University of Cordoba / Universidad de Córdoba

Doctoral Program in Agricultural, Food, Forestry, and Sustainable Rural Development Engineering

Programa de Doctorado en Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible

Ph.D. Thesis / Tesis Doctoral

Biocontrol agents to reduce aflatoxins in nuts: inoculum dynamic studies, varietal resistance to the pathogen, and characterization of the population of *Aspergillus* spp. section *Flavi* in Spain

Estudio de la dinámica de inóculo de los agentes de biocontrol de aflatoxinas en frutos secos, resistencia varietal al patógeno y caracterización de la población de *Aspergillus* spp. sección *Flavi* en España

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TITULO: *Biocontrol agents to reduce aflatoxins in nuts: inoculum dynamic studies, varietal resistance to the pathogen, and characterization of the population of Aspergillus spp. section Flavi in Spain*

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INFORME RAZONADO DEL/DE LOS DIRETOR/ES DE LA TESIS
(Se hará mención a la evolución y desarrollo de la Tesis, así como a trabajos y publicaciones derivados de la misma)

TÍTULO DE LA TESIS: Estudio de la dinámica de inóculo de los agentes de biocontrol de aflatoxinas en frutos secos, resistencia varietal al patógeno y caracterización de la población de *Aspergillus* spp. sección *Flavi* en España

DOCTORANDA: María Teresa García López

Don Juan Moral Moral, investigador Ramón y Cajal de la Universidad de Córdoba y Don Themis J. Michailides, Catedrático del Departamento de Patología Vegetal de la Universidad de California-Davis, como directores de la alumna del programa de doctorado "Ingeniería agraria, alimentaria, forestal y del medio rural sostenible" Dña. María Teresa García López informan que:

Durante su periodo como doctoranda del programa, desde noviembre de 2017, hasta la fecha de emitir este informe, ha cumplido con todas las actividades formativas obligatorias del programa, así como otras actividades voluntarias, con diligencia y dedicación. Entre estas actividades destaca su estancia de investigación de carácter internacional, al comienzo del desarrollo de su Tesis Doctoral, en:

"Kearney Agricultural Research and Extension Center" (KARE), concretamente en el Laboratorio de Patología Vegetal Dirigido por el Prof. Themis J. Michailides y perteneciente a la Universidad de California-Davis, EE. UU. El periodo de esta estancia abarcó desde febrero de 2017 hasta septiembre de 2018.

Gracias a la estratégica localización del centro, ubicado en el Valle de San Joaquín en California, una de las zonas agrícolamente más productivas en el mundo, María Teresa tuvo la oportunidad de atender los siguientes seminarios y "workshops" como actividades complementarias de aprendizaje:

- Fig Meeting, Tulare (9th, May 2017)
- Walnut Tour (6-7 June 2017)
- 2018 Statewide Pistachio Day (17 January 2018)

- Pistachio Workshop (19-20 July 2018)

La finalidad de esta estancia de movilidad internacional, con un total de 10 meses de dedicación, estuvo orientada a que la doctoranda se formara en el laboratorio del Dr. Michailides localizado en el KARE. "Michalidides' Lab" es el grupo de referencia internacional en enfermedades fúngicas que afectan a almendro, pistacho y nogal. Además, esta estancia ha permitido a la doctoranda adquirir una formación adicional sobre distintos aspectos agronómicos de los frutos secos en California, líder mundial en la producción.

Principalmente, su Tesis se ha centrado en la revisión del estado actual y las perspectivas futuras del empleo de productos de biocontrol en aflatoxinas (Capítulo 1); el desarrollo de una herramienta molecular, mediante PCR a tiempo real, para la cuantificación de la cepa de biocontrol AF36 en suelo, frutos y hojas de pistacho (Capítulo 2); el estudio de la resistencia de cultivares y selecciones avanzadas de almendro a la colonización por *Aspergillus flavus* y *A. parasiticus* y la contaminación por aflatoxinas, así como la interacción entre la resistencia varietal y el empleo de la estrategia de biocontrol de aflatoxinas (Capítulo 3).

Estos trabajos han dado lugar a las siguientes publicaciones en revistas científicas indexadas:

1. Present status and perspective on the future use of aflatoxin biocontrol products. Moral, J., Garcia-Lopez, M. T., Camiletti, B. X., Jaime, R., Michailides, T. J., Bandyopadhyay, R., and Ortega-Beltran, A. April 2020. Agronomy. DOI: 10.3390/agronomy10040491

2. Quantification of the aflatoxin biocontrol strain *Aspergillus flavus* AF36 in soil, and nuts and leaves of pistachio by real-time PCR
Garcia-Lopez, M. T., Luo, Y., Ortega-Beltran, A., Jaime, R., Moral, J[*]. and Michailides, T. J[*]. May 2020. Plant Disease. DOI: 10.1094/PDIS-05-20-1097-RE.
[*]Juan Moral and Themis J. Michailides cosupervised this article.

3. Resistance to *Aspergillus flavus* and *A. parasiticus* in almond advanced selections and cultivars and its interaction with the aflatoxins biocontrol strategy
Moral, J.[*], Garcia-Lopez, M. T.[*], Gordon, A., Ortega-Beltran, A., Puckett, R. D., Tomari, K., Gradziel, T. M., and Michailides, T. J. September 2021. Plant Disease. DOI: 10.1094/PDIS-05-21-0892-RE.
[*]Juan Moral and M. Teresa Garcia contributed equally to this article.

Además, durante su estancia en California, la doctoranda trabajó en la evaluación de las inflorescencias masculinas de pistacho como portador alternativo al producto comercial de la cepa de biocontrol AF36 (Capítulo 2); y en el estudio epidemiológico de la cepa de biocontrol (Capítulo 2). Por otro lado, durante el transcurso de su estancia doctoral en España, Mª. Teresa y colaboradores han caracterizado la población natural de *Aspergillus* spp. sección *Flavi*, en las principales zonas de cultivo de frutos secos en Andalucía y Castilla La Mancha en la búsqueda de potenciales agentes de biocontrol para su empleo comercial en España (Capítulo 4).

Estos trabajos ha sido recogidos en los siguientes manuscritos/capítulos:

1. Pistachio male inflorescences as an alternative substrate for the application of atoxigenic strains of *Aspergillus flavus*
Garcia-Lopez, M. T., Gordon, A., Puckett, R., Chevailler, C., Doster, M., Michailides, T.J. *, and Moral, J*
*Juan Moral and Themis J. Michailides cosupervised this article.

Enviado a Plant Disease

2. Sporulation and dispersal of the biological control agent *Aspergillus flavus* AF36 under field conditions in nut crops in California
Garcia-Lopez, M. T., Jaime, R., Doster, Meca, E., Puckett, R., Michailides, T. J., and Moral, J.

Se enviará a Phytopathology

3. Characterization of the *Aspergillus* spp. population in Spanish almond and pistachio orchards and identification of atoxigenic strains of *A. flavus*
Garcia-Lopez, M. T., Velez-Simon, Camiletti, B. X., Priego-Capote, F., M., Ledesma-Escobar, C. A., Diez, C. M., Michailides, T. J.* and Moral, J.*
*Juan Moral and Themis J. Michailides cosupervised this article.

Se enviará a Plant Pathology

Otras aportaciones científicas derivadas directamente de la Tesis Doctoral son:

a) Congresos y ponencias invitadas:

- Comunicación en formato Póster, Congreso Sociedad Española de Fitopatología (Toledo, 2018): 'Pistachio male flowers as substrate for the biological control agent AF36: Epidemiological approaches.'

- Comunicación en formato Póster, Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias (Almería, 2018): 'Quantification of an atoxigenic strain of *Aspergillus flavus* (AF36) using real time PCR'.

- Comunicación en formato Póster, VII Congreso Científico de Investigadores en Formación de la UCO (Córdoba, 2019): 'Cuantificación de la cepa no-tóxica de *Aspergillus flavus* (AF36) usando PCR a tiempo real'.

- Comunicación oral, Congreso de la Sociedad Española de Ciencias Hortícolas (Córdoba, 2021): Resistencia de selecciones avanzadas y variedades de almendro a *Aspergillus flavus* y *A. parasiticus*.

b) Artículos de divulgación:

- Publicación en la revista española Phytoma: Contaminación de aflatoxinas en frutos secos, un problema emergente. N302:38-43.

c) Dirección de Trabajos Fin de Grado y Máster:

- Co-dirección del Trabajo Fin de Grado: 'Identificación de especies de *Aspergillus* procedentes de suelos de cultivo de almendro y pistacho en España.' Alumna: María Vélez Simón. Noviembre de 2019.

- Co-dirección del Trabajo Fin de Grado: 'Efecto del estrés hídrico en el crecimiento micelial, germinación de esclerocios y esporulación de *Aspergillus flavus* y *A. parasiticus*'. María Dolores Ruíz Prados. Julio de 2020.

- Co-dirección del Trabajo Fin de Grado: 'Modelización de la contaminación de los frutos del pistachero por aflatoxinas en california: análisis de sensibilidad y verificación'. Juan Manuel Sánchez Godino. Diciembre de 2020.

- Co-dirección del Trabajo Fin de Máster: 'Caracterización molecular y toxigénica de especies de *Aspergillus* procedentes de suelos de cultivo de almendro y pistacho de España'. Cayetano Marín Palma. Noviembre de 2020.

Otras aportaciones científicas durante el periodo que ha ocupado la Tesis Doctoral, aunque no directamente derivadas de la Tesis son:

- Comunicación en formato Póster, Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias (Almería, 2019): 'Estudio de la competencia entre especies de *Colletotrichum* patogénicas en olivo'

- Comunicación oral en el VIII Congreso Científico de Investigadores en Formación de la UCO (Córdoba, 2020): 'Estudio de la competencia entre especies de *Colletotrichum* patogénicas en olivo'

- Publicación en revista indexada: **Effect of Cultivar Resistance and Soil Management on Spatial–Temporal Development of Verticillium Wilt of Olive: A Long-Term Study.** Ostos, E[*]., Garcia-Lopez, M. T[*]., Porras, R., Lopez-Escudero, F. J., Trapero-Casas, A., Michailides, T. J., and Moral, J. October 2020. Frontiers in plant science, 11. JCR Impact Factor: 5.754; JIF Rank: 17/235; First Quartile, (Q1) in Plant Sciences Category.
[*]Eduardo Ostos and M. Teresa Garcia contributed equally to this article.

- Publicación en revista indexada: **First Report of *Colletotrichum karstii* causing fruit Anthracnose of *Carissa grandiflora* in Spain.** Garcia-Lopez, M. T., Gordon, A., Raya, M. C., Diez, C. M., and Moral, J. September 2020. Plant Disease. JCR Impact Factor: 4.438; JIF Rank: 29/235; First Quartile, (Q1) in Plant Sciences Category.

Por todo ello, **se autoriza la presentación de la Tesis Doctoral con mención internacional.**

Córdoba, 6 de junio de 2022

Firma de los directores

Themis J. Michailides

Fdo. Themis J. Michailides   Fdo. Juan Moral Moral

MENCIÓN DE DOCTORADO INTERNACIONAL EN EL TÍTULO DE DOCTOR

TÍTULO DE LA TESIS: Estudio de la dinámica de inóculo de los agentes de biocontrol de aflatoxinas en frutos secos, resistencia varietal al patógeno y caracterización de la población de *Aspergillus* spp. sección *Flavi* en España

DOCTORANDA: María Teresa García López

Mediante la presentación de esta Memoria se pretende optar a la mención de **Doctorado Internacional**, habida cuenta de que la doctoranda reúne los requisitos exigidos para tal mención, a saber:

1. Estancia de 10 meses en un centro de investigación de otro país realizando trabajos de investigación relacionados con la Tesis Doctoral:

   Laboratorio de Patología Vegetal del *Kearney Agricultural Research and Extension (K.A.R.E.) Center*, perteneciente a la Universidad de California-Davis, en Estados Unidos, bajo la supervisión del Prof. Dr. Themis Michailides. Fecha de estancia: desde septiembre-2017 hasta noviembre-2018.

2. Informes favorables de dos doctores pertenecientes a Instituciones de Enseñanza Superior de otros países:
   - Prof. Alejandro Calderón-Urrea. Fresno State. Department of Biology. Fresno, California, United States.
   - Dr. Kamyar Aram. East Bay Specialty Crops Advisor. University of California Cooperative Extension. Department of Agriculture and Natural Resources. Concord, California, United States.

3. Uno de los miembros del tribunal que ha de evaluar la presente Tesis Doctoral pertenece a un Centro de Enseñanza Superior de otro país:

   Dr. **Ángel Medina Vaya**, responsable del Grupo de Micología Aplicada y director en funciones de Medio Ambiente y Agroalimentación en el Instituto de Suelos y Agroalimentación de Cranfield, Reino Unido.

4. La exposición y la defensa del esta Tesis Doctoral se realizará en una lengua diferente a la materna: **inglés**

INDICIOS DE CALIDAD CIENTÍFICA DE LA TESIS DOCTORAL

TÍTULO DE LA TESIS: Estudio de la dinámica de inóculo de los agentes de biocontrol de aflatoxinas en frutos secos, resistencia varietal al patógeno y caracterización de la población de *Aspergillus* spp. sección *Flavi* en España

DOCTORANDO: María Teresa García López

Publicaciones	**JCR IF**	**JIF Rank**	**Quartile/ Category**
Present status and perspective on the future use of aflatoxin biocontrol products. 2020. Agronomy. DOI: 10.3390/agronomy10040491	3.417	16/91	Q1 Agronomy
Quantification of the aflatoxin biocontrol strain *Aspergillus flavus* AF36 in soil and nuts and leaves of pistachio by real-time PCR. 2020. Plant Disease. DOI: 10.1094/PDIS-05-20-1097-RE.	4.438	29/235	Q1 Plant Science
Resistance to *Aspergillus flavus* and *A. parasiticus* in almond advanced selections and cultivars and its interaction with the aflatoxins biocontrol strategy. 2021. Plant Disease. DOI: 10.1094/PDIS-05-21-0892-RE.	4.438	29/235	Q1 Plant Science
Pistachio male inflorescences as an alternative substrate for the application of atoxigenic strains of *Aspergillus flavus*	Submitted to Plant Disease		
Sporulation and dispersal of the biological control agent *Aspergillus flavus* AF36 under field conditions in nut crops in California	Editing		
Characterization of the *Aspergillus* spp. population in Spanish almond and pistachio orchards and identification of atoxigenic strains of *A. flavus*	Editing		

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I am also immensely grateful for the help of my director, the professor Juan Moral, who boosted my career by offering me my particular "California dream" and involving me, once back in Spain, in the exceptional group UCOLIVO, at the Department of Agronomy of the University of Cordoba. I appreciate all the opportunities I was provided to reach this crucial milestone with the constant funding availability for conducting the research. More importantly, I am grateful for the knowledge gained through the diversity of jobs performed.

Finally, I thank the warmth and support of my family and close dear friends, and the wonderful work colleagues I had the luck to find from the early moments in California till my final steps in Cordoba.

Dedico mi reconocimiento más profundo a mi director, el profesor Themistocles J. Michailides, y al equipo que lidera en *Kearney Agricultural Research and Extension Center*, perteneciente a la Universidad de California, por la oportunidad brindada y el impulso de aprendizaje en las primeras etapas de este doctorado.

También agradezco inmensamente la ayuda de mi director, el profesor Juan Moral, que impulsó mi carrera ofreciéndome mi particular "sueño californiano" e involucrándome, una vez de vuelta en España, en el excepcional grupo UCOLIVO, del Departamento de Agronomía. de la Universidad de Córdoba. Agradezco todas las oportunidades que me brindaron para alcanzar este momento crucial con la disponibilidad constante de fondos para realizar la investigación. Más importante aún, estoy agradecida por el conocimiento adquirido a través de la diversidad de trabajos realizados.

Finalmente, agradezco la calidez y el apoyo de mi familia y amigos muy queridos, y los maravillosos compañeros de trabajo que tuve la suerte de encontrar desde los primeros momentos en California hasta mis últimos pasos en Córdoba.

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ABSTRACT

Almond and pistachio nuts can be occasionally colonized with the fungal species *Aspergillus flavus* and *A. parasiticus* and, concomitantly, contaminated with aflatoxins, potent carcinogenic mycotoxins for humans. As reviewed in chapter I, the most effective pre-harvest management strategy for limiting aflatoxin contamination is the massive release of native atoxigenic strains of *A. flavus* for the competitive displacement (or competitive exclusion) of wild toxigenic isolates from the agroecosystem. Many farmers from the U.S.A., Africa, and Italy and diverse crop industries benefit from using this technology. Thereafter, in temperate and tropical regions, where the biocontrol strategy is still not implemented, the search for native atoxigenic *A. flavus* strains is a necessity.

In California, for example, the US Environmental Protection Agency granted registration of the atoxigenic strain AF36 of *A. flavus* for use in pistachio and almond in 2012 and 2017, respectively. This strain is applied using sorghum grains as the AF36 spores carrier with the commercial name AF36 Prevail®. The use of AF36 Prevail® is a clear competitive advantage for U.S. farmers since commercial biocontrol agents to reduce aflatoxin contamination are not available in other nut-growing regions such as Australia, Spain, or Turkey.

Although AF36 Prevail® was primarily developed for applying to row crops (e.g. maize, cotton), it has also been effective in limiting aflatoxin contamination in nut trees in California. Even so, AF36 Prevail® often fails in nut orchards because of differential characteristics between row and tree crops. Then, we evaluated (chapter II) the sporulation of the biocontrol strain AF36 in pistachio orchards and advised farmers to spread the AF36 Prevail® product in the moist soil area but avoid the site where the irrigation drops fall. Our study about the dynamics of *A. flavus*' spores suggested that AF36 Prevail® could be applied every two rows obtaining an overlapping effect on the non-treated row whether the distance between tree rows is ≤ 10 m. Furthermore, we detected that tree debris in the canopy act as an inoculum source for *Aspergillus* species included in section *Nigri*, ochratoxins producers, and biocontrol strategies may act parallelly to protect against both mycotoxins.

It is essential to monitor how the atoxigenic AF36 strain survives and competes with aflatoxin-producing species populations in the target agroecosystem to understand how it

can displace wild isolates of *Aspergillus* spp. Traditionally, biocontrol strains of *A. flavus* have been monitored through vegetative compatibility assays (VCA), but these are tedious and time-consuming. Thus, we tackled this concern by developing and validating a mismatch-qPCR assay to quantify the proportion of AF36 vs. toxigenic genotypes of *A. flavus* and *A. parasiticus* from diverse soil and plant samples. Our mismatch-qPCR efficiently quantifies AF36 proportions in the *Aspergillus* population.

To overcome the disadvantages (loss of grains, poor sporulation, etcetera) of applying the strain AF36 using sorghum grains as carriers, we studied (chapter II) the pistachio male inflorescences as an inoculum source of atoxigenic strains. Male inflorescences are an abundant and free substrate, regularly distributed in the orchard. In our trials, the density of AF36 spores on the pistachio canopy of the inflorescence-treated trees was similar ($P > 0.05$) to this of Prevail®-treated trees. Furthermore, our results indicated that in pistachio orchards, where biocontrol practices are not conducted, eliminating this critical source of toxigenic *Aspergillus* inoculum is recommended.

In chapter III, we characterized the resistance of various almond cultivars against *A. flavus* and *A. parasiticus* colonization and aflatoxin contamination. Remarkably, we found high variability in response to aflatoxin contamination of almond cultivars caused by both *Aspergillus* species. In addition, the shells were an insurmountable barrier to the pathogen, regardless of their type of shell (hard, semihard, or paper shell). However, natural-opening shells often occur in paper shell almond cultivars in the field. Our results also pointed out the importance of peach for introgressing resistance to the pathogen in almond breeding programs. Finally, we presented the possibility of combining both cultivar resistance and biocontrol, which offers a particularly promising aflatoxin control strategy.

In a final chapter IV, we surveyed two leading Spanish almond- and pistachio-producing regions, Andalusia and Castilla La Mancha. In these surveys, we isolated 78 strains of *Aspergillus* section *Flavi*. Remarkably, four *A. flavus* were identified as atoxigenic (i.e., no-aflatoxin and no-cyclopiazonic acid producers) and, to our knowledge, this is the first report of atoxigenic strains of *A. flavus* native to Spain. Besides, six *A. tamarii* strains resulted, for the first time, described as slightly aflatoxigenic.

With the work advocated in this Ph. D Thesis, we have contributed definitely to the optimized use of this biological control strain in Californian tree nut crops. In addition, we are closer to offering a safe product option to be used infield shortly by the Spanish almond and pistachio producers.

RESUMEN

Las almendras y los pistachos son colonizados ocasionalmente por las especies fúngicas *Aspergillus flavus* y *A. parasiticus* y, por consiguiente, pueden contaminarse con aflatoxinas, potentes micotoxinas cancerígenas para los humanos. En el capítulo I de la presente Tesis Doctoral, revisamos una de las estrategias de control más efectiva para limitar la contaminación por aflatoxinas en campo: la liberación masiva de cepas atoxigénicas (no productoras de micotoxinas) nativas de *A. flavus* para el desplazamiento competitivo (o exclusión competitiva) de los aislados toxigénicos del agroecosistema. Muchos agricultores de EE. UU., África e Italia tienen acceso comercial a este tipo de agentes de biocontrol. En cambio, en las regiones templadas y tropicales donde aún no se implementa esta estrategia de biocontrol es necesaria la búsqueda de cepas atoxigénicas de *A. flavus*.

En California, la Agencia de Protección Ambiental (EPA) de EE. UU. otorgó el registro de la cepa atoxigénica AF36 de *A. flavus* para uso en pistachero y almendro en 2012 y 2017, respectivamente. La cepa AF36 se aplica en campo utilizando granos de sorgo recubiertos de esporas con el nombre comercial AF36 Prevail®. El uso de AF36 Prevail® supone una ventaja competitiva para los agricultores estadounidenses ya que, en otras regiones productoras de frutos secos, como Australia, España o Turquía, no hay agentes de control biológico comerciales disponibles.

El producto AF36 Prevail® se desarrolló para su uso en cultivos extensivos (ej. maíz y algodón), aunque también se ha mostrado eficaz disminuyendo la contaminación por aflatoxinas en frutos secos. Aun así, el control biológico de aflatoxinas en frutos secos mediante el uso de AF36 Prevail® fracasa con frecuencia debido a características agronomicas propias de este tipo de cultivos arbóreos. En el capítulo II, por lo tanto, evaluamos la esporulación y dispersión del producto AF36 Prevail® en campos de pistachero y recomendamos a los agricultores aplicar el producto en el área de suelo irrigada por los microaspersores aunque, evitando la zona donde impactan las gotas del agua de riego ya que afecta negativamente a su esporulación. Según nuestro estudio sobre la dispersión de las esporas de *A. flavus*, las esporas de AF36 fácilmente alcanzan la copa de los pistacheros próximos al punto de aplicación aunque disminuye marcadamente con la distancia a la fuente de inóculo y la altura, ajustándose a distintas ecuaciones de

difusión. Nuestros datos apuntan a que AF36 Prevail® podría aplicarse en filas alternas de pistacheros obteniéndose un efecto de protección (densidad de esporas por árbol) similar en el conjunto de los árboles si la distancia entre filas es $\leq$ 10 m. Además, detectamos que los restos de tejido que quedan en la copa de los pistacheros actúan como fuente de inóculo para las especies de *Aspergillus* de la sección *Nigri*, productores de ocratoxinas, por lo tanto, la estrategia de biocontrol puede actuar de forma paralela contra ambas micotoxinas.

Para comprender cómo la cepa atoxigénica AF36 sobrevive, compite y desplaza a las cepas silvestres de *Aspergillus* spp. productoras de aflatoxinas, es esencial la monitorización en los campos donde ha sido liberada. Tradicionalmente, las cepas de *A. flavus* se han monitoreado mediante ensayos de compatibilidad vegetativa (VCA), pero son tediosos y requieren varias semanas para su ejecución. Por lo tanto, desarrollamos y validamos (capítulo II) un protocolo de qPCR basado en un Mismatch para cuantificar la proporción de AF36 frente a los genotipos toxigénicos de *A. flavus* y la población general de *A. parasiticus*. Nuestra qPCR-Mismatch cuantifica de manera eficiente las proporciones de AF36 respecto a la población de *Aspergillus* en el suelo y la planta.

Debido a los problemas derivados de aplicar la cepa AF36 en granos de sorgo (pérdida de granos, mala esporulación, etcétera), estudiamos (capítulo II) la posibilidad de utilizar las inflorescencias masculinas de pistachero como fuente de inóculo para las cepas atoxigénicas. Las inflorescencias masculinas constituyen un sustrato abundante, gratuito, y que se distribuye regularmente en la plantación. En nuestros ensayos, la densidad de esporas de AF36 en la copa de los pistacheros con inflorescencias del suelo inoculadas con AF36 fue similar ($P > 0.05$) a la de los árboles tratados con AF36 Prevail®. Estos resultados apuntan indirectamente a que, en los campos de pistacheros donde no se llevan a cabo prácticas de biocontrol, es recomendable eliminar las inflorescencias masculinas en el suelo al constituir una importante fuente de inóculo.

En el capítulo III, caracterizamos la resistencia de cultivares y selecciones avanzadas de almendro a la colonización por *A. flavus* y *A. parasiticus* y, la subsiguiente, contaminación por aflatoxinas. Sorprendentemente, encontramos una alta variabilidad en la resistencia/susceptibilidad de los genotipos de almendro a la colonización por ambas especies. Además, la cáscara (endocarpo) intacta resultó ser una barrera infranqueable para el patógeno, independientemente del tipo (dura, semidura o de papel). Sin embargo,

las aperturas de la cáscara, que naturalmente pueden aparecer en cultivares de almendro de cáscara de papel, constituyen un punto de entrada para las esporas del patógeno. En este capítulo, además, destacamos la importancia del melocotonero para la introgresión de genes de resistencia al patógeno en los programas de mejora del almendro. Finalmente, presentamos la posibilidad de combinar tanto la resistencia del cultivar como el biocontrol, lo que ofrece una estrategia de control de aflatoxina particularmente prometedora.

En un capítulo final (IV), caracterizamos la población de *Aspergillus* spp. en dos de las principales regiones españolas productoras de almendras y pistachos, Andalucía y Castilla La Mancha. Durante las prospecciones realizadas, aislamos 78 cepas de *Aspergillus* sección *Flavi* y seis cepas de *A. tamarii* que sorprendentemente fueron caracterizadas como ligeramente aflatoxigénicas. Cabe destacar, que identificamos cuatro cepas de *A. flavus* como atoxigénicas (es decir, no productoras de aflatoxinas ni ácido ciclopiazónico) lo que constituye la primera descripción de cepas atoxigénicas de *A. flavus* españolas.

En la presente Tesis Doctoral, hemos contribuido notablemente a la optimización del control biológico de aflatoxinas en los cultivos de frutos secos de California. Además, estamos más cerca de ofrecer agentes de control biológicos para reducir la contaminación por aflatoxinas que puedan emplear los productores españoles.

OBJECTIVES

The general objective of the current Doctoral Thesis is to improve aflatoxin biological control by i) using atoxigenic strains of *Aspergillus flavus* through studying inoculum dynamics, ii) varietal resistance to the pathogen, and iii) the characterization of the population of *Aspergillus* spp. section *Flavi* in Spain. This general aim has been addressed through the following four specific objectives:

a. Reviewing the current status and discussing the future perspective of aflatoxin biocontrol products.

b. Studying the inoculum dynamic of the biological control agent *A. flavus* AF36 through i) the understanding of sporulation and dispersal under field conditions in nut crops in California, ii) the development of a mismatch-qPCR to quantify proportions of AF36 accurately in different substrates, and iii) exploring pistachio male inflorescences as an alternative substrate for its application.

c. Evaluating the resistance to *A. flavus* and *A. parasiticus* in almond advanced selections and cultivars and their combination with the aflatoxin biocontrol strategy.

d. Characterizing the *Aspergillus* spp. section *Flavi* population in commercial nut orchards in Spain and isolated atoxigenic strains as potential biocontrol agents.

GENERAL INTRODUCTION

Decays or deterioration of grains and nuts are caused by fungal species, among which the genera *Aspergillus* stands out. One of the most troubling effects of these decays is the induction of mycotoxicosis in animals and humans, i.e., diseases caused by the consumption of food and feed contaminated by mycotoxins produced by fungal species (Agrios 2005). Mycotoxins are low-molecular-weight natural compounds produced as a product of the secondary metabolism of filamentous fungi such as *Alternaria, Claviceps*, *Fusarium*, and *Penicillium*, in addition to *Aspergillus* (Bennett and Klich 2003). Mycotoxins found in food and feed include aflatoxins, ochratoxin A, trichothecenes, zearalenone, fumonisins, ergot alkaloids, altenuene, alternariol, alternariol methyl ether, altertoxin, and tenuazonic acid, among others (Bhatnagar et al. 2018; Gil-Serna et al. 2020; Marin et al. 2013). Mycotoxins differ in their chemical formula, the preferent commodities, the conditions under which they are produced, their effects on various animals and humans, and their toxicity.

Noteworthily, aflatoxins are among the most potent carcinogens, being hepatotoxic and mutagenic. Human deaths caused due to the consumption of contaminated food by aflatoxin eventually occur, majorly in impoverished nations where postharvest storages are deficient. For example, deadly outbreaks caused by aflatoxins consumption have been reported in Kenya because of contaminated maize consumption and in India due to contaminated dairy products (Probst et al. 2007; Klingelhöfer et al. 2018). Aflatoxins are difuran-containing, polyketide-derived compounds produced by fungal species of *Aspergillus* (Bennett and Klich 2003). Besides, some species of *Aspergillus* (mainly *A. fumigatus*) are opportunistic pathogens in humans causing aspergillosis, a lung infection that affects immunocompromised patients when exposed to many spores in the air (Hedayati et al. 2007; O'Gorman 2011).

Research into aflatoxins began following the outbreak of an unknown disease called Turkey X disease that caused the sudden death of more than 100,000 poults in England in 1960. These deaths were attributed to toxicity from the ingestion of peanut meal imported from Brazil (Richard 2008). From that moment on, efforts to discover the causal agent(s) involved intensified, beginning a period (1960-1975) known as the "Mycotoxins gold rush" that gave rise to the recognition of 300-400 compounds as mycotoxins, from

which just 3-4% represent real threats for human and animals (Maggon et al. 1977). Soon after the outrage, the species *A. flavus* was identified as the causal agent, and the toxic compound was named aflatoxin (Austwick and Ayerst 1963). Shortly after, a new outbreak in Kenya allowed the identification of *A. parasiticus* as the aflatoxin-producing species causing the disease. Next, four major aflatoxins were isolated and identified by thin-layer chromatography: B_1, B_2, G_1, and G_2 (Armbrecht et al. 1963). Type B, those that have blue fluorescence under ultraviolet light (365 nm), and type G, those whose fluorescence is green. Also relevant is the aflatoxin M_1, derived from B_1, frequently present in the milk of mammals fed with contaminated products (Galvano et al. 1996). Therefore, aflatoxin M_1 can be transferred to infants during lactation (Marchese et al. 2018). For example, in 2013, the Andalusian "Valle de Los Pedroches Dairy Cooperative" (COVAP) suffered a crisis and was forced to destroy 2 million liters of milk contaminated with aflatoxin M_1 and, lamentably, the problem reappeared in 2016 (Caravaca 2013; Pizá 2016). The International Agency for Research on Cancer (IARC) classified B_1 and M_1 as human carcinogens belonging to Group 1 and Group 2B, respectively.

On the other hand, some *Aspergillus* species also produce cyclopiazonic acid (CPA), another mycotoxin that affects animals' nervous systems and gastrointestinal tracts. Only one incident of CPA poisoning has been reported in humans (Burdock and Flamm 2000; Gil-Serna et al. 2020). Aflatoxins and CPA often co-contaminate food and feed products; indeed, many of the typical symptoms associated with "turkey X disease" are attributed to CPA (Cole 1986; Richard 2008). CPA has also been suggested to function as a pathogenicity factor inducing the cell death in plants, fungal colonization of the plant tissues, and the contamination with aflatoxins (Bhatnagar et al. 2018; Chalivendra et al. 2017).

The genus *Aspergillus* contains saprophytic and heterothallic fungi that develop hyaline hyphae and reproduce asexually, with the formation of many airborne, small (3 to 6 µm), light, and dry (hydrophobic) conidia on conidiophores (Abbas et al. 2009; Klich 2002). Occasionally, some species of *Aspergillus* develop airborne sexual ascospores (Horn et al. 2009; Moore et al. 2013). The *Aspergillus* genus shows huge genetic diversity, reflected in morphological, physiological, and molecular differences between species and isolates of the same species (Horn 2009; Olarte et al. 2012). Aflatoxins are produced by species distributed in the genus *Aspergillus* within three sections *Flavi, Ochraceorosei,* and *Nidulantes*. However, the species *A. flavus* and *A. parasiticus —Flavi* section— are

the most common and harmful aflatoxin producers. Both species *A. flavus* and *A. parasiticus* are ubiquitous in temperate zones and are increasing their presence in many agricultural soils due to increased aridity caused by climate change (Baazeem et al. 2021; Cotty and Jaime 2007; Medina et al. 2017). Numerous crops are contaminated by mycotoxins produced by these fungi, highlighting the problems associated with corn (Ortega-Beltran and Cotty 2018; Soni et al. 2020), peanuts (Njoroge 2018; Diao et al. 2014), and cotton (Jaime and Cotty 2004). Although aflatoxins only occasionally contaminate nuts, these entail huge losses for farmers and nut-industry (Ortega-Beltran et al. 2019).

The European Food Safety Authority (EFSA), based on the ALARA (As Low As Reasonably Achievable) principle, which considers consumption habits, establishes restrictive limits through the Commission Regulation. Thus, depending on the product, the maximum permissible levels are established between 0.1 µg/kg (for baby foods) and 8 µg/kg for aflatoxin B_1 and between 4 and 15 µg/kg for the sum of the B and G aflatoxins (EC. No. 1881/2006). In the case of the nuts, the E.U. has the maximum limits of 8 and 10 µg/kg for B_1 and the sum of B and G aflatoxins, respectively. For its part, the U.S. Food and Drug Administration (F.D.A.) sets only one limit of 20 µg/kg for the sum of B and G aflatoxins. These thresholds of aflatoxin contamination cause numerous rejections of shipments of diverse commodities at the international borders. For example, in the European Union, the Rapid Alert System for Food and Feed (RASFF) alerted 24 and 27 products originating in the U.S.A. and Spain, respectively, from 2014-to 2020 (Figure 1). Thus, the most concerning U.S. products were pistachios (16 alerts, 67%), groundnuts (3 alerts, 13%), and almonds (2 alerts, 8%). In the case of Spain, the most problematic commodities were dry figs (59%), almonds (6 alerts, 22%), and pistachios (2 alerts, 7%). Remarkably, even though the volume of almond production shipped by the U.S.A. is higher than the Spanish, the number of alerts in Spanish almonds overcomes the American.

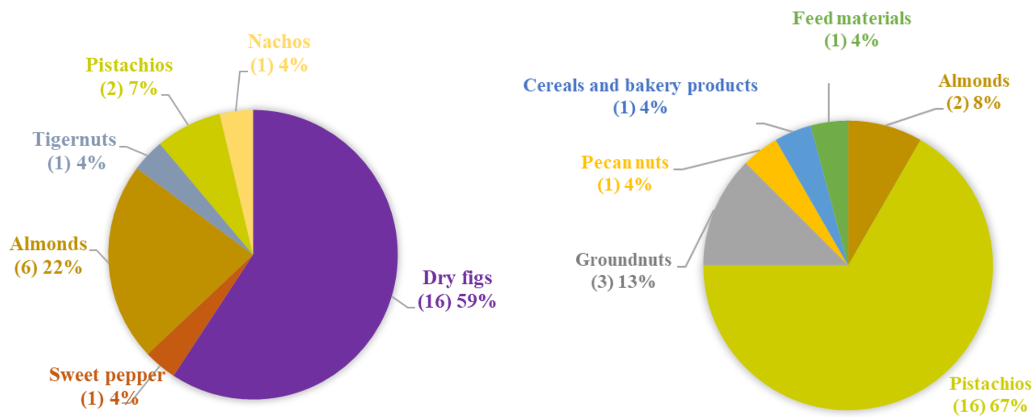

Figure 1. Alerts due to aflatoxin-contaminated commodities originated in Spain (left) and the U.S.A. (right) from 2014 to 2020 (RASFF, E.U.)

Tree nut crops are valuable in California, the major global producer. The primary destinations of nut shipments are the European Union and China (USDA 2022). Both almonds and pistachios grow along the San Joaquin Valley on over 700.000 hectares, characterized by a hot Mediterranean climate. Research conducted during the last three decades allowed the understanding of the *Aspergillus* contamination cycle in nut crops in California, considering the features of the pathogen population (Donner et al. 2015; Doster and Michailides 1995; Ortega-Beltran et al. 2018); identifying the main risking environmental factors such as high temperature and humidity (Abbas et al. 2009; Baazeem et al. 2021; Palumbo et al. 2014; Picot et al. 2018); and the plant characteristics associated with *Aspergillus* contamination, i.e., the presence of early splits in pistachio, and, shell openings and seed resistance in almonds (Dicenta et al. 2003; Doster and Michailides 1994; Moral et al. 2022).

Thus, the species *A. flavus* and *A. parasiticus* saprophytically overwinter in the plant debris, mummies on the canopy or litter on the ground, or as sclerotia in the soil of the nut orchards (Doster and Michailides 1994; Horn 2003). When environmental conditions become conducive (i.e., hot and dry) for the disease in late spring-summer in California, the fungi develop long chains of conidia that can reach nuts susceptible to contamination (Figure 2).

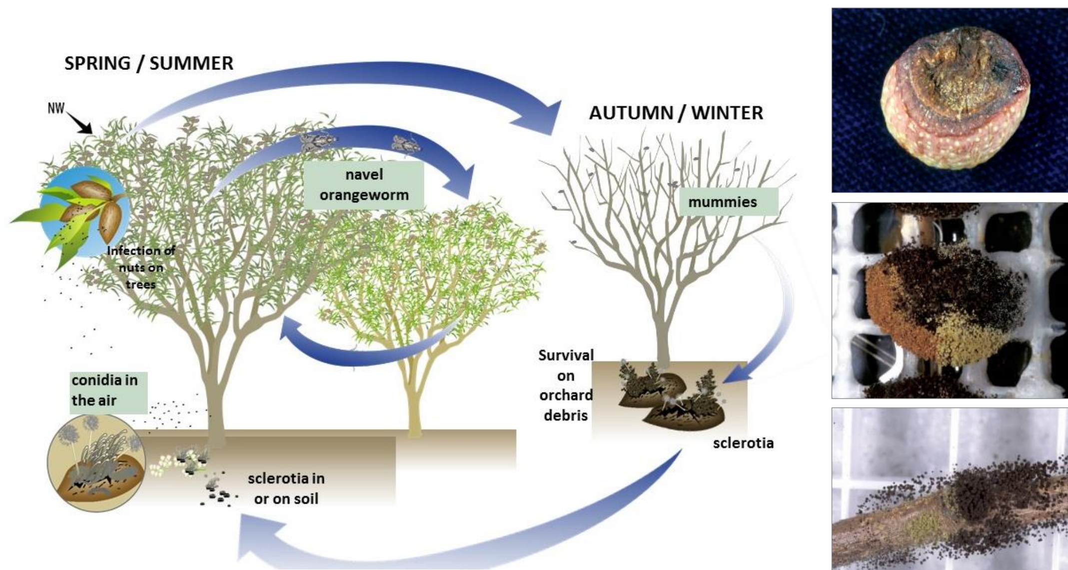

Figure 2. The life cycle of *Aspergillus flavus* and *A. parasiticus* in nut-producing orchards. On the right, the orchard colonized debris (from up to down, pistachio, almond, and almond-twig) by *Aspergillus* spp (T. J. Michailides).

In the case of pistachio, susceptible nuts are those named early splits, which leave the kernel exposed to the pathogen's conidia without the hull protection (Doster and Michailides 1994). The other risk factor is the infestation by pests; remarkably, the navel orangeworm specie (*Amyeloys transitella* L), which larvae feed in the nuts, and the moth carries the pathogen's conidia into the nut kernels in California (Palumbo et al. 2014). Fortunately, this pest is a quarantined species in the E.U. Thus, the control of aflatoxins in Californian pistachios aims to limit early splits nuts (Doster and Michailides 1994a; Doster et al. 2001); the proper control of the navel orangeworm, including early harvest to reduce its number of generations (Wilson et al. 2020); and the use of less susceptible cultivars (Moral et al., 2022). In the case of almonds, the varieties traditionally cultivated in California have soft-shell; conversely, those grown in Spain are hard-shell cultivars. Overall, hardshell varieties show higher tolerance to navel orangeworm infestation and, subsequently, *Aspergillus* colonization and aflatoxins contamination. Different Lepidopteran pests that represent a risk in Spain are *Anarsia lineatella* or *Plodia interpunctella* (Almacellas Gort and Marin Sanchez 2011; Schatzki and Ong 2001).

The use of the biological control strain AF36 rises above the different approaches for reducing aflatoxin contamination of nuts, i.e., an atoxigenic (non-aflatoxin producer) strain of *A. flavus* (Doster et al. 2014). Thus, the AF36 strain displaces the wild toxigenic isolates in the orchard by competitive displacement, when extensively spread in the field,

reducing aflatoxin contamination of the nuts. This intraspecific (*A. flavus* / *A.flavus*) or interspecific (*A. flavus* / *A. parasiticus*) competition can also be considered competitive exclusion since a complete exclusion is rarely observed in natural ecosystems (Wang and Liu 2020). The commercial formulation of this biological control strain (AF36 Prevail®) consists of sorghum grains coated with a spore suspension that is applied on the ground (Ortega-Beltran et al. 2016). In California, the Environmental Protection Agency (E.P.A.) and the California Department of Pesticide Regulation approved AF36 Prevail® for use in pistachio orchards in 2012 and, five years later, in almond and fig orchards (Ortega-Beltran et al. 2019). Thanks to this biocontrol strategy, substantial reductions in aflatoxin contamination in nuts have been achieved in California (Doster et al. 2014; Ortega-Beltran et al. 2018). Internationally accepted is the fact that the atoxigenic strains used in biopesticide formulations should be native to the target agroecosystem since these strains are well adapted. Unfortunately, this technology has not been honed in Spain, where almond and pistachio industries are boosting (Mañas Jiménez 2018), and aflatoxin contamination poses an emerging problem (Garcia-Lopez et al. 2018; Figure 1).

The success of this biocontrol technique by displacement requires a thorough knowledge of the *Aspergillus* population structure in the agroecosystem. Traditionally, the population structures of this fungal genus have been studied by vegetative compatibility tests. Thus, there are some Vegetative Compatibility Groups (VCGs) formed exclusively by atoxigenic members, and those are the ones more prone to be used as biocontrol agents (Mehl et al. 2012; Ortega-Beltran et al. 2019). Changes in *Aspergillus* populations due to the application of the biocontrol agents need to be monitored. Thus, the tracking of the AF36 strain has been done using VCG tests (Doster et al. 2014; Grubisha and Cotty 2015), a resource-intensive and time-consuming methodology.

Faced with all of this, the present Ph.D. Thesis has focused on the optimization of the biological control of aflatoxin in nuts based on the use of atoxigenic strains of *A. flavus* in California by i) studying the sporulation dynamics of AF36, ii) by developing a fast and accurate mismatch-qPCR to quantify this atoxigenic strain; iii) by developing alternative methods for a more efficient application of this isolates; and iv), by combining its effect with the genetic resistance of the almond cultivar. Finally, we have characterized the *Aspergillus* spp. section *Flavi* population present in nut-producing areas of Spain, with a future perspective on developing a native biocontrol product to protect Spanish nut crops.

A. flavus AF36 Prevail® biocontrol product sporulated on the ground of a tree nut orchard

Ph.D. Thesis

Biocontrol agents to reduce aflatoxins in nuts: inoculum dynamic studies, varietal resistance to the pathogen, and characterization of the population of Aspergillus spp. section Flavi in Spain

CHAPTER I

BIOCONTROL OF AFLATOXINS IN NUTS: REVIEW

PRESENT STATUS AND PERSPECTIVE ON THE FUTURE USE OF AFLATOXIN BIOCONTROL PRODUCTS

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Abstract

Aflatoxin contamination of important food and feed crops occurs frequently in warm tropical and subtropical regions. The contamination is caused mainly by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin contamination negatively affects health and trade sectors and causes economic losses to agricultural industries. Many pre- and post-harvest technologies can limit aflatoxin contamination but may not always reduce aflatoxin concentrations below tolerance thresholds. However, the use of atoxigenic (non-toxin producing) isolates of *A. flavus* to competitively displace aflatoxin producers is a practical strategy that effectively limits aflatoxin contamination in crops from field to plate. Biocontrol products formulated with atoxigenic isolates as active ingredients have been registered for use in the US, several African nations, and one such product is in final stages of registration in Italy. Many other nations are seeking to develop biocontrol products to protect their crops. In this review article we present an overview of the biocontrol technology, explain the basis to select atoxigenic isolates as active ingredients, describe how formulations are developed and tested, and describe how a biocontrol product is used commercially. Future perspectives on formulations of aflatoxin biocontrol products, along with other important topics related to the aflatoxin biocontrol technology are also discussed.

Keywords: aflatoxin; atoxigenic isolates; biocontrol agents; biocontrol technology

1. Introduction

1.1. The aflatoxin problem

Aflatoxin contamination of economically important food and feed crops occurs frequently in tropical and subtropical regions (Klich 2007b; JECFA 2018). Susceptible crops include maize, groundnut, cottonseed, tree nuts, figs, and chilies, among others (Bandyopadhyay et al. 2007; Amaike and Keller 2011; Ortega-Beltran et al. 2018). Aflatoxins are synthetized alone or with other mycotoxins by several *Aspergillus* species, most of them belonging to section *Flavi* (Table 1) (Frisvad et al. 2019). However, *A. flavus* and *A. parasiticus* are the species most commonly associated with aflatoxin contamination of crops (Amaike and Keller 2011; Klich 2007a). In general, toxigenic members of *A. flavus* produce aflatoxins B_1 and B_2 (blue fluorescent), while toxigenic members of *A. parasiticus* produce aflatoxins G_1 and G_2 (green fluorescent) in addition to B_1 and B_2 (Klich 2007a). The most potent of the four aflatoxins is B_1. The four types of aflatoxins are associated with a myriad of health threats including stunting, impaired food conversion, immunosuppression, liver cancer, and, under acute exposure, death (Azziz-Baumgartner et al. 2005). Similar negative impacts and low productivity occur in livestock when their feeds contain unsafe aflatoxin levels (Bryden 2012; Monson et al. 2015). Most mycotoxin alerts reported in the European Union by the Rapid Alert System for Food and Feed (RASFF 2019) are raised by crop lots contaminated with aflatoxins, followed by ochratoxins (Figure 1).

The magnitude of the aflatoxin contamination problem differs between developed and developing nations. Regulations and strict limits (tolerance levels) are enforced in developed nations to protect consumers from contaminated foods and feeds (Wu 2015; Van de Perre et al. 2015; Logrieco et al. 2018; Ortega-Beltran et al. 2019). In contrast, regulations in emerging and developing nations, if they exist, are poorly enforced with few exceptions. Examples of exceptions include cases when the contamination levels reach a national health emergency, such as maize destruction in areas of Kenya, and the banning of several brands of maize flour and peanut butter in Kenya, Rwanda, and Uganda (Lubanga and Bii 2019; Mwakio 2019; Omulo 2019; CGTN Africa 2019). Therefore, in emerging and developing nations, most contaminated crops enter the food and feed chains, regardless of their aflatoxin content (Bandyopadhyay et al. 2007; Guan et al. 2011; Probst et al. 2014; Waliyar et al. 2014; Njoroge et al. 2017; Seetha et al. 2017;

Lindahl et al. 2018). The absence of mechanisms to enforce aflatoxin tolerance levels results in chronic aflatoxin exposure with the subsequent lack of access to markets, poverty, low well-being, poor economic growth, being among other constraints in the affected populations (JECFA 2018; Bandyopadhyay et al. 2016; Leroy et al. 2018). For all these reasons, in the developing world, contamination of foods and feeds with aflatoxins has a profound negative impact on personal, social, and national development opportunities.

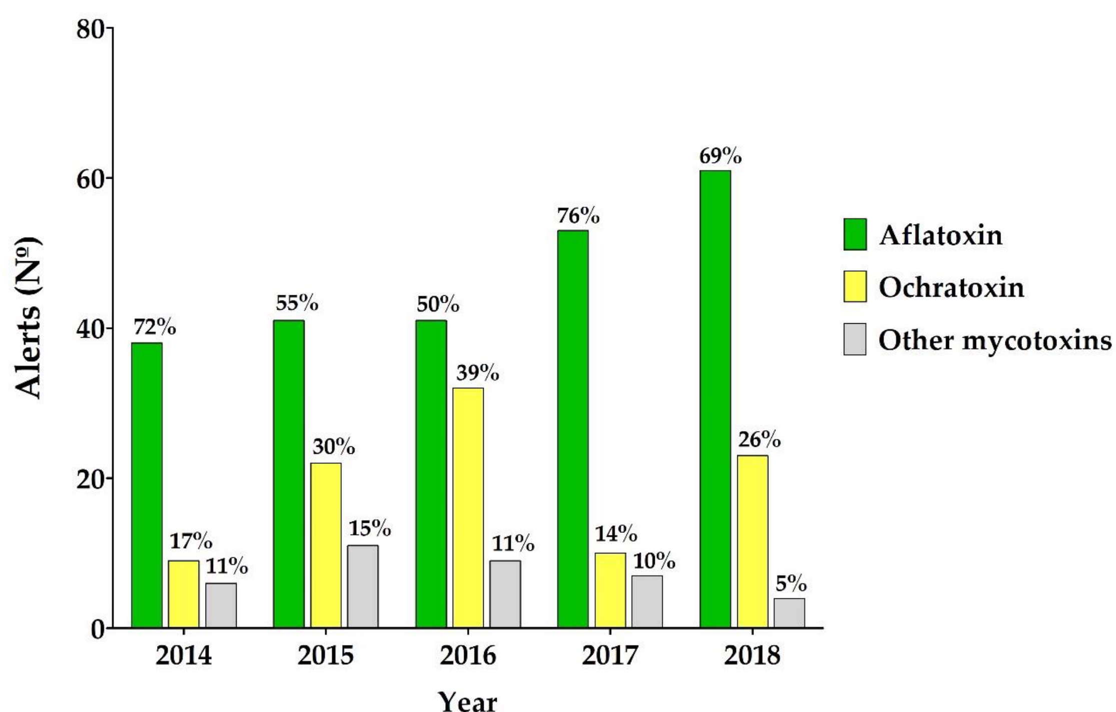

Figure 1. Number and relative percentage of mycotoxin alerts reported during the last five years (2014-2018) by the Rapid Alert System for Food and Feed (RASFF, 2019)

Table 1. Toxigenic profile of aflatoxin-producing species within *Aspergillus* section *Flavi*[1]

Species	Aflatoxin B_1 & B_2	Aflatoxin G_1 & G_2	Aflatrem	CPA[2]	3-Nit-acid[3]	Tenuazonic acid
A. aflatoxiformans	+	+	+	+	-	-
A. arachidicola	+	+	-	-	-	-
A. austwickii	+	+	+	+	-	-
A. cerealis	+	+	+	+	-	-
A. flavus	+	+ [4]	+	+	+	-
A. luteovirescens	+	+	-	-	-	+
A. minisclerotigenes	+	+	+	+	-	-
A. mottae	+	+	-	+	-	-
A. nomius	+	+	-	-	-	+
A. novoparasiticus	+	+	-	-	-	-
A. parasiticus	+	+	-	-	-	-
A. pipericola	+	+	+	+	-	-
A. pseudocaelatus	+	+	-	+	-	+
A. pseudonomius	+	+	-	-	-	+
A. pseudotamarii	+	-	-	+	-	+
A. sergii	+	+	+	+	-	-
A. togoensis	+	-	-	-	-	-
A. transmontanensis	+	+	-	-	-	-

[1] In each species, there are members that may not produce one or several toxins listed in the table.
[2] Cyclopiazonic acid.
[3] 3-Nitropropionic acid
[4] Only few isolates have been reported to produce G-type aflatoxins, data from (Frisvad et al. 2019; Klich 2007a, 2002).

1.2. Technologies to limit aflatoxin crop contamination

The notion that aflatoxin contamination is a problem restricted to post-harvest stages still incorrectly permeates in certain regions and sectors across the globe. Aflatoxin contamination usually starts in the field and, if post-harvest handling is deficient, aflatoxin concentrations can dramatically increase during storage (Waliyar et al. 2014; Seetha et al. 2017; Bandyopadhyay et al. 2016; Cotty et al. 2008; Mahuku et al. 2019). Several cultural practices and technologies that prevent or limit the contamination process in the field are available for both highly mechanized and small-scale agricultural systems. Among the pre-harvest technologies are the use of atoxigenic isolates of *A. flavus* as

biocontrol agents, insect control, timely harvesting, and use of less susceptible cultivars, including, in the case of maize and cotton, Bt-cultivars (Abbas et al. 2008, 2009).

Regarding post-harvest technologies, sorting has been described as a successful practice to reduce aflatoxin contamination in grain (i.e., maize) and nut lots (i.e., groundnut, pistachio, almond). Removal of nuts and grains showing morphological characteristics associated with aflatoxin contamination (e.g., shriveled, discolored, early split, worm galleries) considerably reduces aflatoxin content in a sorted batch (Doster and Michailides 1994; Whitaker et al. 2005; Matumba et al. 2015). Chemical detoxification is very limited to human food, but some compounds (e.g., ammonium, hydrated bentonite, magnetic carbon) can reduce aflatoxin concentration in the final product (Peng et al. 2018). Finally, other practices such as crop drying, improved sanitation, controlled processing, and storage conditions, significantly contribute to reduce aflatoxin accumulation (Waliyar et al. 2014; Seetha et al. 2017; Bandyopadhyay et al. 2016; Mahuku et al. 2019; Campbell et al. 2003; Jaime and Cotty 2004; Hell et al. 2008; Diao et al. 2014).

Due to the complexity and the multitude of factors affecting the aflatoxin contamination process, none of those technologies used individually provides complete protection. Rather than using a single or few somehow effective technologies, it is necessary to integrate aflatoxin management strategies addressing the entire crop production and distribution chain (Logrieco et al. 2018; Ayalew et al. 2017).

1.3. Biocontrol as a tool to decrease crop aflatoxin contamination

Genetic diversity is large and aflatoxin production potential is highly variable within and among aflatoxin-producing species (Klich 2007a; Grubisha and Cotty 2009). For example, populations of *A. flavus* are composed of aflatoxin producers and non-toxin-producing isolates (syn.: atoxigenic) (see 2.2). *A. flavus* is divided into two morphotypes, L and S, according to the size of sclerotia. The L morphotype produces few, large sclerotia (> 400 µm) while the S morphotype produces large numbers of small sclerotia (< 400 µm) (Cotty et al. 1994). Isolates of the L morphotype are highly variable in aflatoxin-producing potential with some of them being atoxigenic, while S morphotype isolates consistently produce high aflatoxin concentrations (Cotty and Jaime 2007). As with several fungal species, *A. flavus* populations can be classified in different vegetative

compatibility groups (VCGs). Isolates belonging to the same VCG are genetically more closely related than isolates belonging to other VCGs. There are certain VCGs composed solely of atoxigenic isolates and those genetic groups are good candidates for biocontrol products that, when applied in the field at the right stage, can result in a decreased aflatoxin content in the crop (Adhikari et al. 2016; Atehnkeng et al. 2016).

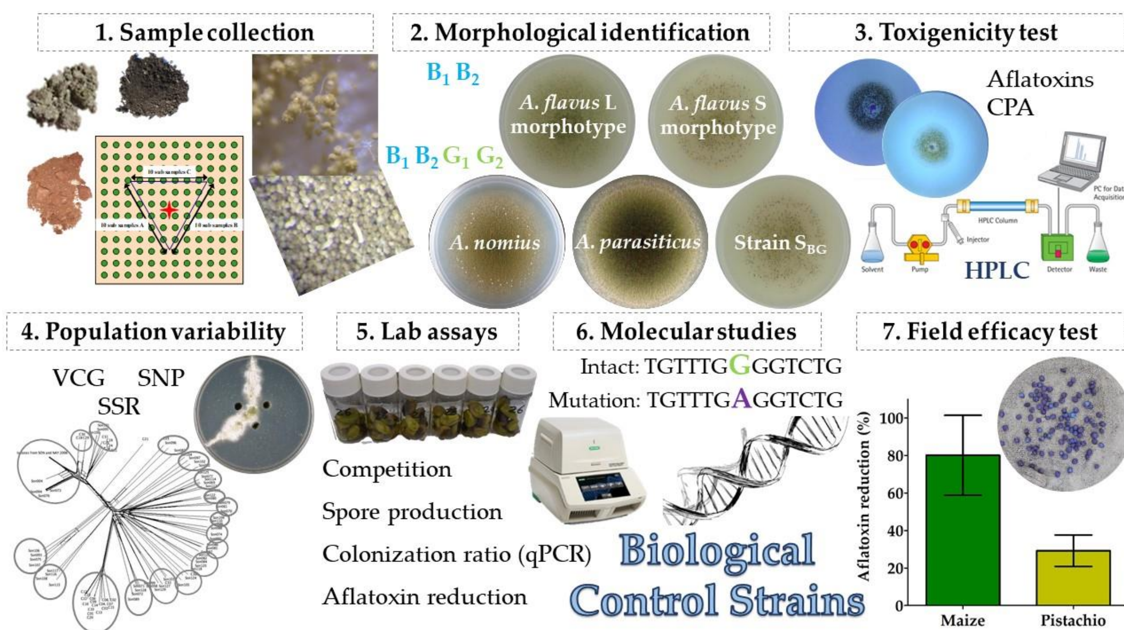

Figure 2. Illustration of a logical test sequence utilized during selection of atoxigenic *Aspergillus flavus* isolates to constitute aflatoxin biocontrol products

Using atoxigenic *A. flavus* isolates as an active ingredient in biocontrol formulations is a safe, low-cost, easy-to-use, and effective technology to protect crops from aflatoxin contamination. Although it is highly effective, the use of biocontrol products must be combined with key aflatoxin management strategies available to farmers in any given region (e.g., technologies to reduce plant stress, to promote rapid drying, and optimal storage of the crop) to minimize the risk of contamination. The process of selecting atoxigenic isolates of *A. flavus* to use as biocontrol agents should follow a series of well-established steps ranging from selecting native and well-adapted isolates, determining the reasons for their inability to produce toxins (e.g., aflatoxins, cyclopiazonic acid [CPA]), and assessing their ability to out-compete aflatoxin producers under controlled and field conditions (Figure 2). Another criterion for selecting the most competitive isolates in field conditions is to evaluate their abilities to spread from soil to crops (Agbetiameh et al. 2019).

When applied in the field, at the right crop phenological stage, atoxigenic biocontrol isolates displace aflatoxin-producers resulting in lowering infection rates by the native toxigenic isolates. Biocontrol treatment allows the production of crops with little to no aflatoxin, even in areas where both the environment and the pathogen population (density and toxicity) are conducive to crop infection and contamination. Frequently, treated crops contain undetectable aflatoxin content. Treatment allows producing crops that meet national and international premium market aflatoxin standards (below tolerance levels) that would be very difficult to achieve in the absence of the biocontrol treatment (Cotty et al. 2007; Mehl et al. 2012; Atehnkeng et al. 2014; Doster et al. 2014; Mauro et al. 2018; Bandyopadhyay, Cardwell, et al. 2019).

1.4. From single to multiple isolates

The United States Department of Agriculture–Agricultural Research Service (USDA–ARS) developed the first biocontrol product, *Aspergillus flavus* AF36, which contains as the active ingredient an atoxigenic isolate native to Yuma, Arizona, US (Brown et al. 1991; Cotty 1989; Cotty and Mellon 2006). The biocontrol product AF36 was initially registered with the United States Environmental Protection Agency (USEPA) for use in cotton fields in Arizona and California, and subsequently the registration label was expanded to treat maize in Arizona and Texas (Martin 2012). AF36 is produced and distributed by the Arizona Cotton Research and Protection Council (Cotty et al. 2007). During a 10-year research effort, it was found both that VCG YV36, to which AF36 belongs, was the atoxigenic VCG most commonly encountered in California and that AF36 was successful in limiting aflatoxin content in commercially produced pistachio. Therefore, USEPA granted registration of AF36 for use in pistachio grown in California and Arizona (2012), and eventually in almond and fig (2017) in California (Ortega-Beltran et al. 2019; Doster et al. 2014). The biocontrol product AF36 is now used in several states across the US, which makes it the most widely used aflatoxin biocontrol product in the world. A second biocontrol product, Afla-guard®, containing a different atoxigenic *A. flavus* isolate (NRRL21882) as an active ingredient, was registered with USEPA for use in maize and groundnut in the US (Dorner 2009). The product Afla-guard® is commercialized by Syngenta Crop Protection, Inc. (Greensboro, NC, US) and has been used in experimental groundnut fields in Turkey (Lavkor et al. 2019).

The International Institute of Tropical Agriculture (IITA) and USDA–ARS, in collaboration with national and international institutions, adapted and improved the aflatoxin biocontrol technology for use in sub-Saharan Africa (SSA). Several aflatoxin biocontrol products that effectively reduce crop aflatoxin content have been developed under the trade name Aflasafe® (Bandyopadhyay et al. 2016, 2019), and several of those are currently commercialized in different nations in Africa (Schreurs et al. 2019). Each Aflasafe® product contains, as active ingredient fungi, four atoxigenic isolates belonging to distinct VCGs native to the target nation. A shift from a single to a multiple-isolate product was made to exploit the repertoire of qualities that diverse atoxigenic isolates have in relationship to competitiveness and adaptation to diverse crops, environments, and agricultural practices (Bandyopadhyay et al. 2016; Mehl et al. 2012; Probst et al. 2011). Using isolates belonging to atoxigenic VCGs widely distributed and successful across environments and crops has proven to be effective in producing crops with low aflatoxin content (Atehnkeng et al. 2016; Agbetiameh et al. 2019). In natural conditions, it has been noted that the dominance of single *A. flavus* VCGs over single and multiple areas, over multiple years, is transient (Ortega-Beltran and Cotty 2018). Thus, use of multiple isolates may increase the chances to promote diverse atoxigenic communities over a longer-term.

USDA–ARS has developed a multi-isolate biocontrol product for use in maize grown in Texas, FourSure™, which contain, as active ingredients, four atoxigenic isolates native to Texas (Shenge et al. 2017). The US Texas Corn Producers Board is seeking the registration of FourSure (USEPA 2016). In California, several atoxigenic VCGs have been identified by the University of California - Davis (UC Davis) in collaboration with USDA-ARS and different tree-nut growing organizations, and isolates in those VCGs are valuable resources as constituents of multi-isolate products to treat nut crops (Ortega-Beltran et al. 2019; Donner et al. 2015; Picot et al. 2018). In North Carolina, combinations of atoxigenic strains have been tested in a field of a research station (Ortega-Beltran and Bandyopadhyay 2019). Research programs at UC Davis and collaborating universities in Argentina and Spain have identified through laboratory tests, atoxigenic *A. flavus* isolates with potential as biocontrol agents for use on those nations, but the efficacy of those isolates has not been tested under field conditions (Camiletti et al. 2017, 2018; Garcia-Lopez et al. 2018). Similarly, studies for identifying potential *A. flavus* biocontrol isolates are being conducted in other countries including, China (Zhou et al. 2015), India

(Hulikunte et al. 2017), Thailand (Pitt et al. 2015; Tran-Dinh et al. 2014) and Serbia (Savić et al. 2020).

2. Basis for selection of atoxigenic isolates as biocontrol agents

2.1. Use of native fungi

Biocontrol formulations containing atoxigenic fungi native to target regions should have a greater chance to be more effective because of their adaptation to the environment, cropping system, and climatic and soil conditions (Atehnkeng et al. 2016; Mehl et al. 2012). Native atoxigenic isolates generally possess superior abilities to compete against other native microorganisms for local resources. Also, native fungi as active ingredients of products allow faster regulatory approval compared to exotic fungi (Bandyopadhyay et al. 2016; Mehl et al. 2012). As there are many atoxigenic *A. flavus* isolates in all nations where aflatoxin-producers thrive, there is no need to introduce exotic organisms. Moreover, native fungi are beneficial germplasm that governments can use/license in a manner considered the most appropriate to reduce aflatoxin contamination and aflatoxin exposure (Mehl et al. 2012; Probst et al. 2011).

2.2. Membership in VCGs that are completely atoxigenic

An important criterion when selecting atoxigenic fungi to compose a biocontrol product is that each candidate isolate must belong to a widely distributed VCG composed only of atoxigenic members (Atehnkeng et al. 2016; Grubisha and Cotty 2015; Mauro et al. 2015). Microbiological, chemical, and molecular tools are employed to find atoxigenic VCGs. Evaluation of aflatoxin-production abilities of a large number of *A. flavus* isolates from a given region is required; thousands of vegetative compatibility tests must be performed, and molecular analyses using simple sequence repeat (SSR) markers must be done to reveal whether a VCG has only atoxigenic members. These activities are both resource intensive and time-consuming but allow detecting VCGs composed exclusively of atoxigenic members.

2.3. Lesions in aflatoxin biosynthesis gene cluster

About 30 clustered genes, and more than 20 enzymatic reactions, are needed for aflatoxin production (Yu et al. 1995; Yu 2012). In all aflatoxin-producing species, the aflatoxin

biosynthesis cluster is located within a 75-kb region of chromosome 3 (Yu et al. 1995). Genes in the cluster may be affected by different types of mutations, including substitutions, insertions, deletions, and frameshifts that can result in atoxigenicity. Deletions can be divided into specific deletions, affecting one gene, or large deletions affecting multiple or all aflatoxin biosynthesis genes (Prieto et al. 1996; Chang et al. 2005). For example, the aflatoxin and CPA biosynthesis gene clusters are entirely deleted in NRRL21882 (Chang et al. 2005) and one of the isolates, *A. flavus* Og0222, in the Aflasafe product used in Nigeria (Adhikari et al. 2016). Sometimes a single nucleotide polymorphism (SNP) is sufficient to confer atoxigenicity. A nonsense mutation (substitution: G-A) in the polyketide synthase gene is enough to confer atoxigenicity to the biocontrol isolate AF36, although there are many other mutations and deletions in the aflatoxin gene cluster of AF36 (Adhikari et al. 2016; Ehrlich and Cotty 2004). Finally, the aflatoxin gene cluster of *A. oryzae*, considered a domesticated species of *A. flavus*, shows several substitutions and frameshift mutations that result in lack of aflatoxin production (Tominaga et al. 2006).

2.4. Area-wide adaptation

Paramount is selection of atoxigenic fungi with known adaptation to target agroecosystems and their cropping systems(Bandyopadhyay et al. 2016). There is large variability among VCGs in abilities to compete for crop substrates (Mehl and Cotty 2010, 2013; Sweany et al. 2011). Numerous studies report isolates with potential as candidate atoxigenic biocontrol agents that were selected from small sets of isolates, and usually from relatively small areas. This can result in selection of isolates that may not be the most appropriate and widely adapted to limit aflatoxin contamination. Area-wide adaptation of atoxigenic VCGs can be revealed by examining several thousand *A. flavus* isolates associated with multiple crops collected in several hundred locations across a target country/region (Ortega-Beltran et al. 2019; Bandyopadhyay et al. 2016; Agbetiameh et al. 2019). Such studies require intensive microbiological, chemical, and molecular tests.

2.5. Superior competitiveness

In controlled conditions, atoxigenic isolates with superior ability to limit aflatoxin contamination are identified by challenging the candidate atoxigenic isolates with high

aflatoxin producers in co-inoculation assays. Typically, these assays are conducted by co-inoculating mature maize grains with both atoxigenic and aflatoxin-producing isolates. Subsequently, production of aflatoxin is quantified, and reduction of aflatoxin contamination is calculated with respect to grains inoculated only with an aflatoxin producing isolate (Probst et al. 2011; Mauro et al. 2015). Competition experiments have also been conducted using almond and pistachio kernels because those were target crops (Ortega-Beltran et al. 2019).

Field testing allows evaluating aflatoxin reduction abilities of candidate fungi under field conditions (Atehnkeng et al. 2014; Doster et al. 2014; Mauro et al. 2018; Brown et al. 1991; Dorner 2009). When selecting atoxigenic fungi for multi-isolate products, field evaluations are done by applying atoxigenic isolates individually (typically 12 isolates are tested) and then, as part of a candidate multi-isolate products (Bandyopadhyay et al. 2016; Agbetiameh et al. 2019; Atehnkeng et al. 2014). An evaluation of this type allows detecting those isolates with the greatest ability to create a founding population in the soil and then to successfully move to the grains or other harvested crop product in the treated fields (Agbetiameh et al. 2019). In Nigeria, field evaluation of a candidate product prior to large-scale efficacy trials revealed that one of the tested isolates was a poor competitor across all tested environments and therefore was replaced with an isolate with superior potential as biocontrol agent (Atehnkeng et al. 2014).

2.6. Efficacy trials

The true value of a biocontrol product composed of single or multiple isolates as active ingredient is revealed when the product is applied in crops managed by the farmers themselves, in multiple fields (usually 300 to 500) in multiple agro-ecological zones, and during multiple years (Doster et al. 2014; Bandyopadhyay et al. 2019; Senghor et al. 2019). Evaluating a product under controlled conditions, in a limited number of locations can result in incorrect interpretation regarding the benefits of biocontrol and its efficacy across environments (Ortega-Beltran and Bandyopadhyay 2019).

Usually, researchers conduct evaluation trials using paired fields, treated vs. untreated. When paired fields are not sufficiently separated (e.g., by a distance of >500 m), untreated fields may be affected by cross-contamination with spores of the biocontrol isolate(s) dispersed from the adjoining treated fields. This effect is much higher in a traditional

block design (Weaver and Abbas 2019). Aflatoxin contamination data of the paired fields often do not meet the requirements (normality and outliers) for applying a statistical paired T-Test. In such cases, transforming the data or using a non-parametric Wilcoxon Signed Rank Test may provide some usable results.

3. Registration of products

Many aflatoxin biocontrol products are currently registered with national biopesticide regulators for use in various crops (Table 2). In the US, AF36 is registered for use on several crops (Ortega-Beltran et al. 2019; Mehl et al. 2012; Doster et al. 2014), while Afla-guard® is registered for use on maize and groundnut (Dorner 2004). In Africa, there are 14 atoxigenic biocontrol products registered under the tradename Aflasafe for use in maize; 13 of those products are also registered for use in groundnut, and two of those are registered for use in sorghum (Bandyopadhyay et al. 2019; Schreurs et al. 2019). The African countries where Aflasafe products are registered are Nigeria, Kenya, Senegal, The Gambia, Burkina Faso, Ghana, Tanzania, Zambia, Mozambique, and Malawi. The atoxigenic biocontrol product AF-X1® is in the final stages of registration for unrestricted use on maize in Italy (http://www.agronomico.com/AFX1.aspx; (Mauro et al. 2018)).

Table 2. List of aflatoxin biocontrol products registered for commercial use[1]

Product	Atoxigenic *Aspergillus flavus* isolate(s)	Responsible Organization or entity	Target country	Crops for use	Reference
Aspergillus flavus AF36 Prevail®	AF36	Arizona Cotton Research and Protection Council	US	Cotton, maize, pistachio, almond, and figs	(Ortega-Beltran et al. 2018; Cotty et al. 2007; Mehl et al. 2012; Doster et al. 2014)
Afla-guard®	NRRL21882	Syngenta®	US	Maize and groundnut	(Dorner 2004)
Aflasafe™	Ka16127, La3279, La3304, Og0222	IITA[3]	Nigeria	Maize and groundnut	(Bandyopadhyay et al. 2019)
Aflasafe KE01™	C6-E, C8-F, E63-I, R7-H	IITA	Kenya	Maize	(Adhikari et al. 2016)
Aflasafe SN01	M2-7, M21-11, Ms14-19, Ss19-14	IITA	Senegal and The Gambia	Maize and groundnut	(Adhikari et al. 2016; Senghor et al. 2019)
Aflasafe BF01	M011-8, G018-2, M109-2, M110-7	IITA	Burkina Faso	Maize and groundnut	(Adhikari et al. 2016)
Aflasafe GH01	GHG079-4, GHG083-4, GHG321-2, GHM174-1	IITA	Ghana	Maize, groundnut, and sorghum	(Agbetiameh et al. 2019)
Aflasafe GH02	GHM511-3, GHM109-4, GHM001-5, GHM287-10	IITA	Ghana	Maize, groundnut, and sorghum	(Agbetiameh et al. 2019)
Aflasafe TZ01	TMS199-3, TMH104-9, TGS364-2, TMH 30-8	IITA	Tanzania	Maize and groundnut	Unpublished registration document
Aflasafe TZ02	TMS64-1, TGS55-6, TMS205-5, TMS137-3	IITA	Tanzania	Maize and groundnut	Unpublished registration document
Aflasafe MWMZ01 [2]	GP5G-8, GP1H-12, MZM594-1, MZM029-7	IITA	Mozambique	Maize and groundnut	Unpublished registration document
Aflasafe MWMZ01 [2]	MW199-1, MW097-8, MW246-2, MW238-2	IITA	Malawi	Maize and groundnut	Unpublished registration document
Aflasafe MZ02	GP5G-8, MZG071-6, MZM028-5, MZM250-8	IITA	Mozambique	Maize and groundnut	Unpublished registration document
Aflasafe MW02	MW258-6, MW332-10, MW248-11, MW204-7	IITA	Malawi	Maize and groundnut	Unpublished registration document
Aflasafe ZM01	110MS-05, 38MS-03, 46MS-02, 03MS-10	IITA	Zambia	Maize and groundnut	Unpublished registration document
Aflasafe ZM02	31MS-12, 12MS-10, 47MS-12, 64MS-03	IITA	Zambia	Maize and groundnut	Unpublished registration document
AF-X1®	MUCL54911	Pioneer ® Int.	Italy	Maize	(Mauro et al. 2018)

[1] All products contain sorghum as the carrier grain, except Afla-guard®, which contains barley as the carrier (Dorner 2004). [2] Aflasafe MWMZ01 is a regional product developed for both Malawi and Mozambique; one native atoxigenic isolate from each of four vegetative compatibility groups co-distributed in both Mozambique and Malawi was selected. [3] IITA, along with several partners, develops Aflasafe products for use in each nation; after registration, IITA licenses biocontrol manufacturing and commercialization responsibilities to private companies or the public sector (Schreurs et al. 2019)

4. Delivery methods

4.1. Grains as carriers and nutritional sources

All commercially available biocontrol products are formulated using sterile grains that serve as carriers and as a nutritive source for the atoxigenic strains. Several formulations were tested at the earlier stages of biocontrol development, including inoculated rice and wheat grains, pasta granules (containing semolina, kaolin, and xanthan gum), maize flour-based granules, and alginate pellets containing several nutrients (Cotty et al. 1994; Dorner et al. 2003). Spraying spore suspensions of the biocontrol agent directly to the canopy of the crop or applied as soil drenches were also tested (Cotty et al. 1994; Dorner et al. 1992). However, the use of grains as carriers have been found to be the best option (Cotty et al. 1994; Dorner et al. 2003). Originally the formulations comprised sterilized grains colonized by the atoxigenic isolates (Cotty et al. 2007; Atehnkeng et al. 2014; Dorner 2004; Bock and Cotty 1999). Although this methodology was effective to deliver the biocontrol agent to the crop, it was expensive and slow to produce. Therefore, formulations using roasted or dehulled grains (to avoid germination) coated with a spore suspension of the biocontrol isolate(s) were developed (Bandyopadhyay et al. 2016; Dorner 2009; Jaime et al. 2014). Coated formulations lower the costs and increase the rate of product manufacture, making it more affordable for farmers.

Grain-based formulations were developed for applications in field crops (cotton, groundnut, maize) with small and closed canopies which provide good conditions for the biocontrol fungus to sporulate, but they might not be adequate for tree nut orchards with more open canopies which do not provide optimal conditions for a fungus to sporulate. Therefore, there is a need to develop alternative formulations and application methods that will deliver the biocontrol isolate(s) more efficiently these less favorable environments.

4.2. Use of bioplastics

To date, all commercial aflatoxin biocontrol products are formulated using grains of either barley, wheat, or sorghum as carrier of the spores of the atoxigenic isolates. Other alternatives have been sought in order to limit the use of food and feed grains. Recent studies have investigated whether coating seeds with a bioplastic containing an atoxigenic isolate may be a useful technology for delivering the biocontrol isolate in maize crops

(Accinelli et al. 2016, 2018). Before planting, maize seeds are film-coated using a starch-based bioplastic previously combined with spores of the biocontrol isolate. Moreover, chemical pesticides (insecticide or fungicides) might be added to the coating slurry for additional protection of seeds in soil. Application of the bioplastic seed-coating with spores favors initial growth of this starch-utilizing fungus, resulting in a decreased frequency of aflatoxin producers in soil. Subsequently, aflatoxin contamination of maize kernels during pre-harvest stages was reported to be reduced (Accinelli et al. 2018). More studies describing key factors that may affect the effectiveness of this technology are needed to improve the performance of this formulation

4.3. Alternative substrates

The delivery method (i.e., barley, sorghum, or wheat grains) is the most expensive component of atoxigenic biocontrol products. In addition to bioplastics (see previous section), other substrates could be employed to reduce the production costs of the biocontrol products. This is particularly important for many smallholder resource-poor farmers, for which the cost to treat 1 ha of a crop may be prohibitive (12-18 USD, depending on the country) if they do not have access to markets paying a premium for safe crops. However, the major drawback of using grains as carriers is that they are frequently predated by ants, birds, and insects. Finding other substrates could reduce the cost of the product, have increased sporulation, and lower vulnerability to predation. The use of cassava peel pellets has been investigated to replace sorghum as a carrier and nutritive source for biocontrol isolates in West Africa, where large quantities of cassava peels are discarded daily (Okike et al. 2015). However, spore yield on cassava peel substrate is low compared to sorghum, meaning a reduction in the effectiveness of the biocontrol product. More research is needed to determine which substrates could provide desirable features of increased sporulation under broader environmental conditions and that would be less prone to predation. Finally, two application methods without carrier have been studied. These are application of aqueous spores suspensions directly to the crop canopy or delivering the active ingredient through the irrigation system but evaluations in commercial orchards remain to be done (Michailides et al. 2018).

5. Effectiveness in crops treated with biocontrol products

5.1. Effectiveness of aflatoxin biocontrol agents in different crops

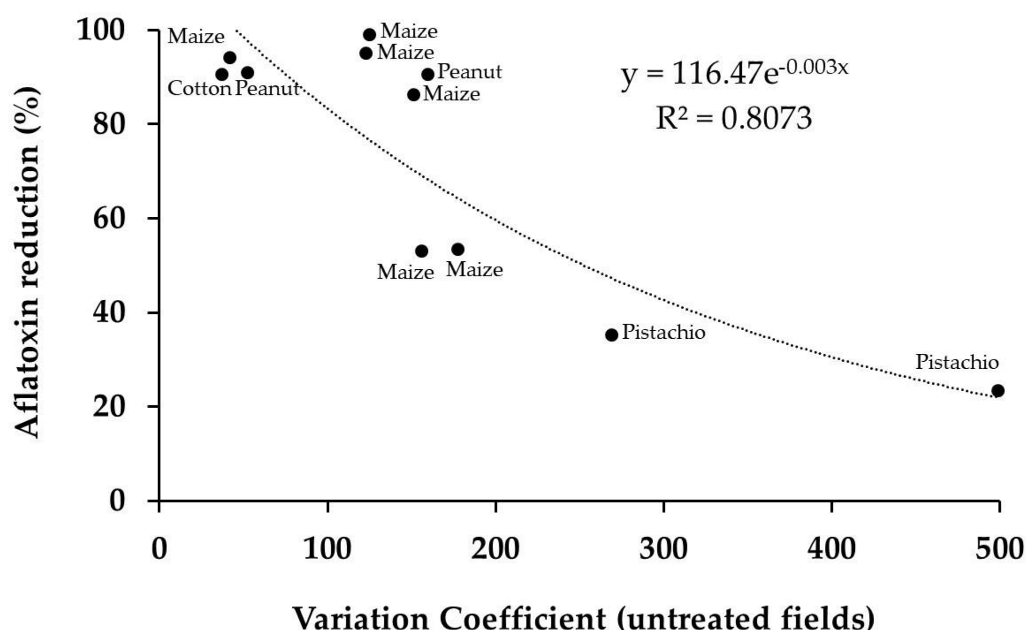

Figure 3. Relationship between aflatoxin reduction (%) as a result of application of atoxigenic *Aspergillus flavus* isolates to different commodities and the variation coefficient of aflatoxin contamination found in untreated fields of these crops.

The efficacy of aflatoxin reduction by atoxigenic isolates of *A. flavus* has been demonstrated in various crops including maize, groundnut, cottonseed, and pistachio in the US, and maize, groundnut, and chili peppers in Africa (Cotty et al. 2007; Doster et al. 2014; Dorner 2004; Ezekiel et al. 2019) (Table 2). Recently, efficacy trials have been conducted in sorghum in Ghana and sunflower in Tanzania and substantial reductions in aflatoxin have been achieved (unpublished results). Results of both field experiments and commercial treatments in cotton, maize, and groundnut fields show reduction in aflatoxin contamination ranging from 70% to 100% compared to adjacent non-treated fields (Cotty et al. 2007; Mauro et al. 2018; Bandyopadhyay et al. 2019; Senghor et al. 2019; Dorner 2002; Atehnkeng et al. 2008). In the commodities in which aflatoxin contamination is more frequent (i.e., the proportion of grains/seeds/fruits that can be contaminated by aflatoxin is higher), the impact of biocontrol isolates on aflatoxin reduction is higher too. Conversely, when the percentage of potential points of infection is meager, which occurs in the case of tree nut crops (i.e., from 1/5,000 to 1/20,000 in pistachio kernels), the

aflatoxin contamination on untreated fields is highly variable (measured as the variation coefficient) and, subsequently, the impact of the biocontrol agents in reducing aflatoxin contamination is frequently low but highly variable. Figure 3 shows the relationship between reduction of aflatoxin contamination as a result of application of atoxigenic *A. flavus* isolates in different commodities and the variation coefficient of aflatoxin contamination in untreated fields of these crops (Cotty et al. 2008; Agbetiameh et al. 2019; Doster et al. 2014; Mauro et al. 2018; R. Bandyopadhyay et al. 2019; Senghor et al. 2019; Dorner et al. 2003; Dorner 2002; Atehnkeng et al. 2008)

5.2. Area-wide treatment for increased efficacy

The overall goal of aflatoxin management with atoxigenic isolates is to change the population structure, typically dominated by aflatoxin producers, by increasing the frequencies of the applied atoxigenic isolates in the target ecosystem. Once the population is composed mainly of atoxigenic isolates, the aflatoxin production potential is greatly reduced.

A single application of an aflatoxin biocontrol product, at the right phenological stage, substantially reduces aflatoxin contamination in treated crops (Cotty et al. 2007; Doster et al. 2014; Bandyopadhyay et al. 2019). However, sometimes the biocontrol product does not completely protect the crop from aflatoxin contamination. Several factors affect the efficacy of treatment, including poor sporulation and product loss, which restrict the full potential of the competitive exclusion and founder effect principles of the technology (Jaime et al. 2014; Michailides et al. 2018). Benefits of aflatoxin biocontrol applications go beyond reducing aflatoxin in the treated crop in a single season. Thus, biocontrol applications also increase the density of atoxigenic isolates in the soil, even in neighboring fields, and the carry-over effect has the potential to displace aflatoxin producers for subsequent seasons. On the other hand, toxigenic fungi residing in soils nearby treated fields can also contaminate the treated crops in subsequent seasons. Studies of the population structure of *A. flavus* at the area-wide scale began in the 1990s (Nelson et al. 1999; Jaime and Cotty 2013) raising the idea that changing the population structure of the fungus at the regional scale will also reduce the aflatoxin-producing potential in the area. Implementing area-wide, long-term biocontrol programs might be the best strategy to reduce the population of aflatoxin-producing isolates for the medium- to long-term (Cotty et al. 2008, 2007). An area-wide and long-term aflatoxin management

program will require treating most aflatoxin susceptible crops growing in the same area. However, effective aflatoxin control might be achieved with partial, booster treatments after initial application for a few years. With area-wide treatment, biocontrol products would need to be reapplied perhaps at a lower dose to maintain a population with low aflatoxin production potential in an area-wide long-term management program. However, the application frequency depends on the characteristics of each area, since areas differ in the time that the biocontrol will persist in the soil (Atehnkeng et al. 2008; Jaime and Cotty 2013).

6. Biocontrol performance in future scenarios

An understanding of the epidemiology of, and the interaction between biocontrol agents and aflatoxin producers under field conditions is essential for successful simulation modelling in future scenarios, particularly under climate change. Simulation models to predict future toxigenic and atoxigenic population scenarios to assist farmers and pest control advisers through Decision Support Systems (DSS) will be important tools. Climate changes that accompany projected increments in atmospheric CO_2 can lead to a reduction in water availability in agricultural areas. Thus, by 2100, predictions indicate a rise in global temperature between 1 and 5 °C, which will affect development of crops and their capacity to adapt along with changes in the current distribution and densities of aflatoxin-producing fungi (Bidartondo et al. 2018). Areas affected by severe water stress (criticality ratio: withdrawals/availability of water > 0.4) will expand and the stress will intensify (Alcamo et al. 2000). Changes in global precipitation, ecological, and crop system patterns may alter the compositions of *Aspergillus* populations and their fitness, since they are primarily influenced by temperature and soil moisture. Jaime and Cotty (Jaime and Cotty 2003) observed spatial and temporal variations in aflatoxin contamination depending primarily on those environmental factors. For example, soil surface temperature influences fungal communities with propagule density decreasing when daily average soil temperature is either below 18°C or above 30°C (Jaime and Cotty 2010). *A. flavus* S morphotype fungi are more likely to occur at a higher proportion during warmer, drier years, and consequently, increased aflatoxin concentrations in crops occurs because of their high aflatoxin-producing potential (Cotty and Jaime 2007). Monitoring climatic parameters and crop aflatoxin accumulation across countries will contribute to a better understanding of the influences of climate change on aflatoxin contamination risks

(Bandyopadhyay et al. 2016). Information on the interaction between the environment and the fungal population size and structure will allow the implementation of aflatoxin management strategies based on weather events, which may include a requirement for selection of atoxigenic VCGs adapted to both hotter, dryer climates and changes in cropping cycles (Cotty and Jaime 2007).

7. Losses for bird, insect, or rodent consumption

Using grains as carriers for biological control isolates consistently reduces aflatoxin contamination in field crops (maize, cotton, groundnut, chili peppers), where closed canopies create conditions of humidity for good sporulation of the biocontrol product when applied at the right time. However, when conditions are not conducive for rapid sporulation, the product is exposed to predation by birds, insects, ants, and rodents (Cotty et al. 2008). Under conditions found in tree nut orchards, predation of the biocontrol product is even greater since the ground is frequently bare of vegetation, leaving the product exposed to animal and insect predation. Preliminary studies conducted in California, at Kearney Agricultural Research and Extension Center, indicate that besides ants, other arthropods (mainly *Oniscidea* spp.) are implicated in the rapid loss of formulated grain carriers (Michailides et al. 2018). Dissimilar agricultural management practices (e.g., crop densities, irrigation strategies, tillage, and maintenance of vegetative cover) are essential factors affecting the fauna found in the orchard. Furthermore, grain carriers could spoil when exposed to excessive moisture in the soil, a condition that favors rapid colonization by other fungi (e.g., *Fusarium* spp.), thus impeding the sporulation of the biocontrol agent(s). Biocontrol efficacy can be further enhanced with innovations in formulations that improve the efficiency of sporulation and reduce the proportion of grains lost due to predation.

8. Concerns posed for using biocontrol products

Isolates used in biocontrol formulations belong to ancient, highly stable atoxigenic VCGs carefully selected through carefully designed and elaborate microbiological, chemical, molecular, and field studies (Agbetiameh et al. 2019; Atehnkeng et al. 2014; Bandyopadhyay et al. 2019; Grubisha and Cotty 2015; Mauro et al. 2015). Well-planned studies have demonstrated that members of an atoxigenic VCG do not exchange genetic material with members of other VCGs (either toxigenic or atoxigenic) despite plenty of

opportunities for exchange of genetic material in both treated and non-treated areas (Grubisha and Cotty 2010, 2015; Ortega-Beltran et al. 2016; Islam et al. 2018). Clonality is the predominant mode of *A. flavus* reproduction. Recombination events between members of toxigenic and atoxigenic VCGs have been reported only in laboratory and field studies under specific conditions (Horn et al. 2009; Horn et al. 2009; Olarte et al. 2012; Moore et al. 2013; Horn et al. 2014) and is rare in nature. Therefore, the risk for recombination and generation of toxigenic variants when applying atoxigenic isolates in the field is minimal.

No major health problems have been reported due to the use and application of biocontrol agents by field workers, but it can cause allergic reaction in some individuals. In general, workers should avoid a prolonged contact with the product through the skin and eyes, avoiding those periods in which a high concentration of spores is expected.

9. Conclusion

Whereas some researchers have critical opinion regarding biocontrol of aflatoxins (Njoroge 2018; Ehrlich et al. 2015; Kagot et al. 2019; Ndemera et al. 2020; Pitt 2019), substantial research conducted with field and tree crops has shown that using atoxigenic isolates of *A. flavus* as biocontrol agents is one of the most effective pre-harvest management strategies for reducing aflatoxin contamination (Figure 3). The extensive research demonstrating the efficacy, safety and benefits of the technology have allowed registration of several aflatoxin biocontrol products with regulatory authorities. Following registration, large-scale use is possible after developing infrastructure to produce the biocontrol product on a large-scale, developing sound commercialization strategies, transferring the technology to appropriate industries, and implementing innovative marketing and distribution channels (Cotty et al. 2007; Schreurs et al. 2019; Bandyopadhyay et al. 2019). It is worth mentioning that this management strategy is especially useful in food-insecure regions with rapidly growing populations in Africa, which also suffer major losses in agriculture due to pests and diseases (Savary et al. 2019), and where farmers' access to other control methods is much more restricted. Hundreds of thousands of farmers and diverse crop industries benefiting from the technology consider biocontrol products as vital tools to produce crops with reduced aflatoxin contamination. The use of biocontrol and other aflatoxin management strategies allow farmers to produce safe crops for their own consumption and/or to sell to premium markets.

Aflatoxin biocontrol products have been developed for use in a fraction of the crops susceptible to aflatoxin contamination. Adapting, testing, and validating the biocontrol technology for use in other susceptible crops (e.g., sesame seed, hazelnut, millet) would provide health, trade, and economic benefits for producers, consumers, and processors of these crops.

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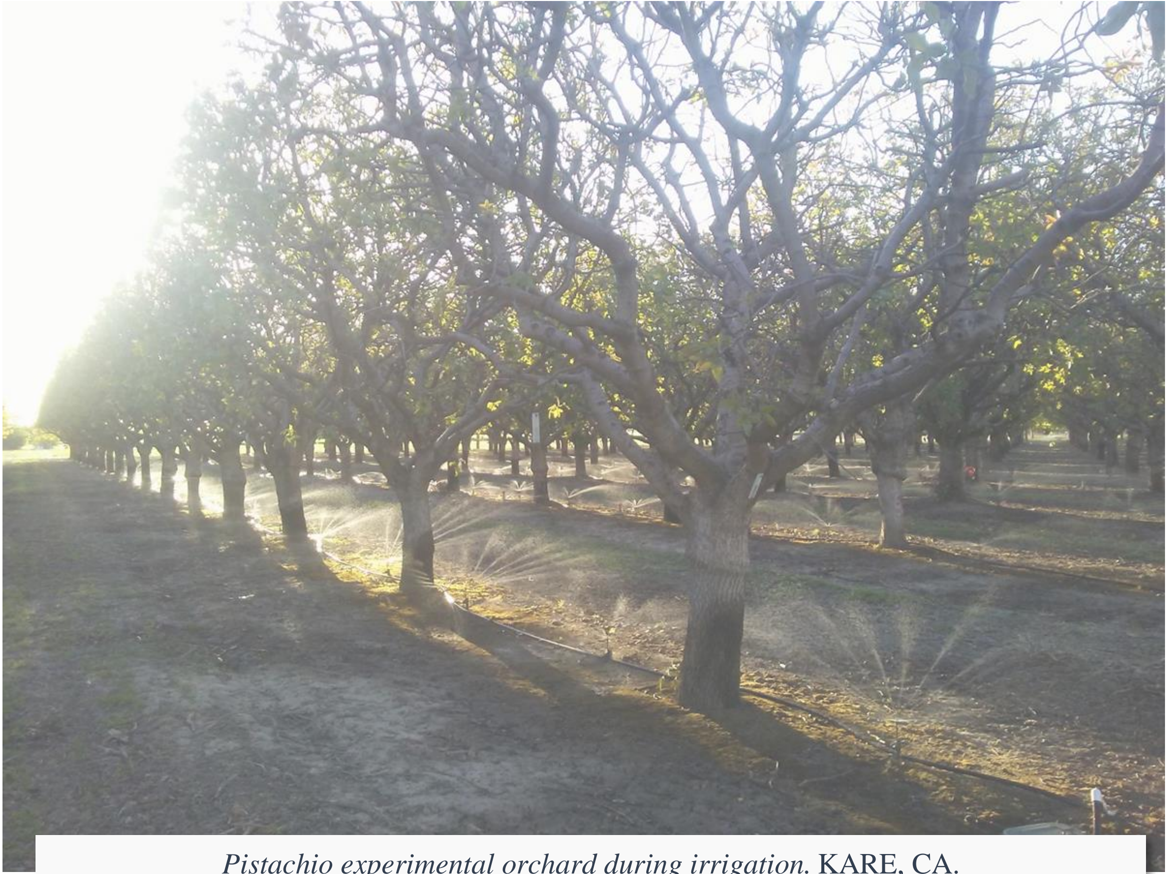

Pistachio experimental orchard during irrigation. KARE, CA.

Ph.D. Thesis

Biocontrol agents to reduce aflatoxins in nuts: inoculum dynamic studies, varietal resistance to the pathogen, and characterization of the population of Aspergillus spp. section Flavi in Spain

CHAPTER II

BIOCONTROL OF AFLATOXINS IN NUTS: INOCULUM DYNAMIC STUDIES

SPORULATION AND DISPERSAL OF THE BIOLOGICAL CONTROL AGENT *ASPERGILLUS FLAVUS* AF36 UNDER FIELD CONDITIONS IN NUT CROPS IN CALIFORNIA

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Abstract

Aflatoxins are secondary fungal metabolites and potent carcinogens produced by *Aspergillus flavus* and *A. parasiticus* that occasionally contaminate pistachio nuts in the field. The international markets reject pistachio nut shipments when aflatoxins exceed permitted maximum levels, causing economic losses for the pistachio processors and farmers. The biological control approach based on the massive spread of the atoxigenic strain AF36 of *A. flavus* stands out among the integrated management practices. Since 2017, the product AF36 Prevail®, sorghum grains coated with AF36 propagules, has been commercially used in pistachio in California. However, many grains of the AF36 Prevail® fail to sporulate in orchards, thus reducing the efficacy of the biocontrol product. Here, we studied the effect of soil moisture on the percentage of sorghum grains showing AF36 sporulation (SG) and the quantity of spores per grain using a sporulation index (SI). Under controlled conditions, SG was higher than 85% when soil moisture was ≥ 13%. Likewise, the SI increased with increasing soil moisture from 8.4 to 21% and then decreased. In the field, the best AF36 sporulation (SG and SI) values corresponded to the sorghum grains located near the micro-sprinklers but were non-impacted by the irrigation water drops. The AF36 Prevail® loss was more pronounced in the non-tilled ground than in the tilled due to quick predation by arthropods. When we studied the dispersal of the AF36 spores, the density of spores decreased markedly with the height and distance from the inoculum source, the pattern of decrease fitting well to diffusion equations. Even so, the spores of AF36 reached easily the canopies of the pistachios located 10 m from the inoculum source. Our results point out that AF36 Prevail® should be applied close to the micro-sprinkler line, in the moist soil area, but avoiding both the area where the irrigation water impacts the grains directly or where there are puddles of water. Due to increased predation in no-tillage fields, an increased rate of the AF36 Prevail® product per hectare is recommended. This work has contributed to optimizing the biocontrol AF36 Prevail® application approach in tree-nut-producing areas of California in terms of retaining more inoculum in the field and treatment-cost savings.

Keywords: AF36 Prevail®, *Aspergillus flavus*, biocontrol, *Pistacia vera*, sporulation

1. Introduction

Aflatoxins are mycotoxins generated from the secondary metabolism of fungal growth and are among the most potent natural carcinogens (IARC 2002). The ubiquitous and saprophytic fungal species Aspergillus flavus and A. parasiticus are the principal aflatoxin producers (Amaike and Keller 2011; Klich 2007). These fungi can colonize and contaminate with aflatoxins numerous crops, including pistachio (Donner et al. 2015; Doster and Michailides 1994a; Ortega-Beltran et al. 2019). Aflatoxins can be classified into Blue (B1 and B2) and Green types (G1 and G2), depending on their fluorescence under UV light (Wu et al. 2011). Aflatoxin B1 is the most important due to its demonstrated carcinogenic properties in humans and its frequent presence in many foods, so the World Health Organization (WHO) classifies this as a class 1 carcinogen (Bbosa et al. 2013).

Californian pistachio nuts, included in California's top-10 valued commodities, are eventually rejected by the international trade when aflatoxins exceed permitted maximum levels, causing substantial economic losses for farmers (USDA 2020). The major exporting destination of Californian pistachios is the EU, which establishes maximum levels for aflatoxins in nuts at 8 and 10 µg/kg for aflatoxin B_1 and a total of aflatoxins (USDA 2018).

The *A. flavus* population comprises aflatoxin-producing isolates, producers mainly of B-type aflatoxins, and non-aflatoxin producers, also known as atoxigenic. Although exceptions, the *A. parasiticus* strains are toxigenic and produce the four types of aflatoxins (Carbone et al. 2007; Frisvad et al. 2019). During the last two decades, researchers have made considerable efforts to understand the populations and biological cycles of *A. flavus* and *A. parasiticus* and to identify the main risking factors in nut trees (Abbas et al. 2009; Abrar et al. 2013; Medina et al. 2017; Palumbo et al. 2014). Overall, both *Aspergillus* species overwinter saprophytically in orchard plant debris, mummies on the tree canopy, litter on the ground, and like sclerotia in/on the soil of nut orchards (Doster and Michailides 1994b; Horn 2003). When environmental conditions become conducive, the fungus develops long chains of airborne asexual spores on a spherical head on top of freestanding conidiophores (Payne 1998); the spherical conidia have a 3 to 6 µm diameter, are light, and dry (hydrophobic) (Abbas et al. 2009).

In the case of pistachio, a low (2-5%, occasionally 10%) of the nuts have their seed (kernel) exposed directly to airborne spores of *Aspergillus* and insect infestation. This natural and premature splitting of both the shell and the hull along the fruit suture results in the development of nuts called "early splits" (Doster and Michailides 1994a). In addition, 1-2% of the nuts can be damaged by pests in California, mainly the Lepidopteran navel orangeworm (*Amyeloys transitella* L.) (Palumbo et al. 2014). Subsequently, the control of aflatoxin in pistachio preharvest should cover cultural practices to reduce the percentage of early splits (e.g., avoiding water stress during the spring and selecting proper rootstocks and cultivars) (Doster and Michailides 1994a; Doster et al. 2001; Moral et al. 2021) and adequate control of pests primarily to reduce the damage (Wilson et al. 2020). Furthermore, the massive spread of the atoxigenic strain AF36 of *A. flavus* stands out among the strategies used to reduce aflatoxin contamination in nuts (Doster et al. 2014; Ortega-Beltran et al. 2018). After releasing in the orchard, the AF36 strain displaces the toxigenic wild isolates of *Aspergillus*, reducing aflatoxin contamination of the pistachio nut shipments (Moral et al. 2022). Thereafter, in 2012, the commercial product *Aspergillus flavus* AF36 on wheat grains was registered for use in pistachio in California. In 2017, an improved application method, sorghum grains coated with AF36 propagules that are applied on the ground at the rate of 11.2 kg/ha (AF36 Prevail®), was approved for the same use (Ortega-Beltran et al. 2016). However, AF36 Prevail® was developed for row crops (i.e., corn and cotton), and it has not been accurately adapted to nut trees, where sorghum grains frequently fail to sporulate at high levels (Garcia-Lopez et al. 2018).

Considering that spores of atoxigenic strain AF36 displace those of the toxigenic wild isolates in the soil and subsequently from the susceptible plant tissues, we need to understand the spores dynamic of the biological control agents in each crop (Horn et al. 2001). In the case of pistachio, the susceptible early splits are located at 2-5 m height from the orchard ground, but no research has been conducted on how the AF36 spores reach these nuts. Knowledge of the dispersal of A36 spores in the field is essential for calculating the AF36 Prevail® rate and the site for the proper application in the field. Recently, Ching'anda et al. (2022) conducted a study in pistachio orchards in Arizona, where AF36 Prevail® was regularly applied at a rate of 11.2 kg/ha, and determined that *A. flavus* propagule densities were maximum during the nut development-maturation

from June to August. These authors also quantified the maximum AF36 propagules in the leaves from the medium-upper pistachio canopies.

This research aimed to optimize the use of AF36 Prevail® in pistachio orchards in California. For that, we studied the effect of soil water content on *A. flavus* AF36 sporulation under controlled conditions. We then took this concept to the field to find the optimum distance placement of AF36 Prevail® from the micro-sprinklers. Subsequently, we studied the dispersal of spores of the atoxigenic *A. flavus* AF36 strain in both the vertical and horizontal directions in a pistachio orchard to determine the ability of this strain of *A. flavus* to reach the susceptible early split pistachio nuts. A preliminary report of this study has been published (Garcia-Lopez et al. 2018).

2. Material and methods

2.1. Effect of soil water content on sporulation of A. flavus AF36 strain

Controlled conditions. To study the effect of the soil water content on the sporulation of AF36 strain on the Prevail® product, we conducted an experiment using 250-ml pots, each containing 150-200 g of natural soil collected at the Agricultural Research and Extension Center (KARE) of the University of California in Parlier, California. Parlier has a cold semi-arid climate (BSk) with hot and dry summers presenting some Mediterranean characteristics (Csa) according to the Köppen-Geiger climate classification (Peel et al. 2007). The KARE's soil is classified as a Typic Xerofluvent with a sandy-loam texture. The soil moisture was gravimetrically determined. Briefly, 10 pots with the soil were oven-dried at 65ºC for 4 days and weighed. Subsequently, the pots were abundantly irrigated, and once the leaching had ceased (field capacity), the pots were again weighed. The water retention capacity of the soil was perceptually calculated by considering the difference in weight of the soil sample at field capacity and dried [WRC = (Soil at field capacity-Dried Soil)/Soil at Field Capacity×100] (Villalobos and Fereres 2016). A group of five 250-ml pots with 130 g of soil were subjected to either 8, 13, 17, 21, or 25% soil moisture levels. From 15 to 20 sorghum grains (AF36 Prevail®) were placed in each pot covered using a folded dense gauze attached with an elastic band. The pots were placed in growth chambers at 30ºC in the dark. The sporulation was recorded after 7 days of incubation using a sporulation index (SI). The SI was calculated for each replicate with the formula $SI = (\Sigma ns \times s)/N$, where s represents sporulation (0 to 4), ns is the number of

sorghum grains with the sporulation of s, and N is the total number of grains. Rating sporulation values were 0 = no visible sporulation; 1 = visible sporulation, covering less than a 25% of the grain surface; and 2 = 25 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of the entire grain surface. Also, we calculated the percentage (sporulated vs. total) of sorghum grains (SG) showing AF36 sporulation. Previously, we showed a linear and positive ($R^2 = 0.811$; $P < 0.001$) relationship between the number of spores produced by AF36 strain on the sorghum grains and the SI (Garcia-Lopez and Moral, *unpublished*). For this reason, and to prevent the spread of AF36 spores because of the handling of Prevail® grains, we used the SI as a dependent variable. There were five replicated pots for each soil moisture, and the experiment was conducted twice. Regression analyses were applied to the pooled means of SI and percentage of seeds showing AF36 sporulation. Both experimental repetitions were individually studied since the data showed different trends. Various linear and non-linear regression models were evaluated to describe the relationship between SI and soil moisture. Among the non-linear equations, we tested the Analytis Beta Model, 3-P and 4-P Gompertz, Gompertz modified by Zwitering, Richards, and Weibull (Hau and Kranz 1990; Campbell and Madden 1990; Zwietering et al. 1990).

The Ratkowsky square-root model (Ratkowsky et al. 1983) was selected because it provided a good fit for the individual experiments and their combination. The Ratkowsky model uses the following equation:

$$\sqrt{SI} = a(W - W_{\min})\left[1 - e^{b(W-Wmax)}\right] \quad \text{(Equation 1)}$$

in which SI = Sporulation Index, W = soil moisture (%), and a and b are unknown shape parameters. W_{min} and W_{max} were the minimum and maximum soil moisture for AF36 sporulation (0 and 30%, respectively). Linear regression was applied to test the relationship between data estimated by non-linear regression and the observed data. In the present study, regression models were chosen from many combinations of terms based on the significance of the estimated parameters ($P \leq 0.05$), Mallow's Cp statistic, Akaike's information criterion modified for small data sets, the coefficient of determination (R^2), R^2 adjusted for degrees of freedom (Ra^2), and the pattern of residuals over predicted and independent variables.

Furthermore, the Gompertz model was selected to evaluate the relationship between soil moisture and the percentage of sorghum grains (SG) showing AF36 sporulation (0 or 1) according to the equation:

$$SG = Y_m e^{-e^{(a-b\times W)}} \quad \text{(Equation 2)}$$

in which W = soil moisture (%), a and b are unknown shape parameters, Y_m is the Y asymptotic. Data from this and the rest of the experiments were analyzed with SPSS (version 14; SPSS Inc., Chicago, IL, United States) or Statistix 10 (Analytical Software, Tallahassee, FL, United States).

Field conditions. Because many grains of the AF36 Prevail® failed to produce sporulation under field conditions (Garcia-Lopez et al., 2018), we studied the optimum distance placement of the product from the micro-sprinklers with a final goal in mind to develop a recommendation to the farmers regarding the proper site for applying the grain inoculum of the biocontrol agent. The study was conducted in an experimental pistachio orchard at the KARE. The pistachios [Kerman (female) and Peters (male)] were planted in 1994 with a spacing of 8 × 6 m. The soil of the experimental plot was managed under no-tillage conditions. No herbicides were applied during this experiment. The plot was irrigated during the growing period of the tree with 6000 m^3 of water per hectare and year using 65-L/h micro-sprinklers. On 28th August 2017, we placed 100 AF36 Prevail® grains into 25-cm circumferences marked on the soil, which centers were at 25, 50, 100, 150, 200, or 250 cm from a micro-sprinkler in both directions north and south, in the perpendicular to the irrigation tube. The experiment was repeated three times, using three different micro-sprinklers located in three other pistachio rows in the same experimental orchard. The SI and the SG were recorded 10 days after applying the sorghum grains. We also measured the gravimetric water content of the soil at each position by weighing soil samples of each position as described above. The experiment was repeated twice in 2018: from 14th June to 12th July and from 12th July 2018 to 31st July (i.e., 28 and 16 days of the grains exposure on the ground, respectively). At the end of the trial, we quantified and collected all the remained grains from the ground and placed them into a 100-mL Erlenmeyer flask with 25 mL of Tween 80 (0.1%) solution in sterile water. Flasks were vigorously shaken for 30 seconds by hand to remove the AF36 conidia from the grains, and different serial water dilutions (from 1:10 to 1:10000) were prepared. Finally, we selected one dilution of each experimental unit attending to the visual density of the

spores in the water, and 300 µl of this were transferred to three 9-cm Petri dishes (100 µl per dish) with SI8 medium (containing 10 g of sucrose, 60 g of NaCl, 1 g of yeast extract, 0.1 g chloramphenicol, 8 ml of a dichloran solution in 2% ethanol, 1 ml of $CuSO_4 \cdot ZnSO_4$ solution, and 15 g of Bacto agar in 1 liter of deionized water). Petri dishes were incubated for 7 days at 30 ºC. We calculated the total AF36 spore quantity, the loss of AF36 Prevail® grains, and the soil moisture at each sampling site, 1 h after the last irrigation. The data of SI as affected by the distance of the sorghum grains from the micro-sprinklers, the total spore production, and the percentage of sporulated grains were analyzed with the nonparametric Kruskal-Wallis' tests, and means were compared with the Dunn's test at P = 0.05. In 2018, both repetitions were compared using a nonparametric two-way Friedman test using the distance to the micro-sprinkler and the repetition as dependent variables.

2.2. Loss of AF36 Prevail® under field conditions

To monitor the loss of AF36 inoculum under field conditions, we placed 300 sorghum grains (AF36 Prevail®) on 3 m^2 of soil surface (1 m^2 in the four cardinal directions with 100 grains each) under the canopy of a 35-year-old pistachios cv. Kerman of a non-tillage plot at KARE Center in August 2017. The grains were quantified at 3, 9, and 11 days after application. The experiment was conducted using two pistachio trees used as experimental blocks. Because most of the grains had disappeared before the 9th day of evaluation, we repeated the experiment in August 2018, but we counted the grains at 0.75 (18 h), 1, 2, 3, and 7 days after application. This latter year, the experiment was conducted using two pistachio trees in the non-tillage plot 64 m away from one another. We repeated the experiment in another experimental plot of the KARE Center. The topsoil layer was mechanically tilled for weed control. Furthermore, a video camera (BirdCam, Wingscapes, Calera, AL, USA) was placed to monitor the feeding behavior of organisms on soils where the biocontrol product was placed.

2.3. Dispersal of spores of the strain A. flavus AF36

Vertical dispersal of spores of the strain A. flavus AF36. The effect of height on spore density of *A. flavus* AF36 was studied in a pistachio orchard at the KARE Center. For that, 200 sorghum grains (AF36 Prevail®) were incubated in humid chambers (100% relative humidity; 30ºC) for 7-10 days until plenty sporulation of the AF36 strain (SI = 4)

on the grains developed. Subsequently, the sporulated grains were placed under the canopy of a 35-year-old pistachio tree cv. Kerman. Two days later, a spore trap was established over the sporulated grains. The spore trap was built using a metallic step ladder with steps at 32, 92, 150, and 234 cm above the ground. Eight 9-cm open Petri dishes with 50 ml Aspergillus Differentiation Agar medium (AFPA Fluka, Steinheim, Germany) were placed at each high using a metallic grid (80 cm × 40 cm) attached to the ladder's steps. After 24 h, the Petri dishes were collected and incubated in the dark for 5 days at 31ºC. Colonies of *Aspergillus* were identified by morphological examination using a dissecting microscope or, when needed, a compound microscope. Isolates of *A. flavus* were confirmed by the reverse distinctive orange reaction on AFPA (Pitt et al. 1983). Isolates unidentified on AFPA plates were transferred to Czapek yeast agar (CYA; containing 49 g of Czapek solution agar, 5 g of yeast extract, 1 ml of $CuSO_4 \cdot ZnSO_4$ solution in 2% ethanol, and 1 liter of deionized water) and identified according to Klich (2002). Only *Aspergillus* colonies of *A. flavus* and those of the section *Nigri* were quantified. Twenty-five isolates of *A. flavus* were confirmed as the AF36 strain by using a specific Mismatch PCR, according to Ortega-Beltran et al. (2016). The experiment was conducted twice in June and July 2017 and again in June 2018. A model adapted to the mass diffusion theory, considering AF36 conidia in the air as a suspension, was selected to evaluate the relationship between the vertical distance from the inoculum source and the sum of AF36 Colonies Forming Units (CFU) quantified at each high (ladder steps) according to the equation:

$$Y = D - \frac{Dz}{\sqrt{r^2+z^2}} \qquad \text{(Equation 3)}$$

in which D = diffusion coefficient, z is the vertical distance from the ground, and r is the initial focus radio.

Horizontal dispersal of spores of the strain A. flavus AF36. Because AF36 strain reduces the aflatoxin contamination in pistachio crops by excluding the toxigenic wild isolates or displacing them from the pistachio canopy (Doster et al. 2014; Moral et al. 2021), we evaluated the horizontal dispersal of its spores in a pistachio orchard [cvs. Kerman (female) and Peters (male)] planted in 1994 at KARE Center. The distance between trees in a row was 8 m, and between rows was 8 m (156 trees ha^{-1}). To this end, we applied 1 kg of Prevail® under a female pistachio tree (the only source of AF36 inoculum in this orchard). The spore densities were quantified in the canopy of this tree (0 m). Likewise,

we quantified the AF36 spores in eight trees planted at distances of 8, 24, 56, or 78 m from the treated tree 10 days after AF36 application. Four pistachio trees were used in every direction from the inoculum source - north, northeast, northwest, south, southeast, southwest, east, and west, including the central tree where the inoculum was placed underneath. In total, 33 pistachios trees were examined.

To quantify the AF36 spores in the canopy of these trees, we collected 10 leaflets of each tree and placed them into a plastic bag with 25 ml of a sterile water solution of 0.1% Tween 80. The bags were vigorously shaken for 30 seconds. The washing water was then transferred to 50-ml Falcon Cone Tubes centrifuged at 2400 rpm for 10 min. The supernatant was discarded, leaving the cone tube with 5 ml of water solution, and the pellet in each tube was re-suspended by Vortexing for 10 seconds. We distributed 2 ml from each tube to 10 Petri dishes (200 µl per plate) containing Si8 medium, and the plates were incubated for a week at 30 ºC. Identification of the fungal colonies was conducted as described above. We conducted five independent evaluations of the density of AF36 spores on the canopy of the pistachio trees. Also, we quantified the density of *A. flavus* spores on the canopy of a pistachio tree cv. Kerman outside of the experimental plot. Finally, the power law, different exponential models, and a model stemming from the mass diffusion equation, which describe the influence of distance from the source of inoculum on the density of trapped conidia, were tested (Aylor 1995, 1999). Finally, we selected a model stemming from diffusion theory to evaluate the relationship between the horizontal distance from the inoculum source and the *A. flavus* AF36 Colonies Forming Units (CFU) quantified according to the equation:

$$Y = \frac{h * T}{2\pi(h^2 + X^2)^{3/2}} \qquad \text{(Equation 4)}$$

in which h = diffusion point height, T is total sporulation time, where the rate of sporulation is considered constant

3. Results

3.1. Effect of soil water content on A. flavus AF36 sporulation

Controlled conditions. We observed sporulated sorghum grains at all soil moisture values studied. However, when the soil moisture was 8%, the incidence of the sporulated grains (SG) was 30.2%, while when the soil moisture was ≥ 13, the SG was 85%. Likewise,

Sporulation Index (SI) increased with increasing soil moisture from 8.4 to 21% and then decreased until a maximum of soil moisture of 25.2%. The Ratkowsky model described well the influence of soil moisture on SI for both experimental replicates (R^2 = 0.810 and R^2 = 0.916, respectively, P < 0.001; Figure 1a).

Furthermore, there was a notable increase in the percentage of SG (from 35 to 85% of SG) when the soil moisture went from 8.4 to 12.6%, respectively. When soil moisture was higher or equaled to 16.8%, the percentage of SG was over 90%. According to the fitted Gompertz model, the maximum percentages of SGs (model asymptotes) were 91.7 and 98.8% for both repetitions (Figure 1b).

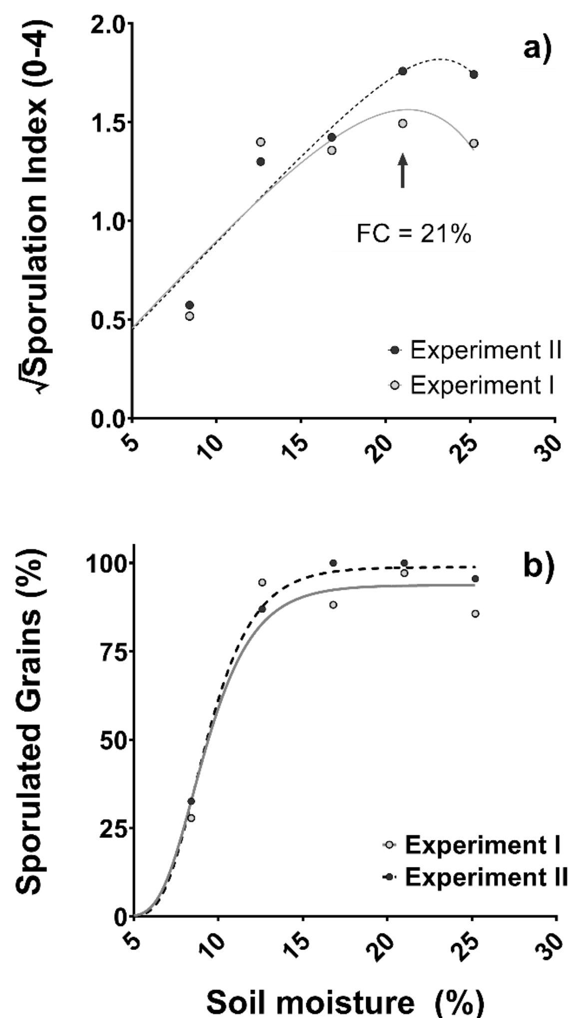

Figure 1. Effect of soil moisture (%) on *Aspergillus flavus* AF36 Sporulation Index (a) and *A. flavus* AF36 Sporulated grains (b). Each experimental data set was treated separately and adapted to the Ratkowsky model (a) and Gompertz (b). Dots are pooled mean of five sub-repetitions of each soil moisture evaluated.

Field conditions. Field soil moisture ranged from 4.7 to 30.7% in the surroundings of the sorghum grains applied to evaluate their sporulation capacity at different distances from the micro-sprinkler in the field. We observed that soil moisture (%) decreased markedly from 150 to 250 cm of distance to the micro-sprinkler in the three experiments. In 2017, sorghum grains (AF36 Prevail®) located at 50 cm from micro-sprinklers showed the maximum SI, significantly ($P < 0.05$) higher than those demonstrated by the grains at 150 and 200 cm, which were in the area of the impact of the irrigation drops. Both the SI and the SG (%) had a similar pattern (Figure 2b). Soil moisture remained constant (19.01±2.66%) from 25 to 150 cm from the micro-sprinkler but dropped (min 12,65%) at 200 and 250 cm (Figure 2a).

In 2018, significant differences were observed between both experiments, with sporulation being much higher (17-fold) in the experiment where the grains were released in mid-July than in those delivered in June ($P < 0.001$); even when the last grains were exposed for 12 days longer than the first ones. In the first experiment of 2018, the AF36 strain developed significantly ($P < 0.05$) more spores in AF36 Prevail® located at 25 and 50 cm from micro-sprinklers than those at 250 cm distance from the sprinkler. The grains situated between 100 and 200 cm from the micro-sprinkler formed an intermediate group according to the sporulation of the biological control agent. In the second trial, the AF36 strain produced a similar ($P = 0.793$) quantity of spores in AF36 Prevail® regardless of its relative position to the micro-sprinkler (Figure 3a). Data pointed out that the best AF36 sporulation values were obtained in the sorghum grains located from 25 to 100 cm from the sprinkler with high soil moisture values but far from the impact of the irrigation droplets.

In 2018, the loss of AF36 Prevail® ranged between 21.5 and 83.7%, with maximum losses at minimum soil moisture values (7.5%). In the first trial of this year, grain losses were similar independently of grain distance from the micro-sprinkler. Conversely, the grain losses increased with distance from the micro-sprinkler as soil moisture dropped in the second trial (Figure 3b). A pattern similar to the one described above was observed when grain losses were expressed per day of exposure (*data not shown*).

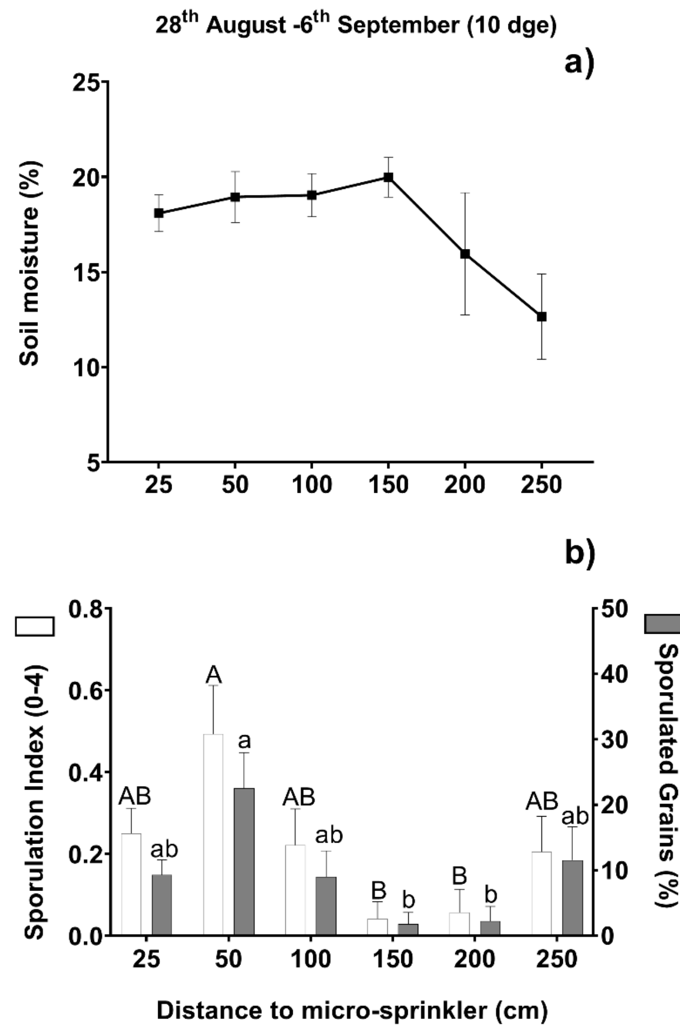

Figure 2. Effect of the placement of AF36 Prevail® at different distances (cm) from the micro-sprinkler with different soil moisture percentages (a) on the *Aspergillus flavus* AF36 Sporulation Index (SI) and percentage of Sporulated Grains (SG) (b) in 2017. Letters on bars indicate statistical differences according to the nonparametric Kruskal-Wallis's tests followed by Dunn's test at P = 0.05.

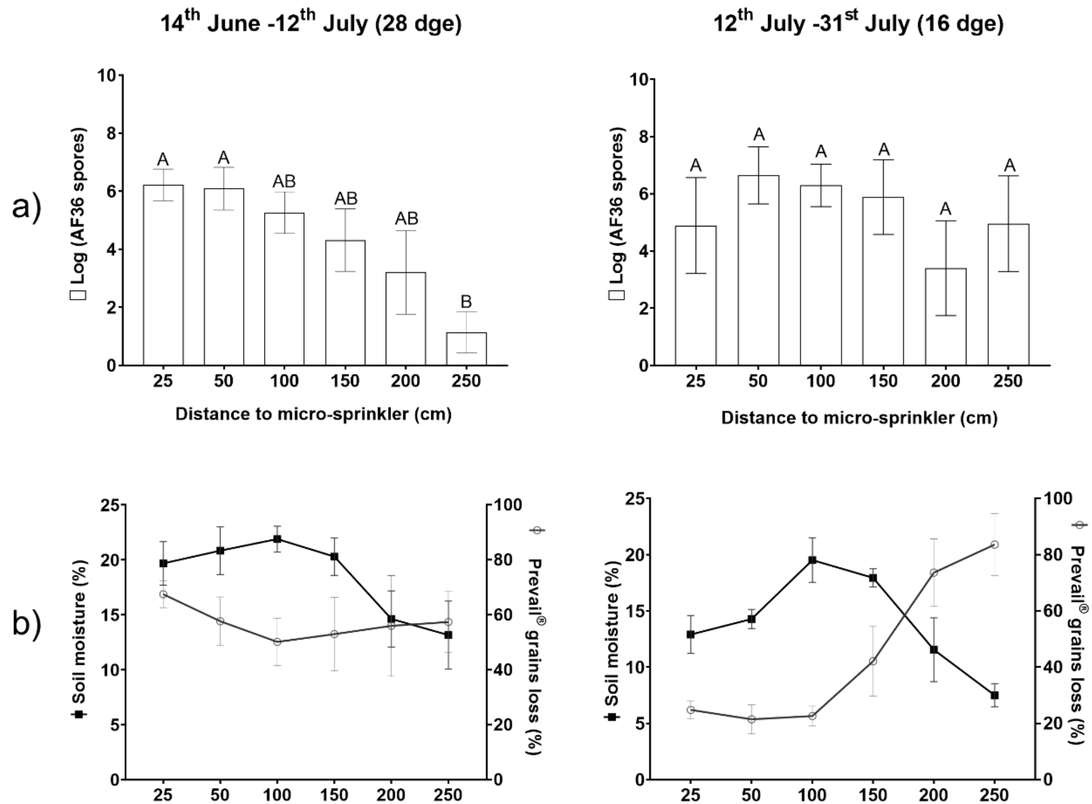

Figure 3. Effect of the placement of AF36 Prevail® at different distances (cm) from the micro-sprinkler on AF36 spores production (a) and the AF36 Prevail® loss (%) related to soil moisture percentage (b) in 2018. Dge refers to the number of days of AF36 Prevail® exposure on the ground. Letters on bars indicate statistical differences according to the nonparametric Kruskal-Wallis's tests followed by Dunn's test at $P = 0.05$.

3.2. Loss of AF36 Prevail® under field conditions

In 2017, when the AF36 Prevail® was applied in a plot whose soil was managed according to no-tillage practices, there was a grain loss of 83.3% at 3 days after application, and no grains were found 3 days later (100% loss). Using shorter observation periods, we found that the loss of grains was more pronounced in the non-tilled ground than in the tilled one during the experiment ($P < 0.001$). Indeed, in the second evaluation, one day after applying the AF36 Prevail® on the ground, more than 90% of the grains had already disappeared on the non-tilled plot. A video camera (BirdCam, Wingscapes) was placed to monitor AF36 Prevail® loss due to the soil organisms' behavior. In non-tilled soils, *Oniscidea* spp. and different ant species (not identified) impacted the residence of the

applied product on the soil. Conversely, both arthropods had a minimum impact on the tilled ground.

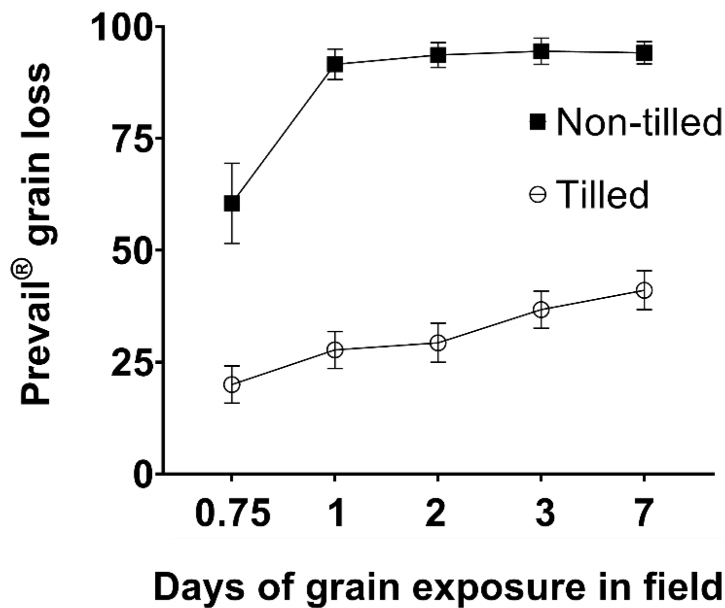

Figure 4. AF36 Prevail® grain loss (%) after the immediate days after placing on the ground under the canopy of pistachio trees cv. Kerman located in an experimental plot with different soil managements, conventional tillage (light dots) versus a non-tilled plot (dark dots).

3.3. Spores dispersal of the strain A. flavus AF36

Vertical spores dispersal of the strain A. flavus AF36. As expected, the density of *A. flavus*' CFU at each height from the ground markedly decreased with the height. The maximum AF36 conidia density, 196 and 347 CFU per Petri dish (June 2017 and 2018), was obtained at 32 cm from the ground. A model stemming from the mass diffusion equation (equation 3) described well the reduction of the density of CFU from the ground to the pistachio canopy (R^2 = 0.652; P < 0.001) (Figure 5). Conversely, the density of spores of the *Aspergillus* spp. section *Nigri* decreased with the high. We detected a similar pattern of spores dispersion in July 2017, with the maximum density of AF36 CFU (148 CFU per Petri dish) at 32 cm from the ground.

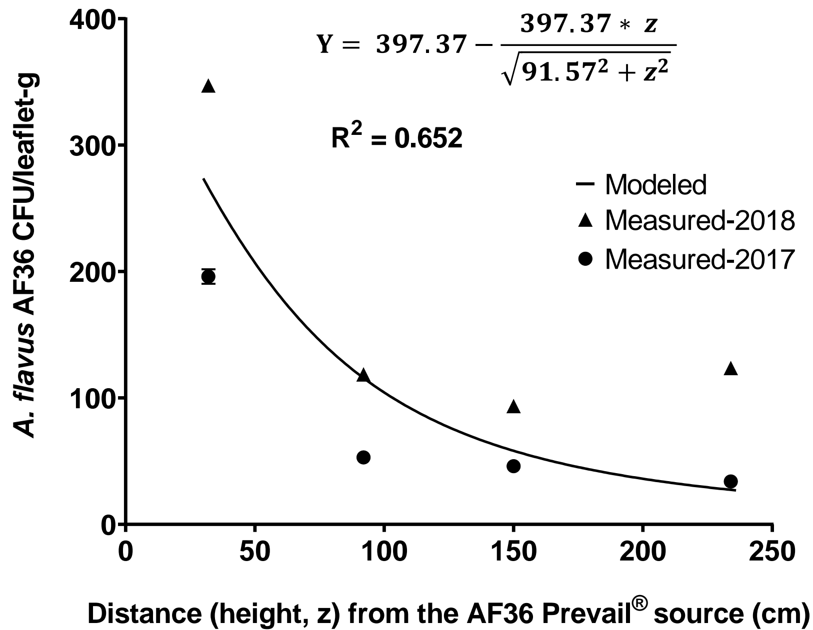

Figure 5. Sum of *A. flavus* AF36 Colonies Forming Units (CFU) per distance from the ground (height, z, in cm) of the AF36 Prevail® inoculum source applied on the ground in June. Two-year study, in 2017 (circular dots) and 2018 (triangular dots).

Horizontal dispersal of the strain A. flavus AF36 spores. A. flavus AF36 spores spread similarly in all directions from the inoculum source. In this case, the *A. flavus* AF36 population algebraically decreased as the distance increased from the AF36 Prevail® inoculum source (Fig. 6) according to equation 4 (R^2 = 0.430; P < 0.001). Thus, the maximum value of CFU per g of pistachio leaflets was obtained on the canopy of the tree underneath which the AF36 Prevail® was applied (421.47 CFU/leaflet-g), but AF36 spores were also caught (24.68 CFU/leaflet-g) on the pistachio trees located at 78 m from the inoculum source. Likewise, dispersion values (i.e., standard error) of AF36 spores caught at different distances were higher (SE = 56.2) close to the inoculum source and close to zero (SE = 6.2) at 78 m, showing that most of the *A. flavus* spores quantified came from the artificial inoculum source.

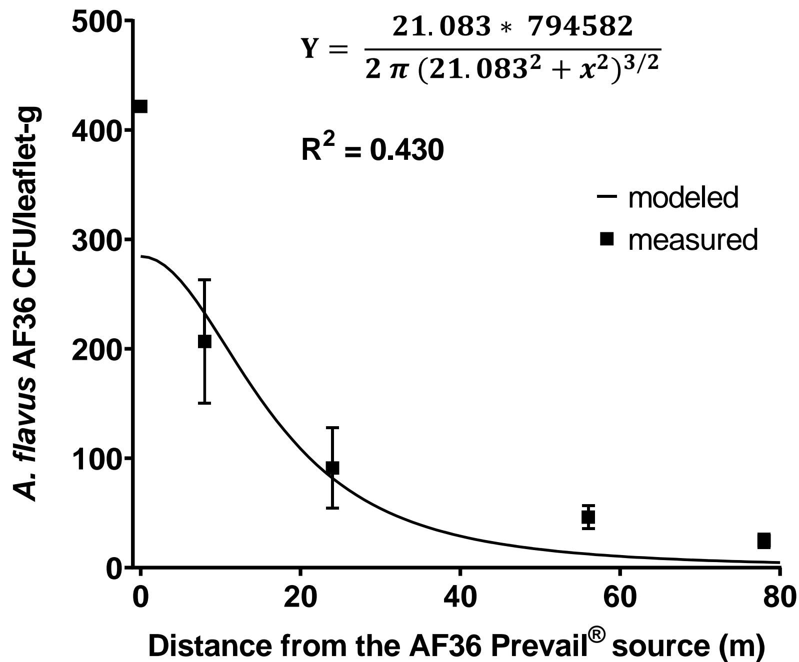

Figure 6. Colonies Forming Units (CFU) of *Aspergillus flavus* AF36 per pistachio leaflet gram as a function of distance (m) from the application site (inoculum source) of the AF36 Prevail®

4. Discussion

Pistachio is a valuable crop in California, the leading producer and exporter worldwide (USDA 2022), while the European is the principal market of this commodity. Occasionally, pistachio nuts are contaminated with aflatoxins (Donner et al. 2015; Doster and Michailides 1994a; Molyneux et al. 2007), which represents a challenge once the product reaches the international trade. Strict thresholds on aflatoxin levels were established worldwide to guarantee human and livestock health (Bui-Klimke et al. 2014), with the European Union market being among the most restrictive (Moral et al. 2020).

Integrated management of the aflatoxin-producer species of *Aspergillus* in the field and post-harvest can maintain aflatoxins under the imposed regulatory levels. In California orchards, this management includes the elimination of inoculum sources, such as pistachio male inflorescences on the ground (Garcia-lopez et al. 2022), avoiding early splits (Doster and Michailides 1994b), and the adequate control of the principal pistachio pest, the navel orangeworm (Palumbo et al. 2014; Pathak et al. 2021; Siegel et al. 2019; Wilson et al. 2020)

Furthermore, one of the most promising actions for limiting aflatoxin contamination is the massive release of the atoxigenic *A. flavus* AF36 strain (Doster et al. 2014; Ortega-Beltran et al. 2018). This biocontrol control agent was commercially applied using wheat coated with AF36's propagules in row crops such as cotton and maize since the 2000s (Cotty and Bayman 1993; Cotty et al. 1994; Cotty and Mellon 2006). In 2012, the US Environmental Protection Agency (USEPA) granted registration of this product for use in pistachio grown in the States of Arizona, California, New Mexico, and Texas since paired field (treated vs. non-treated plots) studies demonstrated the effect of the biocontrol strategy on reducing aflatoxin contamination in pistachio nuts (Doster et al. 2014; Moral et al. 2020). A change in the biocontrol agent carrier to sorghum resulted in registering AF36 Prevail® for use in row crops in 2014; and then in almond, figs, and pistachio in 2017. However, the performance of the biocontrol to reduce aflatoxin contamination in pistachio orchards have differences from the row crops, mainly due to the tree architecture (Doster et al. 2014; Kaminiaris et al. 2020), but still, commercial recommendations on its use are the same. Unlike row crops, for example, AF36 Prevail® applied on the ground in tree nut orchards is: i) more exposed to sunlight; ii) less exposed to wet soil since the micro-sprinklers heterogeneously irrigate the soil, and iii) far (2-5 m) from the susceptible nuts to be protected that are located in the tree canopy.

Trying to minimize these gaps in the biological control strategy in nut-producing fields, through the present research work, we searched for the best performance of AF36 Prevail® in an experimental pistachio orchard, subjected to commercial management practices at the KARE Center. In our experiments in pots, the AF36 Prevail® showed the greatest sporulation values when the soil water content was close to field capacity (21%). Under field conditions, the irrigation guarantees soil moisture close to field capacity, ensuring fungal activity at temperatures between 25 and 37 ºC (Lahouar et al. 2016). Our study allowed us to test AF36 Prevail® performance in the nut fields and provide recommendations on properly applying it in pistachio orchards.

In practical terms, the best values of AF36 sporulation on sorghum grains were reached close to the micro-sprinklers, where high soil moistures were common. However, the application of AF36 Prevail® in soil areas where the irrigation droplets fell directly on the grain was counterproductive due to the drop impact on the grains and the presence of water puddles. In addition, semi-buried sorghum grains and soaked ones colonized by

saprophytic fungi (e.g., *Fusarium* spp.) were common in these flooded zones. In the opposite situation, our experiments showed that grains far from the micro-sprinkler water source did not reach an optimum hydration level, and the AF36 sporulation was reduced or failed. Therefore, our recommendation to farmers is "to spread the AF36 Prevail® product on the moistened soil but avoid the area where the irrigation water impacts using micro-sprinklers". We are aware that many of the new pistachio plantations in the Mediterranean basin, such as in Italy or Spain (Gomez et al. 2022), are rainfed or managed with deficit irrigation by drip (Lansari 2016; Moldero et al. 2022). These agronomical characteristics should be considered when developing formulates for releasing atoxigenic strains of *A. flavus* (Garcia-Lopez et al. Chapter IV present Thesis). In such a case, once the technology is tuned, recommendations for its application will be based on rain forecasting or irrigation management carried out on the plantation.

In our plots, the orchard fauna more actively predating sorghum grains were arthropods of the Formicidae (ants), the Armadillidiidae (pillbugs), and small beetle groups and, much more occasionally, birds, as observed using the BirdCam equipment. As it is well-known, soil diversity is enhanced by non-till practices (Logan et al. 1991). This fact follows our results since sorghum grains disappeared just a few days after applying to the non-tilled plots.

Our dispersal of AF36 spores experiments conducted complemented the insights in this field carried out recently by Ching'anda et al. (2022). Monitoring *Aspergillus* spores is difficult due to their size and dispersion through the air currents (Abdalla 1988; Golan and Pringle 2017). In our experiment, AF36 spores easily reached the canopy of the trees located right above the ground where the grains were applied and decreased by half in the trees at 8 m. These result suggests that the application could be every two rows when the distance between adjacent rows is between 8 and 10 m (a standard distance in pistachio plantations under irrigation conditions), and a similar density of AF36 spore could be obtained on the tree canopies of the non-treated row. However, extending the application distance could lead to inappropriate tree nut canopy protection.

Likewise, we have observed the concentration AF36 spores markedly decreased (thus diminishing their protecting effect) with the vertical and horizontal distance from the inoculum source. Both curves fit well with equations developed according to mass diffusion theory in the absence of flow. This assumption is reasonable given the relatively

short distances in our experiments, even though the effect of the prevailing wind is ignored (Okubo and Levin, 2001).In any case, this assumption allowed us to derive different equations for the horizontal and vertical experiments. To our knowledge, this is the first time diffusion theory has been applied to understand the dispersal of spores as a biological control agent. Therefore, this approach could help us optimize the AF36 Prevail® for area-wide and long-term biological control programs, which are considered the best strategy to reduce the population of aflatoxin-producing isolates for the medium-to long-term (Cotty et al. 2007). For that purpose, however, the diffusion model has to be developed to accommodate turbulent diffusion and the effect of the prevailing winds.

Finally, in our dispersal experiments, we remarkably observed the density of CFU of the *Aspergillus* spp. of the section *Nigri*, considered ochratoxin producers (Cabañes and Bragulat 2018), increased with the height, and it was maximum in the pistachio tree canopy. These results suggest that the debris in the tree canopy may function as a reproduction source for these fungal species, pathogens that, rather sooner than later, will be considered in the biocontrol strategy.

In summary, through this research and considering the differences between row and tree crops, we provided answers to optimize the current biocontrol technique available (AF36 Prevail®) for use in tree nut orchards in California. Also, we modeled the dynamics of the atoxigenic spores when applied to the field by describing the movements from the inoculum source until the susceptible nuts are protected. Finally, we provided valuable recommendations for efficient use of the AF36 Prevail® that led to cost savings related to the biocontrol of aflatoxins in Californian tree nut orchards.

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QUANTIFICATION OF THE AFLATOXIN BIOCONTROL STRAIN *ASPERGILLUS FLAVUS* AF36 IN SOIL, AND NUTS AND LEAVES OF PISTACHIO BY REAL-TIME PCR

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Abstract

The species *Aspergillus flavus* and *A. parasiticus* are commonly found in the soils of nut-growing areas in California. Several isolates can produce aflatoxins that occasionally contaminate nut kernels conditioning their sale. The strain AF36 of *A. flavus*, which does not produce aflatoxins, is registered as a biocontrol agent for use in almond, pistachio, and fig crops in California. After application in the orchards, AF36 displaces aflatoxin-producing *Aspergillus* spp. and thus reduces aflatoxin contamination. Vegetative compatibility assays (VCA) have traditionally been used to track AF36 in soils and crops where it has been applied. However, VCA is labor-intensive and time-consuming. Here, we developed a quantitative real-time PCR (qPCR) protocol to quantify the proportions of AF36 accurately and efficiently in different substrates. Specific primers to target AF36 and toxigenic strains of *A. flavus* and *A. parasiticus* were designed based on a sequence of *aflC*, a gene essential for aflatoxin biosynthesis. Standard curves were generated to calculate proportions of AF36 based on threshold values (Cq). Verification assays using pure DNA and conidial suspension mixtures demonstrated a significant relationship by regression analysis between known and qPCR-measured AF36 proportions in DNA ($R^2 = 0.974$; $P < 0.001$) and conidia mixtures ($R^2 = 0.950$; $P < 0.001$). The tests conducted by qPCR in pistachio leaves, nuts, and soil samples demonstrated the usefulness of the qPCR method to precisely quantify proportions of AF36 in diverse substrates, ensuring important time and cost savings. The outputs of the current study will serve to design better aflatoxin management strategies for pistachio and other crops.

Keywords: aflatoxins, *Aspergillus flavus*, *A. parasiticus*, atoxigenic strain, qPCR

1. Introduction

Aflatoxins are secondary metabolites produced by several *Aspergillus* species, and they are the most toxic and carcinogenic among the currently known mycotoxins (Yu et al. 2004). The four major aflatoxins, B_1, B_2, G_1, and G_2, negatively impact crop quality and its safety. Because aflatoxins are harmful to humans and livestock, crops exceeding the stringent tolerance thresholds are banned from commercialization and consumption in nations that enforce aflatoxin regulations (Grace et al. 2015; Payne 1998).

In California, tree nut crops are economically important commodities destined for both domestic consumption and export, and their production keeps increasing (CDFA 2018; Perez et al. 2017). Tree nut crops such as pistachio and almond are occasionally contaminated with aflatoxins (Doster and Michailides 1994; Palumbo et al. 2014). Aflatoxin contamination in pistachio and almond nuts sometimes exceeds the regulatory limits of 20 or 10 µg/kg for total aflatoxins imposed by the Food and Drug Administration (FDA) in the USA and the European Food Safety Authority (EFSA), respectively. The low tolerance for aflatoxins is a serious concern for Californian growers, and sometimes their nut lots are denied from entering lucrative markets. During the last 10 years, the Rapid Alert System for Food and Feed (RASFF) put in place by the EU reported the border rejection of 86 and 92 loads of almond and pistachio, respectively, coming from the USA (Moral et al. 2020). Therefore, growers in California must implement aflatoxin mitigation strategies to prevent rejection of their crops.

Two fungal species, *A. flavus* and *A. parasiticus*, are the major producers of aflatoxins and both are common in nut-growing areas in California (Donner et al. 2015; Doster and Michailides 1994). The species *A. parasiticus* produces both B and G aflatoxins, while the population of *A. flavus* is composed of both toxigenic isolates, which produce B aflatoxins, and non-toxigenic (i.e., atoxigenic) isolates (Amaike and Keller 2011; Donner et al. 2015; Klich 2007). The populations of each of these species can be divided into vegetative compatibility groups (VCGs) (Bayman and Cotty 1993; Horn and Greene 1995). There are several *A. flavus* VCGs composed exclusively of atoxigenic members and those can be used as biocontrol agents to limit crop aflatoxin contamination in the field (Mehl et al. 2012; Ortega-Beltran et al. 2019).

After several years of research during the 1980s and 1990s, the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) registered the aflatoxin biocontrol product *Aspergillus flavus* AF36 with the US Environmental Protection Agency (USEPA) as the first atoxigenic aflatoxin biocontrol product for use in cottonseed in Arizona (Cotty and Mellon 2006). The active ingredient of that biocontrol product is the strain AF36 (referred from here on as AF36). AF36 was originally isolated in the Yuma Valley, Arizona (Cotty 1989). Subsequently, AF36 was registered for use in maize, pistachio, almond, and fig grown in different USA states (Cotty et al. 2007; Doster et al. 2014; Ortega-Beltran et al. 2019).

Competitive exclusion of toxigenic isolates of *Aspergillus* spp. is the main mechanism through which aflatoxin biocontrol agents decrease aflatoxin contamination in treated crops (Abbas et al. 2011; Cotty et al. 1994; Doster et al. 2014; Mehl and Cotty 2010). Hence, the quantification of AF36 before and after treatment is fundamental for understanding its ability to colonize the target ecosystem and to displace native *Aspergillus* toxigenic isolates. Routinely, tracking of the AF36 strain has been done using vegetative compatibility assays (VCAs) (Doster et al. 2014; Grubisha and Cotty 2015). However, VCA is resource-intensive and time-consuming. Pyrosequencing is another suitable tool to distinguish *A. flavus* genotypes, including AF36, within diverse matrices (Das et al. 2008; Mehl and Cotty 2010; Shenge et al. 2019) but the corresponding equipment is relatively expensive and not commonly used. Quantitative real-time polymerase chain reaction (qPCR) can distinguish various *Aspergillus* species (Luo et al. 2009; Sardiñas et al. 2011) and could be used to distinguish specific genotypes within mixtures of *Aspergillus* genotypes.

Members of VCG YV36, to which AF36 belongs, bear a single nucleotide polymorphism (SNP) that causes an early stop codon in the polyketide synthase (*aflC*) gene, resulting in the inability to produce aflatoxins (Ehrlich and Cotty 2004). Based on the genetic dissimilarity of AF36, that is, having a SNP in *aflC* not present in aflatoxin producers, Ortega-Beltran et al. (2016) honed a multiplex-PCR assay to target the SNP to differentiate AF36 from toxin producers. The assay incorporates an intentional mismatch to destabilize binding by an ‘erroneous’ primer (Kwok et al. 1990) and allows amplification of the PCR product only by isolates containing the SNP. The development of a qPCR assay that builds upon the multiplex assay mentioned above would be an asset

to cope with the shortcomings of VCA. Such method would be valuable to distinguish between crop samples treated with AF36 and those that received no treatment and also to monitor frequencies of the biocontrol agent in any given environment to determine whether the application of the product is necessary.

In the current study, we designed a qPCR protocol to quantify proportions of the aflatoxin biocontrol strain AF36 within samples that contained toxigenic isolates of *A. flavus* and *A. parasiticus*. Results of the current work demonstrates the potential of this specific qPCR assay to be used in a diverse source of substrates (soil, conidial suspensions, fruit or leaf tissues), giving rise to the continuity of epidemiological and competition studies of AF36 in both laboratory and field, since these studies can be conducted using any substrate in a time- and cost-effective manner. Shedding additional light on biology, epidemiology, and ecology of the biocontrol strain will ultimately lead to the design of more efficient aflatoxin mitigation strategies.

2. Materials and methods

2.1. Isolates

The AF36 strain was obtained from the USDA-ARS Laboratory in Tucson, Arizona. The strains *A. flavus* 2A1L-11 and *A. parasiticus* 4C1P-11, native to California, were used as toxigenic strains (Ortega-Beltran et al. 2019). Both toxigenic strains are part of the fungal collection maintained at the University of California, Kearney Agricultural Research and Extension Center (KARE).

2.2. DNA extraction from pure cultures

Each of the three strains (2A1L-11, 4C1P-11, and AF36) were separately grown in Potato Dextrose Broth (PDB) liquid media (Difco Laboratories Inc., Detroit, MI) in Parafilm-sealed volumetric flasks at 25°C for 4 days. Under aseptic conditions, mycelia of each strain were washed with sterile water, air dried, harvested, and transferred into FastDNA tubes containing garnet matrix and a ¼ inch ceramic sphere (as shipped). The FastDNA extraction kit (MP Biomedicals, Irvine, CA) was used for DNA extraction following the method described by Luo et al. (2009). The DNA extracted from each sample was diluted in 35 µl of nucleotide-free water and stored at -20°C for later use.

2.3. DNA extraction from conidia grown on plates and collected from leaves

Conidia of each strain were scraped and harvested from 5-day-old Potato Dextrose Agar (PDA) cultures grown at 30°C to obtain conidial suspensions. In subsequent experiments, conidial suspensions were also obtained from the surface of pistachio leaves by washing them with sterile water. DNA extraction and dilutions were conducted as described above.

2.4. DNA extraction from pistachio nuts

Pistachio nuts were intentionally chosen and picked manually from cv. Kerman trees prior to harvest of a pistachio orchard at KARE. The nuts were split by kernel and hulls and cut into fine pieces using a sterile scalpel, and 0.3 g were placed into a FastDNA extraction tube with 250 µl of the Protein Precipitation Solution (PPS) and 900 µl of the Cell Lysis/DNA Solubilizing Solution for Vegetation (CLS-VF, MP Biomedicals). Samples were ground twice with a homogenizer (MP Biomedicals) for 40 s. DNA extraction and dilution was conducted as described above.

2.5. DNA extraction from soil

Soil samples were collected from treated and non-treated orchards at KARE. Several well-distributed soil subsamples were taken from the first 2 cm of the surface layer to obtain 1 kg of soil. Soil clods were pulverized using a rubber hammer. Then, soil was passed through a sieve No. 20, ATSM E-11 (disinfected with 10% bleach, rinsed, and dried between samples), and stored in clean paper bags until use at 22-30ºC. To extract genomic DNA of *Aspergillus* spp., 500 mg of sieved soil samples were added into the Lysing Matrix E tube (MP Biomedicals). The DNA extraction was performed as described above and DNA of each sample was diluted in 40 µl of nucleotide-free water.

2.6. Specific primer design

We designed two pairs of primers to quantify the proportion of AF36 with respect to toxigenic strains of *A. flavus* and *A. parasiticus*. AF36 contains a naturally occurring mutation conferring atoxigenicity. The single site mutation (G→A) is located at 591 nt in the *aflC* gene (Ehrlich et al. 2004). We used the primer-BLAST designing tool of NCBI. The *aflC* reference sequence for the first pair was that of AF36 and the second was that of toxigenic *A. flavus* strain NRRL3357. Those sequences are publicly available in NCBI and deposited in GenBank as accessions GCA_000006275.2 and GCA_012897275.1,

respectively. The first pair of specific primers, SNP36 Sh2 (5’-GCCTATCGCTGTACAAACTG-3’) and SNP36 Cb (3’-GCTGGGGATCCAGAAC**T**CA-5’, the letter in bold indicates the *aflC* mutation site found in AF36), was used to target AF36 DNA. The SNP36 Cb primer, previously designed by Ortega-Beltran et al. (2016) to identify AF36 using a conventional PCR multiplex assay, incorporates an intentional mismatch at position 593 nt in *aflC* (fifth nt of the primer from the end 5’). The second nt of the SNP36 Cb primer binds to the *aflC* SNP but not to DNA of strains that do not contain the SNP (Figure 1). The combination of SNP36 Cb and the new primer SNP36 Sh2 amplifies a 137 bp amplicon.

Likewise, we designed a new primer pair Fw-nomutB (5’-CTTGGTCTACCATTGTTTG**G**-3’), which first nucleotide, from 3’ end, binds at 591 nt in the *aflC* gene of isolates lacking the AF36 SNP, and Rv-nomut267 (5’-GGTAGGCGTCGTGTCTAG-3’). Isolates lacking the AF36 SNP amplify a 284 bp amplicon.

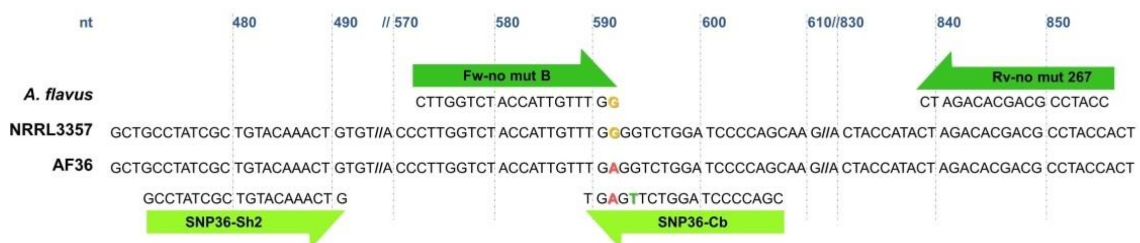

Figure 1. Comparison of portions of the polyketide synthase (*aflC*) gene between a highly toxigenic *Aspergillus flavus* strain NRRL3357 and the biocontrol strain *A. flavus* AF36 showing the single-site mutation (G→A) at the 591 nucleotide that confers atoxigenicity to AF36 and the regions targeted by the two sets of primers used in the current study.

2.7. Conventional PCR

The PCR amplifications were performed in 25 µl volume containing 5 µl of PCR Master Mix (Promega Corp, Madison, WI), 0.8 µl of forward and reverse primer (4 µmol/l each), 2 µl of template DNA (20 ng) and 16.4 µl of water. The following conditions were used: an initial preheat at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. Gradient PCR revealed 64°C as the optimal annealing temperature. PCR products were examined in 1.5% agarose gels in TAE buffer with a reference ladder.

2.8. Real-time quantitative PCR assay (qPCR)

qPCR amplifications were performed with a CFX96 Touch™ instrument (Bio-Rad, Hercules, CA) using SYBR Green I fluorescent dye. Two amplifications for each sample (one for each primer pair) were conducted in 25 µl volume containing 12.5 µl of Brilliant® II SYBR® Green QRT-PCR Master Mix (Stratagene Corp., La Jolla, CA), 2 µl of template DNA extracted from any source described above, 0.25 µl of both forward and reverse primer (4 µmol/l each), and 10 µl of water. The conditions used were the same as described above. After the amplifications were completed, melting curves were obtained based on a standard protocol of the qPCR system (CFX Maestro™ Software) and used to confirm that the signal of the melting curve peak was from the expected target product. The qPCR products were also examined in 1.5% agarose gels in TAE buffer.

2.9. Development of standard curves for DNA and conidia number quantification (qPCR assay calibration)

The primer pair, SNP36 Sh2 / SNP36 Cb (for AF36) and Fw-nomutB / Rv-nomut267 (for 2A1L-11 and 4C1P-11), plus six 10× serial dilutions of DNA of each strain (from 14×10^5 pg to 14 pg) were utilized to generate the standard curves for quantitative detection of the three strains used in the current study. The qPCR conditions described previously were used and the threshold cycle (Cq) values vs the corresponding log quantities (pg) of DNA from two replicates were employed to generate the corresponding standard curve for each strain. The standard curves gave rise to two equations used to calculate the DNA concentration of atoxigenic and toxigenic strains (SCAF36 for AF36 and SCAFP for 2A1L-11 and/or 4C1P-11). We compared samples containing unknown amounts of DNA to our standard curve to calculate DNA concentrations of each strain. Similarly, a standard curve (SCAF36-conidia) was generated by plotting the Cq values obtained from qPCR assays conducted in duplicate with the primer pair SNP36 Sh2 / SNP36 Cb vs the log number of conidia, which was obtained by extracting DNA from serial conidial dilutions of AF36 (from 2.4×10^6 to 24 conidia/ml). A second standard curve (SCAFP-conidia) was obtained by plotting the Cq values from the qPCR assay performed twice with the primer pair Fw-nomutB / Rv-nomut267 vs the log number of conidia, from the extraction of DNA of six serial conidial dilutions of 2A1L-11 and 4C1P-11 (from 3.4×10^6 to 34 conidia/ml). The equations defining these curves allowed estimating the number of conidia/ml of each genotype present in a certain sample.

2.10. Determination of proportion of AF36 in a sample

After qPCR amplification, two Cq values were obtained, one for each of the two primer pairs used in the study. Inserting the Cq value in the pertinent standard curve equation, the amount of DNA for the corresponding genotype present in the sample could be calculated, and the proportion of AF36 was obtained as:

$$\text{AF36 }(\%) = \frac{\text{A}}{\text{A+B}} \times 100 \qquad \text{(Equation 1)}$$

where, "A" is the DNA quantity (pg) calculated from the standard curve generated for AF36 (SCAF36) using the primers SNP36 Sh2 / SNP36 Cb, and "B" is the DNA quantity (pg) calculated by using the standard curve generated for toxigenic *A. parasiticus* and *A. flavus* (SCAFP) by using the primers Fw-nomutB/Rv-nomut267.

2.11. Quantification of the proportion of AF36 (qPCR assay verification)

The designed qPCR was verified using: i) known mixtures of DNA from AF36 and 2A1L-11 or AF36 and 4C1P-11; and ii) known mixtures of conidial suspensions from the same three strains.

DNA mixtures. Different DNA mixtures were prepared by using 14×10^3 pg of DNA of AF36 and 2A1L-11 or 4C1P-11 from pure cultures, comprising 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 99% (wt/wt) of AF36's DNA.

Conidial suspension mixtures. Different conidial mixtures of AF36 and 2A1L-11 or 4C1P-11 were prepared with a final density of 10^7 conidia/ml to further confirm the applicability of the qPCR assay. Nine mixtures with various proportions of AF36 were made of 10, 20, 30, 40, 50, 60, 70, 80, and 90% of AF36's conidia. Then, 200 µl of each mixture were added into a 2 ml FastDNA tube, ground twice with a homogenizer (MP Biomedicals) for 40 s each time, centrifuged at 14,000 rpm for 10 min, and the supernatant was discarded. Next, the DNA of each mixture was extracted as previously described for conidial suspensions.

The qPCR assay was conducted twice to obtain Cq values according to both pairs of primers (SNP36 Sh2 / SNP36 Cb and Fw-nomutB / Rv-nomut267) for each DNA or conidial mixture. SCAF36 and SCAFP equations were used to calculate DNA (pg) with each primer pair for each combination of strains and Equation 1 to determine the

proportion of AF36 in the mixture. Linear regression through the origin between the known proportions of AF36 DNA in mixtures and those quantified with qPCR assays were calculated. In all linear regression analyses, the following were determined: significance of the regression, coefficient of determination (R^2), coefficient of determination adjusted for degrees of freedom (Ra^2), and the pattern of residuals. All data analysis was conducted using Statistix 10.0 Analytical Software (Tallahassee, FL)

Pistachio leaves. Leaves of pistachio cv. Kerman were collected from a 15-year-old experimental pistachio plot located at KARE. The leaves were placed in plastic humid chambers (10 leaves/chamber). Subsequently, the leaves were inoculated using 15 ml of a conidial suspension (10^6 conidia/ml) combining AF36 and 2A1L-11 as: 5:95, 50:50, and 95:5 (AF36 conidia:2A1L-11 conidia). Inoculated leaves were air dried overnight at 18-22ºC. Each 10 leaves group was then placed into a plastic bag with 25 ml of 0.1% TWEEN®80. The plastic bags were vigorously shaken for 1 min, conidial suspensions were collected in 50 ml Falcon tubes and centrifuged for 10 min (2,300 rpm). The 5 ml of the precipitated conidia were separated in four 1.25 ml vials, grinded twice with a homogenizer (MP Biomedicals) for 40 s and centrifuged for 5 min at 14,000 rpm. The supernatant was removed and 500 µl of Cell Lysis/DNA Solubilizing Solution for Fungi (CLS-Y) were added to each vial, which were vortexed for 20 s. DNA extraction and dilution were conducted as described above. The qPCR was conducted twice and the proportion of AF36 DNA in each mixture was calculated using Equation 1 as previously described, based on the quantity of DNA of each strain according to SCAF36 and SCAFP.

Soil samples. Manually infested soil samples were also used to verify the qPCR assay. For that, 1 kg of soil from the 2 cm top surface was acquired by sub-sampling a non-AF36-treated almond plot located at KARE and sieved to be separated in seven 2-g samples as described above. Different combinations of 5 mm diameter discs of PDA with 1-week-old growing colonies (mycelia and conidia) of AF36, 2A1L-11, and 4C1P-11 were properly mixed with 2-g-soil samples (Figure 5). Then, 500 mg of each soil sample were employed for the DNA extraction according to the procedure indicated above. Similarly, the qPCR was conducted twice for each sample and the proportion of AF36 DNA was calculated using Equation 1 as described before.

2.12. Application of the qPCR assay to determine the proportion of AF36 in nut and soil samples collected from pistachio fields

After verifying that the qPCR performed as expected, the equations from standard curves were also used to compare the proportion of AF36 vs *A. flavus* and *A. parasiticus* present in nuts and soil samples collected from fields.

Nut samples. Thirty early split pistachio nuts, i.e., atypical nuts with split hulls, which kernel is exposed to insect and mold invasions, including *Aspergillus* spp. (Doster and Michailides 1994), were harvested from an AF36-treated plot (treated with *Aspergillus flavus* AF36 Prevail®, the new formulation, applied at a rate 10 kg/ha) located at KARE. The early split nuts were divided into two groups of 15 nuts each: those with rough and shriveled hulls, showing a dark and stained suture (symptomatic) of *Aspergillus* spp. contamination and those with smooth hulls and without the stained suture (asymptomatic) (Doster and Michailides 1994). In each group, DNA extraction was conducted as described for pistachio nuts, independently for kernels and hulls, and qPCR assays were performed to obtain the Cq values that allow calculating DNA quantities of each genotype using the equations derived from the standard curves (SCAF36 and SCAFP). Based on the results, AF36 incidence (%) and AF36 molecular severity (MS) were calculated according to the following equations:

$$\text{AF36 Incidence (\%)} = \frac{\text{AF36 samples (Nº)}}{\text{N}} \times 100 \qquad \text{(Equation 2)}$$

$$\text{AF36 molecular severity} = \text{Log}\frac{\text{AF36 DNA (pg)}}{\text{Plant weight (g)}} \qquad \text{(Equation 3)}$$

where, “AF36 samples” is the number (Nº) of samples with presence of AF36, i.e. samples in which a certain amount of AF36 DNA was detected using the qPCR assay, with respect to the total number of samples, “N” (Equation 2). In Equation 3, AF36 DNA is the quantity of DNA according to SCAF36, while the “plant weight (g)” correspond to the amount of tissue used for the DNA extraction. In this latter equation, if AF36 DNA (pg) < 5, we considered AF36 MS $= 0$.

Soil samples. Nine 10-g soil samples were taken from the first 2 cm of the surface layer in the same AF36-treated almond plot at KARE. For comparisons, nine additional samples from AF36 non-treated fields contiguous to the almond plot were collected

adopting the same criteria. Samples were dried in paper bags at room temperature (21-25ºC) for one week, then sieved and DNA extracted as described for soil samples. The qPCR assay was conducted three times. From the results obtained, statistical analyses were conducted to compare AF36 incidence (%), as indicated in Equation 2. Since the presence of *A. flavus* and *A. parasiticus* in soil samples without plant debris taken from the upper cm of the first soil layer is mainly composed of scattered conidia (propagules) into the soil matrix (Abbas et al. 2009; Horn 2003; Luo et al. 2009), we also calculated the conidial density (%) of each plot as follows:

$$\text{Conidial density} = \text{Log}\frac{\text{Conidia (Nº)}}{\text{Soil weight (g)}} \quad \text{(Equation 4)}$$

Being the fungal "Conidia (Nº)" calculated based on the equations SCAF36-conidia and SCAFP-conidia resulted from the qPCR calibration test performed using conidial suspensions of AF36 and 2A1L-11 or 4C1P-11 using for that the Cq values obtained by conducting the qPCR assay as described before.

In both nut and soil assays, the different treatments were compared according to Kruskal-Wallis Test ($\alpha = 0.05$). qPCR assays for both nuts and soil samples were performed three times.

2.12. Evaluation of the competition between AF36 and 2A1L-11 in culture media

Five conidial suspensions (10^8 conidia/ml) were prepared by combining AF36 and 2A1L-11 conidia at proportions 95:5, 80:20, 50:50, 20:80, and 5:95 (AF36:2A1L-11). Subsequently, 100 µl of each conidial suspension was transferred to 9-cm Petri dishes with PDA and incubated at 30ºC to begin the first generation of competition between both strains. After 3 days of incubation, 1 ml of sterile 0.1% TWEEN®80 was added to each plate with the *Aspergillus* colonies, and the conidia were scraped with a sterile plastic rod. From this new conidial suspension, 500 µl were used for DNA extraction, and the proportion of AF36 in each sample was calculated as described above. The remaining 500 µl from the conidial suspension of each treatment were adjusted to 10^8 conidia/ml using a hemocytometer, and 100 µl were transferred to a new PDA plate, which was incubated again as above to be considered as the second conidia generation. This process (culture-conidia and wash-culture) was repeated six times to obtain six conidia generations. The dynamic in the proportions of AF36 over generations was used to study competitive

ability of AF36 and the toxigenic strain over several generations; this may give clues of AF36 behavior in nature after being released in the field. For each generation and treatment, the mean and standard deviation were calculated using the Summary Statistic of Statistix 10.

3. Results

3.1. Primer specificity test

The primer pair SNP36 Sh2 / SNP36 Cb successfully distinguished AF36 from the toxigenic *A. flavus* and *A. parasiticus* used in the qPCR assay. No amplification from AF36 was obtained using the primer pair Fw-nomutB / Rv-nomut267, while this pair was amplified by both *A. flavus* and *A. parasiticus* toxigenic strains.

3.2. Development of standard curves for DNA and conidia number quantification (qPCR calibration)

Figure 2a shows the standard curve generated with the primer pair SNP36 Sh2 / SNP36 Cb by using six serial dilutions of AF36 DNA, from pure culture. Figure 2b shows the standard curve generated with the primer pair Fw-nomutB / Rv-nomut267 by using five serial DNA dilutions of 2A1L-11 and 4C1P-11 DNA.

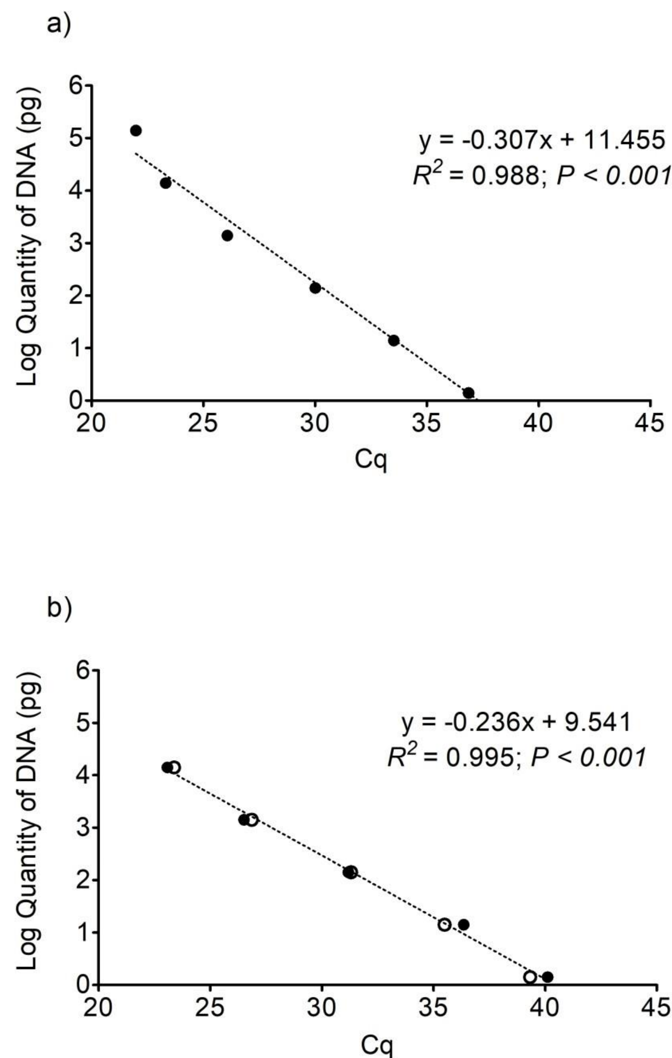

Figure 2. Standard curves from real-time PCR (qPCR) by plotting the threshold cycle (Cq) vs the log quantity of DNA (pg) extracted from pure cultures of *Aspergillus flavus*, AF36 strain, by using SNP36 Sh2/SNP36 Cb specific primers for the detection (a) and *A. flavus* (2A1L-11, white dots) and *A. parasiticus* (4C1P- 832 11, black dots) by using Fw-nomutB/Rv-nomut-267 specific primers (b).

The standard curves for conidia quantification, shown in Figure 3a, were generated with the primer pair SNP36 Sh2 / SNP36 Cb by using DNA extracted from five serial conidia dilutions of AF36 (SCAF36-conidia) as y = -0.270x + 11.672, where "y" is the log of the number of conidia (conidia/ml) of AF36 and "x" is the Cq value from qPCR (R2 = 0.959; P < 0.001). Figure 3b shows the curve for the primer pair Fw-nomutB / Rv-nomut267 by using DNA extracted from four serial dilutions of 2A1L-11 and 4C1P-11 as y = -0.245x + 10.696, where "y" is the log of the number of conidia (conidia/ml) of the toxigenic strain and "x" is the Cq value from qPCR (R2 = 0.879; P < 0.001).

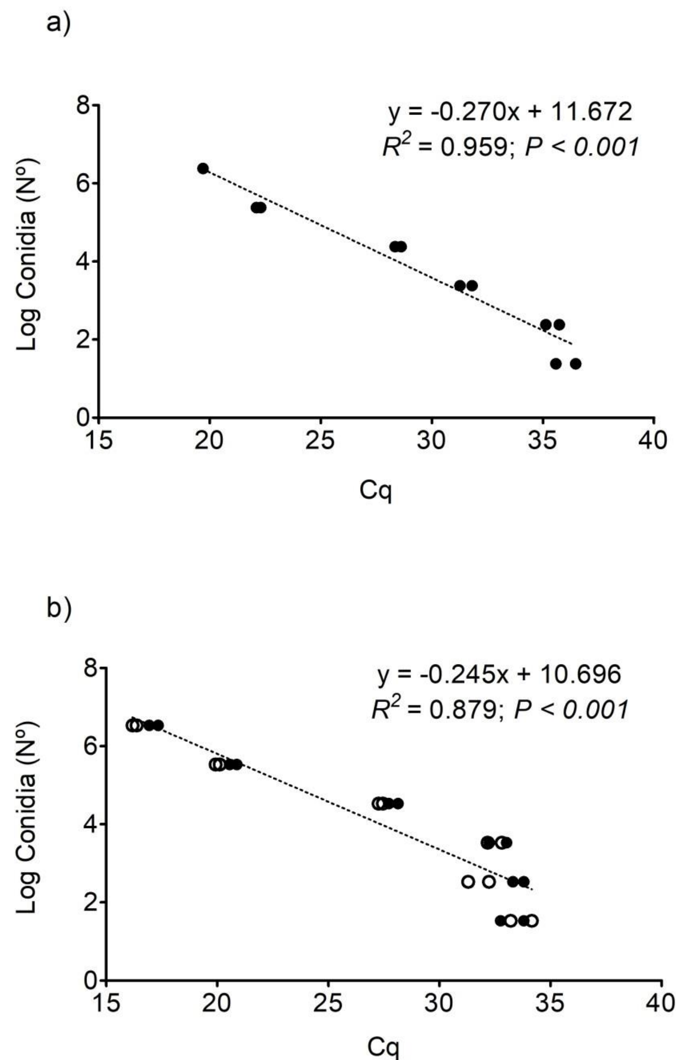

Figure 3. Standard curves from real-time PCR (qPCR) by plotting the threshold cycle (Cq) vs the log Conidia (Nº) using DNA extracted from serial conidial suspensions of *Aspergillus flavus*, AF36 strain, by using SNP36 Sh2/SNP36 Cb primers (a) and *Aspergillus flavus* (2A1L-11, white dots) and *A. parasiticus* (4C1P-11, black dots) by using Fw-no mut B/Rv-no mut-267 primers (b)

3.3. Quantification of the proportion of AF36 (qPCR assay verification)

DNA mixtures. A highly significant (R^2 = 0.974; P < 0.001) regression was obtained between the percentage of DNA of AF36 existent in 11 known pure DNA mixtures and those quantified with the qPCR assay (Figure 4a). Regression was forced through the origin to increase the meaning of the relationship between both variables. The adjusted equation was y = 1.163x, where "x" is the percentage of DNA of AF36 present in the DNA mixture, and "y" is the same value calculated by using the qPCR assay.

Conidial suspension mixtures. Similarly, nine known proportions of conidial suspensions as mixtures of AF36 with toxigenic strains were confronted with those calculated using the qPCR assay (Figure 4b). The linear regression was again forced through the origin, being the relation among variables y = 1.016x highly significant ($R^2 = 0.950$; $P < 0.001$), where "x" is the percentage of conidia of AF36 present in the conidial mixture, and "y" is the same value calculated by using the qPCR assay.

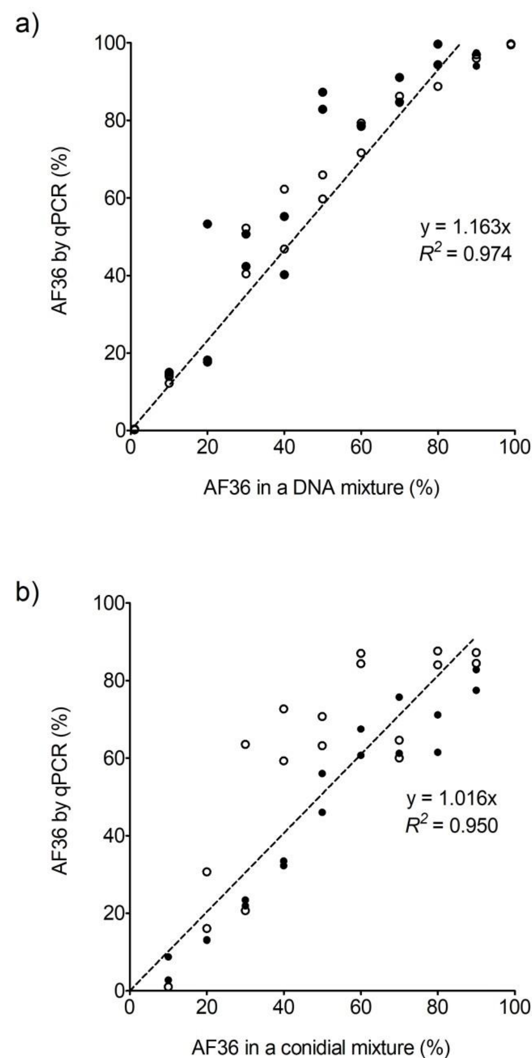

Figure 4. Linear regression between the percentage of *Aspergillus flavus* AF36 in known mixtures of DNA (a) and known conidial mixtures (b) and the percentage of AF36 in the mixture calculated by qPCR. White dots represent values of AF36 mixed with *Aspergillus flavus* 2A1L-11 and black dots represent values of AF36 mixed with *A. parasiticus* 4C1P-11.

Pistachio leaves. When we inoculated pistachio leaves using different combinations of conidia of AF36 and other *Aspergillus* strains, a highly significant ($R^2 = 0.924$; $P < 0.001$) linear regression (y = 1.061x) was obtained between the inoculated AF36 conidia proportions, “x”, and the AF36 proportions quantified from the qPCR assay, “y”.

Soil samples. In the inoculated soil with various known mixtures of the *Aspergillus* genotypes, we detected the presence of 100% of each genotype when added individually to the soil sample and it was possible to identify distinctive proportions when AF36 was mixed in a large (3:1) or a small (1:3) fraction with any of the other genotypes. In the control treatments, the qPCR assay did not detect another Aspergillus strain other than those used to infest the soil samples (Figure 5).

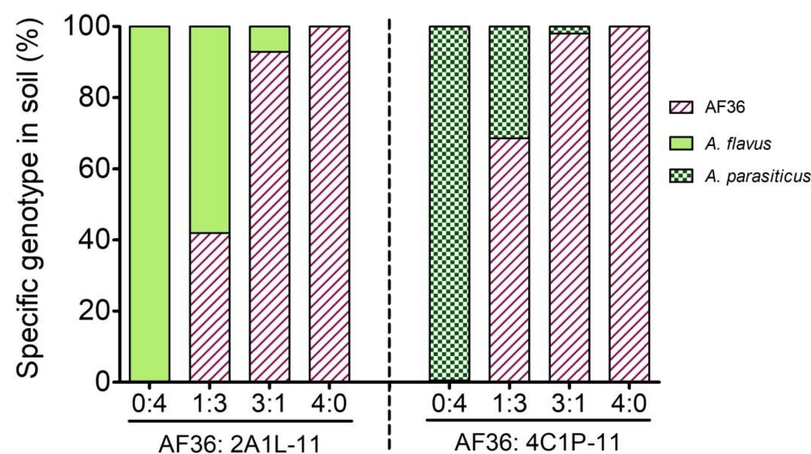

Figure 5. Percentage of each genotype calculated by qPCR assay, using natural soil samples infested with different mixtures of four fungal discs (mycelia and conidia) on Potato Dextrose Agar of only nontoxigenic *Aspergillus flavus* AF36 (0:4) or AF36 combined with the toxigenic genotypes (*A. flavus*, 2A1L-11 or *A. parasiticus*, 4C1P-11) in different proportions.

3.4. Application of the qPCR to determine the proportion of AF36 on various sources of samples collected from fields

Nut samples. The application of the qPCR assay over early split pistachio nut samples collected from commercially treated pistachio plots resulted in similar AF36 incidence (%) (Equation 2) and AF36 MS (Equation 3) values among kernel and hulls tested, but dissimilar ($P = 0.021$) for values of AF36 MS between symptomatic and asymptomatic hulls. No other Aspergillus genotype distinct from AF36 was found in the samples. The

incidences of AF36 contaminating early split pistachio nuts ranged from 0 to 60% in both kernel and hull tissues, and the corresponding MS of AF36 were in a log scale range from 0 to 1.7 pg/g (Figure 6).

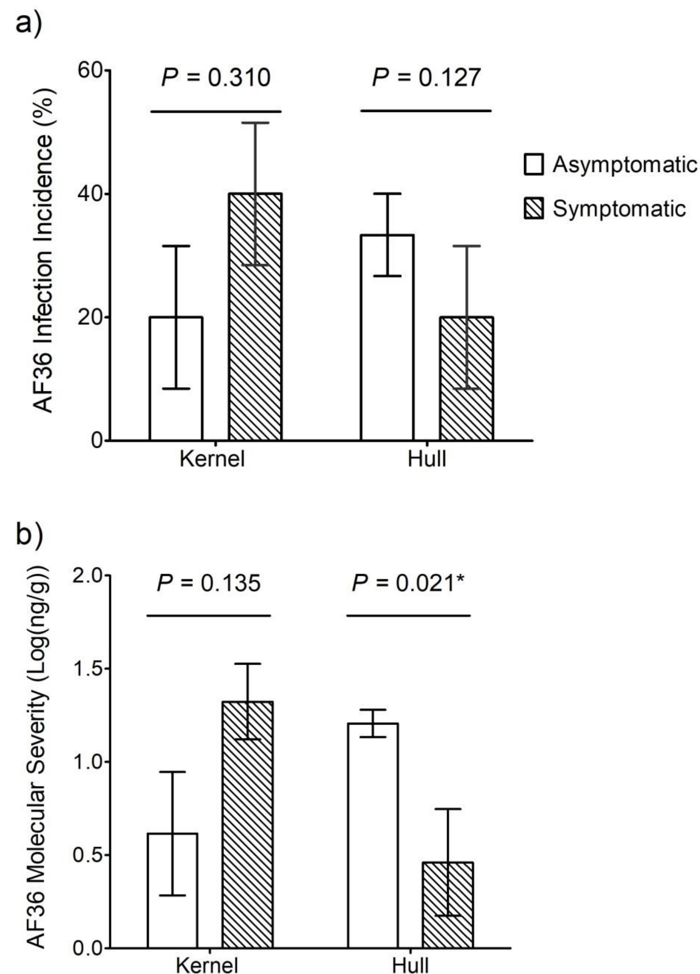

Figure 6. Infection incidence (%) and log molecular severity (pg/g) of *Aspergillus flavus* AF36 strain quantified in early split nuts collected from a commercially AF36-treated pistachio plot. Kernels and hulls were tested independently. Significant differences between asymptomatic (nuts with smooth hulls and without the stained suture) or symptomatic (nuts with rough and shriveled hulls, showing a dark and stained suture) kernels or hulls according to Kruskal-Wallis Test at $P < 0.05^{*}$

Soil samples. In treated soils, AF36 had an incidence of 100% among the examined *Aspergillus* communities and that incidence was higher (P = 0.007) than that of AF36 in untreated soils (62%; Figure 7). In addition, the conidial density (Equation 4) in the soil of the treated plot (2,598 conidia/g) was significantly higher (P = 0.021) than in the untreated plot (39 conidia/g) (Figure 7).

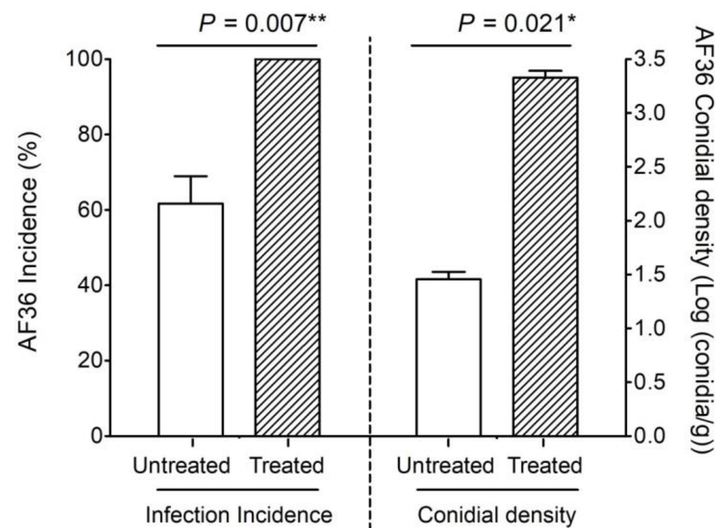

Figure 7. *Aspergillus flavus* atoxigenic AF36 strain incidence (%), left, and conidial density, log (conidia/g), right, quantified in soil samples collected from AF36 commercially treated and contiguous untreated pistachio fields. Significant differences between AF36 treated and untreated soils according to Kruskal- Wallis Test at $P < 0.05^{*}$

3.5. Evaluation of the competition between AF36 and 2A1L-11 in culture media.

The results of the competition experiments demonstrated that after six generations, the proportion of AF36 remained relatively stable when the starting point is at high concentration, close to 100% or in a low concentration at about the 5% in the mixture with 2A1L-11. However, from 80% at the beginning, the percentage of AF36 varies around 60-80%, with a final decline to 20% at 6th generation. Conversely, from 50% and 20% as the starting point, the population of AF36 ends up with an increase of 20% (Figure 8).

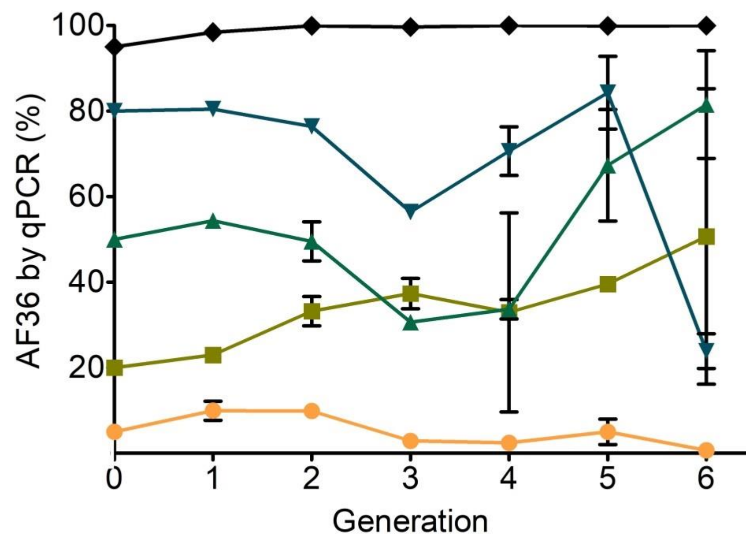

Figure 8. Dynamic in the proportions of *Aspergillus flavus* atoxigenic AF36 strain quantified by the qPCR assay over six three-days-long generations in culture media, starting from different initial proportions of AF36 in mixture with the toxigenic *A. flavus* 2A1L-11 strain.

4. Discussion

Crops such as almond or pistachio are occasionally contaminated with aflatoxins, and this can cause severe economic loss (Bayman et al. 2002; Campbell et al. 2003; Doster 1996; Doster and Michailides 1994; Ortega-Beltran et al. 2019; Palumbo et al. 2014). Overall, an increase in aflatoxin contamination has occurred in the last decade in temperate zones driven by the changing patterns in climate (Battilani et al. 2016; Cotty and Jaime 2007; Medina et al. 2017). These events have bolstered the research and delivery work to mitigate the increment of aflatoxin contamination in regions where susceptible crops have great economic value, or are staple foods, such as in many African nations (Bandyopadhyay et al. 2016). Diverse control strategies have extensively been sought to prevent and reduce aflatoxin contamination in crops, however, biocontrol of aflatoxins using endemic atoxigenic strains has become a useful tool in several parts of the world, since it is efficient in preventing toxin accumulation and safe guards the quality of food and feed before and after harvest (Bandyopadhyay et al. 2016; Camiletti et al. 2017, 2018; Mauro et al. 2015; Moral et al. 2020; Ortega-Beltran et al. 2019; Savic et al. 2020). The atoxigenic biocontrol technology was developed in the US, where it continues to be used and currently, AF36 is the most widely used biocontrol strain to reduce aflatoxin contamination (Moral et al. 2020).

Successful aflatoxin biocontrol is revealed by both aflatoxin reductions in treated crops and displacement of aflatoxin producers (Agbetiameh et al. 2020; Atehnkeng et al. 2014; Camiletti et al. 2018; Dorner 2009; Mauro et al. 2015; Weaver et al. 2015; Zhou et al. 2015). Understanding how the populations of non- aflatoxigenic strains change over the time after being released to the environment allows determining if treatment is being effective (Abbas et al. 2009; Donner et al. 2015; Horn 2003; Jaime and Cotty 2004; Mauro et al. 2013; Moore et al. 2017). It is also important to monitor how the atoxigenic strains survive and compete with populations of aflatoxin-producing species (Cotty et al. 2007; Cotty and Bayman 1993; Mehl and Cotty 2010). It is difficult to answer these questions by using traditional approaches based on fungal culturing methods, such as vegetative compatibility assays (VCA). However, VCA are extensively used despite being a time, resource, and labor intense task (Atehnkeng et al. 2016; Bayman and Cotty 1991, 1993; Camiletti et al. 2018; Horn and Greene 1995; Ortega-Beltran et al. 2018; Ortega-Beltran and Cotty 2018; Probst et al. 2011). The tediousness of VCA provided the necessary impetus to reconsider the way in which epidemiological studies of AF36 are conducted in tree nut orchards. Here we developed a qPCR assay to quantify the proportion of AF36 vs toxigenic genotypes of *A. flavus* and/or *A. parasiticus* from a diverse source of samples, including mycelia from pure cultures, conidial suspensions, soil, and plant tissues. The assay can be used to work with 48 samples at a time, obtaining quality results in less than two hours' time for a reasonable cost (less than 30 USD) (Garcia- Lopez and Michailides, unpublished data). In comparison, it can take months to conduct VCA for the same number of samples.

The strain AF36 belongs to VCG YV36, spread from California to Georgia and also endemic to México (Ehrlich and Cotty 2004; Ortega-Beltran et al. 2016). All YV36 members are atoxigenic because of a SNP in *aflC* although members of the VCG contain additional degeneration in *aflC* and in other genes necessary for aflatoxin production. However, the *aflC* SNP, can be found in atoxigenic isolates belonging to other VCGs (Ehrlich and Cotty, 2004; Grubisha and Cotty 2015). Thus, the assay would also quantify other atoxigenic isolates carrying the SNP, in addition to YV36 members that are native to the area. YV36 is one of the most common atoxigenic VCGs across California tree nut orchards with frequencies of up to 7% of the *Aspergillus* communities (Ortega-Beltran et al. 2019; Picot et al. 2018). However, when AF36 is applied in the orchards, this strain will most likely dominate the communities. Detecting the SNP in treated samples is a

reasonable estimate of presence of AF36. There are hundreds of VCGs in a single area and not all of them are atoxigenic, and among all the atoxigenic not all of them contain the *aflC* SNP (Bayman and Cotty 1991; Ehrlich and Cotty 2004; Ortega-Beltran and Cotty 2018).

The proportion of AF36 in various sources was accurately quantified using established standard curves. Highly significant regressions between known and detected proportions of AF36 using the qPCR assay were obtained from various sources. This demonstrates that the qPCR assay can efficiently quantify AF36 proportions at the population level from different sources and distinguish AF36 from other strains of *A. flavus* or *A. parasiticus*. The standard curves for each primer pair were generated and the R^2 coefficients proved to be good indicators of robust and reproducible assays of this study. Due to the equal conditions of qPCR protocols for both primer pairs, the qPCR can be conducted at the same time with the two primer sets, reducing the time of analysis, allowing simultaneous calculation of proportions of AF36:*A. flavus* + *A. parasiticus*. Based on the standard curves, the normal range from 20 to 35 of the Cq values permits the detection from 5 to 200,000 pg of AF36 DNA and from 19 to 66,000 pg of *A. flavus* and/or *A. parasiticus* DNA that might be present in a sample from pure culture DNA. That sensitivity is appropriate to accurately quantifying a target genotype in each sample. Likewise, our method allows quantifying the number of conidia of each strain in a sample with the limits varying from 170 to 2×10^6 conidia of AF36 and from 250 to 1×10^6 of *A. flavus* and/or *A. parasiticus*. Higher accuracy occurred at densities > 2,000 conidia/g, in both cases while lower accuracy occurred at densities below 250 conidia/g. Use of larger field samples, bearing consequently higher target DNA content, can increase accuracy, as proposed by Luo et al. (2009) and Wang et al. (2006). Keeping consistency of the methodology in sampling processing and quantification is important to systematically guarantee the high accuracy of unknown sample quantification.

The discrimination power for quantification of AF36 vs *A. flavus* and/or *A. parasiticus* was also confirmed using samples containing mixtures of DNA at different proportions. Moreover, the accuracy of the method to quantify these fungi in artificially contaminated matrices was authenticated by an adequate regression coefficient when extracting DNA from washes of inoculated leaves. Besides, the results confirmed the applicability of the method in controlled studies using soil samples. Quantifying fungal levels in field

matrices is essential to continue with epidemiological studies for understanding the biocontrol strain behavior in nature.

The methods to extract *Aspergillus* DNA from a diverse source of samples were successful to obtain the required DNA quality for qPCR. Extraction of fungal DNA directly from soil is more difficult compared with extractions from pure fungal cultures, plant tissues, and some restricted environments by using a commercial kit, especially when the density of pathogen's propagules in soil is very low. Previous reports already described protocols to quantify *A. flavus* and *A. parasiticus* (Carbone et al. 2007; Frisvad et al. 2005; Luo et al. 2009), which helped as references for the DNA extraction method from soil samples used in the current study.

Using the designed primers and the DNA extraction method, a qPCR assay was generated to quantify the proportion of AF36 with respect to *A. flavus* and/or *A. parasiticus* contained in soil samples coming from AF36-treated vs. untreated contiguous plots. As expected, treated fields were dominated by AF36. Since the untreated fields were adjacent, most likely the AF36 strain moved from the treated plot, although AF36 incidence and its conidial density were significantly lower in the untreated plots. Tests were also conducted with early split nuts collected from a commercially AF36-treated plot, allowing the comparison of AF36 infection incidence and its molecular severity (MS) in both kernels and hulls with fungal infection symptoms or not. Symptomatic early split kernels (i.e., showing a dark and stained suture) had higher AF36 MS in the kernel than in the hull. Conversely, the healthy looking (asymptomatic) early split kernels had significantly higher AF36 MS in the hull, indicating that the colonization process was at an early stage. This research also involved a preliminary study on competition between AF36 and other strains which demonstrated the potential applicability of the qPCR assay to track the AF36 survival after being released to the environment as biocontrol agent. Bayman and Cotty (1993) suggested that an atoxigenic strain was able to compete effectively at the same inoculum proportion (50:50) or with even less atoxigenic inoculum on cotton balls or in liquid fermentation systems. Similar findings were reported by Mauro et al. (2015) when examining atoxigenic strains native to Italy. They suggested that atoxigenic strains used two mechanisms of action; by exclusion of the toxigenic strain from the niche, and by competing for nutrients destined for aflatoxin biosynthesis. The mechanisms of AF36 in reducing aflatoxin contamination still need to be intensively

studied. Needed is an efficient quantification method to determine abilities of AF36 to compete with other individuals for infection sites, and how much strength AF36 could express to reduce aflatoxin production process. When AF36 is released into the environment, many factors may affect its survival, growth, and reproduction (Michailides et al. 2018). It is important to quantify such effects so that the faith of biocontrol strains in the environment after release could be accurately modelled and predicted (Abdel-Hadi et al. 2012; Marin et al. 2012).

Understanding the dynamics of AF36 under different scenarios is important to design the best strategies of application of a biocontrol agent to reduce risk of aflatoxin contamination in nut crops (Ortega-Beltran et al. 2018). Determining how AF36 competes with native aflatoxigenic strains under various conditions could greatly help the decision-making process on better and more efficient use of the biocontrol agent. All the above issues rely on an efficient and accurate method to rapidly process samples from various sources to obtain valuable information timely. Our established qPCR assay could be used to handle such difficult, time-sensitive tasks to accelerate aflatoxin management while improving food safety.

In addition, the formulation of AF36 needs to be improved to increase the efficiency of displacement and the qPCR assay developed can be useful to monitor subsequent experiments without the need to conduct the laborious VCA. Another possibility is to monitor the residual effect of AF36 in treated orchards to determine whether partial or complete yearly or every other year treatment is needed. This can considerably save costs to farmers and would give the opportunity to more farmers to access the product. Furthermore, it is expected that mixtures of atoxigenic strains will be used to complement the efficacy of AF36. Assays to quantify the proportions of those strains need to be developed in the future.

Conclusion

A qPCR protocol to quantify proportions of AF36 accurately and efficiently was developed for use in diverse substrates (soil, conidial suspensions, fruit or leaf tissues). The assay will serve to conduct epidemiological and competition studies of AF36 in both laboratory and field studies in a time- and cost-effective manner. Increased knowledge on

the biology, epidemiology, and ecology of the biocontrol strain will ultimately lead to the design of more efficient aflatoxin mitigation strategies.

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PISTACHIO MALE INFLORESCENCES AS AN ALTERNATIVE SUBSTRATE FOR THE APPLICATION OF ATOXIGENIC STRAINS OF *ASPERGILLUS FLAVUS*

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Abstract

Aflatoxins are carcinogens mainly produced by *Aspergillus flavus* and *A. parasiticus* in susceptible crops, including pistachio. The primary inoculum sources of these pathogens are plant debris in the orchard soils. In Californian fields, one approach to controlling aflatoxin contamination is based on releasing the atoxigenic strain of *A. flavus* AF36 in inoculated (coated) sorghum grains (AF36 Prevail®). However, this control method can fail due to poor sporulation of the AF36 strain or sorghum grain losses due to predation. In 2008 and 2018, we showed that toxigenic and atoxigenic isolates of *Aspergillus* spp. frequently colonized fallen inflorescences of male pistachios. Under controlled conditions, AF36 strain profusely colonized pistachio male inflorescences when humidity was higher than 90%. However, there were significant differences (between types of inflorescence (aerial > fallen). In 2016, we considerably (P = 0.015) increased the population of AF36 on the canopies of trees, when fallen inflorescences were inoculated with AF36, compared to non-treated trees. In 2017 and 2018, these differences were not detected (P > 0.05) due to cross-contamination of AF36 strain between seasons and neighboring plots. In any case, the density of AF36 spores on the canopy of the inflorescence-treated trees was similar (P > 0.05) to those of treated trees with the commercial product. Here, we present a new method for applying AF36 strain based on using a natural, abundant, and uniformly distributed substrate in pistachio fields, and we discuss how it can be improved. Furthermore, our results indicate that in pistachio orchards, where biocontrol practices are not conducted, eliminating this important source of toxigenic *Aspergillus* inoculum is recommended.

Keywords: aflatoxin, biological control, mycotoxin, nut crops

1. Introduction

Californian farmers face barriers to marketing pistachio nuts in international trade because of strict regulations for aflatoxin contamination (Bui-Klimke et al. 2014). The latter causes substantial economic losses to the pistachio industry (Bui-Klimke et al. 2014; Rabadan and Triguero 2020), even though aflatoxin contamination of Californian nuts is only occasionally (rarely) (Doster and Michailides 1994).

The cosmopolitan fungal species *Aspergillus flavus* Link and *A. parasiticus* Speare are the major aflatoxin producers contaminating pistachio nuts (Amaike and Keller 2011; Donner et al. 2015; Klich 2007). However, while the population of *A. parasiticus* is only composed of toxigenic isolates (Frisvad et al. 2019), the *A. flavus* population comprises non-toxigenic (i.e., isolates that do not produce aflatoxins) and toxigenic isolates (Klich 2007; Moral et al. 2020). Furthermore, the proportions of non-toxigenic and toxigenic isolates of *A. flavus* vary among regions and seasons (Donner et al. 2015; Ortega-Beltran et al. 2018), determining the risk of crop contamination (Bandyopadhyay et al. 2016; Benkerroum 2020; Mahuku et al. 2019; Seetha et al. 2017).

In pistachio orchards, plant debris that remains on the soil, such as leaves, male inflorescences or nuts are the primary inoculum sources of *A. flavus* and *A. parasiticus* (Doster and Michailides 1994). Also, pathogen can survive buried in the soil more than one year as spores or, even longer as sclerotia (Horn 2003; Wicklow et al. 1993). These fungal species develop many spores on inoculum sources, which are dispersed through the air or insects until reaching the nuts (Doster et al. 2014; Palumbo et al., 2014). However, only a few (1-4%) pistachio nuts, known as early splits, can be colonized by the pathogen spores since they have split hulls, exposing the kernel to invasion by fungal spores and insects (Doster and Michailides, 1994).

In order to reduce aflatoxin nut contamination preharvest, the most promising strategy is the application of the biocontrol *A. flavus* AF36 strain along with reducing the main risk factors: the incidence of early splits and the level of damage by the navel orangeworm pest (*Amyelois transitella*), a lepidopteran insect whose moth acts as vector and its larva feeds from the kernel (Doster et al., 2014; Palumbo et al., 2014; Wilson et al., 2020). The biocontrol strategy comprises a massive application of atoxigenic strain *A. flavus* AF36 on coated sorghum grains (AF36 Prevail®) on the ground to compete for infection sites

(i.e., early splits) and displace the toxigenic-wild isolates (Doster et al., 2014; Moral et al. 2020). After application of the AF36 Prevail® grains, the AF36 strain can be isolated from soil samples of pistachio orchards for years (Doster et al., 2014) but how this strain remains has not been studied yet.

This biocontrol strategy was first developed for row crops such as cotton and maize (Cotty and Bayman 1993; Cotty, 2006) and, in 2012, was registered in pistachio orchards (Doster et al. 2014; Ortega-Beltran et al. 2019). However, the biocontrol of aflatoxin in pistachio orchards has distinctive features (Ching'anda et al. 2022, Doster et al. 2014; Kaminiaris et al. 2020), such as: i) many AF36 Prevail® grains fail to produce sporulation due to low soil moisture conditions; ii) since sorghum grains may remain on the bare ground, product losses due to predation by arthropods and birds are a fact (Garcia-Lopez et al. 2018); iii) the high humidity inside maize and cotton fields are more favorable for AF36 sporulation on the sorghum grains than in nut orchards (Doster et al. 2014); and iv), the early splits to be protected are at 2-5 m from the ground. However, despite these differences, the application dosage of AF36 Prevail® (11 kg/ha) recommended pistachio orchards is based on doses used for row crops. Thus, one could appreciate the self-evident interest in searching for alternative methods to improve the efficiency of biocontrol applications in nut orchards by increasing sporulation and searching for additional substrates to build up the inoculum of non-aflatoxic strains in the orchard.

Here, we studied pistachio male inflorescences as an inoculum source and a substrate for applying the AF36 strain. Thus, we hypothesized that the latter action has a double positive effect: it increases the propagule population of the biological control strain and, at the same time, displaces the wild toxigenic population of *Aspergillus* from these debris. For this, i) we first characterized the different sections of *Aspergillus* spp. in pistachio male inflorescences, nuts and soils of treated and non-treated plots; ii) we evaluated the colonization capacity of the AF36 strain on the pistachio male inflorescences under controlled and field conditions; and iii), we compared the use of pistachio male inflorescences as the substrate for AF36 strain to the commercial product (AF36 Prevail®).

2. Materials and methods

2.1. Characterization of Aspergillus spp. in pistachio male inflorescences and nuts, and soils of treated and non-treated plots

During 2008 and 2018, fallen male inflorescences, nuts, and soils were sampled in two plots of a commercial orchard (35°37’50.5 “N 119°52’42.3 W”) belonging to Paramount Farms Co., the world largest pistachio company, located in Lost Hills, California. One of the plots was treated with AF36 Prevail®, while the other was used as non-treated control. Both plots were separated by 945 m. AF36 Prevail® was applied at the rate of 11 kg/ha once each year in early July.

Fallen male inflorescences and nuts sampling. Male inflorescences and nuts were collected from the ground under the canopy of each of three pistachios cv. Peters and three female ones cv. Kerman, respectively, before and after applying Prevail® grains in the treated plot. Likewise, one male tree cv. Peters and another female tree cv. Kerman were sampled in the non-treated plot. Thus, 120 male inflorescences (30 inflorescences per male tree) were collected using sterilized forceps and placed on salt agar (6% NaCl and 1% agar) in 60-mm diameter Petri dishes in the field (Doster and Michailides, 1994).

Similarly, 80 nuts (20 nuts per ‘Kerman’ tree) were collected and transferred to salt agar in the field. Also, other 80 nuts (20 nuts per ‘Kerman’ tree) were surfaced-disinfected in a laminar-flow hood by dipping the nuts in ethanol (70%) for 15 seconds, followed by 1 min in NaOCl solution (0.5%), and rinsed with sterile distilled water. The surface-disinfected nuts were then transferred to salt agar as before. The Petri dishes containing the inflorescences and nuts were incubated for 7 days at 30 ºC.

After the incubation period, we examined the percentages of samples (inflorescences or nuts) colonized by fungal species belonging to different *Aspergillus* sections (*Flavi*, *Circumdati,* or *Nigri*) by observing the main morphological features of the colonies under a dissecting microscope (10 - 20×). Thus, *Aspergillus* isolates that belonged to section *Nigri* presenting conidial masses in shades of black, to section *Circumdati* presenting yellow, buff, or ochraceous shades conidial masses, and to section *Flavi* presenting yellow-green to deep olive-brown conidia (Klich 2002; Pitt and Hocking 2009). Subsequently, the *A. flavus* isolates were classified according to the size of their sclerotia as S, L or NSP morphotypes ─ S = Small (< 400 μm) or L = Large (> 400 μm) sclerotia

or NSP = Non-Sclerotia Producer– as detailed by Camiletti et al. (2018). The *A. flavus* L-morphotype isolates were then classified according to their Vegetative Compatibility Group (VCG) to discriminate by VCG YV36, the VCG to which AF36 belongs (Ehrlich and Cotty 2004).

Soil sampling. Three 40 g-soil samples (A, B, and C) were taken from treated and non-treated plots each year. Every 40-g soil sample was composed of 30 soil sub-samples (1.2-1.5 g), collected from the first 3-cm top surface layer of the soil under the pistachio tree canopy or the soil in the middle aisle between rows of a dozen trees. The soil samples were collected using an ethanol-sterilized (70%) trowel and mixed thoroughly in a paper bag. The samples were then dried at room temperature (23-26 ºC) for 7 days. The dried soil samples were crushed with a pestle and a mortar and passed through a sieve (0.85 mm mesh). From every sample, 2 g of soil were homogenously distributed and spread on 10 Petri dishes of 9-cm containing Si10 culture medium (10 g of sucrose, 60 g of NaCl, 1 g of yeast extract, 0.1 g chloramphenicol, 10 ml of a 0.2% dichloran solution in ethanol, 1 ml of $CuSO_4 \cdot ZnSO_4$ solution, and 15 g of Bacto agar in 1 liter of deionized water) according to Palumbo et al. 2014. The Si10 medium is a selective medium for *A. flavus* detection that limits the growth of numerous soil-borne fungal species (Henson 1981). After 7 days of incubation at 30 ºC, we identified and quantified the *Aspergillus* isolates section *Flavi,* again according to the morphological features of the colonies, conidiophores, and conidia under a dissecting microscope. Then, we calculated the density of colony-forming units (CFU) of *A. flavus* in the soil (CFU/g soil). To obtain pure cultures of *A. flavus* isolates, putative colonies of the pathogen were recultured twice on Czapek Yeast Extract Agar (CYA). Subsequently, 20 *A. flavus* isolates per subsample (i.e., 60 samples per orchard) were randomly selected and classified according to their type of sclerotia (S, L, or NSP). Afterward, a VCG test was conducted using all the 20-morphotype isolates recovered from each plot to discriminate by VCG YV36 as above.

2.2. Colonization of pistachio male inflorescences by the AF36 strain under controlled and field conditions

Colonization under controlled conditions. Aerial or fallen (i.e., collected from the tree canopy or the ground, respectively) male inflorescences were collected to study their ability to act as an inoculum source for the AF36 strain. Aerial and fallen inflorescences were collected from six 'Peters' trees (five of each type per tree) located at Kearney

Agricultural Research and Extension Center (KARE), 36°35'53.3 "N 119°30'23.4 W" (Parlier, California, U.S.A.). Aerial and fallen inflorescences were then divided into three groups of 10 inflorescences of each type and placed in disinfested plastic containers (30 ×10×10 cm) used as humid chambers. Sterile water (300 ml) was added to each container to maintain high humidity (approx. 100%). The inflorescences were inoculated with a spore suspension (10^6 spores/ml and 0.5 ml per inflorescence) of the *A. flavus* AF36 strain. The inoculated inflorescences in the humid chambers were incubated for a week in an incubator at 30ºC in the dark. The percentage of the inflorescence surface colonized by the AF36 strain was visually estimated.

To study the effect of humidity on the percentage of inflorescence colonization by the AF36 strain, aerial inflorescences were collected from the canopy of five 'Peters' trees, inoculated, and incubated at different levels of relative humidity. Thus, five saturated solutions of salts (300 ml each) were prepared to control the humidity into sterile plastic containers (30×10×10 cm): NaCl, $(NH_4)_2SO_4$, KCl, KNO_3, and K_2SO_4, which generated humidity values of 75, 80, 85, 90, and 95%, respectively (Wexler and Hasegawa, 1954). Moreover, sterile water was used to generate a humidity value of 100%. The relative humidities were also determined using a Hobo Pro RH/Temperature data logger (Onset Computer Co., MA), and solutions were readjusted when necessary. Six chambers were used per treatment. Each plastic chamber contained five inflorescences inoculated with a spore suspension (10^6 spores/ml and 0.5 ml per inflorescence) of the AF36 strain. Then, the percentage of inflorescences colonized by AF36 was visually estimated after 7 days of incubation at 30ºC.

Colonization under field conditions. To study the ability of inflorescences of the male pistachio trees to act as an inoculum source of the AF36 strain, fallen inflorescences under the canopies of four trees of the cvs Peters and Randy were inoculated in May 2016. The cv. Randy was included since most of the new pistachio plantations are being conducted with it due to its higher flowering production (Ferguson and Haviland, 2016). The inoculation was performed by spraying 1500 ml of a spore suspension (10^6 spores/ml) of the AF36 strain per tree. As non-inoculated control, fallen inflorescences of four other pistachio trees of each cultivar were sprayed using sterile water. Twenty days after treatment, 10 inflorescences of each treated tree were collected and incubated in humid chambers for 7 days, as described above. Finally, the percentage of inflorescences

colonized by the biological control strain was visually estimated. For the remaining experiments, we used the cv. Peter since inflorescences from both ('Peters' and 'Randy') had showed similar ability to act as an inoculum source and most of the plantations with 'Randy' trees are still in juvenil phase (no-flowering).

Finally, to monitor the establishment of AF36 strain in male inflorescences under field conditions, male inflorescences from the canopy of 'Peters' trees from an experimental plot located at the KARE were collected in May 2016. Thus, 'Peters' inflorescences were inoculated with a spore suspension (10^6 spores/ml) of the atoxigenic *A. flavus* strain AF36 and placed inside nylon mesh bags (10 inflorescences/bag). Eighteen bags were hung in the canopy of six pistachio trees (three bags per tree), and the same number was distributed on the ground under their canopies. Two months after the inoculation, one bag from the ground and another from the canopy of each tree was collected monthly from July to September. Six inflorescences from each bag were cut into small fragments (≈ 6 mm, 36 pieces per inflorescence) and plated in three Petri dishes containing Si10 medium in sterile conditions at the laboratory. The percentage of inflorescence pieces colonized by *A. flavus* was identified, and the data were expressed as the percentage of pieces colonization by the AF36 strain. The AF36 strain was identified by PCR using specific primers according to Ortega-Beltran et al. (2016).

2.3. Monthly monitoring of A. flavus in soil and canopy after field application using fallen male inflorescences as a substrate of AF36

To evaluate the pistachio male inflorescences as an inoculum source for the AF36 strain, fallen inflorescences of seven trees cv. Peters were sprayed with a 10^5 spore/ml spore suspension (1500 ml per tree) of this strain. The selected seven pistachios are randomly located at KARE in two experimental plots (distance between trees and rows 8 and 5 m, respectively). The spread of the AF36 strain on the sprayed trees was evaluated on both the pistachio canopy and the soil from April to September 2016, 2017, and 2018.

Monitoring of inoculum on pistachio leaflet surfaces. Ten leaflets of each experimental tree were harvested and placed into a plastic bag with 25 ml of 0.1% Tween 80 sterile solution. The bags were vigorously shaken for 30 seconds by hand, and the washing water was poured into 50-ml Falcon Cone Tubes, which were centrifuged at 5,000 rpm for 10 min. The supernatant was removed, leaving the conical base of each tube with 5 ml of

washing water and the pellet. After vortexing for 10 s, 2 ml of the washing water of each tube were transferred to 10 Petri dishes (Si8 medium) that were incubated for 7 days at 30ºC in the dark. The number of *A. flavus* colonies in each Petri dish was quantified under the dissecting microscope according to the morphological features described above. The density of *A. flavus* inoculum on the pistachio canopy was expressed as the number of spores density per leaflet gram (spores/leaflet-g).

Monitoring the AF36 inoculum on the ground. We collected two soil samples, transcribing a circumference of one and two meters apart from the main trunk, to determine the density of AF36 in the ground. Each sample consisted of mixing five subsamples taken from the first 3-cm top surface layer along the trajectory. The soil samples were then dried at room temperature (23-26ºC) for 4 days. One gram of soil of each sample was distributed equally in five Petri dishes (Si10 medium) and incubated for 7 days at 30 ºC. The density of *A. flavus* was expressed as CFU of *A. flavus* per soil gram (CFU/g soil).

Finally, the *A. flavus* isolates from each source (canopy or ground) were classified. Those isolates of the L-morphotypes were cultured on CYA and identified either as AF36 or not using a specific-mismatch qPCR (Garcia-Lopez et al. 2020). The amount of AF36 inoculum present in the soil and the surface of the leaflet of each tree was measured yearly before and after the treatment application.

In 2016, the following treatments were used: i) Peters-treated, seven male pistachios cv. Peters whose fallen inflorescences were sprayed with 1500 ml per tree of the AF36 strain spores (10^5 spores/ ml) suspension; and ii) Peters-non-treated control, five pistachios cv. Peters whose inflorescences were sprayed with water. In 2017 and 2018, the treatments were: i) Peters-treated; ii) Peters-non-treated control; iii) Soil-treated, two female pistachios cv. Kerman, which ground was sprayed with 1500 ml of spores suspension of AF36 strain; and iv) AF36 Prevail®, three female pistachios cv. Kerman with AF36 applied at the commercial dosage (40 g AF36 Prevail® grains per tree) under tree canopies.

The quantities of the AF36 spores on the pistachio tree canopy and soil were determined to evaluate the efficacy of the different application methods. Each year, the AF36 inoculum in the canopy was monitored monthly, from May to September. In 2016, the

soil was evaluated twice, in April and May, i.e., before and after the treatments, respectively, and monthly in 2017 and 2018. Every year, the treatments were performed 15 days before the second sampling, in May.

2.4. Statistical Analyses

All experiments were replicated. The results are presented as mean values with standard errors or with comparison letters when applicable. The percentage of *Aspergillus* sections *Nigri*, *Circundati*, and *Flavi* on the pistachio male inflorescences was studied by Chi-square test using two by two contingency tables. Wilcoxon's rank-sum test compared the percentage of pistachio male inflorescence (%) colonized by the AF36 strain.

The best subset regression process fitted second- or third-order polynomial equations relating to humidity and inflorescence colonization by AF36 strains. The equation was selected according to the *P-value* of the independent variables (X, X^2, and X^3) and the coefficient of determination (R^2). Finally, the derivative of the adjusted equations was used to calculate the optimum humidity for inflorescence colonization by AF36 strain.

The areas under the number of spores density curves (AUSPC) were calculated by trapezoidal integration to study the different AF36 application methods on the number of spores of *A. flavus* on the pistachio tree canopy across the season (Campbell and Madden 1990), since the area under the progress curve is very useful for comparing treatments across seasons (Madden et al. 2007). Treatment areas were subjected to the nonparametric Wilcoxon Rank Test in 2016 data and to Kruskal–Wallis in 2017 and 2018. In these latter years, differences between treatments were studied according to Dunn's comparisons at $P < 0.05$. All the data were analyzed using the softwares Statistix 10 and SPSS21.

3. Results

3.1. Characterization of Aspergillus spp. in pistachio male inflorescences and nuts, and soils of treated and non-treated plots

Characterization of Aspergillus spp. present in male inflorescences and nut samples. The percentage of inflorescences colonized with *Aspergillus* spp. belonging to the commonly found sections *Nigri, Circumdati*, and *Flavi* was quantified in male inflorescences of pistachio in 2008 and 2018, before (mid-June) and after (mid-July) the treatment with AF36 Prevail® in comparison with a non-treated plot. In general, the percentage of male

inflorescences colonized with *Aspergillus* section *Flavi* in the treated plot was higher in 2018 than in 2008 in both months, June (75.6 *vs* 13.3) and July (84.4 *vs* 25.6). After AF36 application, the percentage of inflorescences colonized with *Aspergillus* section *Flavi* increased 12.2% and 8.9% in 2008 and 2018, respectively (Table 1), but not significantly. However, the percentage of inflorescences colonized by isolates of the *Flavi* section significantly ($P < 0.001$) decreased to a 40% during this period in the untreated plot in 2008. After the application of AF36 Prevail® grains, the percentage of inflorescences showing isolates of the section *Flavi* was 22.3% higher in the treated plot than the untreated plot. This effect was not detected in 2018. The distribution of *A. flavus* isolates considering the morphotype was similar in both years. In 2008, morphotype L represented 60% (83% VCG YV36, to which AF36 belongs) in the treated plot and 50% (no YV36 detected) in the non-treated one. In 2018, morphotype L represented 50% (80% VCG YV36) in the treated plot and 30% (33% VCG YV36) in the non-treated plot. Furthermore, the *Aspergillus* population belonging to sections *Circumdati* and *Nigri* colonized more than 50% (range 58.9-100%) of inflorescences in both treated and untreated plots in 2008 and 2018, showing no significant ($P > 0.05$) increase or decrease after the treatment with AF36 Prevail®. Non-disinfested pistachio nuts were mainly (89-100%) colonized with *Aspergillus* section *Nigri* in both years. Moreover, a low percentage (3.3%) of colonies belonging to section *Flavi* was found in 2008 in the treated orchard, which increased by 50% after the AF36 application (from 1.7 to 3.3%). This latter year, section *Circumdati* decreased significantly between observation times in both treated (from 30% to 23.3%) and non-treated orchards (from 25% to 5%). In contrast, no colonies of *Aspergillus* section *Flavi* were found in nuts, and just one isolate was found belonging to section *Circumdati* in the treated orchard in July 2018. Regarding the percentage of nuts colonized by *Aspergillus* spp. we obtained very few colonies of *Aspergillus* section *Flavi* when the nuts were surface-desinfected (1-3), being difficult to compare.

Characterization of Aspergillus spp. present in the soil. The *A. flavus* population within the *Aspergillus* section *Flavi* (%) present in the ground before the application of AF36 Prevail® in 2008 was significantly different (11.7 vs 0.5 CFU/g soil; $P < 0.01$; Table 1) between the treated and the non-treated orchards. In June 2008, 70% of the *A. flavus* isolates of the treated orchard belonged to the morphotype with Large (L) sclerotia; and 80% of these were classified into the VCG YV36, the VCG to which the atoxigenic isolate

AF36 belongs. In June 2018, non-significant (P = 0.527) differences were found between plots mainly because a high increase of the CFU was detected in the non-treated plot (30.2 vs 29 CFU/g). In this latter year, the L- morphotype strains represented 68.3% of the *A. flavus* isolates, and 70% resulted in VCG YV36.

Table 1. Incidence of colonization of pistachio male inflorescences and nuts with *Aspergillus* spp. sections *Flavi, Circumdati,* and *Nigri* from the ground of a treated and a non-treated pistachio orchard, before (June) and after (July) the application of AF36 Prevail® in 2008 and 2018, and presence of *Aspergillus* section *Flavi* in soil (CFU/g soil).

Inflorescences (%)		**2008**			**2018**		
		June	**July**	**Δ%**	**June**	**July**	**Δ%**
Section *Flavi*	Treated	13.3	25.6	12.2^{ns}	75.6	84.4	8.9^{ns}
	Untreated	43.3	3.3	-40***	80	100	20^{ns}
	Δ%	-30**	22.3**		-4.4^{ns}	-15.6^{ns}	
Section *Circumdati*	Treated	60	75.6	15.6^{ns}	58.9	83.3	24.4^{ns}
	Untreated	96.7	96.7	0^{ns}	70	100	30^{ns}
	Δ%	-36.7*	-21.1^{ns}		-11.1^{ns}	-16.7^{ns}	
Section *Nigri*	Treated	76.7	88.9	12.2^{ns}	85.6	93.3	7.8^{ns}
	Untreated	96.7	100	3.3^{ns}	96.7	73.3	-23.3^{ns}
	Δ%	-20^{ns}	-11.1^{ns}		-11.1^{ns}	20^{ns}	
Nuts (non-disinfested) (%)		**June**	**July**	**Δ%**	**June**	**July**	**Δ%**
Section *Flavi*	Treated	1.7	3.3	1.6	0	0	0
	Untreated	0	0	0	0	0	0
	Δ%	1.7	3.3		0	0	
Section *Circumdati*	Treated	30	23.3	-6.7	0	2	2
	Untreated	25	5	-20**	0	0	0
	Δ%	5	18.3*		0	2	
Section *Nigri*	Treated	100	100	0	93	98	5
	Untreated	100	100	0	40	100	60***
	Δ%	0	0		53***	-2	
Soil (CFU/ g soil)		**June**			**June**		
Section *Flavi*	Treated	11.7			30.2		
	Untreated	0.5			29		
	Δ%	11.2**			1.2		

Aspergillus isolates belonging to section *Flavi* presented yellowish green to deep olive-brown conidia, those belonging to section *Circumdati* presented yellow, buff, or ochraceous shades conidial masses and those to section *Nigri* presented conidial masses in shades of black (Klich 2002; Pitt and Hocking 2009). Statistical significance ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) by Pearson's Chi-square test using two by two contingency tables.

3.2. Colonization of pistachio male inflorescences by the AF36 strain under controlled and field conditions

Colonization under controlled conditions. The AF36 strain profusely colonized both aerial and fallen pistachio male inflorescences. Still, the colonization was significantly higher ($P < 0.05$) in the aerial inflorescences than those collected from the ground (95 vs 34.5%) (Figure 1). In addition, other saprophytic fungal species, mainly of the genus *Fusarium*, were frequently observed colonizing the fallen male inflorescences.

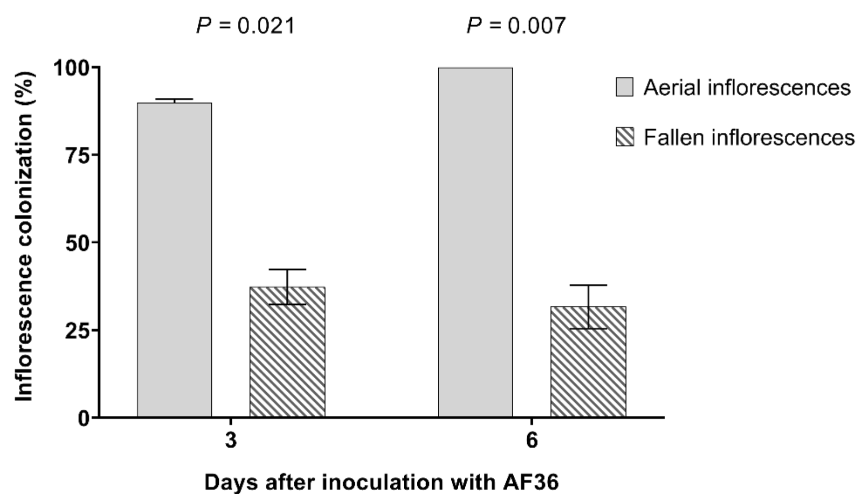

Figure 1. Surface colonization by *Aspergillus flavus* (AF36 strain) of pistachio male inflorescences cv. Peters collected from the tree canopy (aerial) or the soil (fallen). Inflorescences were collected, inoculated with a spores suspension of AF36, and incubated at 30ºC under 100% relative humidity for 7 days. The percentage of inflorescence colonization was visually estimated at 3 and 6 days after inoculation. P-values show differences according to Wilcoxon Rank Sum test.

When aerial inflorescences inoculated with the AF36 strain were incubated at different humidities (75-100%), the humidity significantly affected ($P < 0.001$) the inflorescence colonization by the biological control strain, but no differences were found between evaluation days. The optimum humidity to obtain a maximum percentage of inflorescence colonization was 96.1% (Figure 2).

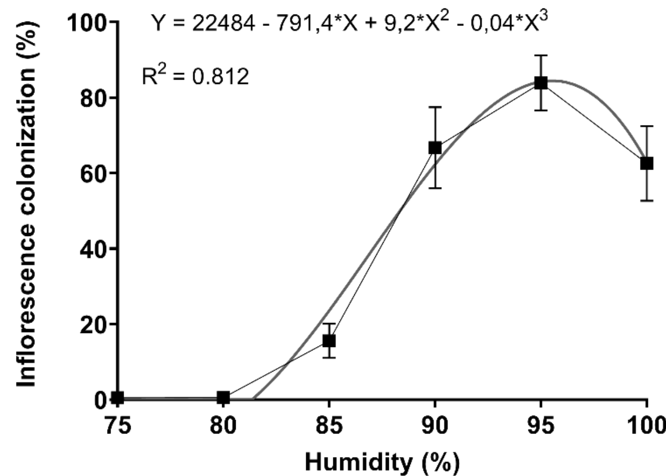

Figure 2. Effect of the humidity on the surface colonization (%) of pistachio male inflorescences cv. Peters by *Aspergillus flavus* (AF36 strain). Inflorescences were collected from the tree canopy, inoculated with a spores suspension of the AF36 strain, and incubated at different humidity levels at 30ºC for 7 days. The best subset regression process fitted third-order polynomial equation relating to humidity and inflorescence colonization by AF36 strains.

Colonization under field conditions. When pistachio inflorescences were inoculated with *A. flavus* AF36, this strain similarly colonized (P = 0.314) the inflorescences of the cvs Randy and Peters (17.5 vs 5%, respectively). As expected, the percentage of inflorescences colonized by *A. flavus* was significantly higher on the inoculated inflorescences than those naturally colonized by the fungus (P = 0.045). In addition, other fungal species, mainly *Fusarium* spp., were found colonizing the inflorescences.

Furthermore, the establishment of AF36 strain in pistachio male inflorescences in the field was also monitored from Many to September. Thus, the AF36 strain was isolated from 80% of the pieces of the aerial inflorescences and 59% of those located on the ground. These differences were significant (P = 0.013) in July, when the AF36 strain was isolated from 63% of the pieces of aerial inflorescences and 35% of the pieces of those from the ground. However, there were non-significant differences in the percentage of colonized inflorescences by the AF36 strain between aerial and ground in August and September (P = 0.394 and P = 0.061, respectively). In general, the percentage of pieces from which the AF36 was isolated increased by 1.5 fold from July to September (Figure 3).

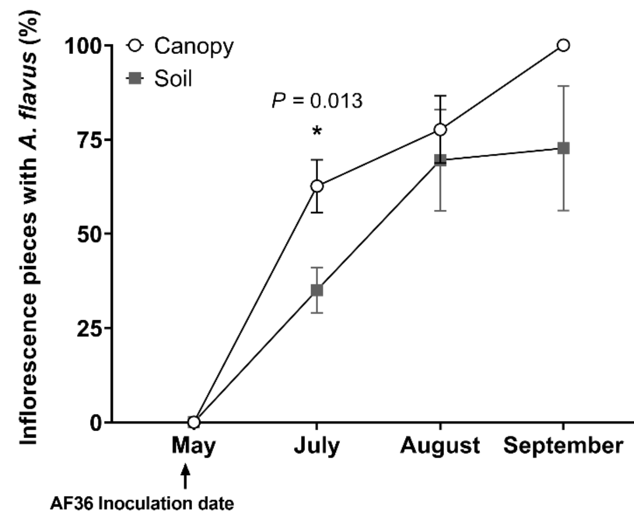

Figure 3. Percentages of male pistachio inflorescences pieces from which *Aspergillus flavus* AF36 was isolated. The inflorescences were previously inoculated using a spore suspension of the AF36 strain placed in the canopy of pistachio trees (aerial) or on the ground (soil). Each point represents the mean value of the percentage of inflorescence colonization taken from six 'Peters' trees, and the bar is the standard error of the mean—significant differences according to the Wilcoxon Rank Sum test.

3.3. Monthly monitoring of A. flavus population in soil and canopy after field application using fallen male inflorescences as a substrate of AF36

Monitoring of inoculum on pistachio leaflet surfaces. Data obtained in both repetitions (plots) were grouped for evaluation after checking for homogeneity of the experimental error variances. In general, the accumulated spores of *A. flavus* remained below five on the pistachio leaflets surface from April to June during the three years. Conversely, an important increment of the *A. flavus* population was detected from July to September during the three seasons.

The maximum accumulated spores of *A. flavus* on leaflets found in the male trees, in which fallen inflorescences were sprayed with AF36 spore suspension (Peters-treated), were 14.47 and 13.38 spores of *A. flavus* per leaflet g in 2016 and 2017, respectively; i.e., the highest data in both years. During the last season (2018), the highest accumulated spore density (18.15 spores/leaflet g) was obtained in the female trees whose ground was directly sprayed with AF36 spores (Soil-treated). Significant differences (P = 0.015) were found between the AUSPCs of the groups Peters-treated (i.e., inoculated inflorescences) and Peters-non-treated control in 2016. However, in the subsequent years, in which the

Prevail® and the Soil-treated were implemented, no significant differences (*P* = 0.547 in 2017 and *P* = 0.188 in 2018) were observed between the different treatments. Nevertheless, the accumulated *A. flavus* spores per leaflet-g obtained in 2017 and 2018 for the alternative application of AF36 using male inflorescences as a substrate was significantly not different (*P* = 0.841 in 2017 and *P* = 0.225 in 2018) than those obtained for the commercial treatment of AF36 using Prevail® grains (Figure 4).

Monitoring the AF36 inoculum on the ground. In general, no differences among treatments were found in any of the years for the CFU/g-soil, and the *A. flavus* population remained oscillating from 0.33 to 43.25 CFU/g-soil. However, when we considered accumulated values along the month, we observed a maximum of 74.90 and 20.67 CFU/g-soil in the treated trees using the male inflorescences as an alternative substrate for AF36.

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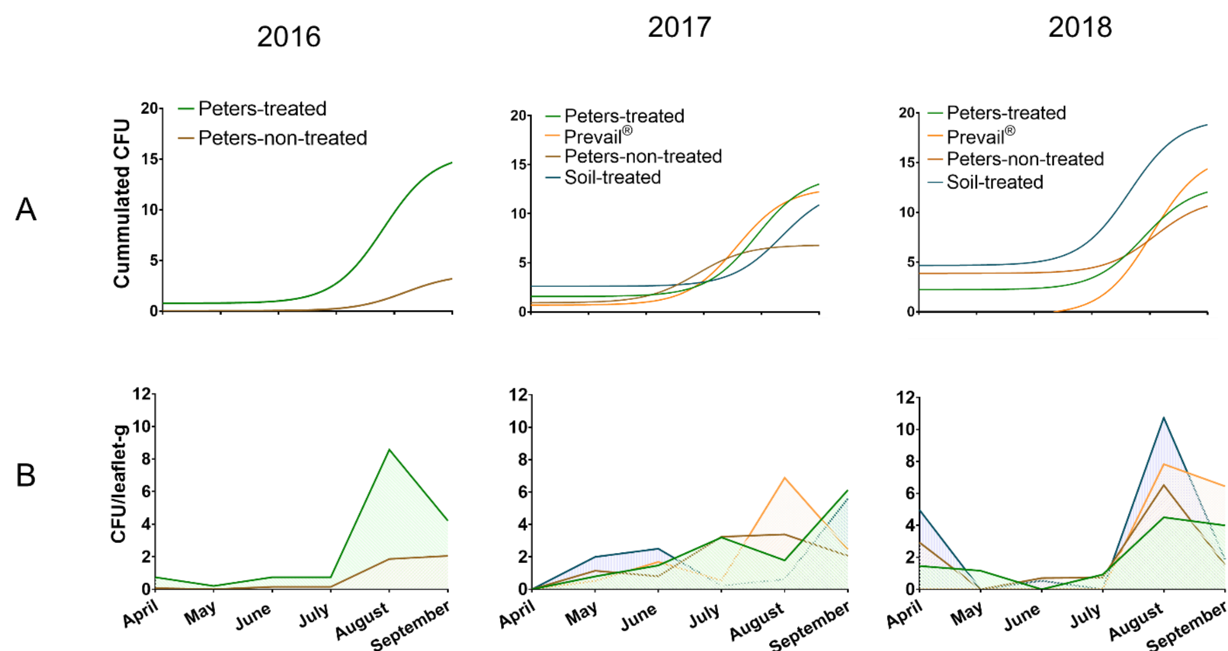

Figure 4. Monthly monitoring of *Aspergillus flavus* population in the canopy of pistachio trees along 2016-2017-2018, from April to September, after field application using fallen male inflorescences as a substrate of *A. flavus* AF36 strain (Peters-treated). Colored lines represent the treatments performed: i) Peters-treated, pistachios cv. Peters whose fallen inflorescences were inoculated by spraying 1500 ml per tree of an AF36 strain spore (10^6 spores/ ml) suspension; ii) Peters-non-treated control, pistachios cv. Peters whose inflorescences were sprayed with water; iii) Soil-treated, pistachios cv. Kerman, which ground was sprayed with 1500 ml of spore suspension of AF36 strain; and iv) Prevail®, represents three female pistachios cv. Kerman with AF36 applied at the commercial dosage (40 g Prevail® grains per tree) under the tree canopy. A) *A. flavus* population in terms of accumulated CFU on the pistachio leaflets surface, tendencies adapted to a sigmoidal curve. B) *A. flavus* population in terms of CFU/leaflet-g quantified monthly. The shaded area corresponds to the Area Under Spore Density Curve.

4. Discussion

Our results highlight the importance of male pistachio inflorescences as an inoculum source of toxigenic isolates of *A. flavus* and *A. parasiticus* in orchards. These results are in concordance with previous field observations by Doster and Michailides (1994) and with the recommendation of eliminating male inflorescences by burying them to limit aflatoxin contamination in nuts (Boutrif 1998). With this in mind, we considered male inflorescences a novel source of inoculum and, simultaneously, an alternative substrate for the biocontrol strain AF36. The male inflorescences are natural (and free) substrates, abundant, and uniformly distributed within the pistachio orchard (Ferguson and Haviland, 2016; Doster and Michailides, 1994). Firstly, we studied the natural population of *Aspergillus* spp. on male inflorescences and its changes before and after applying the AF36 Prevail® inoculum. In general, no significant changes in the density of sections *Circumdati* or *Nigri* were observed. However, changes in *A. flavus* VCG frequencies were expected after the biocontrol application, but no alterations are supposed to be observed in the *Aspergillus* spp. population size (Ortega-Beltran et al. 2020). The species of *Aspergillus* section *Flavi* became more abundant after the first treatment in 2016. Furthermore, the population of isolates of the section *Flavi* in the AF36 Prevail® treated plot was 4-times higher in 2018 than in 2008, but no differences were found between treated and non-treated plots in 2018, which suggests the effect of year applications and the spreading capacity of the biocontrol agent (Cotty and Bayman 1993). Premature-fallen nuts are frequent debris under the ground of female pistachios (Doster and Michailides 1994), but did not represent a good *Aspergillus* spp. inoculum source according to our results.

This early exploration allowed us to compare the effect of AF36 treatment using male inflorescences as an example. Pistachio panicle-inflorescences start to fall on the ground after pollen dehiscence, creating a padded layer under the pollinizer tree (Ferguson and Haviland 2016; Parfitt et al. 2010). Thus, to study the potential of pistachio male inflorescences as an adequate substrate for AF36, we conducted a controlled experiment and demonstrated that the inflorescences from the canopy represent a better inoculum source than those fallen on the ground. It is understandable since inflorescences from the ground decayed due to soil microorganisms. Besides, we determined the most appropriate environmental humidity (about 96%) for the correct sporulation of AF36 in the inflorescences, easily achieved by micro-sprinkler irrigation of pistachio orchards in

California (Marino et al. 2019). Subsequently, fallen inflorescences can act as an AF36 inoculum source, as shown in the experiments performed under field conditions. Fortunately, this substrate is ensured yearly after the yearly bloom of pistachios.

Regarding the male cultivar of choice, the cv. Randy, which is being widely used as a pollinizer of the cvs. Golden Hills and Lost Hills (Parfitt et al. 2010), its inflorescences were an AF36 substrate at least as good as those of the dominant cultivar. Indeed, besides the advantages associated with the cv. Randy, this cultivar offers more abundance due to its high density and large inflorescences (Parfitt et al. 2010).

Finally, we compared both substrates for AF36 strain (pistachio male inflorescences and the commercial product AF36 Prevail®) by monitoring AF36 spores in the pistachio tree canopy. Also, we monitored the inoculum in the soil, where the *A. flavus* population remained constant over time. Therefore, monitoring the inoculum on the tree canopy, where the susceptible nuts are located, represents a more efficient approach to appreciate differences. Based on that, we demonstrated a higher abundance of AF36 inoculum in 2016 in the treated trees when compared with the non-treated ones. Conversely, in 2017 and 2018, no differences were found among treatments because of cross-contamination of the AF36 strains between seasons and adjacent plots (Ching'anda et al. 2022, Moral et al. 2020), so an overlapping of treatment effects might have occurred. Additionally, one must consider that the application of AF36 Prevail® product in pistachio orchards in California has started since the registration of this product was in 2012. In any case, the densities of the AF36 spores in the tree canopies were similar on the commercial (AF36 Prevail®) treated trees than those using male inflorescences. Concomitantly, we showed the importance of the male inflorescence on the soil as a source of inoculum for species of *Aspergillus*.

Further studies should be conducted before adopting this alternative method for a commercial scale to respond to some unanswered questions. For example, can we further increase the AF36 population if the inflorescences are inoculated at various times? Can we improve the establishment of the biocontrol strain on the inflorescence by adding some additives (adjuvants, nutrients, etc.) to the spore suspension? Finally, is it possible to distribute the spores of the biocontrol strains to male inflorescences through the orchard irrigation system? In any case, this study shows that male pistachio inflorescences represent an attractive and accessible inoculum substrate to increase and spread the

biocontrol strain AF36 uniformly in pistachio orchards.

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Almond nut affected by navel orangeworm (*Amyelois transitella* Walker), cultivar Carmel

Ph.D. Thesis

Biocontrol agents to reduce aflatoxins in nuts: inoculum dynamic studies, varietal resistance to the pathogen, and characterization of the population of Aspergillus spp. section Flavi in Spain

CHAPTER III

BIOCONTROL OF AFLATOXINS IN NUTS: VARIETAL RESISTANCE

RESISTANCE TO *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS* IN ALMOND ADVANCED SELECTIONS AND CULTIVARS AND ITS INTERACTION WITH THE AFLATOXIN BIOCONTROL STRATEGY

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Abstract

Aflatoxin contamination of almond kernels, caused by *Aspergillus flavus* and *A. parasiticus*, is a severe concern for growers because of its high toxicity. In California, the global leader of almond production, aflatoxin can be managed by applying the biological control strain AF36 of *A. flavus* and selecting resistant cultivars. Here, we classified the almond genotypes by K-Means cluster analysis into three groups (susceptible [S], moderately susceptible [MS], or resistant [R]) based on aflatoxin content of inoculated kernels. The protective effects of the shell and seedcoat in preventing aflatoxin contamination were also examined. The presence of intact shells reduced aflatoxin contamination >100-fold. The seedcoat provided a layer of protection but not complete protection. In kernel inoculation assays, none of the studied almond genotypes showed a total resistance to the pathogen. However, nine traditional cultivars and four advanced selections were classified as R. Because these advanced selections contained germplasm derived from peach, we compared the kernel resistance of three peach cultivars to that shown by kernels of an R (Sonora) and an S (Carmel) almond cultivar and five pistachio cultivars. Overall, peach kernels were significantly more resistant to the pathogen than almond kernels, which were more resistant than pistachio kernels. Finally, we studied the combined effect of the cultivar resistance and the biocontrol strain AF36 in limiting aflatoxin contamination. For this, we co-inoculated almond kernels of R Sonora and S Carmel with AF36 72 h before or 48 h after inoculating with an aflatoxin-producing strain of *A. flavus*. The percentage of aflatoxin reduction by AF36 strain was greater in kernels of Carmel (98%) than in those of Sonora (83%). Cultivar resistance also affected the kernel colonization by the biological control strain. AF36 strain limited aflatoxin contamination in almond kernels even when applied 48 h after the aflatoxin-producing strain. Our results show that biocontrol combined with the use of cultivars with resistance to aflatoxin contamination can result in a more robust protection strategy than the use of either practice in isolation.

Keywords: aflatoxins, almond, *Aspergillus flavus*, *Aspergillus parasiticus*, cultivar resistance

1. Introduction

Almond [*Prunus dulcis* (Miller D.A. Webb) syn. *Amygdalus dulcis* Mill., or *Amygdalus communis* L.] is one of the most important tree nut crops in California, where >80% of the annual global production occurs (https://www.fas.usda.gov/commodities). In California, aflatoxins contaminate almond nuts at low frequencies. Even so, 22% of RASFF Mycotoxin Notifications for the United States from 2010 to 2019 were reported for aflatoxins in almonds (Alshannaq and Yu 2021). Aflatoxins are a severe concern for growers because shipments exceeding regulatory thresholds are rejected in domestic and international markets (Pigłowski 2019; Whitaker et al. 2010). In California nut-growing areas, the species *Aspergillus flavus* Link. and *A. parasiticus* Speare are the predominant producers of aflatoxins (Doster and Michailides 1994; Ortega-Beltran et al. 2019). Generally, the fungus *A. parasiticus* produces the four types of aflatoxins (B_1, B_2, G_1, and G_2) whereas the population of *A. flavus* consists of toxigenic isolates (producing B_1 and B_2 aflatoxins) and nonaflatoxigenic isolates (Amaike and Keller 2011).

Wild, non-aflatoxigenic isolates of *A. flavus* (hereafter referred to as atoxigenic isolates) have been used to develop aflatoxin biocontrol strategies for many crops, including nut crops (Doster et al. 2014).

The atoxigenic biocontrol technology is based on the release of atoxigenic isolates of *A. flavus* to displace the native toxigenic ones (Mehl et al. 2012). Aflatoxin reduction caused by the introduction of atoxigenic isolates of *A. flavus* increases when the contamination in the target crop is greater (Moral et al. 2020). Although aflatoxin frequency is low for nuts in California, the biocontrol strain AF36 of *A. flavus* (registered for commercial use as AF36 Prevail) has been shown to be an effective strategy for reducing aflatoxin contamination in California pistachios (Doster et al. 2014). Similarly, when applied in almond orchards, the AF36 strain dominated soils and displaced toxigenic strains of both *A. parasiticus* and *A. flavus*. Biocontrol products formulated with atoxigenic isolates as active ingredients have been registered with biopesticide regulatory authorities to protect different crops in the United States, Italy, and several African nations (http://www.agronomico.com/AFX1.aspx; Moral et al. 2020).

The risk of almonds becoming contaminated with aflatoxins in California mainly stems from the infestation by navel orangeworm (NOW; *Amyelois transitella* Walker), a Lepidopteran pest whose larvae damage the almond fruit and can serve as a vector for aflatoxin producers to reach the kernel (Hamby et al. 2011; Palumbo et al. 2014; Wilson

et al. 2020). Weather conditions (Williams et al. 2021) and the composition of pathogen populations also influence aflatoxin content in almonds (Ortega-Beltran et al. 2020). Although different studies have been conducted, there are gaps in knowledge concerning almond resistance to *Aspergillus* spp. For example, the risk of infestation and contamination of almond kernels by aflatoxins could depend on shell type (paper shell, soft, semihard, or hard shell) and cultivar resistance to the pathogen.

In 1994, Gradziel and Wang (1994) showed the protective effect of the seedcoat against *A. flavus* colonization. These authors, and later Dicenta et al. (2003), evaluated the resistance of kernels of various American and European almond cultivars to *A. flavus* through inoculation assays considering the kernel colonization by the pathogen but not the aflatoxin content. In 2000, Gradziel et al. (2000) evaluated the kernel resistance of 13 almond cultivars and four advanced selections (almond × peach backcrosses) to *A. flavus* by inoculating split kernels and evaluating kernel colonization, and they assessed the pathogen's ability to produce aflatoxin on agar plus almond kernel powder from these almond genotypes. In a more recent study (Gradziel 2020), the author described a marked reduction of aflatoxin content in almond cultivars incorporating peach genes.

The studies mentioned above were conducted using the fungus *A. flavus* but not the highly toxigenic *A. parasiticus* (Dicenta et al. 2003; Gradziel 2020; Gradziel et al. 2000). Consequently, it is necessary to evaluate susceptibilities to *A. parasiticus* because this species commonly interacts with almond (Donner et al. 2015). Indeed, *A. parasiticus* is responsible for >50% of the California nut batches exceeding 20 ppb of total aflatoxins (regulatory limit established by the U.S. Food and Drug Administration; Garcia-Lopez et al. 2018).

Studies evaluating several atoxigenic isolates of *A. flavus*, including AF36, have been conducted using a single almond cultivar under controlled conditions (Ortega-Beltran et al. 2019). However, the combined effect of cultivar resistance and atoxigenic fungi, particularly AF36, in limiting aflatoxin contamination in almond kernels has not been studied.

The evaluation of different almond cultivars in their resistance to both *A. flavus* and *A. parasiticus* infection and aflatoxin production was the primary purpose of this study. We also assessed the protective effect of the shell and the seedcoat against pathogen colonization and kernel contamination with aflatoxins and compared the resistance with different peach and pistachio cultivars. Finally, we examined the combined effect of

cultivar resistance with biocontrol using the U.S. Environmental Protection Agency (USEPA)-registered atoxigenic strain AF36.

2. Materials and methods

2.1. Resistance of traditional almond cultivars to Aspergillus spp.

In 2016, we studied the resistance of 10 commercial Californian almond cultivars to colonization and aflatoxin production by *Aspergillus* spp. (Table 1). For that, we considered (i) the cultivar effect, (ii) the protective effect of the shell (inoculating kernels with or without shell), (iii) the protective effect of the seedcoat (inoculating kernels with or without the seedcoat), and (iv) the effect of aflatoxin-producing isolates of *A. flavus* or *A. parasiticus*. Almond kernels were inoculated with a 0.5-ml spore suspension (10^6 spores) per kernel (Ortega-Beltran et al. 2018). In all cases, we inoculated 20 to 24 kernels and 20 to 24 kernels with shell (henceforth nuts) of each cultivar, set in three replicates (three Petri dishes). Before inoculation, both kernels and nuts were surface-disinfected with commercial bleach according to Camiletti et al. (2018). Then, dried kernels and nuts were independently inoculated with two reference isolates of *A. flavus* (2A1L11 and 29C3L11) and one of *A. parasiticus* (10A1P11) (Ortega-Beltran et al. 2019). A small wound (5 × 5 mm) was made by scratching the seedcoat of half of the almond kernel group inoculated to evaluate the protective effect reported by Gradziel and Wang (1994). In this case, we studied the effect of wounding on kernels inoculated just *A. flavus* to reduce the number of experimental units. Petri dishes containing the kernels or nuts were incubated for 7 days at optimal conditions for aflatoxin production (30ºC and 100% humidity) after placing the Petri dishes in humidity chambers (plastic containers measuring 22 × 16 × 10 cm). Colonization (including both amounts of mycelia and sporulation) of each inoculated kernel and nut was quantified using a 0 to 14 scale, where 0 = no mycelium and 14 = kernel or nut completely covered with spores (Dicenta et al. 2003). In addition, the thickness of the seedcoat of 10 kernels of each almond genotype was measured using a Leica compound microscope (Leica DM2000 LED Microscope, Wetzlar, Germany) at ×200 magnification. For that, the frozen kernels were cut using a microtome (Microtome PFM Medical, Koln, Germany) and mounted on glass slides with sterile water.

After assessing kernel colonization, we conducted the aflatoxin extraction as follows. Shells were carefully and aseptically removed and ground as the directly inoculated

kernels. Shells were ground independently. Then, 30 ml of 60% methanol and 5 g of NaCl were added to each kernel set, ground with the solvent, and filtered and processed following the Association of Official Analytical Chemists (Cunniff 1995) for aflatoxin extraction. The concentrations of B_1 and G_1 aflatoxins were calculated using thin-layer chromatography (TLC) and a CAMAG TLC Scanner 3 (Muttenz, Switzerland) following the protocol described by Camiletti et al. (2018). As a reference for calculations, we used aflatoxin standards B_1, B_2, G_1, and G_2, from Sigma-Aldrich (St. Louis, MO).

2.2. Resistance of advanced selections and cultivars of almond to **Aspergillus** *spp.*

In 2016, we used two isolates of *A. flavus* (2A1L11 and 29C3L11) and one of *A. parasiticus* (10A1P11) to evaluate the resistance of kernels of 10 traditional cultivars and nine advanced selections to both fungal growth and aflatoxin accumulation (Table 2). Toward this goal, we inoculated the nonwounded kernels and incubated them in Petri dishes as described above but with an incubation period of 10 days. Also, we measured the thickness of the seedcoat of 10 kernels of each almond genotype as described above. Resistance of almond, peach, and pistachio kernels to *Aspergillus* spp. In previous assays, we observed that almond cultivar with peach pedigree showed high resistance to *Aspergillus* spp. Therefore, in 2017 we compared resistance to kernel rot and aflatoxin accumulation of two almond and four peach cultivars. For comparison, we also included kernels of four pistachio cultivars in the assay (Figure 1). In all cases, we disinfested and inoculated nonwounded kernels using a spore suspension of *A. flavus* 2A1L11. The inoculated kernels were incubated for 7 days, and aflatoxin B_1 was quantified as above. The experiment was repeated in 2018. The data of both repetitions were combined after checking for homogeneity of the experimental error by the F test.

2.3. Combined effect of biocontrol and cultivar resistance

In 2017, we studied the combined effect of cultivar resistance with the atoxigenic *A. flavus* isolate AF36, the active ingredient of the USEPA- registered biocontrol product AF36 Prevail, by coinoculating almonds kernels of cultivars Carmel (S) and Sonora (R). We disinfested 10 almond kernels of each cultivar and coinoculated them using 0.5 ml of a spore suspension (106 spores/ml) each of the atoxigenic isolate AF36 and toxigenic isolate 2A1L11. There were five treatments: AF36 inoculated before (72, 48, and 24 h) inoculating 2A1L11; both isolates inoculated simultaneously (0 h); and AF36 inoculated after (+48 h) 2A1L11 was inoculated. As controls, we used almond kernels inoculated

with only the toxigenic or only the atoxigenic isolate. Kernel disinfestation, inoculation, incubation, and aflatoxin B1 quantification were performed as above. The experiment was repeated. The data of both experiments were combined after checking for homogeneity of the experimental error by the F test.

2.4. Statistical analysis

We arranged all the assays into a randomized design. We subjected data of different independent variables to factorial analysis of variance (ANOVA). When necessary, dependent variables were log- or inverse-transformed for variance homogeneity. After ANOVA, we compared the means by least significant difference at $P = 0.05$. Likewise, we calculated the percentage of the variance attributed to the independent variables calculating eta squared (g^2, an r-type effect) according to $g^2 = [(SS_{effect}/SS_{total}) \times 100]$ (Norman and Streiner 2008). We classified the cultivars using a nonhierarchical K-Means cluster analysis by assuming the groups susceptible, moderately susceptible, and resistant. We used the average concentration of aflatoxins B_1 and G_1 for each combination isolate-cultivar/genotype of inoculated kernels. We selected the optimum number of groups according to the dendrogram's topology, testing the significance of the formed group on the aflatoxin accumulations and checking the presence of extreme values and outliers (Moral et al. 2017). When almond kernels were coinoculated with the biocontrol isolate AF36, we separately studied the significance of differences between inoculated and coinoculated kernels for each cultivar according to the nonparametric Wilcoxon signed rank test at $P = 0.05$. Finally, we analyzed the relationships between the dependent variables by Pearson's correlation. Data from all experiments were analyzed using Statistix 10 and SPSS 16 Software.

3. Results

3.1. Resistance of commercial almond cultivars to Aspergillus spp

Because seedcoat wounds were made only on almond kernels inoculated with the *A. flavus* isolates, two ANOVAs were independently conducted (Table 1). In the first ANOVA, we omitted the isolate *A. parasiticus* to examine the effect of wounding on kernel contamination by aflatoxin B_1. We found significant effects of the almond cultivar ($P = 0.015$; $g^2 = 26\%$ of the total variance), the presence of the wound on the seedcoat ($P < 0.001$; $g^2 = 12\%$; wounded kernels were more susceptible than intact), and the *A. flavus* isolate ($P < 0.001$; $g^2 = 6\%$; 26C3L11 produced more aflatoxin B_1 than 2A1L11), whereas

the double and triple interactions were not significant ($P > 0.05$). We obtained similar results according to the kernel colonization, but the interaction wound × cultivar was significant ($P = 0.006$; $g^2 = 5\%$). For example, wounding increased the colonization of the Sonora kernels significantly by *A. flavus* although it did not affect cultivar Padre kernels (Table 1). Furthermore, when we studied the effect of the cultivar on the inocula tion conducted using the isolate 10A1P11 of *A. parasiticus* (by omitting *A. flavus*), we found significant differences ($P < 0.001$; $g^2 > 70\%$) among almond cultivars for the three dependent variables (B_1 and G_1 contamination, and kernel colonization). Overall, kernels of Sonora allowed the lowest aflatoxin content ($B_1 + G_1 = 60$ ppm) but a similar level as kernels of cultivar Independence ($B_1 + G_1 = 89$ ppm). Wounding the seedcoat of kernels of Sonora markedly increased aflatoxin accumulation. The seedcoat thickness ranged from 111 to 157 µm for cultivars Butte and Independence, respectively. However, there was no correlation ($r = -0.308$; $P = 0.501$) between the thickness of seedcoat and aflatoxin content in the nonwounded inoculated kernels of the different cultivars.

According to the K-Means cluster, cultivars Independence, Nonpareil, Sonora, and Wood Colony formed the aflatoxin resistant group, whereas cultivars Carmel and Frintz were the most susceptible. The remaining four cultivars formed a moderately susceptible group (Table 1). As expected, aflatoxin concentrations (B_1 and G_1) on almond kernels were significantly correlated with fungal colonization ($r = 0.730$; $P < 0.001$).

Independently of shell type (paper shell, soft, semihard, or hard shell), *Aspergillus* spp. reproduced less on the inoculated nuts (i.e., in-shell kernels) and did not produce aflatoxin on the shell (data not shown). However, the examined fungi could overcome the shell barrier and reach the kernel. Even so, the presence of intact shells reduced the aflatoxin contamination by >100-fold, and only one experimental unit (one Petri dish with seven Sonora nuts inoculated with *A. parasiticus*) had >20 ppb of aflatoxin B_1 (96 ppb).

Table 1. Aflatoxin B_1 and G_1 accumulation (ppm) in almond kernels of 10 commercial Californian cultivars independently inoculated with two isolates of *Aspergillus flavus* and one isolate of *A. parasiticus*

Cultivar	Shell texture	Self-fertile	Origin	*Aspergillus flavus* 2A1L11 Intact Coloniz[a] (0-14)	2A1L11 Wound Coloniz (0-14)	2A1L11 Intact B_1 (ppm)	2A1L11 Wound B_1 (ppm)	*Aspergillus flavus* 29C3L11 Intact Coloniz (0-14)	29C3L11 Wound Coloniz (0-14)	29C3L11 Intact B_1 (ppm)	29C3L11 Wound B_1 (ppm)	*Aspergillus parasiticus* 10A1P11 Intact Coloniz (0-14)	10A1P11 Intact B_1 (ppm)	10A1P11 Intact G_1 (ppm)	Combined results Intact Avg. Coloniz (0-14)	Combined results Intact B_1+G_1[b] (ppm)	Reaction[c]
Carmel	Soft	No	US (NP × Mission)	6.3	10.6	61	73	8.4	11.2	106	118	9.0	116	105	**7.9**	**199**	**S**
Fritz	Semi-hard	No	US (Mission × Drake)	5.3	10.1	38	131	7.4	10.4	156	127	8.8	77	74	**7.2**	**164**	**S**
Butte	Semi-hard	No	US (Mission x NP)	6.8	8.8	70	121	7.1	11.1	58	139	8.4	52	44	**7.4**	**104**	**MS**
Mission	Semi-hard	No	US (unknown)	9.2	11.1	127	149	8.0	11.5	78	130	7.0	44	49	**8.1**	**132**	**MS**
Monterey	Semi-hard	No	US (NP x Mission)	6.9	8.3	65	109	6.7	10.1	102	124	6.1	57	77	**6.6**	**152**	**MS**
Padre	Semi-Hard	No	US (Mission x Swanson)	10.6	11.0	90	80	9.5	11.6	137	146	10.9	41	32	**10.3**	**121**	**MS**
Independence	Soft	Yes	US (Zaiger Genetics, CA, US)	4.3	6.9	13	71	5.5	10.9	55	95	2.3	9	12	**4.0**	**38**	**R**
Nonpareil	Soft	No	US (unknown)	3.0	7.0	31	93	3.3	7.1	95	113	5.1	38	43	**3.8**	**98**	**R**
Sonora	Soft	No	US (NP x Eureka)	0.5	7.6	4	72	1.4	8.3	13	55	4.6	20	23	**2.2**	**35**	**R**
Wood Colony	Semi-hard	No	US (unknown)	6.0	8.3	58	64	4.7	10.9	58	101	5.5	37	43	**5.4**	**94**	**R**
Average				**5.9**	**9.0**	**56**	**96**	**6**	**10**	**86**	**115**	**7**	**49**	**50**	**6.3**	**114**	

[a] Colonization.
[b] Sum of the averaged B_1, considering the isolates 2A1L11, 29C3L11, and 10A1P11, plus the G_1 produced by 10A1P11.
[c] Cultivars were classified as susceptible (S), moderately susceptible (MS), or resistant (R) considering the B_1 and G_1 aflatoxin content in intact kernels caused by the three *Aspergillus* isolates according to a nonhierarchical K-Means cluster analysis with the initial cluster center method under the assumption of three groups using the SPSS 16 Software.

Table 2. Aflatoxin B_1 and G_1 accumulation (ppm) in intact kernels of 10 traditional Californian and Spanish cultivars and nine advanced selections independently inoculated with two isolates of *Aspergillus flavus* and one isolate of *A. parasiticus*

				Aspergillus flavus				*A. parasiticus*					
			Isolate	2A1L11		29C3L11		10A1P11			Combined results		
Cultivar	Shell texture	Self-fertile	Origin	Coloniz. (0-14)	B_1 (ppm)	Coloniz. (0-14)	B_1 (ppm)	Coloniz. (0-14)	B_1 (ppm)	G_1 (ppm)	Avg. Coloniz. (0-14)	B_1+G_1[b] (ppm)	Reaction[c]
A95,1-26	Soft	No	U.S.A. (A05-4 × Winters)	8.5	164	8.6	147	8.3	64	80	**8.5**	**205**	**S**
A97,1-232	Soft	Yes	U.S.A. (A25-75 x Winters)	8.8	165	9.7	93	8.1	48	63	**8.9**	**165**	**S**
Aldrich	Soft	No	U.S.A. (unknown)	7.4	34	9.7	100	6.8	95	117	**8.0**	**193**	**S**
Price	Soft	No	U.S.A. (unknown)	8.3	59	9.1	101	8.1	118	139	**8.5**	**232**	**S**
A04,18-20	Soft	Yes	U.S.A. (A05-4 × A11-77)	8.0	91	8.3	63	7.1	67	72	**7.8**	**146**	**MS**
A06,3-319	Soft	Yes	U.S.A. (NP × A96,1-133)	8.1	96	8.6	82	6.7	68	82	**7.8**	**164**	**MS**
Antoñeta	Hard	No	Spain (Tuono × Ferragnes)	8.8	56	9.4	78	9.6	80	92	**9.3**	**163**	**MS**
Marcona	Hard	No	Spain (unknown)	7.7	76	8.9	92	8.3	68	61	**8.3**	**140**	**MS**
Marta	Hard	No	Spain (Tuono × Ferragnes)	9.9	78	9.6	97	7.6	79	61	**9.0**	**146**	**MS**
A04,8-160	Soft	Yes	U.S.A. (NP × A97,1-232)	6.5	35	5.2	81	4.9	24	19	**5.5**	**66**	**R**
A05,6-340	Soft	Yes	U.S.A. (Winters × A97,1-232)	6.0	36	5.4	61	5.8	39	71	**5.9**	**116**	**R**
A97,2-240	Soft	No	U.S.A. (A3-4 x Ferragnes)	7.6	109	8.2	78	6.6	31	36	**7.5**	**109**	**R**
A98,2-305	Soft	Yes	U.S.A. (NP × A3-3)	6.3	86	9.0	109	8.7	30	35	**8.0**	**110**	**R**
Ferraduel	Hard	No	France (Aï × Cristomorto)	7.2	113	6.4	68	5.2	35	48	**6.3**	**120**	**R**
Ferragnes	Semi-hard	No	France (Aï × Cristomorto)	8.2	51	8.8	76	7.7	37	39	**8.2**	**94**	**R**
F5C,7-10	Paper-shell	No	U.S.A. (NP × A3-19)	7.0	65	9.0	91	4.4	24	20	**6.8**	**80**	**R**
Kester	Soft	No	U.S.A. (Tardy Nonpareil × Arbuckle)	8.9	61	1.0	77	9.8	39	43	**6.6**	**102**	**R**
Tarragona	Hard	No	Spain (FLTU18 × Annaneta)	8.0	31	9.6	54	7.3	34	48	**8.3**	**88**	**R**
Winters	Paper-shell	No	U.S.A. (A3-1 x A6-7)	8.4	43	8.1	53	7.8	46	45	**8.1**	**92**	**R**
Average				**7.9**	**76**	**8.2**	**85**	**7.3**	**54**	**62**	**7.7**	**134**	

[a] Colonization.
[b] Sum of the averaged B_1, considering the isolates 2A1L11, 29C3L11, and 10A1P11, plus the G_1 produced by 10A1P11.
[c] Cultivars were classified as susceptible (S), moderately susceptible (MS), or resistant (R) considering the B_1 and G_1 aflatoxin concentrations in intact kernels caused by the three *Aspergillus* isolates according to a nonhierarchical K-Means cluster analysis with the initial cluster center method under the assumption of three groups. Data were analyzed using the SPSS 16 Software.

3.2. Resistance of almond advanced selections and cultivars to Aspergillus spp

We found significant effects of the almond cultivar ($P < 0.001$; $g^2 = 17\%$) and *Aspergillus isolates* (i.e., 2A1L11, 29C3L11, and 10A1P11) ($P < 0.001$; $g^2 = 6\%$) and their interaction ($P < 0.001$; $g^2 = 21\%$) on aflatoxin accumulation in inoculated kernels. For example, 'Aldrich' kernels inoculated with the strain 2A1L11 of *A. flavus* had lower aflatoxin B_1 accummulation (34 ppm) than those inoculated with *A. parasiticus* 10A1P11 (95 ppm). Conversely, 'Ferraduel' kernels accumulated 113 ppm of aflatoxin B_1 when inoculated with 2A1L11 and 35 ppm when inoculated with 10A1P11 (Table 2). For kernels inoculated with *A. parasiticus*, the almond genotype explained 65% of the total variance in aflatoxin G_1 content. ANOVA also showed significant differences among almond genotypes ($P < 0.001$; $g^2 = 81\%$) and between *Aspergillus* isolates ($P < 0.001$; $g^2 = 14\%$) whereas the interaction between both independent variables was not significant ($P = 0.149$). Fisher's least significant difference classified the *Aspergillus* isolates according to their ability to colonize the inoculated kernels as follows: *A. flavus* 29C3L11 > *A. flavus* 2A1L11 > *A. parasiticus* 10A1P11. Kernel colonization was correlated significantly ($r = 0.563$; $P = 0.012$) with aflatoxin concentration.

Overall, kernels of the advanced selection A04,8-160 and the Spanish cultivar Tarragona had the lowest aflatoxin content ($B_1 + G_1$ = 159 and 167 ppm, respectively), forming the resistant group with eight other genotypes. Conversely, the advance selections A95,1-26 and A97,1-132 together with the Californian cultivars Aldrich and Price formed the susceptible group ($B_1 + G_1$ ranged from 455 to 346 ppm). The cultivars Antoñeta, Marta, and Marcona and the advanced selections A4,18-20 and A06,3-319 formed the moder ately susceptible group (Table 2). The seedcoat thickness ranged from 88 to 264 µm for the genotype A05,6-340 and Antoñeta, respectively. Also in this case, there was no significant correlation ($r = 0.196$; $P = 0.4349$) between seedcoat thickness (micrometers) and fungal colonization.

3.3. Resistance of almond, peach, and pistachio kernels to Aspergillus spp

When we evaluated the resistance of kernels of cultivars of various crops to aflatoxin B_1 contamination, we detected significant ($P < 0.001$; $g^2 = 26\%$) differences among them (pistachio 62 ppm > almond 36 ppm > peach 3 ppm), although there were differences among cultivars of each crop. When the variable cultivars were examined as the independent variable (i.e., not considering the crop), it explained 33% of the total variance ($P < 0.001$). Overall, kernels of peach had much lower aflatoxin levels for all cultivars,

although the kernels of cultivar Ryan Sun had similar levels as those of almond Sonora. In the case of almonds, Sonora had lower aflatoxin B_1 levels than the pistachio cultivars, except Aria (Figure 1).

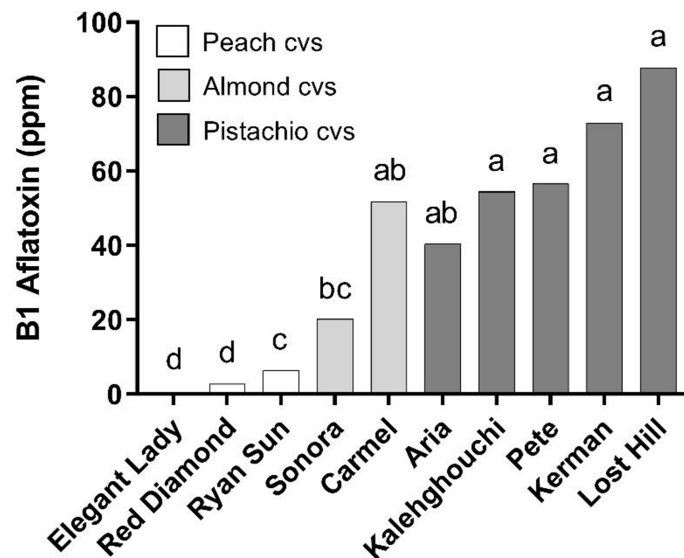

Figure 1. Accumulation of aflatoxin B_1 (ppm) in kernels of different cultivars of almond, peach, and pistachio inoculated with *Aspergillus flavus* isolate 2A1L11. The mean values with different letter are significantly different according to Fisher's protected least significant difference (LSD) test at $P = 0.05$.

3.4. Combined effects of aflatoxin biocontrol and cultivar resistance

As expected, coinoculated kernels of Carmel (susceptible) and Sonora (resistant) had significantly (Wilcoxon signed rank test, $P < 0.001$) lower aflatoxin content than kernels inoculated just with the toxigenic isolates. For example, kernels of Carmel (used as a positive control) had aflatoxin content between 47 and 116 ppm whereas aflatoxin content in coinoculated kernels of this cultivar was consistently below 15 ppm (1.5 ± 3.6 ppm). In the resistant Sonora, these differences were also evident (10 ± 3.8 ppm in inoculation with toxigenic versus 2 ± 3.3 ppm in coinoculation with toxigenic and atoxigenic isolates). Considering just the coinoculated kernels (i.e., using the percentage of aflatoxin reduction as a dependent variable), we found significant differences ($P = 0.032$) between cultivars. Thus, the percentage of aflatoxin reduction was higher in the susceptible Carmel than in Sonora (98 ± 32 versus 83 ± 5.4%, respectively; Figure 2). Conversely, when studying aflatoxin concentration in the coinoculated kernels, we found no significant effects of the cultivar, AF36 application timing, and their interaction. Interestingly, AF36

successfully limited aflatoxin contamination in almond kernels even when applied 48 h after the inoculation with toxigenic isolates.

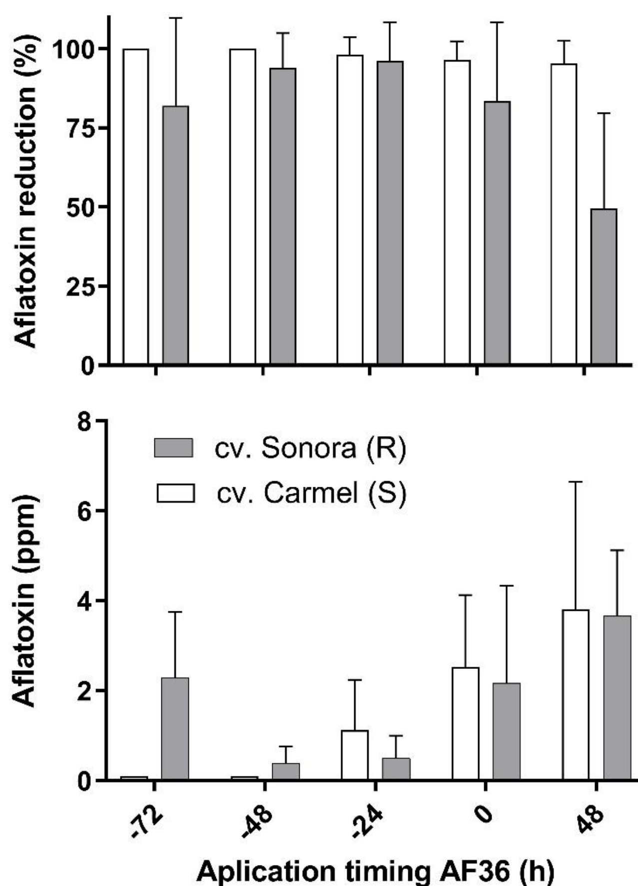

Figure 2. Ability of the atoxigenic isolate of *Aspergillus flavus* AF36 to reduce the accumulation of aflatoxin B_1 in almond kernels of the susceptible cultivar Carmel and resistant cultivar Sonora when coinoculated with two toxigenic isolates of *A. flavus* (data combined; upper panel). Aflatoxin reduction (%) = [1 − (total aflatoxin in coinoculation/total aflatoxin in kernels inoculated only with the toxigenic isolates)] × 100. Aflatoxin accumulation in coinoculated kernels. AF36 was inoculated at different application timing, before (−72, −48, and −24 h) or after (+48 h) the inoculation of the toxigenic isolates (lower panel). Bars and lines represent the mean of the treatment and the standard error, respectively. Aflatoxin concentration in control inoculated kernels of Carmel was significantly ($P < 0.001$) higher than in those of Sonora (average: 76 versus 10 ppm, respectively). The percentage of aflatoxin reduction was greater ($P = 0.032$) in coinoculated kernels of Carmel than in those of Sonora (98 ± 32% versus 83 ± 5.4%, respectively).

4. Discussion

To our knowledge, this is the first time that aflatoxin contamination has been evaluated in kernels of various almond cultivars using both *A. flavus* and *A. parasiticus*. We found high variability in response to aflatoxin contamination of almond cultivars when independently inoculated with three *Aspergillus* isolates. This variation agrees with the variability previously described concerning *A. flavus* colonization of almond cultivars (Dicenta et al. 2003). Unfortunately, none of the studied cultivars were immune to either aflatoxin contamination or fungal growth. Dicenta et al. (2003) reported that kernels of several cultivars were completely resistant to colonization by the pathogen even after 18 days of inoculation. However, we identified exceptional genotypes that show potential for resistance breeding. It is essential to point out that our experimental conditions were highly conducive for aflatoxin production, and the observed levels of resistance are true of practical importance. Thus, cultivar resistance combined with biocontrol may offer the best strategy for limiting aflatoxin contamination.

Our inoculation assays have demonstrated the critical protective effect of the shell in preventing aflatoxin contamination of the kernels, regardless of the type of shell (hard, semihard, or paper shell; Table 1). However, the type of shell can also affect susceptibility to other pests. For example, infestation with NOW, *Amyelois transitella*, a major Lepidopteran pest of almond (and other crops) that causes kernel damage (wounding) and vectoring aflatoxin-producing fungi, has been reported to influence aflatoxin contamination (Palumbo et al. 2014; Picot et al. 2017). Besides, almond shell-seal and hull-split time have been shown to affect susceptibility to NOW damage (Hamby et al. 2011). Intuitively, it may be expected that lower aflatoxin contamination would occur in cultivars protected by hard shells. However, we have detected low aflatoxin contamination in soft and even paper-shell almond cultivars in the field. That was the case for Nonpareil, the most widely grown cultivar (45% of the state production) across California (Gradziel, 2020). This paper-shell variety can be affected by NOW at relatively low levels (Hamby et al. 2011). In our inoculation using unshelled kernels, Sonora, another soft-shell widely grown cultivar, allowed low aflatoxin levels (Table 1). It should be noted that the latter cultivar, under field conditions, is frequently contaminated by other fungi, including species in *Aspergillus* section *Nigri* and *Rhizopus* spp. (T. J. Michailides and J. Moral, unpublished data).

Almond breeding programs use peach germplasm to introduce desirable traits into

traditional almond varieties (Gradziel 2020). In the first experiment, we included a peach-derived almond variety (Independence) and classified it as one of the most resistant tested cultivars to fungal colonization and aflatoxin contamination. Independence is the dominant self-pollinating variety in California, where extensive new orchards of this cultivar can be found, and volumes in the market are expected to increase significantly in the upcoming years (Boyd 2020). Likewise, it might be interesting to evaluate the influence of peach genotype rootstocks for conferring aflatoxin resistance to almond. Nowadays, the rootstock Nemaguard (dominant in traditional San Joaquin Valley almond orchards) provides the attributes of a peach genotype, and its use is limited to specific agricultural scenarios, such as nematode-infested sandy soils. However, the effect of the almond rootstocks on aflatoxin accumulation has not been considered.

In the second inoculation assay, the Spanish cultivar Tarragona showed considerable resistance to accumulation of both B_1 and G_1 aflatoxins. Interestingly, the pollenizers ‘Winters’ and ‘Kester’, recently released genotypes from the University of California-Davis (Gradziel and Lampinen 2019; Gradziel et al. 2007), showed resistance to aflatoxin accumulation (Table 2). In our study, peach kernels were highly resistant to aflatoxin contamination. This supports the hypothesis that almond genotypes that incorporate peach germplasm are more resistant to aflatoxin (Gradziel 2020; Gradziel et al. 2000). In the current study, the highly resistant selections A04,8-160, A05,6-340, A97,2-240, and A98,2-305 all contained germplasm derived from peach or wild peach relatives.

In contrast, pistachio cultivars were significantly more susceptible to aflatoxin contamination than almonds. Indeed, the pistachio crop is more frequently affected by aflatoxin contamination than almond. For example, from 2010 to 2019, pistachios from the United States were the nut commodities with the most rejections, according to the European Rapid Alert System for Food and Feed (Alshannaq and Yu 2021).

In our study, aflatoxin concentrations and kernel colonization were correlated, as intuitively expected. Conversely, Gradziel et al. (2000) did not find correlations between pathogen colonization and aflatoxin contamination. However, these later assays were conducted by inoculating sliced kernels to assess kernel colonization and culture media enriched with the powdered kernel to measure aflatoxin accumulation. These processes do not represent realistic situations.

In the current study, the seedcoat of almond kernels was found to offer a layer of protection (independent of its thickness) but without providing complete protection. Conversely, cultivar susceptibility to aflatoxin contamination depends highly on the shell

seal, as reported by Gradziel and Kester (1994). As mentioned above, in our inoculation assays, the examined fungi had low ability in contaminating kernels in whole, intact nuts. Indeed, the presence of intact shells reduced the aflatoxin contamination by >100-fold compared with nuts with compromised shells. These results reinforce the need for adequate control of NOW infestation or the selection of less susceptible almond cultivars to this pest (Palumbo et al. 2014) as well as prevention of mechanical damage during or after harvest, which may compromise kernel integrity and increase susceptibilities to fungal reproduction and/or aflatoxin accumulation.

Our results indicate that cultivar resistance combined with biocontrol using atoxigenic isolates offers a particularly promising aflatoxin control strategy. Interestingly, the biological control strain AF36 was more efficient in limiting the aflatoxin contamination in coinoculated kernels of the susceptible cultivar (measured as percentage reduction to inoculated kernels) than in those of the resistant genotype because cultivar resistance also reduced kernel colonization by the atoxigenic isolate. However, under field conditions, combining both control approaches could be highly beneficial. Recently, Maxwell et al. (2021) reported aflatoxin degradation by atoxigenic isolates (including AF36) as an additional mechanism through which aflatoxin in treated crops is reduced. Our results further support those reported by Maxwell et al. (2021) because we observed a pronounced curative effect of AF36 in coinoculated kernels even when inoculated 48 h after the toxigenic isolate.

In conclusion, California almond farmers would benefit from using genotypes with low susceptibility to aflatoxin-producing fungi that are also less prone to NOW damage. This resistance strategy can be combined with the atoxigenic biocontrol strain AF36 of *A. flavus*, currently registered with USEPA for almond, cotton, maize, pistachio, and fig. Further reductions could be achieved through standard disease management practices, including on-time harvesting, proper nut drying, sorting out of damaged kernels, and controlled storage.

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Pistachio orchard, cultivar Kerman, finca 'El Jarón', Ciudad Real, España

PhD Thesis

Biocontrol agents to reduce aflatoxins in nuts: inoculum dynamic studies, varietal resistance to the pathogen, and characterization of the population of Aspergillus *spp. section Flavi in Spain*

CHAPTER IV

BIOCONTROL OF AFLATOXINS IN NUTS:

CHARACTERIZATION OF THE NATURAL POPULATION OF *ASPERGILLUS* SPP. SECTION *FLAVI* IN SPAIN

CHARACTERIZATION OF THE *ASPERGILLUS* SPP. POPULATION IN SPANISH ALMOND AND PISTACHIO ORCHARDS AND IDENTIFICATION OF ATOXIGENIC STRAINS OF *A. FLAVUS*

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Abstract

The fungal species *Aspergillus flavus* and *A. parasiticus* are the primary producers of aflatoxins, which are carcinogenic mycotoxins whose consumption poses a health hazard for consumers. Both these *Aspergillus* species grow saprophytically on plant debris, producing many airborne conidia that can infect various agricultural crops, including almond and pistachio, and contaminate them with aflatoxins,. The massive release of atoxigenic (non-aflatoxin-producing) strains of *A. flavus* stands out as the most prominent method for limiting aflatoxin contamination in fields. However, atoxigenic and native strains of *A. flavus* have not been described in Spain, one of the principal almond and pistachio producers worldwide. From 2019 to 2021, we surveyed the populations of *A. flavus* and *A. parasiticus* in soils and nuts of the Andalusia and Castilla La Mancha regions in Spain. In total, 78 *Aspergillus* section *Flavi* isolates were collected from 13 nut fields. These strains were identified at the species level according to their morphological characteristics and genomic regions, internal transcribed spacer (ITS), beta-tubulin (BT2), and calmodulin (CDM). Thus, 51 strains were identified as *A. flavus*, eight as *A. parasiticus,* and eight as *A. tamarii*. In turn, the *A. flavus* isolates were classified according to the size of their sclerotia. Thus, 21 isolates resulted in L-morphotype (large sclerotia, frequently atoxigenic) and 6 in S-morphotype (small sclerotia, primarily toxigenic), while 16 were non-sclerotia producers (NSP). In addition, we conducted a qualitative toxigenic characterization in Coconut Agar Medium (CAM). The atoxigenic isolates according to CAM test were subjected to a second toxigenicity screening by mass spectrometry (LC/MS Q-TOF). L-morphotype strains of *A. flavus* were significantly (P = 0.048) more prevalent than S-morphotype strains in both regions during this 3-years survey. Four *A. flavus* were identified as atoxigenic. Interestingly, 6 *A. tamarii* strains were identified slightly aflatoxigenic. To our knowledge, this is the first report of atoxigenic strains of *A. flavus* native to Spain and of toxigenic isolates of *A. tamarii*.

Keywords: aflatoxins, atoxigenic isolates, *Aspergillus flavus*, *A. parasiticus, A. tamarii*

1. Introduction

Numerous species of the fungal genus *Aspergillus* produce mycotoxins, among which aflatoxins are the most potent carcinogens, hepatotoxic, and immunosuppressive (IARC 2002). Aflatoxin consumption is a health hazard for humans and livestock. The species *A. flavus* and *A. parasiticus* are the primary aflatoxin producers (Horn 2003; Klich 2007). Also, some isolates of the species *A. flavus* produce cyclopiazonic acid (CPA), another mycotoxin that negatively affects different animals (Ostry et al. 2018; Chalivendra et al. 2017).

The species *A. flavus* and *A. parasiticus* are ubiquitous in temperate areas and are expanding due to the drought caused by climate change (Baazeem et al. 2021; Frisvad et al. 2019; Medina et al. 2015). Numerous arable crops (e.g., corn and cotton) are susceptible to aflatoxins contamination, but these mycotoxins can also contaminate woody crops, including almond and pistachio trees (Alves et al. 2020; Bandyopadhyay et al. 2019; Moral et al. 2021). Almond (*Prunus dulcis* L.) and pistachio (*Pistacia vera* L.) are valuable crops in Spain, where the cultivated surfaces with these species are 688,000 and 56,000 ha, respectively. Approximately 70% of the Spanish almond and pistachio production is exported, mainly to the rest of Europe and China (Mañas-Jimenez, 2018).

The most common and critical aflatoxins are classified into four types (B_1, B_2, G_1, and G_2), attending to the fluorescence emitted under 365 nm UV light (**B**lue or **G**reen) , and their abundance is indicated with the subscript 1 (primary) and 2 (secondary) compounds, respectively (Wu et al. 2011). Aflatoxins are strictly regulated in the global trade causing numerous rejections of food shipments at the international borders (Wu 2004). For example, the European Union established the maximum limits at 8 and 10 µg/kg for aflatoxin B_1 and total aflatoxins, respectively. For example, the Rapid Alert System for Food and Feed (RASFF) alerted 27 aflatoxin-contaminated products from Spain from 2014 to 2020; 30% were because of contaminated almond and pistachio products.

Under field conditions, *A. flavus* and *A. parasiticus* produce thousands of small (3-6 µm) airborne spores that cannot directly colonize the seeds of almond and pistachio nuts since the mesocarp (hull) and the endocarp (shell) are undefeatable barriers (Mahoney and Rodriguez 1996; Moral et al., 2022). However, abiotic or biotic stresses can break the nut

hull and expose the seed to air and fungal spores (Doster and Michailides 1994; Garcia-Lopez et al. 2018; Palumbo et al. 2014; Wilson et al. 2020). For example, aflatoxin contamination mainly occurs in early splits in the pistachio crop. In other words, pistachio nuts with exposed seeds due to their hulls breaking as they adhere to the shells, which naturally open during the ripening (Doster and Doster and Michailides 1994; Sommer et al. 1986). Regarding almonds, paper-shell varieties occasionally show a natural opening in the shell, while the hard-shell varieties do not (Moral et al. 2021). Furthermore, different lepidopterans such as *Amyelois transitella*, *Anarsia lineatella* or *Plodia interpunctella*, with nut-mining larvae, can facilitate the colonization of nut seeds by the pathogen (Almacellas Gort and Marin Sanchez 2011; Johnson 2008; Palumbo et al. 2014; Schatzki and Ong 2001).

The composition of both *A. flavus* and *A. parasiticus* populations (density and toxicity) determines the risk of contamination of each crop in a given region (Mehl and Cotty 2013). Remarkably, a variable percentage of the population of *A. flavus* in a given region is composed by non-toxigenic (atoxigenic) isolates; i.e., *A. flavus* isolates that do not produce mycotoxins and that share the ecological niche with toxigenic ones (Adhikari et al. 2016; Atehnkeng et al. 2016). The massive release of competitive atoxigenic strains of *A. flavus* in the field has become the most successful preharvest control method of aflatoxins in nut crops (Doster et al. 2014; Garcia-Lopez et al., 2021). One of the required characteristics of the atoxigenic strains of *A. flavus* as biological control agents is to be native to the target crops since they are considered better adapted to the environment and have a more accessible phytosanitary register (Senghor et al. 2021). Unfortunately, this technology is still not available for Spanish nut farmers.

In the present study, we surveyed and characterized the *A. flavus* and *A. parasiticus* populations in different almond and pistachio cultivation areas in Spain. Ultimately, we plan to select native atoxigenic strains to study their competitive performance in the field. Then, in the medium term, these strains could be used individually or in combination as biocontrol agents (Bandyopadhyay et al. 2016; Moral et al. 2020) to reduce aflatoxins contamination in Spanish tree nut crops.

2. Materials and methods

2.1. Sampling of almond and pistachio orchards

We conducted three surveys during the summers of 2019, 2020, and 2021 in 13 nut orchards in Spain. In Castilla La Mancha (Ciudad Real province, center of Spain), two surveys focused on rainfed pistachio orchards. Also, we surveyed another 11 orchards distributed among Granada, Jaen, Cordoba, and Seville provinces of Andalusia, southern Spain. Nine of the Andalusian orchards were cultivated with almonds (6 orchards with drip irrigation and 3 rainfed cultivated), and two with pistachios with drip irrigation.

Soil. Since *A. flavus* and *A. parasiticus* present an aggregate pattern in the ground (Jaime and Cotty 2004), regular soil sampling was performed. Thus, soil samples were obtained by walking along the sides of an equilateral triangle (approximately 100-m side) in the center of each orchard. Three samples (one per triangle side) were collected per orchard, each composed of 30 subsamples of 10-15 g, taken from the first 3 cm of the topsoil, avoiding gravel. The soil samples were dried at room temperature (22-25ºC) for 7 days in the laboratory. Next, clumps were broken using a rubber hammer, soil samples were hand-mixed for homogenization, passed through a 0.8 mm sieve (disinfested with 10% bleach and dried between samples), and kept in paper bags until its later use (Donner et al. 2015).

Pistachio nuts. Nut samples were obtained randomly from the canopies of the different pistachio trees from two of the prospected orchards located in the Ciudad Real and Jaen provinces. From each orchard, 30 early splits and 30 undamaged pistachio nuts were harvested, taken to the laboratory, and peeled to remove both hull and shell, to keep the kernels (seeds) separately in aseptic conditions. The seeds were then divided into 10- seed groups to be processed for isolation of fungal species.

2.2. Identification and isolation of A. flavus and A. parasiticus

All the methods described in this section were conducted in a Biological Safety cabinet (Burdinola®, OR-ST 1500, Barcelona, Spain).

Soil samples. 10-g samples of each soil were individually added into 250-ml flasks with 100 ml of sterile water and a magnetic bar to prepare soil suspensions. We transferred 10

ml of the soil suspension onto 9-cm Petri dishes (100 µl/plate) with AFPA medium (Aspergillus flavus and parasiticus agar, Merck Life Science S.L.U., Madrid, Spain) (Pitt et al. 1983) (Figure 1). The AFPA medium allows the identification of the species *A. flavus* and *A. parasiticus* because both species excrete aspergillic acid, which reacts with the ferric ions of the ferric citrate of the medium, giving rise to a visible orange color at the base of the plate (Pitt et al. 1983). AFPA Petri dishes with the soil suspension were incubated at 30°C in the dark for 7 days. Finally, we quantified the number of colonies of the species *A. flavus* and *A. parasiticus* and calculated the density of the pathogens in the soil. Identified colonies were re-cultured in Potato Dextrose Agar (PDA, Merck Life Science S.L.U., Madrid, Spain) medium and purified by successive transfers using a sterile needle (Rodrigues et al. 2009).

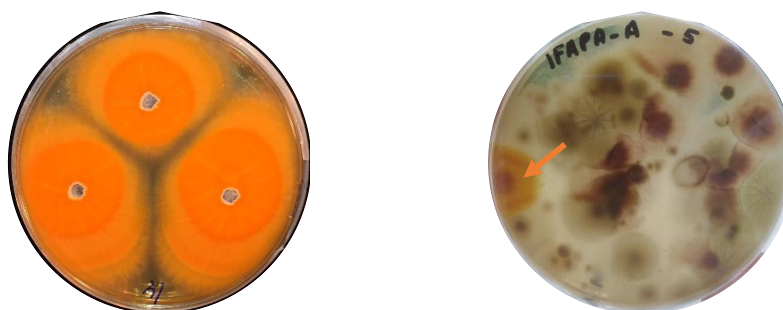

Figure 1. On the left, the orange reaction produced by *A. flavus* strain ASP005 on the back of a Petri dish with Aspergillus flavus and parasiticus agar (AFPA) selective culture medium (Pitt et al. 1983). On the right, colonies of *A. flavus* or *A. parasiticus* in a Petri dish with AFPA cultured with a 100- µl soil suspension after 7 days of incubation.

Once pure colonies of each isolate were obtained, five 0.5-cm-diameter agar discs containing mycelium and spores of each isolate were placed into 5-ml glass vials with sterile water that were stored at 4ºC in the DAUCO collection. In addition, all the strains were preserved in Paraffin oil-coated PDA tubes and included in the collection.

Pistachio nut samples. Each 30-nut sample was transferred to three 250-ml flasks with 100 ml of sterile water (10 nuts per flask). Each flask was kept for 5 min in ultrasonic equipment and then stirred at 150 rpm for 10 min (Ortega-Beltran et al. 2018). One milliliter of the nuts washing water from each flask was taken and evenly dispersed in 10 plates (100 µl/plate) with AFPA medium. After 7 days of incubation at 30°C, colonies of

A. flavus and *A. parasiticus* were identified and quantified (Figure 1). The fungal strains were then transferred to PDA medium, purified, and stored in the DAUCO collection.

2.3. Aspergillus identification

DNA extractions. Isolates identified upstream as *A. flavus* or *A. parasiticus* were cultured in PDA medium, sealed with Parafilm and incubated for 7 days at room temperature (23-25ºC). Subsequently, a 15-µl spore suspension of each isolate was transferred into a 0.5-cm-diameter well in the center of the medium of each PDA plate. After seven days, we added 2 ml of water with Tween 20 (0.1%) to each plate and recovered the fungal spores suspension in a 1.5-ml Eppendorf tube. DNA extractions were conducted according to the OMEGA fungal DNA protocol (E.Z.N.A.® Fungal DNA Mini Kit, Omega Bio-Tek Inc., GA, U.S.A).

Finally, DNA concentrations and absorbance indices (A260/280 and A260/230) were quantified using a spectrophotometer (Maestro-Nano Micro-Volume, V-Bioscience) and DNA was kept at -20°C until further use.

The ITS (*Internal Transcribed Spacer*), BT2 (*β-tubulin*), and CMD (*Calmodulin*) genomic regions were amplified according to Samson and Varga (2009). To conduct the PCR, 1 µl of DNA was used in each reaction mixture. The PCR reaction mix was prepared with 16.87 µl of pure water, 5 µl of PCR buffer (×5 Mytaq Reaction Buffer, Bioline), 1 µl of each pair of primers (ITS1 and ITS4; BT2a and BT2b; or CMD5 and CMD6), and 0.135 µl of polymerase (MyTaq DNA Polymerase, Bioline). The PCR reaction was performed in a volume of 25 µl for each isolate.

In total, three reaction mixtures were prepared for each isolate. First, the ITS region was amplified using the primer pair ITS1 (5'-TCCGTAGGT GAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), a segment of the BT2 gene using primers bT2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and bT2b (5'-ACCCTCA GTGTAGTGACCCTTGGC-3') (Glass and Donaldson 1995) and a segment of the CMD gene using primers CMD5 (5'-CCGAGTACAAGGAGGCCTTC-3') and CMD6 (5'-CCGATAGAGGTCATAACGTGG-3') (Hong et al. 2005).

In the thermocycler (Bio-Rad T100), samples with the PCR mixture were initially preheated at 95°C for 4 min, then 35 cycles were performed at 95°C for 15 s, at 48°C for 20 s, and at 72°C for 1 min. Finally, the PCR product was kept at 4 ºC until removed and kept refrigerated.

PCR products were purified using a MEGAquick-spin™ Plus purification kit and prepared to perform the Sanger sequencing at the Central Research Support Service of the University of Cordoba. Data from each sequence was inspected, assembled, and optimized using MEGA software (v. 10.0.7) (Kumar et al. 2018). Next, each isolate's 'sense' and 'antisense' sequences were aligned using a general-purpose multiple sequence alignment program for DNA or proteins, ClustalW (Hall 2013). Once the sequence of each isolate was obtained, the 'BLAST' tool (Basic Local Alignment Search Tool) of the NCBI (National Center for Biotechnology Information) was used to compare our problem sequence (sequence query) against a large number of sequences found in the NCBI database.

2.4. Sclerotial morphotype

The strains identified as *A. flavus* were incubated in CZ culture medium (Czapek-Dox Agar; Thermo Fisher Scientific Inc., Madrid, Spain) to stimulate the development of the sclerotia resistance structures (Camiletti et al. 2018). For that, 15 µl of a spore suspension from each strain were transferred into a 0.5-cm-diameter well in the center of a CZ plates, which were sealed with Parafilm and incubated at 30ºC in the dark for 14 days (Camiletti et al. 2018). Next, the sclerotia obtained in CZ medium were recovered by adding 1 ml of water with Tween 20 (0.1%) to each plate and, with the help of an inoculating loop, dragged and placed in Petri dishes provided with a paper filter, which were allowed to dry inside the biosafety cabinet.

Finally, the diameters of five sclerotia per isolate were measured under a dissecting optical microscope (Nikon Eclipse 80i; Nikon Corp., Tokyo) at 40× magnification. *Aspergillus flavus* strains were classified as L, S, or NSP (Cotty, 1989; Camiletti et al. 2018).

2.5. Toxins production

Qualitative determination. For a preliminary selection of the atoxigenic strains of *A. flavus*., we transferred 15 µl of a spore suspension of each strain into a centered 0.5-cm well of a 9-cm Petri dish with Coconut Agar Medium [333 ml coconut milk (Dee Thai, Leche de Coco, Hacendado, Spain), 777 ml of deionized water, and 20 g agar] that were incubated for 7 days in the dark at 25°C (Davis et al. 1987). Because of the reaction between the coconut lipids and the aflatoxins, a fluorescence halo is observed around the fungal colony of the toxic isolates under UV light (365 nm) at 7 days of incubation (Giorni et al. 2007; Rodrigues et al. 2009). The dependent variable was considered bimodal: presence or absence of visible halo. The strain ASP010 of *A. flavus*, morphologically characterized as *A. flavus* was used as a positive control.

Quantitative determination. For the extraction of aflatoxins produced in each plate of CAM medium, we used as mobile phase a mixture of the solvents methanol: dichloromethane: ethyl acetate (1:2:3). Five ml of the mobile phase were introduced into 10-ml dark glass vials. We placed five 4-mm-diameter disks from 7-day-old colonies grown in CAM of each *Aspergillus* strain into each glass vial. Subsequently, the vials were subjected to an ultrasonic bath for 10 min and incubated at room temperature (22-24ºC) in the dark (Camiletti et al., 2018). Standard solutions of aflatoxins (2000 ppb AFB_1 and AFB_2 and 500 ppb AFG_1 and AFG_2; Merck Life Science S.L.U., Madrid, Spain) were used for the quantification. Methanol was used to make five 10× serial dilutions in the mass spectrometry equipment. The peak area values obtained with the mass spectrometry equipment were compared to the concentration values of the standards (ppb). Standard lines were generated to aflatoxin quantification (ppb) detected in each sample. Aflatoxins analysis was carried out at the Department of Analytical Chemistry at the University of Córdoba, by using liquid chromatography (Agilent 1200 series; Palo Alto, CA, USA) coupled to time-of-flight mass spectrometer in high resolution mode (Agilent 6540 series; Palo Alto, CA, USA). The separation of metabolites was carried out in a Zorbax Eclipse plus C18 analytical column (150 × 3 mm i.d., 1.8 µm particle; Agilent, Palo Alto, CA, USA). The mobile phases were water (phase A) and acetonitrile (phase B), both solutions acidified with 0.1% (v/v) formic acid as ionization agent. Flow-rate was set at 0.25 mL/min and the injection volume was 2 µL. The LC pump was programmed with the

following elution gradient: 4% to 25% B in 3 min, change from 25% to 60% B in 5 min, from 60% to 100% B in 4 min and constant at 100% B for 10 min (total time 22 min).

Similarly, according to the molecular formula ($C_{20}H_{20}N_2O_3$) and the isotopic mass (336.149), it was also possible to detect the samples in which cyclopiazonic acid was present.

The MassHunter Qualitative Analyses software (version B7.00; Agilent Technologies, Santa Clara, CA) was utilized to process the data obtained by LC–QTOF in MS/MS mode. The MS/MS information of targeted compounds was used to confirm the presence of target metabolites by comparing to the MS/MS spectra obtained by standards or for tentative identification (if as the case) by comparing the experimental MS/MS spectra in the MassBank of North America MS/MS (https://mona.fiehnlab.ucdavis.edu/spectra/search) and METLIN MS/MS (http://metlin.scripps.edu) databases. Once the metabolites were identified, the extracted ion chromatogram of each of them was integrated to obtain the data matrix used in this study.

3. Results and discussion

3.1. Almond and pistachio orchards surveyed

The density of *Aspergillus* section *Flavi* propagules obtained in soil ranged from 0 to 40 CFU/g of soil. The maximum density value was obtained in 2019, 40 CFU of *Aspergillus* section *Flavi* per gram of soil corresponding to a rainfed almond orchard belonging to the Institute for Research and Formation in Agriculture and Fishery (IFAPA in Spanish) at Alameda del Obispo, Cordoba. However, by mean terms, rainfed orchards showed higher inoculum densities than the irrigated (16.7 and 6.7 CFU/g of soil), although no significant differences were observed (Wilcoxon Rank Sum Test, P = 0.108). This information contrasts with the high inoculum density described in soils of arable crops from the Arizona and Texas States of the USA (Horn 2003; Jaime and Cotty 2010) or Nigeria (Donner et al. 2009), where the values of CFU/g of soil ranged from hundreds, in cotton and sorghum, to thousands in corn fields. However, in California's major almond-producing counties, Donner et al. (2015) described a density of *A. flavus* and *A. parasiticus* between 6.1-16.8 CFU/g soil when using a sampling method like the one used

in our studies. These data emphasized the need for soil sampling in arable crops in Spain for comparisons.

Remarkebly, and although the risk factors for aflatoxin contamination in Spanish almond nuts are apparently low (hard-shell cultivars, low pathogen density, and harvesting without contact of the fruit with the soil) (Moral et al., 2022), the RASFF reported eight alerts by aflatoxin contamination in almond nuts from Spain during 2014 to 2020; while only reported two alerts in almond shipments (and 16 alerts in pistachio) from California during the same period, even though almond production in USA is much higher than in Spain.

Concomitantly to *A. flavus* quantification, we evaluated the presence of *Aspergillus* sections *Nigri* and *Terrei* on the surveyed soils. The densities of isolates belonging to these species were generally higher than those belonging to section *Flavi,* and showed the same pattern. *Aspergillus* section *Nigri* propagules were 75 and 66.7 CFU/g of soil in rainfed and irrigated almond orchards, respectively (Pearson's Chi-Square test $P = 0.527$); and 73.3 and 68.7 CFU/g of soil in pistachio and almond orchards, respectively (Pearson's Chi-Square test $P = 0.963$). Similarly, section *Terrei* was similar in rainfed and irrigated fields ($P = 0.418$; 60 and 43 CFU/g of soil) and stood out in pistachio orchards in comparison with almond ones ($P = 0.007$; 100 and 38.7 CFU/g of soil). The species of *Aspergillus* included in these sections are not aflatoxin producers; nevertheless, tracing patterns in their population is valuable due to their close relationship with ochratoxins production (Bayman and Baker 2006), another relevant mycotoxin internationally regulated, and others, such as citrinin (Bennett and Klich 2003).

In pistachio kernels, the density of *Aspergillus* spp. section *Flavi* was 4.5-times higher in kernels from early splits than conventional nuts (Wilcoxon Rank Sum Test $P < 0.001$; 2.15 vs 0.47 CFU/g , respectively). Ortega-Beltran et al. (2018) studied *Aspergillus* in developing almond nuts and also found relatively low densitites, from 0-6 CFU/ g of nut, until the linear increase in August.

3.2. Species identification

A total of 78 isolates of *Aspergillus* belonging to section *Flavi* were obtained in the Spanish almond and pistachio orchards. Fifty one isolates (65%) were identified as *A.*

flavus, 42 of them were isolated from soils (29 almond and 13 from pistachio orchards) and 36 isolated from pistachio kernels, mainly from early split (29 isolates) but also non-damaged nuts (seven isolates). Our results point to the fact that early splits are an excellent substrate for isolation of *Aspergillus* spp., as previously observed by Doster and Michailides (1994). Likewise, seven isolates from soil samples were identified as *A. parasiticus*, but just one *A. parasiticus* was obtained from an early split. This is concordant with the fact that *A. parasiticus* is predominant in peanuts that develop under the ground (Horn and Greene 1995; Horn 2003). However, pistachio nuts contamination with *A. parasiticus* has been indirectly observed in shipments contaminated with B aflatoxins, but also G ones, which are mainly produced by *A. parasiticus* (Garcia-Lopez et al. 2018).

Nineteen strains were classified within section *Flavi*, but as different species than *A. flavus* and *A. parasiticus*. Thus, we isolated seven *A. minisclerotigenes* strains from pistachio early splits and one from soil. This latter species, which produces aflatoxins B_1 and B_2, CPA, kojic acid, and other compounds, was initially reported contaminating peanuts in Argentina (Pildain et al., 2008), but then, affecting maize in Portugal (Soares et al. 2012) and different commodities in several African countries (El Mahgubi et al. 2013; Probst et al. 2014). Remarkably, *A. minisclerotigenes* has been described as causing lung infection in humans in Andalusia region but it has not been associated with any crop (Vidal-Acuña et al., 2019). Besides, we identified eight *A. tamarii* strains from soil samples of almond (6 isolates) and pistachio (2 isolates) orchards; and two isolates of *A. alliaceus,* and one of *A. novoparasiticus* from a pistachio orchard soil.

3.3. Sclerotial morphotype

In other to identify atoxigenic strains of *A. flavus*, we firstly classified them according to their sclerotial morphotypes. Also, this sclerotia classification was extended to *A. minisclerotigenes* and *A. novoparasiticus*.

Twenty-one strains of *A. flavus* (41%) were classified as L-strain morphotype, distributed in almond (14 strains) and pistachio fields (7 strains); while six (12%) isolates belonged to the S-strain morphotype. Aflatoxin-producing L strains were significantly (Pearson's Chi-Square test, $P = 0.035$) more prevalent than atoxigenic isolates. Dominance of the L-morphotypes over S-morphotypes of *A. flavus* is described in crops such as maize, cotton,

and almonds (Donner et al. 2015; Jaime and Cotty 2010; Mutegi et al. 2012). Finally, 14 strains (27%) of *A. flavus* resulted in NSP, predominantly obtained (10 strains) on early splits, which should be rechecked in a more conducive culture media for sclerotia production (Camiletti et al. 2018).

Furthermore, we evaluated the capacity of *A. minisclerotigenes* (minor = small in Latin and sclerotium = hard in Greek) to develop sclerotia in media, and five of them developed L-sclerotia (diameter > 400 µm), in contrast with its original description as S-strain morphotype: 150–300 µm diameter (Pildain et al. 2008). The species *A. novoparasiticus* did not develop sclerotia cultured under our conditions (Gonçalves et al. 2012).

Variations in the spatial distribution of these species were found between the regions, plots and seasons as expected (Jaime and Cotty, 2013). Thus, *A. flavus* was dominat in the Cordoba and Jaen (54% and 34%, respectively) provinces of Andalusia region, *A. parasiticus* was the most frequent species in Granada (60%), and *A. tamarii* was prevalent in Seville (50%). Finally, all the *A. minisclerotigenes* and *A. alliaceus* isolates were obtained in pistachio nuts from Torredelcampo (Jaen province). Pistachio nut contamination with *Aspergillus* isolates of the section *Flavi* was much more frequent in Jaen province of Andalusia region (33/36) than in Ciudad Real province of Castilla La Mancha region.

Indeed, only one *A. tamarii* and one *A. parasiticus* were isolated from the soil in Ciudad Real provience, where the species *A. flavus* and was undetected (Figure 2).

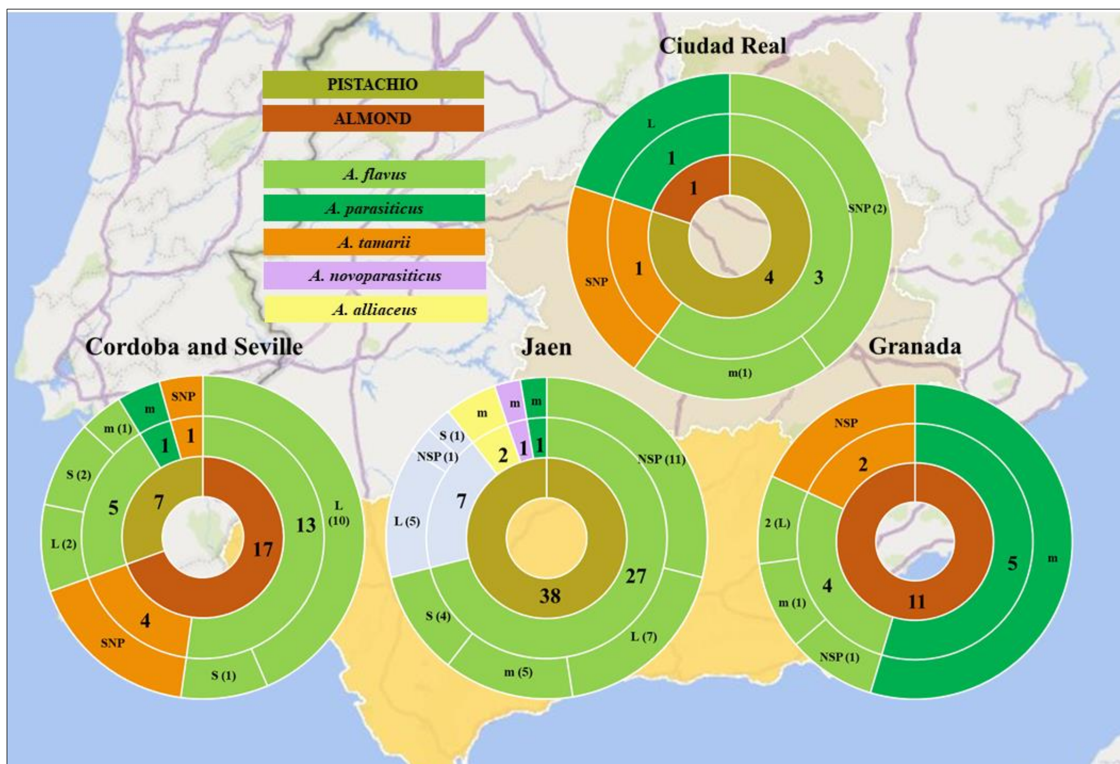

Figure 2. Composition of *Aspergillus* section *Flavi* (N° of isolates) population in Spanish tree nut orchards obtained in yearly surveys (2019, 2020, and 2021)

3.4. Toxins production

In general, the CAM medium allowed the rapid identification of aflatoxigenic isolates of *Aspergillus* spp., lately confirmed by mass spectrometry. Because the presence of false negatives are expected by this screening method (Marin-Palma 2020), the *A. flavus* strains that did not show fluorescence-halo around the growing colony in CAM were re-analyzed by mass spectrometry (LC/MS Q-TOF). Thus, we classified four *A. flavus* strains (morphotype NSP) from pistachio early splits from the Jaen province as atoxigenic. Also, seven *A. flavus* (three L-morphotype, four NSP, and two non-identified-strains) were very scarcely toxigenic (less than 1 ppb of aflatoxins B_1 or B_2). All the *A. flavus* strains with small sclerotia (S-morphotype) produced both B and G aflatoxins. Toxigenicity showed by the *A. flavus* L-strains was very variable according their ability to produce aflatoxins that ranged between 0-1114.29, 0-148.96, 0-41.82, 0-1.71 ppb for aflatoxins B_1, B_2, G_1 and G_2, respectively. The Spanish *A. flavus* population seems to present differences from other geographical regions, where the *A. flavus* strains are described as producers of the B-type aflatoxins (Frisvad et al. 2019; Klich 2002; Moral et al. 2020). Conversely, in

agreement with the literature, all *A. parasiticus* strains were aflatoxigenic and produced either the four types of aflatoxins or only the B type. Compared to the other species, *A. parasiticus* produced significantly (Kruskall-Wallis Test, *P* = 0.002) a higher concentration of aflatoxin B_1 (the most potent carcinogen). Furthermore, and interestingly, three *A. tamarii* strains were identified as aflatoxin producers, but at concentrations below 1 pbb, probably undetectable using other analitiyical methods (Figure 3). To our knowledge, this is the first time reporting aflatoxigenic isolates of *A. tamarii* in Spain. The first description of an aflatoxigenic *A. tamarii* was done in 1996 by Goto et al., but Klich et al. (2000) expressed doubt about the correct identification of this strain. Lately, other researchers have reported *A. tamarii* as non-aflatoxin producers (Agbetiameh et al. 2017; Sserumaga et al. 2020).

Regarding the CPA, another important mycotoxin quantified in our study, 45 *Aspergillus* strains were identified as CPA and aflatoxin-producers, including 30 strains of *A. flavus*, and five of *A. tamarii,* eight *of A. minisclerotigenes*, and the *A. novoparasiticus*. Also, 28 strains were identified as just aflatoxigenic, including 17 of *A. flavus,* the seven strains of *A. parasiticus* and two *A. tamarii*. Finally, we isolated a CPA-producer *A. tamarii* strain.

In the present study, we identified four atoxigenic strains of *A. flavus* natives to Spain for the first time. Interestingly, these strains were also non CPA-producer (Figure 3, left). Therefore, future surveys are needed to increase the number of atoxigenic isolates of this *Aspergillus* species. Finally, a biological control agent will be selected according to its inability to produce aflatoxins or CPA according to mutations in the aflatoxin gene cluster and the ability to reduce the aflatoxin contamination in controlled conditions and then in the field.

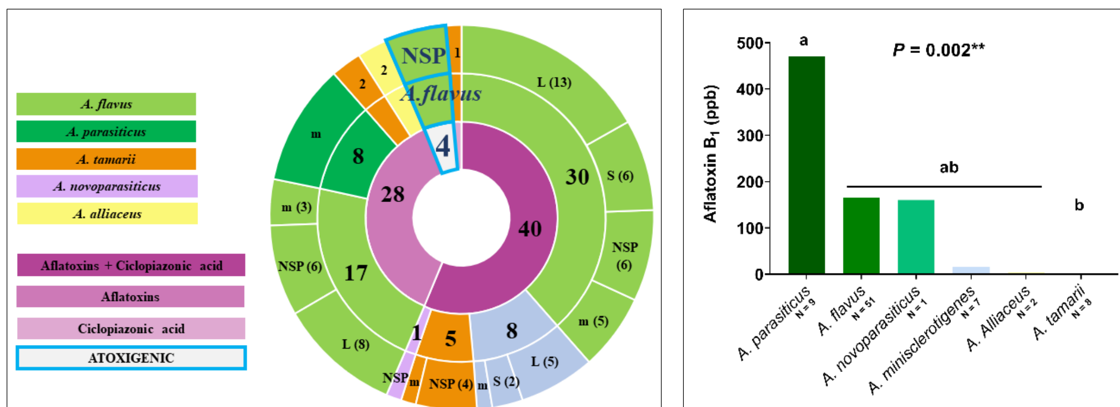

Figure 3. Toxicological composition of *Aspergillus* section *Flavi* population in Spanish tree nut crops obtained from three surveys (2019, 2020, 2021), left; and aflatoxin B_1 concentration detected by mass spectrometry (LC/MS Q-TOF) on Cononut Agar Media where the *Aspergillus* spp. grew, right.

Anex. Summary Table. Characterization of *Aspergillus* spp. section *Flavi* strains obtained from Spanish pistachio and almond orchards

Year	Strains Code	Province	Crop	Origin	Classification of isolates at the species level				Sclerotial Morphotype	Toxins production					
					Species	Query coverage from BLAST in NCBI (%)				CAM	AFB_1	AFB_2	AFG_1	AFG_2	CPA
						ITS	BT2	CMD							
2019	ASP001	Cordoba	Almond	Soil	*A. flavus*	99%			NSP	0	4,12	0,49	0,00	0,00	1
	ASP002	Cordoba	Almond	Soil	*A. flavus*	99%			L	0	0,37	0,14	0,00	0,00	1
	ASP003	Cordoba	Almond	Soil	*A. flavus*	99%			L	1	443,00	61,11	0,10	0,03	1
	ASP004	Cordoba	Almond	Soil	*A. flavus*	99%			L	1	196,03	27,71	0,01	0,00	1
	ASP005	Cordoba	Almond	Soil	*A. flavus*	99%			L	1	87,47	11,73	0,02	0,00	1
	ASP006	Cordoba	Almond	Soil	*A. flavus*	99%			L	1	382,52	61,01	0,01	0,04	1
	ASP007	Cordoba	Almond	Soil	*A. flavus**				L	0	0,07	0,00	0,00	0,00	0
	ASP008	Cordoba	Almond	Soil	*A. flavus**				L	0	0,42	0,03	0,00	0,00	0
	ASP009	Cordoba	Almond	Soil	*A. flavus**				L	0	0,26	0,00	0,00	0,00	0
	ASP010	Cordoba	Almond	Soil	*A. flavus**				NSP	1	2588,36	551,52	0,03	0,04	1
	ASP011	Cordoba	Almond	Soil	*A. flavus**				L	0	0,86	0,00	0,00	0,00	0
2020	ASP012	Cordoba	Almond	Soil	*A. flavus*	100%	100%	100%	L	1	222,45	31,45	0,01	0,00	1
	ASP013	Cordoba	Pistachio	Soil	*A. flavus*	100%	m	m	L	1	1114,29	148,96	0,01	0,06	1
	ASP014	Cordoba	Pistachio	Soil	*A. flavus*	100%	100%	100%	L	1	283,70	41,09	0,00	0,00	1
	ASP015	Cordoba	Pistachio	Soil	*A. flavus*	100%	99%	100%	S	1	63,63	7,85	17,69	1,97	1
	ASP016	Cordoba	Pistachio	Soil	*A. flavus*	100%	100%	100%	m	1	1054,24	174,19	0,01	0,00	1
	ASP017	Cordoba	Pistachio	Soil	*A. flavus*	m	100%	100%	S	1	1516,53	260,90	0,02	0,00	1
	ASP018	Granada	Almond	Soil	*A. flavus*	100%	100%	100%	m	1	1035,04	154,33	6,23	0,57	0
	ASP019	Granada	Almond	Soil	*A. flavus*	99%	100%	100%	L	1	219,86	28,68	0,00	0,03	0
	ASP020	Granada	Almond	Soil	*A. flavus*	100%	100%	100%	NSP	1	176,79	24,88	0,00	0,00	1
	ASP021	Granada	Almond	Soil	*A. flavus*	m	100%	100%	L	1	511,91	99,46	5,56	0,97	0
	ASP022	Jaen	Pistachio	Soil	*A. flavus*	100%			S	1	189,23	29,83	60,56	7,44	1
	ASP023	Cordoba	Almond	Soil	*A. minisclerotigenes*	m	100%	100%	S	1	56,60	8,03	12,12	1,20	1
	ASP024	Jaen	Pistachio	Soil	*A. novoparasiticus*	100%	m	m	NSP	1	160,48	24,85	20,04	2,34	1
	ASP025	Ciudad Real	Almond	Soil	*A. parasiticus*	100%	m	m	m	0	0,65	0,06	0,05	0,01	0
	ASP026	Cordoba	Pistachio	Soil	*A. parasiticus*	m	100%	100%	m	0	514,72	75,70	0,01	0,00	0
	ASP027	Granada	Almond	Soil	*A. parasiticus*	100%	100%	100%	m	0	64,39	10,19	0,00	0,00	0
	ASP028	Granada	Almond	Soil	*A. parasiticus*	99%	100%	100%	m	1	523,59	69,25	0,00	0,00	0
	ASP029	Granada	Almond	Soil	*A. parasiticus*	100%	100%	100%	m	1	507,12	73,69	0,03	0,00	0
	ASP030	Granada	Almond	Soil	*A. parasiticus*	100%	100%	100%	m	1	954,79	153,05	6,01	0,68	0
	ASP031	Granada	Almond	Soil	*A. parasiticus*	99%	100%	100%	m	1	41,45	5,53	0,00	0,00	0

*Morphological characterization

Anex. Summary Table.Characterization of *Aspergillus* spp. section *Flavi* strains obtained from Spanish pistachio and almond orchards

					Classification of isolates at the species level					Toxins production					
Year	Strains Code	Province	Crop	Origin	Species	Query coverage from BLAST in NCBI (%)			Sclerotial Morphotype	CAM	AFB_1	AFB_2	AFG_1	AFG_2	CPA
						ITS	BT2	CMD							
2020	ASP032	Ciudad Real	Pistachio	Soil	*A. tamarii*	100	100	100	NSP	0	0,06	0,00	0,00	0,03	1
	ASP033	Cordoba	Pistachio	Soil	*A. tamarii*	100	100	100	NSP	0	0,13	0,00	0,00	0,00	1
	ASP034	Granada	Almond	Soil	*A. tamarii*	100	100	100	NSP	0	0,00	0,06	0,00	0,00	1
	ASP035	Granada	Almond	Soil	*A. tamarii*	100	100	100	NSP	0	0,00	0,00	0,00	0,00	1
	ASP036	Seville	Almond	Soil	*A. tamarii*	100	100	100	m	0	0,46	0,07	0,00	0,00	0
	ASP037	Seville	Almond	Soil	*A. tamarii*	100	100	100	m	0	0,23	0,25	0,00	0,00	1
	ASP038	Seville	Almond	Soil	*A. tamarii*	100	100	100	NSP	0	0,32	0,14	0,00	0,00	0
	ASP039	Seville	Almond	Soil	*A. tamarii*	100	m	m	NSP	0	0,04	0,00	0,00	0,00	1
2021	ASP040	Jaen	Pistachio	Soil	*A. Alliaceus*		100		m	m	0,00	0,74	0,00	0,00	0
	ASP041	Jaen	Pistachio	Soil	*A. Alliaceus*		100		m	1	6,52	1,90	2,17	0,30	0
	ASP042	Ciudad Real	Pistachio	Undamaged fruit	*A. flavus*	100	100	100	NSP	1	0,46	0,00	0,00	0,00	0
	ASP043	Ciudad Real	Pistachio	Early Splits	*A. flavus*		100		NSP	1	9,22	1,40	0,00	0,42	0
	ASP044	Ciudad Real	Pistachio	Undamaged fruit	*A. flavus*		100		m	m	0,02	0,00	0,00	0,00	0
	ASP045	Jaen	Pistachio	Early Split	*A. flavus*	100	100	98	NSP	0/1	0,00	0,89	0,00	0,00	1
	ASP046	Jaen	Pistachio	Early Split	*A. flavus*	100			m	m	2,99	1,16	8,29	0,13	1
	ASP047	Jaen	Pistachio	Early Split	*A. flavus*	100			m	1	12,29	5,12	8,89	0,51	1
	ASP048	Jaen	Pistachio	Early Split	*A. flavus*	99			L	1	3,37	0,96	9,53	0,13	1
	ASP049	Jaen	Pistachio	Early Split	*A. flavus*	100			L	1	17,76	0,00	19,48	0,91	1
	ASP050	Jaen	Pistachio	Early Split	*A. flavus*	100			S	1	4,65	1,18	22,51	0,29	1
	ASP051	Jaen	Pistachio	Early Split	*A. flavus*	100			L	1	41,66	0,40	41,82	1,71	1
	ASP052	Jaen	Pistachio	Early Split	*A. flavus*	100			m	1	39,31	2,71	0,54	0,92	1
	ASP053	Jaen	Pistachio	Early Split	*A. flavus*	100	100	100	m	m	0,00	3,81	0,00	0,00	0
	ASP054	Jaen	Pistachio	Early Split	*A. flavus*		100	100	NSP	0	0,00	0,91	0,00	0,00	0
	ASP055	Jaen	Pistachio	Early Split	*A. flavus*		100		NSP	0	0,00	0,00	0,00	0,00	0
	ASP056	Jaen	Pistachio	Early Split	*A. flavus*	100	100	100	NSP	0	0,00	0,00	0,00	0,00	0
	ASP057	Jaen	Pistachio	Undamaged fruit	*A. flavus*	100			L	1	0,00	1,90	0,00	0,00	0
	ASP058	Jaen	Pistachio	Early Split	*A. flavus*	100	100		NSP	m	0,00	1,03	0,00	0,00	0
	ASP059	Jaen	Pistachio	Undamaged fruit	*A. flavus*	100			S	1	30,82	2,95	35,39	0,63	1
	ASP060	Jaen	Pistachio	Undamaged fruit	*A. flavus*	100			S	1	4,43	0,00	13,29	0,34	1

Anex. Summary Table.Characterization of *Aspergillus* spp. section *Flavi* strains obtained from Spanish pistachio and almond orchards

					Classification of isolates at the species level					Toxins production					
Year	Strains Code	Province	Crop	Origin	Species	Query coverage from BLAST in NCBI (%)			Sclerotial Morphotype	CAM	AFB_1	AFB_2	AFG_1	AFG_2	CPA
						ITS	BT2	CMD							
2021	ASP061	Jaen	Pistachio	Early Split	*A. flavus*	100	100		NSP	m	0,00	0,00	15,12	0,98	0
	ASP062	Jaen	Pistachio	Early Split	*A. flavus*	100			NSP	1	0,00	0,00	0,00	0,00	0
	ASP063	Jaen	Pistachio	Early Split	*A. flavus*	100	100	100	NSP	1	0,00	0,00	0,00	0,00	0
	ASP064	Jaen	Pistachio	Early Split	*A. flavus*	100	100	100	NSP	0	0,00	0,33	0,00	0,00	0
	ASP065	Jaen	Pistachio	Early Split	*A. flavus*	100			L	1	2,33	2,71	2,50	0,24	1
	ASP066	Jaen	Pistachio	Early Split	*A. flavus*	100			L	1	22,64	0,00	16,23	0,90	0
	ASP067	Jaen	Pistachio	Early Split	*A. flavus*	100	100	99	m	1	0,07	0,00	0,00	0,00	1
	ASP068	Jaen	Pistachio	Undamaged fruit	*A. flavus*	100	100	100	NSP	0	0,00	0,93	0,00	0,00	1
	ASP069	Jaen	Pistachio	Undamaged fruit	*A. flavus*		100		NSP	0	29,68	3,25	22,86	1,08	1
	ASP070	Jaen	Pistachio	Soil	*A. flavus*	100			L	1	5,82	0,00	8,84	0,39	1
	ASP071	Jaen	Pistachio	Early Split	*A. minisclerotigenes*		100		m	1	7,48	0,82	20,50	0,27	1
	ASP072	Jaen	Pistachio	Early Split	*A. minisclerotigenes*		100		S	1	2,28	2,73	8,97	0,12	1
	ASP073	Jaen	Pistachio	Early Split	*A. minisclerotigenes*		100		L	1	59,55	1,22	40,46	1,39	1
	ASP074	Jaen	Pistachio	Early Split	*A. minisclerotigenes*		100		L	1	4,47	6,63	8,25	0,25	1
	ASP075	Jaen	Pistachio	Early Split	*A. minisclerotigenes*		97		L	1	18,12	2,77	20,18	0,63	1
	ASP076	Jaen	Pistachio	Early Split	*A. minisclerotigenes*		99		L	1	8,81	0,00	6,87	0,39	1
	ASP077	Jaen	Pistachio	Early Split	*A. minisclerotigenes*		99		L	1	8,83	0,00	16,50	0,51	1
	ASP078	Jaen	Pistachio	Early Split	*A. parasiticus*	100			m	1	2,21	0,71	0,47	0,11	1

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GENERAL DISCUSSION

Substantial research has been conducted to reduce aflatoxin contamination in food and feed during the last four decades. In the case of the nut trees, their seeds can be occasionally infected by *Aspergillus flavus* and *A. parasiticus*, the major aflatoxin producers (Donner et al. 2015; Doster and Michailides 1994a; Molyneux et al. 2007). If the harvested nuts were dried to specified moisture levels (< 6% moisture) and conditions in postharvest storage were maintained as the ones recommended by each respective industry, aflatoxin would not develop in the postharvest stage, suggesting the importance of reducing aflatoxin contamination pre-harvest, when nut fruits are developing in the field. The most effective pre-harvest management strategy for limiting aflatoxin contamination in nut crops is the use of mixes of native atoxigenic isolates of *A. flavus* for the competitive displacement (or competitive exclusion) of wild toxigenic isolates in the agroecosystem.

In the first chapter of this Ph.D. Thesis, we reviewed the present status and perspective on the future use of aflatoxin biocontrol products (Moral et al. 2020). Many farmers in the U.S.A., Africa, and Italy benefit from using biological control strains of *A. flavus*. Overall, regulatory authorities allow the registration of biocontrol products whose efficacy, safety, and benefits have been demonstrated. During the registration process, one has to consider that *A. flavus* is a human pathogen, and the developed methods for the competitive displacement must keep the density of airborne spores at safe levels for farmworkers (Klich 2007). In California, we have detected significant peaks of *A. flavus* spores on plots treated with the atoxigenic strains AF36 compared to no-treated plots in August 2008 (16 vs 61 spores per m^3 of air) and August 2009 (48 vs 80 spores per m^3 of air) (Michailides, unpublished data). However, no health problems have been detected in this or the other American States, where the atoxigenic *A. flavus* AF36 strain is massively released at 11.2 kg/ha). In addition, caution is needed to keep the density of AF36 spores very low at the time of crop pollination and to secure the safety of the pollinating bees (Ortega-Beltran et al. 2018). These and many other experiments are required by the regulatory authorities before approving the registration of atoxigenic strains of *A. flavus* in different countries.

In temperate regions where the biocontrol strategy is still not implemented for the reduction of aflatoxin contamination, the search for native atoxigenic *A. flavus* strains is

necessary for the development and registration of new biocontrol products to be used in susceptible crops (Moral et al. 2020). An increase in the incidence of aflatoxin contamination can be expected because of climate change (Medina et al. 2017) and the expansion of specialty crops to higher aridity regions (Gitz et al. 2016). That is the case in Spain, where the tree-nut industry is going through a major expansion (Mañas Jiménez 2018) and, thereby, facing new challenges, such as search for sufficient irrigation water (Moldero et al. 2022) and aflatoxin contamination (Garcia-Lopez et al. 2018) in the absence of commercial native atoxigenic strains of *A. flavus* for the development of biological control products. Thus, in our research we focused on searching for native atoxigenic strains of *A. flavus* related to tree nut crops in Spain and, we intend to continue the research work (evaluating density in the orchard, efficacy in co-inoculation studies, ability to sporulate, competitive capacity against frequent toxigenic strains, etc.) in order to develop a biocontrol product for registration and use by Spanish nut growers (unpublished results, chapter IV).

California is the primary producer and exporter of pistachio and almond nuts (USDA 2022), and the European, Chinese, and other Asian markets are the major importers. Aflatoxin contamination represents a challenge, and it can be costly when the product reaches international markets and becomes rejected at the destination. Very low thresholds on aflatoxins levels are established worldwide to guarantee human and animal health (Bui-Klimke et al. 2014). Both China and the European Union have much more stringent standards than those in the U.S.A. In California, since post-harvest technologies of nuts avoid secondary cycles of the pathogen, aflatoxin contamination is attributed mainly to pre-harvest (Campbell et al. 2003; Moral et al. 2020). Infield, along with the control and reduction of damage by the navel orange worm (*Amyelois transitella*), the most promising action taken is the application of the atoxigenic *A. flavus* AF36 strain utilizing sorghum grains as the carrier (AF36 Prevail®) (Doster et al. 2014; Ortega-Beltran et al. 2018). New information on resistant cultivars can also be used when growers decide on new plantings, especially in hot spots where conditions are conducive to aflatoxin contamination (Moral et al. 2021, chapter III). The AF36 Prevail® was registered first for use in row crops, such as cotton and maize (Cotty and Bayman 1993; Cotty 1994), and its registration and use in nut crops is relatively recent. In California, USEPA granted registration of AF36, utilizing wheat as the carrier, for use in pistachio in 2012, and in 2017, AF36 Prevail® registration, utilizing sorghum as the carrier,

included pistachios, almonds, and figs (Moral et al. 2020). In other major nut-producing areas, such as Australia, Spain, or Turkey, commercial biocontrol agents to reduce aflatoxin contamination are not available yet.

In California, even though paired field studies demonstrated the positive effect of the biocontrol strategy on reducing aflatoxin contamination (Doster et al. 2014), the efficacy of the application still needs to be improved. The performance of the biocontrol to limit aflatoxin contamination in nut orchards has striking differences from their analog row crops (Doster et al. 2014; Kaminiaris et al. 2020), but commercial recommendations on its rate and use are the same. The research conducted in chapter II of the present Thesis addressed the sporulation dynamics of the biocontrol strain AF36 in Californian pistachio orchards. Specifically, this chapter was centered on the effect of soil water content on *A. flavus* AF36 Prevail® sporulation, quantified sorghum grain loss, and monitored the strain AF36 spores' dispersal in both vertical and horizontal directions under field (pistachio) conditions. Under controlled conditions, sorghum grains made the strain AF36 sporulate even when ground moisture was as low as 8%, although reaching the optimum of sporulation when the soil was close to field capacity (21%). Our recommendation to farmers is "to spread the AF36 Prevail® product in the moist soil area but avoid the area where the irrigation water impacts the grains, or there is a puddle of water". Grains that fall onto the dry ground will not produce any sporulation. We have detected that there is more predation activity of sorghum grains by the orchard fauna (ants, pillbugs, and birds) in the driest areas of the ground. In our non-tilled plot, the soil was enriched with higher diversity and density of arthropods, as is expected (Logan et al. 1991;). Those removed the sorghum grains, in a relatively short time, reinforcing the need to increase the AF36 Prevail® application rate per ha in non-tilled orchards. When the AF36 strain is applied in pistachio orchards, its spores are intended to protect (contaminate) the target nuts (Ching'anda et al. 2022). In terms of horizontal dispersion, the AF36 spores quickly get into the low canopy of the trees located right above the inoculum source, but the density of spores decreases in the trees 10 m apart from the inoculum source, and more markedly so at longer distances. These dynamics of spore density can imply that AF36Prevail® application could be every other row, and an overlapping effect could occur on the non-treated row. Remarkably, the density of spores of *Aspergillus* spp. section *Nigri* recorded high in the canopy was greater than that determined at ground level. Some species of *Aspergillus* in section *Nigri* are major ochratoxin producers (Cabañes and Bragulat 2018).

Our results showed that the nut tree canopy might function as an inoculum source for *Aspergillus* species such as those included in section *Nigri*, and biocontrol strategies should act in parallel, ideally, to reduce either aflatoxin or ochratoxin mycotoxins. It would also be interesting to identify whether debris found in the tree canopy act as substrates for the toxigenic and atoxigenic *Aspergillus* spp.

Understanding how the populations of atoxigenic *Aspergillus* strains change over time after being released into the environment allows us to determine the treatment efficacy in reducing aflatoxin contamination in susceptible crops (Jaime and Cotty 2004; Mauro et al. 2013; Moore et al. 2017). It is also essential to monitor how the atoxigenic strains survive and compete with populations of aflatoxin-producing strains (Cotty et al. 2007; Cotty and Bayman 1993; Mehl and Cotty 2010). However, the use of the traditional approach of the vegetative compatibility assays (VCA) to identify the various *Aspergillus* strains is a very tedious and time-consuming approach (Atehnkeng et al. 2016; Camiletti et al. 2018; Ortega-Beltran et al. 2018). For this reason, in chapter II (Garcia-Lopez et al. 2021), we tackled this concern by developing and validating a mismatch-qPCR assay to quantify the proportion of AF36 vs the aflatoxigenic genotypes of *A. flavus* and *A. parasiticus* from a diverse source of samples, including pure mycelia or conidia and contaminated soil and plant tissues. Our mismatch-qPCR efficiently quantifies AF36 proportions in the *Aspergillus* population, facilitating our fieldwork. Briefly, this qPCR approach facilitate our research by monitoring the AF36 strain quickly and accurately. Then, it can be used for further searches, for example, to evaluate the sporulation capacity of the biological control product and its efficacy in displacing the toxigenic strains.

The use of AF36 Prevail® product to protect the nuts from aflatoxin contamination has some challenges, such as a failure of the product to produce sufficient sporulation or grain inoculum losses by predation. In chapter II, we studied the pistachio male inflorescences as a source of *Aspergillus* inoculum and simultaneously proposed these as a novel substrate for the AF36 strain (Garcia-Lopez et al., Plant Dis. submitted). The male inflorescences can serve as a natural substrate, abundant, and uniformly distributed within the pistachio orchard, since male trees are planted in every fifth tree in every fifth row (corresponding to a ratio of 1 male: 24 female trees) (Ferguson et al. 1980; Doster and Michailides 1994a). When we studied the natural population of *Aspergillus* spp. on pistachio male inflorescences before and after applying AF36 Prevail® in the years 2008 and, later in 2018, we detected no changes in the density of sections *Circumdati* or *Nigri*.

On the other hand, the species of *Aspergillus* section *Flavi* (and hence also AF36 strain) became more abundant after the first treatment in 2008. However, no differences were found between treated and non-treated plots in 2018, which suggests the effect of year applications and the spreading capacity of the biocontrol agent (Cotty and Bayman 1993). In controlled conditions, we demonstrated that the inflorescences on the tree (aerial) represent a better substrate for a source of inoculum than those fallen on the ground because of the competition with soil microorganisms. Besides, we showed excellent inflorescence colonization by the AF36 strain under high humidities (> 96%), easily achieved by micro-sprinkler irrigation in pistachio orchards in California (Marino et al. 2019). Finally, we compared the male inflorescences and the sorghum grains as substrates for the AF36 strain by quantifying their spores in the tree canopy. We first demonstrated a higher density of AF36 spores in the inflorescences of treated trees than in those of the non-treated ones in 2016. However, in 2017 and 2018, no differences were found between the two treatments, probably because of cross-contamination of the biological control strain between seasons and neighboring plots (Moral et al. 2020). In any case, the densities of the AF36 strain spores in the tree canopy were similar in trees treated with the commercial AF36 Prevail® product and in the tree canopies treated with male inflorescences. In this chapter, we propose and discuss, in addition, some approaches to improve this novel method for applying atoxigenic strains of *A. flavus*.

In Chapter III, we characterized the resistance of various almond cultivars against *A. flavus* and *A. parasiticus* colonization and then aflatoxin contamination (Moral et al. 2021). Almond cultivars presented high variability in response to aflatoxin contamination with three toxigenic *Aspergillus* isolates, independently inoculated. This variation agrees with the variability previously described concerning *A. flavus* colonization of almond cultivars (Dicenta et al. 2003). Unfortunately, none of the studied cultivars were immune to either fungal infection or aflatoxin contamination. However, we identified exceptional genotypes (cvs. Independence, Tarraco, Winters, or Kester) that showed elevated tolerance to the pathogen. It is essential to point out that our experimental conditions were highly conducive to aflatoxin production, and the observed resistance levels are of practical importance. These assays also demonstrated the critical protective effect of the shell in preventing aflatoxin contamination of the kernels, regardless of the type of shell (hard, semihard, or paper-shell). Once we evaluated the resistance to the pathogen of the peach-derived almond cultivars (Gradziel 2020), we demonstrated considerable tolerance

of these genotypes to aflatoxin accumulation. This highlights the importance of peach for introgressing resistance to the pathogen in breeding programs (Gradziel and Lampinen 2019; Gradziel et al. 2007). Finally, the last assay results indicated that cultivar resistance combined with biocontrol using atoxigenic isolates offers a particularly promising aflatoxin control strategy. Under field conditions, a combination of both control approaches could be highly beneficial.

Finally, in chapter IV, we surveyed the populations of *A. flavus* and *A. parasiticus* in soils and nuts of almond and pistachio orchards of Andalusia and Castilla La Mancha, two nut growing areas in Southern and Central Spain, respectively. Unfortunately, Spanish farmers cannot benefit of using atoxigenic strains of *A. flavus* since no native strains have been described. Thus, we obtained 78 strains of *Aspergillus* section *Flav*i from 13 Spanish orchards during 2018 to 2021. These strains were identified according to their morphological and molecular (ITS, BT2, and CDM) characteristics. A total of 51 strains were identified as *A. flavus*, eight as *A. parasiticus,* and eight as *A. tamarii*. In turn, the *A. flavus* isolates were classified according to the size of their sclerotia, of which 21 resulted in L-morphotype (large sclerotia, frequently atoxigenic), 6 in S-morphotype (small sclerotia, primarily toxigenic), and 16 were non-sclerotia producers. According to a toxicological characterization by mass spectrometry (LC/MS Q-TOF) of the strains, four *A. flavus* were characterized as atoxigenic (i.e., no-aflatoxin and no-cyclopiazonic acid producers) and, to our knowledge, this is the first discovery and report of atoxigenic strains of *A. flavus* native to Spain. These strains will be subjected to further competition analyses to evaluate their performance as potential biocontrol agents. Remarkably, we identified six *A. tamarii* strains as slightly aflatoxigenic.

As mentioned above, aflatoxin contamination poses a severe risk to human health. The control technology described here is based on the mix of natural and environmentally-friendly biological control agents: atoxigenic native strains of A. flavus that ensure nuts consumers' safety. Through the work advocated in this Ph.D. Thesis, we have substantially improved the biocontrol of aflatoxins in California and generated the foundation research work on a control technology that nut producers in our country are now closer to using.

KEY CONCLUSIONS

1. Searching for native atoxigenic *Aspergillus flavus* strains is mandatory in many nut-growing regions since aflatoxin contamination will rise due to higher aridity driven by climate change. Also, it is necessary to adapt and optimize the biological control of aflatoxins based on native atoxigenic strains in each nut agroecosystem.

2. The commercial product approved in California, that is sorghum grains coated with the atoxigenic strain AF36 of *A. flavus* (AF36 Prevail®), should be spread on soil areas with high moisture content (close to the micro-sprinklers) while avoiding the direct impact of the irrigation drops. In these wet soil areas, the AF36 Prevail® sporulates excessively, thus displacing the wild toxigenic isolates of *Aspergillus* spp. from the inoculum sources and protecting the susceptible nuts.

3. Increasing the AF36 Prevail® rate per surface is recommended in non-tilled orchards due to their higher density of soil arthropods that feed on the sorghum grains, thus reducing the amount of the biocontrol product.

4. Our studies on the dispersal of spores of *A. flavus* in pistachio orchards show that AF36 spores decrease markedly as a function of height and distance from the inoculum source. However, the AF36 spores can easily reach (and protect) the susceptible nuts of the trees at the max. distance of 10 m.

5. We have developed a mismatch-qPCR protocol to accurately quantify the proportions of AF36 strain in the soil and the plant. This protocol is being used for ecological studies of the AF36 strain in both the laboratory and field.

6. We propose the pistachio male inflorescences as a substrate for the atoxigenic strains of *A. flavus*. Thus, the inoculation of male inflorescences with the AF36 strain has a double impact, on increasing the density of the biological control agent and displacing the wild isolates from them. Further studies are needed to optimize this alternative natural substrate for increasing the inoculum in pistachio orchards.

7. The almond shell has a critical protective effect in preventing aflatoxin contamination of the kernel, regardless of the shell type (hard, semihard, or paper-shell).

8. We have identified nine almond cultivars (e.g., Nonpareil or Sonora) and five advanced selections as highly tolerant to aflatoxin contamination. These assays have also shown the importance of using the peach as a progenitor in the almond breeding programs,

conferring both self-fertility and resistance to *Aspergillus* spp. requirements for the new cultivars.

9. Finally, we have identified four atoxigenic (no aflatoxins and no cyclopiazonic acid producers) strains of *A. flavus* in nut orchards in Spain. This is a crucial first step for the development of biocontrol agents that limit aflatoxin contamination in our country.

CONCLUSIONES PRINCIPALES

1. La búsqueda de cepas atoxigénicas de *Aspergillus flavus* es necesaria en muchas regiones productoras de frutos secos, ya que se espera un incremento de la contaminación por aflatoxinas como consecuencia de un aumento de la aridez debido al cambio climático. Asimismo, es necesario adaptar y optimizar el control biológico de aflatoxinas en cada agroecosistema.

2. El producto comercial aprobado en California, que consiste en granos de sorgo recubiertos con la cepa atoxigénica AF36 de *A. flavus* (AF36 Prevail®), debe aplicarse en áreas del suelo con alto contenido de humedad (cerca de los microaspersores) pero evitando el impacto directo de las gotas de agua de riego. En las áreas de suelo húmedo, el AF36 Prevail® esporula abundantemente, desplazando a los aislados toxigénicos silvestres de *Aspergillus* spp. de las fuentes de inóculo y protegiendo los frutos susceptibles.

3. Se recomienda aumentar la dosis de AF36 Prevail® por superficie en plantaciones sin laboreo debido a su mayor densidad de artrópodos en el suelo, que frecuentemente se alimentan de los granos de sorgo, lo que reduce la cantidad del producto de control biológico.

4. Nuestros estudios sobre la dispersión de esporas de *A. flavus* en plantaciones de pistachero muestran que las esporas de AF36 disminuyen notablemente en función de la altura y la distancia a la fuente de inóculo. Sin embargo, las esporas AF36 alcanzan (y protegen) fácilmente los frutos susceptibles hasta en árboles ubicados a una distancia máxima de 10 m.

5. Hemos desarrollado un protocolo de qPCR-Mismatch para cuantificar con precisión las proporciones de la cepa AF36 en el suelo y en la planta. Este protocolo se está utilizando para estudios ecológicos de la cepa AF36 en el laboratorio y en el campo.

6. Proponemos las inflorescencias masculinas de pistachero como sustrato para las cepas atoxigénicas de *A. flavus.* La inoculación de inflorescencias masculinas con las cepas AF36 tiene un doble impacto, al aumentar la densidad del agente de control biológico y desplazar a los aislados silvestres de éstas. Son necesarios más estudios para optimizar el uso de este sustrato natural alternativo para aumentar el inóculo en plantaciones de pistacho.

7. La cáscara (endocarpo) de la almendra tiene un efecto protector crítico en la prevención de la contaminación de la semilla por aflatoxinas, independientemente del tipo de cáscara (dura, semidura o de papel).

8. Hemos identificado nueve cultivares de almendras (p. ej., Nonpareil o Sonora) y cinco selecciones avanzadas como altamente tolerantes a la contaminación por aflatoxinas. Estos ensayos también han demostrado la importancia de utilizar melocotonero como progenitor en los programas de mejora del almendro, confiriéndole tanto autofertilidad como resistencia a *Aspergillus* spp., que son exigencias para los nuevos cultivares.

9. Finalmente, hemos identificado cuatro cepas atoxigénicas (no productoras de aflatoxinas ni ácido ciclopiazónico) de *A. flavus* en plantaciones de frutos secos en España. Este es un primer paso crucial para el desarrollo de agentes de biocontrol que limiten la contaminación por aflatoxinas en nuestro país.

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ANNEX. ACTIVIDADES FORMATIVAS COMPLEMENTARIAS REALIZADAS DURANTE EL DESARROLLO DEL PLAN DE FORMACIÓN

- ✓ *Febrero-2018*. Certificación nivel de inglés. TOEFL iBT Test (ETS), en California State University, Fresno. Total Score: 95
- ✓ *Marzo-2018*. Actividad 3 obligatoria del Programa de Doctorado. Mejora de la empleabilidad y orientación laboral. Universidad de Córdoba.
- ✓ *Octubre-2018*. XIX Congreso de la Sociedad Española de Fitopatología (Toledo)
- ✓ *Diciembre-2018*. Curso de Introducción a Programación R para el análisis de datos (2ª Edición). Programa de Formación Permanente-Universidad de Córdoba.
- ✓ *Diciembre-2018*. Primer Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias (Almería)
- ✓ *Febrero-2019*. VII Congreso Científico de Investigadores en Formación (Córdoba)
- ✓ *Marzo-2019*. Actividad 2 obligatoria del Programa de Doctorado. Visita a la Estación Experimental del Zaidín.
- ✓ *Marzo-junio-2019*. Curso Online: Seguridad Alimentaria. Análisis de Peligros y Puntos Críticos de Control. Universidad de Salamanca.
- ✓ *Junio-2019*. Actividad 1 obligatoria del Programa de Doctorado. Organización de un Seminario de Actualidad sobre los retos de la investigación en la Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible: 'Ciencia Abierta'.
- ✓ *Octubre-2019*. II Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias (Almería)
- ✓ *Noviembre-2019*. Curso presencial perteneciente al CeiA3, Training Network Course 2019: 'Genomic - Assisted Breeding of Vegetable Crops'. (Almería).
- ✓ *Enero-2020*. Curso presencial perteneciente al Máster Oficial en Biotecnología de la UCO. 'Genómica Funcional en Investigación Biomédica' (Córdoba).
- ✓ *Febrero-2020*. VIII Congreso Científico de Investigadores en Formación (Córdoba)
- ✓ *Abril-julio-2021*. Coordinación y realización de tutorías en el Curso Virtual: 'Conocimientos Básicos de Olivicultura Sostenible', organizado por el Área de Cooperación y Solidaridad de la Universidad de Córdoba, como parte del proyecto

"Consumo Responsable, Soberanía alimentaria y Comercio Justo, con enfoque de Economía Social y Solidaria, en la Universidad Mayor de San Simón en Cochabamba". Constante comunicación con los docentes del grupo UCOLIVO y con la responsable técnica de proyectos del ACyS.

- ✓ *Octubre-Noviembre-2021*. Curso Online a través de la UPOV (*Union for the Protection of New Varieties of Plants*). 'DL-205. Introducción al Sistema de la UPOV de Protección de las Variedades Vegetales en Virtud del Convenio de la UPOV'.
- ✓ *Marzo-Abril-2022*. Curso Online a través de la UPOV (*Union for the Protection of New Varieties of Plants*). 'DL-305. Examen de solicitudes de derechos de obtentor.