Estudio de la biología reproductiva del cultivar de olivos 'Arbequina' (*Olea europaea L.*) en las condiciones de Uruguay

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RESUMEN

En las últimas dos décadas el cultivo del olivo ha experimentado un importante crecimiento en el Uruguay, posicionándose con 7.000 hectáreas implantadas como segundo rubro frutícola a nivel nacional. Sin embargo, los rendimientos obtenidos no son los esperados, asociado a un bajo cuajado de frutos. Las condiciones edafoclimáticas nacionales son contrastantes con las mediterráneas, zona típica de producción y origen del cultivo. Se postula como hipótesis que estas condiciones ambientales, pueden condicionar la biología reproductiva y afectar el período efectivo de polinización (PEP) limitando el cuajado. En este trabajo se determinó el PEP para 'Arbequina', principal cultivar de olivos en Uruguay, utilizando las dos metodologías tradicionales, la respuesta en cuajado a polinizaciones secuenciales en función de la edad de la flor y el análisis por microscopía de fluorescencia de sus componentes (receptividad estigmática, viabilidad de óvulos y crecimiento de tubos polínicos). El PEP para las condiciones de Uruguay presentó una duración de al menos una semana sin diferencias entre los métodos de estimación. La receptividad estigmática se mantuvo por al menos 20 días post antesis (DPA). Más del 50 % de las flores mantuvieron al menos un óvulo viable 23 DPA. A los tubos polínicos les tomó entre dos y tres días crecer desde el estigma hasta alcanzar un óvulo. No se observaron diferencias en el crecimiento de los tubos polínicos en función de la edad de la flor, incluso en flores de 20 días de edad. Se confirma la autoincompatibilidad del cultivar 'Arbequina' y su compatibilidad con los cultivares 'Picual' y 'Coratina'. Los resultados obtenidos señalan que el PEP en Uruguay no es una limitante para el cuajado y por lo tanto los aspectos a evaluar son la disponibilidad de polen compatible en las quintas y su distribución en el tiempo y espacio en relación a las condiciones ambientales.

Palabras clave: receptividad estigmática, viabilidad de óvulos, crecimiento tubo polínico, microsatélites (SSRs), análisis de paternidad

Study of the reproductive biology of 'Arbequina' olive cultivar (*Olea europaea* L.) in the Uruguay-conditions

SUMMARY

In the last two decades, Uruguayan olive cultivation has experienced an important expansion, reaching 7,000 hectares planted and becoming the second fruit crop at national level. However, yields obtained are lower than expected associated with a low fruit set. The national edaphoclimatic conditions are in contrast to those of the Mediterranean region, the typical production region and origin of the crop. It is hypothesized that these environmental conditions may influence the reproductive biology and affect the effective pollination period (EPP) limiting fruit set. In this work, after three year of experiments, we determined the EPP for 'Arbequina', the main olive cultivar in Uruguay, by means of the two traditional methodologies, as a response in fruit setting to sequential pollinations depending on the age of the flower, and by fluorescence microscopy analysis of its components (stigmatic receptivity, ovule viability and pollen tube growth). The EPP duration for the Uruguayan conditions was consider to be at least 7 days, with no differences between the estimation methods. Stigmatic receptivity was maintained for at least 20 days post anthesis (DPA). More than 50 % of the evaluated flowers kept at least one viable ovule 23 DPA. Pollen tubes required between two and three days to growth from the stigma until reach an ovule. No differences were found for the pollen tube growth in relation with the pistil age. Self-incompatibility of 'Arbequina' and its compatibility with 'Picual' and 'Coratina' were confirmed. Results obtained for 'Arbequina' EPP in Uruguay indicate that it is not limiting, therefore, it is important to evaluate the lack of compatible pollen in the orchards and their spatial and temporal distribution in relation to environmental conditions.

Keywords: stigmatic receptivity, ovule viability, pollen tube growth, microsatellites (SSRs), paternity analysis

1 INTRODUCCIÓN GENERAL

1.1 IMPORTANCIA DE LA OLIVICULTURA PARA URUGUAY

La producción de aceites a nivel nacional se encuentra basada fundamentalmente en la utilización de cultivos anuales como la soja, colza y girasol (MGAP.OPYPA, 2019), en modelos productivos cuestionados por sus impactos tanto sociales como ambientales (Barri, 2009). En ese contexto, el olivo se presenta como una excelente alternativa para la producción de aceite. Su condición de cultivo perenne, que puede mantener su vida productiva por décadas en condiciones económicamente viables (Barranco et al., 2008), conlleva grandes beneficios ecosistémicos. Como cultivo intensivo en el uso de los recursos, específicamente del trabajo, emplea gran cantidad de familias en el medio rural. Además de esto, el aceite de oliva, extraido fisicamente, es nutricionalmente superior a otros aceites debido a su característico perfil lipídico rico en grasas monoinsaturadas y sus propiedades antioxidantes (Ruiz-Gutiérrez et al., 1998) asociados a beneficios sobre la presión arterial y el colesterol (Storniolo et al., 2017), la reducción del riesgo de contraer ciertos tipos de cáncer (Reboredo-Rodríguez et al., 2018) y la prevención de enfermedades cardiovasculares (Salas-Salvadó et al., 2018).

1.2 DESARROLLO Y DESAFÍOS DE LA OLIVICULTURA EN UN CONTEXTO MUNDIAL DE EXPANSIÓN

En las últimas décadas, el incremento en la demanda de aceites de oliva se refleja en el aumento del 17,4 % en las exportaciones a nivel mundial entre 2013 y 2018 (García Galán et al., 2019). Esto incentivó la expansión desde la tradicional zona de producción mediterránea a nuevas áreas, tanto en el hemisferio norte como en el sur (Seifi et al. 2015, Ayerza y Sibbett 2001). La olivicultura en zonas no tradicionales viene presentando importantes problemas productivos, asociados en general a pobres cuajados de frutos que resultan en rendimientos insuficientes y/o inestables (Aybar et al. 2015, Zhu et al. 2013), o producciones alternantes (Conde-Innamorato et al. 2019,

Beyá-Marshall y Fichet 2017, Navarro-Ainza y López-Carvajal 2013). Dichos resultados han sido asociados a problemas de biología floral y/o de dispersión del polen (Vuletin Selak et al., 2014b), como consecuencia de efectos climáticos negativos como lluvias o vientos durante la floración y temperaturas extremas (Orlandi et al. 2010, Ayerza y Sibbett 2001, Connor y Fereres 2005), y a la limitación de polen compatible que puede representar una barrera a la fertilización y por consiguiente al cuajado (Ayerza y Coates 2004, Guerin et al., 2000). Estas condiciones son propias de las nuevas zonas de producción y también se presentan asociadas al cambio climático en las zonas tradicionales (Benlloch-González et al., 2018).

Con el objetivo de determinar el impacto del clima se han realizado trabajos en zonas no tradicionales pero de clima árido, dónde se focalizó en el posible impacto de las temperaturas extremas (Ayerza y Sibbett, 2001). Especialmente durante la floración, las temperaturas extremas podrían actuar reduciendo la duración del tiempo de vida de la flor (Cuevas et al., 2009), afectando las relaciones de compatibilidad entre los cultivares (Navarro-Ainza y López-Carvajal, 2013) y modificando procesos fenológicos (Aybar et al., 2015). La expansión de la olivicultura hacia nuevas regiones requiere información sobre la biología reproductiva y la fenología de los diferentes cultivares en respuesta a los principales factores ambientales (Aguilera et al. 2014, Orlandi et al. 2010). Sin embargo, poco se conoce sobre el comportamiento del olivo en ambientes caracterizados por la alta variabilidad interanual en cuanto a regímenes térmicos y de precipitaciones, dónde la ocurrencia de primaveras lluviosas, baja heliofanía y bajas temperaturas son frecuentes.

1.3 IMPORTANCIA DE LA DETERMINACIÓN DEL PERÍODO EFECTIVO DE POLINIZACIÓN PARA EL OLIVO EN REGIONES NO TRADICIONALES

El pasaje de una flor a fruto (cuajado) en olivos es dependiente de la fecundación. Para que la fecundación ocurra deben satisfacerse ciertos procesos previos que van desde el transporte del polen compatible hasta un estigma receptivo, pasando por la adhesión y posterior germinación del polen en el estigma, el crecimiento del tubo

polínico a través del estilo hasta alcanzar un óvulo viable. El Período Efectivo de Polinización (PEP) fue descripto por Williams (1965) como el período de tiempo durante el cual la flor está funcional para que la polinización pueda resultar en el cuajado de un fruto (Sanzol y Herrero, 2001). Este autor propuso que el PEP podía ser estimado a través del estudio de los componentes vinculados a la interacción polen-pistilo, como la longevidad del óvulo (LO) menos el tiempo requerido por el tubo polínico para crecer (CTP) desde el estigma hasta el óvulo, sin que este valor supere el período con receptividad estigmática (RE). El estudio de estos tres componentes necesarios para determinar el PEP puede ser realizado mediante la observación de ciertas características empleando microscopía de fluorescencia (Cuevas et al. 1994b, Stösser y Anvari 1982). Williams (1965) propuso que cuando no es posible determinar el PEP a través del estudio de sus componentes, éste podría inferirse por la respuesta de la planta a polinizaciones secuenciales, polinizando grupos de flores de una misma edad, en días sucesivos, y evaluando luego el cuajado resultante para cada fecha. El PEP entonces es determinado al comparar la tasa de cuajado en función de la edad de las flores y se considera finalizado cuando las flores ya no son capaces de transformarse en fruto. Si bien este método permite determinar la duración del PEP no logra identificar el componente que lo limita (Sanzol y Herrero, 2001).

El PEP fue estudiado para muchas especies frutales como manzano (Williams, 1965), peral (Sanzol et al., 2003), cerezo (Stösser y Anvari, 1982), damasco (Egea y Burgos, 1992), grosella (Tromp y Borsboom, 1994) y kiwi (González et al., 1995), en diversas condiciones climáticas, donde se observó importante variación entre años (Sanzol y Herrero, 2001) y entre cultivares (Cuevas et al. 2009, Brevis et al. 2006, Burgos et al. 1991, Williams 1965). Diferentes componentes han sido identificados como limitantes en diferentes situaciones. La receptividad estigmática ha sido identificada como limitante del PEP en perales (Sanzol et al., 2003) y cerezo (Hedhly et al., 2003) sometidos a altas temperaturas. La viabilidad de los óvulos se vio reducida con el aumento de la temperatura, limitando el PEP en ciruelos (Cerović et al., 2000). El crecimiento de tubos polínicos en duraznero se vio acelerado al aumentar la temperatura (Hedhly et al., 2004), aunque una menor cantidad de tubos polínicos alcanzaron la base del estilo en cerezo (Hedhly et al., 2005).

En olivos el PEP ha sido extensamente estudiado para las condiciones del mediterráneo. Para el cultivar 'Picual' se reportan variaciones de entre 6 a 12 días en condiciones mediterráneas (Cuevas et al., 2009) y de 4 a 8 días en Irán (Arzani y Javady, 2002), en tanto para el cultivar 'Manzanillo' fue estimado entre 3 y 4 días en las condiciones de California (Cuevas et al., 2009). Las diferencias obtenidas en los valores del PEP, están relacionadas al método utilizado para su estimación, ya que diferentes métodos han producido resultados contrastantes independientemente del cultivar analizado. El PEP estimado para los cultivares 'Lastovka', 'Leccino' y 'Levantinka' creciendo en las condiciones de Croacia y basado en el método de polinizaciones secuenciales fue de 2 a 3 días, mientras que las estimaciones en función del estudio microscópico de sus componentes fue de 5 a 12 días, dependiendo del cultivar (Vuletin Selak et al., 2014b). Se ha sugerido que la senescencia de los tejidos del estilo podría afectar negativamente el desarrollo de los tubos polínicos y fecundación de los óvulos. Esto explicaría las diferencias reportadas entre ambos métodos, sin embargo esto aún no ha sido confirmado (Vuletin Selak et al. 2014b, Cuevas et al. 2009).

1.4 NECESIDAD DE GENERAR CONOCIMIENTOS NACIONALES

Uruguay es un país nuevo como productor de aceites de oliva, con casi 7 mil hectáreas implantadas en las últimas dos décadas. En base a los requerimientos climáticos propuestos por Moriondo et al. (2008) Uruguay sería apto para el desarrollo de la olivicultura por su régimen térmico, aunque se desconoce si su amplia variabilidad interanual de regímenes térmicos y de precipitaciones podría llevarlo a situaciones alejadas del óptimo. En plantaciones experimentales con riego, el rendimiento anual en kilogramos de fruta superó las 8 toneladas por hectárea (incluyendo años "on" y "off"), con un contenido de aceite superior al 36 % en base seca (Conde-Innamorato et al., 2019). Estos valores son incluso superiores a los reportados en Chile para los mismos cultivares (Tapia et al., 2009). Los valores para el índice de alternancia (ABI por su sigla en inglés) muestran variaciones entre 0 y 25 toneladas por hectárea por año, similares a los mencionados para condiciones de Israel

por Lavee (2007). La alternancia se explica por factores endógenos, donde la carga de fruta determina una regulación negativa sobre el retorno a la floración. Los años de alta floración son los años de alta carga y los años de baja floración determina baja carga a pesar de un buen cuajado (Lavee, 2007). Sin embargo, factores del ambiente asociados a efectos bióticos o abióticos pueden hacer desaparecer la floración directamente o anular el cuajado, repitiendo ciclos "off" y acentuar la alternancia (Haberman et al. 2017, Guitton et al. 2012) o inclusive generar que el proceso se sincronice para toda una región (Conde-Innamorato et al., 2019). El clima en Uruguay se caracteriza por una alta variabilidad interanual en términos de temperatura y régimen hídrico con una temperatura media de 16 º a 19 º C y una precipitación media anual de entre 1100 y 1600 mm (Vaughan et al., 2017). Si bien no se registran temperaturas extremas, la probabilidad de tener 50 % de días con precipitaciones y humedad relativa superior a los 60-70 % entre octubre y noviembre es alta.

En Uruguay, 'Arbequina' es el cultivar más difundido, representa el 50 % del área olivícola y presenta en general muy bajos rendimientos o producciones erráticas. Muchos productores aún mantienen plantaciones monovarietales en el entendido de que 'Arbequina' es un cultivar autocompatible, si bien fue recientemente demostrada su autoincompatibilidad (Sánchez-Estrada y Cuevas, 2018). En otros casos se encuentra plantada con el cultivar 'Coratina' sin que existan reportes de su inter-compatibilidad. Adicionalmente, estudios sobre la auto e inter-compatibilidad en olivos han producido resultados ambiguos, sumado al hecho de que la compatibilidad puede ser influenciada por condiciones ambientales (Rejón et al. 2013, Koubouris et al. 2009, Lavee et al. 2002, Mekuria et al. 1999, Griggs et al. 1975).

Muchos aspectos de este cultivo se han investigado extensamente para su zona típica de producción en la cuenca del Mediterráneo. Dadas las diferencias climáticas y edáficas entre dicha zona y el Uruguay, no es posible la transferencia de los conocimientos y tecnologías generados en la zona clásica de cultivo y resulta necesario el desarrollo de estudios a nivel local. El siguiente trabajo se enmarca dentro del proyecto "Competitividad del cultivo del Olivo: Análisis tecnológico y económico", financiado por la Comisión Sectorial de Investigación Científica - Sector Productivo, donde se coordinan esfuerzos de equipos de investigación de Facultad de Agronomía,

Facultad de Ciencias y el Instituto Nacional de Investigación Agropecuaria, con el objetivo central de estudiar la adaptabilidad del cultivo a nuestras condiciones edafoclimáticas en favor de la consolidación del rubro. Específicamente, el proyecto busca conocer cómo se comportan, es decir, cómo florecen y cuajan y qué calidad de aceite producen los principales cultivares plantados en el país, cuáles son los rendimientos necesarios para cubrir los costos de producción, qué impacto tienen medidas de manejo como poda, aplicación de fitorreguladores y riego en el rendimiento. Parte del trabajo se realizó además en el marco de la asistencia técnica a la empresa olivícola "Nuevo Manantial S.A." cuyo objetivo fue realizar un seguimiento de los procesos involucrados en el cuajado del cultivar 'Arbequina' de manera de poder explicar los rendimientos obtenidos.

1.5 OBJETIVOS

En este trabajo se estudió la biología floral del cultivar 'Arbequina' en una región no tradicional para la olivicultura. Se focalizó en la interacción polen-pistilo, considerando: 1) la disponibilidad de polen compatible en el estigma, 2) la duración del período efectivo de polinización y 3) la funcionalidad de los tejidos del pistilo durante el tiempo de vida de la flor.

Específicamente se propone confirmar la autoincompatibilidad de 'Arbequina' en condiciones locales y su compatibilidad con los cultivares 'Coratina' y 'Picual', frecuentemente utilizados en las parcelas. Se propone estimar la duración del período efectivo de polinización mediante los dos métodos tradicionales utilizados en olivo y establecer si el crecimiento de los tubos polínicos depende de la edad de la flor.

2 THE EFFECTIVE POLLINATION PERIOD OF THE OLIVE CULTIVAR 'ARBEQUINA' (*Olea europaea* L.) IN A NON-TRADITIONAL REGION

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2.1 ABSTRACT

An increase in olive oil demand in the last decades, determined an expansion from the Mediterranean basin to new regions, in the northern and southern hemispheres. Olives in non-traditional producing regions are facing important problems, associated to poor fruit-set resulting in unstable fruit yields. That is the case of Uruguay in South America, where olive cultivation has experienced an important expansion, reaching 7000 hectares planted and becoming the second fruit crop at national level. However, yields obtained are lower than expected associated with a low fruit set. Edaphoclimatic conditions in Uruguay, characterized by a high interannual variability in thermal and rainfall regime with mean temperatures between 16° to 19°C and annual mean precipitation between 1,100 to 1,600 mm, are in contrast to those of the Mediterranean region. It is hypothesized that these environmental conditions may influence the reproductive biology and affect the effective pollination period (EPP) limiting fruit set. In this work, after three year of experiments, we determined the EPP for 'Arbequina', the main olive cultivar in Uruguay, by means of the two traditional methodologies, as a response in fruit setting to sequential pollinations depending on the age of the flower, and by fluorescence microscopy analysis of its components (stigmatic receptivity, ovule viability and pollen tube growth). The EPP duration for

the Uruguayan conditions was consider to be at least 7 days, with no differences between the estimation methods. Stigmatic receptivity, assessed by the capacity to support pollen adhesion and germination, was maintained for at least 20 days post anthesis (DPA). More than 50 % of the evaluated flowers kept at least one viable ovule 23 DPA. Pollen tubes required between two and three days to grow from the stigma until reaching an ovule. No differences were found for the pollen tube growth in relation with the pistil age. Results obtained for 'Arbequina' EPP in Uruguay indicate that it is not limiting and it is interpreted that the lack of compatible pollen in the orchards could limit fruit set.
Key words: stigmatic receptivity, ovule viability, pollen tube growth, microsatellites (SSRs)

2.2 INTRODUCTION

An increase in olive oil demand in the last decades, reflected in world exports of olive oil that increased 17.4 % between 2013 and 2018 (García Galán et al., 2019), determined an expansion from the Mediterranean basin to new regions, in the northern and southern hemispheres (Ayerza and Sibbett, 2001; Seifi et al., 2015). Olives in non-traditional producing regions, where little information about crop function is available, are facing important problems, associated to poor fruit-set resulting in unstable fruit yields (Zhu et al., 2013; Aybar et al., 2015, Torres et al., 2017), in addition to the generalized alternate bearing (Navarro-Ainza and López-Carvajal, 2013; Beyá-Marshall and Fichet, 2017, Conde-Innamorato et al., 2019). These problems, in the Mediterranean basin, have been associated with floral biology and/or problems in pollen dispersal (Vuletin Selak et al., 2014b). In order to assess the impact of climate, some works have been done in arid non-traditional regions, focusing on the possible impacts of extreme temperatures (Ayerza and Sibbett, 2001). Extreme temperatures especially during flowering, could shorten the life span of flowers (Cuevas et al., 2009), by reducing ovule longevity or pollen tube growth rate which could affect as a consequence the compatibility relations among cultivars (Navarro-Ainza and López-Carvajal, 2013) or the flowering time (Aybar et al., 2015). Expansion of olive

production to new regions requires information about the flowering response of different cultivars to key environmental factors (Orlandi et al., 2010; Aguilera et al., 2014). However, little is known about the behavior of olive in environments with high interannual variability in thermal and rainfall regimes such as those of Uruguay where the occurrence of rainy springs with low heliophany and low temperatures is usual.

Fruit set depends on a number of combined reproductive processes. These processes range from the pollen transfer to receptive stigmas, through pollen adhesion and germination in the stigma and pollen tube growth in the pistil until it reaches a viable ovule. This leads to the concept of Effective Pollination Period (EPP), described by Williams in 1965, as the period of time during which pollination can result in fruit set (Sanzol and Herrero, 2001). This author proposed that the EPP could be estimated by the study of the components of pollen-pistil interaction. The EPP is the result of ovule longevity (OL) minus the time needed for a pollen tube to grow from the stigma until it reaches a viable ovule (PTG), provided that this value does not exceed the period of stigmatic receptivity (SR). The duration of the EPP can be estimated by the analyses of these three components by fluorescence microscopy (Vuletin Selak et al., 2014b). Williams (1965) proposed that the EPP could also be inferred by plant response to sequential pollinations of successive cohorts of flowers of increasing age and evaluating the resulting fruit set. EPP is determined by comparing the rates of fruit set of the different cohorts of flowers, until they produced a reduced fruit set. Although this method allows to determine the duration of EPP, it does not allow to identify which component is limiting the EPP (Sanzol and Herrero, 2001). The EPP has been studied for many species in different climatic conditions with important variation among years and cultivars. The EPP in olives has been largely studied for the Mediterranean basin, for cv. 'Picual' it may vary between 6 and 12 days (Cuevas et al., 2009) or between 4 and 8 days (Arzani and Javady, 2002). For cv. 'Manzanillo' in California it has been estimated to last between 3 and 4 days (Cuevas et al., 2009). The differences in the EPP, are attributed to the method used, since different methods provide contrasting results regardless of the cultivar analyzed. The EPP estimated, for cultivars 'Lastovka', 'Leccino' and 'Levantinka' growing in Croatia conditions, based on initial fruit (IFS) set and final fruit set (FFS) was between 2 and 3 days, while based on the microscopic

study of EPP components it was between 5 and 12 days, depending on the cultivar (Vuletin Selak et al., 2014b). Although it has been suggested that style senescence should be taken into account as a new component in the EPP study, as could prevent pollen tubes from reaching and fertilizing the ovules this has not been confirmed (Cuevas et al., 2009; Vuletin Selak et al., 2014b, 2014a).

Uruguay, in South America, is a new olive oil producer country, with around 7 thousand hectares planted in the last two decades, and it is considered suitable for the development of olive production in terms of thermal requirements with a humid subtropical climate marked by strong inter-annual variability (Moriondo et al., 2008). In our experimental conditions under irrigation, annual average fruit yields including "on" and "off" years, exceeded 8 t/ha with oil contents above 36 % dry weight basis (Conde-Innamorato et al., 2019), but they identified alternate bearing was identified as the main productive constraint, evidenced by alternate bearing index (ABI) values above 0.60, 60 % higher than those previously reported for the same cultivars (Tapia et al., 2009). These alternate bearing index (ABI) values imply fruit yield variations between 0 and 25 t/ha per year, similar to those mentioned by Lavee (2007) in Israel. The years of high flowering are the years of high load and the years of low flowering determine low load despite a good fruit setting (Lavee, 2007). However, environmental factors associated with biotic or abiotic effects could cause massive flower dropping which cancels fruit set, repeating "off" cycles and accentuating alternate bearing (Haberman et al. 2017, Guitton et al. 2012) or even generate an alternate bearing synchronized in a region (Conde-Innamorato et al., 2019). The climate of the experimental area is characterized by a high interannual variability in thermal and rainfall regime with a mean temperature between 16° to 19°C and annual mean precipitation between 1,100 to 1,600 mm (Vaughan et al., 2017). Unlike in other non-traditional regions with arid climates, extreme temperatures are not common; however, the probability of having 50 % of the days with rain, and relative humidity above 60-70 % in October and November, during the flowering period, is considerable. In Uruguay, 'Arbequina' represents 50 % of the total olive area, and even though it has recently classified as self-incompatible (Sánchez-Estrada and Cuevas, 2018), many producers still have monovarietal orchards or combined plantations with cultivars like

‘Coratina’ without knowledge of their inter-compatibility. As a consequence, in many of those situations, ‘Arbequina’ is showing low yields or erratic productions. In addition, studies of self and inter-compatibility between olive cultivars have produced ambiguous results, suggesting that compatibility is often influenced by environmental conditions (Griggs et al., 1975; Mekuria et al., 1999; Lavee et al., 2002; Koubouris et al., 2009; Rejón et al., 2013). In this work, we studied the flower biology of ‘Arbequina’ growing in the climatic conditions of a humid subtropical non-traditional olive producing region. We focused on the pollen-pistil interaction, considering: 1) the compatibility relationships with the most commonly available pollen donor cultivars, 2) the EPP duration by different methodologies and 3) the functionality of the pistil tissue during the life-span of the flower.

2.3 MATERIALS AND METHODS

2.3.1 Evaluation of cultivars compatibility relations and pollen availability

A compatibility trial was carried out during 2017 in a commercial orchard located in Rocha-Uruguay (34º 04’ S; 54º 24’ W; altitude 125 m). We used a randomized complete block design (RCBD) with ten three-year-old ‘Arbequina’ trees (blocks) from a rainfed orchard with ‘Coratina’ as pollinizer in a 3:1 relation. All trees were selected for their good sanitary and nutritional status and good development of annual shoots. Four treatments were compared: self-pollination (SP), open-pollination (OP), cross-pollination with ‘Coratina’ (XC) and cross-pollinations with ‘Picual’ (XP). Annual shoots with at least 100 perfect flowers were selected and two shoots per treatment were tagged in each of the ten ‘Arbequina’ trees. Open flowers and stages previous to stage defined by Vuletin Selak et al. (2014b) as white balloon were removed (Fig. 1A). Unwanted cross-pollination was prevented by enclosing shoots within rayon fabric bags, except for the open-pollination treatment where shoots were not enclosed. On treatments XC and XP, shoots were pollinated by using directly shoots with open flowers of the pollen donor cultivar as paint brushes. This procedure was repeated three times every three days. For all pollination treatments, pollen viability was previously checked with 2,3,5-triphenyl tetrazolium chloride (TTC)

(Cook and Stanley, 1960). Initial fruit set (IFS) and final fruit set (FFS) was expressed as the percentage of enlarging fruits (Fig. 1C) relative to the initial number of flowers in the branch, recorded 20 (IFS) and 45 (FFS) days after pollination (DAP) respectively. The percentage of fruit set was analyzed in R Software (R Core Team, 2018) by a Generalized Linear Model (GLM) with a quasibinomial approach (due to overdispersion), including date and pollinizer as fixed effects and plant as a random effect, using packages lme4 (Bates et al., 2015), emmeans (Searle et al., 1980), multcomp (Hothorn et al., 2008), multcompView (Graves et al., 2012), ordinal (Christensen, 2019), blmeco (Korner-Nievergelt et al., 2015), RVAideMemoire (Hervé, 2015). The significance of fixed effects was tested with an analysis of deviance and a Tukey test was used for multiple mean comparisons ($p < 0.05$).

For paternity analysis, fruits were collected on February and kept at -18°C until the molecular analysis. A total of 88 fruits, 20 from the treatment XC, 18 from XP, 30 from OP and 20 from SP, were analyzed to determine the pollen donor. The DNA extraction was made directly from ungerminated seeds. For this, the mesocarp, endocarp and seedcoats were removed. When two embryos were found inside the same fruit they were treated as different samples. DNA extraction was performed following Doyle and Doyle (1987) and Collins and Symons (1992) protocols. In addition, DNA from mature leaves of the different cultivars presented in the field (putative fathers) was extracted in order to obtain their profiles for fourteen microsatellite loci (Sefc et al., 2000; Carriero et al., 2002; Cipriani et al., 2002). After that, the probability of non-exclusion of each marker was calculated using CERVUS v.3.0.7 (Kalinowski et al., 2007) and four of them (ssrOeUA-DCA9, ssrOeUA-DCA16, UDO99–011, UDO99-043), with a combined probability of no-exclusion for identity of 6,84E-06, were selected for paternity analysis based on their discrimination power.

PCR amplification for each microsatellite was performed in a reaction volume of 15 µL containing PCR buffer (ThermoFisher) 1x, $MgCl_2$ 1.5 mM, dNTPs 0.15 mM, M13 0.40 µM, forward primer 0.30 µM, reverse primer 4.5 µM (both fluorescent phosphonamidites FAM, HEX), BSA 0.80 µg, Taq 0.60 U/µL, ADN 150 µg. Volume was adjusted to 15 µL with ultrapure water. Amplification was performed following a temperature cycling parameter initial denaturation at 95 ºC for 5 min, 35 cycles of

95°C for 20 s, 30 s at the published annealing temperatures (Sefc et al., 2000; Carriero et al., 2002; Cipriani et al., 2002) and 72°C for 30 s and a final extension at of 72°C for 8 min. Amplifications were checked in an agarose gel (1 %) stained with ethidium bromide. Sequencing was performed in the DNA fragment service provided by Yale University and data were analyzed with the software Geneious R9© and Peak Scanner™ Version: 1.0. Paternity tests of the seeds were performed with Cervus (Kalinowski et al., 2007), using the paternity analysis (mother known/father unknown) with the maximum likelihood approach (LOD) (Marshall et al., 1998; Kalinowski et al., 2007). The most likely father was determined from the log-likelihood ratios (LOD score) based on the genotypes of the offspring, known mother, and each candidate father (including the mother itself as a putative father), putative fathers were considered as the cultivars with the highest LOD score and an interval of confidence of at least 95 %.

2.3.2 EPP determination by the analysis of components and by fruit set resulting from sequential pollinations

The EEP study and analysis of its components was performed on 12-year-old 'Arbequina' olive trees growing in a multivarietal orchard at INIA "Las Brujas" Experimental Station in Southern Uruguay (34°40' S; 56°20' W; altitude 21 m). The study was conducted during the 2018 and 2019 flowering seasons where 4 and 3 trees were selected, respectively. Two trees of 'Coratina' and another two of 'Picual' located in the same orchard were used as pollinizers. Field work started in October 20th and 24th for 2018 and 2019 respectively in coincidence with the beginning of the flowering period defined when trees bore 10 % of flowers at anthesis, according with the phenological phase BBCH 61 (Sanz-Cortés at al., 2002). Components of the EPP, stigma receptivity (SR), pollen tube growth rate (PTW), and ovule longevity (OL) in 'Arbequina' trees were determined using fluorescence microscopy. In local conditions, bloom is usually asynchronous and not all the flowers in the panicles are in the same stage at the same time, so it was decided to isolate them and work on individual flower, prioritizing apical flowers or in the subsequent levels of the panicle. Individual flowers were tagged, emasculated at the white balloon stage and covered with an aluminum

cap to prevent unwanted pollination (Fig. 1B). Pollination was performed at the scheduled day, fixed in FAA (formaldehyde, acetic acid, ethanol 70 %, 5: 5: 90), changed to ethanol 70 % after 24 h, until microscopy observation. Fluorescence microscopy was performed according to the methodology proposed by Martin (1958) with hypochlorite as softener. The microscope used (Nikon Labophot, Tokyo, Japan) was equipped with a U filter (exciting 330-380, dichroism 400 and cut-off 420). Given that stigmatic receptivity and ovule longevity are independent processes and take place in different places, both determinations were carried out in the same flower. Stigmatic receptivity and ovule longevity were determined on 20 flowers per treatment, 5 flowers from XC and 5 from XP, per tree (4), per sampling date (7). After emasculation, flowers were pollinated directly using the anther of the pollen donor from the date of anthesis (day 1) until day 7 DPA. Stigmatic receptivity evaluation was performed by observing pollen grains adhered and germinated on the stigma by fluorescence microscopy. Since when samples are softened with hypochlorite, pollen grains in the stigma become discolored and cannot be individualized, a stigma was considered receptive when a mass of pollen tubes was observed growing on it. Ovule longevity was assessed by fluorescence as described for Stösser and Anvari (1982). Flowers were only squashed to separate the ovules from the rest of the gynoecium after staining. Each ovule was classified as 0: viable o 1: non-viable based on the presence of yellow rings or areas in the ovule (Cuevas et al., 1994c). An individual flower was considered fertile when at least one of its four ovules was viable (Cuevas et al., 2009). Results were expressed as the proportion of flowers with at least one viable ovule per sample date and the proportion of non-viable ovules was analyzed using a GLM, assuming binomial distribution. The significance of the fixed effects was tested with an analysis of deviance and a Tukey test was used for multiple mean comparisons ($p < 0.05$).

Figure 1: 'Arbequina' panicle showing A: the 'white balloon stage' (circle) in which emasculation was performed, B: aluminum cap used to prevent unwanted pollination after emasculation and C: fruits setting

Pollen tube growth was determined in 20 flowers per treatment (5 flowers from XC and 5 XP per tree (4) per sampling date (7). Flowers were fixed in FAA from day 1 to 7 after pollination. A self-pollination treatment was also applied by pollinating flowers with their own pollen, and some other remained unpollinated to assess the occurrence of pollen contamination under the aluminum cap. Flowers were processed for observation by fluorescence microscopy as previously described. Pollen tube growth was evaluated for each flower in a scale from 0 to 6 where 0 corresponds to non-germination of pollen grains, 1: growth at the level of the stigma, 2: pollen tubes do not exceed 15 % of the style, 3: 50 %, 4: 100 %, 5: growth into the ovary, and 6: at least one ovule penetrated by a pollen tube. For pollen tube growth a Cumulative Link Model (CLM) was used and a Tukey test was used for multiple mean comparisons ($p < 0.05$).

The duration of the EPP inferred by fruit set resulting from sequential pollination at anthesis and at increasing days after was performed in 2018. Shoots with at least 100 perfect flowers of consistent age were used for pollination at each date. Consistent flower age was assured by removing open flowers one given day and by eliminating closed flowers the following day. Unwanted cross-pollination was prevented by

enclosing shoots within fabric bags until the scheduled pollination date. Flowers were not emasculated in this experiment to minimize damage to the shoots. Two shoots per pollination day (from day 1 to 7 after anthesis) were pollinated with 'Coratina' and another two with 'Picual'. Shoots were pollinated by directly using shoots with open flowers of the pollen donor cultivar as paint brushes. Two shoots per tree remained bagged to determine the level of fruit set in self-pollinated while two remain un-bagged as controls (OP treatment). Initial and final fruit set were determined and analyzed following the same procedure described for the compatibility trial. To corroborate the correct pollination, a subset of 5 flowers from each branch were fixed in FAA 24 h after pollination and the presence of pollen tube growth observed by means of fluorescence as described above.

In order to confirm the identity of the fruit obtained and rule out the occurrence of pollen contamination, fruits from each branch pollinated on date 7 (with highest risk of pollen contamination), as well as that from open and self- pollination, were harvested and kept in separated bags at -18 ºC until the DNA extraction. The analyzed fruit were 49 from XC; 49 from XP; 50 from OP and 16 from SP treatment. The protocol used for paternity testes was the same that the one described above for the compatibility trial, with the only difference that six more SSRs (ssrOeUA-DCA3, ssrOeUA-DCA11, ssrOeUA-DCA18, GAPU101, GAPU71B, GAPU103A) were used the reduce possible errors of identifications for the presence of more possible pollen donor in the experimental field.

2.3.3 Evaluation of pistil aging effects

The effect of pistil aging in pollen tube growth was evaluated in 30 flowers per date. Flowers were emasculated at the white balloon stage, covered with aluminum cap and pollinated with 'Coratina' pollen at 3, 6, 9, 12, 14, 17 and 20 DPA, after fixed in FAA 3 DAP. To rule out the occurrence of pollen contamination under the aluminum cap, some flowers remained unpollinated. Pollen tube growth, stigmatic receptivity and ovule viability were determined by fluorescence microscopy. Results for ovule longevity and stigmatic receptivity were expressed as above, and the proportion of flowers where pollen tubes reached the ovary or penetrated an ovule was

compared between sample dates using a GLM, assuming a binomial distribution. A Tukey test was used for multiple mean comparisons ($p < 0.05$).

2.4 RESULTS AND DISCUSSION

2.4.1 Evaluation of compatibility relations and pollen availability

Trees used in trials had their identity verified as SSR profiles matched those reported for each cultivars in the world bank of olive germplasm (Table 1) (Trujillo et al., 2013). High fruit sets were obtained after pollinations with 'Coratina' and 'Picual' (Fig. 2). No statistical differences were found between the fruit set under OP relative to the fruit set of the treatment XC. Pollination with 'Coratina' resulted in a statistically higher fruit set than pollination with 'Picual'. On the other hand, SP yielded the lowest fruit set percentage (less than 5 %). Even though IFS was always higher than FFS, the ranking between treatments was the same in IFS and FFS.

Table 1: SSRs profiles obtained for plants used as mothers and as pollen source

SSR	Arbequina	Coratina	Picual
ssrOeUA-DCA3	231-241	237-241	237-247
ssrOeUA-DCA9	182-204	180-192	160-182
ssrOeUA-DCA11	140-178	130-130	140-178
ssrOeUA-DCA15	246-265	246-246	246-265
ssrOeUA-DCA16	122-144	148-171	122-124
ssrOeUA-DCA18	164-174	172-176	166-172
GAPU59	206-220	210-210	210-220
GAPU 71B	121-141	121-141	118-127
GAPU 101	183-206	197-217	191-199
GAPU 103	147-157	133-159	133-133
UDO99-011	116-129	114-131	116-119
UDO99-011	175-175	175-198	208-212

Percentages of fruit set were around 25 and 20 % for initial and final fruit set in 'Arbequina' shoots in OP as well as in XC pollinations. Although this kind of study involves small and manipulated shoots and therefore, extrapolation to the orchard level

is limited, the fruit set rates obtained are in agreement with those reported in similar works for the Mediterranean condition (Cuevas et al., 2009; Vuletin Selak et al., 2014b, 2014a). OP proved to be enough to obtain a high fruit set with a high proportion of pollinizers in the orchard (3:1 relation). In these conditions, XC matched with the optimum usually represented by open unrestricted pollination in multivarietal orchards, the supplementation with pollen grains in cross-pollinations with 'Coratina' (the pollinizer of the orchard) did not improve fruit set. This orchard had a higher pollinizer proportion than the recommend 50-50 pollinizer relation (Sánchez-Estrada and Cuevas, 2020), and pollinizers were located at distances smaller than 30-40 m which have been shown to assure a correct pollination (Griggs et al., 1975).

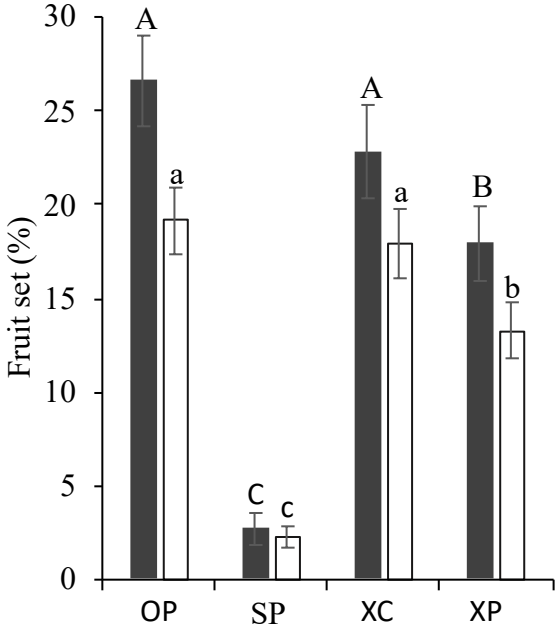

Figure 2: Percentage of initial and final fruit set in dark and white bars respectively for 'Arbequina' trees after open-pollination (OP), self-pollination (SP), cross-pollination with 'Coratina' (XC) and cross-pollination with 'Picual' (XP). Bars show standard error. Different letters indicate significant differences ($p < 0.05$) among treatments for IFS (capital letters) and FFS (lower case letters)

Paternity analyses confirmed that fruits harvested from the shoots cross-pollinated with 'Picual' and 'Coratina' were all consequence of the cross-pollination made (Table 2). Non pollen contamination was found in the shoots pollinated with

‘Coratina’ and ‘Picual’. None of the sixteen fruits obtained in the SP shoots were authentically self-fathered, then 80 % were the product of contamination with ‘Coratina’ (the pollinizer cultivar of the orchard), the remaining one fruit corresponded with another cultivar presented in field at greater distances than ‘Coratina’, ‘Koroneiki’, and only one could not be assigned to any cultivar.

Table 2: Percentage of olive embryos assigned to each putative father after SP: self-pollination, OP: open-pollination, XC: cross-pollination with ‘Coratina’ and XP: cross-pollination with ‘Picual’

Putative father	SP	OP	XC	XP
Arbequina	0	0	0	0
Coratina	80	100	100	0
Picual	10	0	0	100
Other	10	0	0	0

*Number of embryos analyzed per treatment: SP = 20, OP = 36, XC = 20, XP = 18, putative fathers were assigned with at least 95 % of confidence; error rate = 0, mismatch for the trio loci = 0

Self-incompatibility in olive represents a major obstacle for sexual reproduction, the existence of a diallelic self-incompatibility system involving only two compatibility groups has been suggested, such that successful fruit production requires pollination between cultivars from different groups (Mariotti et al., 2020). Consequently, we have shown that ‘Coratina’ and ‘Arbequina’ are inter-compatible. Self-incompatibility in ‘Arbequina’ based on fruit set and analysis of pollen-pistil interactions was verified in this work as previously reported (Sánchez-Estrada and Cuevas, 2018) in agreement with previous works where molecular information (SSRs) was used (Díaz et al., 2006; Shemer et al., 2014; Marchese et al., 2016). Inter-compatibility between ‘Arbequina’ and 'Picual', previously reported by Díaz et al. (2006), was also verified. These results do not provide evidence for environmental changes in compatibility relationships (Seifi et al., 2011; Marchese et al., 2016). We showed that the low fruit set or fruit number obtained on self-pollinated shoots was completely caused by pollen contamination, reinforcing the utility of paternity tests as

reported by different authors (Mookerjee et al., 2005; Díaz et al., 2006; Seifi et al., 2012; Saumitou-Laprade et al., 2017; Breton and Bervillé, 2018; Farinelli et al., 2018; Montemurro et al., 2019; Besnard et al., 2020).

2.4.2 EPP determination by the analysis of components

2.4.2.1 Stigmatic receptivity

According to the fluorescence microscopy observations, flowers of 'Arbequina' were able to support pollen grain adhesion and germination for the full sampling period of one-week. The total number of analyzed flowers supported abundantly these processes (Fig. 3). The high amount of pollen grains adhered made it impossible to count the exact number. No differences between 'Picual' or 'Coratina' as pollinizers were observed. Low pollen grain adhesion was observed in SP flowers, but absence of pollen tube growth.

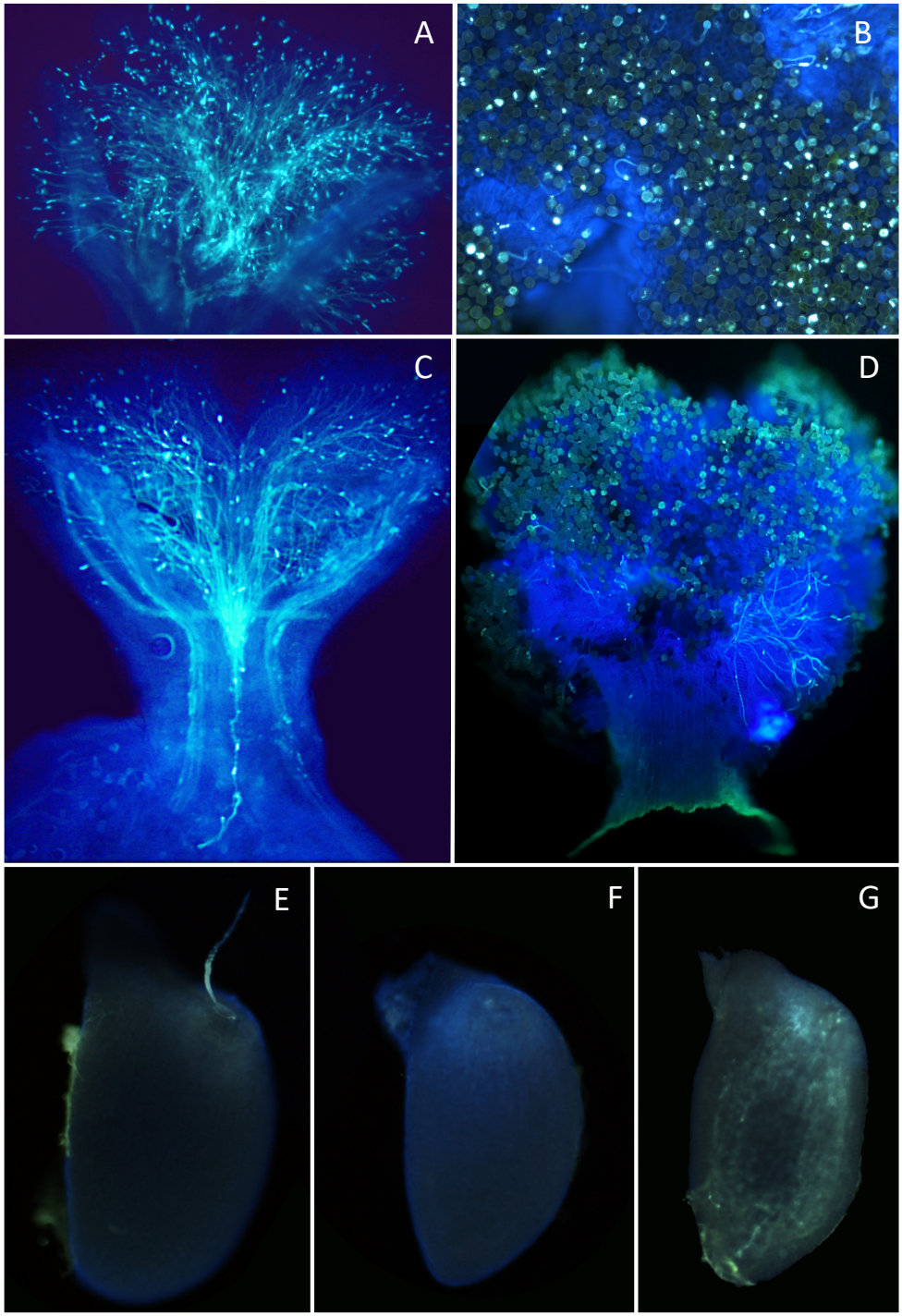

Figure 3: Pistils and ovules of 'Arbequina' stained with methyl blue. A: stigma with abundant pollen tubes growing. B: stigma with pollen grains adhered. C: pollen tube growth through the style. D: Squashed stigma with pollen grains adhered and pollen tubes growing. E: ovule reached by a pollen tube. F: viable ovule. G: non-viable ovule. A and C softened with hypochlorite, B, D-G with NAOH

2.4.2.2 Pollen tube growth

Pollen tube growth evaluations showed that no pollen tube reached an ovule, for both cultivars used as pollinizers, during the first day after pollination. The first ovule penetrated by a pollen tube was observed 2 and 3 DAP for XC and XP, respectively. Statistical differences were found for pollen tube growth on XP, between the first and second DAP, while non statistical differences were found after 3 and 7 DAP, maybe due to the fact that most pollen tubes had already passed the base of the style 3 DAP (Fig. 4). Results obtained on XC were very similar to XP; nevertheless, pollen tube growth was significantly lower on the first DAP, and values 2 DAP were lower than values 5 to 7 DAP. On the SP treatment, no pollen tube or stylar tissue growth was observed. In this case, growth was reduced to some pollen tubes germinated with pollen tubes growing only at the level of the stigma (data not shown).

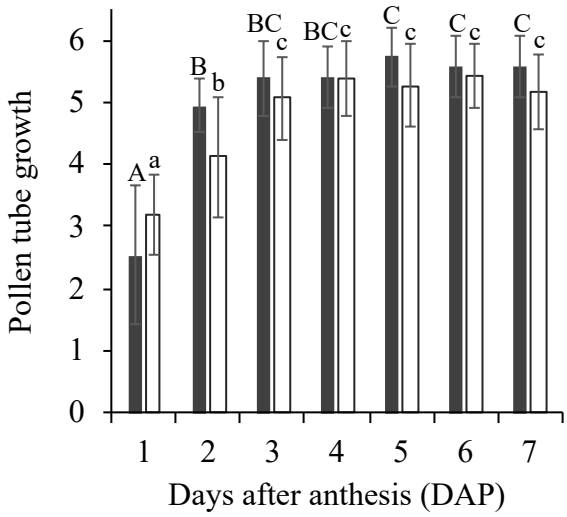

Figure 4: Pollen tube growth in 'Arbequina' flowers pollinated with 'Coratina' (dark bars) or 'Picual' (white bars) evaluated in an ordinal scale where 0 means no growth, 1: growth at the level of the stigma, 2: pollen tubes do not exceed 15 % of the style, 3: pollen tubes do not exceed 50 % of the style, 4: pollen tubes reached the base of the style, 5: pollen tube growth into the ovary, 6: observation of at least one penetrated ovule. The flowers were fixed at increasing days after pollination (DAP). Values are means, n=20. Error bars show standard error. Different letters represent significant

differences between dates for ‘Coratina’ (capital letters) and ‘Picual’ (lower case) ($p < 0.05$)

2.4.2.3 Ovule longevity

In 2018, no significant differences were found in the proportion of flowers with at least one viable ovule during the one-week sampling period (more than 80 %) (Fig. 5A). Also, no differences were found for the proportion of non-viable ovules per sampling date (Fig. 5B).

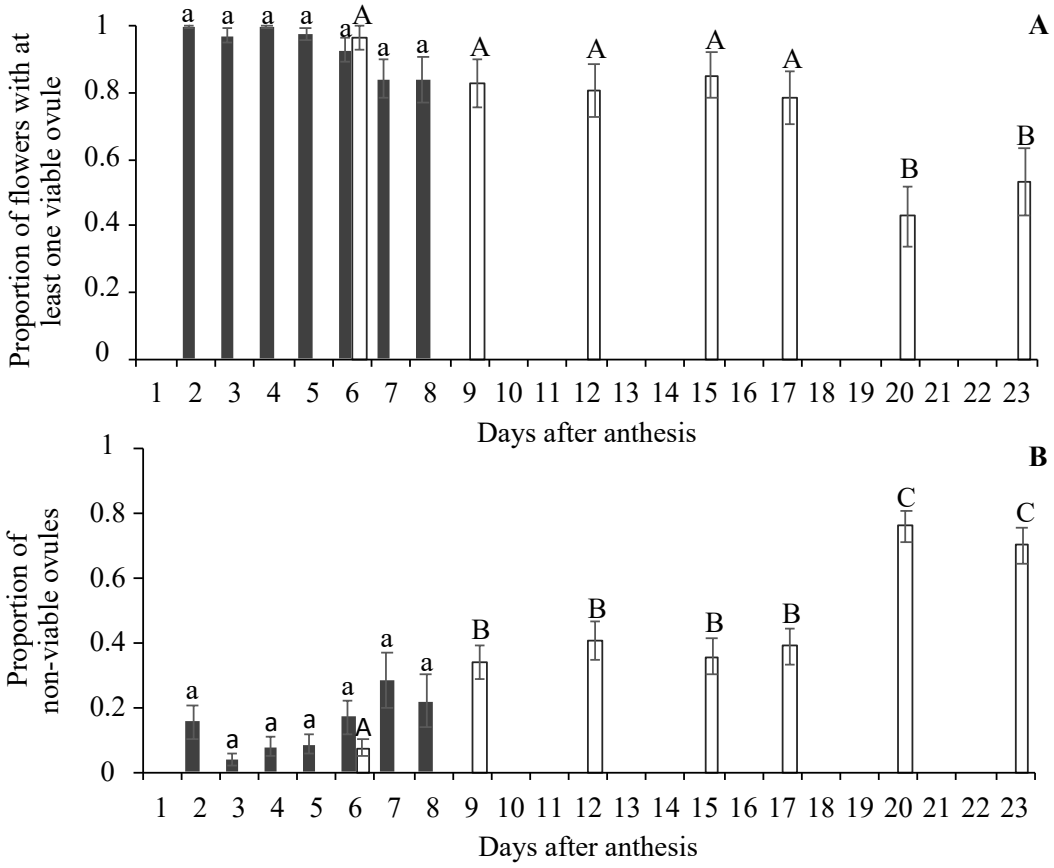

Figure 5: A: Proportion of flowers with at least one viable ovule at increasing days after anthesis in 2018 (dark bars) and 2019 (white bars). B: Proportion of non-viable ovules at increasing days after anthesis for 2018 (dark bars) and 2019 (white bars). Bars show standard error. Different letters show significant differences between dates for 2018 (lower case) and 2019 (capital letters) ($p < 0.05$)

2.4.3 EPP determination by fruit set resulting from sequential pollinations

IFS and FFS for 'Arbequina' trees after sequential pollinations for a period of one-week did not differ between XC and XP (Fig. 6). FFS percentage was lower than IFS in all of the treatments. Fruit set in OP shoots (not shown) was similar to cross-pollinations for both IFS and FFS. Only one of the 6 shoots from SP exhibited fruit set (16 fruits in total), the rest contained only some "shotberries". In all flowers fixed 24 h after pollination from each pollinated branch, stigmatic receptivity was confirmed and pollen tube growth was observed. No-statistical differences were found between pollinizers or dates. No penetrated ovules were found in these flowers 24 h after pollination. Maximum pollen tube growth reached the base of the style. No stylar growth was observed in self-pollinated shoots.

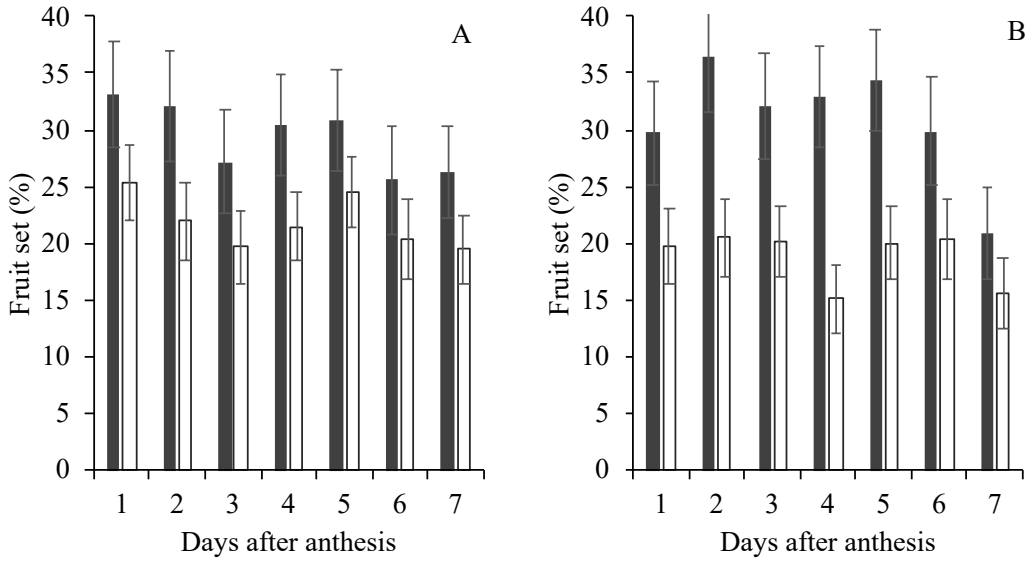

Figure 6: Percentage of Initial (dark bars) and final (white bars) fruit set for 'Arbequina' trees after pollinations at the day of anthesis (day 1) and at increasing days after anthesis. A: Pollination with 'Picual' and B: pollination with 'Coratina', error bars show standard error, no significant differences were observed among dates

Paternity analysis performed on the fruits harvested from shoots pollinated 7 DAP confirmed that these fruits were all the product of the cross-pollination done (Table 3). Pollen grain contamination observed was very low, corresponding to 4 % and 16 % for XP and XC respectively. In XC, 10 % corresponded to a single branch

with the total fruit assigned to ‘Picual’ as the most probable father, so we infer that this can be attributed to a human error. This behavior was similar to the observed for the only one SP branch where almost 70 % of the fruits were attributed to ‘Coratina’ as putative father. No fruits from the 164 analyzed had ‘Arbequina’ as father and 34 % of the fruits from OP shoots did not have any of the cultivars used in the work as father.

Table 3: Percentage of embryos per branch and total (T) assigned to each putative father after SP: self-pollination, OP: open-pollination, XC: cross-pollination with ‘Coratina’ and XP: cross-pollination with ‘Picual’.

Putative father	SP	OP							XC							XP				
		Branch							Branch							Branch				
	T	1	2	3	4	5	6	**T**	1	2	3	4	5	6	**T**	1	2	3	4	**T**
Arbequina	**0**							**0**							**0**					**0**
Coratina	**68**			16		4	6	**26**	8	12		12	29	23	**84**		2			**2**
Picual	**13**	2			20	6	12	**40**			10			2	**12**	27	20	29	20	**96**
Other	**19**	2	10	8	2	2	10	**34**				2		2	**4**				2	**2**

*Number of embryos analyzed per treatment: SP = 16, OP = 50, XC = 49, XP = 49, putative fathers were assigned with at least 95 % of confidence; error rate = 0, mismatch for the trio loci = 0

2.4.4 Evaluation of pistil aging effects

No-statistical differences between the proportions of flowers with at least one viable ovule were found for the first 17 DAP, where the proportions were always higher than 80 % (Fig. 5). The proportion observed 20 and 23 DAP was lower than that recorded in the previous sampling dates, but it was higher than 50 %, even for the last sampling date (23 DAP). A progressive increase in the proportion of non-viable ovules was observed. The proportion of non-viable ovules showed a marked increase 20 and 23 DAP, with more than 70 % of the analyzed ovules showing symptoms of senescence (Fig. 5B). Massive pollen grain adherence and germination was observed in all flowers analyzed, for all the flower ages. No statistical differences were found

between the proportion of flowers where pollen tubes reached or exceeded the base of the style relative to flower age, even for 20-day-old flowers.

2.4.5 Integration of EPP estimation methods results

Our results showed that EPP estimated by fruit set resulting after sequential pollinations was maintained for at least 7 days, in agreement with EPP reports for olives in the Mediterranean basin which range between 4 and 12 days (Bini, 1984; Arzani and Javady, 2002; Pinillos and Cuevas, 2009). Longer than reports for EPP in California conditions of 3 or 4 days (Cuevas et al., 2009) or even for studies carried out in Mediterranean conditions of only 2 - 4 days (Vuletin Selak et al., 2014b, 2014a). Good correlations between EPP estimation methods were found in other fruit species (González et al., 1995; Sanzol et al., 2003). Nevertheless, previous studies in olive trees reported that the duration of the EPP estimated from delayed pollinations was shorter than that estimated from the components of the EPP (Cuevas et al., 2009; Vuletin Selak et al., 2014b, 2014a). These authors suggest that discrepancies could be due to the loss of functionality of the style because senescence might prevent the growth of pollen tubes on their way to the ovules, even though viable ovules are still present in the ovary. This highlights the importance of considering the role played by the style in the growth of the pollen tubes toward the ovules, as was observed in some species (Egea and Burgos, 1992, Nepi and Pacini, 2001). In the present study, no differences in pollen tube growth were found. Although we focused on the role played by each one of the EPP components to identify the limiting factor, as proposed for Sanzol and Herrero (2001), we could not identify any limiting EPP components since all stigmas analyzed remained receptive until the end of the sampling period which extended to 7 and 20 Days After Anthesis (DAA) each year respectively.

For the estimation of ovule longevity, an ovule was considered senescent when full rings of yellowish fluorescence was observed, which indicates an obstruction of conductive tissues with the corresponding loss of functionality (Cuevas et al., 1994c). In this work, no differences in the proportion of flowers with at least one viable ovule were found for the period of 7 days studied in the first year. In the second year, a significant proportion of non-viable ovules appeared 20 and 23 DAA although the

percentage of flowers with at least one viable ovule was greater than 50 %. Even if in other species this component appears as a limiting factor, it seems that olive trees are less sensitive since in several cultivars it was found to last beyond the end of the sampling period (>9 DAA or 7 DAA) (Vuletin Selak et al., 2014a), 16-20 days for 'Picual' (Cuevas et al., 2009), more than 50 % of ovaries with viable ovules 20 DAA (Fernández-Escobar et al., 2008), or 22 DAA in 'Arbequina' (Cuevas, 1992). Previous works argue that this method may overestimate ovule longevity in olive due in part to the difficulty in assessing the beginning of senescence (Cuevas et al., 2009; Vuletin Selak et al., 2014b).

Pollen tube growth was fast for both cultivars used as pollinizers. The first penetrated ovule was observed 2 days after pollination for XC and 3 DAP for XP. Most pollen tubes were growing in the ovary or already penetrating an ovule 3 DAP for XP and 2 - 3 DAP for XC. Our results for pollen tube growth agree with reports for cultivar 'Manzanillo' and 'Picual' growing in Mediterranean conditions, where first ovule penetrated are observed 1 - 2 days after pollination (Cuevas et al., 2009; Vuletin Selak et al., 2014b), and levels around 50 % of penetrated ovules were found 3 - 4 days after pollination (Villemur et al., 1984; Arzani and Javady, 2002). Temperatures during the experimental trials were around 20 ºC (Supplementary Figure 1), five degrees lower than the optimum temperature reported as the best for pollen tube growth (Cuevas et al., 1994b). Although we observed ovule penetration, our approach did not allow us to observe the fusion of the gametes.

Our results for stigmatic receptivity in 'Arbequina' agree with reports for other cultivars growing the Mediterranean basin were SR was maintained for 16 and 20 days (Cuevas et al., 2009), or more than 7 days (Vuletin Selak et al., 2014a). Because degraded stigmas in pear may support pollen adhesion but not pollen tube growth (Sanzol et al., 2003) we assessed stigma receptivity in terms of its capacity to support abundant pollen adhesion and pollen tube growth (Fig. 2). These extended periods of stigmatic receptivity could be due to the fact that flowers were covered to prevent unwanted pollination in most of these studies. In natural conditions (uncovered flowers) floral longevity in general (Stead, 1992) and stigmatic integrity in particular (Suárez et al., 2012) were observed to decrease after pollination. Additionally, SR

period is reported to be prolonged when pollen grains arrival to the stigma is prevented (Serrano and Olmedilla, 2012), like we do in our studies.

2.4.6 Final considerations

To sum up, EPP determined for 'Arbequina' was at least 7 days, as no reductions were observed in stigmatic receptivity, ovule viability, or differences in growth of pollen tubes as a function of flower age, consistent with the determination of EPP by sequential pollinations we conclude that the length of the EPP is not responsible for the low fruit that underlie the low yields in spite of the climatic conditions different from those of the Mediterranean basin. We could not identify limitations due to any of the components of the EPP during the first week after anthesis, and we showed that pistil tissues remain functional for a long time. The low fruit set of 'Arbequina' could be better explained by the low levels of compatible pollen present in the environment. Including compatible pollinizers in orchards of self-incompatible cultivars such as 'Arbequina' is key to obtaining a good level of fruit set. Furthermore, the influence of climatic conditions in non-traditional regions on pollen dispersal may be of particular importance particularly in new production regions with low cultivar diversity and monovarietal orchards.

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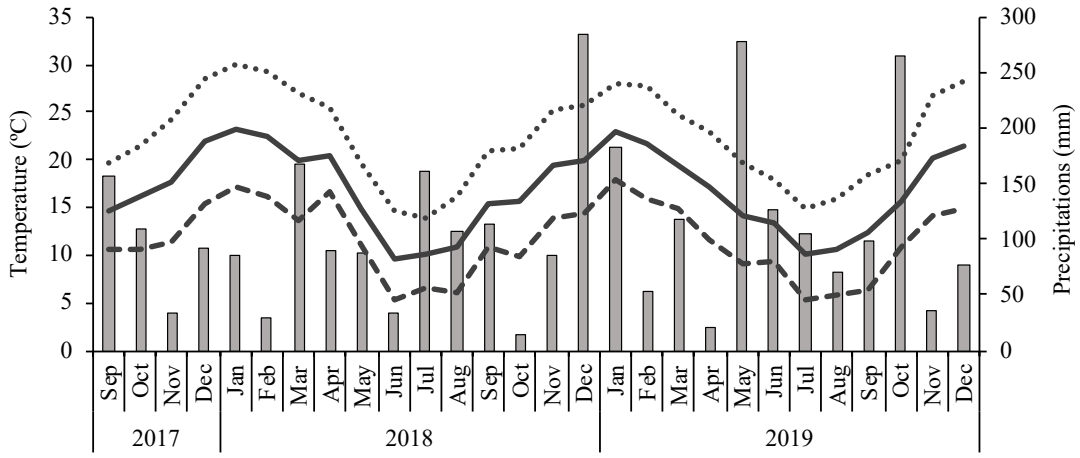
2.7 SUPPLEMENTARY DATA



Supplementary Figure 1: Average monthly maximum (dotted line), average (solid line), and minimum (dashed line) temperature (in ºC); and monthly accumulated precipitation (bars) in millimeters from September 2017 to December 2019 in INIA Las Brujas-Canelones.

3 DISCUSIÓN GENERAL

En este estudio se determinó el PEP para ‘Arbequina’, el cultivar de olivos más utilizado en Uruguay, una zona no tradicional para el cultivo con un clima templado húmedo. El PEP fue determinado mediante dos abordajes metodológicos, como respuesta en cuajado frente a polinizaciones secuenciales desde el momento de antesis hasta el día 7 días post antesis, y mediante el estudio de la funcionalidad de sus componentes. ‘Coratina’ y ‘Picual’, los dos cultivares más usados en la región en plantaciones polivarietales fueron utilizados como polinizantes. El PEP determinado para ‘Arbequina’ fue de al menos 7 días para ambos métodos de estimación, ya que durante dicho período no se observó una reducción significativa del porcentaje de cuajado en función de la edad de la flor, ni reducciones en la funcionalidad de sus componentes, para ambos polinizantes.

El PEP de al menos 7 días reportado en este trabajo para ‘Arbequina’ estimado a través de polinizaciones secuenciales, es mayor a los 3 - 4 reportados para otros cultivares de olivos en condiciones áridas como las de California, utilizando metodologías similares (Cuevas et al., 2009). Si bien concuerda con algunos reportes para la zona mediterránea donde se reportan estimaciones del PEP de entre 4 y 12 días (Pinillos y Cuevas 2009, Arzani y Javady 2002, Bini 1984), son mayores a los 2 - 4 días reportados también para condiciones del mediterráneo (Vuletin Selak et al., 2014b, 2014a).

Para varias especies se han reportado buenas correlaciones entre los resultados obtenidos para la estimación del PEP por ambos métodos (Sanzol et al. 2003, González et al. 1995). Sin embargo, otros estudios reportan que la duración del PEP estimada por polinizaciones secuenciales es más corta que por el análisis de sus componentes (Vuletin Selak et al. 2014a, 2014b, Cuevas et al. 2009). Los mismos autores sugieren que las discrepancias podrían deberse a la pérdida de funcionalidad del estilo en el tiempo, lo que limita el crecimiento de los tubos polínicos, tal como fue observado en especies de Cucurbitáceas (Nepi y Pacini, 2001) o en damascos (Egea y Burgos, 1992). Contrariamente, en este trabajo no se observaron diferencias en el crecimiento de los

tubos polínicos en función de la edad de la flor, aún en flores de 20 días post antesis. Sanzol y Herrero (2001) proponen que el mayor interés del análisis microscópico de los componentes del PEP es poder determinar el factor limitante del proceso, sin embargo, el estudio de los componentes del PEP realizado no mostró en este trabajo ningún factor limitante, lo que sugiere que la problemática de cuajado observada se deba a otro factor.

La autoincompatibilidad de 'Arbequina' basada en análisis de cuajado y estudios de la interacción polen-pistilo (Sánchez-Estrada y Cuevas, 2018) fue reportada durante la realización de este trabajo, en concordancia con reportes previos de trabajos a nivel molecular (Marchese et al. 2016, Shemer et al. 2014, Díaz et al. 2006,). En este trabajo se realizaron conjuntamente determinaciones de cuajado confirmadas por análisis de paternidad con SSRs y estudios de la observación de la interacción polen-pistilo en una zona no tradicional para el cultivo. Este trabajo permitió confirmar la autoincompatibilidad de 'Arbequina', su inter-compatibilidad con 'Picual' previamente reportada (Díaz et al., 2006) y con 'Coratina', sobre lo cual no existían reportes previos. Los bajos porcentajes de cuajado obtenidos en ramas de 'Arbequina' auto-polinizadas fueron atribuidos a contaminaciones con polen dentro de la bolsa, lo cual fue corroborado mediante la aplicación de técnicas moleculares. Esto demuestra la utilidad de llevar adelante análisis de paternidad en este tipo de abordajes con polinizaciones manuales, como fuera sugerido en otros trabajos (Besnard et al. 2020, Montemurro et al. 2019, Farinelli et al. 2018, Breton y Bervillé 2018, Saumitou-Laprade et al. 2017, Seifi et al. 2012, Díaz et al. 2006, Mookerjee et al. 2005).

La polinización libre en las condiciones del ensayo con alta presión de polinizante (25 % polinizante en el cuadro) fue suficiente para producir altos porcentajes de cuajado final superiores al 15 %. Los valores de cuajado obtenidos son similares a los reportados en trabajos similares para otros cultivares en condiciones del Mediterráneo (Vuletin Selak et al. 2014a, 2014b, Cuevas et al.2009). En las condiciones del ensayo, el agregado de polen en los tratamientos de polinizaciones manuales no logró incrementar los porcentajes de cuajado, lo que sugiere que en estas condiciones el polen no fue limitante. Dichos resultados coinciden con reportes donde se sugiere que para minimizar el déficit de polinización y mejorar los niveles de

fecundación se deben utilizar presiones de polinizante del 50 % (Sánchez-Estrada y Cuevas, 2020) o distancias menores a los 30 - 40 metros con respecto al cultivar polinizante (Griggs et al., 1975). No se observó un menor cuajado en las ramas embolsadas polinizadas con 'Coratina' y las ramas en polinización libre lo que descarta el posible efecto negativo del embolsado de las ramas sobre el nivel de cuajado.

Los resultados obtenidos van en concordancia con las problemáticas identificadas por productores olivícolas a nivel nacional. En 2018, nuestro equipo de investigación realizó una encuesta a cerca de 20 productores integrantes de la Asociación Olivícola Uruguaya (ASOLUR). En dicha encuesta se identificó que las principales limitantes para el rubro están asociadas a 1) costos de producción, 2) problemas sanitarios, 3) condiciones climáticas que repercuten en la productividad, el cuajado y el estado sanitario. Los productores reconocen la necesidad de generar conocimiento a nivel local tendientes a 1) mejorar los rendimientos, ya sea a través del incremento del cuajado o la reducción de la alternancia, 2) trabajar en la adaptación de cultivares a nuestras condiciones, y 3) manejar la sanidad del cultivo, destacando la necesidad por algunos productores de realizar un manejo ecológico. La mayor parte de los productores nacionales encuestados (más del 80 %) recomendaría invertir en nuevas plantaciones de olivos, pero debido a los bajos rendimientos obtenidos en muchas situaciones productivas, no es 'Arbequina' el cultivar que recomendarían en primer lugar. Para las últimas floraciones de 'Arbequina' (2017 y 2018), la mayoría de los productores encuestados coinciden en que los cuadros que presentaban cultivares polinizantes obtuvieron mayores rendimientos que los cuadros puros, independientemente del nivel de floración inicial. Sin embargo, existe un grupo de productores que plantaría montes puros (un único cultivar), sin visualizar la necesidad de incluir polinizantes.

Si bien gran parte de los montes de olivo en Uruguay fueron instalados considerando a 'Arbequina' como autocompatible, en este trabajo se confirma que 'Arbequina' es un cultivar autoincompatible y por lo tanto requiere de la presencia de polen compatible en los cuadros para poder cuajar. Se sugiere que en el diseño de nuevas plantaciones se incorporen cultivares polinizantes inter-compatibles. La autoincompatibilidad representa uno de los mayores obstáculos para la producción en

las plantaciones de olivos, Mariotti et al. (2020) establecen la existencia de un sistema de autoincompatibilidad esporofítico dialélico que determina la existencia de dos grupos de compatibilidad y por lo tanto, se requiere de la polinización entre cultivares de los diferentes grupos para obtener producción de frutos exitosa. Se considera fundamental promover trabajos de investigación locales para establecer cuáles son las combinaciones óptimas de cultivares inter-compatibles y las proporciones a incorporar. En plantaciones ya instaladas dónde se hayan registrados problemas de cuajado, se recomienda la implantación de cultivares polinizantes o la posible aplicación artificial de polen exógeno compatible. Esta última metodología podría ser utilizada también en floraciones durante primaveras lluviosas y húmedas dónde las condiciones climáticas no sean propicias para el transporte de polen. La característica observada en este trabajo sobre la amplia extensión de la vida de la flor cuando no llega polen al estigma, es información útil para el manejo de las polinizaciones artificiales en el campo.

Los resultados obtenidos en este trabajo muestran la necesidad de la generación de conocimientos asociados a nuestra realidad productiva que respondan a problemáticas propias expresadas por los productores, así como también, la necesidad de la extensión de dichos resultados a través de un vínculo estrecho con el sector productivo.

4 CONCLUSIONES GENERALES

En este trabajo se determinó el período efectivo de polinización (PEP), para el cultivar de olivos ‘Arbequina’, mediante las dos metodologías disponibles, a través polinizaciones secuenciales, y mediante el análisis de sus componentes. El PEP para las condiciones de Uruguay presentó un largo de al menos una semana sin diferencias entre los métodos de estimación. La receptividad estigmática se mantuvo por al menos 20 días desde la antesis. La viabilidad de los óvulos fue también muy prolongada, no se observaron diferencias en la proporción de óvulos no viables hasta 17 días post antesis, y más del 50 % de las flores mantuvieron al menos un óvulo viable 23 DPA. En tanto a los tubos polínicos les toma entre 2 y 3 días crecer desde el estigma hasta alcanzar un óvulo.

No se observaron diferencias en el crecimiento de los tubos polínicos en función de la edad de la flor incluso en flores de hasta 20 DPA lo cual descarta el envejecimiento del pistilo como responsable de las diferencias reportadas en la bibliografía entre los métodos de estimación del PEP.

Nuestros resultados no muestran limitaciones en el PEP en una zona no tradicional para el cultivo del olivo como lo es Uruguay. Por esta razón, descartamos que el largo del PEP para estas condiciones como responsable del bajo cuajado obtenido.

Confirmamos la condición de auto-incompatibilidad para el cultivar ‘Arbequina’ lo cual determina su dependencia de la llegada del polen compatible (mientras las flores mantienen su funcionalidad) para poder cuajar un fruto. Hemos comprobado la compatibilidad de ‘Arbequina’ con ‘Coratina’, el cultivar más frecuentemente utilizado en Uruguay como polinizante. Los resultados obtenidos señalan que el PEP en Uruguay no es una limitante para el cuajado, se hace oportuno el estudio de otros aspectos involucrados como a la disponibilidad de polen compatible en las quintas y su relación con las condiciones climáticas locales caracterizadas por la alta variabilidad y elevada humedad y precipitaciones.

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