Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias

Caracterización morfológica, bioquímica y agronómica de variedades tardías de mandarinas (*Citrus reticulata* Blanco)

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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF LATE-SEASON VARIETIES OF MANDARIN GROWING IN SPAIN UNDER HOMOGENEOUS GROWING CONDITIONS

Autores: Alejandro Andy Maciá-Vázquez, Dámaris Núñez-Gómez, Juan José Martínez-Nicolás, Pilar Legua & Pablo Melgarejo

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INFLUENCE OF ROOTSTOCK ON YIELD, MORPHOLOGICAL, BIOCHEMICAL AND SENSORY CHARACTERISTICS OF ’AFOURER’ VARIETY MANDARINS

Autores: Alejandro Andy Maciá-Vázquez, Juan José Martínez-Nicolás, Dámaris Núñez-Gómez, Pablo Melgarejo & Pilar Legua

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Mandarin Variety Significantly Affects the Metabolites Present in the Leaves

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CERTIFICAN:

Que la Tesis Doctoral Titulada "**Caracterización morfológica, bioquímica y agronómica de variedades tardías de mandarinas (*Citrus reticulata* Blanco)**" de la que es autor el graduado en Ciencias Ambientales, D. Alejandro Andy Maciá Vázquez, ha sido realizada bajo nuestra dirección y autorizamos a que sea presentada para optar a la obtención del grado de Doctor por la Universidad Miguel Hernández.

Y para que conste a los efectos oportunos se firma el presente certificado en Orihuela, a

Firmado:

Dr. Dña. Juana Fernández López, Catedrática de Universidad y Coordinadora del Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias (ReTos-AAA) de la Universidad Miguel Hernández de Elche (UMH),

CERTIFICA:

Que la Tesis Doctoral titulada "**Caracterización morfológica, bioquímica y agronómica de variedades tardías de mandarinas (*Citrus reticulata* Blanco)"** de la que es autor el graduado en Ciencias Ambientales **D. Alejandro Andy Maciá Vázquez**, ha sido realizada bajo la dirección del **Dr. Pablo Melgarejo Moreno** y la codirección de la **Dra. Pilar Legua Murcia**, actuando como tutor de la misma el Dr. Juan José Martínez Nicolás. Considero que la Tesis es conforme, en cuanto a forma y contenido, a los requerimientos del Programa de Doctorado ReTos-AAA, siendo por tanto apta para su exposición y defensa pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a

Dra. Dña. Juana Fernández López

Coordinadora del Programa Doctorado ReTos-AAA

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ÍNDICE

ABREVIATURAS Y SÍMBOLOS

^{1}H NMR	Resonancia magnética nuclear de protón
ABTS	2.20'-radical method Azinobis (3-ethylbenzothiazolin-6-sulfonic)
AGE	Ácido gálico equivalente
ANOVA	Análisis de la varianza
AT	Acidez titulable
AVASA	Agrupación de Viveristas de Agrios S.A.
CC	Citrange Carrizo
CM	*Citrus macrophylla*
CTV	Virus de la tristeza de los cítricos
DPPH	2.20'-Diphenyl-1-Picrylhydrazyl
DSPC	Correlación parcial dispersa desfasada
FA	Forner Alcaide nº 5
HPLC	Cromatografía líquida de alta eficacia
HSD	Diferencias honestamente significativas
IC	Índice de color
MC	Mandarino Cleopatra
PCA	Análisis de componentes principales
PLSD-DA	Análisis discriminante de mínimos cuadrados parciales
SAM	Análisis de Significación de Microarrays
SST	Sólidos solubles totales
VIP	Variable de Importancia en la proyección

ESTRUCTURA DE LA TESIS

Para la elaboración de la presente tesis doctoral se ha seguido la metodología basada en la publicación de un compendio de artículos de investigación, con la finalidad de obtener el título de doctor, para ello, en la redacción de la misma se ha seguido la normativa vigente de la Universidad Miguel Hernández de Elche.

La Tesis Doctoral se estructura en las siguientes partes:

1. Introducción: Se expone el estado del arte y la importancia del estudio de las mandarinas tardías.
2. Objetivos: Se describen tanto los objetivos generales, como los objetivos específicos de la investigación.
3. Resumen de la metodología: Se explican las condiciones experimentales de los ensayos realizados, así como las distintas determinaciones realizadas y los equipos y técnicas utilizadas para su medición.
4. Resumen de los resultados y discusión: Análisis de los resultados y conclusiones obtenidos en cada uno de los ensayos realizados.
5. Conclusiones generales: Conclusiones globales de los estudios que forman parte de la tesis doctoral.
6. Investigaciones futuras: Posibles nuevas líneas de trabajo para investigaciones futuras.
7. Publicaciones científicas: Artículos publicados que componen la base de la tesis doctoral
   - En la primera publicación (Maciá-Vázquez *et al.*, 2023) se caracterizó y se comparó tres variedades tardías de mandarinas (‘Afourer’, ‘Orri’ y ‘Tango’) y se estudió el efecto del portainjerto en los frutos de la variedad ‘Tango’, en condiciones homogéneas de cultivo.
   - En la segunda publicación (Maciá-Vázquez *et al.*, 2024) se estudió el efecto del portainjerto en los frutos de la variedad tardía de mandarina ‘Afourer’, en condiciones homogéneas de cultivo.
   - En la tercera publicación (Maciá-Vázquez *et al.*, 2024) se analizaron los metabolitos presentes en las hojas de tres variedades tardías de

mandarinas (‘Afourer’, ‘Orri’ y ‘Tango’) y su relación con las características agronómicas.

RESUMEN

Las mandarinas son uno de los cítricos más importantes en cuanto a producción y exportaciones a nivel mundial. Existen una amplia variedad de mandarinas comerciales, pero de las más destacadas son las recolectadas en la época invernal, las variedades tardías. Estas tienen un alto valor económico, por tener altos rendimientos de producción de frutos, por recolectarse en una época en la que existen pocas variedades comerciales y por estar sometidas a estrictas limitaciones de cultivo por los obtentores. Por ello, el análisis detallado de las variedades comerciales tardías, el efecto que tiene el portainjerto en la calidad final del fruto y la adaptación de los árboles a las condiciones bióticas y abióticas cada vez más extremas por el cambio climático, son de gran interés para la agricultura.

Con todo esto, el objetivo de esta Tesis Doctoral es caracterizar morfológica, bioquímica y agronómicamente tres variedades tardías de mandarinas (*Citrus reticulata* Blanco) en condiciones homogéneas de cultivo. Específicamente se caracterizaron y compararon los frutos de las variedades tardías 'Afourer', 'Orri' y 'Tango', se analizó la influencia del portainjerto en los frutos de variedades tardías de mandarinas, y se analizaron los metabolitos presentes en las hojas de las variedades tardías 'Afourer', 'Orri' y 'Tango' y su influencia en las características agronómicas.

Los resultados de la caracterización de estas variedades tardías mostraron que la variedad 'Afourer' destacó por tener un 20% más de tamaño y peso en comparación con las otras variedades. La variedad 'Orri' destacó por tener una gran cantidad de zumo y un mayor brillo, así como una concentración de azúcares un 18% mayor, además de mostrar una mayor cantidad de ácido málico en comparación con el ácido cítrico. Las mandarinas 'Tango' injertadas sobre Forner Alcaide nº5 presentaron un mayor peso y tamaño, así como una tonalidad más anaranjada y una mayor cantidad de fenoles totales. El análisis metabolómico reveló que todas las muestras de mandarina analizadas contenían predominantemente aminoácidos no esenciales. Los portainjertos mandarino Cleopatra y Citrange Carrizo fueron los más interesantes en función del conjunto de características morfológicas, bioquímicas y sensoriales, destacando por la

producción de frutas de mayor tamaño, mejor rendimiento en zumo, y una mayor calidad organoléptica. El análisis de los metabolitos en las hojas mostró que la variedad 'Afourer' destaca, desde el punto de vista agronómico, por su mayor crecimiento vegetativo. Por otro lado, la variedad 'Tango' mostró una mayor resistencia al estrés abiótico. No obstante, la variedad 'Orri' sobresalió sobre las otras dos por su resistencia tanto a estreses abióticos como bióticos, y por producir frutos más dulces y de mayor calidad, a pesar de tener un menor crecimiento vegetativo.

ABSTRACT

Mandarins are among the most significant citrus fruits in terms of production and exports worldwide. There is a diverse range of commercial mandarins, but the most notable are those harvested during the winter season, the late varieties. These have a high economic value due to their high fruit yields, their harvesting at a time when there are few commercial varieties, and their subjection to strict cultivation limitations by breeders. Consequently, the detailed analysis of late commercial varieties, the effect of rootstock on final fruit quality, and the adaptation of trees to increasingly extreme biotic and abiotic conditions due to climate change are of great interest to agriculture.

The objective of this PhD Thesis is to characterize morphologically, biochemically, and agronomically three late varieties of mandarin (Citrus reticulata Blanco) under homogeneous growing conditions. Specifically, the fruits of the late varieties 'Afourer', 'Orri' and 'Tango' were characterized and compared, the influence of the rootstock on the fruits of late mandarin varieties was analyzed, and the metabolites present in the leaves of the late varieties 'Afourer', 'Orri' and 'Tango' were analyzed and their influence on the agronomic characteristics was determined.

The results of the characterization of three late varieties revealed that the 'Afourer' variety exhibited the greatest size and weight, with an average of 20% greater than the other varieties. The 'Orri' variety exhibited a higher juice quantity and brightness, as well as an 18% higher sugar concentration, and a higher malic acid to citric acid ratio. The 'Tango' mandarins grafted on Forner Alcaide nº5 exhibited greater weight and size, as well as a more orange hue and a higher

amount of total phenols. Metabolomic analysis revealed that all mandarin samples analyzed contained predominantly non-essential amino acids. The rootstocks Cleopatra mandarin and Carrizo citrange exhibited the most notable morphological, biochemical, and sensory characteristics. These rootstocks were associated with the production of larger fruits, higher juice yield, and superior organoleptic quality. The analysis of metabolites in the leaves revealed that the 'Afourer' variety exhibited greater vegetative growth, from an agronomic perspective, in comparison to the other varieties. In contrast, the 'Tango' variety demonstrated enhanced resilience to abiotic stress. Nevertheless, the variety 'Orri' exhibited the most notable resistance to both abiotic and biotic stresses, as well as the highest quality fruit, despite displaying the lowest vegetative growth.

1. INTRODUCCIÓN

Esta Tesis Doctoral estudia las características morfológicas, bioquímicas y agronómicas de tres variedades de mandarinas tardías ('Afourer', 'Orri' y 'Tango') cultivadas en condiciones homogéneas de cultivo, injertadas con varios portainjertos, en tres parcelas, con condiciones agroclimáticas similares, localizadas en el Sureste de España. Con este estudio se busca determinar queé variedades tardías y portainjertos son los más interesantes para su cultivo por los citricultores. Para ello, en primer lugar, se expondrá a continuación el estado del arte y una revisión bibliográfica sobre el tema objeto de esta Tesis.

1.1. Características de las mandarinas

El mandarino (*Citrus reticulata* Blanco) es un árbol frutal perteneciente a la familia *Rutaceae*. Presenta porte redondeado, con hojas perennes unifoliadas y nervios reticulados, mientras que sus ramas están provistas comúnmente de espinas. Presenta flores hermafroditas pequeñas y de color blanco, con 5 pétalos ligeramente solapados y 18 estambres. Sus raíces son sólidas y de color blanco, desarrollando una gran cantidad de pelos radiculares (Agustí *et al.*, 2020; Matheyambath *et al.*, 2016; Talon *et al.*, 2020).

Produce frutos hesperidios carnosos llamadas mandarinas, cuyas características varían considerablemente según la variedad. Las mandarinas presentan un color entre naranja intenso y naranja rojizo dependiendo de la variedad y su estado de madurez. Suelen presentar un diámetro ecuatorial de entre 4 y 8 cm. La base del cuello puede ser de corte bajo a alto, mientras que su ápice está profundamente deprimido. Las distintas variedades pueden presentar diferentes grosores de corteza, con glándulas de aceites esenciales hundidas y una superficie desde lisa hasta algo rugosa. Del mismo modo los frutos pueden presentar semillas o no según la variedad (Agustí *et al.*, 2020; Forsyth, 2003; Matheyambath *et al.*, 2016; Talón *et al.*, 2020).

Las mandarinas cuentan con un amplio número de cultivares e híbridos que difieren enormemente en cuanto a sus rasgos morfológicos, bioquímicos y agronómicos (Al-Saif *et al.*, 2022). Las mandarinas se pueden clasificar, según la época del año en la que se cosechen, en tempranas, de media estación o tardías (Forsyth, 2003) (Figura 1). Algunas de las variedades de mandarina tardía más destacadas que se pueden encontrar actualmente en el mercado son 'Afourer', 'Orri' y 'Tango'. La variedad ‘Afourer’, también conocida como 'Nadorcott', fue obtenida en 1988 en Marruecos y su origen podría deberse a la hibridación entre la variedad 'Murcott' y un parental polinizador desconocido ('Murcott' es un tangor, híbrido de mandarina y naranja, *Citrus reticulata* Blanco × *Citrus sinensis* (L.) Osbeck) (Nadori, 1998). La variedad ‘Orri’ es otra mandarina de temporada tardía originada en Israel a partir de yemas irradiadas del cultivar ‘Orah’ [‘Orah’ es un híbrido de ‘Temple’ (*Citrus temple* hort. ex Y. Tanaka) × ‘Dancy’ (*Citrus tangerina* hort. ex Tanaka)] (Vardi *et al.*, 2003). La mandarina 'Tango' es un cítrico híbrido que se desarrolló en la Universidad de California partir de una yema irradiada del híbrido diploide de mandarina 'Murcott' (Roose & Williams, 2007).

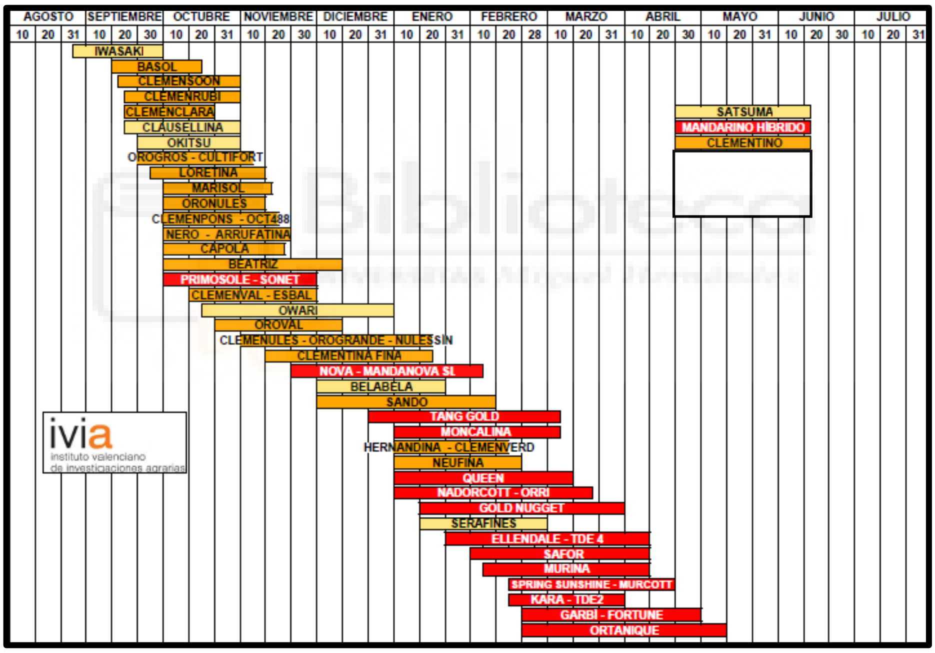

Figura 1. Calendario de cosecha de las distintas variedades de mandarinas, clasificadas como Satsumas, Manarino Híbrido y Clementinas. Fuente: Editada a partir del original de IVIA (2024).

1.2. Importancia económica de las mandarinas

Las mandarinas son uno de los cítricos más importantes del mundo en términos de toneladas producidas. Asia ha sido históricamente el mayor productor de esta fruta, con el 71,33% del total de la producción mundial; en 2019 China produjo el 52,62% de las mandarinas del mundo. Otros grandes productores de mandarinas en el mundo son Turquía (3,74%), Marruecos (3,67%) o Egipto

(2,93%). Sin embargo, el mayor exportador de mandarinas del mundo es España, que representa el 26,77% de las toneladas comercializadas de este cítrico (FAO, 2021). En España, las mandarinas suponen casi el 20% del total de cítricos producidos. Su elevada producción y exportación hacen de esta fruta un elemento económico clave para este sector (MAPA, 2020).

Más específicamente, las variedades tardías tienen un gran interés agro-económico, por recolectarse en un periodo invernal en el que hay pocas variedades comerciales y por tener mayores producciones. Además, los agricultores deben pagar altos royalties para cultivar estas variedades, y ajustarse a la limitación impuesta por los obtentores de las patentes de las variedades, en el número de árboles que se puede cultivar o dónde pueden ser comercializados los frutos (Smith, 2016). Como ejemplo, se estima que existen 3 millones de árboles de la variedad 'Afourer' cultivados en España con permiso del obtentor. En la variedad "Orri", el número de árboles que se pueden cultivar en España está limitado a 1,3 millones y con la condición de que esta variedad no se pueda comercializar fuera de la Unión Europea. En cuanto a la variedad "Tango", por el momento no existe limitación en el tamaño de la plantación y, aunque no se dispone de información sobre el número de árboles cultivados en España, se sabe que el cultivo de esta variedad ha experimentado un crecimiento [según datos no publicados facilitados por la Agrupación de Viveristas de Agrios S.A. (AVASA)].

1.3. Compuestos bioquímicos y calidad de las mandarinas

Las mandarinas tienen un alto valor nutritivo. Los principales componentes bioquímicos de estos frutos son azúcares, ácidos orgánicos, carotenoides, polifenoles (flavonoides y ácidos fenólicos), limonoides y vitaminas, destacando la vitamina C (Lado *et al.*, 2016). El ácido ascórbico (vitamina C) es un ácido orgánico que juega un papel importante en la prevención de muchas enfermedades, gracias a su capacidad antioxidante, antiinflamatoria y anticancerígena (Benavente-García & Castillo, 2008; Daud *et al.*, 2016; Y.S. Huang & Ho, 2010).

La calidad de las mandarinas y su atractivo para los consumidores vienen determinados por una combinación de propiedades morfológicas y bioquímicas.

Desde una perspectiva morfológica, los consumidores valoran atributos como el color, la facilidad de pelado, la presencia o ausencia de semillas o la cantidad de zumo. Mientras que el sabor y las propiedades nutricionales de los frutos vendrán dados por parámetros bioquímicos como la acidez, el índice de madurez, la actividad antioxidante, el contenido en azúcares o la concentración de ácidos orgánicos (Boudries, 2012; Legua *et al.*, 2014; Sdiri *et al.*, 2012; Zhao *et al.*, 2017).

1.4. Importancia del portainjerto en las mandarinas

Los portainjertos o patrones se utilizan ampliamente en la agricultura porque se ha demostrado que facilitan el enraizamiento, aumentan la absorción de nutrientes y agua, afectan a la maduración de la fruta y mejoran la calidad de los frutos (Chen *et al.*, 2022; de Carvalho *et al.*, 2021; Forner-Giner *et al.*, 2023; Legua *et al.*, 2011). A lo que hay que sumar que aumentan la resistencia a factores abióticos como la salinidad, la sequía, el tipo de suelo o la asfixia radicular. Además de factores bióticos como la resistencia a nemátodos o infecciones fúngicas (Behzadi Rad *et al.*, 2021; de Carvalho *et al.*, 2021; Jaimez *et al.*, 2023; Vahdati *et al.*, 2021). Específicamente en cítricos, los portainjertos suelen centrarse en la resistencia frente al Virus de la Tristeza de los Cítricos (CTV, por sus siglas en inglés), la resistencia a nemátodos, el rendimiento de producción de frutos, la época de maduración o la tolerancia a diversos estreses abióticos (Martínez-Cuenca *et al.*, 2016).

El estudio, hibridación y creación de nuevos portainjertos ha sido de gran interés para la citricultura durante décadas, con el objetivo de encontrar portainjertos con características agronómicas y cualitativas mejoradas (Continella *et al.*, 2018; Fu *et al.*, 2016). Algunos de los portainjertos más utilizados en el cultivo de mandarinos son *Citrus macrophylla* Wester (CM), Mandarino Cleopatra (*Citrus reshni* Hort. ex Tan.) y Citrange Carrizo (*Citrus sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.). CM es conocido por su alto rendimiento, y resistencia/tolerancia a la caliza y a la salinidad, aunque es sensible a las heladas y al CTV (Maciá-Vázquez *et al.*, 2023; Martínez-Cuenca *et al.*, 2016) (Tabla 1). Mandarino Cleopatra (MC), por su parte, destaca por su tolerancia al CTV, resistencia a la caliza, salinidad y heladas, aunque es algo sensible a la asfixia radicular (Martínez-Cuenca *et al.*, 2016; Morales *et al.*, 2023). Citrange Carrizo (CC) se caracteriza por su tolerancia al CTV, un alto rendimiento de producción, una maduración temprana y resistencia a la asfixia radicular; aunque es más sensible a la caliza y a la salinidad (Martínez-Cuenca *et al.*, 2016; Morales Alfaro *et al.*, 2023).

Tabla 1. Efecto de los portainjertos a estudio en las características agronómicas y sus respuestas frente a diferentes estreses abióticos y al CTV. MS=Muy Sensible, S=Sensible, R=Resistente, Rm=Resistencia media, MR=Muy Resistente, T=Tolerante m=Media, MA=Muy Alta, N=Normal, A=Adelantado. Fuente: Martínez-Cuenca et al. (2016); Morales Alfaro et al. (2023).

	Caliza	Salinidad	Asfixia radicular	Heladas	Sequía	CTV	Producción	Maduración
Citrus macrophylla	R	R	S	MS	Rm	S	MA	N
Mandarino Cleopatra	R	MR	S	R	Rm	T	m	N
Citrange Carrizo	S	S	S	S	S	T	m	A
Forner Alcaide nº5	Rm	Rm	Rm	Rm	Rm	T	MA	A

Además, la elección del portainjerto tiene importantes implicaciones en la calidad final del fruto. El portainjerto puede influir en gran medida en las características morfológicas del fruto, como el peso, el diámetro, el color o el contenido de zumo (Forner-Giner *et al.*, 2023; Legua *et al.*, 2011; Morales *et al.*, 2021). Los portainjertos también influyen en las propiedades bioquímicas, organolépticas y nutricionales del fruto, como los sólidos solubles totales (SST), los fenoles totales, las antocianinas, los ácidos orgánicos, los azúcares y la actividad antioxidante total (Chen *et al.*, 2024; Legua *et al.*, 2014; Modica *et al.*, 2022).

1.5. Análisis metabolómico de las hojas

Los estudios realizados sobre mandarinas se han centrado en mostrar la alta calidad y los notables beneficios nutricionales de sus frutos (Maciá-Vázquez *et al.*, 2024; Simón-Grao *et al.*, 2014; Tarancón *et al.*, 2021). Sin embargo, los estudios centrados en otros tejidos vegetales, como las hojas de mandarina, son mucho más limitados. Las hojas pueden proporcionarnos información sobre sus características agronómicas y sus mecanismos de respuesta a diferentes estreses abióticos y bióticos (Asai *et al.*, 2017; Hildebrandt, 2018). Por tanto, el conocimiento de estas características es de vital importancia, más aún en variedades tardías, ya que no existen publicaciones previas que analicen metabolómicamente sus hojas.

Las técnicas de análisis metabolómico han mejorado significativamente en los últimos años debido a los recientes avances en instrumentación más potente y precisa, así como a importantes mejoras en el procesamiento de datos y la capacidad computacional (Aharoni & Brandizzi, 2012; Rolin *et al.*, 2013). Las mejoras en las técnicas de análisis de datos y análisis estadístico permiten analizar y procesar los resultados obtenidos de forma más eficiente, lo que conduce a una mejor comprensión de los resultados observados (Okada *et al.*, 2010). La resonancia magnética nuclear es actualmente una técnica muy potente y precisa para la identificación y cuantificación de metabolitos primarios y secundarios en tejidos vegetales (Kumar, 2016; Rolin *et al.*, 2013). La resonancia magnética nuclear se utiliza actualmente en un gran número de estudios para determinar con precisión los metabolitos presentes en muestras frescas de cualquier tejido vegetal (Aharoni & Brandizzi, 2012; Gogna *et al.*, 2015; Melgarejo *et al.*, 2022; Sousa *et al.*, 2022; Tristán *et al.*, 2022).

2. OBJETIVOS

El objetivo principal de esta Tesis Doctoral es caracterizar morfológica, bioquímica y agronómicamente tres variedades tardías de mandarinas (*Citrus reticulata* Blanco) en condiciones homogéneas de cultivo.

Los objetivos específicos marcados para esta Tesis Doctoral son:

- Caracterizar y comparar los frutos de las variedades tardías 'Afourer', 'Orri' y 'Tango'.
- Analizar la influencia del portainjerto en los frutos de variedades tardías de mandarinas.
- Analizar los metabolitos presentes en las hojas de las variedades tardías 'Afourer', 'Orri' y 'Tango' y su influencia en las características agronómicas.

3. RESUMEN DE MATERIALES Y MÉTODOS

3.1. Material vegetal

Para este estudio se recolectaron mandarinas (*Citrus reticulata* Blanco) y hojas de tres fincas localizadas en el sureste de la Península Ibérica que comparten las mismas condiciones edafoclimáticas:

- La primera parcela se localiza en Torre-Pacheco (Murcia, España; 37º46′11,2″ N 1º01′07,4″ O). Los árboles, de 14 años de edad, tenían una altura comprendida entre 3 y 4 m y estaban plantados a un marco de 5,5 × 4 m. Allí se recolectaron los frutos y las hojas de las variedades tardías 'Afourer', 'Orri', y 'Tango', cultivadas sobre el portainjerto *Citrus macrophylla* Wester (CM).

- La segunda parcela se ubica en el término municipal de Torremendo (Alicante, España; 37º58′47,8″ N 0º50′24,5″ O). Los árboles de esta explotación tenían 11 años y estaban plantados con un marco de 5,5 × 4 m, la altura de los árboles variaba entre los 2,5 y los 3 m. Allí se recolectaron los frutos de la variedad "Tango" injertada sobre el patrón Forner Alcaide nº5 (FA).

- La tercera parcela se halla en el municipio de Abanilla (Murcia, España; 38º11′13.7" N 1º01′15.6" O). Los árboles están plantados con un marco de 6 × 5 m, sobre mesetas de 45 cm de altura. Allí se recolectaron mandarinas de la variedad 'Afourer' injertadas sobre los portainjertos *Citrus macrophylla* Wester (CM), Mandarino Cleopatra (MC) y Citrange Carrizo (CC). En cuanto a la edad y altura de los árboles, los árboles con el portainjerto CM tenían 6 años, con alturas comprendidas entre 2,4 y 3,0 m. Por el contrario, los árboles injertados sobre los portainjertos MC y CC tenían 11 años de edad y alturas comprendidas entre los 3,5 y los 4,5 m (Figura 2).

Figura 2. Fotografía de finca de Abanilla donde se recolectaron las muestras.

Todos árboles estudiados se cultivaron siguiendo prácticas tradicionales de manejo del cultivo para la producción comercial. Se aplicaron tratamientos fitosanitarios y se subsanaron las deficiencias de nutrientes según lo requiriese el cultivo. En el momento de la recolección de la fruta, todos los árboles presentaban un buen desarrollo y condiciones fitosanitarias adecuadas.

Todas las mandarinas se recolectaron manualmente cuando alcanzaron su estado de madurez comercial, según la definición DOUE-L-2021-81443 (European Union, 2021). Se recolectaron cincuenta frutos de cada combinación variedad/portainjerto, procedentes de árboles diferentes. La selección de los frutos se hizo al azar, garantizando la representación de la muestra. Para mantener la coherencia, los frutos se recolectaron a la misma altura, y en todas las orientaciones de los árboles. Posteriormente, las mandarinas se transportaron al laboratorio y se almacenaron a una temperatura de 4 ºC hasta su análisis.

En cuanto a las hojas, éstas se recolectaron por triplicado de los mismos árboles escogidos para los frutos. Las hojas eran todas adultas y presentaban un desarrollo correcto, sin deficiencias nutricionales, enfermedades o plagas. Las muestras se llevaron rápidamente al laboratorio, donde se limpiaron con abundante agua destilada para eliminar posibles restos de polvo o impurezas. A continuación, todas las muestras de hojas se cortaron en trozos homogéneos y se congelaron inmediatamente utilizando nitrógeno líquido. Una vez congeladas las muestras, se almacenaron a -80 °C en recipientes de propileno hasta su liofilización. Las muestras se liofilizaron (Christ Alpha 2-4 LSCplus, Martin Christ, Osterode am Harz, Alemania) durante 48 h. A continuación, se molieron y se tamizaron hasta obtener un polvo fino homogéneo que se almacenó en tubos de 2 mL a -20 °C hasta que se realizó el análisis metabolómico.

3.2. Rendimiento de frutos

El rendimiento se ha calculado a partir de los datos recogidos durante tres cosechas, durante tres años. Para ello se seleccionaron al azar cincuenta árboles representativos de cada variedad/portainjerto, se pesaron todos los frutos recolectados y a partir de los datos recogidos se calculó el rendimiento medio por árbol. Los resultados se expresaron en kilogramos por árbol.

3.3. Parámetros físicos y morfológicos de los frutos

Para la determinación de los parámetros físicos y morfológicos de los frutos, se midió el peso del fruto (g), el diámetro ecuatorial (mm) y el diámetro polar (mm). Las mandarinas se cortaron ecuatorialmente y se midió el espesor de la corteza, el número de carpelos y el número de semillas. Posteriormente se extrajo el zumo y se registraron con precisión el volumen de zumo y el peso de la corteza. Tras extraer el zumo, se pesó la piel, y se midió el volumen (mL) y peso (g) del zumo obtenido. El rendimiento en zumo, expresado en porcentaje, se calculó dividiendo el volumen de zumo por el peso del fruto.

Para evaluar el color de la corteza y del zumo se midió por triplicado utilizando un colorímetro (espectrofotómetro portátil CM- 700d, Konica Minolta, Osaka, Japón) con un ángulo de visión de 10º e iluminante estándar D65 (Figura 3). A partir de los valores de color medidos se calculó el índice de color de Jiménez Cuesta para cítricos de [IC=(1000*a)/(L*b)] (Legua *et al.*, 2018).

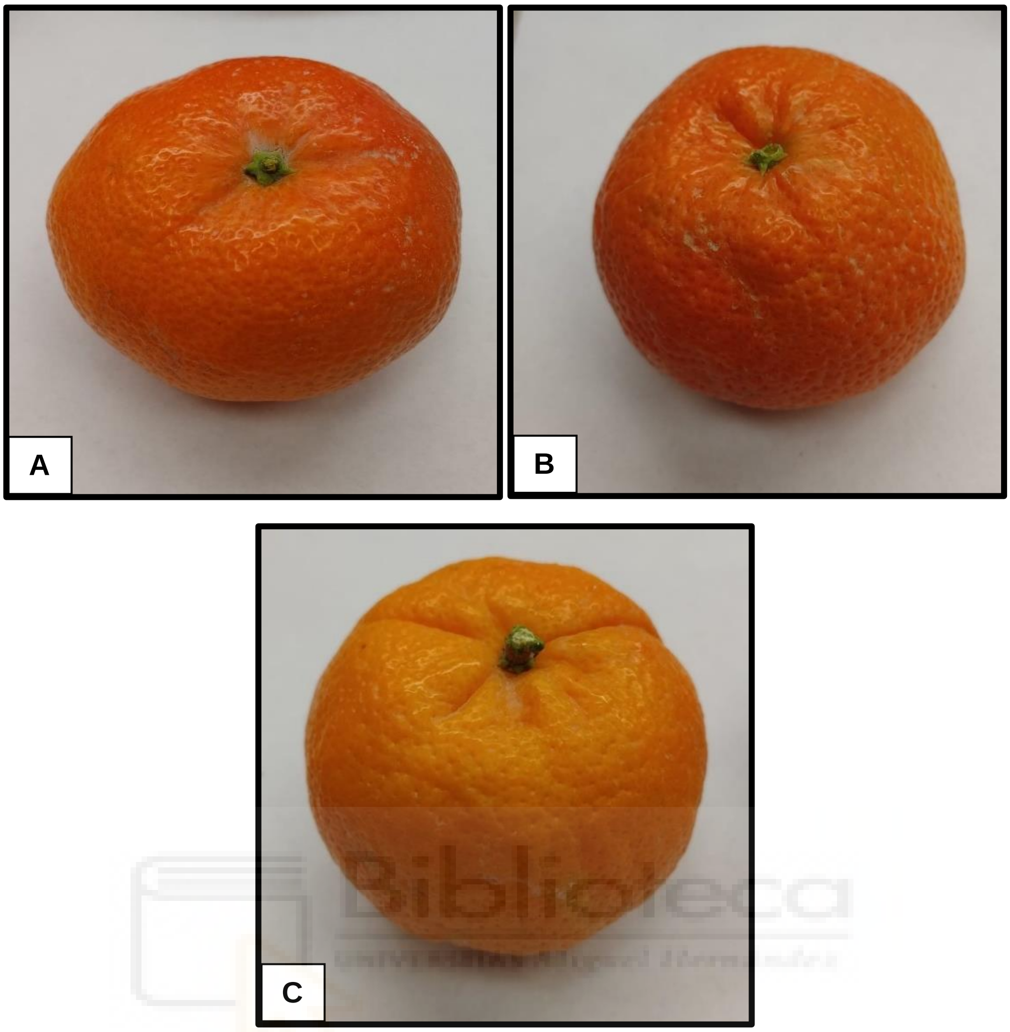

Figura 3. Apariencia externa de las mandarinas 'Afourer' (A), 'Tango' (B) y 'Orri' (C).

3.4. Parámetros químicos de los frutos

Como parámetros químicos se analizó la acidez titulable (AT), expresada en g de ácido cítrico/L, y el pH del zumo se midió con un valorador automático de acidez (877 Titrino plus, Metrohm, Herisau, Suiza) empleando NaOH 0,1 N hasta alcanzar un pH de 8,1 y utilizando 5 mL de zumo de mandarina diluido en 50 mL de agua destilada. Los sólidos solubles totales (SST) se determinaron con un refractómetro manual (modelo N1, Atago, Tokio, Japón) y los resultados se expresaron en ºBrix a 20 ºC. El índice de madurez se calculó como la relación SST/TA.

3.5. Actividad antioxidante del zumo de los frutos

Los fenoles totales de las muestras se midieron por triplicado según el método de Folin-Ciocalteu (Singleton *et al.*, 1999). Se utilizó una curva de calibración de ácido gálico y los resultados se expresaron como mg de ácido gálico equivalente/litro de zumo (mg AGE/L). La actividad antioxidante total se evaluó según el protocolo descrito por Martínez-Nicolas *et al.* (2022). Se emplearon los métodos de los radicales ABTS+ [2.20'-radical method Azinobis (3-ethylbenzothiazolin-6-sulfonic)] y de DPPH (2.20'-Diphenyl-1-Picrylhydrazyl). Los resultados de la actividad antioxidante se expresaron como miligramos de equivalente de Trolox por mililitro de zumo (mg Trolox/mL). Las mediciones de fenoles y la actividad antioxidante total de la muestra se realizaron por triplicado a 760 nm utilizando un espectrofotómetro (Helios γ, Thermo Spectronic, Cambridge, Reino Unido).

3.6. Ácidos orgánicos y azúcares de los frutos

La detección y cuantificación de los ácidos orgánicos y azúcares presentes en el zumo de mandarina se realizó mediante cromatografía líquida de alta resolución (HPLC, por sus siglas en inglés), siguiendo la metodología descrita por Legua *et al.* (2014). El zumo centrifugado se filtró empleando un filtro de poro de 0,45 µm. El zumo se inyectó por triplicado en un cromatógrafo Hewlett Packard (serie 1100, Wilmington, DE, EE.UU.), empleando una fase móvil de ácido fosfórico al 0,1 % a 0,5 mL/min, con una columna Supelcogel [C-610H (30 cm × 7,8 mm ID), Supelco] y una precolumna Supelguard [C610H (5 cm × 4,6 mm), Supelco]. Se utilizó un detector de matriz de diodos ultravioleta (210 nm) para cuantificar los ácidos orgánicos y un detector de índice de refracción para los azúcares. Para hallar la concentración en las muestras se utilizaron curvas estándar de ácidos orgánicos y azúcares puros. Los resultados se expresaron en g/100 mL.

3.7. Análisis metabolómico de las hojas y el zumo de los frutos

Los metabolitos presentes en el zumo y las hojas de mandarina se midieron mediante resonancia magnética nuclear de 1 protón (^1H NMR) siguiendo la metodología descrita por Melgarejo *et al.* (2022) y van der Sar *et al.* (2013). Por separado, las muestras de hoja liofilizada y de zumo se mezclaron cada una de ellas con una mezcla hidro-metanólica (1:1, MeOH:H_2O). A continuación, las muestras se sometieron a sonicación durante 30 minutos y a centrifugación (11.000 rpm) durante 20 minutos. El sobrenadante se sometió a speed-vacuum hasta que se evaporó toda la fase líquida y el sólido obtenido se resuspendió en 800 µL de tampón fosfato potásico junto con 0,58 mM de TPS. Finalmente, se tomaron 600 µL de la muestra para su análisis cuantitativo mediante ^{1}H NMR (Ascend NMR Magnet 500 MHz, Bruker, Billerica, MA, EE.UU.) (Figura 4). La identificación y cuantificación final de todos los metabolitos detectados se realizó mediante la integración de los picos de los espectros obtenidos directamente de la resonancia magnético nuclear. Los resultados se expresaron en mM.

Figura 4. Ascend NMR Magnet 500 MHz con el que se realizó ^{1}H NMR.

3.8. Análisis sensorial

El análisis sensorial de las mandarinas se llevó a cabo siguiendo las directrices de Gacula (2008). Se utilizó un panel de catadores compuesto por 10 jueces de edades comprendidas entre 20 y 70 años (6 hombres y 4 mujeres). La evaluación sensorial se realizó en una sala correctamente acondicionada con cabinas separadas para cada catador. Las frutas se valoraron externa e internamente en una escala hedónica de 1 a 10, siendo 1 muy desagradable y 10 muy agradable. La valoración externa incluía el tamaño, el color, la forma y la facilidad de pelado. La valoración interna consistía en el sabor, el dulzor, la presencia de semillas, la sensación en boca de la pulpa y la acidez de la fruta.

3.9. Análisis estadístico

Los resultados de todos los análisis se sometieron a un análisis de la varianza (ANOVA) de un factor, acompañado de una prueba HSD de Tukey ($p \leq 0,05$) para la separación de las medias, utilizando el programa Statgraphics-Centurion 18 (Chicago, IL, EE.UU.).

Para el análisis estadístico multivariante de los metabolitos de las hojas se empleó MetaboAnalyst 5.0, una tecnología JavaServer Faces de código abierto (Wishart Research Group, University of Alberta, Edmonton, AB, Canadá). Se usó el Análisis de Componentes Principales (PCA, por sus siglas en inglés), el Análisis Discriminante de Mínimos Cuadrados Parciales (PLSD-DA, por sus siglas en inglés) y su Variable de Importancia en la Proyección (VIP), el Análisis de Significación de Microarrays (SAM, por sus siglas en inglés), el mapa de calor de agrupación jerárquica y la Correlación Parcial Dispersa Desfasada (DSPC, por sus siglas en inglés) se calcularon siguiendo la metodología descrita por Melgarejo *et al.* (2023).

4. RESUMEN DE LOS RESULTADOS Y DISCUSIÓN

4.1. PUBLICACIÓN 1: Morphological and Biochemical Characterization of Late-Season Varieties of Mandarin Growing in Spain under Homogeneous Growing Conditions

4.1.1. Objetivos

Los objetivos de este estudio fueron por un lado caracterizar las principales propiedades morfológicas y bioquímicas de las variedades de mandarina tardía 'Afourer', 'Orri' y 'Tango', en condiciones homogéneas de cultivo. Con el fin de obtener información sobre las variedades más atractivas para el cultivo en función de la calidad de su fruta. Por otro lado, se buscó comparar las principales características de las mandarinas 'Tango' cuando se cultivan utilizando dos portainjertos diferentes: *Citrus macrophylla* y Forner Alcaide nº5. Las mandarinas empleadas en este estudio se cultivaron en múltiples fincas situadas en el Sureste de la Península Ibérica con condiciones homogéneas de cultivo.

4.1.2. Resumen de resultados y discusión

La caracterización física mostró diferencias significativas en parámetros como el peso de los frutos. En ese sentido, la variedad 'Afourer' presentó un peso significativamente mayor (120,75 g), superando en hasta un 20% a las otras dos variedades. Este peso es considerablemente superior al descrito por Tarancón *et al.* (2021) para la misma variedad, acercándose a las variedades más pesadas de híbridos de mandarina descritas en su estudio. A pesar de que el diámetro ecuatorial de 'Afourer' fue mayor, no hubo diferencias significativas en el diámetro polar entre las tres variedades. El número de carpelos por fruto fue similar entre las variedades, con un promedio de 9 carpelos por fruto. La mayoría de los frutos carecían de semillas, un rasgo deseable para los consumidores. El grosor de la piel no mostró diferencias significativas, variando entre 3,25 y 4,13 mm. No obstante, la cáscara de la variedad 'Afourer' fue más pesada (51,91 g por fruto), hasta un 18% más que las cáscaras de 'Orri' y 'Tango'. En cuanto al zumo, el peso y el volumen fueron superiores en 'Afourer' y 'Orri' en comparación con 'Tango'. Sin embargo, el rendimiento en zumo, calculado como porcentaje del volumen de zumo respecto al peso del fruto, fue mayor en 'Orri' (53,47%). Estos resultados difieren de otros estudios de híbridos de mandarina, sugiriendo variaciones significativas en el rendimiento del zumo entre estas variedades (Tarancón *et al.*, 2021). La diferencia en el rendimiento se atribuye a que, aunque 'Afourer' tiene un mayor peso, contiene más pulpa. Así, es probable que los consumidores prefieran 'Orri' por tener unos carpelos más jugosos. En cuanto a la variación de las propiedades físicas de la variedad Tango con el portainjerto, hay que destacar que las mandarinas cultivadas sobre FA pesaron 13 g más que las cultivadas sobre CM y tuvieron un mayor diámetro ecuatorial. No se observaron diferencias en los carpelos y semillas. El peso y volumen de zumo fue estadísticamente superior al presente en el patrón CM, aunque no se presenciaron diferencias en el rendimiento de zumo por mandarina.

Según los análisis sensoriales, el color del fruto es un parámetro importante para la percepción de la calidad, especialmente el brillo y el tono anaranjado (Gámbaro *et al.*, 2021). Los resultados mostraron que la variedad 'Orri' presentó un mayor brillo, no obstante, tuvo un menor tono anaranjado tanto en la piel como en el zumo, con respecto a las otras variedades estudiadas. En cuanto al efecto del portainjerto se observó que las mandarinas 'Tango' cultivadas sobre CM tuvieron un mayor brillo, mientras que el patrón FA presentó un color más anaranjado en piel y zumo.

El análisis de los parámetros químicos evidenció que la variedad 'Afourer' tuvo el menor pH (3,69) y la variedad 'Orri' el mayor (4,24). La acidez valorable fue hasta 2 g de ácido cítrico/L superior en las variedades "Afourer" y "Orri" que en la variedad "Tango". Los sólidos solubles totales fueron mayores en los frutos de 'Orri' (13,72 ºBrix), lo que nos revelaría que esta variedad tiene un mayor dulzor debido a que el 80% de los SST son azúcares (Shorbagi *et al.*, 2022). No se observaron diferencias significativas en el índice de madurez entre las tres variedades estudiadas. En todos los casos se alcanzó el IM mínimo de 6,5 para la comercialización de mandarinas de ciclo tardío (Quinza-Guerrero & López-Marcos, 1978). En lo referente al efecto del patrón en las propiedades químicas, la variedad 'Tango' cultivada sobre FA mostró un mayor pH (4,00) y acidez titulable (9,30 g de ácido cítrico/L) que en el patrón CM. Los SST de FA fueron 11,72 ºBrix, similares a los presentes en CM, aunque ligeramente inferiores a los mostrados para esta misma variedad por Morales *et al.* (2021).

La cuantificación de fenoles totales en el zumo nos permite estimar la actividad antioxidante de los frutos (Kumar & Goel, 2019). Los resultados mostraron que la variedad ‘Orri’ (1097,21 mg AGE/L) tuvo hasta un 40% más de fenoles que la variedad ‘Tango’ (752,14 mg AGE/L). Los resultados de estas variedades tardías fueron inferiores a los descritos para clementinas en otro estudio (Roussos *et al.*, 2019). Por otro lado, mediante los métodos ABTS y DPPH no se observaron diferencias significativas en la actividad antioxidante. En lo que respecta al efecto del portainjerto, los frutos de la variedad 'Tango' cultivados en FA tuvieron hasta un 28% más de fenoles que en CM.

Mediante HPLC se detectó en el zumo la presencia de los ácidos orgánicos cítrico, málico, ascórbico y succínico. El perfil de ácidos orgánicos varió entre las variedades estudiadas. Mientras que las variedades ‘Afourer’ (1,02 g/100 mL) y ‘Tango’ (0,79 g/100 mL) tuvieron el ácido cítrico como ácido mayoritario, siendo esto lo común en las mandarinas, la variedad ‘Orri’ presentó mayores concentraciones el ácido málico (0,61 g/100 mL) (Karadeniz, 2004). Esta mayor cantidad de ácido málico podría deberse a una adaptación de ‘Orri’ a climas secos (He *et al.*, 2022). Aunque la mayor presencia de ácido málico puede dar lugar a un sabor ligeramente diferente, éste no debería influir en el sabor característico de estas mandarinas (Shi *et al.*, 2022). En cuanto al resto de ácidos, la variedad ‘Orri’ tuvo una menor cantidad de ácido succínico, pero una mayor cantidad de fórmico. Los valores de ácido succínico en 'Afourer' y 'Tango' son sustancialmente superiores que en otras mandarinas tardías (Al-Saif *et al.*, 2022). No hubo diferencias significativas en las concentraciones de ácido ascórbico. La variedad 'Orri' (1,44 g/100 mL) tuvo una menor cantidad de ácidos en comparación con 'Afourer' y 'Tango' (2,3 g/100 mL, 2,07 g/100mL). Por otro lado, el patrón FA en la variedad ‘Tango’ produjo una mayor concentración de ácido cítrico (0,99 g/100 mL).

Los azúcares detectados por HPLC fueron sacarosa, fructosa y glucosa. La relación de concentración de sacarosa, fructosa y glucosa es normalmente 2:1:1, una relación similar fue identificada en todos los frutos analizados (Kelebek & Selli, 2011). La variedad ‘Orri’ destacó por una concentración de azúcares 18% mayor, observándose cantidades significativamente mayores de sacarosa y glucosa. Los valores de glucosa y fructosa fueron superiores a los descritos por

Sdiri *et al.* (2012) en otras variedades de mandarinas tardías. No se demostró un efecto significativo del portainjerto en las concentraciones de azúcares en las mandarinas ‘Tango’.

El análisis metabolómico con ^{1}H NMR del zumo, mostró la presencia de 7 aminoácidos: GABA, alanina, arginina, asparagina, aspartato, glutamina, leucina, isoleucina, prolina, tirosina y valina, siendo el aspartato el más abundante. Aunque encontramos algunos aminoácidos esenciales como la leucina, la mayoría de ellos no son considerados esenciales (Liu *et al.*, 2012). No se encontraron diferencias significativas entre las variedades exceptuando la prolina. Este aminoácido que está estrechamente ligado a la respuesta de la planta frente al estrés abiótico, se encontró en mayor concentración en la variedad ‘Orri’ (Hosseinifard *et al.*, 2022). Paralelamente, la variedad 'Tango' cultivada sobre el patrón CM presenta una mayor cantidad de GABA, asparagina, ácido aspártico y tirosina. Estos metabolitos están implicados en la respuesta al estrés biótico y abiótico, pudiendo indicar que este patrón puede tener una mayor resistencia a ciertos tipos de estrés (Bown *et al.*, 2006; Okumoto *et al.*, 2021).

Otros metabolitos detectados del zumo fueron colina, etanol y trigonelina, no encontrándose diferencias entre las variedades. Sin embargo, se observó una mayor cantidad de etanol en la variedad 'Tango' cultivada en FA que en CM. El etanol fue el metabolito secundario más abundante, con concentraciones entre 1,08 mM y 5,9 mM, estando relacionado con la maduración del fruto y en la generación de compuestos volátiles que dan los aromas (Pesis, 2005). En menor cantidad encontramos la colina, una biomolécula que interviene en la formación de las membranas celulares y del neurotransmisor acetilcolina (Blusztajn, 1998). La trigonelina es un alcaloide que se acumula en las plantas en condiciones de estrés biótico y abiótico, y junto con la colina puede proteger a la planta de infecciones fúngicas (Mohamadi *et al.*, 2018; Schoeppner & Kindl, 1979).

4.1.3. Conclusiones

La caracterización de tres variedades tardías de mandarina en condiciones de cultivo homogéneas nos muestra diferencias significativas entre ellas. 'Afourer' destaca por tener un 20% más de tamaño y peso, además de ser más anaranjada. 'Orri' destacó por tener una gran cantidad de zumo, un mayor brillo y una concentración un 18% mayor de azúcares. Su perfil de ácidos orgánicos destaca por tener una mayor cantidad de ácido málico que de cítrico. No hubo diferencias en actividad antioxidante entre las variedades. Paralelamente, se analizaron mandarinas 'Tango' cultivadas sobre el portainjerto FA, presentando un mayor peso y tamaño, así como una tonalidad más anaranjada que los cultivados sobre CM, no observándose grandes diferencias en los ácidos orgánicos y azúcares. La actividad antioxidante total fue similar, aunque se constató hasta un 28% más de fenoles en el portainjerto FA. Todo ello indica que los frutos de ambos portainjertos tendrán una calidad organoléptica muy similar. El análisis metabolómico reveló que todas las muestras de mandarina analizadas contenían predominantemente aminoácidos no esenciales, detectándose otros metabolitos como etanol, colina o trigonelina. El portainjerto afectó significativamente en las concentraciones de aminoácidos como GABA o el ácido aspártico.

4.2. PUBLICACIÓN 2: Influence of rootstock on yield, morphological, biochemical and sensory characteristics of ’Afourer’ variety mandarins

4.2.1. Objetivos

El objetivo de este estudio es investigar la influencia del portainjerto sobre el rendimiento, las características morfológicas, la composición bioquímica y los atributos sensoriales de la variedad de mandarina tardías 'Afourer' (*Citrus reticulata* Blanco). Para ello, se recolectaron frutos de esta variedad cultivados sobre tres portainjertos diferentes (CM, MC y CC), en condiciones homogéneas de cultivo.

4.2.2. Resumen de resultados y discusión

El portainjerto tiene un impacto significativo en los parámetros agronómicos, incluyendo el rendimiento de fruta (de Carvalho *et al.*, 2021) y por ello se comparó el rendimiento de producción de mandarinas 'Afourer' entre los patrones MC y CC, los árboles cultivados sobre CC produjeron hasta 77 kg más de fruta que en MC. Este hallazgo es corroborado por Martínez-Cuenca *et al.* (2016). Estos resultados resaltan el valor del portainjerto CC.

La caracterización morfológica de los frutos cultivados sobre diferentes portainjertos evidenció que las mandarinas cultivadas sobre CC tuvieron hasta un 11% más de peso. El diámetro ecuatorial fue mayor en las mandarinas cultivas en MC y CC. El mayor peso observado en el patrón CC también fue reportado en naranjas 'Navelina' (Forner-Giner *et al.*, 2003). Al abrir los frutos, los cultivados en CM tuvieron de media un gajo más por mandarina y un mayor grosor de piel. Ninguna mandarina estudiada presentó semillas. El análisis de los parámetros del zumo reveló que las mandarinas cultivadas sobre el patrón CM tuvieron un menor peso y volumen de zumo, encontrándose los valores más altos en CC. Los frutos cultivados sobre portainjertos CC y MC serán más jugosos y atractivos para los consumidores, al tener hasta un 17% más de zumo. Estas variaciones en el contenido de zumo se atribuyen al mayor peso y diámetro de los frutos observados en CC y MC. Por otro lado, el portainjerto sobre el que se cultiva los cítricos puede influir en el color externo e interno del fruto (Aguilar-Hernández *et al.*, 2020; Morales *et al.*, 2021). La tonalidad anaranjada de la mandarina es un factor importante que influye en la percepción de los consumidores. Los resultados obtenidos muestran un mayor tono anaranjado en CC. Esto es corroborado por otro estudio sobre naranjas 'Navelina' (M. A. Forner-Giner *et al.*, 2003). En cuanto al color del zumo se observó un tono más anaranjado en los frutos cultivados sobre CM.

Los valores de pH, acidez titulable, SST e índice de madurez determinan en gran medida el sabor y la calidad del fruto (Goldenberg *et al.*, 2018). No se encontraron diferencias significativas entre los pH zumo obtenido de los frutos de los tres portainjertos, con valores entre 3,68 y 4,01, valores de pH muy similares a los descritos por (Simón-Grao *et al.*, 2014). La acidez titulable de los frutos cultivados sobre CM fue de 3 g ácido cítrico/L superior. Esto indicaría que los frutos en MC tienden a reducir su acidez más rápidamente a medida que maduran, provocando que su sabor sea más insípido, ya que la acidez se diluye con el aumento del contenido de agua (Kimball, 1984; Morales *et al.*, 2021). Esto se constata también en el hecho de que los frutos cultivados sobre CM tuvieron un mayor índice de madurez (14,51). Las mandarinas cultivadas en CC (13,02 ºBrix) también presentaron un SST mayor que los cultivados en CM (10,04 ºBrix). Los SST son mayoritariamente azúcares, por lo que esto podría indicar que los frutos cultivados en CC se percibirán más dulces (Shorbagi *et al.*, 2022).

Algunos estudios han confirmado que el portainjerto es capaz alterar las concentraciones de compuestos fenólicos y la actividad antioxidante total en cítricos (Rapisarda *et al.*, 2022). En este caso, no se observaron diferencias significativas en la cantidad de fenoles entre los tres portainjertos, algo que contrasta con los resultados encontrados por Legua *et al.* (2014) en mandarinas 'Clemenules'. Por otro lado, de acuerdo con los resultados obtenidos por el método ABTS, el portainjerto CM mostró una mayor actividad antioxidante.

Mediante HPLC se identificaron los ácidos orgánicos cítrico, málico, ascórbico y succínico. El ácido mayoritario en los tres portainjertos fue el ácido cítrico, con valores que oscilaron entre 0,99 g/100 mL y 1,02 g/100 mL. En otro estudio, con mandarinas 'Clemenules', el portainjerto MC tenía una cantidad mayor de este ácido (Legua *et al.*, 2014). Se detectaron 0,02 g/100 mL de ácido ascórbico. Las cantidades de ácido málico y succínico fueron estadísticamente superiores en los frutos cultivados sobre CM (0,32 g/100 mL, 0,85 g/100 mL, respectivamente). El ácido fórmico, sin embargo, se encontró en concentraciones más bajas en los frutos cultivados sobre MC que CC. Aún con las variaciones anteriormente descritas, no se observaron diferencias significativas en los ácidos orgánicos totales.

En total se identificaron 3 azúcares, la sacarosa, la glucosa y la fructosa mediante HPLC. Existen evidencias en mandarino y naranjo de que el portainjerto sobre el que se cultivan influye directamente en las concentraciones de azúcares en los frutos (Barry *et al.*, 2004; Legua *et al.*, 2014). Nuestros resultados no mostraron diferencias en los valores de sacarosa, con concentraciones de entre 5,34 y 5,67 g/100 mL. Sin embargo, el patrón CM presentó hasta un 13% más de fructosa. Los valores de glucosa en los tres portainjertos fueron similares. Tampoco hubo diferencias significativas en la suma total de azúcares.

El análisis metabolómico del zumo mediante ^1H NMR nos aporta información valiosa sobre las propiedades nutricionales y organolépticas de las mandarinas, así como información sobre su adaptación al medio. Los aminoácidos más abundantes detectados fueron ácido aspártico, arginina, asparagina, prolina y tirosina; aminoácidos no esenciales para el ser humano (Liu *et al.*, 2012). El ácido aspártico estaría implicado en el correcto crecimiento de la planta y en su respuesta al estrés abiótico (Okumoto *et al.*, 2021; Rizwan *et al.*, 2017). Los resultados indican que el portainjerto no alteró las concentraciones medias de la mayoría de aminoácidos y otros metabolitos. La Leucina no fue detectada en los portainjertos CM y MC, posiblemente por encontrarse en concentraciones muy bajas al igual que en CC. La prolina se encontró menor cantidad en el patrón CM. Esto podría deberse a que la prolina es uno de los principales aportadores de Nitrógeno orgánico e interviene en la maduración del fruto; a mayor madurez, más prolina se habrá metabolizado (Xiong *et al.*, 2023).

Otros metabolitos detectados en el zumo fueron el etanol, la colina y la trigonielina. Compuestos implicados en conferir olor a los frutos, ser precursor del neurotransmisor acetilcolina y ayudar a las plantas contra fuentes de estrés biótico y abiótico (Blusztajn, 1998; Mohamadi *et al.*, 2018; Pesis, 2005).

El análisis sensorial proporciona información valiosa sobre la percepción del consumidor de la calidad del fruto, ya que muchas de las diferencias en los parámetros morfológicos y bioquímicos que pueden detectarse mediante análisis de laboratorio pueden no ser perceptibles para el consumidor medio. En este estudio los consumidores realizaron una valoración externa e interna de estas mandarinas. En la evaluación externa, los frutos cultivados sobre MC fueron mejor valorados en cuanto a tamaño y facilidad de pelado. En comparación con los resultados de nuestro estudio, las mandarinas de mayor tamaño son mejor valoradas. En cuanto al color y la forma, no se observaron diferencias significativas, lo cual contrasta con los resultados sobre el color obtenidos en la caracterización física de este estudio, las diferencias en la tonalidad anaranjada no fueron valoradas por los jueces. En cuanto a la evaluación interna, una vez consumidos los portainjertos MC y CC tuvieron mejores calificaciones en sabor, dulzor y pulpa que el portainjerto CM, que se caracterizó por tener una menor acidez, por lo que resaltan por ser más insípidos.

4.2.3. Conclusiones

Se analizó la influencia del portainjerto en la producción de fruta y en las principales características morfológicas, bioquímicas y sensoriales de las mandarinas 'Afourer'. El portainjerto CC fue el más productivo, con hasta 77 kg/árbol más que el portainjerto MC, haciéndolo más interesante para el agricultor. Los frutos cultivados sobre portainjertos MC y CC tuvieron un mayor tamaño, hasta un 17 % más de rendimiento en zumo y un tono más anaranjado. Estos portainjertos también presentaron una mayor acidez titulable y SST. Por otra parte, el patrón CM mostró una mayor actividad antioxidante total por el método ABTS. Destacó el ácido cítrico como el ácido mayoritario. Las concentraciones de azúcares fueron similares en los tres portainjertos a excepción de la fructosa, que presentó hasta un 13 % más de este azúcar en CM. Los resultados de la metabolómica del zumo muestran que todas las muestras de mandarina analizadas en este estudio contenían principalmente aminoácidos no esenciales; el ácido aspártico fue el mayoritario. Otros metabolitos implicados en el olor y la resistencia al estrés abiótico y biótico como el etanol, la colina y la trigonielina fueron detectados. En las condiciones de cultivo estudiadas, los portainjertos más interesantes según el conjunto de características estudiadas, y el análisis sensorial, fueron MC y CC.

4.3. PUBLICACIÓN 3: Mandarin Variety Significantly Affects the Metabolites Present in the Leaves

4.3.1. Objetivos

El objetivo de este estudio fue determinar y comparar los metabolitos presentes en las hojas de tres variedades comerciales de mandarina tardías ('Afourer', 'Orri' y 'Tango') bajo condiciones homogéneas de cultivo. El análisis de metabolitos se realizó mediante resonancia magnética nuclear (^{1}H NMR) y los resultados obtenidos se analizaron mediante análisis multivariante de datos.

4.3.2. Resumen de resultados y discusión

Los principales metabolitos identificados mediante resonancia magnética nuclear de las hojas de mandarina fueron aminoácidos, ácidos orgánicos y azúcares. Se identificaron diez aminoácidos diferentes en las hojas de las variedades "Afourer" y "Tango", mientras que solo nueve en la variedad "Orri"; en esta última no se detectó asparagina. La prolina destacó por ser el más abundante. Se identificándose cinco ácidos orgánicos en las hojas: citrato, fórmico, fumarato, malato y quinato. El malato fue el ácido orgánico mayoritario en las variedades 'Afourer' y 'Tango', mientras que el quinato lo fue en 'Orri'. En lo referente a los azúcares el más abundante fue la sacarosa, seguida de la glucosa y el mio-inositol; también se detectó maltosa en cantidades menores. Otros metabolitos identificados fueron la colina y la trigonelina.

El análisis de los metabolitos proporciona información valiosa sobre las características agronómicas, el desarrollo vegetativo y la resistencia a estreses abióticos y bióticos de los mandarinos (Asai *et al.*, 2017; Hildebrandt, 2018). La presencia de prolina como principal aminoácido, junto con la colina y trigonelina, demuestran que estas tres variedades están ampliamente adaptadas al cultivo en zonas con un elevado estrés abiótico de sequía y salinidad (Cho *et al.*, 1999; Ghosh *et al.*, 2022; Yamada *et al.*, 2009). Estreses presentes en el Sureste de la Península Ibérica, lugar donde se recogieron las muestras, y una de las zonas con mayor producción mundial de mandarinas (FAO, 2021). La elección de una variedad con mayor resistencia a los factores abióticos es crucial en el escenario de cambio climático al que nos enfrentamos, el cultivo de mandarinas más resistentes ahorrará costes de cultivo y asegurará mayores rendimientos. El cultivo de las variedades 'Tango' y 'Orri' resulta de mayor interés por sus altas concentraciones de metabolitos como GABA en las ‘Tango’ e isoleucina en las ‘Orri’ (Cheng *et al.*, 2023; Pandita, 2022).

Paralelamente, también es importante que los agricultores cultiven variedades resistentes a estreses bióticos, como a plagas de insectos. En este sentido, la variedad 'Orri' destaca por sus mayores concentraciones de quinato e isoleucina, los cuales le confieren una mayor protección frente a las plagas (Pandita, 2022). Por otro lado, 'Afourer' también sería de gran interés debido sus altas concentraciones de malato (Surekha *et al.*, 2013).

El desarrollo vegetativo es otro factor importante; un mayor crecimiento de los árboles permitirá a los agricultores disponer antes de plantaciones maduras. La variedad 'Afourer' destaca aquí por sus mayores concentraciones de asparagina, metabolito estrechamente relacionado con el crecimiento vegetativo (Gaufichon *et al.*, 2010).

Por último, la calidad final de la fruta es fundamental para los citricultores. Según los resultados obtenidos, la variedad 'Orri' destacará por su mayor dulzor, ya que se detectó una cantidad mucho mayor de sacarosa y glucosa en las hojas que en las otras dos variedades, lo que se correlaciona con el azúcar que estará presente finalmente en el fruto (Katz *et al.*, 2007; Lourkisti *et al.*, 2023).

Adicionalmente, se estudiaron con más detenimiento y con diversos análisis estadísticos las propiedades funcionales de los metabolitos presentes en las hojas. Solo se hallaron diferencias significativas en cuatro aminoácidos (GABA, asparagina, aspartato e isoleucina). Se observó una concentración 53% mayor de GABA en las hojas de la variedad ‘Tango’. Esto nos indica que esta variedad esta mejor adaptada a la sequía y al cambio climático, presentes en zonas con altas producciones de mandarinas, como en el sureste español (Huang *et al.*, 2022; Zhang *et al.*, 2023). Las concentraciones de GABA en hojas están estrechamente relacionadas con la conversión de ácido cítrico en azúcares durante el proceso de maduración del fruto (Lourkisti *et al.*, 2023).

Por otra parte, las hojas de la variedad 'Afourer' contienen un 28% más de asparagina que las de la variedad 'Tango', mientras que este aminoácido no se detectó en la variedad 'Orri'. La asparagina desempeña un papel crucial en hojas de plantas adultas, participando principalmente en la absorción y movilización de nitrógeno inorgánico durante el crecimiento, la floración y la senescencia de la planta (Gaufichon *et al.*, 2010). Una mayor concentración de este aminoácido en las hojas implica un mayor crecimiento vegetativo del árbol (Antunes *et al.*, 2008; Gaufichon *et al.*, 2010; Potel *et al.*, 2009). Su ausencia en 'Orri' podría deberse a que no había sido metabolizado en el momento del muestreo, ya que se detectaron precursores de este metabolito, como el aspartato (Potel *et al.*, 2009).

La variedad "Tango" también tenía un 64% más de aspartato. La función de este aminoácido en las plantas es formar otros nuevos, lo que aumenta la eficiencia del ciclo de Krebs y en consecuencia aumenta el crecimiento vegetativo de las plantas (Fait *et al.*, 2006; Galili, 2011; Gaufichon *et al.*, 2010).

Por otro lado, la variedad 'Orri' presentó hasta el doble de isoleucina que en 'Afourer' y 'Tango'. La presencia de isoleucina en las hojas está relacionada con la biosíntesis de jasmonato, un compuesto implicado en la respuesta fisiológica al estrés biótico y abiótico (Pandita, 2022).

No se observaron diferencias significativas en el resto de aminoácidos detectados, mostrando valores dentro del rango esperado para los cítricos (Sousa *et al.*, 2022).

El malato y el quinato fueron los ácidos más abundantes. La concentración de malato detectada fue hasta un 43% mayor en la variedad 'Afourer'. La presencia de malato en las plantas contribuye a procesos de crecimiento y desarrollo de las plantas; es uno de los principales compuestos implicados en la fotorrespiración (Dao *et al.*, 2022; Lindén *et al.*, 2016; Schneidereit *et al.*, 2006). El ácido málico en las hojas puede reducir el ataque de algunas plagas de lepidópteros (Surekha *et al.*, 2013 citando a Narayanamma *et al.*, 2007 y Yoshida *et al.*, 1997)

El quinato estuvo más presente en 'Orri', siendo el ácido orgánico mayoritario en esta variedad. Está relacionado con la resistencia de las plantas al estrés biótico, siendo un precursor de varios ácidos orgánicos que contribuyen a la protección de las hojas frente al ataque de insectos (Carrington *et al.*, 2018, citando a Leiss *et al.*, 2009). Además, está implicado en las vías metabólicas del Shikimato, que son esenciales en la biosíntesis de aminoácidos aromáticos (Carrington *et al.*, 2018; Rocha-Santos & Duarte, 2014).

El principal azúcar detectado en las hojas fue la sacarosa, seguida de la glucosa y el mio-inositol; también se detectó maltosa en cantidades menores. Esto contrasta con otros estudios realizados en hojas de limonero, que muestran que la maltosa es el azúcar mayoritario (Melgarejo *et al.*, 2022). La sacarosa estaba más presente en las variedades 'Orri' y 'Tango'. Este azúcar es el principal proveedor energético de estas plantas, crucial para su metabolismo y desarrollo (Kelebek & Selli, 2011). La presencia de este azúcar está ligada con la calidad final del fruto, ya que durante el proceso de maduración la sacarosa se moviliza desde las hojas al fruto, aportando gran parte del sabor dulzor característico. (Katz *et al.*, 2007; Kelebek & Selli, 2011; Leiss *et al.*, 2009; Lourkisti *et al.*, 2023).

Por otro lado, 'Orri' mostró hasta 2,3 veces más glucosa que las otras dos variedades estudiadas. Una mayor concentración de glucosa se asocia con un mayor dulzor en el fruto (Katz *et al.*, 2011). Un estudio previo realizado 'Orri' tiene un mayor contenido en azúcar (Maciá-Vázquez *et al.*, 2023).

Las concentraciones de colina fueron mayores en las hojas de 'Orri'. La colina es una biomolécula sintetizada por las plantas, precursora de la betaína, un compuesto relacionado con la respuesta de las plantas al estrés biótico y abiótico (Chen *et al.*, 2024).

Mientras tanto, la presencia de trigonelina en las muestras de ‘Afourer’ y ‘Tango’ alcanzó el doble de concentración que en ‘Orri’. Este biocompuesto actúa como osmolito y se acumula en las plantas en condiciones de estrés biótico y abiótico (Mohamadi *et al.*, 2018). Los estudios muestran que este compuesto junto con la colina está implicado en la respuesta de las plantas frente estrés salino (Cho *et al.*, 1999; Nuccio *et al.*, 2000; Yamada *et al.*, 2009). Esto es de gran interés para la agricultura debido al estrés por salinidad en las regiones mediterráneas, siendo este uno de los principales problemas para los cultivos de cítricos (Syvertsen & Garcia-Sanchez, 2014). El problema de la salinidad suele resolverse mediante la elección de portainjertos mejor adaptados (Pérez-Pérez *et al.*, 2007; Rodríguez-Gamir *et al.*, 2010). Sin embargo, este estudio muestra cómo la elección de la variedad es también de gran importancia, ya que observamos diferencias en metabolitos implicados en la respuesta al estrés, incluso utilizando el mismo portainjerto (*Citrus macrophylla*).

El análisis de los resultados mediante PCA es un método de análisis multivariante que nos permite visualizar las varianzas dimensiones más pequeñas mediante una representación gráfica (Jolliffe & Cadima, 2016). El PCA muestra que el Componente 1 representa el 79,7% de la varianza total entre variedades, mientras que el Componente 2 representa el 19,3% de la variabilidad total.

La regresión PLS-DA y el VIP nos muestran qué metabolitos son significativos y diferenciadores entre las tres variedades (Janková & van de Geer, 2015; Lee *et al.*, 2018). Los resultados muestran que sólo el malato, la prolina y la sacarosa fueron los metabolitos que tuvieron un VIP mayor que 1, por lo que estos compuestos tuvieron las mayores diferencias significativas entre las tres variedades.

Por otra parte, el mapa de calor de agrupación jerárquica muestra visualmente con una escala de colores las concentraciones relativas de cada uno de los metabolitos detectados (Wilkinson & Friendly, 2009). El mapa de calor mostró que la variedad 'Orri' se caracterizaba por mayores cantidades de colina, quinato y formico, así como del azúcar glucosa y los aminoácidos treonina, alanina o isoleucina. La variedad "Afourer" por una mayor concentración de malato y citrato, azúcar maltosa y los aminoácidos asparagina y tirosina. La variedad "Tango" por una mayor concentración de los aminoácidos GABA, aspartato y prolina.

El análisis de significación de microarrays (SAM) nos proporciona información sobre qué metabolitos presentan las mayores diferencias significativas (Tusher *et al.*, 2001). El SAM y el análisis cluster de nuestros resultados muestran que el malato y el quinato en mayor medida y la sacarosa y la prolina en menor, fueron los metabolitos con mayor significanciá. A través del cluster, también encontramos que las variedades 'Afourer' y 'Tango' están más correlacionadas entre sí que la variedad 'Orri', lo que se debe principalmente a que la variedad 'Tango' se obtuvo de la hibridación de la variedad 'Afourer' (Roose & Williams, 2007).

El análisis de las rutas metabólicas se llevó a cabo aplicando el algoritmo de Correlación Parcial Desviada (DSPC) a los resultados obtenidos en ^{1}H NMR. Este análisis permite identificar gráficamente la correlación, mediante rutas metabólicas, entre los diferentes metabolitos presentes en las hojas (Basu *et al.*, 2017). El análisis muestra que la asparagina tiene el mayor grado de interrelación, ya que está implicado en muchas de las rutas metabólicas del resto de compuestos detectados. Esta molécula está relacionada positivamente con la trigonelina, el malato, el quinato y el citrato, y negativamente con el fumarato, la valina, la glucosa, la colina, la isoleucina y la treonina.

La isoleucina también tuvo una presencia importante en las interrelaciones metabolómicas. La mayoría de sus relaciones fueron positivas (leucina, alanina, treonina, asparagina, valina y glucosa), y sólo tres fueron negativas (malato, citrato y quinato). La isoleucina participa en el ciclo de Krebs, lo que implica que una mayor cantidad de este compuesto está relacionada con una menor cantidad de malato y citrato, mientras que está directamente relacionada con la glucosa (Gorissen & Phillips, 2019).

La valina y la glucosa tuvieron también una alta interrelación, ya que son compuestos relacionados con el ciclo de Krebs. Estos compuestos están relacionados con la síntesis y degradación de una amplia variedad de aminoácidos, ácidos y azúcares (Duggleby *et al.*, 2008; Kumari, 2023).

4.3.3. Conclusiones

En este estudio, se realizó un análisis metabolómico de las hojas de tres variedades de mandarina tardías ('Afourer', 'Orri', y 'Tango') cultivadas en condiciones homogéneas sobre *Citrus macrophylla* para caracterizar los metabolitos presentes en las hojas de estas mandarinas y las diferencias entre variedades. Los metabolitos más abundantes encontrados fueron ácidos orgánicos y azúcares, principalmente malato, quinato y sacarosa. También se identificaron diez aminoácidos, destacando por su concentración la prolina. El análisis de correlación mostró que la asparagina y la isoleucina estaban implicadas en la mayoría de las rutas metabólicas. El análisis estadístico multivariante realizado muestra claramente que existen diferencias significativas entre las concentraciones de metabolitos presentes en las tres variedades estudiadas, especialmente en compuestos como el malato, el quinato y la sacarosa. La variedad "Orri" tiene un perfil metabolómico más diferenciado en comparación con las otras variedades estudiadas. Desde el punto de vista agronómico, la variedad "Afourer" destaca sobre las otras dos variedades por su mayor crecimiento vegetativo. La variedad 'Tango' destaca por su mayor resistencia al estrés abiótico. Pero, sobre todo, la variedad 'Orri' destaca sobre las otras dos variedades porque, aunque tiene un menor crecimiento vegetativo que las otras dos variedades, tiene una buena resistencia tanto a estreses abióticos como bióticos y sus frutos suelen ser más dulces y de mayor calidad.

5. CONCLUSIONES GENERALES

Una vez presentados los resultados y con conclusiones relativas a cada una de las publicaciones. Como conclusiones generales de esta Tesis Doctoral, se podría afirmar que:

- Las características de las mandarinas varían significativamente con la variedad y el portainjerto utilizado; en condiciones homogéneas de cultivo.
- La selección de la variedad y el portainjerto adecuado es importante para obtener mandarinas de alta calidad.
- La variedad 'Afourer' presenta frutos más grandes, de mayor peso y con un color más anaranjado, que las otras variedades estudiadas.
- La variedad 'Orri' tiene el mayor rendimiento de zumo, brillo de la piel, cantidad de azúcares, y un perfil de ácidos orgánicos único.
- El ácido málico es el ácido orgánico mayoritario en las mandarinas 'Orri' y no el cítrico.
- El análisis metabolómico del zumo demostró ser una herramienta útil para estudiar la calidad de la mandarina.
- Para todas las variedades y combinaciones de portainjertos, el zumo de las mandarinas contiene principalmente aminoácidos no esenciales.
- La mandarina 'Tango' cultivada en el portainjerto FA fue más grande, pesada, anaranjada y con mayor contenido de fenoles totales que cultivada CM.
- El portainjerto CC en la variedad 'Afourer' produce una mayor cantidad de fruta que el patrón MC.
- Los frutos cultivados sobre portainjertos MC y CC son tuvieron un mayor tamaño, rendimiento de zumo, acidez titulable, SST y un tono más anaranjado.
- El patrón CM mostró una mayor actividad antioxidante total por el método ABTS y una mayor concentración de fructosa.

- Los portainjertos más interesantes para su uso en la agricultura, según el conjunto de características estudiadas y el análisis sensorial, fueron MC y CC.
- Los metabolitos más abundantes en las hojas de mandarino fueron ácidos orgánicos (malato y quinato) y azúcares (sacarosa).
- La variedad "Afourer" destaca desde el punto de vista agronómico por tener metabolitos en sus hojas implicados en un mayor crecimiento vegetativo.
- La variedad 'Tango' destacó agronómicamente por tener metabolitos en sus hojas relacionados con una mayor resistencia al estrés abiótico.
- La variedad 'Orri' se diferencia de las otras dos variedades por tener un perfil metabolómico único, relacionado con una mayor resistencia al estrés abiótico y biótico, y una mayor calidad de fruto.
- La variedad 'Orri' es la más recomendable para su cultivo, debido a que presenta características morfológicas, bioquímicas y agronómicas más destacables.

6. INVESTIGACIONES FUTURAS

Con base en los estudios, sus resultados y las conclusiones generales alcanzadas en esta Tesis Doctoral, sería de gran interés estudiar a continuación:

- El análisis de aún más combinaciones de variedad/portainjerto en condiciones homogéneas de cultivo.
- Realizar un análisis más exhaustivo de las cualidades nutricionales y nutraceúticas de las variedades tardías, como el análisis de compuestos fenólicos.
- Los compuestos volátiles presentes en las variedades tardías de mandarinas para investigar que influencia tiene el portainjerto en ellos.
- Analizar la influencia del portainjerto en los metabolitos presentes en las hojas.
- Realizar un análisis morfológico y fotosintético de las hojas de las variedades tardías y relacionarlo con los resultados obtenidos en compuestos metabolómicos.
- Estudiar metabolómicamente otros tejidos vegetales de los mandarinos tardíos.

7. REFERENCIAS

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ANEXO. COPIA DE LAS SEPARATAS DE LOS ARTÍCULOS

PUBLICACIÓN 1
Morphological and Biochemical Characterization of Late-Season Varieties of Mandarin Growing in Spain under Homogeneous Growing Conditions
Alejandro Andy Maciá-Vázquez, Dámaris Núñez-Gómez, Juan José Martínez-Nicolás, Pilar Legua and Pablo Melgarejo
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PUBLICACIÓN 2
Influence of rootstock on yield, morphological, biochemical and sensory characteristics of ’Afourer’ variety mandarins
Alejandro Andy Maciá-Vázquez, Juan José Martínez-Nicolás, Dámaris Núñez-Gómez Pablo Melgarejo and Pilar Legua
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Factor de Impacto: 4.3 (2021) (Categoría Horticulture)

PUBLICACIÓN 3
Mandarin Variety Significantly Affects the Metabolites Present in the Leaves
Alejandro Andy Maciá-Vázquez, Dámaris Núñez-Gómez, Juan José Martínez-Nicolás, Pilar Legua and Pablo Melgarejo
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Communication

Morphological and Biochemical Characterization of Late-Season Varieties of Mandarin Growing in Spain under Homogeneous Growing Conditions

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Abstract: Mandarins are one of the most important citrus fruits in the world in terms of tons produced. The late-season varieties of mandarin have a great economic value due to their high production in a season with few mandarin varieties. The objective is to carry out a preliminary study of characterization and comparison of the morphological and biochemical properties of the late varieties 'Afourer', 'Tango', and 'Orri'. The characterization consisted of physicochemical parameters related to the quality of the fruits, highlighting the total antioxidant activity using ABTS and DPPH, the organic acids and sugars using HPLC and the metabolomics of the juice by ^{1}H-NMR. Afourer' mandarins were heavier and larger (120.75 g, 67.60 mm) than the other two varieties studied. Mandarins of the 'Orri' variety showed a different organic acid profile compared to the other varieties studied, and a higher amount of sugars (13.49 g/100 mL). 'Tango' variety mandarins grown on the Forner-Alcaide rootstock stood out for having a larger weight (113.52 g), a more intense color, and a greater amount of phenolic compounds (966.85 mg AGE/L Forner) than the fruits grown on *Citrus macrophylla*. The metabolomics analysis showed that these mandarin varieties had mainly non-essential amino acids.

Keywords: mandarin; late-season; Citrus rootstock; morphology; HPLC; metabolomics

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1. Introduction

Mandarins are one of the most important citrus fruits in the world in terms of tons produced. Asia has historically been the largest producer of this fruit, with 71.33% of the total world production, in 2019 China produced 52.62% of the world's mandarins [1]. Other large producers of mandarins in the world are Turkey (3.74%), Morocco (3.67%) or and Egypt (2.93 %) [1]. The largest exporter of mandarins in the world is Spain, which represents 26.77% of the traded tons of this citrus [1]. In Spain, mandarins account for almost 20% of the total citrus produced. Its high production and export make this fruit a key economic element for this sector [2].

The genus *Citrus* has a large number of varieties, cultivars, and hybrids that differ greatly in terms of their morphological and genetic traits, as well as in terms of their growth patterns and yield production [3]. Mandarins, *Citrus reticulata*, therefore have a wide number of varieties, among which the late-season varieties stand out. Some outstanding examples of late mandarin varieties are 'Afourer', also known as 'Nadorcott', was bred in 1988 in Morocco and its origin could be the hybridization between 'Murcott' variety and an unknown pollinator parent ('Murcott' is a tangor hybrid of tangerine and orange, *Citrus reticulata* Blanco × *Citrus sinensis* (L.) Osbeck) [4]. 'Orri' is a another late-season mandarin originated in Israel from irradiated bud wood of the cultivar 'Orah' ['Orah' is hybrid of 'Temple' (*Citrus temple* hort. ex Y. Tanaka) × 'Dancy' (*Citrus tangerina* hort. ex Tanaka)] [5]. 'Tango' mandarin is a hybrid citrus late-season fruit that was developed at the University of California, Riverside from an irradiated bud of the diploid mandarin hybrid 'Murcott' [6].

Mandarins, according to their ripening, can be classified into early, mid and late-season varieties. Late-season varieties are of great economic interest, for being collected in a winter period in which productions are limited and for their higher productivity. This late-season harvest provides the product with a high value, so it is of great interest to know the characteristics and agronomic-commercial advantages that each one of them presents. So that citrus growers can choose those varieties or variety/rootstock combinations that best suit their objectives.

The quality of mandarins and their appeal to consumers are determined by a combination of morphological and biochemial properties. From a morphological perspective, consumers value attributes such as color, the ease of peeling, the presence or absence of seeds or the amount of juice. While the flavor and nutritional properties of the fruits will be given by biochemical parameters such as acidity, maturity index, antioxidant activity, sugar content or organic acid concentration [7–10].

Mandarins have a high nutritional value. The main biochemical components of these fruits are sugars, organic acids, carotenoids, polyphenols (flavonoids and phenolic acids), limonoids and vitamins, highlighting vitamin C of the latter [11]. Ascorbic acid (vitamin C) is an organic acid that plays an important role in the prevention of many diseases, thanks to its antioxidant, anti-inflammatory and anticancer capacity [12–14].

The biochemical and morphological properties of the fruits can be different depending on the rootstock on which it is grown [15]. Citrus growers commonly choose the *Citrus macrophylla* rootstock to grow citrus because of its fast and abundant production [16]. However, this rootstock is sensitive to CTV (citrus tristeza virus) and to the attack of nematodes, which is why some farmers decide to cultivate their trees on a rootstock resistant to these diseases, such as Forner-Alcaide nº5 [16].

The objectives of this preliminary study were twofold. Firstly, to characterize the morphological and biochemical attributes of the late mandarin varieties 'Afourer', 'Orri', and 'Tango' under homogeneous growing conditions. This analysis aimed to compare their key characteristics and provide valuable information regarding the preferred varieties for cultivation based on fruit quality. Secondly, the study aimed to compare the main characteristics of 'Tango' mandarins when cultivated using two different rootstocks: Citrus macrophylla and Forner Alcaide nº5. The mandarins included in this study were grown across multiple farms situated in the Southeast of the Iberian Peninsula.

2. Materials and Methods

2.1. Plant Material

For this study, mandarin trees were selected from two different fields located in Southeast Spain, both having similar edaphoclimatic conditions. The three mandarin varieties ('Afourer', 'Orri', and 'Tango'), grafted on the Citrus macrophylla rootstock, were cultivated in a field situated in Torre-Pacheco (Murcia, Spain; 37°46′11.2″ N 1°01′07.4″ W). The agrometeorological conditions recorded in 2021 for this area were an average temperature of 17.97 °C, average humidity of 69.76%, wind speed of 1.14 m/s, rainfall of 249.40 mm, and evapotranspiration of 1172 mm [17]. The trees, which were 14 years old, ranged in height from 3 to 4 m and were planted within a frame measuring 5.5 × 4 m.

In contrast, the 'Tango' variety grafted on the Forner-Alcaide nº5 rootstock (*C. reshni* × *P. trifoliata*) was cultivated in a farm located in the municipality of Torremendo (Alicante, Spain; 37°58′47.8″ N 0°50′24.5″ W). In 2021, the average temperature in this area was 18.61 °C, average humidity was 68.71%, wind speed was 4.09 m/s, rainfall was 441.72 mm, and evapotranspiration was 1158.36 mm [18]. The trees in this farm were 11 years old and had a similar crop frame of 5.5 × 4 m but varied in height between 2.5 and 3 m.

For all the trees, traditional crop management practices were carried out for commercial production, including the following: water application was done through a drip irrigation system with an average consumption ranging between 5500 and 6000 m^3/ha/year. Fertilization was performed with approximately 180–200 units of NPK per year, 100 units of P_2O_5 per year, and 50 units of K_2O per year. Annual pruning was conducted for all the trees. Phytosanitary treatments and addressing nutrient deficiencies were applied as needed, varying accordingly. At the time of fruit harvest, all the trees exhibited good development and phytosanitary conditions.

All the studied mandarin varieties were manually harvested when they reached their state of commercial maturity, as defined by DOUE-L-2021-81443 [19]. This state is determined based on several criteria: the juice content (>33%), the ratio of sugars to acids (7.5:1), the coloration (typical coloration of the variety on at least one-third of the fruit's surface), and the caliber (>45 mm). These criteria ensure that the mandarins meet the required quality standards for commercial purposes.

Fifty fruits were carefully collected from each variety/rootstock combination, sourced from five different trees. The selection of fruits was done randomly, ensuring an unbiased representation. To maintain consistency, the fruits were collected at the same height and from all sides of the trees. Subsequently, the mandarin fruits were transported to the laboratory and stored at a controlled temperature of 4 °C until the analysis and process. The fruits were analyzed on the same collected day.

2.2. Biometric and Physical Parameters

Fruit weight (g), equatorial diameter (mm), and polar diameter (mm) were the physical parameters measured of the fruit samples. The fruits were cut along the equatorial region and the peel thickness, number of carpels, and number of seeds were measured. For juice extraction, an electric juicer (Mpz22, Braun, Spain) was employed, and the volume of juice and the weight of the peel were accurately recorded. The weight of the juice was determined by calculating the difference between the fruit weight and the peel weight. Juice yield was calculated and expressed as the percentage of juice volume per fruit weight.

To evaluate the peel color, measurements were taken at three equidistant points from the equatorial region of the fruit using a colorimeter (CM-700d portable spectrophotometer, Konica Minolta, Osaka, Japan). The colorimeter used a view angle of 10º and standard illuminant D65, conforming to the standards set by the Commission Internationale de l'Eclairage (CIE) [20]. The values obtained included L*(brightness; where black = 0, white = 100), a*(↓a*: green, ↑a*: red), b*(↓b*: blue, ↑b*: yellow), h[h = arctang (b*/a*), where red = 0, yellow = 90, teal = 180, and blue = 270], and the C* [color intensity or saturation, C* = (a*2 + b*2)/2]. In addition, the Jimenez Cuesta citrus color index was calculated using the formula (CI) [CI = (1000*a)/(L*b)] [21]. The color evaluation of the juice was conducted separately. Measurements were performed in triplicate using a colorimetry cuvette, ensuring consistent and reliable results. The color evaluation followed the standardized guidelines established by the Commission Internationale de l'Eclairage (CIE).

2.3. Chemical Parameters

The titratable acidity (TA) expressed in g of citric acid/L and the pH were measured with an automatic acidity titrator (877 Titrino plus, Metrohm, Herisau, Switzerland) with 0.1 N NaOH until reaching a pH of 8.1, using 5 mL of mandarin juice diluted in 50 mL of distilled water. With the same device, the pH was also measured. Total soluble solids (TSS) were determined using a handheld refractometer (N1 model, Atago, Tokyo, Japan) and the results were expressed in ºBrix at 20 °C. The Maturity Index was calculated as the SST/TA ratio.

2.4. Antioxidant Activity

Total phenols were quantified following the methodology described by Singleton et al. [22]. The Folin-Ciocalteu reagent was utilized, and samples were analyzed in triplicate at a wavelength of 760 nm using a spectrophotometer (Helios γ, Thermo Spectronic, Cambridge, UK). The concentration of phenols was determined by constructing a calibration curve using gallic acid as the reference compound. The results were expressed as milligrams of gallic acid equivalent per liter of juice (mg GAE/L).

Total antioxidant activity was assessed according to the protocol outlined by Martínez-Nicolas et al. [23]. The ABTS+ (Azinobis [3-ethylbenzothiazolin-6-sulfonic] radical) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical methods were employed. Measurements were conducted in triplicate at 760 nm using a spectrophotometer (Helios γ, Thermo Spectronic, Cambridge, UK). The antioxidant activity results were expressed as milligrams of Trolox equivalent per milliliter of juice (mg Trolox/mL).

2.5. Organic Acids and Sugars

Organic acids and sugars in the mandarin juice were quantified as described by Legua et al. [10], briefly: filtered mandarin juice was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered through a cellulose nitrate membrane filter (0.45 µm). Afterwards, it was analysed by High-Performance Liquid Chromatography (HPLC, Hewlett Packard 1100 series, Wilmington, DE, USA), using a Supelcocolumn column [Supelcogel C-610H column (30 cm × 7.8 mm ID), Supelco] and a Supelguard precolumn [C610H (5 cm × 4.6 mm), Supelco]. A 0.1% phosphoric acid mobile phase was used at a flow rate of 0.5 mL/min. Organic acids were detected by an ultraviolet diode detector at 210 nm, for sugars a refractive index detector was used. The quantification of organic acids and sugars was performed by comparing the retention times and the area of the peaks with a standard curve of pure acids and sugars. The results of acids and sugars were expressed in g/100 mL. All the chemical reagents and standards used in the HPLC analysis were appropriate and sourced from Sigma Aldrich. These high-quality materials ensure accuracy and reliability in the analytical process.

2.6. Juice Metabolomics

The metabolomics of mandarin juice was measured by nuclear magnetic resonance (1H-NMR) following the methodology described by Melgarejo et al. [24].

2.7. Statistical Analysis

For the statistical analysis, the Statgraphics -Centurion 18 (Chicago, IL, USA) program was used. An analysis of variance was performed using an ANOVA of one factor for the locations of Abanilla and Campo de Cartagena separately. For the separation of means, Tukey's HSD test was used with a confidence level of 95%.

3. Results and Discussion

3.1. Biometric and Physical Parameters

At first sight and externally, these three varieties differ slightly in color tones or size (Figure 1). When the physical characterization was carried out in the laboratory, differences were observed in some parameters such as the weight of the fruits (Table 1). The 'Afourer' variety exhibited significantly higher weight (120.75 g) compared to the other two, with a noticeable difference of up to 20%. The weight of the 'Afourer' variety was much higher than that described by Tarancon et al. for this same variety, even our results are close to the heaviest varieties of mandarin hybrids described in their study [25]. While the equatorial diameter of the 'Afourer' variety was significantly larger, no significant differences were observed in the polar and equatorial diameters among the three varieties studied.

The number of carpels per fruit showed no significant variation among the three varieties, averaging around 9 carpels per fruit. Moreover, most of the fruits in all three varieties were found to be seedless, which is considered a desirable trait by consumers. The thickness of the peel did not differ significantly among the three varieties, with values ranging between 3.25 and 4.13 mm. However, the 'Afourer' variety exhibited a higher peel weight (51.91 g), up to 18% heavier than the peels of 'Orri' and 'Tango' varieties.

In terms of juice characteristics, both weight and volume were higher in the 'Afourer' and 'Orri' varieties compared to 'Tango'. However, the juice yield, calculated as a percentage of juice volume to fruit weight, was higher in the 'Orri' variety (53.47%) than in the other two varieties. In contrast to other studies of mandarin hybrids, our results showed that there can be significant differences in juice yield between these varieties [25]. This difference in yield can be attributed to the fact that although 'Afourer' has a higher fruit weight than 'Orri', it contains a larger amount of pulp. Consequently, the 'Orri' variety, known for its juicy carpels, is likely to be preferred by consumers.

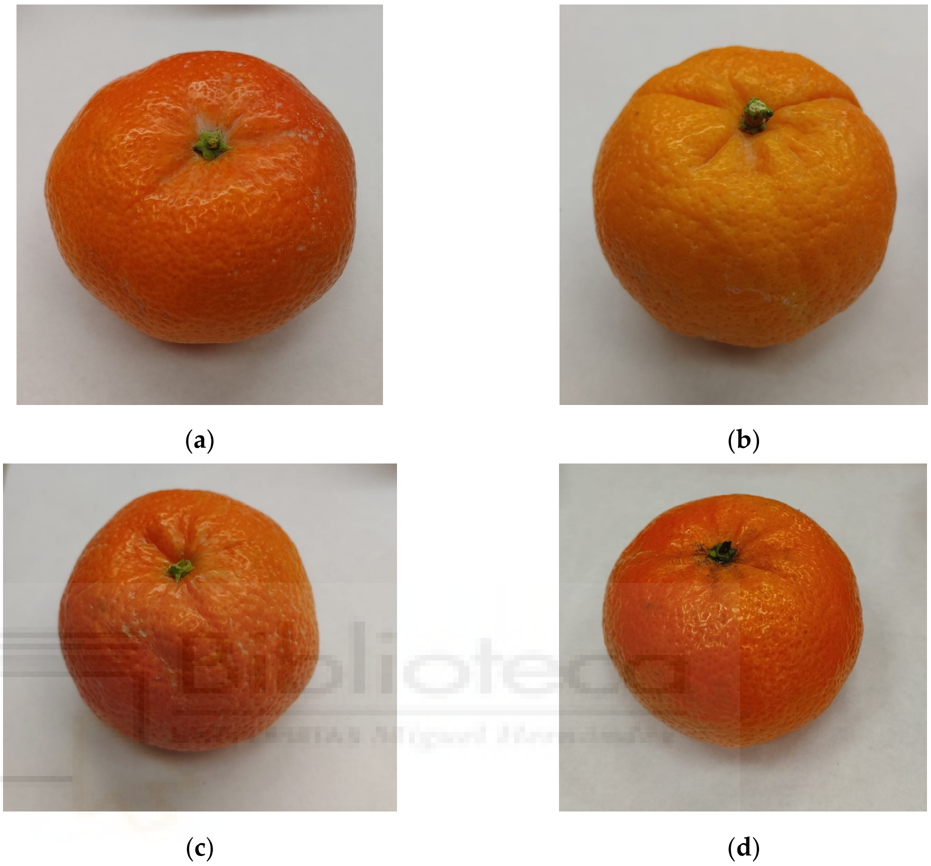

Figure 1. External photographs of the 3 mandarin varieties studied. (**a**) 'Afourer' variety; (**b**) 'Orri' variety; (**c**) 'Tango' variety cultivated on the *Citrus macrophylla* rootstock; (**d**) 'Tango' variety cultivated on the Forner-Alcaide nº5 rootstock.

Table 1. Physical properties of the three varieties of mandarins, cultivated on the *Citrus macrophylla* (CM) rootstock. Values are expressed as mean ± SE (n = 50).

Parameters	'Afourer'	'Orri'	'Tango'
Fruit weight (g)	120.75 ± 3.53 a	110.35 ± 2.59 b	99.91 ± 2.28 c
Equatorial diameter (mm)	67.60 ± 0.77 a	63.59 ± 0.53 b	62.90 ± 0.54 b
Polar diameter (mm)	48.80 ± 0.49 a	48.61 ± 0.48 a	47.33 ± 0.42 a
Number of carpels	9.44 ± 0.15 a	9.52 ± 0.15 a	9.38 ± 0.13 a
Number of seeds	0.24 ± 0.09 a	0.24 ± 0.09 a	0.02 ± 0.02 a
Peel thickness (mm)	3.31 ± 0.09 a	4.13 ± 0.72 a	3.25 ± 0.08 a
Peel weight (g)	51.91 ± 2.04 a	43.95 ± 1.38 b	44.78 ± 1.46 b
Juice weight (g)	68.84 ± 1.78 a	66.40 ± 1.47 a	55.13 ± 1.15 b
Juice volume (mL)	59.10 ± 2.09 a	58.72 ± 1.55 a	47.44 ± 1.51 b
Juice yield (%)	49.05 ± 1.07 b	53.47 ± 1.03 a	47.41 ± 1.04 b

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

Table 2 presents the results of the physical characterization of 'Tango' mandarins grown on two different rootstocks, Forner-Alcaide nº5 (FA) and *Citrus macrophylla* (CM). The average weight of mandarins cultivated on FA rootstock was 113.52 g, which was approximately 13 g heavier than those grown on CM rootstock. While there were no significant differences in the polar diameter between the two rootstocks, the equatorial diameter of mandarins cultivated on FA rootstock was found to be statistically greater than those grown on CM. The number of carpels per fruit did not differ significantly between the two rootstocks, with an average of 9 carpels per fruit. Additionally, neither rootstock exhibited seeds in the 'Tango' mandarins.

Table 2. Physical properties of 'Tango' variety cultivated on the Forner-Alcaide nº5 rootstock (FA) compared to the *Citrus macrophylla* rootstock (CM). Values are expressed as mean ± SE (n = 50).

Parameters	CM	FA
Fruit weight (g)	99.91 ± 2.28 b	113.52 ± 3.07 a
Equatorial diameter (mm)	62.90 ± 0.54 b	66.46 ± 0.67 a
Polar diameter (mm)	47.33 ± 0.42 a	47.71 ± 0.49 a
Number of carpels	9.38 ± 0.13 a	9.40 ± 0.12 a
Number of seeds	0.02 ± 0.02 a	0 a
Peel thickness (mm)	3.25 ± 0.08 a	3.13 ± 0.08 a
Peel weight (g)	44.78 ± 1.46 b	49.42 ± 1.75 a
Juice weight (g)	55.13 ± 1.15 b	64.10 ± 1.73 a
Juice volume (mL)	47.44 ± 1.51 b	55.98 ± 1.64 a
Juice yield (%)	47.41 ± 1.04 a	49.43 ± 0.74 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

When it comes to juice characteristics, both the weight and volume of juice obtained from mandarins grown on FA rootstock (64.10 g, 55.98 mL) were higher compared to those grown on CM rootstock (55.13 g, 47.44 mL). However, no significant differences were observed in the juice yield between the two rootstocks.

Color is an important parameter in the quality of the fruits for consumers. In Table 3 we can see the results of peel color and juice in the three varieties studied. According to sensory analyzes carried out by Gámbaro et al. to consumers, the greater brightness and orange hue in peel is better valued by consumers [26]. There were significant differences in all peel color parameters among the three varieties. The peel of the 'Orri' variety was brighter (L*) than the other two varieties. According to the CI, the 'Afourer' variety showed the most orange color (14.32), followed by the 'Tango' variety (13.41) and the 'Orri' variety with less tonality (12.03). According to the CI, all the fruits displayed a vibrant orange color, which is characteristic of this citrus. Regarding the color of the juice, we see from the CI that the varieties of 'Afourer' (9.18) and 'Tango' (9.55) had a more orange color than the variety 'Orri' (7.50).

Table 3. CIE parameters and color index (CI) of the peel and juice of the three varieties of mandarins, cultivated on the *Citrus macrophylla* (CM) rootstock. Parameters: L* (brightness; where black = 0, white = 100), a* (↓a*: green, ↑a*: red), b* (↓b*: blue, ↑b*: yellow), h (where red = 0, yellow = 90, teal = 180, and blue = 270), C* (color intensity or saturation) and CI (Color Index). Values are expressed as mean ± SE (n = 50 peel samples, n = 5 juice samples).

	Parameters	'Afourer'	'Orri'	'Tango'
Peel	L*	58.70 ± 0.26 b	62.65 ± 0.20 a	59.45 ± 0.24 b
	a*	37.89 ± 0.27 ab	38.18 ± 0.22 a	37.02 ± 0.36 b
	b*	45.45 ± 0.42 c	50.89 ± 0.28 a	46.73 ± 0.41 b
	C*	59.37 ± 0.34 b	63.69 ± 0.17 a	59.76 ± 0.39 b
	h	59.37 ± 0.34 a	53.09 ± 0.28 b	51.60 ± 0.37 c
	CI	14.32 ± 0.24 a	12.03 ± 0.16 c	13.41 ± 0.22 b
Juice	L*	40.77 ± 0.44 b	43.90 ± 0.13 a	41.11 ± 0.50 b
	a*	3.92 ± 0.33 a	4.70 ± 0.12 a	4.30 ± 0.38 a
	b*	10.42 ± 0.58 b	14.32 ± 0.46 a	10.90 ± 0.81 b
	C*	11.13 ± 0.66 a	15.07 ± 0.46 a	11.72 ± 0.89 a
	h	69.49 ± 0.52 b	71.79 ± 0.46 a	68.57 ± 0.32 b
	CI	9.18 ± 0.16 a	7.50 ± 0.21 b	9.55 ± 0.07 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

According to the color data, the external appearance of 'Tango' variety fruits cultivated on the CM rootstock (Table 4) showed higher brightness (L*) but a less orange color compared to those grown on the FA rootstock. In contrast, the fruits cultivated on the FA rootstock displayed a more intense orange color. Additionally, the juice obtained from the FA rootstock exhibited a richer, more vibrant orange hue.

Table 4. CIE parameters and color index (CI) of the peel and juice of 'Tango' variety cultivated on the Forner-Alcaide nº5 rootstock (FA) compared to the Citrus macrophylla rootstock (CM). Parameters: L* (brightness; where black = 0, white = 100), a* (↓a*: green, ↑a*: red), b* (↓b*: blue, ↑b*: yellow), h (where red = 0, yellow = 90, teal = 180, and blue = 270), C* (color intensity or saturation) and CI (Color Index). Values are expressed as mean ± SE (n = 50 peel, n = 5 juice).

	Parameters	CM	FA
Peel	L*	59.45 ± 0.24 a	58.05 ± 0.21 b
	a*	37.02 ± 0.36 b	38.95 ± 0.25 a
	b*	46.73 ± 0.41 a	43.35 ± 0.32 b
	C*	59.76 ± 0.39 a	58.36 ± 0.33 b
	h	51.60 ± 0.37 a	48.02 ± 0.24 b
	CI	13.41 ± 0.22 b	15.54 ± 0.18 a
Juice	L*	41.11 ± 0.50 b	42.84 ± 0.03 a
	a*	4.30 ± 0.38 b	5.77 ± 0.13 a
	b*	10.90 ± 0.81 b	13.31 ± 0.19 a
	C*	11.72 ± 0.89 b	14.51 ± 0.22 a
	h	68.57 ± 0.32 ab	14.51 ± 0.22 a
	CI	9.55 ± 0.07 b	10.12 ± 0.10 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

3.2. Chemical Parameters

Analysis of the chemical parameters among the three varieties studied are shown in Table 5. The pH was lower in the variety 'Afourer' (3.69) and higher in 'Orri' (4.24), the results obtained in 'Tango' variety were intermediate between the other two varieties. The titratable acidity was up to 2 g of citric acid/L higher in 'Afourer' and 'Orri' varieties than in 'Tango' variety. Soluble solids are linked to the sweetness of mandarin because 80% of these are sugars [27]. The total soluble solids were higher in the fruits of 'Orri' (13.72 ºBrix), followed by those of 'Afourer' (12.36 ºBrix) and in lower quantity in those of 'Tango' (10.80 ºBrix). The total soluble solids of 'Orri' variety was similar to that observed in other commercial mandarin varieties, such as 'Fortuna' (13.8 ºBrix) [28]. No significant differences were seen in the maturity index among the three varieties studied. In all cases, the minimum MI of 6.5 was reached for the commercialization of late-season mandarins [29].

Table 5. Chemical properties of the three varieties of mandarins, cultivated on the *Citrus macrophylla* (CM) rootstock. Parameters: pH, acidity titratable (TA), total soluble solids (TSS), maturity index (MI). Values expressed as mean ± SE (n = 5).

Parameters	'Afourer'	'Orri'	'Tango'
pH	3.69 ± 0.09 b	4.24 ± 0.19 a	3.83 ± 0.03 ab
TA (g citric acid/L)	9.25 ± 0.55 a	9.51 ± 0.29 a	7.40 ± 0.38 b
TSS (ºBrix)	12.36 ± 0.25 b	13.72 ± 0.22 a	10.80 ± 0.31 c
MI	13.52 ± 0.68 a	14.47 ± 0.39 a	14.74 ± 0.87 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

About the chemical properties of 'Tango' variety mandarins cultivated on the FA rootstock (Table 6), the pH was 4.00, significantly higher than that observed in 'Tango' cultivated on CM. The titratable acidity was 9.30 ± 0.41 g of citric acid/L, being statistically higher than the acidity observed on CM. 11.72 ºBrix detected in the juice, these data were similar to those obtained by this same study in CM, although slightly lower than those shown by Morales et al. for this same variety [30]. The MI of the fruits in FA reached the minimum required for its commercialization [29].

Table 6. Chemical properties of 'Tango' variety cultivated on the Forner-Alcaide nº5 rootstock (FA) compared to the *Citrus macrophylla* rootstock (CM). Parameters: pH, acidity titratable as citric acid (TA), total soluble solids (TSS), maturity index (MI). Values expressed as mean ± SE (n = 5).

Parameters	CM	FA
pH	3.83 ± 0.03 b	4.00 ± 0.03 a
TA (g citric ac./L)	7.40 ± 0.38 b	9.30 ± 0.41 a
TSS (ºBrix)	10.80 ± 0.31 a	11.72 ± 0.34 a
MI	14.74 ± 0.87 a	12.66 ± 0.42 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

3.3. Antioxidant Activity

The quantification of total phenols in juice enables us to estimate the antioxidant activity of the fruits, because these compounds have the ability to neutralize free radicals by donating hydrogen atoms [31]. In Table 7 we observed a higher amount of total phenols in 'Orri' variety (1097.21 mg AGE/L), up to 40% more than those observed in 'Tango' variety (752.14 mg AGE/L), 'Afourer' variety (857.31 mg GAE/L) did not show significant differences with Orri or Tango. The values of 'Tango' and 'Afourer' were similar to those described by Rekha et al. for mandarins (*Citrus reticulata*) (800 µg GAE/mL) [32]. The results in total phenols of these three late-season varieties were lower than those reported for clementines in another study [33]. On the other hand, no significant differences were observed in the total antioxidant activity among the three varieties with any of the two methods.

Table 7. Total phenols and antioxidant activity of the three varieties of mandarins, cultivated on the *Citrus macrophylla* (CM) rootstock. Parameters: Total phenols (TP), and total antioxidant activity according to the ABTS and DPPH methods. Values are expressed as mean ± SE (n = 3).

Parameters	'Afourer'	'Orri'	'Tango'
TP (mg AGE/L)	857.31 ± 79.70 ab	1097.21 ± 97.90 a	752.14 ± 35.62 b
ABTS (mg Trolox/mL)	3.32 ± 0.40 a	2.35 ± 0.04 a	3.85 ± 0.48 a
DPPH (mg Trolox/mL)	4.79 ± 0.08 a	3.56 ± 0.49 a	4.14 ± 0.08 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

Regarding the fruits of 'Tango' variety cultivated on FA (Table 8), they had significantly more total phenols than those described for this same variety in CM, 28% more of these biomolecules. The AAT results in AF with the ABTS method were shown to be statistically inferior to those obtained in the fruits of 'Tango' cultivated on CM. However, no differences were observed between both rootstocks using the DPPH method.

Table 8. Total phenols and antioxidant activity of 'Tango' variety cultivated on the Forner-Alcaide nº5 rootstock (FA) compared to the *Citrus macrophylla* rootstock (CM). Parameters: Total phenols (TP), and total antioxidant activity according to the ABTS and DPPH methods. Values expressed as mean ± SE (n = 3).

Parameters	CM	FA
TP (mg AGE/L)	752.14 ± 35.62 b	966.85 ± 24.88 a
ABTS (mg Trolox/mL)	3.85 ± 0.48 a	2.61 ± 0.10 b
DPPH (mg Trolox/mL)	4.14 ± 0.08 a	4.23 ± 0.21 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

3.4. Organic Acids and Sugars Content

Table 9 shows the organic acids detected by HPLC. Citric, malic, ascorbic, succinic, and formic acids were detected in all the samples studied. When comparing the three mandarin varieties studied, a significant difference was observed in the organic acid profiles. Citric acid was the majority in 'Afourer' variety (1.02 g/100 mL), it was in large quantities in 'Tango' (0.79 g/100 mL), but it was in low quantities in 'Orri' (0.11 g/100mL). The results in 'Afourer' and 'Tango' are similar to those detected in tangerines [34]. Malic acid was the majority in 'Orri' variety (0.61 g/100 mL) and was also statistically higher than in 'Afourer' and 'Tango' varieties (0.28 g/100 mL, 0.27 g/100mL). The very high values of malic acid obtained in 'Orri' variety are like those we would find in apples [35]. 0.02 g/100 mL of ascorbic acid was detected in 'Afourer' and 'Tango' varieties, and 0.01 g/100 mL in 'Orri' variety. A significantly higher amount of succinic acid was observed in the varieties 'Afourer' and 'Tango' than in the variety 'Orri'. While the variety 'Orri' shows similar values to other late-season mandarins, the values of 'Afourer' and 'Tango' are substantially higher [3]. A significantly higher amount of formic acid was found in 'Orri' variety, somewhat lower in 'Afourer' fruits and a lower amount in 'Tango'. The total amount of organic acids show a lower amount of acids in 'Orri' variety (1.44 g/100 mL) compared to 'Afourer' and 'Tango' varieties (2.3 g/100 mL, 2.07 g/100mL). The organic acid profile of 'Orri' variety is remarkable as it does not resemble two other mandarin varieties. This variation could be due to an adaptation of this variety to dry climates, as indicated in a study on Orah mandarins, a variety phylogenetically close to 'Orri' variety, growing in dry climates would increase the presence of malic acid [36]. Such a high presence of malic acid in 'Orri' would cause differences in flavour concerning the other two varieties, although the characteristic flavour of citrus fruit would be maintained since this acid together with the citric acid gives the characteristic mandarin flavour [37].

Table 9. Organic acids and sugars (g/100 mL) of the three varieties of mandarins, cultivated on the *Citrus macrophylla* (CM) rootstock. Values are expressed as mean ± SE (n = 3).

	Parameters	'Afourer'	'Orri'	'Tango'
Organic acids	Citric acid	1.02 ± 0.03 a	0.11 ± 0.01 c	0.79 ± 0.02 b
	Malic acid	0.28 ± 0.02 b	0.61 ± 0.02 a	0.27 ± 0.01 b
	Ascorbic acid	0.02 a	0.01 b	0.02 a
	Succinic acid	0.76 ± 0.06 a	0.37 ± 0.04 b	0.87 ± 0.03 a
	Formic acid	0.22 ± 0.02 b	0.34 ± 0.01 a	0.12 c
	Total acids	2.3 ± 0.03 a	1.44 ± 0.07 c	2.07 ± 0.02 b
Sugars	Sucrose	5.58 ± 0.07 ab	5.99 ± 0.16 a	5.27 ± 0.03 b
	Fructose	2.95 ± 0.05 a	2.91 ± 0.03 a	2.78 ± 0.16 a
	Glucose	3.87 ± 0.06 b	4.59 ± 0.11 a	3.43 ± 0.09 c
	Total sugars	12.4 ± 0.11 b	13.49 ± 0.29 a	11.48 ± 0.27 b

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

As for 'Tango' variety cultivated on Forner-Alcaide nº5 (Table 10), we see that citric acid is the majority in these mandarins with 0.99 g/100 mL, unlike those analysed in this study on the CM rootstock. Succinic acid was also found in large quantities (0.81 g/100 mL). Malic, ascorbic and formic acids were detected in smaller quantities, in quantities similar to those obtained in 'Tango' on CM. The amount of organic acids was considerably higher than those described by Morales et al. (12.07–13.69 g/L) in this same variety and rootstock [30].

Table 9 also shows the results of the sugars detected by HPLC. In mandarins, the concentration ratio of sucrose, fructose and glucose is usually 2:1:1, a similar ratio was identified in all our analysed fruits [38]. The sugar contents among the three varieties studied showed that 'Orri' variety (13.49 g/100 mL) has a higher amount of sugars than in the other two varieties, with a significant difference of up to 18% between the values of 'Orri' and 'Tango'. The sucrose values reached 5.99 g/100 mL in 'Orri' variety, being statistically higher than those observed in 'Tango' (5.27 g/100 mL), with the concentrations of sucrose remaining in intermediate statistical values than the variety 'Afourer'. No significant differences were seen in the amounts of fructose among the three varieties, with values between 2.78 g/100 mL and 2.95 g/100 mL. As for glucose, 'Orri' mandarins again had the highest amount of this sugar (4.59 g/100 mL), followed by 'Afourer' variety and finding less of this sugar in 'Tango' variety (3.43 g/100 mL). The values of each of the sugars were similar to those that we could find in clementine varieties [33]. The glucose and fructose values were higher than those described by Sdiri et al. in other late-season mandarin varieties [7].

Table 10. Organic acids and sugars (g/100 mL) of 'Tango' variety cultivated on the Forner-Alcaide nº5 rootstock (FA) compared to the *Citrus macrophylla* rootstock (CM). Values are expressed as mean ± SE (n = 3).

	Parameters	CM	FA
Organic acids	Citric acid	0.79 ± 0.02 b	0.99 ± 0.02 a
	Malic acid	0.27 ± 0.01 a	0.25 ± 0.02 a
	Ascorbic acid	0.02 a	0.02 a
	Succinic acid	0.87 ± 0.03 a	0.81 ± 0.1 a
	Formic acid	0.12 a	0.13 ± 0.02 a
	Total acids	2.07 ± 0.02 a	2.21 ± 0.09 a
Sugars	Sucrose	5.27 ± 0.03 a	5.47 ± 0.09 a
	Fructose	2.78 ± 0.16 a	2.65 ± 0.06 a
	Glucose	3.43 ± 0.09 a	3.62 ± 0.06 a
	Total sugars	11.48 ± 0.27 a	11.74 ± 0.16 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

This study failed to identify any significant difference in the concentration of the three sugars between the fruits of 'Tango' variety cultivated on the FA and CM rootstocks (Table 10). The sweetness in the fruits of both rootstocks resulted identical. These results show a higher amount of sugars than those described by Morales et al. for this same variety [30].

3.5. Juice Metabolomics

Metabolomic analysis with 1H-NMR (Tables 11 and 12) showed the presence of the amino acids GABA, Alanine, Arginine, Asparagine, Aspartate, Glutamine, Leucine, Isoleucine, Proline, Tyrosine, Valine in all the samples analysed. The most abundant amino acid was aspartate, with values ranging from 21.75 mM ('Tango' on FA rootstock) to 108.2 mM ('Orri'). No significant differences were found in any amino acid among the three varieties studied, except for proline, which was found in higher amounts in the 'Orri' variety. Proline is closely linked to plant response to abiotic stress, so this higher concentration in 'Orri' variety suggests greater adaptability to stress [39]. As for the analysis of the 'Tango' variety grown on different rootstocks, there were no significant differences in most amino acids, except for GABA, Asparagine, Aspartic Acid and Tyrosine. The greater amount of these metabolites in the CM rootstock, which is known to be involved in the response to biotic and abiotic stress, this could indicate that this rootstock may have a higher resistance to certain types of stresses [40,41]. In general, citrus fruits have a low amount of amino acids and those present are usually non-essential such as alanine, arginine, asparagine, glutamine, aspartic acid, tyrosine or proline [42] presents in our analysis. Although we also found some essential amino acids in our samples such as leucine, isoleucine, or valine, these are found in the lowest concentrations.

Table 11. Amino acids and other metabolites (mM) of the three varieties of mandarins, cultivated on the *Citrus macrophylla* (CM) rootstock. Values are expressed as mean ± SE (n = 3).

	Parameters	'Afourer'	'Orri'	'Tango'
Amino acids	GABA	2.68 ± 0.6 a	3.43 ± 1.04 a	1.92 ± 0.08 a
	Alanine	2.36 ± 0.69 a	1.35 ± 0.41 a	1.37 ± 0.07 a
	Arginine	12.05 ± 1.93 a	19.02 ± 3.47 a	7.32 ± 0.13 a
	Asparagine	14.15 ± 6.25 a	15.51 ± 4.51 a	6.42 ± 0.05 a
	Aspartic Acid	36.73 ± 17.56 a	108.2 ± 18.8 a	36.61 ± 0.69 a
	Glutamine	2.03 ± 0.39 a	5.15 ± 1.18 a	1.81 ± 0.13 a
	Isoleucine	0.07 ± 0.02 a	0.08 ± 0.02 a	0.05 a
	Leucine	0.04 a	0.06 ± 0.02 a	0.04 a
	Proline	10.55 ± 0.83 ab	37.7 ± 8.66 a	5.97 ± 0.13 b
	Tyrosine	1.12 ± 0.2 a	2.4 ± 0.59 a	1.06 a
	Valine	0.22 ± 0.05 a	0.19 ± 0.04 a	0.16 a
Other metabolites	Choline	0.49 ± 0.17 a	0.65 ± 0.14 a	0.53 ± 0.12 a
	Ethanol	3.77 ± 1.09 a	1.62 ± 0.13 a	1.63 ± 0.42 a
	Trigonelline	0.14 ± 0.03 a	0.16 ± 0.05 a	0.09 ± 0.01 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

Table 12. Amino acids and other metabolites (mM) of 'Tango' variety cultivated on the Forner-Alcaide nº5 rootstock (FA) compared to the *Citrus macrophylla* rootstock (CM). Values are expressed as mean ± SE (n = 3).

	Parameters	CM	FA
Amino acids	GABA	1.92 ± 0.08 a	0.65 ± 0.13 b
	Alanine	1.37 ± 0.07 a	2.46 ± 0.52 a
	Arginine	7.32 ± 0.13 a	8.7 ± 0.32 a
	Asparagine	6.42 ± 0.05 b	7.14 ± 0.02 a
	Aspartic Acid	36.61 ± 0.69 a	21.75 ± 0.05 b
	Glutamine	1.81 ± 0.13 a	1.92 ± 0.23 a
	Isoleucine	0.05 a	0.05 a
	Leucine	0.04 a	0.06a
	Proline	5.97 ± 0.13 a	10.77 ± 1.35 a
	Tyrosine	1.06 a	0.89 ± 0.03 b
	Valine	0.16 a	0.17 a
Other metabolites	Choline	0.53 ± 0.12 a	0.36 ± 0.01 a
	Ethanol	1.63 ± 0.42 b	4.59 ± 0.37 a
	Trigonelline	0.09 ± 0.01 a	0.16 ± 0.02 a

The different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

Other metabolites detected (Tables 11 and 12) in the mandarin juice were choline, ethanol and trigonelline. No significant differences were found between the three varieties in any metabolite; however, we observed a higher amount of ethanol in 'Tango' variety grown on FA than on CM. Ethanol was the most abundant secondary metabolite, with concentrations between 1.08 mM and 5.9 mM. Ethanol is a secondary metabolite produced by anaerobic respiration, it is involved in fruit ripening and in the generation of volatile compounds that give the aromas [43]. In smaller amounts, we find choline, with values between 0.76 mM and 0.36 mM and trigonelline 0.28 mM and 0.09 mM. Choline (also known as vitamin B4) is essential for humans, this biomolecule is involved in the formation of cell membranes and is a precursor of the neurotransmitter acetylcholine [44]. Trigonelline is an alkaloid present in plants, commonly found in coffee in concentrations of around 1%, giving this fruit part of its characteristic aroma [45]. Trigonelline accumulates in plants under biotic and abiotic stress conditions, and together with choline it can protect the plant from fungal infections [46,47]. Trigonelline as a biomolecule has antioxidant and antidiabetic effects that could be of interest to the pharmaceutical industry.

4. Conclusions

Three late varieties of mandarin have been characterized and compared under homogeneous growing conditions. 'Afourer' variety stood out for being larger and weighing up to 20% more than the rest of the varieties. 'Afouer' also contains a large volume of juice and is more orange. Its main organic acid was citric. 'Orri' variety stood out for having a large amount of juice, higher brightness, and a sugar concentration 18% higher than in the other two sugar concentration varieties. However, its organic acid profile was different from that of the other varieties, with malic as the main acid. The total antioxidant activity was not significantly different between the three varieties.

'Tango' mandarins cultivated on two different rootstocks were analyzed, highlighting that the fruits cultivated on FA have a greater weight and size, as well as a more orange hue than those cultivated on the CM rootstock. The HPLC results showed that the organic acid profile was quite similar between both rootstocks, citric acid was found in high amounts in both rootstocks. The total antioxidant activity was similar, although up to 28% more phenols were observed in the FA rootstock. All this indicates that the fruits in both rootstocks will have a very similar organoleptic quality.

The metabolomics analysis revealed that all the analyzed mandarin samples contained predominantly non-essential amino acids such as aspartate, alanine, or proline. Other metabolites such as ethanol, choline or trigonelline were detected. Significant differences were observed in some amino acids such as GABA or aspartic acid in 'Tango' fruits grown on different rootstocks.

The results presented in this preliminary study are based on data collected from a single year. However, comprehensive analyses over multiple years will be conducted in the future to validate and reinforce the findings reported in this study.

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Research Paper

Influence of rootstock on yield, morphological, biochemical and sensory characteristics of 'Afourer' variety mandarins

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ABSTRACT

Rootstock's can affect the characteristics and quality of citrus fruit. Knowing which rootstocks produce better quality fruit is very important in late mandarin varieties, due to their high economic value. The objective of this study is to know the effects on yield and the main morphological, biochemical and sensory properties of the late mandarin variety 'Afourer' when cultivated on three different rootstocks [*Citrus macrophylla* Wester (CM), Mandarino Cleopatra (MC) and Citrange Carrizo (CC)]. The morphological properties (weight and dimensions) and colorimetric characteristics (external and internal fruit color) were analyzed. The main biochemical characteristics, including total antioxidant activity analyzed by ABTS and DPPH methods, organic acids and sugars determined by HPLC, and juice metabolomics by ^{1}H-NMR, were also investigated. In addition, the juice quality was assessed by a sensory panel. The CC rootstock produced up to 77 kg of fruit/tree more than MC. Fruits cultivated on MC and CC had significantly larger dimensions, a greater orange coloration and up to 17 % more juice than fruits cultivated on CM. In terms of biochemical properties, there were no significant differences in most of the properties analyzed among the three rootstocks, highlighting only that the CM rootstock induced 13 % more fructose in the fruits than the rest of the rootstocks. Fruits cultivated on the CC and MC rootstocks had a better taste when evaluated by sensory analysis than those cultivated on the CM rootstock.

1. Introduction

Late-season mandarin varieties hold significant economic importance due to their high yield and the fact that they are harvested during a period when fewer varieties are available in the market. The variety 'Afourer', also known as 'Nadorcott', is a late-season variety originating in Morocco from the hybridization between the variety 'Murcott' and an unknown pollinator (Nadori, 1998; Garmendia et al., 2019). Farmers are required to pay a high royalty to cultivate this variety, and there is a limited number of trees that can be grown (Smith, 2016). In Spain, it is estimated that there are 3 million 'Afourer' variety trees cultivated with the breeder's permission (according to a personal report provided by Agrupación de Viveristas de Agrios S.A., Madrid, Spain).

Rootstocks are widely used in agriculture because they have been shown to facilitate rooting, increase nutrient and water uptake, affect fruit ripening, improve crop quality, and increase resistance to abiotic factors such as salinity, drought, soil type and waterlogging, as well as biotic factors such as resistance to nematodes or fungal infections (Behzadi Rad et al., 2021; de Carvalho et al., 2021; Jaimez et al., 2023; Vahdati et al., 2021). Specifically in citrus, rootstocks are usually focused on sensitivity to CTV (citrus tristeza virus), resistance to nematodes, yield, ripening date, or tolerance to various abiotic stresses (Martínez-Cuenca et al., 2016). The study, hybridization, and creation of new rootstocks have been of great interest to citriculture for decades, aiming to find rootstocks with improved agronomic and qualitative characteristics (Continella et al., 2018; Fu et al., 2016).

Some of the most commonly used rootstocks in mandarin cultivation are *Citrus macrophylla* Wester (CM), Mandarino Cleopatra (*Citrus reshni* Hort. ex Tan.), and Citrange Carrizo (*Citrus sinensis* (L.) Osb. x Poncirus trifoliata (L.) Raf.). CM is known for its high yield and resistance to limestone and salinity, although it is sensitive to frost and CTV (Martínez-Cuenca et al., 2016; Maciá-Vázquez et al., 2023). Mandarino Cleopatra (MC), on the other hand, is notable for its tolerance to CTV, resistance to limestone, salinity, and frost, although it is somewhat

Abbreviations: CM, *Citrus macrophylla* wester; MC, mandarino cleopatra; CC, citrange carrizo; TSS, total soluble solids.

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sensitive to root asphyxia (Martínez-Cuenca et al., 2016; Morales et al., 2023). Citrange Carrizo (CC) is characterized by its tolerance to CTV, high yield, early ripening, and resistance to root asphyxia, although it is more sensitive to limestone and salinity (Martínez-Cuenca et al., 2016; Morales et al., 2023).

Furthermore, the choice of rootstock has significant implications for the final quality of the fruit (Lee & Keremane, 2024). The rootstock can greatly influence the morphological characteristics of the fruit, such as weight, diameter, color, or juice content (Forner-Giner et al., 2023; Legua et al., 2011; Morales et al., 2021). Rootstocks also impact the fruit's biochemical, organoleptic, and nutritional properties, including total soluble solids (TSS), total phenols, anthocyanins, organic acids, sugars, and total antioxidant activity (Chen et al., 2022; Legua et al., 2014; Modica et al., 2022).

The objective of this study is to investigate the influence of rootstock on the yield, morphological characteristics, biochemical composition, and sensory attributes of the late-season mandarin variety 'Afourer' (*Citrus reticulata* Blanco). To achieve this, fruits of this variety cultivated on three different rootstocks (CM, MC, and CC) were collected under standardized growing conditions.

2. Materials and methods

2.1. Plant material

In this work, 'Afourer' variety mandarin fruits (*Citrus reticulata* Blanco) grafted on three different rootstocks were studied. The rootstocks used were i) *Citrus macrophylla* Wester (CM); ii) Mandarin Cleopatra (*Citrus reshni* Hort. ex Tan.); and iii) Citrange Carrizo (*Citrus sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.). All the trees are located in a commercial mandarin plantation in the municipality of Abanilla in Murcia, Spain (38°11′13.7"N 1°01′15.6"W). The trees are planted with a 6×5 frame on 45 cm raised beds.

The crop irrigation was by a drip irrigation system (4 drippers per tree, with a flow rate of 4 L/h) and total annual water consumption around 6,200 m^3/ha. The fertilization was applied through irrigation water, with an annual consumption of 215 functional units (UF) of nitrogen, 67 UF of P_2O_5, and 125 UF of K_2O. Beside the grown conditions, the soil properties (sandy-loam soil, 42.4 % $CaCO_3$, 11.2 % active calcium carbonate, 5.75 mS/cm, and pH = 7.59) were also kept consistent for all variety/rootstock combinations studied in order to reduce the environmental impact on the parameters studied.

In terms of tree age, the trees grafted onto CM rootstock were 6 years old, with heights ranging between 2.4 and 3.0 m and crown widths between 2.0 and 2.3 m. In contrast, the trees grafted onto MC rootstock (with heights between 3.5 and 4.5 m and crown widths between 4.7 and 5.3 m) and CC rootstock (with heights between 3.7 and 4.1 m and crown widths between 5.0 and 5.7 m) were 11 years old. Due to this significant age difference, the comparison of fruit yield was conducted exclusively between Mandarin Cleopatra and Citrange Carrizo rootstocks, as these trees were of the same age. This decision was made because fruit yield is a parameter closely associated with tree age.

In this way, the analysis of fruit yield was carried out with the data obtained during the three years of study for these two patterns, while the complete characterization of the mandarins and their fruit quality was carried out during two seasons (two years) for the three rootstocks studied.

The mandarins were manually collected at the optimal state of maturity for marketing, as regulated in Spain by DOUE-L-2021-81443 (Commission Delegate Regulation, 2021). At the time of harvesting, all the trees were in good phytosanitary condition. For the fruit characterization analysis, a total of one hundred mandarins were collected over the two years (fifty per year). The mandarins were collected from 10 different trees for each variety/rootstock combination distributed across the farmlands. Once collected, the fruits were transported to the laboratory and stored at 4 ºC until analysis.

2.2. Mandarin yield

Data on mandarin yield were collected over a period of three harvesting seasons, spanning three years. For each season, a total of fifty trees were randomly selected from the field, and all the harvested fruits from each tree were weighed. The average yield per tree was then calculated based on the collected data. In particular, the yield of trees cultivated on the MC and CC rootstocks was compared, as they were of the same age (11 years). The results were expressed in kilograms per tree, providing a measure of the average yield achieved by each rootstock.

2.3. Morphological parameters of the fruit

The morphological parameters of the mandarin fruit were measured, including weight, equatorial and polar diameter, peel thickness, number of carpels, and number of seeds. To obtain these measurements, the fruit was cut in half at the equatorial region. To extract the juice, the mandarins were squeezed using an electric juicer (Mpz22, Braun). After juicing, the peel was weighed, and the volume of juice obtained was measured. The juice yield, expressed as a percentage, was calculated by dividing the juice volume by the fruit weight.

Furthermore, the color of both the peel and the juice was measured in triplicate using a colorimeter (CM-700d portable spectrophotometer, Konica Minolta, Osaka, Japan) with a view angle of 10º and standard illuminant D65. The Jimenez Cuesta citrus color index [CI=(1000a)/(Lxb)] was calculated based on the measured color values (Legua et al., 2018).

2.4. Chemical parameters of the fruit

Titratable acidity, expressed in grams of citric acid per liter (g citric acid/L), and pH were measured using an automatic acid titrator (877 Titrino plus, Metrohm, Herisau, Switzerland). The titration was performed with 0.1 N NaOH until a pH of 8.1 was reached, allowing for the determination of the titratable acidity. Total soluble solids (TSS), measured in degrees Brix (°Brix) at 20° C, were determined using a handheld refractometer (model N1, Atago, Tokyo, Japan). TSS provides an indication of the sugar content in the fruit. The maturity index was calculated as the ratio of TSS to titratable acidity. This index provides a measure of the fruit's maturity, with a higher value indicating a higher sugar-to-acid ratio. It serves as an indicator of the fruit's flavor profile and readiness for consumption.

2.5. Antioxidant activity

Total phenols in the samples were measured in triplicate according to the Folin-Ciocalteu method at 760 nm in a spectrophotometer (Helios γ, Thermo Spectronic, Cambridge, UK) (Singleton et al., 1999). A gallic acid calibration curve was used to determine the concentration of this biomolecule. The results were expressed as mg gallic acid equivalent/liter of juice (mg AGE/L).

The total antioxidant activity of the juice samples was measured in triplicate at 760 nm, using the ABTS (2.20′-radical method Azinobis [3-ethylbenzothiazolin-6-sulfonic]) and DPPH (2.20′-Diphenyl-1-Picrylhydrazyl) methods described by Martínez-Nicolas et al. (2022). A spectrophotometer (Helios γ, Thermo Spectronic, Cambridge, UK) was used. Results were expressed as mg Trolox equivalents/mL of juice (mg Trolox/mL).

2.6. Organic acids and sugars

Detection and quantification of organic acids and sugars present in mandarin juice was performed by high performance liquid chromatography (HPLC), following the methodology described by Legua et al. (2014). The centrifuged juice was filtered using a cellulose nitrate filter with a pore size of 0.45 µm. The juice was injected in triplicate into a chromatograph (Hewlett Packard 1100 series, Wilmington, DE, USA), employing a 0.1 % phosphoric acid mobile phase at 0.5 mL/min, with a Supelcocolumn [Supelcogel C-610H column (30 cm × 7.8 mm ID), Supelco] and a Supelguard precolumn [C610H (5 cm × 4.6 mm), Supelco]. An ultraviolet diode array detector (210 nm) was used to detect organic acids and a refractive index detector for sugars. Standard curves for organic acids and pure sugars were used to quantify the concentration in our samples. Results for acids and sugars were expressed in g/100 mL.

2.7. Juice metabolomics

To perform the metabolomics analysis, the juice was centrifuged and prepared, according to the methodology described for Melgarejo et al. (2022), to be analyzed by nuclear magnetic resonance (^{1}H-NMR). The results were expressed in mM.

2.8. Sensory analysis

Sensory analysis of 'Afourer' mandarins cultivated on three rootstocks was carried out based on guidelines provided by Gacula Jr (2008). A panel of tasters composed of 10 judges aged 20 to 70 years (6 males and 4 females) was utilized for sensory evaluation. Sensory evaluation was conducted in a comfortable, temperature-controlled and odor-free room with separate booths for each judge on the panel. The fruits were rated externally and internally on a hedonic scale of 1 to 10, with 1 being very unpleasant and 10 being very pleasant. The external rating included fruit size, color, shape, and ease of peeling. The internal rating consisted of flavor, sweetness, presence of seeds, pulp mouthfeel, and fruit acidity.

2.9. Statistical analysis

Statgraphics centurion 18 (StatPoint Technologies, Chicago, IL, USA) was used to perform an analysis of variance using ANOVA for one factor and Tukey's HSD test ($p \leq 0.05$) was used for the separation of means to analyze the significant differences among the three rootstocks.

3. Results and discusion

3.1. Yield

Rootstock can significantly impact agronomic parameters, including fruit yield (de Carvalho et al., 2021). In this study, the yield of 'Afourer' mandarins was compared between two rootstocks, MC and CC, with both plantations having the same age of 11 years. The results, as shown in Table 1, revealed that trees cultivated on CC rootstock produced up to 77 kg more fruit per tree compared to those on MC rootstock. This finding corroborates the higher yield associated with CC rootstock, as previously mentioned by Martínez-Cuenca et al. (2016). These results highlight the value of CC rootstock for growers when selecting the appropriate rootstock for cultivating mandarin trees. However, it is important to consider other factors such as resistance to abiotic and biotic stresses when making the final decision.

Table 1
Yield of 'Afourer' variety mandarins cultivated on MC and CC rootstocks. Values expressed as mean±SE. (n=3).

Parameter	MC	CC
Yield (kg/tree)	168.40 ± 32.31 a	245.96 ± 16.05 b

Different letters in the same row indicate significant differences according to Tukey's test ($p<0.05$).

3.2. Morphological parameters of the fruit

The results of the morphological characterization of the fruit are presented in Table 2. Significantly higher fruit weight was observed in mandarins cultivated on CC rootstock (134.27 g), which were up to 11 % heavier compared to those cultivated on CM and MC rootstocks. A similar trend was observed in a study on 'Lane Late' oranges, where CM rootstock exhibited higher fruit weight than MC (Legua et al., 2011). In terms of equatorial diameter, significant differences were found among mandarins cultivated on the three rootstocks, being higher in MC and CC rootstocks than in CM. However, no significant differences were observed in polar diameter. The greater fruit weight and diameter observed in CC rootstock were also reported in another study on 'Navelina' oranges (Forner-Giner et al., 2003).

Regarding the number of carpels, on average, mandarins on CM rootstock had approximately one more carpel compared to those on the other two rootstocks. The presence of seeds in mandarins is an important factor affecting consumer preference, as it may decrease the overall enjoyment of the fruit (Gámbaro et al., 2021). The 'Afourer' variety is characterized by being seedless, and our results confirm the absence of seeds in the majority of fruits across all three rootstocks (Barry et al., 2020).

In terms of peel thickness, mandarins cultivated on CM exhibited greater thickness (4.20 mm) compared to those on MC and CC (3.22 mm, 3.25 mm). No significant differences were observed in peel weight among the three rootstocks. Analysis of the morphological parameters of the juice revealed significant differences among fruits cultivated on the three rootstocks. Juice weight and volume were lower in mandarins on CM rootstock, while somewhat higher values were observed in those on MC, with the highest values found in CC fruits (Table 2). Juice yield was up to 17 % higher in CC and MC fruits compared to CM fruits. This suggests that fruits cultivated on CC and MC rootstocks are juicier and more appealing to consumers than those cultivated on CM. These variations in juice content are primarily attributed to the greater fruit weight and diameter observed in CC and MC. The juice yield values obtained in this study align closely with those reported by Tarancón et al. (2021).

Fruit color and shade are important factors influencing consumer preference, as mandarins with more orange shades are generally more appealing to buyers. It is known that the rootstock on which a citrus variety is cultivated can influence biochemical compounds, resulting in differences in both external and internal fruit color (Aguilar-Hernández et al., 2020; Morales et al., 2021). The results obtained for the fruit peel color index demonstrated that mandarins cultivated on CC rootstock exhibited significantly more orange coloration, although all rootstocks achieved a high orange hue. This finding is consistent with another

Table 2
Morphological properties of 'Afourer' mandarins cultivated on CM, MC and CC rootstocks. Values are expressed as mean±SE (n=100, except for the color index in the juice n=10).

Parameters	CM	MC	CC
Fruit weight (g)	121.23 ± 3.20 a	120.60 ± 2.81 a	134.27 ± 3.21 b
Equatorial diameter (mm)	67.50 ± 0.53 a	70.47 ± 0.66 b	70.53 ± 0.69 b
Polar diameter (mm)	51.09 ± 0.58 a	51.09 ± 0.56 a	51.42 ± 0.56 a
Number of carpels	10.30 ± 0.15 b	9.34 ± 0.16 a	9.61 ± 0.16 a
Number of seeds	0.08 ± 0.07 a	0.24 ± 0.11 a	0.28 ± 0.16 a
Peel thickness (mm)	4.20 ± 0.09 b	3.22 ± 0.10 a	3.25 ± 0.11 a
Peel weight (g)	57.59 ± 1.96 a	57.84 ± 1.70 a	59.26 ± 2.25 a
Juice weight (g)	53.98 ± 1.60 a	66.97 ± 2.44 b	72.23 ± 1.83 c
Juice volume (mL)	49.41 ± 1.74 a	62.39 ± 2.44 b	67.28 ± 1.82 c
Juice yield (%)	41.82 ± 1.07 a	45.97 ± 1.27 b	49.10 ± 1.06 b
Color index [Peel]	12.28 ± 0.13 a	13.78 ± 0.15 b	14.30 ± 0.20 c
Color index [Juice]	13.55 ± 0.31 b	12.33 ± 0.29 a	12.49 ± 0.17 a

Different letters in the same row indicate significant differences according to Tukey's test ($p<0.05$).

study on 'Navelina' oranges, where fruits cultivated on MC rootstock had a lower color index compared to CC (Forner-Giner et al., 2003). However, in the juice, a more orange hue was observed in fruits cultivated on CM rootstock.

3.3. Chemical parameters of the fruit

pH, titratable acidity, TSS and maturity index values largely determine the flavor and quality of the fruit (Goldenberg et al., 2018). In this study (Table 3), no statistically significant differences were found among the pH's of the juice of the three rootstocks, with values between 3.68 and 4.01, pH values very similar to those described by Simón-Grao et al. (2014) in 'Afourer' fruits cultivated in the same geographical area. Regarding titratable acidity, less acidity was detected in fruits cultivated on CM, up to 3 g citric acid/L less than in fruits cultivated on MC and CC. This higher acidity in MC and CC rootstocks is corroborated by another study in 'Clemenules' mandarins (Legua et al., 2014). Fruits cultivated on CM tend to reduce their acidity more rapidly as they ripen, this causes these fruits to have a blander flavor as the acidity is diluted with increasing water content (Kimball, 1984; Morales et al., 2021). Fruits cultivated on CC (13.02 °Brix) also had significantly higher TSS than those cultivated on CM (10.04 °Brix), while those cultivated on MC had intermediate values. The results were similar to those obtained by Simón-Grao et al. (2014) and those described by Barry et al. (2020) in other mandarin studies. TSS are mostly sugars, so this could indicate that fruits cultivated on CC will be perceived sweeter (Shorbagi et al., 2022). Fruit maturity index also showed different among rootstocks, the mean values ranged from 12.08 in MC to 14.51 in CM. The maturity index exceeded 6.5, the minimum value to be marketed in Spain (Quinza-Guerrero & López-Marcos, 1978).

3.4. Antioxidant activity

Rootstock is also responsible for altering the concentrations of phenolic compounds and total antioxidant activity in citrus fruits (Rapisarda et al., 2022). Total phenols are biochemical compounds produced by plants that have a high nutritional value because they possess a high antioxidant capacity and are involved in the prevention of human diseases (Hasler, 1998). No significant differences were observed in the amount of phenols among the three rootstocks studied (Table 4), the values ranged from 869.63 to 893.23 mg AGE/L. However, these results contrast with those found by Legua et al. (2014), in which he demonstrated a higher amount of total phenols in 'Clemenules' mandarins cultivated on MC than on CM and CC. On the other hand, according to the results obtained by the ABTS method, the CM rootstock showed higher antioxidant activity than the other two rootstocks studied. Nevertheless, no significant differences in total antioxidant activity were observed among the three standards by the DPPH method (Table 4).

Table 3
Chemical properties of 'Afourer' mandarins cultivated on CM, MC and CC rootstocks. Values expressed as mean±SE (n=10).

Parameters	CM	MC	CC
pH	3.68 ± 0.09 a	3.93 ± 0.09 a	4.01 ± 0.07 a
Acidity titratable (g citric acid/L)	7.72 ± 0.37 a	10.53 ± 0.46 b	10.74 ± 0.29 b
TSS (°Brix)	10.04 ± 0.12 a	12.60 ± 0.07 b	13.02 ± 0.12 c
Maturity index	14.51 ± 0.31 b	12.08 ± 0.47 a	12.19 ± 0.31 a

Different letters in the same row indicate significant differences according to Tukey's test ($p<0.05$).

Table 4
Total phenols and total antioxidant activity of 'Afourer' variety mandarins cultivated on CM, MC and CC rootstocks. Values are expressed as mean±SE (n=6).

Parameters	CM	MC	CC
Total phenols (mg AGE/L)	893.23 ± 14.39 a	869.63 ± 16.96 a	889.68 ± 75.42 a
ABTS method (mg Trolox/mL)	3.61 ± 0.31 b	2.68 ± 0.17 a	2.51±0.15 a
DPPH method (mg Trolox/mL)	4.23 ± 0.28 a	3.92 ± 0.23 a	4.26 ± 0.19 a

Different letters in the same row indicate significant differences according to Tukey's test ($p<0.05$).

3.5. Organic acids and sugars

HPLC analysis detected citric, malic, ascorbic, ascorbic, succinic and formic organic acid were detected in all the samples studied (Table 5). The major acid in the three rootstocks was citric acid, with values ranging from 0.99 g/100 mL to 1.02 g/100 mL, with no significant differences among the three rootstocks. In another study, with 'Clemenules' mandarins, it was shown that the MC rootstock had a significantly higher amount of citric acid (Legua et al., 2014). 0.02 g/100 mL ascorbic acid was detected in all three rootstocks. The amounts of malic and succinic acid were statistically higher in fruits cultivated on CM (0.32 g/100 mL malic, 0.85 g/100 mL succinic) than in those cultivated on MC and CC rootstocks. Formic acid, however, was found in lower concentrations in fruits cultivated on CM. Even with the differences previously described, no significant differences were observed in the total organic acids among the three rootstocks analyzed, with values between 2.21 g/100 mL and 2.29 g/100 mL of organic acids in the mandarin juice.

In addition, sucrose, glucose and fructose sugars were identified by HPLC (Table 5). There is evidence in mandarin and orange trees that the rootstock on which they are cultivated has a direct influence on fruit sugar concentrations (Barry et al., 2004; Legua et al., 2014). Our results showed no differences in sucrose values among the three rootstocks, with values between 5.34 and 5.67 g/100 mL. Significant differences were observed in fructose values among rootstocks, with the CM rootstock giving the highest amount of this sugar, up to 10 % more than in the CC rootstock and 13 % more than in fruits cultivated on MC. The

Table 5
Organic acids and sugars of 'Afourer' mandarins cultivated on CM, MC and CC rootstocks. Values are expressed as mean±SE (n=6).

	Parameters	CM	MC	CC
Organic acids	Citric acid (g/100 mL)	0.99 ± 0.02 a	0.99 ± 0.05 a	1.02 ± 0.05 a
	Malic acid (g/100 mL)	0.32 ± 0.01 b	0.27±0.01 a	0.26 ± 0.01 a
	Ascorbic acid (g/100 mL)	0.02 a	0.02 a	0.02 a
	Succinic acid (g/100 mL)	0.85 ± 0.04 b	0.05 ± 0.01 a	0.6 ± 0.01 a
	Formic acid (g/100 mL)	0.15 ± 0.02 a	0.27 ± 0.01 b	0.29 ± 0.01 b
	Total acids (g/100 mL)	2.27 ± 0.08 a	2.21 ± 0.04 a	2.29±0.07 a
Sugars	Sucrose (g/100 mL)	5.51 ± 0.20 a	5.34 ± 0.31 a	5.67 ± 0.26 a
	Fructose (g/100 mL)	3.08 ± 0.05 c	2.72 ± 0.01 a	2.80 ± 0.02 b
	Glucose (g/100 mL)	3.87 ± 0.07 a	3.74 ± 0.12 a	3.94 ± 0.10 a
	Total sugars (g/100 mL)	12.15 ± 0.41 a	11.57 ± 0.40 a	12.12 ± 0.20 a

Different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

glucose values in the three rootstocks were similar. In another study on blood oranges, the results showed significant differences in the amount of sucrose, with the CC rootstock having a higher concentration, but no differences were observed in fructose levels (Morales et al., 2021). There were also no significant differences in the total sum of sugars among the three rootstocks studied, with values between 11.57 g/100 mL and 12.15 g/100 mL.

3.6. Metabolomic analysis

The metabolomic analysis of the juice by 1H-NMR (Table 6) gives us valuable information on the nutritional and organoleptic properties of the mandarins, as well as information on their adaptation to the environment. The most abundant amino acids detected in these mandarins were aspartic acid, arginine, asparagine, proline, or tyrosine; non-essential amino acids for humans (Liu et al., 2012). The most abundant amino acid, with concentrations between 47.13 and 50.93 mM, has been aspartic acid, an amino acid that would be involved in the correct growth of the plant and in its response to abiotic stress such as drought or salinity (Okumoto et al., 2021; Rizwan et al., 2017). The results of this study indicate that the rootstock did not alter the mean concentrations of any amino acid or other metabolite, except for the non-detection of Leucine in CM and MC rootstocks, possibly because it was found in very low concentrations as in CC, and proline which was found in lower concentrations in CM rootstock. This lower amount of proline could be due to the fact that this amino acid is one of the main contributors of organic N in plants and is involved in fruit ripening; the greater the maturity, the more proline will have been metabolized and in the fruit it will be detected in lower concentrations (Xiong et al., 2023).

Other metabolites detected in mandarin juice are ethanol, the most abundant, a biomolecule involved in the generation of volatile compounds that give the fruits their odor (Pesis, 2005). Another metabolite detected in lower concentrations is choline, a biomolecule essential for humans as the precursor of the neurotransmitter acetylcholine (Blusztajn, 1998). Also detected in low concentrations was trigonelline, a compound that helps plants against sources of biotic and abiotic stresses (Mohamadi et al., 2018).

Table 6
Amino acids and other metabolites of 'Afourer' variety mandarins cultivated on CM, MC and CC rootstocks. Values are expressed as mean±SE (n=6). ND=Not Detected, LOD ≥ 10 μM.

	Parameters	CM	MC	CC
Amino acids	GABA (mM)	1.95 ± 0.52 a	3.13 ± 0.33 a	3.12 ± 0.40 a
	Alanine (mM)	2.56 ± 0.45 a	3.20 ± 0.40 a	3.80 ± 0.43 a
	Arginine (mM)	11.75 ± 1.78 a	14.12 ± 0.50 a	14.83 ± 0.31 a
	Asparagine (mM)	9.13 ± 1.61 a	12.18 ± 4.03 a	12.12 ± 3.95 a
	Aspartic Acid (mM)	48.92 ± 1.37 a	50.93 ± 1.42 a	47.13 ± 1.85 a
	Glutamine (mM)	2.32 ± 0.33 a	2.62 ± 0.10 a	2.84 ± 0.20 a
	Isoleucine (mM)	0.09 ± 0.02 a	0.10 ± 0.01 a	0.09 ± 0.01 a
	Leucine (mM)	ND	ND	0.02 ± 0.04 a
	Proline (mM)	7.13 ± 1.52 a	14.23 ± 1.34 b	17.86 ± 2.80 b
	Tyrosine (mM)	4.26 ± 2.52 a	1.52 ± 0.50 a	1.48 ± 0.23 a
	Valine (mM)	0.24 ± 0.04 a	0.33 ± 0.05 a	0.34 ± 0.08 a
Other metabolites	Choline (mM)	0.59 ± 0.19 a	0.67 ± 0.18 a	0.62 ± 0.09 a
	Ethanol (mM)	1.06 ± 0.11 a	3.12 ± 1.42 a	5.5 ± 1.48 a
	Trigonelline (mM)	0.12 ± 0.05 a	0.23 ± 0.07 a	0.25 ± 0.08 a

Different letters in the same row indicate significant differences according to Tukey's test ($p<0.05$).

3.7. Sensory analysis

Sensory analysis provides valuable information on consumer perception of fruit quality since many of the differences in morphological and biochemical parameters that can be detected by laboratory analysis may not be perceptible to the average consumer. In this study, consumers were given 'Afourer' mandarins cultivated on the three rootstocks under study. In the external evaluation (Table 7), fruit cultivated on MC rootstock were rated higher for size and ease of peeling than the other two rootstocks. In relation to the results of our study, the larger mandarins are better valued (Section 3.2.). In terms of color and shape, no significant differences were observed in the tasters' evaluations. This contrasts with the results on color obtained in the physical characterization of this study (Section 3.2.), the differences in the orange hue are not appreciated or are not valued by the judges. Regarding the internal evaluation of the fruits, once consumed, the MC and CC rootstocks had better qualifications in flavor, sweetness, and pulp than the CM rootstock, which was characterized by having a lower acidity, but being more tasteless. The absence of seeds in the fruit gave all mandarins the highest score.

4. Conclusion

We analyzed the influence of rootstock on fruit yield and on the main morphological, biochemical and sensory characteristics of late-season 'Afourer' mandarins. The CC rootstock was the most productive, with up to 77 kg/tree more than the MC rootstock, making it more interesting for the grower. Fruits cultivated on MC and CC rootstocks had a larger size, up to 17 % higher juice yield and a higher orange hue than mandarins cultivated on CM, which could be more attractive to consumers. These rootstocks also had a higher titratable acidity and TSS. On the other hand, the CM rootstock showed higher total antioxidant activity by the ABTS method. No significant differences were observed in the total concentration of organic acids; the major acid was citric acid. Sugar concentrations were similar in the three rootstocks except for fructose, which had up to 13 % more of this sugar in the CM rootstock. The metabolomics results show that all mandarin samples analyzed in this study contained mostly non-essential amino acids. The major amino acid was aspartic acid. Other metabolites involved in odor and resistance to abiotic and biotic stress such as ethanol, choline and trigonelline were

Table 7
Sensory analysis by tasters of 'Afourer' variety mandarins cultivated on CM, MC and CC rootstocks. Values are expressed as mean±SE (n=10).

	Parameters	CM	MC	CC
External evaluation	Size	7.15 ± 0.24 a	8.25 ± 0.27 b	7.30 ± 0.30 a
	Color	7.80 ± 0.29 a	8.20 ± 0.29 a	8.10 ± 0.23 a
	Shape	7.90 ± 0.23 a	8.00 ± 0.21 a	8.40 ± 0.22 a
	Peeling	7.40±0.30 a	8.30 ± 0.15 b	7.30 ± 0.33 a
Internal evaluation	Flavor	6.70±0.21 a	7.70 ± 0.36 b	7.70±0.21 b
	Sweetness	6.50 ± 0.54 a	7.90 ± 0.35 b	7.80 ± 0.25 b
	Seeds	10.00 a	10.00 a	10.00 a
	Pulp	7.00±0.36 a	7.70 ± 0.22 b	7.90 ± 0.13 b
	Acidity	8.30±0.26 b	7.30 ± 0.30 a	7.20 ± 0.32 a

Different letters in the same row indicate significant differences according to Tukey's test ($p<0.05$).

detected. Under the growing conditions studied, the most interesting rootstocks according to the set of characteristics studied, and the sensory analysis, were MC and CC.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Article

Mandarin Variety Significantly Affects the Metabolites Present in the Leaves

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Abstract: Late-season varieties of mandarin (*Citrus reticulata* Blanco) have a high economic value, so their study, characterization, and comparison among different commercial varieties is of great interest for agriculture. Detailed metabolomic analysis of mandarin leaves can provide valuable information on agronomic characteristics, vegetative development, and tree response to abiotic and biotic stresses. In this study, an analysis of the main metabolites presents in the leaves of three late-season mandarin orange varieties ('Afourer', 'Orri' and 'Tango'), cultivated under homogeneous conditions, was carried out using nuclear magnetic resonance (^{1}H NMR) and multivariate statistical analysis techniques. The results show that organic acids and sugars are the metabolites with the highest presence in mandarin leaves, especially malate and sucrose. Ten amino acids and other metabolites such as choline and trigonelline were also detected. Metabolites such as asparagine and isoleucine were widely implicated in the metabolic pathways of the detected compounds. The 'Orri' variety showed significantly more differences in metabolite concentrations compared to the other two varieties studied. Malate and sucrose were shown to be the metabolites with the greatest significant differences between the varieties compared. From an agronomic point of view, the 'Orri' variety differs from the other two varieties because it has concentrations of metabolites that provide good resistance to abiotic and biotic stresses and fruits of higher quality and sweetness.

Keywords: ^{1}H NMR; mandarins; late season; metabolomic footprint; leaves

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1. Introduction

Mandarins (*Citrus reticulata* Blanco) are one of the most economically important citrus fruits in terms of tons produced and exported in the world [1]. A large number of mandarin varieties and hybrids are cultivated, which have very different agronomic, morphological, and biochemical properties [2]. Late-season mandarin varieties are characterized by a high economic value due to the small number of varieties that are harvested in the winter season and the high production of these varieties [3]. Some of the most outstanding late-season mandarin varieties that can be found in the market today are 'Afourer', 'Orri', and 'Tango'.

In terms of production extension, it is estimated that there are 3 million trees of the 'Afourer' variety cultivated in Spain with the breeder's permission [according to unpublished data provided by Agrupación de Viveristas de Agrios S.A. (AVASA)]. Regarding the 'Orri' variety, the number of trees that can be cultivated in Spain is limited to 1.3 million and with the condition that this variety cannot be commercialized outside the European Union (according to unpublished data provided by AVASA). As for the 'Tango' variety, for the moment, there is no limitation on the size of the plantation and, although there is no information available on the number of trees grown in Spain, it is known that the cultivation of this variety has experienced growth (according to unpublished data provided by AVASA).

Regarding the origins of the variety 'Afourer', also known as 'Nadorcott', it was first cultivated in Morocco in 1988; it's the origin of this variety could have occurred from the hybridization of the variety 'Murcott', which is also known as 'Nadorcott' [*Citrus reticulata* Blanco × *Citrus sinensis* (L.) Osbeck] [4]. On the other hand, the 'Orri' variety is derived from irradiated cultivars of the 'Orah' variety (*Citrus temple* hort. ex Y. Tanaka) × (*Citrus tangerina* hort. ex Tanaka) in Israel [5]. The 'Tango' mandarin variety was developed at the University of California from hybridization via irradiation of the "Murcott" variety [6].

Previous studies of the fruits of the 'Afourer' variety indicate that it has a larger size and weight and a greater orange color if we compare them with 'Orri' and 'Tango' varieties [3]. Regarding the fruits of the 'Orri' variety, previous studies have identified that these are characterized by having a higher amount of juice and sugars than the other two varieties; in addition, it was identified that the main organic acid was malic acid and not citric acid as is usually common [3]. Finally, 'Tango' showed morphological and biochemical characteristics, very similar to the 'Afourer' variety [3].

Although few studies have been conducted on these varieties, they have also focused on showing the high quality and remarkable nutritional benefits of the mandarin fruit [7–9]. However, studies focused on other plant tissues, such as mandarin leaves, are much more limited. Leaves can provide us with information on their agronomic characteristics and their response mechanisms to different abiotic and biotic stresses [10,11]. Therefore, knowledge of these characteristics is of vital importance, since they are very important varieties and for which there are no previous publications that metabolomically analyze their leaves.

Metabolomic analysis techniques have improved significantly in recent years due to recent advances in more powerful and accurate instrumentation, as well as significant improvements in data processing and computational capacity [12,13]. Improvements in data analysis and statistical analysis techniques allow the results obtained to be analyzed and processed more efficiently, leading to a better understanding of the observed results [14]. Nuclear magnetic resonance is now a very powerful and accurate technique for the identification and quantification of primary and secondary metabolites in plant tissues [13,15]. Nuclear magnetic resonance is currently used in a large number of studies to accurately determine the metabolites present in fresh samples of any plant tissue [12,16–19].

The objective of this study is to determine and compare the metabolites present in the leaves of the three most important commercial late-season mandarin varieties ('Afourer', 'Orri', and 'Tango') in the Spanish market, under homogeneous growing conditions. Metabolite analysis was performed using nuclear magnetic resonance (^{1}H NMR) and the results obtained were analyzed via multivariate data analysis.

2. Materials and Methods

2.1. Plant Material and Experimental Design

For this study on leaf metabolomics, the leaves of three late-season varieties of mandarin trees were used (*Citrus reticulata* Blanco)—'Afourer', 'Orri', and 'Tango'—cultivated on *Citrus macrophylla* rootstock. In a previous study, mandarins from these same trees were morphologically and biochemically characterized, showing their quality and good growing conditions [3]. All varieties were placed in homogeneous growing conditions, cultivated on plateaus, with a planting frame of 5.5 × 4 m; all trees were 12 years old, with an average height of 376 cm ('Afourer'), 336 cm ('Orri'), and 254 cm ('Tango'), as well as a cup diameter of 316 cm ('Afourer'), 350 cm ('Orri'), and 216 cm ('Tango'). The commercial plots where the varieties were grown are located in the southeast of Spain, in the municipality of Torre-Pacheco (Murcia, Spain, 37°46′11.2″ N 1°01′07.4″ W). The region in which the study was conducted is characterized by a semi-arid climate, with high inter-annual variability in rainfall and long periods of drought with short episodes of very intense torrential rainfall [20]. The average agrometeorological conditions recorded during the last 10 years in the study area were as follows: temperature of 18.43 ± 0.15 °C, humidity of 68.90 ± 0.92%, wind speed of 2.01 ± 0.03 m/s, rainfall of 330.07 ± 44.26 mm, and evapotranspiration of 1283.63 ± 20.38 mm [21].

These mandarin trees were grown following cultivation practices aimed at their commercialization both nationally and internationally. The irrigation system used is a drip irrigation system; each tree had 4 L/h drippers (four drippers per tree), which provided the ferti-irrigation needs. The water consumption in the plots where these mandarin trees were grown was between 5500 and 6000 m^3/ha/year. Fertilization comprised between 180 and 200 units of NPK, 100 units of P_2O_5, and 50 units of K_2O per year. The irrigation and fertilizer doses were based on those used by local citrus growers, adapted to the needs of the crop, and continuously monitored using substrate probes and periodic foliar analysis to ensure optimal agrometeorological conditions. The trees were pruned annually and phytosanitary products were applied according to the needs and the appearance of different pests in the crop.

A total of 25 adult leaves of each mandarin variety were collected in triplicate, taking 5 leaves for each orientation (north, south, east, and west), from 5 trees at random (different trees for each replicate), in the 5 × 5 m plot. The leaves collected presented a correct development, highlighting the absence of nutritional deficiencies as well as diseases or pests. The leaves had an average length with leafstalks of 107.94 cm, 123.18 cm, and 102.09 cm; an average leaf width of 46.30, 41.73, and 42.37 cm; and an average leaf surface of 36.20, 34.30, and 30.32 cm^2 for 'Afourer', 'Orri', and 'Tango', respectively. The samples were quickly taken to the laboratory, to avoid their degradation, where they were cleaned with abundant distilled water to remove possible traces of dust, soil, and other elements. Then, all leaf samples were cut into homogeneous pieces and immediately frozen using liquid nitrogen. Once the samples were frozen, they were stored at −80 °C in propylene containers until lyophilization. The samples were freeze-dried (Christ Alpha 2-4 LSCplus, Martin Christ, Osterode am Harz, Germany) for 48 h. The samples were then ground (TSM6A013, Taurus, Oliana, Spain) and sieved to a fine homogeneous powder that was stored in 2 mL tubes at −20 °C until metabolomic analysis was performed.

2.2. Metabolomics Analysis of Leaves by ^{1}H NMR

Metabolomic analysis was performed in triplicate via nuclear magnetic resonance (^{1}H NMR) following the methodology described by Van der Sar et al. [22] and Melgarejo et al. [17]. A total of 0.5 mg of freeze-dried leaf sample was mixed with a hydromethanolic mixture (1:1, MeOH:H_2O). The samples were then sonicated for 30 min, centrifuged at 11,000 rpm for 20 min, and the supernatant was extracted. It was then subjected to speed-vacuum until all the liquid phase was evaporated; the solid obtained was resuspended in 800 µL of potassium phosphate buffer together with 0.58 mM TPS. Finally, 600 µL of the sample was taken for quantitative analysis via ^{1}H NMR (Ascend NMR Magnet 500 MHz, Bruker, Billerica, MA, USA). Subsequently, the identification and quantification of all detected metabolites were performed by integrating the peaks of the spectra obtained directly from the MNR (see Figure S1 in Supplementary Materials). For this purpose, chemical shifts obtained from an internal database of amino acids, organic acids, sugars, and other metabolites were used (Table 1).

Table 1. ^{1}H NMR chemical shifts identified in the samples of the three mandarin varieties.

Types of Compounds	Compounds	Chemical Shift (ppm) [1]
Amino acids	GABA	2.3 (t)
	Alanine	1.46 (d)
	Asparagine	2.94 (dd)
	Aspartate	2.81 (dd)
	Isoleucine	0.98 (d)
	Leucine	0.95 (t)
	Proline	2.0 (m)
	Threonine	1.3 (d)
	Tyrosine	6.9 (d)
	Valine	1.0 (d)

Table 1. *Cont.*

Types of Compounds	Compounds	Chemical Shift (ppm) [1]
Organic acids	Citrate	2.69 (d)
	Formate	8.43 (s)
	Fumarate	6.53 (s)
	Malate	2.7 (d)
	Quinate	1.7 (dd)
Sugars	Glucose	5.2 (d)
	Maltose	5.44 (d)
	myo-inostol	3.3 (t)
	Sucrose	5.4 (d)
Other metabolites	Choline	3.2 (s)
	Trigonelline	9.1 (s)

[1] Where the letter represents the multiplicity: s, singlet; d, doublet; t, triplet; dd, double of doublets; m, multiplet. Confidence level of 95%.

2.3. Statistical Analysis

A one-factor analysis of variance (ANOVA) was performed using the Statgraphics centurion 18 (StatPoint Technologies, Chicago, IL, USA), and Tukey's HSD test ($p \leq 0.05$) was applied for the contrast and separation of means. For the multivariate statistical analysis, MetaboAnalyst 5.0, an open-source JavaServer Faces Technology (Wishart Research Group, University of Alberta, Edmonton, AB, Canada), was employed. So, Principal Component Analysis (PCA), Partial Least Squares-Discriminant Analysis (PLSD-DA) and its Variable Importance in Projection (VIP), Significance Analysis of Microarray (SAM), hierarchical clustering heatmap, and Debiased Sparse Partial Correlation (DSPC) were calculated following the methodology described by Melgarejo et al. [23]. These statistical analyses are among the most effective for metabolomic studies in plants [24,25]. ANOVA with Tukey's test allows us to see the variances between the means of each sample with high statistical precision. The PCA allows us to visualize the variances between the metabolites of the samples in a graph of small dimensions. Using the PLSD-DA, we can easily see which metabolites vary the most between samples. The hierarchical clustering heatmap allows easier graphical visualization due to a color scale within which metabolites are in higher concentrations in all samples. The SAM is similar to the previous one, but, in this analysis, the color scale shows the metabolites that are more differentiated among the samples. Finally, the DSPC is one of the most powerful tools in metabolomic analysis, since it allows us to see graphically and with great quality what the correlation is between all the detected metabolites and how strong this correlation is.

3. Results and Discussion

The main metabolites identified via nuclear magnetic resonance of mandarin leaves were amino acids, organic acids, and sugars (Table 2). First, significant differences were observed between the number of amino acids present in the leaves of the different varieties (Table 2). Ten different amino acids were identified in the leaves of the 'Afourer' and 'Tango' varieties and nine in the 'Orri' variety; asparagine was not detected in the latter. The most abundant amino acid in all samples analyzed was proline. In addition, five amino acids were identified in the leaves: citrate, formic, fumarate, malate, and quinate. Malate was the major organic acid in the varieties 'Afourer' and 'Tango', while quinate was the major organic acid in 'Orri'. On the other hand, four sugars were detected: sucrose, glucose, myo-inositol, and maltose. The main sugar detected in all samples was sucrose, followed by glucose and myo-inositol; maltose was also detected in smaller amounts. Other metabolites detected were choline and trigonelline.

Table 2. Metabolite concentration (mM) in the leaves of three late-season mandarins varieties grown under homogeneous conditions. Values are expressed as mean ± SE (n = 3).

Types of Compounds	Compounds	'Afourer'	'Orri'	'Tango'
Amino acids	GABA	0.58 ± 0.01 a	0.62 ± 0.03 a	0.89 ± 0.03 b
	Alanine	0.11 ± 0.01 a	0.21 ± 0.06 a	0.16 ± 0.01 a
	Asparagine	0.09 ± 0 b	ND	0.07 ± 0 a
	Aspartate	0.3 ± 0.02 a	0.28 ± 0.02 a	0.46 ± 0 b
	Isoleucine	0.03 ± 0 a	0.06 ± 0 b	0.04 ± 0 a
	Leucine	0.03 ± 0.01 a	0.12 ± 0.04 a	0.11 ± 0 a
	Proline	5.26 ± 0.05 a	5.01 ± 0.65 a	6.39 ± 0.05 a
	Threonine	0.06 ± 0.01 a	0.09 ± 0.02 a	0.07 ± 0.01 a
	Tyrosine	1.67 ± 0.04 a	1.65 ± 0.01 a	1.66 ± 0.06 a
	Valine	0.04 ± 0 a	0.08 ± 0.01 a	0.04 ± 0.02 a
Organic acids	Citrate	1 ± 0.15 a	0.72 ± 0.09 a	0.91 ± 0.02 a
	Formate	0.02 ± 0 a	0.03 ± 0.01 a	0.02 ± 0 a
	Fumarate	0.08 ± 0.01 a	0.09 ± 0.02 a	0.09 ± 0.01 a
	Malate	15.46 ± 0.05 c	10.8 ± 0.04 a	12.51 ± 0.38 b
	Quinate	9.34 ± 0.05 a	17.28 ± 1.01 b	8.72 ± 0.29 a
Sugars	Glucose	1.57 ± 0.02 a	3.33 ± 0.16 b	1.45 ± 0.15 a
	Maltose	0.29 ± 0.06 a	0.23 ± 0 a	0.16 ± 0.02 a
	myo-inostol	1.09 ± 0.01 a	1.17 ± 0.03 a	1.25 ± 0.24 a
	Sucrose	8.26 ± 0.13 a	9.02 ± 0.11 b	9.35 ± 0.12 b
Other metabolites	Choline	0.37 ± 0 b	0.56 ± 0.01 c	0.31 ± 0.02 a
	Trigonelline	0.07 ± 0 b	0.04 ± 0 a	0.08 ± 0 b

Different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$), with "a" being the lowest average values, "c" the highest, and "b" are the values between "a" and "c". ND = Not Detected.

A quantitative analysis of the different metabolites present in leaves of the three varieties studied ('Afourer', 'Orri', and 'Tango') provides a highly accurate characterization and comparison of the samples studied. The analysis of the metabolites provides valuable information on the agronomic characteristics, vegetative development, and resistance to abiotic and biotic stresses of mandarin trees [10,11]. Therefore, from an agronomic point of view, the results obtained can be interpreted as follows.

With the presence of proline as the main amino acid, choline, and trinonieline, the three varieties are widely adapted to cultivation in areas with high abiotic stresses of drought and salinity [26–28]. Such stresses include those found in the southeast of the Iberian Peninsula or, more generally, in the Mediterranean basin, areas where a large part of the world's mandarin production is found [1]. The choice of a variety with greater resistance to abiotic factors is of great interest to growers because, in the scenario of climate change that we are facing, the cultivation of more resistant mandarins will save cultivation costs and ensure higher yields. Regarding this aspect, the cultivation of the varieties 'Tango' and 'Orri' could be of greater interest due to their ability to withstand abiotic stress due to the higher concentrations of GABA and isoleucine, respectively [29,30].

In parallel, it is also important for farmers' crops to have resistance to biotic stresses, such as insect pests, which cause additional costs in pesticide treatment or loss of production. In this sense, the 'Orri' variety would be of great interest, since its higher concentrations of quinate and isoleucine give it greater protection against pests than the other two varieties [30]. Although, it should be noted that the variety 'Afourer' would also be of great interest due to its higher concentration of malate, a compound also involved in the protection against pests [31].

Vegetative development is another important factor to consider from an agronomic point of view. Greater growth of the trees will allow growers to have mature plantations sooner and, thus, achieve higher yields as soon as possible. In this respect, we highlight the 'Afourer' variety for its higher concentrations of asparagine, a metabolite closely linked to vegetative growth [32]. In comparison, the 'Tango' variety has lower concentrations; in the 'Orri' variety, it is not even detectable.

Finally, the final quality of the fruit is fundamental to citrus growers. The tastier and sweeter varieties will be more appreciated in the market, which will allow the growers to obtain greater economic profitability. According to the results obtained, the 'Orri' variety will stand out for its greater sweetness, since a much higher amount of sucrose and glucose was detected in the leaves than in the other two varieties, which correlates with the sugar that will be present in the fruit [33,34].

With this in mind, the results of the various statistical analyses used are analyzed and discussed in more detail below.

3.1. Analysis of Variance by ANOVA

3.1.1. Amino Acids

The most abundant amino acid in the three varieties was proline (Table 2), a metabolite closely related to citrus fruit ripening and responses to biotic and abiotic stresses [26,35]. Other amino acids also found in high concentrations were tyrosine, GABA, and aspartate.

However, of all the amino acids detected, there were only significant differences in four of them (GABA, asparagine, aspartate, and isoleucine). A significantly higher concentration of GABA was observed in the leaves of the 'Tango' variety, with up to 53% more of this amino acid than in the other two varieties. The presence of this amino acid in greater quantities provides us with information on the response of this variety to climatic conditions, influencing the response of the plants to drought [24,36]. This is of great interest due to the fact that plantations of this citrus are usually carried out in areas commonly affected by drought, such as southeastern Spain. In the same way, this amino acid provides us with information on the final quality of mandarins, since GABA concentrations in leaves are closely related to the conversion of citric acid Into sugars during the fruit ripening process [33].

On the other hand, the leaves of the 'Afourer' variety contain 28% more asparagine than those of the 'Tango' variety, while this amino acid was not detected in the 'Orri' variety. Asparagine is a metabolite that plays a crucial role in various plant biochemical processes [32]. In adult plant leaves, it primarily participates in the uptake and mobilization of inorganic nitrogen during plant growth, flowering, and senescence. Additionally, it facilitates the mobilization of nitrogen to the seeds of fruits [32]. A higher concentration of this amino acid in the leaves implies a higher mobilization of nitrogen, which contributes to higher vegetative growth of the tree [32,37,38]. The absence of this amino acid in the 'Orri' variety could be due to the fact that it had not been metabolized at the time of sampling, since precursors of this metabolite were detected, such as aspartate [37].

The 'Tango' variety also had 64% more aspartate. The function of this amino acid in plants is to form new amino acids such as lysine, threonine, isoleucine, methionine, and glycine to increase the efficiency of the Krebs cycle and, consequently, to intervene in the vegetative growth of plants [32,39,40]. This would indicate that the 'Tango' variety has less vegetative growth than the other two varieties studied [32,40].

On the other hand, the variety 'Orri' presented up to twice the amount of isoleucine than the rest of the varieties analyzed. The presence of isoleucine in the leaves is related to the biosynthesis of jasmonate, a compound involved in the physiological response of the leaves (stomata opening and closing) to biotic and abiotic stresses [30].

No significant differences were observed in the remaining amino acids detected in the samples of the three varieties, which showed values within the expected range for citrus fruits [18].

3.1.2. Organic Acids

Malate and quinate were the most abundant acids in all samples and showed significant differences between varieties (Table 2). The concentration of malate detected was up to 43% higher in the 'Afourer' variety than in the other two varieties. The presence of malate in plants contributes to a wide variety of metabolic processes affecting plant growth and development; it is one of the main compounds involved in photorespiration and is closely related to the mobilization of nitrogen and phosphorus within plants [41–43]. Some studies have shown that a higher amount of malic acid in leaves reduces the attack of some lepidopteran pests such as *Helicoverpa armígera* (Surekha Devi et al. [31] quoting Narayanamma et al. [44] and Yoshida et al. [45]).

Quinate is present in a significantly higher concentration in the 'Orri' variety, being its most important organic acid. Quinate is related to plant resistance to biotic stress, being a precursor of several organic acids that contribute to the protection of leaves against insect attack (Carrington et al. [46], quoting Leiss et al. [47]). In addition, this metabolite is involved in the metabolic pathways of Shikimate, which are essential in the biosynthesis of aromatic amino acids such as tyrosine or tryptophan in plants [46,48].

3.1.3. Sugars

As highlighted above, the main sugar detected in all samples was sucrose, followed by glucose and myo-inositol; maltose was also detected in smaller amounts (Table 2). This is in contrast to other studies conducted on lemon tree leaves, which show that maltose is one of the major sugars in lemon tree leaves [17].

Choline is a biomolecule synthesized by plants whose main function is usually to be the precursor of betaine, a compound linked to the response of plants to biotic and abiotic stresses [49]. Sucrose was significantly more present in the 'Orri' and 'Tango' varieties than in the 'Afourer' variety. Sucrose, the most abundant disaccharide in mandarins, is the main energy supplier for these plants, crucial for their metabolism and development [50]. The presence of this sugar is of great importance for the final quality of the fruit, since during the ripening process sucrose is mobilized from the leaves to the fruit, providing a large part of the characteristic sweet taste. [33,34,47,50].

On the other hand, the 'Orri' variety showed up to 2.3 times more glucose than the other two varieties studied. A higher concentration of glucose is also associated with a higher amount of sugar in the fruit and, therefore, higher sweetness [51]. A previous study carried out on the fruit of these three mandarin varieties shows that the fruit of the 'Orri' variety has a higher sugar content [3].

3.1.4. Other Metabolites

The concentrations of choline were significantly different among the three varieties, being higher in the leaves of the 'Orri' variety, followed by the 'Afourer' variety, and lower in the 'Tango' variety. The presence of trigonelline in the 'Afourer' and 'Tango' samples reached twice the concentration of the 'Orri' variety (Table 2).

On the other hand, trigolienin is a plant alkaloid that acts as an osmolyte and accumulates in plants under biotic and abiotic stress conditions [52]. Studies show that these two metabolites are involved in pathways related to the response of plants to salt stress [27,28,53]. This is noteworthy for agriculture because salinity stress in Mediterranean regions, caused mainly by frequent droughts and low irrigation water quality, is one of the main problems for citrus crops [54]. The problem of salinity is usually solved by choosing rootstocks better adapted to abiotic stresses [55,56]. However, this study shows how the choice of variety is also of great importance, since we observed significant differences in several of the metabolites present and involved in the response to stress, even when using the same rootstock (*Citrus macrophylla*).

3.2. Principal Component Analysis and Partial Least Squares-Discriminant Analysis

PCA (Principal Component Analysis) is a multivariate analysis method that allows us to visualize the data in smaller dimensions through a graphical representation, helping us to identify the variances and variations among the samples of the study [57]. The PCA of our study (Figure 1) shows that Component 1 represents 79.7% of the total variance among varieties, while Component 2 represents 19.3% of the total variability.

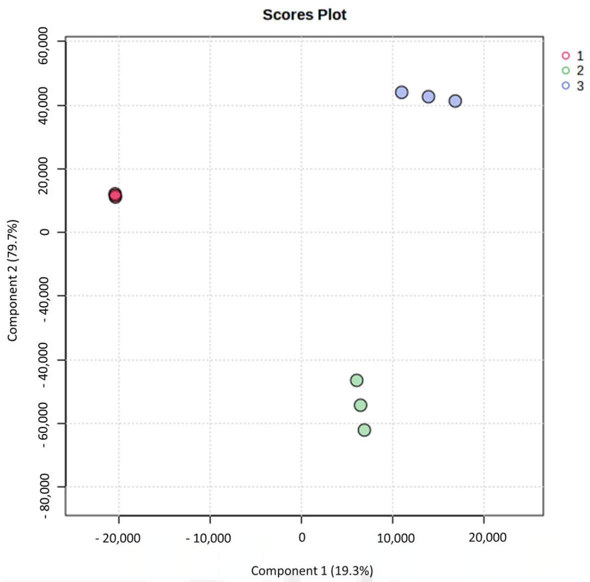

Figure 1. Principal Component Analysis (PCA) graph of three late-season mandarin varieties (1. 'Afourer'; 2. 'Orri'; 3. 'Tango').

Using PLS-DA (Partial Least Squares-Discriminant Analysis) regression, the VIP (Variable Importance in Projection) was estimated, which shows us which metabolites are significant and differentiating among the three mandarin varieties [58,59]. The results (Figure 2) show that only malate, proline, and sucrose were the metabolites that had a VIP greater than 1, so these compounds had the highest significant differences among the three varieties.

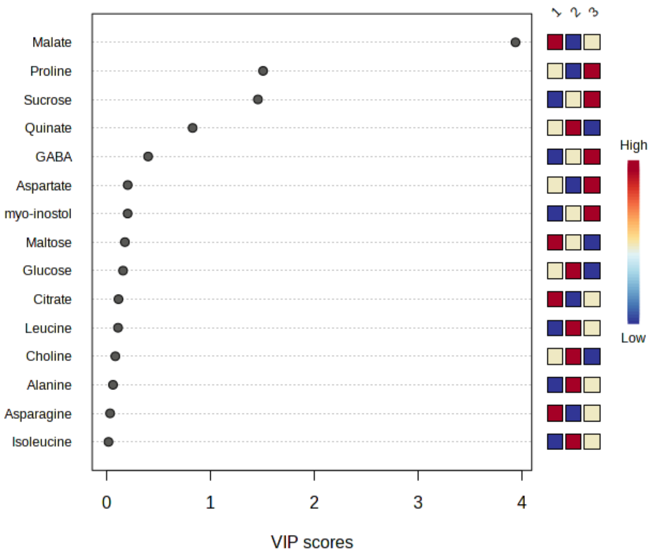

Figure 2. Partial Least Squares-Discriminant Analysis (PLS-DA), graphically represented using a Variable Importance in Projection (VIP) plot, of the metabolites detected in the leaves of three late-season mandarin varieties (1. 'Afourer'; 2. 'Orri'; 3. 'Tango'). The red color represents a higher concentration of a certain metabolite.

3.3. Hierarchical Clustering Heatmap

On the other hand, the hierarchical clustering heatmap shows visually with a color scale the relative concentrations of each of the detected metabolites [60]. The heatmap (Figure 3) showed that the 'Orri' variety was characterized by higher amounts of the metabolites choline, quinate, and formate acids, as well as the sugar glucose and the amino acids threonine, alanine, or isoleucine. The 'Afourer' variety was characterized by a higher concentration of malate and citrate acids, maltose sugar, and the amino acids asparagine and tyrosine. The 'Tango' variety was characterized by higher concentrations of the amino acids GABA, aspartate, and proline.

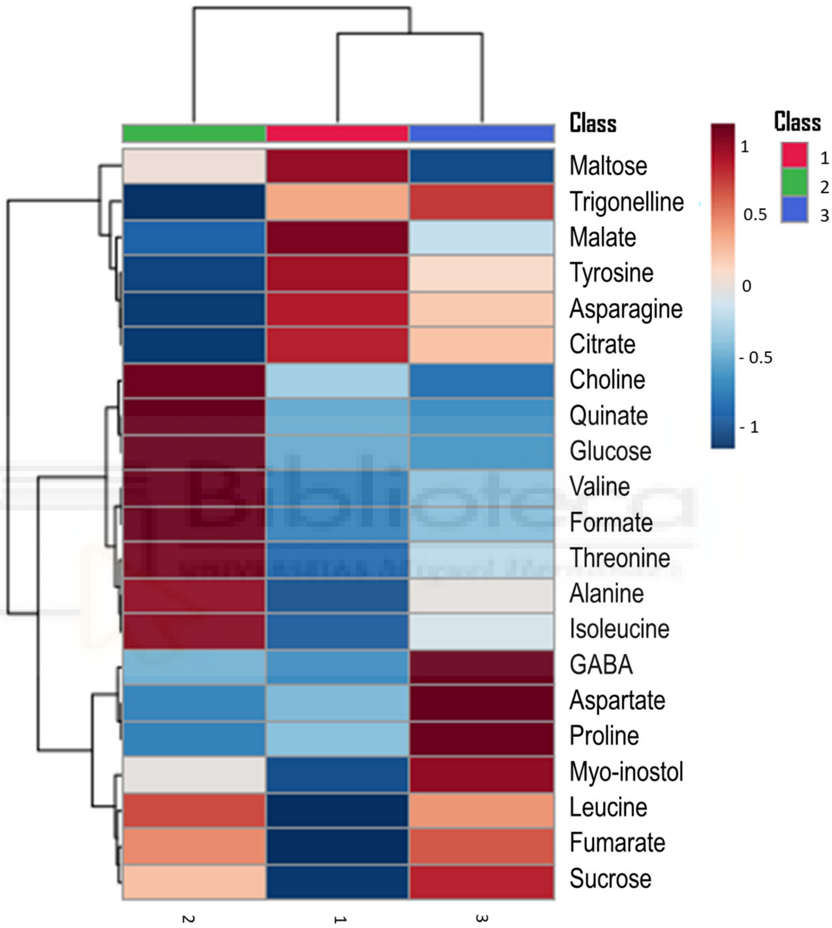

Figure 3. Hierarchical clustering heatmap of metabolites detected in the leaves of three late-season mandarin orange varieties (1. 'Afourer'; 2. 'Orri'; 3. 'Tango'). The red color represents a higher relative concentration of a given metabolite.

3.4. Significance Analysis of Microarray

Significance Analysis of Microarray (SAM) provides us with information on which metabolites have the greatest significant difference among all the samples analyzed [61]. SAM and cluster analysis (Figure 4) of our results show that malate and quinate to a greater extent and sucrose and proline to a lesser extent were the metabolites with the highest significance. Through the cluster, we also found that the varieties 'Afourer' and 'Tango' are more correlated with each other than the variety 'Orri', which is mainly due to the fact that the variety 'Tango' was obtained from the hybridization of the variety 'Afourer' [6].

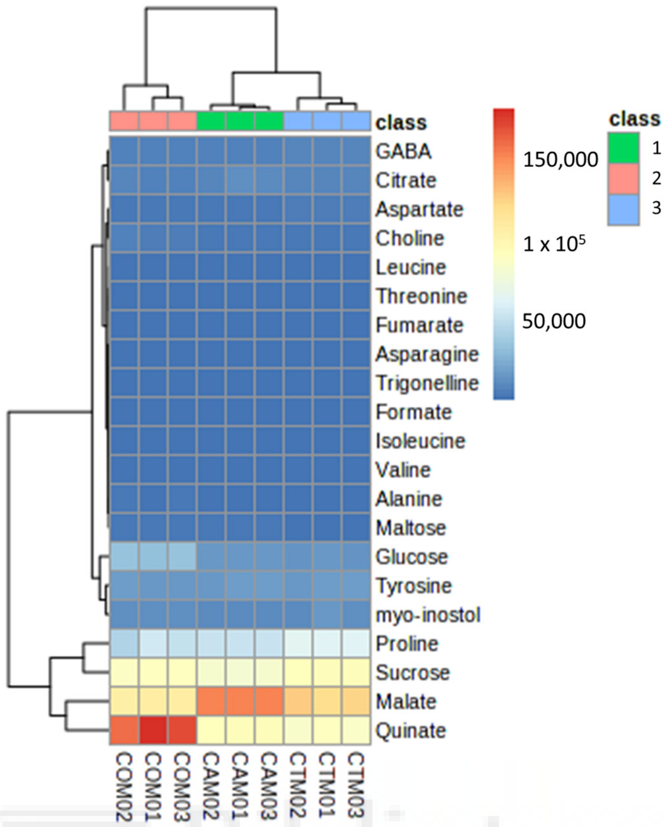

Figure 4. Significance Analysis of Microarray (SAM) of metabolites detected in leaves of three late-season mandarin varieties (1. 'Afourer'; 2. 'Orri'; 3. 'Tango'). The red color represents a higher significance of a given metabolite.

3.5. Deviated Scattered Partial Correlation

Analysis of the metabolic pathways was carried out by applying the Deviated Scattered Partial Correlation (DSPC) algorithm to the results obtained in ^{1}H NMR. This algorithm was formulated by Janková and van de Geer [58] using graphical loop modeling. This analysis allows us to graphically identify the correlation between different metabolites present in the leaf samples to be analyzed [62]. The graphical model (Figure 5) represents the metabolites in a weighted node network, where the partial correlation coefficients and *p*-values of each pair of metabolites that are correlated by metabolic pathways are represented. Two nodes joined by a red line represent a positive relationship, while those joined by a blue line represent a negative relationship.

The analysis of the metabolic pathways carried out on the mandarin varieties under study using the DSPC algorithm shows that asparagine has a grade 10 and an interrelation of 29.73, which shows that it is an important metabolite, as it is involved in many of the metabolic pathways of the rest of the detected compounds. Asparagine is positively related to trigonelline, malate, quinate, and citrate and negatively related to fumarate, valine, glucose, choline, isoleucine, and threonine. This amino acid in plants is involved in several metabolic pathways related to the process of photorespiration, in addition to being the main reservoir and transporter of nitrogen from the roots to the leaves [32,63].

Isoleucine also had an important presence in the metabolomic interrelationships with an interrelationship of 34.63 and a grade of 9. Most of its relationships were positive (leucine, alanine, threonine, asparagine, valine, and glucose), with only three being negative (malate, citrate, and quinate). Isoleucine is involved in the Krebs cycle, which implies that a higher amount of this compound is related to a lower amount of malate and citrate, while it is directly related to glucose [64].

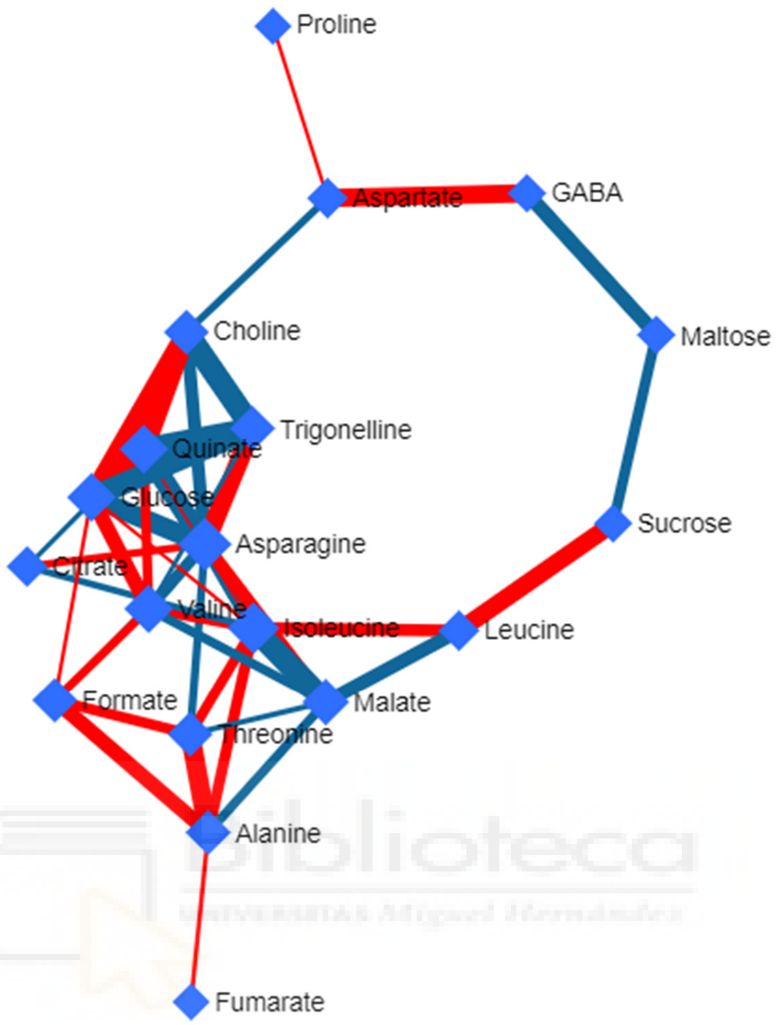

Figure 5. Correlation network formed using the Debiased Sparse Partial Correlation (DSPC) algorithm of the metabolites detected via ^{1}H NMR in the leaves of three late-season mandarin varieties ('Afourer', 'Orri', and 'Tango'). Red lines represent positive relationships, while blue lines represent negative relationships. The size of the nodes shows the direction of change. The color of the lines is adjusted to a p-value < 0.05 and the false discovery rate (FDR) is adjusted to a p-value < 0.2.

Valine and glucose play direct and indirect roles in the Krebs cycle, which directly relates them to the synthesis and degradation of a wide variety of amino acids, acids, and sugars [65,66]. Quinate, on the other hand, is mainly involved in metabolic pathways for the synthesis of aromatic amino acids [46].

4. Conclusions

In this study, a metabolomic analysis of the leaves of three late-season mandarin varieties ('Afourer', 'Orri', and 'Tango') grown under homogeneous conditions on *Citrus macrophylla* rootstock was performed to characterize the metabolites present in the leaves of these mandarins and the differences between varieties. The most abundant metabolites found were organic acids and sugars, mainly malate, quinate, and sucrose. Ten amino acids were also identified in the leaves of these mandarins, with proline, tyrosine, and GABA standing out in terms of concentration, as well as other metabolites such as choline and trigonieline. Correlation analysis showed that asparagine and isoleucine were involved in many of the pathways of the other metabolites detected. The multivariate statistical analysis performed clearly shows that there are significant differences between the concentrations of metabolites present in the three varieties studied, especially in compounds such as malate, quinate, and sucrose. In summary, the results show that the 'Orri' variety has a more differentiated metabolomic profile compared to the other varieties studied. This indicates differentiated vegetative growth, different adaptation to different biotic and abiotic stresses (mainly pests and salt stress), and a direct correlation with the final quality of mandarins. Therefore, from an agronomic point of view, the variety 'Afourer' stands out from the other two varieties due to its greater vegetative growth. The variety 'Tango' stands out for its greater resistance to abiotic stress. But, above all, the variety 'Orri' stands out from the other two varieties because, although it has a lower vegetative growth than the other two varieties, it has a good resistance to both abiotic and biotic stresses and its fruits tend to be sweeter and of higher quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10040359/s1, Figure S1: ^{1}H NMR spectra of the three varieties studied ('Afourer', 'Orri' and 'Tango').

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