ECOLOGÍA DE LOS TERPENOS VOLÁTILES DE LA PIEL DE LA NARANJA: COMUNICACIÓN PLANTA-FRUGÍVORO

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A mons pares i ma filla

Abstract

Ecology of volatile terpenes from the orange peel: animal-fruit communication

For decades many authors have investigated how frugivores have configured the shape, size, nutritional content, color, etc. of the fleshy fruits along their evolution. More recently, the interest of the scientific community has increased in establishing the role of certain fruit chemical compounds in the interactions between frugivores and fleshy fruits.

Secondary metabolites of plants are synthesized in small quantities and perform different specialized functions in the biotic and abiotic interactions of plants with their environment, such as defense against herbivores, attraction of pollinating insects, communication between different organs within a plant, etc. Citrus fruits mainly produce terpenes as characteristic secondary metabolites, which are stored in essential oil glands in all their organs except roots. In the fruit flavedo, monoterpenes are produced predominantly, being D-limonene the most abundant one.

The general objective of this thesis is to investigate the mechanisms of citrus fruit/frugivore communication mediated by the volatile compound D-limonene abundantly accumulated in the fruit favedo. For this, we make various citrus fruit offerings in orchards in both tropical (Sâo Paulo, Brazil) and Mediterranean (Valencia, Spain) environments and follow frugivores behavior through the use of photo-trapping and fingerprint cameras. By using genetically-modified oranges with reduced content of D-limonene in their flavedo compared to conventional oranges with high D-limonene content, we investigate the specific role (attraction / repellence) of this compound in fruit interactions with vertebrate and invertebrate frugivores. The juice of these fruits, modified or not, is subjected to human panels that, though detecting differences in their smell, do not find any of them as depreciable or better than the other.

The infection of citrus fruits by the specialized fungus *P. digitatum* modifies the physicochemical characteristics of the fruits making them more attractive to vertebrate frugivores. The study of the content and emission of volatile organic compounds of healthy and infected fruits reveals the chemical changes in the profile of volatile compounds resulting from fungal infection that are attractive to vertebrate

frugivores. The detection of pronounced emission peaks of D-limonene in genetically-modified fruits with reduced content in this compound infected by *P. digitatum* leads us to investigate whether the fungus is capable of producing this hydrocarbonated monoterpene.

Finally, the citrus-frugivores system is studied from the point of view of its effect on the ecosystem. Specifically, we evaluate the possibility that the interaction between cultivated citrus and wild boar introduced from outside its native area may contribute to the naturalization of citrus in the American continent.

Resumen

Ecología de los terpenos volátiles de la piel de la naranja: comunicación planta-frugívoro

Desde hace décadas muchos autores han investigado cómo a lo largo de la evolución, los frugívoros han configurado la forma, el tamaño, el contenido nutricional, color, etc. de los frutos carnosos. Más recientemente se ha intensificado el interés de la comunidad científica por establecer el papel de determinados compuestos químicos en la interacción entre frugívoros y frutos carnosos.

Los metabolitos secundarios de las plantas se sintetizan en pequeñas cantidades y pueden realizar diferentes funciones especializadas en las interacciones bióticas y abióticas de las plantas con su entorno, como defensa frente a herbívoros, atracción de insectos polinizadores, comunicación entre diferentes órganos de una planta, etc. Los cítricos producen principalmente terpenos como metabolitos secundarios característicos, que se almacenan en las glándulas de aceites esenciales de todos sus órganos excepto raíces. En el flavedo de los frutos se producen sobretodo monoterpenos, siendo el más abundante el D-limoneno.

El objetivo general de la tesis es investigar los mecanismos de comunicación cítricos-frugívoros mediados por el compuesto volátil D-limoneno del flavedo. Para ello realizamos diversos ofrecimientos de frutos cítricos en campos de cultivo de ambientes tropicales (São Paulo, Brasil) y Mediterráneo (Valencia, España) y mediante el uso de cámaras de fototrampeo y huellas, estudiamos la influencia que este compuesto ejerce en el comportamiento de los frugívoros. Gracias al uso de naranjas con reducido contenido de D-limoneno en su flavedo obtenidas mediante técnicas de ingeniería genética junto a naranjas convencionales con alto contenido de D-limoneno, investigamos el papel específico (atracción/repelencia) de este compuesto en las relaciones de los frutos con los frugívoros vertebrados e invertebrados. El zumo de estos frutos, modificados o no, se somete a paneles de catadores humanos que, aunque detectan diferencias en el olor de los mismos, no son clasificadas como depreciables.

La infección de los frutos cítricos por el hongo especialista *P. digitatum* modifica las características fisicoquímicas de los frutos haciéndolos más atrayentes para los frugívoros vertebrados. El estudio del contenido y emisión de los compuestos orgánicos volátiles de los

frutos sanos e infectados revela los cambios químicos en el perfil de los compuestos volátiles como consecuencia de la infección fúngica que resultan atrayentes a los frugívoros vertebrados. La detección de picos importantes de emisión del volátil D-limoneno en frutos con reducido contenido en este compuesto infectados por *P. digitatum* nos conduce a investigar si el hongo es capaz de emitir este monoterpeno hidrocarbonado.

Finalmente se estudia el sistema cítricos-frugívoros desde el punto de vista de su efecto en los ecosistemas. En concreto, evaluamos la posibilidad de que la interacción entre cítricos cultivados y jabalíes introducidos fuera de su distribución nativa pudiera contribuir al asilvestramiento de los cítricos en el continente americano.

Resum

Ecologia dels terpens volàtils de la pell de la taronja: comunicació planta-frugívor

Molts autors proposen que al llarg de l'evolució, els animals frugívors han configurat la forma, la grandària, el contingut nutricional, la textura, etc. dels fruits carnosos. L'estudi de la composició de les característiques específiques dels fruits i la seua interacció amb els animals frugívors en el seu ambient natural està despertant molt interés en la comunitat científica, per a intentar establir el paper de determinats compostos químics en la interacció entre frugívors i fruits carnosos.

Els metabòlits secundaris de les plantes se sintetitzen en xicotetes quantitats i poden realitzar diferents funcions especialitzades en les interaccions biòtiques i abiòtiques de les plantes, com a defensa enfront d'herbívora, atracció d'insectes pol·linitzadors, comunicació entre diferents òrgans d'una planta, etc. En els cítrics s'emmagatzemen principalment en les glàndules d'olis essencials del flavedo dels fruits i són principalment monoterpens, sent el més abundant el D-limonè.

L'objectiu general de la tesi és investigar els mecanismes de comunicació cítrics-frugívors mediats pel compost volàtil D-limonè. Per a això realitzem diversos oferiments de fruits cítrics en camps de cultiu d'ambients tropicals (São Paulo, Brasil) i Mediterrànies (València, Espanya) i mitjançant l'ús de cambres de fototrampeig i petjades, estudiem la influència que aquest compost exerceix en el comportament dels frugívors. Gràcies a l'ús de taronges amb reduït contingut de D-limonè al seu flavedo obtingudes mitjançant tècniques d'enginyeria genètica, investiguem el paper específic (atracció/repel.lència) d'aquest compost determinat en les relacions amb els frugívors vertebrats i invertebrats. El suc d'aquests fruits se sotmet a un panell de tastadors humans que, encara que detecten diferències en l'olfacte del suc, aquestes no són classificades com nocives.

La infecció dels fruits cítrics pel fong especialista *P. digitatum* modifica les característiques fisicoquímiques dels fruits fent-los més atraients per als frugívors vertebrats. L'estudi del contingut i emissió dels compostos orgànics volàtils dels fruits sans i infectats revela els canvis químics en el perfil dels compostos volàtils com a conseqüència de la

infecció que resulten atraients als frugívors vertebrats. La detecció de pics importants d'emissió del volàtil D-limonè en fruits amb reduït contingut en aquest compost infectats per *P. digitatum* ens condueix a investigar si el fong és capaç d'emetre aquest terpé hidrocarbonat.

Finalment s'estudia la interacció de dues espècies natives del continent asiàtic (porcs senglar i cítrics) interactuant fora de la seua distribució nativa en el continent americà baix noves condicions ecològiques.

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1. INTRODUCCIÓN

1.1. Interacciones planta-frugívoros.

Las plantas son sésiles, no pueden moverse. Su mecanismo de desplazamiento se limita prácticamente al movimiento de polen y semillas (Jordano et al. 2011). Este hecho tan simple ha sido el motor evolutivo de numerosas estrategias en las plantas para poder colonizar nuevos territorios, conocidas como estrategias de dispersión. Janzen (1970) y Connell (1971) sugirieron que las semillas dispersadas cerca de la planta madre tienen una mayor mortalidad (causada por la competencia, la depredación y las enfermedades) que aquellas dispersadas lejos de la planta madre. Las especies de plantas han evolucionado distintas estrategias para ser dispersadas exitosamente. Las plantas pueden dispersar sus semillas mediante el viento, el agua, en el interior de los animales (endozoocoria), adheridas al exterior de los animales (exozoocoria) o por otros medios (Pijl 1982). La mayoría de las plantas que crecen en zonas templadas y tropicales tienden a ser dispersadas por animales (Howe y Smallwood 1982). Aunque muchas plantas son capaces de reproducirse sin la ayuda de agentes externos, se calcula que frugívoros y granívoros dispersan aproximadamente el 60-80% de todas las especies de plantas (Wang y Smith 2002). Para que el proceso de dispersión sea efectivo las semillas deben permanecer viables después de ser manipuladas o consumidas por los animales, es decir, después de pasar por sus tractos digestivos en el caso de la endozoocoria. La probabilidad de que una semilla se convierta en una planta adulta dependerá del número de semillas dispersadas, del número de visitas realizadas por un dispersor, del tratamiento en el aparato digestivo y de la calidad de la deposición (Schupp 1993, Schupp et al. 2010). En ocasiones, el paso por el sistema digestivo del animal incluso les beneficia haciéndolas menos atractivas a los predadores, eliminando patógenos o escarificándolas y estimulando

así la germinación (Janzen 1981, Traveset 1998, Robertson et al. 2006). Existen evidencias de algunas especies perennes leñosas como *Castanea crenata, Olea europea, Persea americana* y *Phoenix dactylifera* dispersadas por personas (Fuler 2018). Los humanos hemos sido importantes frugívoros dispersores de frutales del Centro y Sudamérica (Zonneveld et al. 2018). Un caso concreto de estudio es el de la manzana (*Malus domestica*) donde, como consecuencia del uso de técnicas de injerto e hibridación por humanos se seleccionaron las especies con frutos grandes tal y como ocurrió durante el Mioceno tardío con la megafauna que seleccionó las especies de *Malus* sp. de frutos grandes (Spengler 2019).

Está asumido que hace 140 millones de años, cuando probablemente se originaron las angiospermas, las semillas de los frutos eran pequeñas y carentes de señales para los animales, indicando que quizá no se habían desarrollado todavía las interacciones planta-dispersores (Tiffney 2004). Durante el Terciario, hace 65 millones de años, las plantas y sus frutos aumentaron considerablemente en tamaño y la producción de frutos carnosos comenzó a evolucionar debido a las presiones selectivas ejercidas por vertebrados frugívoros dispersores de semillas (Fleming y Kress 2011). La dispersión de frutos carnosos por aves ha sido más habitual que por mamíferos en todas las angiospermas y se sugiere que la evolución de los frutos dispersados por aves ha facilitado la evolución de la frugivoría en primates (Fleming y Kress 2011). Con algunas excepciones, se cree que la mayoría de las familias botánicas dispersadas por vertebrados frugívoros son más ricas en especies y más antiguas geológicamente que el resto de angiospermas. Las semillas de los frutos carnosos tienden a ser más grandes que las semillas dispersadas por otros medios (Hughes et al. 1994) y las semillas de las perennes leñosas dispersadas por personas tienden a ser más largas, delgadas y con

ápices más puntiagudos que las no dispersadas por personas (Fuler 2018).

Las plantas invierten muchos recursos en forma de señales y semillas para ser dispersadas efectivamente. Por su parte, los frugívoros están bajo presiones selectivas por conseguir los recursos nutritivos necesarios para sobrevivir y reproducirse (Jones y Wheelwright 1987). El resultado es una alta variación de tamaños, formas, colores, aromas y sabores de frutos y semillas adaptados a los diversos animales frugívoros (Cipollini y Levey 1997a). Esto, a su vez, ha facilitado la evolución de una enorme diversidad de animales, con distintos hábitos alimentarios (Thompson 2005). No obstante, la mayor parte de los vertebrados frugívoros son generalistas tróficos, alimentándose también de otras partes vegetales (semillas, hojas, etc.) y presas animales (Jordano 2000). Casi todas las plantas dispersadas por animales tienden a ser dispersadas por numerosos animales y casi todos los frugívoros se alimentan de frutas de muchas especies y de otros recursos, lo que indica interacciones generalizadas (Howe 1993, Karban 2015). Por ejemplo, Snow (1981) halló que solo 17 familias de aves frugívoras tropicales podían considerarse estrictamente frugívoras, 21 familias consumían una dieta mixta con una gran proporción de frutas y una pequeña variedad de presas animales y 23 familias mezclaban frutas y otros recursos en sus dietas. La frugivoría estricta entre los mamíferos es prácticamente inexistente (Jordano 2000). Entre los murciélagos, solo los géneros Pteropodidae y los Phyllostomidae pueden considerarse mayoritariamente frugívoros (Gardner 1977, Marshall 1983, Fleming 1986), complementando su dieta de frutas con el consumo de insectos (Courts 1998). Los frutos carnosos son el tipo de alimento más consumido entre los primates (Corlett 1998), la corzuela (*Mazama* spp.) y los pequeños antílopes africanos (*Cephalophus* spp.), los

cuales pueden incluir hasta el 85% de frutas en su dieta (Dubost 1984, Bodmer 1990).

Los frugívoros perciben la madurez de los frutos en base principalmente a rasgos visuales, olfativos y táctiles (Rodríguez et al. 2013). Las personas seleccionamos los frutos por su contenido en azúcares entre otros parámetros (Fuler 2018). Los frutos maduros dispersados por aves diurnas suelen ser rojos o negros mientras que los dispersados por mamíferos (con frecuencia nocturnos) suelen ser verdes, amarillos o marrones (Janson 1983, Willson y Whelan 1990). Los frutos dispersados por frugívoros diurnos tienden a cambiar de color cuando maduran mientras que los dispersados por nocturnos no cambian de color cuando maduran y frecuentemente liberan fuertes olores (Lomáscolo y Schaefer 2010). Además del color, las características morfológicas (p.e. tamaño) también informan a los frugívoros sobre el estado de madurez (Schaefer et al. 2004). La dureza y el olor de un fruto son las señales más utilizadas por los primates frugívoros para detectar la madurez de un fruto (Dominy 2004). El olor de un fruto es el resultado de la emisión de una mezcla de diferentes compuestos volátiles; cada fruto tiene un olor característico y los animales necesitan receptores nerviosos específicos para poder identificarlos. Así, en algunos casos es posible establecer una comunicación privada entre un frugívoro determinado y los frutos que habitualmente consume, aunque, en la mayoría de casos, estas señales olfativas suelen ser percibidas por muchos frugívoros generalistas. El olor es particularmente útil para los mamíferos frugívoros de hábitos nocturnos para localizar los frutos maduros (Fedriani y Boulay 2006, Schaefer y Ruxton 2011).

En las últimas décadas la comunicación entre plantas de frutos carnosos y frugívoros ha despertado el interés de ecólogos y bioquímicos interesados en su posible co-evolución (Snow 1971, Mckey 1975, Herrera 1982, Jordano 2000, Lomáscolo et al. 2010), y

más concretamente en el papel de los metabolitos secundarios producidos por los frutos en las interacciones con sus depredadores y dispersores (Cipollini y Levey 1997b, Fedriani y Boulay 2006, Whitehead et al. 2016).

Todavía hay pocas evidencias empíricas sobre las funciones de los metabolitos secundarios, aunque todo parece indicar que juegan un importante papel en las interacciones bióticas y abióticas de las plantas. Se cree que los compuestos orgánicos volátiles (COVs) eran originalmente compuestos defensivos utilizados por las plantas para combatir plagas y microorganismos (Turlings y Tumlinson 1992). Entre las principales funciones actuales de los COVs destaca su papel defensivo contra herbívoros, atracción de dispersores de semillas, repelencia de predadores de semillas, comunicación entre distintos órganos de una planta y entre diferentes plantas, atracción de polinizadores y predadores de plagas (Farmer y Ryan 1990, Lerdau et al. 1997, Peñuelas y Llusià 2001, Ariza et al. 2002, Gershenzon y Dudareva 2007, Papadopoulus et al. 2015, Chen et al. 2018). Se cree que su primera función mientras los frutos están inmaduros es defenderlos frente cualquier tipo de amenaza y con ello proteger a las semillas aún inmaduras (Mack 2000). Cipollini y Levey (1997a) describen los posibles efectos de los metabolitos secundarios existentes en los frutos carnosos sobre los dispersores vertebrados de semillas, que pueden ser atracción, repelencia, asociación, indigestión, toxicidad, asimilación de proteínas e inhibición de la germinación (véase también Fedriani et al. 2012, Whitehead et al. 2016). Aharoni et al. (2003) demostraron que terpenos específicos emitidos por hojas podían intoxicar, repeler o desanimar a herbívoros, o atraer predadores naturales y parásitos de los herbívoros (Kappers et al. 2005). Los trabajos de COVs acumulados en frutos son mucho menos abundantes que los referidos a flores y hojas. En general, flores y frutos liberan una amplia variedad de COVs especialmente en dos

picos máximos antes de la polinización y durante la maduración de los frutos (Dudareva et al. 2004). Flores, hojas y frutos emiten diferentes perfiles de COVs, sugiriendo posibles funciones diferentes en sus interacciones con el entorno.

A medida que un fruto madura se producen cambios en los COVs junto con otros cambios en la textura del fruto, tamaño, sabor, aroma y color. La influencia de la maduración del fruto en la síntesis de COVs ha sido ampliamente documentada (Villatoro et al. 2008, Yang et al. 2011, Lebrun et al. 2008, Lavilla et al. 2002). Los frugívoros son capaces de captar dichos cambios. Se han realizado muchos estudios centrados en el color de los frutos como señal de maduración y sus interacciones con animales dispersores de semillas (Schaefer 2011, Valido et al. 2011). Sin embargo, los cambios producidos en el perfil de COVs como señal de maduración de los frutos para sus frugívoros siguen siendo poco estudiados (pero véase Nevo et al. 2018, 2019, Valenta et al. 2017).

Los COVs comprenden una amplia diversidad de metabolitos secundarios con bajo peso molecular que se encuentran en forma gaseosa a temperatura ambiente debido a su baja presión de vapor. Algunos COVs son comunes en diferentes géneros de plantas mientras que otros son específicos de uno o pocos taxones. Desde el punto de vista químico los COVs se pueden clasificar en ésteres, alcoholes, aldehídos, cetonas, lactonas y terpenos. Se acumulan en tejidos y órganos vegetales específicos, como es el caso de las glándulas de aceite del flavedo de los frutos cítricos (Rodríguez et al. 2013). Los aceites esenciales de la piel de la naranja juegan un papel esencial en las interacciones de los frutos con microorganismos especializados (Rodríguez et al. 2011a; 2014). Estos resultados indican que la acumulación de D-limoneno en el flavedo de los frutos cítricos está implicada en las interacciones tróficas entre frutos, insectos y microorganismos, lo cual revela las importantes funciones de los

terpenos en la naturaleza. En el caso de animales, tanto los terpenos emitidos por los frutos cítricos como su color y textura pueden representarse como señales de comunicación con distintos gremios de frugívoros (p.e. dispersores y depredadores de semillas, despulpadores, hongos y bacterias patógenas; Rodríguez et al. 2013, Fedriani y Delibes 2013). En los Capítulos II y III se aborda la importancia de los COVs del flavedo de los frutos cítricos en la atracción o repelencia de frugívoros generalistas.

1.2. Importancia de los ecosistemas agrícolas

En algunas ocasiones, el ritmo pausado de ciertas intervenciones humanas con el medio ambiente desde la antigüedad ha permitido una adaptación entre las prácticas agrícolas y los ecosistemas seminaturales (Sans 2007) generando agroecosistemas simplificados como los pastizales o más complejos como las dehesas mediterráneas extremeñas y los cafetales sudamericanos tropicales (Perfecto et al. 1996, Moguel y Toledo 1999). Los agroecosistemas son ecosistemas agropogénicos ya que su origen y mantenimiento va asociado a la actividad humana, básicamente dirigida a la producción de alimentos, pastos, fibras y leñas, creando ecosistemas seminaturales con elevado grado de diversidad de especies mantenidas en el espacio y el tiempo (Vega et al. 1997, Bugalho et al. 2011). Actualmente ocupan más de la cuarta parte de la superficie terrestre mundial (50 millones km^2, Estrada et al. 2012) y pueden desempeñar un papel importante en la conservación de la biodiversidad al proporcionar hábitat para muchas especies en paisajes dominados por la presencia humana (Bhagwat et al. 2008).

Los agroecosistemas tienen una importancia capital tanto como hábitat de refugio, alimentación y reproducción de numerosas especies de vertebrados e invertebrados como por las interacciones, procesos, y servicios ecosistémicos que tienen lugar en estos hábitats

(McNeely y Schroth 2006, Bugalho et al. 2011). Un claro ejemplo es el manejo intencionado de los sistemas de árboles de sombra nativos en asociación con las plantaciones de café en muchos ambientes tropicales húmedos (agrosilvicultura), ya que reducen la deforestación al tiempo que abastecen y mejoran las necesidades de las comunidades locales (Ashley 2006) aliviando la presión sobre los recursos de los bosques nativos, proporcionando hábitat para muchas especies fuera de las reservas naturales protegidas (Perfecto 2005) y aumentando la conectividad entre ecosistemas tropicales (McNeely y Schroth 2006). Estrada et al. (2012) revisaron la utilización de los agroecosistemas por primates de Centro y Sudamérica, África subsahariana y Asia sudoriental. Estos autores encontraron que 57 taxones de primates usaron 38 tipos de agroecosistemas como hábitats temporales o permanentes, poniendo de manifiesto la importancia que este tipo de hábitats tienen en la conservación a gran escala. Los estudios de Castro-Luna y Galinfo-Gonzáles (2012) sobre murciélagos frugívoros en tres agroecosistemas cafetales de Veracruz (México) demostraron que a mayor diversidad de especies frutales mayor abundancia y riqueza de murciélagos, poniendo de manifiesto la utilidad de los agroecosistemas en la conservación de la fauna.

El establecimiento exitoso de la vida silvestre en las tierras de cultivo y los mecanismos utilizados por animales y plantas para adaptarse a estos nuevos entornos son de particular interés ya que proporciona información útil para el manejo de la biodiversidad en un mundo cambiante (Pimm y Gittleman 1992). En particular, el estudio de los procesos exaptativos que permiten a los frugívoros vertebrados adaptarse a los agroecosistemas es fundamental desde el punto de vista de la conservación, ya que muchas especies de plantas son dispersadas por frugívoros (Rey 2011). Desde el punto de vista ecológico, los agroecosistemas ofrecen excelentes entornos logísticos donde investigar las 'interacciones noveles' entre plantas y frugívoros

a través de experimentos de campo fácilmente replicables (Peris et al. 2017). La existencia de cultivos extendidos mundialmente como los cítricos, permiten estudiar las interacciones de un mismo cultivo con diferentes grupos funcionales de diferentes ambientes y continentes. Esta posibilidad ofrece una excelente oportunidad para estudiar la importancia ecológica de los diferentes grupos funcionales de frugívoros (i.e. dispersores y depredadores de semillas, despulpadores) así como el efecto de diferentes tratamientos (p.e. infección o no por *Penicillium*).

Pero los agroecosistemas también pueden causar impactos negativos sobre el medio (Perfecto y Vandermeer 2008) como consecuencia de un uso excesivo de productos fitosanitarios (Wilson 1987, Perfecto et al. 1996), contaminación de los suelos (Metcalf 1980), aumento de la erosión por el uso de maquinaria (Giller et al. 1997), reducción de la biodiversidad (Salafsky 1994), fragmentación del hábitat (Gascon et al. 2000, Ferraz et al. 2003), extinciones locales (Strier 1999, Relyea 2005), contaminación de aguas por abonos nitrogenados, e introducción y naturalización de especies exóticas (Stampella et al. 2014). Dado el origen asiático de los cítricos, el clima tropical ofrece temperaturas y régimen hídrico óptimo para la germinación, crecimiento y desarrollo de las plantas cítricas. En el Capítulo V se estudió la posible naturalización del naranjo dulce en el bosque tropical seco de la *Mata Atlântica* de Brasil dispersado principalmente por el jabalí (*Sus scrofa*).

1.3. Cítricos

1.3.1. Origen y distribución

Aunque existen diversas teorías sobre el origen de los cítricos, está comúnmente aceptado que se originaron en las regiones subtropicales y tropicales del Sudeste Asiático y del Archipiélago Malayo (Webber et al. 1967, Chapot 1975, Calabrese 1992, Davies y Albrigo 1994).

Diferentes autores (Scora 1975, Barret y Rhodes 1976, Nicolosi et al. 2000, Wu et al. 2014, 2016) indican que los diferentes genotipos de interés comercial que hoy conocemos del subgénero *Citrus* provienen de tres taxones principales, que son el pummelo o zamboa (*Citrus maxima* Bur. Merr.), el cidro (*Citrus medica* L.) y el mandarino (*Citrus reticulata* Blanco). A partir de estos taxones y debido a hibridaciones y retrocruzamientos naturales, selección natural, mutaciones espontáneas y la selección artifical, se originaron el resto de híbridos que hoy conocemos del género *Citrus* (Swingle y Reece 1967).

Las primeras referencias escritas de cítricos se remontan al año 2.400 a.C. en China (texto *Tribute to Yu*), y 800 a.C. en la colección de textos sagrados Brahma en India (texto *Vajasaneyi Samhita*), donde se citan las mandarinas, la zamboa, el yuzu (*Citrus junos* Sieb. ex. Tan) y el kumquat (*Fortunella* spp., Peña et al. 2008).

El cidro fue posiblemente el primer cítrico que llegó al continente europeo hacia el año 300 a.C. (Swingle y Reece 1967) y se supone que fue el único cultivado durante siglos hasta su difusión en Grecia (s. III a.C.) e Italia (s. I. d.C.) por los judíos. El primer cítrico que llegó a la Península Ibérica fue el cidro en el s. VII introducido probablemente por los romanos en Andalucía (Zaragoza 2007). El naranjo amargo (*Citrus aurantium* L.) fue distribuido por los comerciantes árabes en el s. X desde la India hasta Irak, Siria, Palestina y Egipto y, desde allí, a Sicilia, España y Cerdeña a finales del s. XI (Zaragoza 2007). Se cree que las primeras variedades de naranjo dulce (*Citrus sinensis* L. Osb.) fueron traídas al continente europeo por comerciantes genoveses o venecianos en el siglo XV y a través de las travesías portuguesas al continente chino (Zaragoza 2007) coincidiendo con el comercio con las colonias inglesas entre los siglos XV y XVI (Ramón-Laca 2003). Cristóbal Colón introdujo los cítricos en Haití en 1493. Posteriormente se distribuyeron por el continente americano llegando a México en 1518, Brasil en 1540,

Florida en 1565, Perú en 1609, California en 1769 y Texas en 1890. Desde Brasil y Tenerife llegaron los primeros cítricos a Australia con los primeros colonos en 1788 (Grigg 1974).

Hasta la llegada del naranjo dulce a la Península Ibérica, los cítricos se empleaban en jardinería como plantas ornamentales y se utilizaban sus frutos en medicina y cosmética. Su consumo en fresco o exprimidos no era habitual pero se utilizaban en la elaboración de confituras. No fue hasta el s. XVI cuando se popularizó su cultivo a pequeña escala, estableciéndose las primeras plantaciones comerciales para consumo en fresco en Castellón y Valencia a finales del s. XVIII. Posteriormente, las plantaciones de naranjo dulce se expandieron debido al creciente comercio con Francia hacia finales del s. XIX (Zaragoza 2007).

Actualmente los cítricos son el primer cultivo frutal leñoso del mundo en cuanto a superficie cultivada y producción. En el año 2016 la producción mundial de cítricos ascendió a más de 124 millones de toneladas ocupando una extensión cultivada de 8.7 millones de hectáreas (FAO statistics 2016), siendo el naranjo dulce el principal genotipo cultivado seguido de mandarinos, limoneros y limeros, pomelos y otros cítricos. En España constituyen el principal frutal, con una superficie total de cultivo de unas 300.000 hectáreas (Ministerio de Agricultura 2017) y una producción superior a los 6 millones de toneladas en el año 2016 (FAO statistics 2016). Los principales productores mundiales son China, Brasil, EEUU, India, México y España, por ese orden. Los últimos avances en investigación permiten, mediante biotecnología, inducir mejoras en diferentes variedades de cítricos ya existentes y bien conocidas (Peña et al. 2007).

1.3.2. Clasificación botánica

La taxonomía de *Citrus* es complicada y liosa debido a la compatibilidad sexual entre *Citrus* y los géneros afines, la facilidad con la que se producen mutaciones espontáneas, la larga historia de su cultivo y su dispersión silvestre (Nicolosi et al. 2000). Actualmente existen clasificaciones distintas propuestas por diferentes autores. Aun así, todos coinciden en que el género *Citrus* pertenece al orden Geraniales, familia Rutaceae y subfamilia Aurantioideae. La clasificación taxonómica sobre la distribución en tribus, subtribus, géneros y especies difiere para cada autor. Kubitzki et al. (2011) reconoce al menos 2100 genotipos en la familia Rutaceae.

Por una parte, Swingle y Reece (1967) subdividen la familia en las tribus *Clauseneae*, con 3 subtribus y 5 géneros, y *Citreae*, con 3 subtribus y 28 géneros. Scora (1975) y Barrett y Rhodes (1976) sugieren la existencia de tres cítricos verdaderos a partir de los cuales y mediante hibridaciones, se han generado los demás genotipos de cítricos cultivados. Swingle y Reece (1967) clasifican dentro de la subtribu *Citreae* los tres cítricos verdaderos: cidro, mandarino y pummelo.

Por otra parte, Tanaka (1961, 1977) propone una clasificación más adaptada a las cualidades agronómicas de los diferentes tipos cultivados e identifica más de 160 especies en diferentes grupos y subgrupos, tomando especial consideración los principales genotipos cultivados, distinguiendo diferentes tipos de mandarinos como clementinos (*C. clementina* Hort. ex. Tan.) y satsumas (*Citrus unshiu* Mak. Marc.), entre otros.

Más recientemente, mediante el uso de marcadores moleculares de genes cloroplásticos, se ha propuesto una nueva clasificación de la subfamilia Aurantioideae, en la que se encuentran los cítricos

verdaderos incluyendo a los géneros *Citrus*, *Clymenia*, *Feroniella*, *Fortunella*, *Microcitrus*, *Oxanthera* y *Poncirus* (Bayer et al. 2009).

Sin embargo, a efectos prácticos, se siguen utilizando las taxonomías de Swingle y Reece (1967) y de Tanaka (1977) en algunos casos para diferenciar entre los distintos tipos de cítricos cultivados.

1.3.3. Biología de los cítricos

1.3.3.1. Descripción botánica

Los cítricos son árboles o pequeños arbustos, raramente hierbas, de forma y tamaño variable que pueden alcanzar hasta los 15 metros de altura en algunos genotipos. La característica más particular de la familia es que contienen cavidades secretoras con glándulas de aceites esenciales volátiles presentes en todos los genotipos. Estas se sitúan en hojas, peciolos, brotes, flores, pericarpio y cotiledones y en general en todos los tejidos parenquimáticos (Kubitzki et al. 2011). En algunos géneros las cavidades secretoras producen resinas. En las hojas, las glándulas de aceite aparecen como pequeños puntos translúcidos.

Los árboles forman un tronco principal o ramificado desde la base, a partir del cual brotan ramas que forman la copa de forma circular o piramidal. Crecen mediante brotaciones simpodiales que se dan en función de las condiciones ambientales a partir de las yemas axilares y terminales.

Las hojas son perennes (excepto en el género *Poncirus*), alternas u opuestas, compuestas por 3 o más foliolos o unifoliadas según genotipos, impares o paripinnadas mayormente, de color verde oscuro en el haz y verde claro en el envés, de forma oval a oblonga, y están unidas al tallo mediante el pecíolo, simple, articulado o expandido. Poseen un nervio central prominente. En algunos casos son aladas,

trifoliadas o unifoliadas generalmente. En la unión con el tallo se encuentra la axila con una o varias yemas axilares y frecuentemente aparecen una o dos espinas a uno u ambos lados de la axila, rectas o curvadas (Kubitzki et al. 2011). En la mayoría de los casos las espinas solo aparecen durante la fase juvenil. Cuando germina la semilla emite una raíz pivotante que crece en profundidad. Esta se ramifica en raíces secundarias y terciarias, donde se forman los pelos radicales responsables de absorber agua y nutrientes.

Las Rutáceas comprenden muchos tipos diferentes de flores incluyendo panículas, racimos y flores solitarias. Son bisexuales o unisexuales, pentacíclicas y actinomorfas generalmente. En muchos géneros solo dos de los cinco estambres son fértiles y se sitúan en la parte superior de la corola. En *Citrus* son más grandes que en el resto de Rutáceas y pueden llegar a desarrollarse hasta 40 estambres. Las anteras poseen apéndices basales a menudo fusionados entre sí, están dispuestas al final del filamento del estambre y generalmente, presentan dos sacos polínicos. El gineceo es apocárpico (formado por una o más hojas carpelares no soldadas) o sincárpico (hojas carpelares soldadas entre sí), está unido al estilo y éste se desprende en la madurez del fruto. Se compone de ovario, estilo y estigma. El desarrollo de los estigmas forma el *compitum*, espacio interior en el que el tubo polínico se conecta con todos los carpelos centralizados. El tubo polínico se desarrolla por el interior del estilo, donde se encuentran tantos canales estilares como hojas carpelares. En gineceos apocárpicos el polen germina en los estigmas y los tubos polínicos se desarrollan en carpelos separados. En algunos géneros (*Choisya*) el tubo polínico continúa hasta los ovarios, compuestos por lóculos sobre el disco nectarífero. Los estigmas pueden se secos o húmedos y generalmente poseen papilas (excepto en *Citrus*, *Erythrochiton* y *Ptelea*). En algunas especies de *Citrus* y *Fortunella* los granos de polen germinan en la superficie del estigma. Este segrega

una gota seminal mucilaginosa que estimula la germinación de los granos de polen. Los ovarios se elevan por encima de la base del estilo. El disco nectarífero y los ginóforos son los principales órganos secretores de néctar y están intercalados entre gineceo y androceo. Los granos de polen se encuentran rodeados por las tecas, epidermis compuesta por dos o tres capas intermedias y una capa glandular de células multinucleadas. Dentro de las tecas existen dos celdas que se abren liberando el polen cuando está maduro. Los granos de polen combinan poros esféricos, reticulados o microperforados y poseen un tectum reticulado. Generalmente se encuentran dos óvulos en cada carpelo encerrados por una tapa característica de la familia. El desarrollo del saco embrionario es de tipo monospórico (migración y fusión de dos núcleos al centro del saco embrionario) y el desarrollo del endospermo de tipo nuclear. La polinización es entomófila siendo insectos de las familias Hymenoptera y Diptera los principales polinizadores (Kubitzki et al. 2011).

En condiciones tropicales los cítricos no tienen latencia invernal mientras que en el clima mediterráneo se da un reposo invernal sin llegar a perder las hojas (Spiegel-Roy y Goldchmidt 1996).

1.3.3.2. Biología reproductiva de los cítricos

La mayoría de las especies del género *Citrus* y sus afines son diploides (Arumuganathan y Earle 1991). Casi todos los cítricos presentan apomixis facultativa. La apomixis consiste en la generación de progenie con características idénticas a la planta madre al desarrollarse embriones únicamente a partir de células madre nucelares sin que haya fecundación ni meiosis (Frost y Soost 1968). En el género *Citrus* normalmente hay fecundación y se desarrollan tanto, un embrión sexual a partir de óvulos fecundados (monoembrionía) o varios embriones nucelares (poliembrionía) (hasta 13 embriones se encontraron en una única semilla, Kubitzki et al.

2011), al menos uno de ellos de origen sexual. Por ello se dice que presentan apomixis facultativa, pero al ser el embrión sexual normalmente muy poco vigoroso, en la práctica se producen casi exclusivamente plantas nucelares (Frost y Soost 1968). Algunos genotipos cítricos, entre los que se encuentran zamboas, cidros y algunos mandarinos, son sin embargo monoembriónicos, desarrollando únicamente embriones sexuales (Iwamasa 1966). La polioembrionía nucelar está muy extendida en toda la familia pero en el género *Citrus* la fertilización es esencial para el desarrollo embrionario (Schneider 1968).

Las semillas están rodeadas por la testa o tegumento exterior, de textura dura y leñosa y color crema, e, interiormente, por un tegumento delgado o tegmen con un extremo de células más gruesas y oscuras conocido como chalaza (Frost y Soost 1968). En el interior del tegmen se encuentra el embrión compuesto por dos cotiledones y un eje embrionario. Los cotiledones son tejidos carnosos donde se almacenan los nutrientes necesarios para la germinación. El número de semillas en un fruto fluctúa desde ninguna (partenocarpia) hasta más de 20.

Los cítricos obtenidos por semilla poseen un largo periodo juvenil. Algunas especies de cítricos pueden tardar más de 10 años en florecer por primera vez. Durante este periodo la planta posee mucho vigor y se caracteriza por tener una tasa de crecimiento vegetativo muy alta, creciendo en altura sin apenas ramificaciones laterales (Davies y Albrigo 1994). A medida que la planta va alcanzando su madurez se produce una pérdida de la dominancia apical. La aparición de las primeras yemas florales indica el final del periodo juvenil. Algunos genotipos presentan espinas durante el periodo juvenil que gradualmente van desapareciendo a medida que se alcanza la madurez sexual.

El desarrollo del fruto en cítricos puede inducirse a partir de la fecundación o mediante la partenocarpia en aquellas variedades que son autoincompatibles y estériles. Existen dos tipos de partenocarpia, la facultativa y la obligada. La partenocarpia facultativa ocurre en especies autoincompatibles o que presentan esterilidad masculina por lo que no desarrollan semillas a menos que sean polinizadas por polen de otras especies fértiles y compatibles. La partenocarpia obligada se da en las especies con esterilidad femenina por lo que los frutos nunca presentan semillas. Existen otros casos en los que es necesaria la polinización para estimular el desarrollo del fruto aunque no se fecunde el ovario. También hay genotipos en los que el óvulo se fecunda pero posteriormente aborta el embrión con lo que no se desarrollan semillas en los frutos. Algunas especies de cítricos comerciales presentan diferentes tipos de autoincompatibilidad y autoesterilidad por lo que son incapaces de producir semillas propias (Janick 2004, Ollitrault et al. 2007).

1.3.3.3. Desarrollo y maduración de los frutos cítricos

El crecimiento del ovario inicia el desarrollo del fruto que es una baya modificada en forma de hesperidio, compuesta por exocarpo, mesocarpo y endocarpo.

El exocarpo o flavedo es la parte exterior del fruto y es muy rico en glándulas de aceites y en carotenoides, que son los pigmentos que le dan su color característico (Figura 1). El mesocarpo o albedo se sitúa entre la pulpa y el flavedo y se compone de tejido blanquecino de textura esponjosa, relativamente seco (Kubitzki et al. 2011). El endocarpo o pulpa se dispone en segmentos o gajos radiales alrededor de un eje floral, encerrados en una membrana carpelar, donde se encuentran las semillas y las vesículas especializadas de zumo (Schneider 1968). En el género *Citrus* las vesículas son grandes

estructuras originadas a partir de las paredes dorsales de los carpelos, y se rellenan de zumo derivado de las capas subepidérmicas (Kubitzki et al. 2011). El zumo está compuesto de agua (80%), azúcares (glucosa y fructosa principalmente, también sacarosa, galactosa y arabinosa), polisacáridos, ácidos orgánicos (mayoritariamente cítrico, también málico, tartárico, ascórbico), constituyentes nitrogenados y lípidos (Nicolosi-Asmundo et al. 1987). Los pigmentos contribuyen al color (carotenoides, antocianos, hesperidina y naringina); vitaminas (C, A, E), minerales (potasio, fósforo, calcio, magnesio, sodio, yodo, selenio, hierro y zinc), flavonoides, limonoides y componentes volátiles que contribuyen al aroma (Kefford 1960, Moreiras et al. 2013).

Según Bain (1958), se diferencian tres fases de crecimiento del fruto:

La Fase I se caracteriza por un aumento del número de células de todos los tejidos por división celular, lo que provoca un rápido crecimiento del fruto. Al final de esta fase se produce la división celular de los septos y el engrosamiento de los lóculos formando lo que serán las vesículas posteriormente. El desarrollo de los carpelos originará los gajos. Esta fase finaliza con la caída fisiológica de los frutos no cuajados.

La Fase II se prolonga durante varios meses y en ella se produce elongación celular, lo que provoca la expansión de los tejidos. En el mesocarpo se forman los espacios intercelulares esponjosos y el exocarpo alcanza su máximo espesor. Se produce un aumento de tamaño como consecuencia del desarrollo de los lóculos. Las vesículas se llenan de zumo a través del pedúnculo vesicular alcanzando su máxima longitud. El principal cambio químico que se produce en la piel de los frutos es una drástica reducción del compuesto monoterpénico oxigenado linalol de las glándulas de aceite mientras que el D-limoneno se incrementa considerablemente a medida que el

fruto madura llegando a ser el compuesto mayoritario (Attaway et al. 1967). El inicio del cambio de color indica el fin de esta fase.

En la Fase III sigue aumentando el tamaño del fruto debido al crecimiento de los segmentos de pulpa, el eje central y la corteza. Se produce el cambio de color de la piel en los climas templados como consecuencia de la degradación de la clorofila y la síntesis de carotenoides en piel y pulpa. Los azúcares y compuestos nitrogenados aumentan en la pulpa a la par que se produce una reducción de los ácidos, principalmente cítrico.

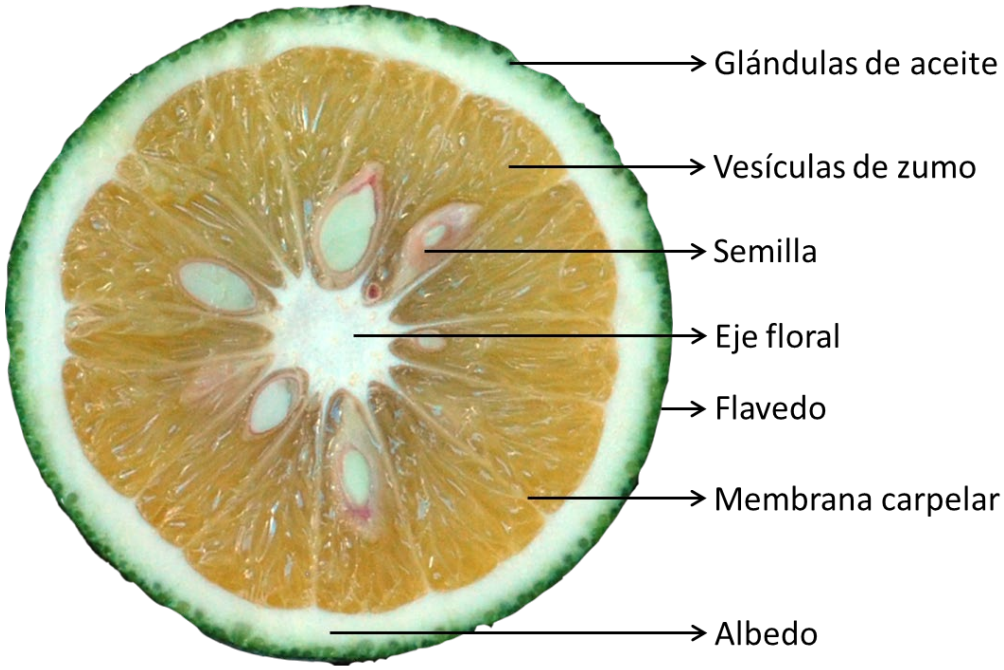

Figura 1. Sección ecuatorial de naranjo dulce en la Fase II de crecimiento.

1.3.3.4. Fitoquímica

Las plantas utilizan nitrógeno, carbono y energía para realizar funciones vitales como la fotosíntesis, la respiración, asimilar nutrientes, sintetizar lípidos, carbohidratos y proteínas imprescindibles para su metabolismo. Estos son los metabolitos primarios. También

destinan grandes cantidades de energía y otros recursos a la síntesis de otras moléculas no relacionadas directamente con sus necesidades vitales estrictas. Estos son los metabolitos secundarios, que generalmente se sintetizan en pequeñas cantidades, realizando diferentes funciones especializadas en las plantas. Las Rutáceas son una de las familias más ricas y diversas en cuanto a metabolitos secundarios producidos se refiere (Price 1963). Algunos metabolitos comparten una estructura química muy similar y su ruta de biosíntesis deriva del ácido antranílico, que es un compuesto estrechamente restringido a las Rutáceas derivado del benceno y precursor químico del indol, intermediario en múltiples reacciones químicas.

Los metabolitos secundarios se agrupan en cuatro tipos en función de su origen biosintético: **terpenos** (hormonas, pigmentos y aceites esenciales), **compuestos fenólicos** (acetofenonas, cumarinas, flavonoides, lignina y taninos), **glicósidos** (saponinas, glicósidos y glucosinolatos) y **alcaloides** y **fenilpropanoides** (acridinas, quinolonas, pirano y furano, alcaloides heterocíclicos aromáticos altamente identificativos de la familia; Kubitzki et al. 2011). Las cumarinas, alcaloides, acetofenonas, flavonoides y limonoides contribuyen al perfil fitoquímico de las Rutáceas (Waterman 1993).

Los terpenos son los compuestos más variados de todos los productos naturales producidos por las plantas. Hay descritos más de 36.000 y todos derivan de fusiones repetitivas de unidades de 5 carbonos (Buchanan et al. 2000). Las plantas producen más variedad de terpenos que los animales y los microorganismos. La producción, almacenamiento y emisión de terpenos en las plantas se realiza en estructuras altamente especializadas, como los tricomas. Los monoterpenos son conocidos como las esencias volátiles de las plantas y están presentes en flores, frutos y otros tejidos. En los cítricos se almacenan principalmente en las glándulas de aceites esenciales del flavedo de los frutos (Sinclair 1984).

1.3.4. Propagación comercial de cítricos

Los árboles cítricos en las plantaciones comerciales se componen de dos genotipos diferentes. El portainjertos constituye el sistema radicular del árbol. Para ello se usan plantas nucelares (somáticas; idénticas a la planta madre) procedentes de semilla de genotipos seleccionados por sus características agronómicas, la productividad que confieren a la copa, elevada poliembrionía facultativa, y su resistencia a enfermedades y estreses abióticos. El paso por semilla garantiza que las plántulas generadas estarán libres de prácticamente todos los patógenos que afectan a los cítricos, incluyendo hongos, bacterias, fitoplasmas y la mayoría de virus y viroides. Todos los portainjertos se obtienen de semillas a partir de *árboles madre* productores de semillas o pipeteros. Estas se cultivan en viveros hasta que alcanzan una edad apropiada para el injerto, donde se retiran las plantas zigóticas si aparecen. La copa es la parte aérea del árbol, en la que se producen los frutos de los distintos genotipos de interés comercial, que se propagan vegetativamente mediante injerto de yemas clonales adultas. De esta manera se consigue evitar el largo periodo de juvenilidad de los genotipos de copa aprovechando las características vigorosas de los portainjertos juveniles (Frost y Soots 1968). Las plantas madre de las variedades a propagar se mantienen en recintos de malla protegidos y libres de patógenos transmisibles por injerto. Mediante esta técnica se propagan la totalidad de cítricos cultivados comercialmente.

En el transcurso de la realización del trabajo de campo de la tesis se han utilizado frutos cítricos de diferentes genotipos comerciales: Clemenules, Bernalina, Owari, Navelina, Pineapple y Pera.

Clemenules es un mandarino del grupo Clementinos, con fruta fácil de pelar, cosechada para consumo en fresco por su excelente calidad organoléptica, elevado contenido en zumo, tamaño mediano y corteza

fina. Sus hojas son lanceoladas, largas y estrechas con el ápice agudo, de peciolo corto y sin alar (Soler y Soler 2006). Presenta autoincompatibilidad por interrupción del desarrollo del tubo polínico en los canales estilares (Soost 1956) por lo que no es capaz de autopolinizarse, razón por la cual sus frutos no presentan semillas en ausencia de polen fértil de otras variedades compatibles.

Bernalina es un naranjo dulce del grupo Blancas con frutos medianos y corteza ancha y rugosa. Posee un alto porcentaje de contenido de zumo y ausencia de semillas en sus frutos. Sus hojas son lanceoladas, anchas, grandes y coriáceas con ápice agudo y peciolo corto. Es una variedad partenocárpica obligada.

Satsuma Owari es un mandarino del grupo Satsumas con frutos de tamaño pequeño a mediano, color amarillo-naranja, forma aplanada, corteza gruesa y algo rugosa, elevado contenido en zumo pobre en azúcares y ácidos totales. Sus hojas son lanceoladas y su limbo agudo, coriáceas con el nervio central muy marcado y color verde oscuro, peciolo largo y poco alado (Soler y Soler 2006). Presenta baja fertilidad en el polen, es decir, incapacidad de producción de polen fértil.

Navelina es un naranjo dulce del grupo Navel, caracterizado por presentar un rudimentario fruto pequeño en el interior estilar del fruto principal, denominado ombligo. Los frutos son de tamaño medio y de excelentes cualidades organolépticas, piel lisa y fácilmente pelable. Variedad partenocárpica que no presenta semillas por esterilidad femenina al no tener óvulos fértiles debido a la degeneración del saco embrionario (Iwamasa 1966), aunque si es polinizada a mano puede producir pocas semillas (Ollitrault et al. 2007). El fruto se desarrolla gracias a la acumulación de giberelinas en el ovario.

Pineapple es un naranjo dulce del grupo Blancas con frutos de tamaño mediano a grande, forma esférica a ligeramente achatada, color

naranja intenso, corteza delgada con la superficie ligeramente picada. La pulpa es de color naranja claro, tierna, jugosa con semillas y excelente aptitud para la industria del zumo. Árbol vigoroso, de tamaño mediano a grande, sin espinas y muy productivo (Citrus Variety Collection 2018)

Pera es un naranjo del grupo Blancas con frutos de tamaño medio a pequeño y forma ligeramente ovalada a elipsoide, corteza delgada de superficie lisa y color naranja claro cuando está madura. Pulpa naranja intenso, de textura fina, jugosa y firme, con semillas. Árbol vigoroso, muy productivo, de crecimiento vertical y mucho follaje con algunas hojas aladas (Citrus Variety Collection 2018). Se cultiva ampliamente en el estado de São Paulo (Brasil) y se destina a la elaboración de zumos.

1.3.5. Parámetros de calidad de los frutos cítricos

La calidad de las frutas cítricas orientadas al mercado del consumo en fresco viene determinada por sus características sensoriales y propiedades fisicoquímicas. En estos atributos juegan un papel importante los sentidos humanos a la hora de elegir una fruta (Capítulo IV). Mediante la vista el consumidor aprecia el color, tamaño y forma del fruto. El tacto advierte sobre su textura, peso y residuo de la pulpa ingerida. Con el olfato sentimos los aromas de la piel y la pulpa, y con el gusto percibimos la relación entre azúcares y ácidos. Las cualidades organolépticas de los frutos cítricos se determinan mediante métodos analíticos estándar, como por ejemplo los descritos por el USDA (2013), que determinan principalmente textura y sabor, presencia de semillas, cantidad de azúcares solubles y acidez (índice de madurez), contenido de zumo, dimensiones y forma del fruto, firmeza, época de maduración, contenido de zumo y facilidad de pelado. Recientemente se han incorporado como parámetro de calidad

las propiedades beneficiosas para la salud de diferentes compuestos como los flavonoides y carotenoides.

El color del flavedo se determina mediante un colorímetro midiendo las coordenadas *a* (mide los cambios de color desde el verde hasta el rojo) y *b* (mide los cambios de color desde el azul al amarillo) y se expresa como la relación de coordenadas *a/b* (Stewart y Leuenberger 1976).

La dureza de la piel se mide mediante la fuerza que ejerce ésta ante la rotura. Se mide con un penetrómetro y se expresa en kg/superficie. El procedimiento es rápido: se coloca un émbolo en el cabezal de lectura y se presiona lenta y uniformemente contra la fruta hasta que se produzca la rotura. En este momento, la báscula registra el máximo peso ejercido. Otro método para medir la firmeza de los frutos es mediante el texturómetro que somete el fruto a la compresión de un plato de diámetro conocido (Abbott 1999).

El sabor viene determinado por la relación azúcares-acidez. Los azúcares solubles en el zumo de naranja se miden con refractómetro digital y se expresan como porcentaje de sólidos disueltos. Para determinar la acidez se realiza una volumetría con hidróxido sódico (NaOH 0.1N) sobre 5 mL de zumo hasta obtener el viraje a rosa-violeta de la fenolftaleína una vez neutralizados los ácidos (principalmente cítrico) a pH 8.2. Del cociente entre los azúcares solubles y la acidez se obtiene el índice de madurez (IM).

El olor a frutas cítricas es único. Se debe a una compleja combinación de compuestos volátiles solubles, principalmente terpenos (monoterpenos y sesquiterpenos) almacenados en las glándulas de aceite del flavedo. En los últimos años el estudio del aroma ha recibido mucha atención aunque todavía se desconocen muchos aspectos de la síntesis y metabolismos de los compuestos responsables del mismo (Sharon-Asa et al. 2003). Para el estudio del

contenido de compuestos volátiles del flavedo es necesaria su extracción mediante el uso de disolventes orgánicos (pentano, hexano, heptano) o destilación en corriente de vapor hasta obtener la fase orgánica. Para el estudio de los compuestos volátiles emitidos del flavedo es necesaria su extracción mediante el uso de una fibra recubierta con polidimetilsiloxano (PDM) capaz de extraer diferentes tipos de moléculas con un umbral de detección muy bajo (partes por trillón) y sin necesidad de usar solventes. Esta técnica es conocida como microextracción en fase sólida (HS-SPME). En ambos casos se identifican en un cromatograma las diferentes moléculas existentes en cada muestra mediante cromatografía de gases-espectrofotometría de masas (GC-MS). Para asegurar la uniformidad del procedimiento se usó 2-octanol como patrón interno.

1.3.6. Mejora genética de cítricos con reducido contenido de D-limoneno.

En la literatura existen trabajos que estudian la interacción del compuesto D-limoneno de la piel de la naranja con diversos organismos. Gonçalves et al. (2006) demostraron que los machos de la mosca mediterránea de las fruta, *Ceratitis capitata,* almacenan en las glándulas salivares y liberan compuestos químicos similares al D-limoneno con la finalidad de atraer a las hembras. Stensmyrs et al. (2012) identificaron un receptor antenal específico del D-limoneno en la hembra de la mosca *Drosophila* que utiliza para la elección del fruto huésped donde ovipositar. Asimismo, los machos de *Drosophila* en contacto con una naranja tienen mayor éxito en la atracción de las hembras para copular. Dweck et al. (2013) mostraron que en una prueba de elección de oviposición binaria la mosca *Drosophila* eligió claramente naranjas sin pelar frente a naranjas peladas, lo que implica que los productos químicos volátiles presentes en el flavedo son importantes, proponiendo que la presencia de D-limoneno es

necesaria para la elección del lugar donde ovipositar. Milet-Pinheiro et al. (2015) demostraron que el D-limoneno y otros 5 monoterpenos y derivados alcohólicos provocaban respuestas de atracción en hembras de *Anastrepha fraterculus* para la cópula (Lima et al. 2001).

Todos estos estudios sugieren que la acumulación de D-limoneno en la piel de las naranjas maduras así como el importante gasto energético que supone su síntesis, no es casual y responde claramente a un papel elemental en la comunicación entre frutas e insectos.

A partir de distintas especies de plantas se han clonado los genes que codifican la síntesis de importantes enzimas implicadas en el metabolismo de monoterpenos tales como el linalol o el D-limoneno. Algunos de estos genes se han utilizado en plantas transgénicas (tomate, tabaco y menta) para modular el contenido de este tipo de compuestos. Además, se ha publicado que la incorporación de una linalol/neloridol sintasa procedente de fresa en plantas de *Arabidopsis* hace que las plantas atraigan predadores de hervíboros que resultan insectos plaga (Kappers et al. 2005). El gen de una D-limoneno sintasa de Satsuma fue clonado por el grupo del Dr. Shimada (NIFTS, Japón). Este gen se ha utilizado para generar naranjos genéticamente modificados (GM) con fruta con contenido bajo de D-limoneno en la piel, de manera que se acumula hasta 85 veces menos de este compuesto en el flavedo de las naranjas GM. Como consecuencia de ello, la fruta resultó resistente a distintos patógenos fúngicos y bacterianos y mucho menos atrayente de la mosca del mediterráneo (Rodríguez et al. 2011a).

Para investigar la interacción de los terpenos volátiles del flavedo de naranjas GM y control con microorganismos especializados se utilizaron por una parte el hongo *Penicillium digitatum*, causante de las mayores pérdidas de cítricos en poscosecha, y por otra la bacteria *Xanthomonas citri* subespecie *citri* causante del cancro cítrico que

reduce el rendimiento y la calidad de los frutos cítricos. En el caso del hongo, pasados 8 días desde la inoculación, la superficie infectada por el hongo alrededor de las heridas ocupaba el 60.8 y 54.9% en los frutos control pero solo un 18.5 y 7.4% en las líneas transgénicas ($P<0.05$). En el caso de la bacteria, los tejidos vegetales muestran mayor susceptibilidad a partir de la Fase II de crecimiento del fruto. Por esta razón, se inocularon frutos verdes obteniéndose superficies de infección alrededor de las heridas del 65.7% en frutos control y solo pequeñas heridas en las líneas transgénicas ($P<0.05$). En ambos casos, los resultados sugieren que la presencia de D-limoneno es necesaria para el establecimiento y desarrollo de la infección. Por tanto la reducción del contenido de D-limoneno en el flavedo de los frutos cítricos podría ser una estrategia útil para evitar enfermedades mediante técnicas de ingeniería genética (Rodríguez et al. 2011a).

Para estudiar las interacciones entre terpenos volátiles del flavedo de naranjas GM y control con insectos plaga, se realizaron ensayos de túnel de viento con la mosca mediterránea de la fruta, *Ceratitis capitata*. Esta mosca está considerada la mayor plaga de los cítricos en ambientes mediterráneos (Papachristos y Papadopoulos 2009). Los ensayos de túnel de viento consisten en la liberación de insectos adultos en un receptáculo cerrado con diferentes aperturas por donde se liberan distintos compuestos volátiles con la finalidad de detectar el efecto comportamental del insecto en función de su vuelo al sentirse atraído o repelido por cada compuesto. En estos ensayos los machos de la mosca de la fruta se sintieron fuertemente atraídos cuando se liberaron discos de D-limoneno puro frente a los discos control con agua. Cuando se colocaron las frutas maduras en el ensayo, los machos se sintieron más atraídos por los frutos control (32% de los vuelos) que por los transgénicos (2%, $P<0.05$), indicando que el D-limoneno es un potente atrayente de machos de la mosca de la fruta. En un último ensayo se liberaron machos en el campo en presencia de

frutas control y transgénicas y estos acudieron preferentemente a las líneas control (Rodríguez et al. 2011a).

Cuando el gen de la D-limoneno sintasa se sobre-expresó en naranjas GM, se consiguió una mayor acumulación de este compuesto en la piel de los frutos y con ello éstos resultaron más sensibles a la infección por *Penicillium* y más atractivos para la mosca *C. capitata* (Rodríguez et al 2011b).

En un trabajo posterior, se descubrió usando diferentes líneas GM que el efecto sobre patógenos y plaga se correlacionaba inversamente con la cantidad de D-limoneno contenida en el flavedo de las frutas de cada línea GM y control, de manera que a menor cantidad de D-limoneno mayor era la protección de las naranjas (Rodríguez et al. 2015). Además, la bajada de D-limoneno en los frutos GM se relacionaba directamente con una activación generalizada de la respuesta de defensa de las plantas frente a patógenos en esos tejidos, de manera que se establecía en el flavedo de la naranja una compensación cruzada a nivel metabólico entre defensa y acumulación de terpenos volátiles (Rodríguez et al. 2014).

Estos trabajos planteaban por primera vez en frutales la posibilidad de alterar los niveles de acumulación de terpenos en las glándulas de aceite de la piel de los frutos como estrategia efectiva de control frente a otros hongos, plagas y bacterias patógenas, pudiendo minimizar o incluso evitar el uso de costosísimos tratamientos fitosanitarios, tanto desde el punto de vista económico como ambiental.

1.3.7. Análisis sensorial de las naranjas con reducido contenido de D-limoneno

Con todo lo anterior, resulta imprescindible que las naranjas GM sean percibidas como apetecibles para el consumidor humano (Capítulo IV), ya que si no fuese así perderían su interés comercial. Al tratarse de

frutos GM, la normativa europea es muy estricta con las posibilidades de realizar experimentos de consumo por humanos, por lo que no se han podido evaluar posibles diferencias en sabor y aroma.

Sin embargo, dado que lo que se ha modificado en las naranjas ha sido particularmente su olor, al alterar los niveles de contenido y emisión de D-limoneno y otros monoterpenos volátiles asociados, sería importante realizar catas de olor con grupos de catadores de naranja de diferentes instituciones. Aunque los frutos carnosos generalmente comparten muchos compuestos volátiles, cada fruta tiene un olor distintivo que es función de la proporción de volátiles clave y la presencia o ausencia de componentes únicos (Baxter et al. 2005). Se sabe que en muchos casos solo un número limitado de componentes contribuye al carácter de un olor (Heath y Reineccius 1986). Los compuestos volátiles de los alimentos percibidos por el sistema sensorial olfativo proporcionan la base para la diversidad de olores y sabores seleccionados y encontrados en la dieta humana (Goff y Klee 2006).

El análisis sensorial de los alimentos es el uso de los sentidos humanos para analizar objetivamente los mismos, en busca de propiedades particulares relacionadas con el olor, la vista, el sonido, el aroma, el sabor y la textura. Se utiliza para evaluar la calidad de los productos y para comparar productos nuevos con los ya existentes. En el caso del olor de un fruto o alimento, para poder evaluarlo científicamente resulta imprescindible realizar catas utilizando procedimientos aceptados internacionalmente, a través del cumplimiento de normas ISO, y categorías hedónicas de amplia utilización. Con ello, podremos interpretar su aceptación, sobre todo al comparar con otros olores de la fruta o el alimento control. Para poder realizar las catas de aromas de frutos, se requiere de equipos de catadores de alimentos (frutos cítricos en nuestro caso) experimentados que pueden evaluar las características de olor de los

productos. Luego, el análisis estadístico avanzado permite comparar los olores de los frutos y cuantificar sus similitudes y diferencias de manera que resulten significativas o no.

1.4. Terpenos y D-limoneno

Las plantas producen una amplia gama de COVs en numerosos tejidos vegetales y mediante distintos procesos metabólicos (Peñuelas 2008). En los frutos cítricos los terpenos son el grupo químico más abundante (Dugo y Giacomo 2002). En los frutos han sido identificados cientos de COVs y el aroma y sabor depende de la combinación de COVs producidos y almacenados en los frutos. Los COVs pueden dividirse en cuatro clases principalmente según su origen metabólico (Negre-Zakharov et al. 2009): terpenos, fenilpropanoides/benzenos, derivados de los ácidos grasos y derivados del ácido amino. La regulación de la expresión de dichos compuestos depende de las relaciones existentes entre los órganos emisores y su interacción con distintos insectos polinizadores, herbívoros y sus parásitos, frugívoros y predadores de semillas (Vickers et al. 2009, Bednarek y Osbourn 2009), pudiéndose sintetizar en abundancia frente a situaciones de estrés biótico (Dudareva et al. 2006), abiótico (Duhl et al. 2008) o multiestreses (Holopainen y Gershenzon 2010).

En cítricos se han identificado COVs específicos de hojas y frutos (Dugo y Giacomo 2002). Todos los terpenos derivan de un precursor de 5 carbonos, el isopentenil difosfato (Buchanan et al. 2000). Los terpenos son una de las clases más amplias en los COVs de los frutos cítricos, especialmente monoterpenos, sesquiterpenos y terpenos irregulares de bajo peso molecular. Monoterpenos y ésteres son los principales responsables del aroma y sabor del fruto maduro (Knudsen et al. 2006). En cítricos, los monoterpenos cíclicos son los volátiles más representativos (50-97%) y se encuentran en las glándulas de aceites esenciales. Los monoterpenos se sintetizan a partir del geranil

difosfato a través de la ruta plastídica del 2C-metil-D-eritritol-4-fosfato (Dudareva et al. 2013). En general, se trata de productos de alto valor económico ya que se usan como aditivos en alimentación y cosmética. Los compuestos terpénicos volátiles son utilizados por las plantas como señales olfativas para la comunicación con el entorno (Rodríguez et al. 2011b). Diversos autores han identificado y cuantificado los compuestos volátiles contenidos en la piel de los frutos cítricos, tanto ancestrales como cultivados, siendo los monoterpenos los terpenos principales del flavedo, y el D-limoneno el más abundante (hasta el 97% en la naranja dulce; Rodríguez et al. 2011a). En la Tabla 1 se muestra el contenido de D-limoneno en diferentes genotipos cítricos (obtenida de Dugo y Di Giacomo 2002).

Tabla 1. Contenido total de D-limoneno en diferentes variedades de cítricos (Dugo y Giacomo 2002).

		D-limoneno (%)
Cítricos verdaderos	*Citrus grandis*	48.9-95.6
	Citrus medica	51.2-93.6
	Citrus reticulata	87.4-91.7
Papedas	*Citrus hystrix*	2.8-14.2
Híbridos	*Citrus aurantium*	80.1-95.8
	Citrus paradisi	83.4-93.8
	Citrus aurantifolia	38.4-50.0
	Citrus clementina	83.0-95.1
	Citrus bergamia	24.1-54.9
	Citrus limon	59.6-76.2
	Citrus junos	60.4-82.4
	Citrus unshiu	41.2-90.7
	Citrus sinensis	91.0-97.0

La síntesis de estos compuestos resulta energéticamente muy cara a las plantas. Los terpenos son más caros de fabricar por gramo de compuesto que la mayoría de otros metabolitos primarios y secundarios (Gershenzon 1994). Por esta razón, es razonable pensar que deben tener alguna importante función adaptativa relacionada con la producción, supervivencia y/o dispersión de semillas.

1.5. Consumidores de cítricos

1.5.1. Vertebrados

Las interacciones entre las plantas y animales son muy frecuentes en la mayoría de los ecosistemas y son de suma importancia para la dinámica y evolución de las poblaciones y comunidades (Fedriani y Delibes 2013). La naturaleza de dichas relaciones varía a lo largo del continuo mutualista-antagonista. Las diferencias de los frugívoros en su tamaño, agudeza visual, capacidad para acceder y manipular la fruta (Wehncke y Reyes-Amaya 2010), y en los hábitos y preferencias alimentarias ha permitido agrupar a los vertebrados consumidores de frutas en diversos gremios funcionales de frugívoros como predadores de semillas (granívoros antagonistas), despulpadores y dispersores de semillas (relaciones mutualistas), entre otros (herbívoros, patógenos, oportunistas). De manera resumida y en general, los patógenos y predadores de semillas (y plántulas) no favorecen la dispersión de las especies ya que al consumirlas destruyen su capacidad germinativa. Los despulpadores son los frugívoros que consumen la pulpa de los frutos sin dispersar generalmente sus semillas. Por último, los dispersores de semillas consumen la pulpa de los frutos incluyendo sus semillas y dispersándolas habitualmente lejos de la planta madre. Entre los hábitos de consumo y en especial en los frutos cítricos, se han detectado los tres gremios de frugívoros. Por ejemplo, durante el desarrollo de esta tesis hemos comprobado como el jabalí es un dispersor de semillas de cítricos ya que, al consumir los frutos ingiere las semillas y estas son viables después de pasar por el tracto digestivo mientras que, la mayoría de aves pequeñas, se comportan como despulpadoras ya que consumen únicamente las vesículas de los gajos discriminando las semillas. Por el contrario, tanto el ratón como la rata en el ambiente mediterráneo consumen semillas de cítricos despreciando las testas y destruyendo el embrión, por lo que actuan como predadores de semillas.

A pesar de que el cultivo de los cítricos está extendido mundialmente, no hemos encontrado estudio alguno centrado en las interacciones ecológicas entre cítricos y vertebrados frugívoros. No obstante, tras realizar una búsqueda minuciosa en bibliografía (Capítulo I), hemos registrado 48 casos de vertebrados frugívoros consumidores de frutos cítricos, en diferentes ecosistemas a nivel mundial. En concreto, 35 especies de mamíferos (10 murciélagos, 8 carnívoros, 7 primates, 3 roedores y 7 cérvidos, lagomorfos, armadillos, marsupiales, elefantes y tapires), 7 de aves (cacatúas, loros, tucanes y paseriformes) y 5 reptiles (aligátores, tortugas y lagartijas). De manera general, los mamíferos parecen ser los principales vertebrados consumidores de frutos cítricos a nivel mundial, aunque también existan numerosos casos documentados de aves y reptiles.

Además de los resultados obtenidos en la búsqueda bibliográfica, durante nuestros experimentos de campo hemos detectado otros frugívoros consumidores de frutos cítricos, tanto en ambientes mediterráneos como tropicales. En concreto, en el ambiente tropical de Matão, São Paulo (Brasil) los vertebrados frugívoros consumidores de frutos cítricos fueron, por orden de importancia, el jabalí (*Sus scrofa* L.), cuatí de cola anillada (*Nasua nasua* L.), paca común (*Cuniculus paca* Brisson), agutí de Azara (*Dasyprocta azarae* Lichtenstein), tapetí (*Sylvilagus brasiliensis* L.), urraca de cresta rizada (*Cyanocoraz cristatellus* Temminck), zorzal colorado (*Turdus rufiventris* Vieillot), tortolita (*Columbina talpacoti* Temminck), guacalate (*Euphractus sexcintus* L.), cotarra chiricote (*Aramides cajaneus* L.) y el mono capuchino negro (*Cebus nigritus* Goldfuss). En el ambiente mediterráneo, en las localidades de Moncada (Valencia) y Villareal (Castellón) se observó, por orden de importancia, el conejo de campo (*Oryctolagus cuniculus* L.), ratones (*Apodemus silvaticus* Kaup y *Mus spretus* Lataste) y rata (*Rattus rattus* L.), lirón careto

(*Eliomys quercinus* L.), mirlo (*Turdus merula* L.), urraca (*Pica pica* L.), gorrión (*Passer domesticus* Illiger) y lavandera (*Motacilla alba* L.).

Según nuestras observaciones y las citas bibliográficas, se han clasificado los frugívoros observados o descritos en los siguientes gremios funcionales:

- Dispersores de semillas: *Sanguinus leucopus, Canis latrans, Canis mesomelas, Cerdocyon thous, Chrysocyon brachyurus, Cebus sp., Macata fuscata, Prebytis melalophos, Muntiacus muntjak, Chiroptera sp., Cuniculus paca, Didelphis albiventris, Elephas maximus, Pan troglodytes, Eleumur coronatus, Martes martes, Martes melampus, Nasua nasua, Pteropus sp., Rousettus aegyptiacus, Tapirus bardii, Paguma larvata, Sus scrofa*.
- Predadores de semillas: *Funambulus pennantii, Elyomys quercinus, Rattus rattus, Mus spretus, Apodemus sylvaticus, Hydrochoerus hydrochaeris, Sylvilagus brasiliensis*.
- Despulpadores: *Passer domesticus, Motacilla alba, Turdus sp., Columbina talpacoti, Oryctolagus cuniculus, Pica pica, Melanerpes carolinus, Podarcis erhardii, Ramphocelus carbo, Aramides cajaneus*.

1.5.2. *Penicillium digitatum* Sacc.

Como se ha visto anteriormente, las plantas producen una gran diversidad de COVs que, a menudo, median la comunicación entre especies con otros organismos. Dos de las principales funciones de los COVs emitidos por las plantas son atraer polinizadores y frugívoros dispersores de semillas (Baldwin 2010). Poco se sabe, sin embargo, sobre si los COVs emitidos por los propios microorganismos podrían ser también utilizados por los frugívoros como señales para localizar frutos infectados (Peris et al. 2017) a la vez que facilitar la dispersión

de los microorganismos (Schmidt et al. 2015, Capítulos II y III). Es por esta razón por la que se explican a continuación las relaciones entre los frutos cítricos y el hongo especialista *P. digitatum*.

Penicillium es un género de hongos necrotrofos perteneciente al reino *Fungi*, filo *Ascomycota*, clase *Eurotiomycetes*, orden *Eurotiales*, familia *Trichocomaceae*. Está compuesto por más de 300 especies. Se reproduce asexualmente mediante conidios y sexualmente mediante esporas. Éstas se distribuyen por la atmósfera de forma que es el hongo más abundante en los suelos (Domsch et al. 1993, Cannon et al. 2007). *Penicillium* es el hongo que mayores pérdidas económicas causa en la poscosecha de la industria de los cítricos (Eckert y Eaks 1989). Existen dos especies de *Penicillium* especialistas de los frutos cítricos causantes de la podredumbre verde y azul, *P. digitatum* Sacc. y *P. italicum* Wehmer, respectivamente, y raramente se encuentran colonizando otros sustratos. Solo en frutos cítricos completa su ciclo biológico como hongo necrotrofo (Raper y Thom 1949, Barkai-Golan 2001). Infectan los frutos a través de pequeñas heridas en el flavedo producidas durante la maduración o el manejo de los frutos. Las emisiones de D-limoneno y otros monoterpenos (pineno y mirceno principalmente) estimulan la germinación de las esporas y el crecimiento del tubo germinal de *P. digitatum* y *P. italicum* (Eckert y Ratnayake 1994, Droby et al. 2008). A su vez, las emisiones de terpenos volátiles inhiben la germinación y crecimiento de otros patógenos no especializados como *Botrytis cinerea* y *Penicillium expansum* (Droby et al. 2008), patógenos no específicos de los frutos cítricos. Estas relaciones parecen indicar que, entre los compuestos volátiles de los frutos cítricos, el D-limoneno juega un papel importante en el reconocimiento del huésped por parte de los hongos *P. digitatum* y *P. italicum* ya que facilita el proceso de infección (Figura 2).

Por otra parte, existe una estrecha relación entre los cítricos, *Penicillium* y diferentes insectos. Experimentos de emergencia de adultos de *Drosophila* a partir de larvas en frutos cítricos infectados por *P. digitatum* y *P. italicum* llevadas a cabo por Atkinson et al. (1981) muestran que la mosca *Drosophila immigrans* se beneficia con la infección de las frutas cítricas por *P. digitatum* y *P. italicum*, prefiriendo ovipositar sobre frutos infectados (Dweck et al. 2013). Esto podría ser consecuencia de adaptaciones específicas de *D. immigrans* a los metabolitos secundarios originados como consecuencia de la infección por *Penicillium*. Por otra parte, las hembras de la mosca de la fruta *Anastrepha* dispersan las esporas de *Penicillium* spp. adheridas a su cuerpo (Machota et al.2013; Figura 2).

Penicillium es un hongo ubicuo. Estudios realizados por Marcet-Houben et al. (2012) tras secuenciar el ADN de dos cepas de *P. digitatum* procedentes de España y China, concluyen que las cepas aisladas en España divergieron muy recientemente de las cepas chinas, probablemente coincidiendo con la reciente expansión del cultivo comercial de los frutos cítricos en el sur de Europa (Webber 1967), sugiriendo que ambos co-evolucionaron en el sudeste asiático.

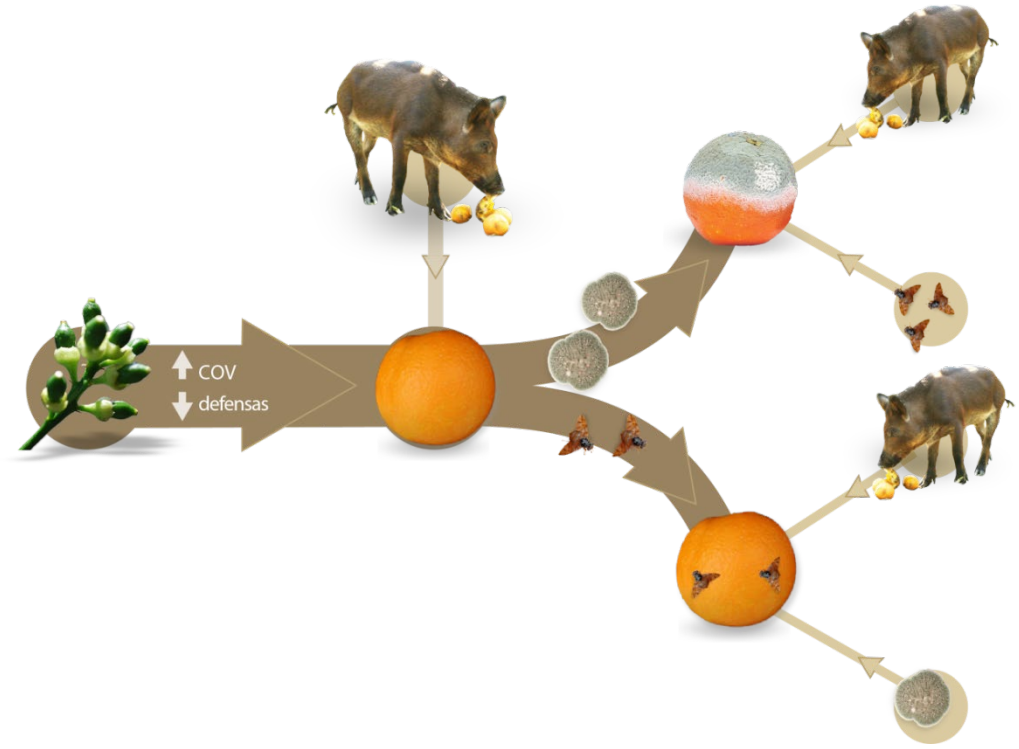

Figura 2. Esquema explicativo del aumento de COVs en frutos maduros y los posibles destinos para un fruto maduro: consumido sano por un frugívoro, infectado por microorganismos y consumido por frugívoro o colonizado por insectos, y/o colonizado por insectos y consumido por frugívoros o infectado por microorganismos.

2. OBJETIVOS

El objetivo general de esta tesis es investigar los mecanismos de comunicación cítrico-frugívoro mediados por el monoterpeno volátil D-limoneno así como la evaluación sensorial en humanos y el papel del hongo *P. digitatum* en las interacciones tritróficas planta-hongo-frugívoro. También se identifican las posibles consecuencias ecológicas de estas interacciones en el naranjo dulce (*C. sinensis*). Este objetivo general comprende los siguientes objetivos específicos que han sido abordados en 5 capítulos.

I. Identificación de vertebrados frugívoros consumidores de cítricos. Experimentos preliminares y revisión bibliográfica.

II. Influencia de *P. digitatum* en la emisión y contenido de compuestos orgánicos volátiles y en la interacción entre frugívoros y frutos cítricos.

III. ¿Es el D-limoneno un terpeno clave en la interacción entre frugívoros y frutos cítricos? Relación entre D-limoneno y *P. digitatum*.

IV. Evaluación sensorial en humanos de las naranjas modificadas genéticamente.

V. Naturalización cítricos en ambientes tropicales.

3. CAPÍTULOS

La tesis está estructurada en cinco capítulos coincidiendo con los 5 objetivos planteados y correspondientes cada uno de ellos a artículos científicos publicados (4) o en preparación (1).

I. Los mamíferos frugívoros prefieren frutos de cítricos infectados por *Penicillium digitatum*: ¿se equivocaba Janzen?

II. Fungal infestation boosts fruit aroma and fruit removal by mammals and birds.

III. A fungus manipulates the interaction of a fleshy fruit with vertebrate frugivores by transforming a deterrent compound into an attractant volatile.

IV. Impact of D-limonene synthase up- or down-regulation on sweet orange fruit and juice odor perception.

V. Reunion in the overseas: introduced wild boars and cultivated orange trees interact in the Mata Atlântica (Brazil).

CAPÍTULO I

LOS MAMÍFEROS FRUGÍVOROS PREFIEREN FRUTOS DE CÍTRICOS INFECTADOS POR *PENICILLIUM DIGITATUM*: ¿SE EQUIVOCABA JANZEN?

FRUGIVOROUS MAMMALS PREFER CITRUS FRUIT INFECTED BY *PENICILLIUM DIGITATUM*: WAS JANZEN WRONG?

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RESUMEN

Janzen (1977) propuso que los vertebrados frugívoros prefieren los frutos sanos frente a los infectados por hongos y bacterias dado que los microbios producen compuestos tóxicos y antibióticos y, además, reducen el valor nutritivo de los frutos infectados. Valoramos dicha hipótesis mediante experimentos de campo en los que ofrecimos tres variedades comerciales de frutos del género *Citrus* sanos e infectados

por *P. digitatum*. Sorprendentemente, los frugívoros (principalmente conejos *Oryctolagus cuniculus* y roedores como la rata negra *Rattus rattus* y ratones, probablemente, *Mus spretus* y *Apodemus sylvaticus*) prefirieron siempre los cítricos infectados a los frutos "control" sanos. En concreto, el consumo de frutos infectados de las tres variedades estudiadas fue hasta 32 veces mayor en comparación con el consumo de frutos sanos. Proponemos tres hipótesis no excluyentes que podrían explicar la preferencia de los frutos infectados por mamíferos y otros vertebrados frugívoros.

Palabras clave: hongo; metabolitos secundarios; tríada; interacciones noveles, relación competitiva; relación facilitativa.

ABSTRACT

Janzen (1977) suggested that frugivorous vertebrates prefer healthy fruit against infected by fungi and bacteria because microbes produce toxic compounds and antibiotics, and also reduces the nutritional value of infected fruit. We evaluated this hypothesis by field experiments in which we offered three commercial varieties of *Citrus* fruits, both healthy and infected by *P. digitatum*. Surprisingly, frugivores (mainly rabbits *Oryctolagus cuniculus* and rodents such as black rats *Rattus rattus* and mice, probably, *Mus spretus* y *Apodemus sylvaticus*) always preferred infected as compared with "control" uninfected fruits. In particular, the consumption of infected fruits of all three varieties studied was up to 32 times higher compared with healthy fruit consumption. We propose three non-mutually exclusive hypotheses that could explain the revealed strong preference of infected fruit by mammals and other frugivores.

Key words: fungus; secondary metabolites; triad; novel interactions, competitive relationship; facilitative relationship.

INTRODUCCIÓN

Numerosas especies vegetales han evolucionado frutos carnosos que atraen y son ingeridos por vertebrados frugívoros, principalmente mamíferos y aves. Con frecuencia, las semillas adheridas a la pulpa son también ingeridas, transportadas internamente y, finalmente, liberadas lejos de la planta madre (i.e. endozoocoria). Estas interacciones mutualistas bipartitas, sin embargo, en ocasiones son alteradas por terceras especies que favorecen o perjudican el mutualismo planta-dispersor (Herrera 1982, Fedriani y Delibes 2013). Un caso relativamente frecuente de dichas "tríadas" (*sensu* Herrera 1984a) es el formado por plantas con frutos carnosos, sus dispersores de semillas y distintos hongos patógenos que se nutren principalmente de pulpa (p.e. Janzen 1977, Buchholz y Levey 1990, Cipollini y Stiles 1993). De forma pionera, Janzen (1977) afirmó que los vertebrados frugívoros prefieren los frutos sanos frente a los infectados por hongos y bacterias dado que los microbios producen compuestos tóxicos y antibióticos y, además, reducen el valor nutritivo de los frutos infectados. Los frutos en fermentación raramente serían comidos por vertebrados excepto en condiciones de inanición extrema o cuando el contenido de compuestos metabolitos secundarios no fuera lo suficientemente alto como para enmascarar los azúcares y otros nutrientes presentes (Janzen 1977).

La mayoría de investigaciones sobre estos sistemas han concluido, de acuerdo con Janzen (1977), que los frugívoros seleccionan frutos sanos frente a infectados o en mal estado, por lo que los hongos interferirían negativamente en el mutualismo planta-dispersor de semillas. Muchas especies de aves muestran mayor preferencia de consumo por frutos frescos que por frutos en putrefacción (Borowicz

1988, Buchholz y Levey 1990, Cipollini y Stiles 1993, García et al. 1999). Por ejemplo, varias especies de aves, habituales dispersoras de semillas de *Vaccinium ovalifolium* y *Juniperus communis*, mostraron preferencia por los frutos maduros sanos frente a los atacados por plagas e insectos, inmaduros, o abortados (Traveset et al. 1995, García et al. 1999). Otros estudios han mostrado que pájaros dispersores de semillas rechazan frutos atacados por insectos (Krischik et al. 1989, Manzur y Courtney 1994). También los mamíferos evitan normalmente los frutos infectados (Dominy 2004). Los primates salvajes generalmente consumen frutos frescos evitando los frutos demasiado maduros (Milton 2004). En general, se acepta comúnmente que los frugívoros de mayor tamaño tienden a repeler los frutos infectados, así como las semillas y pulpa en mal estado (Sherratt et al. 2006).

Existen pocos estudios sobre frugívoros que consumen frutos fermentados o en mal estado (Dudley 2004). Fitzgerald et al. (1990) demostraron la intoxicación y posterior mortalidad de *Bombycilla cedrorum* (Bombycillidae) al ingerir frutos de espino, *Crataegus* sp. con elevado contenido de etanol en su pulpa. Asimismo, monos sifacas, *Propithecus diadema candidus*, en Madagascar consumieron semillas de frutos caídos muy maduros, posiblemente con contenido elevado de etanol en la pulpa (Milton 2004). Por tanto, algunos vertebrados frugívoros, y entre ellos mamíferos, pueden alimentarse de frutos en descomposición e incluso pudieran haber evolucionado adaptaciones específicas para ello. Asimismo, algunos autores indican que la infección de frutos por larvas de insectos puede incrementar el contenido nutricional de los frutos (Piper 1986, Drew 1987) y hacerlos más atractivos para mamíferos y aves (Redford et al. 1984).

En resumen, microorganismos y mamíferos frugívoros parecen tener una relación fundamentalmente competitiva (i.e. se limitan la disponibilidad de alimento mutuamente). En este sentido, la

acumulación de metabolitos secundarios en los frutos jugaría un papel primordialmente protector de los mismos frente al ataque de microorganismos (Janzen 1977, Cipollini y Stiles 1993). Sin embargo, no conocemos ningún estudio que haya evaluado si la naturaleza de dicha relación (competitiva, neutra, o facilitativa) varía entre distintas especies de frugívoros y/o variedades de frutos. Existe la interesante posibilidad, no considerada hasta la fecha, de que hongos y otros microorganismos reblandezcan la piel de los frutos infectados y degraden sus metabolitos secundarios protectores, con lo que ésta perdería parcialmente su capacidad protectora. Ello facilitaría el acceso de frugívoros vertebrados incapaces de acceder a la pulpa de los frutos sanos. En este caso, la interacción entre el hongo y los mamíferos frugívoros podría ser facilitativa. La falta de estudios probablemente se relacione con dificultades logísticas de realizar experimentos de campo suficientemente replicados con frutos infectados y sanos en similar estado de maduración.

Muchas especies de mamíferos consumen frecuentemente frutos domésticos en sistemas agrícolas de todo el mundo (Fedriani et al. 2001, Rosalino y Santo-Reis 2009; Tabla 2). Estas "interacciones noveles" (*sensu* Wood et al. 2015), que se dan en sistemas agrícolas logísticamente apropiados, pueden ser de gran ayuda para progresar en nuestro conocimiento sobre algunas cuestiones ecológicas, tales como el carácter competitivo, neutro o facilitador de los hongos en su interacción con mamíferos frugívoros (véase también Thompson 2013). Actualmente los cítricos (*Citrus sp*.) son el primer cultivo frutal del mundo tanto en superficie (más de 8 millones de Hectáreas cultivadas) como en producción (131 millones de Tm producidas en el año 2012) (FAO 2012). Se cultivan en más de 100 países en la franja comprendida entre los 40° de latitud al norte y sur del ecuador. Pese a su importancia económica y agronómica, llama enormemente la atención el vacío existente acerca del conocimiento de las

interacciones ecológicas con otras especies y sus potenciales consecuencias. Aunque no conocemos ningún estudio centrado en la ecología de los cítricos y sus interacciones con vertebrados frugívoros, sí se han citado casos de frugívoros consumidores de frutos cítricos en diferentes ecosistemas y partes del mundo (véase Tabla 2).

En este estudio evaluamos experimentalmente la posible atracción o repelencia de frutos infectados por hongos por parte de los vertebrados consumidores de cítricos. Para ello, realizamos una serie de experimentos de campo en los que se ofrecieron frutos sanos e infectados por *P. digitatum* de tres variedades de cítricos en una parcela de cultivo de cítricos en Valencia. En concreto, nuestro objetivo fue contestar a las siguientes dos preguntas: (*i*) ¿Cuál es la naturaleza (competitiva *vs*. facilitadora) de la interacción entre hongos y mamíferos frugívoros?, y (*ii*) ¿es la naturaleza de la interacción consistente entre especies de frugívoros y variedades de cítricos?

Tabla 2. Especies animales consumidoras de frutos cítricos, parte del fruto consumida, y localización geográfica de los estudios.

Table 2. Animal species consumers of citrus fruit, part of the fruit consumed, and geographical location.

Nombre común	Nombre científico	Familia	Localización	Parte consumida	Referencia
Paca común	*Cuniculus paca*	Cuniculidae	Floresta Atlántica, Brasil	Pulpa y semillas	Zuracatto et al. 2010
Comadreja overa	*Didelphis albiventris*	Didelphiadae	Curitiba, Brasil	Semillas en sus heces	Cáceres 2002
Aguara guazú	*Chrysocyon brachyurus*	Canidae	Itapetininga, Sao Paulo, Brasil	Pulpa	Motta-Junior y Martins 2002
Mono carablanca	*Cebus capucinus*	Cebidae	Costa Rica	Pulpa	Baker 1996
Coyote	*Canis latrans*	Canidae	California	Semillas en sus heces	Silverstein 2005
Coyote	*Canis latrans*	Canidae	México	Pulpa	Monroy et al. 2003
Elefante asiático	*Elephas maximus*	Elephantidae	Tailandia	Frutos	Kitamura et al. 2002
Muntíaco de la India	*Muntiacus muntjak*	Cervidae	Tailandia	Frutos	Kitamura et al. 2002
Marta de Japón	*Martes melampus*	Mustelidae	Japón	Semillas en sus heces	Tsuji et al. 2011
Macaco de Japón	*Macata fuscata*	Cercopithecidae	Isla Yakushima, Japón	Frutos	Otani y Shibata 2000
Ardilla de palmera	*Funambulus pennantii*	Sciuridae	Perth, Australia	Pulpa	Long 2003, Palmer et al. 2007
Agutí negro	*Dasyprocta mexicana*	Dasyproctidae	México	Semillas y pulpa	Chambé 2012
Chimpancé	*Pan troglodytes schweinfurthii*	Hominidae	Mahale, Tanzania	Frutos de limón (*C. limon*)	Takahata et al. 1986
Chimpancé	*P. troglodytes verus*	Hominidae	Cantanhez National Park, Guinea Bissau	Frutos de lima (*C. aurantifolia*)	Bessa et al. 2015
Chimpancé	*P. troglodytes verus*	Hominidae	Bossou (Guinea)	Frutos de limón, pomelo y amargo	Sugiyama & Koman
Surili de Sumatra	*Presbytis melalophos*	Cercopithecidae	Indonesia	Pulpa	Ungar 1995
Lémur coronado	*Eulemur coronatus*	Lemuridae	Madagascar	Frutos de *C. madagascariensis*	Chen et al. 2015
Lirón careto	*Eliomys quercinus*	Gliridae	España	Pulpa	Gil-Delgado et al. 2010.
Gualacate	*Euphractus sexcinctus*	Dasypodidae	Mato Grosso, SP, Brasil	Pulpa y semillas	Dalponte y Tavares-Filho 2004
Chacal de lomo negro	*Canis mesomelas*	Canidae	Reserva Natural Monkolodi, Botswana	Frutos	Kaunda y Skinner 2003
Marta	*Martes martes*	Mustelidae	Mallorca, España	Pulpa	Clevenger 1996
Murciélago egipcio	*Rousettus aegyptiacus*	Pteropodidae	Turquía	Pulpa	Albayrak et al. 2008

Murciélago de Ryukyu	*Pteropus dasymallus*	Pteropodidae	Japón	Frutos de naranja	Vincenot et al. 2015
	Pteropus conspicillatus	Pteropodidae	Queensland, Australia	Frutos de naranja y mandarina	Richards 1990
Zorro volador	*Saguinus leucopus*	Callitrichidae	Mariquita, Tolima, Colombia	Frutos	Poveda y S.-Palomino 2004
Tití gris	*Tapirus bairdii*	Tapiridae	Gran Pantanal, Brasil	Fruto y semillas de *C. aurantium*	Olmos 1997
Tapir silvestre	*Paguma larvata*	Viverridae	Japón	Pulpa	Torii 1986
Paguma	*Pteroglossus torquatus*	Ramphastidae	Panamá	Frutos	Leck 1972
Tucancillo collarejo	*Amazona arausiaca*	Psittacidae	Dominica	Frutos	Douglas et al. 2013
Amazona gorgirroja	*Thraupis episcopus*	Thraupidae	Cordillera de la Costa, Venezuela	Pulpa	Verea et al. 2009
Tangara azulada	*Ramphocelus carbo*	Thraupidae	Cordillera de la Costa, Venezuela	Pulpa	Verea et al. 2009
Toche negro	*Cacatua galerita*	Cacatuidae	Australia	Frutos de naranja y limón	White 2011
Cacatúa galerita	*Larus michahellis*	Laridae	Algeria	Semillas de cítricos	Moulaî et al. 2008
Gaviota patiamarilla	*Melanerpes carolinus*	Picidae	Florida	Frutos	Beal 1911
Carpintero de Carolina	*Geochelone elephantopus*	Testudinidae	Santa Cruz, Islas Galápagos, Ecuador	Frutos	Cayot 1987
	Gopherus polyphemus	Testudinidae	Algeria	Frutos	Reuther et al. 1978
Tortuga gigante	*Alligator mississippiensis*	Alligatoridae	Carolina del Norte	Frutos directamente del árbol	Platt et al. 2013
Tortuga de Florida	*Alligator mississippiensis*	Alligatoridae	Carolina del Norte	Frutos caídos	Brueggen 2002
Caimán del Misisipi	*Nasua nasua*	Procyonidae	Iguazú, Argentina	Pulpa y semillas	Hirsch 2009
Caimán del Misisipi	*Pteropus dasymallus*	Pteropodidae	Japón	Pulpa	Vincenot et al. 2015
Coati de cola anillada	*Pteropus conspicillatus*	Pteropodidae	Australia	Pulpa	Waples 2002
	Pteropus poliocephalus	Pteropodidae	Australia	Pulpa	Rogers 2002
Murciélago Ryukyu	*Rousettus aegyptiacus*	Pteropodidae	Israel	Pulpa	Moran y Keider 1993
Zorro volador	*Rousettus aegyptiacus*	Pteropodidae	Egipto	Pulpa	Madkour 1977
Murciélago gris	*Rousettus aegyptiacus*	Pteropodidae	Turquía	Pulpa	Albayrak et al. 2008
Murciélago egipcio	*Chiroptera sp.*	Chiropterae	India	Pulpa	Sharma et al. 2004
Murciélago egipcio	*Elephas sp.*	Elephantidae	India	Frutos	Sharma et al. 2004
Murciélago egipcio	*Podarcis erhardii*	Lacertidae	Grecia	Pulpa	Brock et al. 2014
Murciélago Elefantes					
Lagartija de Erhard					

MATERIAL Y MÉTODOS

Área de estudio

El estudio fue realizado en las parcelas cultivadas del Instituto Valenciano de Investigaciones Agrarias (IVIA) entre los meses de julio a diciembre de 2013. El clima es Mediterráneo cálido con veranos secos y calurosos e inviernos templados. La parcela donde se realizó el estudio tiene una superficie de 0.4 Hectáreas, con diferentes variedades de cítricos entre las que predomina la clementina Clemenules (*Citrus clementina* Hort. ex Tan.). La parcela está rodeada por campos de cultivo comerciales y experimentales de cítricos. En un extremo de la parcela existen varias madrigueras habitadas por conejos.

Para los experimentos de campo, seleccionamos tres variedades de cítricos (clementina Clemenules [*Citrus clementina* Hort. ex Tan.], satsuma Owari [*Citrus unshiu* Marc.] y naranja Bernalina [*C. sinensis* L. Osbeck]) que difieren en sus propiedades físicoquímicas y nutritivas. Clemenules es una mandarina del grupo clementinas, con fruto de tamaño mediano, corteza fina, algo rugosa, fácil de pelar, elevado contenido en zumo, equilibrio en la relación ácidos – azúcares, pulpa fundente (Soler y Soler 2006), índice de madurez (IM) 11,5 y acidez 13 g/L (datos consultados en Variedades comerciales de cítricos para noviembre, http://www.ivia.es 2015). La Bernalina es una naranja del grupo Blancas que produce frutos de tamaño mediano a grande, corteza espesa, elevado contenido en zumo, IM = 13 y acidez 9 g/L (elaboración propia, análisis realizados en julio de 2013). La satsuma Owari es una mandarina con frutos de tamaño mediano a pequeño, color naranja poco intenso y forma aplanada, con alto contenido en zumo (Soler y Soler 2006), IM = 7 y acidez 16 g/L (datos consultados en Variedades comerciales de cítricos para octubre, http://www.ivia.es 2015). Clementinas y satsumas son mandarinas, por tanto, con corteza más fina que la

naranja Bernalina, lo cual puede tener implicaciones en sus interacciones con distintos vertebrados frugívoros. No obstante, el D-limoneno es el compuesto volátil predominante en las glándulas de aceite de la piel de las tres variedades.

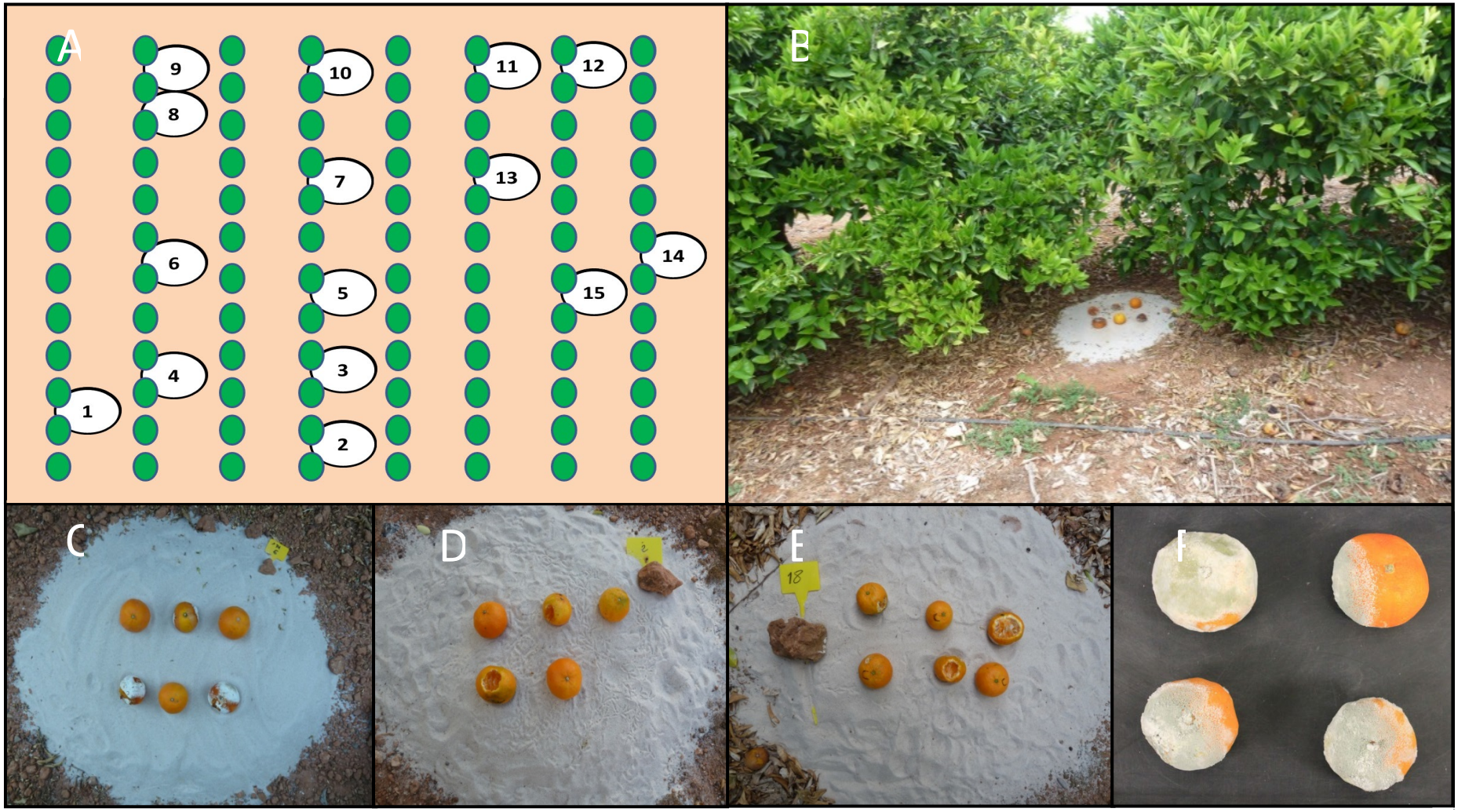
Diseño experimental

Para evaluar la preferencia por frutos de cítricos sanos e infectados por hongos por parte de mamíferos y otros vertebrados frugívoros, se ofrecieron ambos tipos de frutos sobre camas de arena (i.e. areneros, ver Figura 3C) de 1 m de diámetro en la misma parcela de cultivo de cítricos. Se recolectaron frutos que no habían recibido tratamientos fitosanitarios en los últimos 3 meses. Los frutos se infectaron con *P. digitatum* cepa NAV-7 obtenida del Laboratorio de Patología de Hongos del Instituto Valenciano de Investigaciones Agrarias (IVIA). Los frutos se desinfectaron mediante inmersión durante 1 minuto en una solución de hipoclorito sódico 4 gL^{-1}. Posteriormente se enjuagaron con agua y secaron al aire. Para infectar los frutos se realizaron 2 incisiones opuestas en la corteza de los frutos, practicadas en el ecuador de cada fruto y posterior inmersión en una solución de agua con Tween 80 y esporas de *P. digitatum* NAV-7 (Palou et al. 2002) durante 2 horas. Los frutos se incubaron a 25°C con elevada humedad relativa. Pasados 7 días desde la infección, se observó un halo de crecimiento activo de las hifas de *P. digitatum* en la piel de los frutos (Figura 3F). En cada arenero se colocaron 3 frutos sanos y 3 frutos infectados dispuestos de manera alterna y separados por ~10 cm. En la parcela se montaron 15 areneros distribuidos aleatoriamente (ver Figura 3A). Los areneros se montaron debajo de los árboles de cítricos simulando la caída natural de los frutos maduros (Figura 3B). Otros detalles sobre la metodología empleada son descritos por Fedriani y Delibes (2009a, 2013). Cada variedad fue ofrecida en el momento en que los frutos se encontraban maduros. Durante 5 días consecutivos y a primera hora de la mañana, se

anotaron los frutos consumidos *in situ* y los faltantes. Los frugívoros visitantes se identificaron por medio de sus huellas y otras señales (heces, tipo de manipulación de los frutos, etc. Ver Figuras 3D y 3F). Los frutos consumidos o faltantes se repusieron cada mañana. Las naranjas se ofrecieron en julio, las satsumas en octubre y las clementinas en noviembre de 2013, coincidiendo con las fechas de maduración de sus frutos.

Figura 3. A) Representación esquemática de la distribución de los quince ofrecimientos de frutos en la parcela de estudio; B) colocación de los ofrecimientos debajo de la copa simulando la caída natural de los frutos; C) cama de arena (i.e. areneros) con frutos sanos e infectados por *P. digitatum* dispuestos de manera alterna; D) arenero con huellas y frutos infectados de clementino comidos por conejo y pájaros ; E) arenero con huellas y frutos infectados de clementino comidos por conejo; F) frutos de clementino infectados por el hongo *P.digitatum* antes de ser ofrecidos en los areneros.

Figure 3. A) Schematic representation of the random distribution of the fifteen fruit offerings in the orchard.; B) we placed fruits on sand bedsunderneath orange trees simulating natural fruit drop; C) sand bed with healthy and infected by *P. digitatum* fruits alternately arranged; D) sand bed with footprints and clementino infected eaten by birds and rabbits; E) sand bed with footprints and clementino infected fruits eaten by rabbit; F) clementino fruits infected by the fungus *P. digitatum* before to be offered in the sand beds.


A
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Análisis estadísticos

Tanto la probabilidad de visita como el porcentaje de frutos de cada tratamiento colectados por los distintos frugívoros se analizaron mediante modelos mixtos lineales generalizados con error binomial y función de enlace logit usando el procedimiento GLIMMIX de SAS (Littell et al. 2006). El tipo de frugívoro (conejo, roedor, ave), la variedad de cítrico (clementina, satsuma y naranja), el tratamiento (sano o infectado por *P. digitatum*), y sus interacciones de segundo orden fueron especificados como factores fijos. La fecha, el número del experimento y el bloque (anidado en experimento) fueron tratados como efectos aleatorios (Bolker et al. 2009). Las medias ajustadas y los errores estándar se calcularon utilizando la opción LSMEANS y fueron luego transformados a escala lineal mediante la serie de Taylor apropiada (Littell et al. 2006). Cuando la interacción entre dos factores fue significativa, se realizaron tests del efecto de un factor en cada nivel del otro factor ("pruebas de efectos principales simples") utilizando la opción "Slice" de LSMEANS (Littell et al. 2006).

RESULTADOS

Mamíferos y otros frugívoros visitantes

Los ofrecimientos de frutos cítricos fueron visitados frecuentemente por conejos (*Oryctolagus cuniculus*), roedores y aves frugívoras. Las diferencias en las tasas de visita entre grupos de frugívoros fueron altamente significativas ($F_{2,\ 810} = 55.39$, $P<0.0001$; Figura 4). Concretamente, los conejos fueron con mucho los frugívoros más frecuentes (89.2 % ± 2.7 [media±1ES] de los ofrecimientos). La probabilidad de visitas por conejos fue 9.7 y 1.6 veces mayor que las de roedores (ratas *Rattus rattus* y ratones *Mus spretus* y/o *Apodemus*

silvaticus) y aves (mirlos *Turdus merula*, urracas *Pica pica*, gorriones *Passer domesticus* y lavanderas *Motacilla alba*), respectivamente. Las aves fueron, de media, 6.1 veces más frecuentes que los roedores (Figura 4). También hubo notables diferencias en las tasas de visitas entre distintas variedades de cítricos ($F_{2, 810} = 5.87$, $P<0.003$). De media, los ofrecimientos de clementina atrajeron ~1.8 veces más frugívoros en comparación con los de satsuma y naranja (Figura 4). La interacción entre tipo de frugívoro y variedad de fruto fue significativa ($F_{4, 810} = 15.04$, $P <0.0001$), indicando que las probabilidades de visitas de los distintos frugívoros no fue consistente entre variedades de frutos cítricos. Por ejemplo, mientras que en los ofrecimientos de clementina y satsuma las aves fueron visitantes más frecuentes que los roedores, en los ofrecimientos de naranja Bernalina las frecuencias de visitas de esos dos mismos grupos de frugívoros fueron similares (29.4%±6.9 y 34.8%±8.2, respectivamente; Figura 4).

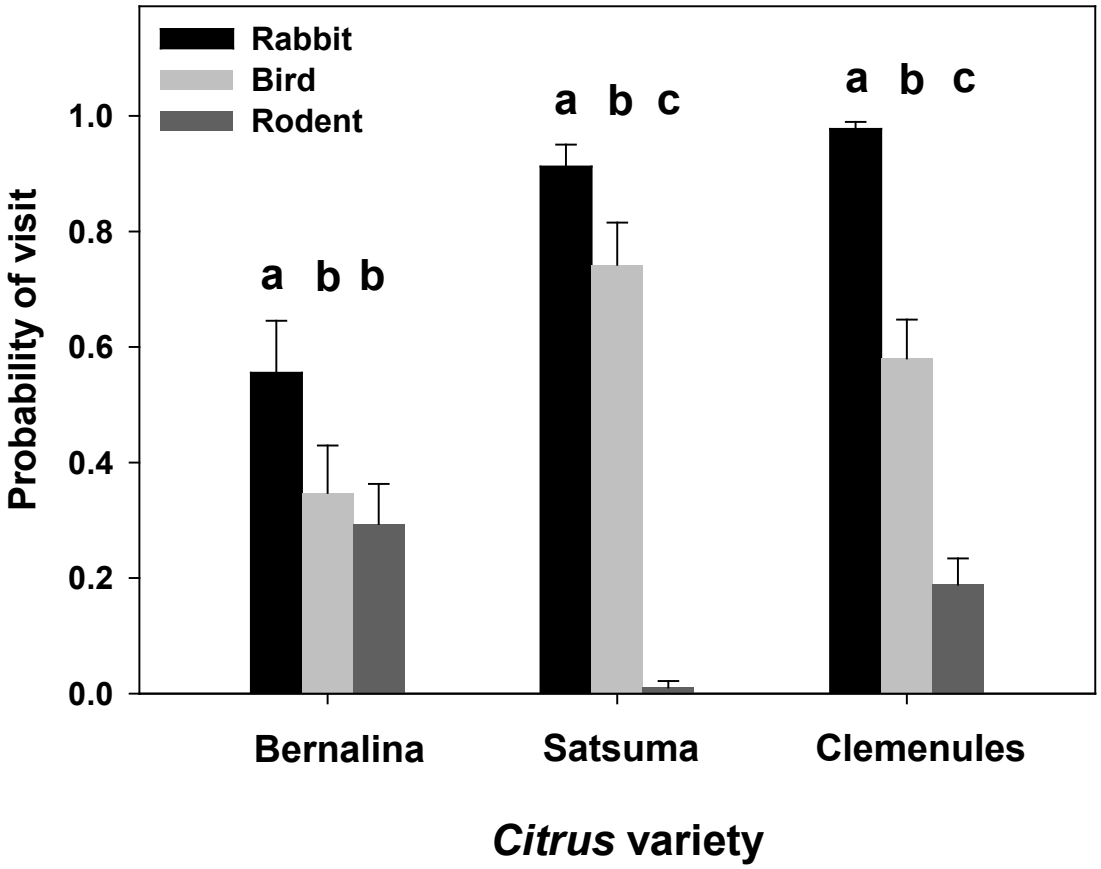

Figura 4. Medias ajustadas y errores estándares en las tasas de visitas según grupo frugívoro y variedad de cítrico. Las letras distintas encima de las barras indican diferencias significativas ($P< 0.05$) entre grupos de frugívoros para cada variedad cítrica.

Figure 4. Adjusted means and standard errors of rates of visits by different frugivore groups and citrus variety. Different letters on top of bars indicate significant differences ($P< 0.05$) between frugivore types for each citrus variety.

Efecto de la infección por *P. digitatum* en la preferencia de frutos

La ingestión de las tres variedades de frutos cítricos por mamíferos y otros frugívoros siguió un patrón similar al de las visitas, siendo la naranja Bernalina la variedad menos consumida (Figura 5). En concreto, el consumo de mandarinas satsuma y clementina fue 3.5 y 2.3 veces mayor que el de naranja, siendo estas diferencias significativas ($F_{2, 514}$= 3.73, *P* = 0.025; Figura 5). La infección de frutos cítricos con *P. digitatum* tuvo un efecto altamente significativo sobre la probabilidad de consumo por parte de mamíferos (conejo, rata y ratón) y otros frugívoros vertebrados (mirlo, gorrión, lavandera, urraca) ($F_{1, 514}$= 204.08, *P* <0.0001). De media, los frutos infectados fueron 17.5 veces más consumidos que los sanos. No obstante, la interacción significativa entre infección por *P. digitatum* y variedad cítrica($F_{2, 514}$ = 9.87, *P* <0.001) indicó que la magnitud de las diferencias en probabilidad de consumo de frutos infectados y sanos cambió entre variedades. En concreto, para mandarinas clementina y satsuma el consumo de frutos infectados fue 32.5 y 17.8 veces mayor, respectivamente, en comparación con las no infectadas, mientras que en naranja Bernalina la preferencia por los frutos infectados fue menos marcada (6.2 veces mayor; Figura 5). Dicha preferencia por los frutos infectados ocurrió para todos los frugívoros vertebrados y para las tres variedades de cítricos (Figura 6). Para naranja Bernalina y clementina no hubo diferencias entre el tipo de frugívoro vertebrado en el consumo de frutos infectados y sanos (Test exacto de Fisher, *P*> 0.107). Para satsuma, la preferencia por los frutos infectados fue ligeramente menor en los conejos en comparación con las aves (Test exacto de Fisher, *P* = 0.027), no habiendo suficientes casos para evaluar la preferencia por los roedores.

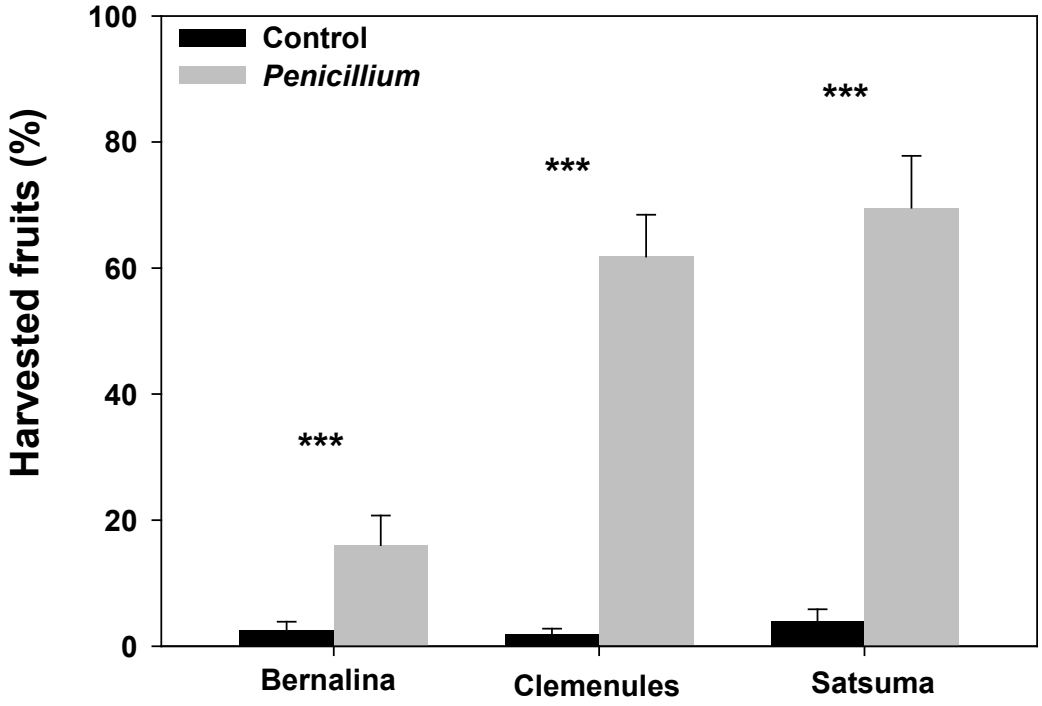

Figura 5. Medias ajustadas y errores estándares de los porcentajes de consumo de frutos según variedad de fruto y tratamiento (infectado o sano).***, *P*< 0.0001

Figure 5. Adjusted means and standard errors of percentage of fruit consumption and treatment depending on fruit variety and treatment (infected or non-infected). ***, *P*< 0.0001

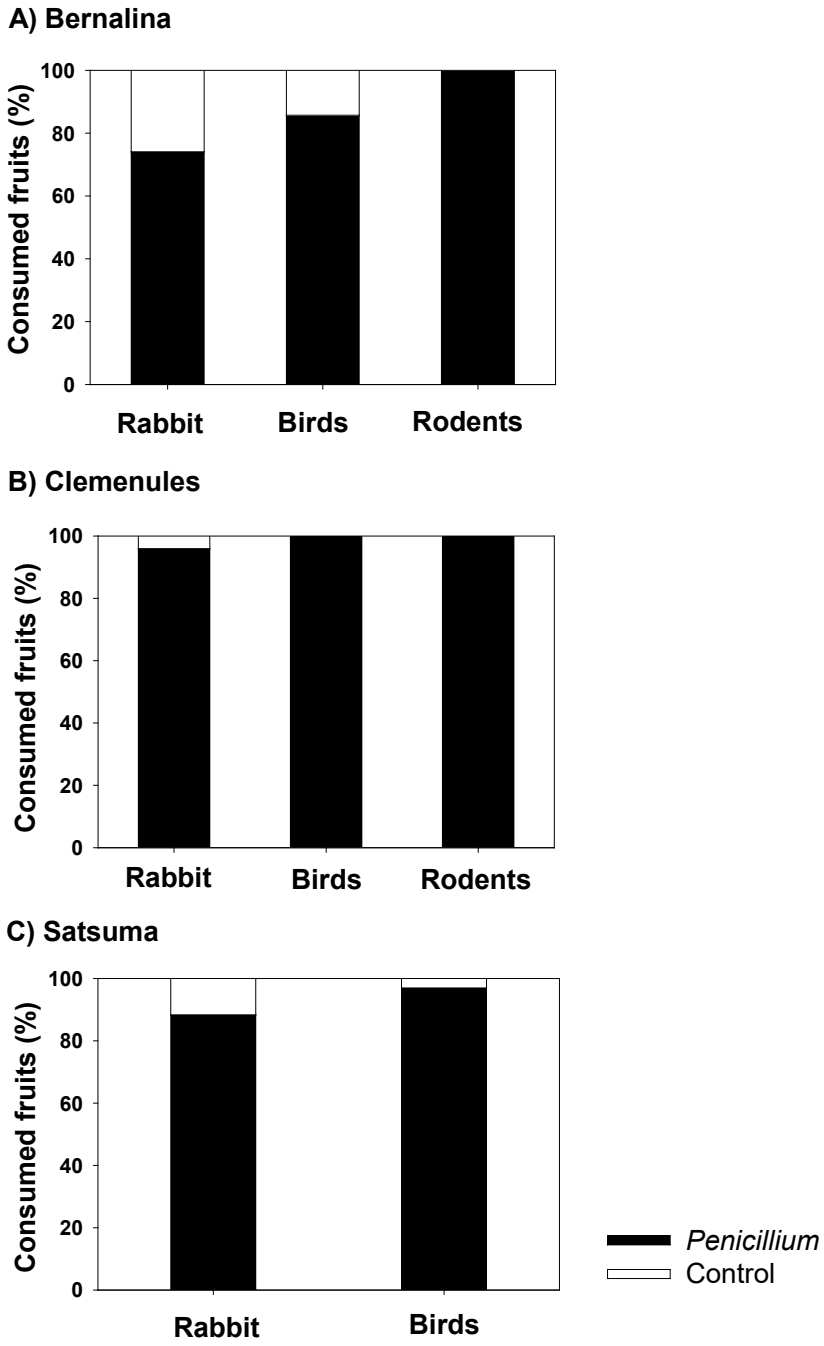

Figura 6. Porcentajes observados de consumo de frutos por distintos frugívoros según variedad de fruto y tratamiento (infectado o sano).

Figure 6. Observed percentage of fruit consumption by different frugivore groups depending on fruit variety and treatment (infected or non-infected).

DISCUSIÓN

Mamíferos y otros frugívoros visitantes

A nivel mundial, los mamíferos de tamaño grande y medio parecen ser los principales consumidores de cítricos, aunque también existen documentados algunos casos de aves y reptiles (Tabla 2). Durante nuestros ofrecimientos de frutos, los visitantes más frecuentes fueron los conejos, seguidos muy de lejos por aves y roedores. La alta frecuencia de visitas de conejos probablemente se relaciona con la cercanía de numerosas madrigueras de conejos (en un mismo día se han llegado a avistar hasta 32 individuos distintos).

Las frecuencias relativas de visitas por los distintos grupos de frugívoros variaron con respecto a las variedades de frutos cítricos ofrecidos. Las aves visitaron mucho más frecuentemente las mandarinas que las naranjas. Esto puede deberse a las diferencias de grosor de la piel entre mandarinas y naranjas, y a las diferencias en las características organolépticas de cada variedad. Las mandarinas tienen una piel más fina en comparación con las naranjas, hecho que puede facilitar el acceso de las aves a la pulpa. En cuanto a las características organolépticas, Clemenules tiene mayor contenido de azúcares en pulpa en comparación con satsuma Owari y naranja Bernalina (datos sin publicar). Estos dos hechos creemos que pueden explicar las diferencias en las tasas de visitas dado que sería esperable que los frutos más atractivos sean aquellos con alto contenido en azúcar y menor dificultad para acceder a ella (Herrera 1984b). No obstante, dado que los ofrecimientos de las tres variedades no se realizaron simultáneamente, no puede descartarse que algunas de las diferencias en las frecuencias de visitas se relacionen con variaciones estacionales en las abundancias relativas de los mamíferos y otros vertebrados frugívoros.

Efecto de la infección por *P. digitatum* en la preferencia de frutos

Los resultados indican que los distintos grupos de frugívoros prefieren consumir frutos cítricos infectados por *P. digitatum* en comparación con los sanos (Figura 5). Este novedoso resultado, que contradice a Janzen (1977), sugiere la existencia de diferencias físicas y/o químicas importantes entre frutos infectados y sanos, que resultan en una fuerte preferencia de los vertebrados frugívoros por los frutos infectados. Observaciones realizadas en Borneo muestran que algunos elefantes (*Elephas maximus*) recorren grandes distancias en busca de frutos fermentados del género *Durio,* Bombacaceae (Siegel 2005). También algunos monos y murciélagos consumen frutos fermentados de *Durio* (Siegel 2005). Algunos animales repiten con asiduidad el consumo de frutos fermentados y en mal estado. En África se han observado elefantes que, junto con ganado, recorren grandes distancias a través de las sabanas para consumir frutos maduros de palmera *Hyphaene thebaica*, de marula (*Sclerocarya birrea*), de mongongo (*Schinziophyton rautanenii*), y dátiles de palmera del género *Borassus,* Arecaceae. Estos frutos fermentan rápidamente una vez maduros generando moléculas aromáticas que atraen animales (Siegel 2005). Estas observaciones junto con otros estudios (Dudley 2000, Dominy 2004, Sherratt et al. 2006) parecen indicar que, aunque no de manera generalizada, sí existen diversas especies de mamíferos y aves habituados al consumo de frutos fermentados, infectados o en mal estado.

A continuación, proponemos tres hipótesis no excluyentes que explicarían la preferencia de los mamíferos y otros vertebrados por los frutos de cítricos infectados frente a los sanos:

1. **Los hongos reblandecen la piel de los frutos infectados y así aumentan la accesibilidad de los frugívoros a la pulpa**: una vez

iniciada la infección del fruto por *Penicillium sp.*, las hifas del hongo segregan una enzima que degrada la lignina de la pared celular (Marcet-Houben et al. 2012) reblandeciendo los tejidos. Este mecanismo de facilitación del acceso de los frugívoros a la pulpa sería especialmente crítico para algunas aves y otros frugívoros de menor tamaño incapaces de atravesar la gruesa piel de cítricos no infectados.

2. **Biotransformación en nuevas sustancias atrayentes o menos repelentes para los frugívoros.** Esta hipótesis comprende dos componentes:

   A) **Los hongos liberan metabolitos secundarios atrayentes de frugívoros**: la infestación de una naranja por *Penicillium* posiblemente conlleve la fermentación de los azúcares que son transformados en alcoholes y aldehídos (Tuset 1987). Por ejemplo, a medida que se desarrolla el hongo *P. digitatum* se produce etileno en los frutos cítricos infectados (Achilea et al. 1985). Estos nuevos compuestos generados a partir de la infección son muy volátiles y emiten aromas que podrían atraer más frugívoros que los frutos no infectados.

   B) **Los hongos degradan las sustancias repelentes de la piel del fruto y posiblemente biotransforman estas sustancias en otras atrayentes de** f**rugívoros.** Las sustancias de la piel posiblemente sean defensoras frente a predadores, plagas y patógenos (Cipollini y Levey 1997b, Rodríguez et al. 2013), y ejerzan un papel protector sobre pulpa y semillas del fruto por lo que podrían repeler a los frugívoros. La infección de una naranja por *Penicillium* origina una serie de procesos bioquímicos (i.e. Biotransformación) que transforma los compuestos existentes en otros compuestos derivados. Por

ejemplo, la infección de un fruto por *P. digitatum* transforma el D-limoneno existente en las glándulas oleicas de la piel en α–terpineol (Tan et al. 1998, Demittenaere et al. 2001). Asimismo, la infección por hongos transforma el citral en timol, geranial, nerol, D-limoneno, α-pineno y geraniol (Esmaeili y Tavassoli 2010). Estos compuestos generados son altamente volátiles y podrían resultar atractivos para mamíferos y otros frugívoros.

3. **Los hongos producen sustancias con propiedades medicinales que atraen a los frugívoros**: algunos autores indican que determinados animales (i.e. chimpancés *Pan troglodytes schweinfurthii* en Uganda, Krief et al. 2005) son capaces de automedicarse de manera intencionada imitando y observando el comportamiento de otros animales adultos. Se han observado individuos de mono carablanca, *Cebus capucinus*, frotando corteza de frutos cítricos sobre su cuerpo y posteriormente aplicándose el zumo de la pulpa en Costa Rica (Baker 1996). Algunos animales consumen frutos con moderados contenidos de etanol probablemente debido al alto contenido calórico de dicho alcohol o a sus propiedades purgantes (Dominy y Lucas 2004). El consumo de frutos infectados por *Penicillium sp.* podría reportarles beneficios para la salud desconocidos hasta la fecha.

Se desconocen muchas de las transformaciones bioquímicas que se dan en un fruto desde que se inicia la infección por *P. digitatum*, por lo que es necesario seguir investigando para determinar qué compuesto o compuestos explican la preferencia de los frugívoros por consumir frutos infectados frente a frutos sanos.

Naturaleza de la interacción entre hongos y mamíferos

La infección de los cítricos por *Penicillium sp.* parece formar parte de una tríada ecológica fruto – hongo – vertebrados frugívoros en la que cada parte interactúa simultáneamente con las otras dos (Herrera 1984a). Las plantas producen frutos carnosos que atraen a los frugívoros y estos dispersan sus semillas, los hongos colonizan tejidos del fruto óptimos para esporular, y los frugívoros se alimentan de pulpa rica en azúcares.

Las hipótesis propuestas sobre el papel de *P. digitatum* pueden ayudar a entender mejor las relaciones ecológicas en interacciones similares entre especies nativas. Contrariamente a la idea de que los microbios colonizan rápidamente los frutos produciendo compuestos tóxicos y antibióticos y reduciendo el valor nutritivo de los frutos infectados (Janzen 1977), nuestros datos experimentales indican que *P. digitatum*, en el caso de los cítricos, tiene un papel facilitador del acceso a la pulpa para los frugívoros (Figura 5). Estos resultados también se ven apoyados por puntuales estudios en otros ecosistemas documentando un alto consumo de frutos infectados (Dominy 2004, Dudley 2004, Milton 2004) probablemente debido a la presencia de bajos niveles de etanol. Esta nueva concepción es opuesta a la visión más generalizada sobre la naturaleza competitiva de las interacciones entre vertebrados frugívoros y los microorganismos (Cipollini y Stiles 1993, Tewksbury 2002) y, ciertamente, invita a que este tipo de sistemas sea detalladamente investigado con renovada atención. Dichas investigaciones también deberían considerar los probables efectos demográficos de estas interacciones. Por ejemplo, algunos frugívoros consumidores de cítricos probablemente dispersen sus semillas viables y faciliten la colonización en hábitats tropicales (Baskaran y Desai 2013, Campos-Arceiz et al. 2012, García-Morales et al. 2012, Montaldo 1993, Milton 2008). Por su

parte, el aporte de fruta de forma predecible y abundante probablemente permite a los frugívoros mantener altas densidades poblaciones (e.g. Fedriani et al. 2001). Asimismo, de manera análoga a lo encontrado en otros sistemas comparables (Johnson 1994, Janos y Sahley 1995, Maser et al. 1978), *Penicillium* y otros hongos que infectan los frutos cítricos se beneficiarían de la dispersión de esporas por parte de los frugívoros tanto de forma exozoócora como endozoócora.

En los frutos cítricos, los monoterpenos son los principales componentes de las glándulas del aceite esencial de la cáscara (flavedo), siendo el D-limoneno el más abundante (hasta 95% en la naranja, Rodríguez et al. 2011). Estudios realizados en naranjas con bajo contenido de D-limoneno (Rodríguez et al. 2011) indican que *P. digitatum* se desarrolla y crece más rápido en frutos con elevados contenidos de D-limoneno que en frutos con bajos contenidos de limoneno en la cáscara. Por tanto, la existencia de D-limoneno en los frutos favorece su colonización por *P. digitatum*, lo que a su vez favorece el consumo por frugívoros vertebrados y, probablemente, la dispersión de semillas. Nuevos experimentos son necesarios para poder identificar la importancia relativa de la infección por *P. digitatum* y de la concentración de D-limoneno y otros compuestos volátiles sobre la selección de frutos por mamíferos y otros vertebrados frugívoros. Asimismo, dado que el consumo de cítricos por mamíferos y otros vertebrados frugívoros parece ser un fenómeno global (Tabla 2), son necesarios nuevos experimentos en distintas localidades y ecosistemas que permitan comprobar la consistencia espacial y temporal de las interesantes tendencias aquí descritas para la tríada cítricos-mamíferos-hongos.

Conviene recordar que las variedades de cítricos utilizadas, así como las condiciones bióticas y abióticas de nuestra parcela experimental, son

lógicamente diferentes a las ancestrales de los cítricos en el Sureste Asiático. Por ejemplo, las especies de frugívoros involucradas en nuestros experimentos no tienen una historia coevolutiva común con los cítricos. A pesar de dichas necesarias cautelas, hay una serie de evidencias que sugieren la generalidad de nuestras conclusiones. Por una parte, el D-limoneno es el compuesto predominante en la mayoría de los cítricos tanto cultivados (p.e. *Citrus clementina* [Dugo y Di Giacomo 2002], *Citrus limon* [Caccioni et al. 1998]) como silvestres (p.e. *Citrus ichangiensis* [Sawamura et al. 1999], *Citrus macroptera* [Rana y Blazquez 2012]) y parece ser el compuesto clave que media las interacciones entre cítricos y otros organismos (Rodríguez et al. 2013). Por otra parte, los resultados de nuestros experimentos muestran de forma inequívoca que los hongos facilitan el acceso a la pulpa a frugívoros tan distintos como conejos, roedores y diversas aves, por lo que es altamente probable que tenga un efecto similar, al menos, en varios de los frugívoros de áreas donde los cítricos son originales.

CONCLUSIÓN

Janzen (1977) afirmó que los vertebrados frugívoros prefieren los frutos sanos frente a los infectados por hongos y bacterias dado que los microbios producen compuestos tóxicos y reducen el valor nutritivo de los frutos infectados. Para evaluar dicha hipótesis realizamos una serie de experimentos de campo ofreciendo frutos cítricos infectados y sanos por *P. digitatum.* Nuestros resultados en un sistema agrícola contradicen a Janzen dado que los frugívoros (conejos, roedores, y aves) prefirieron consumir los frutos infectados por *P. digitatum*. Proponemos que la infección por *P. digitatum* puede transformar algunas sustancias de los frutos aumentando su atractivo. No obstante, más estudios

experimentales en otros ecosistemas y contexto ecológicos son ciertamente necesarios para poder evaluar la generalidad de nuestros resultados.

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CAPÍTULO II

FUNGAL INFESTATION BOOSTS FRUIT AROMA AND FRUIT REMOVAL BY MAMMALS AND BIRDS

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ABSTRACT

For four decades, an influential hypothesis has posited that competition for food resources between microbes and vertebrates selects for microbes to alter these resources in ways that make them unpalatable to

vertebrates. We chose an understudied cross kingdom interaction to experimentally evaluate the effect of fruit infection by fungi on both vertebrate (mammals and birds) fruit preferences and on ecologically relevant fruit traits (volatile compounds, toughness, etc). Our well-replicated field experiments revealed that, in contrast to previous studies, frugivorous mammals and birds consistently preferred infected over intact fruits. This was concordant with the higher level of attractive volatiles (esters, ethanol) in infected fruits. This investigation suggests that vertebrate frugivores, fleshy-fruited plants, and microbes form a tripartite interaction in which each part could interact positively with the other two (e.g. both orange seeds and fungal spores are likely dispersed by mammals). Such a mutualistic view of these complex interactions is opposed to the generalized idea of competition between frugivorous vertebrates and microorganisms. Thus, this research provides a new perspective on the widely accepted plant evolutionary dilemma to make fruits attractive to mutualistic frugivores while unattractive to presumed antagonistic microbes that constrain seed dispersal.

INTRODUCTION

Human activity creates numerous opportunities for the appearance of so-called 'novel interactions' (Parker and Gilbert 2004) between species that otherwise would not coexist. Novel interactions arising from biological invasions have been deeply investigated (Traveset and Richardson 2014) and pair-wise interactions between native and crop species have also received some attention (Ellstrand 2003, Schroth et al. 2004, Bhagwat et al. 2008). Surprisingly, novel cross-kingdom (e.g. plant-vertebrate-microbe) interactions remain largely understudied despite their pervasiveness and potential ecological, evolutionary, and economical relevance in natural and humanized ecosystems (Desprez-Loustau et al. 2007, Traveset and Richardson 2014). In particular, novel cross-kingdom interactions taking place in agro-ecosystems provide excellent logistical settings to investigate, through easily replicated field experiments, intriguing ecological and evolutionary questions.

Interactions between fruiting groves and frugivorous vertebrates are widespread worldwide (Fedriani et al. 2001, Rey 2011, Peris et al. 2015). Seeds of domestic species are often ingested by vertebrates and eventually released away from the mother plant (i.e. endozoochory), potentially leading to naturalization of such cultivated plants (Richardson et al. 2000). Interestingly, these bipartite interactions can be joined by microorganisms that feed on the pulp and seeds, thus potentially interfering with the domestic plant-seed disperser interaction. Janzen (1977) suggested that fruits infected by microbes are rarely eaten by vertebrates because microbes produce toxic compounds and reduce the nutritional value of infected fruit. Most empirical investigations of these cross-kingdom interactions (Buchholz and Levey 1990, Cipollini and Stiles 1993, Borowicz 1998) as well as recent theoretical evidence have supported Janzen's famous prediction (Ruxton et al. 2014), but see

(Sherratt et al. 2006). Nonetheless, there exist a handful of studies showing that frugivores prefer infected fruits (Dominy and Lucas 2004, Milton 2004, Asplund et al. 2016). Also, recent paleogenetic data suggest that during the middle Miocene (~16 MA ago), apes evolved the ability of ingesting fallen microbe-infected fruit (Carrigan 2015). Such disparate findings are potentially related to frugivore response to fruit infestation changing with vertebrate, plant, and/or pathogen species, as well as with the ecological context (Cipollini and Stiles 1993, Perea et al. 2013). Surprisingly, however, most available empirical evidence is restricted to interactions involving small fruits and small frugivorous birds (see Supplementary Table S1), and extensively replicated field experiments assessing the spatial and temporal consistency of frugivore responses to microbe infestation are lacking. Furthermore, whether different frugivore functional groups with contrasting foraging modes (e.g. seed disperses, pulp feeders, seed predators; Fedriani and Delibes 2011, 2013) respond similarly to microbe infestation is unknown. Though species involved in novel interactions do not share a common evolutionary history, these tripartite interactions could shed light on how plants solve the evolutionary dilemma of making their fruits attractive to seed dispersers while unattractive to antagonistic microbes (Buchholz and Levey 1990, Ruxton et al. 2014).

We also know very little concerning the mechanisms underlying vertebrate responses to microbe fruit infestation. In particular, plant volatile organic compounds (VOCs) are secondary metabolites that mediate the attraction of seed dispersers and the avoidance of seed predators (Lomáscolo et al. 2010, Rodríguez et al. 2013). VOCs emitted by fruits and altered by microorganism infestation are known in some domestic species (Vikram et al. 2004, Droby et al. 2008). Whether these microbe-induced VOCs changes together with other potential changes in

fruit physical and chemical proprieties (e.g. toughness, sweetness, pH) have a significant effect on subsequent fruit interactions with native vertebrate frugivores remains a puzzle (Asplund et al. 2016).

Frugivorous vertebrates, domestic *Citrus* trees, and microorganisms form widespread though understudied tripartite interactions (Droby et al. 2008, Stampella et al. 2014, Peris et al. 2015). In particular, *P. digitatum* Sacc. infects large fractions of harvested orange fruits worldwide (Droby et al. 2008). In this study, we chose the interaction among the sweet orange tree (*C. sinensis* L. Osb.), several mammalian and avian frugivores, and *P. digitatum* in tropical and Mediterranean groves to experimentally address the following four questions: *i*) Does infection by *Penicillium* alter orange fruit preferences by different vertebrate frugivores (mammals, birds)? If so, and given their contrasting frugivore faunas, *ii*) is such effect consistent between tropical and Mediterranean orange groves? Also, since different frugivore guilds (i.e. seed dispersers, pulp feeders, granivore rodents) often show contrasting foraging behaviors (Fedriani et al. 2012, 2013), *iii*) is the effect of fungal infestation consistent among frugivore guilds? Finally, *iv*) does *Penicillium* infestation alter physical and/or chemical orange fruit parameters (e.g. toughness, VOC profiles, acid/sugar and ethanol concentrations) and, if so, are such changes consistent with frugivore fruit preferences?

RESULTS

Effect of *Penicillium* infestation on fruit harvesting

Frugivore tracks and/or other signs such as feces were found by the fruit in all experimental orange trees both in the Mediterranean and the

tropical experimental groves. Whereas in the Mediterranean groves, we recorded visits by pulp feeders (mostly rabbits) and rodents, in the tropical groves, in addition to those two frugivore guilds, we also recorded frequent visits by seed dispersers (mostly wild boars).

Our mixed model indicated that, once the effects of random factors were controlled for, overall fruit harvesting was 8.2-fold higher in the tropical than in the Mediterranean groves ($F_{1, 2897} = 118.90$, $P < 0.0001$; Figure 7). Frugivores from both regions strongly preferred *Penicillium*-infected as compared with intact fruits ($F_{1, 2897} = 551.31$, $P < 0.0001$; Figure 7). Nevertheless, there was a significant interaction between *Penicillium* infestation and region ($F_{1, 2896} = 93.65$, $P < 0.0001$) indicating that the effect of infestation on fruit harvesting was stronger in the Mediterranean orange groves as compared with the tropical ones (Figure 7). Specifically, in the Mediterranean groves, harvesting percentage for *Penicillium*-infected fruit was 21.1-fold higher than for intact fruit (tests of slices, $F_{1, 2896} = 369.01$, $P < 0.0001$), whereas in Brazil fruit harvesting percentage for *Penicillium*-infected fruit was 2.6-fold higher than for intact fruit ($F_{1, 2897} = 187.42$, $P < 0.0001$). We undertook a second set of analyses to identify guild-specific effects of *Penicillium*-infestation on fruit harvesting in each region (Table 3). In the Mediterranean groves, there were strong overall significant differences between guilds in the percentages of fruit harvesting ($P< 0.0001$; Table 3), with pulp feeders harvesting 8.90-times more fruits than rodents (Figure 8A). Also, we found a strong significant *Penicillium* infestation effect on fruit harvesting, being 17.23-times higher for infected as compared to intact orange fruits (Table 3; Figure 8A). The non-significant interaction between infestation and guild (Table 3) indicated that the marked preference for infected fruits was consistent on sign and magnitude for both frugivore guilds (Figure 8A).

In the tropical groves, seed dispersers (6.70±0.88%) harvested significantly more fruits than both seed-eating rodents (1.09±0.27%) and pulp feeders (4.87±0.68%; Table 4). As in the Mediterranean groves, overall fruit harvesting was significantly (Table 3) higher for *Penicillium*-infected as compared with intact fruit (Figure 8B). Interestingly, however, there was a strongly significant interaction between guild and *Penicillium*-infestation (Table 3) indicating that the magnitude and/or direction of *Penicillium* infestation effect on fruit harvesting varied among frugivore guilds (Figure 8B). In particular, test of slices showed no significant *Penicillium* infestation effect on rodent fruit harvesting ($F_{1, 4215} = 1.10$, $P = 0.294$), whereas seed dispersers ($F_{1, 4215} = 38.32$, $P < 0.0001$) and, particularly, pulp feeders ($F_{1, 4215} = 60.54$, $P < 0.0001$) markedly selected infected fruit (Figure 8B). Overall, our results indicated a strong vertebrate frugivore preference for *Penicillium*-infected fruits. Furthermore, such fruit preference took place in frugivores as contrasting as wild boars, ring-tailed coatis, curl-crested jays and ruddy ground doves in the tropical groves, or European rabbits, field mice, blackbirds, and magpies in the Mediterranean groves.

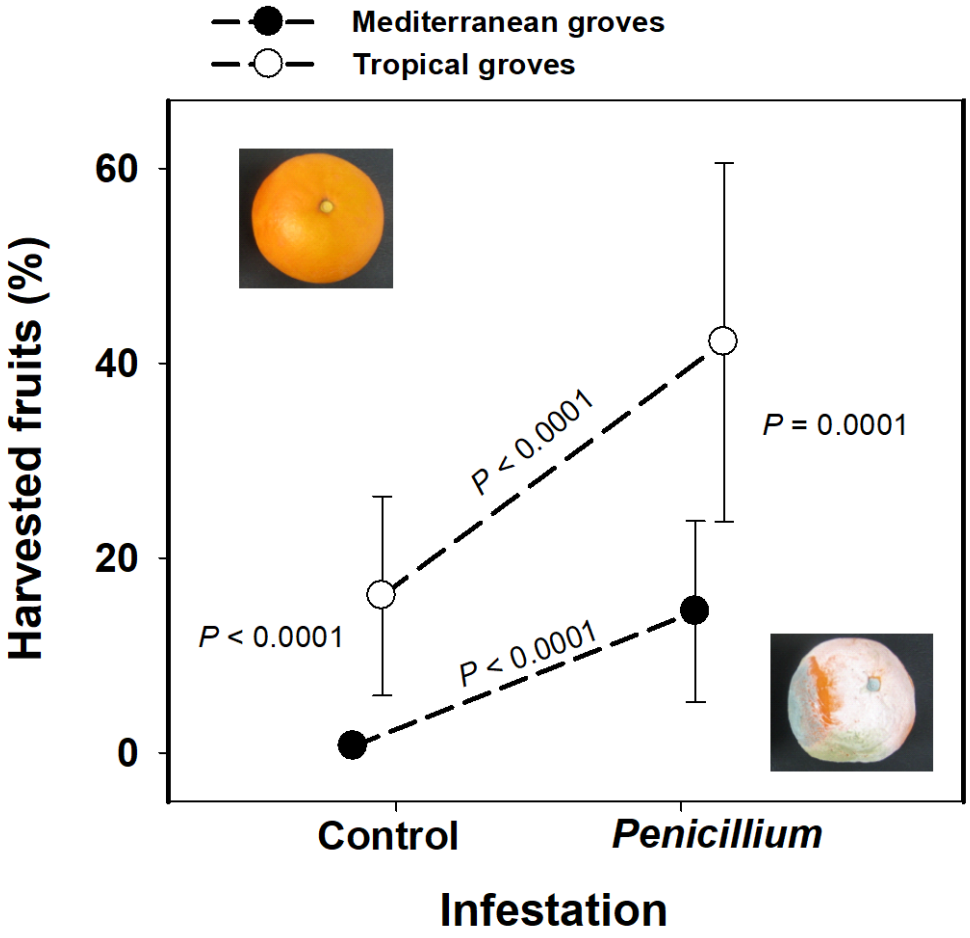

Figure 7. Harvesting by vertebrate frugivores (mammals and birds) of intact and *Penicillium*-infected oranges. Graphical representation of statistically significant interaction between *Penicillium* infestation (intact vs. *Penicillium*-infected) and region (tropical vs. Mediterranean) found for overall fruit harvesting by vertebrate frugivores of sweet orange (*C. sinensis*) fruit in our experimental Mediterranean and tropical groves. The *P*-values of the tests for the four simple main effects involved in the interaction are shown.

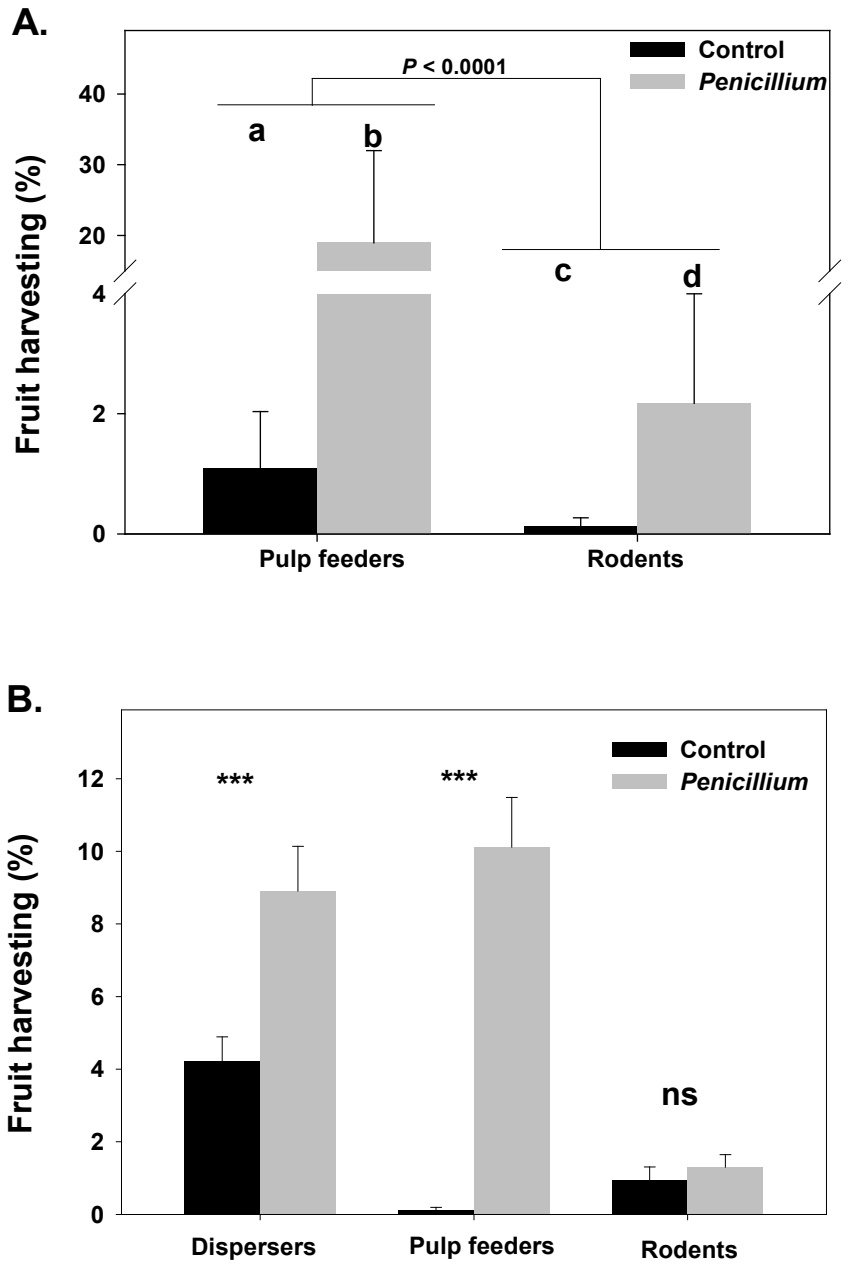

Figure 8. Fruit harvest by different frugivore guilds (i.e. seed dispersers, pulp feeders, granivore rodents). Model corrected mean percentages (±1SE) of sweet orange (*C. sinensis*) fruit harvest by different frugivore guilds as a function of *Penicillium* infestation in the Mediterranean (A) and tropical groves (B). Different lowercase letters among *Penicillium* infestation levels denote significant ($P< 0.05$) differences. ***, $P< 0.0001$; ns, not significant ($P> 0.05$).

Table 3. Results of main effect tests using generalized linear mixed models on the effects of *P. digitatum* infestation (P) and consumer guild (G), as well as their second-order interaction, on guild-specific percentages of fruit harvesting in Mediterranean and tropical sweet orange (*C. sinensis*) groves.

	Mediterranean groves			Tropical groves		
	F	d.f.	*P*	F	d.f.	*P*
Penicilliu m (P)	138.25	1, 3062	**<.0001**	63.64	1, 4215	**<.0001**
Guild (G)	80.30	1, 3062	**<.0001**	49.20	2, 4215	**<.0001**
P *G	0.29	1, 3062	0.561	32.51	2, 4215	**<.0001**

Effect of *Penicillium* infestation on VOC emission and on fruit physical and chemical traits

Our VOC analyses of intact and infected oranges identified 55 and 89 different compounds in intact and *Penicillium*-infected fruits, respectively (the entire list can be found as Supplementary Table S2). Both types of fruits differed significantly in mean percentages of volatile compounds, both for all compound groups considered individually (i.e. GLM univariate tests) and when all volatiles were treated simultaneously in a multivariate analysis of variance (MANOVA, $F_{5, 8} = 63.23$, $P < 0.0001$). For instance, percentage of esters in infected fruits was 5.7-fold higher

as compared with intact fruits (F_1 = 169.88, P<0.0001), whereas percentages of hydrocarbons was 1.3-fold higher in intact as compared to infected fruits (F_1 = 249.6, P<0.0001; Figure 7). The differences between fruit types in percentages of alcohols was small but significant (F_1 = 6.67, P< 0.05). In particular, the percentage of ethanol was 5.26-fold higher in infected as compared to intact fruits. The amount of unidentified volatile compounds in *Penicillium*-infected (7.02%) was similar to that in intact fruits (7.64%). However, when we repeated these analyses considering unidentified volatiles as a sixth compound group, both multivariate and univariate analyses yielded results very similar to those outlined above and, thus, are not detailed here.

We also found overall differences in toughness, pH, and ºBrix between intact and infected fruits ($F_{1, 36}$ = 131.55, P < 0.0001). Specifically, toughness of intact fruits (3.70 ± 0.12 kg) was 4.16 times higher than that of infected fruits (0.89±0.06 kg; F_1 = 412.90, P < 0.0001). The pH of intact fruits (2.47±0.03) was slightly, but significantly, lower than for infected fruits (2.67±0.04; F_1 = 17.14, P < 0.001). Finally, sweetness of intact fruits (15.48±0.25 ºBrix) was 12% higher than that of *Penicillium*-infected fruits (13.81±0.16 ºBrix; F_1 = 31.70, P < 0.0001).

DISCUSSION

We chose a novel interaction to experimentally evaluate the effect of fruit infestation by fungi on both fruit appeal to frugivores and vertebrate fruit preferences. Interestingly, and in contrast with most previous work, frugivores consistently preferred infected over intact fruits. Furthermore, the preference for infected fruits was correlated with the higher esters and ethanol levels, but lower sugar levels, in infected as compared to

intact fruits (Lomáscolo et al. 2010, Fedrinani et al. 2012). This investigation thus challenges the widely accepted idea of vertebrate avoidance of microbe-infected fruits and reveals a probable mechanism by which microbes facilitate the naturalization of non-native plant species.

Our experimental results show clearly that all three functional groups of frugivores (seed dispersers, pulp feeders, and rodents) preferred fungi-infected over uninfected orange fruits. Such fruit preference took place in very contrasting frugivores differing in many traits such as body sizes (e.g. wild boar *vs*. ruddy ground dove in the tropical groves) and feeding habits (e.g.rabbit *vs*. magpie in the Mediterranean groves). Preference for infected fruits was not significant for rodents in the tropical groves. However, our camera traps and field observations indicated that lowland pacas, a frequent rodent visitor, consumed *in situ* infected fruits whereas they usually carried to the jungle uninfected ones. This observation opens the possibility that some of these uninfected fruits were stored (Fedriani and Boulay 2003) till they become spoiled and their physical and chemical traits altered (see below). The pattern of preference for fungi-infected fruits also held true during the three studied fruiting seasons, supporting the robustness of our findings and suggesting that similar results could be expected for comparable systems.

After Janzen (1977), it has been often reported that frugivores prefer ripe, uninfected fruits over spoiled ones (Cipollini and Stiles 1993) (see Supplementary Table S1). Nevertheless, changes in fruit preference by frugivores can be expected to vary with vertebrate, plant, and microspecies (Cipollini and Stiles 1993). Regrettably, most studies reporting preference for intact fruits have relied on feeding trials with captive small frugivorous birds and plant species with small-sized fruits

(usually ≤1cm in diameter; Supplementary Table S1). Conversely, apart from the current study, very little is known about the effect of microbe infection on large or medium sized fruits. Fruit size might be consequential for frugivore preferences in relation to infestation due to several causes. Large fruit size allows the possibility of fungal infection altering just the outermost fruit parts (i.e. exocarp) and not the pulp, which may be infected in small-sized fruits. In orange fruits, and probably also in other relatively large fruits, *Penicillium* and other microbes thrive in the exocarp, leaving most of the inner pulp much less infected (authors *personal observation*). As we revealed, fungal infection softens fruit exocarps, there by facilitating foraging by some frugivores. We propose that research bias towards small frugivores birds and towards small-sized fruit with thin exocarps have often led to the wrong perception that microbe infection generally lessens vertebrate fruit consumption (Aplund et al. 2016). Further research on plant-vertebrate-microbe systems is undoubtedly needed. Also, though the infected fruit we offered in our field experiments, exhibited well-developed fungal growth, the question remains as to whether, as the fruit continues to rot, it will become less attractive to frugivores.

Frugivore preference for infected orange fruits opens the non-trivial question of what traits (nutritive, chemical, and physical), caused such pattern of fruit selection (Cipollini and Levey 1997a, Fedriani and Boulay 2006, Lomáscolo et al. 2010, Rodríguez et al. 2013), despite the lower sugar content of infected compared to control fruits. Many vertebrate frugivores are able to perceive and respond to some odours in the environment through odorant receptors, which are activated by sets of VOCs (Lomáscolo et al. 2010, Borges et al. 2011, Valenta et al. 2013). In particular, ethanol accumulation in fruits has been long identified as potential signal of high reward (Schaefer and Ruxton 2011) and, in our

study, infected orange fruit showed a five-fold increase of accumulated ethanol in the endocarp. Thus, high ethanol concentration of infected orange fruits may explain, in part, frugivore preferences in our tropical and Mediterranean groves (Dudley 2000, Milton 2004, Siegel 2005).

Other candidate traits are monoterpene hydrocarbons, which were emitted predominantly by intact as compared to infected fruit (69% *vs*. 54%); however, these compounds were partly transformed during *Penicillium* infestation to alcohols and esters. Interestingly, these two chemical classes were further transformed into ethyl esters, which went from representing only 2% of total VOCs in intact fruits to ~20% in infected ones (i.e. a 10-fold increase). Animals so diverse as humans, monkeys, rats and flies are known to perceive and respond positively to esters at very low odour thresholds (Laska and Seibt 2002, Keller and Vosshall 2007, Gómez-Marin et al. 2011). Our results thus suggest that esters (within a mixed VOC blend) were perceived as attractive cues by vertebrate frugivores in both tropical and Mediterranean experimental groves. Also the fungus secretes about 50 enzymes involved in plant cell wall degradation (Marcet-Houben et al. 2012), thus softening plant tissues, as indicated by toughness evaluations. This softening probably facilitated frugivore access to fruit pulp, chiefly in the case of small pulp-feeding birds and some rodents unable to penetrate the thick pericarp of intact orange fruits (Balcomb and Chapman 2003, Fedrinani and Delibes 2013, Grant and Grant 2014). Finally, we cannot rule out the possibility of frugivore self-medication with *Penicillium*-produced antibiotics, as it has been documented in other similar systems (Baker 1996).

Our study supports that vertebrate frugivores, fleshy-fruited plants, and microbes may form a mutualistic ecological triad (Herrera 1996). In orange fruits, monoterpene hydrocarbons are accumulated at very high

levels in oil glands of the exocarp, and these compounds are known to favour fruit colonization by *Penicillium* (Droby et al. 2008, Rodríguez et al. 2011). Spoiled fruit and fungus emit VOCs (e.g. esters) that attract frugivore vertebrates which prefer feeding on soft, alcohol -and sugar-rich fruits. Vertebrates frequently disperse viable orange seeds (e.g. wild boars; authors *unpublished data*) facilitating tree naturalization in tropical habitats (Stampella et al. 2014, authors *personal observation*). Furthermore, recent progress in fungal dispersal has revealed that passage through vertebrate guts can provide a mechanism of dispersal for fungi as well as seeds (Andras et al. 2013, Tesson et al. 2016). Therefore, *Penicillium* and other microbes infecting oranges and other large fruits are likely to benefit from spore dispersal by frugivores. New investigations disentangling the direct and indirect effects likely taking place in these complex multitrophic systems are crucial (Fedriani and Delibes 2013).

On the other hand, because fungal infection has tens fruit abscission, it has been generally assumed that it has a negative effect on seed dispersal (i.e. fallen fruits are equated to undispersed ones; Janzen 1977, Cipollini and Stiles 1993). This is probably the case for most bird-dispersed plants, since birds tend to forage on the canopy and not underneath fruiting trees. Conversely, tree species dispersed to some significant extent by non-arboreal mammals, such as lagomorphs, pigs, other ungulates, and carnivores require fruit dropping to archive seed dispersal, and thus fungal infection also enhances in this way tree dispersal success. Such a cooperative relationships in these cross kingdom interactions is opposed to the more generalized view of the competitive nature of interactions between frugivorous vertebrates and microorganisms (Cipollini and Stiles 1993, Tewksbury 2002, Ruxton et al.

2014) which certainly invites to study in detail other similar systems involving large-fruited plants and contrasting frugivores.

To conclude, we show that frugivores consistently preferred infected over intact orange fruits and that such pattern concords with a high level of attractive volatile compounds in infected fruits. We predict that plant species with large fruits and seeds and dispersed mostly by mammals with acute sense of smell are the most likely to experience enhanced consumption of infected fruits, aided by microbe-induced conspicuous aromas and softened peels. Though some of our target species lack a common evolutionary history, this investigation illustrates a way by which microbes can maintain mutualistic interactions with fleshy-fruited plants and, thus, questioning whether there really is a plant evolutionary dilemma of making their fruits attractive to some frugivores while unattractive to microbes (Buchholz and Levey 1990, Cipollini and Levey 1997a, Ruxton et al. 2014).

MATERIAL AND METHODS

Study species

The genus *Citrus* (Rutaceae) comprises several species whose origin is Asian. The orange tree (*C. sinensis* L. Osb.) is an evergreen, flowering tree, with an average height of 9 to 10 m. The fruit is a special type of berry named hesperidium, consisting of fleshy parts divided by segments, the whole being surrounded by a separable skin. This is composed of two major regions: the pericarp, commonly known as the peel, and the endocarp, often called the pulp. The pericarp is composed of external coloured peel known as flavedo (exocarp; rich in oil sacs containing volatile compounds), and the internal usually white and

spongy layer known as albedo (mesocarp). The inner flesh or pulp consists of segments surrounding the central axis of the fruit enclosed in a locular membrane in which seeds and juice sacs (vesicles formed by highly vacuolated cells containing juice) grow. The acidity of the juice of citrus fruits is largely due to high contents of citric acid, malic acid and fumaric acid, in order of abundance. *P. digitatum* Sacc. causes the most damaging postharvest disease of sweet orange fruits (Droby et al. 2008). Dormant *Penicillium* spores present on the fruit's surface germinate rapidly and colonize injured peel tissue before and during harvesting and processing.

Study sites

The study was conducted from June 2013 to May 2015 in two very distinct geographical regions, a Mediterranean in eastern Spain and a Tropical in southern Brazil. In both regions we used several experimental groves where frugivore activity was known. In Spain, we selected two Mediterranean sites within the Valencia province in the municipalities of Moncada and Sagunto. In Moncada, we used a 0.6 ha experimental field within the Instituto Valenciano de Investigaciones Agrarias (IVIA; latitude 39°35'N, longitude 0°23'W; 50m a.s.l.). The Sagunto field site is located near the Sierra Calderona Natural Park (latitude 39°42'N, longitude 0°15'W; 30 m a.s.l), within extensive orange monocultures. Within this site, we used two different orange groves (0.2 and 0.4 ha, respectively) 250 meters apart. The most common frugivore species in these two Mediterranean sites were rabbits (*Oryctolagus cuniculus* L.) and small birds that acted as pulp feeders (i.e. they consume the fruit pulp but without ingesting the seeds; Fedriani and Delibres 2013). Granivore rodents (e.g. *Mus spretus* L.) and rats (*Rattus rattus* L.) were also relatively frequent visitors. The tropical field site (called Cambuhy;

latitude 21°38’S, longitude 48°31’W, 600 m above sea level; 14,083 ha) is located next to a dry tropical forest in Matão, São Paulo, southern Brazil. Inside the farm there is a large (2,168 ha) semi-deciduous forest of dry Mata Atlantica called Mata da Virgínia. We selected two orange groves (8.1 and 11.5 ha, respectively) about 1.3 km apart of each other. The most frequent frugivore visitors were introduced wild boars (*Sus scrofa* L.) which together with the scarce ring-tailed coati (*Nasua nasua* L.) ingested whole fruits and delivered intact viable seeds (Authors *unpublished data*). Several species of small rodents were also frequent visitors and together with lowland pacas (*Cuniculus paca* Brisson) comprised the guild seed-eating rodents. Pulp feeding bird species such as curl-crested jay (*Cyanocorax cristatellus* Temminck), pale-breasted thrush (*Turdus leucomelas* Vieillot), and ruddy ground dove (*Columbina talpacoti* Temminck) frequently visited and picked the pulp of our experimental fruits. Further detail on the study sites and their frugivore assemblages are documented in Supplementary materials and methods. All field experiments were done under permission of IVIA, Fundecitrus, and all orange grove owners both in the tropical and the Mediterranean sites.

Field experimental design

To evaluate vertebrate frugivore preference in the field, intact and *Penicillium*-infected fruit types were simultaneously offered underneath orange trees simulating natural fruit drop on circular sand beds (1-meter of diameter; Fedriani and Delibes 2013). All frugivores (e.g. mammals, birds) had access to fruit since no exclusion system was implemented (Fedriani and Delibes 2013). Intact and infected fruits (2 or 3 per type) were alternately arranged, with ~10 cm spacing, beneath each experimental tree. In the experiments of Sagunto and Moncada, 15 fruit depots haphazardly distributed in the field during the months of April to June 2014 and June to December 2013, respectively, coinciding with the ripening seasons. In Brazil, fruits were offered in two parallel rows (10 fruit depots each). Within each row, fruit depots were 60 m apart. Fruit harvesting was recorded during seven and twelve consecutive days in July 2014 and May 2015, respectively. Every one or two days early in the morning the numbers of fruits either consumed *in situ* or removed were recorded and replaced by new fruits of the corresponding treatment. Frugivore identification was based on frugivore tracks on fine sand (Balcomb and Chapman 2003, Fedriani and Delibes 2009a, 2013,) and on the way fruits were manipulated and eaten by different frugivores. To confirm the origin of some animal traces and signs, some Bushnell Trophy Cameras with motion sensors were used in Brazil fields. The timing of our experiments was intended to coincide with the ripening of offered fruits.

To inoculate fruit with *P. digitatum*, we used fruits not sprayed with any insecticide and fungicide during at least the previous three months. Fully developed orange fruits were taken to the lab and then their surfaces were disinfected with 1-min immersion in a sodium hypochlorite solution

(4 gL^{-1}), rinsed with fresh water and left to air dry at room temperature. *P. digitatum* isolated NAV-7 was obtained from the culture collection of the Laboratory of Pathology, Postharvest Technology Center (IVIA, Moncada, Spain). Oranges were inoculated by wounding the rind with a stainless steel tip and introducing 2 µL of a known concentration of $1x10^6$ *P. digitatum* NAV-7 spores mL^{-1} in two opposite incisions in the equator of the fruit (Rodríguez et al. 2011). Inoculated fruit were placed on closed plastic bags and incubated at 20ºC and 80% relative humidity (RH). Infected fruits were used in the field experiment 7-11 days after inoculation, when the halo of the fungus covered the entire fruit surface (see Figure 7). A second set of fully developed oranges were used as controls and were treated as infected fruits except that were no wounded and inoculated with *P. digitatum*. We evaluated the possibility that the tiny wounds induce changes on VOC profiles and thus were partly responsible of possible differences between control and *Penicillium*-infected fruits. However, our results unmistakably showed no differences between wounded and unwounded (control) orange fruits for all volatile classes (see Supplementary Figure S1).

Fruit volatile emissions, physical and chemical traits

To assess how VOC profiles relate to frugivore fruit preferences, chemical analysis of volatiles emitted from intact and *Penicillium*-infected fruits were performed. For *Penicillium*-infected fruits, volatile analyses were conducted in days 7, 8, 9, 10, and 11 after infection, with two fruits per day. The volatile compounds were extracted by headspace solid-phase microextraction (HS-SPME) and analysed by GC-MS essentially as described in Rodríguez et al. (2011). Briefly, samples were introduced into glass beakers of 1 L volume (Labbox Labware) closed with foil. 10 µg of 2-octanol (Aldrich, purity ≥ 99.5%) was added as internal

standard. After 1 h of equilibration at room temperature, a 100 µm fiber coated with polydimethylsiloxane (PDMS, Supelco, USA), previously conditioned in the GC injector as indicated by the manufacturer, and was inserted into the glass and exposed for 40 min. The adsorbed volatiles were injected to a gas chromatograph-mass spectrometer (GC-MS) by desorption at 250ºC during 1 min in splitless mode in the injection port of a 6890N gas chromatograph (Agilent Technologies). Volatile compounds were separated on an Agilent JandW DB-5ms GC Column (60 m x 0.25 mm x 1.00 µm) coupled to a Termo-DSQ mass spectrometer. The GC interface and MS source temperatures were 260ºC and 230ºC, respectively. Oven programming conditions were 40ºC for 2 min, 5ºC/min ramp until 250ºC, and a final hold at 250ºC for 5 min. Helium was the carrier gas at 1.5 mL/min in the splitless mode. Data was recorded in a 5975B mass spectrometer (Agilent Technologies) in the 35-250 m/z range at 7 scans, with electronic impact ionization at 70 eV. Chromatograms were processed by means of the Enhanced ChemStation E.02.02 software (Agilent Technologies). Compounds in HS-SPME extractions were identified by matching the acquired mass spectra with those stored in the reference library (National Institute of Standards and Technology) and/or by comparison with authentic standard compounds when available. The relative emission rate of every compound in each sample was calculated as its corrected peak area (by fruit weight) divided by the recovery rate of the internal standard. The results are reported as the mean values of peak area percent ± standard error. Infected fruits showed comparable VOC profiles independently of the day of sampling after *Penicillium* infection and thus data were pooled. All identified volatile compounds were grouped into five main types (ethers, alcohols, ketones, hydrocarbons, and esters). The results in Figure 9 are reported as the mean values of peak area percent. For individual

volatiles, the results (Supplementary Table S2) are reported as the mean values of peak area ± standard error and the correspondent peak area percent. GC-MS was performed at the Metabolomics Service in Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universidad Politécnica de Valencia (Spain).

In addition, 20 intact and 20 *Penicillium*-infected orange fruits harvested from 10 trees (2 fruit per tree) were chosen to perform sugar/acid analyses of the juice of each fruit, according to Citrus Handbook (1998). Acidity was measured using a pH-meter (CRISON Basic 207) and sugar content using a refractometer (HANNA HI 96811, expressed in ºBRIX ± standard error). Fruit resistance to pressure was measured using a hand-held penetrometer (Penetrometer Fruit Pressure Tester FT011) and expressed in kg as the mean of the peak force at rupture ± standard error following Shmulevich et al. (2003). Two measurements were performed on each fruit with the penetrometer.

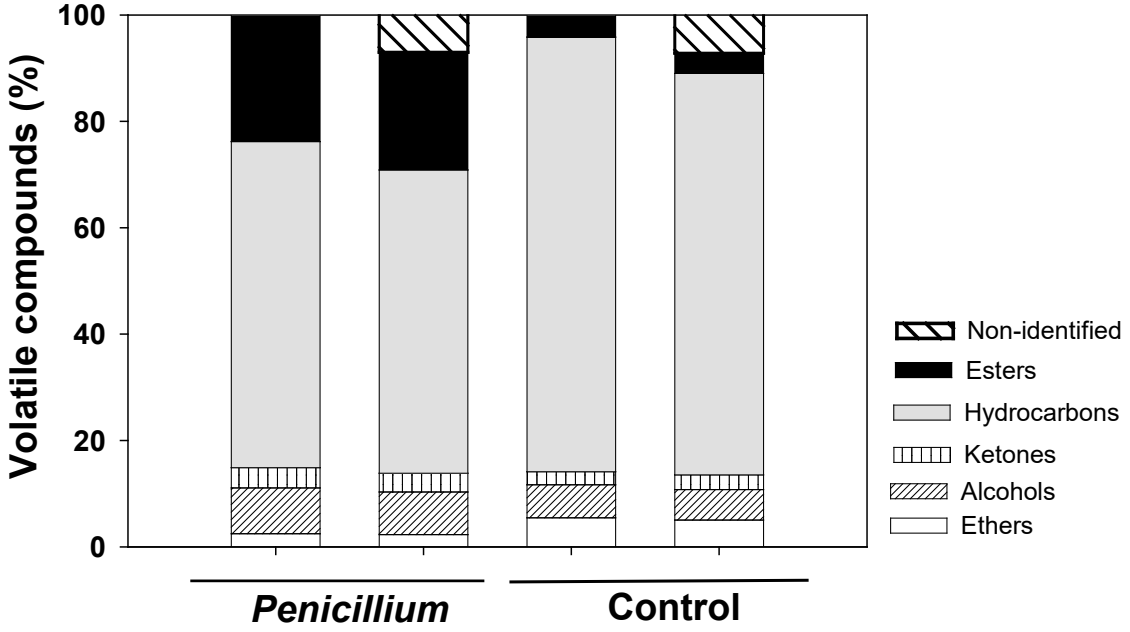

Figure 9. GCMS analysis of the volatile compounds emission in control and *Penicillium*-infected sweet orange (*C. sinensis*) fruits. Constituents are classified by chemical class: alcohols, esters, hydrocarbons, ketones, aldehydes, ethers and epoxides. For each treatment (control and *Penicillium*-infected) percentages are shown first without considering non-identified compounds and, then, considering them as an additional class.

Statistical analyses

The results were analysed by fitting generalized linear mixed models using the Proc Glimmix from SAS (2014), which allows the modelling of non-normal response variables as well as the usage of both fixed and random factors (Bolker et al. 2009). We first evaluated overall frugivore preference for infected *vs*. intact fruits. In this model, the percentage of fruit harvesting was the response variable, whereas fruit infestation and region (tropical, Mediterranean) were specified as fixed factors. Then, we

evaluated guild-specific fruit preferences by fitting a second model with frugivore guild (seed dispersers, seed predators, and pulp consumers) and fruit infestation (infected *vs*. intact fruits) as fixed factors. To evaluate the consistence of the effect of one factor at the different levels of other factors, in both models we also included second-order interactions among main effects. When the interaction between any two factors was significant, tests for the effect of a given factor at the different levels of the other factor (i.e. tests of slices) were performed using the SLICE option in the LSMEANS statement of the MIXED procedure (SAS 2014). Season (2013, 2014, and 2015) and block (nested within parcel) were included as random factors in both models. To compare the effects of different levels of any significant main factor, we calculated the difference between their least-square means. Because of the binomial nature of the response variables (percentage of fruit harvested), binomial error and logit link function were specified.

Identified VOCs emitted by orange fruits were classified into one of five main groups (ethers, alcohols, ketones, monoterpenes hydrocarbons, and esters). Multivariate analysis of variance (MANOVA) in GLM procedure (SAS 2014) were done to test overall differences in the percentages of the five main types of VOCs between *Penicillium*-infected and intact orange fruits. Once overall significant differences were detected, we applied univariate analyses for each group of VOCs. Differences in toughness, pH, and Brix degrees were also tested with multivariate and univariate analyses.

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Author contributions

LP and JMF conceived the study, JEP performed the field experiments, JEP and AR performed biochemical analyses, JMF analysed the data, LP JMF JEP wrote the first draft, and all authors contributed substantially to reviewing the manuscript.

Competing interests. The authors declare no competing financial interests.

Supplementary Table S1. Details concerning studies documenting vertebrate frugivore preference for intact or microbes-infected fruits. Note how most available studies concerning the effect of fruit infestation on frugivore preference have focussed on small birds and on small fruited plants. Also, note how previous studies have documented vertebrate preference of intact fruits as compared to microbe-infected fruits.

[1]*Alternaria, Aspergillus, Botrytis, Colletotrichum, Cladosporium, Fusarium, Geotrichum, Penicillium, Pestalotiopsis, Phoma, Rhizopus, Saccharomyces, Phomopsis*

Frugivore.	Frugivore Family	Frugivore Order	Frugivore length (cm)	Feeding on	Fruit Family	Type Fruit	Fruit length	Infestation organism	Fugivore fruit preference	Reference
Bombycilla cedrorum	*Bombycillidae*	*Passeriformes*	15-18	*Ilex opaca*	*Aquifoliaceae*	Small red drupe	6-12mm	Small darkened or fungal spot	Intact Fruits	Buchholz and Levey 1990
				Loniceramaackii maxim	*Caprofoliaceae*	Bright red to black berry	2-6mm			
				Prunus caroliniana	*Rosaceae*	Tiny black cherries	1cm			
				Phytolacca americana	*Phytolaceae*	Shiny dark purple berry	1cm			
				Cornus florida	*Cornaceae*	Cluster of two to ten separate drupe	10-15mm			
Dumetella carolinensis	*Mimidae*	*Passeriformes*	22-30	*Vaccinium corymbosum*	*Ericaceae*	Blue-black berry	0.7-1.5cm	14 fruit rot fungi[1]	Intact Fruits	Cipollini y Styles 1993
Catharus fuscescens	*Turdidae*	*Passeriformes*	16-18	*Vaccinium vacillans*	*Ericaceae*	Blue to shiny black berry	1.2cm			
Hylocichla mustelina	*Turdidae*	*Passeriformes*	19-21	*Gaylussacia frondosa*	*Ericaceae*	Blue, black or white drupe	1cm			
				Vaccinium macrocarpon	*Ericaceae*	Red berry	9-14mm			
				Arctostaphylo suva-ursi	*Ericaceae*	Red berry	7-10mm			
Turdus migratorius	*Turdidae*	*Passeriformes*	23-28	*Gaultheria procumbens*	*Ericaceae*	Dry red capsule	6-9mm			
Mimus polyglottos	*Mimidae*	*Passeriformes*	25	*Cornus amomum*	*Cornaceae*	Small blue drupe	0.6cm	Microbe-infested fruits	Intact Fruits	Borowicz 1988
Dumetella carolinensis	*Mimidae*	*Passeriformes*	22-30							
Zonotrichia albicollis	*Emberizidae*	*Passeriformes*	17							
Poecilehud sonicus	*Paridae*	*Passeriformes*	12.5-14.5	*Cornus canadiensis*	*Cornaceae*	Red drupes	5mm	Microbes	Intact Fruits	Burger 1987
Turdus migratorius	*Turdidae*	*Passeriformes*	23-28							
Poecilea tricapillus	*Paridae*	*Passeriformes*	12-15							
Bombycilla garrulus	*Bombycillidae*	*Passeriformes*	32-35.5	*Sorbus aucuparia*	*Rosaceae*	Small red pomes	2cm	Fermented fruits	Ethanol preference	Eriksson and Nummi 1983
Pyrrhula pyrrhula	*Fringillidae*	*Passeriformes*	15-17.5	*Crataegus monogyna*	*Rosaceae*	Oval dark red pome	1cm			
Dasyprocta punctata	*Dasyproctidae*	*Rodentia*	42-62	*Astrocaryum standleyanum*	*Arecaceae*	Ovoid dates	2.5-6cm	Fermented fruits	Ethanol preference	Dudley 2004

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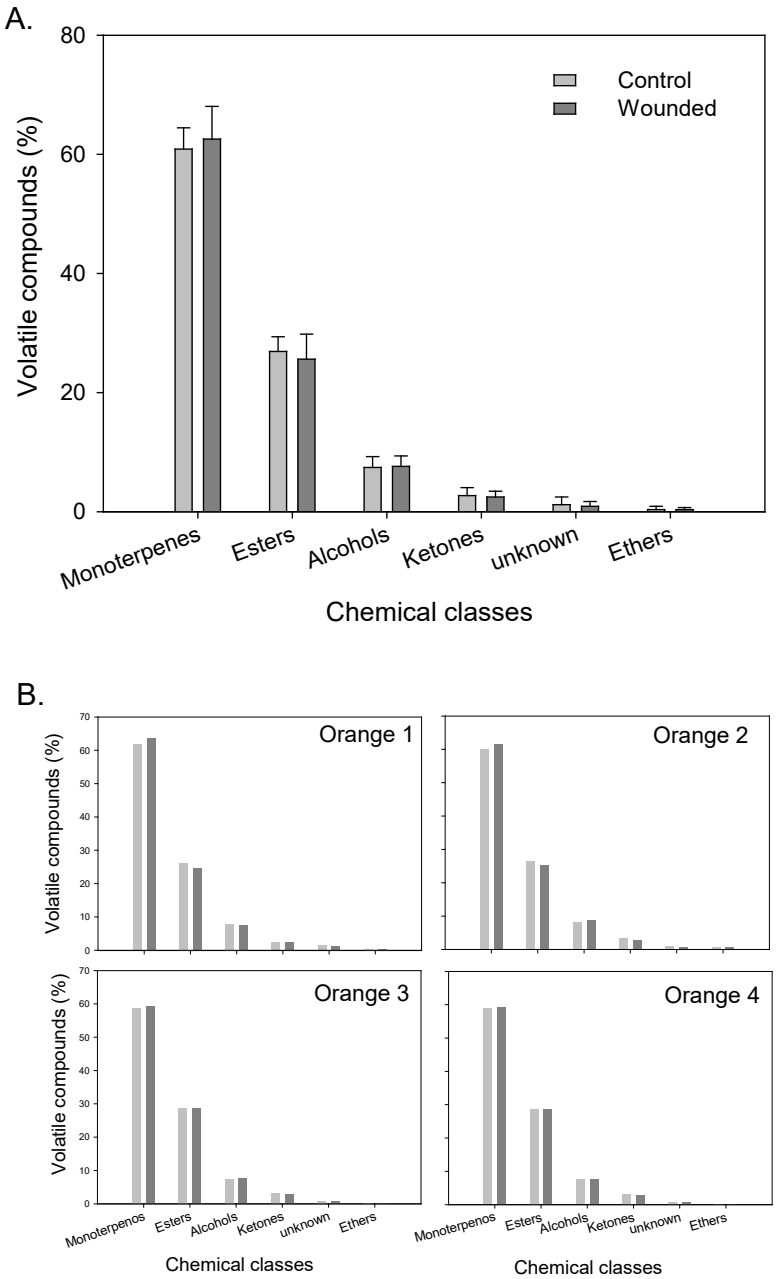
Supplementary Figure S1. GCMS analysis of the volatile compounds emission of four sweet orange (*C. sinensis*) fruits both before (i.e. control) and after being wounded (but not inoculated with *Penicillium*). These four fruits collected in March 2017 from four different trees. Though these fruits showed a somewhat atypical ripening phenology, they were valid samples to evaluate the potential effect of wounding on fruit VOC profile. Volatile compounds were classified as: alcohols, esters, hydrocarbons, ketones, aldehydes, ethers and epoxides. Note how for both values averages across all four orange samples (A) and for individual oranges (B) there were not noticeable differences for any volatile class.



Supplementary materials and methods.

Orange tree visitation by frugivores.

1. Methods

To evaluate vertebrate frugivore preference, intact and *Penicillium*-infected fruit types were simultaneously offered on circular sand beds (1-meter of diameter) underneath tree crowns simulating natural fruit drop (e.g. Fedriani and Delibes 2013). Frugivore identification was based on frugivore tracks on fine sand (e.g. Fedriani and Delibes 2013) and on the way fruits were manipulated and eaten by different frugivores. To confirm the origin of some animal traces and signs, some Busnhell Trophy Cameras with motion sensors were used in Brazil fields.

The field experiments were carried out in Mediterranean and tropical orange groves. In the Mediterranean Moncada site, we used a 0.6 ha experimental field within the Instituto Valenciano de Investigaciones Agrarias (IVIA; latitude 39°35'N, longitude 0°23'W; 50m a.s.l.). The most common frugivore species are rabbit (*Oryctolagus cuniculus* L.), black rat (*Rattus rattus* L.), mice (probably Algerian mouse *Mus spretus* Lataste and *Apodemus sylvaticus* L.), common blackbird (*Turdus merula* L.), Eurasian magpie (*Pica pica* L.), house sparrow (*Passer domesticus* L.) and white wagtail (*Motacilla alba* L.). The Sagunto Mediterranean site is located near the Sierra Calderona Natural Park (latitude 39°42'N, longitude 0°15'W; 30 m a.s.l), within extensive orange monocultures. The most common frugivore species in the area are rabbit, garden dormouse (*Eliomys quercinus* L.), black rat, Algerian mouse, house sparrow, common blackbird and European turtle dove (*Streptopelia turtur* L.; Gil-Delgado *et al.*, 2009).

The tropical field site (called Cambuhy; latitude 21°38'S, longitude 48°31'W, 600 m asl) is located next to a dry tropical forest in Matão, São Paulo, southern Brazil. This is a large (14.083 ha) farm of coffee (*Coffea arabica* L.), orange, corn (*Zea mays* L.) and rubber (*Hevea brasiliensis* (Willd. ex A, Juss.) Mull. Arg.). Inside the farm there is a large (2.168,32 ha) semi-deciduous forest of dry Mata Atlantica called Mata da Virgínia. We selected two orange groves (8.1 and 11.5 ha, respectively) adjacent to the forest and 1.3 Km apart of each other. The main local mammalian frugivores there are ring-tailed coati (*Nasua nasua* L.), wild boar (*Sus scrofa* L.), armadillo (*Dasypus novemcinctus* L., *Euphractus sexcinctus* L. and *Cabassous tatouay* L.), azara's agouti (*Dasyprocta azarae* Lichtenstein), tapeti (*Sylvilagus brasiliensis* L.), lowland paca (*Cuniculus paca* Brisson) and black capuchin monkey (*Cebus nigritus* Goldfuss). The most common frugivore birds are curl-crested jay (*Cyanocorax cristatellus* Temminck), pale-breasted thrush (*Turdus leucomelas* Vieillot), ruddy ground dove (*Columbina talpacoti* Temminck), grey-necked wood rail (*Aramides cajanea* L.), red-eye vireo (*Vireo olivaceus* L.) and rufous-collared sparrow (*Zonotrichia capensis* Muller). Wild boar comes from genetic crosses between European wild boar and domestic pigs (Giménez *et al.*, 2003).

The results concerning frugivore visitation were analyzed by fitting generalized linear mixed models using the Proc Glimmix from SAS (SAS Institute, 2014), which allows the modeling of non-normal response variables as well as the usage of both fixed and random factors (Bolket *et al.*, 2009). We modeled, for each sort of grove (i.e. Mediterranean, tropical) separately, the probability of frugivore visit as a function of consumer guild (seed dispersers, rodents, and pulp feeders). Because of the binomial nature of the response variables (probability of visit), binomial error and logit link function were specified (Bolket *et al.*, 2009).

2. Results on frugivore visitation

Frugivore tracks and/or other signs such as feces were found by the fruit in all experimental orange trees and on a large fraction of trials both in the tropical (43.85%; n = 707 night-trees) and Mediterranean (58.60%; n = 884 night-trees) experimental groves. Overall, we recorded 828 frugivore visits to target orange trees (minimum estimate, since occasionally more than one individual could be involved in a single visit). Each visitation was undertaken by one or two frugivore guilds (1.15 ± 0.02 and 1.14 ± 0.01 in the tropical and the Mediterranean groves, respectively). Whereas in the Mediterranean groves we recorded visits by pulp feeders and rodents, in Brazil, in addition to those two frugivore guilds, we also recorded frequent visits by seed dispersers (mostly introduced wild boars).

In the Mediterranean groves, the probability of visit strongly and significantly varied among frugivore guilds ($F_{2, 2097} = 53.97$, $P < 0.0001$). Specifically, the probability of visit by pulp feeders (0.65±0.16) was 5.9-fold higher as compared with that for seed-eating rodents (0.11± 0.08). In the tropical groves we also found overall significant differences among guilds in their probability of visit ($F_{2, 2097} = 53.75$, $P < 0.0001$), with pulp feeders being again the most frequent visitors (0.28±0.13), followed by seed dispersers (0.18±0.09), and then by seed-eating rodents (0.05± 0.03).

Supplementary Table S2. Volatile terpene compounds identified by GC-MS of *P. digitatum*-infected (A) and control (B) oranges grouped by chemical class, compound, relative percent area and their correspondent standard error.

A) Infected fruit volatile emission					
Chemical class	**Nº**	**Compound***	**% area**	**Mean area**	**Standard error**
Alcohols	**1**	Ethanol	0.13	68775114	30045196
	2	Isopentyl alcohol	0.09	48556170	29688983
	3	3-Penten-1-ol, 4-methyl	0.02	11179669	8851827
	4	1-Hexanol	0.08	41419066	12393808
	5	1-Heptanol	0.05	28046648	7815629
	6	2-octanol	1.28	698993310	349002760
	7	Eucalyptol	0.07	40533186	15241533
	8	1-Octanol	2.07	1132421270	305158804
	9	β-Linalool	1.29	704129765	208394232
	10	cis-β-Terpineol	0.07	40600870	18644233
	11	trans-*p*-Mentha-2, 8-dienol	0.23	124665695	73774734
	12	2-Cyclohexen-1-ol	0.06	32917460	20275412
	13	1-Nonanol	0.55	301495215	84344319
	14	α-Terpineol	1.26	687858439	200462900
	15	2-Oxabicyclol [2, 2, 2] octan-6-ol	0.71	388739208	75393939
	16	*n*-Tridecyl alcohol	0.07	35576587	13416933
	17	1-Butanol, 2-methyl	0.03	17111691	13801211
Esters	**18**	Acetic acid, methyl ester	0.07	38888783	7713610
	19	Acetic acid, ethyl ester	2.49	1362157384	272881095
	20	Propanoic acid, 2-oxo, ethyl ester	0.08	44953622	13187921
	21	Acetic acid, propyl ester	0.04	20071972	5059717
	22	Acetic acid, isobutyl ester	0.08	44397459	11772852
	23	Hexanoic acid, 3-methyl-2-butenyl ester	2.19	1195957102	385082615
	24	Acetic acid, 2-pentyl ester	0.00	1790451	1000092

	25	Acetic acid, isopentyl ester	0.23	125964727	42516352
	26	1-Butanol, 3-methyl, acetate	0.07	38280113	14694910
	27	Acetic acid, pentyl ester	0.07	38096257	9240312
	28	Acetic acid prenyl ester	0.04	22780388	6708573
	29	Acetic acid, hexyl ester	2.54	1385907728	230174101
	30	Acetic acid, heptyl ester	0.67	366754155	56990247
	31	2-Octanol, acetate	2.22	1210988156	156312252
	32	Octanoic acid, ethyl ester	0.12	65392475	12541945
	33	Acetic acid, octyl ester	9.22	5039849225	929405744
	34	Acetic acid, nonyl ester	1.33	724508221	131669190
	35	Acetic acid, decyl ester	0.24	129123836	27304243
Hydrocarbons	**36**	3-Thujene	0.01	4691267	1954280
	37	α-Pinene	0.22	117933393	28069804
	38	α-Phellandrene	0.78	424351286	162799871
	39	β-myrcene	0.03	17498472	6896961
	40	β-Pinene	0.03	15649064	6901425
	41	3-Carene	0.38	206091485	54357909
	42	Terpinolene	0.16	86242684	20274489
	43	D-limonene	45.09	24650082264	6005091974
	44	β-Phellandrene	0.77	420369538	129055675
	45	δ-Elemene	0.47	259556140	69880261
	46	β-Elemene	6.16	3367554055	734103156
	47	β-Caryophyllene	0.45	244204324	48206373
	48	β-Cubebene	0.06	33018265	11337883
	49	α-Caryophyllene	0.06	33006668	11532878
	50	Valencene	1.67	910260434	230739460
	51	α-Selinene	0.55	299244022	126664886
	52	α-Panansinsen	0.13	73558563	14024364
	53	Cyclohexene, 5, 6-diethenyl-1-methyl	0.41	222828236	126951112
Ketones	**54**	2-Octanone	3.41	1862238523	545997797
	55	*p*-Mentha-1, 8-dien-3-one (+)	0.12	64997105	13864282

Ethers and epoxides	**56**	Linalool oxide	0.94	516479536	178712749
	57	Limonene oxide (*Z*)	0.60	325896335	119717026
	58	Limonene epoxide	0.28	153538393	80225045
	59	Epoxylinalool	0.42	230952744	50481910
	60	Caryophyllene oxide	0.05	27077876	9050790
Non identified	**61**		0.05	26243299	11469879
	62		0.07	36649388	22616062
	63		0.15	83921314	18252507
	64		0.06	33858549	7770893
	65		0.21	113293842	75175554
	66		0.10	53953297	21419616
	67		0.46	252512914	61734942
	68		0.20	108852073	28418960
	69		0.24	131164082	20492624
	70		0.03	16692535	6953733
	71		0.05	25620158	4588383
	72		0.34	185653470	73542612
	73		2.51	1370580891	403618689
	74		0.02	9822100	4013921
	75		0.02	12421740	5789233
	76		0.76	415511459	84802331
	77		0.09	46924640	37258000
	78		0.12	63312455	21957553
	79		0.04	24572441	11639679
	80		0.03	17870747	6737993
	81		0.04	21640439	13323840
	82		0.09	49791874	18737342
	83		0.35	188648677	74855967
	84		0.11	59877165	18657619
	85		0.11	61706739	20431045
	86		0.15	80929946	33176421
	87		0.33	177884942	38981940
	88		0.19	104915805	29437529
	89		0.12	65240495	30563167

B) Control (healthy) fruit volatile emission					
Chemical class	**Nº**	**Compound***	**% area**	**Mean area**	**Standard error**
Alcohols	**1**	Ethanol	0.02	12176283	5262407
	2	2-octanol	4.38	2230080660	284887823
	3	3-Hexen-1-ol	1.00	508873323	137059267
	4	β-Linalool	0.27	139936827	40323886
	5	α-Terpineol	0.07	37114326	9229008
Esters	**6**	Pentanoic acid, ethyl ester	0.02	11681879	4844224
	7	Acetic acid, hexyl ester	0.50	253431893	46017774
	8	Butanoic acid, ethyl ester	0.12	58800990	22790163
	9	Octanoic acid, ethyl ester	1.27	646075995	198336980
	10	Nonanoic acid, ethyl ester	0.04	19314823	7849216
	11	Butanoic acid, 2-octyl ester	0.09	45473008	14722265
	12	9-Octadecynoic acid, metyl ester	1.73	880230166	231255992
	13	5-methyl-hexanoic acid ethyl ester	0.06	31783913	8978856
Hydrocarbons	**14**	α-Pinene	0.04	20944566	8779958
	15	α-Phellandrene	0.11	54450431	9216313
	16	β-Myrcene	1.34	681887061	166084502
	17	3-Carene	0.07	34645597	13962043
	18	D-Limonene	5.47	2788835078	464274942
	19	Terpinolene	0.05	26988462	11278907
	20	Eremophilene	0.32	165235657	25344936
	21	β-Elemene	3.70	1883429265	435857914
	22	Isocaryophyllene	0.32	161903012	60344311
	23	β-Caryophyllene	24.19	12323401625	1661686759
	24	β-Cubebene	0.55	279810006	72267589
	25	α-Caryophyllene	2.23	1133531350	204471777

	26	Selinene	0.37	187568444	34005476
	27	Azulene	1.08	547820288	104001617
	28	Valencene	29.55	1505350516 9	1814072266
	29	α-Selinene	2.66	1352453010	322428678
	30	(-)-α-Panasinsen	3.43	1748366811	364050539
Ketones	**31**	2-Octanone	2.27	1158851291	173987756
Ethers and epoxides	**32**	Limonene epoxide	0.36	185697615	48580452
	33	Epoxylinalool	0.11	54952923	23567410
	34	Diepicedrene-1-oxide	0.05	27607001	7662008
	35	Caryophyllene oxide	0.64	323817924	65984395
	36	Calarene epoxide	0.03	13651599	4933129
	37	Caryophyllene epoxide	0.06	32994378	9335181
	38	α-Cedrene epoxide	0.09	43939556	8502018
Aldehydes	**39**	Cyclohexane	3.46	1761760013	508002713
	40	β-Cyclocitral	0.20	103953892	40677234
	41	Longifolene aldehyde	0.03	16644849	4413986
Non identified	**42**		0.06	32712519	7131162
	43		0.02	8782552	3348264
	44		0.05	25475204	8341801
	45		0.11	58321839	11494879
	46		0.04	19342209	5996364
	47		0.02	9683733	4594622
	48		2.04	1040266405	202277652
	49		0.07	36781243	7847909
	50		4.25	2165777524	497072767
	51		0.82	415735295	164754063
	52		0.04	18601033	7881858
	53		0.03	13807958	6201223
	54		0.02	8213705	3681012
	55		0.08	41816551	12777436

* The chemical structure of the compounds can be found in Knudsen et al. 2006.

CAPÍTULO III

A FUNGUS MANIPULATES THE INTERACTION OF A FLESHY FRUIT WITH VERTEBRATE FRUGIVORES BY TRANSFORMING A DETERRENT COMPOUND INTO AN ATTRACTANT VOLATILE

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En preparación

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Abstract

The importance of fruit volatile organic compounds (VOCs) as a signal for either attraction or repellence of frugivore animals has been investigated but without determining the actual role of any specific metabolite in such interactions. Moreover, the participation of VOCs in their frequent interplay with specialized microorganisms infecting fruits remains largely unexplored. D-limonene is the predominant VOC from the peel oil glands of citrus fruits. Using genetically modified orange fruit with blocked D-limonene production (LSAS) and its D-limonene-rich control counterpart (C) for frugivore offerings in the fields, we show that the main function of this monoterpene is preventing fruit consumption by animal generalists. When LSAS and C fruit was offered intact and also infected by the specialized fungus *P. digitatum*, mammal frugivores preferred to consume infected LSAS fruit. Intriguingly, VOC analysis revealed that D-limonene emission was increased 16.3 times in LSAS fruit. Dissection of *P. digitatum* genome and emitted VOCs allowed us to determine that the fungus has a functional monoterpene synthase that uses the orange peel for VOC production, including D-limonene. Overall, this indicates that the D-limonene deterrent in intact fruit is not only exploited but also produced by the fungus in infected fruit as a VOC to attract mammals, likely to favor fungal dispersal but it may also have evolved to benefit fruit consumption as well as seed dispersal.

INTRODUCTION

Many vertebrate-dispersed plants have evolved edible seed coverings or appendages to attract frugivores. Frugivorous vertebrates frequently ingest the package made up of seeds plus the rewarding flesh, transport

the seeds internally (i.e., endozoochory), and often release them away from the parental environment, where survival may be higher. A much less studied interaction in this frugivorous interaction are the microorganisms, which are the most abundant frugivores of fleshy fruits. Fungi naturally infect a wide range of wild fruits and softening during ripening facilitates the establishment of opportunistic microbial infections. Hundreds of VOCs accumulate in fruits and, although the effect of VOCs has been studied extensively, interactions of VOCs with microorganisms in an ecological context have rarely been investigated.

The exocarp is the first line of contact of fruits with both frugivores and the surrounding environment (Eigenbrode and Espelie 1995), playing important roles in protecting the fruits and seeds against biotic (restricting microbial and pest infections) and abiotic (preventing desiccation, resistance to UV irradiation, etc.) stresses. The exocarp is often fundamental in maintaining fruit palatability and thus promoting vertebrate seed dispersal (Kerstiens 1996, Martin and Rose 2014). The external cuticle of fleshy fruits from plant species may vary substantially but mainly comprises waxes and also volatile organic compounds (VOCs), predominantly terpenoids, and fatty acid derivatives such as aldehydes, carboxylic acids, alcohols, and aliphatic esters (Martin and Rose 2014, Nevo et al. 2018). Fruit color is provided by either chlorophylls, carotenoids, anthocyanins or betalains (Chen 2015).

Such visual and volatile cues from the peel are detected by frugivores and determine the efficiency of fruits in attracting animals (Rodríguez et al. 2013, Nevo et al. 2016, Valenta et al. 2017). Then, once the fruit is located, texture, size, and morphology, together with flesh chemical characteristics determine fruit palatability or deterrence (Cipollini and Levey 1997a). Although there are recent reports showing the importance

of fruit aromas on selection by animals (Lomáscolo and Schaefer 2010, Nevo et al. 2018), the proof that a specific VOC attracts or deters frugivores has not been verified in the field. A unique approach to determine how a particular compound functions (e.g. repellent *vs*. attractant) would be measuring the performance of fleshy fruits altered genetically in the production of such a compound (and its derivatives) when exposed to frugivores and compare it to the performance of control fruits. Sweet orange (*C. sinensis* L. Osb.) represents an excellent model to perform such studies because it produces a monoterpene compound, D-limonene, which represents more than 97% of VOCs accumulated in oil glands from fruit peel. Although orange is not a species but a thousand-year old hybrid between *C. grandis* and *C. reticulata* ancient species, these two species also contain D-limonene as the most predominant compound in their fruit peels (95.6 and 91.4%, respectively; Dugo and Di Giacomo 2002), indicating that high D-limonene content in the peel has been conserved in with citrus fruits.

We have transformed sweet orange plants with a D-limonene synthase gene in antisense configuration (LSAS) to obtain fruits that accumulate up to 85-fold less D-limonene in fruit peels than empty vector (EV) or untransformed orange fruits (controls). As a consequence of this huge metabolic modification, specific derived monoterpene alcohols (i.e. β-citronellol, nerol and geraniol) and esters (i.e. Geranyl acetate) increased their contents in LSAS fruit peels (more than 10 and 3-fold, respectively) and aldehydes (i.e. *Z*-citral) decreased (more than 5-fold) (Rodríguez et al. 2011a). LSAS fruits were more resistant than EV and non-modified counterparts to specialist pathogens, demonstrating the importance of D-limonene for monophagous microorganisms to establish efficient compatible interactions with their fruit hosts (Rodríguez et al. 2013).

Worldwide, where sweet orange trees are widely grown (Davies and Albrigo 1999), overmatured (wounded) fruits as well as those abscised below the trees are usually infected by the specialist fungus *P. digitatum,* causing fruit decay (Palou 2014). The fact that this fungus exhibits a high degree of host specificity and has not been described naturally occurring and complet in gits biological cycle in other pathosystems (Frisvad and Samson 2004), indicates ancestral associations of these fruits with *P. digitatum* (Marcet-Houben et al. 2012). We have previously shown in different agroecosystems that vertebrate frugivores prefer *Penicillium*-infected over intact orange fruit, suggesting that changes in emission of attractive VOCs and texture induced by the fungal infection were responsible of such choices (Peris et al. 2017). However, the actual role of *P. digitatum* in altering volatile profiles in oranges as well as in general how microorganism alters fleshy fruits chemistry to modulate animal preferences in other tritrophic cross-kingdom interactions is largely unknown.

Here, orange fruits from plants differing just in a single gene controlling the expression and accumulation of the most abundant compound of their peels offered an excellent experimental system to test first whether D-limonene in fruit peels is actually a deterrent or an attractant compound to animal frugivores in the field. Then, LSAS *vs*. control fruits were used to investigate the perception of D-limonene by *P. digitatum* and how their interactions in both fruit genotypes affect frugivores choices. Third, we investigated whether preferred VOC profiles in infected fruits were manipulated by the fungus to facilitate its dispersal or VOC transformations just occurred as a chemical consequence of the pathogenic infection (Ariza et al. 2002, Ben-Yehoshua et al. 2008). Results from these experiments may have implications for our current understanding of how fleshy fruit/frugivores/phytopathogen interactions

have evolved with each element of the triad playing an active role in the outcome of beneficial interactions for all partners.

RESULTS AND DISCUSSION

D-limonene in fruit peel is deterrent for generalist frugivores

Control and LSAS sweet orange fruits from three different genetically modified (GM) events (AS3, AS5 and AS7, Rodríguez et al. 2011) were offered to frugivores at two experimental locations, Moncada and Villareal. Frugivore tracks and feces were often found in most orange fruit piles. Rabbits were by far the most frequent frugivore mammal visitors, with visitation frequencies of 96.7% and 72.4% in Moncada and Villarreal orchards, respectively. Birds were also frequent in Moncada (80.0%) but seldom visited fruit piles in Villarreal (0.95%). Rodents occurred in Moncada (16.7%) but were not observed in Villarreal. Results from our mixed model for data from both orange orchards indicated that, once the effect of random factors was controlled for, overall fruit harvesting was 12-fold higher for the LSAS lines (11.48 ± 7.51%) as compared with the control lines (0.96 ± 0.77%; $F_{1,\ 189} = 44.73$, $P < 0.0001$) in the first season of evaluation (Figures 10A and 10B). To identify potential differences between LSAS lines (AS3, AS5, AS7), in a second analysis we used data from three other seasons collected from Villarreal and Moncada orchards. Intact LSAS lines showed harvesting percentages between 7 to 22-fold higher as compared to intact control fruit. The differences between LSAS and control lines were highly significant ($F_{3,\ 75} = 19.63$, $P < 0.0001$).

To identify other potential orange fruit consumers, we also evaluated fruit harvesting by invertebrates (i.e. snails). Snails consumed 13.5 ± 1.1%, 16.6 ± 5.2% and 15.2 ± 4.1% of the AS3, AS5 and AS7 fruits, respectively, whereas only 2.6 ± 0.4 % of the control fruit was consumed in Villarreal in the first season (Figure 12C). Additionally, total peel area consumed by snails was higher in LSAS than in control fruits

(Figure 12D and 12E). Similar results were observed in the other seasons (Supplementary Figure S2).

When VOC content was evaluated, fruit genotype had significant effect as a main factor on content percentages of all chemical groups except for alcohols (Supplementary Table S3) showing also significant differences when all VOCs were analyzed simultaneously (MANOVA, $F_{6, 11} = 173.9$, $P < 0.0001$). However, the principal change in VOC content was the amount of D-limonene accumulated in control fruit that was on average 75.0-fold higher than in LSAS fruits (Figure 12F, Supplementary Table S4). Minor changes were observed for most of the other chemical groups. For example, control fruits had 4.4- and 5.6-fold more aldehydes and hydrocarbons (other than D-limonene), respectively, compared to LSAS fruit, while the esters content was 4.8-fold higher in LSAS than in control fruits (Supplementary Table S4). All these changes are likely consequences of the drastic reduction of D-limonene accumulation in LSAS fruit. Similar VOC profiles were observed for the other seasons (Supplementary Tables S3 and S4).

When D-limonene content was reduced as much as 75-fold in LSAS fruit peels, all vertebrate and invertebrate generalist consumers preferred eating them, envisaging its role as a deterrent compound of generalist frugivores. Thus, the role of D-limonene contained in the oil glands from oranges peels is consistent with the primary function proposed for secondary metabolites (and particularly terpenes) in ripe fruits, which is defense against potential consumers (Cipollini and Levey 1997b, Phillips and Croteau 1999, Mack 2000). To our knowledge, these results are the first genetic evidence of the function of a specific highly abundant terpene as a fleshy fruit deterrent demonstrated under field conditions

using isogenic lines that only differ in the production of this compound and related metabolites.

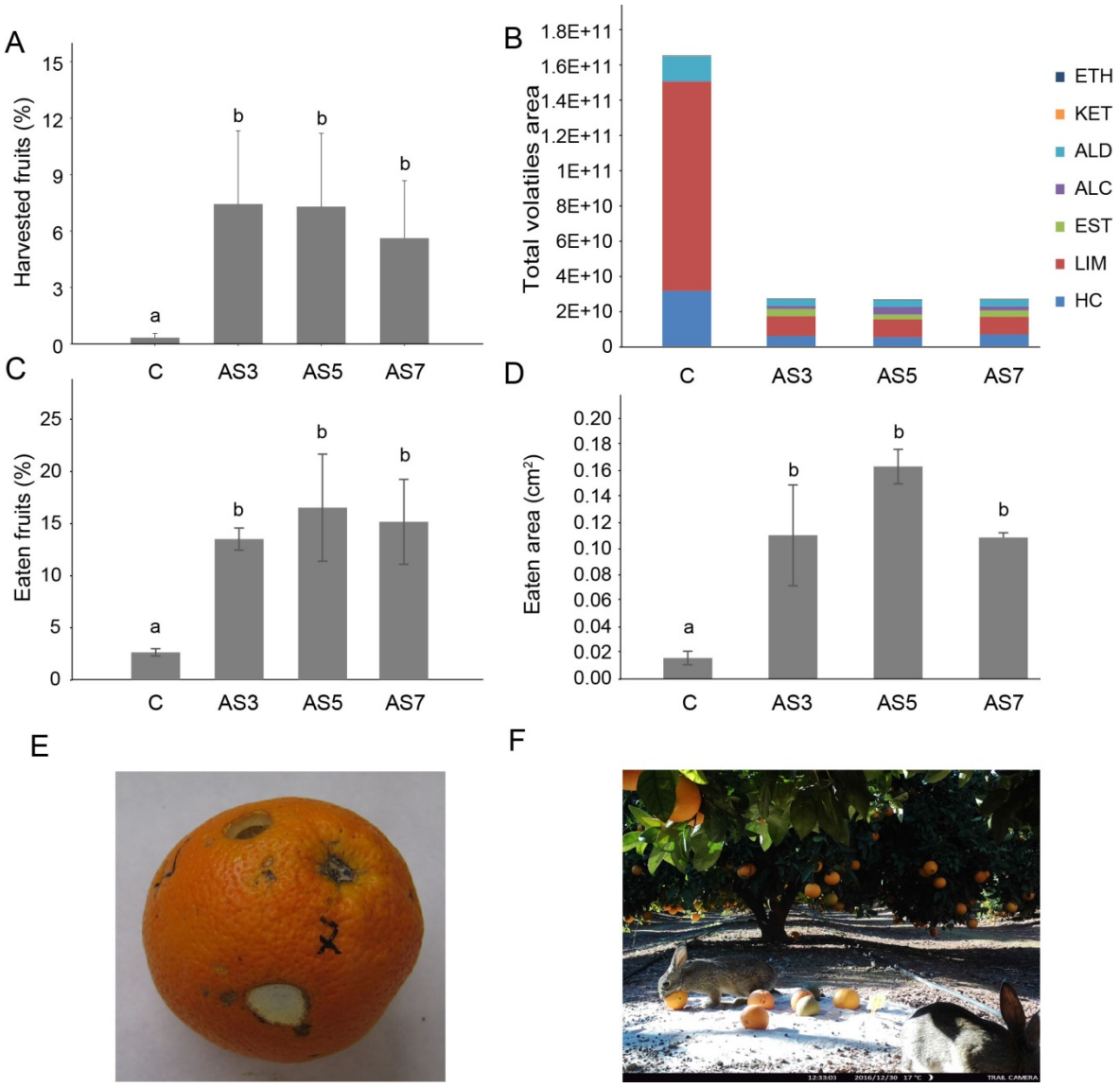

Figure 10. Fruit consumption by frugivorous of intact LSAS (AS3, AS5 and AS7) and control (C) oranges. A) Percentage of consumption of intact LSAS and control oranges by specialized frugivores. B) Total volatile content area of intact LSAS and control oranges. C and D) Percentage of fruits (C) and total area (D) eaten by opportunistic frugivores: snails. E) AS7 line with the peel injured by snails. F) A rabbit eating an intact orange of the AS5 line in a sandbox. LIM: limonene, KET: ketones, HC: hydrocarbons, ETH: ethers, EST: esters, ALD: aldehydes, ALC: alcohols.

VOCs are the main factor explaining frugivores preference for *Penicillium*-infected LSAS fruits over *Penicillium*-infected control ones.

On the other side, when control and LSAS fruit were infected or not by *P. digitatum* and offered together with non-infected LSAS and control fruits, frugivores clearly preferred *P. digitatum*-infected (7.33 ± 5.15%) over intact fruits (1.56 ± 1.22%; $F_{1, 189}$ = 17.79, P < 0.0001; Figure 11A). This is consistent with our previous report showing frugivores preference of *Penicillium*-infected over intact orange fruit (Peris et al. 2017). Interestingly, there was a significant interaction between genotype and *P. digitatum* infection ($F_{1, 189}$ = 5.49, P < 0.05) indicating that the effect of infection on fruit harvesting was not consistent between LSAS and control lines. Specifically, whereas for the control fruit *P. digitatum*-infection did not have a significant effect on harvesting percentage because all treatments were offered together (tests of slices, $F_{1, 189}$ = 0.99, P = 0.320), for LSAS lines harvesting percentage was 22.8-fold higher for *P. digitatum*-infected than for intact fruit ($F_{1, 189}$ = 95.22, P < 0.0001; Figure 11A, Video S1). This large difference in harvesting percentages, led us to identify potential differences in physicochemical properties of the fruits such as toughness and VOCs contents/emissions from LSAS and control fruits.

We also found overall differences among treatments in physical properties. Specifically, toughness of intact fruits (3.16 ± 0.05 kg) was 12.1 times higher than that of infected fruits (0.26±0.06 kg; $F_{1,155}$ = 1338.14, P < 0.0001). However, there were not differences in toughness between control (1.73 ± 0.07 kg) and LSAS fruits (1.69 ± 0.04 kg; $F_{1,155}$ = 0.16, P = 0.693), and the interaction between *Penicillium*-infection and fruit genotype was not significant ($F_{1,155}$ = 2.70, P = 0.103) (Figure

13B). These results are in agreement with our previous report showing that *Penicillium*-infected soft fruits are generally chosen by frugivores (Peris et al. 2017), but did not explain why *Penicillium*-infected LSAS fruits were preferred over *Penicillium*-infected control ones.

When we compared control and LSAS oranges using the VOCs emission dataset, fruit genotype had significant effect as the main factor on percentages of alcohols, esters, and D-limonene when considered individually and also when all VOCs were considered simultaneously in a multivariate analysis of variance (MANOVA, $F_{6, 45} = 17.46$, $P<0.0001$) (Supplementary Tables S3 and S4). Nonetheless, there was a significant interaction between *Penicillium*-infection and fruit genotype for all compound groups considered individually (Supplementary Table S3) and when all VOCs were considered simultaneously (MANOVA, $F_{7, 45} = 6.01$, $P<0.0001$), indicating that the effect of *Penicillium*-infection was inconsistent between both fruit genotypes. For example, *Penicillium*-infection led to a marked increase (13.3-fold) of alcohols in control fruits whereas such increase was much less marked (3.4-fold) in LSAS fruits while the surge in D-limonene was less marked in control fruits (3.5-fold) than in LSAS fruits (16.3-fold) (Figure 13C). Results from univariate and multivariate analyses using data on emission from a second season confirmed largely these patterns (Supplementary Tables S3 and S4 and Supplementary Figure S3).

Overall, content of VOCs was less variable than emission in relation to fruit genotype and *Penicillium*-infection. Nonetheless, the first season content dataset indicated that fruit genotype had significant effect as the main factor on content percentages of almost all chemical groups (Supplementary Table S3) showing significant differences when all VOCs were analyzed simultaneously (MANOVA, $F_{6, 11} = 173.9$, $P<0.0001$). The

strong reduction in D-limonene content (Figure 11D) together with the surge in emission of specific VOCs (mainly D-limonene) would explain the preference of frugivore mammals for *Penicillium*-infected LSAS fruit compared to the *Penicillium*-infected control counterpart.

We have shown previously that the presence of D-limonene in orange fruit peels facilitates *P. digitatum* germination, growth and colonization when the concentration of D-limonene was high in intact orange fruit peels (Rodríguez et al. 2011a). Below toxic concentrations, microorganisms have the ability to transform monoterpenes to other VOCs involving the introduction of functional groups, oxidation reactions, and molecular rearrangements catalyzed by various enzymes (Marmulla and Harder 2014). In a detailed analysis of the infected orange fruit VOC emissions, we observed that, as *P. digitatum* infection progressed in fruit peels, D-limonene increased from 14% before infection to 34% of total emitted VOCs 9 days after infection (Supplementary Table S4). Moreover, *P. digitatum* transformed other mono- and sesquiterpene hydrocarbons which decreased from 66% to 22% of the total VOCs 9 days after infection, mainly into alcohols and esters. Alcohols increased in percentages of emissions from 2% before infection to 29% 6 days after infection, and esters increased from 1% before infection to 17% 9 days after infection (Supplementary Table S4). Coincidently, VOCs from alcohol and ester chemical groups are generally characteristic of mature fleshy fruits (Rodriguez et al. 2013). These VOCs are recognized by different mammal frugivores (Laska 1990, Laska and Seibt 2002), and likely used to locate ripe fruits suggesting that such changes in VOC profiles may be acting as an attractant. Moreover, D-limonene over-accumulation in orange fruits after fungal infection may derive in part from monoterpene hydrocarbon transformations (Larsen and Frisvad 1995, Nilsson et al. 1996, Stoppacher et al. 2010) as D-limonene

emission increased in *Penicillium*-infected over intact control fruit (Supplementary Table S4). However, in the case of LSAS fruit, this hypothesis is unlikely because silencing of D-limonene synthase activity would preclude the surge in emission of this compound from infected fruit peels barely producing it. We then wondered whether *P. digitatum* would contain a monoterpene synthase able to produce D-limonene at high levels using orange peels as substrate.

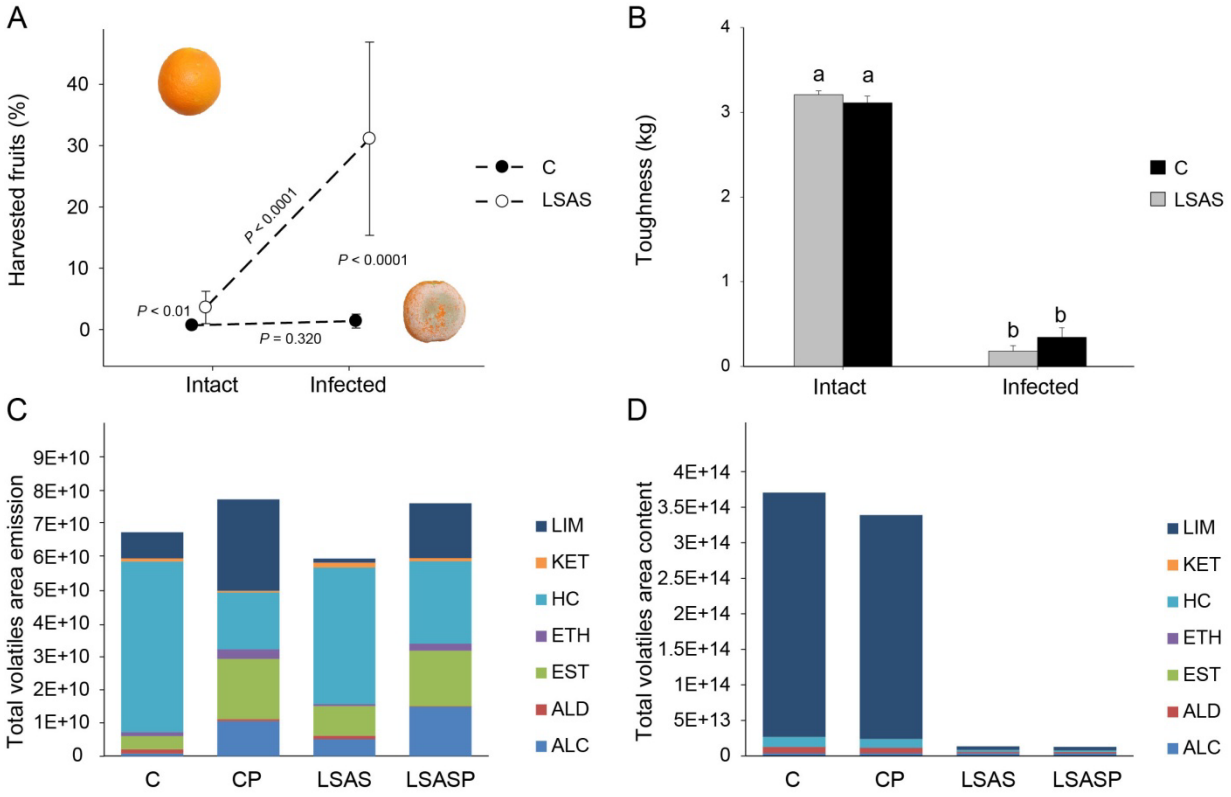

Figure 11. Fruit consumption by frugivores and physico-chemical characteristics of intact (control C and LSAS) and *P. digitatum*-infected (CP and LSASP) fruits. A) Percentage of intact and *P. digitatum*-infected harvested fruits. B) Toughness of intact and *P. digitatum*-infected harvested fruits. C) Total volatiles emission area of intact and *P. digitatum*-infected fruits. D) Total volatiles content area of intact and *P. digitatum*-

infected fruits. Toughness was measured in at least 20 fruits of each line with two measurements per fruit. Volatiles emission and content were measured in at least 10 fruits per plant. LIM: limonene, KET: ketones, HC: hydrocarbons, ETH: ethers, EST: esters, ALD: aldehydes, ALC: alcohols.

Identification and functional characterization of terpene synthase (TPS) genes in *P. digitatum* and *C. sinensis*

It is known that several *Penicillium* species are able to produce D-limonene when cultured *in vitro* in different rich agar media (Sunesson et al. 1995, Lippolis et al. 2016). To identify putative *P. digitatum* genes with homology to TPS genes, the genome of *P. digitatum* PHI26 was screened. A total of 20 putative TPS genes were selected belonging to the isoprenoid synthase domain superfamily (IPR008949), terpenoid cyclases/protein prenyltransferase alpha-alpha toroid superfamily protein (IPR08930) and acyclic terpene utilization family protein (AtuA, IPR010839) (Supplementary Table S5). After removing sequences corresponding for putative prenyl transferases, a total of 10 putative TPS gene models were selected in the *P. digitatum* genome for phylogenetic analysis together with other functionally characterized fungal terpene synthases (Figure 12A, Supplementary Figure S4).

Some of the predicted peptides (PDIG_00600, PDIG_00550 and PDIG_52740) represent domains described in Pfam resources as TPS-characteristic, such as PF03936, described as TPS C-terminal domain. All TPS putative genes were annotated as predicted to be aristolochene synthases (PDIG_83020 and PDIG_47830), pentalenene synthases (PDIG_04920 and PDIG_50820), trichodiene synthases (PDIG_00600 and PDIG_52740), other terpenoid synthases-related (PDIG_05850 and PDIG_00550) and two members of the acyclic terpene utilization family proteins (AtuA; PDIG_23670 and PDIG_44920). The putative trichodiene synthases (PDIG_00600 and PDIG_52740) were amplified and cloned from flavedo of 7 day-infected oranges (Figure 12). The cloned sequences coincided exactly with the sequences annotated in the databases.

A BLAST search of the D-limonene synthase cloned from the sweet orange control identified a sequence of 99% similarity at the nucleotide and amino acid level with a previously characterized D-limonene synthase gene from orange (NCBI accession KU746814, Morehouse et al. 2017). The translated protein sequence consisted of 607 amino acids with an estimated molecular weight of 70.4 kDa. Both sequences only differed in a substitution of an Ala for Val at position 245 (Supplementary Figure S4). The *Arabidopsis thaliana* caryophyllene synthase gene (At5g23960) as the control for the sesquiterpene reactions using farnesyl diphosphate (FPP) as substrate was cloned previously in our laboratory and the sequence coincided exactly with the sequence annotated in the databases.

Recombinant proteins were functionally characterized using *in vitro* enzyme assays with geranyl pyrophosphate (GPP) and FPP as substrates after expression in *Escherichia coli*. The production of sufficient soluble protein was confirmed by SDS-PAGE for all constructs (data not shown). The different products produced by the recombinant proteins were analyzed by GC-MS. Analysis of the products generated by conversion of FPP did not show any sesquiterpene in the putative TPS genes analyzed except for the *A. thaliana* caryophyllene synthase positive control that converted FPP to β-caryophyllene (75.8 ± 6.4%), α-humulene (19.6 ± 4.1%) and α-copaene (4.6 ± 2.4%) (Figure 12C). The *P. digitatum* putative TPS PDIG_00600 did not produce any monoterpene compound after the addition of GPP (Figure 14D) but the analysis of the monoterpene products generated by PDIG_52740 showed that it is a multiproduct enzyme producing a mixture of α-thujene (5.3 ± 0.2%), α-pinene (4.1 ± 0.1%), β-myrcene (3.1 ± 0.3%), α-phellandrene (4.7 ± 0.9%), (*Z*)-β-ocimene (6.5 ± 0.8%), D-limonene (20 ± 0.6%), eucalyptol (18.5 ± 0.9%), γ-terpinene (3.4 ± 0.3%), (*Z*)-β-terpineol

(6.8 ± 0.5%), terpinolene (14.6 ± 0.4%), α-terpineol (5.5 ± 0.2%) and α-terpineol acetate (7.7 ± 0.9%), plus other minor compounds (Figure 14E). The positive control *C. sinensis* D-limonene synthase construct converted mainly GPP to D-limonene (94.4 ± 1.9 %) and the minor products β-myrcene (4.2 ± 0.2%) and (*E*)-β-ocimene (1.1 ± 0.1%) (Figure 12F), which basically coincides with the previously characterized D-limonene synthase from orange (Morehouse et al. 2017).

D-limonene and terpinolene emissions strongly increased 7 days after *P. digitatum* infection (14.2 % and 66.9 %) in LSAS fruits, respectively, and 3.5% and 8% in control fruits, respectively, when fungal sporulation was initially visible in infected fruit (Supplementary Table S4). An increase of these compounds was observed also for season 2 (Supplementary Table S4). Moreover, eucalyptol was only detected in *P. digitatum*-infected fruits (Supplementary Table S4), which strongly indicates these VOCs were mainly produced by the action of the fungal monoterpene synthase.

Although monoterpene synthases have been identified previously in fungal endophytes (Shaw et al. 2015, Wu et al. 2016), they had been never characterized in phytopathogenic fungi. This discovery led us to consider the possible origin of this terpene synthase. Phylogenetic analysis revealed that *P. digitatum* monoterpene synthase was more closely related to other fungal terpene synthases than to other plant limonene synthases (Figure 14A), which coincides with the results from the functional characterization of the monoterpene 1,8-cineole in *Hypoxylon* sp (Shaw et al. 2015). Our results suggest that this may be a case of convergent evolution in the biosynthesis of ecologically-relevant monoterpenes in which *Penicillium* is able to sequester D-limonene from its citrus fruit host as well as to produce it *de novo* in fruit peels, as it

has been shown previously for cyanogenic defence compounds in Burnet moth specialized insects and their food plant (Jensen et al. 2011).

If the fungus has a monoterpene synthase capable of using fruit prenyl transferases as precursors to produce D-limonene and other monoterpenes and the fruit infected by *P. digitatum* emits many more monoterpenes than the intact one and those VOCs emitted (mostly D-limonene) are important quantitatively in the attraction of frugivores, our conclusion is that the fungus manipulates the interaction of the fruit with its frugivores to attract them, possibly to disperse the fungus itself. In addition, the triad could also be beneficial for the other partners since the fleshy fruits may be an essential source of nutrients and water for frugivores and the orange tree would also profit from seed dispersal by frugivorous vertebrates. Therefore, we show here that a deterrent compound for vertebrate and invertebrate generalist frugivores is metabolized by a specialized frugivore (*P. digitatum*) to emit VOCs typical of mature fruit while it is also produced and emitted by the fungus itself in the fruit to attract different frugivorous animals. At the same time, the fungal infection would facilitate physical access to the pulp by the frugivorous vertebrates (Peris et al. 2017) and thus possibly promote dispersal of at least the spores of the fungus with these animals (Dighton et al. 1992).

Plant dispersal would be likely affected by such tritrophic interaction. Ideally, to know how the emission of D-limonene and other volatile monoterpene compounds by the infected fruit would affect the dispersal of citrus (through their seeds) as well as the post-digestion of the infected fruit by natural herbivores once ingested, it would be necessary establish other experimental settings in which fruit from an ancestral citrus genotype (always rich in D-limonene at the fruit peel) were offered

in natural settings, infected or not with *P. digitatum*, and exposed to frugivorous wild animals from their areas of origin and diversity (South China and Southeast Asia). Nowadays natural citrus areas are almost non-existent and most megafaunal mammals dispersing them in the past are now extinct (Corlett 2017). Nevertheless, citrus fruit/*P. digitatum* relations would likely be a remnant of pre-domestication ages, as indicated by the presence of a fully functional monoterpene synthase in the fungal genome and also further suggested by the high identity of mitochondrial genomes from *P. digitatum* strains of China and Spain as well as by their low gene content compared to other *Penicillium* species (Marcet-Houben et al. 2012).

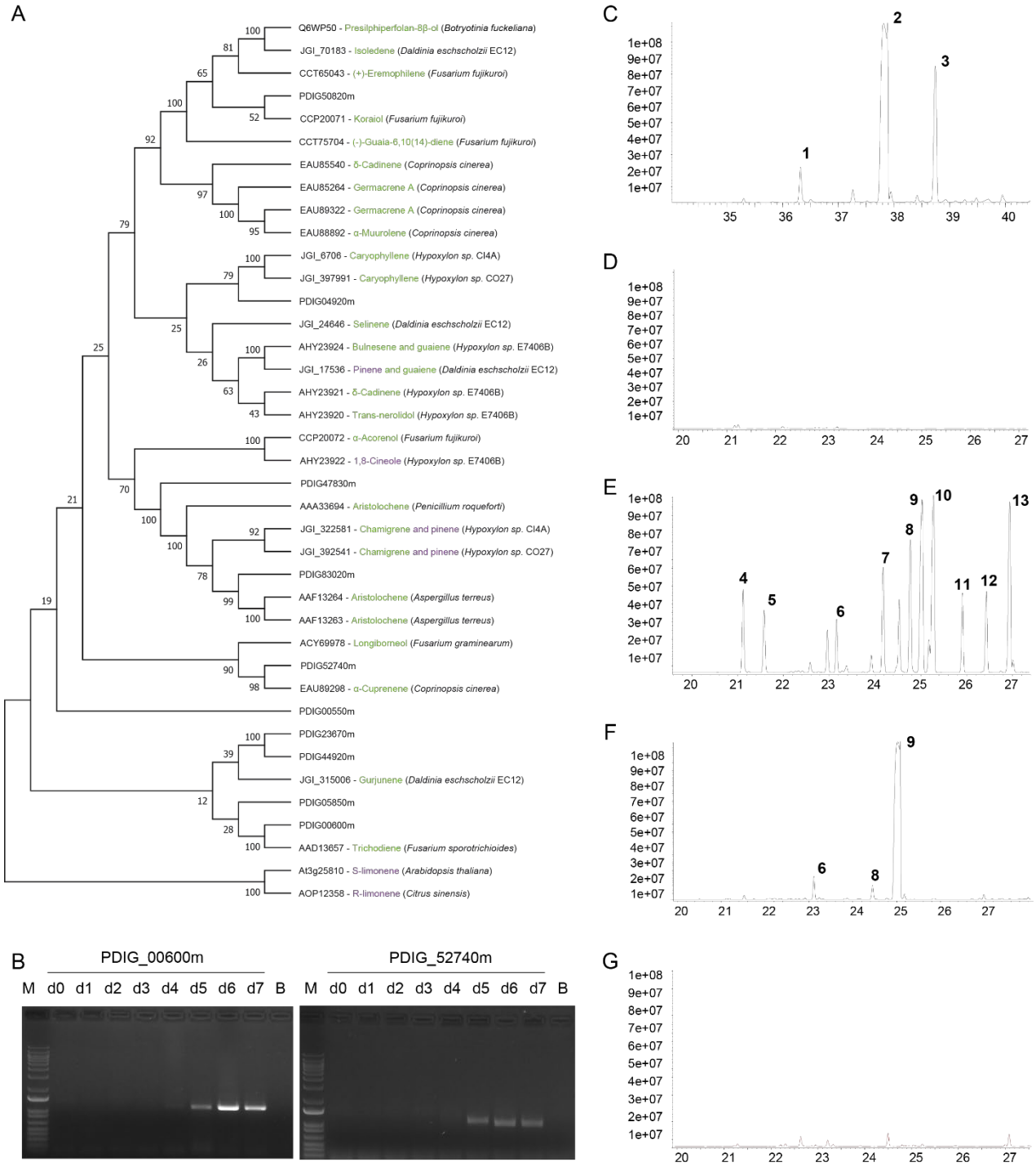
A
Q6WP50 - Presilphiperfolan-8β-ol (Botryotinia fuckeliana)
JGI_70183 - Isoledene (Daldinia eschscholzii EC12)
CCT65043 - (+)-Eremophilene (Fusarium fujikuroi)
PDIG50820m
CCP20071 - Koraiol (Fusarium fujikuroi)
CCT75704 - (-)-Guaia-6,10(14)-diene (Fusarium fujikuroi)
EAU85540 - δ-Cadinene (Coprinopsis cinerea)
EAU85264 - Germacrene A (Coprinopsis cinerea)
EAU89322 - Germacrene A (Coprinopsis cinerea)
EAU88892 - α-Muurolene (Coprinopsis cinerea)
JGI_6706 - Caryophyllene (Hypoxylon sp. CI4A)
JGI_397991 - Caryophyllene (Hypoxylon sp. CO27)
PDIG04920m
JGI_24646 - Selinene (Daldinia eschscholzii EC12)
AHY23924 - Bulnesene and guaiene (Hypoxylon sp. E7406B)
JGI_17536 - Pinene and guaiene (Daldinia eschscholzii EC12)
AHY23921 - δ-Cadinene (Hypoxylon sp. E7406B)
AHY23920 - Trans-nerolidol (Hypoxylon sp. E7406B)
CCP20072 - α-Acorenol (Fusarium fujikuroi)
AHY23922 - 1,8-Cineole (Hypoxylon sp. E7406B)
PDIG47830m
AAA33694 - Aristolochene (Penicillium roqueforti)
JGI_322581 - Chamigrene and pinene (Hypoxylon sp. CI4A)
JGI_392541 - Chamigrene and pinene (Hypoxylon sp. CO27)
PDIG83020m
AAF13264 - Aristolochene (Aspergillus terreus)
AAF13263 - Aristolochene (Aspergillus terreus)
ACY69978 - Longiborneol (Fusarium graminearum)
PDIG52740m
EAU89298 - α-Cuprenene (Coprinopsis cinerea)
PDIG00550m
PDIG23670m
PDIG44920m
JGI_315006 - Gurjunene (Daldinia eschscholzii EC12)
PDIG05850m
PDIG00600m
AAD13657 - Trichodiene (Fusarium sporotrichioides)
At3g25810 - S-limonene (Arabidopsis thaliana)
AOP12358 - R-limonene (Citrus sinensis)
B
PDIG_00600m
PDIG_52740m
M d0 d1 d2 d3 d4 d5 d6 d7 B
M d0 d1 d2 d3 d4 d5 d6 d7 B
C
D
E
F
G

Figure 12. Functional characterization and classification of putative *P. digitatum* terpene synthase genes (PdTPS). A) Phylogenetic analysis of putative PdTPSs (PDIG), previously functionally characterized fungi terpene synthases and the plant terpene synthases R-limonene synthase ((+)-LS) from *C. sinensis* (AOP12358) and S-limonene synthase from Arabidopsis thaliana (At3g25810) and its products (green: sesquiterpenes, purple: monoterpenes). B) Expression of putative *P. digitatum* terpene synthase genes (PDIG_00600m and PDIG_52740m) in flavedo of *P. digitatum*-infected control oranges at different days post-inoculation (d0 to d7). C to G) Total ion chromatograms of the products of the recombinant proteins using geranyl pyrophosphate (GPP) or farnesyl pyrophosphate (FPP) as substrate. C) *Arabidopsis thaliana* caryophyllene synthase + FPP. D) PDIG_00600m + GPP. E) PDIG_52740m + GPP. F) (+)-LS + GPP. G) pET45b empty vector + GPP. Putative PdTPS genes based on sequence similarity with characterized proteins: PDIG_00550m (terpenoid synthase), PDIG_00600m (trichodiene synthase), PDIG_04920m (pentalenene synthase), PDIG_05850m (terpenoid synthase), PDIG_47830m (aristolochene synthase), PDIG_50820m (pentalenene synthase), PDIG_52740m (trichodiene synthase), PDIG_83020m (aristolochene synthase), PDIG_23670m (AtuA), PDIG_44920m (AtuA). M: Molecular marker 1 Kb Plus DNA Ladder, Invitrogen. B: Blank control. Only a portion of the chromatogram of each sample is shown. Peak identification: (1) Copaene, (2) β-caryophyllene, (3) α-caryophyllene, (4) α-thujene, (5) β-pinene, (6) β-myrcene, (7) α-phellandrene, (8) (E)-β-ocimene, (9) Limonene, (10) eucalyptol, (11) α-terpinene, (12) (Z)-β-terpineol, (13) terpinolene. Peaks without a label represent terpenes that could not be unambiguously identified or PDMS fiber residues.

MATERIAL AND METHODS

Plant material

Regular mature Navelina sweet orange control and LSAS fruits (Rodríguez et al. 2011a) were collected in the Estación Experimental Agraria of Villarreal (Castellón). Intact and *P. digitatum*-infected LSAS lines (AS3, AS5 and AS7) and control fruits were offered to frugivores. Navelina belongs to the Navel Orange Group, characterized by the formation of a navel inside the stylar end of the fruit. The fruit is medium-large size, round or spherical, high in acidity (acidity = 16 g/L), with a maturity index (MI) of 8 and high juice content of 50-54% (data accessed in commercial citrus varieties in December 2017; http://www.ivia.gva.es/variedades). The Navelina tree is vigorous, medium size, with the spherical and leafy crown, without thorns and dark green leaves. It is an early variety, with an intense orange color peel (Soler and Soler 2006).

Study areas

The study was conducted from November to December during the maturity season period in different years (2013-2017) at two different locations (Moncada and Villarreal) in a Mediterranean climate region in València and Castelló provinces of Spain, respectively, where the main fruit tree crop is sweet orange (aerial views of each plot can be seen in Supplementary Figure S5). In Moncada, a 0.6 ha experimental field within the Instituto Valenciano de Investigaciones Agrarias (IVIA; 39°34’ N and 0°23’ W, ~17 m.a.s.l.) was used. The most common frugivore species in the area are rabbit (*Oryctolagus cuniculus* L.), black rat (*Rattus rattus* L.), mice (probably Algerian mouse *Mus spretus* Lataste and *Apodemus sylvaticus* L.), common blackbird (*Turdus merula* L.),

Eurasian magpie (*Pica pica* L.), house sparrow (*Passer domesticus* L.) and white wagtail (*Motacilla alba* L.). In Villarreal, a 0.7 ha experimental citrus orchard (39°56’ N and 0°8’ W, 19 m.a.s.l.) was used, within the Estación Experimental Agraria of Villarreal (Castellón). The main frugivore species there are rabbit, black rat, house sparrow, common blackbird, Eurasian magpie and spotless starling (*Sturnus unicolor* Temminck).The most frequent snail species are garden snail (*Helix aspersa* Müller), white garden snail (*Theba pisana* Müller) and land snail (*Otala punctata* Müller and *Iberus gualtieranus alonensis* Ferussac).

Experimental design

Circular beds of fine sand (i.e. sandboxes; Fedriani and Delibes 2013) were mounted under the canopy on the soil to allow the animal footprints to be engraved (the distribution of sandboxes can be seen in Supplementary Figure S5). To prioritize the consumption of oranges in the sandboxes, all other oranges dropped from the trees of the orchard were eliminated. In the first experiments, control and LSAS intact oranges were offered simultaneously in each sandbox (10 cm separated) simulating natural fruit drop. In the second experiment, control (C) and LSAS (AS3, AS5 and AS7) intact oranges and *P. digitatum*-infected control (CP) and LSAS (LSASP) oranges were simultaneously offered, placed alternately above each sandbox (10 cm separated) simulating natural fruit drop (Supplementary Figure S5). Between 15 and 22 sandboxes (1-meter in diameter) were randomly distributed in each plot. Offer duration was between 4 and 11 consecutive and alternate days. Every day, each sandbox was visited early in the morning, results were annotated and pictures were taken. Eaten or missing fruits were replaced by new fruits. Frugivores were identified by their footprints and other signs (feces or type of fruit manipulation, Supplementary Figure S6). In

Villarreal a wildlife camera Ltl Acorn 5310A with motion sensor was used to photograph and film the frugivores.

Fruit inoculation with *P. digitatum*

Fruits unsprayed at least in the three previous months were infected with *P. digitatum* NAV-7 obtained from Laboratory of Fungal Pathology of IVIA (Moncada, Spain). Fruits were disinfected by immersion for 1 minute in a 4 gL^{-1} sodium hypochlorite solution, rinsed with water and air dried. To inoculate the fruits, two opposite incisions were made with a stainless steel tip and 10 µL of a concentration of 1 x 10^6 mL^{-1} *P. digitatum* spore suspension were inoculated in the equatorial region of the fruit (Rodríguez et al. 2011a). The fruits were incubated as previously described (Peris et al. 2017) at 25ºC and high humidity in closed plastic bags. Fruits with a halo of at least 5 cm in diameter of *P. digitatum* hyphae growing around the wounds were used for offerings (6 days after inoculation in the case of control fruits and 9 days in the case of LSAS fruits, to homogenise infestation because the latter are more resistant to *P. digitatum* than the former; Rodriguez et al. 2011a).

Evaluation of fruit flavedo consumption by snails

To assess the role of D-limonene in the preference of opportunistic frugivores, the consumption of fruit by snails was evaluated. For this, intact LSAS and control fruits were offered alternately in the sandboxes and the number of fruits with flavedo injuries caused by snail meals were registered daily during three seasons in Villarreal and Moncada. The results are presented as percentage of fruits and total area eaten (Figures 10 and Supplementary Figure S2).

Identification of putative *P. digitatum* Terpene Synthases (PdTPSs)

In order to identify putative TPS genes in the *P. digitatum* PHI26 genome, searches in Ensembl and JGI Genome portals (Kersey et al.

2018, Marcet-Houben et al. 2012) were performed using as queries the keywords "terpene" and "terpenoid". The best matches corresponded in most cases to other fungal genes automatically annotated (Supplementary Table S5).

Multiple sequence alignment of PdTPS, other functionally characterized fungal terpene synthases and limonene synthase proteins (Supplementary Figure S4) was performed using ClustalO (version 1.2.4, Madeira et al. 2019). A phylogenetic tree was constructed using the MEGA 7.0 software after removing sequences corresponding for putative prenyl transferases, with the neighbor-joining (NJ) method (Saitou and Nei 1987) and bootstrap analysis (1,000 replicates).

RNA Isolation, cDNA Synthesis and TPS Isolation *from P. digitatum* and *C. sinensis*

Total RNA from intact and from *P. digitatum*- infected flavedo tissues, was isolated using the Nucleospin RNA plant (Macherey-Nagel) kit according to manufacturer instructions. Total RNA (0.5 µg) was reversed transcribed using 200 U of SuperScript II Reverse Transcriptase (Invitrogen) and 500 ng of oligodT primer. Full length cDNA clones were amplified from intact and 7d-infected oranges using KAPA HiFi polymerase (KAPA Biosystems). As a positive control, the coding sequence of a putative D-limonene synthase (Cs3g04360) annotated in the *C. sinensis Annotation Project* (CAP, Wang et al. 2014) database was selected and cloned from mature Navelina sweet orange flavedo by following Alquézar et al. (2017). The complete coding sequences of PDIG_00600 and PDIG_52740 were cloned into the bacterial expression vector pET-45b(+) (Novagen) using an Infusion HD cloning kit (Clontech) following manufacturer instructions. As controls, the complete coding sequence of *Arabidopsis thaliana* caryophyllene synthase gene

(AT5G23960) and *C. sinensis* D-limonene synthase gene from mature flavedo, also cloned in pET-45b(+), were used. Gene-specific primer pairs were designed based on the sequences available in the databases (Supplementary Table S4).

Functional Analysis of putative TPSs

One microliter of the reaction mix was used to transform *E. coli* DH5competent cells. Identity of the clones was confirmed by performing a blast against the JGI and Ensembl databases. Selected clones and pET-45b(+) empty vector as control were transformed into *E. coli* BL21(DE3) strain (Invitrogen, Carlsbad, CA, USA) for induction experiments.

Experiments of induction and protein expression were performed basically as previously described (Alquézar et al. 2017). Briefly, single colonies were used to inoculate 5 mL Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/mL) and grown overnight at 37 ºC and 200 r.p.m. One-hundred µL of these cultures were used to inoculate 100 mL of LB containing carbenicillin (50 µg/mL) and grown at 28 ºC and 200 r.p.m. to an OD600 was between 0.5 and 0.8. Induction of protein expression was performed by addition of IPTG (isopropyl β-D-thiogalacto-pyranoside) to the culture to a final concentration of 1 mM, cultured overnight at 18 ºC and 100 r.p.m and confirmed by SDS-PAGE. Cells were pelleted by centrifugation for 10 min at 4,000 r.p.m. and resuspended in 4 mL of chilled buffer (monoterpenes buffer: 25 mM HEPES, pH 7.5, 5 mM DTT, 10% [v/v] glycerol, 1 mM $MnCl_2$ and 100 mM KCl; sesquiterpenes buffer: 25 mm HEPES, pH 7.3, 10 mM $MgCl_2$, 10mM DTT and 10% [v/v] glycerol; Martin et al. 2002) and disrupted by 4 × 30 s treatments with a ultrasonic processor (UP200S, Hielscher). Cell debris was pelleted by centrifugation at 15,000 r.p.m. for 30 min at 4 °C. The supernatant, which contained the expressed recombinant His-tagged

soluble proteins, was purified over HisTrap affinity columns (GE Healthcare) according to the manufacturer's instructions. The purity of the recombinant proteins was checked by SDS-PAGE. The purified protein (50 µL) was transferred to 10 mL Teflon sealed glass tubes, supplemented with 20 µg of geranyl pyrophosphate ammonium salt (GPP, Sigma) or 10 µg (E,E)-farnesyl-diphosphate (FPP, Sigma), and incubated O/N at 28 ºC.

Physical fruit parameters

Fruit strength at break was measured using a hand-held penetrometer (Penetrometer Fruit Pressure Tester FT011) with a 2,01 cm^2 tip and expressed in kg required for the rupture ± standard error. Two measurements were made with the penetrometer for each fruit and at least 20 fruits of each LSAS and control line were chosen for the analyses.

Fruit volatile emissions and content

Chemical analysis of the total VOC emission by flavedo of intact and *P. digitatum*-infected fruits were extracted by headspace in a closed 1L-beaker by solid-phase microextraction (HS-SPME, 100µm poly(dimethyl) siloxane, Supelco, Bellefonte, PA) and analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) using 10 individual fruits from each of the intact and *P. digitatum*-infected lines. All samples were collected from different trees of the same plot cultivated under the same agronomic conditions. Analyses were conducted in days 1 to 3 after harvesting (intact) and days 6 to 9 after *P. digitatum* inoculation, under equal temperature conditions (22°C) in the seasons 1 and 2. Additionally, the progress of the VOC emission from a control orange at 0, 3, 6 and 9 days after the infection was followed to know how VOCs change during the

infection process. For the functional analyses of the terpene synthase genes, the SPME fiber was placed into the headspace of the 10mL-tube. The fiber was exposed during 45-min at 22 ºC and immediately transferred to GC injector (220 ºC) and thermal desorption was prolonged to 4 min.

For the total VOC content of flavedo of intact and *P. digitatum*-infected fruits, extractions were performed as explained before (Rodríguez et al. 2011a). Briefly, flavedo pieces were ground in liquid nitrogen and frozen at -80 ºC until extraction, with pentane and 2-octanol used as internal standards.

GC-MS was performed at the Metabolomics Service in Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universidad Politécnica de Valencia, basically as described in Peris et al. 2017. Compounds in the chromatograms were identified by matching the acquired mass spectra with those stored in the reference libraries (National Institute of Standards and Technology, NIST) or from authentic standard compounds when available (Rodríguez et al. 2011a) and checking with their retention time. The area of all peaks was calculated including unidentified VOCs to which a number was assigned. The total area of all peaks was integrated and results are expressed as total area of each chemical group in the chromatogram. The compounds were grouped by chemical groups as alcohol; esters; aldehydes; ethers and epoxides, hydrocarbons (other than D-limonene), ketones, D-limonene and non-identified/others.

Statistical analyses

Most results were analyzed by fitting generalized linear mixed models using the Proc Glimmix from SAS (SAS Institute 2014) which allows the

modelling of non-normal response variables as well as the usage of both fixed and random factors (Bolker et al. 2009). First, we evaluated overall frugivore preference for LSAS versus control oranges and *P. digitatum*-infected versus intact fruits. In this model, the percentage of fruit harvesting was the response variable, whereas orange type and fruit infection were specified as fixed factors. To evaluate the consistence of the effect of one factor at the different levels of other factors, we also included in the model the second-order interactions between orange type and infection. When the interaction between these two factors was significant, tests for the effect of a given factor at the different levels of the other factor (i.e. tests of slices) were performed using the SLICE option in the LSMEANS statement of the MIXED procedure (SAS Institute 2014). Study site (Villarreal, Moncada) and block (nested within study site) were included as random factors in both models. To compare the effects of different levels of any significant main factor, we calculated the difference between their least-square means. Because of the binomial nature of the response variable (percentage of fruit harvested), binomial error and logit link function were specified. Second, we evaluated potential differences between LSAS lines (AS3, AS5, AS7) fitting a new generalized linear mixed model (Proc Glimmix from SAS, Bolker et al. 2009) where only data from intact fruit in Villarreal and Moncada orchards were considered. The percentage of fruit harvesting was the response variable and orange type was specified as the only one fixed factor. All other procedures and conventions were those as specified above.

Identified orange fruit VOCs content or emission were classified into one of seven main groups (alcohols; esters; aldehydes, ethers and epoxides; hydrocarbons; ketones; D-limonene and non-identified/others). Multivariate analysis of variance (MANOVA) in GLM procedure (SAS

Institute 2014) was done to test overall differences in the percentages of the seven main types of VOCs between LSAS versus control lines and between *P. digitatum*-infected versus intact fruits. Once overall significant differences were detected, we applied univariate analyses for each group of VOCs. Differences in toughness were also tested with univariate analyses.

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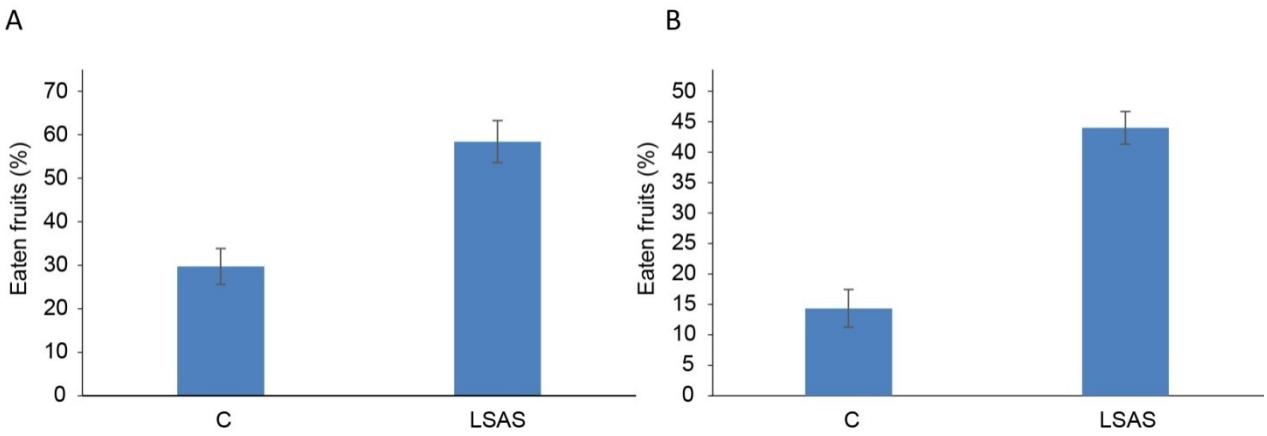
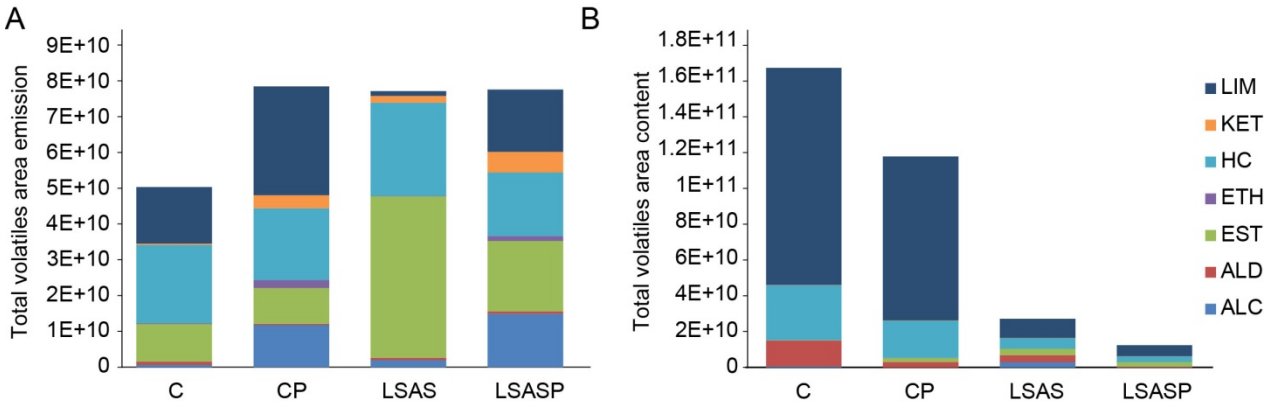
Author contributions

L.P. and J.M.F. conceived the study; J.E.P. and A.R. performed the field experiments and biochemical analyses; J.M.F. analyzed the data; L.P., J.M.F., J.E.P. and A.R. wrote the first draft, and all authors contributed substantially to reviewing the manuscript.

Competing interests. The authors declare that they have no competing interests.



Supplementary Figure S2. Percentage of intact LSAS and control (C) orange fruits eaten by snails in two consecutive seasons. A) Season 2. B) Season 3.



Supplementary Figure S3. Volatile organic compounds abundance emission and content in *P. digitatum*-infected control and LSAS oranges grouped by their chemical class. A) Volatiles emission area. B) Volatiles content area. LIM: limonene, KET: ketones, HC: hydrocarbons, ETH: ethers, EST: esters, ALD: aldehydes, ALC: alcohols.

>PDIG23670m (*P. digitatum*)

MENTLIKASANSTDGIKGDPAYEMYRQATLGDVDFITGDYLAEVNMANNAQAFQRGEHPGYEQTA
WEGLQQTIDVIAEKGIKVVLNGGALNPKGLALKVHELINQKGLALRVAYLSGDDLYTKLGPNMPQS
ADGLQHLDAENMSVKPRPLTYAFTNITKPVPMVSAHAYLGARGIVDGLRRGADIIICGRVADASPVI
AAAWYWHSWSESDYDRLAGSLVAGHLIECSAYVTGGNFPGFDQHPVDTFLEPGFPIAEIDAGGSC
VIVKHPGTGGMVDVDTVRCQLLYELQGNVYLNSDVSAILDDVVIEPVGKDRVRIHGIRGYAPPPTT
KLAVFYPGGFEAQILLNATGYGTDEKWDLIERQIRHFIPKESIDRIDTLEFQRIGIPAPNPSSQRQST
TYLRIFVAAGEADAVLAVGKAMKDISLKHFSGFHSSLDMRTAVPRPILAYYPAIVKQDDLNEEINFID
GPDSITSFPTGHPPAYKALQPRASYNHSPSSDEVALLQSPTRSIRLGDIALARSGDKGSNLNFGIFV
PNATHWPWLRSYMSIERMREMLADDDDWDESFFVERVEFPHIHAVHFVVYGILGRGVSSSSRLD
GFGKGFADYVRDKTVEVPVNILD

>PDIG44920m (*P. digitatum*)

MMLSKRDIRIGNVSGATGDSPHAMLRMAQDGNVDVIVGDWLSEMNIAWNAITKDQDPSLGYEP
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KCENLLHLDHPEWALRDWLLEPYCGVAYIGAWGIVEGMKAGADIIICGRVTDASPVIGAAAWWHD
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LRSKVVPIPVQFLERGCI

>PDIG50820m (*P. digitatum*)

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VLASVPIWGEETDRQVLKFINLWRDMVLGNLHWSFRTTRYLGNEGGEVHRTRVLHLPC

>PDIG83020m (*P. digitatum*)

MSLEQGSAYNEKLIPISRGDVLPDRSVPVEYITYDLWESMRAKDRSMANEILEPVFVFMRAQTDRT
RIRPMGLGSYLEYRERDVGKALLAALMRFSMALTISPVELTLLGEIDANCSKHLSVINDVYSYEKELR

ASKTAHAEGGALCTSVRILADEIAISIEAAKRVLIFMCRELESRHLVLVEELRANGRQSASLAAYVEG
LEFQMSGNEEWSKTTLRYNNLV

>PDIG00600m (*P. digitatum*)

MTRQAFPFEDYLNSVVRFLDTMEYYDTNYTAEERVSKLHYVYTNTAKHLAHYDRQKQIKMSPNNLQ
ALIQVTVAMVVYGWATVSENVMVDLSIHFTYVLILDNSTDDNPAKSLESFHNTMLAGLPQEHPWW
QMVNNHFPLVLRHYGPYCGLTLIRSTADCSYSSSFPTTHVTDIVACKVFQRCLVEQHNFMGFPGSL
NYPIVLRRMNPLGQCIGSSIFPKERFDENELFSEITAGIAEMENWMVSVTGLLSFYKEFDEPRDQAN
LVDNYAWSNKITPEGALENLTTDTIVDSEQLLAAFRDKDPRISQTLSTFCQGYVTWHLCDPRYRLEE
LHSFGGESTEVSAKFHNYLQSAKEAGSVDPEAWAYPSVASLAADDLAYWSDGL

>PDIG47830m (*P. digitatum*)

MSLTEVLSYWDRVMKIVLGTASPDRSICLEWMIHDTVMVMRSMDEVLAYDVAQGFCQLLQAQTS
QERTKIETLGSYLKFREIDVDRPLYTALIRFGTKLDLTTAELEKTAALESTAFRHASVMNDIYSWERE
WKAYQANPADGARLVSAIYILANETGLPHSACKRLMYSYCRELELALKQSTDEMRHKSMGSLTPEL
EMYIKGLAYLMCGIELWSQWTQRYQQ

>PDIG05850m (*P. digitatum*)

MVECVHANAQSRSPSYVDSDSIVFKNERNDLALRFLTDAKKAQKAMEDCSDVCAIYFPADLDVAF
NSEIENRLAADICEWRFFTCLSKSLKDLAISMSRLCSLQVNLSYERRTLLHRHLAWIYIMDEVCERL
PVYGLHDTVEKTYLENLKNITRNLPIEDLNQYKGICPDDLLHMALDVQKILAEDLMPLKRELLEESHV
QKCSETLCLFIDYQYEEGKIFLERPTSHETMPTRVYTIGINMVFLLSLQTPIVEVYNANDMGLVQLSII
GALYHDFIGLQKDLNCRDLKLDGSIGLNLVVASMKESGYNEKEAMQAMVRRLNSYCHDLQFFMSA
YPPLYKQFYQAGLQIVFALHDYHLMGATESSNSRYGWHRVSDYKHSESSQDPRAP

>PDIG04920m (*P. digitatum*)

MDYVTASLRALFEGLDGHELIIPSRAETNPGWEVTEHDLSNKSIKAELTSWVESWWSDGPNPAMI
EALNLPNWVALLYPNVKYEKRLTIAKSLSWIFAWDEPLDDGEFTHDPVGAINYCNETIKFMELLRDP
SQVSSACHPNPNIASFKDSFVSIWESHPVCTGRYLDECLRFVQGTSEAVGRREKNQVPTLADYFD
WRVLNLGFEIYFTLVEYSQDLKVPDECSKPNVFTQIIFREASLILSVYNDLLSLPKELDAGQFESCVPI
KMFELGLSLDETINSFGQMIHECAKRFNKAEQDLYSHTSPDSLADVQAFVQGIKQQVVGTNKHFY
SMDRYVRKGVNQPDGSIKFKIALEQ

>PDIG00550m (*P. digitatum*)

MATCDYLSSQHDGIVSPLSGIRLPNNVTGKIPIYKRLNEEHIPDYNIVLVDSVPQSAVISSHAKFKAS
INPENAGDAQDFHESCFAEAATVNLLFPDVRSESIRICLAAWLAAICTADDLLEAMPPAAATANLKE
LILKFQGRKADVSATNKLASIFLFFIDHCNQQLDLDESISRQLNNDLCNICESWLDELRFRQGILPN
NFETYMQFRGKTMAIQPFFTLMRTMYKPIEGEYLSGLQDLLNQISLVLGLQNDLVGLEKDRRDGET
MNAVLLSLKEKAEMDADHMEIEFRRKIEEICDLHNLYVSAAVEMYQALHVSMNEDAHDPTLETAIL
ALADTHLKWCTSYKRYQAKIE

>PDIG52740m (*P. digitatum*)

MDPISAENTLAYHGLWMKSTTTDGVTSAHTPPDQIKSLIRTFLEDIGFDRSIHVVQDHEMTRAVW
QYFQSLELGEKTEQSVQQTLHPSVNFAYHGYTTLPFQVRVLGAIQFLYMFLVDDVAEEFIEDLQAFG
QNFVLNQQHKHPVLAGLDSHLRNLSHYYGPYCHSVMIKGMLDYINGRIIEHKIKVSKFQFSSASRL
MPMFLRSKVGGAEIMIHFLYPNSVFPEEEFVMKYFPVTLELVLFIDFTNDILSYYKEFCLSNETGNFVS
NFADTHHVQHVDVLRYLTSYTPEVIKSAYKQLQSNPSLLALIKDFTQGMIMLFTAHRRYHLVELFAE
EQYLPPYDEDA

>EAU89322 - Germacrene A (*Coprinopsis cinerea*)

MVNLSWYWQGQGNISKSIGPPSQTYTKSVLREQSMTFRMLALQSGLKSAASDHVSTSGSGILRFL
SRILPANTTRRCSACCTEMSSLDATIHPVLNFEDKKIVLPDLVSHCNFKLRVSRHRKRITGETKRWL
FKGDNLVGPARNKYHGLKAGLLTAMTYPDAAYPQLRLCNDFLTYLFHIDNLSDDMDNRGTWSTAN
EVLNSLYHPYTYHGQARVGRMTRDYWRRMILTASPGSQQRFIETFDFFFQSVTQQAIDRLTGEIPD
LESYIALRRDTSGCKPCWALIEYANNLDLPDEVMDHPVVRSLGEAANDLVTWSNDIFSFNVEQSK
GDTHNMIPVVMHQEGLDLQSAVDFVGEMLDHTSTCCGLESPLWLTPPLRIRRNAPRLYRVFL

>EAU85264 - Germacrene A (*C. cinerea*)

MPSPAGALPKSFILPDLVNDCPFPLRVNPLCDEVGRLSEQWFLRHANYSPPRAVAFMALKAGELTAA
CYPDADAFHLRVSDDFMNFLFNADDWLDDFDIEDTYGLANCTVRALRDPVNFITDKRAGLMTKSY
FSRFLKTAGPRCTERFIQTLALYFESVVTQKQARNNGTLPDLESYITIRRNNSGCKPCYALIEFCAGI
DLPDEVINHPIIQSLEDASNDLIAWSNDIFSFNREQSRHDSFNMVSIVMHQKGFALQEAVNFVGEL
CKKAMERFQADKRNLPSWGPEIDGEVAMYVDGLQNWIVGSLNWSIDGTERYFGKDGPGIKKHRK
VKLFPKRPLKTPAVRVLA

>EAU88892 - α-Muurolene (*C. cinerea*)

MSTPSSSLTTDESPASFILPDLVSHCPFPLRYHPKGDEVAKQTVHWLDSNCPDLTAKERKAMYGLQ
AGELTGYCYPYTTPERLRVVADFLNYLFHLDNISDGMMTRETAVLADVVMNALWFPEDYRPTKGQA

AEELNPGKLARDFWSRCIPDCGPGTQARFKETFGSFFEAVNIQARARDEGVIPDLESYIDVRRDTS
GCKPCWVLIEYALGIDLPDFVVEHPVIAALNQGTNDLVTWSNDIFSYNVEQSKGDTHNMIIILMEH
HGHTLQSAVDYVGSLCQQTINTFCENKQQLPSWGPEIDDMVAKYVQGLEDWIVGSLHWSFQTRR
YFGDEGQEIKQHRLVKLLTVAPPPPPPPTPPPQSSDADTKKQKVKAQDGKGPVSDEEVWALVRAE
QSKGSILESLFGFLTTSLSRIFFGYFFAYSH

>EAU85540 - δ-Cadinene (*C. cinerea*)

MRPTARQFTLPDLFSICPLQDATNPWYKQAAAESRAWINSYNIFTDRKRAFFIQGSNELLCSHVYAY
AGYEQFRTCCDFVNLLFVVDEISDDQNGQDARATGRIFVNAMRDAHWDDGSILAKITHEFRERFV
RLAGPKTVRRFADLCESYTDCVAREAELRERNQVLGLNDFIALRRQNSAVLLCYSLVEYILGIDLDD
EVYEDPTFAKAYWAACDFVCWANDVYSYDMEQAKGHTGNNVVTVLMKEKDLSLQEASDYIGREC
EKQMRDYLEAKSQLLQSTDLPQEAVRYIEALGYWMVGNLVWSFESQRYFGAQHERVKATHVVHL
RPSSVLEASCDSDSDSDC

>EAU89298 - α-Cuprenene (*C. cinerea*)

MPAALPYNVSRDNKWDIKKIIQDFFKRCDVPYQVIPYDTELWNACLKRAKEKGYPVEPDSPMSLYR
SFKVGVVITRTSYGHIQDYEILIWVATFTAFVTYADDAFQEDIQHLHSFARTFLQNEKHEHPVLEAF
AQFLRESSIRFSHFVANTVVSSALRFMMSIALEFEGQNVSVSTEAREYPGYIRILSGLSDIYALFAFP
MDLPRSTYIQAFPEQIDYINGTNDLLSFYKEELDCETVNFISAAATSQQVSKLEVLRNAAEKAAYSY
DVVVNVLKPYPEALAAWKSFARGFCYFHTSSPRYRLGEMFHDFEHDLVCKCASCTEI

>ACY69978 - Longiborneol (*Fusarium graminearum*)

MLATPTLSYFDKPSLPSSEGGDPALAARLQPLYSRFLMDLDLQPEYRRHESEKLMEEVLKFAKSTGV
PHDLNSHSYQSLMVGYTYADNCLPYHDIEVKVYVAIYTWLATICDDAEALGIIDDVQLFEQRFILGE
EQPTVLLRAFADQLKLTYKLYHPLVANLILCSSLNLLTSTSLVARKGIKEKGDHPSKGGNYFAWYIRE
RDGVGEAYSWFTFPKRQFPNLDIPIEAIEDMTRFIAYLNDVLSFYKESLAGETHNYINHTAAYEGVD
SDAALHKTAQDTIDCARRIESVLAGKGEYEKAWRLHASGYLLMHVQRGRYRLIEVGVGDAPDVHE
VIKKI

>AAD13657 - Trichodiene (*Fusarium sporotrichioides*)

MENFPTEYFLNTTVRLLEYIRYRDSNYTREERIENLHYAYNKAAHHFAQPRQQQLLKVDPKRLQASL
QTIVGMVVYSWAKVSKECMADLSIHYTYTLVLDDSKDDPYPTMVNYFDDLQAGREQAHPWWALV
NEHFPNVLRHFGPFCSLNLIRSTLDFFEGCWIEQYNFGGFPGSHDYPQFLRRMNGLGHCVGASLW
PKEQFNERSLFLEITSAIAQMENWMVWVNDLMSFYKEFDDERDQISLVKNYVVSDEISLHEALEKL

TQDTLHSSKQMVAVFSDKDPQVMDTIECFMHGYVTWHLCDRRYRLSEIYEKVKEEKTEDAQKFCK
FYEQAANVGAVSPSEWAYPPVAQLANVRSKDVKEVQKPFLSSIELVE

>CCP20072 - α-Acorenol (*Fusarium fujikuroi*)

MPHKDLPIRPLVRAFDPVGPDTLGPPDLDFASLFRERNVPEDAPLTLYPEQLNVPWHTSLPWTRQS
KWWVQGEAAGRDLVNRISADKASERGALPVEFMDERRKGKIDELVEDAVSCAVYLYPSSSPTRIE
LLTQALLLLFFHDDVMERGATQDDATVCDDFVTMIPKNKHMKRYFAEVLECDPILGPGLLRAIGLFV
NAGRKKSPFKQDKYATLAEYLDYRRHDIAKPFMIAAIRFGSGVRQTPEETAPFAELEDLYVQHSILIN
DLYSYDKEMYEARTINGSVVNAVHVIEKLMCVPPHLAKTITRTMSFDVEKKYYAESERFMRDPALN
DKQRTYVIALFDCLTGNLFHHATLGRYSRYAEYVFDCKT

>AHY23922 - 1,8-Cineole (*Hypoxylon sp*. E7406B)

MRPITCSFDPVGISFQTESKQENFEFLREAISRSVPGLENCNVFDPRSLGVPWPTSFPAAAQSKYW
KDAEEAAAELMDQIVAAAPGEQGSLPAELAVSDKKAAKRRELLDTSVSAPMNMFPAANAPRARIM
AKANLLIFMHDDVCEYQSVQSTIIDSALADTSTPNGKGADILWQNRIFKEFSEETNREDPVVGPQF
LQGILNWVEHTRKALPASMTFRSFNEYIDYRIGDFAVDFCDAAILLTCEIFLTPADMEPLRKLHRLYM
THFSLTNDLYSFNKEVVAEQETGSAVINAVRVLEQLVDTSTRSAKVLLRAFLWDLELQIHDELTRLK
GTDLTPSQWRFARGMVEVCAGNIFYSATCLRYAKPGLRGI

>AAA33694 - Aristolochene (*Penicillium roqueforti*)

MATSTETISSLAQPFVHLENPINSPLVKETIRPRNDTTITPPPTQWSYLCHPRVKEVQDEVDGYFLEN
WKFPSFKAVRTFLDAKFSEVTCLYFPLALDDRIHFACRLLTVLFLIDDVLEHMSFADGEAYNNRLIPI
SRGDVLPDRTKPEEFILYDLWESMRAHDAELANEVLEPTFVFMRAQTDRARLSIHELGHYLEYREK
DVGKALLSALMRFSMGLRLSADELQDMKALEANCAKQLSVVNDIYSYDKEEEASRTGHKEGAFLC
SAVKVLAEESKLGIPATKRVLWSMTREWETVHDEIVAEKIASPDGCSEAAKAYMKGLEYQMSGNE
QWSKTTRRYN

>AAF13263 - Aristolochene (*Aspergillus terreus*)

MKKPNGTNGASSSLEPPPSTFQPLCHPLVEEVSKEVDGYFLQHWNFPNEKARKKFVAAGFSRVTCL
YFPKALDDRIHFACRLLTVLFLIDDLLEYMSFEEGSAYNEKLIPISRGDVLPDRSIPVEYIIYDLWESM
RAHDREMADEILEPVFLFMRAQTDRTRARPMGLGGYLEYRERDVGKELLAALMRFSMGLKLSPSEL
QRVREIDANCSKHLSVVNDIYSYEKELYTSKTAHSEGGILCTSVQILAQEADVTAEAAKRVLFVMCR
EWELRHQLLVARLSAEGLETPGLAAYVEGLEYQMSGNELWSQTTLRYSVVVD

>AAF13264 - Aristolochene (*A. terreus*)

MKKPNGTNGASSSLEPPPSTFQPLCHPLVEEVSKEVDGYFLQHWNFPNEKARKKFVAAGFSRVTCL
YFPKALDDRIHFACRLLTVLFLIDDLLEYMSFEEGSAYNEKLIPISRGDVLPDRSIPVEYIIYDLWESM
RAHDREMADEILEPVFLFMRAQTDRTRARPMGLGGYLEYRERDVGKELLAALMRFSMGLKLSPSEL
QRVREIDANCSKHLSVVNDIYSYEKELYTSKTAHSEGGILCTSVQILAQEADVTAEAAKRVLFVMCR
EWELRHQLLVARLSAEGLETPGLAAYVEGLEYQMSGNELWSQTTLRYSVVVD

>AHY23924 - Bulnesene and guaiene (*Hypoxylon sp*. E7406B)

MKSSKMMRTTLLRLARRARSRLLSILSPHSVPAAQEVQRTSEKPSAQQGLCGEALVLASQLDGKTF
HVPDLWKVFSDWPLAANPHAQRLDALVDSLLERIITNEKKLKALKQANFGRLISLWYPDAEWSELE
IAAAYSVWIFVWDDEVDANDTDVSNDEELSRAYYQKSLRTIHNLLGLDPVEDGQEPVFEDDQSLH
PNMALFADVGRGMRATTDKIQRERFYRELENFMVQVGVEHVHRMRGSIPSVEKYIEIRSGSVGCA
PQIAITDAMLRIRLPESIMECAAMKALWRETVVICFILNDVYSVQKEIAQDSLLNLVPVMYKNLDPEK
QSLDTVTRDIEVLLQDTVRKFEEAAKSLSEMTSNDAQVSKDVQAFIKWCRYFITGVQQWSLESRR
YGMAKCVNEDGSLSIVL

>AHY23921 - δ-Cadinene (*Hypoxylon sp*. E7406B)

MAATIQGNERSGLNPQLLPFSVNTREQLLTDTRGSRVMIPDLQSMISHWPQRTNTDVERLDEYVE
KALTCFSSLSNNEARVRRLKATNVAFIAATWWPYASYKALEVLTSLLLWLFAWDDETDSPEFSAVI
NDWDKASTFRQRTTNYLQQSLLKNSKSNLANMSTDPINALFGPVAEAISESCDDRQVGTFLDELLF
YVKMCGEEQKLQVAHRLPTVEEYVRLRLGSGAVRVCFATIEYAYGITLSQKIMDDEAMQRIWHEAN
IIIHTTNDILSVKKEVAQSQVDSLVPLLALELGSMQAAMNHAVDIVRSSIQRFDTAAIEILERYATTP
KVQEDIRKSIDACRYACTSNLNWSLVSGRYKLNCQSMEGGLYITL

>AHY23920 - Trans-nerolidol (*Hypoxylon sp*. E7406B)

MYDYREKELLAKRLKGQRLVIPDMRPIFAHWPCGQNEKYQEMKDMIDTRLASQPMKEESRRAFND
MNPTLLAATWWPTSSMNQYRVLVDLIIWFGYWDDLIESLASDPGAAEGLRSATKTLVRQSLDLGG
LEEDMSINNPLILGFKNIAEEVCKVYDEEQRRVLLGHFDRYIDATQLEAEADLSETLPSLKRYWEVR
VLTSGMGTPLSFTEFAAGVKLPSQIVSSAAYESLWVTTVLINSIVNDLVSFKKEMKAGSVLSSVAIL
YHEVDNLDAAVQMSLAHLRILVDEFDRTANAILSKFPLGIDEVESVSKAIDVLRMVNTGNLEWSLQ
SKRYGVGQFMTPNGQIELVL

>CCT65043 - (+)-Eremophilene (*Fusarium fujikuroi*)

MIATINGDTKINGKGHPTEVRIPDMFGSIMSATPMVNPHHFKVKAAADAFIADYLKMDKHEATKNR
KADFCFCASAMAPHADAEALRTMVDWLNWIFYFDDDFDEGQLDRDPVAAEKEIRHTLAVLEEGAE
IPDRELHPLRYLFRTIWDRVKERAYPDVQTQFKITHKRYLDGLLHQVEATRDGNGQPRTEEDYIRM
RRRTVGGYPCISLIAYAHNVDLSQEAFEHPSVQECIAVGCDLAWIHNDIVSYKKDVKSGIEHNFITV
LKKNGFTTQQAMDRAGELQDECYRRWYLALASMPIWGESIDREVLRYIEACHSFPLGDLLWSFQT
GRYLGATEGYKLHETRVLDLSDLEPIAV

>CCT75704 - (-)-Guaia-6,10(14)-diene (*F. fujikuroi*)

MVKFDSGSESEMTNGDELHINSKHEVKSRMANGNGVHNVPDHDQFQDRAEMEVLILPDLFSSLM
SVPARENPHYASVKADADEWISFVINADAKWASRNKRVDFTYLASIWAPDCSAFALRTSADWNS
WAFLFDDQFDEGHLSNDLEGAINEIARTREIMEGTAPRYTADSEHPIRYVFQTLCDRVKQNPEGFY
AGKPSSERFYRRWMWAHELYWEGLVAQVRTNVEGRSFTRGPEEYLAMRRGSLGAYPALVNNEWA
YGIDLPEEVADHPLVFEIMIIMSDQILLVKDILSYEKDLRLGVDHNMVRLLKAKGLSTQQAINEVGV
MINNCYRRYYRALSELPCFGEEADRALLGYLEVEKNHALGSLLWSYNTGRYFKSKEDGARVRKTRE
LLIPKKMAAL

>Q6WP50 - Presilphiperfolan-8β-ol (*Botryotinia fuckeliana*)

MAIPALEPQLHDADTSSNNMSSNSTDSGYDTNSTTPLEKSEKPNTQELKQQQLDPKRPPFVRVPDL
FGSIMSTKPVVNPNYFAAKARGDRWIARVMNFNKAVAARNSKVDLCFLASMWAPDAPEDRLVMM
LDWNHWVFLFDDQFDEGHLKEDPAAAAEEVKQTIAIMGGNAPRYTAESNPIRYVFQQCWDRLKA
VSSQEMQQRWIDQHKRYFDQLLVQVDQQVGGENFTRDVEAYMDLRRGTIGVYPAISLSEYGAGV
NVPQHVYDHPSLQECMKVSADLVTLVNDVLSYRKDLELGVDHNLMSLLMQRDNLSAQQAVDVIG
DMVNECYRRWYLALAELPSYGEKIDYNVMKFVEICRAVAQGNLYWSFQTGRYLGPEGHEVHETGI
MYLPPAANLVVA

>CCP20071 - Koraiol (*F. fujikuroi*)

MVPSLITPPPSRSGEATPQKDACLNPVNIAEPEGHWIKLPEALFSSIMAVEPEVNPLYRTSKALSDE
WLKTALRMNDKTAVIWSRLDIAYMSAICAPHADLETLKLMNDWNGWVFAFDDPFDEGTFANDPIK
AAEEVIYTLATLDNIHPVVSPDENPLRHTLQSCWMRFRERSSPSLQYRWKKHLTMYCVGVLQQVG
VQHRATRPTIEEYMDMRAGCVGAYPCIGLMEFAEGIDIPQNVMDHPSMQAISRITCDLVTLQNDLC
SYRKDLIQGEESNIIFILKDQGMTDQQAVDQIGEMLYDCYRRWHMALANLPFWGEGIDRDVIKFV
TGCRNIALGNLHWSLYTFRYLGNDGPEVKRTRMMKLP

>JGI_322581 - Chamigrene and pinene (*Hypoxylon sp*. CI4A)

MSLAPSSGDYPSSHWTPLIHPLSEKVTREVDGYYLQHWPFPDERSRKKFVAAGFSRVTCFYFPKAL
NDRIHFACRLLTVLFLIDDLLEYMSLEDGKAYNEKLIPISRGDVLPDRSVPVEYITYDLWESMRAHD
RIMADDILEPVFTFMRAQTDSVRLEAMDLGRYLEYRERDVGKALLGALMRFSMGLVVPPEDLAIVRP
IDFNCSRHLSVINDIWSFEKELLASKNAHEEGGVLCSAVSVLADQVGISIDGSKRILYYLCREWEH
RHETLVKEMLQVRDTPALRSYVKGLEYQMSGNEMWSRTTMRYLAPKD

>JGI_392541 - Chamigrene and pinene (*Hypoxylon sp*. CO27)

MAPMAEECVSASPNQGHAKPVATPMRRAVHIPSSEWTAQIHPLHEKVIAEVDGYFLQHWPFPSEK
TRKKFVAAGFSRVTCLYFPKALDDRIHFACRLLTLLFLVDDILEHMSLEDGRAYNERLMPLFRGSVLP
DRSVPVEWISYDLWESMRAHDRDMADEIIEPVFTFMWAQTDPARLTEMGLGQYLEYRERDVGKAL
LAALMRFSMALIVSPSDLEMVRPVDRNCSKHLSVINDIWSYEKEVLAAQTLHEEGGMLCTAVAVLS
KEAEISTDASKRVLYHLCREWEDEHRILVADILAQNDTPVLRAYLQGLEFQMSGNELWSRTTLRYV
QPRP

>JGI_17536 - Pinene and guaiene (*Daldinia eschscholzii* EC12)

MKSQTLSPLFRLAELVHYKLLSIFPRKPLAQTVEPTANPDLRGDASILAAQLDGKTFRLPDLWKVFSE
WPLAANPHAKRLEGLVDSMLERIITNEKKLKALKKADFGRLMSLWYPDAEWPELEIATAYSVWIFV
WDDEVDAGDTDVSNDEELARAYYRKSLSTVHCLLGLDESEGAEERIAREEASLHPNMALFADVGR
GLRNSTDRIQRERFYRELENFMISVGVEHGHRMRGSIPTVEKYLHIRSGSVGCAPQIALTDHMLKIR
LPESIMECAPMKELWKETVVMCLILNDVYSVQKEIAQASLFNLVPVMYKNCSPEKQTLDTVTRGVE
AALQESMRGFEDAAKALGEMASDDAQVSRDVQAFIKWCRYFITGVLQWSLESKRYGMADCRHKD
GSLSIVL

>JGI_6706 - Caryophyllene (*Hypoxylon sp*. CI4A)

MAPDIDQIWASTSDVPASAVDERKALINRALNQKVLVPNILSLMPTWTSALQPDLDEINKEIDEWL
PTVNVAEAKKAKHRARGNYAFLTAVYYPHCKKDKMLTLSKFLYWIFFWDDEIDNGGELTDDEEGT
QQCCDETNKCIDDCLGPNPNYTPPSNARGTVEMFYPILRDLRAGLSPISTERLRLELHDYVNGVGR
QQKVRQGDHLPDPWYHFQIRSDDVGVIPSITQNEYAMEFELPEYVRRHEAMEAIVQECTKLTVLLN
DVLSLQKEFRVSQLENIVLLFMNKYDLSLQAAIDKILDLIREHYQICVAAEERLPWSKDDEKLNEDIR
EYVRGCQRLATGTAYWSYSCERYFKQTQVNDKWEVLLDLSYE

>JGI_397991 - Caryophyllene (*Hypoxylon sp*. CO27)

MAPDIDHIWSTTSDVATSSIDERKNLIKRALNQKVLVPSILSLMPEWPSDVQPDVDEINKEIDEWL
PTVNVAEKKKVKHRARGNYTLLAAIYYPHCKKDKMLTLSKFLYWIFFWDDEIDTGGDLTEDEEGTL
QCCQETLNCVDDCLGPNPNYTPPPNSRGTVEMFYPILRDLRAGLGPVSTERLRLELHDYVNGVGKQ
QQVRQGDHLPDPWYHFQIRSDDVGVIPSITQNEYAMEFELPEYVRRHEAMEFIVQECTKITVLLND
VLSLQKEFRVSQLENIVLLFMNKYNISLSKAIDKVLQLIREHYAICVEAEERLPWSKDDEKLNDNIRE
YVRGCHRLATGTAFWSYSERYFKQTQVNDKWEVLLDLSYE

>JGI_315006 - Gurjunene (*Daldinia eschscholzii* EC12)

MVVTTRSKKRVSEDAPETTVKRPRLEEKTDLRRWRMLDEKGRHTWHYLEDDEAVRKWPQSYADK
WYLGLDTGLPTLPKPQKPLDAVVNGLTFFEKLQLPSGQWGCEYGGPMFLLPGIVFTWYATKTPIPW
YVATEIKNYLFARAHPEDGGWGLHIEGESTVFGTALNYAVLRIVGLDPEHPVMVKARGTLHKLGGA
TYAPHWAKFWLSVLGVCKWDIVNPVPPELWLLPDWVPFAPWRWWIHMRQVFLPMSYIYEKKWSC
EETDIVRALRQELFVEPWEKIDWLGNRNSICSVDNYHPKSWLLNTANWFLVHIWNPYLRTKGLAQ
KAEAWVGKLIDMEDENTDFADLAPVNAAMNTIVCYIRDGPGSYSVRRHIERLEDSMWVNGDGML
CNGTNGVQCWDTSFLIQALTDAGLEQDPRWKPMLNKALIFLDNQQIRENCKDQDICYRQQRKGA
WAFSTRDQGYAVCDCVSEALKSVILLQHTPGFPQLLEDQRIFDAVDTLLTYQNKSGACSSYEPTRG
SELLEMLNAAEVFGKIMVEYDYVECTTAVVTALSLFQKHWPDYRPKEIEAFIGRSVKAVKSLQQPD
GSWYGNWAICYTYATMFALESLKSIGETYGNSSYSKRGCDFLISKQREDGGWSESYRSCERMIYT
EHPTGSQVVMTAWALIGLMKADYPDIKPLKKGIKLIMDRQQPNGEWKQEAIEGVFNKSCMISYPN
YKFTFTMKALGMFATKYPNETVV

>JGI_24646 - Selinene (*D. eschscholzii* EC12)

MSILDTKTDFDLLLGKCIGQRVEIPDLFALCPWGLEVSPLDEKLTMEVELWRSRWINDPTSLKRNRI
VESCLFARGIAPKAALNELITLAKYQAWLFYWDDVYDFGDFNDKYEEIVSHQEQTIELLHRSLFEKD
PGSIDPAKIAPNYLTVQSIYEWASVVREKSVSSSLKIWLLKVLVDFCTATFYLQSAFDKRRILDLETY
RKIRMDSSAVFPTLGMVLFTDQVAFPPWFFDHVSIKKAAELVNIIVWVTNDIVSARQELQCKHLDN
LIPLLVHHRGITLQEAIREASKITHQAYLDFEELEPQLMQLGENRGVVYEMQRFVASCRHVCTGIFN
WTYHIKRYILWEPGMTRSGLSTVLGEDLLKK

>JGI_70183 - Isoledene (*Daldinia eschscholzii* EC12)

MLDSSELAEPHEGRRSVRIPDLFSSIMATKPVVNPNYFKVKAAGDRWIKRIMKMDEKASDKNSKV
DFCYMICIWAPDADEEALRIMLDWNNWIFLFDDQFDEGHLKDDPVAAQQEVNATMAVMEDDSPL
VRPEESPILYVFQTCWLRLKQRAPTEIQQRYKERHKRYFDQLVAQVQEIARGQVLTGDVVTYLEAR

RRTIGVYPAITLAEYGEGVRLSDSVLSHHSLQECMRITADLVILVNDILSYKKDLDLGVDYNLITLLM
KQNLSLQESMDKIGALIESCYRNWYLTLAELPLYGEETDNEVLRFVEACRCVALGNLYWSFKTGRYL
GSEGHDLHKTRTMYL

>At3g25810 - S-limonene (*A. thaliana*)

MATLCIGSAPIYQNACIHNFRLQRPRRFISKSMTKTMPDANPLDLRRRSGNYQPSSWDHSYLLSIE
NKYVNEKEVITRHVLKKKVKKMLEEVETKSRLEKLELIDDLQKLGVSYHFEQEINNILTNFHLENGK
NIWKCDKEEDLHATALEFRLLRQHGFGVSEDIFDVIIDKIESNTFKSDNITSIITLYEASYLSTKSDT
KLHKVIRPFATEQIRNFVDDESETYNIMLREMAIHALEIPYHWRMRRLETRWYIDAYEKKHDMNLFL
AEFAKIDFNIVQTAHQEDVKYVSCWWKETGLGSQLHFVRDRIVENYFWTVGMIYEPQFGYIRRIVA
IVAALITVIDDIYDIYGTPEELELFTAMVQNWDINRLDELPEYMKLCFLTLFNEINAMGCDVLKCKNI
DVIPYFKKSWADLCKAYLVEAKWYKGGYKPSVEEYMQNAWISISAPTMLIHFYCAFSGQISVQILE
SLVQQQQDVVRCSATVLRLANDLATSPDELARGDVLKSVQCYMHETGVSEEEARTHVQQMISHT
WDEMNYEARTAARSSSLLSRRFVETAMNLARMSQCMYQHGDGHGCPDKAKIVDRVQTLLVDPIP
LD

>AOP12358 - R-limonene (*C. sinensis*)

MSSCINPSTLATSVNGFKCLPLATNRAAIRIMAKNKPVQCLVSTKYDNLTVDRRSANYQPSIWDHD
FLQSLNSNYTDETYKRRAEELKGKVKTAIKDVTEPLDQLELIDNLQRLGLAYHFEPEIRNILRNIHNH
NKDYNWRKENLYATSLEFRLLRQHGYPVSQEVFSGFKDDKVGFICDDFKGILSLHEASYYSLEGES
IMEEAWQFTSKHLKEMMITSNSKEEDVFVAEQAKRALELPLHWKAPMLEARWFIHVYEKREDKNH
LLLELAKLEFNTLQAIYQEELKDISGWWKDTGLGEKLSFARNRLVASFLWSMGIAFEPQFAYCRRVL
TISIALITVIDDIYDVYGTLDELEIFTDAVARWDINYALKHLPGYMKMCFLALYNFVNEFAYYVLKQQ
DFDMLLSIKHAWLGLIQAYLVEAKWYHSKYTPKLEEYLENGLVSITGPLIITISYLSGTNPIIKKELEFL
ESNPDIVHWSSKIFRLQDDLGTSSDEIQRGDVPKSIQCYMHETGASEEVAREHIKDMMRQMWKK
VNAYTADKDSPLTRTTAEFLLNLVRMSHFMYLHGDGHGVQNQETIDVGFTLLFQPIPLEDKDMAFT
ASPGTKG

Supplementary Figure S4. Amino acid sequences of putative terpene synthase genes of *P. digitatum*, other functionally characterized fungal terpene synthases and its products (NCBI and JGI accesions) and limonene synthase genes from plants (*C. sinensis* and *Arabidopsis thaliana*) used to perform the multiple sequence alignment and phylogenetic tree using ClustalO and MEGA 7.0 softwares.

Supplementary Figure S5. Aerial views and sketch of the random distribution of the sandboxes in each plot. A and B) Aerial view of Moncada (A) and Villarreal (B) plots. C and D) Positioning of the sandboxes in the plots under the tree canopy in Moncada (C) and Villarreal (D) plots. E and F) Sandbox positioning under the tree canopy with intact and infected LSAS and control oranges showing the action of the frugivores in the field.

Green circles represent the trees and white circles with numbers represent the sandboxes.

Supplementary Figure S6. Pictures of footprints and ways of orange eating by frugivores. A) Rabbits eat all the fruit, avoiding the seeds when present. B) Birds eat the fruits avoiding the septa of the segments. C) Rats or mice (*Muridae* family) eat the fruits and leave peel residues.

Supplementary Table S3. Main results of generalized linear mixed models on the effects of *Penicillium digitatum* infection (intact *vs*. infected) and fruit genotype (control vs. LSAS), as well as their second-order interaction, on percentages of emission and content of different chemical classes present on sweet oranges (*Citrus sinensis*) during season 1 and season 2.

	Emission						**Content**					
	Fruit genotype (F)		**Penicillium (P)**		**F*P**		**Fruit genotype (F)**		**Penicillium (P)**		**F*P**	
	F_1	***P***	**F_1**	***P***	**F_1**	***P***	**F_1**	***P***	**F_1**	***P***	**F_1**	***P***
Season 1												
Alcohols	13.04	**0.001**	57.47	**<.0001**	4.61	**0.037**	0.45	0.514	0.01	0.922	0.01	0.936
Aldehydes, ethers and epoxides	3.69	0.060	25.75	**<.0001**	17.2	**0.0001**	65.7	**<.0001**	0.05	0.831	0.04	0.853
Esters	7.85	**0.001**	31.67	**<.0001**	8.8	**0.01**	158.55	**<.0001**	18.93	**0.001**	9.48	**0.01**
Hydrocarbones	1.52	0.223	218.27	**<.0001**	7.71	**0.01**	63.74	**<.0001**	0.25	0.621	0.01	0.933
Ketones	1.38	0.246	10.52	**0.01**	0.51	0.480	68.25	**<.0001**	51.11	**<.0001**	9.19	**0.01**
Limonene	49.36	**<.0001**	269.96	**<.0001**	0.05	0.821	68.01	**<.0001**	0.06	0.803	0.0	0.982
Non identified	4.56	**0.05**	9.56	**0.01**	7.32	**0.01**				**-**		

Season 2

Alcohols	2.68	0.113	534.28	**<.0001**	0.12	0.737	49.39	**<.0001**	126.2	**<.0001**	37.87	**<.0001**
Aldehydes, ethers and epoxides	81.55	**<.0001**	170.37	**<.0001**	10.44	**0.01**	0.83	0.365	36.06	**<.0001**	5.31	**0.01**
Esters	521.72	**<.0001**	246.36	**<.0001**	81.82	**<.0001**	72.33	**<.0001**	46.01	**<.0001**	2.22	0.140
Hydrocarbones	187.24	**<.0001**	109.37	**<.0001**	28.09	**<.0001**	5.81	**0.0185**	0.05	0.828	0.93	0.337
Ketones	12.47	**0.05**	121.88	**<.0001**	0.47	0.497	33.68	**<.0001**	1.66	0.201	0.17	0.681
Limonene	121.6	**<.0001**	39.86	**<.0001**	22	**<.0001**	86.35	**<.0001**	5.65	**0.05**	4.89	**0.05**
Non identified	1.81	0.189	116.41	**<.0001**	1.63	0.212	16.68	**0.0001**	38.2	**<.0001**	32.94	**<.0001**

Supplementary Table S4. Terpene synthase putative genes identified in Ensembl and JGI Genome portals selected based in relevant keywords as queries and pertaining to the isoprenoid synthase domain superfamily protein (IPR008949), terpenoid cyclases/protein prenyltransferase alpha-alpha toroid superfamily protein (IPR08930) and acyclic terpene utilization family protein (AtuA, IPR010839).

Gene	Annotations	Description (if known)
PDIG_33940m	IPR008930, PF00432	Oxidosqualene-lanosterol cyclase and related proteins
PDIG_39630m	IPR008930, PF00432	Beta subunit of farnesyltransferase
PDIG_48940m	IPR008930, PF00432	Protein geranylgeranyltransferase type II, beta subunit
PDIG_59090m	IPR008930. PF00432	Protein geranylgeranyltransferase Type I, beta subunit
PDIG_00550m	IPR008949	Hypothetical protein
PDIG_00600m	IPR008949, PF06330	Trichodiene synthase
PDIG_04920m	IPR008949	Hypothetical protein. Pentalenene synthase
PDIG_05850m	IPR008949	Hypothetical protein. Terpenoid synthase
PDIG_10690m	IPR008949	Phytoene/squalene synthetase
PDIG_17200m	IPR008949, PF00494, PF01044, PS01045	Squalene synthetase. Farnesyldiphosphate farnesyltransferase
PDIG_19740m	IPR008949, PF00348, PF00444	Geranylgeranyl pyrophosphate synthase/Polyprenyl synthetase
PDIG_47830m	IPR008949	Hypothetical protein. Aristolochene synthase
PDIG_50820m	IPR008949, PF03936	Hypothetical protein. Pentalenene synthase
PDIG_52740m	IPR008949	Hypothetical protein. Trichodiene synthase
PDIG_53170m	IPR008949, PF00348, PF00444, PF00723	Pharnesyl pyrophosphate synthase
PDIG_76910m	IPR008949,	Geranylgeranyl pyrophosphate

	PF00348, PF00444	synthase/Polyprenyl synthetase
PDIG_78010m	IPR008949, PF00348, PF00444, PF00723	Geranylgeranyl pyrophosphate synthase/Polyprenyl synthetase
PDIG_83020m	IPR008949, PF03936	Hypothetical protein. Aristolochene synthase
PDIG_23670m	IPR010839, PF07287	Hypothetical protein. AtuA
PDIG_44920m	IPR010839, PF07287	Hypothetical protein. AtuA

Table S5. Primer pairs used to clone TPSs genes from *P. digitatum*, *Arabidopsis thaliana* caryophyllene synthase and (+)-limonene synthase from *C. sinensis* in pET45b(+) with Infusion HD cloning kit (Clontech).

Gene	**Oligo Name**	**Sequence**	**Length (bp)**
PDIG_00600m	00600-F	GACTCGAGTGCGGCCTCAAAGCCCATCCGACCAATAC	37
	00600-R	GACAAGCTTGCGGCCACGAGACAAGCCTTCCCTTTTG	37
PDIG_52740m	52740-F	CTCCCAATTGGGATCCTATGCATCCTCGTCATAAGGTGG	39
	52740-R	ACAAGAGTCCGGATCCTGATCCTATTTCCGCCGAAAACAC	40
A. thaliana caryophyllene synthase (At5g23960)	B201	GCAAGCTTGTCGACCTGCAGTTAAATGGGTATAGTTTCAATG	42
	B202	ACAAGAGTCCGGATCCGGGGAGTGAAGTCAACCGTC	36
C.sinensis (+)-limonene synthase	LIM_F	GACTCGAGTGCGGCCTCAGCCTTTGGTGCCAGG	33
	LIM_R	GACAAGCTTGCGGCCTCTTCTTGCATTAATCCCTCAACC	39

CAPÍTULO IV

IMPACT OF D-LIMONENE SYNTHASE UP- OR DOWN-REGULATION ON SWEET ORANGE FRUIT AND JUICE ODOR PERCEPTION

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ABSTRACT

Citrus fruits are characterized by a complex mixture of volatiles making up their characteristic aromas, being the D-limonene the most abundant one. However, its role on citrus fruit and juice odor is controversial. Transgenic oranges engineered for alterations in the presence or concentration of few related chemical groups enable asking precise questions about their contribution to overall odor, either positive or negative, as perceived by the human nose. Here, either down- or up- regulation of a D-limonene synthase allowed us to infer that a decrease of as much as 51 times in D-limonene and an increase of as much as 3.2 times in linalool in juice were neutral for odor perception while an increase of only 3 times in ethyl esters stimulated the preference of 66% of the judges. The ability to address these questions presents exciting opportunities to understand the basic principles of selection of food.

Keywords: D-limonene, genetically-modified fruits, sensory panel, alcohols, ethyl esters, orange odor perception, OAV, *C. sinensis.*

INTRODUCTION

Citrus types are the most economically relevant and extensively grown fruit tree crops in the world and their fruits are an important source of secondary metabolites for nutrition, health, and industrial applications. Moreover, they are one of the most aromatic edible fruits available (Sharon-Asa et al. 2003). Citrus fruit odor results from a complex combination of soluble and volatile compounds, the latter consisting mostly of mono - and sesquiterpenes, which are accumulated in specialized oil glands in the peel (flavedo) and oil bodies in the juice sacs. Among citrus, sweet orange fruits are the most popular ones (Dugo and Di Giacomo 2002), as they are consumed both fresh and processed into juice. Additionally, orange peels containing abundant fragrant substances are widely used for extracting essential oils which are commercialized for flavoring foods, beverages, perfumes, cosmetics, etc. (Qiao et al. 2008).

The fruit quality attributes are classified into two groups: *i*) internal quality attributes, including texture/mouthfeel, seed presence and number, juice percentage, juice color, flavor (governed by the balance between sugar: acid content plus the concentration of volatile compounds); and *ii*) external quality attributes, related to the appearance and especially important for fruit intended for fresh consumption, such as size, shape, peel color, presence of alterations and defects on the surface (blemishes, puffing,…), etc.; this also includes attributes related to post-harvest shelf life of the fruit, such as antifungal wax treatments, cold storage time and conditions, etc. Quality attributes have strong economical relevance because they are related to consumer perception and ultimately determine marketability, price and use of fruits. They may eventually constrain the success of a citrus industry (Moufida and Marzouk 2003). Nowadays, many quality attributes are evaluated by subjective

methods, but it would be desirable to develop objective standards of human liking.

Although different fruits often share many volatile compounds, each fruit has a distinctive odor that is a function of the proportion of key volatiles and the presence or absence of unique components (Baxter et al. 2005). It is known that in many cases only a limited number of flavor components contribute to the character of an odor (Heath and Reineccius 1986). The olfactory sensory system and the food volatiles with which they interact provide the basis for the diversity of odors and flavors selected by men and found in the human diet (Goff and Klee 2006).

Citrus fruits can be distinguished from other kinds of fruits by a characteristic "citrus-like" odor, but each citrus fruit type differs in cultivars, hybrids and genotypes according to its specific odor attributes. While esters are the most important aroma compounds responsible of the odor in several fruits (Jordán et al. 2001), the oxygenated terpenes and medium length aldehydes are generally considered the primary volatile compounds contributing to odor in citrus fruits and juices (Ahmed et al. 1978). In general, in citrus, oxygenated compounds comprising alcohols and aldehydes, but also ketones, acids, and esters occur in relatively small amounts, though they are widely responsible for the odor and flavor profiles of fruits. D-limonene is the most abundant volatile component of all commercially grown citrus fruits and together with other monoterpene hydrocarbons makes up about 96% of total volatile compounds (Dugo and Di Giacomo 2002). However, its role on citrus fruit and juice odor is controversial. There are reports indicating that it is a relatively important contributor (Buettner and Schieberle 2001, Lin and Rouseff 2001) but others report a minimal active effect on odor and flavor (Baxter et al. 2005, Plotto et al. 2008). Högnadóttir and Rouseff

(2003) suggested that D-limonene might play an odor activity by co-eluting other minor hydrophobic volatiles because it has a low odor threshold (Plotto et al. 2004).

Odors and flavors are major determinants of fruit quality, but these traits are often genetically complex and difficult to score (Galili et al. 2002), making them difficult targets for breeding. Natural variation and genetic engineering in flavor-associated odor volatiles have been used to evaluate the chemistry of tomato fruits, creating a predictive model of liking (Tieman et al. 2012). We have modified the volatile profile of sweet orange fruits by down-regulating a citrus D-limonene synthase gene under the control of the CaMV 35S promoter (Rodríguez et al. 2011a and b). Antisense (AS) down-regulation of D-limonene synthase expression led to reduction in the accumulation of different monoterpene hydrocarbons (up to 100 times less D-limonene in the peel of downregulated fruits) and (likely due to a partial redirection of the pathway) to the accumulation of monoterpenes alcohols, further transformed into aldehydes and ethyl esters, which were only present in low concentrations in empty vector (EV) control fruits (Rodríguez et al. 2011a). AS fruits were found to be more resistant to important diseases caused by bacteria and fungi, such as *Xanthomonas citri* subsp *citri* and *P. digitatum*, respectively, and less attractant to an important citrus pest, the Mediterranean fruit fly *Ceratitis capitata* (Rodríguez et al. 2011a). These fruits are a promising tool for generating broad spectrum resistance against the most important pests and pathogens in citrus worldwide, allowing to reduce the use of highly toxic pesticides.

The availability of these transgenic fruits with the same genetic background in two different orange varieties, Navelina and Pineapple, were used here to assess whether the quantitative or qualitative alteration of several terpenoid volatile organic compounds (VOCs) in

their fruits contributed positively, negatively or were neutral for fruit and juice odor perception.

MATERIAL AND METHODS

Plant materials

Sweet orange transformants used in this work were generated previously in our laboratory (Rodríguez et al. 2011a and b). Briefly, *A. tumefaciens* EHA 105 containing the binary plasmid pBI121 FLM with the D-limonene synthase gene from satsuma mandarin (*Citrus unshiu* Mark) in antisense (AS) orientation under the control of the *Cauliflower mosaic virus* 35S promoter and the nopaline synthase gene (NOS) terminator was used in the different experiments as a vector for the transformation of two sweet orange types: Navelina and Pineapple sweet orange (*C. sinensis* L. Osb.). AS3, AS5 and EV Navelina, and AS11 and EV Pineapple transgenic lines were chosen for our experiments based on their efficient and stable down-regulation (AS) of the limonene synthase gene and low transgene loci number. Ten plants per transgenic line were transferred to orchard conditions in 2008, together with their respective controls (EV; plants transformed with the pBI121 FLM plasmid alone). The experimental orchard was located at Villarreal, Spain (latitude 39°56'40.4''N, longitude 0°08'11.0''W and elevation of 67 m; typical Mediterranean climate), and was approved by the biosafety regulatory authorities (permit B/ES/08/02). All scions were grafted onto Carrizo citrange rootstock and grown in a loamy clay soil using drip irrigation. The orchard was managed as for normal citrus cultivation in the Mediterranean region.

Navelina orange fruits are seedless and they reach optimum maturity in the second half of December, when the ratio of sugars/acids of the fruits reach more than eight, although they can be harvested from mid-October until the end of January depending on the year.

Pineapple orange fruits are seeded and they reach optimum maturity in Spain in the second half of January, when the ratio of sugars/acids of the fruits reach nine, although they can be harvested from second half of December until the end of March depending on the year. For the first season, fruits were harvested on 24th November of 2011 for Navelina sweet orange and on 10th January 2012 for Pineapple sweet orange. For the second season analyzed, fruits were harvested on 17th January of 2013 for Navelina sweet orange and on 28th March 2013 for Pineapple sweet orange.

Phenology

The phenological cycle of every tree in the orchard was evaluated through weekly observations. The predominant phenological stage of development according to BBCH codifications was recorded and grouped into phases stressing flowering and fruit development stages as described in Pons et al. (2012). A visual representation of the phenological cycle of each line was produced by generating phenological calendars (Supplementary Figure S7).

Supplementary Figure S7. Schematic representation of the phenological cycle of trees from the transgenic sweet orange lines Navelina AS3, AS5 and EV, and Pineapple AS11 and EV. Phenological stages were recorded weekly according to the BBCH codification for citrus and grouped into 3 main phases including shoot formation and flowering (yellow), fruit development (green) and maturation (orange) stages.

Analysis of fruit quality

The assessment of fruit quality for the sweet orange lines was performed for the same 2 seasons in which the sensory analyses were performed. Thirty fully mature fruits per tree (grouping in bags of 5 fruits each) were harvested and immediately processed. The following fruit quality parameters were measured and averaged for each sample: total soluble solids (TSS), titratable acidity (TA) and maturity index (MI). The juice with pulp was extracted from the fruit using a rotary citrus squeezer (the same used for sensorial evaluation; Lomi model 4) and, immediately, the TSS was determined in terms of Brix degrees using a refractometer (Atago PR-101 model 0-45 %, Tokyo, Japan). TA of the juice was determined by titration with 0.1 mol L^{-1} NaOH and expressed as the percentage of anhydrous citric acid by weight, using phenolphthalein as a visual endpoint indicator, according to AOAC methods (AOAC. 1980. Official Methods of Analysis, 13th ed. N°46024 and N° 22061. Association of Official Analytical Chemists, Washington. DC). MI was estimated as the TSS/TA ratio.

Extraction of Volatiles and Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Flavedo and juice with pulp tissue was obtained from orange fruits, immediately frozen in liquid nitrogen, and stored at -80 ºC until extraction. The extraction of flavedo volatiles was performed as reported before (Rodríguez et al. 2011a). A Thermo Trace GC Ultra coupled to a Thermo DSQ mass spectrometer with electron ionization mode at 70 eV was used. Frozen ground material (200 mg) was weighed in screw-cap Pyrex tubes and then immediately 3 mL of cold pentane and 25 g of 2-octanol (Fluka; internal standard) were added. Samples were homogenized on ice for 30 s with a Yellowline homogenizer (model DI 25). The suspension was vortexed for 15 s, and 3 mL of MilliQ water were added. The sample was further vortexed for 30 s and centrifuged at 1,800g for 10 min at 4 ºC. The

organic phase was recovered with a Pasteur pipette, and the aqueous phase re-extracted two more times with 3 mL of pentane. A 2 µL aliquot of the pooled organic phases was directly injected into the gas chromatograph-mass spectrometer (GC-MS) for volatile analysis; at least two extractions for each sample were performed.

The volatile compounds of juice with pulp were extracted by headspace solid-phase microextraction (HS-SPME) and analyzed by GC-MS. A 100 µm fiber coated with polydimethylsiloxane (PDMS, Supelco, USA) was used. The fiber was conditioned in the GC injector as indicated by the manufacturer prior to use. 1.5 g of the ground juice with pulp sample was placed in a 7 mL headspace vial containing a stirring bar and sodium chloride (0.45 g) and capped with a 13 mm diameter PFTE/silicone septum. 10 µg of 2-octanol was added as internal standard. The sample was then equilibrated at 37 °C for 10 min under stirring (500 rpm). Afterwards, the vial was incubated with the fiber at 40 ºC for 30 min without stirring. After sampling the headspace volatiles, the fiber was retracted into its sheath and then immediately transferred to the injector port of the GC–MS at 220 ºC and 4 min. Each analytical sample was measured in triplicate. The ion source and the transfer line were set to 200 ºC and 260 ºC, respectively. Volatile compounds were separated on a HP-INNOWax (Agilent JandC Columns) column (30 m x 0,25 mm x 0,25 µm) coupled to a Termo DSQ mass spectrometer. The column temperatures were programmed as follows: 40 ºC for 5 min, raised to 150 ºC at 5 ºC min^{-1}, then raised to 250 ºC at 20 ºC min^{-1} and held for 2 min at 250 ºC. The injector temperature was 220 ºC. Helium was the carrier gas at 1.5 mL min^{-1} in the splitless mode. Electron impact mass spectra were recorded in the 30 to 400 amu range with a scanning speed of 0.5 $scans^{-1}$. Compounds in both pentane or HS-SPME extractions were identified by matching the acquired mass spectra with those stored in the reference libraries (Wiley6, MAINLIB,

REPLIB and National Institute of Standards and Technology) and/or by comparison with authentic standard compounds when available. Data were analyzed by integrating the peak areas of total ion chromatograms using Xcalibur 1.4.z software and quantified by using calibrating curves previously obtained in the laboratory of authentic chemical compounds. The recovery rate of each extraction was calculated with the internal standard (2-octanol) to assure the uniformity of the procedure. The amount of every compound in each sample was calculated as its corrected peak area (by weight and volume) divided by its response factor and recovery rate of the internal standard. The results are reported as the mean values of peak area percent ± SE or in ng/g ± SE from the total identified volatiles in each case.

Published odor thresholds in an orange juice matrix (Plotto et al. 2004, 2008) were used to determine the contribution of the identified compounds to the orange juice aroma by calculating their odour activity values (OAVs). Thus, the interaction between the orange juice matrix and the volatile compound is considered. The OAV is the ratio between a compound concentration and its odor threshold. An OAV higher than 1 is assumed to contribute to that juice aroma.

Preparation of samples for sensory evaluation

Navelina and Pineapple sweet oranges were harvested in the morning of the day of the odor testing and immediately selected for uniformity in size and absence of defects. Navelina is consumed as fresh fruit while Pineapple is used for juice processing.

Fresh fruits. Right after harvesting, Navelina oranges were cut transversely and each half was immediately placed/faced down in a white dish that was completely tasteless and odorless and presented to the panelists at a uniform room temperature.

Fresh juice with pulp. In each analysis, at least 200 fruits were harvested in the morning of the day of the odor testing and groups of 20 oranges each were taken for every juice evaluation session. The juice from each group was extracted using a rotary citrus squeezer with a strainer (Lomi model 4) and immediately pour (including the pulp that passed through filters) into 15 mL-aliquots in a 40 mL-flask with cup and served at a uniform room temperature.

Each sample was identified by a random 3-digit number, different for every assay and the order in which the sample appeared for each level was also random and balanced among subjects.

Sensorial evaluation

Each panel consisted of volunteers (n=54–70, males and females, age range 20-65 years old) from two Research Institutes: Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Spain) and Instituto de Agroquímica y Tecnología de Alimentos (IATA, Paterna, Spain) being all of them frequent citrus fruit and juice consumers. Most panelists participated in all tests, and have performed the same task for the two seasons analyzed. Panels took place in individual booths under white light at room temperature (ISO 8595:2007), usually from 10:00 a.m. to 14:00 p.m. Samples were prepared within 1 h prior to evaluation. Panelists were able to make comments after the evaluation session.

For cut fruit (flavedo and pulp with juice) odor evaluation, a paired comparison was performed (ISO 5495:2005). Panelists were presented with two halves of unpeeled fresh Navelina oranges, one of them being the EV control line (AS3 or AS5 vs. EV halves). They were asked to choose which of the samples they preferred or whether they were able to differentiate between them. In another test, they were asked to choose which sample between both was more intense.

Panelists were first instructed to peel a piece of flavedo of each sample, smell both of them and answer the question. After that, they were instructed to smell the juice with pulp and answer the question. If they could not perceive a difference, they were instructed to guess (forced choice).

For juice with pulp odor evaluation, a ranking test was performed (ISO 4121:2003). Panelists were presented with 3 flasks, corresponding to juice from the three transgenic lines tested of each variety (AS3, AS5 and EV for Navelina or AS11 and EV for Pineapple juice comparison). Panelists were first instructed to uncap the flaks in the appropriate order near their nose and smell. Orange juice odor was scored on a 9-point hedonic category scale varying from 1 (extremely dislike) to 9 (extremely like). For the Friedman tests, the acceptability scores (1 to 9) given by each consumer were converted into rank order numbers (1,2,3 = low quality; 4,5,6 = acceptable quality and 7,8,9 = high quality).

Statistical analysis

For the analysis of the parameters of fruit quality, the variables were checked for normality, and those that deviated were transformed appropriately. Means were compared by the least significance difference (LSD) test. The statistical analyses were all performed using the software package Statgraphics v.5.1 software (Manugistics Inc.) and a significance level (α) of 0.01 was taken into consideration to protect against Type I errors.

For the analysis of data obtained in the paired comparison test of sensory panels, tables based on binomial distribution were used, in which the minimum number of correct judgments to establish significance at various probability levels are given (Roessler et al. 1978). Discrimination tests (paired comparisons) and hedonic ranking

score were analyzed using Fizz Calculations software (Biosystemes, France). A Friedman test was also applied to data obtained from ranking tests (sensory evaluation of juice). In this case the acceptability scores (1 to 9) given by each panelist to the evaluated samples were converted into rank order numbers.

Juice with pulp volatile emission data were compared among lines and together with sensorial evaluations served to establish correlations between chemistry and liking. Flavedo volatile content was tested just for Navelina fruits, as the panelists were taught to cut transversally the flavedo of oranges from this variety, disrupting oil glands and thus releasing the oils directly to the nose.

RESULTS

Phenological calendars and fruit quality attributes were comparable in transformants showing suppressed accumulation of D-limonene and empty vector controls.

Making use of comparative analyses of phenology conducted over two years, we evaluated the equivalence of field-grown D-limonene synthase down-regulated transgenic sweet orange trees relative to their EV controls in terms of plant growth and fruit development. The comparison between AS3, AS5 and EV Navelina and AS11 and EV Pineapple transgenic lines showed that the expression of D-limonene transgenes did not cause any alteration of the main phenotypic and agronomic plant and fruit characteristics (Supplementary Figure S7). Therefore, the modification of D-limonene accumulation in fruit tissues *per se* did not affect the morphological appearance or phenological cycle of the trees.

During ripening there is a decline in titratable acidity of fruits (TA) mostly due to catabolism of citric acid in citrus juice and an increase

in sugars, usually expressed as total soluble solids (TSS). The typical taste and aroma of citrus fruits is determined, besides the accumulation of volatile compounds, by the maturity index (MI) that is the TSS/TA ratio. To assess whether the modification of D-limonene accumulation affected the quality of the transgenic fruits, TSS, TA and MI were evaluated in fruit samples from the orchard-grown transgenic trees of the two varieties in two different harvest seasons. We found no significant differences for any of the parameters analyzed with $P < 0.01$ in Navelina fruits (Table 5A). For Pineapple, we only found a significant difference in TSS between AS11 and EV, but not influencing the final MI (Table 5B). Small differences in TSS and MI values between the first and second season for both cultivars are explained by the fact that fruits were harvested at the beginning and the end of the season, respectively, for both varieties. In this way, we could infer that specific differences in VOC profiles for a given season were mostly attributable to the influence of environmental conditions on fruit development and maturation (within a range of standard commercial MIs for fruit harvesting) and that common differences in both seasons were attributable to the genetic modification performed. We had previously shown that morphological and biochemical characteristics of the orange fruit flavedo were not altered in transformants showing constitutive down-regulation of the D-limonene synthase gene (Rodríguez et al. 2014, 2015). Chlorophyll and total carotenoid contents in EV control green and mature flavedo from Navelina and Pineapple oranges were similar to those found in AS lines (Rodríguez et al. 2014).

Table 4. Average values for the fruit quality variables evaluated for oranges cv. Navelina (4A) and Pineapple (4B). Means separation done by the least significance difference (LSD) test. Means in a column with different letters are statistically different ($P < 0.05$).

Table 4A.

Season	Transgenic line	TA (%)	SSC (ºBrix)	MI (SSC/TA)
First	AS3	1.1 ± 0.17a	8.9 ± 0.06a	8.3 ± 0.19a
First	AS5	1.2 ± 0.13a	9.0 ± 0.16a	7.8 ± 0.06a
First	EV	1.1 ± 0.22a	8.7 ± 0.16a	8.1 ± 0.19a

Season	Transgenic line	TA (%)	SSC (ºBrix)	MI (SSC/TA)
Second	AS3	1.3 ± 0.04a	11.0 ± 0.1a	8.7 ± 0.3a
Second	AS5	1.4 ± 0.06a	12.1 ± 0.1a	8.8 ± 0.4a
Second	EV	1.3 ± 0.03a	11.1 ± 0.3a	8.4 ± 0.2a

TA = titratable acidity; SSC = soluble solids content; MI = maturity index

Table 4B.

Season	Transgenic line	TA (%)	SSC (ºBrix)	MI (SSC/TA)
First	AS11	1.1 ± 0.17a	9.37 ± 0.12a	8.31 ± 0.15a
First	EV	1.1 ± 0.10a	9.33 ± 0.12a	8.71 ± 0.16a

Season	Transgenic line	TA (%)	SSC (ºBrix)	MI (SSC/TA)
Second	AS11	1.0 ± 0.03a	11.0 ± 0.1a	10.8 ± 0.3a
Second	EV	1.3 ± 0.10a	11.8 ± 0.1b	9.5 ± 0.8a

TA = titratable acidity; SSC = soluble solids content; MI = maturity index

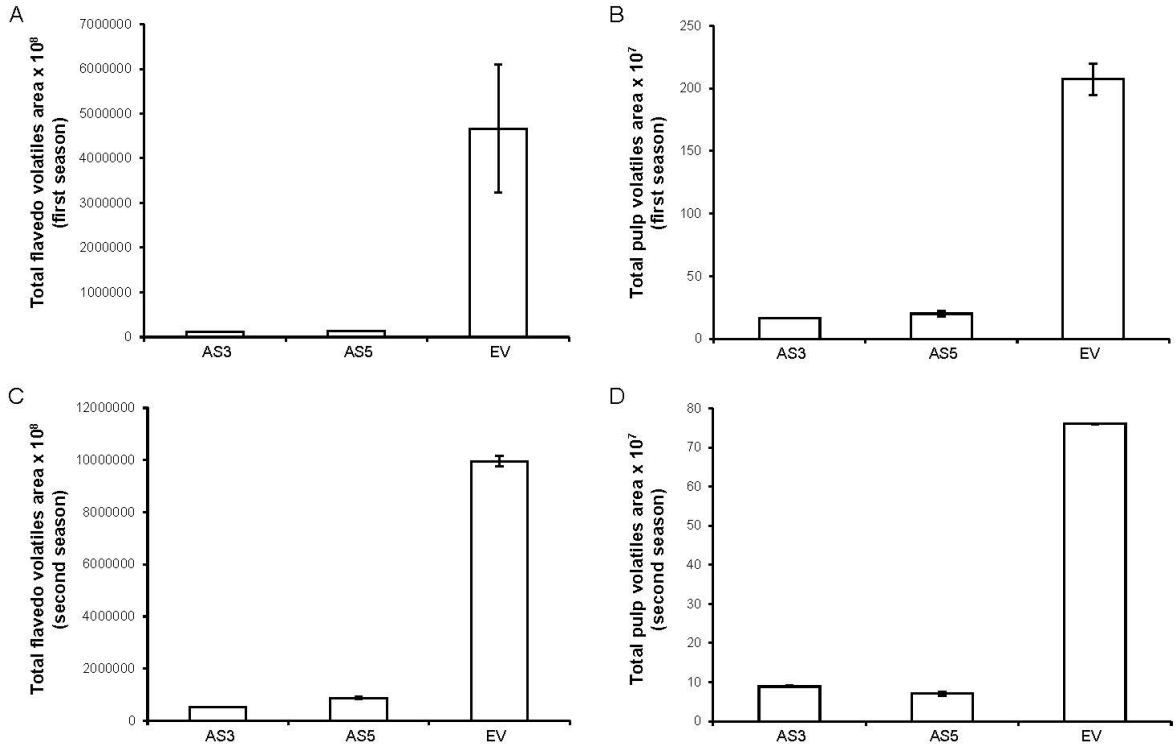
Different and distinctive VOC profiles were found in fruits from D-limonene synthase antisense vs. empty vector control transformants

As a whole in Navelina, EV fruits contained and emitted much more total VOCs than AS fruits (Supplementary Figure S8). For Pineapple juice with pulp, there were quantitative differences between the first and second years for VOC emission in the two transgenic lines, but AS11 always emitted much less VOCs than EV for a same year (Supplementary Figure S9).

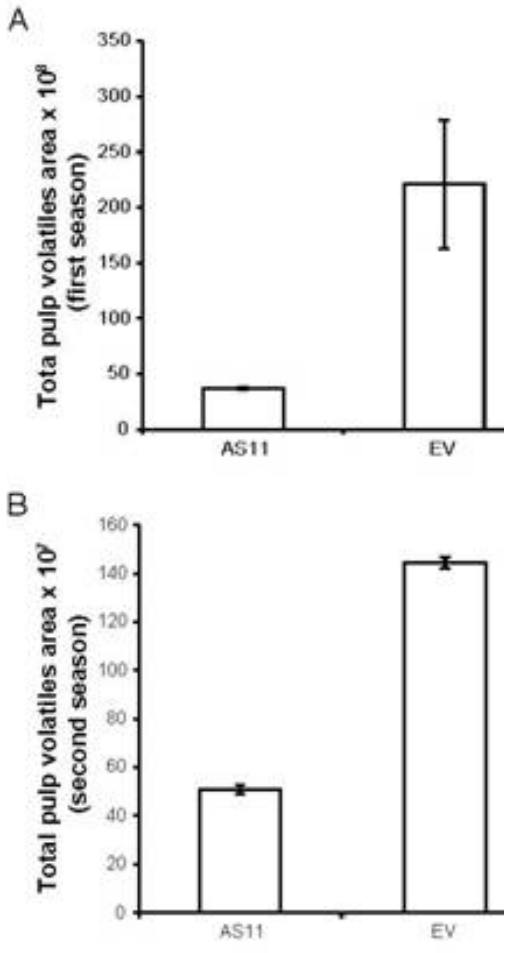
For both sweet orange juice with pulp types, the most conspicuous difference between AS and EV samples was the 2.6 to over 51-fold decrease in emission of D-limonene and the very much reduction in the emission of related monoterpene hydrocarbons including α- and β-myrcene and β-pinene to levels which made some of them undetectable for specific transgenic lines/seasons (Tables 5 and 6). D-limonene synthase down-regulation led to partially blocked accumulation of D-limonene, which caused a diversion of the pathway leading to the about two- to more than three-fold enhanced emission of linalool and additionally, in some samples, related monoterpene alcohols such as α-citronellol and nerol (Tables 5 and 6; Supplementary Tables S6 and S7). As a consequence of this, monoterpene and aliphatic aldehyde emission levels were also generally altered, particularly for both (*Z*)- and (*E*)-citral forms together with hexanal, octanal, nonanal and decanal, especially in the second season evaluated for both sweet orange varieties. Derived from aldehydes, esters and their levels were also modified slightly in some samples. Somehow unrelated sesquiterpene hydrocarbons as valencene, and other terpenes as β-ciclocitral and nootkatone showed significantly lower concentrations in AS than EV samples (Tables 7 and 8).

Therefore, AS juice was characterized by the higher influence of the oxygen fraction (Tables 7 and 8).

Regarding Navelina sweet orange peel, AS samples generally showed a strong decrease in the accumulation of D-limonene and β-myrcene, enhanced levels of linalool and other alcohols (nerol, geraniol and β-citronellol) but reduced concentrations of α-terpineol, and reduced levels of aldehydes, both monoterpene (citral) and aliphatic (octanal, nonanal and decanal) ones when compared with EV controls, resembling major differences found in AS vs. EV juices with pulp. However, valencene and β-ciclocitral were only detected in both AS peels and not in EV samples the second season evaluated (Table 7; Supplementary Table S8).



Supplementary Figure S8. Total normalized volatiles peak areas of Navelina fruits for flavedo (A, C) and juice with pulp (B, D) in the first (A, B) and second (C, D) seasons analyzed.



Supplementary Figure S9. Total normalized volatiles peak areas of Pineapple fruits for juice with pulp in the first (A) and second (B) seasons analyzed.

To assess whether these distinctive VOC profiles could lead to different odor activity values (OAV) for the citrus juices and peel, we evaluated which of these compounds were present in concentrations higher than their threshold value (Tables 5, 6 and 7). In Navelina sweet orange juice, the monoterpene hydrocarbons D-limonene and β-myrcene contributed to odor perception only in the case of EV control fruits, while reaching values much lower than 1 in AS juices. The alcohol linalool was the only compound important in juice odor for all the three AS3, AS5 and EV juices for both seasons analyzed, showing higher OAV usually in AS juices.

Additionally, ethyl hexanoate contributed to odor of only AS5 juice the first season and the aliphatic aldehydes octanal, nonanal and decanal had an impact on odor of EV juices just the second season (Table 5).

In Pineapple sweet orange juices, D-limonene contributed to the odor perception of the two juices types, but OAVs were much lower in

AS11, compared to EV (Table 6). The other major monoterpene hydrocarbon β-myrcene (plus α-pinene the second season) as well as the ethyl esters ethyl butyrate and ethyl hexanoate (just the second season) were affecting odor perception of EV, but not AS11 juices. As in Navelina juices, linalool was the most influential alcohol for AS odor juice perception, especially the first season in which it was contributing to global OAV of only AS11 juice. Moreover, the second season, one of the aliphatic aldehydes, either nonanal or decanal, had an impact on the OAV of AS11 juices, while both compounds enriched the OAV of EV controls. Additionally, valencene had a positive OAV in EV but not AS11 juices the second season (Table 6).

In the case of Navelina sweet orange flavedo, almost all the compounds mentioned before and represented in Table 8 had a positive influence on global OAV, but values were generally much reduced in AS compared to EV fruits, in such a way for minor compounds that α-terpineol (both seasons) and (*E*)-citral (the second season) enriched the global OAV of only EV samples. However, the second season, valencene and β-ciclocitral contributed to global OAV of AS but not EV fruits (Table 8).

The odor thresholds in an orange juice matrix are higher than those obtained in water, but some VOCs showing highly divergent concentrations in AS vs. EV transgenic juices did not show positive OAVs (Tables 5, 6, 7; Supplementary Tables S6, S7 and S8). The possible contribution of VOCs such as the alcohols nerol, β-citronellol or geraniol to odor and flavor perception in AS fruits and juices remains to be further investigated.

Table 5. Orthonasal odor activity values (o-OAVs) calculated as the ratio between a compound concentration and its odour threshold for Navelina sweet orange juices in two consecutive seasons using published thresholds values from a reconstituted pump-out matrix[a,b]

Season 1	Concentration (ng/g)						Odor threshold (ppb)	o-OAVs					
	AS3		AS5		EV			AS3		AS5		EV	
Compound	Media	SE	Media	SE	Media	SE		Media	SE	Media	SE	Media	SE
Monoterpene hydrocarbons													
β-myrcene	122.8a	20.5	102.5 a	34.8	3667.8b	375.5	773	0.16	0.03	0.13	0.04	**4.74***	0.49
Limonene	1976.7a	330.3	2505.3a	15.9	101617.8b	9975.2	13700	0.14	0.02	0.18	0.00	**7.42***	0.73
Monoterpene alcohols													
Linalool	1049.7b	76.8	1277.6b	203.1	400.0a	30.1	113	**9.29***	0.68	**11.31***	1.80	**3.54***	0.27
Monoterpene aldehydes													
(*Z*)-citral	19.3a	0.6	22.2a	3.1	9.9a	4.3	1230	0.02	0.00	0.02	0.00	0.01	0.00
(*E*)-citral	0.0a	0.0	0.0a	0.0	37.7b	6.4	1230	0.00	0.00	0.00	0.00	0.03	0.01
Nonanal	22.3a	1.1	27.7a	0.2	36.8a	16.9	312	0.07	0.00	0.09	0.00	0.12	0.05
Decanal	69.9a	6.0	74.6a	4.6	123.0a	32.1	204	0.34	0.03	0.37	0.02	0.60	0.16
Ethyl esters													
Ethyl hexanoate	0.0a	0.0	10.8a	4.3	0.0a	0.0	3.3	0.00	0.00	**3.26***	1.29	0.00	0.00
Aliphatic esters													
Octyl acetate	19.8a	0.6	13.2a	2.0	25.2a	7.9	2767	0.01	0.00	0.00	0.00	0.01	0.00
Sesquiterpene hydrocarbons													
Valencene	190.0a	7.2	331.1ab	58.0	501.4b	82.7	4756	0.04	0.00	0.07	0.01	0.11	0.02
Others/irregular													
β-cyclocitral	8.3a	0.9	9.7ab	2.1	17.4b	2.2	190	0.04	0.00	0.05	0.01	0.09	0.01

Season 2	Concentration (ng/g)						Odor threshold (ppb)	PROMEDIOS OAVs					
	AS3		AS5		CNA			AS3		AS5		CNA	
Compound	Media	SE	Media	SE	Media	SE		Media	SE	Media	SE	Media	SE
Monoterpene hydrocarbons													
α-pinene	33.8a	2.7	49.0a	39.2	727.2b	39.3	1650	0.02	0.00	0.03	0.02	0.44	0.02
β-myrcene	0.0a	0.0	0.0a	0.0	9376.0b	392.9	773	0.00	0.00	0.00	0.00	**12.13***	0.51
Limonene	5639.8a	147.1	5185.9a	347.1	85038.1b	415.8	13700	0.41	0.01	0.38	0.03	**6.21***	0.03
Monoterpene alcohols													
Linalool	216.4a	21.4	682.7c	46.6	516.0b	6.3	113	**1.91***	0.19	**6.04***	0.41	**4.57***	0.06
α-terpineol	17.5a	1.0	37.2b	2.3	139.3c	0.9	25900	0.00	0.00	0.00	0.00	0.01	0.00
Monoterpene aldehydes													
(*Z*)-citral	0.0a	0.0	0.0a	0.0	20.4b	3.4	1230	0.00	0.00	0.00	0.00	0.02	0.00
(*E*)-citral	0.0a	0.0	6.5a	0.2	22.8b	5.8	1230	0.00	0.00	0.01	0.00	0.02	0.00
Aliphatic aldehydes													
Octanal	0.0a	0.0	0.0a	0.0	969.5a	779.5	233	0.00	0.00	0.00	0.00	**4.16***	3.35
Nonanal	185.7a	13.3	246.2ab	26.8	325.7b	34.5	312	0.60	0.04	0.79	0.09	**1.04***	0.11
Decanal	0.0a	0.0	0.0a	0.0	763.3b	33.8	204	0.00	0.00	0.00	0.00	**3.74***	0.17
Ethyl esters													
Ethyl butanoate	0.0a	0.0	0.0a	0.0	1.1b	0.1	1.71	0.00	0.00	0.00	0.00	0.63	0.06
Aliphatic esters													
Octyl acetate	0.0a	0.0	0.0a	0.0	25.4b	5.6	2767	0.00	0.00	0.00	0.00	0.01	0.00
Sesquiterpene hydrocarbons													
Valencene	834.8c	6.6	284.0a	42.3	572.0b	7.6	4756	0.18	0.00	0.06	0.01	0.12	0.00

*SignificantOAVs

[a,b]Superscript numbers are reference numbers for published thresholds (Plotto et al. 2004, 2008) in an orange juice matrix used to calculate the OAVs.

Table 6. Orthonasal odor activity values (o-OAVs) calculated as the ratio between a compound concentration and its odour threshold for Pineapple sweet orange juices in two consecutive seasons using published thresholds values from a reconstituted pump-out matrix[a,b]

A. Season 1

	Concentration (ng/g)				Odor threshold (ppb)	o-OAVs			
	AS11		EV			AS11		EV	
Compound	Media	SE	Media	SE		Media	SE	Media	SE
Monoterpene hydrocarbons									
α-pinene	0.0a	0.0	912.5b	124.3	1650	0.00	0.00	0.55	0.08
β-myrcene	460.2a	71.5	3603.6ab	1274.3	773	0.60	0.09	4.66*	1.65
Limonene	17376.7a	394.9	102471.5b	27769.4	13700	1.27*	0.03	7.48*	2.03
Monoterpene alcohols									
Linalool	188.1b	16.6	67.2a	1.3	113	1.66*	0.15	0.59	0.01
Monoterpene aldehydes									
(Z)-citral	1.1b	0.1	0.0a	0.0	1230	0.00	0.00	0.00	0.00
Aliphatic aldehydes									
Hexanal	0.0a	0.0	144.8a	74.0	151	0.00	0.00	0.96	0.49
Nonanal	12.1a	1.8	16.4a	11.6	312	0.04	0.01	0.05	0.04
Decanal	7.3a	1.2	4.7a	4.7	204	0.04	0.01	0.02	0.02
Ethyl esters									
Ethyl hexanoate	0.0a	0.0	0.0a	0.0	3.3	0.00	0.00	0.00	0.00
Ethyl-3-hydroxyhexanoate	0.0a	0.0	2.0a	2.0	10716	0.00	0.00	0.00	0.00
Aliphatic esters									
Octyl acetate	1.4b	0.1	0.0a	0.0	2767	0.00	0.00	0.00	0.00
Sesquiterpene hydrocarbons									
Valencene	137.7a	9.7	1689.1b	372.1	4756	0.03	0.00	0.36	0.08
Others/irregular									
β-cyclocitral	0.4a	0.4	4.5b	1.4	190	0.00	0.00	0.02	0.01
Nootkatone	17.8a	13.3	42.1a	13.9	2240	0.01	0.01	0.02	0.01

B. Season 2

	Concentration (ng/g)				Odor threshold (ppb)	o-OAVs			
	AS11		CPI			AS11		CPI	
Compound	Media	SE	Media	SE		Media	SE	Media	SE
Monoterpene hydrocarbons									
α-pinene	556.3a	71.2	3217.5b	10.9	1650	0.34	0.04	**1.95***	0.01
β-myrcene	707.4a	20.8	3058.3b	43.1	773	0.92	0.03	**3.96***	0.06
β-pinene	0.0a	0.0	739.0b	20.1	37200	0.00	0.00	0.02	0.00
Limonene	50407.7a	2710.0	131557.5b	2745.5	13700	**3.68***	0.20	**9.60***	0.20
Monoterpene alcohols									
Linalool	1663.0c	51.3	884.2b	45.9	113	**14.72***	0.45	**7.82***	0.41
α-terpineol	35.2a	1.3	123.7b	10.9	25900	0.00	0.00	0.00	0.00
Monoterpene aldehydes									
(*E*)-citral	0.0a	0.0	33.8b	1.8	1230	0.00	0.00	0.03	0.00
Aliphatic aldehydes									
Nonanal	581.2b	95.3	388.4ab	10.6	312	**1.86***	0.31	**1.24***	0.03
Decanal	131.4a	22.9	451.1c	4.4	204	0.64	0.11	**2.21***	0.02
Ethyl esters									
Ethyl butanoate	0.0a	0.0	4.9a	0.3	1.71	0.00	0.00	**2.88***	0.20
Ethyl hexanoate	0.0a	0.0	397.3b	16.0	3.3	0.00	0.00	**120.39***	4.84
Ethyl-3-hydroxyhexanoate	0.0a	0.0	7.2a	0.6	10716	0.00	0.00	0.00	0.00
Aliphatic esters									
Octyl acetate	0.0a	0.0	202.5b	9.6	2767	0.00	0.00	0.07	0.00
Sesquiterpene hydrocarbons									
Valencene	921.3a	36.9	5293.0c	15.4	4756	0.19	0.01	**1.11***	0.00
Others/irregular									
Nootkatone	0.0a	0.0	24.5b	0.4	2240	0.00	0.00	0.01	0.00

*Significant OAVs

[a,b]Superscript numbers are reference numbers forpublished thresholds (Plotto et al. 2004, 2008) in an orange juice matrix used to calculate the OAVs

*Significant OAVs

[a,b]Superscript numbers are reference numbers for published thresholds (Plotto et al. 2004, 2008) in an orange juice matrix used to calculate the OAVs

Table 7. Orthonasal odor activity values (o-OAVs) calculated as the ratio between a compound concentration and its odour threshold for Navelina sweet orange flavedo in two consecutive seasons using published thresholds values from a reconstituted pump-out matrix[a,b]

Season 1	Concentration (ng/g)						Odor threshold (ppb)	PROMEDIOS OAVs					
	AS3		AS5		CNA			AS3		AS5		CNA	
planta	Media	SE	Media	SE	Media	SE		Media	SE	Media	SE	Media	SE
Monoterpene hydrocarbons													
β-myrcene	1555.4a	332.7	1693.2a	194.9	78067.6b	25212.5	773	2.01	0.43	2.19	0.25	100.99	32.62
Limonene	232620.4a	29094.3	225536.1a	36178.6	21095748.4b	6488717.9	13700	16.98	2.12	16.46	2.64	1539.84	473.63
Monoterpene alcohols													
Linalool	91585.9a	29767.4	94434.8a	32616.4	118665.7a	31068.5	113	810.49	263.43	835.71	288.64	1050.14	274.94
α-terpineol	5146.1a	1113.1	7451.7a	1192.5	74666.9b	25544.5	25900	0.20	0.04	0.29	0.05	2.88	0.99
Monoterpene aldehydes													
(*Z*)-citral	3637.1a	994.0	2381.4a	261.7	19087.6b	5659.8	1230	2.96	0.81	1.94	0.21	15.52	4.60
(*E*)-citral	18989.1a	122.6	19608.0a	496.3	28359.7a	7371.3	1230	15.44	0.10	15.94	0.40	23.06	5.99
Aliphatic aldehydes													
Octanal	18369.4a	7584.9	17528.6a	8425.7	376878.3b	60999.1	233	78.84	32.55	75.23	36.16	1617.50	261.80
Nonanal	3385.4a	2167.6	3917.4a	1635.6	38156.4b	7221.1	312	10.85	6.95	12.56	5.24	122.30	23.14
Decanal	15418.6a	6227.7	14703.0a	6943.3	98116.4b	19151.2	204	75.58	30.53	72.07	34.04	480.96	93.88
Sesquiterpene hydrocarbons													
Valencene	13941.3a	4491.1	22699.5a	4267.1	25221.3a	9439.9	4756	2.93	0.94	4.77	0.90	5.30	1.98
Others/irregular													
3-hexen-1-ol	0.0a	0.0	242.3a	242.3	0.0a	0.0	348	0.00	0.00	0.70	0.70	0.00	0.00
β-cyclocitral	592.3a	179.9	493.9a	278.3	385.9a	385.9	190	3.12	0.95	2.60	1.46	2.03	2.03

Season 2	Concentration (ng/g)						Odor threshold (ppb)	PROMEDIOS OAVs					
	AS3		AS5		CNA			AS3		AS5		CNA	
Compound	Media	SE	Media	SE	Media	SE		Media	SE	Media	SE	Media	SE
Monoterpene hydrocarbons													
β-myrcene	5573.6a	203.9	11006.6a	1331.5	204995.3b	2820.2	773	7.21	0.26	14.24	1.72	265.19	3.65
Limonene	757216.4a	14079.0	1949894.1a	119416.3	44778501.0b	1102475.8	13700	55.27	1.03	142.33	8.72	3268.50	80.47
Monoterpene alcohols													
Linalool	290981.4b	4905.0	381192.8c	10477.3	175885.3a	4224.0	113	2575.06	43.41	3373.39	92.72	1556.51	37.38
α–terpineol	8869.1a	527.9	20723.4a	1309.9	114698.4b	4988.4	25900	0.34	0.02	0.80	0.05	4.43	0.19
Monoterpene aldehydes													
(*Z*)-citral	10679.2a	628.7	10883.4a	974.6	49009.4b	723.3	1230	8.68	0.51	8.85	0.79	39.85	0.59
(*E*)-citral	0.0	0.0	0.0	0.0	63523.8	3113.2	1230	0.00	0.00	0.00	0.00	51.65	2.53
Aliphatic aldehydes													
Octanal	18379.8a	141.0	87895.8b	4749.7	854846.4c	5799.3	233	78.88	0.61	377.24	20.38	3668.87	24.89
Nonanal	6859.8a	290.5	22350.4b	2010.1	83023.1c	589.7	312	21.99	0.93	71.64	6.44	266.10	1.89
Decanal	33660.6a	563.9	99169.2b	3831.7	306443.0c	8072.1	204	165.00	2.76	486.12	18.78	1502.17	39.57
Sesquiterpene hydrocarbons													
Valencene	83520.3b	1337.7	92392.5b	5985.0	0.0a	0.0	4756	17.56	0.28	19.43	1.26	0.00	0.00
Others/irregular													
3-hexen-1-ol	1163.2a	132.6	1744.5a	901.4	3768.1a	642.2	348	3.34	0.38	5.01	2.59	10.83	1.85
b-cyclocitral	2255.5c	62.1	877.3b	67.3	0.0a	0.0	190	11.87	0.33	4.62	0.35	0.00	0.00
Nootkatone	54616.1	6729.0	43152.2	3111.9	47393.9	4923.8	2240	24.38	3.00	19.26	1.39	21.16	2.20

* Significant OAVs

[a,b]Superscript numbers are reference numbers for published thresholds (Plotto et al., 2004, 2008) in an orange juice matrix used to calculate the OAVs

Sensory panelists made fruit and juice with pulp choices correlated with the lack or presence and abundance of certain specific volatile compounds

We next attempted to correlate the different VOC and OAV profiles with sensory responses of citrus cut fruit and juice with pulp of the panelists to generate an estimate of the overall impact of specific VOCs or VOC groups on odor perception. Half-cut fruits or orange juices with pulp were offered to panels from two different research centers consisting of 54-70 volunteers, who were used to consume and evaluate citrus fruits and juices.

In spite of the great differences found in the accumulation of total VOCs and OAVs (mainly D-limonene) in Navelina AS compared to EV fruits (Tables 5 and 7, and Supplementary Table S7), the members of both panels did not perceive any significant difference in the odor intensity of flavedo or juice with pulp between AS3 and EV fruits in any of the two seasons analyzed at $P<0.01$ (Figure 13). They significantly distinguished the odor of the EV cut fruits from that of AS5 ones in the first season but odor choices were comparable between these two lines for the second season (Figure 13). As there were not differences in the total OAVs of AS3 and AS5 vs. EV samples, and the only conspicuous difference in the VOC profile of AS5 peel between the first and second years was a higher accumulation of β-citronellol, nerol and geraniol the first year and this difference was additionally observed when compared to AS3 peels, these compounds may explain panelists' perceptions. Alternatively, much higher OAV for linalool in AS5 vs. EV together with the contribution of ethyl hexanoate to the global OAV of AS5 (and not AS3 and EV) juice with pulp may have also influenced panelists' discriminations. Panelists also found a higher intensity of the juice with pulp odor of AS5 vs. EV fruits in the second season and were able to differentiate between them

(Figure 13G and 13H). That season, AS5 juice with pulp emission was characterized by a higher contribution of linalool to total OAV when compared to AS3 one. Additionally, D-limonene and β-myrcene were lacking in the global OAV of AS5 when compared to that EV juices and the opposite occurred for aliphatic aldehydes (Table 5), which as a whole may explain consumers' discrimination of both juices.

However, all AS3, AS5 and EV fruits were considered to have an "acceptable quality" in a 9-point hedonic evaluation of the juice with pulp odor (results not shown). Some panel members noticed a similarity between AS fruits peel odor and lemon-like or sour orange-like odor, likely related to the increased accumulation of linalool in peel and juice with pulp of AS fruits. Most panelists described the odors associated with AS fruits as with rose or geranium-like notes, in accordance with their VOC composition (Supplementary Tables S6 and S8). Overall, the sweet aroma derived from linalool (and perhaps other alcohols as nerol, β-citronellol or geraniol) would not contribute in AS fruits to any "off-odor" when accumulated and emitted at levels similar to those found in the AS lines.

For Pineapple orange juices with pulp, panelists did not find statistically significant differences at $P<0.01$ between AS11 and EV control juices and their hedonic ratings were also comparable (Figure 13), even when AS11 juice showed a much reduced OAV for D-limonene and lacked β-myrcene (and α-pinene the second year) when compared with OAVs of EV juices. As in the case of Navelina sweet orange AS juices, AS11 emitted much more linalool than EV juice, making both qualitative (1st season) and quantitative (2nd season) contributions to its global OAV. The higher production of linalool (and other alcohols; see Supplementary Table S7) did not affect negatively to panelist scores in this case.

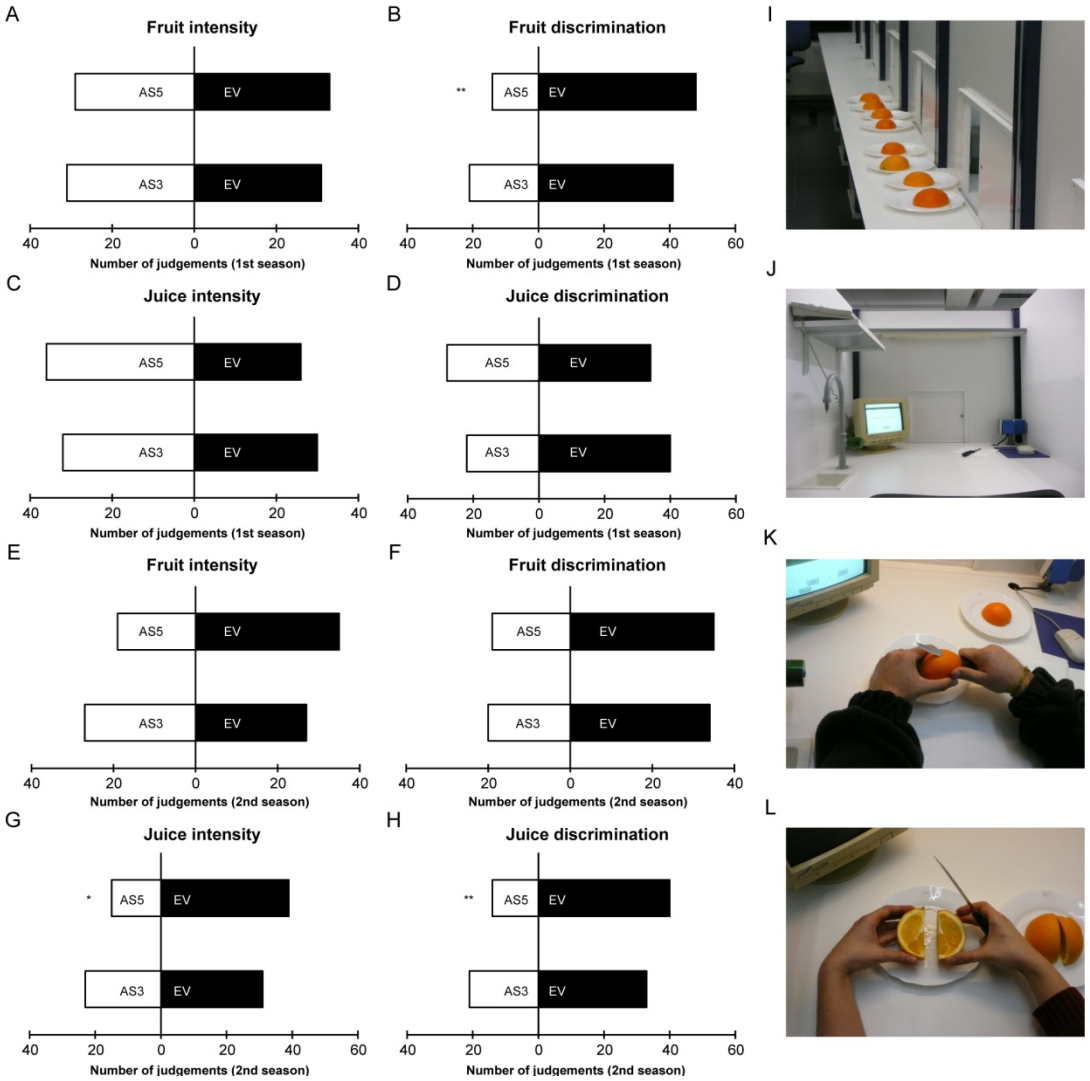


Figure 13. Organoleptic evaluation of fresh-cut fruit and juice with pulp of transgenic Navelina sweet oranges. (A-H) Smell (orthonasal route) evaluations for the odor intensity and discrimination (perceived as different) in fresh-cut fruit and juice with pulp in the comparison of Navelina AS5 vs. EV *vs*. EV samples performed by panelists for two different seasons (n=62 for the first season (A-D) and n=54 for the second season (E-H)). Differences found are statistically significant by two-tailed paired comparisons at $P \leq 0.01$ (*) and $P \leq 0.001$ (**). (I-L) Details of the sensory facility for the odor tests. (I) Individual booths with the two-paired samples presented to the panelists. (J) Situation of the panelist inside the booth. (K) A panelist cutting a Navelina orange fruit before smelling the peel. (L) A panelist before smelling the fresh juice with pulp of a Navelina orange.

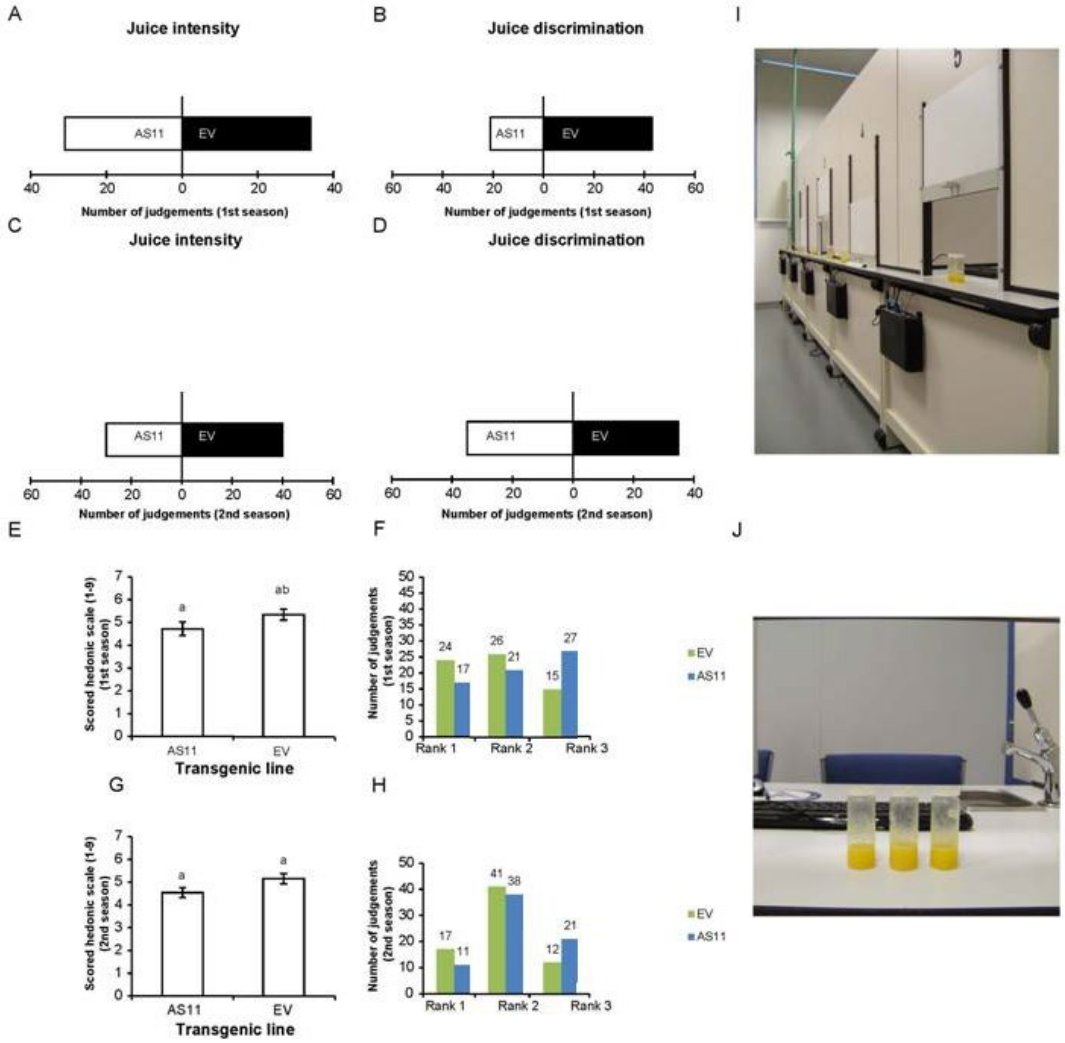


Figure 14. Organoleptic evaluations of fresh-juice with pulp of transgenic Pineapple sweet oranges. (A-D) Smell (orthonasal route) evaluations for the juice-odor intensity and discrimination (perceived as different) in the comparison of Pineapple AS11 *vs.* EV vs. EV samples performed by panelists for two different seasons (n=65 for the first season (A, B) and n=70 for the second season (C, D)). Differences found are statistically significant by two-tailed paired comparisons at $P \leq 0.01$ (*) and $P \leq 0.001$ (**). (E-H) Mean hedonic scores and ranking (Friedman tests) after the sensory evaluation of the fresh juice from different transgenic Pineapple oranges using an hedonic scale where 1=dislike extremely to 9=like extremely. Scaled values were grouped using ranks where Rank 1 included values 7 to 9, Rank 2 included values 4 to 6 and Rank 3 included values 1 to 3 in Friedman tests (F and H). Means followed by the same letter are not significantly different ($P \leq 0.01$). (I-J) Details of the sensory facility for the smelling tests. (I) Individual booths with the

juice samples presented to the panelists for the juice-odor intensity and preference tests. (J) Juice samples presented to the panelists for the hedonic tests.

DISCUSSION

In the context of plant genetics, breeding for quality means improving traits such as flavor, nutrition, appearance and postharvest processing. In citrus fruits, genetic engineering have been already used to achieve resistance to an important postharvest disease as the green mold rot caused by *P. digitatum*, fruit resistance to citrus canker caused by the bacterium *Xanthomonas citri subsp. citri* and less attraction to the Medfly pest *Ceratitis capitata* (Rodríguez et al., 2011a), and to increase β-carotene content of the juice, thus enhancing its antioxidant properties *in vivo* (Pons et al. 2014). The potential for plant metabolic engineering to increase the accumulation and emission of specific fruit odor compounds could allow transferring such desirable quality traits into mature tissues of elite genotypes. However, before that, it is essential uncovering chemical groups of compounds that may be discriminated by our olfactory sensory system from complex mixtures and either improve or decrease the quality of a blend. In tomato, fruit-specific geraniol synthase over-expression led to a highly increased accumulation of monoterpene alcohols, aldehydes, esters and oxides as well as hydrocarbons as expense of reduced lycopene, but these fruits were preferred over control counterparts by panelists (Davidovich-Rikanati et al. 2007). In another work, transgenic tomato plants were modified to no longer express a 13-lipoxygenase gene (*LoxC*) whose product catalyzes the first step in the metabolic pathway that converts 18:2 and 18:3 fatty acids to C6 volatiles such as cis-3-hexenal, hexanal, cis-3-hexen-1-ol,hexyl alcohol and hexyl acetate. Consumers were able to distinguish the transgenic (unable to produce C6 volatiles) from control fruits but it did not affect their preferences (Tieman et al. 2012).

D-limonene synthase down-regulated orange fruits offer an unprecedented tool to study the influence of D-limonene and related terpene compounds (mainly qualitatively but also quantitatively altered) in whole cut fruit and juice quality as perceived by odor panelists. D-limonene is the most abundant terpene compound in sweet orange as well as in most citrus fruits (Dugo and Di Giacomo 2002). In AS fruits, its concentration was reduced at least 90 times in the peel, reaching very low OAVs, and 6 times in the juices, thus lacking OAV, when compared to EV controls. However, panelists did not differentiate and neither find significant differences in intensity between both AS and EV transgenic types and in both orange cultivars, Navelina and Pineapple. In spite of its high accumulation, the role that D-limonene plays in orange fruit and juice odor is not clear. It was rated as a prominent contributor of citrus juice aromas (Selli and Kelebek 2011), a barely aroma active compound (Perez-Cacho and Rouseff 2008), a mid-potency VOC (Choi 2005) and a negative contributor to citrus juice aromas (Tietel et al. 2011). In flavor modeling studies, D-limonene was considered to be important to mimic orange juice odor (Ahmed et al. 1978, Buettner and Schieberle 2001). Our results indicate that D-limonene contributed little to sweet orange odor but we cannot discard the idea that it is acting in the complex VOC mixture through additive or synergistic effect with other orange odor components, serving as a solvent for the other compounds (Perez-Cacho and Rouseff 2008).

Apart from drastically reduced D-limonene concentrations, AS juices showed higher accumulation of monoterpene alcohols, mainly linalool, which strongly contributed both quantitatively and qualitatively to their total OAVs. Other alcohols asnerol, β-citronellol and geraniol also showed increased concentrations in AS vs. EV juices thought none of them reached OAVs above 1. However, floral notes generally provided by them were perceived by most panelists. Although their

accumulation levels varied between transgenic lines and seasons (but not much between varieties), some of these alcohols reached concentrations typically found in certain sour orange, lemon and lime genotypes and such distinctive blend was also noticed by panelists. It is possible that having a much reduced amount of D-limonene as a solvent in AS juices would increase the volatility of these compounds thus influencing their perception. Nevertheless, typical AS odor had not influence on panelist differentiations, odor intensities and hedonic scores, considering that they were chosen or classified at comparable rates to EV control fruits and juices for both Navelina and Pineapple varieties. However, in the specific case of Navelina AS5 samples panelists perceived them as different, less intense than EV ones in the first season for the cut fruit and in the second season for the juice. In the first case, it coincided with the important contribution of linalool together with ethyl hexanoate to the global OAV of AS5 (and not AS3) juice with pulp as well as with the lack of OAV for D-limonene and other monoterpene hydrocarbons. However, panelists did not find the odor of AS5 whole cut fruit or juice unpleasant, but different, being considered by some panelists as oranges smelling like lemons or limes. Considering that TSS and TA of AS5 fruit was characteristic of mature oranges and comparable to those of EV and AS3 fruits, it worth testing how panelists would feel the taste and aroma of AS5 fruit and its juice compared to EV counterparts.

It is widely considered that the alcohol linalool has a substantial contribution to orange fresh fruit and juice flavor (Ahmed et al. 1978, Bazemore et al. 2003), being pondered as one of the three most prominent constituents of good quality peel oil and orange juice (Macleod et al. 1988). It also characterizes the floral odor of fresh and processed mandarins and the peel oil of clementines (Buettner et al. 2003, Schieberle et al. 2003) and contributes to the refreshing floral aroma of orange peel and juice (Macleod et al. 1988, Qiao et al.

2008). Other terpene alcohols such as β-citronellol and geraniol have also been found to add fruity aromas to the essence oils of oranges (Högnadóttir and Rouseff 2003). Therefore, it could be expectable that the relative increase in the concentration of these alcohols, especially linalool, in orange fruits may lead to generation of new varieties with more pleasant odor and aroma, similar to those of lemons, limes or bergamots. Our results seem to contradict in part these expectations, although in our transgenic fruits linalool increases were generally correlated to D-limonene strong decreases and vice versa. It is possible that a better compensated concentration of both compounds may generate more pleasant fruits.

We have previously shown that antisense down-regulation of D-limonene synthase in the sweet orange peel induced a drastic decrease in the accumulation of D-limonene plus related monoterpene hydrocarbons while concentrations of other terpene compounds including monoterpene alcohols, aldehydes and esters were also altered (Rodríguez et al. 2011a). This led to constitutive activation of plant natural defenses and consequently to resistance to diverse fungal and bacterial pathogens as well as less attraction to an important citrus pest (Rodríguez et al. 2011a, 2014). Here, we have been interested in investigating whether differences in the accumulation and emission of terpene compounds by these genetically modified sweet orange fruits would affect negatively odor perception by potential consumers, thus precluding further development of this promising biotechnological product. Moreover, the availability of AS fruits and juices with null OAVs for D-limonene and related monoterpene hydrocarbons as well as much higher OAVs for linalool and their isogenic counterparts with regular concentrations and OAVs for these compounds, allowed us to study the role of specific VOCs or VOC groups in the odor of orange fruit and juice. We show here that the lack of D-limonene and monoterpene hydrocarbons in the global

OAV of sweet orange juices was neutral for intensity and panelists did not perceive them as different to regular controls. Conversely, in spite of the important role widely attributed to linalool as well as other oxygenated terpenes as positive contributors to orange odor, in our case, the unbalance of not only linalool but also D-limonene and other minor compounds in the same fruit and juice backgrounds could be responsible of the consideration of increased linalool concentrations as neutral. More studies are needed to assess whether linalool and/or the other oxygenated terpenes may play a different role in flavor panels. Our data provide clues for understanding which specific chemical groups influence odor juice and fruit perception. This is essential to better select targets for molecular engineering of aroma and flavor.

In conclusion, our results indicate that AS down-regulation of D-limonene synthase and the consequent modification of fruit odor by genetic engineering did not affect negatively sweet orange fruit and juice intensity and discrimination. Moreover, as AS fruits have antimicrobial and pesticide activities, such modifications may also improve shelf-life of stored fruits and/or reduce synthetic pesticide use, which could influence positively to the consumers perception.

Acknowledgements

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Supplementary Table S6. Volatile components identified (%) in juice with pulp of cv. Navelina fruits analyzed by GC-MS in the first season (S6A) and second season (S6B).

S6A.

COMPOUND	COMPOUND NAME	AS3		AS5		EV	
		M	S	M	S	M	S
Monoterpene hydrocarbons (MH)							
1	Sabinene	3	0	4	0	0	0
2	δ-3-carene	0	0	0	0	0	0
3	β-myrcene	0	0	0	0	1	0
4	Limonene	2	3	2	3	9	0
5	τ-terpinene	0	0	0	0	0	0
7	β-ocimene	0	0	0	0	0	0
8	α-terpinolene	0	0	0	0	0	0
Sesquiterpene hydrocarbons (SH)							
16	α-copaene	0	0	0	0	0	0
18	β-cubebene	0	0	0	0	0	0
21	β-elemene	0	0	1	0	0	0
27	β-selinene	0	0	0	0	0	0
28	α-caryophyllene	0	0	0	0	0	0
31	τ-selinene	0	0	0	0	0	0
33	Eudesmadiene	0	0	0	0	0	0
34	τ-gurjunene	0	0	0	0	0	0
35	Germacrene-D	0	0	0	0	0	0
36	Valencene	9	0	1	0	1	0
37	α-selinene	0	0	0	0	0	0
42	δ-cadinene	0	0	0	0	0	0
Alcohols (ALC)							
10	1-hexanol	0	0	0	0	0	0
11	(*Z*)-3-hexen-1-ol	0	0	0	0	0	0
14	1-heptanol	0	0	0	0	0	0
19	Linalool	2	1	2	0	0	0
20	1-octanol	4	0	2	0	0	0
22	4-terpineol	1	0	1	0	0	0
29	1-nonanol	0	0	0	0	0	0
26	*p*-mentha-trans-2,8-dien-	0	0	0	0	0	0
41	(*Z*)-carveol	0	0	0	0	0	0
44	1-decanol	1	0	0	0	0	0
45	β-citronellol	2	0	2	0	0	0
47	Nerol	7	0	7	0	0	0
49	(*E*)-carveol	0	0	0	0	0	0
48	3-cyclohexene ethanol	0	0	0	0	0	0
50	Geraniol	1	0	0	0	0	0
51	(*Z*)-carveol	0	0	0	0	0	0
53	*p*-menth-1-en-9-ol	0	0	0	0	0	0
55	Perilla alcohol	0	0	0	0	0	0

Aliphatic aldehydes (AA)							
12	Nonanal	0	0	0	0	0	0
17	Decanal	1	0	0	0	0	0
23	Undecanal	0	0	0	0	0	0
Monoterpene aldehydes (MA)							
32	(*Z*)-citral	0	0	0	0	0	0
40	(*E*)-citral	0	0	0	0	0	0
46	Perilla aldehyde	0	0	0	0	0	0
Ethyl esters (EE)							
6	Ethyl hexanoate	0	0	0	0	0	0
13	Ethyl octanoate	0	0	0	0	0	0
Aliphatic and monoterpene esters (AME)							
25	Octyl butyrate	0	0	0	0	0	0
15	*n*-octyl acetate	1	0	0	0	0	0
30	Citronellyl acetate	0	0	0	0	0	0
39	Neryl acetate	7	0	4	0	0	0
43	Geranyl acetate	1	0	0	0	0	0
52	Perilla acetate	0	0	0	0	0	0
Other compounds (OC)							
9	2-octanone	3	0	3	0	0	0
24	β-cyclocitral	0	0	0	0	0	0
38	δ-carvone	0	0	0	0	0	0
54	Caryophyllene oxide	0	0	0	0	0	0

S6B.

COMPOUND	COMPOUND NAME	AS3		AS5		EV	
		ME	S	ME	S	ME	S
Monoterpene hydrocarbons							
4	α-pinene	0,1	0,	0,2	0,	0,3	0,
6	Sabinene	1,3	0,	1,7	0,	0,3	0,
7	β-myrcene	0,0	0,	0,0	0,	3,8	0,
9	α-	0,0	0,	0,0	0,	0,1	0,
10	δ-3-Carene	0,0	0,	0,0	0,	0,2	0,
11	α-terpinene	0,0	0,	0,0	0,	0,1	0,
12	Limonene	48,	0,	55,	0,	84,	0,
13	β-terpinene	0,5	0,	0,7	0,	0,0	0,
14	β-	0,0	0,	0,0	0,	1,0	0,
15	τ-terpinene	0,7	0,	1,0	0,	0,2	0,
16	Terpinolene	0,2	0,	0,3	0,	0,2	0,
Sesquiterpene hydrocarbons							
33	α-cubebene	0,3	0,	0,2	0,	0,0	0,
35	α-copaene	0,9	0,	0,9	0,	0,4	0,
36	β-elemene	0,5	0,	0,3	0,	0,3	0,
37	Germacrene-	0,5	0,	0,4	0,	0,1	0,
39	β-	1,3	0,	2,3	0,	0,2	0,
40	τ-muurolene	0,0	0,	0,0	0,	0,0	0,
41	β-selinene	0,7	0,	0,3	0,	0,0	0,

42	α-	0,3	0,	0,3	0,	0,0	0,
43	γ-selinene	0,4	0,	0,1	0,	0,0	0,
44	γ-gurjunene	0,0	0,	0,0	0,	0,0	0,
45	τ-selinene	2,1	0,	0,6	0,	0,2	0,
46	Valencene	30,	0,	12,	0,	2,4	0,
47	α-selinene	1,8	0,	0,7	0,	0,1	0,
48	(-)-	0,6	0,	0,2	0,	0,0	0,
49	δ-Cadinene	0,8	0,	0,8	0,	0,2	0,
50	α-panasinsen	1,4	0,	0,4	0,	0,1	0,
Alcohols (ALC)							
5	Heptanol	0,0	0,	0,1	0,	0,0	0,
17	Linalool	3,4	0,	13,	0,	0,9	0,
19	4-Terpineol	1,2	0,	0,6	0,	0,1	0,
22	α-terpineol	0,3	0,	0,8	0,	0,2	0,
23	β-citronellol	0,0	0,	0,2	0,	0,0	0,
24	Nerol	0,0	0,	0,3	0,	0,0	0,
38	*p*-menth-1-	0,2	0,	0,2	0,	0,0	0,
Aliphatic aldehydes (AA)							
2	(*E*)-2-Hexenal	0,1	0,	0,4	0,	0,0	0,
8	Octanal	0,0	0,	0,0	0,	0,6	0,
18	Nonanal	1,1	0,	1,8	0,	0,2	0,
21	Decanal	0,0	0,	0,0	0,	0,5	0,
Monoterpene aldehydes (MA)							
26	(*Z*)-citral	0,0	0,	0,0	0,	0,0	0,
27	(*E*)-citral	0,0	0,	0,3	0,	0,1	0,
28	Perilla	0,0	0,	0,0	0,	0,0	0,
Ethyl esters (EE)							
1	Ethyl butyrate	0,0	0,	0,0	0,	0,0	0,
Aliphatic and monoterpene							
3	Methyl	0,0	0,	0,0	0,	0,0	0,
20	Octyl acetate	0,0	0,	0,0	0,	0,1	0,
25	Linalool	0,0	0,	0,5	0,	0,0	0,
29	(*E*)-carvyl	0,0	0,	0,0	0,	0,2	0,
30	(*Z*)-carvyl	0,0	0,	0,0	0,	0,0	0,
31	Citronellyl	0,0	0,	0,0	0,	0,0	0,
32	Nerol acetate	0,0	0,	0,0	0,	0,0	0,
34	Geraniol	0,0	0,	0,0	0,	0,0	0,

TI Tentative Identification

Supplementary Table S7. Volatile components identified (%) in juice with pulp of cv. Pineapple fruits analyzed by GC-MS in the first season (S7A) and second season (S7B).

S7A.

COMPOUND NUMBER	COMPOUND NAME	AS11		EV	
		MEAN	SE	MEAN	SE
Monoterpene hydrocarbons (MH)					
1	α-pinene	0,00	0,00	0,44	0,04
3	Sabinene	0,38	0,11	0,28	0,01
4	β-myrcene	0,96	0,13	1,25	0,08
5	Limonene	87,93	0,06	87,91	0,50
Sesquiterpene hydrocarbons (SH)					
15	α-cubebene	0,00	0,00	0,03	0,01
20	α-copaene	0,32	0,03	0,11	0,06
23	β-cubebene	0,26	0,02	0,22	0,07
26	β-elemene	0,18	0,02	0,17	0,00
27	β-caryophyllene	0,05	0,00	0,08	0,01
31	Aromandrene	0,04	0,01	0,10	0,00
32	β-cadinene	0,00	0,00	0,03	0,01
33	α-humulene	0,04	0,00	0,02	0,00
35	τ-selinene	0,02	0,00	0,05	0,00
38	β-maaliene	0,22	0,02	0,35	0,01
39	α-gurjunene	0,04	0,01	0,10	0,00
40	Germacrene-D	0,13	0,00	0,08	0,01
41	Valencene	2,94	0,28	6,18	0,18
42	α-selinene	0,19	0,01	0,40	0,03
45	Eremophilene	0,00	0,00	0,16	0,00
48	δ-cadinene	0,52	0,00	0,55	0,04
Alcohols (ALC)					
10	1-hexanol	0,02	0,00	0,00	0,00
11	(*Z*)-3-hexen-1-ol	0,05	0,01	0,02	0,00
16	1-heptanol	0,17	0,04	0,04	0,01
17	6-methyl-hept-5-	0,08	0,02	0,02	0,00
24	Linalool	1,76	0,11	0,11	0,02
25	1-octanol	0,03	0,00	0,00	0,00
28	4-terpineol	0,03	0,01	0,01	0,00
30	(*E*)-*p*-mentha-2,8-	0,00	0,00	0,01	0,00
50	β-citronellol	0,31	0,02	0,01	0,00
52	Nerol	0,07	0,01	0,00	0,00
53	*p*-menth-3-en-1-ol	0,01	0,00	0,04	0,00
54	Geraniol	0,02	0,00	0,00	0,00
Aliphatic aldehydes (AA)					
2	Hexanal	0,00	0,00	0,08	0,01
6	(*E*)-2-hexenal	0,07	0,03	0,09	0,01
12	Nonanal	0,04	0,01	0,01	0,00
22	Decanal	0,05	0,01	0,01	0,00
Monoterpene aldehydes (MA)					

19	Citronellal	0,04	0,00	0,00	0,00
37	(*Z*)-citral	0,02	0,00	0,00	0,00
51	Perilla aldehyde	0,00	0,00	0,08	0,01
Ethyl esters (EE)					
7	Ethyl hexanoate	0,00	0,00	0,00	0,00
13	Ethyl octanoate	0,00	0,00	0,01	0,01
36	Ethyl 3-	0,00	0,00	0,01	0,00
Aliphatic and monoterpene esters					
18	*n*-octyl acetate	0,00	0,00	0,11	0,00
34	Citronellyl acetate	0,16	0,03	0,00	0,00
44	Neryl acetate	0,52	0,07	0,00	0,00
47	(*E*)-carvyl acetate	0,00	0,00	0,02	0,00
49	Geranyl acetate	0,08	0,02	0,02	0,00
55	(*E*)-geranylacetone	0,46	0,00	0,21	0,01
56	Limonen-10-yl	0,00	0,00	0,03	0,01
Other compounds (OC)					
8	2-octanone	1,19	0,09	0,19	0,03
9	Cyclohexane, 2-ethenyl-1,1-	0,10	0,02	0,02	0,00
14	(*E*)-limonene oxide	0,00	0,00	0,05	0,02
21	1-hexanol-2-ethyl	0,02	0,01	0,02	0,00
29	β-cyclocitral	0,00	0,00	0,01	0,00
43	δ-carvone	0,00	0,00	0,05	0,01
46	2-Cyclohexen-1-one, 2,4,4-	0,08	0,01	0,00	0,00
57	β-ionone	0,01	0,00	0,03	0,00
58	Nootkatone	0,37	0,27	0,18	0,06

S7B.

COMPOU	COMPOUND NAME	AS11		EV	
		M	S	M	S
Monoterpene hydrocarbons (MH)					
4	α-thujene	0	0	0	0
5	α-pinene	0	0	0	0
8	Sabinene	0	0	0	0
9	β-myrcene	3	0	5	0
11	β-pinene	0	0	0	0
12	α-phellandrene	0	0	0	0
13	δ-3-Carene	0	0	0	0
14	α-terpinene	0	0	0	0
15	Limonene	7	1	6	0
16	β-phellandrene	0	0	1	0
17	τ-terpinene	0	0	0	0
18	Terpinolene	0	0	0	0
Sesquiterpene hydrocarbons (SH)					

40	α-cubebene	0	0	0	0
43	α-copaene	0	0	0	0
44	β-elemene	0	0	0	0
45	Germacrene-D	0	0	0	0
50	β-caryophyllene	0	0	0	0
51	β-cubebene	0	0	0	0
52	β-selinene	0	0	0	0
55	γ-selinene	0	0	0	0
56	γ-gurjunene	0	0	0	0
57	τ-selinene	0	0	0	0
58	Valencene	5	0	1	0
59	α-selinene	0	0	0	0
60	(-)-	0	0	0	0
61	δ-Cadinene	0	0	0	0
62	α-panasinsen	0	0	0	0
53	α-caryophyllene	0	0	0	0
64	β-panasinsene	0	0	0	0
Alcohols (ALC)					
7	Heptanol	0	0	0	0
19	Linalool	4	0	0	0
23	(*Z*)-*p*-Mentha-1,8-	0	0	0	0
24	(*Z*)-carveol	0	0	0	0
28	4-Terpineol	0	0	0	0
30	α-terpineol	0	0	0	0
31	β-citronellol	0	0	0	0
32	Nerol	0	0	0	0
46	*p*-menth-1-en-9-ol	0	0	0	0
Aliphatic aldehydes (AA)					
2	(E)-2-Hexenal	0	0	0	0
6	(*Z*)-2-heptenal	0	0	0	0
20	Nonanal	0	0	0	0
29	Decanal	0	0	0	0
Monoterpene aldehydes (MA)					
34	(*E*)-citral	0	0	0	0
35	Perilla aldehyde	0	0	0	0
Ethyl esters (EE)					
1	Ethyl butyrate	0	0	0	0
10	Ethyl hexanoate	0	0	0	0
22	Ethyl 3-	0	0	0	0
Aliphatic and monoterpene esters					
3	Methyl hexanoate	0	0	0	0
25	Butyl hexanoate	0	0	0	0
26	Ethyl octanoate	0	0	0	0
27	Octyl acetate	0	0	0	0
42	Decyl acetate	0	0	0	0
36	(*E*)-carvyl acetate	0	0	0	0
37	(*Z*)-carvyl acetate	0	0	0	0
38	Citronellyl acetate	0	0	0	0

39	Neryl acetate	0	0	0	0
41	Geraniol acetate	0	0	0	0
48	Perilla acetate	0	0	0	0
Other compounds (OC)					
21	Geraniol formate	0	0	0	0
33	δ-carvone	0	0	0	0
47	α-ionone	0	0	0	0
49	Geranyl acetone	0	0	0	0
54	β-ionone	0	0	0	0
63	Caryophyllene oxide	0	0	0	0
65	Nootkatone	0	0	0	0

Supplementary Table S8. Volatile components identified (%) in flavedo of cv. Navelina fruits analyzed by GC-MS in the first season (S8A) and second season (S8B).

Table S8A.

COMPOUND NUMBER	COMPOUND NAME	AS3		AS5		EV	
		ME	SE	ME	SE	ME	SE
Monoterpene hydrocarbons (MH)							
1	Sabinene	1	0	1	0	1	0
2	δ-3-carene	0	0	0	0	0	0
3	β-myrcene	0	0	1	0	1	0
4	Limonene	3	3	2	6	9	0
5	β-ocimene	0	0	2	0	0	0
6	α-terpinolene	0	0	0	0	0	0
Sesquiterpene hydrocarbons (SH)							
15	α-copaene	0	0	0	0	0	0
21	Germacrene-D	0	0	0	0	0	0
22	β-elemene	0	0	1	0	0	0
28	α-humulene	0	0	0	0	0	0
30	β-farnesene	0	0	0	0	0	0
33	Valencene	2	0	6	0	0	0
34	α-muurolene	0	0	0	0	0	0
36	τ-muurolene	0	0	0	0	0	0
Alcohols (ALC)							
8	(*Z*)-3-hexen-1-ol	0	0	0	0	0	0
12	1-heptanol	0	0	0	0	0	0
13	(*Z*)-sabinene hydrate	0	0	0	0	0	0
17	(*E*)-sabinene hydrate	0	0	0	0	0	0
19	Linalool	2	6	2	6	0	0
20	1-octanol	0	0	2	0	0	0
23	4-terpineol	0	0	0	0	0	0
26	(*E*)-*p*-mentha-2,8-dienol	0	0	0	0	0	0

32	α-terpineol	0	0	0	0	0	0
38	1-decanol	0	0	0	0	0	0
39	β-citronellol	1	0	3	0	0	0
41	τ-isogeraniol	0	0	0	0	0	0
42	Nerol	3	0	9	2	0	0
45	(*E*)-carveol	0	0	0	0	0	0
46	Geraniol	0	0	1	0	0	0
48	(*Z*)-carveol	0	0	0	0	0	0
51	Perilla alcohol	0	0	0	0	0	0
52	Nerolidol	0	0	0	0	0	0
53	Elemol	0	0	0	0	0	0
56	Farnesol	0	0	0	0	0	0
Aliphatic aldehydes (AA)							
7	Octanal	1	0	0	0	1	0
9	Nonanal	0	0	0	0	0	0
16	Decanal	2	1	1	1	0	0
24	Undecanal	0	0	0	0	0	0
27	2-decanal	0	0	0	0	0	0
44	Decadienal	0	0	0	0	0	0
Monoterpene aldehydes (MA)							
14	Citronellal	2	0	1	0	0	0
31	(*Z*)-citral	1	0	0	0	0	0
35	(*E*)-citral	6	0	6	0	0	0
40	Perilla aldehyde	0	0	0	0	0	0
Aliphatic and monoterpene esters							
29	Citronellyl acetate	0	0	0	0	0	0
37	Geranyl acetate	0	0	1	0	0	0
47	Limonen-10-yl acetate	0	0	0	0	0	0
Other compounds (OC)							
10	(*Z*)-limonene oxide	0	0	0	0	0	0
11	(*E*)-limonene oxide	0	0	0	0	0	0
18	(*Z*)-sabinene hydrate TI	0	0	0	0	0	0
25	β-cyclocitral	0	0	0	0	0	0
43	(+)-isopiperitenone	0	0	0	0	0	0
49	Caryophyllene oxide TI	0	0	0	0	0	0
50	(-)-Caryophyllene oxide	0	0	0	0	0	0
54	β-sinensal	0	0	0	0	0	0
55	α-sinensal	0	0	0	0	0	0

Table S8B.

COMPOUND	COMPOUND NAME	AS3		AS5		EV	
		ME	S	ME	S	ME	S
Monoterpene hydrocarbons (MH)							
1	Sabinene	7,0	0,	8,9	0,	1,1	0,
2	δ-3-carene	0,0	0,	0,0	0,	0,4	0,
3	β-myrcene	1,0	0,	1,1	0,	1,9	0,

4	Limonene	29,	0,	45,	0,	91,	0,
5	τ-terpinene	0,0	0,	0,0	0,	0,0	0,
6	β-ocimene	0,4	0,	0,4	0,	0,1	0,
7	*p*-cymene	0,0	0,	0,0	0,	0,0	0,
Sesquiterpene hydrocarbons							
16	α-copaene	0,0	0,	0,0	0,	0,0	0,
18	β-cubebene	0,0	0,	0,0	0,	0,0	0,
21	Germacrene	0,0	0,	0,0	0,	0,0	0,
22	(*E*)-	0,5	0,	0,4	0,	0,0	0,
27	β-selinene	0,0	0,	0,0	0,	0,0	0,
29	α-humulene	0,0	0,	0,0	0,	0,0	0,
31	β-farnesene	0,0	0,	0,0	0,	0,0	0,
35	Valencene	4,9	0,	3,2	0,	0,0	0,
39	τ-muurolene	0,3	0,	0,2	0,	0,0	0,
Alcohols (ALC)							
9	2-nonen-1-ol	0,0	0,	0,0	0,	0,0	0,
10	(*Z*)-3-hexen-	0,0	0,	0,0	0,	0,0	0,
14	(*E*)-sabinene	0,1	0,	0,2	0,	0,0	0,
19	Linalool	14,	0,	11,	0,	0,4	0,
20	1-octanol	0,8	0,	3,1	0,	0,1	0,
23	4-terpineol	0,0	0,	0,0	0,	0,0	0,
26	(*E*)-*p*-	0,0	0,	0,0	0,	0,0	0,
34	α-terpineol	0,2	0,	0,3	0,	0,1	0,
41	β-citronellol	4,8	0,	3,3	0,	0,0	0,
43	Nerol	3,8	0,	3,3	0,	0,0	0,
45	(*E*)-carveol	0,0	0,	0,0	0,	0,0	0,
46	Geraniol	1,8	0,	1,6	0,	0,0	0,
47	(*Z*)-carveol	0,0	0,	0,0	0,	0,0	0,
48	Farnesol	0,0	0,	0,0	0,	0,0	0,
50	Torreyol	0,0	0,	0,0	0,	0,0	0,
53	Epiglobulol	0,0	0,	0,0	0,	0,0	0,
54	Nerolidol	0,0	0,	0,0	0,	0,0	0,
55	Elemol	0,0	0,	0,0	0,	0,0	0,
59	Farnesol	0,7	0,	0,2	0,	0,0	0,
Aliphatic aldehydes (AA)							
8	Octanal	0,4	0,	1,2	0,	1,0	0,
11	Nonanal	0,1	0,	0,3	0,	0,1	0,
17	Decanal	1,8	0,	3,1	0,	0,8	0,
24	Undecanal	0,1	0,	0,1	0,	0,0	0,
28	2-decenal	0,0	0,	0,0	0,	0,0	0,
36	Dodecanal	0,0	0,	0,0	0,	0,4	0,
49	Tetradecanal	0,0	0,	0,0	0,	0,0	0,
56	Hexadecanal	0,0	0,	0,0	0,	0,0	0,
Monoterpene aldehydes (MA)							
15	Citronellal	4,0	0,	2,2	0,	0,0	0,
32	(*Z*)-citral	0,8	0,	0,5	0,	0,2	0,
37	(*E*)-citral	0,0	0,	0,0	0,	0,2	0,
42	Perilla	0,0	0,	0,0	0,	0,0	0,

Aliphatic and monoterpene esters							
30	Citronellyl	1,8	0,	0,8	0,	0,0	0,
33	*n*-decyl	0,4	0,	0,3	0,	0,0	0,
38	Nerol	10,	0,	3,5	0,	0,0	0,
40	Geranyl	4,2	0,	1,1	0,	0,0	0,
Other compounds (OC)							
12	(*Z*)-limonene	0,1	0,	0,1	0,	0,0	0,
13	(*E*)-limonene	0,0	0,	0,0	0,	0,0	0,
25	β-cyclocitral	0,1	0,	0,0	0,	0,0	0,
44	(+)-	0,0	0,	0,0	0,	0,0	0,
51	Caryophyllen	0,0	0,	0,0	0,	0,0	0,
52	(-)-	0,2	0,	0,4	0,	0,0	0,
57	β-sinensal	0,2	0,	0,2	0,	0,0	0,
58	α-sinensal	0,1	0,	0,1	0,	0,0	0,
60	Dimethoxy-	0,0	0,	0,0	0,	0,0	0,
61	Nootkatone	3,2	0,	1,5	0,	0,1	0,
62	Isopimpinelli	0,0	0,	0,0	0,	0,0	0,

TI Tentative Identification

CAPÍTULO V

REUNION IN THE OVERSEAS: INTRODUCED WILD BOARS AND CULTIVATED ORANGE TREES INTERACT IN THE MATA ATLÂNTICA (BRAZIL)

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Abstract

Little is known concerning novel interactions between species that typically interact in their native range but, as a consequence of human activity, are also interacting out of their original distribution under new ecological conditions. We investigate the interaction between the orange tree and wild boar, both of which share Asian origins and have been introduced to the Americas (i.e. the overseas). Specifically, we assessed whether *i*) wild boars consume orange (*C. sinensis*) fruits and seeds in orchards adjacent to a remnant of the Atlantic Forest of Brazil, *ii*) the orange seeds are viable after passing through boar's digestive tract and *iii*) whether the orange tree may naturalise in the forest remnant assisted by wild boars. Our camera surveys indicated that wild boar was by far the most frequent consumer of orange fruits (40.5% of camera trap-days). A considerable proportion of sown orange seeds extracted from fresh boar feces emerged seedlings (27.8%, N = 386) under controlled greenhouse conditions. Further, 37.6% of sown seeds (N = 500) in the forest remnant emerged seedlings in July 2015; however, after ~4 years (March 2019) only 9 seedlings survived (i.e. 4.8%, N = 188). Finally, 52 sweet orange seedlings were found during surveys within the forest remnant which is intensively used by wild boars. This study indicates a high potential of boars to act as effective seed dispersers of the sweet orange. However, harsh competition with native vegetation and the incidence of lethal diseases, which quickly kill sweet orange trees under non-agricultural conditions, could seriously limit orange tree establishment in the forest. Our results have important implications not only because the wild boar could be a vector of potential invasive species, but also because they disperse seeds of some native species (e.g. the queen palm, *Syagrus romanzofiana*) in defaunated forests, where large native seed dispersers are missing; thus, wild boars could exert critical ecological functions lost due to human activity.

Keywords: agroecosystems; *Citrus*; frugivory; invasions; naturalization; novel interactions; seed dispersal; *Sus scrofa.*

Resumen

Se conoce relativamente poco sobre las llamadas 'interacciones noveles' entre especies que típicamente interactúan en su área de distribución nativa pero que, como consecuencia de la actividad humana, también interactúan fuera de su distribución original bajo nuevas condiciones ecológicas. Investigamos la interacción entre el naranjo y el jabalí, ambos con origen asiático e introducidos en las Américas (es decir, en ultramar). Específicamente, evaluamos si *i*) los jabalíes consumen frutas y semillas del naranjo (*C. sinensis*) en naranjales adyacentes a un parche remanente del Bosque Atlántico de Brasil, *ii*) las semillas de naranja son viables tras pasar por el tracto digestivo del jabalí, y *iii*) si el naranjo puede llegar a naturalizarse en el parche de bosque gracias a los jabalíes. Los resultados de nuestro fototrampeo indicaron que el jabalí fue, con mucho, el consumidor más frecuente de las naranjas (40.5% cámaras trampa-días). Una proporción considerable de semillas de naranjo extraídas de heces de jabalí frescas y sembradas emergieron plántulas bajo condiciones de invernadero controladas (27.8%, N = 386). Además, el 37.6% de las semillas sembradas (N = 500) en el parche remanente de bosque emergieron plántulas en julio de 2015; sin embargo, después de aproximadamente 4 años (marzo de 2019) solo sobrevivieron 9 plántulas (es decir, 4.8%, N = 188). Finalmente, se encontraron 52 plántulas de naranja dulce durante varias prospecciones dentro del parche de bosque que es utilizado intensivamente por los jabalíes. Este estudio indica un alto potencial de los jabalíes para actuar como dispersores eficaces de semillas del naranjo dulce. Sin embargo, la

severa competencia con la abundante vegetación nativa y la incidencia de enfermedades letales, que matan rápidamente los naranjos dulces en condiciones no agrícolas, podrían limitar seriamente el establecimiento de naranjos en el bosque. Nuestros resultados tienen implicaciones importantes no solo porque el jabalí podría ser un vector de posibles especies de plantas invasoras, sino también porque dispersan semillas de algunas especies nativas (p.e., la palmera reina, *Syagrus romanzofiana*) en estos bosques defaunados, donde faltan dispersores nativos de semillas de gran tamaño. Por ello, los jabalíes podrían ejercer funciones ecológicas críticas que se han perdido debido a la actividad humana.

Palabras clave: agroecosistemas; *Citrus*; frugivoría; invasiones; naturalización; interacciones noveles; dispersión de semillas; *Sus scrofa*.

INTRODUCTION

Novel interactions usually take place between species that interact only because of human activity and would otherwise not even coexist (Hobbs et al. 2006, Turcotte et al. 2017). Thus, most typically novel interactions arise from 'introduced species' (i.e. those living outside their native distributional range due to human activity; Traveset and Richardson 2014, Woodet al. 2015) as well as from interactions between native and crop species (Bhagwat et al. 2008). Less studied are novel interactions between species that originally interact in their native range but, as consequence of human activity (domestication, hunt introductions, etc.; Parker and Gilbert 2004), are now interacting out of their original distribution under new ecological conditions. Here, we report one such case focused on the interaction between sweet orange tree *C. sinensis* L. Osbeck, native to Asia but cultivated worldwide, and wild boar *Sus scrofa* L., which is native to Eurasian and has been introduced in the Americas (i.e. the overseas) as game species. Investigating this novel interaction is important to understand the chances of naturalization of sweet oranges mediated by introduced wild boars (e.g. García et al. 2014).

The cultivation of citrus fruits is widespread worldwide in regions with optimal edaphoclimatic conditions for their development (30-40º North and South latitude). Although it is one of the most important fruit crops in the world, there are few studies about the basic ecology of citrus. Of particular interest is determining which animals eat their fruits and disperse their seeds. Only anecdotal evidence has been found about animals that consume cultivated citrus which has recently reviewed by Peris et al. (2015). Very little is known about whether the interactions between *Citrus* species and its fruit consumers (both native and introduced) are mutualistic (i.e. seed dispersal) or antagonistic (seed predation; but see Gade 1976, Ungar 1995). For instance, we are not aware of any study that has evaluated whether

Citrus seeds ingested by frugivores are viable, whether they emerge as seedlings, and how long they survive under the field conditions of non-native ranges. Such information is critical to assess the chances of *Citrus* species to become naturalized outside of its original range.

The wild boar has been naturalized in many countries after being introduced as a game species and due to escapes of domestic pigs with which it hybridizes (Gimenez et al. 2003). These naturalized populations are causing major environmental issues around the world (Barrios-García and Ballari 2012, Pedrosa et al. 2015). Wild boar adapts to a variety of habitat types and can rapidly increase its population (Massei and Genov 2004). It is a threat to native species of flora and fauna (Bratton 1974) and is considered one of the 100 most 'invasive species' (i.e. introduced species with a tendency to spread and to cause environmental damages; Lowe et al. 2000). The boar is omnivorous, eating everything from grain to carrion, including fruit and acting as seed disperser of many large-fruited plants (e.g. Fedriani and Delibes 2009b). Consequently, wild boar has great potential to act as an effective seed disperser (*sensu* Schupp et al. 2010; see also Schupp et al. 2017) of *Citrus* spp. and other cultivated species, exerting thus marked ecological impacts outside of its original geographical range. Nonetheless, introduced wild boars could also act as seed dispersers of some native plant species in areas where their regular seed dispersers have been extirpated (Dirzo et al. 2014) having thus potential positive effects for some plant populations.

In this study, we evaluate the potential of introduced wild boars to act as seed dispersers of *C. sinensis* in the Atlantic Forest of Brazil. *Citrus* are native to the tropical and subtropical regions of Southeast Asia (Webber 1967), with edaphoclimatic conditions similar to those of Brazilian Atlantic Forest, indicating the suitability for *Citrus* in our study area (A. Juliano, *unpublished*). To evaluate the importance of wild boars as consumers of *Citrus* fruits, we made camera surveys

within a large orange orchard adjacent to natural forest. Then, to evaluate whether boars disperse viable seeds within their feces, we conducted emergence trials with seeds extracted from boar feces under controlled greenhouse conditions. Finally, to assess orange seedlings recruitment and establishment under local field conditions we surveyed the forest remnant searching for putative orange seedlings, and also conducted seedling emergence and survival trials in the forest remnant. Specifically, we addressed the following three questions: *i*) Are wild boars freqüent consumers of orange fruits and their seeds? *ii*) Do viable orange seeds appear after passing through boar's digestive tract? *iii*) Is the forest remnant an appropriate habitat for orange seedling recruitment and establishment? Based on the opportunistic feeding habits of wild boars and in their known ability to disperser large-fruited species (e.g. Fedriani and Delibes 2009b), we hypothesized they will be effective dispersers of the sweet orange tree. However, because strong competition with native vegetation and because of the incidence of lethal diseases, which quickly kill sweet orange trees under non-agricultural conditions, we expected a low recruitment of sweet orange trees within the forest remnant.

MATERIALS AND METHODS

Study site and system

The study was carried out in Cambuhy Agrícola Ltda. (located at Matâo, Sâo Paulo, southern Brazil; 21°38' S and 48°31' W, ~600 m.a.s.l.) between April 2014 and March 2019. The agricultural farm of Cambuhy has an area of 14,083 ha where sugarcane (*Saccharum officinarum* L.), rubber (*Hevea brasiliensis* (Willd. ex A., Juss.) Müll. Arg.), corn (*Zea mays* L.) and citrus are grown. A nature reserve of 2,168 ha is found inside the farm corresponding to the Cerrado and Atlantic forest domains, called Mata da Virgínia. The predominant

native vegetation is the semi-deciduous seasonal forest with small portions of woodland savanna, Cerrado domain (Barros et al. 2017). The climate is warm and humid tropical with a maximum temperature of 30.1±0.37ºC, a minimum temperature of 17.3±0.52ºC, and an average temperature of 22.8±0.40ºC (data obtained in the Cambuhy's weather station during 2014-2016). The annual rainfall during 2010-2016 was 1386.5 + 61.06 mm, with a maximum of 1601.1 mm (in 2015) and a minimum of 1172.3 mm (in 2011).

The genus *Citrus* (Rutaceae) comprises perennial green trees originated in Southeast Asia. They produce hesperidium fleshy fruits divided into segments surrounded by peel that contains oil glands rich in volatile compounds. Orange is one of the most important fruit crops in the world. They are grown in more than 140 countries and, in 2015, the area planted with citrus fruits in the world was 8.7 million ha and citrus production was approximately 121 million tons (FAO statistics, 2016). An orange of the Pera cultivar matures between late May and June in Cambuhy, weighs 277.80 ± 21.37 g, measures 8.2 ± 0.12 cm in diameter and has a sugar pulp content of 9.67 ± 0.13 ºBRIX. The estimated production of a tree is 2000 oranges per year. Our studies were performed when the fruit was already mature but just before operational harvesting for commercial activities of the farm. Sweet orange seeds are polyembryonic, recalcitrant, non-dormant, with sizes ranging from 1.2-1.8 cm in length (Spiegel-Roy and Goldschmidt 1996).

Camera trap surveys of orange fruit consumers

To identify citrus frugivores and estimate their relative importance as fruit consumers, night-vision camera traps with motion sensors were installed on the trunks of orange trees and focused on mature oranges on the ground that had fallen from the tree in the cultivation plots near the forest remnant. The cameras (5 Bushnell Trophy Cam model

119446, USA and 1 Wildlife Trail model 02B, UK) were installed on cultivated trees along two rows with 3 cameras each at a distance of 6 and 76 m from the edge of the forest remnant, respectively. The cameras were checked daily to download the recorded images and verify their correct operation. Recording of frugivores was done during April-May of 2014 and 2015 in 5 consecutive days periods. Since some cameras eventually failed, overall we undertook a sampling effort of 42 camera trap-days and attained 9924 files (photos and videos) recording 679 vertebrate frugivores. Careful observations of all these files allowed us to estimate the relative importance of each recorded vertebrate as fruit consumer measured as the percentage of camera trap-days.

Occurrence viable orange seeds in boar feces and seedling emergence under controlled conditions

To estimate how often orange seeds occur in boar feces, during May 2014 we walked along three transects in the forest (two of 2000 m and one of 1500 m) located 5-8 m from the orchard. Each transect was searched for mammal feces once, and we covered a width of about 10 m (e.g. Suárez-Esteban et al. 2013). At the same time, feces were sought in the first 10 m of the forest remnant near these citrus orchards (two transects of 1000 meters). In total, 7.5 km in length were covered in 2014 (Supplementary Figure S10).

Fresh boar feces (N=19) were identified (Bang and Vivó 1975), bagged individually, and transferred to the laboratory the same day. They were measured, fresh weighed, and then sieved by a different-sized wire mesh sieves with warm water to extract the seeds (e.g. Fedriani et al. 2001). Once cleaned, seeds were counted and stored in a refrigerator (4-6ºC, to avoid deterioration and fungal infectation; e.g. King et al. 1981) for a maximum of 7 days. Then, they were sowed under greenhouse conditions in separate pots with citrus soil

(50% Sphagnum peat, 50% coconut fiber), irrigated twice a week, 166 W/m^2 of natural irradiation, (average temperature of 23ºC, with a minimum of 18°C and a maximum of 28°C), to evaluate their viability (e.g. Fedriani et al. 2015). Together with citrus seeds, 53 seeds of queen palm (*Syagrus romanzofiana*) found with in boar feces were sown.

Orange seedling recruitment and establishment in the forest remnant

We evaluated whether the forest remnant is an appropriate habitat for seedling recruitment and establishment by means of three approaches. Firstly, three people separated by three meters and walking in parallel straight lines through the forest remnant searched for citrus plants. During 2014, we walked two 900 m transects within the forest remnants (using machetes to open a path, see Supplementary Figure S10). During 2015, we surveyed a 3 km transect parallel to the line of citrus orchards but inside the forest remnant. In all cases, the width of the area surveyed along transects was 10 m approximately. Secondly, eight quadrants were made in the forest delimiting 60m^2 (6x10m) each, taking as a reference the method proposed by Braun-Blanquet (1979). To build the quadrants, four wooden stakes nailed to the ground were used, on which threads were tied to delimit the perimeter of the study. To facilitate the work, the threads were tied every 1.5 m lengthwise and every 1 m crosswise, forming a grid. In each grid a thorough analysis of the flora resembling citrus plants was performed. Eight quadrants were made, five in 2014 and three in 2015. Finally, we experimentally assessed whether orange seeds germinate and whether seedlings survive in the forest remnant. To this end, in May 2015, sweet orange fruits were collected from four fruiting trees. Seeds were extracted and washed to remove traces of pulp and dried for 1 day at room temperature. Seeds were sown in five quadrants (2 x 1 m each one) arranged along two

lines 3 km apart. Whereas in the first line we set 3 quadrants at 0, 15, and 30 m inside the forest remnant, in the second one, due to logistic limitation, only two quadrants (at 0 and 15 m inside the forest) were set. As 500 seeds were required to perform this experiment, and it is extremely difficult to get those numbers from boar feces, we decided to use seeds extracted from fruits. Before seed sowing, the existing vegetation in each quadrant was removed and the soil was loosened with a hoe. The quadrants were subdivided into 50 grids (20 x 20 cm). In each grid center, a hole of 1 cm in depth was made and 2 seeds were deposited, so a total of 100 seeds per quadrant were planted. Sown seeds were covered with the local soil (dried leaves were removed) and then watered every 2 weeks during the first 2 months after sowing. Thereafter, the sowings were subjected to natural conditions. Seedling emergence and growth was registered every two months.

Molecular typing of citrus plants found in the forest

A sample of three suspected *Citrus* seedlings found in the forest were subjected to microsatellite Simple Sequence Repeat analysis (following Pons et al. 2011) to verify whether they belonged to the genus *Citrus*. As there was no unique marker to unequivocally distinguish among different citrus species, a multilocus analysis was made by choosing 3 different markers. These markers were *CIR07C07* (Froelicher et al. 2008), *CIR01C06* (Cuenca et al. 2011) and *mest86* (Luro, unpublished).

RESULTS

Relative importance of orange fruit consumers

Table 8 shows the percentage of camera trap-days in which each frugivore species was recorded, the mean number (±1SE) of different

individuals recorded per day and camera, the maximum number of individuals recorded, and the functional group of visitors (seed dispersers, seed predators, and pulp consumers). Among citrus seed dispersers, non-native wild boars were the most frequently recorded (40.5%; Figure 15), followed by Azara agouti (21.4%) and Lowland agouti (16.7%; Table 8), being these differences significant (χ^2 = 6.90, d.f. = 2, P < 0.05). Further, we detected up to 6 wild boars per day and per camera, while only up to 2 agoutis (Table 8). Thus, we considering the number of recorded individuals of each disperser species the predominance of wild boars as seed dispersers (58.9%) when compared to Azara agouti (25.0%) and Lowland agouti (16.1%) was larger (χ^2 = 25.8, d.f. = 2, P < 0.0001). The most abundant pulp consumers were unidentified small birds (that were recorded almost every camera trap-day) and that were often recorded consuming pulp and vesicle remains scattered by wild boars. Seed predators (small rodents, capybara, and tapeti) were infrequent visitors (Table 8).

Figure 15. Wildboars (*Sus scrofa* L.) eating mature sweet orange fruits under the canopy of a tree in an orchard near the forest remnant. Because the species often hybridizes with domestic pigs, the possibility exists that the photographed individuals are hybrids.

Table 8. Percentage of animal observations in Cambuhy (Brazil) through the use of camera-traps, the mean number of animals observed per day and per camera, the maximum number of animals recorded and the functional guild of each frugivore. Our sampling effort (42 camera trap-days) rendered 9,924 files (photosandvideos) of 679 different vertebrate frugivores.

Species	% of days filmed	Mean±S.E.	Maximum	Functional group	Reference
Susscrofa	40.5	1.94±2.5	6	Seed disperser	Barrios-García and Ballarri, 2012
Dasyprocta azarae	21.4	1.55±0.2	2	Seed disperser	Ribeiro and Vieira, 2014
Cuniculus paca	16.7	1.28±0.3	2	Seed disperser	Wenny, 2000
Nasua nasua	7.1	1±0.0	1	Seed disperser	Alves-Costa andEterovick, 2007
Cerdocyon thous	4.8	1±0.0	1	Seed disperser	Rocha *et al*., 2004
Small birds	97.6	11.63±11.6	50	Pulp feeders	Personal observation
Turdus leucomelas	76.2	1.71±1.8	4	Pulp feeders	Personal observation
Columbina talpacoti	59.5	1.84±2.2	4	Pulp feeders	Personal observation
Aramides cajaneus	28.6	1.91±0.1	3	Pulp feeders	Personal observation
Hydrochoerus hydrochaeris	2.4	10±0.0	10	Seed predator	Personal observation
Sylvilagus brasiliensis	4.8	1±0.0	1	Seed predator	Personal observation
Small rodents	4.8	1±0.0	1	Seed predator	Personal observation
Dasypus novemcinctus	2.4	1±0.0	1	Unknown	
Iguana spp.	2.4	1±0.0	1	Unknown	
Penelope spp.	2.4	1±0.0	1	Unknown	

Wild boar dispersal of viable orange seeds

Most boar feces (68.4%, N =19) contained citrus seeds. The mean number of citrus seeds per boar feces was 29.1± 7.9, ranging from 4-101 (overall, 386 seeds in 14 feces). Citrus seedlings started to emerge one week after planting. Emergence percentage of citrus seedlings three months after sowing was 27.8% (Table 9). No seedling emerged later than 3 months after planting. In addition to citrus seeds, two out of 53 seeds of queen palm found within boar feces emerged seedlings within the first 2 months after sowing (Table 9).

Table 9. Fresh weight and number of seeds (*Citrus* sp., *Syagrus romanzofiana*, *Zea mays* and unidentified species) found in wild boar feces collected at the grove-forest remnant ecotone. The emergence percentage of citrus seedlings under greenhouse conditions is also shown.

Sample	Fresh weight (g)	Number of seeds and plant species found				Citrus seedling emergence	
		Citrus sp.	*Syagrus romanzofiana*	*Zeamays*	Non-identified	Total	%
1	62.1	79				8	10.1
2	78.2	101	2			17	16.8
3	69.3	43				12	27.9
4	69.4	32				14	43.8
5	50.3	9			9	7	77.8
6	23.5	4	2			0	0
7	103.7	4	15			0	0
8	124.1	-	3			-	-
9	109.3	28				15	53.6
10	133.2	-				-	-
11	63.5	-		1		-	-
12	33.8	9				0	0
13	152.6	38	1	12		15	39.5
14	45.9	3				0	0
15	98.6	19				17	89.5
16	38.3	9				0	0
17	99.1	-	22			-	-
18	33.4	8				0	0
19	180.4	-	53			-	-

Orange seedling emergence and establishment in the forest remnant

No citrus seedlings were found in the three transects searched in 2014. However, 48 citrus seedlings were found within the 3-km transects searched in 2015 (they were grouped into 5 clusters within 50 m^2). The mean height of these seedlings (including roots) was 10.60±1.53 cm, ranging from 5-40 cm. Their estimated age was between 6 months and 2 years. In addition, we found four seedlings in one (out of 8) 60m^2 established quadrants. All 52 seedlings were taken to the laboratory, measured, and analysed to confirm their genetic identity.

Three of the presumed citrus seedlings found in the forest remnant were verified as citrus types based on molecular markers. Citrus seedlings were identified as sweet oranges according to their SSR profiles, clearly distinguishable for those of mandarin (*Citrus reshni* Hort. ex Tan.) and clementine (*Citrus clementina* Hort. ex Tan.) controls. This result is expected as citrus orchards at Cambuhy consisted basically of sweet orange trees.

Sweet orange sowing in the forest remnant

Two months after sowing seeds in the forest remnant (i.e. July 2015), 37.6% of sown seeds (N =500) emerged seedlings. Seedling survival rates decreased over the following years, being 72.3% (N = 188) one year later (July 2016), 46.8% (N = 188) two years later (March 2017) and 26.6% after three years (May 2018) and 4.8% after four years (March 2019). In May 2018, wild boars completely destroyed two quadrants. In March 2019, the 9 surviving orange seedlings were from 9 to 42 cm long, with an average height of 27.72±3.41 cm.

DISCUSSION

Peris et al. (2015) recently reviewed citrus fruit vertebrate consumers and reported up to 28 vertebrate species. Other authors have detailed citrus fruit consumption by wild mammals and birds, as well as by livestock, both in areas where it is cultivated (e.g. Argentina, Dominica, Tanzania and Pakistan; Din and Ghazanfar 1980, Shafi et al. 1986, Navarro et al. 1991, Wiley 1993, Corp and Byrne 2004, Stampella et al. 2014) and where it is native (e.g. Torii 1986, Ungar 1995, Kitamura et al. 2002). Surprisingly, however, none of these studies have evaluated whether citrus frugivores acted as seed dispersers or as predators. To our knowledge, this is the first study evaluating the qualitative seed dispersal effectiveness (Schupp et al. 2010) of *Citrus* species, either in its native range or in areas where the species is cultivated. Based on our camera survey (Table 8) and field observations (e.g. fruit remains found underneath trees), wild boar was the only frequent species (40.5%) that visited fruiting orange trees, consumed whole fruits, and that could act as an effective seed disperser (Figure 15). Other mammals that could act as seed dispersers (e.g. Azara agouti, Lowland agouti, crab-eating fox) were much less frequent (5-21%; Table 8). For example, Zuracatto, et al. (2010) documented Lowland agouti eating citrus fruits (including their seeds) in the Atlantic Forest of Brazil. For example Jansen et al. (2010) indicates that Central American agouti (*Dasyprocta punctata*) are of great ecological importance as seed predators and seed dispersers. Zuracatto et al. (2010) found that Lowland agouti consume both the endocarp and the seeds of *C. sinensis*, *Citrus limon* and *Citrus deliciosa*. However, Smythe (1978) indicate that agoutis (*Dasyprocta* spp.) eat, but do not disperse, seeds of certain Citrus species. In our study, we also very frequently recorded several bird species underneath orange trees; however, they were always picking pulp remains from fruits previously processed by boars or from

fungus-infected fruits (which soften the fruit peel allowing birds to access the pulp; Peris et al. 2017) and no evidence of seed ingestion (and thus potential dispersal) was found during this study. Thus, the potential role of other mammals on citrus seed dispersal deserves further research.

The emergence experiments in the greenhouse and in the forest remnant, as well as our field surveys, where we found several citrus seedlings within the forest remnant, strongly support the conclusion that wild boars are acting as relatively effective dispersers of *C. sinensis* seeds. Surprisingly, however, we have not found any adult orange trees in the forest despite intensive searches, which could be explained in at least two ways. Firstly, since *C. sinensis* has a long period of juvenility, ranging from 7 to more than 10 years (Spiegel-Roy and Goldschmidt 1996), it could be due to a lack of time to recruit into adult trees. However, this seems unlikely since the orange tree orchards in Cambuhy Agrícola Ltda. were established in the 1970's. Secondly, although sweet orange seeds reach the forest, germinate, and seedlings emerge, perhaps they might not find the appropriate conditions to establish as adult individuals. Although the soil and climate conditions were optimal for the emergence and growth of citrus trees, competition with the abundant wild plants for water, light and nutrients could prevent citrus establishment. In addition, there are certain lethal and ubiquitous diseases for sweet orange trees such as Tristeza (caused by *Citrus tristeza* virus) or gummosis (caused by *Phytophthora* spp.) which quickly kill sweet orange trees under non-agricultural conditions (Spiegel-Roy and Goldschmidt 1996, Moreno et al. 2008). Indeed, most cases of citrus and Rutaceae naturalization have been reported in areas near their centers of origin and diversity, such as China and Southeast Asia (see Hong and Blackmore 2015). In the Americas, as a result of their introduction for cultivation as a combination of rootstocks and scions of two different genotypes, only

rootstock types have been found naturalized (Gade 1979, Nesom 2014). Therefore, it seems that the sweet orange has never been found naturalized in South America, which may explain the lack of success in our attempts either to find or to establish sweet orange adult in the forest remnants.

The wild boar is becoming a major matter of concern for the conservation of biodiversity in many areas around the world (Massei and Genov 2004, Barrios-García and Ballari 2012, Pedrosa et al. 2015). In spite of its increasing distribution and density, there is a lack of information about their interactions with other species in the new distribution. Specifically, wild boars are becoming widespread and ubiquitous in several areas previously occupied by Atlantic forests of Brazil (Pedrosa et al. 2015). Here we show that they act as effective seed dispersers of the sweet orange tree in southern Brazil, which could be environmental issue if this exotic plant establish and become invasive. On the other hand, boars also acted as seed dispersers of the native queen palm. This finding could be especially relevant in defaunated areas of the Brazilian Atlantic forest (Chiarello 1999, Galetti et al. 2017) where wild boars, through their novel interactions with native plants, could provide them with lost seed dispersal services. Boars could be dispersing many cultivated *Citrus* species in different areas of the world where boths species have been introduced and favoured by human influences through citrus orchard abandonment, farming or lumbering practices (Gade 1976). Thus, further studies concerning the pervasiveness and potential ecological outcomes of such novel interactions in other areas are clearly needed.

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Supplementary Figure S10. Perimeter of the Cambuhy Farm, location of the citrus orchards, and details of the four transects made. The greenhouse is located in the farm, about 0.5-2 kilometers from transects.

4. DISCUSIÓN GENERAL DE LOS RESULTADOS

En esta tesis nos hemos centrado en investigar la 'tríada' (Herrera 1984a) formada por una planta de frutos carnosos, vertebrados consumidores de sus frutos, y un hongo, supuestamente patógeno, que se nutre principalmente de pulpa (p.e. Janzen 1977, Buchholz and Levey 1990, Cipollini and Stiles 1993). El estudio de este sistema, *a priori* relativamente sencillo, se complica debido a la complejidad estructural y a la multifuncionalidad de los distintos tejidos de los frutos. En concreto, el exocarpo de los frutos carnosos es una barrera que, entre otras funciones, evita su deshidratación, previene las infecciones microbianas (Yeast y Rose 2013), mantiene la palatabilidad y promueve la dispersión de las semillas por frugívoros (Martin y Rose 2014).

El exocarpo está impregnado y recubierto por diversos compuestos orgánicos solubles en solventes conocidos como ceras y comprenden una mezcla de ácidos grasos de cadena muy larga y sus derivados: alcanos, aldehídos, cetonas, alcoholes y ésteres, junto con compuestos cíclicos, como terpenoides y esteroles (Jetter et al. 2006). Algunos de estos compuestos, como los terpenos, son metabolitos secundarios que, en ocasiones, se acumulan en grandes cantidades en la piel de los frutos carnosos y están involucrados en las interacciones con los frugívoros tanto por contacto con la piel, como mediante su emisión en forma de compuestos orgánicos volátiles que son percibidos a distancia (COVs; Rodríguez et al. 2013). Además, estos COVs son compuestos clave que determinan las características de aroma y sabor de los frutos (Goff y Klee 2006).

En el caso de los cítricos, sus frutos acumulan grandes cantidades de monoterpenos en su exocarpo o flavedo, y el COV mayoritario es el monoterpeno D-limoneno, llegando a alcanzar, en el caso de la naranja dulce (*C. sinensis*), hasta el 97% del total de los COVs acumulados (Dugo y Di Giacomo 2002). La síntesis de D-limoneno le supone mucho coste

energético a la planta (Gershenzon 1994) por lo que es esperable que tenga importantes funciones para su desempeño (supervivencia, éxito reproductivo). En buena medida, durante esta tesis nos hemos centrado en diseccionar las posibles funciones del D-Limoneno en la comunicación entre cítricos y los consumidores de sus frutos (vertebrados, hongos). A continuación discutimos sobre la omnipresencia de la interacción entre cítricos y vertebrados frugívoros, sobre algunas de las consecuencias ecológicas de estas interacciones noveles y, de forma más detallada, sobre la principales funciones del D-Limoneno en la interacción entre cítricos, hongos y vertebrados frugívoros.

Los frutos cítricos son consumidos por vertebrados frugívoros mundialmente

En esta investigación nos ha interesado saber qué especies animales consumen los frutos cítricos, qué características físico-bioquímicas favorecen su consumo y si estas podrían beneficiar a su naturalización en ambiente tropical. Hemos documentado varios casos en los que se ha descrito, siempre de manera anecdótica, el consumo de frutos cítricos (Peris et al. 2015). Sin embargo, ningún trabajo ha investigado antes de manera exhaustiva el ensamble de consumidores de cítricos y el papel funcional (dispersores, depredadores, despulpadores) de los distintos gremios de frugívoros. Los resultados de nuestros numerosos y bien replicados ofrecimientos de cítricos en ambientes tropicales y Mediterráneos permiten concluir que éstos son consumidos por vertebrados frugívoros allí donde se cultivan. El conejo fue el mayor consumidor de frutos cítricos en ambiente Mediterráneo y el jabalí en el tropical.

El jabalí es el mayor dispersor efectivo de semillas de naranjo dulce en ambiente tropical

La expansión mundial de las actividades humanas (agricultura, ganadería, caza, etc.) ha llevado a cultivar y diseminar multitud de plantas (también animales vertebrados, insectos, microorganismos, etc.; Zonneveld et al. 2018) fuera de sus regiones nativas, posibilitando el establecimiento de nuevas interacciones entre diferentes organismos que, de otro modo, no existirían, y que son conocidas como interacciones noveles. Además, en el caso de los cítricos, su cultivo cercano a áreas naturales en climas tropicales y subtropicales proporciona un entorno logístico excelente donde estudiar estas nuevas interacciones entre las frutas y los frugívoros.

En nuestros estudios realizados en ambiente tropical (Brasil) interactuaron el jabalí con la naranja dulce fuera de su región de procedencia ancestral (ambos comparten origen asiático) en un agroecosistema ajeno como es la Mata Atlántica. El jabalí resultó ser el frugívoro vertebrado que más frutos cítricos consumió y semillas dispersó en el bosque remanente junto a los campos de cultivo (Peris et al. 2019), pudiendo llegar a favorecer la naturalización de plantas alóctonas en agroecosistemas noveles, que sin la presencia del jabalí probablemente nunca se producirían. Esta tesis reseña el alto potencial del jabalí como dispersor efectivo no solo de semillas de naranjo dulce, sino también de especies nativas, un resultado confirmado recientemente en otras áreas tropicales (Pedrosa et al. 2019).

El D-limoneno de la piel de las naranjas es un compuesto defensivo frente a frugívoros generalistas

Aunque se ha propuesto que los metabolitos secundarios que se acumulan en el exocarpo de los frutos carnosos juegan un papel primeramente

defensivo frente a depredadores (Cipollini y Levey 1997a; Mack 2000; Aharoni et al. 2003; Fedriani et al. 2012; Whitehead et al. 2016), no hay estudios que lo demuestren para ningún metabolito específico comparando plantas isogénicas que producen o no dicho metabolito en la piel de sus frutos. En concreto, hemos utilizado variedades de naranjos genéticamente modificadas, a las que se les ha bloqueado la capacidad de producir D-limoneno mediante una estrategia antisentido (AS), y de sus isolíneas control no alteradas en la producción de este compuesto. Ello nos ha permitido comparar la preferencia de frugívoros por frutos que acumulan D-limoneno a altos niveles (control) frente a aquellos que lo acumulan a niveles mucho más bajos (AS; ~85 veces menos) en condiciones de campo. La clara preferencia de los frugívoros por la fruta AS demuestra de manera inequívoca el papel del D-limoneno en la protección de la naranja frente a posibles consumidores generalistas, tanto vertebrados como invertebrados y sugiere que este tipo de metabolitos son primordialmente defensivos. De hecho, no se acumulan exclusivamente en el exocarpo de las frutas sino también en distintos órganos de las plantas que incluyen hojas, tallos y flores, aunque a mucho menores concentraciones que en el flavedo. Turlings y Tumlinson (1992) proponen que los COVs ejercían originariamente funciones antimicrobianas proporcionando así un tipo de sistema inmunitario primigenio a las plantas. Sin embargo, los VOCs que se producen y acumulan en estos distintos órganos de las plantas generalmente no son los mismos o si lo son, se acumulan, a muy diferentes concentraciones en distintos órganos, lo cual sugiere que podrían tener papeles diferentes o complementarios en dichos tejidos. Por ejemplo, algunos metabolitos que se acumulan y son emitidos por las flores tienen un papel defensivo pero también los hay atrayentes de polinizadores, los cuales han contribuido de manera fundamental en la coevolución de las plantas y sus polinizadores (síndrome de dispersión floral; Faegri y Pijl 1980).

En contra de lo observado para vertebrados frugívoros en condiciones de campo, las catas realizadas con humanos en condiciones de laboratorio indican que el olfato humano, siendo capaz de detectar diferencias entre las frutas con muy diferentes contenidos de D-limoneno, no muestran ninguna preferencia (Rodríguez et al. 2017). Ello es probablemente debido a nuestra menor capacidad olfativa comparada con la de la mayoría de otros frugívoros vertebrados estudiados. Además, el hecho de que las catas fuesen solo de olores y no interviniese su consumo podría influir en la toma de decisiones por parte de los catadores. Sería necesario por tanto realizar experimentos más completos con humanos para poder comparar de forma más fiable con los resultados obtenidos con frugívoros en condiciones de campo.

El contenido de D-limoneno se incrementa en las glándulas de aceites esenciales durante el desarrollo del fruto hasta que las semillas están formadas

Durante el desarrollo de los frutos cítricos, las glándulas de aceites esenciales del flavedo van acumulando D-limoneno hasta alcanzar niveles muy altos (comparados con los de otros terpenos que también se acumulan en dichas glándulas), al menos hasta que la semilla ya está prácticamente formada. La siembra de semillas de distintas variedades de cítricos en el momento en que se produce el máximo contenido de D-limoneno en el flavedo nos ha permitido confirmar que más del 50% de las mismas eran viables y germinaron. Como a partir de ese momento del desarrollo, el contenido de D-limoneno en el flavedo de los cítricos se mantiene o incluso comienza a bajar ligera y progresivamente (Attaway et al. 1967; Peris et al. resultados no publicados), sugerimos que la planta no precisa de seguir produciendo D-limoneno y por tanto reduce su inversión en la producción de compuestos de defensa del flavedo.

Con ello, los niveles de acumulación de D-limoneno en el flavedo de los frutos cítricos continúan siendo muy altos incluso en la fruta madura. ¿Cómo entonces consiguen acceder los vertebrados frugívoros a la pulpa y a las semillas?

Los microorganismos frugívoros especialistas de la naranja utilizan el contenido de D-limoneno para desarrollarse y/o evitar la entrada de otros frugívoros en ese nicho.

La alta acumulación de D-limoneno en la piel de los frutos maduros es necesaria para la atracción eficiente de frugívoros especializados de los cítricos (hongos, bacterias, etc.) que lo usan/metabolizan en su propio beneficio. Concretamente, en el caso de *P. digitatum* la presencia de D-limoneno estimula la germinación de esporas y elongación de sus hifas (Droby et al. 2008; Rodríguez et al. 2011a). Además, naranjas AS, con contenido reducido de D-limoneno en la piel son más resistentes a la infección que las naranjas control, con contenido de D-limoneno muy alto (Rodríguez et al. 2011ab, 2015). También las naranjas AS resultan más resistentes a otros microorganismos especializados como la bacteria *Xanthomonas citri* subs *citri* y el hongo *Phyllosticta citricarpa* (responsable de la mancha negra de los cítricos) que fueron prácticamente incapaces de infectar los frutos con reducido contenido de D-limoneno (Rodríguez et al. 2015, 2018). El hecho de que estos microorganismos sean específicos de cítricos y no se hayan descrito como capaces de completar su ciclo biológico en otras especies (Barkai-Golan 2001; Gómez-Sanchis et al. 2012) implica un alto grado de especialización con el huésped. Al desarrollarse sobre tejidos vegetales ricos en D-limoneno se aseguran un nicho que otros organismos no son capaces de colonizar (por ejemplo, *Staphylococcus aereus, Escherichia coli* y *Pseudomonas aeruginosa* son incapaces de desarrollarse en tejidos ricos en D-limoneno; Bourgou et al. 2012). Esta especialización ha favorecido la dispersión de estos

microorganismos con los cítricos por prácticamente todas las áreas citrícolas del mundo.

La infección de los frutos cítricos por *Penicillium* no resulta deletérea para las semillas

En el Mediterráneo, los frugívoros vertebrados consumen mayoritariamente los frutos cítricos una vez se han desprendido del pedúnculo y caído al suelo. Antes, durante y después de la abscisión, se producen pequeñas heridas necesarias para que *P. digitatum* (hongo ubicuo; Marcet-Houben y Gabalgón 2009) penetre y colonice rápidamente el tejido vegetal (Marcet-Houben et al. 2012). Allí donde se cultivan cítricos hay *P. digitatum* y *P. italicum* colonizando los frutos maduros caídos al suelo. Sabemos que la presencia de D-limoneno en el flavedo de los frutos cítricos estimula la germinación de las esporas de *Penicillium* (Eckert y Ratnayake 1994, Droby et al. 2008) y que el hongo coloniza el flavedo y el albedo aunque, raramente, *Penicillium* llega a desarrollarse en los lóculos que contienen las vesículas de zumo y en las semillas contenidas en el fruto (Figura 16). En cualquier caso, para investigar si la infección de las semillas por *P. digitatum* afectaría a su germinación, hemos forzado la infección de semillas durante 60 días en condiciones de alta humedad de distintas variedades de cítricos con *P. digitatum*. Sorprendentemente, entre un 50 y 72% de las semillas infectadas continuaban germinando normalmente (Figura 17, Tabla 10), lo cual indicaba que muy probablemente las dos capas protectoras de la semilla estaban protegiendo eficazmente a la misma de la infección por *P. digitatum* (aunque no podemos descartar que las semillas produzcan compuestos fungicidas) (nuestros resultados no publicados). Aunque desde un punto de vista fitopatogénico *P. digitatum* y *P. italicum* son considerados importantes patógenos en pos-cosecha; desde un punto de

vista ecológico se trata más bien de hongos simbiontes, ya que no dificultan la supervivencia ni la reproducción de su huésped.

Figura 16. Naranja Navelina infectada con *P. digitatum* después de 11 días de su inoculación en laboratorio y detalle de las hifas colonizando el flavedo.

Figura 17. Semillas de citrange Carrizo (*C. sinensis* L. Osb. X *Poncirus trifoliata* L. Raf.) sanas (abajo derecha) e infectadas (abajo izquierda) con *P. digitatum* y semilleros correspondientes después de 110 días de cultivo en invernadero.

Tabla 10. Tratamiento (sana o infectada de *P. digitatum*), fecha de siembra, número de semillas sembradas y germinadas y porcentaje de germinación de semillas cítricas cultivadas en invernadero.

Especie	Tratamiento	Fecha de siembra	Nº de semillas	Evaluación (días)	Semillas germinadas	% de Germinación
Citrange carrizo	Sanas	28/12/2014	96	110	87	90.63
Citrange	Infectadas	28/12/2014	96	110	48	50.00

carrizo Citrus reticulata	Sanas	02/01/2015	147	144	133	90.48
Citrus reticulata	Infectadas	02/01/2015	136	144	98	72.06

La infección por *Penicillium* de los frutos cítricos altera su contenido y emisión de volátiles y sus características físicas haciéndolos más apetecibles para los frugívoros

La clara preferencia de los frugívoros por fruta infectada por *Penicillium* se podría explicar por su mayor contenido de ésteres y etanol en las frutas infectadas y menor contenido de monoterpenos hidrocarbonados en las frutas intactas. Toda fermentación de un fruto fresco rico en azúcares produce altos niveles de etanol (principalmente junto con otros alcoholes) y ésteres, compuestos volátiles que liberan aromas fácilmente detectables por frugívoros. La infección de frutos por hongos altera el perfil de volátiles de los frutos (Holopainen y Gershenzon 2010) y los volátiles son importantes en la comunicación planta-frugívoro (Schaefer y Ruxton 2011). Estos cambios en el perfil de COVs junto con la disminución del contenido de monoterpenos hidrocarbonados (incluido el D-Limoneno) en los frutos infectados podría explicar la elección de los frugívoros por consumirlos (Trantallidi et al. 2015). Por tanto, nuestra investigación reveló que los cambios físicos y químicos producidos por la infección de *P. digitatum* facilitan el forrajeo de los frugívoros, al permitir mejor acceso a la pulpa (ya que reblandecen su piel) y transformar gran parte los monoterpenos hidrocarbonados en alcoholes y esteres. Nuestros resultados son consistentes con Dudley (2000), quien sugiere que el etanol en la fruta podría resultar atrayentes de frugívoros dispersores de semillas, y también con Laska y Seibt (2002) que mostraron cómo algunos frugívoros tienen una excelente sensibilidad olfativa para ésteres acéticos.

P. digitatum **es capaz de producir y disparar la emisión de D-limoneno en la piel de las naranjas infectadas, haciéndolas más atractiva para los frugívoros especializados**

El hecho de que en estado natural *P. digitatum* solo colonice frutos maduros podría ser una señal utilizada por los frugívoros para elegir inequívocamente frutos con suficiente contenido de D-limoneno en la piel y máximo contenido de nutrientes en la pulpa.

Cuando se ofrecieron frutos AS y control ambos infectados por *P. digitatum* junto con frutos AS y control intactos, los frugívoros prefirieron consumir primeramente los frutos AS infectados y después los AS intactos. Los frutos control fueron los menos consumidos, de forma que no encontramos diferencias estadísticas de preferencia entre los que estaban infectados y los no infectados por el hongo. En cualquier caso, los frutos AS infectados fueron con mucho los más consumidos (entre 7 y 22 veces más preferidos). Al tratar de identificar las diferencias en contenido y emisión de COVs en los frutos ofrecidos, observamos que se incrementaron las emisiones de ésteres y alcoholes en los frutos infectados (Laska y Seibt 2002, Rodríguez et al. 2013). Sin embargo, lo que más nos llamó la atención fue la elevada emisión de D-limoneno por los frutos AS infectados, cuyo contenido de D-limoneno era bajo. Por ello nos preguntamos si *P. digitatum* contendría una monoterpeno sintasa capaz de producir grandes cantidades de D-limoneno utilizando el flavedo de los frutos cítricos como sustrato.

El genoma de ***P. digitatum*** **tiene el gen precursor de una monoterpeno sintasa que es activa en la piel de la naranja e induce la síntesis de D-limoneno y otros monoterpenos volátiles**

De acuerdo con nuestra predicción, encontramos un gen candidato en el genoma del hongo. Un detallado análisis funcional reveló que se trataba efectivamente de una monoterpeno sintasa con capacidad para producir D-limoneno y otros monoterpenos. Se trata de la primera monoterpeno sintasa caracterizada de un hongo fitopatógeno. En las frutas AS, la emisión de D-limoneno aumentaba fuertemente después de 7 días de infección coincidiendo con el momento de esporulación del hongo.

Concluimos que el hongo ha evolucionado una estrategia de supervivencia muy efectiva que le ha facilitado sobrevivir durante miles de años y dispersarse junto con los cítricos por todos los continentes. Produciendo D-Limoneno en forma volátil manipula la interacción de la fruta con sus frugívoros, aumentando el consumo de estos frutos y, con ello, favoreciendo la dispersión de sus esporas (Dighton et al. 1992). Esto también beneficiaría a los frugívoros (los frutos son una fuente de nutrientes y agua) y a los cítricos (que son dispersados por vertebrados frugívoros). Es la primera vez que se descubre que un microorganismo modifica las relaciones entre frugívoros vertebrados y plantas con el beneficio de todos los actores. Por lo tanto, un compuesto en principio disuasorio para los frugívoros generalistas (vertebrados e invertebrados) se metaboliza por un frugívoro especializado (*P. digitatum*),emitiendo COVs típicos de la fruta madura. Además, el propio hongo produce y emite COVs en la fruta que atraen a diferentes animales frugívoros.

El hecho de que la monoterpeno sintasa de *P. digitatum* esté filogenéticamente relacionada con otras terpeno sintasas de microorganismos pero no de plantas indica que no ha sido adquirida de plantas mediante transferencia horizontal, sino que probablemente el hongo tenía la capacidad de producir monoterpenos incluso antes de haber encontrado a los cítricos como huésped más adecuado. Nuestros resultados sugieren que la presencia de una monoterpeno sintasa

funcional en el genoma de *Penicillium* puede haber sido la razón de que este hongo infecte frutos cítricos, al encontrar en su flavedos el nicho ecológico adecuado para vivir, multiplicarse y también diseminarse. Se trataría de un caso de evolución convergente en el que dos organismos muy diferentes comparten la ruta de biosíntesis de un mismo tipo de compuestos ecológicamente relevantes (en defensa y dispersión de la semilla), de manera que el hongo es capaz de producir dichos compuestos usando a su huésped como sustrato apropiado. Una estrategia similar se ha propuesto para explicar la producción de compuestos cianogénicos de defensa por polillas especializadas y sus huéspedes vegetales (Jensen y col. 2010).

Por otra parte, nuestros resultados también sugieren que sería de interés buscar monoterpenos sintasas en el genoma de otros frugívoros especialistas (como la bacteria *Xanthomonas citri* subs. *citri* o el hongo *Phyllosticta citricarpa*), que viven y se reproducen en un entorno rico en D-limoneno y otros monoterpenos.

Las funciones del monoterpeno D-limoneno no se limitan a mediador de interacciones del fruto cítrico con microorganismos sino que más bien se trata de un compuesto clave en las relaciones entre los frugívoros generalistas dispersores de semillas, los microorganismos especializados y los frutos cítricos, fundamental en la coevolución de dichas interacciones y probablemente también en la supervivencia de cítricos, frugívoros y microorganismos en la naturaleza.

5.CONCLUSIONES

1. El consume de frutos cítricos por vertebrados frugivores está generalizado en ambientes tropical y Mediterráneo de distintas partes del mundo dando lugar a interacciones noveles.

2. En contra de las evidencias teóricas anteriores hemos demostrado que distintos grupos funcionales de frugívoros vertebrados (despulpadores, predadores de semillas y dispersores) prefirieron consumir frutos cítricos infectados por *P. digitatum* antes que frutos intactos tanto en ambiente tropical como Mediterráneo.

3. La infección de las naranjas por *P. digitatum* altera el perfil de los COVs en emisión (aumenta la emisión de ésteres y alcoholes en detrimento de la emisión de terpenos hidrocarbonados) y en contenido (disminución del contenido de terpenos hidrocarbonados).

4. Los cambios físico-químicos producidos después de la infección por *P. digitatum* podrían explicar las preferencias de los frugívoros vertebrados por consumir frutos infectados.

5. Hemos revelado por primera vez el papel ecológico defensivo del metabolito secundario volátil D-limoneno. Los frugívoros vertebrados e invertebrados de ambiente Mediterráneo prefirieron consumir frutos sanos con reducido contenido de D-limoneno en su flavedo utilizando isolíneas genéticamente idénticas que solo difieren en la producción de este compuesto.

6. Los frugivores de ambiente Mediterráneo prefirieron consumer naranjas AS infectadas frente a AS sanas y controles sanos e infectados, siendo la única condición diferente que las AS

infectadas emiten 22 veces más compuestos volátiles (D-limoneno) que las otras. Por tanto, D-limoneno es el compuesto esencial que determina la preferencia de los frugívoros.

7. Hemos descubierto una terpeno sintasa en el genoma de *P. digitatum* capaz de producir D-limoneno utilizando la piel de los frutos cítricos como sustrato, alterando con ello las relaciones entre frutos, vertebrados frugívoros y *Penicullium*.

8. En los análisis sensoriales realizados con zumo de naranjas AS y control, los catadores detectaron diferencias pero no preferencias por ningún tipo de zumo y las diferencias no fueron clasificadas como depreciables.

9. El jabalí (especie introducida e Brasil), actúa como un dispersor efectivo de otra especie introducida (*C. sinensis*) pero también de una especie nativa (*S. romanzofyana*) por lo que puede favorecer la dispersión de plantas nativas mediante interacciones noveles.

10. Solo un 5% de las semillas germinadas de *C. sinensis* en condiciones silvestres siguen vivas tras 46 meses. Ningún naranjo dulce silvestre adulto fue encontrado en los numerosos transectos. Esto evidencia que, aunque el naranjo dulce puede germinar y crecer en condiciones tropicales, no es un ambiente propicio para completar su crecimiento y desarrollo.

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