

			Agronomical		Antioxidants				Basic Fruit Quality			Individual Sugars					Individual Organic Acids						
Nº	**LG**	**Marker name**	**Harvest**	**Peel Color**	**RAC**	**TFC**	**TPC**	**AsA**	**SSC**	**TA**	**RI**	**Suc**	**Glu**	**Fru[1]**	**Sor**	**Sug**	**Oxa**	**Cit**	**Tar**	**Mal**	**Qui**	**Succ-Shi**	**Acids**
1	**1**	**CH-Vf1**										***							*			**	
2	**1**	**Hi02c07**								**	**							*		*			
3	**2**	**CH02c06**				***	**		*	*	*	**	*		***	*	***	*	*		*	***	
4	**3**	**GD12[1]**																					
5	**3**	**MdSWEET2a**			**	*	*			*							***	*	**	*	*		*
6	**4**	**MdSWEET9b**			*										*		**				*		
7	**5**	**CH05f06**	*		**	***	***		**	**	*					*		***	*	*	***	**	**
8	**5**	**MdSWEET2d**	*				*			**								**		**	*		**
9	**6**	**MdSWEET7b**			***	**	***				*								*				
10	**7**	**CH04e05**						**			**							*				***	
11	**8**	**CH01h10**															**						
12	**9**	**CH01f03b**	*							**	*							**		**	*	***	**
13	**10**	**CH02c11**		**	**		*	*															
14	**10**	**MdSWEET2e[1]**																					
15	**11**	**CH02d08[1]**																					
16	**11**	**MdSWEET2b**								*							*	*	*	*	*		*
17	**11**	**MdSWEET7a**		*			*			**	*					*		**					
18	**12**	**CH01f02**			*		**			***	*				***			*					*
19	**13**	**GD147**													**								
20	**14**	**CH04c07**	*		*	*	**	*		**	***		*				*	*		*	*	*	*
21	**14**	**MdSWEET12a**	***						**			*			**	***							
22	**15**	**CH02c09**							*		*								**				
23	**17**	**CH01h01**			*		*				*	*							**				

[1]: No association found for this SSR or trait. Statistical significance at *: $P \leq 0.01$; **: $P \leq 0.001$; ***: $P \leq 0.0001$; Abbreviations: RAC, relative antioxidant content; TFC, total flavonoids content; TPC, total phenolics content; AsA, Ascorbic acid; SSC, soluble solids content; TA, titratable acidity; RI, ripening index; Suc, sucrose; Glu, glucose; Fru, fructose; Sor, sorbitol; Sug, total sugars; Oxa, oxalate; Cit, citrate; Tar, tartarate; Mal, malate; Qui, Quinate; Succ-Shi, succinate and shikimate; Acids, total organic acids.

5.5. Discussion

Phenotypic Characterization

As expected, a large variability for the different traits assessed was found among accessions, and the concentrations of biochemical compounds observed in the present study were in line with previous reported values (Aprea et al., 2017; Castel et al., 2020; Donahue et al., 2021; Kistechok et al., 2022; Reig et al., 2015; Slatnar et al., 2019; Yang et al., 2021; Zhang et al., 2022; Zhen et al., 2018). Nonetheless, the larger sample size of this study (118 accessions) resulted in a greater range of concentrations with significant differences in the values of traits assessed.

Moreover, significant correlations were found between the different traits evaluated. Positive and significant correlations were found as previously reported in other studies between phenolics and flavonoids or RAC (Mignard et al., 2021b; Preti & Tarola, 2020; Raudone et al., 2017). Many of the correlations observed could be explained by the photosynthetic activity (Kistechok et al., 2022; Pandey et al., 2017). Indeed, the photosynthetic products will act as substrates for many of metabolic pathways. The sum of individual sugars, and main products of the primary metabolism by the photosynthesis, and SSC shown significant negative correlations with bioactive compounds (TPC and TFC) and positive with all the organic acids, substrates and products of the secondary metabolism in plants (Ruan et al., 2014). These correlations, could be explained as a possible response to the phenolic compounds biosynthesis. Indeed, carbohydrates as fructose increase the erythose-4-phosphate production that constitute, together with the phosphoenolpyruvate (PEP), a substrate for phenolic compounds through the shikimate pathway (Vallarino & Osorio, 2019). Furthermore, positive and significant correlations were also found between the antioxidants and the organic acids (Mignard et al., 2022). The decarboxylation of the dicarboxylates such as malate and other organic acids is linked to the degradation of organic acids and thus, this decarboxylation permits PEP production. The PEP is closely associate to the activation of gluconeogenesis and thus resulting in glucose production in fruits (Vallarino & Osorio, 2019). These correlations showed thus, the linkage between the primary and the secondary metabolism (Li et al., 2012). In fact, the secondary metabolism (antioxidant compounds) is connected to primary metabolism (sugars and acids) as substrates are supplied from primary pathways and drove into the secondary biosynthetic routes. Indeed, through the polyol or sorbitol-aldose reductase pathway, a two-step mechanism converting glucose into fructose, the positive and significant correlations between the different individual sugars could be explicated.

Moreover, high and significant correlations were found between harvest date, and the concentration in the fruit of soluble solids content, sorbitol, sugars, and as expected, with the ripening index. These results show that when fruits are harvested late in the season but each accession at its optimum commercial maturity, they are sweeter, in general. In contrast, significant and negative correlations were found between the harvest date and individual/total organic acids, titratable acidity, the antioxidant compounds (TPC and TFC) and the relative antioxidant capacity (RAC) (Li et al., 2012). Furthermore, the non-Spanish accessions seemed to present less organic acids concentrations and less antioxidant compounds (TPC, TFC, and RAC) compared with the Spanish well adapted accessions as ‘Camuesa Fina de Aragón’, ‘Reguard – 2’, ‘Transparente’, and ‘Verde Doncella’ accessions, as previously reported (Mignard et al., 2021b; 2022). These results highlights the importance of the germplasm characterization (Swarup et al., 2020) in the aim to boost autochthone accessions and phytogenetic resources for breeding programs.

Genetic Identity and Overall Diversity

The successful amplification and polymorphism obtained using 23 SSR markers covering the whole genome used to screen the 186 apple accessions (Pop1) confirmed previously reported results for cultivar identification and genetic mapping in apple trees (Bakır et al., 2022; Ferreira et al., 2016; Khachtib et al., 2022; Pereira-Lorenzo et al., 2017; Marconi et al., 2018; Urrestarazu et al., 2012; Van Treuren et al., 2010; Zhen et al., 2018).

Although some ancient apple Spanish accessions, relevant in the past in Spain (Herrero et al., 1964), were assessed in this study, there was any proof that they were true-to-type accessions (Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2012). Indeed, the different accessions labelled as ‘Camosa-1’/‘Camosa-2’, ‘Reguard-2’/‘Reguard-4’, ‘Mañaga-1’/‘Mañaga-2’ or ‘Verde Doncella-1’/‘Verde Doncella-3’ did not show the same SSR profiles. In contrast, ‘Verde Doncella-2’ and ‘Verde Doncella-3’ exhibited exactly the same allelic profiles. Moreover, the apple genetic resources analysis using SSR markers should be studied with attention because of the incidence of mutations, the genome structure variations or the epigenetic alterations which could engender phenotypic modifications no distinguishable using SSR markers (Lassois et al., 2016; Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2017). Consequently, the phenotypic and the molecular characterizations of apple accessions, both complementary approaches, could determine whether the accessions with the same SSR profiles would be the same.

Among the 23 different SSR markers, the level of genetic diversity and expected/observed heterozygosity were high. As apple is a self-incompatible cross pollination specie (Pereira-Lorenzo et al., 2017), the high diversity observed indicated that the apple genetic resources and thus, the accessions, preserved in the germplasm bank of the Aula Dei – CSIC were highly diverse. The high genetic diversity found was in agreement with the variability exhibited in other studies (Bühlmann et al., 2015; Ferreira et al., 2016; Lassois et al., 2016; Liang et al., 2015; Patzak et al., 2012; Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2012; Van Treuren et al., 2010). All SSR loci analyzed in this study displayed a high degree of polymorphism with 4 to 35 alleles per locus and with 1.5 to 8.98 effective number of alleles per locus. Indeed, the mean value found in this study was of 15.65 number of alleles per locus for the population 1, which is slightly lower that the 16.69 observed by Urrestarazu et al. (2012) studying 493 accessions and the 18.62 reported by Pereira-Lorenzo et al. (2017) with 1453 accessions in Spain and sharing respectively 16 SSRs (13 markers out of 16 were used in this study) and 13 SSR markers (12 out of 13 were included in the present work). Moreover, 18 alleles in average per locus were showed in a Turkish germplasm of 206 accessions (Bakır et al., 2022) and 19.5 in a collection of 2163 accessions in France (Lassois et al., 2016). Nevertheless, Marconi et al. (2018) assessing 175 accessions in Italy and Ferreira et al. (2016) studying 87 accessions in Portugal observed a mean value for the number of alleles per locus of 14.6 and 11.5 respectively. The observed heterozygosity averaged for the all dataset (0.75) over the 23 SSR loci was exactly the same as reported by Meland et al. (2022), studying 171 accessions and using seven SSRs markers also used in the present study (CH02c06, GD12, CH01h10, CH02c11, CH02d08, CH02c09, and CH01h01 markers). Moreover, the H_0 was higher than the 0.67 observed by Ferreira et al. (2016) but slightly lower than reported values of 0.78 (Marconi et al., 2018), 0.83 (Lassois et al., 2016), 0.76 (Bakır et al., 2022), and 0.81 (Urrestarazu et al., 2012). The differences found in those studies could be due to the different plant material, the ploidy level or even the number of accessions assessed in each study.

According to the triploid accessions, it is worth mentioning that the amplification of three alleles in a single locus is not an evidence for categorizing an accession as triploid (Liang et al., 2015; Ferreira et al., 2016). In fact, in diploid accessions, a third fragment could be amplified as the result of duplication or a somatic mutation (Liang et al., 2015). However, if more than two alleles were found at several loci (several SSRs markers) the accessions were categorized as triploids. Furthermore, in the present study, a confirmation of the ploidy level by flow cytometry for the whole dataset was assessed as described by Reig et al. (2015).

Population Structure

The STRUCTURE results showed that two main subpopulations (K = 2) with some degree of admixture within both of them (Spanish and non-Spanish accessions) were exhibited. Similar studies in apple also reported two unstructured populations, indicating a strong subpopulation structure using respectively 1453 (Pereira-Lorenzo et al., 2017) and 493 accessions (Urrestarazu et al., 2012). Indeed, Pereira-Lorenzo et al. (2017) reported that the analysis of 1453 apple accessions, conserved in the Spanish collections, permitted the discrimination of an Iberian gene pool of apple accessions separated from an extensive set of non-Spanish reference cultivars.

The UPGMA cluster analysis grouped all the 118 apple accessions into a dendrogram. The cluster analysis was able to group all genotypes into two large clusters, with the first one (cluster 1) containing four subgroups (groups 1 to 4) and the second one (cluster 2), with two subgroups (groups 5 and 6). The UPGMA analysis also showed that one of the two subpopulations encountered was quite-exclusively formed by non-Spanish accessions (30 out of 33). Indeed, the cluster 1 included 16 non-Spanish cultivars (‘Astrakan Red’, ‘Baujade’, ‘Belleza de Roma’, ‘Blackjon’, ‘Bofla’, ‘Florina’, ‘Fuji’, ‘Granny Smith-1’, ‘Granny Smith-2’, ‘Idared’, ‘Jonadel’, ‘Jonathan-1’, ‘Jonathan-2’, ‘MacIntosh’, ‘Red Rome Beauty’, and ‘Redaphough’) and the cluster 2 included all the 30 remaining non-Spanish reference cultivars and only three Spanish accessions (‘Biscarri-1’, ‘De Valdés’, and ‘Valsaina’). Nevertheless, in the cluster 1, 15 non-Spanish accessions were included in the subgroup 1 and only one was in the subgroup 4 (‘Jonadel’). The groups 2 and 3 did not show any non-Spanish accession.

The results for genetic subpopulation obtained suggested that the non-Spanish reference cultivars were slightly more similar among them than with the Spanish accessions. In fact, several studies have showed that the European germplasm core collections shared a lot of the non-Spanish reference plant material conserved (Cornille et al., 2012; Jung et al., 2020; Marconi et al., 2018; Pereira-Lorenzo et al., 2017; Swarup et al., 2020; Urrestarazu et al., 2012; 2016). Moreover, according to the autochthone accessions from the different germplasm collections around the world, domestication and breeding could have cause diversity loss. Nevertheless, regardless of the many decades of domestication of *Malus x domestica* and its clonal propagation, there is no proof that domesticated apple has showed a genetic bottleneck in contrast with *Malus x sieversii* (Gross et al., 2014; Wedger et al., 2021).

The same Spanish/non-Spanish cluster segregation was also distinguishable in previous studies regarding the influence of climate parameters on basic fruit quality (SSC, TA, and RI), antioxidants traits (TPC, TFC, AsA, and RAC), individual sugars (glucose, fructose, sucrose, and sorbitol) and organic acids (citric, malic, oxalic, quinic, succinic, shikimic, and tartaric) contents of 155 accessions out of the 186 included in the Pop1 assessed in this study. Moreover, the range of the results was larger for the Spanish accessions than for the non-Spanish ones (Mignard et al., 2021b; 2022) highlighting more similar profiles for the non-Spanish cultivars. Indeed, Spanish autochthone accessions reported a higher biodiversity and, in general, higher contents for some basic fruit quality traits, antioxidants and individual sugars and organic acids than non-Spanish accessions over five years of study. Higher contents of antioxidants and organic acids were observed, in general, in the groups 1, 2, 3, and 4 of the clustering analysis, while the sugar profile was more heterogeneous according to the accession's origins. These results strengthen the consciousness of the importance of autochthone phytogenetic resources and underline the high biodiversity found in germplasm core collections (Swarup et al., 2021). Moreover, climatic traits such as precipitation, solar radiation, and temperature strongly influenced the antioxidant and metabolite profiles of the accessions studied and depended on its origin (Donahue et al., 2021; Kistechok et al., 2022; Li et al., 2012; Mignard et al., 2021b; 2022; Reig et al., 2015). Nevertheless, Pereira-Lorenzo et al. (2017) reported the same segregation with a total of 1453 accessions including part of the accessions of the present work and from different germplasm collections from Spain. Moreover, Mignard et al. (2021b; 2022) showed a segregation between Spanish local accessions and modern non-Spanish cultivars according to the biochemical contents (SSC, TA, RI, TPC, TFC, AsA, RAC, sucrose, glucose, fructose, sorbitol, oxalate, quinate, malate, citrate, tartarate, succinate and shikimate) and to the climatic factors influence (high solar radiation and low temperatures). This highlight the fact that further the climatic parameters influence in the metabolite profiles of the apple fruits (Li et al., 2020), the genetics have a stronger influence on the biochemical contents and explained why two subpopulations were found in previous fruit quality studies (Mignard et al., 2021b; 2022).

Linkage Disequilibrium and Association Mapping

The present assay is the first study reported in apple to find association with agronomical and biochemical traits in a germplasm collection using both Spanish and non-Spanish apple genetic resources and assessed by 23 SSR molecular markers. Moreover, 21 traits of interest such as basic fruit quality, antioxidant parameters, individual sugars and organic acids were assessed all together. Despite being the first approximation made to date in apple between biochemical traits and SSRs markers for 21 parameters assessed, significant associations have been found and will be of great help in future studies. Regarding the bibliography, scarce studies of genetic/phenotypic associations using SSRs can be found. The published studies analyzed exclusively several organoleptic parameters based on SNPs markers (Gutierrez et al., 2018; Jung et al., 2020; Larsen et al., 2017; McClure et al., 2018; 2019; Urrestarazu et al., 2017). However, Tsykun et al. (2017) reported that the multi-allelic SSRs markers seemed to be best suited for detecting genetic structure than SNPs markers because the SSRs markers had a higher discrimination power than bi-allelic SNP markers (Guichoux et al., 2011).

Zhen et al. (2018) reported five associations between the SWEET SSRs markers and four individual sugars (sucrose, glucose, fructose, and sorbitol). Indeed, the MdSWEET2e marker was significantly associated with sucrose, fructose and total sugars (Zhen et al., 2018), but it did not in the present study. The MdSWEET9b marker showed significant association with the contents of sorbitol and was not linked significantly with fructose and total sugars as reported by Zhen et al. (2018). Nevertheless, the MdSWEET9b marker also showed associations with RAC, oxalate and quinate. In fact, Zhen et al. (2018) did not found association between the other SWEETs gene SSRs markers (MdSWEET7b, MdSWEET2d, MdSWEET2b, MdSWEET2a, MdSWEET12a, MdSWEET17a) and the individual sugars. In the present study, more phenotypic traits were assessed and thus, more significant associations were found.

Apart from the MdSWEETs markers, the SSR markers assessed in the present work have not been used for association mapping with biochemical traits in previous studies. Nevertheless, Quantitative Trait Loci (QTLs) with different phenotypic traits have been encountered and these results may be very useful because many of the associated markers were located in common regions where major genes or QTLs have been previously identified on the apple genome (Chagné et al., 2012; Guan et al., 2015; Howard et al., 2019; Kenis et al., 2008; Kunihisa et al., 2014; McClure et al., 2019; Sun et al., 2015; Zhang & Han, 2021).

According to the agronomical parameters, harvest date showed a significant association with the CH01f03b marker. Kenis et al. (2008) reported a QTL with harvest date located in the linkage group LG9 as well as the CH01f03b marker. Peel color trait was associated with CH02c11 marker at LG10 and with MdSWEET7a marker at LG11. Howard et al. (2019) found QTLs in the LG2, LG5, LG8, and LG9 regarding the peel color. A higher number of significant associations with the basic fruit quality was found than on previous published works (Guan et al., 2015; Kunihisa et al., 2014; Zhang & Han, 2021). Moreover, Kenis et al. (2008) also reported several QTLs for basic fruit quality. Significant association between SSC and TA with the CH02c06 marker at LG2 were found. Guan et al. (2015), Kunihisa et al. (2014), and Zhang & Han (2021) reported the same QTL for SSC in the LG2. Indeed, SSC was linked to the CH02c06 marker (LG2), the CH05f06 marker (LG5), the MdSWEET12a marker (LG14), and the CH02c09 marker (LG15) while TA was associated with SSR markers at LG1, LG2, LG3, LG5, LG9, LG11, LG12, and LG14. Antioxidant compounds were highly linked to several SSR markers assessed. Indeed, at least one antioxidant trait was associated with SSR markers located in the LG2, LG3, LG4, LG5, LG6, LG7, LG10, LG11, LG12, LG14, or LG17. Chagné et al. (2012) and McClure et al. (2019) also showed associations between antioxidant parameters and QTLs at several LGs, in agreement with those observed in the present study. Twenty six significant associations were found between the antioxidant compounds and SSR markers in the LG2, LG3, LG4, LG5, LG6, LG7, LG10, Lg11, LG12, LG14, and LG17. The high content of total phenols was correlated with high radiation and low temperatures, as shown by Mignard et al. (2021). These results are of vital importance taking into account that extreme weather conditions will affect the fruit quality, being the Ebro Valley one of the most vulnerable area for apple production and also one of the most affected regions influenced by these changes.

According to the individual sugars, sucrose was highly linked to the CH-Vf1 marker (LG1). Guan et al. (2015) and Sun et al. (2015) reported a QTL in the LG1 with high linkage to sucrose. They also reported other QTLs for fructose, glucose and sorbitol but in different LGs. Nevertheless, in our study sorbitol did show any association with CH02c06 (LG2), MdSWEET9b (LG4), CH01f02 (LG12), GD147 (LG13), and MdSWEET12a (LG14) markers.

Regarding the organic acids, in the present work, a significant association with the CH04c07 marker (LG14) was found. Chagné et al. (2012) reported a QTL in the LG14 linked to the quinic acid content. Moreover, for organic acids, significant associations between CH04e05 (LG7), CH01h10 (LG8), and CH02c09 markers (LG15) with oxalate, citrate, succinate, shikimate, and tartarate was found. Sun et al. (2015) showed several QTLs in the LG7, LG8, and LG15, being the same LGs as the SSRs markers linked to organic acids in the present study.

The results obtained provided a strong base for further association mapping with agronomical and biochemical traits that could be applied in other species because of the synteny (Gharghani et al., 2009; Raja et al., 2022; Urrestarazu et al., 2017) inside the *Rosaceae* family. Moreover, the 126 significant trait-marker associations ($P \leq 0.01$) found in the present study, could be a potential information for effective Marker Assisted Selection (MAS) in apple breeding programs. Additionally, future studies are needed in this area with a view to undergoing candidate genes identification and fine mapping.

5.6. Conclusions

The present study provided new details about the EEAD-CSIC core collection population structure according to their ploidy level and their origin (Spanish/non-Spanish). The population structure analysis showed two subpopulations in agreement with the phenotypic segregation observed in previous studies. These results highlight the awareness into the genetic architecture of important fruit agronomical and biochemical traits because of the intrinsic correlation between the genetics and the metabolite profiles of the apple accessions. A total of 126 significant associations were observed between the 23 SSR markers assessed and the 21 phenotypic traits evaluated.

These results would help in breeding programs with Marker Assisted Selection (MAS) for fruit quality traits in apple, and in particular, the possible simultaneous selection for agronomical and biochemical parameters. The content of some nutritional compounds, as total phenols content, also associated with some SSR markers, have turned out to be correlated with extreme climatic factors, being a point to take into account in future fruit quality breeding programs. This study also identified many new marker-locus trait associations such as with antioxidant or organic acids compounds, which may be useful for predictions and for a better understanding of the apple genome. Finding specific regions of the genome will provide further information regarding candidate genes involved in apple fruit quality.

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Capítulo 6

Assessment of watercore disorder and related genes in different apple accessions

6.1. Abstract

Watercore is an internal physiological disorder of apple which occurs only when fruit is still on the tree. It is generally expressed as sorbitol rich fluid accumulation in intercellular spaces of the fruit storage parenchyma tissues. In the present work, four apple cultivars consisting in three susceptible for watercore, ‘Helada’, ‘Tempera’, and ‘Verde Doncella’, and one tolerant for watercore, ‘Gala’, all harvested from the EEAD - CSIC apple germplasm collection, were assessed. Four different moments were chosen for sampling aiming to evaluate the evolution along with the influence of the watercore disorder in apple. According to the phenotypic traits, fruit density, bioactive compounds (total phenols and flavonoids contents and relative antioxidant capacity) as well as individual sugars (sucrose, glucose, fructose and the alcohol sugar, sorbitol) were analysed in watercore affected and sound apple flesh. Moreover, this study aims to evaluate the differential expression of ten sorbitol transporters from the *MdSOT* family transport genes, as well as with genes involved in oxidative stresses response as phenylalanine ammonia lyase (*MdPAL*), polyphenol oxidase (*MdPPO*) and peroxidase (*MdPOX*). Furthermore, a PCA was carried out to understand how the studied phenotypic traits and the relative genetic expression of different genes contribute to variability among watercore affected/non-affected tissues. For all the susceptible accessions, higher fruit density and much higher contents of sorbitol were found in watercore affected tissues. The expression of sorbitol transporters *MdSOT1*, *MdSOT2*, *MdSOT2.2, and MdSOT5.2* was lower while *MdSOT5.3* showed higher expression in watercored apple tissues than in sound apple. In general, the antioxidants contents were higher in watercored apples and this result was supported by a generally increased expression of antioxidant related genes.

Keywords: Antioxidants, *Malus x domestica* Borkh, *MdSOT* transporters, *PAL* genes, *PPO*, *POX*, phenolics, sugars

6.2. Introduction

Watercore is an internal physiological disorder of apple or pear fruits which occurs only when they are still on the tree (Buccheri et al., 2020; Cebulj et al., 2021; Liu et al., 2022; Melado-Herreros et al., 2013). If not severe, there are no visible symptoms of this disorder on the fruit skin. Watercore can be observed in the apple core, flesh or even peel and it is caused by the intercellular spaces of the affected tissue being filled with a sorbitol-rich fluid (Kasai & Arakawa, 2010). As the liquid accumulated between the cells of the fruit reduces the scattering of light passing through the tissue, the watercored flesh seemed to be translucent (Itai, 2015; Kasai & Arakawa, 2010). Sorbitol is the main transported polyol in the intercellular spaces of apple flesh tissue (Itai, 2015; Kasai & Arakawa, 2010; Tanaka et al., 2020; Yamada et al., 2006; Yang et al., 2018). Moreover, due to lower intercellular air space volume, watercore tissues suffer oxidative and abiotic stresses and the formation of reactive oxygen species (ROS) increases (Anwar et al., 2021; Cebulj et al., 2021). The antioxidant enzymes as the phenylalanine ammonia lyase (PAL), the polyphenol oxidase (PPO) and peroxidase (POX), would be thus more active in watercore tissues because of the oxidative stress, and the antioxidant substrates such as phenolics are consumed (Buccheri et al., 2020; Zupan et al., 2016). Nevertheless, Cebulj et al. (2021) observed an increase in some antioxidant compounds in peel tissues, as hydroxycinnamic acids and flavonols, included in the phenylpropanoid pathway.

Watercore can be divided into two types: early and late watercore (Itai, 2015; Yamada et al., 2005). It has been reported that watercore development is related to sugar metabolism during the fruit maturation process (Aprea et al., 2017; Charles et al., 2019). Indeed, early watercore is observed in immature fruits and is often detected after warm summers (Yamada et al., 2006). Positive correlations between watercored fruit intensity and temperatures were also demonstrated in apple (Yamada et al., 2004). Nevertheless, early watercore, in general, disappears in the next months. The other type, the late watercore, is more problematic because of its persistence in the fruit and the dramatic economic loss caused by physiological disorders, such as browning and breakdown during postharvest (Itai, 2015; Kasai & Arakawa, 2010). Indeed, watercore in apples is related with negative acceptability from consumers mainly because of these undesirable disorders that occur in watercored fruits (Tanaka et al., 2020). Nevertheless, in some countries like Japan for instance, watercored fruits are truly appreciated because of the flavor. Indeed, watercored fruit is characterized with a pineapple-like taste and a rich aroma. This rich aroma of watercore trait has become a target in recent breeding programs (Kunihisa et al., 2016; Tanaka et al., 2020). Moreover, as the watercore is directly related to fruit maturity (Charles et al., 2019; Itai, 2015; Yamada, 2004), it is, for the consumers, a proof that fruits have been fully mature on the tree (Tanaka et al., 2020).

It is well known that sugar profiles affect the flavor, color, aroma, and texture of the fruit and thus influence on consumer acceptance (Charles et al., 2019; Zhu et al., 2021). Certainly, sorbitol increases the sweet flavour of apples (Aprea et al., 2017) and as the watercore is caused by an over-accumulation of sorbitol, apple flesh is sweeter and its flavor is richer. In the *Rosacea* family, sorbitol is the main primary photosynthetic product. Sorbitol is loaded into the phloem for long-distance transport, and then unloaded into sink organs through symplastic or apoplastic pathways (Patrick et al., 2013). The symplastic pathway uses symplast structures, and the concentration gradient moves the sugars across the membranes. This process is complex and requires sugar transporters encoded by multiple gene families (Wei et al., 2014; Zhu et al., 2022). The apoplastic pathway is thus mostly mediated by sugar transporters and contribute to the high accumulation of sugar in mature fruits. Evidence for sorbitol apoplastic phloem accumulation was also reported in apple (Pleyerová et al., 2022; Yang et al., 2019; Zhang et al. 2004).

Sorbitol is synthesized in leaves via the aldose-6-phosphate reductase (A6PR). After being unloaded, sorbitol can be taken up into the cytosol of parenchyma cells by a sorbitol transporter (MdSOT) and converted to fructose by sorbitol dehydrogenase (Li et al., 2018). The sugar alcohol sorbitol pathways in apple plays a crucial role in efficient carbohydrate use (Ruan et al., 2014; Yang et al., 2018), sugar accumulation (Li et al., 2018), and abiotic stresses resistance (Yang et al., 2019, Zhao et al., 2020).

The expression of sorbitol transporters in *Malus x domestica* Borkh (*MdSOT*) was lower in watercored apple tissues than in sound apple (Gao et al., 2005). Deficiencies in these sorbitol transporters are directly responsible for watercored apple tissues (Gao et al., 2005). Indeed, Li et al. (2012) reported that *MdSOT1* and *MdSOT2* were expressed in all sink tissues excluding watercored fruits. Their expression were significant in fruits meanwhile relatively low in leaves. It has been reported that they were important during sorbitol unloading in sinks, whereas some other *MdSOT* transporters are most likely to be involved in phloem loading (Pleyerová et al., 2022). Nevertheless, expressions of *MdSOT3*, *MdSOT4* and *MdSOT5* were valuable mainly in vegetative organs meanwhile fruits showed little or only weak expression of these MdSOTs (Gao et al., 2005; Li et al., 2012). *MdSOTs* apple transporters are substrate-specific and their affinity for sorbitol is really high (Pleyerová et al., 2022).

A better knowledge of the different mechanisms causing watercore development would help to design storage and marketing strategies for susceptible accessions. Environmental and genetic factors contributing to watercore development are still not well defined (Itai, 2015) and thus, this study aims to evaluate individual sugars and antioxidant compounds as well as the relative antioxidant capacity of three susceptible accessions for watercore from the EEAD-CSIC germplasm bank. The differential expression of ten sorbitol transporters from the *MdSOT* family transport genes and genes involved in oxidative stresses response as phenylalanine ammonia lyase (*MdPAL*), polyphenol oxidase (*MdPPO*) and peroxidase (*MdPOX*) will be determined. The main objectives were thus to characterize metabolite profiles, increase the knowledge of organoleptic and nutritional fruit quality of watercored fruits, and assess the expression of sorbitol transporters and their influence on the watercore susceptible/resistant apple tissues.

6.3. Materials and methods

6.3.1. Field trial and plant material

For this study, four apple accessions (*Malus x domestica* Borkh) from the apple germplasm core collection established at the Experimental Station of Aula Dei (EEAD-CSIC, Zaragoza, NE Spain: 41° 43′ 42.7″ N, 0° 48′ 44.1″ W) were assessed. This germplasm collection is located in the central Ebro Valley's, on a heavy and calcareous soil and under a cold-semiarid Mediterranean climate. The accessions assessed (Table 6.1) were classified according to their susceptibility to develop watercore in mature fruit. Indeed, the 'Helada', 'Tempera' and 'Verde Doncella' accessions were considered as susceptible to watercore and the 'Gala' was chosen as a non-susceptible/reference variety (Figure 6.1). Each accession has three-tree replications established in a unique block design and are budded on the MM106 rootstock. Trees were trained to a low-density open-vase system (6 m × 5 m). Cultural management practices, such as fertilization, winter pruning, and pest or disease control were conducted as in a commercial orchard. Trees were hand-thinned at 40–45 days after full bloom (DAFB), leaving one fruit per cluster. The average daily temperature (tmd), average minimum daily temperature (tmn), and average maximum daily temperature (tmx), as well as solar radiation (rad), mean daily relative humidity (rh) and total precipitation (pre) were recorded as described by Mignard et al. (2021), from the Aula Dei meteorological station, nearby the orchard, which belongs to the official network of the Spanish meteorological service (AEMET).

Table 6.1. Basic information of the four apple accessions from the EEAD-CSIC germplasm bank assessed on this study.

Accession	EEAD Code	Classification	Origin	Watercore	Skin color	Full Bloom Date	Harvest Date	Ploidy
Gala	3197	Non-Spanish	New Zeland	Absence/Ref	Bicolor	96	243	2
Helada	3368	Spanish	Baleares, Sp	Presence	Green	97	277	2
Tempera	3334	Spanish	Lugo, Sp	Presence	Green	104	265	2
Verde Doncella	310	Spanish	Zaragoza, Sp	Presence	Green	98	267	2

Sp: Spain; Ref: Reference variety. Harvest and Full Bloom dates are means of five years and are expressed in Julian Days.

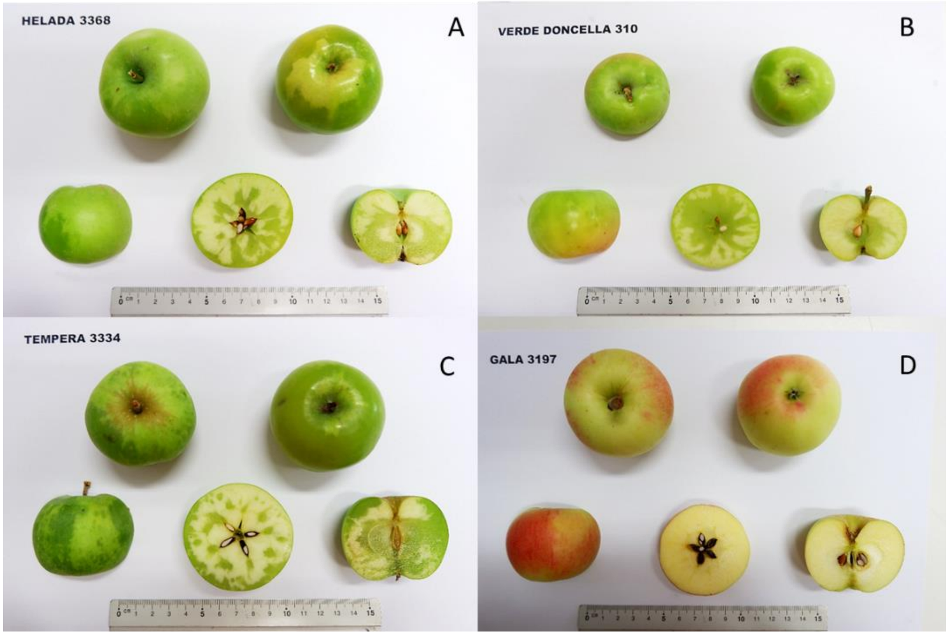


Figure 6.1. The four accessions assessed in this study: Helada (A), Verde Doncella (B), Tempera (C), and Gala (D). A, B and C showed presence of watercored tissues meanwhile Gala was chosen as the non-susceptible/reference variety.

6.3.2. Fruit sampling

Four different moments were chosen for sampling in 2020: 27th of July (209 Julian Days - JD), 17th of August (230 JD), 7th of September (251 JD) and 21st of September (265 JD). These dates corresponded to 113, 134, 155, and 169 days after full bloom (DAFB) for ‘Gala’, to 112, 133, 154, and 168 DAFB for ‘Helada’, to 105, 126, 147, and

161 DAFB for ‘Tempera’, and to 111, 132, 153, and 167 DAFB for ‘Verde Doncella’. Apples were separated into two groups: apples without watercore (sound apples) and apples presenting watercore (watercored apples). At each sampling period, a representative random sample of 12 sound fruits (four fruits per tree × three replicates) and 12 visually watercored fruits were sampled. For all the samples, the watercore was scored as presence/absence of watercored tissue. The volume and the weight of each fruit were also measured and the density according to the weight/volume ratio was calculated.

After peeling the fruits, a flesh sample composite of five g was built by mixing all pieces from the four selected fruits for each DAFB sampling replicate, accession and type of fruit (Sound/Watercored). Three biological replicates per accession were prepared. Only watercored zones of flesh tissue of watercored apple were sampled as ‘Watercored’ while healthy flesh tissues from only sound apple were sampled as ‘Sound’. Then, samples were frozen in liquid nitrogen and kept at -80°C until further analysis. The different fruit traits (antioxidant compounds and individual sugars) were measured for each accession, sampling period and fruit type (Sound/Watercored) during at least three times within the four sampling periods and means for each period, accession and fruit type.

6.3.3. Individual sugars analysis and phytochemical traits

Samples were analyzed by HPLC for the content of individual sugars as described by Mignard et al. (2022). Sugars found in fruit flesh were assessed using an Aminex HPX-87C column (300 mm x 7.8 mm, Biorad) with a refractive index detector at 35°C (Waters 2410, Waters Corporation, Milford, USA) with filtered and degassed Milli-Q water as mobile phase at 85°C and flow rate was fitted at 0.6 mL/min. The individual sugars (glucose, fructose, sucrose, and sorbitol) were identified by their retention time characteristics using the adequate standards. All chemicals were of analytical grade. Concentrations were expressed as g per kg of fresh weight (FW).

For the analysis of the total phenolics content (TPC), total flavonoids content (TFC), and the relative antioxidant capacity (RAC), samples were analyzed using a 96-well microplate spectrophotometer photodiode array detector (Asys UVM 340 microplate reader; Biochrom, Cambridge, UK) as described by Mignard et al. (2021). The standard calibration curves were prepared on each microplate. For TPC, absorbance was measured at 725 nm and the results expressed in mg of gallic acid equivalent (GAE) per 100 g of FW. According to TFC, absorbance was measured at 510 nm. The results were then expressed in mg catechin equivalent (CE) per 100 g FW. The RAC was carried out using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, absorbance was measured at 515 nm and the results were expressed in mg Trolox per 100 g FW.

6.3.4. Gene expression

For the four periods of sampling, total RNA was isolated from 100 mg of lyophilized samples as previously reported by Amri et al. (2021) with modifications. Final RNA was resuspended in DEPC water and stored at -80ºC until further analysis. The concentration of RNA was determined by an UV spectrophotometer (NanoDrop ND-2000, Thermo Fisher Scientific, Wilmington, DE, United States). Additionally, quality of RNA was confirmed by electrophoresis.

Using the DNase/RNase-free (EN0521, Thermo Scientific), DNA was digested from an aliquot containing 2 µg of RNA. The RNA free from DNA was then reverse transcribed using a First Strand cDNA synthesis kit (K1631, Thermo Scientific) according to manufacturer's instructions. The cDNA was resuspended (1:20) and aliquoted reaching a concentration of 2 ng/µL approximately.

Quantitative polymerase chain reaction (qPCR or real time PCR) was used to analyse the expression of ten selected genes (QuantStudio 3, Real-Time PCR System). The Real-Time qPCR amplifications were performed using specific primers for ten target sorbitol transporter genes described by Wei et al. (2014): *MdSOT1*, *MdSOT2, MdSOT2.2*, *MdSOT3*, *MdSOT3.2*, *MdSOT3.3*, *MdSOT4*, *MdSOT5*, *MdSOT5.2*, and *MdSOT5.3*; and five target *MdPAL* genes described by Zhang et al. (2018): *MdPAL1*, *MdPAL2*, *MdPAL3/4/6/7*, *MdPAL5*, and *MdPAL8* and finally two more specific primers related to the phenylpropanoide pathway *MdPPO* and *MdPOX* (Table 6.2). The reactions included a total of 20 µL, 10 µL of the SYBR® Green PCR Master Mix (4367659, Applied Biosystems), 10 ng of cDNA and 500 nM of corresponding primer. The RT-qPCR experiments were carried out with two technical replicates. In addition, two negative controls were included in each plate and gene: one without cDNA and the other without primer. Three candidate reference genes transcripts, described by Bowen et al. (2014), were tested to identify the most stable reference gene for the normalization of gene expression and standardize the different cDNA samples: StaR-related lipid transfer protein (LTL1); Phytochrome protein phosphatase 3 (FYPP3); and Casein kinase II subunit beta-4 (CKB4).

Table 6.2. Sequences and description of the primers used in the RT-qPCR for identification of watercore candidate genes and the three reference genes assessed in this study.

Gene name	Category	Tm	Eff	Oligo sequence (5' to 3')	Source
MdSOT1	Gene	85.0	1.77	F GGTTAGAATGACGTGGGCAGTTAT R TCATCAACCTATTCACGGCCAC	Wei et al., (2014)
MdSOT2	Gene	76.8	1.81	F ACAAGGGTCAAGTCCACTAGGCA R CCGATAAGATGTAATTGGCAACAG	
MdSOT2.2	Gene	84.4	1.82	F CATCTGTTGTTCTTGCCATCGGT R CCTGCGGCTCCTTTGATGTCAT	
MdSOT3	Gene	ne	ne	F AGAGCATGACGGCAGTGGAC R ATCCTCTGGAGATTCACACACAA	
MdSOT3.2	Gene	ne	ne	F CTGCCCGGACAGCGGAAGAG R CTTTCTTTGATAAAGAGTGATGCC	
MdSOT3.3	Gene	82.9	1.83	F ATGGCTGACCAGAGGGCGAAC R AGTGACGCTCCACTCATTACACC	
MdSOT4	Gene	ne	ne	F ATCGGCACCACTAACTTATCTCC R TAACCTGTTCCGCTGTCTGC	
MdSOT5	Gene	ne	ne	F AGATGGCTGACCGGACAACT R AGCAGAGTAAGACGAGGAACAT	
MdSOT5.2	Gene	84.5	1.83	F CGCTGTGGGCCTTACGATCATC R TCCGATACTGCATCCTTGAGCG	
MdSOT5.3	Gene	84.1	1.84	F TTGGTGTCGCCCTTACAATCATT R TCCATACTGCAGCCTTGGGCA	
MdPAL_1	Gene	83.0	1.81	F TGCCGAAAGAGGTCGAAACCAC R GCACCATTCCACTCCTTCAAGC	Zhang et al., (2018)
MdPAL_2	Gene	85.0	1.85	F TCTCTGGCAGACAGGACCCAAG R CCCAACGCCTTCGACCATTAGA	
MdPAL_3/4/6/7	Gene	82.5	1.81	F GCACAAGTTGAAGCACCACCCT R GTGATGTTCGGAGAGCGTAGCG	
MdPAL_5	Gene	84.1	1.86	F TGGCAGCAGCACAGAATCAACC R GGCAATGTACGAGAGCGGAACC	
MdPAL_8	Gene	84.2	1.75	F CGGGCTCAAGGGTGTGGAGATA R AGCCTGGCAAAGTGCTACCAAA	
MdPOX	Gene	79.5	1.79	F CCTCCTCAAAACAAGCGTTC R CACAGCACACACAAACAATTTC	Vilanova et al., (2014)
MdPPO	Gene	78.3	1.84	F CCTACTCACAAAGCCCAAGC R CCTCCAAGACCAAGAAGCAC	Di Guardo et al., (2013)
LTL1	Ref. Gene	80.9	1.88	F CCATATCCAGGCTTGCCTAA R ACGAGGGTAACCTCACATGC	Bowen et al., (2014)
FYPP3	Ref. Gene	79.0	1.82	F TCGAGCACACCAACTTGTTC R GATAGAAGCCACATTCCCACA	
CKB4	Ref. Gene	78.5	1.85	F GTTGAATCAGCAGCGGAGAT R AAACTCTTGGGCATCTTCCA	

The GeNorm (V. 3.5), a tool from Microsoft Excel, was used to select the best candidate reference gene for this study. The PCR reactions were identical for all primers sets: 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s and 60ºC for 1 min. The melting curve was performed with gradient as 95ºC for 15 s, 60ºC for 1 min and 95ºC for 1 s. The efficiencies (E) and the quantification cycles (Cq) values were determined at constant fluorescence using the LingRegPCR software. The relative expression ratio (R) of the target genes were calculated based on a control and expressed in comparison to a reference gene as described by Plaffl (2001).

6.3.5. Data analysis

All statistical analyses were carried out using SPSS software (IBM Corp. Statistics V.28) and R language (R Development Core Team, 2019). A two-way analysis of variance (ANOVA) was run to determine whether there were any statistically significant differences between the means of the evaluated traits. Means were separated using Duncan's multiple range test at $P \leq 0.05$. Pearson's correlations and a principal component analysis (PCA) were executed to understand how the studied traits contribute to variability among accessions.

6.4. Results and Discussion

6.4.1. Phenotypic characterization

The statistical analysis was carried out with the four accessions presented in the Table 6.1 ('Gala', 'Helada', 'Tempera', and 'Verde Doncella'). The ANOVA analysis showed significant differences ($P \leq 0.01$) among all the different apple accessions according to all the phenotypic traits evaluated, except for glucose content (Table 6.3). Additionally, significant differences were also obtained between traits and susceptibility for watercore, between traits and the DAFB sampling and finally, between the traits and the interaction with watercore and DAFB. Moreover, the bilateral Pearson's correlations were carried out first with sound fruits and then with watercored fruits.

Table 6.3. ANOVA two – way results for the effect of the watercore and the DAFB on biochemical traits of the four apple cultivars assessed.

Trait	Accession	Watercore	DAFB	W x DAFB
Density	Gala	-	ns	-
	Helada	**	ns	ns
	Tempera	***	ns	ns
	Verde Doncella	**	ns	ns
RAC	Gala	-	***	-
	Helada	ns	***	ns
	Tempera	ns	ns	**
	Verde Doncella	ns	**	ns
TPC	Gala	-	**	-
	Helada	ns	**	ns
	Tempera	ns	ns	*
	Verde Doncella	ns	*	ns
TFC	Gala	-	***	-
	Helada	ns	***	ns
	Tempera	ns	ns	*
	Verde Doncella	ns	ns	ns
Sucrose	Gala	-	***	-
	Helada	*	***	**
	Tempera	***	*	ns
	Verde Doncella	ns	***	ns
Glucose	Gala	-	ns	-
	Helada	*	ns	ns
	Tempera	*	ns	*
	Verde Doncella	ns	ns	ns
Fructose	Gala	-	ns	-
	Helada	**	ns	ns
	Tempera	ns	ns	ns
	Verde Doncella	**	ns	ns
Sorbitol	Gala	-	***	-
	Helada	***	***	***
	Tempera	***	ns	ns
	Verde Doncella	***	*	*

Statistical significance at *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; Abbreviations: A x W, interaction accession x watercore; DAFB, day after full bloom; W x DAFB, interaction watercore x DAFB; TPC, total phenolics content; TFC, total flavonoids content; RAC, relative antioxidant content.

6.4.1.1. Climatic and Agronomical traits

The climatic traits ranges observed in the conditions of the central Ebro Valley (Figure 6.2), and thus in the orchard, between 96 JD (first full bloom date for 'Gala') and 265 JD (last harvest date of the present study for 'Helada' and 'Verde Doncella') indicated a region with a climate considered as arid or semi-arid. And average annual precipitation amounts to 345 mm, warm temperatures on summer and cold in winter, and diurnal temperature variation amounts to 13.3 °C (Salvador et al., 2011). Thus, the central Ebro basin is considered a vulnerable region for apple production (Reig et al., 2015). Indeed, the temperatures were high with an average for the mean, maximum and minimum temperatures fitted at 21.5ºC, 29.4ºC, and 14.1ºC respectively. The total precipitations scored 179.4 mm in the period 96-265 JD and the daily mean precipitation was insignificant with an average of 1.1 mm while the mean daily water needed for a reference crop in summer is about 4–6 mm (Ferrante and Mariani, 2018). The average relative humidity (RH) for the period of study was relatively low with 62.4%. The RH, which at a given temperature is the water content of the atmosphere expressed as a percentage of the saturated water content, plays a crucial role in the osmoregulation for plants by regulating the transpiration at the leaf level (Ferrante and Mariani, 2018). Moreover, the solar radiation was high with an average of 271.5 W/m^2. Furthermore, Melado-Herreros et al. (2013) found significant and positive linkage between the watercore disorder and the solar radiation.

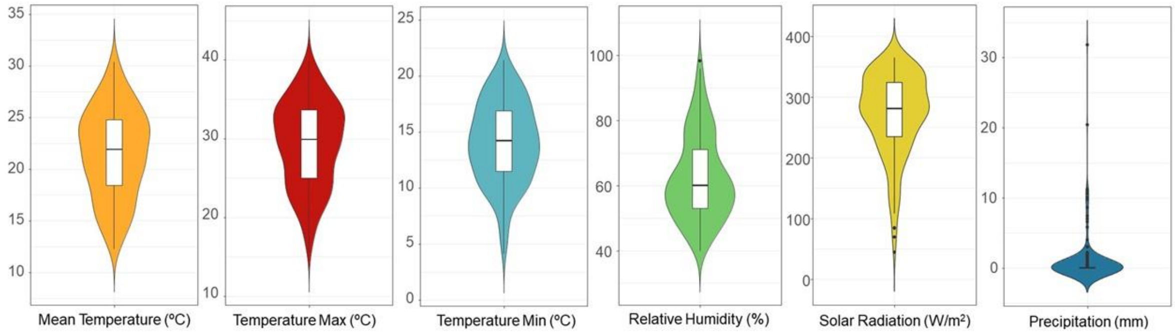


Figure 6.2. Violin plots showing the variability of the climate parameters in 2020, computed between 96 (first full bloom date) and 265 Julian Days (last harvest date).

Moreover, in the present study, the only agronomical parameter assessed, apart from the biochemical susceptibility for watercore, was the fruit density. Indeed, the density varied greatly (Figure 6.3) from 0.71 ('Tempera', sound fruits at 126 DAFB) to

0.95 (‘Tempera’, watercored fruits at 147 DAFB) and showed significant differences for all the accessions between watercore affected fruits and sound fruits (Table 6.3). Indeed, watercored fruits showed higher values for the fruit density as previously reported by other studies in apple (Baranowski et al., 2008; Herremans et al., 2014). Nevertheless, the period of sampling (DAFB) and the interaction between the sampling (DAFB) and the susceptibility for watercore did not show any differences in the fruit density, demonstrating the strong influence on the density by the physical disorder watercore (Herremans et al., 2014).

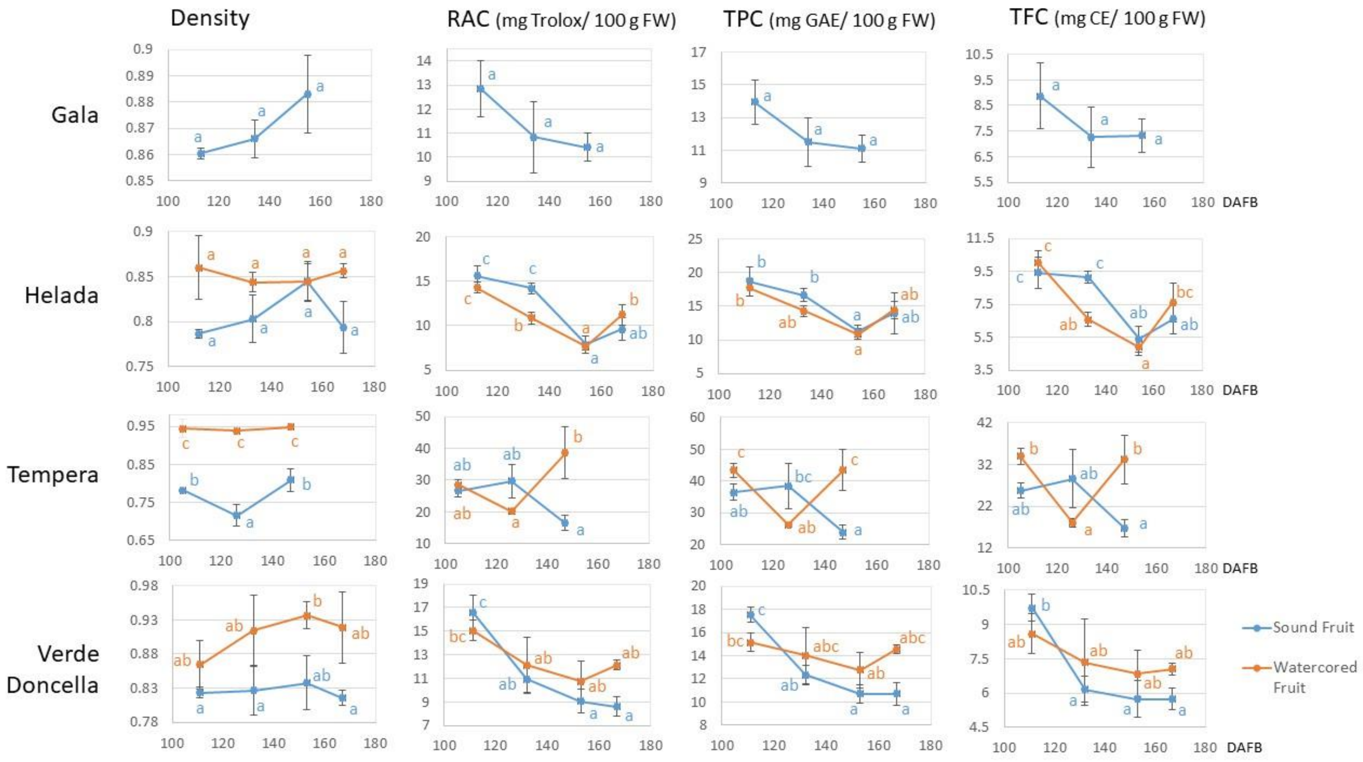


Figure 6.3. Phenotypic density and antioxidant traits for the accessions assessed (‘Gala’ – reference accession, ‘Helada’, ‘Tempera’, and ‘Verde Doncella’) according to the presence/absence of watercore and the DAFB. Error bars represent the standard error of the mean.

6.4.1.2. Antioxidant compounds

The antioxidant compounds showed differences between accessions and the DAFB (Tables 6.3 and 6.4 and Figure 6.3). The total phenolics content (TPC) ranged among apple accessions and watercore disorder from 10.71 (‘Verde Doncella’, sound fruits at 153 DAFB) to 43.46 (‘Tempera’, watercored fruits at 147 DAFB) mg gallic acid equivalents (GAE)/100 g FW. The total flavonoids content (TFC) varied from 4.91

(‘Helada’, watercored fruits at 154 DAFB) to 33.99 (‘Tempera’, watercored fruits at 105 DAFB) mg catechin equivalents (CE)/100 g FW. The relative antioxidant capacity (RAC) values ranged from 7.66 (‘Helada’, watercored fruits at 154 DAFB) to 38.65 (‘Tempera’, watercored fruits at 147 DAFB) mg trolox/100 g FW. Finally, the standard deviation for TPC, TFC, and RAC were fitted at 10.4, 8.9 and 7.8 respectively (data not shown).

Considering all the sampling sates (DAFB) and stages of development studied, the total phenols (TPC) and flavonoids (TFC) contents, as well as the relative antioxidant capacity (RAC) were similar in sound fruits and watercored apples for the three accessions studied (‘Helada’, ‘Tempera’, and ‘Verde Doncella’) (Table 6.3). Nevertheless, ‘Tempera’ showed an increase in biochemical compounds for the watercore affected fruits in their last sampling date (147 DAFB) showing a clear difference with the sound apples (Figure 6.3). Indeed, the study of the accessions in the last state of development showed significant differences between healthy and watercored fruits (Table 6.4). Watercored fruits presented higher content, in general, of antioxidant compounds (RAC, TPC and TFC) but this increase was strongly influenced by the cultivar assessed, because the ‘Tempera’ showed the greatest sound-watercore influence (Table 6.4). These results were in agreement with those reported by Cebulj et al. (2021) because they assessed individual phenolics compounds for the ‘Fuji’ cultivar and observed higher antioxidants content in the watercored tissues.

Moreover, RAC and TPC decreased along with the ripening of the fruit (increased DAFB sampling) in the accessions ‘Gala’, ‘Helada’, and ‘Verde Doncella’. The TFC also decreased for ‘Gala’ and ‘Helada’ (Figure 6.3). In general, the phenolics and flavonoids contents and the relative antioxidant capacity tended to decrease according to the maturity of the fruit as reported by Duda-Chodak et al. (2011). The ‘Tempera’ accession did not show correlations with the DAFB sampling for the antioxidant parameters (TPC, TFC, and RAC) but it did with the interaction between the DAFB and the watercore disorder (Table 6.3).

Table 6.5. Pearson's bivariate correlation coefficients between phenotypic traits studied and relative expressions of the gene activity assessed for sound fruits.

Sound	**RAC**	**TPC**	**TFC**	**Sucrose**	**Glucose**	**Fructose**	**Sorbitol**	**SOT1**	**SOT2**	**SO2.2**	**SOT5.2**	**SOT5.3**	**PAL2**	**PAL 3/4/6/7**	**PAL5**	**PAL8**	**PPO**	**POX**
DAFB	-,581**	-,479**	-,436**	,429**														-,360*
Density	-,574**	-,600**	-,578**	,493**		-,310*	-,365*	,358*			,415**							
RAC		,972**	,963**	-,331*		,523**	,472**						-,391*					
TPC			,981**	-,308*		,588**	,584**						-,389*			-,319*		
TFC						,614**	,591**						-,480**			-,406**		
Sucrose					-,455**			,603**		-,412**	,719**		-,420**			-,463**		-,492**
Glucose									,326*	,506**			,678**		-,327*	,686**	-,322*	,324*
Fructose							,838**											
Sorbitol																		
SOT1											,772**	,519**		,526**	,488**	-,322*	,401*	-,484**
SOT2										,669**		,341*	,536**			,515**		,542**
SO2.2												,592**	,635**			,760**		,455**
SOT5.2													-,329*		,423**	-,316*		
SOT5.3																		
PAL1														,576**			,729**	
PAL2																,919**		,393*
PAL3/4/6/7																	,707**	
PAL5																		
PAL8																		,509**

Statistical significance at *: $P \leq 0.05$; **: $P \leq 0.01$; Abbreviations: DAFB, day after full bloom; TPC, total phenolics content; TFC, total flavonoids content; RAC, relative antioxidant content.

Table 6.6. Pearson's bivariate correlation coefficients between phenotypic traits studied and relative expressions of the gene activity assessed for watercore affected fruits.

Watercore	RAC	TPC	TFC	Sucrose	Glucose	Fructose	Sorbitol	SOT1	SOT2	SO2.2	SOT5.2	SOT5.3	PAL1	PAL2	PAL 3/4/6/7	PAL5	PAL8	PPO	POX
DAFB			-,348*	,509**	,355*						,581**							-,518**	
Density	,462**	,446**	,454**	-,466**			,467**	,462*					,384*		,375*				-,461*
RAC		,952**	,948**	-,551**		,734**	,621**	,832**	-,507**					-,660**	,603**	,475**	-,571**	,686**	-,479**
TPC			,998**	-,598**		,684**	,662**	,831**	-,536**					-,721**	,650**	,523**	-,644**	,747**	-,495**
TFC				-,619**		,665**	,664**	,843**	-,522**					-,716**	,669**	,534**	-,631**	,756**	-,490**
Sucrose								-,599**					-,477**		-,510**	-,538**		-,627**	
Glucose						,367*								,463*			,482**	-,442*	
Fructose							,652**	,537**											
Sorbitol								,631**		-,585**	,424*			-,529**	,425*		-,510**		-,596**
SOT1														-,443*	,694**	,522**		,649**	
SOT2												,373*		,534**	-,393*		,607**	-,427*	,560**
SO2.2												,460*		,626**			,628**		,672**
SOT5.2																			
SOT5.3													,405*	,497**			,640**		
PAL1																			
PAL2																	,890**	-,556**	,521**
PAL3/4/6/7																,484**		,587**	
PAL5																		,627**	
PAL8																		-,467*	,617**

Statistical significance at *: P ≤ 0.05; **: P ≤ 0.01; Abbreviations: DAFB, day after full bloom; TPC, total phenolics content; TFC, total flavonoids content; RAC, relative antioxidant content.

Table 6.4. Relative antioxidant capacity, total phenolics and total flavonoids content (n=3) in sound and watercored fruits harvested at maturity state (DAFB = 155, 168, 147, and 167 for 'Gala', 'Helada', 'Tempera', and 'Verde Doncella' accessions respectively).

	RAC	**TPC**	**TFC**
Main factors			
Watercore			
Sound	11.29 a	14.92 a	9.11 a
Watercored	20.67 b	24.16 b	15.95 b
Accession			
Gala	10.41 a	11.09 a	7.33 a
Helada	10.43 a	14.20 b	7.10 a
Tempera	27.58 b	33.68 c	24.98 b
Verde Doncella	10.36 a	12.65 ab	6.39 a
Interaction			
Sound			
Gala	10.41 b	11.09 a	7.33 a
Helada	9.62 a	13.95 a	6.59 a
Tempera	16.51 b	23.89 b	16.79 b
Verde Doncella	8.60 a	10.73 a	5.74 a
Watercored			
Gala			
Helada	11.24 a	14.44 a	7.61 a
Tempera	38.65 c	43.46 c	33.17 c
Verde Doncella	12.12 b	14.57 a	7.05 a
Significance			
Watercore	**	**	**
Cultivar	***	***	***
Watercore*Accession	*	*	**

Statistical significance at *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; Abbreviations: DAFB, day after full bloom; TPC, total phenolics content; TFC, total flavonoids content; RAC, relative antioxidant content.

Tables 6.5 and 6.6 showed the Pearson's correlation coefficients between phenotypic traits studied and relative expressions of the genes activity assessed. According to the antioxidant traits, the RAC was highly and positively correlated with TPC ($r_{Watercored} = 0.95$; $r_{Sound} = 0.97$) and TFC ($r_{Watercored} = 0.95$; $r_{Sound} = 0.96$). Moreover, TPC and TFC were highly correlated ($r_{Watercored} = 0.998$; $r_{Sound} = 0.98$) as the flavonoids is a subgroup of the polyphenols (Lattanzio et al., 2013). These correlations were similar for healthy and watercore affected fruits and they were in agreement with those previously reported in other fruit studies in apple (Mignard et al., 2021; 2022; Preti & Tarola, 2020; Raudone et al., 2017), peach and nectarines (Font i Forcada et al., 2019) and apricot fruits (Ezzat et al., 2021). The density of the fruit was negatively correlated with RAC, TPC, and TFC of sound fruits (r_{Sound} = -0.57; r_{Sound} = -.60; r_{Sound} = -0.58, respectively) but positively with RAC, TPC, and TFC of watercored fruits showing that the watercored fruits exhibited more density and more antioxidant content, as published before (Cebulj et al., 2021; Melado-Herreros et al., 2013). Moreover, the DAFB showed significant correlation with the antioxidant traits of watercored fruit only for TFC ($r_{Watercored}$ = -0.35) however the sounds apples showed correlations between the DAFB sampling and RAC, TPC, and TFC (Tables 6.5 and 6.6) indicating the well-known tendency of the bioactive compounds to decrease along with the fruit maturity (Duda-Chodak et al., 2011).

6.4.1.3. Individual sugars

The individual sugars values ranged significantly among apple accessions and watercore disorder (Table 6.3 and Figure 6.4). Indeed, sucrose concentrations varied considerably, ranging from 5.59 (‘Tempera’, watercored fruits at 105 DAFB) to 26.27 (‘Gala’ at 155 DAFB) g kg-1 FW. Glucose values ranged from 4.78 (‘Tempera’, sound fruits at 105 DAFB) to 16.30 (‘Verde Doncella’, sound fruits at 111 DAFB) and fructose values ranged from 31.97 (‘Gala’ at 134 DAFB) to 50.22 (‘Tempera’, sound fruits at 147 DAFB) g kg-1 FW. Finally, the alcohol sugar, sorbitol contents varied considerably from 0.54 (‘Gala’ at 113 DAFB) to 24.88 (‘Tempera’, watercored fruits at 147 DAFB) g kg-1 FW. The standard deviations for sucrose, glucose, fructose and sorbitol, were fitted at 4.4, 2.7, 5.0 and 7.0, respectively (data not shown).

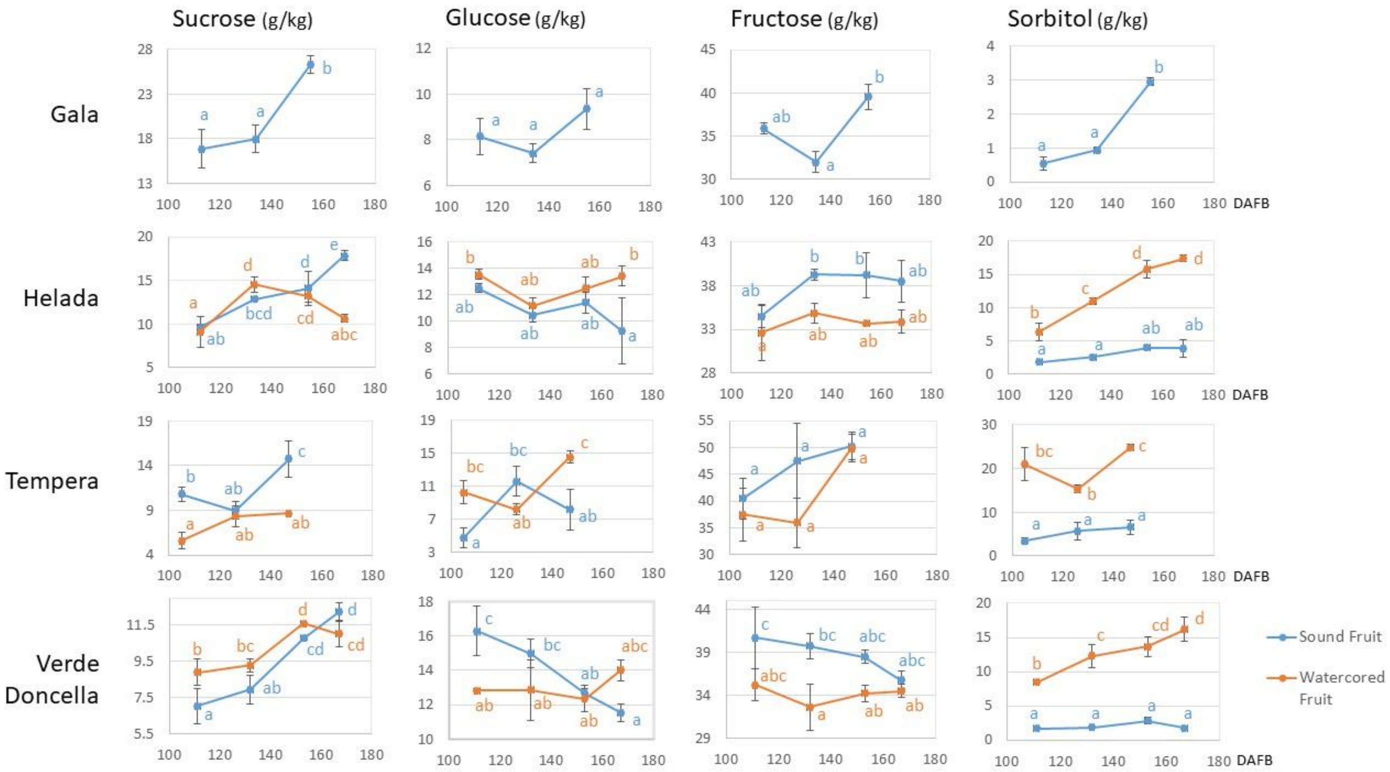


Figure 6.4. Individual sugars assessed for the accessions assessed ('Gala' – reference accession, 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. Error bars represent the standard error of the mean.

Comparing the individual sugar content, when harvested fruits were at commercial maturity, between sound and watercored fruits, in general, for the four accessions studied, a higher concentration of glucose and sorbitol, and lower of sucrose, was observed in the watercore affected apples compared with the healthy fruits (Table 6.7). The fructose content was similar for sound ant watercored fruits in the present study but Melado-Herreros et al. (2013) observed lower levels of fructose in watercored fruits compared to sound apples in the 'Esperiega' cultivar. Nevertheless, Melado-Herreros et al. (2013) also observed the decrease in sucrose content. In the present work, differences were also observed in the fructose content according to the affected/sound factor several weeks before the commercial maturity for the accessions 'Helada' and 'Verde Doncella'. However, their content was not different at the end of the sampling period and higher maturity (Table 6.3 and Figure 6.3).

Table 6.7. Sucrose, glucose, fructose and sorbitol content (n=3) in sound and watercored fruits apple accessions (Gala, Helada, Tempera and Verde Doncella), in maturity state (DAFB = 155, 168, 147, and 167 for 'Gala', 'Helada', 'Tempera', and 'Verde Doncella' accessions respectively).

	Sucrose	Glucose	Fructose	Sorbitol
Main factors				
Watercore				
Sound	17.77 b	9.58 a	41.01	3.78 a
Watercored	10.11 a	13.97 b	39.40	19.49 b
Accession				
Gala	26.27 c	9.34 ns	39.54 b	2.95 a
Helada	14.24 b	11.34 ns	36.19 a	10.61 b
Tempera	11.71 ab	11.32 ns	50.04 c	15.71 b
Verde Doncella	11.63 a	12.78 ns	35.12 a	9.00 b
Interaction				
Sound				
Gala	26.27 e	9.34	39.54	2.95
Helada	17.84 d	9.27	38.50	3.84
Tempera	14.75 c	8.16	50.22	6.53
Verde Doncella	12.23 bc	11.55	35.77	1.79
Watercored				
Gala				
Helada	10.64 ab	13.42	33.88	17.39
Tempera	8.67 a	14.47	49.85	24.88
Verde Doncella	11.03 ab	14.01	34.47	16.21
Significance				
Watercore	***	**	ns	***
Accession	***	ns	***	***
Watercore*Accession	**	ns	ns	ns

Statistical significance at *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; Abbreviations: DAFB, day after full bloom.

In contrast to our results, higher sucrose concentrations in watercored fruits was found in 'Delicious', 'Gloster', and 'Fuji' cultivars (Bowen and Watkins, 1997; Zupan et al., 2016). Tanaka et al. (2020) also showed higher values of sucrose for the 'Fuji' cultivar but a decrease for other cultivars as 'Gloster', 'Delicious', and 'Esperiega'. In the present work, the 'Verde Doncella' also showed a tendency to exhibit a higher concentration in sucrose for watercored fruits, in agreement with other studies. Sucrose was linked to the maturity of fruit and showed significant differences for all the accessions assessed according to the DAFB sampling. Moreover, the glucose content was higher in the affected fruits compared with sound tissues for the ‘Helada’ and ‘Tempera’ accessions. These results were in good accordance with those reported by Melado-Herreros et al. (2013) in the ‘Esperiega’ cultivar. In contrast, Zupan et al. (2016) observed a tendency of glucose to decrease in ‘Delicious’ and ‘Fuji’ cultivars. Both glucose and fructose did not showed any significant differences according to the DAFB sampling dates of the present work.

Furthermore, for all the accessions studied, sorbitol contents were much higher in watercored fruits compared with sound tissue of flesh apples (Table 6.3, Figure 6.4) as previously reported in apple studies (Cebulj et al., 2021; Melado-Herreros et al., 2013; Zupan et al., 2016). Indeed, it is well-known that watercore is a physical disorder with typical sorbitol accumulation in the intercellular spaces of the fruit (Cebulj et al., 2021; Gao et al., 2005; Tanaka et al., 2020).

Moreover, individual sugars as fructose and sucrose showed moderate but significant correlations (Tables 6.5 and 6.6) with the antioxidant traits assessed. Indeed, sucrose exhibited negative correlations with TPC ($r_{\text{Watercored}}$ = -0.60; r_{Sound} = -0.31), TFC ($r_{\text{Watercored}}$ = -0.62), and RAC ($r_{\text{Watercored}}$ = -0.55; r_{Sound} = -0.33). The alcohol sugar sorbitol was positive and highly correlated with fructose ($r_{\text{Watercored}}$ = 0.65; r_{Sound} = 0.84) and with all the other individual sugars and antioxidant parameters, and showed positive correlations with TPC ($r_{\text{Watercored}}$ = 0.68; r_{Sound} = 0.58), TFC ($r_{\text{Watercored}}$ = 0.67; r_{Sound} = 0.59), and RAC ($r_{\text{Watercored}}$ = 0.73; r_{Sound} = 0.47). The correlations were significantly different in healthy and watercored fruits for these parameters highlighting the influence of the watercore on antioxidant and sugar traits as previously reported (Cebulj et al., 2021, Melado-Herreros et al., 2013; Tanaka et al., 2020; Zupan et al., 2016).

Furthermore, significant correlation was found between sorbitol and the fruit density. The correlation was positive when fruits were affected by watercore ($r_{\text{Watercored}}$ = 0.47) and negative in the case of sound fruits (r_{Sound} = -0.37). Indeed, the density of watercored fruits was significantly higher than in sound fruits (Table 6.3) and sorbitol was the parameter most affected by watercore as reported in previous studies (Baranowski et al., 2008; Herremans et al., 2014; Itai, 2015; Tanaka et al., 2020).

Table 6.8. MdSOTs, MdPALs and MdPOX expression genes relative to 'Gala' at 113 DAFB (n=3) in sound and watercored fruits apple accessions harvested at maturity state (DAFB = 155, 168, 147, and 167 for 'Gala', 'Helada', 'Tempera', and 'Verde Doncella' accessions respectively).

	SOT1	SOT2	SO2.2	SOT5.2	SOT5.3	PAL1	PAL2	PAL3/4/6/7	PAL5	PAL8	PPO	POX
Main factors												
Watercore												
Sound	0.56	0.98	0.83 b	0.41 a	0.71 b	0.90 a	17.25 b	1.91	0.39	13.72	0.56	21.64
Watercored	0.40	0.70	0.41 a	1.05 b	0.27 a	6.98 b	17.07 a	5.94	0.87	16.06	2.75	65.39
Accession												
Gala	1.00 b	1.00 bc	1.00 b	1.00 b	1.00 c	1.00 a	1.00 a	1.00	1.00	1.00 a	1.00 ab	1.00 a
Helada	0.30 a	0.77 ab	0.47 a	0.99 b	0.11 a	4.97 b	28.17 b	5.77	0.83	24.26 b	0.48 ab	76.10 b
Tempera	0.57 a	0.26 ab	0.17 a	0.25 a	0.39 b	3.36 ab	0.01 a	5.04	0.45	2.52 a	2.88 b	29.41 ab
Verde Doncella	0.32 a	1.52 c	1.16 b	0.53 ab	0.83 c	2.83 ab	33.07 b	1.12	0.18	25.29 b	0.21 a	33.04 ab
Interaction												
Sound												
Gala	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 b	1.00
Helada	0.38	0.60	0.53	0.29	0.16	0.43	35.94	4.89	0.31	27.32	0.69 ab	39.23
Tempera	0.44	0.31	0.21	0.19	0.49	1.32	0.01	1.95	0.16	1.79	0.46 ab	22.69
Verde Doncella	0.35	1.88	1.47	0.13	0.99	0.70	38.28	0.80	0.04	29.30	0.13 a	29.51
Affected												
Gala												
Helada	0.24	0.88	0.42	1.45	0.07	7.99	22.99	6.36	1.18	22.21	0.08 a	100.68
Tempera	0.77	0.18	0.10	0.35	0.24	6.42	0.00	9.68	0.89	3.61	6.52 c	39.50
Verde Doncella	0.28	0.97	0.71	1.14	0.58	6.02	25.26	1.59	0.38	19.27	0.32 a	38.35
Significance												
Watercore	ns	ns	*	***	*	***	*	ns	ns	ns	ns	ns
Accession	**	*	**	**	***	ns	***	ns	ns	***	*	ns
Watercore*Accession	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns

Statistical significance at *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; Abbreviations: DAFB. day after full bloom.

6.4.2. Gene expression Analysis

The four accessions studied (‘Gala’, ‘Helada’, ‘Tempera’, and ‘Verde Doncella’) and the expression of 12 candidate genes showed significant differences according to the different factors: ‘Accession’, ‘Watercore’, the DAFB sampling and the interactions between them (Table 6.8).

The GeNorm tool from Microsoft Excel was used to determine the most stable gene to be used as a reference gene. Consequently, the *LTL1* was the most stable gene among the three tested genes and thus, the reference gene used in the present work.

6.4.2.1. Genes involved in sorbitol transports – MdSOTs genes

In Rosaceae, photosynthesis-derived carbohydrates are transported mainly as sorbitol, which is mediated by *MdSOTs* transporters (Wei et al., 2014). According to the *MdSOT1* (Figure 6.5), *MdSOT2* (Figure 6.6), and *MdSOT2.2* (Figure 6.7) relative gene expression, the tendency was similar for the three accessions showing watercore (‘Helada’, ‘Tempera’, and ‘Verde Doncella’). Indeed, the *MdSOTs* genes were less expressed when statistically significant, in the watercore affected fruits (Figure 6.8). These results were in agreement with those reported by Loescher et al. (2005). Moreover, Gao et al. (2005) studied the gene expression of *MdSOT1* and *MdSOT2* and reported that the observed decreased ability to transport sorbitol into fruit storage parenchyma tissues would lead to a sorbitol accumulation in the intercellular space.

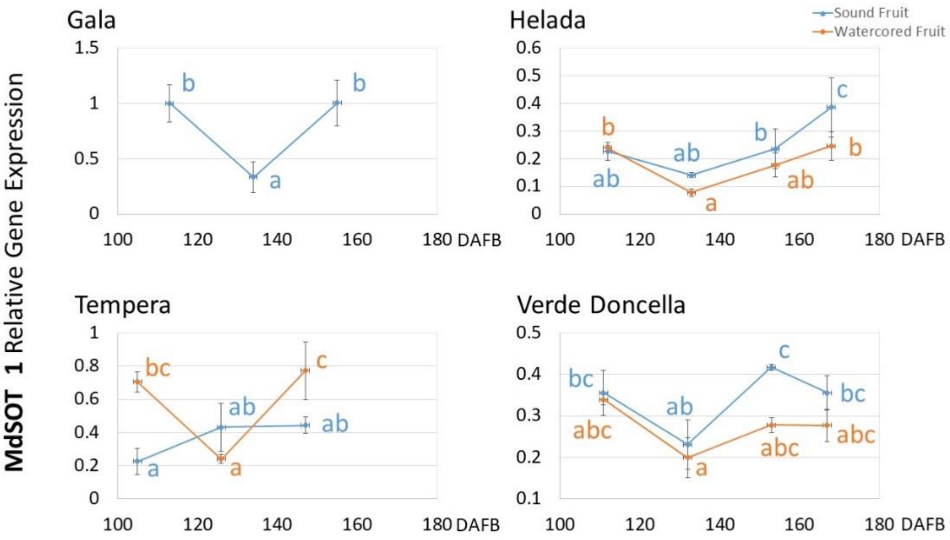


Figure 6.5. *MdSOT 1* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

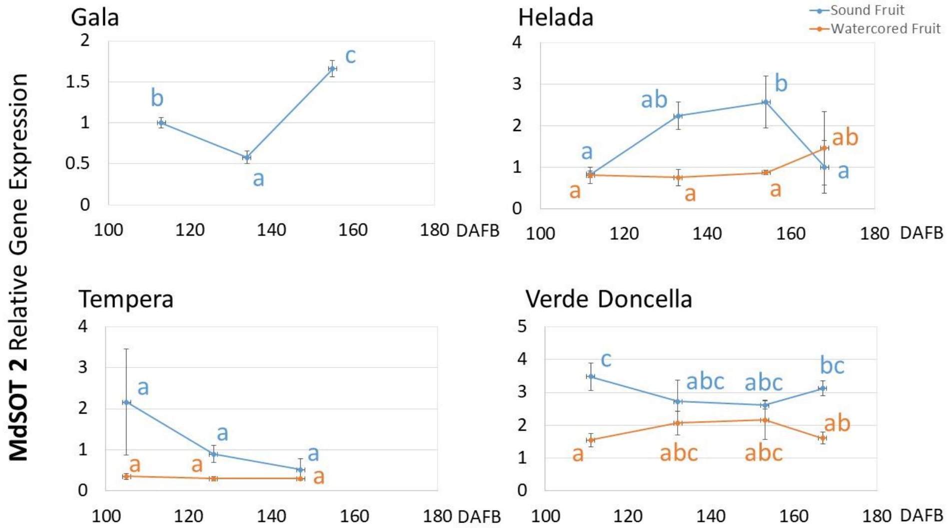


Figure 6.6. *MdSOT 2* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

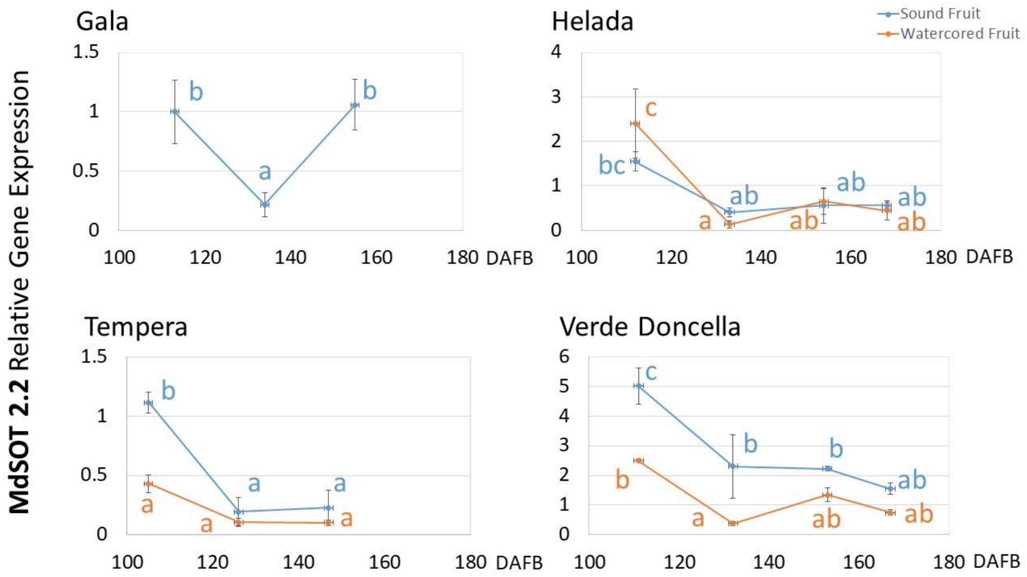
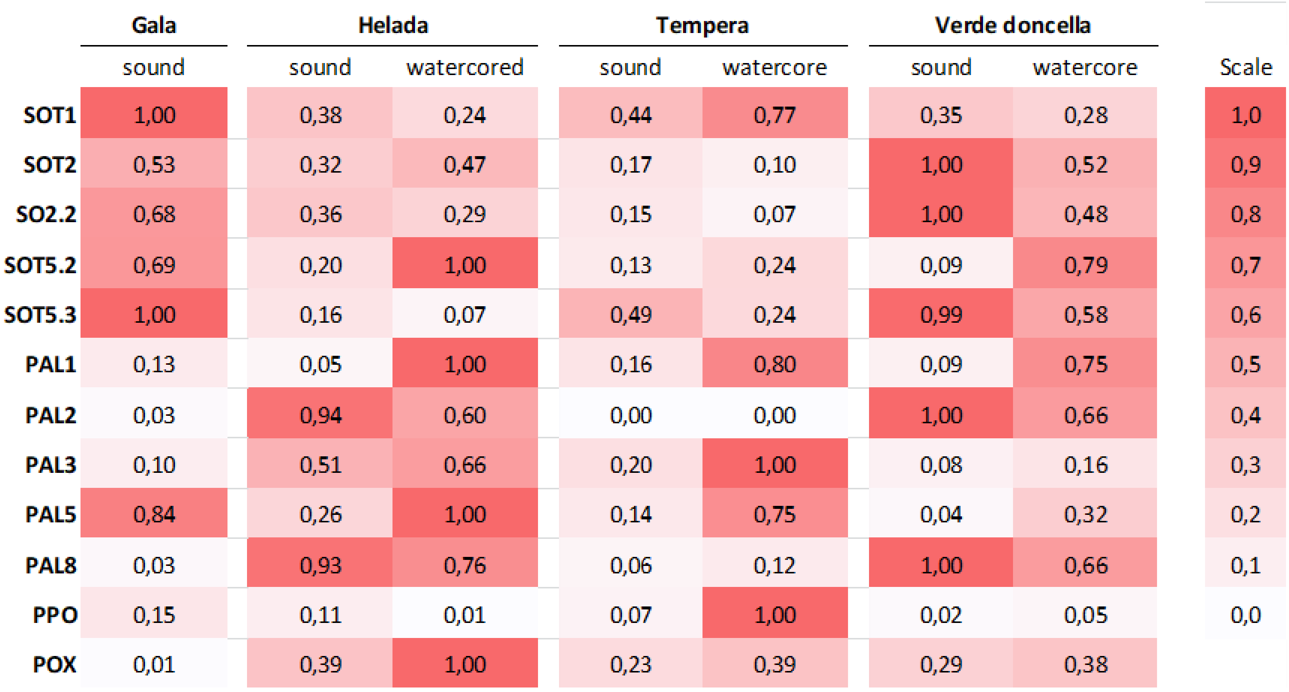


Figure 6.7. *MdSOT 2.2* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.



	Gala	Helada		Tempera		Verde doncella		
	sound	sound	watercored	sound	watercore	sound	watercore	Scale
SOT1	1,00	0,38	0,24	0,44	0,77	0,35	0,28	1,0
SOT2	0,53	0,32	0,47	0,17	0,10	1,00	0,52	0,9
SO2.2	0,68	0,36	0,29	0,15	0,07	1,00	0,48	0,8
SOT5.2	0,69	0,20	1,00	0,13	0,24	0,09	0,79	0,7
SOT5.3	1,00	0,16	0,07	0,49	0,24	0,99	0,58	0,6
PAL1	0,13	0,05	1,00	0,16	0,80	0,09	0,75	0,5
PAL2	0,03	0,94	0,60	0,00	0,00	1,00	0,66	0,4
PAL3	0,10	0,51	0,66	0,20	1,00	0,08	0,16	0,3
PAL5	0,84	0,26	1,00	0,14	0,75	0,04	0,32	0,2
PAL8	0,03	0,93	0,76	0,06	0,12	1,00	0,66	0,1
PPO	0,15	0,11	0,01	0,07	1,00	0,02	0,05	0,0
POX	0,01	0,39	1,00	0,23	0,39	0,29	0,38	

Figure 6.8. Relative gene expression for the four accessions assessed according to the presence/absence of watercore and evaluated at 155, 168, 147, and 167 DAFB for 'Gala', 'Helada', 'Tempera', and 'Verde Doncella' accessions respectively. Gene expression were normalized per gene against the highest expression, which was set as '1'.

The relative expression of the *MdSOT5.3* gene (Figure 6.9) was less consistent. In the case of the 'Tempera' and 'Verde Doncella' accessions, the tendency was similar to the other *MdSOTs* genes cited before (*MdSOT1*, *MdSOT2*, and *MdSOT2.2*) and its expression decreased. However, for the 'Helada' accession, the MdSOT5.3 gene was more expressed in the watercore affected fruits than in the sound apples, with less mature fruits (112 DAFB) and increasing later with the maturity of the fruit. Furthermore, for the three susceptible accessions to develop watercore ('Helada', 'Tempera', and 'Verde Doncella'), the relative expression of the *MdSOT5.2* (Figure 6.10) was higher in the watercored apples than in the control unaffected tissues.

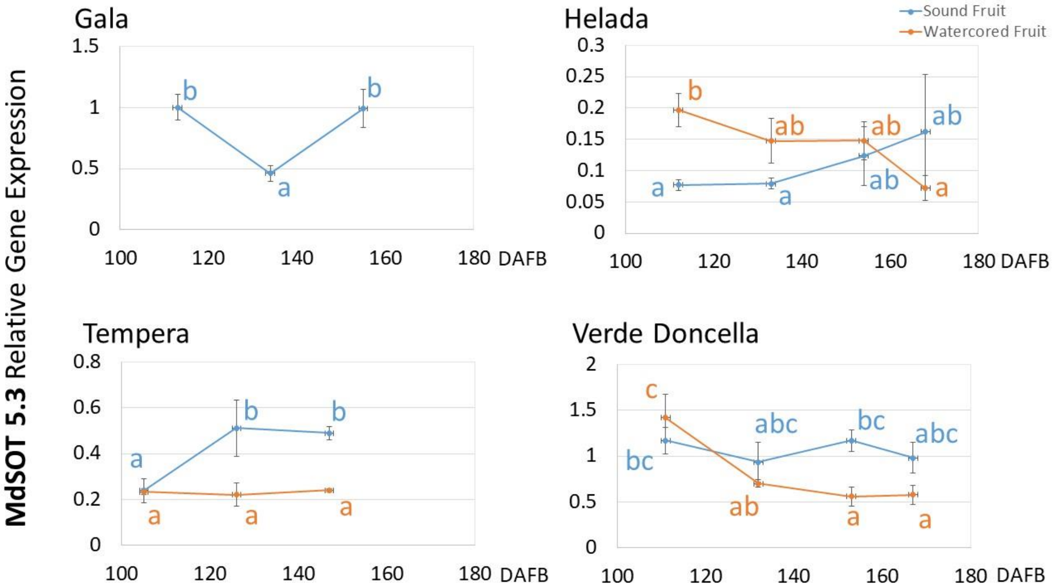


Figure 6.9. *MdSOT 5.3* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

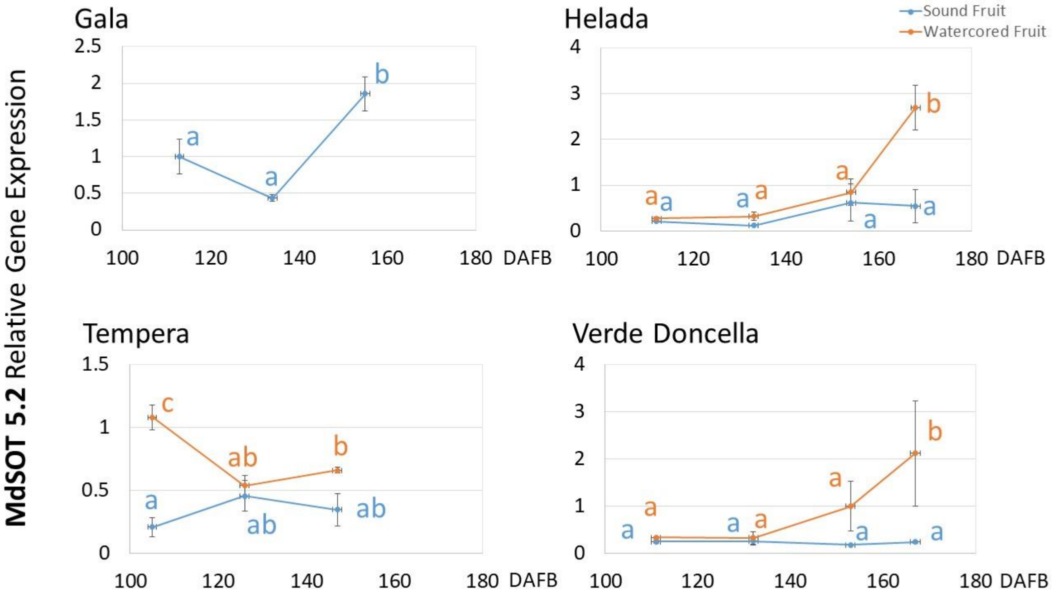


Figure 6.10. *MdSOT 5.2* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

6.4.2.2. Genes involved in the Phenylpropanoid pathway – MdPALs genes

The phenylalanine ammonia lyase (PAL) is the main enzyme studied of the phenylpropanoid metabolism pathways (Anwar et al., 2021; Cebulj et al., 2021). It is involved in an extensive spectrum of resistance or tolerance against different diseases and biotic or abiotic stresses, as well as other stresses as scion/rootstock graft incompatibility (Amri et al., 2021; Cebulj et al., 2021; You et al., 2020). Zhang et al. (2018) reported five *MdPAL* genes isoforms in apple. They also reported that apple maturity stages influenced on these five *MdPAL* relative expression tendencies. In the present study, the possible role of *MdPAL* genes in response to watercore disorder is studied.

Among them, the *MdPAL1* was the most expressed *MdPAL* isoform in fruits and its relative expression was significantly higher in the watercored fruits at the final maturity stage (Table 6.8 and Figure 6.11). The increase in *MdPAL1* expression and consequently, the increase in the phenylalanine ammonia lyase enzyme concentration could be a defence mechanism as response against the oxidative stress caused by the watercore disorder (Cebulj et al., 2021; Zupan et al., 2016).

The relative gene expression of the *MdPAL3/4/6/7* isoforms and *MdPAL5* showed a similar tendency to *MdPAL1* for all the accessions assessed. However, no significant differences depending on the sampling DAFB period were found (Figures 6.12 and 6.13).

Furthermore, the tendency of the relative expression for the *MdPAL2* (Figure 6.14) and *MdPAL8* (Figure 6.15) genes seemed to be the opposite of those before cited. Indeed, the tendency was, in general, to exhibit less expression of these *MdPAL* genes in the watercore affected tissues (Table 6.6 and Figure 6.8), in agreement with Cebulj et al. (2021). However, for the 'Tempera' accession, the *MdPAL8* relative expression was lower in sound fruits compared to the watercored fruits.

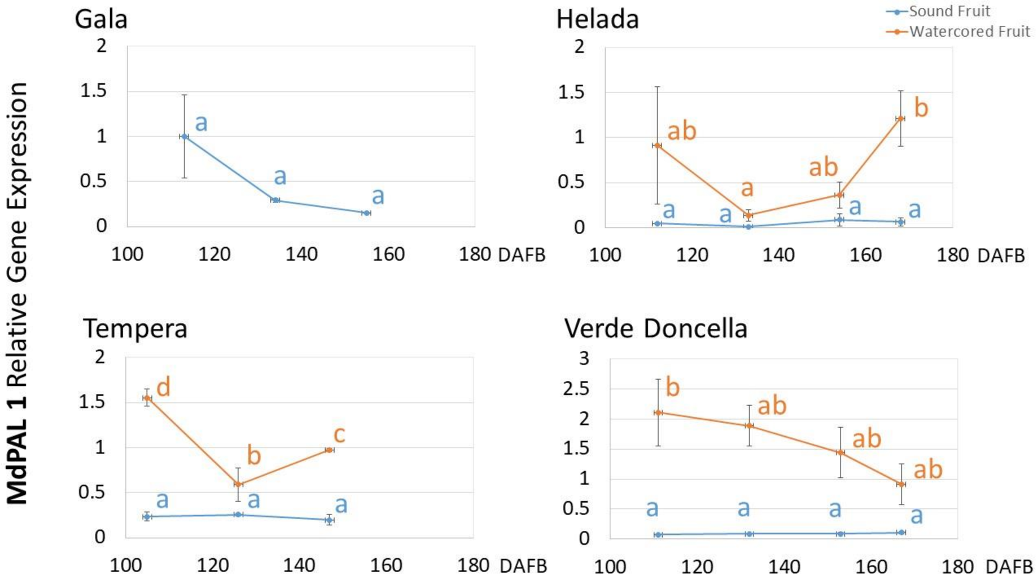


Figure 6.11. *MdPAL 1* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

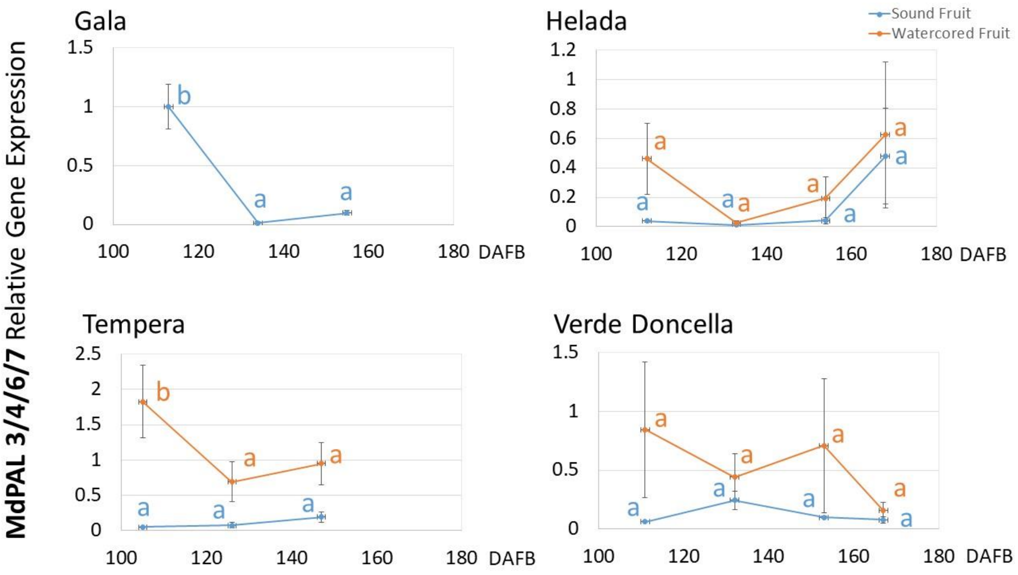


Figure 6.12. *MdPAL 3/4/6/7* relative gene expression for the accessions assessed (‘Gala’, ‘Helada’, ‘Tempera’, and ‘Verde Doncella’) according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

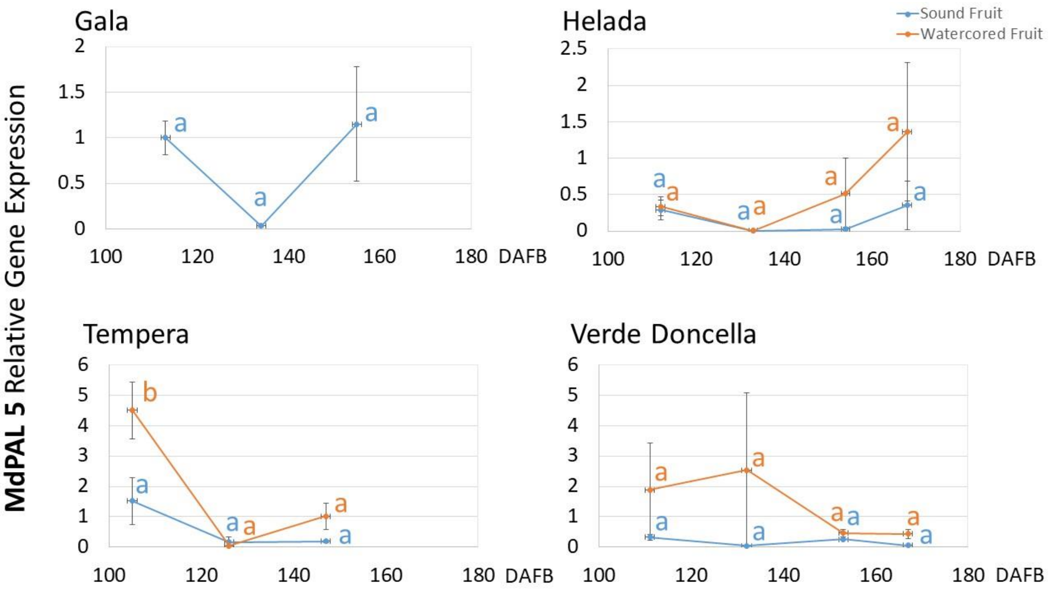


Figure 6.13. *MdPAL 5* relative gene expression for the accessions assessed (‘Gala’, ‘Helada’, ‘Tempera’, and ‘Verde Doncella’) according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

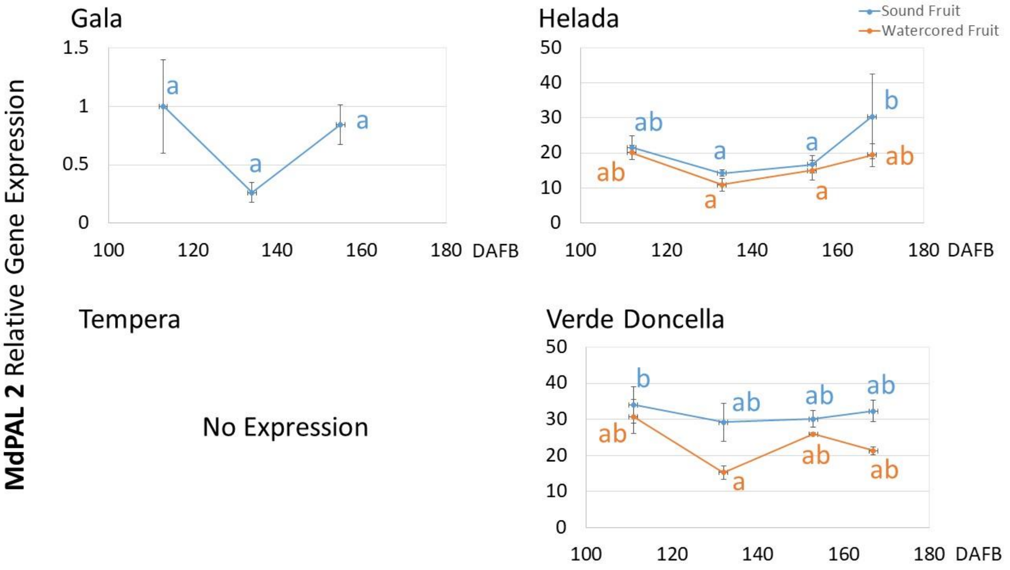


Figure 6.14. *MdPAL 2* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

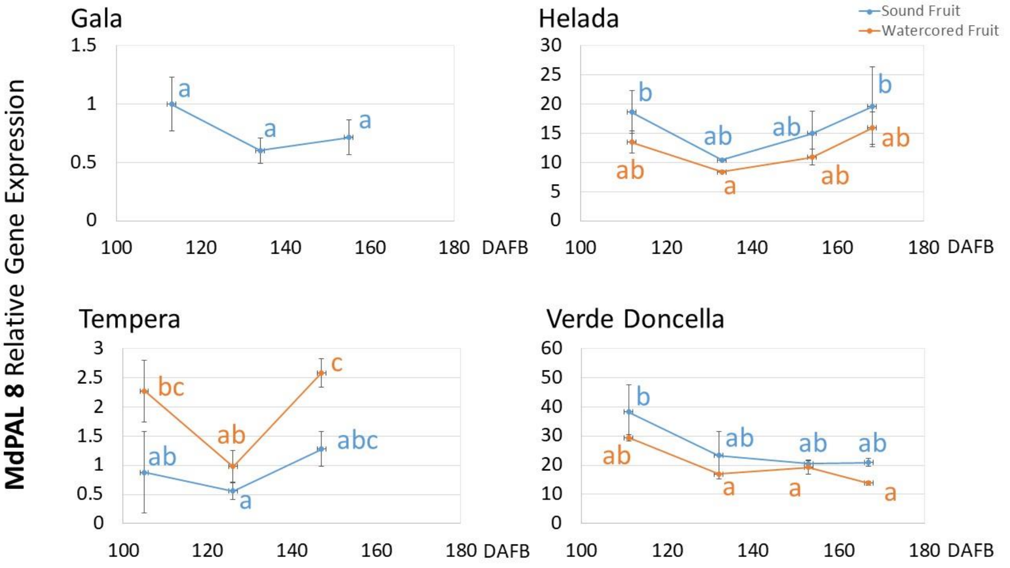


Figure 6.15. *MdPAL 8* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

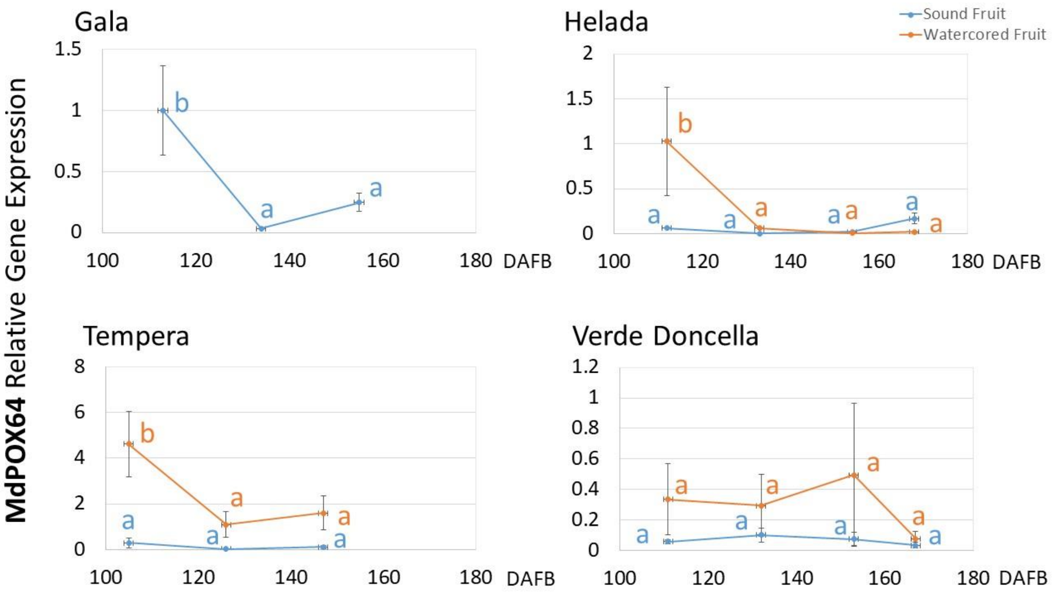


Figure 6.16. *MdPOX64* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

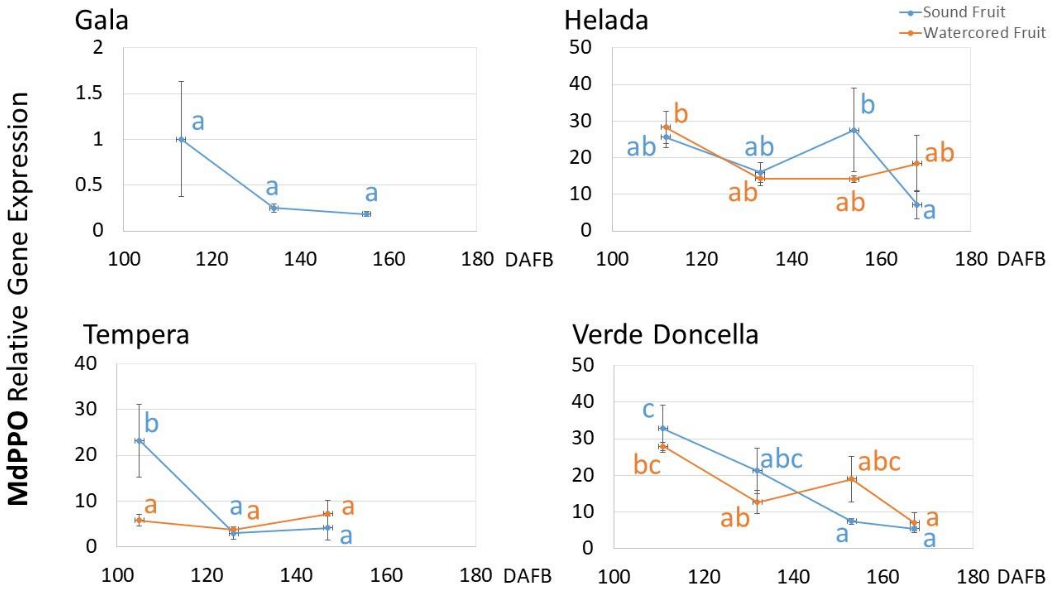


Figure 6.17. *MdPPO* relative gene expression for the accessions assessed ('Gala', 'Helada', 'Tempera', and 'Verde Doncella') according to the presence/absence of watercore and the DAFB. The reference gene was *LTL1* gene. Error bars represent the standard error of the mean.

6.4.2.3. Polyphenol Oxidase and Peroxidase genes – MdPPO and MdPOX genes

Antioxidant compounds and related enzymes contribute in great extend to the quality in fruits and vegetables (Lattanzio, 2013). The main enzymes responsible for quality loss in apples are polyphenol oxidase (PPO) and peroxidase (POX), which could be linked to the phenolic degradation, during fruit ripenning (Hutabarat and Halbwirth, 2019).

In the present work, no significant differences have been observed between the watercored and the sound fruits according to the relative expression for the *MdPPO* and *MdPOX* genes (Figures 6.16 and 6.17, Table 6.8). Moreover, previous studies have described the correlation between PPO and POX enzymes and the tolerance or resistance to pest and diseases (Suzuki et al., 2012). Indeed, PPO contributes to lignification and pigmentation and together with POX it consumes oxygen and produce quinones, which may reduce plant digestibility by several pests. Watercore induces sugar accumulation in the intercellular spaces, causing a physiological disorder (Melado-Herreros et al., 2013; Tanaka et al., 2020). Nevertheless, Zupan et al. (2016) reported a higher POX activity in watercored apples indicating that watercore has an influence on apple fruit quality even before internal breakdown and browning occur.

6.4.2.4. Pearson's correlations according to the gene expression

According to the relative gene expression, significant correlations were found (Tables 6.5 and 6.6), between the antioxidant trait with the relative gene expression of the watercored apples, demonstrating the oxidative stress caused by the watercore disorder (Cebulj et al., 2021; Zupan et al., 2016). Moreover, the sorbitol concentration was the main phenotypic trait affected by the watercore disorder in all the accessions ('Helada', 'Tempera', and 'Verde Doncella'). Indeed, correlations between sorbitol and gene expression were found for watercored apples. The *MdSOT1* and the *MdSOT5.2* genes expression were correlated positively with the sorbitol content (r = 0.63 and r = 0.42 respectively) while the *MdSOT2.2* was negatively correlated (r = -0.58). These results would indicate the role of the different *MdSOTs* genes, accumulation of sorbitol in the fruit (*MdSOT5.2*) or in the phloem before reaching sink accumulation tissues (MdSOT1 and *MdSOT2.2*). For the antioxidant genes, the *MdPAL2* (r = -0.53), the *MdPAL8* (r = -0.51), and the *MdPOX* (r = -0.60) were negatively correlated and *MdPAL3/4/6/7* (r = 0.43) was positively correlated with sorbitol content.

6.4.3. Principal components analysis - PCA

A principal component analysis (PCA) was carried out to understand how watercore could influence on the accessions' performance (Figure 6.18). Indeed, the first two PCs of the PCA, PC1 and PC2, accounted respectively for 34.8% and 20% explaining thus, a 54.8% of the total variability. The PC1 loadings suggested that separation on this component was mainly due to antioxidant contents (TPC, TFC, and RAC), relative expression of *MdSOT1*, *MdSOT2*, *MdSOT2.2*, *MdPAL2*, *MdPAL3/4/6/7*, *MdPAL5*, *MdPAL8*, and *MdPPO* genes and, to sorbitol (Figure 6.19). Moreover, the PC2 mainly contributed to the variability of the relative expression of *MdPAL1* gene, sucrose, glucose and other *MdPAL* genes but in a minor proportion than in the PC1. The fruit density were in both PC1 and PC2 loadings significant (Figure 6.19). Fruit density, glucose, and sorbitol parameters were observed as the three major phenotypic traits exhibiting significant differences between watercored and sound fruits. According to the relative gene expressions, the *MdPAL* genes seemed to be the most influenced by the watercore disorder.

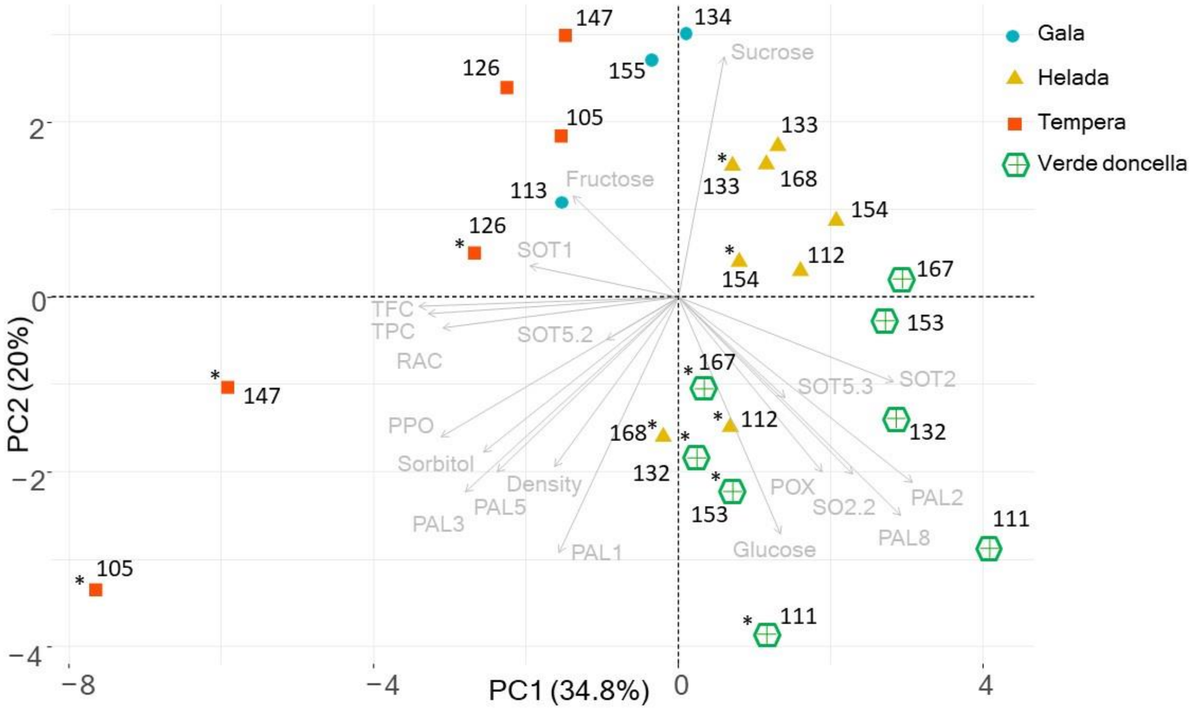


Figure 6.18. Principal components analysis (PCA) for the biochemical antioxidant traits, the individual sugars, and the relative gene expression evaluated on the four apple accessions assessed. The numbers corresponded to the DAFB of each accession and type (watercored/sound). Each point corresponding to four fruits sampled. Abbreviations: DAFB, day after full bloom; TPC, total phenolics content; TFC, total flavonoids content; RAC, relative antioxidant content; PAL3, PAL3/4/6/7.

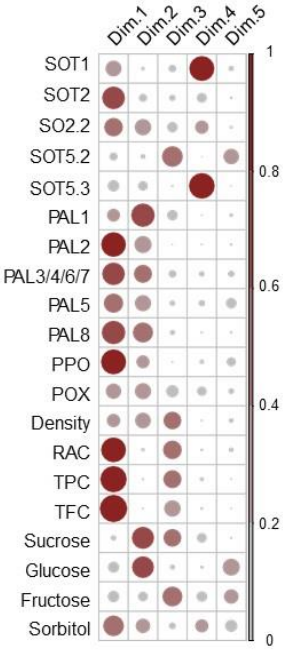


Figure 6.19. Correlations plot indicating the contributions of the phenotypic parameters and the relative expression gene assessed according to the five first dimensions of the PCA.

The samples in the negative side of the PC1 showed higher antioxidant contents. Indeed, all the 'Tempera' samples were observed in the negative side of the PC1 because of their high levels for the TPC, TFC, and RAC concentrations. Moreover, the samples on the negative side of PC2 induced higher values for fruit density and higher concentrations for glucose and sorbitol. It is easily noticed (Figure 6.18) that the watercored fruits for each accession were grouped and separated from the sound apple fruits. Accessions on the negative side of PC2, corresponding to most of the watercore affected samples, except for the 'Verde Doncella' accession, showed, in general higher sorbitol content and fruit density. Moreover, the non-susceptible reference cultivar ('Gala') fruits were grouped, in general, with the other sound fruits of the susceptible accessions ('Helada', 'Tempera', and 'Verde Doncella') in the positive side of both PC1 and PC2. These results indicated low relative gene expression of *MdPAL* and *MdPPO* genes, a lower fruit density, less concentration in sorbitol, and more sucrose and fructose contents, in healthy fruits and 'Gala' cultivar.

A clear cluster including the watercored samples with specific fruit quality values according to the fruit density, the sorbitol concentrations and specific higher expression of genes involved in oxidative stresses could therefore be identified through the PCA analysis.

6.5. Conclusions

The watercored apple fruits showed higher fruit density, higher contents of sorbitol and antioxidant compounds. In watercored apple fruits, the expression of sorbitol transporters *MdSOT1*, *MdSOT2*, *MdSOT2.2*, and *MdSOT5.3* was lower while the expression of *MdSOT5.2* increased along with the maturity of the fruit and was higher at the commercial maturity stage (last DAFB) compared with sound apples.

In general, the higher antioxidants contents in watercored apples were supported by a generally increased expression of antioxidant related genes (*MdPAL*, *MdPPO* and *MdPOX*). The present study permitted a better knowledge of the different genes expression involved in watercore development and will help to screen or improve for more tolerant cultivars.

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Capítulo 7

Postharvest analisis of 17 apple accessions from the EEAD – CSIC germplasm bank

7.1. Abstract

Apple fruit is available all year round in the market. In this work, 17 accessions from the Apple Germplasm Bank of the EEAD-CSIC were harvested and stored in a cold room during six months, at low temperature (1-1.5ºC) and controlled relative humidity (85-90%). The objective of this study was to evaluate differences among accessions at biochemical level between harvest and after a long-term cold storage, defining their suitability for storage. After storage, the basic parameters of fruit quality were analyzed and antioxidant compounds were evaluated. Individual sugars and major organic acids were also determined. Differences were observed between genotypes and significant correlations between traits studied were detected. A PCA was carried out explaining 70% of the total variability. ‘Evasni’ seemed to be the cultivar with the less decrease in fruit quality during postharvest and thus presented the best performance under cold storage. The concentrations of organic acids and phenolic compounds, in general, decreased after long-term conservation. The accessions responded to preservation differently. Triploid accessions with presence of russeting highlighted for their higher content in bioactive compounds after cold storage. Nevertheless, the russeting seemed to be related with cell damages and increased chilling injuries as browning or dehydration. Spanish accessions (‘Reneta’ and ‘Solafuente’) and other non-Spanish bred cultivars for their quality and marketability, showed their suitability for conservation with no chilling injuries after storage. The present work highlighted the interest of native accessions and non-Spanish cultivars to be considered in future breeding programs thanks to their best marketability and consumer acceptability.

Keywords: basic fruit quality, chilling injury, *Malus x domestica* Borkh, organic acids, phenolics, sugars

7.2. Introduction

Apple (*Malus x domestica* Borkh) has a great importance on the market since it is among the most extensively consumed fresh fruits in human nutrition. Indeed, apple fruit is available on the marketplace all year round for consumers. Moreover, it is considered as one of the major temperate fruit crops cultivated worldwide (Mushtaq et al., 2020). In 2020, more than 86 M tonnes of apple were produced globally (FAOSTAT, 2022) and more than 617 thousands of tonnes were produced in 2021, in Spain (MAPAMA, 2022). Catalonia, Galicia and Aragón represented the 48.4%, 17% and 13.1% respectively of the total Spanish production.

It is well known that fruits and vegetables represent a major source of biomolecules for humans (Boeing et al., 2012; Gibney et al., 2019; Ho et al., 2020; Michalska & Łysiak, 2015; Zhang et al., 2016). Apple fruit is also a source of micro-nutrients, sugars, organic acids, oligosaccharides, minerals, phytochemical and antioxidant compounds, and dietary fiber. Biomolecules as antioxidant compounds could improve the quality and the shelf life of vegetables and reduce the risk of postharvest diseases and/or chilling injuries (Bui et al., 2019; Davey et al., 2007; Khan et al., 2021; Lattanzio, 2013; Singh et al., 2018). The content of the bioactive compounds differs considerably depending on genetics, environmental conditions, farming practices, ripening date or even postharvest storage conditions (Alhaj Alali et al., 2020; Quiles et al., 2005; Mignard et al., 2021b; 2022; Neri et al., 2020; Wu et al., 2012; Zhang et al., 2021). Biomolecules from fruits are very vulnerable to degradation, mostly due to certain handling practices, chilling injuries, and other several stresses (Rivera-Pastrana et al., 2010). These aspects can reduce the antioxidant capacity and, therefore, the nutritional and organoleptic quality of fresh apples (Villa-Rodriguez et al., 2015). The quality of fruits and vegetables after a long-term cold storage has been traditionally well-defined in terms of sensorial qualities (freshness, color, and nonappearance of chilling injuries), texture (firmness, juiciness, and crispness) and care (pathogenic microorganisms) (Villa-Rodriguez et al., 2015). Nevertheless, most of the studies in this field has not focused the effect of cold storage on the nutritional quality. Nonetheless, fruits and vegetables biomolecules are subject to qualitative and quantitative changes during long-term cold storage (Leja et al., 2003; Zhang et al., 2021).

Postharvest technologies aim to reduce the levels of weight loss of the fruit once harvested and to maintain its nutritional quality. The constantly growing demand for fresh fruit on the market highlights the critical need to maintain a high level of both aesthetic and organoleptic quality of fruits, even for a long time after harvest, in order to guarantee consumer satisfaction (Cainelli et al., 2019). Low-temperature storage reduce the metabolic level and slow the deterioration of fresh fruits (Leja et al., 2003; Mushtaq et al., 2020). Cold storage is extensively used to extend shelf life of postharvest fruits since both metabolic activity and deterioration process are accelerated after harvest because of fruit ripening. Indeed, antioxidants metabolism is closely connected to chilling injuries in postharvest (Bui et al., 2019; Davey et al., 2007; Foyer and Noctor, 2005; Mushtaq et al., 2020). The main effect of low temperature is a significant decrease of the overall metabolism of the fruit, causing a slower evolution of the fruit ripening during postharvest and a delay of senescence as the apple fruit is considered a climacteric fruit. Nevertheless, long period of cold storage could negatively influence the final apple quality and thus, its acceptability by consumers and marketability. In fact, improper low-temperatures often cause chilling injuries and result in an economic loss for the producers. Sub-optimal temperatures, depending on the accession, the ripening and the time of storage, could engender an excess of reactive oxygen species (ROS) in the fruit cells, resulting in the decrease of antioxidant substances such as ascorbic acid or total phenolics (Bui et al., 2019; Cainelli et al., 2019; Han et al., 2021; Kevers et al., 2007; Khan et al., 2021).

Postharvest shelf life for apples is very susceptible to low temperature and humidity variations. These are the major causes of deterioration in apple products through the supply chain (Han et al., 2021). Aesthetic and physiological properties of apples as membrane permeability or enzyme activity are degraded if the apple storage temperature decreases below the threshold temperature for more than a definite period. This threshold depend on the cultivar and the ripening of the apple stored (Cainelli et al., 2019). Chilling injuries appear when these degradations occurs and make the apple unmarketable (Han et al., 2021).

Typical symptoms of chilling injuries in apple are browning (peel and/or flesh), dehydration or biomolecules degradation and thus, a decrease of fruit quality (Al-Abbasy et al., 2021; Cebulj et al., 2021; Khan et al., 2021; Rasouli & Saba, 2018; Singh et al., 2018). Chilling injuries depend on the intensity of the abiotic stress caused by low temperatures, the genetic susceptibility of the accession to cold exposure, and the length of time of the cold storage (Cainelli et al., 2019). Fruits stored at low temperatures and high relative humidity not only show less water loss, but also suffer fewer chilling injuries. Nonetheless, high humidity could easily leads to a more susceptible fruit against infection by pathogenic microorganisms as reported in apple and other fresh material (Fahmy and Nakano, 2013; Grammen et al., 2019; Zhang et al., 2016). Therefore, to guarantee safe and good looking apples with high organoleptic and functional quality, fresh apples must be stored in the appropriate environment during cold storage and transportation (Di Guardo et al., 2013).

This work focuses on evaluating the fruit quality for 17 accessions and observing the values of different bioactive compounds, such as antioxidants, individual sugars and major organic acids during a long-term storage at 1-1.5ºC. The characterization of these compounds could contribute to increase the knowledge about adaptation of different apple cultivars to their postharvest storage and their shelf life (Leja et al., 2003; Wu et al., 2012). Indeed, this work aims to improve our understanding on how different apple cultivars respond after six months of cold storage. The application of this knowledge to the fruit industry could offer different options for fruit management, achieving more health benefits and best organoleptic quality to consumer.

7.3. Materials and methods

7.3.1. Plant material and field trial

The apple accessions [*Malus x domestica* Borkh] of the present study were obtained from the apple germplasm bank of the Experimental Station of Aula Dei (EEAD-CSIC, Zaragoza, NE Spain: 41° 43′ 42.7″ N, 0° 48′ 44.1″ W). A total of 17 accessions, consisting of five local Spanish accessions and 12 non-Spanish accessions were assessed (Table 7.1). Most of the non-Spanish accessions were commercial cultivars meanwhile the Spanish accessions were traditional and/or autochthone cultivars. The 17 accessions were classified according to their skin color as bicolor or red (11 accessions), green (3), yellow (1) and brown (2), with brown corresponding to apples 100% russeted covered. They were also classified according to their ploidy level as diploids (13 accessions) and triploids (4 accessions).

7.3.2. Fruit sampling and storage

A representative sample of 40 fruits per accession were harvested when they exhibited the ground representative color of each accession. Samples were washed and then stored for six months in a commercial cold room. The storage temperature was 1-1.5°C and the humidity fixed at 85-90%. Regarding to the fruit sampling for each accession, after the six months of cold storage, 12 apples that were similar in size, with a uniform color and no pest damage or mechanical injuries were selected for the subsequent analyses. Fruit traits were measured by three replicates (four fruits per replication) and means for each accession were calculated.

Table 7.1. Information of the 17 apple accessions used on this study.

Accession	Nº	EEAD Code	Classification	Origin	Skin Color	Flesh Color	Ploidy
Averdal	1	882021	Non-Spanish	-	Red	Cream	2
Baujade	2	923284	Non-Spanish	France	Green	Greenish	2
Bossost - MRF 76	3	3627	Spanish	Spain	Brown	Cream	3
Cripps Pink	4	933540	Non-Spanish	UK	Bicolor	Cream	2
Cul de Cirio - MRF 39	5	3551	Spanish	Spain	Bicolor	Cream	2
Delcon	6	2896	Non-Spanish	France	Bicolor	White	2
Evasni - Scarlet Spur	7	933554	Non-Spanish	France	Bicolor	Cream	2
Florina	8	3633	Non-Spanish	France	Bicolor	White	2
Golden Paradise	9	3739	Non-Spanish	Spain	Yellow	Cream	2
Granny Smith	10	2614	Non-Spanish	Australia	Green	Greenish	2
Morro de Liebre	11	3256	Spanish	Spain	Bicolor	White	2
Red Delicious	12	3085	Non-Spanish	US	Bicolor	Cream	2
Red Elstar	13	882002	Non-Spanish	Netherlands	Bicolor	White	2
Reineta Blanca del Canadá	14	308	Non-Spanish	France	Green	Cream	3
Reineta Gris	15	2883	Non-Spanish	France	Brown	Cream	3
Reneta	16	3408	Spanish	Spain	Bicolor	White	2
Solafuente	17	3559	Spanish	Spain	Bicolor	White	3

EEAD, Experimental Station of Aula Dei; US, United States; UK, United Kingdom.

7.3.3. Basic fruit quality and phytochemical traits

Soluble solids content (SSC) and titratable acidity (TA) were determined on flesh juice as described by Mignard et al. (2021a) after six months of cold storage. SSC was expressed as ºBrix and TA as g malic acid per liter. Ripening index (RI) was also calculated based on the SSC/TA ratio.

For the analysis of the total phenolics content (TPC), total flavonoids content (TFC), vitamin C (ascorbic acid – AsA) and the relative antioxidant capacity (RAC), three replicates per accession were sampled and prepared as described by Mignard et al. (2021a).

Individual sugars and organic acids were analysed by HPLC as previously described by Font i Forcada et al. (2019) and Mignard et al. (2022). The individual sugars (glucose, fructose, sucrose), the sugar-alcohol (sorbitol) and organic acids (citric, malic, succinic, shikimic and tartaric) were identified by their retention time characteristics using the adequate standards. Concentrations were expressed as g per kg of fresh weight

(FW) as described by Mignard et al (2022). All chemicals and standards were of analytical grade.

7.3.4. Data analysis

All statistical analyses were carried out using R language (R Development Core Team, 2019). Data were reported as means ± standard deviation of the mean (SD). The one-way analysis of variance (ANOVA) was run to determine whether there were any statistically significant differences between the means of the evaluated traits among accessions and the date of analysis (harvest time or after six months of cold storage). The Pearson's correlations and Principal Component Analysis (PCA) were performed to understand how biochemical traits contribute to variability among accessions.

7.4. Results and Discussion

7.4.1. Statistical analysis

The ANOVA analysis showed significant differences ($P \leq 0.001$) among the different apple accessions, presented in the Table 7.1, for all traits evaluated at harvest (Mignard et al., 2021b; 2022) and after six months of cold storage (Figures 7.1 and 7.2). Minimums, maximums, means and standard deviations are shown in the Table 7.2 for traits analyzed both at harvest and postharvest periods. For all parameters studied, values were in similar ranges to those published in other apple studies (Aprea et al., 2017; Castel et al., 2020; Lattanzio, 2013; Moon et al., 2020; Oszmiański et al., 2020). Moreover, means for the 17 apple accessions at harvest time and after six months of cold storage are presented in Figures 7.1 and 7.2.

Table 7.2. Average values for basic quality and bioactive compounds traits over 17 accessions at harvest and postharvest: units, minimum, maximum, mean values, and standard deviation (SD).

Trait	**Units**	**Period**	**Minimum**	**Maximum**	**Mean**	**SD**
SSC	ºBrix	Harvest	11.58	17.14	13.58	1.85
		Postharvest	9.83	24.23	15.85	3.92
TA	g malic acid L-1	Harvest	3.29	14.03	6.73	3.33
		Postharvest	2.22	10.07	5.18	2.51
RI	-	Harvest	1.28	4.51	2.54	1.50
		Postharvest	1.57	8.06	3.69	1.80
TPC	mg GAE 100 g FW-1	Harvest	18.48	57.04	33.51	19.50
		Postharvest	6.14	49.84	23.00	11.59
TFC	mg CE 100 g FW-1	Harvest	6.87	32.86	17.05	18.52
		Postharvest	5.83	39.01	16.87	10.57
AsA	mg AsA 100 g FW-1	Harvest	1.71	3.64	2.59	1.63
		Postharvest	0.53	3.54	1.46	0.81
RAC	mg Trolox 100 g FW-1	Harvest	7.13	21.21	13.74	6.59
		Postharvest	5.57	19.46	13.40	4.70
Sugars	g kg^{-1}	Harvest	71.13	112.18	90.60	17.76
		Postharvest	63.77	138.72	89.74	21.43
Sucrose	g kg^{-1}	Harvest	11.47	42.14	29.56	9.14
		Postharvest	5.97	38.61	21.56	10.57
Glucose	g kg^{-1}	Harvest	6.38	18.99	12.62	5.66
		Postharvest	5.15	23.53	15.69	5.06
Fructose	g kg^{-1}	Harvest	31.39	53.03	43.76	8.96
		Postharvest	26.04	65.20	46.59	9.81
Sorbitol	g kg^{-1}	Harvest	2.10	11.43	4.66	3.41
		Postharvest	2.04	19.80	5.90	4.82
Acids	g kg^{-1}	Harvest	3.63	11.26	6.63	2.34
		Postharvest	1.52	7.76	3.40	1.88
Malic	g kg^{-1}	Harvest	2.78	10.13	5.68	2.12
		Postharvest	0.96	7.04	2.90	1.90
Citric	g kg^{-1}	Harvest	0.02	0.11	0.05	0.03
		Postharvest	0.01	0.24	0.04	0.06
Tartaric	g kg^{-1}	Harvest	0.03	0.05	0.04	0.02
		Postharvest	0.02	0.07	0.04	0.01
Succ+Shi	g kg^{-1}	Harvest	0.19	0.86	0.45	0.28
		Postharvest	0.18	0.81	0.43	0.17

SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total acid; flavonoids content; AsA, Ascorbic RAC, relative antioxidant content; Sugars, total sugars; Acids, total organic acid; Succ+Shi, succinic + shikimic.

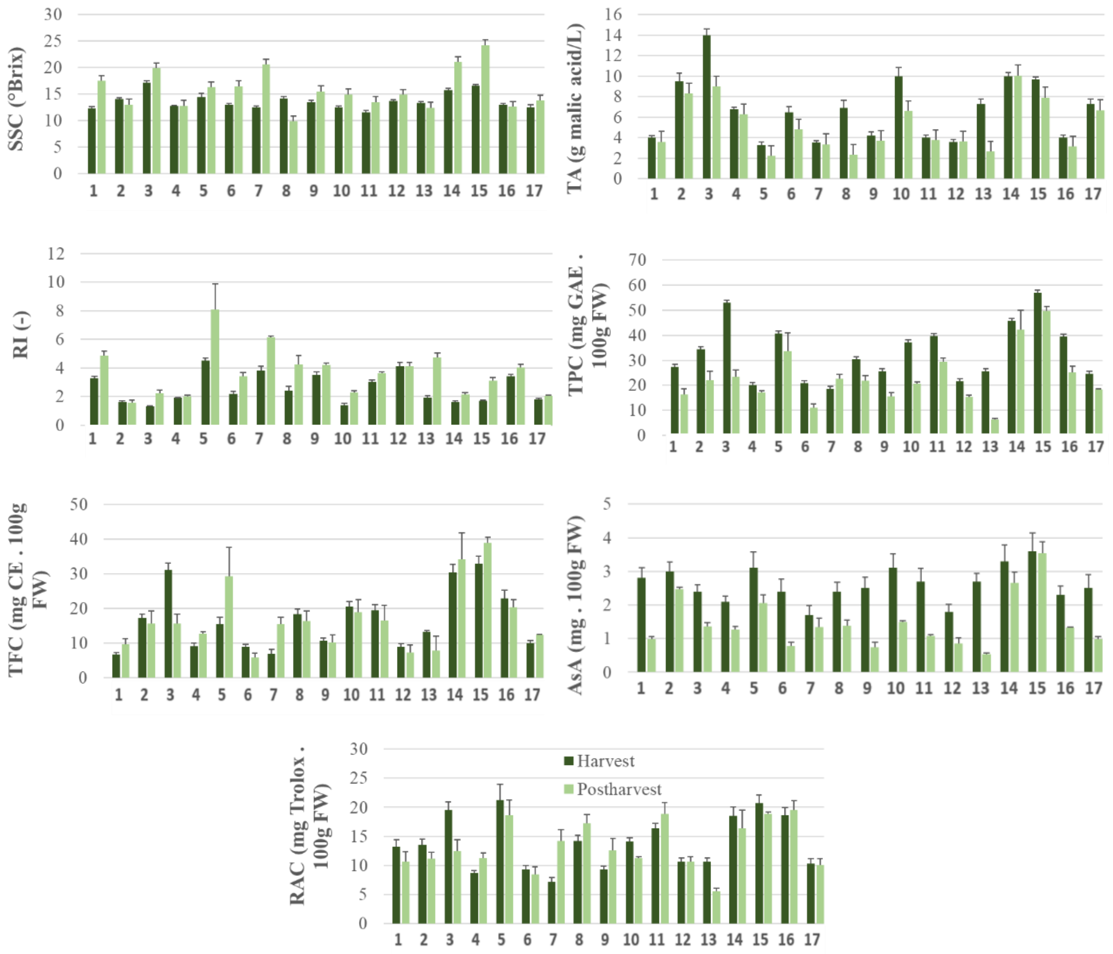

Figure 7.1. Changes in basic fruit quality and antioxidant traits between harvest and postharvest date for the 17 apple accessions assessed.

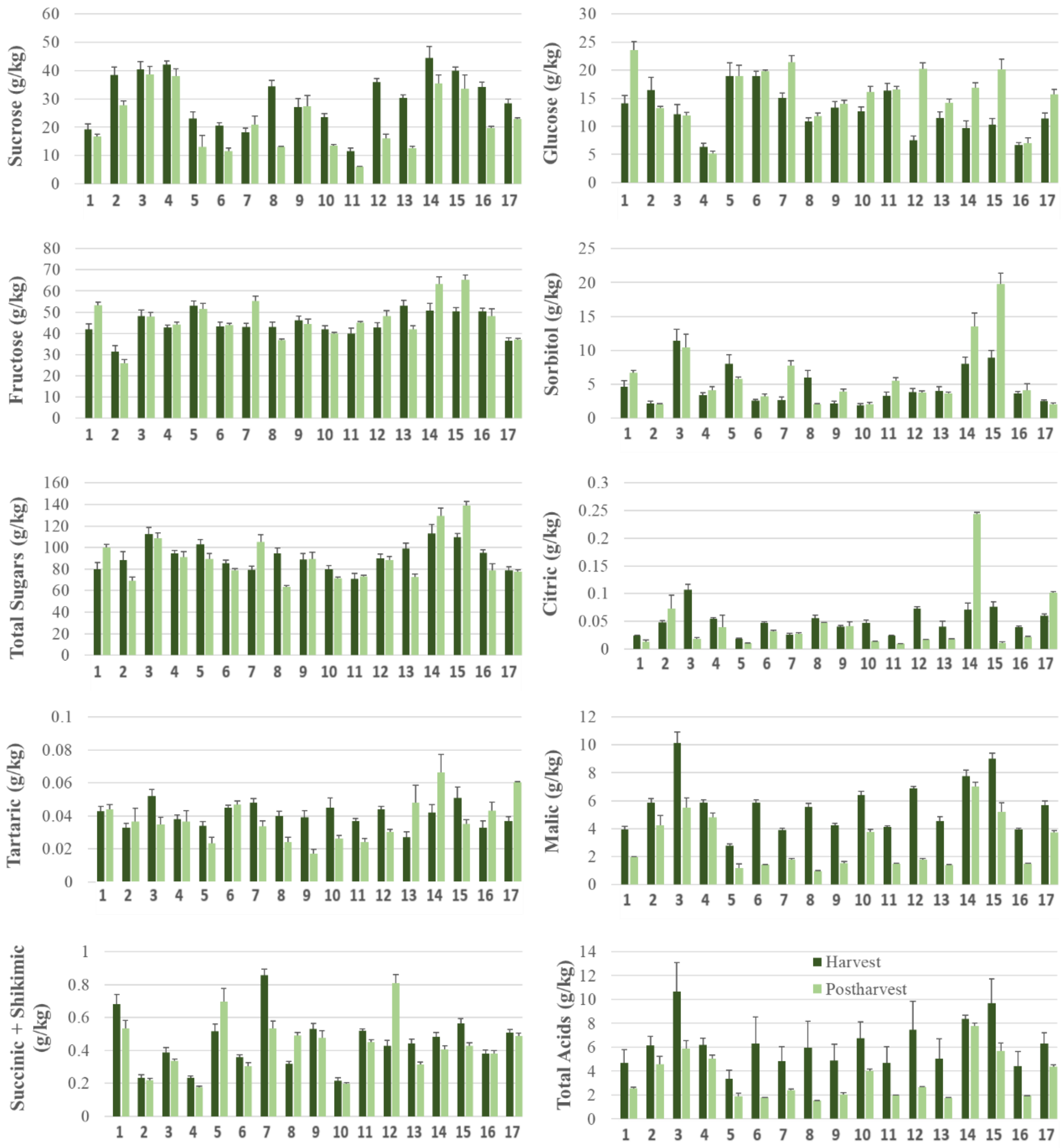

Figure 7.2. Changes in individual sugars and organic acids between harvest and postharvest date for the 17 apple accessions studied in this study.

7.4.2. Basic fruit quality traits and antioxidant compounds

According to the basic fruit quality traits, values varied greatly in this study as reported in other apple studies at harvest as well as during postharvest long-term storage (Alhaj Alali et al., 2020; Castel et al., 2020; Corollaro et al., 2014; Mignard et al., 2021a; 2022; Moon et al., 2020; Yang et al., 2021). In fact, the soluble solids content (SSC) ranged among apple accessions in postharvest from 9.8 ('Florina') to 24.2 ('Reineta Gris') ºBrix, compared to a less variation from 11.6 ('Morro de Liebre') to 17.1 ('Bossost – MRF 76') at harvest date. The titrable acidity (TA) postharvest values varied greatly ranging from 2.2 (‘Cul de Cirio – MRF 39’) to 10.1 (‘Reineta Blanca del Canadá’) g malic acid per liter. Meanwhile, at harvest time, data ranged from 3.3 (again for the ‘Cul de Cirio – MRF 39’ accession) to 14.0 (‘Bossost – MRF 76’) g malic acid per liter. Regarding the ripening index (RI=SSC/TA), values ranged from 1.6 (‘Baujade’) to 8.1 (‘Cul de Cirio – MRF 39’) after six months of cold storage, compared to a less variation from 1.3 (‘Bossost – MRF 76’) to 4.5 (‘Cul de Cirio – MRF 39’) at harvest, because metabolic pathways causing ripening of fruits continue during postharvest (Ehsani-Moghaddam et al., 2013). The standard deviations for SSC, TA and RI after six months of cold storage were respectively fitted at 3.9, 2.5 and 1.8 while at harvest, they were fitted at 1.9 for SSC, 3.3 for TA and 1.5 for RI (Table 7.2).

The antioxidant compounds varied greatly in this study as previously reported by other studies (Castel et al., 2020; Kevers et al., 2011; Oszmiański et al., 2020; Lopez et al., 1994; Raudone et al., 2017). Indeed, the total phenolics content (TPC) varied significantly among apple accessions, both in postharvest and harvest period. Values ranged from 6.1 (‘Red Elstar’) to 49.8 (‘Reineta Gris’) in postharvest, compared with a slightly lower variation at harvest, from 18.5 (‘Evasni’) to 57 (‘Reineta Gris’) mg gallic acid equivalents (GAE)/100 g FW. Total phenolics thus decreased in average from 33.5 at harvest to 23 GAE/100 g FW after a long-term cold storage. López et al. (1994) reported that the decrease of phenolics could be the result of their oxidation associated with browning. However, phenols could be also synthesized as antioxidant factors against abiotic stresses (Foyer & Noctor, 2005; Lattanzio, 2013).

For the total flavonoids content (TFC), values also varied significantly ranging in postharvest from 5.8 (‘Delcon’) to 39.0 (‘Reineta Gris’) and at harvest from 6.7 (‘Averdal’) to 32.9 (‘Reineta Gris’) mg catechin equivalents (CE)/100 g FW. Regarding the ascorbic acid (AsA – Vitamin C), values ranged from 0.5 (‘Red Elstar’) to 3.5 (‘Reineta Gris’) mg AsA/100 g FW in postharvest and from 1.7 (‘Evasni’) to 3.6 (again, the ‘Reineta Gris’ accession) mg AsA/100 g FW at harvest. Finally, relative antioxidant capacity (RAC) values ranged from 5.6 (‘Red Elstar’) to 19.5 (‘Reneta’) mg trolox/100 g FW in postharvest and from 7.1 (‘Evasni’) to 21.2 (‘Cul de Cirio – MRF 39’) at postharvest. The standard deviations for TPC, TFC, AsA and RAC in postharvest analysis were fitted at 11.6, 10.6, 0.8 and 4.7 respectively (Table 7.2). According to Mignard et al. (2021a), the standard deviations at harvest were, in general, higher with 19.4 for TPC, 18.4 for TFC, 1.6 for AsA and 6.6 for RAC.

For the different basic quality traits, biochemical compounds and relative antioxidant capacity, comparison of means between the harvest date and after six months of cold storage (Figure 7.1) showed significant differences among accessions. Thus, the SSC values tended to be slightly increased after six months of cold storage, unless for the ‘Florina’ accession. In contrast, the TA, when the evolution was significant, decreased for all the accessions assessed probably due to the process of fruit ripening after harvest. Indeed, it is well known that when fruits are harvested, they continue their ripening during the postharvest storage. Changes in biomolecules describe the ripe of the fruits as peel color or fruit flavour (Ehsani-Moghaddam et al., 2013; Singh et al., 2018). Indeed, as the soluble solids content (SSC) and the individual sugars tended to increase while the titratable acidity (TA) as well as the organic acids decreased considerably, the ripening index (RI=SSC/TA) increased and the biomolecules profiles changed during postharvest.

Moreover, the flavonoids and the relative antioxidant capacity (RAC) did not show a general pattern in their increase or decrease but the evolution depended more on the studied accession. In the case of phenols, with the exception of the cultivar ‘Evasni - Scarlet Spur’, the values decreased significantly after the six months of storage as previously reported in other studies (Alhaj Alali et al., 2020; Kevers et al., 2007). Indeed, phenolics are not stable and undergo a clear metabolic turnover during cold storage, while flavonoids do not seem to be affected in this way as their contents were more constant between harvest and postharvest values. Several studies have shown significant fluctuations in the content of antioxidants in apple at low temperatures (Alhaj Alali et al., 2020; Bui et al., 2019; Villa-Rodriguez et al., 2015). Increased levels of bioactive molecules should be considered as an important guarantee against the impact of storage evolution of phenolics on the nutritional value of apples. Nevertheless, Kevers et al. (2011) reported that total phenolics content showed an increase after three months of storage at cold temperature, followed by a decrease. The decrease continued over the following months, explaining the lower values observed after six months in the cold room (Kevers et al., 2011). In the present work, the cultivar ‘Evasni – Scarlet Spur’ seemed to have a best performance under a long-term storage because of its increase in TPC, TFC and RAC.

Storage induces noteworthy metabolic changes in apples, such as a decrease in the ascorbic acid level (Kevers et al., 2011). Similarly, the loss of ascorbic acid observed in the present work could be an indicator of oxidative stress during storage (Foyer & Noctor, 2005; Kevers et al., 2007; Rivera-Pastrana et al., 2010; Van der Sluis et al., 2001). Indeed, AsA plays a major role in defense to biotic and abiotic stresses in plants against free radicals inducing peroxidation.

7.4.3. Individual sugars and organic acids profiles

According to the total sugars values (Sugars) and the major sugar for apple, fructose, values varied significantly among apple accessions and harvest/postharvest periods as previously reported (Aprea et al., 2017; Castel et al., 2020; Leja et al., 2003; Mignard et al., 2022; Zhang et al., 2021). Indeed, total sugars ranged from 63.8 ('Florina') to 138.7 ('Reineta Gris') g kg^{-1} FW in postharvest and from 71.1 ('Morro de Liebre') to 112.8 ('Reineta Blanca del Canadá') at harvest time. Moreover, fructose (Fru) values in postharvest varied considerably from 26.0 ('Baujade') to 65.2 ('Reineta Gris') g kg^{-1} FW, while values at harvest date ranged from 31.4 ('Baujade') to 53.0 ('Cul de Cirio – MRF 39') g kg^{-1} FW. The standard deviations for total sugars and fructose in postharvest were fitted at 21.4 and 9.8 respectively (Table 7.2). For the harvest data, the standard deviations were fitted at 17.8 for total sugars and 9.0 for fructose (Table 7.2).

Regarding the total acids (Acids) among accessions, levels were within the range previously reported (Castel et al., 2020; Mignard et al., 2022; Vallarino & Osorio, 2019; Yang et al., 2021; Zhu et al., 2022). Indeed, values ranged from 1.5 ('Florina') to 7.8 ('Reineta Blanca del Canadá') g kg^{-1} FW in postharvest, and 3.3 ('Cul de Cirio – MRF 39') to 10.7 ('Bossost – MRF 76') g kg^{-1} FW at harvest time. Regarding the malic acid, which is the major acid in apple, values ranged considerably, compared with the other acids, from 1.0 ('Florina') to 7.0 ('Reineta Blanca del Canadá') g kg^{-1} FW in postharvest and from 2.8 ('Cul de Cirio – MRF 39') to 10.1 ('Bossost – MRF 76') at harvest.

The profiles of the individual sugars and organic acids for the 17 apple accessions studied after six months of cold storage compared with harvest time are shown in Figure 7.2. Firstly, it is easily noticed the great variability found in this study for all the traits assessed among accessions. According to the individual sugars (sucrose, glucose, and fructose) and the sugar-alcohol (sorbitol), the changes observed in postharvest were diverse. Indeed, sucrose values tended to drop slightly for all accessions unless for the 'Florina', 'Red Delicious' and 'Red Elstar' cultivars. Meanwhile, fructose values generally increased or maintained the harvest level except for 'Baujade', 'Florina' and 'Red Elstar', which showed a loss of fructose. Sorbitol showed different performance after a long-term cold storage. It is noteworthy the great increase in sorbitol for the two Reinette accessions, 'Reineta Blanca del Canadá' and 'Reineta Gris'. Moreover, glucose values increased with greater intensity, except for ‘Baujade’ and ‘Cripps Pink’ cultivars. Additionally, the sum of the individual sugars showed different performance after six months of cold conservation. Indeed, for the 'Averdal', 'Evasni', 'Reineta Blanca del Canadá' and 'Reineta Gris' accessions, the sum of individual sugars increased while for ‘Baujade’, ‘Cul de Cirio’, ‘Delcon’, ‘Florina’, ‘Granny Smith’, ‘Red Elstar’ and ‘Reneta’, the total sugars tended to decreased. For the rest of accessions, the differences observed between harvest and postharvest periods were not significant.

Citric, tartaric, succinic and shikimic acids showed more irregular profiles according to accessions, but malic, the main acid in the apple, and total acids decreased considerably as reported before (Vallarino & Osorio, 2019; Zhu et al., 2022). Titratable acidity decreased slightly in postharvest as before mentioned. Moreover, previous studies showed that organic acids or sourness of the fruit negatively correlated with sensory acceptability of professional panellists (Keenan et al., 2012). In general, the equilibrium of sweetness and sourness determine consumer satisfaction for apple flavor. All the bioactive molecules assessed in this study influence the organoleptic perception of sweetness, sourness, and aroma (Aprea et al., 2017; Yang et al., 2021) and contribute to the quality of the fruit and its acceptance by consumers. Because of the decrease of acids and the increase of SSC and some sugars like glucose or sorbitol during cold storage, fruits as for the ‘Averdal’, ‘Bossost’, ‘Cul de Cirio’, ‘Delcon’, ‘Evasni’, ‘Golden Paradise’, ‘Granny Smith’, ‘Morro de Liebre’, ‘Red Delicious’, and ‘Reineta Gris’ accessions after long-term cold storage should have a better acceptability by consumers. It is also important to select and breed cultivars (‘Cripps Pink’, ‘Cul de Cirio’, ‘Evasni’, ‘Florina’, ‘Reineta Blance del Canadá’, and ‘Reineta Gris’) maintaining their fruit quality as total phenolics or flavonoids.

7.4.4. Pearson’s correlations between traits at postharvest

Significant bilateral correlations between the traits assessed at postharvest (Figure 7.3, Table S1) were found ($P \leq 0.01$). Indeed, the relative antioxidant capacity (RAC) was greatly and positively correlated with TPC (r=0.79) and TFC (r=0.73). TPC was also significantly and highly positive correlated with TFC (r=0.96). AsA likewise presented a significant and positive correlation with TPC (r=0.88) and TFC (r=0.89). Previous studies reported similar high and positive correlations between the antioxidant compounds at harvest (Preti & Tarola, 2020; Mignard et al., 2021a; Raudone et al., 2017; Wang et al., 2015).

Moreover, significant and positive correlations were found between TA and total acids (r=0.95) and between SSC and total sugars (r=0.92). SSC was also correlated with glucose (r=0.54), fructose (r=0.80) and sorbitol (r=0.87) while TA was also correlated with malic acid (r=0.96) and citric acid (r=0.57). These correlations highlight the common utilization of SSC and TA as approximate values for fruit sweetness and sourness, respectively (Mignard et al., 2022). Nevertheless, fruit sweetness or sourness cannot be defined only by SSC and TA but each biochemical traits of individual sugars and organic acids are really important (Aprea et al., 2017).

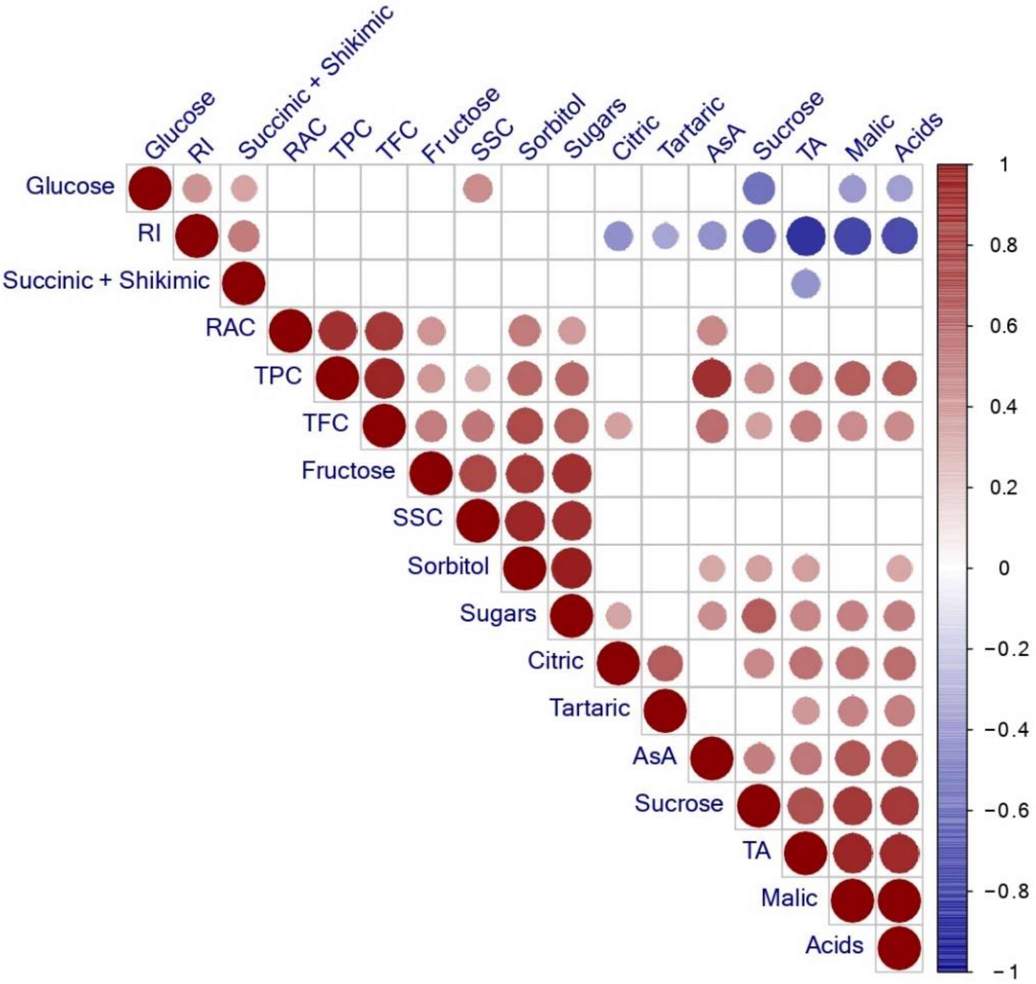

Figure 7.3. Pearson's correlation coefficients for the traits studied for the 17 accessions assessed in this study. Abbreviations: SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; RAC, relative antioxidant content; Sugars, total sugars; Acids, total organic acid; Succ+Shi, succinic + shikimic. The size of the circle for each correlation and the color depicts the significance and the magnitude of the correlation coefficient, respectively.

Furthermore, metabolic pathways could explicate the negative correlation (Figure 7.3, Table S1) between glucose and total organic acids (r=-0.34) and malic acid (r=-0.53). Indeed, organic acids degradation can occur through dicarboxylates decarboxylation (Ruan, 2014). This permits phosphoenolpyruvate (PEP) production, which plays a role in gluconeogenesis activation, resulting in increased glucose production and organic acids degradation (Vallarino & Osorio, 2019; Zhu et al., 2022). As explained above, 15 accessions showed the tendency to show an increase in glucose after six months of cold storage, while ‘Baujade’ and ‘Cripps Pink’ decreased their values. Moreover, the sum of organic acids and malic acid, for all the 17 accessions assessed, decreased significantly while for almost all of them, glucose and fructose values remained or tended to increase. Rymenants et al. (2020) reported that the perceived sweetness by consumer was importantly and significantly influenced by the fruit acidity and vice versa. Indeed, Aprea et al. (2017) reported a negative correlation between malic acid and the perceived sweetness of fruits. We could easily highlight the increase of sweetness sensation in apple after long-term cold storage than at harvest because of its significantly decrease in malic acid for more acid cultivars.

7.4.5. Principal components analysis

A principal component analysis (PCA) was carried out to appreciate how traits could influence on the 17 different accessions assessed after a long-term cold storage (Figure 7.4). The first two components, PC1 and PC2, accounted respectively for 46.2% and 23.8% of the total variability. Indeed, 70% of the total variance could be explained according to the first two components. Firstly, the PC1 loadings suggested that separation on this component was principally due to acids and sugars parameters, TA and SSC for the basic fruit quality traits and mostly all the antioxidant compounds. Moreover, the PC2 mostly contributed to the ripening index (RI), the succinic and shikimic acids and some sugars as fructose or glucose, but with less significance. Accessions on the positive side of PC1 corresponding mainly to triploid accessions, with presence of russeting, induced in general, higher values of sugars, organic acids and antioxidant biomolecules as previously described (Busatto et al., 2019; Wang et al., 2014). Indeed, only five accessions were on the positive side of PC1 and three were triploids: ‘Reineta Gris’, ‘Reineta Blanca del Canadá’, and ‘Bossost’. These three accessions showed the higher values for total acids, total sugars and TPC (Figures 7.1 and 7.2). However, most apple accessions exhibiting russeting are triploids (Busattto et al., 2019; Reig et al., 2015), and are less suitable in breeding programs, even though russeted apples could improve bioactive molecules in apple. In contrast, accessions on the negative side of PC1, corresponding to most of the well-known foreign cultivars such as ‘Granny Smith’, ‘Red Delicious’ or ‘Averdal’, showed, in general, lower values for those biochemical compounds (Mignard et al., 2021a).

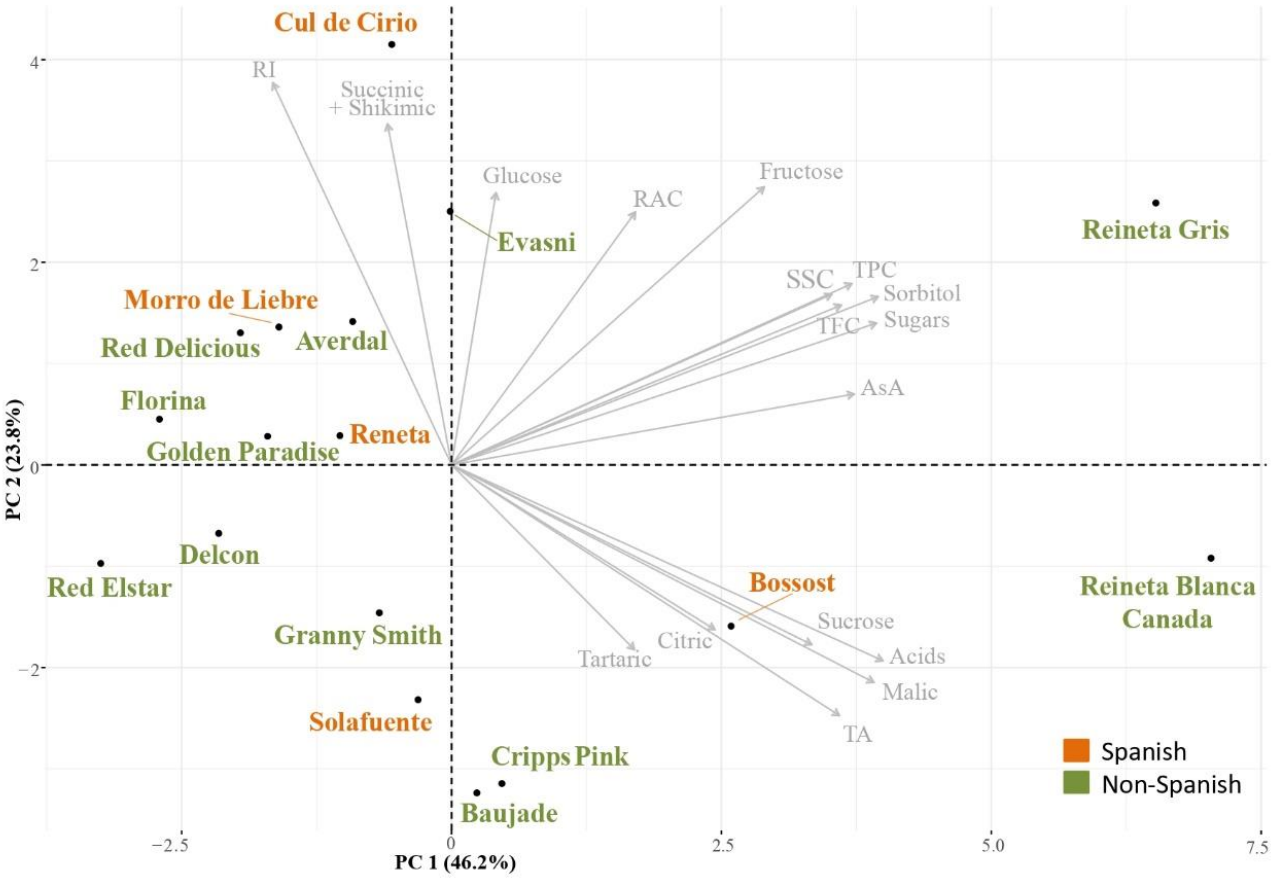

Figure 7.4. Bidimensional distribution (PCA) showing the relation among the evaluated traits on the 17 apple accessions after six months of cold storage (postharvest). Abbreviations: SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; RAC, relative antioxidant content; Sugars, total sugars; Acids, total organic acid.

The accession ‘Cul de Cirio’ stood out because of its high value for RI. Indeed, this local Spanish accession showed a value of 4.5 for RI at harvest date and increased to 8.1 after six months of cold storage (Figure 7.1). This increase was the major evolution that could be noticed in this study and we can see that the ‘Cul de Cirio’ accession maintained glucose, fructose and sorbitol values high after cold storage, while tartaric, citric and malic acids values were low. This means ‘Cul de Cirio’ could be a good accession for its marketability due to its low acidity and high sweetness after six months of storage. A panellists group should confirm the sensorial acceptance of this accession (Symoneaux et al., 2012).

The observed values for the different fruit quality traits, antioxidants and both individual sugars and organic acids studied for 'Morro de Liebre' and 'Reneta' accessions, both originated from Spain, were similar to the well-known commercial cultivars or cultivars resulting from breeding programs such as 'Averdal', 'Delcon', 'Florina', 'Golden Paradise', 'Red Delicious' or 'Red Elstar'.

7.4.6. Visual symptoms for susceptibility to cold storage and relationship with fruit quality traits

The susceptibility of the 17 accessions of this study to present chilling injury symptoms such as browning (skin or pulp) or dehydration of the fruits during long-term storage was presented in the Table 7.3 and the Figures 7.5 and 7.6.

Table 7.3. Susceptibility for the 17 apple accessions assessed following six months of cold storage.

Accession	N	General Appearance	Browning	Dehydration	Conservation
Averdal	1	Bad	2	2	Unsuitable
Baujade	2	Good	1	1	Suitable
Bossost - MRF 76	3	Bad	2	2	Unsuitable
Cripps Pink	4	Good	1	1	Suitable
Cul de Cirio - MRF 39	5	Good	2	1	Unsuitable
Delcon	6	Good	1	1	Suitable
Evasni - Scarlet Spur	7	Good	1	1	Suitable
Florina	8	Good	1	1	Suitable
Golden Paradise	9	Good	2	1	Unsuitable
Granny Smith	10	Good	1	1	Suitable
Morro de Liebre	11	Good	2	1	Unsuitable
Red Delicious	12	Good	1	1	Suitable
Red Elstar	13	Bad	2	1	Unsuitable
Reineta Blanca del Canadá	14	Bad	2	2	Unsuitable
Reineta Gris	15	Bad	2	2	Unsuitable
Reneta	16	Good	1	1	Suitable
Solafuente	17	Good	1	1	Suitable

1: inexistent; 2: presence of chilling injury symptoms.

After six months of conservation, nine accessions (‘Baujade’, ‘Cripps Pink’, ‘Delcon’, ‘Evasni’, ‘Florina’, ‘Granny Smith’, ‘Red Delicious’, ‘Reneta’, and ‘Solafuente’) showed no apparent cold damage (Table 7.3, Figure 7.5). Out of these nine accessions characterized as suitable for long-term conservation, two were Spanish accessions (‘Reneta’ and ‘Solafuente’). The other seven non-Spanish accessions were all well-known cultivars in the market and/or exhibiting similar characteristics among them (‘Baujade’, ‘Cripps Pink’, ‘Delcon’, ‘Evasni - Scarlet Spur’, ‘Florina’, ‘Granny Smith’ and ‘Red Delicious’). These accessions, as a result of long time of breeding and selection, confirmed their suitability to permit the marketability of apple all year round.

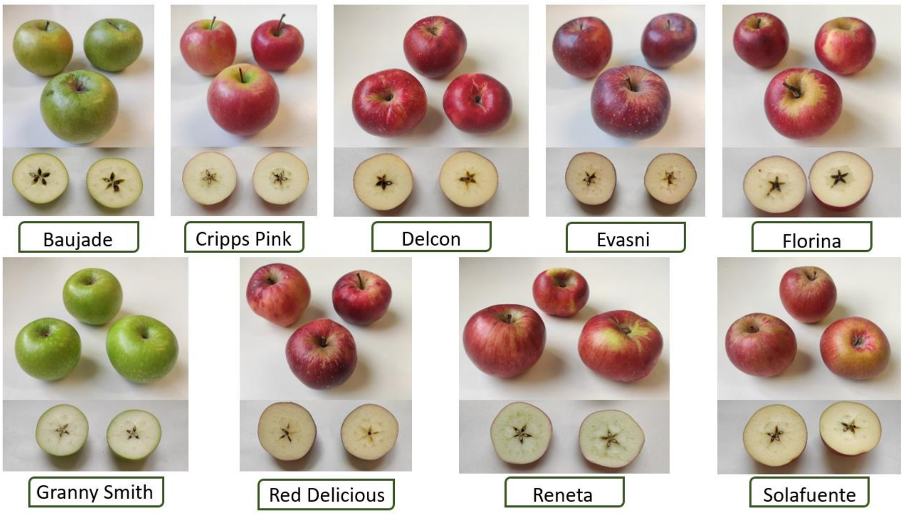

Figure 7.5. The nine suitable accessions for long-term cold storage assessed in this study.

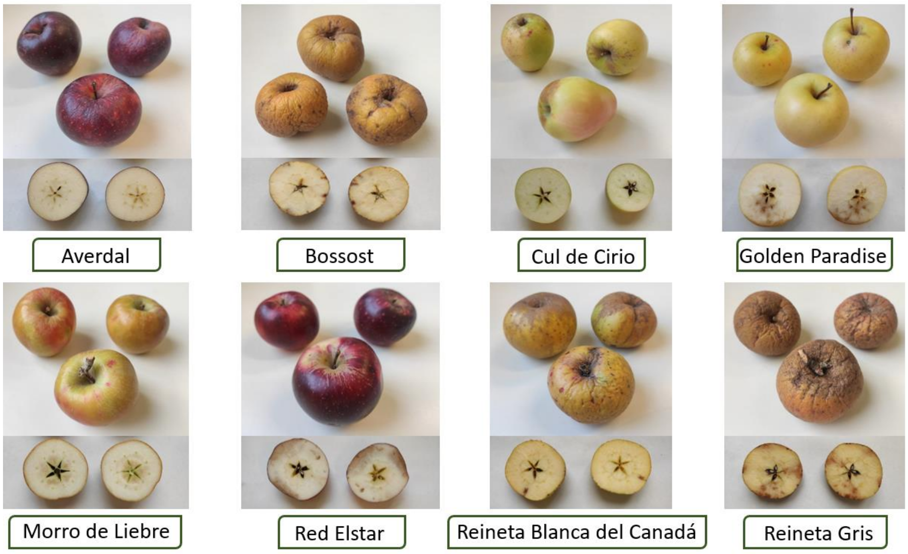

Figure 7.6. The eight unsuitable accessions for long-term cold storage studied.

Among the accessions assessed, eight (‘Averdal’, ‘Bossost’, ‘Cul de Cirio’, ‘Golden Paradise’, ‘Morro de Liebre’, ‘Red Elstar’, ‘Reineta Blanca del Canadá’, and ‘Reineta Gris’) were classified as unsuitable for long-term cold storage because of browning and/or dehydration after six months of cold storage (Table 7.3, Figure 7.6). The modern organization for fruit distribution and marketability aim to guarantee fruit quality also during storage, allowing high quality standards to be maintained from the orchard to the consumer (Di Guardo et al., 2013). Indeed, browning is associated with a lower fruit quality and freshness, and major deterioration. Its prevention by selecting tolerant cultivars, has taken a great effort in horticultural and food research for years (Cebulj et al., 2021). It is well known that the browning is caused by oxidation of the phenolics compounds in damaged cells (Cebulj et al., 2021). Browning could thus occurr by improper handling or processing, cutting, peeling or grinding. Nevertheless, cells could also be damaged by cold storage (Cebulj et al., 2021; Rasouli & Saba, 2018). The phenolics, when the cell is damaged enter in contact with the polyphenol oxidase enzyme (PPO) resulting into an oxidized phenol causing the appearing browning. In fact, in intact cells, PPO seems to have little activity towards phenolics compounds. PPO interacts with phenolics as substrates (Rasouli & Saba, 2018).

Table 7.4 showed the means for the different traits assessed in this study when the accessions were affected by browning or not. A T-test was thus carried out in the aim to know if the differences observed between sound accessions and the affected accessions by browning were significant. Firstly, the differences were significant for the main antioxidant compounds responsible for browning (Al-abbasy et al., 2021; Moon et al., 2020; Singh et al., 2018). Indeed, phenolic compounds can protect the fruit from oxidative stress at low temperatures, but they can also be responsible for greater browning (Singh et al., 2018). TPC, TFC, AsA and RAC means were higher in accessions exhibiting browning than in sound accessions. Moreover, Khanizadeh et al. (2006) described that the lack of substrate for PPO enzyme may be the cause of non-browning apples. Indeed, in other species, it has been reported that the more phenolics content the more PPO activity could be found and thus, more browning would be observed (Khan et al., 2021; Singh et al., 2018).

There were also significant differences between groups for fructose and sorbitol. Nonetheless, the accessions exhibiting higher levels of fructose and sorbitol as well as antioxidants compounds, were the russeted apples. The accessions with russeting (‘Bossost’, ‘Reineta Blanca del Canadá’ and ‘Reineta Gris’), visually appeared to be more dehydrated than the others. Busatto et al. (2019) reported that russeting is a genetic-controlled disorder resulting from the periderm coat consisting of a network of suberized cells straight above the skin. This disorder shows up as a brown and rough matrix deposition. Dehydration should be easier for a russeted apple due to this periderm disorder and the damage in cells making the possible contact between antioxidants and PPO enzyme causing browning (Khan et al., 2021; Moon et al., 2020; Singh et al., 2018). Furthermore, dehydration could explain the increase in bioactive compounds as antioxidants, sugars, sorbitol or tartaric acid (Figures 7.1 and 7.2) by their concentration (Quiles et al., 2005).

Table 7.4. Means for both groups, sound fruits or affected by browning apple.

Trait	Sound apple	Browning	Significance
SSC	13.1	14.3	ns
TA	6.5	7.1	ns
RI	2.5	2.6	ns
TPC	27.5	39.4	*
TFC	13.6	20.0	*
AsA	2.4	2.9	*
RAC	11.8	16.2	*
Sucrose	30.6	29.9	ns
Glucose	11.8	13.3	ns
Fructose	41.6	47.9	*
Sorbitol	3.2	6.3	*
Sugars	87.3	97.0	ns
Citric	0.05	0.05	ns
Tartaric	0.04	0.04	ns
Malic	5.55	5.82	ns
Succ + Shi	0.39	0.51	ns
Acids	6.03	6.43	ns

Statistical significance at *: P ≤ 0.05; ns: no significant. SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total acid; flavonoids content; AsA, Ascorbic RAC, relative antioxidant content; Sugars, total sugars; Acids, total organic acid; Succ+Shi, succinic + shikimic.

7.5. Conclusions

The quality traits of fruit (levels of sugars, organic acids and antioxidants) of the accessions studied showed a great diversity and different performances after the postharvest period. This work showed the importance of the biochemical quality in postharvest and the visual quality (dehydration or browning). Russeted accessions (‘Bossost’, ‘Reineta Blanca del Canadá’ and ‘Reineta Gris’), all triploid accessions, showed more chilling injuries (browning and dehydration) than the other accessions. Additionally, ‘Reneta’ and ‘Solafuente’ were the Spanish accessions best conserved based on the visual symptoms and fruit quality, while ‘Evasni’ seemed to be the less affected by long-term cold storage in the antioxidant profile. These results highlighted the importance of the autochthone accessions (‘Reneta’ and ‘Solafuente’) for its use in future breeding programs. Moreover, other non-Spanish cultivars, resulting from long time selection processes, as ‘Baujade’, ‘Cripps Pink’, ‘Delcon’, ‘Evasni’, ‘Florina’, ‘Granny Smith’, and the ‘Red Delicious’, confirmed their suitability for long-term storage periods with no chilling injuries after six months of cold storage.

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Capítulo 8

Discusión general

La manzana (*Malus x domestica* Borkh) es una de las frutas más consumidas en el mundo y de las más producidas en climas templados. Sin embargo, los problemas de calidad del fruto, mala adaptación en condiciones limitantes del cultivo y menor aceptación del consumidor en las últimas décadas, para algunas variedades autóctonas, han llevado al arranque de plantaciones y, por ello, a una creciente dependencia de las importaciones en España (Iglesias et al., 2016). El genotipo de las variedades es el que determina principalmente la calidad de la fruta que llega al consumidor, pero dicha calidad se ve también muy influida por otros factores. Entre ellos, los factores climáticos del área de cultivo (radiación solar, precipitación, temperaturas, altitud) (Iglesias et al., 2018; Mignard et al., 2021; 2022) y agronómicos (patrón utilizado, marco e intensidad de plantación, riego) (Donahue et al., 2021; Reig et al., 2018; Slatnar et al., 2019), la influencia de fisiopatías (mancha amarga o 'bitter pit', vitrescencia, etc.) (Cebulj et al., 2021; Melado-Herreros et al., 2013) y la conservación en postcosecha (Kevers et al., 2011; Singh et al., 2018) pueden afectar a la calidad organoléptica y nutricional de la fruta.

En esta tesis se han estudiado un total de 186 variedades de manzano. De ellas, 94 son accesiones autóctonas españolas y 92 son variedades extranjeras, algunas utilizadas como referencia. Todas ellas están incluidas en la colección de germoplasma de manzano de la Estación Experimental de Aula Dei (EEAD - CSIC). Fue establecida en condiciones típicas del Valle Medio del Ebro, considerada como una región vulnerable para el cultivo del manzano (Beguería et al., 2003; Reig et al., 2015), debido a las condiciones edafoclimáticas y sobre todo a los factores limitantes que se ven más acentuados en las últimas décadas, en el marco del cambio climático (Parajuli et al., 2019; Parry, 2019).

Se ha llevado a cabo la caracterización fenotípica (morfológica, pomológica y bioquímica) de las accesiones estudiadas y se ha evaluado la influencia de los factores climáticos antes mencionados, de una fisiopatía (vitrescencia) y conservación en postcosecha en cámara frigorífica (seis meses). Para evaluar la calidad organoléptica y nutricional de la fruta, se determinaron parámetros como el contenido en sólidos solubles y acidez, el índice de madurez, los compuestos antioxidantes y capacidad antioxidante relativa, los azúcares individuales y los ácidos orgánicos mayoritarios. Por otra parte, se ha estudiado la estructura poblacional y el desequilibrio de ligamiento de la colección de manzano, mediante 23 marcadores del tipo SSRs. También se ha llevado a cabo un estudio genético por mapeo de asociación con los caracteres fenotípicos previamente evaluados.

Evaluación de parámetros de calidad organoléptica y nutricional de la fruta e influencia de diferentes factores climáticos

Los resultados obtenidos tras la evaluación de 155 accesiones de manzana (99 autóctonas españolas y 56 cultivares extranjeros) en el período de cinco años (2014-2018), demostraron una gran diversidad fenotípica para los parámetros de la calidad básica del fruto (acidez valorable, contenido en sólidos solubles e índice de madurez), compuestos bioquímicos (fenoles y flavonoides totales, vitamina C, azúcares individuales y ácidos orgánicos mayoritarios), así como para la capacidad antioxidante relativa. Además, las diferencias entre accesiones y años de cosecha, indicaron que tanto el genotipo de la accesión como el año, presentaban efectos significativos sobre los parámetros evaluados. Este efecto también se demostró en otras colecciones de germoplasma de frutales como melocotonero y nectarina (Font i Forcada et al., 2014, 2019), albaricoquero (Gómez-Martínez et al., 2021) y manzano (Castel et al., 2020; Reig et al., 2015).

En el presente trabajo, el rango de los parámetros de calidad determinados varió en mayor medida que en otros estudios debido al mayor número de accesiones estudiadas. No obstante, los valores encontrados estuvieron dentro de los descritos para los parámetros de calidad básica del fruto (Guan et al., 2015; Slatnar et al., 2019), de los compuestos antioxidantes (Boyer y Liu, 2004; Raudone et al., 2017; Yuri et al., 2009), así como de los azúcares individuales y ácidos orgánicos mayoritarios (Aprea et al., 2017; Bureau et al., 2012; Castel et al., 2020; Yang et al., 2021). Como es bien sabido, el azúcar más abundante en manzana es la fructosa, seguida de la sacarosa, la glucosa y el sorbitol (Aprea et al., 2017; Yang et al., 2021). Igualmente, el ácido málico es el principal ácido orgánico en manzana, con valores que oscilaron entre 10 y 100 veces mayor que algunos de los restantes ácidos orgánicos (Bureau et al., 2012; Mignard et al., 2022).

Las accesiones españolas tendieron a mostrar, en general, mayor contenido en sólidos solubles, acidez total y otros compuestos bioquímicos como antioxidantes, sorbitol, y ácidos orgánicos, comparados con los cultivares extranjeros de referencia. El mayor contenido en compuestos antioxidantes de las accesiones españolas es probablemente debido a su mejor adaptación a las condiciones agroclimáticas de la zona de cultivo (Beguería et al., 2003; Reig et al., 2015). No obstante, las variedades comerciales mostraron, en general, unos valores de acidez más equilibrada con los valores de azúcares o de sólidos solubles, garantizando así una sensación de dulzor que facilita la mayor aceptación del consumidor, como se menciona en otros estudios (Jakobek et al., 2020; Musacchi et al., 2018). Las accesiones locales españolas, como la ‘Camuesa Fina de Aragón’, ‘Prau Riu – 5’ y ‘Transparente’, mostraron unos rangos con valores más amplios, en cuanto al contenido de algunos compuestos de calidad básica del fruto, como para los sólidos solubles o la acidez total. Además, las accesiones locales ‘Camuesa Fina de Aragón’, ‘Les_1’, ‘Pero Pardo’, ‘Prau Riu_5’, ‘Transparente Blanca’ y ‘Transparente’, destacaron por sus mayores contenidos en fenoles (TPC) y flavonoides totales (TFC) y capacidad antioxidante relativa (RAC), con respecto a cultivares extranjeros como ‘Akane’, ‘Deljeni’, ‘Delorgue Festival’, ‘Granny Smith’, ‘Red Rome Beauty’, ‘Reineta Blanca del Canadá’ y ‘Reineta Gris’, que presentaron valores más bajos que las accesiones autóctonas antes citadas. Muchos estudios destacan a los compuestos antioxidantes de la fruta por encontrarse entre las biomoléculas más beneficiosas para la salud del consumidor (Boeing et al., 2012; Boyer et al., 2004; Lattanzio, 2013), y por ello, la importancia de caracterizar la calidad nutricional en los bancos de germoplasma de frutales existentes (Swarup et al., 2021). En las variedades resultantes de los programas de mejora genética, el sabor, el dulzor, el aroma y la textura de la fruta, se vieron más destacados en el proceso de selección, comparado con su calidad nutricional y su contenido en compuestos bioactivos como el de algunos antioxidantes (Gómez-Martínez et al., 2021; Preti y Tarola, 2020).

Por otra parte, cabe destacar las accesiones triploides con presencia de ‘russeting’ o rugosidad en su piel, como las variedades ‘Bossost_2’, ‘Reineta Gris’, ‘Reineta Blanca Canadá’ y ‘Terrera’, que en este trabajo, mostraron mayor contenido en compuestos antioxidantes, azúcares y ácidos orgánicos. Busatto et al. (2019) ya destacaron que el ‘russeting’ en la piel de la manzana aumentaba sus perfiles de antioxidantes como el de los fenoles totales. Sin embargo, el ‘russeting’ se considera, en general, un rasgo negativo en determinadas variedades y no es apreciado por los consumidores, con la excepción de algunas variedades concretas como las del grupo ‘Reineta’ (Wang et al., 2014). Mediante el uso de algunas variedades con presencia de ‘russeting’, se podría aumentar los perfiles en compuestos fenólicos, azúcares y ácidos orgánicos y, por lo tanto, la calidad organoléptica y nutricional de las variedades resultantes de los programas de mejora genética del manzano. Sin embargo, también hay que tener en cuenta las correlaciones entre algunos compuestos bioquímicos, como los fenoles y su mayor potencial de pardeamiento en rutas metabólicas, por lo que deberían considerarse en la selección de nuevas variedades. Los ácidos orgánicos son degradados vía su decarboxilación y, después de la producción de fosfoenolpiruvato (PEP) vinculado a la activación de la gluconeogénesis que, a su vez, sintetiza carbohidratos como puede ser la glucosa (Vallarino y Osorio, 2019). Por otra parte, las correlaciones positivas entre TPC, TFC y RAC que se encontraron en este trabajo también se han mencionado en otros trabajos (Preti y Tarola, 2021; Raudone et al., 2017; Wang et al., 2015). Asimismo, los flavonoides son un grupo importante de los compuestos fenólicos en las manzanas y, por ello, ambos contribuyen significativamente a la capacidad antioxidante relativa de los frutos (Bui et al., 2019; Lattanzio, 2013).

Por otra parte, el análisis de componentes principales mostró una mayor variabilidad entre las accesiones autóctonas españolas, y con respecto a las accesiones extranjeras. Los cultivares resultantes de programas de mejora genética se vieron, en general, más estrechamente relacionados entre sí para los parámetros fenotípicos estudiados. Sin embargo, algunos cultivares extranjeros, como ‘Fuji’ y ‘Astrakan Red’, destacaron separándose de la tendencia de agrupación anterior, debido a su mayor contenido en azúcares individuales (‘Fuji’) y en ácidos orgánicos y compuestos antioxidantes (‘Astrakan Red’). El cultivar ‘Fuji’ es el resultado de un cruzamiento entre dos variedades americanas (‘Red Delicious’ y ‘Ralls Janet’) (Mandal et al., 2021).

Con el análisis fenotípico realizado, se relacionaron los perfiles de calidad básica del fruto, compuestos bioactivos, capacidad antioxidante relativa, azúcares individuales y ácidos orgánicos mayoritarios con las condiciones climáticas de la zona de cultivo. El Valle del Ebro se caracteriza por tener un clima árido o semiárido con veranos cálidos, alta radiación, temperaturas cálidas de día y noche y muy baja precipitación anual (Beguería et al., 2003; Reig et al., 2015; Salvador et al., 2011). Se sabe que los factores climáticos, también influyen directamente en la fisiología y desarrollo de la fruta y en su calidad, así como en el contenido en antioxidantes, azúcares y ácidos orgánicos, y los perfiles de metabolitos resultantes (Gitea et al., 2019; Iglesias et al., 2018; Stewart et al., 2020). En el presente trabajo, el análisis de regresión realizado con modelos de efectos mixtos, permitió determinar la mayor influencia de algunos factores climáticos sobre los parámetros fenotípicos previamente estudiados para 155 accesiones y varias cosechas consecutivas en el período de cinco años considerado. Además, se hicieron distintos análisis de agrupamiento para ordenar las accesiones evaluadas según la influencia de los factores climáticos estudiados. La segregación entre accesiones mediante este análisis de agrupamiento basado en los coeficientes del análisis de regresión con modelos de efectos mixtos, reforzó las similitudes entre los cultivares extranjeros de referencia y sus diferencias con respecto a las accesiones autóctonas españolas. En efecto, en los diferentes análisis de agrupamiento se observó que las variedades extranjeras se agrupaban más entre ellas, mostrando contenidos más similares en los parámetros fenotípicos estudiados, mientras que las accesiones locales mostraban mayores rangos en sus perfiles metabólicos (Mignard et al., 2021; 2022).

También se determinó que la radiación solar y las temperaturas afectaron en mayor medida a los contenidos en antioxidantes, azúcares y ácidos orgánicos (Mignard et al., 2021; 2022). En este estudio, los compuestos bioactivos (fenoles y flavonoides totales) tendieron a disminuir, en general, con temperaturas más elevadas, mientras que aumentaban con una mayor precipitación y radiación solar (Mignard et al., 2021). En otros trabajos también se ha visto la influencia de factores climáticos como la radiación solar sobre la textura y el sabor de la fruta (Fischer et al., 2016).

Los fenoles totales están entre los principales compuestos que contribuyen a proteger los frutos (Lattanzio, 2013) de los daños causados por los rayos ultravioleta de la luz solar y su acumulación podría estar relacionada con esta función foto-protectora (Li et al., 2013). En el caso de la capacidad antioxidante relativa, hay que destacar que la radiación solar influyó positivamente sobre los valores para las accesiones locales, pero negativamente en el caso de los cultivares extranjeros, probablemente debido a la mejor adaptación de las variedades autóctonas españolas, frente a los factores climáticos predominantes en la región de cultivo. En el presente trabajo, se encontró una influencia significativa de las temperaturas más bajas y un mayor contenido en algunos compuestos bioquímicos, como el de los flavonoides y los fenoles totales. Lattanzio et al. (2001) ya destacaron que las temperaturas más bajas, durante el desarrollo del fruto, implicaban una estimulación de la actividad de la fenilalanina amonio-liasa (PAL) en la manzana, así como de otras enzimas importantes de la ruta metabólica de la biosíntesis de los compuestos fenólicos. Dichos compuestos fenólicos son metabolitos secundarios implicados en el desarrollo del fruto y de las plantas, y por ello, serían fundamentales para generar una respuesta bioquímica de adaptación frente a estreses bióticos y/o abióticos, como se ha visto en otros trabajos, tanto en órganos vegetativos o estructurales del árbol (Amri et al., 2021), como en fruto (McClure et al., 2019).

Asimismo, el metabolismo secundario (compuestos antioxidantes en el caso de este trabajo) está vinculado al metabolismo primario (azúcares solubles y ácidos orgánicos), ya que los productos de las rutas metabólicas primarias actúan como sustrato de las rutas secundarias (Santos-Sánchez et al., 2019). El aumento del contenido en fructosa podría incrementar la producción de eritrosa-4-fosfato que constituye, junto con el fosfoenolpiruvato (PEP), un sustrato para compuestos fenólicos a través de la vía del shikímico (Ibrahim y Jaafar, 2011). Además, el contenido en azúcares individuales (sacarosa, glucosa y fructosa) se vio, en general, negativamente correlacionado con la precipitación, pero aumentaba con el rango de temperaturas y la radiación solar. Sin embargo, el sorbitol aumentó con la precipitación y la radiación solar para la mayoría de las accesiones. Li et al. (2013) también observó que la piel de la manzana, en el lado más expuesta al sol, tenía un mayor contenido en sorbitol. Este azucar es el principal producto de la fotosíntesis en la familia de las rosáceas y tiene un papel importante en la osmoregulación en el fruto (Dietrich et al., 2007; Zhang et al., 2011). Así, los valores de sorbitol podrían estar relacionados con la adaptabilidad de las plantas a diferentes estreses, ligados a las condiciones climáticas como el frío o también a otros estreses, como el déficit en agua o la salinidad del suelo, a través de la regulación osmótica (Dietrich et al., 2007; Gao et al., 2005).

Esta influencia significativa de algunos factores climáticos, afectó más a las accesiones españolas comparado con las variedades extranjeras, demostrando su mejor adaptación a las condiciones del Valle del Ebro aumentando, por ejemplo, sus compuestos antioxidantes, para paliar al estrés oxidativo causado por algunos factores climáticos, como la radiación solar y las temperaturas altas. Además, esta mejor adaptabilidad muestra la importancia de los recursos fitogenéticos locales. Por todo ello, este trabajo enfatiza la importancia de la región geográfica donde se cultiva el árbol frutal.

Caracterización genética, estructura de población y asociación genética mediante microsatélites

El estudio genético de 186 accesiones de manzana mediante 23 marcadores moleculares de tipo microsatélites (Single Sequence Repeat - SSR), mostraron una gran diversidad entre accesiones como ya se refiere en trabajos anteriores para distintas colecciones de manzano (Pereira-Lorenzo et al., 2017; Marconi et al., 2018; Urrestarazu et al., 2012; Zhen et al., 2018).

Se encontraron accesiones que compartían el mismo nombre pero con distintos alelos para algunos de los marcadores evaluados. Las accesiones etiquetadas como

‘Camosa-1’/‘Camosa-2’, ‘Reguard-2’/‘Reguard-4’, ‘Mañaga-1’/‘Mañaga-2’ o ‘Verde Doncella-1’/‘'Verde Doncella-3’ mostraron distintos perfiles alélicos para algunos de los 23 SSRs utilizados en el presente trabajo. Por el contrario, ‘Verde Doncella-2’ y ‘Verde Doncella-3’, tuvieron los mismos perfiles alélicos. Las caracterizaciones tanto fenotípicas como moleculares, con enfoques diferentes y complementarios, podrían determinar si las accesiones con los mismos perfiles de SSR son, efectivamente, la misma variedad.

Además, el estudio de caracterización genética, mediante los 23 SSRs, mostró una elevada diversidad genética y heterocigosis esperada/observada en el material vegetal evaluado, de acuerdo con la variabilidad observada en otros estudios para manzano (Bühlmann et al., 2015; Ferreira et al., 2016; Lassois et al., 2016; Liang et al., 2015; Patzak et al., 2012; Pereira-Lorenzo et al., 2017; Urrestarazu et al., 2012; Van Treuren et al., 2010). Asimismo, en el presente trabajo, el número promedio de alelos por locus (15,65) para las 186 accesiones de manzano, fue ligeramente menor al observado por Urrestarazu et al. (2012) evaluando 493 accesiones mediante 16 SSRs (16,69) y Pereira-Lorenzo et al. (2017) con 1453 accesiones mediante 13 SSRs (18,62), en colecciones españolas de manzano. Ambos estudios compartían 13 SSRs de los 23 utilizados en este trabajo. En otros países, también se encontró un mayor número promedio de alelos por locus. Bakır et al. (2022) indicaron un valor de 18 evaluando 206 accesiones de Turquía, mientras que Lassois et al. (2016), en Francia, observaron un valor medio igual a 19,5 en una colección de 2163 accesiones. No obstante, Marconi et al. (2018) evaluando 175 accesiones en Italia y Ferreira et al. (2016) estudiando 87 accesiones en Portugal, observaron valores inferiores (14,6 y 11,5 respectivamente).

La heterocigosidad observada promedio para las 186 accesiones evaluadas alcanzó un valor igual (0,75) al de Meland et al. (2022) en un estudio de 171 accesiones mediante siete SSRs compartidos con el presente estudio. Además, la heterocigosidad observada en el banco de germoplasma de la EEAD fue superior al observado por Ferreira et al. (2016) y ligeramente inferior a los valores mencionados por Marconi et al. (2018), Lassois et al. (2016), Bakır et al. (2022) y Urrestarazu et al. (2012). La explicación a estas diferencias podría encontrarse en los diferentes materiales vegetales evaluados, el nivel de ploidía y sobre todo el número de accesiones evaluadas.

Los estudios de estructura de población mostraron que para las dos poblaciones diploides estudiadas, la división en dos subpoblaciones principales (K = 2) era la más significativa. Pereira-Lorenzo et al. (2017) y Urrestarazu et al. (2012) también reportaron dos subpoblaciones en estudios similares, indicando una fuerte estructura de población.

Además, el análisis de agrupamiento por pares, según la distancia genética entre perfiles moleculares de las 118 accesiones diploides de manzana, agrupó los genotipos en dos clusters principales. El primero se dividía en cuatro subgrupos mientras que el segundo se dividía en dos. El análisis de agrupamiento, al igual que el estudio de estructura de población de 118 accesiones, mostró que uno de los dos clusters estaba formado casi exclusivamente por accesiones no españolas. En efecto, en este cluster se incluían 30 accesiones no españolas y tan solo había tres accesiones locales españolas (‘Biscarri-1’, ‘De Valdés’ y ‘Valsaina’).

El análisis de la estructura de población de 1453 accesiones de manzano, conservadas en las principales colecciones de germoplasma de España (incluida la colección de la EEAD – CSIC), permitió la discriminación de un extenso conjunto genético ibérico de accesiones de manzano, separándose de las no españolas (Pereira-Lorenzo et al., 2017). Además, los genotipos de los cultivares de referencia no españoles eran más similares entre ellos comparado con las accesiones españolas. Por otra parte, las colecciones europeas de germoplasma comparten gran parte del material vegetal de referencia no español conservado (Cornille et al., 2012; Jung et al., 2020; Marconi et al., 2018; Pereira-Lorenzo et al., 2017; Swarup et al., 2021; Urrestarazu et al., 2012; 2016).

Además, se observó un mayor contenido en antioxidantes y ácidos orgánicos, en general, en los grupos del análisis de agrupación, sobre todo en los formados por accesiones españolas locales. Una segregación similar a la encontrada en el presente estudio entre accesiones españolas y no españolas o de referencia, también fue observada al estudiar la influencia de los factores climáticos en la calidad de la fruta, compuestos antioxidantes, azúcares individuales y ácidos orgánicos para 155 accesiones de las 186 evaluadas en este estudio (Mignard et al., 2021; 2022). Además, las accesiones españolas mostraron mayor diversidad en los compuestos bioactivos y perfiles metabólicos, y fue menor para los cultivares no españoles. Parámetros y factores climáticos como la precipitación, la radiación solar y la temperatura influyeron también diferencialmente en los perfiles en antioxidantes, azúcares y ácidos orgánicos de las accesiones estudiadas.

Este trabajo es el primer estudio con SSRs en manzana para análisis de asociación con 21 parámetros agronómicos y bioquímicos evaluados en una colección de germoplasma. A pesar de ser la primera aproximación hasta la fecha entre parámetros bioquímicos y marcadores moleculares del tipo SSRs para dichos parámetros, se encontraron 126 asociaciones significativas entre los marcadores moleculares y los 21 parámetros fenotípicos de interés. Los estudios publicados disponibles analizaron exclusivamente parámetros organolépticos basados en marcadores moleculares del tipo SNPs (Gutiérrez et al., 2018; Jung et al., 2020; Larsen et al., 2017; McClure et al., 2018; 2019; Urrestarazu et al., 2017). Sin embargo, los marcadores SSRs multi-alélicos podrían ser más adecuados para detectar la estructura genética de población comparados con los marcadores bi-alélicos SNPs (Guichoux et al., 2011; Tsykun et al., 2017).

Zhen et al. (2018), en un estudio de 188 cultivares de manzana, encontraron asociaciones entre los marcadores moleculares denominados SWEETs y los azúcares individuales (sacarosa, glucosa, fructosa y sorbitol). En efecto, encontraron una correlación entre el marcador MdSWEET2e y la sacarosa, la fructosa y los azúcares totales. No obstante, en el presente estudio, no se correlacionaron estos parámetros fenotípicos con el marcador MdSWEET2e. Sin embargo, hay que destacar que el marcador MdSWEET9b mostró asociaciones significativas con el contenido en sorbitol, oxálico y quínico, y con la capacidad antioxidante relativa. En efecto, Zhen et al. (2018), que diseñaron estos marcadores moleculares (MdSWEETs), vieron asociaciones únicamente para los azucares individuales. En el presente estudio, se encontraron más asociaciones con dichos marcadores, al evaluar más parámetros fenotípicos.

Con la excepción de los MdSWEETs, los restantes marcadores SSRs del presente trabajo, no se han utilizado en estudios anteriores para el mapeo de asociación con parámetros bioquímicos. Sin embargo, se han encontrado loci de caracteres cuantitativos (Quantitative Trait Loci - QTLs) en relación con los 21 parámetros fenotípicos evaluados en el presente estudio. Estos resultados pueden ser muy útiles debido a que muchos de los marcadores asociados se encontraban en regiones comunes, donde los genes principales o QTLs ya se han identificado previamente en manzano (Chagné et al., 2012; Guan et al., 2015; Howard et al., 2019; Kenis et al., 2008; Kunihisa et al., 2014; McClure et al., 2019; Sun et al., 2015; Zhang & Han, 2021).

La fecha de cosecha, como parámetro agronómico, mostró estar relacionado con el marcador CH01f03b. Kenis et al. (2008) encontraron un QTL relacionado con la fecha de cosecha en el grupo de ligamiento 9 (LG9), donde se encontraba el marcador CH01f03b. El color de la piel del fruto se asoció con el marcador CH02c11 en el LG10 y con el marcador MdSWEET7a en el LG11. No obstante, Howard et al. (2019) encontraron QTLs en otros grupos de ligamiento con respecto al color de la piel.

También se encontró asociación significativa con el marcador CH02c06 en el LG2 para el contenido en sólidos solubles y la acidez valorable. En el mismo grupo de ligamiento, Guan et al. (2015), Kunihisa et al. (2014), y Zhang & Han (2021) también mencionaron un QTL para SSC.

Se encontraron asociaciones significativas con los antioxidantes evaluados en el presente trabajo (fenoles y flavonoides totales, vitamina C y capacidad antioxidante relativa). Chagné et al. (2012) y McClure et al. (2019) también mostraron asociaciones entre antioxidantes, fenoles y QTLs en varios grupos de ligamiento, de acuerdo con los observados en el presente estudio. En total, se encontraron 26 asociaciones significativas entre los compuestos antioxidantes y los marcadores SSRs. Además, el contenido en fenoles totales aumentó con una mayor radiación y baja temperatura (Mignard et al., 2021). Esta observación, en conjunto con las asociaciones genéticas de este trabajo, convierten estos resultados en información muy importante para los programas de mejora del manzano, teniendo en cuenta que los factores climáticos afectan la calidad de la fruta, siendo el Valle del Ebro una de las zonas más vulnerables para la producción de manzanas y también una de las regiones más afectadas por el cambio climático.

En cuanto a los azúcares individuales, la sacarosa se vió muy vinculada al marcador CH-Vf1 (LG1). Guan et al. (2015) y Sun et al. (2015) reportaron un QTL en el LG1 también con alta vinculación a la sacarosa. Además, el sorbitol mostró asociación con los marcadores CH02c06 (LG2), MdSWEET9b (LG4), CH01f02 (LG12), GD147 (LG13) y MdSWEET12a (LG14). En futuros trabajos, estas asociaciones podrían ser útiles para la evaluación y la caracterización de fisiopatías como la vitrescencia, dado el papel tan importante de algunos azucares en esta fisiopatía.

Se encontraron asociaciones significativas entre los marcadores CH04e05 (LG7), CH01h10 (LG8) y CH02c09 (LG15) y los ácidos orgánicos (oxálico, cítrico, succínico, shikimico y tartárico). Sun et al. (2015) mostraron varios QTLs en los mismos grupos de ligamiento donde se encontraban dichos marcadores SSRs vinculados a los ácidos orgánicos mayoritarios.

El presente estudio proporcionó nuevos detalles sobre la estructura de la población de manzano de la EEAD-CSIC, según el nivel de ploidía y origen de las accesiones (español/no español). El análisis de la estructura poblacional mostró dos subpoblaciones de acuerdo con la segregación también observada en estudios previos. Estos resultados destacan el conocimiento de la arquitectura genética de importantes parámetros agronómicos y bioquímicos de la fruta, debido a la correlación intrínseca entre la genética y los perfiles metabólicos de las accesiones estudiadas. Además, los resultados obtenidos proporcionaron una base sólida para un mejor mapeo por asociación con parámetros fenotípicos que podrían aplicarse en otras especies, debido a la sintenia encontrada dentro de la familia de las Rosaceae. Las asociaciones encontradas podrían ser utilizadas en programas de mejora genética de manzano mediante Selección Asistida por Marcadores (MAS). Este estudio se complementará con la búsqueda de genes candidatos relacionados con las asociaciones encontradas.

Estudio fenotípico y de la expresión diferencial de genes relacionados con la 'vitrescencia'

Los datos climáticos de la estación meteorológica situada al lado de la plantación de la colección de manzano, indican un clima considerado como árido o semiárido (Salvador et al., 2011). Las precipitaciones son extremadamente bajas y las temperaturas son muy altas en verano y bajas en invierno, y la variación de la temperatura diurna muy amplia. Además, el valle Medio del Ebro se considera una región vulnerable para el cultivo y producción de manzana (Reig et al., 2015). También, se sabe que los factores climáticos influyen en los perfiles metabólicos del fruto (fenoles y flavonoides totales, capacidad antioxidante relativa, azúcares individuales y ácidos orgánicos) (Mignard et al., 2021; 2022). Melado-Herreros et al. (2013) encontraron una vinculación significativa y positiva entre la vitrescencia y la radiación solar.

Se observó que los frutos con vitrescencia mostraban una mayor densidad del fruto, como ya se mencionó en estudios previos en manzana (Baranowski et al., 2008; Herremans et al., 2014). No obstante, considerando la última fecha de cosecha analizada (madurez comercial), el contenido en fenoles y flavonoides totales, así como la capacidad antioxidante relativa aumentó en las manzanas presentando vitrescencia en su pulpa, para las tres accesiones estudiadas ('Helada', 'Tempera', y 'Verde Doncella'), comparadas con frutos sanos de dichas variedades y de la variedad 'Gala', utilizada de referencia. Estos resultados estuvieron de acuerdo con los de Cebulj et al. (2021) que evaluaron compuestos fenólicos individuales para el cultivar 'Fuji' y observaron un mayor contenido en compuestos fenólicos especificos en los frutos vitrescentes. En general, tanto en frutos sanos como afectados, la tendencia observada en el presente trabajo para el contenido en fenoles y flavonoides totales y la capacidad antioxidante relativa fue de disminuir de acuerdo con la madurez de la fruta (Duda-Chodak et al., 2011).

Para las tres accesiones estudiadas ('Helada', 'Tempera', y 'Verde Doncella'), el contenido en sorbitol fue mucho mayor en los frutos con vitrescencia en comparación con los frutos sanos y con la variedad utilizada de referencia (‘Gala’) dado que la vitrescencia es un trastorno físico de la fruta, con acumulación típica de sorbitol en los espacios intercelulares del fruto (Cebulj et al., 2021; Gao et al., 2005; Melado-Herreros et al., 2013; Tanaka et al., 2020; Zupan et al., 2016).

Además, en el presente trabajo, el contenido en fructosa tendía, antes de su madurez comercial, a ser inferior en los frutos con vitrescencia comparado con frutos sanos, como lo observado en el cultivar ‘Esperiega’ por Melado-Herreros et al. (2013). Asimismo, la concentración en sacarosa era más alta en frutos con vitrescencia, como ya lo reportaron varios estudios en los cultivares ‘Delicious’, ‘Gloster’ y ‘Fuji’ (Bowen y Watkins, 1997; Zupan et al., 2016). Tanaka et al. (2020) también mostraron valores más altos de sacarosa para el cultivar ‘Fuji’, pero una disminución para otros cultivares como ‘Gloster’, ‘Delicious’ y ‘Esperiega’. Las diferencias en el contenido en sacarosa, entre frutos con vitrescencia y frutos sanos, estaban vinculadas a la madurez de la fruta. Por otra parte, el contenido en glucosa fue mayor en las frutas afectadas por la vitrescencia en comparación con las frutas sanas de las accesiones ‘Helada’ y ‘Tempera’, de acuerdo con Melado-Herreros et al. (2013) para la variedad ‘Esperiega’. Por el contrario, Zupan et al. (2016) observaron una tendencia a la disminución de la glucosa en dos cultivares (‘Delicious’ y ‘Fuji’).

En las rosáceas, los carbohidratos derivados de la fotosíntesis se transportan principalmente como sorbitol, vía los denominados transportadores *MdSOTs* (Wei et al., 2014). De acuerdo con los genes *MdSOT1*, *MdSOT2*, *MdSOT2.2* y *MdSOT5.3* la tendencia fue similar para las tres accesiones susceptibles a la vitrescencia (‘Helada’, ‘Tempera’ y ‘Verde Doncella’) y su expresión fue menor en los frutos afectados por la fisiopatía, coincidiendo con los resultados observados por Gao et al. (2005) y Loescher et al. (2005). Además, Gao et al. (2005) estudiaron la expresión genética de *MdSOT1* y *MdSOT2* e informaron que la disminución observada de la capacidad de transportar sorbitol a los tejidos del parénquima de almacenamiento de fruta conduciría a una acumulación de sorbitol en el espacio intercelular. Por lo contrario, para las tres accesiones (‘Helada’, ‘Tempera’ y ‘Verde Doncella’), la expresión relativa del gen *MdSOT5.2* fue mayor en las manzanas con vitrescencia que en los frutos no afectados utilizados como control.

La fenilalanina amonio liasa (PAL) es la principal enzima estudiada de las vías del metabolismo fenilpropanoide (Anwar et al., 2021; Cebulj et al., 2021). Está involucrada en un amplio espectro de resistencias o tolerancias frente a diferentes enfermedades y estreses bióticos o abióticos, así como en otros estreses, como la incompatibilidad de injerto variedad/patrón (Amri et al., 2021; Cebulj et al., 2021; You et al., 2020). Zhang et al. (2018) publicaron cinco isoformas de genes *MdPAL* en manzana. Entre ellos, el *MdPAL1* fue la isoforma *PAL* más expresada en los frutos y su expresión relativa fue significativamente mayor en los frutos con vitrescencia en la etapa final de madurez. El aumento en la expresión de *MdPAL1* así como, el aumento en la concentración de enzima fenilalanina amonio-liasa, podría ser un mecanismo de defensa como respuesta al estrés oxidativo causado por los desórdenes metabólicos asociados a la vitrescencia (Cebulj et al., 2021; Zupan et al., 2016).

La expresión génica relativa de las isoformas *MdPAL3/4/6/7* y *MdPAL5* mostró una tendencia similar a *MdPAL1* para todas las accesiones evaluadas. Además, la tendencia de la expresión relativa para los genes *MdPAL2* y *MdPAL8* parecía ser la opuesta a las citadas anteriormente y su expresión era menor en los frutos afectados por vitrescencia. Cebulj et al. (2021) reportaron resultados similares.

Los compuestos antioxidantes y las enzimas relacionadas contribuyen en gran medida a la calidad de las frutas y verduras (Lattanzio, 2013). Las principales enzimas responsables de la pérdida de calidad en las manzanas son la polifenol oxidasa (PPO) y la polifenol peroxidasa (POX), que podrían estar relacionadas con la degradación fenólica, durante la maduración de los frutos (Hutabarat y Halbwirth, 2019). En el presente trabajo no se han observado diferencias significativas entre frutos afectados o no para la expresión relativa de los genes *MdPPO* y *MdPOX*. Sin embargo, Zupan et al. (2016) observaron una mayor actividad de la enzima POX en las manzanas con vitrescencia.

Análisis en postcosecha tras seis meses de almacenamiento en cámara frigorífica

La evaluación de la calidad básica del fruto (contenido en sólidos solubles, acidez valorable e índice de madurez) y de diferentes compuestos bioactivos (antioxidantes, azúcares individuales y ácidos orgánicos mayoritarios) de 17 accesiones de manzana tras seis meses de almacenamiento en cámara frigorífica, a baja temperatura y con niveles de humedad controlada, mostró diferencias de comportamiento entre las accesiones evaluadas para todos los parámetros considerados.

En este trabajo, los parámetros de calidad básica del fruto en postcosecha variaron respecto al momento de la cosecha. Mientras que el contenido en sólidos solubles tendía a aumentar ligeramente en postcosecha (salvo para la variedad ‘Florina’), la acidez valorable tendía a disminuir para todas las accesiones. Por ello, el índice de madurez también aumentó. Como es previsible, los procesos metabólicos implicados en la maduración de los frutos continúan durante el almacenamiento (Ehsani-Moghaddam et al., 2013; Singh et al., 2018).

Respecto a los contenidos en compuestos antioxidantes, los fenoles disminuyeron significativamente (con la excepción del cultivar ‘Evasni - Scarlet Spur’) tras la conservación en postcosecha, como también se menciona en otros estudios (Alhaj Alali et al., 2020; Kevers et al., 2007). Además, los flavonoides totales disminuyeron para todos las variedades, con la excepción de ‘Cul de Cirio’, ‘Evasni’ y ‘Red Elstar’. Los compuestos fenólicos también pueden evolucionar durante el almacenamiento en frío. Kevers et al. (2011) observó que el contenido total en fenoles aumentaba a los tres meses de almacenamiento a bajas temperaturas, para disminuir posteriormente durante los meses siguientes. Los valores más bajos, observados en este trabajo a los seis meses, podrían responder a dicha tendencia durante un período de tiempo de conservación más largo. López et al. (1994) explicaron que la disminución de los fenoles, tras un almacenamiento de larga duración, podría ser el resultado de su oxidación y estar asociada al pardeamiento. Asimismo, la pérdida de ácido ascórbico, observada en el presente trabajo, también podría ser un indicador de estrés oxidativo durante el almacenamiento, como ya lo comentaron otros autores (Foyer & Noctor, 2005; Kevers et al., 2007). Así, en este estudio, los fenoles y flavonoides totales, la vitamina C y la capacidad antioxidante relativa mostraron mayores contenidos para las accesiones que presentaban mayor pardeamiento y deshidratación de la pulpa, sobre todo las que tenían ‘russeting’ como ‘Bossost’, ‘Reineta Blanca Canadá’ y ‘Reineta Gris’, ya que también eran las más afectadas por los daños por frío. En efecto, los compuestos fenólicos protegen la fruta de los estreses oxidativos causados por bajas temperaturas, pero también son responsables de un mayor pardeamiento (Singh et al., 2018).

Por el contrario, la variedad ‘Evasni - Scarlet Spur’ parece tener un mejor comportamiento en su conservación a largo plazo, debido al aumento en fenoles, flavonoides y capacidad antioxidante relativa. En este caso, los fenoles también podrían sintetizarse como factores antioxidantes contra estreses abióticos durante la conservación en frio de la fruta (Foyer y Noctor, 2005). Sin embargo, los flavonoides, en general, no parecen verse afectados, con valores similares observados en cosecha y postcosecha. En efecto, los flavonoides y la capacidad antioxidante relativa (RAC) no mostraron un patrón general, dependiendo mucho más del genotipo de la accesión estudiada. Sin embargo, hay que destacar que las accesiones ‘Cripps Pink’, ‘Cul de Cirio’, ‘Evasni’, ‘Florina’, ‘Reineta Blanca Canadá’ y ‘Reineta Gris’ mantuvieron sus valores y mostraron niveles más estables en los compuestos antioxidantes totales (TPC, TFC y RAC). Es importante seleccionar cultivares cuyos niveles de compuestos bioactivos y por lo tanto, su calidad nutricional, se mantengan en postcosecha. No obstante, también debería estudiarse el perfil más específico de los diferentes compuestos fenólicos en futuros trabajos.

Los contenidos en azúcares totales y ácidos orgánicos mayoritarios en postcosecha y en el momento de la cosecha estuvieron dentro de los rangos publicados (Castel et al., 2020; Zhu et al., 2022). Sin embargo, los valores de azúcares totales variaron significativamente entre accesiones y respecto al momento del análisis (cosecha/postcoseha). Para las accesiones ‘Averdal’, ‘Evasni’, ‘Reineta Blanca Canadá’ y ‘Reineta Gris’, el total de azúcares en fruto aumentó respecto a los valores medios en cosecha, mientras que para ‘Baujade’, ‘Cul de Cirio’, ‘Delcon’, ‘Florina’, ‘Granny Smith’, ‘Red Elstar’ y ‘Reneta’, los azúcares totales tendieron a disminuir. Respecto a los azúcares individuales, cabe destacar el aumento del sorbitol para las accesiones ‘Reineta Blanca Canadá’ y ‘Reineta Gris’, las dos con ‘russeting’ en su piel. En cuanto a los ácidos orgánicos, los ácidos cítrico, tartárico, succínico y shikímico mostraron perfiles menos consistentes y su aumento o disminución se vió más influenciado según el genotipo de las accesiones. No obstante, el ácido málico, mayoritario en la manzana, y los ácidos totales, disminuyeron considerablemente en postcosecha, como se menciona en otros trabajos (Vallarino y Osorio, 2019; Zhu et al., 2022). Debido a la disminución de los ácidos y el aumento del contenido en sólidos solubles y de algunos azúcares individuales como la glucosa o el sorbitol durante el almacenamiento en frío, algunas accesiones como ‘Averdal’, ‘Bossost’, ‘Cul de Cirio’, ‘Delcon’, ‘Evasni’, ‘Golden Paradise’, ‘Granny Smith’, ‘Morro de Liebre’, ‘Red Delicious’ y ‘Reineta Gris’ podrían ser más atractivas para el consumidor y para su comercialización, tras un largo período de almacenamiento en frio.

Cabe destacar las tres accesiones triploides con presencia de russeting, ‘Reineta Gris’, ‘Reineta Blanca Canadá’ y ‘Bossost’, que mostraron, en general, los valores más altos para los ácidos orgánicos, azúcares individuales y los fenoles y flavonoides totales, como se menciona en otros estudios (Busatto et al., 2019; Wang et al., 2014). Otros cultivares extranjeros, como ‘Granny Smith’, ‘Red Delicious’ y ‘Averdal’, mostraron en general, valores más bajos para algunos compuestos bioquímicos (antioxidantes y ácidos orgánicos) en cosecha, como ya se describió en la bibliografía (Castel et al., 2020; Mignard et al., 2021; 2022). Además, la accesión española ‘Cul de Cirio’ destacó por mostrar la mayor evolución de índice de madurez tras su conservación, con valores más altos en glucosa, fructosa y sorbitol tras el almacenamiento, mientras que los ácidos tartárico, cítrico y málico disminuyeron en sus frutos. ‘Cul de Cirio’ podría, por todo ello, ser una accesión local española de interés para su comercialización debido a su menor acidez y mayor dulzor tras seis meses de almacenamiento. No obstante, se debería confirmar su aceptación sensorial mediante expertos panelistas, como se menciona en algunos casos para nuevas variedades (Gracia y Cantín, 2022; Symoneaux et al., 2012).

Además, la variedad ‘Cul de Cirio’, presentó algunos síntomas (bastante leves) de daños por frio. En efecto, ocho accesiones (‘Averdal’, ‘Bossost’, ‘Cul de Cirio’, ‘Golden Paradise’, ‘Morro de Liebre’, ‘Red Elstar’, ‘Reineta Blanca Canadá’ y ‘Reineta Gris’) fueron clasificadas como no aptas para largos períodos de conservación por presentar pardeamiento y/o deshidratación tras seis meses de almacenamiento. El pardeamiento en la pulpa de la fruta es asociado con una menor frescura de la fruta y deterioro de la misma (Di Guardo et al., 2013). Es una causa importante de pérdida de calidad y disminuye la aceptación por parte del consumidor. Este problema no solo reduce la calidad visual sino que también resulta en cambios indeseables en el sabor y el valor nutricional de los productos de manzana (Rasouli y Saba, 2018). Su prevención mediante selección de cultivares tolerantes, es un aspecto muy importante en la investigación alimentaria (Cebulj et al., 2021). El pardeamiento es causado por la oxidación de los compuestos fenólicos mediante enzimas como la fenilalanina amonio liasa (PAL), la polifenol oxidasa (PPO) y las peroxidasas (POX) (Al-Abbasy et al., 2021; Khan et al., 2021; Moon et al., 2020). El almacenamiento en frío también podría dañar las paredes celulares debido al estrés oxidativo generado (Cebulj et al., 2021; Rasouli y Saba, 2018) y así, permitir el contacto entre los fenoles con la enzima polifenol oxidasa (PPO), entre otras, y causar mayor pardeamiento. En efecto, la PPO interactúa con los fenoles como sustratos (Rasouli y Saba, 2018) y un mayor contenido en fenoles podría causar más pardeamiento (Khan et al., 2021; Singh et al., 2018). En efecto, enzimas como la PPO, la POX o la PAL catalizan la hidroxilación de monofenoles a o-difenilos y la oxidación de o-difenoles a o-quinonas altamente reactivas que pueden dar lugar a pigmentos oscuros, marrones o rojizos, también comúnmente llamados melaninas (Tomas-Barberán y Espin, 2001). Además, esta oxidación podría verse acentuada por problemas de manipulación y/o de procesamiento inadecuados (Singh et al., 2018).

Por otra parte, tras seis meses de conservación en frío, nueve accesiones (‘Baujade’, ‘Cripps Pink’, ‘Delcon’, ‘Evasni’, ‘Florina’, ‘Granny Smith’, ‘Red Delicious’, ‘Reneta’ y ‘Solafuente’) no mostraron síntomas visuales de daños por frío, es decir que no presentaban ni pardeamiento de la pulpa ni aparente deshidratación del fruto. Los cultivares resultantes de procesos de mejora y selección como ‘Cripps Pink’, ‘Evasni’, ‘Florina’, ‘Granny Smith’ y ‘Red Delicious’, confirmaron su mayor idoneidad para largos periodos de almacenamiento, con menor incidencia de daños por frio. Además, también hay que destacar que las accesiones españolas ‘Reneta’ y ‘Solafuente’ mostraron un comportamiento similar, con ausencia de síntomas visuales de daños por frio y buena calidad del fruto.

Este trabajo mostró la relación de los parámetros bioquímicos y visuales (deshidratación y/o pardeamiento) en postcosecha y la influencia del genotipo de las accesiones. Dos accesiones locales (‘Reneta’ y ‘Solafuente’) resaltaron la importancia de los recursos fitogenéticos autóctonos al comportarse como variedades comerciales y no presentar ningún síntoma de daños por frío y al conservar su calidad nutricional. La caracterización, para un mayor número de accesiones contribuirá a la selección de variedades con mejor comportamiento para su adecuada comercialización.

Este trabajo potenciará las variedades locales mejor adaptadas en el área de cultivo y aportará información y herramientas de trabajo de gran interés para su uso en los trabajos de selección y mejora genética de manzano.

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Capítulo 9

Conclusiones

1- El estudio de 155 accesiones de manzana del banco de germoplasma de la Estación Experimental de Aula Dei (EEAD – CSIC) en el período de cinco años (2014-2018) mostró una gran diversidad para los diferentes parámetros pomológicos (color de la piel y de la pulpa, forma y tamaño del fruto), los caracteres de calidad básica (contenido en sólidos solubles, acidez valorable e índice de madurez) y compuestos bioquímicos y bioactivos del fruto (antioxidantes, azúcares individuales y ácidos orgánicos mayoritarios). Se demuestra así la importancia de conservar estos recursos fitogenéticos y caracterizar esta variabilidad por su potencial interés en los programas de mejora genética.

2- El estudio fenotípico de 99 accesiones españolas y 56 variedades extranjeras mostró el gran interés de los recursos fitogenéticos autóctonos españoles, dado que, en general, mostraron un mayor contenido en compuestos bioactivos, y en algunos ácidos orgánicos individuales y acidez valorable. Las variedades autóctonas ‘Camuesa Fina de Aragón’ y ‘Urarte’ destacaron por su mayor contenido en compuestos antioxidantes y en ácidos orgánicos respectivamente.

3- Las accesiones con piel rugosa completamente recubierta de ‘russeting’ (‘Bossost_2’, ‘Reineta Gris’ y ‘Terrera’) o parcialmente cubierta (‘Reineta Blanca Canadá’) mostraron mayores valores para algunos parámetros de calidad básica del fruto, de antioxidantes, de azúcares individuales y de ácidos orgánicos mayoritarios. No obstante, el carácter triploide de estas variedades dificulta su uso en los programas de mejora genética, pese a su gran interés para la selección de variedades con mayor calidad organoléptica y nutricional.

4- Los factores climáticos influyeron significativamente en los perfiles de los diferentes metabolitos estudiados en el período de cinco años considerado. Los compuestos bioactivos (fenoles y flavonoides totales) tendieron a disminuir, en general, con temperaturas más altas, mientras que aumentaron con mayor precipitación y radiación solar. El contenido en azúcares, se vio inversamente relacionado con la precipitación y aumentó con el rango de temperaturas y la radiación solar, lo que indica la importancia de la región geográfica del cultivo y sus condiciones climáticas para optimizar la calidad organoléptica y nutricional del fruto.

5- Los estudios de caracterización genética y de estructura de población de 186 accesiones (150 diploides y 36 triploides), mediante el uso de 23 microsatélites (SSRs), determinaron dos subpoblaciones para las accesiones diploides y cuatro para las triploides. Además, el análisis de agrupamiento por pares, según la distancia genética entre perfiles moleculares, mostró una clasificación según su origen (español/no-español). Además, según dichos estudios se observó una gran diversidad que facilitará la diferenciación genética y fenotípica entre las accesiones autóctonas y los cultivares de referencia.

6- El estudio genético por mapeo de asociación para 118 accesiones evaluadas con 21 caracteres agronómicos y bioquímicos reveló un total de 126 asociaciones significativas. Las asociaciones con parámetros tales como fecha de cosecha, color de la piel, calidad básica del fruto, contenido en antioxidantes, en azúcares o en ácidos orgánicos, muestran el potencial interés de los marcadores utilizados (SSRs) para la selección asistida en los programas de mejora genética.

7- El estudio de tres variedades susceptibles (‘Helada’, ‘Tempera’ y ‘Verde Doncella’) frente a una fisiopatía denominada ‘vitrescencia’, mostró mayor densidad del fruto, mayor contenido en sorbitol y en compuestos antioxidantes en los frutos vitrescentes. La menor expresión diferencial de algunos genes relacionados con el transporte del sorbitol (*MdSOT1*, *MdSOT2*, *MdSOT2.2* y *MdSOT5.3*) se observó relacionada con la mayor acumulación de sorbitol en los frutos vitrescentes. Por otra parte, la mayor expresión de genes relacionados con los antioxidantes (*PAL*, *PPO* y *POX*) también estuvo relacionada con el mayor contenido de antioxidantes en las manzanas con vitrescencia.

8- El estudio de 17 accesiones de manzana en postcosecha mostró que tras seis meses de conservación en cámara frigorífica, los síntomas visuales de daños por frio (deshidratación y/o pardeamiento) y de calidad básica del fruto y compuestos bioquímicos (niveles de azúcares, ácidos orgánicos y antioxidantes) estaban relacionados. Las accesiones triploides con presencia de ‘russeting’ en su piel (‘Bossost’, ‘Reineta Blanca Canadá’ y ‘Reineta Gris’), presentaron más pardeamiento intenso y deshidratación del fruto, posiblemente debido a su mayor contenido en fenoles y flavonoides totales, y características de la piel corchosa causada por el ‘russeting’.

9- Los cultivares resultantes de procesos de mejora genética y selección, como ‘Baujade’, ‘Cripps Pink’ (‘Pink Lady’), ‘Delcon’, ‘Evasni’, ‘Florina’, ‘Granny Smith’, y ‘Red Delicious’, confirmaron su mayor idoneidad para su conservación en frio, dada la menor incidencia de los daños observados tras seis meses de almacenamiento en cámara frigorífica, con niveles de humedad controlados. No obstante, las accesiones españolas ‘Reneta’ y ‘Solafuente’ mostraron un comportamiento similar, con ausencia de síntomas visuales de daños por frio y una buena calidad del fruto tras su almacenamiento.

Capítulo 10

Anexos

10.1. Material suplementario correspondiente al capítulo 1

10.1.1. La manzana en la historia, la religión y el arte

10.2. Material suplementario correspondiente al capítulo 3

10.2.1. Supplementary file 1. Violin plots showing the variability between accessions (dots) and years of the harvest date and the climate parameters, computed between June 1st each year and the harvest day.

10.2.2. Supplementary File 2. ANOVA results for the effect of accession and year on the seven traits studied for the average of the five years of study.

10.2.3. Supplementary File 3. Mean for all years of study 2014-2018 of the 155 apple accessions for the seven different traits studied.

10.2.4. Supplementary File 4. Eigenvalues of the principal components analysis, and variance explained by each component.

10.2.5. Supplementary File 5. Eigenvectors and accumulative variance of the 5 principal components (PCs).

10.3. Material suplementario correspondiente al capítulo 4

10.3.1. Supplementary File 1. Basic information of the 155 apple accessions used on this study.

10.3.2. Supplementary File 2. Statistical significance of accession (A), year (Y), and their interaction (A x Y) on individual sugars and organic acids.

10.3.3. Supplementary File 3. Violin plots showing the variability between accessions (dots) and years of the sugars and organic acids parameters.

10.3.4. Supplementary File 4. Individual sugars and organic acids mean values (2014-2018) for the 155 apple accessions studied.

10.3.5. Supplementary File 5. Pearson's correlation coefficients between traits.

10.3.6. Supplementary File 6. Hierarchical clustering heatmap of Pearson correlation coefficients over basic quality parameters, biochemical antioxidant traits, sugars, and organic acids pairs (using Pearson distance, and average linkage).

10.3.7. Supplementary File 7. Eigenvalues of the ten principal components of the PCA, and variance explained by each component.

10.3.8. Supplementary File 8. Eigenvectors of the five principal components (PCs).

10.3.9. Supplementary File 9. Correlations between the different sugars and organic acids of the 155 accessions studied and the climatic traits.

10.3.10. Supplementary File 10. Mean values of the mixed-effects model coefficients (int, intercept; pre, precipitation; trg, temperature range; rad, solar radiation) for the different sugar traits. Results are shown for each cluster, according to the accession's origin, and according to the skin colour.

10.3.11. Supplementary File 11. Mean values of the mixed-effects model coefficients (int, intercept; pre, precipitation; trg, temperature range; rad, solar radiation) for the different organic acid traits. Results are shown for each cluster, according to the accession's origin, and according to the skin colour.

10.4. Material suplementario correspondiente al capítulo 7

10.4.1. Tabla S1. Pearson's correlation coefficients for the traits studied for the 17 accessions assessed in this study.

10.1. Material suplementario correspondiente al capítulo 1

10.1.1. LA MANZANA EN LA HISTORIA, LA RELIGIÓN Y EL ARTE

Las manzanas son las grandes protagonistas de nuestra historia. Se considera una fruta con mucha personalidad y carisma a lo largo de los siglos. Las manzanas nos han acompañado desde hace miles de años alimentando teorías revolucionarias e historias sorprendentes. Desde el origen del mundo, la literatura, el arte, la ciencia o las matemáticas, hicieron referencia a la manzana. Esta fruta inspiró a grandes personajes de la historia de muchos ámbitos. Así, se dice que el físico Isaac Newton (1643-1727) "descubrió su Sistema de Gravitación, cuyo primer indicio se lo dio el ver caer una manzana de un árbol" según John Conduitt su asistente personal. Las manzanas aparecen en muchas tradiciones religiosas y mitológicas, en general, como una fruta mística o prohibida. Por tanto, en la antigua Grecia la manzana era considerada sagrada según Afrodita. Tirar una manzana a alguien era como declararle de manera simbólica su amor. Atraparla era entonces aceptar este amor.

"Te tiro la manzana, y si estás dispuesta a amarme, tómala y comparte conmigo tu niñez; pero si tus pensamientos son lo que ruego que no sean, tómala y considera cuán efímera es la belleza." (Platón, 427-347 ac, Epigrama VII).

En la religión católica también la manzana tiene un papel importante. En efecto, en el Génesis, Dios les dice a Adán y Eva, que no deberán comer el fruto del "árbol del bien y del mal" que se encuentra en el Edén. No obstante, una serpiente engaña a Eva para que ella lo pruebe y le dé también de probar a Adán. Esto es conocido universalmente como el primer "pecado original" (Figura 1.1). Aunque no se identifica el fruto prohibido del Edén en el Libro del Génesis, la tradición cristiana popular ha sostenido que fue una manzana con la que Eva embaucó a Adán.

Figura 1.1. Adán y Eva, Pedro Rubens, 1628-1629. Copia de Tiziano, Museo del Prado.

A partir de allí, la manzana fue convertida en un símbolo para el conocimiento, la inmortalidad, la tentación y la caída del hombre en el pecado. Por ello, la laringe en la garganta se llama comúnmente la "manzana de Adán", en el sentido de que es porque la fruta prohibida, la manzana, se le quedó atascada en la garganta al probarla. También, como para mostrar esta imagen negativa de la manzana, Alan Turing, matemático y reconocido como precursor de la informática moderna, se quitó la vida en 1954 con una manzana envenenada con cianuro, tras ser condenado a castración química en un juicio por su homosexualidad. Según se rumorea, la manzana mordida de Apple®, marca creada por Steve Jobs, le haría tributo años después.

Los artistas y pintores se vieron influenciados por todas estas “historias de manzanas”. Dentro de los cuadros de pintura se encuentran a las naturalezas muertas de manzanas de Paul Cézanne (1839-1906). Hay una cantidad increíble de cuadros de manzana con la firma de Cézanne (Figura 1.2).

Figura 1.2. Ejemplos de cuadros de Paul Cézanne de naturaleza muerta con manzanas.

Muchos otros artistas se inspiraron de la manzana en sus obras. Se puede decir que la manzana era una de las frutas más presente en las naturalezas muertas. La podemos apreciar en el trabajo de famosos pintores como Ambrosius Bosschaert (Figura 1.3), Henri Fantin-Latour (Figura 1.4), René Magritte (Figura 1.5) o Claude Monet (Figura 1.6) entre muchos otros.

Figura 1.3. Ambrosius Bosschaert the Elder. *Peaches, apples, apricots and grapes*.

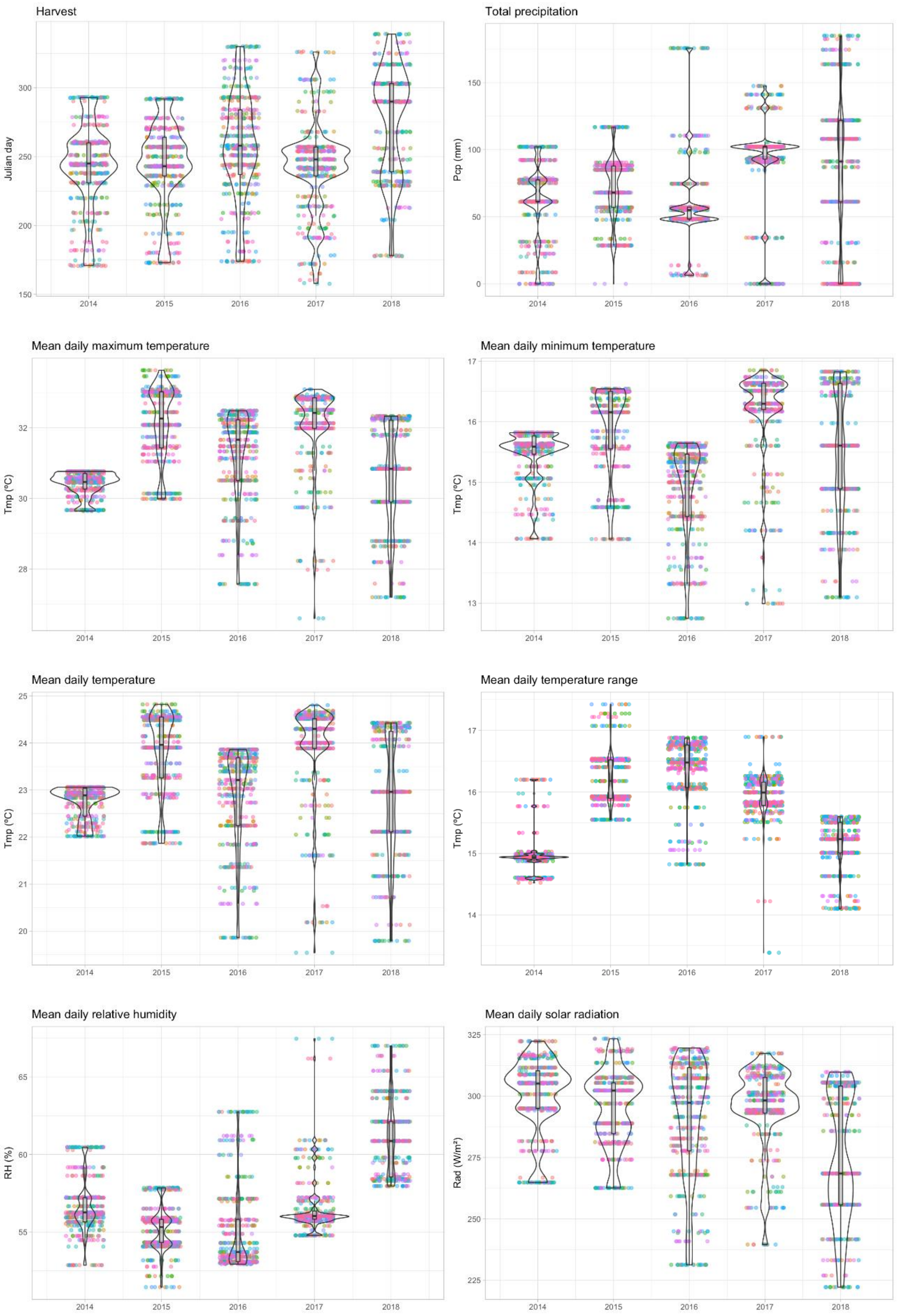
Figura 1.4. Henri Fantin-Latour. *The apples in the basket*, 1888.

Figura 1.5. René Magritte: *Ceci n'est pas une pomme*, 1954.

Figura 1.6. Claude Monet: *Manzanas y uvas*, 1879-1880.

10.2. Material suplementario correspondiente al capítulo 3

10.2.1. Supplementary File 1. Violin plots showing the variability between accessions (dots) and years of the harvest date and the climate parameters, computed between June 1st each year and the harvest day.



10.2.2. Supplementary File 2. ANOVA results for the effect of accession and year on the seven traits studied for the average of the five years of study.

Source of variation	**Units**	**Accession (A)**	**Year (Y)**	**A x Y**
SSC	ºBrix	***	***	***
TA	g malic acid L-1	***	***	***
RI	-	***	***	***
TPC	mg GAE 100 g FW-1	***	***	***
TFC	mg CE 100 g FW-1	***	***	***
AsA	mg AsA 100 g FW-1	***	***	***
RAC	mg Trolox 100 g FW-1	***	***	***

Data were evaluated by two-way variance (ANOVA); *** $P \leq 0.001$.

10.2.3. Supplementary File 3. Mean for all years of study (2014-2018) of the 155 apple accessions for the seven different traits studied.

Variety	Nº	SSC	TA	RI	TPC	TFC	AsA	RAC	Class.
Aciprés	1	16.1	2.8	5.9	35.1	16.6	2.4	18.0	Local
Akane	2	14.1	7.1	2.0	55.6	34.7	2.7	21.4	Foreign
Almenar-2 (MRF 46)	3	14.0	3.0	5.2	39.8	25.6	2.6	19.8	Local
Ascara 1	4	12.0	7.6	1.5	49.9	31.1	2.7	15.0	Local
Ascara 2	5	13.2	8.0	1.7	47.1	29.1	2.7	17.4	Local
Astrakan Red	6	12.3	12.2	1.1	74.2	55.0	2.7	26.4	Foreign
Audiena de Oroz	7	12.8	7.4	1.9	46.4	25.9	2.2	16.5	Local
Augüenta	8	12.8	6.9	1.9	51.4	32.5	2.8	19.2	Local
Averdal_1	9	12.3	4.0	3.3	27.4	6.7	2.8	13.2	Foreign
Averdal_2	10	12.9	3.1	4.1	17.7	13.1	2.0	7.8	Foreign
Baujade	11	14.0	9.5	1.6	34.5	17.2	3.0	13.5	Foreign
Bellaguarda Lardero (MSV 27)	12	10.1	11.6	0.9	61.9	43.9	4.6	22.5	Local
Belleza de Roma	13	14.3	5.9	2.5	31.8	17.0	2.5	13.5	Local
Biscarri-1 (M 107)	14	12.8	5.0	2.7	15.2	6.0	3.4	6.5	Local
Blackjon	15	13.6	7.4	1.9	27.9	16.4	2.5	10.7	Foreign
Bofla	16	13.2	9.1	1.5	34.9	19.4	2.8	12.2	Local
Boluaga	17	14.1	7.9	1.8	34.1	16.4	2.8	12.5	Local
Bossost-1 (MRF 75)	18	12.6	10.7	1.3	57.9	35.7	3.0	20.4	Local
Bossost-2 (MRF 76)	19	17.1	14.0	1.3	53.1	31.1	2.4	19.5	Local
Bossost-4 (MRF 78)	20	15.1	6.1	2.5	37.5	17.0	4.0	12.8	Local
Bossost-5 (MRF 79)	21	13.4	10.5	1.3	72.9	58.7	3.4	20.9	Local
Bost Kantoia	22	13.1	5.5	2.5	29.0	12.6	3.2	10.9	Local
Cabdellà-2 (MRF 49)	23	13.9	12.6	1.1	53.3	34.5	4.6	17.4	Local
Cabello de Angel	24	12.2	4.4	2.9	34.8	17.3	2.3	13.3	Local
Calvilla de San Salvador	25	12.6	8.9	1.4	33.0	18.0	4.8	13.4	Local
Camosa (MRF-42)	26	15.5	3.3	4.7	72.3	46.5	4.5	25.0	Local
Camosa (MRF-60)	27	12.5	5.9	2.4	46.8	30.9	3.7	21.5	Local
Camuesa de Daroca	28	12.5	2.5	5.9	40.4	21.6	3.2	20.1	Local
Camuesa de Llobregat	29	14.4	3.9	4.0	28.8	9.8	2.6	9.2	Local
Camuesa Fina de Aragón	30	10.6	12.3	0.9	98.1	89.0	3.4	29.5	Local
Carapanón	31	13.4	7.8	1.8	39.2	24.2	2.5	14.3	Local
Carrió	32	13.0	5.6	2.3	26.5	15.2	2.7	12.1	Local
Cella	33	14.8	2.9	5.5	40.0	22.7	2.4	19.0	Local
Ciri Blanc	34	13.4	3.3	4.1	38.7	15.2	2.2	17.9	Local
Cirio (MRF-52)	35	14.7	8.8	1.8	44.9	27.7	2.1	16.1	Local
Cox's Orange Pippin	36	14.8	9.1	1.7	33.1	17.2	4.3	12.1	Foreign
Cripps Pink	37	12.7	6.8	1.9	20.0	9.1	2.1	8.7	Foreign
Cuallarga	38	13.4	6.9	2.0	48.9	33.2	2.2	17.8	Local
Cul de Cirio (MRF 39)	39	14.4	3.3	4.5	40.8	15.4	3.1	21.2	Local
De Agosto (MRF 57)	40	12.7	6.9	2.0	54.8	32.3	3.2	22.2	Local
De Pera	41	17.0	10.5	1.7	40.9	19.7	2.7	15.7	Local
De Valdés	42	14.3	5.0	3.1	38.1	21.7	4.1	14.4	Local
Delciri	43	12.9	2.1	6.2	30.5	13.8	2.8	12.8	Local
Delcon	44	13.0	6.5	2.2	20.9	8.8	2.4	9.3	Foreign
Delgared Infel	45	12.6	4.4	2.9	18.6	7.8	1.4	5.9	Foreign
Deljeni	46	13.6	6.7	2.1	41.9	23.4	2.7	15.6	Foreign
Delkistar	47	12.3	6.6	1.9	23.3	8.4	2.7	9.6	Foreign
Delorgue Festival	48	12.7	4.7	2.7	41.4	23.4	2.2	15.3	Foreign
Elista	49	12.2	9.2	1.4	28.9	13.9	2.1	12.6	Foreign
Esperiega	50	13.4	8.5	1.7	37.9	20.8	2.3	13.9	Local
Esperiega de Olba (M 106)	51	14.2	2.8	5.6	43.9	21.7	3.1	20.6	Local
Eugenia	52	17.0	8.8	2.0	54.8	37.4	4.2	25.6	Local
Evasni (Scarlet Spur)	53	12.5	3.5	3.8	18.5	6.9	1.7	7.1	Foreign
Florina	54	14.2	6.9	2.4	30.5	18.4	2.4	14.2	Foreign
Fuji	55	16.9	4.2	4.1	25.5	9.9	2.7	10.5	Foreign

Variety	**Nº**	**SSC**	**TA**	**RI**	**TPC**	**TFC**	**AsA**	**RAC**	**Class.**
Gala	56	13.6	4.8	3.2	29.6	16.8	2.4	13.0	Foreign
Galaxy	57	12.2	3.9	3.2	21.7	8.2	1.7	11.2	Foreign
Golden Delicious 675	58	15.2	5.1	3.2	25.2	11.8	2.9	10.5	Local
Golden Delicious Infel 972	59	15.0	4.7	3.3	24.3	16.7	2.7	12.7	Foreign
Golden Paradise	60	13.5	4.2	3.5	25.5	10.7	2.5	9.3	Local
Golden Smoothee	61	15.1	4.9	3.1	33.1	15.4	3.9	11.2	Foreign
Granny Smith_1	62	12.3	10.3	1.3	34.4	20.3	2.8	13.0	Foreign
Granny Smith_2	63	12.5	10.0	1.4	37.1	20.6	3.1	14.1	Foreign
Guillemes	64	12.6	6.5	2.0	57.4	36.3	4.7	18.0	Local
Hared	65	12.5	4.0	3.5	25.9	10.1	2.4	13.5	Foreign
Helada	66	12.9	4.2	3.3	34.3	16.1	2.4	13.6	Local
Hierro	67	14.5	11.9	1.4	29.0	13.7	4.1	11.5	Local
Idared	68	12.2	7.5	1.8	26.7	12.1	1.9	11.6	Foreign
Irgo-2 (MRF 66)	69	13.2	4.7	2.9	35.2	17.0	3.7	14.0	Local
Jonadel	70	13.8	6.7	2.1	25.9	11.0	2.9	9.8	Foreign
Jonagored	71	13.5	6.1	2.5	28.0	12.2	2.2	12.5	Foreign
Jonathan_1	72	14.1	7.5	1.9	30.5	19.0	2.9	11.5	Foreign
Jonathan_2	73	14.8	7.9	1.9	29.1	13.6	2.8	11.3	Foreign
Jubilee	74	12.3	7.2	1.8	25.4	10.4	2.2	10.1	Foreign
Landetxo	75	12.9	12.2	1.1	70.5	51.1	3.6	26.3	Local
Les-1 (MRF 73)	76	13.0	4.3	3.0	84.6	72.8	3.0	30.8	Local
Les-2 (MRF 74)	77	13.4	6.9	2.0	49.2	38.7	2.5	18.0	Local
Magaña (MRF 43)	78	12.2	4.6	2.8	26.3	11.9	3.2	10.2	Local
Mañaga	79	12.5	5.5	2.5	30.5	16.1	5.2	12.5	Local
Marinera	80	10.7	8.8	1.3	61.5	46.1	2.1	20.7	Local
Marquinez	81	10.2	11.7	0.9	54.1	40.2	2.4	19.0	Local
McIntosh	82	13.8	6.8	2.1	31.3	17.6	1.9	12.6	Foreign
Médulas-1 (MSV 38)	83	14.4	7.5	2.1	41.5	22.7	3.3	15.1	Local
Melrose	84	12.9	6.7	2.0	26.5	15.3	2.0	11.2	Foreign
Merrigold	85	10.3	9.3	1.3	34.8	17.9	2.1	14.9	Foreign
Montcada-1 (MRF 82)	86	14.6	4.7	3.4	29.7	14.3	2.0	12.8	Local
Morro de Liebre	87	11.6	4.0	3.0	39.8	19.5	2.7	16.4	Local
Nesple	88	13.5	6.7	2.1	52.9	31.9	2.5	19.0	Local
Normanda	89	13.1	7.2	2.3	28.4	12.2	3.1	11.7	Local
Nueva Starking	90	14.1	3.0	4.9	20.8	9.4	1.7	9.8	Foreign
Ortell	91	13.8	4.7	3.3	25.1	8.4	2.3	10.1	Local
Ortell (MSV 24)	92	14.5	4.6	3.5	31.3	14.1	2.6	12.3	Local
Pera 2	93	17.0	10.3	1.6	37.4	20.4	4.3	13.9	Local
Pera de Sangüesa	94	14.9	7.4	2.1	42.4	22.1	2.3	16.0	Local
Pero Pardo	95	12.8	11.4	1.4	45.6	26.9	2.9	18.5	Local
Peromingan	96	14.8	8.0	2.0	47.5	34.8	2.7	21.1	Local
Peruco de Caparroso	97	12.3	2.9	4.6	59.8	47.2	2.2	28.1	Local
Plaona	98	11.7	4.4	2.7	25.5	11.5	1.8	11.4	Foreign
Poma de San Juan (MRF 47)	99	11.3	4.6	2.2	38.0	22.5	1.8	15.4	Local
Prau Riu 3	100	14.8	6.3	2.4	50.2	24.5	2.8	21.7	Local
Prau Riu 4	101	13.3	9.0	1.4	70.0	50.0	2.9	22.0	Local
Prau Riu 5	102	14.1	11.5	1.2	87.0	81.0	3.9	24.9	Local
Prima	103	11.1	7.9	1.4	20.4	8.4	2.0	11.0	Foreign
Rebellón	104	13.6	7.3	1.9	46.8	31.3	3.1	17.4	Local
Red Chief	105	13.2	3.1	4.4	21.6	9.3	2.4	11.2	Foreign
Red Delicious	106	13.7	3.6	4.1	21.7	8.9	1.8	10.6	Foreign
Red Elstar	107	13.3	7.3	1.9	25.7	13.2	2.7	10.6	Foreign
Red Rome Beauty	108	14.2	4.9	3.2	38.1	19.9	2.7	14.7	Foreign
Redaphough	109	12.7	9.6	1.4	29.2	12.3	2.1	11.6	Foreign
Regal Prince_1	110	12.9	3.7	3.6	23.7	9.8	2.0	13.2	Foreign
Regal Prince_2	111	13.3	3.7	3.7	31.0	14.9	2.0	10.6	Foreign
Reguard-1 (MRF 53)	112	15.1	4.8	3.3	50.3	30.0	5.9	24.9	Local
Reguard-2 (MRF 54)	113	13.2	17.3	0.8	40.9	13.6	3.0	11.0	Local

Variety	Nº	SSC	TA	RI	TPC	TFC	AsA	RAC	Class.
Reguard-4 (MRF 56)	114	13.2	4.5	3.0	35.3	20.7	2.0	13.8	Local
Reina de Reinetas	115	14.0	6.6	2.2	40.2	18.6	2.4	17.4	Foreign
Reineta Blanca Canada_1	116	15.7	10.0	1.6	45.6	30.3	3.3	18.5	Local
Reineta Blanca Canada_2	117	15.3	9.6	1.6	48.0	32.8	3.7	15.7	Foreign
Reineta Blanca Canada_3	118	14.2	9.8	1.5	53.1	31.3	2.9	16.3	Local
Reineta Encarnada	119	14.2	10.1	1.6	56.5	30.7	3.6	18.0	Local
Reineta Gris	120	16.6	9.7	1.7	57.0	32.9	3.6	20.7	Foreign
Reineta Inesita Asua	121	13.5	8.3	1.5	46.7	28.0	2.1	17.2	Local
Reineta Regil	122	17.2	9.7	1.8	49.8	32.9	5.1	18.5	Local
Reneta	123	13.0	4.0	3.4	39.4	22.9	2.3	18.6	Local
Roja Valle Benejama	124	13.0	3.6	3.6	34.7	18.1	2.1	17.2	Local
Roser de la Reula (MRF 40)	125	14.2	5.5	2.7	62.1	34.6	3.5	25.1	Local
Royal Red Delicious	126	15.6	5.4	3.1	26.6	10.2	1.9	13.4	Foreign
Rubinete	127	15.3	7.7	2.2	31.1	13.4	2.1	10.2	Foreign
Ruixou-1 (MRF 51)	128	15.6	3.5	4.8	45.5	27.4	1.8	21.7	Local
San Felipe	129	11.8	3.9	3.1	34.5	19.3	2.6	14.5	Local
San Miguel	130	14.2	4.1	3.4	27.7	13.3	2.3	10.8	Local
Sandia	131	12.6	7.9	1.6	61.9	48.0	4.4	20.9	Local
Sant Jaume	132	11.5	11.9	1.0	53.8	35.3	2.3	19.0	Local
Sant Joan	133	10.4	8.1	1.3	48.9	24.3	3.1	16.1	Local
Santa Margarida	134	10.3	12.2	0.9	66.4	42.9	2.9	23.1	Local
Signatillis	135	11.1	9.0	1.3	42.7	24.0	2.7	12.6	Local
Solafuente	136	12.5	7.3	1.8	24.5	9.9	2.5	10.3	Local
Starking_1	137	14.3	3.8	4.0	24.9	13.9	2.2	13.8	Foreign
Starking_2	138	14.1	3.9	4.0	30.2	14.5	2.3	12.5	Foreign
Starkrimson_1	139	14.2	3.1	5.2	25.0	11.6	2.2	12.8	Foreign
Starkrimson_2	140	14.8	3.3	4.9	28.9	14.0	2.5	14.3	Foreign
Taüll-1 (MRF 67)	141	12.3	9.6	1.3	45.8	23.1	2.0	17.3	Local
Telamon	142	13.0	4.9	2.5	30.0	15.8	2.1	10.4	Foreign
Tempera	143	13.1	7.1	1.9	53.9	34.7	2.9	19.2	Local
Terrera	144	17.8	12.8	1.4	51.1	33.3	3.2	18.2	Local
Top Red Delicious	145	15.6	3.5	4.5	26.1	11.5	1.8	14.2	Foreign
Totxa	146	15.9	6.9	2.4	42.2	20.7	3.5	13.0	Local
Transparente	147	11.7	12.5	0.9	73.1	55.0	5.3	24.2	Local
Transparente Blanca	148	10.4	12.8	0.8	66.9	47.9	4.5	23.6	Local
Urarte	149	10.5	16.2	0.7	62.8	40.4	2.2	21.1	Local
Urtebete	150	12.6	7.0	1.6	47.5	33.0	2.2	17.2	Local
Valsaina	151	14.3	7.8	2.0	45.3	26.3	2.4	16.1	Local
Verde Doncella (MRF 36)	152	13.5	1.8	8.6	78.4	45.9	2.6	20.4	Local
Verde Doncella_1	153	13.4	2.2	6.6	22.2	11.4	1.8	11.9	Local
Verde Doncella_2	154	12.2	10.8	2.3	20.7	9.3	3.1	10.9	Local
Vinçada Tardía (MRF 61)	155	14.2	9.0	1.7	48.7	29.2	2.4	15.3	Local

SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; RAC, relative antioxidant content; Class., classification. Units: SSC:ºBrix; TA: g malic acid L^{-1}; TPC: mg GAE 100 g FW^{-1}; TFC: mg CE 100 g FW^{-1}; Ascorbic acid: mg AsA 100 g FW^{-1}; RAC: mg Trolox 100 g FW^{-1}.

10.2.4. Supplementary File 4. Eigenvalues of the principal components analysis, and variance explained by each component.

Principal Component	Eigenvalue	Variance (%)	Cumulative variance (%)
1	3.53	50.5	50.5
2	1.49	21.3	71.8
3	1.08	15.4	87.2
4	0.61	8.7	95.9
5	0.14	1.9	97.8
6	0.12	1.7	99.5
7	0.03	0.5	100

10.2.5. Supplementary File 5. Eigenvectors and accumulative variance of the five principal components (PCs).

Variable	PC 1	PC 2	PC 3	PC 4	PC 5
SSC	-0.15	0.41	0.80	0.41	-0.04
TA	0.69	-0.61	0.21	0.18	0.26
RI	-0.56	0.77	-0.14	-0.10	0.25
RAC	0.83	0.45	-0.15	0.08	-0.04
TFC	0.92	0.24	-0.17	0.08	-0.04
TPC	0.93	0.28	-0.13	0.09	0.02
AsA	0.55	0.10	0.55	-0.61	0.00

SSC, soluble solids content; TA, titratable acidity; RI, ripening index; RAC, relative antioxidant content; TFC, flavonoids; TPC, total phenollics content; AsA, ascorbic acid.

10.3. Material suplementario correspondiente al capítulo 4

10.3.1. Supplementary File 1. Basic information of the 155 apple accessions used on this study.

Accession	Nº	Classification	Skin color	Flesh color
Aciprés	1	Spanish	Bicolor	Cream
Akane	2	Non-Spanish	Bicolor	White
Almenar_2 - MRF 46	3	Spanish	Bicolor	Cream
Ascara_1	4	Spanish	Bicolor	Cream
Ascara_2	5	Spanish	Bicolor	Cream
Astrakan Red	6	Non-Spanish	Bicolor	White
Audiena de Oroz	7	Spanish	Green	White
Augüenta	8	Spanish	Green	Greenish
Averdal_1	9	Non-Spanish	Red	Cream
Averdal_2	10	Non-Spanish	Red	Cream
Baujade	11	Non-Spanish	Green	Greenish
Bellaguarda Lardero - MSV 27	12	Spanish	Yellow	White
Belleza de Roma	13	Non-Spanish	Bicolor	Cream
Biscarri_1 - M 107	14	Spanish	Bicolor	White
Blackjon	15	Non-Spanish	Bicolor	White
Bofla	16	Spanish	Green	Cream
Boluaga	17	Spanish	Bicolor	Cream
Bossost_1 - MRF 75	18	Spanish	Bicolor	Greenish
Bossost_2 - MRF 76	19	Spanish	Brown	Cream
Bossost_4 - MRF 78	20	Spanish	Bicolor	Greenish
Bossost_5 - MRF 79	21	Spanish	Bicolor	Cream
Bost Kantoia	22	Spanish	Yellow	Cream
Cabdellà_2 - MRF 49	23	Spanish	Bicolor	Cream
Cabello de Angel	24	Spanish	Yellow	Cream
Calvilla de San Salvador	25	Spanish	Bicolor	Greenish
Camosa - MRF 42	26	Spanish	Bicolor	Cream
Camosa - MRF 60	27	Spanish	Bicolor	Cream
Camuesa de Daroca	28	Spanish	Green	White
Camuesa de Llobregat	29	Spanish	Green	White
Camuesa Fina de Aragón	30	Spanish	Bicolor	White
Carapanón	31	Spanish	Bicolor	White
Carrió	32	Spanish	Bicolor	Greenish
Cella	33	Spanish	Green	Cream
Ciri Blanc	34	Spanish	Green	White
Cirio - MRF 52	35	Spanish	Green	Cream
Cox's Orange Pippin	36	Non-Spanish	Bicolor	Cream
Cripps Pink	37	Non-Spanish	Bicolor	Cream
Cuallarga	38	Spanish	Green	Cream
Cul de Cirio - MRF 39	39	Spanish	Bicolor	Cream
De Agosto - MRF 57	40	Spanish	Bicolor	Cream
De Pera	41	Spanish	Yellow	Cream
De Valdés	42	Spanish	Bicolor	Cream
Delciri	43	Spanish	Yellow	White
Delcon	44	Non-Spanish	Bicolor	White
Delgared Infel	45	Non-Spanish	Red	White
Deljeni	46	Non-Spanish	Yellow	White
Delkistar	47	Non-Spanish	Bicolor	Cream
Delorgue Festival	48	Non-Spanish	Bicolor	Cream
Elista	49	Non-Spanish	Bicolor	White
Esperiega	50	Spanish	Yellow	Cream
Esperiega de Olba - M 106	51	Spanish	Bicolor	Cream
Eugenia	52	Spanish	Bicolor	Greenish
Evasni - Scarlet Spur	53	Non-Spanish	Bicolor	Cream
Florina	54	Non-Spanish	Bicolor	White

Fuji	55	Non-Spanish	Bicolor	Cream
Gala	56	Non-Spanish	Bicolor	White
Galaxy	57	Non-Spanish	Bicolor	Cream
Golden Delicious_675	58	Non-Spanish	Yellow	Cream
Golden Delicious Infel_972	59	Non-Spanish	Yellow	Cream
Golden Paradise	60	Non-Spanish	Yellow	Cream
Golden Smoothee	61	Non-Spanish	Yellow	Cream
Granny Smith_1	62	Non-Spanish	Green	Greenish
Granny Smith_2	63	Non-Spanish	Green	Greenish
Guillemes	64	Spanish	Bicolor	White
Hared	65	Non-Spanish	Bicolor	White
Helada	66	Spanish	Green	White
Hierro	67	Spanish	Bicolor	White
Idared	68	Non-Spanish	Bicolor	White
Irgo_2 - MRF 66	69	Spanish	Bicolor	Cream
Jonadel	70	Non-Spanish	Bicolor	White
Jonagored	71	Non-Spanish	Bicolor	Cream
Jonathan_1	72	Non-Spanish	Bicolor	Cream
Jonathan_2	73	Non-Spanish	Bicolor	Cream
Jubilee	74	Non-Spanish	Bicolor	Cream
Landetxo	75	Spanish	Bicolor	White
Les_1 - MRF 73	76	Spanish	Bicolor	Cream
Les_2 - MRF 74	77	Spanish	Bicolor	White
Mañaga	78	Spanish	Green	Greenish
Mañaga - MRF 43	79	Spanish	Bicolor	Cream
Marinera	80	Spanish	Bicolor	White
Marquinez	81	Spanish	Bicolor	White
McIntosh	82	Non-Spanish	Bicolor	Cream
Médulas_1 - MSV 38	83	Spanish	Bicolor	Cream
Melrose	84	Non-Spanish	Bicolor	Cream
Merrigold	85	Non-Spanish	Yellow	Greenish
Montcada_1 - MRF 82	86	Spanish	Bicolor	Cream
Morro de Liebre	87	Spanish	Bicolor	White
Nesple	88	Spanish	Bicolor	Cream
Normanda	89	Spanish	Bicolor	White
Nueva Starking	90	Non-Spanish	Red	White
Ortell	91	Spanish	Bicolor	White
Ortell - MSV 24	92	Spanish	Bicolor	White
Pera_2	93	Spanish	Yellow	Cream
Pera de Sangüesa	94	Spanish	Green	Cream
Peromingan	95	Spanish	Green	Greenish
Pero Pardo	96	Spanish	Green	Cream
Peruco de Caparroso	97	Spanish	Bicolor	White
Plaona	98	Non-Spanish	Green	Cream
Poma de San Juan - MRF 47	99	Spanish	Bicolor	White
Prau Riu_3	100	Spanish	Bicolor	Greenish
Prau Riu_4	101	Spanish	Bicolor	White
Prau Riu_5	102	Spanish	Green	Greenish
Prima	103	Non-Spanish	Red	White
Rebellón	104	Spanish	Bicolor	White
Red Delicious	105	Non-Spanish	Bicolor	Cream
Red Elstar	106	Non-Spanish	Bicolor	White
Red Rome Beauty	107	Non-Spanish	Bicolor	Cream
Redaphough	108	Non-Spanish	Red	Cream
Red Chief	109	Non-Spanish	Bicolor	White
Regal Prince_1	110	Non-Spanish	Bicolor	Cream
Regal Prince_2	111	Non-Spanish	Bicolor	Cream
Reguard_1 - MRF 53	112	Spanish	Bicolor	Cream
Reguard_2 - MRF 54	113	Spanish	Bicolor	Greenish
Reguard_4 - MRF 56	114	Spanish	Bicolor	White

Reina de Reinetas	115	Non-Spanish	Bicolor	White
Reineta Blanca Canada_1	116	Spanish	Green	Cream
Reineta Blanca Canada_2	117	Non-Spanish	Green	Cream
Reineta Blanca Canada_3	118	Spanish	Green	Cream
Reineta Encarnada	119	Spanish	Bicolor	Greenish
Reineta Gris	120	Spanish	Brown	Cream
Reineta Inesita Asua	121	Spanish	Bicolor	White
Reineta Regil	122	Spanish	Green	Greenish
Reneta	123	Spanish	Bicolor	White
Roja Valle Benejama	124	Spanish	Bicolor	White
Roser de la Reula - MRF 40	125	Spanish	Bicolor	Cream
Royal Red Delicious	126	Non-Spanish	Bicolor	White
Rubinete	127	Non-Spanish	Bicolor	Cream
Ruixou_1 - MRF 51	128	Spanish	Bicolor	Cream
San Felipe	129	Spanish	Bicolor	Greenish
San Miguel	130	Spanish	Bicolor	Cream
Sandia	131	Spanish	Bicolor	White
Sant Jaume	132	Spanish	Bicolor	Greenish
Sant Joan	133	Spanish	Bicolor	Greenish
Santa Margarida	134	Spanish	Bicolor	Greenish
Signatillis	135	Spanish	Green	White
Solafuente	136	Spanish	Bicolor	White
Starking_1	137	Non-Spanish	Bicolor	Cream
Starking_2	138	Non-Spanish	Bicolor	Cream
Starkrimson_1	139	Non-Spanish	Red	Cream
Starkrimson_2	140	Non-Spanish	Red	Cream
Taüll_1 - MRF 67	141	Spanish	Green	Cream
Telamon	142	Non-Spanish	Bicolor	Cream
Tempera	143	Spanish	Green	Greenish
Terrera	144	Spanish	Brown	Cream
Top Red Delicious	145	Non-Spanish	Red	Cream
Totxa	146	Spanish	Green	White
Transparente	147	Spanish	Green	White
Transparente Blanca	148	Spanish	Yellow	Greenish
Urarte	149	Spanish	Green	Greenish
Urtebete	150	Spanish	Green	Cream
Valsaina	151	Spanish	Bicolor	Cream
Verde Doncella_MRF 36	152	Spanish	Green	Cream
Verde Doncella_1	153	Spanish	Green	Cream
Verde Doncella_2	154	Spanish	Green	Cream
Vinçada Tardía - MRF 61	155	Spanish	Green	Cream

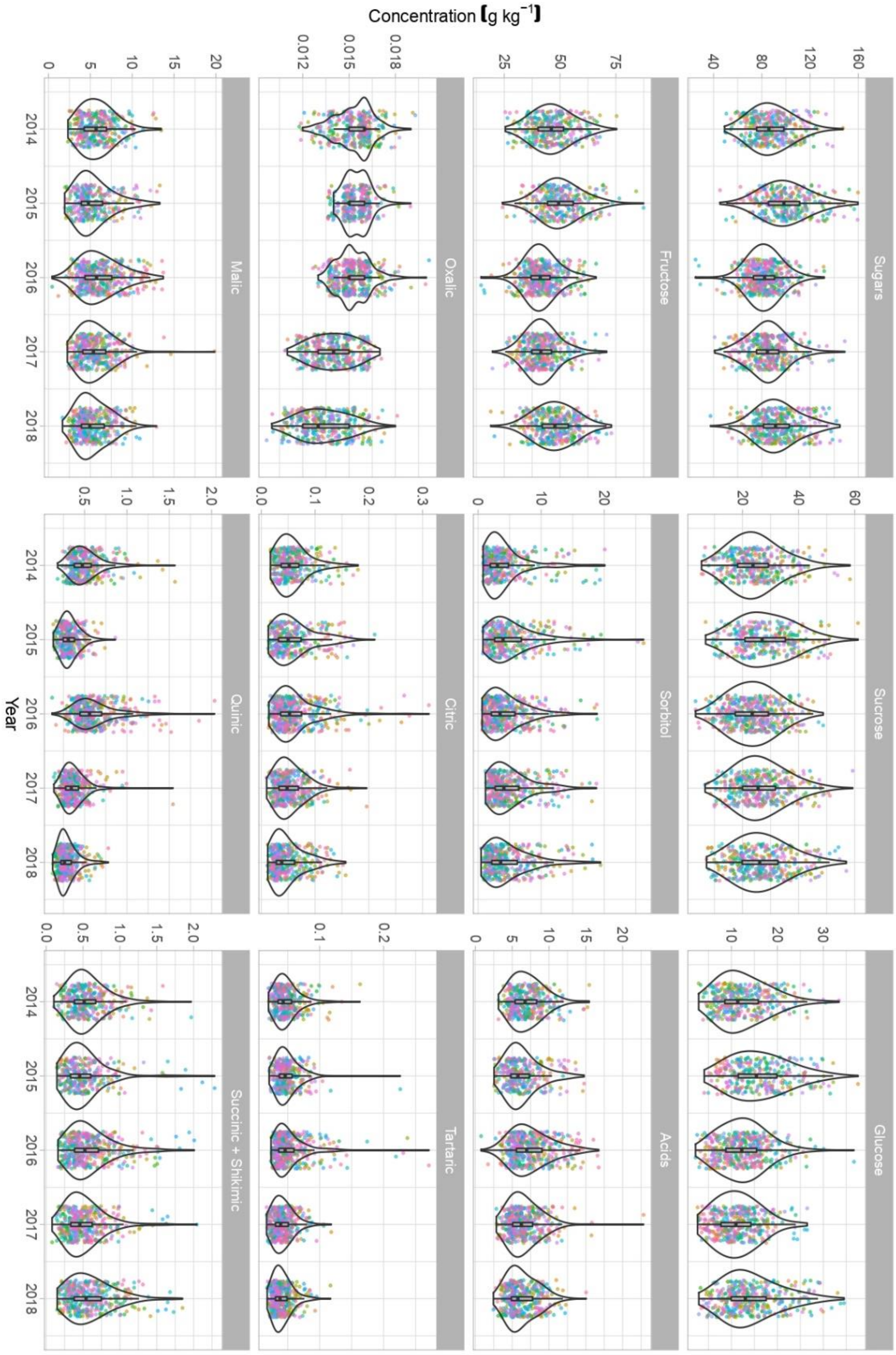
This table includes characteristics of the plant material previously mentioned in Mignard et al. (2021).

10.3.2. Supplementary File 2. Statistical significance of accession (A), year (Y), and their interaction (A x Y) on individual sugars and organic acids.

Source of variation	Units	Accession (A)	Year (Y)	A x Y
Sucrose	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Glucose	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Fructose	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Sorbitol	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Sugars	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Oxalic	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Citric	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Tartaric	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Malic	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Quinic	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Succinic + Shikimic	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Acids	g kg^{-1}	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

Data were evaluated by two-way variance (ANOVA)

10.3.3. Supplementary File 3. Violin plots showing the variability between accessions (dots) and years of the sugars and organic acids parameters.



10.3.4. Supplementary File 4. Individual sugars and organic acids mean values (2014-2018) for the 155 apple accessions studied.

Nº	Sucrose	Glucose	Fructose	Sorbitol	Total Sugars	Oxalic	Citric	Tartaric	Malic	Quinic	Succinic + Shikimic	Total Acids
1	36.31	13.37	43.97	6.17	99.82	0.015	0.037	0.036	2.879	0.239	0.457	3.657
2	10.29	18.61	61.41	5.01	95.32	0.015	0.072	0.047	6.974	0.478	0.303	7.880
3	22.90	17.23	45.11	5.52	90.76	0.016	0.035	0.053	3.536	0.373	0.756	4.762
4	26.48	6.82	41.91	3.36	78.57	0.015	0.076	0.045	7.180	0.472	0.632	8.413
5	31.19	6.98	45.27	4.72	88.16	0.015	0.097	0.035	5.486	0.355	0.417	6.399
6	20.19	13.70	41.58	1.84	77.31	0.015	0.108	0.069	9.529	0.800	0.875	11.389
7	26.38	17.74	40.50	2.76	87.38	0.015	0.055	0.071	5.716	0.567	0.681	7.098
8	13.72	13.86	50.54	7.83	85.95	0.015	0.061	0.069	6.206	0.503	0.728	7.575
9	19.14	14.08	42.03	4.62	79.87	0.014	0.024	0.043	3.956	0.374	0.680	5.084
10	15.52	13.30	44.04	2.94	75.80	0.014	0.022	0.045	4.170	0.451	0.768	5.464
11	38.48	16.47	31.39	2.17	88.51	0.014	0.048	0.033	5.836	0.348	0.233	6.505
12	16.89	6.57	41.74	2.23	67.43	0.015	0.136	0.064	9.031	0.653	0.746	10.638
13	34.00	8.51	47.75	3.04	93.30	0.015	0.054	0.037	5.243	0.352	0.625	6.317
14	30.32	10.13	43.00	4.23	87.68	0.014	0.033	0.031	4.409	0.380	0.400	5.260
15	24.02	14.22	42.55	3.20	83.99	0.015	0.063	0.043	6.672	0.453	0.468	7.708
16	32.27	9.53	38.45	3.27	83.52	0.015	0.060	0.050	6.447	0.451	0.569	7.583
17	30.67	14.22	36.12	6.73	87.74	0.015	0.066	0.041	6.254	0.485	0.496	7.350
18	19.94	12.31	44.83	4.20	81.28	0.015	0.087	0.057	7.833	0.658	0.786	9.429
19	40.37	12.17	48.21	11.43	112.18	0.014	0.107	0.052	10.131	0.574	0.387	11.259
20	29.07	19.40	45.19	7.51	100.64	0.014	0.051	0.045	5.115	0.402	0.625	6.244
21	20.04	12.72	41.98	4.54	79.28	0.016	0.100	0.061	8.718	0.700	0.518	10.106
22	40.75	8.30	44.76	2.42	96.23	0.015	0.036	0.040	4.780	0.314	0.623	5.799
23	26.78	13.12	48.44	6.56	94.90	0.015	0.130	0.063	9.521	0.628	0.295	10.645
24	27.38	7.97	37.41	4.10	76.85	0.015	0.021	0.030	3.409	0.385	1.116	4.967
25	16.78	17.99	45.46	2.60	82.83	0.015	0.081	0.062	7.640	0.472	0.518	8.781
26	28.26	9.78	51.76	9.98	99.78	0.016	0.032	0.080	3.846	0.369	0.545	4.880
27	22.61	14.62	45.70	4.07	87.00	0.016	0.058	0.055	5.288	0.567	0.637	6.614
28	27.17	11.07	36.77	4.20	79.22	0.015	0.033	0.057	3.390	0.410	1.171	5.068
29	28.01	9.55	55.40	4.06	97.01	0.015	0.038	0.091	4.604	0.481	0.801	6.023
30	15.49	14.58	32.97	3.19	66.24	0.015	0.107	0.060	8.059	0.592	0.603	9.430
31	27.79	13.37	41.90	2.78	85.84	0.015	0.082	0.048	6.776	0.463	0.396	7.773
32	25.33	15.81	39.31	4.17	84.61	0.014	0.053	0.048	4.938	0.379	0.848	6.275
33	38.70	8.56	41.17	6.30	94.72	0.015	0.036	0.049	3.264	0.296	0.502	4.157
34	24.33	16.99	50.61	5.67	96.64	0.016	0.020	0.040	2.920	0.329	0.547	3.864
35	30.70	13.49	47.90	9.85	101.94	0.014	0.049	0.055	7.086	0.355	0.698	8.251
36	37.48	9.20	43.40	8.20	97.74	0.015	0.089	0.065	7.502	0.462	0.203	8.329
37	42.14	6.38	42.70	3.43	94.65	0.014	0.054	0.038	5.864	0.338	0.232	6.533
38	20.82	17.85	50.67	2.44	91.77	0.015	0.094	0.080	6.145	0.465	0.913	7.705
39	23.05	18.99	53.03	8.06	103.12	0.015	0.018	0.034	2.781	0.272	0.515	3.628
40	24.96	12.53	40.67	8.90	87.07	0.015	0.078	0.059	5.392	0.497	0.576	6.611
41	38.51	10.50	47.19	7.09	103.30	0.015	0.095	0.053	8.241	0.502	0.491	9.391
42	32.16	14.43	54.88	3.91	105.39	0.014	0.034	0.033	4.725	0.425	0.625	5.850
43	18.08	16.90	46.69	4.65	86.33	0.016	0.029	0.045	2.683	0.282	0.705	3.752
44	20.48	18.93	43.36	2.61	85.38	0.015	0.047	0.045	5.850	0.411	0.357	6.688
45	22.80	15.54	49.49	2.95	90.79	0.015	0.050	0.039	5.390	0.377	0.283	6.121
46	27.39	9.48	54.99	2.26	94.12	0.014	0.054	0.046	6.076	0.402	0.263	6.848
47	20.24	11.91	43.42	2.28	77.85	0.014	0.047	0.036	5.873	0.431	1.152	7.546
48	16.83	15.88	52.78	1.46	86.95	0.015	0.038	0.033	4.745	0.327	0.353	5.504
49	29.53	7.64	40.87	3.01	81.04	0.014	0.099	0.050	7.528	0.438	0.507	8.630

50	34.85	11.13	41.92	4.54	92.44	0.015	0.055	0.052	6.153	0.404	0.483	7.155
51	27.19	10.73	44.36	10.07	92.35	0.014	0.028	0.040	3.361	0.263	0.409	4.109
52	23.33	20.71	58.46	11.51	114.01	0.015	0.103	0.065	6.668	0.615	0.684	8.144
53	18.27	15.12	43.09	2.71	79.18	0.015	0.026	0.048	3.898	0.397	0.857	5.234
54	34.40	10.91	42.90	5.97	94.18	0.015	0.056	0.040	5.531	0.390	0.319	6.343
55	28.50	22.70	55.80	8.26	115.27	0.014	0.037	0.053	4.511	0.355	0.268	5.231
56	32.40	9.62	44.38	4.11	90.52	0.014	0.048	0.030	4.605	0.331	0.585	5.606
57	27.39	10.01	40.53	2.02	79.95	0.014	0.029	0.033	3.794	0.318	0.694	4.875
58	27.52	13.45	53.91	3.73	98.61	0.014	0.046	0.042	5.399	0.385	0.293	6.172
59	26.66	15.60	49.71	3.24	95.21	0.014	0.039	0.040	4.918	0.302	0.356	5.662
60	27.21	13.32	46.24	2.10	88.88	0.014	0.040	0.039	4.256	0.384	0.533	5.259
61	26.24	13.90	52.64	2.94	95.73	0.015	0.045	0.052	4.673	0.364	0.256	5.396
62	23.46	12.71	41.85	1.87	79.89	0.014	0.047	0.045	6.428	0.397	0.217	7.142
63	27.66	12.87	41.09	2.34	83.97	0.014	0.054	0.039	6.644	0.395	0.195	7.334
64	26.06	12.96	46.49	6.07	91.59	0.015	0.059	0.038	5.523	0.398	0.651	6.677
65	21.23	15.99	42.38	4.52	84.12	0.015	0.026	0.050	4.016	0.343	0.780	5.223
66	23.77	14.09	46.39	7.83	92.08	0.014	0.023	0.036	4.147	0.313	0.367	4.893
67	35.58	8.22	46.32	3.71	93.82	0.015	0.066	0.029	6.904	0.465	0.433	7.904
68	23.19	9.18	44.98	1.75	79.11	0.015	0.044	0.036	5.492	0.358	0.279	6.217
69	26.38	13.86	41.94	4.70	86.89	0.015	0.045	0.033	4.185	0.261	0.373	4.904
70	24.85	14.79	42.91	7.18	89.73	0.015	0.041	0.042	5.210	0.357	0.515	6.172
71	28.14	14.32	49.01	3.02	94.49	0.014	0.048	0.042	5.261	0.347	0.300	6.004
72	25.23	19.07	48.23	5.46	97.99	0.015	0.063	0.053	7.072	0.367	0.428	7.991
73	25.16	16.63	47.06	4.14	93.00	0.015	0.062	0.038	7.065	0.418	0.451	8.041
74	22.12	10.89	49.18	2.73	84.92	0.015	0.042	0.040	5.571	0.369	0.427	6.432
75	22.63	15.35	42.97	2.89	83.83	0.016	0.101	0.053	8.539	0.679	0.696	10.077
76	10.94	20.42	45.58	4.91	81.85	0.015	0.066	0.090	3.661	0.456	0.638	4.918
77	16.41	16.30	42.50	5.46	80.67	0.015	0.071	0.050	5.968	0.464	0.535	7.096
78	25.94	12.40	41.71	3.93	83.98	0.015	0.058	0.050	4.353	0.282	0.379	5.131
79	25.67	11.14	40.40	3.31	80.52	0.015	0.039	0.037	3.937	0.265	0.395	4.680
80	17.45	11.82	40.48	1.79	71.54	0.015	0.061	0.057	6.565	0.555	1.623	8.870
81	14.93	10.51	37.29	1.94	64.67	0.015	0.092	0.053	8.302	0.617	1.039	10.112
82	17.99	7.53	50.71	2.44	78.68	0.014	0.058	0.026	6.551	0.396	0.230	7.270
83	24.55	8.89	55.60	6.55	95.59	0.014	0.045	0.035	6.438	0.422	0.525	7.472
84	15.44	18.55	50.45	3.12	87.56	0.014	0.045	0.040	5.571	0.375	0.405	6.444
85	19.10	10.69	45.33	2.09	77.21	0.015	0.100	0.054	7.152	0.377	0.540	8.230
86	34.56	8.97	50.23	7.97	101.73	0.015	0.032	0.031	4.436	0.355	0.629	5.491
87	11.47	16.34	40.00	3.32	71.13	0.015	0.024	0.037	4.114	0.324	0.520	5.026
88	19.39	23.84	41.93	3.68	88.84	0.014	0.047	0.045	5.195	0.485	0.660	6.440
89	21.86	14.90	44.42	5.13	86.31	0.015	0.039	0.031	4.996	0.362	0.389	5.825
90	25.57	14.81	45.25	5.06	90.70	0.014	0.026	0.038	3.766	0.389	0.700	4.927
91	21.26	18.95	47.62	4.21	92.05	0.015	0.033	0.041	4.173	0.361	0.354	4.970
92	21.42	22.45	51.69	5.28	100.84	0.014	0.030	0.035	3.913	0.366	0.335	4.685
93	41.08	9.26	49.93	6.86	107.13	0.015	0.096	0.052	8.602	0.550	0.556	9.864
94	26.42	12.22	50.74	5.51	94.89	0.015	0.060	0.052	6.124	0.340	0.573	7.158
95	18.86	11.52	35.84	3.51	69.73	0.015	0.106	0.040	9.523	0.622	0.543	10.845
96	28.73	12.96	50.83	7.85	100.38	0.014	0.050	0.052	5.760	0.371	0.518	6.758
97	15.63	16.71	42.59	2.86	77.79	0.015	0.038	0.077	3.239	0.397	0.924	4.683
98	24.33	11.30	37.50	1.20	74.33	0.014	0.029	0.037	4.091	0.314	0.480	4.960
99	17.77	9.00	45.88	2.31	74.97	0.015	0.067	0.076	3.779	0.381	1.677	5.988
100	25.72	18.71	47.07	11.96	103.46	0.015	0.057	0.049	5.181	0.417	0.476	6.191
101	24.45	13.30	43.03	6.18	86.96	0.016	0.080	0.061	7.188	0.589	0.386	8.312
102	40.50	8.71	41.07	4.20	94.48	0.018	0.054	0.078	8.862	0.707	0.615	10.326
103	17.24	15.31	49.17	1.66	83.39	0.015	0.057	0.042	6.104	0.389	0.291	6.891

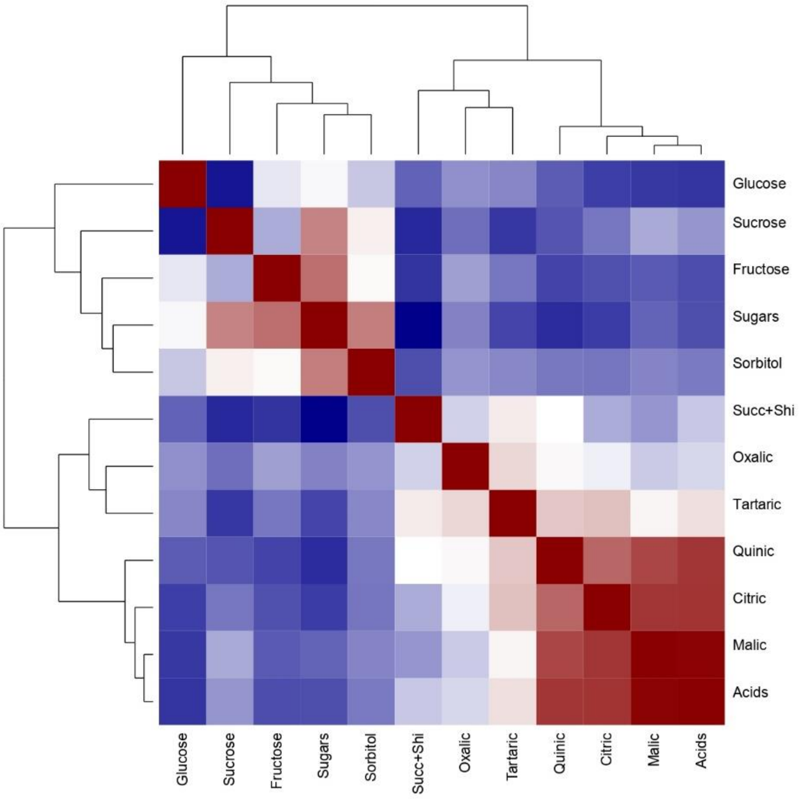
104	31.08	11.11	39.35	4.75	86.29	0.014	0.061	0.039	6.617	0.481	0.622	7.828
105	23.93	14.74	44.63	4.11	87.40	0.014	0.027	0.042	3.635	0.370	0.661	4.742
106	35.81	7.55	42.87	3.86	90.09	0.014	0.073	0.044	6.913	0.450	0.429	7.917
107	30.31	11.51	52.97	4.01	98.80	0.014	0.040	0.027	4.541	0.331	0.442	5.388
108	20.00	18.49	39.74	3.22	81.44	0.014	0.070	0.045	7.337	0.454	0.404	8.319
109	25.81	17.09	47.82	3.59	94.00	0.014	0.021	0.041	3.465	0.336	0.676	4.546
110	28.70	12.18	47.96	2.99	91.83	0.015	0.039	0.033	4.301	0.333	0.705	5.418
111	33.04	11.63	49.89	2.33	96.90	0.015	0.038	0.033	3.974	0.336	0.602	4.990
112	26.46	22.41	56.90	6.21	111.98	0.016	0.041	0.032	4.055	0.337	0.314	4.788
113	31.83	18.53	50.35	3.92	104.62	0.016	0.143	0.075	9.814	0.461	0.498	11.000
114	29.02	10.07	44.43	2.08	85.60	0.014	0.039	0.032	4.436	0.360	0.779	5.654
115	27.97	18.68	44.03	3.31	93.98	0.015	0.051	0.042	5.553	0.372	0.424	6.451
116	44.47	9.69	50.60	8.04	112.80	0.015	0.071	0.042	7.765	0.439	0.482	8.808
117	32.87	8.18	42.45	8.33	91.83	0.014	0.068	0.035	8.310	0.504	0.460	9.384
118	37.51	8.20	45.41	7.36	98.48	0.014	0.065	0.042	7.651	0.579	0.438	8.783
119	36.26	10.85	41.08	4.65	92.84	0.015	0.057	0.040	5.945	0.432	0.668	7.149
120	39.88	10.38	50.33	8.91	109.51	0.015	0.076	0.051	9.013	0.579	0.563	10.289
121	29.29	13.18	54.30	4.44	101.21	0.015	0.089	0.052	7.303	0.491	0.539	8.484
122	32.86	17.79	60.60	10.36	121.61	0.014	0.036	0.041	7.267	0.447	0.443	8.243
123	34.24	6.72	50.32	3.66	94.94	0.015	0.039	0.033	3.960	0.361	0.382	4.782
124	32.17	6.23	47.99	4.94	91.34	0.014	0.037	0.027	3.737	0.312	0.356	4.476
125	25.23	13.26	47.48	8.97	94.93	0.015	0.076	0.037	4.930	0.381	0.332	5.764
126	24.15	17.60	51.14	8.51	101.40	0.014	0.029	0.051	4.600	0.412	0.575	5.675
127	32.76	15.53	49.30	3.69	101.28	0.014	0.055	0.054	5.839	0.336	0.248	6.541
128	24.41	24.29	44.82	10.97	104.49	0.015	0.041	0.038	3.908	0.354	0.331	4.681
129	19.72	7.07	49.98	3.14	79.90	0.015	0.043	0.077	3.931	0.322	0.632	5.012
130	21.82	12.61	45.34	8.87	88.64	0.014	0.035	0.061	4.636	0.419	0.242	5.401
131	26.51	9.97	43.72	2.06	82.26	0.015	0.060	0.040	6.468	0.693	0.782	8.051
132	18.54	10.03	41.96	3.57	74.10	0.015	0.107	0.075	8.126	0.507	0.950	9.772
133	17.31	14.62	35.51	2.44	69.87	0.016	0.050	0.061	5.769	0.612	0.924	7.423
134	20.19	9.05	36.83	2.13	68.20	0.016	0.119	0.079	10.234	0.717	0.903	12.061
135	20.77	8.29	45.89	3.30	78.25	0.015	0.069	0.049	6.364	0.531	0.574	7.595
136	28.46	11.43	36.35	2.49	78.72	0.014	0.060	0.037	5.672	0.397	0.510	6.684
137	24.04	14.40	45.85	4.49	88.41	0.014	0.027	0.044	4.208	0.432	0.656	5.374
138	27.31	13.75	46.11	5.32	92.49	0.014	0.025	0.040	3.707	0.362	0.622	4.764
139	26.55	18.43	46.80	7.56	99.34	0.014	0.025	0.037	3.467	0.395	0.692	4.623
140	27.08	18.01	46.11	7.50	98.71	0.015	0.028	0.039	3.657	0.353	0.663	4.747
141	14.66	15.84	47.65	3.50	81.65	0.015	0.053	0.043	6.897	0.644	0.790	8.434
142	11.91	16.08	50.51	1.88	80.39	0.015	0.063	0.038	5.196	0.458	0.344	6.106
143	18.69	7.74	44.74	8.72	79.88	0.015	0.053	0.058	5.828	0.445	1.064	7.456
144	41.95	10.64	47.22	12.29	112.10	0.015	0.093	0.044	9.768	0.724	0.426	11.065
145	23.70	16.04	49.55	5.34	94.63	0.015	0.032	0.049	4.479	0.469	0.648	5.684
146	30.94	10.98	52.26	6.72	100.90	0.015	0.046	0.039	5.922	0.531	0.461	7.008
147	16.21	10.97	43.28	2.00	72.46	0.016	0.148	0.090	9.240	0.740	0.612	10.840
148	15.38	7.72	38.24	2.35	63.69	0.016	0.140	0.066	9.141	0.764	0.734	10.790
149	16.07	11.17	32.03	2.57	61.85	0.015	0.109	0.040	10.686	0.873	0.864	12.581
150	26.50	11.91	37.35	3.14	78.90	0.014	0.048	0.073	6.053	0.389	0.544	7.114
151	30.48	17.07	41.43	2.45	91.43	0.014	0.064	0.058	5.933	0.455	0.403	6.921
152	18.64	8.24	44.01	2.26	73.15	0.015	0.120	0.058	9.137	0.724	0.684	10.731
153	24.85	12.45	43.18	7.40	87.88	0.014	0.043	0.041	3.206	0.288	0.316	3.901
154	22.21	10.33	38.55	7.31	78.40	0.014	0.037	0.036	2.842	0.235	0.266	3.424
155	35.76	8.63	40.83	7.62	92.84	0.015	0.059	0.057	7.945	0.545	0.581	9.195

10.3.5. Supplementary File 5. Pearson’s correlation coefficients between traits.

	Glu	Fru	Sor	Total Sugars	Oxa	Cit	Tar	Mal	Qui	Succ+ Shi	Total Acids
Suc	-.340**	ns	.339**	.639**	ns	ns	-.237**	ns	-.161*	-.291**	ns
Glu	-	.234**	ns	.285**	ns	-.218**	ns	-.245**	ns	ns	-.253**
Fru			.311**	.691**	ns	-.176*	ns	ns	-.218**	-.257**	-.187*
Sor	-	-	-	.651**	ns	ns	ns	ns	ns	-.188*	ns
Sugars					ns	-.231**	-.182*	ns	-.279**	-.402**	-.185*
Oxa	-	-	-	-	-	.344**	.452**	.266**	.415**	ns	.297**
Cit							.480**	.856**	.724**	ns	.861**
Tar	-	-	-	-	-	-	-	.347**	.464**	.349**	.404**
Mal									.803**	ns	.990**
Qui	-	-	-	-	-	-	-	-	-	.275**	.853**

Statistical significance at **: $P \leq 0.01$; *: $P \leq 0.05$; ns: not significant; Abbreviations: Suc, sucrose; Glu, glucose; Fru, fructose; Sor, sorbitol; Oxa, oxalic acid; Cit, citric acid; Tar, tartaric acid; Mal, malic acid; Qui, quinic acid; Succ+Shi, succinic + shikimic acid.

10.3.6. Supplementary File 6. Hierarchical clustering heatmap of Pearson correlation coefficients over basic quality parameters, biochemical antioxidant traits, sugars, and organic acids pairs (using Pearson distance, and average linkage).



10.3.7. Supplementary File 7. Eigenvalues of the ten principal components of the PCA, and variance explained by each component.

Principal Component	Eigenvalue	Variance (%)	Cumulative variance (%)
1	4.27	35.59	35.59
2	2.39	19.90	55.49
3	1.55	12.94	68.43
4	1.07	8.94	77.37
5	7.65	6.37	83.74
6	6.50	5.42	89.16
7	5.31	4.43	93.59
8	4.36	3.64	97.23
9	2.18	1.81	99.04
10	1.14	0.95	99.99

10.3.8. Supplementary File 8. Eigenvectors of the five principal components (PCs).

Variable	PC 1	PC 2	PC 3	PC 4	PC 5
Sucrose	-0.23	0.64	-0.52	0.39	-0.01
Glucose	-0.31	0.00	0.66	-0.51	0.17
Fructose	-0.40	0.51	0.43	-0.09	-0.20
Sorbitol	-0.27	0.66	0.21	0.19	0.43
Sugars	-0.52	0.81	0.16	0.07	0.06
Oxalic	0.35	0.09	0.51	0.45	-0.51
Citric	0.86	0.29	-0.02	-0.15	-0.08
Tartaric	0.58	0.00	0.49	0.28	0.03
Malic	0.85	0.42	-0.16	-0.22	0.01
Quinic	0.88	0.18	0.10	-0.07	0.13
Succinic + Shikimic	0.37	-0.44	0.23	0.47	0.46
Acids	0.90	0.35	-0.11	-0.15	0.07

Abbreviations: Sugars, total sugars; Acids, total organic acids.

10.3.9. Supplementary File 9. Correlations between the different sugars and organic acids of the 155 accessions studied and the climatic traits.

	Suc	Glu	Fru	Sor	Total Sugars	Oxa	Cit	Tar	Mal	Qui	Succ+Shi	Total Acids
pre	0.305***	ns	ns	0.255***	0.226***	-0.363***	-0.364***	-0.377***	-0.294***	-0.478***	-0.239***	-0.353***
pre_day	ns	-0.149***	-0.101***	ns	-0.116***	-0.156***	ns	-0.086***	ns	-0.161***	ns	ns
tmx	-0.094***	0.062**	ns	ns	ns	0.05*	0.105***	0.105***	0.081***	ns	-0.054*	0.072**
tmn	-0.067**	0.066**	0.139***	ns	0.06*	-0.084***	ns	ns	ns	-0.127***	-0.056*	ns
tmd	-0.107***	0.054*	0.078**	ns	ns	ns	0.098***	0.084***	0.082***	ns	ns	0.07**
trg	-0.089***	ns	-0.091***	ns	-0.08***	0.199***	0.157***	0.188***	0.105***	0.191***	ns	0.116***
rh	0.174***	ns	0.053*	0.079***	0.128***	-0.353***	-0.241***	-0.276***	-0.191***	-0.386***	ns	-0.216***
rad	-0.313***	-0.056*	ns	-0.218***	-0.226***	0.228***	0.331***	0.307***	0.279***	0.346***	0.145***	0.314***

Statistical significance at ***: $P \leq 0.001$; **: $P \leq 0.01$; *: $P \leq 0.05$; ns: not significant. Abbreviations: Suc, sucrose; Glu, glucose; Fru, fructose; Sor, sorbitol; Oxa, oxalic acid; Cit, citric acid; Tar, tartaric acid; Mal, malic acid; Qui, quinic acid; Succ+Shi, succinic + shikimic acid; pre, total precipitation; pre_day, mean daily precipitation; tmx, mean daily maximum temperature; tmn, mean daily minimum temperature; tmd, mean daily temperature; trg, mean daily temperature range; rh, relative humidity; rad, solar radiation.

10.3.10. Supplementary File 10. Mean values of the mixed-effects model coefficients (int, intercept; pre, precipitation; trg, temperature range; rad, solar radiation) for the different sugar traits. Results are shown for each cluster, according to the accession's origin, and according to the skin colour.

		Cluster				**Origin**		**Skin Colour**				
		1	**2**	**3**	**4**	**Non-Spanish**	**Spanish**	**Bicolour**	**Brown**	**Green**	**Red**	**Yellow**
Suc	**int**	0.000^{3}	-0.155^{4}	-0.036	0.352^{2}	-0.026	0.015	-0.035^{Br}	$1.382^{Bi,Gr,Re}$	-0.030^{Br}	-0.345^{Br}	0.236
	pre	$0.109^{2,3}$	0.304^{1}	0.300^{1}	0.263	0.184	0.270	0.196^{Gr}	0.337	0.376^{Bi}	0.156	0.225
	trg	0.044^{2}	0.203^{1}	0.113	0.077	0.112	0.111	0.110	0.084	0.107	0.111	0.137
	rad	-0.172	-0.147	-0.081	-0.181	-0.156	-0.132	-0.131	-0.187	-0.121	-0.146	-0.241
Glu	**int**	0.170^{4}	-0.073	0.108^{4}	$-0.414^{1,3}$	0.041	-0.023	0.112^{Ye}	-0.417	-0.189	0.323	-0.422^{Bi}
	pre	-0.327^{2}	$0.155^{1,3,4}$	-0.132^{2}	-0.258^{2}	-0.006^{*}	-0.201^{*}	-0.184	-0.007	-0.107	0.008	0.056
	trg	0.114	0.087	0.038	0.033	0.104	0.055	0.077	0.051	0.041	0.034	0.151
	rad	-0.277^{2}	$0.045^{1,3,4}$	-0.135^{2}	-0.258^{2}	-0.137	-0.152	-0.183	-0.105	-0.094	-0.146	-0.039
Fru	**int**	$0.244^{2,3,4}$	-0.091^{1}	-0.096^{1}	-0.137^{1}	0.025	-0.014	0.051	0.213	-0.156	-0.085	0.045
	pre	$-0.448^{2,3}$	$0.222^{1,3,4}$	$-0.028^{1,2}$	-0.250^{2}	-0.076	-0.141	-0.174	-0.155	0.027	-0.102	-0.093
	trg	-0.067	-0.134	-0.163	-0.143	-0.099	-0.137	-0.103	-0.250	-0.160	-0.230	-0.081
	rad	$-0.314^{2,3}$	$0.179^{1,3,4}$	$-0.050^{1,2,4}$	$-0.634^{2,3}$	-0.081	-0.131	-0.140	-0.186	-0.018	-0.148	-0.119
Sor	**int**	0.088	-0.209	-0.015	0.235	-0.361^{*}	0.204^{*}	-0.043^{Br}	$1.451^{Bi,Gr,Re,Ye}$	0.091^{Br}	-0.104^{Br}	-0.170^{Br}
	pre	$-0.003^{2,3}$	$0.519^{1,4}$	$0.496^{1,3}$	$0.173^{2,3}$	0.368	0.271	0.301	-0.007	0.333	0.166	0.431
	trg	0.109^{2}	$0.338^{1,3}$	0.143^{2}	0.209	0.273^{*}	0.151^{*}	0.199	0.303	0.186	0.325	0.092
	rad	-0.061^{3}	0.157	$0.278^{1,4}$	-0.057^{3}	0.069	0.110	0.107	-0.326	0.147	-0.178	0.150
Sugars	**int**	$0.216^{2,3}$	-0.168^{1}	-0.060^{1}	-0.010	-0.032	0.018	0.042^{Br}	$0.856^{Bi,Gr,Re,Ye}$	-0.164^{Br}	-0.096^{Br}	-0.004^{Br}
	pre	$-0.314^{2,3,4}$	$0.390^{1,4}$	0.191^{1}	$-0.055^{1,2}$	0.088	0.042	-0.016	-0.047	0.247	0.007	0.158
	trg	0.039	0.103	0.003	-0.008	0.072	0.021	0.050	0.008	0.006	-0.016	0.085
	rad	$-0.414^{2,3}$	$0.018^{1,4}$	$-0.095^{1,4}$	$-0.390^{2,3}$	-0.208	-0.199	-0.232	-0.319	-0.087	-0.293	-0.198

$^{1; 2; 3; 4}$ Significant differences among clusters. The number corresponding to the cluster that showed a significant difference. *Significant differences among the origin. $^{Bi; Br; Gr; Re; Ye}$ Significant differences among skin colour. The Bi, bicolour; Br, brown; Gr, green; Re, red and Ye, yellow corresponding to the skin colour group which showed the significant difference. Abbreviations: Suc, sucrose; Glu, glucose; Fru, fructose; Sor, sorbitol; Sugars, total sugars; int, intercept; pre, total precipitation; trg, mean daily temperature range.

10.3.11. Supplementary File 11. Mean values of the mixed-effects model coefficients (int, intercept; pre, precipitation; trg, temperature range; rad, solar radiation) for the different organic acid traits. Results are shown for each cluster, according to the accession's origin, and according to the skin colour.

		Cluster				**Origin**		**Skin Colour**				
		1	**2**	**3**	**4**	**Non-Spanish**	**Spanish**	**Bicolour**	**Brown**	**Green**	**Red**	**Yellow**
Oxa	**int**	-0.015	-0.036	0.027	0.042	-0.130^{*}	0.073^{*}	0.001	-0.014	0.038	-0.135	-0.004
	pre	-0.612	-0.632	-0.671	-0.638	-0.635	-0.640	-0.629	-0.759	-0.664	-0.659	-0.596
	rad	-0.103	-0.080	-0.069	-0.093	-0.053^{*}	-0.104^{*}	-0.093	0.007	-0.085	-0.027	-0.094
Cit	**int**	$-0.352^{3,4}$	$-0.588^{3,4}$	$0.406^{1,2}$	$1.002^{1,2,3}$	-0.291^{*}	0.165^{*}	-0.024	1.009^{Re}	0.107^{Re}	$-0.691^{Br,Gr}$	0.128
	pre	-0.210	-0.207	-0.252	-0.207	-0.192^{*}	-0.237^{*}	-0.224	-0.173	-0.227	-0.197	-0.209
	trg	-0.056	0.017	-0.026	0.005	-0.034	-0.010	-0.016	-0.023	-0.032	-0.076	0.031
	rad	0.122^{4}	$0.070^{3,4}$	$0.151^{2,4}$	$0.242^{1,2,3}$	0.135	0.133	0.126	0.280	0.144	0.104	0.149
Tar	**int**	-0.057^{3}	$-0.335^{3,4}$	$0.236^{1,2}$	0.276^{2}	-0.231^{*}	0.131^{*}	-0.074^{Gr}	0.112	0.238^{Bi}	-0.228	0.048
	pre	-0.380	-0.287	-0.385	-0.354	-0.311	-0.376	-0.365	-0.448	-0.385	-0.167	-0.285
	trg	0.010	0.054^{3}	-0.014^{2}	0.003	0.038^{*}	0.001^{*}	0.017	0.013	-0.011	0.050	0.034
	rad	0.008	$-0.030^{3,4}$	0.096^{2}	0.128^{2}	-0.008^{*}	0.068^{*}	0.013	-0.002	0.093	0.077	0.083
Mal	**int**	$-0.426^{3,4}$	$-0.668^{3,4}$	$0.453^{1,2}$	$1.204^{1,2,3}$	-0.146	0.082	-0.096^{Br}	$1.560^{Bi,Gr,Re,Ye}$	0.260^{Br}	-0.492^{Br}	0.004^{Br}
	pre	-0.160	-0.135	-0.192	-0.192	-0.151	-0.176	-0.165	-0.222	-0.161	-0.155	-0.191
	trg	$-0.094^{3,4}$	-0.074^{4}	$-0.054^{1,4}$	$-0.005^{1,2,3}$	-0.073	-0.059	-0.066	0.003	-0.048	-0.091	-0.088
	rad	$0.142^{2,4}$	$0.098^{1,3,4}$	0.170^{2}	$0.198^{1,2}$	0.152	0.143	0.140	0.205	0.158	0.125	0.162
Qui	**int**	$-0.277^{3,4}$	$-0.188^{3,4}$	$0.232^{1,2}$	$0.456^{1,2,3}$	-0.182^{*}	0.103^{*}	-0.078^{Gr}	0.591	0.226^{Bi}	-0.052	-0.112
	pre	$-0.539^{3,4}$	$-0.669^{3,4}$	$-0.872^{1,2}$	$-0.865^{1,2}$	-0.631^{*}	-0.766^{*}	-0.685^{Br}	-1.171	-0.827	-0.638^{Br}	-0.626^{Br}
	trg	-0.058	-0.064	-0.057	0.050	-0.036	-0.047	-0.043	$0.131^{Bi,Re,Ye}$	-0.065	-0.095	0.009
	rad	0.164^{3}	0.057	0.042^{1}	0.093	0.126	0.070	0.078	0.018	0.083	0.107	0.195
Succ+Shi	**int**	$-0.347^{2,4}$	0.235^{1}	0.059	0.153^{1}	-0.256^{*}	0.145^{*}	-0.019	-0.265	0.118	0.013	-0.111
	pre	-0.095^{3}	-0.213	-0.334^{1}	-0.238	-0.156	-0.250	-0.202	-0.275	-0.320^{Re}	0.046^{Gr}	-0.212
	trg	-0.080	-0.143	-0.149	-0.114	-0.112	-0.128	-0.120	-0.146	-0.143	-0.078	-0.108
	rad	-0.044^{3}	-0.132	-0.178^{1}	-0.082	-0.102	-0.117	-0.111	-0.175	-0.138	-0.049	-0.078
Acids	**int**	$-0.468^{3,4}$	$-0.601^{3,4}$	$0.449^{1,2}$	$1.175^{1,2,3}$	-0.180^{*}	0.102^{*}	-0.097^{Br}	$1.480^{Bi,Re,Ye}$	0.263	-0.456^{Br}	-0.004^{Br}
	pre	-0.223^{3}	$-0.209^{3,4}$	$-0.290^{1,2}$	-0.291^{2}	-0.224	-0.262	-0.241	-0.334	-0.259	-0.215	-0.269
	trg	$-0.109^{3,4}$	$-0.097^{3,4}$	$-0.055^{1,2}$	$0.013^{1,2,3}$	-0.083	-0.066	-0.075	0.024	-0.055	-0.108	-0.093
	rad	$0.120^{2,4}$	$0.085^{1,3,4}$	0.136^{2}	$0.166^{1,2}$	0.127	0.119	0.117	0.164	0.131	0.109	0.134

$^{1; 2; 3; 4}$ Significant differences among clusters. The number corresponding to the cluster, which showed the significant difference. *Significant differences among the origin. $^{Bi; Br; Gr; Re; Ye}$ Significant differences among skin colour: Bi,bicolour; Br, brown; Gr, green; Re, red and Ye, yellow corresponding to the skin colour group, showing significant differences. Abbreviations: Oxa, oxalic acid; Cit, citric acid; Tar, tartaric acid; Mal, malic acid; Qui, quinic acid; Succ+Shi, succinic + shikimic acid; Acids, total organic acid; pre, total precipitation; trg, mean daily temperature range.

10.4. Material suplementario correspondiente al capítulo 7

10.4.1. Tabla S1. Pearson's correlation coefficients for the traits studied for the 17 accessions assessed in this study.

Table S1. Pearson's correlation coefficients for the traits studied for the 17 accessions assessed in this study.

	RI	TPC	TFC	AsA	RAC	Sugars	Sucrose	Glucose	Fructose	Sorbitol	Acids	Citric	Tartaric	Malic	Succ+Shi
SSC	ns	0.603*	0.549*	0.549*	ns	0.918**	ns	0.544*	0.803**	0.874**	0.528*	ns	ns	0.502*	ns
TA	-0.786**	ns	ns	0.576*	ns	0.494*	0.744**	ns	ns	ns	0.947**	0.565*	ns	0.958**	ns
RI	-	ns	ns	ns	ns	ns	-0.510*	ns	ns	ns	-0.667**	ns	ns	-0.701**	0.651**
TPC			0.960**	0.882**	0.793**	0.669**	ns	ns	0.621**	0.791**	0.530*	ns	ns	0.502*	ns
TFC	-	-	-	0.893**	0.731**	0.623**	ns	ns	0.591*	0.741**	0.525*	ns	ns	0.505*	ns
AsA					0.543*	0.596*	ns	ns	ns	0.704**	0.626**	ns	ns	0.616**	ns
RAC	-	-	-	-	-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sugars							0.643**	ns	0.874**	0.934**	0.637**	ns	ns	0.604*	ns
Sucrose	-	-	-	-	-	-	-	ns	ns	0.525*	0.817**	ns	ns	0.820**	ns
Glucose									ns	ns	-0.338*	ns	ns	-0.532*	0.543*
Fructose	-	-	-	-	-	-	-	-	-	0.837**	ns	ns	ns	ns	ns
Sorbitol											0.585*	ns	ns	0.562*	ns
Acids	-	-	-	-	-	-	-	-	-	-	-	0.619**	ns	0.996**	ns
Citric													0.662**	0.585*	ns
Tartaric	-	-	-	-	-	-	-	-	-	-	-	-	-	ns	ns
Malic															ns

SSC, soluble solids content; TA, titratable acidity; RI, ripening index; TPC, total phenolics content; TFC, total flavonoids content; AsA, Ascorbic acid; RAC, relative antioxidant content; Sugars, total sugars; Acids, total organic acid; Succ+Shi, succinic + shikimic.

Capítulo 11

Artículos

11.1. Artículos publicados, aceptados o enviados en revistas internacionales (SCI) durante el transcurso de esta tesis doctoral

P. Mignard, Beguería, S., Reig, G., Font i Forcada, C., Moreno, M.A. (2021). Genetic origin and climate determine fruit quality and antioxidant traits on apple (*Malus x domestica* Borkh). *Scientia Horticulturae*, 285, 110142. DOI: 10.1016/j.scienta.2021.110142.

P. Mignard, Beguería, S., Giménez, R., Font i Forcada, C., Reig, G., Moreno, M.A. (2022). Effect of Genetics and Climate on Apple Sugars and Organic Acids Profiles. *Agronomy*, 12(4), 827. DOI: 10.3390/agronomy12040827.

11.2. Artículos publicados en revistas no SCI durante el transcurso de esta tesis doctoral

P. Mignard, G. Reig, C. Font i Forcada, M.A. Moreno Sánchez (2020). Antioxidantes y calidad del fruto de variedades de manzana del Banco de Germoplasma de la Estación Experimental de Aula Dei-CSIC. *Actas Portuguesas de Horticultura* 30: 689-694.

P. Mignard, Beguería, S., Reig, G., Font i Forcada, C., Moreno, M.A. (2021). Phenotypic analysis of fruit quality traits and effect of climate in an apple (*Malus*× *domestica* Borkh) germplasm bank of Aragón, Spain. *Acta Horticulturae*, 1307: 109-114. DOI: 10.17660/ActaHortic.2021.1307.17.

G. Reig, **P. Mignard**, C. Font i Forcada, J. Val, M.A. Moreno (2018). El Banco de Germoplasma de manzano de la Estación Experimental de Aula Dei como fuente de diversidad de calidad de fruto. *Revista de Fruticultura*. Nº63 Julio/Agosto 2018.

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11.3. Otros artículos SCI publicados en revistas internacionales

C. Font i Forcada, G. Reig, R. Giménez, **P. Mignard**, L. Mestre M.A. Moreno (2019). Sugars and organic acids profile and antioxidant compounds of nectarine fruits influenced by different rootstocks. *Scientia Horticulturae* 248, pages 145-153. DOI: 10.1016/j.scienta.2018.12.010.

C. Font i Forcada, G. Reig, L. Mestre, **P. Mignard**, J.A. Betrán, M.A. Moreno (2020). Scion× Rootstock Response on Production, Mineral Composition and Fruit Quality under Heavy-Calcareous Soil and Hot Climate. *Agronomy*, 10(8), 1159. DOI: 10.3390/agronomy10081159.

11.4. Otros artículos no SCI publicados en revistas internacionales

A. E. Salazar González, C. Font i Forcada, **P. Mignard**, G. Reig Córdoba, J. Val Falcón, M.A. Moreno Sánchez (2020). Compatibilidad de diferentes patrones *Prunus* injertados con ciruelo japonés y albaricoquero. *Actas Portuguesas de Horticultura*, 30: 252-259.

L. Mestre, O. Fayos, M.A. Moreno Sánchez, **P. Mignard**, C. Mallor, A. Garcés-Claver (2020). Estudio de compuestos antioxidantes en distintas especies del género *Capsicum*. *Actas Portuguesas de Horticultura*, 30: 684-688.

C. Font i Forcada, L. Mestre, A.E. Salazar González, **P. Mignard**, J. Pinochet, G. Reig Córdoba, M.A. Moreno Sánchez (2020). Comportamiento de distintos patrones *Prunus* injertados con la nectarina 'Big Top' en condiciones de asfixia de raíces. *Actas Portuguesas de Horticultura*, 30: 236-241.

C. Font i Forcada, G. Reig, **P. Mignard**, J. Val, M.A. Moreno (2018). Calidad del fruto en variedades de melocotonero y nectarina del Banco de Germoplasma de la Estación Experimental de Aula Dei. *Revista de Fruticultura*. Nº63 Julio/Agosto 2018.

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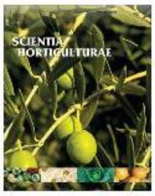

Genetic origin and climate determine fruit quality and antioxidant traits on apple (*Malus x domestica* Borkh)

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ABSTRACT

Apples are highly appreciated in terms of flavor, nutritional and health-promoting compounds and are associated with a reduced risk to develop age-related and chronic diseases. In this study, 155 accessions of *Malus x domestica* Borkh from the apple germplasm collection, located at the Experimental Station of Aula Dei in NE Spain, were analyzed during five consecutive years (2014–2018). Basic fruit quality traits including soluble solids content (SSC), titratable acidity (TA) and the ratio SSC/TA (ripening index - RI) were obtained. In addition, biochemical compounds such as total phenolics content (TPC), flavonoids (TFC), vitamin C (Ascorbic acid - AsA) and relative antioxidant capacity (RAC) were determined. Statistical analysis was used to determine differences in trait values among accessions and years. The well adapted local accessions showed, in general, higher average content of antioxidants and RAC compared with the foreign and commercial ones. A multivariate model was fitted with the accessions and climate features of each year as independent variables. A cluster analysis was then performed on the model coefficients space to classify the 155 accessions within five groups. The cluster analysis showed that foreign cultivars (those not originating from Spain) were concentrated in two groups while local accessions could not be segregated and had very different profiles. Furthermore, they increased the concentration of bioactive compounds tended to decrease with higher temperatures, while increased with higher solar radiation. Statistical analyses emphasized differences between groups and highlighted accessions and climate as main factors affecting metabolite profiles and fruit characteristics.

1. Introduction

Apple (*Malus x domestica* Borkh, family *Rosaceae*, tribe *Pyreae*) is among the most widely consumed fresh fruits in the world. It has a special importance as one of the major temperate fruit crops cultivated globally. In 2019, more than 87 M tonnes were produced worldwide (FAOSTAT, 2021) and it ranked second in production after peaches and nectarines, and before pears, in Spain with more than 638 thousands of tonnes. Since its consumption is widespread in many countries and it is available on the market for the whole year, apple is, among fruits and vegetables, a major source of nutrients and bioactive compounds for humans (Michalska and Łysiak, 2015). The content of antioxidants present in apples is also important because of their contribution to the sensory quality of fresh fruit and processed apple products (Khanizadeh et al., 2008). Bioactive compounds improve the quality and the shelf life of vegetables and reduce the risk of post-harvest diseases (Bui et al., 2019; Davey et al., 2007). They also provide important health benefits to humans (Boeing et al., 2012; Gibney et al., 2019; Ho et al., 2020). In fact, apples are an important dietary source of potentially healthy biomolecules such as antioxidants. Moreover, there is an increasing evidence of a relationship between the consumption of fruits and vegetables and a reduced risk of human diseases such as cancer, heart coronary, cardiovascular, diabetes, Alzheimer's diseases, and age-related functional decline (Zhang et al., 2016).

Currently, there are more than 7000 documented apple cultivars in the world (Urrestarazu et al., 2016; Pereira-Lorenzo et al., 2017). However, the global production is dominated by relatively few well-adapted cultivars ('Fuji', 'Gala', 'Golden', 'Granny Smith' and 'Delicious'), many of which are closely related (Urrestarazu et al., 2016; Ordidge et al., 2018), to the detriment of the locally well-adapted apple

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Effect of Genetics and Climate on Apple Sugars and Organic Acids Profiles

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Abstract: Apple is widely cultivated in temperate regions. The beneficial properties of apple for preventing several illnesses are widely known. Nevertheless, qualitative variables such as sweetness or sourness may influence consumer satisfaction; they are critical factors for fruit consumption and essential in plant breeding. In the present work, 155 apple accessions were assessed during five consecutive years (2014–2018). Four individual sugars and seven organic acids were analysed by HPLC. A mixed-effects model was fitted with accessions and the years' climatic features as independent variables. A cluster analysis was applied on the mixed-effects model coefficients. Four groups were considered as optimum. Genetics seemed to have the strongest effect and showed clear differences between accession groups, while climate effects were strong only for certain compounds and had a more horizontal behaviour equally affecting the different accession groups. In fact, non-Spanish cultivars tended to concentrate, while autochthone accessions had a much wider spread. Individual sugars and acids concentrations correlated negatively with precipitation and positively with temperature range and solar radiation in all accession groups. The geographic region where the orchard is grown is thus very important in the resulting metabolites profiles. Moreover, apple genetics would also play a decisive role as highlighted in the cluster analysis.

Keywords: *Malus* × *domestica* Borkh.; fruit quality; temperature; solar radiation; HPLC; fructose; sorbitol; malic acid

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1. Introduction

Apple has been cultivated in Europe and Asia since antiquity [1] and it is currently the largest fruit crop in temperate regions. Globally, apple production ranked 86.4 million tons worldwide [2]. Therefore, its availability in markets all year around makes apple fruit the most consumed fresh fruit in the world.

Apple is well known for being a source of dietary fiber, micro-nutrients such as vitamin C, sugars, organic acids, minerals, and phytochemical compounds [3–6] and its beneficial properties for human health is also well established [7–10]. Qualitative traits such as sweetness, sourness, skin colour, fruit firmness, and other organoleptic characteristics strongly influence consumer satisfaction [11–13]. Among the latter, sourness and sweetness are the main factors influencing fruit palatability and consumption. Therefore, they are among the most relevant traits considered in plant breeding programs [14]. A combination of organic and mineral acids such as citric, malic, tartaric, quinic, or succinic are responsible for sourness [15]; however, fruit acidity not only affects the overall apple flavour but also influences the perception of other organoleptic traits such as sweetness