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TESIS DOCTORAL

Gestión integral del proceso productivo del higo seco que asegure un producto de máxima calidad higiénico-sanitaria

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PHD THESIS

Integrated management of the fig production process maximises hygienic and sanitary quality

ANA ISABEL GALVÁN ROMERO

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ÍNDICE DE CONTENIDO

RESUMEN

El **cultivo de la higuera** (*Ficus carica* L.) ha experimentado en la última década un gran incremento en superficie de cultivo y producción de higo fresco y especialmente de higo seco. En España, Extremadura lidera la producción nacional con 37.382 t con una superficie aproximada de 7.034 ha. El principal destino de esta producción es para consumo en seco, siendo las principales variedades Calabacita y Cuello Dama Blanco. Tradicionalmente, la producción de higo seco se ha llevado a cabo en plantaciones en secano con amplios marcos de plantación, lo que ha dado lugar a rendimientos entre 1.000 y 3.500 kg/ha en función de la fertilidad del suelo. Hoy en día, la fruticultura moderna exige, para aumentar la productividad y el beneficio de las explotaciones, implementar sistemas de producción más eficientes en cuanto a manejo e insumos. Por ello, los sistemas en superintensivo se están introduciendo en regadío en un gran número de especies frutícolas ya que garantizan todo lo mencionado anteriormente. En el caso de la higuera, estos sistemas aún no han sido desarrollados y debido a su introducción en regadío favorecido por el incremento de la demanda y a la escasa mano de obra necesaria para la recolección de higos se hace necesario su estudio. Además, estos sistemas podrían implementarse con mallas que faciliten la recogida de los higos.

El secado de los higos se realiza de manera natural al sol, iniciándose en el árbol para luego terminar de secarse en el suelo, de donde son recogidos a mano cada 7-10 días. Este proceso presenta, entre sus principales inconvenientes, que el higo seco tarda en alcanzar un contenido de humedad <26% de acuerdo con la norma DDP-14 sobre la comercialización y el control de calidad de los higos secos y requiere forzosamente de un periodo de secado extra de, entre 4-5 días, bajo túnel en paseras. Por tanto, esta gestión tradicional de la producción puede dar lugar a un alto riesgo de contaminación fúngica por mohos toxigénicos y, como consecuencia, la potencial

aparición de metabolitos secundarios tóxicos llamados micotoxinas. Además, durante el posterior procesado industrial del higo seco, éste pasa por diferentes etapas, siendo el escaldado una de las etapas más críticas, ya que supone una rehidratación de los higos secos que puede favorecer la germinación de las esporas de mohos toxigénicos debido a las condiciones favorables de temperatura y humedad. Asímismo, el secado, el almacenamiento a temperatura ambiente y posteriormente la comercialización a esta temperatura, pueden favorecer la proliferación de mohos toxigénicos y la producción de micotoxinas.

Entre los principales géneros de mohos toxigénicos que aparecen en el higo seco destacan *Aspergillus, Penicillium, Alternaria* y *Fusarium* y, dentro de estos, destaca *Aspergillus* spp. por producir aflatoxinas, principalmente B_1, y ocratoxina A que son las micotoxinas más frecuentes en este producto y el principal problema para su comercialización.

En los últimos años, las investigaciones se han centrado en la caracterización ecofisiológica de los mohos productores de micotoxinas. Sin embargo, no hay literatura científica disponible sobre el momento de infección de los higos secos, así como el impacto del manejo del cultivo sobre el desarrollo de estos mohos toxigénicos y, en consecuencia, la aparición de micotoxinas. Por tanto, el **objetivo** de esta **Tesis Doctoral** es la aplicación de acciones que engloben buenas prácticas agronómicas, así como estrategias para incrementar la producción, evitar la contaminación y el desarrollo de mohos toxigénicos y la producción de micotoxinas, tanto en campo como en industria, para asegurar un producto con la máxima calidad higiénico-sanitaria y, por tanto, seguro para el consumidor.

Los **resultados** de esta **Tesis Doctoral** han demostrado que la productividad obtenida bajo sistemas superintensivos en regadío es muy superior a los sistemas tradicionales. Además, presenta otras ventajas adicionales como facilitar el proceso de la recolección mediante la utilización de mallas suspendidas que evita el contacto directo de los higos secos con el suelo, disminuyendo de este modo el riesgo de contaminación fúngica.

Por otro lado, cabe destacar que la contaminación con mohos toxigénicos comienza en el árbol durante el proceso de maduración de los higos. Los principales géneros de mohos identificados pertenecían a *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp. y *Cladosporium* spp. Además, del proceso de maduración, se ha podido constatar que la localización geográfica tiene un impacto significativo en la calidad físico-química del producto, así como en la diversidad de especies fúngicas y en la contaminación de aflatoxinas y ocratoxina A. Sin embargo, se pudo comprobar que el sistema de producción en secano o regadío tiene un impacto limitado.

Por otra parte, el procesado de los higos secos en la industria conlleva una serie de etapas, curado, calibrado, escaldado, almacenado y producto final, que presentan un riesgo importante de infección por mohos toxigénicos y, por consiguiente, contaminación por micotoxinas. Después de evaluar cada una de estas etapas en tres industrias diferentes, se pudo observar una alta incidencia de mohos productores de aflatoxinas y ocratoxina A en la mayoría de ellas.

Finalmente, el control de la temperatura (>30ºC) durante el proceso de producción, así como el mantenimiento de las bajas temperaturas (<10ºC) durante el almacenamiento y la comercialización de los higos secos es crucial para evitar el crecimiento y la producción de micotoxinas. Asimismo, además de controlar la

temperatura de almacenamiento, se ha visto también que el uso de compuestos volátiles, como el acetato de 2-feniletilo y el acetato de furfurilo son una buena estrategia para aplicar en diferentes etapas del procesado de los higos secos para el control de las aflatoxinas y ocratoxina A en higos secos.

En **conclusión**, los resultados obtenidos en esta **Tesis Doctoral** ponen de manifiesto que **la producción de higos secos en condiciones controladas desde la fase de precosecha hasta la fase de postcosecha** permite la **obtención de higos secos con una elevada calidad higiénico-sanitaria**, ayudando de este modo a la comercialización y sostenibilidad de este producto.

ABSTRACT

Fig tree (*Ficus carica* L.) cultivation has undergone a tremendous increase in the last decade regarding crop planted area and yield of fresh figs, especially dried figs. In Spain, Extremadura leads the national fig production with 37.382 t with an approximate crop area of 7,.034 ha. The leading destination of this production is dried figs, the main varieties being Calabacita and Cuello Dama Blanco. Traditionally, dried fig production has been carried out in rainfed orchards with wide spacing, which has resulted in yields of between 1.000 and 3.500 kg/ha depending on soil fertility. Modern orchards need to implement more efficient production systems in terms of management and inputs to increase yields and farm profits.

For this reason, superintensive systems are being introduced in irrigation for many fruit species to ensure those mentioned earlier. These systems have not yet been implemented in the case of fig trees. Due to their introduction to irrigation, favoured by the increase in demand and the lack of labour for harvesting, it is necessary to study them. Furthermore, these systems could be implemented with systems that facilitate fig harvesting.

Figs are sun-dried naturally, starting on the tree and then drying on the ground, from where they are harvested manually every 7-10 days. One of the main disadvantages of this process is that dried figs do not reach <26% moisture content following the DDP-14 standard on the marketing and quality control of dried figs, and an additional drying period of 4-5 days under a tunnel in raisins is necessary. Therefore, this traditional production management can lead to a high risk of fungal contamination by toxigenic moulds and, consequently, the potential appearance of toxic secondary metabolites called mycotoxins. Furthermore, during the subsequent industrial processing of dried figs, they undergo different stages, with blanching being

one of the most critical, as it involves rehydration of dried figs that may encourage the spore germination of toxigenic moulds due to temperature and humidity optimised conditions. Likewise, drying, storage at room temperature and subsequent marketability at this temperature may promote toxigenic mould proliferation and mycotoxin production.

Toxigenic mould genuses that grow on dried figs include *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium*. *Aspergillus* spp. stands out for producing aflatoxins, mainly B1, and ochratoxin A, which are the most frequent mycotoxins in this product and the main problem for its marketability.

In recent years, research has focused on the ecophysiological traits of mycotoxin-producing moulds. However, there is no scientific literature available on the infection timing of dried figs and the impact of crop management on the development of these toxigenic moulds and, consequently, mycotoxin production. Therefore, the **aim** of this **PhD Thesis** is the application of actions that include good agronomic practices, as well as strategies to increase yield, avoid contamination and the development of toxigenic moulds and mycotoxin production, both in orchards and industries, in order to ensure a product with the highest hygienic-sanitary quality and, therefore, consumer safety.

The r**esults** of this **PhD Thesis** have shown that the yield obtained under superintensive systems is much higher than in traditional systems. Moreover, it has other additional advantages, such as facilitating the harvesting operation by using suspended meshes that prevent direct contact of dried figs with the ground, thus preventing the fungal contamination risk.

On the other hand, it should be highlighted that contamination with toxigenic moulds began on the tree during the fig ripening process. The principal genera of moulds identified were *Penicillium* spp., *Aspergillu*s spp., *Alternaria* spp. and *Cladosporium* spp. Furthermore, apart from the ripening process, it was found that the geographic location has a significant impact on the physicochemical quality of figs, as well as on the diversity of fungal species and aflatoxin and ochratoxin A production. However, water management was shown to have a relatively limited impact.

The processing of dried figs in the industry involves several stages, curing, sorting, blanching, storage and final product, which have a significant risk of infection by toxigenic moulds and, consequently, mycotoxin production. After assessing these stages in three different industries, most of them could detect a high incidence of aflatoxin and ochratoxin A producing moulds.

Finally, temperature control (>30ºC) during the production process, as well as the maintenance of low temperatures (<10ºC) during storage and marketability of dried figs, is crucial to avoid the growth and mycotoxin production. Also, besides controlling the storage temperature, volatile compounds such as 2-phenethyl acetate and furfuryl acetate are an excellent strategy to apply at different stages of dried fig processing to control aflatoxins and ochratoxin A in dried figs.

In **conclusion**, the results obtained in this **PhD** thesis show that the integrated management of the dried fig production from preharvest to postharvest allows the production of dried figs with **high hygienic-sanitary quality**, thus supporting the commercialisation and sustainability of this product.

INTRODUCCIÓN

1. La higuera

1.1. Botánica

La higuera (*Ficus carica* L., 2n=26) es una especie frutal perteneciente a la familia *Moraceae*, que agrupa a más de 40 géneros, siendo el género *Ficus* el de mayor importancia. Comprende aproximadamente 750 especies distribuidas en su mayoría en las regiones tropicales y subtropicales del planeta (Berg, 2003). Es un árbol caducifolio, de crecimiento rápido y de dimensiones variables en función de factores tales como genotipo, riego, fertilización, enfermedades, suelo, etc. Algunos árboles pueden llegar a medir hasta 8-10 m de diámetro y pueden alcanzar una altura en su madurez que puede oscilar entre 3 y 10 m (Figura 1). La edad media de los árboles está aproximadamente entre 50 y 60 años (Janick, 2006).

Figura 1. Porte de una higuera (*Ficus Carica* L.) en plantación regular para la producción de higo seco. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

El tronco de la higuera es grueso, provisto de una corteza lisa y de color gris. La madera es de poca densidad por lo que es muy sensible a la radiación solar. La altura va a depender del uso de su producción. Los árboles destinados para la producción de fresco se forman más bajos, a unos 50-70 cm del suelo para facilitar las labores de recolección, mientras que los destinados a la producción de higo seco se suelen formar a un metro de altura para favorecer el acceso a la base del tronco, así como la aireación y el secado de los frutos. En el tronco y ramas aparecen hinchazones nodales por debajo o a ambos lados de las cicatrices peciolares en árboles a partir del tercer o cuarto verde (Pereira, 2016). También podemos encontrar

protuberancias corticales en forma de tubérculo, generalmente esféricas y suele ser propias de árboles con más de tres años.

Las ramas primarias no suelen ser numerosas, y se caracterizan por ser glabras, lisas, más o menos nudosas y de color gris claro. Presentan mucha elasticidad y, a veces, pueden agrietarse debido a la incidencia directa del sol. Sin embargo, el número de ramas secundarias es muy variable en función del genotipo. En las ramas se localizan las yemas o botones, que pueden ser de madera, de flor y adventicias. Las dos primeras son axilares y se sitúan por encima de la inserción del peciolo de la hoja. Las adventicias se sitúan en la base de ramas y en troncos, y serán el origen de rebrotes en circunstancias favorables. Los botones florales son casi esféricos y se localizan sobre las ramas del año en variedades uníferas, mientras que en variedades bíferas aparecen tanto sobre ramas del año como del año anterior. Las de madera son globosas puntiagudas y se sitúan en todos los nudos. Al final de cada rama del año, se sitúa una yema apical a partir de la cual se inicia el crecimiento anual de la rama. Su color varía desde marrón claro hasta verde amarillento dependiendo del genotipo.

Las hojas de la higuera son grandes, coriáceas y muy variables en cuanto a forma y tamaño. Son palmatipartidas o palmatilobuladas, con un número de lóbulos entre 3 y 7. La base de las hojas puede ser decurrente, truncado, corbado, moderadamente calcáreo o fuertemente calcáreo y sus lóbulos puden ser lineales, espatulados, liriados, triangulares, rómbicos y sin lóbulos (UPOV, 2010). El tamaño de las hojas oscila entre 10 y 20 cm. En el haz presentan un color verde brillante, de tacto áspero con pilosidad corta y rígida. El envés es de color más blanquecino, con

pelos largos y fuertes. Con el frío las hojas empiezan a amarillear y caer (Pereira, 2016).

Las flores y los frutos se localizan en el interior de un receptáculo piriforme o redondeado de aspecto carnoso que es un tipo de infrutescencia denominado sicono. Esta infrutescencia contiene numerosas flores de tamaño muy pequeño y al madurar dan lugar a unos frutos secos denominados aquenios y a un periantio carnoso y dulce. Cuando el sicono se localiza en la axila de la hoja de los brotes del año anterior se denomina primera cosecha o breva, mientras que la cosecha principal o higos son los siconos localizados en los crecimientos del año (Pereira, 2016).

Por otro lado, la higuera es una especie ginodioica con dos morfologías diferentes: los árboles femeninos (*Ficus carica sativa* L.) que producen siconos con flores femeninas de estilo largo que se convertirán en frutos comestibles y los árboles masculinos o cabrahigos (*Ficus carica caprificus* L.) que producen siconos con flores masculinas y femeninas de estilo corto. Dado que solamente los cabrahigos producen polen, el sistema reproductivo es funcionalmente dioico (Kjellberg y col., 1987). El cabrahigo va a permitir completar la polinización denominada caprificación. Este proceso es realizado por el himenóptero *Blastophaga psenes* L. que transporta pasivamente el polen desde las flores masculinas, situadas en los frutos profichi del cabrahigo, hasta las flores femeninas de estilo largo de los higos. Aparte de la polinización natural, la caprificación por parte de los fruticultores se ha practicado en el área mediterránea durante siglos (Condit, 1955). Para ello, los fruticultores colocaban algunos frutos de profichi, que contenían polen y polinizadores, en los árboles femeninos para facilitar la polinización de las flores femeninas de los higos. Realizar esta tarea en plantaciones comerciales de higuera es laboriosa y costosa, por

lo que este proceso ha caído en desuso en favor de variedades que no requieren caprificación. La erradicación de la caprificación ha provocado en las vareidades que requieren polinización una erosión de la biodiversidad de esta especie (Marcoluti y col., 2020)

1. 2 Variedades de higuera

De acuerdo con su compleja biología floral, comercialmente se cultivan tres tipos de higueras femeninas (Storey, 1976).

- **Tipo común**: Son aquellas higueras que desarrolla el fruto de forma partenocárpica (sin necesidad de polinización). Se dividen en dos grupos: uníferas (solo producen una cosecha) y bíferas (producen dos cosechas, brevas e higos). Los higos pueden consumirse tanto frescos como secos.
- **Tipo San Pedro**: Son higueras bíferas, que presentan una primera cosecha partenocárpica (brevas) y necesitan polinización para la producción de higos.
- **Tipo Esmirna**: Son higueras uníferas, que requieren polinización obligatoria para la producción de higos.

Debido a la facilidad de propagación de la higuera por estaquilla leñosa, este cultivo ha sido ampliamente distribuido y cultivado en muchas partes del mundo, obteniendo una gran variabilidad fenotípica debida a las mutaciones naturales dentro de una misma variedad (Flaishman y col., 2008). Esto ha dado lugar a un elevado número de variedades. Condit (1955) describió 607 variedades, sin embargo, la producción comercial se centra en unas pocas variedades (Polat y Siddiq, 2012). En España, el panorama varietal está constituido por variedades locales o autóctonas que son el resultado de la selección natural promovida por las distintas condiciones edafoclimáticas, pero también de una selección artificial por parte de los fruticultores.

La mayoría de estas variedades locales presentan una distribución muy restringida y su cultivo está poco extendido, mientras que unas pocas son variedades ancestrales que se han extendido dentro y fuera de nuestras fronteras, tomando distintos nombres. En la revisión realizada por López- Corrales y col. (2016) se muestran, por Comunidades Autónomas, las principales variedades locales, resaltando aquellas que localmente se han considerado de mayor importancia. La mayoría corresponden al grupo de las partenocárpicas, ya sean bíferas o uníferas, y en menor medida las de tipo San Pedro. Las de tipo Esmirna no son utilizadas por el elevado coste que supone la mano de obra para la caprificación.

Cabe destacar que, en el Banco Nacional de Germoplasma de higuera, localizado en el Centro de Investigaciones Científicas y Tecnológicas de Extremadura-Finca La Orden, en Guadajira (Badajoz), se conservan más de 350 variedades distintas, de las cuales el 85% son representativas de la diversidad cultivada en España. Además, a nivel nacional, también se encuentra otra importante colección de higueras en el Campo Experimental de "Son Mut Nou", ubicado dentro de la Marina de Llucmajor (Mallorca), que cuenta con un total de 834 variedades diferentes, de las cuales 255 corresponden a variedades autóctonas de las Islas Baleares. Muchas de las variedades existentes debido a su escasa producción, su pequeño tamaño o su mala calidad organoléptica no presentan interés comercial. En la elección de la variedad para el establecimiento de nuevas plantaciones, se deben tener en cuenta aspectos como el tipo de producción (brevas y/o higos), el destino de dicha producción (consumo en fresco o seco), la fecha de maduración (temprana, media o tardía) y la preferencia del mercado (coloración de la piel y pulpa) (López-Corrales y col., 2012).

En la actualidad, el Registro Oficial de variedades comerciales de higuera de España, dependiente del Ministerio de Agricultura, Pesca y Alimentación incluye 51 variedades. Las primeras 50 aparecen descritas y caracterizadas por López- Corrales y col. (2011). En base al destino de su producción, se pueden agrupar en variedades para la producción de fresco, seco o de doble aptitud. Para la producción en fresco destacan las variedades Albacor, Coll Dame negre, San Antonio y Dalmatie, entre otras, mientras que para la producción de seco destacan 'Calabacita' y 'Cuello Dama Blanco', aunque esta última se caracteriza por tener doble aptitud (López-Corrales y col., 2011; Pereira, 2016).

La variedad **Calabacita** se caracteriza por ser una variedad bífera, de origen extremeño, con una escasa producción de brevas, pero con una elevada producción de higos. Es la variedad más utilizada para la producción de higo seco en Extremadura debido a la elevada calidad organoléptica de su fruto (Villalobos, 2015), cultivándose principalmente en la zona de Montánchez, en los municipios de Almoharín, Arroyomolinos de Montánchez y Valdefuentes. El período de recolección de los higos secos abarca desde finales de julio hasta finales de septiembre, situándose la máxima producción en la segunda quincena de agosto. Los higos de la variedad Calabacita se caracterizan por tener un tamaño medio entre 35 y 45 mm de diámetro, forma esférica y un pedúnculo largo (Figura 2). En fresco, su piel es de color verde amarillento, fina y consistente, mientras que la pulpa es de color ámbar, muy jugosa y de sabor muy dulce. Esta variedad está perfectamente adaptada a las condiciones de cultivo tanto en secano como en regadío (Pereira, 2016).

Figura 2. Higos secos (Izquierda) y frescos (Derecha) de la variedad Calabacita. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

En cuanto a la variedad **Cuello Dama Blanco**, es una variedad bífera, cultivada principalmente en el norte de Extremadura y municipios del sur de la provincia de Ávila. Presenta una doble aptitud, es decir, los higos se utilizan tanto para consumo en fresco, por su buen tamaño, como para secado. La producción de brevas es escasa en comparación con la de higos. El período de recolección del higo seco abarca desde primeros de agosto hasta mediados de septiembre, alcanzándose la máxima producción a mediados de agosto. Los higos de esta variedad son de tamaño medio entre 50 y 55 mm de diámetro y forma esférica con pedúnculo grueso y muy corto. En fresco, la piel es gruesa y resistente y presenta un color amarillo verdoso (Figura 3). Su pulpa es de color ámbar con una leve tonalidad rosa y presenta muy buena calidad organoléptica (Pereira, 2016).

Figura 3. Higos frescos de la variedad Cuello Dama Blanco. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

1.3. Importancia económica del cultivo de la higuera

La producción mundial de higos se estima en torno a 1.264.943 t en el año 2020 (FAOSTAT, 2020), con una superficie de cultivo de 281.522 ha. El principal país productor es Turquía con un volumen de producción de 320.000 t, seguido de Egipto y Marruecos con 201.212 y 144.246 t respectivamente. El cuarto, quinto y sexto puesto lo ocupan Argelia, Irán y España (Figura 4) (FAOSTAT, 2020).

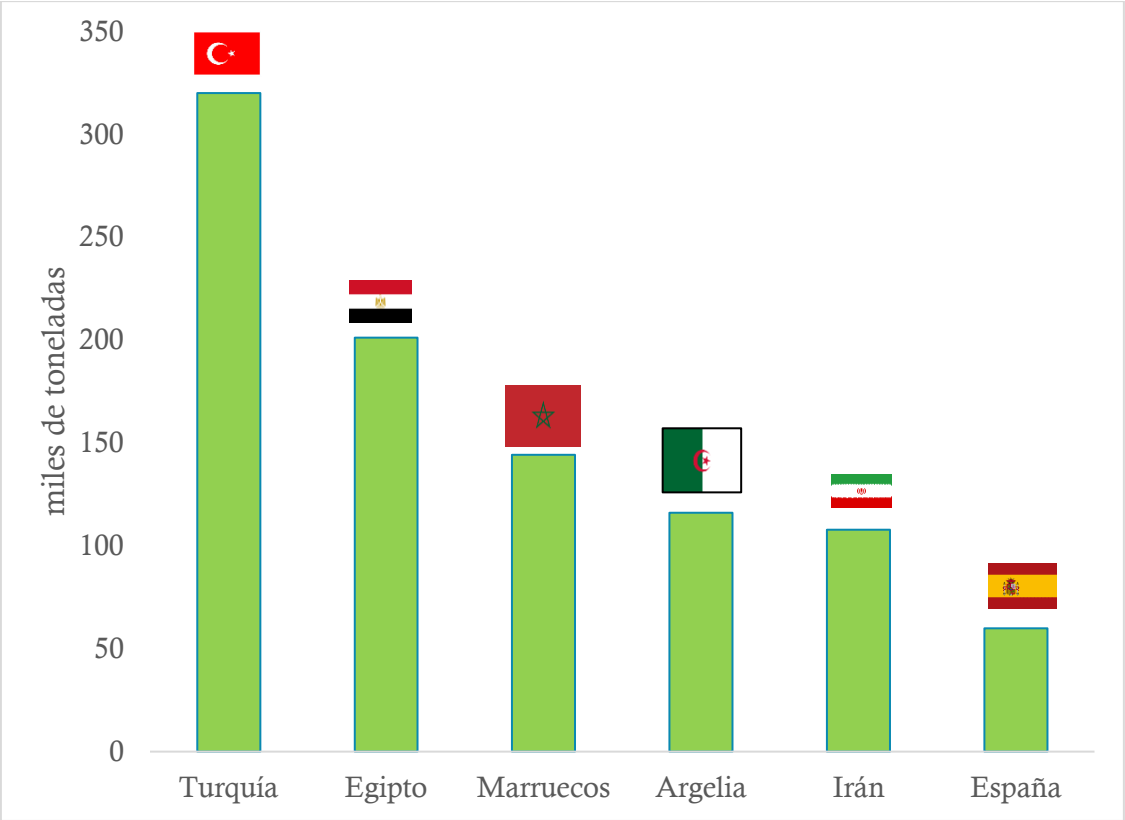

Figura 4. Principales países productores de higos a nivel mundial. Fuente: FAOSTAT, 2020.

De este volumen de producción mundial, durante la campaña 2020/2021 se produjeron unas 148.000 t de higos secos (ICN, 2021). Turquía fue el principal productor con un 58% del total, seguido de Irán (17%), España (7%), Estados Unidos (6%), Grecia (4%), Afganistán (3%) y en último lugar Italia (1%) (ICN, 2021) (Figura 5).

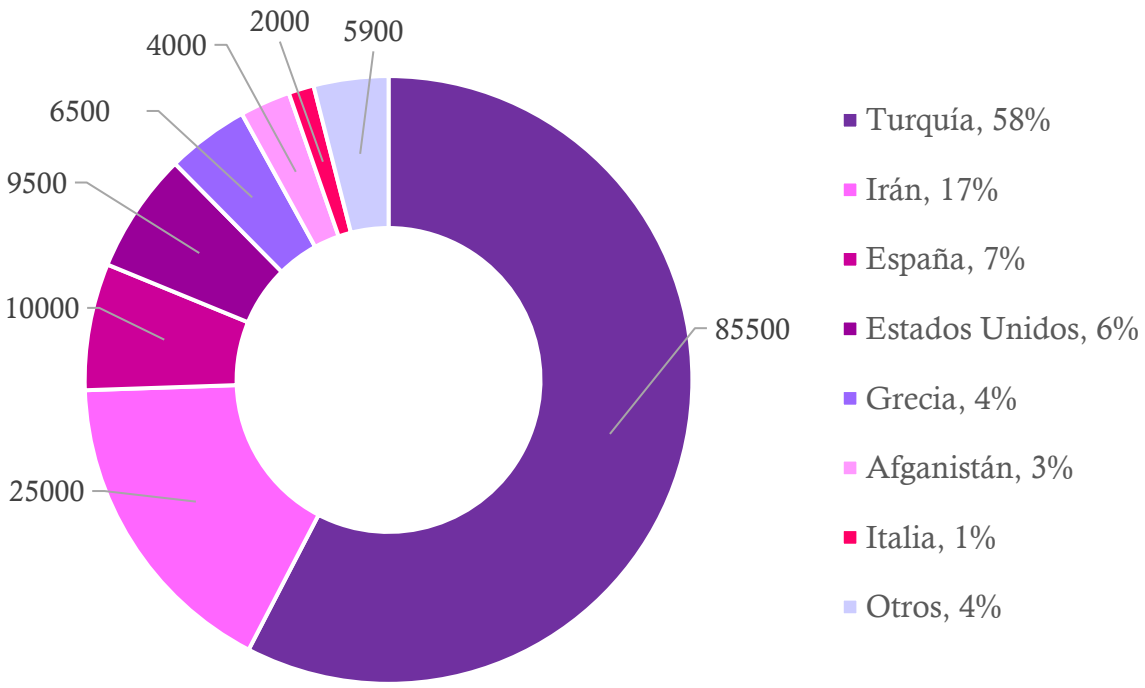

Figura 5. Producción (t) de higo seco a nivel mundial en la temporada 2020/2021. Fuente: INC, 2021.

De los países europeos, España es el principal productor con 15.720 ha y una producción de 59.900 t, lo que supone el 46,7% de la producción europea y el 4,7% de la producción mundial. De esta producción, el 77,68% se cultiva en secano.

A nivel nacional, Extremadura lidera la superficie y producción con 7.034 ha y 37.382 t, muy por encima de otras comunidades como Cataluña (5.834 t), Galicia (3.075 t), Comunidad Valenciana (2.932 t) y Castilla La Mancha (2.663 t) (MAPA, 2020) (Figura 6).

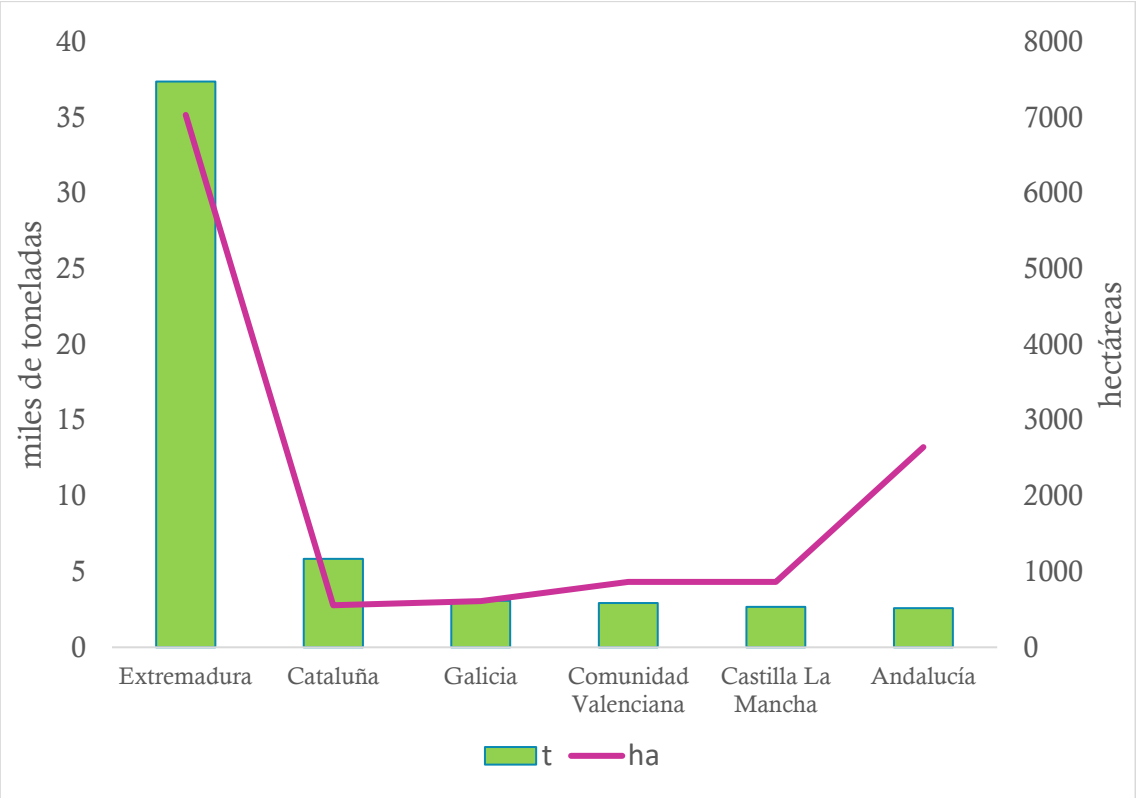

Figura 6. Principales comunidades productoras de higos a nivel nacional. Fuente: MAPA, 2020.

En la última década, este cultivo ha experimentado un incremento de superficie y producción. Por un lado, debido a la necesidad de búsqueda de cultivos alternativos ante la crisis de precios y de consumo del sector de la fruta de hueso y, por otro lado, por el incremento de la demanda a nivel nacional e internacional de higos tanto para consumo en fresco como para seco. Además de los precios competitivos percibidos por los fruticultores. Por ello, a nivel nacional se ha pasado de 11.629 ha de superficie en 2010 a 15.720 ha en 2020 (MAPA, 2020).

En el caso de Extremadura, el análisis de estos datos pone de manifiesto la introducción de este cultivo en regadío a partir de 2015, contabilizándose en el año 2020 unas 1.241 ha, de las cuales el 70,1% corresponden a la provincia de Badajoz.

En cuanto al flujo de comercio, en el año 2020, los principales países exportadores de higos fueron Turquía (46,5%), Afganistán (21,1%), Austria (3,57%), Alemania (3,48%) y España (3,46%) (OEC, 2020). España exportó un total de 4.536 t de higos frescos y 2.404 t de higos secos (FAOSTAT, 2020). Entre, los principales países importadores de higos en 2020 se encuentran India (20,7%), Alemania (13%), Francia (9,32%), Estados Unidos (6,73%) y Países Bajos (3,95%) (OEC, 2020). España importó un total de 669 t de higos frescos y 603 t de higos secos (FAOSTAT, 2020).

1.4. Técnicas de cultivo: Sistemas de producción en intensivo y superintensivo

Tradicionalmente, en España se han cultivado millones de higueras diseminados por toda la geografía nacional en plantaciones bajo condiciones de secano con amplios marcos de plantación (10 x 10 m e incluso 12 x 12 m). Estos árboles alcanzan una altura de hasta 6 m y un gran volumen de copa, con las consiguientes dificultades de manejo sobre todo para la producción en fresco. El rendimiento medio en secano se estima en torno a los 1.500 kg/ha, mientras que para las plantaciones de regadío el rendimiento se eleva a unos 6.600 kg/ha (López-Corrales y Balas, 2014).

En la actualidad, se buscan sistemas de formación más eficientes en los que las técnicas de cultivo como la poda, la fertilización y el riego juegan un papel fundamental, ya que aumentan la productividad y calibre de los frutos. Así, en California, las higueras son podadas horizontalmente a una determinada altura para evitar la recolección con escaleras y las plantaciones existentes son de alta densidad (4 x 2 m), incluyendo espalderas en sistemas de producción en 'cordón' similar a la

viña. Este sistema puede ser empleado para producir dos cosechas de higos al año en regiones de climas muy cálidos como Israel (Flaishman y col., 2008).

En España, se han llevado a cabo estudios con marcos de plantación en intensivo con variedades para la producción de higo fresco, con árboles formados en vaso bajo para facilitar la recolección desde el suelo y con un marco de plantación de 5 x 4 m. Bajo este sistema, se han obtenido rendimientos anuales entre 15 y 40 t/ha en el quinto verde de la plantación considerando las dos cosechas y la variedad (Pereira y col., 2015). Estos datos suponen triplicar los rendimientos establecidos para regadío en España por el Ministerio de Agricultura, Pesca y Alimentación (MAPA, 2020).

El sistema de formación en superintensivo permite aumentar el número de árboles por ha, con marcos típicos de 5 x 2 m o 4 x 4 m y en dos disposiciones posibles: en seto o espaldera. Para formar una espaldera, los árboles se disponen formando un eje vertical del que partirán varias ramas primarias entutoradas por alambres de acero, a modo de "pisos" a distintas alturas. Finalmente, los frutales acaban uniéndose entre sí, formando la espaldera o muro vegetal (Figura 7). En el caso del seto (típicamente empleado en cultivos como el olivo o el almendro), los árboles únicamente se establecen muy próximos entre sí hasta unir sus copas. La espaldera se ha optimizado en otros frutales como la vid, por las ventajas que aporta: reducción de costes y mayor efectividad en la aplicación de fitosanitarios, mayor insolación y aireación de la copa del árbol, optimización del tiempo en las podas y recolecciones (manuales en el caso de la higuera), aplicación más efectiva de los riegos, etc. (Iglesias, 2019) No obstante, favorece la propagación de ciertas plagas y enfermedades por la corta distancia entre árboles, implica mayores costes iniciales

para el establecimiento de la plantación y requiere patrones enanizantes, aunque en el caso de la higuera no es necesario.

Estos sistemas superintensivos podrían implementarse en el cultivo de la higuera, tanto para la producción de higo fresco como de seco, si bien, es necesario tener en cuenta aspectos relativos a la variedad como el hábito de crecimiento o la densidad de ramificación y el tipo productivo. En general, en las plantaciones para la producción en fresco tanto de brevas como de higos, las ramas principales (entre 3 y 5 ramas) deben insertarse a partir de unos 50- 60 cm del tronco y es necesario alcanzar en la poda de invierno un equilibrio que asegure ambas cosechas (si se poda demasiado disminuye la producción de brevas al año siguiente). En el caso de plantaciones para secado, la altura de inserción de las ramas principales debe ser próxima a los 90-100 cm para facilitar la recogida de los higos desde el suelo y facilitar la aireación y la pérdida de humedad de los frutos. Estudios relativos a fechas e intensidades de poda en variedades tipo San Pedro, cultivados para la producción de brevas, los autores encontraron diferencias significativas tanto en la producción como en la productividad dependiendo de la fecha y el tipo de corte. Las producciones más elevadas se obtuvieron cuando los árboles se podaron en fechas tempranas, después de la producción de higos, ya que estas podas tempranas incrementaron la longitud de los nuevos brotes productores de brevas en la campaña siguiente (Caetano y col., 2005; Puebla y col., 2003). Estas técnicas no van ligadas únicamente a alcanzar mayores rendimientos sino también a lograr una sostenibilidad a largo plazo, para lo cual la Unión Europea se ha implicado activamente a través de sus políticas frente al cambio climático. Así, en 2019 se firmó el Green Deal o Pacto Verde, un paquete de medidas económicas que tiene como objetivos frenar el cambio climático, reducir la huella de carbono y la pérdida de la

biodiversidad, entre otros. Este pacto, incluye una serie de estrategias, siendo "De la Granja a la Mesa" la que más influye al sector agropecuario. La Comisión Europea apuesta por una Agricultura Intensiva Sostenible, apoyándose en la tecnología, o lo que se ha venido a denominar como "Agricultura de Precisión" o "Smart Farming". En el caso de los frutales, esta intensificación busca reducir el volumen de copa hasta conseguir una estructura bidimensional, facilitando un uso eficiente de los inputs y optimizando el periodo improductivo a coste de una mayor inversión inicial.

Un aspecto muy interesante de estos sistemas superintensivos en higueras para la producción de higo seco, es la posibilidad de mejorar la recolección de los frutos que tradicionalmente son recolectados a mano del suelo, normalmente una vez a la semana (Aksoy, 1997). Como resultado, estos frutos pueden ser contaminados por insectos u mohos toxigénicos que están presentes de forma natural en el entorno. El método tradicional de recolección y producción de higos secos se ve comprometido por la presencia de micotoxinas producidas por mohos de los géneros *Asperguilllus spp*., *Fusarium spp*. y *Penicillium spp*. (Gilbert y Senyuva, 2008). Dada la creciente importancia de la seguridad alimentaria en los mercados mundiales y la demanda de los consumidores de productos de alta calidad y seguros, es necesario garantizar una manipulación adecuada de los higos secos durante el proceso de producción de los higos secos en campo. Por tanto, el estudio de nuevas plantaciones superintensivas más productivas y eficientes que permitan satisfacer el aumento de la demanda de los consumidores también tiene que garantizar la obtención de un producto con la máxima calidad higiénica y sanitaria. Para ello, estos sistemas pueden permitir la instalación de unas estructuras para el soporte de mallas que eviten que el higo seco entre en contacto con el suelo y, a la vez, facilitar su recolección.

Figura 7. Sistema intensivo en espaldera para la producción de higos frescos. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

2. Higo seco

El higo seco es uno de los productos más antiguos e importantes en la región mediterránea (Veberic y col., 2015). El secado del higo es un proceso mediante el cual el peso y el volumen del fruto se reduce y se incrementa el contenido en azúcares. El principal objetivo de la producción de higos secos es prolongar su vida útil mediante la disminución de la actividad de agua (a_w) a niveles que inhiban el crecimiento microbiano y la actividad enzimática (Cano-Chauca y col., 2004; Villalobos y col., 2016). Existen dos tipos de secado: el natural, que obtiene su fuente de calor a través del sol, y el artificial, basados en sistemas de generación de calor mediante equipos artificiales (Hansen y col., 1993; Mat Desa y col., 2019).

El secado natural al sol es el método más utilizado en las explotaciones agrícolas para la producción de higos secos, dado que los frutos se secan al aire libre, bajo el sol y con un bajo coste de producción (Villalobos y col., 2016). Este tipo de secado es posible en la mayoría de las regiones que presenten un clima adecuado. Para el secado tradicional al sol, los higos se cosechan del árbol o se recogen del suelo y, posteriormente, el secado final se realiza en bandejas bajo cubierta o sin ella (Haug y col., 2013). En los últimos años, también se han puesto en práctica el uso de sistemas artificiales, entre ellos, se encuentran los métodos convectivos, que utilizan fuentes de energías tradicionales como la electricidad u otros combustibles y/o fuentes renovables como la energía solar, las microondas y otras formas de calentamiento por radiación para la generación de calor (Maghoumi y col., 2022). Estos métodos permiten un secado rápido y uniforme. Sin embargo, la mayoría de los métodos de secado asistidos por equipos, excepto el secado solar, tienen un elevado coste energético y, en consecuencia, aumentan el coste del producto final (Sharma y col., 2009). Los secaderos de alta temperatura que se abastecen con electricidad se utilizan en los países más desarrollados con un fin comercial (Tiwari, 2016). En los últimos años, se han llevado a cabo numerosos estudios relacionados con el secado artificial. Konak y col. (2017) estudiaron diferentes tipos de secado en variedades de higos tanto de piel negra como de piel verde recogidos parcialmente secos y observaron que el secado al sol requería de 2-3 días, mientras que el secado artificial mediante horno, ajustado a 60 °C, tardó 12 h. En este mismo sentido, Xanthopoulos y col. (2009) en un estudio también sobre secado artificial de higos, los frutos enteros tardaron una media de 48, 36 y 22 h a temperaturas del aire de 45, 55 y 65 °C, respectivamente. En el secado artificial tiene el inconveniente de que es muy importante controlar las condiciones de secado, debido a que, si se aplica una

temperatura alta durante la primera etapa de secado, se produce la formación de una "costra" (Cemeroğlu y Özkan, 2009). También es necesario en caso de las variedades de piel verde hacerlo bajo condiciones de luz para favorecer la destrucción de las colorfilas (Villalobos y col., 2016). En Extremadura, tradicionalmente, el secado de los higos se ha llevado a cabo mediante secado natural al sol, debido entre otros aspectos, a que permite el secado a granel y los costes de inversión y energía son bajos.

2.1. Producción y procesado del higo seco

En la figura 8 se muestra el diagrama de flujo de la producción y procesado del higo seco. El higo seco pasa por diferentes etapas, primero en el campo y paseras hasta alcanzar una humedad del 26% y, posteriormente, en la industria hasta obtener el producto final. Cuando los higos secos llegan a la industria, pasan por distintas etapas que comprenden desde el curado/fumigación para el control de las plagas hasta el enharinado y envasado final. Este proceso no es idéntico en todas las industrias, ya que pueden existir variaciones en el tiempo, la temperatura de conservación o en cómo se lleven a cabo las diferentes etapas. Durante el procesado, se deben tener en cuenta diferentes tipos de peligros físicos, químicos y biológicos para su prevención o reducción. Entre los peligros físicos, se encuentran restos de tierra y piedras, mientras que entre los principales riesgos químicos destacan la presencia de cloraminas y cloratos en el producto final como consecuencia del uso de productos clorados para la desinfección del producto y del agua de lavado. Finalmente, entre los principales riesgos biológicos destacan el crecimiento de mohos toxigénicos y la producción de micotoxinas, aunque también plagas de almacenamiento como los ácaros.

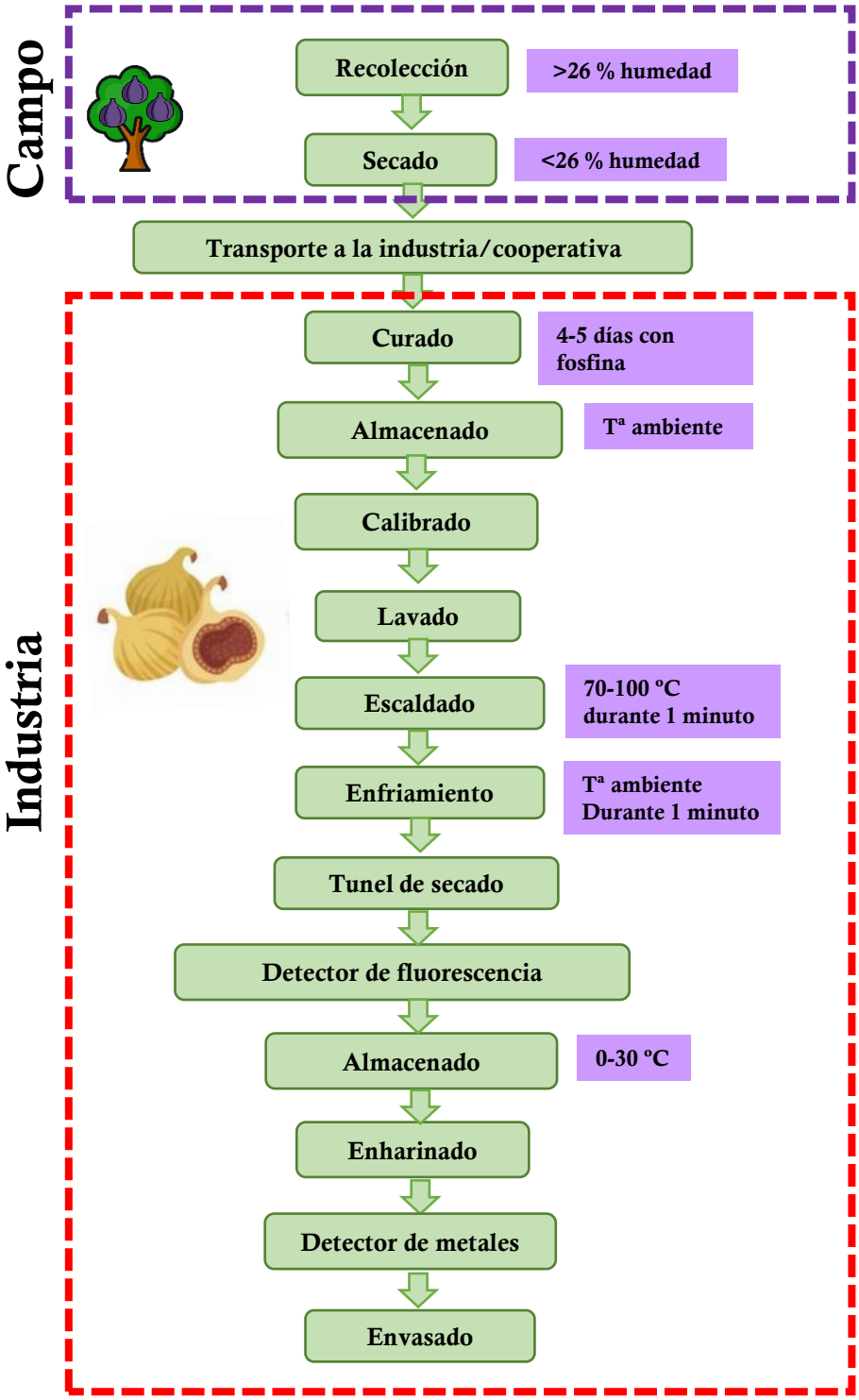

Figura 8. Diagrama de flujo del proceso de producción del higo seco.

- **Recolección:** los higos secos se recolectan en campo durante los meses de agosto y septiembre. Tradicionalmente, se deja que los higos maduren por completo, se deshidratan parcialmente en el árbol y se caen de forma natural

al suelo donde continúan secándose y se recogen periódicamente (2-3 veces durante la campaña) de forma manual o mediante el uso de sopladores y rastrillos y/o palas (Figura 9). En este momento, los higos están parcialmente secos, con un contenido de humedad que oscila entorno al 30-60% (Şen y col., 2017; Senyuva y col., 2008). La duración de esta primera fase viene determinada por el tamaño del higo, el caudal de aire, así como de la temperatura y humedad relativa (HR) del ambiente (Aksoy, 2017).

Figura 9. Recolección de higos secos con el uso de sopladores. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

- **Secado:** Los higos son llevados a almacenes o invernaderos, en donde termina de secarse en paseras al sol hasta adquirir un contenido de humedad inferior al 26% de acuerdo con la norma de calidad DDP-14 relativa a la

comercialización y control de calidad de los higos secos (UNECE, 2016) (Figura 10). Durante este segundo secado, se recomienda espaciar o separar los higos entre sí y voltear los frutos para obtener un secado homogéneo en toda la superficie. El tiempo de secado de un higo depende en gran medida de las condiciones climatológicas. Desde el momento en que los higos comienzan a secarse hasta su recogida en el suelo y secado final, los frutos están expuestos a enfermedades fúngicas, plagas, daños mecánicos y, en consecuencia, pérdidas de calidad.

Figura 10. Secado de higos al sol en paseras bajo invernadero. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

Posteriormente, se realiza una primera **selección manual** y **limpieza** de los frutos y los higos secos, que están teóricamente sanos, son entregados en la empresa transformadora o **industria** y/o **cooperativa**.

- **Curado:** Los higos secos son fumigados con fosfuro de aluminio y/o magnesio durante 4-5 días en condiciones de estanqueidad, para la eliminación de plagas procedentes del campo y prevención de plagas durante el almacenamiento (Athanassiou y col., 2016). Para su aplicación, los higos secos se meten en contenedores a temperatura ambiente durante 3-5 días. Durante este tiempo, el fosfuro de aluminio reacciona con la humedad y se produce el fosfuro de hidrógeno, denominado "fosfina". De esta manera el higo seco queda libre de plagas durante un período máximo garantizado de 3 meses.
- **Almacenado:** Los higos secos son almacenados en palés a temperatura ambiente o bajo refrigeración, dependiendo de la industria y procesado (Figura 11).

Figura 11. Etapa de almacenado del higo seco en palés en cámaras de refrigeración. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

- **Calibrado:** Los higos secos son clasificados por tamaño y peso en diferentes categorías comerciales mediante el uso de una clasificadora automática con

tamices de diferentes diámetros con el objetivo de conseguir una calidad homogénea del lote a procesar (Figura 12). Su tamaño se determinará por el número de frutos por kilogramo o por el diámetro del fruto. Los higos secos se clasifican según la norma DDP-14 (UNECE, 2016) en 11 tamaños distintos, que van desde el tamaño 1 que tiene menos de 40 frutos hasta el tamaño 11 que tiene más de 120 frutos por kilogramo. Las categorías de calidad son "Extra", Categoría I y Categoría II. El número de frutos por kilogramo está limitado a 65 para la categoría "Extra" y a 120 para la Clase I. Hay ciertas tolerancias para el rango de tamaño en cada categoría de calidad si los higos secos se clasifican por su diámetro, el diámetro mínimo debe ser de 18 mm para las variedades de higos negros y de 22 mm para las variedades de color claro. Durante este proceso también se eliminan los materiales extraños que puedan existir como piedras, palos, tierra y objetos de plástico entre otros. Posteriormente, los higos secos son transportados mediante cintas hacia la lavadora.

Figura 12. Trabajadores en la calibradora de la cooperativa Regadhigos de Almoharín (Cáceres). Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

- **Lavado:** Los higos secos son lavados con agua a temperatura ambiente (de calidad potable o con agua hiperclorada dependiendo de la industria) en una lavadora industrial de frutas durante 1 minuto aproximadamente. Durante el lavado, se incrementa el contenido de humedad de los higos secos un 2-3%, especialmente en la piel exterior. Posteriormente, los higos son transportados mediante cintas hasta la escaldadora.
- **Escaldado:** El escaldado es un tratamiento térmico corto que se aplica a los productos vegetales para la inactivación enzimática. Los higos secos entran en balsas de agua que están entre 70 y 100ºC de temperatura, aquí son mantenidos durante 1 minuto aproximadamente, dependiendo de la industria. Algunas industrias realizan este escaldado con vapor, es decir, los higos secos pasan a través de una atmósfera de vapor saturado. A diferencia

de otros tratamientos, el escaldado no destruye los microorganismos ni alarga la vida útil. El objetivo del escaldado es mejorar la apariencia y textura del producto. Posteriormente, son transportados en cintas hasta las duchas de enfriamiento.

- **Enfriamiento:** Los higos pasan por debajo de duchas de agua a temperatura ambiente durante 1 minuto, para bajar su temperatura de manera rápida. A continuación, los higos pasan a un túnel de secado.
- **Secado:** Se lleva a cabo en un túnel mediante soplado de aire caliente (45-50ºC) con la ayuda de ventiladores sobre una cinta transportadora que se mueve a velocidad constante. El objetivo es reducir el contenido de humedad del producto. Seguidamente, los higos son transportados mediante cintas a la cabina donde se encuentra el detector de fluorescencia mediante lámparas de luz ultravioleta para detectar mediante fluorescencia la presencia de higos secos contaminados con aflatoxinas (AFs).
- **Detector de fluorescencia:** Para controlar las AFs la industria utiliza lámparas ultravioletas (UV) de 365 nm de longitud de onda, que permiten visualizar la presencia o no de fluorescencia amarilla verdosa brillante (BGYF) producido por el ácido kójico, indicador de contaminación con AFs en los higos secos (Kılıç y İnner, 2022; Sulyok y col., 2020), ya que los mohos productores de AFs también producen este ácido. Para ello, los higos secos pasan bajo las lámparas UV instaladas y los positivos BGYF se eliminan de la línea por los operarios (Figura 13). Esta fluorescencia se detecta en la parte externa del higo y cuando existe una contaminación elevada de AFs. Sin embargo, si las AFs se encuentran principalmente en la parte interior del higo,

este método es poco eficaz para reducir la contaminación por AFs en higo seco (Şen, 2022).

Figura 13. Higos secos fluorescentes bajo luz UV. Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

- **Almacenado:** Los higos secos son almacenados en cámaras a temperaturas entre 0 y 30ºC, dependiendo de la industria o cooperativa, hasta su comercialización (3 a 12 meses). A veces, se realiza una fumigación adicional con fosfina en el producto contra las plagas de almacenamiento.
- **Enharinado:** Cuando se van a comercializar, los higos secos son enharinados en una tolva con harina de arroz (Figura 14). De esta forma, se consigue conservar mejor el producto y evitar que se muestren los defectos de color y otros tipos de defectos. Además, la harina también sirve para captar la humedad superficial que pueda aparecer.

Figura 14. Enharinado de higos secos en la Agrupación de Cooperativa del Valle del Jerte S.C.L. (Cáceres). Fuente: Dpto. Fruticultura Mediterránea (CICYTEX).

- **Detector de metales:** Los higos secos enharinados pasan a través de un arco o detector de metales para garantizar que están libres de cualquier resto metálico.
- **Envasado:** Una vez enharinados, son envasados mediante una envasadora automática con pesaje automático. Los higos secos suelen comercializarse en bolsas de plástico de aproximadamente 400 g. Aunque existen otros formatos de envasado como a granel en cajas de cartón, tarrinas, barquetas, bolsa de yute o al vacío (Figura 15), que pueden contener envases de consumo que oscilan, en su mayoría, entre los 250 g y los 2.500 g. Tras el envasado los higos se comercializan a temperatura ambiente.

Figura 15. Diferentes presentaciones comerciales de higos secos. Fuente: Ecoficus (http://www.ecoficus.com) e Higos & Figs (https://higosandfigs.com/tag/almoharin/).

Además del consumo directo de los frutos, también existe una gama de productos de alto valor añadido en las que se utiliza el higo seco como base. En los mercados se pueden encontrar pasta de higo, barritas energéticas, bombones de higos, licores, mermeladas, entre otros. Estos productos llevan un procesado industrial diferente. Sin embargo, en España, la principal vía de comercialización es para consumo directo.

2.2. Características nutricionales y de calidad del higo seco

La relación entre la alimentación y la salud es cada vez más importante, ya que los consumidores exigen alimentos saludables, sanos, sabrosos, naturales y seguros (Abbasi y col., 2013). Los higos frescos son ricos en hidratos de carbono, predominantemente fructosa y glucosa, fibra, magnesio, potasio, calcio y vitaminas K, α-tocoferol y D_3 (Lim, 2012; Sadia y col., 2014). Durante la desecación de los higos frescos, su contenido en agua se reduce produciendo una concentración de los nutrientes. En particular, los higos secos son una de las mayores fuentes vegetales de fibra, magnesio, potasio, calcio y vitamina K para los seres humanos (Arvaniti y col., 2019; Lim, 2012). Además, son una buena fuente de ácidos orgánicos, compuestos fenólicos y compuestos volátiles que proporcionan un aroma característico (Bekatorou y col., 2002; Slatnar y col., 2011; Veberic y col., 2015; Vemmos y col., 2013). Numerosos estudios han informado sobre la eficacia del consumo de higos frescos y secos para prevenir las enfermedades cardiovasculares (Alamgeer y col., 2017; Gholami y col., 2012), el cáncer (Marrelli y col., 2012; Purnamasari y col., 2019), la inflamación (Yang y col., 2015) y el estreñimiento (Rtibi y col., 2018). La composición de los higos depende de varios factores como el genotipo, las condiciones edafoclimáticas y el estado de maduración del fruto, entre otros (Ercisli y col., 2012; Flaishman y col., 2008; Pereira y col., 2017). Pourghayoumi y col. (2016) informaron que los contenidos en sólidos solubles totales (SST) en higos secos oscilaban entre 60 y 84 °Brix. Se ha informado que la radiación a la que se exponen los frutos durante el proceso de secado puede influir en el contenido de SST y, por tanto, de azúcares (Cucunubo Bosa y col., 2019; Li y col., 2020). Además, debido a la pérdida del contenido de humedad, se produce una concentración de azúcares y fibra. Esto hace que el higo seco sea un producto muy apreciado por los

consumidores ya que la fibra dietética desempeña un papel importante en la salud humana como se ha mencionado anteriormente.

Por otro lado, la calidad de los higos secos también viene determinada por el contenido de humedad, la firmeza, el color y el tamaño de los frutos. El tamaño y el color son los principales factores de comercialización de los higos secos, especialmente para consumo directo (Ansari y col., 2014; İrget y col., 2008; Polat y Siddiq, 2012). En un estudio llevado a cabo en Irán mostraron que los árboles regados con 2000 L/árbol tenían rendimientos más altos y frutos con diámetros >22 mm. Sin embargo, observaron un color más oscuro de los frutos. Además, informaron también que el riego temprano de primavera dio lugar a un mayor número de frutos pequeños (Abdolahipour y col., 2019). En cuanto al color, se ha demostrado que el secado en invernadero mejora el color amarillo, el brillo y la intensidad de color (Lachtar y col., 2022). Abul-Fadl y col. (2015) observaron que los higos secos que presentan elevados niveles de azúcares reductores interactúan con los aminoácidos y dan lugar a compuestos marrones. Otros factores que también pueden afectar al color son la temperatura y la humedad del aire, la incidencia de luz solar, el método de secado, el tipo de suelo y la cantidad de agua del suelo (Flaishman y col., 2008). Además, estos factores también pueden influir en el contenido final de humedad de los frutos y, por tanto, en la firmeza, parámetro muy apreciado por el consumidor en este tipo de fruto. Lachtar y col. (2022) demostraron que la humedad y la a_w se correlacionan positivamente y ambos parámetros se correlacionan negativamente con la firmeza. En este sentido, Ansari y col. (2014) confirmaron la relación entre la firmeza de la fruta seca y el contenido de humedad. Uno de los métodos más utilizados en el procesado industrial del higo seco para mejorar la firmeza y apariencia de estos es el escaldado. Villalobos y col. (2019) obtuvieron valores de

firmeza en torno a 1 N/mm^{-1} en higos secos de la variedad Calabacita. En general, los higos secos más apreciados por los consumidores son de color amarillo, de firmeza blanda y con sabor dulce (Lachtar y col., 2022).

2.3 Peligros microbiológicos durante la producción de higo seco

2.3.1 Población fúngica

La contaminación y el crecimiento fúngico es un problema característico de una gran variedad de alimentos con una humedad de baja a intermedia, como cereales, frutos secos, frutas desecadas, harinas, especias y pan (Figura 16). Los mohos se caracterizan por crecer a bajas a_w y son más competitivos en estas condiciones que otros microorganismos (Aydin y col., 2008; Hammami y col., 2014; Jahanmard y col., 2014). El contenido de humedad es un factor clave ya que afecta directamente al desarrollo de mohos toxigénicos (Aksoy, 2017; Perera, 2005). En este sentido, para los higos secos, la norma DDP-14 establece que el contenido máximo de humedad debe ser inferior al 26% (UNECE, 2016) para prevenir el crecimiento de mohos.

Figura 16. Higo fresco con crecimiento fúngico en superficie. Fuente: Dpto. Nutrición y Bromatología (Escuela de Ingenierías Agrarias).

Los higos secos presentan un contenido de a_w bajo y, debido a sus características intrínsecas de pH y contenido en azúcares, los mohos son los principales contaminantes microbiológicos presentes en este fruto (Drusch and Ragab, 2003). Por otro lado, las condiciones de temperatura y humedad durante la maduración y el marchitamiento de los frutos en el árbol son favorables para el crecimiento de mohos. Además, el contacto de los higos con el suelo durante 7-10 días y los sistemas de recolección como barrido o el uso de sopladores, hacen que se favorezca la contaminación y el desarrollo de mohos toxigénicos. Estos mohos puedan desarrollarse sobre la piel o en la cavidad interna del fruto a donde llegan a través de vectores como los insectos. Asimismo, las condiciones de humedad y temperatura alcanzadas durante el procesado industrial de los higos secos son favorables para el crecimiento fúngico (Trucksess y Scott, 2008). Se trata por tanto de un producto muy susceptible de ser contaminado por mohos, por lo que la presencia fúngica ha sido ampliamente estudiada en este tipo de fruto. Heperkan y col. (2012) informaron que la micobiota que se encuentra con más frecuencia en los higos secos es *Aspergillus* sección *Nigri*, especies de *Fusarium*, *Aspergillus* sección *Flavi*, y especies de *Penicillium*. Javanmard (2010) informó también que las especies más frecuentes en los higos secos de Irán fueron *A. niger* (90,9%), *A. flavus* (63,7%) y *Acremonium* spp. (54,6%) y en menor frecuencia *Alternaria* spp. y *Penicillium* spp. (9,1%). Hedawoo y Bijwe. (2018) aislaron 15 especies fúngicas a partir de higos secos e informaron que el género *Aspergillus* fue el aislado más dominante, con un total de seis especies diferentes. En Yemen, *Aspergillus* fue de nuevo el género más frecuentemente aislado en higos secos, siendo *A. niger* la especie predominante, mientras que *Penicillium* spp. ocupó el segundo lugar (Alghalibi y Shater, 2004). Özay y Eke, (1988) observaron que el crecimiento de mohos pertenecientes al género

Aspergillus era más propio de las zonas tropicales y subtropicales, debido a sus mayores niveles de temperatura y humedad. Por el contrario, los mohos pertenecientes al género *Penicillium* se encuentran sobre todo en regiones de clima templado, mientras que los mohos pertenecientes al género *Fusarium* prevalecen en las regiones más frías. La mayoría de las especies fúngicas descritas anteriormente se caracterizan por la producción de forma natural de metabolitos secundarios tóxicos llamados micotoxinas (Samson y col., 2019). En base a todo lo mencionado con anterioridad, el desarrollo de mohos toxigénicos productores de micotoxinas es uno de los principales riesgos biológicos que presenta el consumo de higo seco y sus derivados, de forma que cada año se notifica a través del Sistema de Alerta Rápida para Aimentos y Piensos (RASFF_ Rapid Alert System for Food and Feed) un elevado número de alertas sanitarias en higos secos por presencia de micotoxinas, especialmente por AFs.

2.3.2. Micotoxinas

Son metabolitos secundarios de bajo peso molecular (Pm<700) producidos por ciertos géneros de mohos filamentosos y pueden encontrarse en una amplia gama de productos básicos (Jeswal y Kumar, 2015; Malachová y col., 2014). La mayoría de las micotoxinas son químicamente estables y persisten tras el procesado de los alimentos (Scott, 1991). En la actualidad, se conocen más de 400 micotoxinas (Turkoz Bakırcı, 2020). Sin embargo, el número de micotoxinas que suelen encontrarse en los alimentos no llega a 30 micotoxinas diferentes (Bennet y Klich, 2003; Santos, 2011). Las más frecuentes encontradas en alimentos y piensos son: AFs, citrinina (CIT), ácido ciclopiazónico (CPA), fumonisinas (FBs), ocratoxina A (OTA), patulina (PAT), tricotecenos (principalmente nivalenol (NIV),

deoxinivalenol (DON), toxina T-2 (T2) y toxina HT-2 (T2), alternariol (AOH), ácido tenuazónico (TeA) y zearalenona (ZEA) (Kumar y col., 2020; Samson y col., 2019). La mayoría de las micotoxinas conocidas son producidas por especies de los géneros *Aspergillus*, *Penicillium*, *Alternaria* y *Fusarium* (Tabla 1).

Las micotoxinas más importantes descritas en higo seco son las AFs y la OTA (Heperkan y col., 2012; Heshmati y col., 2017; Karbancıoglu-Güler y Heperkan, 2009; Senyuva y col., 2008). Además, también se han informado de la aparición de otras micotoxinas en estos frutos como las fumonisinas (FBs) (Karbancioğlu-Güler y Heperkan, 2009), CPA (Heperkan, 2011), ácido kójico, esterigmatocistina, ácido tenuazónico, ácido fusárico, y la beauvericina, entre otros (Di sanzo y col., 2018; Sulyok y col., 2020).

Tabla 1. Micotoxinas producidas por las principales especies fúngicas potencialmente toxigénicas de *Aspergillus*, *Penicillium*, *Alternaria* y *Fusarium*.

Género	Especies	Micotoxinas	Referencias
Aspergillus	***A. flavus***	Aflatoxinas (tipo B) Ácido ciclopiazónico Ácido kójico Ácido 3-nitropropiónico	Samson y col. (2019); Varga y col. (2015)
	A. parasiticus	Aflatoxinas (tipo B y G) Ácido kójico	Varga y col. (2015)
	A. tamarii	Ácido ciclopiazónico Ácido kójico Ácido tenuazónico	Samson y col. (2019);
	A. oryzae	Ácido ciclopiazónico Ácido kójico Ácido 3-nitropropiónico	Chang y col. (2009); Samson y col. (2019)
	A. niger	Ocratoxina A Fumonisinas	Frisvad y col. (2018)
	A. welwitschiae	Ocratoxina A Fumonisinas	Frisvad y col. (2011)
	A. tubingensis	Ocratoxina A	Lasram y col. (2012)
	A. ochraceus	Ocratoxinas	Magan y Aldred (2006)
	A. carbonarius	Ocratoxina A	Samson y col. (2019)
Penicillium	***P. commune***	Ácido ciclopiazónico	Frisvad y Samson, (2004); Samson y col. (2004)
	P. expansum	Patulina Citrinina	Frisvad y Samson, (2004); Samson y col. (2004)
	P. griseofulvum	Patulina	Frisvad y Samson, (2004); Samson y col. (2004)
	P. verrucosum	Citrinina Ocratoxina A	Frisvad y Samson, (2004), Schmidt-Heydt y col. (2018)
	P. citrinum	Citrinina	Houbraken y col. (2010, 2011)
	P. chrysogenum	Ácido secalónico	Frisvad y Samson (2004);
Alternaria	***A. alternata***	Ácido tenuazónico	Samson y col. (2019)
	A. arborescens	Ácido tenuazónico	Samson y col. (2019)
Fusarium	***F. graminearum***	Fusarina C Tricotecenos Zearalenona	Samson y col. (2019)
	F. verticillioides	Fumonisinas Ácido fusárico Moniliformina	Samson y col. (2019)
	F. proliferatum	Fusaproliferina	Ramos (2011)

Las micotoxinas son metabolitos secundarios que se sintetizan por diversas rutas enzimáticas, se han contabilizado hasta un total de 27, a partir de diferentes componentes como polipéptidos, aminoácidos, fenoles y terpenoides (Caceres y col., 2020). Una determinada micotoxina puede ser producida por diferentes especies y una determinada cepa puede producir diferentes micotoxinas (Atalla y col., 2003). Cuanto más compleja es la ruta biosintética de una micotoxina, menor es el número de especies fúngicas capaces de elaborarla (Moss, 1991). El principal problema de las micotoxinas es que son potentes tóxicos, tienen grandes consecuencias para la salud humana y animal y, por ello, han sido clasificadas por La Agencia Internacional para la Investigación del Cáncer de la Organización Mundial de la Salud como unas de los mayores carcinógenos humanos (OMS-IARC). La ingesta de elevados niveles de micotoxinas en la dieta puede causar efectos adversos agudos o crónicos, afectando a distintos órganos, aparatos o sistemas, especialmente al hígado, riñón, sistema nervioso, endocrino e inmunitario (Cáceres y col., 2020). Los síntomas causados por las micotoxinas suelen ser diferentes unos de otros como lo son las propias estructuras químicas de dichos metabolitos secundarios. Además, su toxicidad depende en gran medida de las cantidades ingeridas, del tiempo de exposición y de las posibles sinergias que puedan derivarse de la ingestión de diferentes micotoxinas al mismo tiempo. Se sabe también que diferentes especies tienen distintas sensibilidades a las micotoxinas y que, por lo general, la edad, el sexo y su estado fisiológico son cruciales en su nivel de toxicidad. Según Bullerman (1979) cuando se ingiere micotoxinas en grandes cantidades pueden causar toxicidad aguda en los animales con resultado de muerte (micotoxicosis). Cuando los animales son expuestos a niveles ligeramente por debajo de los letales causan disminución de peso y de la producción de leche y huevos, mientras que cuando se exponen a pequeñas

concentraciones provocan supresión de la función inmune y disminución de la resistencia a la infección. Sin embargo, cuando se exponen los animales a bajas concentraciones, aunque prolongadas, las micotoxinas favorecen la formación de tumores y el desarrollo de enfermedades crónicas en los órganos vitales. En la forma subaguda, los individuos jóvenes pueden presentar retardo en el crecimiento, pérdida del apetito, se compromete el sistema inmunitario, se aumenta la fragilidad capilar afectando al tiempo de coagulación sanguínea y de ahí, la presencia de hematomas, postración y muerte. Datos circunstanciales vinculan también enfermedades crónicas como el cáncer de hígado y esófago al consumo de alimentos contaminados con AFs (Villalobos, 2015).

El consumo de piensos preparados con ingredientes como las tortas de semillas oleaginosas de maní, algodón, coco o granos de maíz contaminados con AFs no solo provocan micotoxicosis en los animales, sino que crean también problemas de residuos de micotoxinas en productos animales, tales como la leche, la carne y los huevos.

2.3.2.1. **Principales micotoxinas en higos secos**

Entre las principales **micotoxinas** que pueden contaminar los higos secos como consecuencia del desarrollo fúngico se encuentran:

-<u>Aflatoxinas</u>: Las AFs son derivados de la difuranocumarina, por vía de los policéticos. Los genes responsables de la producción AFs se localizan en el clúster 54 que incluye 30 genes y cuya activación está regulado principalmente por aflR y aflS (Caceres y col., 2020). Son producidas por las especies *A. flavus* y *A. parasiticus* principalmente, pero también por otras especies como *A. bombycis*, *A. pseudotamarii*, *A. ochraceoroseus*, *A. nomius*, *A. toxicarius* (Klich, 2007; Peterson y col., 2001). Se conocen más de 16 derivados de AFs, cuatro de ellos considerados como los más importantes AFB_1, AFB_2, AFG_1, AFG_2 (Pitt y Hocking, 1997) (Figura 17), produciendo *A. flavus* AFs del tipo B, mientras que *A. parasiticus* produce AFs del tipo B y G.

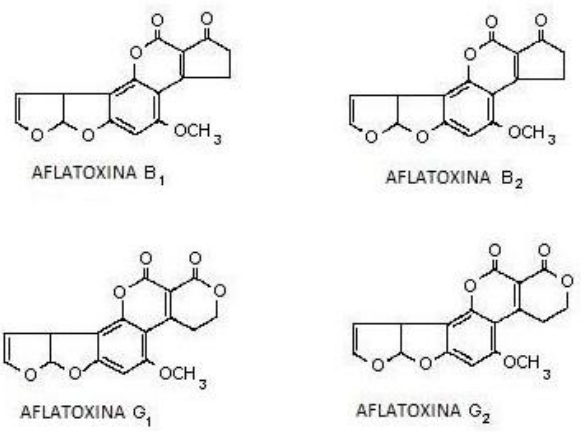

Figura 17. Estructuras químicas de AFs de los tipos B y G. Fuente: Cruz y col. (2014).

Dentro del grupo de las AFs, la AFB_1 ha sido clasificada como carcinógeno humano del grupo 1 por IARC (IARC, 1993).

Los mohos productores de AFs son ubicuos y han sido descritos como contaminantes de muchos alimentos como frutos secos, maíz, semillas oleaginosas, frutas desecadas, café, cereales, etc. Por ello, en interés de la salud pública, es necesario mantener el contenido AFs en niveles seguros para garantizar la salud de los consumidores. Los niveles máximos de AFs permitidos en productos alimenticios están determinados por el Reglamento (EU) Nº 165/2010, mientras que en el caso de los higos secos, el contenido máximo de AFs en higos secos está regulado por el Reglamento (UE) Nº 1058/2012 (Tabla 2).

Tabla 2. Límites máximos de AFB_1 y AFs en frutos e higos secos establecidos por el Reglamento (UE) Nº 1058/2012

Alimentos	AFB_1 (µg/kg)	AFs (µg/kg)
Frutos secos, distintos de los higos secos, destinados a ser sometidos a un proceso de selección u otro tratamiento físico, antes del consumo humano o de su uso como ingredientes de productos alimenticios	5,0	10,0
Frutos secos, distintos de los higos secos, y productos derivados de su transformación, destinados al consumo humano directo o a ser usados como ingredientes en los productos alimenticios	2,0	4,0
Higos secos	6,0	10,0

- <u>Ocratoxina A</u>: La OTA aislada orginalmente del *A. ochraceus* en 1965 es la más tóxica de las OTA producidas por mohos toxigénicos (Van Der Merwe y col., 1965). La OTA es un derivado de la fenilalanina-dihidroisocumarina (Figura 18), y es muy estable tanto a la temperatura como a la hidrólisis.

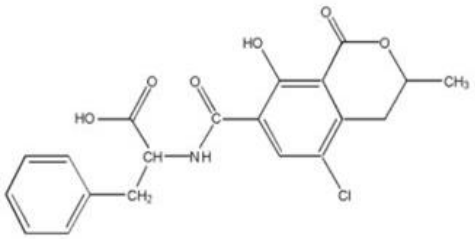

Figura 18. Estructura química de OTA. Fuente: Cruz y col. (2014).

Es sintetizada por una gran variedad de especies del género *Aspergillus*, en particular *A. westerdijkiae*, *A. steynii*, *A. ochraceus* y *A. niger* (Gil-Serna y col., 2011; Karolewiez y Geisen, 2005). Entre las especies de *Penicillium*, *P. verrucosum* y *P. nordicum* son la principal fuente de OTA. Esta micotoxina ha sido clasificada por la IARC como un compuesto carncerígeno 2B (IARC, 1993), por sus propiedades

carcinógenas, nefrotóxicas, teratógenas, inmunotóxicas y, posiblemente, neurotóxicas (Drusch y Ragab, 2003). Esta micotoxina se encuentra a menudo en los piensos y en alimentos como el trigo, el centeno, el café, los frutos secos y en las bebidas como la cerveza, el vino y los zumos de uva (Marín y col., 2013; Ravelo Abreu y col., 2011)

Para la protección de la salud pública, el Reglamento (UE) Nº 2022/1370 establece un contenido máximo de OTA en algunos alimentos donde se había observado un contenido muy elevado de la misma. Además, este nuevo reglamento incluye a los higos secos con un nivel máximo de 2 µg/kg.

-<u>Ácido ciclopiazónico</u>: El ácido ciclopiazónico (CPA) es químicamente un ácido indol tetrámico (Figura 19). Sirve como toxina debido a su capacidad para inhibir las ATPasas dependientes de calcio que se encuentran en los retículos endoplasmático y sarcoplasmático (Chang y col., 2009). Los principales productores de CPA pertenecen a los géneros *Penicillium* (*P. camembertii*, *P. chrysogenum*, *P. commune*, *P. crustosum*, *P. griseofulvum*, *P. hirsutum*, *P. verrucosum*, *P. melanoconidium* y *P. viridicatum* (Frisvad y Samson, 2004) y *Aspergillus* (*A. oryzae*, *A. tamarii*, *A. versicolor* y *A. flavus* (Larsen y col., 2007; Taniwaki y col., 2012; Varga y col., 2011). El CPA ha sido detectado en diferentes productos alimenticios como son cereales, frutos secos, maíz, leche, frutas, carne, huevos (Burdock y Flamm, 2000; Navale y col., 2021).

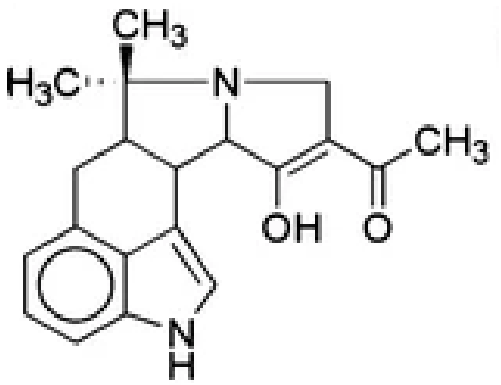

Figura 19. Estructura química del CPA. Fuente: Durán y col. (2007).

Las micotoxinas pueden interactuar entre ellas, aumentando el efecto sinérgico de las mismas (Maragos, 2009). Esto sucede muy frecuente con el CPA, ya que puede ser producido también por *A. flavus* que es productor de AFs (Cole, 1986).

-<u>Fumonisinas</u>: Las fumonisinas (FBs) son micotoxinas producidas por especies del género *Fusarium*, concretamente *Fusarium verticillioides*, *Fusarium proliferatum*, *Fusarium nygamai*, y también por *Alternaria alternata* f. sp *lycopersici* (Marasas y col., 2001; Rheeder y col., 2002). De todas estas, la principal especie de importancia económica es *F. verticillioides*, ya que está presente en prácticamente todas las muestras de maíz (Marasas y col., 2001), si bien, la mayoría de las cepas no producen la toxina.

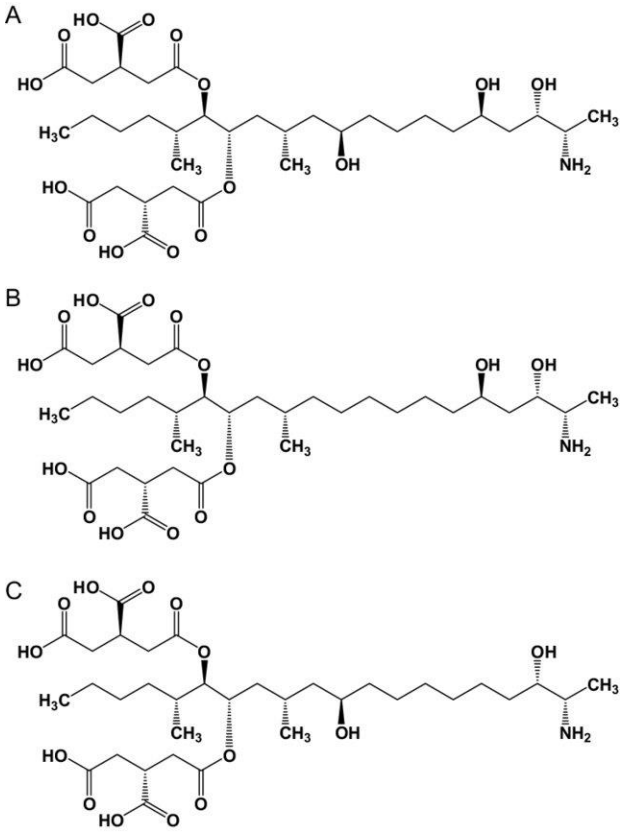

Figura 20. Estructura química de la FB_1 (A), FB_2 (B) y FB_3 (C). Fuente: Ren y col. (2011).

Las FBs del grupo B son las más comunes en la naturaleza y la más frecuente del grupo es la FB_1, aunque también se han detectado FB_2, FB_3 y FB_4 en los alimentos. La estructura química de las FBs se muestra en la Figura 20. La IARC clasifica a las fumonisinas en el grupo 2B, como posiblemente carcinogénicas para las personas (IARC, 1987).

-<u>Ácido kójico</u>: El ácido kójico (KA) es un derivado de la pirona que inhibe la tirosinasa (Figura 21), por lo que es un inhibidor de la formación de pigmentos en los tejidos vegetales y animales (Varga y col., 2015).

El KA es producido principalmente por especies del género *Aspergillus* pertenecientes a la sección *Flavi* (*A. arachidicola*, *A. bombycis*, *A. caelatus*, *A. flavus*, *A. lanosus*, *A. nomius*, *A. oryzae*, *A. parasiticus*, *A. parvisclerotigenus*, *A. pseudocaelatus*, *A. pseudonomius*, A. *pseudotamarii*, *A. sojae*, *A. tamarii*) (Varga y col., 2011).

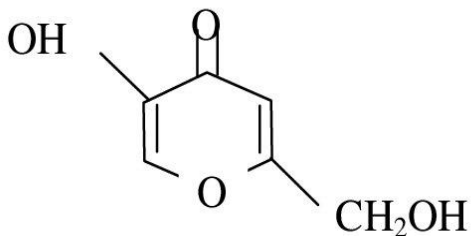

Figura 21. Estructura química del KA. Fuente: Abdel-Naser, 2007.

-<u>Esterigmatocistina</u>: La esterigmatocistina (ST) es un metabolito fúngico que tiene estructura de policétido (Figura 22). Fue aislado por primera vez en 1954 a partir de cultivos de *Aspergillus versicolor* (Soriano del Castillo, 2007). Esta micotoxina es producida principalmente por especies del género *Aspergillus* (*A. chevalieru*, *A. ruber*, *A. amstelodami*, *A. versicolor*, *A. aureolatus, A. quadrilineatus* y *A. sydowi* (Frisvad y col., 2005).

La STE es un precursor de la AFB_1 en aquellos casos en los que los cereales y/o los alimentos están contaminados con mohos capaces de producir AFs. La STE presenta una toxicidad aguda relativamente baja, aunque tiene un efecto tóxico crónico relevante. La IARC la ha clasificado como un carcinógeno del tipo 2B (IARC, 1987).

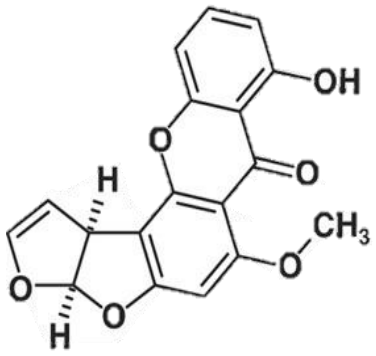

Figura 22. Estructura química de la STE. Fuente: Veršilovskis y De Saeger

Según el informe anual del RASFF, en el año 2020 se han notificado 400 alertas de micotoxinas. Las AFs son las más frecuentemente notificadas en los alimentos (367 notificaciones), de las cuales se han detectado especialmente en higos secos procedentes de Turquía (58 notificaciones). Además, la OTA se encontró sobre todo en frutas y verduras, en particular en higos secos de Turquía (RASFF, 2020).

No obstante, la presencia de micotoxinas en higos secos no solo se trata de un problema sanitario, sino que existen implicaciones económicas y comerciales muy importantes que afectan tanto a los países desarrollados como a los que están en vías de desarrollo. De este modo, las exportaciones de los países productores y exportadores de este tipo de producto se ven cada vez más afectadas, a medida que las legislaciones que regulan los niveles permitidos de micotoxinas en estos países más desarrollados se van haciendo cada vez más restrictivas (Cabañes, 2000).

2.3.2.2 Factores que influyen en la producción de micotoxinas

El crecimiento de los mohos toxigénicos y, en consecuencia, la producción de micotoxinas están determinados tanto por la interacción del individuo (edad, cepa,...), como por las características del sustrato colonizado (pH, composición nutricional,…) y por el entorno en el que crece (temperatura, a_w, HR, radiación solar,...) (Frisvad y Samson, 1991; Njumbe Ediage y col., 2015). Además, las diferencias genéticas entre las especies pueden contribuir a la producción de micotoxinas (Nicholson y col., 2003). De hecho, podemos encontrar dentro de una misma especie cepas productoras y no productoras (Cabañes y col., 2013). Del mismo modo, que diferentes cepas de una misma especie aisladas de diferentes sustratos o en diferentes estaciones pueden producir cantidades diferentes de la misma micotoxina (Dobson y col., 2006).

Los factores más estudiados que influyen en la producción de micotoxinas son la temperatura y a_w. El rango de temperatura y a_w para el crecimiento de los mohos está entre 10 y 35ºC para la temperatura y entre 0,85 y 1 para a_w, con algunas pocas especies capaz de crecer por debajo y por encima de estos rangos (Pitt y Hocking, 2009). En general, las condiciones de dichos factores son más estrictas para la producción de micotoxinas que para el desarrollo del hongo. En general, en algunos casos, la temperatura óptima para la producción de micotoxinas está por debajo de la óptima de crecimiento. Asimismo, también es importante considerar en la producción de micotoxinas la interacción entre los microorganismos presentes en el alimento tanto interespecífica como intraespecífica. Generalmente, las especies toxigénicas en los alimentos cohabitan con bacterias y levaduras, cuya presencia puede inhibir o favorecer la producción de micotoxinas (Gil-Serna y col., 2011; Marín y col., 2011). Esto ha dado lugar a plantear estrategias de biocontrol de mohos toxigénicos basadas en levaduras o en mohos no toxigénicos (Moral y col., 2020; Tejero y col., 2021).

Otros parámetros ambientales, como las fuentes de nutrientes ya sea de carbono o nitrógeno, el pH y la exposición a la luz también interfieren directamente en la producción de micotoxinas (Cáceres y col., 2020). Por otro lado, debido al calentamiento global, a consecuencia del cambio climático, se pueden ver alteradas las etapas y tasas de desarrollo de los mohos toxigénicos (Moretti y col., 2019). Se ha demostrado que las condiciones de estrés ambiental tales como sequía, temperatura, precipitaciones o humedad excesiva pueden promover el crecimiento fúngico y la producción de micotoxinas en cultivos en crecimiento (Medina y col., 2015; Medina y col., 2017). De hecho, los cambios en la agricultura, las prácticas en las últimas décadas pueden resultar en un aumento del estrés en las plantas y, por lo tanto,

potencian la contaminación por mohos toxigénicos y la producción de micotoxinas. Marroquín-Cardona y col. (2014) observaron que el estrés hídrico junto con elevadas temperaturas daba lugar a una mayor colonización de mohos productores de AFs. Bircan y col. (2008), también pudieron comprobar que, debido al estrés hídrico por altas temperaturas, observaron un aumento de la incidencia de AFs en higos secos en Turquía durante la campaña 2017 en comparación con las campañas anteriores.

En higo seco, la infección por mohos toxigénicos y la aparición de micotoxinas ocurren principalmente en el campo, siendo la etapa de mayor riesgo cuando el higo aún está en el árbol (Aksoy y col., 2013; Heperkan, 2006). Debido a su alto contenido en carbohidratos y a los niveles de a_w mostrados durante la sobremaduración y el secado, los higos secos proporcionan un sustrato adecuado para el desarrollo de diferentes tipos de mohos (Gilbert y Senyuva, 2008; Heperkan y col., 2012; Özay y Alperden, 1991). Además, la presencia de estos mohos también se ve influenciada por las condiciones meteorológicas anuales (Özer Meyvacı, 2009). Así, Karaca (2008) informó que tiempos largos de secado aumentan el riesgo de contaminación fúngica y, en consecuencia, la aparición de micotoxinas. Durante el secado tradicional al sol, cuando los higos semisecos caen al suelo, el riesgo de contaminación aumenta ya que en este estado de maduración los higos tienen un contenido de humedad elevado y están expuestos a la infestación por plagas y mohos toxigénicos transmitidos por insectos o presentes en el suelo (Flaishman y col., 2008; Marín y col., 2013; Taniwaki y col., 2018). Durante el secado final en paseras, la proliferación y el desarrollo fúngico depende del contenido de humedad, tiempo de secado y carga microbiana (Flaishman y col., 2008). Şen y col. (2017) observaron que durante el secado al sol de los higos de la variedad Sarılop, la a_w fue de 0,91 en los higos frescos maduros, 0,79 en los higos semisecos, y 0,62 en los higos

completamente secos. Por lo tanto, el higo, por sus características endógenas, es un sustrato que favorece el crecimiento de mohos y la formación de micotoxinas durante el proceso de secado natural. Este riesgo es posible minimizarlo sólo después de que la fruta está completamente seca (a_w<0,70) (Aksoy y col., 2013; Codex Alimentarius, 2008). Asimismo, otros factores tales como la calidad higiénico-sanitaria del material de cosecha y transporte deben tenerse en cuenta. Además, si los higos secos presentan algún daño, el riesgo de contaminación es mayor.

Durante el procesado industrial, los higos secos pasan por una serie de etapas que suponen un riesgo importante de contaminación fúngica. Una de las etapas más críticas para el desarrollo fúngico y la producción de micotoxinas es el escaldado y posterior secado, ya que se generan condiciones de temperatura y humedad que favorecen la proliferación de mohos toxigénicos. Karaca y col. (2010) señaló que la rehidratación del producto seco en condiciones inadecuadas de almacenamiento puede favorecer el crecimiento fúngico y la consiguiente contaminación por micotoxinas. López y col. (2016) informaron que un escaldado inadecuado puede provocar el desarrollo de mohos toxigénicos (*Aspergillus* spp, *Penicillium* spp. y *Alternaria* spp.). Asimismo, tiempos largos de almacenamiento y comercialización con temperatura y HR no adecuadas conducen a un mayor riesgo de contaminación fúngica. En este sentido, De Mello y Scussel (2007) en un estudio sobre la evaluación de micotoxinas en frutos secos, observaron que la contaminación fúngica se debía a sus características intrínsecas de humedad y contenido de nutrientes, largos periodos de almacenamiento y altos valores de a_w. Por otro lado, los equipos y utensilios, así como las cámaras de almacenamiento, pueden suponer un riesgo de crecimiento de mohos si no se realiza una buena desinfección.

2.4 Métodos de control

Para el control de los peligros microbiológicos por mohos toxigénicos es necesario llevar a cabo diferentes estrategias que abarquen desde el campo a la mesa. Así, un conjunto integral de acciones que engloben buenas prácticas agronómicas, control integrado de plagas y enfermedades, implementar sistemas de recolección que eviten el contacto con el suelo, almacenamiento bajo condiciones de temperatura y humedad adecuadas, así como el desarrollo de métodos basados en el control biológico o biocontrol podrían contribuir a la obtención de higos secos sanitariamente seguros. Sin embargo, en la bibliografía consultada pocos son los trabajos que han estudiado el control higiénico-sanitario durante la producción y procesado de higo seco.

Las estrategias de control aplicadas en el período de precosecha son importantes para disminuir la infección fúngica y la formación de micotoxinas (Amézqueta y col., 2012). En este sentido, los sistemas de cultivo en intensivo podrían favorecer un crecimiento más homogéneo de los higos y, por tanto, una recogida más uniforme. Para desarrollar y aplicar las **buenas prácticas agrícolas** (BPA) como enfoque preventivo para reducir la presencia de mohos micotoxigénicos en el cultivo de la higuera, el primer paso es llevar a cabo un seguimiento de las poblaciones de mohos prevalentes, así como de las concentraciones de micotoxinas producidas. El siguiente paso podría ser el diseño **de Análisis de Peligros y Puntos de Control Crítico** (APPCC). El laboreo justo antes de la cosecha puede dispersar los mohos toxigénicos, aumentando el riesgo de contaminación. Asimismo, una gestión eficaz de las plagas es crucial para evitar la transmisión de esporas a la cavidad de los higos. Además, el cuidado en la cosecha es también muy importante

y se ha demostrado que eliminar los frutos dañados antes del secado y el procesado industrial reduce la cantidad de OTA en uvas (Meyvaci y col., 2012). Por otro lado, el desarrollo de un sistema de recolección mediante el uso de mallas es fundamental para evitar el contacto del higo con el suelo y facilitar la recolección. Otra técnica que también puede ayudar a reducir el desarrollo fúngico y la formación de micotoxinas es el **secado artificial**, ya que se acorta el tiempo durante el cual los higos tienen niveles de a_w elevadas (Benalia y col., 2015; Mutlu-Ingök y Karbancıoğlu-Güler, 2015; Petric y col., 2018). Abul-Fadl y col. (2015) observaron que el uso de secaderos artificiales garantiza un secado controlado y evita el desarrollo de micotoxinas. Zare y Jalili (2020) en un estudio con higos secos recogidos en red y secados en un secadero solar observaron también una reducción en la contaminación microbiana de los higos del 3,7%.

Finalmente, otra estrategia que ha mostrado resultados prometedores para el control de mohos toxigénicos ha sido el **control biológico** o **biocontrol** basados en el uso de bacterias, levaduras o mohos no toxigénicos o atoxigénicos. En todos los casos, los estudios de biocontrol están dirigidos a reducir el crecimiento fúngico en las fases previas a la cosecha. En cuanto al uso de bacterias, Öztopuz y col. (2018) observaron que seis cepas de *Bacillus thuringiensis* y *Bacillus mojavensis*, aisladas de suelos de plantaciones regulares de higueras, mostraron potencial para actuar como agentes de biocontrol contra mohos toxigénicos. Ben Taheur y col. (2019) también observaron que las bacterias lácticas, concretamente *Lactobacillus kefiri*, redujo el crecimiento de *A. flavus* y *A. carbonarius* y, en consecuencia, la contaminación por AFB_1, AFB_2 y OTA en almendras y cacahuetes inoculados. Por otro lado, en cuanto al uso de levaduras para el control de mohos, Ruiz-Moyano y col. (2016) informaron que las levaduras *Hanseniaspora opuntiae* L479 y *Metschnikowia pulcherrima* L672,

aisladas a partir de higos, tuvieron efectos antagónicos contra *Penicillium expansum*, *Cladosporium cladosporioides*, *Botrytis cinerea* y *Monilinia laxa*. Jaibangyang y col. (2020) también redujeron el crecimiento de *A. flavus* (64,9%) y la producción de AFs (78,4%) en granos de maíz asociados a la producción de Compuestos Orgánicos Volátiles (COVs) usando la levadura *Candida nivariensis*. En esta misma línea, Tejero y col. (2021) identificaron varios COVs producidos por las levaduras *Hanseniaspora uvarum* y *Hanseniaspora opuntiae* que, in vitro, disminuyeron el crecimiento de *A. flavus* y la producción de AFs.

En cuanto al uso de mohos no toxigénicos, esta estrategia se basa en la introducción repetida de cepas no toxigénicas de mohos para reemplazar a la población toxigéncia, empleando dos mecanismos de acción; por exclusión de la cepa toxigénica del nicho, y por competencia por los nutrientes destinados a la biosíntesis de micotoxinas.

La eficacia de la reducción de AFs mediante aislados atoxigénicos de *A. flavus* se ha demostrado en diversos cultivos, como el maíz, el cacahuete, la semilla de algodón y el pistacho en Estados Unidos, y el maíz, el cacahuete y los pimientos en África (Cotty y col., 2007; Dorner, 2004; Doster y col., 2014; Ezekiel y col., 2019). Así mismo Moore y col., (2021) vieron que ciertas cepas no toxigénicas de *A. flavus* producen COVs antifúngicos, como la 3-octanona y trans-2-metil-2-butenal, lo que da lugar a una reducción significativa en la producción de AFs.

Por otro lado, ya se dispone de productos comerciales de biocontrol para su aplicación en el suelo y por vía foliar. En EE.UU. ha sido aprobado por la Agencia de Protección Ambiental dos productos comerciales basados en cepas atoxigénicas de *A. flavus* (Afla-guard® (NRRL21882) y AF36®) para la prevención de AFs en cacahuetes, maíz y semillas de algodón (Dorner, 2009).

OBJETIVOS

El **objetivo general** de esta **Tesis Doctoral** es la búsqueda de estrategias que permitan obtener higos secos de elevada calidad y sanitariamente seguros.

Para la consecución de este objetivo general son necesarios los siguientes **objetivos específicos**:

- Analizar la **producción y calidad** de los higos secos en plantaciones **superintensivas** con riego localizado. Evaluar el impacto de un sistema de **recogida en malla** sobre las características higiénicas del producto.

- Identificar los **peligros microbiológicos** y evaluar la **calidad** de los higos asociados a la **zona de producción** y al **manejo del agua.**

- Evaluar la **flora fúngica** y la presencia de micotoxinas durante el procesado en la **industria** del higo seco.

- Buscar nuevas estrategias que permitan **controlar el crecimiento fúngico** y la **producción de micotoxinas** a lo largo del proceso productivo del higo seco.

MATERIAL Y MÉTODOS

Esta **Tesis Doctoral** se estructura en **cuatro capítulos**, titulados:

CAPÍTULO 1. Impacto de sistemas de producción en **superintensivo** sobre las características **agronómicas** y de **calidad** de higo seco.

CAPÍTULO 2. Análisis de los **peligros microbiológicos** durante el **desarrollo** y la **producción** del higo seco en el **campo.**

CAPÍTULO 3. Evaluación de los **peligros microbiológicos** en las diferentes etapas del procesado del higo seco en **industria**.

CAPÍTULO 4. Ecofisiología de ***Aspergillus*** **spp.** y **control** de la producción de **micotoxinas.**

En la figura 23 se describe la **metodología general** de los cuatro capítulos, los artículos que incluyen cada uno de ellos y los objetivos que se pretenden abordar. Este trabajo forma parte del proyecto de investigación **RTA2017-00032-C02-00** titulado: **Gestión integral del proceso productivo del higo seco que asegure un producto de máxima calidad higiénico-sanitaria**.

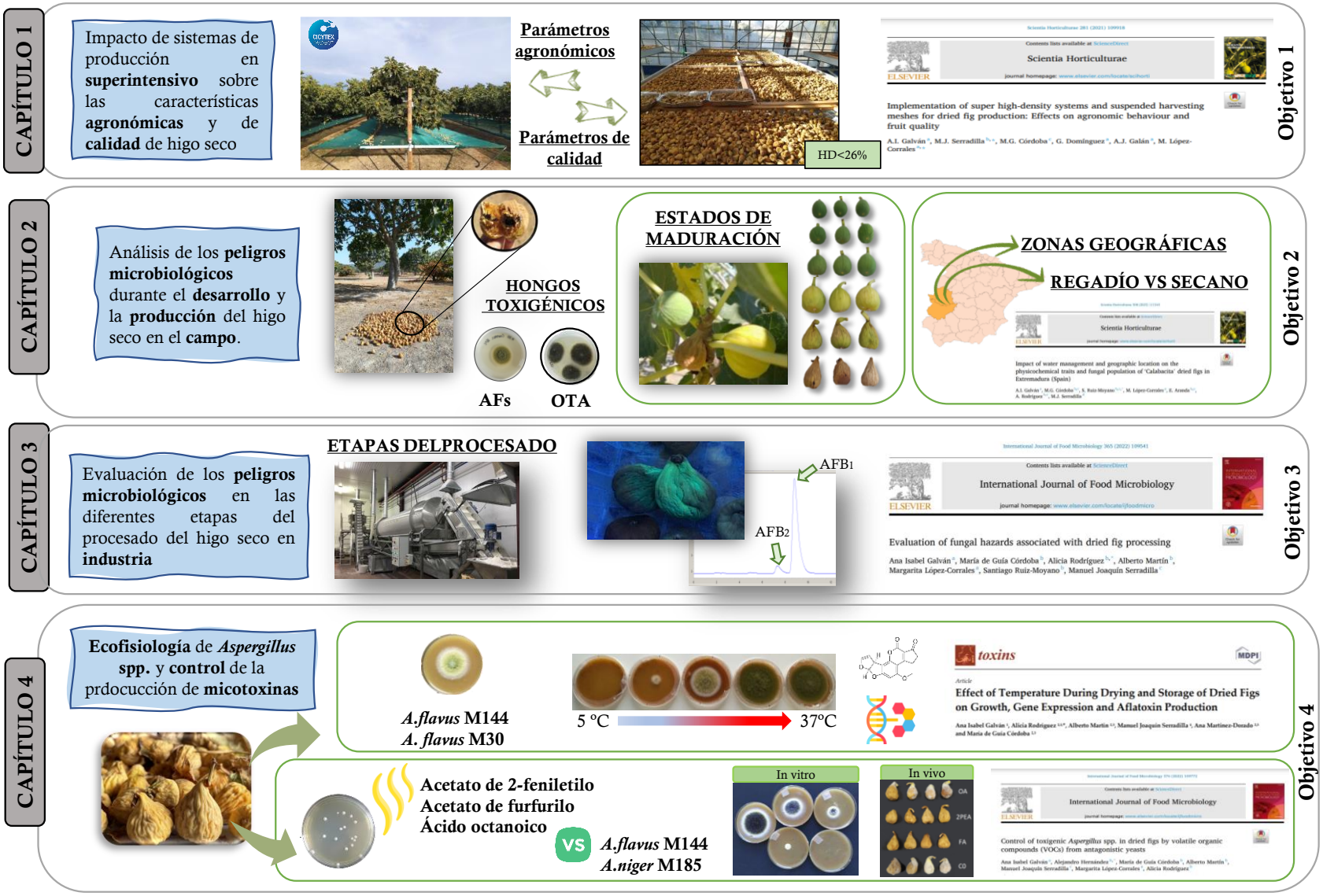

Figura 23. Metodología general de la Tesis Doctoral

CAPÍTULO 1

Para el desarrollo del **Capítulo 1** (figura X), se llevó a cabo un estudio de la variedad **Calabacita**, tradicionalmente cultivada en secano para la producción de higos secos en el suroeste de España, en **superintensivo** (1000 árboles/ha), durante 5 años consecutivos, con un marco de plantación de 5 × 2 m, y un sistema de **recolección con malla** suspendida, con el objetivo de ver la adaptación de esta variedad a este sistema de producción, aumentar la producción, reducir los costes, facilitar la recolección y además evitar el contacto directo de los higos secos con el suelo (**Publicación 1**) (Figura 24).

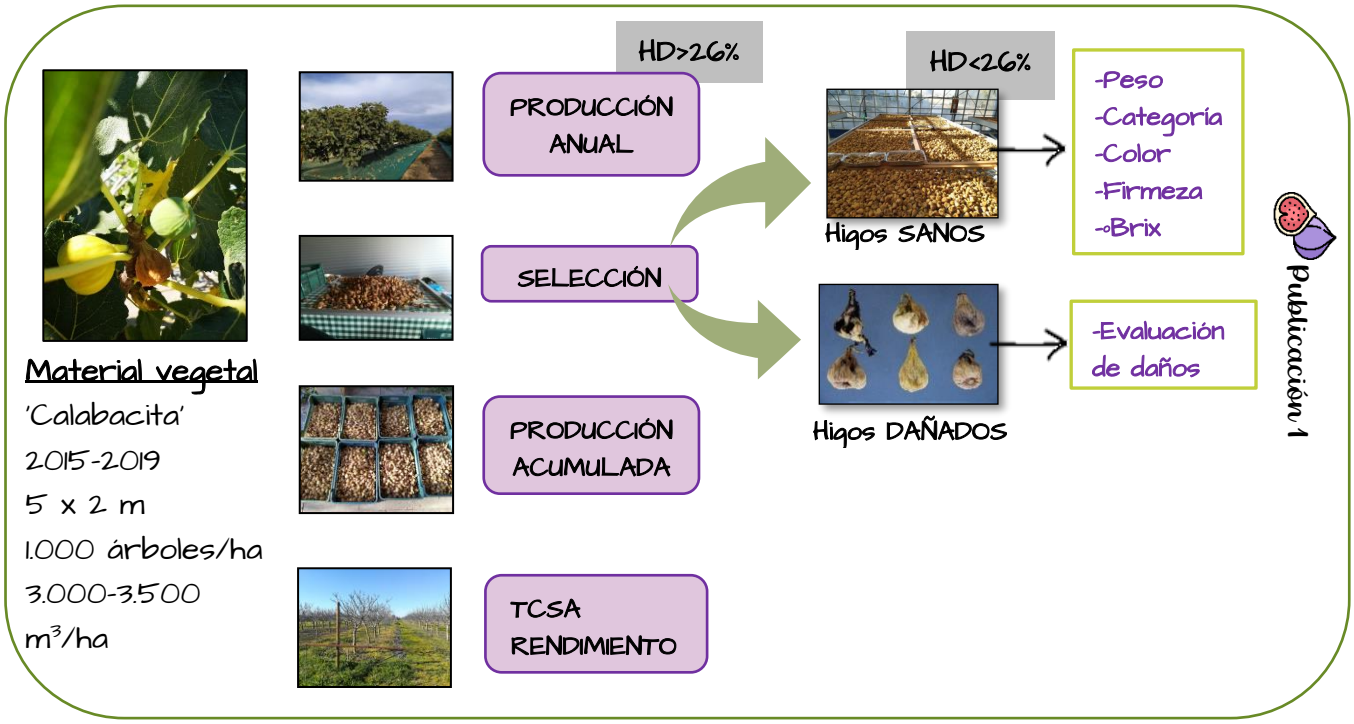

Figura 24. Diseño experimental del capítulo 1, publicación 1.

CAPÍTULO 2

En el **Capítulo 2**, el objetivo fue analizar los **peligros microbiológicos** durante el **desarrollo** y la **producción** del higo seco en el campo. Para ello, se llevaron a cabo dos estudios:

En el primero se evaluaron los peligros microbiológicos y los parámetros físico-químicos de dos variedades de higo, Cuello Dama Blanco y Calabacita, a lo largo de los diferentes estados de maduración (H1-H10) por los que pasa el higo en el árbol hasta su secado final, con el fin de determinar en qué momento o estado de maduración es más susceptible el fruto a ser infectado por mohos micotoxigénicos (**Publicación 2**) (Figura 25).

Por otro lado, en el segundo estudio se determinó la calidad físico-química y microbiológica de higos secos producidos en las tres principales zonas productoras de Extremadura (Guadajira, Guareña y Almoharín). Al mismo tiempo, también se evaluó el impacto del manejo del agua (secano y regadío) en la zona de Almoharín, con el objetivo de evaluar cómo influye la localización geográfica y el manejo del agua en la calidad físico-química y microbiológica (**Publicación 3**) (Figura 26).

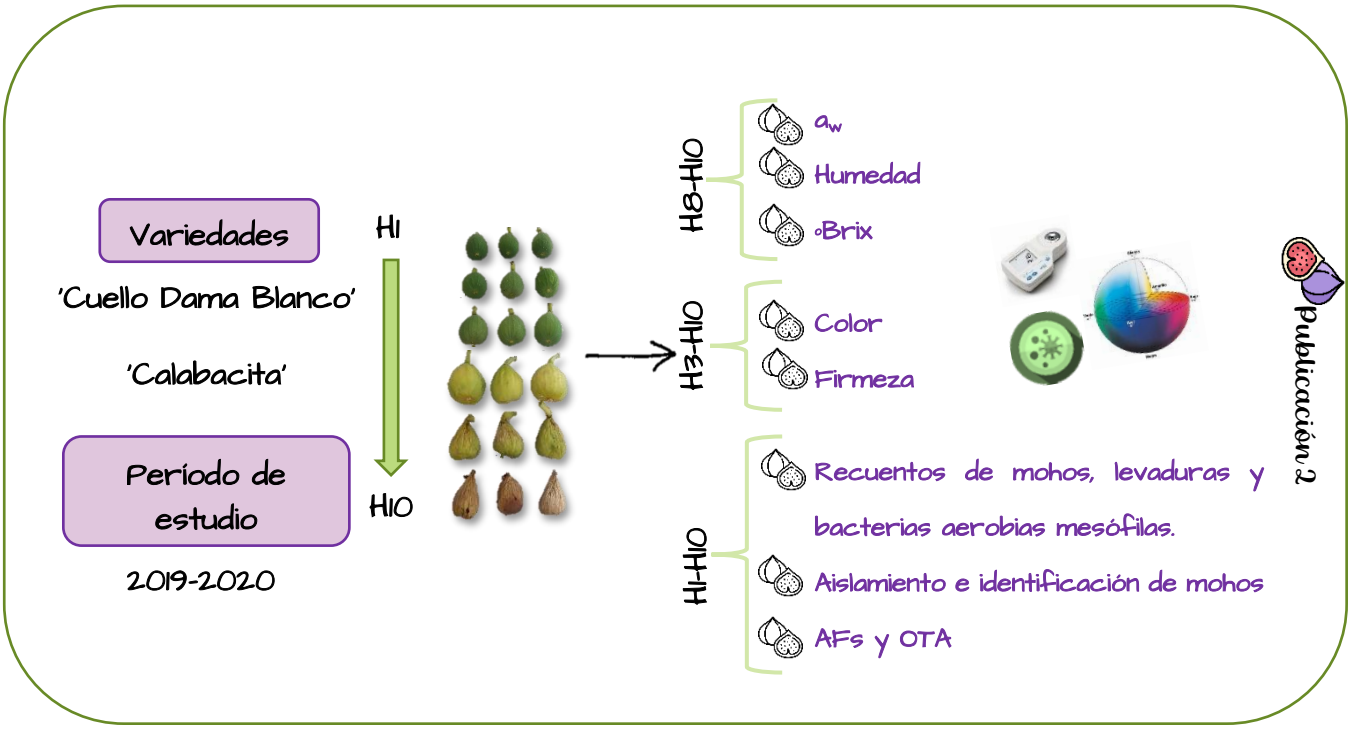

Figura 25. Diseño experimental del capítulo 2, publicación 2.

Figura 26. Diseño experimental del capítulo 2, publicación 3.

CAPITULO 3

En el **Capítulo 3** se estudiaron los **peligros microbiológicos** durante el **procesado en industria**. Para ello, se llevó a cabo un estudio en tres industrias de Extremadura (A, B, C) para evaluar el impacto que tienen las diferentes etapas del procesado (**curado**, **calibrado**, **lavado**, **escaldado**, **almacenado** y **producto final**) sobre el crecimiento de mohos toxigénicos y la producción de AFs y OTA en higos secos, con el fin de establecer medidas de control eficaces para garantizar la producción de higos secos de forma segura (**Publicación 4**) (Figura 27).

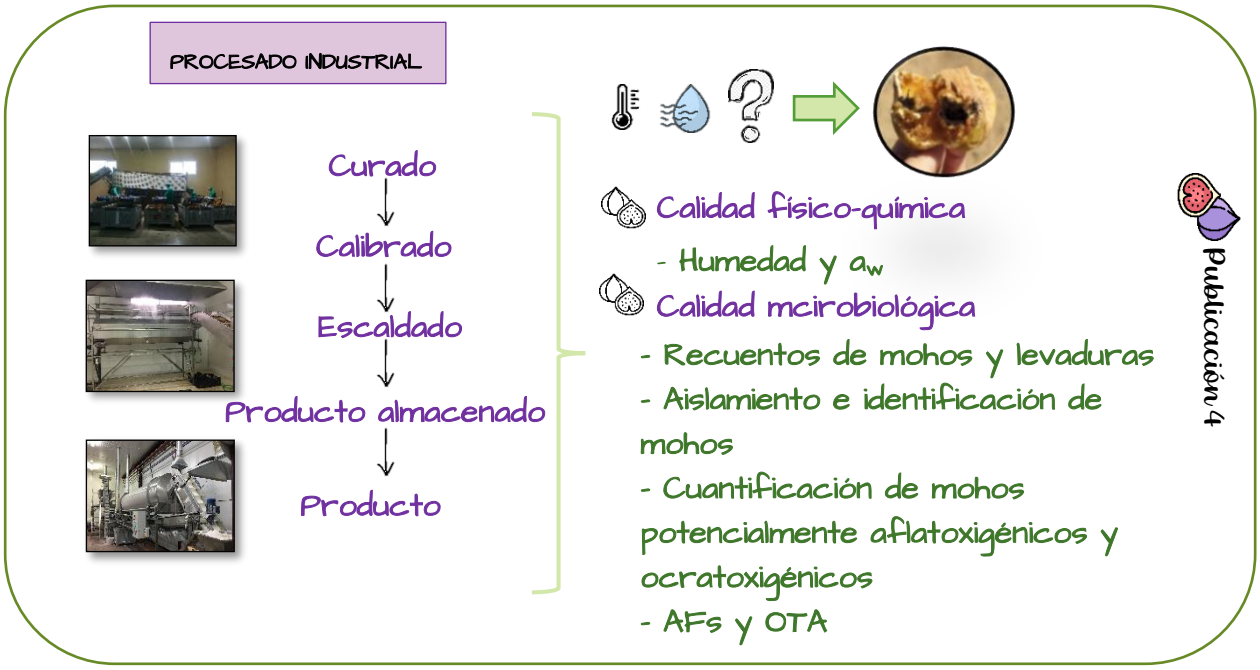

Figura 27. Diseño experimental del capítulo 3, publicación 4.

CAPÍTULO 4

El **Capítulo 4** analiza la ecofisiología del género *Aspergillus* y el control de la producción de micotoxinas. Para ello, se realizaron dos estudios:

El primero consistió en evaluar el crecimiento (tiempo de retardo y tasa de crecimiento) y la producción de AFs a 25ºC de 11 cepas de *A. flavus* aisladas de higos tanto frescos como secos. Para ello, elaboramos un agar de higo con una a_w de 0,96 con un 10% de higos secos, intentando simular unas condiciones lo más parecidas posible al producto original. Posteriormente, se seleccionaron dos cepas de *A. flavus* (M30 y M144) y se trabajó con las temperaturas relacionadas con el procesado del higo seco en industria (5, 16, 25, 30 y 37ºC), evaluando tanto el crecimiento como la producción de AFs, así como estudiar el nivel de expresión de los genes que regulan la ruta de biosíntesis de AFs (aflR) (**Publicación 5**) (Figura 28).

Por otro lado, el segundo trabajo consistió en evaluar los efectos de **tres COVs** (ácido octanoico, acetato de 2-feniletilo y acetato de furfurilo), producidos por levaduras antagonistas *H. uvarum* y *H. opuntiae* aisladas de higos, sobre el crecimiento, la germinación, la expresión genética y la producción de AFs y OTA en el control del desarrollo de *A. flavus* (M144) y *A. niger (M185)* en agar de higo seco (*in vitro*) y las tasas de incidencia en higos secos (*in vivo*) con el propósito de evitar las micotoxinas en los mismos (**Publicación 6**) (Figura 29).

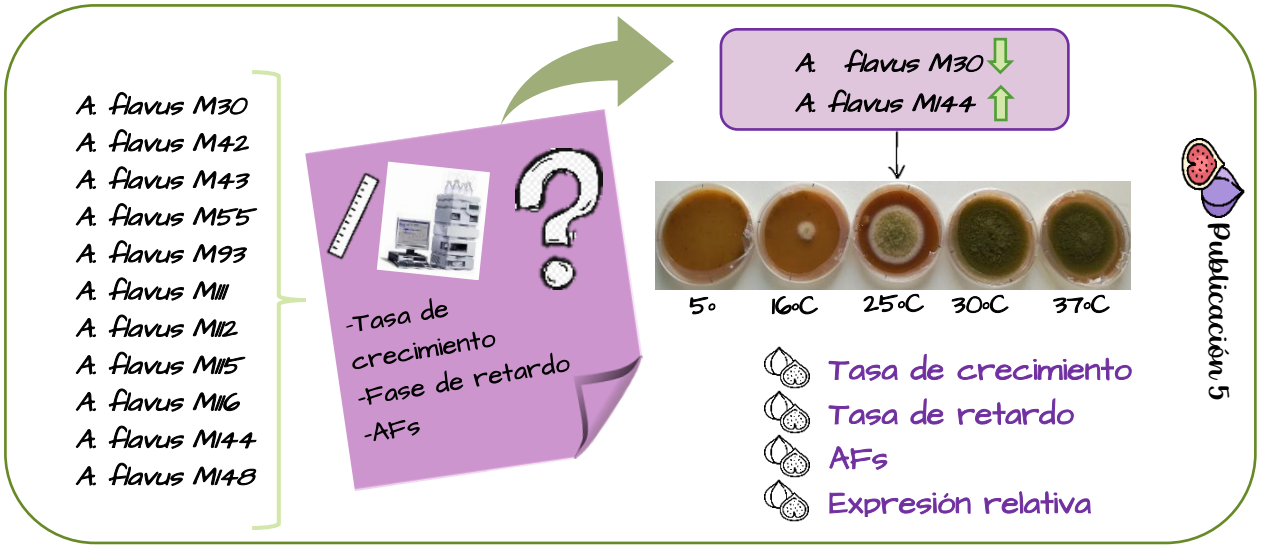

Figura 28. Diseño experimental del capítulo 4, publicación 5.

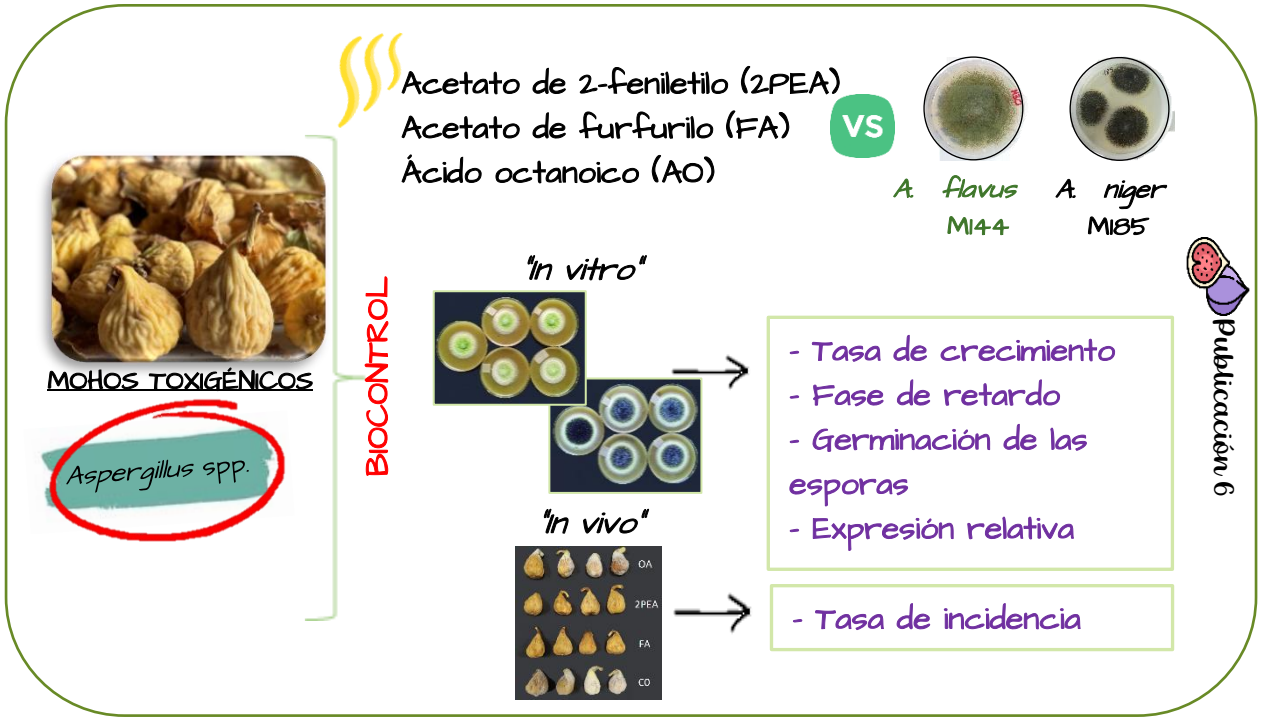

Figura 29. Diseño experimental del capítulo 4, publicación 6.

RESULTADOS Y DISCUSIÓN

CAPÍTULO 1

PUBLICACIÓN 1

Implementation of super high-density systems and suspended harvesting meshes for dried fig production: Effects on agronomic behaviour and fruit quality

Ana Isabel Galván, Manuel Joaquín Serradilla, María de Guía Córdoba, Guadalupe Domínguez, Antonio Jesús Galán, Margarita López- Corrales

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Implementation of super high-density systems and suspended harvesting meshes for dried fig production: Effects on agronomic behaviour and fruit quality

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ABSTRACT

The demand for dried figs has increased in recent years. This, together with rising production costs and difficult access to labour for harvesting, has made it necessary to seek alternatives to the traditional systems. The fig tree is a fruit species with a high adaptive capacity. In this work, we studied for 5 consecutive years the adaptation of the Calabacita variety, traditionally used for dried fig production in the southwest of Spain, under high density conditions (1000 trees per hectare with a planting frame of 5 × 2 m). In addition, a suspended mesh netting structure was installed to facilitate the harvesting process. Compared to the traditional system, the results obtained with this system are higher. In addition to improving the harvesting operation, use of the suspended mesh netting may also reduce damage caused by insects. The dried figs obtained were firmer and had a darker brown colour than those using the traditional system, probably because the suspended meshes favour the drying process by facilitating air circulation. Finally, use of the suspended mesh netting also constitutes an important advance from a sanitary perspective, as the lower moisture content of the figs harvested this way impedes the proliferation of mycotoxigenic fungi and, consequently, the possible presence of mycotoxins.

1. Introduction

In traditional production systems, the fig tree has always been cultivated in low-density orchards (less than 100 trees/ha), characterised by very limited management and irrigation, and with generally low yields (López-Corrales et al., 2012). In modern fruit orchards, to increase profitability, new high-density formation systems have begun to be introduced using dwarfing rootstock and training systems that ensure maximum sunlight interception to guarantee higher fruit quality and yield, as well as better adaptation of these systems to facilitate operations such as harvesting, pruning, etc. (Iglesias, 2019). Thus, nowadays, in other fruit species such as olive, apple or citrus, intensive and super-intensive density systems have acquired a high degree of popularity (Iglesias, 2019). Previous studies on olive trees, for example, have shown that yield and fruit quality are higher with high-density plantations and irrigation systems (Ahumada Orellana et al., 2018; Martorana et al., 2017). A study carried out by Milosevic et al. (2008) showed greater precocity and higher yield and return on investment in super-intensive plum cultivation compared to the traditional system. In similar traditional crops such as almond, irrigation is considered the main limiting factor of yield in terms of number of fruits and quality (Gutiérrez-Gordillo et al., 2019).

Fig cultivation is strongly affected by climatic conditions. For this reason, 70 % of the world's fig production is concentrated in countries on the Mediterranean coast (Arpaci, 2017), with Turkey the leading producer with 305.698 t. Spain is Europe's leading producer with 47.750 t. Extremadura, in the southwest of Spain, has the country's largest cultivated area (6.104 ha), mostly for the production of dried figs, although figs can also be also consumed fresh, processed or in fig paste (Vebric et al., 2008). In this area, only 14 % of the area is

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Table 1
Maximum, average and minimum temperature in degrees Celsius at the harvest period for the study period (2015-2019).

Years	Maximum Temperature	Average temperature	Minimum Temperature
5th green (2015)	29.47	21.46	14.96
6th green (2016)	32.72	23.68	14.54
7th green (2017)	33.61	23.90	14.41
8th green (2018)	33.20	24.19	15.46
9th green (2019)	32.40	23.33	15.05

cultivated under irrigated conditions (MAPA, 2018). Most of the trees planted for cultivation of dried figs in the southwest of Spain correspond to the Calabacita variety. These figs are well known for their quality in all foreign markets.

With traditional dried fig production, after the figs ripen and fall they are picked by hand from the ground usually once a week (Aksoy, 1997). As a result, figs can be contaminated by insects or mycotoxigenic fungi that are naturally present in the environment. In recent years, the question of food safety has acquired more and more importance in consumer purchases. There is a growing demand for products with a high level of hygienic and sanitary quality (Isin et al., 2007). The traditional dried fig collection and production method is therefore compromised as the result of the presence of mycotoxins produced by fungi of the genus *Asperguilllus* spp., *Fusarium* spp. and *Penicillium* spp. (Gilbert and Senyuva, 2008). Given the increasing importance of food safety in world markets and consumer demand for high quality products, there is a need to ensure proper handling of dried figs during the production process. The development of more productive and efficient methods are required to meet the increase in consumer demand whilst at the same time guaranteeing a product of high hygienic and sanitary quality.

To our knowledge, no studies have been published in the literature on dried fig yield under super high-density systems. Among other advantages of these systems is the possibility of installing mesh structures that prevent the dried fig from coming into contact with the ground and facilitate its harvesting process. Therefore, the main objective of this study is to determine the agronomic behaviour of the Calabacita variety and the quality parameters of its dried figs in a super-high density system with drip irrigation and a suspended mesh netting structure in the southwest of Spain.

2. Materials and methods

2.1. Plant material

The present study was carried out in super high-density fig tree orchards located in the southwest of Spain at the Scientific and Technological Research Centre of Extremadura, Finca La Orden-Valdesequera (CICYTEX), Guadajira, Badajoz, (latitude 38° 51′ 7.78" N; longitude 6° 40′ 16.59" W), at an altitude of 217 m above sea level. The temperatures in this area during the harvest period are shown in Table 1 (REDAREX, 2021).

The variety used for this study was 'Calabacita'. This is a common type, which develops parthenocarpic fruits. The trees were established in the field in 2010. The data were collected over the course of five consecutive seasons, starting in 2015. The spacing between trees is 5 × 2 m with a density of 1000 plants/ha. The experimental design of this study for dried consumption used three blocks with forty trees per block.

Irrigation was by drip. During the first three years only one dripper/tree was installed and from the fourth year two drippers/tree. The annual volume of water applied was estimated at about 3000−3500 m^3/ha between June and September, although this value varied depending on the climate of each spring and summer.

Fig. 1. Fig orchard mesh netting system.

2.2. Agronomic parameters

2.2.1. Yield and vegetative growth

In July each season, iron structures were installed along the rows. These were spaced 2 m apart and extended approximately 50 cm above the ground. Raschel-type 50 % windbreak shade mesh netting was mounted on the structures (Fig. 1).With this type of mesh, the figs do not bunch together when they fall,and the mesh size of 2.5 × 2.5 cm ensures that the dried fig peduncles do not get stuck and make harvesting easier.

As the figs ripened and fell onto the meshes, they were hand-picked by block once every ten days throughout the harvest period. At this specific time, the moisture content of dried figs is around 30−40% depending on the weather, with normally a higher moisture content towards the end of the season. After the figs have been harvested, the whole and undamaged dried figs are selected and spread out in a greenhouse on tables to dry for four or five days until their moisture content falls below 26 % in accordance with Standard DDP-14 concerning the marketing and commercial quality control of dried figs (UNECE, 2016).

In each year of the study, annual yield, cumulative yield, yield efficiency, undamaged dried figs, undamaged dried figs with moisture content <26 %, and total losses of dried figs were measured per block in the different harvests throughout the season.

Annual yield was calculated as kilos harvested during a season (from the beginning of August to the beginning of October) and expressed in kg/tree. Cumulative production was calculated as the sum of annual production over the study years and also expressed in kg/tree. Yield efficiency of each block was computed from the harvest data as the ratio between cumulative production per final trunk cross-sectional area (TCSA) and expressed in kg/cm^2. Total losses of damaged dried figs in relation to annual production were expressed in % terms. Undamaged dried figs production consisted of dried figs with a moisture content between 30−40% (before greenhouse) and was expressed in kg/tree. Undamaged dried figs production with a moisture content <26 % (after greenhouse) was also expressed in kg/tree.

The study period in this work was 2015−2019, corresponding to the 5^{th} to 9^{th} green.

2.2.2. Fruit size, categories and damaged dried figs

For each block and harvest, 100 fruits were randomly selected and weighed using an AE-166 balance (Mettler Toledo. Madrid. Spain). Dried figs were classified according to Standard DDP-14 (UNECE, 2016) into the following classes: "Extra" Class, Class I and Class II. The size for whole dried figs is determined by the number of fruit per kilogram. The number of fruit per kilogram is limited to 65 for "Extra" Class and 120 for Class I.

Total losses were due to excess moisture, bird pecking or microorganisms and were expressed as %.

Table 2
Yield (kg/tree), total losses (%), undamaged dried figs (kg/tree) and undamaged dried figs with moisture content <26 % (kg/tree) from the three different blocks for the 5 consecutive seasons of the study period (2015-2019).

Years	Blocks	Annual Yield	Total losses of harvest day	Undamaged moisture content 30−40%	Undamaged moisture content<26 %
	1	5.04	22.95	3.89	3.19
5th green (2015)	2	3.95	14.67	3.37	2.85
	3	4.91	16.03	4.12	3.54
	Mean	4.63[a]	17.88[b]	3.79[a]	3.19[a]
	1	7.29	15.52	6.16	4.35
6th green (2016)	2	4.98	15.99	4.18	2.73
	3	5.58	15.93	4.69	3.63
	Mean	5.95[b]	15.81[a]	5.01[b]	3.57[b]
	1	7.99	22.56	6.18	4.87
7th green (2017)	2	5.58	15.93	4.69	3.63
	3	7.44	33.96	4.91	3.68
	Mean	7.01[c]	24.15[c]	5.26[c]	4.06[c]
	1	13.31	18.61	10.83	9.33
8th green (2018)	2	9.42	15.25	7.98	6.31
	3	10.69	18.09	8.75	7.53
	Mean	11.14[d]	17.32[b]	9.19[e]	7.72[e]
	1	12.87	33.42	8.57	7.85
9th green (2019)	2	10.78	25.90	7.99	7.55
	3	9.30	21.07	7.34	7.00
	Mean	10.99[d]	26.80[d]	7.97[d]	7.47[d]

* Different letters in superscript indicate a significant difference in the mean values of the blocks between the different study years ($p<0.05$).

2.3. Quality parameters

From the fruit hand-harvested from meshes per block on each harvest date with moisture content <26 %, 25 fruits were sampled randomly and were immediately used for quality evaluation of the year of study.

2.3.1. Firmness

Firmness was measured on both sides of the 25 fruits in each block and harvest using a TA.XT2i Texture Analyzer (Stable Micro Systems, Godalming, UK) connected to a computer. Force was applied to produce a 6% deformation and the force distance was 40 mm. The slope was determined in the linear zone of the force-deformation curve and the results expressed as N mm^{-1}.

2.3.2. Colour

The following colour coordinates were determined: lightness (L*), redness (a*, red ± green) and yellowness (b*, yellow ± blue) using a CR-400 tristimulus colorimeter (Minolta, Tokyo, Japan). Additionally, the saturation index or chroma (C*), defined as ($C= (a^{*2}+b^{*2})^{*}0.5$) and hue angle (h*) as arctangent (b*/a*) was determined on the skin of 25 fruits on opposite sides for each block and harvest.

2.3.3. Soluble solids

Total soluble solids (TSS) content was measured for each block and harvest date using an RM40 digital refractometer (Mettler Toledo, Madrid, Spain). For this purpose, 10 fruits were homogenized and 3 measures were taken. Results are expressed as °Brix.

2.4. Statistical analysis

Statistical analysis was performed using SPSS for Windows, 21.0. Data were treated to a multivariate one-way analysis of variance (ANOVA), with year as the fixed factor. Statistical significance was assessed by Tukey's HSD test, and the level of significance was set at $p \leq 0.05$. The least significant difference post-hoc test was used to compare groups. In addition, the effect of temperature on the fruit characteristic was analyzed using Pearson's correlation coefficient. A principal component analysis (PCA) was carried out to check the effect of year on agronomic and quality parameters.

3. Results and discussion

3.1. Yield and vegetative growth

3.1.1. Yield, cumulative production, and yield efficiency

In general, fig trees require a warm climate with hot summers and mild winters for good growth and fruit production (Gaaliche et al., 2011). The 'Calabacita' is characterised by its thin, fleshy skin which is suitable for sun-drying (Aksoy, 2017), the traditional fig drying method used in Extremadura. In addition, it is characterised as an early variety whose dried fig production begins in the second week of August and extends to the first week of October just before the first rains begin. The fact that it is an early variety makes it an interesting option to increase the farmers income. The annual yield (kg/tree), total losses at harvest, undamaged fruit and undamaged fruit with less than 26 % moisture are shown in Table 2. In terms of annual yield, a steady increase was observed throughout the study period, rising from an average production of 4.63 kg/tree (5^{th} green) to 10.99 kg/tree (9^{th} green), which corresponds to a net increase of 58 %. This result is consistent with that obtained by Kumar et al. (2014). The application of traditional agricultural techniques is increasingly limiting the profitability of fig tree plantations and their competitiveness, especially due to rising labour costs and low yield. However, the use of super high-density systems for dried fig production is totally unknown. The potential production reported in this study using such a system was higher than the values obtained with rainfed systems in previous studies carried out on dried figs in Spain (Balas and López Corrales, 2014). Total losses ranged from an average value of 17.88 % (5^{th} green) to 26.80 % (9^{th} green), representing an increase of 33 %. As for undamaged fruit, an average value of 3.79 kg/tree was obtained during the 5^{th} green, representing a decrease of 18 % compared to yield per tree, whilst in the 9^{th} green the average value was 7.97 kg/tree, representing a decrease of 27 % compared to yield per tree. In addition, of the undamaged fruits, 3.19 kg/tree (5^{th} green) had a moisture content below 26 %, compared to 7.47 kg/tree for the 9^{th} green (Table 1). The use of meshes makes it easier to harvest these fruits and may mitigate the impact of damage caused by birds such as starlings (*Sturnus unicolor*), considered one of the most serious causes of fig damage (Pereira et al., 2017). Furthermore, the super high-density system could facilitate the use of nets to cover the trees and reduce bird pecking damage. The trees of Block 1 had the highest cumulative production (46.50 kg/tree) and those of Block 2 the lowest (34.70 kg/tree) after the 5 consecutive years of the study, coinciding with the blocks

Table 3
Cumulative yield (kg/tree), undamaged cumulative yield (kg/tree), undamaged cumulative yield with moisture content <26 % (kg/tree), TCSA (cm^2), and yield efficiency (kg/cm^2) of the dried figs from the three different blocks for the whole study period (2015-2019).

Blocks	Cumulative yield (2015−2019)	Undamaged cumulative yield (2015−2019)	Undamaged cumulative yield with moisture content<26 % (2015−2019)	TCSA	Yield efficiency
1	46.50	35.63	29.59	105.49	0.44
2	34.70	28.21	23.06	79.47	0.44
3	37.92	29.82	25.38	98.10	0.39

Table 4
Fruit weight (g) and commercial classes (%) of the dried figs from the three different blocks for the 5 consecutive seasons of the study period (2015-2019).

Years	Blocks	Fruit weight (g)	Class II	Class I	Extra class
5th green (2015)	1	10.66	15.67	82.67	1.67
	2	10.90	13	84.67	2.33
	3	10.35	19.83	78.83	1.33
	Mean	10.64^{e}	16.17^{a}	82.06^{e}	1.78^{d}
6th green (2016)	1	8.30	50	50	0
	2	8.72	45.20	54.40	0.40
	3	8.14	51.50	48.50	0
	Mean	8.39^{b}	48.9^{d}	50.96^{b}	0.13^{b}
7th green (2017)	1	9.05	37	62	1
	2	9.27	34	65.25	0.75
	3	8.15	52.25	47	0.75
	Mean	882^{c}	41.08^{c}	58.08^{c}	0.83^{c}
8th green (2018)	1	9.71	27.75	72.25	0
	2	9.79	28	70.50	1.50
	3	8.97	40.75	59	0.25
	Mean	949^{d}	31.97^{b}	67.25^{d}	0.875^{c}
9th green (2019)	1	7.86	58.30	41.70	0
	2	8.15	54.85	45.15	0
	3	7.64	65.55	34.45	0
	Mean	7.88^{a}	59.57^{e}	40.43^{a}	0^{a}

*Different letters in superscript indicate significant differences in the mean values of the blocks between the different study years ($p<0.05$).

with the highest and lowest TCSA (105.49 cm^2 vs. 79.47 cm^2) (Table 3). The differences among blocks could be attributed to the strong competition between the trees, with those in block 1 having a higher vegetative growth. Factors such as weeds absorbing soil moisture, solar radiation and intensive evaporation of water from the soil surface can affect fig tree development (Jafari et al., 2012). However, despite the differences between blocks, yield efficiency was very similar between them, ranging from 0.39 to 0.44 kg/cm2 (Table 3). In terms of kg/ha, Block 1 had a cumulative yield of 46.500 kg/ha compared to the 34.700 kg/ha of Block 2. However, when considering only undamaged dried figs with moisture content below 26 %, the cumulative yield of Block 1 was 29.590 kg/ha, representing a decrease of 36 %, whilst in Block 2 this reduction was around 33 %, as was the case with Block 3 with a corresponding cumulative final yield of 25.380 kg/ha. The Calabacita variety in irrigated conditions and with intensive tree-spacing for fresh production had a production of 6.000 kg/ha in the 8th green (Balas and López Corrales, 2014), whilst in super high-density conditions the production of dried figs increased to 11.140 kg/ha in the same green. This is a similar result to that obtained by Chithiraichelvan et al. (2017) for the Poona variety, with 14.000 kg/ha at the same tree-spacing of 5 × 2 m and 1.000 plants/ha. There are two key factors that condition fig production, tree-spacing and canopy management through pruning and training (Kumar et al., 2014). With regard to tree-spacing, it has been found that fig trees in planting spaces of 5 × 2 m or 5 × 2.5 m are able to produce more figs and therefore better yield, as well as greater vigour,

Table 5
Breakdown of dried fig losses from the three different blocks for the 5 consecutive seasons of the study period (2015-2019).

Years	Blocks	Excess moisture	Bird pecking	Damaged by microorganisms
5th green (2015)	1	19.72	54.43	25.85
	2	23.49	54.01	22.50
	3	24.09	31.88	44.03
	Mean	$22,43^{a}$	46.77^{c}	30.79^{d}
6th green (2016)	1	53.29	38.54	8.17
	2	53.24	36.21	10.55
	3	47.85	43.10	9.05
	Mean	51.46^{d}	39.28^{b}	10.31^{a}
7th green (2017)	1	39.35	54.75	5.90
	2	32.15	56.80	11.05
	3	35.51	52.29	12.20
	Mean	35.67^{c}	54.61^{d}	9.71^{a}
8th green (2018)	1	46.29	31.64	22.07
	2	49.91	26.89	23.20
	3	53.18	29.36	17.46
	Mean	49.79^{d}	29.30^{a}	20.91^{c}
9th green (2019)	1	32.61	51.46	15.94
	2	22.20	61.02	16.78
	3	27.10	54.50	18.41
	Mean	27.30^{b}	55.66^{d}	17.04^{b}

*Different letters in superscript indicate significant differences in the mean values of the blocks between the different study years ($p<0.05$).

because negative water potentials are generated that favour water translocation available in the soil through the transpiration rate, gas exchange and stomatal conductance (Kumar et al., 2014; Chithiraichelvan et al., 2017). In relation to the canopy, suitable pruning management, whilst maintaining an adequate leaf-to-fruit ratio, favours solar radiation and thus increases the photosynthetic rate, which in turn results in better yield and even fruit quality (Chithiraichelvan et al., 2017).

3.1.2. Fruit size, categories and damaged dried figs

The results for fruit size, categories and damaged dried figs are shown in Tables 4 and 5.

The weight of the dried figs is a critical factor when determining the quality of the product. The different commercial classes are established according to Standard DDP-14 (UNECE, 2016). Fruit weight of the Calabacita variety ranged in this study from 7.88 g (9th green) to 10.64 g (5th green) (Table 4), lower than the reported average weight for the Kadota variety of 15−18 g (Genna et al., 2008), but larger than the 6.44 g of the Sabz variety (Rahemi and Jafari, 2008). Dried figs from the 5th green had both the highest average weight and the highest percentage (around 82 %) in Class I, whereas in the 9th green almost 60 % of the dried figs were in Class II. Although the increase in production from the 5th to 9th green obviously resulted in a greater number of fruits, the fruits produced were smaller in size. This may also be due to the fact that the average temperature in 2015 was lower than in 2019, 21 °C and 23 °C respectively during the harvest period. It is known that colder environments during ripening and fruit development favour larger fruit size (Aksoy, 1994).

Dried fig producers annually suffer major economic losses due to excess moisture, bird pecking and damage caused by microorganisms. In this study, the percentages of each type of loss were evaluated (Table 5). In general, losses were primarily the result of bird pecking and excess moisture content, with microorganism damage less of a factor. In specific reference to excess moisture content, the percentages ranged from 22.43 % (5th green) to 51.46 % (6th green). The quality standard for dried figs states that the moisture content should not exceed 26 %, among other reasons because when figs are semi-dry (31–36 %) the risk of fungal growth and thus mycotoxin production is increased (Gilbert and Senyuva, 2008). As mentioned above, bird pecking, especially by starlings, represented a high percentage of the losses (Table 5), ranging

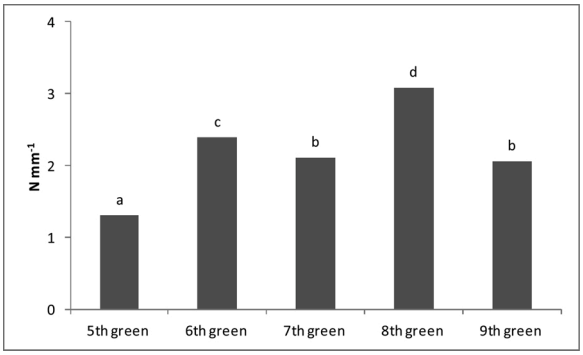

Fig. 2. Mean values for firmness (N mm^{-1}) of dried figs of the three 'Calabacita' blocks for 5 consecutive seasons (2015-2019). Different letters between columns indicate significant differences ($p < 0.05$).

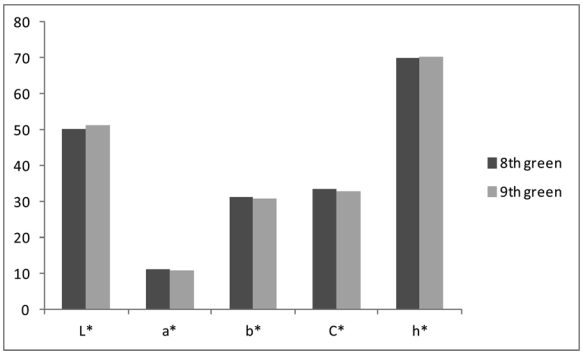

Fig. 3. Mean values of the skin colour of the dried figs from the three 'Calabacita' blocks from the last two years of the study.

between 29.30 % (8th green) and 55.66 % (9th green) of losses over the 5 consecutive years of the study. In the case of fresh figs, losses of this type of around 11 % have been reported (Pereira et al., 2017). Undoubtedly, the fact that the dried fig has a ripening period favours the incidence of damage by birds. Consequently, the development of strategies to minimize the impact of these damages is essential to ensure the profitability of this crop. In this respect, harvesting in meshes elevated above the ground at least makes it difficult for insects, which can be carriers of microorganisms, to gain access.

3.2. Fruit quality

3.2.1. Firmness

Dried fig firmness was studied in the three 'Calabacita' blocks during the 5 consecutive years of the study (2015−2019). Significant differences between years were found (Fig. 2).The firmest dried figs were obtained in the 8th green, with an average value of 3.09 N/mm^{-1}. It should be noted that the year 2018 was characterised by the highest minimum temperature of 15 °C. The lowest firmness values were obtained during the 5th green with an average 1.31 N/mm^{-1}, coinciding with the year of lowest average temperature (21 °C) and lowest maximum temperature (29 °C). Indeed, a positive correlation was observed between the maximum, minimum and average temperature, being significant for the last (0.880; $p < 0.05$). In previous works, figs of this same variety dried traditionally have been characterised by average firmness values of around 1 N/mm^{-1} (Villalobos et al., 2018). It can be concluded that the use of meshes for harvesting favours a more homogeneous drying process as the fruit is more aerated and does not have any contact with the ground. Although the edible quality of dried figs is greatly affected by moisture content (Polat and Siddiq, 2012), the dried figs obtained in this study showed similar quality to the dried figs

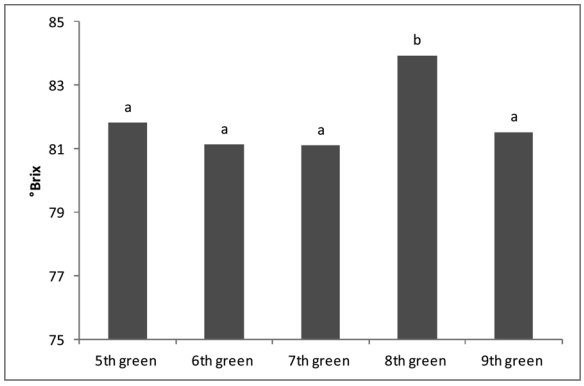

Fig. 4. Mean values for total soluble solids (°Brix) of dried figs of the three 'Calabacita' blocks for 5 consecutive seasons (2015-2019). Different letters in columns indicate significant differences ($p < 0.05$).

traditionally collected from the ground, with dried figs collected in meshes showing greater firmness probably due to greater moisture loss. This higher moisture loss is a guarantee of sanitary quality as it prevents the development of mycotoxigenic moulds such as *Aspergillus flavus*, which require a minimum water activity of 0.78 (Gilbert and Senyuva, 2008).

3.2.2. Colour objective

Skin colour was determined during the last two years of the study (Fig. 3). The dried figs were characterised by a more intense, homogeneous brown colour. Similar values were obtained in the two years studied, with no significant differences observed. Unlike figs dried traditionally without the use of meshes, these dried figs presented a darker brown colour than those obtained by Genna et al. (2008) for the Dottato variety, showing average L*, a* and b* values of 60, 4.7 and 26.8 respectively. This darkening may be due to the fact that figs harvested on meshes have a higher water loss and, in addition, the fact of being more exposed to air currents together with high temperatures may favour browning due to the enzymatic oxidation reactions of the phenolic compounds (Beveridge and Harrison, 1984).

3.2.3. Total soluble solids

The TSS values in dried 'Calabacita' figs ranged from 81.11°Brix in the7th green to 83.94°Brix in the 8th green, (Fig. 4). The TSS values in all the study years were considerably higher than the average value of 37.88°Brix reported by Villalobos et al. (2018) for the same variety. These values were also higher than those reported for different varieties under traditional drying conditions (Genna et al., 2008; Naikwadi et al., 2010; Noutfia et al., 2018). In these studies, the TSS content did not exceed 69°Brix, 55 and 71.9°Brix, respectively. Differences in the sugar content of fruit and vegetables depend on genotype, training system, climate conditions and geographical location (Matthews et al., 1987; Darjazi and Larijani, 2012; Chithiraichelvan et al., 2017). Several authors (Kumar et al., 2014; Mano and Hamada, 2005; Hosomi et al., 2013; Chithiraichelvan et al., 2017) have reported no significant differences in soluble solids content due to planting density per hectare, although Mano et al. (2011) did observea decrease in soluble solids content as planting space narrowed. However, in this study, a significant increase in soluble solids content was observed between the traditional system and the high-density system. Additionally, the highest concentrations of soluble solids were obtained during the warmest year (2018). In order to favour the drying process, in addition to having a fleshy and thin skin, a high content of sugars and soluble solids is necessary to guarantee a high quality organoleptic dried fig (Slatnar et al., 2011). The results obtained in this study show the high suitability of the Calabacita variety for drying under high-density systems and with harvesting mesh netting, providing high physicochemical and sanitary quality dried figs.

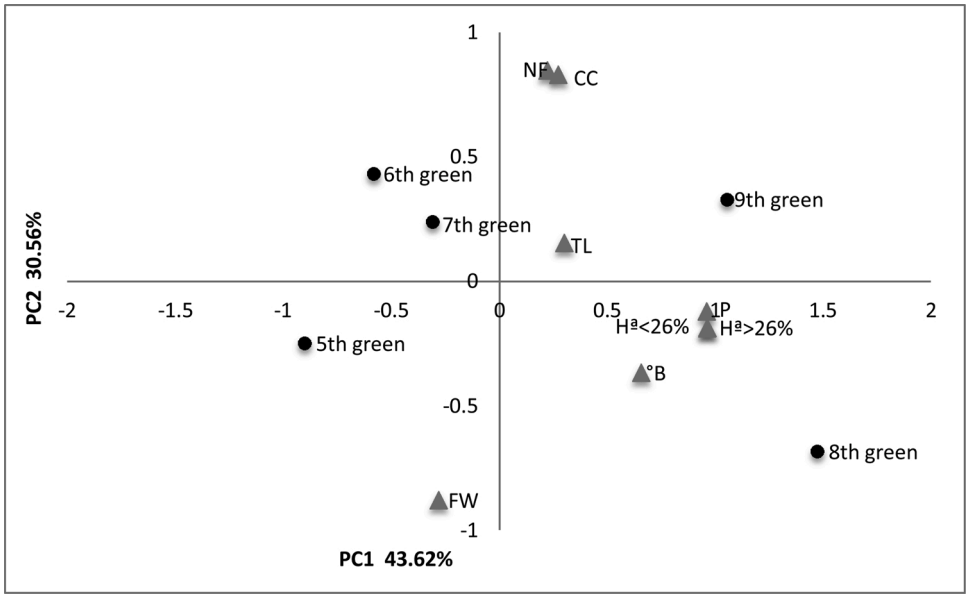

Fig. 5. Projection of the samples with sample grouping according to the year of study (●) and variables (▲) onto the space defined by the first two principal components (PC1/PC2). Total losses (TL), ºBrix (ºB), Number of fruit (NF), Commercial classes (CC), Production (P), Undamaged dried figs Hª>26 % (Hª>26 %), Undamaged dried figs Hª<26 % (Hª<26 %).

3.3. Principal component analysis

The overall effect concerning year of study (Fig. 5) showed some differences in the sample distribution, mainly along the principal component (PC) 1, which represented 43.62 % of the variability. Thus, 2018 and 2019 provided samples with positive scores on the PC1, which could be explained by the positive loadings of the variables yield, °Brix and undamaged dried figs with moisture less than 26 % on the same axis. The opposite behaviour was observed for the samples of 2015, 2016 and 2017, which were located on the negative values of the PC1. Our results support the hypothesis that as the tree grows the production and quality of the fruits increases at the expense of dried fruit weight

4. Conclusions

In dried fig production, labour short ages and the goal of increasing yield efficiency to alleviate production costs has led to the search for more profitable and sustainable production systems. This is the first known report in dried figs on the application of a high-density system with irrigation. Using this type of system and a suspended mesh netting infrastructure, yield of the Calabacita variety, traditionally used for natural drying in rainfed conditions, was increased 3.67-fold, from approximately 3000 kg/ha to 11,000 kg/ha in the 9th green. In addition, the use of suspension meshes was found to facilitate harvesting operations and mitigate the damage caused by insect bites. From the point of view of quality, the dried figs obtained were mostly in Classes I and II. These figs were characterised by a more intense brown colour and greater firmness than the traditional drying system. This was due to the fact that the suspended mesh favoured air circulation and, therefore, the drying process, obtaining dried figs with a lower moisture content and, therefore, safer from a health point of view. Future studies on the impact of this system of harvesting suspended meshes will focus on the reduction of the growth of mycotoxigenic fungi.

CRediT authorship contribution statement

A.I. Galván: Conceptualization, Investigation, Formal analysis, Writing - original draft. **M.J. Serradilla:** Writing - review & editing. **M. G. Córdoba:** Writing - review & editing. **G. Domínguez:** Methodology. **A.J. Galán:** Methodology. **M. López-Corrales:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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CAPÍTULO 2

PUBLICACIÓN 2

Evaluation of physicochemical parameters of two fig varieties throughout the different phenological phases of ripening on toxigenic fungi population

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Evaluation of physicochemical parameters of two fig varieties throughout the different phenological phases of ripening on toxigenic fungi population

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Abstract

The fig tree, belonging to the genus *Ficus*, is a typical crop of the Mediterranean area. At national level, Extremadura is the leading autonomous community in terms of surface area and production of figs, with 7.034 ha and an annual production of 37.382 t. Figs can be eaten fresh, dried and/or processed. Dried figs have the peculiarity that, due to their sun-drying process, they are susceptible to alteration by the growth of moulds. Moreover, under favourable conditions, certain species of moulds can produce mycotoxins, which pose a health risk. Therefore, the aim of this work was to evaluate the physicochemical and microbiological parameters of two fig varieties, Cuello Dama Blanco and Calabacita, throughout the different ripening stages. Physico-chemical changes of figs at different ripening stages influence mould counts. A high presence of toxigenic mould species was observed from the early stages of growth in the two varieties studied, although mycotoxin production was only observed in H9 and H10. Therefore, the implementation of antifungal strategies at this intermediate stage of fruit development is necessary to prevent the presence of mycotoxins in the final product.

Keywords: Figs, ripening stages, moulds, Aflatoxins, Ochratoxin A

1. Introduction

Fig consumption has increased in the last few years, probably because of their high nutritional composition and natural sweetness (Pereira et al., 2015). Figs can be consumed in different ways: fresh, dried, and processed fig products. World fig production is estimated to be around 1.264.943 tons in 2020 (FAOSTAT, 2020). Spain leads European production with 59.900 tons. Of this production, 62.41% is carried out in Extremadura, mainly destined for dried consumption (MAPA, 2020).

Fig fruit is a climacteric fruit (Marei and Crane, 1971) characterized by a double-sigmoid growth curve composed of three phases: phase I (first 6 weeks of growth) consists of a rapid increase in fruit size, with a consequent increase in fresh and dry weight, sugar and moisture content; phase II (from 7 to 11 weeks), the fruit remains almost the same size, colour and firmness; and phase III (four weeks prior to commercial maturity) is characterized by a significant increase in sugar and moisture content, fruit growth and fresh and dry weight. This last stage of ripening includes both the change of colour and the softening of the fruit (Crane and Brown, 1950, Crisosto et al., 2010). The major problem in fig cultivate

on is that not all fruit ripen at the same time, this causes management issues and harvesting of unripe or overripe fruit, which in turn involves fruit quality defects and the appearance of fungal diseases which are later difficult to remove. This is further aggravated in the production of dried figs, which fall to the ground at different times and are not harvested daily, which makes them remain in contact with the ground for a long time, which favors insect attack and development of toxigenic fungi, causing decreases in safety and quality of the fruit (Gilbert and Senyuva, 2008; Guirguis, 2018; Villalobos et al., 2016).

Fungi can colonise fig during pre-harvest, harvest and post-harvest. It has been reported that the most common fungi encountered on dried figs belong to genera *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* (Heperkan, 2006; Turkoz Bakirci, 2020). These genera have some species producers of mycotoxins which can be synthesised under propitious environmental and nutritional conditions. Once fig is contaminated with mycotoxins, they are extremely difficult to eliminate. *Aspergillus* section *Flavi¸* producer of aflatoxins (AFs), and *Aspergillus* section *Nigri*, producer of ochratoxin A (OTA), are the main fungal species colonizing dried figs (Bircan, 2009; Heperkan 2006; Senyuva et al., 2008; Heshmati et al., 2017), although other mycotoxins have been detected in dried figs. Wang et al. (2018) detected *Alternaria* toxins, in addition to ochratoxin B, HT-2, beauvericin and AFs in dried figs from local markets in China. Karbancioglu-Güler and Heperkan (2009) informed about the presence of fumonisins in dried figs from orchards. Sulyok et al. (2020) reported the accumulation in dried figs of other important mycotoxins such as cyclopiazonic, kojic and fusaric acids. Because of the toxicity and prevalence of mycotoxins in dried figs, the European Union has set a maximum permissible AFs content in dried figs, 6 μg/kg for aflatoxin B_1 (AFB_1) and 10 μg/kg for the sum of AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Regulation (EC) 1058/2012).

Mycotoxin accumulation in dried figs is known to occur during harvesting, drying, industrial processing and prolonged storage (Javanmard, 2010). However, they can also be produced while the fruit is on the tree when growing conditions are appropriate (Gilbert and Senyuva, 2008). Fungal and mycotoxin contamination are influenced by both intrinsic and extrinsic factors such as fig variety, water activity (a_w), ripening stage, geographical area, and processing (Villalobos et al., 2019). In this context, Galván et al. (2021) reported the influence of the temperature on the growth,

gene expression and aflatoxin production of *A. flavus* in a dried fig model system. Although it has been shown that dried figs harvested from the ground can be easily infected externally by fungi, some inner parts of figs can be also infected (Ait Mimoune et al., 2018). Therefore, it seems possible that some fungi can colonise fungi during ripening on the tree. However, to the best of our knowledge, despite extensive research on dried figs, no studies have been conducted on the mechanisms occurring in natural fungal infection of fig throughout the fig growth phases.

The objective of this study was to evaluate the physicochemical and microbiological parameters of two fig varieties, Cuello Dama Blanco and Calabacita, throughout the different growth phases of ripening, from on the tree to their complete drying.

2. Materials and Methods

2.1 Plant material and sampling

Figs were harvested from 9-year-old trees grown at the Center of Agricultural Research Finca La Orden-Valdesequera (latitude 38° 85' 19'' N, longitude −6° 68' 28'' W, Guadajira, Badajoz, Spain) belonging to the Scientific and Technological Research Centre of Extremadura (CICYTEX, Spain). Two fig varieties, Cuello Dama Blanco and Calabacita, were used for this study. Samples were collected every 15 days between June and August of two years (2019 and 2020). Three random blocks were used for each cultivar with a tree spacing of 5 × 2 m and drip irrigation (3000-3500 m^3/ha per year applied from June to September) and located at an altitude of 223 m above sea level. The key meteorological parameters during the fig production months were obtained from the standard weather station placed near to the sampling orchards (REDAREX, 2022) (Figure 1). Ten phenological stages were established according to size and ripening: H1) 5-8 cm; H2) 8-12 cm; H3) 12-16 cm;

H4) 16-20 cm; H5) 20-25 cm; H6) 25-30 cm; H7) 30-35 cm; H8) 35-45 cm; H9) Dried figs on the tree; H10) Dried figs on the ground (Figure 2). It should be note that figs have a staggered harvesting pattern. It means that at each sampling, all the growth phases can be present on the tree.

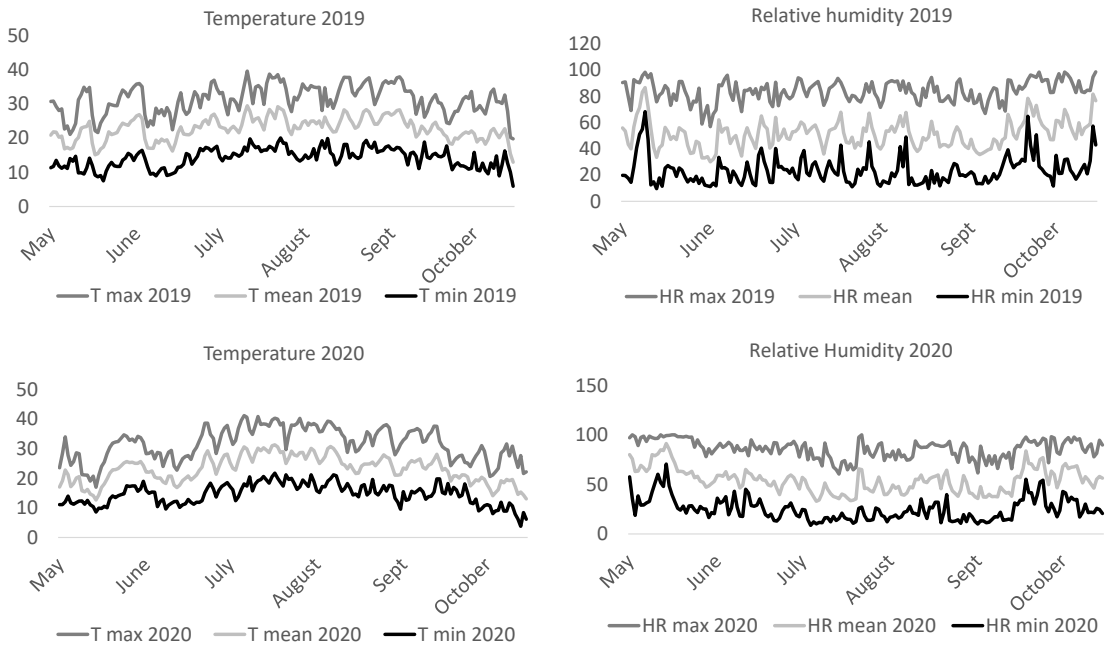


Figure 1. Maximum (RH max), minimum (RH min) and mean (RH mean) relative humidity (%) and maximum (T max), minimum (T min) and mean (T mean) temperature daily from 1 May to 15 October in Guadajira during the study period, 2019 and 2020.

Figure 2. Different ripening stages (H1-H10) of the Calabacita variety.

2.2 Quality parameters

The fruits were collected by hand under sterile conditions. A total of 30 fruits (10 from each block), were taken from each of the two varieties studies at each stage of ripening and used to determine physicochemical and microbiological parameters.

2.2.1 Physicochemical parameters

2.2.1.1 Moisture content and water activity

Moisture content was determined by oven drying for 24 h at 104 °C according to the AOAC Official Method 934.01 and expressed as a percentage (%) (AOAC, 2005). The a_w was measured using a Novasina water activity meter (LabMASTER-aw neo-Novasina AG, Lachen, Switzerland). Both determinations were made in triplicate from ripening stages H8, H9 and H10 in the two varieties studied in each sampling.

2.2.1.2 Total soluble solids

Total soluble solids (TSS) were measured at the ripening stages H8, H9 and H10 in the two varieties studied in each sampling using a RM40 digital refractometer at 20 °C (Mettler Toledo, Madrid, Spain). Ten random fruits of each variety were homogenised, and three replicates were made. The results were expressed in °Brix

2.2.1.3 Firmness

Texture measurements were conducted on both sides of the 10 fruits at the ripening stages from H3 to H10 in the two varieties studied in each sampling. The texture analysis was performed using a TA-XT 2i Texture Analyser of Aname (Stable Micro Systems Godalming, UK) texturometer. Force was applied to produce a 6% deformation and the slope in the linear zone of the force-deformation curve was determined and the results were expressed as N mm^{-1}.

2.2.1.4 Colour

Colour measurements were carried out on both sides of 10 fruits at the ripening stages from H3 to H10 in the two varieties studied in each sampling. A Minolta CR-400 colorimeter (Minolta Camera, Osaka, Japan) was used. The colour coordinates determined were: L*(lightness), the saturation index or chroma (C*), defined as $C* = (\frac{a*2+b*2)}{2}$, and hue angle (H◦), defined as $H◦ = \arctan = \frac{a*}{b*}$.

2.2.2 Microbiological parameters

2.2.2.1 Mesophilic aerobic bacteria, yeast and mould counts

Microbiological counts were carried out at all the ripening stages (H1-H10) in the two varieties studied in each sampling. Ten g of sample were homogenised in 90 mL

of sterile 0.1 % (w/v) peptone water in a Stomacher (Lab Blender, Model 4001, Seward Medical, London, UK) for 30 s. Serial 10-fold dilutions were performed and 0.1 mL of aliquots were inoculated on specific media. Mesophilic aerobic bacteria counts were performed on Plate Count Agar (PCA, Oxoid, Basingstoke, UK) for 48 h at 30 ± 1 °C. Moulds and yeasts counts were performed on Potato Dextrose Agar (PDA, Scharlab, Barcelona, Spain) acidified to pH 3.5 with a sterile solution of tartaric acid (10%). Plates were incubated at 25 ± 1 °C for 5 days. Results were expressed as log cfu/g. Three independent replicates were performed.

2.2.2.2 Isolation and identification of moulds

For isolation of moulds before their identification, mould colonies were taken from each acidified PDA plate and transferred to a new acidified PDA plate to obtain pure colonies after several repetitions of this step. Isolates were stored in 50% glycerol at -80 °C. Every mould strain was inoculated on PDA and incubated for 5 days at 25 °C. The mycelium of each strain was scraped off the agar and used for genomic DNA extraction which was carried out using the quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, California, USA).

Moulds isolated were identified by amplification and sequencing the internal transcribed spacer ITS1-5.8S rRNA-ITS2 region using the primers ITS1/ITS4 (White et al., 1900) and the β-tubulin gene using the primers Bt2a/Bt2b (Glass and Donaldson, 1995). PCR amplification was carried out in a reaction volume of 50 µL in a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA). PCR amplification products were visualized by electrophoresis on a 1% (w/v) agarose gel, purified and sequenced by the Service of Bioscience Applied Techniques (STAB) at the University of Extremadura (Badajoz, Spain). Sequences were analysed using Bioedit version 7.2

and checked by the basic local alignment search tool (BLAST) based on maximum similarity.

2.2.2.3 Extraction and quantification of aflatoxins and ochratoxin A

AFs and OTA were extracted from figs belonging to different ripening stages, from H3 to H10, in the two varieties studied in each sampling day using multi-mycotoxin immunoaffinity columns AFLAOCHRA PREP® (R-Biopharm AG, Germany) according to the manufacturer's recommendations. Quantification of AFs and OTA was performed using an Angilet 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with Agilent 1200 series FLD detector (G1321A) using a LC-18 column (15 cm x 4.6 mm, 5 μm particle size; Supelco, Bellefonte, PA) with the same conditions described by Galván et al. (2022). AFs calibrations were performed with a standard mixture purchased from Sigma-Aldrich (San Luis, EEUU), and OTA calibrations were carried out with a standard from Thermo Fisher Scientific (Waltham, USA). All solvents used were HPLC grade and purchased from Thermo Fisher Scientific.

2.3 Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics for Windows 25.0. One way analysis of variance (ANOVA) was carried out to determine significant differences within a variety between the different ripening stages (H1-H10). Tukey's test was applied to compare the mean values obtained and the significance level was set at $p \leq 0.05$.

3. Results and discussion

3.1 Physicochemical fruit parameters

3.1.1 Moisture content, water activity and total soluble solids

Figure 3 shows moisture content and a_w values of figs at the final ripening stages (H8, H9 and H10) in the two varieties studied in order to study their influence on microbiological counts and mould population and further mycotoxin production. Moisture content values ranged between 42.14 and 78.77% in Cuello Dama Blanco variety (Figure 3A) and 33.20 and 78.28% in Calabacita variety (Figure 3B). These values are similar to those obtained by Galván et al. (2021), who reported moisture content values between 30 and 40% in dried figs collected on meshes. Gilbert and Senyuva (2008) described a moisture content in semi-dried figs between 31 and 36%. Regarding the a_w values of figs of the different stages ranged from 0.83 to 0.98 in 'Cuello Dama Blanco' (Figure 3A) and from 0.74 to 0.98 in 'Calabacita' (Figure 3B). Gilbert and Senyuva (2008) reported a_w values between 0.80 and 0.87 in semi-dried figs. As the figs start to ripen, a gradual decrease in moisture content and a_w was observed from H8 to H10 ($p \leq 0.05$) probably due to the natural drying of the figs (Figure 3). This drying process takes approximately 10 days for Cuello Dama Blanco variety and 8 days for Calabacita variety, since the fruits belonging to the last variety are smaller and therefore, need less time to dry. During this time, figs are exposed to mould contamination, and consequently to mycotoxin production. The decrease of the a_w of the fruits favour a longer shelf-life (Aksoy, 2017). Furthermore, to ensure the microbiological quality of dried figs, a_w values below 0.65-0.60 (Gilbert and Senyuva, 2008; Perera, 2005) and moisture content values below 26% are recommended by the quality standard DDP-14 (UNECE, 2016).

On the other hand, Figure 3 shows the TSS content for the two varieties studied. The TSS content increased as moisture content and a_w decreased throughout fruit ripening. Significant differences ($p \leq 0.05$) were found in the TSS content at different ripening stages for the two varieties studied (Figure 3A and 3B). The TSS content of fresh figs at the stage H) was 23.5 °Brix for Cuello Dama Blanco variety (Figure 3A) and 20.4 °Brix for Calabacita variety (Figure 3B). These values are similar to those reported by other authors. Pereira et al. (2015) reported in a study about the quality of six fig cultivars for fresh consumption a TSS content ranging from 17.5 to 21.4 °Brix for varieties Banane and Cuello Dama Blanco, respectively. Caliskan and Polat (2012) in a study on fig genotypes grown in Turkey reported a TSS content between 20.2 °Brix for Bursa Siyahı variety and 24 °Brix for Morgüz variety. The TSS content of the semi-dried figs (H10) was 61.35 °Brix for Cuello Dama Blanco variety (Figure 3A) and 69.6 °Brix for Calabacita variety (Figure 3B). Sedaghat and Rahemi, (2018) reported that glucose and fructose content increases during growth and ripening of figs. Furthermore, the TSS content throughout ripening was also observed in other fruits such as plum (Díaz-Mula et al., 2008) and cherry (Serradilla et al., 2011). Bremer (2008) reported that TSS content is highly relevant to the sensory quality of dried figs, as it is directly related to favourable consumer ratings.

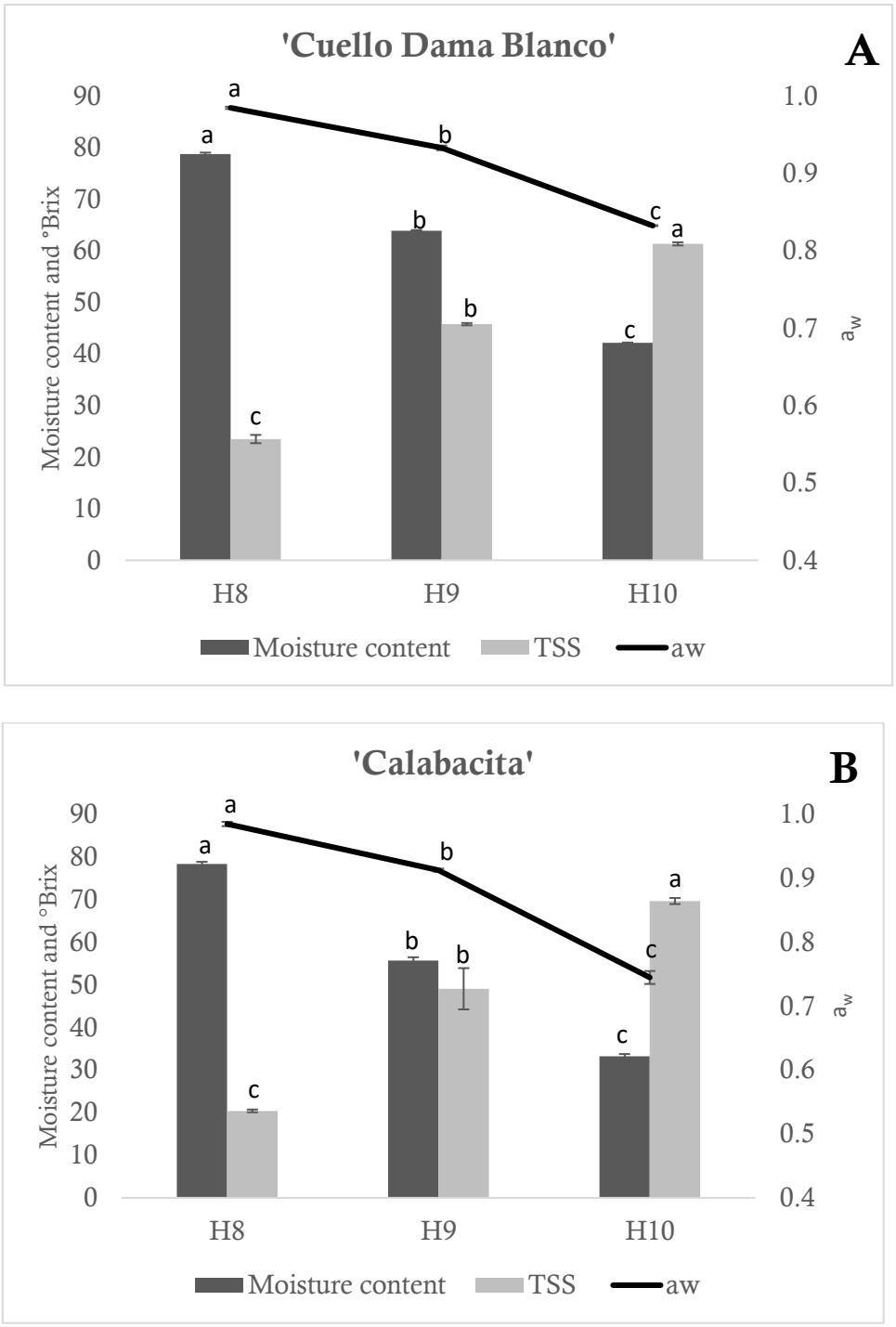


Figure 3. Mean values ± SD of moisture content (%), total soluble solids (TSS) content and water activity (a_w) of the last three ripening stages (H8, H9 and H10) of the two studied varieties Cuello Dama Blanco (A) and Calabacita (B) during the two years of study (2019 and 2020). Different letters (^{a,b,c}) indicate significant differences between the different ripening stages.

3.1.2 Firmness

Figure 4 shows the firmness results of the different fig ripening stages (H3-H10). The results showed significant differences between the different ripening stages in the two varieties studied (Figure 4). The highest values were reached at the ripening stage H6 with a value of 8.45 N/mm for Cuello Dama Blanco variety (Figure 4A) and 8.87 N/mm for Calabacita variety (Figure 4B). However, fruit firmness decreases throughout ripening. This is due to softening by the degradation activity of the cell wall (Valero and Serrano, 2010) and it is observed that from fresh fig (H8) onwards, the values significantly decreased ($p \leq 0.05$) in the two varieties studied (Figure 4A and Figure 4B). These results are in line with those obtained by other authors. Crisosto et al. (2010) in a study of four fresh fig cultivars harvested at two ripening stages found that firmness decreased with ripening. Pereira et al. (2017) reported a reduction in firmness throughout ripening in nine varieties of fresh consumption brebas. Pereira et al. (2015) in a quality study of six varieties of figs for fresh consumption obtained a firmness between 1.2 for the variety Colar Elche and 0.9 for Cuello Dama Blanco variety.

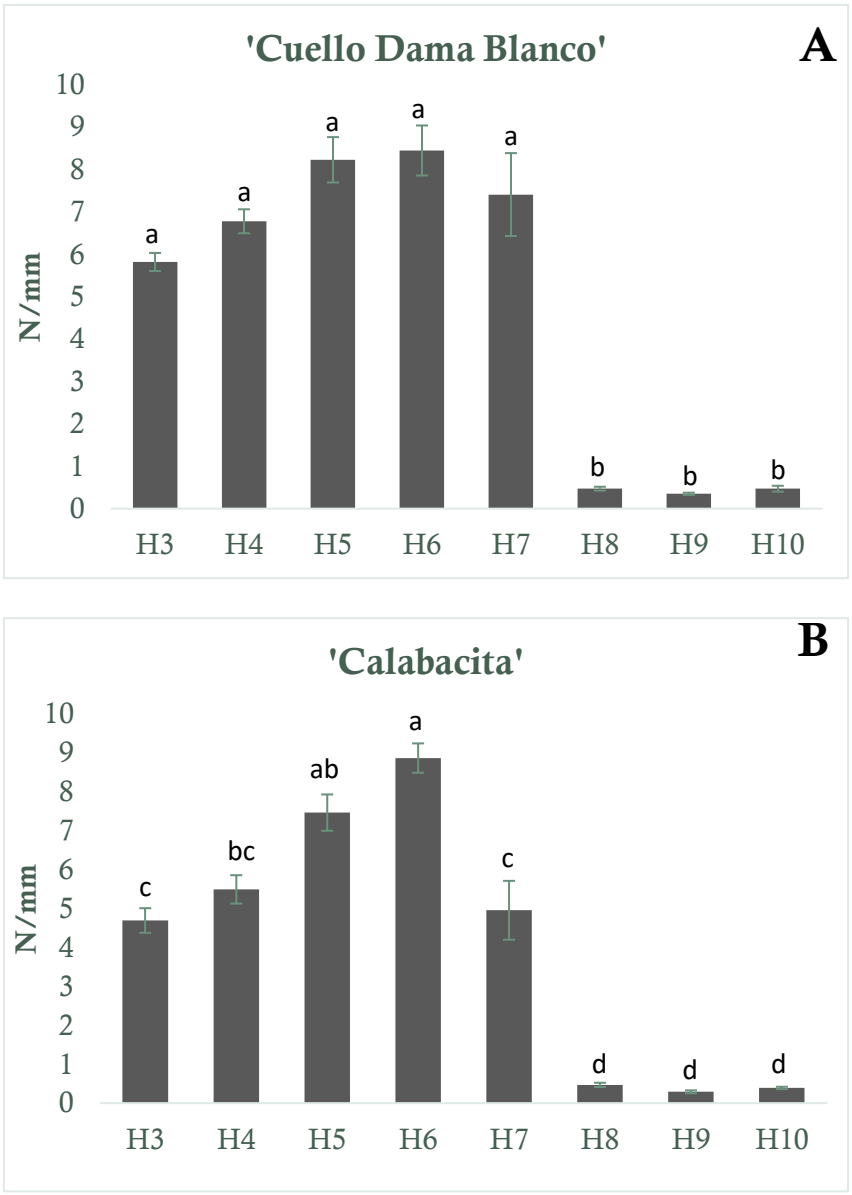
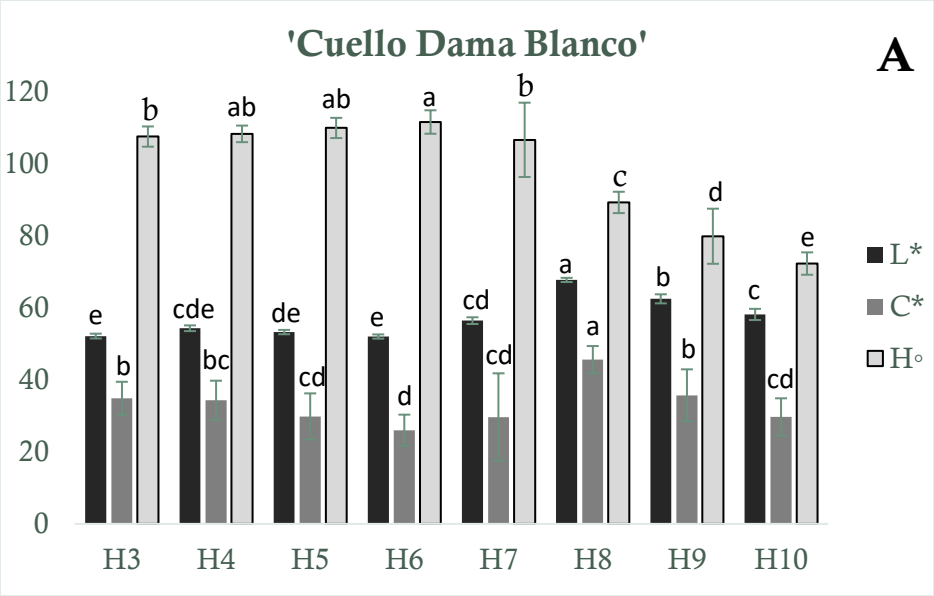


Figure 4. Mean values ± SD of firmness (N/mm), of the different ripening stages (H3 to H10) of the two studied varieties Cuello Dama Blanco (A) and Calabacita (B) during the two years of study (2019 and 2020). Different letters (^{a,b,c,d}) indicate significant differences between the different ripening stages.

3.1.3 Colour

Regarding colour parameters (L*, C* and H°), significant differences ($p \leq 0.05$) were observed at the different ripening stages (H3-H10) in the two varieties studied (Figure

5). The L*, C* and H° values ranged from 52 to 67.7 (L*), from 26 to 45.5 (C*) and from 72.2 to 111.5 (H°) for the Cuello Dama Blanco variety (Figure 5A) and from 47.3 to 62.5 (L*), from 25.2 to 38.3 (C*) and from 72 to 113.2 (H°) for the variety Calabacita (Figure 5B). In both varieties, for the lightness parameter (L*), significantly higher values ($p \leq 0.05$) were observed in H8, H9 and H10. Likewise, a progressive decrease in the colour parameters (L*, C* and H°) was observed from the H8 stage onwards (Figure 5). This means that figs lose lightness, brightness and have a lighter brown colour as ripening progresses. Changes in pre-harvest fruit colour depend on environmental conditions and variety (Zare and Jalili, 2020). Yellow skin colour is desirable for dried figs and the yellow-brown discolouration starts on the tree. One of the most important quality parameters in crops that contain a large amount of total sugars is colour, as reducing sugars are able to react with amino acids and lead to non-enzymatic browning reactions and the formation of brown colour (Abul-Fadl et al., 2015). Besides, skin colour is one of the main quality factors in the marketing of dried figs and indicates the ripening stage of the fruit (İrget et al., 2008).



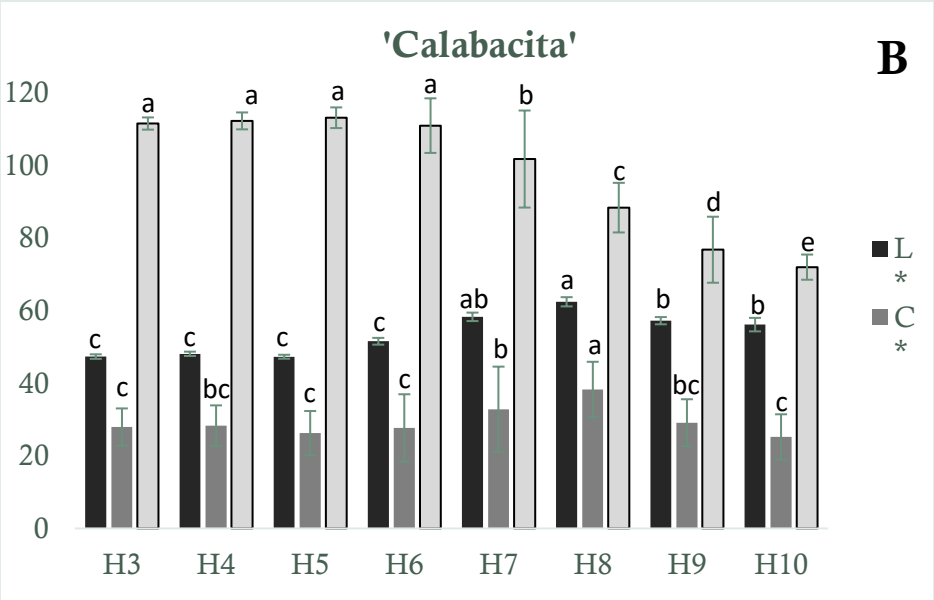


Figure 5. Mean values ± SD of colour (L*, C*, H°), of the different ripening stages (H3 to H10) of the two studied varieties Cuello Dama Blanco (A) and Calabacita (B) during the two years of study (2019 and 2020). Different letters (^{a,b,c,d,e}) indicate significant differences between the different ripening stages.

3.2 Microbiological parameters

3.2.1 Mesophilic aerobic bacteria, yeast and mould counts

The counts of moulds, yeasts and mesophilic aerobic bacteria at the different ripening stages (H1-H10) of the two varieties studied, Cuello Dama Blanco and Calabacita, are shown in Table 1. The mesophilic aerobic bacteria counts ranged from 2.7 to 7.96 log ufc/g in 'Cuello Dama Blanco' and 2.82 to 5.68 log ufc/g in 'Calabacita'. In both varieties, the counts were more numerous at stages H8, H9 and H10 ($p \leq 0.05$), when the fig starts to dry on the tree (Table 1). These results are slightly higher than those found by Guirguis et al. (2018) in commercial dried figs. Villalobos et al. (2019) found that the total number of mesophilic aerobic bacteria in sun-dried figs was 5.2 log cfu/g. In another study, Büyüksirit Bedir et al. (2021) encountered aerobic

mesophilic bacteria counts at the beginning of storage of 5.13 log cfu/g for sun-dried figs and 6.57 log cfu/g for freeze-dried figs. Regarding yeast counts, they ranged from 2.06 log cfu/g (H6) to 7.83 log cfu/g (H9) in 'Cuello Dama Blanco' and 2.26 log cfu/g (H3) to 5.74 log cfu/g (H9) in 'Calabacita' (Table 1). In the same way of the mesophilic aerobic bacteria counts, the counts at stages H8, H9 and H10 were higher than at the initial stages ($p \leq 0.05$). These results are in accordance with those found in the literature. On the other hand, mould counts in the two varieties studied were lower than 3 log cfu/g. This was not expected since moulds are considered the main biological hazard of dried figs during ripening on trees, storage and processing (Karaca and Nas, 2008). In the Cuello Dama Blanco variety the mould counts ranged from 2.02 log cfu/g (H5) to 2.84 log cfu/g (H10) and in the Calabacita variety from 2.11 log cfu/g (H5) to 2.92 log cfu/g (H10) (Table 1). In both varieties, the most numerous mould counts were obtained at the stage H10 ($p \leq 0.05$). This may be due to the fact that in the H10 stage, figs are in contact with the ground. These mould counts are similar to those obtained by Villalobos et al. (2019) on sun-dried figs (control treatment) on the same varieties.

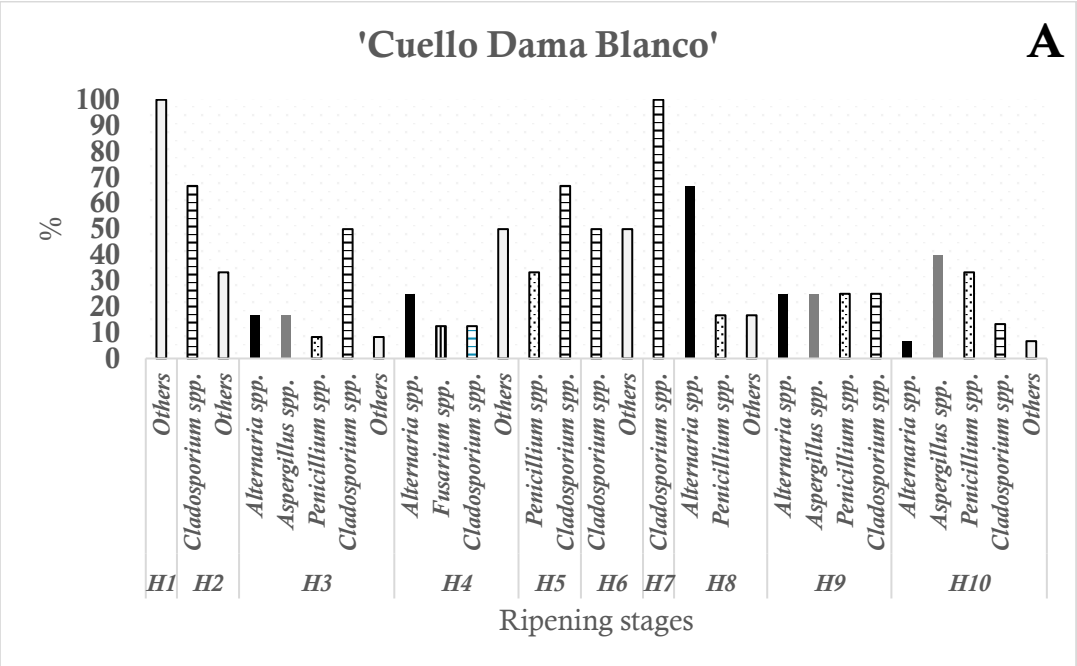
3.2.2 Identification of moulds

The identification of moulds during fruit ripening on the tree provides relevant information about the real risk of the presence of toxigenic species and further mycotoxin production. This is also of utmost importance to look for efficient and appropriate biocontrol strategies to counteract the risk associated with the accumulation of mycotoxins in figs. The incidence of the different mould genera identified in 'Cuello Dama Blanco' and 'Calabacita' is shown in figure 6. In general, a total of 74.2% of the isolates belonged to one of the four mould genera: *Aspergillus* (26.6%), *Cladosporium* (22.7%), *Alternaria* (14.1%) and *Penicillium* (10.9%). In the

Cuello Dama Blanco variety, the most abundant genus was *Cladosporium* spp. followed by *Alternaria* spp. However, in Calabacita variety, the prevailing genus was *Aspergillus* spp. followed by *Alternaria* spp. The impact of ripening on the toxigenic mould genera was remarkable as the fruit ripened in the two varieties studied. In Cuello Dama Blanco variety, *Alternaria* spp. was the major genus at H8 (66.6%), and *Aspergillus* spp. at H10 (40%). In H9, *Alternaria* spp., *Aspergillus* spp. and *Penicillium* spp. were detected in equal abundance (Figure 6A). In Calabacita variety, in the last three stages of ripening, H8, H9 and H10, the genus *Aspergillus* predominated with 33.4, 50 and 50%, respectively. Buchanan et al. (1975) reported that green fruits are more resistant to colonisation by *Aspergillus* spp., but their resistance to these species is loosen during ripening. It is important to know, that the genus *Aspergillus* is one of the major problems associated with mould infection on dried figs, due to the toxigenic potential of some species (Ait Mimoune et al., 2018). In addition, these results are similar to those obtained by other authors. Javanmard (2010) found that *Aspergillus* spp. comprised 34.4% of the total fungal population in Iranian dried figs. Cantín et al. (2011) reported that the most abundant pathogenic species recovered from the surface of fresh figs were *Alternaria* and *Rhizopus* spp. Heperkan et al. (2012) reported that *Aspergillus* spp. was the dominant genus among dried fig samples.

Regarding species identification, the relative abundance of mould species at different stages of ripening for Cuello Dama Blanco and Calabacita varieties is shown in table 2 and 3, respectively. In Cuello Dama Blanco variety 14 different especies were detected (Table 2) and in 'Calabacita' a total of 16 (Table 3). The highest species diversity in 'Cuello Dama Blanca' was found at stage H10 (Table 2) and in 'Calabacita' at H1 and H2 (Table 3). Most of the species found at different ripening stages are potentially toxigenic species. It has been described that differences mould

species prevalence are influenced by the interaction of abiotic factors such as a_w and temperatura (Burm Lee et al., 2015). In this regard, Galván et al. (2021) reported that the optimal growth rates for *A. flavus* on dried fig-based medium were at 30 and 37°C at 0.96 a_w. These temperature and a_w conditions are similar to those of this study during the fig ripening process (Figure 2 and 3). The predominant species in 'Cuello Dama Blanco' are *Alternaria* section *alternata*, *A. niger* and *C. cladosporioides* and in 'Calabacita' *Alternaria* section *alternata*, *A. flavus* and *A. niger*. These results are in line with other authors. Heperkan (2006) reported that the most common mycobiota found colonising dried figs are *Aspergillus* section *Nigri*, *Aspergillus* section *Flavi*, *Fusarium* spp. and *Penicillium* spp. Villalobos et al. (2019) found that the mycobiota of figs were dominated by *C. cladosporioides* and different species of the genus *Penicillium*.



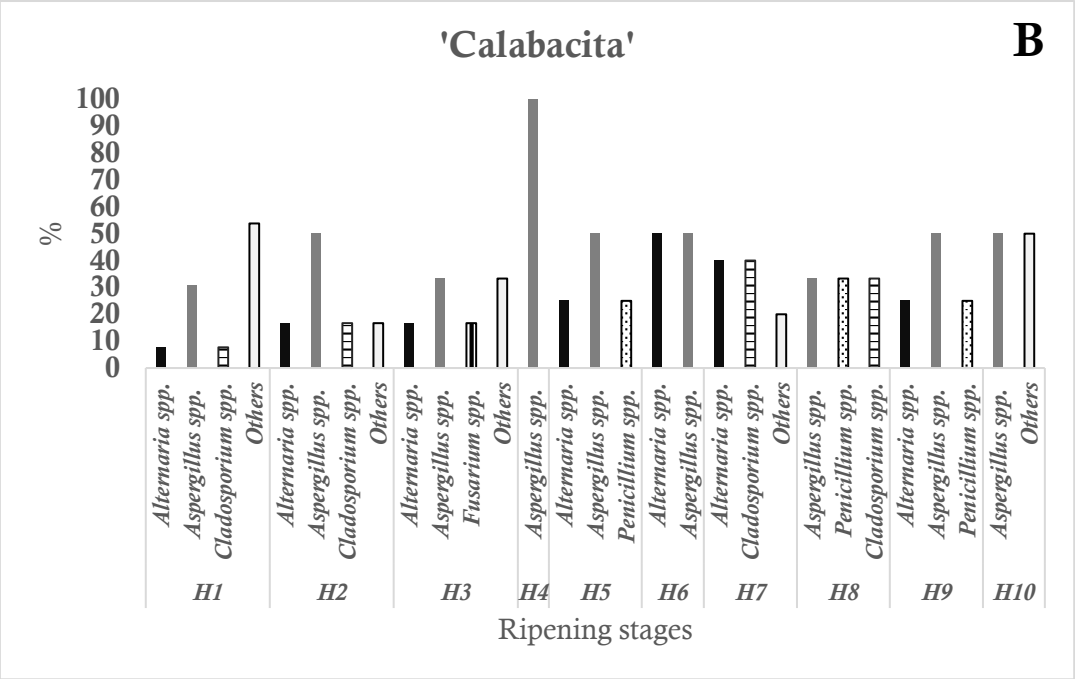


Figure 6. Relative abundance of mould genera of different ripening stages (H1-H10) of the two varieties Cuello Dama Blanco (A) and Calabacita (B) during the two years of study (2019 and 2020).

3.2.3 Aflatoxin and Ochratoxin A

Table 4 shows the percentage of samples contaminated with AFs and OTA at the different ripening stages (H1-10) of the two varieties studied. The most important mycotoxins found in dried figs are AFs and OTA (Celik and Kabak, 2022; Turkoz Bakirci, 2020; Bircan, 2009; Senyuva et al., 2005). The presence of AFs and OTA during the production process of dried figs is one of the main problems. AFs and OTA were detected in 1.9% and 3.8 % of the fig samples, respectively (Table 4). This percentage is lower than those reported in other studies of dried figs. Kabak (2016) in a study of dried figs taken from small scale farmers, retail shops and supermarkets in different cities of Turkey, found AFs in 12.3% of the samples. Bircan et al. (2008) analysed 4917 samples of dried figs destined for export from Turkey to the EU and found that 32% of the samples contained AFs. Celik and Kabak (2022) detected in

Turkish commercial dried figs an incidence of AFs and OTA of 14% and 8%, respectively. In a study on the different stages of the industrial processing of dried figs, Galván et al. (2022) reported that about 10% of the samples were contaminated with AFs and 6% with OTA. AFs were found at the H10 stage in the Cuello Dama Blanco variety (10-25 µg/kg). As for OTA, at H9 stage it was detected in 'Cuello Dama Blanco' with a concentration ranging between 10-25 µg/kg and it was also detected in 'Calabacita' at H10 stage in the 25-50 µg/kg range. While in the early maturity stages (H1-H8) there was no presence of AFs and OTA (Table 4) although potentially producing species of both mycotoxins were detected. This may be because the figs are green in the early stages of ripening and do not have the necessary requirements for mycotoxin production. The maximum limit set by the European Commission (EC) for total AFs (AFB_1, AFB_2, AFG_1 and AFG_2) in dried figs is 10 µg/kg, and for AFB_1 is 6 µg/kg (EC, 2012). Recently, a maximum level for OTA in dried figs has been set at 2 µg/kg (EC, 2022). In this study, the AFs and OTA samples detected were above these limits.

4. Conclusions

The physicochemical parameters of figs at the different ripening phases and the variety have influence on the mould population pattern of the figs. Most of the species identified in both fig varieties are toxigenic although no mycotoxins were found at the initial ripening stages of the fruits (from H1 to H8). Based on results, implementation of good agriculture practices together with other strategies including biocontrol at pre-harvest and a more efficient and faster drying of figs using meshes for fruit collection at harvest should be advisable.

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Table 1. Mean values of mould and yeast counts at the different ripening stages (from H1 to H10) of the two studied varieties, Cuello Dama Blanco and Calabacita, during the two years of study (2019 and 2020).

Variety	Microbial counts (log cfu/g)	Stages of maturity									
		H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**
Cuello Dama Blanco	**Mould**	2.63^{ab*}	2.36^{b}	2.13^{b}	2.12^{b}	2.02^{b}	2.60^{ab}	2.30^{b}	2.47^{b}	2.60^{ab}	2.84^{a}
	Yeast	2.86^{d}	2.57^{d}	2.17^{d}	2.07^{d}	2.10^{d}	2.06^{d}	2.19^{d}	4.29^{c}	7.83^{a}	6.33^{b}
	Mesophilic Aerobic bacteria	3.35^{bc}	2.91^{bc}	2.53^{c}	2.61^{c}	2.62^{c}	2.17^{c}	2.9^{bc}	4.35^{b}	7.79^{a}	7.96^{a}
Calabacita	**Mould**	2.68^{ab}	2.72^{ab}	2.24^{ab}	2.40^{ab}	2.11^{b}	2.22^{ab}	2.16^{b}	2.40^{ab}	2.17^{b}	2.92^{a}
	Yeast	2.82^{b}	2.79^{b}	2.26^{b}	2.42^{b}	2.43^{b}	2.35^{b}	2.78^{b}	3.20^{b}	5.74^{a}	3.14^{b}
	Mesophilic Aerobic bacteria	2.95^{b}	2.82^{b}	2.85^{b}	3.30^{b}	3.05^{b}	2.99^{b}	3.19^{b}	3.79^{b}	5.68^{a}	3.77^{b}

*Different letters (^{a,b,c,d}) indicate significant differences between the different ripening stages.

Table 2. Relative abundance of mould species isolated from the different ripening stages (H1-H10) of the Cuello Dama Blanco variety, during the two years of study (2019 and 2020).

Genus	Species	Stages of Maturity										GenBank accession number ITS	GenBank accession number β-tubulin
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		
***Alternaria* spp.**	***Alternaria* sect. *Alternata***	—	—	9.1	25	—	—	—	60	25	—	MT174142.1	KY814627.1
	Alternaria* sect. *Infectoriae	—	—	9.1	25	—	—	—	20	—	7.1	MK460886.1	KY965830.1
***Aspergillus* spp.**	***A. flavus***	—	—	9.1	—	—	—	—	—	—	7.1	MH864264.1	CP059871.1
	A. niger	—	—	—	—	—	—	—	—	25	28.8	MT447518.1	LC387870.1
	A. nidulans	—	—	9.1	—	—	—	—	—	—	7.1	MH864362.1	MN594520.1
***Penicillium* spp.**	***P. glabrum***	—	—	9.1	—	25	—	—	20	—	—	MH854998.1	GQ367502.1
	P. citrinum	—	—	—	—	—	—	—	—	25	7.1	MH858380.1	GU944545.1
	P. simplicissimum	—	—	—	—	—	—	—	—	—	7.1	KM979508.1	-
	P. corylophilum	—	—	—	—	—	—	—	—	—	14.4	MH861216.1	GU944519.1
	P. commune	—	—	—	—	—	—	—	—	—	7.1	KX674626.1	-
***Fusarium* spp.**	***F. proliferatum***	—	—	—	25	—	—	—	—	—	—	MT428196.1	MT966795.1
***Cladosporium* spp.**	***C. cladosporioides***	—	100	54.5	—	75	100	100	—	25	7.1	NR_119839.1	MH780075.1
	C. herbarum	—	—	—	25	—	—	—	—	—	—	KU182498.1	-
	C. ramotenellum	—	—	—	—	—	—	—	—	—	7.1	MF804502.1	MT881914.1

Table 3. Relative abundance (%) of mould species isolated from the different ripening stages (H1-H10) of the Calabacita variety, during the two years of study (2019 and 2020).

Genus	Species	Stages of Maturity										GenBank accession number ITS	GenBank accession number β-tubulin
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		
Alternaria spp.	*Alternaria* sect. *Alternata*	16.7	20	25	—	25	50	—	—	—	—	MT174142.1	KY814627.1
	Alternaria sect. *Infectoriae*	—	—	—	—	—	—	50	—	25	—	MK460886.1	KY965830.1
Aspergillus spp.	*A. flavus*	—	20	—	60	—	—	—	22.2	50	33.4	MH864264.1	CP059871.1
	A. tamarii	33.4	—	25	—	—	—	—	—	—	—	MH865259.1	EF661474
	A. niger	16.7	20	25	40	50	—	—	—	—	—	MT447518.1	LC387870.1
	A. welwitschiae	—	—	—	—	—	50	—	—	—	33.3	MH374611.1	MG832179.1
	A. chevalieri	—	20	—	—	—	—	—	—	—	—	MT316337.1	KU872178.1
	A. melleus	—	—	—	—	—	—	—	11.2	—	—	MH864931.1	MT410177.1
	A. fumigatus	—	—	—	—	—	—	—	—	—	33.3	MH865793.1	KU935623.1
	A. europaeus	16.7	—	—	—	—	—	—	—	—	—	FJ531070.1	MT452519.1
Penicillium spp.	*P. simplicissimum*	—	—	—	—	—	—	—	33.3	25	—	KM979508.1	-
	P. citrinum	—	—	—	—	25	—	—	—	—	—	MH858380.1	GU944545.1
Fusarium spp.	*F. verticillioides*	—	—	25	—	—	—	—	—	—	—	MH864460.1	MF662672.1
Cladosporium spp.	*C. cladosporioides*	16.7	—	—	—	—	—	50	—	—	—	NR_119839.1	MH780075.1
	C. tenuissimum	—	—	—	—	—	—	—	33.3	—	—	JQ781836.1	-
	C. oxysporum	—	20	—	—	—	—	—	—	—	—	NR_152267.1	MF175220.1

Table 4. Percentage of samples contaminated with AFs and OTA and the concentration (µg/kg) of the two varieties studied (Cuello Dama Blanco and Calabacita) at the different stages of ripening (H1-H10) during the two years of study (2019 and 2020).

Variety	Mycotoxins	µg/kg	Stages of maturity		
			H1-H8	H9	H10
Cuello Dama Blanco	AFs	<LOD*	100	100	66.7
		10-25			33.3
		25-50			
	OTA	<LOD	100	75	100
		10-25		25	
		25-50			
Calabacita	AFs	<LOD	100	100	100
		10-25			
		25-50			
	OTA	<LOD	100	100	66.7
		10-25			
		25-50			33.3

*LOD = AFB_1 (0.07 µg/kg), AFB_2 (0.3 µg/kg) and OTA (0.38 µg/kg)

CAPÍTULO 2

PUBLICACIÓN 3

Impact of water management and geographic location on the physicochemical traits and fungal population of 'Calabacita' dried figs in Extremadura (Spain)

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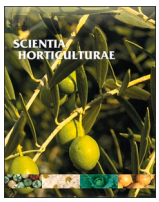

Impact of water management and geographic location on the physicochemical traits and fungal population of 'Calabacita' dried figs in Extremadura (Spain)

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ABSTRACT

The traditional process of sun-drying figs involves a high risk of toxigenic mold contamination and, as a consequence, potential mycotoxin occurrence. In this study, the influence of the environmental conditions in the three main production areas of 'Calabacita' dried figs in Extremadura (Spain), more precisely Almoharín, Guadajira, and Guareña, on physicochemical traits and microbiological quality was evaluated. Simultaneously, the impact of water management, irrigated and rainfed conditions, in the Almoharín area was also determined. For this purpose, dried fig samples were collected in two consecutive seasons (2018 and 2019) at different drying steps, harvesting from the ground and after final drying under greenhouse conditions until the final moisture content was reached. All physicochemical parameters were significantly influenced by geographic location whereas water management had only a significant impact on firmness, total soluble solids and fruit size. Changes in moisture content and a_w of dried figs from different geographic locations as well as water management modified yeast counts, while mold counts did not show significant changes. However, the mold population was complex, with 40 species identified, mainly belonging to *Penicillium* spp. (29.4%), *Aspergillus* spp. (24.5%), *Cladosporium* spp. (18.9%) and *Alternaria* spp. (17.3%). The occurrence of these mold species was strongly influenced by geographic location while the influence of water management was minimal. Furthermore, a substantial co-occurrence of samples contaminated with aflatoxin- and ochratoxin A-producing *Aspergillus* species was found. Of the total samples of dried figs analysed, 10.8% were contaminated with aflatoxins ranging from 0.1 to >70 ppb and 12.5% with ochratoxin A in the range from 10 to >70 ppb. The edaphoclimatic conditions specific to each geographic location set the physicochemical and microbiological quality of dried figs in Extremadura. In contrast, rainfed conditions had a limited impact beyond fruit size and higher level of AFs-producing mold under water stress. These findings are crucial to minimize the risks associated with the occurrence of toxigenic molds on dried figs.

1. Introduction

The fig tree (*Ficus carica* L.) has become an economically important crop in recent years in the Mediterranean area. Dried figs have gained growing importance as they are attractive to the industry, traders and individual consumers, offering a longer shelf life and higher economic benefit and consumer-friendliness compared to using the fruit in fresh form (Ansari et al., 2014). In addition, they are an excellent nutritional source, being particularly rich in fibre, minerals, vitamins, amino acids and phenolic compounds (Wojdyło et al., 2016). World dried fig

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production is concentrated in the Northern Hemisphere, specifically in countries of the Mediterranean basin such as Turkey, Egypt, Morocco, Algeria, Iran and Spain (FAOSTAT, 2020). Spain is the main producer in the European Union with about 62.4% of production localized in the Extremadura area under rainfed and irrigated conditions (MAPA, 2020). However, in recent years, in order to increase revenue from the dried fig crop, growing conditions and horticultural practices have been improved by applying super-intensive cultivation with localized irrigation (Galván et al., 2021b).

Sun-drying has been one of the most widely used methods of fig processing and preservation (Sridhar et al., 2019). Figs intended for sun-drying are left on the tree until fruits reach full ripening and/or are overripe, partially desiccate and eventually fall to the ground, the fruit being picked from the ground usually once a week (Flaishman et al., 2008). At this stage, the moisture content of most of them is between 30% and 60% (Aksoy, 1997; Ural, 1997; Galván et al., 2021b). Therefore, sun-drying is carried out during a period of 4 to 6 days, depending on ambient temperature and relative humidity, to reduce the moisture content to below 26% in accordance with Standard DDP-14 on the marketing and commercial quality control of dried figs (UNECE, 2016). This traditional management of dried fig production results in a high risk of infestation with pests and toxigenic molds that may result in mycotoxin contamination. Delayed harvesting from the ground, too slow drying owing to weather conditions or deficient storage conditions may enhance the contamination by toxigenic fungi (Flaishman et al., 2008). In addition, the high sugar content of dried figs, as well as their low water activity, favor mold growth (Gilbert and Senyuva, 2008).

The most common mycobiota found colonizing dried figs belong to the genera *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. and *Alternaria* spp. (Heperkan, 2006; Bakirci, 2020). These molds can produce mycotoxins, which are the major issue for the commercialization of dried figs and their derivatives, as they cause serious toxicological effects on human health and large-scale economic losses for traders and farmers (Amirahmadi et al., 2018; Di Sanzo et al., 2018). Several relevant mycotoxins have been reported in dried figs (Gilbert and Senyuva, 2008; Di Sanzo et al., 2018; Sulyok et al., 2020), suggesting that a wide diversity of toxigenic molds may contaminate them from the field to the market. The most important mycotoxins described in dried figs are aflatoxins (AFs) produced by *Aspergillus* section *Flavi* species and ochratoxin A (OTA) produced by *Aspergillus* section *Nigri* species (Senyuva et al., 2008; Heperkan et al., 2012; Heshmati et al., 2017). In this respect, Galván et al. (2021a) demonstrated the influence of temperature in dried fig model systems on growth, gene expression and AF production. Aldars-García et al. (2018) also compared the impact of different steady-state temperatures (15–35 °C) on the relative initial growth of *A. flavus* strains. On the other hand, Gilbert et al. (2018) and Verheecke-Vaessen et al. (2019) have suggested that environmental stress may lead to increases in mycotoxin production.

In the literature, most of the studies have been focused on the occurrence of mycotoxins in dried figs. However, as far as we know, there is no scientific literature on how water management (irrigated or rainfed) and geographic location (environmental conditions) affect the physicochemical traits and microbiological quality of dried figs, as well as AF and OTA occurrence in dried figs. Moreover, characterization of the fungal diversity in dried figs under these factors could help to implement strategies to guarantee their hygienic quality by controlling toxigenic mold species and consequently reducing the health risks associated with the exposure to mycotoxins. Within this context, the objective of this study was to determine the physicochemical traits and microbiological quality of dried figs from three geographical locations where the crop is widely grown in Extremadura. At the same time, this research also aims to evaluate the impact of water management, i.e. both rainfed and irrigated conditions, on the same features mentioned above, in one of these locations, where the crop coexists in both rainfed and irrigated conditions.

2. Materials and methods

2.1. Sampling of dried figs

The experimental design and sampling locations are graphically presented in Fig. 1. Dried fig samples of the variety 'Calabacita' were collected during two consecutive seasons (2018 and 2019) from commercial orchards established in 2010 located in the representative production areas Almoharín, Guadajira and Guareña of the Autonomous Community of Extremadura (Spain). In all sampling orchards, the trees were located in irrigated plantations, whereas in the Almoharín area, samples were also obtained from trees in rainfed plantations separated by approximately 800 m from irrigated orchard (Fig. 1). Specifically, the Guadajira plot is superintensive with a spacing of 5 × 2 m (1000 trees/ha), Guareña, is intensive with a spacing of 5 × 5 m (400 trees/ha) and in Almoharín, the irrigated plot with a spacing of 8 × 8 m (156 trees/ha) and the rainfed plot with 10 × 10 m (100 trees/ha). Water application by micro-sprinkler irrigation and accumulated rainfall during the months of growth and production of dried figs in the three geographical locations studied are shown in Table S1. Rainfed land depended exclusively on rainfall. Key meteorological parameters of these geographic locations during the months of growth and production of dried figs were obtained from three standard weather stations placed near to the sampling orchards (REDAREX 2022) (Fig. S1 and S2). To evaluate the impact of water management, the Almoharín area was selected, with trees in irrigated and rainfed orchards, while the Guadajira, Guareña and Almoharín orchards, all under irrigated conditions, were used to evaluate the impact of geographical location.

Dried fig samples were picked by hand directly from the ground (harvest), where they remained for approximately 6 to 7 days. Two samplings were carried out during the fig harvesting season (from early August to early October) in each study year. In each sampling, 9 representative trees were selected from the orchard in each location. The total production of each tree was collected, ranging from 3 to 4 kg for irrigated trees and from 1 to 2 kg for rainfed trees. Each tree represented a biological repetition and determinations were carried out in duplicate on each sample. The samples were transported under refrigeration to the laboratory. A proportional sample of each tree's production was randomly taken and homogenized using a Mortar Grinder Pulverisette 2 (Fritsch, Germany) for harvesting determinations (Fig. 1). Overall, dried figs are often harvested when their moisture content is > 30–40%, so they require an extra drying process. For this reason, the remaining samples from each tree was placed in a drying facility or greenhouse (after final drying) (under conditions of 60% humidity and average temperature 30–40 °C) where they were kept until the moisture content was below 26% according to the Standard DDP-14 (UNECE, 2016) (3–6 days), followed by final drying. After determinations on whole fruits, each sample was homogenized with a Mortar Grinder Pulverisette 2 (Fritsch).

2.2. Determination of physicochemical traits

Water activity (a_w) and moisture content were measured at harvest time and after final drying by an a_w meter (LabMASTER-aw, Novasina AG, Lachen, Switzerland) and according to AOAC Official Method 934.01, respectively. In addition, after final drying, fruit weight, total soluble solids, firmness and colour were determined as described in our previous work (Galván et al., 2021b).

2.3. Mold and yeast counts

Microbial counts were performed at harvest time and after final drying. Approximately 25 g of the sample was diluted 10-fold in peptone water (Condalab, Madrid, Spain) and homogenized for 30 s in a Stomacher 400 (Lab Blender, Model 4001, Seward Medical, London, UK). Subsequently, 10-fold serial dilutions were performed in peptone water

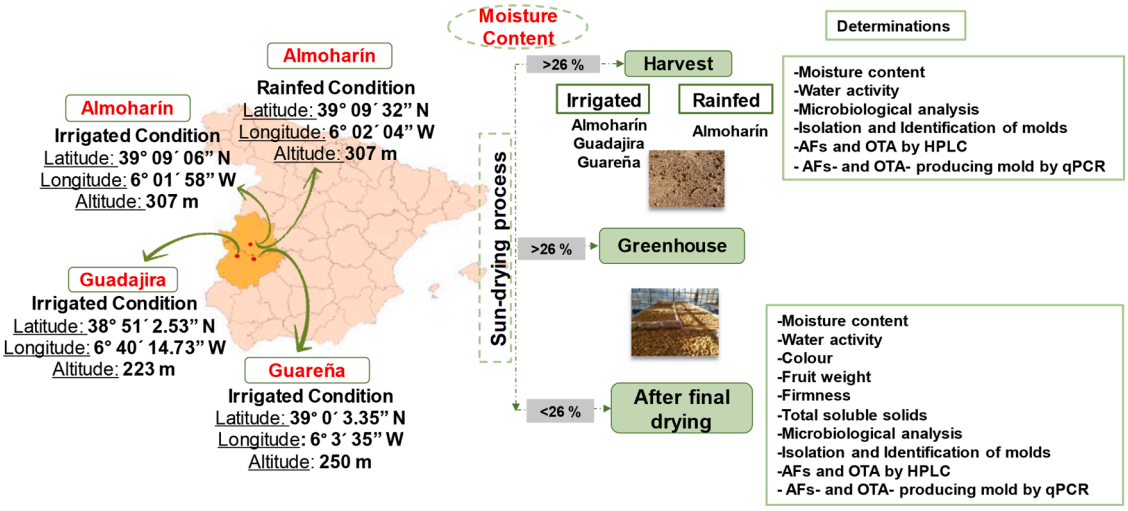

Fig. 1. Experimental design of the three geographic locations studied in Extremadura (red spots) during 2018 and 2019. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 0.1 mL aliquots were inoculated onto acidified potato dextrose agar (PDA, Condalab) plates. PDA was acidified to pH 3.5 with a sterile 10% (w/v) tartaric acid solution. After incubation at 25 ± 1 °C for 5 days, yeast and molds were counted, and the results were expressed as log cfu/g.

2.4. Isolation and identification of molds

2.4.1. Isolation

Three mold colonies were randomly selected from the highest dilutions and isolated onto acidified PDA plates until a pure culture was obtained. Conidial suspensions were obtained by swabbing the colony with 10 mL of sterile distilled water containing 0.05% (v/v) Tween 80 (Scharlab, Barcelona, Spain). The conidial suspension of each pure mold isolate was stored at −80 °C in glycerol solution (50% v/v).

2.4.2. Mold identification

Genomic DNA from mold isolates was extracted with a quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA concentration and purity were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

DNA from each isolate was amplified by inter simple sequence repeat PCR (ISSR-PCR) using the $(GTG)_5$ primer (Table S2). Amplifications were performed in a 50 μL reaction mixture containing 10 ng of DNA, 0.2 mM of each dNTP, 50 pmol of $(GTG)_5$ primer, 0.1 vol of 10 × PCR buffer and 1.25 U DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). PCR was run in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) with an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min 30 s, with a final extension period at 72 °C for 10 min. Amplification products were separated by electrophoresis in 2% agarose gels and visualized by staining with Midori Green Advance (Nippon Genetics, Tokyo, Japan). ISSR-PCR products were analysed as previously described by Merchán et al (2020) using the Generuler 100 bp plus DNA ladder (Thermo Fisher Scientific) as a reference. Two representative isolates from each ISSR-PCR pattern were identified to species level by sequencing the internal transcribed spacer ITS1/ITS2-5.8 S rDNA using the primer pairs ITS1 and ITS4 (White et al., 1990) and the β-tubulin gene using the primers Bt2a and Bt2b (Glass and Donaldson, 1995) as described by Galván et al. (2022) (Table S2). Moreover, for the specific identification of isolates belonging to *Aspergillus* section *Flavi* (*A. flavus, A. parasiticus* and *A. oryzae*), their AF profile was determined by HPLC-FLD after growing them on acidified PDA following the methodology also described by Galván et al. (2021a).

2.5. Quantification of potential aflatoxin- and ochratoxin-producing molds by a culture-independent method

DNA from dried fig samples at harvest and after final drying was extracted using a quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) according to the methodology described in our previous work (Galván et al., 2022). DNA concentration and purity (A_{260}/A_{280}) were determined with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and stored at −20 °C until required.

Quantification of *Aspergillus* section *Nigri* OTA producers and *Aspergillus* section *Flavi* AF producers was performed by SYBR Green qPCR assay on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, USA) according to the methodology described by Galván et al. (2022) using primer pairs ANPKSFRT and ANPKSRRT (Table S2) for OTA producers (Castellá and Cabañes, 2011) and primers F-omt and R-omt (Table S2) for Afs producers (Rodríguez et al., 2012), respectively.

2.6. Aflatoxin and ochratoxin A quantification

AFs (AFB_1, AFB_2, AFG_1 and AFG_2) and OTA were extracted from the samples at harvest and after final drying using AFLAOCHRA PREP® multi-mycotoxin immunoaffinity columns (R-Biopharm AG, Germany) according to the manufacturer's recommendations. Afterwards, the concentration of AFs and OTA in the samples was determined using an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1200 series G1321A fluorescence detector using a SUPELCOSIL LC-18 column (15 cm × 4.6 mm, 5 μm particle size; Supelco, Bellefonte, PA, USA) according to the chromatography conditions described by Galván et al. (2022).

2.7. Data analysis

The physicochemical and microbiological data were treated with a two-way analysis of variance (ANOVA) using SPSS for Windows, 25.0. Subsequently, the Bonferroni test was applied to compare the mean values obtained and the significance level was set at $p \leq 0.05$. The percentage of mold isolates identified at genus level was studied by the Chi-square test using contingency tables. Statistically significant differences were established by the Z-test of two proportion. Finally, principal component analysis (PCA) was performed on the correlation matrix of variables.

3. Results and discussion

3.1. Impact on physicochemical traits

Tables 1 and 2 show the moisture content and a_w of dried figs at harvest and after final drying by each geographic location and water management, respectively. Among geographic locations, moisture content ranged between 32.4% and 34.49%, the Almoharín area showing the highest mean value in both years of testing, significantly different ($p > 0.05$) from the Guareña area. Moreover, this production area is highlighted for showing the highest values of environmental relative humidity and the lowest temperature during the harvest period of both years of study: 67.1% and 22.4 °C in 2018 and 62.4% and 21.5 °C in 2019 (Fig. S1 and S2). In contrast, figs from the Guareña area, in the southern part of Extremadura, had the lowest moisture content (32.4%), significantly different ($p > 0.05$) to that of those from the Almoharín area, but not the Guadajira area with 32.99% (Table 1). Both areas were characterized by an ambient humidity between 53% and 57% (Fig S2). Drying conditions are determined by day–night temperature regime, drying duration, air flow rate and relative humidity (Aksoy, 2017). As expected, independently of cultivation type, dried figs having a higher moisture content is dependent on high relative humidity and low temperatures during the drying process. These findings are close to those shown in previous studies (Senyuva et al., 2008; Galván et al., 2021b), who also reported a moisture content of 30–40% in dried figs produced under traditional sun-drying. Nevertheless, according to Arvaniti et al. (2019), with this drying method it is possible to achieve a low moisture content of between 20% and 26%. As all samples tested at harvest in this study had a moisture content above the 26% established by the Standard DDP-14 (UNECE, 2016), a final drying was carried out under a greenhouse tunnel until the threshold value of 26% was achieved. After this drying process, the moisture content was around 21% without significant differences among geographic locations and water management. Regarding a_w, values among geographic locations ranged from 0.71 to 0.76 at harvest and from 0.51 to 0.56 after drying. Likewise, in terms of a_w, the Almoharín area showed the highest mean values at harvest and after final drying, with no significant differences ($p > 0.05$) between rainfed and irrigated cultivation (Tables 1 and 2). Moisture content and a_w are two key intrinsic factors that directly affect the quality of dried figs, as well as the presence of toxigenic mold and potential production of mycotoxins (Perera, 2005; Aksoy, 2017). To ensure the safety of dried figs throughout their supply chain, an a_w value of 0.65 or less is necessary, as at this a_w there is unlikely to be any fungal growth and mycotoxin formation triggered (Perera, 2005). Therefore, an a_w below 0.6 after final drying contributes to preventing the development of toxigenic molds (Gilbert and Senyuva, 2008), ensuring the microbiological quality of dried figs.

On the other hand, the impact of different geographic locations and water management on the firmness, skin colour, total soluble solids (TSS) and fruit weight of dried figs is shown in Table 3. Overall, all these parameters were affected by geographic location and water management ($p \leq 0.05$), although in the latter instance the colour was not affected ($p > 0.05$). These parameters play a decisive role in the final sensory quality and thereby determine consumer acceptance (Polat and Siddiq, 2012; Ansari et al., 2014). Thus, a high soluble solid content along with a high pectin content and low acidity allows a fast-drying and consequently higher-quality dried fruit (Aksoy, 2021).

Among geographic locations tested under irrigated conditions, significantly higher firmness values ($p \leq 0.05$) were obtained for the Guadajira area, with a mean value of 1.75 N. In terms of TSS and fruit weight, the Guareña area showed the lowest and the highest values respectively, without statistical differences between Guadajira and Almoharín areas for these two parameters. These traits are related to moisture content and fruit size, which in turn depend in part on the environmental conditions of production area (Crisosto et al., 2020). The Guadajira area, despite obtaining the largest water supply (Table S1), recorded the highest average temperature and lowest relative humidity (Fig. S1 and S2), which may explain its highest values of firmness and TSS. As expected, desiccation is enhanced by a hot and dry climate (Aksoy, 2021). Moreover, the radiation to which fruits are exposed can influence TSS content (Cucunubo Bosa et al., 2019; Li et al., 2020). On the impact of water management in Almoharín, dried figs grown under irrigated conditions showed a mean weight of 9.93 g compared to 7.56 g under rainfed conditions (Table 3). Additionally, dried figs grown under irrigated conditions were also softer. Increased water supply, e.g. through rainfall or irrigation, has been reported to increase fruit size and weight (Naor et al., 2000).

Regarding colour parameters, in this study, they were only significantly affected by geographic location ($p \leq 0.05$). The lightness (L*) varied from 53.75 to 60.13, while chromaticity (C*) ranged from 30.74 to 36.30 and hue angle (h*) from 71.49 to 74.36 (Table 3). Colour, together with firmness, is considered one of the main attributes of dried products (Perera, 2005). Figs are characterized by phenolic compounds that, among other factors, are conditioned by the geographic location (Ercisli et al., 2012; Pereira et al., 2017; Karantzi et al., 2021). These compounds during the drying process act as a substrate for enzymes such as polyphenol oxidase, resulting in oxidized forms of them, which subsequently polymerize into brown compounds (Perera, 2005). Moreover, the characteristic brown colour of dried figs may also be due to non-enzymatic or chemical reactions such as the Maillard reaction; the high sugar content of figs and high temperatures favor this reaction, resulting in a browning reaction due to the reduction of these sugars to amino acids, as well as caramelization and ascorbic acid browning (Perera, 2005; Yemis et al., 2012). As expected, these reactions may be favoured by environmental conditions such as more intense and warmer air currents and high temperatures (Beveridge and Harrison, 1984; (Echavarría et al., 2012). In this way, dried figs from the Almoharín area, exposed to lower temperatures and higher relative humidity

Table 1
Mean values ± SD of moisture content and water activity of fruit from the three geographic locations studied (Guadajira, Guareña and Almoharín) both at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019).

	Geographic location						Sig.		
	Guadajira		Guareña		Almoharín				
	H	AFD	H	AFD	H	AFD	*p* drying	*p* geographic location	*p* drying–geographic location
Water activity (a_w)	0.71 ± 0.05^{b1}	0.51 ± 0.40^{a}	0.72 ± 0.04^{b12}	0.53 ± 0.04^{a}	0.76 ± 0.06^{b2}	0.56 ± 0.11^{a}	0.031	0.006	0.851
Moisture content (%)	32.99 ± 3.52^{b12}	20.59 ± 1.87^{a}	32.4 ± 2.16^{b1}	21.06 ± 3.06^{a}	34.49 ± 4.97^{b2}	20.98 ± 5.71^{a}	0.017	0.001	0.232

Different superscript letters indicate significant differences between before and after drying within the same production area. Different superscript numbers indicate significant differences between geographic locations for the same drying stage.

Table 2
Mean values ± SD of moisture content and water activity of fruit grown under different water management (irrigated (AI) and rainfed (AR)) both at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019).

	H		AFD		Sig.		
	AI	AR	AI	AR	*p* drying	*p* water management	*p* drying–water management
Water activity (a_w)	0.76 ± 0.06	0.80 ± 0.13	0.56 ± 0.11	0.58 ± 0.06	0.918	0.398	0.27
Moisture content (%)	34.49 ± 4.97	36.12 ± 8.37	20.98 ± 5.71	23.74 ± 3.27	0.665	0.152	0.4

Table 3
Mean values ± SD of firmness, colour, soluble solids content and weight of fruit from the three geographic locations studied (Guadajira (GDI), Guareña (GRI) and Almoharín) and under different water management in the Almoharín area (irrigated (AI) and rainfed (AR)) in the samples after final drying during the two years of study (2018 and 2019).

		GDI			GRI			AI			AR			*p* geographic location	*p* water management
	Firmness (N)	1.75	±	0.86^{b}	1.43	±	0.3^{a}	1.48	±	0.49^{a}	2.53	±	1.17	0.028	0.000
Colour	L*	54.61	±	6.44^{a}	53.75	±	6.48^{a}	60.13	±	4.86^{b}	58.56	±	8.22	0.000	0.208
	C*	36.30	±	3.93^{b}	30.74	±	2.07^{a}	35.24	±	3.63^{b}	36.37	±	5.65	0.000	0.054
	h*	71.49	±	3.23^{a}	73.51	±	2.68^{b}	74.36	±	2.09^{b}	74.88	±	2.71	0.000	0.366
	Total soluble solids (°Brix)	84.45	±	1.53^{b}	78.29	±	2.76^{a}	83.18	±	2.27^{b}	80.89	±	4.18	0.000	0.004
	Fruit weight (g)	10.11	±	2.29^{a}	12.18	±	1.62^{b}	9.93	±	1.27^{a}	7.56	±	2.50	0.000	0.000

Different superscript letters indicate significant differences among the three irrigated geographic locations studied.

(Fig. S1 and S2), showed higher mean values of L* and hue angles (h*), indicating a lighter brown colour. In contrast, dried figs from the Guadajira and Guareña areas, exposed to higher temperature and lower relative humidity, were characterized by lower L* and h* values, showing darker brown figs.

3.2. Mold and yeast counts

The occurrence of mycotoxins due to toxigenic fungal growth is the main problem in conventional direct sun-drying of dried figs due to exposure to the open air and the critical range of a_w they experience during drying (Gilbert and Senyuva, 2008; Mat Desa et al., 2019). Mold and yeast counts at harvest and after final drying in the different geographic locations studied are shown in Table 4. Mold counts ranged from 2.43 to 2.85 log cfu/g at harvest and from 2.40 to 2.68 log cfu/g after final drying. At harvest, dried figs from the Guareña area showed significantly different ($p \leq 0.05$) mold counts with respect to the other two locations. Nevertheless, after final drying, no significant differences were found among the geographic locations studied ($p \geq 0.05$). The drying process only led to a significant decrease in the mold counts in dried figs from Guareña ($p \leq 0.05$). According to Magan and Aldred (2007), intrinsic factors such as nutrient composition define fungal spoilage. Dried figs from the Guareña area after final drying showed the significant lowest TSS ($p \leq 0.05$). This could explain the lower fungal growth. Additionally, these mold counts are in accordance with those described by Villalobos et al. (2019) in sun-dried figs of the same cultivar.

Table 4
Mean values ± SD of mold and yeast counts in figs from the three geographic locations studied (Guadajira, Guareña and Almoharín) both at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019).

Geographic location	Drying time	Mold (log cfu/g)			Yeast (log cfu/g)		
Guadajira	H (> 26%)	2.43	±	0.69^{1}	4.43	±	1.11^{b3}
	AFD (< 26%)	2.68	±	0.58	2.80	±	1.03^{a2}
Guareña	H (> 26%)	2.85	±	0.22^{b2}	2.39	±	0.50^{1}
	AFD (< 26%)	2.40	±	0.29^{a}	2.05	±	0.15^{1}
Almoharín	H (> 26%)	2.55	±	0.66^{1}	3.28	±	1.29^{b2}
	AFD (< 26%)	2.49	±	0.49	2.17	±	0.44^{a1}
p geographic location		0.042			0.000		
p drying		0.014			0.39		
p drying–geographic location		0.025			0.17		

Different superscript letters indicate significant differences between harvest and after drying within the same geographic location. Different superscript numbers indicate significant differences between geographic locations for the same drying stage.

Yeast counts ranged from 2.39 to 4.43 log cfu/g at harvest and from 2.05 to 2.80 log cfu/g after final drying. Significant differences ($p \leq 0.05$) were observed among the three geographic locations, yeast counts being significantly higher in Guadajira area at harvest and after final drying ($p \leq 0.05$). This may be explained by the fact that the Guadajira area showed the highest average temperatures and relative humidity (Fig. S1 and S2), closest to the optimal growth temperature for yeast growth (Walker and Dijck, 2006). Moreover, contrary to the general impact of drying on mold counts, yeast counts decreased significantly in dried figs from Almoharín and Guadajira after drying ($p \leq 0.05$). As expected, molds are better adapted than yeasts to low moisture and a_w conditions (Iamanaka et al., 2005).

With regard to the impact of water management, mold and yeast counts at harvest and after final drying are shown in Fig. 2. Overall, mold counts were around 2.5 log cfu/g, with no significant differences ($p > 0.05$) between rainfed and irrigated conditions, both at harvest and after final drying. In contrast, for yeast counts, significant differences ($p \leq 0.05$) were observed between rainfed and irrigated conditions. At harvest, the highest yeast counts were obtained under irrigation (3.28 log cfu/g), suggesting that these conditions encourage yeast growth. Likewise, for geographic location, final drying led to a decrease of yeast counts, although it was only significant for irrigated conditions ($p \leq 0.05$). For the first time, this study provides insights about counts of yeast growing on dried figs as a function of the impact of water management and climatic conditions which, along with mycobiota, are responsible for the quality properties of this product.

3.3. Identification of molds

Dried figs are suitable for contamination and growth of a wide range of molds during their ripening process. This is due to their intrinsic

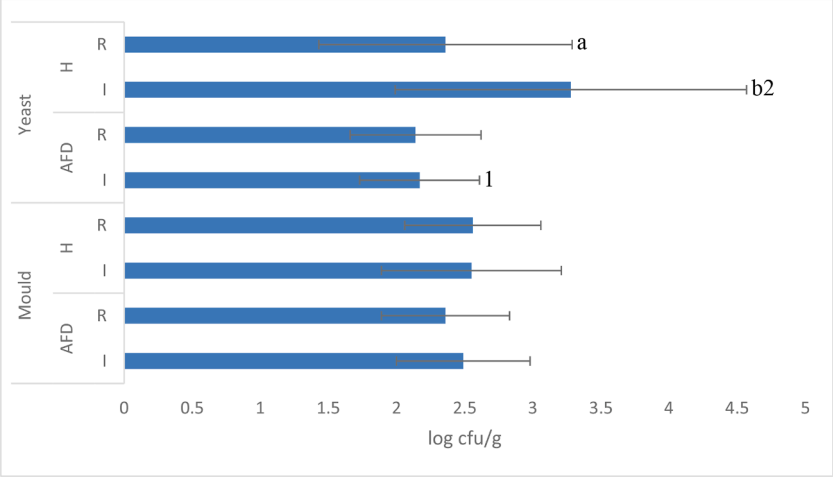

Fig. 2. Mean values ± SD of mold and yeast counts (log cfu/g) in Almoharín under rainfed and irrigated conditions both at harvest (H) and after final drying (AFD). Different letters (a,b) indicate significant differences between irrigated and rainfed conditions within drying time. Different numbers (1,2) indicate significant differences between drying times within the same water management.

physicochemical and nutritional traits (Iamanaka et al., 2005; Karaca and Nas, 2008) as well as other processing and extrinsic factors such as water management and climatic conditions (Aksoy, 2017; Crisosto et al., 2020). A total of 810 mold isolates were obtained, identified and clustered by ISSR-PCR using the $(GTG)_5$ primer into 40 different band profiles (P1–P40) (Table S3). Subsequent sequencing of the partial ITS and β-tubulin genetic markers of members from each cluster allowed the identification of 40 different species in the different geographic locations studied. The incidence of different fungal genera identified in the dried figs in both years is shown in Table S3. Four mold genera comprised 90.1% of the isolates: *Penicillium* (29.4), *Aspergillus* (24.5%), *Cladosporium* (18.9%) and *Alternaria* (17.3%). These genera have been identified as the most common mycobiota isolated from dried figs (Heperkan, 2006; Gilbert and Senyuva, 2008; López et al., 2016; Villalobos et al., 2019; Galván et al., 2022). In addition, the genera *Penicillium, Aspergillus* and *Alternaria* include most of the main toxigenic species. So, their specific identification provides relevant information for developing preharvest control strategies to prevent mycotoxin contamination in dried figs. Tables 5 and 6 show the relative abundance of mold genera by geographic location and water management at harvest and after final drying under greenhouse conditions, respectively. With respect to geographic location, similar mycobiota dynamics were observed in both Guadajira and Almoharín areas. At harvest, *Penicillium* spp. was the dominant isolates in both areas, with 37.8% and 32% respectively, followed by *Aspergillus* spp., *Cladosporium* spp. and *Alternaria* spp. (Table 5). On the contrary, the Guareña area at harvest time recorded a significantly higher level ($p \leq 0.05$) of *Aspergillus* spp. (43.5%) and *Alternaria* spp. (34.8%) and the lowest level of *Penicillium*

Table 5
Relative abundance of mold genera in dried figs from the three geographic locations studied Guadajira (GDI), Guareña (GRI) and Almoharín (AI)) in the samples at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019).

Mold genera	GDI		GRI		AI	
	H	AFD	H	AFD	H	AFD
Alternaria spp.	13.5^{a*}	18.2^{ab}	34.8^{b}	18.8^{ab}	12^{a}	24^{ab}
Aspergillus spp.	24.3^{ab}	20.5^{a}	43.5^{b}	43.8^{b}	24^{ab}	12^{a}
Cladosporium spp.	21.6^{ab}	11.4^{a}	8.7^{a}	6.3^{a}	22^{ab}	26^{b}
Fusarium spp.	2.7^{a}	2.3^{a}	0^{a}	0^{a}	0^{a}	2^{a}
Penicillium spp.	37.8^{c}	36.4^{c}	13^{a}	12.5^{a}	32^{bc}	24^{ab}
Others	0^{a}	11.4^{b}	0^{a}	18.8^{c}	10^{b}	12^{bc}

*Different superscript letters indicate significant differences among the three irrigated geographic locations studied ($p < 0.05$).

Table 6
Relative abundance of mold genera in dried fig obtained from Almoharín under different water management (irrigated (AI) and rainfed (AR)) both at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019).

Mold genera	AI		AR	
	H	AFD	H	AFD
Alternaria spp.	12^{a*}	24^{b}	7.1^{a}	18.5^{ab}
Aspergillus spp.	24^{b}	12^{a}	23.8^{ab}	25.9^{b}
Cladosporium spp.	22^{a}	26^{a}	19^{a}	25.9^{a}
Fusarium spp.	0^{a}	2^{a}	0^{a}	0^{a}
Penicillium spp.	32^{bc}	24^{ab}	40.5^{c}	18.5^{a}
Others	10^{a}	12^{a}	9.5^{a}	11.1^{a}

* *Different superscript letters indicate significant differences among the three irrigated geographic locations studied ($p < 0.05$).

spp. (13%). In the literature, differences in the prevalence of mold genera among geographic locations may be associated with climate conditions, among which temperature, relative humidity and rainfall play a key role in fungal colonization (García-Cela et al. 2014; Medina et al., 2015). However, in this study, no clear relationship was found between the climatic conditions, temperature and relative humidity, of the three geographic locations and the mycobiota. The Almoharín area recorded in both study years the lowest mean temperature (approximately 22 °C) and the highest mean relative humidity (between 62 and 67 %). However, the most widely divergent mycobiota was found in Guareña (Fig. S1 and S2). García-Cela et al. (2014) also observed in vineyards in two different Spanish agroclimatic regions (south and northeast) that coexistence and natural competition between fungal species is determined by environmental conditions such as temperature, relative humidity and rainfall. Thereby, in this work, other factors such as irrigation water supply and rainfall, which were different among the three geographic locations studied (Table S1), as well as soil water potential, soil type and agronomic management may also be involved. These factors determine the availability of water and consequently the fungal colonization (Marroquín-Cardona et al., 2014; Nji et al., 2022). In this context, where dried figs were collected from the ground, it can be stated that the soil-specific mycobiota from each geographical location may be the main source of contamination.

After final drying sampling, substantial changes in the complex mycobiota of dried figs mainly due to the impact of decreasing moisture content as well as a_w. These factors are well known to have a different influence on species of the same genus (Medina et al., 2015), boosting the final mycobiota. In particular, dried figs from Almoharín and

Guadajira areas showed an increase in the mean values of *Alternaria* spp. and a decrease in *Aspergillus* spp. and *Penicillium* spp., although these changes were not significant ($p > 0.05$). By contrast, a different dynamic was observed in the Guareña area, where the mean values of *Alternaria* spp. decreased, whereas the rates of *Aspergillus* spp. and *Penicillium* spp. maintained constant.

Conversely, with respect to water management, data suggest that irrigation had no significant impact on mycobiota, which at harvest and after final drying were similar in both systems (Table 6). Specifically, in both production systems at harvest sampling, *Penicillium* spp. was dominant, followed by *Aspergillus* spp., *Cladosporium* spp. and *Alternaria* spp. After final drying sampling, the mycobiota was dominated in rainfed by *Cladosporium* spp. and *Aspergillus* spp. which represented 25.9% of isolated molds each, followed by *Alternaria* spp. and *Penicillium* spp. at the same rate, 18.5%. However, under irrigated, significant differences were observed in *Aspergillus* spp ($p \leq 0.05$) with respect to rainfed, being this genus the fourth after *Cladosporium* spp. (26%), *Penicillium* spp. (24%) and *Alternaria* spp. (24%). Thus, the drying process affected the mycobiota. In particular, under irrigated condition, a significant increase of *Alternaria* spp. and decrease of *Aspergillus* spp. was found, whereas under rainfed only a significantly decline of *Penicillium* spp. was observed ($p \leq 0.05$).

Regarding the dominant mold species, Table 7 shows the relative abundance of mold species by geographic location and water management at harvest and after final drying. Overall, a wide diversity of species was found. For all variables studied, at least 10 different species were detected, suggesting that sun-dried figs have a complex mycobiota. Similar to what was observed at the genus level, geographic location had a great impact on the mold species population, suggesting the relevance of environmental conditions in shaping it. This study supports evidence from previous observations (Ozer et al., 2012; García-Cela et al., 2014). By geographic location under irrigated conditions, as well as at genus level, the mycobiota of dried figs from Guadajira and Almoharín areas were the most similar to each other, while the Guareña area was the most divergent. At harvest, the current study found that in dried figs from Guadajira, five species comprised approximately 67% of the total isolates, specifically *Penicillium citrinum* (19%), *Aspergillus tubingensis* and *Alternaria* section *Alternaria* (13.5% each), and *P. spinulosum* and *Cladosporium cladosporioides* (10.8% each). Four of them were also the dominant species in the dried figs from Almoharín: *C. cladosporioides* (17.7%), *P. spinulosum* (15.5%), *Alternaria* section *Alternaria* (13.4%) and *P. citrinum* (11.1%). In this latter area, but under rainfed conditions, the mold species identified and rates were similar to those under irrigated conditions. The main difference was associated with the prevalence of *Alternaria* spp. found, which were higher under irrigation condition. Irrigation decreases soil temperature and increases water availability that are more suitable conditions for *Alternaria* spp. colonization in hot and dry summers like those found in Extremadura (Lee et al., 2015). In contrast, in the Guareña area, the mold species diversity was lower and only two species, *Alternaria* section *Alternaria* (34.8%) and *A. tubingensis* (17.3%), comprised around 50% of isolates. In accordance with the present results, Villalobos et al. (2019) showed that the mycobiota of dried figs produced under different drying methods was dominated by *C. cladosporioides* and different species of the genus *Penicillium*, including *P. citrinum* and *P. spinulosum*. Their high prevalence could be due to the fact that they are considered as highly ubiquitous and easily spread fungal species (Egbuta et al., 2016). Furthermore, several authors have described the dominance of

Table 7
Relative abundance of mold species isolated from dried figs from the three geographic locations studied (Guadajira (GDI), Guareña (GRI) and Almoharín) and under different water management in the Almoharín area (irrigated (AI) and rainfed (AR)) both at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019).

	GDI			GRI			AI			AR		
	H	AFD	Total	H	AFD	Total	H	AFD	Total	H	AFD	Total
Alternaria sect. *Alternaria*	13.5	15.4	14.4	34.8	7.7	25.0	13.4	20.5	16.9	-	16.7	4.9
Alternaria sect. *Infectoriae*	-	5.2	2.6	-	15.4	5.6	-	6.9	3.3	7.9	4.1	6.5
Aspergillus flavus	8.1	7.7	7.9	8.8	15.3	11.1	8.8	4.6	6.8	2.7	4.1	3.2
Aspergillus oryzae	-	-	-	-	-	-	2.2	-	1.1	-	-	-
Aspergillus tamarii	-	-	-	-	-	-	-	2.2	1.1	-	-	-
Aspergillus welwitschiae	2.7	5.2	4.0	4.3	7.7	5.6	4.5	2.2	3.4	7.8	8.4	8.2
Aspergillus tubingensis	13.5	7.7	10.6	17.3	7.7	13.9	4.5	2.2	3.4	2.7	-	1.7
Aspergillus niger	-	-	-	4.3	7.7	5.6	2.2	2.2	2.2	-	-	-
Aspergillus ochraceus	-	-	-	-	-	-	-	-	-	2.7	4.1	3.2
Aspergillus melleus	-	-	-	4.3	7.7	5.6	-	-	-	-	-	-
Aspergillus nidulans	-	2.5	1.3	-	-	-	2.2	-	1.1	-	-	-
Aspergillus chevalieri	-	-	-	-	-	-	-	-	-	5.2	-	3.2
Aspergillus lentulus	-	-	-	-	-	-	-	-	-	2.7	12.6	6.5
Aspergillus niveus	-	-	-	-	-	-	2.2	-	1.1	2.7	-	1.7
Aspergillus fumigatus	-	-	-	4.3	-	2.7	-	-	-	-	-	-
Aspergillus europaeus	-	-	-	-	7.7	2.7	-	-	-	-	-	-
Penicillium citrinum	19.0	12.9	15.9	8.8	15.4	11.1	11.1	9.1	10.1	10.5	4.1	8.2
Penicillium sizovae	-	2.5	1.3	-	-	-	-	-	-	5.2	-	3.2
Penicillium expansum	-	-	-	-	-	-	2.2	-	1.1	-	4.1	1.7
Penicillium spinulosum	10.8	2.5	6.7	-	-	-	15.5	6.9	11.4	21.0	-	13.2
Penicillium glabrum	-	2.5	1.3	-	-	-	2.3	6.9	4.5	-	8.4	3.2
Penicillium thomii	-	-	-	-	-	-	4.4	-	2.2	-	-	-
Penicillium crustosum	8.1	5.1	6.5	-	-	-	-	-	-	5.2	-	3.2
Penicillium raistrickii	-	-	-	-	-	-	-	2.2	1.1	-	-	-
Penicillium chrysogenum	-	2.5	1.3	-	-	-	-	-	-	-	-	-
Penicillium corylophilum	-	12.9	6.5	-	-	-	-	2.2	1.1	-	4.1	1.7
Penicillium vanoranjei	-	-	-	-	-	-	-	-	-	2.7	-	1.7
Penicillium oxalicum	-	-	-	4.3	-	2.7	-	-	-	-	-	-
Fusarium proliferatum	-	-	-	-	-	-	-	2.2	1.1	-	-	-
Fusarium verticillioides	2.7	2.5	2.6	-	-	-	-	-	-	-	-	-
Cladosporium cladosporioides	10.8	12.9	11.9	8.8	7.7	8.4	17.7	22.8	20.3	15.8	25.1	19.7
Cladosporium ramotenellum	2.7	-	1.3	-	-	-	4.4	6.9	5.6	2.6	-	1.7
Cladosporium tenuissimum	5.4	-	2.6	-	-	-	2.3	-	1.1	2.6	-	1.7
Cladosporium limoniforme	2.7	-	1.3	-	-	-	-	-	-	-	-	-
Cladosporium oxysporum	-	-	-	-	-	-	-	-	-	-	4.2	1.7

Aspergillus from section *Nigri* in dried figs of different origin (Senyuva et al., 2008; Javanmard, 2010; Galván et al., 2022). However, *Alternaria* section *Alternaria* is commonly associated with fresh figs (Doster and Michailides, 2007; Villalobos et al., 2017), while in dried figs its reported occurrence is lower (Javanmard, 2010; Galván et al., 2022).

Regarding the influence of final drying, this mainly influenced the rates of certain dominant species; to a lesser extent, variations were also found in the subdominant population. For instance, the prevalence of *A. tubingensis* and *P. spinulosum* decreased in all geographic locations studied under different water management. Contrary to what was observed for harvest sampling, *Alternaria* section *Infectoriae* was detected under irrigated conditions in all geographic locations and *Alternaria* section *Alternaria* only in the Almoharín area under rainfed conditions. Finally, among geographic locations, the most relevant variations were observed in Guareña, where there was an increase of relevant toxigenic species such as *Aspergillus flavus, A. welwitschiae, A. niger* and *P. citrinum*. This geographic location is where the highest rainfall was recorded, which may favor soil colonization of toxigenic molds (Medina et al., 2015).

In terms of mycotoxin mold species occurrence, several toxigenic species were detected from the genera *Penicillium, Aspergillus, Alternaria* and *Fusarium*. The most important mycotoxins associated with dried figs include AFs, especially aflatoxin B_1 (AFB_1), and OTA (Flaishman et al., 2008; Di Sanzo et al., 2018). *Aspergillus flavus*, well known for its capacity to produce AFB_1, AFB_2 and cyclopiazonic acid (CPA), was detected after final drying for all variables studied at rates from 4.1% to 15.3% (Table 5). In the case of OTA producers, *Aspergillus welwitschiae* and *A. tubingensis* were also found widely distributed at rates below 10%, whereas other producer species such as *A. niger* and *A. ochraceus* were less prevalent. In the case of *A. tubingensis*, its OTA-producing capacity remains controversial and it appears to be strain-dependent (García--Cela et al., 2014; Gil-Serna et al., 2019). Additionally, *A. welwitschiae* and *A. niger* can also produce fumonisin B_2 (Gil-Serna et al., 2019). Among that of other toxigenic species, the occurrence of *P. citrinum* and *Alternaria* section *Alternaria* at final drying is noteworthy. *P. citrinum* produces citrinin (Perrone and Susca, 2017), while *Alternaria* section *Alternaria* produces several mycotoxins with toxic activity and has been reported to possess a higher mycotoxin potential than *Alternaria* section *Infectoriae* (Lee et al., 2015; Tralamazza et al., 2018). Sporadically, other toxigenic species were also found at low rates, such as *Aspergillus tamarii* and *A. oryzae* which are CPA producers, the citrinin producer *A. niveus*, patulin and citrinin producer *P. expansum* and fumonisin producer *Fusarium verticillioides*. Therefore, dried figs may be susceptible to contamination by a wide range of mycotoxins. In fact, Sulyok et al. (2020) detected a wide diversity of mycotoxins in Turkish dried figs, indicating the complexity of the mycobiota associated with this product.

3.4. Culture-independent quantification of potential aflatoxin- and ochratoxin A-producing molds

To date, the maximum legal limits for AFs according to Commission Regulation (EC) No. 1058/2012 (EC [European Commission] 2012) for dried figs subjected to sorting or other physical treatment before consumption have been set at 6 ppb for AFB_1 and 10 ppb for total AFs. However, other mycotoxins remain unregulated. Among them, in recent years, OTA has attracted attention due to its high occurrence in this product (EFSA [European Food Safety Authority] 2020). Thus, the molds producing these mycotoxins and factors that favor their presence are of particular interest to the dried fig sector. Fig. 3 shows the percentage of samples from the different geographic locations and water

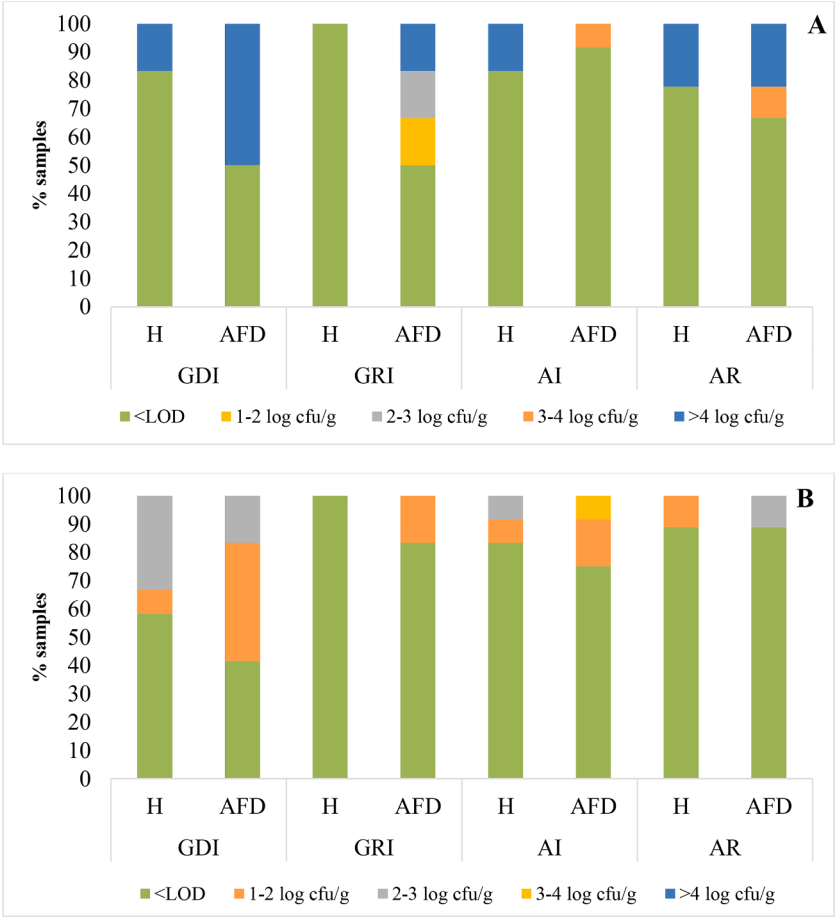

Fig. 3. Percentage of samples with levels of AF-producing molds (A) and OTA-producing molds (B) obtained in the three geographic locations studied (Guadajira, Guareña and Almoharín) and under different water management (irrigated and rainfed) in Almoharín both at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019). Limit of detection (LOD) = 1 cfu/g (0 log cfu/g). GDI (Guadajira irrigated), GRI (Guareña irrigated), AI (Almoharín irrigated), AR (Almoharín rainfed).

management contaminated with molds producing AFs (3A) and OTA (3B) at different levels (log cfu/g). A high co-occurrence of samples contaminated by AF- and OTA-producing *Aspergillus* species was observed. These findings agree with our previous results on the identification of these mycotoxin-producing molds by a culture-dependent technique. In addition, mold species associated with AF and OTA production have been reported in the literature as among the main hazards of dried figs (Saadullah and Abdullah, 2015; Di Sanzo et al., 2018; Galván et al., 2022). As for AF-producing molds, they were detected at different levels for all the variables studied except in the Guareña area at harvest, where were below the detection limit. By geographic location, the occurrence at harvest was lower than after final drying in two of the three geographic locations evaluated. After final drying sampling, the highest occurrence was observed in Guadajira and Guareña areas, with 50% of the samples contaminated with AF-producing molds (> 1 cfu/g), whereas in the Almoharin area only 8.3%. Between water management in Almoharin, rainfed conditions showed the highest occurrence in both harvest and after final drying with 22.2% and 33.3% contaminated, respectively. It is well known that water stress is one of the main factors involved in aflatoxin contamination (Medina et al., 2017). In fact, water stress together with high temperatures are more prone conditions for AF-producing molds colonization (Marroquín-Cardona et al., 2014). In this regard, Bircan et al. (2008) observed an increased incidence of AF in dried figs from Turkey in 2007 compared to previous years. This was associated with water stress, high temperatures and low relative humidity recorded in 2007. Thus, in the current climate change scenario, where in the Mediterranean basin is expecting an increase in temperature and drought events is expected (Medina et al., 2017), suitable irrigation management may contribute to reduce AFs contamination.

OTA-producing molds were also detected at different levels for all the variables studied, except in the Guareña area at harvest. By geographic location, the occurrence at harvest was lower than after final drying for all variables evaluated. After final drying under irrigated conditions, as for AF-producing molds, the highest occurrence was observed in Guadajira with 58% of the samples positive, followed by Almoharín (25%) and Guareña (17%). These counts were lower than those for AF-producing molds and ranged from < LOD to 3–4 log cfu/g. In positive samples after final drying, counts varied among geographic locations. The Almoharín area presented counts of 1–4 log cfu/g, Guadajira of 1–3 log cfu/g and Guareña of 1–2 log cfu/g. In this case of Almoharín under rainfed conditions, although the occurrence was slightly lower than for irrigated conditions (11%), the counts obtained were higher (2–3 log cfu/g). These findings suggest that the occurrence and level of AF- and OTA-producing molds are strongly influenced by geographic location and water management. It is well documented in the literature that the colonization of these molds is highly influenced by different extrinsic and intrinsic factors, temperature and moisture being among the most relevant ones (Abdel-Hadi et al., 2012). Higher temperature and humidity can favor mold growth, colonization and mycotoxin production (Medina et al., 2015). This may explain the higher prevalence of AF- and OTA-producing molds in Guadajira, where a higher irrigation dose and temperature were recorded. On the other hand, dried figs obtained under rainfed conditions showed a high incidence of AF-producing molds. This finding is consistent with those of Giorni et al. (2007) who reported the better adaptation of *A. flavus* over other species under very dry and warm conditions.

3.5. Aflatoxin and ochratoxin A quantification

Fig. 4 shows the percentage of dried fig samples from the different

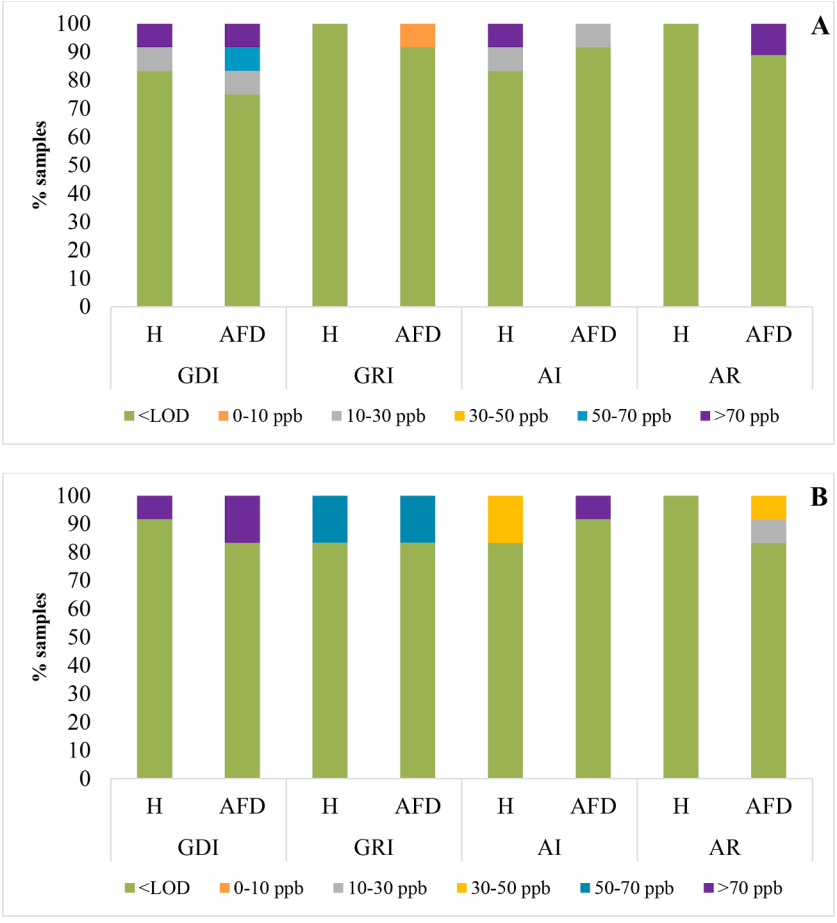

Fig. 4. Percentage of samples contaminated with different amounts (ppb = parts per billion) of AFs (A) and OTA (B) in the three geographic locations studied (Guadajira, Guareña and Almoharín) and under different water management (irrigated and rainfed) in Almoharín both at harvest (H) and after final drying (AFD) during the two years of study (2018 and 2019). LOD = AFB_1 (0.07 ppb), AFB_2 (0.3 ppb) and OTA (0.38 ppb). GDI (Guadajira irrigated), GRI (Guareña irrigated), AI (Almoharín irrigated), AR (Almoharín rainfed).

geographic locations as well as water management contaminated with different amounts of AFs (4A) and OTA (4B). The percentage of samples positive for AFs and OTA detected was equal or lower to that for the mycotoxin-producing molds determined by an independent technique (qPCR). This is because the presence of the mycotoxin-producing mold does not necessarily lead to the production of mycotoxins but represents a potential hazard. Across the different geographic locations and water management, the mean percentages of AFs and OTA above the threshold of detection were 10.7% and 12.5%, respectively. These results are in line with those of previous reports on the occurrence of mycotoxins in dried figs, where both mycotoxins are commonly found (Di Sanzo et al., 2018; Bakirci, 2020; Sulyok et al., 2020). Furthermore, it was generally observed that AFs and OTA levels were equal or higher after final drying samples than harvest samples, except for Almoharín irrigated (AI). It appears that toxigenic molds infest fruit in the orchard and the slowness of the traditional sun-drying process, depending on the climatic conditions, may provide conditions for mycotoxin occurrence. Therefore, it is essential to develop alternative artificial drying strategies to reduce aflatoxin and OTA contamination in order to ensure the hygienic quality of dried figs (Villalobos et al., 2019).

The results from this study confirm that the AF contamination was in concordance with the abundance of AF-producing molds (Fig. 3A and 4A). Among geographic locations, the highest production of AFs was in the Guadajira area where around 16.6% and 25% of samples produced more than 10 ppb at harvest and after final drying, respectively. In contrast, the lowest AF accumulation was in the Guareña area, where all samples were below the maximum legal limit established by European Commission (EC, 2012). Concerning water management in the Almoharín area, the percentage of contaminated samples under irrigated conditions at harvest was higher than those under rainfed. Nevertheless, after final drying, the tendency was different, the occurrence and accumulation of AFs under rainfed conditions (11% with > 70 ppb) was higher than under irrigated. This could be due to the higher occurrence of AF-producing molds after final drying under rainfed conditions (Fig. 4A).

On the other hand, with respect to OTA, although it is not regulated by the EU in dried figs, a legal limit of 10 ppb applies to dried grapes and nuts (EC [European Commission], 2005), which is taken as a reference. OTA contamination greater than 10 ppb was detected in between 8.3% and 16.7% of samples in all geographic locations at harvest and after final drying (Fig. 4B). In this case, no good correlation was found between the abundance of OTA-producing molds and OTA accumulation (Fig. 3B and 4B). For instance, although after final drying the Guadajira area clearly showed a higher abundance of OTA-producing molds, OTA

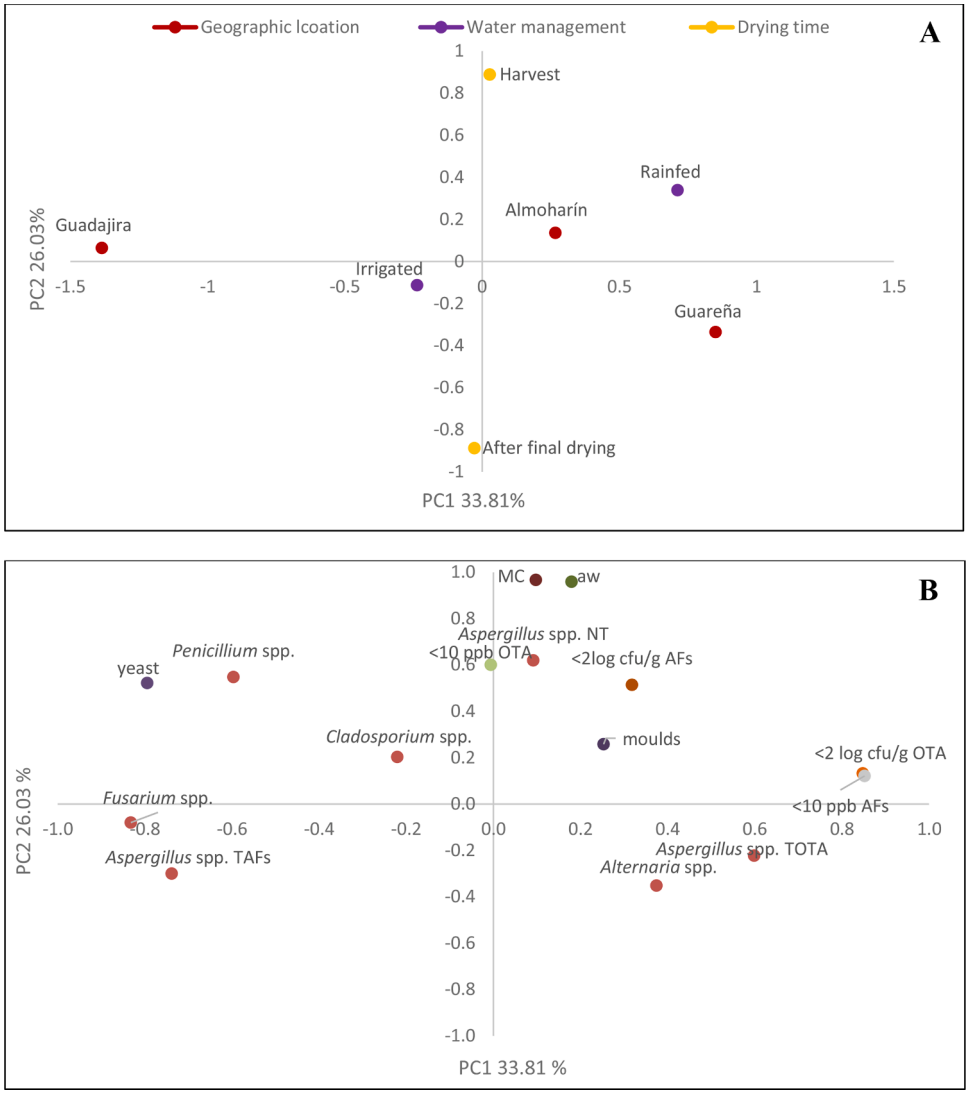

Fig. 5. Projection of samples grouped according to geographic location, water management and drying time (A) and variables (B) in the space defined by the first two components (PC1/PC2). MC (moisture content), a_w (water activity), OTA (ochratoxin A), AFs (aflatoxins), *Aspergillus* spp. TOTA (OTA-producing *Aspergillus*), *Aspergillus* spp. TAFs (AF-producing *Aspergillus*), *Aspergillus* spp. NT (non-toxigenic *Aspergillus*).

contamination was similar to that in the other geographic locations. Likewise, among water management after final drying, a higher percentage of OTA-positive samples was recorded under rainfed conditions, while the abundance of OTA-producing molds was slightly higher under irrigated conditions. This may be due to the fact that OTA production is affected by several factors such as the species and strains of *Aspergillus* section *Nigri* present, the level of fungal contamination, climate conditions, substrate composition and competition with other contaminant fungal species (Astoreca et al., 2010; García-Cela et al., 2014).

3.6. Principal component analysis

The results of the factor analysis according to geographic location showed a clustering of the samples mainly along PC1, which explained the highest percentage of the variance (33.81%), while PC2 explained 26.03% (Fig. 5). Thus, the mean of Guadajira area samples was to the left of PC1 (Fig. 5A). This behaviour is explained by the negative loadings of AF-producing *Aspergillus* and *Fusarium* spp. on the same axis (Fig. 5B). In contrast, samples from the Guareña area tended to have a positive PC1 score (Fig. 5A), which can be attributed to the greater presence of OTA-producing *Aspergillus* and *Alternaria* spp., represented by the positive loadings of these variables on PC1 (Fig. 5B). It is noteworthy that the samples from the Guadajira area, which had a higher occurrence of AF-producing *Aspergillus*, also had a higher AF content than those from Guareña and Almoharín. These results provide further support for the hypothesis that this mycotoxin production is affected by geographic location and, therefore, by the climatic conditions of each area.

The results of the factorial analysis according to water management (irrigated and rainfed), did not show a clustering according to the variables related to microbiological quality, suggesting that water management has a limited influence on the studied variable (Fig. 5B). Finally, the influence of final drying is mainly explained by PC2, which explains that figs are drier after this process, showing its great influence on moisture content and a_w parameters, shaping the mycobiota (Fig. 5A).

4. Conclusions

The results of this study suggest that all physicochemical parameters of dried figs evaluated were significantly influenced by the climate conditions in each geographic location, whereas water management had only a significant impact on firmness, total soluble solids and fruit size. Regarding mycobiota, this was complex, with 40 species in total identified, mainly belonging to *Penicillium* spp. (29.4%), *Aspergillus* spp. (24.5%), *Cladosporium* spp. (18.9%) and *Alternaria* spp. (17.3%). The occurrence of these mold species and aflatoxin and OTA contamination were affected by geographic location, although other factors such as soil type and soil water potential may also be involved. In contrast, water management had limited impact on mycobiota. However, water stress under rainfed conditions led to a higher level of AFs-producing mold. So, the results gathered here can be used as a starting point for further studies on the impact of other agronomic variables on toxigenic mold contamination. Finally, our findings confirm that scientific studies on the impact of environmental conditions on dried fig quality are crucial for the development of agronomic strategies in order to improve production and quality by minimizing the risks associated with the occurrence of toxigenic molds, which may positively influence the marketability of dried figs.

CRediT authorship contribution statement

A.I. Galván: Conceptualization, Investigation, Formal analysis, Writing – original draft. **M.G. Córdoba:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – review & editing. **S. Ruiz-Moyano:** Methodology, Visualization, Supervision, Writing – review & editing. **M. López-Corrales:** Conceptualization, Project administration, Funding acquisition. **E. Aranda:** Methodology, Formal analysis, Investigation. **A. Rodríguez:** Methodology, Visualization, Supervision. **M.J. Serradilla:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

None.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.111543.

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CAPÍTULO 3

PUBLICACIÓN 4

Evaluation of fungal hazards associated with dried fig processing

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Evaluation of fungal hazards associated with dried fig processing

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ABSTRACT

The processing of dried figs in the industry involves a number of stages that present a significant risk of filamentous fungal infection of the fruit and subsequent mycotoxin contamination, due to the changes in temperature and water activity (a_w) to which dried figs are exposed. In this study, the environmental conditions and the physicochemical parameters of dried figs at different processing stages were evaluated in 3 different industries, and were associated with fungal counts and the presence of toxigenic moulds and their mycotoxins. For this, dried figs at 5 relevant stages of industrial processing (curing, sizing, blanching, storage, and final product) in 3 industries located in Extremadura (Spain) were sampled. Changes in moisture content and a_w of dried figs during processing were observed and they influenced the mycological quality of figs. Among the fungal genera, *Aspergillus* spp. predominated in most stages except blanching, where *Penicillium* spp. prevailed. About 10% of the dried fig samples were contaminated with aflatoxins (AFs) and 6% with ochratoxin A (OTA). Based on findings, longer drying times are necessary after blanching to reduce a_w and to avoid the development of toxigenic moulds. In addition, all stages covering industry processing, final storage, and retailing of dried figs are advisable to be conducted at refrigeration conditions and controlled relative humidity to avoid mycotoxin production. The enumeration of AFs- and OTA- producing moulds by real-time PCR seems to be a good indicator for integration into prevention strategies to control filamentous fungal hazards and subsequent mycotoxin synthesis during the processing of dried figs.

1. Introduction

Commercial fig production takes place in areas with a predominantly Mediterranean climate (Mat Desa et al., 2019) such as California, Australia, South America, and the Mediterranean countries (Ersoy et al., 2017). Spain is the leader in European production being the region of Extremadura the main national producer of dried figs (MAPA, 2020). Calabacita is the most cultivated variety in this area due to its sweet taste and small size, allowing a uniform drying (Galván et al., 2021). Calabacita's characteristics favours a lower moisture content, and this is essential to minimize the fungal contamination and spoilage of dried figs. Traditional figs drying starts with the fruit senescence in the tree and continues to the ground, being harvested by hand or mechanically partially dried. Once harvested, the drying process is completed in solar dehydrators or in domestic storage rooms until the fig reaches a moisture content of ≤26%, in accordance with Standard DDP-14 on the commercial and marketing quality control of dried figs (UNECE, 2016). At industrial level in Extremadura, dried figs are first cured with aluminium or magnesium phosphide (phosphine) for pest control, and the stored at room temperature without humidity control until they are processed. Dried figs are usually blanched, hot-air dried, stored at room temperature and then placed onto the market to be commercialized with a shelf-life of around 6 months at room temperature.

The temperature and humidity conditions during the processing and storage of dried figs can favour the proliferation of toxigenic fungi and further mycotoxin production. In fact, mycotoxins are the most hazardous compounds that can occur in dried figs, throughout both pre- and postharvest (Bircan, 2009), being aflatoxins (AFs) and ochratoxin A (OTA) the most frequent mycotoxins found (Bircan, 2009; Drusch and Aumann, 2005; Kabak, 2016; Scott and Trucksess, 2009; Senyuva et al.,

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2005). The incidence of these toxic fungal metabolites leads to economic losses of producers due to the rejection of exported contaminated products (Heperkan et al., 2012). In addition, other relevant mycotoxins reported in dried figs are, fumonisin B_1, kojic acid, fusaric acid, and tenuazonic acid (Di Sanzo et al., 2018; López et al., 2016b; Sulyok et al., 2020).

Due to their toxicity and frequency in figs, several countries have set up regulations for mycotoxins in order to protect the consumers' health (Trucksess and Scott, 2008). The limits for these toxin compounds may vary between countries, as well as the type of mycotoxin. The maximum levels established by European Union (EU) for AFs in dried figs are 6 μg/kg for aflatoxin B_1 (AFB_1) and 10 μg/kg for total AFs (AFB_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2) (EU, 2012). In the case of OTA, although no limit has been yet established in dried figs, the EU has started to discuss the products that must be subjected to a maximum level of this metabolite, including dried figs (EFSA, 2020). In other products like dried figs such as dried vine fruits (currants, sultanas) the maximum level of OTA has been set up 10 μg/kg (EC, 2006).

Therefore, from a food safety perspective, it is of utmost importance to identify the hazards that may occur during the processing of dried figs in order to design control mechanisms if necessary (Ropkins and Beck, 2000). The different stages in the processing of dried figs in the industry, such as blanching and long storage periods, combined with inadequate temperatures and humidity, could favour the growth of toxigenic moulds and greater mycotoxin contamination of dried figs both at storage and at fruit retailing. De Mello and Scussel (2007) in a study on the assessment of mycotoxins in dried fruits observed that fungal contamination was due to their intrinsic characteristics of moisture and nutrient content, long storage time, and high water activity (a_w) values. The contamination of dried fruits starts on the tree and continues during storage as a result of poor drying and storage conditions, including both high temperatures and relative humidities. Besides, rehydration of the dried product under unsuitable conditions of storage may reactivate the fungal growth with subsequent mycotoxin contamination (Karaca et al., 2010). Once mycotoxins are produced, they cannot be removed from the product during processing. In most industries, bright greenish-yellow fluorescence is commonly used in a preliminary screening of AFs contaminated dried figs (Mat Desa et al., 2019) despite certain types of AFs could not be detected (Trucksess and Scott, 2008). In this sense, a study of the processing stages of dried figs would be useful to evaluate which are the critical stages in relation to their microbiological quality. Thus, an effective preventive system could be established leading to a safer production of dried figs. In order to implement a HACCP system, it is essential to have a good understanding of the processing of the product and to determine which critical steps need to be controlled. To the best our knowledge, there is no scientific literature that evaluates the safety and quality of dried figs throughout the manufacturing process. Within this framework, the aim of this study was to evaluate the impact that different processing stages of dried figs has on fungal growth and mycotoxin production in order to establish efficient control actions to ensure the production of safely dried figs.

2. Materials and methods

2.1. Sampling

Dried fig samples were collected along the industry's processing line from three different producers during two consecutive seasons (2018 and 2019) located at the main producing areas of Extremadura such as Almoharín (39°10′35″N; 6°02′39″O, 39°10′35″N; 6°02′39″O) (Industry A), Villar de Rena (−5.81178 39° 4′ 36″ N; 5° 48′ 42″ O) (Industry B) and Jerte Valley (40°08′23″N, 5°52′50″O) (Industry C) (Spain). Five kg of sample were collected from the main processing stages in each industry: curing, sizing, blanching, stored product, and final product (Table 1). Sampling was carried out by triplicate in each industry and each year of study from different random batches, taking two samples from each stage. The samples were immediately transported to the laboratory facilities and homogenized using a Mortar Grinder Pulverisette 2 (Fritsch, Germany). From these homogenates, instrumental triplicates were carried out for each analysis.

2.2. Physico-chemical determinations

Water activity was determined using an a_w meter (LabMASTER-a_w, Novasina AG, Lachen, Switzerland). Moisture content was determined according to the AOAC Official Method 934.01 (AOAC, 2005) by oven drying for 24 h at 104 °C and expressed as a percentage (%).

2.3. Mould and yeast counts

Ten grams of sample were homogenized in 90 mL of sterile peptone water. Then, serial dilutions were carried out with 0.1% (*w/v*) peptone water, and 0.1 mL aliquots were placed in acidified potato dextrose agar (PDA, Scharlab, Barcelona, Spain) to pH 3.5 and rose bengal plates with chloramphenicol (RBCA; Oxoid). Plates were incubated at 25 °C for 5 days, and the results were expressed as log cfu/g.

2.4. Isolation and identification of moulds

2.4.1. Isolation

Four-five isolates were randomly taken from each acidified PDA plate from the highest dilutions. Each colony was transferred onto a new acidified PDA plate for obtaining twice pure colonies. The isolates were stored in 50% sterile glycerol (*v/v*) at −80 °C until required.

Table 1
Production process of dried figs and differences between the three industries studied (A, B, C).

Industry	Dried figs production process										
	Curing	Storage product	Sizing	Washing	Blanching	Cooling shower	Drying tunnel	BGY-F and metal detector	Stored product	Floured and Packing	Final product
A	Phosphine fumigation (1 g phosphine/ t) (~ 7 days of exposure at room temperature)	Room temperature until product processing (1–8 weeks)	Sorting dried figs by size using special equipment	Washing with tap water for 1 min	100 °C for 1 min.	Showers with running water for 1 min	Hot air drying of figs for 1 min	Detection of metals	Room temperature (± 25 °C)	Figs with flour and packed in bags	Dried figs with rice flour
B					70 °C for 1 min.			Detection of aflatoxins and metals	Refrigeration chamber (± 0 °C)		
C					100 °C for 1 min.			Detection of aflatoxins and metals	Refrigeration chamber (± 8 °C)		

2.4.2. Identification

2.4.2.1. DNA extraction. Genomic DNA from mould isolates was extracted using the quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions. To measure the concentration (μg/μL) and purity (A_{260}/A_{280}) of DNA, a Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used.

2.4.2.2. PCR. Inter-simple sequence repeat (ISSR)-PCR was performed using the primer $(GTG)_5$ as described by (Cabañas et al., 2020), following the PCR conditions described by (Gallardo et al., 2014). Amplification products were visualized after electrophoresis in 1.5% agarose gels, by staining with Midori Green Advance (Nippon, Japan). The GeneRuler 100 bp plus DNA ladder (Thermo Fisher Scientific, San Jose, CA, USA) was used as a reference. The fragment profiles obtained were grouped into operational taxonomic units (OTUs). Cluster analysis of the bands of each isolate was carried out using the unweighted pair group method with arithmetic averages (UPGMA). The analysis was performed using the software NTSYSpc version 2.0. Three representative isolates of each OUT were identified to species level by sequencing the ITS1–5.8 rDNA ITS2 region using the primers pairs ITS1 and ITS4 described by (White et al., 1990) and β- tubulin genes using the forward and the reverse primers Bt2a and Bt2b described by (Glass and Donaldson, 1995). PCR reactions were run in a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA). The DNA obtained was purified using the GeneJET PCR purification kit (Thermo Fisher Scientific, USA) and sequenced by the Service of Bioscience Applied Techniques (STAB) at the University of Extremadura (Badajoz, Spain).

2.4.2.3. Sequence analysis. Sequences were analyzed using Bioedit version 7.2 and checked by nucleotide-nucleotide BLAST comparison at the NCBI database. The identities of the isolates were determined on the highest score and > 97% similarity was used as the criterion for species identification. For the confirmation of *Aspergillus* section *Flavi* species, specific PCR assays were carried out using primers FLA1/FLA2 for *A. flavus* as described by González-Salgado et al. (2008). *A. niger* and *A. welwitschiae* species belonging to *Aspergillus* section *Nigri* species could be distinguished using the *β-tubulin* gene as described (Perrone et al., 2011).

2.5. qPCR assays to quantify potentially toxigenic moulds

2.5.1. DNA extraction

DNA extraction from dried figs was carried out with the quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) with some changes. Five grams of dried figs were weighed and homogenized in a Stomacher 400 (Lab Blender, Model 4001, Seward Medical, London, UK) with 20 mL of Tris Buffer Ph 8. Two mL of this mixture was then transferred to an Eppendorf and centrifuged in an Eppendorf Centrifuge 5424 R (Eppendorf AG, Germany) at 15,871g/10 min. The supernatant was discarded, and the pellet was resuspended in 300 mL of nuclease-free water (Thermo Fisher Scientific, USA). The protocol of the kit was then followed according to the manufacturer's instructions, except that just before beating with the 1600 MiniG mixer (Automated Tissue Homogenizer and Cell Lyser, SPEX, Metuchen) for 5 min and 1500 rpm, 10 μL of proteinase K were added (20 mg/mL, Thermo Fisher Scientific, USA), followed by an incubation step at 65 °C for 45 min. The DNA concentration and quality were assessed by the Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). DNA was stored at −20 °C until use.

2.5.2. qPCR reactions

The Applied Biosystems 7300 Fast real-time PCR system (Applied Biosystems, USA) was used for qPCR amplification and detection. To detect the presence of the AFs producers, primer pairs F-omT and R-omT (200 nM) were used to amplify the *aflP* gene of *Aspergillus* section *Flavi* (Rodríguez et al., 2012). The primers ANPKSFRT and ANPKSRRT (300 nM) were used to amplify the *pks* gene of *Aspergillus* section *Nigri* (Castellá and Cabañes, 2011). The SYBR Green methodology was used. Reaction mixtures were prepared in triplicate of 12.5 μl reaction mixture in 96-well optical MicroAmp reaction plates and sealed with optical adhesive caps (Applied Biosystems). The reaction mixture consisted of 7.5 μL NZY qPCR Green Master Mix 2× (NZYTech, Lisbon, Portugal), different concentrations of each primer, and 2.5 μL of DNA template. Three negative controls without DNA were included in the runs. An initial denaturation step of 10 min at 95 °C, was followed by 40 cycles performed according to the following temperature regime: 95 °C for 15 s and 60 °C for 30 s. After the last PCR cycle, melting curve analysis of the PCR products was carried out by heating to 72–95 °C and continuous measurement of the fluorescence to verify the PCR product. Quantitative cycle (Cq) determinations were automatically performed by the 7300 System Software (Applied Biosystems).

2.5.3. Potential aflatoxigenic and ochratoxigenic mould quantification

Quantification of potential OTA- and AFs- producing moulds was conducted by using standard curves relating Cq values and tenfold dilutions of potential ochratoxigenic and aflatoxigenic moulds (log cfu/g) of known standards, respectively. qPCR reactions were conducted as described in Section 2.5.2. Finally, the ochratoxigenic and aflatoxigenic mould counts were extrapolated from their respective standard curves.

2.6. Mycotoxin analyses

2.6.1. Extraction

For the extraction of AFs and OTA, 100 g of dried figs were mixed with 400 mL extraction solution (MeOH: H_2O, 8:2, *v*/v) and 5 g of NaCl, shaken in the dark for 30 min, and filtered through filter paper (Whatman no.4). Next, 36 mL of phosphate buffer solution (PBS) (Thermo Fisher Scientific, USA) was added to the 4 mL filtrate. The pH was adjusted to 7.4 with NaOH (2 M). The solution was eluted onto the immunoaffinity column AFLAOCHRA PREP® (R-Biopharm AG, Germany) at 1–2 drops/min, followed by washing with 20 mL PBS. The elution was carried out with 1 mL of HPLC grade methanol (Thermo Fisher Scientific, USA) followed by 1 mL of water.

2.6.2. Quantification

AFs and OTA were analyzed on an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with Agilent 1200 series fluorescence detector G1321A. The separation was achieved using a Supelcosil LC-18 column, 15 cm × 4.6 mm, 5 μm (Supelco, Bellefonte, USA). For analysis of AFs, a mobile phase containing a mixture of MeOH: ACN:water (20:20:60 *v*/v/v/) was used at an isocratic flow rate of 1 mL/min. In addition, post-column derivatization with 0.05% pyridinium bromide (*w/v*; Sigma) was pumped at 0.3 mL/min on an Agilent 1100 Series HPLC device. For the analysis of OTA, a mobile phase in an isocratic mode composed of water/acetonitrile/acetic acid (41:57:2 *v*/v/v) was used. Finally, 100 μL of extract were injected into HPLC for both AFs and OTA analyses. Fluorescence (FLD) detection was carried out using 333 nm (excitation) and 460 nm (emission). Calibration curves for AFs and OTA were built with a standard mixture (Sigma-Aldrich, St. Louis, MO). The detection limits were 0.07 μg/kg for AFB_1, 0.3 μg/kg for AFB_2, and 0.38 μg/kg for OTA.

2.7. Statistics

Microbiological count data were treated to a one-way analysis of variance (ANOVA) using SPSS for Windows, 25.0. Afterward, Tukey's test was applied to compare the mean values obtained and the level of significance was set at $p \leq 0.05$.

3. Results and discussion

To the best of our knowlewdge, this is the first study that in-depth analyzes the processing of dried fig to evaluate the filamentous fungal hazards that may suppose an important risk in consumer's health. In this work, the environmental conditions and physicochemical parameters of dried figs at different processing stages in 3 different industries were evaluated and associated with fungal counts and the presence of toxigenic moulds and their mycotoxins.

3.1. Moisture content and water activity

As shown in Fig. 1, a similar pattern of moisture content (%) and a_w values were observed in the industries studied, finding the main differences in the blanching and storage stages. The blanching temperature in industry B was 70 °C while in industries A and C was 100 °C (Table 1). Regarding storage temperatures, they were 25 °C in industry A, 8 °C in industry C and 0 °C in industry B (Table 1). Moisture content values ranged from 22.88 to 34.55% and their values increased slowly throughout the sequential stages of the dried fig processing (from curing to blanching) reaching the highest ones ($p \leq 0.05$) at the blanching stage, especially in industry C with a value of 34.55% (Fig. 1). Then, moisture content values dropped slightly until obtaining the final product values between 30.85% for industry C and 26.76% for industry B. According to the standard DDP-14 on the control of the commercial quality and marketing of dried figs (UNECE, 2016) the moisture content should be less than 26% in the final product. However, in this work, the moisture content of dried figs in the three industries studied was higher than 26%, probably due to the short time of drying (1 min) after the blanching stage for the three industries studied. In a study about the influence of blanching on the drying and rehydration of banana slices, the drying time was 6 h at 50, 60, and 80 °C and the moisture content losses ranged between 30% and 45%, depending on the temperature (Taiwo and Adeyemi, 2009). In another study focused on the effect of sun-drying on some quality parameters in dried figs, the moisture content in dried yellow-skinned figs was 17.3% (Kamiloglu and Capanoglu, 2015). Blanching is one of the most widely used methods (Kidmose and Martens, 1999), mainly to inactivate enzymes and reduce the microbial load (Xu et al., 2012). However, it has been described that changes in environmental conditions during processing or inadequate blanching can lead to the development of mycotoxigenic moulds (*Aspergillus* spp., *Penicillium* spp. and *Alternaria* spp.) (Heperkan et al., 2012; López et al., 2016a) and further mycotoxin production such as AFs and OTA, which are the most frequently found in dried figs (Bircan, 2009; Gilbert and Senyuva, 2008; Rahimi and Shakerian, 2013; Turkoz Bakirci, 2020).

With respect to a_w values, their evolution was similar to the moisture content (Fig. 1). After a slow rise in the a_w values until the blanching stage, there was a steady decline until the final product was obtained, reaching a_w values between 0.65 a_w in industry B and 0.69 a_w in industry A. These a_w values are higher than those recommended by Jay et al. (2005) who reported that dried foods should be stored at a_w values <0.60, In addition, the relative humidity conditions of the chamber should be controlled in order to avoid increases in a_w value of the product favouring mould growth on the surface of the product. Again, the greatest a_w values in the three industries were observed at the blanching stage, ranging from 0.65 a_w for industry B to 0.72 a_w for industries A and C.

3.2. Mould and yeast counts

The mean values of mould and yeast counts (log cfu/g) at the different stages of dried fig processing revealed significant differences between stages and industries (Table 2). In dried fig processing, several factors can occur that lead to fungal or yeast proliferation (Gilbert and Senyuva, 2008). Regarding mould counts, significant increases ($p \leq 0.05$) were found at the curing and final product stages in the industries. Besides, a significant decrease ($p \leq 0.05$) in the counts from the sizing to the blanching stage in industries A and B was observed. The highest mould counts were encountered in the stored product, with values ranging from 2.88 to 3.13 log cfu/g, although no significant effect of the storage temperature on mould counts was observed. Neme and Mohammed (2017) showed that low or cold temperatures have fungistatic properties, not fungicides, so fungal growth and metabolism are enormously minimized. However, it should be noted that mould counts in the final product in industry B, with a storage temperature of 0 °C, were higher ($p \leq 0.05$) than in the other two industries. This may be because microbes surviving the drying process may remain inactive for long periods and become active once the food is rehydrated (Prabhakar and Mallika, 2014). Therefore, it seems clear that proper refrigeration conditions of dried figs are extremely necessary to avoid fungal hazards in this product. Mould counts of this work are similar or slightly lower than those found in other studies. Guirguis (2018) in a study based on the microbiological quality of commercial dried figs found mould and yeast counts of 4.6 log cfu/g. Villalobos et al. (2019) in a study about the influence of different fig drying methods reported similar mould counts in the control treatment (completely dehydrated figs). However,

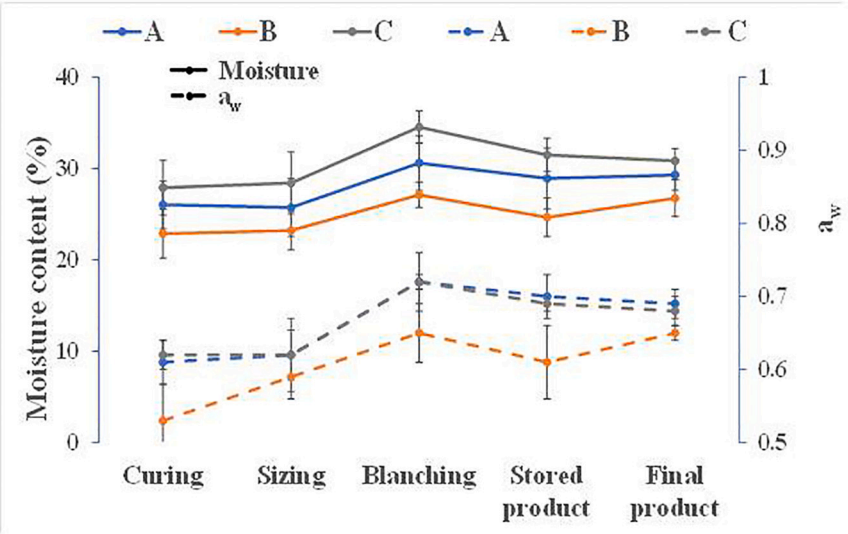

Fig. 1. Mean values of moisture content (%) and water activity (a_w) at the different stages of the dried fig production process in the three industries evaluated.

Table 2
Mean values of mould and yeast counts (log cfu/g) at the different stages of dried fig processing in the three industries evaluated.

	Industries	Stages					*p*
		Curing	Sizing	Blanching	Stored product	Final product	
Mould	A	3.05^{a}A	2.96^{a}	2.21^{b}	3.13^{a}	2.27^{b}B	0.000
	B	2.65^{ab}B	2.96^{a}	2.03^{b}	2.81^{a}	3.10^{a}A	0.000
	C	2.16^{ab}C	2.73^{ab}	2.40^{ab}	2.88^{a}	2.03^{b}B	0.030
	p	0.000	0.639	0.138	0.068	0.000	
Yeast	A	2.25B	2.71	2.54	2.57AB	2.44B	0.539
	B	2.11^{b}B	2.16^{ab}	3.08^{a}	2.00^{b}B	2.44^{ab}B	0.006
	C	3.26^{ab}A	2.46^{b}	2.83^{ab}	2.91^{ab}A	3.61^{a}A	0.008
	p	0.001	0.285	0.189	0.021	0.000	

Limit of detection: 2 log cfu/g.
Different capital letters (A,B) indicate significant differences between industries within the same stage **p** means statistical significance.
Different letters (a, b, c) indicate significant differences between stages within the same industry.

(Öztekin et al., 2006) encountered initial yeast and mould counts in dried figs around 1.46 log cfu/g.

Regarding yeast counts, significant differences were found between industries and processing stages at the curing, stored product, and final product stages ($p \leq 0.05$). In addition, significant differences between some of them were observed in both industries B and C ($p \leq 0.05$). The highest yeast counts were found in industry C in all stages studied where there were significant differences between industries. This fact may be because products from industry C possessed the highest moisture content at all stages.

3.3. Identification of moulds

The prevalence of mould species in the three studied industries and in the processing stages (curing, sizing, blanching, stored, and final products) are shown in Figs. 2 and 3. A total of 231 strains were identified, 88 belonging to industry A, 71 to industry B and 72 to industry C. The identification of moulds is especially important to provide information on which mycotoxins might be present. Identification was done by sequencing DNA regions amplified with the primers pairs ITS1 and ITS4 and Bt2a and Bt2b. Dried figs provide a suitable medium that favours the growth of a wide range of moulds. The predominant genus in industries A and B was *Aspergillus* spp. with prevalence rates of 54.6% and 76%, respectively. Next, *Penicillium* spp., *Cladosporium* spp. and *Alternaria* spp. were the most important ones with 25%, 13.6%, and 6.8%, respectively, in industry A and 18.3%, 4.3%, and 1.4% in industry B (Fig. 2). However, in industry C the genus *Penicillium* spp. was the predominant with a prevalence rate of 50%, followed by *Aspergillus* spp., *Alternaria* spp. and *Cladosporium* spp. with 33.3%, 11.1%, and 5.6%, respectively (Fig. 2). Regarding the processing stages of dried figs, the principal genus was *Aspergillus* spp. in all stages except in the blanching

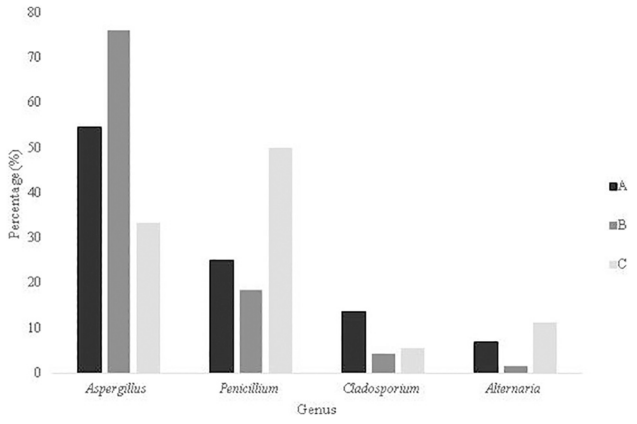

Fig. 2. Percentage of mould genus frequencies in the three industries (A, B and C) studied.

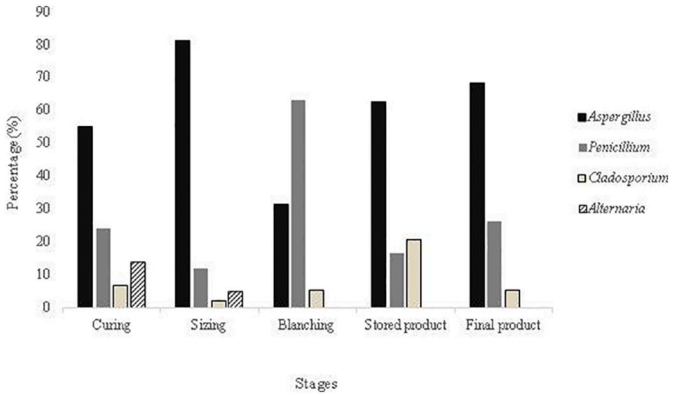

Fig. 3. Percentage of mould genus frequencies in the analyzed dried fig processing stages (Curing, Sizing, Blanching, Stored product, Final product) in the three industries (A, B and C). The values reflect the mean of the three sampled companies.

stage, where the genus *Penicillium* spp. prevailed (Fig. 3). The highest percentages of *Aspergillus* spp. were found in the sizing and final product stages with 81% and 68.4%, respectively. *Alternaria* spp. was only observed in the curing and sizing stage with 13.8% and 4.8%, respectively (Fig. 3). In dried date fruits collected in Perugia (Italy) the presence of mould species belonging to *Aspergillus*, *Penicillium* and *Cladosporium* had been previously described (Quaglia et al., 2020). Besides, Javanmard (2010) in a study regarding the occurrence of mould species on Iranian dried figs at different stages of production and processing, observed that *Aspergillus* spp. comprised 34.4% of the total fungal population.

The main mycotoxin-producing fungal genera found were *Aspergillus* spp., *Penicillium* spp. and *Alternaria* spp. (Table 3). In the case of *Aspergillus* spp., *A. tubingensis*, *A. niger*, *A. welwitschiae* and *A. flavus* were the prevailing mould species in the three industries. In industry A the predominant species of the genus *Aspergillus* spp. were *A. tubingensis* (36.8%) and *A. flavus* (7.9%). In industry B, *A. tubingensis* was the most predominant (42.6%) followed by *A. welwitschiae* and *A. flavus* (10.3%), while in industry C, *A. welwitschiae* and *A. flavus* were the main *Aspergillus* species with 11.8%. Heperkan et al. (2012) reported that *A. flavus* was the dominant species among dried fig samples. Senyuva et al. (2008) observed that *A. niger* is the most isolated fungus in Turkish dried figs. Out of these species, *A. flavus* produces AFB_1, AFB_2 and cyclopiazonic acid (CPA) (Vaamonde et al., 2003), *A. niger* and *A. welwitschiae* have been described as OTA producers (Perrone et al., 2011). In the case of *A. tubingensis,* this species seems to produce OTA in some experimental conditions (Chiotta et al., 2011; Lasram et al., 2012); however, Storari et al. (2012) reported that such species is not a mycotoxin producer. Although in California, the main source of OTA contamination in

Table 3
Identification of the mould species isolated in the three industries and the different stages of dried fig processing studied.

Number of isolates (%)													
			Industries				Stages					GeneBank accession numbers	
Genus	Species	Primers	A	B	C	TOTAL	Curing	Sizing	Blanching	Stored product	Final product	ITS	Btub
	A. welwitschiae	2*	5.3	10.3	11.8	8.9	3.7	9.8	–	5.3	27.7	MH374611.1	MG832179.1
	A. niger	2*	5.3	57.4	5.9	6.5	–	12.3	–	10.5	5.6	MT447518.1	LC573662.1
	A. tubingensis	2*	36.8	42.6	5.9	35.8	33.3	51.3	16.6	42	16.5	LC573618.1	LC387873.1
	A. ochraceus	2*	2.6	–	–	0.8	3.7	–	–	–	–	MH864932.1	KJ775051.1
Aspergillus	*A. flavus*	1*	7.9	10.3	11.8	9.8	3.7	7.3	16.6	10.5	16.5	CP044617.1	CP059871.1
	A. tamarii	2*	5.3	2.9	–	3.3	3.7	2.4	–	10.5	–	MH865259.1	EF661474
	A. calidoustus	4*	–	1.5	–	0.8	3.7	–	–	–	–	–	MN735972.1
	A. foetidus	2*	–	1.5	–	0.8	3.7	–	–	–	–	MH854624.1	DQ768454.1
	A. transmontanensis	2*	–	1.5	–	0.8	–	–	–	–	5.6	JF412771	HM803101
	P. citrinum	2*	10.5	4.4	5.9	6.5	14.8	–	11.1	–	11.1	MH864240.1	GU944545.1
	P. expansum	2*	–	8.8	11.8	6.5	–	–	33.3	5.3	5.6	DQ339562.1	AY674399.1
	P. corylophilum	2*	2.6	1.5	–	1.6	–	2.4	5.6	0	–	MF475922.1	GU944519.1
	P. glabrum	2*	7.9	–	11.8	4	7.5	2.4	5.6	5.3	–	MH854998.1	GQ367502.1
Penicillium	*P. crustosum*	2*	5.3	–	11.8	3.3	–	2.4	5.6	5.3	5.6	MH857883.1	AY674351.1
	P. chrysogenum	3*	2.6	2.9	–	2.5	3.7	2.4	–	5.3	–	MH865982.1	–
	P. menonorum	2*	–	–	5.9	0.8	–	–	5.6	–	–	HQ646591.1	HQ646573.1
	P. janczewskii	3*	–	1.5	–	0.8	–	2.4	–	–	–	MK179261.1	–
	P. bilaiae	2*	0	–	5.9	0.8	–	–	–	–	5.6	AF033402.1	KC773834.1
Alternaria	*A. alternata*	2*	7.9	1.5	11.8	4.9	14.8	4.9	–	–	–	MW326089.1	KY814627.1

1*ITS1F/ITS4, Bt2a/Bt2b, FLA1/FLA2 2* ITS1F/ITS4, Bt2a/Bt2b 3* ITS1F/ITS4 4* Bt2a/Bt2b.

dried figs was *Aspergillus alliaceus* (Bayman et al., 2002), this mould has not been isolated in any of the three industries studied in Extremadura.

Regarding the *Penicillium* genus, the most predominant species found in dried figs were *P. citrinum*, *P. expansum*, *P. glabrum* and *P. crustosum* with differences between industries. *P. citrinum* (10.5%) and *P. expansum* (8.8%) were the main species encountered in industries A and B, respectively; whereas in industry C, *P. expansum* (11.8%), *P. glabrum* (11.8%) and *P. crustosum* (11.8%) were the most prevailing ones. *P. citrinum* produces citrinin (Perrone and Susca, 2017), *P. expansum* is the most important source of patulin and can also produce citrinin (Perrone and Susca, 2017; Watanabe, 2008) and *P. crustosum* produces roquefortine C (Perrone and Susca, 2017).

With respect to *Alternaria* spp. genus, only *A. alternata* was found, being more found in industry C (11.8%), followed by industries A and B with 7.9% and 1.5%, respectively. Heperkan (2006) reported that the dominant mould population on Turkish dried figs belonged to *Aspergillus* section *Nigri*, *Aspergillus* section *Flavi*, *Fusarium* spp., and *Penicillium* spp.

Regarding the predominant moulds in the different stages studied, *A. tubingensis* was the prevailing species of *Aspergillus* genus in all stages except in the final product stage, where *A. welwitschiae* was predominant. Concerning *Penicillium* genus, the species *P. citrinum* was the most frequently encountered at the curing and final product stages while *P. expansum* was at the blanching stage. *A. alternata* was only identified at the curing (14.8%) and sizing (4.9%) stages. In the last years, some reports have also informed about the presence of *Alternaria* spp. in dried figs (López et al., 2016a). The differences between the prevalence and the heterogenicity of mould species found in the three industries studied could be due to the different geographical areas of dried fig production as reported by Ozer (2008). In addition, differences in blanching and storage temperatures with respect to the prevalence of mould species between industries may be associated with the temperature changes to which dried figs are exposed and storage times (Kesho et al., 2019). In fact, there was no treatment during processing able to remove the presence of moulds on the product surface.

3.4. Detection and quantification of aflatoxin- and ochratoxin A-producing moulds by qPCR

Results regarding detection and quantification of AFs- and OTA-producing moulds by qPCR by using SYBR Green methodology are shown in Table 4. The analysis revealed the high co-occurrence of samples contaminated by AFs- and OTA- producing *Aspergillus* species. AFs-producing moulds were detected in the three industries studied at the curing, blanching and storage stages. In addition, in industry C, they were also detected at the sizing stage. In general, *A. flavus* were found in samples where AFs-producing moulds were detected. The counts determined by qPCR ranged between <LOD and 5 log cfu/g, obtaining the highest values (3–5 log cfu/g) in industry C. This fact may be due to a higher moisture content of dried figs at all stages in industry C, which can influence their a_w, promoting fungal growth. In addition,

Table 4
Detection and quantification of aflatoxin- and ochratoxin A- producing moulds (log cfu/g) at different stages of dried fig processing with the specific primers F-omt/R-omt and ANPKSFRT/ANPKSRRT in the three industries studied.

	Industries					
	A		B		C	
Stages	Aflatoxin	Ochratoxin A	Aflatoxin	Ochratoxin A	Aflatoxin	Ochratoxin A
Curing	[c]12.5%	[b]37.50%	[b]25%	[b]25%	[c]37.5%	[c]37.5%
Sizing	[a]<LOD	[a]<LOD	[a]<LOD	[c]25%	[c]12.5%	[a]<LOD
Blanching	[c]12.5%	[a]<LOD	[c]50%	[c]12.5%	[c]25%	[a]<LOD
Stored product	[b]25%	[a]<LOD	[c]25%	[a]<LOD	[c]25%	[c]12.5%
Final product	[a]<LOD	[b]12.5%	[a]<LOD	[a]<LOD	[a]<LOD	[a]<LOD

[a] LOD = 1 cfu/g (0 log cfu/g).
[b] Aflatoxigenic mould counts (1–3 log cfu/g) and ochratoxigenic mould counts (0–1.5 log cfu/g).
[c] Aflatoxigenic mould counts (3–5 log cfu/g) and ochratoxigenic mould counts (1.5–3 log cfu/g).

environmental conditions during processing and storage are well known to influence the growth and biosynthesis of toxins (Hope and Magan, 2003). High relative humidity and temperature values can favour mould growth in dried figs (Rodríguez et al., 2012).

Regarding the detection of OTA-producing moulds in the dried fig samples, the counts quantified by qPCR ranged from <LOD to 3 log cfu/g. The incidence of OTA producers was higher at the curing stage in the three industries studied. Additionally, the counts were higher in industry B in the sizing and blanching stages and in industry C in the curing and stored product stages. Industry A had the lowest incidence of both OTA- and AFs- producing moulds. Overall, our results indicated a high incidence of toxigenic *Aspergillus* in dried figs. Similarly, other studies have also observed high counts of these mycotoxin-producing mould species (Iamanaka et al., 2005; Saadullah and Abdullah, 2015).

3.5. Aflatoxin and ochratoxin A detection

The percentage of samples contaminated with AFs and OTA at the different processing stages of the dried fig in the three industries studied are shown in Table 5. Although the presence of mould does not always indicate the presence of mycotoxins, it does indicate a potential mycotoxin hazard. In this context, approximately 10% of the dried fig samples were contaminated with AFs and 6% with OTA. AFs concentrations ranged from < LOD to 75 μg/kg for AFB_1 and < LOD to 22 μg/kg for AFB_2. About OTA concentrations, they varied from < LOD to 50 μg/kg. In the EU, the legislation setting limits for mycotoxins in food is Regulation (EC, 2006). The maximum allowed content for total AFs (B_1, B_2, G_1 and G_2) in dried figs is 10 μg/kg while for aflatoxin B_1 is 6 μg/kg. However, although other dried fruits such as dried grapes and nuts are covered by this European Regulation with regard to OTA, maximum amounts of this mycotoxin in dried figs are currently not regulated. Nevertheless, some countries such as Germany have set OTA limits in dried figs at 8 μg/kg (Bundesgesetzblatt Jahrgang, 2004).

The highest amounts of AFs (AFB_1 50–75 μg/kg and AFB_2 6–12 μg/kg) were detected at the blanching and final product stages in industry B. OTA amounts were mainly detected at the curing stage in industries A (37,5%) and C (12,5%), and at the sizing (25%) and blanching (12,5%) stage in industry B. The highest amounts of OTA (25–50 μg/kg) were found at the curing stage in industry A and at the sizing stage in industry B. The incidence of AFs and OTA observed in this study in dried figs agrees with the results of recent studies. Bircan (2009) analyzed the presence of AFs in dried figs destined for exportation from Turkey and found that 32% of the samples contained AFs with levels ranging from 0.2 to 260 μg/kg. In a similar study, dried fig samples collected from the Aegean region of Turkey were found to be contaminated with AFs with a frequency similar to that found in this study (10%). Özay and Alperden (1991) detected OTA in only 3% of the dried fig samples from the Aegean Region at levels 5.2–8.3 μg/kg in the harvest during 1988. A high incidence of OTA contamination was reported by Karbancıoğlu-Güler and Heperkan (2008), 44.3% of the samples contained OTA within the range of 0.12–1 μg/kg, while 3.5% had levels above 8 μg/kg. Bircan (2009))detected that 18% of dried fig samples collected from different exporting companies prior to the packaging were contaminated with detectable levels of OTA in the range of 0.87–24.37 μg/kg. Senyuva et al. (2005) reported the presence of 14–15% OTA contaminated samples with a maximum concentration of 26 μg/kg. In industry A, OTA had a higher incidence than AFs. The optimum temperature for OTA production by *Aspergillus* section *Nigri* is 15 °C–30 °C in peanut seeds, and the maximum concentration was observed at 25 °C (Astoreca et al., 2010).

In the curing stage, dried figs were kept approximately for 4–5 days at environmental temperature. This could result in the development of moulds and consequently the production of mycotoxins at later stages. In general, AFs occurred with a relatively greater incidence than OTA in industry B, whereas both AFs and OTA had the same incidence in industry C. However, industry A had a higher incidence of OTA than AFs (Table 5).

Table 5
Percentage of samples contaminated with aflatoxins (AFs) and ochratoxin A (OTA) and the concentration (μg/kg) in the different processing steps of dried figs studied in the three industries.

	Industries					
	A		B		C	
Stages	AFs	OTA	AFs	OTA	AFs	OTA
Curing	[a]<LOD	[c]37.5%	[b]25%	[a]<LOD	[a]<LOD	[b]12.5%
Sizing	[a]<LOD	[a]<LOD	[a]<LOD	[c]25%	[a]<LOD	[a]<LOD
Blanching	[c]12.5%	[a]<LOD	[d]50%	[b]12.5%	[b]12.5%	[a]<LOD
Stored product	[a]<LOD	[a]<LOD	[b]25%	[a]<LOD	[a]<LOD	[a]<LOD
Final product	[a]<LOD	[a]<LOD	[d]12.5%	[a]<LOD	[a]<LOD	[a]<LOD

[a] LOD = AFB_1 (0.07 μg/kg), AFB_2 (0.3 μg/kg) and OTA (0.38 μg/kg).
[b] Aflatoxin B_1 values (0–25 μg/kg) and B_2 values (0–6 μg/kg)/ochratoxin A values 0–10 μg/kg.
[c] Aflatoxin B_1 values (25–50 μg/kg) and B_2 values (6–12 μg/kg)/ochratoxin A values of 25–50 μg/kg.
[d] Aflatoxin B_1 values (50–75 μg/kg) and B_2 values (12–22 μg/kg).

AFs were found in all three industries at the blanching stage. This fact could be justified because mycotoxins are heat resistant (Bullerman and Bianchini, 2007) and this stage was conducted during a short time (1 min). High heat treatments would have to be applied for longer periods of time and this may lead to alterations in the organoleptic characteristics of the products (Kabak et al., 2006). Therefore, short blanching periods would favour the production of mycotoxins since fungi can resist this treatment and can activate secondary metabolic routes in order to favour its growth and survival by synthesizing these secondary metabolites (Patriarca et al., 2019). In addition, in industry B, AFs were also detected at the stored product and final product stages. Blanching and storage are two important critical points in the processing of dried figs for mycotoxin production. Blanching can lead to the breakdown of structures and disposal of nutrients, and in the absence of rapid cooling, it leads to the development of toxigenic moulds. In addition, during the storage of dried figs, there are favourable relative humidity conditions for the development of toxigenic moulds.

4. Conclusions

The results indicate that the changes in a_w and temperature that dried figs undergo during the different stages of industrial processing have great relevance to the mycological quality of dried figs. A high incidence of *Aspergillus* spp. capable of producing AFs and OTA was found in most of the processing stages studied regardless of the industry evaluated. Based on findings, blanching should be homogeneous and efficient with a longer subsequent drying stage to reduce the dried fig moisture content below 26% in order to minimize or even avoid the development of toxigenic moulds. In addition, postharvest of dried figs during all stages covering industry processing, final storage and retailing are advisable to be conducted at refrigeration conditions and controlled relative humidity to avoid mycotoxin production. The early detection of AFs- and OTA- producing moulds by qPCR can be considered as a good indicator for integration into prevention strategies to control filamentous fungal hazards and subsequent mycotoxin synthesis during the processing of dried figs.

Declaration of competing interest

None.

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CAPÍTULO 4

PUBLICACIÓN 5

Effect of Temperature During Drying and Storage of Dried Figs on Growth, Gene Expression and Aflatoxin Production

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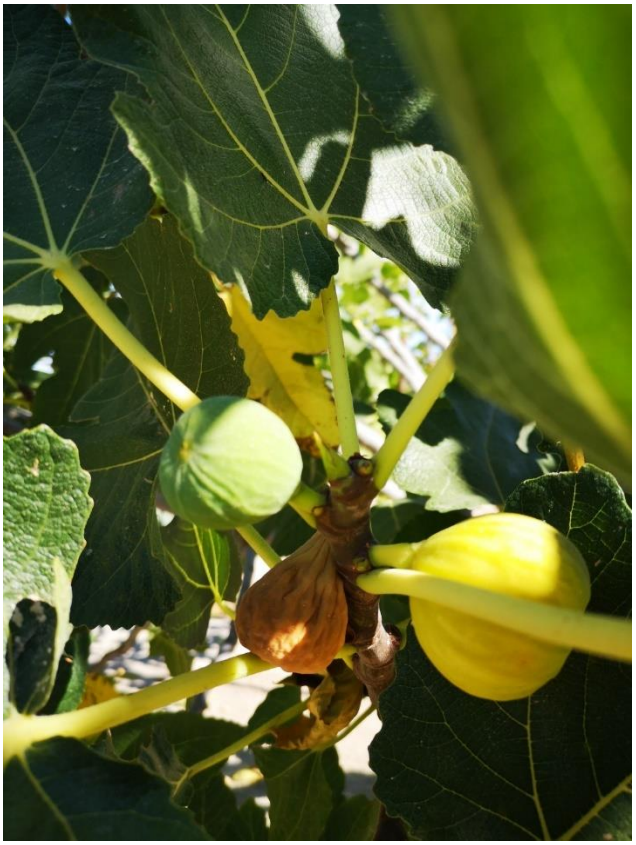

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Article

Effect of Temperature During Drying and Storage of Dried Figs on Growth, Gene Expression and Aflatoxin Production

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Abstract: Dried fig is susceptible to infection by *Aspergillus flavus*, the major producer of the carcinogenic mycotoxins. This fruit may be contaminated by the fungus throughout the entire chain production, especially during natural sun-drying, post-harvest, industrial processing, storage, and fruit retailing. Correct management of such critical stages is necessary to prevent mould growth and mycotoxin accumulation, with temperature being one of the main factors associated with these problems. The effect of different temperatures (5, 16, 25, 30, and 37 °C) related to dried-fig processing on growth, one of the regulatory genes of aflatoxin pathway (*aflR*) and mycotoxin production by *A. flavus*, was assessed. Firstly, growth and aflatoxin production of 11 *A. flavus* strains were checked before selecting two strains (M30 and M144) for in-depth studies. Findings showed that there were enormous differences in aflatoxin amounts and related-gene expression between the two selected strains. Based on the results, mild temperatures, and changes in temperature during drying and storage of dried figs should be avoided. Drying should be conducted at temperatures >30 °C and close to 37 °C, while industry processing, storage, and retailing of dried figs are advisable to perform at refrigeration temperatures (<10 °C) to avoid mycotoxin production.

Keywords: mycotoxin; toxigenic moulds; food safety; figs

Key Contribution: Correct management and control of temperature during drying, storage, industrial processing, and fruit retailing avoid infection of dried fig by *A. flavus* and their toxic metabolites (aflatoxins).

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1. Introduction

The fig tree originates from the Middle East where it has been cultivated for millennia, probably because of well adaptation to high temperatures and low water regimes, so it has traditionally been cultivated in marginal soils under rain-fed conditions. Its fruit, the common fig (*Ficus carica* L.), is a typical species of the tropic and subtropic areas, being one of the most important agricultural products in the Middle East and Mediterranean region [1]. Fig is a seasonal fruit that can be harvested twice a year, either during the spring and summer season or in the early and late summer, depending on the cultivar [2,3]. Both fresh and dried figs are extensively consumed worldwide due to their organoleptic characteristics, important nutritional value, and natural sweetness [4]. In addition, in the last decade, production of fresh and dried figs has increased by 44% [5].

However, the high perishability of fresh fruit extremely limits the increase of area and production of this crop in the Mediterranean basin and further exportation to third countries. For this reason, the production of dried fig has been dramatically rising during the last years [5], since drying is a potential agricultural preservation technique, regardless of geographical and other challenges. Drying has proven to be a reliable preservation method for figs, in terms of technical feasibility and nutritional quality [6]. However, when temperature and duration of drying are not extremely controlled, as occurs in natural sun-drying, the hygienic-sanitary quality of figs may be affected.

Natural sun-drying has been practiced widely in tropical and subtropical countries since ancient times [7], with the main objective of ensuring the conservation of figs and extending their shelf life [8]. Apart from inconveniences caused by the uncontrolled temperature and time, the absence of meshes implies drying of figs on the ground, which in turn can lead to their infection by filamentous fungi [9]. The most predominant toxigenic fungi in dried figs are *Aspergillus* section *Nigri*, *Aspergillus* section *Flavi, Fusarium* spp., and *Penicillium* species [10–12]. Recently, some reports have also informed about the presence of *Alternaria* spp. in dried figs [13,14]. Some of these filamentous fungi may produce mycotoxins when the environmental factors, especially temperature and water activity (a_w), are propitious [15–17]. In addition, other critical stages of dried fig processing to take into account are storage, and even during fruit retailing, since when figs are at this phase they are also susceptible to fungal colonisation and further mycotoxin production [11,18].

There are various mycotoxins found in figs including ochratoxin A (OTA), alternariol (AOH), tenuazonic acid (TeA), fumonisin B_1, and aflatoxins [11,13,14,19–21]. Aflatoxins are the most important and with the highest prevalence found in figs. These mycotoxins have been found in dried figs from Turkey [11,19], Cyprus [22], and China [21]. Among the aflatoxins, aflatoxin B_1 is recognized as one of the most potent carcinogens in foods and has been classed by the International Agency of Research for Cancer (IARC) in group 1A [23]. Due to the high toxicity of the aflatoxins and its high incidence in dried figs, the European Union has established maximum limits for aflatoxin contamination in this product at 6 μg/kg AFB_1 and 10 μg/kg total aflatoxins (sum of AFB_1, AFB_2, AFG_1, AFG_2) [24].

In spite of these precedents, no investigations have yet been conducted about the ecophysiology of *A. flavus*, mould species producer of aflatoxins, in figs [7,25,26], under different environmental conditions occurring during fig processing. For this reason, this study is of great interest in order to investigate the capacity of *A. flavus* to grow and produce aflatoxins in a dry fig-based (DFB) medium from both phenotypic and genotypic points of view. These kinds of studies could pave the way to understand changes in the ecological status during the fig drying to comprehend the environmental conditions which favour the growth of *A. flavus* and aflatoxin production. Thus, the objective of this study was to evaluate the effect of temperature related to fig processing on growth, one of the regulatory genes of aflatoxin pathway (*aflR*) and mycotoxin production of *A. flavus* on a DFB agar at 0.96 a_w.

2. Results

2.1. Selection of Two Aflatoxigenic Strains: Initial Screening

Initial experiments were performed using eleven *A. flavus* strains (M30, M42, M43, M55, M93, M111, M112, M115, M116, M144, and M148) to evaluate differences and similarities in their growth, lag time, and mycotoxin production capacity. For this, the *A. flavus* strains were inoculated on DFB agar 0.96 a_w and incubated at 25 °C for 7 days.

Figure 1 shows the combined effect of temperature, a_w, and nutritional composition of the DFB agar on lag times prior to growth of the *A. flavus* strains tested. The lag times fluctuated between 0.11 (*A. flavus* M148) and 1.01 (*A. flavus* M30) days. Although it may

appear that they were quite similar, some significant intra-strain differences ($p \leq 0.05$) were found.

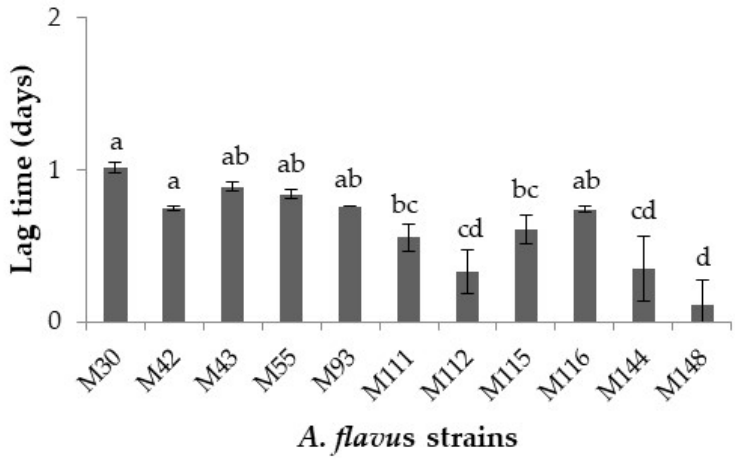

Figure 1. Lag time prior to growth (days) of the 11 *Aspergillus flavus* strains over the 7 day incubation period. Different letters indicate significant differences ($p \leq 0.05$).

Regarding the mean growth rates of the strains of *A. flavus*, they are displayed in Figure 2. Growth rates ranged from 5.15 (M115) to 6.49 (M43) mm radius/day. The strains M43, M55, and M93 grew faster than the remaining *A. flavus* strains checked, excluding the strain M30 ($p \leq 0.05$). The strains M111, M112, M115, M116, and M148 showed the slowest growth of the strains evaluated.

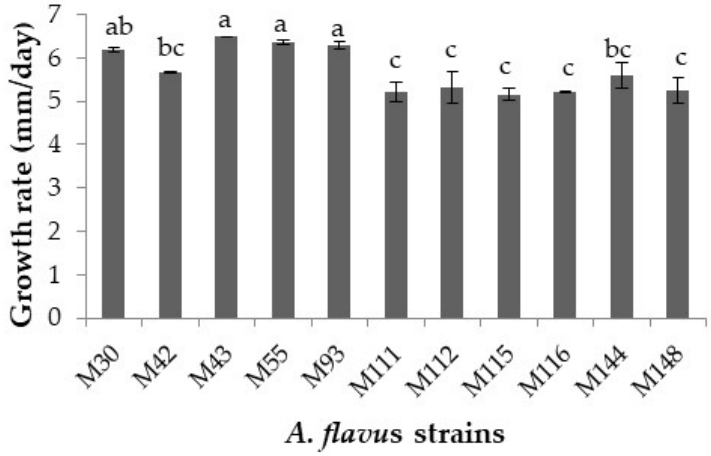

Figure 2. Growth rate (mm/day) of the 11 *Aspergillus flavus* strains over the 7 day incubation period. Different letters indicate significant differences ($p \leq 0.05$).

With respect to aflatoxin production by the *A. flavus* strains at the specific environmental and nutritional conditions evaluated, higher intra-strain differences compared to the other two parameters analysed (lag phase and growth rates) were observed. In Figure 3, it can be observed that, in general, all the strains produced much higher amounts of aflatoxin B_1 than aflatoxin B_2; even in three of the strains, no aflatoxin B_2 production was detected above the limit of detection of the technique (M30, M115, and M148). Regarding the aflatoxin B_1, the strains M30, M115, and M148 produced aflatoxin B_1 quantities lower than 1 ppb. Three other strains (M93, M111, and M112) synthesised this mycotoxin at

levels between 2 and 9 ppb, while the remaining 5 strains produced aflatoxin B_1 quantities higher than 17 ppb, the strains M144 and M116 being the highest producers of this mycotoxin. With regard to aflatoxin B_2, the maximum amount synthesised was 1.28 ppb by the strain M144. All the other *A. flavus* produced this mycotoxin at levels below 1 ppb.

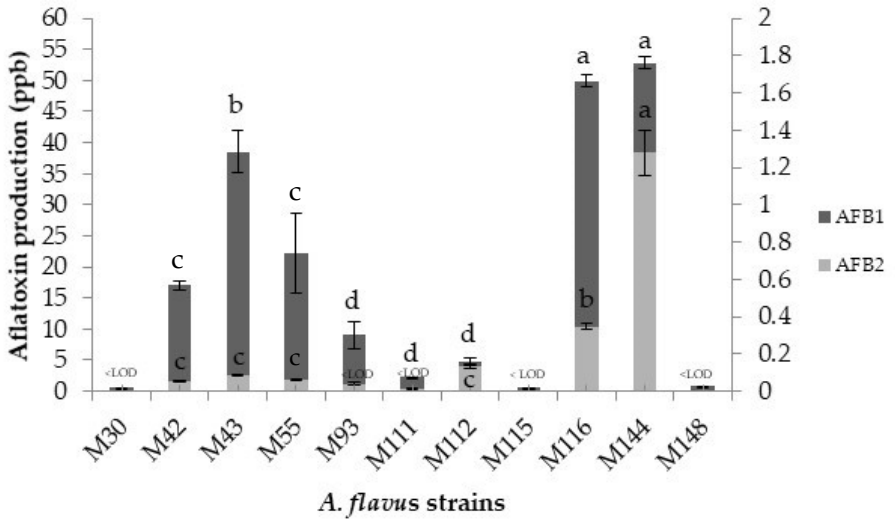

Figure 3. Aflatoxin production (ppb) of the 11 *Aspergillus flavus* strains over the 7 day incubation period. Different letters indicate significant differences for the same aflatoxins ($p \leq 0.05$). *LOD means Limit of Detection.

Based on the results obtained, the strains *A. flavus* M30 and *A. flavus* M144 were selected to carry out a more detailed study to study the lag time, growth, aflatoxin contamination, and related gene expression of *A. flavus* in relation to ecophysiological parameters linked to dried-fig production. These two strains were selected based on their lowest and highest aflatoxin production of the 11 strains isolated from dried figs.

2.2. Effect of Temperature on Lag Times, Growth Rates, Mycotoxin Production and Aflatoxin-Related Gene Expression

2.2.1. Lag Times Prior to Growth

Figure 4 shows the effect of temperature related to the dried-fig processing on lag times prior to growth for both strains of *A. flavus* (M30 and M144). For both strains, no growth occurred at 5 °C. At the warmer temperatures tested (37 and 30 °C), *A. flavus* M30 had shorter lag phases than *A. flavus* M144, while at 25 °C, the latter showed the shortest lag time. At the lowest temperature evaluated, no differences were found between both strains. In addition, for both strains, the length of the lag phase rose substantially as temperature decreased.

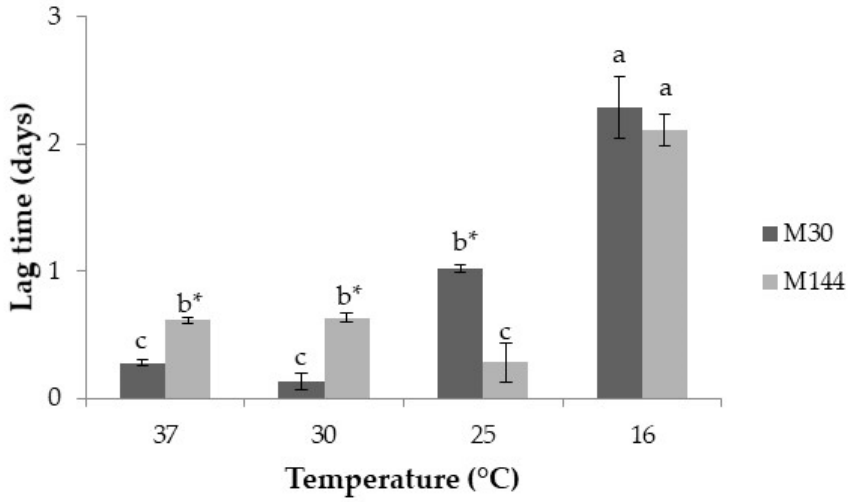

Figure 4. Lag time prior to growth (days) of the *Aspergillus flavus* M144 and *A. flavus* M30 at the different temperatures studied over the 12 day incubation period. Different letters indicate significant differences at the different temperatures for the same strain ($p \leq 0.05$). Asterisk (*) means significant differences between both strains at the same temperature ($p \leq 0.05$).

2.2.2. Growth

The influence of temperature on the growth of both strains of *A. flavus* is shown in Figure 5. *A. flavus* M144 grew faster than *A. flavus* M30 in most of the conditions tested ($p \leq 0.05$), although no significant differences were found at 16 °C ($p > 0.05$). Optimum growth rates (≈11 and 8 mm/day for *A. flavus* M144, and *A. flavus* M30, respectively) were observed at 30 and 37 °C in both strains. Besides, no intra-strain differences were encountered at 30 and 37 °C. Furthermore, the growth of both strains declined as the temperature fell down.

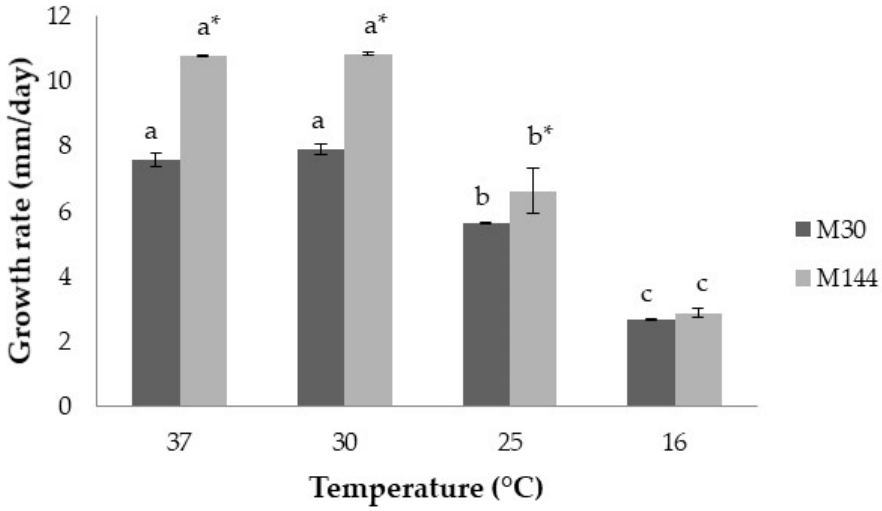

Figure 5. Growth rate (mm/day) of the *Aspergillus flavus* M144 and *A. flavus* M30 at the different temperatures studied over the 12 day incubation period. Different letters indicate significant differences at the different temperatures for the same strain ($p \leq 0.05$). Asterisk (*) means significant differences between both strains at the same temperature ($p \leq 0.05$).

2.2.3. Aflatoxin Production

Table 1 shows the effect of temperature on AFB_1 and AFB_2 production by *A. flavus* M144 and *A. flavus* M30 after 3, 5, 7, and 12 days of incubation. Neither AFB_1 nor AFB_2 was produced by the strain *A. flavus* M30 at the conditions and times evaluated (<LOD: Limit of Detection). Regarding the strain *A. flavus* M144, it produced much higher quantities of AFB_1 than AFB_2 in all the conditions tested. However, it should be emphasized that, in spite of the fact that there were differences regarding both mycotoxins produced by the strain *A. flavus* M144, the tendency was quite similar. Thus, maximum AFB_1 and AFB_2 production were detected at 25 °C at the four days tested; being detected in general higher amounts as the incubation period increased. At the remaining temperatures studied, AFB_1 and AFB_2 production was observed at 16 °C by day 12 of incubation.

Table 1. Aflatoxin B_1 and B_2 production (ppb) of *Aspergillus flavus* M144. [1]

Aflatoxin	Days of incubation	37 °C	30 °C	25 °C	16 °C
B_1	12	<LOD [2]	0.25 ± 0.35^{a3}	60.63 ± 7.70^{a1}	10.15 ± 1.56^{a2}
	7	<LOD	0.03 ± 0.01^{c3}	58.39 ± 1.93^{a1}	0.10 ± 0.07^{b2}
	5	<LOD	0.12 ± 0.04^{b3}	2.68 ± 0.51^{b1}	0.03 ± 0.01^{b2}
	3	<LOD	0.02 ± 0.01^{c2}	1.26 ± 0.83^{b1}	<LOD
B_2	12	<LOD	<LOD	0.02 ± 0.01^{b}	<LOD
	7	0.10 ± 0.01^{a2}	0.06 ± 0.00 [2]	0.15 ± 0.06^{a1}	0.13 ± 0.01^{1}
	5	0.02 ± 0.01^{b}	<LOD	<LOD	<LOD
	3	<LOD	<LOD	<LOD	<LOD

[1] The strain M30 did not produce detectable amounts of aflatoxin B_1 and B_2. [2]LOD: Limit of detection. Different letters along a column indicate significant differences at the different incubation times for the same temperature and for each aflatoxin (B_1 and B_2) ($p \leq 0.05$). Different numbers along a row indicate significant differences at the different temperatures for the same incubation time and the same aflatoxin ($p \leq 0.05$).

2.2.4. Gene Expression Studies

The effect of incubation days on *aflR* gene expression of *A. flavus* M144 at different temperatures is shown in Figure 6. The incubation temperature of 25 °C was used as a calibrator in this study. As shown in Figure 6, in the case of the expression of *aflR* gene is inhibited in most cases at temperatures of 16, 30, and 37 °C and all incubation times evaluated, with the exception of day 7 at 16 °C. In the case of the strain M30, no changes in the expression of the tested regulatory gene at the different temperatures evaluated regarding the control occurred (data not shown).

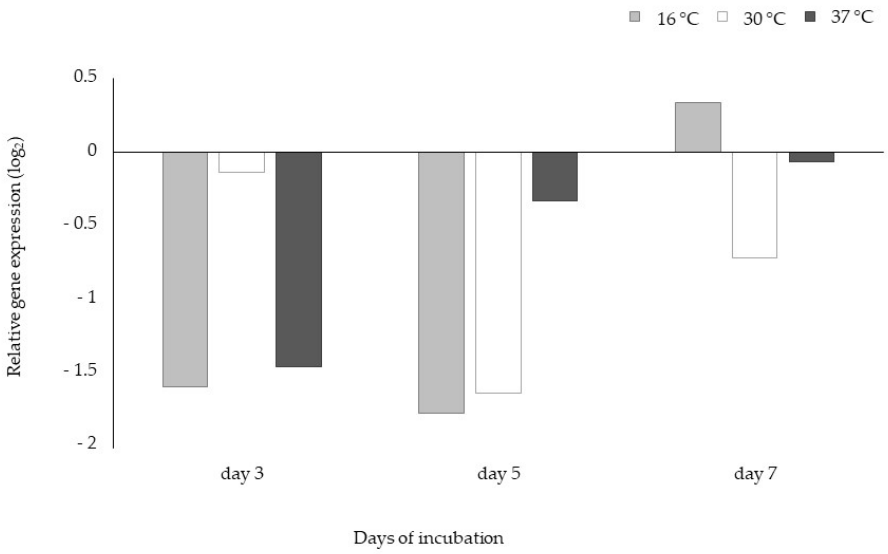

Figure 6. Effect of the temperature on the expression of the *aflR* by *Aspergillus flavus* M144 at different incubation times. The calibrator corresponded to *A. flavus* when grown at 25 °C.

3. Discussion

This is the first study to examine the impact of temperature on growth, *aflR* gene expression, and aflatoxin production by *A. flavus* in a dry fig-based matrix. This species has been encountered in dried figs and can cause accumulation of aflatoxins in this commodity [7,9,11]. Özlüoymak [27] has reported that the critical period for *A. flavus* for starting to grow is when the ripening of the figs is occurring on the tree and it continues during the over-ripening period. Besides, environmental conditions occurring during processing of dried fig and storage when dried figs are launched into the market where temperatures are rarely controlled also favored growth and development of *A. flavus*. Once this species colonizes fig, it may synthesise aflatoxins, both on the surface and the inner part of the fig without damaging the skin [28]. It is thus important to understand the ecological conditions for growth, aflatoxin-related gene expression and aflatoxin production by this species in this matrix. This can be useful for targeting control strategies to minimize mycotoxin contamination within the HACCP framework in the dried fig industry.

At first, the growth behavior and mycotoxin synthesis ability of 11 *A. flavus* strains isolated from figs at a fixed temperature (25 °C) in a DFB agar were screened in order to further select 2 strains based on the initial results obtained for in-depth ecophysiological studies. The initial experiment results showed that there were relatively few interspecies significant differences on lag phase and growth, whilst this was not true for aflatoxin production. Regarding the two parameters related to mould growth, the lag phases ranged between 0.11 and 1 days, while mean growth rates varied from 5.15 to 6.49 mm/day. These values indicate that *A. flavus* starts to grow immediately on a DFB medium and the nutritional composition of this medium based on fig favors the rapid growth of this toxigenic species. This is supported by the comparison of the results of the present study with previous reports informing about the lag phases and growth of *A. flavus* in different food-based model systems. For instance, Peromingo et al. [29] demonstrated that two strains of *A. flavus* had little differences on both lag phase prior to growth and growth when growing on two dry-cured meat product-based medium at 25 °C. Casquete et al. [30] observed little differences between three strains of *A. flavus* at different a_w in a cheese model system. With regard to aflatoxin synthesised by the 11 *A. flavus* strains, there were higher significant differences at strain level, varying aflatoxin B_1

amounts produced between 0.6 and 50 ppb, while for aflatoxin B_2 they were in the range from <LOD to 1.28 ppb. Previous studies have also shown differences in aflatoxin synthesis by various *A. flavus* strains at 25 °C in different media [29,31]. Besides, in general, they produced much higher quantities of aflatoxin B_1 than aflatoxin B_2 in DFB agar. In this study, two *A. flavus* strains were selected based on their mycotoxin production capacity, being the strains M144 (aflatoxin-producing strain) and M30 (non-aflatoxin-producing strain) used for examining the impact of temperature on growth, aflatoxin-related gene expression and mycotoxin production by *A. flavus* in DFB agar.

Temperature represents a key environmental factor in the growth and production of aflatoxins [32,33]. For this reason, five different temperatures, which were selected due to their importance during the drying, processing, and retailing of fig fruits, were assessed. For this: 5 °C represents the advisable household and industrial storage temperature; 16, 25 and 30 °C are common minima, average and maximum temperatures during harvest stage at night, respectively, and 37 °C represents extreme temperatures that can occur in the field during the harvest of the fruits (Extremadura a southwest Spanish region in the high summer season; http://redarexplus.gobex.es/RedarexPlus). In addition, 16 and 25 °C are usual intermediate ambient temperatures utilized by both consumers and producers to store dried figs. Also, 25 °C is the usual temperature in the dried fig postharvest. Finally, 37 °C also represents the optimum condition for *A. flavus* growth [32].

When studying the influence of temperature on growth parameters of the two selected *A. flavus* strains, overall, both strains were unable to grow at 5 °C over the 12 day incubation period of our experiments. These results are consistent with several investigations that suggest that growth at a temperature below 10 °C does not occur [29,34]. Regarding the other temperatures, despite some differences found between the two strains, in general, the lag phases were shorter and mean growth rates faster as temperature increased ($p \leq 0.05$). These results are in accordance with those published by Mohale et al. [35], who investigated the growth of toxigenic and atoxigenic *A. flavus* strains at 20, 25 and 30 °C, and also with those published by Schmidt-Heydt et al. [32], who showed that the growth optimum for *A. flavus* was at 37 °C. Pitt and Miscamble [36] reported that the optimum temperature for *A. flavus* growth was 25 °C in the range from 0.96 to 0.98 a_w, 30 °C at 0.985 a_w and 37 °C at 0.96 a_w. Other previous studies on *A. flavus* growth on groundnuts suggest a_w optima of 0.94 a_w at 34 °C [37]. Abdel-Hadi et al. [16] found that optimum growth of *A. flavus* was 0.99 a_w and 35 °C on conducive YES medium. Surprisingly, the strain M144 (aflatoxin-producing strain) initiated its growth slightly later than the other strain tested (M30, non-aflatoxin-producing strain), but its mean growth rate was more rapid at temperatures warmer than 25 °C. Probably, in the case of the strain M144, the synthesis of aflatoxins itself would have been of great help for its adaptation and colonisation of the DFB agar. This phenomenon has been described before [38,39].

Findings from aflatoxins produced by the two strains of interest showed enormous differences at strain and species levels. The aflatoxin produced by both *A. flavus* at 5 °C was not tested since growth was not observed. The strain M30 did not produce aflatoxins either in temperature or incubation day evaluated. The strain M144 produced both aflatoxin B_1 and aflatoxin B_2, but the quantities produced of the most carcinogenic were much higher ($p \leq 0.05$). As expected, the largest aflatoxin B_1 and quantities detected were at 25 °C ($p \leq 0.05$); however, also important amounts of such toxin would have been contemplated at 30 °C according to the results reported by Schmidt-Heydt et al. [32], who evaluated the effect of a wide range of a_w and temperatures on *A. flavus*, although this was not observed in this work. At the warmest temperature checked (37 °C), no aflatoxin production was observed, while at 16 °C, at the end of the incubation time the strain synthesised aflatoxin B_1 amounts > 10 ppb. These results correlate with those published with Schmidt-Heydt et al. [32]. In the same manner, aflatoxin B_2 was more produced by this strain at 25 °C and later at 16 °C. So, it seems that the temperature enormously affects aflatoxin production by *A. flavus* independently of the substrate where the mould grows.

In general, it should be emphasised that the amounts of aflatoxin found in the DFB agar are higher than those found in other culture media, food-based model systems, or food matrices [29–31]. The explanation may be that the preferred carbon sources for aflatoxin production are sugars [40], and dried figs provide a rich source of glucose and fructose [7]. Furthermore, the temperature of 25 °C and a 0.96 a_w are optimal for the growth *of A. flavus* [41].

Regarding the assessment of the expression of the *aflR* gene of the strain M144, the major regulatory gene in the aflatoxin pathway, which activates the aflatoxin structural genes [42], it was observed that, in general, this gene expression was repressed throughout the incubation time and at any of the temperatures evaluated with respect to the calibrator (25 °C). This is in accordance with results obtained in the phenotypic mycotoxin production, where maximum amounts were found at 25 °C. These findings are reasonable since the *aflR* gene controls are well-correlated with aflatoxin production by *A. flavus* [32,43,44]. Unsurprisingly, a basal expression of the regulatory gene occurred with no differences between conditions checked in the case of the non-producing strain (*A. flavus* M30).

4. Conclusions

The effect of temperature during drying and storage of dried figs has a profound effect on lag times prior to growth, relative growth rates, *aflR* gene expression and aflatoxin production by strains of *A. flavus* isolated of such fruit. In general, the capacity of colonisation of the dried fig-based model system was similar to all the strains tested; however, their ability to produce aflatoxins varied between strains. Concretely, there are some important differences between the two selected *A. flavus* (M144, important producing-strain and M30, non-producing strain). Based on the results, mild temperatures and changes in temperature during drying and storage of dried figs should be avoided. Drying should be conducted at temperatures > 30 °C and close to 37 °C, while industry processing, storage, and retailing of dried figs are advisable to perform at refrigeration temperatures (<10 °C) to avoid mycotoxin production.

5. Material and Methods

5.1. Mould Strains

Eleven strains belonging to *A. flavus* previously isolated from dried figs (*Ficus carica* L.) from different geographical areas of Extremadura (a southwest region of Spain) were used in this study. Information about the isolate codes, origin, geographical area, and moisture content of the strains is shown in Table 2. Isolation of the strains was made following the protocol described by Ruiz-Moyano et al. [45]. For this, genomic DNA from the 11 moulds isolated was extracted with the quick-DNA Fungal/Bacterial Miniprep Kit (Zymo research) according to the manufacturer's instructions. The ITS rDNA region was amplified using the primer pairs ITS1 and ITS4 described by White et al. [46]. PCR products were sequenced at the Facility of Bioscience Applied Techniques of SAIUEX (University of Extremadura, Spain) with the same primers used in the amplification steps. Sequencing was performed from both the 5′ and the 3′ ends of each PCR product. The obtained sequences were edited and assembled into a consensus sequence of the corresponding amplicon. To determine the closest known relatives of the obtained ITS rDNA sequences of the isolates, searches were performed against the NCBI nucleotide (nr/nt) database with the Basic Local Alignment Search Tool (BLAST) tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences were separately analysed and > 95% similarity was used as the criterion for species identification. The isolates were maintained by regular subculturing in Potato Dextrose Agar (PDA) at 25 °C for 7 days and then kept at 4 °C for short-term storage until required.

Table 2. Codes, geographical area, moisture content, and origin of the 11 strains of *Aspergillus flavus* used in the present study.

Isolate Code	Geographical Area	Origin [1]	Moisture Content (%)
A. flavus M30	South of Extremadura	Field	16.78
A. flavus M42	South of Extremadura	Field	16.78
A. flavus M43	South of Extremadura	Field	16.39
A. flavus M55	South of Extremadura	Field	16.78
A. flavus M93	South of Extremadura	Field	20.46
A. flavus M111	South of Extremadura	Field	20.46
A. flavus M112	South of Extremadura	Field	19.01
A. flavus M115	South of Extremadura	Industry	27.62
A. flavus M116	South of Extremadura	Industry	27.62
A. flavus M144	North of Extremadura	Field	36.20
A. flavus M148	South of Extremadura	Field	16.39

[1] Field or industry.

5.2. Culture Medium Preparation

DFB agar was prepared with 30 g of lyophilised dried fig which were added to 300 mL of deionised sterile water and blended with a hand mixer. The remaining deionised sterile water was added to complete 1 L and it was brought to a boil. Subsequently, 20 g of bacteriological agar (Pronadisa, Madrid, Spain) were added and mixed vigorously. The culture medium was sterilised by autoclaving at 121 °C for 20 min (103 KPa). After autoclaving, the DFB agar was shaken, and poured into 9 cm diameter Petri plates. The a_w of the DFB agar was measured by using a Novasina LabMaster-a_w meter (AG, Lachen, Switzerland).

5.3. Inoculum, Inoculation, and Experimental Settings

For inoculum preparation, the isolates were inoculated by spreading on PDA and incubated at 25 °C for 7 days. The spores of each mould isolate were collected using 10 mL deionised water containing 0.05% Tween 80 and rubbing the surface with a glass rod. The spore suspensions were quantified with the aid of a microscope (Olympus CX 400, Tokyo, Japan) and a Neubauer chamber before their adjustment to 10^6 spores/mL by diluting with deionised water to be used as inoculum. The spore suspensions were maintained for long-term storage at −80 °C in glycerol solution (50% *v*/*v*). New starter cultures were used for each experiment.

Firstly, an initial screening of the mould isolates were done. For this, DFB agar was centrally inoculated with 2 µL of the inoculum of each of the 11 mould isolates and incubated at 25 °C for a period of up to 7 days. The growth assessment and aflatoxin production were tested. The two isolates which obtained the highest (*A. flavus* M144) and the lowest (*A. flavus* M30) aflatoxin production were selected to carry out detailed studies on the relationship between ecophysiological factors, growth, gene expression, and aflatoxin contamination.

Secondly, the *A. flavus* M144 and M30, selected from the initial screening experiment, were 2-point inoculated on DFB agar with 2 µL of each inoculum for growth and aflatoxin production. For gene expression studies, sterile cellophane overlays (Packaging Limited, UK) were placed onto DFB agar before inoculation. The agar plates were incubated at 5, 16, 25, 30, and 37 °C for up to 12 days to simulate the wide range of conditions throughout the sun-drying process, industrial processing, storage, and retailing of dried figs. The a_w of the medium kept constant during the experiment period. All experiments were done with three replicates per treatment and repeated once.

5.4. Lag Time Prior to Growth and Growth Assessment

Growth was daily recorded by measuring two right angles diameters. Data were analysed using a primary model by plotting colony diameter against time. Data plots showed, after a lag phase, a linear trend with time. The linear part of this graph (linear phase) was used to calculate growth rate (μ, mm/d) [47]. To calculate the lag times (days), the formula of the regression line was equalised to the original inoculum size (diameter, mm).

5.5. Gene Expression Analysis

5.5.1. Sampling and Sample Preparation

For gene expression analysis, samples from strains M144 and M30 were taken at 3, 5, and 7 days of incubation. All experiments were made in triplicate.

After each incubation time, the cellophane disks containing the whole colonies were collected under sterile conditions and quickly frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

5.5.2. RNA Extraction

For RNA extraction, frozen mycelia were ground to fine powder in a pre-frozen mortar and pestle. Next, approximately 50 mg of frozen mycelia were weighed in a sterile Eppendorf, and the RNA extraction was carried out using the Spectrum™ Plant Total RNA Kit (SigmaAldrich, St. Louis, MO, USA). The RNA concentration and purity (A_{260}/A_{280} ratio) were determined spectrophotometrically using a 1.5 μL aliquot on a NanoDrop (Thermo Scientific™ *NanoDrop* 2000). Samples were diluted to a concentration of 0.1 μg/μL and treated with DNAse I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in order to remove genomic DNA. Then RNA was kept at −80 °C until reverse transcription (RT) reaction.

5.5.3. RT-qPCR Reactions and Relative Quantification

RT-qPCR assays were used to amplify the *aflR* gene as target gene, and the *β-tubulin* gene as endogenous gene.

1. Primers

The primer pair aflRtaq1/aflRtaq2 previously designed from the *aflR* gene associated with the aflatoxin biosynthesis pathway [43], and the primer pair F-TubJD/ R-TubJD designed from the *β-tubulin* gene [43] were used.

2. cDNA synthesis

The RT reaction was conducted by using 5 μL of total RNA (100 ng) according to the instructions of PrimeScript™ RT Reagent Kit (Takara Bio Inc., Kusatsu, Shiga, Japan). cDNA samples were stored at −20 °C for subsequent qPCR analysis.

3. Real-time PCR reactions

The real-time PCR (qPCR) reactions were performed in the 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using the SYBR Green system. Reaction mixtures were dispensed into wells of MicroAmp Optical 96-Well Reaction Plates and sealed with optical adhesive covers (Applied Biosystems). Three replicates of a RNA control sample together with a template-free negative control were also included in the runs. The reaction mixture for each gene consisted of 7.5 μL NZY qPCR Green Master Mix 2x (NZYTech, Lisbon, Portugal), 300 nM of each primer and 2.5 μL of cDNA in a final volume of 12.5 μL. PCR reaction conditions included a first step of 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After the final PCR cycle, the melting curve of the PCR products was analysed according to the following protocol: slow ramp between 60 and 95 °C in 0.5 °C increments for 5 s. The value of the quantification cycle (Cq), which corresponds to the intersection between each fluorescence curve and a

threshold line was automatically calculated by the 7300 Fast System Software (Applied Biosystems). Three technical repetitions were made.

4. Relative gene expression

Relative quantification of the expression of the *aflR* gene expression was calculated following the $2^{-\Delta\Delta C_T}$ method [48]. The *β-tubulin* gene was used as the endogenous control to normalise the quantification of the cDNA target added to each reaction. The calibrator corresponded to *A. flavus* when grown at 25 °C, a usual temperature in the dried fig postharvest, storage, and harvesting.

5.6. Mycotoxin Analysis

5.6.1. Sampling and Sample Preparation

After 3, 5, 7, and 12 days of incubation, the agar plates containing the whole colonies were immediately stored at −20 °C until use. Aflatoxin content could not be determined at 5 °C since no growth of *A. flavus* occurred.

5.6.2. Aflatoxin Extraction and Quantification

All solvents used for aflatoxin were HPLC grade and purchased from Thermo Fisher Scientific (Runcorn, UK). The isolation and purification of aflatoxins was conducted following the method described by Rodríguez et al. [49]. Then, the dry extracts were redissolved in 1 mL of HPLC-grace acetonitrile (Fisher Scientific) and filtered through a 0.22 PTFE membrane filter, in vials for quantification. The aflatoxin analysis was performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a FLD detector (Agilent G1321A) fitted at 360 nm and using a C18 HPLC column (250 × 4.6 mm, 5 μm particle size; Supelco, Bellefonte, PA). The injection volumen was 100 μL and the flow rate was 1 mL/min. The mobile phase used for the separation contained HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B), in a gradient mode established from 15% B in the initial phase to 100% B after 30 min. Standard curves for calibration purpose were performed using standards of aflatoxin B_1 and B_2 acquired from Sigma-Aldrich.

5.7. Statistical Análisis

Data on lag phase, growth rates, *aflR* gene expression and toxin production were tested for normality using the Shapiro–Wilk test. A statistical analysis of the parameters was performed using one-way ANOVA. The differences among means values were separated by Tukey's honestly significant difference test ($p \leq 0.05$) in SPSS for Windows version 21.0.

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CAPÍTULO 4

PUBLICACIÓN 6

Control of toxigenic *Aspergillus* spp. in dried figs by volatile organic compounds (VOCs) from antagonistic yeasts

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Control of toxigenic *Aspergillus* spp. in dried figs by volatile organic compounds (VOCs) from antagonistic yeasts

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ABSTRACT

Aspergillus flavus and *Aspergillus niger* are fungi which can contaminate dried figs before and after harvest and consequently produce aflatoxins (AFs) and ochratoxin A (OTA). Many approaches have been applied to minimise the growth of these filamentous fungi, mainly involving the use of synthetic fungicides which are limited due to their negative impact on human health and the environment. In this context, biocontrol is a recent approach that needs to be explored. This study evaluated the potential of three volatile organic compounds (VOCs), octanoic acid (OA), 2-phenylethyl acetate (2PEA) and furfuryl acetate (FA), produced by *Hanseniaspora uvarum* and *Hanseniaspora opuntiae* yeasts on the growth, germination, gene expression and production of AFs and OTA by *A. flavus* M144 and *A. niger* M185 on dried fig-based agar and the incidence rates in dried figs. Two of the three VOCs evaluated (2PEA and FA) effectively controlled *A. flavus* M144 and *A. niger* M185 by using at least amounts of 50 μL (715 μL/L in the headspace) for FA and 100 μL (1430 μL/L in the headspace) for 2PEA in dried figs. One of the mode of actions of both compounds consists in early repressing the expression of genes involved in the biosynthesis of AFs (*aflR*) and OTA (*pks*) of *A. flavus* and *A. niger*, respectively. The results of this study support the application of 2PEA and FA at the early post-harvest stages of dried figs to control mycotoxin accumulation.

1. Introduction

Dried fig is the product obtained by dehydrating the fruits of the fig tree (*Ficus carica* L.). According to FAOStat (2020) (http://www.fao.org/faostat/en/#home), the main exporter countries in 2020 were Turkey, with more than 85.000 t, followed by Afghanistan and Germany. The production of dried figs is carried out in a traditional way; handling and harvesting of the crop are prone to fungal contamination as dehydration is a lengthy process and starts with fruit ripening and senescence in the tree and continues on the ground. Once the figs are on the ground, they are manually harvested and placed on wooden trays or cement floors. The dehydration process of figs ends with their drying under the sun for 3–5 days (Javanmard, 2010; Villalobos et al., 2019) until they reach a moisture content ≤26%, according to Standard DDP-14 (UNECE, 2016). After drying, the figs can be stored for several months until commercialisation.

The production process, the long periods of drying and storing, as well as the physicochemical characteristics of dried figs favour filamentous fungi contamination and mycelia development (Flaishman et al., 2008). The mould population on dried figs varies, with counts ranging from 1.46 to 6.74 $\log_{10}$ CFU/g (Javanmard, 2010; Öztekin et al., 2006; Villalobos et al., 2016, 2019). This mould population is mainly composed of different species of the genera *Cladosporium*, *Acremonium*, *Mucor*, *Fusarium*, *Penicillium* and *Aspergillus* (Galván et al., 2021; Heperkan et al., 2012a; Javanmard, 2010; Villalobos et al., 2016). The presence of mycotoxigenic species in dried figs is common, resulting in contamination with different mycotoxins. The most important and frequent mycotoxins encountered in dried figs are aflatoxins (AFs) and ochratoxin A (OTA) (Bakirci, 2020; Bircan, 2009; Senyuva et al., 2005), although other important mycotoxins have also been reported. For example, Wang et al. (2018) detected toxins of *Alternaria* (alternariol and alternariol monomethyl ether), ochratoxin B, HT-2 toxin and

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beauvericin (BEA), besides B and G-group AFs (AFB_1, AFB_2, AFG_1 and AFG_2) in dried figs from different areas of China. In samples from Tunisia and Spain (most of them imported from Turkey), the most prevalent mycotoxins were enniatins and BEA, belonging to the *Fusarium* toxin group (Azaiez et al., 2015). Besides, OTA, AFG_1, HT-2 toxin and diacetoxyscirprenol were identified in such samples (Azaiez et al., 2015). Karbancıoglu-Güler and Heperkan (2009) determined that more than 71% of dried figs samples from the Aegean region (Turkey) presented detectable amounts of fumonisin B_1. The contamination of dried figs with fumonisins, OTA and cyclopiazonic acid, in co-occurrence with AFs, has also been reported from this region (Heperkan et al., 2012a; Heperkan et al., 2012b).

From the point of view of toxicology and toxin regulation, the most concerning mycotoxins in dried figs are AFs and OTA. The European Commission (EC) has set a maximum tolerable limit of 6 μg/kg of AFB_1 and 10 μg/kg for the sum of AFB_1, AFB_2, AFG_1 and AFG_2 in dried fruits (EC, 2012). Although to date, the EC has not established a maximum limit of OTA for marketed dried figs, the European Food Safety Authority indicates that figs are important contributors to OTA dietary exposure (EFSA, 2020). The great public health impact of AFs and OTA contamination in dried figs has been supported by 138 notifications in the Rapid Alert System for Food and Feed (RASFF) portal (https://webgate.ec.europa.eu/rasff-window/screen/list) since January 2020 to January 2022; most of the figs were from Turkey. For this reason, it is important to control and minimise the presence of AF- and OTA- producing species in figs.

So far, there are numerous methods to control pathogenic mould growth and mycotoxin contamination in dried figs and other dried products, with biocontrol and biological detoxification being an emerging and prominent strategies. Efficient biodegradation of AFB_1 has been reported in non-toxigenic strains of *Aspergillus niger* (Qiu et al., 2021). Also, lactic-acid bacteria, specifically *Lactobacillus kefiri*, reduced *A. flavus* and *A. carbonarius* growth and AFB_1, AFB_2 and OTA contamination in inoculated almonds and peanuts (Taheur et al., 2019). Among the microorganisms that can act as biopreservatives, yeasts are the most suitable ones to counteract risks provoked by the growth of filamentous fungi (Sharma et al., 2009). Ruiz-Moyano et al. (2016) reported the antagonistic capacity of two yeast strains, *H. opuntiae* L479 and *M. pulcherrima* L672, isolated from fig and breva crops to control the development of common postharvest pathogens.

Among the mechanism of action of antagonistic microbes (Spadaro and Droby, 2016), the application of antagonistic microbial producers of antifungal volatile organic compounds (VOCs) or the compounds themselves is one of the less explored ways to mitigate toxigenic moulds, although the results, so far, are promising. For instance, *Candida nivariensis* reduced *A. flavus* growth (64.9%) and AF production (78.4%) in corn grains associated with the production of VOCs, with the main one being 1-pentanol (Jaibangyang et al., 2020). In another study, non-toxigenic *A. flavus* strains produced antifungal VOCs such as 3-octanone and trans-2-methyl-2-butenal, which resulted in a significant reduction in AF production (Moore et al., 2021). Tejero et al. (2021) identified several VOCs produced by *Hanseniaspora uvarum* and *Hanseniaspora opuntiae* which, *in vitro*, diminished *A. flavus* growth and AF production.

In this context, the aim of this work was to evaluate the effects of three antifungal VOCs produced by the antagonistic yeasts *H. uvarum* and *H. opuntiae* in controlling the development of *Aspergillus* spp. in dried figs to prevent AF and OTA accumulation in dried figs.

2. Materials and methods

2.1. Moulds strains and inoculum

Two common fungal pathogens of dried figs, belonging to the genus *Aspergillus*, were selected for this study: *A. flavus* M144 (previously characterised by Galván et al. (2021) and *A. niger* M185. These moulds were isolated from dried figs (*Ficus carica* L.) harvested from fields in Extremadura (Spain), following to protocol described by Ruiz-Moyano et al. (2009). Mould identification was carried out as described by Galván et al. (2021).

The fungal inocula were prepared by growing both mould strains on potato dextrose agar (PDA, Scharlab, Barcelona Spain) plates at pH 3.5 with a sterilised solution of tartaric acid at 10% (w/v) and incubated for 7 days at 25 °C. Subsequently, 10 mL of sterile deionised water was added to each plate, and the conidia were freed by rubbing the surface with a glass rod. The conidia concentration was adjusted to 10^5 conidia/mL using a microscope (Olympus CX 400, Tokyo, Japan) and a Neubauer chamber.

2.2. Dried fig-based medium preparation

The dried fig samples used in this study are of the Calabacita variety and were obtained from the Scientific and Technological Research Centre of Extremadura, Finca La Orden-Valdesequera (CICYTEX), Guadajira, Spain. A dried fig-base (DFB) medium was essentially prepared following the protocol described by Galván et al. (2021), but the percentage of lyophilised dried figs in the media was 10%.

2.3. Volatile organic compounds

Three antifungal VOCs were used in this work, namely octanoic acid (OA), 2-phenethyl acetate (2PEA) and furfuryl acetate (FA). All compounds were purchased from Sigma-Aldrich (San Luis, EEUU) and were previously identified as produced from antagonistic yeast isolates and potentially associated with antimould properties (Ruiz-Moyano et al., 2020). To evaluate the antagonistic activities of the three VOCs against two moulds, *A. flavus* M144 and *A. niger* M185, *in-vitro* assays with DFB plates and *in-vivo* assays with dried figs were carried out.

2.4. In-vitro antifungal activity of volatile organic compounds

2.4.1. "Conidial" and conidial suspensions

To determine the involvement of the VOCs in the inhibition of conidium germination, 100 μL of conidium suspensions were seeded on a PDA plate. After that, VOCs were applied as described in Section 2.4, and the plates were incubated at 25 °C for 12 h. Subsequently, a glass slide was placed on top of the agar and observed under 40× magnification with sub-stage illumination (DMLS, Leica, Buccinasco, MI, Italy), using a Leica DM 2000 LED microscope. For each plate, the percentage of spore germination was estimated from the observation of 100 spores. When the germ tube was at least as long as the width of the spore, it was considered germinated (Gougouli and Koutsoumanis, 2013). The lengths of 50 germinative tubes per plate were measured and compared with those of the control plates. Three plates were performed for treatment, and the assay was repeated twice.

2.4.2. Effects of VOCs on lag phase, growth rate, relative gene expression and aflatoxin production

2.4.2.1. Effects of VOCs on lag phase and growth rate. The *in-vitro* evaluation of VOCs effects on lag phase and growth rate was carried out by using DFB plates. To perform the assay, a 1 × 1-cm square of sterilised filter paper was placed on one side of the plate, on which different quantities of 10, 25, 50 and 100 μL (which are 143, 357, 715 and 1430 μL/L of headspace) of the different three VOCs were added. In the centre of the plate, 5 μL of the spore solution of each mould was inoculated. Three control samples were made with each mould without adding VOCs. Subsequently, the lid of the petri dish was discarded, and the bottom of another empty petri dish was placed on the top of the previous one, and both parts were fixed with parafilm. To avoid an increase in the CO_2 concentration to detrimental levels (Contarino et al., 2019), four holes were drilled in the sides just between the two plates with an

incandescent seeding loop. The plates were then incubated at room temperature (≈ 25 °C) for 8 days. The doses of each compound in each of the moulds were studied in triplicate, and the assay was repeated twice.

Radial growth was daily recorded by measuring two right-angled diameters with a scaled rule. Growth curves were obtained by plotting the mycelial diameter (mm) against the incubation period (days). The lag phase (λ; days) was determined from linear regression by equating the formula of the regression line with the original inoculum size (diameter, mm), and the growth rate (μ; mm/day) was determined from the slope of the growth curve (Rodríguez et al., 2014).

2.4.2.2. Quantification of the relative gene expression. The two moulds *A. flavus* M144 and *A. niger* M185 were sampled on days 5, 7 and 8 of the incubation periods for gene expression analysis. After each incubation time, mycelium was scraped from the surface, collected under sterile conditions, flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

- RNA extraction

The RNA was extracted from frozen mycelium using the Spectrum™ Plant Total RNA kit (SigmaAldrich), following the manufacturer's instructions. The RNA concentration (ng/μL) and purity (A260/A280 ratio) were determined spectrophotometrically in a NanoDrop (Thermo Scientific™ NanoDrop 2000). Samples were diluted to a concentration of 0.1 μg/μL and treated with DNAse I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove genomic DNA traces. The obtained RNA was kept at −80 °C until the reverse transcription (RT) reaction.

- Two-step reverse-transcription real-time PCR

First, the RT reaction was conducted using 5 μL of total RNA (≅ 100 ng) with the PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan), as described by the manufacturer. Complementary DNA (cDNA) samples were stored at −20 °C until analysis. The real-time PCR (qPCR) reactions were performed in the 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA), using the SYBR® Green technology. Amplification of the *aflR*, *pks* and *β-tubulin* genes was carried out following the methodology described by Rodríguez et al. (2012) and the methodology described in this study. Briefly, the reaction mixture for the amplification of each gene consisted of 6.25 μL of NZY qPCR Green Master Mix 2x (NZYTech, Lisbon, Portugal), 2.5 μL of cDNA template (2 ng/μL) and different concentrations of each primer. For the *aflR* gene, the final concentration of the primer pair AflRTaq1 and AflRTaq2 was 300 nM each, for the primers pks-F1/pks-R1, which amplify the *pks* gene, the concentration as 300 nM each, whereas for the primers F-TUBjd/R-TUBjd, which amplify the *β-tubulin* gene, the concentration was 400 nM each. The thermal cycling conditions for amplification of the three genes included one initial denaturation step at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After the final PCR cycle, melting curve analyses of the PCR products were conducted, and the values of the melting temperatures were checked to ensure the reliability of the results and the specificity of the primers. The quantification cycle (Cq), the cycle in which the fluorescence reaches a defined threshold, was automatically obtained by the instrument, using the default parameters of the 7300 Fast System Software (Applied Biosystems).

- Calculation of the relative gene expression

The relative quantification of the expression of the *aflR* and *pks* genes was calculated using the $2^{-\Delta\Delta CT}$ method described elsewhere (Livak and Schmittgen, 2001). The *β-tubulin* gene was used as an endogenous control to normalise the amount of target cDNA added to each reaction. The calibrator used for the calculations was the non-treated control sample (*A. flavus* or *A. niger*) at each sampling time.

2.4.2.3. Aflatoxin and ochratoxin A analysis. The AF content produced by *A. flavus* M144 and the OTA content produced by *A. niger* M185 were determined on days 5, 7 and 8 of incubation. Agar plates were immediately stored at −20 °C until extraction.

- Standards and solvents

All solvents used were of HPLC grade and purchased from Thermo Fisher Scientific. The AF calibrations were carried out with a standard mixture of AFB_1, AFB_2, AFG_1 and AFG_2, purchased from Sigma-Aldrich, and OTA calibrations were performed with a standard from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

- Mycotoxin extraction

Both AFs and OTA were extracted according to the method described by Ruiz-Moyano et al. (2009) with modifications. The contents of an agar plate with the whole colonies were transferred to a plastic filter bag and homogenised with 80 mL of chloroform. The protocol was followed stepwise, except that filtering was performed at the end, once the residue was resuspended in 1 mL methanol and 1 mL water, through a 0.22-μM pore size filter into HPLC vials for quantification.

- Mycotoxin quantification

The quantification of AFs and OTA was performed using an Angilet 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with an Agilent 1200 FLD detector (G1321A); excitation and emission wavelengths were 330 and 460 nm, respectively, using a C-18 column (15 cm × 4.6 mm, 5 μm particle size; Supelco, Bellefonte, PA). The injection volume was 100 μL. To quantify AFs, a post-column derivatisation with pyridinium bromide at 0.05% (*w/v*; Sigma), pumped at 0.3 mL/min, was performed. Separation of AFs was achieved with a mobile phase containing a mixture of MeOH: ACN: water (20:20:60 *v/v/v*), which was delivered at an isocratic flow rate of 1 mL/min. The OTA quantification was performed with the mobile phase composed of water: ACN: acetic acid (41:57:2) at an isocratic flow rate of 1 mL/min.

2.5. Antifungal activity of volatile organic compounds on dried figs

Based on the results of the *in-vitro* assay, a further experiment was carried out on dried figs with the three VOCs. The dried figs were immersed in a spore suspension of 10^5 spores/mL for 2 min, and each compound was studied in triplicate with each of the moulds. Each replicate consisted of 20 dried figs, which were placed in 3-L plastic boxes with the respective VOC in glass culture dishes, with 4.3 mL of each VOC to reproduce the same concentrations as used in Section 2.4. Three control samples were run with each mould without adding VOCs. Plastic boxes were incubated at 25 °C for 30 days.

A daily visual assessment was carried out, where the percentage of dried figs with the presence or absence of mould contamination in each plastic box was quantified.

2.6. Statistical analysis

The growth parameters, spore germination, AF and OTA production and relative gene expression in the *in-vitro* assay, as well as the percentage damage in the *in-vivo* assay, were analysed using one-way ANOVA. Differences between mean values were separated by Tukey's honestly significant difference test ($p \leq 0.05$). All analyses were performed in SPSS for Windows version 21.0.

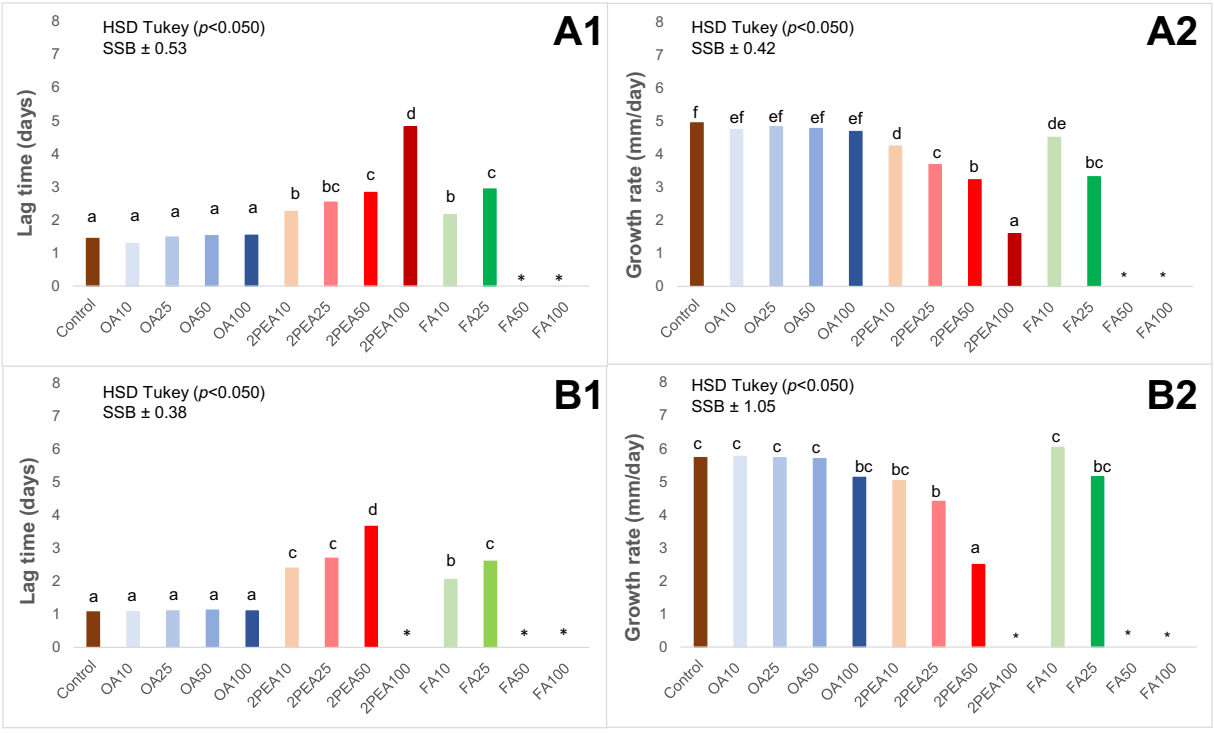

Fig. 1. Growth parameters in dried fig-based agar at 25 °C of (A) *A. flavus* M144 and (B) *A. niger* M185, represented by values of (1) lag phase (days) and (2) growth rate (mm/day). OA: octanoico acid; 2PEA: 2-phenylethyl acetate; FA: furfuryl acetate. The number (10, 25, 50, 100) indicates the amount (μL) of compounds applied in the plates. "*" indicates complete inhibition of mycelial growth. SSB: Statistical Significance Bar.

3. Results and discussion

3.1. Effects of VOCs on lag phase and growth rate

In recent years, several studies have reported the production of VOCs by antagonistic microorganisms which control the development of *Aspergillus sp.* Bacterial species such as *Streptomyces alboflavus*, *Streptomyces yanglinensis* and *Bacillus licheniformis* presented high efficacy for the growth inhibition of *Aspergillus ochraceus*, *A. westerdijkiae*, *A. carbonarius*, *A. niger* and *A. flavus*. The antagonistic activities have been related to compounds of different chemical families, such as dimethyl trisulfide, benzenamine, methyl 2-methylbutyrate, 2-phenylethanol, β-caryophyllene and 3-methyl-1-butanol (Lyu et al., 2020; Ul Hassan et al., 2019; Yang et al., 2018). Some species of yeasts produce VOCs with similar properties, such as *Candida nivariensis*, which produces 1-pentanol (Jaibangyang et al., 2020), or *Candida intermedia* and *Lachancea thermotolerans*, which produce 2-phenylethanol (Farbo et al., 2018). In recent works, *H. uvarum* and *H. opuntiae*, which produce 2PEA, FA and OA, were effective in the control of *Botrytis cinerea* and *A. flavus* (Ruiz-Moyano et al., 2020; Tejero et al., 2021). The effects of these three VOCs on the *in-vitro* growth of *A. flavus* and *A. niger* on DFB agar are shown in Figs. 1 and 2. The lag phase prior to the growth of *A. flavus* M144 in the control plates was 1.44 ± 0.07 days. The application of OA, in any of the different amounts, did not modify this parameter ($p > 0.050$). The application on 2PEA and FA extended the lag phase in a dose-dependent manner ($p < 0.001$). The lowest amounts of VOCs presented values of 2.26 ± 0.24 and 2.16 ± 0.08 days for 2PEA and FA, respectively, whereas 100 μL of 2PEA resulted in 4.82 ± 0.26 days of lag phase, and 25 μL of FA delayed the start of the mycelium growth to 2.93 ± 0.14 days (Fig. 1.A1). Higher amounts of FA (50 and 100 μL) did not allow mycelial growth (Fig. 2). Similar results were obtained in the case of *A. niger* M185. The lag phase in control plates was 1.08 ± 0.06 days, achieving values of 3.66 ± 0.22 days after treatment with 50 μL of 2PEA and 2.61 ± 0.15 days with 25 μL of FA (Fig. 1.B1).

The graphs showed, after a lag phase, a linear trend with time. The growth rate of *A. flavus* M144 in the control plates was 4.95 ± 0.10 mm/day and was not affected by treatment with OA ($p > 0.050$). However, treatment with amounts ≥25 μL of 2PEA and FA reduced or even impeded mycelial development ($p \leq 0.001$). In the case of *A. niger*, its growth rate was 5.73 ± 0.03 mm/day in control plates. The growth rate was reduced to 5.13 ± 0.08 mm/day with 100 μL of OA and was completely inhibited with 100 μL of 2PEA and 50 μL of FA. The overall lack of antifungal activity of OA was unexpected because of the previous reports about its control capacity for hyphal growth and cell aggregation in *Candida albicans* (Lee et al., 2021) and the disorder in cell membranes of several filamentous fungi (Pohl et al., 2011). The compound 2PEA is a highly valued flavouring compound used in the fragrance, cosmetic and food industries. However, its antifungal properties have been less exploited than those of its precursor phenylethyl alcohol, which is used as a disinfectant, pest control agent, cleaning agent and in personal care products (Martínez-Avila et al., 2018). Finally, some furan derivates with microbial origin have been associated with antifungal activities, such as 2,3-dihydrofuran produced by atoxigenic strains of *A. flavus* (Moore et al., 2021) and furan-tetrahydro-2,5-dimethyl produced by *Bacillus subtillis* and effective against *Botrytis cinerea* (Mu et al., 2017).

Treatment with 2PEA and FA produced changes at the macroscopic level (Fig. 2) on *A. flavus*. The mycelia presented a white colour, and sporulation was reduced or inhibited, mainly in FA applications. Similar effects have been observed when *A. carbonarius* and *A. ochaceus* were exposed to VOCs produced by *Lachancea thermotolerans*, *Candida intermedia*, *Candida friedrichii* and *Cyberlindnera jadinii* (Farbo et al., 2018).

3.2. Effects of VOCs on conidium germination

The influences of the three compounds on spore germination are shown in Table 1. Control treatments of *A. flavus* M144 presented 74.08 ± 8.11% of germinated spores after 12 h of incubation, with a medium germ tube size of 22.45 ± 2.74 μm. A non-significant ($p > 0.050$)

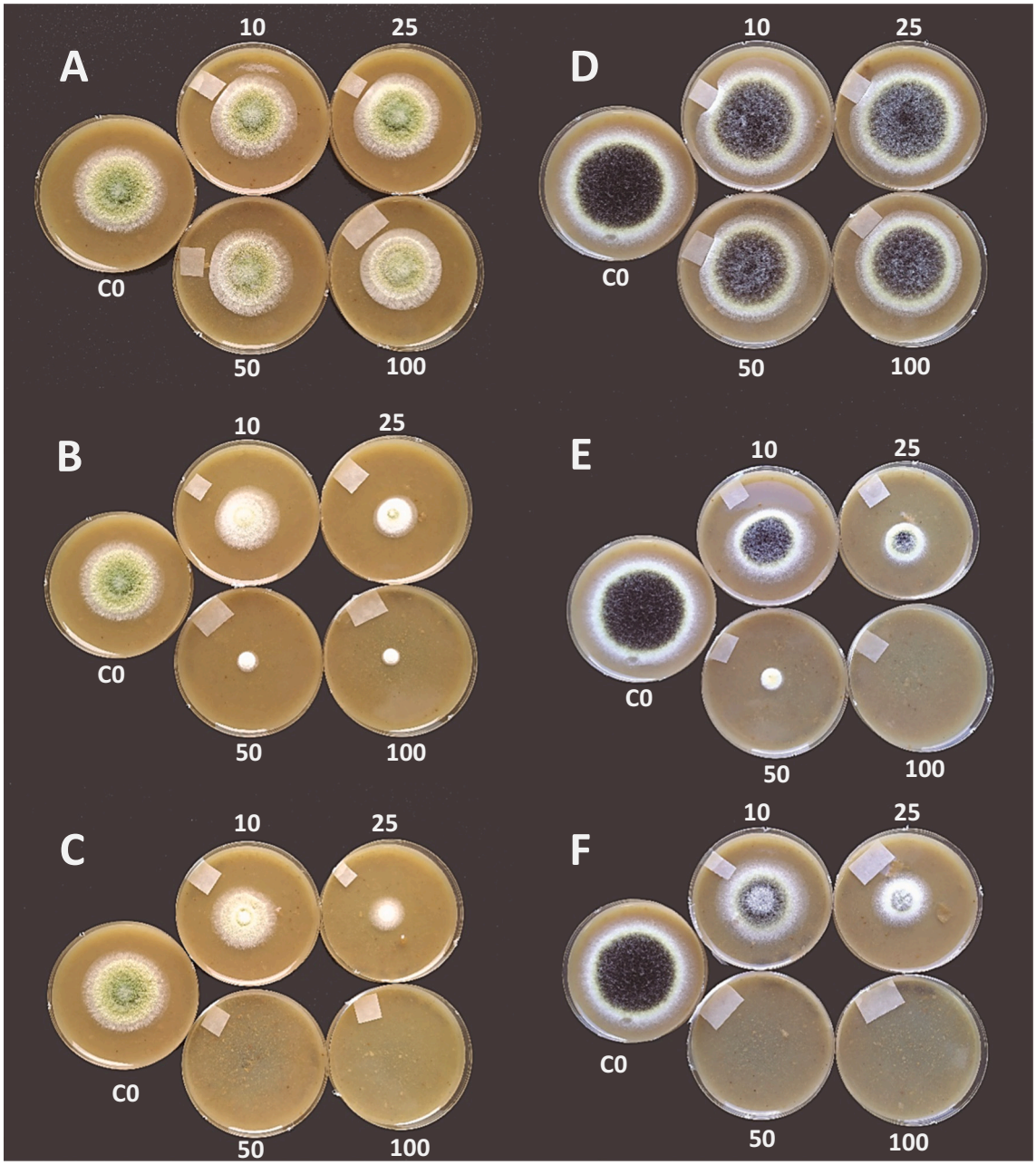

Fig. 2. Growth on dried fig-based agar at 25 °C *of A. flavus* M144 (A, B, C) and *A. niger* M185 (D, E, F) against the three volatile organic compounds, octanoic acid (A and D), 2-phenylethyl acetate (B and E) and furfuryl acetate (C and F) on day 5 of the study at the four treatments studied (10, 25, 50 and 100) and the control (C0).

decrease in the percentage of germination and the size of the germ tube was observed when OA was applied. Germination diminished ($p \leq 0.050$) to values of 49.10 ± 3.41% and 25.63 ± 3.26% with 10 and 100 μL of 2PEA, respectively. However, the size of the germ tube was not significantly modified when 2PEA was added, with mean values ranging from 20.23 to 18.36 μM. The FA inhibited the germination from 33.46 ± 7.30% at 10 μL to complete the inhibition at 100 μL. The size of the germ tube was approximately half (11.34 ± 0.38 μM) of that of the control at 10 μL of FA.

Regarding *A. niger* M185, it presented 85.23 ± 4.30% of germination, with a size of germ tubes of 17.41 ± 1.11 μm. The addition of OA reduced mean values of % germination (71.15 ± 2.57% at 100 μL) and size of the germ tube (13.86 ± 2.57 μM at 100 μL), but no statistical differences were found ($p > 0.050$). The addition of 2PEA allowed a significant reduction of the percentage of germination with treatments ≥50 μL, but germ tube size was not impacted. Finally, FA diminished the germination at all treatments and completely inhibited the germination of *A. niger* M185 at ≥50 μL.

The inhibition of conidium germination is an important mechanism of controlling toxigenic fungi because it impedes colonisation and toxin production in the agri-food products. In this sense, VOCs produced by *Streptomyces alboflavus* TD-1, mainly dimethyl trisulfide and benzenamine, strongly inhibited the spore germination of *A. ochraceus* (Yang et al., 2018). In the abovementioned study, dimethyl trisulfide and benzanamide completely inhibited mycelial growth at 10 and 1000 μL/L, respectively. In our case, the concentrations for the complete inhibition of spore germination with FA were 715 μL/L for *A. niger* and 1430 μL/L for *A. flavus*. In a previous study, phenylethyl alcohol and 1-pentanol produced by *Enterobacter asburiae* Vt-7 presented the minimal inhibitory concentrations of spore germination for *A. flavus* of 100 and

Table 1
Effects of treatment with octanoic acid (OA), 2-phenethyl acetate (2PEA) and furfuryl acetate (FA) at different doses of application (μL) and control (C0) on spore germination of *A. flavus* M144 and *A. niger* M185.

Strain	Compound	Treatment (μL)	Size of germ tube (μm)	% Germination
A. flavus M144	C0	0	22.45[a]* ± 2.73	74.08[a] ± 8.11
	OA	10	19.11[a] ± 1.34	74.62[a] ± 2.81
	OA	25	19.22[a] ± 0.63	69.86[a] ± 2.94
	OA	50	17.90[a] ± 2.03	68.12[a] ± 2.35
	OA	100	18.53[a] ± 1.34	61.61[a] ± 4.85
	2PEA	10	20.23[a] ± 1.32	49.10[b] ± 3.41
	2PEA	25	19.51[a] ± 0.47	34.65[b] ± 0.77
	2PEA	50	18.83[a] ± 1.95	33.43[b] ± 7.45
	2PEA	100	18.36[a] ± 3.23	25.63[c] ± 3.25
	FA	10	11.34[b] ± 0.36	33.46[b] ± 7.30
	FA	25	11.49[b] ± 0.18	18.81[c] ± 1.91
	FA	50	7.02[b] ± 1.04	9.19[c] ± 1.06
	FA	100	0.00[c] ± 0.00	0.00[d] ± 0.00
A. niger M185	C0	0	17.41[a] ± 1.11	85.23[a] ± 4.30
	OA	10	17.11[a] ± 0.51	85.63[a] ± 5.72
	OA	25	16.91[a] ± 1.29	78.46[a] ± 2.14
	OA	50	14.12[a] ± 0.84	74.92[a] ± 0.53
	OA	100	13.86[a] ± 2.57	71.15[a] ± 5.53
	2PEA	10	14.25[a] ± 2.12	84.39[a] ± 1.16
	2PEA	25	13.31[a] ± 1.91	77.63[a] ± 3.45
	2PEA	50	12.11[ab] ± 2.06	55.12[b] ± 10.11
	2PEA	100	15.19[a] ± 2.03	27.94[c] ± 4.39
	FA	10	10.61[b] ± 1.37	66.20[b] ± 7.40
	FA	25	8.64[b] ± 0.3	57.29[b] ± 9.06
	FA	50	0.00[c] ± 0.00	0.00[d] ± 0.00
	FA	100	0.00[c] ± 0.00	0.00[d] ± 0.00

* In a column, mean values with different letters indicates statistical differences ($p \leq 0.050$) for each mould specie.

200 μL/L, respectively (Gong et al., 2019).

3.3. Effects of VOCs on gene expression

The effects of the three VOCs, at different amounts, on the relative expression of the *aflR* gene by *A. flavus* at 5, 7 and 8 days of incubation are shown in Fig. 3A. The expression of the regulatory *aflR* gene is one of the main factors in the AF pathway (Georgianna and Payne, 2009), and its expression has been associated with AF production under different environmental and nutritional conditions (Peromingo et al., 2017; Tejero et al., 2021). The relative expression of the target gene was evaluated and compared with that of the control batch (*A. flavus* inoculated on DFB agar plates in the absence of compounds) at each incubation time. The impact of the FA at 50 and 100 μL on the AF-related gene expression could not be evaluated since no *A. flavus* growth was observed. First, changes in the relative *aflR* gene expression could be observed during the incubation time; these results coincided with previous studies that demonstrated that the expression of this regulatory gene may fluctuate over time, depending on experimental conditions (Lozano-Ojalvo et al., 2013; Peromingo et al., 2017; Tejero et al., 2021). On day 5, overexpression of the AF regulator gene was provoked by the three VOCs at all the amounts tested, except in the case of OA at 10 μL. The impact on gene expression was more pronounced in FA and 2PEA than in OA. Regarding OA, amounts ranging from 25 to 100 μL had a similar influence on the expression of the *aflR* gene (≈1 $\log_2$). With respect to 2PEA, the lowest and the highest amounts of this compound increased the expression of the target gene more significantly (≈ 4 $\log_2$) than the intermediate quantities (≈ 1–2 $\log_2$). Finally, in relation to FA, the two quantities evaluated, 10 and 25 μL, stimulated the expression of the *aflR* gene at levels of 3 and 5 $\log_2$, respectively. After 7 days of incubation, an inhibition of the AF regulator gene expression was only observed when OA was applied at 25 μL, and there was no effect either at 50 or 100 μL. The remaining compounds and concentrations stimulated the expression of the mycotoxin-biosynthetic gene at levels of about 1

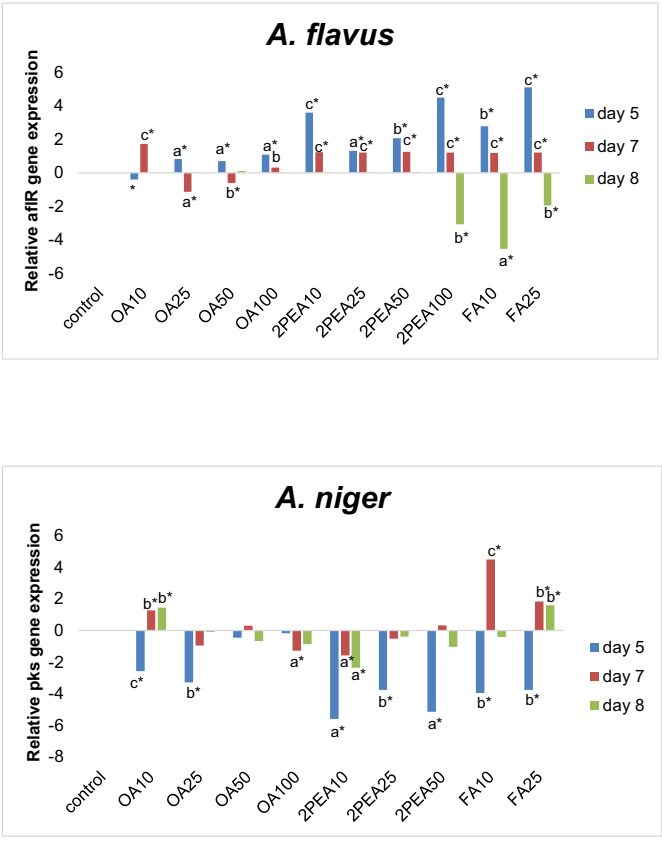

Fig. 3. Effects of volatile organic compounds on the relative expression of the *aflR* gene in *A. flavus* (A) and the *pks* gene in *A. niger* (B) on days 5, 7 and 8. Calibrators (non-treated control samples at each incubation time) always take the value of 0. OA: octanoic acid; 2PEA: 2-phenylethyl acetate; FA: furfuryl acetate. The number next to the compounds (10, 25, 50, 100) indicates the amount (μL) of compounds applied on the plates. [a,b,c] Different letters above the bars indicate significant differences ($p \leq 0.050$) within-day among the different compounds and concentrations. (*) Asterisk means significant difference ($p \leq 0.050$) regarding the calibrator.

$\log_2$. On the last day of incubation (day 8), no influence of most combinations of VOCs × amounts on gene expression was found, only at the highest 2PEA amount tested (100 μL) and the two of FA quantities (10 and 25 μL), which noticeably decreased the expression of the target gene at levels of ≈ 2–4 $\log_2$. Overall, it is important to point out that in general, the expression of this mycotoxin biosynthetic gene in the presence of VOCs was more repressed at longer incubation periods. Tejero et al. (2021) also found that two yeasts (*H. opuntiae* L479 and *H. uvarum* L793), producers of VOCs, were able to inhibit the expression of *aflR* gene by *A. flavus* at day 9 of incubation.

The influence of the combinations of the three VOCs × four amounts on the relative expression of the *pks* gene by *A. niger* at 5, 7 and 8 days of incubation is displayed in Fig. 3B. The *pks* gene is an important gene intimately linked to OTA synthesis (Gil-Serna et al., 2018; O'Callaghan et al., 2003). The relative expression of the target gene was evaluated and compared with that of the control batch (*A. niger* inoculated on DFB agar plates in the absence of compounds) at each incubation time. The impacts of 2PEA at 100 μL and FA at 50 and 100 μL on the OTA-related gene expression could not be evaluated since no *A. niger* growth was observed. At the shortest incubation time (day 5), the expression of the *pks* gene dropped when the three VOCs at the different amounts were applied, with the exception of OA at 50 and 100 μL, which had no effect on gene expression. The highest effect on *pks* gene expression was found when either 2PEA or FA was used, which provoked a considerable decline in the expression (≈4–6 $\log_2$). On day 7 of incubation, repression

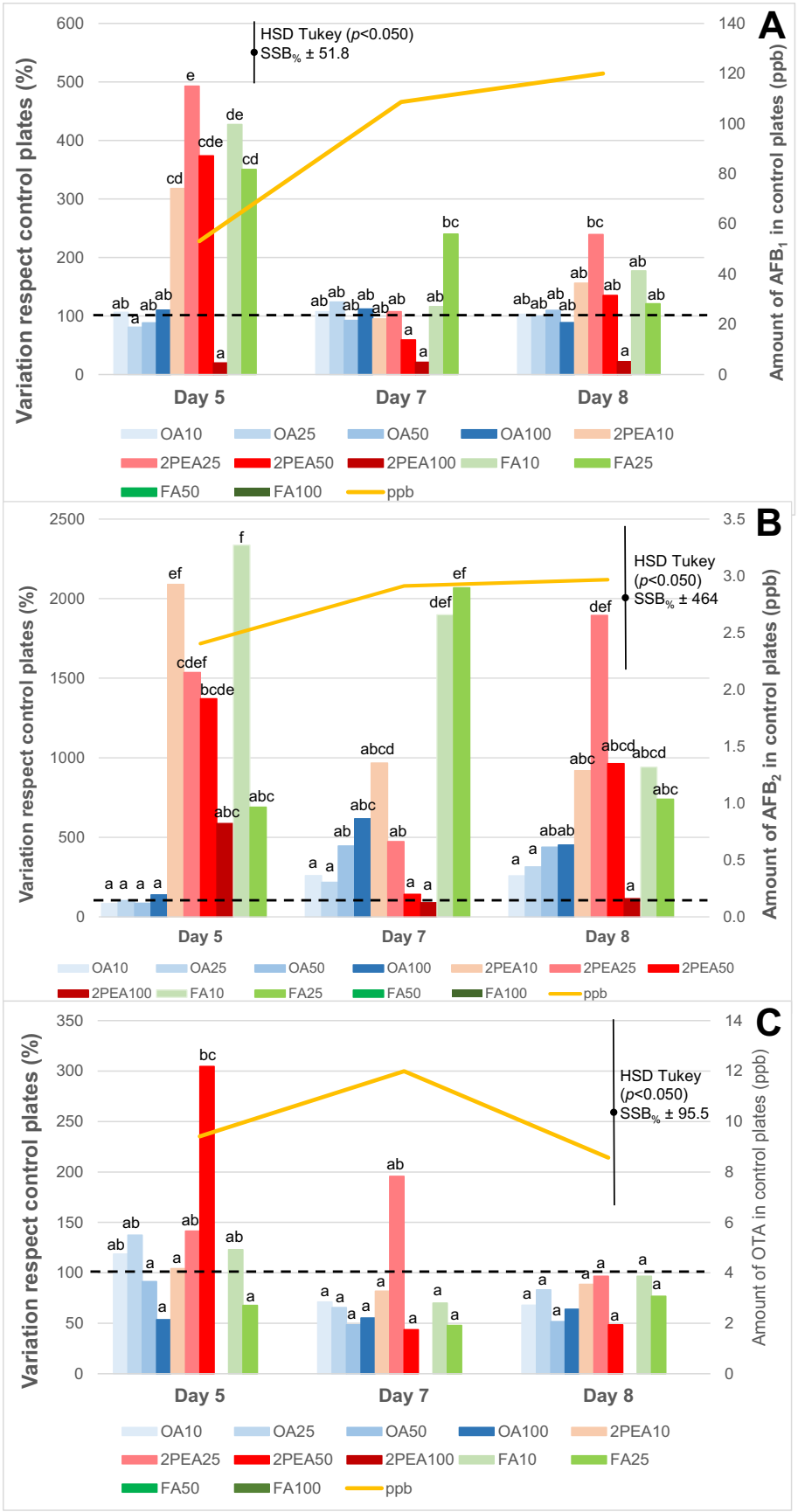

Fig. 4. Variation (%) with respect to control plates of aflatoxin B_1 (A) and aflatoxin B_2 (B) by *A. flavus* M144 and ochratoxin A (C) by *A. niger* M185 on days 5, 7 and 8 of the study of the three volatile organic compounds (OA: octanoic acid; 2PEA: 2-phenylethyl acetate; FA: furfuryl acetate). The number next to the compounds (10, 25, 50, 100) indicates the amount (μL) of compounds applied on the plates. The amounts of aflatoxin B_1, B_2 and ochratoxin A of the control plates (ppb) are shown on the yellow line (A, B, C). ^{a,b,c,d,e,f} Different letters above the bars indicate significant differences ($p < 0.050$) within-day among the different compounds and concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of gene expression was encountered when OA and 2PEA were applied at the highest (100 μL) and lowest (10 μL) amounts, respectively, at levels of about 1 log_2. Activation of the expression of the *pks* gene was observed when spreading out FA at 10 and 25 μL and OA at 10 μL. At the last sampling day, only three combinations VOCs × amount exerted a significant impact on gene expression, with two of them (OA at 10 μL and FA at 25 μL) increasing it and the other one (2PEA at 10 μL) reducing it. Taking into account the results for the ochratoxigenic species in relation to gene expression studies, a contrasting effect regarding aflatoxigenic species behaviour was found. The most pronounced inhibition of the *pks* gene expression was observed at a shorter incubation period (day 5). No previous studies regarding the influences of antifungal compounds on this OTA-biosynthetic gene expression have been conducted so far, rendering any comparisons impossible.

3.4. Effects of VOCs on aflatoxin and ochratoxin A production

The presence of AFs and OTAs during the production and storage of dried figs is a major concern for health and food safety authorities. Some yeast strains can produce VOCs, and in several cases, volatile compounds have been reported as the main factors responsible for the antagonistic capacity (Farbo et al., 2018). During the *in-vitro* assays using DFB agar plates, AFB_1 and AFB_2 produced by *A. flavus* M144 and OTA synthesised by *A. niger* M185 were significantly influenced by the three VOCs, 2PEA, FA and OA, produced by *H. uvarum* and *H. opuntiae* (Fig. 4). The percentages of mycotoxin amount variation with respect to the control DFB agar plates for AFB_1 ranged from 19.74 to 493% (Fig. 4A). The mean AFB_1 concentrations for *A. flavus* M144 (without VOCs exposure) were 53.16 ± 22.69 ppb on day 5, 108.68 ± 14.74 ppb on day 7 and 120.03 ± 30.73 ppb on day 8 (Fig. 4A). The effects of the VOCs varied at different application concentrations. Generally, the OA had no effect on AFB_1 produced by *A. flavus* compared to the control. The highest variation, with respect to the control plates, of AFB_1 was observed with 2PEA at 25 μL on days 5 and 8, with 492.97 and 239.77%, respectively, and with FA at 25 μL on day 7 (239.89%). However, the AFB_1 content was significantly diminished on days 5, 7 and 8 with 2PEA at 100 μL ($p \leq 0.050$); the percentages of variation, with respect to the control, were 19.74, 20.93 and 22.10%, respectively. In the case of AFB_2 production (Fig. 4B), the percentages of variation regarding the DFB control agar ranged from 83.56 to 2336.48%. The mean AFB_2 concentrations for the *A. flavus* M144 control agar plates on days 5, 7 and 8 ranged between 2.40 ± 1.24 and 2.87 ± 2.14 ppb. The highest percentages of variation, with respect to the control, were found on day 5 with FA at 10 μL, with 2335.48%, on day 7 with FA at 25 μL, with 2067.52%, and on day 8 with 2PEA at 25 μL, with 1793.59% ($p \leq 0.050$).The addition of FA at 50 and 100 μL effectively inhibited the production of AFB_1 and AFB_2 by *A. flavus* M144. Chang et al. (2015) demonstrated that the volatile 2-phenylethanol, produced by *Pichia anomala*, can reduce AF production when applied to pistachios. In a similar study of Wang et al. (2019), cinnamaldehyde inhibited aflatoxin production in *A. flavus*. Moore et al. (2021) reported that the volatile compounds decane and 2,3-dihydrofuran reduced the AF production by three aflatoxigenic strains and completely inhibited the production of cyclopiazonic acid by one strain.

Regarding OTA, in terms of variation with respect to the DFB control plates (Fig. 4C), the percentages varied from 43.72% in 2PEA at 50 μL on day 7 to 304.69% in 2PEA at 50 μL on day 5. The average OTA concentrations for *A. niger* M185 control plates were 9.41 ± 2.72 ppb on day 5, 12.00 ± 1.12 ppb on day 7 and 8.57 ± 0.52 ppb on day 8 (Fig. 4C). The greatest variation with respect to the OTA control plates was observed with 2PEA at 50 μL on day 5 and at 25 μL on day 7 ($p \leq 0.050$). The OTA production was effectively inhibited with FA at 50 and 100 μL and 2PEA at 100 μL on all days of the study. In other studies, VOCs produced by different strains of *Pichia anomala*, *Pichia kluyveri* and *Hanseniaspora uvarum* inhibited the growth of *A. ochraceus* and OTA production during the production of *Coffea arabica* (Masoud and Kaltoft, 2006; Masoud et al., 2005). Farbo et al. (2018) demonstrated that 2-phenylethanol, produced for four yeasts, plays an important role in the antagonistic activity against *A. carbonarius* MPVA566 and *A. ochraceus* MPVA703.

Table 2
Effects of treatment with octanoic acid (OA), 2-phenethyl acetate (2PEA) and furfuryl acetate (FA) and control (C0) on the incidence rates of *A. flavus* M144 and *A. niger* M185 on days 5, 6, 7, 8, 9 and 30.

Day	Compound	Incidence rate of *A. flavus* M144	Incidence rate of *A. niger* M185
5	C0	$96.25^{a} \pm 4.14$*	$52.50^{a} \pm 16.77$
5	OA	$70.00^{a} \pm 10.00$	$72.50^{a} \pm 12.50$
5	2PEA	$0.00^{b} \pm 0.00$	$1.67^{b} \pm 2.36$
5	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
6	C0	$94.86^{a} \pm 6.12$	$65.42^{a} \pm 26.90$
6	OA	$97.22^{a} \pm 2.78$	$91.67^{a} \pm 8.33$
6	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
6	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
7	C0	$96.25^{a} \pm 6.50$	$70.83^{a} \pm 26.46$
7	OA	$100^{a} \pm 0.00$	$94.44^{a} \pm 5.56$
7	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
7	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
8	C0	$100^{a} \pm 0.00$	$86.63^{a} \pm 10.52$
8	OA	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
8	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
8	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
9	C0	$100^{a} \pm 0.00$	$94.44^{a} \pm 5.56$
9	OA	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
9	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
9	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
30	C0	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
30	OA	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
30	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
30	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$

* In a column, mean values with different letters indicates statistical differences ($p \leq 0.050$) for each mould specie.

3.5. Antifungal activities of volatile organic compounds on dried figs

Based on the *in-vitro* results, the highest amount of VOCs was selected for the *in-vivo* test (100 μL; 1430 μL/L of headspace). Since lower concentrations of VOCs could stimulate mycotoxin production, according to the results displayed in Fig. 4, the sporestatic treatment is the best choice in the use of VOCs as biofumigants. Table 2 shows the evolution of the percentages of incidence of *A. flavus* M144 and *A. niger* M185 on inoculated dried figs exposed to OA, 2PEA and FA. After 5 days of storage, control treatments achieved 96.25 ± 4.14% of infection for *A. flavus* M144, whereas a complete incidence was observed at day 8. The application of 1430 μL/L of OA produced a non-significant delay of infection at 5 days of storage ($p > 0.050$), with an incidence of 70.00 ± 10.00%. However, differences disappeared on the next day of exposure (day 6). This result could be associated with the effects of OA on spore germination and the size of the germ tube observed in *in-vitro* analyses. In the case of *A. niger* M184, a certain non-significant stimulus ($p > 0.050$) of mould development was observed at the first days of storage. Control treatments produced 52.50 ± 16.77% of infection in 5 days, whereas 72.50 ± 12.50% of infected figs were observed in OA treatments. These differences were observed until day 9, when both treatments achieved 100% incidence. Although the antifungal properties of OA on *A. flavus* and *A. niger* have previously been established (Huang et al., 2010), presenting minimal inhibitory concentrations similar to those of conventional antifungals such as sorbic acid, in our case, the application of OA did not effectively control the development of *Aspergillus sp.* in figs (Fig. 5).

The application of 2PEA and FA could effectively control both strains of *Aspergillus*. The 2PEA completely inhibited infections by *A. flavus* M144 (Table 2) during the 30 days of storage, and no symptoms of mycelium development were observed (Fig. 5). In contrast, 2.08 ±

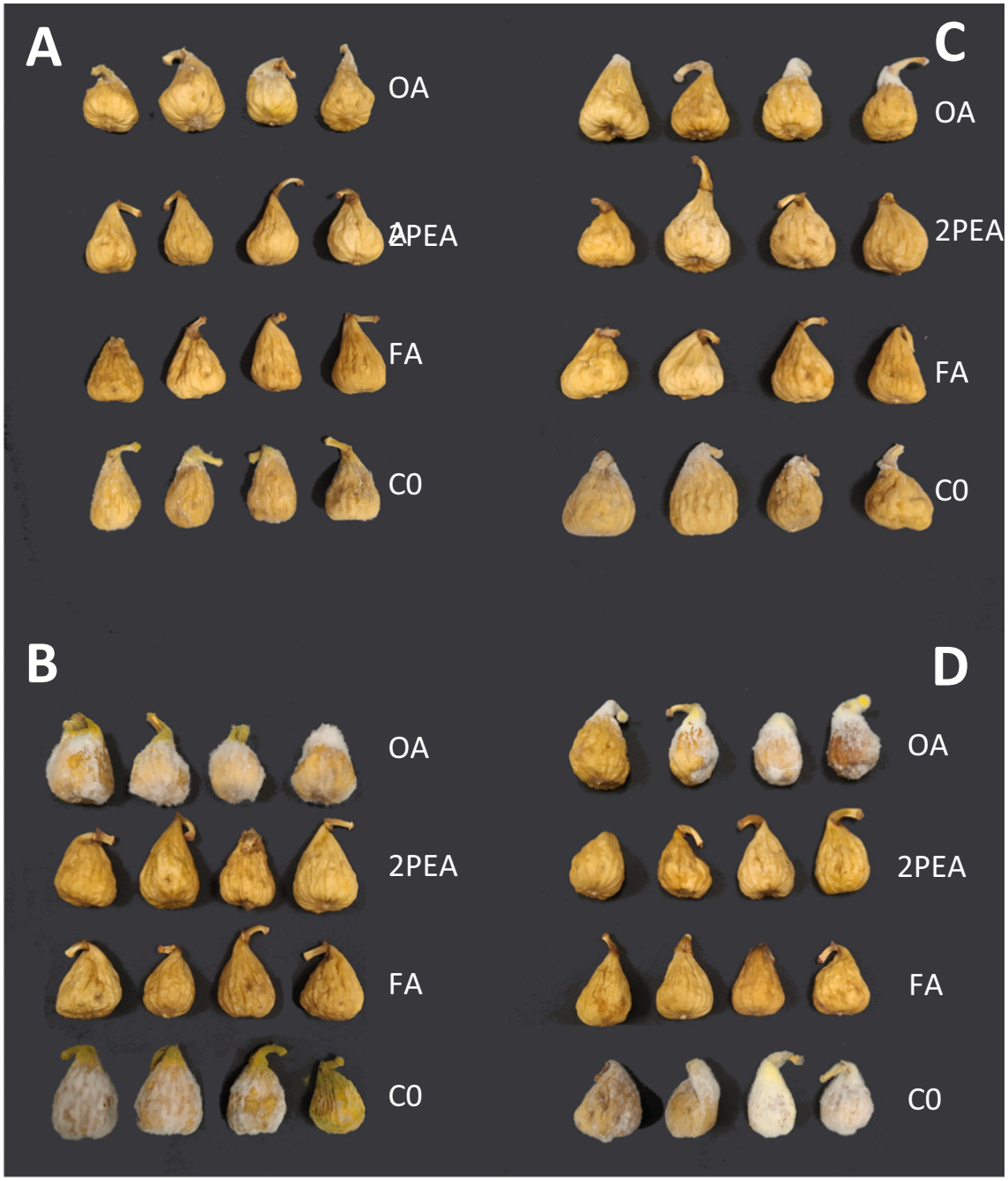

Fig. 5. Growth at 25 °C of *A. flavus* M144 (A and B) and *A. niger* M185 (C and D) on dried figs against the three volatile organic compounds, octanoic acid (OA), 2-phenylethyl acetate (2PEA) and furfuryl acetate (FA), on days 6 (A and C) and 9 (B and D) of the study at the concentration of 1413 μL/L of headspace and the control (C0).

2.95% of the figs presented a visible mycelium when *A. niger* M184 was inoculated. The application of FA avoided mycelium appearance during the 30 days of storage for both *Aspergillus* species (Table 2; Fig. 5).

4. Discussion

The findings of the *in-vitro* (growth, germination, mycotoxin biosynthetic-gene expression and toxin production) and *in-vivo* analyses (incidence rate) carried out in this study proved that two of the three VOCs tested (2PEA and FA) were effective in the control of *A. flavus* and *A. niger* either in a dried fig model system or in dried figs. In addition, the highest effect of VOCs on AFs and OTA reduction was observed on day 8, when the synthesis of VOCs by two yeasts (*H. opuntiae* L479 and *H. uvarum* L793) reaches its maximum (Tejero et al., 2021). In the case of aflatoxigenic species, this coincides with the fact that both compounds repress the expression of the regulator gene at this time, in contrast to the ochratoxigenic species. Probably, the early inhibition of the *pks* gene expression of *A. niger* by the action of VOCs is sufficient to keep the OTA levels low. The results of this study support the application of 2PEA and FA at the early post-harvest stages of dried figs to control mycotoxin accumulation.

Previous studies have pointed out the convenience of using VOCs as biofumigants because of their rapid conversion to gaseous state (Passone and Etcheverry, 2014) and the absence of organoleptic modifications on products (Schotsmans et al., 2008). In this sense, this work has proved the high effectivity of FA and/or 2PEA to control *A. flavus* and *A. niger*

infections in dried figs. Moreover, these compounds presented pleasant fruity and floral odours, respectively; and are considered as safe (GRAS) flavouring agents by the Flavor and Extract Manufacturers' Association (FEMA) and the Food and Drug Administration (FDA). Thus, there are no safety concerns when using biofumigants to control toxigenic moulds during dried fig production and commercialisation.

The selection of the concentrations of these compounds used in biofumigation treatments is key in order to ensure mycotoxin concentrations are kept below legal limits. It has been found that at low concentrations of 2-PEA and FA there is an increase in aflatoxins and OTA synthesis. In this regard, Klich (2007) reported that certain biological treatments using yeasts, and chemicals such as pesticides and volatile compounds have similar effects on mycotoxin production in *Aspergillus* spp. Specifically, among the volatile compounds tested, C6 to C9 alkenals stimulate aflatoxins synthesis at low concentrations (Zeringue, 1991), and similar effect was found when applying 3-methyl-1-butanol (Greene-McDowelle et al., 1999). Therefore, the most relevant findings of the current study related to the use of VOCs, should be focused on their ability to inhibit spore germination, as shown in *in vitro* and *in vivo* tests at the highest concentrations tested. Additionally, these findings also question the suitability of using the VOC-producing yeasts of the *Hanseniaspora* genus in products susceptible to contamination by toxigenic fungal species. De Paiva et al. (2017) and Ruiz-Moyano et al. (2016, 2020) showed the feasibility of using antifungal VOC-producing yeasts on several fruit species. By contrast, the concentrations of VOCs produced by these antagonists may cause the opposite effect after application on dried figs, which means increasing mycotoxin synthesis. Therefore, 2-PEA and FA can be considered as potential treatments for the control of *Aspergillus* spp. growth in dried figs; however, the concentrations applied must ensure spore inhibition to avoid potential risks of mycotoxin formation.

CRediT authorship contribution statement

Ana Isabel Galván: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Alejandro Hernández:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition. **María de Guía Córdoba:** Writing – review & editing, Funding acquisition. **Alberto Martín:** Visualization, Writing – review & editing. **Manuel Joaquín Serradilla:** Writing – review & editing. **Margarita López-Corrales:** Project administration, Funding acquisition. **Alicia Rodríguez:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

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

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CONCLUSIONES/CONCLUSIONS

1. La **variedad Calabacita** mostró un gran potencial productivo en **superintensivo** con riego localizado y sistema de malla suspendida para la producción de higos para consumo en seco, con las ventajas de un **mayor rendimiento, facilidad de recolección** y **menor riesgo de contaminación** de los higos secos, favoreciendo la producción de higos de elevada calidad higiénico-sanitara.

   The Calabacita variety showed great productive potential in superintensive with drip irrigation and suspended mesh system for the production of figs for dry consumption, with the advantages of higher yields, easy harvesting and reducing contamination risk of dried figs, encouraging the production of hygienic-sanitary quality figs.

2. El estudio de los diferentes **estados de maduración** a lo largo del crecimiento del higo en la higuera mostró la presencia de **especies fúngicas toxigéncias** desde las primeras etapas del crecimiento en las dos variedades estudiadas, Calabacita y Cuello de Dama Blanco, aunque no se encontraron micotoxinas en las fases iniciales de maduración de los frutos (de H1 a H8). Esto indica la importancia de la **aplicación de medidas de control eficientes desde las primeras etapas del desarrollo** de los higos.

   The study of the different ripening stages throughout fig fruit growth in fig trees revealed the occurrence of toxigenic fungal species from the early ripening stages in both varieties studied, Calabacita and Cuello de Dama Blanco, although no mycotoxins were found in the first stages of fruit ripening (from H1 to H8). These results indicate the importance of applying efficient control measures from the early stages of fig growth.

3. Las condiciones climáticas en las diferentes **localizaciones geográficas** estudiadas mostraron una influencia significativa sobre los parámetros físico-químicos como la humedad, la actividad de agua, el color, los sólidos solubles totales y el peso de los frutos. Sin embargo, el **manejo del agua** solo afectó significativamente a la firmeza, los sólidos solubles totales y el peso de los frutos.

   Climatic conditions in the different geographic locations studied significantly influenced physicochemical parameters such as moisture content, water activity, colour, total soluble solids and fruit weight. However, water management only significantly influenced firmness, total soluble solids and fruit weight.

4. La diversidad de **especies fúngicas** y la **contaminación por aflatoxinas y ocratoxina A** en los higos secos se vio influida por la localización geográfica. En cambio, la gestión del agua tuvo un impacto limitado en la micobiota, aunque el estrés hídrico en condiciones de secano condujo a un mayor nivel de mohos productor de aflatoxinas. Estos resultados son cruciales para el desarrollo de estrategias agronómicas que permitan minimizar los riesgos asociados a la aparición de mohos micotoxigénicos en los higos secos.

   Fungal species diversity and aflatoxin and ochratoxin A production in dried figs was influenced by geographic location. Conversely, water management had a limited impact on the mycobiota, although water stress under rainfed conditions led to a higher level of aflatoxin-producing moulds. These results are essential for developing of agronomic strategies to minimise the risks associated with mycotoxigenic moulds on dried figs.

5. Las principales **especies fúngicas** encontradas durante el procesado en la industria fueron ***A. tubingensis, A. flavus*** y ***A. welwitschiae*** con una alta incidencia en la producción de aflatoxinas y ocratoxina A en la mayoría de las etapas del procesado independientemente de la industria estudiada. En base a estos resultados, un **secado** y **enfriamiento** eficaz después del escaldado, junto con un **almacenamiento en condiciones de refrigeración y humedad relativa controlada** podrían ser medidas de control adecuadas para disminuir la incidencia de mohos y la producción de micotoxinas en higos secos.

   The major fungal species found during processing in the industry were *A. tubingensis*, *A. flavus* and *A. welwitschiae*, with a high occurrence of aflatoxins and ochratoxin A production in most processing stages irrespective of the industry studied. Based on these results, effective drying and cooling after blanching, combined cooled storage and controlled relative humidity could be suitable control measures to reduce the occurrence of moulds and mycotoxin production in dried figs.

6. El estudio del efecto de la temperatura sobre el crecimiento de ***A. flavus*** y la producción de micotoxinas, mostró una mayor incidencia de la producción de **aflatoxina B_1** y **aflatoxina B_2** a la temperatura de 25ºC. Sin embargo, no se obtuvo crecimiento a 5ºC. Estos resultados muestran la importancia de mantener las **bajas temperaturas** (<10°C), durante el **almacenamiento** y la comercialización de los higos secos para evitar el desarrollo de mohos y la consiguiente producción de micotoxinas.

   The effect of temperature on *A. flavus* growth and mycotoxin production showed a higher occurrence of AFB_1 and AFB_2 production at 25ºC.

Nevertheless, no growth was obtained at 5ºC. These results highlight the importance of maintaining low temperatures (<10°C) during storage and marketability of dried figs to prevent mould growth and subsequent mycotoxin production.

7. Los compuestos orgánicos volátiles estudiados, **acetato de 2-feniletilo** (2PCA) y el **acetato de furfurilo** (FA), mostraron una buena eficacia en el control de *A. flavus* M144 y *A.niger* M185 utilizando concentraciones de al menos 715 µL/L para el FA y 1430 µL/L para el 2PCA. La aplicación de estos compuestos podría ser una adecuada **estrategia de control en la producción de higos secos** sanitariamente seguros.

   The volatile organic compounds studied, 2-phenylethyl acetate (2PCA) and furfuryl acetate (FA), effectively controlled *A. flavus* M144 and *A.niger* M185 using concentrations at least 715 µL/L for FA and 1430 µL/L for 2PCA. Applying these compounds could be a suitable control strategy in safe dried fig production

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ANEXOS

INDICADORES DE CALIDAD CIENTÍFICA DE LA TESIS DOCTORAL

Publicaciones	JCR Impact Factor	JIF Rank	Quartile/ Category
Implementation of super high-density systems and suspended harvesting meshes for dried fig production: Effects on agronomic behaviour and fruit quality. DOI: 10.1016/j.scienta.2021.109918	**4.342**	**4/36**	**Q1 Horticulture**
Evaluation of physicochemical parameters of two fig varieties throughout the different phenological phases of ripening on toxigenic fungi population	**Editing**		
Impact of water management and geographic location on the physicochemical traits and fungal population of 'Calabacita' dried figs in Extremadura (Spain). DOI: 10.1016/j.scientia.2022.111543	**4.342**	**4/36**	**Q1 Horticulture**
Evaluation of fungal hazards associated with dried fig processing. DOI: 10.1016/j.ijfoodmicro.2022.109541	**5.911**	**31/143**	**Q1 Food science & technology**
Effect of Temperature During Drying and Storage of Dried Figs on Growth, Gene Expression and Aflatoxin Production. DOI: 10.3390/toxins13020134	**5.075**	**40/143**	**Q2 Food science & technology**
Control of toxigenic *Aspergillus* spp. in dried figs by volatile organic compounds (VOCs) from antagonistic yeasts. DOI: 10.1016/j.ijfoodmicro.2022.109772	**5.911**	**31/143**	**Q1 Food science & technology**