CHARACTERIZATION AND IMPROVEMENT OF PLANT-ASSOCIATED LACTOBACILLUS PLANTARUM. NOVEL BIOCONTROL AGENT FOR FIRE BLIGHT DISEASE

Gemma Roselló Prados

Universitat de Girona

PhD Thesis

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Programa de Doctorat:

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Dirigida per:

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Memòria presentada per optar al títol de doctora per la Universitat de Girona

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DECLAREM:

Que el treball titulat "Characterization and improvement of plant-associated *Lactobacillus plantarum*. Novel biocontrol agents for fire blight disease", que presenta Gemma Roselló Prados per 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, signen aquest document a Girona, el 5 d'abril de 2016.

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Anna Bonaterra Carreras, professora Titular de l'Àrea de Producció Vegetal de la Universitat de Girona, i directora dels projectes de recerca "Control biotecnológico del fuego bacteriano. Utilización de cepas bacterianas Gram-positivas productoras de bacteriocinas y ciclolipopéptidos" (Ref. AGL2009-13255-C02-01/AGR) i "Nuevas estrategias de control del fuego bacteriano. Bacterias del ácido láctico productoras de bacteriocinas (BALFUEBA)" (Ref. AGL2012-39880-C02-01) de la Comisión Interministerial de Ciencia y Tecnología, en els que es circumscriu la tesi doctoral titulada "Characterization and improvement of plant-associated *Lactobacillus plantarum*. Novel biocontrol agents for fire blight disease" realitzada per **Gemma Roselló Prados**.

DECLARA QUE:

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

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Dra. Anna Bonaterra Carreras

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Anònim

List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMP	Antimicrobial Peptide
AU	Arbitrary Units
BCA	Biological Control Agent
C	Cytosine
CC	Clonal Complex
CFU	Colony-Forming Unit
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Agency
ELISA	Enzyme-linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
FDA	US Food and Drug Administration
G	Guanine
GRAS	Generally Recognized As Safe
h	hour
kb	kilobase
kDa	kilo Dalton
LAB	Lactic Acid Bacteria
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of Flight
mes	mesentericin
ml	millilitre
MLST	Multilocus Sequence Typing
MRS	Man, Rogosa and Sharpe medium
MST	Minimum Spanning Tree
nis	nisin
orf	open reading frame
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction

PFGE	Pulsed-Field Gel Electrophoresis
pln	plantaricin
QPS	Qualified Presumption As Safe
RAPD-PCR	Random Amplified Polymorphic DNA-PCR
RD	Royal Decree
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RH	Relative Humidity
RT-PCR	Reverse Transcription PCR
SAR	Systemic Acquired Resistance
ST	Sequence Type

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List of Publications

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

Roselló G., Bonaterra A., Francés J., Montesinos L., Badosa E. and Montesinos E. (2013). Biological control of fire blight of apple and pear with antagonistic *Lactobacillus plantarum*. *European Journal of Plant Pathology*, 137:621-633.

European Journal of Plant Pathology has an impact factor of 1.707 and it is in the first quartile (Q1) in the "Horticulture" category (2013 Journal Citation Reports Science Edition, published by Thompson Reuters).

Roselló G., Daranas N., Badosa E., Trias R., Francés J., Montesinos E. and Bonaterra A. (2015). Diversity of plant-associated *Lactobacillus plantarum* and antagonistic potential against plant pathogenic bacteria. *Applied and Environmental Microbiology*. Submitted.

Applied and Environmental Microbiology has an impact factor of 3.668 and it is in the first quartile (Q1) in the "Biotechnology and Applied Microbiology" and "Microbiology" categories (2014 Journal Citation Reports Science Edition, published by Thompson Reuters).

Roselló G., Francés J., Daranas N., Montesinos E. and Bonaterra A. (2015) Control of fire blight with mixed inocula of two *Lactobacillus plantarum* strains and lactic acid. *Phytopathology*. Submitted.

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

A Patent derived from this work:

Montesinos, E., Bonaterra, A., Francés, J.M., Badosa, E., Montesinos, L., Roselló, G. "Cepa de *Lactobacillus plantarum* para el control del fuego bacteriano". Spain Patent P2013300685. May 29, 2013.

Summary

Fire blight is a disease caused by *Erwinia amylovora*, considered a harmful quarantine organism in the European Union (EU), which affects plants of the rosaceous family, including several fruit trees and ornamental species of great economic interest. In most EU countries, the control of fire blight is done exclusively with copper products because antibiotics are not allowed. In addition, the effectiveness of these is limited due to the emergence of resistance. For this reason and together with the new regulations for the sustainable use of pesticides, biological control is considered a good alternative for the control of fire blight. Currently, there are available biocontrol agents (BCA) for this disease, either commercial or experimental, but the efficacy lack consistency. Therefore, it is still necessary to search new species or strains of biocontrol agents.

Lactic acid bacteria (LAB) could be good candidates for biocontrol agents of this disease. These bacteria have been traditionally used as biopreservatives in food and also have shown activity against phytopathogen bacteria. In addition, LAB are considered as safe for food safety agencies.

Aiming to find LAB strains as BCA able to inhibit *E. amylovora* infections in detached plant organs, the ability of 100 LAB isolates selected in a former study has been determined in detached pear organs (flowers, leaves and immature fruits). This screening procedure was a good strategy to select candidates of BCA. Eight strains (AC73, CM209, FC560, PC40, PM366, PM411, TC54 and TC92) have been selected because they exhibited inhibition of the infections in most of the performed experiments in the different detached plant organs. These strains were identified, their spectrum of antagonistic activity has been characterized and the presence of bacteriocin biosynthetic genes has determined. Also studies of colonization and efficacy trials in pear-potted plants and in mixed field-lab assays have been made. Three out of eight selected strains, which were identified as *Lactobacillus plantarum* TC54, TC92 and PM411, were considered excellent BCA candidates due to their effectiveness in fire blight biocontrol in various experiments, both in detached plant organs and in pear-potted plants, as well as in mixed field-lab blossom assays. Besides, they efficiently colonized and survived in pear and apple flowers under different conditions of relative humidity. They also showed a

broad spectrum of antibacterial activity against plant pathogenic bacteria and the presence of plantaricin biosynthethic genes.

The selection of *L. plantarum* strains as BCA candidates confirmed the potential of certain strains of this species to be used as active ingredients of microbial pesticides for fire blight control. Therefore, with the aim to find leader strains that could be used as BCA, all *L. plantarum* strains present in our LAB collection have been identified and characterized.
A total of 45 isolates have been genotypically characterized by means of Multilocus Sequence Typing (MLST) and Random Amplified Polymorphic DNA (RAPD-PCR), and were analyzed for the presence of plantaricin biosynthetic genes. A low heterogeneity in plant-associated *L. plantarum* strains has been observed. In MLST analysis, the strains presented eight different sequence types (ST), but the majority of strains shared the same ST. The population structure of the *L. plantarum* strains of this study with other strains described in other studies has been analysed by means of a minimum spanning tree (MST). The study suggested that there was a clonal structure. In the analysis of the plantaricin genes, most of the strains presented the same gene profile. Interestingly, a relationship between the genotypic profile obtained by MLST and RAPD and the source of isolates has been described, separating isolates from raw vegetables from those obtained from fruits.
The phenotypic characterization was performed according to the antimicrobial activity against different phytopathogenic bacteria and more specifically in the case of *E. amylovora* by the inhibition of infections in detached plant organs. Isolates showed a high heterogeneity in the antagonistic potential against bacteria, with a group of 15 isolates that exhibited a wide range and strong activity of *in vitro* antagonism. Moreover, this heterogeneity has been confirmed by the levels of inhibition of infections caused by *E. amylovora*. A relationship between the antagonistic activity and the genotypic profiles (MLST, RAPD and plantaricin genes) has not been found. Using a multivariate statistical analysis combining the phenotypic and genotypic properties, it was determined that strains TC92 and PM411 were candidates to be used as active ingredients in future formulations of biopesticides against fire blight.

In previous efficacy assays of fire blight biocontrol, these two strains showed variable efficacy. Therefore, it was necessary to increase the biocontrol

efficacy and get more consistent results throughout the experiments. To achieve this objective, we used a mixture of the two strains (TC92 and PM411) combined with lactic acid, because it was observed that lactic acid inhibited *E. amylovora* and did not affect *L. plantarum* strains. The combination of lactic acid and strains TC92 and PM411 increased efficacy and reliability in the biocontrol of fire blight in all experiments performed. This strategy would be appropriate for the future development of new formulations of lactic acid bacteria as biopesticides for integrated management of fire blight.

Resum

El foc bacterià és una malaltia causada pel bacteri *Erwinia amylovora*, considerat un microorganisme nociu de quarantena en la Unió Europea (UE), que afecta a plantes de la família de les rosàcies, en la que s'inclouen arbres fruiters i varies espècies ornamentals de gran interès econòmic. En la majoria dels països de la UE, el control del foc bacterià es realitza exclusivament amb productes cúprics, ja que els antibiòtics no estan permesos. A més, l'eficàcia d'aquests està limitada degut a l'aparició de resistències. Per aquesta raó i unit a les noves normatives d'ús sostenible dels productes fitosanitaris, el control biològic es considera una bona alternativa pel control del foc bacterià. Actualment hi ha bioplaguicides disponibles per aquesta malaltia basats en agents de biocontrol (ABC), ja siguin comercials o experimentals, però d'eficàcia moderada i poc consistents. Per tant, segueix sent necessària la recerca de noves espècies o soques d'agents de biocontrol.

Els bacteris de l'àcid làctic (BAL) podrien ser uns bons candidats a agents de biocontrol d'aquesta malaltia. Aquests bacteris han estat utilitzats tradicionalment com a bioconservants en aliments i també s'ha demostrat la seva activitat front a bacteris fitopatògens. A més, els BAL estan qualificats com a segurs per les agències de seguretat alimentària.

Amb l'objectiu de cercar soques de BAL capaces d'inhibir les infeccions causades per *E. amylovora*, es va determinar aquesta capacitat en els 100 aïllats escollits en estudis previs en diferents òrgans vegetals (flors, fulles i fruits immadurs). Aquest tipus d'assaigs van ser una bona estratègia per seleccionar els candidats a ABC. Es van seleccionar vuit soques (AC73, CM209, FC560, PC40, PM366, PM411, TC54 i TC92) ja que presentaven inhibició de les infeccions en la majoria dels assajos realitzats en els diferents òrgans vegetals. Aquestes soques van ser identificades, es va caracteritzar el seu espectre d'activitat antagonista i es va determinar la presència de gens biosintètics de bacteriocines. També es van realitzar estudis de colonització i assajos d'eficàcia en pereres en contenidor i en assajos mixtes de camp-laboratori en corimbes florals. Tres de les vuit soques seleccionades, les quals van ser identificades com a *Lactobacillus plantarum* TC54, TC92 i PM411, van ser considerades excel·lents candidats a ABC tenint en compte la seva eficàcia en el control de les infeccions del foc bacterià en els diferents assajos realitzats, tant en diferents òrgans vegetals com en planta en contenidor i en

corimbes florals. A més, van colonitzar i sobreviure eficientment en flors de pomera i perera en diferents condicions d'humitat relativa. També van presentar un elevat i ampli espectre d'activitat antagonista i els gens de síntesi de plantaricines.

La selecció de soques de *L. plantarum* com a candidates a ABC va confirmar el seu potencial com a ingredients actius de plaguicides microbians pel control del foc bacterià. Amb l'objectiu de cercar soques líders que poguessin ser utilitzades com a ABC, es van identificar i caracteritzar totes les soques de l'espècie *L. plantarum* presents en la nostra col·lecció de BALs. En total es van caracteritzar genotípicament 45 aïllats mitjançant "Multilocus Sequence Typing" (MLST) i "Random Amplified Polymorphic DNA" (RAPD) i se'ls va determinar la presència dels gens biosintètics de plantaricines. Es va observar que els *L. plantarum* aïllats de productes vegetals presentaven una baixa heterogeneïtat genotípica. En el MLST, els aïllats van presentar 8 seqüències tipus diferents (ST) però la majoria presentaven la mateixa. Mitjançant un anàlisi "Minimum Spanning Tree" (MST) dels ST obtinguts en el MLST, es va estudiar el mode d'evolució dels *L. plantarum* d'aquesta tesi juntament amb els descrits en altres estudis suggerint que aquest seria clonal. En l'anàlisi dels perfils RAPD, la majoria dels aïllats es van agrupar en tres grups. En l'estudi de la presència dels gens de plantaricines, la majoria de les soques van presentar el mateix perfil. Es va observar una relació entre els perfils genotípics obtinguts per MLST i RAPD, i l'origen dels aïllats, que va permetre separar els aïllats que provenien d'hortalisses dels de fruites. La caracterització fenotípica va consistir en determinar l'activitat antimicrobiana d'aquests aïllats front a diferents bacteris fitopatògens i més específicament, en el cas d'*E. amylovora*, en la inhibició de les seves infeccions causades en diferents òrgans de perera. Els aïllats van presentar una elevada heterogeneïtat en el potencial antagonista, amb un grup de 15 soques que exhibien un ampli i elevat espectre d'antagonisme *in vitro*. A més, aquesta heterogeneïtat va ser confirmada en els nivells d'inhibició de les infeccions causades per *E. amylovora*. No es va observar cap relació entre l'activitat antagonista i els perfils genotípics (MLST, RAPDs i gens de plantaricines). Mitjançant un anàlisi estadístic multivariant, combinant les propietats genotípiques i fenotípiques estudiades, es va determinar que les soques TC92 i PM411 eren candidates a ser utilitzades com a ingredients actius en futures formulacions de bioplaguicides contra el foc bacterià.

Els resultats obtinguts en estudis previs d'eficàcia d'aquestes dues soques en el biocontrol del foc bacterià, mostraven una eficàcia variable. Per tant, es va plantejar una estratègia per millorar aquesta eficàcia i obtenir uns resultats més consistents al llarg dels diferents experiments. Per aconseguir-ho, es va utilitzar la barreja de les dues soques seleccionades (TC92 i PM411) combinada amb l'àcid làctic. L'àcid làctic va inhibir *E. amylovora* tant *in vitro* com en plantes en contenidor sense afectar a les soques de *L. plantarum*. La combinació de l'àcid làctic i de les soques TC92 i PM411 va incrementar l'eficàcia i la fiabilitat en el control del foc bacterià en tots els assajos realitzats. Aquesta estratègia seria l'adequada pel futur desenvolupament de noves formulacions dels bacteris de l'àcid làctic com a bioplaguicides pel maneig integrat del foc bacterià.

Resumen

El fuego bacteriano es una enfermedad causada por la bacteria *Erwinia amylovora*, considerada un microorganismo nocivo de cuarentena en la Unión Europea (UE), que afecta a plantas de la familia de las rosáceas, en la que se incluyen árboles frutales y varias especies ornamentales de gran interés económico. En la mayoría de los países de la UE, el control del fuego bacteriano se realiza exclusivamente con productos cúpricos, ya que los antibióticos no están permitidos. Además, la eficacia de estos está limitada debido a la aparición de resistencias. Por esta razón y unido a las nuevas normativas de uso sostenible de productos fitosanitarios, el control biológico se considera una buena alternativa para el control del fuego bacteriano. Actualmente hay bioplaguicidas disponibles para esta enfermedad basados en agentes de biocontrol (ABC), ya sea comerciales o experimentales, pero de eficacia moderada y poco consistentes. Por lo tanto, sigue siendo necesario la búsqueda de nuevas especies o cepas de agentes de biocontrol.

Las bacterias del ácido láctico (BAL) podrían ser unas buenas candidatas a agentes de biocontrol de esta enfermedad. Estas bacterias han sido utilizadas tradicionalmente como bioconservantes en alimentos y también se ha demostrado su actividad frente a bacterias fitopatógenas. Además, las BAL están calificadas como seguras por las agencias de seguridad alimentaria.

Con el objetivo de buscar cepas de BAL capaces de inhibir las infecciones causadas por *E. amylovora,* se determinó esta capacidad en los 100 aislados escogidos en estudios previos en diferentes órganos vegetales (flores, hojas y frutos inmaduros). Este tipo de ensayos fueron una buena estrategia para seleccionar los candidatos a ABC. Se seleccionaron ocho cepas (AC73, CM209, FC560, PC40, PM366, PM411, TC54 y TC92) ya que presentaban inhibición de las infecciones en la mayoría de los ensayos realizados en los distintos órganos vegetales. Estas cepas fueron identificadas, se caracterizó su espectro de actividad antagonista y se determinó la presencia de genes biosintéticos de bacteriocinas. También se realizaron estudios de colonización y ensayos de eficacia en perales en contenedor y en corimbos florales en ensayos mixtos de campo-laboratorio. Tres de las ocho cepas seleccionadas que fueron identificadas como *Lactobacillus plantarum* TC54, TC92 y PM411, se consideraron excelentes candidatas a ABC por su eficacia en el control de las infecciones del fuego bacteriano en los distintos ensayos realizados, tanto en

distintos órganos vegetales como en planta y en corimbos florales en ensayos mixtos de campo-laboratorio. Además, estas cepas colonizaron y sobrevivieron eficientemente en flores de peral y manzano en distintas condiciones de humedad relativa. También presentaron un amplio espectro de actividad antibacteriana y los genes de síntesis de las plantaricinas.

La selección de las cepas de *L. plantarum* como candidatas a ABC confirmó su potencial para ser utilizadas como ingredientes activos de plaguicidas microbianos para el control del fuego bacteriano. Con el objetivo de buscar cepas líderes que puedan ser utilizadas como ABC, se identificaron y caracterizaron todas las cepas de la especie *L. plantarum* presentes en nuestra colección de BALs. En total se caracterizaron genotípicamente 45 aislados mediante "Multilocus Sequence Typing" (MLST) y "Random Amplified Polymorphic DNA" (RAPD) y se les determinó la presencia de los genes biosintéticos de plantaricinas. Se observó que los *L. plantarum* aislados de productos vegetales presentaban una baja heterogeneidad genotípica. En el MLST, los aislados presentaron 8 secuencias tipo (ST) diferentes pero la mayoría presentaban la misma. Mediante un análisis "Minimum Spanning Tree" (MST) de los ST obtenidos en el MLST, se estudió el modo de evolución de los *L. plantarum* de esta tesis juntamente a los descritos en otros estudios sugiriendo que éste sería clonal. En el análisis de perfiles RAPD, la mayoría de los aislados se agruparon en tres grupos. En el estudio de la presencia de los genes de plantaricinas, la mayoría de las cepas presentaron el mismo perfil. Se observó una relación entre los perfiles genotípicos obtenidos por MLST y RAPD, y el origen de los aislados, que permitieron separar los aislados que provenían de hortalizas de los de frutas. La caracterización fenotípica consistió en determinar la actividad antimicrobiana de estos aislados frente a diferentes bacterias fitopatógenas y más específicamente, en el caso de *E. amylovora*, en la inhibición de sus infecciones causadas en distintos órganos de peral. Los aislados presentaron una elevada heterogeneidad en el potencial antagonista, con un grupo de 15 cepas que exhibían un amplio y elevado espectro de antagonismo *in vitro*. Además, esta heterogeneidad fue confirmada en los niveles de inhibición de las infecciones causadas por *E. amylovora*. No se observó ninguna relación entre la actividad antagonista y los perfiles genotípicos (MLST, RAPDs y genes de plantaricinas). Mediante un análisis estadístico multivariante combinando las propiedades genotípicas y fenotípicas estudiadas, se determinó que las cepas TC92 y PM411 eran candidatas a ser

utilizadas como ingredientes activos en futuras formulaciones de bioplaguicidas contra el fuego bacteriano.

Los resultados obtenidos en estudios previos de eficacia de estas dos cepas en el biocontrol del fuego bacteriano, mostraron una eficacia variable y en algunos casos baja. Por lo tanto, se estudió desarrollar una estrategia para mejorar esta eficacia y obtener unos resultados más consistentes a lo largo de los distintos ensayos. Para lograrlo, se utilizó la mezcla de las dos cepas seleccionadas (TC92 y PM411) combinada con ácido láctico. El ácido láctico inhibió a *E. amylovora* tanto *in vitro* como en plantas en contenedor y no afectó a las cepas *L. plantarum*. La combinación de ácido láctico y de las cepas TC92 y PM411 incrementó la eficacia y la fiabilidad en el control del fuego bacteriano en todos los ensayos realizados. Esta estrategia seria la adecuada para el futuro desarrollo de nuevas formulaciones de las bacterias del ácido láctico como bioplaguicidas para el manejo integrado del fuego bacteriano.

Chapter I:

General Introduction

1. Fire blight disease

Fire blight is one of the most serious bacterial diseases of the pomaceous fruit trees in the world which has caused important economic losses. The causative agent is *Erwinia amylovora*, a quarantine bacteria in the European Union (EU). This bacteria affects mostly plants belonging to the Maloideae subfamily (e.g. apple, pear, cotoneaster, hawthorn, quince and loquat), including also other ornamental species members of the *Rosaceae* family (Malnoy *et al.*, 2012).
The impact of fire blight on production is highly variable, depending mostly on climatic conditions during spring. Whereas, when there is a severe outbreak, it can disrupt orchard production for several years (Vanneste, 2000).
This disease is highly contagious and difficult to control, causing the progressive death of the sensitive varieties of trees, not only affecting the production, but modifying the variety structure and orchard management and increasing the production costs (Palacio-Bielsa & Cambra, 2009). Fire blight is best controlled using an integrated approach that combines horticultural practices designed to minimize tree susceptibility and disease spread, and sprays of bactericides to protect against infection. However, the non-authorization of use of antibiotics in agriculture in the EU (only as exceptional authorization), and the limited efficacy of alternative control agents provide a chance to develop novel pesticides.

1.1. History and geographical distribution

Fire blight was described for the first time in 1780 in Hudson Valley of New York State (USA) and was spread to Canada and the rest of United States, both Atlantic and Pacific zones. In 1919, this disease was detected in New Zealand and in Europe, in South England, in 1957. In 1964, it was identified in Egypt and two years later, in Netherlands. Since then, it has not stopped to spread and nowadays it is affecting the majority of countries of the Northern and Central Europe, the Mediterranean area and Middle East (Figure 1).

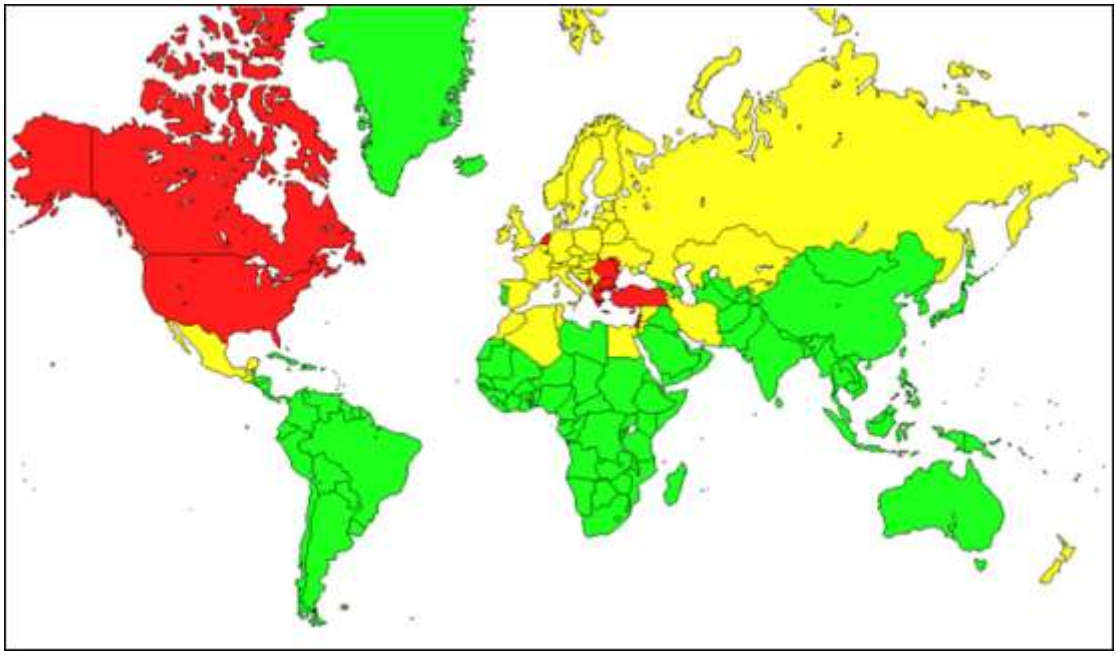

Figure 1. Fire blight world distribution based on data reviewed by EPPO (2015). Countries where fire blight is widely distributed, where is partially distributed and where has still not been described.

In Spain, fire blight was first detected in 1995 in Guipuzkoa (Euskadi), close to the Atlantic French border (Butrón, 1995). Next, other single outbreaks have been detected and eradicated in different regions of Spain, for example: Navarra (1996), Castilla y León (Segovia, 1996), Castilla-La Mancha (Guadalajara, 1998), Catalunya (Lleida, 1998), Aragón (1998), La Rioja (2002) (Donat *et al.*, 2005). Nevertheless, Spain was considered a protected zone against fire blight because the pathogen was absent and the outbreaks were eradicated. However, since 2011, some regions (Castilla y León, Extremadura, La Rioja, Navarra, Castilla-La Mancha and Madrid) or part of them (Catalunya, Comunitat Valenciana and Euskadi) have lost this consideration because the disease is established (MAGRAMA, 2015) (Figure 2).

Figure 2. Distribution of protected regions against fire blight in Spain. Regions where are protected zone and where have lost this consideration (From MAGRAMA, 2015).

1.2. Symptomatology and diagnosis

The name "fire blight" is descriptive of the most typical symptoms of disease: a blackening of shoots, flowers and foliage as thought they had been swept by fire. Depending on the plant part affected many names such as blossom, shoot, trunk and rootstock blight are frequently used (van der Zwet & Keil, 1979).

The first symptoms of fire blight usually appear in spring, during bloom and bud break because the most susceptible organs are flowers and young shoots. In the early stages of infection, blossoms appear water-soaked, then wilt, turn brown to black and die. Generally, the entire cluster becomes blighted and killed. Infected blossoms may fall or remain attached to the tree, being useful symptom in detecting blighted trees from distance (Figure 3.A). Although, the most obvious symptom of the disease is the shoot blight phase, which first appears one to several weeks after the petal fall. In young shoots, the first symptoms are the presence of drops of sticky bacterial ooze on the surface of

shoots (Figure 3.B), whereas old infections are characterised by wilted and necrotized tissues in the leaves and stem on young, succulent shoot tips that turn brown or black. The infected growing shoots often exhibit a typical curling at the end, called a shepherd's crook (Figure 3.C). Blighted leaves remain attached to the tree (Figure 3.D). Fruits may appear dark, dried and shrivelled if infected when they are young, or show red, brown or black lesions when infected later. Infected fruits often present droplets of ooze and usually remain attached to the tree, taking on a mummified appearance. Ooze consists of bacterial cells embedded in a polysaccharide matrix, this viscous substance protects cells from desiccation and other abiotic stress factors and is attractive to insects, such as flies, that can disseminate bacterial cells to flowers (Malnoy *et al.*, 2012). Bacterial cankers can be formed when the infection progresses into the woody tissue and are the sites where the bacteria overwinter (Figure 3.E). Active fire blight holdover cankers have a dark, water-soaked appearance. Rootstock o collar blight is an important phase of fire blight, but only when susceptible trees are grown on highly susceptible rootstock (van der Zwet & Beer, 1995). The pathogen can migrate internally downward from shoots or directly infect rootstock wounds. In rootstock blight, symptoms are similar that in shoot blight, being able to observe the same types of cankers.

The fire blight symptoms are similar in all host species, but the most spectacular symptoms are observed in pear trees. Infected pear trees can dead acquiring the typical aspect of burned trees that gives the name to the disease. In apple trees, loquat, *Crataegus*, *Cotoneaster*, *Pyracantha* and other wild or ornamental rosaceous plants, the colouring of the infected plant tissues is red to dark brown (Palacio-Bielsa & Cambra, 2009).

The symptoms of fire blight can be confused with those of other diseases caused by bacteria and fungi, by insect attacks or physiological alterations (Palacio-Bielsa & Cambra, 2009). Thus, the diagnosis of the disease is difficult and a laboratory analysis is required. The identification procedure for *E. amylovora* in plant material samples usually includes combination of microbiological (semi-selective media, nutritional and enzymatic tests), immunological (ELISA) and PCR approaches (López *et al.*, 2009). The detection of *E. amylovora* by means of highly sensitive and reliable methods

provides new tools to study transmission pathways and the biological cycle of the disease.

Figure 3. Fire blight symptoms. (A) Blossom infection. (B) Drops of exudates on shoot. (C) Infected growing shoot showing the shepherd's crook symptom. (D) Shoot blight. (E) Bacterial canker in woody tissue (Photos of J. Francés).

1.3. Disease cycle

The development of fire blight is associated with the seasonal development of the host plant and hence appears cyclic in nature (van der Zwet & Beer, 1995). *E. amylovora* remains during winter in a small number of the annual holdover cankers that were formed on diseased branches in the previous season (Figure 4). In spring, with the warm temperatures, the pathogen becomes active in

the margins of holdover cankers. The bacterial cells are released onto the bark surface, sometimes as visible ooze. This inoculum, considered as primary inoculum, is disseminated by insects or wind driven rain from the canker to blossoms (Johnson, 2000).

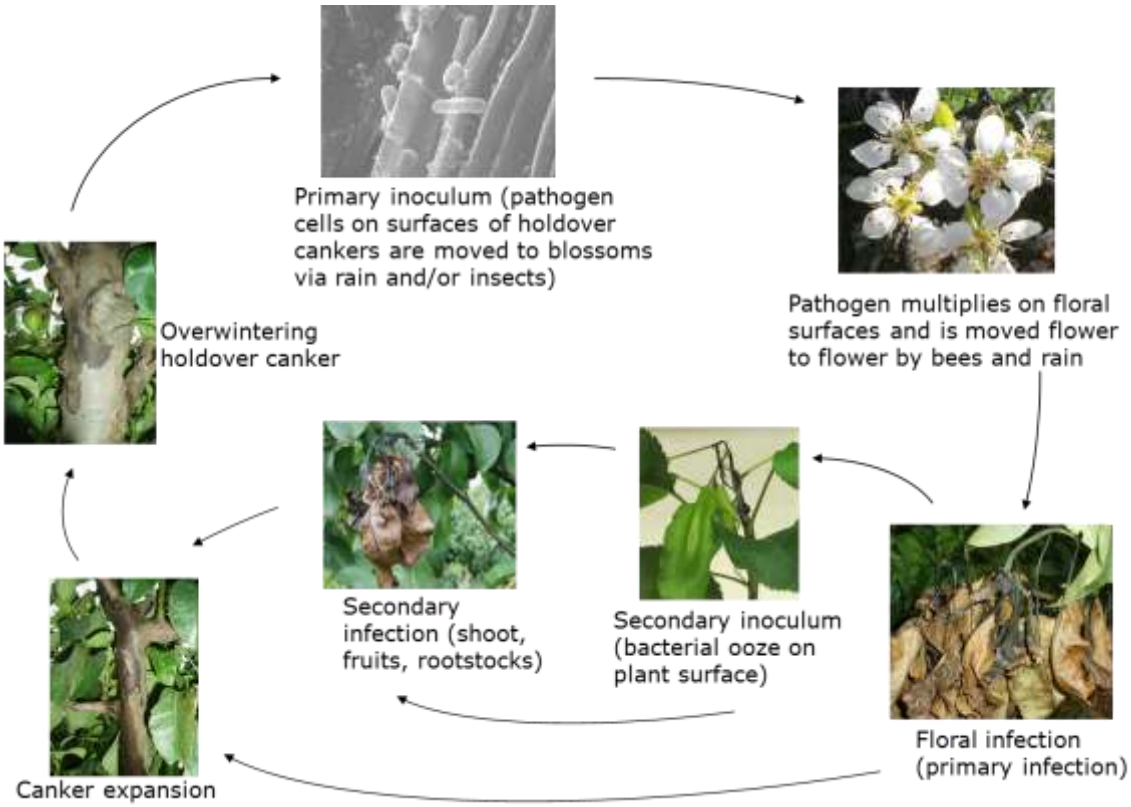

Figure 4. Disease cycle of fire blight (Modified from Johnson, 2000).

Once on the blossoms, *E. amylovora* has the ability to multiply very rapidly in an epiphytic phase on floral surface, including stigmas, anthers and the hypanthium (Johnson & Stockwell, 1998). Dissemination of bacteria among flowers of different plants can be caused by wind and rain, but is most likely influenced by the action of pollinating insects, such as bees (Malnoy *et al.*, 2012). Blossom blight (primary infection) is initiated when the pathogen enters into the host plant through nectarthodes, which are the main entrance pathway (endophytic phase). Stigmas, anthers and stomata are other natural opening also used by *E. amylovora* to infect plant. Secondary infections include shoot, fruit and rootstock blight. These phases are initiated by the inoculum

(secondary inoculum) produced on diseases tissues as a result of primary infection. The pathogen needs openings on plant surface to produce shoot and fruit blight. Even though there are natural entries, is more commonly the entrance through wounds, which are caused by sucking insects, wind whipping, hail or pruning. Once *E. amylovora* penetrates the plant, multiplies into intercellular spaces and moves rapidly through the cortical parenchyma, the phloem and the xylem vessels, infecting shoots (Thomson, 2000). Both primary and secondary infections can expand throughout the summer, if the environmental conditions, temperature and humidity are favourable. Although secondary infections are usually more numerous than primary ones and generally cause more serious injury to the trees (van der Zwet & Beer, 1995). Toward the end of the growing season, multiplication of bacteria decreases and cankers can appear in trunk and branches, which are the bacterial reservoir during winter.

1.4. The pathogen: *Erwinia amylovora*

Erwinia amylovora is a Gram negative plant pathogenic bacterium, belonging to Proteobacteria division, γ subdivision and the family of *Enterobacteriaceae*. It is rod shaped (0.3 x 1-3 μm) and has an exopolysaccharide capsule.

E. amylovora is facultative anaerobe and produces mainly ethanol and carbon dioxide, with small amounts of lactic acid and acetic acid, formic acid, succinic acid, acetoin and 2,3-butanediol from glucose fermentative metabolism. Besides, this bacteria uses citrate, lactate and formate, but not tartrate, galacturonate nor malonate as a carbon source (Paulin, 2000).

This pathogen is motile by peritrichous flagella and its motility has been shown to be associated with a specific chemotaxis, which is temperature- and pH-dependent, being the optimal conditions 20ºC and pH=6.8 (Raymundo & Ries, 1980a,b; Paulin, 2000).

The nicotinic acid is required for *E. amylovora* growth. This requirement is not common among the genus *Erwinia*, and it has been included in the biochemical profile test for *E. amylovora* characterization (Starr & Mandel, 1950; Holt *et al.*, 1994; Paulin, 2000)

Although *E. amylovora* is capable to growth between 3-5ºC and 37ºC, the optimal temperature is 25-37ºC (Billing et al., 1961; Paulin, 2000).

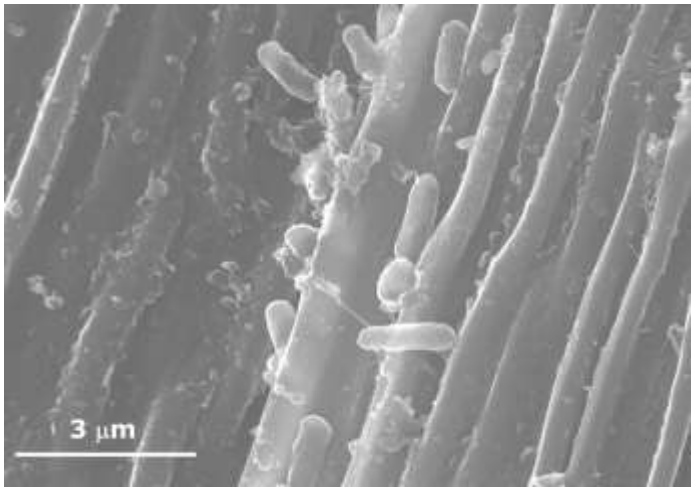

Figure 5. Scanning electron micrographs of Conference pear leaf inoculated with *E. amylovora.*

E. amylovora can be present as an epiphyte, on the surface of various host tissues, for example on pear leaves surface (Figure 5) and/or as an endophyte, inside the vascular system of the plant (van der Zwet *et al.*, 1988).

1.5. Fire blight management

Fire blight is a disease difficult to manage because the control methods available are in general of low efficacy, therefore a preventive strategy is the best solution. An effective management of fire blight should reduce the amount of inoculum that is available to initiate new infections, should impose barriers to successful establishment of the pathogen on the host and should reduce host susceptibility to infection (Norelli *et al.*, 2003). For these reasons, an integrated management of fire blight is required, in which the use of different strategies and tactics achieve to control the disease. These strategies include from regulatory and agronomic measures or resistant plant selection to different control methods.

1.5.1. Regulatory measures

Fire blight is considered a quarantine disease in European Union, so it was included in the Council Directive 2000/29/EC (OJ L, 2000), related to the protective measures against the introduction of harmful organisms to the plants or the crop products into the Community and against their spread. For this reason, a phytosanitary passport was regulated by the Commission Directive 2005/17/EC (OJ L, 2005), to assure that plants comply with regulations concerning quarantine organisms and which allows them to be transported freely in the EU. Furthermore, specific regions within the EU have been designated as protected zone against certain pests and diseases (EFSA Panel on Plant Health, 2014).

In Spain, the measures to control the fire blight were regulated by the Royal Decree (RD) 58/2005 (BOE, 2005) about preventive measures against the introduction and the dissemination of the disease (Cambra & Díaz, 2009). As well as, the establishment of the National Program of Eradication and Fire Blight Control of the Rosaceae, regulated by RD 1201/1999 (BOE, 1999), amended by RD 1786/2011 (BOE, 2012; MAGRAMA, 2015).

1.5.2. Agronomic measures

Agronomic measures try to reduce the infection risk, the incidence and the severity of infections and control the disease spreading. These measures will be a complement of the other strategies.

First, the orchards should be located in rich and well-drained soils without overhead irrigation systems. Then, the practises that promote the early cessation of growth without an excessive reduction of tree vigour are also carried out, such as applying nitrogen fertilizer early without exceeding and cultivating no later than in midsummer. Also, it is important to prevent secondary flowering (Steiner, 2000; EFSA Panel on Plant Health, 2014).

Another important practise is pruning of trees during dormancy to remove infected tissue and the whole tree, when it is necessary. Also, the disinfection of the tools and the removing from the orchard and the destruction of pruned plant material is necessary (Steiner, 2000). Also, the wild hosts in the vicinity of the orchard should be removed (van Teyligen, 2002).

1.5.3. Resistant/tolerant varieties

The use of fire blight-resistant cultivars could be one of the most effective methods available to manage the disease. Unfortunately, there is not plant material completely resistant to fire blight and the most varieties currently available still have moderate to low susceptibility to fire blight (EFSA Panel on Plant Health, 2014). Pear cultivars are generally more susceptible than table apples. For example, the more resistant varieties of apple cultivars are "Golden Delicious", "Royal Gala", "Reineta Blanca" or "Red Chief", while resistant pear cultivars are "Harrow", "Ercolini (Coscia)" or "Rome" (EFSA Panel on Plant Health, 2014).

Nevertheless, the introduction of disease resistance genes through conventional breeding methods is very difficult and costly, due to the apple and pear's heterozygosis, long generation time and self-incompatibility. The use of genetic engineering can overcome these obstacles by introducing resistance genes directly into commercial cultivars without losing desirable characteristics (Norelli *et al.*, 2003). The genes more extensively used and studied in apple and pear code mainly for antimicrobial compounds, inhibition of bacterial pathogenicity factors, and silencing or overexpression of related defence genes of the plant (Malnoy *et al.*, 2012).

Although, cultivars of apple and pear with significant levels of fire blight resistance have been obtained and some of them have been studied from different aspects both genetic and agronomic, none is commercialized or authorized in Europe (Montesinos *et al.*, 2009; EFSA Panel on Plant Health, 2014). Some examples of these apple trees are a transgenic derivate from Royal Gala and a cisgenic Gala derivate (Norelli *et al.*, 2003; Broggini *et al.*, 2014; EFSA Panel on Plant Health, 2014).

1.5.4. Physical control

One of the physical methods that can be used for prevention of fire blight in plant material for propagation is thermotherapy (Keck *et al.*, 1995). This technique has been applied to fruit and ornamental plants by treatment with dry heat at 45°C for 60 minutes. A significantly reduction of the population of a virulent *E. amylovora*, which was artificially inoculated, and without affecting

the viability of the plant material have been achieved (Ruz *et al.*, 2003). However, this method is still experimental.

1.5.5. Chemical control

Traditionally, the most commonly used strategy for fire blight management has been chemical control. Chemical pesticides are oriented to eliminate or inactivate the pathogen before it penetrates the host tissue by destroying the source of inoculum or by protecting potential invasion sites, such as flowers or wounds. Most of chemicals available are not systemic and have not curative action (Psallidas & Tsiantos, 2000).

A large number of chemicals have been tested against fire blight, but only the copper compounds and the antibiotics control efficiently the disease.

The pesticides consisting on copper as active ingredient are based on the controlled liberation of copper ion, which are commercialised with different formulations, such as sulphate, hydroxide, oxychloride, oxide or Bordeaux mixture. The disadvantages of these products are the phytotoxicity during bloom period and, generally, the low persistence and plant penetration. Copper products are authorized in deciduous fruit trees for the control of various bacterial diseases, including fire blight. Their action against *E. amylovora* is direct, due to their microbicide activity (Montesinos *et al.*, 2009).

The antibiotics have a direct effect, because they inhibit the growth of the pathogen. From all the antibiotics evaluated against fire blight, only streptomycin, oxytetracyclin and kasugamycin have the necessary requirements to be used in field applications (Psallidas & Tsiantos, 2000). Although streptomycin is considered the most effective bactericide against fire blight, its use in agriculture has been prohibited in many countries, mainly due to the development of resistance by *E. amylovora* (Psallidas & Tsiantos, 2000; McManus *et al.*, 2002). Oxytetracycline is used as the second line of defence against fire blight, when streptomycin-resistant *E. amylovora* have been detected (McManus *et al.*, 2002; Duffy *et al.*, 2014).

The use of antibiotics is not allowed by the legislation of the European Union. In Spain, only kasugamycin has been authorized until 2007 to control bacterial disease in apple and pear trees (Montesinos *et al.*, 2009).

There are some novel compounds, which do not have direct inhibitory action against the pathogen but they can control the fire blight, such as fosetyl-aluminium (Fosetyl-Al), harpin, prohexadione-Ca or benzothiadiazole. These compounds induce systemic acquired resistance (SAR) in the host plant or are plant growth regulators that decrease plant susceptibility to *E. amylovora* infection (Montesinos *et al.*, 2009). Also the induction of SAR has been reported in apple trees, which have been treated by trunk injection, which is a method that utilizes tree xylem to distribute injected compounds, such as oxytetracycline (Aćimović *et al.*, 2015).

Interestingly, there are also antimicrobial peptides (AMPs) as novel pesticides. AMPs are natural compounds produced by animals and plants as a first line of defence, and by microorganisms in antibiosis as a competitive factor. AMPs have a wide range of activity against fungal and bacterial plant pathogens and have been involved in the control of several plant diseases. They are short sequence peptides, generally less than 50 amino acids residues. Their mechanism of action is usually based on the membrane disruption, but some AMPs interact with intracellular targets and inhibit of nucleic acid or protein synthesis or have enzymatic activity inside the cell. Although some naturals AMPs do not have a good efficacy in the plant disease control and have phytotoxicity, they can be used as the basis for the development of new AMPs with better qualities by synthetic procedures (Montesinos, 2007). The efficacy to fire blight control has been described in synthetic AMPs and it is comparable to streptomycin in *ex vivo* test, greenhouse and even in field test (Badosa *et al.*, 2014)

1.5.6. Fire blight risk assessment models

Risk assessment systems were developed to ensure optimal timing for applications of pesticides and to guide prospections in relation to risk infection. Fire blight evolution depends on the amount and virulence of the present pathogen, favourable environmental conditions and the degree of the host susceptibility to the disease.

The moments of highest risk of infection occurs during flowering and when the climatic factors cause injury in the plants. In these moments, a favourable weather conditions are determinant for the disease development, mainly

temperature and moisture. Moderate temperature (>18ºC) and high relative humidity (RH) allow a quick bacterial multiplication.

The knowledge of several of these relationships has allowed in recent years the development of epidemiological models that predict the likelihood that the disease occurs. Within all existing models, Maryblight and Cougarblight are the most used. These models are being used in the stations of phytosanitary notices in United States and in various European countries (Montesinos *et al.*, 2009).

2. Biological control of fire blight

Chemical control of fire blight is strongly limited by the scarce number of bactericides available as active ingredients caused by new European regulations, like the Commission Directive 2009/128/EC and the Regulation EC 1107/2009 (OJ L, 2009a, b). For this reason, biological control emerges as a complementary strategy in order to control plant disease.

2.1. Biological control

According to the definition proposed by the National Academy of Sciences, biological control is the use of natural or modified organisms, genes, or gene products to reduce the effects of undesirable organisms and to favour desirable organisms such as crops, trees, animals and beneficial insects and microorganisms (NAS 1987, cited by Thomashow & Weller, 1996).

The ideal biological control agent (BCA) should have the following characteristics (Wilson & Wisniewski, 1994; Trias, 2008):

- genetic stability,
- efficacy at low concentrations and against a wide range of pathogens on a variety of hosts,
- simple nutritional requirements,
- survival in adverse environmental conditions,
- growth on cheap substrate in fermenters,
- lack of pathogenicity for the plant and no production of metabolites potentially toxic to humans,

- resistance to the most frequently used pesticide, and compatibility with other treatments.

The development of BCAs requires many different stages (Figure 6), but usually including the following steps: selection of possible antagonists by means of screening methods able to analyse a high number of isolates, development of a mass production method and an appropriate formulation which allow to increase biocontrol activity and ensure its stability, determination of the mechanisms of biocontrol to improve its activity, development of a monitoring system to detect and quantify the BCA in the environment and to make more extensive toxicology tests or environmental impact studies with the aim to register for use (Trias, 2008).

2.1.1. Selection of potential biocontrol agents

Selection consists of the isolation and screening of microorganisms able to inhibit the plant pathogen and to reduce disease levels. An appropriate sampling can increase the probability of obtaining useful microorganisms, therefore the nature of samples, media composition and enrichment-isolation techniques are very important (Montesinos, 2003).
An interesting environment described by some authors to find candidates as BCAs is near the pathogen infection site (Handelsman *et al.*, 1990) or where there is evidence of presence of beneficial microorganisms (Montesinos, 2003). Moreover, BCAs could be isolated from the same natural environment where will be introduced or applied afterwards to ensure the ecological adaptation. As the presence of microorganisms with high antagonistic activity is relatively rare in the environment, the isolation of a high number of candidates is recommended.

The next stage of biocontrol development is to choose a screening method to select the suitable candidates. This step is critical because the type of selected microorganisms depends on the used method (Montesinos, 2003).

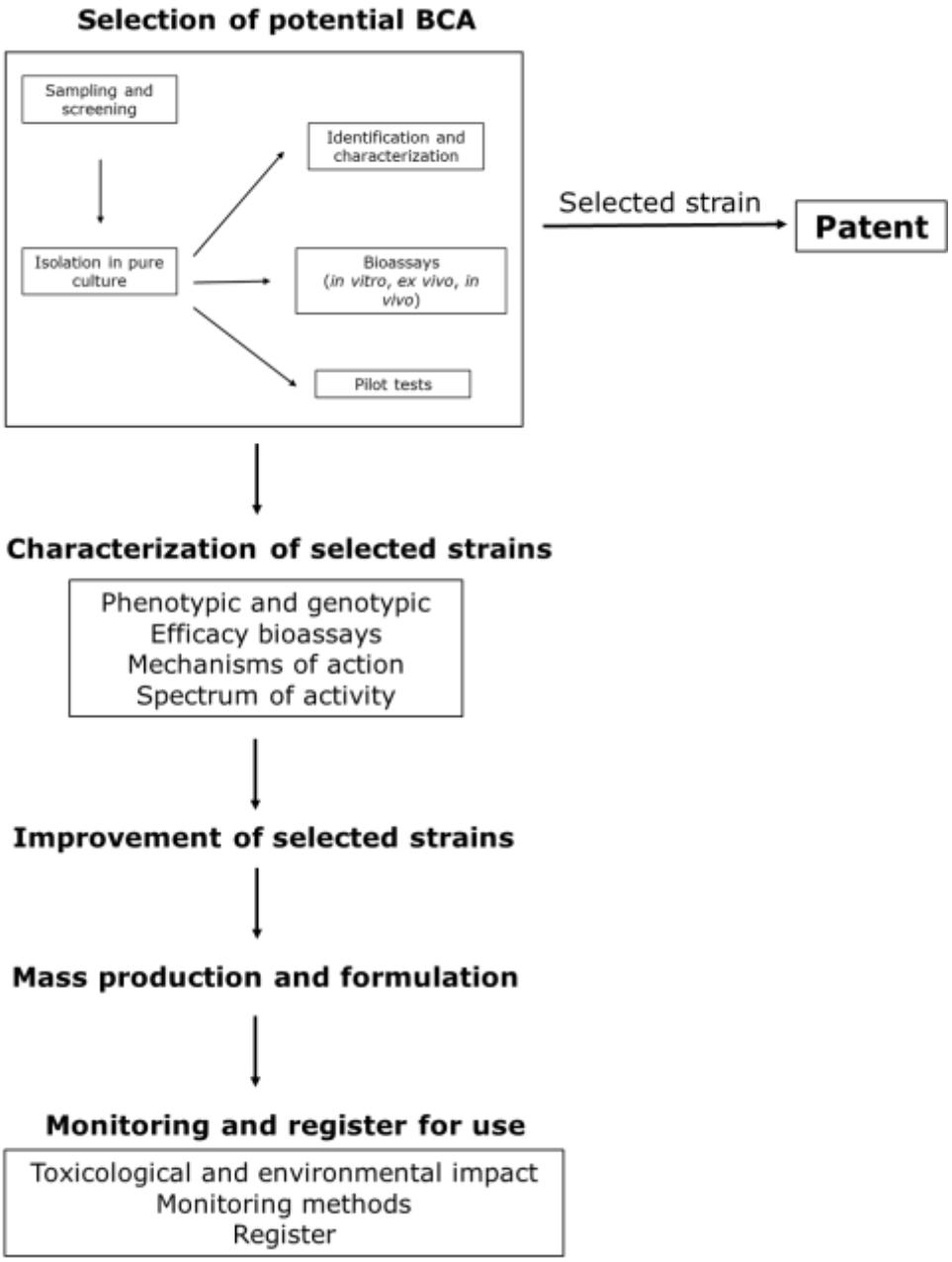

Figure 6. Procedure for the selection and development of BCA (Adapted from Montesinos, 2003 and Trias, 2008).

A suitable method to select antagonists should meet two requirements. First, it has to be rapid and simple in order to screen many potential biocontrol agents, and second, the interaction between the host, the pathogen and the antagonist should be taken into account because all components are involved

in the efficacy of disease suppression in the field (Andrews, 1985; Handelsman *et al.*, 1990).

For this reason, the best protocol is the combination of an *in vitro* stage to determine the potentiality to produce antagonism, and a screening *ex vivo* procedure (using detached plant organs) under controlled environment conditions, to prevent discarding of isolates without *in vitro* antagonism activity (Andrews, 1992). Furthermore, several studies have shown a low or no correlation between the selected BCA obtained in both strategies (*in vitro* and using plant material). It might be due to the difficulties of the BCA to colonize and survive in the plant tissue, but also to the reduction of the production of antimicrobial compounds in this environment (Mercier & Wilson, 1994).

In the case of fire blight control, the selection of effective antagonist strains has been traditionally made *in vitro*, in media-based assays, and in *ex vivo* assays, performed in immature pear fruits (Cabrefiga, 2004). These procedures favoured those antagonists which exhibited antibiosis as a major mechanism of action. However, it has been not always correlated with the efficacy of the selected antagonists to inhibit *E. amylovora* in blossoms or in plants (Wilson *et al.*, 1990; Wilson *et al.*, 1992). Therefore, the evaluation of the efficacy of isolates should be also performed in blossoms because a correlation between the ability of BCA to control *E. amylovora* on this plant organ and their effectiveness in field has been reported (Mercier & Lindow, 2001). In this way, procedures have been developed to test antagonist strains in blossoms even during non-seasonal availability of flowers, by forcing bloom under controlled environment (Pusey, 1997).

In general, the efficacy assays of BCAs should be performed *in vitro* and *ex vivo*, and finally in pilot trials under real conditions of application (field and greenhouse) (Montesinos & Bonaterra, 1996).

Currently, the use of molecular techniques makes possible to select the potential BCA by means of genetic markers. The knowledge of the genes that have importance in the biocontrol activity, may be useful to develop suitable genetic markers. Nowadays, specific markers for antimicrobial compounds have been already described, and it is possible to detect these marker genes by PCR, and to identify bacteria with the putative capacity to synthesize these

compounds. For example, the detection of the genes encoding antibiotics such as phenazine-1-carboxylic acid or 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* (Raaijmakers *et al.*, 1997) or genes encoding AMPs, such as cyclic lipopeptides in *Bacillus* spp., which have been related with antagonism (Mora *et al.*, 2011).

2.1.2. Characterization of biocontrol agents

Once a suitable BCA candidate has been selected, it is necessary to proceed to its identification and characterization by phenotypic and genotypic analysis. The interest for the agronomic or industrial application of certain microbial species, has contributed to the need to identify microorganisms at the strain level because most properties, like technological or antimicrobial attributes, are strain specific (Sánchez *et al.*, 2004). In the case of BCAs, the identification at strain level is necessary to evaluate the fate, behaviour and the impact in the environment. Moreover, this information would be required for patenting and pesticide registration (Bonaterra *et al.*, 2012).

Morphological and cultural characteristics alone are not sufficient to distinguish between strains of the same species (Schena *et al.*, 2000), besides many have similar nutritional and growth requirements and it is difficult to identify them using classical methods (Tanganurat *et al.*, 2009). Therefore, it is necessary the use of genotypic methods.

Genotypic methods used for typing lactic acid bacteria are generally PCR-based methods like macrorestriction analysis of DNA by pulsed-field gel electrophoresis (PFGE) (Sánchez *et al.*, 2004), ribotyping and restriction fragment length polymorphism (RFLP) analysis of the PCR-16S rDNA intergenic spacer region (Rodas *et al.*, 2005) and randomly amplified polymorphic DNA (RAPD – PCR) (Bringel *et al.*, 2001). Besides that, multilocus sequence typing (MLST) has recently been shown to be a useful technique for bacterial typing. MLST involves the sequencing of internal fragments of house-keeping genes for each strain of a particular species. Since 1998, MLST has been used to characterize many bacterial pathogens (de las Rivas *et al.*, 2006; Tanganurat *et al.*, 2009).

Phenotypic methods for typing lactic acid bacteria are mainly based in the physiological and biochemical characteristics of the strains, for example

carbohydrate fermentation patterns, antibiotic susceptibility or antagonist activity patterns (Trias *et al.*, 2008b,c; Tanganurat *et al.*, 2009).

Moreover, the knowledge of the control mechanisms involved in the biocontrol activity is crucial for the BCA development and for improving its efficacy. Most BCAs do not use a single mode of action, but a combination of several mechanisms, which allow the pathogen inhibition. The most important mechanisms are the production of antimicrobial substances, nutrient and space competition, biofilm formation, induction of host resistance, direct interaction between the antagonist and the pathogen, and quorum sensing interference (Pal & Gardener, 2006). The production of antimicrobial substances is based on the synthesis of compounds such as enzymes, antibiotics, bacteriocins or toxins that kill or have detrimental effect on the pathogen. Competition for nutrients and space is based on the capacity to exclude other microorganisms in the plant tissues (Andrews, 1992).

2.1.3. Improvement of biocontrol agents

A general problem in the development of biocontrol agents is the low consistency in the efficacy when they are applied under field conditions, where biotic (host species, nutritional status, pathogen) and abiotic (temperature, wetness, relative humidity) factors affect their colonization and their survival (Johnson *et al.*, 2000; Sundin *et al.*, 2009; Bonaterra *et al.*, 2012). This problem can be solved by increasing the ability of the BCAs to colonize and survive in the plant environment. To achieve this objective, different strategies can be used.

One strategy is based in the nutritional enhancement, which consists of the addition of nutrients in the formulation that are preferentially used by the BCA and not by the pathogen. This strategy has been reported to enhance survival and adaptability in the plant environment as well as biocontrol efficacy in several fungal and bacterial plant pathogens (Janisiewicz *et al.*, 1992; El-Ghaouth *et al.*, 2000; Guetsky *et al.*, 2002a; Cabrefiga *et al.*, 2011).

Another possible strategy to improve the biocontrol efficacy is the amendment of BCAs with low toxic antimicrobial compounds. Several studies reported that the efficacy of BCAs could be improved with the combination with compounds

such as bioregulators (Yu *et al.*, 2006; Spinelli *et al.*, 2011) , organic acids (Yu *et al.*, 2007; Seo *et al.*, 2013) or essential oils (Arrebola *et al.*, 2010; Zamani-Zadeh *et al.*, 2014). Currently, *Aureobasidium pullulans* strains supplemented with citric acid are the components of a commercial microbial pesticide for fire blight control (Kunz & Donat, 2014).

The modification of the physiology of the BCA to adapt themselves to adverse situations after their application in natural environments is another approach. A physiological improvement by osmoadaptation can be achieved by cultivation under osmotic stress, which cause the intracellular accumulation of compatible solutes, including sugars, polyols, heterosides, amino acids and amino acids derivates. This strategy increase the ecological fitness of BCAs, allowing for a better tolerance to adverse conditions, such as drought or salinity, freezing or high temperatures, and increasing the efficacy of disease control (Bonaterra *et al.*, 2005, 2007, 2012; Cabrefiga *et al.*, 2011). Sometimes, osmoadaptation is combined with nutritional enhancement (Cabrefiga *et al.*, 2011).

Another approach to overcome the lack of consistency of biological control is the use of mixtures of strains or species of BCAs, which are compatible and that complement each other with different mechanisms of action. These mixtures increase the range of effectiveness and ecological performance (Janisiewicz, 1988; Guetsky *et al.*, 2001, 2002b; Spadaro & Gullino, 2005; Agustí *et al.*, 2011; Stockwell *et al.*, 2011; Yang *et al.*, 2015).

Finally, the improvement of BCAs can also be achieved by means of genetic modification. Biotechnology can be used to manipulate genes present in BCAs or to introduce new traits to increase its efficacy by enhancing colonizing ability or by potentiation of the production of antimicrobial compounds. For example, the overexpression of a proteinase in *Trichoderma harzianum* (Flores *et al.*, 1997) or the overproduction of antimicrobial polyketides, such as pyoluteorin and 2,4-diacetylphloroglucinol, in *P. fluorescens* CHA0 (Girlanda *et al.*, 2001) have improved the efficacy of disease control.

2.1.4. Mass production and formulation of biocontrol agents

For the commercial development of a microbial pesticide, the preparation of BCAs include industrial scale production (fermentation), formulation and preservation of the strains.

Mass production should be rapid, efficient and inexpensive, besides it has to assure the maintenance of the antagonistic efficacy of the BCA. For this reason, several studies about production at industrial scale have to be performed to get a high quantity of microorganism in a cheap medium in a short period of time and a stable product through time for delivery.

The development of a suitable preservation and formulation methodology is essential for obtaining a long shelf-life of microbial pesticides. In order to get it, it is necessary to maintain the viability and the activity of the BCAs. Formulation can be in liquid state and maintained by refrigeration, or by keeping as dehydrated product not dependent on refrigeration. Dehydration methods such as lyophilisation and spray-drying allow optimum conditions of storage, handling and formulation of the microorganism. The use of additives compatible with the BCA in the formulation can increase its survival, improve the application and stabilization of the final product (Montesinos, 2003; Bonaterra *et al.*, 2012). Many microbial biopesticides used in the control of fire blight are formulated as dried products (wettable powders or granules) (Montesinos & Bonaterra, 2009; Cabrefiga *et al.*, 2014). An example of this type of product is a wettable powder of *P. fluorescens* A506 at a concentration of $1x10^{10}$ CFU g^{-1} (Stockwell & Stack, 2007).

2.1.5. Monitoring and registration for use

The development of reliable monitoring methods that assure the identification and quantification of the BCA after field release is needed for pesticide registration. These methods will provide information about the population dynamics of the biocontrol strain in the target organism such as plants. Such knowledge is essential, since the efficacy of the BCA depends on its colonisation on plant surfaces and population size in the pathogen entrance site. The monitoring methods can be microbiological (e.g. morphology or

antibiotic-resistance), direct (e.g. bioluminescence) or molecular (e.g. use of molecular markers) (Pujol, 2006). Examples of monitoring methods developed for strains of BCAs of fire blight are real-time PCR for *P. fluorescens* EPS62e (Pujol *et al.*, 2005, 2006, 2007) or for *P. agglomerans* E325 (Braun-Kiewnick *et al.*, 2012).

Before registration, also it is important to know that the BCAs do not have harmful effects on plants, non-target microorganisms, animals and environment. Thus it is necessary to guarantee the human health doing toxicological studies (Montesinos, 2003), but also an analysis of environmental impact is required by the regulations of many countries (Bonaterra *et al.*, 2012).
The register of plant protection products in EU for commercialization is regulated by the Regulation EC 1107/2009 (European Commission, 2015).

2.2. Biological control of fire blight

In the last 30 years, several studies have been done for the development of biological control strategies for fire blight management. For this purpose, several strains have been selected and studied because of their ability to prevent or suppress the progress of the disease (Table 1).

There are some BCAs that are currently available for fruit growers and others are in process of approval of their commercialisation in different countries. In the European Union, *Bacillus subtilis* strain QST713 and *A. pullulans* strains DSM14940 and DSM14941 are authorised as fungicides and bactericides (European Commission, 2015).

It is possible the combination of some authorised BCA with different strategies, such as the mixture of strains or the combination with antimicrobial compounds to achieve a higher biocontrol efficacy. An example is a commercial product that is a combination of a mixture of two *A. pullulans* strains combined with citric acid (Kunz & Donat, 2014).

P. fluorescens EPS62e, a strain isolated by our research group of the University of Girona from a Conference pear in Spain, has been developed and has also

been the subject of several studies, obtaining a dried formulation that offered promising results in Mediterranean agroclimatic conditions (Pujol *et al.*, 2005, 2006, 2007; Cabrefiga *et al.*, 2007, 2011, 2014). However, this strain is not still commercialised (Montesinos *et al.*, 2009).

Table 1. Biological control agents of fire blight.

Organism	Mode of action	Commercial name	Source
Aureobasidium pullulans DSM14940 and DSM14941	Competitive exclusion	Blossom Protect™	Kunz *et al.*, 2011
Bacillus amyloliquefaciens subsp. *plantarum* D747	Antibiosis	Double Nickel 55™	Highland *et al.*, 2012
Bacillus pumilis QST2808	Antibiosis	Sonata®	Bayer CropScience
Bacillus subtilis QST713	Antibiosis	Serenade®	Aldwinckle *et al.*, 2002
B. subtilis BS-F3	Antibiosis	-	Alexandrova *et al.*, 2002
B. subtilis BD170	Antibiosis	Biopro®	Broggini *et al.*, 2005
Erwinia tasmaniensis DS08	Competitive exclusion	-	Huebert *et al.*, 2014
Pantoea vagans C9-1	Antibiosis	BlightBan C9-1	Ishimaru *et al.*, 1988
Pantoea agglomerans E252	Antibiosis	-	Vanneste *et al.*, 1992
P. agglomerans E325	Competition, antibiosis	Bloomtime® Biological FD	Pusey *et al.*, 2008b
P. agglomerans Eh24	Competitive exclusion	-	Özaktan *et al.*, 1999
P. agglomerans Eh112Y	Antibiosis	-	Wodzinski *et al.*, 1994
P. agglomerans Eh318	Antibiosis	-	Wright & Beer, 1996
P. agglomerans EhHI9NI13	Antibiosis	-	Wilson *et al.*, 1990
P. agglomerans Eh1087	Antibiosis	-	Kearns & Hale, 1996
P. agglomerans P10c	Competition, antibiosis	Blossom Bless™	Vanneste *et al.*, 2002
Pseudomonas fluorescens A506	Competitive exclusion, antibiosis	BlightBan® A506	Wilson & Lindow, 1993
P. fluorescens EPS62e	Competitive exclusion	-	Cabrefiga *et al.*, 2007
Pseudomonas graminis 49M	Antibiosis	-	Mikicinski *et al.*, 2011
Pseudomonas sp. R1	Antibiosis	-	Laux *et al.*, 2002
Rahnella aquatilis Ra39	Competition	-	Laux *et al.*, 2002

It should be noted that the BCAs are more effective when they are applied in blossoms because their activity is based on the prevention of the blossom infection and colonization by *E. amylovora*, which needs to multiply on the stigmatic surfaces prior to penetrate and infect the plant (Johnson & Stockwell, 1998). During blossom, the chemical products available to control fire blight such as copper compounds usually have more phytotoxicity, therefore the biological control may be good alternative or complement (Montesinos *et al.*, 2009).

Although the amount of available BCAs, some have limitations due to the inconsistent performance in field tests (Sundin *et al.*, 2009; Ngugi *et al.*, 2011). For this reason, it is necessary to find new strains or species of BCAs that have to fulfil the current strict authorization requirements in most countries for microbial pesticides (Montesinos & Bonaterra, 2009). Good candidates for biocontrol of fire blight would be lactic acid bacteria (LAB).

3. Lactic acid bacteria: novel biocontrol agents

3.1. Taxonomy and physiology of LAB

Lactic acid bacteria (LAB) are a group of Gram positive rods and cocci, non-spore forming, non-motile, catalase-negative, devoid of cytochromes but aerotolerant, fermentative with lactic acid as the major end-product of sugar fermentation. Typically, LAB are mesophilic bacteria, but they are also able to grow at temperatures ranging from 5 to 45ºC, and they tolerate both acid and alkaline environments (Axelsson, 2004).

LAB have two different metabolic pathways for sugar fermentation, the homo- and heterofermentative pathway. The homofermentative pathway follows the glycolysis (Embden-Meyerhof-Parnas pathway), resulting under standard conditions in lactic acid as the end product. This pathway occurs among *Lactococcus*, *Streptococcus*, *Pediococcus* and homofermentative *Lactobacillus* species. The heterofermentative or the 6-phosphogluconate / phosphoketolase pathway results in significant amounts of other end products such as ethanol, acetate and CO_2 in addition to lactic acid. This pathway occurs in *Leuconostoc*,

Oenococcus, *Weissella* and on the other *Lactobacillus* species (Stiles & Holzapfel, 1997; Axelsson, 2004). Besides these products, some LAB can produce different types of antimicrobial compounds (Reis *et al.*, 2012).

LAB are generally associated with nutrient-rich habitats, such as various food products (milk, meat, beverages, vegetables) and some are members of the normal microbiota of mucosal surfaces of mammals. Interestingly, LAB are also found in the phyllosphere of plants, being *Leuconostoc* and *Lactobacillus* the most abundant genera (Trias *et al.*, 2008b; Zwielehner *et al.*, 2008; Leveau & Tech, 2011; Leff & Fierer, 2013; Williams *et al.*, 2013). Within these genera, the most common species are *Leuconostoc mesenteroides* and *Lactobacillus plantarum* (Di Cagno *et al.*, 2008, 2010)

L. plantarum (Figure 7) is one of the most widespread LAB species in the environment, with presence in different ecological niches, both in fermented foods and plant material as well as in gastro-intestinal tract of human and animals. This species is used in the production of fermented products, but also it is widely employed in novel foods and health applications, such as probiotics (Siezen *et al.*, 2010).

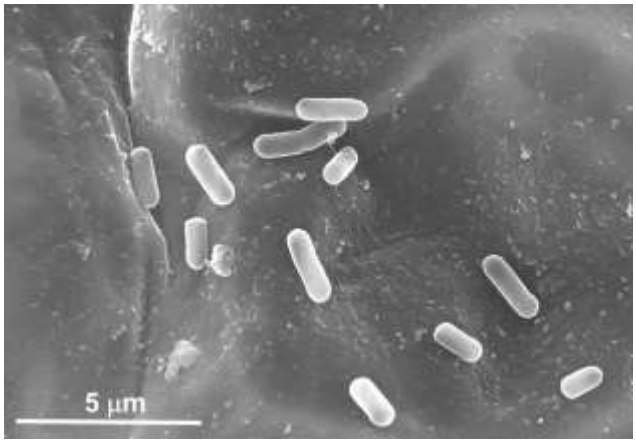

Figure 7. Scanning electron micrographs of Conference pear leaf inoculated with *L. plantarum* TC92.

Leuconostoc spp. have their natural ecological niche in green vegetation and roots, and they are also found in fermented and refrigerated food products (Hemme & Foucaud-Scheunemann, 2004).

3.2. Antimicrobial activity of LAB

The antimicrobial activity of LAB is due to the combined action of different mechanisms. Such as nutrient and space competition, production of antimicrobial compounds like organic acids, hydrogen peroxide, carbon dioxide, diacetyl, broad-spectrum low molecular weight compounds (e.g. reuterin) and bacteriocins (Caplice & Fitzgerald, 1999).

The production of organic acids (lactic, acetic, benzoic and propionic acids) has an antagonistic effect on the microbiota thanks to the inhibition of the active transport processes and the modification of their membrane potential (Caplice & Fitzgerald, 1999). Moreover, organic acids cause a decrease of the environmental pH (Reis *et al.*, 2012).

The accumulation of hydrogen peroxide because of LAB are unable to produce catalase, oxidises the lipid membrane and cellular proteins of target microorganisms (Dalié *et al.*, 2010). The carbon dioxide produced during heterofermentative pathway create an anaerobic environment which is toxic for some bacteria (Caplice & Fitzgerald, 1999).

Most genera of LAB are able to produce protein compounds with a noteworthy antimicrobial effect, which are known as bacteriocins (Ouwehand & Verterlund, 1998)

3.2.1. Bacteriocins

Bacteriocins are classified as ribosomal-synthesized peptides, as biologically active proteins or proteins complexes with antimicrobial activity against different species, and they are produced by different groups of bacteria, both Gram positive and negative bacteria. In Gram positive bacteria, LAB are an especially important group due to the high number of bacteriocins producer strains (Reis *et al.*, 2012).

Bacteriocins synthesized by LAB constitute a heterogeneous group of peptides and proteins, which are grouped into four classes based on chemical structure,

molecular weight and thermal stability (Reis *et al.*, 2012). In table 2, the characteristics of the four groups and subgroups of bacteriocins are described.

Table 2. Classification of bacteriocins synthesized (Caplice & Fitzgerald, 1999; Cotter *et al.*, 2005; Drider *et al.*, 2006; Todorov, 2009; Reis *et al.*, 2012)

Class	Characteristics	Subclass	Examples
I Lantibiotic bacteriocins	Small (less than 5 kDa*, with 19 to 38 aa), heat-stable, containing lanthionine and B-methyl lanthionine of post-translational modification	Ia: elongated, cationic and pore forming peptides	Nisin – *Lactococcus lactis*
		Ib: compact, with globular structures, enzyme inhibitors and immunologically actives	Mersacidin – *Bacillus* spp.
II Non-lantibiotic bacteriocins	Small (less than 10 kDa with 30-100 aa) heat-stable	IIa: antilisterial pediocin-like bacteriocins with the conserved amino acid sequence YGNGV	Pediocin PA-1 – *Pediococcus acidilactici* Sakacin G – *Lactobacillus sakei*
		IIb: two-peptides bacteriocins	Plantaricin EF – *L. plantarum* Plantaricin JK – *L. plantarum*
		IIc: *sec*-dependent secretion of bacteriocins	Lactococcin 972 – *Lactococcus lactis* IPLA 972
		IId: unclassified small heat-stable non-lanthionine bacteriocins	Enterocin B – *Enterococcus faecium* Carnobacteriocin A – *Carnobacterium piscicola*
III Bacteriolysins	Non-bacteriocin lytic proteins, large (more than 30 kDa), heat-labile		Helveticin J – *Lactobacillus helveticus* 481 Lysostaphin – *Staphylococcus simulans*
IV	Undefined mixture of proteins, lipids and carbohydrates, heat-stable		Leuconocin S – *Leuconostoc paramesenteroides*

*kDa= kilodalton, unit of atomic mass

The action mechanism of bacteriocins differ depending on the class, but generally, these protein compounds modify the selective permeability of the cell membrane with the pore formation (class I, II and IV) or destroy the cell wall (class III or bacteriolysins), causing the cell death (Figure 8) (Cotter *et al.*, 2005).

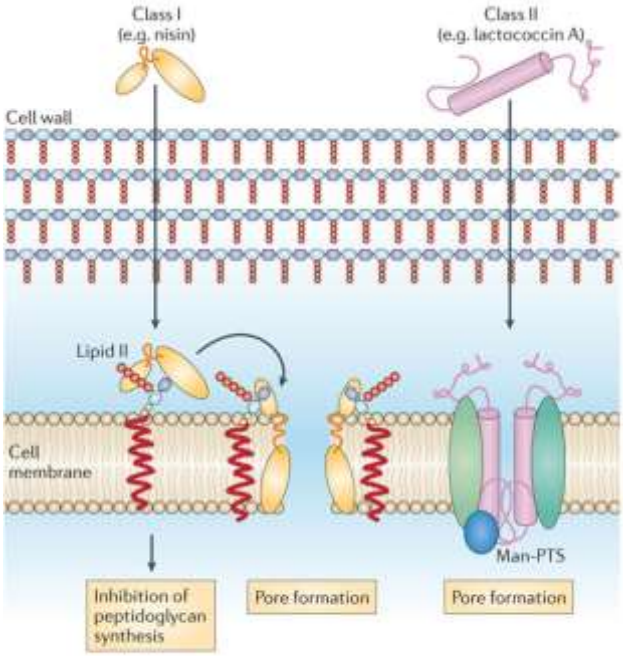

Figure 8. Mode of action of some bacteriocins in Gram positive bacteria (From Cotter *et al.*, 2013).

A high number of bacteriocins produced by LAB have been isolated and characterized. Even though these molecules mainly have activity against phylogenetically related bacteria, also bacteriocins against Gram negative bacteria, molds, yeasts and fungi have been reported (Magnusson *et al.*, 2003; Ben Omar *et al.*, 2008).

Although the potential of bacteriocins as food preservatives and their antagonistic effect against important food pathogens (Cotter *et al.*, 2005; Drider *et al.*, 2006; Jofre *et al.*, 2008; Trias *et al.*, 2008a), the interest in the use of bacteriocins in the control of phytopathogens has been increased and several examples of bacteriocins that inhibit plant pathogenic bacteria and fungi have been reported (Lavermicocca *et al.*, 2002; Pharm *et al.*, 2004; Parret *et al.*, 2005; Hammami *et al.*, 2012; Grinter *et al.*, 2012; Mouloud *et al.*, 2013).

Within the bacteriocins produced by LAB, nisin is the most characterized and used in a commercial scale as food preservative in processed cheese, meats and beverages. Besides, nisin was the first LAB bacteriocin described (Cotter

et al., 2005). This bacteriocin belongs to the class I bacteriocin or lantibiotic. The importance of this bacteriocin is due to a wide spectrum of activity against Gram-negative and Gram-positive, including also the prevention of the outgrowth of spores of many *Clostridium* and *Bacillus* spp. Its mechanism of action is through disruption of membrane instigated by formation of pores in the bacterial cell membrane (Figure 8) (Reis *et al.*, 2012).

In the most common plant-associated LAB, *L. mesenteroides* and *L. plantarum*, the production of bacteriocins have also been described (Héchard *et al.*, 1992; Todorov, 2009).

L. mesenteroides strains can produce mesentericins, which usually belong to the class IIa bacteriocins that are pediocin-like and active against *Listeria* (Cotter *et al.*, 2005). An example of this type of bacteriocins is mesentericin Y105 produced by *L. mesenteroides* Y105 (Héchard *et al.*, 1992). The genes that encoded this mesentericin are located on plasmid pHY30 and are organised in two gene clusters. One cluster contains the genes *mesY* and *mesI*, encoding the bacteriocin and the putative immunity peptide respectively. The other cluster includes genes *mesC*, *mesD* and *mesE*, in which *mesD* encodes the ABC transporters and *mesE*, their accessory factors (Fremaux *et al.*, 1995). Interestingly, the production of mesentericins has been previously reported in LAB isolates from plant sources. *L. mesenteroides* CM160 and CM135 produce mesentericin CM160 and CM135 respectively, which showed a complete sequence homology with mesentericin Y105 (Trias *et al.*, 2008a).

L. plantarum strains can produce bacteriocins, which are called plantaricins. The antimicrobial activity of some of these strains is due to the synthesis of these protein compounds. Several plantaricins have been identified and characterized such as W, C19, 4.23, EF, JK, S, NC8 and 1.25 plantaricins (Holo *et al.*, 2001; Maldonado *et al.*, 2003; Todorov, 2009; Cho *et al.*, 2010) and often the same strain can synthesize multiple plantaricins.

The genes, which code for the synthesis of plantaricins, are organized in different operons within a loci, the *pln* loci. This loci has been characterized from different *L. plantarum* strains, such as C11 strain isolated from fermented cucumber (Daeschel *et al.*, 1990), WCFS1 from human saliva (Kleerebezem *et al.*, 2003), NC8 from grass silage (Aukrust & Blom, 1992), J51 and J23 from

grape must and wine respectively (Rojo-Bezares *et al.*, 2007; Navarro *et al.*, 2008). From the knowledge provided by these strains studies, five different *pln* loci have been described and characterized, which are represented in Figure 9.

Each locus has a length of 18-19 kb and contains about 25 genes, which are organized into 5-6 operons. The genetic organization of *pln* loci presents a conserved part between the studied *L. plantarum* strains, which contains one bacteriocin operon (*plnEFI*) and one transport operon (*plnGHSTUVW*). While the less conserved part includes a regulatory operon (*plnABCD* or *plnNC8IFHK-D*), two or three bacteriocin operons and one or two operons with unknown function (Diep *et al.*, 2009).

Using the *pln* loci of strain C11 as an example, the function of the different operons is detailed below. The regulatory operon (*plnABCD*) encodes an inducing peptide (PlnA) which induces the transcription of five operons, a histidine protein kinase (PlnB) and two response regulators (PlnC and PlnD). The *plnEFI* and *plnJKLR* operons encode two-peptide bacteriocins, PlnEF and PlnJK, and their corresponding immunity proteins. The *plnMNOP* operon contains genes related with plantaricin production and immunity but the function of some genes is unknown. Finally, *plnGHSTUVWXY* operon encodes proteins of an ABC transport system that secretes and processes the bacteriocin precursors (Diep *et al.*, 2009; Sáenz *et al.*, 2009).

The *pln* loci of WCSF1 strain is almost identical to the C11- *pln* loci, however the *pln* loci from NC8, J51 and J23 strains present more differences (Diep *et al.*, 2009). *L. plantarum* strain NC8 presents the *plnNC8βαc* operon, which produces the inducible two-peptide plantaricin NC8βα and besides, the regulator operon (*plnNC8IFHK-D*) is different to the one described in C11-*pln* (*plnABCD*).

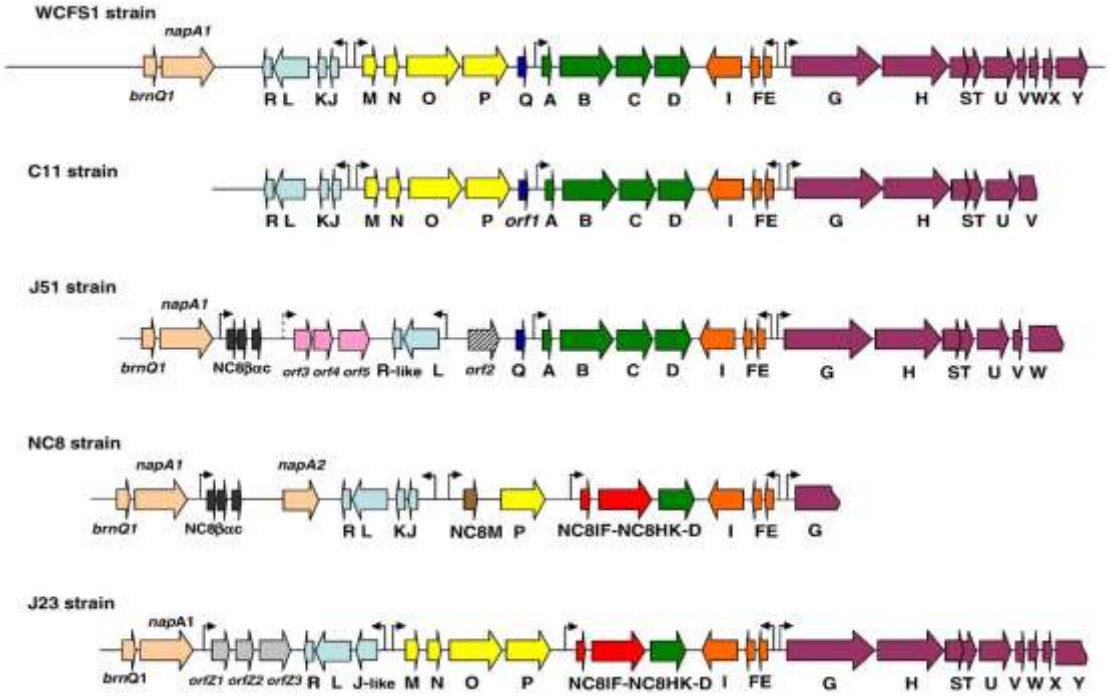

Figure 9. Genetic map of *pln* loci of *L. plantarum* type strains C11, WCFS1, J51, NC8 and J23. The *pln* genes are represented by arrows and the colour code for *pln* operons: regulatory operon (green/red); *plnEFI* operon (orange); *plnMNOP* operon (yellow); *plnJKLR* operon (pale blue); *plNC8βαc* operon (black) and ABC transporter system genes (purple) (From Sáenz *et al.*, 2009).

The *pln* loci of J51 and J23 strains have a different organization of genes and new open reading frames (orfs) comparing with C11 and NC8 *pln* loci. In addition, J51 *pln* locus contains two biosynthetic operons of plantaricins, *plnNC8βαc* and *orf3-4-5*, which are not present at C11-*pln* (Maldonado *et al.*, 2003; Rojo-Bezares *et al.*, 2007; Diep *et al.*, 2009; Sáenz *et al.*, 2009)

3.3. LAB as biopreservatives and biocontrol agents

LAB have been traditionally used in food production and they have been widely described as biopreservatives to be applied to meat or dairy products (Holzapfel *et al.*, 1995; Hugas *et al.*, 1998; Vermeirem *et al.*, 2004), and also in fermented vegetables and fruit juices (Ruiz-Barba *et al.*, 1994; Gomez *et al.*, 2002).

The interest in LAB as potential antimicrobial agents in food technology is due to that they produce several antagonist compounds, which are capable to prevent the growth and the activity of spoiling and/or pathogenic microorganisms. In addition, LAB have been considered with the status of generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA) and with the qualified presumption as safe (QPS) status by the European Food Safety Agency (EFSA).

The use of LAB, which were isolated from fresh plant products, as biopreservatives of fruits and vegetables has been reported to control food-borne human pathogenic bacteria (*E. coli*, *Salmonella typhimurium* and *Listeria monocytogenes*) and postharvest fungi (*Penicillium expansum* and *Botrytis cinerea*) (Trias *et al.*, 2008a,b,c). Also, several LAB strains of different origins have been reported to control of mycotoxigenic moulds, such as *Penicillium*, *Aspergillus* and *Fusarium* (Magnusson *et al.*, 2003; Dalié *et al.*, 2010). Moreover, some strains of LAB have been studied as antagonists against plant pathogenic bacteria, such as *Pectobacterium carotovorum*, *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* (Visser *et al.*, 1986; Trias *et al.*, 2008c; Shrestha *et al.*, 2014). Interestingly, some of the most promising strains pertained to *L. plantarum*.

Chapter II:

Context and Approach of this PhD thesis

1. Context of this work

The current trend of crop protection has been reoriented to a rational use of pesticides and to a reduction of the number of registered active ingredients to those certainly unavoidable, more selective, less toxic and with a lower negative environmental impact (Montesinos, 2003; Montesinos & Bonaterra, 2009). In this context, the interest in new active ingredients based on microorganisms has increased. For this reason, the group of Plant Pathology-CIDSAV of the University of Girona focused its research nearly twenty years ago in the development of new innovative and sustainable technologies to control plant diseases, such as biocontrol. In this sense, the group has been working in beneficial Gram negative bacteria, especially strains of *P. fluorescens* and *P. agglomerans* species, to control plant diseases of economic interest in agriculture, like fire blight and brown spot of pear, but also in postharvest fungal rot diseases. Different aspects of biocontrol have been studied, such as the analysis of the mechanisms of action (Cabrefiga *et al.*, 2007), dose-response relationships (Montesinos & Bonaterra, 1996; Francés *et al.*, 2006), colonization and traceability (Pujol *et al.*, 2005, 2006, 2007) and physiological improvement of strains in formulations (Bonaterra *et al.*, 2005, 2007; Cabrefiga *et al.*, 2011, 2014). In the last decade, the group started a new research focused on Gram positive bacterial strains as BCAs, including lactic acid bacteria and *Bacillus* (Mora *et al.*, 2011, 2015), with an interesting profile of biosafety, production and formulation. The group created a LAB collection, in which certain species of LAB isolated from fresh plant products have been reported to control foodborne bacterial pathogens and postharvest fungi (Trias *et al.*, 2008a,b,c). Accordingly, the present PhD thesis is focused to study the application of LAB strains as BCAs against plant bacterial diseases specifically to fire blight control.

2. Approach of the PhD thesis

The possibility of using LAB to develop BCAs of fire blight disease of rosaceous plants has been addressed in the present study. A summary of the steps involved in this study is shown in Figure 10 and includes an initial screening procedure to select the most interesting LAB candidates as BCAs, a characterization step addressed to have robust criteria to select putative biocontrol agents and a final step of improvement of lead strains in a formulation.

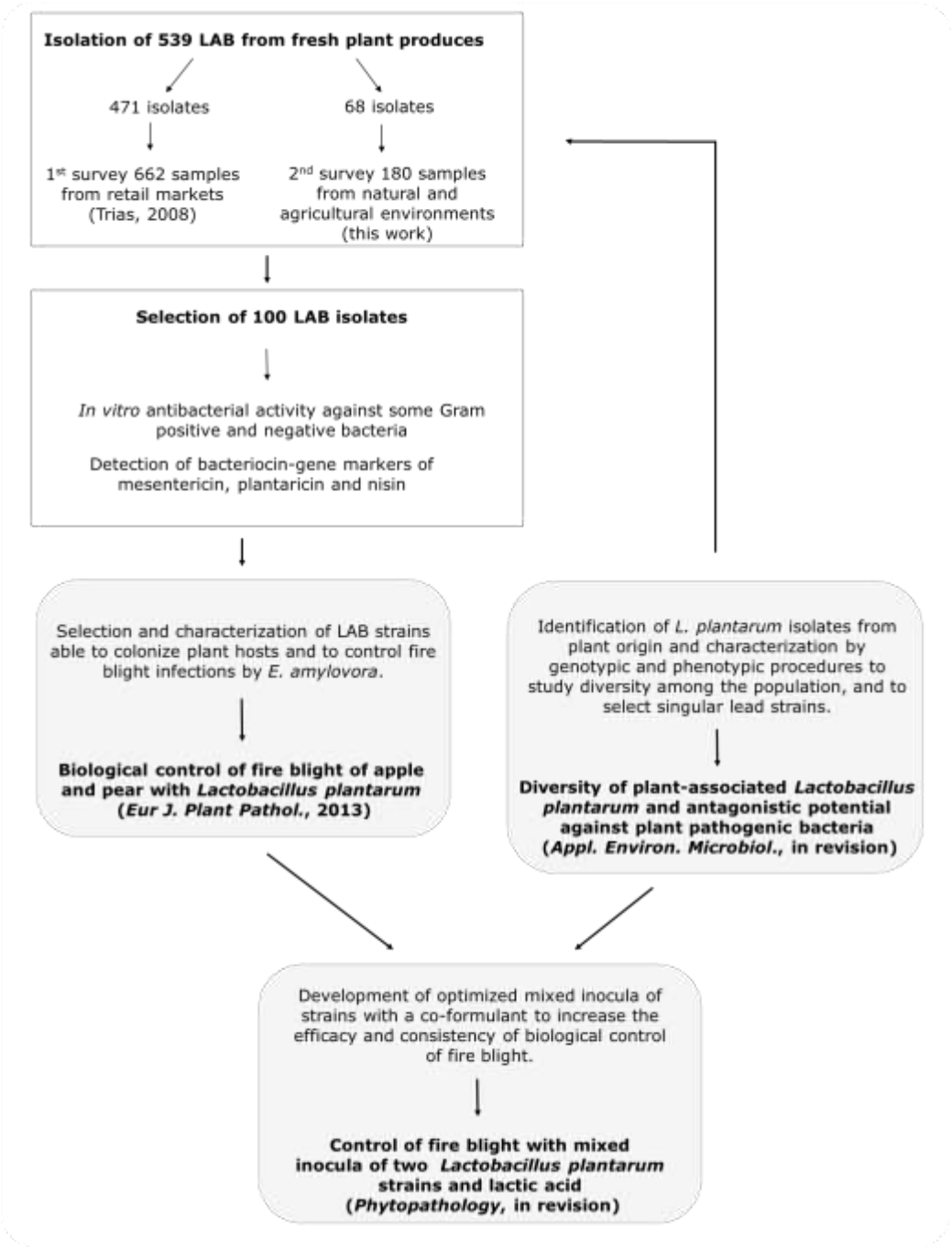

Figure 10. Summary of steps involved in the present work.

A LAB collection made of a batch previously reported (Trias, 2008), has been enriched in the present work with new strains. The final collection consisted of 539 LAB isolates from 842 samples from fresh plant sources collected in two surveys (471 from retail markets, obtained in the first survey and 68 from wild or agricultural environments, obtained in the second survey). In the first survey, the presence of LAB has been confirmed in the 71% of the samples (Trias *et al.*, 2008b). In contrast, in the second survey, only 38% of the samples presented LAB (this work). Interestingly, products from retail markets had a higher incidence and density of LAB than those obtained from wild environments (Table 3).

Table 3. Incidence of lactic acid bacteria in samples of fresh plant produce. Samples were collected from different crops in retail markets and in wild environments, and were obtained in two surveys in different years (obtained from Trias *et al.*, 2008 and this work).

Survey	Type		Number of samples	Number of LAB isolated	Incidence of LAB (%)
Retail Markets					
	Crop/product	Fresh fruits	286	160	55.9
		Packet and ready-to-eat	94	94	100.0
		Raw vegetables	282	217	76.9
Natural/agricultural environments					
	Crop/product	Fresh fruits	103	30	29.1
		Forest vegetation	33	11	33.3
		Oilseed and protein	10	7	70.0
		Raw vegetables	34	20	58.8

Previously to this study, 100 LAB isolates were selected to have antibacterial activity against target bacteria combined with the presence of some selected gene markers. The screening procedure was used to select, among the isolates, candidates to inhibit *E. amylovora* and control fire blight. *In vitro* antibacterial activity against some Gram positive and negative bacteria was studied in agar medium, specifically in a spot test (Figure 11). Ninety six out of 539 isolates had a wide spectrum of antagonism against a full range of target bacteria and interestingly, 226 isolates exhibited antagonism activity against *E. amylovora.*

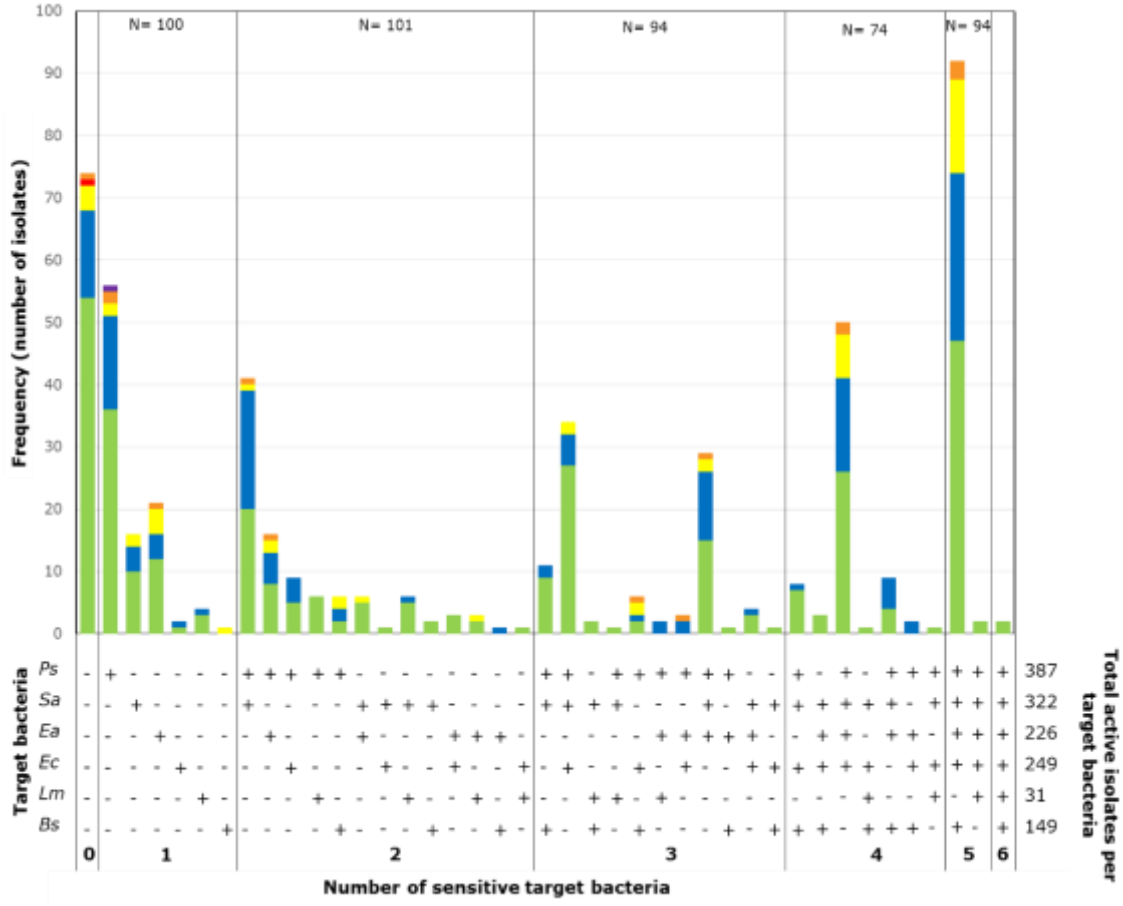

Figure 11. Frequency distribution of patterns of antibacterial activity of 539 LAB isolates from plant sources against 6 target bacteria in spot test. The target bacteria are *Pseudomonas syringae* (*Ps*), *Staphylococcus aureus* (*Sa*), *E. amylovora* (*Ea*), *Escherichia coli* (*Ec*), *L. mesenteroides* (*Lm*) and *B. subtilis* (*Bs*) The number of isolates (N) within each group with antibacterial activity against the same number of target bacteria is indicated in the upper part of the panels. Total numbers of active isolates per bacteria was showed in the right part of the panel. The proportion of isolates that present the biosynthetic genes of bacteriocins is indicated in each bar: no bacteriocin genes (green), *mes* (blue), *pln* (yellow), *nis* (red), *mes* plus *pln* (orange) and *mes* plus *nis* (purple).

The molecular markers used to perform the screening stage were *plnEF*, *mes* and *nis* genes encoding respectively for plantaricin, mesentiricin and nisin. One tenth of LAB isolates (62 strains) presented the *plnEF* gene, 168 presented the *mes* gene and only two presented the *nis* gene.

In the present PhD thesis, a screening procedure based on the suppressive effect against the effectiveness *E. amylovora* infections have been used to select candidates of BCA from 100 LAB strains, selected in the previous stage. The inhibition of *E. amylovora* infections was determined in different detached plant organs such as flowers, leaves and fruits. The most interesting strains were identified as *L. plantarum* and characterized, and their efficacy in the control of *E. amylovora* was confirmed in whole plants and in mixed field-lab blossom assay.

After that, all LAB isolates belonging to *L. plantarum* species from the LAB collection were identified and characterized using genotypic and phenotypic features. The genotypic characterization has been addressed by means of MLST, RAPD-PCR and gene profiling, detecting the presence of genes related with plantaricin biosynthesis (plantaricin-genes profiling). Besides, a phenotypic characterization has been addressed determining the antagonism against different bacterial plant pathogens (*E. amylovora*, *Pseudomonas syringae pv. syringae*, *P. syringae pv. actinidiae*, *Xanthomonas fragariae* and *X. arboricola pv. pruni*) and more specifically, in the case of *E. amylovora*, the antagonism of cells and cell-free culture supernatants and the inhibition of infections in detached plant organs was also studied. The main objective of this part of the study was to characterize plant-associated *L. plantarum* isolates to select lead strains that can be used as active ingredients of biopesticides.

Finally, an improvement step has been included to enhance the level of biocontrol against *E. amylovora* achieved by selected lead strains. Two strategies have been used that included the use of a mixture of these selected lead strains and their combination with a non-toxic antimicrobial compound, co-formulant, that may improve their efficacy. This final study provides an example of the application of the multibarrier theory in fire blight control for future development of biopesticide formulations.

Chapter III:

Objectives

The main objective of this thesis was to study lactic acid bacteria as biological control agents for fire blight disease of rosaceous plants.

To accomplish the main goal, the following specific objectives were defined:

- Selection and characterization of LAB strains able to colonize plant hosts and to control fire blight infections by *E. amylovora*.

- Identification of *L. plantarum* isolates from plant origin and characterization by genotypic and phenotypic procedures to study diversity among the population, and to select singular lead strains.

- Development of optimized mixed inocula of strains with a co-formulant to increase the efficacy and consistency of biological control of fire blight.

Chapter IV:

Biological control of fire blight of apple and pear with antagonistic *Lactobacillus plantarum*

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Abstract

Lactic acid bacteria (LAB) can be a source of biological control agents (BCA) of fire blight disease. Several species of LAB are inhabitants of plants and are currently used as biopreservatives of food because of their antagonistic properties against bacteria, and are considered as generally safe. Candidates to BCA were selected from a large collection of LAB strains obtained from plant environments. Strains were first chosen based on the consistency of the suppressive effect against *E. amylovora* infections in detached plant organs (flowers, fruits and leaves). *Lactobacillus plantarum* strains PC40, PM411, TC54 and TC92 were effective against *E. amylovora* in most of the experiments performed. Besides, strains PM411, TC54 and TC92 had strong antagonistic activity against *E. amylovora* and also other target bacteria, and presented genes involved in plantaricin biosynthesis (*plnJ*, *plnK*, *plnL*, *plnR* and *plnEF*). The strains efficiently colonized pear and apple flowers; they maintained stable populations for at least 1 week under high RH conditions, and survived at low RH conditions. They were effective in preventing fire blight on pear flowers, fruits and leaves, as well as in whole plants and in a semi-field blossom assay. The present study confirms the potential of certain strains of *L. plantarum* to be used as active ingredient of microbial biopesticides for fire blight control that could be eventually extended to other plant bacterial diseases.

Keywords

Fire blight; Erwinia amylovora; Biocontrol agents; Lactic acid bacteria

Chapter V:

Diversity of plant-associated *Lactobacillus plantarum* and antagonistic potential against plant pathogenic bacteria

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G. Roselló participated in the design of the experimental work, performed it, participated in the writing of the manuscript and read and approved the final manuscript.

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Abstract

The genotypic and phenotypic diversity of *Lactobacillus plantarum* from plant environments was studied. Genotypic analysis was performed by multilocus sequence typing (MLST), randomly amplified polymorphic DNA (RAPD-PCR) and presence of plantaricin genes. Phenotypic analysis consisted of antimicrobial activity against the plant pathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *actinidiae*, *Xanthomonas fragariae* and *Xanthomonas arboricola* pv. *pruni*, and more specifically inhibition of infection of host-plant organs by *E. amylovora*. MLST analysis of six housekeeping genes (*pgm*, *ddl*, *gyrB*, *purK1*, *gdh* and *mutS*) revealed eight sequence types (ST), with ST16 as the dominant among isolates. Six new allele sequences were identified. The population structure was analysed with the minimum spanning tree method (MST) using the six housekeeping genes (71 strains including other reports). Strains were grouped into three clonal complexes (CCs) with isolates of the present study in all CCs, but mainly in CC1 (ST16). RAPD-PCR analysis confirmed this heterogeneity. However, plantaricin genes showed the same profile in the majority of isolates (42 out of 45 with 20 *pln* genes). A marked heterogeneity among isolates in the antagonistic potential against bacterial plant pathogens was found, with a group of 15 strains showing intense and wide range of *in vitro* antagonism. A principal component analysis depending on their inhibitory activity against *E. amylovora* allowed a good discrimination between *L. plantarum* strains by combining genotypic and phenotypic fingerprinting analysis. Strains TC92 and PM411 appeared as lead strains for further development of novel microbial biopesticides.

Chapter VI:

Control of fire blight with mixed inocula of two *Lactobacillus plantarum* strains and lactic acid

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Emilio Montesinos and Anna Bonaterra

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for publication in *Phytopathology*

G. Roselló participated in the design of experimental work, performed it, wrote the manuscript and read and approved the final manuscript.

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Roselló G., Francés J., Daranas N., Montesinos E. and Bonaterra A. (2015). Control of fire blight with mixed inocula of two Lactobacillus plantarum strains and lactic acid. Manuscript submitted for publication

Abstract

Lactobacillus plantarum strains TC92 and PM411 were able to grow *in vitro*, and to colonize and survive on pear plants, attaining the same population level either individually or mixed, thus indicating their compatibility. Lactic acid strongly inhibited *E. amylovora* both *in vitro* and when applied to plant surfaces, but had no effect on *L. plantarum*. Both, lactic acid or a mixture of strains TC92 and PM411 reduced infections by *E. amylovora* in pear plants. Interestingly, a combination of lactic acid and the two strains increased the efficacy and reliability of biocontrol of fire blight under controlled greenhouse conditions. The present study provides an example of the application of the multibarrier theory in fire blight control for future development of biopesticide formulations of lactic acid bacteria for fire blight management.

Chapter VII:

General Discussion

Crop protection has been reoriented to a sustainable use of pesticides and it is strongly affected by a reduction in the number of authorized active ingredients, especially in Europe. This fact together with the scarce availability of effective alternative strategies to control bacterial plant diseases has increased the interest in the development of biocontrol agents as active ingredients of pesticides.

Among the bacterial diseases of rosaceous plants, fire blight caused by *E. amylovora* is one of the most widespread, which has an important economic impact because it is difficult to control. The effective control requires the combination of different strategies being the application of active ingredients based on chemicals the most commonly used. However, the restrictions in the use of some products (e.g. antibiotics) or the development of resistance to active compounds, makes necessary to find novel methods such as biological control. Even though, there are several available commercial biological control agents (BCAs), their efficacy for fire blight control is generally low and variable (Sundin *et al.*, 2009; Ngugi *et al.*, 2011). Therefore, it is necessary to find new species and strains for BCAs of this disease.

The development of BCAs requires several steps such as isolation, selection, production, formulation and delivery as a commercial product, and finally the implementation in the plant protection management programs. For these purposes, the selection of lactic acid bacteria (LAB) strains as potential BCAs, their characterization and the improvement of their biocontrol efficacy have constituted the main objectives of this PhD thesis.

We have proposed LAB strains as novel BCAs for fire blight control. LAB have been traditionally used in food technology for the preservation of meat, dairy products and fermented vegetables (Ruiz-Barba *et al.*, 1994; Stiles & Holzapfel, 1997). Moreover, certain LAB strains efficiently control food-borne pathogens and postharvest fungi (Trias *et al.*, 2008a,b,c) or plant pathogenic bacteria (Visser *et al.*, 1986; Trias *et al.*, 2008c). In addition, LAB fulfil desired characteristics concerning biosafety. This aspect is very important in order to get the permit from the authorities at the time of registration and commercialization of a microbial pesticide (Montesinos & Bonaterra, 2009).

However, LAB have been rarely studied as BCA against plant pathogens, the majority of reported BCA for the fire blight biocontrol belong to *Bacillus*, *Pantoea* and *Pseudomonas* species, which represent some of the most common plant-associated epiphytes (Pusey *et al.*, 2009; Williams *et al.*, 2013). The typically used cultivation methods in surveys of plant microbiota have underestimated microbial diversity, but recent findings using different types of growth media or cultivation independent methods based in metagenomics allowed to uncover a wider range of microbial diversity (Yashiro *et al.*, 2011; Aleklett *et al.*, 2014). In this sense, recent investigations have shown a much broader diversity of microorganisms in the phyllosphere than previously known, including the presence of LAB species (Zwielehner *et al.*, 2008; Di Cagno *et al.*, 2008, 2010; Caponigro *et al.*, 2010; Leveau & Tech, 2011; Yashiro & McManus, 2012; Shade *et al.*, 2013; Williams *et al.*, 2013), thus open the possibility to explore these plant-associated microorganisms as BCA. In agreement with these reports our group obtained a collection of 539 LAB isolates from 842 samples from fresh plant sources collected in two surveys.

In a former study, a selection of isolates based in the antibacterial activity combined with the presence of several gene markers was used. The objective of the screening was to select, among the isolates, candidates able to inhibit *E. amylovora* and control fire blight in its plant host. Combining the results obtained in the *in vitro* assays and in the detection of bacteriocin-gene markers, 100 isolates were selected. The isolates had wide spectrum of antibacterial activity against target plant pathogenic bacteria, or specific antagonism activity against *E. amylovora*, and the presence of bacteriocin biosynthetic genes.

In the present study, a screening procedure based on the suppressive effect against *E. amylovora* infections in detached plant organs (flowers, fruits and leaves) has been used to select candidates of BCA from the above mentioned large collection of LAB (100 previously selected strains).

This type of assays allow the interaction between the host, the pathogen and the antagonist and have high correlation with the biocontrol ability in the field (Andrews, 1985; Handelsman *et al.*, 1990). This protocol has been also used

in other studies that considered detached flowers the best model to select microorganisms with inhibitory activity against *E. amylovora* infections. In other reports, a correlation between the ability of biocontrol of *E. amylovora* on flowers and their effectiveness in field has been reported (Mercier & Lindow, 2001). The methodology used in these assays in the present work has been optimized (phenological stage of plant organ, concentration of the BCA and the pathogen and application schedule). Disease incidence and severity depends on the phenological stage of the plant organ, being usually higher in younger than in older ones. In the present study, young leaves (leaves located 3-5 cm of the shoot apex) inoculated with *E. amylovora* exhibited a 75.5% of severity and a 93.3% of disease incidence, while old leaves reached only a 22.2% and 40% respectively. Other authors reported that the flower age also plays a role in infection (Pusey & Curry, 2004; Malnoy *et al.*, 2012). Consequently, young leaves and flowers, and immature fruits were used as detached plant organs in the assays performed. LAB isolates were applied at a concentration of 10^8 CFU ml^{-1}, which is the concentration reported generally as suitable in similar studies (Pusey, 1997; Mercier & Lindow, 2001; Cabrefiga *et al.*, 2007). Moreover, LAB were applied 24 h prior to the pathogen inoculation, to allow bacteria to colonize the surface of the plant organs, previously to the establishment of the pathogen, to prevent the increase of its population, as previously reported (Wilson & Lindow, 1993; Johnson & Stockwell, 1998, 2000; Stockwell *et al.*, 1999).

Eight strains of LAB (AC73, CM209, FC560, PC40, PM366, PM411, TC54 and TC92) have been selected because they showed a consistent suppressive activity against *E. amylovora* in most detached pear organs, in almost all experiments performed. Taking into account that the initial number of LAB isolates was 539, the yield of the screening process was of only a 1.5%. This yield is in accordance with another study performed with *Pseudomonas fluorescens* using *ex vivo* assays against *E. amylovora* with an efficiency of 1.5% (Cabrefiga, 2004), but lower than those achieved in other studies performed such as against *Stemphylium vesicarium* in pear leaves (7%) (Montesinos *et al.*, 1996) or against *Sclerotinia sclerotiorum* in bean buds (3%) (Yuen *et al.*, 1994).

Selected strains AC73, PC40, TC54, TC92 and PM411 have been identified by 16S rDNA sequencing as *L. plantarum* and harboured the plantaricin genes *plnEF* and *plnJK*. Besides, strains CM209 and PM366 were identified as *L. mesenteroides* and had the *mes* gene. However, strain FC560 could not be identified and did not present neither *pln* genes nor *mes* gene.

Four strains PM366, PM411, TC92 and TC54 exhibited a high *in vitro* antagonism activity against *E. amylovora* and to the other target bacteria, including both Gram positive and Gram negative bacteria. These antagonistic properties of LAB from plant origin have been previously reported (Visser *et al.*, 1986; Trias *et al.*, 2008c).

The applicability of selected strains as BCA was studied in more detail by means of colonization and control efficacy studies in whole plants and in mixed field-lab blossom experiments. Three of the eight selected strains, which have been identified as *L. plantarum* TC54, TC92 and PM411, were excellent candidates considering their effectiveness to control fire blight infections in pear potted plants and in mixed field-lab blossom assays, as well as their ability to colonize and survive in pear and apple flowers. Moreover, the efficacy of control of infections of *E. amylovora* in detached plant organs, the wide spectrum of antibacterial activity and the presence of the genes related with bacteriocin biosynthesis support and confirmed that would be excellent candidates as BCA.

The selection of *L. plantarum* strains as BCA candidates confirms the potential of certain strains of this species to be used as active ingredients of microbial pesticides for fire blight control. This fact encouraged us to search and characterize all plant-associated *L. plantarum* strains from our LAB collection using genotypic and phenotypic features to select lead strains that can be used as active ingredients of biopesticides.

L. plantarum species was found at a relatively low frequency, only 45 isolates out of 539 LAB from the collection. Interestingly, all of them were obtained from fresh vegetables and fruits but none from ready-to-eat produce. After that, this collection of isolates was characterized in order to search for robust criteria to select putative BCA with singularities. The commercial interest of

this species has stimulated studies to explore its diversity and to find lead strains. Each strain has different attributes, which are mainly strain-specific and confer to the strain its uniqueness (Di Cagno *et al.*, 2010). Furthermore, characterization at strain level is necessary prior to exploitation or patenting. In several studies, LAB strains have been phenotypically characterized by physiological and biochemical features, for example carbohydrate fermentation patterns. However, many LAB had similar nutritional requirements, and thus molecular techniques are necessary (Tanganurat *et al.*, 2009). Studies performing a genotypic characterization, used pulsed-field gel electrophoresis (PFGE) (Sánchez *et al.*, 2004), ribotyping and restriction fragment length polymorphism (RFLP) analysis of the PCR-16S rDNA intergenic spacer region (Rodas *et al.*, 2005), randomly amplified polymorphic DNA (RAPD – PCR) (Bringel *et al.*, 2001) or multilocus sequence typing (MLST) (de las Rivas *et al.*, 2006; Tanganurat *et al.*, 2009).

In the present study, the genotypic characterization has been addressed by means of MLST, RAPD-PCR and gene profiling, detecting the presence of genes related with plantaricin biosynthesis. Besides, a phenotypic characterization has been addressed determining the antagonism against different bacterial plant pathogens (*E. amylovora*, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *actinidiae*, *Xanthomonas fragariae* and *X. arboricola* pv. *pruni*) and more specifically, in the case of *E. amylovora,* the antagonism of cells and cell-free culture supernatants and the inhibition of infections in detached pear organs was also studied.

The MLST methodology used has been already reported with *L. plantarum* strains from wine and fermented food products that presented different profiles (de las Rivas *et al.*, 2006; Tanganurat *et al.*, 2009; Xu *et al.*, 2015). In the present work, MLST of the internal fragments of six reported housekeeping loci has been used to analyse selected 45 *L. plantarum* isolates from plant origin. Housekeeping genes are preferred because an analysis of mutations in these genes is more likely to reflect the phylogeny of strains since they evolve slowly. Therefore can indicate the genetic relationships among strains more accurately than genes under selective pressure (Picozzi *et al.*, 2010). The 45 isolates of *L. plantarum* of the present study exhibited eight different allelic combinations or sequence types (ST), but most of the isolates

(60%) were included in the same ST, which is according to a recent study of MLST in *L. plantarum* with different housekeeping genes, where the 39.7% of the isolates shared ST (Xu *et al.*, 2015). In a previous report, it has been suggested that strains that shared the same ST could be the same strain (de las Rivas *et al.*, 2006). In contrast, in our study, the strains of the same ST have been isolated from different samples (vegetables, location, time), thus probably are not siblings. This is in agreement with other reports that have found *Lactobacillus casei* or *Lactobacillus sanfranciscensis* strains sharing the same ST that came from different surveys (Cai *et al.*, 2007; Picozzi *et al.*, 2010).

However, a relationship between the MLST profile and the source of isolation has been found since strains sharing the same or similar ST were obtained from similar environmental sources (e.g. fruits or vegetables). This is in agreement with recent reports of *L. plantarum*, where isolates from the same environmental sources have similar nucleotide profile (Xu *et al.*, 2015). Also in a study of *Lactobacillus fermentum*, isolates were separated according to their origin, dairy products or acidic gruel (Dan *et al.*, 2015). However, this relationship has not been observed in other reports in *L. plantarum* (de las Rivas *et al.*, 2006; Tanganurat *et al.*, 2009), *L. sanfranciscensis* (Picozzi *et al.*, 2010) or *L. casei* (Cai *et al.*, 2007).

To study the origin of the allelic diversity of *L. plantarum* in the present work, a minimum spanning tree (MST) analysis was used using the MLST profiles from 71 strains, including 45 strains of the present study and 26 previously reported (de las Rivas *et al.*, 2006; Tanganurat *et al.*, 2009), that represented a total of 21 different STs. In this analysis, STs were mainly grouped into three clonal complexes (CCs) and 12 singletons. Interestingly, CC1 presented ST5 that is considered the primary founder. This supports a clonal mode of evolution in *L. plantarum*, which is reported in strains isolated from pickled vegetables (Tanganurat *et al.*, 2009). Moreover, in other LAB species a clonal population has been also reported, such as *L. fermentum* (Dan *et al.*, 2015), *L. sanfranciscensis* (Picozzi *et al.*, 2010) or *Leuconostoc gelidum* (Rahkila *et al.*, 2015).

However, the above mentioned reports contrast with a study that suggested a panmitic mode of evolution of *L. plantarum* because most of the STs were represented by a single strain (de las Rivas *et al.*, 2006). Moreover, these

authors consider that panmitic population may be so variable that identical ST are only found among isolates in direct contact. This hypothesis is contrary to the fact that the same ST has been found in strains from different origins (isolated in different places, times and sources).

To complement the genotypic characterization of the *L. plantarum* strains studied in the present work, a RAPD-PCR analysis was performed. Isolates have been assembled into three main groups and nine singletons according to their RAPD-PCR profile. In this case a relationship between the RAPD-PCR profile groups and the source of isolation has also been observed. This is in agreement with a report in *L. plantarum* (Di Cagno *et al.*, 2010) and in *Lactobacillus delbrueckii* (Cebeci & Gürakan, 2011).

The grouping of our strains obtained using RAPD-PCR profile are in concordance with STs of MLST analysis. Moreover, both techniques group the majority of the strains according to the type of isolation source. The concordance between clusters obtained using different genotypic methods has been already reported using RAPD-PCR and Amplified Fragment Length Polymorphism (AFLP) (Di Cagno *et al.*, 2010) and MLST and AFLP (Diancourt *et al.*, 2007).

The RAPD-PCR method used in the present study allowed also to distinguish *L. plantarum* strains because the combined use of five primers has given a high discriminatory power. In contrast, MLST analysis presented lower discriminatory power than RAPD-PCR. This fact also happened in a report that used the same techniques (Cebeci & Gürakan, 2011) or in another study that compared MLST with PFGE analysis (Picozzi *et al.*, 2010). However, some reports have stated MLST as a good discriminatory technique (de las Rivas *et al.*, 2006; Bilhère *et al.*, 2009). A possible optimization of the MLST analysis could be the addition of more housekeeping genes to the study.

The presence of genes involved in the plantaricin biosynthesis was studied in *L. plantarum* strains, in addition to MLST and RAPD analysis. This analysis revealed a high homogeneity in the plantaricin-genes profile (plantaritype) within the strains, with a dominant plantaritype identical to the reference strain *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003). This is in agreement

with a study of oenological isolates, which has reported that the plantaritype of strain WCFS1 was the most frequent (Sáenz *et al.*, 2009). However, other authors described more plantaritypes in isolates from fermented plant products (Ben Omar *et al.*, 2008; Knoll *et al.*, 2008; Hurtado *et al.*, 2011a).

The majority of the strains from our collection (42 out of 45) harbour the main operons involved in the plantaricin synthesis: regulatory operon (*plnABCD*), plantaricin operons (*plnEFI* and *plnJKLR*), transport operon (*plnGHSTUV*) and *plnMNOP* operon. Only three strains lacked some of selected genes (CC85, PC67 and CM450). Strain CC85 lacked *plnK* that would cause that plantaricin J loses the synergistically effect of plantaricin K, thus could reduce its antimicrobial activity (Diep *et al.*, 2009). Strain PC67 did not present the *plnM* gene which function is unknown (Diep *et al.*, 2009), although other authors suggested that encoded for an immunity protein (Rojo-Bezares *et al.*, 2008). Strain CM450 only presented the *plnGHSTUV* operon as has been previously reported in other *L. plantarum* strains from oenological origin (Sáenz *et al.*, 2009).

This homogeneity of plantaritype within the strains of the present study is not due to a common origin because strains with the same plantaritype pertain to different MLST or RAPD-PCR groups and originated from diverse plant sources. For example, strains FC248 and TC54 share the same plantaritype, but different MLST and RAPD-PCR groups, moreover FC248 was isolated from fruit of retail markets and TC54 came from raw vegetables.

Therefore, it is hypothesized that the prevalence of *pln* genes in plant-associated *L. plantarum* strains may confer a competitive advantage in this environment. Moreover, the production of bacteriocins may play a protective role and act to prohibit the invasion of other strains or species into an occupied niche (Riley & Werzt, 2002). This prevalence of *pln* genes could be explained by the transfer of genes between different strains of the same niche. Accordingly, it has been demonstrated that bacteriocin production in LAB is frequently associated to mobile genetic elements that may facilitate the transfer of genes between strains or species sharing the same niche (Dykes, 1995). For example, some plantaricin genes have been found in *L. fermentum* isolated in the same niche as *L. plantarum* (Ben Omar *et al.*, 2008). In addition, the base composition (G+C content) of the fully *pln* loci was lower

(Navarro *et al.*, 2008) than the overall G+C content reported for the whole chromosome of *L. plantarum* (Kleerebezem *et al.*, 2003), which suggest a high plasticity in this region (Navarro *et al.*, 2008). Regions encoding plantaricin biosynthesis were included in lifestyle adaptation islands and they gain and loss genes by horizontal genetic transfer (Molenaar *et al.*, 2005).

In relation to the phenotypic characterization, there was a strong diversity among isolates in the antibacterial profiles against the bacterial plant pathogens studied, and 15 strains presented a broad range and strong activity against all the target bacteria. This in agreement with reports that have demonstrated a high antagonistic activity of certain strains of *L. plantarum* against Gram positive and Gram negative bacteria (Elegado *et al.*, 2004; Trias *et al.*, 2008b; Ben Omar *et al.*, 2008; Knoll *et al.*, 2008). In these reports, *L. plantarum* strains tend to inhibit more Gram positive bacteria than Gram negative (Elegado *et al.*, 2004; Knoll *et al.*, 2008). This could explain why in the present study some of *L. plantarum* strains do not presented a strong antagonistic activity against the target bacteria, which are Gram negative.

The specific study of antagonistic and infection inhibitory activity against *E. amylovora* has confirmed the phenotypic heterogeneity among the strains. Most of *L. plantarum* strains exhibited moderate antagonistic activity in MRS medium, while only half of strains showed inhibition in LBP medium. As reported, the use of two media in the *in vitro* test increase the chance to obtain strains with antagonistic activity (Trias *et al.*, 2008b). All cell-free culture supernatants obtained from *L. plantarum* strains showed inhibitory activity against *E. amylovora*. The inhibitory activity is probably due to antimicrobial compounds, which are produced and excreted in the culture medium, such as organic acids, hydrogen peroxide or bacteriocins, as reported (Reis *et al.*, 2012). The differences of activity between strains could be due to the different quantities of antimicrobial compounds produced, as reported, the resistance to the antimicrobial compounds action of the target bacteria is dose-dependent (Elegado *et al.*, 2004).

The ability to inhibit *E. amylovora* infection in detached pear organs was very different among the studied *L. plantarum* strains. Some of them reduced infections consistently in all organs, whereas others had low activity or were only effective in one organ. Some of these strains (AC73, PM411, TC54 and TC92) had been previously chosen in the initial screening experiments in detached pear organs as good antagonists against *E. amylovora*, thus their efficacy has been confirmed. However, only two strains (TC92 and PM411) showed high and consistent efficacy against *E. amylovora* infections in detached pear organs together with high *in vitro* activity against bacterial plant pathogens. No relationship was observed between the *in vitro* activity and the inhibition of infections in detached pear organs. This indicates that in the inhibition of infections, besides the antibiosis, other mechanisms of action can be involved, as shown by previous studies in the biological control of *E. amylovora* (Vanneste *et al.*, 1992; Wilson *et al.*, 1992; Nuclo *et al.*, 1998; Cabrefiga, 2004).

No relationship was found between the presence of *pln* genes in the strains and its antagonistic activity. Strains that harboured the same plantaritype (including 20 genes involved in plantaricin biosynthesis) have different level of antagonistic activity, against *E. amylovora* and the other bacterial plant pathogens. The low antagonism could be related to the lack of some *pln* genes, but only strains CC85, PC67 and CM450 lacked some of these genes. In the case of CC85, this hypothesis could be right. However, the lack of *plnM* does not appear to affect the activity of strain PC67. Interestingly, strain CM450 exhibited a low activity against *E. amylovora*, which could be due to the lack of *pln* genes. Nevertheless, this strain exhibited antagonism against the other bacterial plant pathogens studied. The different antibacterial activity observed in CM450 against the phytopathogenic bacteria could be due to the resistance of some target bacteria to antimicrobial compounds produced by the strain. It has been reported that the resistance to bacteriocin action is species or strain-specific and dose-dependent (Elegado *et al.*, 2004). Thus, strain CM450 could produce enough amounts of organic acids or other antibacterial compounds that affect the studied target bacteria, but not *E. amylovora*. Also, it could be possible that strain CM450 presented other genes that encode bacteriocin different to those studied here. Other studies reported *L. plantarum* strains obtained from fruit and vegetables, that harbour different bacteriocins such as

plantaricin NA or C19, among others (Todorov, 2009). Moreover, the presence of plantaricin biosynthetic genes could not be always associated with the synthesis of the gene product. In other species such as *Bacillus* spp., specifically isolated from plant sources, it has been also described the lack of relationship between genes and their products for some genes (Mora *et al.*, 2015). A differential production of bacteriocins could be due to different expression levels of the genes or growth conditions (Hurtado *et al.*, 2011b; Doulgeraki *et al.*, 2013). Also, the lack of relationship between the presence of some genes and the production of bacteriocins could be due to the gene mutation as it has been described in *L. plantarum* PCS20 that fails to produce the functional two-peptide bacteriocin, plantaricin EF, due to a deletion of one nucleotide causing a frame shift of the open reading frame (Cho *et al.*, 2010). However, to confirm the production of plantaricin in the *L. plantarum* strains studied, it would be necessary to determine the expression of *pln* genes using Real-Time RT-PCR. Also, plantaricins should be purified and identified according to their mass using for example, matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF). In addition, the role of plantaricin production in the antagonistic activity in *L. plantarum* should be studied by mutagenesis of the genes that encoded the plantaricins.

Therefore, as a conclusion, any relationship was found between antagonistic activity and genotypic patterns studied. Finding a relationship between the antagonism and a genotypic trait, would be useful as a strategy to design a genetic marker for screening to select putative BCA. In this way, the selection and screening of isolates having a high antagonistic activity would be easier and faster, such as in *Bacillus* spp., where the presence of biosynthetic genes of antimicrobial peptides (AMP) have been related with the antagonism (Mora *et al.*, 2015).

The combination of genotypic and phenotypic methods has been useful to allow a higher discrimination between the strains. This is in agreement with a previous study that recommended the combination of biochemical and genotypic characterization as a better option to characterize isolates (Tanganurat *et al.*, 2009). To find lead strains candidates as active ingredients of biopesticides it would be useful the multivariate statistical analysis

combining phenotypic and genotypic characterization. Multivariate statistical analysis has been used to detect groups of isolates showing interesting patterns of properties, which have not been detected with univariate analysis of distribution (Piraino *et al.*, 2008). Principal component analysis (PCA) and other multivariate techniques, such as cluster analysis, have been successfully used to summarize the diversity of characteristics of the studied LAB. For example, in other reports PCA has been used to study the properties of several species of LAB isolated of different cheeses (Piraino *et al.*, 2008) or the microbial diversity of *Streptococcus thermophilus* (Giraffa *et al.*, 2001). In our study, combining the results of antagonism and infection inhibitory activity against *E. amylovora* in a PCA, strains with a good profile of activity (e.g. TC54, TC92 and PM411) or with low activity (e.g. CC85 and CM450) were clearly separated. This confirms that the graphical representation in the PCA biplot has allowed the easy selection of strains with interesting combination of properties, confirming the value of this technique in screening studies (Piraino *et al.*, 2008).

Besides, we observed that strain TC54 and strain TC92 were genotypically similar because both presented the same ST in MLST (ST16) and belong to the same RAPD-PCR group (G_A). Whereas, strain PM411 showed different profile both in MLST and in RAPD-PCR, ST21 and G_B respectively. With respect to antibacterial activity against plant pathogenic bacteria, strains TC92 and PM411 exhibited a higher activity than strain TC54. Due to the clear genotypic differences and to the broad antagonistic activity against plant pathogenic bacteria, strains TC92 and PM411 have been selected as suitable candidates for future development of biopesticides for fire blight management and this application has been protected with a patent (Patent Spain, Nº P201330685, 2013).

Besides the use of lead strains as suitable BCA for fire blight control, these strains could be utilised to control other plant pathogenic bacteria different from *E. amylovora*. These strains also exhibited antagonism against *P. syringae* pv. *syringae*, *P. syringae* pv. *actinidiae*, *X. fragariae* and *X. arboricola* pv. *pruni*. The antagonistic activity of LAB against plant pathogenic bacteria was previously reported (Trias *et al.*, 2008c; Shrestha *et al.*, 2014), as well as their activity against postharvest fungi and moulds (Magnusson *et al.*, 2003; Trias *et al.*, 2008c; Dalié *et al.*, 2010).

However, there are currently few reports about *L. plantarum* strains against plant pathogenic bacteria (Visser *et al.*, 1986), but there are more in fungi and mould control (Zamani-Zadeh *et al.*, 2014; Baffoni *et al.*, 2015). Therefore, the use of *L. plantarum* strains active against phytopathogens could be a novel approach for plant disease control.

In the last part of the present work, we have tried to enhance the level of biocontrol of *L. plantarum* strains, TC92 and PM411, against *E. amylovora* following two strategies: the mixture of these *L. plantarum* strains and their combination with lactic acid. This improvement step is usually required in the development of a BCA to increase the biocontrol activity and reliability. In our case, the efficacy of strains TC92 and PM411 in fire blight control, both in whole pear plants and in mixed field-lab blossom assays, was significant, but suffered of certain inconsistency from trial to trial.

The compatibility of TC92 and PM411 strains has been studied *in vitro* and in plant surfaces. According to other authors strains have to be compatible in the nutrients and space utilization, and have to complement their antagonistic activity, without interfering (Lutz *et al.*, 2004; Agustí *et al.*, 2011).

Thus, to determine the *in vitro* compatibility between strains TC92 and PM411, a defined medium was used instead of the rich MRS medium, to simulate the growth conditions in plant environments. This defined medium contained glucose as a main carbon source, casamino acids as the main nitrogen source and some salts, which are present in the most of suitable mediums for *L. plantarum* (de Man *et al.*, 1960; Schillinger & Lücke, 1989; Wegkamp *et al.*, 2010). Glucose is one of the most abundant sugars in the stigmatic secretions of pomaceous flowers, together with fructose (Pusey *et al.*, 2008a). Therefore, this monosaccharide was used in the defined medium to simulate the chemical composition of plant surfaces. Even though the quantities of free amino acids detected in stigma exudates are lower than sugar levels (Pusey *et al.*, 2008a), in defined medium the quantity of the nitrogen source are higher than glucose. This is because *L. plantarum*, as the majority of LAB, could be auxotrophic for multiple amino acids and vitamins, which can vary remarkably between different strains (Wegkamp *et al.*, 2010), thus it is necessary to include them in the media.

L. plantarum TC92 and PM411 showed compatibility *in vitro* and in plant surfaces, and these strains can cohabit the same ecological niche. This agrees with the fact that some LAB mixtures have been traditionally used in the field of food technology as starters, bioprotectives or probiotics (Seo *et al.*, 2013; Tirloni *et al.*, 2014; Toscano *et al.*, 2014; Xiong *et al.*, 2014).

Mixed application of *L. plantarum* strains, TC92 and PM411, has improved biocontrol of *E. amylovora* and was more consistent in the reduction of infections compared to the individual strain treatment, but not in all assays. These results are in agreement with other studies performed on different pathogens and plant hosts suggesting that the application of more than one antagonist would increase biocontrol efficacy (Janisiewicz, 1988; Spadaro & Gullino, 2005; Stockwell *et al.*, 2011). In the majority of the reported BCAs mixtures, strains of different species are combined, that increases the probability to have different mechanisms of biocontrol (Duffy & Weller, 1995; Guetsky *et al.*, 2001, 2002; Lutz *et al.*, 2004; Stockwell *et al.*, 2010, 2011). However, there are also BCAs mixtures of strains of the same species, such in the present study (de Boer *et al.*, 2003; Agustí *et al.*, 2011; Kunz & Donat, 2014; Yang *et al.*, 2015). The antagonistic capacity of our two LAB strains could be due to different mechanisms of action, including antibiosis and competition for nutrients and space (Alakomi *et al.*, 2000; Cleveland *et al.*, 2001; Trias *et al.*, 2008a).

We have additional evidences that *L. plantarum* strains, TC92 and PM411, have differential traits related to plant surface colonization. Particularly, differences in cell attachment to leaves surfaces were observed. Both strains present structures attaching cells to the leaf surface in cell envelope, but interestingly, strain PM411 has more attaching structures than TC92. Taking into account the phenotypic, genotypic and morphological differences between strains PM411 and TC92, it could be hypothesized that might complement each other, as in other studies with mixtures of compatible BCA (Duffy & Weller, 1995; Guetsky *et al.*, 2001, 2002; Pusey & Curry, 2004; Spadaro & Gullino, 2005; Agustí *et al.*, 2011; Stockwell *et al.*, 2011).

Although the mixture of strains, TC92 and PM411, has sometimes improved the biocontrol, it was judged not sufficient for field application. Thus, another strategy has been studied consisting of the combination of the mixture of strains with a non-toxic compound, as co-formulant, such as lactic acid. *L.*

plantarum TC92 and PM411 are tolerant to the lactic acid, due to the fact that these bacteria produce and tolerate lactic acid and other organic acids produced during fermentation (McDonald *et al.*, 1990; Reis *et al.*, 2012). In contrast, *E. amylovora* was strongly inhibited by lactic acid, even though that *E. amylovora* can produce small amounts of this acid from glucose in fermentative metabolism (Paulin, 2000). The capacity of lactic acid to inhibit the growth of many Gram positive and negative bacteria has already been reported and it causes physiological and morphological adverse changes in bacterial cells (Alakomi *et al.*, 2000; Wang *et al.*, 2015).

The methodology used to study the tolerance of lactic acid by *L. plantarum* and *E. amylovora* strains consisted of the application of different concentrations of lactic acid *in vitro* and on the pear plant assays. The concentration of lactic acid affecting survival of *E. amylovora*, but not of *L. plantarum* strains, was 0.05%. However, on pear plants assay the suitable concentration was 0.5%, 10-fold higher than those used *in vitro*. Nevertheless, the concentration of lactic acid required in our studies was lower than the doses reported for food borne pathogens control (Park *et al.*, 2011; Seo *et al.*, 2013). Indeed, we have not used such high concentrations because a concentration higher than 0.5% caused phytotoxicity in the younger leaves of the pear plants.

Lactic acid alone reduced fire blight infections in plants, but their effect was not always consistent. However, when lactic acid was combined with *L. plantarum* strains, a clear improvement in fire blight control was observed. Lactic acid might reduce the environmental pH and might inhibit the *E. amylovora* growth, allowing for a better LAB colonization of plant surfaces. A similar action has been reported by the citric acid in combination with two strains of *A. pullulans* (Kunz *et al.*, 2011). In this report, the addition of citric acid enhanced the efficiency of the mixture of *A. pullulans* strains, as we have observed with the addition of lactic acid in our study.

The combination of lactic acid with a *L. plantarum* strains mixture is an effective strategy to improve the control of fire blight and would be suitable for future development of novel biopesticide formulations, providing an example of the multibarrier theory.

Although the combination of lactic acid with the mixture of two *L. plantarum* strains is a suitable strategy for future development of a novel biopesticide for fire blight control, it would be necessary to evaluate their efficacy, in field tests under different agricultural and climatic conditions. Moreover, more experiments on colonization and survival studies in the field are also needed. Furthermore, the population levels of BCAs should remain stable or should exceed a concentration limit, below which inhibition of *E. amylovora* is not achieved. In *L. plantarum* strains, this threshold concentration is unknown, as the time that they remain in the plant tissue above this limit. Both data are important to decide the best moment for the BCA application. Probably, multiple applications of *L. plantarum* strains may be necessary to optimize biocontrol, like in the case of other BCAs for fire blight control (Nuclo *et al.*, 1998).

Additional strategies different to mixtures of strains amended with lactic acid can be used to improve the efficacy of *L. plantarum* strains in the fire blight control. Possible strategies might be the physiological improvement by osmoadaptation and nutritional enhancement that improve the survival and fitness. The usefulness of this approach has been reported in the fire blight biocontrol agent *P. fluorescens* EPS62e (Bonaterra *et al.*, 2007; Cabrefiga *et al.*, 2011).

Finally, to develop a microbial biopesticide with *L. plantarum* strains, a suitable formulation as a dried product is required to increase shelf-life, but also to allow a good storage and transportation.

As a general conclusion of the present work, *L. plantarum* strains TC92 and PM411 are suitable candidates as BCA of fire blight because they are potent antagonists of *E. amylovora* and efficient colonizers of plant hosts. The improvement of the efficacy and reliability in fire blight control with the mixture of these strains combined with lactic acid, encourage us to continue working to develop of a novel biopesticide formulation for fire blight management. Moreover, they exhibited intense and broad antagonistic activity against other plant pathogenic bacteria, which allows to follow the research to use these strains for the control of other plant bacterial diseases.

Chapter VIII:

Conclusions

1. The screening procedure based on the consistency of the suppressive effect against *Erwinia amylovora* infections in detached plant organs (flowers, fruits and leaves) was a good strategy to select candidates of biocontrol agents. LAB strain FC560, *Lactobacillus plantarum* AC73, *Leuconostoc mesenteroides* CM209, *L. plantarum* PC40, *L. mesenteroides* PM366, *L. plantarum* PM411, *L. plantarum* TC54 and *L. plantarum* TC92 have been selected to have a consistent suppressive effect against *E. amylovora*.

2. *Lactobacillus plantarum* strains TC54, TC92 and PM411 have potential to be used as biological control agents of fire blight disease because they were effective in preventing fire blight infections and they efficiently colonized and survived in pear and apple flowers. Also, they exhibited a high antagonistic activity against *E. amylovora* and other target bacteria and presented the genes involved in plantaricin biosynthesis.

3. Plant-associated *L. plantarum* strains have a low genotypic heterogeneity. The majority of strains shared the same sequence type profile of six housekeeping genes (*pgm*, *ddl*, *gyrB*, *purK1*, *gdh* and *mutS*) analysed using MLST. They clustered into three main groups depending on the combined RAPD-PCR analysis. Moreover, the majority of the isolates shared the same plantaritype when the *pln* cluster was analysed.

4. A clonal mode of evolution of plant-associated *L. plantarum* has been suggested using minimum spanning tree analysis over the six housekeeping loci *pgm*, *ddl*, *gyrB*, *purK1*, *gdh* and *mutS* used in MLST analysis.

5. A relationship between genotypic patterns (MLST and RAPDs) and the source of isolation of *L. plantarum* strains has been found. In particular isolates obtained from raw vegetables were clearly separated from those obtained from fruits. However, no relationship between antagonistic activity and genotypic patterns (MLST, RAPDs and plantaritypes) has been found.

6. A high heterogeneity was found among *L. plantarum* strains in the antagonistic potential against bacterial plant pathogens (*E. amylovora*, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *actinidiae*, *Xanthomonas fragariae* and *X. arboricola* pv. *pruni)* and in the antagonism and infection inhibition activity against *E. amylovora*.

7. Strains TC92 and PM411 appeared as lead candidates to be used as active ingredients for future biopesticides. Their singularities have been revealed using a principal component analysis based on the inhibitory activity against *E. amylovora*, and by combining the genotypic and phenotypic fingerprinting analysis.

8. The application of the multibarrier theory, based on the combination of lactic acid (as co-formulant) and the mixture of strains TC92 and PM411, increased the efficacy and reliability of fire blight control. Therefore, this strategy would be a suitable approach for future development of novel biopesticide formulations for fire blight management.

Chapter IX:

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