Universitat de Girona

SYNTHETIC ANTIFUNGAL PEPTIDES FOR CONTROLLING BROWN SPOT OF PEAR CAUSED BY STEMPHYLIUM VESICARIUM. ACTIVITY, MODE OF ACTION AND FIELD EVALUATION

Mireia Puig Garcia

Universitat de Girona

Doctoral Thesis

Synthetic antifungal peptides for controlling brown spot of pear caused by *Stemphylium vesicarium*. Activity, mode of action and field evaluation

Mireia Puig Garcia

2015

Universitat de Girona

Doctoral Thesis

Synthetic antifungal peptides for controlling brown spot of pear caused by *Stemphylium vesicarium*. Activity, mode of action and field evaluation

Mireia Puig Garcia

2015

Doctoral Programme in Technology

Supervised by:

Dr. Isidre Llorente Cabratosa

Dra. Concepció Moragrega Garcia

Mireia Puig Dr. Isidre Llorente Dra. Concepció Moragrega

Dissertation submitted to apply for the Doctoral degree by the Universitat de Girona

Universitat de Girona

Dr. Isidre Llorente Cabratosa i **Dra. Concepció Moragrega Garcia**, Professors Titulars de l'Àrea Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària, i Investigadors de l'Institut de Tecnologia Agroalimentària de la Universitat de Girona,

DECLAREM:

Que el treball titulat "*Synthetic antifungal peptides for controlling brown spot of pear caused by* Stemphylium vesicarium. *Activity, mode of action and field evaluation*", que presenta **Mireia Puig Garcia** per a l'obtenció del títol de doctora per la Universitat de Girona, ha estat realitzat sota la nostra direcció.

I, perquè així consti i tingui els efectes oportuns, signem aquest document:

Dr. Isidre Llorente Cabratosa

Dra. Concepció Moragrega Garcia

Girona, 22 d' octubre 2015

This work was supported by the Spanish Ministerio de Economía y Competitividad (project AGL2009-09829/AGR) and by a BR grant from Universitat de Girona (BR 10/17)

Als meus pares i a en Denis

Acknowledgments

Ja està, per molt increïble que em sembli ja s'ha acabat. Ara toca escriure les últimes paraules de la tesi, però no les menys importants. Fa cinc anys vaig decidir emprendre un camí totalment desconegut per a mi i crec que no hagués pogut arribar al final sense vosaltres. Durant aquests anys he après coses noves, tant científiques com personals, i he pogut conèixer persones excepcionals que sempre portaré amb mi. I ara em toca donar-vos les gràcies per tot el que m'heu ensenyat, pel vostre recolzament i els vostres ànims. Amb vosaltres aquest camí ha estat menys feixuc. Per tot això MOLTES GRÀCIES.

De fet, aquest camí va començar quan el Dr. Isidre Llorente i la Dra. Concepció Moragrega, els directors d'aquesta tesi, van donar-me l'oportunitat de fer el treball final de carrera i de començar a treballar en el grup de Patologia Vegetal. En aquell moment no m'imaginava que arribaria fins aquí. Isidre i Cun, moltes gràcies per haver confiat en mi per i haver-me iniciat en el món de la recerca, per les vostres hores de dedicació, per la vostra supervisió, per les vostres hores de dedicació, vostra supervisió, paciència, visió més crítica, per la correcció de la tesi i sobretot per tot el que he après al llarg d'aquests set anys! Moltíssimes gràcies.

Agrair al Dr. Emilio Montesinos deixar-me formar part del grup de Patologia Vegetal i per escoltar-me i ajudar-me sempre que ho he necessitat. Emili, dir-te que t'estic molt agraïda per haver confiat en mi pel projecte STREP, el meu primer projecte professional. Moltes gràcies de tot cor.

Un especial agraïment a la Lídia Ruz pel seu suport i per la seva ajuda en tot moment, pels seus consells, per venir a camp a fer les valoracions i sobretot pels seus ànims. Moltes gràcies per ser la meva germana gran en la ciència!

En Josep Pereda gràcies pel manteniment del material vegetal. Enric moltes gràcies per ajudar-me en les valoracions de camp i per les estones compartides en els microscopis, sempre amb RAC1 de fons.

Agrair a la Carme, en Jordi, en Vicens i en Dani dels Serveis tècnics de recerca de la UdG la seva ajuda amb els microscopis i per la realització d'algunes de les fotografies de la tesi. Moltes gràcies a la Natàlia per la seva ajuda en l'estadística i per la seva amistat.

Quiero dar las gracias al Dr. Francisco Cazorla y al Dr. Antonio de Vicente por aceptarme en su laboratorio de Microbiología de la Universidad de Málaga durante 3 meses. Francis, quiero agradecerte todo lo que me enseñaste, todo el apoyo que recibí, las risas, las presentaciones..., pero sobretodo por haberme dado la oportunidad de conocer a la gente maravillosa de tu grupo. Gracias a Irene, Nacho, Mariki, Nuria, Alvarito, Conchita, Jesús, Houda, Joaquín, Eloy, Alejandro y Diego. Un especial agradecimiento para Claudia, David, Carmen, Cristian, Guti y María, sois geniales, sois muy grandes, muchísimas gracias por todo! De verdad, MUCHAS GRACIAS! Siempre seréis mis andaluces favoritos y os llevaré en mi corazón!

A tots els membres del grup de Patologia Vegetal: Emili, Jordi, Esther, Lídia, Anna, Jesús, Isidre, Cun, Josep, Enric, Laura, Isa, Bea, Gemma, Gerard, Núria i Miquel per estar sempre disposats a donar un cop de

mà, i pels vostres ànims. Moltes gràcies a tots els estudiants que han anat passant pel laboratori de PV durant tots aquests anys. Gràcies a tota la gent del menjador i amb qui també he compartit esmorzars i celebracions: Pere, Dolors, Mª Àngels, Mònica, Maria, Núria, Anna, Alberto, Helena, Chang, Nuri, Cristina, Carme, Elena, Gemma, Nuri i Anna Maria.

Moltes gràcies al Sr. Bas i a l'Adrià Bas per confiar en mi i acceptar-me dins Agrolac. De veritat, moltíssimes gràcies! I gràcies a l'Eva, l'Aina i la Montse per la seva simpatia i perquè des del primer dia m'heu fet sentir com a casa!

De les meves companyes de laboratori: Nuri, Gemma i Cristina què puc dir, moltíssimes gràcies pels vostres somriures, rialles, consells, per la vostra ajuda; moltes gràcies! Gemma, que ràpid han passat aquests anys, estic molt contenta d'haver compartit el despatx i la gran quantitat d'hores amb tu. La tesi no hagués estat el mateix sense tu! No canviïs mai i et desitjo el millor!

A les meves compis de Màster i grup de teràpia tesil: Irene, Eli, Eva, Gemma, Nuri, Chang i Cristina. Moltes i moltes gràcies per les bones estones que hem compartit, des de Granada, a Màlaga, algunes fins a Aachen, dinars al Parc, sopars, berenars, casaments, bebès, etc. Sempre us portaré dins meu i no us oblidaré mai per molts Km que ens separi. Us desitjo molta sort a totes!!!

Laura, Isa i Bea, dir-vos que sou la canya, moltes gràcies per tot el suport que m'heu donat aquest any! Fa un any no m'esperava tenir unes companyes de feina com vosaltres i gràcies per compartir canyes,

futbol, maquillatge, sopars i per sempre voler-ho celebrar tot! Sou una alegria i gràcies per deixar-me formar part de les vostres vides.

Xavi i Nai, no sé si ho sabeu però sou un pilar molt important en la meva vida, que espero que no caigui mai. Fa molts anys que ens coneixem i sé que aquest últim any us he ratllat fins a l'infinit amb la meva tesi, moltes gràcies per haver-me suportat i donat suport. Espero estar present en els moments més importants de les vostres vides com vosaltres heu estat sempre en els meus. I gràcies pel vostre amor incondicional.

A la meva família, tant pels que hi són com pels que sempre hi seran, moltes gràcies pel vostre amor i per ser sempre present. La gent no sé si ho sap però les Garcia som collonudes jajajaja. Agrair especialment a la iaia Juanita, pel suport que sempre m'has donat, per ser present en tots els moments i per desitjar-me sempre el millor, moltes gràcies.

Als meus pares agrair-vos tot l'amor que sempre m'heu donat, tot el suport, sou el meu consell de savis, sou de qui he aprés més en la vida i qui m'estima més del món. Aquesta tesi és tant vostra com meva! Us estimo moltíssim! Moltes gràcies per tot i crec que tinc els millors pares que em podrien haver tocat en aquest món! A la meva germaneta dir-li que val un imperi, tant com a persona com a professional i que no seria la mateixa sense ella en la meva vida. Espero que siguis molt feliç! T'estimo!

Denis, Denis, Denis...què dir de tu. Sé que aquesta tesi potser et fa més il·lusió a tu que a mi. Moltes gràcies per les hores dedicades a la portada. La veritat és que no tinc paraules per agrair-te el que has fet

per mi durant aquest últim any. Fa molt temps que compartim les nostres vides i t'he de dir que em fas molt feliç. Gràcies per calmar-me, per esverar-me, per ajudar-me, per animar-me, per estimar-me però sobretot per estar al meu costat. Gràcies per permetre'm ser una part més de la teva família i donar-me l'oportunitat de ser la mama d'en Neo i la tieta de la Clara i en Nil, dues personetes molt especials per mi. T'estimo moltíssim.

Neo! No m'oblido de tu, moltes gràcies per fer-me companyia al cantó de l'ordinador durant les infinitats d'hores que ha durat la redacció d'aquest tesi.

List of abbreviations

Aa	Amino acid
APD	Antimicrobial peptide database
AFLP	Amplified fragment length polymorfism
AMPs	Antimicrobial peptides
AS	Acetosyringone
ATMT	*Agrobacterium tumefaciens*-mediated transformation
AUDPC	Area under the disease progress curve
BCA's	Biological control agents
BP15-FITC	BP15 labeled with Fluorescein 5-isothiocyanate
BP15-Rh	BP15 labeled with Rhodamine-B
BSP	Brown spot of pear
BSPcast	Brown spot of pear forecasting system
CDD	Cumulative degree days
CFW	Calcofluor white dye
CLSM	Confocal laser scanning microscope
CMI	Concentració mínima inhibitòria
CPPs	Cell-penetring peptides
CR	Cumulative daily infection risk
DNA	Deoxyribonucleic acid
EU	European Union
FITC	Fluorescein 5-isothiocyanate
IPM	Integrated pest management
ITS	Internal transcribed spacer
GFP	Green fluorescent protein
gpd	Glyceraldehyde-3-phosphate dehydrogenase
hph	hygromycin resistance gene
LB	Luria Bertani
LD_{50}	Median lethal dose
LLD	Limit lethal dose
L/W	Lenght to width ratio
MIC	Minimum inhibitory concentration
OD_{600}	Optical density at 600 nm
PAMcast	*Pleospora allii* maturation forecast
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
P/L	Peptide and lipid ratio
R	Relative daily infection risk
RAPD	Random amplified polymorphic DNA
RH	Relative humidity

Rh	Rhodamine-B
RIPs	Ribosome-inactivating proteins
S	Disease severity
SEM	Scanning electron microscopy
SG	SYTOX green
T	Temperature during the wetness period
T-DNA	Transfer DNA
USA	United States of America
W	Wetness duration

Amino acid abbreviations are indicated according to the IUPAC/IUBMB code (International Union of Pure Applied Chemistry/ International Union of Biochemistry and Molecular Biology).

List of figures

Figure 1. Location of areas affected by brown spot of pear in Europe, including Spain, Italy, France, the Netherlands, Belgium and Portugal **21**

Figure 2. Morphology of *Stemphylium vesicarium* conidia (A) and mature asci with eight ascospores of *Pleospora allii* (B)..... **22**

Figure 3. Brown spot of pear characteristic symptoms: (A) necrotic lesions on leaves, (B and C) lesions on pear fruit **25**

Figure 4. Pathogenic and saprophytic phases in the biological cycle of *Stemphylium vesicarium* and *Pleospora allii* in pear orchards. Pathogenic phase occurs on the aerial pear organs during spring and summer, causing plant infection, and the saprophytic phase occurs on herbaceous plants and pear leaf litter on the orchard floor **28**

Figure 5. Protein models representing the secondary structures of the four classes of antimicrobial peptides: (A) α-helical (magainin), (B) ß-sheet (protegrin), (C) extended (indolicin) and (D) loop (lactoferricin B) **42**

Figure 6. Models proposed for the permeabilization of target cell membrane by membrane active AMPs (A,B,C) and mechanism of action for AMPs with intracellular activity (1,2,3,4,5,6) **50**

Figure 7. Edmunson wheel projection of the 11-mer peptides that were synthesized **55**

List of tables

Table 1. Pear production (in tonnes) in the major pear growing countries in 2012 (FAOSTAT, 2014) 19

Table 2. Classification and mode of action of fungicides used in control of brown spot of pear 35

Table 3. Synthetic antimicrobial peptides active against plant pathogenic bacteria and fungi 41

Table 5. Plant pathogenic bacteria controlled by synthetic antimicrobial peptides 52

Table 5. Plant pathogenic fungi controlled by synthetic antimicrobial peptides 53

Table of contents

Acknowledgments
List of abbreviations i
List of Figures iii
List of Tables iv

List of Papers 1

Summary 3
Resum 7
Resumen 11

Chapter 1. General Introduction 17

1. Brown spot of pear 20
   1.1. Causal agent and pathogenicity 21
   1.2. Disease symptoms 24
   1.3. Epidemiology and inoculum production 25
   1.4. Inoculum assessment 28

2. Brown spot of pear management 30
   2.1. Forecasting models 30
   2.2. Chemical control 33
   2.3. Biological control 36
   2.4. Sanitation and cultural practices 37
   2.5. Integrated pest management of BSP 37

3. Antimicrobial peptides: candidates for controlling plant diseases 38
   3.1. Classification of antimicrobial peptides 40
   3.2. Mode of action of antimicrobial peptides 44
   3.3. AMPs for controlling plant pathogens 51
   3.4. Peptide collections: CECMEL11 chemical library 54

Chapter 2. Objectives 57

Chapter 3. Postinfection activity of synthetic antimicrobial peptides against *Stemphylium vesicarium* in pear 61

Chapter 4. Controlling brown spot of pear by a synthetic antimicrobial peptide under field conditions 73

Chapter 5. Interaction of antifungal peptide BP15 with *Stemphylium vesicarium*, the causal agent of brown spot of pear 99

Chapter 6. General discussion 129

Chapter 7. Conclusions 143

References 147

List of publications

This PhD thesis is presented as a compendium of three publications:

Puig M, Moragrega C, Ruz L, Montesinos E and Llorente I, 2014. Postinfection activity of synthetic antimicrobial peptides against *Stemphylium vesicarium* in pear. *Phytopathology*, 104: 1192-1200. DOI: 10.1094/PHYTO-02-14-0036-R

Phytopathology has an impact factor of **3.119** and it was situated in the first quartile (**Q1**) in the Plant Sciences category in 2014 (© Journal Citation Reports Science Edition, published by Thompson Reuters).

Puig M, Moragrega C, Ruz L, Montesinos E and Llorente I, 2015. Controlling brown spot of pear by a synthetic antimicrobial peptide under field conditions. *Plant disease*. Published *on line* DOI:10.1094/PDIS-03-15-0250-RE

Plant disease has an impact factor of **3.020** and it is situated in the first quartile (**Q1**) in the Plant Sciences category in 2014 (© Journal Citation Reports Science Edition, published by Thompson Reuters).

Puig M, Moragrega C, Ruz L, Calderón CE, Cazorla FM, Montesinos E and Llorente I, 2015. Interaction of antifungal peptide BP15 with *Stemphylium vesicarium*, causal agent of brown spot of pear. *Fungal biology.* Submitted.

Fungal biology has an impact factor of **2.342** and it is situated in the second quartile (**Q2**) in the Mycology category in 2014 (© Journal Citation Reports Science Edition, published by Thompson Reuters).

Summary

Brown spot of pear (BSP) is a fungal disease caused by *Stemphylium vesicarium*. The disease has been reported in pear growing areas of Europe where produces serious economic losses. Currently, disease control is achieved by fungicides systematically applied or scheduled by the predictive model BSPcast. Chemical control can be combined with sanitation methods to reduce the inoculum produced on the orchard ground; the most common procedures are leaf litter removal during the winter and treatments using biological control agents (*Trichoderma* sp.). However, the disease control efficacy is limited, due to the characteristics of the disease and the reduction of the allowed fungicides. Therefore, the research is focused on finding new chemical compounds to be applied alternatively or as complements to the existing fungicides.

In the last decade, antimicrobial peptides have been considered to be used in the control of plant diseases, as they have demonstrated high activity against some bacterial and fungal phytopathogens. In addition, the linear antimicrobial peptides are biodegradable and present low toxicity, both in plants and mammals. The CIDSAV and LIPPSO (University of Girona) have developed the chemical library CECMEL11, a 125-member peptide library, which is composed of *de novo* designed and synthetically produced cecropin A-melittin hybrid undecapeptide. These peptides have demonstrated a high inhibitory activity against several plant pathogens.

This thesis is part of a research project focused on the use of synthetic antimicrobial peptides for controlling the brown spot of pear. The main objective was to identify peptides from the CECMEL11 collection with the ability to control the brown spot of pear. Thus, twelve synthetic

antifungal peptides selected from CECMEL11 library were evaluated for their efficacy of inhibition of *S. vesicarium* spore germination and growth *in vitro*, and the most effective peptides were assessed for their ability to reduce fungal infection on detached pear leaves. Finally, peptides with potential for disease control were evaluated under field conditions applied scheduled by the forecasting model BSPcast. In parallel and in order to elucidate the mechanisms of action, the interaction of the most effective peptide with *S. vesicarium* was analyzed by live-cell imaging techniques.

Firstly, twelve peptides from the CECMEL11 library were screened *in vitro* for fungicidal activity against *S. vesicarium*. The *in vitro* experiments showed that eight antimicrobial peptides (BP15, BP21, BP22, BP23, BP24, BP25, BP35 and BP38) applied at different concentrations (5, 10, 25, 50 and 100 μM) significantly reduced the germination of conidia and the fungal growth. The most effective peptides were BP15 and BP22 with a minimum inhibitory concentration (MIC) of 10 and 50 μM, respectively. Most tested peptides produced morphological alterations to new germ tubes and hyphae, causing inhibition of fungal growth. Also, peptides BP15, BP22 and BP25 significantly reduced the sporulation of *S. vesicarium* (from 50 to 95 %). However, none of the tested peptides showed sporicidial activity against *S. vesicarium* conidia.

Two peptides (BP15 and BP22) were selected to be tested for their efficacy of control of *S. vesicarium* infections under controlled conditions. First of all, BP15 and BP22 were evaluated following two strategies of application: preventative (3 h before *S. vesicarium* inoculation) and postinfective (15 h after the pathogen inoculation). The two peptides applied following the postinfective strategy significantly reduced the severity of the disease, while the preventative treatments did not reduce infections of *S. vesicarium*. Additionally, the effect of

delayed applications of peptides BP15 and BP22 at 6, 12, 15, 18 and 24 hours after pathogen inoculation was determined. Treatments with BP15 at the different times after pathogen inoculation significantly reduced the disease, and the results obtained with this peptide were more consistent than those obtained with BP22.

The efficacy of disease control of BP15 and BP22 was further evaluated on potted pear plants under field conditions. The experiments were realized in two different orchards, in one orchard the plants were artificially inoculated with *S. vesicarium* strain EPS26 and in the other orchard the pear plants were exposed to natural inoculum. With the purpose to test the efficacy of peptides applied at different times after BSPcast risk threshold for treatment was reached, sprays were done the same day that the risk threshold was reached, and 24 h and 48 h later. BP15 significantly reduced BSP disease severity between 42 % and 60 %, while the efficacy of BP22 was lower and less consistent. No significant differences were observed in the efficacy of BP15 for BSP control whatever the time application after the BSPcast risk threshold was reached (0, 24 or 48 h).

Finally, the peptide BP15 was evaluated for BSP disease control in a pear orchard for two consecutive years. The applications of peptide on pear trees were scheduled by the model BSPcast and BP15 was applied on tree shoots the same day that the model risk threshold was reached. In the two years, trials began in May and ended in September. In three out of four trials a significant reduction of disease on shoots treated with BP15 was observed and the disease control efficacy (38.2 %) was similar or slightly lower than efficacy of fungicide thiram.

The mechanism of action of the peptide BP15 on *S. vesicarium* was analyzed both *in vitro* and *ex vivo* experiments using live-cell imaging techniques. A double staining method based on the combination of

SYTOX green and calcofluor white, coupled to epifluorescence microscopy was used to investigate fungal-membrane permeabilization and alterations in fungal growth induced by BP15. *S. vesicarium* membrane permeabilization by BP15 resulted to be peptide-concentration dependent. At low concentrations of BP15 (MIC and subMIC), the exposure time required to induce the cells permeabilization was approximately 6 hours, whereas fungal cell membrane disruption was immediately induced by BP15 at 100 µM (10X MIC). GFP-transformants of *S. vesicarium* were obtained and exposed to Rhodamine-labelled BP15 to study the internalization of BP15 by fungal cells. Confocal laser scanning microscope images revealed that Rhodamine-labelled BP15 was localized inside the germ tubes and hyphae of GFP-transformants of *S. vesicarium*. Also, a decrease in green fluorescence emission of vital marker GFP was observed in hyphae cells that internalized Rhodamine-labelled BP15, related to the lethal effects of peptide on fungal cells. Confocal laser scanning microscope observations confirmed that ungerminated conidia are not a target for BP15, since no interaction between peptide and conidia was observed.

This PhD Thesis demonstrates the potential of peptide BP15 to be used in BSP disease control based on the BSPcast model for scheduling the treatments under pear integrated pest management programs.

Resum

L'estemfiliosi o taca bruna de la perera és una malaltia causada pel fong *Stemphylium vesicarium* que provoca importants pèrdues econòmiques en determinades zones productores de pera a Europa. Actualment el control de la malaltia es realitza mitjançant fungicides aplicats a cadència fixa o guiats pel model predictiu de risc d'infecció BSPcast. El control químic de les infeccions es focalitza en la part aèria dels arbres i es pot combinar amb mètodes de reducció d'inòcul del patogen en les restes vegetals en el sòl de les plantacions, com l'eliminació de les restes vegetals i l'aplicació d'agents de biocontrol (*Trichoderma* sp). Tot i això, l'eficàcia de control és limitada, a causa de les característiques de la malaltia i la reducció de les matèries actives permeses. Una de les línies d'investigació per incrementar l'eficàcia de control de l'estemfiliosi de la perera és l'obtenció de nous compostos químics, tant com a alternativa o com a complement dels fungicides utilitzats actualment.

En aquest sentit, en els darrers anys s'ha incrementat l'interès en la utilització dels pèptids antimicrobians per al control de malalties de plantes, ja que alguns han mostrat activitat enfront de bacteris i fongs fitopatògens en diferents patosistemes. Dins d'aquest grup, els pèptids antimicrobians lineals serien una bona opció, atès que són compostos biodegradables i presenten baixa toxicitat, tant en plantes com en animals. La llibreria CECMEL11, dissenyada per química combinatòria (CIDSAV-LIPPSO, Universitat de Girona), consta de 125 undecapèptids lineals híbrids de la cecropina A i la melitina, els quals han mostrat activitat inhibitòria enfront de bacteris i fongs fitopatògens.

Aquesta tesi s'emmarca en un projecte de recerca orientat a la utilització de pèptids antimicrobians sintètics en el control de l'estemfiliosi de la

perera. L'objectiu general va ser identificar els pèptids de la llibreria CECMEL11 que tinguessin la capacitat de controlar la malaltia. Per a això, en primer lloc es va avaluar la capacitat d'inhibició de *S. vesicarium* de dotze pèptids escollits de la llibreria CECMELL11 mitjançant assajos *in vitro*. Els pèptids més eficaços van ser avaluats *ex vivo* per determinar l'efecte en les infeccions del fong en fulles de perera. Finalment, els pèptids amb potencial per controlar la malaltia van ser provats en condicions de camp i es van aplicar de forma guiada pel model de predicció BSPcast. Paral·lelament i a fi de proposar els mecanismes d'acció, es va analitzar la interacció del pèptid amb major potencial de control de la malaltia amb *S. vesicarium*.

En els assajos realitzats *in vitro*, dels dotze pèptids avaluats a diferents concentracions (5, 10, 25, 50 i 100 µM), vuit (BP15, BP21, BP22, BP23, BP24, BP25, BP35 i BP38) van reduir significativament la germinació dels conidis de *S. vesicarium*. Els pèptids més eficaços van ser el BP15 i el BP22 amb una concentració mínima inhibitòria (CMI) de 10 i 50 µM, respectivament. Els pèptids capaços d'inhibir la germinació dels conidis de *S. vesicarium* van produir alteracions morfològiques als nous tubs germinatius i a les hifes, provocant l'aturada del creixement del fong. Els pèptids BP15, BP22 i BP25 també van reduir significativament l'esporulació del fong, entre un 50 i un 95%. No obstant això, cap dels pèptids avaluats van mostrar activitat esporicida contra als conidis de *S. vesicarium*.

Els pèptids BP15 i BP22 van ser avaluats en assajos *ex vivo* per determinar l'eficàcia de control de les infeccions *S. vesicarium* en condicions d'ambient controlat. Primer, els pèptids es van aplicar seguint dues estratègies d'aplicació: preventiva (abans de la inoculació

del patogen) i postinfectiva (15 hores després de la inoculació del patogen), per determinar amb quina estratègia els pèptids eren més eficaços per al control de la malaltia. Els dos pèptids aplicats després de la inoculació del fong van reduir significativament la severitat de la malaltia, mentre que en l'aplicació preventiva l'eficàcia de control va ser molt baixa. Posteriorment es va estudiar l'efecte de l'aplicació dels pèptids BP15 i BP22 a les 6, 12, 15, 18 i 24 hores després de la inoculació del patogen. Les aplicacions de BP15 als diferents temps assajats van reduir significativament la malaltia, i els resultats obtinguts amb el pèptid BP15 van ser més consistents que els obtinguts amb el BP22.

A continuació, es va avaluar l'eficàcia de control de la malaltia dels pèptids BP15 i BP22 sota condicions de camp en plantes de perera en contenidor. Es van realitzar assajos en dues finques diferents, una amb plantes inoculades amb *S. vesicarium* EPS26 i en l'altra les plantes van ser sotmeses a inòcul natural present en la plantació. Les aplicacions dels pèptids es van guiar pel model de predicció de risc d'infecció BSPcast i els tractaments es van fer el mateix dia o a les 24 o 48 hores després d'haver-se superat el llindar de tractament. El pèptid BP15 va reduir significativament la severitat de la malaltia, entre el 42 % i el 60 %, mentre que pel BP22 va ser inferior i amb menor consistència. Utilitzant el pèptid BP15, l'eficàcia de control de l'estemfiliosi es mantenia tant si s'aplicava al mateix dia o a les 24 o 48h d'haver-se donat l'avís de tractament.

Posteriorment, el pèptid BP15 es va avaluar a camp en arbres de perera durant dos anys consecutius i en una finca amb dos nivells d'inòcul. En aquest cas, les aplicacions del pèptid van ser guiades pel model BSPcast i es realitzaven en brots dels arbres al mateix dia que es

superava el llindar de tractament. En els dos anys els assajos van començar al maig i van finalitzar al setembre. En tres dels quatre assajos, en els arbres tractats amb BP15 es va observar una reducció significativa de la malaltia, amb una eficàcia de control similar o lleugerament inferior a la del fungicida tiram.

Paral·lelament, es va analitzar el mecanisme d'acció del pèptid BP15 enfront *S. vesicarium*, tant en condicions *in vitro* com *ex vivo*, mitjançant tècniques d'anàlisi d'imatges de cèl·lules vives. Amb aquest objectiu, es van obtenir transformats de *S. vesicarium* per l'expressió de la proteïna verd fluorescent (GFP), i es van utilitzar fluorocroms específics per marcar el pèptid i microscòpia de fluorescència i làser confocal. Es va posar de manifest la capacitat del pèptid per permeabilitzar la membrana cel·lular del fong depenent de la concentració i el temps d'exposició. A concentracions baixes (CMI i subCMI) es van requerir exposicions d'aproximadament 6 hores per induir la permeabilització, mentre que a concentracions elevades (10xMIC) una hora va ser suficient. Es va evidenciar que el pèptid és internalitzat per les cèl·lules de les hifes i tubs germinatius del fong. D'altra banda, es va confirmar que el pèptid no interacciona amb els conidis no germinats de *S. vesicarium*.

Aquesta tesi demostra el potencial del pèptid BP15 per ser utilitzat en programes de maneig de l'estemfiliosi de la perera basats en el model de predicció de risc d'infecció BSPcast.

Resumen

La estemfiliosis o mancha marrón del peral es una enfermedad fúngica causada por *Stemphylium vesicarium*. Es una de las principales enfermedades que afectan al cultivo del peral en determinadas zonas productoras de Europa pudiendo provocar importantes pérdidas económicas. Actualmente, el control de la enfermedad se realiza mediante la aplicación de fungicidas a cadencia fija o guiada por el modelo predictivo BSPcast. El control químico se puede combinar con métodos de reducción del inoculo en el suelo, eliminando los restos vegetales de la plantación y/o la aplicación de agentes de biocontrol, como *Trichoderma* sp. No obstante, la eficacia de control es limitada, debido a las características de la enfermedad y a los mecanismos de acción de los fungicidas autorizados. Una de las líneas de investigación para incrementar la eficacia de control de la estemfiliosis del peral es la obtención de nuevos compuestos químicos, ya sea como alternativa o como un complemento a los fungicidas actualmente utilizados.

En los últimos años, se ha incrementado el interés en la utilización de péptidos antimicrobianos en el control de las enfermedades de plantas, ya que algunos han mostrado actividad frente a bacterias y hongos fitopatógenos en distintos patosistemas. Concretamente, los péptidos antimicrobianos lineales serían una buena opción, ya que son compuestos biodegradables y presentan baja toxicidad, tanto en plantas como en mamíferos. La librería CECMEL11, diseñada por química combinatoria (CIDSAV-LIPPSO, Universitat de Girona) consta de 125 undecapéptidos lineales híbridos de la cecropina A y la melitina, los cuales han mostrado actividad inhibitoria frente a bacterias y hongos fitopatógenos.

Esta tesis se enmarca en un proyecto de investigación orientado a la utilización de péptidos antimicrobianos sintéticos en el control de la estemfiliosis del peral. El objetivo general fue identificar entre algunos de los péptidos de la librería CECMEL11 aquellos que mostraran capacidad para controlar la mancha marrón del peral. Para ello, en primer lugar se evaluó la capacidad de inhibición de *S. vesicarium* mediante ensayos *in vitro* de doce péptidos escogidos de la librería CECMELL11, que previamente habían mostrado actividad antifúngica. Los péptidos más eficaces se evaluaron *ex vivo* para determinar el efecto en las infecciones del hongo en hojas de peral. Finalmente, los péptidos con potencial para controlar la enfermedad fueron evaluados en condiciones de campo, aplicados de forma guiada por el modelo de predicción BSPcast. Paralelamente y a fin de proponer los mecanismos de actuación, se analizó la interacción del péptido con mayor potencial de control de la enfermedad con *S. vesicarium*.

En los experimentos realizados *in vitro*, de los doce péptidos evaluados a diferentes concentraciones (5, 10, 25, 50 y 100 µM) ocho (BP15, BP21, BP22, BP23, BP24, BP25, BP35 y BP38) redujeron significativamente la germinación de las conidias de *S. vesicarium*. Los dos péptidos más eficaces fueron el BP15 y el BP22 con una concentración mínima inhibitoria (CMI) de 10 y 50 µM, respectivamente. Los péptidos capaces de inhibir la germinación de las conidias produjeron alteraciones morfológicas en los nuevos tubos germinativos y en las hifas, provocando la parada del crecimiento del hongo. Los péptidos BP15, BP22 y BP25 también redujeron significativamente, entre un 50 % y un 95%, la esporulación del hongo. Sin embargo, ninguno de los péptidos evaluados mostró actividad esporicida frente a las conidias de *S. vesicarium*.

Con el objetivo de determinar la eficacia de control de las infecciones de S. *vesicarium* bajo condiciones de ambiente controlado, los péptidos BP15 y BP22 fueron evaluados en ensayos *ex vivo*. Primero los dos péptidos fueron aplicados siguiendo dos estrategias: preventiva (antes de la inoculación del patógeno) y postinfectiva (15 horas después de la inoculación del patógeno), para determinar con qué estrategia los péptidos eran más eficaces para el control de la enfermedad. Los péptidos BP15 y BP22 aplicados de manera postinfectiva redujeron significativamente la severidad de la enfermedad; no obstante, cuando fueron aplicados preventivamente la eficacia de control fue muy baja. Posteriormente se determinó el efecto de la aplicación de los péptidos a las 6, 12, 15, 18 y 24 horas de la inoculación del patógeno. Los resultados obtenidos con el péptido BP15 fueron más consistentes que los del BP22. Los tratamientos con BP15 aplicados en los diferentes tiempos ensayados redujeron significativamente la enfermedad.

A continuación se evaluó la eficacia de control de la enfermedad de los péptidos BP15 y BP22 bajo condiciones de campo en plantas de peral en contenedor. Se realizaron ensayos en dos fincas diferentes, una con plantas inoculadas con *S. vesicarium* EPS26 y en la otra las plantas eran sometidas al inoculo natural de la plantación. Las aplicaciones de los péptidos se guiaron por el modelo de predicción de riesgo de infección BSPcast y los tratamientos se realizaron el mismo día o en las 24 o 48 horas posteriores a la superación del umbral de tratamiento. El péptido BP15 redujo significativamente la severidad de la enfermedad entre el 42 y el 60%, mientras que la eficacia en el control de la enfermedad por el BP22 fue inferior y con menor consistencia. La eficacia de control de la estemfiliosis utilizando el BP15 se mantenía tanto si se aplicaba el

mismo día o a las 24 o 48 horas de haberse dado el aviso de tratamiento.

Finalmente el péptido BP15 se evaluó en campo en árboles de peral durante dos años consecutivos y en una finca con dos niveles de inóculo. Las aplicaciones del péptido fueron guiadas por el modelo BSPcast y se realizaron en brotes de los perales el mismo día que se superaba el umbral de tratamiento. En los dos años los ensayos comenzaron en mayo y finalizaron en septiembre. En tres de los cuatro ensayos se observó una reducción significativa de la enfermedad en los brotes tratados con el BP15, con una eficacia de control similar o ligeramente inferior a la del fungicida comercial tiram.

Paralelamente, se analizó el mecanismo de acción del péptido BP15 frente a *S. vesicarium*, tanto *in vitro* como *ex vivo*, mediante técnicas de análisis de imágenes de células vivas. Para ello, se obtuvieron transformados de *S. vesicarium* para que expresasen la proteína verde fluorescente (GFP), y, además, se utilizaron fluorocromos específicos y la microscopía de fluorescencia y láser confocal. Se demostró la capacidad del péptido para permeabilizar la membrana celular del hongo dependiendo de la concentración y el tiempo de exposición. A concentraciones bajas (CMI y subCMI) se requirieron exposiciones de aproximadamente 6 horas para inducir la permeabilización, mientras que a concentraciones elevadas (10xCMI) una hora en contacto fue suficiente. También se evidenció que el péptido es internalizado por las células de las hifas y los tubos germinativos del hongo. Finalmente, se confirmó que el péptido no interacciona con las conidias no germinadas de *S. vesicarium*.

Esta tesis demuestra el potencial del péptido BP15 para ser utilizado en programas de manejo de la estemfiliosi del peral basados en el modelo de predicción de riesgo de infección BSPcast.

Chapter 1

General Introduction

Pear production (*Pyrus communis* L.) is present throughout the world as a result of the wide range of pear varieties available and their ability to adapt to different soils and climatic conditions. Nowadays, pear production and consumption is on the increase. Bananas, apples and pears feature among the fruits most widely consumed in the European Union (EU) and the United States of America (USA). This fact is attributed to their texture, flavor and nutritional value (Prognosfruit, 2014; World Association Apple and Pear, 2014). China, the USA, Argentina, Italy, Turkey and Spain (Table 1) are the major pear producers in the world today. The most cultivated pear varieties in Europe are Conference, Abate Fetel, Williams BC and Rocha.

In 2013, pear production in Catalonia was estimated to be 262.290 tonnes with Girona and Lleida being the most important production areas. Despite this increase in production, pear consumption in Catalonia actually fell by 16.5% in 2013 and it was rated as the third most consumed fresh fruit (AFRUCAT, 2014 and DAAM, 2014).

Table 1. Pear production (in tonnes) in the major pear growing countries in 2012 (FAOSTAT, 2014)

Country	Production
China	16,100,000
United States of America	778,582
Argentina	700,000
Italy	645,540
Turkey	439,656
Spain	400,600
Republic of Korea	394,596
India	340,000
South Africa	338,584
Japan	299,000

World-wide pear production is limited by pests and diseases and significant economic losses in pear production are caused by not only insects, such as fruit moth (*Cydia pomonella*), the pear psylla (*Cacopsylla pyri L.*) or the Mediterranean fruit fly (*Ceratitis capitata*)(Mazor & Erez, 2004; Sciarretta & Trematerra, 2011; Civolani, 2012; Jones *et al.*, 2013; Manoukis & Hoffman, 2014); but also mites such as the pear leaf blister mite (*Eriophyes pyri*) and the pear rust mite (*Epitrimerus pyri*) (Jeppson *et al.*, 1975; Bergh & Weiss, 1993; Lindquist *et al.*, 1996; Michalska *et al.*, 2010). The main bacterial diseases affecting pear trees include fire blight (*Erwinia amylovora*), and bacterial blast and blossom blight (*Pseudomonas syringae* pv. *syringae*) (Vannest, 2000; Llorente et al., 2003; Palacio-Bielsa & Cambra Álvarez, 2009), while the fungal disease pear scab (*Venturia pirina*) is an economically important disease throughout the world and can cause serious losses on susceptible cultivars (Shabi *et al.*, 1973; Villalta *et al.*, 2000). Besides, brown spot of pear disease, caused by *Stemphylium vesicarium*, is one the worst pear diseases in Europe and its negative consequences have increased in recent years (Llorente & Montesinos, 2006; Rossi & Pattori, 2009; Llorente et al., 2011a).

1. Brown spot of pear

Brown spot of pear (BSP) was first reported in 1975 in the Emilia-Romagna region of Italy (Ponti *et al.*, 1982). The disease was later observed in Spain and France in the 1980s, with BSP initially being reported in Catalonia in 1984 (Vilardell, 1988). Nowadays, BSP has spread not only throughout Italy and Spain (Catalonia, Euskadi and La Rioja), but also to other pear growing areas in Europe, such as Portugal and, in Northern Europe, the Netherlands and Belgium where it is fast becoming a fungal disease with an enormous economic impact (Fig. 1)

(Ponti *et al.*, 1982; Vilardell, 1988; Blancard *et al.*, 1989; Heijne & Van Mourik, 2001; Llorente & Montesinos, 2006).

Figure 1. Location of areas affected by brown spot of pear in Europe (red points), including Spain, Italy, France, the Netherlands, Belgium and Portugal.

The severity of disease varies depending on the pear cultivar, the geographic area and meteorological conditions. Brown spot of pear can affect from 1 to 10 % of the production despite control measures being in place and in severe cases it can cause complete crop loss (Llorente & Montesinos, 2006; Llorente *et al.*, 2011a).

1.1. Causal agent and pathogenicity

The BSP pathogen is characterized by its two sexual states: the anamorph, *Stemphylium vesicarium,* produces conidia, and the teleomorph, *Pleospora allii,* develops ascospores within pseudothecia (Fig. 2) (Simmons, 1969).

Stemphylium vesicarium (Wallrs.) E. Simmons (1969) is a Deuteromycete that has erect conidiophores with a single terminal conidium. Mature conidia are oblong to oval in shape, dark brown in color, with a densely verrucose external wall, multicellular and with three to five transverse septa and one to three series of longitudinal septa, constricted at one or more commonly three of the major transverse

septa (Fig. 2A) (Simmons, 1969). While the size of conidia depends on the strain and the growth medium, conidia can range from 21 to 48 µm in length and from 10 to 22 µm in width. Generally, the length to width ratio (L/W) is from 1.5 to 2.7 for conidia developed in host tissue, whereas in growth media L/W ratio can be 2.5-3.0. The aerial mycelium is filamentous, sparse and hyaline (Fig. 2) (Simmons, 1969; Llorente & Montesinos, 2006; Puig *et al.*, 2015).

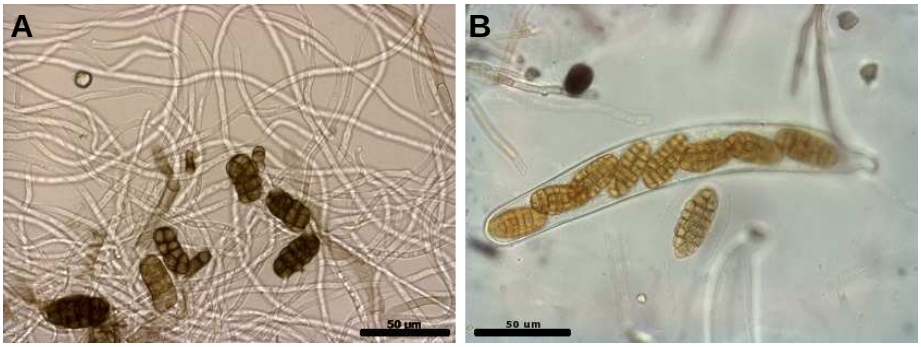

Figure 2. Morphology of *Stemphylium vesicarium* conidia **(A)** and mature asci with eight ascospores of *Pleospora allii* **(B)**. Image B courtesy of P. Loshuertos.

The teleomorph *P. allii* (Rabenh.) Ces & De Not belongs to the Class Ascomycetes, Order Pleosporales. The pseudotechia of *P. allii* are brown to black in color, coriaceous, globose and ostiolate. The size (with a mean diameter of 100 to 500 µm) depends on the substrate. Asci are bitunicate, cylindric-clavate (131 × 26 µm) and contain eight ascospores (Fig. 2B). Ascospores are yellow-brown, ellipsoidal or oblong to clavate in shape, with three to seven transverse septa and one longitudinal septum in each of most initial transverse divisions of the spore and with secondary septa in the transverse sub-divisions with approximate size of 32 × 14 µm (Fig. 2B) (Simmons, 1969; Llorente & Montesinos, 2006).

Many species of *Stemphylium* are economically important pathogens in agricultural crops. Saprophytic and pathogenic forms of *Stemphylium*

occur in a wide range of plants. *Stemphylium* species are characteristically colonizers of dead plant tissues, but there is limited information on the relationship between saprophytic and pathogenic populations of *Stemphylium* spp. or on the possible host-specificity of pathogenic isolates (Köhl *et al*., 2008). Some species are opportunistic pathogens found among a large variety of crops. Besides the pear tree, *Stemphylium vesicarium* has been described as pathogenic on herbaceous crops such as *Allium cepa* (onion), *Allium sativum* (garlic), *Asparagus officinalis* (asparagus), *Medicago sativa* (alfalfa) and on non-crop plants, such as *Aster* sp. (Falloon *et al*., 1984, 1987; Shishkoff & Lorbeer, 1989; Irwin & Bray, 1991; Aveling & Snyman, 1993; Chaisrisook *et al*., 1995; Prados-Ligero *et al*., 1998; Basallote-Ureba *et al*., 1999).

In the specific case of the pear, differences in levels of BSP susceptibility have been reported among pear cultivars. The most susceptible pear cultivars are the economically important and frequently cultivated varieties such as Conference, Abate Fetel, Passe Crassane and Alexandrine. The cultivars Williams BC, Blanquilla, Beurre Hardy, Louis Bonne, Grand Champion and Highland are slightly susceptible to BSP disease (Montesinos *et al*., 1995b). Furthermore, ontogenic susceptibility to BSP has been described in pear trees as the young leaves and immature fruit are more susceptible than the mature ones (Montesinos *et al*., 1995b).

The pathogenicity of *S. vesicarium* is directly correlated with the ability to produce SV-toxins, namely SV-toxin I and SV-toxin II. The plasma membrane modifications made by SV-toxins play an important role in the infection and colonization of host tissues by *S. vesicarium*. These toxins were found in culture filtrates of isolates of *S. vesicarium*

pathogenic to pear and the severity of necrosis was demonstrated to increase with toxin concentration and the susceptibility of the pear varieties (Singh *et al.*, 1999). Additionally, depending on the phenological stage and cultivar of the pear, different toxin effects were observed among pear tissues, for example younger leaves are more sensitive to toxins than older ones are (Singh *et al.*, 1999, 2000).

1.2. Disease symptoms

Disease symptoms consist of necrotic lesions on fruit and leaves and, sometimes, on petioles and twigs. The first leaf symptoms, 1 to 5 mm brown spots (Fig. 3A), are observed from late April or May, but are more frequently detected in June. Wide necrotic areas may be observed if the infection reaches leaf veins and extreme disease severity can cause premature tree defoliation. The first symptoms on fruit may appear at the end of May, but more usually in June when fruit are highly susceptible and the weather conditions are favorable. Initially, the spots on young fruits are circular, brown, range from 1 to 2 mm in diameter and are sometimes surrounded by a red halo (Fig. 3B). Later, on mature fruit, spots increase to 10 to 20 mm in size (Fig. 3C), and internal rot may occur if lesions are colonized by saprophytic fungi. Lesions are observed more frequently on the upper surface and on the calyx of young fruit, and their number increase until harvest. Affected fruit are unmarketable and can drop prematurely (Montesinos *et al.*, 1995b; Alberoni *et al.*, 2005; Llorente & Montesinos, 2006; Llorente *et al.*, 2011a).

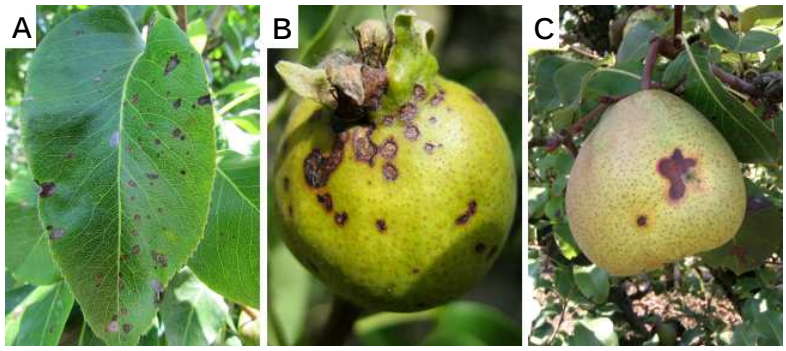

Figure 3. Brown spot of pear characteristic symptoms: **(A)** necrotic lesions on leaves, **(B and C)** lesions on pear fruit. Image C was courtesy of P. Loshuertos.

1.3. Epidemiology and inoculum production

The disease cycle is divided into two phases: 1) *saprophytic phase* in which the fungus grows on plant debris on the orchard floor (throughout the whole year) and 2) a *pathogenic phase* on the aerial pear organs during the pear growing period (spring and summer) (Fig. 4). As noted earlier, the causal agent of BSP produces two types of inoculum: sexual ascospores within pseudothecia and asexual conidia.

Pseudothecia are produced on both the fallen infected pear leaves and on the saprophytic colonized residues of grasses and herbaceous plants on the orchard floor (Rossi *et al.*, 2008; Llorente *et al.*, 2011a). Ascospores mature and disperse mainly from late summer to spring and can infect pear leaves or, more commonly, initiate the saprophytic colonization of the ground plant residues, which then persists throughout the year (Rossi *et al.*, 2005a,b, 2008; Llorente & Montesinos, 2006; Llorente *et al.*, 2010b). The development of pseudothecia of *P. allii* is influenced by relative humidity (RH) and temperature. Relative humidity is a significant limiting factor governing maturation, because pseudothecia only mature when RH is close to saturation point. The optimal temperature for the maturation of pseudothecia is between 10 and 15°C, whereas

maturation rates decrease when temperatures are 5°C or lower. No maturation was observed when temperatures reached above 25°C (Llorente & Montesinos, 2004; Llorente *et al*., 2006). Ascospores of *P. allii* are released mainly from February to June, and from August to October; but ascospores have been also trapped in July in some pear orchards (Llorente & Montesinos, 2006; Rossi *et al*., 2008). Conidia are also produced abundantly on the ground-cover litter from spring to autumn. Once produced, conidia become airborne and infect pear leaves and fruit (Rossi *et al*., 2005b, 2008; Llorente *et al*., 2011a). Plant residues on the orchard floor are considered to be a constant source of conidia potentially causing infections on pear leaves and fruits during the entire season. Lesions on infected leaves and fruit on pear trees are also considered a source of conidia of *S. vesicarium*. Temperatures between 15 and 25°C and a high relative humidity, particularly wetness duration longer than 10 h per day, are favorable to conidia release. Most of the conidia are released during the morning and at midday, i.e. when the dew disappears and the wind velocity increases, thus the conidia can disengage from conidiophores. During rainy days, *S. vesicarium* conidia do not show the typical daily periodicity, as the rain prevents liberation of conidia by surface-tension effects (Rossi *et al*., 2005a,b, 2008; Giosuè *et al*., 2006; Llorente *et al*., 2006; Llorente & Montesinos, 2006; Köhl *et al*., 2009). Both conidia and ascospores also play an important role in the constant spread of the pathogen population on plant residues on the orchard floor (Rossi *et al*., 2005b, 2008; Köhl *et al*., 2008; Llorente *et al*., 2011a).

Both kinds of inoculum (conidia of *S. vesicarium* and ascospores *of P. allii*) are capable of producing infections on pear fruit and leaves, but the majority of infections are produced by conidia. The optimal temperature

for conidial germination ranges from 20 to 30°C when a high rate of germination occurs. Additionally, RH is an important limiting factor for conidial germination as *S. vesicarium* conidia need wetness to germinate, and consequently a high relative humidity (RH>98%) (Cugier & Humbert, 1991; Montesinos & Vilardell, 1992). Thus, both the wetness duration and temperature during the period of wetness are the most important weather parameters that affect *S. vesicarium* infection on fruit and leaves. A minimum of 6 hours of wetness on pear tree organs is required to initiate an infection and the optimal temperatures during the wetness period are between 20°C and 25°C with the most favorable being 22.5°C (Montesinos *et al.*, 1995a; Llorente & Montesinos, 2002). Several studies demonstrated that the infection process is irreversibly stopped if low RH dry periods occur between wetness periods. However, those dry periods with a high RH do not stop infections that have already been initiated. Nowadays, it is conceived that wetness periods can be considered interrupted if the length of interruption at low RH is ≥ 3 h (Llorente & Montesinos, 2002; Llorente *et al.*, 2011b). Favorable temperature for ascospores germination range from 15 to 20°C, lower than the optimal temperature for conidial germination, since *P. allii* has adapted to winter or spring conditions with their colder temperatures (Llorente *et al.*, 2006). Additionally, ascospores germinate more slowly than conidia, with 70% of germination occurring after 5 h of incubation under favorable conditions.

S. vesicarium and *P. allii* are highly infective because conidia and ascospores are multicellular and thus each cell can develop a germ tube, which in turn increases the likelihood of infection through lenticels in the fruit and stomata the in leaves (Montesinos & Vilardell, 1992; Llorente & Montesinos, 2006; Llorente *et al.*, 2011a).

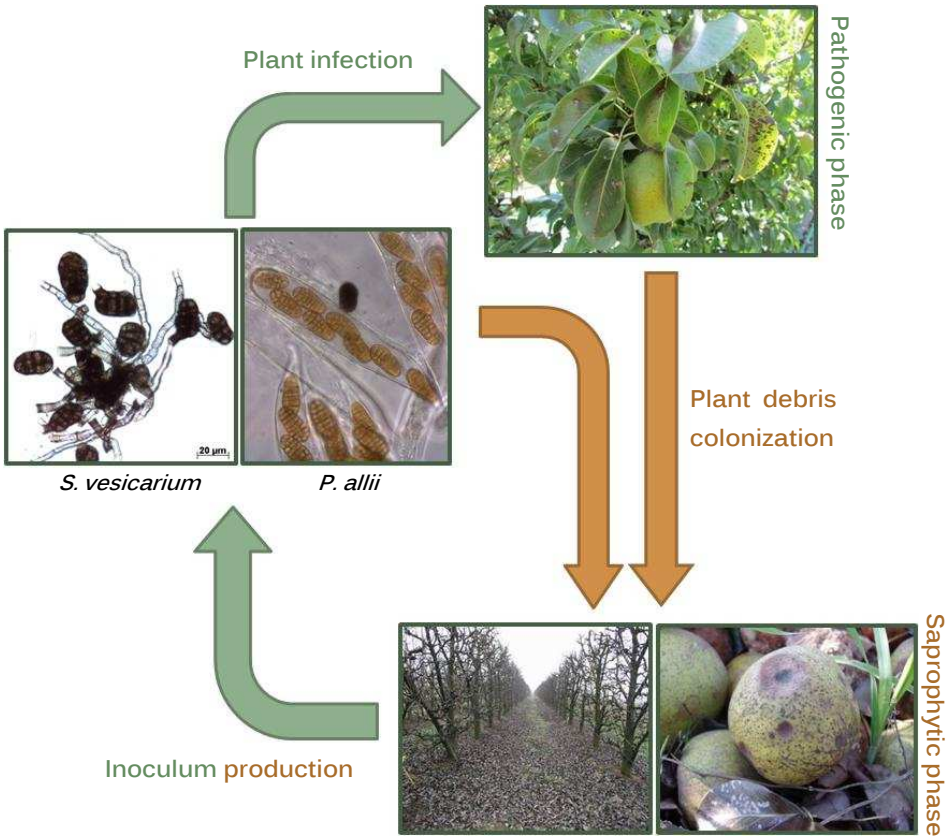

Figure 4. Pathogenic and saprophytic phases in the biological cycle of *Stemphylium vesicarium* and *Pleospora allii* in pear orchards. Pathogenic phase occurs on the aerial pear organs during spring and summer, causing plant infection, and the saprophytic phase occurs on herbaceous plants and pear leaf litter on the orchard floor. Some images are courtesy of P. Loshuertos. Modified from Llorente *et al.,* 2011a.

1.4. Inoculum assessment

By carrying out an assessment of inoculum potential in the orchard environment, the efficacy of disease control methods is increased. Generally, inoculum monitoring is achieved through spore traps, while the identification of *Stemphylium* species relies on morphological and developmental characters such as variation in conidia, conidiophore and ascospores morphology (Simmons, 1969; Puig *et al.*, 2015). However, many of these characteristics overlap among species in the

genus, making it difficult to distinguish *S. vesicarium* from other *Stemphylium* species (Simmons, 1969, 1985, 2004; Rossi *et al.*, 2008; Köhl *et al.*, 2008, 2009; Puig *et al.*, 2015). Correspondence among ITS and *gpd* sequences and morphological traits have been evaluated in order to determine their applicability in the identification of *S. vesicarium* (Puig *et al.*, 2015). Unambiguous identification of field isolates in the *S. vesicarium* species group is only achieved with combined morphological and molecular data. A recent study revealed that several species of *Stemphylium* coexist in pear orchards with *S. vesicarium* and only isolates identified as *S. vesicarium* are pathogenic on pear. Consequently, direct measurements of the airborne inoculum using volumetric spore traps and based on conidia morphology may overestimate the actual pathogen population and its pathogenic potential (Puig *et al.*, 2015).

Estimating of pathogenic potential inoculum in a pear orchard requires a proper differentiation among pathogenic and nonpathogenic isolates. However, the conidia and ascospores of pathogenic populations cannot be morphologically distinguished from conidia and ascospores of nonpathogenic populations, so the pathogenic potential may also be overestimated. Recently, qualitative and quantitative molecular tools have been developed for specific analysis of *S. vesicarium*. Pear pathogenic isolates of *S. vesicarium* in pear orchards can be distinguished from nonpathogenic isolates on the basis of distinctive Amplified Fragment Length Polymorphism (AFLP) fingerprinting profiles. A TaqMan PCR has been developed on the sequence of specific DNA fragments in pathogenic isolates from pear. This PCR demonstrates a very high sensitivity with quantification from 1 ng to 100 ng of DNA (Köhl *et al.*, 2008, 2009, 2013). Additionally, *S. vesicarium* isolates from pear

orchards were characterized with RAPD and AFLPs techniques and several fragments obtained by PCR-based techniques were selected to design a duplex PCR specific to *S. vesicarium* and to pear pathogenic isolates (Ruz *et al*., 2012).

2. Brown spot of pear management

BSP management is focused on reducing infections during the growing season as well as reducing inoculum production, through chemical sprays programs and sanitation practices.

2.1. Forecasting models

Forecasting models are used to schedule chemical spraying to ensure efficient disease control and to avoid the use of unnecessary fungicide sprays. Two different forecasting models have been developed for BSP disease control.

PAMcast model

The PAMcast model (*Pleospora allii* Maturation forecast) was developed to establish the relationship between the environmental conditions and the development of pseudothecia of *P. allii* (Llorente & Montesinos, 2004). Under high RH conditions the development of *P. alli* is dependent on temperature. This model estimates the percentage of mature pseudothecia in relation to the cumulative degree days (CDD). The PAMcast consists of a monomolecular function that predicts the percentage of mature pseudothecia on the basis of temperature and RH during the winter.

The monomolecular function is:

$$ln\frac{1}{1-y} = 0.12550 + 0.005048 \cdot CDD$$

Where *y* is the proportion of mature pseudothecia and *CDD* is the cumulative degree days (0°C base).

According to this model, 750 CDD are required for a 98% maturation of *P. allii* pseudothecia. For several years now, PAMcast has been validated under field conditions in pear orchards and it may be used to determine when exactly to initiate measures to prevent primary infections by inoculum released from debris colonization (Llorente & Montesinos, 2004; Llorente *et al*., 2006).

BSPcast model

The BSPcast model (Brown Spot of Pear forecasting system) was developed specifically to predict the infection risk of *S. vesicarium* on pear (Montesinos *et al*., 1995a; Llorente, 1997; Llorente *et al*., 2000). This model quantifies the effect of daily wetness duration and mean temperature of wetness periods have on the BSP disease (Montesinos *et al*., 1995a; Llorente *et al*., 2000). Daily wetness duration (W) and mean air temperature during the wetness periods (T) are used to compute the daily disease severity (S) according to the following equation (Montesinos *et al*., 1995a; Llorente *et al*., 2000):

$$\text{Log}_{10}(S) = -1.70962 + 0.0289\text{T} + 0.04943\text{W} + 0.00868\text{TW} - 0.002362\text{W}^2 - 0.000238\text{T}^2\text{W}$$

The relative daily infection risk (R) is calculated with W and T values from 8:01 GMT of the previous day to 8:00 GMT of the current day. Since

the maximum daily disease severity predicted by the equation is 3.7942, then R is calculated as:

$$R=S/3.7942 \qquad (0 \leq R \leq 1)$$

Next, the cumulative daily infection risk (CR) is obtained by the sum of the R values of the previous 3 days. Values of $CR \geq 0.4$ or $CR \geq 0.5$ are commonly used as threshold for spraying fungicides. Instead of the use of cumulative risk ($CR \geq 0.4$), the daily infection risk $R \geq 0.2$ can be used as the action threshold to spray with the same efficacy of control (Llorente *et al.*, 2011b).

For a number of years now, the BSPcast model has been evaluated over a wide range of conditions and validated as an advisory system to schedule fungicide spraying in disease management programs in Spain and Italy (Llorente & Montesinos, 2006; Llorente *et al.*, 2011a). Some changes were introduced to improve its effectiveness and to refine its accuracy. The effect of interrupted wetness periods and RH during the interruption of wetness was incorporated into the BSPcast model (Llorente *et al.*, 2011a,b).

Currently, BSPcast is implemented as a warning system in the agrometeorological network of the Plant Health Services of Catalonia (Spain) and Emilia-Romagna (Italy). Successful results were obtained when the BSPcast was assessed in Belgium, the Netherlands and Portugal (Llorente & Montesinos, 2006; Llorente *et al.*, 2011a). Spraying fungicides according to the BSPcast data not only reduces between 30% to 40% the number of treatments required, but also demonstrates a similar efficacy to the fixed schedule fungicide applications when the

disease level is moderate to low (Llorente *et al.*, 2000; Llorente & Montesinos, 2006).

2.2. Chemical control

Chemical control of BSP is needed to reduce *S. vesicarium* infection in pear orchards. Fungicides can be classified according to different characteristics i.e. type of chemical (inorganic or organic), mobility in plant (contact or systemic), role in protection, application timing (preventative, postinfection and curative) and range of activity (Table 2) (Llorente, 1997; Ogle, 1997; Oerke *et al.*, 1999; McGrath, 2004; Agrios, 2005; Hollomon, 2009; FRAC, 2014). Fungicides that can be used against *S. vesicarium*, their characteristics and mode of action are listed in Table 2. Dithiocarbamates (thiram, mancozeb), triazoles (tebuconazole) and strobilurins (kresoxim-methyl, trifloxystrobin) are the most commonly used. Captan, chlorothalonil, fludioxonil and difenoconazole are also applied in BSP disease control (Table 2) (Ponti *et al.*, 1993; Llorente & Montesinos, 2006; Llorente *et al.*, 2011a).

Chemical control of BSP is currently achieved with preventative spraying applied every 7 to 14 days from petal fall to a few weeks prior to harvest. Typically, 15 to 25 fungicide sprays are required to maintain low levels of disease incidence in fruit when the disease pressure is moderate or high (Singh *et al.*, 1999; Llorente & Montesinos, 2006; Llorente *et al.*, 2011a). However, some applications of fungicides may be unnecessary because environmental conditions are not always conducive to *S. vesicarium* infections. The indiscriminate use of fungicides is not suitable for integrated production systems and the large amounts of fungicide used increase yield costs. Non-target negative effects of indiscriminate chemical usage, include soil and water pollution in the actual orchards

themselves or in the surrounding areas, along with the selection of fungicide resistant pathogen isolates of *S. vesicarium*; as reported in orchards in Italy (Khan, 1980; Vighi & Funari, 1995; Gevao, 2000; Taube *et al.*, 2002; Alberoni *et al.*, 2005, 2010; Harman *et al.*, 2006; Naik & Prasad, 2006). Consequently, BSPcast is used as a tool for scheduling fungicide spraying and for reducing the number of treatments. BSPcast is currently implemented in pear production areas in Spain, Portugal, Italy, Belgium and the Netherlands.

Under high disease pressure, fungicides are not sufficiently effective and the disease is not reduced to economically acceptable levels (Llorente, 1997; Llorente *et al.*, 2000, 2011b; Llorente & Montesinos, 2006). Accordingly, the application of fungicides should be combined with other control methods such as biological control and sanitation practices. In addition, the European Union (Directives 91/414/CEE and 2009/128/CE) and the USA (the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)) have carried out regulatory changes in pesticide registration requirements based on environmental impact, toxicology and traceability. As a result, several fungicides that were once used to control plant disease have been banned (Montesinos & Bardají, 2008; Montesinos *et al.*, 2012). Hence, very few fungicides are authorized for BSP control and even fewer in Integrated Pest Management (IPM) (Table 2). Due to EU and USA regulations concerning agrochemical registration and application, nowadays many efforts are focused on controlling pests through new chemical compounds, biological control agents and varietal innovation (Montesinos, 2003, 2007; Agrios, 2005; Leadbeater & Gisi, 2009; Dehne *et al.*, 2011; Montesinos *et al.*, 2012).

Table 2. Classification and mode of action of fungicides used in control of brown spot of pear (Llorente, 1997; Ogle, 1997; Gullino *et al.*, 2000; Agrios, 2005; Russell, 2005; Oerke, 2006; Morton & Staub, 2008; Smith, *et al.*, 2009; Hollomon, 2009; Leadbeater & Gisi, 2009; Dehne *et al.*, 2011; Yang *et al.*, 2011; FRAC, 2014).

Fungicide	Group name	Chemical group	Mobility	Activity	Mode of action	IPM*	Resistance Risk**
Thiram	Dithiocarbamates	Dimethyl dithiocarbamate	Contact	Multi-site	Inactivation of –SH groups in amino acids, proteins and enzymes.	No	Low
Mancozeb		Ethylene bisdithiocarbamate				No	Low
Tebuconazole	DMI-fungicides (DeMethylation Inihibtors)	Triazoles	Systemic	Single-site	Inhibition of sterol biosynthesis in membranes	Yes	High
Difenoconazole						No	High
Kresoxim-methyl	QoI-fungicides (Quinone outside Inhibition)	Strobilurins	Systemic	Single-site	Inhibition of mitochondrial respiration, blocking the cytochrome bc1 complex and inhibiting ATP formation.	Yes	High
Trifloxystrobin						Yes	High
Pyraclostrobin		Methoxy-carbamates				Yes	High
Captan	Phtalimide	Phtalimide	Contact	Multi-site	Inhibits the production of essential compounds containing —NH2 and —SH groups (amino compounds and enzymes).	Yes	Low
Chlorothalonil	SDHI (Succinate dehydrogenase inhibitors)	Chloronitriles (phthalonitriles)	Contact	Multi-site	Reacts with —NH2 and —SH groups and inactivate enzymes that have such groups.	Yes	Low
Boscalid		Pyridine-carboxamides	Contact	Single-site		Yes	High
Fluopyram		Pyridinyl-ethyl-benzamides	Contact	Single-site	Inhibition of mitochondrial respiration in fungi (inhibition of complex II)	Yes	High
Fludioxonil	PP-fungicides (Phenylpyrroles)	Phenylpyrroles	Contact	Single-site	Affects the signal transduction	Yes	Medium
Cyprodinil	AP - fungicides (Anilino-Pyrimidines)	Anilino-pyrimidines	Mainly contact	Single-site	Inhibition of amino acids and protein synthesis (methionine biosynthesis)	Yes	High

* Fungicides allowed for being used in the integrated pest management. **Level of intrinsic risk for resistance evolution to fungicide (FRAC Code List, 2014).

2.3. Biological control

Several biological control agents (BCA's) have been evaluated for BSP disease control on aerial pear tree organs. The first studies were conducted with spore suspensions of *Trichoderma koningii* and *T. viride* applied on leaves. However, the efficacy was low and no reduction in disease was observed (Ponti *et al.*, 1993). The strain *Pseudomonas fluorescens* EPS288 showed moderate control efficacy in greenhouse conditions when it was applied as a preventative foliar spray on potted pear plants. However, when it was tested in field conditions its control activity decreased due to the low survival rate, poor colonization of pear leaves and fruit surface and insufficient competition with autochthonous microorganisms in the phyllosphere (Montesinos & Bonaterra, 1996; Montesinos *et al.*, 1996; Llorente, 1997).

On the other hand, strategies based on reducing inoculum using BCA's were evaluated with the purpose of disrupting the biological cycle at the saprophytic phase. The treatments consisted of applications of *Trichoderma* sp. formulations on leaf debris on the orchard floor in an attempt to reduce the overwintering inoculum of *P. allii*. When these BCA's were applied at the beginning of pseudothecia maturation a 57% to 96% reduction in inoculum was observed (Llorente *et al.*, 2006). The combination of sanitation practices, including pear leaf litter removal, with two to three applications of *Trichoderma* sp. from February to April resulted in reducing disease incidence at harvest time by approximately 30% (Llorente *et al.*, 2010a,b). In addition, treatments with *Trichoderma* spp. during the growing season reduced the *S. vesicarium* conidia on leaf debris in more than 99% six weeks after application (Rossi & Pattori, 2009). Recently, several strains of *Bacillus subtilis* to reduce *S. vesicarium* inoculum production on soil have been evaluated *in vitro*

and *ex vivo*. Three *B. subtilis* strains reduced inoculum production from 40% to 70% (Ruz *et al.*, 2013).

2.4. Sanitation and cultural practices

Sanitation and cultural practices during the growing season help to increase the efficacy of control and pear production. To disrupt the biological cycle, sanitary methods to prevent pseudothecia formation during autumn and winter are recommended. Methods based on crushing or removing leaf debris from the orchard floor before February have been shown to be highly efficient in reducing ascospore production (Llorente *et al.*, 2006; Llorente & Montesinos, 2006). In the case of sanitation methods, and as has been previously described, applying BCA's based on *Trichoderma* spp. can be included to reduce inoculum production.

The irrigation system and tree nutrition can affect the incidence and severity of BSP. Proper soil drainage and a drip irrigation system are recommended to minimize disease levels. Avoiding nutrient deficiencies and excesses of nitrogen is recommended as this may stimulate the pear trees' vegetative growth (Llorente & Montesinos, 2006).

2.5. Integrated Pest Management of BSP

Integrated Pest Management (IPM) considers all the available crop information i.e. pathogens, the history of disease in previous years, varietal resistance to diseases, environmental conditions, locality, availability of materials, land, labor and costs, and different methods of control (Agrios, 2005).

Within the Catalan IPM guidelines, the authorized fungicides for controlling BSP disease are boscalid+pyraclostrobin, cyprodinil+fludioxonil, fluopyram+tebuconazole, captan, kresoxim-methyl, tebuconazole and trifloxystrobin (DAAM, 2014). However, the efficacy of disease control is limited, especially when disease pressure is high. New strategies and methods to control BSP, ones which follow an IPM program, are essential to enhance effectiveness.

The trends in plant disease control are oriented towards a rational use of plant protection chemicals and a reduction in the number of registered active ingredients used. In this context, interest in new active ingredients has increased, and as a result scientists and agrochemical companies are focusing their research on the development of new agrochemicals which are more selective, less toxic and have a much lower negative environmental impact (Montesinos, 2003; Leadbeater & Gisi, 2009; Dehne *et al.*, 2011). New antimicrobial compounds, which fulfill the requirements of EU and USA regulations, may provide an alternative or be the essential complement in plant disease protection.

3. Antimicrobial peptides: candidates for controlling plant diseases

Antimicrobial peptides (AMPs) are evolutionarily conserved components present in bacteria, fungi, invertebrates, plants, amphibians, birds and mammals, and even in humans. Organisms use AMPs against invading pathogens, thereby, they are found in sites where pathogen attack and infect, such as the mouth, skin, leaves, flowers, seeds, etc. (Hancock & Lehrer, 1998; DeLucca & Walsh, 1999; Hancock & Chapple, 1999; Hancock *et al.*, 2006; Montesinos, 2007).

In 1922 the peptide lysine was discovered by Alexander Fleming and, nowadays, more than 2000 peptides have been described (Wang, 2003). Since the 1980s, when Han Boman, Michael Zasloff and Robert Lehrer, isolated and purified insect cecropins, amphibian magainins and mammalian defensins, respectively, the number of known AMPs has steadily increased year after year (Steiner *et al.*, 1981; Ganz *et al.*, 1990; Wang *et al.*, 2010). Currently, the Antimicrobial Peptide Database (APD) contains 2579 entries with 935 antifungal, 2131 antibacterial, 183 anticancer and 170 antiviral (105 anti-HIV) peptides (*http://aps.unmc.edu/AP/main.php*, Wang *et al.*, 2009). According to the APD, the main sources of peptides are animals, plants and bacteria (Wang, 2003; Wang *et al.*, 2009, 2010).

In accordance with their characteristics, AMPs are short peptides (< 50 amino acids), positively charged at physiological pH (net charge of +2 to +9) with the ability to assume an amphipathic structure conferred by their amino acid composition with differentiated hydrophobic and hydrophilic domains (Yeaman & Yount, 2003; Marcos *et al.*, 2008). The most abundant residues in AMPs are cationic (arginine and lysine) and hydrophobic (tryptophan, phenylalanine, leucine, and isoleucine). The net positive charge and the ability to be amphipathic are the essential properties for their mode of action against microorganisms (Oren & Shai, 1998; Giangaspero *et al.*, 2001; Powers & Hancock, 2003; Keymanesh *et al.*, 2009; Pasupuleti *et al.*, 2012).

AMPs have been considered as a new generation of compounds that have a huge potential to be applied in different fields such as, clinical treatments against resistant bacteria and opportunistic fungal infection, veterinary use, food preservation, crop protection and other industrial

uses (Hancock & Chapple, 1999; Zasloff, 2002; Hancock & Sahl, 2006; Keymanesh *et al.*, 2009; Montesinos *et al.*, 2012; Perez Espitia *et al.*, 2012). Although a lot of natural AMPs have exhibited antimicrobial activity, some of them do present the following drawbacks: a) obtaining them involves high production costs because AMPs are naturally produced in small amounts, b) they can cause toxicity in plant and human cells, and c) they are rapidly degraded by proteases. Consequently, many strategies have been formulated for designing shorter, non-toxic and more effective and stable synthetic AMPs.

Synthetic AMPs are designed and prepared based on the modification of known natural AMPs or on de *novo* design strategies and are produced by synthetic or biotechnological methods (Monroc *et al.*, 2006; Marcos *et al.*, 2008; Montesinos & Bardají, 2008; Montesinos *et al.*, 2012). In recent decades, several synthetic AMPs have been evaluated in the control of plant pathogens (Table 3).

3.1. Classification of antimicrobial peptides

Natural and synthetic AMPs show a great diversity in the number of residues, primary structures, positioning of charged residues and secondary structures (Yeaman & Yount, 2003; Powers & Hancock, 2003; Brogden, 2005). The two most widely used classifications are based on AMPs biosynthesis pathways and their secondary structures.

A. Biosynthesis pathway

Natural synthesis of AMPs is usually induced in response to an infection and the large majority of synthesized AMPs by multicellular organism are encoded by genome (Epand & Vogel, 1999). Based on their biosynthesis pathway, AMPs are divided into two categories:

nonribosomally synthesized peptides and *ribosomally synthesized peptides.*

Table 3. Synthetic antimicrobial peptides active against plant pathogenic bacteria and fungi.

Peptide	Origin	#Aa	Activity*	References
BP76	Cecropin A-melittin hybrid	11	F+B	(Ferre *et al.*, 2006; Badosa *et al.*, 2007, 2009)
BP100	Cecropin A-melittin hybrid	11	B	(Badosa *et al.*, 2007)
BP194	Novo design (cyclic)	10	B	(Monroc *et al.*, 2006a)
CAMEL	Cecropin A-melittin hybrid	15	B	(Kamysz *et al.*, 2005)
C14-*KLLK*	Lipopetide	14	F+B	(Makovitzki *et al.*, 2007)
D2A21	Novo design	23	F+B	(Rioux et al., 2000)
D32R	Thionin	47	F+B	(Vila-Perelló *et al.*, 2003)
D4E1	Novo design	17	F+B	(DeLucca & Walsh, 1999; Mentag *et al.*, 2003; Oard *et al.*, 2004)
ESF1	Novo design	20	F+B	(Powell *et al.*, 1995)
ESF12	Novo design	18	F+B	(Powell *et al.*, 1995)
GR7	Novo design	20	F+B	(Dykes *et al.*, 1998)
$LfcinB_{17-31}$	Bovine lactoferricin	15	F	(Muñoz & Marcos, 2006)
$LfcinB_{20-25}$	Bovine lactoferricin	6	F	(Muñoz & Marcos, 2006)
Iseganan	Protegrin I	17	B	(Chen *et al.*, 2000)
MB-39	Cecropin B	39	F+B	(Owens & Heutte, 1997)
MBG01	Rs-AFPs	19	F	(Schaaper *et al.*, 2001)
MsrA1	Cecropin A-melittin hybrid	34	F+B	(Osusky *et al.*, 2000; Rustagi *et al.*, 2014)
MrsA2	Dermaseptin B1	32	F+B	(Osusky *et al.*, 2005)
MSI-99	Magainin	23	F+B	(Alan & Earle, 2002)
Myp30	Magainin	24	F+B	(Li *et al.*, 2001)
P18	Cecropin-magainin hybrid	18	F+B	(Lee *et al.*, 2004)
PAF26	Novo design	6	F	(López-García *et al.*, 2002)
Pc87	Novo design	9	F	(Bishop-Hurley *et al.*, 2002)
Pep7	Magainin	7	F+B	(Zasloff, 1987; Ali & Reddy, 2000)
Pep3	Cecropin A-melittin hybrid	11	F+B	(Cavallarin *et al.*, 1998; Ali & Reddy, 2000)
Pexiganan	Magainin	22	B	(Chen *et al.*, 2000)
phor21	Novo design	21	F+B	(Javadpour *et al.*, 1996; Oard *et al.*, 2004)
PPD1	Novo design	5	F	(Reed *et al.*, 1997)
Rev4	Indolicin	13	F	(Li *et al.*, 2002)
Shiva-1	Cecropin B	38	B	(Jaynes *et al.*, 1993)
10R	Indolicin	13	F+B	(Bhargava *et al.*, 2007)
11 R	Indolicin	13	F+B	(Bhargava *et al.*, 2007)
66-10	Novo design	7	F	(Gonzalez *et al.*, 2002)

* B, antibacterial; F, antifungal; F+B, antifungal and antibacterial.

The first group includes peptaibols, cyclopeptides and pseudopeptides produced by bacteria, fungi and streptomycetes. Some examples of nonribosomally AMPs are gramicidin A, polymyxin B, alamethicin, daptomycin and vancomycin. On the other hand, ribosomally synthesized peptides are produced by most living organisms with defensins, small bacteriocins, cecropin A, magainin, melittin and lactoferricin belonging to this group (Hancock & Chapple, 1999; Yeaman & Yount, 2003; Montesinos, 2007; Montesinos *et al.*, 2012).

B. Secondary structure

The most common classification of AMPs is based on their conformational structure in membrane-like environments. According to the conformational structure, AMPs are divided into four categories: α-helical, ß-sheet, peptides with extended structures and peptides with loop structures. The conformations most commonly found in nature are α-helical and ß-sheet (Boman, 1995; Epand & Vogel, 1999; Van't Hof *et al.*, 2001; Yeaman & Yount, 2003; Powers & Hancock, 2003; Jenssen & Hamill, 2006). The representative structures from each of these classes are indicated in figure 5.

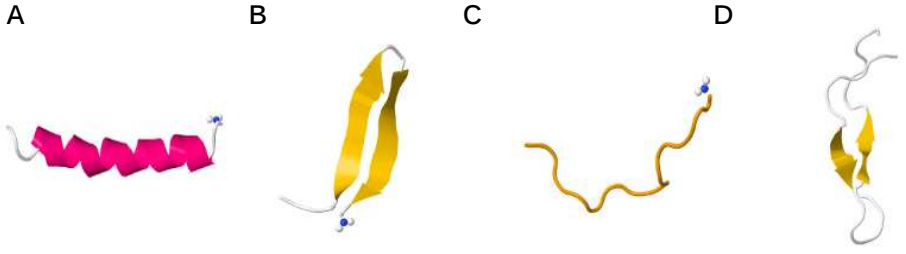

Figure 5. Protein models representing the secondary structures of the four classes of antimicrobial peptides: **(A)** α-helical (magainin), **(B)** ß-sheet (protegrin), **(C)** extended (indolicin) and **(D)** loop (lactoferricin B). All structures were obtained freely from the RCSB Protein Data Bank (PDB) (*http://www.pdb.org*, Berman *et al.*, 2000)

i. **Linear peptides with amphipathic α-helical structure** adopt a disordered structure and are highly flexible in aqueous solution. When interacting with membrane surface and hydrophobic solvents these peptides transform into an α-helical conformation. The α-helical peptides are the most abundantly distributed and widely studied group of AMPs. They are easy to find in the extracellular fluids of insects and frogs and they constitute about 27% of all AMPs with known secondary structure (Pasupuleti *et al.*, 2012). Most of them are cationic and exhibit selective toxicity for microorganisms. The AMPs in this group generally kill the microorganisms by forming membrane defects, which result in electrolyte loss (Oren & Shai, 1998; Pasupuleti *et al.*, 2012). Recently, these AMPs are also suspected of being able to kill microorganisms through the inhibition of their intracellular targets (Peters *et al.*, 2010; Muñoz *et al.*, 2013a). Cecropin A, buforin II, magainin, LL-37, melittin and synthetic peptides Pep3, BP76, BP100, MSI-99 and PMAP-23 belong to this group.

ii. **ß-sheet peptides** are produced in many animals and plants, some of them show antifungal and antibacterial activity. These AMPs are characterized by the presence of an antiparallel ß-sheet generally stabilized by disulfide bonds. As a rule they adopt a ß-sheet conformation in aqueous solution and they can be stabilized by interacting with surface membrane lipids. ß-sheet peptides are, in contrast to α-helical peptides, much more ordered in aqueous solution and membrane environments due to the constraints imposed by disulfide bonds or cyclization of the peptide backbone. Protegrins, tachyplesin, ß-defensins, polymixin, heliomicin and gramicidin are some examples of this class of AMPs (DeLucca &

Walsh, 1999; Epand & Vogel, 1999; Powers & Hancock, 2003; Brogden, 2005; Hancock & Sahl, 2006).

iii. **Linear peptides with extended structures** exhibit a lack of classical secondary structures due to the high content of proline and/or glycine. These peptides form their final structures by hydrogen bond and Van der Waals interaction with membrane lipids. As with the α-helical peptides, they are flexible in aqueous solution although when they interact with membranes they adopt an amphipathic structure. The best characterized peptides in this class are indolicidin, PR-39, tenecin-3 and bactenecin-5 (Powers & Hancock, 2003; Jenssen & Hamill, 2006; Hancock *et al.*, 2006; Hancock & Sahl, 2006; Van der Weerden *et al.*, 2013).

iv. **Looped peptides** are characterized by their loop structure given by the presence of a single disulfide bridge, amide or isopeptide. There are a few known peptides exhibiting a loop structure some examples of which are thanatin, lactoferricin B, bactenecin-1, nisin and mersacidin.

3.2. Mode of action of antimicrobial peptides

Since studies began, AMPs and their antimicrobial activity have been largely associated to their interaction and disruption of host/target cell membranes. AMPs require a net positive charge and the ability to assume an amphipathic structure in order to interact with the cell membranes of the target microorganisms and to bind to them. In addition, the interaction with cell membranes depends on the membrane composition and the structure of both the microorganisms and the AMPs. Thus, the antibacterial and antifungal peptides may have

different mode of action (Oren & Shai, 1998; Van't Hof *et al.*, 2001; Zasloff, 2002; Shai, 2002; Yeaman & Yount, 2003; Powers & Hancock, 2003; Theis & Stahl, 2004; Ferre *et al.*, 2009; Pasupuleti *et al.*, 2012; Muñoz *et al.*, 2012; Van der Weerden *et al.*, 2013). Recent studies have suggested that not all AMPs kill their target cell through membrane damage, but rather some AMPs also block essential intracellular processes after translocation across the cell membrane and so provoke cell death (Oren & Shai, 1998; Lundberg & Langel, 2003; Yeaman & Yount, 2003; Theis & Stahl, 2004; Brogden, 2005; Nicolas, 2009; Peters *et al.*, 2010; Mania *et al.*, 2010; Muñoz *et al.*, 2012, 2013a,b). Hence, membrane permeabilization is not the only cause of pathogen death. As a result of this, AMPs can be classified into two mechanistic categories: *membrane disruptive AMPs* and *non membrane disruptive AMPs.*

A. Membrane disruptive AMPs

Membrane disruptive AMPs generally belong to the α-helical structural class (Powers & Hancock, 2003). These AMPs are able to permeabilize and form membrane pores that then inhibit microbial growth. The first step in the AMPs mode of action is the physical interaction with the outer structures that surround the microbial cell: 1) at low peptide/lipid ratio (P/L), AMPs are bound parallel to a lipid bilayer; 2) when the P/L ratio increases, AMPs are orientated perpendicular to the membrane and 3) at higher P/L ratio they insert themselves into the bilayers forming transmembrane pores (I state). Three mechanistic models have been developed to explain membrane disruption, namely the *barrel-stave model*, the *carpet model* and the *toroidal pore model* (Fig. 6) (Powers & Hancock, 2003; Theis & Stahl, 2004; Brogden, 2005; Perez Espitia *et al.*, 2012; Pasupuleti *et al.*, 2012; Van der Weerden *et al.*, 2013).

i. The **barrel-stave model** (also called the helical bundle model) describes the pore formation through the binding of peptides on membranes. The hydrophobic surface of the peptide interacts with the lipid core of the cell membrane, while the hydrophilic surface of the AMPs is directed inside thus producing an aqueous pore (Fig. 6A). The progressive insertion of additional peptides into the membrane surface enlarges the size of these pores. Transmembrane pores can contain from 3 to 11 parallel helical molecules. To allow pore formation the inserted molecules should have distinct structures, such as an amphipathic or hydrophobic α-helix, a ß-sheet or both α-helix and ß-sheet structures. Pores disrupt the membrane potential and allow the leakage of cytoplasmic components. This model has been proposed to explain the activity of antimicrobial peptides, such as magainins, melittin, protegrin-1, LL-37 and MSI-78 (Powers & Hancock, 2003; Brogden, 2005; Perez Espitia *et al.*, 2012; Pasupuleti *et al.*, 2012).

ii. The **toroidal pore model** is considered a variant of the barrel-stave model. The α-helix peptides are initially oriented parallel to the membrane surface, then, the hydrophobic residues of the bound peptides displace the polar head groups, creating a breach in the hydrophobic region and inducing positive curvature in the membrane. The lipid bilayer bends back on itself, so the top and bottom monolayers form a continuous bend from one side of the membrane to the other (Fig. 6B). The resulting pore is sufficient to allow the peptides to move across the membrane. In this model lipids are intercalated with peptides in the transmembrane channel, even when they are perpendicularly inserted into the lipid bilayer. Some AMPs displaying this mechanism are magainin, melittin, LL-37 and

protegrins (Yeaman & Yount, 2003; Brogden, 2005; Jenssen & Hamill, 2006; Milani *et al.*, 2009; Nicolas, 2009; Perez Espitia *et al.*, 2012; Pasupuleti *et al.*, 2012).

iii. The **carpet model.** The AMPs in this group orientate themselves parallel to the membrane surface and cover the bilayer surface in a carpet-like manner. At high concentrations, AMPs are in contact with phospholipids located on the outer surface of the cell membrane which, in turn, allows the peptide to permeabilize the membrane (Fig. 6C). According to this model, AMPs exhibit a preferential binding with the phospholipid groups. The binding step is followed by the alignment of the AMPs on the membrane surface where the hydrophilic surface of AMPs is in contact with phospholipid or water molecules, causing a reorientation of hydrophilic residues and creating a hydrophobic core. The pores formed may enable the passage of low molecular weight molecules prior to complete membrane lysis. Finally, AMPs fragment the membrane through the deformation of membrane curvature. Dermaseptin, cecropin, ovesprin, caerin 1.1 and melittin have been proposed as following this mechanism (Brogden, 2005; Jenssen & Hamill, 2006; Perez Espitia *et al.*, 2012).

B. Non membrane disruptive AMPs

The implementation of cell and molecular biology methods (live-cell imaging techniques, genome-wide screens of mutant collections or transcriptomics) has facilitated the knowledge of the interaction between AMPs and target cells (Nicolas, 2009; Muñoz *et al.*, 2013a). These studies have demonstrated that several peptides kill microorganisms by inhibiting their intracellular targets (Park *et al.*, 1998;

Lundberg & Langel, 2003; Brogden, 2005; Deshayes *et al.*, 2005; Nicolas, 2009; Peters *et al.*, 2010; Hilpert *et al.*, 2010; Mania *et al.*, 2010; Muñoz *et al.*, 2013a; Van der Weerden *et al.*, 2013). The first step in destabilizing the membrane is the association of AMPs with the cell membrane. The AMPs destabilize the membrane and penetrate the cells to inhibit the intracellular processes. Most of these AMPs share characteristics with natural cell-penetrating peptides (CPPs) such as the charge, the structure and the host cell membrane interactions (Lundberg & Langel, 2003; Deshayes *et al.*, 2005). CPPs can be translocated across membranes without disrupting them, entering into the cell via endocytosis, however, the endocytotic pathway is not the usual route of AMPs as their microbial targets lack the necessary factors to carry out endocytosis (Guaní-Guerra *et al.*, 2010; Madani *et al.*, 2011).

Following membrane permeabilization, AMPs can activate or lock intracellular targets. Once the AMPs penetrate the cell membranes they may alter cell functions by the inhibiting DNA, RNA or protein synthesis, or by inhibiting some enzymatic activities and/or altering the cytoplasmic membrane and cell wall synthesis (Fig. 6). In these cases, cell death is the result of multiple inhibitory effects. Additionally, ribosomes are a key intracellular target for some AMPs. Such the peptides that inactivate ribosmes are called ribosome-inactivating proteins (RIPs) (Epand & Vogel, 1999; Brogden, 2005; Nicolas, 2009; Peters *et al.*, 2010; Hilpert *et al.*, 2010; Mania *et al.*, 2010; Muñoz *et al.*, 2013a). Several natural or synthetic AMPs are non membrane disruptive; for instance Cc-GRP, ABP-CM4, AFP, Cecropin B, PAF26, Sub 5 and lactoferricin. Briefly, AFP inhibits chitin synthesis; Cc-GRP and Sub5 are localized in intracellular structures and in the fungal cell wall and PAF26 interacts with the fungal cell wall and/or plasma membrane, enters into

the cells and produces specific intracellular effects associated with cell death (Reed *et al.*, 1997; Park *et al.*, 1998; Oard *et al.*, 2004; Muñoz & Marcos, 2006; Muñoz *et al.*, 2006, 2013a; Hagen *et al.*, 2007; Zhang *et al.*, 2008; Mania *et al.*, 2010; Zottich *et al.*, 2013). Even though many antifungal peptides have been studied, there is still not enough information available about their mode of action, especially against filamentous fungi associated with plant diseases (DeLuca & Walsh, 2000; Selitrennikoff, 2001; Rajasekaran *et al.*, 2012; Van der Weerden *et al.*, 2013).

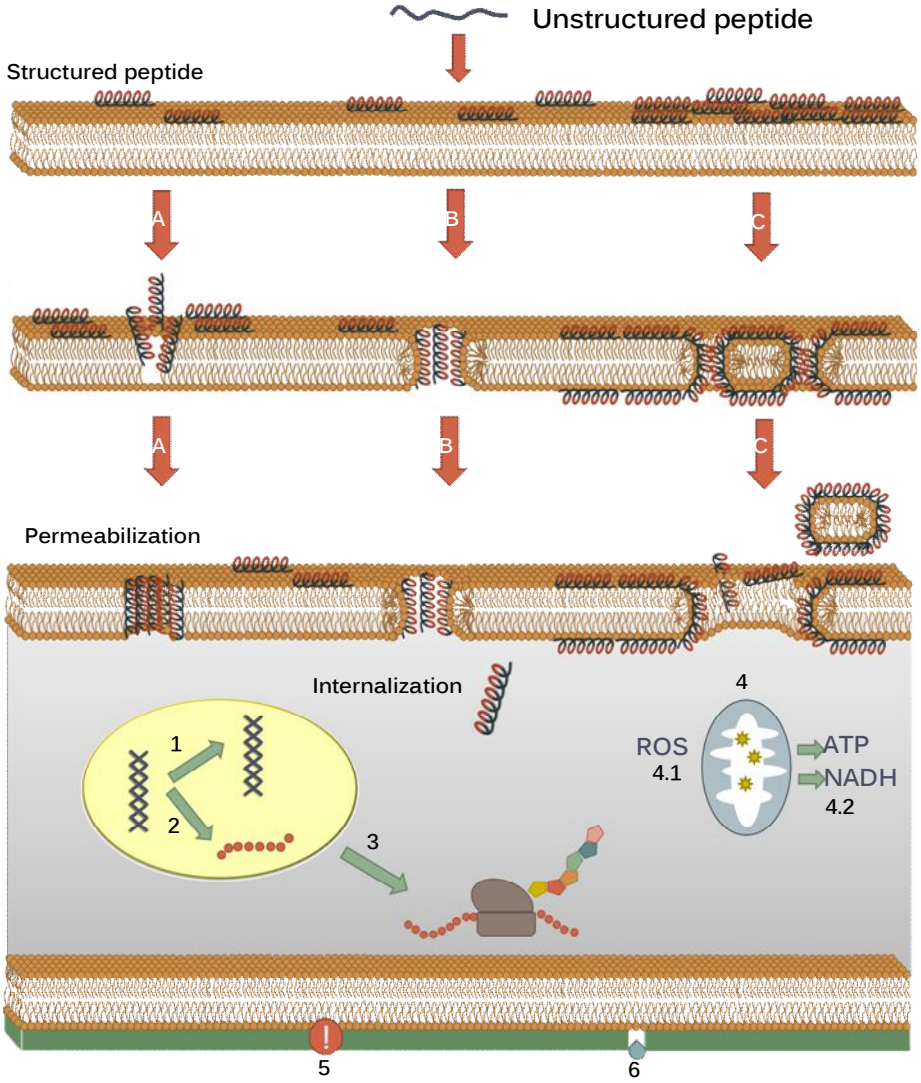

Figure 6. Models proposed for the permeabilization of target cell membrane by membrane active AMPs (A, B and C) and mechanism of action for AMPs with intracellular activity (1,2,3,4,5 and 6). Peptides are unstructured before binding to the membranes, but they rapidly adopt a favorable structure for the translocation into the membrane. Later, peptides destabilize the membranes either by the (A) barrel stove model, (B) toroidal pore model or (C) carpet model. Once the peptide is inside the cell it can inhibit DNA synthesis (1), block RNA synthesis (2) or inhibit ribosomal function and protein synthesis (3). Moreover, AMPs can alter the mitochondria (4) resulting in inhibition of cellular respiration and induction of reactive oxygen species (ROS) formation (4.1) and disruption of mitochondrial cell membrane integrity and efflux of ATP and NADH (4.2). Some AMPs inhibit the enzymes required for maintaining the cell wall structure (5), sometimes, associated to a leakage of cellular content (6). Modified from Jenssen & Hamill (2006) and Peters *et al.* (2010).

3.3. AMPs for controlling plant pathogens

When searching for AMPs for agricultural use, the main goal is to find the shortest, most active and least toxic peptide (Marcos *et al.*, 2008; López-García *et al.*, 2012; Montesinos *et al.*, 2012). The most popular strategies when designing AMPs for plant protection are based on the modifying known sequences with antimicrobial activity (usually natural AMPs) through: a) shortening bioactive part(s) of a large protein or linear peptides with an α-helical structure (such as cecropin A and magainin), b) constructing peptide hybrids with fragments of natural peptides, c) modifying natural AMPs by adding, deleting or replacing one or more residues and/or d) truncating N- or C-terminus (Montesinos, 2007; Marcos *et al.*, 2008; Montesinos & Bardají, 2008; Montesinos *et al.*, 2012).

The first step in selecting the most effective AMPs against the target pathogens is the *in vitro* screening of known natural or synthetic peptides. These *in vitro* tests are essential because they allow large collections of peptides to be screened quantitatively using a minimum amount of peptide. Once the selection of leader peptides has been made, the following stage involves the *ex vivo* tests. These experiments are only a close approximation of the activity in field conditions as several factors can reduce peptide activity when AMPs interact with plants. The main limiting factors that can influence the activity of AMPs are host, pathogen components, strategy of treatment and environmental factors (Montesinos & Bardají, 2008; Montesinos *et al.*, 2012). Consequently, the *ex vivo* concentration is always higher than the *in vitro* Minimum Inhibitory Concentration (MIC). Then, the assays *in planta* under controlled conditions or in the field are conducted with only one or two AMPs as it is essential to be able to verify the efficacy of AMPs in field conditions. Some difficulties can appear when AMPs are

applied to the field, for instance their inhibition by proteases or ultraviolet light. Otherwise, other parameters to take into account when choosing the appropriate AMP are cytotoxicity to nontarget cells, treatment strategy and the protease susceptibility of leader AMPs.

Once an effective AMP has been selected, optimizing synthesis processes and reducing production costs come into play. Developing formulations and verifying then in field conditions are essential procedures before any large scale production of the selected AMPs. Finally, any industrial exploitation and/or commercialization of AMPs proposed as being a registered product for plant protection, will require further testing in accordance with the specific legislation and guidelines of particular countries (Montesinos *et al.*, 2012). Tables 4 and 5 depict the inhibition of plant pathogenic microorganisms by synthetic AMPs.

Table 4. Plant pathogenic bacteria controlled by synthetic antimicrobial peptides.

Bacterial plant pathogens	Peptides	References
Erwinia amylovora	BP76 ,BP100, BPC194, ESF12	(Powell *et al.*, 1995; Monroc *et al.*, 2006a; Badosa *et al.*, 2007; Güell *et al.*, 2011)
Erwinia carotovora subsp. *carotovora*	MSI-99, CecP1, MrsA2	(Alan & Earle, 2002; Osusky *et al.*, 2005; Zakharchenko *et al.*, 2005)
Pseudomonas solanacearum	Shiva-1	(Jaynes *et al.*, 1993)
Pseudomonas syringae pv. *syringae*	BP100, BP76, BPC194, C14-*KLLK*, MSI-99,	(Alan & Earle, 2002; Badosa *et al.*, 2007; Makovitzki *et al.*, 2007; Güell *et al.*, 2011)
Pseudomonas syringae pv. *tabaci*	MSI-99	(Alan & Earle, 2002)
Pseudomonas syringae pv. *tomato*	MSI-99	(Alan & Earle, 2002)
Xanthomonas campestris pv. *populi*	D4E1	(Mentag *et al.*, 2003)
Xanthomonas campestris pv. *vesicatoria*	MSI-99	(Alan & Earle, 2002)

Table 5. Plant pathogenic fungi controlled by synthetic antimicrobial peptides.

Fungi plant pathogens	Peptides	References
Alternaria brassicae	MsrA1	(Rustagi *et al.*, 2014)
Alternaria solani	AFP, MSI-99, C14-*KLLK*	(Vila *et al.*, 2001; Alan & Earle, 2002; Makovitzki *et al.*, 2007)
Botrytis cinerea	AFP, C14-*KLLK*, $LfcinB_{17–31}$, $LfcinB_{20–25}$, PAF26, Pen4-1	(López-garcía *et al.*, 2002; Moreno *et al.*, 2003; Cuthbertson *et al.*, 2004; Muñoz & Marcos, 2006; Makovitzki *et al.*, 2007)
Ceratocystis fagacearum	Pep3, PPD1	(Reed *et al.*, 1997)
Fusarium moniliforme	AFP	(Vila *et al.*, 2001)
Fusarium oxysporium	AFP, Cc-GRP, ESF1, Pen4-1, Pep3, Pep6, PAF26	(Powell *et al.*, 1995; Reed *et al.*, 1997; Cavallarin *et al.*, 1998; López-García *et al.*, 2002; Cuthbertson *et al.*, 2004; Hagen *et al.*, 2007; Zottich *et al.*, 2013)
Fusarium sambucinum	66-10, 77-3	(Gonzalez *et al.*, 2002)
Magnaporthe grisea	AFP, $LfcinB_{17–31}$, $LfcinB_{20–25}$	(Vila *et al.*, 2001; Muñoz & Marcos, 2006)
Penicillium digitatum	BM0, BP15, MSI-99, $LfcinB_{17–31}$, $LfcinB_{20–25}$, PAF26	(López-García *et al.*, 2002; Alan & Earle, 2002; Muñoz & Marcos, 2006; Muñoz *et al.*, 2007)
Penicillium expansum	BP15, BP22, PAF26	(López-García *et al.*, 2007; Badosa *et al.*, 2009)
Penicillium italicum	$LfcinB_{17–31}$, $LfcinB_{20–25}$, PAF26	(López-García *et al.*, 2002; Muñoz & Marcos, 2006)
Peronospora tabacina	Myp30, Rev4	(Li *et al.*, 2001, 2002)
Phytophthora capsici	Pc87	(Bishop-Hurley *et al.*, 2002)
Phytophthora infestans	MSI-99, Pep3,	(Cavallarin *et al.*, 1998; Alan & Earle, 2002)
Pythium ultimum	Pep3	(Reed *et al.*, 1997)
Rhizoctonia solani	Cecropin B, D4E1, D2A21, Pep3, phor21	(Reed *et al.*, 1997; Oard *et al.*, 2004)
Sclerotinia sclerotiorum	MsrA1	(Rustagi *et al.*, 2014)

In recent years, in spite of the high production cost, studies of AMPs to be applied in agriculture and food preservation are on the increase (Marcos *et al.*, 2008; Montesinos & Bardají, 2008; López-García *et al.*, 2012; Montesinos *et al.*, 2012; Perez Espitia *et al.*, 2012; Badosa *et al.*, 2013; Company *et al.*, 2013). However, production cost must be significantly reduced for AMPs to become as cheap as conventional antibiotics, and thus become a very real alternative to conventional disease control treatments. Nowadays, low-cost AMPs production is difficult to achieve with standard organic chemistry procedures based on solid-phase. One such way to reduce production costs in obtaining AMPs for plant protection would be through the synthesis of short AMPs

(4–6 amino acid residues) with solution organic synthesis and/or chemoenzymatic approaches. Recently, new research has been focused on the recombinant expression of AMPs in microorganism or plants as biofactories (Montesinos & Bardají, 2008; López-García *et al.*, 2012; Montesinos *et al.*, 2012; Nadal *et al.*, 2012; Badosa *et al.*, 2013; Company *et al.*, 2013). Cecropin, lactoferricin and indolicidin have been expressed in *E. coli*; and penaedin, CAMA and ABP-CM4 have been expressed in *Pichia pastoris* (Montesinos & Bardají, 2008). BP100 and its derivatives have been expressed in rice and tobacco plants and in *Arabidopsis thaliana* (Nadal *et al.*, 2012; Company *et al.*, 2013) and cecropin A has also been expressed in rice plants (Coca *et al.*, 2006).

3.4. Peptide collections: CECMEL11 chemical library

Peptide libraries or peptide collections have been developed with the emergence of efficient methods for synthesizing peptides and their analogs. These peptide libraries exhibit advances in the identification and characterization of bioactive peptides. They allow peptide modifications to be tested as to identify and understand their effects on bioactivity and on their new properties. Moreover, hundreds of compounds can now be screened far more rapidly than was possible with the traditional methods (Montesinos, 2007; Marcos *et al.*, 2008; Montesinos & Bardají, 2008; Güell *et al.*, 2011; Montesinos *et al.*, 2012).

A set of peptides derived from Pep3 (WKLFKKILKVL-NH_2) (Cavallarin *et al.*, 1998) was designed to improve its antimicrobial activity with less cytotoxicity and less susceptibility to protease degradation (Ferre *et al.*, 2006; Badosa *et al.*, 2007; Montesinos *et al.*, 2012). The first 30 peptides exhibited good results, with BP76 being the peptide with the highest antimicrobial activity and performance. These good results prompted

CIDSAV (Centre d'Innovació i Desenvolupament en Sanitat Vegetal) and LIPPSO (Laboratori d'Innovació en Processos i Productes de Síntesi Orgànica), groups from the Universitat de Girona, to design a combinatorial library to improve antibacterial activity and reduce the cytotoxicity and susceptibility to the protease degradation of BP76. Research resulted in a 125-member peptide library, composed of synthetic linear undecapeptides cecropin A(1-7)-melittin hybrids. The CECMEL11 library was developed using combinatorial chemistry and was designed by combining five variations at each R, X1 and X10 positions (Fig 7.). Combinatorial chemistry enables the simultaneous synthesis of a large number of variations of a previously reported peptide and results in the development of large libraries. Three important parameters were taken into account when designing these peptides: the charge, the amphipathicity and the α-helical structure (Badosa *et al*., 2007).

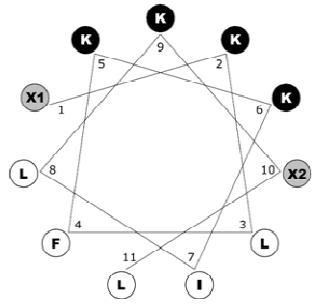

Figure 7. Edmunson wheel projection of the 11-mer peptides that were synthesized. Black background, hydrophilic amino acids; white background, hydrophobic amino acids; grey background, residues that can be either hydrophilic or hydrophobic, depending on the sequence (Montesinos *et al*., 2012).

The AMPs in this library were evaluated for antibacterial activity against *Erwinia amylovora, Xanthomonas axonopodis* pv. *vesicatoria*, *Pseudomonas syringae*, for antifungal activity against *Pencillium expansum* and for hemolytic activity and proteolytic susceptibility. Briefly, fifteen peptides presented high antibacterial activity while the other

fifteen peptides exhibited antifungal profile, and only four peptides were found in both sets (Badosa *et al.*, 2007; Montesinos *et al.*, 2012). In a subsequent work, the antifungal activity of a set of fifteen CECMEL11 sequences was tested *in vitro* against *Fusarium oxysporum*, *P. expansum*, *Aspergillus niger* and *Rhizopus stolonifer*. Some peptides highly active against *F. oxysporum* (MIC= 2.5 μM) and *P. expansum* (MIC= 12.5 μM) were detected. These works resulted in the classification of the tested CECMEL11 peptides according to their antimicrobial activity, so that peptide BP100 (H-KKLFKKILKYL-NH_2) showed strong antibacterial activity but low antifungal activity, BP21 (Ac-FKLFKKILKVL-NH_2) was strongly antifungal but poorly antibacterial and BP15 (KKLFKKILKVL-NH_2) shared antibacterial and antifungal activities (Badosa *et al.*, 2007, 2009). Additionally, the peptide BP22 (Ts-FKLFKKILKVL-NH_2) demonstrated a high *in vitro* antifungal activity against *P. expansum* and significantly decreased apple rot lesion size when it was applied at 300 μM to apple fruit (Badosa *et al.*, 2009).

On the other hand, the best linear and cyclic AMPs exhibited very low hemolytic activity, with minimal hemolytic concentrations in the range of 100–150 μM and the degradation times by proteases were in the range of 45–60 min, for the best linear (BP100) and cyclic (BPC194) antibacterial peptides. The cyclic peptides were more stable for proteinase K digestion than the linear ones (Badosa *et al.*, 2007; Montesinos & Bardají, 2008; Güell *et al.*, 2011; Montesinos *et al.*, 2012). Some of the AMPs from the CECMEL11 library have been submitted to oral toxicity testing in mice. BP100, BP76 and BP15, showed a low limit lethal dose (LLD) and a median lethal dose (LD_{50}) higher than 1000-2000 mg/kg of body weight and exhibited a very low oral toxicity (Montesinos & Bardají, 2008).

Chapter 2

Objectives

The **main** objective of this PhD thesis was to identify any antifungal peptides from the CECMELL11 library with the potential to control brown spot of pear (BSP) disease. Aimed towards this goal, the following specific objectives were proposed:

1. To evaluate twelve antifungal peptides from the CECMEL11 library for *in vitro* inhibition of growth and sporulation of *Stemphylium vesicarium*.

2. To determine the efficacy of *in vitro* most effective peptides for controlling *S. vesicarium* infection on detached pear leaves using preventative and postinfective application strategies.

3. To evaluate the efficacy of the two most effective peptides applied in a disease management program to control brown spot of pear under field conditions.

4. To analyze the interaction and the effects of the most effective peptide on *S. vesicarium* and to propose a possible mode of action.

Following the same order as the aims, the research developed in this thesis is organized into the following chapters:

Chapter 3. Postinfection activity of synthetic antimicrobial peptides against *Stemphylium vesicarium* in pear.

Chapter 4. Controlling brown spot of pear by a synthetic antimicrobial peptide under field conditions.

Chapter 5. Interaction of antifungal peptide BP15 with *Stemphylium vesicarium*, causal agent of brown spot of pear.

Chapter 3

Postinfection activity of synthetic antimicrobial peptides against *Stemphylium vesicarium* in pear

(Phytopathology 104:1192-1200)

Puig M, Moragrega C, Ruz L, Montesinos E and Llorente. “Postinfection activity of synthetic antimicrobial peptides against Stemphylium vesicarium in pear”. Phytopathology. Vol. 104, Issue 11 (2014) : 1192-1200

http://dx.doi.org/10.1094/PHYTO-02-14-0036-R
http://apsjournals.apsnet.org/doi/abs/10.1094/PHYTO-02-14-0036-R

Accepted for publication 7 May 2014

Abstract

Brown spot of pear is a fungal disease of economic importance caused by Stemphylium vesicarium that affects the pear crops in Europe. Due to the characteristics of this disease and the moderate efficacy of available fungicides, the effectiveness of control measures is very limited; however, synthetic antimicrobial peptides (AMPs) may be a complement to these fungicides. In the present study, 12 AMPs of the CECMEL11 library were screened for fungicidal activity against S. vesicarium. In vitro experiments showed that eight AMPs significantly reduced the germination of conidia. The most effective peptides, BP15, BP22, and BP25, reduced fungal growth and sporulation at concentrations below 50 µM. Leaf assays showed that preventive application of BP15 and BP22 did not reduce infection; however, when the peptides were applied curatively, infection was significantly reduced. The use of a BP15 fluorescein 5-isothiocyanate conjugate revealed that the peptide binds to hyphae and germ tubes and produces malformations that irreversibly stop their development.

Chapter 4

Controlling brown spot of pear by a synthetic antimicrobial peptide under field conditions

(Plant disease DOI:10.1094/PDIS-03-15-0250-RE)

Controlling Brown Spot of Pear by a Synthetic Antimicrobial Peptide under Field Conditions

Mireia Puig, Concepció Moragrega, Lídia Ruz, Emilio Montesinos and Isidre Llorente*.

Institute of Food and Agricultural Technology-XaRTA-CIDSAV, University of Girona, Spain
*Corresponding author

Abstract

Brown spot of pear is a fungal disease caused by *Stemphylium vesicarium* of increasing importance in several pear-growing areas of Europe. Disease control measures include the application of fungicides and sanitation methods. Antimicrobial peptides may be a complement or alternative to conventional fungicides used to manage brown spot disease. In a previous study, the synthetic peptide BP15 showed post-infection fungicidal activity against *S. vesicarium* in *in vitro* and detached leaf assays. In the present study the efficacy of BP15 (KKLFKKILKVL-NH_2) in controlling brown spot of pear was evaluated under field conditions using potted plants and pear trees in orchards. In field trials the treatments with BP15 or with the fungicide thiram were scheduled according to the infection risk predicted by the BSPcast model. Potted pear plants treated with BP15 showed a disease reduction about 42% to 60% in five out of seven trials. In three out of four tree trials the disease severity on shoots treated with BP15 was significantly lower than in the non-treated controls with a mean efficacy of 38.2%. It was concluded that BP15 is a good candidate to be further developed as a fungicide for controlling brown spot of pear.

Introduction

Brown spot of pear (BSP) caused by the fungus *Stemphylium vesicarium* (Wallr.) E. Simmons has been reported in the most important pear-growing areas of Europe, which are located in Italy, Spain, The Netherlands, Belgium and Portugal (Blancard et al. 1989; Heijne and Van Mourik 2001; Llorente and Montesinos 2006; Ponti et al. 1982; Vilardell 1988). The symptoms of BSP include necrotic lesions on fruits, leaves, petioles and twigs. High disease incidence can provoke premature defoliation in trees and cause fruit drop. The severity of BSP varies from year to year and with climatic conditions, and yield losses may range between 1 and 10% of the total production despite the control measures

applied (Llorente and Montesinos 2006; Llorente et al. 2011a). BSP control is mainly based on the use of fungicides applied at fixed schedules or according to the Brown Spot of Pear forecasting system (BSPcast) (Llorente et al. 2000; Montesinos et al. 1995).

The BSPcast model predicts infection risk and quantifies the effect of daily wetness duration and temperature during wetness periods on BSP disease (Llorente and Montesinos 2006; Llorente et al. 2000; Montesinos et al. 1995). The use of BSPcast reduces significantly the number of fungicide treatments compared with standard fungicide schedules (Llorente et al. 2000). To increase the efficacy in disease control, different sanitation methods have been proposed for reducing inoculum production on orchard ground, such as by leaf litter removal during winter and biological control applications based on *Trichoderma* spp. (Llorente et al. 2008; Llorente et al. 2010).

However, the use of chemical control with fungicides is currently the main strategy for managing BSP, which still faces several problems for a sustainable use. An accurate timing application of fungicides before or at the beginning of the infection process is very important because *S. vesicarium* produces SV-toxins during its growth. If fungicides are not applied during the start of infection process, toxins have already been released and then necrosis will appear on the leaves or fruit and control will be not effective (Llorente and Montesinos 2006; Singh et al. 1999; Singh et al. 2000). Unfortunately, no curative fungicides are available for controlling BSP (Llorente et al. 2011a). Resistant strains of *S. vesicarium* to strobilurins and dicarboximide fungicides have been detected (Alberoni et al. 2010; Alberoni et al. 2005). Additionally, the high frequency of fungicide sprays needed to maintain disease levels below tolerance

thresholds may result in high residue levels in fruits and strong environmental impacts on orchards. Thus, there is a need for new fungicides for BSP control.

Synthetic antimicrobial peptides (AMPs) have attracted attention as novel fungicides (Keymanesh et al. 2009; López-García et al. 2002; Montesinos 2007; Montesinos et al. 2012; Montesinos and Bardají 2008). Several peptides of the CECMEL11 library (Ferre et al. 2006), comprising cecropin A-melittin hybrid undecapeptides, were found to be active in preventing post-harvest infections in apple caused by the blue-mold fungus *Penicillium expansum* (Badosa et al. 2009). Interestingly, one of these peptides, BP15, showed fungicidal effects against *S. vesicarium* with post-infection activity in detached pear leaves (Puig et al. 2014). However, the performance of BP15 has not been tested under field conditions in whole plants or orchards.

The goal of the present study was to evaluate the efficacy of BP15 for controlling BSP under orchard conditions. The experiments were performed in potted pear plants and in pear trees in experimental orchards. The treatment applications were timed according to BSPcast model schedules.

Materials and methods

Peptide synthesis

The peptide BP15 (KKLFKKILKVL-NH_2) was obtained at >90% purity from LIPPSO (University of Girona, Spain). It was synthesized using solid-phase methodology with 9-fluorenylmethoxycarbonyl (Fmoc)-type chemistry, tert-butyloxycarbonyl side-chain protection for Lys and Trp and tert-butyl for Tyr, as previously described (Badosa et al. 2009; Ferre et al. 2006).

Weather parameters measurement

Environmental parameters were monitored with a CR10X datalogger (Campbell Scientific Ltd., Leicester, UK) connected to temperature-relative humidity (model HMP35AC), wetness (model 236) and rainfall (model ARG100) sensors. Automatic weather stations were placed on the experimental orchards. Temperature and relative humidity were measured every 10 min, and leaf wetness duration and rainfall were measured every 20 s. Mean temperature, mean relative humidity, total duration of wetness and total rainfall were recorded every hour.

Potted plant trials in pear orchards

Trials were performed in two locations during July and September of 2011 in Catalonia (north-eastern of Spain). Orchard A, located in Girona (41°57'37.16"N 2°49'51.84"E), comprised 0.5 ha of different pear cultivars; orchard B, located in Campllong (Girona) (41°54'27.2"N 2°49'12.0"E), comprised 2 ha of pear cv. Passe Crassane. The disease incidence of BSP on leaves in the preceding year in orchard A was very low (<5%), whereas in orchard B it was high (80%). According to this, the amount of natural inoculum during the trials was expected to be low on orchard A and high on orchard B.

Self-rooted, potted cv. Conference pear plants grown in the greenhouse were pruned, leaving only one shoot. When plants reached the height of 0.75-1 m, they were used for the experiments. These plants were transported to orchards A and B for exposure to natural conditions. Plants used in the experiments performed in orchard A were artificially inoculated with *S. vesicarium* strain EPS26 due to the low level of natural inoculum in this orchard, whereas plants used in the trials performed in orchard B were exposed to natural inoculum in the orchard.

To obtain inoculum for the experiments in orchard A, *S. vesicarium* EPS26 was grown on tomato agar plates (800 ml of distilled water, 10 g of tomato concentrated juice, 2.3 g of $CaCO_3$ and 12.8 g of agar) for 7 to 10 days at 22.5˚C with a 16 h-light photoperiod in a growth cabinet (I-30BLL Percival Plant Biology Chamber, Percival Scientific Inc., USA). Then, conidia were collected from the agar surface, transferred to distilled water and filtered. The concentration of the suspension was determined by counting the conidia in 5 drops (10 µl each) under microscope. Finally, the conidial concentration was adjusted to 10^3 conidia/ml. Potted pear plants were inoculated with the conidia suspension until runoff by using an airbrush after been placed on the orchard.

The experimental factors consisted of treatment with peptide BP15 and a non-treated control. Twenty milliliters of BP15 at 100 µM (135.7 mg/liter) were applied per plant. Three plants were used per treatment and control. Sprays were scheduled according to the infection risk level determined by BSPcast model (Llorente et al. 2000; Montesinos et al. 1995). The threshold for BP15 application was the cumulative risk CR≥0.4. Different sets of six plants were transported to each experimental orchard and randomly distributed among the pear trees in July and September. Different code numbers were assigned to trials to facilitate easy identification of them in this report (Table 1).

With the purpose to test the efficacy of BP15 applied at different times, sprays were done the same day when CR≥0.4 threshold was reached on trials A1, A3, A4, B1 and B3, or after 24 h (A2, A5, B2 and B4) or 48 h (A6 and B5).

In trials performed in orchard B corresponding to pear plants exposed to natural inoculum, plants were left in the orchard for an initial period of 5 days to ensure natural inoculation with the pathogen before the treatments started.

In each trial, the peptide BP15 was applied to the corresponding potted plants using an airbrush. After the treatment, the plants remained for 24 h in the orchard to be exposed to natural conditions aimed to let that the peptide–*S. vesicarium* interact under field conditions. Then, plants were transported to the laboratory and incubated for seven days in a controlled-environment chamber for the expression of symptoms (16 h-light photoperiod, 22.5°C, 70% RH). Disease severity was recorded by visually determining the number of lesions per pear leaf on the 5 youngest leaves in each treated and control plant. Disease severity was assessed on the day when the treatment was applied and 7 days after, and only new lesions were considered. In total, 11 sets of plants were used.

Table 1. Characteristics of trials performed for evaluation of peptide BP15 efficacy in controlling brown spot of pear on potted pear plants incubated under natural conditions.

Code	Orchard[x]	Input data[y]	Treatment data[z]
A1	A	06/07/2011	13/07/2011
A2	A	06/07/2011	14/07/2011
A3	A	14/07/2011	20/07/2011
A4	A	20/07/2011	27/07/2011
A5	A	08/09/2011	12/09/2011
A6	A	08/09/2011	13/09/2011
B1	B	06/07/2011	13/07/2011
B2	B	06/07/2011	14/07/2011
B3	B	07/09/2011	12/09/2011
B4	B	07/09/2011	13/09/2011
B5	B	07/09/2011	14/09/2011

[x] orchard A was located in Girona and orchard B in Campllong

[y] data when potted plants where placed on the orchard

[z] data of BP15 applications. Plants remained under natural conditions for 24 h after the treatment.

Data analysis

Data were analyzed using R statistical software (R Core Team, 2014). When the disease severity observed in non-treated control plants was lower than one lesion/leaf, this trial was excluded from the analysis. Accordingly, the data finally analyzed corresponded to four plant sets out of six from orchard A (trials A3, A4, A5 and A6) and three out five from orchard B (B3, B4 and B5).

The datasets were tested for equality of variances (Bartlett) and normality (Shapiro-Wilk). The effect of peptide on the disease severity was determined by analysis of variance using mixed models with the lme4 and lmerTest packages. The treatment was considered as a fixed factor, the plants as random factor and the data of each leaf nested within plants. Mean comparisons were performed with Tukey's contrast test. Efficacy of treatments was calculated as $E=[1-(y_t/y_{nt})]*100$, where: E is the efficacy in disease control; y_{nt} the disease severity in the non-treated control; and y_t the disease severity in treated plants. The effect of trial on the efficacy in disease control of BP15 was evaluated using one way analysis of variance. Since no significant differences were observed between trials (P=0.9183), pooled data were analyzed. The one way analysis of variance was used to determine the effect of application time (0, 24 or 48 h) after BSPcast threshold was achieved on the efficacy in disease control. The datasets were tested for equality of variances (Bartlett) and normality (Shapiro-Wilk).

Climatic conditions during the trials

The variation of temperature, rainfall and wetness duration recorded during the trials are presented in Figure 1. The monthly average daily mean temperatures during July and September were 20.1 and 22.5°C,

respectively, in the orchard A and 22.5 and 23.5°C, respectively, in the orchard B (Fig. 1). Rainfall occurred only from the middle to the end of July, with an intense rainfall of 114 mm in orchard A. No rainfall events were observed in September in the orchard B. The wetness period varied along July, when 26% of days in orchard A and 10% of days in orchard B had a wetness duration longer than 12 h. In September, 50% of days in orchard A and 67% of days in orchard B experienced long wetness periods.

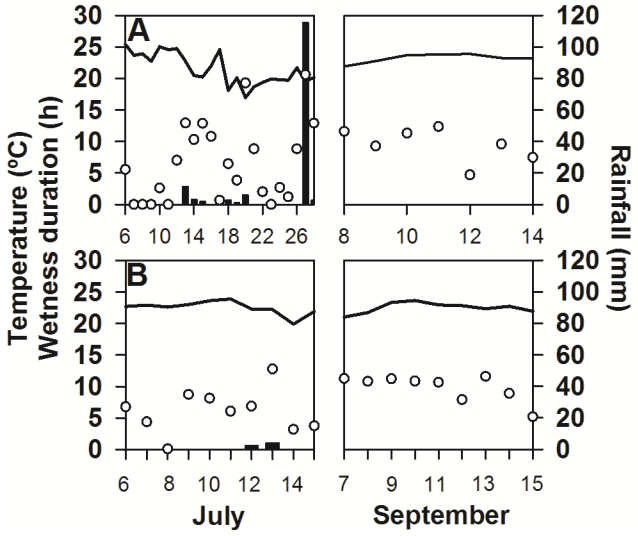

Figure 1. Variation of mean temperature (solid line), daily wetness duration (circles) and rainfall (bars) during the trial periods in 2011. Upper panels correspond to orchard A, and lower panels to orchard B. Trials were performed using potted plants incubated under natural conditions.

Tree trials in pear orchards

Four field trials were conducted in orchard B (Campllong, 41˚54'27.2"N 2˚49'12.0"E), in cv. Passe Crassane pear during the growing seasons of 2012 (trials I and II) and 2013 (trials III and IV). This orchard was chosen because the incidence of BSP in the preceding years had been very high (70% on fruit and 80% on leaves). Previously, a sanitation method of control was applied in a part of the orchard to obtain two levels of *S.*

vesicarium inoculum in the same orchard (Llorente et al. 2010). This sanitation method involved covering part of the orchard ground (300 m^2) with a plastic foil to reduce the production and release of inoculum by leaf litter. In the rest of the orchard area (approximately 1,200 m^2) no sanitation method was applied, and the orchard remained unaltered.

Trials I and III (high inoculum pressure) were conducted in an area of the orchard where no sanitation method was applied, and trials II and IV (medium inoculum pressure) were placed in the area with plastic foil covering the ground. In all trials, a complete randomized block design with three blocks was used. Each block consisted of 12 trees (trials II and IV) or 18 trees (trials I and III) of which four or six were used. In each of the trees used, a total of six shoots (two per treatment) with at least 10 leaves, located in the middle of canopy were labeled at the beginning of the season, in May. The trials started in May and ended in September.

The treatments evaluated were the peptide BP15 at 100 μM (135.7 mg/liter), thiram (2,000 mg a.i./liter) as a reference fungicide and the non-treated control. Peptide and fungicide applications were scheduled according to the BSPcast model. Sprays were applied when the daily infection risk was R≥0.2 or the cumulative infection risk was CR≥0.4 (Llorente et al. 2011b). Peptide or thiram solutions were applied on labeled shoots until runoff (16 ml per shoot) using a commercial portable sprayer. It was assumed that the fungicide thiram provided seven-day protection except when rainfall was higher than 20 mm. For peptide BP15 one-day protection was considered according to a previous report (Puig et al. 2014).

Disease severity was assessed on all leaves of each labeled shoot by counting the number of lesions per leaf every 15 to 20 days and at the end of the trials. Mean disease incidence and severity were calculated per shoot.

Data analysis

Data were analyzed using R statistical software (R Core Team, 2014). The data sets were tested for equality of variances (Bartlett) and normality (Shapiro-Wilk).

The effect of treatments on disease severity during the whole epidemic was determined with repeated-measures analysis of variance using mixed models with Restricted Maximum Likelihood Comparisons (RMEL). The analysis was performed with Proc GLM using SAS 9.2 software program (SAS Institute Inc., Cary, NC, USA). The variance-covariance matrix structure was compared according to different models, and compound symmetry structure was chosen because it showed the lowest AIC statistic. Comparison of means was performed with Tukey's contrast test.

The effect of peptide and thiram fungicide treatments on disease severity at the end of the trials was determined by analysis of variance using mixed models obtained with the lmr function and Restricted Maximum Likelihood Comparisons (RMEL) with R statistical software (R Core Team, 2014). One fixed factor (treatment), random blocks and sub-sampling within the experimental unit were considered because data from the shoots and trees were nested within blocks. Comparisons of the means were performed with Tukey's contrast test.

Climatic conditions during the trials

During the years 2012 and 2013, July and August were characterized as the warmest months (Fig. 2). The average daily mean temperature for 2012 (trials I and II) was 20.9°C with a minimum temperature of 11.8°C and a maximum temperature of 27.5°C. The range of temperatures in 2013 (trials III and IV) was from 10.7°C to 26°C, with an average daily mean temperature of 19.6°C. The rainfall in 2013 was double that in 2012, with an accumulated rainfall of 341 mm by the end of the study. In 2012, the period from June to August was dry with few rain events. Days with longer wetness duration periods in both years were concentrated in May and September; additionally, in 2013 some long wetness duration days occurred in July and August. The variation of meteorological parameters in 2012 and 2013 showed a relationship between days with rain and long leaf wetness periods.

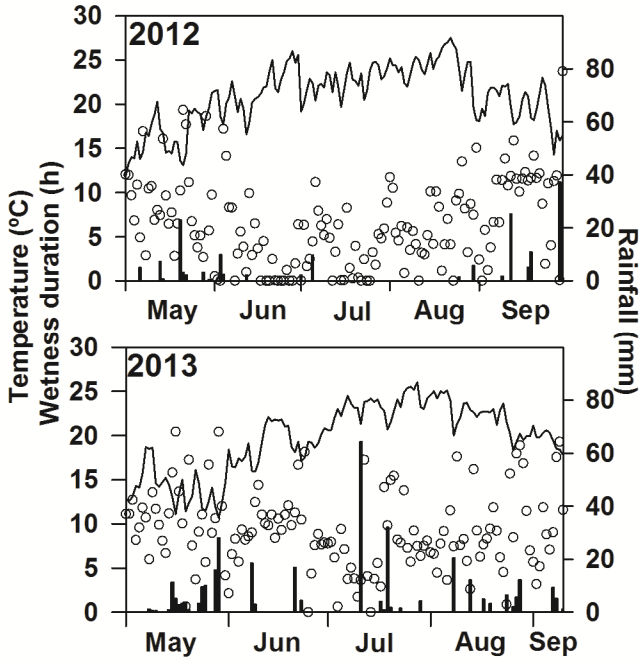

Figure 2. Variation of mean temperature (solid line), daily wetness duration (circles) and rainfall (bars) during field trials performed in 2012 and in 2013 in the experimental pear orchard Campllong.

Results

Potted plant trials in pear orchards

The variation of disease risk forecasted by BSPcast for each trial is shown in Figure 3. Data from trials A1, A2, B1 and B2 were not used in the analysis because the disease severity in the non-treated controls was lower than one lesion per leaf. Also, in these trials the number of days with infection risk CR≥0.4 was very low. In the remaining useful trials, the BSP risk predicted for the days before peptide application was high. In general, the disease severity in non-treated plants observed in artificially inoculated plants (orchard A) was higher (4.1 to 27.6 lesions/leaf) than that observed in the plants exposed to natural inoculum (orchard B) (2.6 to 4.1 lesions/leaf) (Fig. 4). The effect of BP15 treatments in reducing disease severity was significant in five out of seven trials (A4, A6, B3, B4 and B5) compared with the non-treated control (Fig. 4). The efficacy for disease control of BP15 in orchard A was 42.5% in trial A6 and 48.1% in trial A4; and in orchard B was 59.5, 59.7 and 53.3% in trials B3, B4 and B5 respectively.

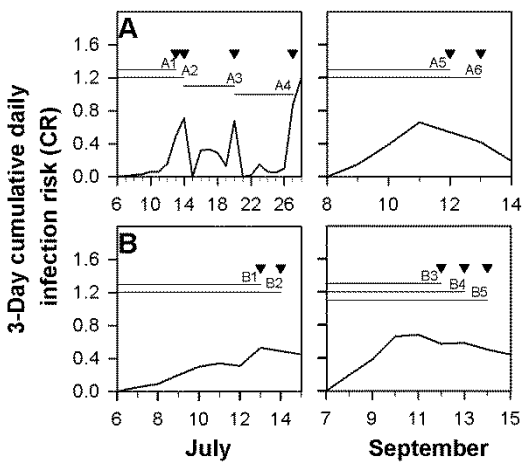

Figure 3. Variation of 3-day cumulative daily infection risk (CR) in trials performed using potted plants incubated in pear orchards during July and September 2011. Upper panels show trials A1 to A6 performed in orchard A and lower panels show trials B1 to B5 performed in orchard B. The horizontal lines indicate the period of time when the plants were incubated in the orchard for each trial until treatments with BP15 (▼) were done. Treatments were applied the same day when CR≥0.4 threshold was reached on trials A1, A3, A4, B1 and B3; after 24 h on trials A2, A5, B2 and B4; and after 48 h on trials A6 and B5.

No significant differences (P=0.938) were observed in the efficacy of BP15 for BSP control whatever the time application after the CR threshold was reached (0, 24 or 48 h), with a mean efficacy of 48.5, 47.9 and 54.4 % respectively.

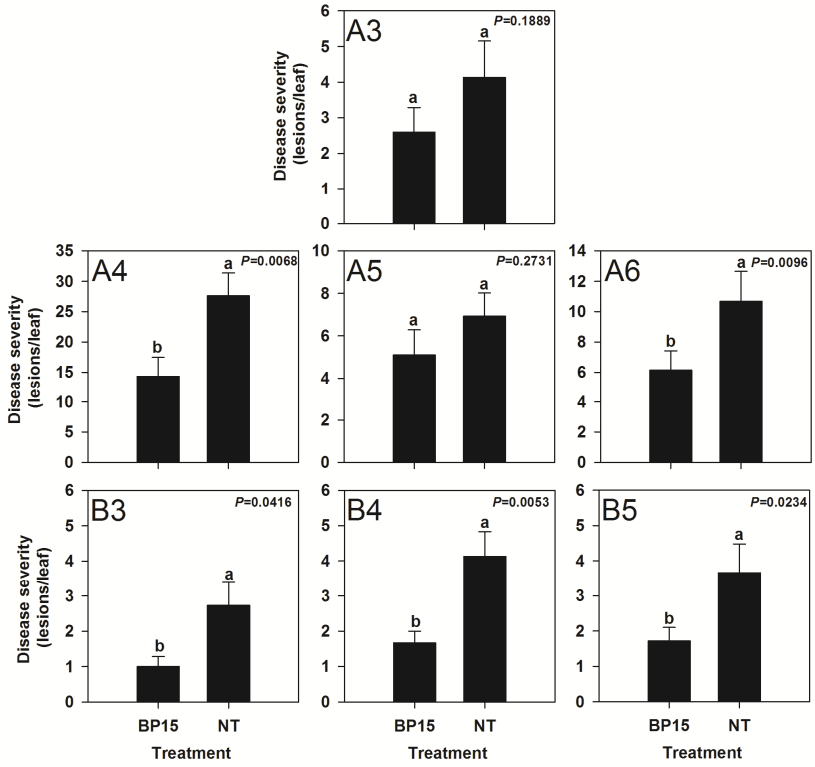

Figure 4. Effect of peptide BP15 treatments and the non-treated control (NT) on disease severity (lesions/leaf) in seven trials performed using potted plants in pear orchards. Panels show trials A3 to A6 performed in orchard A, and trials B3 to B5 performed in orchard B. Treatments were applied the same day when CR≥0.4 threshold was reached on trials A3, A4 and B3; after 24 h on trials A5 and B4; and after 48 h on trials A6 and B5. Values of each bar are the mean of three replicates with 5 leaves per replicate. The standard error of the mean is also indicated. Values followed by the same letter are not significantly different according to Tukey's test (P≤0.05). P-values are presented

Tree trials in pear orchards

In trials performed during 2012 and 2013, the treatments were sprayed according to the daily infection risk index (R≥0.2) or the cumulative

infection risk index (CR≥0.4). The first high value of R and CR occurred in May (Fig. 5) in both years, after days with rain. In 2012, the R and CR values were high in May, August and September. In the period of study, R values reached the action threshold level of 0.2 in 27 days and the CR≥0.4 in 42 days (until the 24th of September); in total, 19 sprays of BP15 and 14 of thiram were applied (Fig. 5). In 2013, the number of days that R≥0.2 was 27, and the number of days with CR≥0.4 was 60 (until 4th of September). However, the number of sprays in the five month trials was 15 for BP15 and 12 for thiram (Fig. 5).

The progression of disease severity in shoots treated with the peptide BP15 or thiram was compared with that in non-treated controls for the four trials over two years (Fig. 5). In all cases, the epidemic started in May after periods with high R and CR values. In 2012, the rate of disease progress was high at the beginning and at the end of the epidemic (May and September). During July and August the disease did not progress due to the lack of rain and short periods of wetting (Fig. 5). Contrarily, in 2013 the epidemic increased during the entire period, especially after July, and the R and CR indexes showed high values during all months due to the rainfall events, including those that occurred in July and August. In all trials, the increases in disease severity coincided with periods with high values of R and CR. The final disease severity in shoots treated with BP15 was significantly lower than in the non-treated shoots in three out of four trials (I, III and IV) (Table 2). In these trials (I, III and IV) the mean disease severity observed in shoots treated with BP15 was 8.5, 8.2 and 6.6 lesions/leaf, respectively, whereas 13.4, 13.8 and 10.6 lesions/leaf were observed in the non-treated shoots. The mean disease severity observed when BP15 or thiram were applied was similar in two trials (III and IV) with 8.2 and 6.6 lesions/leaf in shoots

treated with BP15, and 8.1 and 6.1 lesions/leaf in shoots treated with thiram. Contrarily, the disease severity differed significantly between shoots treated with BP15 and thiram in trials I and II, with an average of 8.5 and 5.5 lesions/leaf for BP15 and 5.6 and 2.1 lesions/leaf for thiram. In all trials the disease severity was significantly lower in shoots treated with thiram in comparison with the non-treated control. The efficacy of disease control observed in trials I, II, III and IV was 37.1, 9.2, 40.4 and 37.1% with BP15 and 58.1, 65.6, 40.9 and 42.2% with thiram, respectively.

Similar results were obtained upon analyzing the whole epidemic by means of a repeated-measures analysis (Table 3). In trials I, III and IV the disease severity was reduced significantly by 20.8, 31.5 and 33.3% respectively on shoots treated with BP15 in comparison with the non-treated shoots. No significant differences in disease severity were observed between leaves treated with BP15 and with thiram in trials II, III and IV. In all trials thiram reduced significantly the disease severity by 45.3, 49.2, 45.9 and 53.4% compared with the non-treated control.

Table 2. Effect of different treatments on disease severity (lesions/leaf) in four field trials performed in Campllong experimental orchard in 2012 (trials I and II) and 2013 (trials III and IV). Data corresponds to linear contrasts for disease severity at the end of the trials.

	2012		2013	
	Trial I	Trial II	Trial III	Trial IV
Treatment contrasts	*P>z*	*P>z*	*P>z*	*P>z*
BP15 vs non-treated	<0.001[z] ***	0.8111 ns	<0.001 ***	0.0055 **
BP15 *vs* thiram	0.0138 *	0.0127 *	0.9960 ns	0.9040 ns
thiram *vs* non-treated	<0.001 ***	0.0021 **	<0.001 ***	0.0012 **

[z] *, ** and ***: Significant at 0.05, 0.01 and <0.001 probability levels, respectively; *ns*=not significant at $P \leq 0.05$. Adjusted *P*-values according to Tukey's contrast test.

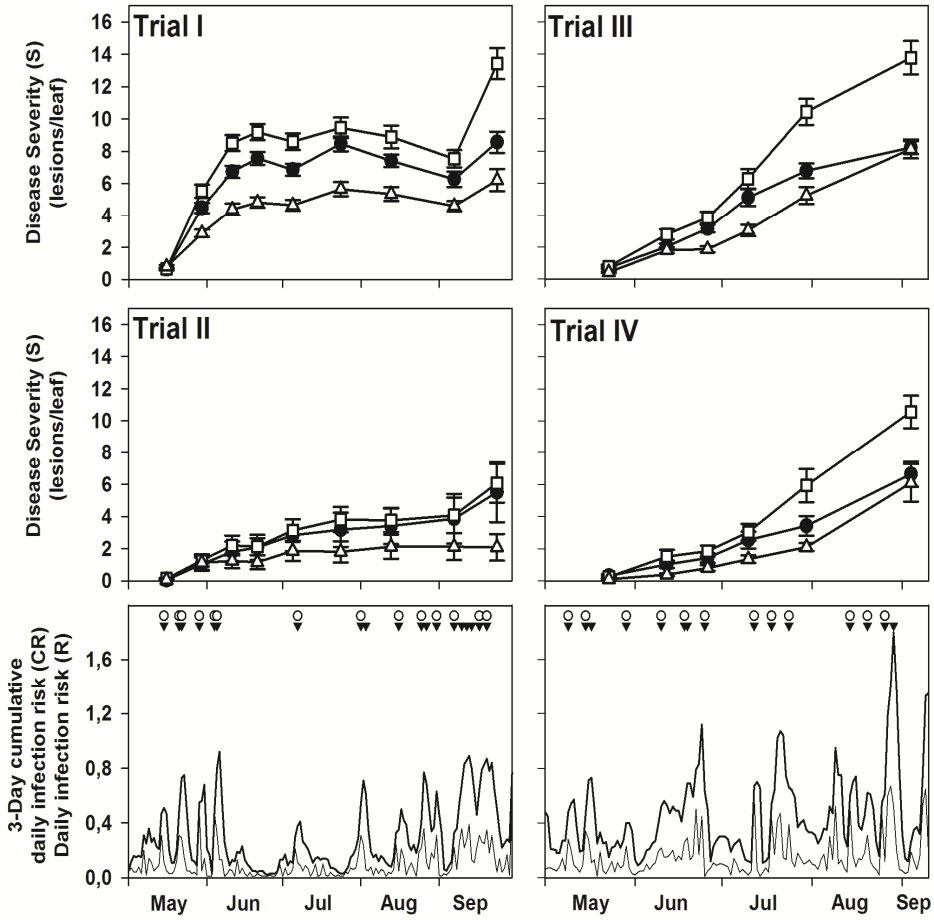

Figure 5. Brown spot disease severity progress curves on leaves for non-treated control (□), leaves treated with the peptide BP15 (■) and leaves treated with thiram (Δ) at R≥0.2 or CR≥0.4, in relation to the daily infection risk (R) (fine line) and the variation of 3-day cumulative daily infection risk (CR) (bold line). Field trials were performed in the experimental pear orchard Campllong in 2012 (left) and 2013 (right). Dates of treatments with BP15 (▼) and thiram (o) are indicated. Values of disease severity are the mean of three replicates with four shoots per replicate in trials I and III and two shoots per replicate in trials II and IV. Bars represent the standard error of the mean.

Table 3. Effect of applications of BP15 and thiram for controlling brown spot of pear during the whole epidemics. Field trials were performed in Campllong experimental orchard during 2012 (trial I and II) and 2013 (trial III and IV). Data corresponds to linear contrasts for disease severity by repeated measures.

	2012		2013	
	Trial I	Trial II	Trial III	Trial IV
Treatment contrasts	*P>z*	*P>z*	*P>z*	*P>z*
BP15 *vs* non-treated	0.0488[z]	0.7806	<0.001	0.0019
	*	*ns*	***	***
BP15 *vs* thiram	0.0147	0.0611	0.1225	0.0979
	*	*ns*	*ns*	ns
Thiram *vs* non-treated	<0.001	0.0098	<0.001	<0.001
	***	***	***	***

[z] *, ** and ***: significant at 0.05, 0.01 and <0.001 probability levels, respectively; *ns*=not significant at $P \leq 0.05$. Adjusted *P*-values according to Tukey's contrast test.

Discussion

In recent years, several synthetic antimicrobial peptides have been reported with antifungal activity. For example, the peptides BP76, BP21 and BP22, from the CECMELL11 library, were effective in controlling blue-mold rot caused by *Penicillium expansum* on apple fruits; PAF19, PAF26 and $LfcinB_{4-9}$ inhibited *P. digitatum* and *P. italicum* on orange fruits; C14-KLLK avoided the development of *Botrytis cinerea* on cucumber leaves and fruits and AFP controlled *Magnaporthe grisea* infection in rice (Badosa et al. 2009; López-García et al. 2003; Makovitzki et al. 2007; Vila et al. 2001).

In one of our previous studies it was demonstrated that BP15 stopped the growth of *S. vesicarium* under *in vitro* and *ex vivo* conditions (Puig et al. 2014). In the present work, the efficacy of BP15 in the control of brown spot of pear was evaluated under field conditions.

The results presented here are most likely the first evidence of the effectiveness of a synthetic peptide in the control of a plant disease under field conditions. In potted plants BP15 significantly reduced the intensity of BSP with a mean efficacy ranging from 45.3% (orchard A) to 57.5% (orchard B). The levels of disease in non-treated controls were higher in orchard A than in orchard B, but no significant differences on the efficacy of disease control were observed among trials. BP15 treatments were not always performed on the day the BSPcast action threshold was reached. Consequently, in some cases the potted plants remained unprotected for 24 or 48 h after the *S. vesicarium* infection had occurred. However, BP15 treatments showed a high efficacy in disease control whatever the time of application after the risk advice. Similar results were obtained on previous research on detached leaves treated with BP15 from 6 to 24 h after the infections started. These results can be explained by the post-infection activity of BP15, which binds directly to the hyphae of *S. vesicarium*; thus, fungal growth is rapidly inhibited (Puig et al. 2014).

To evaluate the efficacy of BP15 on BSP control in pear trees, the peptide was applied over two years on shoots in trees with different levels of disease as measured in the non-treated controls (between 6.1 to 13.8 lesions/leaf). The results obtained upon treatment of tree shoots were consistent in most of the trials. In three out of four trials the disease severity on leaves, assessed at the end of the trials or during the whole epidemic, was reduced when shoots were treated with the peptide in comparison with the non-treated control. These results state that BP15 is an effective fungicide against *S. vesicarium* infections applied according to the BSPcast model under different climatic conditions and different disease levels. The efficacy of disease control on leaves using fungicides

such as thiram alone or complemented with sanitation measures has been described to be between 30 to 60% (Antoniacci et al. 2006; Llorente et al. 2010). Interestingly, these values are in the same range of efficacy observed in three out of four trials presented herein using BP15 (38.2%). The efficacy of disease control using thiram was higher (47.1%). Unfortunately, disease control was incomplete using either thiram or BP15, probably related to the production of two host-specific toxins (SV-toxins I and II) by *S. vesicarium* during the infection process before the fungicides have controlled the fungal growth (Singh et al. 2000; Singh et al. 1999).

Treatment with BP15 could have additional benefits in comparison with thiram or other fungicides: (i) the peptide is composed of proteinogenous amino-acids and already degradable by proteases; thus, it is not expected to produce residues on fruits; (ii) the mechanism of action is by membrane damage and then it is expected also a low probability of resistance in the target pathogen (Güell et al. 2011; Puig et al. 2014). Antifungal peptides are normally degraded within a few minutes when exposed to the natural environment due to the activity of proteases from leaves and fruits (Badosa et al. 2009; Ferre et al. 2006; Güell et al. 2011; Keymanesh et al. 2009; Montesinos et al. 2012; Montesinos 2007). The high post-infection activity of BP15 against *S. vesicarium* indicates that the persistence of the peptide in the leaf environment is sufficient to be effective. Additionally, BP15 does not exhibit cytotoxicity in erythrocytes or on plant tissues at effective antifungal concentrations (Badosa et al. 2009; Ferre et al. 2006; Puig et al. 2014). In addition, toxicity in animals is very low according to oral ingestion tests in mice, with a median lethal dose (LD_{50}) in the range of 1,000-2,000 mg/kg of body weight (Montesinos et al. 2012).

The results obtained in field experiments over two years provide evidences that BP15 is a good candidate for developing new pesticides for BSP management as an alternative or complement to conventional fungicide treatments scheduled with the BSPcast warning system. The post-infection activity of BP15 against *S. vesicarium* offers new possibilities in an Integrated Pest Management strategy to control the BSP. On pear orchards with low levels of disease the use of BP15 may be enough to control the BSP applied according to the risk predicted by BSPcast model. On orchards with higher disease levels, BP15 should be combined with conventional fungicides. Nowadays the weather forecast is accurate enough to apply fungicides preventively, few hours before the infections really begin. Then, once the BSPcast model confirms that climatic conditions are favorable to trigger the infections, the BP15 should be applied to inhibit the growth of conidia that remain viable. This strategy can increase the efficacy of BSP control without increase the fungicide residue levels on fruit.

One of the main limitations for commercial use of peptides as pesticides is the high economic costs of production. Additional efforts to obtain and optimize formulation are necessary to develop antimicrobial peptides for agricultural uses. Research to better understand the mechanisms of action and to optimize mass production systems for field testing against BSP and other fungal diseases of crops is ongoing.

Acknowledgments

This research was supported in part by grant from the Ministerio de Educación y Ciencia (AGL2009-09829/AGR) of Spain. Mireia Puig was the recipient of a predoctoral fellowship from Universitat de Girona (BR10-17). We acknowledge the helpful assistance in field work by E. Agustí and J. Pereda.

Literature Cited

Alberoni, G., Cavallini, D., Collina, M., and Brunelli, A. 2010. Characterisation of the first *Stemphylium vesicarium* isolates resistant to strobilurins in Italian pear orchards. Eur. J. Plant Pathol. 126:453–457.

Alberoni, G., Collina, M., Pancaldi, D., and Brunelli, A. 2005. Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards. Eur. J. Plant Pathol. 113:211–219.

Antoniacci, L., Bugiani, R., and Rossi, R. 2006. Control of brown spot of pear in organic pear orchard. In *Orchard diseases International workshop; 7th, Orchard diseases*, p. 117–122.

Badosa, E., Ferre, R., Francés, J., Bardají, E., and Feliu, L. 2009. Sporicidal activity of synthetic antifungal undecapeptides and control of *Penicillium* rot of apples. Appl. Environ. Microbiol. 75:5563–5569.

Blancard, D., Allard, E., and Brest, P. 1989. La stemphyliose du poirier ou “macules brunes”. Phytoma 406:35–37.

Ferre, R., Badosa, E., Feliu, L., Planas, M., Montesinos, E., and Bardají, E. 2006. Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides. Appl. Environ. Microbiol. 72:3302–3308.

Güell, I., Cabrefiga, J., Badosa, E., Ferre, R., Talleda, M., Bardají, E., Planas, M., Feliu, L., and Montesinos, E. 2011. Improvement of the efficacy of linear undecapeptides against plant-pathogenic bacteria by incorporation of D-amino acids. Appl. Environ. Microbiol. 77:2667–2675.

Heijne, B., and Van Mourik, J. 2001. Zwart- vruchtrot op peer neemt toe. Fruitt. Den Haag 91:18–19.

Keymanesh, K., Soltani, S., and Sardari, S. 2009. Application of antimicrobial peptides in agriculture and food industry. World J. Microbiol. Biotechnol. 25:933–944.

Llorente, I., and Montesinos, E. 2006. Brown spot of pear: an emerging disease of economic importance in Europe. Plant Dis. 90:1368–1375.

Llorente, I., Moragrega, C., Ruz, L., and Montesinos, E. 2011a. An update on control of brown spot of pear. Trees. 26:239–245.

Llorente, I., Vilardell, A., Vilardell, P., and Montesinos, E. 2008. Evaluation of new methods in integrated control of brown spot of pear (*Stemphylium vesicarium*, Teleomorph *Pleospora allii*). Acta Hortic. 825–831.

Llorente, I., Vilardell, A., Vilardell, P., Pattori, E., Bugiani, R., Rossi, V., and Montesinos, E. 2010. Control of brown spot of pear by reducing the overwintering inoculum through sanitation. Eur. J. Plant Pathol. 128:127–141.

Llorente, I., Vilardell, P., Bugiani, R., Gherardi, I., and Montesinos, E. 2000. Evaluation of BSPcast disease warning system in reduced fungicide use programs for management of brown spot of pear. Plant Dis. 84:631–637.

Llorente, I., Vilardell, P., and Montesinos, E. 2011b. Evaluation of a revision of the BSPcast decision support system for control of brown spot of pear. Phytopathol. Mediterr. 50:139–149.

López-García, B., Pérez-Payá, E., and Marcos, J. F. 2002. Identification of novel hexapeptides bioactive against phytopathogenic fungi through screening of a synthetic peptide combinatorial library. Appl. Environ. Microbiol. 68:2453–2460.

López-García, B., Veyrat, A., Pérez-Payá, E., González-Candelas, L., and Marcos, J. F. 2003. Comparison of the activity of antifungal hexapeptides and the fungicides thiabendazole and imazalil against postharvest fungal pathogens. Int. J. Food Microbiol. 89:163–170.

Makovitzki, A., Viterbo, A., Brotman, Y., Chet, I. and Shai, Y. 2007. Inhibition of fungal and bacterial plant pathogens in vitro and in planta with ultrashort cationic lipopeptides. Appl. Environ. Microbiol. 73:6629-6636.

Montesinos, E. 2007. Antimicrobial peptides and plant disease control. FEMS Microbiol. Lett. 270:1–11.

Montesinos, E., Badosa, E., Cabrefiga, J., Planas, M., Feliu, L., and Bardají, E. 2012. Antimicrobial peptides for plant disease control. From discovery to application. In *Small Wonders: Peptides for Disease Control*, eds. K. Rajasekaran, J.W. Cary, J.M. Jaynes, and E. Montesinos. p. 235–261.

Montesinos, E., and Bardají, E. 2008. Synthetic antimicrobial peptides as agricultural pesticides for plant-disease control. Chem. Biodivers. 5:1225–1237.

Montesinos, E., Moragrega, C., Llorente, I., Vilardell, P., Bonaterra, A., Ponti, I., Bugiani, R., and Brunelli, A. 1995. Development and evaluation of an infection model for *Stemphylium vesicarium* on pear

based on temperature and wetness duration. Phytopathology 85:586–592.

Ponti, I., Cavani, P., and Brunelli, A. 1982. “Maculatura bruna” delle pere: eziologia e difesa. Inf. Fitopatol. 32:35–40.

Puig, M., Moragrega, C., Ruz, L., Montesinos, E., and Llorente, I. 2014. Postinfection activity of synthetic antimicrobial peptides against *Stemphylium vesicarium* in pear. Phytopathology 104:1192–1200.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Online:http://www.R-project.org/.

Singh, P., Bugiani, R., Cavanni, P., Nakajima, H., Kodama, M., Otani, H., and Kohmoto, K. 1999. Purification and biological characterization of host-specific SV-toxins from *Stemphylium vesicarium* causing brown spot of European pear. Phytopathology 89:947–953.

Singh, P., Park, P., Bugiani, R., Cavanni, P., Nakajima, H., Kodama, M., Otani, H., and Kohmoto, K. 2000. Effects of host-selective SV-toxin from *Stemphylium vesicarium*, the cause of brown spot of European pear plants, on ultrastructure of leaf cells. J. Phytopathol. 148:87–93.

Vila, L., Lacadena, V., Fontanet, P., Martinez del Pozo, A., San Segundo, B. 2001. A protein from the mold *Aspergillus giganteus* is a potent inhibitor of fungal plant pathogens. Mol. Plant. Microbe. Interact. 14:1327–1331.

Vilardell, P. 1988. *Stemphylium vesicarium* en plantaciones de peral. Frutic. Prof. 18:51–55.

Chapter 5

Interaction of antifungal peptide BP15 with *Stemphylium vesicarium*, the causal agent of brown spot of pear

(Submitted in *Fungal Biology*)

Interaction of antifungal peptide BP15 with *Stemphylium vesicarium*, the causal agent of brown spot of pear

M. Puig[a], C. Moragrega[a,*], L. Ruz[a], C.E. Calderón[b], F.M. Cazorla[b], E. Montesinos[a] and I. Llorente[a].

[a]Institute of Food and Agricultural Technology- XaRTA-CIDSAV, University of Girona.
[b]Departamento de Microbiología, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", IHSM-UMA-CSIC, Universidad de Málaga.
*Corresponding author.

ABSTRACT

BP15, a short cecropin A-melittin hybrid peptide, has shown antifungal activity against several plant pathogenic fungi, including *S. vesicarium,* the causal agent of brown spot of pear. BP15 inhibits the germination, growth and sporulation of *S. vesicarium* and displays post-infection activity by stopping fungal infection in pear leaves. In the present work, live-cell imaging was undertaken to understand the antifungal mechanism of BP15. A double-staining method based on the combination of calcofluor white and SYTOX green coupled with epifluorescence microscopy was used to investigate fungal cell permeabilization and alterations in fungal growth induced by BP15. GFP-transformants of *S. vesicarium* were obtained and exposed to rhodamine-labelled BP15. Confocal laser microscopy provided evidence of peptide internalization by hyphae, resulting in fungal cell disorganization and death. *S. vesicarium* membrane permeabilization by BP15 was found to be peptide-concentration dependent. BP15 at MIC and sub-MIC concentrations (10 and 5 μM, respectively) inhibited *S. vesicarium* growth and produced morphological alterations to germ tubes, with slow and discontinuous compromise of fungal cell membranes. Fungal cell membrane disruption was immediately induced by BP15 at 100 μM, and this was accompanied by rapid peptide internalization by *S. vesicarium* hyphae. Peptide BP15 interacted with germ tubes and hyphae of *S. vesicarium* but not with conidial cells.

KEY WORDS

Agrobacterium tumefaciens-mediated transformation; green fluorescent protein; growth inhibition; membrane permeabilization; mode of action.

ABBREVIATIONS

AMPs: Antimicrobial peptides
ATMT: *Agrobacterium tumefaciens*-mediated transformation
BSP: Brown spot of pear
CFW: Calcofluor white
CLSM: Confocal laser scanning microscope
GFP: Green fluorescent protein
MIC: Minimal inhibitory concentration.
SG: SYTOX Green

1. Introduction

Stemphylium vesicarium (Wallr.) E. Simmons is the causal agent of brown spot of pear (BSP), a fungal disease of economic importance in pear-production areas in Europe (Llorente and Montesinos 2006). Since the first outbreaks reported in Italy in 1975 (Ponti *et al.* 1982), the relative importance of BSP has increased significantly and economic losses range from 1 to 10 % of the total production (Llorente and Montesinos 2006; Llorente *et al.* 2011). The disease symptoms consist of necrotic lesions on fruits, leaves and shoots. Infected fruits are unmarketable and severe outbreaks can result in premature defoliation and fruit abscission prior to harvest. Chemical control of BSP is based on preventative fungicide sprays applied at a fixed schedule or according to the BSPcast forecasting system (Llorente *et al.* 2000; Montesinos *et al.* 1995). The efficacy of the available fungicides (dithiocarbamates, strobilurins or captan) is moderate, especially when disease pressure is high (Llorente and Montesinos, 2006), and limited by the emergence of fungicide-resistant *S. vesicarium* isolates (Alberoni *et al.* 2005; 2010). Recently, antimicrobial peptides (AMPs) have emerged as an alternative or complement to conventional compounds used to control plant diseases (Keymanesh *et al.* 2009; Montesinos 2007; Montesinos *et al.* 2012; Zasloff 2002). Their effectiveness at low doses, and their ability to specifically target microorganisms without damaging plant cells, make AMPs suitable for overcoming the undesirable effects of conventional fungicides and bactericides (Montesinos and Bardají 2008; Montesinos *et al.* 2012). Several peptides from the CECMEL11 library (LIPPSO-CIDSAV, University of Girona, Girona, Spain), which is composed of *de novo* designed and synthetically produced cecropin A-melittin hybrid undecapeptides, were evaluated for *S. vesicarium* infection control in pear. Peptides BP15, BP22 and BP25 inhibited *S. vesicarium* growth and

sporulation, and peptide BP15 (KKLFKKILKVL-NH_2) showed post-infection activity on pear leaves (Puig *et al*. 2014). The curative activity of BP15 makes it suitable for field application according to the BSPcast forecasting system once favourable conditions for infection have been detected and infections are initiated (Puig *et al*. 2015). Knowledge regarding the mode of action of BP15 in the target organism *S. vesicarium* would contribute to the design of efficient application strategies and increase its efficacy in BSP control.

The mechanisms of action of AMPs in fungal cells are related to disruption of cell membranes and alteration of the structure of the cell wall, cytoskeleton and nucleus (Mania *et al*. 2010; Muñoz *et al*. 2006; Muñoz and Marcos 2006; Oard *et al*. 2004; Reed *et al*. 1997; Zhang *et al*. 2008; Zottich *et al*. 2013). Some AMPs are able to translocate across the plasma membrane non-disruptively and affect intracellular processes (Brogden 2005; Mania *et al*. 2010; Muñoz *et al*. 2013a, 2013b; Nicolas 2009); hence, it is suspected that the membrane permeabilization is not the only cause of pathogen cell death. Cecropin A, melittin and their hybrids have been widely studied for their antibacterial mode of action (Ferre *et al*. 2006; 2009; Glättli *et al*. 2006; Hristova *et al*. 2001; Hancock 2001; Makovitzki *et al*. 2007; Raghuraman and Chattopadhyay 2007; Shai 2002), whereas their mode of action on fungal cells has been less investigated with most reports focusing on their activity on yeast cells (Lee *et al*. 1997). The mechanisms by which cecropin A-melittin hybrid peptides, such as BP15, interact with plant pathogenic filamentous fungi are still unclear.

In this work, the interaction of synthetic peptide BP15 with *S. vesicarium* structures was analysed using differential dyes and biotechnological

tools. The abilities of BP15 to compromise fungal cell membranes and alter fungal growth were visualized with SYTOX Green probe and Calcofluor White dye, respectively. *Agrobacterium*-mediated GFP transformants of *S. vesicarium* were obtained to assess the internalization of rhodamine-labelled BP15 by *S. vesicarium* cells *in vitro* and in pear leaves.

2. Materials and methods

2.1. Fungal strain and culture conditions

Strain *S. vesicarium* EPS26 isolated from infected *Pyrus communis* fruit and deposited in the Culture Collection of Institute of Food and Agricultural Technology (INTEA, Girona, Spain) was used. Fresh cultures were obtained by growing the strain on tomato agar plates (800 ml of distilled water, 10 g of tomato concentrate juice, 2.3 g of $CaCO_3$ and 12.8 g of agar) for 7 to 10 days at 22.5°C with a 16 h-light photoperiod in a growth cabinet (I-30BLL Percival Plant Biology Chamber, Percival Scientific Inc., USA). Conidial suspensions were collected by adding 5 ml sterile distilled water with 0.002% Tween-20 to mycelium surface and scraping with a sterile L-shaped cell spreader. Suspensions were filtered before counting in a Thoma cell chamber and adjusted to 10^3 conidia ml^{-1}. *S. vesicarium* EPS26 stock cultures were maintained in PDA slants at 4°C.

2.2. Antifungal peptides

Peptide BP15 (> 90 % purity) was acquired from LIPPSO (University of Girona, Girona, Spain), and BP15 labelled with rhodamine-B (BP15-Rh) by covalent modification of the N-terminus was purchased from CASLO Laboratory (CASLO ApS, Lyngby, Denmark). The minimal inhibitory concentration (MIC) of BP15 against *S. vesicarium* growth and

sporulation was 10 and 50 µM, respectively. BP15 inhibited the fungal infection process on pear leaves at 50-100 µM (Puig *et al*. 2014). In this work, BP15 was applied at concentrations of 5 µM (subMIC), 10 µM (MIC) and 100 µM, depending on the experiment.

2.3. Generation and characterization of stable *S. vesicarium*-GFP transformants

Green fluorescent protein (GFP) is a vital marker widely used for *in vivo* visualization of fungal development and fungal-plant interactions. *S. vesicarium*-GFP transformants were obtained and used to visualize the peptide's effects on fungal structures under CLSM (Table 1). *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *S. vesicarium* was performed by using two plasmids, pTAS5-PgpdA-GFP-TtrpC (Meyer *et al*. 2007) and pPK2-HPHGFP (Michielse *et al*. 2009) designed to express enhanced GFP. *Agrobacterium tumefaciens* strain LBA4404 harbouring the plasmid was provided by Dr. Paul Hooykaas' Lab (University Biology of Leiden, Leiden, The Netherlands). Transformation of *S. vesicarium* EPS26 was carried out as described by Michielse *et al.* (2008) using *S. vesicarium* conidial suspensions. The stability of GFP expression was evaluated for five selected transformants by inoculation onto PDA plates containing 50 µg ml^{-1} hygromycin B of serial subcultures up to ten times. Colonies were examined under a Multizoom Nikon microscope (AZ100, Nikon Instruments, Melville, NY, USA) with the B filter (excitation, 460-500 nm; emission, 510-560 nm). Images were taken with a Nikon Digital Sight DS-5Mc and recorded and processed using the image analysis software Nis-Elements (Nikon Instruments Inc., Melville, NY, USA). Transformants were maintained at 4°C in PDA plates containing 100 µg ml^{-1} hygromycin B. To confirm the integration of the T-DNA into the genome of selected transformants, the

presence of the hygromycin resistance gene (*hph*) and the *hph-gfp* sequence were analysed. Mycelia harvested from fresh cultures of each *S. vesicarium*-GFP transformant and the wild-type strain EPS26 were ground in liquid nitrogen. DNA extractions were performed using the DNeasy™ Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Aliquots of DNA extracts were quantified with a spectrophotometer (Nanodrop ND-1000, UV-Vis Spectrophotometer, ThermoFisher Scientific, USA), and the samples were stored at -20°C. PCR amplification of the *hph* gene was performed using primers *hphF* and *hphR* (Pliego et al. 2009), which amplify a 400-bp sequence. The *hph-gfp* sequence was amplified by primers HGF (5'-AGGTCGCCAACATCTTCTTC-3') and GFPR (5'-CTTCAGCACGTGTCTTGTAG-3'), which amplify a 681-bp sequence.

Colony morphology, growth rate, and pathogenicity on pear were assessed and compared among the selected transformants and the wild-type strain EPS26. For growth evaluation, each GFP-transformant was co-cultured with the wild-type strain by placing 5-mm side agar blocks containing actively growing mycelia, collected from 5 day old fresh cultures, onto PDA and tomato agar plates. The cultures were incubated at 22.5°C and 16 h-light photoperiod for 6 days. Three replicates of five plates per growth medium were inoculated with each transformant/wild-type strain pair. Growth was assessed by measuring colony diameter 6 days after inoculation. The pathogenicity of the five transformants and the wild-type strain was determined by inoculation onto detached cv. Conference pear leaves as described in Puig *et al.* (2014). For each strain, three replicates of three leaves per replicate were inoculated with four 30-µl drops of 10^3 conidia ml^{-1} deposited on the leaf surface. The inoculated leaves were incubated at 22.5°C and 16-hour

light photoperiod for 5 days. Disease severity was assessed at the end of the incubation period using a 1-4 severity index based on the diameter of necrotic lesions (Puig *et al.* 2014). The statistical significance of data on colony diameter and disease severity were analysed using the SAS 9.2 software program (SAS Institute Inc., NC, USA). In all experiments, datasets were tested for equality of variances (Bartlett) and normality (Shapiro-Wilk). Data were evaluated using one-way ANOVA with Proc GLM, and means were compared with Fisher's protected least significant difference test at P=0.05.

To test whether the GFP-fluorescence of transformant strains could be detected on inoculated pear leaves, 5-day infected leaf segments were placed directly onto glass slides and examined under an inverted CLSM (TCS-NT, Leica Microsystems, Bensheim, Germany) equipped with filter blocks with spectral properties matching those of GFP (488 nm excitation and emission from 500 to 560 nm). Images were acquired and edited using the LAS-AF software (Leica microsystems, Wetzlar, Germany).

2.4. Alterations of *S. vesicarium* cell membrane integrity and morphology by BP15 peptide

Fungal cell membrane integrity assessment was based on the uptake of the fluorescent dye SYTOX Green (SG) (Molecular Probes; Invitrogen Corp, Carlsbad, CA, USA), which only penetrates cells with leaky plasma membranes and fluoresces when it interacts with nucleic acids (Table 1). Fungal cell wall alterations were assessed as abnormal chitin accumulation. Chitin deposition on fungal cells was analysed with Calcofluor White dye (CFW) (Fluorescent Brightener 28, Sigma-Aldrich,

St. Louis, MO, USA) which binds specifically to chitin (Pringle, 1991) (Table 1).

Table 1. Description of probes and markers used to assess the effects of peptide BP15 on *S. vesicarium*.

Probe/Marker	Function	Peptide effects analysed
SYTOX Green (SG)	Fluorescent nucleic acid stain that does not penetrate living cells. Assessment the integrity prokaryote and eukaryote cell membranes.	Disruption of *S. vesicarium* cell membrane.
Calcofluor White (CFW)	Fluorescent stain that binds strongly to structures containing cellulose and chitin.	Alteration of structures of cell wall in *S. vesicarium*.
Green fluorescent protein (GFP)	Vital marker. Used in fungal development, fungal-plant interaction and control studies. GFP-tagged *S. vesicarium* obtained for visualization of peptide-*S. vesicarium* interaction under CLSM.	Internalization of peptide BP15 by fungal cells.
Rhodamine-labelled BP15 (BP15-Rh)	Fluorescent dye for cellular localization of fluorochrome conjugated compounds using fluorescence microscopy. Visualization of peptide-*S. vesicarium* interaction under CLSM.	Internalization of peptide BP15 by fungal cells.

Effects of BP15 were assessed on ungerminated conidia and on actively growing germlings of *S. vesicarium* EPS26. Germlings were obtained after a 15-hour incubation of conidial suspensions at 22.5ºC for germination. Aliquots of 90 µl of either conidial or germling suspension (10^3 conidia ml^{-1}) were dispensed into 1.5 ml light-safe microcentrifuge tubes, and 10 µl of BP15 was added to final concentrations of 5, 10 or 100 µM. The same volume of sterile distilled water was added to suspensions of non-treated controls. The mixtures were incubated for 24 h at 22.5ºC. After different times of exposure to BP15 (0, 3, and 6 h), the

fungal suspensions were stained with SG and CFW. First, 5 µl of SG stock solution (4 µM) was added to reach a final concentration of 0.2 µM. After 5 min of incubation in the dark, 50 µl of CFW 0.1% (w/v) was added and the samples were incubated in the dark for an additional 5 min. The experiment consisted of three replicates of each treatment. Localization of fluorescent dye in the conidia and germ tubes was visualized by fluorescence microscopy (AXIO SCOPE A1, Carl Zeiss, Gottingen, Germany) with the filter set 38 (excitation, 470/40; emission, 525/50) for SG detection and filter set 49 (excitation, 365; emission, 445/50) for CFW detection. Three 20-µl aliquots were visualized per sample (≈150 conidia/germling per treatment). Photomicrographs were taken with an AxioCam digital camera (Carl Zeiss Microscopy GmbH, Munich, Germany) mounted on the microscope.

2.5. Internalization of BP15 and fungal cell viability

Internalization of BP15 by *S. vesicarium* was studied *in vitro* and during the fungal infection process on pear leaves (*ex vivo*). For this purpose, BP15 peptide labelled with the fluorochrome rhodamine and GFP expressing *S. vesicarium* transformants were used (Table 1). In *in vitro* assays, 270 µl of conidial suspension (10^3 conidia ml^{-1}) of each of five *S. vesicarium*- GFP transformants (MAL1, MAL2, MAL3, MAL4 and MAL5) was placed in a 1.5-ml light safe microcentrifuge tube and incubated for germination at 22.5°C for 15 h. After incubation, 30 µl of BP15-Rh was added to a final concentration of 100 µM, and the mixture was incubated for 6 additional hours at 22.5°C. In the non-treated controls, 30 µl of sterile distilled water was added instead of the peptide. Three tubes, corresponding to three replicates, were incubated per transformant strain. Ten minutes after the peptide treatment, and at one-hour time intervals, three 20 µl-aliquots (≈60 germlings) from each tube were

placed directly on a glass slide and examined using an inverted CLSM (TCS-NT, Leica Microsystems, Bensheim, Germany) equipped with filter blocks with spectral properties matching those of GFP (488 nm excitation and emission from 500 to 560 nm) and rhodamine B (543.5 nm excitation and emission from 590 to 625 nm).

Ex vivo studies were carried out with the MAL5 transformant strain, which exhibited stable and highly intense green fluorescence. Conidial suspensions (10^3 conidia ml^{-1}) were obtained from fresh cultures as described above and sprayed until runoff onto the surface of detached cv. Conference pear leaves. Three replicates of three leaves per replicate were inoculated, placed on moistened filter paper into sterile plates, and incubated at 22.5°C in darkness for 15 h to initialize the infection process. Then, the leaves were sprayed with 100 µM BP15-Rh solution or sterile distilled water (non-treated control) and incubated at 22.5°C for 5 days for disease development. Samples of inoculated leaves were excised for microscopic observations 1 and 24 h after peptide treatment. Small leaf sections were placed in a water droplet and mounted and observed under an inverted microscope (TE2000U Eclipse, Nikon, Japan) equipped with argon ion and HeNe lasers. The excitation and emission wavelengths were as follows: excitation at 488 nm and emission at 515-530 nm to visualize GFP fluorescence and excitation at 543.5 nm and emission at 590-595 nm to visualize BP15-Rh fluorescence. Simultaneous bright-field images were captured with a transmitted light detector. Filter settings were adjusted to achieve the maximum signal from the fluorescent protein and fluorochrome and minimum autofluorescence from the plant tissue. The imaging was carried out at room temperature. Maximum projections of an appropriate number of

optical slices were applied to visualize the hyphal sections (≈50 germlings per sample).

3. Results

3.1. Isolation and characterization of stable S. vesicarium-GFP transformants

Transformations with both plasmids were successful, resulting in five stable transformants expressing GFP (MAL1, MAL2, MAL3, MAL4, and MAL5). PCR confirmed the acquisition of the hygromycin resistance gene and the *hph-gfp* sequence in these strains. Transformant MAL5 showed a PCR product band more intense than the other ones (Fig. 1). All of the transformants exhibited green fluorescence, whereas no green autofluorescent background was observed in the wild-type strain (Fig. 2A). Microscopic analysis of the GFP-tagged strains revealed homogeneity of the fluorescence signal, which was clearly visible in conidia and hyphae and was stable for the duration of the observations (up to 6 hours) (Fig. 2B and 2C). The expression of GFP did not affect the growth or morphology of the transformant strains. All of them were able to grow on PDA and tomato agar similarly to the wild-type strain EPS26. Growth rates were not significantly different among strains (0.527-0.677 mm day^{-1}), and the conidial and hyphal morphology and pigmentation of the transformants were identical. The five GFP-transformants were pathogenic on pear and produced the same typical brown spot symptoms as the wild type strain. Disease severity on pear leaves inoculated with the GFP-transformants or the wild-type strain EPS26 was high (95-100%) and not significantly different (P=0.7001). When leaf sections of infected areas were observed under CLSM, the fungus was easily detected and hyphal and conidial cells expressed GFP during the infection process (Fig. 2D). Transformant MAL5 showed the strongest

and most stable fluorescence when visualized under CLSM, and it was selected for *ex vivo* studies on the internalization of BP15 by *S. vesicarium*.

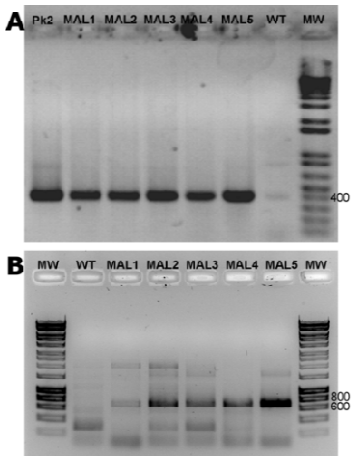

Figure 1. Molecular characterization of ATMT *S. vesicarium* strains (MAL1 to MAL5) using the plasmid pPK2-HPHGFP (Pk2). (A) PCR-amplified product of the *hph* gene. (B) PCR-amplified product of the *hph-gfp* gene. MW: DNA molecular size markers, 1 kb Plus DNA Ladder from Invitrogen (A) and HyperLadder 1 kb from Bioline (B).

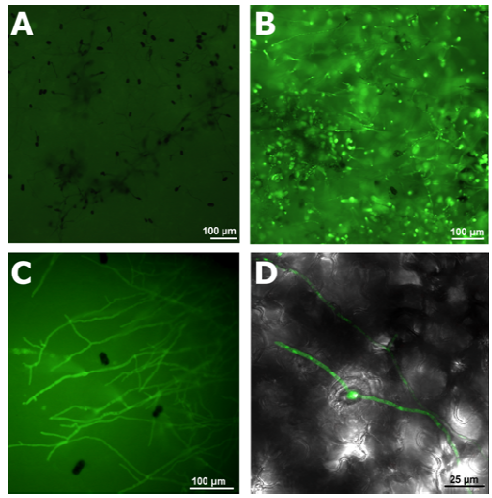

Figure 2. Detection of green fluorescence from 5-day-old cultures of *S. vesicarium* GFP-transformant MAL5 on PDA. No autofluorescence was observed in the wild-type strain (A). Note the intense fluorescence from young conidia and tips of long filaments (B) and the homogeneous fluorescence distributed in the cytoplasm of emerging hyphae (C). (D) Germinating conidium of MAL5 on a pear leaf.

3.2. Alterations of *S. vesicarium* cell membrane integrity and morphology by BP15 peptide

No fluorescent signal was observed in cells of *S. vesicarium* incubated with the SG probe without BP15 pre-treatment (Fig. 3A1 and 3A2). Ungerminated conidia exposed to BP15 did not emit SG fluorescence,

thus indicating that the conidial cell membranes were not altered by BP15 (data not shown).

Differences in SG staining were observed in germling cells of *S. vesicarium* exposed to BP15 depending on the peptide concentration. Germlings exposed to BP15 at 100 µM for 3 or 6 h showed intense SG staining, and continuous green fluorescence all along the hyphae, related to the absence of a defined nucleus and extensive cell permeabilization (Fig. 3D1, 3D2). Germlings of *S. vesicarium* exposed to subMIC (5 µM) and MIC (10 µM) concentrations of BP15 for 3 h showed bright green nuclei as well as discontinuous cytoplasmic fluorescence, with stained cells close to non-stained ones (Fig. 3B1 and 3C1, respectively). After 6 h of exposure to BP15 at subMIC and MIC concentrations confluent SG fluorescence was visualized through the hyphae (Fig. 3B2 and 3C2, respectively).

Non-treated germlings and conidia of *S. vesicarium* incubated with CFW, a chitin-specific fluorescent dye, showed prevalent staining of the intercellular septa (Fig. 4A1 and 4A2). BP15 peptide treatment altered the chitin deposition pattern in hyphal cells at all concentrations tested (Fig. 4). CFW fluorescence in germ tubes treated with BP15 at 100 µM was uniformly distributed along the hyphae, and the septum separation between cells was not evident due to wall disorganization (Fig. 4D1 and 4D2). After 3 h of exposure to BP15 at 5 or 10 µM, CFW staining of chitin was observed in intercellular septa and also scattered along the germ tubes, as a result of partial disorganization of the fungal cell wall (Fig. 4B1 and 4C1). CFW staining of germlings exposed for 6 h to subMIC or MIC concentrations of BP15 occurred all along the hyphae, whereas the septa of germ tubes were less intensely stained (Fig. 4B2 and 4C2).

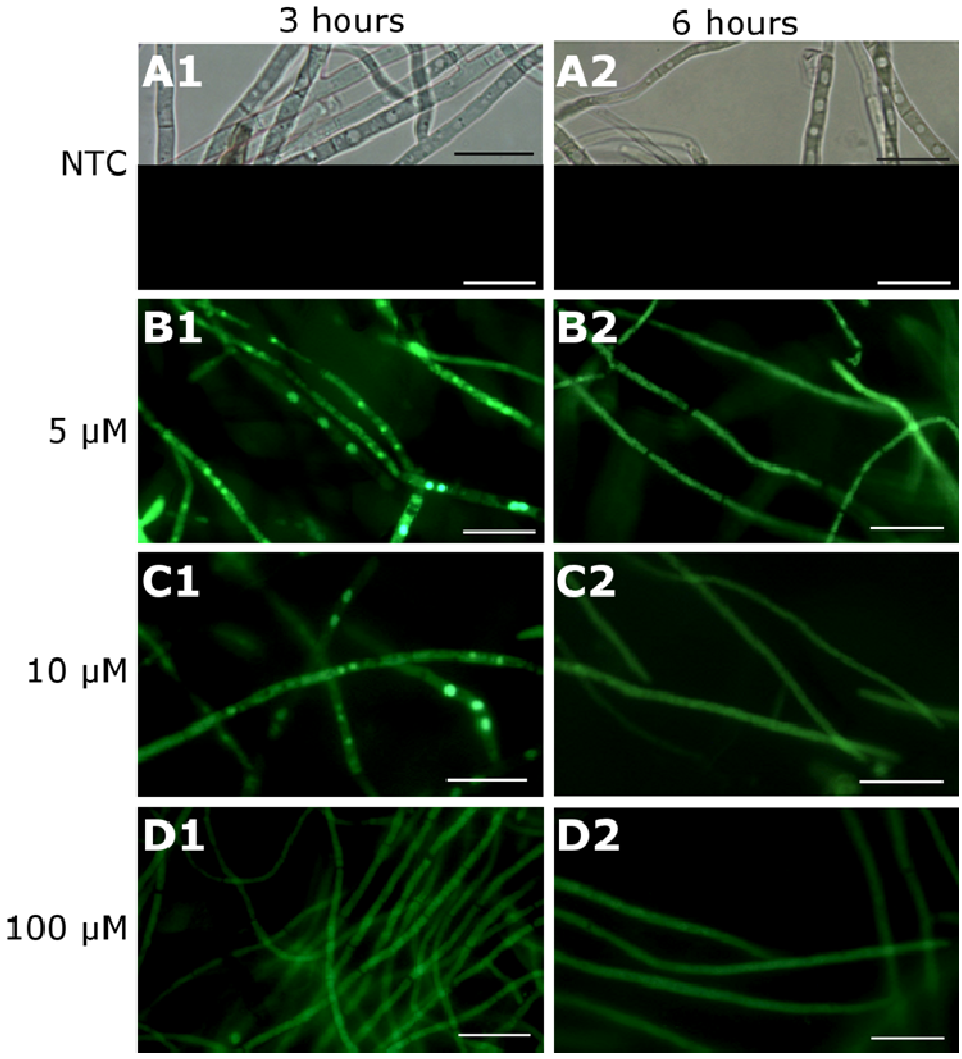

Fig. 3. Alteration of fungal membrane integrity in *S. vesicarium* by peptide BP15. Fluorescence microscopic images of wild-type strain EPS26 germlings exposed to BP15 at 5 µM, 10 µM or 100 µM for 3 to 6 h, or to sterile distilled water (NTC) stained with SYTOX Green dye. Panels in A1 and A2 show the same area under bright field (top) and fluorescence emission from SYTOX Green (bottom). Bars = 20 µm.

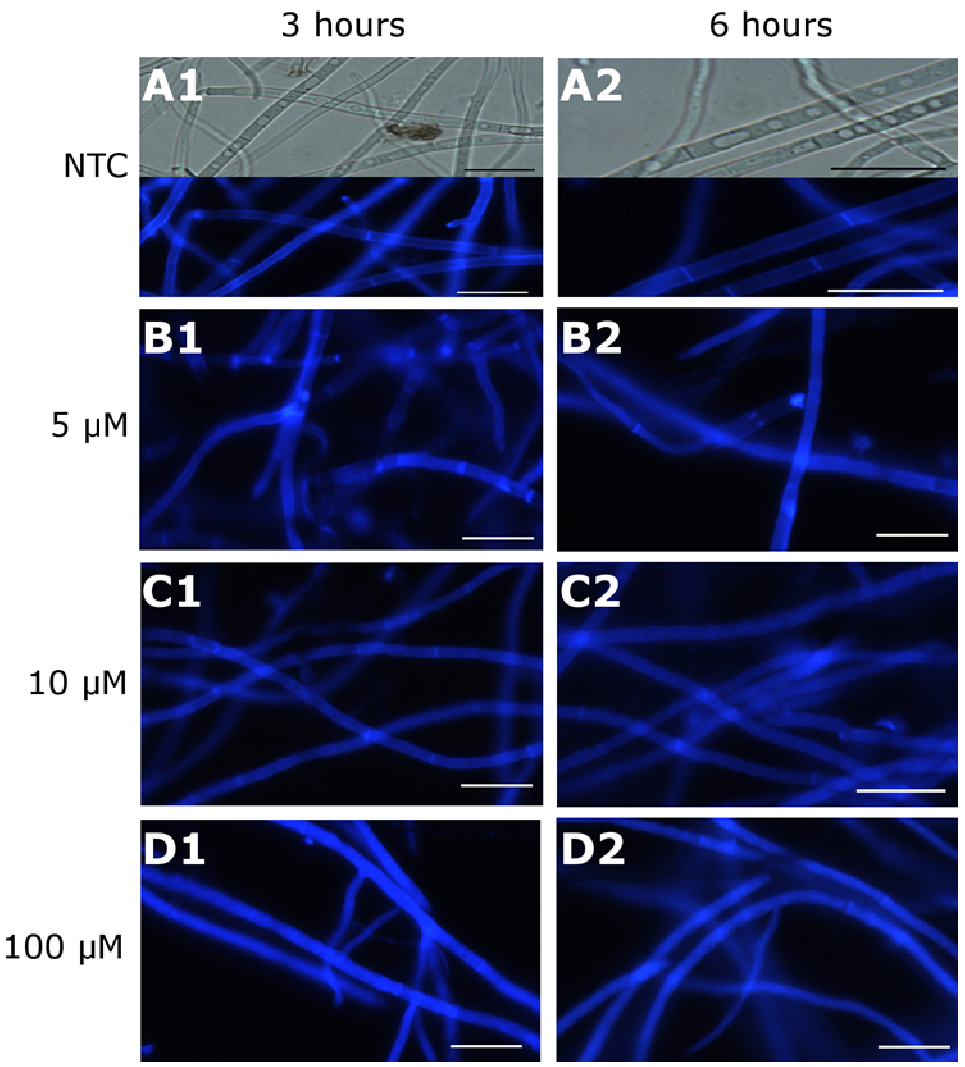

Fig. 4. Alteration of chitin deposition in *S. vesicarium* by peptide BP15. Fluorescence microscopic images of wild-type strain EPS26 germlings exposed to BP15 at 5 µM, 10 µM or 100 µM for 3 to 6 h, or to sterile distilled water (NTC) stained with Calcofluor White dye. Panels in A1 and A2 show the same area under bright field (top) and fluorescence emission from CFW (bottom). Bars = 20 µm.

3.3. Internalization of BP15 and fungal cell viability

Active growth of fungal germ tubes was observed in suspensions of the five GFP-transformants not exposed to BP15-Rh. Conidia and germ tubes of non-treated controls emitted green fluorescence under CLSM

throughout the 6 h of incubation (Fig. 5A). *In vitro* internalization of BP15 was similar by the five GFP-transformants; micrographs presented in Figure 5 correspond to MAL5 transformant as representative of all of them. The *S. vesicarium* growth inhibition activity of BP15-Rh did not differ from that of non-labelled BP15 peptide (data not shown). When suspensions of actively growing GFP-transformant germlings were exposed to BP15-Rh at 100 µM, rhodamine red fluorescence was visualized in germ tube cells after 10 min of exposure. After 2 h of exposure to BP15, the red fluorescence was localized in the cytosol of germ tubes, and an outflow of cell material was observed (Fig. 5B). Sites of GFP and BP15-Rh co-localization were detected by overlap in cell labelling, which resulted in yellow or orange coloured regions (Fig. 5B). After 6 h of exposure, a decrease in green GFP fluorescence was observed in hyphal cells that had internalized the peptide BP15, probably related to cell death (Fig. 5D, 5E and 5F). Additionally, exposure of germlings to 100 µM BP15-Rh arrested polarized hyphal growth, and induced tip swelling and bifurcation (Fig. 5C). All during the experiment, no red fluorescence was observed inside the conidia (Fig. 5B, 5E and 5F).

The intracellular localization of BP15-Rh was further investigated on detached pear leaves infected by the GFP-transformant strain MAL5. Transverse and vertical sections of non-treated leaves showed intense green fluorescence emitted by fungal hyphae and an absence of BP15-Rh red labelling (Fig 6A). Internalization of BP15 by fungal cells after the infection started was confirmed by observing transverse leaf sections 1 and 24 h after peptide treatment. One hour after peptide treatment, a red cytoplasmic signal was co-localized with GFP emission in some hyphal cells (Fig. 6B). Twenty-four hours after peptide application, a decrease of

green fluorescence within MAL5 hyphae was observed together with the emission of intense BP15-Rh red fluorescence (Fig. 6C, 6D). Red fluorescence from rhodamine was not observed inside the conidial cells (Fig. 6B). BP15-Rh reduced BSP disease in pear leaves when applied after the infection had initiated. Necrotic spots from 10-15 mm wide were observed 5 days after fungal inoculation on non-treated leaves (Fig. 6E), whereas 0-3 mm necrotic spots developed on BP15-Rh treated leaves (Fig. 6F).

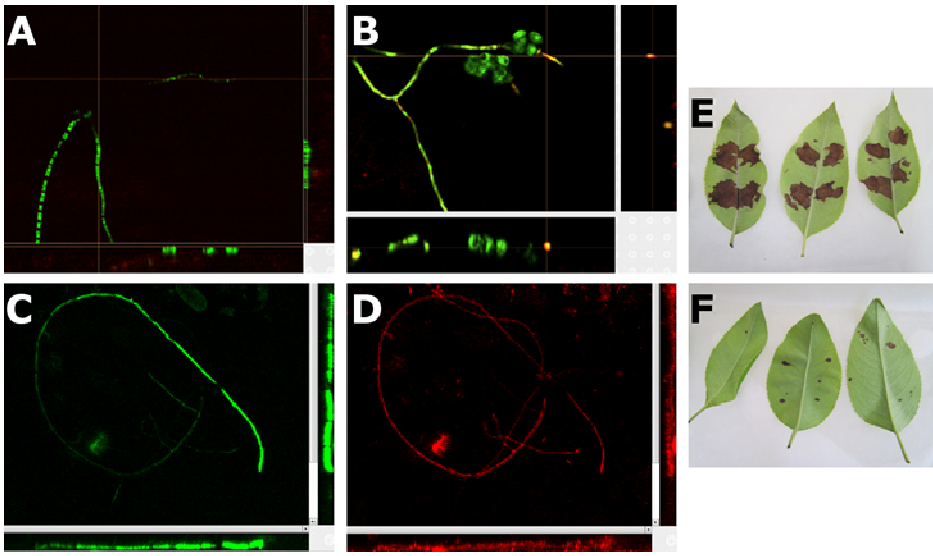

Fig. 5. Confocal laser scanning microscopy of *S. vesicarium* GFP-transformant MAL5 exposed to BP15-Rh peptide in *in vitro* assays. (A) Non-treated control germlings showed normal growth and green fluorescing cytoplasm. (B-F) Representative confocal images showing the localization of BP15-Rh (in red) in germ tubes (green), but not in conidial cells. (B) Co-localization of red and green fluorescence (circles) in germlings exposed for 2 h to BP15 at 100 μM and leakage of cytoplasmic content (arrowhead). (C) After 5 h of exposure to 100 μM BP15, the germ tube growth had stopped and alterations of branch emergence were observed (arrows). Germlings exposed for 24 h to BP15-Rh internalization excited with wavelengths of 488 nm for GFP (D), 543.5 nm for rhodamine (E), and co-excited with both wavelengths for co-localization of markers (F).

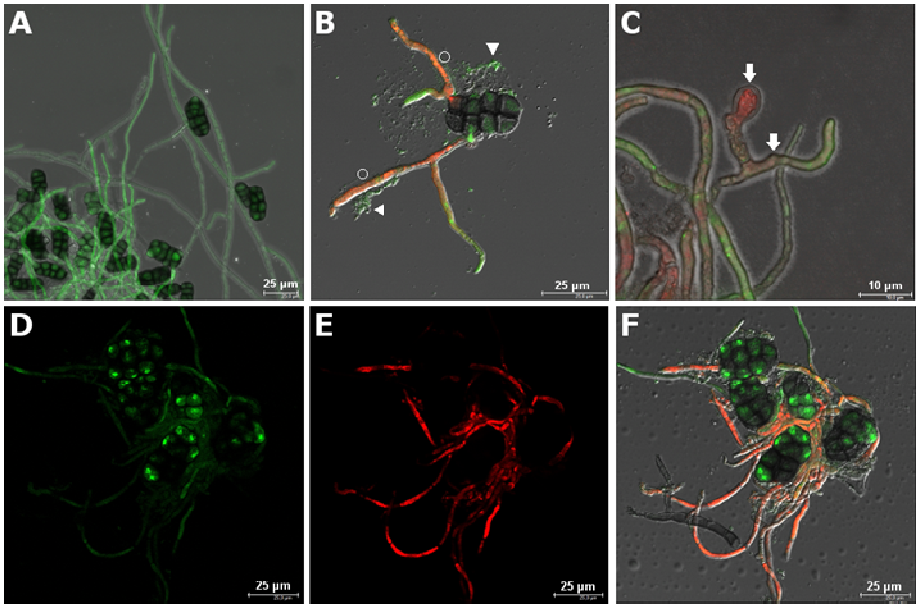

Fig 6. (A-D) Confocal laser scanning microscopy of BP15-Rh at 100 µM on germlings of *S. vesicarium* MAL5 inoculated onto cv. Conference pear leaves. (A) Transverse section of non-treated control 24 h after fungal inoculation, only GFP green fluorescence emission from fungal cells is observed. (B) Co-visualization of GFP green fluorescence and rhodamine red fluorescence can be observed 1 h after peptide treatment in vertical sections taken at the points indicated by the orange lines. GFP fluorescence emission (C) and red fluorescence of BP15-Rh internalized by hyphal cells of *S. vesicarium* MAL5 (D) 24 h after peptide treatment; on the right side and below the images, sections show the lateral maximum projection of hyphae. Sequential pictures were taken at 0.5-µm intervals. BSP disease symptoms 5 days after inoculation with *S. vesicarium* MAL5on non-treated control pear leaves (E) and in leaves treated with peptide BP15-Rh at 100 µM applied 15 h after pathogen inoculation (F).

4. Discussion

In the present work multiple experimental approaches based on live-cell imaging were undertaken to understand the mode of action of peptide BP15 against *S. vesicarium*.

The use of SG revealed that peptide BP15 induces membrane permeabilization in *S. vesicarium* hyphal cells and that this process is peptide-concentration dependent. The most rapid and intense SG uptake, together with diffusion of nuclear contents to the cytoplasm, was

observed with BP15 at 100 μM. Low and irregular SG uptake was observed after short exposure times with BP15 at sub-MIC and MIC concentrations, but longer times of exposure at these low concentrations induced complete membrane permeabilization in *S. vesicarium* hyphal cells. The specific structure of BP15, a small cationic peptide bearing a charge of +5 conferred by the presence of the positively charged amino acid lysine, increases the possibility that the charged residues will interact with the hydrophobic chains of negatively charged phospholipids of cell membranes and compromise their integrity, as determined in this work. Further analysis involving transmission electron microscope observations could provide more detailed information regarding the effects of BP15 on *S. vesicarium* plasma membrane integrity and whether pore formation or other mechanisms are involved in the process.

Previous studies had shown the inhibition of *S. vesicarium* hyphal growth after 15 hours of exposure to BP15 (10-100 μM) (Puig *et al.* 2014). Alterations on hyphal growth have been visualized in this study when *S. vesicarium* germlings were treated with subMIC, MIC and supraMIC concentrations of BP15, together with alterations in cell wall structure and abnormal deposition of chitin, revealed by CFW staining. Morphological alterations in *S. vesicarium* by peptide BP15 included reduction in germ tube length, thickening, and dichotomous tip branching, in agreement with previous studies (Puig *et al.* 2014). The abnormal cell growth could be related to alterations in cell polarity and/or wall deposition, similarly as described for other antifungal peptides that provoke cell wall stress at the hyphal apex and inhibit the maintenance of polarity (Muñoz *et al.* 2006).

Enhanced green fluorescent protein was used as a vital marker to detect changes that occurred in fungal cells after treatment with peptide BP15. The *Gfp* gene was successfully transformed into *S. vesicarium* and stably expressed in hyphae and conidia both *in vitro* and *ex vivo*. The expression of GFP by the fungus did not affect its morphology, growth or pathogenicity compared to the wild-type strain.

In previous studies FITC-labelled BP15 was found to distribute uniformly surrounding *S. vesicarium* hyphae, indicating that the initial interaction with the fungal cell takes place at the cell periphery (Puig *et al*. 2014). In the present paper, BP15-Rh was localized inside of actively growing GFP-transformant cells after short exposure times. GFP fluorescence in fungal cells decreased with exposition time to BP15-Rh, thus suggesting that BP15 internalization precedes BP15-induced cell death. CLSM observations and leaf assays confirmed that BP15-Rh at 100 µM stops the fungal growth and the infection process in pear leaves. Postinfection activity of BP15 in control of BSP disease has been demonstrated under laboratory and field conditions. Disease severity was reduced on detached pear leaves treated with BP15 from 6 to 24 h after the infections started (Puig *et al.* 2014). Moreover, BP15 field treatments showed a high efficacy in BSP disease control whatever the time of application (0 to 48 h) after the BSPcast risk advice (Puig et al., 2015). The present work confirms the direct interaction of BP15 with *S. vesicarium* hyphae infecting pear leaves, and provides evidence on the mechanisms by which BP15 exerts its curative activity against this pathogen.

Our previous studies stated that BP15 does not have sporicidal activity against *S. vesicarium* conidia, but it inhibits conidial germination at a

concentration of 10 μM. At this concentration, *S. vesicarium* conidia initiated the germination but the newly formed germ tubes remained shorter than the appropriate conidial length for considering them as fully germinated (Puig *et al*. 2014). The present study confirms that conidia of *S. vesicarium* are not a target for BP15 because BP15 did not permeabilize the conidial cell membranes, nor was it internalized by conidial cells. These findings suggest that the previously reported inhibition of germination of *S. vesicarium* conidia by this peptide could be due to inhibition of germ tube elongation after conidial wall breakdown rather than to direct action on conidia. These observations support the low efficacy of preventive applications of BP15 in control of BSP disease (Puig *et al.* 2014). However, BP15 and other antifungal peptides from the CECMEL11 library, BP20, BP33, and BP76, have sporicidal activity against *Fusarium oxysporum* and *Penicillium expansum* (Badosa *et al.* 2009). The specificity of BP15 towards *S. vesicarium* hyphae may be related to both the peptide and the microorganism. Mainly, the features of the target membrane and especially of the conidial cell wall (lipid composition, structure, length and complexity of the hydrophilic polysaccharide) may influence peptide specificity. Conidia of *S. vesicarium* are multicellular, darkly pigmented, and have a densely verrucose thick external wall. These characteristics could play a role in protecting them from the direct action of BP15. Additionally, peptide degradation by conidial extracellular proteases could also explain peptide selective activity, as they do in *Aspergillus* spp. conidial resistance to Cecropin A (Bland and De Lucca, 1998). Knowledge on the specific factors involved in selective activity of peptide BP15 on fungal structures could improve disease control efficacy.

5. Conclusions

The results presented here provide evidence that the mode of action of BP15 against *S. vesicarium* could be attributed to the combined effect of membrane permeabilization and cell penetration ability, which results in alterations to fungal growth and fungal cell death.

Acknowledgments

This research was supported in part by grants from the Ministerio de Educación y Ciencia (AGL2009-09829/AGR and AGL2011-30354-C02-01) of Spain. Mireia Puig was the recipient of a predoctoral fellowship from Universitat de Girona (BR10-17). We acknowledge the helpful assistance of Carla Massó and the Research Technical Services from Universitat de Girona. The authors thank Dr. Clara Pliego for her help with the ATMT protocols.

References

Alberoni G, Collina M, Pancaldi D, Brunelli A, 2005. Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards. *European Journal of Plant Pathology* **113**, 211–219.

Alberoni G, Cavallini D, Collina M, Brunelli A, 2010. Characterisation of the first *Stemphylium vesicarium* isolates resistant to strobilurins in Italian pear orchards. *European Journal of Plant Pathology* **126**, 453–457.

Badosa E, Ferre R, Francés J, Bardají E, Feliu L, 2009. Sporicidal activity of aynthetic antifungal undecapeptides and control of penicillium rot of apples. *Applied and Environmental Microbiology* **75**, 5563–5569.

Bland J M, De Lucca AJ, 1998. Identification of Cecropin A proteolytic cleavage sites resulting from *Aspergillus flavus*

extracellular protease(s). *Journal of Agricultural and Food Chemistry* **46**, 5324–5327.

Brogden KA, 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* **3**, 238–250.

Ferre R, Badosa E, Feliu L, Planas M, Montesinos E, Bardají E, 2006. Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides. *Applied and Environmental Microbiology* **72**, 3302–3308.

Ferre R, Melo MN, Correia AD, Feliu L, Bardají E, Planas M, Castanho M, 2009. Synergistic effects of the membrane actions of cecropin-melittin antimicrobial hybrid peptide BP100. Biophysical Journal 96, 1815–1827.

Glättli A, Chandrasekhar I, van Gunsteren WF, 2006. A molecular dynamics study of the bee venom melittin in aqueous solution, in methanol, and inserted in a phospholipid bilayer. *European Biophysics Journal* **35**, 255–267.

Hancock RE, 2001. Cationic peptides: effectors in innate immunity and novel antimicrobials. *The Lancet Infectious Diseases* **1**, 156–164.

Hristova K, Dempsey CE, White SH, 2001. Structure, location, and lipid perturbations of melittin at the membrane interface. *Biophysical Journal* **80**, 801–811.

Keymanesh K, Soltani S, Sardari S, 2009. Application of antimicrobial peptides in agriculture and food industry. *World Journal of Microbiology & Biotechnology* **25**, 933–944.

Llorente I, Vilardell P, Bugiani R, Gherardi I, Montesinos E, 2000. Evaluation of BSPcast disease warning system in reduced fungicide use programs for management of brown spot of pear. *Plant Disease* **84**, 631–637.

Llorente I, Montesinos E, 2006. Brown Spot of Pear: An emerging disease of economic importance in Europe. *Plant Disease* **90**, 1368–1375.

Llorente I, Moragrega C, Ruz L, Montesinos E, 2011. An update on control of brown spot of pear. *Trees* **26**, 239–245.

Makovitzki A, Viterbo A, Brotman Y, Chet I, Shai Y, 2007. Inhibition of fungal and bacterial plant pathogens *in vitro* and in planta with ultrashort cationic lipopeptides. *Applied and Environmental Microbiology* **73**, 6629–6636.

Mania D, Hilpert K, Ruden S, Fischer R, Takeshita N, 2010. Screening for antifungal peptides and their modes of action in *Aspergillus nidulans*. *Applied and Environmental Microbiology* **76**, 7102–7108.

Meyer V, Damveld RA, Arentshorst M, Stahl U, van del Hondel CA, Ram AF. 2007. Survival in the presence of antifungals: genome-wide expression profilling of *Aspergillus niger* in response to sublethal concentrations of caspofungin and fenpropimorph. *Journal of Biological Chemistry* **282**, 32935–32948.

Michielse CB, Hooykaas PJ, van den Hondel CA, Ram AF, 2008. Agrobacterium-mediated transformation of the filamentous fungus *Aspergillus awamori*. *Nature Protocols* **3**, 1671–1678.

Michielse CB, van Wijk R, Reijnen L, Cornelissen B, Rep M. 2009. Insight into the molecular requirements for pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici* through large-scale insertional mutagenesis. *Genome Biology* **10**, R4.

Montesinos E, Moragrega C, Llorente I, Vilardell P, Bonaterra A, Ponti I, 1995. Development and evaluation of an infection model for *Stemphylium vesicarium* on pear based on temperature and wetness duration. *Phytopathology* **85**, 586–592.

Montesinos, E, 2007. Antimicrobial peptides and plant disease control. *FEMS Microbiology Letters*, **270**: 1–11.

Montesinos E, Bardají E, 2008. Synthetic antimicrobial peptides as agricultural pesticides for plant-disease control. *Chemistry & Biodiversity* **5**, 1225–1237.

Montesinos E, Badosa E, Cabrefiga J, Planas M, Feliu L, Bardají E, 2012. Antimicrobial peptides for plant disease control. From discovery to application. In: Rajasekaran K, Cary JW, Jaynes JM, Montesinos E (eds), *Small Wonders: Peptides for Disease Control*. American Chemical Society, Washington DC, pp. 235–261.

Muñoz A, López-García B, Marcos JF, 2006. Studies on the mode of action of the antifungal hexapeptide PAF26. *Antimicrobial Agents and Chemotherapy* **50**, 3847–3855.

Muñoz A, Marcos JF, 2006. Activity and mode of action against fungal phytopathogens of bovine lactoferricin-derived peptides. *Journal of Applied Microbiology* **101**, 1199–1207.

Muñoz A, Gandía M, Harries E, Carmona L, Read ND, Marcos JF, 2013a. Understanding the mechanism of action of cell-penetrating antifungal peptides using the rationally designed hexapeptide PAF26 as a model. *Fungal Biology Reviews* **26**, 146–155.

Muñoz A, Harries E, Contreras-Valenzuela A, Carmona L, Read ND, Marcos JF, 2013b. Two functional motifs define the interaction, internalization and toxicity of the cell-penetrating antifungal peptide PAF26 on fungal cells. *PloS One* **8**, e54813.

Nicolas P, 2009. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS Journal* **276**, 6483–6496.

Oard S, Rush MC, Oard JH, 2004. Characterization of antimicrobial peptides against a US strain of the rice pathogen *Rhizoctonia* solani. *Journal of Applied Microbiology* **97**, 169–180.

Pliego C, Kanematsu S, Ruano-Rosa D, de Vicente A, López-Herrera C, Cazorla, FM, Ramos, 2009. GFP sheds light on the infection process of avocado roots by *Rosellinia necatrix*. *Fungal Genetics and Biology* **46**, 137–145.

Ponti I, Cavani P, Brunelli A, 1982. “Maculatura bruna” delle pere: eziologia e difesa. *Informatore Fitopatologico* **32**, 35–40.

Pringle JR, 1991. Staining of bud scars and other cell wall chitin with calcofluor. *Methods in Enzymology* **194**, 732–735.

Puig M, Moragrega C, Ruz L, Montesinos E, Llorente I, 2014. Postinfection activity of synthetic antimicrobial peptides against Stemphylium vesicarium in pear. *Phytopathology* **104**, 1192–1200.

Puig M, Moragrega C, Ruz L, Montesinos E, Llorente I, 2015. Controlling brown spot of pear by a synthetic antimicrobial peptide under field conditions. *Plant disease* http://dx.doi.org/10.1094/PDIS-03-15-0250-RE

Raghuraman H, Chattopadhyay A, 2007. Melittin: a membrane-active peptide with diverse functions. *Bioscience Reports* **27**, 189–223.

Reed JD, Edwards DL, Gonzalez CF, 1997. Synthetic peptide combinatorial libraries: a method for the identification of bioactive peptides against phytopathogenic fungi. *Molecular Plant-Microbe Interactions* **10**, 537–549.

Shai Y, 2002. Mode of action of membrane active antimicrobial peptides. *Biopolymers* **66**, 236–248.

Zasloff M, 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389–395.

Zhang J, Wu X, Zhang SQ, 2008. Antifungal mechanism of antibacterial peptide, ABP-CM4, from *Bombyx mori* against *Aspergillus niger*. *Biotechnology Letters* **30**, 2157–2163.

Zottich U, Da Cunha M, Carvalho AO, Dias GB, Casarin N, Vasconcelos IM, Gomes VM, 2013. An antifungal peptide from *Coffea canephora* seeds with sequence homology to glycine-rich proteins exerts membrane permeabilization and nuclear localization in fungi. *Biochimica et Biophysica Acta* **1830**, 3509–3516.

Chapter 6

General Discussion

This work focuses on the use of synthetic AMPs to control brown spot of pear disease (BSP), caused by the fungus *Stemphylium vesicarium*. BSP control is based on not only on applying fungicides during the pear growing period, but also combining these with sanitation practices aimed at reducing inoculum production. However, the efficacy of disease control is not absolute (Llorente et al., 2011a) thus, new compounds are needed to be used either as alternatives or complements to the current commercial fungicides in integrated disease management. AMPs are a new generation of compounds with a huge potential to be used in crop protection. In recent years peptide libraries or peptide collections have been developed at the same time as the emergence of efficient methods for synthesizing peptides and their analogs, and studies into AMPs for application in agriculture are on the increase. Along these lines, CIDSAV and LIPPSO, from the Universitat de Girona, designed a combinatorial library composed of synthetic linear undecapeptides cecropin A-melittin hybrids (CECMEL11 library) with potential antimicrobial activity. Previous studies into the antifungal activity of synthetic AMPs from the CECMEL11 library demonstrated that some peptides inhibited the growth of the plant pathogenic fungi *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium expansum* and *Rhizopus stolonifer* (Badosa et al., 2009). These results prompted us to evaluate the effectiveness of these peptides in controlling *S. vesicarium* infections on pear.

Twelve AMPs from the CECMEL11 library were screened *in vitro* to determine whether they would be able to inhibit *S. vesicarium* growth and sporulation. The most effective peptides were then selected and evaluated for their efficacy in controlling *S. vesicarium* infections on pear in *ex vivo* experiments. The optimal peptide application timing for

disease control was determined and feasible mechanisms of action were proposed. Peptides BP15 and BP22 were selected on the basis of their *in vitro* and *ex vivo* efficacy and evaluated for BSP disease control under field conditions. Finally, peptide BP15 was further evaluated in a disease management program for brown spot of pear control.

In vitro screening demonstrated that some antimicrobial peptides from the CECMEL11 library are active against *S. vesicarium*. Eight out of twelve peptides inhibited *S. vesicarium* germination and growth at concentrations lower than 100 µM. Peptides BP15, BP22 and BP25 were the most effective with MIC for conidia germination of 10 µM (BP15) and 50 µM (BP22 and BP25). These MIC values are within the range of MICs determined for other AMPs active against plant pathogenic fungi, such as pep20 against *Alternaria solani* and *Phytophthora infestans* (Ali & Reddy, 2000), C16-KLLK, C14-KLLK, C12-KLLK for *A. alternata*, *Botrytis cinerea* and *Cochliobolus heterostrphus* (Makovitzki *et al.*, 2007), PAF20, PAF26 against *Penicillium italicum* and *P. digitatum* (López-García *et al.*, 2002) and the natural peptide Cc-GRP for *Colletotrichum lindemuthianum* and *Fusarium oxysporum* (Zottich *et al.*, 2013). Additionally, the MIC values of peptide BP15 agree with the values previously obtained for this peptide in inhibiting the growth of *Fusarium oxysporum* and *Penicillium expansum* (Badosa et al., 2009).

Morphological alterations were observed under an optical microscope on *S. vesicarium* hyphae exposed to peptides BP15, BP22 and BP25 at doses lower than their respective MICs. As well as a reduction in hyphae length, when compared to the nontreated control, constricted regions, thicker cells and closer septa were some of peptide effects on *S. vesicarium*. In addition, condensed mycelial aggregates around the

conidia were observed. These observations agree with fungal modifications caused by antimicrobial peptides C14-KLLK on *A. alternata* and *B. cinerea* (Makovitzki *et al.*, 2007), and pep6, pep7 and pep20 on *A. solani* and *P. infestans* (Ali & Reddy, 2000). Visualization of fungal cell walls stained with calcofluor white dye confirmed alterations in cell wall structure caused by BP15, suggesting that the peptide could induce cell wall stress predominately at the hyphal apex and inhibit the maintenance of polarity. The malformations caused on newly formed germ tubes and hyphae by BP15 could result in the loss of ability to develop the asexual reproductive structures, i.e. conidiophores and conidia. In fact, peptides BP15 and BP22 also reduced the sporulation of *S. vesicarium*. Accordingly, new infections on leaves and fruit would be reduced by the decrease in new inoculum produced on infected tissues.

None of the tested peptides, BP15, BP22 or BP25, showed sporicidal activity against *S. vesicarium*. Conidia that had been in contact with AMPs at 100 µM for 48 h were able to develop mycelia and grow on tomato agar plates. Fluorescence microscope observations confirmed that BP15 targeted *S. vesicarium* hyphal cells but not conidial cells. On the other hand, several AMPs from the CECMEL11 library, including BP15, demonstrated sporicidal activity on the microconidia of *F. oxysporum* and the conidia of *P. expansum* (Badosa *et al.*, 2009). It has been reported that the mode of action of AMPs depends on the particular AMP involved and on the composition and physical properties of the target bilayer (lipid composition, structure, length and complexity of the hydrophilic polysaccharide) (Sato & Feix, 2006). Thus, the differences in BP15 activity would be explained by the morphological difference in conidia. Concretely, conidia of *S. vesicarium* are multicellular, with a densely thick verrucose external wall and are

melanin-like pigmented. These characteristics could prevent BP15 adhesion to *S. vesicarium* conidia.

On the basis of *in vitro* results, BP15 and BP22 were selected to be tested for controlling *S. vesicarium* infections on pear under laboratory and field conditions. Therefore, an evaluation of fungicide efficacy in disease control is able to be conducted under different scenarios of peptide-pathogen interaction on the host plant: a) co-inoculation, when peptide and pathogen are applied at the same time on the host plant, b) preventative, when peptide is applied before the pathogen inoculation, c) curative or postinfection, when the pathogen is inoculated first and then the peptide is applied and, finally, d) pre-incubate inoculum, when the peptide and pathogen are mixed and applied after a fixed incubation time (Alan & Earle, 2002; Moreno *et al.*, 2003; Muñoz *et al.*, 2006; Badosa *et al.*, 2009; Montesinos *et al.*, 2012). In most studies AMPs have been applied preventative or have been co-inoculated with the pathogen. For example, C14-KLLK was effective in controlling *B. cinerea* on cucumber leaves and fruit when it was applied immediately after pathogen inoculation (Makovitzki *et al.*, 2007) and the co-inoculation of PAF26 with *P. digitatum* conidia inhibited the development of disease in citrus fruits (Muñoz *et al.*, 2007). In this work, *ex vivo* assays on detached pear leaves were conducted to determine the efficacy of BP15 and BP22 for controlling *S. vesicarium* infections on pear when applied under preventative and postinfective strategies. While results indicated that BP15 and BP22 were effective in reducing *S. vesicarium* infection on pear leaves when applied in a postinfective strategy whatever the concentration tested, this was not so when applied preventatively. The efficacy of a postinfective application of these peptides may be explained by their interaction with fungal hyphae and germ tubes,

resulting in altering or inhibiting fungal growth and consequently, stopping the infection progress. Observing the infected leaves under fluorescence microscope it was confirmed that BP15 targeted germ tubes immediately after the treatment. The non-sporicidal effect of BP15 and BP22 may explain the preventative treatments' lack of efficacy. Additionally, the degradation or inactivation of peptides by some compounds on leaf surface, such as the phenolic compounds and proteases may reduce their antifungal activity. According to our experiments, the peptide degradation would have occurred within less than three hours contact with plant surfaces as when *S. vesicarium* was inoculated three hours after the peptide application, the disease was not controlled. In fact, previous works reported that peptides from the CECMEL11 library are either degraded or inactivated by proteinase K or plant leaf extracts within 45 minutes (Ferre *et al*., 2006; Güell *et al*., 2011).

Despite the efficacy of the peptides on postinfective treatments, some small lesions not related to progressive infections did appear on leaves. These small necrotic spots may be related to the host-specific toxins SV I and II produced by *S. vesicarium* (Llorente & Montesinos, 2006), which have an early effect on the plasma membranes of host leaves and appear to be the key or central event responsible for early pathogenesis on pear (Singh *et al*. 1999).

Furthermore, BP15 significantly reduced the severity of the disease when applied for up to 24 hours once *S. vesicarium* germination on the pear leaves had begun. Similar results were observed for peptide BP22 but these were not consistent in some of the repetitions. Until now, fungicides, scheduled in commercial orchards with the BSPcast model for controlling BSP disease, have few or no curative effects on BSP

infections and their efficacy is moderate (Llorente, 1997; Llorente & Montesinos, 2006). BP15's postinfective activity is a key advantageous feature of this peptide as its application can be guided by the forecasting model BSPcast when the infections have already started and as a result offers new potential in BSP disease management.

Once peptides BP15 and BP22 from the CECMEL11 library were identified for their efficacy in controlling the BSP disease, the next step in this work was to evaluate their effectiveness under field conditions. Field assays were performed over two years on both potted pear plants exposed to orchard conditions and on pear trees. In accordance with postinfection activity, peptide treatments were timed on the basis of the BSPcast forecasting model. This model determines when environmental conditions are favorable for *S. vesicarium* infections on pear. Field assays with the potted pear plants demonstrated that when BP15 was applied according to the BSPcast model disease severity was significantly reduced with an efficacy of control from 40% to 60%. Even though BP15 was not applied the same day that BSPcast warned of favorable conditions for *S. vesicarium* infections, a high efficacy of control was obtained, thus demonstrating that BP15 could be applied from 24 to 48 hours after the model has raised the alert of the BSP infections beginning. These results confirm the capacity of BP15 to stop the progress of the disease observed in the *ex vivo* experiments. Results obtained using BP22 showed a low efficacy of BSP control and in some repetitions the results were not consistent. This may well be related to the structure of BP22, as it rapidly degrades when applied under field conditions. Accordingly, BP22 was not tested further in field trials.

On pear trees assays, disease severity was reduced in 3 out of 4 trials when shoots were treated with either BP15 or the fungicide thiram. In Trial II the results were inconsistent, which may be due to the considerable variability observed. Moreover, in 2 out of 4 trials the disease reduction on shoots treated with BP15 was similar to that observed in trees treated with thiram. Interestingly, the efficacy obtained in the three trials using BP15 (37.1%, 40.4% and 37.1%) was similar to previously described efficacies for commercial fungicides i.e. among 30% to 60% (Antoniacci et al., 2006; Llorente *et al.*, 2010). Although the efficacy of BP15 is similar to that of some commercial fungicides, according to the warning system in Dutch orchards better effectiveness (close to 80%) has been obtained when difenoconazole and trifloxystrobin are sprayed in combination with thiram (Jong & Heijne, 2005). However, difenoconazole use is not permitted under the Catalan Integrated Pest Management (IPM) guidelines (DAAM, 2014). Unfortunately, disease control was incomplete when shoots were treated using either BP15 or thiram, probably due to the production of the two host-specific toxins, as was observed with the *ex vivo* tests.

In the two years of study, BP15 application combined with plastic foil on the ground (to reduce inoculum production) significantly decreased (by approximately 50%) disease severity when compared to the untreated controls without ground coverage. These results suggest that to improve disease control efficacy, treatment addressed at controlling infection (on the aerial parts of the trees) using BP15 must be complemented with sanitation methods to reduce inoculum production (on the orchard floor) such as removing fallen leaves from the orchard ground, and/or complementing treatment with biocontrol agents such as *Trichoderma* sp. (Rossi *et al.*, 2005; Llorente & Montesinos, 2006; Llorente *et al.*, 2011a).

The results presented in this work probably provide the first hard evidence of the efficacy of a synthetic peptide in the control of a plant disease under field conditions. AMP sprays, and more specifically BP15, would appear to be extremely promising in reducing any brown spot of pear forecast by the BPScast model. One benefit of using BP15 to control BSP disease is its rapid degradation, which means low residue levels on fruit and a reduced pre-harvest interval. However, this characteristic could be a drawback as the number of BP15 treatments is higher than that of other commercial fungicides because BP15 must be applied each time the threshold risk is reached. It is supposed that the persistence of AMPs in the ecosystem is lower than that of commercial fungicides due to their rapid degradation, although, as far as we know, no study into the potential impact on the microbial populations and/or ecosystems has been undertaken. It would be interesting to focus future research on elucidating the impact of BP15 and other CECMEL11 peptides with potential use in crop protection on the agroecosystem, and in particular on microbial populations in the phyllospher.

Finally, knowing how an antifungal compound such as BP15 acts contributes to improving the effectiveness of disease control treatments. Different experimental approaches based on live-cell imaging were used to understand what BP15 mode of action was against *S. vesicarium*.

A SYTOX Green assay provided evidence that BP15 permeabilizes the membrane of *S. vesicarium* in a time (and concentration) dependent manner; as do the antifungal peptides PAF26 and LfcinB17-31 against *P. digitatum* (Muñoz & Marcos, 2006; Muñoz *et al.*, 2006). Fluorescent microscope observations of BP15-*S. vesicarium* interaction suggest that membrane permeation is BP15's primary mode of action. In other words,

α-helical peptides destabilize and disrupt the cell membrane by forming membrane defects, which result in electrolyte loss and cell death (Oren & Shai, 1998; Pasupuleti et al., 2012). Among the three ways described for peptides with α-helix structure to permeate the microbial cell membrane (Chapter 1 p. 46-47), BP15 may well follow the carpet model and disturb the cell membrane of *S. vesicarium* hyphae. To cause damage to the cell membrane, as evidenced by the SYTOX experiments, BP15 seemed to need a high P/L ratio as the peptide was observed causing more damage at high concentrations and when contact time was increased. Apart from compromising the integrity of the cell membrane, BP15 destabilized the nuclear membrane at any concentration tested, although at low BP15 concentration the nuclear membrane permeabilization occurred later. The effect on nuclei could be explained by either the damage to the cytoplasmic membrane, which would cause the cell death and the subsequent nuclear degradation, or by the internalization of BP15 by fungal cells and the interaction with the nuclear membrane. Internalization of BP15 by fungal cells could disrupt normal cell functions, by inhibiting nucleic acids, suppressing synthesis of macromolecules, affecting the cell wall growth and enzymatic activities, etc.; as has been described for the natural and synthetic α-helical peptides, Cc-GRP, ABP-CM4, AFP, Cecropin B, PAF26, Sub 5 and lactoferricin (Reed *et al.*, 1997; Park *et al.*, 1998; Oard *et al.*, 2004; Muñoz & Marcos, 2006; Muñoz *et al.*, 2006, 2013; Hagen *et al.*, 2007; Zhang *et al.*, 2008; Mania *et al.*, 2010; Zottich *et al.*, 2013). Recent studies indicate that many α-helical peptides have complex mechanisms of action involving several targets and they kill microorganisms by inhibiting intracellular targets, blocking essential intracellular processes after translocation across the cell membrane and, thus, provoking cell death (Peters *et al.*, 2010; Hilpert *et al.*, 2010; Muñoz *et al.*, 2013).

Ferrer *et al*. (2009) demonstrated that peptide BP100 clearly showed a translocation behavior for both high and low P/L ratios, so in order to clarify whether BP15 localizes on the fungal cell membrane or is internalized by fungal cells, the green fluorescent protein (GFP) was used as a vital marker. The successful transformation of *S. vesicarium* provides a new tool for studying the activity and mode of action of not only other fungicides, but also many aspects of fungal-plant interaction or the interaction with biocontrol agents. In this work the *S. vesicarium-GFP* transformants were used to detect changes that occur in fungal cells after treatment with the peptide BP15 labeled with red fluorochrome Rhodamine B (BP15-Rh) *in vitro* and during the infection process. Confocal laser microscope observations confirmed that BP15 is internalized by *S. vesicarium* cells, since the hyphae showed an overlapping signal of BP15-Rh with GFP of *S. vesicarium* in the cytoplasm of hyphae cells. In addition, the green fluorescence emitted by the living GFP-*S. vesicarium* cells decreased in the regions where BP15-Rh was localized. This fact could be related to the effects of BP15 on fungal cells, resulting in the inhibition of green protein synthesis or in cell death. The rapid localization of BP15 in cells and the reduction of green fluorescence could explain the postinfection activity of BP15, as it stops the hyphae elongation and, as a consequence, the infection's progress is interrupted. Identifying intracellular targets could lead to a better understanding of BP15's mode of action against *S. vesicarium*. In summary, we hypothesize that the antifungal activity of BP15 may involve different antifungal mechanisms; and the combination of cell permeabilization and its cell-penetrating ability would determine its postinfection activity against *S. vesicarium*.

The results obtained in this thesis offer new possibilities in the use of AMPs in crop protection in general and in BSP management in particular.

Chapter 7

Conclusions

According to the studies enclosed in this thesis, the following conclusions can be drawn:

1. Eight out of the twelve tested peptides from the CECMEL11 library (BP15, BP21, BP22, BP23, BP24, BP25, BP35, and BP38) reduce *in vitro* the germination and growth of *Stemphylium vesicarium* at concentrations lower than 100 µM. BP15 and BP22 are the most active peptides with MIC values of 10 and 50 µM, respectively. None of the peptides tested have sporicidal activity against *S. vesicarium* conidia.

2. Peptides BP15, BP22 and BP25 significantly reduce (50% to 95%) the sporulation of *S. vesicarium* grown in a Petri dish under controlled conditions.

3. Preventative applications of BP15 and BP22 on pear leaves in *ex vivo* experiments under controlled conditions and *in planta* under field conditions do not reduce *S. vesicarium* infections.

4. Postinfection applications of BP15 on pear leaves in *ex vivo* experiments under controlled conditions and *in planta* under field conditions consistently reduces *S. vesicarium* infections. Disease control using applications of BP22 is lower than that of BP15.

5. Applications of peptide BP15 according to the BSPcast forecasting model significantly reduce brown spot of pear severity in pear orchards. The efficacy of disease control is similar or slightly lower than that obtained using the fungicide thiram. Consequently, BP15 would be a good candidate to be included in the brown spot of pear management program using the BSPcast warning system.

6. Peptide BP15 interacts with the hyphae of *S. vesicarium* and alters their development. BP15 is rapidly degraded once applied to leaves.

7. Peptide BP15's mode of action against *S. vesicarium* involves fungal cell membrane permeabilization in a time and dose-dependent manner. BP15 internalization by *S. vesicarium* hyphal cells results in internal cell disorganization and cell death.

Chapter 8

References

A

Agrios GN, 2005. *Plant Pathology*. London, Unite Kingdom. Academic Press.

Alan AR, Earle ED, 2002. Sensitivity of bacterial and fungal plant pathogens to the lytic peptides, MSI-99, magainin II and cecropin B. *Molecular Plant-Microbe Interactions* **15**, 701–708.

Alberoni G, Cavallini D, Collina M, Brunelli A, 2010. Characterisation of the first *Stemphylium vesicarium* isolates resistant to strobilurins in Italian pear orchards. *European Journal of Plant Pathology* **126**, 453–457.

Alberoni G, Collina M, Pancaldi D, Brunelli A, 2005. Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards. *European Journal of Plant Pathology* **113**, 211–219.

Ali GS, Reddy ASN, 2000. Inhibition of fungal and bacterial plant pathogens by synthetic peptides: in vitro growth inhibition, interaction between peptides and inhibition of disease progression. *Molecular Plant-Microbe Interactions* **13**, 847–859.

Antoniacci L, Bugiani R, Rossi R, 2006. Control of brown spot of pear in organic pear orchard. In: *Orchard diseases International workshop; 7th, Orchard diseases*. 117–122.

Aveling TAS, Snyman HG, 1993. Infection studies of *Stemphylium vesicarium* on onion leaves. *Mycological Research* **97**, 984–988.

B

Badosa E, Ferre R, Francés J, Bardají E, Feliu L, 2009. Sporicidal activity of aynthetic antifungal undecapeptides and control of *Penicillium* rot of apples. *Applied and Environmental Microbiology* **75**, 5563–5569.

Badosa E, Ferre R, Planas M *et al*., 2007. A library of linear undecapeptides with bactericidal activity against phytopathogenic bacteria. *Peptides* **28**, 2276–2285.

Badosa E, Moiset G, Montesinos L *et al.*, 2013. Derivatives of the antimicrobial peptide BP100 for expression in plant systems. *PloS one* **8**, e85515.

Barna B, Leiter E, Hegedus N, Bíró T, Pócsi I, 2008. Effect of the *Penicillium chrysogenum* antifungal protein (PAF) on barley powdery mildew and wheat leaf rust pathogens. *Journal of Basic Microbiology* **48**, 516–520.

Basallote-Ureba MJ, Prados-Ligero AM, Melero-Vara JM, 1999. Aetiology of leaf spot of garlic and onion caused by *Stemphylium vesicarium* in Spain. *Plant Pathology* **48**, 139–145.

Bergh JC, Weiss CR, 1993. Pear rust mite, *Epitrimerus pyri* (Acari: Eriophyidae) oviposition and nymphal development on Pyrus and non-Pyrus hosts. *Experimental & Applied Acarology* **17**, 215–224.

Berman HM, Westbrook, Feng Z *et al.*, 2000. The protein data bank. *Nucleic Acids Research* **28**, 235–242.

Bhargava A, Osusky M, Hancock RE, Forward BS, Kay WW, Misra S, 2007. Antiviral indolicidin variant peptides: Evaluation for broad-spectrum disease resistance in transgenic *Nicotiana tabacum*. *Plant Science* **172**, 515–523.

Bishop-Hurley SL, Mounter SA, Laskey J *et al.*, 2002. Phage-displayed peptides as developmental agonists for *Phytophthora capsici* zoospores. *Applied and Environmental Microbiology* **68**, 3315–3320.

Blancard D, Allard E, Brest P, 1989. La stemphyliose du poirier ou “macules brunes”. *Phytoma* **406**, 35–37.

Boman HG, 1995. Peptide antibiotics and their role in innate immunity. *Annual Review of Immunology* **13**, 61–92.

Brogden KA, 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* **3**, 238–250.

C

Cavallarin L, Andreu D, San Segundo B, 1998. Cecropin A-derived peptides are potent inhibitors of fungal plant pathogens. *Molecular Plant-Microbe Interactions* **11**, 218–227.

Chaisrisook C, Stuteville DL, Skinner DZ, 1995. Five *Stemphylium* spp. pathogenic to alfalfa: occurrence in the United States and time requirements for ascospore production. *Plant Disease* **79**, 369–372.

Chen J, Falla TJ, Liu H *et al.*, 2000. Development of protegrins for the treatment and prevention of oral mucositis: structure-activity relationships of synthetic protegrin analogues. *Biopolymers* **55**, 88–98.

Civolani S, 2012. The past and present of pear protection against the pear psylla, *Cacopsylla pyri* L. In: Perveen F, ed. *Insecticides - Pest Engineering*. InTech. DOI: 10.5772/28460.

Coca M, Peñas G, Gómez J *et al.*, 2006. Enhanced resistance to the rice blast fungus *Magnaporthe grisea* conferred by expression of a cecropin A gene in transgenic rice. *Planta* **223**, 392–406.

Company N, Nadal A, La Paz J-L *et al.*, 2013. The production of recombinant cationic α-helical antimicrobial peptides in plant cells induces the formation of protein bodies derived from the endoplasmic reticulum. *Plant Biotechnology Journal*, DOI: 10.1111/pbi.12119

Cugier JP, Humbert W, 1991. Stemphyliose du poirier. Etude de la biologie du parasite et recherches des fongicides actifs. *Phytoma Défense Végétaux* **431**, 47–50.

Cuthbertson BJ, Büllesbach EE, Fievet J, Bachère E, Gross PS, 2004. A new class (penaeidin class 4) of antimicrobial peptides from the Atlantic white shrimp (*Litopenaeus setiferus*) exhibits target specificity and an independent proline-rich-domain function. *The Biochemical Journal* **381**, 79–86.

D

Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H, (eds) 2011. *Modern Fungicides and Antifungal Compounds VI*. Germany.

DeLucca AJ, Bland JM, Jacks TJ, Grimm C, Cleveland TE, Walsh TJ, 1997. Fungicidal activity of cecropin A. *Antimicrobial agents and chemotherapy* **41**, 481–483.

DeLuca AJ, Walsh TJ, 2000. Antifungal peptides: origin, activity, and therapeutic potential. *Revista Iberoamericana de Micología* **17**, 116–120.

DeLucca A, Walsh T, 1999. Antifungal peptides: novel therapeutic compounds against emerging pathogens. *Antimicrobial Agents and Chemotherapy* **43**, 1–11.

Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural (DAAM), 2014. Norma tècnica per a la producció integrada de fruita de llavor. Annexos de control integrat de plagues, malalties i de matèries actives (Revisió 03.04.2014). Generalitat de Catalunya. Online: http://agricultura.gencat.cat/ca/ambits/agricultura/produccio-integrada/normes-quaderns/normes-tecniques-produccio/fruita-llavor [Accessed: June , 2014]

Deshayes S, Morris MC, Divita G, Heitz F, 2005. Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cellular and Molecular Life Sciences: CMLS* **62**, 1839–1849.

Dykes GA, Aimoto S, Hastings JW, 1998. Modification of a synthetic antimicrobial peptide (ESF1) for improved inhibitory activity. *Biochemical and Biophysical Research Communications* **248**, 268–272.

E

Epand RM, Vogel HJ, 1999. Diversity of antimicrobial peptides and their mechanisms of action. *Biochimica et Biophysica Acta - Biomembranes* **1462**, 11–28.

F

Falloon PG, Fallon LM, Grogan RG, 1987. Etiology and epidemiology of *Stemphylium* leaf-spot and purple apot of asparagus in California. *Phytopathology* **77**, 407–413.

Falloon P., Falloon L., Grogan RG, 1984. Purple spot and *Stemphylium* leaf spot or asparagus. *California Agriculture*, 21.

Ferre R, Badosa E, Feliu L, Planas M, Montesinos E, Bardají E, 2006. Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides. *Applied and Environmental Microbiology* **72**, 3302–3308.

Ferre R, Melo MN, Correia AD *et al*., 2009. Synergistic effects of the membrane actions of cecropin-melittin antimicrobial hybrid peptide BP100. *Biophysical Journal* **96**, 1815–1827.

Fungicide Ressitance Action Committee (FRAC), 2014. FRAC Code List ©2014: Fungicides sorted by mode of action. Online: http://www.frac.info [Accessed: May , 2014]

Food and Agriculture Organization of the United Nations (FAOSTAT), 2014. Statistics Division of the Food and Agriculture Organization of the United Nations. Online: <http://faostat.fao.org [Accessed: April , 2014]

G

Ganz T, Selsted ME, Lehrer R., 1990. Defensins. *European Journal of Haematology* **44**, 1–8.

Gevao B, 2000. Bound pesticide residues in soils: a review. *Environmental Pollution* **108**, 3–14.

Giangaspero A, Sandri L, Tossi A, 2001. Amphipathic α-helical antimicrobial peptides. A systematic study of the effects of structural and physical properties on biological activity. *European Journal of Biochemistry* **268**, 5589–5600.

Giosuè S, Rossi V, Buginai R, Mazzoni C, 2006. Modelling dynamics of airborne conidia of *Stemphylium vesicarium*, the causal agent of brown spot of pear. In: *7th International workshop Orchard diseases*. 169–176.

Gisi U, 1996. Synergistic interactions of fungicides in mixtures. *Phytopathology* **86**, 1273 – 1279.

Gonzalez CF, Provi EM, Zhu L, Ebbole DJ. 2002. Independent and synergistic activity of synthetic peptides against thiabendazole-resistant *Fusarium sambucinum*. *Phytopathology* **92**, 917 – 924.

Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. 2010. Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clinical immunology* **135**, 1-11.

Güell I, Badosa E, Talleda M *et al*., 2010. Antimicrobial peptides containing D-amino acids with in vivo activity against plant pathogenic bacteria. *Journal of Peptide Science* **16**, 139.

Güell I, Cabrefiga J, Badosa E *et al*., 2011. Improvement of the efficacy of linear undecapeptides against plant-pathogenic bacteria by incorporation of D-amino acids. *Applied and Environmental Microbiology* **77**, 2667–2675.

Gullino ML, Leroux P, Smith CM, 2000. Uses and challenges of novel compounds for plant disease control. *Crop Protection* **19**, 1–11.

H

Hagen S, Marx F, Ram AF, Meyer V, 2007. The antifungal protein AFP from *Aspergillus giganteus* inhibits chitin synthesis in sensitive fungi. *Applied and Environmental Microbiology* **73**, 2128–2134.

Hancock REW, Brown KL, Mookherjee N, 2006. Host defence peptides from invertebrates--emerging antimicrobial strategies. *Immunobiology* **211**, 315–322.

Hancock REW, Chapple DS, 1999. Peptide Antibiotics. *Antimicrobial Agents and Chemotherapy* **43**, 1317–1323.

Hancock REW, Lehrer R, 1998. Cationic peptides: a new source of antibiotics. *Trends in Biotechnology* **16**, 82–88.

Hancock REW, Sahl H-G, 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology* **24**, 1551–1557.

Harman GE, Nelson EB, Ondik KL, 2006. Effects of fungicide application on nontarget microbial populations. *Turfgrass and Environmental Research Online* **5**, 102–105.

Heijne B, Van Mourik J, 2001. Zwart- vruchtrot op peer neemt toe. *Fruitteelt Den Haag* **91**, 18–19.

Hilpert K, McLeod B, Yu J *et al.*, 2010. Short cationic antimicrobial peptides interact with ATP. *Antimicrobial Agents and Chemotherapy* **54**, 4480–4483.

Hollomon, DW 2009. Fungicides for Plant Diseases. *eLS*. DOI: 10.1002/9780470015902. a0021312

I

Irwin JAG, Bray RA, 1991. Variation in virulence within the cool temperature biotype of *Stemphylium vesicarium* (Wallr.) Simmons, a lucerene leaf spot pathogen. *Australian Journal of Experimental Agriculture* **31**, 793–795.

J

Javadpour MM, Juban MM, Lo WC *et al.*, 1996. De novo antimicrobial peptides with low mammalian cell toxicity. *Journal of Medicinal Chemistry* **39**, 3107–3113.

Jaynes JM, Nagpala P, Destéfano-Beltrán L et al., 1993. Expression of a Cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*. *Plant Science* **89**, 43–53.

Jenssen H, Hamill P, 2006. Peptide antimicrobial agents. *Clinical Microbiology Reviews* **19**, 491-511.

Jeppson LR, Keifer HH, Baker EW, 1975. *Mites injurious to economic Plants*. University of California Press.

Jones VP, Hilton R, Brunner JF *et al*., 2013. Predicting the emergence of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), on a degree-day scale in North America. *Pest Management science* **69**, 1393–1398.

Jong PF, Heijne B, 2005. Test of fungicides against *Stemphylium vesicarium* on pear with or without a warning system. In: *ISHS Acta Horticulturae 671: IX International Pear Symposium*. 615–620.

K

Kamysz W, Krolicka A, Bogucka K, Ossowski T, Lukasiak J, Lojkowska E, 2005. Antibacterial activity of synthetic peptides against plant pathogenic *Pectobacterium* species. *Journal of Phytopathology* **153**, 313–317.

Keymanesh K, Soltani S, Sardari S, 2009. Application of antimicrobial peptides in agriculture and food industry. *World Journal of Microbiology & Biotechnology* **25**, 933–944.

Khan SU, 1980. *Pesticides in the soil environment*. Elsevier Scientific Publishing Co., Amsterdam, The Netherlands.

Köhl J, Groenenboom-de Haas B, Goossen-van de Geijn H *et al*., 2008. Pathogenicity of *Stemphylium vesicarium* from different hosts causing brown spot in pear. *European Journal of Plant Pathology* **124**, 151–162.

Köhl J, Groenenboom-de Haas BH, Kastelein P, Rossi V, Waalwijk C, 2009. Quantitative detection of pear-pathogenic *Stemphylium vesicarium* in orchards. *Phytopathology* **99**, 1377–1386.

Köhl J, Jong P-F, Kastelein P *et al*., 2013. Dynamics of pear-pathogenic *Stemphylium vesicarium* in necrotic plant residues in Dutch pear orchards. *European Journal of Plant Pathology* **137**, 609–619.

L

Leadbeater A, Gisi U, 2009. The Challenges of chemical control of plant diseases. In: Gisi U, Chet I, Gullino ML, (eds). *Recent Developments in Management of Plant Diseases*. Dordrecht: Springer Netherlands, 3–17.

Lee DG, Hahm KS, Shin SY, 2004. Structure and fungicidal activity of a synthetic antimicrobial peptide, P18, and its truncated peptides. *Biotechnology Letters* **26**, 337–341.

Li Q, Lawrence CB, Maelor Davies H, Everett NP, 2002. A tridecapeptide possesses both antimicrobial and protease-inhibitory activities. *Peptides* **23**, 1–6.

Li Q, Lawrence CB, Xing HY *et al.*, 2001. Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco. *Planta* **212**, 635–639.

Lindquist EE, Bruin J, Sabelis MW, 1996. Eriophyoid mites- Their biology, natural enemies and control. Elsevier Science Publ., Amsterdam, the Netherlands.

Liu Z, Zeng M, Dong S, Xu J, Song H, Zhao Y, 2007. Effect of an antifungal peptide from oyster enzymatic hydrolysates for control of gray mold (*Botrytis cinerea*) on harvested strawberries. *Postharvest Biology and Technology* **46**, 95–98.

Llorente I, 1997. Desenvolupament d'un sistema de previsió de risc d'infecció per *Stemphylium vesicarium*. Avaluació, validació i implementació en parcel·les experimentals en camps comercials de perera. Universitat de Girona. Online: http://www.tdx.cat/handle/10803/7800

Llorente I, Manceau C, Montesinos E, 2003. Susceptibility of European pear cultivars to *Pseudomonas syringae* pv . *syringae* using immature fruit and detached leaf assays. *European Journal of Plant Pathology* **109**, 319–326.

Llorente I, Montesinos E, 2002. Effect of relative humidity and interrupted wetness periods on brown spot severity of pear caused by *Stemphylium vesicarium*. *Phytopathology* **92**, 99–104.

Llorente I, Montesinos E, 2004. Development and field evaluation of a model to estimate the maturity of pseudothecia of *Pleospora allii* on pear. *Plant Disease* **88**, 215–219.

Llorente I, Montesinos E, 2006. Brown spot of pear: an emerging disease of economic importance in Europe. *Plant Disease* **90**, 1368–1375.

Llorente I, Moragrega C, Ruz L *et al*., 2010a. Basis for new strategies in integratred control of brown spot of pear (*Stemphylium vesicarium*, teleomorph *Pleospora allii*). In: *IOBC/WPRS Bulletin* **54**, 35–39.

Llorente I, Moragrega C, Ruz L, Montesinos E, 2011a. An update on control of brown spot of pear. *Trees* **26**, 239–245.

Llorente I, Vilardell P, Bugiani R, Gherardi I, Montesinos E, 2000. Evaluation of BSPcast disease warning system in reduced fungicide use programs for management of brown spot of pear. *Plant Disease* **84**, 631–637.

Llorente I, Vilardell A, Montesinos E, 2006. Infection potential of *Pleospora allii* and evaluation of methods for reduction of the overwintering inoculum of brown spot of pear. *Plant Disease* **90**, 1511–1516.

Llorente I, Vilardell P, Montesinos E, 2011b. Evaluation of a revision of the BSPcast decision support system for control of brown spot of pear. *Phytopathologia Mediterranea* **50**, 139–49.

Llorente I, Vilardell A, Vilardell P *et al*., 2010b. Control of brown spot of pear by reducing the overwintering inoculum through sanitation. *European Journal of Plant Pathology* **128**, 127–141.

López-García B, González-Candelas L, Pérez-Payá E, Marcos JF, 2000. Identification and characterization of a hexapeptide with activity against phytopathogenic fungi that cause postharvest decay in fruits. *Molecular Plant-Microbe Interactions* **13**, 837–846.

López-García B, Pérez-Payá E, Marcos JF, 2002. Identification of novel hexapeptides bioactive against phytopathogenic fungi through screening of a synthetic peptide combinatorial library. *Applied and Environmental Microbiology* **68**, 2453–2460

López-García B, San Segundo B, Coca M, 2012. Antimicrobial peptides as a promising alternative for plant disease protection. In: Rajasekaran K, Cary JW, Jaynes JM, Montesinos E (eds). *Small Wonders: Peptides for Disease Control*. 263–294.

López-García B, Ubhayasekera W, Gallo RL, Marcos JF, 2007. Parallel evaluation of antimicrobial peptides derived from the synthetic PAF26 and the human LL37. *Biochemical and Biophysical Research Communications* **356**, 107–113.

López-García B, Veyrat A, Pérez-Payá E, González-Candelas L, Marcos JF, 2003. Comparison of the activity of antifungal hexapeptides and the fungicides thiabendazole and imazalil against postharvest fungal pathogens. *International Journal of Food Microbiology* **89**, 163–170.

Lundberg P, Langel Ü, 2003. A brief introduction to cell-penetrating peptides. *Journal of Molecular Recognition* **16**, 227–233.

M

Madani F, Lindberg S, Langel U, Futaki S, Gräslund A, 2011. Mechanisms of cellular uptake of cell-penetrating peptides. *Journal of Biophysics* **2011**, 414729.

Makovitzki A, Viterbo A, Brotman Y, Chet I, Shai Y, 2007. Inhibition of fungal and bacterial plant pathogens in vitro and in planta with ultrashort cationic lipopeptides. *Applied and Environmental Microbiology* **73**, 6629–6636.

Mania D, Hilpert K, Ruden S, Fischer R, Takeshita N, 2010. Screening for antifungal peptides and their modes of action in *Aspergillus nidulans*. *Applied and Environmental Microbiology* **76**, 7102–7108.

Manoukis NC, Hoffman K, 2014. An agent-based simulation of extirpation of *Ceratitis capitata* applied to invasions in California. *Journal of Pest Science* **87**, 39–51.

Marcos JF, Muñoz A, Perez-Paya E, Misra S, Lopez-Garcia B, 2008. Identification and rational design of novel antimicrobial peptides for plant protection. *Annual Review of Phytopathology* **46**, 273–301.

Mazor M, Erez A, 2004. Processed kaolin protects fruits from Mediterranean fruit fly infestations. *Crop Protection* **23**, 47–51.

McGrath, MT, 2004. What are fungicides? *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2004-0825-01.

Mentag R, Luckevich M, Morency M, Seguin A, 2003. Bacterial disease resistance of transgenic hybrid poplar expressing the synthetic antimicrobial peptide D4E1. *Tree Physiology* **23**, 405–411.

Michalska K, Skoracka A, Navia D, Amrine JW, 2010. Behavioural studies on eriophyoid mites: an overview. *Experimental & Applied Acarology* **51**, 31–59.

Milani A, Benedusi M, Aquila M, Rispoli G, 2009. Pore forming properties of cecropin-melittin hybrid peptide in a natural membrane. *Molecules* **14**, 5179–5188.

Monroc S, Badosa E, Besalú E *et al*., 2006a. Improvement of cyclic decapeptides against plant pathogenic bacteria using a combinatorial chemistry approach. *Peptides* **27**, 2575–84.

Monroc S, Badosa E, Feliu L, Planas M, Montesinos E, Bardaji E, 2006b. De novo designed cyclic cationic peptides as inhibitors of plant pathogenic bacteria. *Peptides* **27**, 2567–2574.

Montesinos E, 2003. Development, registration and commercialization of microbial pesticides for plant protection. *International microbiology : the official journal of the Spanish Society for Microbiology* **6**, 245–252.

Montesinos E, 2007. Antimicrobial peptides and plant disease control. *FEMS microbiology letters* **270**, 1–11.

Montesinos E, Badosa E, Cabrefiga J, Planas M, Feliu L, Bardají E, 2012. Antimicrobial Peptides for Plant Disease Control. From Discovery to Application, pp. 235–261. In: Rajasekaran K, Cary JW, Jaynes JM, Montesinos E (eds). *Small Wonders: Peptides for Disease Control*. Volume 1095. American Chemical Society/ Oxford University Press Inc. Washington, USA.

Montesinos E, Bardají E, 2008. Synthetic antimicrobial peptides as agricultural pesticides for plant-disease control. *Chemistry & Biodiversity* **5**, 1225–1237.

Montesinos E, Bonaterra A, 1996. Dose-response models in biological control of plant pathogens: An empirical verification. *Phytopathology* **86**, 464–472.

Montesinos E, Bonaterra A, Ophir Y, Beer S V, 1996. Antagonism of selected bacterial strains to *Stemphylium vesicarium* and biological control of brown spot of pear under controlled environment conditions. *Phytopathology* **86**, 856–863.

Montesinos E, Moragrega C, Llorente I *et al*., 1995a. Development and evaluation of an infection model for *Stemphylium vesicarium* on pear based on temperature and wetness Duration. *Phytopathology* **85**, 586–92.

Montesinos E, Moragrega C, Llorente I, Vilardell P, 1995b. Susceptibility of selected european pear cultivars to infection by *Stemphylium vesicarium* and influence of leaf and fruit age. *Plant Disease* **79**, 471–473.

Montesinos E, Vilardell P, 1992. Evaluation of FAST as a forecasting system for scheduling fungicide sprays for control of *Stemphylium vesicarium* on pear. *Plant Disease* **76**, 1221–1226.

Moreno AB, del Pozo AM, Borja M, San Segundo B, 2003. Activity of the antifungal protein from *Aspergillus giganteus* against *Botrytis cinerea*. *Phytopathology* **93**, 1344–1353.

Morton V, Staub T, 2008. A short history of fungicides. Online, APSnet Features. DOI: 10.1094/APSnetFeature-2008-0308

Muñoz A, Gandía M, Harries E, Carmona L, Read ND, Marcos JF, 2013a. Understanding the mechanism of action of cell-penetrating antifungal peptides using the rationally designed hexapeptide PAF26 as a model. *Fungal Biology Reviews* **26**, 146–155.

Muñoz A, Harries E, Contreras-Valenzuela A, Carmona L, Read ND, Marcos JF, 2013b. Two functional motifs define the interaction, internalization and toxicity of the cell-penetrating antifungal peptide PAF26 on fungal cells. *PloS one* **8**, e54813.

Muñoz A, López-García B, Marcos JF, 2006. Studies on the mode of action of the antifungal hexapeptide PAF26. *Antimicrobial Agents and Chemotherapy* **50**, 3847–3855.

Muñoz A, López-García B, Marcos JF, 2007. Comparative study of antimicrobial peptides to control citrus postharvest decay caused by *Penicillium digitatum*. *Journal of Agricultural and Food Chemistry* **55**, 8170–8176.

Muñoz A, Marcos JF, 2006. Activity and mode of action against fungal phytopathogens of bovine lactoferricin-derived peptides. *Journal of Applied Microbiology* **101**, 1199–1207.

Muñoz A, Marcos JF, Read ND, 2012. Concentration-dependent mechanisms of cell penetration and killing by the de novo designed antifungal hexapeptide PAF26. *Molecular Microbiology* **85**, 89–106.

N

Nadal A, Montero M, Company N *et al.*, 2012. Constitutive expression of transgenes encoding derivatives of the synthetic antimicrobial peptide BP100: impact on rice host plant fitness. *BMC plant biology* **12**, 159.

Naik SN, Prasad R, 2006. Pesticide residue in organic and conventional food-risk analysis. *Journal of Chemical Health and Safety* **13**, 12–19.

Nicolas P, 2009. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *The FEBS journal* **276**, 6483–6496.

O

Oard S, Rush MC, Oard JH, 2004. Characterization of antimicrobial peptides against a US strain of the rice pathogen *Rhizoctonia solani*. *Journal of Applied Microbiology* **97**, 169–180.

Oerke EC, 2006. Crop losses to pests. *The Journal of Agricultural Science* **144**, 31–43.

Oerke EC, Dehne HW, Schönbeck F, Weber A (eds), 1999. *Crop production and crop protection: estimated losses in major food and cash crops*. Elsevier, Amsterdam, the Netherlands.

Ogle HJ, 1997. Disease Management: Chemical. In: Brown J, Ogle HJ (eds). *Plant Pathogens and Plant Diseases*. Berringer, Australia. Rockvale Publications.

Oren Z, Shai Y, 1998. Mode of action of linear amphipathic α-helical antimicrobial peptides. *Biopolymers* **47**, 451–463.

Osusky M, Osuska L, Kay W, Misra S, 2005. Genetic modification of potato against microbial diseases: in vitro and in planta activity of a dermaseptin B1 derivative, MsrA2. *Theoretical and Applied Genetics* **111**, 711–722.

Osusky M, Zhou G, Osuska L, Hancock RE, Kay WW, Misra S, 2000. Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens. *Nature biotechnology* **18**, 1162–1166.

Owens LD, Heutte TM, 1997. A single amino acid substitution in the antimicrobial defense protein cecropin B is associated with diminished degradation by leaf intercellular fluid. *Molecular Plant-Microbe Interactions* **10**, 525–528.

P

Palacio-Bielsa A, Cambra Álvarez MA, 2009. El fuego bacteriano de las rosáceas (*Erwinia amylovora*). Ministerio de Medio Ambiente y Medio Rural y Marino(ed). Madrid, Spain

Park CB, Kim HS, Kim SC, 1998. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochemical and Biophysical Research Communications* **244**, 253–257.

Pasupuleti M, Schmidtchen A, Malmsten M, 2012. Antimicrobial peptides: key components of the innate immune system. *Critical Reviews in Biotechnology* **32**, 143–171.

Perez Espitia PJ, de Fátima Ferreira Soares N, dos Reis Coimbra JS, de Andrade NJ, Souza Cruz R, Alves Medeiros EA, 2012. Bioactive peptides: synthesis, properties, and applications in the packaging and preservation of food. *Comprehensive Reviews in Food Science and Food Safety* **11**, 187–204.

Peters BM, Shirtliff ME, Jabra-Rizk MA, 2010. Antimicrobial peptides: primeval molecules or future drugs? *PLoS pathogens* **6**, e1001067.

Ponti I, Brunelli A, Tosi C *et al.*, 1993. Verifica dell'attività di diversi preparati contro la maculatura bruna del pero. *Informatore Fitopatologico* **43**, 45–52.

Ponti I, Cavani P, Brunelli A, 1982. "Maculatura bruna" delle pere: eziologia e difesa. *Informatore Fitopatologico* **32**, 35–40.

Powell WA, Catranis CM, Maynard CA, 1995. Synthetic antimicrobial peptide design. *Molecular Plant-Microbe Interactions* **8**, 792–794.

Powers J-PS, Hancock REW, 2003. The relationship between peptide structure and antibacterial activity. *Peptides* **24**, 1681–1691.

Prados-Ligero AM, Gonzalez-Andujar JL, Melero-Vara JM, Basallote-Ureba MJ, 1998. Development of *Pleospora allii* on garlic debris infected by *Stemphylium vesicarium*. *European Journal of Plant Pathology* **104**, 861–870.

Prognosfruit, 2014. Prognosfruit. Online: http://www.prognosfruit.eu [Accessed: May , 2014]

Pryor S, Gibson D, Bergstrom G, Walker L, 2007. Minimization of between-well sample variance of antifungal activity using a high-throughput screening microplate bioassay. *BioTechniques* **42**, 168–172.

Puig M, Ruz L, Montesinos E, Moragrega C, Llorente I, 2015. Combined morphological and molecular approach for identification of *Stemphylium vesicarium* inoculum in pear orchards. *Fungal Biology*, **119**, 136-144.

R

Rajasekaran K, Cary JW, Chlan CA, Jaynes JM, Bhatnagar D, 2012. Strategies for controlling plant diseases and mycotoxin contamination using antimicrobial synthetic peptides. In: Rajasekaran, K. Cary, J.W. Jaynes, J.M. Montesinos E (eds). *Small Wonders: Peptides for Disease Control*. 295–315.

Reed JD, Edwards DL, Gonzalez CF, 1997. Synthetic peptide combinatorial libraries: a method for the identification of bioactive peptides against phytopathogenic fungi. *Molecular Plant-Microbe Interactions* **10**, 537–549.

Rioux D, Jacobi V, Simard M, Hamelin RC, 2000. Structural changes of spores of tree fungal pathogens after treatment with the designed antimicrobial peptide D2A21. *Canadian Journal of Botany* **78**, 462–471.

Rossi V, Bugiani R, Giosué S, Natali P, 2005a. Patterns of airborne conidia of *Stemphylium vesicarium*, the causal agent of brown spot disease of pears, in relation to weather conditions. *Aerobiologia* **21**, 203–216.

Rossi V, Pattori E, 2009. Inoculum reduction of Stemphylium *vesicarium*, the causal agent of brown spot of pear, through application of Trichoderma-based products. *Biological Control* **49**, 52–57.

Rossi V, Pattori E, Bugiani R, 2008. Sources and seasonal dynamics of inoculum for brown spot disease of pear. *European Journal of Plant Pathology* **121**, 147–159.

Rossi V, Pattori E, Giosue S, Bugiani R, 2005b. Growth and sporulation of *Stemphylium vesicarium*, the causal agent of brown spot of pear, on

herb plants of orchard lawns. *European Journal of Plant Pathology* **111**, 361–370.

Russell PE, 2005. A century of fungicide evolution. *The Journal of Agricultural Science* **143**, 11–25.

Rustagi A, Kumar D, Shekhar S, Yusuf MA, Misra S, Sarin NB, 2014. Transgenic *Brassica juncea* plants expressing MsrA1, a synthetic cationic antimicrobial peptide, exhibit resistance to fungal phytopathogens. *Molecular Biotechnology* **56**, 535–545.

Ruz L, Moragrega C, Puig M, Montesinos E, Llorente I, 2012. Characterization of *Stemphylium vesicarium* populations from Spanish pear orchards. In: In: *IOBC/WPRS Bulletin* **84**, 11–15.

Ruz L, Puig M, Moragrega C, Montesinos E, Llorente I, 2013. Control of brown spot of pear (*Stemphylium vesicarium*) with selected *Bacillus subtilis* strains. In: 5th Congress of European Microbiologists/ Federation of European Microbiological Societies.

S

Sato H, Feix JB, 2006. Peptide-membrane interactions and mechanisms of membrane destruction by amphipathic α-helical antimicrobial peptides. *Biochimica et Biophysica Acta* **1758**, 1245–1256.

Schaaper WMM, Posthuma GA, Meloen RH *et al.*, 2001. Synthetic peptides derived from the beta2-beta3 loop of *Raphanus sativus* antifungal protein 2 that mimic the active site. *Journal of Peptide Research* **57**, 409–418.

Sciarretta A, Trematerra P, 2011. Spatio-temporal distribution of *Ceratitis capitata* population in a heterogeneous landscape in central Italy. *Journal of Applied Entomology* **135**, 241–251.

Selitrennikoff CP, 2001. Antifungal proteins. *Applied and Environmental Microbiology* **67**, 2883–2894.

Shabi E, Rotem J, Loebenstein G, 1973. Physiological races of *Venturia pirina* on pear. *Phytopathology* **63**, 41-43

Shai Y, 2002. Mode of action of membrane active antimicrobial peptides. *Biopolymers* **66**, 236–248.

Shishkoff N, Lorbeer JW, 1989. Etiology of *Stemphylium* leaf blight of onion. *Phytopathology* **79**, 301–304.

Simmons EG, 1969. Perfect stages of *Stemphylium*. *Mycologia* **61**, 1–26.

Simmons EG, 1985. Perfect states of *Stemphylium*.II. *Mycologia1* **38**, 284–293.

Simmons EG, 2004. Novel dematiaceous hyphomycetes. *Studies in Mycology*, 109–18.

Singh P, Bugiani R, Cavanni P *et al*., 1999. Purification and biological characterization of host-specific SV-toxins from *Stemphylium vesicarium* causing brown spot of European pear. *Phytopathology* **89**, 947–953.

Singh P, Park P, Bugiani R *et al*., 2000. Effects of host-selective SV-toxin from *Stemphylium vesicarium*, the cause of brown spot of European pear plants, on ultrastructure of leaf cells. *Journal of Phytopathology* **148**, 87–93.

Smith IM, Dunez J, Phillips DH, Lelliott RA, Archer SA (eds), 1988. European handbook of plant diseases. Oxford, United Kingdom. Blackwell Scientific Publications.

Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG, 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**, 246–248.

T

Taube J, Vorkamp K, Förster M, Herrmann R, 2002. Pesticide residues in biological waste. *Chemosphere* **49**, 1357–1365.

Theis T, Stahl U, 2004. Antifungal proteins : targets , mechanisms and prospective applications. *Cellular and Molecular Life Sciences* **61**, 437–455.

V

Van't Hof W, Veerman EC, Helmerhorst EJ, Amerongen A V, 2001. Antimicrobial peptides: properties and applicability. *Biological Chemistry* **382**, 597–619.

Vannest JL, 2000 (ed). Fire blight: the disease and its causative agent, *Erwinia amylovora*. Wallingford, United Kingdom. CABI.

Vighi M, Funari E, 1995. *Pesticide risk in groundwater.* Lewis Publishers Inc, Chelsea, United Kingdom

Vila L, Lacadena V, Fontanet P, Martinez del Pozo a, San Segundo B, 2001. A protein from the mold *Aspergillus giganteus* is a potent inhibitor of fungal plant pathogens. *Molecular Plant-Microbe Interactions* **14**, 1327–1331.

Vila-Perelló M, Sánchez-Vallet A, García-Olmedo F, Molina A, Andreu D, 2003. Synthetic and structural studies on *Pyrularia pubera* thionin: a single-residue mutation enhances activity against Gram-negative bacteria. *FEBS letters* **536**, 215–219.

Vilardell P, 1988. *Stemphylium vesicarium* en plantaciones de peral. *Fruticultura Profesional* **18**, 51–55.

Villalta O, Washington WS, Rimmington GM, Taylor PA, 2000. Influence of spore dose and interrupted wet periods on the development of pear scab caused by *Venturia pirina* on pear (*Pyrus communis*) seedlings. *Australasian Plant Pathology* **29**, 255–262.

W

Wang Z, 2003. The Antimicrobial Peptide Database. Online: http://aps.unmc.edu/AP/ main.php [Accessed: June , 2014]

Wang G, Li X, Wang Z, 2009. APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic Acids Research* **37**, (Database issue):D933-7. doi: 10.1093/nar/gkn823.

Wang G, Li X, Zasloff M, 2010. A database view of naturally occurring antimicrobial peptides: nomenclature, classification and amino acid sequence analysis. In: Wang G, (ed). *Antimicrobial peptides: discovery, design and novel therapeutic strategies*. Oxfordshire, United Kingdom. CABI, 1–21.

van der Weerden NL, Bleackley MR, Anderson MA, 2013. Properties and mechanisms of action of naturally occurring antifungal peptides. *Cellular and Molecular Life sciences* **70**, 3545–3570.

World Association Apple and Pear, 2014. WAPA. Online: http://www.wapa-association.org [Accessed: May , 2014]

Y

Yang C, Hamel C, Vujanovic V, Gan Y, 2011. Fungicide: Modes of Action and Possible Impact on Nontarget Microorganisms. *ISRN Ecology* **2011**, 1–8.

Yeaman MR, Yount NY, 2003. Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews* **55**, 27–55.

Z

Zakharchenko NS, Rukavtsova EB, Gudkov AT, Buryanov YI, 2005. Enhanced resistance to phytopathogenic bacteria in transgenic tobacco plants with synthetic gene of antimicrobial peptide cecropin P1. *Russian Journal of Genetics* **41**, 1187–1193.

Zasloff M, 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389–395.

Zasloff M, 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 5449–5453.

Zhang J, Wu X, Zhang S-Q, 2008. Antifungal mechanism of antibacterial peptide, ABP-CM4, from *Bombyx mori* against *Aspergillus niger*. *Biotechnology Letters* **30**, 2157–2163.

Zottich U, Da Cunha M, Carvalho AO *et al.*, 2013. An antifungal peptide from *Coffea canephora* seeds with sequence homology to glycine-rich proteins exerts membrane permeabilization and nuclear localization in fungi. *Biochimica et biophysica acta* **1830**, 3509–3516.