UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTMENT OF AGRO-FORESTRY ECOSYSTEMS

MASTER'S DEGREE IN

PLANT HEALTH IN SUSTAINABLE CROPPING SYSTEMS

Co-funded by the
Erasmus+ Programme
of the European Union

OCCURRENCE AND MOLECULAR CHARACTERIZATION OF RESISTANCE TO QoI FUNGICIDES IN *Plurivorosphaerella nawae*, CAUSAL AGENT OF CIRCULAR LEAF SPOT OF PERSIMMON

2021

LUAN VITOR NASCIMENTO

Academic tutor: Dr Mónica Berbegal Martínez

Co-tutor: Dr Antonio Vicent Civera

Valencia, September 2021

RESUMEN

Ocurrencia y caracterización molecular de la resistencia a los fungicidas QoI en *Plurivorosphaerella nawae*, agente causal de la mancha foliar del caqui.

Uno de los principales problemas bióticos que afectan al cultivo de caqui en la Comunidad Valenciana es la enfermedad de la mancha foliar, causada por el Ascomycete *Plurivorosphaerella nawae* (≡ *Mycosphaerella nawae*). El manejo de la enfermedad se basa en gran parte en tratamientos químicos, especialmente con los fungicidas del grupo de los inhibidores de la quinona (QoIs). A medida que los agricultores empezaron a observar una reducción en la eficacia de los fungicidas en 2019, las autoridades decidieron tomar medidas e investigar lo que se consideraba como señales de que *P. nawae* estaba desarrollando resistencia a los fungicidas. Teniendo en cuenta que los QoIs se describen como fungicidas con alto riesgo inherente de desarrollo de resistencia, este trabajo tuvo como objetivos (i) aislar *P. nawae* de hojas sintomáticas muestreadas de diferentes zonas de la Comunidad Valenciana, (ii) detectar por medios moleculares la presencia de la resistencia y escalar su prevalencia dentro de la población, y (iii) estimar la sensibilidad de la población al compuesto activo piraclostrobin, uno de los más utilizados para controlar la enfermedad en esta región. Para ello, se sembraron fragmentos de hojas afectadas en medio de cultivo patata dextrosa agar (PDA) con sulfato de estreptomicina, que se incubaron posteriormente para aislar el patógeno. Se obtuvieron colonias puras de hojas sintomáticas de las que se extrajo el ADN genómico para la identificación de la especie fúngica usando cebadores específicos y mediante secuenciación de la región ITS. La resistencia a los fungicidas QoIs se analizó amplificando y secuenciando un fragmento de 223 bp del gen del citocromo b. Para la caracterización de la sensibilidad se obtuvo la concentración capaz de inhibir 50% del crecimiento fúngico, a partir de la exposición de los aislados a concentraciones crecientes de la materia activa. Encontramos que la baja eficacia de los fungicidas QoI para el control de *P. nawae* en la Comunidad Valenciana está relacionada con una elevada prevalencia de la mutación G143A. La sensibilidad al fungicida piraclostrobin de la población mutante portadora del G143A fue, en promedio, 545 veces más baja que la población de aislados sin esta mutación.

Palabras clave: *Mycosphaerella*, resistencia, estrobilurina.

ABSTRACT

Occurrence and molecular characterization of resistance to QoI fungicides in *Plurivorosphaerella nawae*, causal agent of circular leaf spot of persimmon.

One of the main biotic problems affecting persimmon in the Valencian Community is the circular leaf spot disease, caused by the Ascomycete *Plurivorosphaerella nawae* (≡ *Mycosphaerella nawae*). Management of the disease relies heavily on chemical treatments, especially with fungicides from the group of quinone outside inhibitors (QoIs). As farmers reported reduced efficacy of fungicides in 2019, authorities decided to take measures and investigate what was being considered as signs of *P. nawae* developing resistance to the most commonly used fungicide in the region. Bearing in mind that QoIs are described as compounds with inherent high risk of resistance development, this study aimed to (i) isolate *P. nawae* from symptomatic leaves sampled from different areas throughout the Valencian Community, (ii) detect by molecular methods the presence of resistance and estimate its prevalence in the population, and (iii) calculate population's sensitivity to the active compound (pyraclostrobin) most frequently applied to control the disease in the region. For such purposes, pure colonies were obtained from symptomatic leaves. Genomic DNA of each isolate was extracted, and identification of fungal species performed using *P. nawae* specific primers and sequencing the ITS region. Resistance to QoI fungicides was analyzed amplifying and sequencing a 226-bp fragment of the cytochrome b gene. Sensitivity was calculated with the effective concentration able to inhibit 50% of fungal growth after exposure to increasing concentrations of the active ingredient. We found that the primary cause of the reduced efficacy of the QoI fungicides in the Valencian Community was associated with the great prevalence of the G143A mutation in the *P. nawae* population. Fungicide sensitivity in the mutant population carrying the G143A was, on average, 545 times lower than the population of wild type individuals.

Key words: *Mycosphaerella*, resistance, strobilurin.

ACKNOWLEDGMENTS

To God who is merciful enough to have forgiven me and blessed me with so much that I can't even express.

To my beloved family who always stands there for me no matter the occasion, the result, or the sentiment. My mother, Julia Elza, and my father, Rui Francisco, who give me nothing but support and encouragement when I board on to new challenges. And my brother Lucas Emanuel who has been by my side since our womb-sharing days, regardless of the mileage between us.

To the many new friends I have made throughout the program, especially those who are part of this incredible and lovely Plant Health community.

To the whole group of professors and lecturers of the Georg-August-Universität Göttingen and the Universitat Politècnica de València, and all the invited speakers, who contributed significantly to my academic formation and have considerably influenced the way I perceive all issues connected, on one way or the other, to an area so comprehensive as the sustainable agriculture.

To the team of coordinators of the Plant Health project, with a very special thank you to Dr Susanne Weigand from the Georg-August-Universität Göttingen, and Dr Josep Armengol from the Universitat Politècnica de València. The experience at both institutions was surely much better because of their support and kindness.

To my master thesis' project supervisors, Dr Mónica Berbegal from the Instituto Agroflorestal Mediterráneo of the Universitat Politècnica de València, and Dr Antonio Vicent from the Instituto Valenciano de Investigaciones Agrárias. First, for accepting my participation in the project, and also for their crucial support and help.

And, to many others who actively played part in this journey my most sincere gratitude.

This research work has been developed as a result of a mobility stay funded by the Erasmus+ - KA1 Erasmus Mundus Joint Master Degrees Programme of the European Commission under the PlantHealth Project.

TABLE OF CONTENTS

1. INTRODUCTION 7
1.1. Persimmon crop 7
1.2. Persimmon diseases 8
1.3. Circular leaf spot of persimmon 9
1.4. Causal agent and epidemiology 9
1.5. Disease management 11
1.6. Quinone Outside Inhibitors 13
1.7. Fungicide resistance 14
2. OBJECTIVES 17
3. RESEARCH METHODOLOGY 18
3.1. Sampling and fungal isolation 18
3.2. Screening for resistance in the population 19
3.3. Identification and molecular detection of resistance to QoI fungicides 20
3.4. Phenotypic assessment of resistance in *P. nawae* population 22
4. RESULTS 22
4.1. Identification and molecular detection of resistance to QoI fungicides 25
4.2. Phenotypic assessment of resistance in *P. nawae* population 26
5. DISCUSSION 28
6. CONCLUSIONS 32
BIBLIOGRAPHIC REFERENCES 33

LIST OF ABBREVIATIONS

EC_{50} - 50% Effective Concentration

IBMCP - Instituto de Biología Molecular y Celular de Plantas

IVIA - Instituto Valenciano de Investigaciones Agrarias (Valencian Institute of Agrarian Research)

PCR - Polymerase Chain Reaction

PDA - Potato Dextrose Agar

PGR - Percentage of Growth Reduction

QoI - Quinone Outside Inhibitor

SBI - Sterol Biosynthesis Inhibitor

1. INTRODUCTION

1.1. Persimmon crop

Diospyros kaki Thunb., commonly known as persimmon or kaki, is a deciduous fruit tree native to China. With traditional cultivation started in ancient times, over 2000 years ago, China appears atop of the list of major producers of the fruit, responsible for about 80% out of the 4 million tonnes of annual persimmon production worldwide. South Asian countries currently hold great part of the global production of the fruit, approximately 95%. South Korea (with 0.4 mi t) and Japan (0.2 mi t) follow China as important producers of persimmon in Asia (Martínez las Heras, 2017). In these countries, local consumers are actually the main market which retains most of their fruits, with no significant exportation (Llácer and Badenes, 2002).

In Europe, the introduction of persimmon occurred in the 17th century (Llácer and Badenes, 2002). The first report of persimmon in Spain dates back to the 19th century. In that period, persimmon was mainly used as ornamental and appreciated for the quality of its wood (Naval *et al.*, 2012). Its importance and cultivation as a fruit crop was limited due to the astringency of fruits obtained on those varieties grown locally. Nevertheless, starting from the early 1990's the Mediterranean region reported an important expansion in cultivated areas, particularly in Valencian Community and Andalusia. The rapid progress in persimmon cropping reported increments figures of 300 times in the production in 15 years. Different factors played role and helped to drive this development culminating in such massive growth, but the most significant ones that are worth mentioning were the introduction of astringent cultivars 'Triumph' in Andalusia, and, most importantly, 'Rojo Brillante', in Valencian Community (Palou *et al.*, 2015), combined with the deployment of new techniques that made possible the removal of astringency after harvest while maintaining fruit firmness.

Particularly, the cultivar 'Rojo Brillante' gained popularity among Spanish growers because of the excellent quality of its fruits, with attractive shape, aroma and flavor. Additionally, the cultivar is highly productive with orchards able to reach productivity of up to 60 t/ha (Llácer and Badenes, 2002; Berbegal *et al.*, 2010a). The introduction of CO_2-

enriched atmosphere in postharvest to remove fruit astringency as a replacement for the traditional procedure of over-ripening substantially improved shelf life of fruits by avoiding the loss of firmness and, therefore, favoring commercialization with external markets (Perucho, 2016).

Led by the outstanding performance of ‘Rojo Brillante’ that ended up replacing most of the other less profitable and traditional varieties throughout Valencian Community and Andalusia, Spain became the worldwide leader in persimmon exports. In 2020, the market of persimmon generated a revenue of US$ 234.4 million for Spanish producers. Countries such as Germany, Italy, France, The Netherlands, and Poland are net importers, and correspond to about 35% of the Spanish persimmon international trade (Tridge, 2021).

1.2. Persimmon diseases

Key phytosanitary concerns in persimmon production in the Mediterranean region include problems in postharvest and in field conditions. Reportedly, the occurrence of black spot disease (*Alternaria alternata* (Fr.) Keissl.) in postharvest increased after the introduction of those technologies that enabled the removal of fruit astringency and extended shelf life (Vicent *et al.*, 2020). Concerning postharvest life, this is the most important disease of persimmon in Spain. Other less frequent postharvest diseases are anthracnosis (*Colletotrichum* spp.), gray mold (*Botrytis cinerea* Pers.: Fr.), blue mold (*Penicillium* spp.), and peduncular rot (*Neopestalotiopsis clavispora* (G.F. Atk.) Maharachch., K.D. Hyde & Crous) (Palou *et al.*, 2015).

At field level, some important generalist soilborne pathogens, such as *Rosellinia necatrix* Berl. ex Prill. and *Armillaria* sp., can induce root and crown rots in different woody plants, including persimmon trees. Nonetheless, currently the most problematic disease in field conditions affecting orchards in Spain is the circular leaf spot, caused by *Plurivorosphaerella nawae* (Hiura & Ikata) O. Hassan & T. Chang (≡ *Mycosphaerella nawae* Hiura & Ikata), which has become a major problem ever since its first detection in the Valencian Community (Vicent *et al.*, 2020).

1.3. Circular leaf spot of persimmon

The fungus *P. nawae* is the causal agent of the circular leaf spot (CLS) disease of persimmon. This species is one of the most important pathogens affecting this crop worldwide and is present in important producing regions of South Korea and Japan in Southeast Asia, and Spain in Europe (Vicent *et al.*, 2012). The occurrence of *P. nawae* in persimmon growing areas in Spain was first detected in 2008 in fields located in Valencian Community (Berbegal *et al.*, 2010b), which was also reported as the first case of the pathogen occurring in semi-arid conditions.

1.4. Causal agent and epidemiology

The ascomycete *P. nawae* was previously known under the synonym *M. nawae*. The fungus has been recently reclassified to a new taxonomic group within the family *Mycosphaerellaceae*, creating the novel genus *Plurivorosphaerella* (Hassan and Chang, 2019). Some members of *Mycosphaerellaceae* are important plant pathogens associated with disease of economic importance, including Sigatoka disease on banana, angular leaf spot of bean, tomato leaf mold and cercospora leaf spot of olives (Videira *et al.*, 2017). The reference for the new name was a combination of *Plurivorous*, due to its wide host range, and *Mycosphaerella*.

The fungus *P. nawae* reproduces sexually through the formation of spindle-shaped ascospores in cylinder- or banana-shaped sac-like structures (asci) inside dark fruiting bodies called pseudothecia (Lee *et al.*, 2016). The asexual form of *P. nawae* may develop two morphologically different conidia from lateral or terminal conidiogenous cells. They are either solitary or in chains, light brown, in different shapes (cylindrical to ovoid) with one or no septa, or long septate conidia with 3 to 6 cells, constricted at the septa, and basal swollen cells (Hassan and Chang, 2019).

On persimmon trees infected with *P. nawae*, primary symptoms of CLS on leaves appear as visible small circular necrotic spots surrounded by a green halo that quickly develop to chlorosis taking over the whole leaf area and culminating on premature defoliation of the tree (Figure 1A, B) (Vicent *et al.*, 2011b). The leaf infection leads to early

maturation and severe fruit drop, which are the most significant damages induced by *P. nawae* in persimmon, causing serious yield and economic losses (Figure 1C). In Spain, total yield loss has been observed in experimental plots and commercial orchards with poor disease management practices (Bassimba *et al.*, 2017).

Figure 1. Persimmon leaves infected with *Plurivorosphaerella nawae* showing characteristic symptoms (A, B) of circular leaf spot, and severe fruit drop (D) observed in a commercial orchard.

The overwintering stage of *P. nawae* is in the form of pseudothecia, which develop on lesions of decaying leaf litter. These fruiting body structures require specific conditions of temperature and humidity to release new ascospores. The increasing temperature in spring prompts the maturation of ascospores, which are forcibly ejected from the pseudothecia and wind-dispersed when suitable conditions of 10 to 16 °C and presence of free water (from rain or irrigation) are met. Upon landing on persimmon leaves, the airborne ascospores will also need moisture and appropriate temperature to germinate and infect the tissue (Figure 2). In Spain, infections are most likely to occur during the period of April to June (Vicent *et al.*, 2011b). Although plants may become infected with

P. nawae in early spring, CLS symptoms will begin to appear only in late summer (end of August and early September) when the fungus emerges from a long period of incubation – up to 4 months (Vicent *et al.*, 2012).

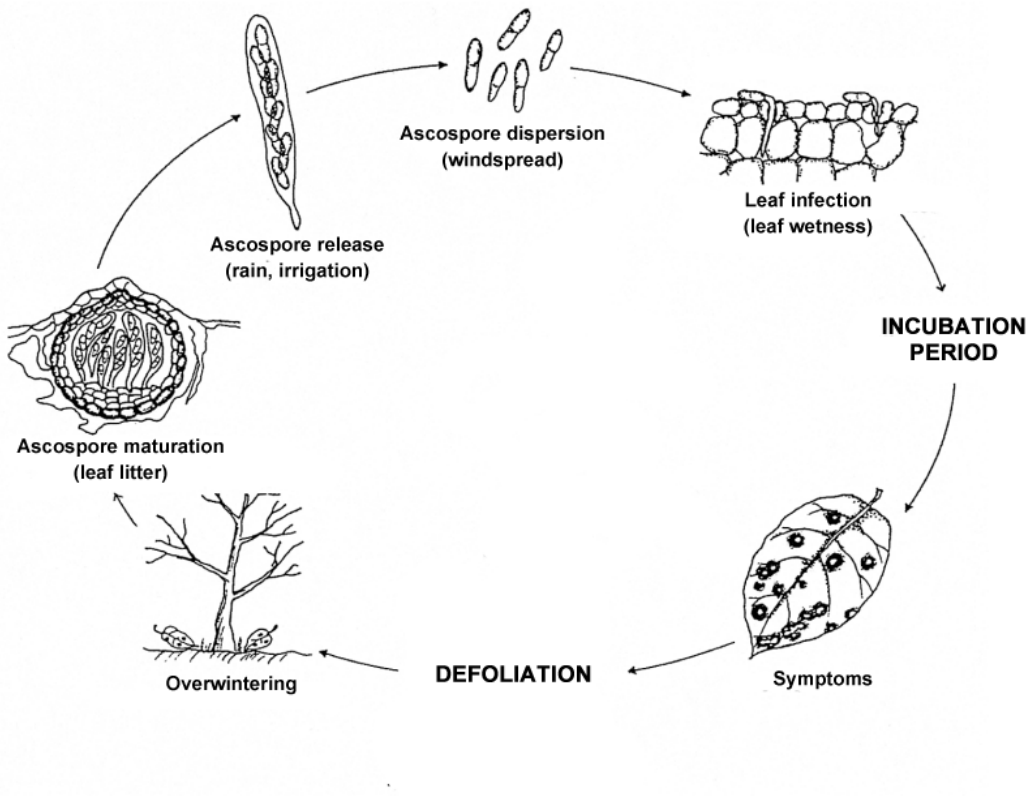

Figure 2. Disease cycle of *Plurivorosphaerella nawae*, causing circular leaf spot in persimmon crop (Kwon and Park (2004), adapted).

Cases of asexual reproduction of *P. nawae* on leaf lesions have only been reported in South Korea, with production of *Ramularia*-like conidia. However, the role of these spores for development of new epidemics in the field is not well-comprehended yet (Kwon and Park, 2004). Nevertheless, secondary infections are not believed to occur in Spanish persimmon producing areas since conidial spores have never been detected in the region (Vicent *et al.*, 2011b) and therefore CLS is considered a monocyclic disease.

1.5. Disease management

Decaying foliage of persimmon trees is the primary source of inoculum for new epidemics in areas where *P. nawae* is present because of the overwintering

pseudothecia. Consequently, sanitation measures consisting in the removal of fallen leaves is an important strategy to reduce initial inoculum. Leaf litter may either be incorporated into the soil by revolving the superficial layers of the orchard, or disposed outside the orchard and utilized for composting or incinerated in order to limit the availability of overwintering material (Vicent *et al.*, 2011b). With cultivation under irrigated systems, as it occurs in the semi-arid conditions of Spain, localized irrigation systems such as dripping should be opted instead of flooding, as a way to prevent suitable conditions for pseudothecium maturation and ascospore release (Vicent *et al.*, 2011a, 2012)

Nevertheless, fungicide treatments are necessary to maintain disease level under economic thresholds during the cropping season. In important growing areas in South Korea, multiple sprays applied on a schedule-basis protect leaves from infection between mid-July and early August (Bassimba *et al.*, 2017). In Spain, preventive spring applications (April to June) with two to four sprays provide good control. The decision to introduce the recommended treatment takes into account other aspects of the crop and environment, including phenological stage of trees, inoculum availability and suitable weather conditions for fungal infection (Vicent *et al.*, 2011b). Post-infection treatments in summer did not succeed in controlling CLS in persimmon. For this reason, only preventive control is recommended for CLS management, which can also help to reduce the risks of *P. nawae* developing resistance to authorized fungicides and avoid problems with residue later during harvest (Bassimba *et al.*, 2017; Martínez-Minaya *et al.*, 2019). Bearing in mind the restrictions regulating the maximum limit of fungicide residues allowed for commercialization, official recommendations also suggest that treatments should not be applied after mid-June (Generalitat Valenciana, 2017).

In Spain, there are few fungicides authorized for the management of CLS in persimmon. To date, the list comprises of one multi-site contact fungicide (mancozeb) and four single-site chemicals from the groups of Quinone outside inhibitors (QoI), commonly known as strobilurins (azoxystrobin and pyraclostrobin) and sterol biosynthesis inhibitors (difenoconazole). Following the guidelines of the Fungicide Resistance Action Committee (FRAC), official regulations recommend alternation of fungicides from different chemical groups in the spraying programs with limitation of

number of applications of those active compounds classified under the high resistance risk label and also applied in mixtures with multi-site fungicides (Berbegal *et al.*, 2011). Considering the range of fungicides authorized for CLS control in Spain, for instance, those from the group of QoIs were restricted to a maximum of two applications during the critical period of infection (Generalitat Valenciana, 2017).

1.6. Quinone Outside Inhibitors

QoIs are a relatively recent class of fungicides and considered as the most important compounds in modern agriculture (Agrios, 2005). They were first identified naturally occurring in fungal organisms from the division of Basidiomycetes. The wood-decaying fungus *Strobilurus tenacellus* (Pers.) Singer was the source of the first QoIs, namely A and B. After these natural compounds were demonstrated to negatively affect growth of other fungi, further research enabled the improvement of Strobilurin A with synthetic molecules that were more stable and were then introduced to the agroindustry as protectants against plant pathogens (Ypema and Gold, 1999).

QoIs integrate a group of chemicals characterized by its unique mode of action that inhibits the electron transport transfer in the mitochondrial respiration. This chemical group targets the quinol oxidation site of cytochrome b, and also comprises other fungicides such as famoxadone, fenamidone and pyribencarb, all of which are susceptible to cross-resistance events. Although of different chemistry, they all affect the same process of energy production in the fungus (Brent and Hollomon, 2007).

The fungicidal activity of QoIs strongly affects the initial phases of fungal development. When the inhibitory compound binds to the target it blocks mitochondrial electron flow, halting NADH and ATP production, which in turn leads to energy deficiency during these highly energy demanding stages of the fungus life cycle (Bartlett *et al.*, 2002). In this sense, spore germination and the period of germ tube elongation are the most sensitive stages. Fungicide effect becomes less severe after fungus enters the mycelial growth phase. Taking these characteristics into account, for maximum effectivity of QoI fungicides protectant applications are more appropriate rather than curative/eradicative treatments (Leadbeater, 2012).

The spectrum of activity with a vast array of hosts was one of the main aspects responsible for making QoIs one of the most widely used class of fungicides, and essential components in disease management programs, controlling important plant diseases caused by Ascomycetes, Basidiomycetes and Oomycetes in many crops (Agrios, 2005). This unprecedent feature, for instance, provided growers for the first time the protection against two of the most important diseases affecting grapevine fields, powdery mildew (*Erysiphe necator* Schwein.) and downy mildew (*Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni), using a single active ingredient (Fernández-Ortuño *et al.*, 2010). Several other major crops have also benefited from the broad spectrum of QoIs to control important diseases simultaneously (e.g. wheat, barley, potato, soybean, rice).

Another special characteristic of the QoIs that contributed to the fungicide's success in the market was the secondary effects on crop quality and yield resulted from the induction of delayed senescence and extended grain-filling period. This boost in yield reported in wheat, corn, soybean and other crops when treated with QoIs is known as "greening effect" (Kanungo and Joshi, 2014). A number of studies have investigated several changes in plant biochemistry that explain this improved performance, regardless of the protection against diseases: different physiological processes being affected by QoIs such as leaf senescence, ethylene biosynthesis, stomatal aperture, plant antioxidant enzyme activity, and hormonal balance; and the reduction of host-defense elicited energy-demanding processes by preventing germination of spores of both pathogenic and non-pathogenic fungi (Bartlett *et al.*, 2002).

1.7. Fungicide resistance

Following the trend of popularity of the newly released fungicides of modern chemistry, populations of QoI-resistant pathogens did not take long to be noticed after such compounds were introduced to the market in 1996. It took no longer than two years for the first reports of resistance in populations of powdery mildew of cereals (northern Germany) and cucurbits (Japan), and in black Sigatoka on banana (Costa Rica) (Bartlett *et al.*, 2002; Sierotzki, 2015). Until today, more than 50 different plant pathogenic fungal

species have been described to have developed resistance to QoI fungicides (FRAC, 2020). This inherent risk of development of resistance in QoI fungicides comes from the nature of these chemicals acting on one single specific site (Balba, 2007, Fernández-Ortuño *et al.*, 2010).

For most pathogens, the major mechanism conferring resistance to QoI fungicides involves point mutations in the mitochondrial cytochrome *b* gene (*cyt b* gene). The loss of effectivity of such range of fungicides is caused by changes in the peptide sequence due to spontaneous nonsynonymous mutations. When these specific mutations happen, they promote a substitution in amino acids that prevents the fungicidal compound from binding to its specific site of action (Bartlett *et al.*, 2002; Fernández-Ortuño *et al.*, 2008).

Three point mutations resulting in amino acid substitutions have been associated to the resistance to QoI fungicides in plant pathogens: from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L), and from glycine to arginine at position 137 (G137R). The G143A substitution is the most frequent mutation and is commonly associated to a high (complete) level of resistance which leads to complete failure of fungicide treatment, whilst the other two (F129L and G137R) are described as moderate (partial) resistance which may be compensated depending on the levels of fungicide applied (FRAC, 2006). This mechanism of resistance has been widely studied in two major *Mycosphaerellaceae* species of global economic importance: *Zymoseptoria tritici* (Roberge ex Desm.) Quaedvl. & Crous (≡ *Septoria tritici* Roberge ex Desm.) causing Septoria leaf blotch on wheat and *Mycosphaerella fijiensis* M. Morelet. causing black Sigatoka on banana. In both species, the main point mutation associated to the resistance is the G143A (Sierotzki *et al.*, 2000; Amil *et al.*, 2007; McCartney *et al.*, 2007; Torriani *et al.*, 2009; Siah *et al.*, 2010).

The evolution of fungicide resistance in fungal populations is dependent on the fitness of isolates that affects the dynamics of competition between resistant and sensitive isolates, and this has important implications for management (Parnell *et al.*, 2005; Hawkins and Fraaije, 2018). The *cyt b* gene is part of the mitochondrial genome, which indicates that progenies of the sexual reproduction will inherit any mutation and therefore, in the particular case of the G143A substitution, the ability to develop in the presence of the fungicide (Sierotzki, 2015). The highest fitness cost is associated with G143A in

species where codon 143 is followed by an intron, as the mutation prevents proper splicing and is therefore lethal (Vallières *et al.*, 2011). Other penalties result from functional trade-offs at the protein level, however, in most cases G143A has not been associated with deleterious effects (Hawkins and Fraaije, 2018). Frequent and repeated QoI compounds application together with the apparent resistant individuals quickly spreading to dominate the area driven mostly by the strong selection pressure of fungicide activity. For instance, control of powdery mildew of cucurbits (*Podosphaera fusca* (Fr.) U. Braun & Shishkoff) in Spain is practically ineffective if QoIs are applied, due to the high prevalence of resistance in the population (Fernández-Ortuño *et al.*, 2006).

In 2011 a special authorization for the use of QoIs was announced in Spain to control CLS in persimmon. Pyraclostrobin and azoxystrobin has been since then integrated in control programs following strict management practices aiming to reduce the risk of resistance development in *P. nawae* populations. In a study with several isolates collected in 2015 from persimmon orchards where QoIs were frequently used to control epidemics, the presence of resistant individuals was discarded by Martínez *et al.* (2017) after the surveyed population demonstrated relatively high levels of sensitivity to pyraclostrobin in *in vitro* assays. In 2015, 92 isolates obtained from symptomatic leaves were evaluated for mycelial growth using 1.5 mg L^{-1} pyraclostrobin amended media as discriminant concentration (Berbegal *et al.*, 2011; Berbegal *et al.*, 2017). Results showed that all the isolates in the population studied were sensitive to the fungicide and this result was corroborated with the absence of the G143A mutation (Berbegal *et al.*, 2017). However, more recently, after surveys conducted by official phytosanitary Spanish agencies during 2019, an area of high risk within Valencian Community, where poor control of CLS was detected, has been indicated and stablished for closer monitoring and to follow special precaution measures (Generalitat Valenciana, 2020). According to the report, the lack of control was presumably linked to the appearance of resistance in *P. nawae* rather than inadequate spray timing.

2. OBJECTIVES

Bearing in mind the recurring problematic of QoI fungicides and its risk of resistance development in plant pathogenic fungi, this study aimed to (i) isolate *P. nawae* from symptomatic leaves sampled from different areas throughout the Valencian Community, (ii) detect by molecular methods the presence of resistance and estimate its prevalence in the population, and (iii) calculate population's sensitivity to the active compound (pyraclostrobin) most frequently applied to control the disease in the region.

3. RESEARCH METHODOLOGY

3.1. Sampling and fungal isolation

The isolates of *P. nawae* were recovered from symptomatic leaves showing characteristic necrotic lesions of CLS, collected in distinct orchards throughout 25 locations (Figure 3) covering the most important persimmon producing areas in the region of 'Ribera Alta', Valencian Community, Spain. Leaves were sampled between September and November 2020. The affected portion of the leaf tissue was cut, surface disinfected in 1.5% sodium hypochlorite and rinsed in sterile distilled water. Fragments of the lesions were plated in potato dextrose agar (Liofilchem, Italy) supplemented with 0.5 g L^{-1} of streptomycin sulfate (Sigma-Aldrich Co., USA) (PDA+S), with five plates per sample leaf, and stored at 23 °C in the dark. After purification in PDA, isolates were subcultured also in PDA and preserved at room temperature in mycelial discs, and at -10 °C in colonized filter paper in the culture collection of the Valencian Institute of Agrarian Research (IVIA, Spain).

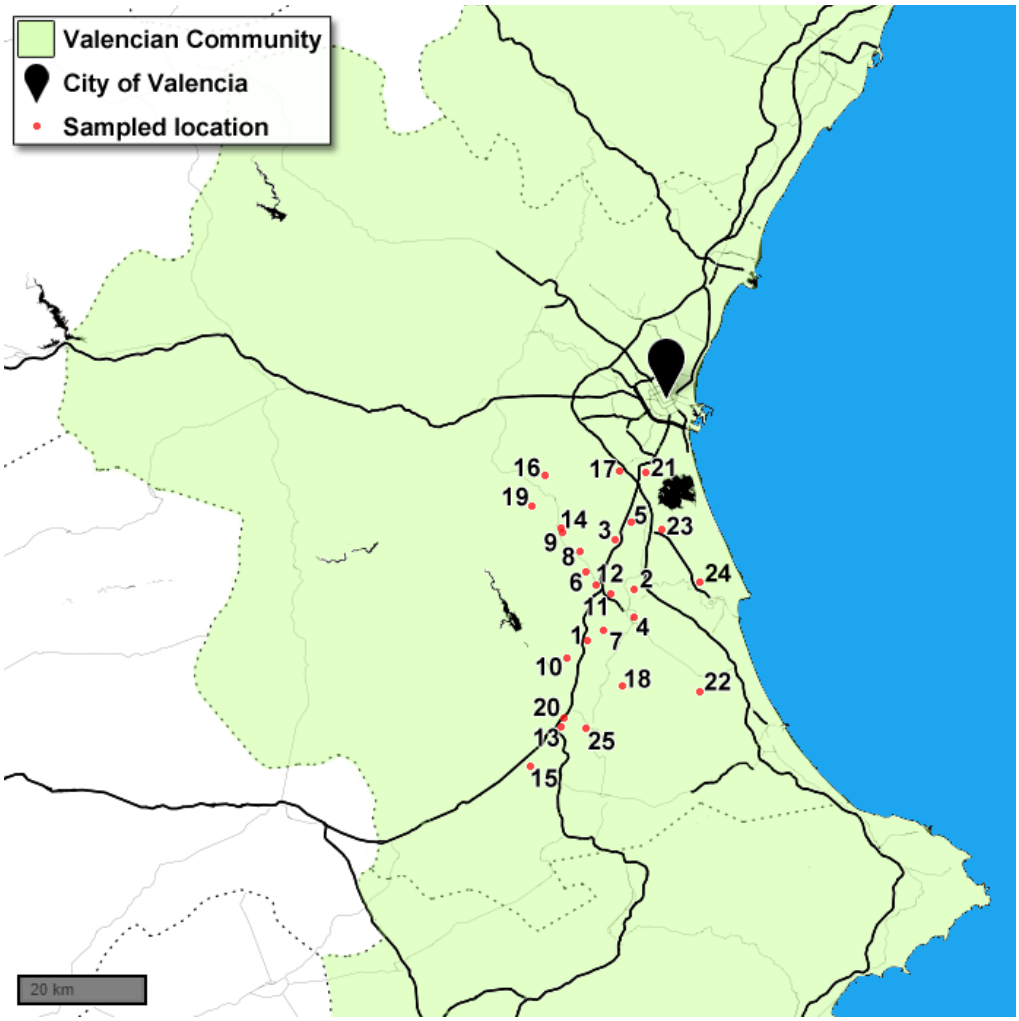

Figure 3. Location of orchards within the Valencian Community from where diseased persimmon leaves showing characteristic symptoms of circular leaf spot (*Plurivorosphaerella nawae*) were collected for assessment. (Municipalities from 1 to 25: Alberic, Algemesí, Alginet, Alzira, Benifaió, Benimodo, Benimuslem, Carlet, Catadau, Gavarda, Guadassuar, L'Alcúdia, Llanera de Rana, Llombai, Montesa, Monteserrat, Picassent, Rafelguaraf, Real, Rotglà i Corberà, Silla, Simat de la Valldigna, Sollana, Sueca and Xativa)

3.2. Screening for resistance in the population

Resistance to QoIs was first tested using a discriminant concentration of 10 mg L^{-1} of the active ingredient pyraclostrobin. The concentration was selected based upon result from previous studies that evaluated sensitivity of *P. nawae* (*unpublished data*). Pyraclostrobin (Cabrio®, BASF España) was diluted in sterile distilled water and

amended to molten PDA to obtain specific concentration of 10 mg L^{-1} of active ingredient. Mycelial discs (8 mm in diameter) of each isolate were taken from pure colonies and placed in direct contact with the agar in the center of PDA plates amended with fungicide and non-amended PDA (control). Isolates of known resistance phenotype collected in 2019 and used in previous studies (*unpublished data*), were obtained from the culture collection of the IVIA and included in the assay as reference of low and high sensitivity to pyraclostrobin. Plates were sealed with parafilm and stored at 23 °C in the dark. After 17 days of incubation, colony diameter was measured in two perpendicular axes and data were converted to percentage of growth reduction (PGR) in comparison to the respective controls.

3.3. Identification and molecular detection of resistance to QoI fungicides

The molecular identification of the isolates sampled from symptomatic persimmon trees was performed following a protocol with primers designed to specifically detect *P. nawae*, and further sequencing of the ITS rDNA region. The genomic DNA of each isolate was extracted from pure colonies grown on PDA, using the E.Z.N.A.® Plant DNA Kit (Omega Bio-tek Inc., USA), following manufacturer's instructions.

Isolates were first screened with species-specific primers, MNf (5' – CTGGCACTGTTGCCCATT – 3') and MNr (5' – GAGATCCGTTGTTGAAAGTTTTG – 3'), described by Berbegal *et al.* (2013). The polymerase chain reaction (PCR) amplification was performed in a final reaction volume of 25 µL containing 2 µL of fungal DNA, 0.4 µM of each primer, and 12.5 µL of Speedy Supreme NZYTaq Master Mix (NYZTech Lda., Portugal), with thermocycler program set for an initial step at 95 °C for 5 min, followed by 50 cycles with denaturation at 94 °C for 2 sec, annealing at 60 °C for 5 sec and extension at 72 °C for 5 sec, and a final extension phase of 2 min at 72 °C.

Additionally, isolates' identity was confirmed based on their sequence of ITS region, amplified using the universal primers ITS1F/ITS4 (Gardes and Bruns, 1996; White *et al.*, 1990). PCR reactions (final volume of 25 µL) consisted of 2 µL of fungal DNA, 0.4 µM of each primer, and 12.5 µL of Speedy Supreme NZYTaq Master Mix. Cycling conditions consisted of a pre-heat at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C

for 2 sec, annealing at 55 °C for 5 sec and extension at 72 °C for 5 sec, and a final extension phase of 2 min at 72 °C.

The detection regarding the presence of the specific G143A point mutation that confers *P. nawae* resistance to QoI fungicides was analyzed based on the partial sequence of the *cyt b* gene, amplified using the primers CBF1 (5'-TAT TATGAG AGA TGT AAA TAA TGG-3') and CBR2 (5'-AAC AAT ATC TTG TCC AAT TCA TGG-3') (Ma *et al.*, 2003). PCR reactions (final volume of 25 µL) were prepared with 2 µL of fungal DNA, 0.4 µM of each primer, and 12.5 µL of Speedy Supreme NZYTaq Master Mix. The program consisted of a pre-heat at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 2 sec, annealing at 50 °C for 5 sec and extension at 72 °C for 5 sec, and a final extension phase of 2 min at 72 °C.

Separation and confirmation of all PCR products were done by electrophoresis in 1% agarose gel stained with RealSafe Nucleic Acid Staining Solution 20,000x (Real-Durviz s.l., Spain) in TRIS-acetate buffer, using 5 µL of each PCR product, and observed under UV light (gelONE, Cleaver Scientific Ltd, UK). A 100-bp ladder (GeneRuler™, Thermo Scientific, USA) was used as a molecular weight marker.

For sequencing of the ITS region and *cyt b* gene, PCR products were purified by using Illustra™ Exonuclease I and Alkaline Phosphatase enzymes in a cycle of 15 min at 37 °C and 15 min at 80 °C. Products were diluted for ideal concentration and sequenced by the DNA Sequencing Service of the *Instituto de Biología Molecular y Celular de Plantas* (IBMCP, Universitat Politècnica de València, Spain).

Edition and assembly of sequences were performed using Sequencher software for DNA analysis (Gene Codes Corp., USA, http://www.genecodes.com/). The obtained sequences were screened against the nucleotide collection of the NCBI database (http://ncbi.nlm.nih.gov) using BLASTn tool. Sequences of the partial *cyt b* gene from QoI-resistant and sensitive isolates of *Mycosphaerella fijiensis* (accession Nos. AF343069 as resistant, and AF343070 as sensitive) were obtained from the NCBI GenBank database and compared to the sequences of the studied isolates.

3.4. Phenotypic assessment of resistance in *P. nawae* population

Isolates of *P. nawae* were evaluated with regard to its sensitivity to pyraclostrobin (QoI) in an assay evaluating the percentage of growth reduction. PDA plates amended with Pyraclostrobin was prepared as previously described to obtain final concentrations of 0.01, 0.1, 1, 10 and 100 mg L^{-1} of the active ingredient. Isolates were transferred from pure colonies to PDA plates following the same methodology as described in the previous section. Plates were stored for 20 days in the dark, at 23 °C. Colony growth was measured as previously described. The effective fungicide concentration to inhibit 50% of fungal growth (EC_{50}) was determined for each isolate. Estimates were calculated with the R statistical software using the *ec50estimator* package (R Core Team, 2015; Alves, 2020).

4. RESULTS

A total of 183 isolates of *P. nawae* were obtained from symptomatic persimmon leaves with characteristic lesions of CLS (Table 1), collected in 2020. The preliminary assessment of these isolates with a discriminant concentration of 10 mg L^{-1} of pyraclostrobin reported a very diverse population relative to its sensitivity, with growth reduction ranging from 0 to 100% (Figure 4). Resistant reference isolates from 2019 exhibited reduction of growth below 26%, while sensitive were reduced by at least 69%.

Table 1. Municipalities within Valencian Community from where leaves with symptoms of circular leaf spot were collected and *Plurivorosphaerella nawae* isolates were tested for G143A point mutation and 50% effective concentration (EC_{50}) of pyraclostrobin.

Municipality	Number of plots surveyed	Number of isolates	Isolates carrying the G143A mutation	Incidence of mutation (%)
Alberic	3	6	6	100
Algemesí	3	8	8	100
Alginet	10	33	24	73

Alzira	4	10	9	90
Benifaió	2	6	0	0
Benimodo	4	16	13	81
Benimuslem	1	3	3	100
Carlet	3	10	6	60
Catadau	1	1	0	0
Gavarda	1	2	0	0
Guadassuar	5	15	15	100
L'Alcúdia	2	4	4	100
Llanera de Rana	1	8	1	13
Llombai	1	3	0	0
Montesa	1	5	0	0
Monteserrat	2	12	0	0
Picassent	6	17	6	35
Rafelguaraf	1	1	0	0
Real	2	6	0	0
Rotglà i Corberà	1	1	0	0
Silla	1	1	0	0
Simat de la Valldigna	1	4	1	25
Sollana	3	5	2	40
Sueca	1	2	2	100
Xativa	1	4	1	25
TOTAL	61	183	101	55

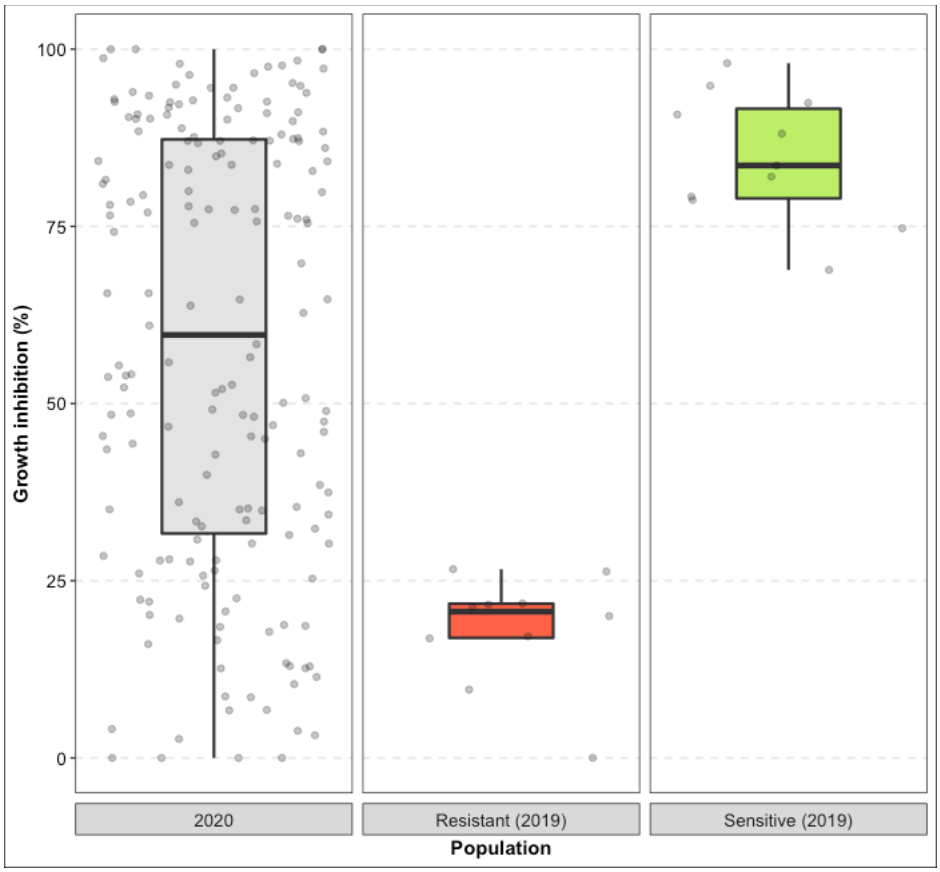

Figure 4. Growth reduction response of isolates of *Plurivorosphaerella nawae* exposed to 10 mg L^{-1} of pyraclostrobin.

Specifically, 46% of the *P. nawae* population demonstrated similar growth inhibition to the group of reference isolates of 2019 with high sensitivity, whilst 20% were within the same range of the resistant isolates. Overall, we observed that a large proportion of isolates (n = 57, 31%) presented growth inhibition between 30 and 70%, which could not be grouped together with neither of the reference groups (Figure 5).

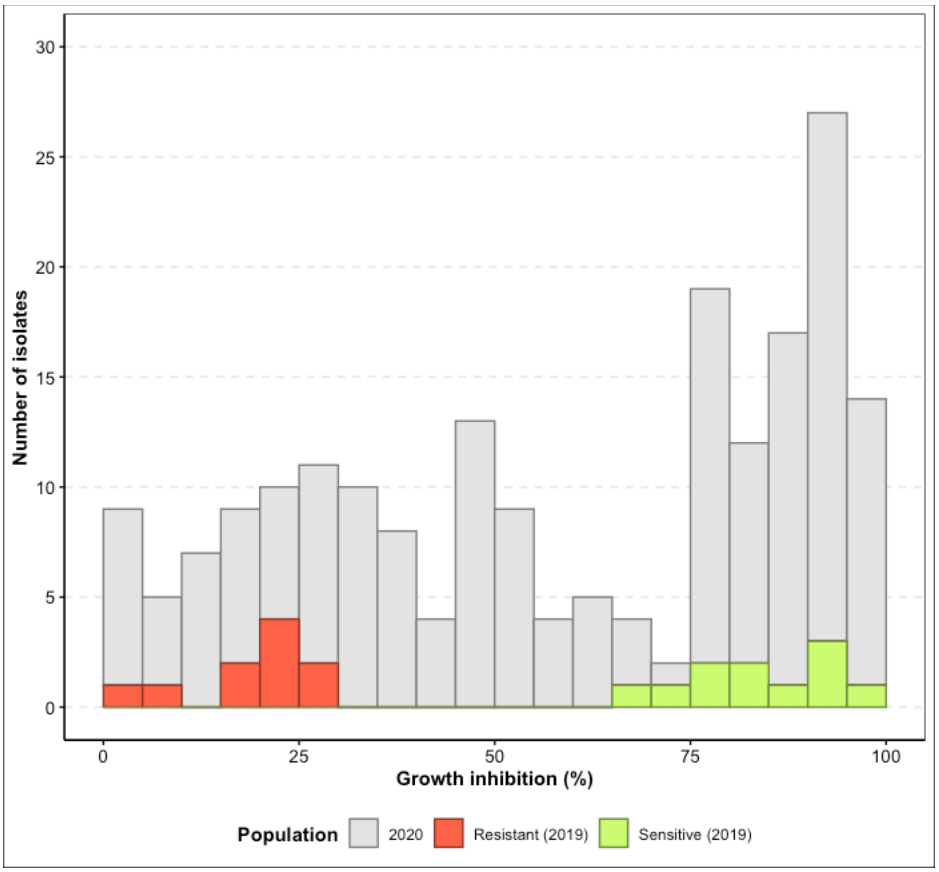

Figure 5. Distribution of number of isolates based on ranges of growth inhibition within the three populations of *Plurivorosphaerella nawae*, after exposure to 10 mg L^{-1} of pyraclostrobin.

4.1. Identification and molecular detection of resistance to QoI fungicides

The use of molecular approaches to characterize our population confirmed that all isolates collected from symptomatic persimmon leaves belonged to the species *P. nawae*. All of them yielded 165 bp amplicon using the species-specific primers and about 665-bp product using the ITS primers. The obtained ITS sequences were searched in the GenBank NCBI database, using the BLASTn tool, which showed 100% identity with DNA sequences of *P. nawae* (≡ *M. nawae*).

The presence of the G143A mutation, inducing QoI-sensitivity loss in plant pathogenic fungi, was analyzed by sequencing the *cyt b* gene. All 183 isolates yielded fragments of

280 bp, regardless of its sensitivity. With the alignment of the sequences and comparison with reference sequences of resistant and sensitive *M. fijiensis* strains, we observed that 101 isolates carried the specific mutation, in which a guanine is replaced by a cytosine at codon 143, thereafter changing the amino acid translation from glycine to alanine (Figure 6). The number represents 55% of the total population, and not surprisingly, this percentage is rather similar to the proportion of isolates showing growth reduction below 70% with the discriminant concentration. In fact, all but one isolate within this range of growth inhibition were confirmed to be carrying the G143A. The exception was an isolate that had growth reduced by 65% but lacked the mutation.

SPECIES	GenBank code	SEQUENCE	GENOTYPE
Mycosphaerella fijiensis	AF343070.1	GYVLPYGQMSL*GATVITNL	WILD TYPE
Mycosphaerella fijiensis	AF343069.1	GYVLPYGQMSL*AATVITNL	MUTANT
Plurivorosphaerella nawae	-	GYVLPYGQMSL*GATVITNL	WILD TYPE
Plurivorosphaerella nawae	-	GYVLPYGQMSL*AATVITNL	MUTANT

Figure 6. Partial alignments of the deduced amino acid sequences of the cytochrome b gene from QoI-sensitive and QoI-resistant isolates of *Mycosphaerella fijiensis* (references) and two representative isolates of *Plurivorosphaerella nawae*. Amino acid substitution (G143A) is highlighted in red.

4.2. Phenotypic assessment of resistance in *P. nawae* population

Our *P. nawae* population was arranged into two homogeneous groups according to the results of the molecular characterization (mutant and wild type genotypes), in order to better comprehend the performance of different genotypes and the specific effect of the mutation over the sensitivity to QoI fungicides. Each isolate was assessed in an *in vitro* assay with increasing concentrations of pyraclostrobin (0 – 100 mg L^{-1}). Data of the wild type population (n = 82) showed EC_{50} values ranging from 0.009 mg L^{-1} to 1.51 mg L^{-1} (Figure 7). Within the group of mutant isolates (n = 101) a much wider range was observed, with EC_{50} values as low as 31.9 mg L^{-1} and up to 1117 mg L^{-1}. Mean EC_{50} value of the resistant population (169 mg L^{-1}) is 545 times higher than the mean of the sensitive population (0.31 mg L^{-1}).

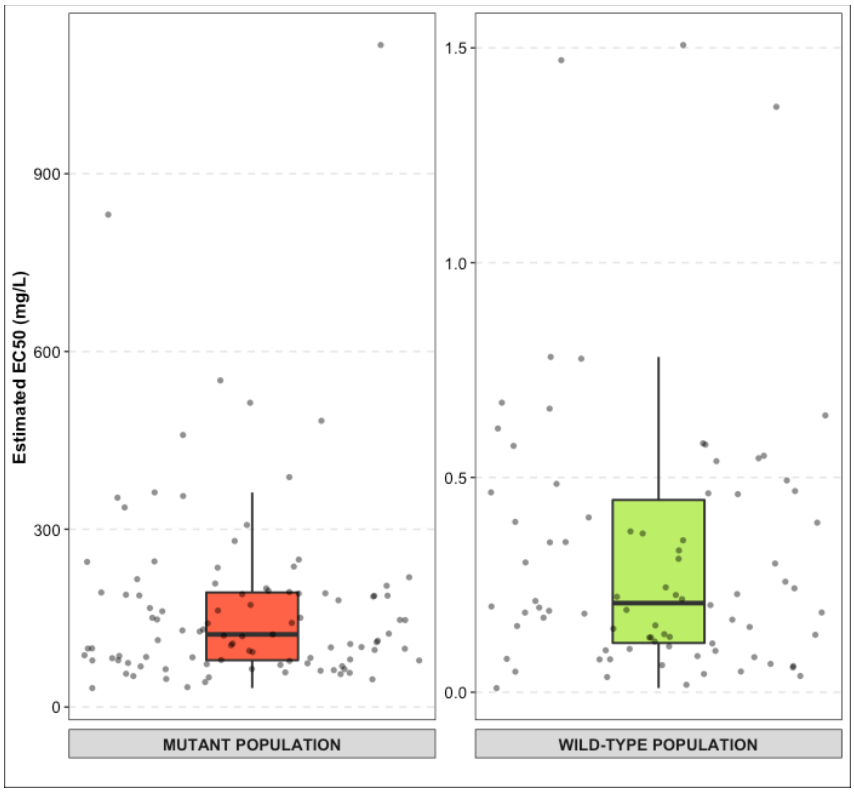

Figure 7. Range of estimated values of the 50% effective concentration (EC_{50}) in mutant and wild-type populations of *Plurivorosphaerella nawae*.

5. DISCUSSION

Management of CLS in the Valencian Community is mostly dependent of preventive applications of fungicide due to the high incidence of the disease and the epidemiological aspects that hinders early detection of infection. With few fungicides authorized for treatment in persimmon crop, QoIs have become the most effective measure, used for nearly 10 years now. However, in the long run, constant application of QoIs is commonly linked to loss of efficacy as the pathogen population coevolving with the pesticide develops mechanisms of resistance.

In 2019, decreased efficacy of QoI fungicides towards the control of *P. nawae* was observed in several locations along the main persimmon producing region of the Valencian Community. Isolates collected in 2020 were assessed using a discriminant concentration of 10 mg L^{-1} of pyraclostrobin. In a previous study, with a different population of *P. nawae*, isolates collected in 2019 exposed to this same specified concentration performed significantly different, with which it was possible to tell apart between highly sensitive and insensitive isolates (*unpublished data*). In the present study, at this discriminant concentration, we were able to obtain the prevalence of isolates with low sensitivity to pyraclostrobin in our population. Although the growth inhibition in some isolates was initially not comparable with any of the two groups from the previous study, it was clear that a shift in the sensitivity to QoIs was present. We came to that conclusion after noticing that at 55% of the population showed growth reductions below 70% after being exposed to 10 mg L^{-1} of pyraclostrobin.

The mechanisms by which a fungicide loses its efficacy against a particular pathogen may fall under two main categories: the quantitative insensitivity, that is a result of different metabolic adaptations improving fungicide degradation and reducing intracellular concentration of the active compound to very low levels; or the qualitative insensitivity, which is a mutation-based complete loss of sensitivity to the active ingredient (Bowness *et al.*, 2016). The latter is generally a common cause of QoI insensitivity in agricultural pathogens. Molecular analyses of the *cyt b* were carried out and revealed that the G143A mutation was present in the resistant isolates, suggesting that the shift in fungicide sensitivity and loss of disease control efficacy were related to the prevalence of a resistant

population in the field. Indeed, this outcome reflects the general situation encountered across the sampled locations, with frequent exposure of the pathogen to QoI fungicides over the last 10 years. Such practice exerted a strong selection pressure on the population and what, at first, might have seemed as a sporadic and incidental lack of fungicide efficacy, due to other factors such inappropriate spray timing or extreme weather events, turned out to be a general situation as the resistant population grew and spread throughout the region.

The breakdown of CLS control with QoI fungicide due to an extensive distribution of the resistant population obligate farmers to completely modify their management program. In such conditions, not only loss of efficacy becomes a frequent scenario if treatments continue to follow label recommendations, increasing the amount of fungicide or frequency of application will not result in any improvement of disease control. *In vitro* assays are widely used to study and evaluate pathogen's sensitivity to fungicidal compounds, allowing an estimation of the effective concentration capable of reducing fungal growth by 50%. In our study, we determined the EC_{50} values for both mutant and wild-type populations to understand the effect of the point mutation on the shifting of pathogen's sensitivity.

EC_{50} mean value of the wild-type population (0.31 mg L^{-1}) was found to be even lower than the 1.8 mg L^{-1} reported by Berbegal *et al.* (2011) for two sensitive isolates of *P. nawae* collected in the same region covered by our study. With remarkable higher sensitivity in the wild-type population in comparison to the mutant population, the likelihood of resistance progressing through a quantitative path is very low. Reduced sensitivity to QoI fungicides in non-mutant strains of *Venturia inaequalis* (Cooke) G. Winter, for instance, is thought to be linked to other mechanisms such as alternative respiration (Fontaine *et al.*, 2009; Fiaccadori *et al.*, 2011). However, in our case, with such response from the wild-type population, displaying high sensitivity, associated with the observation of high values of EC_{50} in the mutant population, it is reasonable to presume that the decreased sensitivity of the overall population is unrelated to secondary metabolic pathways that promote fungicide detoxification.

Furthermore, none of the isolates from the resistant population have shown EC_{50} values anywhere near the range of the sensitive population. With EC_{50} values > 30 mg L^{-}

[1], and mean of 169 mg L^{-1} in the resistant population, it is evident that, under field conditions, effective control of CLS with QoI fungicides is impracticable. If under controlled *in vitro* conditions mutant individuals were on average 545 times less sensitive than wild type individuals, natural environments would certainly implicate in much higher and unfeasible rates of fungicide to effectively reduce fungal growth. Many other similar cases of highly insensitive pathogen populations have been described in the literature. Among important horticultural crops, populations of *Botrytis cinerea* Pers. (in vegetables and cucurbits), *Mycosphaerella pinodes* (Berk. & A. Bloxam) Vestergr. (peas), and *Podosphaera fusca* (peas) assayed *in vitro,* were reported to have EC_{50} values well above label rate (Fernández-Ortuño *et al.*, 2006, Bardas *et al.*, 2010, Bowness *et al.*, 2016).

Altogether, results from both molecular and phenotypic characterization provide sufficient evidence to demonstrate that the primary cause of the reduced efficacy against CLS of the QoI fungicides applied in persimmon orchards in Spain arises from the high prevalence of the resistant population of *P. nawae* carrying the G143A mutation. Whether this prevalence will result in a complete domination of the resistant population and elimination of the sensitive individuals depends on both environmental conditions and biological factors. With apparent little to no fitness cost of G143A mutation, the selection pressure on *P. nawae* populations imposed by intense fungicide application should be the major component driving this growth and shift towards a dominance of resistant populations at field level (Parnell *et al.*, 2005; Hawkins and Fraaije, 2018), and therefore strategies of disease management in what concerns the application of fungicides should prioritize control without QoI fungicides.

Moreover, the widespread distribution of QoI resistance brings about another concern for the management of CLS in persimmon in Spain. As both pyraclostrobin and azoxystrobin cannot be included in the spray programs due to the lack of efficacy, the list of available fungicides shrinks dramatically. As of today, mancozeb, a broad-spectrum fungicide, and difenoconazole (from the group of SBIs) are the only two alternatives registered for field treatment. However, it is noteworthy that in 2020 the European Commission has decided not to renew the authorization of mancozeb. This decision means that Member States of the European Union will soon withdraw authorizations of all products formulated with mancozeb as their active compound and products will be

removed from the market (European Commission, 2020). It is thus encouraged that chemical companies and official bodies work together to provide growers with new and effective alternatives. Otherwise, shortly the protection against CLS will solely rely on a single active ingredient (difenoconazole). This, by no means, comply with schemes of good agricultural practices that target the reduction of pathogen resistance development through the rotation of fungicides with different modes of action. Although SBIs are considered by FRAC as of medium-risk to resistance development in contrast to the high-risk QoIs, still difenoconazole and similar compounds are affected by this problem. FRAC guidelines for fungicide resistance management strongly advises against repeated application of SBIs, especially in those areas with high disease pressure, as it is the case of CLS in the Valencian Community (FRAC, 2021). If this situation persists for too long, it is very likely that in near future loss of sensitivity and resistance will arise after the repeated use of fungicides from the group of the SBI.

Taking into consideration that cultivar diversity is virtually nonexistent across persimmon orchards in the Valencian Community and the fact that the causal agent *P. nawae* has been stablished in the region for more than a decade (Berbegal *et al.*, 2010), the current situation presents an increased risk of seasonal disease outbreaks. The cv. ‘Rojo Brillante’ alone represents about 90% of the planted area in this region (Iglesias Valera, 2018), and this lack of genetic variability among highly susceptible plants, together with the reduced efficacy of the available control methods poses a serious threat to the persimmon industry. Consequences of a severe outbreak of CLS due to infective preventive control could reduce supply of persimmon to a level that would harm markets even beyond borders, as Spain appears as the major exporter of persimmon worldwide (46% of global share) (Global Trade, 2021).

6. CONCLUSIONS

Resistance to QoI fungicides in Plurivorosphaerella nawae, the causal agent of circular leaf spot of persimmon, is widespread across the main persimmon-producing regions in the Valencian Community, Spain. The resistance is conferred by the G143A mutation in the cytochrome b, which changes the amino acid sequence right at the binding site of QoI fungicides. Out of the 183 isolates analyzed from symptomatic leaves, 101 carried the mutation.

Sensitivity to QoI fungicides is seriously compromised due to the high prevalence of resistant isolates, observed with the estimated EC_{50} values ranging from 31.9 mg L^{-1} to 1117 mg L^{-1} in the mutant population, while wild-type individuals showed values between 0.009 mg L^{-1} and 1.51 mg L^{-1}. This confirms that, as of today, application of QoI fungicides for the control of circular leaf spot in the Valencian Community should be discontinued and a search for alternatives have to be initiated.

BIBLIOGRAPHIC REFERENCES

Agrios, G. N. (2005). Plant pathology (5th edition), p. 922.

Alves, K. S. (2020). ec50estimator: An automated way to estimate EC50 for stratified datasets. R package version 0.1.0. https://CRAN.R-project.org/package=ec50estimator

Amil, A. F., Heaney, S. P., Stanger, C., & Shaw, M. W. (2007). Dynamics of QoI sensitivity in *Mycosphaerella fijiensis* in Costa Rica during 2000 to 2003. Phytopathology, 97(11), 1451-1457.

Balba, H. (2007). Review of strobilurin fungicide chemicals. Journal of Environmental Science and Health Part B, 42(4), 441-451.

Bardas, G. A., Veloukas, T., Koutita, O., & Karaoglanidis, G. S. (2010). Multiple resistance of *Botrytis cinerea* from kiwi fruit to SDHIs, QoIs and fungicides of other chemical groups. Pest Management Science, 66(9), 967-973.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. Pest Management Science, 58(7), 649-662.

Bassimba, D. D. M., Mira, J. L., Sedano, M. E., & Vicent, A. (2017). Control and yield loss modelling of circular leaf spot of persimmon caused by *Mycosphaerella nawae*. Annals of Applied Biology, 170(3), 391-404.

Berbegal, M., Armengol, J., & García-Jiménez, J. (2011). Evaluation of fungicides to control circular leaf spot of persimmon caused by *Mycosphaerella nawae*. Crop Protection, 30(11), 1461-1468.

Berbegal, M., Mora-Sala, B., García-Jiménez, J. (2013). A nested-polymerase chain reaction protocol for the detection of *Mycosphaerella nawae* in persimmon. European Journal of Plant Pathology, 137, 273-281.

Berbegal, M., Pérez-Sierra, A., Armengol, J., & Jiménez, J. G. (2010a). La necrosis foliar causada por *Mycosphaerella nawae* Hiura & Ikata: una nueva enfermedad del caqui (*Diospyros kaki* Lf) en España. Boletín de Sanidad Vegetal. Plagas, 36(2), 213-224.

Berbegal, M., Pérez-Sierra, A., Armengol, J., Park, C. S., & García-Jiménez, J. (2010b). First report of circular leaf spot of persimmon caused by *Mycosphaerella nawae* in Spain. Plant Disease, 94(3), 374-374.

Bowness, R., Gossen, B. D., Chang, K.-F., Goswani, R., Willenborg, G. J., Holtz, M., and Strelkov, S. E. (2016). Sensitivity of *Mycosphaerella pinodes* to pyraclostrobin fungicide. Plant Disease, 100(1), 192-199.

Brent, K. J., & Hollomon, D. W. (2007). Fungicide resistance in crop pathogens: how can it be managed? (p. 56). Brussels: FRAC.

European Commission (2020). Commission Implementing Regulation (EU) 2020/2087. Official Journal of the European Union. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32020R2087&qid=1608132304092 (Accessed on 14th June 2021)

Fernández-Ortuño, D., Pérez-García, A., López-Ruiz, F., Romero, D., De Vicente, A., & Torés, J. A. (2006). Occurrence and distribution of resistance to QoI fungicides in populations of *Podosphaera fusca* in south central Spain. European Journal of Plant Pathology, 115(2), 215-222.

Fernández-Ortuño, D., Torés, J. A., De Vicente, A., & Pérez-García, A. (2008). Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. International Microbiology, 11(1), 1.

Fernández-Ortuño, D., Torés, J. A., De Vicente, A., & Pérez-García, A. (2010). The QoI fungicides, the rise and fall of a successful class of agricultural fungicides. Fungicides, 203-220.

Fiaccadori, R., Cicognani, E., Alberoni, G., Collina, M., & Brunelli, A. (2011). Sensitivity to strobilurin fungicides of Italian *Venturia inaequalis* populations with different origin and scab control. Pest Management Science, 67(5), 535-540.

Fontaine, S., Remuson, F., Fraissinet-Tachet, L., Micoud, A., Marmeisse, R., & Melayah, D. (2009). Monitoring of *Venturia inaequalis* harbouring the QoI resistance G143A mutation in French orchards as revealed by PCR assays. Pest Management Science, 65(1), 74-81.

FRAC – Fungicide Resistance Action Committee (2006). Mutations associated with QoI-resistance. https://www.frac.info/docs/default-source/working-groups/qoi-quick-references/mutations-associated-with-qo-resistance.pdf?sfvrsn=c9db449a_4 (Accessed on 12th June 2021)

FRAC – Fungicide Resistance Action Committee (2020). List of first confirmed cases of plant pathogenic organisms resistant to disease control agents. https://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-first-confirmed-cases-of-plant-pathogenic-organisms-resistant-to-disease-control-agents_05_2020.pdf?sfvrsn=7073499a_2 (Accessed on 12th June 2021)

FRAC – Fungicide Resistance Action Committee (2021). https://www.frac.info/frac-teams/working-groups/sbi-fungicides/recommendations-for-sbi (Accessed on 12th June 2021)

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology, 2(2), 113-118.

Generalitat Valenciana (2017). Sanitat Vegetal. La mancha foliar del caqui (*Mycosphaerella nawae* Hiura & Ikata). https://agroambient.gva.es/documents/163214705/163899388/Mancha+foliar+del+caqui+%28Mycosphaerella+nawae%29.pdf/51ffd3bf-5cc4-422e-9947-02552543be1b (Accessed on 12th June 2021)

Generalitat Valenciana (2020). La mancha foliar del caqui – *Plurivorosphaerella nawae* (=*Mycosphaerella nawae*). https://agroambient.gva.es/documents/163214705/164587757/Recomendaciones+e

strategia+control+Mancha+Foliar+del+caqui+2020.pdf/dc267add-59be-4ff4-910f-11cdc5af2a6e (Accessed on 12th June 2021)

Global Trade (2021). The global persimmon market slipped back slightly to $7.1B. https://www.globaltrademag.com/the-global-persimmon-market-slipped-back-slightly-to-7-1b/ (Accessed on 12th July 2021)

Hassan, O., & Chang, T. (2019). Phylogenetic and morphological reassessment of *Mycosphaerella nawae*, the causal agent of circular leaf spot in persimmon. Plant Disease, 103(2), 200-213.

Hawkins, N. J., & Fraaije, B. A. (2018). Fitness penalties in the evolution of fungicide resistance. Annual Review of Phytopathology, 56, 339-360.

Iglesias Valera, G. (2018). Optimización de la elaboración de vinagre de caqui Rojo Brillante (*Diospyros kaki*). (Master dissertation, Universitat Politècnica de València).

Kanungo, M., & Joshi, J. (2014). Impact of pyraclostrobin (F-500) on crop plants. Plant Science Today, 1(3), 174-178.

Kwon, J. H., & Park, C. S. (2004). Ecology of disease outbreak of circular leaf spot of persimmon and inoculum dynamics of *Mycosphaerella nawae*. Research in Plant Disease, 10(4), 209-216.

Leadbeater, A. (2012). Resistance risk to QoI fungicides and anti-resistance strategies. In: Thind, T. S. (Ed) Fungicide resistance in crop protection: Risk and management, 141.

Lee, S.-Y., Lim, Y.-S., & Jung, H.-Y. (2016). Molecular phylogeny and morphology of *Mycosphaerella nawae*, the causal agent of circular leaf spot on persimmon. Mycobiology, 44(4), 191–201. doi:10.5941/myco.2016.44.4.191

Llácer, G., & Badenes, M.L. (2002). Persimmon production and market. Options Méditerranéennes, Série A, 51, 9-21.

Ma, Z., Felts, D., Michailides, T. J. (2003). Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. Pesticide Biochemistry and Physiology, 77(2), 66-74.

Martínez las Heras, R. (2017). Valorización del cultivo del caqui (Doctoral dissertation, Universitat Politècnica de València).

Martínez-Minaya, J., Conesa, D. V., López-Quílez, A., Mira, J. L., & Vicent, A. (2019). Modelling inoculum availability of *Plurivorosphaerella nawae* in persimmon leaf litter with Bayesian beta regression. Phytopathology (First look).

Martínez, M. B., Albalat, A. R., Armengol, J., & Ginér, J. (2017). Evaluación de la sensibilidad a mancozeb y piraclostrobin de *Mycosphaerella nawae*, agente causal de la mancha foliar del caqui. Phytoma España, 294, 50-53.

McCartney, C., Mercer, P. C., Cooke, L. R., & Fraaije, B. A. (2007). Effects of a strobilurin-based spray programme on disease control, green leaf area, yield and development of fungicide-resistance in *Mycosphaerella graminicola* in Northern Ireland. Crop Protection, 26(8), 1272-1280.

Naval, M. M., Martínez-Calvo, J., Badenes, M. L., & Llácer, G. (2012). Los programas de mejora genética de frutales en el Instituto Valenciano de Investigaciones Agrarias (IVIA) IV. Caqui. Revista de Fruticultura, (20), 4-13.

Palou, L., Montesinos-Herrero, C., Tarazona, I., Besada, C., & Taberner, V. (2015). Incidence and etiology of postharvest fungal diseases of persimmon (*Diospyros kaki* Thunb. cv. Rojo Brillante) in Spain. Plant Disease, 99(10), 1416-1425.

Parnell, S., Gilligan, C. A., & Van den Bosch, F. (2005). Small-scale fungicide spray heterogeneity and the coexistence of resistant and sensitive pathogen strains. Phytopathology, 95(6), 632-639.

Perucho, R. (2016). Evolution of production of the 'Rojo Brillante' cultivar in Spain and its impact on markets. In VI International Symposium on Persimmon 1195 (pp. 1-8).

R Core Team (2015). R: A Language and Environment for Statistical Computing. http://www.R-project.org/.

Siah, A., Deweer, C., Morand, E., Reignault, P., & Halama, P. (2010). Azoxystrobin resistance of French *Mycosphaerella graminicola* strains assessed by four in vitro bioassays and by screening of G143A substitution. Crop Protection, 29(7), 737-743.

Sierotzki, H. (2015). Respiration inhibitors: complex III. In Fungicide resistance in plant pathogens (pp. 119-143). Springer, Tokyo.

Sierotzki, H., Parisi, S., Steinfeld, U., Tenzer, I., Poirey, S., & Gisi, U. (2000). Mode of resistance to respiration inhibitors at the cytochrome bc1 enzyme complex of *Mycosphaerella fijiensis* field isolates. Pest Management Science, 56(10), 833-841.

Torriani, S. F., Brunner, P. C., McDonald, B. A., & Sierotzki, H. (2009). QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. Pest Management Science, 65(2), 155-162.

Tridge (2021). Exportaciones mundiales de caqui. http://www.tridge.com/intelligences/persimmon/export (Accessed on 12th July 2021)

Vallières, C., Trouillard, M., Dujardin, G., & Meunier, B. (2011). Deleterious effect of the Qo inhibitor compound resistance-conferring mutation G143A in the intron-containing cytochrome b gene and mechanisms for bypassing it. Applied and Environmental Microbiology, 77(6), 2088-2093.

Vicent, A., Bassimba, D. D. M., & Intrigliolo, D. S. (2011a). Effects of temperature, water regime and irrigation system on the release of ascospores of *Mycosphaerella nawae*, causal agent of circular leaf spot of persimmon. Plant Pathology, 60(5), 890-908.

Vicent, A., Bassimba, D. D., Hinarejos, C., & Mira, J. L. (2012). Inoculum and disease dynamics of circular leaf spot of persimmon caused by *Mycosphaerella nawae* under semi-arid conditions. European Journal of Plant Pathology, 134(2), 289-299.

Vicent, A., Bassimba, D., Hinarejos, C., & Mira, J. L. (2011b). La mancha foliar del caqui, una nueva enfermedad en un cultivo en expansión. Vida Rural, 336, 16-19.

Vicent, A., Mira, J. L., Taberner, V., & Palou, L. (2020). Principales enfermedades fúngicas del caqui en España. Vida Rural, (480), 12-18.

Videira, S. I. R., Groenewald, J. Z., Nakashima, C., Braun, U., Barreto, R. W., de Wit, P. J., & Crous, P. W. (2017). Mycosphaerellaceae–chaos or clarity? Studies in Mycology, 87, 257-421.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., & White, J. T. (Eds) PCR protocols: a guide to methods and applications (pp. 315-322). San Diego, California: Academic Press.

Ypema, H. L., & Gold, R. E. (1999). Kresoxim-methyl: modification of a naturally occurring compound to produce a new fungicide. Plant Disease, 83(1), 4-19.