TESIS DE DOCTORADO

Estudios etiológicos, epidemiológicos y de control de *Colletotrichum* spp. asociado a atizonado de flor y podredumbre de frutos en el cultivo del olivo

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ESTRUCTURA DE LA TESIS

El documento de tesis se estructura bajo el formato de Compendio de Artículos (TCA) y está compuesto por una introducción general, cuatro artículos científicos, discusión general y conclusiones generales.

La recopilación de los artículos científicos son los siguientes:

- **Artículo 1**- Olive anthracnose caused by *Colletotrichum* in Uruguay: symptoms, species diversity and flowers and fruits pathogenicity. Publicado en: **European Journal Plant Pathology** (2021) 160:663-68. https://doi.org/10.1007/s10658-021-02274-z

- **Artículo 2**- Incidence of *Colletotrichum* latent infections during olive fruit development under Uruguayan environmental conditions. Publicado en: **International Journal of Pest Management** (2022) 68:286-294. https://doi.org/10.1080/09670874.2022.2119490

- **Artículo 3**- *Colletotrichum* infections during flower development and fruit ripening in four olive cultivars Con revisiones en: **Phytopathologia Mediterranea.**

- **Artículo 4**- Assessment of fungicides efficacy against *C. acutatum* s.s., *C. nymphaeae,* and *C. fioriniae* causing olive anthracnose in Uruguay. A enviar a: **Crop Protection.**

TABLA DE CONTENDIO

RESUMEN

La antracnosis del olivo ocasionada por *Colletotrichum* spp., es la enfermedad de mayor relevancia del olivo en Uruguay. Los síntomas principales que causa esta enfermedad son la podredumbre de fruto y el atizonado de las panículas florales. Además de disminuir el rendimiento, afecta negativamente la calidad de los aceites cuando son elaborados con frutos afectados. Los objetivos de esta tesis fueron clarificar la etiología de la antracnosis del olivo en Uruguay, conocer las características epidemiológicas en condiciones locales y evaluar medidas de control que contribuyan al diseño de un sistema de manejo integrado para esta enfermedad.

Se determinó la presencia de cinco especies causando la antracnosis del olivo en Uruguay. *Colletotrichum acutatum* s.s. resulto ser la especie predominante (82%) y en segundo lugar *C. nymphaceae* (13%), estas dos especies junto a *C. fioriniae* (1%) pertenecen al complejo de especies de *C. acutatum* s.l. Las restantes dos especies identificadas fueron *C. theobromicola* (3%) y *C. alienum* (1%) pertenecientes al complejo de especies de *C. gloesporioides* s.l. Además, se comunicó por primera vez la presencia de la especie de *C. alienum* asociada a la antracnosis del olivo en el mundo.

Con relación a la ocurrencia de infecciones latentes por *Colletotrichum* spp. en frutos verdes, se confirmó su presencia en todas las etapas de desarrollo del fruto desde cuajado a cosecha. Por otra parte, se encontró una mayor incidencia de infecciones latentes en los frutos producidos en la región Sureste comparado con la región Centro-sur. A partir de los índices creados en base a las condiciones ambientales que favorecen la ocurrencia de infecciones por *Colletotrichum* spp., se verificó que durante la evaluación la región Sureste presentó mayor número de días con alta humedad relativa (mayor a 90 %) y precipitaciones ligeras (<5 mm) solas o combinados con precipitaciones abundantes y una temperatura media favorable (entre 20 y 30ºC) en comparación con la región Centro-sur.

Respecto a la susceptibilidad a la infección por *Colletotrichum* durante el desarrollo de la floración y madurez de fruto, se comprobó que las infecciones comienzan tempranamente durante el desarrollo de la flor y aumentan sustancialmente durante la floración. En el caso de los frutos, la susceptibilidad también se incrementó sustancialmente a medida que avanzó la madurez de los mismos.

Finalmente, la evaluación de fungicidas in vitro mostró que tebuconazole y propiconazole dentro del grupo de los triazoles, pyraclostrobin dentro del grupo de las estrobilurinas y ziram dentro del grupo de los ditiocarbamatos, tuvieron el mejor comportamiento en la

inhibición del crecimiento micelial de *Colletotrichum* spp. En el caso de los cobres, no se observaron diferencias entre los tres principios activos evaluados. Tebuconazole, pyraclostrobin y ziram fueron evaluados sobre panículas florales y los tres cúpricos (caldo bórdeles, oxicloruro y oxido cuproso) sobre los frutos para el control de la antracnosis. Sobre las panículas florales, los tres fungicidas retrasaron la manifestación de los síntomas entre 24 a 48h comparado con el control, mientras que, en general, ziram y pyraclostrobin lograron la mayor reducción en la incidencia del atizonado de las flores. Por otro lado, los fungicidas cúpricos, en general, mostraron una baja eficacia en el control de la antracnosis en los frutos.

Palabras claves: Antracnosis, control químico, infecciones latentes, *Olea europea*, susceptibilidad de cultivares

INTRODUCCIÓN GENERAL

El olivo (*Olea europaea* ssp. *europaea* L.) es un cultivo originario de la cuenca mediterránea que se expandió a diferentes zonas, entre ellas a Uruguay (Pereira, 2016). Si bien la producción olivícola en el país se conoce desde principios del siglo XX, fue recién a partir del 2002 que sufrió un fuerte crecimiento pasando de 500 ha a 6000 ha plantadas con un sistema de plantación intensivo (285-400 árboles/ha) y de secano (DIEA 2020).

La mayoría de los cultivares sembrados en Uruguay son de origen español e italiano. Respecto a su importancia, casi la mitad pertenecen al cultivar Arbequina con un 47% de la superficie plantada, seguida por Coratina con el 21% y Picual junto con Frantoio con el 11%. La zona de producción más importante en Uruguay es la zona Este donde se concentra el 80% de la superficie sembrada, le siguen el Centro y Litoral sur con un 11%, y por último la zona Norte del país con un 9% (DIEA 2020).

El clima de Uruguay se caracteriza por presentar frecuentes días con alta humedad relativa, precipitaciones recurrentes (alrededor de 1.100 mm por año) y temperaturas medias a lo largo del año (Conde-Innamorato et al. 2019) lo que favorece el desarrollo de enfermedades fúngicas en el olivar. Estas condiciones climáticas son muy diferentes a la zona de origen del olivo en el mediterráneo, que se caracteriza por tener un clima más bien seco debido a las escasas precipitaciones (Tous et al., 2005).

La aceituna jabonosa o antracnosis causada por especies del género *Colletotrichum*, es la principal enfermedad que afecta al olivo y se encuentra ampliamente distribuida alrededor del mundo (Talhinhas et al. 2018). En Uruguay, esta enfermedad es la de mayor prevalencia y la que causa mayor daño en los olivares de todo el país (Conde y Leoni, 2007; Leoni et al., 2018; Montelongo et al., 2013). El principal síntoma que causa es la podredumbre del fruto que consiste en una lesión deprimida de color marrón oscuro que rápidamente se cubre de una masa muscilaginosa de color salmón-anaranjada formada por conidios del hongo (Moral et al. 2014; Mosca et al. 2014; Talhinhas et al. 2018). Los frutos afectados se momifican y pueden permanecer en la copa de los árboles o caer al suelo (Moral et al. 2009; Moral y Trapero 2012). La podredumbre de fruto no solo disminuye el rendimiento de la cosecha, sino que también afecta negativamente la calidad del aceite elaborado a partir de frutos infectados, alterando el color, la acidez y la

calidad organoléptica entre otras propiedades (Trapero y Blanco 2008; Moral et al. 2014; Leoni et al., 2015).

Otros síntomas de esta enfermedad son las manchas foliares, la muerte de ramitas y el atizonado de las panículas florales (Oliveira et al, 2005; Moral et al., 2009; Talhinhas et al. 2018). Además, es conocida la ocurrencia de infecciones en frutos jóvenes que permanecen latentes hasta que los frutos alcanzan la madurez cuando se activan manifestándose el síntoma típico de podredumbre (Moral et al., 2009; Moral y Trapero 2012; Sergeeva, 2014; Talhinhas et al 2018).

Hasta la fecha se han anunciado 18 especies de *Colletotrichum* causantes de la antracnosis del olivo agrupadas en los complejos de especies de *C. acutatum, C. gloesporioides* y *C. boninense* (Schena et al. 2014; Chattaoui et al. 2016; Moral et al. 2017; Talhinhas et al. 2018, Moral et al., 2021). Dentro del complejo de especies de *C. acutatum* las especies identificadas son *C. acutatum* s.s, *C. godetiae, C. nymphaeae, C. fiorinae, C. lupini, C. rhombiforme* y *C. simmondsii* (Moral et al. 2014; Materatski et al. 2018; Msairi et al. 2020). Dentro del complejo de especies de *C. gloesporioides* están *C. aenigma, C. gloeosporioides* s. s., *C. cigaro, C. quenslandium*. *C. siamense, C. theobromicola, C. fructicola, C. perseae* (Schena et al. 2014; Moral et al. 2014; Moral et al., 2021) y *C. alienum* informa a partir de este estudio. Por último, dentro del complejo de especies *C. boninense* se ha identificado a *C. karstii y C. boninense* (Schena et al. 2014; Moral et al., 2021).

Durante la última década en Uruguay, ocurrió un importante brote epidémico de la antracnosis que duró varios años teniendo su pico en el año 2017 provocando pérdidas casi totales de producción. Durante este brote epidémico además de la conocida podredumbre de frutos se registró una alta incidencia del atizonado de panículas florales, sobre todo en la zona este del país. El ataque a la flor constituye un daño directo ya que las flores atizonadas no producen fruta, pero además, las panículas afectadas actúan como una importante fuente de inóculo secundario promoviendo la ocurrencia de infecciones latentes en frutos verdes y podredumbres en frutos maduros.

Debido a que el olivo es un cultivo emergente en la producción agrícola uruguaya, los estudios locales sobre etiología, epidemiología y manejo de las enfermedades que lo afectan son escasos. Respecto a la identificación de especies de *Colletotrichum* asociadas a la antracnosis del olivo, existe un único antecedente que es el trabajo de Montelongo y

colaboradores (2013). En dicho trabajo se obtuvieron aislados de frutos maduros con síntomas de podredumbre y se analizó exclusivamente la región ITS identificando a los aislados analizados como pertenecientes a los complejos *C. acutatum* o *C. gloeosporioides*.

Es sabido que filogenias basadas en varias regiones génicas con alto poder resolutivo son imprescindibles para obtener una identificación precisa y fiable de las especies de *Colletotrichum* (Damm et al. 2012a, 2012b; Weir et al. 2012, Vieira et al. 2017). Por otra parte, conocer con exactitud las especies de *Colletotrichum* presentes en cada región olivícola, es fundamental para la evaluación e implementación de estrategias de control efectivas. Esto es debido a que se han visto diferencias en la agresividad entre diferentes especies de *Colletotrichum* (Dowling et al., 2020). También, se han encontrado diferencias en la sensibilidad a fungicidas entre complejos de especies de *Colletotrichum* e incluso entre especies pertenecientes al mismo complejo (Dowling et al., 2020; Chen et al. 2016; Munir et al. 2016). Por otra parte, es importante conocer si existe algún tipo de asociación entre especies y tipos de síntomas. Por ejemplo, la caída de las flores de los citrus se ha asociado fundamentalmente a *C. acutatum* mientras que en la postcosecha predomina *C. gloeosporioides* (Freeman et al., 1998; Zulfiqar et al.,1996). En la antracnosis del olivo no se sabe si son las mismas especies las que están ocasionando el atizonado de panículas florales y luego la podredumbre de frutos o si se trata de especies diferentes.

Asimismo, se ha visto que la susceptibilidad de los cultivares de olivo a la antracnosis es variable (Moral y Trapero 2009; Moral et al. 2014; 2017) y que también, dentro de un mismo cultivar, la susceptibilidad varía en función de la especie de *Colletotrichum* que esté infectando (Talhinhas et al. 2015). Otro factor que influye el grado de susceptibilidad es el estadio de desarrollo del órgano afectado. Trabajos previos han demostrado que la susceptibilidad de los frutos se incrementa a medida que avanza la madurez (Moral et al., 2009; Talhinhas et al. 2011) aunque la incidencia final depende, en gran medida, de las condiciones ambientales de esa temporada (Leoni et al.2018; Conde-Innamorato et al. 2019). Por otra parte, hasta lo que conocemos, no se ha estudiado con detalle cómo evoluciona la susceptibilidad en los diferentes estadios de desarrollo de las panículas florales a *Colletotrichum* spp. Esta información es fundamental al momento de desarrollar estrategias de manejo debido a que permite, por ejemplo, ajustar con precisión el inicio de las aplicaciones preventivas con fungicidas.

El manejo de la antracnosis del olivo se lleva adelante diseñando estrategias de Manejo Integrado en las que se combinan medidas de control genético (utilización de variedades menos sensibles), cultural (diseño de la plantación, conducción y poda, fertilización equilibrada) y químico (aplicación de fungicidas). La selección de cultivares menos susceptibles, la poda de los árboles para favorecer la ventilación, las cosechas tempranas y las aplicaciones de fungicidas principalmente a base de cobre, han sido las medidas más utilizadas (Talhinhas et al. 2018). En cuanto a la susceptibilidad varietal, en general se ha visto que Picual, Coratina, Frantoio son cultivares menos susceptible a la antracnosis que otros como Arbequina, Galega vulgar y Barnea (Bartolini y Cerreti, 2017; Moral et al., 2017; 2015; Talhinhas et al., 2015). Respecto a la poda de árboles para favorecer la ventilación, esta medida es especialmente importante en aquellas regiones de clima húmedo como Uruguay. En relación con la cosecha temprana, esta medida se realiza con el fin de reducir la incidencia de la enfermedad debido a que el fruto maduro presenta mayor susceptibilidad a la antracnosis. Esta medida a su vez tiene efectos positivos en la producción, ya que el aceite obtenido a partir de fruta verde posee mayor calidad respecto al obtenido de fruta madura mejorando la calidad sensorial, estabilidad oxidativa, así como también aumentando su valor nutricional (Brkić Bubola et al., 2012; El Qarnifa et al., 2019).

En cuanto al uso de fungicidas para el control de la antracnosis del olivo, existen trabajos que muestran diferencias en la sensibilidad a los fungicidas entre los diferentes complejos de especies de *Colletotrichum*, así como entre especies dentro del mismo complejo tanto en olivo como en otros frutales (Chen et al., 2016; Munir et al., 2016; Zhang et al., 2020; Schoeneberg y Hu 2022). Así, por ejemplo, estudios realizados por Chen et al. (2016) con aislados causantes de la antracnosis del durazno encontraron que *C. nymphaeae* manifestó resistencia a los fungicidas flutriafol y fenbuconazol, mientras que *C. fioriniae* se comportó como sensible. Por otra parte, Moral et al. (2018) encontraron que aislados de *C. nymphaeae* fueron más sensibles al sulfato de cobre que los de *C. godetieae*, mientras que los de *C. godetiae* fueron más sensibles a kresoxim-metil que los de *C. nymphaeae*.

El único antecedente de evaluación de fungicidas para el control de la antracnosis en Uruguay se basó en determinar la efectividad in vitro de oxicloruro de cobre, el sulfato de cobre y el óxido cuproso en la reducción del crecimiento micelial de *Colletotrichum* spp. En este trabajo se evaluó la inhibición del crecimiento micelial de aislados de los

complejos de especies de *C. acutatum* y *C. gloesporioides* y, en general, el fungicida que más inhibió el crecimiento micelial fue el oxicloruro de cobre (Montelongo et al., 2013). Sin embargo, no se desarrollaron estudios posteriores para evaluar la eficacia de estos fungicidas en el control de la antracnosis en condiciones de campo.

Desafortunadamente la aplicación de fungicidas sobre la fruta está restringida solamente a aquellos principios activos a base de cobre. Los fungicidas orgánicos como los inhibidores de la biosíntesis del ergosterol, las estrobilurinas o dithiocarbamatos entre otros, están limitados debido a que son liposolubles y los residuos pueden aparecer tanto en los frutos como en los aceites obtenidos a partir de estos (Moral and Trapero, 2009; Moral et al., 2014; 2018).

Finalmente, y con el fin de realizar un correcto manejo de la antracnosis del olivo en Uruguay, en esta tesis se propuso generar conocimientos locales acerca de la etiología de la antracnosis, así como aportar elementos que permitan elucidar aspectos epidemiológicos de esta enfermedad. También conocer la eficacia de diversos fungicidas que podrían utilizarse para su control durante la floración y madurez de fruto. El objetivo final es proporcionar información útil que permita desarrollar un paquete de manejo racional y ambientalmente sostenible para la antracnosis del olivo en Uruguay.

HIPÓTESIS

1. En Uruguay la antracnosis del olivo es ocasionada por diferentes especies de *Colletotrichum* pertenecientes a más de un complejo de especies.

2. En las condiciones de producción de Uruguay, las especies de *Colletotrichum* ocasionan infecciones latentes en frutos verdes de olivo durante todo su desarrollo.

3. Los aislados de *Colletotrichum* asociados a la antracnosis del olivo ocasionan tanto atizonado de las panículas florales como podredumbre de fruto en los cultivares producidos en Uruguay.

4. La susceptibilidad de las panículas florales y de los frutos se incrementa a medida que avanza el desarrollo de estos órganos.

5. Es posible controlar la antracnosis del olivo mediante la aplicación de fungicidas durante la floración y madurez de fruto.

OBJETIVOS

Objetivo general

Generar conocimientos sobre la etiología y epidemiología de *Colletotrichum* spp. así como evaluar medidas de control para el diseño e implementación de sistemas de Manejo Integrado en el cultivo de olivo en Uruguay.

Objetivos específicos

1. Identificar las especies de *Colletotrichum* causantes de la antracnosis del olivo en Uruguay, mediante estudios morfológicos y análisis filogenéticos de varias regiones génicas y verificar su patogenecidad.

2. Verificar la ocurrencia de infecciones latentes causadas por especies de *Colletotrichum* en frutos verdes de diferentes cultivares y evaluar su evolución durante el desarrollo del fruto en dos regiones olivícolas.

3. Analizar la evolución de la susceptibilidad de flores y frutos de olivo durante su desarrollo a las especies de *Colletotrichum* en diferentes cultivares.

4. Evaluar la efectividad de diferentes fungicidas para el control del atizonado de panículas florales y podredumbre de frutos de olivo causado por especies de *Colletotrichum.*

ARTÍCULO 1

Olive anthracnose caused by *Colletotrichum* in Uruguay: symptoms, species diversity and flowers and fruits pathogenicity

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Olive anthracnose caused by *Colletotrichum* in Uruguay: symptoms, species diversity and flowers and fruits pathogenicity

ABSTRACT

Olive anthracnose caused by *C. acutatum*, *C. gloeosporioides* and *C. boninense* species complexes, is the most spread and economically important olive disease worldwide. The most traditional symptom is fruit rot, but more recently a high incidence of blossom blight emerged in some olive world regions as Uruguay, causing significant losses. In this work, a collection of *Colletotrichum* isolates obtained from different organs with symptoms attributable to anthracnose, with special emphasis in blighted flowers, was characterized. Based in the analysis of six partial gene regions (GADPH, ACT, βTUB2, HIS3, APN2/MAT-IGS and GAP2-IGS) and phenotypical characters, five *Colletotrichum* species were identified. *C. acutatum* s.s. was found as the prevalent species (n=89) followed by *C. nymphaeae* (n=14) and *C. fioriniae* (n=1) all belonging to *C. acutatum* species complexes. The other two species found were *C. theobromicola* (n=3) and *C. alienum* (n=1) both belonging to *C. gloeosporioides* species complexes SC. To our knowledge, this study represents the first report of *C. alienum* causing olive anthracnose worldwide. Phenotypical characteristics differed mainly between the species complexes, validating their utility to separate the isolates to complex level. The tested isolates caused necrosis involving all floral organs and the typical "soapy fruit" symptom appeared regardless of the *Colletotrichum* species inoculated. Due the olive anthracnose is an extremely dangerous disease, further studies to development an effective management strategy are necessary to mitigate its incidence.

Key word

blossom blight, *Colletotrichum* complex, fruit rot, *Olea europea, Colletotrichum acutatum* s.s.

1. INTRODUCTION

Olive (*Olea europaea* subsp. *europaea* L) activity in Uruguay is known since early in the 1900s, but it was in the last two decades when commercial production had a strong expansion. Since 2000s several orchards with appropriated varieties used for virgin olive oil production, were massively planted using an intensive rainfed plantation system (285 - 400 trees/ha), increasing the olive area from 500 to 7000 hectares (MGAP-DIEA, 2020). Contrary to the Mediterranean traditional regions, Uruguay is characterized by frequent high relative humidity days and recurrent rainfalls (around 1.100 mm per year) along the year (Conde-Innamorato et al., 2019) favouring disease development.

Olive anthracnose is the most spread and economically important olive fruit disease worldwide (Talhinhas et al., 2018) and the most devastating olive disease in Uruguay (Montelongo et al., 2014; Leoni et al., 2018). When environmental conditions are favourable for epidemics development, olive anthracnose cause severe outbreaks lending to significant yield losses, especially in super-high-density planting system (Talhinhas et al., 2011; Moral et al., 2012; 2014; Kolainis et al., 2020). In addition, olive oil quality considerably decreases when olive fruits are affected by anthracnose. Olive oil elaborated with infected fruits acquires a reddish colour followed by an increase of acidity and decrease of organoleptic properties (Moral et al., 2014; Leoni et al., 2018).

The most common symptom caused by olive anthracnose is fruit rot. *Colletotrichum* infects the fruit mostly in a mature state generating a brown lesion which are rapidly covered with orange spore masses giving the typical "soapy fruit" name (Moral et al., 2014; Mosca et al., 2014; Talhinhas et al., 2018). Affected fruits can drop prematurely or remain in the tree, finally they mummify (Moral et al., 2009; Moral and Trapero, 2012). Eventually and under favourable conditions, *Colletotrichum* can infect leaves and branches causing necrosis, defoliation, and death of branches (Talhinhas et al., 2018; Moral et al., 2014). Also, *Colletotrichum* infecting flowers from early stages causing blossom blight and reducing fruit set appeared in South Africa and more recently in Australia and Greece (Gorter 1956; Seergeeva et al., 2008; Iliadi et al., 2018, Talhinhas et al., 2018).

Olive anthracnose is caused by at least 14 *Colletotrichum* species belonging to the species complex *Colletotrichum acutatum* sensu lato (s. l.), *C. gloeosporioides* s. l. and *C. boninense* s. l. (Talhinhas et al., 2011; Schena et al., 2014; Chattaoui et al., 2016; Moral et al., 2017; Talhinhas et al., 2018). Inside *C. acutatum* s. l. species complex *C. acutatum* s.s, *C. godetiae* (=*C. clavatum*) and *C. nymphaeae* have been reported as the most frequent. Other species reported into this species complex are *C. fiorinae*, *C. lupini*, *C. rhombiforme* and *C. simmondsii* (Moral et al., 2014; Materatski et al., 2018, Msairi et al., 2020). The importance of each species of *Colletotrichum* varies according to the olive growing region. In Andalusia (south Spain), Italy and in most countries of the Mediterranean Basin, the species *C. godetiae* prevails (Moral and Trapero, 2009; Moral et al., 2014, Talhinhas et al., 2018), while in Portugal predominates *C. nymphaeae* and it seems that this species is restricted to the southwest of the Iberian Peninsula (Talinhas et al., 2005, Moral et al., 2014, Materatski et al,, 2018; Talhinhas et al., 2018). Finally, *C. acutatum* s. s. predominates in the Southern hemisphere (Moral et al., 2014, Talhinhas et al., 2018). Within *C. gloeosporioides* s. l. species complex the species associated with the anthracnose are *C. aenigma*, *C. gloeosporioides* s. s., *C. kahawe* sbp. *ciggaro*, *C. quenslandium*. *C. siamense* and *C. theobromicola* (Schena et al., 2013, Moral et al., 2014). Although this species complex is known to be spread in all the olive growing regions worldwide, it is always less frequent than the *C. acutatum* s.l. (Talhinhas et al., 2018). Finally, within the *C. boninense* s. l. species complex, only *C. karstii* has been associated with olive anthracnose disease, this species was found in Italy (Schena et al., 2014).

In Uruguay, olive anthracnose disease infects the commercial orchards causing the typical symptoms of fruit rot and mummies (Montelongo et al., 2014, Leoni et al., 2018; Conde et al., 2019). In the last years, a consistently high incidence of blossom blight has been observed, with 2017 the year being the highest incidence of this symptom.

Studies of etiology, epidemiology and management of olive anthracnose disease are scarce in Uruguay. Montelongo et al. (2013) isolated *Colletotrichum* strains from typical symptoms of anthracnose on mature fruit and found isolates belonging to the *C. acutatatum* and *C. gloeosporioides* species complex. However, the species identification was based exclusively on the ITS phylogenetic analysis. In addition to this gene region, other loci must be incorporated to a multi-locus phylogenetic analysis to obtain a reliable identification of *Colletotrichum* species (Damm et al., 2012a; 2012b; Weir et al., 2012;

Kolainis et al., 2020). Furthermore, Vieira et al. (2020) found that the best sets of molecular markers vary according to the *Colletotrichum* species complex. Particularly to *C. acutatum* s.l., the more appropriated loci are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (TUB2), histone (HIS3) or Actine (ACT), while for *C. gloeosporioides* s.l. are glutamine synthetase (GS), β-tubulin (TUB2), DNA lyase (APN2), hypothetical protein (GAP2-IGS) or intergenic region between DNA lyase and the mating type Mat1-2 (APN2/MAT-IGS) the best gene regions. Also, the GAPDH locus has shown to be suitable for species complexes differentiation and for a primary approximation to the species identification.

Correct identification of *Colletotrichum* species causing olive anthracnose is crucial due to the potential aggressiveness variation or fungicide susceptibility among the species as well as other characteristics (Moral et al., 2018). Besides, having a proper identification of the causal agents of anthracnose will facilitate future studies of resistance programs as well as of the bests control strategies for management disease. Thus, the objective of this study was to characterize a collection of *Colletotrichum* isolates obtained from olive fruits, flowers, branches and leaves with symptoms of anthracnose in Uruguay, based on DNA phylogenetic analysis, phenotypical characteristics and pathogenicity tests.

2. MATERIAL AND METHODS

2.1.*Field symptoms and fungal isolates*

Between 2017 and 2018, twenty-five orchards of nine farmers situated in the south-eastern, south, and south-western of Uruguay were surveyed. The varieties sampled were ‘Arbequina’, ‘Coratina’, ‘Picual’, ‘Frantoio’, ‘Manzanilla’, ‘Arbozana’, ‘Leccino’, and ‘Pandolina’ (Fig. 1). Symptoms attributable to anthracnose in flower, leaves, branches, and fruits were carefully observed and registered. When present, samples of all organs affected by *Colletotrichum* spp. were collected from different trees.

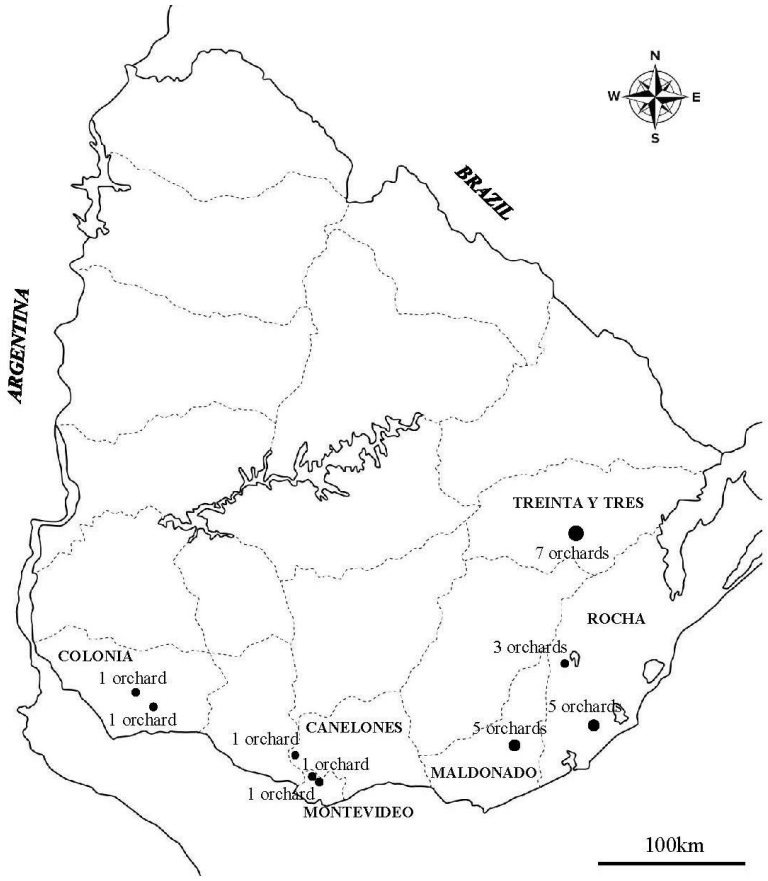

Fig. 1 Geographical localization of the nine olive farmers survey (25 orchards) in the south-eastern, south, and south-western of Uruguay

To obtain monoconidial isolates, a *Colletotrichum* spp. conidial mass was taken from each organ collected and placed in a microtube of 1.5 ml containing sterile water and vortexed to dislodge the conidia. Immediately, serial dilutions were performed to achieve a concentration of 10^3 conida/ml and a 100 µl aliquot was scattered on agar water medium (Oxoid Ltd., Hampshire, England) containing 0.4 g/l of streptomycin sulphate (Sigma-Aldrich, China). When the organ collected did not present *Colletotrichum* spp. conidia, a humid chamber was made to induce sporulation. After 24 h of incubation at 25 °C in the dark a germinated conidia was transferred to a new Petri dish containing potato dextrose agar (PDA) (PDA, Oxoid Ltd., Hampshire, England) and maintained in the same conditions. A maximum of eight fungal isolates per organ and commercial orchard were selected (Table 1).

Monoconidial isolates were grown in sterile filter papers, dried with silica-gel and stored at -20°C. The isolates were deposited in the fungal culture collection at the Plant Protection Department, Faculty of Agronomy, University of the Republic, Uruguay.

2.2. *Phylogenetic analysis*

Isolates were grown on PDA for one week at 25ºC in the dark. Total DNA was extracted from mycelium and reproductive structures using the commercial kit ADN Quick-DNA™ Fungal/ Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer instructions. DNA suspensions were stored at -20°C.

To obtain a primary identification, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene region was amplified and sequenced in the 108 *Colletotrichum* isolates generated in this study, using GDF1/GDR1 primers (Guerber et al., 2003). The sequences were compared with those deposited in the GenBank using the BLAST source (blast.ncbi.nlm.nih.gov/Blast.cgi). To improve the knowledge of phylogenetic relationships among the isolates, βtubuline (βTUB2), actin (ACT) and histone (HIS3) gene regions were amplified in a subset of 32 isolates identified as *C. acutatum* s.s (representing different geographical origins, affected olive organs and GAPDH haplotypes) and the remaining 15 isolates belonging *C. acutatum* complex. The primers used were BT2Fd/BT4Rd (Woudenberg et al.,2009), ACT-512F/ACT-783R (Carbone and Kohn, 1999) and CYLH3F/CYLH3R (Crous et al., 2004), respectively. For the four isolates identified belonging *C. gloeosporioides* complex, the gene regions amplified were βTUB2, ACT, mating type Mat1-2 (APN2/MAT-IGS) and a hypothetical protein (GAP2-IGS). The primers used for the last two gene regions were CgDL_F6/CgMAT1_F2 and GAP1041/GAP/IGS-2044 respectively (Vieira et al., 2017).

Each PCR reaction contained 1x PCR buffer, 2.5 mM MgCl2, 0.4 mM of each dNTP, 0.4 μM of each primer, 1 U of DNA polymerase (Bioron, Germany) and 1 μL of template DNA. The PCR reaction was adjusted to a final volume of 20 μl with MQ water. The amplifications were performed on a MultiGene™ Mini (Labnet International, Inc., USA). The program for GADPH, ACT, βTUB2 and HIS3 consisted in an initial step of 94 °C for 5 min followed by 34 cycles 94 °C for 45 s, 52 °C for 30 s (60 ºC HIS3), and 72 °C for 45 s and for APN2/MAT-IGS and GAP2-IGS 3 min at 95°C, followed by 35 cycles of 95°C for 30 s, 62°C (APN2/MAT-IGS) or 58ºC (GAP2-IGS) for 45 s, and 72°C

for 1 min 30 s. A final extension of 72 °C for 10 min was performed in all amplifications. PCR products were analysed in 1.5% agarose gels stained with GelRedTM and visualized in a transiluminator under UV light. A GeneRuler 100-bp DNA ladder plus was used as a molecular weight marker (Thermo, Lithuania). PCR products were purified and sequenced in Macrogen Inc., Seul, Korea.

The sequences for each gene region, were aligned using ClustalW program, available within MEGA 10.01.8 program (https://www.megasoftware.net/). The alignments were manually edited when necessary. Related sequences as well as sequences of the phylogenetically closest species (including ex-type) obtained from the GenBank, were incorporated in the alignments (Supplemental Table S1). Multilocus phylogenetic analyses were performed separately using the four amplified gene regions for C. *acutatum* complex (GAPDH, βTUB2, ACT and HIS3), whereas to *C. gloeosporioides* complex the five amplified gene regions (GAPDH, βTUB2, ACT, APN2/MAT-IGS and GAP2-IGS) respectively. Multilocus alignments were built using Sequence Matrix v.1.8 (http://www.ggvaidya.com/taxondna/).

Phylogenetic trees were constructed using Bayesian inference (BI) and Maximum likelihood (ML) methods. BI and ML analyses were inferred with MrBayes 3.2.7 (Ronquist et al., 2012) and RAxML 8.2.12 (Stamatakis, 2014) programs respectively, implemented in CIPRES Science Gateway v 3.3 (http://www.phylo.org/). For BI phylogenetic analysis, the best-fit model of each locus was selected according to Akaike information criterion corrected (AICc) in MEGA 10.01.8 program. Four Marko Chain Monte Carlo (MCMC) chains were run simultaneously starting from a random tree to 10 million of generations. Trees were sampled every 1000 generations, and the first 2500 were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 7500 trees. For the ML analysis, generalized time-reversible with gamma correction (GTR + GAMMA) nucleotide substitution model and 1000 bootstrap iterations were indicated. The other parameters were used as default settings.

Sequences generated in this study were deposited in the GenBank (Table 1) and the alignment files and trees in TreeBase (www.treebase.org) under the accession number 27270 (http://purl.org/phylo/treebase/phylows/study/TB2:S27270).

Table 1 Uruguayan isolates of *Colletotrichum* spp. obtained from olive

	Fungal species	Isolate*	Olive cultivar	Geographical origin Departament/Area	Affected tissue	GenBank Accesion number					
						GADPH	ACT	BTUB2	HIS	APN2/MAT-IGS	GAP2-IGS
CaSC[1]	*C. acutatum* s.s.	OL1	Arbequina	Rocha/19 de Abril	Flower	MW038944	MW038893	MW038842	MW038995	-	-
		OL5	Arbequina	Rocha/19 de Abril	Flower	MW038945	MW038894	MW038843	MW038996	-	-
		OL6	Arbequina	Rocha/19 de Abril	Flower	MW038946	MW038895	MW038844	MW038997	-	-
		OL15	Arbequina	Rocha/Velázquez	Flower	MW038947	MW038896	MW038845	MW038998	-	-
		OL16	Coratina	Rocha/19 de Abril	Leaf	MW038948	MW038897	MW038846	MW038999	-	-
		OL18	Arbequina	Maldonado/Garzón	Flower	MW038949	MW038898	MW038847	MW039100	-	-
		OL24	Arbequina	Montevideo/La Paz	Flower	MW038950	MW038899	MW038848	MW039101	-	-
		OL30	Arbosana	Montevideo/Melilla	Flower	MW038951	MW038900	MW038849	MW039102	-	-
		OL31	Pandolina	Montevideo/Melilla	Flower	MW038952	MW038901	MW038850	MW039103	-	-
		OL34	Arbequina	Montevideo/Melilla	Flower	MW038953	MW038902	MW038851	MW039104	-	-
		OL36	Arbequina	Montevideo/Melilla	Flower	MW038954	MW038903	MW038852	MW039105	-	-
		OL42	Arbequina	Treinta y Tres/Mendizabal	Flower	MW038955	MW038904	MW038853	MW039106	-	-
		OL47	Arbequina	Colonia/Tarariras	Flower	MW038956	MW038905	MW038854	MW039107	-	-
		OL50	Arbosana	Colonia/Artilleros	Flower	MW038957	MW038906	MW038855	MW039108	-	-
		OL51	Arbequina	Rocha/19 de Abril	Leaf	MW038958	MW038907	MW038856	MW039109	-	-
		OL53	Arbequina	Rocha/19 de Abril	Branch	MW038959	MW038908	MW038857	MW039110	-	-
		OL57	Arbequina	Rocha/19 de Abril	Fruit	MW038960	MW038909	MW038858	MW039111	-	-
		OL60	Arbequina	Rocha/19 de Abril	Fruit	MW038961	MW038910	MW038859	MW039112	-	-
		OL61	Picual	Rocha/19 de Abril	Fruit	MW038962	MW038911	MW038860	MW039113	-	-
		OL63	Coratina	Rocha/19 de Abril	Fruit	MW038963	MW038912	MW038861	MW039114	-	-
		OL65	Arbequina	Montevideo/La Paz	Fruit	MW038964	MW038913	MW038862	MW039115	-	-
		OL72	Picual	Montevideo/La Paz	Fruit	MW038965	MW038914	MW038863	MW039116	-	-
		OL74	Picual	Rocha/19 de Abril	Fruit	MW038966	MW038915	MW038864	MW039117	-	-
		OL78	Arbequina	Montevideo/La Paz	Fruit	MW038967	MW038916	MW038865	MW039118	-	-

		OL82	Picual	Rocha/19 de Abril	Fruit	MW038968	MW038917	MW038866	MW039119	-	-
		OL89	Picual	Rocha/19 de Abril l	Fruit	MW038969	MW038918	MW038867	MW039120	-	-
		OL90	Arbequina	Rocha/19 de Abril	Fruit	MW038970	MW038919	MW038868	MW039121	-	-
		OL92	Coratina	Maldonado/Garzón	Fruit	MW038971	MW038920	MW038869	MW039122	-	-
		OL94	Arbequina	Montevideo/La Paz	Fruit	MW038972	MW038921	MW038870	MW039123	-	-
		OL97	Arbequina	Montevideo/La Paz	Fruit	MW038973	MW038922	MW038871	MW039124	-	-
		OL103	Coratina	Treinta y Tres/Mendizabal	Fruit	MW038974	MW038923	MW038872	MW039125	-	-
		OL106	Arbequina	Treinta y Tres/Mendizabal	Fruit	MW038975	MW038924	MW038873	MW039126	-	-
	C. nymphaeae	OL9	Arbequina	Rocha/Velázquez	Flower	MW038976	MW038925	MW038874	MW039127	-	-
		OL12	Arbequina	Rocha/Velázquez	Flower	MW038977	MW038926	MW038875	MW039128	-	-
		OL19	Arbequina	Maldonado/Garzón	Flower	MW038978	MW038927	MW038876	MW039129	-	-
		OL21	Picual	Maldonado/Garzón	Flower	MW038979	MW038928	MW038877	MW039130	-	-
		OL27	Coratina	Treinta y Tres/Mendizabal	Flower	MW038980	MW038929	MW038878	MW039131	-	-
		OL28	Arbequina	Treinta y Tres/Mendizabal	Flower	MW038981	MW038930	MW038879	MW039132	-	-
		OL29	Arbequina	Treinta y Tres/Mendizabal	Flower	MW038982	MW038931	MW038880	MW039133	-	-
		OL40	Leccino	Canelones/Las Brujas	Flower	MW038983	MW038932	MW038881	MW039134	-	-
		OL43	Coratina	Treinta y Tres/Mendizabal	Flower	MW038984	MW038933	MW038882	MW039135	-	-
		OL56	Arbequina	Maldonado/Garzón	Branch	MW038985	MW038934	MW038883	MW039136	-	-
		OL96	Arbequina	Montevideo/La Paz	Fruit	MW038986	MW038935	MW038884	MW039137	-	-
		OL107	Picual	Treinta y Tres/Mendizabal	Fruit	MW038987	MW038936	MW038885	MW039138	-	-
		OL109	Arbequina	Maldonado/Garzón	Branch	MW038988	MW038937	MW038886	MW039139	-	-
		OL113	Arbequina	Canelones/Las Brujas	Fruit	MW038959	MW038938	MW038887	MW039140	-	-
	C. fioriniae	OL23	Arbequina	Montevideo/La Paz	Flower	MW038990	MW038939	MW038888	MW039041	-	-
CgSC	*C. theobromicola*	OL110	Manzanilla	Canelones/Las Brujas	Fruit	MW038991	MW038940	MW038889	-	MW039046	MW039042
		OL111	Manzanilla	Canelones/Las Brujas	Fruit	MW038992	MW038941	MW038890	-	MW039047	MW039043
		OL112	Arbequina	Canelones/Las Brujas	Fruit	MW038993	MW038942	MW038891	-	MW039048	MW039044
	C. alienum	OL98	Arbequina	Montevideo/Melilla	Fruit	MW038994	MW038943	MW038892	-	MW039049	MW039045

*Subset of 51 isolated selected to perform multilocus phylogenetic analyses and morphological and physiological characterization

[1]CaSC: *Colletotrichun acutatum* species complex, CgSC: *C. gloeosporioides* species complex

2.3. *Morphological and physiological characteristic*

The subset of 51 isolates (37 of *C. acutatum* complex and four of *C. gloeosporioides* complex), were utilized to study phenotypical characteristics as colony colour, shape and size of conidia, sporulation capacity and temperature effect on colony growth.

The *Colletotrichum* isolates were grown in PDA media at 25 °C in darkness. Ten days later, anverse and reverse colour and presence of zonation were registered for each isolate. The length and width of 30 randomly selected conidia per isolate were registered and rated as fusiform, oblong or obclavate at 400X magnification using a digital camera (Microscope-eye-piece-camera, AM-4023X, Taiwan) incorporated to the microscope. To determine the conidia production capacity, each isolate was grown on three PDA plates and incubated in darkness at 25°C. After 10 days, two 4 mm agar plugs with mycelia and spores, were cut from the growing edge of each colony and placed in a 2.0-ml microtube containing 1 ml of sterile water. Microtube were vortexed for 5 seconds and the number of conidia per ml was counted using a hemacytometer.

Daily growth rates from 5 °C to 35 °C with intervals of 5 °C were determined. For this, mycelial plugs of 5 mm were cut from the growing edge of 7-day-old colonies growing on PDA at 25 °C and placed in the centre of PDA plates. For each isolate and temperature, three replicates were performed. After seven days, the colony diameter was measured along two perpendicular axes, the two measures were averaged, and radial growth in millimetres per day was calculated. The experiment was performed under a completely randomized design with three repetitions. The effect of temperature on mycelial growth was analysed using a non-linear regression and the generated data was fit to a cubic equation. The statistical analyses and graph drawings were performed in the software SigmaPlot 10.0.0.54 (http://www.sigmaplot.co.uk/).

2.4. *Flower and fruit pathogenicity test*

The pathogenicity of selected isolates of the five *Colletotrichum* species identified as *C. acutatum* s.s. morphotype 1 (OL24), *C. acutatum* s.s. morphotype 2 (OL78) *C. nymphaeae* (OL19), *C. fiorinae* (OL23), *C. theobromicola* (OL112) and *C. alienum* (OL98), was evaluated in flower and fruit of olive cv Arbequina.

Inoculum was prepared by flooding the agar surface of each colony grown at 25 °C on PDA with 12-h photoperiod, with 10 ml of sterile distilled water (SDW) and scraping with a Drigalski-spatel. The resulting spore suspension was filtered through two layers of cheesecloth and diluted with SDW. Conidial concentration was adjusted with a hemacytometer to 1×10^6 conidia/ml.

Flowers at the phenological stage of final differentiation and healthy, were collected from a commercial orchard without any antecedent of *Colletotrichum* incidence. The flowers were surface disinfested by dipping them 1 min in 1.0% NaClO solution and rinsed twice with SDW. After dried, the flowers were dipped in the conidial suspension for 30s and placed in transparent plastic bags containing moistened sterile paper towels and incubated at 25°C with 12-h photoperiod. Three repetitions were used per isolate, and each repetition consisted in the middle part of two inflorescences with approximately 20 flowers each one. Inflorescences dipped in SDW were used as controls. After six days, the number of flowers with symptoms of *Colletotrichum* spp. was registered.

Fruits at phenological stage of veraison and healthy, were collected from the same commercial orchard. The fruits were washed with water and soap and rinsed with SDW. Then, were dipped 1 min in 1.0% NaClO solution and rinsed twice with SDW. After dried, fruits were dipped in the conidia suspension for 30s and placed into plug seedling trays, one fruit in each hole. The plug seedling trays were moistened and enclosed in transparent nylon bags and incubated at 25°C with 12-h photoperiod. Twenty fruit were used per isolate. Twenty fruits dipped in SDW were used as controls. After ten days, the number of fruits with symptoms caused by *Colletotrichum* spp. was registered.

Each experiment was performed under a completely randomized essay with three repetitions. Incidence of *Colletotrichum* spp. in flower and fruit data were analyzed through a non-parametric Kruskal-Wallis test using InfoStat version 2016. (http://www.infostat.com.ar).

3. RESULTS

3.1. *Field symptoms*

A high incidence of necrotic flower, fruit rot and mummy symptoms were observed in most of the olive orchards surveyed (Fig. 2). Necrosis in flowers were visible from the early stage of flowering and involved all organs, calyx, petals, stamens, and pistils (Fig. 2a and b). When the symptoms were severe, the flowers dropped, and the necrosis of the rachis was usually observed (Fig. 2c). Orange conidia masses of *Colletotrichum* spp. were usually present over or into the infected flowers (Fig. 2d). Fruit rot symptoms were visible from green stage, but the incidence was more notorious in mature fruit. The fruit rot was usually covered by abundant large orange conidia masses of *Colletotrichum* spp. (Fig. 2g and h). Mummified fruits were present over the tree as well as on the ground (Fig. 2i). Other symptoms registered were dead branches and, more rarely, leaf lesions (Fig. 2e and f).

One-hundred and eight monosporic *Colletotrichum* spp. isolates were obtained from the olive samples collected, giving a collection of forty-five isolates from fruits, forty-three from flowers, six from branches and five from leaves (Table 1).

3.2. *Phylogenetic analysis*

The BLAST search implemented with GAPDH sequences, placed all isolates generated in this study into two *Colletotrichum* species complex, *C. acutatum* complex (n = 104) and *C. gloeosporioides* complex (n = 4). It also showed a high identity (99 to 100%) of the strains with four *Colletotrichum* species, except the isolate OL98 that was positioned between *C. alienum*, *C. fructicola* and *C. chrysophilum*. The four species were *C. acutatum* s.s. (n = 89), *C. nymphaeae* (n = 14) and *C. fioriniae* (n = 1) belonging to *C. acutatum* complex and *C. theobromicola* (n = 3) belonging to *C. gloeosporioides* complex respectively.

The individual sequence data sets of species belonging *C. acutatum* and *C. gloeosporiodes* species complex, did not show significant conflicts in tree topology indicating that the genes can be combined. The multilocus alignments of *C. acutatum* and *C. gloeosporioides* species complex phylogenetic analyses performed with the subset of 51 isolates (32 of the 89 defined as *C. acutatum* s.s. and the remaining isolates), confirmed

the identity of the isolates and was useful for accurate resolution of species limits of Uruguayan strains (Fig. 3 and 4). The *C. acutatum* complex dataset contained 91 taxa (47 from this study) and 1234 characters including gaps, corresponding to four loci (GAPDH: 1-209, ACT: 210-417, βTUB2: 418-882, and HIS3: 883-1234) of which 842 were constant and 266 parsimony informative. The *C. gloeosporioides* complex dataset consisted of 46 taxa (4 generated in this study) and 2474 characters including gaps, comprising five loci (ACT: 1-237, BTUB: 238-718, GADPH: 719-931, GAP-IGS: 932-1750 and APN2/MAT-IGS: 1751-2474) of which 1153 were constant and 987 parsimony informative. In both *Colletotrichum* species complex, the topologies of the inferred trees with BI and ML analyses were congruent among themselves. Thus BI trees are shown with the support node value of the two phylogenetic methods used in this study.

The phylogenetic analysis performed with the 47 selected strains attributable to *C. acutatum* complex showed that 32 of them were clustered in a well-supported clade (BS/PP: 1/100) together with *C. acutatum* s.s. Other 14 isolates of this species complex were clustered in another well supported clade (BS/PP: 0.95/70) with *C. nymphaeae*. Finally, the last isolate was clustered with *C. fioriniae* in a third well supported clade (BS/PP: 1/96) (Fig. 3). Regarding the four strains defined as belonging to *C. gloeosporioide*s complex, three of them were grouped in a strong supported clade with *C. theobromicola* (BS/PP: 1/100) and the remaining isolate in another well supported clade with *C. alienum* (BS/PP: 0.83/96) (Fig. 4). To our knowledge, this is the first report of *C. alienum* causing anthracnose in olive worldwide.

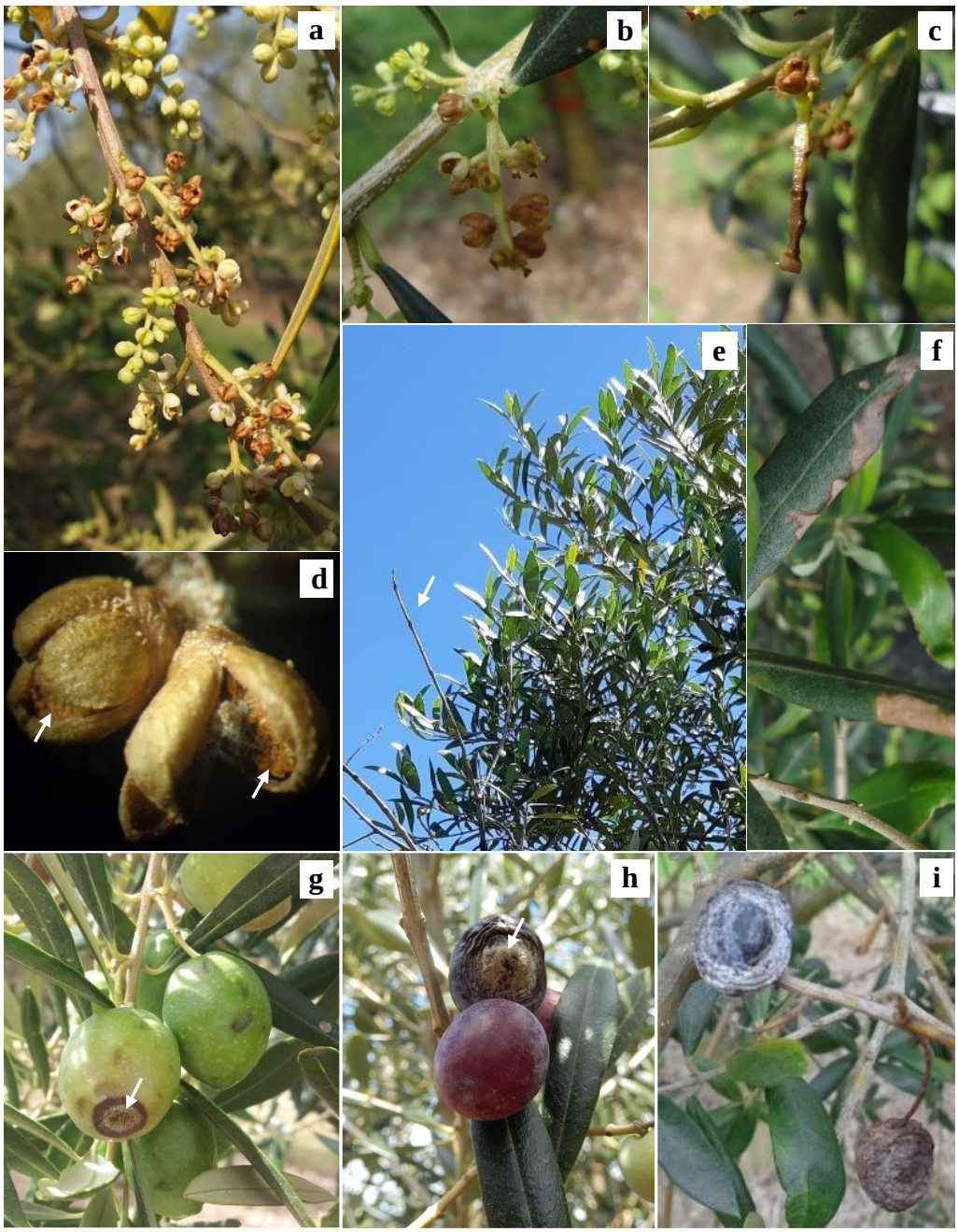

Fig. 2 Symptoms of anthracnose on olive tree caused by *Colletotrichum* spp. **a-d.** blighted flowers; **c**. necrosis on inflorescence with dropping off flowers and necrotic rachis; **d.** detail of muscilaginous orange conidial masses of *Colletotrichum* spp. on the inside of bud flowers (indicate with arrows); **e.** terminal dead branch (indicate with arrow); **f.** leaves with necrotic lesion; **g-h.** green and mature fruits rot with typical conidial masses of *Colletotrichum* spp. (indicate with arrows); **i.** mummified fruits.

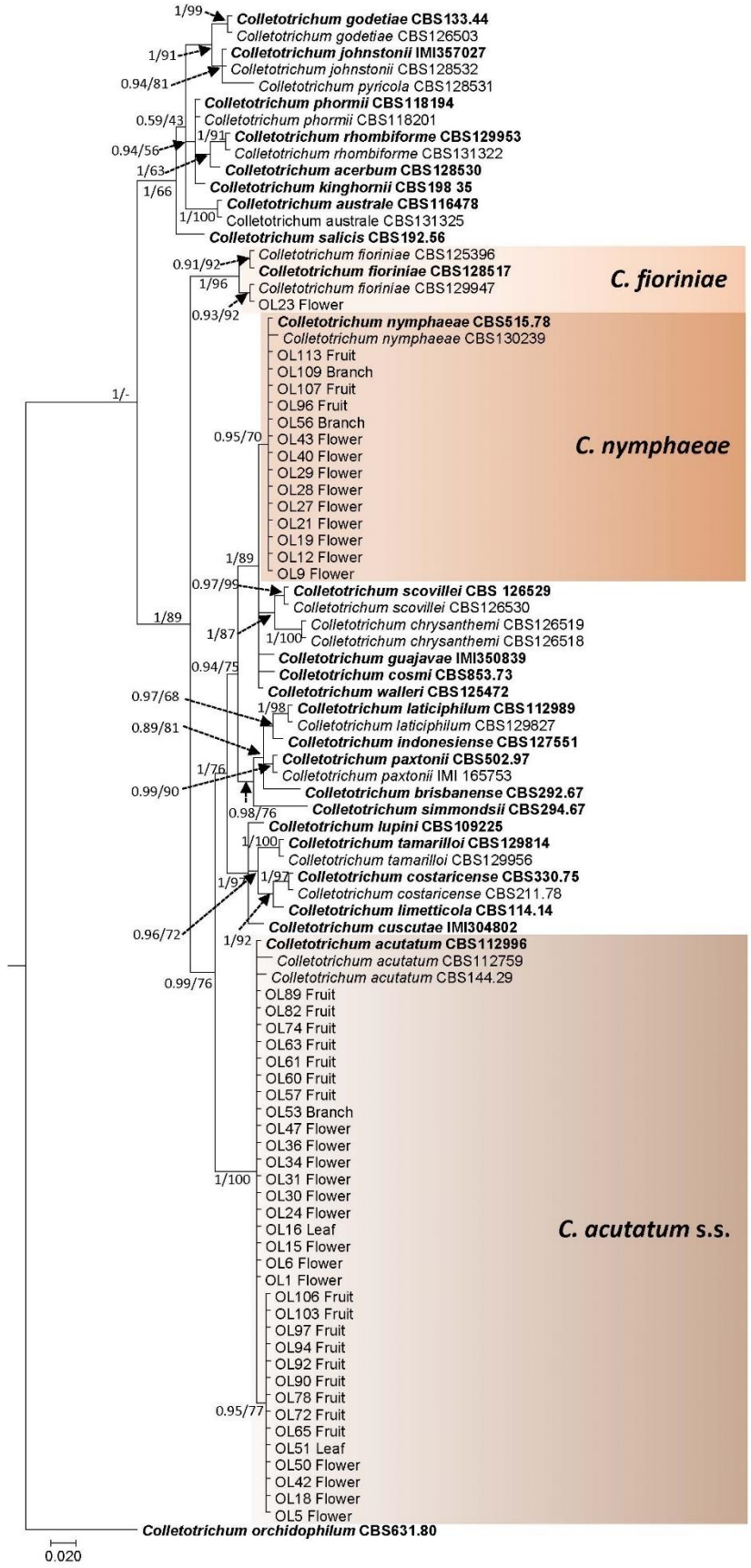

Fig. 3 Bayesian inference phylogenetic tree of 47 *Colletotrichum acutatum* species complex isolates obtained from olive organs with symptoms attributable to anthracnose in Uruguay. The tree was built using concatenated sequences of the GAPDH, ACT, Btub2 and HIS3 genomic regions. *Colletotrichum orchidophilum* CBS631.80 was used as outgroup. Bootstrap support values of posterior probability and maximum likelihood are shown at the nodes before and after the bar, respectively. Ex-type isolates are indicated in bold. Scale bar represents the estimated number of substitutions per site

1.1.

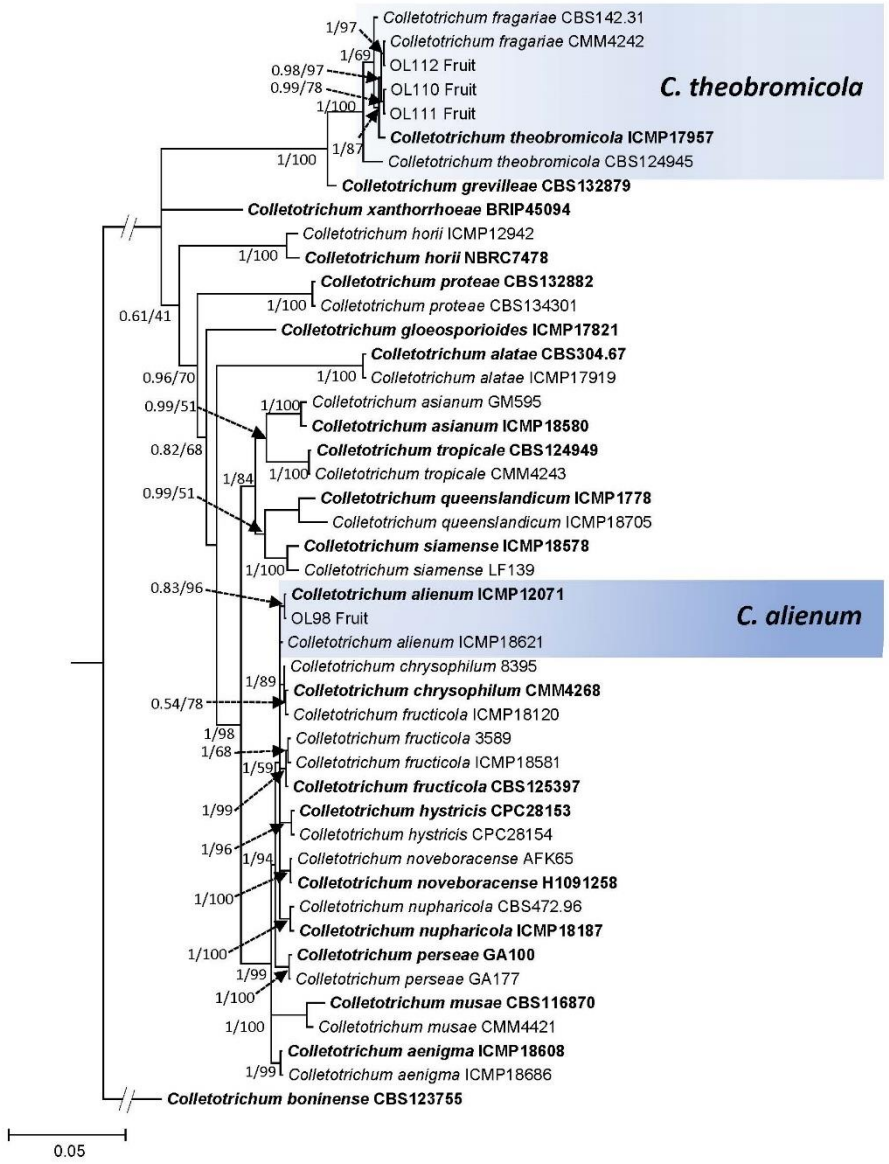

Fig. 4 Bayesian inference phylogenetic tree of four *Colletotrichum gloeosporioides* species complex isolates obtained from olive organs with symptoms attributable to anthracnose in Uruguay. The tree was built using concatenated sequences of the ACT, βTUB2 GAPDH, GAP-IGS and APN2/MAT-IGS genomic regions. *Colletotrichum boninense* CBS123755 was used as outgroup. Bootstrap support values of posterior probability and maximum likelihood are shown at the nodes before and after the bar, respectively. Ex-type isolates are indicated in bold. Double hash marks indicate branch lengths shortened at least 2-fold to facilitate visualization. Scale bar represents the estimated number of substitutions per site

3.3. *Morphological and physiological characteristic*

Isolates belonging to the same *Colletotrichum* species had a similar appearance in PDA medium except those of the *C. acutatum* s. s., in this species two morphotypes were registered. The isolates from morphotype one, were characterized by having a light salmon colour, while those of morphotype two showed a dark salmon colour (Fig. 5). The other species of *C. acutatum* complex, the isolates of *C. nymphaeae* mainly presented greyish white to cream-beige colour and the isolate of *C. fioriniae* light salmon colour. Regarding the two species of *C. gloeosporioides* complex, *C. theobromicola* and *C. alienum*, grey or light gray to white were the common colours. In addition, the isolates of *C. theobromicola* frequently exhibited a marked zonation on its growth patterns. (Table 2, Fig. 5).

Isolates of species belonging to *C. acutatum* complex mainly had conidia with two pointed ends, except for *C. nymphaeae*, in this species the most frequent conidia shape had a rounded. Isolates of the species belonging to the *C. gloeosporioides* complex showed to have a conidia shape with two rounded ends for *C. alienum* and one rounded end and one pointed end for *C. theobromicola*. The conidia size was notoriously smaller in the isolates of the species of *C. acutatum* complex (10.89 to 12.17 μ in length) that in those of *C. gloeosporioides* complex (14.93 to 17.52 μ in length). About the sporulation capacity, the isolates of both morphotype of *C. acutatum* s. s. produced substantially the largest number conidia/ml, followed by the isolates of *C. fiorinae* and *C. nymphaeae*. Isolates of the two species of *C. gloeosporioides* complex, *C. theobromicola* and *C. alienum*, showed to have the lowest capacity to produce conidia) (Table 2).

All isolates of the five *Colletotrichum* species grew at all temperatures compressed between 10 and 30 °C. The temperature of 5 °C caused the death of the isolates. At 35 °C only the three isolates of *C. theobromicola* as well as the *C. alienum* isolate were able to grow. The growing rate average was 8.0 mm/day in the isolates of the species of *C. acutatum* s.l. and 11.12 mm/day in those species belonging to *C. gloeosporioides* s.l. The optimal temperature of growing was around 25 °C for all species (Fig. 6).

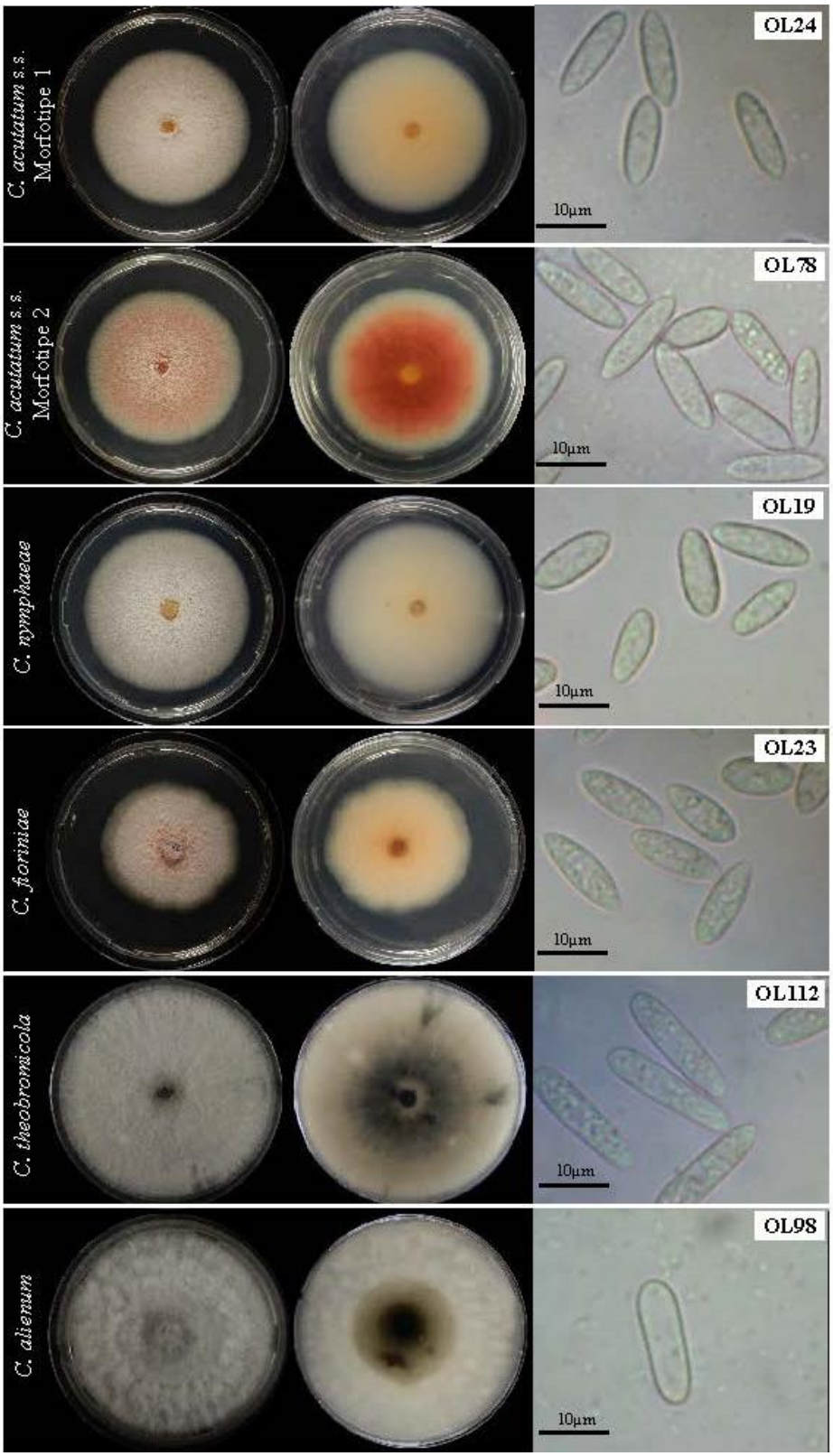

Fig. 5 Anvers (left) and reverse (right) of colonies and conidia aspect of selected isolates of the five *Colletotrichum* species obtained from olive tree in Uruguay. The strains were growing in PDA medium for seven days. The two *C. acutatum* s.s. morphotype defined are showed.

Table 2 Morphological and micro-morphological characteristics of the olive Uruguayan isolates analyzed

Species Complex	Species		Colony colour (PDA)		Sporulation capacity* (conidia/ml)	Conidia size (µ)[2]			Conidia shape (%)[2,3]		
			Upper side	Reverse side		Length (L)	Width (W)	L/W	0	1	2
CaSC[1]	*C. acutatum* s.s.	Morfotipe 1 (22 isolates)	Light salmon, increasing to salmon towards the center	Salmon	7.95E+05	11.98 ± 1.22	3.9 ± 0.35	3.07	1.2	21.4	77.4
		Morfotipe 2 (10 isolates)	Dark salmon	Dark salmon	8.80E+05	10.89 ± 1.06	4.21 ± 0.41	2.59	0.8	17.4	81.8
	C. nymphaeae	(14 isolates)	Greyish white	Cream-beige	2.55E+05	10.96 ± 1.33	4.01 ± 0.47	2.74	1.4	74.6	23.9
	C. fioriniae	(1 isolate)	Light salmon	Light salmon	5.50E+05	12.17 ± 0.82	5.05 ± 0.33	2.41	0	17.1	82.9
CgSC	*C. theobromicola*	(3 isolates)	Grey	Light gray with dark centre	1.20E+05	17.52 ± 1.97	4.47 ± 0.43	3.9	2	67	31.1
	C. alienum	(1 isolate)	Greyish white (grey centre)	White with dark olive gray	3.33E+03	14.93 ± 1.78	5.27 ± 0.57	2.83	66.7	26.7	6.7

[1] CaSC= *C. acutatum* s. l. species complex, CgSC= *C. gloeosporioides* s. l. species complex

[2] Values represent the average registered in the isolates into each species or morphotype of *Colletotrichum* and the ± SD

[3] 0=two rounded ends, 1=one rounded end and one pointed end, 2= two pointed ends

3.4. *Flower and fruit pathogenicity test*

The six isolates of the five *Colletotrichum* species inoculated were pathogenic and causes similar symptoms in the olive flowers and fruits. The first symptoms observed on inoculated flowers at final differentiation state were registered two days after the inoculation, and on the sixth day, they achieved almost 100% of incidence. In case of the fruits inoculated in veraison state, the first symptoms appeared at the fourth day and reached between 70 and 95% of incidence after ten incubation days (Fig. 7).

The symptoms developed have the same appearance as those observed on the olive commercial orchards. The flowers showed the typical blight that involved the entire organ, and the fruits manifested the characteristic "soapy fruit" symptoms. In some blighted flowers and fruit rots, conidial masses of *Colletotrichum* spp. were developed. The symptoms registered in non-inoculated flowers and fruits can be attributable to *Colletotrichum* sp. latent infection expression.

Cultural and conidial characteristics of isolates recovered from inoculated flowers and fruits with the *Colletotrichum* spp. isolates, were corroborated on PDA as previously described, fulfilling the Koch's postulates.

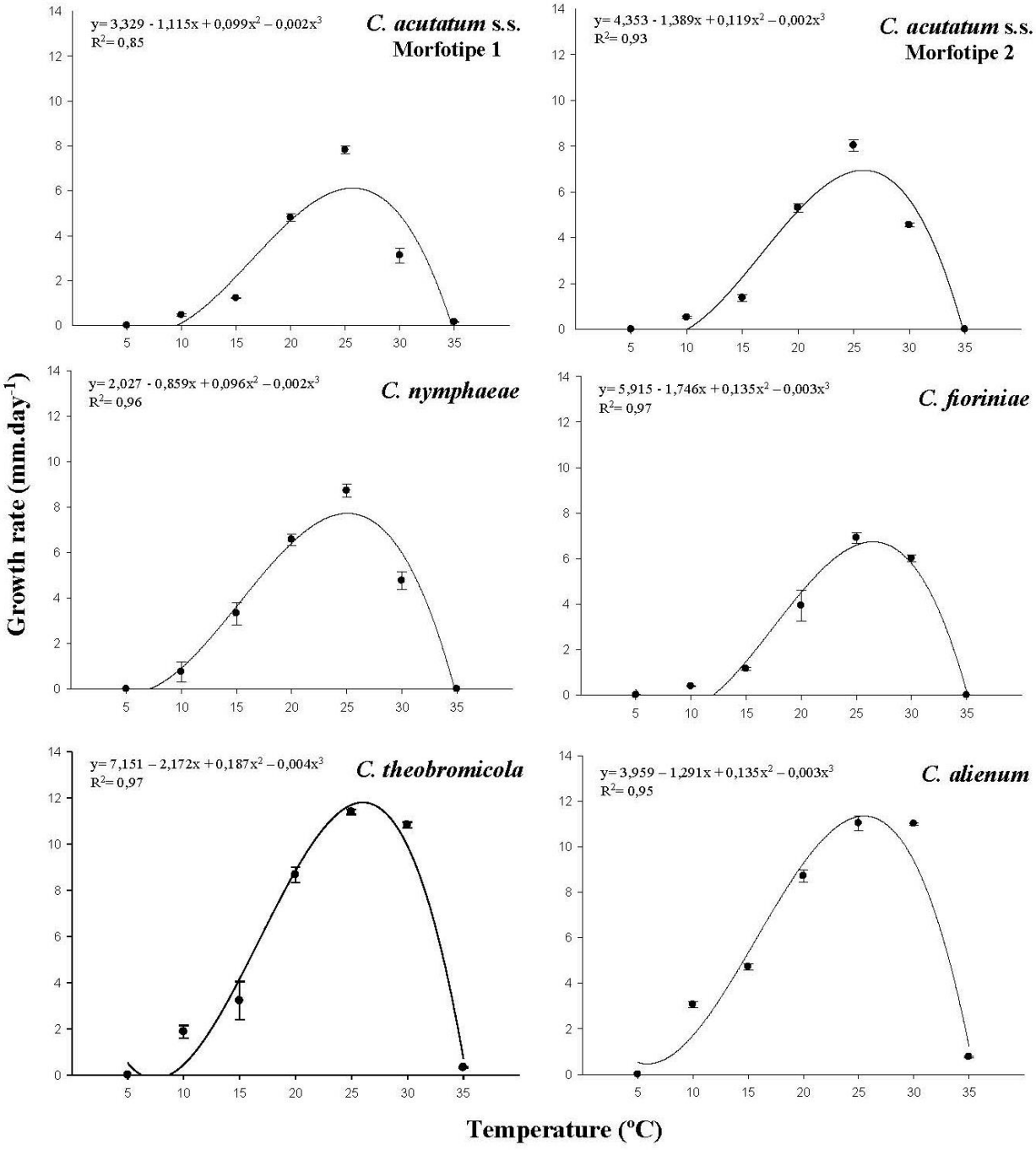

Fig. 6 Effect of temperature on mycelial growth rate (mm/day) of each specie or morphotype of *Colletotrichum* growing on potato dextrose agar at 5, 10, 15, 20, 25, 30 and 35 ºC per seven days. The data represent the overage of the isolates of each species. Temperatures were adjusted nonlinear regression curve. Vertical bars are the standard error of the means

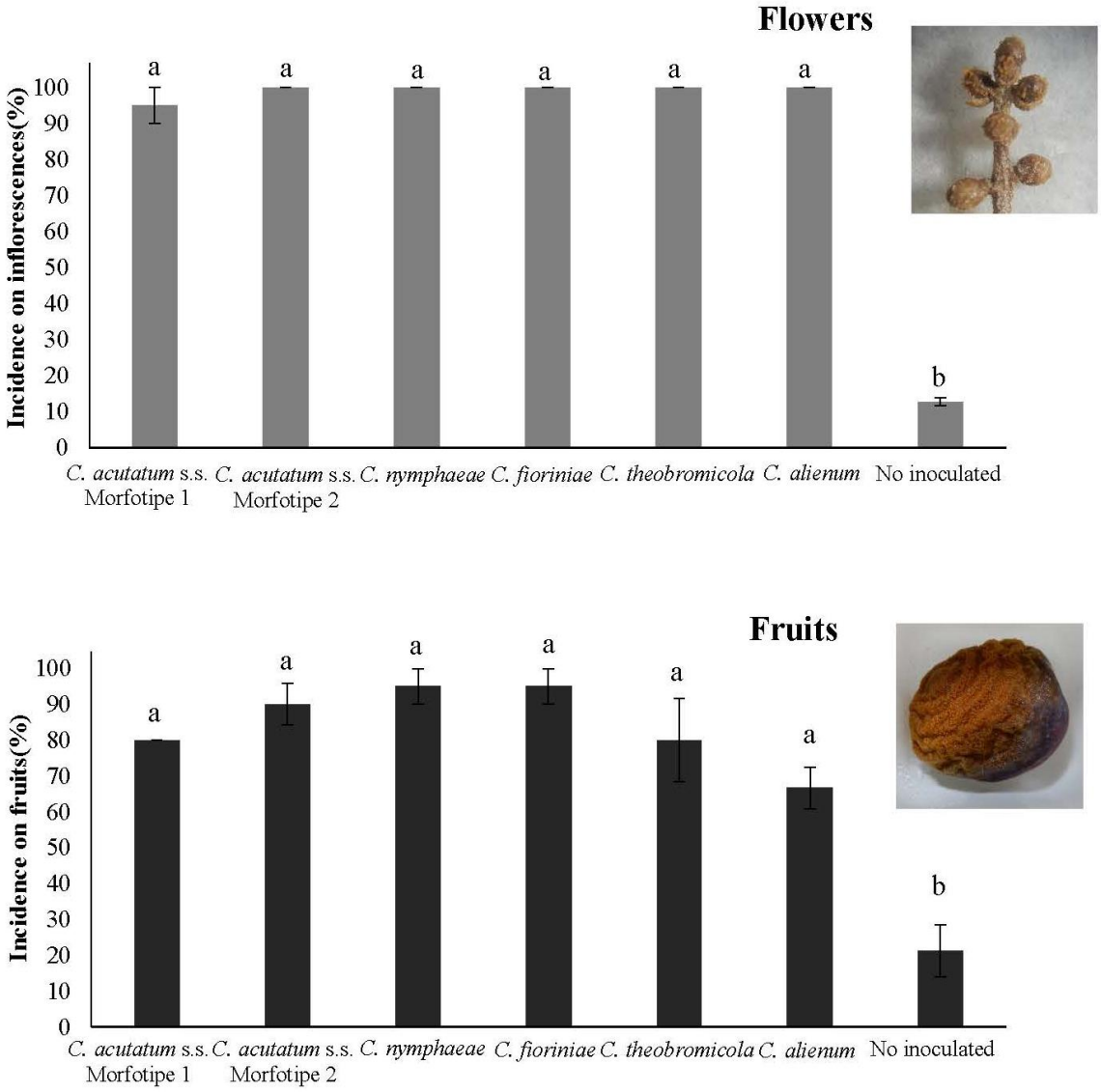

Fig. 7 Necrotic and rot incidence developed on flowers at final differentiation state and fruits at veraison stage, after six and ten days of incubation, respectively. The organs were disinfested and inoculated with selected isolate of five species or morphotype of *Colletotrichum* obtained from Uruguayan olive tree. The incidence registered in the no inoculated treatments can be attributable to the latent infection of *Colletotrichum* spp. expression. The same letter does not differ significantly according to Kruskal-wallis test ($p \leq 0.05$). Vertical bars are the standard error of the mean.

4. DISCUSSION

The olive anthracnose caused by numerous species of *Colletotrichum* is a well-known disease in most olive-growing areas. Numerous studies have been conducted to elucidate different aspects of this disease, mainly in the Mediterranean region where the world olive production is concentrated (Moral and Trapero, 2009; Moral et. al., 2009; Schena et al., 2013; Chattaoui et al., 2016; Moral et al., 2017; Talhinhas et al., 2018). Although anthracnose has been investigated for several years, there are numerous aspects that are still not fully understood, mainly at the non-traditional olive production areas such as the Atlantic coast of South America.

Olive anthracnose can affect various organs, but the most frequent and economically important symptom is fruit rot. Olive orchards with fruit rot generate yield losses and lead to poor oil quality (Moral et al., 2014; Leoni et al., 2018) In Uruguay, the fruit rot caused by anthracnose is a well-known symptom and studies of how it can affect the oil quality have been conducted (Leoni et al., 2018). In this work, we confirm the widely distribution of the fruit rot throughout the olive-growing areas, principally mature fruit rot (Moral and Trapero, 2009; Talhinhas et al., 2011). In the present study, we found abundant mummy fruits over both tree and ground. The main role of the mummies in the anthracnose cycle is to provide an inoculum reservoir of *Colletotrichum* sp. for future infections (Moral and Trapero, 2009; Talhinhas et al., 2018).

Another widely distributed symptom found was blossom blight. This less-known symptom was observed in all the orchards surveyed, and in some of them was the most important symptoms detected. Blossom blights is present in the olive in South Africa (Gorter,1956) Australia (Sergeeva et al., 2008) in Greece (Iliadi et al., 2018). In other countries as Portugal (Talhinhas et al., 2011) and Spain (Moral et al., 2009) *Colletotrichum* spp. was found in flowers without causing scarce visible symptoms. Apparently, flowers can be infected by *Colletotrichum* spp. from early states in the floral differentiation (Sergeeva et al., 2008). Thus, if during flowering time environmental conditions are favorable to disease development, the result can be devastating as occurred in 2017 in the eastern region of Uruguay. In this region where approximately 80% of olive production is concentrated (MGAP-DIEA, 2020) abundant rains and long periods of high humidity were registered during the flowering. As consequence, an increased incidence and severe blossom blight was recorded causing yield losses of almost 100% (2017, data no published).

Leaf lesions and dead branches were also observed. Leaf lesions were found occasionally but dead branches were relatively frequent in all orchards. Some works mention that death branches are important inoculum reservoirs of *Colletotrichum* (Talhinhas et al., 2011; 2018) as well as the mummified fruits, the leaves, and the weed (Moral and Trapero, 2009). The consistency in which dead branches were found in Uruguayan olive orchards, indicate that the importance of this symptom in the anthracnose cycle should be studied with more detail.

In this work, *C. acutatum* s.s. was found to be the most prevalent species causing the olive anthracnose disease in Uruguay. The phylogenetic analyses determined that 82% of the isolates studied belong to this species. Other species of *C. acutatum* complex isolated from symptomatic organs were *C. nymphaeae* and less frequently *C. fioriniae*. Species belonging to *C. gloeosporioides* complex were also found but much less frequently; these are *C. theobromicola* and *C. alienum*. The predominance of species of *C. acutatum* complex causing olive anthracnose has been demonstrated in numerous works (Chattaoui et al., 2016; Thalhinhas et al., 2018). In a previous study conducted in Uruguay, isolates of both *Colletotrichum* species complexes were found associated with olive anthracnose and *C. acutatum* s.l. was also the most frequent. But despite the species were determined only based on morphology and ITS phylogeny analysis, apparently C. *acutatum* s.s. was not found (Montelongo et al., 2013). In the present work, we used four of the more informative gene regions proposed to identify the isolates into *C. acutatum* complex. (GAPDH, BTUB, ACT, HIS3) and five of those recommended for isolates that belong to *C. gloeosporioides* complex. (GAPDH, βTUB2, ACT, APN2/MAT-IGS and GAP2-IGS) (Viera et al., 2020) what guarantees the reliability of our results.

The explosive emergence of *C. acutatum* s.s. affecting olive crops has already been mentioned in other countries as Greece (Iliadi et al., 2018), Italy (Mosca et al., 2014) and Tunisia (Chattaoui et al., 2016). Before that, in the Mediterranean Basin isolates belonging to *C. acutatum* complex (presumably *C. acutatum* s.s.) had been reported only in the south of Portugal (Talhinhas et al., 2005) and in other regions of the world as Australia (Sergeeva et al., 2008), Brazil (Duarte et al., 2010) and South Africa (Gorter, 1956). The causes that could explain why this pathogen turns from undetected to be the most frequent, are still unknown. There is evidence that when *C. acutatum* s.s. is introduced in a region, is capable to produce explosive epidemics as occurred in Uruguay in the recent years. But the advantages that may explain this behaviour are still unknown.

Regarding the other species identified in this work, *C. nymphaeae* and *C. fioriniae* haves been isolated from olive in Portugal (Thalhinas et al., 2005) and *C. theobromicola* in Australia

(Shena et al., 2014), but to our knowledge, the species *C. alienum* is reported for the first time associated with olive crop worldwide.

Although phenotypical characteristics are not enough to delimit *Colletotrichum* species accurately, they provide an excellent approximation to species complex level and are valuable information of the biology of these fungi (Alaniz et al., 2015; Phoulivong et al., 2010; Talhinhas et al., 2018). The light cream to salmon colors of the colonies, conidia shape with predominately one or two pointed ends and the high ability to produce conidia, are attributes of species that belong to *C. acutatum complex*. Whereas colonies with grey to light grey or white colours, conidia shape mostly with two and eventually one rounded end and low ability to produce conidia, are attributes of species of *C. gloeosporioides* complex. In addition, species of *C. acutatum* complex grew slower than those of *C. gloeosporioides* complex. The presence of zonation is a characteristic described by the specie *C. theobromicola* (Brooks, 1931) and it was consistently observated on colonies of this species.

Uruguayan isolates were virulent on both types of olive tissues. Typical anthracnose symptoms were visible on fruits and flowers and pathogen signs were developed over both affected organs few days after inoculation with isolates of the five species identified as *Colletotrichum*. The virulence of *Colletotrichum* spp. has also been mentioned in other works (Moral and Trapero, 2009; Schena et al., 2014; Moral et al., 2017). This explosive behaviour of *Colletotrichum* spp. could explain the devasting epidemic observed in commercial olive orchards when weather conditions are favourable to anthracnose development (Moral and Trapero, 2012). Symptoms present in non-inoculated organs can be attributable to *Colletotrichum* infection originated in the olive orchard. Latent infections of *Colletotrichum* are usual in the olive fruit (Talhinhas et al., 2018) and apparently also in the olive flowers.

Finally, in this work we confirmed that the olive anthracnose is an extremely dangerous disease. Species of *Colletotrichum* can severely affect the olive yield and can cause total loses when environmental conditions are favorable to disease development. Finding an effective management strategy that mitigates its incidence is a challenge due its explosive behavior, especially when the orchards are produced in intensive systems as Uruguay. Preventive strategies based on cultural management such as elimination of affected organs, improve the tree ventilation or overtake the harvest, combined with the use of less susceptible varieties and effective fungicides treatments, should be carefully evaluated and validated to minimize the olive anthracnose incidence.

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6. SUPPLEMENTARY INFORMATION

Table S1. GenBank accession and collection details used in the *C. acutatum* and *C. gloeosporioides* species complexes phylogeny

Species Complex	Species	Accession No.	Host	Country	GenBank No.						Reference
					GADPH	ACT	TUB2	HIS3	APN2/MAT-IGS	GAP2-IGS	
CaSC[1]	*C. acerbum*	CBS 128530, ICMP 12921, PRJ 1199.3*	*Malus domestica,*	New Zealand	JQ948790	JQ949780	JQ950110	JQ949450	-	-	Damm et al. 2012b
	C. acutatum	CBS 112996, ATCC 56816, STE-U 5292*	*Carica papaya*	Australia	JQ948677	JQ005839	JQ005860	JQ005818	-	-	Damm et al. 2012b
	C. acutatum	CBS 112759, STE-U 4470	*Hakea sericea*	South Africa	JQ948722	JQ949712	JQ950042	JQ949382	-	-	Damm et al. 2012b
	C. acutatum	CBS 144.29	*Capsicum annuum*	Sri Lanka	JQ948732	JQ949722	JQ950052	JQ949392	-	-	Damm et al. 2012b
	C. australe	CBS 116478, HKUCC 2616*	*Trachycarpus fortunei*	South Africa	JQ948786	JQ949776	JQ950106	JQ949446	-	-	Damm et al. 2012b
	C. australe	CBS 131325, CPC 19820	*Hakea sp.*	Australia	JQ948787	JQ949777	JQ950107	JQ949447	-	-	Damm et al. 2012b
	C. brisbanense	CBS 292.67, DPI 11711*	*Capsicum annuum*	Australia	JQ948621	JQ949612	JQ949942	JQ949282	-	-	Damm et al. 2012b
	C. chrysanthemi	CBS 126519, PD 85/694	*Chrysanthemum coronarium*	Netherlands	JQ948602	JQ949593	JQ949923	JQ949263	-	-	Damm et al. 2012b
	C. chrysanthemi	CBS 126518, PD 84/520	*Carthamus sp.,*	Netherlands	JQ948601	JQ949592	JQ949922	JQ949262	-	-	Damm et al. 2012b
	C. cosmi	CBS 853.73, PD 73/856*	*Cosmos sp.*	Netherlands	JQ948604	JQ949595	JQ949925	JQ949265	-	-	Damm et al. 2012b
	C. costaricense	CBS 330.75*	*Coffea arabica, cv. Typica*	Costa Rica	JQ948510	JQ949501	JQ949831	JQ949171	-	-	Damm et al. 2012b
	C. costaricense	CBS 211.78, IMI 309622	*Coffea sp., twig*	Costa Rica	JQ948511	JQ949502	JQ949832	JQ949172	-	-	Damm et al. 2012b
	C. cuscutae	IMI 304802, CPC 18873*	*Cuscuta sp*	. Dominica	JQ948525	JQ949516	JQ949846	JQ949186	-	-	Damm et al. 2012b
	C. fioriniae	CBS 125396, GJS 08-140A	*Malus domestica*	USA	JQ948629	JQ949620	JQ949950	JQ949290	-	-	Damm et al. 2012b
	C. fioriniae	CBS128517*	*Persea Americana*	Australia	JQ948622	JQ949631	JQ949961	JQ949301	-	-	Damm et al. 2012b
	C. fioriniae	CBS 129947, CR46, RB022	*Vitis vinifera*	Portugal	JQ948673	JQ949664	JQ949994	JQ949334	-	-	Damm et al. 2012b
	C. godetiae	CBS 126503, PD 88/859, BBA 70342	*Fragaria × ananassa*	UK	JQ948751	JQ949741	JQ950071	JQ949411	-	-	Damm et al. 2012b
	C. godetiae	CBS 133.44*	*Clarkia hybrida*	Denmark	JQ948733	JQ949723	JQ950053	JQ949393	-	-	Damm et al. 2012b
	C. guajavae	IMI 350839, CPC 18893*	*Psidium guajava*	India	JQ948600	JQ949591	JQ949921	JQ949261	-	-	Damm et al. 2012b
	C. indonesiense	CBS 127551, CPC 14986*	*Eucalyptus sp.*	Indonesia	JQ948618	JQ949609	JQ949939	JQ949279	-	-	Damm et al. 2012b
	C. johnstonii	IMI 357027, CPC 18924, PRJ 1125.005	*Citrus sp.*	New Zealand	JQ948774	JQ949764	JQ950094	JQ949434	-	-	Damm et al. 2012b

	C. johnstonii	CBS 128532, ICMP 12926, PRJ 1139.3*	*Solanum lycopersicum*	New Zealand	JQ948775	JQ949765	JQ950095	JQ949435	-	-	Damm et al. 2012b
	C. kinghornii	CBS 198.35*	*Phormium sp.*	UK	JQ948785	JQ949775	JQ950105	JQ949445	-	-	Damm et al. 2012b
	C. lacticiphilum	CBS 112989, IMI 383015, STE-U 5303*	*Hevea brasiliensis*	India	JQ948619	JQ949610	JQ949940	JQ949280	-	-	Damm et al. 2012b
	C. lacticiphilum	CBS 129827, CH2	*Hevea brasiliensis*	Colombia	JQ948620	JQ949611	JQ949941	JQ949281	-	-	Damm et al. 2012b
	C. limetticola	CBS 114.14*	*Citrus aurantifolia*	USA, Florida	JQ948523	JQ949514	JQ949844	JQ949184	-	-	Damm et al. 2012b
	C. lupini	CBS 109225, BBA 70884*	*Lupinus albus*	Ukraine	JQ948485	JQ949476	JQ949806	JQ949146	-	-	Damm et al. 2012b
	C. nymphaeae	CBS 515.78*	*Nymphaea alba*	Netherlands	JQ948527	JQ949518	JQ949848	JQ949188	-	-	Damm et al. 2012b
	C. nymphaeae	CBS 130239	*Fragaria × ananassa*	Netherlands	JQ948580	JQ949571	JQ949901	JQ949241	-	-	Damm et al. 2012b
	C. orchidophilum	CBS 631.80	*Ascocenda sp.*	USA	JQ948482	JQ949473	JQ949803	JQ949143	-	-	Damm et al. 2012b
	C. paxtonii	CBS 502.97, LARS 58	*Musa nana*	"West Indies"	JQ948616	JQ949607	JQ949937	JQ949277	-	-	Damm et al. 2012b
	C. paxtonii	IMI 165753, CPC 18868*	*Musa sp.*	Saint Lucia	JQ948615	JQ949606	JQ949936	JQ949276	-	-	Damm et al. 2012b
	C. phormii	CBS 118194, AR 3546*	*Phormium sp.*	Germany	JQ948777	JQ949767	JQ950097	JQ949437	-	-	Damm et al. 2012b
	C. phormii	CBS 118201, MEP 1334	*Phormium sp.*	New Zealand	JQ948780	JQ949770	JQ950100	JQ949440	-	-	Damm et al. 2012b
	C. pyricola	CBS 128531, ICMP 12924, PRJ 977.1*	*Pyrus communis*	New Zealand	JQ948776	JQ949766	JQ950096	JQ949436	-	-	Damm et al. 2012b
	C. rhombiforme	CBS 129953, PT250, RB011*	*Olea europaea*	Portugal	JQ948788	JQ949778	JQ950108	JQ949448	-	-	Damm et al. 2012b
	C. rhombiforme	CBS 131322, DAOM 233253, C10, MS1L34	*Vaccinium macrocarpum*	USA, Florida	JQ948789	JQ949779	JQ950109	JQ949449	-	-	Damm et al. 2012b
	C. salicis	CBS 607.94* CBS192.56	*Salix sp.*	Germany	JQ948793	JQ949783	JQ950113	JQ949453	-	-	Damm et al. 2012b
	C. scovillei	CBS 126529, PD 94/921-3, BBA 70349*	*Capsicum sp.*	Indonesia	JQ948597	JQ949588	JQ949918	JQ949258	-	-	Damm et al. 2012b
	C. scovillei	CBS 126530, PD 94/921-4	*Capsicum sp*	Indonesia	JQ948598	JQ949589	JQ949919	JQ949259	-	-	Damm et al. 2012b
	C. simmondsii	CBS 122122, BRIP 28519*	*Carica papaya*	Australia	JQ948607	JQ949598	JQ949928	JQ949268	-	-	Damm et al. 2012b
	C. tamarilloi	CBS 129814, T.A.6*	*Solanum betaceu*	Colombia	JQ948514	JQ949505	JQ949835	JQ949175	-	-	Damm et al. 2012b
	C. tamarilloi	CBS 129956, Tom-9, RB112	*Solanum betaceum*	Colombia	JQ948520	JQ949511	JQ949841	JQ949181	-	-	Damm et al. 2012b
	C. walleri	CBS 125472, BMT(HL)19*	*Coffea sp.*	Vietnam	JQ948605	JQ949596	JQ949926	JQ949266	-	-	Damm et al. 2012b
CgSC	*C. aenigma*	ICMP 18608*	*Persea americana*	Israel	JX010044	JX009443	JX010389	-	KM360143	-	Khodadadi et al 2020
	C. aenigma	ICMP 18686	*Pyrus pyrifolia*	Japan	JX009913	JX009519	JX010390	-	-	-	Khodadadi et al 2020
	C. alatae	CBS 304.67, ICMP 17919*	*Dioscorea alata*	India	JX009990	JX009471	JX010383	-	-	-	Khodadadi et al 2020
	C. alatae	ICMP 18122	*Dioscorea alata*	Nigeria	JX010011	JX009470	JX010449	-	-	-	Khodadadi et al 2020
	C. alienum	ICMP 12071*	*Malus domestica*	New Zealand	JX010028	JX009572	JX010411	-	KC888927	-	Vieira et al., 2017; Weir et al 2012

C. alienum	ICMP 18621	*Persea americana*	New Zealand	JX009959	JX009552	JX010386	-	-	-	Vieira et al., 2017; Weir et al 2012
C. asianum	ICMP 18580, CBS 130418*	*Coffea arabica*	Thailand	JX010053	JX009584	JX010406	-	FR718814	-	Khodadadi et al 2020, Vieira et al., 2017.
C. asianum	GM595, MTCC 11680	*Mangifera indica*	India	JQ894623	JQ894545	JQ894601	-	JQ894554	-	Khodadadi et al 2020
C. chrysophilum	CMM 4268*, URM 7362	*Musa sp.*	Brazil	KX094183	KX093982	KX094285	-	KX094325	KX094125	Vieira et al., 2017.
C. chrysophilum (C. ignotum)	8395	*Theobroma cacao*	Panama	KX094176	KX093976	GU994473	-	GU994444	KX094126	Vieira et al., 2017.
C. fragariae	CBS 142.31,ICMP 17927	*Fragaria × ananassa*	USA	JX010024	JX009516	JX010373	-	JQ807844	-	Vieira et al., 2017; Weir et al 2012
C. fragariae	CMM 4242	*Musa sp.*	Brazil	KX094173	KX093971	KX094278	-	KX094320	KX094111	Vieira et al., 2017.
C. fructicola	3589	*Theobroma cacao*	Panama	KX094175	KX093975	KX094280	-	GU994440	KX094122	Vieira et al., 2017.
C. fructicola	CBS 125397*, ICMP 18646	*Tetragastris panamensis*	Panama	JX010032	JX009581	JX010409	-	JQ807839	-	Vieira et al., 2017; Weir et al 2012
C. fructicola	ICMP 18120	*Discorea alata*	Nigeria	JX010041	JX009436	JX010401	-	-		Weir et al 2012
C. fructicola	ICMP 18581*, CBS 130416	*Coffea arabica*	Thailand	JX010033	FJ907426	JX010405	-	JQ807838	-	Vieira et al., 2017; Weir et al 2012
C. gloeosporioides	IMI 356878*, ICMP 17821, CBS 112999	*Citrus sinensi*	Italy	JX010056	JX009531	JX010445	-	JQ807843	-	Khodadadi et al 2020
C. grevileae	CBS 132879*, CPC 15481	*Grevillea sp.*	Italy	KC297010	KC296941	KC297102	-	-	-	Khodadadi et al 2020
C. horii	ICMP 12942	*Diospyros kaki*	New Zealand	GQ329685	JX009533	JX010375	-	-	-	Weir et al 2012
C. horii	NBRC 7478*, ICMP 10492	*Diospyros kaki*	Japan	GQ329681	JX009438	JX010450	-	JQ807840	-	Vieira et al., 2017; Weir et al 2012
C. hystricis	CBS 142411 = CPC 28153*	*Citrus hystrix*	Italy	KY856274	KY856023	KY856532	-	-	-	Khodadadi et al 2020
C. hystricis	CBS 142412 = CPC 28154	*Citrus hystrix*	Italy	KY856275	KY856024	KY856533	-	-	-	Khodadadi et al 2020
C. musae	CBS 116870*, ICMP 19119	*Musa sp.*	USA	JX010050	JX009433	HQ596280	-	KC888926	-	Vieira et al., 2017; Weir et al 2012
C. musae	CMM 4421	*Musa sp.*	Brazil	KX094194	KX093970	KX094297	-	KX094335	KX094118	Vieira et al., 2017.
C. nupharicola	CBS 472.96, ICMP 17940	*Nymphaea ordorata*	USA	JX010031	JX009582	JX010399	-	JX145320	-	Vieira et al., 2017; Weir et al 2012
C. nupharicola	CBS 470.96*, ICMP 18187	*Nuphar lutea subsp. polysepala*	USA	JX009936	JX009486	JX010397	-	JX145319	-	Vieira et al., 2017; Weir et al 2012
C. perseae	GA100 TCBS141365*	*Persea americana*	Mikve Israel Orchard, Central Israel	KX620242	KX620145	KX620341	-	KX620177	-	Sharma, et al., 2017
C. perseae	GA177	*Persea americana*	Mikve Israel Orchard, Central Israel	KX620245	KX620148	KX620344	-	KX620180	-	Sharma, et al., 2018
C. proteae	CBS 132882, CPC 14859*	*Protea sp.*	South Africa	KC297009	KC296940	KC297101	-	-	-	Khodadadi et al 2020
C. proteae	CBS 134301, CPC 14860	*Protea sp.*	South Africa	KC842379	KC842373	KC842387	-	-	-	Khodadadi et al 2021
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	JX009934	JX009447	JX010414	-	KC888928	-	Khodadadi et al 2020
C. queenslandicum	ICMP 18705	*Coffea sp.*	Fiji	JX010036	JX009490	JX010412	-	-	-	Khodadadi et al 2020

	C. siamense	ICMP 18578*, CBS 130417 LC2931, CGMCC 3.17353,	*Coffea arabica*	Thailand	JX009924	FJ907423	JX010404	-	JQ899289	-	Khodadadi et al 2020
	C. siamense	LF139	*Camellia sp.*	China	KJ954788	KJ954369	KJ955236	-	KJ954503	-	Khodadadi et al 2020, Vieira et al., 2017.
	C. theobromicola	CBS 124945*,ICMP 18649	*Theobroma cacao*	Panama	JX010006	JX009444	JX010447	-	KC790726	-	Vieira et al., 2017; Weir et al 2012
	C. theobromicola (syn. C. gloeosporioides f.stylosanthis)	MUCL 42294(*), ICMP 17957, CBS 124251	*Stylosanthes viscosa*	Australia	JX009962	JX009575	JX010380	-	-	-	Weir et al 2012
	C. tropicale	CMM 4243	*Musa sp.*	Brazil	KU213601	KU213596	KU213604	-	KU213597	KX094120	Vieira et al., 2017; Weir et al 2012
	C. tropicale	CBS 124949*, ICMP 18653	*Theobroma cacao*	Panama	JX010007	JX009489	GU994454	-	GU994425	-	Khodadadi et al 2020
	C. xanthorrhoeae	BRIP 45094*, ICMP 17903,CBS 127831	*Xanthorrhoea preissii*	Australia	JX009927	JX009478	JX010448	-	-	-	Weir et al 2012
	C.noveboracense	AFK65	*Apple/Empire Columbia/ Apple/Idared*	NY	MN812243	MN701178	MN701196	-	MN701183	-	Khodadadi et al 2020
	C.noveboracense	AFKH109 *	*Columbia/ Crinum*	NY	MN640567	MN640565	MN640569	-	MN640564	-	Khodadadi et al 2020
CbSC	*C. boninense*	MAFF 305972, CBS 123755*	*asiaticum var. sinicum*	Japan	JQ005240	JQ005501	JQ005588	-	-	-	Damm et al, 2012

ARTÍCULO 2

Incidence of *Colletotrichum* latent infections during olive fruit development under Uruguayan environmental conditions

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Incidence of *Colletotrichum* latent infections during olive fruit development under Uruguayan environmental conditions

ABSTRACT

Anthracnose caused by *Colletotrichum* is the most important olive disease in humid climate regions worldwide. *Colletotrichum* produce latent infections in green fruits lending to fruit rot at mature stage. We evaluated the incidence of *Colletotrichum* latent infections during fruit development in two agroclimatic regions of Uruguay, south-eastern and south-central, and three cultivars, Arbequina, Cortina and Picual. *Colletotrichum* latent infections were present at all stages of olive fruit development, with a substantially higher incidence in the south-eastern (60.5%) than the south-central region (1.2%). Environmental indices indicated that during the experiment the south-eastern had substantially greater number of days with high relative humidity (> 80%) and light rainfall (< 5 mm) alone or combined with abundant rainfall and mean temperature (20 to 30ºC), that the south-central region. Arbequina and Picual presented the highest incidence of latent infections in both regions. Thus, we demonstrated that the south-eastern, the main agroclimatic region of olive production in Uruguay, is highly conducive to *Colletotrichum* latent infections development, and that Arbequina, the major cultivar planted, is highly susceptible to this pathogen.

Key word: anthracnose, environmental conditions, latent infection, soapy rot

1. INTRODUCTION

The genus *Colletotrichum* (teleomorph *Glomerella*) is considered the eighth most important plant pathogen worldwide (Dean et al., 2012; Dowling et al., 2020). Within this genus, fifteen species complexes have been reported causing destructive diseases in more than 3200 plants, including agricultural crops. Species of *Colletotrichum* are associated with anthracnose symptoms in fruit and others aerial parts of the plants produced in tropical, subtropical, and temperate regions, especially those with humid climate (O'Connell et al., 2012; Moraga et al., 2018; Da Silva et al., 2020; Dowling et al., 2020; Gomes et al., 2021). Also, *Colletotrichum* infections can remain latent during fruit grow until fruits ripening when this pathogen cause decay (Prusky et al., 2013; Whenneker and Thomma, 2020).

For olive crop, anthracnose is recognized the most important disease in all olive-growing regions with humid climate in the world (Cacciola et al., 2012; Moral et al., 2014; Talhinhas et al., 2018). Environmental conditions such as relative humidity and frequent rainfall during flowering and fruit development, have been associated with severe epidemic outbreaks (Sergeeva 2011, Moral and Trapero, 2012). Consequently, devastating losses occur in both susceptible cultivars and those considered less susceptible to *Colletotrichum* species (Talhinhas et al., 2011; Moral and Trapero, 2012; Seergeva, 2014; Talhinhas et al., 2015; 2018; Moreira et al., 2021).

The *Colletotrichum* species infect many parts of the olive tree, such as shoots, stems, branches, leaves, flowers, and fruits, producing blossom blight, defoliation, or death of branches, but the most important symptom is fruit rot at maturity stage (Cacciola et al., 2012; Moral and Trapero, 2012; Seergeva 2014; Talhinhas et al, 2018). Rotten fruit generate direct yield losses, but also undesirable physicochemical and organoleptic parameters in the oil, reducing its quality and increasing economic losses (Moral et al., 2014; Leoni et al., 2018; Talhinhas et al., 2018; Peres et al., 2021).

Furthermore, *Colletotrichum* species can produce asymptomatic or latent infections in various olive tree organs such as leaves, stems and green fruits (Moral et al., 2009; 2012; Sergeeva, 2014; Talhinhas et al 2018). In this phase, known as biotrophic, the pathogen produces primary intracellular hyphae that infect host cells without affecting them. It is followed by the necrotrophic phase, in which the pathogen produces secondary

hyphae and secrets toxins and enzymes, colonizing the cells and degrading the plant tissue, causing the characteristic anthracnose symptoms. This behavior explains the hemibiotrophic lifestyle of most of *Colletotrichum* known species (Prusky et al., 2013; Sergeeva, 2014; Baroncelli et al., 2017; Da Silva et al., 2020; Gomes et al., 2021). Duration of the two phases varies among *Colletotrichum* species and depends on the stage of host development and weather conditions (Peres et al., 2005; De Silva et al., 2017; Da Silva et al., 2020).

When the fruits begin to ripen and the meteorological condition are favorable to disease development, reactivation of latent infection occur and field symptoms are observed (Moral et al., 2009; 2012; Romero et al., 2018; Talhinhas et al., 2018). Also, some studies indicate that the latent infection incidence in green olive fruit is directly affected by the cultivar, the environmental conditions, and fruit maturity (Moral et al., 2012; Romero et al., 2021).

Anthracnose is the most important olive disease in Uruguay. Although olives can be infected by Colletotrichum every year, final incidence mainly depends on environmental conditions (Leoni et al, 2018; Conde-Innamorato et al., 2019, Moreira et al, 2021). Species belonging to *C. acutatum* species complex, predominately *C. acutatum* s.s, and *C. gloesporioides* complex, have recently been identified as the causal agents of this disease. In addition, it was corroborated that fruit rot and blossom blight are the most prevalent and destructive symptoms for Uruguayan olive crops (Moreira, et al 2021).

However, more studies are needed to better understand the epidemiology of olive anthracnose under the environmental conditions of Uruguay. For example, there is little information about the importance of latent infections in olive fruits. Furthermore, rainfall, relative humidity and temperature influence and different cultivars susceptibility to *Colletotrichum* latent infections incidence under local conditions has not been investigated yet. Thus, to advance in the knowledge of olive anthracnose behavior in Uruguay, the objective of this work is to study the incidence of *Colletotrichum* latent infections in the three widest planted olive cultivars during fruit development, in two of the main agroclimatic regions of olive production.

2. MATERIALS AND METHODS

2.1.*Plant material*

This study was conducted during the 2017-2018 growing season in commercial farms situated in two different olive-producing agroclimatic regions in Uruguay, separated by 200 km. One of them is in the south-eastern region, Rocha department (34°18'38.7"S 54°01'49.4"W), the most important olive production area of the country (76% of total production) and the other farm is located in the south-central region, Montevideo department (34°43'54.7"S 56°10'00.5"W). In the south-eastern region the evaluated cultivars were Arbequina, Coratina and Picual whereas in the south-central were Arbequina and Picual. Both orchards had an intensive rainfed planting system (285-400 trees/ha).

Samplings with a frequency of one month were conducted from after fruit set (November-December) to beginning of harvest (February-March), in both production regions and three cultivars. Each sample consisted of 20 healthy fruits collected per tree, from five randomly selected trees per orchard. A total of 100 fruits for each cultivar, region and sampling moment were collected.

2.2.*Colletotrichum latent infection*

The collected olive fruits were surface disinfected by dipping them into distilled water containing 0.02% Twen-20 for 1 min, then in 70% alcohol for 30s and finally in 1% NaClO for 3 min. Immediately, the fruits were rinsed three times with sterile distilled water to eliminate residues. After that, and to induce the expression of *Colletotrichum* latent infections, the olive fruits were dipped in a 0.05% NaOH solution for 96 hs (Romero et al., 2018). Then, the fruits were placed into plug seedling trays, one fruit in each hole. The plug seedling trays were enclosed in moistened transparent nylon bags and incubated at 24 °C with 12-h photoperiod for 30 days.

The final incidence of *Colletotrichum* latent infections was evaluated 30 days after the induction described above. Fruits were considered infected when typical symptom and sign of anthracnose were observed, which are depressed brown lesion, covered with orange-colored mucilaginous spore masses.

2.3.*Meteorological variables*

Meteorological variables were analyzed every ten days from October 2017 (start of fruit set) to March 2018 (beginning of harvest) in the two agroclimatic regions evaluated. These variables were mean (Tx) and maximum (Tmax) temperature, mean (RHx) and maximum (RHmax) relative humidity and accumulated rainfall (PP).

For south-central region, the meteorological data was obtained using an automatic weather station located the National Research Institute INIA-Las Brujas, in Canelones department (data available at http://www.inia.uy/gras/Clima/Banco-datos-agroclimatico), located at 15 km from the experimental field. For the south-eastern region, data was obtained with an automatic weather station (iMETOS® 3.3) located in the same field where the experiment was carried out.

Using the meteorological data, a total of 23 indices were created based on favorable conditions for *Colletotrichum* infection. These conditions were, average daily temperature between 20 to 30°C, high relative humidity (HRmax >80%, HRmax >90% and HRmax >95%) and rainfall (PP <5 mm and PP >5 mm) (Moral and Trapero 2012, Talhinhas et al., 2018; Moreira et al., 2021). The indices were estimated for each olive agroclimatic region considering the number of days per month in which each one of the favorable environmental conditions was present alone or as a combination of them.

2.4.*Statistical analysis*

Data were analyzed with the statistical software R (R Core Team, 2021), with a primary descriptive analysis of incidence results to evaluate data quality.

A linear mixed model (using "lme" function in the R package; Pinheiro et al. 2021) was applied to assess the effect of the region, sampling time and cultivars and their interactions as follow:

$$Y_{ijklm} = \mu + \alpha_i + \gamma_{j(i)} + \delta_k + \alpha\gamma_{ij} + \alpha\delta_{ik} + \gamma\delta_{jk} + \varepsilon_{ijkl} + \eta_{ijklm}$$

Where μ is the overall mean, α_i is the *i-th* region effect, $\gamma_{j(i)}$ is the *j-th* variety effect nested in the *i-th* region, δ_k is effect of the *k-th* sampling time, $\alpha\gamma_{ij}$ is the region by variety interaction effect, $\alpha\delta_{ik}$ is the region by sampling time interaction effect, $\gamma\delta_{jk}$, ε_{ijkl} is the

experimental error, and η_{ijklm} is the subsampling error. $\varepsilon_{ijkl} \sim N(0, \sigma_{\varepsilon}^2)$ and $\eta_{ijklm} \sim N(0, \sigma_{\eta}^2)$. When significant differences were found, Tukey test with a 95% confidence level was performed using the "emmeans" and "cld" functions from the *emmeans* (Lenth et al., 2018) and *multcomp* (Graves et al. 2018) packages, respectively.

Monthly average indices between regions were compared through t-test at 95% confidence level. Then radar plots were implemented with the "radarchart" function of the "fmsb" package (Nakasawa, 2019) of R to graphically show differences between indices as total number of days during the evaluation period.

3. RESULTS

3.1.*Colletotrichum latent infection*

The latent infections incidence of *Colletotrichum* was assessed in olive fruits collected during four sampling times, three cultivars and both agroclimatic regions. The observed symptoms consisted of depressed lesions on the fruit covered by the typical salmon orange gelatinous masses of the fungus, corresponding to the conidia of *Colletotrichum* (Figure 1).

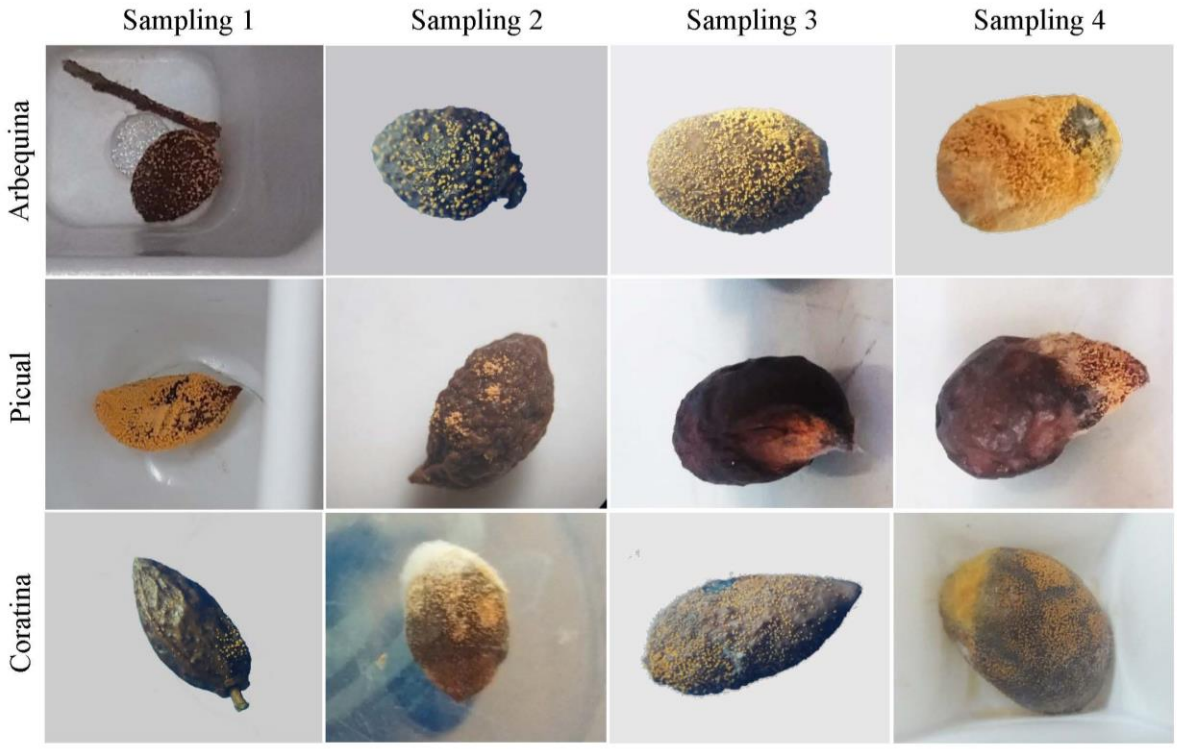

Fig. 1. Symptoms and signs expression of *Colletotrichum* latent infections in olive fruits according to sampling time and cultivar.

The statistical analysis indicated significant effect of latent infections incidence for the main effects region (P= 0.0004), cultivar (P= 0.0175), and sampling time (P= 0.025), but not for the interaction among them (Table 1). The south-eastern region had a substantially higher average of *Colletotrichum* latent infection incidence (60.5%) compared with the south-central region (1.2%). Regarding the olive cultivars, no significative differences were observed between Arbequina and Picual, within each agroclimatic evaluated regions. The *Colletotrichum* latent infection incidence for Arbequina and Picual was 66.4% and 72.7%, respectively, in the south-eastern region and 10.2% and 10.7%, respectively, for the south-central region. However, in the south-eastern, the only region in which Coratina was evaluated, this cultivar presented a significative lowest latent infection incidence (42.3%) compared with Arbequina and Picual. For sampling time, the highest *Colletotrichum* incidence values were detected on the first and fourth sampling times (41.8% and 44.4%, respectively), differing significantly from the second and third sampling times (18.8% and 18.6%, respectively) (Table 2 and Figure 2).

Table 1. Analysis of variance of *Colletotrichum* latent infections incidence in two olive agroclimatic regions, three cultivars and four sampling times evaluated.

	Sum Sq	Mean Sq	Num DF	Den Df	F value	Pr(>F)[1]
Regions	31155	31155	1	3	284.5	0.0004
Cultivar	4528.5	2264.3	2	3	20.7	0.0175
Sampling	5085.7	1695.2	3	3	15.5	0.0249
Regions*Cultivar	86.3	86.3	1	3	0.8	0.4400
Cultivar*Sampling	824.1	137.4	6	3	1.2	0.4611

Type III analysis of variance table using Satterthwaite's method.

Table 2. Adjusted means and standard errors of *Colletotrichum* latent infections for the two olive agroclimatic regions, samplings times and cultivars evaluated.

		Adjusted mean (standard error) *	
Regions	South-central	10.4 (3.7) ^{B}	
	South-eastern	60.6 (3.1) ^{A}	
Cultivar**		**South-eastern**	**South-Central**
	Arbequina	66.4 (5.3) ^{A}	10.5 (5.3) ^{C}
	Picual	72.7 (5.3) ^{A}	10.2 (5.3) ^{C}
	Coratina	42.2 (5.3) ^{B}	-
Sampling	Sampling 1	46.4 (4.8) ^{A}	
	Sampling 2	23.4 (4.8) ^{B}	
	Sampling 3	23.2 (4.8) ^{B}	
	Sampling 4	49.0 (4.8) ^{A}	

* Means with the same letter are not significantly different at 95% of confidence level according to Tukey test
** Due nested effect, cultivar means were adjusted by olive agroclimatic regions

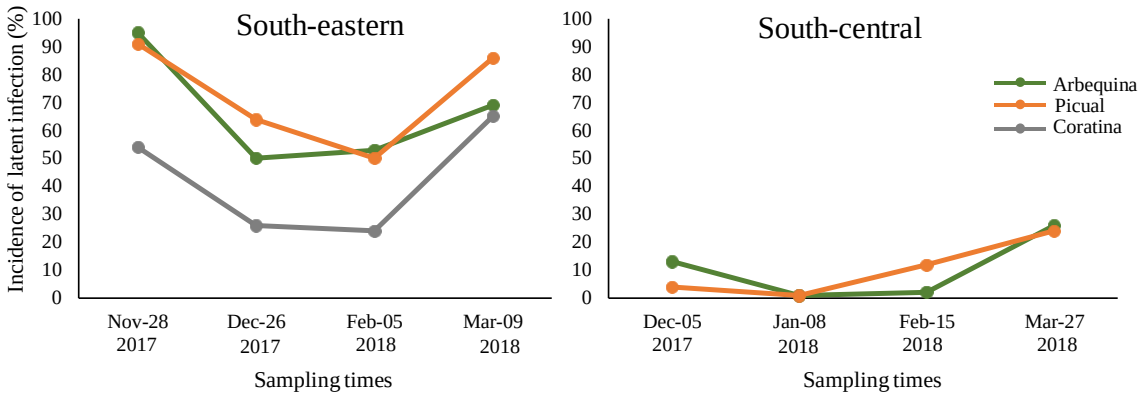

Fig. 2. Evolution of *Colletotrichum* latent infection incidence in olive fruits per cultivar registered in two agroclimatic regions of olive production in Uruguay.

3.2. *Meteorological variables*

In general, the meteorological conditions registered from October 2017 to March 2018 were similar for the two agroclimatic regions. The mean temperature ranged from 16 to 24°C during the whole period. Rainfalls were frequent in each month, and during the entire period analyzed, an accumulated rainfall of 566 mm was recorded from the south-eastern and 518 mm from the south-central regions. Relative humidity registered in the south-eastern region during the entire analyzed period showed values of RHx between 66 to 80% and between 88 to 98% of RHmax. Nevertheless, for the south-central region, the recorded RHx values were between 54 to70% and RHmax values between 84 to 90% (Figure 3).

From the 23 indices created, 10 of them showed significant differences between the two olive agroclimatic regions (Figure 4). The PP<5mm, RH>90%, T*RH>95%, PP<5mm*RH>80%, PP<5mm*RH>90%, PP<5mm*RH>95%, PP<5mm*RH>95%*T, RH>95%, PP>5mm*RH>95% and PP>5mm*RH>95%*T indices were significantly higher in the south-eastern region (Table 3 and Figure 4).

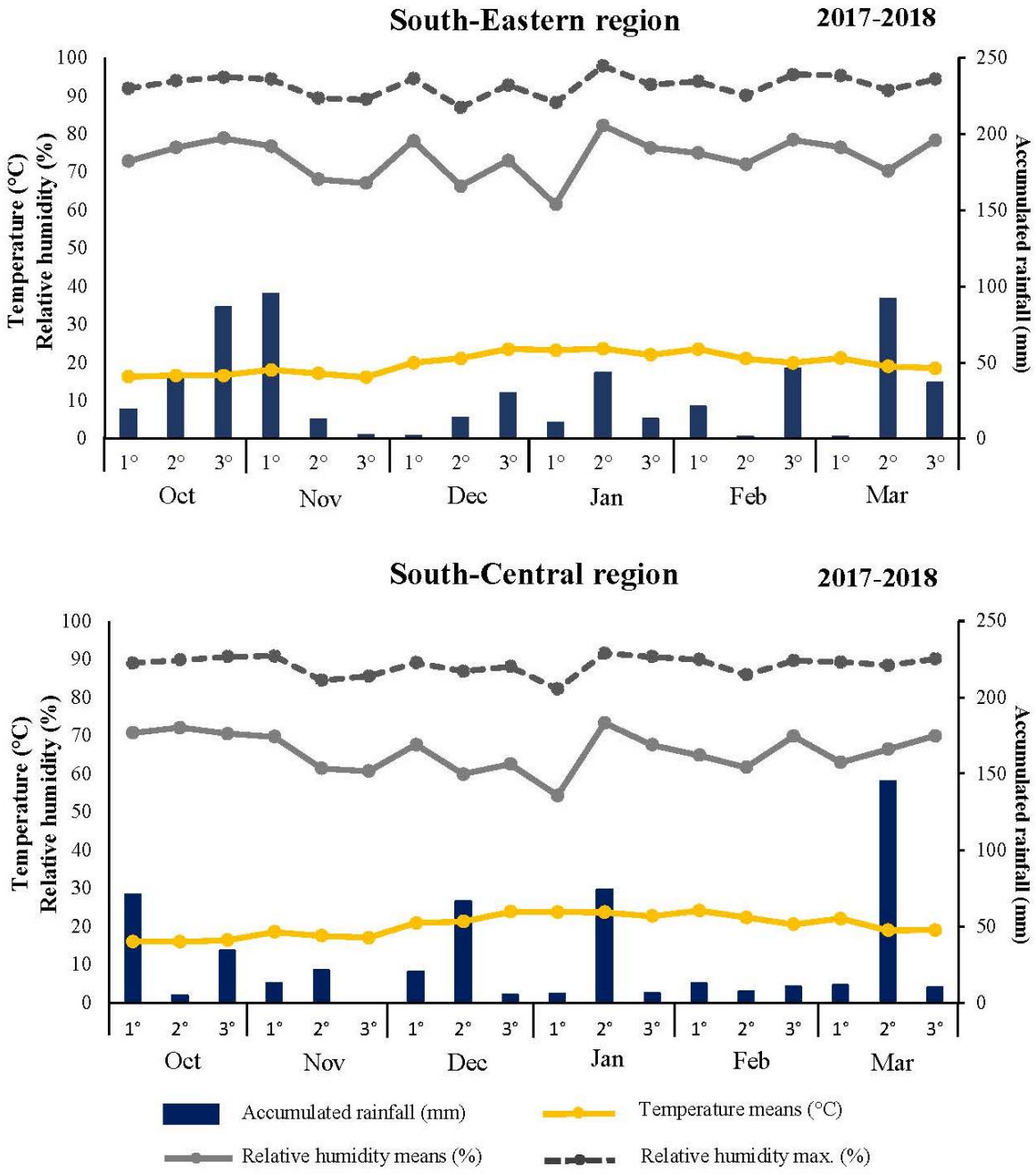

Fig. 3. Meteorological data in south-eastern and south-central regions during October 2017 to March 2018. Average of temperature, relative humidity and accumulated rainfall every 10 days are presented.

Table 3. T-test for the comparison of monthly average number of days with favorable weather conditions for *Colletotrichum* infection in olive fruit for the south-eastern and south-central regions of Uruguay.

Index[1]	South-eastern	South-central	*p*-value
Tx 20 to 30°C	14.2	16.2	0.75
PP <5 mm	8.0	3.0	**<0.01**
PP >5 mm	3.7	3.3	0.59
HRmax >80%	28.7	28.2	0.66
HRmax >90%	22.2	14.7	**<0.01**
HRmax >95%	12.8	0.5	**<0.001**
Tx * HRmax>80%	10.6	12.6	0.67
Tx * HRmax>90%	11.3	8.3	0.48
Tx * HRmax>95%	7.8	0.3	**0.02**
Tx * PP<5mm	4.2	1.5	0.1
PP<5mm * Hrmax>80%	7.8	3.0	**<0.01**
PP<5mm * Hrmax>90%	7.5	1.5	**0.02**
PP<5mm * Hrmax>95%	5.8	0.0	**<0.01**
PP<5mm * HRmax>80% * Tx	4.2	1.5	0.1
PP<5mm * HRmax>90% * Tx	3.8	0.7	0.06
PP<5mm * HRmax>95% * Tx	3.2	0.0	**0.03**
Tx * PP>5mm	1.5	2.3	0.35
PP>5mm * Hrmax>80%	3.7	3.3	0.59
PP>5mm * Hrmax>90%	3.5	2.7	0.32
PP>5mm * Hrmax>95%	3.3	0.2	**<0.001**
PP>5mm * HRmax>80% * Tx	1.5	2.3	0.35
PP>5mm * HRmax>90% * Tx	1.5	1.8	0.71
PP>5mm * HRmax>95% * Tx	1.5	0.2	**<0.001**

[1] Monthly average elaborated for the period October 2017 to March 2018. Days with mean Temperature 20 to 30°C (Tx), maximum relative humidity >80%, 90% and 95% (RHmax), rainfall accumulated <5mm and >5mm (PP). Index with p-value <0.05 are indicated in bold.

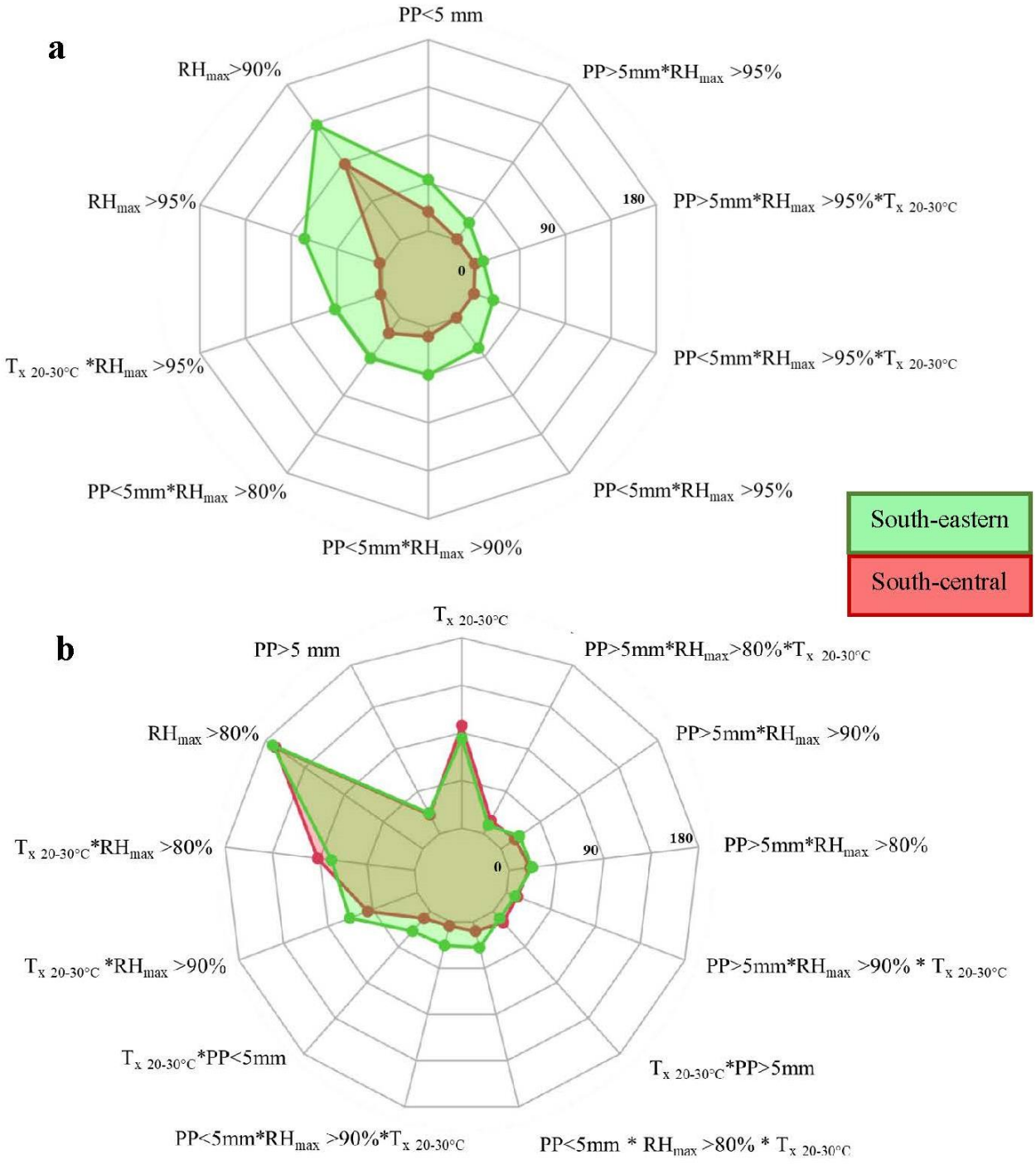

Fig. 4. Radar plots for meteorological indices registered in the South-eastern and South-central agroclimatic regions of olive production grouped according to statistical significant (a) and non-significant (b) differences between regions.

4. DISCUSSION

In the present work, we examined the incidence of *Colletotrichum* latent infections throughout the development of olive fruit. The study was conducted in two agroclimatic regions of olive production in Uruguay, the south-eastern and south-central, and in three cultivars, Arbequina, Coratina and Picual. We also analyzed the influence of environmental conditions on the incidence of *Colletotrichum* latent infection.

Colletotrichum latent infections were recorded in the three cultivars evaluated, but differences among regions, cultivars and samplings moments were observed. Considering all cultivars, the incidence of *Colletotricum* latent infections was substantially higher in the south-eastern region compared with south-central. It can be explained by the environmental conditions, which were more conducive to the disease development during the experiment. Uruguay is characterized by a humid temperate climate with annual rainfall means around 1,100 mm and an annual mean temperature of 17.7°C (Conde-Innamorato et al., 2019). During the evaluated period, frequent rainfall and high relative humidity were present in both regions. Nevertheless, in the south-central region, a higher average and maximum relative humidity was registered than the south-eastern.

Indices created based on environmental favorable conditions for *Colletotrichum* infections showed that, during the evaluation period, the south-eastern region presented substantially a greater number of days with high relative humidity and light rainfall (<5mm) alone or combined with abundant rainfall and favorable mean temperature than the south-central. These indices would explain the prominent higher incidence of latent infections of *Colletotrichum* observed in that region. These results are in accordance with other authors who mention that rainfall, combined with mild temperatures, create a humid and warm environment that favors *Colletotrichum* infection (Sergeeva 2011, Moral and Trapero, 2012). Although further research is needed, present study shows that the main region of olive production (76%) in Uruguay (MGAP, 2020) presents extremely favorable environmental conditions for the development of *Colletotrichum* infections. This appreciation is aligned with the observed in recent years, particularly in 2017, when the highest epidemic outbreaks occurred in the south-eastern region (Moreira et al., 2021).

Regarding cultivars behavior, we observed a similar incidence of *Colletotrichum* latent infections in Arbequina and Picual in both agroclimatic regions, but a substantially lower incidence in Coratina in the south-eastern the only region where this cultivar was evaluated. These results are partly in agreement with other studies which found that Arbequina is susceptible to anthracnose whereas Coratina is characterized as moderately susceptible (Moral and Trapero, 2009; Bartolini and Cerreti, 2013). Nevertheless, Picual is considered moderately resistant to *Colletotrichum* (Moral and Trapero 2009; Talhinhas et al., 2015; Moral et al., 2017) and in the present study did not show differences with Arbequina. This difference could be attributed to the fact that genetic resistance of this cultivar to *Colletotrichum* may be poorly related to the incidence of latent infections.

About the sampling time, we recorded latent infections of *Colletotrichum* in all sampling times evaluated, confirming that olive fruits can be infected from early stages of development (Moral et al., 2009) Curiously, the incidence of latent *Colletotrichum* infections substantially decreased between the first and second sampling, especially in the south-eastern region, where the highest incidence was recorded. According to Rallo (1994), the period of natural physiological fruit drop lasts from six to seven weeks after flowering. It is probably that the olive plants were still in fruit drop stage at the first sampling time, and that the plant possibly selects diseases fruits for discard. Furthermore, falling of infected fruits is mentioned as one of the symptoms of anthracnose disease (Moral et al., 2009; Sergeeva, 2014; Talhinhas et al, 2018). In general, the incidence of latent infections remained constant between the second and third sampling moment (December to February). During these months, days with high temperatures (30ºC) and low relative humidity predominated, environmental conditions that do not favor *Colletotrichum* infections. Finally, at the last sampling time, the incidence of latent infections substantially increased in all evaluations of both agroclimatic regions. During this time (February to March) moderate temperatures and long periods of high humidity increased. In addition, the fruits were closely to maturity, the stage at which susceptibility to anthracnose increases (Moral et al 2008, Moral and Trapero 2012).

In summary, in this work we demonstrated that *Colletotrichum* latent infections can occur at all stages of olive fruit development, although its incidence depends on environmental conditions, cultivar, and fruit developmental stage, as previously report by Moral et al. (2014). Unfortunately, we found that environmental conditions in the south-eastern region, the main olive production region of Uruguay, are very conducive to the

occurrence of latent infections. In addition, we confirmed that Arbequina, the main olive cultivar produced in Uruguay, is one of the most susceptible to be infected by *Colletotrichum*.

Future research should be focused on better understanding how the incidence of *Colletotrichum* latent infections during fruit development correlates with visible anthracnose symptoms in the field. This information would allow to know the phytosanitary status of olive fruits during their development and the potential damages that anthracnose could cause at harvest. Also, this will contribute to improve management practices for this disease, for example in a scenario of high incidence of latent infections during fruit development, minimizing crop losses by earlier harvest or by the application of chemical treatments.

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ARTÍCULO 3

Colletotrichum infections during flower development and fruit ripening in four olive cultivars

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Con revisiones en: ***Phytopathologia Mediterranea***

***Colletotrichum* infections during flower development and fruit ripening in four olive cultivars**

ABSTRACT

Olive anthracnose caused by *Colletotrichum* is considered the major olive disease in olive-growing regions worldwide. The most destructive symptoms caused by this fungus are fruit rot and blossom blight. Susceptibility of fruit to *Colletotrichum* increases with fruit maturity, but differences between cultivars and *Colletotrichum* species have been reported. Also, the information available about flower susceptibility during their development is scarce. In this study, the susceptibility of Arbequina, Coratina, Frantoio and Picual cultivars were evaluated during flower development and fruit maturity to five *Colletotrichum* species, *C. acutatum* s.s., *C. nymphaeae*, *C. fioriniae*, *C. theobromicola* and *C. alienum*. Susceptibility to anthracnose begins in the early stages during flower development and increases during the blossom. In that organ, Arbequina, Coratina and Picual were found as susceptible whereas Frantoio as moderately susceptible. Green fruit presented substantially less anthracnose severity than mature fruit. At green fruit stage, Arbequina and Frantoio were the most susceptible, Coratina presented an intermediate behavior and Picual was moderately susceptible, while no differences were found among cultivars at mature fruit stage. Regards to *Colletotrichum* species, no mayor differences were found with exception of *C. theobromicola*, which caused the highest severity at green fruit stage. Future research should focus on developing anthracnose management strategies to minimize the disease progress from early stages of flower development and fruit ripening, especially in those more susceptible olive cultivars.

Key word: Olive anthracnose, cultivar susceptibility, blossom blight, soapy rot

1. INTRODUCTION

Anthracnose is considered the most important olive disease in olive-growing regions worldwide, especially in those with humid climate (Azevedo-Nogueira et al. 2020; Cacciola et al. 2012; Moral et al. 2014; Talhinhas et al. 2018) such as South Africa (Gorter 1956), Australia (Sergeeva et al., 2008), Brazil (Filoda et al. 2021) and Uruguay (Moreira et al., 2021). Currently, 18 *Colletotrichum* species belonging to the species complexes *C. acutatum*, *C. gloeosporioides* and *C. boninense* have been reported to be associated to this disease (Chattaoui et al. 2016; Moral et al. 2017; Moreira et. al 2021; Schena et al. 2014; Talhinhas et al. 2011; 2018). In Uruguay, *C. acutatum* s.s. was found as the prevalent species followed by *C. nymphaeae* and *C. fioriniae* belonging to *C. acutatum* species complex, and *C. theobromicola* and *C. alienum* belonging to *C. gloeosporioides* species complex (Moreira et al. 2021).

The most known symptom caused by anthracnose is the fruit rot at the ripening stage. Fruit rot causes considerable yield losses and a significant deterioration in oil quality (Leoni et al. 2018; Moral et al. 2014). Additionally, infections can occur from flowering to fruit ripening. During the bloom, species of *Colletotrichum* can infect the calyx, petals, stamens, and pistils causing the collapse of the flowers known as blossom blight (Filoda et al. 2021; Moral et al. 2009; Moreira et al. 2021; Talhinhas et al. 2018). The infected flower is usually covered with an orange gelatinous mass of *Colletotrichum* conidia (Sergeeva et al. 2008; Moreira et al. 2021). If the flower is not destroyed, *Colletotrichum* infect the fruit set remaining as latent until fruit ripens when the typical soapy fruit is expressed (Moral et al., 2009; Talhinhas et al., 2018) although their importance in yield loss is unknown (Moral et al. 2009; Talhinhas et al. 2018).

Infected fruits show a depressed brown lesion that quickly is covered with abundant orange gelatinous mass of *Colletotrichum* conidia. The fruit can be infected from green stages and remain as latent infections until maturation. The infected fruit can fall or remain mummified in the tree serving as a primary inoculum source for subsequent infections in the next year (Moral et al. 2008; 2014; Mosca et al. 2014; Talhinhas et al. 2018).

The Uruguayan climate characterized by persistent relatively high humidity, frequent rainfalls of about 1.100 mm per year and moderate temperatures (Conde-Innamorato et.al. 2019; Leoni et al. 2018) favor olive anthracnose development. In addition, 50% of the plantations (currently 2788ha) belong to the Arbequina cultivar (MGAP-DIEA, 2021), which is considered moderately susceptible to this disease (Moral, et. al, 2014; 2017; Leoni et al. 2018). The remaining 50% of the area is planted mostly with cultivars which have shown no resistance under these climatic conditions, such as Coratina, Picual and Frantoio (Leoni et al. 2018). The main destination of these four cultivars is oil production for export (MGAP-DIEA, 2020).

Many studies have shown that the susceptibility of olive cultivars to anthracnose can be variable (Cacciola et al. 2012; Moral and Trapero 2009; Moral et al. 2014; 2017) and depends on the *Colletotrichum* species (Talhinhas et al. 2015). Also, it is widely known that fruit susceptibility varies according to their developmental stage (Moral and Trapero 2009; Moral et al. 2008; 2017; Talhinhas et al. 2015). Unfortunately, the information available about the susceptibility of different fruit maturity stages and different cultivars to *Colletotrichum* is limited. In addition, although it is known that flowers can be infected by *Colletotrichum* (Kolainis et al. 2020; Moral et al. 2014; Moreira et al. 2021), there are no studies that indicate from what moment of flower differentiation this organ is susceptible. Knowing when the first infections occur at flowering and their potential incidence is crucial to adjust management strategies, for example those based on fungicide application.

Based on these antecedents, in this study we focused on evaluating the susceptibility of Arbequina, Coratina, Picual and Frantoio, the main four olive cultivars produced in Uruguay, during the flower development and fruit ripening to five *Colletotrichum* species which were previously identified associated to olive anthracnose in this country.

2. MATERIAL AND METHODS

2.1.*Plant and fungal material*

Olive flowers panicles and fruits apparently healthy were collected from commercial orchards with scarce anthracnose antecedent, of Arbequina, Coratina, Picual and

Frantoio, the four most widely planted cultivars in Uruguay. The flower panicles were collected at three different flowering stages, 1- swollen bud (BBCH51), 2- final differentiation (BBCH55), and 3- beginning of flowering (BBCH61) and the fruits at two physiological maturity stages, 1- green fruit (BBCH80) and 2- ripe fruit (BBCH89) according to the phenology scale developed by Sanz-Cortes et al. (2002). The collected samples were stored in nylon bags into coolers for their preservation until to be processed in the laboratory.

Fifteen *Colletotrichum* isolates belonging to *C. acutatum*. s.s (n=8), *C. nymphaeae* (n=3), *C. fioriniae* (n=1), *C. theobromicola* (n=2) and C. *alienum* (n=1) were used in this study (Table 1). The strains were selected from the olive *Colletotrichum* collection previously identified and deposited in the Plant Protection Department, Facultad de Agronomía, Universidad de la República, Uruguay (Moreira et al. 2021).

Table 1. Uruguayan Colletotrichum isolates used to evaluate the susceptibility of four olive cultivars at different phenological stages during flower development and fruit ripening.

Species Complex	Fungal species	Isolate	Olive cultivar	Geographical origin		Organ
C. acutatum species complex	*C. acutatum*	OL18	Arbequina	Maldonado	Garzón	Flower
		OL36	Arbequina	Montevideo	Melilla	Flower
		OL42	Arbequina	Treinta y Tres	Mendizabal	Flower
		OL51	Arbequina	Rocha	Nuevo Manantial	Leaf
		OL53	Arbequina	Rocha	Nuevo Manantial	Branch
		OL74	Picual	Rocha	Nuevo Manantial	Fruit
		OL92	Coratina	Maldonado	Garzón	Fruit
		OL97	Arbequina	Montevideo	La Paz	Fruit
	C. nymphaeae	OL28	Arbequina	Treinta y Tres	Mendizabal	Flower
		OL96	Arbequina	Montevideo	La Paz	Fruit
		OL113	Arbequina	Canelones	Las Brujas	Fruit
	C. fioriniae	OL23	Arbequina	Montevideo	La Paz	Flower
C. gloesporioides species complex	*C. theobromicola*	OL110	Manzanilla	Canelones	Las Brujas	Fruit
		OL112	Arbequina	Canelones	Las Brujas	Fruit
	C. alienum	OL98	Arbequina	Montevideo	Melilla	Fruit

For inoculum preparation, each isolate was grown on Potato Dextrose Agar (Oxoid Ltd., Hampshire, England) at 24 ºC under near UV-light with a 12-h photoperiod. At seven days of incubation, colony surface was flooded with 10 ml of sterile distilled

water (SDW) and scraped with a sterile Drigalski-spatel. The resulting spore suspension was filtered through layers of cheesecloth and the conidium concentration was adjusted to $1x10^6$ conidia mL^{-1} with a hemacytometer.

2.2. *Flower inoculation*

Flowers in the swollen bud and final differentiation stage (BBCH51 and BBCH55 respectively), were surface-disinfected by dipping 1 min in 1.0% NaClO solution and then rinsed three times with SDW. For beginning of flowering (BBCH61) stage, the surface-disinfection was not possible due the extremely sensitive of flower petals to NaClO. After air-dried, the flowers were dipped in the corresponding isolate conidial suspension for 30s, placed in transparent plastic trays containing moistened filter paper and incubated at 24 °C with 12-h photoperiod. A control treatment per cultivar and flower phenological stage inoculated with SDW was included. Three repetitions per cultivars, phenological stage and *Colletotrichum* isolate were implemented. Each repetition consisted of at least eight swollen buds for Stage 1, two panicles with at least 15 undeveloped flower buds each one for Stage 2, and two panicles with at least 10 open or semi-open flowers for Stage 3. Each experiment was performed according with a completely randomized design with factorial arrangement.

Anthracnose incidence was assessed periodically until the plant material was destroyed or until 100% incidence was achieved, 12 days for swollen bud, 6 days for final differentiation and 4 days for beginning of flowering stage. Incidence was calculated as the percentage of affected buds or flowers in the total number of buds or flowers evaluated. In each evaluation the initial symptoms and their evolution was registered as the number of necrotic buds or flowers and the presence of gelatinous mass of *Colletotrichum* conidia.

2.3. *Fruit inoculation*

Fruits at the Stage 1- green fruit and Stage 2- ripe fruit (BBCH80 and BBCH89 respectively) were surface-disinfected by dipping 3 min in 1.0% NaClO solution and then rinsed three times with SDW. After drying, the fruits were dipped in the corresponding isolate conidial suspension for 30s and placed into plug seedling trays, one fruit in each

hole. The plug seedling trays were enclosed in moistened transparent nylon bags and incubated at 24 °C with 12-h photoperiod. A control treatment per cultivar and fruit phenological stage inoculated with SDW was included. Four repetitions per cultivar, phenological stage and *Colletotrichum* isolate were used. Each repetition consisted of five fruits and every experiment was performed according to a completely randomized design with factorial arrangement.

Anthracnose severity was periodically assessed during 50 days for green fruit and 18 days for mature fruit. The 0-5 rating scale proposed by Moral et al. (2008) was used, where 0 = no visible lesions, 1 = visible lesions affecting <25% of the fruit surface, 2 = 25–49%, 3 = 50–74%, 4 = 75–100%, and 5 = soapy fruit (fruit completely covered with gelatinous mass of Colletotrichum spores). Additionally, the presence of gelatinous mass with *Colletotrichum* conidia was registered.

2.4. *Statistical analysis*

Flower anthracnose incidence values at the three-flower phenological stages of the four olive cultivars were plotted. A regression curve was fitted considering the significance of the regression and the coefficient of determination (R^2) based on average incidence of the 15 *Colletotrichum* isolates and evaluation moment. In addition, anthracnose incidence values were used to calculate the area under the disease progress curve (AUDPCi) for each *Colletotrichum* species according to the following formula:

$$AUDPCi = \sum_{i=1}^{n}[(I_{i+1}+I)/2]\ (t_i+1-t_i)$$

where I is the incidence (%) at i*th* observation, ti is the time (days) at the i*th* observation, and *n* is the total of number of observations.

The AUDPC data were analyzed for normality with the Shapiro-Wilk test, and for homogeneity with the Levene test. Then, they were subjected to ANOVA analysis with a factorial arrangement ($p \leq 0.05$), with olive cultivar and *Colletotrichum* species as factors. In case of significance, the treatments means were compared using Tukey's test ($p \leq 0.05$).

Fruit anthracnose severity values were used to calculate the McKinney's Index according to Moral et al. (2017) using the following formula:

$$McKinney's\ Index = \frac{\sum(n_i\ x\ i)}{5\ x\ N}\ \text{x}\ 100$$

where i represents the severity of symptoms (0–5), n_i is the number of fruits with the severity of i, and N is the total number of evaluated fruits.

The McKinney's Index values at the two-maturity stages and for the four olive cultivars were plotted. A regression model was adjusted for each *Colletotrichum* species considering the significance of the regression and the coefficient of determination (R^2). Anthracnose severity values were used to calculate the AUDPC for each *Colletotrichum* species using the same formula indicated above. The AUDPC data were analyzed for normality with Shapiro-Wilk test, and for homogeneity with the Levene test. The AUDPC was transformed to $\sqrt{\text{AUDPC}/100}$ when necessary to comply with normality assumptions. Then, the AUDPC data were subjected to ANOVA analysis with a factorial arrangement ($p \leq 0.05$), with olive cultivar and *Colletotrichum* species as factors. In case of significance, the treatments means were compared using by Tukey's test ($p \leq 0.05$).

Results were statically analyzed using InfoStat version 2016. (http://www.infostat.com.ar) and SigmaPlot version 12.0 (http://www.sigmaplot.co.uk) programs.

3. RESULTS

3.1.*Flowers infections*

Typical anthracnose symptoms and signs developed in flowers at the three phenological stages in the four cultivars inoculated with the *Colletotrichum* species. No symptoms were observed in control treatments (Figure 1 a-c). Initial symptoms consisted of a brownish coloration of the bud swollen or flower bud at the stage of final differentiation, and necrotic lesions on flower petals (Figure 1 a.2-c.2). Then, the affected organs quickly blighted and were covered with orange-salmon-colored gelatinous masses containing abundant *Colletotrichum* conidia (Figure 1 a.3-c.3). Additionally, an

accelerated detachment of buds swollen, flower buds and open flowers was observed in comparison with the control treatments, which remained attached for a longer period.

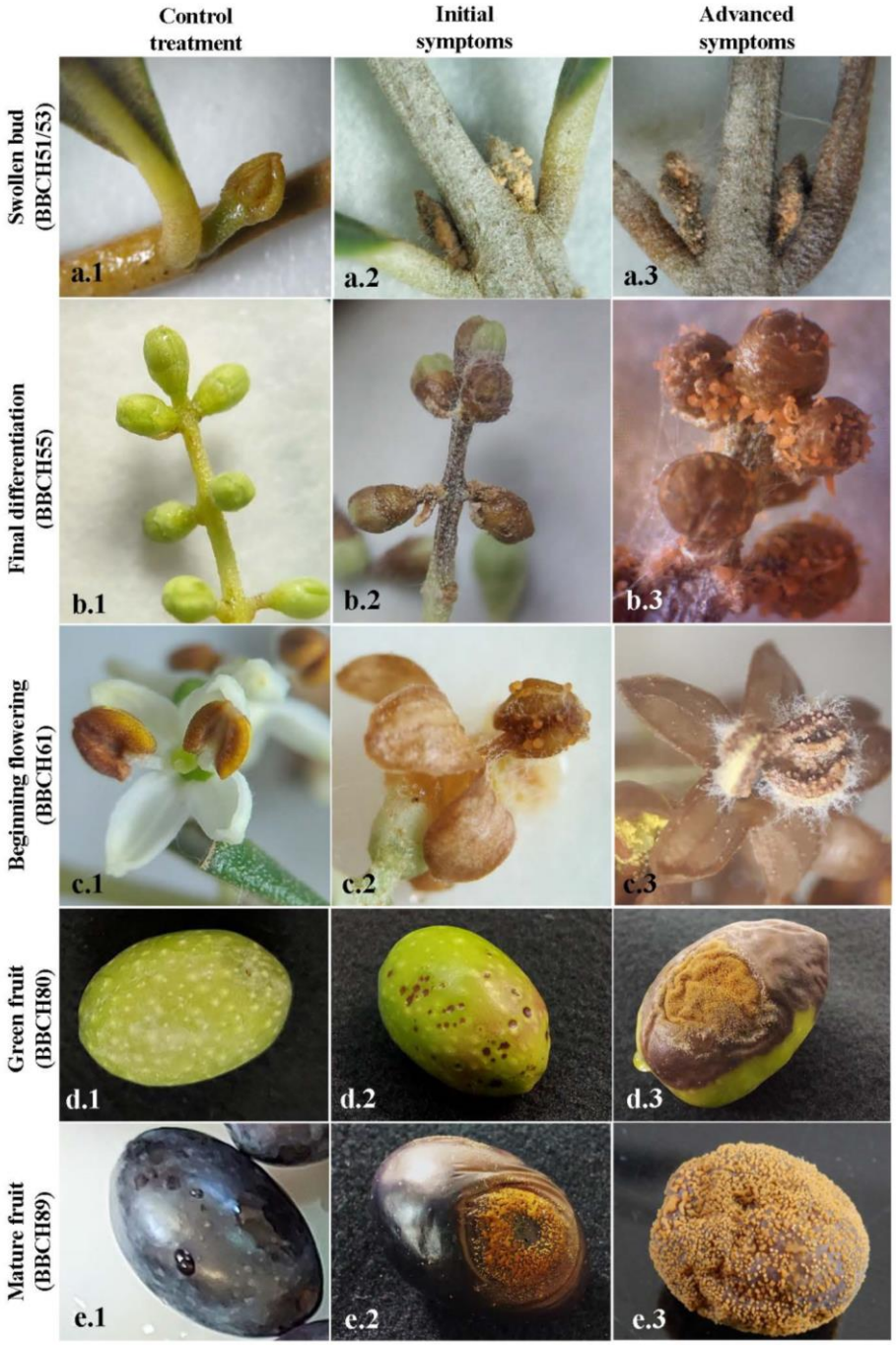

Fig. 1. Initial and advanced anthracnose symptoms developed on olive flower and fruits at different phenological stages inoculated with species of *Colletotrichum*, **a-** swollen buds (BBCH51/53) 7 and 12 days after inoculation, respectively, **b-** final differentiation (BBCH55), 3 and 6 days after inoculation, respectively, **c**-beginning of flowering (BBCH61), 2 and 4 days after inoculation, respectively, **d-** green fruit (BBCH80) 7 and 26 days after inoculation, respectively and **e-** mature fruit (BBCH89) 6 and 12 days after inoculation, respectively.

Almost all graphs of blossom blight incidence were fitted to an exponential curve type (Figure 2 and Tabale S1). For the AUDPC variable, a significant interaction was found between olive cultivars and *Colletotrichum* species inoculated in the three flowering phenological stages **(**Table 2). The lowest anthracnose incidence was observed at the swollen bud stage and no major differences were registered among cultivars. In this stage, the first symptoms appeared approximately between 4 and 7 days after inoculation, whereas the highest anthracnose incidence was recorded at 12 days and ranged between 20 and 40% (Figure 2).

At final differentiation stage, the first symptoms were observed at 3 days after inoculation, with the lowest average incidence in the Frantoio cultivar (2.0%) and the highest in the Arbequina cultivar (17%). Then, the incidence progressed quickly and 6 days after inoculation reached values between 59 and 100%, being Frantoio and Arbequina the least and most affected cultivar, respectively. Finally, at Stage 3- beginning flowering the highest anthracnose incidence was recorded. Two days after inoculation, the incidence values ranged from 8% in Frantoio to 50% in Picual, and 2 days later between 45% in Frantoio and 91% in Arbequina (Figure 2, Table 3).

Except *C. alienum* when was inoculated on Arbequina cultivar at bud swollen stage, all five *Colletotrichum* species were able to infect flowers at all three phenological stages. Nevertheless, in some specific combination species-cultivar-phenological stage, anthracnose symptoms were not visible until the second time of evaluation**.** For example, Coratina and Picual inoculated with all *Colletotrichum* species at swollen bud stage (Figure 2).

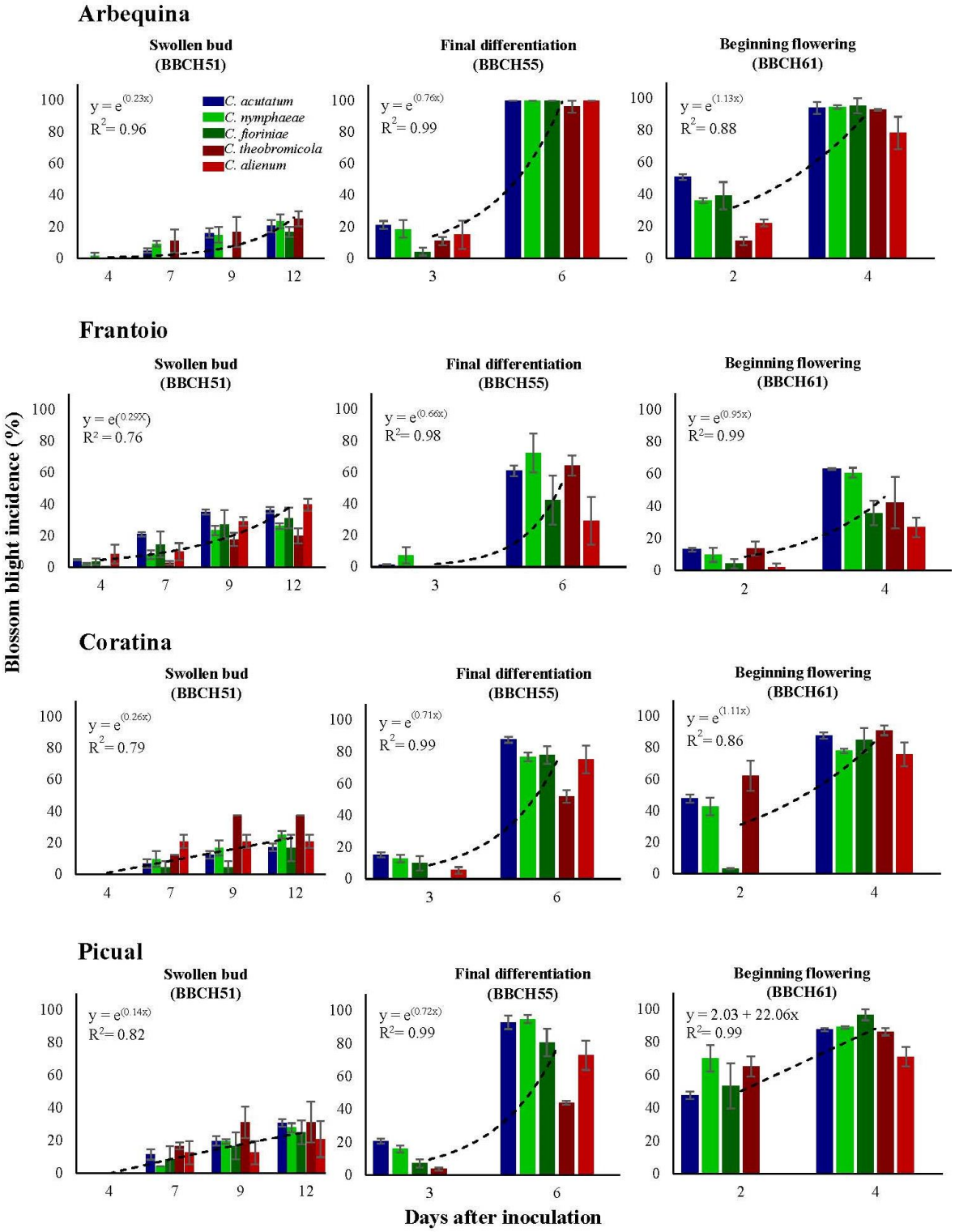

Fig. 2. Evolution of blossom blight incidence in flower of four olive cultivars inoculated with five *Colletotrichum* species at swollen buds (BBCH51/53), final differentiation (BBCH55) and beginning of flowering (BBCH61) phenological stages. The vertical bars indicate the standard error of the mean calculated based on the three replicates used. A trend curve was graphed based on the average incidence of each evaluation time. The R^2 and the estimation of the trend curve were performed using SigmaPlot version 12.0 software.

Table 2. Analysis of variance of Area Under Disease Progress Curve (AUDPC) data estimated based on anthracnose incidence and severity developed in flower and fruits, respectively, of four olive cultivars inoculated with five *Colletotrichum* species. The flowers were inoculated in three and fruit in two phenological stages.

Source	SS[1]	df	MS	F	p-value	CV
Swollen bud (BBCH51/53)						
Model	441.44	19	23.23	3.77	0.0003	42.61
Cultivar	118.23	3	39.41	6.4	0.0013	
Species	103.16	4	25.79	4.18	0.0068	
Cultivar x species	211.8	12	17.65	2.86	**0.0069**	
Error	228.01	37	6.16			
Total	669.45	56				
Final differentation (BBCH55)						
Model	4518.11	19	237.8	14.51	<0.0001	17.46
Cultivar	2425.35	3	808.45	49.31	<0.0001	
Species	1264.55	4	316014	19.28	<0.0001	
Cultivar x species	828.22	12	69.02	4.21	**0.0003**	
Error	655.76	40	16.39			
Total	5173.87	59				
Begining flowering (BBCH61)						
Model	15598.22	19	820.96	23.28	<0.0001	16.92
Cultivar	8356.56	3	2785.5	78.99	<0.0001	
Species	2926.24	4	731.56	20.75	<0.0001	
Cultivar x species	4315.43	12	356.62	10.2	**<0.0001**	
Error	1410.57	40	35.26			
Total	17008.79	59				
Green fruit (BBCH80)						
Model	2.1	19	0.11	138.49	<0.0001	5.34
Cultivar	1.08	3	0.36	451.37	<0.0001	
Species	0.84	4	0.21	261.74	<0.0001	
Cultivar x species	0.17	12	0.01	18.18	**<0.0001**	
Error	0.04	53	8.0E-0.4			
Total	2.14	72				
Mature fruit (BBCH89)						
Model	5114.24	19	269.17	8.51	<0.0001	15.98
Cultivar	924.51	3	308.17	9.74	<0.0001	
Species	1593.17	4	398.29	12.59	<0.0001	
Cultivar x species	2453.88	12	204.49	6.49	**<0.0001**	
Error	1866.42	59	31.63			
Total	6980.66	78				

[1]SS: sum of squares, df: degrees of freedom, MS: mean squares, F: teste F, CV: coefficient of variation

Table 3. Table 3. Area Under Disease Progress Curve (AUDPC) data estimated based on anthracnose incidence and severity developed in flower and fruits, respectively, of four olive cultivars inoculated with five Colletotrichum species. The flowers were inoculated in three and fruit in two phenological stages.

Cultivar	Specie	Phenological stage				
		Flowers			Fruit	
		Swollen bud (BBCH51/53)[1]	Final differentition (BBCH55)	Beginning flowering (BBCH61)	Green Fruit (BBCH80)	Mature Fruit (BBCH89)
Arbequina	*C. acutatum*	4.66 **abcd**[2]	35.62 **h**	48.93 **cde**	35.94 **ef**	41.44 **de**
	C. nymphaeae	5.35 **abcd**	34.29 **gh**	34.47 **bcd**	29.28 **de**	38.90 **cde**
	C. fioriniae	1.39 **ab**	27.09 **defgh**	43.40 **cde**	46.70 **gh**	44.38 **e**
	C. theobromicola	5.94 **abcd**	29.53 **efgh**	31.16 **bc**	67.78 **j**	44.30 **e**
	C. alienum	0.00 **a**	32.49 **fgh**	47.90 **cde**	36.26 **fg**	13.05 **a**
Frantoio	*C. acutatum*	11.94 **d**	15.83 **abcd**	22.23 **ab**	24.77 **cd**	43.24 **de**
	C. nymphaeae	6.85 **abcd**	21.82 **bcdefg**	20.13 **ab**	35.72 **ef**	37.72 **bcde**
	C. fioriniae	4.58 **abcd**	10.62 **ab**	11.06 **a**	23.60 **cd**	44.44 **e**
	C. theobromicola	4.55 **abcd**	16.31 **abcd**	17.44 **ab**	61.98 **ij**	37.72 **bcde**
	C. alienum	6.66 **abcd**	4.90 **a**	7.85 **a**	17.97 **cd**	26.5 **abc**
Coratina	*C. acutatum*	4.11 **abcd**	29.91 **efgh**	45.75 **cde**	30.04 **de**	32.13 **bcde**
	C. nymphaeae	5.75 **abcd**	25.59 **defgh**	40.91 **cde**	29.28 **de**	31.33 **bcde**
	C. fioriniae	2.55 **abc**	24.49 **cdefgh**	22.27 **ab**	20.04 **bc**	40.72 **cde**
	C. theobromicola	10.07 **cd**	13.00 **abc**	53.81 **e**	51.39 **hi**	40.59 **cde**
	C. alienum	7.52 **abcd**	21.58 **bcdef**	18.95 **ab**	16.96 **bc**	39.88 **cde**
Picual	*C. acutatum*	6.90 **abcd**	34.62 **h**	55.61 **e**	6.26 **a**	23.11 **ab**
	C. nymphaeae	5.60 **abcd**	31.72 **fgh**	56.98 **e**	11.95 **b**	31.35 **bcde**
	C. fioriniae	5.56 **abcd**	23.75 **cdefgh**	50.89 **de**	4.56 **a**	31.55 **bcde**
	C. theobromicola	9.26 **bcd**	12.84 **abc**	54.24 **e**	34.13 **ef**	31.35 **bcde**
	C. alienum	7.82 **abcd**	18.21 **bcde**	17.78 **ab**	5.64 **a**	23.22 **bcd**

[1] Phenological scale according to Sanz-Cortes et al., (2002)

[2] In each column, mean values followed by the same letter are not significantly different according to Tukey's HSD test at p=0,05

3.2.*Fruit infection*

Characteristic anthracnose symptoms and signs developed in fruits at the two phenological stages (green and ripe) of four cultivars inoculated and also in some fruit not inoculated with *Colletotrichum* species (Figure 1 d-e). Symptoms developed in not inoculated fruits can be attributable to the natural latent infection of *Colletotrichum*. On green fruit stage, the initial symptoms consisted of small, depressed, 1-2 mm necrotic lesions scattered throughout the fruit (Figure 1 d.2). Then, progressed into depressed brown lesions that were quickly covered with orange gelatinous masses of *Colletotrichum*

conidia, known as soapy fruit (Figure 1 d.3). On ripe fruit, symptoms developed more quickly and consisted of the typical soapy fruit described above (Figure 1 e2-3).

Anthracnose fruit severity in both phenological stages, green and ripe fruit, were fitted to a curve of sigmoidal type (Figure 3 and Table S1). For the AUDPC variable, a significant interaction was found between olive cultivars and *Colletotrichum* species inoculated at both green and ripe fruit phenological stages (Table 2). On green fruit, the first symptoms were observed between 5 and 14 days after inoculation. In this stage, Arbequina and Frantoio reached severity values close to 50% 30 days after inoculation and Coratina 35 days after inoculation, whereas Picual reached 50% of severity 50 days after inoculation (Figure 3). At mature fruit stage, the first symptoms were observed four days after inoculation. Then, the anthracnose disease evolution occurred more quickly compared to green fruit stage. Considering all cultivars and *Colletotrichum* species, the severity value was in average 77% and 92% after 12 and 18 days from inoculation, respectively (Figure 3).

The five *Colletotrichum* species were able to infect fruits at both phenological stages in the four olive cultivars. Nevertheless, *C. theobromicola* caused significantly the highest AUDPC values at green fruit stage (Table 3). The green fruit inoculated with this species, substantially developed symptoms earlier (5 days after inoculation) in comparison with the other *Colletotrichum* species (14 days after inoculation) and reached almost 100% of severity about 15 days earlier than those inoculated with the other *Colletotrichum* spp. (Figure 3). In ripe fruit, the behavior among the *Colletotrichum* species was similar. Anthracnose index values close to 100% were reached at about day 15 after inoculation, except for Arbequina and Frantoio inoculated with *C. alienum* in which the severity index was close to 50%.

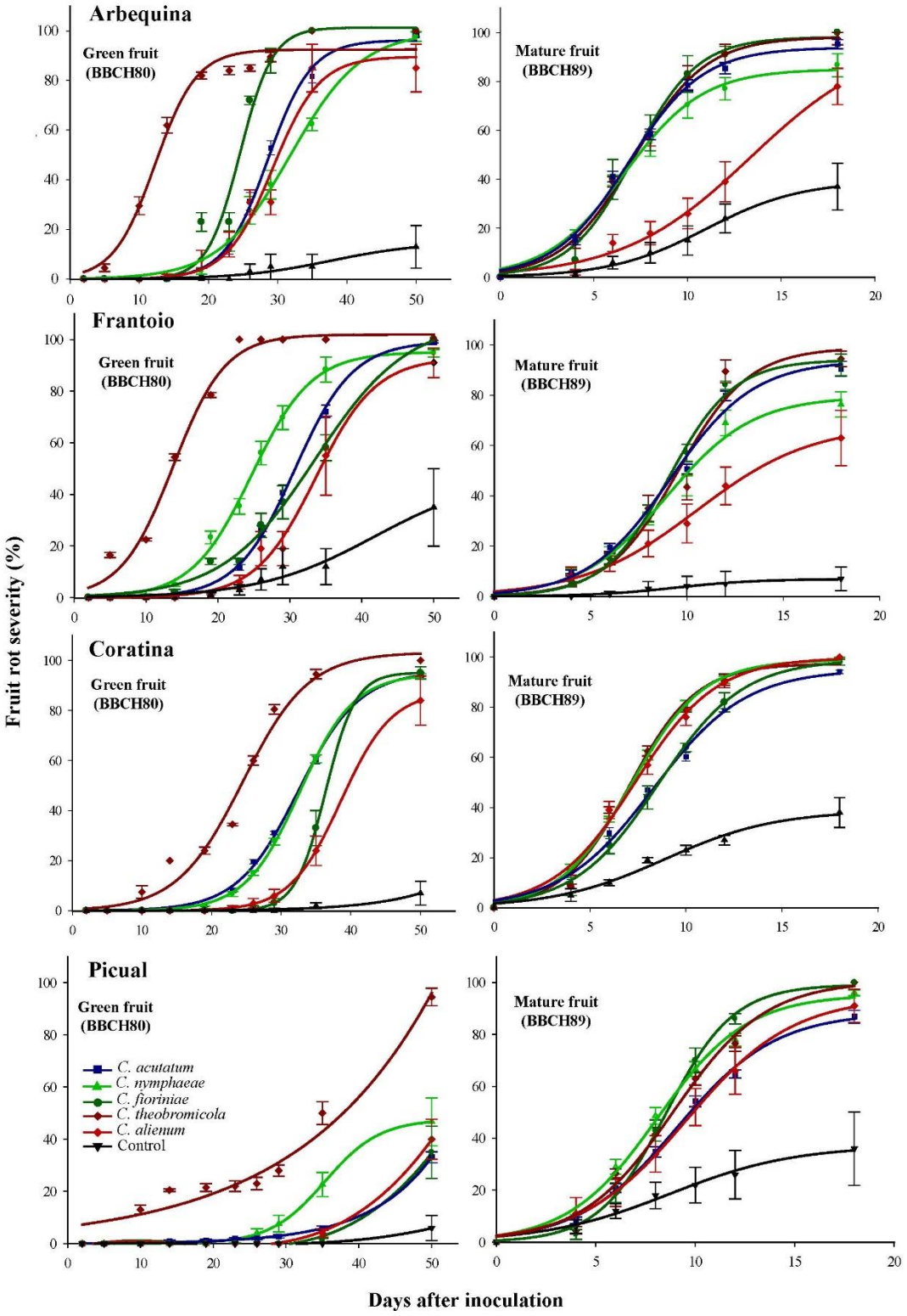

Fig. 3 Evolution of anthracnose severity index in fruit of four olive cultivars inoculated with five *Colletotrichum* species at green (BBCH80) and ripe fruit (BBCH89) phenological stage. Disease severity values were used to calculate the McKinney index. The incidence registered in the control treatments can be attributable to the latent infection of *Colletotrichum* spp. expression. The vertical bars correspond to the standard error of the mean calculated based on the four replicates used. The R2 and the estimation of the trend curve were performed using SigmaPlot version 12.0 software.

4. DISCUSSION

In the present work we study the anthracnose susceptibility during flower development and fruit ripening in the main olive cultivars produced in Uruguay, Arbequina, Frantoio, Coratina and Picual. That study was conducted by artificial inoculations of detached olive panicles and fruits against five *Colletotrichum* species. Overall, our results indicated that the four cultivars studied are susceptible to *Colletotrichum* at all phenological stages evaluated, although differences in phenological stages, olive cultivars, and *Colletotrichum* species were registered.

In the present study we observed that the swollen bud stage is susceptible to be infected by *Colletotrichum* species. Although the susceptibility of this phenological stage to anthracnose was low, we demonstrated that *Colletotrichum* can infect flowers from early stages during flower development. In later stages of flower development, susceptibility increases and symptoms and signs progress more quickly. Symptoms include brown coloration of the flower organs that progresses to the collapse of the entire inflorescences, known as blossom blight. Then, affected organs are quickly covered by typical orange-salmon-colored gelatinous masses, corresponding to *Colletotrichum* conidia (Iliadi et al. 2018; Moreira et al. 2021; Sergeeva et al. 2008).

Our results are in accordance with Kolainis et al. (2020) who observed that in Koroneiki and Kalamon cultivars, the first anthracnose symptoms appeared two days after inoculation of detached flowers at beginning of flowering stage. Conversely, Moral et al. (2009) inoculated flowers of Arbequina, Hojiblanca and Picual cultivars at the same phenological stage, but the first symptoms were visible five days after inoculation. Possibly, these differences could be explained because of Moral et al. (2009) utilized attached flowers whereas in our experiment and those developed by Kolainis et al. (2020) detached flowers were used.

Regarding the olive cultivars evaluated, some differences were observed in the susceptibility to anthracnose at different flowering stages. At swollen bud stage no major variations were observed, but at final differentiation and beginning of flowering stages, Frantoio was the least susceptible cultivar and Arbequina the most susceptible cultivar to *Colletotrichum*, whereas Coratina and Picual presented an intermediate behavior. Similar

results were found by Moral et al. (2009) who found that Arbequina was more susceptible than Hojiblanca and Picual when inoculated at the beginning of flowering.

In recent research, we demonstrated that the species *C. acutatum* s.s., *C. nymphaeae*, *C. fioriniae*, *C. theobromicola* and *C. alienum* cause typical blossom blight at beginning of the flowering stage (Moreira et al. 2021). In the present study we observed that, although the incubation period can be variable, isolates of these five *Colletotrichum* species can infect olive from early stages of flower development.

The results recorded in our study with respect to the behavior of inoculated green and ripe fruit are generally in agreement with previous research. Olive fruits can be infected by *Colletotrichum* at different stages during fruit ripening, but the susceptibility substantially increases with the ripens (Chattaoui et al. 2016; Moral et al. 2008; 2009; 2017; Sergeeva 2014). Moreover, in this study we observed differences in symptoms development. While on ripe fruit the typical soapy olive fruit was observed from the beginning, on green fruit the initial symptoms consist of small and depressed necrotic lesions that later progress into the typical soapy fruit. In addition, at green stage the first symptoms were visible between five and seven days after inoculation, whereas on ripe fruit they were observed between one and four days earlier. Similar results were obtained by Moral et al. (2008), who observed that first symptoms appear at seven and four days on detached green and ripe fruit inoculated with *Colletotrichum*, respectively.

Regarding the susceptibility of the different olive cultivars, at green fruit stage Picual was the least susceptible and Arbequina and Frantoio the most susceptible cultivars to *Colletotrichum* spp., while Coratina developed an intermediate behavior. These results are in accordance with other studies in which Arbequina appears as susceptible to moderately susceptible, Coratina as moderately susceptible (Andres 1991; Moral and Trapero 2009; Bartolini and Cerreti 2013) and Picual as moderately resistant or resistant to anthracnose (Moral et al. 2017; Moral and Trapero 2009; Talhinhas et al. 2015).

On mature fruit, the four olive cultivars developed symptoms in a similar way, being all highly susceptible to anthracnose. This finding presents some discrepancies with other studies in which Frantoio was found highly resistant to anthracnose in Spain (Moral et al. 2008; 2017; Moral and Trapero 2009). Nevertheless, in accordance with our result,

Frantoio showed high susceptibility to anthracnose in Argentina (Andres 1991) and Italy (Loprieno and Tenerini 1960).

Although the differences in anthracnose susceptibility found among cultivars on green fruit stage were not recorded on ripe fruit stage, this was not surprising. Moral and Trapero (2009) mentioned that green fruits are more resistant to be infected by *Colletotrichum* than ripe fruit, probably because of the higher concentration of phenolic compounds in comparison with ripe fruits. However, when the maturity is reached, all cultivars can become diseased and show a complete rot regardless of their susceptibility (Moral et al. 2008).

Colletotrichum theobromicola differed substantially from the other *Colletotrichum* species inoculated in this study, being the most aggressive at green fruit stage. This finding was surprising since this species, together with *C. alienum*, was isolated in a very low proportion from olives with typical anthracnose symptoms in Uruguay (Moreira et al. 2021). In Australia, inoculated detached fruit on green stage was more affected by *C. theobromicola* and *C. gloesporioides* s.s than *C. aenigm*a, *C. ciggaro*, *C. queenslandicum*, *C. siamense* and *C. karstii* (Schena et al. 2014). Regarding to *C. theobromicola* world distribution, it has been reported affecting olive in Argentina, Australia, and Uruguay (Schena et al. 2014; Lima et al. 2020; Moreira et al. 2021), but not in countries of the Mediterranean basin, where most of the olives are cultivated. Schena et al. (2014) mentioned that new diseases are expected to emerge as consequence of climate change and suggested that *C. theobromicola* could play an important role in olive anthracnose disease.

On ripe fruit, the behavior of the five *Colletotrichum* species was similar, except for *C. alienum* which was less aggressive when it was inoculated on Arbequina and Frantoio cultivars. In Portugal Talhinhas et al. (2015), found that *C. acutatum* s.s. and *C. nymphaeae* were more aggressive than *C. gloeosporioides* s.s. and *C. rhombiforme* on ripe fruit and in Italy Schena et al. (2017), observed that *C. acutatum* s.s. was more aggressive than *C. godetiae* in that organ. In our work *C. acutatum* s.s., *C. nymphaeae* and *C. fioriniae* seem to be similar in aggressiveness.

In summary, to the best of our knowledge, this is the first study in which anthracnose susceptibility is evaluated at different flower development stages. Based on

our results, we concluded that the risk of anthracnose occurrence starts at early stages of flower development. This finding would allow us to take more appropriate management decisions. For example, the most opportune time to initiate preventive fungicide applications should be at early stages of flower development to help minimize yield losses caused by olive anthracnose. Regards to cultivars, Frantoio appears as moderately susceptible whereas Picual, Coratina and Arbequina as susceptible cultivars during flowering. However, our results indicate that at green fruit stage, Frantoio and Arbequina are the most susceptible cultivars, Coratina has an intermediate behavior and Picual appear as the less susceptible cultivar to anthracnose. In mature fruit no differences were found among cultivars. Unfortunately, in this study we confirmed that Arbequina, the main olive cultivar produced in Uruguay, is one of the most susceptible to *Colletotrichum* during both flowering and fruit ripening. Future research should focus on improving anthracnose management strategies to minimize the impact of this disease during flower development and fruit maturity, especially in those olive cultivars more susceptible.

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6. SUPPLEMENTARY INFORMATION

Table S1. Fitted models based on anthracnose severity evolution as a function of time, cultivar and *Colletotrichum* species inoculated on green and ripe olive fruit.

Cultivar/specie	Phenological stage								
	Green Fruit (BBCH80)					Mature Fruit (BBCH89)			
Arbequina	a	b	x0	c	R^2	a	b	x0	R^2
C. acutatum s.s.	96.26[1] <0.0001	3.14 <0.0001	28.58 <0.0001	-	0.99	93.86[1] <0.0001	1.97 0.0007	6.84 <0.0001	0.99
C. nymphaeae	99.87[1] <0.0001	5.17 <0.0001	31.84 <0.0001	-	0.99	85.05[1] <0.0001	2.09 0.0008	6.63 <0.0001	0.99
C. fioriniae	101.35[1] <0.0001	2.35 0.0031	24.56 <0.0001	-	0.97	98.15[1] <0.0001	1.69 0.003	7.11 <0.0001	0.98
C. theobromicola	92.54[1] <0.0001	3.00 0.0009	12.29 <0.0001	-	0.98	98.14[1] <0.0001	1.90 0.0005	7.13 <0.0001	0.99
C. alienum	89.67[1] <0.0001	3.35 0.0031	29.31 <0.0001	-	0.97	98.02[1] 0.0019	3.42 0.0043	13.36 0.0005	0.99
Control	14.90[1] 0.002	6.61 0.0226	37.67 <0.0001	-	0.95	39.35 <0.0001	2.54 0.0006	10.96 <0.0001	0.99
Frantoio									
C. acutatum s.s.	99.26[1] <0.0001	4.04 <0.0001	30.68 <0.0001	-	0.99	93.82[1] <0.0001	2.16 0.0018	9.15 <0.0001	0.98
C. nymphaeae	95.15[1] <0.0001	4.16 <0.0001	24.69 <0.0001	-	0.99	79.46[1] <0.0001	2.28 0.0029	8.94 <0.0001	0.99
C. fioriniae	107.46[1] <0.0001	6.46 <0.0001	33.55 <0.0001	-	0.99	94.08[1] <0.0001	1.69 0.003	9.04 <0.0001	0.99
C. theobromicola	101.91[1] <0.0001	3.62 0.0001	13.74 <0.0001	-	0.98	98.94[1] 0.0005	1.84 0.0202	9.51 0.0001	0.96
C. alienum	92.94[1] <0.0001	4.19 <0.0001	33.59 <0.0001	-	0.99	68.35[1] <0.0001	2.98 0.0005	10.51 <0.0001	0.99
Control	47.38[1] 0.0133	8.03 0.0095	41.78 <0.0001	-	0.97	9.36[1] 0.0002	2.16 0.0072	9.36 <0.0001	0.98
Coratina									
C. acutatum s.s.	95.65[1] <0.0001	4.38 <0.0001	32.44 <0.0001	-	0.99	95.09[1] <0.0001	2.39 0.001	8.29 <0.0001	0.99
C. nymphaeae	94.60[1] <0.0001	3.90 <0.0001	32.63 <0.0001	-	0.99	98.92[1] <0.0001	1.86 <0.0001	7.15 <0.0001	0.99
C. fioriniae	95.17[1] <0.0001	2.16 <0.0001	36.37 <0.0001	-	1	99.01[1] <0.0001	2.18 <0.0001	8.56 <0.0001	0.99
C. theobromicola	103.22[1] <0.0001	4.72 <0.0003	24.48 <0.0001	-	0.98	97.38[1] <0.0001	1.74 0.002	7.05 <0.0001	0.99
C. alienum	87.71[1] <0.0001	3.66 <0.0001	38.57 <0.0001	-	1	99.85[1] <0.0001	2.118 0.0005	7.33 <0.0001	0.99
Control	-0.17[2] 0.0013	0.0058 <0.0001	0.75 0.04	-	0.97	38.80[1] 0.0001	2.87 0.003	8.89 0.0001	0.98

Picual

C. acutatum s.s.	-0.69[2] 0.0051	0.02 0.0001	3.34 0.04	-	0.96	88.36[1] <0.0001	2.48 0.0002	9.08 <0.0001	0.99
C. nymphaeae	47.61[1] <0.0001	3.77 <0.0001	35.33 <0.0001	-	0.99	95.33[1] <0.0001	2.24 0.0005	8.08 <0.0001	0.99
C. fioriniae	0.0002[3] 0.0123	0.24 <0.0001	-	-	0.99	99.18[1] <0.0001	1.69 0.0001	8.52 <0.0001	0.99
C. theobromicola	6.57[3] <0.0006	0.053 <0.0001	-	-	0.96	100.53[1] <0.0001	2.31 0.002	8.89 <0.0001	0.99
C. alienum	0.11[4] <0.0006	-	-	-	0.98	94.44[1] <0.0001	2.66 <0.0001	9.57 <0.0001	0.99
Control	0.15[5] <0.027	-0.01 0.0049	-0.45 0.04	0.0002 0.0006	0.98	37.16[1] 0.0001	3.13 0.001	8.71 <0.0001	0.98

[1] $y = \frac{a}{1+e^{-((x-xo)/b)}}$

[2] $y = x_0+ax+bx^2$

[3] $y = ae^{(bx)}$

[4] $y = ae^{(ax)}$

[5] $y = x_0+ax+bx^2+cx^3$

ARTÍCULO 4

Assessment of fungicides efficacy against *C. acutatum* s.s., *C. nymphaeae*, and *C. fioriniae* causing olive anthracnose in Uruguay

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A enviar a: **Crop Protection**

Assessment of fungicides efficacy against *C. acutatum* s.s., *C. nymphaeae*, and *C. fioriniae* causing olive anthracnose in Uruguay

ABSTRACT

Olive anthracnose, caused by *Colletotrichu*m, is the most destructive olive disease worldwide. In this study, the in vitro effect of 14 fungicides from six chemical groups on the mycelial growth inhibition of *C. acutatum* s.s., *C. nymphaeae* and *C. fioriniae* isolates was evaluated. Tebuconazole, pyraclostrobin and ziram significantly inhibited the mycelial growth of *Colletotrichum* spp. with EC_{50} values between 0.02 to 0.15 mg a.i L, 0.16 to 0.43 mg a.i L and 9.29 and 26.23 mg a.i L^{-1}, respectively. These three fungicides were then evaluated on detached olive flowers of Arbequina, Frantoio, Coratina and Picual cultivars inoculated with the three *Colletotrichum* species, while copper oxychloride, cuprous oxide and copper calcium sulfate were evaluated on detached fruits from the same cultivars. The three fungicides applied over flower delayed 24 to 48 h the initial symptoms and reduced the anthracnose incidence by more than 60%, being ziram and pyraclostrobin the most effective. In contrast, copper-based fungicides showed low efficacy in reducing the anthracnose severity in fruit. Only the three copper-based fungicides in Picual and copper calcium sulfate in Frantoio were able to reduce between 24 and 33% the anthracnose severity. This work presents the potential of some fungicides to be used for the control of olive anthracnose in an integrated management strategy.

Keyword: soap fruit, blossom blight, inhibition mycelial growth, chemical control, *Olea europaea*

1. INTRODUCTION

Olive anthracnose, also known as soap fruit, is the most destructive olive disease worldwide (Sergeeva et al. 2008; Cacciola et al., 2012, Talhinhas et al., 2018; Moral et al., 2021; Filoda et al., 2021). In humid olive-growing areas as Uruguay, severe epidemic outbreaks occur causing devastating economic damages due yield losses and the low quality of oils produced with infected fruits (Moral et al., 2009; 2014; Leoni et al., 2018; Moreira et al., 2021).

Fruit rot is the main symptom of anthracnose and consists of circular, depressed lesions covered by orange gelatinous masses of conidia. Fruits are infected at any stage of their development, but the maximum susceptibility occurs at the ripening stage when the color changes from green to black (Moral et al., 2009; 2014). Also, from early flowering stages, all parts of the flower can be infected lending blossom blight (Sergeeva et al., 2008; Moral et al., 2009; Iliadi et al. 2018; Filoda et al., 2021; Moreira et al., 2021). Other symptoms such as chlorosis, leaf necrosis, defoliation, and regressive branch dieback have been associated with this disease (Moral et al., 2009; Cacciola et al., 2012; Sergeeva, 2014). Blossom blight and fruit rot are the most frequent and destructive symptoms affecting olive production in Uruguay (Moreira et al., 2021).

Olive anthracnose is caused by 18 species belonging to *Colletotrichum* genus which are distributed in three species complexes, *C. acutatum*, *C. boninense* and *C. gloeosporioides* (Talhinhas et al. 2018; Moral et al, 2021; Moreira et al., 2021; Riolo et al., 2022). In Uruguay *C. acutatum* species complex was recently found as the predominating complex causing olive anthracnose with *C. acutatum* s.s. as the most important species followed by *C. nymphaeae* and *C. fiorinie* (Moreira et al., 2021).

The management of this disease is based on spraying fungicides combined with cultural practices. The most commonly used fungicides are copper-based applied during autumn in a preventive strategy to protect the fruits. Field applications of bordeaux mixture or copper oxychloride during the fall have demonstrated to be effective in controlling olive anthracnose (Martelli and Piglionica, 1961; Pennisi et al., 1993; Cacciola et al., 2012). Nevertheless, copper-based fungicides help control disease mainly under low disease pressure, low conducive climate and low inoculum level. Also,

Colletotrichum spp. has been reported as tolerant to this fungicide chemical group (Cacciola, et al., 2012; Roca et al., 2019) reducing its efficiency.

Fungicides belonging to other chemical groups such as the systemics strobilurins or triazoles have proven to be efficient in controlling olive anthracnose. For example, the strobilurins trifloxystrobin (Moral et al., 2014) and azoxystrobin applied prior to flowering and at early fruit set (Sergeeva, 2011; Talhinhas et al., 2018) showed good performance to control anthracnose. Moral et al. (2018) found that tebuconazole and trifloxystrobin were effective in controlling *C. godetiae* on inoculated detached red-purple fruit of Arbequina cultivar. In addition, contact fungicides of dithiocarbamate, guanidine or phthalimide chemical groups were evaluated to control *Colletotrichum* affecting different fruit crops with variable results (Freeman et al., 1997; Gao et al., 2017; Moral et al., 2018). Unfortunately, the use of that organic fungicides after fruit set is unrecommended because most of them are liposoluble and their residues have been detected in oil (Moral and Trapero, 2009; Moral et al., 2014; 2018).

In addition, some formulated mixtures of cupric with other fungicides have also proven to be efficient. Moral et al. (2018) found that a high efficacy for anthracnose control was observed when the mixture of copper hydroxide with folpet was applied on detached green-yellowish olive fruits of Arbequina cultivar inoculated with *C. godetiae.* In the same study, the mixture of copper hydroxide with folpet applied on detached ripe (black) olive fruits of Hojiblanca cultivar showed a significant reduction in the severity of symptoms. Nigro et al. (2018) observed that the mixtures of tebuconazole with trifloxystrobin applied in pre-flowering followed by Mancozeb applied at veraison, reduced the incidence of anthracnose on olive drupes.

Differences in fungicide sensitivity have been found between *Colletotrichum* species complexes, as well as between species within the same complex affecting olive or other fruit crops such as apple, peach, and strawberry (Chen et al., 2016; Munir et al., 2016; Zhang et al., 2020; Schoeneberg and Hu 2022). For example, olive strains of *C. nymphaeae* seem to be more sensitive to copper sulfate than those of *C. godetieae*, while *C. godetiae* strains were more sensitive to kresoxim-methyl than *C. nymphaeae* strains (Moral et al., 2018). Differences have also been observed between isolates of different morphotypes—within the *C. acutatum* species complex to kresoxim-methyl, pyraclostrobin, trifloxystrobin, difenoconazole and tebuconazole (Kolainis et al., 2020).

In Uruguay, the efficacy of copper oxychloride, copper sulfate and cuprous oxide in reducing mycelial growth of *Colletotrichum* spp. has been previously evaluated in vitro. In that study, differences in mycelial growth inhibition of *C. acutatum* and *C. gloesporioides* species complex strains were recorded (Montelongo et al., 2013). Nevertheless, no studies were established to verify the sensitivity of *Colletotrichum* spp. to these fungicides under field conditions.

To develop an efficient strategy for olive anthracnose management in Uruguay, it is crucial to know the sensitivity to fungicides of the *Colletotrichum* spp. present in the region, as well as the appropriate timing for their application.. For this purpose, the objectives of this study were: i) to evaluate the in vitro efficacy of 14 fungicides, belonging to six chemical groups, to inhibit the mycelial growth of *C. acutatum* s.s., *C. nymphaeae* and *C. fioriniae* species causing olive anthracnose in Uruguay, ii) to determine the protectant efficacy of the three best fungicides, according to the in vitro assay, applied on detached olive flower at initial-final differentiation stage, and iii) to determine the protectant efficacy of three copper-based fungicides applied on detached olive fruits at veraison stage. Trials were conducted on Arbequina, Coratina, Frantoio and Picual cultivars employing isolates of the three *Colletotrichum* species.

2. MATERIALS AND METHODS

2.1.*Colletotrichum isolates and fungicides*

Twenty *Colletotrichum* monosporic strains belonging to *C. acutatum* s.s., (15), *C. nymphaeae* (4), and *C. fioriniae* (1) isolated from olive anthracnose symptoms in Uruguay, were used in this study (Table 1). The isolates were obtained from the culture collection identified and deposited at the Department of Plant Protection, Facultad de Agronomía, Universidad de la República, Uruguay (Moreira et al., 2021).

Commercial formulations of 14 fungicides belonging to six chemical groups (Table 2) were selected to evaluate their capability to inhibit mycelial growth of the 20 *Colletotrichum* isolates selected.

2.2. *Mycelial growth inhibition*

Stock solutions of each fungicide were prepared in sterile distilled water (SDW). Subsequently, appropriate volumes of each fungicide were added to sterilized potato dextrose agar (PDA) at 45°C. Final concentrations of 0.1, 1, 10 and 100 μgL^{-1} of each active ingredient (a.i.) were obtained, except for copper-based fungicides where final concentrations used were 100, 250, 500 and 1000 $\mu g\ mL^{-1}$ of metallic copper.

Table 1. Uruguayan *Colletrotrichum* spp. isolates obtained from olive and used in this study.

Fungal specie	Isolate	Geographical origin Departament/ Area	Olive cultivar	Isolation organ
C. acutatum s.s.	OL1	Rocha/ 19 de Abril	Arbequina	Flower
	OL5	Rocha/ 19 de Abril	Arbequina	Flower
	OL10	Rocha/ Velázquez	Arbequina	Flower
	OL16	Rocha/ 19 de Abril	Coratina	Leaf
	OL18	Maldonado/ Garzón	Arbequina	Flower
	OL20	Maldonado/ Garzón	Picual	Flower
	OL24	Montevideo/ La Paz	Arbequina	Flower
	OL31	Montevideo/ Melilla	Pandolina	Flower
	OL34*	Montevideo/ Melilla	Arbequina	Flower
	OL42	Treinta y Tres/ Mendizábal	Arbequina	Flower
	OL45	Treinta y Tres/ Mendizábal	Coratina	Flower
	OL47	Colonia/ Morrito Tarariras	Coratina	Flower
	OL49*	Colonia/ Astilleros	Arbosana	Flower
	OL51*	Rocha/ 19 de Abril	Arbequina	Leaf
	OL53	Rocha/ 19 de Abril	Arbequina	Branch
C. nymphaceae	OL9*	Rocha/ Velázquez	Arbequina	Flower
	OL19*	Maldonado/ Garzón	Arbequina	Flower
	OL36	Montevideo/ Melilla	Arbequina	Flower
	OL43	Treinta y Tres/ Mendizábal	Coratina	Flower
C. fiorinae	OL23*	Montevideo/ La Paz	Arbequina	Flower

* Isolates selected for trials on detached flower and fruits of olive

* Isolates selected for trials on detached flower and fruits of olive

Table 2. Fungicides selected for in vitro sensitivity testing against olive *Colletotrichum* spp.

Chimical Group	Active ingredient	Trade name	Manufacturer	Formulation[1]
Triazoles	Difenoconazole	Escozate	Tafirel	250 g L^{-1} EC
	Tebuconazole	Calypso 430	Saudu	430g L^{-1} SC
	Propiconazole	Bumper 25	Lanafil S.A.	23% 250 g L^{-1} EC
Strobilurins	Azoxistrobin	Quadris	Syngenta	22,81% 250 g L^{-1} SC
	Trifloxystrobin	Flint 50	Bayer	50% WG
	Kresoxym-methyl	Squeeze	Cibeles	44%/ 500 g L^{-1} SC
	Pyraclostrobin	Tapyr	Proquimur	25%- 250 g L^{-1} EC
Ditiocarbamates	Mancozeb	Mancozate	Tafirel	80% WP
	Ziram	Ziram	Tafirel	80% WG
Guanidines	Dodine	Relampago	Saudu	500 L^{-1} SC
Phthalimides	Captan	Captan 80	Agroregional	80% WG
Copper	Copper oxychloride	Oxicloruro de cobre	Agroregional	85% (Cu 50%) WP
	Cuprous oxide	Cobre Nordox	Lanafil S.A.	86% (Cu 75%) WG
	Copper calcium sulfate	Caldo Bordales	Fanaproqui	CuSO4 neut. c/ Cal 67,8% (Cu 20%) WP

[1]SC: Suspension concentrate, EC: emulsifiable concentrate, WG: Water dispersible granule, WP: Wettable powder.

Mycelial agar plugs of 5 mm in diameter obtained from the margins of *Colletotrichum* colonies with 7-day-old actively growing, were transferred to the center of plates amended with fungicide. Three replicates per isolate, fungicide and concentration were used. PDA plates amended with sterile water instead of fungicide solution were used as controls. Plates were incubated at 25 °C in the dark. After seven days, two perpendicular diameters of each colony were measured and averaged using an electronic digital caliper (KAMASA® Professional, model KM-447).

Percentage of mycelial growth inhibition was estimated for each isolate, fungicide and concentration dividing the mean mycelial growth on fungicide-amended PDA by the mean mycelial growth on control plates, multiplied by 100. These values were transformed into probits and plotted against $\log_{10}$ values of the fungicide concentration. Subsequently, the effective concentration values that inhibited 50% of mycelial growth (EC_{50} values) were determined from the adjusted probit regression analysis.

2.3. *Efficacy of fungicides on detached flower*

Based on mycelial growth inhibition results, the fungicides pyraclostrobin, tebuconazole and ziram were selected to evaluate their efficacy in controlling olive anthracnose on detached flowers. A total of six isolates belonging to *C. acutatum* s.s., (3) *C. nymphaeae* (2), and *C. fioriniae* (1) were selected for this study (Table 1).

Olive flowers apparently healthy of Arbequina, Coratina, Frantoio and Picual cultivars were collected from commercial orchards at initial-final differentiation stage (BBCH54-55) according to scale of Sanz-Cortes et al. (2002). Collected flowers were stored in nylon bags and maintained in coolers for their preservation until to be processed in the laboratory.

Fungicides suspensions of pyraclostrobin (0.4 ml $a.i.l^{-1}$), tebuconazole (0.3 ml $a.i.l^{-1}$) and ziram (3 g $a.i.l^{-1}$) were prepared in SDW according to label recommendations. Immediately, the olive flowers were sprayed with the fungicides suspensions until runoff using a hand sprayer. Flowers were maintained at room temperature for 24h to air-dried and then sprayed with the conidial suspension ($1x10^6$ $conidia.ml^{-1}$) of the corresponding isolate until runoff. Immediately, flowers were placed in transparent plastic trays containing sterile filter paper moistened with SDW and incubated at 24ºC with 12h photoperiod for seven days.

Three repetitions were used per cultivar, fungicide and isolate combination. Each repetition consists in one tray containing two flower panicles with at least 15 flower buds in each one. Three trays containing flower panicles, sprayed with SDW and subsequently inoculated with the conidial suspension were used as control. Anthracnose incidence was daily evaluated registering symptoms and/or signs of *Colletotrichum* in each flower.

Incidence data for each *Colletotrichum* species were averaged within each cultivar and fungicide combination. These values were plotted and a regression model was adjusted for each fungicide into each cultivar considering the significance of the regression and the coefficient of determination (R^2) in SigmaPlot version 12.0 (http://www.sigmaplot.co.uk) program. In addition, incidence values were used to calculate the area under the disease progress curve (AUDPCi) according to the following formula:

$$AUDPCi = \sum_{i=1}^{n}[(I_{i+1}+I)/2]\ (t_i+1-t_i)$$

where I is the incidence (%) at i*th* observation, ti is the time (days) at the i*th o*bservation, and *n* is the total number of observations.

The normality of the AUDPC data was analyzed with the Shapiro-Wilk test, and the homogeneity with the Levene test. The AUDPCi was transformed to √y to conform to normality assumptions. The values were then subjected to ANOVA analysis with a factorial arrangement ($p \leq 0.05$), with olive cultivar, fungicide and *Colletotrichum* species as factors. Means of the treatments were compared using Duncan test ($p \leq 0.05$). Data tests were analyzed using InfoStat version 2016 (http://www.infostat.com.ar) program.

2.4. *Efficacy of copper-based fungicides on detached fruit*

The three copper-based fungicides were evaluated to determine their efficacy in controlling olive anthracnose on detached fruit (Table 2) using the same isolates that on detached flower essay (Table 1). Olive fruit apparently healthy of Arbequina, Coratina, Frantoio and Picual cultivars were collected from commercial crops at the veraison stage (BBCH85) according to Sanz-Cortes et al. (2002). Collected samples were stored in nylon bags and maintained in coolers for their preservation until to be processed in the laboratory.

Olive fruits were washed with SDW and 0.02% Tween 20 for 1 min, immersed in 1% NaClO for 1 min, rinsed with SDW and air-dried at room temperature. Fungicides suspensions of copper oxychloride (3.5 g a.i.l^{-1}), cuprous oxide (2.0 g a.i.l^{-1}) and Copper calcium sulfate (7.5 g a.i.l^{-1}) were prepared in SDW according to label recommendations. Immediately, the olive fruits were immersed for 30s in the suspensions of each fungicide. Olive fruits were maintained at room temperature for 24h to air-dried and then sprayed with the conidial suspension ($1x10^6$ conidia.ml^{-1}) of the corresponding isolate until runoff. Olive fruits were placed into plug seedling trays, one fruit in each hole, enclosed in moistened transparent nylon bags and incubated at 25 °C with 12-h photoperiod for 15 days. Four repetitions with five olive fruit per each combination of cultivar, fungicide, and isolate, were used. Four repetitions with five olive fruit of each cultivar sprayed with SDW and subsequently inoculated with the corresponding conidial suspension were used as control. The severity of anthracnose was evaluated every three days using the scale

from 0-5 suggested by Moral et al. (2008), where 0 = no visible lesions, 1 = visible lesions affecting <25% of the fruit surface, 2 = 25–49%, 3 = 50–74%, 4 = 75–100%, and 5 = soapy fruit (fruit completely covered with gelatinous mass of *Colletotrichum* spores).

Fruit severity was used to calculate the McKinney Index (MKI) according to Moral et al. (2017), using the following formula:

$$McKinney's\ Index = \frac{\sum(n_i\ x\ i)}{5\ x\ N}\ \text{x}\ 100$$

where i represents the severity of symptoms (0–5), n_i is the number of fruits with the severity of i, and N the total number of evaluated fruits.

McKinney's Index values of each *Colletotrichum* species were averaged within each cultivar and fungicide combination. These values were plotted and a regression model was adjusted for each fungicide into each cultivar considering the significance of the regression and the coefficient of determination (R^2) in SigmaPlot version 12.0 (http://www.sigmaplot.co.uk) program. In addition, McKinney's Index values were used to calculate the AUDPC for each fungicide using the same formula indicated above.

The AUDPC data were analyzed for normality and the homogeneity of the data was verified and then subjected to ANOVA analysis with a factorial arrangement ($p \leq 0.05$). Means of treatments were compared using Duncan's test ($p \leq 0.05$). Data tests were analyzed with InfoStat version 2016 (http://www.infostat.com.ar) program.

3. RESULTS

3.1. *Mycelial growth inhibition*

Differences in mycelial growth inhibition among the *Colletotrichum* spp. isolates caused by the fungicides evaluated were found (Fig. 1). Overall, isolates showed high sensitivity to the triazoles tebuconazole and propiconazole, with EC_{50} values ranged from 0.16 to 0.43 and 0.27 to 0.93 mg a.i L^{-1}, respectively. The EC_{50} values of difenoconazole ranged between 0.07 to 18,9 mg a.i L^{-1} due the presence of two isolates with reduced sensitivity, OL5 and OL10 whose EC_{50} were 18.9 and 3.58 mg a.i L^{-1}, respectively (Fig. 1).

Within strobilurin chemical group, only pyraclostrobin presented high efficacy in inhibiting the mycelial growth of all isolates with EC_{50} values ranging from 0.02 to 0.15 mg a.i L^{-1}. However, most of the isolates were found to be insensitive to the three other strobilurins tested. In the case of kresoxym methyl, the isolates presented EC_{50} values between 0.22 and >100 mg a.i L^{-1}, whereas for azoxystrobin ranged between 0.31 to >100 mg a.i L^{-1} and for trifloxystrobin between 5.8 x 10^{-6} and >100 mg a.i L^{-1} (Fig. 1).

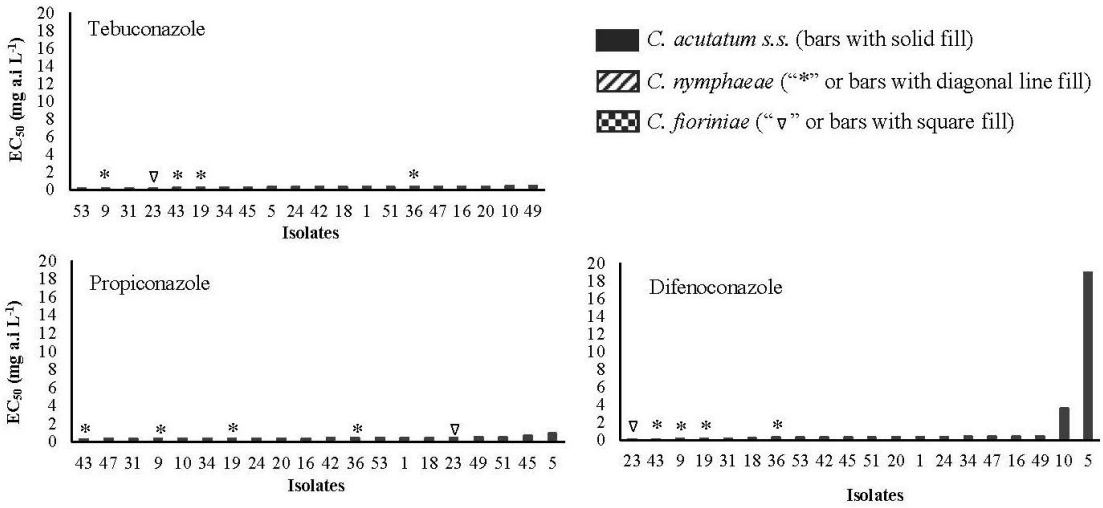
Respect to fungicides belonging to dithiocarbamate chemical group, all isolates were sensitive to ziram with EC_{50} values ranging from 9.29 to 26.23 mg a.i L^{-1} while to mancozeb, six showed reduced sensitivity with EC_{50} values ranging from 55.66 to 92.62 mg a.i L^{-1} and 14 were insensitive (EC_{50} values >100 mg a.i L^{-1}). With respect to dodine, most of the isolates were insensitive (EC_{50} values>100 mg a.i L^{-1}) except the isolates OL23 of *C. fioriniae* and OL42 of *C. acutatum,* which presented EC_{50} values of 5.42 and 6 x10^{-4} mg a.i L^{-1}, respectively. In the case of captan, most of the isolates also were insensitive (EC_{50} values>100 mg a.i L^{-1}) whereas three showed reduced sensitivity, with EC_{50} values ranging from 49.22 to 62.77 mg a.i L^{-1} (Fig. 1).

Finally, about the copper-based fungicides copper oxychloride, cuprous oxide and copper calcium sulfate, no major differences were observed among them. In general, all the isolates behaved as moderately sensitive to these fungicides with average EC_{50} values between 92.93 and 355.19 mg a.i L^{-1} (Fig. 1).

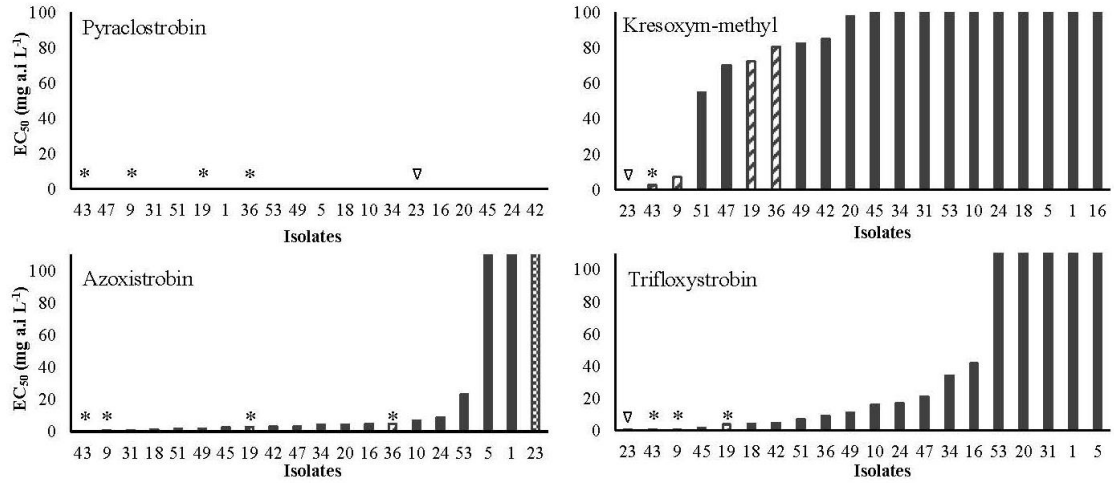
3.2. *Efficacy of fungicides on detached flower*

The evolution of blossom blight incidence fitted to an exponential type of curve in all cultivars, regardless of the *Colletotrichum* species or fungicide applied (Fig. 2 and Sup 1). On olive flowers sprayed with fungicides, blossom blight symptoms were visible at least 24 to 48 hours later than those unsprayed. Overall, the incidence of anthracnose in all treatments sprayed with fungicides was lower than those unsprayed.

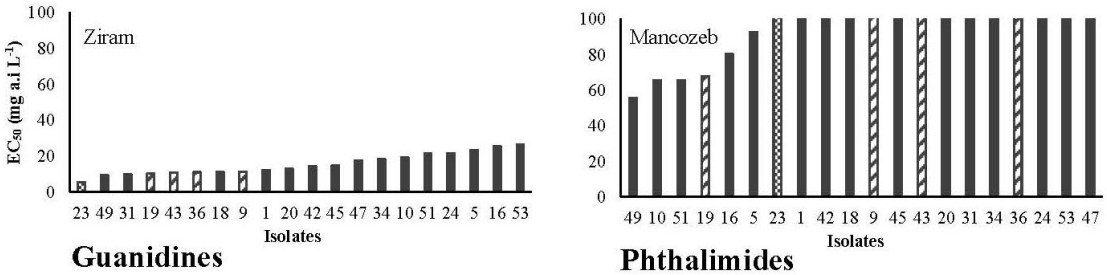
Triazoles



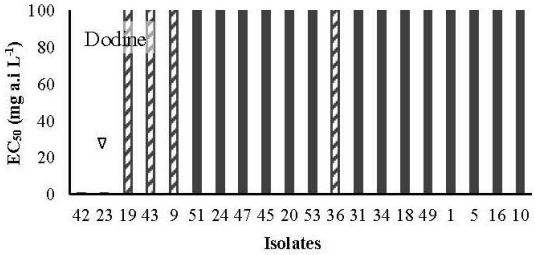
Strobilurins



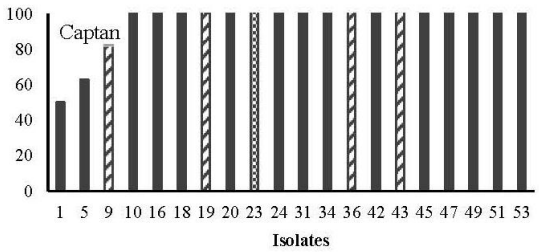
Dithiocarbamates



Guanidines



Phthalimides



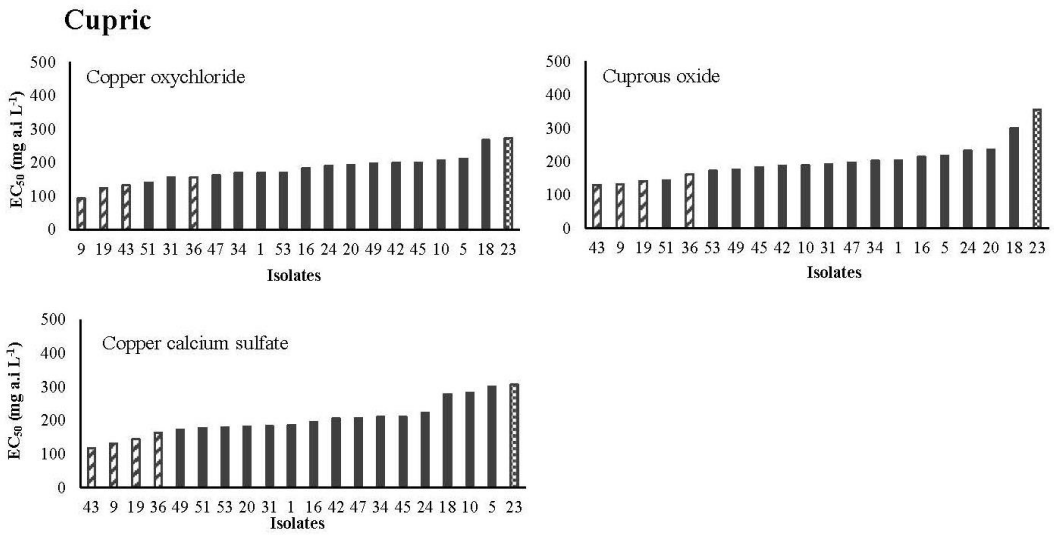


Fig. 1 Average effective concentration (mg a.i L-1) of 14 fungicides that inhibit 50% of mycelial growth (EC_{50}) of *Colletotrichum acutatum* s.s., *C. nymphaeae* and *C. fioriniae* isolates causing anthracnose in olive trees.

For the variable AUDPC, significant interaction was found between olive cultivars, *Colletotrichum* species and fungicides (P= 0.0003) (Table 3). Although not always statistical differences were found, on all combinations of olive cultivars and *Colletotrichum* species the fungicides tebuconazole, pyraclostrobin and ziram, applied on flowers panicles, reduced AUDPC compared with the control unsprayed. Ziram was able to reduce on average 83.5% the AUDPC with values between 56% and 100% respect to unsprayed control, whereas pyraclostyrobin reduced on average 79% the AUDPC with values between 48% and 100% with respect to unsprayed control and tebuconazole reduced on average 60% the AUDPC with values between 2 % and 97% with respect to unsprayed control (Table 4).

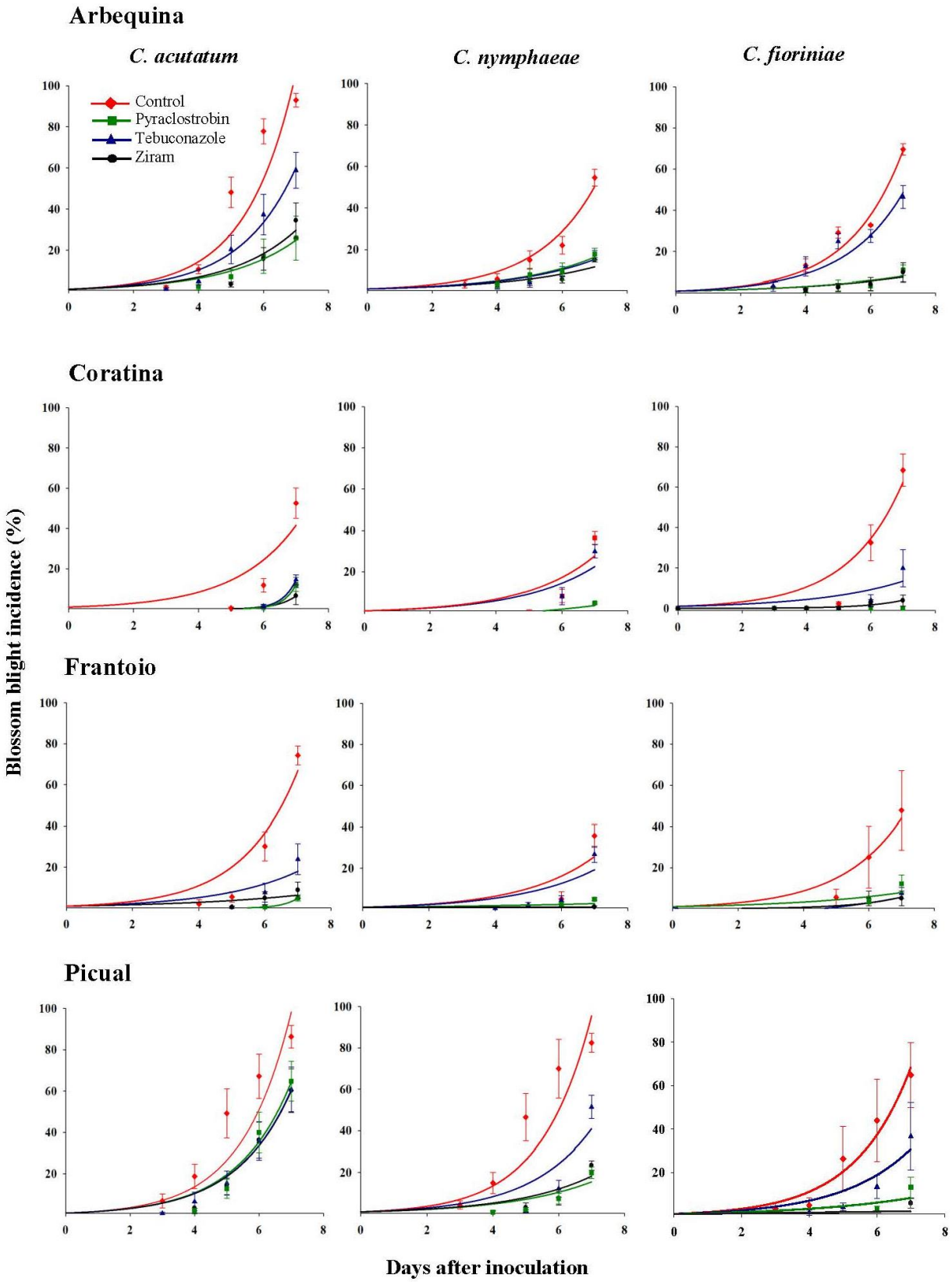


Fig. 2 Evolution of blossom blight incidence in flower at initial-final differentiation (BBCH54-55) phenological stage of four olive cultivars sprayed with Pyraclostrobin, Tebuconazole and Ziram and inoculated with *Colletotrichum acutatum* s.s., *C. nymphaeae* and *C. fioriniae.*

Table 3. Analysis of variance of Area Under Disease Progress Curve (AUDPC) data estimated based on anthracnose incidence developed in flower at initial-final differentiation (BBCH54-55) phenological stage of four olive cultivars sprayed with three fungicides and inoculated with three *Colletotrichum* species.

Source[1]	SS[2]	df	MS	F	p-value	CV
Model	569.77	47	12.12	13.51	<0.0001	47.99
Cultivar	146.15	3	48.72	54.3	<0.0001	
Fungicide	167.82	3	55.94	62.35	<0.0001	
Specie	34.3	2	17.15	19.12	<0.0001	
Cultivar x Fungicide	22.31	9	2.48	2.76	0.0043	
Cultivar x Specie	24.11	6	4.02	4.48	0.0003	
Fungicide x Specie	2.6	6	0.43	0.48	0.8202	
Cultivar x Fungicide x specie	44.28	18	2.46	2.74	0.0003	
Error	212.64	237	0.9			
Total	782.41	284				

Table 4. Area Under Disease Progress Curve (AUDPC) data estimated based on anthracnose incidence developed in flower at initial-final differentiation (BBCH54-55) phenological stage of four olive cultivars sprayed with three fungicide and inoculated with three *Colletotrichum* species.

Cultivar	Fungicide	Species		
		C. acutatum s.s.	***C. nymphaeae***	***C. fioriniae***
Arbequina	**Control**	**26.69 a**	**10.91 bcd**	**13.44 b**
	Tebuconazole	13.44 bc	3.54 efgh	13.00 b
	Pyraclostrobin	5.62 efgh	4.17 fgh	1.98 fghij
	Ziram	5.19 cdef	2.45 fghij	1.94 fghij
Coratina	**Control**	**4.07 defg**	**3.52 efgh**	**10.28 bcde**
	Tebuconazole	1.24 fghij	1.68 fghij	0.44 ghij
	Pyraclostrobin	1.01 fghij	0.35 ghij	0.00 j
	Ziram	0.38 hij	0.00 j	0.64 ghij
Frantoio	**Control**	**11.68 bc**	**3.98 efgh**	**2.08 fghij**
	Tebuconazole	1.83 Fghij	3.90 efgh	0.55 fghij
	Pyraclostrobin	0.44 ghij	0.69 fghij	1.40 fghij
	Ziram	0.54 ghij	0.08 ij	0.60 ghij
Picual	**Control**	**27.42 a**	**25.92 a**	**16.37 b**
	Tebuconazole	12.85 bcd	5.59 cdef	0.42 ghij
	Pyraclostrobin	12.49 bc	2.90 fghi	1.39 fghij
	Ziram	11.96 bcde	3.28 fgh	5.36 cdef

[1]Mean values followed by the same letter are not significantly different according to Duncan test at p<0,05

3.3. *Efficacy of copper-based fungicides on detached fruit*

In all cultivars evaluated, the evolution of olive fruit rot severity fitted to a sigmoid type of curve, regardless *Colletotrichum* species or fungicide combinations (Fig. 3 and Sup 2). In general, fruit rot symptoms were visible almost simultaneously in olive fruit treated with copper-based fungicides than in those not treated with fungicides (Fig. 3).

For the AUDPC variable, a significant interaction was found between olive cultivars and *Colletotrichum* species (P=0.0046) and between olive cultivars and fungicides (P= 0.0055) (Table 5). As the objective of this study was to evaluate the efficacy of fungicides, only the interaction between cultivars and fungicides was analyzed. On Arbequina and Coratina cultivars, no statistical differences were found in AUDPC between olive fruit immersed in copper-based fungicides and those untreated with these fungicides. In Frantoio cultivar only copper calcium sulfate was able to statistically reduce the AUDPC by 23% on overage compared with the control treatment without fungicide. Finally, in Picual the three copper-based fungicides were able to statistically reduce AUDPC by 33%, 26% and 24% on overage for cuprous oxide, copper calcium sulfate and copper oxychloride, respectively, compared with the control treatment without fungicide.

4. DISCUSSION

The environmental conditions in Uruguay, characterized by usual rainfall and frequent days with high humidity, favor olive anthracnose development (Conde-Innamorato et al., 2019; Moreira et al., 2021), thus, the use of fungicides as part of the disease management strategy, is crucial to achieve acceptable control. In this study, we screened 14 systemic and contact fungicides by in vitro mycelial growth inhibition assays. The effect of each fungicide was evaluated against isolates of *C. acutatum* s.s., *C. nymphaeae* and *C. fioriniae* belonging to *C. acutatum* species complex, the main *Colletotrichum* species complex causing olive anthracnose in Uruguay (Moreira et al, 2021).

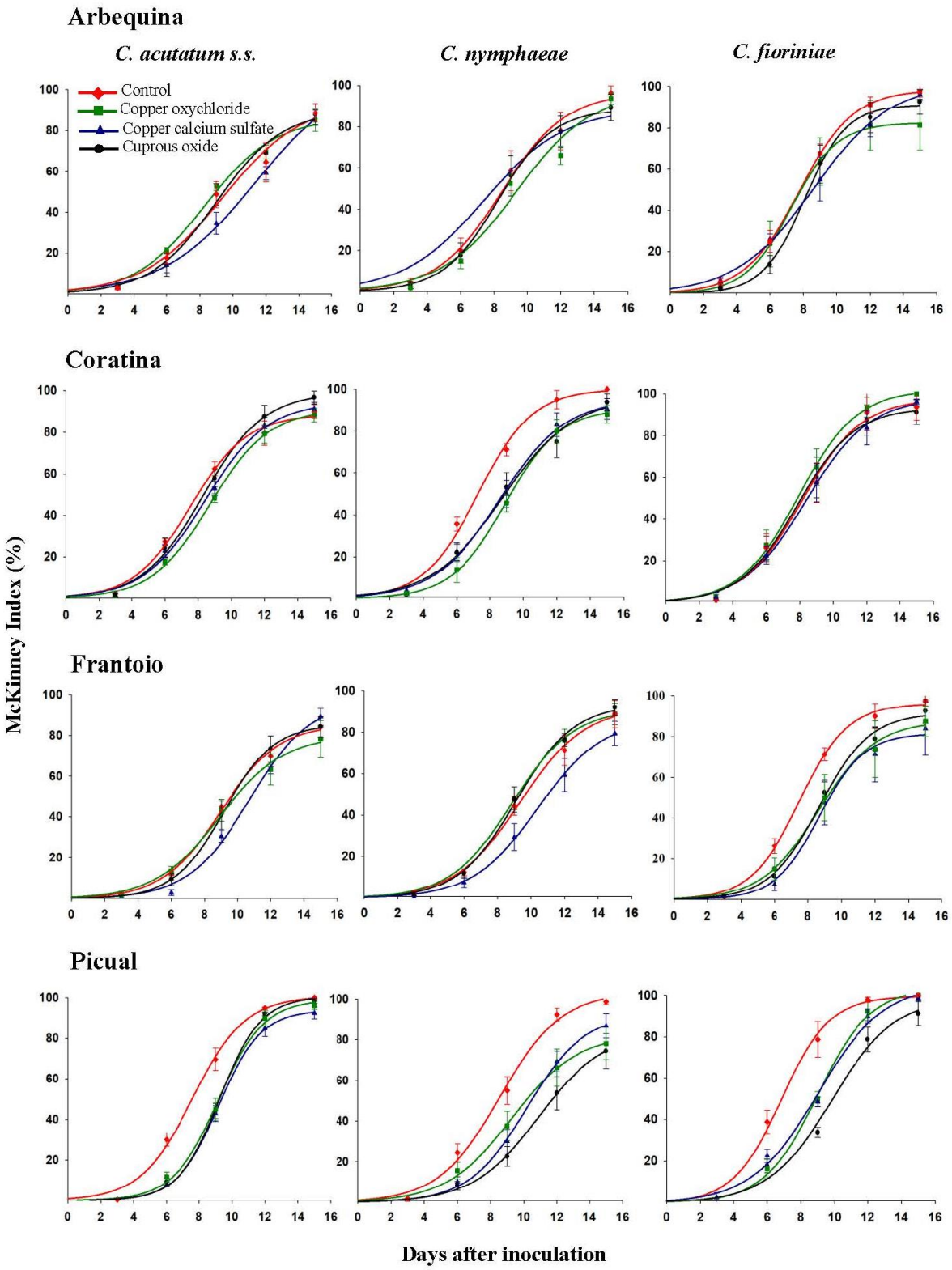


Fig. 3 Evolution of anthracnose severity index in fruit at veraison (BBCH85) phenological stage of four olive cultivars treated with three copper-based fungicides and inoculated with *Colletotrichum acutatum* s.s., *C. nymphaeae* and *C. fioriniae*. Disease severity values were used to calculate the McKinney index.

Table 5. Analysis of variance of Area Under Disease Progress Curve (AUDPC) data estimated based on anthracnose severity developed in fruit at veraison (BBCH85) phenological stage of four olive cultivars treated with three copper-based fungicide and inoculated with three *Colletotrichum* species.

Source	SS[1]	df	MS	F	p-value	CV
Model	13538.09	47	288.04	3.24	<0.0001	24.51
Cultivar	2466.36	3	822.12	9.23	<0.0001	
Specie	1396.01	2	698.01	7.84	0.0005	
Fungicide	1913.62	3	637.87	7.16	0.0001	
Cultivar x Specie	1708.13	6	284.69	3.2	**0.0046**	
Cultivar x Fungicide	2127.56	9	236.4	2.66	**0.0055**	
Fungicide x Specie	559.3	6	93.22	1.05	0.3946	
Cultivar x Fungicide x Specie	1828.58	18	101.59	1.14	0.3104	
Error	29912.91	336	89.03			
Total	43451	383				

[1]SS: sum of squares, df: degrees of freedom, MS: mean squares, F: teste F, CV: coefficient of variation

Table 6. Area Under Disease Progress Curve (AUDPC) data estimated based on anthracnose severity developed in fruit at veraison (BBCH85) phenological stage of four olive cultivars treated with three copper-based fungicide and inoculated with three *Colletotrichum* species.

	Cultivar			
	Arbequina	**Coratina**	**Frantoio**	**Picual**
Fungicides				
Control	41.1 bcde	44.59 ab	38.89 bcde	49.35 a
Cuprous oxide	39.76 bcde	42.68 bc	35.95 def	32.78 fg
Copper calcium sulfate	40.86 bcde	41.87 bcd	29.76 g	36.53 cdef
Copper oxychloride	40.93 bcde	41.01 bcde	35.17 efg	37.47 cdef

[1]Mean values followed by the same letter are not significantly different according to Duncan test at p<0,05

Triazoles fungicides are one of the major groups used to control anthracnose in different fruit crops worldwide (Dowling, et al., 2020; Chen et al. 2016; Kolainis et al 2020; Gama et al 2020, Chechi et al., 2019; Schoeneberg and Hu, 2022). However, due the extensive use, different populations of *Colletotrichum* spp. have become less sensitive to triazoles leading to control failures in the field (Hu et al., 2015; Chen et al., 2016; Forcelini et al., 2016; Zhang et al., 2017; Dowling, 2020). In this study, the sensitivity of isolates of the three *Colletotrichum* species evaluated to tebuconazole and propiconazole were consistently high, whereas difenoconazole resistant isolates were found. This result was not surprising because these fungicides act at a specific site in the fungus, thus, the risk of generating resistance to these fungicides is high (Forcelini et al., 2016, FRAC, 2022). The presence of difenoconazole-resistant isolates is possibly due to the history of use of this fungicide, in fact, some of them have usually been applied in Uruguayan olive orchards during the last decade for anthracnose control. Therefore, this phenomenon would explain the finding of these resistant individuals. Furthermore, the development of resistance in *Colletotrichum* spp. to these fungicides has already been reported in multiple commercial crops such as apple, strawberry, peach, blueberry and pepper (Chen et al., 2016; Dowling, 2020).

Strobilurins are an important chemical group of agricultural fungicides and are currently being utilized in a wide range of crops throughout the world (Dowling, 2020). Strobilurins control a wide range of Ascomycetes fungi including species of the *Colletotrichum* genus. In our study, pyraclostrobin showed high efficacy in inhibiting the mycelial growth of all isolates evaluated. This result is in accordance with previous studies in which this fungicide was able to effectively inhibit the mycelial growth of *C. acutatum* isolates from strawberry (Forcelini et al., 2016) and chili (Gao et al., 2017) and *C. nymphaeae* isolates from peach (Usman et al., 2022). In contrast, most of our *Colletotrichum* isolates were resistant to the other three strobilurins evaluated, kresoxym methyl, azoxystrobin and trifloxystrobin with EC_{50} values higher than 100 µg mL^{-1} in most of cases. These results were not surprising because these fungicides also have a high risk of generating resistance (Forcelini et al., 2016; Dowling, 2020; FRAC, 2022). Forcelini et al. (2016) mention that isolates of *C. acutatum* showing EC_{50} between 3 and 100 µg mL^{-1} of azoxystrobin are considered moderately resistant, while those with EC_{50} higher than 100 µg mL^{-1} are completely resistant. In addition, Zhang et al. (2020) indicate that *C. siamense* and *C. fructicola* are resistant to azoxystrobin when they have EC_{50}

greater than 100 µg mL^{-1}. On the other hand, our results confirm that no positive cross-resistance occur between pyraclostrobin and azoxystrobin, kresoxim-methyl or trifloxystrobin, even when they are members of the same fungicide chemical group.

Regarding to Dithiocarbomate chemical group, all our *Colletotrichum* isolates were sensitive to ziram, but were insensitive to mancozeb. Gao et al. (2017) found that mancozeb was effective in inhibiting spore germination of strawberry *C. acutatum* isolates but was not efficient in inhibiting mycelial growth of them, in agreement with our results. The guanidine dodine and the phtahalimide captan were also ineffective in inhibiting the mycelial growth of Uruguayan *Colletotrichum* isolates. Freeman et al. (1997) found that captan was not effective in controlling strawberry anthracnose on stolons naturally infected with *C. acutatum.*

The three copper-based fungicides showed intermediate efficacy with similar behavior between them. Our data is in accordance with the study conducted by Moral et al. (2018) where copper hydroxide, copper oxychloride, cuprous oxide and copper calcium sulfate ingredient actives were the least effective in inhibiting mycelial growth of *C. godetiae* and *C. nymphaeae* olive isolates. Other authors also observed that copper sulfate was not effective in inhibiting mycelial growth of strawberries *C. acutatum* isolates (Es-Soufi et al., 2018) and that copper oxychloride proved the lowest mycelial inhibition in avocado *C. gloesporioides* isolates (Kimaru et al., 2018)

Regarding to assay on detached flowers, the three organic fungicides, particularly ziram and pyraclostrobin, were effective in controlling olive anthracnose. These fungicides delayed the manifestation of blossom blight symptom by 24 to 48h and overall reduce the anthracnose incidence through the experiment compared with the control. The fungicides pyraclostrobin and tebuconazole showed to have good performance controlling anthracnose disease in other crops such as citrus (Piccirillo et al., 2018), chili (Gao et al., 2017) or strawberry (Turechek et al., 2008).

In contrast, copper-based fungicides applied on fruit at veraison stage using the doses suggested by the manufacturers, in general failed to reduce the anthracnose severity. Only in Picual cultivar the three copper-based fungicides, and in Frantoio the copper calcium sulfate, were able to reduce the disease in some grade. This is in line with trials conducted by Moral et al. (2018), where the efficacy of copper-based fungicides

alone or in mixtures with other fungicides were evaluated and only copper hydroxide mixed with folpet reduce the disease in ripe olive fruits of Hojiblanca cultivar. Previous studies suggest that copper applied alone is inefficient to control *Colletotrichum*, while when is combined with other systemic fungicides, the efficacy increase (Pennisi et al., 1993). According to Moral et al. (2018), *Colletotrichum* species can be considered more resistant to metallic copper than other olive pathogens such as *Spilocaea oleagina* and *Pseudocercospora cladosporioides*.

In conclusion, our study allows us to optimize the selection of the most effective fungicides for the management of olive anthracnose disease. We demonstrated that ziram and pyraclostrobin are effective in controlling blossom blight. We also confirmed the low efficacy of copper-based fungicides applied in fruit to control anthracnose. Nevertheless, although some fungicides have shown effectiveness, efficient anthracnose management requires the integration of different strategies (Cacciola et al., 2012; Talhinhas et al., 2018). For example, the use of less susceptible cultivars, early harvests to avoid the development of infections during the mayor fruit susceptibility or tree pruning to improve ventilation, combined with fungicide applications during flowering and fruit development, should be implemented.

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6. SUPPLEMENTARY INFORMATION

Table S1. Fitted models for the evolution of *Colletotrichum* spp. incidence as a function of time by cultivar, specie inoculated and fungicide application in flower at initial-final differentiation (BBCH54-55) phenological stage.

Cultivar/fungicide	**Species**					
	C. acutatum s.s.		*C. nymphaeae*		*C.fioriniae*	
Arbequina	a	R^2	a	R^2	a	R^2
Control inoculted	0.66691 <0.0001	0.85	0.3893 <0.0001	0.86	0.6052 <0.0001	0.96
Pyraclostrobin	0.4591 <0.0001	0.92	0.3994 <0.0001	0.91	0.5549 <0.0001	0.93
Tebuconazole	0.5855 <0.0001	0.97	0.3893 <0.0001	0.97	0.3009 0.0002	0.69
Ziram	0.4848 <0.0001	0.83	0.3512 <0.0001	0.73	0.2912 0.0001	0.73
Coratina						
Control inoculted	0.5326 <0.0001	0.74	0.4750 <0.0001	0.7	0.5070 <0.0001	0.88
Pyraclostrobin	0.2733 0.008	0.36	-[2]	-	-	-
Tebuconazole	0.3071 0.0031	0.41	0.4451 <0.0001	0.68	0.33714 0.0005	0.56
Ziram	-	-	-	-	-	-
Frantoio						
Control inoculted	0.6007 <0.0001	0.91	0.4637 <0.0001	0.62	0.5407 <0.0001	0.89
Pyraclostrobin	-	-	-	-	0.2964 0.017	0.5
Tebuconazole	0.4130 <0.0001	0.68	0.4226 0.0001	0.64	-	-
Ziram	0.2647 0.0017	0.54	-	-	-	-
Picual						
Control inoculted	0.6554 <0.0001	0.84	0.6513 <0.0001	0.83	0.6037 <0.0001	0.96
Pyraclostrobin	0.5450 <0.0001	0.95	0.3915 <0.0001	0.74	0.3038 0.0019	0.48
Tebuconazole	0.5842 <0.0001	0.96	0.4149 <0.0001	0.75	0.4898 <0.0001	0.83
Ziram	0.5862 <0.0001	0.98	0.5304 <0.0001	0.75	-	-

[1] $y = e^{(a*x)}$

[2] Not adjusted model

Table S2. Fitted models for the evolution of *Colletotrichum* spp. incidence as a function of time by cultivar, specie inoculated and fungicide application in fruit at veraison (BBCH85) phenological stage.

Cultivar/fungicide	Species					
	C. acutatum s.s.		*C. nymphaeae*		*C.fioriniae*	
Arbequina	a	R^2	a	R^2	a	R^2
Control inoculted	0.66691 <0.0001	0.85	0.3893 <0.0001	0.86	0.6052 <0.0001	0.96
Pyraclostrobin	0.4591 <0.0001	0.92	0.3994 <0.0001	0.91	0.5549 <0.0001	0.93
Tebuconazole	0.5855 <0.0001	0.97	0.3893 <0.0001	0.97	0.3009 0.0002	0.69
Ziram	0.4848 <0.0001	0.83	0.3512 <0.0001	0.73	0.2912 0.0001	0.73
Coratina						
Control inoculted	0.5326 <0.0001	0.74	0.4750 <0.0001	0.7	0.5070 <0.0001	0.88
Pyraclostrobin	0.2733 0.008	0.36	-[2]	-	-	-
Tebuconazole	0.3071 0.0031	0.41	0.4451 <0.0001	0.68	0.33714 0.0005	0.56
Ziram	-	-	-	-	-	-
Frantoio						
Control inoculted	0.6007 <0.0001	0.91	0.4637 <0.0001	0.62	0.5407 <0.0001	0.89
Pyraclostrobin	-	-	-	-	0.2964 0.017	0.5
Tebuconazole	0.4130 <0.0001	0.68	0.4226 0.0001	0.64	-	-
Ziram	0.2647 0.0017	0.54	-	-	-	-
Picual						
Control inoculted	0.6554 <0.0001	0.84	0.6513 <0.0001	0.83	0.6037 <0.0001	0.96
Pyraclostrobin	0.5450 <0.0001	0.95	0.3915 <0.0001	0.74	0.3038 0.0019	0.48
Tebuconazole	0.5842 <0.0001	0.96	0.4149 <0.0001	0.75	0.4898 <0.0001	0.83
Ziram	0.5862 <0.0001	0.98	0.5304 <0.0001	0.75	-	-

[1] $y = e^{(a*x)}$

[2] Not adjusted model

DISCUSIÓN GENERAL

La importancia de la antracnosis del olivo en las diferentes regiones olivareras radica en las pérdidas en rendimientos debido a la podredumbre de los frutos, así como a la reducción de la calidad de los aceites elaborados con lotes de frutos infectados (Cacciola et al., 2012; Moral et al., 2014, Leoni et al, 2018). Sumado a esto, la producción olivícola uruguaya se ubica en una región con condiciones climáticas que suelen ser extremadamente conducentes al desarrollo de esta enfermedad. Por otra parte, dado que el olivar es un cultivo emergente en Uruguay, los antecedentes de investigación acerca de las enfermedades que lo afectan eran escasos por lo que fue necesario realizar estudios que involucren diferentes aspectos de la enfermedad. Por ejemplo, conocer con exactitud los síntomas y daños ocasionados durante la etapa de floración y desarrollo de fruto, la etiología de la enfermedad, así como clarificar aspectos relacionados con la epidemiología y evaluar medidas de manejo que aporten al desarrollo de estrategias eficientes para el control de esta enfermedad.

En este trabajo se constató la presencia de frutos con síntomas de podredumbre por *Colletotrichum* spp. en las diferentes zonas olivícolas del país. A esto se sumó la presencia un nuevo síntoma que consiste en el atizonado de las panículas florales causando pérdidas tempranas al impedir el cuajado de los frutos. La incidencia de flores atizonadas fue mayor en la región Este del país, donde se concentra la mayor producción de olivo (80%). El síntoma del atizonado de panículas florales tiene una distribución mundial más restringida, hasta el momento fue observado en Sudáfrica (Gorter 1956) y más tarde en Australia (Sergeeva et al. 2008), Grecia (Iliadi et al.2018) y recientemente en Brasil (Filoda et al., 2021) causando daños importantes. Por otro lado, en España (Moral et al. 2009) y Portugal (Talhinhas et al. 2011) también fue detectado, pero sin causar daños importantes.

Con el fin de conocer que especies de *Colletotrichum* estaban ocasionando la antracnosis del olivo en Uruguay, se caracterizó morfológica y molecularmente una colección de 108 aislados de *Colletotrichum* obtenidos predominantemente de panículas florales atizonadas y de frutos con podredumbre típico. Las muestras se colectaron incluyendo los diferentes cultivares plantados y las diferentes zonas de producción. Entre las cinco especies identificadas aparece *C. acutatum* s.s. como la predominante con 82% de los aislados, un 13% como *C. nymphaceae* y 1% como *C. fioriniae,* estas tres especies

pertenecen al complejo de especies de *C. acutatum*. Las restantes dos especies identificadas, fueron *C. theobromicola* (3%) y *C. alienum* (1%), quienes pertenecen al complejo de especies de *C. gloesporioides.* Esta predominancia de la especie *C. acutatum* s.s. ya ha sido mencionada en otras zonas olivícolas del mundo acompañando la ocurrencia de epidemias explosivas de antracnosis como la ocurrida en Uruguay, en Grecia (Iliadi et al. 2018), Italia (Mosca et al. 2014) y Tunes (Chattaoui et al. 2016). Respecto a *C. nymphaeae* y *C. fioriniae,* estas especies fueron encontradas en Portugal (Talhinhas et al. 2005) mientras que *C. theobromicola* en Australia (Schena et al., 2014). Sin embargo, es la primera vez en el mundo que se anuncia a *C. alienum* causando la antracnosis del olivo. Asimismo, en los ensayos de patogenicidad realizados se comprobó que las cinco especies son capaces de ocasionar tanto el atizonado de las panículas florales como la podredumbre de los frutos en las cuatro cultivares más plantadas en nuestro país Arbequina, Coratina, Frantoio y Picual.

Durante la temporada 2017/18 se determinó la incidencia de infecciones latentes de *Colletotrichum* durante el proceso de desarrollo del fruto en los cultivares Arbequina, Coratina y Picual en las dos principales regiones uruguayas de producción de olivo, Sureste y Centro-sur del país. Como resultado se comprobó la presencia de infecciones latentes en todas las etapas del desarrollo del fruto desde cuajado a cosecha y en todos los cultivares evaluados. Estas infecciones fueron sustancialmente mayores en la región Sureste en comparación con la región Centro-sur. Para explicar estas diferencias se analizó la evolución de diferentes variables climáticas durante el periodo de desarrollo del fruto. Utilizando esas variables se crearon índices en base a las condiciones ambientales que favorecen la ocurrencia de infecciones por *Colletotrichum* spp. El análisis de estos índices para el año del muestreo reveló que la región Sureste presentó un mayor número de días con alta humedad relativa (mayor a 90 %) y precipitaciones ligeras (<5 mm) solas o combinados con precipitaciones abundantes y temperaturas medias favorables (entre 20 y 30ºC) en comparación con la región Centro-sur. Estos resultados coinciden con lo publicado previamente por diferentes autores que mencionan que las precipitaciones, combinadas con temperaturas suaves, crean un ambiente húmedo y cálido que favorece la infección por *Colletotrichum* (Sergeeva 2011; Moral y Trapero 2012). Por lo tanto y si estas condiciones ambientales se mantienen, en la principal región olivícola de Uruguay donde se concentra el 80% de la producción de olivo (DIEA, 2020) estarían ocurriendo condiciones ambientales más conducentes al desarrollo de la

antracnosis del olivo que en la región Centro-sur, lo que explicaría porque esta región fue la más afectada durante brote epidémico ocurrido hace algunos años.

Respecto a la proporción de frutos con infecciones latentes de *Colletotrichum*, en los diferentes cultivares se observó que Arbequina y Picual presentaron similar incidencia, mientras que en Coratina la incidencia fue menor. Estos resultados coinciden con otros estudios en los que se encontró que Arbequina es susceptible a la antracnosis, mientras que Coratina se caracteriza por ser moderadamente susceptible (Moral et al. 2009; Bartolini y Cerreti 2013). Por otra parte, Picual que en estudios previos fue considera como un cultivar moderadamente resistente a *Colletotrichum* (Moral et al. 2009; Talhinhas et al. 2015; Moral et al., 2017), en este estudio no se diferenció de Arbequina.

Con el fin de conocer cómo evoluciona la susceptibilidad de las flores durante su desarrollo y de los frutos durante su maduración a la infección por *Colletotrichum*, se realizaron inoculaciones de flores y frutos a lo largo de los diferentes estadios fenológicos de los cultivares Arbequina, Coratina, Frantoio y Picual con las cinco especies de *Colletotrichum* encontradas en Uruguay. Se demostró que estas cinco especies de *Colletotrichum* tienen la capacidad de infectar a las flores desde etapas tempranas de desarrollo, y que, la susceptibilidad aumenta a medida que avanza el desarrollo de las panículas florales. En trabajos anteriores ya se había observado que, en estadios avanzados de desarrollo, los síntomas y signos se manifiestan rápidamente (Iliadi et al. 2018; Kolainis et al. 2020; Sergeeva et al. 2008). Nuestros resultados indican que, si bien los estadios avanzados de la flor son más susceptibles a la antracnosis, el periodo de riesgo de que ocurran infecciones por *Colletotrichum* inicia tempranamente. Este resultado es de gran importancia al momento de diseñar estrategias de manejo para esta enfermedad. Impedir la ocurrencia de infecciones tempranas es un elemento clave para el manejo de esta enfermedad ya que estas infecciones producirán inóculo secundario favoreciendo que la epidemia pase a la fase de desarrollo exponencial si no son controladas a tiempo.

Comparando la susceptibilidad de los cultivares evaluados en los estadios de diferenciación final e inicio de floración, Frantoio fue el cultivar que se comportó como menos susceptible y Arbequina como el más susceptible a *Colletotrichum* spp., mientras que Coratina y Picual presentaron un comportamiento intermedio. La mayor susceptibilidad de Arbequina coincide con los resultados obtenidos previamente por Moral et al. (2009) quienes encontraron que Arbequina fue más susceptible que

Hojiblanca y Picual cuando fueron inoculadas al inicio de la floración. En cuanto a las especies de *Colletotrichum* inoculadas, las cinco especies fueron capaces de infectar la flor desde los primeros estadios de su desarrollo.

Respecto a la susceptibilidad de los frutos, ésta también se incrementó a medida que avanzó su madurez, confirmando los resultados obtenidos en trabajos previos donde se constató un progreso de la enfermedad más lento en frutos verdes que en frutos maduros (Chattaoui et al. 2016; Moral et al. 2008; 2009; 2017; Sergeeva 2014). Con respecto a los cultivares, Arbequina y Frantoio fueron las más susceptibles en la etapa de fruto verde, Coratina tuvo un comportamiento intermedio y Picual se comportó como moderadamente resistente. En cambio, no se observaron diferencias en la susceptibilidad entre cultivares en la etapa de fruto maduro. Estos resultados son coincidentes con estudios anteriores en los que Arbequina aparece como susceptible a moderadamente susceptible, Coratina como moderadamente susceptible (Andrés 1991; Moral y Trapero 2009; Bartolini y Cerreti 2013) y Picual como moderadamente resistente o resistente a la antracnosis (Moral et al. 2017; Moral y Trapero 2009; Talhinhas et al. 2015). Si bien algunos autores consideran al cultivar Frantoio como resistente (Moral et al. 2008; 2017; Moral y Trapero 2009), en este estudio se mostró como susceptible al igual que lo observado en investigaciones desarrolladas en Argentina (Andrés 1991) e Italia (Loprieno y Tenerini 1960).

Con respecto a la agresividad de las especies de *Colletotrichum*, en nuestro trabajo *C. thebromicola* fue la más agresiva cuando se inoculo en frutos verdes coincidiendo con resultados obtenidos en Australia. En dicho trabajo, *C. theobromicola* y *C. gloesporioides* s.s fueron más agresivos que *C. aenigma*, *C. kahawae*, *C. queenslandicum*, *C. siamense* y *C. karstii* cuando fueron inoculados en frutos verdes desprendidos (Schena et al. 2014). Por otra parte, no encontramos diferencias en la agresividad entre las cinco especies cuando se inocularon en fruto maduro, a diferencia de los resultados obtenidos por Talhinhas et al. (2015) quienes encontraron que las especies *C. acutatum* s.s. y *C. nymphaeae* fueron más agresivas que *C. gloeosporioides* s.s. y *C. rhombiforme*, dos especies que no han sido encontradas en Uruguay.

Para racionalizar el manejo de la antracnosis del olivo en Uruguay mediante fungicidas es indispensable conocer la sensibilidad de las especies de *Colletotrichum* predominantes, así como determinar el momento adecuado para su aplicación. En este trabajo se realizó el primer estudio exhaustivo de evaluación de la efectividad de

fungicidas para el control de esta enfermedad. Se evaluaron 14 fungicidas pertenecientes a seis grupos químicos mediante ensayos de inhibición del crecimiento micelial in vitro. El efecto de cada fungicida fue evaluado frente a tres de las especies de *Colletotrichum* encontradas en Uruguay causando la antracnosis del olivo. Se procuro incluir las dos especies principales *C. acutatum* s.s. y *C. nymphaeae*, mientras que la tercera especie evaluada fue *C. fioriniae*.

Los fungicidas más eficaces en inhibir el crecimiento micelial fueron tebuconazol y propiconazol (triazoles) y pyraclostrobin (estrobilurina). La eficacia de los triazoles en el control de especies de *Colletotrichum* ya ha sido demostrada en estudios anteriores. Aislados de *C. acutatum* procedentes de olivo y de cítricos fueron muy sensibles al tebuconazol (Kolainis et al 2020; Gama et al 2020), mientras que aislados de *C. fioriniae* procedentes de manzano y aislados de *C. nymphaeae* y *C. fiorinie* obtenidos de frutilla al propiconazol (Chechi et al., 2019; Schoeneberg y Hu, 2022). Respecto al pyraclostrobin, también existen trabajos que mencionan su eficacia inhibiendo el crecimiento micelial aislados de *C. acutatum* de frutilla (Forcelini et al 2016), pimiento (Gao et al, 2017) y de aislados de *C. nymphaeae* de duraznero (Usman et al 2022).

Por el contrario, la mayoría de nuestros aislados fueron resistentes a las otras tres estrobilurinas evaluadas, kresoxym metil, azoxistrobin y trifloxistrobin con valores de EC_{50} superiores a 100 µg mL^{-1} en la mayoría de los casos. Forcelini et al. (2016) mencionan que los aislados de *C. acutatum* que muestran EC_{50} entre 3 y 100 µg mL^{-1} de azoxistrobin se consideran moderadamente resistentes, mientras que aquellos con EC_{50} superior a 100 µg mL^{-1} como completamente resistentes. Por otro lado, Zhang et al. (2020) indican que *C. siamense* y *C. fructicola* son resistentes a la azoxistrobin cuando presentan una EC50 superior a 100 µg mL-1. No se observó resistencia cruzada positiva entre pyraclostrobin y azoxistrobin, krexoxim metil o trifloxistrobin a pesar de pertenecer al mismo grupo químico de fungicidas, las estrobilurinas.

La baja sensibilidad encontrada en nuestros aislados de *Colletotrichum* spp. a difenoconazole, azoxistrobin, krexoxim metil o trifloxistrobin puede deberse a la historia de uso de esos fungicidas ya que se trata de fungicidas con sitio de acción específico, por lo tanto, el riesgo de generar resistencia a estos fungicidas es alto (Forcelini et al., 2016, FRAC, 2022). De hecho, tenemos certeza de que algunos de estos fungicidas han sido aplicados habitualmente en los olivares uruguayos durante la última década para el control de antracnosis, lo que explicaría el hallazgo de estos individuos resistentes. Por

otra parte, existen antecedentes donde se relata el desarrollo de resistencia en poblaciones de *Colletotrichum* spp. en otros cultivos como manzana, frutilla, durazno, arándano y pimiento a estos fungicidas (Hu et al., 2015; Chen et al., 2016; Forcelini et al., 2016; Zhang et al., 2017; Dowling, 2020).

En cuanto a los fungicidas orgánicos y de contacto evaluados, el ziram (ditiocarbamato) fue el único que inhibió satisfactoriamente el crecimiento micelar de los aislados de *Colletotrichum* spp. Mientras que entre los fungicidas cúpricos (inorgánicos) no se encontraron mayores diferencias entre ellos, mostrando una eficacia intermedia. La baja efectividad del Mancozeb y del captan en inhibir el crecimiento micelial de estos hongos ya fue comunicada anteriormente por diferentes autores. Gao et al. (2017) y Freeman et al. (1997) encontraron que mancozeb y captan no fueron eficientes en la inhibición del crecimiento micelial de aislados de *C. acutatum* en frutilla.

Con respecto a los fungicidas cúpricos, otros autores obtuvieron resultados similares a los nuestros donde el hidróxido de cobre, el oxicloruro de cobre, el óxido cuproso y el sulfato cálcico de cobre fueron relativamente poco eficaces en inhibir el crecimiento micelial de aislados de *Colletotrichum* spp. obtenidos de olivo (Moral et al., 2018). Tampoco fue eficaz el sulfato de cobre en la inhibición del crecimiento micelial de aislados de *C. acutatum* s.s. en frutilla (Es-Soufi et al., 2018) ni el oxicloruro de cobre en aislados de *C. gloesporioides* en palta (Kimaru et al., 2018).

Por su buen comportamiento en inhibir el crecimiento micelial de *Colletotrichum* spp., pyraclostrobin, tebuconazole y ziram fueron seleccionados para evaluar su eficiencia en el control del atizonado de las panículas florales. En tanto que, solamente los tres fungicidas cúpricos fueron evaluados por su eficiencia del control de la podredumbre en los frutos. Esto último se debió a que no es recomendable aplicar fungicidas orgánicos luego de la floración porque al ser en su mayoría liposolubles, existe un alto riesgo de que sean retenidos en el aceite del fruto (Moral et al., 2014).

Los tres fungicidas aplicados sobre las panículas florales, pyraclostrobin, ziram y tebuconazole, lograron retrasar el inicio de la enfermedad entre 24 a 48 horas respecto al control no tratado con fungicida. Además, en todos los cultivares evaluados la incidencia de la antracnosis durante todo el ensayo fue sustancialmente menor en las panículas tratadas con fungicidas que las no tratadas. En otros cultivos como los cítricos (Piccirillo et al, 2018), morrón (Gao et al 2017) o frutilla (Turechek et al, 2008) las aplicaciones de

pyraclostrobin y tebuconazol también se mostraron eficaces en el control de la antracnosis. En cambio, los fungicidas a base de cobre aplicados sobre frutos en estado de envero fueron poco eficientes en reducir la severidad de la antracnosis. Estudios anteriores sugieren que aplicaciones solo de cobre son ineficaces para controlar *Colletotrichum*, mientras que cuando se combina con otros fungicidas orgánicos la eficacia en el control de la antracnosis aumenta sustancialmente (Pennisi et al., 1993; Moral et al. 2018). Este resultado es muy desalentador dado que luego de la floración las herramientas químicas disponibles están muy limitadas.

CONCLUSIONES GENERALES

- La antracnosis del olivo en Uruguay es causada por cinco especies de *Colletotrichum* pertenecientes a dos complejos de especies, la predominante es *C. acutatum* s.s, seguida por *C. nymphaeae* y *C. fioriniae* correspondientes al complejo de especies de *C. acutatum* y en menor proporción *C. theobromicola* y *C. alienum* dentro del complejo de especies de *C. gloesporioides*,
- Se anuncia por primera vez a *C. alienum* causando la antracnosis del olivo en el mundo.
- Las infecciones latentes por *Colletotrichum* spp. ocurren en todas las etapas de desarrollo del fruto desde cuajado a cosecha, con una mayor incidencia en la zona sureste del país debido a que las condiciones climáticas en esta región serían más favorables a la ocurrencia de infecciones de este patógeno.
- Las panículas florales de olivo son susceptibles a ser infectadas por *Colletotrichum* spp desde etapas tempranas de su desarrollo, en tanto que la susceptibilidad aumenta sustancialmente con el desarrollo de la flor.
- La susceptibilidad de los frutos de olivo a *Colletotrichum* spp se incrementa sustancialmente a medida que avanza su madurez.
- Durante la floración, el cultivar Arbequina fue el más susceptible y Frantoio el menos susceptible a la antracnosis, mientras que en fruto verde ambos cultivares se comportaron como susceptibles y Picual como moderadamente resistente, en fruto maduro no se observaron diferencias entre cultivares.
- Los fungicidas más eficaces en inhibir in vitro el crecimiento micelial de *Colletotrichum* spp. fueron los triazoles tebuconazol y propiconazol y la estrobilurina pyraclostrobin. Dentro de los fungicidas orgánicos de contacto el ditiocarbamato ziram fue el más eficaz.
- Los fungicidas pyraclostrobin, ziram y tebuconazole lograron retrasar el inicio de la enfermedad en las panículas florales, en tanto que los dos primeros fueron los que lograron la mayor reducción en la incidencia del atizonado de las flores. En cambio, los fungicidas a base de cobre aplicados sobre frutos fueron poco eficientes en reducir la severidad de la antracnosis.

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