Universitat de Girona

EXPERIMENTAL BASIS FOR THE CONTROL OF PLANT DISEASES CAUSED BY *Xylella fastidiosa* USING SYNTHETIC ANTIMICROBIAL PEPTIDES

Aina Baró Sabé

Universitat de Girona

DOCTORAL THESIS

Experimental basis for the control of plant diseases caused by *Xylella fastidiosa* using synthetic antimicrobial peptides

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Doctoral Programme in Technology

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Thesis submitted to apply for the Doctoral degree
by the University of Girona

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DECLAREM:

Que el treball titulat "Experimental basis for the control of plant diseases caused by *Xylella fastidiosa* using synthetic antimicrobial peptides", que presenta Aina Baró Sabé per l'obtenció del títol de doctora per la Universitat de Girona, ha estat realitzat sota la nostra direcció i que compleix els requisits per poder optar a Menció Internacional.

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DECLARA QUE:
Aquesta tesi està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de Patologia Vegetal i de l'Institut de Tecnologia Agroalimentària de la Universitat de Girona que participen en els esmentats projectes (Article 2. Apartat 2. RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996).

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Girona, 23 de juny de 2021.

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LIST OF ABBREVIATIONS

µM	micromolar
aa	Amino acid
ABA	Abscisic acid
Acyl-CoA	Acyl-coenzime A
ALS	Almond Leaf Scorch
AMPs	Antimicrobial peptides
Cu	Copper
$CuSO_4$	Copper(II) sulfate
CVC	Citrus Variegated Chlorosis
DNA	Deoxyribonucleic acid
DSF	Diffusible signal factor
ED_{50}	Median effective dose
EFSA	European Food Safety Authority
EPPO	European and Mediterranean Plant Protection Organization
ETI	Effector triggered immunity
EU	European Union
GMO	Genetically modified organism
GWSS	Glassy-winged sharpshooter
ha	Hectare
H2020	Horizon 2020
HR	Hypersensitive response
IDM	Integrated disease management
ISR	Induced systemic resistance
km	Kilometer
LPS	Lipopolysaccharide
m	Meter
Mb	Megabase
MeSA	Methyl salicylate
MIC	Minimum inhibitory concentration
MID	Minimal infective dose
MLST	Multilocus sequence typing
NAC	*N*-Acetylcysteine
OMV	Outer membrane vesicles

OQDS	Olive Quick Decline Syndrome
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PD	Pierce's Disease
Peps	Plant elicitor peptides
PlgA	Polygalacturonase A
POnTE	Pest Organisms Threatening Europe
PR1	*Pathogenesis-related* 1 gene
PTI	Pattern triggered immunity
qPCR	Real-time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
ST	Sequence type
TEM	Transmission electron microscopy
USA	United States of America
VBNC	Viable-but-non-culturable
v-qPCR	Viability-qPCR
XF-ACTORS	*Xylella Fastidiosa* Active Containment Through a multidisciplinary-Oriented Research Strategy
Zn	Zinc

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LIST OF PUBLICATIONS

This Ph.D. Thesis is presented as a compendium of three publications:

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Baró A., Badosa E., Montesinos L., Feliu L., Planas M., Montesinos E., Bonaterra A. (2020) Screening and identification of BP100 peptide conjugates active against *Xylella fastidiosa* using a viability-qPCR method. **BMC Microbiology**, 20:229.

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SUMMARY

Xylella fastidiosa is one of the most harmful bacterial plant pathogens worldwide, causing a variety of diseases, with huge economic impact to agriculture and environment. Until 2013, the pathogen was mostly detected in the American continent, causing mainly Pierce's disease (PD) of grapevines, citrus variegated chlorosis (CVC) and coffee leaf scorch (CLS). Nevertheless, the arrival of different subspecies of this pathogen to the European Union (EU) changed its distribution worldwide. At present, *X. fastidiosa* is importantly affecting olive trees in Apulia region in Southern Italy, *Polygala myrtifolia* in the PACA region in Southern France and Corsica, and almond trees in Alicante province and Balearic Islands in Spain.

The management of *X. fastidiosa*-related diseases is currently based on measures for the prevention of its spread to other regions, such as the use of pathogen-free propagation plant material, exclusion and quarantine measures, and vector control. Up to now, no therapeutic solutions are available for the control of *X. fastidiosa* in diseased plants and trees. Therefore, there is a need for novel bactericides against this pathogen. Antimicrobial peptides (AMPs) represent a promising alternative to traditional compounds due to their activity against a wide range of plant pathogens, low cytotoxicity, mode of action and availability for creating sustainable pesticides and disease resistant crops. Accordingly, the objective of the present Ph.D. Thesis focused on the study of AMPs as a sustainable alternative for the control of *X. fastidiosa*-related diseases.

Previous results obtained by our group on the antimicrobial activity of newly described peptides, served for the selection of lead peptides with potential for being tested *in vitro* against *X. fastidiosa*. The peptide BP178 reported the highest bactericidal activity against different *X. fastidiosa* strains, which was associated to cell lysis. Pore formation and disruption of the bacterial membrane was observed by transmission electron microscopy (TEM) ultrastructural analysis, demonstrating that direct killing is the main mechanism of action of BP178 peptide. Time-course and dose-effect experiments revealed that the bactericidal activity of BP178 happens within the first 10 to 20 min post-exposure and it is

significantly affected by the peptide dose and the *X. fastidiosa* strain and concentration. Nevertheless, a threshold ratio around 10^9 BP178 molecules/cell was estimated to be necessary for the lysis of all *X. fastidiosa* strains tested. The bactericidal activity of BP178 was also significantly affected by the diluent used, indicating that the complex environment under *in vivo* conditions might affect the interaction between AMPs and bacterial cell membranes.

In order to have a large number of peptides with potential to be effective against *X. fastidiosa in planta*, a methodology for the screening of AMPs based on their bactericidal activity against this pathogen was developed. The designed contact test followed by a viability-qPCR (v-qPCR) overcomes the quarantine status of *X. fastidiosa* in the EU and its fastidious nature and slow growth, and quantifies the whole fraction of viable cells, either culturable or not. The methodology allowed the identification, in addition to the BP178, of five new AMPs (BP171, BP175, BP170, BP176 and BP180) active against *X. fastidiosa*. Moreover, it was used to determine the differential susceptibility of *X. fastidiosa* strains to the peptide BP178, as well as differences in the induction of the viable-but-non-culturable (VBNC) state.

The study of pathogenicity related parameters is important to better predict and manage pathogen outbreaks, but also to optimize treatment guidelines in terms of time and dose of application, particularly in the case of AMPs. In this sense, the aggressiveness of different Spanish isolates in almond plants was investigated. All *X. fastidiosa* isolates (IVIA 5387.2, IVIA 5901.2, IVIA 5235.4 and IVIA 5770) were able to cause almond leaf scorch (ALS) in three almond cultivars (*Avijor*, *Soleta* and *Penta*) that are currently being produced as smarttrees. However, differences in aggressiveness were observed between strains, being the strain from the subspecies *multiplex*, IVIA 5901.2, the one reporting low disease severities and colonization rates of new growing shoots. Dose-response relationships revealed differences in important pathogenicity parameters between the two almond isolates of *X. fastidiosa* studied. While IVIA 5387.2 reached high population levels but its median effective dose (ED_{50}) was also high, IVIA 5901.2 attained population levels below the ones of IVIA 5387.2 but its ED_{50} was low. Minimal infective doses (MID) values were also different between strains, but in both cases were below 3000

CFU/plant, indicating that vectors could easily transmit the disease as *X. fastidiosa* concentrations reported in insect heads range from 30 to 10^5 copies/head.

To sum up, highly bactericidal peptides against the plant pathogenic bacterium *X. fastidiosa* have been identified in the present work, revealing important parameters of their activity. Moreover, the aggressiveness of Spanish isolates of *X. fastidiosa* has been studied in order to increase knowledge for the design of efficient treatment guidelines for the control of ALS and other *X. fastidiosa*-related diseases.

RESUM

Xylella fastidiosa és un dels bacteris fitopatògens que provoca més danys a nivell mundial, causant una varietat de malalties amb un gran impacte econòmic en l'agricultura i l'entorn. Fins el 2013, *X. fastidiosa* s'havia detectat majoritàriament al continent americà, causant principalment la malaltia de Pierce a les vinyes, la clorosis variegada dels cítrics i el socarrat de la fulla del cafè. No obstant, l'arribada de diferents subespècies d'aquest patogen a la Unió Europea (UE) ha canviat la seva distribució arreu. Avui en dia, *X. fastidiosa* afecta severament les oliveres a la regió d'Apulia al sud d'Itàlia, *Polygala myrtifolia* a la regió de PACA al sud de França i a Còrsega, i els ametllers a la provincia d'Alacant i a les illes Balears a Espanya.

Actualment, el maneig de les malalties causades per *X. fastidiosa* està basat en l'ús de mesures per prevenir la seva propagació a altres regions, com ara l'ús de material vegetal de propagació lliure de patògens, mesures d'exclusió i quarantena, i el control dels vectors. Fins al moment, no s'ha trobat cap solució terapèutica pel control de *X. fastidiosa* en plantes i arbres afectats. És per això que sorgeix la necessitat de trobar nous bactericides contra aquest patogen. Els pèptids antimicrobians (PAMs) representen una alternativa prometedora als compostos tradicionals degut a la seva activitat contra una àmplia gamma de patògens vegetals, baixa toxicitat, mode d'acció, i disponibilitat per crear pesticides sostenibles i cultius resistents a les malalties. Així doncs, l'objectiu de la present tesi doctoral es va centrar en l'estudi dels PAMs com a alternativa sostenible pel control de les malalties causades per *X. fastidiosa*.

Els resultats obtinguts prèviament pel nostre grup sobre l'activitat antimicrobiana de pèptids recentment descrits, van servir per a la selecció de pèptids líders amb potencial per ser provats *in vitro* contra *X. fastidiosa*. El pèptid BP178 va mostrar l'activitat bactericida més elevada contra diferents soques de *X. fastidiosa*, la qual va ser associada a la lisi cel·lular. La formació de porus i la lisis de la membrana bacteriana es van observar mitjançant l'anàlisi ultraestructural utilitzant el microscopi electrònic de transmissió (MET), demostrant que aquesta acció directa sobre les membranes és la que condueix a la mort bacteriana, essent el principal

mecanisme d'acció del pèptid BP178. Les dinàmiques poblacionals al llarg del temps i els experiments de dosi-efecte van revelar que l'activitat bactericida de BP178 es produeix dels 10 als 20 minuts després de l'exposició, i que aquesta es veu afectada significativament per la dosi de pèptid i la soca i concentració de *X. fastidiosa*. No obstant, es va estimar que era necessari un llindar al voltant de 10^9 molècules de BP178/cèl·lula per a la lisi de totes les soques de *X. fastidiosa* provades. L'activitat bactericida del BP178 també es va veure afectada significativament pel diluent utilitzat, fet que indica que l'entorn en condicions *in vivo* podria afectar la interacció entre els PAMs i les membranes cel·lulars bacterianes.

Amb l'objectiu de disposar d'un elevat nombre de pèptids potencialment eficaços contra *X. fastidiosa in planta*, es va desenvolupar una metodologia pel cribratge de PAMs basada en la seva activitat bactericida contra aquest patogen. La prova de contacte dissenyada acoblada a una qPCR de viabilitat (v-qPCR) supera l'estat de quarantena de *X. fastidiosa* a la UE, la seva naturalesa *fastidiosa* i el seu creixement lent, a més de quantificar tota la fracció de cèl·lules viables, cultivables o no. Aquesta metodologia va permetre identificar, a més del BP178, cinc nous PAMs (BP171, BP175, BP170, BP176 i BP180) actius contra *X. fastidiosa*. També es va utilitzar per determinar la susceptibilitat diferencial de les soques de *X. fastidiosa* al pèptid BP178, així com les diferències en la inducció de l'estat de viables però no cultivables.

L'estudi dels paràmetres relacionats amb la patogenicitat és important per predir i gestionar millor els brots de malalties associades al patogen, però també per optimitzar les pautes de tractament pel que fa el temps i la dosi d'aplicació, especialment en el cas dels PAMs. En aquest sentit, es va investigar l'agressivitat de diferents aïllats espanyols en plantes d'ametller. Tots els aïllats de *X. fastidiosa* (IVIA 5387.2, IVIA 5901.2, IVIA 5235.4 i IVIA 5770) van ser capaços de causar el socarrat de la fulla d'ametller (ALS) en tres cultivars (*Avijor*, *Soleta* i *Penta*) que actualment es produeixen com a *smarttrees*. Tot i així, es van observar diferències en l'agressivitat entre soques, essent la soca de la subespècie *multiplex*, IVIA 5901.2, la que va mostrar una severitat de la malaltia i una taxa de colonització de brots nous en creixement més baixes. Les relacions dosi-resposta van revelar diferències en paràmetres importants de la patogenicitat entre els dos aïllats d'ametller estudiats. Tot i que l'aïllat IVIA

5387.2 va assolir nivells de població alts, la seva dosi efectiva mitjana (ED_{50}) també es va estimar alta. Per altra banda, IVIA 5901.2 va assolir nivells de població inferiors als de IVIA 5387.2, però la seva ED_{50} es va estimar baixa. La dosi infectiva mínima (DIM) també va resultar ser diferent entre les soques, però en tots dos casos era inferior a 3000 UFC/planta, cosa que indica que els vectors podrien transmetre fàcilment la malaltia ja que les concentracions de *X. fastidiosa* detectades en caps d'insectes oscil·len entre 30 i 10^5 còpies/cap.

En resum, en el present treball s'identifiquen pèptids altament bactericides contra el fitopatogen *X. fastidiosa* i es posen de manifest paràmetres importants de la seva activitat. A més, s'estudia l'agressivitat d'aïllats espanyols de *X. fastidiosa*, incrementant el coneixement pel disseny de pautes de tractament eficients en el control de ALS i altres malalties causades per *X. fastidiosa*.

RESUMEN

Xylella fastidiosa es una de las bacterias fitopatógenas que provoca más daños a nivel mundial, causando una variedad de enfermedades con un gran impacto económico en la agricultura y el entorno. Hasta el 2013, *X. fastidiosa* se había detectado mayoritariamente en el continente americano, causando principalmente la enfermedad de Pierce en la vid, la clorosis variegada de los cítricos y la quemadura de la hoja del café. No obstante, la llegada de diferentes subespecies de este patógeno a la Unión Europea (UE) ha cambiado su distribución. Hoy en día, *X. fastidiosa* afecta severamente los olivos de la región de Apulia en el sur de Italia, *Polygala myrtifolia* en la región de PACA en el sur de Francia y en Córcega, y los almendros en la provincia de Alicante y en las islas Baleares en España.

Actualmente, el manejo de las enfermedades causadas por *X. fastidiosa* está basado en el uso de medidas para prevenir su propagación hacia otras regiones, como seria el uso de material vegetal de propagación libre de patógenos, medidas de exclusión y cuarentena, y el control de los vectores. Hasta el momento, no se ha encontrado ninguna solución terapéutica para el control de *X. fastidiosa* en plantas y árboles afectados. Es por este motivo que surge la necesidad de encontrar nuevos bactericidas contra este patógeno. Los péptidos antimicrobianos (PAMs) representan una alternativa prometedora a los compuestos tradicionales debido a su actividad contra una amplia gamma de patógenos vegetales, baja toxicidad, método de acción, y disponibilidad para crear pesticidas sostenibles y cultivos resistentes a enfermedades. Así pues, el objetivo de la presente tesis doctoral se centró en el estudio de los PAMs como alternativa sostenible para el control de enfermedades causadas por *X. fastidiosa*.

Los resultados obtenidos previamente en nuestro grupo sobre la actividad antimicrobiana de péptidos recientemente descritos, sirvió para la selección de péptidos líderes con potencial para ser probados *in vitro* contra *X. fastidiosa*. El péptido BP178 mostró la mayor actividad antimicrobiana contra diferentes cepas de *X. fastidiosa*, la cual fue asociada a la lisis celular. La formación de poros y la lisis de la membrana bacteriana se observaron mediante el análisis ultraestructural utilizando el microscopio electrónico de transmisión (MET), demostrando que esta

acción directa sobre las membranas es la que conduce a la muerte bacteriana, siendo el principal mecanismo de acción del péptido BP178. Las dinámicas poblacionales a lo largo del tiempo y los experimentos de dosis-efecto revelaron que la actividad bactericida del BP178 se produce de los 10 a los 20 minutes después de la exposición, y que esta se ve afectada significativamente por la dosis de péptido y la cepa y concentración de *X. fastidiosa*. No obstante, se estimó que era necesario un umbral alrededor de 10^9 moléculas de BP178/célula para la lisis de todas las cepas de *X. fastidiosa* probadas. La actividad bactericida del BP178 también se vio afectada significativamente por el diluyente utilizado, hecho que indica que el entorno en condiciones *in vivo* podría afectar la interacción entre los PAMs y las membranas celulares bacterianas.

Con el objectivo de disponer de un nombre elevado de péptidos potencialmente eficaces contra *X. fastidiosa in planta*, se desarrolló una metodología para el cribado de PAMs basada en su actividad bactericida contra este patógeno. La prueba de contacto diseñada acoblada a una qPCR de viabilidad (v-qPCR) supera el estado de cuarentena de *X. fastidiosa* en la UE, su naturaleza fastidiosa y su crecimiento lento, además de cuantificar toda la fracción de células viables, cultivables o no. Ésta metodología permitió identificar, además del BP178, cinco nuevos PAMs (BP171, BP175, BP170, BP176 y BP180) activos contra *X. fastidiosa*. También se utilizó para determinar la susceptibilidad diferencial de las cepas de *X. fastidiosa* al péptido BP178, así como las diferencias en la inducción del estado de viables pero no cultivables.

El estudio de los parámetros relacionados con la patogenicidad es importante para predecir y gestionar mejor los brotes de enfermedades asociadas al patógeno, pero también para optimizar las pautas de tratamiento en relación al tiempo y la dosis de aplicación, especialmente en el caso de los PAMs. En este sentido, se investigó la agresividad de diferentes aislados españoles en plantas de almendro. Todos los aislados de *X. fastidiosa* (IVIA 5387.2, IVIA 5901.2, IVIA 5235.4 y IVIA 5770) fueron capaces de causar la quemadura de la hoja del almendro (ALS) en tres cultivares (*Avijor*, *Soleta* y *Penta*) que actualmente se producen como *smarttrees*. No obstante, se observaron diferencias en la agresividad entre cepas, siendo la cepa de la subespecie *multiplex*, IVIA 5901.2, la que

mostró una severidad de la enfermedad y una tasa de colonización de brotes nuevos en crecimiento más bajas. Las relaciones dosis-respuesta revelaron diferencias en parámetros importantes de la patogenicidad entre los dos aislados de almendro estudiados. Aunque el aislado IVIA 5387.2 alcanzó niveles de población altos, su dosis efectiva media (ED_{50}) también se estimó alta. Por otra parte, IVIA 5901.2 alcanzó niveles de población inferiores als de IVIA 5387.2, pero su ED_{50} se estimó baja. La dosis infectiva mínima (DIM) también resultó diferente entre las cepas, pero en los dos casos fue inferior a 3000 UFC/planta, hecho que indica que los vectores podrían transmitir fácilmente la enfermedad ya que las concentraciones de *X. fastidiosa* detectadas en las cabezas de los insectos oscilan entre 30 y 10^5 copias/cabeza.

En resumen, en el presente trabajo se identifican péptidos altamente bactericidas contra *X. fastidiosa*, revelando parámetros importantes de su actividad. Además, se estudia la agresividad de aislados españoles de *X. fastidiosa*, incrementando el conocimiento para el diseño de pautas de tratamiento eficientes en el control de ALS y otras enfermedades causadas por *X. fastidiosa***.**

CHAPTER I

General introduction

Plant pathology is the science that study all concepts involved in plant disease, such as the interactions between pathogens and host plants in specific environments, the factors and mechanisms responsible for disease, their epidemiology, their effects on the ecosystem, and the methods for preventing and controlling them. In agriculture, the diagnosis of plant diseases and its efficient management are essential for the control of plant epidemics. In this sense, crop protection is the discipline mainly focused on protecting crops from yield losses due to biological causes, both in the field and during storage (Oerke, 2006). Although some pathogens only affect one variety of crop plant, others can affect a wide range of plant species. Moreover, different pathogens can invade one single crop plant, which, on average, can be affected by a hundred or more plant diseases (Agrios, 2005).

Plant protection has always had an important role in meeting the food demands, but nowadays, this role is even more crucial due to the growing demand for food quality and quantity, as a consequence of an increase in the world population (Savary et al., 2012). It has been estimated that world food production should increase at least 60 % by 2050 to cover all the population needs (Van Esse et al., 2020). However, climate change, the accelerated evolution of pathogens, and the use of modern agronomic practices, such as the monoculture, have created ecological environments favorable for pathogen infection, with reduced plant host immunity and absence of natural and diverse enemies. In fact, it has been reported that direct yield losses caused by plant pathogens still range between 16 and 18 % of the global agricultural productivity (Oerke 2006). This indicates that causes and mechanisms behind epidemic development are not yet fully understood, hindering the application of adequate strategies to efficiently manage them (Dun-chun et al., 2016).

The term epidemic can be described in many ways, but in general it implies an increase in disease intensity in a host population over time and space (Vanderplank, 2017). In 1880, almost 14,000 ha of vineyards in California (United States of America (USA)) were heavily affected by an unknown disease, bringing most of the grapevines to death (Landa et al., 2019). In 1887, the pathologist Newton B. Pierce described in detail the

disease, which was named Pierce's Disease (PD) of grapevines. Nevertheless, it was not until the 1980s when the causal agent was firstly isolated (Davis et al., 1978), and formally described and classified as *Xylella fastidiosa* (Wells et al., 1987). One century after its emergence, this plant pathogenic bacteria is still an important threat for the grapevine production in California (Tumber et al., 2012). Although *X. fastidiosa* was detected in Taiwan in 1994 (Su et al., 2016), it was though that this dangerous bacterium was restricted to the American continent, as outbreaks were only detected in other regions of that continent, like Brazil and Costa Rica (Chang et al., 1993; Rocha et al., 2010). Nevertheless, since 2013, the European Union (EU) is facing an important threat for its agriculture due to the emergence of *X. fastidiosa* in Apulia (Italy) (Saponari et al., 2019). From that moment on, outbreaks of different intensity have been detected in other regions of the EU, such as Corsica and PACA and Occitanie regions in France, Balearic Islands, Alicante province and Madrid in Spain, and Oporto in Portugal (Denancé et al., 2017; Olmo et al., 2017; European Commission, 2021). It has been estimated that a situation of full *X. fastidiosa* spread across the EU has the potential of affecting between 35 and 70 % of the EU production value of olive trees, 11 % of citrus, 13 % of almond and between 1-2 % of grape. In addition to direct impacts on production, this situation would have significant indirect effects like an increase in the unemployment within the agricultural sector; it has been estimated that nearly 300,000 jobs across EU currently involved in agriculture could be at risk (Sánchez et al., 2019).

1. The causal agent *Xylella fastidiosa*, and diseases

1.1. *Xylella fastidiosa*

X. fastidiosa is a Gram-negative bacterium belonging to the *Xanthomonadaceae* family, which inhabits in the xylem vessels of 595 host plants from 85 taxonomic families (EFSA, 2020). While many host plants are asymptomatic carriers of the bacterium, *X. fastidiosa* causes important diseases in many others, such as grapevines (*Vitis vinifera*), almond (*Prunus dulcis*), citrus (*Citrus* spp.) and olive (*Olea europaea*) (Baldi and La

Porta, 2017). Even though symptoms are slightly different depending on the host species, *X. fastidiosa*-related diseases are characterized by a generalized decline of the plant, mostly with stunting of leaves and fruits and leaf scorching, but also with the drying of different plant parts (Fig. 1). If *X. fastidiosa* is completely established throughout the whole vascular system, it is able to completely block off the xylem vessels, leading to the death of the plant (EPPO, 2016). The comprehension of why *X. fastidiosa* causes serious diseases in specific host plants but not in others is still poorly understood (Almeida et al., 2019). Nevertheless, the understanding of its biological cycle (Fig. 2) and its virulence traits (Table 1) are important to further knowledge in this direction.

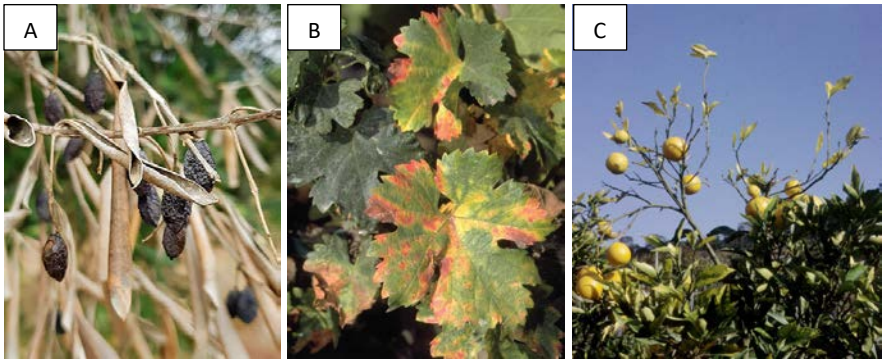

Figure 1. *X. fastidiosa*-related diseases symptoms. (A) Olive Quick Decline Syndrome (OQDS), dry leaves and fruits (image courtesy of S. White, UK Centre for Ecology & Hydrology, Lancaster); (B) PD of grapevines, necrotic leaves (image courtesy of J. Clark, University of California, Berkeley); (C) Citrus Variegated Chlorosis (CVC), twig dieback and reduced dimension fruits (image courtesy of M. Scortichini, Istituto Sperimentale per la Frutticoltura, Rome).

X. fastidiosa has the capacity to adapt to insect vectors, which are responsible for carrying the bacterium from an infected plant to a healthy one, and into host plants (Fig. 2). Once a susceptible host plant is infected, *X. fastidiosa* starts to move through the vascular system by twitching motility triggered by the presence of pili type IV and the production of anti-adhesion factors, such as the outer membrane vesicles (OMV) (Ionescu et al., 2014). Movement from one vessel to the other is feasible because *X. fastidiosa* has the capacity of degrading pit membranes by the production of cell wall degrading enzymes, like polygalacturonase A (PlgA) and

xylanases (Roper et al., 2007). Once the plant is colonized, *X. fastidiosa* has the capacity to produce biofilm, which is the mechanism that permits its acquisition by insect vectors and, consequently, its transmission to other plants. The presence of pili type I, the production of afimbrial adhesines, such as HxfA, HxfB and Xat A, and the synthesis of exopolysaccharides like gumH and gumB, are the main mechanisms responsible for this biofilm formation. Calcium (Ca^{2+}) has also been associated with higher rates of biofilm formation (Parker et al., 2016), but a recent study has revealed that this mineral element regulates *X. fastidiosa* behavior differently depending on its stage of infection (Chen and De La Fuente, 2019). *X. fastidiosa* is considered a plant pathogen when its biofilm causes the blockage of xylem vessels and, therefore, the death of the plant (Bucci, 2018).

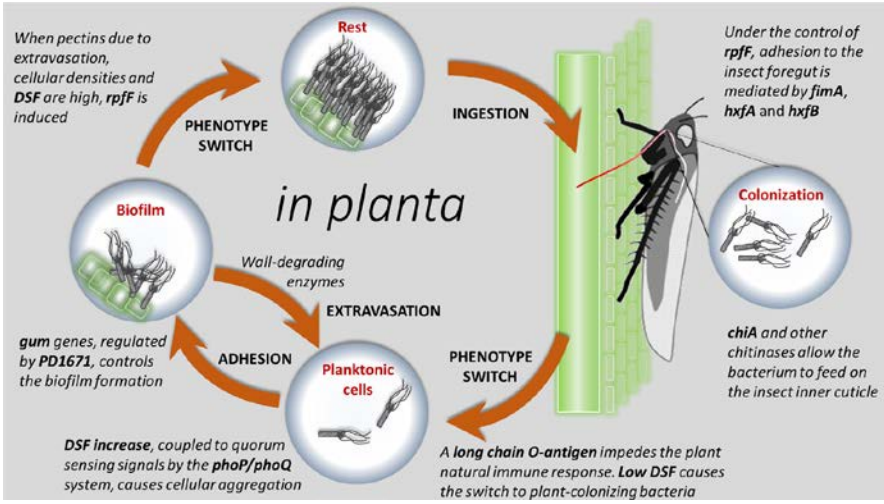

Figure 2. Biological cycle of *X. fastidiosa* (Bucci, 2018).

X. fastidiosa cells communicate through cell-to-cell signaling in order to know if they should colonize plants or form biofilm. In fact, quorum sensing, the ability to detect and to respond to cell density by gene regulation, is the main mechanism that lead the biological cycle of this pathogen (Sicard et al., 2018). The signaling molecule responsible for this communication is a fatty acid called diffusible signal factor (DSF), and it is transported by the OMV produced by the *X. fastidiosa* cells themselves (Ionescu et al., 2016). Abundance of DSF induces the insect acquisition

phase, which is the formation of biofilm, whereas low levels of DSF trigger the mobility of the pathogen for the colonization of the whole plant (Chatterjee et al., 2008a). This equilibrium is related with the fact that high colonization rates result in excessive symptoms, which would decrease attractiveness to insects and consequently, a low transmission rate.

Table 1. Virulence traits of *X. fastidiosa.*

Mobility	Twitching – Pili type IV
	Cell wall degrading enzymes – PglA, xylanases
	Anti-adhesion factors – OMV
	Mineral elements – Ca^{2+}
Biofilm formation	Pili type I
	Afimbrial adhesines – HxfA, HxfB, Xat A
	Exopolysaccharides – gumH, gumB
	Mineral elements – Ca^{2+}
Cell-cell signaling	DSF
	Transport of DSF - OMV
Pathogen-Plant interaction	LPS O-antigen
	Plant defense response – tyloses, gels, crystals

It is important to mention the lipopolysaccharide (LPS) O-antigen as a virulence factor of *X. fastidiosa*. This molecule has the ability to mask LPS, which act as pathogen-associated molecular patterns (PAMPs), delaying the plant innate immune recognition of the pathogen during the first stage of infection (Rapicavoli et al., 2018). This strategy gives advantage to *X. fastidiosa* in the sense that it can start the infection without any plant reaction. When the plant senses that it is being colonized by a pathogen, it activates its immune system and starts producing tyloses, gels and crystals to try to control the infection. Nevertheless, what happens is that this reaction enhances the blockage already started by the bacterial biofilm (Choi et al., 2013).

As *X. fastidiosa* is a xylem limited bacterium, its transmission is by insect vectors, mainly spittlebugs and sharpshooter leafhoppers, through their sucking mouthparts and their feeding habits. It is a propagative and persistent bacterium, which multiplies and remains inside the foregut of the insect vectors, but its transmission is non-circulative neither transovarial (Almeida and Nunney, 2015). No latent period is necessary to

infect other host plants, so vectors are competent immediately after acquisition. Both processes, acquisition and transmission, are generally fast and generally last for less than 1 h. Although nymphs can acquire *X. fastidiosa*, they lose their ability to transmit it after moulting. Therefore, adults are the only vectors and once infected, they remain infective its whole life (Cornara et al., 2016; Overall and Rebek, 2017).

In the EU, 95 potential vectors of *X. fastidiosa* have been identified, mainly from the families of *Cicadellidae*, *Cercopidae*, *Aphrophoridae* and *Cicadidae*. Differently from the USA, where the main vectors described are sharpshooter leafhoppers (Almeida et al., 2005), the transmission of *X. fastidiosa* in Southern Italy has been ascribed to *Philaenus spumarius*, a species of insect belonging to the spittlebug family *Aphrophoridae* (Cornara et al., 2017a). Other species, such as *Neophilaenus campestris* and *Philaenus italosignus*, have demonstrated their capacity to acquire and transmit the bacterium from different hosts, although less effectively compared to *P. spumarius* (Cavalieri et al., 2019). This reflects that transmission depends on the insect vector species, in addition to the *X. fastidiosa* genotype, the host plant and the tissue of acquisition, the bacterial load inside the plant, the present symptomatology and the environmental conditions. Until now, no specificity between species of insect vector and subspecies of *X. fastidiosa* has been found (Sicard et al., 2018). Because most of these potential vectors of *X. fastidiosa* are not considered a pest for any agricultural system, in addition to the late onset of symptoms in susceptible host plants, the spread of *X. fastidiosa* is very difficult to control. Moreover, the presence of asymptomatic plants acting as a reservoir of the pathogen increases the chances of dispersal from one region to another.

X. fastidiosa is currently divided into three major subspecies *(X. fastidiosa* subsp. *fastidiosa, multiplex,* and *pauca*) (Schaad et al., 2004), although additional subspecies have been suggested (i.e. *X. fastidiosa* subsp*. sandyi* and *morus*) (Schuenzel et al., 2005; Nunney et al., 2014). The genome of *X. fastidiosa* subsp. *pauca* strain 9a5c was the first bacterial plant pathogen genome sequenced (Simpson et al., 2000). It is composed of the main chromosome (2.68 Mb), which shows an important gene

reduction compared to the genome of other species of the genera *Xanthomonas* (Pieretti et al., 2009), and of two plasmids encoding for proteins potentially involved in gene transfer. In fact, homologous recombination has been described as one of the main drivers of *X. fastidiosa* adaptation to novel hosts (Vanhove et al., 2019; Denancé et al., 2019), as *X. fastidiosa* host specificity is a genetically-controlled character (Killiny and Almeida, 2011). Although in the past, geographical isolation of *X. fastidiosa* subspecies led to an initial host specificity (Vanhove et al., 2019), globalization has added more genetic diversity in the regions where new *X. fastidiosa* subspecies have been introduced. This rise in diversity, in addition to the genomic plasticity of *X. fastidiosa*, has led to an increase in the genomic variability of this pathogen by homologous recombination (Nunney et al., 2014) and the emergence of new genotypes with different traits.

Because host shifts have been observed, demonstrating host specialization not only between subspecies but also within the same subspecies (Sanderlin, 2017), a more accurate categorization of all *X. fastidiosa* strains has been required. Nowadays, each new isolate is being classified not only by subspecies but also into sequence type (ST) using the multilocus sequence typing (MLST) (Landa et al., 2020). This genetic technic, based on the identification of differences in the nucleotide sequence of seven housekeeping genes (*leuA, petC, malF, cysG, holC, nuoL, gltT*), is widely used to assign strains to genetic groups, independently of its subspecies classification (Mazzaglia et al., 2020). As December 2020, 87 ST profiles of *X. fastidiosa* have been identified worldwide (Jolley et al., 2018), some of them newly described in the EU territory since 2013. This supports the fact that *X. fastidiosa* is a highly recombinant bacterium, as MLST technic is based on conserved genomic regions and yet differences are found in these genomic regions (Raffini et al., 2020).

1.2. Diseases caused by *X. fastidiosa* in Mediterranean crops

The agricultural sector is of vital importance for the Mediterranean region, as it presents particular climatologic conditions that highly favors the cultivation of certain crop products with relevant nutritional, commercial,

environmental and social value. Nevertheless, since 2013, part of the EU agricultural production is at risk due to the outbreak of *X. fastidiosa* detected in Southern Italy. A genome-wide analysis study revealed that it was due to the import of plant material infected with *X. fastidiosa* from Central America (Giampetruzzi et al., 2017). From that moment on, other outbreaks have appeared in other EU regions, mostly associated with ornamental plant trade and movement of plant material for breeding purposes (Olmo et al., 2021). At present, *X. fastidiosa* is mostly distributed through the Mediterranean region (Fig. 3).

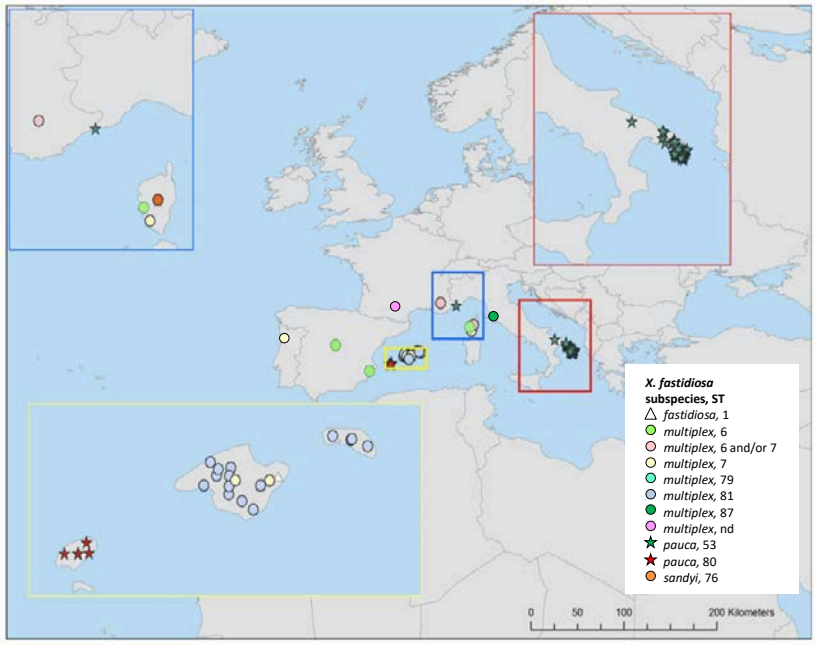

Figure 3. Distribution of the different *X. fastidiosa* subspecies and STs reported in the EU (actualized and modified from EFSA PLH Panel, 2018).

In 2017, 1.9 % of the EU total agricultural land was used for the production of fruit, being Spain the country with the highest proportion of area (40.1 %), mainly dedicated to the production of nuts and citrus fruits. In fact, the 55.8 % of EU orange production and the 85.2 % of the EU almond production were in Spain, which is still the world's third largest almond producer, after USA and Australia, accounting for the 10.5 % of the world's almond production (De Cicca, 2018). Additionally, Spain is the

largest producer of olives for olive oil, ingredient mainly produced in EU (two-thirds of the world's production). This is because a large proportion of olive trees are cultivated in the Mediterranean region (Eurostats, 2018), where climatic conditions are optimal for their growth.

1.2.1. Olive quick decline syndrome

Olive Quick Decline Syndrome (OQDS) is the disease caused by *X. fastidiosa* in olive trees. First symptoms appear in the top of the tree canopy, when leaves, young twigs and branches start to show significant signals of desiccation. From that moment on, disease severity rapidly increases and symptoms expand to the rest of the crown, conferring a scorched aspect. In most cases, this accelerated appearance of symptoms ends with the death of the tree, indicating its full colonization by *X. fastidiosa* (Fig. 4) (Saponari et al., 2017). In the EU, unusual desiccation symptoms were first observed between 2008 and 2010 in Apulia (Italy), but it was not until 2013 that *X. fastidiosa* subsp. *pauca* ST 53 was detected as the causal agent of OQDS (Saponari et al., 2019). Since then, its rapid spread has already affected a large proportion of olive orchards in Southern Italy, even though precautionary measures and lots of efforts are being made to stop the epidemic.

Figure 4. Different stages of OQDS in olive trees in Southern Italy. (A) Initial stage (image courtesy of E. Montesinos, University of Girona, Girona); (B) intermediate stage (Martelli et al., 2016); (C) final stage (image courtesy of E. Montesinos, University of Girona, Girona).

Spain was the second EU region in reporting OQDS, specifically in Balearic Islands. *X. fastidiosa* strains found to be responsible for the outbreaks were different from the ones detected in Apulia. In Mallorca and Menorca, the subspecies *multiplex* (ST 81) was related to wild olive

trees, whereas the subspecies *pauca* (ST 80) was associated to olive deadly dieback in Ibiza. Although symptoms are very similar to those observed in Italy, the survival time in Balearic Islands seems longer than in Apulia, as well as the mortality rate seems lower. Nevertheless, data analysis has to be performed in order to confirm these observations, indicating lower aggressiveness of the Balearic strains compared to the Italian ones (Olmo et al., 2021).

In mainland Spain, OQDS was detected in an orchard of Picual cultivar olive trees in the autonomous community of Madrid in April 2018. The eight years old olive tree presented characteristic symptoms of *X. fastidiosa* infection, and the presence of the bacterium was confirmed by the Official Plant Health Laboratory of the Community of Madrid and the National Reference Laboratory for Phytopathogenic Bacteria (bacteriology laboratory of Instituto Valenciano de Investigaciones Agrarias). The strain isolated corresponded to the subspecies *multiplex*. In order to avoid the spread, eradication measures were adopted, and the elimination of the infected tree and other potential hosts was carried out, as well as sampling of all specified hosts. Additionally, treatments against insect vectors were applied in the infected area during the whole month. By the end of the year, another sampling was performed, with all samples collected testing negative (EPPO, 2021).

1.2.2. Almond leaf scorch

Almond leaf scorch (ALS) is the disease caused by *X. fastidiosa* in almond trees. Because almonds are deciduous trees, symptoms first appear on individual leaves in early June or mid-July, but by the end of July it is totally noticeable the infection as the symptoms are fully developed. Once infected, trees develop the disease each year and *X. fastidiosa* slowly progresses towards other parts of the tree. In general, when trees are fully colonized by the pathogen, they die. This fact importantly affects the regions where there is an important production of almonds, such as California in USA or Spain in the EU. For example, it has been described that ALS in California affects the yield of infected almond trees, reducing it by 20 to 40 %, depending on the cultivar (Sisterson et al., 2012).

In Spain, ALS caused by *X. fastidiosa* subsp. *fastidiosa* ST 1 was first detected in Mallorca (Moralejo et al., 2019). Even though containment measures are being implemented in order to reduce the spread, almond orchards are currently one of the most affected crops in all Balearic Islands (Fig. 5A), with ST 80 and ST 81 also associated to ALS in Ibiza and Menorca, respectively. Specifically in Mallorca, ALS is affecting the 81 % of almond trees, with a registered reduction of the total surface of almond orchards going from 29,789 ha to 11,814 ha (Olmo et al., 2021). Apart from Balearic Islands, another outbreak of *X. fastidiosa* in almond trees was detected in Alicante in 2017 (Giampetruzzi et al., 2019), this time caused by a strain from the subspecies *multiplex* ST 6. Because the outbreak occurred in mainland Spain, eradication measures are being adopted in order to avoid the spread towards the country (Fig. 5B). From the first detection and until November 2019, around 21,662 almond trees from 431 different plots were cut out in Alicante, as well as, part of the forestland undergrowth (EPPO, 2021). These decisions are controversial, as it has been reported that ALS-affected trees still produce, although with reduced yields. In this sense, a balance between economic benefit of retaining ALS-affected trees and the risk of these almond trees of serving as a source of inoculum has to be performed.

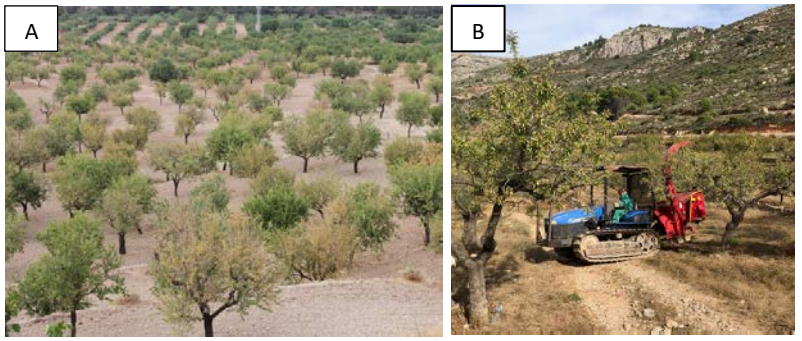

Figure 5. (A) An almond grove in Mallorca (Puigpunyent ~70 km from the main focus at Son Carrió) showing infected trees with systemic symptoms of ALS. Image taken in July 2017 (Moralejo et al., 2020). (B) Eradication in an almond orchard in Alicante after the detection of a *X. fastidiosa* infected tree (image courtesy of E. Montesinos, University of Girona, Girona).

2. Control of *X. fastidiosa* diseases

The control of *X. fastidiosa*-related diseases is still an important threat for the agriculture all over the world, mainly due to the high levels of *X. fastidiosa* genetic plasticity and the fact that host plants do not show symptoms during the first stages of the disease (Kyrkou et al., 2018). In this context, the EU is constantly reporting new insights into the management of *X. fastidiosa* outbreaks, in order to avoid the introduction and spread of the pathogen through the entire continent. Actions are mainly focused on exclusion and quarantine measures, and on the control of insect vectors and alternative hosts.

2.1. Exclusion and quarantine

In general, trade in plants and derived products follows a strict EU regulation that implies the obligatory possession of a phytosanitary passport that guarantees its proper inspection and the accomplishment of the plant health requirements of the EU (EU, 2019). Because *X. fastidiosa* is classified as one of the most harmful plant pathogens for the EU agricultural sector (Sánchez et al., 2019), a specific regulation was implemented (EU, 2020).

The measures that are being adopted nowadays at borders aim to prevent the entry of the pathogen through plant material and insects, which have been reported as the main pathways of *X. fastidiosa* introduction (EFSA, 2015). Seeds, fruits, cut flowers, ornamental foliage and detached wood, have been considered unlikely to be ways of entry, although some references of contaminated seeds from *Citrus* spp. have been reported, but without observation of any transmission of the bacterium to the plants (Coletta-Filho et al., 2014). One of the most important measures adopted is the restriction in the movement of plant material from infected areas, which are different depending on the *X. fastidiosa* status of the country or region of emission. Establishing a quarantine period prior to the entry of possible asymptomatic plant material has been proposed, although the asymptomatic period of many *X. fastidiosa* diseases is unknown and very variable within the already

known ones. The thermotherapy of grapevine cuttings for planting have been recommended, as it has been observed to be effective in avoiding the presence of both, *X. fastidiosa* and insect vectors (EFSA, 2019b).

Because the EU requires an official phytosanitary certificate from the countries or regions of emission indicating that the country is free of the pathogen, annual prospections have been implemented in order to detect any *X. fastidiosa* entry. During the appropriate time of the year, sample collecting from orchards, greenhouse, nurseries… is mandatory, as well as their analysis using molecular based technics, which are already described by the European and Mediterranean Plant Protection Organization (EPPO) (EPPO, 2019). Once a positive is confirmed, the country has to inform the EU about the presence of *X. fastidiosa* in its territory and establish a demarcated area. This area consists of an infected zone (50 m around the infected plant) and a buffer zone, which has different characteristics depending on the territory situation (2.5 km in eradication or 5 km in containment) (Fig. 6).

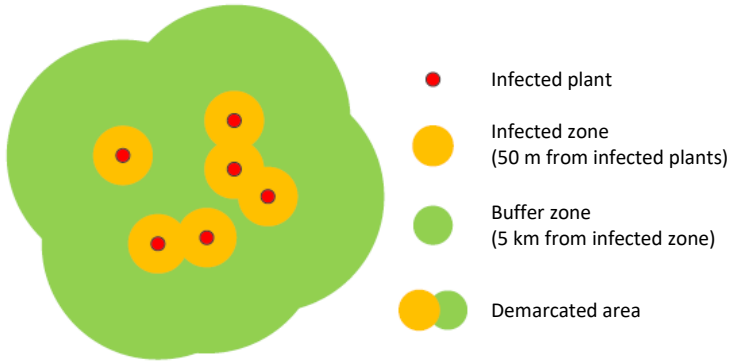

Figure 6. Representation of a demarcated area in a region where containment measures are being adopted (modified from EFSA, 2019b).

In regions where eradication is established as the main activity, all infected or symptomatic plants have to be removed from the infected zone, as well as plants belonging to the same species, irrespective of their health status. Moreover, other regulated specified plants known to be susceptible to that specific subspecies of *X. fastidiosa* shall also be removed if sampling has not been performed immediately, in exception of plants with historic value, which can be preserved if they are not found

infected (following strict control measures). Additionally, treatments using chemical, biological or mechanical approaches have to be performed against insect vectors. In the buffer zone, all host plants have also to be tested for the presence of the bacterium, implementing the same procedure if a positive sample is found, as well as an insect vector.

On the other hand, in regions where there is a wide spread of *X. fastidiosa* and eradication measures would not be feasible, containment measures are adopted. In this case, intensive surveillance and immediate removal of the infected plants are mandatory measures implemented in the infected zone, being plants with historical or social value an exception depending on the member state. In the buffer zone, annual prospections have to be performed, in addition to the analysis of insect vectors. In all the demarcated area, treatments using chemical, biological or mechanical approaches have to be carried out against insect vectors.

2.2. Control of insect vectors and alternative hosts

The efficient control of insect vectors is essential for the management of *X. fastidiosa*-related diseases. In order to achieve it, strategies should be focused on the reduction of the adult vector population density, as well as on the avoidance of their spread, either from alternative hosts and between orchards (primary spread), or between trees from the same orchard (secondary spread) (EFSA, 2019b). In this sense, and as above-mentioned, the control of insect vectors is mandatory in all demarcated areas of the EU, whether the region is under containment or eradication. Also, the application of insecticide treatments to the plant material at borders is required.

The application of insecticides approved for use in the EU is the most common strategy used nowadays, as it has been observed to be an effective measure in the control of *P. spumarius* in Italy. The most effective insecticides, which are the neonicotinoids (e.g. acetamiprid) and the pyrethroids (e.g. deltametrin), showed a 75 to 100 % of mortality (Dongiovanni et al., 2018a). Other compounds, such as essential oils containing volatile organic compounds, have been proposed as

sustainable tools for the control of insect vectors (Ganassi et al., 2020). In olive orchards in Italy, field trials using sweet orange essential oil have been performed showing initial insect vector mortality, but persistence of the product was low or nonexistent (Dongiovanni et al., 2018a). The application of kaolin, a particle film technology, was also tested in olive orchards in Italy, as it reported a reduction in the transmission rate of *X. fastidiosa* in grapevines in USA (Tubajika et al., 2007). Nevertheless, preliminary results did not show a significant reduction on *X. fastidiosa* transmission in treated plots compared to control plots (EFSA, 2019b).

Biological control has also been proposed for the control of *X. fastidiosa* insect vectors. Nevertheless, information regarding their natural enemies is still very limited, and the introduction of non-indigenous species has several regulatory issues (EPPO, 2014, 2018). In the EU, the impact of predators, parasitoids and entomopathogenic fungi has been assessed against Aphrophoridae eggs, showing mortality rates that could be significant for the control of *X. fastidiosa* vectors (Reis et al., 2018). Also, the use of *Zelus renardii*, a natural enemy of *P. spumarius*, has reported promising results, as the strategy applied was able to control the spread and population density of the insect vector (Liccardo et al., 2020). In the USA, other approaches using genetic engineering have been investigated, such as the modification of a symbiotic bacterium associated with *Homalodisca vitripennis* expressing antimicrobial peptides (AMPs) (melittin and scorpine-like molecule), which blocks the transmission of *X. fastidiosa* (Arora et al., 2018).

Agricultural practices, such as the removal of ground vegetation from orchards by soil tillage, mowing or herbicides, have also been related with the control of *X. fastidiosa* insect vectors (Cornara et al., 2018). This is because herbaceous host plant and weeds are a preferred ovoposition site and favored place for nymphal development for most of the *X. fastidiosa* insect vectors (Bodino et al., 2020). Also in Italy, a two-year field trial, in which soil tillage was carried out in winter and spring, showed an efficacy of 99.6 % in reducing the abundance of *P. spumarius* and *N. campestris* compared to control plots (Dongiovanni et al., 2018b). Another important practice to be considered, which has been very

effective in controlling CVC in Brazil, is the adoption of a certification program in nurseries, in which plants must be grown in a screen-house free of insect vectors with regular monitoring of *X. fastidiosa* infections (Coletta-Filho et al., 2020).

2.3. Control of *X. fastidiosa* on host plants

Since the identification and characterization of *X. fastidiosa* in 1987, this bacterium has been the subject of an intensive research with the aim of obtaining effective measures for its full control. Different approaches, such as chemical and biological compounds or genetic engineering, have been explored in both, greenhouse and field conditions (Table 2). Even though promising results have been obtained during the past decades, there are still no available strategies to cure *X. fastidiosa* infected plants (Kyrkou et al., 2018; EFSA, 2019a).

2.3.1. Chemical control

Chemical control has been the dominant approach used in agriculture since many years ago. For the control of *X. fastidiosa* diseased plants, different strategies have been investigated under greenhouse and field conditions. The application of oxytetracycline via trunk injections into leaf scorched almond trees was tested in field in Iran. Although a significant reduction of disease symptoms was observed (73 %), the need of repeating the treatment every year and the fact that oxytetracycline is an antibiotic, increased the concerns related with environmental and human health about its use (Amanifar et al., 2016).

Another chemical compound that has been extensively tested is *N*-Acetylcysteine (NAC), an analogue of cysteine that affects the production of extracellular polysaccharides and consequently the biofilm formation, in addition to the disruption of mature biofilms by means of disulphide bonds disruption. Under greenhouse conditions, a significant reduction of symptoms and bacterial growth rate was observed in infected sweet orange plants (*Citrus sinensis*) after NAC application by hydroponics,

Table 2. Summary of strategies tested to prevent and/or cure *X. fastidiosa* infections in different host plants.

Type of control	Strategy	Status of strategy	Reference
Chemical control	Oxytetracycline	Tested in almond orchards	Amanifar et al., 2016
	NAC	Tested in citrus plants under greenhouse conditions and in olive and citrus orchards	Muranaka et al., 2013; De Souza et al., 2019; Dongiovanni et al., 2017
	Bioactive detergent	Tested in old olive orchards	Bruno et al., 2021
	Zn, Cu and citric acid fertilizer	Tested in olive orchards	Scortichini et al., 2018
	$CuSO_4$	Tested in tobacco plants under greenhouse conditions	Ge et al., 2020
	Menadione and benzethonium chloride	Tested in grapevines under greenhouse conditions	Zhang et al., 2019
	ABA	Tested in grapevines under greenhouse conditions	Meyer and Kirkpatrick, 2011
	DSF, palmitoleic acid and macadamia nut oil	Tested in grapevines under greenhouse conditions	Lindow et al., 2017
Biological control	*Curtobacterium flaccumfaciens*	Tested in Madagascar periwinkle plants under greenhouse conditions	Lacava et al., 2007
	Methylobacterium mesophilicum	Tested in Madagascar periwinkle plants under greenhouse conditions	Lacava et al., 2006
	Paraburkholderia phytofirmans	Tested in grapevines under greenhouse conditions	Baccari et al., 2019
	Weakly virulent *X. fastidiosa* strain	Tested in vineyards	Hopkins, 2005
	Avirulent mutant of *X. fastidiosa*	Tested in grapevines under greenhouse conditions	Hao et al., 2017
	Cocktail of lytic phages	Tested in grapevines under greenhouse conditions	Das et al., 2015

Table 2. (continued)

Type of control	Strategy	Status of strategy	Reference
Genetic engineering	GMO expressing the RpfF gene	Tested in vineyards and in tobacco and citrus plants under greenhouse conditions	Lindow et al., 2014; Caserta et al., 2017
	GMO expressing a SA carboxyl methyltransferase	Tested in tobacco plants under greenhouse conditions	Gómez et al., 2020
	GMO expressing AMPs	Tested in vineyards	Li et al., 2015; Dandekar et al., 2019
	GMO expressing pPGIP	Tested in vineyards	Dandekar et al., 2019
Agricultural practices	Weed management and regular rototilling	Tested in olive orchards	EFSA PLH Panel, 2015; EFSA, 2016
	Strategically localize new orchards	Tested in vineyards and almond orchards	Sisterson et al., 2010
	Pruning	Tested in vineyards	Hopkins and Purcell, 2002; Daugherty et al., 2018
	Remove plants that are unlikely to recover	Simulation approach	Sisterson and Stenger, 2013
	Reduce irrigation	Tested in almond and citrus orchards	Krugner and Backus, 2014
	Replanting	Tested in young almond orchards	Sisterson et al., 2012
	Reuse rootstocks to form a new healthy canopy by grafting healthy scions	Tested in citrus orchards	Lopes et al., 2020
	Host resistance/tolerance	Tested in vineyards, citrus, almond and olive orchards	Krivanek et al., 2005; Garcia et al., 2012; Sisterson et al., 2008 and 2012; EFSA, 2017

fertigation and adsorbed fertilizer. Nevertheless, symptoms reappeared after treatment stopped, indicating that *X. fastidiosa* was still present in plants (Muranaka et al., 2013). Although the antibacterial effect of NAC against pathogens has been reported (Picchi et al., 2016), *in vitro* experiments using non-lethal concentrations of NAC against *X. fastidiosa* subsp. *pauca* strain DeDonno showed a shift of the bacterium into a hyper-attachment phenotype (Cattò et al., 2019). This could explain why plants treated with NAC showed a reduction in disease severity, as a hyper-attachment phenotype of *X. fastidiosa* has been associated with reduced virulence *in planta* (Chatterjee et al., 2008b). NAC treatments in field conditions have also been conducted in affected areas in Brazil and Italy, within the framework of H2020 projects POnTE and XF-ACTORS. In new olive plantations and olive orchards with initial *X. fastidiosa* incidence, one trunk injection application per year was the only treatment that resulted in some reduction of branch desiccation and dieback, although no decrease in *X. fastidiosa* population levels was observed by real-time PCR (qPCR) (Dongiovanni et al., 2017). On the other hand, a two years field trial in severe diseased citrus trees showed that the application of NAC-fertilizer improves their fitness and their fruit size (De Souza et al., 2019).

A recent study using a bioactive detergent composed of plant oil and water extracts from different botanical species, sulphur, sodium and calcium hydroxide, was also evaluated in highly infected olive orchards in Italy. Although a limited number of samples were tested and only over a period of 2 to 4 years, results showed that spray treatments to the canopies and trunks induced recovery from *X. fastidiosa* in old olive trees from cv. Ogliarola Salentina and cv. Cellina di Nardò. So, it has been suggested that treatments with this product reduce bacterial cells, as well as activate the plant immune system. Moreover, this product acts as growth promoter and protects plant cell membrane integrity against damage caused by *X. fastidiosa* virulence factors (Bruno et al., 2021).

It was hypothesized that manipulation of the plant mineral levels could be an efficient disease management strategy because *in vitro* experiments showed that zinc (Zn) detoxification is required for full *X. fastidiosa* virulence (Navarrete and De La Fuente, 2015), and copper (Cu)

influences its growth and biofilm formation (Cobine et al., 2013). In this sense, treatments with a Zn- and Cu-containing citric acid biocomplex fertilizer, obtained through natural fermentation processes using soil fungi, were applied in infected olive orchards in Italy. Although preventive spray application to tree canopies, in addition to a pest (insects and weeds) control and a routine fertilization and harrowing of the soil, reduced disease severity and bacterial population levels, the treatment was not effective in eliminating the pathogen. Satisfactory re-sprouting of new shoots was observed, compared to non-treated plants, but *X. fastidiosa* was still detected in the new vegetation. The same was observed for trunk injection treatments applied to the already infected trees (Scortichini et al., 2018). The administration of Cu via soil drench using Cu(II) sulfate ($CuSO_4$) was also investigated in tobacco plants under greenhouse conditions. While similar Cu concentrations were detected in leaves compared to leaves from olive trees treated with this fertilizer, no reduction of disease severity nor pathogen growth was observed in this study. Contrary, an enhanced *X. fastidiosa* virulence was noted in treated plants, with an increase of symptoms and bacterial population levels (Ge et al., 2020).

In greenhouse conditions, the application of menadione and benzethonium chloride treatments, abscisic acid (ABA) and DSF related components in grapevines for the control of PD has also been explored. While menadione acts as a plant defense activator, inducing the production of reactive oxygen species (ROS), benzethonium chloride is a biocidal component with a broad spectrum of microbiocidal activity. Both chemicals administrated via soil drench were detected systemically in plants and were efficient in reducing PD symptoms over a period of three months (Zhang et al., 2019). The fall application of ABA via drench reported a curing effect in *X. fastidiosa* infected grapevines, but did not prevent plants of being re-infected with the pathogen. The highest curing percentages were correlated with plants showing the highest concentration of total phenolic compounds in sap (Meyer and Kirkpatrick, 2011). The direct application to plant surfaces of DSF molecules, palmitoleic acid and macadamia nut oil (which has a 23 % of palmitoleic acid) via spray was adopted aiming the confusion of the pathogen,

changing its phenotype from planktonic to biofilm formation. Because all components tested resulted in the decrease of PD severity, further investigation is being pursued as it is a practical and inexpensive strategy (Lindow et al., 2017).

2.3.2. Biological control

A potential alternative or complement to chemical control is the use of biological control, which consists in the management of plant diseases using other organisms (Gurr and You, 2016). There are three main strategies: (i) the classical, in which a natural enemy of the pathogen is newly introduced in order to achieve disease control; (ii) the inductive, where the population levels of an already existing natural enemy are increased; (iii) and the inoculative, which consists in maintaining an already existing natural enemy through regular reestablishment.

For the biological control of *X. fastidiosa*, some of these strategies have been explored using either endophytes, avirulent or weakly virulent *X. fastidiosa* strains, or phages. In the case of endophytes, *Curtobacterium flaccumfaciens* and *Methylobacterium mesophilicum* (isolated from asymptomatic CVC plants) have been proposed as biological control agents due to their characterization as activators of systemic acquired resistance (SAR) and their antagonistic activity against *X. fastidiosa* (Azevedo et al., 2016). Madagascar periwinkle (*Catharanthus roseus*) plants co-inoculated with both, *C. flaccumfaciens* and *X. fastidiosa*, under greenhouse conditions, showed no appearance of symptoms as well as comparable fitness to non-infected plants (Lacava et al., 2007). The same experiment was performed using *M. mesophilicum*, resulting in a reduction of the *X. fastidiosa* population (Lacava et al., 2006). These observations were related with a down-regulation of genes related to bacterial growth in *X. fastidiosa*, which was observed when it was co-cultured *in vitro* with *M. mesophilicum* (Dourado et al., 2015). Similar results were obtained using another endophyte, *Paraburkholderia phytofirmans*, which was able to easily colonize xylem vessels of grapevines. When inoculated simultaneously or after *X. fastidiosa*, it conferred protection to plants by activation of their induced systemic

resistance (ISR) (Baccari et al., 2019). Recently, studies of the microbiome in grapevines (Deyett and Rolshausen, 2019) and olive (Vergine et al., 2020; Giampetruzzi et al., 2020; Zicca et al., 2020) have been performed in order to identify more endophytes with potential to be used in biological control, as differneces between resistant and susceptible cultivars and between seasons have been observed.

The inoculation of grapevines with avirulent or weakly virulent *X. fastidiosa* strains has also been investigated. In greenhouse conditions, several weakly virulent strains reported promising results regarding reduction of PD symptoms. Nevertheless, once they were tested under field conditions, only the strain EB92-1 showed a significant reduction of PD incidence. It was hypothesized that these differential results are due to multiple *X. fastidiosa* inoculations by insect vectors in the field, in addition to the non-controlled climatic conditions and the differences in grapevine cultivar resistance (Hopkins, 2005). The assessment of an avirulent mutant strain of *X. fastidiosa*, in which a gene encoding a putative acyl-coenzyme A (acyl-CoA) synthetase was delated, was also performed under greenhouse conditions. Results showed that its application, two weeks prior the inoculation of a virulent *X. fastidiosa*, controlled PD disease progression, probably due to the induction of SAR in plants (Hao et al., 2017).

Phages are also proposed as biological control agents due to their specificity in infecting only prokaryotic organisms (bacteria or archaea) and their exponential propagation. One of the main concerns of their use is their capacity to integrate into the bacterial genome as a prophage. For this reason, only lytic phages can be used for biological control (Buttimer et al., 2017). For the control of *X. fastidiosa*, a cocktail of four lytic phages has been tested *in vitro* against different *X. fastidiosa* strains (Ahern et al., 2014) and in grapevines under greenhouse conditions (Das et al., 2015) reporting positive results. While phage population levels decreased in healthy plants, *X. fastidiosa* infected plants had a higher phage concentration and showed reduced PD symptoms and lower population levels of *X. fastidiosa* compared to non-treated plants. Prior to field trials, it is important to understand the potential of phage transmission by insect

vectors. Some experiments have revealed that the uptake of this cocktail of phages is highly efficient by glassy-winged sharpshooter (GWSS), but its transfer to plants is low, probably due to a dilution effect during feeding activity (Bhowmick et al., 2016).

2.3.3. Genetic engineering

The manipulation of genomes has always been performed, indirectly with the application of traditional breeding methods or directly with the emergence of new genetic engineering techniques. All procedures aim to obtain offspring with desired traits, such as increased resistance to diseases, to abiotic stresses, or increased productivity. Even though genetically modified organisms (GMO) still have a strict authorization process in the EU, new genome editing technics, such as CRISPR/Cas9, might change their regulation in the future as these GMO cannot be differentiated from conventionally breed varieties or naturally occurring variants (Wasmer, 2019).

Different strategies have been used in order to obtain genetically modified plants resistant to *X. fastidiosa.* As mentioned, the growth behavior of *X. fastidiosa* is mainly regulated by the production of DSF. In this sense, different host plant species have been genetically modified with the introduction of the RpfF gene, in order to obtain organisms that synthetize the DSF molecule by themselves. In grapevines, the GMO was tested under greenhouse and field conditions, showing a reduction of disease incidence compared to the wild type varieties (Lindow et al., 2014). Similar results were observed in genetically modified tobacco and citrus plants. Under greenhouse conditions, plants presented a reduced rate of disease development, including decreased symptomatology and low *X. fastidiosa* population levels (Caserta et al., 2017). These findings are in agreement with the ones already obtained using direct application of DSF to grapevine plants, indicating that DSF modifies the *X. fastidiosa* behavior by reducing its mobility through the plant, and consequently its capacity to colonize and cause disease. In addition, the production of DSF has been associated with the activation of ROS production and overexpression of *pathogenesis-related 1* (PR1), indicating a possible

effect of this molecule in the activation of the plant immune system (Kakkar et al., 2015).

Recently, transgenic *Nicotiana tabacum* lines overexpressing a gene from *Citrus reticulata* encoding a salicylic acid carboxyl methyltransferase (involved in the production of methyl salicylate (MeSA)) were obtained. The upregulation of PR1 gene and the increase of MeSA, detected by gas chromatography, confirmed that MeSA is responsible for the activation of SAR in transgenic lines and also in neighboring wild-type plants due to its volatile nature. *X. fastidiosa* infections were carried out in these plants, and a significant reduction of disease severity in transgenic lines was observed compared to wild-type plants (Gómez et al., 2020).

The transgenic expression of AMPs into crop species has been reported to confer resistance against various plant pathogens (Montesinos E., 2007). In this direction, the production and evaluation of genetically modified grapevines expressing the AMP LIMA-A, able to kill *X. fastidiosa* cells at low concentrations *in vitro*, was performed under greenhouse conditions. Some lines remained symptomless after several *X. fastidiosa* inoculations and over a period of 10 years, showing low bacterial population levels in sap. Nevertheless, these promising results were not confirmed in the field, probably due to increased levels of biotic and abiotic stresses and the dormancy of plants, which reduced the expression of the LIMA-A gene (Li et al., 2015). Differently, grapevine lines expressing either a chimeric protein, composed of an elastase and a fragment of the AMP Cecropin B, or a polygalacturonase-inhibiting protein showed reduced mortality due to *X. fastidiosa* compared to wild type lines under field conditions (Dandekar et al., 2019). The genetically modified grapevines, used as rootstocks, also provided trans-graft protection to the scion, due to the target of both proteins into the xylem lumen and their capacity to move through the graft union (Agüero et al., 2005; Dandekar et al., 2012). The activity of both transgenic proteins has been associated to the activation of the plant immune system, as plants were in good health in spite of the infection (Dandekar et al., 2019).

The discovery of the LPS O-antigen mechanism that *X. fastidiosa* uses to avoid the activation of the plant immune system (Rapicavoli et al., 2018), prompted research lines focused on the expression of LPS in grapevines. The aim is to obtain varieties with their defenses activated and, consequently, more resistant to PD. Additionally, and because it is being observed that the elicitation of the plant immune system reduces *X. fastidiosa* population levels in plants, RNA-seq analysis are ongoing in order to understand the time course of defense activation and detect important genes during the process, which could be modified using the new genome editing technics (Research progress reports CDFA, 2019).

2.3.4. Agricultural practices

Good agricultural practices are generally described as a collection of principles to apply for the economic, social and environmental sustainability of the on-farm production and post-production processes. Their introduction into a wide range of farming systems and at different scales aims the reduction of the overuse of pesticides, resulting in safe and healthy food and non-food agricultural products.

In order to avoid the presence of *X. fastidiosa* in an orchard, different agricultural practices should be taken into account. For example, weed management and regular rototilling is highly recommended to avoid undesirable plants to serve as reservoirs of *X. fastidiosa* (EFSA PLH Panel, 2015; EFSA, 2016). Moreover, it is important to strategically localize new orchards, which should be far from weeds that enhance the spread of the pathogen (Sisterson et al., 2010).

Once an orchard is infected with *X. fastidiosa*, in addition to the above-mentioned practices, a regular summer and autumn pruning of symptomless plants has been recommended in vineyards (Hopkins and Purcell, 2002), although it has been observed that severe pruning is not efficient (Daugherty et al., 2018). Removing plants that are unlikely to recover or produce is also highly recommended (Sisterson and Stenger, 2013), as well as reducing irrigation in order to water-stress plants, making them less attractive for insect vectors (Krugner and Backus, 2014). In the

case of ALS-affected orchards, it has been suggested that replanting is a good management if almond trees are young (Sisterson et al., 2012). This practice has also been used in partially CVC-affected orchards, but the reuse of the rootstock to form a new healthy canopy by grafting healthy scions has also been suggested, as new scions grow free of CVC and faster than new plantings (Lopes, 2020).

The use of resistant/tolerant cultivars is another relevant practice that increases the probabilities of achieving a *X. fastidiosa* control. Various degrees of resistance/tolerance have already been observed in grape (Krivanek et al., 2005), citrus (Garcia et al., 2012), and almond (Sisterson et al., 2008, 2012). In the EU, differences in susceptibility between olive varieties infected with *X. fastidiosa* subsp. *pauca* ST 53 have been identified. The varieties Leccino and FS-17® showed lower bacterial population levels compared to susceptible olive cultivars, as well as a reduced disease severity (EFSA, 2017). In the case of Leccino, these observations have been related with a different transcriptomic profile in comparison with the susceptible cv. Ogliarola Salentina, suggesting that this resistant variety might harbor regulatory elements that mitigate *X. fastidiosa* infections (Giampetruzzi et al., 2016).

2.4. Integrated disease management of *X. fastidiosa*

The concept of integrated disease management (IDM) got importance after the increasing concern regarding the use of pesticides and the difficulties in registering new chemical compounds (Razdan and Grupta, 2009). Programs emphasizing sustainable agriculture started to enhance the application of IDM, defined as the sustainable approach for managing disease using a combination of biological, cultural, host-resistance and chemical strategies, in order to minimize economic, health and environmental risks (El Khoury and Makkouk, 2010). In fact, it was proved that IDM is a more sustainable and effective approach compared to the one single control strategy. This is because disease control is generally partially effective and high amounts of the one single product were being used, increasing the environmental damage and the development of resistances (Thomas, 1999).

In order to achieve an optimized IDM (Fig. 7), it is fundamental to have a more ecological approach, in a sense that the fully understanding of every strategy adopted and their interactions is required for determining their compatibility in a local agrosystem (Thomas, 1999). For this reason, in addition to find new strategies, research should be focused on (i) the biochemistry and molecular biology of the pathogens and their genetics, in order to understand resistance and discover new target sites and metabolites, and (ii) on epidemiology, which would give knowledge for the probability of spread and the optimal timing application of control strategies (Waard, 1993).

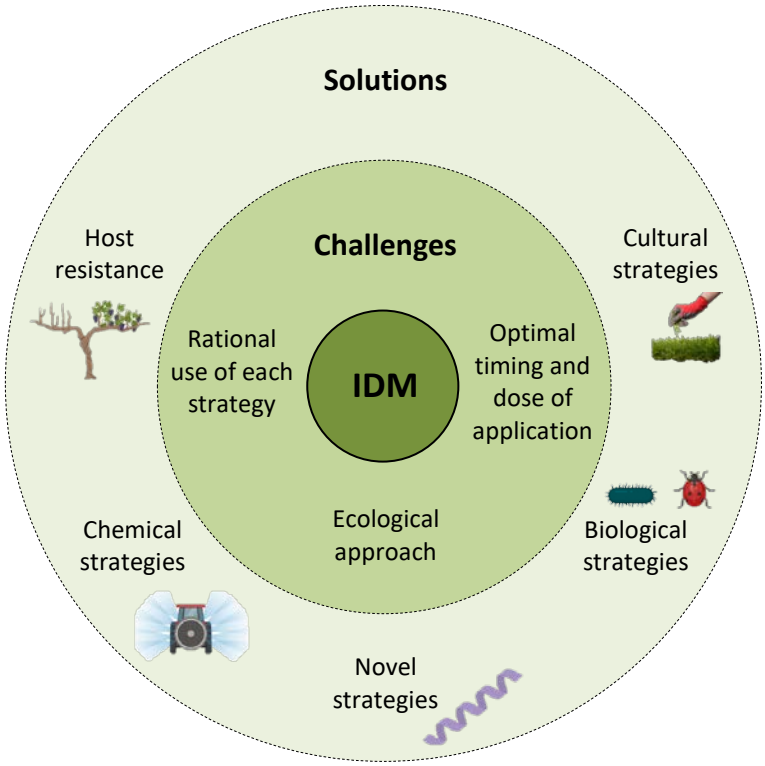

Figure 7. Challenges and strategies for achieving an optimized IDM.

Even though there is still few information available regarding *X. fastidiosa* in the EU, different modelling approaches have been developed to capture its epidemiological process and to assess its probability of spread. Until now, it has been observed that the most important factors

for a successful local eradication are the application of a highly effective insect vector control and the early detection of the pathogen, in addition to the rapid implementation of control measures (e.g. removing plants) (EFSA, 2019b). In this sense, the annual prospections implemented by the EU commission are of vital importance, as it is the most effective way to rapidly detect the presence of *X. fastidiosa* in a region.

When considering a long-range spread, modelling of data from olive orchards in Italy revealed different parameters as the most relevant ones in controlling the disease. In the model developed by Fierro et al., 2019, the number of insect vectors and trees was reported as the main parameter to consider, indicating the need for an efficient management of insect vectors and the elimination of infected trees. Differently, other key components were considered as possible targets for the application of control strategies in the model developed by Brunetti et al., 2020. Within them, the partial removal of weeds around olive orchards, instead of the removal of the productive olive trees, was considered the most promising practice that should be applied for the achievement of an effective and cost-efficient control of OQDS.

Even though different strategies could be implemented for an IDM of *X. fastidiosa*, what it is common within them is the efficient control of insect vectors, either by chemical or biological control or by removal of weeds, where naiades and young adult spittlebugs inhabit (Cornara et al., 2016; Fierro et al., 2019).

3. Peptides as a novel strategy for plant disease control

Nowadays, plant disease control is mostly achieved by combining improved breeding lines and the rational use of synthetic chemical pesticides based on disease forecasts, systems that predict the occurrence or change in severity of a plant disease. Nevertheless, environmental and human health issues regarding the application of some of these pesticides, such as the presence of multi-drug resistant strains, are still a major concern (Goyal and Mattoo, 2016). For this reason, in the last years,

modifications in the pesticide regulations have been undertaken by many countries with the aim of retaining only more selective compounds with low toxicity and reduced negative environmental impact (Zeitler et al., 2013). In this context, and taking into account that climate change is increasing the probabilities of plant disease emergence and spread, research directed to the development of novel plant protection products is necessary (Datta et al., 2015).

Over the past decades, AMPs have continuously been proposed as a potential tool to create both, more sustainable pesticides and disease resistant crops. Although there are no pesticides or transgenic plants based on AMPs commercialized up to date, the careful design and selection of suitable peptides is essential for their improved development (Montesinos E., 2007; Montesinos E. et al., 2012; Van Esse et al., 2020).

3.1. Antimicrobial peptides

AMPs are short (generally less than 50 amino acid (aa)), cationic and amphiphilic sequences produced not only by animal and plant species as key components of their innate immune system, but also by unicellular organisms like bacteria and yeast (Jung et al., 2014; Breen et al., 2015). It has been suggested that they are critical for the successful evolution of organisms, probably because they take part in a wide range of signaling processes and have an important role in competition between organisms (Zeitler et al., 2013; Meneguetti et al., 2017).

They are structurally diverse, showing not only α-helical and β-sheet structures but also hairpin and loops, and they display a broad spectrum of action, suggesting their interfere with structural and functional cellular components. In fact, their main mechanism of action is membrane disruption due to the formation of pores. Their cationic nature enables an initial electrostatic binding with negatively charged membranes, which generally turns to a membrane permeabilization and disruption. Different models have been proposed, according to the interaction established depending on the peptide structure (Fig. 8) (Goyal and Mattoo, 2016). Another possibility after the binding is the

translocation of the peptide to the cytoplasm by permeabilization or passive peptide transport. In these situations, AMPs have the capacity to interfere central cellular processes like cell wall synthesis, protein synthesis and folding, or gene expression (Spohn et al., 2019).

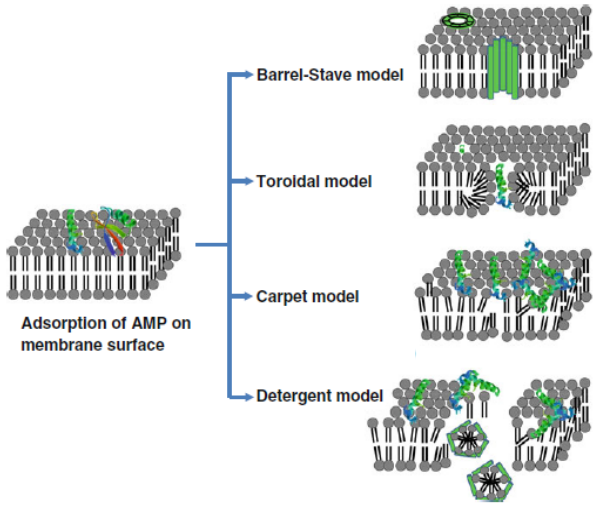

Figure 8. Membrane permeabilization models proposed after the binding of AMPs to the membrane surface (Goyal and Mattoo, 2016).

Differently from eukaryotic cells, which have a more complex and generally non-charged cell wall, the outer membrane of prokaryotic cells is partially composed of highly negative charged molecules, corresponding to LPS in Gram-negative bacteria and teichoic and teichuronic acids in Gram-positive ones. This membrane architecture makes AMPs selective towards microorganisms (Zeitler et al., 2013). Even though resistance against AMPs can be acquired by microorganisms, through gene transfer or combination of resistance mechanisms, it has been reported that probabilities are low compared to other antimicrobials. This is due to their multi-targeting characteristics and to the fact that there is limited availability of resistance mutations against AMPs (Spohn et al., 2019).

In addition to the direct interaction with microorganisms, AMPs can act as PAMPs or plant elicitor peptides (Peps), eliciting the plant immune system (Goyal and Mattoo, 2016; Ruiz et al., 2018). In general, PAMPs and Peps activate the primary innate immunity, which is

constituted by different types of responses, both local and systemic (Fig. 9). Local responses are mainly aimed at activating the hypersensitive response (HR), which is characterized by an increase in the production of ROS and a programmed cell death of infected cells in order to avoid the spread of the pathogen. On the other hand, systemic responses are responsible for the activation and preparation of the plant immune system in healthy tissues distant from the infection site (Bektas and Eulgem, 2015). This process is called priming, and it has been described as the mechanism that induces the plant to a new physiological state, conferring a higher level of resistance against pathogens. In this state, the plant is able to respond quickly and robustly when exposed to biotic stresses (Conrath, 2009).

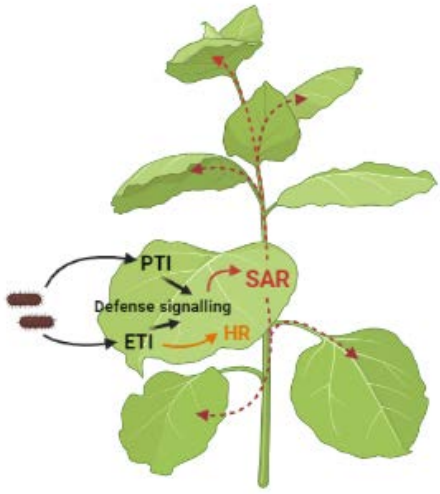

Figure 9. Schematic representation of the activation of primary innate immunity by bacterial pathogens. PTI, pattern triggered immunity; ETI, effector triggered immunity; HR, hypersensitive response; SAR, systemic acquired resistance. Created with BioRender.com.

A wide range of AMPs have been reported and tested for their antimicrobial and elicitor activities (Boto et al., 2018; Malik et al., 2020). For example, bacteriocins are secreted by a large number of bacterial species and have been described as potential antimicrobials against several plant pathogens (Grinter et al., 2012; Subramanian and Smith, 2015; Rooney et al., 2020). In plants, AMPs showing inhibitory activity against plant pathogens have also been reported (Breen et al., 2015).

AMPs from animals, mainly linear peptides, have been extensively studied as they display highly activity against important plant pathogens such as *X. fastidiosa* (Table 3).

Table 3. AMPs from animals active against *X. fastidiosa*.

AMPs	Source	Activity	Reference
Magainin I	*Xenopus laevis*	*In vitro*	Li and Gray, 2003
Cecropin A	*Hyalophora cecropia*	*In vitro*	Andersen et al., 2004
Cecropin B			
PGQ	*Xenopus laevis*	*In vitro*	Kuzina et al., 2006
Indolicin	*Bos taurus*		
Magainin II	*Xenopus laevis*		
Dermaseptin	*Phyllomedusa*		
Gomesin	*Acanthoscurria gomesiana*	*In vitro* and *in planta*	Fogaça et al., 2010
Scorpine-like molecule	*Vaejovis mexicanus*	*In vitro*	Arora et al., 2018
Melittin	*Apis mellifera*		

3.2. Process of development of antimicrobial peptides

AMPs from natural sources are generally produced in low amounts and their extraction and purification processes are normally complex and expensive (Montesinos E. et al., 2012). Additionally, they tend to be unstable and highly toxic against human and plant cells, especially the ones that show significant antimicrobial activity (Montesinos E., 2007; Zeitler et al., 2013). In this sense, the chemical synthesis of AMPs has advantages, as it gives the possibility to incorporate modifications in order to improve the bioactivity of natural peptides or synthetize *de novo* ones (Meneguetti et al., 2017).

As in the pharma sector, the process of development of AMPs by chemical synthesis follows a series of steps that starts with its design, either using natural peptide sequences as models or *de novo* designed peptides using rational design. The screening of these new peptides using different methodologies, able to determine their bactericidal or

bacteriostatic properties, is the second step, followed by the selection of peptides showing the best profile. Secondary libraries using these lead peptides are designed by combinatorial approaches or the incorporation of modifications already used to improve their characteristics. Screening of these new peptides is performed with the aim of selecting the best peptides to continue the process. Once a good candidate is obtained, toxicity tests in animals, synthesis optimization and formulation are required in order to start greenhouse and field tests (Fig. 10) (Montesinos E. et al., 2012).

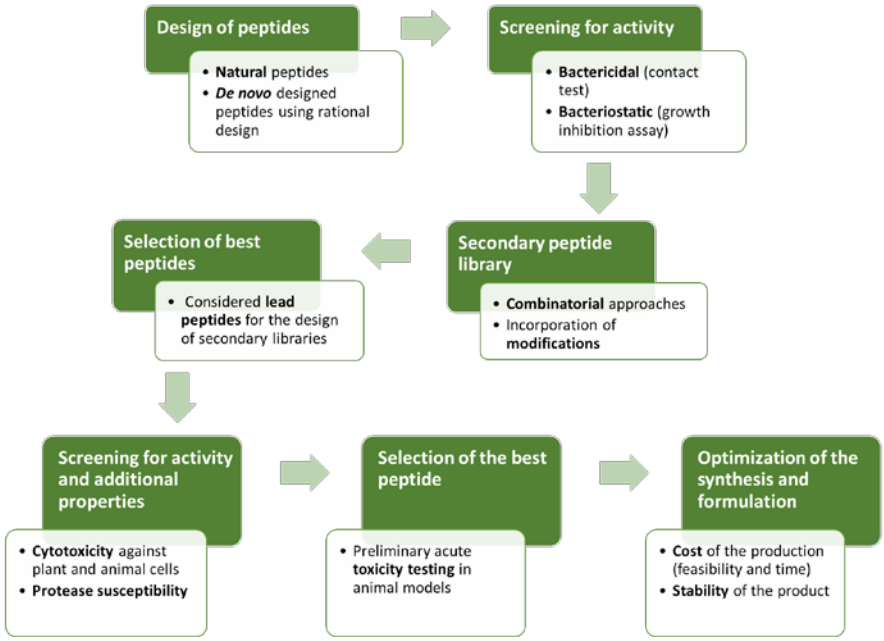

Figure 10. Flow diagram of the development of AMPs by chemical synthesis (modified from Montesinos E. et al., 2012).

When designing new AMPs, it is important to know that at least seven parameters are relevant for their biological activity: size, sequence, degree of structuring, charge, hydrophobicity, amphipathicity and width of the helix. A good balance between these factors, and in particular a minimum threshold of hydrophobicity and net positive charge, confers to the peptide significant antimicrobial activity (Badosa et al., 2007). So,

rather than the precise aa sequence, the activity of AMPs depends on their physicochemical properties. This fact makes the activity of AMPs susceptible to the environment conditions, as their properties can change due to, for example, an increase of ionic strength produced by the presence of salt ions in the medium (Montesinos E. et al., 2012).

In order to increase their antimicrobial activity and/or improve their stability to achieve an extended effect over time, modifications are generally done in AMPs produced by chemical synthesis. For example, the production of fusion proteins, tandem polypeptides or small truncated sequences containing minimal domains for activity, and the addition of D-aa in the sequence have been used to synthetize more stable peptides (Badosa et al., 2013; Puig et al., 2014; Boto et al., 2018). To increase the antimicrobial activity, the juxtaposition of active fragments from natural peptides and the incorporation of an acyl group are modifications that have been successfully exploited (Badosa et al., 2009; Oliveras et al., 2018).

A wide range of screening methodologies have been used over time in order to determine the antimicrobial activity of different compounds. Most methods have been focused on determining their bacteriostatic activity, which is the inhibitory activity observed when the compound is present (Badosa et al., 2007). This is the case of diffusion tests or broth or agar dilution assays, in which known amounts of peptide are deposited in an active growing culture of the target microorganism. Contrary, methods consisting of a contact test followed by culture or molecular techniques based on cell viability are able to determine the bactericidal activity of the compounds. This type of assay is more suitable for the selection of AMPs, as their inhibitory effect persists due to the fact that the compound kills the cells (Montesinos E. et al., 2012).

In order to compare the activity of different AMPs, and to understand their kinetics of inactivation, it is important to perform dose-response relationships as well as to determine useful parameters such as the minimum inhibitory concentration (MIC) and the median effective dose (ED_{50}). This information gives an estimation of the peptide potency,

although it is important to notice that *in vitro* results do not necessarily reflect the efficacy of peptides *in vivo*. In this sense, it is important to have a large number of AMPs with potential to become good antimicrobial compounds (Badosa et al., 2007).

3.3. Perspectives on the use of antimicrobial peptides for plant disease control, and against *X. fastidiosa* diseases

The interest of using AMPs for plant disease control is mainly due to their mechanisms of action targeting both, pathogens and host plants. Moreover, the fact that they can be produced by microorganisms that act as biocontrol agents or by the plant itself expands their ways of administration. GMO expressing them can be develop, either to be cultivated or to be used as biofactories (Montesinos E., 2007; Montesinos L. et al., 2017).

In our group, in coordination with the *Laboratori d'Innovació en Processos i Productes de Síntesi Orgànica* group (LIPPSO), extensive research on AMPs has been performed with the aim of obtaining good candidates for plant protection. The development of different peptide libraries by combinatorial approaches and the evaluation of their antimicrobial activity against different plant pathogens and their elicitor activity against different host plants has led to the discovery of promising lead peptides. This is the case of BP100, a linear undecapeptide departing from the CECMEL11 library, which was developed from the combination of cecropin A and melittin fragments (Ferré et al., 2006). This peptide has reported highly antimicrobial activity *in vitro* against a wide range of plant pathogens, like *Pseudomonas syringae* pathovars, *Erwinia amylovora* and *Xanthomonas axonopodis* pv. *vesicatoria*. Moreover, assays using either pear and apple flowers or potted pear plants reaffirmed its activity against *E. amylovora*, showing results comparable to streptomycin (Badosa et al. 2007; Badosa et al., 2017). *In planta* experiments also demonstrated the efficacy of preventive BP100 treatments in controlling phytoplasms, compared to BTH (Rufó et al., 2017), indicating a possible role in the activation of the plant immune system. This was also hypothesized when the induction of some defense-related genes in tomato was observed 24

h after the treatment with the peptide (Oliveras et al., 2018). Another interesting feature of BP100 is its capacity to act as cell-penetrating peptide. This confers to the peptide the ability to act as carrier of functional active cargo, such as DNA, RNA, drugs, nanoparticles and antibodies into cells without membrane disruption (Eggenberger et al., 2011).

Due to the fact that BP100 showed great properties as antimicrobial agent, derivatives for being expressed in plants were designed, always considering important structural requirements like minimal threshold size and targeting to subcellular organelles. These requirements are important to guarantee stability, avoid toxicity and ensure the peptide accumulation in a specific tissue (Streatfield, 2007). Tandemly arranged BP100 units targeted to the endoplasmic reticulum, where toxicity and degradation by proteolytic enzymes were expected to be lower, were designed and transformed into rice plants. Although plants expressing BP100 inverted repeats had increased resistance to some pathogens and their agronomic performance was comparable to untransformed plants, the expression of BP100 direct tandems showed a strong negative impact on the plant fitness, as well as a poor efficiency of transformation (Nadal et al., 2012). This was also observed with the direct expression of BP100, fused to the fluorescent marker protein DsRed, in *Nicotiana benthamiana* and *Arabidopsis thaliana* plants. The transiently expression of the peptide had no harmful effects, but long-term exposure to BP100 had deleterious impact on the plants (Company et al., 2014).

The heterologous production of proteins in rice endosperm has been extensively investigated (Takaiwa et al., 2007; Boothe et al., 2010), as is the place with the optimal biochemical environment for proteins to long-term accumulate (Bundó et al., 2014). From another library of BP100 derivatives designed for being expressed in plants (Badosa et al., 2013), the derivate BP178 was successfully produced in rice seeds, specifically in the protein bodies of the endosperm tissue. Rice seeds accumulating the peptide showed resistance to bacterial and fungal infections. The purification of the peptide was also achieved, being the purified product highly active against *Dickeya* sp. (Montesinos L. et al., 2017).

4. Context and approach of this Ph.D. Thesis

The emergence of the plant pathogen *X. fastidiosa* in the EU has focused the attention on understanding its epidemiology and finding novel solutions for its prevention and cure. Since the start of the 21th century, the Plant Pathology group (CIDSAV) of the University of Girona, in coordination with the LIPPSO group of the University of Girona, are working on the development of synthetic AMPs against important plant pathogens, such as *E. amylovora*, *P. syringae* and *X. vesicatoria*, within others. The obtainment of high active AMPs with low toxicity and resistant to proteases has increased their interest in the production of more sustainable pesticides, as well as disease resistant crops by genetic engineering.

In the framework of the European project XF-ACTORS (H2020 – SFS-09-2016) and the national projects E-RTA INIA - INTEROLIVO (Ref. 2017-00004-C06-03 and Ref. 041/18) and PEP-XYL (RTI Ref. 2018-099410-B-C21), the present Ph.D. Thesis has focused on developing a screening methodology for the identification of highly active peptides against *X. fastidiosa*, studying the bactericidal activity of lead peptides against different pathogen strains and characterizing their main mechanism of action, as well as on determining the aggressiveness of four Spanish isolates of *X. fastidiosa* in different almond cultivars, and characterizing their population dynamics and their dose-response relationships. This Thesis has been presented as a compendium of three scientific papers that compile the research performed to achieve the mentioned goals.

CHAPTER II

Objectives

The main objective of this Ph.D. Thesis was to study the potential of synthetic AMPs for the control of *X. fastidiosa*-related diseases, and to establish the experimental basis for the design of treatment guidelines using these compounds.

The specific objectives were:

i. To evaluate the bactericidal activity of lead AMPs against different *X. fastidiosa* strains and to elucidate their main mechanism of action.

ii. To develop a screening methodology, based on molecular technics, for the identification of highly active AMPs against *X. fastidiosa*.

iii. To assess the aggressiveness of four Spanish isolates of *X. fastidiosa* in causing ALS in different almond cultivars, and to characterize their population dynamics and dose-response relationships over time.

This Thesis is presented as a compendium of three publications that correspond to chapters III, IV and V, and follows the same order as the objectives.

CHAPTER III. Differential susceptibility of *Xylella fastidiosa* strains to synthetic bactericidal peptides. *Phytopathology*, 110:1018-1026.

CHAPTER IV. Screening and identification of BP100 peptide conjugates active against *Xylella fastidiosa* using a viability-qPCR method. *BMC Microbiology*, 20:229.

CHAPTER V. Aggressiveness of Spanish isolates of *Xylella fastidiosa* to almond plants of different cultivars under greenhouse conditions. *Phytopathology*, https://doi.org/10.1094/PHYTO-02-21-0049-R.

CHAPTER III

Differential susceptibility of *Xylella fastidiosa* strains to synthetic bactericidal peptides

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Disease Control and Pest Management

Differential Susceptibility of *Xylella fastidiosa* Strains to Synthetic Bactericidal Peptides

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ABSTRACT

The kinetics of cell inactivation and the susceptibility of *Xylella fastidiosa* subspecies *fastidiosa*, *multiplex*, and *pauca* to synthetic antimicrobial peptides from two libraries (CECMEL11 and CYCLO10) were studied. The bactericidal effect was dependent on the relative concentrations of peptide and bacterial cells, and was influenced by the diluent, either buffer or sap. The most bactericidal and lytic peptide was BP178, an enlarged derivative of the amphipathic cationic linear undecapeptide BP100. The maximum reduction in survivors after BP178 treatment occurred within the first 10 to 20 min of contact and at micromolar concentrations (<10 μM), resulting in pore formation in cell membranes, abundant production of outer membrane vesicles, and lysis. A threshold ratio of 10^9 molecules of peptide per bacterial cell was estimated to be necessary to initiate cell inactivation. There was a differential susceptibility to BP178 among strains, with DD1 being the most resistant and CFBP 8173 the most susceptible. Moreover, strains showed a proportion of cells under the viable but nonculturable state, which was highly variable among strains. These findings may have implications for managing the diseases caused by *X. fastidiosa.*

Keywords: antimicrobial peptides, bactericidal activity, bacteriology, biochemistry and cell biology, disease control and pest management, v-qPCR, viable but nonculturable state, *Xylella fastidiosa*

Xylem- and phloem-limited plant bacterial diseases, caused by phytoplasms, *Candidatus* Liberibacter, and *Xylella fastidiosa*, are among the most important threats limiting crop productivity worldwide (Kumari et al. 2019; Saponari et al. 2019; Wang et al. 2017). Diseases caused by *X. fastidiosa* produce leaf scorch and wilting symptoms, and sudden dead of infected plants, mainly due to interference with xylem vessel performance and sap flow (De Benedictis et al. 2017; Roper et al. 2007; Sicard et al. 2018). Although this pathogen is well established in several areas of the North and South American continents (e.g., Pierce's disease of grapevine and citrus variegated chlorosis) (Garcia et al. 2012; Purcell 2013), *X. fastidiosa* diseases have emerged in recent years as several outbreaks of considerable intensity and extension in Europe (e.g., Italy, France, and Spain), affecting mainly olive, almond, and also wild vegetation (Denancé et al. 2017; Olmo et al. 2017; Saponari et al. 2013).

The management of diseases caused by *X. fastidiosa* is currently based on the use of pathogen-free propagation plant material, quarantine and eradication measures, and vector control (Sicard et al. 2018). However, no cure for the disease exists up to now due to the lack of effective bactericides and the difficulties in accessing xylem vessels, where the pathogen establishes (EFSA Panel on Plant Health 2019; Kyrkou et al. 2018). Therefore, there is a need for new chemical compounds against *X. fastidiosa*, because current antibacterial compounds did not provide effective disease control (EFSA Panel on Plant Health 2019).

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The author(s) declare no conflict of interest.

Antimicrobial peptides have been identified as potential candidates for plant disease control (Marcos et al. 2008; Montesinos 2007; Montesinos et al. 2012) and have a strong potential for the control of *X. fastidiosa* (Kuzina et al. 2006). Chimeras of naturally occurring or de novo synthetized peptide sequences have been obtained by structure-function approaches to improve their properties like antimicrobial activity, toxicity, protease susceptibility, and stability (Montesinos et al. 2012). Members of a CECMEL11 library of linear undecapeptides departing from cecropin A and melittin (Ferré et al. 2006) (e.g., peptide BP100) are active, both in vitro (lytic activity) and in planta at low micromolar concentrations, against several bacterial plant pathogens, like *Erwinia amylovora*, *Pseudomonas syringae* pathovars, *Xanthomonas-vesicatoria*, *Xanthomonas arboricola* pv. *pruni*, *Xanthomonas fragariae*, and phytoplasms (Badosa et al. 2007; Rufo et al. 2017). Based on these interesting properties, several derivatives of BP100 have been developed to find new biological properties (arylhistidines, peptidotriazoles, and carbopeptides) (Güell et al. 2011, 2012; Ng-Choi et al. 2014), or for plant expression (Badosa et al. 2013). Another library, the CYCLO10, consisting of de novo designed cyclo-decapeptides was developed (Monroc et al. 2006), and peptide BPC194 and several histidine and lipopeptide derivatives showed interesting bactericidal properties (Vilà et al. 2016).

Studies on inhibition of *X. fastidiosa* by antimicrobial peptides have received little attention in the past, probably due to the difficulties of working with this fastidious bacterium. Though, a few reports have provided the minimal inhibitory concentration (MIC) of growth for cecropin/magainin derivatives (Li and Gray 2003), cecropins A/B (Andersen et al. 2004), gomosin (Fogaça et al. 2010), indolicidin and dermaseptin derivatives (Kuzina et al. 2006), and radicinin (Aldrich et al. 2015). However, it was not determined if the effect observed was simply bacteriostatic or bactericidal. To assess the bactericidal activity, a "killing assay" (contact test) has to be performed (Lambert 2004), as it is done with several bacterial pathogens of clinical, food, and water interest (Abdelbaqi et al. 2016; Pini et al. 2005). In addition, there is a need to analyze dose-response relationships and to know if there are differences in susceptibility to peptides among strains of *X. fastidiosa.*

The aims of the present work were (i) to screen lead peptides from CECMEL11 and CYCLO10 libraries for their bactericidal activity against *X. fastidiosa*, (ii) to relate peptide activity with cell damage at ultrastructural level, (iii) to study the kinetics of cell survival and dose-effect relationships, and (iv) to analyze the effect of the most active peptide, BP178, on the viability and culturability of different *X. fastidiosa* strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Several strains of *X. fastidiosa* were used (Table 1) depending on the experiment. All bacteria were stored in Pierce disease (PD2) (Davis et al. 1980) broth supplemented with glycerol (30%) and maintained at −80°C. When required, strains were cultured in buffered charcoal yeast extract (Coletta-Filho and Machado 2003) and colonies scrapped from the agar surface after growing for 5 to 7 days at 28°C. The cell material was suspended in sterile succinate citrate phosphate (SCP) buffer (EPPO 2018) to obtain a suspension of an optical density (OD) at 600 nm (Spectro Direct, Tintometer GmbH, Lovibond Water Testing, Germany) of 0.3 to 0.35 (depending on *X. fastidiosa* strain), corresponding to 10^8 CFU/ml. Previously, standard curves relating OD to cell concentration were obtained for all strains.

Peptides. Three linear peptides from the CECMEL11 library and one cyclic peptide from the CYCLO10 library were used. Peptides BP100 and BP15 were linear undecapeptides consisting of cecropin A (amino acids from 1 to 7) and melittin (amino acids from 2 to 9) hybrids (Ferré et al. 2006). Peptide BP178 is a derivative from BP100 (Badosa et al. 2013). All of them were synthesized in the Laboratory of Organic Chemistry from the University of Girona (LIPPSO) by means of solid-phase synthesis methods. Cyclic peptide BPC194 (Monroc et al. 2006) was purchased from Caslo Laboratory ApS (Lyngby, Denmark). Lyophilized peptides were solubilized in sterile Milli-Q water to a stock concentration of 2 mM and filter sterilized through a 0.22-µm-pore-size filter. Dilutions of the stock solution were made to obtain the desired final concentrations. Characteristics of the peptides are shown in Table 2.

General conditions for the contact test. The activity of the antimicrobial peptides was tested by a contact exposure test (Russell et al. 1982). Twenty microliters of the corresponding peptide dilution was mixed in a multiwell plate with 180 µl of a *X. fastidiosa* suspension (final cell concentration of 10^6, 10^7, or 10^8 CFU/ml, depending on the assay) to a total volume of 200 µl. In all experiments, three replicates for each concentration, peptide, and strain were used, and a nontreated control was also included. Multiwell plates were incubated at 25°C and samples were taken at the desired contact time. Depending on the experiment, bactericidal activity was evaluated through quantification of (i) culturable cells by plate counting and/or (ii) viable cells by viable quantitative PCR (v-qPCR) (described below). Plate counting was performed by plating 20 µl of 10-fold dilutions on PD2 agar plate, and colony forming units were determined after the incubation of Petri plates at 28°C for 7 to 21 days. To quantify viable cells, 200 µl of each bacterial suspension was treated with PEMAX (GenIUL, Terrassa, Spain) at 7.5 µM and after DNA extraction (GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, U.S.A.), DNA samples were analyzed in duplicate by a TaqMan-based qPCR assay using the target-specific XF16S-3 primer pair (reverse GTAGGA GTCTGGACCGTGTCTC, forward CGGCAGCACGTTGGTAGTAA, probe FAM/-CATGGGTGGCGAGTGGC-/TAMRA).

Standard curves were made by preparing 10-fold serial dilutions of a homogeneous cell suspension of 10^8 CFU/ml, until 10^2 CFU/ml, obtained by scraping the surface of an actively growing Petri plate culture. Then, culturable cells, assessed by plate counting, and C_T values, determined by v-qPCR as described previously, were used to calculate a calibration curve. The amount of viable cells, expressed as $\log_{10}$ CFU/ml, was obtained by interpolating the C_T values from each sample against the respective standard curve for each strain.

Bactericidal activity of lead peptides. The bactericidal activity of four lead peptides (BPC194, BP15, BP100, and BP178) was tested against cell suspensions of two strains of *X. fastidiosa* subsp. *fastidiosa* (Temecula and LMG 17159) and one strain of *X. fastidiosa* subsp. *pauca* (DD1), adjusted to 10^7 CFU/ml. A 3 h contact test was performed at final peptide concentrations of 1, 10, and 50 µM. Culturable cells were quantified by plate counting as described previously.

Effect of the diluent solution or sap on the activity of peptide BP178. The effect of SCP buffer, phosphate buffered saline (PBS) (EPPO 2018), and xylem sap (undiluted and half diluted in distilled water) on the antibacterial activity of BP178 was studied. SCP buffer was composed of 1.0 g of trisodium citrate, 1.0 g of disodium succinate, 1.5 g of dipotassium phosphate, and 1.0 g of potassium phosphate per 1.0 liter of distilled water. PBS was

TABLE 1. Strains of *Xylella fastidiosa* used in the present work

Strain	Subspecies	Source[a]	Origin	Reference
DD1 (De Donno)	*X. fastidiosa* subsp. *pauca*	CNR-Bari	Olive, Apulia (Italy)	Saponari et al. 2017
Temecula (ATCC 700964)	*X. fastidiosa* subsp. *fastidiosa*	ATCC	Grapevine, Riverside, California (U.S.A.)	Rodrigues et al. 2003
IVIA 5235.4	*X. fastidiosa* subsp. *fastidiosa*	IVIA	Cherry, Mallorca (Spain)	Olmo et al. 2017
IVIA 5387.2	*X. fastidiosa* subsp. *fastidiosa*	IVIA	Almond, Mallorca (Spain)	E. Marco (*personal communication*)
IVIA 5901.2	*X. fastidiosa* subsp. *multiplex*	IVIA	Almond, Alicante (Spain)	Giampetruzzi et al. 2019
CFBP 8430	*X. fastidiosa* subsp. *multiplex*	CFBP	NA[b]	EPPO 2018
CFBP 8173	*X. fastidiosa* subsp. *multiplex*	CFBP	*Prunus*, Georgia (U.S.A.)	Schaad et al. 2004
LMG 17159	*X. fastidiosa* subsp. *fastidiosa*	LMG	Grapevine, Florida (U.S.A.)	NA[b]

[a] CNR-Bari, Consiglio Nazionale delle Ricerche; ATCC, American Type Culture Collection; IVIA, Instituto Valenciano de Investigaciones Agrarias; CFBP, International Center for Bacterial Resources, INRA; and LMG, Laboratory of Microbiology from Ghent University.
[b] Not accounted.

TABLE 2. Characteristics of the antimicrobial peptides used in this work, and reported activity against representative plant pathogenic bacteria

Peptide	Sequence	MIC (µM)[a]			Reference
		Xav	*Pss*	*Ea*	
BP15	KKLFKKILKVL-NH_2	12.0–15.0	2.0–5.0	5.0–7.0	Ferré et al. 2006
BP100	H-KKLFKKILKYL-NH_2	5.0–7.5	2.5–5.0	2.5–5.0	Badosa et al. 2007
BP178	KKLFKKILKYL-AGPA-GIGKFLHSAK-KDEL-OH	2.5–5.0	2.5–5.0	2.5–5.0	Badosa et al. 2013
BPC194	c(KKLKKFKKLQ)	3.1–6.2	3.1–6.2	6.2–12.5	Monroc et al. 2006

[a] Minimal inhibitory concentration. *Xav*, *Xanthomonas arboricola* pv. *vesicatoria*; *Pss*, *Pseudomonas syringae* pv. *syringae*; *Ea*, *Erwinia amylovora*.

composed of 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.15 g of disodium phosphate, 0.2 g of potassium phosphate per 1.0 liter of distilled water. The above buffers were sterilized with autoclave. Xylem sap was obtained from grapevine cultivar Carinyena by pruning six plants before bud break and collecting sap bleeding directly at the cutting edge of the shoots. Sap was sterilized by filtration and preserved at −80°C until use. A cell suspension of *X. fastidiosa* subsp. *fastidiosa* Temecula adjusted to 10^7 CFU/ml was prepared, and 180 µl was centrifuged for 10 min at 13,000 rpm to collect all the cells. The pelleted cells were resuspended with the same volume of the appropriate solution (SCP, PBS, sap, or diluted sap), and 20 µl of the peptide dilutions was added to a final volume of 200 µl. A 3 h contact test was performed at final peptide concentrations of 1.5, 3.1, 12.5, 50, 100, and 150 µM. After the incubation period, viable cells were quantified by v-qPCR, and the proportion of viable cells reduction (N_0/N_t) was used as a measure of the peptide activity. N_0 and N_t were the initial and final concentration of viable cells, respectively. The experiment was performed two times.

Death kinetics and dose-response relationships. The effect of BP178 on the survival (culturable cells) of *X. fastidiosa* subsp. *fastidiosa* Temecula, *X. fastidiosa* subsp. m*ultiplex* IVIA 5901.2, and *X. fastidiosa* subsp. *pauca* DD1 was tested over time. Peptide concentrations tested were 5, 10, 25, and 50 µM. The initial cell concentration was 10^7 CFU/ml, and sampling was performed at different times depending on the experiment (from 0 to 180 min). Plate counting was performed as described above and the proportion of survivors reduction (N_0/N_t) was used as a measure of the activity of peptide. The experiment was performed two times, one for the kinetics and another for the effect of the concentration at 120 or 180 min contact time.

Alteration of cell membrane integrity by the antimicrobial peptides. The cell envelope integrity of *X. fastidiosa* subsp. *fastidiosa* Temecula and *X. fastidiosa* subsp. *pauca* DD1 after peptide treatment was observed by transmission electron microscopes (TEM), using samples taken from the previously described experiments. After 3 h of contact exposure to BP178 at different concentrations (i.e., 5, 20, and 100 µM), cells were harvested by centrifugation at 10,000 × *g* for 10 min. For preservation of the structure, cells were fixed with glutaraldehyde (2.5%) in sodium cacodylate buffer (0.1 M, pH 7.4) for 4 h at 4°C. Fixed pellets were washed in sodium cacodylate buffer (0.1 M, pH 7.4) two times during 30 min at 4°C, and stored at the same temperature until postfixing. Postfixation was performed in 1% OsO_4 osmium tetraoxide in sodium cacodylate buffer (0.1 M, pH 7.4) for 3 h at room temperature. Then, the samples were washed again in sodium cacodylate buffer and dehydrated in ethanol series. The inclusion step in epoxy resin was performed at room temperature using a Noria shaker. Then, ultra-thin sections of 60 to 80 nm were obtained. Contrast of sections was performed with uranium acetate 2% and then lead citrate (Reynolds 1963). The samples were observed either with a Zeiss EM-910 or with a JEOL JEEM1400 transmission electron microscope. TEM micrographs were taken and used to determine the frequency of live, affected, and lysed cells for the nontreated control and for cells exposed to 20 µM BP178. Live cells were considered when the ultrastructure did not show signs of damage as in most cells of the nontreated control; affected cells showed cytoplasm retraction or intracellular condensation bodies; and lysed cells showed envelope anomalies (e.g., membrane pores and outer membrane vesicles) or free cell membrane material.

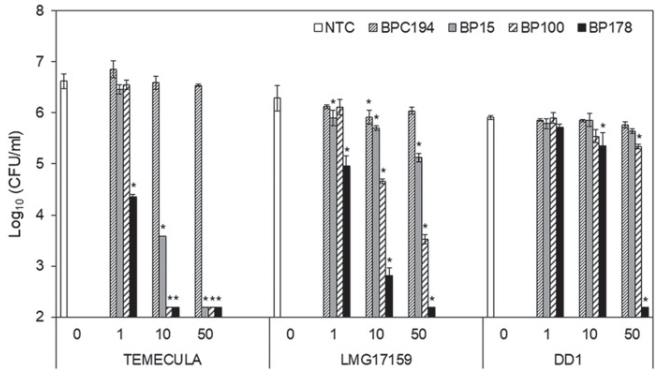

Fig. 1. Effect of peptides BPC194, BP15, BP100, and BP178 on survival (culturable cells) of *Xylella fastidiosa* subsp. *fastidiosa* strains Temecula and LMG 17159, and *X. fastidiosa* subsp. *pauca* DD1. A contact test was performed at 0 (NTC, nontreated control), 1, 10, or 50 µM peptide concentrations. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Asterisks indicate treatments significantly different from NTC within each strain according to Tukey's test ($P < 0.05$).

Effect of BP178 on viable and culturable cells. Four contact test experiments with BP178 were performed as described above against seven *X. fastidiosa* strains (Table 1). The experiments were performed by combining cell concentration at 10^7 or 10^8 CFU/ml with peptide concentration at 10 or 25 µM. After 3 h incubation, culturable (plate counting) and viable (v-qPCR) cells were quantified as described above, and the percentage of survival ($100 \times N_t/N_0$) was used as a measure of the strain susceptibility to the peptide.

Data analysis. Shapiro-Wilk and Leven's tests were performed to assess data normality and homoscedasticity. To test the significance of the effect of strain, peptide and peptide concentration in the experiments, two-way analysis of variance was performed. Means of $\log_{10}$ survivors, reduction in survivors, or percentage of survival were separated according to the Tukey's test at a *P* value of ≤0.05.

RESULTS

Bactericidal activity of peptides. The cyclic decapeptide BPC194, the CECMEL11 peptides BP15 and BP100, and the BP100 derivative peptide, BP178, were assessed for their bactericidal activity against three strains of *X. fastidiosa*, at three peptide concentrations (Fig. 1). Survival was significantly affected by the type and dose of peptide. However, the effect of the peptide was dependent on the strain ($P < 0.001$). The Temecula strain significantly decreased culturability by peptides BP178, BP100, and BP15 at 10 and 50 µM, followed by LMG 17159 which also undergo a reduction of culturable cells after the contact test with these three peptides. The DD1 strain was susceptible to peptide BP178 at 10 and 50 µM, but only to BP100 at 50 µM. Globally, the most active peptide was BP178, followed by BP100 and BP15. No significant activity was observed for peptide BPC194.

Effect of the diluent solution or sap on the activity of BP178. The effect of different buffer solutions or plant sap on the activity of BP178 against *X. fastidiosa* subsp. *fastidiosa* Temecula is shown in Figure 2. The diluent had a significant effect on the reduction of viable cells after exposure to peptide BP178 at all concentrations assessed ($P < 0.001$). BP178 had higher activity when cells were suspended in buffer solutions than in sap. At concentrations above 50 µM, the reduction in viable cells was of 1.5 to 3 log when using buffer solutions, and of 0.3 to 1 log when cells were suspended in sap (diluted or undiluted). SCP buffer allowed the highest activity of the BP178 peptide.

Lytic effects of peptide BP178 on *X. fastidiosa*. BP178 peptide had clearly lytic effects on Temecula and DD1 strains as it is shown by TEM micrographs (Figs. 3 and 4). The untreated control cells showed the typical cristate ultrastructure of a well-organized cell envelope, containing the outer membrane (OM) and cytoplasmic membrane (Figs. 3A and B and 4B). In the case of Temecula strain, peptide treatment at 5 and 20 µM already resulted in strong cell damage (Fig. 3C and E) with cell lysis and abundant production of typical outer membrane vesicles (OMV) observed as spheroid particles ranging in size from approximately 20 to 250 nm, produced through the blebbing and pinching off of portions of the OM

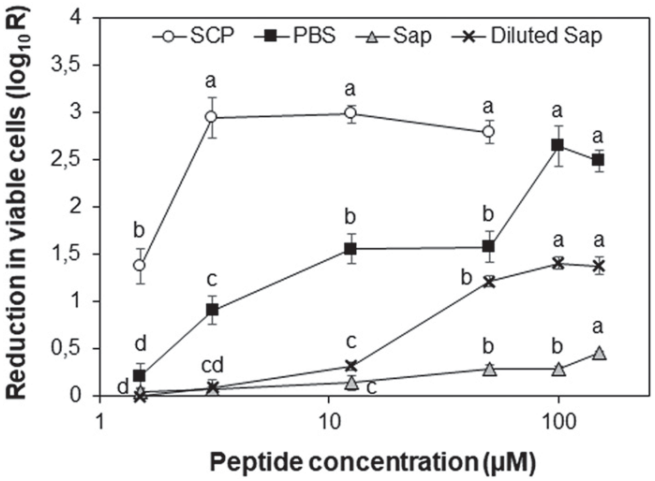

Fig. 2. Effect of the diluent solution on the activity of BP178 against *Xylella fastidiosa* subsp. *fastidiosa* Temecula. Different peptide concentrations and diluent solutions were evaluated. SCP, succinate citrate phosphate buffer; PBS, phosphate buffered saline; Sap, xylem sap from *Vitis* sp.; and Diluted sap, half diluted sap. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Different letters within the same diluent indicate significant differences between peptide concentrations according to Tukey's test ($P < 0.05$).

(Fig. 4E). Peptide treatment also resulted in pore formation in cell membranes (Fig. 4C and D). Moreover, condensation of intracellular material and presence of free cell membranes were observed (Fig. 4F). On the contrary, DD1 strain showed lower cell lysis than Temecula strain even at 20 or 100 µM (Fig. 3D and F). Despite this, cells showed frequently intracellular material condensation at 100 µM (Fig. 3F). Untreated controls of DD1 showed the presence of an exopolysaccharide barrier around the cells, observed as a lighter halo, which was not detected in the case of Temecula cells (Fig. 4A). Based on their ultrastructure, cells from both strains were classified as live (normal structure), affected (cytoplasm retraction or presence of intracellular condensation bodies), or lysed cells (empty or strongly disorganized with membrane pores or OMVs). Temecula and DD1 strains significantly differed in their susceptibility to the lytic effect of peptide BP178 ($P < 0.001$). As shown in Figure 5, BP178 at 20 µM had a strong and significant effect in decreasing live cells and increasing the frequency of affected and lysed cells in Temecula strain. However, in DD1 strain there was a slight but significant effect in increasing the frequency of affected cells and no significant effect was observed in the frequency of live and lysed cells.

Time-course of cell survival upon peptide treatment. The time-course of cells survival was studied as culturable cells at four BP178 peptide concentrations (Fig. 6). The death kinetics showed two stages, a fast first stage (10 to 20 min) followed by a second

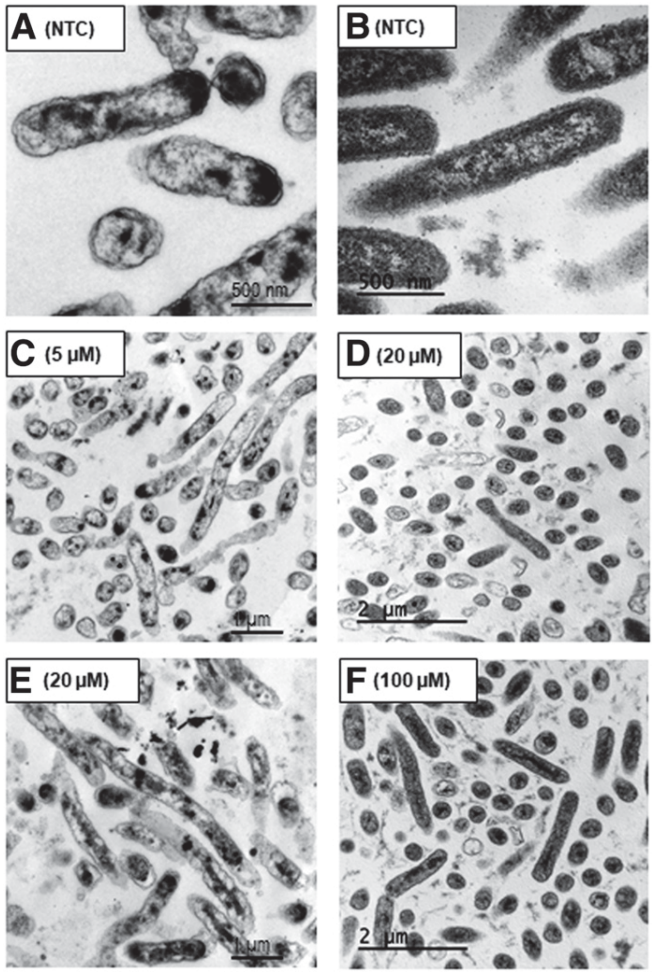

Fig. 3. Ultrastructure of *Xylella fastidiosa* subsp. *fastidiosa* Temecula and *X. fastidiosa* subsp. *pauca* DD1 after exposure to peptide BP178 at different concentrations. **A, C, and E,** Temecula strain; and **B, D, and F,** DD1 strain. Peptide concentrations are shown in brackets.

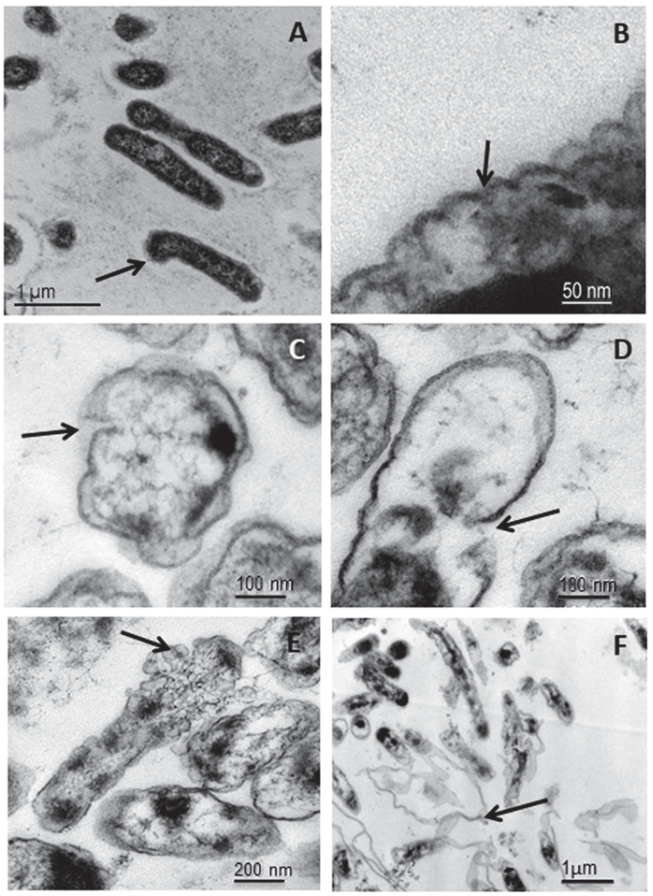

Fig. 4. Relevant structures of *Xylella fastidiosa* observed with transmission electron microscopes (indicated with arrows). **A,** Presence of an exomucopolysaccharide envelope in strain DD1; **B,** cristate structure of the cell wall; **C and D,** initial stages of pore formation in the cell membranes by peptide BP178; **E,** burst of cell membranes with abundant outer membrane vesicles in Temecula strain; and **F,** rest of cell membranes and presence of condensed intracellular material after BP178 treatment at high concentrations.

stage consisting of a plateau of maximal survival reduction. Reduction in survivors was significantly affected by the peptide concentration in Temecula ($P < 0.001$) and IVIA 5901.2 ($P < 0.001$); in the case of DD1 strain, this effect was lower although significant ($P < 0.001$). Low peptide concentrations (5 and 10 μM), reduced 2 log survival in Temecula strain, only 1 log in IVIA 5901.2, but much less in DD1 strain. High peptide concentrations (25 and 50 μM), reduced 4 to 6 log survival in Temecula strain, 2 to 4 log in IVIA 5901.2 and less than 1 log in DD1.

Dose-effect relationships. The relationship between the reduction in survivors as culturable cells, at several peptide concentrations, is shown in Figure 7. There were significant differences in this parameter between strains ($P < 0.001$), with Temecula strain showing the highest reduction, slightly followed by IVIA 5901.2. *X. fastidiosa* subsp. *pauca* strain DD1 was the least susceptible among the three strains studied. Reduction in survivors showed a direct relationship to peptide concentration in all strains, but a hyperbolic pattern (typical saturation kinetics) was observed. No significant increase of bactericidal effect was evidenced at peptide concentrations higher than 25 μM. In addition, cells survival (culturable cells) at different peptide and initial cell concentrations obtained from several contact test experiments (data from Figs. 1, 6, and 7) were used to calculate the ratio between the number of molecules of BP178 per cell. The plot of the reduction in survivors against the ratio is shown in Figure 8. Interestingly, a minimal threshold ratio to have a bactericidal effect was observed at 9 $\log_{10}$ number of molecules of BP178 per cell.

Effect of BP178 treatment on viability and culturability of cells. The effect of the treatment with peptide BP178 on cell viability (v-qPCR) and culturability (plate counting) was studied in seven strains of *X. fastidiosa*. The experiment was repeated but, at two different peptide and cell concentrations (Fig. 9). It was observed that there were significant differences in survival (culturable and viable cells) among strains ($P < 0.001$). For the strains of the subspecies *fastidiosa* (Temecula, IVIA 5235.4, and IVIA 5387.2) and *multiplex* (IVIA 5901.2, CFBP 8173, and CFBP 8430) there was no particular pattern in the effect of peptide BP178 on survival associated to the subspecies. The most susceptible strain was CFBP 8173 with a reduction in survival to $\log_{10}$ of −3 (log reduction in survivors of 5), and the least susceptible strain was DD1 with $\log_{10}$ of 0 (log reduction in survivors of 2) at 25 μM of peptide and 10^7 CFU/ml (Fig. 9). This pattern was observed in the four experiments performed.

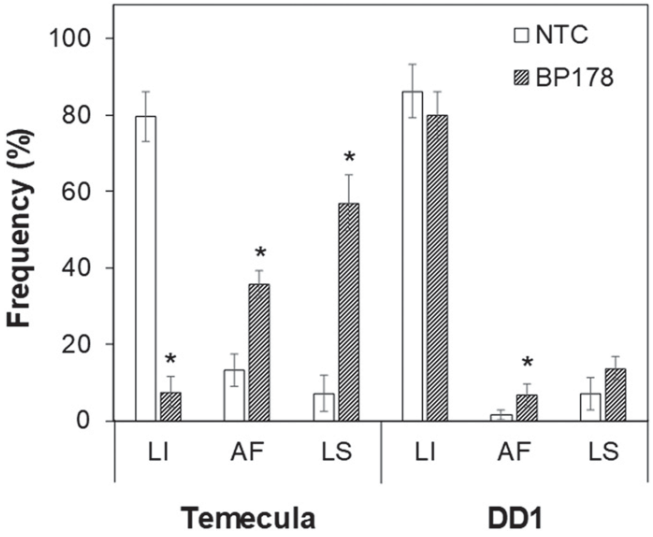

Fig. 5. Effect of BP178 peptide on the frequency of live, affected, and lysed cells in Temecula and DD1 strains compared with a nontreated control (NTC). Data correspond to transmission electron microscope pictures taken in the same experiments of Figures 3 and 4. Peptide concentration was 20 μM. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Asterisks indicate significant differences between BP178 treatment and NTC according to Tukey's test ($P < 0.05$).

By comparing v-qPCR and plate counting for each strain, it was observed that after the peptide treatment, as expected, more viable than culturable cells were observed in all strains ($P < 0.001$). This difference can be attributed to the development of a viable but nonculturable state (VBNC). The strains response to BP178 in terms of VBNC levels was very different. For example, in DD1 practically all viable cells were culturable at the two cell and peptide concentrations studied. However, in other strains most viable cells were nonculturable (IVIA 5235.4 at 10^7 CFU/ml and 10 and 25 μM of BP178; IVIA 5901.2 at 10^7 CFU/ml and 25 μM of BP178).

DISCUSSION

In the present study we used a contact test ("killing assay") for the assessment of the kinetics of viable/culturable cells during

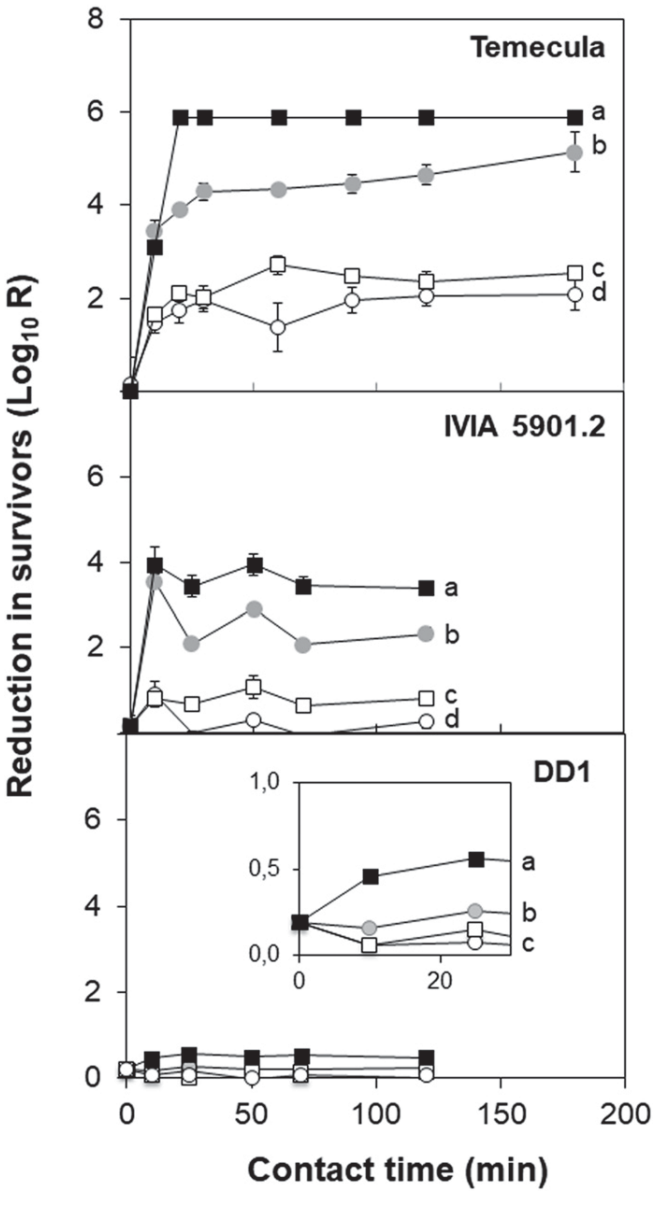

Fig. 6. Kinetics of survival of *Xylella fastidiosa* subsp. *fastidiosa* Temecula, *X. fastidiosa* subsp. *multiplex* IVIA 5901.2, and *X. fastidiosa* subsp. *pauca* DD1 in the presence of peptide BP178 at different concentrations. Concentrations of peptide were 5 μM (white circle), 10 μM (white square), 25 μM (gray circle), and 50 μM (black square). Values are the means of three replicates, and error bars represent the standard deviation of the mean. Different letters within the same strain panel indicate significant differences between peptide concentrations in survival at the end time points, according to Tukey's test ($P < 0.05$).

the exposure to synthetic antimicrobial peptides, and we evaluated the bactericidal effect of peptides on different *X. fastidiosa* strains. Most studies on the activity of antimicrobials against *X. fastidiosa* used growth inhibition assays to determine the MIC (Aldrich et al. 2015; Andersen et al. 2004; Fogaça et al. 2010; Kuzina et al. 2006; Li and Gray 2003). However, in growth inhibition assays, the antimicrobial agent is continuously in contact with the microorganism cells and the inhibitory activity cannot be distinguished between biostatic and biocidal effects (Lambert 2004).

The strongest bactericidal activity among the tested CECMEL11 and CYCLO10 leads or derivative peptides was observed in BP178. Peptide BP178 was originally designed as a derivative from BP100 for plant expression and it has been successfully produced in rice seed endosperm (Montesinos et al. 2017). Its sequence included an N-terminal ER retention signal (KDEL) and a hinge (AGPA) to separate BP100 from a sequence of magainin (GIGKFLHSAK) (Badosa et al. 2013).

BP178 is more active in SCP than in PBS and grapevine xylem sap. As in other amphipathic peptides, its antimicrobial activity is affected by the net charge, and the pH and salts composition of the diluent (Nguyen et al. 2011). In particular, salts decrease activity while low pH moderately increase it, as in cecropin P1 and magainin 1 (Kacprzyk et al. 2007; Schwab et al. 1999), but this effect depends on the target microorganism (Andersen et al. 2004; Lee et al. 1997). Total salts contents in SCP buffer is lower (4.5 g/liter) than in PBS buffer (9.5 g/liter), and this is in agreement with the above mentioned report. Unfortunately, we did not measure salts contents in the grapevine xylem sap used for the experiments, but the fact that upon dilution peptide activity have increased indicates that the inhibitory effect is probably due to its solutes concentration. The total solute contents in xylem sap of most plants range from 0.5 to 20 g/liter and the main components in grapevine have been reported to be inorganic ions, amino acids (mainly glutamine) and organic acids (Andersen and Brodbeck 1989; Lima et al. 2017). Because xylem sap composition strongly changes with plant species, phenological stage, and environmental conditions (Tyree and Zimmermann 2002), the activity of peptide BP178 against *X. fastidiosa* in plant hosts may have limitations under certain conditions, but could be favored in others.

The bactericidal activity of peptide BP178 on *X. fastidiosa* was due to lytic effects. The mechanism of action of amphipathic cationic peptides, like CECMEL11 (e.g., BP100 and BP178), is based on its interaction with lipid components of the cell membranes, leading to membrane permeabilization or disorganization with the consequence of cell lysis (Nguyen et al. 2011). We have shown that cells of *X. fastidiosa* subsp. *fastidiosa* Temecula and *X. fastidiosa* subsp. *pauca* strain DD1, after contact with the peptide, showed strong damage in the cell envelope as was observed by TEM ultrastructural analysis. This effect was observed in the Temecula strain at low peptide concentration, affecting both outer and inner membranes, and at 20 µM cells were completely lysed releasing extracellular materials and showing OMVs. OMVs have been reported in practically all gram-negative bacteria (Jan 2017; Solé et al. 2015), including *X. fastidiosa* (Ionescu et al. 2014), in many cases as a response to envelope stress (McBroom and Kuehn 2007). Cell lysis was also observed in *X. fastidiosa* subsp. *pauca* strain DD1 but at higher peptide concentration (100 µM) and to a lesser extend compared with the Temecula strain. The same cristate structure of a well-organized cell envelope was observed in DD1, but in addition, a capsule of exopolysaccharides around DD1 cells was shown. This is in agreement with the reports of exopolysaccharide secretion (fastidian gum) in other *X. fastidiosa* strains (Da Silva et al. 2001; Roper et al. 2007). Resistance to antimicrobial peptides in human pathogens has been associated to exomucopolysaccharide capsules (Joo et al. 2016). Thus, the presence of the extracellular matrix in DD1 could explain its greater tolerance to peptides compared with the other strains studied in the present work.

The maximum reduction in survivors under the effect of lytic peptides was observed at 10 to 20 min at all concentrations tested, which implicates a rapid lytic action of these type of peptides on *X. fastidiosa*. However, a plateau on reduction in survivors was also observed in all cases. The plateau was dependent on the peptide concentration and this is similar to what has been reported for several disinfectants against human pathogens (Ioannou et al. 2007; Lambert et al. 1999). This is because the antimicrobial compound binds to target cells and therefore is being consumed or degraded by

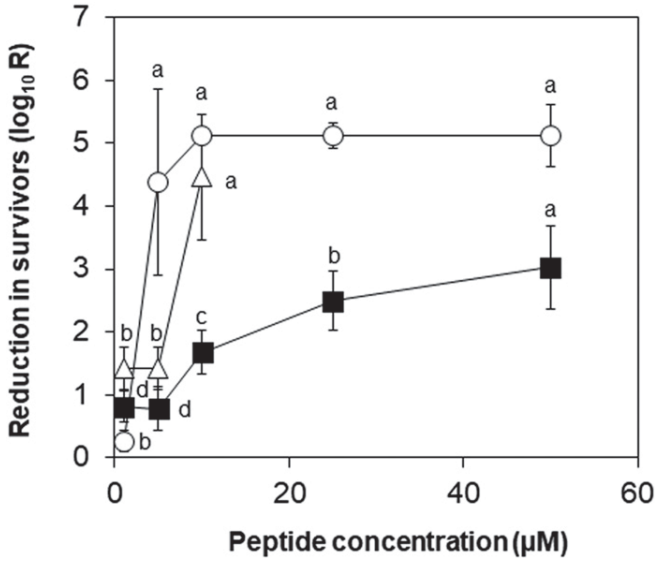

Fig. 7. Effect of the concentration of peptide BP178 on survival of *Xylella fastidiosa* subsp. *pauca* DD1 (black square), *X. fastidiosa* subsp. *fastidiosa* Temecula (white circle), and *X. fastidiosa* subsp. *multiplex* IVIA 5901.2 (white triangle) at an initial cell concentration of 10^7 CFU/ml. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Different letters within the same strain indicate significant differences between peptide concentrations according to Tukey's test ($P < 0.05$).

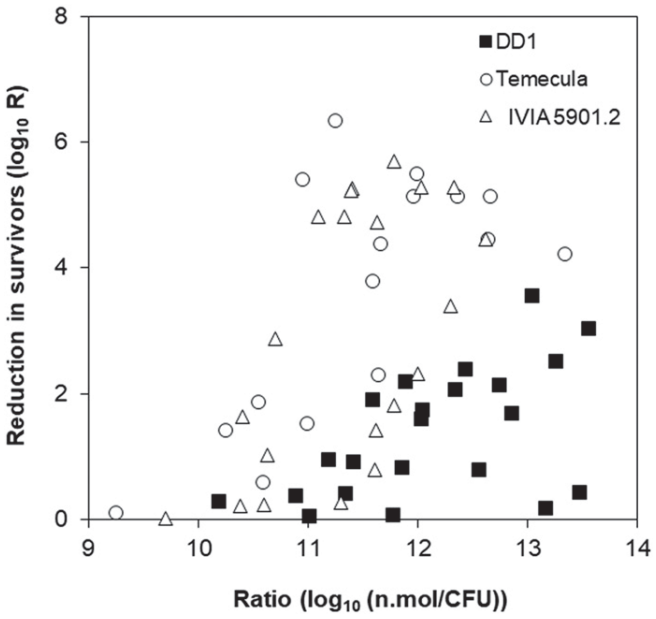

Fig. 8. Scatter plot of the reduction in survivors with the ratio of the number of molecules of peptide BP178 per cell in *Xylella fastidiosa* subsp. *pauca* DD1, *X. fastidiosa* subsp. *fastidiosa* Temecula, and *X. fastidiosa* subsp. *multiplex* IVIA 5901.2. Data combined from experiments shown in Figures 1, 6, and 7. Reduction in survivors was calculated at the end time points during the contact tests, performed at different cell and peptide concentrations.

the bacterial cells during the interaction. Lambert and Johnston (2000) have considered this effect in their intrinsic quenching model of bacterial death kinetics in the presence of a disinfectant. In our study, we observed that the reduction in survivors decreased when cell concentration increased, and this fact agreed with the assumption of the abovementioned authors due to a cell quenching of the peptide.

A threshold of bactericidal action for BP178 peptide against *X. fastidiosa*, in terms of molecules per cell, is reported in the present study. We have found for the three strains of *X. fastidiosa* a threshold ratio BP178/cell of 10^9 in the killing assay. Using growth inhibition assays, the minimum inhibitory concentration of BP178 against *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae*, and *Xanthomonas axonopodis* pv. *vesicatoria* was in the range of 2.5 to 5 µM (Badosa et al. 2013). Considering these results, the threshold ratio for growth inhibition of these plant pathogenic bacteria can be estimated as 10^7 molecules per cell. Although information provided by growth inhibition and killing assays is different, *X. fastidiosa* seems to be less susceptible to peptide BP178 than other plant pathogenic bacteria. Additionally, for synthetic vesicles mimicking bacterial cell membranes exposed to the peptide BP100 (a CECMEL11 peptide), it has been reported that 1.1×10^4 BP100/vesicle were needed for leakage of their contents (Manzini et al. 2014). Taking into account an average radius of the vesicles of 50 nm, this effective dose corresponds to a ratio of 0.3×10^7 BP100/µm^2. From the TEM observation of cells size of *X. fastidiosa*, the average surface of a cell can be calculated as 45 µm^2 (data not shown). Then, a threshold action for BP178 in *X. fastidiosa* is around 2.2×10^7 BP178/µm^2, which is seven times the value reported for synthetic vesicles. Therefore, there is higher threshold action ratio of this lytic peptide in *X. fastidiosa* than in other bacteria or in synthetic membrane vesicles. The existence of a bactericidal threshold action for BP178 has implications in disease management. Under high inoculum of *X. fastidiosa*, a given dose of peptide for plant treatment could be noneffective if the ratio peptide to cells is below the threshold. For this reason, it is important to know this threshold action and adjust the treatment dose to the inoculum of the pathogen. However, this is not only an issue with the peptide but it also happens with other antimicrobials.

In addition, we reported a differential susceptibility to the BP178 peptide among a set of seven strains of *X. fastidiosa* being the subsp. *pauca* DD1 the most tolerant, and subsp. *multiplex* CFBP 8173 the most susceptible. It can be speculated that these difference could be due to changes in the cell envelope structure and composition. In fact, the species *X. fastidiosa* presents a large flexible gene pool that is described to be strain-specific, resulting in genomes of variable

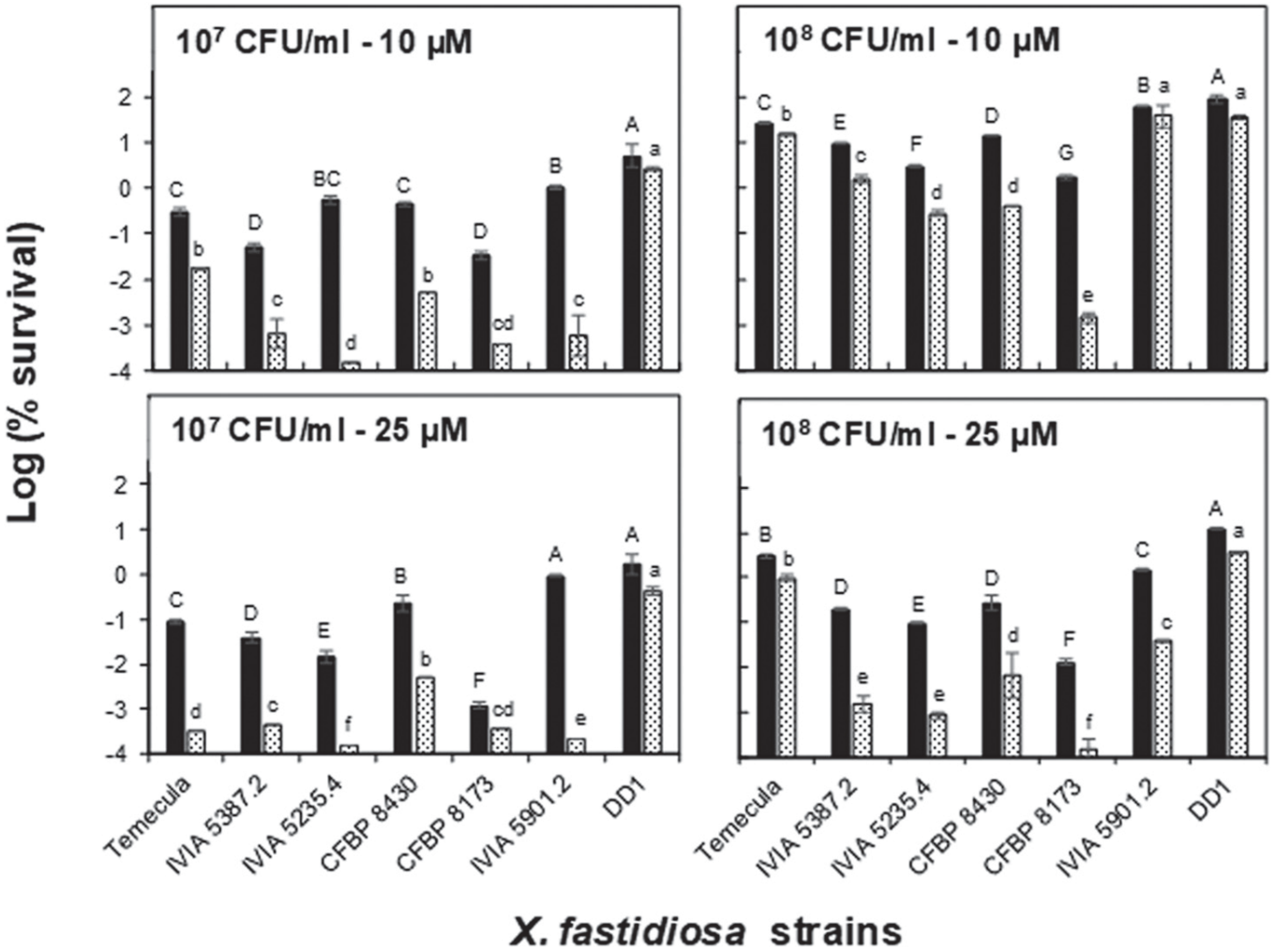

Fig. 9. Effect of peptide BP178 on survival of seven strains of three subspecies of *Xylella fastidiosa*. Survival was measured as viable (v-qPCR, black columns) and culturable cells (plate counting, dotted columns). The experiments correspond to low (10^7 CFU/ml) and high (10^8 CFU/ml) cell concentration suspensions that were exposed to 10 or 25 µM peptide. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Capital letters correspond to comparison of viable cells survival between strains. Lowercase letters correspond to comparison of culturable cells survival between strains. Comparisons within each panel were performed according to Tukey's test ($P < 0.05$).

sizes between strains and containing specific gene sets. Most of this genomic diversity is related with environmental response factors, such as drug-resistance enzymes or transcriptional factors, but also to variations associated with structural compounds of the cell wall and the outer membrane (Nunes et al. 2003). Particularly, a recent genomic analysis has revealed that the subspecies *pauca* have 175 GO terms overrepresented compared with the other subspecies, and 20 of them have been associated with the bacterial envelope, cell wall and plasma membrane (Denancé et al. 2019). Also, different levels of OMV production have been observed (Feitosa-Junior et al. 2019), which can be another key factor to explain the different susceptibility of the strains to the peptides. Further experiments with BP178 are needed to know in more detail additional mechanisms of action (e.g., possible intracellular targets) and detoxification pathways (protease degradation, sequestering by capsular structures, and detoxification with OMVs) in *X. fastidiosa*, to develop and improve strategies for the use of this peptide in disease management.

During the killing assay after the exposure to BP178, the amount of viable cells (v-qPCR) was higher than of culturable cells in all strains of *X. fastidiosa* studied. The difference can be attributed to the induction of VBNC. Our finding with peptide BP178 is in agreement with the report that *X. fastidiosa* subsp. *fastidiosa* Temecula enters into a viable but nonculturable state under the exposure to Zn (Navarrete and De La Fuente 2014), and extends the ability to develop this stage to other strains of the subspecies *multiplex* and *pauca*. In the VBNC state, bacteria fail to grow and to produce colonies on agar media, although they remain alive (Oliver 2000). The VBNC state has been reported in other plant pathogenic bacteria like *Erwinia amylovora*, *Ralstonia solanacearum*, and *Xanthomonas axonopodis* pv. *citri* as a survival mechanism to stressful conditions (e.g., starvation and Cu compounds) (Del Campo et al. 2009; Grey and Steck 2001; Ordax et al. 2006). Interestingly, response of *X. fastidiosa* strains to treatment was very different in terms of VBNC levels, because in DD1 practically all viable cells were culturable. The difference could be attributed to the different performance and capacity to grow in the synthetic media that has been reported (Almeida and Purcell 2003).

Conclusions. The present study provides additional knowledge on the mechanisms of action of antimicrobials against *X. fastidiosa*, specifically of lytic synthetic antimicrobial peptides. Findings about death kinetics, dose-response relationships, and threshold action ratio, and the differential susceptibility among strains would have important consequences for managing the diseases in infected plant host strategies.

ACKNOWLEDGMENTS

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CHAPTER IV

Screening and identification of BP100 peptide conjugates active against *Xylella fastidiosa* using a viability-qPCR method

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Screening and identification of BP100 peptide conjugates active against *Xylella fastidiosa* using a viability-qPCR method

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Abstract

Background: *Xylella fastidiosa* is one of the most harmful bacterial plant pathogens worldwide, causing a variety of diseases, with huge economic impact to agriculture and environment. Although it has been extensively studied, there are no therapeutic solutions to suppress disease development in infected plants. In this context, antimicrobial peptides represent promising alternatives to traditional compounds due to their activity against a wide range of plant pathogens, their low cytotoxicity, their mode of action that make resistance more difficult and their availability for being expressed in plants.

Results: Peptide conjugates derived from the lead peptide **BP100** and fragments of cecropin, magainin or melittin were selected and tested against the plant pathogenic bacteria *X. fastidiosa*. In order to screen the activity of these antimicrobials, and due to the fastidious nature of the pathogen, a methodology consisting of a contact test coupled with the viability-quantitative PCR (v-qPCR) method was developed. The nucleic acid-binding dye PEMAX was used to selectively quantify viable cells by v-qPCR. In addition, the primer set XF16S-3 amplifying a 279 bp fragment was selected as the most suitable for v-qPCR. The performance of the method was assessed by comparing v-qPCR viable cells estimation with conventional qPCR and plate counting. When cells were treated with peptide conjugates derived from **BP100**, the observed differences between methods suggested that, in addition to cell death due to the lytic effect of the peptides, there was an induction of the viable but non-culturable state in cells. Notably, a contact test coupled to v-qPCR allowed fast and accurate screening of antimicrobial peptides, and led to the identification of new peptide conjugates active against *X. fastidiosa*.

Conclusions: Antimicrobial peptides active against *X. fastidiosa* have been identified using an optimized methodology that quantifies viable cells without a cultivation stage, avoiding underestimation or false negative detection of the pathogen due to the viable but non-culturable state, and overestimation of the viable population observed using qPCR. These findings provide new alternative compounds for being tested *in planta* for the control of *X. fastidiosa*, and a methodology that enables the fast screening of a large amount of antimicrobials against this plant pathogenic bacterium.

Keywords: Antimicrobial peptides, *Xylella fastidiosa*, Viability-qPCR, PEMAX, Plant pathogens

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Background

Xylella fastidiosa (Xf) is a xylem-limited Gram-negative bacterium transmitted by insect vectors that causes economically important plant diseases. Pierce's disease of grapevine and Citrus Variegated Chlorosis were the most important diseases caused by Xf worldwide for many years [1, 2]. However, Xf recently emerged as a potential threat to European agriculture [3]. The outbreak of Xf in 2013 in Apulia (Italy) in oleander, almond and olive trees [4], and the detections in Corsica and Provence Alpes-Côte d'Azur (France), Alicante and the Balearic Islands (Spain), Tuscany (Italy), and Vila Nova de Gaia (Portugal) [5, 6] constitute an important change to its geographical distribution and adds new host plants.

The measures adopted in Europe are eradication of the infected plants to reduce inoculum sources to prevent the spread of the bacterium, the use of insecticides to control the vector population, and the use of pathogen-free plant material. However, these methods have been only partially successful and different strategies are being explored in order to find alternatives to achieve the management of diseases caused by Xf [7]. Direct strategies to control disease in affected hosts, based on chemical compounds like antibiotics, copper compounds, or biofilm inhibitors, either applied by sprays, drench or endotherapy, have failed to cure infected trees [8]. Therefore, there is a need for new and safe compounds for Xf disease management. Among the new compounds, antimicrobial peptides (AMPs) could be considered good candidates because they display activity against a wide range of plant pathogens, exhibit low cytotoxicity and their mode of action make more difficult the development of resistance [9–12]. In particular, a few AMPs with bactericidal activity against Xf have been reported, including cecropin A and B, magainin I and II, Shiva-1, indolicidin, PGQ, dermaseptin and gomesin [13–15]. Most of these peptides cause disruption of the cytoplasmic membrane, but also some of them have been described to interact with intracellular targets causing the inhibition of key processes [16]. Within our search for new AMPs to control plant diseases, we reported peptide conjugates incorporating units of the lead peptide **BP100** and fragments of cecropin A, magainin II or melittin, which were specifically designed to be expressed in plants [17, 18]. In fact, the peptide conjugate **BP178** was successfully expressed in rice endosperm, showing resistance against some plant pathogens [19]. This demonstrates the availability of these peptides for being produced by the plant itself, which could overcome the difficulties in accessing the vascular location of Xf observed by other treatment strategies. This family of peptides exhibited high antibacterial activity in vitro against plant pathogenic bacteria such as *Xanthomonas axonopodis* pv. *vesicatoria*, *Pseudomonas syringae* pv. *syringae* and *Erwinia amylovora*, were low haemolytic, and were able to control infections in plant hosts caused by these bacteria or even due to phytoplasms [10, 17, 20]. In the case of Xf, only **BP178** has been tested in vitro, showing high antibacterial activity against a collection of Xf strains. Its lytic activity upon Xf cells was identified as the main mode of action, with pore formation and disorganization of the cell membrane [21]. Therefore, we envisaged that this biological activity profile makes peptide conjugates derived from **BP100** good candidates to be tested against Xf. Since any sequence modification may influence their antimicrobial activity against Xf, as well as their stability and toxicity, a wide range of peptides must be screened to obtain the best candidates to be tested in plants.

Currently, there is a need for rapid, reliable and efficient methods useful for the screening of antimicrobial compounds against Xf due to the difficulties of culture of most of the strains and their slow growth [22]. Conventional methods, such as disk-diffusion test, broth or agar dilution assays, as well as antimicrobial gradient and automated instrument systems, rely on measuring growth inhibition using culture-based methods that are time consuming and unreliable for Xf [23]. Moreover, these methods may overestimate the antimicrobial activity of the tested compounds against Xf considering that its cells can enter in a viable-but-non-culturable state (VBNC) in response to harsh environments [24, 25]. Several methods have already been proposed to analyze only viable cells, such as ATP bioluminescence [23], direct microscopy or flow cytometry such as LIVE/DEAD® *Bac*light™ [26], DAPI combined with SYTOX Green [27], or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) that evaluates respiratory activity [28]. However, these methods are not able to specifically quantify viable target cells in mixture cultures. Alternative non culture-based methods would be more suitable to evaluate the efficacy of new compounds to inhibit Xf. Nucleic acid-based techniques such as quantitative PCR (qPCR) are commonly used to quantify total specific bacteria, as they can specifically detect target cells. All methods mentioned require specific sample preparation, training, and equipment. Nevertheless qPCR is particularly popular because it has been used for a wide number of applications and has become a standard equipment in researcher laboratories, so methods that use qPCR are easier to be performed anywhere.

A limitation of the qPCR is the overestimation of alive cells. Due to the fact that DNA can persist for an extended period after cell death [29], the DNA of both viable and dead cells is amplified. In contrast, the viable quantitative PCR (v-qPCR) allows the quantification of

only viable cells. Generally, v-qPCR uses the nucleic acid-binding dyes propidium monoazide (PMA or PMAxx) or ethidium monoazide (EMA) in combination with qPCR for selectively detecting and enumerating viable cells. Both PMA and EMA bind to the free DNA and the DNA of dead cells with damaged membranes. In addition, EMA binds to the DNA of non-metabolically active cells with an intact membrane, avoiding its subsequent amplification by qPCR. In the PEMAX reagent, an optimized mixture of PMA (≥20 μM) and EMA (< 10 μM) is used [30]. This low level of EMA is accumulated inside non-metabolically active cells that still have an intact cell membrane, while it is eliminated from viable cells through active transport. Therefore, after treatment with PEMAX, only the DNA of viable cells remains unlabelled and is detected by qPCR [31, 32]. This methodology has already been used for foodborne pathogenic bacteria in different matrices [33], to monitor biological control agents in field studies [34] and, in the case of Xf, to differentiate viable cells under stressing conditions [35, 36]. Nevertheless, v-qPCR using the PEMAX reagent has never been optimized as a screening methodology for the identification of antimicrobials active against Xf.

For the development of a v-qPCR assay for the detection and quantification of Xf it is necessary to find a molecular marker species-specific suitable to be used with PEMAX. Different primer pairs and probes specific for Xf detection have been described and validated [37–40]. Primer pairs normally show different amplification efficiencies and levels of sensitivity depending on the target site, the nature of the primers and the length of the amplicon. Moreover, suppression of dead cells amplification after PEMAX treatment is also dependent on the length of the DNA fragment amplified by qPCR, as the probability of dye binding increases in longer target regions [34].

The aim of the present work was to find peptide conjugates derived from **BP100** highly active against Xf in vitro. To accomplish this purpose, firstly a screening methodology based on a contact test combined with a v-qPCR method was optimized for representative strains of Xf and for an accurate and reliable evaluation of the antimicrobial activity of peptides. Afterwards, a set of peptide conjugates derived from **BP100**, designed for being expressed in plant systems and active against other plant pathogens, were selected and screened using the optimized methodology to evaluate its antimicrobial activity against Xf.

Results

Amplification efficiency and sensitivity of qPCR assays

Eight TaqMan based qPCR assays amplifying three different gene sequence targets of Xf and producing different amplicon lengths were checked in order to study their suitability for v-qPCR (Table 1). Standard curves of the eight qPCR assays showed good linearity over 7-log range, from 1×10^2 to 1×10^8 CFU/ml, reporting R^2 values over 0.99 (Additional file 1). Table 1 shows the amplification efficiency and the sensitivity of each qPCR assay. All amplification efficiencies were higher than 94%, and did not vary between qPCR assays having the same target gene, except for the Elongation factor Tu (EFTu), which ranged from 95 to 98%. The three qPCR assays amplifying part of the 16S rRNA gene (XF16S) displayed the best amplification efficiencies (97%).

Regarding the sensitivity, the eight qPCR assays were very different at 5×10^3 CFU/ml cycle threshold values (C_T), ranging from 27.2 to 33.9. Again, the three assays amplifying part of the XF16S gene displayed the higher sensitivity. In all cases, qPCR assays amplifying larger DNA fragments (311, 279, and 307 bp) were less sensitive than the ones generating shorter amplicons. The XF16S-3 design (279 bp) showed sensitivity values comparable to the ones obtained with the qPCR assays amplifying fragments of less than 100 bp. Due to the fact that higher amplicon lengths are more suitable when using PEMAX, the qPCR assay with XF16S-3 was selected for further experiments.

V-qPCR

The effect of different PEMAX concentrations on the amplification of DNA targets of viable and dead Xf subsp. *fastidiosa* strain Temecula (Xff) cells was studied by determining the signal reduction value (SR), defined as the difference of C_T value between PEMAX and non-PEMAX treated samples (ΔC_T) (Additional file 2). On viable cells, no significant differences on SR values were observed when using a PEMAX concentration of 2.5, 5, 7.5 and 50 μM. However, at 10 μM, the SR value was significantly higher compared to 5 and 7.5 μM. Regarding dead cells, significant differences were observed between PEMAX concentrations, being 7.5 and 10 μM the concentrations with highest SR values. Based on these results, a PEMAX concentration of 7.5 μM was chosen for further experiments, as it was the lowest concentration that allowed a better discrimination between viable and dead cells.

Standard curves were performed using cell suspensions of Xff, Xf subsp. *pauca* (Xfp) and Xf subsp. *multiplex* (Xfm) to evaluate the suitability of the v-qPCR method to quantify viable cells. PEMAX and non-PEMAX-treated standard curves showed good linearity between 1×10^2 and 1×10^7 CFU/ml, with R^2 values above 0.985. In all cases, a shift of around 2 cycles was observed when comparing PEMAX treated and non-treated samples from the same subspecies (Fig. 1). This

Table 1 Primers and TaqMan probes used for qPCR analysis, amplification efficiency and sensitivity analysis

qPCR assay	Primer/ probe	Sequence	Amplicon length (bp)	Slope	R^2	Efficiency (%)	Sensitivity[a]	Reference of source
HL-1	rev-2	GGTTTTGCTGACTGGCAACA	221	−3.47	0.99	94	30.8	37
HL-2	rev-3	CACTTGTGGTAAGCATCCTGAG	307	−3.49	0.99	94	31.8	This study
	for	AAGGCAATAAACGCGCACTA						37
	probe	FAM/−TGGCAGGCAGCAACGATACGGCT−/BHQ						37
XF16S-1	rev-1	CCGATGTATTCCTCACCCGTC	62	−3.39	0.99	97	27.2	39
XF16S-2	rev-2	CTAATCGGACATCGGCTCAT	181	−3.39	0.99	97	28.1	This study
XF16S-3	rev-3	GTAGGAGTCTGGACCGTGTCTC	279	−3.39	0.99	97	29.7	21
	for	CGGCAGCACGTTGGTAGTAA						39
	probe	FAM/−CATGGGTGGCGAGTGGC−/TAMRA						39
EFTu-1	rev-1	GGCGAGCCAACAAAATGTGTT	77	−3.21	0.99	95	28.4	38
EFTu-2	rev-2	ATCACCAGGAAAATCATACTTGCT	202	−3.38	0.99	98	29.4	This study
EFTu-3	rev-3	GAATGTGGGTATCCAATGCTTC	311	−3.21	0.99	95	33.9	This study
	for	GGATGGTGCGATTTTAGTATGTTCT						38
	probe	FAM/−TGATGGTCCGATGCCTCAGACTCGT−/TAMRA						38

[a] C_T value at a concentration of 5×10^3 CFU/ml

variation was already observed in the optimization of the PEMAX concentration (Additional file 2). Amplification efficiencies of all standard curves were around 80% and values were comparable among subspecies (88.1% without PEMAX and 80% with PEMAX for Xff, 83.2% without PEMAX and 77.1% with PEMAX for Xfp, and 79.5% without PEMAX and 80.8% with PEMAX for Xfm). In dead cells, samples ranging from 1×10^3 to 1×10^7 CFU/ml treated with PEMAX displayed C_T values higher than 37.5, indicating, as expected, an inhibition of their amplification (Fig. 1). In mixtures of viable cells (from 1×10^3 to 1×10^7 CFU/ml) and dead cells (fixed quantity of 1×10^6 CFU/ml), standard curves showed a high correlation coefficient (R^2 values above 0.99) when samples were treated with PEMAX (Fig. 1). Amplification efficiencies calculated were similar to the ones obtained in the standard curves of only viable cells (92.9% for Xff, 80.9% for Xfp and 80.8% for Xfm), indicating that presence of DNA from dead cells do not interfere in the amplification of DNA from viable cells.

Antimicrobial activity of peptide conjugates derived from BP100: optimization of the contact test

To develop a method for screening antimicrobial activity of AMPs against Xf, different contact test conditions were studied, such as Xff cell concentration, contact test time and AMP concentration. Loss of viability after the contact test was assessed by v-qPCR and compared with plate counting (culturable cells) and qPCR (total cells).

The antimicrobial activity of **BP178** at 1.6, 12.5 and 50 μM was studied against two different Xff cell concentrations in a 3 h contact test (Fig. 2). At all peptide concentrations, Xff cells showed higher loss of viability (expressed as log reduction of cell viability) at 1×10^7 CFU/ml than at 1×10^8 CFU/ml, indicating a significant effect of the initial cell concentration ($P < 0.001$). Specifically, treatment of Xff cells at 1×10^7 CFU/ml with **BP178** at 1.6 μM caused a significant reduction of viable cells (1.5 log), while no significant reduction was observed at 1×10^8 CFU/ml. At 12.5 μM, significant reduction of viable cells was observed in both cases, being 3 log reduction at 1×10^7 CFU/ml whereas 2 log reduction at 1×10^8 CFU/ml. A peptide concentration of 50 μM, both Xff cell concentrations exhibited a similar reduction of viability of around 3 log.

The effect of peptide **BP178** on viability and culturability at different contact test times (from 1.5 to 48 h) was studied (Fig. 3). **BP178** at 50 μM reduced ($P < 0.001$) viable and culturable cells of Xff in all exposure times. There were significant differences ($P < 0.001$) between v-qPCR (viable cells) and plate counting (culturable cells) in Xff suspensions mixed with **BP178**. In the case of v-qPCR, a progressive viability reduction occurred up to a contact test time of 6 h (between 2 and 3.5 log reduction), practically reaching the detection limit of the method (3 log CFU/ml). In contrast, the culturability of the cells mixed with the peptide dropped abruptly to levels near the detection limit (1.5 log CFU/ml) after 1 h of incubation. Xff cells maintained similar levels of both, cell viability (v-qPCR) and cell culturability, in the non-treated control (without **BP178**) over 48 h.

The AMP concentration was evaluated by assessing the loss of viability of Xff suspensions mixed with **BP178** at 3.1, 6.2, 12.5, 25 and 50 μM at a contact time of 3 and 24 h (Fig. 4). In this experiment, there were also significant differences ($P < 0.001$) between v-qPCR

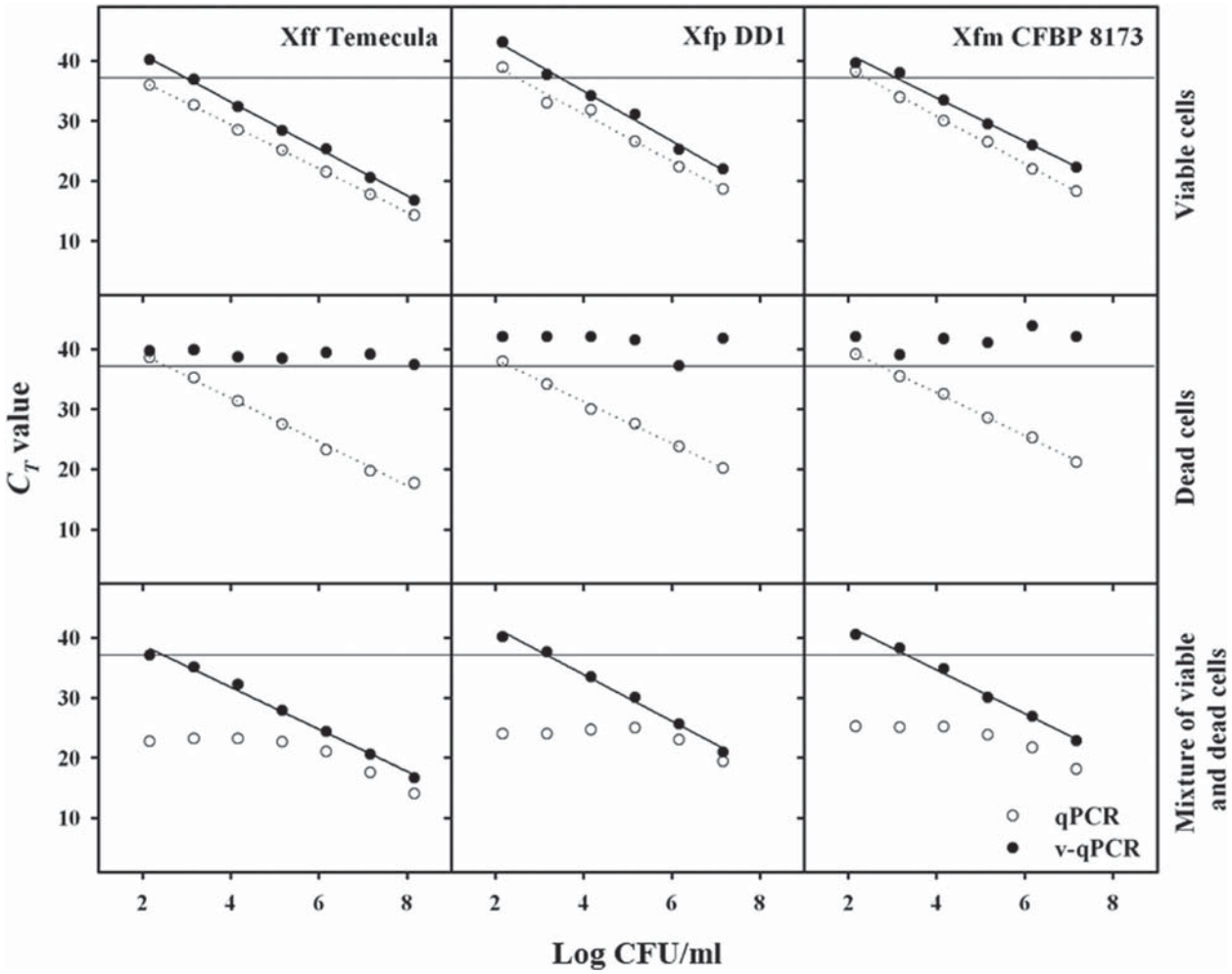

Fig. 1 Relationship between C_T values and cell concentration in three strains of Xf using conventional qPCR (white symbols) and v-qPCR (black symbols), for viable cells, dead cells, and a mixture of viable cells with a fixed concentration of dead cells (1×10^6 CFU/ml). TaqMan-based qPCR assay done with XF16S-3 primers. The thin line represents the detection limit at $C_T = 37.5$

(viable cells), plate counting (culturable cells) and qPCR (total cells) in Xff suspensions in the presence of different concentrations of **BP178**. In the case of v-qPCR, a similar reduction of cell viability was observed (around 3 log reduction) for all **BP178** concentrations in both contact test times (3 and 24 h), and only differences between the incubation periods were observed at 3.1 μM and 50 μM ($P < 0.001$). Comparing peptide concentrations, a progressive viability reduction occurred in the contact test of 3 h and significant differences were observed between 0, 3.1 and 12.5 μM. The culturability of Xff cells was also reduced without significant differences in almost all peptide concentrations at both incubation periods (around 5 log cell culturability reduction). The minimal bactericidal concentration (MBC) of **BP178** was determined to be 3.1–6.25 μM, which corresponds to 10–20 μg/ml.

Screening of peptide conjugates derived from BP100 against Xff

Eleven selected conjugates derived from **BP100** were tested against Xff at 3.1 and 12.5 μM (Table 2). Peptide **tag54**, an epitope tag designed for being used in peptide detection and purification, was included as a control to check the effect upon Xff cells of a peptide that previously showed no antimicrobial activity against other plant pathogenic bacteria [17]. **BP100** and **BP178** were also assayed for comparison purposes. Peptide **tag54** did not show antimicrobial activity against Xff cells. **BP100** led to a log reduction (N_0/N) of cell viability of 1.39 at 3.1 μM and of 3.27 at 12.5 μM. At 3.1 μM, all peptide conjugates showed antimicrobial activity with a log reduction of cell viability between 0.91 and 2.95. At 12.5 μM, a higher effect was observed for all peptides, leading to an Xff viability reduction between 1.33 and

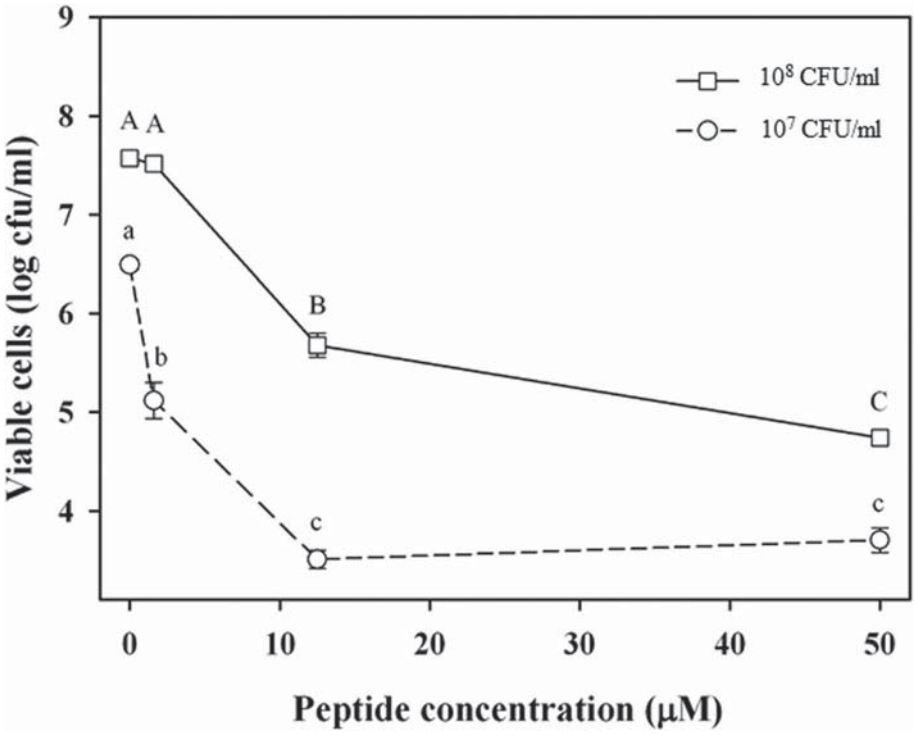

Fig. 2 Effect of peptide **BP178** on viability of Xff strain Temecula estimated by v-qPCR at different peptide concentrations (1.6, 12.5 and 50 μM). Two assays were performed at different initial Xff cell concentrations, 1×10^7 CFU/ml (circles) and 1×10^8 CFU/ml (squares). The exposure time to the peptide was 3 h. Xff concentration in non-treated cells was estimated after 3 h by v-qPCR. The detection limit of the v-qPCR is 3 log CFU/ml. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Lowercase letters correspond to the means comparison of viable cells in 1×10^7 CFU/ml. Capital letters correspond to the means comparison of viable cells in 1×10^8 CFU/ml. Means sharing the same letters are not significantly different ($P < 0.05$), according to the Tukey's test

3.79 log. **BP171**, **BP175** and **BP178** were highly active, with a 3.5–4 log reduction of cell viability at 12.5 μM of peptide concentration and 2.5–3 log reduction at 3.1 μM. **BP170**, **BP176** and **BP180** were moderately active, with a 3–3.5 log reduction at 12.5 μM and 2–3 log reduction at 3.1 μM. **BP181**, **BP188**, **BP192**, **BP198** and **BP213** were low active, with less than 3 log reduction at 12.5 μM and less than 2 log reduction at 3.1 μM. Highly and moderately active peptides have a significantly different log reduction compared to low active peptides (according to the mean separation test). The highly active peptides were conjugates incorporating a **BP100** unit and a melittin or a magainin fragment. In particular, **BP171**, containing **BP100** and a melittin fragment, led to a log reduction of 3.79 at 12.5 μM and of 2.91 at 3.1 μM, while **BP175** and **BP178,** which incorporate **BP100** and a magainin fragment, led to a log reduction of 3.52 and 3.54 at 12.5 μM, and of 2.65 and 2.95 at 3.1 μM, respectively.

The antibacterial activity of **BP171** and **BP198** was also evaluated at different peptide concentrations by v-qPCR and plate counting (Additional file 3). Results showed that, as expected, both methods classified **BP171** as highly active against Xff and **BP198** as a low active peptide against this pathogen. After **BP171** treatment, the Xff viable and culturable cells reached the detection limit in both technics, whereas **BP198** was not able to completely inactivate Xff, neither using plate counting nor v-qPCR.

Discussion

The difficulties in managing diseases caused by Xf have stimulated the search for novel bactericides. Several antimicrobial compounds, such as toxins, antibiotics, phenolic acids and AMPs, have been reported to be active against several Xf strains with MBC or minimal inhibitory concentrations (MIC) ranging from 8 to 800 μM [14, 15, 41–44]. Interestingly, the AMPs magainin I and II, and dermaseptin have been reported to display low MIC or MBC values against Xf [14]. In addition, AMPs such as the lytic peptides LIMA-A and cecropin B have been expressed in grapevines resulting in a successful control of Xf in greenhouse conditions [45, 46]. So, their antibacterial activity and their availability for being expressed in plants make AMPs good candidates for the control of this plant pathogen, either using transgenic

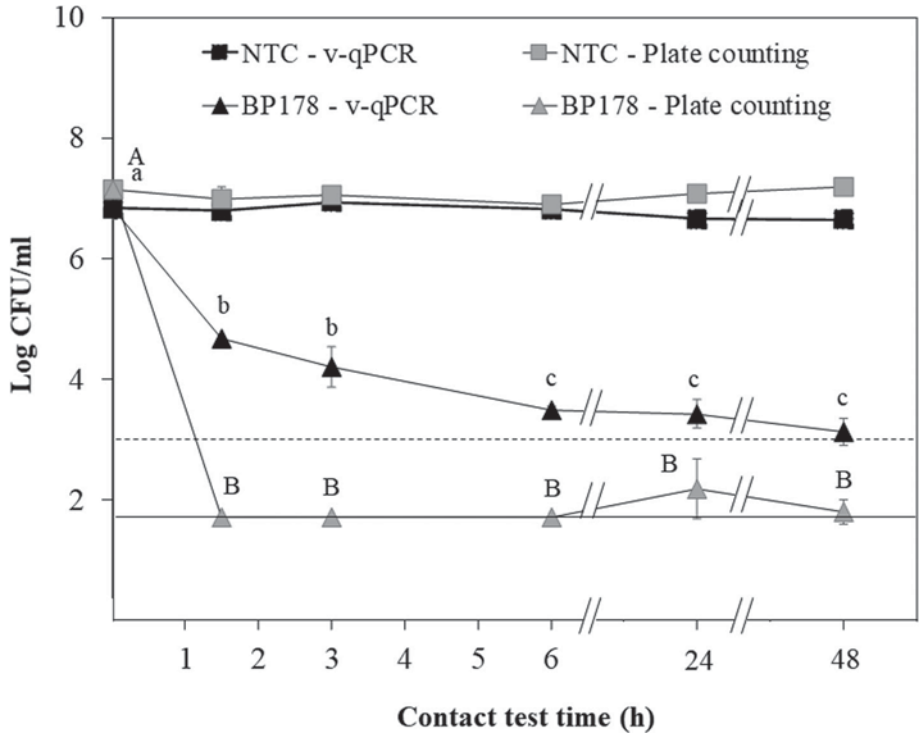

Fig. 3 Effect of peptide **BP178** on viability and culturability of Xff strain Temecula at different exposure times. Cell viability was estimated by v-qPCR (black symbols) and cell culturability by plate counting (grey symbols). Initial cell concentration was 1×10^7 CFU/ml and the **BP178** concentration used was 50 μM. Non-treated controls (NTC) were also performed by adding the corresponding volume of sterile distilled water. The dash line represents the detection limit of v-qPCR, whereas the normal line indicates the detection limit of the plate counting technic. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Lowercase letters correspond to the means comparison of viable cells treated with **BP178** (black triangles). Capital letters correspond to the means comparison of culturable cells treated with **BP178** (grey triangles). Means sharing the same letters are not significantly different ($P < 0.05$), according to the Tukey's test

expression or other delivery strategies such as endotherapy. In the present study, a set of 11 peptide conjugates derived from the lead peptide **BP100** and a fragment of cecropin, magainin or melittin, previously reported by our group as active against several plant pathogenic bacteria and with low toxicity to eukaryotic cells (moderate to low hemolysis) (Table 3) [10, 17, 20], were screened for their activity against Xf. One of these peptide conjugates (**BP178**) has been produced in transgenic rice [18, 19], and has also been tested in vitro against Xf and other plant pathogens showing high antibacterial activity [17, 21].

A methodology consisting of a contact test combined with a v-qPCR method was developed in the present work in order to screen the activity of AMPs against the fastidious bacterium Xf. The v-qPCR method has the advantage to allow the quantification of viable cells, including VBNC and culturable cells, without a cultivation stage. Other studies used a variety of culture-dependent methods to evaluate the antimicrobial activity against Xf. Nevertheless, for the screening of large amounts of antimicrobial peptides, these methodologies are time consuming, as they require incubation periods of several days for Xf to grow. While the v-qPCR can be performed in less than 1 day, about 4–7 days are required for the agar plate dilution assay, for the contact test followed by plate counting or for the agar disc diffusion method [14, 15, 42, 44].

The v-qPCR has been efficiently used for the monitoring of microorganisms with biotechnological potential [31], and for the detection and quantification of human pathogens in food [47] or in the environment [48]. In particular, in the case of Xf, different PCR assays are commonly used for the detection and quantification, and v-qPCR methods in combination with EMA or PMAxx reagents were also reported to discriminate between viable and membrane-damaged cells [35, 36]. In our work the PEMAX reagent, an optimized mixture of EMA and PMA that has been previously proven to be efficient in discriminating viable from dead cells in a biological control agent was used [34]. The effect of PEMAX concentration was optimized in order to detect only viable Xf cells. PEMAX at 7.5 μM was the lowest concentration showing good results as inhibited the DNA amplification of dead Xff cells at 1×10^7 CFU/ml while viable cells were not affected. Lower concentrations, 2.5 and 5 μM,

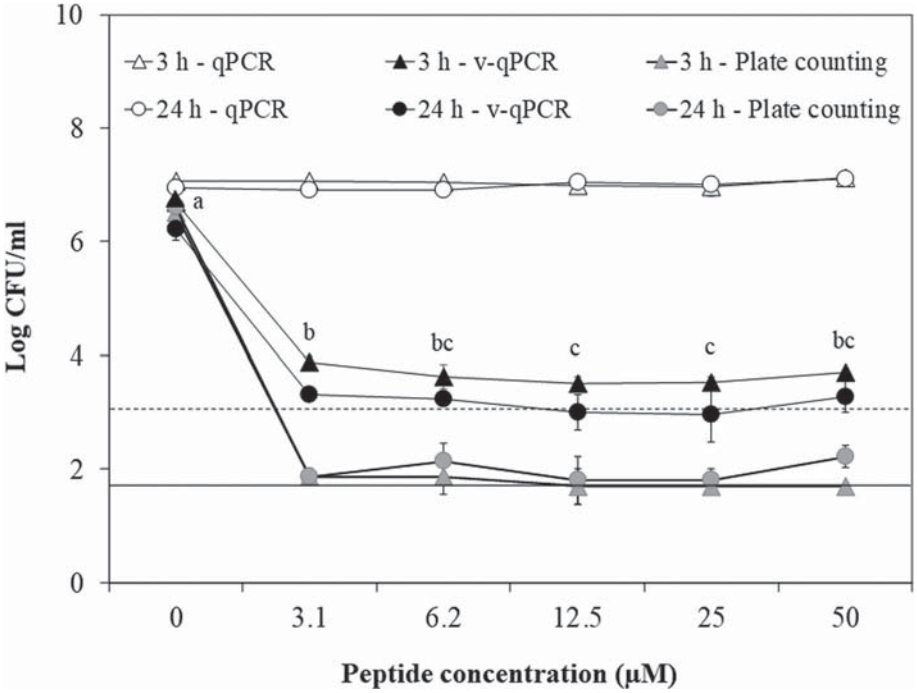

Fig. 4 Effect of peptide **BP178** on viability and culturability of Xff strain Temecula at different peptide concentrations. Total cell concentration was estimated by conventional qPCR (white symbols), cell viability was estimated by v-qPCR (black symbols), and cell culturability by plate counting (grey symbols). Exposure times of 3 h (triangles) and 24 h (circles) were used. Cell concentration was 1×10^7 CFU/ml in both cases. The dash line represents the detection limit of v-qPCR, whereas the normal line indicates the detection limit of the plate counting technic. Values are the means of three replicates, and error bars represent the standard deviation of the mean. Letters correspond to the means comparison of viable cells treated with **BP178** at exposure time of 3 h. Means sharing the same letters are not significantly different ($P < 0.05$), according to the Tukey's test

Table 2 Screening of conjugate peptides derived from **BP100** against Xff, compared with **BP100** and **tag54,** by means of a contact test combined with v-qPCR method

Peptide type	Code	Sequence	N° of AA	Log N_0/N[a]			
				3.1 μM		12.5 μM	
Reference peptides	**tag54**	KDWEHLKDWEHLKDWEHL-OH	18	0 ± 0	g	0 ± 0	G
	BP100	KKLFKKILKYL-NH_2	11	1.39 ± 0.07	d	3.27 ± 0.09	ABC
BP100 (dimer)	**BP192**	KKLFKKILKYL - **AGPA** - KKLFKKILKYL - **KDEL**-OH	30	0.91 ± 0.13	e	1.33 ± 0.11	F
	BP198	KKLFKKILKYL - KKLFKKILKYL - **KDEL**-OH	26	1.37 ± 0.04	d	1.94 ± 0.08	DE
	BP213	KKLFKKILKYL - **AGPA** - LYKLIKKFLKK - **KDEL** - OH	30	1.17 ± 0.02	de	1.34 ± 0.10	F
BP100 - Melittin [10–19]	**BP170**	KKLFKKILKYL - TTGLPALISW - OH	21	2.89 ± 0.09	ab	3.11 ± 0.06	BC
	BP171	KKLFKKILKYL - **AGPA** - TTGLPALISW-OH	25	2.91 ± 0.02	ab	3.79 ± 0.15	A
BP100 - Magainin [4–10]	**BP180**	KKLFKKILKYL - KFLHSAK-OH	18	2.29 ± 0.27	c	2.96 ± 0.19	C
	BP181	KKLFKKILKYL - **AGPA** - KFLHSAK-OH	22	1.30 ± 0.08	de	2.40 ± 0.37	D
BP100 - Magainin [1–10]	**BP175**	KKLFKKILKYL - **AGPA** - GIGKFLHSAK-OH	25	2.65 ± 0.18	abc	3.52 ± 0.16	AB
	BP176	KKLFKKILKYL - GIGKFLHSAK-OH	21	2.47 ± 0.12	bc	3.34 ± 0.11	ABC
	BP178	KKLFKKILKYL - **AGPA** - GIGKFLHSAK - **KDEL**-OH	29	2.95 ± 0.22	a	3.54 ± 0.05	AB
BP100 - Cecropin A [25–37]	**BP188**	KKLFKKILKYL - AVAVVGQATQIAK - **KDEL**-OH	28	0.89 ± 0.04	e	1.60 ± 0.09	EF

[a]Log reduction of Xff cell viability after the treatment with the peptides (at 3.1 and 12.5 μM) for 3 h was calculated as log N_0/N, where N_0 is the initial number of viable cells and N is the number of viable cells after the treatment, as estimated by v-qPCR. Values are the mean of three replicates, the confidence intervals are indicated. Lowercase letters correspond to the means comparison of Log N_0/N in 3.1 μM. Capital letters correspond to the means comparison of Log N_0/N in 12.5 μM. Means sharing the same letter indicate no significant differences between peptides ($P < 0.05$), according to the Tukey's test

Table 3 MIC against different plant pathogens and hemolysis percentage displayed by the peptide conjugates derived from **BP100** tested in this study

Peptide type	Code	MIC (μM)			Hemolysis[d] (%)		
		Xav[a]	*Psa*[b]	*Ea*[c]	50 μM	150 μM	250 μM
Reference peptides	**tag54**	> 100	> 100	> 100	0	0	1
	BP100	10–20	7.5–10	7.5–10	1	8	18
BP100 (dimer)	**BP192**	7.5–10	7.5–10	7.5–10	51	69	69
	BP198	10–20	10–20	10–20	59	72	72
	BP213	1.25–2.5	2.5–5.0	2.5–5.0	90	92	98
BP100 - Melittin [10–19]	**BP170**	1.25–2.5	2.5–5.0	2.5–5.0	82	93	98
	BP171	2.5–5.0	1.25–2.5	2.5–5.0	5	16	34
BP100 - Magainin [4–10]	**BP180**	2.5–5.0	2.5–5.0	2.5–5.0	12	54	58
	BP181	2.5–5.0	1.25–2.5	2.5–5.0	0	0	0
BP100 - Magainin [1–10]	**BP175**	1.25–2.5	2.5–5.0	5.0–7.5	6	14	32
	BP176	2.5–5.0	2.5–5.0	5.0–7.5	3	44	59
	BP178	2.5–5.0	2.5–5.0	2.5–5.0	0	3	25
BP100 - Cecropin A [25–37]	**BP188**	2.5–5.0	1.25–2.5	2.5–5.0	10	24	42

[a]*Xav*, *Xanthomonas axonopodis* pv. vesicatoria; [b]*Psa*, *Pseudomonas syringae* pv. syringae; [c]*Ea*, *Erwinia amylovora*; [d]Percent hemolysis plus confidence interval (α = 0.05)

were less effective in preventing DNA amplification of high cell concentrations, probably due to the lack of available reagent. In contrast, higher concentrations of PEMAX, 10 and 50 μM, caused a slight toxicity effect on Xff cells. In other studies, a PEMAX concentration of 50 μM has been reported to be the optimal to detect *Lactobacillus* and *Salmonella* using a v-qPCR assay [31, 34]. However, it has also been described that excessive concentrations of these two dyes causes toxicity in some microorganisms [36, 49]. Therefore, the concentration of PEMAX has to be optimized for each species in order to allow DNA amplification of only viable cells, without being toxic to the bacteria.

In order to choose the best conditions for v-qPCR, eight Xf-specific qPCR assays with different amplification sites and lengths were compared. All assays showed acceptable and similar efficiency percentages that were in agreement with those observed in other qPCR designs used for the quantification of Xf [50, 51]. In contrast, the assays differed in the sensitivity values. The primer pair (XF16S-3), chosen for further assays with a length of 279 bp, exhibited sensitivity values similar to those previously reported [50, 51]. As it has been described [34, 52], the amplicon length is an important parameter to consider when optimizing a v-qPCR because there is a higher probability of dye intercalation in cell-free DNA when using long length amplicons compared to short length amplicons. The reliability of the v-qPCR when using XF16S-3 as primer pair and a PEMAX concentration of 7.5 μM was evaluated and validated on viable and dead cells, and on a mixture of viable and dead cells of Xff, Xfp and Xfm. v-qPCR method developed showed acceptable amplification efficiencies and correlation coefficient values. Although both, the use of longer amplicons and the presence of PEMAX, decreased the sensitivity of the qPCR, a C_T value corresponding to 1×10^3 CFU/ml viable cells was determined as the detection limit of the developed v-qPCR.

To set up the conditions of the contact test, the initial cell concentration of Xff, the contact test time and the peptide concentration were optimized. Considering other studies, which employed Xf cell concentrations ranging from 1×10^5 to 1×10^8 CFU/ml to test antimicrobial compounds [14, 41, 42], a Xff concentration of 1×10^7 CFU/ml was chosen as it brought out the effect of the AMP at low concentration and enabled a viability reduction of 4 log before reaching the detection limit of the v-qPCR method (1×10^3 CFU/ml). At 1×10^8 CFU/ml, a different viability reduction pattern was observed. As described, antimicrobial peptides (and other antimicrobials) are quenched during interaction with target cells due to their binding to the cell through time. Because there is a threshold number of peptide molecules necessary to kill a target cell, the viability reduction is not only dependent on the antimicrobial concentration but also on the target bacteria concentration [53, 54]. Regarding the contact test time, a lethality percentage around 99.8% was observed after 3 h of contact test with the peptide. Against Xf, a contact test time of 18 h has been employed [15] but as reported, the bactericidal effect of an antimicrobial compound is time-dependent and a lethality percentage of 90% after 6 h is equivalent to a 99.9% of dead cells after 24 h [23]. Therefore, in our study a contact test of 3 h allows fast screening of AMPs

against Xf with similar results than longer contact test times. Finally, peptide concentrations of 3.1 and 12.5 μM were the ones selected for the screening of AMPs because it was envisaged that they would allow the classification of the peptides according to their activity against Xf.

The suitability of the v-qPCR method to estimate the viability of Xf cells after the contact test was studied by comparing it with qPCR and plate counting onto PD2 agar plates. No significant differences were observed between the three methods in untreated Xff cells. However, in cells treated with the peptide conjugates derived from **BP100**, qPCR overestimated viable cells (around 4 log units) compared to v-qPCR, indicating the presence of DNA from dead cells, and plate counting underestimated the viability of Xff (around 2 log units). While previous research has focused on determining the activity of antimicrobial compounds using methodologies that report information about the culturable cells [14, 15, 41, 42], v-qPCR offers the possibility of determining the amount of viable cells, irrespective of their culturability. In the present study, it was observed that viability of Xf cells was progressively reduced after the treatment with AMPs, while culturability dropped abruptly to levels near the detection limit. This fact is probably due to the formation of metabolically active persistent cells (VBNC state). It has been described that Xf cells enter in the VBNC state when they are exposed to inhibitory concentrations of antimicrobial compounds [25, 35, 55, 56]. In other plant pathogens, VBNC cells have been reported to have the capacity to revert its physiological state and acquire again its virulence, being widely responsible for recalcitrant infections [57, 58]. Taking this into account, the quantification of the whole viable fraction (including VBNC and culturable cells) is necessary to determine the antimicrobial activity of compounds because the presence of these cells can play a significant role in terms of defining their pathogenicity and epidemiology.

Remarkably, the use of the above described contact test coupled with the v-qPCR for the screening of peptides allowed a rapid and reliable identification of sequences among the peptide conjugates derived from **BP100** active against Xf, and their classification as: (i) highly active (**BP171**, **BP175**, **BP178**), (ii) mid active (**BP170**, **BP176**, **BP180**), and (iii) low active (**BP181**, **BP188**, **BP192**, **BP198**, **BP213**). The best peptides **BP171**, which incorporates **BP100** and a melittin fragment, and **BP175** and **BP178**, which result from the conjugation of **BP100** with a magainin II fragment, showed higher activity than **BP100**. Other AMPs, such as gomesin, dermaseptin or magainin II, have been reported to be active against Xf (MIC or MBC of 4.5–9, 8–32 and 8–64 μg/ml, respectively) [14, 15]. Unfortunately, it is not possible to compare these activity values obtained using v-qPCR because the methods used in other works to assay the antibacterial activity were different. However, the activity values of **BP171** and **BP178** determined in our work using plate counting, which attained a MBC between 1.5 and 3.1 μM (~ 5–10 μg/ml) and 3.1 and 6.1 μM (~ 10–20 μg/ml) respectively, can be compared and are similar to the gomesin values, since the method used in both cases was a contact test followed by plate counting.

Conclusions

This work has allowed the fast screening and identification of five new bactericidal peptide conjugates (**BP171**, **BP175**, **BP170**, **BP176**, **BP180**) active against Xf, in addition to the previously described **BP178**. All of them can be considered as candidates for the development of new agents to treat the plant diseases caused by this bacterium. The contact test combined with v-qPCR method has the advantage of quantifying only viable Xf cells, therefore the evaluation of the antimicrobial effect of AMPs is more precise. Moreover, considering the European Union rules for quarantine organisms, and particularly for Xf, the method minimizes the risk of dissemination of the pathogen, as it allows working in more safe conditions (shorter periods of time with manipulating living cells), compared to the culture-based methods. Apart from testing AMPs and other antimicrobials against Xf in vitro, the method could also be used in plants, as the Xf population quantified in naturally infected olive trees and in artificially inoculated grapevines is around 10^7–10^8 CFU/ml [36, 59]. This would be of interest to confirm the antimicrobial activity of the AMPs against the pathogen in their hosts. In addition, the fact that these conjugates were designed to be expressed in plants extends their possible technological use by means of transgenic plant hosts producing peptides to kill the pathogen [45, 46, 60, 61].

Methods

Xf strains, growth conditions and DNA extraction

Xff strain Temecula 1 ATCC 700964 [62], Xfp strain DD1 [63] and Xfm strain CFBP 8173 [64] were used. All strains were grown in BCYE agar [65] at 28 °C for 1 week and were stored in PD2 broth [66] with 30% glycerol at − 80 °C. Cell suspensions were prepared in sterile succinate-citrate-phosphate (SCP) buffer [40] at 1×10^8 CFU/ml (optical density at 600 nm being 0.3, confirmed by colony counts) and diluted to appropriate concentrations. DNA was extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, USA) following the specific protocol for Gram-negative bacterial suspensions. Briefly, 200 μl were centrifuged at 15,900 x g during 10 min, the pellet was resuspended in 180 μl of digestion solution and 20 μl of proteinase K. Samples were incubated at 56 °C for 30 min, then 20 μl of

RNase solution was added and another incubation step of 10 min at room temperature was carried out. Next, 200 μl of lysis solution were added, followed by 400 μl of 50% ethanol, and all the volume was transferred to a GeneJET Genomic DNA Purification Column. Two washes were performed using two different wash buffers, and finally DNA was re-suspended with 30 μl of PCR-grade water. DNA was stored at − 20 °C for further analysis.

qPCR design: evaluation of the amplification efficiency and sensitivity

qPCR assays were conducted using the primer pairs and TaqMan probe sets described in Table 1. Primer3Plus software was used to obtain amplicons with different length that shared the described forward primers and probes but with new different reverse primers. All qPCR were performed using 96-well plates containing 12.5 μl 2X TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, USA), final concentrations of 400 nM for each forward and reverse primer and of 150 nM for TaqMan probe with dye, 8.46 μl of PCR-grade water and 2 μl of template DNA in each well. Serial 10-fold dilutions of Xff covering a 7-log range (from 1×10^2 to 1×10^8 CFU/ml) were prepared in sterile SCP buffer and each concentration was performed in triplicate. DNA extraction from each suspension was performed as described above. All reactions were performed in duplicate and carried out in a QuantStudio 5 real-time PCR system (Applied Biosystems, Foster City, CA, USA). qPCR conditions were 95 °C for 10 min for enzyme activation followed by denaturation at 95 °C for 1 min, and extension and annealing at 59 °C for 1 min. The qPCR was run for 45 cycles. Standard curves were developed to check the sensitivity and efficiency of the qPCR assays. C_T values were plotted against the logarithm of the initial number of CFU/ml to determine the amplification efficiency of each design using the following equation.

$$\mathbf{E}(\%) = \left(10^{-1/slope} - 1\right) \times 100$$

V-qPCR: optimization of the PEMAX concentration

A stock solution of 2000 μM of PEMAX reagent (GenIUL, Terrassa, Spain) was prepared and stored as described [32]. To optimize the concentration of PEMAX, 20 μl of PEMAX stock solutions at 25, 50, 75, 100 or 500 μM were added into 180 μl of viable or dead Xff cell suspension, both adjusted to 1×10^7 CFU/ml in SCP. Dead cells were obtained by heating the cell suspension at 95 °C for 10 min (ThermoMixer F1.5; Eppendorf, Hamburg, Germany), and the suspension was plated on PD2 agar and incubated for 1 week at 28 °C to check the absence of growth. PEMAX treated samples were thoroughly mixed and incubated for 30 min in the dark at room temperature with manual shaking every 10 min. Next, samples were photoactivated with the PhAST Blue photoactivation system (GenIUL, Barcelona, Spain) for 15 min with intensity of 100%. Each PEMAX treated sample was transferred into DNA low-binding 1.5 ml tube (Sarstedt, Nümbrecht, Germany) and collected by centrifugation at 15,900 x g for 10 min. A washing step to eliminate the excess of PEMAX was required, so supernatant was eliminated and 500 μl of sterile SCP buffer was added. Samples were collected under the same centrifugation conditions. Non-PEMAX treated samples, prepared with 20 μl of SCP buffer plus 180 μl of viable and dead cells, were also analysed. DNA extraction of all samples was carried out as described above and qPCR was performed according to the conditions described initially and using the primer pair XF16S forward and probe and its reverse 3 (XF16S-3). Signal reduction (SR), defined as the difference between cycle threshold values (ΔC_T) of non-PEMAX treated and PEMAX treated samples, was calculated to determine the effect of PEMAX concentration on DNA amplification suppression by qPCR assay. Three biological replicates were performed.

Evaluation of v-qPCR with Xff, Xfp and Xfm strains

The v-qPCR sensitivity and amplification efficiency was evaluated with standard curves. Suspensions of viable and dead Xff, Xfp and Xfm cells were prepared in SCP as described above. Samples were prepared to cover a 7-log range (from 1×10^2 CFU/ml to 1×10^8 CFU/ml) in Xff and a 6-log range (from 1×10^2 CFU/ml and up to 1×10^7 CFU/ml) in Xfp and Xfm. Mixture suspensions were also prepared, with the same concentration range of viable Xf cells in addition to a constant number of dead cells (1×10^6 CFU/ml). From each suspension, 180 μl were treated with PEMAX at 7.5 μM according to the procedure described previously, and 180 μl were used as non-PEMAX treated sample. DNA extraction was performed as described in both PEMAX treated and non-PEMAX treated samples. qPCR was performed as described previously, each reaction per duplicate and using XF16S-3 as the primer pair. Standard curves were generated plotting C_T values obtained against the logarithm of the initial number of CFU/ml, and the amplification efficiency was calculated as described above.

Evaluation of v-qPCR for antimicrobial activity assessment

The contact test conditions were optimized for the antimicrobial activity assessment of AMPs against Xf. Xff cell concentration, contact test time and peptide concentration were evaluated. The peptide **BP178** (Table 2) was used [19]. Lyophilized **BP178** was solubilized in sterile Milli-Q water to a final concentration of 1 mM, filter sterilized through a 0.22 μm pore filter and 10X

stock solutions of the desired concentrations were prepared in sterile distilled water. Suspensions of Xff cells prepared in sterile SCP buffer were used, and 20 μl of each **BP178** stock concentration were mixed in 1.5 ml tubes with 160 μl of the corresponding Xff cell suspension and incubated for 1.5, 3, 6, 24, or 48 h depending on the experiment. After the incubation period, 20 μl of PEMAX or SCP buffer were added to the samples for v-qPCR or qPCR, respectively, before DNA extraction.

In a first experiment, suspensions of Xff cells at 1×10^7 and 1×10^8 CFU/ml were tested to determine the differences in log reduction when using **BP178** final concentrations of 1.6, 12.5 and 50 μM. A second experiment was used to evaluate different contact test times in order to select the most suitable one for the assays. Additionally, in a third experiment, **BP178** concentrations of 3.1, 6.2, 12.5, 25 and 50 μM were incubated with Xff at 1×10^7 CFU/ml for 3 and 24 h to determine the most informative peptide concentrations to screen the peptides.

A non-treated control (Xff cells without peptide) using SCP buffer instead of peptide was also included in all the experiments, and three replicates for each Xff cell concentration, contact test time and peptide concentration were used. Xff $\log_{10}$ CFU/ml of the initial cell suspensions and of the contact tests, with or without the peptide, was determined using qPCR (total cells), v-qPCR (viable cells) and plate counting (culturable cells). For assessment of total, viable and culturable cells, aliquots were taken from the contact test wells at given times.

For qPCR and v-qPCR, DNA was isolated from two individual samples of 200 μl of each contact test, in the case of v-qPCR, previously the sample was treated with PEMAX at final concentration of 7.5 μM as described above. DNA extraction, qPCR analysis using the TaqMan-based qPCR assay XF16S-3 and quantification were performed as described above. The amount of total and viable cells was obtained by interpolating the C_T values from each sample against the respective standard curve and expressed as $\log_{10}$ CFU/ml. For plate counting, each sample was serially diluted, and appropriate dilutions were seeded onto PD2 agar plates. Plates were incubated at least for 1 week at 28 °C, colonies were counted and CFU/ml value was determined for each sample. The MBC of **BP178** was determined in order to compare with other described peptides. The MBC corresponds to the lowest concentration where no growth was detected in plate counting after exposure to the peptide in the contact test.

Screening of peptide conjugates derived from BP100 against Xff

A set of peptide conjugates derived from peptide **BP100** reported by our group as AMPs (Table 2) [17] were selected to be screened against Xff. The epitope tag peptide **tag54** was used as a negative control and **BP100** and **BP178** was included for comparison purposes. All AMPs were evaluated as described above, at final concentrations of 3.1 and 12.5 μM and against a suspension of Xff at 1×10^7 CFU/ml using a 3 h contact test. After the incubation period, Xff population level was assessed using v-qPCR as described above. Loss of viability after the contact test was calculated and expressed as logarithmic reduction of Xff population. Three replicates for each AMP and concentration were used. After the screening, **BP171** and **BP198**, showing different antibacterial activity against Xff, were selected to assess the performance of the v-qPCR methodology for the quantification of the viable Xff population. Peptide concentrations of 1.5, 3.1, 6.2, 12.5 and 25 μM were incubated with Xff at 1×10^7 CFU/ml for 3 h to determine viable and culturable cells by v-qPCR and plate counting respectively. Three replicates for each AMP and concentration were used.

Statistical analysis

To test the significance of the effect of PEMAX concentration in the suppression of DNA amplification (signal reduction) on dead and viable cells of Xff, a one-way analysis of variance (ANOVA) was performed. To test the significance of the parameters studied to set up the conditions of the contact test (initial Xff cell concentration, contact test time and peptide concentration) and of the cell quantification method, a two or three-way ANOVA were performed. To test the effect of AMPs on Xff viability reduction a one-way ANOVA was performed. In all cases, means were separated according to the Tukey's test at a P value of ≤0.05.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-020-01915-3.

Additional file 1. Standard curves of the eight qPCR assays studied. Each set of primer pairs amplifying the same target gene with different amplicon lengths are shown in the same box, (A) 16S rRNA gene (XF16S), (B) EFTu gene (EFTu), and (C) conserved hypothetical protein (HL). The equations of the curves are shown for each primer pair.

Additional file 2. Signal reduction (SR) in the qPCR of viable (white) and dead (grey) cells after treatment with different PEMAX concentrations. SR is the difference between the C_T value of non-PEMAX and PEMAX treated cells. Cell concentration was 1×10^7 CFU/ml. TaqMan-based qPCR assay XF16S-3 (amplicon length of 279 bp) was used for this experiment. The results are shown as the mean from three independent replicates, and error bars represent standard deviation of the means. Lowercase letters correspond to the means comparison of SR in viable cells. Capital letters correspond to the means comparison of SR in dead cells. Means sharing the same letters are not significantly different ($P < 0.05$), according to the Tukey's test.

Additional file 3. Effect of peptides **BP171** (circles) and **BP198** (triangles) on viability and culturability of Xff strain Temecula at different peptide concentrations. Cell viability was estimated by v-qPCR (black

symbols), and cell culturability by plate counting (grey symbols). An exposure time of 3 h and a cell concentration of 1×10^7 CFU/ml were used in both cases. The dash line represents the detection limit of v-qPCR, whereas the normal line indicates the detection limit of the plate counting technic. Values are the means of three replicates, and error bars represent the standard deviation of the mean.

Abbreviations

Xf: *Xylella fastidiosa*; AMPs: Antimicrobial peptides; VBNC: Viable-but-non-culturable; CTC: 5-cyano-2,3-ditolyl tetrazolium chloride; qPCR: Quantitative PCR; v-qPCR: Viable quantitative PCR; PMA or PMAxx: Propidium monoazide; EMA: Ethidium monoazide; CFU: Colony forming units; C_T: Cycle threshold; Xff: *Xylella fastidiosa* subsp. *fastidiosa*; Xfp: *Xylella fastidiosa* subsp. *pauca*; Xfm: *Xylella fastidiosa* subsp. *multiplex*; SR: Signal reduction; ΔC_T: Difference between cycle threshold values; MBC: Minimal bactericidal concentration; MIC: Minimal inhibitory concentration; PD2: Pierce Disease 2; BCYE: Buffered charcoal yeast extract; SCP: Succinate-citrate-phosphate; ANOVA: Analysis of variance

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Authors' contributions

ABa performed the main experiments and data analyses, and wrote the paper. EB, EM and ABo designed the research, analysed the data, and contributed in the writing process. MP and LF provided the AMPs. LM assisted in laboratory experiments and data analyses. EM, ABo, EB, MP and LF obtained the financial support. All authors read, review and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

CHAPTER V

Aggressiveness of Spanish isolates of *Xylella fastidiosa* to almond plants of different cultivars under greenhouse conditions

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Aggressiveness of Spanish isolates of *Xylella fastidiosa* to almond plants of different cultivars under greenhouse conditions

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ABSTRACT

The aggressiveness of Spanish isolates of *X. fastidiosa*, representing different sequence types, were studied in almond plants of several cultivars by means of the dynamics of the population levels and symptoms, colonization and spread, and dose-response relationships.

Pathogen dynamics in almond plants under greenhouse conditions showed doubling times of 2.1 to 2.5 days during the exponential growth phase, with a maximum population size around 35 dpi. A differential pattern in population dynamics was observed between sap and xylem tissue after the exponential growth, as population levels in the xylem tissue remained stable while viable cells in sap decreased. Population levels were higher in two upwards zones than in downwards zones, with respect to the inoculation area. The first symptoms were observed between 20 and 60 dpi, and disease severity increased over time at doubling times of 30 days, with a maximum observed at 120 dpi.

Strains tested showed differences in population levels in the cultivars studied and were able to spread with different intensity from contaminated plant parts to new growing shoots after pruning.

Two almond isolates showed a different performance in dose-response relationships when inoculated in Avijor cultivar. While IVIA 5387.2 reached

higher population levels but showed high ED_{50} and MID values, IVIA 5901.2 showed low population levels as well as low ED_{50} and MID values.
This study raises implications for the epidemiology of *X. fastidiosa* in almond crops, estimating doubling times of the pathogen *in planta* and of symptoms development, as well as showing differential aggressiveness between strains.

Keywords: *Xylella fastidiosa*, *Prunus dulcis*, aggressiveness, growth dynamics

INTRODUCTION

Diseases caused by *Xylella fastidiosa* are considered one of the most threatening in agriculture all over the world in recent years, causing serious economic losses in important crops (Almeida et al. 2019). Until 2013, the pathogen was present in the American continents, causing mainly Pierce's disease (PD) of grapevines in California and citrus variegated chlorosis (CVC) and coffee leaf scorch (CLS) diseases in Central and South America. However, the arrival of different subspecies of *X. fastidiosa* to the European Union (EU) changed its distribution worldwide (EFSA 2020). At present, *X. fastidiosa* is importantly affecting olive trees in Apulia region in Southern Italy (Saponari et al. 2019), *Polygala myrtifolia* in the PACA region in Southern France and Corsica (Denancé et al. 2017), and almond trees in Alicante region and Balearic Islands in Spain (Giampetruzzi et al. 2019). However, the potential to infect nearly 600 plant hosts, including cultivated and wild species, makes this pathogen one of the main concerns in plant protection (Sicard et al. 2018).

As the majority of diseases caused by *X. fastidiosa*, almond leaf scorch (ALS) disease is characterized by an initial leaf scorching followed by a general decline of the tree, which leads to a reduction of its health and productivity and, in some cases, to its death, directly or indirectly related to *X. fastidiosa* infections (Cao et al. 2013; Krugner and Ledbetter 2016). Although almond trees can remain asymptomatic from the start of bloom until kernel enlargement, it has been described that ALS affects yield, reducing it by 20 to 40 %, depending on the cultivar (Sisterson et al. 2012). Spain is the number one almond producer in the EU (83.9 % of its total almond crops) and accounts for 10.5 % of the world's almond

production (De Cicco 2018). ALS was first detected in Mallorca in 2017 caused by *X. fastidiosa* subsp. *fastidiosa*, and containment measures implemented were unable to reduce disease spread in almond crops, which are currently one of the most affected crops in Balearic Islands (Moralejo et al. 2019). In Alicante, ALS caused by *X. fastidiosa* subsp. *multiplex* was also detected in 2017, and strong eradication measures are currently being adopted (Giampetruzzi et al. 2019).

Although the epidemiology of *X. fastidiosa* diseases have been the object of research for many years (Purcell 2013; Almeida et al. 2019), one aspect that requires more studies is the explanation for the different intensity between outbreaks in a similar climatic area and crop, as well as the existence of asymptomatic plants or asymptomatic stages within an infected plant. Among several reasons, there is the possibility that these situations are due to differences in pathogenicity of the subspecies or strain responsible for the outbreak.

Pathogenicity is generally defined as the ability of a pathogen to infect a host and cause disease (De Wolf and Isard 2007; Surico 2013). It has been reported that pathogenicity is composed of virulence and aggressiveness, both genetically-controlled components (Bos and Parleviet 1995). However, while virulence is classified as a qualitative trait dependent on the presence/absence of virulence/R-genes, aggressiveness is generally defined as the quantitative variation of pathogenicity on susceptible hosts and is polygenically determined (Pariaud et al. 2009). Aggressiveness can be assessed as the capacity of a pathogen to migrate from the point of inoculation to other parts of the plant host, the progression of infection, and the amount of pathogen inoculum needed to produce a given effect on the host (Cabrefiga and Montesinos 2005). For this reason, the levels of disease severity and the pathogen population size after inoculating a host with a specific strain can be used to estimate its aggressiveness (Pariaud et al. 2009, Nutter et al. 2006).

In the case of *X. fastidiosa,* it is important to know if there is different aggressiveness among strains and species in a given plant host, because it can reveal important epidemiological consequences for the management of this quarantine pathogen (Lopes et al. 2010). Parameters

related to aggressiveness can be estimated from dose-response relationships such as the median effective dose (ED_{50}) and maximum population level (P_{max}), or the growth rate of the pathogen after its inoculation in the host plant (Dt, doubling times) (Cabrefiga and Montesinos 2005). Other interesting parameters include the capacity of spread of the pathogen from the point of inoculation to other plant parts (e.g. upwards or downwards spread from the inoculated shoot), as it has been used in other bacterial plant pathogens (Caires et al. 2020).

There are already studies about the pathogenicity of *X. fastidiosa* strains in different plant hosts, such as in grapevine and almond (Almeida and Purcell 2003; Cao et al. 2013; Rashed et al. 2013; Rogers and Ledbetter 2015; Sanderlin 2017), or in alfalfa (Lopes et al. 2010). However, due to the relevance of almond crops in the EU, especially in Spain, and the emergence of new genotypes of *X. fastidiosa* affecting this crop (Landa et al. 2020), it is necessary to study the aggressiveness of Spanish isolates of *X. fastidiosa* in almond cultivars of economic importance.

The aim of the present work was to study the aggressiveness of four strains of *X. fastidiosa* representing different subspecies and sequence types (STs) in three almond cultivars. In more detail, the objectives were: (1) to study the population dynamics of *X. fastidiosa* subsp. *fastidiosa* ST 1 isolated from almond plants in Mallorca and analyse symptoms development for a long period of time, (2) to determine differences in aggressiveness of four *X. fastidiosa* Spanish isolates against three almond cultivars and study their capacity to spread from the point of inoculation to adjacent tissues and new growing shoots, and (3) to determine dose-response relationships in order to estimate important parameters of aggressiveness of two new almond isolates of *X. fastidiosa* in a cultivar.

MATERIALS AND METHODS

Plant hosts and greenhouse conditions

One-year old almond plants (*Prunus dulcis*) from the cv. Avijor, Soleta and Penta provided by Agromillora S. L. U. (Spain) were used for the experiments. All plants were maintained in 0.8 l pots in an environmentally controlled greenhouse maintained at 25±2 ºC (day) and 18±2 ºC (night),

with a minimum relative humidity of 60 %, and with a photoperiod of 16 h light and 8 h dark. Prior and during the experiments, plants were watered to saturation every three days, and fertilized with a 200 ppm solution of NPK (20:10:20) once a week. Also, all along the experiments, standard treatments with insecticide and acaricide were performed to avoid presence of insect vectors or pests. One day before inoculation of *X. fastidiosa*, plants were not watered to enhance the uptake of the bacterial suspension. Infected plants were cultivated in a biosafety level II+ quarantine greenhouse according to EPPO recommended containment conditions (EPPO 2006) and taking into account the consideration of *X. fastidiosa* as a quarantine pathogen in the EU (EFSA 2018).

X. fastidiosa Spanish isolates

Three strains of *X. fastidiosa* subsp. *fastidiosa* (IVIA 5770, IVIA 5235.4 and IVIA 5387.2) and one strain of *X. fastidiosa* subsp. *multiplex* (IVIA 5901.2) isolated in years 2016 and 2017 from different outbreaks in Spain (Table 1), were used within the different experiments. Strains were maintained at -80 °C in PD2 broth supplemented with 30 % glycerol. When required, cryoconserved stock cultures were plated on BCYE solid medium and grown at 28 °C for two passages of 5 days each.

Table 1. *Xylella fastidiosa* Spanish isolates used in the present work.

Strain	Subspecies	Source[a]	Origin	Reference
IVIA 5235.4	*X. fastidiosa* subsp. *fastidiosa* ST 1	IVIA	cherry, Mallorca (Spain)	Landa et al. 2018
IVIA 5387.2	*X. fastidiosa* subsp. *fastidiosa* ST 1	IVIA	almond Mallorca (Spain)	E. Marco (pers. comm.)
IVIA 5770	*X. fastidiosa* subsp. *fastidiosa* ST 1	IVIA	grapevine, Mallorca (Spain)	E. Marco (pers. comm.)
IVIA 5901.2	*X. fastidiosa* subsp. *multiplex* ST 6	IVIA	almond, Alicante (Spain)	Giampetruzzi et al., 2019

[a] IVIA, Instituto Valenciano de Investigaciones Agrarias

Inoculation of *X. fastidiosa*

Suspensions of *X. fastidiosa* were prepared in phosphate buffered saline (PBS) to the required concentration, confirmed by plate counting. Because we observed cell aggregation inside the microsyringe during inoculation when using PBS and to ensure cell viability, cells were pelleted (10 min at 13,000 rpm) and resuspended in PD2 broth. Depending on the experiment, concentrations of 10^9 CFU/ml ($OD_{600} \cong 1$) or 10^8 CFU/ml ($OD_{600} \cong 0.3$) were used, as well as a non-treated control (PD2 broth).

Almond plants were inoculated using a high precision microinjector (NanoJet, Chemyx, Stafford, USA) provided with a Hamilton 250 µl syringe with a thin needle with bevel tip (Bonaduz, Switzerland) (Supplementary Fig. S1). The needle end was introduced until approximately one half the plant stem diameter to inoculate the vascular system. Three inoculations of the *X. fastidiosa* suspension of 10 µl each (30 µl of total inoculum/plant) were applied at the same side of the stem in a section of 3 cm at around 15 cm above the substrate level (Fig. 1). Inoculated plants were maintained in the above mentioned biosafety greenhouse.

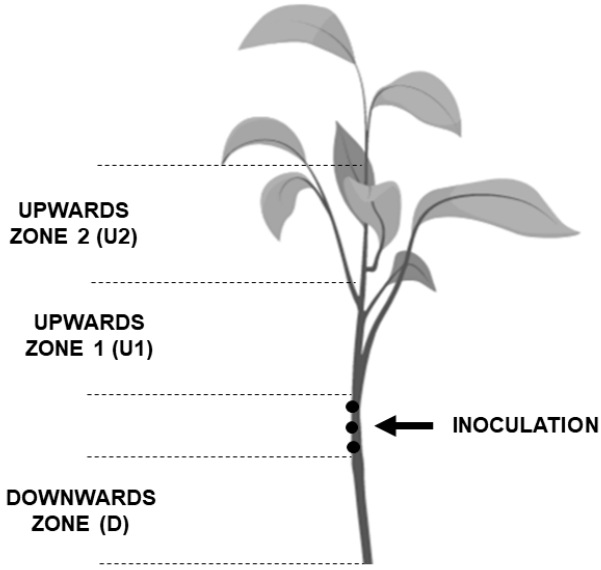

Figure 1. Scheme of the inoculation points and the sampled zones in almond plants. Upwards zones (U1 and U2) and downwards zone (D).

Sample processing, DNA extraction and qPCR analysis

The levels of *X. fastidiosa* in inoculated almond plants were analysed either by viability-qPCR (v-qPCR) or qPCR, depending on the plant material. To determine the movement of the pathogen from the inoculated area, 16 cm of shoot material were sampled above the inoculation points (U1 and U2 zones, 8 cm each zone), and 8 cm below (D zone) (Fig. 1). Before sample processing, bark was removed from each 8 cm fragment, to analyse vascular tissue material (including mostly xylem tissue).

v-qPCR was used to assess planktonic viable cells in sap, whereas conventional qPCR was utilized to quantify the total number of cells remaining in the stem, after sap extraction. For planktonic viable cells, sap was obtained from each 8 cm fragment, cutting the fragment in three parts and putting them in 2 ml centrifuge tube with a hole at the bottom. The 2 ml tubes were inserted inside 5 ml tubes, and the assembly was centrifuged at 13,000 rpm for 25 min. Sap collected in the 5 ml tube was diluted to a final volume of 500 µl with sterile PBS. 180 µl were treated with PMAxx (Biotum) at 7.5 µM while other 180 µl were used as a non-PMAxx treated sample. The PMAxx treatment was performed as previously described for the PEMAX reagent (Baró et al. 2020), modifying the dark incubation period to 7 min with manual shaking every 2 min.

To quantify the total number of remaining cells in the stem (biofilm/attached), the sap extracted xylem tissue was placed in extraction bags (Bioreba AG, Switzerland) with sterile PBS (ratio 1 ml / 0.2 g) and homogenized using a hammer followed by the Homex 6 semi-automated homogenizer (Bioreba AG, Switzerland). Bags were incubated 20 min at 4 °C, and 180 µl from the homogenate were collected and centrifuged for 10 min at 13,000 rpm. Then, the supernatant was discarded and the pelleted material containing cells was kept at -20 °C for further DNA extraction. In xylem tissue, the PMAxx reagent was not used because of interference of abundant plant DNA in the extract.

DNA extraction of all the samples (sap and xylem tissue) was performed using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) and analysed in duplicate. TaqMan-based qPCR was used as

described previously (Baró et al. 2020). The number of viable cells in sap, expressed as $\log_{10}$ CFU/ml, and of total cells in the xylem tissue, expressed as $\log_{10}$ CFU/g, was obtained by interpolating C_T values in the corresponding standard curves, made either with sap or with a plant extract from a healthy almond plant cv. Avijor, fortified with known concentrations of *X. fastidiosa*.

For some experiments (Figures 5, 6 and 7), the whole bark peeled shoot portion was used (combined sap and xylem tissue), and the material was analysed by TaqMan-based qPCR.

Population dynamics of *X. fastidiosa* in inoculated almond plants

A set of 30 almond plants cv. Avijor was inoculated with *X. fastidiosa* subsp. *fastidiosa* IVIA 5387.2, to determine its population dynamics inside the plant. Two experiments were performed. In the first experiment, plants were inoculated with 30 µl of a suspension adjusted to 10^8 CFU/ml ($OD_{600} \cong 0.3$), equivalent to a total of $3x10^6$ CFU/plant. In the second experiment, 30 µl of a turbid cell suspension corresponding to 10^9 CFU/ml ($OD_{600} \cong 1$), equivalent to $3x10^7$ CFU/plant, was used. The movement (U1, U2 and D zones) and the population of *X. fastidiosa* in sap or xylem tissue were determined by v-qPCR and qPCR in sap and by qPCR in xylem tissue as described above, at 10 different times (10, 20, 30, 40, 50, 60, 90, 120, 150 and 180 days post-inoculation (dpi)). Three plants for each time were used to determine the levels of *X. fastidiosa* in sap and xylem tissue at the U1, U2 and D sampling zones, and each plant was analysed individually. Symptoms were also evaluated following a severity scale adapted from the one already described for grapevines (Fig. 2) (Clifford et al. 2013).

Aggressiveness of *X. fastidiosa* isolates in different almond cultivars

The four *X. fastidiosa* Spanish isolates were inoculated in almond plants of each cultivar selected (*Penta*, *Soleta* and *Avijor*) in order to determine their aggressiveness. Each cultivar and strain combination consisted of 9 plants. Plants were inoculated with a suspension of the corresponding strain adjusted to 10^8 CFU/ml, confirmed by plate counting. After 60 dpi, symptoms were assessed and *X. fastidiosa* was analysed in six out of the nine plants inoculated, using bark peeled stem fragments without previous sap extraction (xylem tissue including sap). Disease intensity was

evaluated following the severity scale as described above. The remaining three plants were kept to continue symptomatology observation over time. To study the spread of *X. fastidiosa* in new shoots after pruning for sampling, the sampled plants were additionally maintained in the greenhouse. The levels of *X. fastidiosa* in the 60 days-new shoots were quantified by qPCR (expressed as $\log_{10}$ CFU/g) following the procedure described above for the first sampling. The assay was repeated twice, although in the first experiment three plants for each strain-cultivar combination were used.

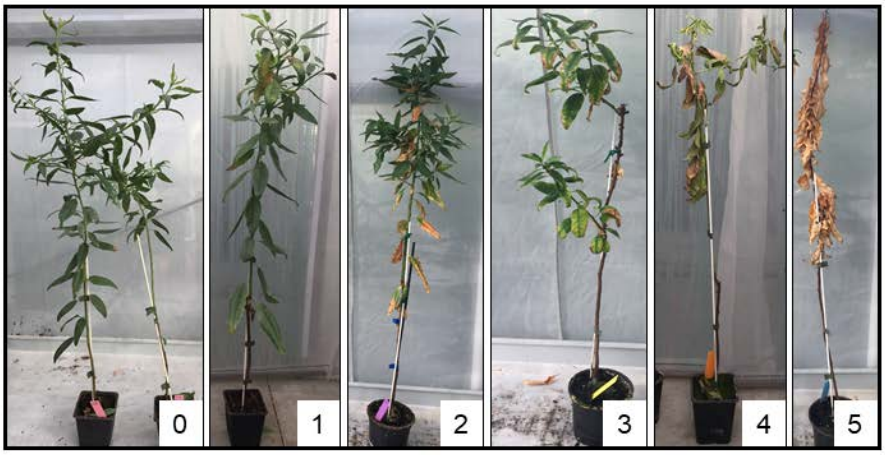

Figure 2. Severity scale used to rate almond plants for ALS disease development. Adapted from Clifford et al. (2013) described for grapevines, 0 = no ALS symptoms, 1 = from one to five leaves just beginning to show marginal necrosis, 2 = from five to ten leaves showing significant marginal necrosis, 3 = one-half or more of the leaves showing marginal necrosis, 4 = all of the leaves showing heavy scorching or fallen, 5 = dead plant.

Dose-effect relationships of two *X. fastidiosa* strains in almond plants

X. fastidosa subsp. *fastidiosa* IVIA 5387.2 and *X. fastidosa* subsp. *multiplex* IVIA 5901.2, isolated from almond, were inoculated in almond plants cv. Avijor to determine their aggressiveness. For both strains, suspensions containing 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 CFU/ml were prepared. Nine plants per dose were inoculated as described above, and the total inoculated amounts (3x10 µl injections) were $3x10^1$, $3x10^2$, $3x10^3$, $3x10^4$, $3x10^5$, $3x10^6$, and $3x10^7$ CFU per plant. For each strain, six plants of each

dose were analysed individually 40 dpi, quantifying the levels of *X. fastidiosa* on xylem tissue (including sap) as described above. The remaining three plants were kept for symptoms observation over time.

Data analysis

To test the differences in disease severity at different time points after inoculation (from 10 days and up to 6 months post-inoculation), a one-way analysis of variance (ANOVA) was performed. To test the differences between *X. fastidiosa* strains affecting the same almond cultivar, and the differences between almond cultivars once inoculated with the same *X. fastidiosa* strain, a one-way analysis of variance (ANOVA) was performed. In all cases, means were separated according to the Tukey's test at a *P* value of ≤0.05.

The effect of the inoculation dose of strains IVIA 5387.2 and IVIA 5901.2 was tested using a one-way analysis of variance (ANOVA). Data on dose-response relationships were adjusted by non-linear regression to the following log-logistic model to determine the maximum population level (P_{max} asymptote), and the median infective dose (ED_{50}):

$$Y = c + \frac{d - c}{1 + exp(b(X - e))}$$

where, *d* is the higher asymptote (P_{max}), *c* is the lower asymptote, *e* is the ED_{50}, and *b* is the slope of the effect-dose curve. The *c* value was fixed to the level of detection of our qPCR method (10^3 CFU/g tissue).

To test the differences in the P_{max}, ED_{50} and minimal infective dose (MID) parameters between strains, a student's unpaired *t*-test was performed with the estimated values and the standard error obtained from the model.

RESULTS

Dynamics of *X. fastidiosa* and disease progression in almond plants

The dynamics of the population levels of *X. fastidiosa* IVIA 5387.2 and disease symptoms progression after inoculation of almond plants cv. Avijor for the two experiments performed is presented in Figure 3.

Quantification of total and viable cells in sap was performed by qPCR (C_T = - 3.6 log CFU · ml^{-1} + 46.6 and R^2 = 0.99) and v-qPCR (C_T = - 3.6 log CFU · ml^{-1} + 47.3 and R^2 = 0.99), respectively. Because the data obtained by v-qPCR and qPCR were similar (Supplementary Fig. S2), it was considered that all cells in sap were viable.

The population dynamics showed two clearly distinguishable phases in the three plant zones (U1, upwards zone 1; U2, upwards zone 2; D, downwards zone). The first stage (from 0 to 40 dpi) showed an exponential growth of *X. fastidiosa* in both experiments, and either in sap and xylem tissue (after sap extraction), with a maximum population at c.a. 35 dpi. The growth rate during this first stage was similar in the upwards (0.34 ± 0.03 day-1 (d-1)) and in the downwards zones (0.28 ± 0.02 d-1), corresponding to doubling times of 2.06 ± 0.16 and 2.44 ± 0.16 days, respectively. During the second stage (from 40 to 180 dpi) the population levels and dynamics were also similar in the three zones sampled and for the two experiments. However, sap and tissue levels differed significantly in this stage (2 to 3 log CFU), because *X. fastidiosa* remained stable in tissue levels whereas it decreased exponentially in sap levels through time. The rate of decay of the sap population in upwards zones was of 0.044 ± 0.007 d-1, corresponding to a decimal reduction time of 22.94 ± 3.78 days, while it ranged from 0.023 to 0.039 d-1 in downwards zone, equivalent to a range of decimal reduction time from 25 and up to 44 days.

The symptoms development was also evaluated all along the experiment. Up to 60 dpi, only few leaves started showing marginal necrosis, without significant differences over time. However, after 60 dpi, disease severity increased, and more leaves started to show marginal necrosis with leaf scorching, passing from green to yellow-red, and in some cases, starting to present significant marginal necrosis. Symptoms were maximum at 120 dpi. At the end of the experiment, almost all plants from both sets had more than one half of their leaves showing significant marginal necrosis. The maximum rate of disease progression happened between 60 to 120 dpi, and was similar in both experiments, being 0.026 d-1 in experiment 1 (doubling time of 27 days) and of 0.024 d-1 in experiment 2 (doubling time of 28 days).

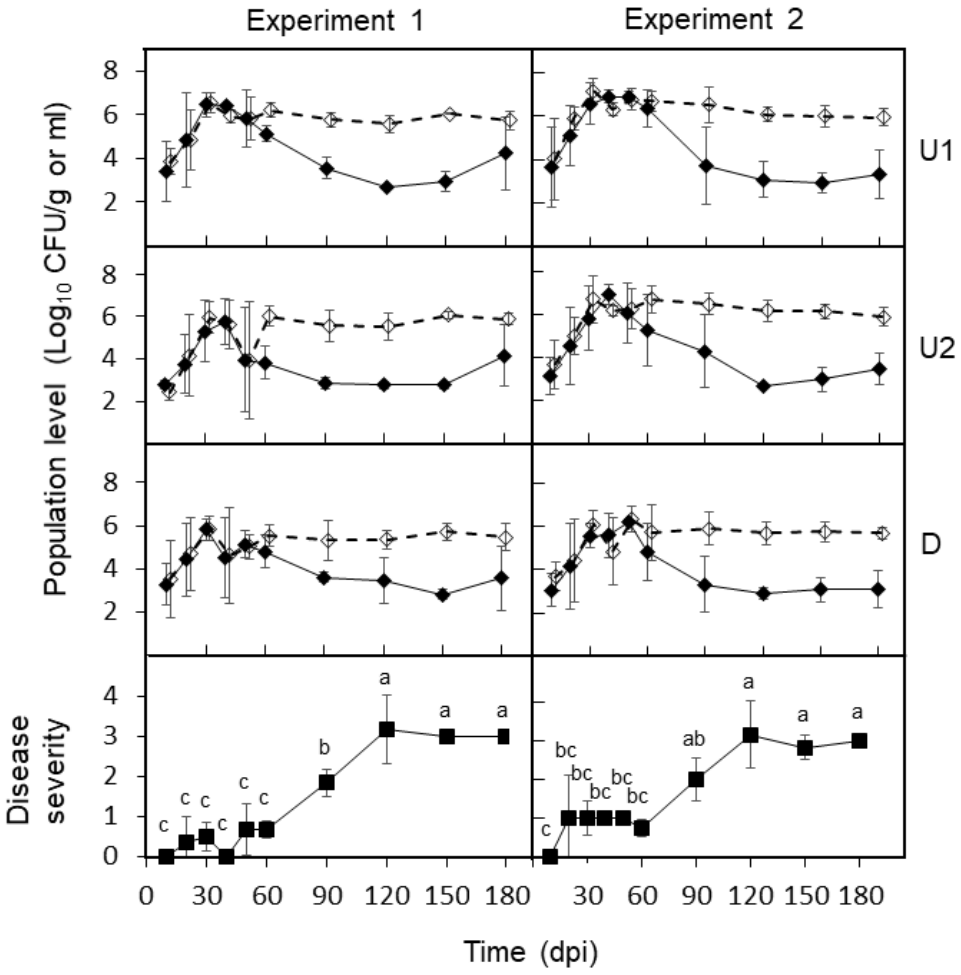

Figure 3. Dynamics of *X. fastidiosa* subsp. *fastidiosa* IVIA 5387.2 and symptoms development in inoculated almond plants of the cv. Avijor. Two independent assays were performed at different inoculum concentrations ($3x10^6$ CFU/plant in experiment 1, and $3x10^7$ CFU/plant in experiment 2). The plants were sampled at 10, 20, 30, 40, 50, 60, 90, 120, 150 and 180 dpi. Viable cells (qPCR) in sap (black symbols – solid line) and remaining cells (qPCR) in xylem tissue (white symbols – dash line) in the three plant zones: U1 (diamonds), U2 (circles) and D (triangles). Disease severity is shown in the bottom panels. Values are the means of three plants, and error bars represent the standard deviation of the mean. Different letters within each experiment indicate significant differences between symptoms according to the Tukey's test ($P < 0.05$).

Colonization and symptoms development in almond cultivars

The aggressiveness of four *X. fastidiosa* strains isolated from recent outbreaks in Spain was studied in three almond cultivars. The population levels after 60 dpi are shown in Figure 4. The cv. Penta was the most susceptible to all *X. fastidiosa* strains tested, showing levels between 10^8 and 10^9 CFU/g in almost all zones of the plant sampled. *Soleta* was the least susceptible to *X. fastidiosa* IVIA 5901.2, while *Avijor* was less susceptible to strain IVIA 5770. Although differential population levels were observed, the bacterial load fluctuated between 10^6 and 10^8 CFU/g among the cultivars, indicating that all strains were able to colonize the three almond cultivars. In general, the downwards zone was the one with lower concentration of *X. fastidiosa* compared to upwards zones. Among strains, and globally within the three cultivars, the strain IVIA 5387.2 acquired the highest population levels, followed by IVIA 5235.4. The strain IVIA 5770 was also highly infective in *Penta* and *Soleta.* The strain IVIA 5901.2 was the one with lower bacterial load, especially in cv. Soleta.

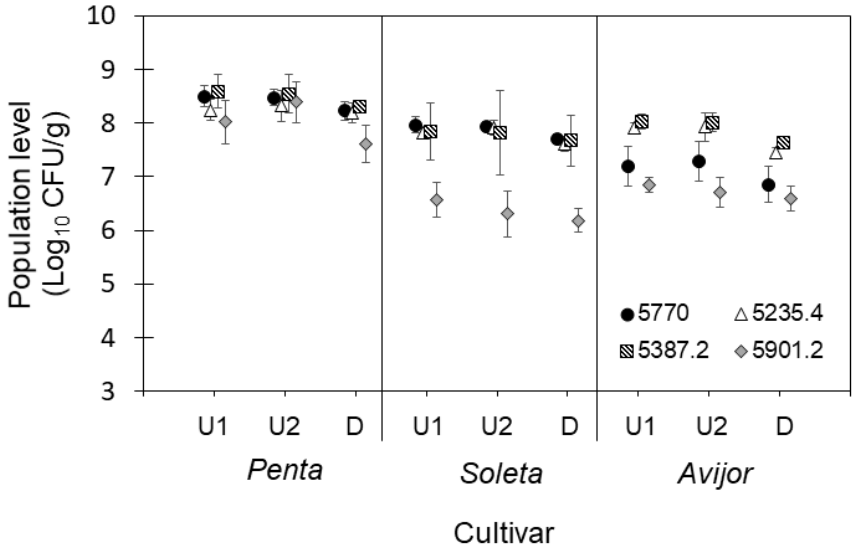

Figure 4. Population levels of four strains of *X. fastidiosa* (IVIA 5770, ST 1; IVIA 5235.4, ST 1; IVIA 5387.2, ST 1; IVIA 5901.2, ST 6) in the different plant zones (U1, U2 and D) of three almond cultivars (*Penta*, *Soleta* and *Avijor*) at 60 dpi. The inoculation dose was $3x10^6$ CFU/plant. The analysis was performed in combined sap and xylem tissue. Values are the means of six plants, and error bars represent the standard deviation of the mean.

Disease severity is shown in Figure 5. There was also a different susceptibility to the four *X. fastidiosa* strains between the three cultivars. Cv. Penta was the most susceptible, whereas *Avijor* was less susceptible. Consistently, strain IVIA 5770 caused the highest disease levels, and IVIA 5901.2 caused very low symptoms in *Penta* and *Soleta* and none in cv. Avijor.

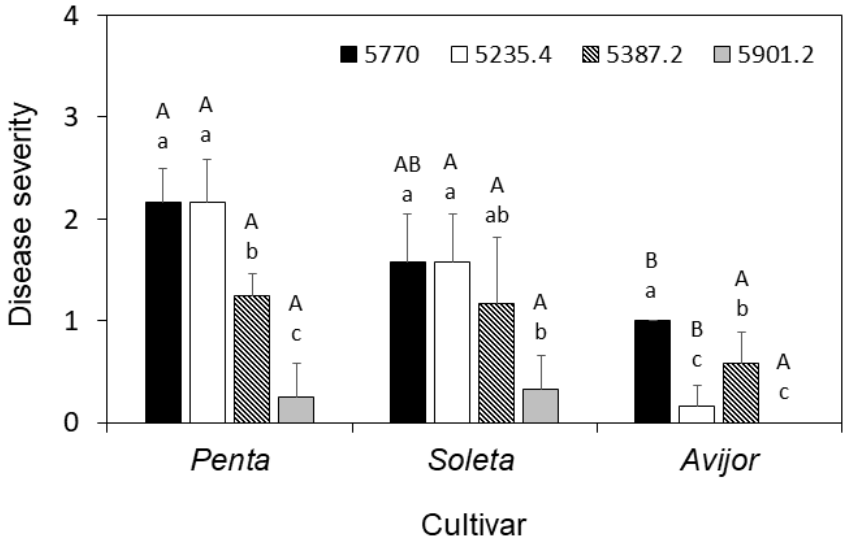

Figure 5. Disease severity in three almond cultivars, 60 days after being inoculated with different *X. fastidiosa* strains (IVIA 5770, ST 1; IVIA 5235.4, ST 1; IVIA 5387.2, ST 1; IVIA 5901.2, ST 6). Values are the means of six plants, and error bars represent the standard deviation of the mean. Capital letters correspond to the comparison between almond cultivars inoculated with the same strain. Lowercase letters correspond to the comparison between *X. fastidiosa* strains inoculated in the same cultivar. Means sharing the same letters are not significantly different ($P < 0.05$), according to the Tukey's test.

Spread of *X. fastidiosa* from below the basal zone to new shoots grown after pruning

The *X. fastidiosa* population levels detected in the new shoots that emerged 60 days after sampling (pruning) the infected plants inoculated with the four strains of the pathogen is shown in Figure 6. Cv. Penta showed the highest levels while cvs. Avijor and Soleta had the lowest levels for the four pathogen isolates. IVIA 5901.2 showed lower population levels in cv. Penta compared to the other strains, and was not detected in the

new shoots of Avijor and Soleta. Strains IVIA 5770, IVIA 5387.2 and IVIA 5235.4 were detected in the three cultivars, with IVIA 5387.2 being the one with the highest population levels. Thus, X. fastidiosa that has been inoculated 60 days before, spreads from below the basal zone to new growing shoots after pruning, but there was a differential capacity to colonize new plant parts depending on the strain and almond cultivar.

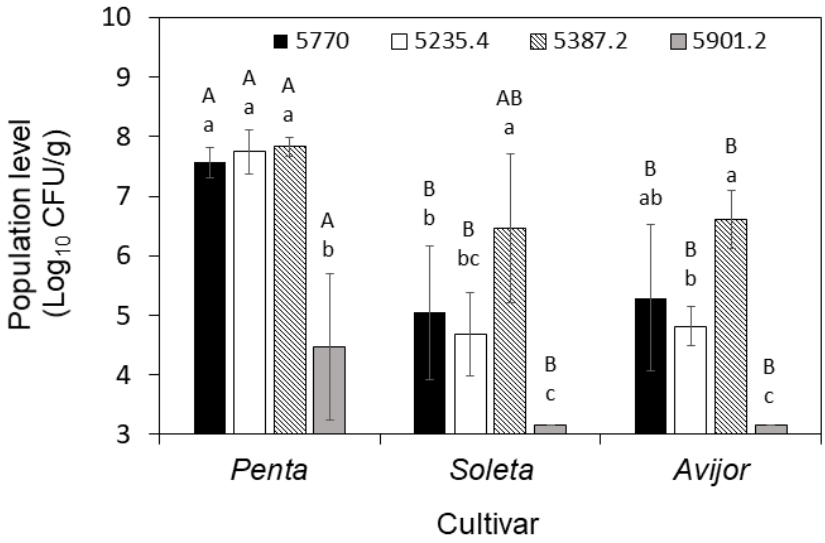

Figure 6. Spread of *X. fastidiosa* (IVIA 5770, ST 1; IVIA 5235.4, ST 1; IVIA 5387.2, ST 1; IVIA 5901.2, ST 6) from above the D zone of inoculated almond plants to the newly grown shoots in three almond cultivars. Inoculated plants were sampled 60 dpi by cutting the main axis shoot at the very down part of D zone, for the analysis. Then, plants were left to grow for 60 additional days and the presence of *X. fastidiosa*, in combined sap and xylem tissue, was quantified. Values are the means of six plants, and error bars represent the standard deviation of the mean. Capital letters correspond to the comparison between almond cultivars inoculated with the same strain. Lowercase letters correspond to the comparison between *X. fastidiosa* strains inoculated in the same cultivar. Means sharing the same letters are not significantly different ($P < 0.05$), according to the Tukey's test.

Dose-effect relationships in two *X. fastidiosa* isolates

The effect of inoculation doses (from 30 and up to $3x10^7$ CFU per plant) in the colonization of strains IVIA 5387.2 and IVIA 5901.2 in almond plants of the cv. Avijor is presented in Figure 7. The relationship between the dose of inoculum and population levels at 40 dpi showed similar saturation

patterns in all plant zones for the same *X. fastidiosa* strain. In the case of IVIA 5387.2, significant differences were observed between high ($3x10^5$ to $3x10^7$ CFU/plant), intermediate ($3x10^3$ and $3x10^4$ CFU/plant) and low (300 CFU/plant) inoculation doses in all plant zones. Differently, IVIA 5901.2 only showed significant differences between the lowest inoculation dose (30 CFU/plant) and the highest inoculation dose ($3x10^7$ CFU/plant).

To estimate the ED_{50} and P_{max}, data sets were adjusted to the 4 parameter log-logistic model. Additionally, a range of values were estimated for the MID (Table 2). When comparing strains, IVIA 5387.2 reached a higher P_{max} than strain IVIA 5901.2 in all plant zones. The P_{max} mean was 7.25 ± 0.53 log CFU/g for the IVIA 5387.2 strain, and 6.31 ± 0.78 log CFU/g for IVIA 5901.2 strain. The ED_{50} estimated for IVIA 5387.2 was the same in U1 and D zones, with a value of 2.5 log CFU/plant. In the U2, this parameter was significantly higher than in the other plant zones, being estimated at 3.75 log CFU/plant. Contrarily, ED_{50} estimated for IVIA 5901.2 was not significantly different between plant zones, ranging from 0.98 to 1.35 log CFU/plant. Globally, strain IVIA 5387.2 acquired higher population levels than IVIA 5901.2, whereas IVIA 5901.2 had a lower ED_{50} than IVIA 5387.2.

Table 2. Estimation of the parameters of aggressiveness, according to a log logistic model, in two strains of *X. fastidiosa* inoculated in *Avijor* almond cv. (data from Fig. 6). Values are followed by the standard error.

Strain	P_{max}[a]			ED_{50}[b]			MID[c]
	U1	U2	D	U1	U2	D	
IVIA 5387.2	7.22 ± 0.11	7.73 ± 0.55	6.8 ± 0.14	2.5 ± 0.05	3.75 ± 0.89	2.5 ± 0.07	300-3000
IVIA 5901.2	6.5 ± 0.53	6.89 ± 1.54	5.55 ± 1.06	1.35 ± 0.42	1.20 ± 0.84	0.98 ± 1.03	30-300
Significance	*	n.s.	*	*	*	*	*

[a] P_{max}, maximum population level (log_{10} CFU/g)

[b] ED_{50}, median effective dose (log_{10} CFU/plant)

[c] MID, minimal infective dose (CFU/plant)

U1, upwards zone 1; U2, upwards zone 2; D, downwards zone

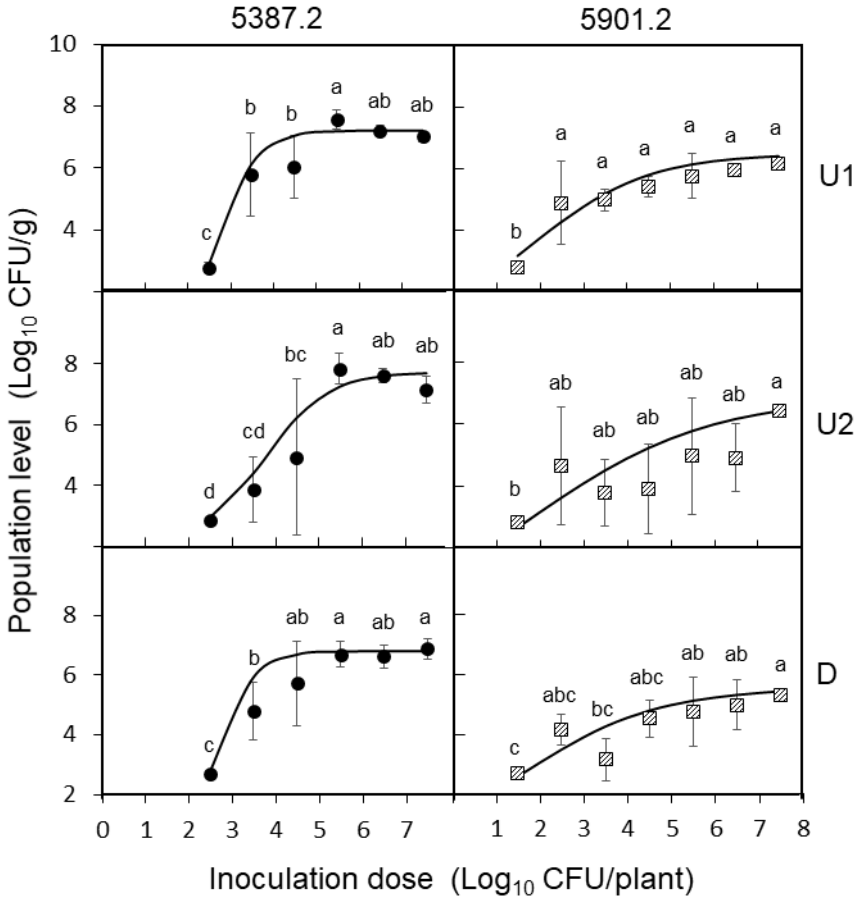

Figure 7. Effect of the inoculation dose in the colonization of almond plants of the cv. Avijor by *X. fastidiosa*. Two assays were performed, one with *X. fastidiosa* subsp. *fastidiosa* IVIA 5387.2 and another with *X. fastidiosa* subsp. *multiplex* IVIA 5901.2. The presence of *X. fastidiosa* in plant material (combined sap and xylem tissue) was quantified in the different plant zones: upwards (U1, U2) and downwards (D). Values are the means of six plants, and error bars represent the standard deviation of the mean. Different letters within the same panel indicate significant differences between inoculation doses according to the Tukey's test ($P < 0.05$). The continuous line in each panel represents the fit of data to the log-logistic model described in the materials and methods.

DISCUSSION

The epidemiology of diseases caused by *X. fastidiosa* is complicated and uncertain, mainly because of its wide range of host species, its genetic variation and the need of vectors (Occhibove et al. 2020). *X. fastidiosa* strains have been reported to differ in infectivity, population growth rate, movement within hosts, and capacity to cause disease in the same host (Lopes et al. 2010). Because information about pathogenicity has important implications in epidemiology (Morris et al. 2019), research on strains of *X. fastidiosa* in almond was performed in the present study.

The microinjection system, used for inoculation of *X. fastidiosa*, was notably different from the systems currently utilized in other reports, which are the pin-prick and the injection methods (Almeida et al. 2001, EPPO 2019). In the more frequently used pin-prick inoculation method, a drop of a highly concentrated *X. fastidiosa* suspension is placed on a leaf axil and punctured several times with an entomological needle, whereas in the microinjection method a microprocessor controller is connected to a microsyringe with a thin needle with bevel tip, to infiltrate known volumes of the bacterial inoculum. In the pin-prick method, the inoculum absorption is dependent on the evapotranspiration rate of the plant (physiological state and environmental conditions), while in the microinjection method, this parameter is relatively independent from evapotranspiration rates as the inoculum volume is placed into the vascular system, mimicking the insect vector feeding/inoculation process. Although the pressure during the injection process may affect plant tissue or pathogen cell performance in the area of puncture, or may cause inoculum cell aggregation in the syringe, the flow (from 10 to 1000 µL/min), injection time, syringe injection volumes and needle thickness can be adjusted to prevent adverse effects. Thus, we recommend the microinjection method for dose-response assays, where accurate doses have to be inoculated in a large number of plants. However, the relatively high cost of the microinjector has to be taken in account, as well as the requirement of two people for performing the inoculation.

Dynamics of *X. fastidiosa* subsp. *fastidiosa* strain IVIA 5387.2 in young almond plants showed doubling times of around 2 days during the exponential phase, which was established from 0 to 40 dpi. As far as we

know, no references related with the growth rate of other *X. fastidiosa* strains *in planta* have been reported yet, but multiplication of *X. fastidiosa* cells in grapevines was hypothesized to take place between 3 and 9 weeks (Sicard et al. 2020). In our case, using young almond plants, the latent period before the start of the exponential phase was not as long as it seems to be in grapevine. However, the doubling times observed by us in almond with a strain of *X. fastidiosa* were in agreement with the 0.45 to 1.9 days reported in a rich culture medium and at optimal temperature (Wells et al 1987; De la Fuente et al. 2008). Our findings demonstrate that, in young almond plants under greenhouse conditions, the bacteria can grow nearly the growth potential in rich culture media.

It is worth to consider that this experimental approach does not represent the agronomic conditions in most of the current almond plantations, and that the actual growth rate in the field might be different, as it is affected by many factors such as cultural practices, tree age and weather conditions, within others. Nevertheless, young shoots are still developed during the vegetative growth of old trees, being the preferred source of feeding and for pathogen inoculation by the insect vectors of *X. fastidiosa* (Bi et al. 2007). Moreover, our results with young plants can also be comparable to the situation in nurseries and in super-intensive almond orchards, where juvenile trees with extensive growing shoots are planted and optimal rates of irrigation and fertilization are being used (Casanova-Gascón et al. 2019). Our interest in using optimal conditions for both, the pathogen and the host plant, was to investigate the maximum ALS disease potential, that is, under the worst epidemiological scenario. So, despite the differences between our approach and most field conditions, doubling times determined in the present study could serve as a baseline for estimating the growth rate in the field, which is still needed for the management of ALS and of other diseases caused by *X. fastidiosa*.

At the end of the exponential phase, around 35 dpi, a maximum population size in almond ranging from 10^6 to 10^8 CFU/g or ml (taking into account both experiments and the three plant parts analysed) was observed in both, sap and xylem tissue, prior to symptoms development. This peak of *X. fastidiosa* population prior to full disease symptoms observation was also reported in other studies with grapevine (Sicard et

al. 2018). Additionally, similar population levels were detected in inoculated grapevines (Fritschi et al. 2008; Sicard et al. 2019) and in naturally infected olive trees (Saponari et al. 2017), indicating that population sizes observed in different plant hosts are of the same order.

A differential pattern in population dynamics was observed between viable cells in sap and total cells in xylem tissue, after the exponential growth phase in the inoculated almond plants. While population levels in the xylem tissue remained stable until the end of the experiment, viable cells in sap decreased after achieving the maximum population size, with a decay rate from 22 to 45 days. These observations are in agreement with the already predicted model for *X. fastidiosa* plant colonization, which describes a first phase of systemic spread and multiplication through the whole plant xylem system, corresponding to the plant invasion phase, and a second one in which cells start to become locally abundant and more "sticky" to the xylem vessels, ready to be acquired and transmitted by insect vectors (Chatterjee et al. 2008).

As in other studies using grapevines and almond plants (Fritschini et al. 2008; Cao et al. 2011; Rashed et al. 2013), in our study the first symptoms were reported between 20 and 60 dpi, always closely located near the inoculation points and progressively spreading above. We have shown that disease severity increased over time at doubling times of 27-28 days, being maximum between 60 to 120 dpi, and the highest disease severity was observed from 120 dpi. At the end of the experiment plants had one-half or more of the leaves showing marginal necrosis but still actively growing. This interruption of the disease progression is in accordance with the hypothesis that *X. fastidiosa* imposes self-limiting behaviours even in its parasitic form, presenting characteristic traits of commensal bacteria (Roper et al. 2019).

From a general perspective, taking together the dynamics of cells population size and disease severity, our findings with *X. fastidiosa* subsp. *fastidiosa* IVIA 5387.2 in young almond plants are in accordance with other studies using grapevines and citrus, reporting a period of time (around 80 to 90 dpi) with a stable or slightly decreased population size preceding the increase in disease severity (Rashed et al. 2013; Niza et al. 2015).

Once we determined the time-course of the population levels and symptoms in strain IVIA 5387.2 and Avijor cultivar, we studied the aggressiveness of four isolates of *X. fastidiosa* corresponding to subspecies *fastidiosa* and *multiplex* in almond cultivars. Although significant differences in the population levels of *X. fastidiosa* were observed between the strain-cultivar combinations, the spread through the whole plant was similar within cultivars, as it was observed in other studies using almond and grapevine (Fritschi et al. 2008; Cao et al. 2011; Rashed et al. 2013). Generally, in all experiments performed in the present study, similar population levels were detected in both upwards zones (U1 and U2), while lower concentrations were found in downwards zone (D). This is in agreement with other studies reporting that *X. fastidiosa* spread is more likely to upwards zones because the direction of xylem sap flow is acropetal, achieving longer-distance travels upstream than downstream (Meng et al. 2005).

Although strain IVIA 5770 was isolated from grapevines, it was able to colonize three almond cultivars, showing population levels similar to the ones detected for the other *X. fastidiosa* strains isolated from *Prunus* spp. (IVIA 5235.4, IVIA 5387.2 and IVIA 5901.2). In other studies, strains isolated from grapevines were able to cause ALS symptoms in several almond cultivars (Almeida and Purcell 2003; Cao et al. 2011). Additionally, we observed that IVIA 5770 and more intensely IVIA 5901.2, showed significant differences in population levels between the three almond cultivars. This is in agreement with the report that the concentration achieved by *X. fastidiosa* in plants is dependent on the host and on the strain type (Almeida and Purcell 2003). A similar situation was observed in the case of disease severity, which was dependent on the strain of *X. fastidiosa.* The grapevine isolate IVIA 5770, the cherry isolate IVIA 5235.4 and the almond isolate IVIA 5387.2 caused stronger symptoms in each almond cultivar studied compared to IVIA 5901.2, which was isolated from almond. This has also been observed in alfalfa, where grape isolates caused more severe stunning than almond isolates, indicating that *X. fastidiosa* genetic variability plays a role in disease severity more than the isolation host (Lopes et al. 2010).

The capacity of spread of *X. fastidiosa* to new shoots from a contaminated plant part was proven in the present study in all almond cultivars tested. In *Penta*, similar levels of the four strains were observed in the new shoots in comparison with the levels observed in the first sampling (60 dpi from the *X. fastidiosa* inoculation), except for the IVIA 5901.2. For the other two cultivars, *Soleta* and *Avijor*, only strains belonging to the subspecies *fastidiosa* (IVIA 5235.4, IVIA 5387.2 and IVIA 5770) were detected. Our findings in inoculated almond plants agreed with pruning experiments in diseased olive trees (Carlucci et al. 2016) and with PD affected grapevines (Kirkpatrick 2008), in which pruning, alone or combined with several bioactive compounds, induced quickly regeneration of new branches and the trees remained free of symptoms for a number of years, but finally disease symptoms reappeared due to the persistent inoculum of *X. fastidiosa*. Moreover, the higher attractiveness of new shoots to insect vectors due to their symptomless aspect (Daugherty et al. 2011), can make them an important source of disease spread (EFSA 2016).

The dose-effect relationships established for IVIA 5387.2 and IVIA 5901.2 almond isolates clearly showed a different strain performance: while IVIA 5387.2 reached high population levels but its ED_{50} was also high, IVIA 5901.2 attained population levels below the ones of IVIA 5387.2 but its ED_{50} was low. Yet, in both cases, higher inoculum doses lead to higher population levels over time, as it was also observed in alfalfa (Lopes et al. 2010). Although differences in the ED_{50} and P_{max} between strains revealed different strain aggressiveness in the same almond cultivar, in both strains, values were comparable to other bacterial plant pathogens. For example, the ED_{50} of several *Erwinia amylovora* strains ranged between 1 to 6 Log_{10} CFU/fruit in immature fruits (Cabrefiga and Montesinos 2005), while in this study ED_{50} has been estimated between 0.98 and 1.35 Log_{10} CFU/plant for IVIA 5901.2 and between 2.50 and 3.75 Log_{10} CFU/plant for IVIA 5387.2.

The MID was estimated to be for the strain IVIA 5901.2 between 30 and 300 CFU/plant and for IVIA 5387.2 between 300 and 3000 CFU/plant. These data can be compared with the *X. fastidiosa* load found in vectors and their potential for deliver infective inoculum. Studies with leafhoppers and spittlebugs vectors have reported *X. fastidiosa*

concentrations in the insect heads ranging from 30 to $5x10^5$ CFU or copies/head (Ramirez et al. 2008; Cornara et al. 2016). These values found in insect heads and the ED_{50} and MID observed for the strains in our study indicate that vectors could easily transmit the disease. However, transmission of *X. fastidiosa* is not only impacted by dose but also by host plant, pathogen strain and vector species.

CONCLUSIONS

This study raises implications for the epidemiology of *X. fastidiosa* directly in almond crops, but also to other host plants. Although, population dynamics of *X. fastidiosa* in almond plants follows the already predicted patterns, the latent period observed prior to exponential phase was shorter compared to the ones reported for other host plants such as grapevines or olive. During the exponential phase, the growth rate observed in young almond plants is of the same order than the one observed in rich culture medium. Additionally, differential aggressiveness among Spanish strains isolated from different plant hosts was observed in almond, in which parameters such as the P_{max}, ED_{50} and MID dose were estimated. Finally, re-infection of new growing shoots also occurs in almond plants, indicating that pruning of the affected branches reduces the population of *X. fastidiosa*, but do not eliminate the pathogen from the plant.

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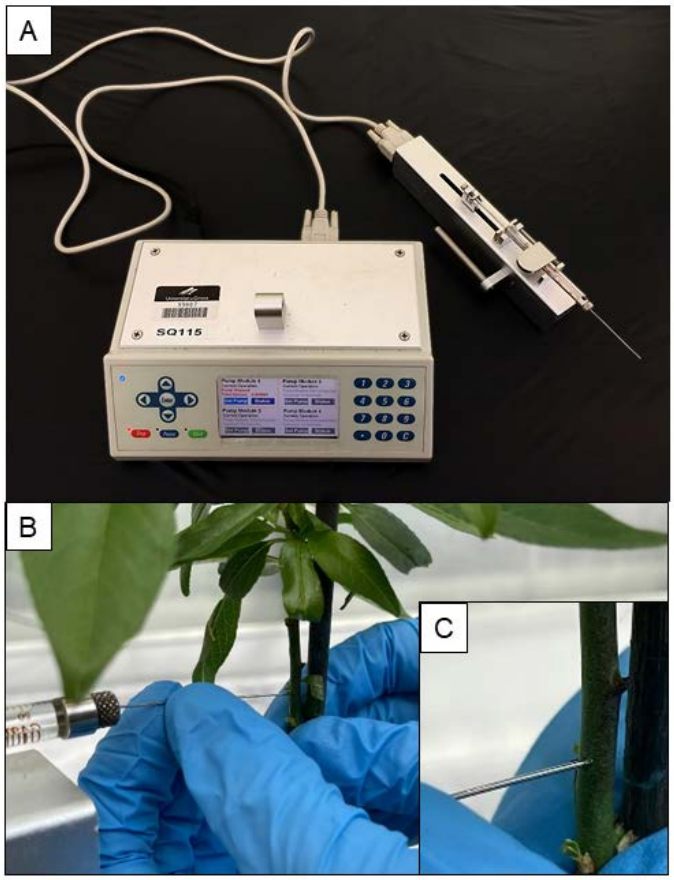
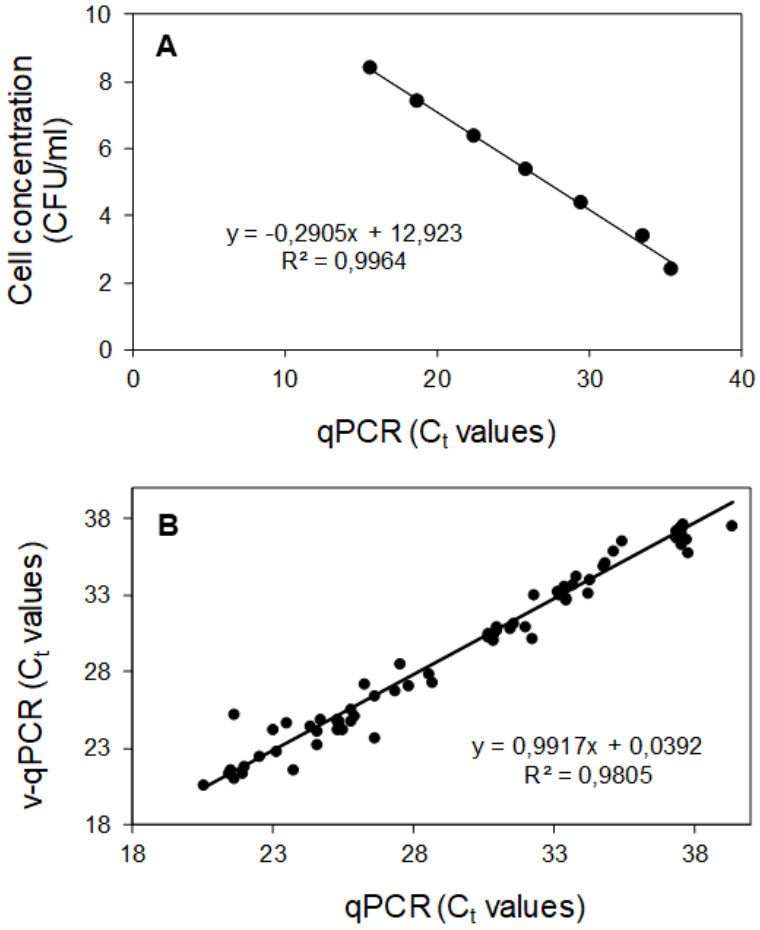
SUPPLEMENTARY FIGURES



Supplementary figure S1. Overview of the microinjection system for the inoculation of *X. fastidiosa*. (A) High precision microinjector (NanoJet, Chemyx, Stafford, USA) provided with a Hamilton 250 µl syringe connected to a needle with bevel tip (Bonaduz, Switzerland); (B) Inoculation process in one-year old almond plants; (C) Detail of the inoculation point.



Supplementary figure S2. (A) Relationship between culturable cells of *X. fastidiosa* (Log_{10} CFU/ml) and qPCR data (C_t values). (B) Regression between C_t values of v qPCR (viable cells) and qPCR (total cells) of *X. fastidiosa* in sap.

CHAPTER VI

General discussion

Agriculture and food related industries have always been sectors of social and economic importance in the EU, one of the world's leading producers and exporters of agricultural products. Since 2013, these sectors are facing an important threat due to the detection of *X. fastidiosa* in Apulia (Italy), one of the most harmful plant pathogens worldwide. In fact, an annual production loss of 5.5 billion euros was estimated in a scenario of full *X. fastidiosa* spread across the EU, affecting around 300,000 jobs associated with this production (Sánchez et al., 2019). So, even though nowadays *X. fastidiosa* is only present in specific regions of Italy, France and Spain, its control is of vital importance as distribution models have predict a large amount of climatically suitable areas throughout the continent (Godefroid et al., 2019).

A wide range of strategies, including chemical and biological control and genetic engineering, have been investigated in order to manage *X. fastidiosa*-related diseases (Kyrkou et al., 2018). Nevertheless, the pathogen is still an important challenge worldwide, as no effective measures are available for its full control (EFSA, 2019a). Currently, in regions where *X. fastidiosa* is present, IDM programs based on the control of insect vectors, the elimination of infected plants and the management of weeds, in addition to the annual prospections and the restrictions in the movement of specified plants, are being implemented (EFSA, 2019b; EU, 2020).

In recent years, AMPs have represented a promising alternative to conventional bactericides based on chemical compounds due to their excellent biocompatibility, low immunogenicity, biodegradation capability, and biological activity (Rong et al., 2018). They present different modes of action, such as selective and direct killing of prokaryotic organisms, interaction with intracellular targets causing inhibition of key processes, and elicitation of the plant immune system (Meneguetti et al., 2017). Their strong potential is also due to their availability for creating both, sustainable pesticides and disease resistant crops (Rajasekaran et al., 2012; Montesinos L., 2014).

For the control of *X. fastidiosa*, AMPs have already been tested *in vitro* showing highly bactericidal activity against different strains of this pathogen (Li and Gray, 2003; Andersen et al., 2004; Kuzina et al., 2006; Fogaça et al., 2010; Arora et al., 2018). Additionally, genetically modified grapevines expressing two different AMPs have been produced and evaluated under greenhouse and field conditions, showing mild PD symptoms and reduced mortality due to *X. fastidiosa* (Li et al., 2015; Dandekar et al., 2019).

In our group, newly designed AMPs, showing antibacterial and plant immune system elicitor activities, have been proposed as good candidates for the control of important plant diseases (Badosa et al., 2007; Badosa et al., 2013). Moreover, their efficacy *in planta* has been demonstrated using both, direct application (Puig et al., 2015; Badosa et al., 2017) and transgenic expression (Montesinos L. et al., 2017).

In this context, the present Ph.D. Thesis aimed to contribute to the *in vitro* evaluation of these newly designed AMPs for the control of *X. fastidiosa*. Moreover, because little is known about the pathogenicity of *X. fastidiosa* Spanish isolates, *X. fastidiosa*-almond interactions were studied in order to gain essential knowledge for the design of an optimized treatment guideline, including AMPs, important for the implementation of efficient disease control programs.

The *in vitro* evaluation of AMPs for the control of *X. fastidiosa* required (i) the elucidation of their main mechanism of action (ii) the study of important activity parameters that influence their activity, and (iii) the development of a rapid and reliable screening methodology for the identification of highly active peptides against this pathogen.

The bactericidal activity against *X. fastidiosa* and the mechanism of action of lead AMPs from different libraries previously described by our group, and reported as active against important plant pathogenic bacteria, such as *E. amylovora*, *X. vesicatoria* and *P. syringae* pv. *syringae* (Monroc et al., 2006; Ferré et al., 2006; Badosa et al. 2013), were investigated. The peptide BP178 was the one showing the highest bactericidal activity

against different *X. fastidiosa* strains, and was chosen for performing further experiments on the mechanism of action and the understanding of important parameters influencing its activity.

As above-mentioned, AMPs present different modes of action. Within them, their bactericidal activity through direct killing is the most reported one, as the peptide-lipid interaction due to charge differences between the molecules results in disruption and/or pore formation in the bacterial membrane (Goyal and Mattoo, 2016). This mechanism of action was observed by transmission electron microscopy (TEM) ultrastructural analysis in the case of peptide BP178 against two strains of *X. fastidiosa* (subsp. *fastidiosa* strain Temecula and subsp. *pauca* strain DD1). Pore formation and disruption of the bacterial membrane were observed, indicating cell lysis. Because BP178 is a BP100-magainin derivative peptide (Badosa et al., 2013), these observations are in agreement with the already reported main mechanism of action of BP100 and magainin peptides (Ferré et al., 2009; Nguyen et al., 2011). Moreover, these findings are supported by the fact that BP100 is an analogue of the peptide BP76 (Badosa et al., 2007), which in turn is an analogue of the hybrid peptide Pep3 derived from cecropin A and melittin peptides (Ferré et al., 2006), already described as lytic peptides (Steiner et al., 1981; Giacometti et al., 2004).

Elucidating the main mechanism of action of AMPs *in vitro* is important not only for the rational design of new and improved sequences, but also for unrevealing the basis of an effective treatment application. In addition, determining dose-response relationships and death kinetics is important for a better understanding of the interactions between a particular peptide and bacterial cell membranes, which depend on their specific molecular properties (Hollmann et al., 2018). The bactericidal activity of BP178 was observed within the first 10 to 20 min post-exposure and it was significantly affected by both, the peptide concentration and the *X. fastidiosa* strain. Nevertheless, a threshold ratio around 10^9 BP178 molecules/cell was estimated to be necessary for the lysis of the *X. fastidiosa* strains studied.

During the *in vitro* evaluation of antimicrobial compounds, it should be taken into account that the complex environment under *in vivo* conditions might also affect the interaction between compounds and bacterial cell membranes, varying their potential (Nguyen et al., 2011; Zeitler et al., 2013). In the case of AMPs, parameters such as pH and salt content of the diluent are prone to affect their bactericidal activity (Montesinos E. et al., 2012). In fact, the bactericidal activity of BP178 against *X. fastidiosa* was significantly affected by the diluent used, indicating that the threshold ratio BP178/cell estimated should be reconsidered when thinking about applying this peptide into the vascular system of a plant, as the environment characteristics might affect its activity. Salt content of the xylem sap, as well as other physicochemical characteristics like pH, should be considered in order to optimize the peptide dose.

Even though *in vitro* studies are the first step on the discovery of new and effective bactericides, it is important to keep in mind that the results obtained do not necessarily reflect the *in vivo* activity (Badosa et al., 2007). In this sense, being able to have a large number of peptides with potential to be effective against *X. fastidiosa* is important. Nevertheless, due to the quarantine status of this pathogen in the EU and its fastidious nature and slow growth (Campanharo et al., 2003), the screening of large amounts of peptides using conventional methods, which are mainly culture-based methods (Balouiri et al., 2016), is time consuming and unreliable for *X. fastidiosa*. Additionally, the induction of a viable-but-non-culturable (VBNC) state of cells has been described for this pathogen in response to antimicrobial stress (Martins et al., 2018; this Thesis, Baró et al., 2020), making conventional methods overestimate the activity of peptides. When considering the development of a screening methodology, it is important to choose a method able to quantify the whole fraction of viable cells, either culturable or not, as the recovery of the VBNC cells pathogenicity has been reported in other bacteria (Grey and Steck, 2001; Ordax et al., 2006; Kan et al., 2019).

The methodology developed in the present work for the screening of AMPs against *X. fastidiosa* was based on a 3 h contact test followed by

a viability-qPCR (v-qPCR) using the nucleic acid-binding dye PEMAX (Codony, 2014). This methodology was able to classify a set of 11 peptides based on their bactericidal activity, allowing a rapid and reliable identification of highly active sequences with potential for being tested *in planta*. Moreover, the methodology was used to determine the differential susceptibility of *X. fastidiosa* strains to the peptide BP178, as well as the differences in the induction of the VBNC state. These findings are important because the capacity of inducing the VBNC state under antimicrobial stress conditions can be an indicator of how resistant is a specific strain to antimicrobials and other stressful conditions (Oliver, 2010). Additionally, they reflect the significant genetic and biological variability that exists, not only between subspecies of *X. fastidiosa* but also between strains of the same subspecies (Potnis et al., 2019; Feitosa-Junior et al., 2019), highlighting the importance of studying each specific *X. fastidiosa* strain separately.

Even though *X. fastidiosa* is a generalist pathogen, infecting over 600 plant species, only a few of them develop disease symptoms, indicating that host and strain diversity interact to determine pathogenicity and host competence (Lopes et al., 2010). Knowledge on these parameters, such as dose-response relationships and dynamics of the bacterial population over time, is essential for establishing the experimental basis for the control of *X. fastidiosa* on host plants. So, as well as it is important to understand the characteristics of the interactions between the pathogen and the antimicrobial compound, it is also necessary to study the pathogenicity of the pathogen for the optimization of treatment guidelines in terms of time and dose of application (Montesinos E. and Bonaterra, 1996; Cabrefiga and Montesinos E., 2005). Moreover, information on pathogenicity can help to develop disease forecasts, which can be very useful to increase the efficacy of application of control strategies (e.g. AMPs), reducing their negative impact on the environment (Thomson et al., 1977; Llorente et al., 2000). In this sense, the pathogenicity of different Spanish isolates of *X. fastidiosa* in almond plants was investigated.

Almond was the host plant chosen in our study because almond orchards are the highest agricultural extension affected by *X. fastidiosa* in Spain, and ALS is nowadays a major concern for the Spanish agriculture. Moreover, the country accounts for the 83.9 % of the total EU almond crops, being the third almond producer in the world (De Cicco, 2018). In Mallorca, for example, the presence of *X. fastidiosa* is changing the agricultural landscape, as almond orchards are being substituted by carob orchards that are resistant to the pathogen (Olmo et al., 2021). In Alicante, eradication measures have been taken since the first detection in 2017, and so far more than 40,000 almond trees have been removed and shredded (Ferrer et al., 2019).

Our findings on the pathogenicity of four Spanish isolates are based on young almond plants under greenhouse conditions. This experimental approach does not represent the agronomic conditions in most of the current almond plantations affected in Spain, but it can be comparable to the situation in nurseries and in high density almond orchards, which are emerging as a new highly productive strategy. This type of cropping system is gaining attention due to its advantages regarding (i) early yields, (ii) smaller and more efficient canopies which make harvest mechanization easier, and (iii) increased efficiency of water, fertilizers and phytosanitary treatments (Casanova-Gascón et al., 2019). Although no references regarding long-term behaviour are available in almond orchards, high-density systems are already being used in apple and olive orchards (Robinson et al., 2013; Martinez-Guanter et al., 2017).

All Spanish isolates of *X. fastidiosa* (IVIA 5387.2, IVIA 5901.2, IVIA 5235.4 and IVIA 5770) were able to cause ALS in three almond cultivars (*Avijor*, *Soleta* and *Penta*) that are currently being produced as smarttrees, a particular plant format created for being adapted to high-density orchards to enter rapidly into production (Agromillora Iberia, S.L.U, Barcelona, Spain). Using this experimental approach, differences in aggressiveness were observed between strains. The grapevine isolate IVIA 5770 was able to colonize the three almond cultivars studied, causing ALS levels similar, or even higher, to the ones detected for the other *X. fastidiosa* strains isolated from *Prunus* spp. (IVIA 5235.4, IVIA 5387.2 and

IVIA 5901.2). These findings have important implications in epidemiology, as they reveal a high capacity of *X. fastidiosa* to spread throughout a region and its ability to adapt to novel hosts, independently of its host of origin. The strain from the subspecies *multiplex*, IVIA 5901.2 was the one reporting low disease severities and colonization rates of new growing shoots, compared to the other three strains studied. These observations are in agreement with a recent phenotypic characterization of this same isolate in comparison with other strains from the subspecies *multiplex* and *fastidiosa*, where IVIA 5901.2 showed the lowest motility rate and caused low disease severity in tobacco plants (Román-Écija et al., 2019).

Differences in disease severity and colonization of new growing shoots between the *X. fastidiosa* strains could also be explained by the almond cultivar genetic structure. In general, plants can either be resistant to pathogens, meaning that the pathogen population is reduced, or tolerant, when the plant limits the pathogen's impact to its own phenotype (Rashed et al., 2013). In our case, although more studies should be performed to confirm this hypothesis, the cv. Avijor seems to better tolerate *X. fastidiosa* infections compared to the other almond cultivars, as similar pathogen population levels resulted in lower disease severity. The cv. Penta was more susceptible than the cv. Soleta, which is in agreement with the differential susceptibility of these cultivars observed against *Colletotrichum* spp. (López-Moral et al., 2020).

Dose-response relationships revealed differences in important pathogenicity parameters related to aggressiveness, such as ED_{50} and MID values, between the two almond isolates of *X. fastidiosa*. While IVIA 5387.2 reached high population levels but its ED_{50} was also high, IVIA 5901.2 attained population levels below the ones of IVIA 5387.2 but its ED_{50} was low. Low values of ED_{50} mean a high infection efficiency. Interestingly, in both strains, the ED_{50} values were lower (from 10 to 10^4 CFU/plant) than the ones reported for citrus and coffee isolates from Brazil, estimated to be around 10^5 CFU/plant (Prado et al., 2008). This could be associated to differences in the interaction between *X. fastidiosa* strains and the corresponding host plants, as well as to differences in the

methodology used for inoculation and analysis during the experiment in our work and the Brazilian study.

Regarding MID values, although they were different between strains, in both cases were below 3000 CFU/plant. These results are in agreement with the fact that few *X. fastidiosa* cells are required for plant inoculation by insect vectors, as transmission is generally fast (less than 1 h after acquisition) and a limited concentration of bacterial cells is expected to be found in the foregut of spittlebugs and leafhoppers (Almeida et al., 2005). Indeed, *X. fastidiosa* concentrations detected in insect heads range from 30 to $5x10^5$ copies/head (Ramirez et al., 2008; Cornara et al., 2017b). This data indicates that both strains of *X. fastidiosa*, IVIA 5387.2 and IVIA 5901.2, are highly prone to be disseminated by insect vectors, although transmission is not only impacted by dose but also by host plant, pathogen strain and vector species.

The differential strain performance observed between both almond isolates could be related with the higher impact of ALS observed in Balearic Islands (IVIA 5387.2) compared to Alicante (IVIA 5901.2). In this sense, functional genomic analysis would be useful to identify pathogenicity related genes, as aggressiveness and virulence are genetically-controlled components (Bos and Parleviet, 1995). In fact, this analysis would be interesting because it has already been observed that IVIA 5901.2 lacks two entire plasmids when compared to another *X. fastidiosa* isolate from Alicante (ESVL strain) (Giampetruzzi et al., 2019). Nevertheless, since it has been hypothesized that *X. fastidiosa* was already present in Mallorca in 1993 (Moralejo et al., 2020) while the introduction in Alicante seems to be relatively recent (Landa et al., 2020), time of pathogen introduction in the region could also be another factor explaining these differences regarding ALS impact.

In general, our findings reaffirmed the fact that *X. fastidiosa* is a highly recombinant bacterium, as variability between strains was observed regarding their susceptibility to AMPs and their aggressiveness in infecting almond plants. Because homologous recombination has been described as one of the main drivers of *X. fastidiosa* adaptation to novel

hosts (Vanhove et al., 2019; Denancé et al., 2019), it could also be associated with the resistance to AMPs. Nevertheless, due to their mechanism of action (membrane disruption) and their multi-targeting characteristics, probabilities of acquiring resistance against these bactericides are low compared to other antimicrobials (Spohn et al., 2019). In this sense, and because some of the AMPs screened showed significant bactericidal activity *in vitro* against *X. fastidiosa*, we consider that it is important to continue the process of development of AMPs as novel and more sustainable bactericides. *In planta* experiments should be performed in order to (i) determine phytotoxicity effects, (ii) discover additional mechanisms of action, such as plant immune system elicitation activity, and (iii) test their efficacy in controlling *X. fastidiosa* infections.

In most of the EU countries, an efficacy evaluation is required before a plant protection product is commercialized, and this will be the case of AMPs due to their potential in plant disease control. Since 1977, EPPO is publishing guidelines on this topic with the aim of harmonizing the efficacy evaluation of a specific product within the registration procedure. Although their use is not obligatory, they have constituted a reference in the EU regarding the data requirements for plant protection products. Within them, specific requirements related with the effectiveness, such as dose and time of application, have to be recommended taking into account the Directive 2009/128/EC, which establishes a framework for community action to achieve the sustainable use of pesticides. We trust that knowledge obtained during the development of this Ph.D. Thesis provides information for the design of guidelines for the efficacy evaluation of AMPs in the control of plant diseases, particularly the ones caused by *X. fastidiosa*.

CHAPTER VII

Conclusions

1. A screening methodology for the identification of active antimicrobial compounds against *X. fastidiosa* has been developed, based on a 3 h contact test followed by a v-qPCR using the nucleic acid-binding dye PEMAX. Using this methodology, six new AMPs (BP170, BP171, BP175, BP176, BP178 and BP180) have been classified as highly active against *X. fastidiosa*.

2. Among the CECMEL11 and CYCLO10 lead and derivative peptides studied, the strongest bactericidal activity was observed in BP178.

3. The main mechanism of action of BP178 against *X. fastidiosa* strains was direct killing through pore formation and disruption of the bacterial membrane, indicating cell lysis and confirming its bactericidal activity.

4. The bactericidal activity of BP178 was observed within the first 10 to 20 min post-exposure and it was significantly affected by the peptide concentration and the *X. fastidiosa* strain and concentration. A threshold ratio around 10^9 BP178 molecules per cell was estimated to be necessary for the lysis of all *X. fastidiosa* strains studied.

5. The bactericidal activity of BP178 was significantly affected by the diluent used due to differences in their salt content, net charge and pH. Because xylem sap composition strongly changes with plant species, phenological stage and environmental conditions, the activity of peptides against *X. fastidiosa* in host plants may have limitations under certain conditions, but could be favored in others.

6. Differences in susceptibility and in the induction of the VBNC state, as a consequence of the treatment with BP178, were observed among a set of *X. fastidiosa* strains belonging to the subspecies *fastidiosa*, *multiplex* and *pauca*. *X. fastidiosa* subsp. *pauca* DD1 was the most tolerant to the peptide and subsp. *multiplex* CFBP 8173 the most susceptible. *X. fastidiosa* subsp. *fastidiosa* IVIA 5235.4 was the one showing the highest capacity to induce the VBNC state under the conditions studied.

7. *X. fastidiosa* subsp. *fastidiosa* IVIA 5387.2 in young almond plants showed doubling times of around 2 days during the exponential phase, which was observed from 0 to 40 days post-inoculation. At the end of this phase, a maximum population size ranging from 10^6 to 10^8

CFU/g or ml was observed in both, sap and xylem tissue, prior to symptoms development.

8. The Spanish isolates of *X. fastidiosa* studied were able to infect three almond cultivars produced as smarttrees. Differences in aggressiveness were observed, as population levels, disease severity and colonization of new growing shoots varied between strains infecting the same cultivar. Among strains, and globally within the three cultivars, the strain IVIA 5387.2 acquired the highest population levels and the highest rate of colonization of new growing shoots. The strain IVIA 5770 caused the most severe disease symptoms in almond. The strain IVIA 5901.2 was the one showing the lowest bacterial load, colonization rates and disease severity.

9. Infectivity titration using dose-response relationships revealed differences in important pathogenicity parameters, such as P_{max}, ED_{50} and MID values, between two almond isolates of *X. fastidiosa.* Strain IVIA 5387.2 acquired higher population levels than IVIA 5901.2 (means of 7.25 and 6.31 $\log_{10}$ CFU/g respectively). IVIA 5901.2 had lower ED_{50} and MID values (between 10-10^2 CFU/plant) compared to IVIA 5387.2 (between 10^2-10^4 CFU/plant).

10. The present study raises implications for the epidemiology and management strategies of *X. fastidiosa* directly in almond crops, but also in other host plants. Moreover, it establishes the experimental basis for the control of *X. fastidiosa* on host plants using synthetic AMPs.

CHAPTER VIII

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