Telma Maria Sampaio Azevedo

LOQUAT (*ERIOBOTRYA JAPONICA* LINDL) MICROBIOTA: SCREENING OF POTENTIAL BIOCONTROL AGENTS AGAINST *ERWINIA AMYLOVORA*

MICROBIOTA DE NESPEREIRA (*ERIOBOTRYA JAPONICA* LINDL): PESQUISA DE POTENCIAIS AGENTES DE CONTROLO BIOLÓGICO CONTRA *ERWINIA AMYLOVORA*

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Professora Doutora María Milagros López

E co-orientação das
Professora Doutora Luísa Moura

Professora Doutora Ester Marco-Noales

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ABSTRACT

Fire blight, caused by the bacterium *Erwinia amylovora*, is one of the most destructive diseases that affect crops of the Rosaceae family, representing a serious threat to orchards and ornamental species of a great economic interest. Due to its pathogenicity, *E. amylovora* is considered a quarantine organism in the European Union. Portugal is, since 2008, recognized as a “Protected Area” for this bacterium.

The lack of available efficient phytochemicals and antibiotics, as well as their high toxicity and resistance, have led to the innumerous research work to screen for new organisms with a biological control action. Nowadays biological control active ingredients are already present in some commercialized products, used as a preventive measure against *E. amylovora*. However, further studies are necessary in investigating new strains or species with biopesticide action for specific crops.

The main objective of this work was to select for possible antagonists against *E. amylovora* from microbiota obtained from loquat (*Eriobotrya japonica*) flowers and leaves. For this reason, distinct isolation methodologies, characterization and ex vivo assays were performed in fruits and flowers in order to evaluate the efficacy of the strains in study on delaying the onset of symptoms caused by pathogen infection, as well as the incidence and severity of this infection.

Out of the 173 bacterial isolates studied, 4 species (*Rosenbergiella epipactidis, Pseudomonas rhizosphaerae, Curtobacterium flaccumfaciens* and *Enterobacter cancerogenus*) were selected since they delayed the onset of fruit necrosis, being associated with low levels of incidence and severity of disease infection (high efficacy of antagonistic activity), as well as no pathogenicity. Since flower stigmas are the more appropriate organs to *E. amylovora* colonization and development, two flower bioassays were conducted to assess the antagonistic activity of these bacterial species: in complete flowers (*R. epipactidis, P. rhizosphaerae, C. flaccumfaciens* and *E. cancerogenus*) and in flowers only with hypanthium and peduncle (*R. epipactidis* and *P. rhizosphaerae*).

The molecular analysis by real-time PCR to quantify *E. amylovora* concentrations after treating the flowers with a selected bacterial antagonistic

species did not show a relationship between the *E. amylovora* concentration and the observed symptoms in both flower bioassays.

Key-words:

Fire blight, *Erwinia amylovora*, pathogen, bacteria, microbiota, Spain, Alicante, biocontrol, loquat, *Eriobotrya japonica*, fruit, wound, flower, blossom, hypanthium, peduncle, leaf, infection, symptom, necrosis, incidence, severity, severity index, efficacy, efficacy scale, antagonism, inhibition, bioassay, inoculation, *ex vivo*, *in vitro*, King's medium B, isolate, strain, morphology, morphotypes, Gram reaction, oxidase reaction, API system, hypersensitive response, tobacco, PCR, *Bacillus*, 16s rRNA gene, real-time PCR, qPCR, quantification, growth curve, *Rosenbergiella epipactidis*, *Pseudomonas rhizosphaerae*, *Curtobacterium flaccumfaciens*, *Enterobacter cancerogenus*.

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RESUMO

O fogo bacteriano, causado pela bactéria *Erwinia amylovora*, é uma das doenças mais destrutivas de inúmeras culturas da família das Rosáceas, representando uma séria ameaça a árvores de fruto e espécies ornamentais de grande interesse económico. Devido à sua elevada patogenicidade, *E. amylovora* é considerada um organismo de quarentena na União Europeia. Portugal é, desde 2008, reconhecido como "Zona Protegida" para a bactéria.

Os resultados pouco satisfatórios obtidos pela utilização de fitoquímicos e antibióticos, acrescidos da elevada toxicidade e resistências dos mesmos no combate à bactéria, conduziram a diversas pesquisas por novos organismos com modo de ação biológica. Atualmente já são comercializados alguns produtos que possuem como substâncias ativas agentes de controlo biológico, usados como ação preventiva contra *E. amylovora*. Contudo, são necessários mais estudos na procura de novas estirpes ou espécies com ação biopesticida.

Este trabalho teve como principal objetivo selecionar possíveis antagonistas contra *E. amylovora* a partir da microbiota de flores e folhas de magnórios (*Eriobotrya japonica*). Para isso, foram realizadas diversas metodologias de isolamento, caracterização e ensaios *ex vivo* em frutos e flores para avaliação da eficácia das estirpes em estudo na redução do aparecimento de sintomas resultantes da infeção pelo patogéneo, bem como incidência e severidade desta infeção.

Em 173 isolados bacterianos estudados, 4 espécies (*Rosenbergiella epipactidis, Pseudomonas rhizosphaerae, Curtobacterium flaccumfaciens* e *Enterobacter cancerogenus*) foram selecionadas uma vez que atrasaram mais o aparecimento de necrose em fruto, tendo conduzido a níveis de incidência e severidade baixos da doença (elevada eficácia de atividade antagonista), além de não apresentarem patogenicidade. Sendo os estigmas das flores os órgãos mais propícios para a colonização e desenvolvimento de *E. amylovora,* procedeu-se à realização de dois bioensaios em flores para testar a ação antagonista destas espécies: em flores completas (*R. epipactidis, P. rhizosphaerae, C. flaccumfaciens e E. cancerogenus*) e em flores apenas com o hipanto e pedúnculo (*R. epipactidis, P. rhizosphaerae).*

A análise molecular através de PCR em tempo real, para quantificar a concentração de *E. amylovora*, após inoculação de flores com as espécies antagonistas selecionadas não evidenciou uma relação entre a concentração de *E. amylovora* e os sintomas observados em ambos os bioensaios realizados em flores.

Palavras-chave:

Fogo bacteriano, *Erwinia amylovora*, patogéneo, bactéria, microbiota, Espanha, Alicante, bio controlo, magnório, *Eriobotrya japonica*, fruto, incisão, flor, hipanto, pedúnculo, folha, infeção, sintoma, necrose, incidência, severidade, índice de severidade, eficácia, escala de eficácia, antagonismo, inibição, bioensaio, inoculação, *ex vivo*, *in vitro*, meio King B, isolado, estirpe, morfologia, morfotipo, reação Gram, reação oxidase, sistema API, resposta hipersensitiva, tabaco, PCR, *Bacillus*, gene 16s rRNA, PCR em tempo real, qPCR, quantificação, curva de crescimento, *Rosenbergiella epipactidis*, *Pseudomonas rhizosphaerae*, *Curtobacterium flaccumfaciens*, *Enterobacter cancerogenus*.

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Abbreviations

ANOVA – Analysis of Variance (also referred to as AOV)

API – Analytical Profile Index

BCA – Biological Control Agent

bp – Base pairs

CECT – Colección Española de Cultivos Tipo

CFBP – Collection Française des Bactéries Phytopathogénes

CFU – Colony-forming units

Cp – Crossing Point in quantitative PCR (also known as Ct, threshold cycle)

DNA – Deoxyribonucleic acid

Ea – *Erwinia amylovora*

EPPO – European and Mediterranean Plant Protection Organization

HR – Hypersensitive Response

IPVC – Instituto Politécnico de Viana do Castelo

IVIA – Instituto Valenciano de Investigaciones Agrarias

KB – King’s medium B

OD – Optical density

PCR – Polymerase Chain reaction

PDO – Protected designation of origin

Pv – Pathovar

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

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CHAPTER I

GENERAL INTRODUCTION

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1 Fire blight disease

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill 1882) Winslow *et al.,* 1920, is the most devastating necrotic disease that affects more than 180 plant species from 39 genera of the *Rosaceae* family. It represents an enormous threat to fruit and ornamental rosaceous plants in many parts of the world (Malnoy et al., 2012; van der Zwet and Keil, 1979).

The most economically important hosts are pear (*Pyrus communis*), apple (*Malus domestica*), loquat (*Eriobotrya japonica*), quince (*Cydonia* oblonga), *Cotoneaster* spp., hawthorn (*Crataegus* spp.), firethorn (*Pyracantha* spp.) and rowan (*Sorbus* spp.). Other hosts include *Chaenomeles* spp., *Mespilus* spp., *Photinia* spp. and other species that grow wild or are used as ornamental plants in landscape plantings. This disease causes progressive death of the host, being highly contagious and difficult to control. The impact of fire blight on production is highly variable, depending mostly on climatic conditions during spring. A severe outbreak of this disease can disrupt orchard production for several years, increasing production costs (EFSA, 2014; Thomson, 2000; van der Zwet and Keil, 1979).

Despite numerous studies and hundreds of publications on the epidemiology of fire blight, there is a constant emergence of new information about this organism (Vrancken et al., 2013).

1.1 History and geographical distribution

Fire blight was believed to be indigenous to North America (presumably from *Crataegus* hosts), from where it is has spread world-wide. The earliest known observations of the disease were made in 1780, on apple, pear and quince, in the Hudson valley of New York State (USA). In 1919 it was identified in New Zealand, and in 1957 was detected in South England, Europe. Fire blight invaded a large area around the Mediterranean Sea. It most probably spread from an initial outbreak detected in the Nile delta region of Egypt in 1964 (van der Zwet and Keil, 1979). The disease was later found in Crete (Greece), Israel, Turkey, Lebanon, Iran and countries of Central Europe (Thomson, 2000).

The introduction of *E. amylovora* in England and Egypt resulted in one continuous zone infected by fire blight, which encompasses most of Western Europe and most of the Mediterranean region. In 1998, all countries belonging to the European Union (except Portugal) had fire blight on pears, apples or ornamentals, either widespread (England,

Belgium, Germany), localized (France, Switzerland) or in restricted spots, under control and local eradication (Spain, Italy, Austria) (Thomson, 2000). Nowadays the situation has changed and fire blight is spread by the majority of countries of the northern and central Europe, the Mediterranean area and Middle East. Nevertheless, large areas of the world seem to be free of fire blight (South America, most of Africa and Asia), although the disease has recently been described in Morocco (CABI, 2016).

Specific regions within the EU have been designated as protected zone against certain pests and diseases. In Spain, fire blight was first detected in 1995, in Guipúzcoa (Basque country). The disease was found in a cider apple orchard close to the Atlantic French border. Other single outbreaks have been detected and eradicated in different regions of Spain: Navarra, Castilla y León (1996), Castilla – La Mancha, Catalunya, Aragón, La Rioja (1998). Since these outbreaks were eradicated, Spain was considered for many years to be a Protected Area. However, in 2001 certain regions (Castilla y León, Extremadura, Castilla la Mancha, La Rioja, Navarra, Guipúzcoa, Aragón, Murcia and Valencia) lost the recognition of the status of Protected Zone for fire blight, because the disease had been established in all or part of its territory (López et al., 1999; MAGRAMA, 2016) (Fig. 1).

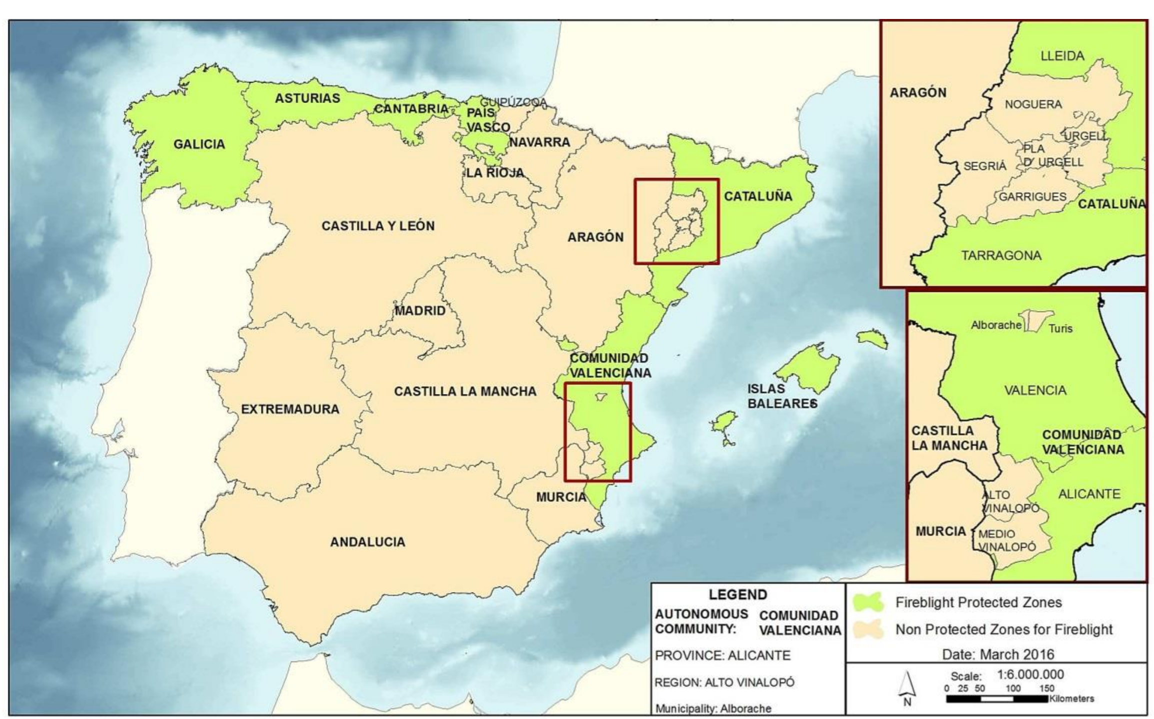

Figure 1 – Distribution of protected regions against fire blight in Spain.
In green colour, protected zones and in yellow non-protected ones.
[Adapted from (MAGRAMA, 2016)]

In Portugal, some fire blight outbreaks were detected in Fundão (2006), west region in pear and apple orchards (2010) and in Centre Region (Guarda, Viseu) and Alentejo in apple, pear and quince (2011), then control and eradication measures (DGADR, 2011). However, according to Regulation (EC) No 690/2008 of the Commission of 4 July 2008, the entire country is now recognized as a Protected Area for *E. amylovora*, recognition obtained as a result of official surveys.

1.2 The pathogen: *Erwinia amylovora*

Erwinia amylovora is a Gram-negative, facultatively anaerobic and pathogenic bacterium, belonging to *Enterobacteriaceae* family, Proteobacteria division and ϒ subdivision. Cells are rod shaped, about 0.3 μm × 1–3 μm in size, non-sporulated and produce an exopolysaccharide capsule. They are motile by peritrichous flagella (Fig. 2) and its mobility has been shown to be associated with specific chemotaxis, being the optimal conditions 20ºC and a pH of 6,8 (Raymundo and Ries, 1981, 1980). Although *E. amylovora* is capable of growing between 3-5ºC and 37ºC, the optimal temperature is 25-37ºC (Paulin, 2000). The first strains of *E. amylovora* that were sequenced were *E. amylovora* 273 (ATCC 49946) and *E. amylovora* CFBP 1430 (EFSA, 2014).

E. amylovora was the first bacterium identified as a plant pathogen and the first shown to be spread by insects (Baker, 1971; Malnoy et al., 2012; Thomson, 2000). Furthermore, is the only bacterium capable of inducing fire blight (Eastgate, 2000). The pathogen can survive as an epiphyte (on the surface of various host tissues), as endophyte (inside the vascular system of the plant) or in latent infections for variable periods, depending on environmental factors (Van der Zwet et al., 1988).

In contrast to most plant pathogenic bacteria that induce necrosis, *E. amylovora* can travel rapidly and extensively from the point of infection. Also remarkable is the ability of the pathogen to spread and survive within host tissues (Thomson, 2000). *E. amylovora* is considered as a quarantine bacterium, classified as a regulated organism in the European Union (EU Council Directive 2000/29, Annex designation II/A2) (Gottsberger, 2010; Vrancken et al., 2013)

Figure 2 – Electron micrograph of an *Erwinia* cell showing peritrichous flagella.
[Source: Johnson (2000)]

1.3 Pathogenicity

The exopolysaccharide (EPS) capsule of *E. amylovora* has been suggested to play a key role in its pathogenicity, by passing the plant defence system, in disturbing and obstructing the vascular system of the plant, and in protecting the bacteria against water and nutrient loss during dry conditions. The major exopolysaccharide produced by *E. amylovora* is amylovoran, which is the main constituent of bacterial ooze. Koczan et al. (2009) discovered that EPS of *E. amylovora* are also involved in biofilm formation, which enables the bacteria to attach to several surfaces and each other (Koczan et al., 2009).

Several studies have identified two essential components for pathogenesis: *ams* genes that are involved in the biosynthesis of amylovoran, and *hrp* genes that are necessary for the regulation, secretion and production of proteins, in particular, those called *harpins*. These proteins seem to interact with the plant cell wall and are necessary for pathogenicity in host plants and the induction of a hypersensitive reaction (HR) on non-host plants (Eastgate, 2000; Khan et al., 2012; Thomson, 2000).

1.4 Disease cycle

The development of fire blight symptoms appears cyclic in nature because it follows the seasonal growth development of the host plant.

E. amylovora overwinters in cankers on twigs, branches, or trunks that were formed on diseased host trees in previous years, thus providing the inoculum for the following season. Cankers are discoloured or depressed lateral areas in the bark, which upon debarking often exhibit reddish-orange and humid tissues in the cortical parenchyma. In spring, when the weather is sufficiently warm and moist and trees resume growth, the pathogen becomes active in the margins of the cankers (Fig. 3) (Thomson, 2000).

Masses of bacterial population are released in the bark surface, forming bacterial ooze (exudates), producing the primary inoculum (Stockwell et al., 1998). Ooze consists of bacterial cells embedded in a polysaccharide matrix that protects cells from desiccation and other abiotic stress factors and is attractive to insects, that can disseminate bacterial cells to flowers (Malnoy et al., 2012).

The primary inoculum is spread by wind driven rain or disseminated by insects (mostly bees, flies and ants) to open blossoms or to wounded shoots. Once on blossoms, *E. amylovora* has the ability to rapidly multiply in an epiphytic phase on floral surface, including stigmas, anthers, hypanthia and stomata (Johnson and Stockwell, 1998; Thomson, 2000). Stigma associated bacteria can be washed into the nectary where a readily available nutrient supply facilitates *E. amylovora* growth. Stigma exudates can support pathogen growth to densities as large as 10^5–10^6 cells per flower (EFSA, 2014).

The bacterium is then transmitted from blossom to blossom by rainfall or more likely by the action of pollinating insects (Malnoy et al., 2012). The primary infection (blossom blight) begins when *E. amylovora* infects the host plant through nectarhodes, which are the main entrance pathway (endophytic phase), from the same orchard or from surrounding areas. A secondary inoculum is produced on infected tissues as a result of the primary infection, originating shoot, fruit and rootstock blight (secondary infection).

The pathogen needs openings on plant surface to produce shoot and fruit blight. Even though there are natural entries, is more common the entrance through wounds, which are caused by sucking insects, wind whipping, hail or pruning (Johnson and Stockwell, 1998). Once the pathogen penetrates the plant, multiplies into intercellular spaces and moves rapidly through the cortical parenchyma, phloem and xylem vessels, infecting shoots (Thomson, 2000).

In summer, secondary infections are favoured by a combination of wounds produced by hail or strong thunderstorms and the presence of contaminated pollen and insect pollinators. Both primary and secondary infections can occur during spring or summer, if the environmental conditions, temperature and humidity are favourable. Despite this, secondary infections occur more often and can cause more damage to trees. In autumn and winter, plant tissues are less susceptible to infections, and generally the progression of infections slows down or stops due to low temperatures and decreased vegetative growth (Thomson, 2000).

Infection events only occur when the host is in a susceptible condition, pathogen inoculum level is adequate and environmental conditions are suitable. Orchard workers

can also facilitate both pathogen dissemination and plant infection by means of hands, clothing, or use of contaminated pruning tools (van der Zwet and Keil, 1979; EFSA, 2014).

Finally, cultivar susceptibility plays a major role in the amount of disease caused by same inoculum pressure (Bastas and Maden, 2007).

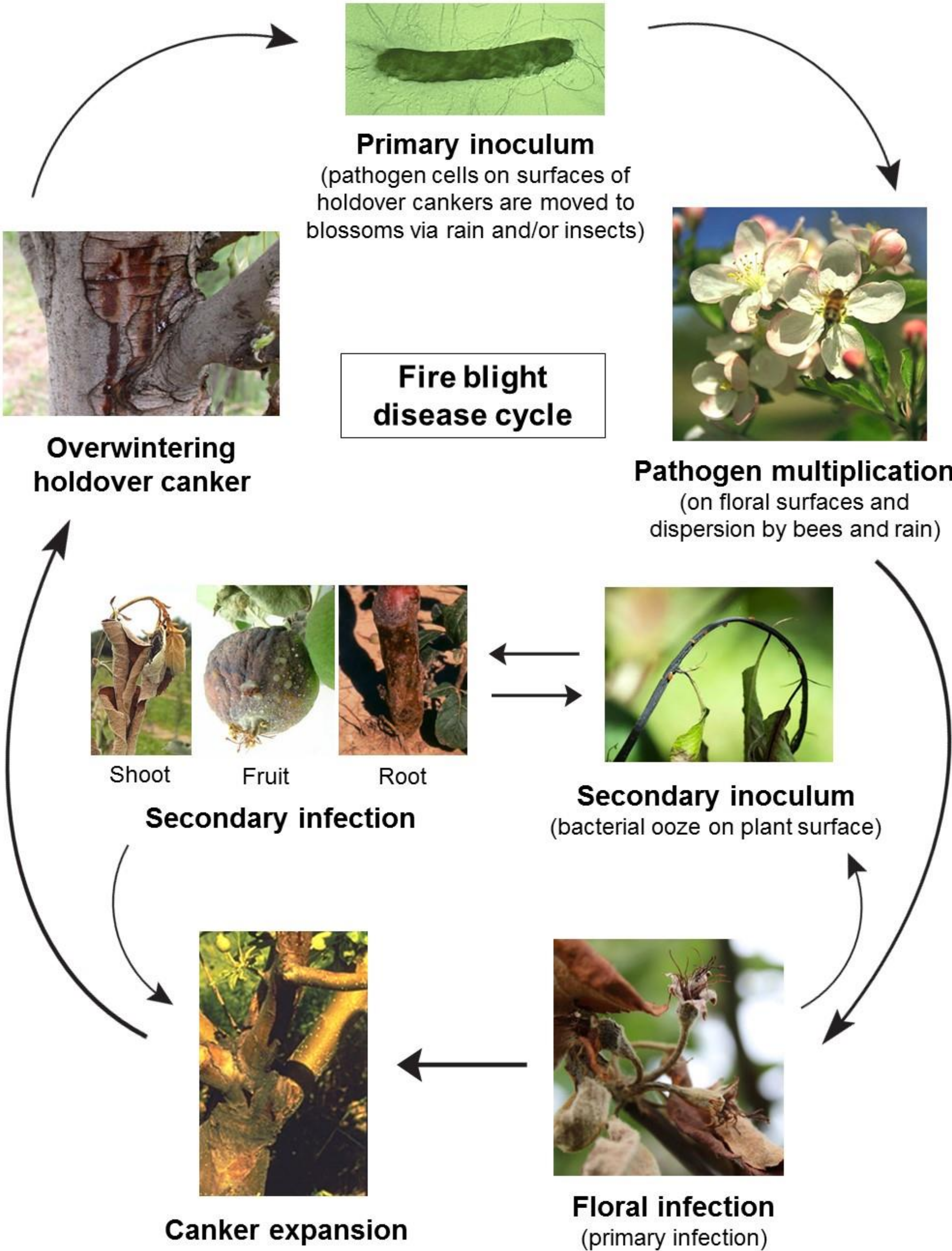

Figure 3 – Disease cycle of fire blight.
[Based on Johnson (2000)]

1.5 Symptomatology

The name “fire blight” is descriptive of the major characteristics of the disease: the brownish appearance of twigs, flowers and leaves as thought they had been swept by fire. Symptoms of fire blight have been extensively described in several existing reviews and books. Primary outbreaks of fire blight in newly planted orchards occasionally occur in the first year of growth, on trees both with and without flowers (Bastas and Maden, 2007).

Blossoms are considered to be the most susceptible organ to *E. amylovora* infections. Initially, blossoms appear water-soaked, then wilt, later show a brownish discoloration and finally have a blackish necrotic aspect. The infection may progress through the peduncle and affect the whole corymb (flower cluster). Infected blossoms may fall or remain attached to the tree. Blossom infection can spread undetected to the rootstock (Figure 4A).

Twigs, branches and the trunk may also be infected, resulting in limited nutrition of subordinate tree branches, which in turns leads to a rapid wilt of the leaves, but not defoliation. This stage often ends with formation of cankers, which appear in summer or autumn, thus providing a survival site for the pathogen during winter. In infected growing shoots the first symptoms are presence of drops of sticky bacterial ooze (Figure 4B) on its surface and often exhibit a typical curling at the end, called a shepherd´s crook (Figure 4C).

Fruits infected by *E. amylovora* in the immature stages appear dark, dried and shrivelled, and later red, brown or with black lesions. Fruits often appear oily or water-soaked, exuding droplets of bacterial ooze, and can remain hanged mummified on the tree (Figure 4D). Leaves can be infected via the vascular system of the shoot or by direct penetration via stomata or wounds (Figure 4E). The typical symptoms of leaves on affected branches start with initial turgor loss followed by a brown to black discoloration. Leaves wilt and shrivel, but remain attached to the tree for some time. The pathogen can migrate internally downward from shoots or directly infect rootstock wounds.

In rootstock blight, symptoms are similar than in shoot blight (Figure 4F), being able to observe the same types of cankers. Bacterial cankers can be formed when the infection progresses into the woody tissue. Active fire blight holdover cankers have a dark, water-soaked appearance (EPPO, 2013; van der Zwet and Keil, 1979) (Figure 4G). The symptoms of fire blight are similar in all host plants, apart from some specificities. Infected pear trees show the most characteristic symptoms, with flowers, leaves, shoots and fruits dark coloured or blackish, as if they have been burnt.

In apple, loquat and other susceptible rosaceous plants, the colour of the plant foliage may be reddish to dark brown (EFSA, 2014). Under favourable conditions symptoms progress very rapidly in a few days. In highly susceptible hosts or during severe disease outbreaks, bacteria can spread systemically resulting in death of the entire tree in a single growing season (Eastgate, 2000).

Symptoms of fire blight can be confused with other diseases (especially at the start of disease development) caused by bacteria and fungi, by insect attack or physical disorders. Fungal cankers might be sometimes confused with fire blight cankers, especially when observed on apple. Thus, diagnosis of the disease is difficult, and requires a laboratory analysis (Bastas and Maden, 2007; Khan et al., 2012).

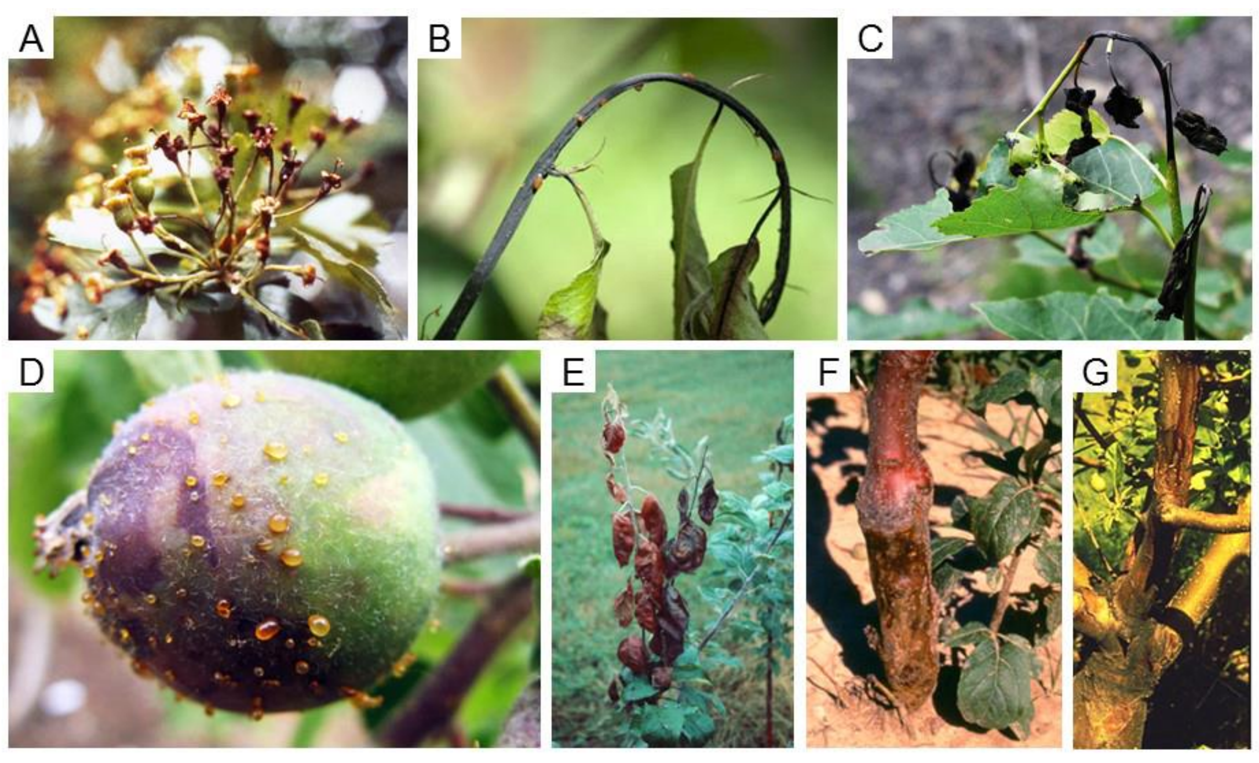

Figure 4 – Symptoms of fire blight disease.
(A) Blossom infection; (B) drops of sticky bacterial ooze in shoots; (C) shepherd´s crook symptom in infected growing shoot; (D) Fruit infection; (E) Leaf infection; (F) Rootstock blight; and (G) Active bacterial canker.
[Source: CABI (2016) and Johnson (2000)]

1.6 Diagnosis

Fire blight diagnostic requires symptom recognition and isolation and identification of *E. amylovora* from plant material disorders (EFSA, 2014). There is an official protocol by (EPPO, 2013), which is usually followed for diagnosis of fire blight.

The identification procedure for *E. amylovora* in plant material samples usually includes at least two tests based on different characteristics of the pathogen: combinations of

microbiological (semi-selective and selective media, nutritional and enzymatic tests), serological (ELISA) and molecular tests (PCR approaches) (López et al., 2009; Pirc et al., 2009).

Several complementary tests can be required, such as the tobacco hypersensitivity reaction, the immature fruit inoculation or the inoculation of shoots of host species to demonstrate pathogenicity of the isolated cultures (EPPO, 2013).

1.7 Fire blight management

Fire blight is a disease difficult to manage, since the available control methods generally have a low efficacy (Bastas and Maden, 2007). Effective management relies on prevention and requires an integrated approach of several practices that promote the reduction of the amount of inoculum available to initiate new infections, imposing barriers to successful establishment of the pathogen on the host, and reducing host susceptibility to infection (Norelli et al., 2003).

Based on these reasons, it is advisable the combination of integrated management strategies, including regulatory and agronomic measures, the use of resistant/tolerant plant cultivars, and the application of control products according to predictions by fire blight forecasting models.

1.7.1 Prevention of introduction

The main risk of introduction and spread of fire blight over medium and long distances is through plant material contaminated with *E. amylovora*, and mainly through plant nursery materials (López et al., 1999). Due to this reason, a phytosanitary passport was regulated by the Commission Directive 2005/17/EC, to ensure that plants comply with quarantine organism regulations, which allows them to be transported freely in the EU. Even in countries without fire blight, *E. amylovora* is a regulated organism, and trade of nursery stock and fruit is restricted. Careful plant management with good quarantine measures might prevent the disease (McManus and Jones, 1995; Zhang and Geider, 1997).

Owing to the phytosanitary risk posed by latent infections, protocols for analysis of asymptomatic material have been developed, especially for nursery material to prevent spread of the disease (EFSA, 2014).

1.7.2 Regulatory measures

Since fire blight is considered as a quarantine disease in European Union (EPPO A2 quarantine pest), the host plants of *E. amylovora* were included in Annex II-A-II (Annex II, Part A, Section II, point 3, and in Annex II, Part B, point 2) of Council Directive 2000/29/EC. This Directive lays down especially on the technical phytosanitary provisions, regulated by the Commission Directive 2005/17/EC, on plants and plant products, as well as on the control at the place of origin of those ones, destined for the EU or to be moved within the EU. Furthermore, is related to protective measures against the introduction of organisms harmful to plants, or crop products into the Community, and against the spread of the disease.

Specific regions within the EU have been designated as protected zone against certain pests and diseases (EFSA, 2014). In Spain, measures to control fire blight were regulated by the RD (Royal Decree) 58/2005, about prevention measures against the introduction and dissemination of the disease, as well as the establishment of the National Program of Eradication and Fire Blight Control of the *Rosaceae*, regulated by RD 1201/1999, amended by RD 1786/2011 (MAGRAMA, 2016).

In Portugal, measures to fire blight control were regulated by the Regulation (EC) 690/2008 of the Commission of 4 July 2008, recognizing protected zones exposed to particular plant health risks (DGADR, 2011).

1.7.3 Agronomic measures

Agronomic measures aim to reduce the infection risk, incidence and severity of infections as well as to control the spread of the disease. As mentioned before, the most receptive stages of the host to fire blight are the flowering and active vegetative growth periods. Consequently, in order to prevent early excessive growth, without compromising tree vigour, practices such as applying lower levels of nitrogen fertilizer during an early stage are common.

Also, it is important to prevent secondary flowering, because secondary flowers that may be produced in late spring or summer are more prone to infections than the ones produced during the main bloom, since warm temperatures favour pathogen multiplication (Thomson, 2000). The pruning of trees during dormancy (in winter time) is another important technique to eliminate cankers and remove infected tissue (or the whole tree, when necessary), even knowing that pruning can disrupt the equilibrium between

vegetative and reproductive growth, leading to a considerable impact on productivity. Additionally, it is crucial to disinfect all of the pruning tools, destroy pruned plant material, and remove wild hosts in the vicinity of the orchard.

Furthermore, orchards should be located in rich and well-drained soils, avoiding overhead irrigation systems that favour wetness and inoculum dissemination (Van Teylingen, 2002).

1.7.4 Chemical and physical control

Traditionally, chemical control had been the most widely used strategy against fire blight. However, these methods have not advanced significantly in the last 50 years.

Chemical pesticides are oriented to eliminate or inactivate *E. amylovora* before penetration in the host tissue by destroying the source of inoculum or by protecting potential invasion sites, such as blossoms or wounds. Most of chemicals available are not systemic and have no curative action. The use of chemical products can prevent infection, and sanitation methods applied to infected plants can control the disease to a certain extent (Thomson, 2000).

There are a limited number of chemical products available, generally with moderate efficacy, such as copper compounds and certain antibiotics that are applied preventatively (Johnson and Stockwell, 1998). Copper products have a direct action against *E. amylovora*, due to their microbicide activity. They are used out of the blooming period since they are highly phytotoxic to flowers. Streptomycin, oxitetracycline and kasugamicin are the only antibiotics with necessary requirements to be used in field applications (McManus et al., 2002). As mentioned, the use of antibiotics is not authorized by the legislation of the EU (EFSA, 2014; Norelli et al., 2003).

One of the physical methods that can be used for prevention of fire blight in plant material for propagation is thermotherapy. This technique can be useful to ensure the absence of *E. amylovora* in nursery plant material, and has been applied to fruit and ornamental plants by treatment with dry heat at 45ºC for 60 minutes, with minimal damage to plant material. Other physical technique relies on disinfectant treatments (e.g. quaternary ammonia, chlorine), however they do not affect endophytic inoculum (KECK et al., 1995; Ruz et al., 2008).

There are also antimicrobial peptides (AMP) 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 been the object of attention in past years as candidates for plant protection products. They are short sequence peptides, with generally fewer than 50 amino acid residues reported in living systems. AMPs have a wide range of activity against fungal and bacterial plant pathogens and have been involved in the control of several plant diseases.

Although some naturals AMPs do not have a good efficacy in the plant disease control, and additionally have phytotoxicity, they can be used as the basis for the development of new AMPs with better qualities by synthetic procedures (Montesinos, 2007). According to Badosa et al. (2014) synthetic AMPs are potential candidates for fire blight control, because they can be designed and produced by peptide chemistry approaches with optimized activity, toxicity and biodegradability.

1.7.5 Resistant / Tolerant varieties

The use of host resistant material is only a restrictedly reliable strategy to control fire blight. Unfortunately, there is no plant material completely resistant to the pathogen and the most resistant varieties currently available still have moderate to low susceptibility to fire blight (EFSA, 2014).

1.7.6 Fire blight risk assessment models

Risk assessment systems were developed to ensure optimal timing for applications of pesticides and to evaluate the potential risk of infection by *E. amylovora*.

In recent years epidemiological models were constructed to identify key infection periods. These models assign a level of risk to various orchards and weather situations.

Within all existing models, Maryblyt and Cougarblight are the most used (UC-IPM, 2014). As computer programs, they predict specific infection events and symptom development for most phases of fire blight epidemics in apples and pears, and can be operated in real time to assess the current risks or progress of an epidemic, or in a simulation mode for predicting future events using forecasted weather data. Information generated in both modes of the program provides a basis for making decisions concerning when to make specific control treatments and when it is reasonably "safe" to delay them (Smith, 1996).

2 Biological Control

There is no single, accepted definition of biological control. In plant pathology, biological control applies to the use of microbial antagonists to suppress diseases as well as the use of host-specific pathogens to control weed populations. The organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA) (Pal and Gardener, 2006).

According to Garrette (1965), biological control can be defined as “any condition under which a practice whereby survival or activity of a pathogen is reduced through the agency of another living organisms (except by man himself) with the result there is a reduction in incidence of disease caused by pathogens” (Garrette, 1965).

As defined by the National Academy of Sciences, and taking into account modern biotechnological developments, biological control refers to 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 (Thomashow and Weller, 1996).

More specifically, biological control refers to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens (Pal and Gardener, 2006). The ideal biological control agent (BCA) should have the following characteristics (Wilson and Wisniewski, 1994):

- Resistance to the most frequently used pesticide, and compatibility with other treatments
- 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 substratum in fermenters
- Lack of pathogenicity for the plants and no production of metabolites potentially toxic to humans

Successful use of biocontrol requires a greater understanding of the biology of both the disease and its antagonist as well as of the physiology of the host plants and its cultivation.

2.1 Biological control of plant bacterial diseases

There are several mechanisms involved in biological control of plant bacterial diseases, by which a microorganism may limit the growth of another: antibiosis, competition for space and limited resources, induction of host resistance, biofilm formation, direct interaction between the antagonist and the pathogen (Whipps, 2001) and quorum sensing interference.

Antibiosis is based on the synthesis of compounds such as antibiotics, enzymes, bacteriocins or toxins that kill or have a detrimental effect on the pathogen. Antibiotics produced by microorganisms are very diverse in molecular structure and mode of action. Usually the same microorganism can produce several antibiotics. One example is *P. fluorescens* CHA0 that produce several compounds with inhibiting activity against different soil pathogens (Keel et al., 1989).

Competition for nutrients and space is based on the capacity to exclude other microorganisms in the plant tissue. Disease inhibition is produced when the antagonistic microorganism is a better competitor for nutrients or space than the pathogen. Space and nutrients must be limiting to produce the competition and these limitations vary in function of the environment where the interaction takes place (Andrews, 1992).

Induction of host resistance is based on the capacity of certain bacteria to induce physiological changes within the plant which provides systemic protection against a broad range of pathogens. This systemic protection is called systemic acquired resistance (SAR) when the induction is mediated by non-pathogenic aerial colonizators while is called induced systemic resistance (ISR) when the elicitation is mediated by rhizobacteria (Van Loon, 1997). Direct antagonism results from physical contact and/or a high-degree of selectivity for the pathogen by the mechanism(s) expressed by the BCA(s) (Pal and Gardener, 2006).

Biofilms can be defined as communities of microorganisms that are attached to a surface. They can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces. Biofilm development is initiated by bacteria in response to specific environmental cues, such as nutrient availability (Toole et al., 2000).

Quorum sensing signals are involved in regulation of a range of important biological functions, including luminescence, antibiotic production, plasmid transfer, motility, virulence and biofilm formation (Zhang and Dong, 2004).

However, it is generally assumed that most biocontrol agents do not strictly use a single control mechanism but a combination of several ones, with a synergic effect, which allows the pathogen inhibition (Whipps, 2001). Studying the complex interactions that take place between the host, pathogen, antagonist and possibly other microorganisms present in the site of interaction can be difficult.

The determination of the action mechanisms need the knowledge of many aspects such as the dynamics of populations, disease cycle, epidemiology and interactions that take place between the biocontrol agent, pathogen, host and other microorganisms Therefore, understanding these mechanisms of action can permit the establishment of optimum conditions between the pathogen and the biocontrol agent and is important for implementing biocontrol in a given pathosystem. An ideal biocontrol strategy introduces the antagonists only when and where they are needed or are most effective, and minimizes wasteful application of inoculum to non-targets (Bonaterra et al., 2003).

2.2 Selection of potential biocontrol agents

Selection of potential biocontrol agents consists on the isolation and screening methods of microorganisms that are able to inhibit the plant pathogen and reduce disease levels. It is recommended the isolation of a significant number of possible candidates since microorganisms with high antagonistic activity are relatively rare.

The ultimate success of biocontrol depends on how well the searching and screening process is done. There is no single, correct way to search or screen. Both depend on the target pathogen, the crop, and the cropping system (Fravel, 2005).

Some authors have described that interesting environments to find potential biocontrol candidates are near the pathogen infection site (Handelsman and Stabb, 1996). Potential BCAs can be isolated from the same natural environment where they will be introduced, or applied afterwards, to ensure ecological adaptation. The effectiveness in selecting microorganisms to increase the probability of obtaining useful microorganisms depends on the nature of samples, media composition techniques for enrichment-isolation and the methodology used (Montesinos, 2003).

The best methodology to screen possible antagonists has to be rapid and simple in order to select as many candidates as possible, and allow reproducing as much as possible the natural conditions where the host, pathogen and antagonist interact.

Before the adoption of a screening method, different aspects should be taken into account like the target disease (foliar or root diseases) or the type of pathogen implicated (fungi, bacteria, viruses). According to the literature, the best candidates to biocontrol agents are epiphytic bacteria because these ones are adapted to live and survive on plant surfaces. These bacteria are naturally adapted to the environmental stresses and for this reason present some characteristics to become biocontrol agents of aerial plant pathogens. Additionally, it is important to understand the interaction between the plant host, the antagonist and the pathogen, since this is the major key to the efficacy of disease reduction in the field (Handelsman and Stabb, 1996).

Designing a screening strategy is further complicated by the limited knowledge of the phenotypic features that determine success as a biocontrol agent because involves many properties of the antagonist. The best protocol to select bacterial antagonists is a combination of an *in vitro* stage to determine the potentiality to produce an antagonism effect, and a screening *ex vivo* procedure (using detached plant organs) under controlled environment conditions (Andrews, 1992).

However, these assays may not reflect what happen under field conditions. Possibly due to the difficulties of the antagonist to colonize and survive in the plant tissue or to the reduction of the production of antimicrobial compound, which is the base of their control. Field assays are the most determinant step in the biocontrol development, and the results in this stage will determine the potentiality of candidates to be exploited as biocontrol agents (Mercier and Wilson, 1994).

2.3 Characterization of biocontrol agents

Once a suitable BCA candidate has been selected, it is necessary to proceed with its identification and characterization of the isolates by phenotypic and genotypic analysis to select strains with desired traits or discard deleterious or pathogenic species, to improve its biocontrol activity and to determine the mode of action that will affect the application strategy. Besides, these steps can improve selection procedures as well.

It is important to evaluate the efficacy, growth rate and substrate colonization at an early stage of the screening process (Glare et al., 2012). The knowledge of these characteristics can help in the improvement of the efficacy and consistency of control, as well as in the production, formulation and delivery with the finality to produce the biocontrol agent at large-scale for commercially distribution. 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).

2.3.1 Examples of biochemical techniques used in this thesis

2.3.1.1 Phenotypic tests: Gram and oxidase reactions

The Gram reaction is one of the most essential of the so-called genus criteria or 'first stage criteria'. This distinction has an important role for the decision as to which criteria should be used in the further identification of the strain.

The cytochrome oxidase is an enzyme that oxidizes the reduced cytochrome C and is thus transformed itself into the reduced and inactive form. Through transfer of the electrons to molecular oxygen, the reduced cytochrome oxidase is transformed again into the active form. In the presence of molecular oxygen, the cytochrome oxidase/ cytochrome c-system can reduce a whole series of organic substances, among them, the so-called NaDi reagent (1-naphthol + dimethylparaphenylene diamine), with formation of the condensation molecule indophenol blue.

This reaction is used for the classification and identification of bacteria.

In the case of cytochrome oxidase-positive germs, the reaction zone is coloured blue to blue-violet.

2.3.1.2 Analytical Profile Index (API) system

API 20E is a standardized identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods which uses 21 miniaturized biochemical tests.

API 20NE is used for the identification of non-fastidious, non-enteric Gram-negative rods, combining 8 conventional tests and 12 assimilation. Both systems contain 20 microtubes with dehydrated substrates.

API 20 E is a 18-24 hour and API 20 NE is a 24-48 hour test.

API 50CH strips consists on 50 biochemical tests used to study fermentation of substrates belonging to the carbohydrate family and its derivates (heterosides, polyalcohols, uronic acids).

A preliminary study of the microorganism is necessary to choose the right API test (gram coloration, oxidase test, catalase test, etc.).

2.4 Improvement of biocontrol agents

The main problem of biocontrol is the limited efficacy when biocontrol agents are applied under field conditions, where biotic (host species, pathogen nutritional status) and abiotic (temperature, relative humidity) factors affect their colonization and survival (Bonaterra et al., 2012; Thomson, 2000).

Thus, it is necessary to understand the mechanisms involved in the interaction between the pathogen and the antagonist, and also to understand the factors that influence growth of both microorganisms in the natural environment, to develop techniques that provide optimal conditions to the antagonistic activity when the antagonist is introduced on a concrete environment.

Different strategies can be used to improve the ability of BCAs to colonize and survive in the environment. A possible strategy is the combination of several strains or species of biocontrol agents to obtain synergic or complementary effects. These mixtures improve the colonization of antagonistic agents because they have a better adaptability to environmental variations. Besides, the development of these mixtures increases the range of action and ecological performance against pathogens and in different hosts (Janisiewicz et al., 1992; Spadaro and Gullino, 2005; Yang et al., 2014).

One is based on the nutritional enhancement, by incorporating nutritional additives in the formulation that are often limiting on the environment and that are preferentially used by the antagonist (Cabrefiga et al., 2011). An additional strategy is the use of BCAs with low toxic antimicrobial compounds, such as bioregulators, organic acids or essential oils (Arrebola et al., 2010; Yu et al., 2006).

Another approach is the modification of the physiology of the BCA by osmoadaptation, to adapt themselves to adverse situations after their application in natural environments. This physiological improvement can be achieved by cultivation under osmotic stress, causing the intracellular accumulation of compatible solutes, including sugars, heterosides and amino acids. This strategy increase the ecological fitness of BCAs by allowing 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., 2007, 2005).

2.5 Formulation and production of biocontrol agents

The commercial development of biocontrol agents to use as biopesticides includes large-scale production of the microorganism and formulation for optimizing its efficiency, preservation and applicability.

Furthermore, a formulated product should be rapid, inexpensive, with easy preparation and should maintain its antagonistic capacity. Moreover, it should have stability during transportation and storage, good shelf-life, and acceptable cost (Andrews, 1992). A critical factor that must be considered when selecting a BCA for commercial development is the availability of a cost-effective production and stabilization technology. Besides, the commercialization of biocontrol agent needs to overcome toxicity tests and registration procedures.

Formulation can be in liquid state and maintained by refrigeration, or by keeping as dehydrated product not dependent on refrigeration (Spadaro and Gullino, 2005).

Many biopesticides used in the control of fire blight are formulated as dried products (wettable powders or granules). A dry product is less weight to ship and at lower risk of possible contamination (Fravel, 2005).

Dehydration methods, such as lyophilization and spray-drying allow optimum conditions of storage, handling and formulation of the microorganism. The use of additives compatible with the BCAs in the formulation can increase its survival, improve the application and stabilization of the final product (Cabrefiga et al., 2014; Montesinos, 2003).

2.6 The case of fire blight

The earliest reports on biological control of fire blight have been reviewed by van der Zwet and Keil (1979) beginning in the early 1930s when bacterial antagonistic strains of *E. amylovora* were isolated and shown to have a tendency to reduce the percentage of fire blight infection when applied to blossoms (van der Zwet and Keil, 1979). Over the last 30 years, integrated disease management programs, including several biological control strategies, have been studied to prevent or suppress the progress of fire blight (Table 1).

Biological control of fire blight is produced when a bacterial antagonist establishes and develops a large population prior to the establishment of *E. amylovora* (Johnson and Stockwell, 1998; Thomson, 2000). For this reason, BCAs are more effective when applied in blossoms, in order to multiply on stigmatic and hypanthial surfaces. Suppression of floral infections reduces the inoculum of *E. amylovora* available for other phases and cycles of

the disease, including shoot infections during the same season and floral infections in the following season (Stockwell et al., 1998; Thomson, 2000). In this system the selection of antagonists is independent of the mode of action developed by these ones (Pusey, 1997).

The advance in biological control of fire blight has been propelled by selection of effective antagonist strains, by enhanced knowledge of the mechanisms by which these strains suppress disease and by increased understanding of the ecology of bacterial epiphytes on plant surfaces (Thomson, 2000). The need to develop and implement biocontrol of fire blight has resulted from several factors, including the increasing importance of the disease, the moderate efficacies of existing control measures, and social demand to enhance the safety and sustainability of agricultural production systems.

However, it is necessary to find new strains or species of BCAs adapted to specific hosts as loquat that have to fulfil strict authorization requirements in most countries for microbial pesticides.

3 Plant Microbiota and strains for Biological Control

3.1 *Bacillus subtilis*

Bacillus subtilis has been reported as a successful control of many plant diseases (Gardener, 2004). *Bacillus subtilis* and related species have been the object of particular interest because of their safety, their widespread distribution in very diverse habitats, their remarkable ability to survive adverse conditions due to the development of endospores, and their production of compounds that are beneficial for agronomical purposes.

Several strains of *Bacillus* have been shown to control plant diseases by different mechanisms of action, including antibiosis, the induction of defence responses in the host plant, and competition for nutrient sources and space. Antimicrobial peptides (AMPs) produced by *Bacillus* spp. have been implicated in the biocontrol of several plant pathogens causing aerial, soil, and postharvest diseases and in the promotion of plant growth.

Table 1 – Biological control agents of fire blight.
[Adapted from Prados (2015)]

Organism	Mode of action	Commercial name	Source
Aureobasidium pullulans DSM14940 and DSM14941 combined with citric acid (as an additive)	Competitive exclusion	Blossom Protect™	Kunz et al., 201l
Bacillus amyloliquefaciens subsp. *plantarum* 0747	Antibiosis	Double Nickel SS™	Highland et al., 2012
Bacillus pumilis QST2808	Antibiosis	Sonata®	Bayer CropScience
Bacillus subtilis QST173	Antibiosis	Serenade®	Aldwinckie et al., 2002
B. subtilis BS-F3	Antibiosis	-	Alexandrova et al., 2002
B. subtilis BD 170	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., 2008
P. agglomerans Eh24	Competitive exclusion	-	Ozaktan et al., 1999
P. agglomerans Eh112Y	Antibiosis	-	Wodzinski et al., 1994
P. agglomerans Eh318	Antibiosis	-	Wright and Beer, 1996
P. agglomerans EhHI9NI13	Antibiosis	-	Wilson et al., 1990
P. agglomerans Eh1087	Antibiosis	-	Kearns and Hale, 1996
P. agglomerans P10c	Competition, antibiosis	Blossom Bless™	Vanneste et al., 2002
Pseudomonas fluorescens A506	Competitive exclusion, antibiosis	BlightBan® A506	Wilson and Lindow, 1993
P. fluorescens EPS62e	Competitive exclusion	-	Cabrefiga et al., 2007
Pseudomonas graminis 49M	Antibiosis	-	Mikicinski et al., 2011
Pseudomonas sp. Rl	Antibiosis	-	Laux et al., 2002
Rahnella aquatilis Ra39	Competition	-	Laux et al., 2002

3.2 *Curtobacterium flaccumfaciens*

Several reports have indicated that *C. flaccumfaciens* or *Curtobacterium* spp*.* can function as a biological control agent against many pathogens, and may function either by the triggering of induced systemic resistance or by antibiosis (Lacava et al., 2007).

Strains of *C. flaccumfaciens* cause bacterial diseases but they or maybe closely related species also have been recovered from the phyllosphere of wheat and have been shown to be a biocontrol agent for cucumber (Raupach and Kloepper, 1998). Recent data indicate that *C. flaccumfaciens* interacted with *Xylella fastidiosa* in *C. roseus,* and reduced the severity of the disease symptoms induced by *X. fastidiosa*. Lacava et al. (2007) suggested, on the basis of *in vitro* interaction experiments, that the growth of *X. fastidiosa* could be inhibited by endophytic *C. flaccumfaciens* (Lacava et al., 2007).

3.3 *Enterobacter cancerogenus*

Enterobacter cancerogenus MSA2 is a plant growth promoting ɤ-proteobacterium that was isolated from the rhizosphere of *Jatropha cucas*. Soil bacteria are very important in biogeochemical cycles and have been used for crop production for decades because their interactions in the rhizosphere are important indicators of plant health and soil fertility. Interaction of plant growth promoting rhizobacteria (PGPR) with host plants involves not only the two partners but other biotic and abiotic factors of the rhizosphere region, developing sustainable systems in crop production (Jha et al., 2012).

3.4 *Pseudomonas fluorescens* CHA0

Pseudomonas fluorescens strain CHA0 colonizes plant roots and suppresses a variety of soilborne diseases (Mascher et al., 2000). Suppression of soilborne plant pathogens by fluorescent pseudomonas depends on complex interactions between the bacteria and their biotic and abiotic environments. To function effectively as biocontrol agents, the fluorescent pseudomonads should have the ability to colonize the roots and to produce certain secondary metabolites.

This bacterium supresses *Thielaviopsis basicola*-induced black root rot of tobacco and *Gaeumannomyces graminis var. tritici*-induced take-all of wheat. Root inoculation of *Arabidopsis thaliana* ecotype Columbia with *Pseudomonas fluorescens* CHA0 was proved to induce ISR in leaves to pathogenic strains of *Peronospora parasitica* (Iavicoli et al.,

2003). Strain CHA0 produces 2,4-diacetylphloroglucinol, a metabolite with antifungal, antibacterial and phytotoxic activity (Keel et al., 1992).

Pseudomonas fluorescens strain CHA0 was also proven to produce iron-chelating metabolites–pyoverdine, salicylic acid pyochelin. Antibiotic metabolites synthesized by this strain - hydrogen cyanide (HCN) and pyoluteorin (Pit) also play an important role in disease suppression. In the same study, it was demonstrated that *P. fluorescens* strain CHA0 produces extracellular protease, phospholipase C and lipase. *gacA gene* function was found to be required for the expression of the former two activities (Sacherer et al., 1994).

3.5 Pseudomonas rhizosphaerae

Pseudomonas rhizosphaerae is a novel species, that was first isolated from the rhizosphere of grasses and selected for its capacity to actively solubilize phosphates *in vitro* (Paternoster et al., 2010). Krimm et al. (2005) showed that *P. rhizosphaerae* is one of the prominent epiphytic bacteria of the phyllosphere of strawberry plants, where it significantly increases the permeability of the plant's cuticle (Krimm et al., 2005). Studies indicate that *P. rhizosphaerae* is also an epiphytic inhabitant of apple blossoms (Paternoster et al., 2010).

3.6 Rosenbergiella epipactidis

Rosenbergiella is a recently described new genus of Enterobacteriaceae family that was previously isolated from floral nectar of two cultivated plant species in Israel, *Amygdalus communis* (almond) and *Citrus paradisi* (grapefruit) (Lenaerts et al., 2014). Junker et al. (2011) demonstrated that Enterobacteriaceae bacteria dominate the epiphytic bacterial communities in petals (Junker et al., 2011).

The genus *Rosenbergiella* (named after Prof. E. Rosenberg, an Israeli microbiologist) has adapted to the conditions of nectar, being able to withstand high sugar concentrations (up to 50%, w/v) and to oxidize the major sugars of nectar (i.e. sucrose, glucose and fructose). Amygdalin, caffeine, nicotine and anabasine were found in the nectar of plants tested in a study conducted by (Fridman et al., 2012).

Rosenbergiella epipactidis, referring to the genus name of the host (Epipactis - terrestrial orchids consisting of approximately 70 species) was the first strain of this bacterial species found in nectar of this host plant. Cells are Gram-negative rods that are

facultative anaerobic, motile and catalase positive. Floral nectar is regarded as the key component in the mutualism between animal-pollinated plants and their pollinators, which use this sugar-rich solution as a reward for their pollination services.

Floral microbes can have a negative impact on plant–pollinator mutualisms by decreasing floral attractiveness through a reduction of nectar nutritional value and interfering with pollen germination and damaging pollen tubes (Lenaerts et al., 2014). Floral nectar was suggested to be not suitable as bacterial habitat and was demonstrated to have antimicrobial properties (Sasu et al., 2010), due to several chemical components that were suggested to limit growths of microflora in the nectar, because of its high sugar concentration (high osmotic pressure), its protein-defensive mechanism against microorganism, and its antimicrobial secondary metabolites (Fridman et al., 2012).

However, it has also been suggested that microbial communities in nectar enhance pollination by producing volatiles or fermentation by-products that attract pollinators and by raising flower temperature, and consequently indirectly governing plant fitness. The ecological role of *Rosenbergiella* species in the multi-kingdom interactions taking place within and around floral nectar and the effect of the bacteria on the nectar remains unclear to date, and deserves further studies (Lenaerts et al., 2014).

4 Loquat (*Eriobotrya japonica*)

Loquat (*Eriobotrya japonica* Lindl) was the chosen plant species used in the experiments of this thesis to select potential biological control strains from the microbiota of its fruit and leaves. The following strains from the list above were studied in more detail (see Results and Discussion): *E. cancerogenus*, *C. flaccumfaciens*, *P. rhizosphaerae* and *R. epipactidis*.

Loquat is a subtropical, evergreen, fruit-bearing tree, of the Rosaceae family, sub-family Pomoideae. The Dadu River Valley of China is considered the origin of the genus *Eriobotrya*. Although most authors believe that the loquat species originated in China, a definitive region of origin is as yet unknown (Lin et al., 1999).

Loquat cultivation is very ancient in Eastern Asia but the crop’s spread to Europe occurred in 1784, when it was introduced into the Botanical Gardens of Paris. In the 20th century, the crop has spread to India, Southeastern Asia, South Africa, as well as Central and South America. Loquats have formed various ecological types in different zones over the course of their cultivation and acclimatization (Vilanova et al., 2001).

Loquat has very different cycle than other fire blight hosts, because it blooms in fall, develops its fruits during winter, and ripens them in early spring. Its unusual phenology allows loquat to reach the market before any other spring fruit (Cuevas et al., 2009). Cultivation of the species has led to a large number of cultivars, due to different selection pressures applied by growers. Loquat production in the Mediterranean area substantially increased from 1985 to 2000, due mainly to intensive cultural practices (Amorós et al., 2003).

Only *E. japonica* is cultivated for its fruit. Other species of the genus are used as rootstocks or as ornamentals. The *Algerie* cultivar is one of the most widespread commercial varieties. Total production in the Mediterranean area is currently about 65,000 tons, with more than 50% produced in Spain, mainly in the province of Alicante, where the crop has met very good environmental conditions for its development.

5 Main Objectives of this thesis

- Isolate epiphytic bacteria from loquat microbiota (blossoms and leaves);
- Use *ex vivo* antagonism assays to select potential biocontrol agents of *Erwinia amylovora* in immature loquat fruit and pear blossoms;
- Select potential biological control agents based on their efficacy index, and on disease incidence and severity of *E. amylovora* infections in immature loquat fruits and pear blossoms.

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CHAPTER II

MATERIALS AND METHODS

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1 Plant material collection and sampling areas

A total of 223 samples of loquat (*Eriobotrya japonica*) blossoms were collected in November 2015 from two distinct orchards of loquat trees (*Algerie* cultivar) (Fig. 5) in the province of Alicante (eastern Spain): 128 samples from the location Callosa d´En Sarrià and 95 samples from La Nucia (Fig. 6).

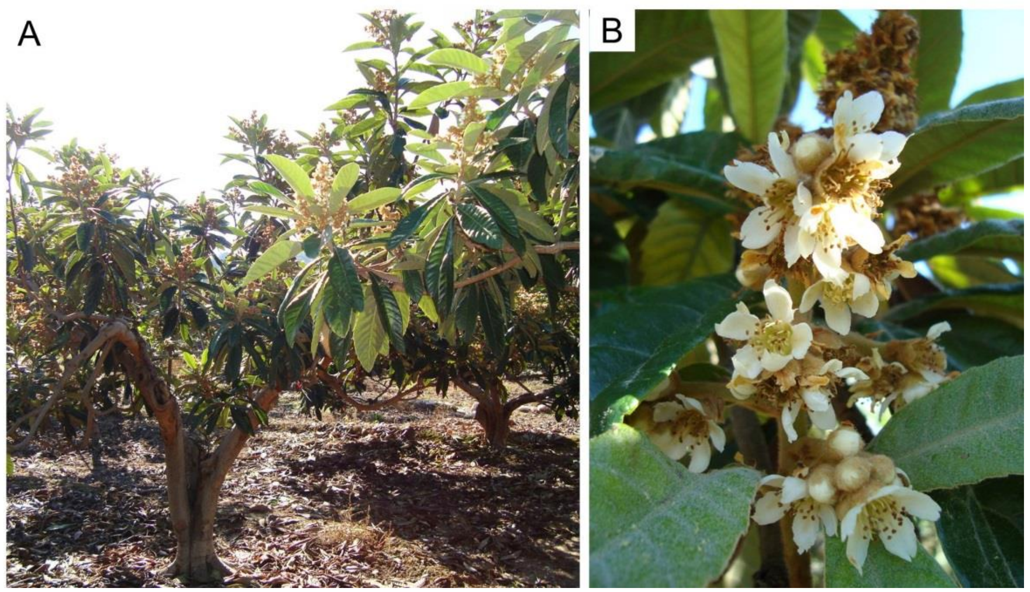

Figure 5 – Loquat (*Eriobotrya japonica*) (*Algerie* cultivar) in Callosa d'En Sarrià orchard. (A) Loquat tree; and (B) loquat blossoms and leaves.

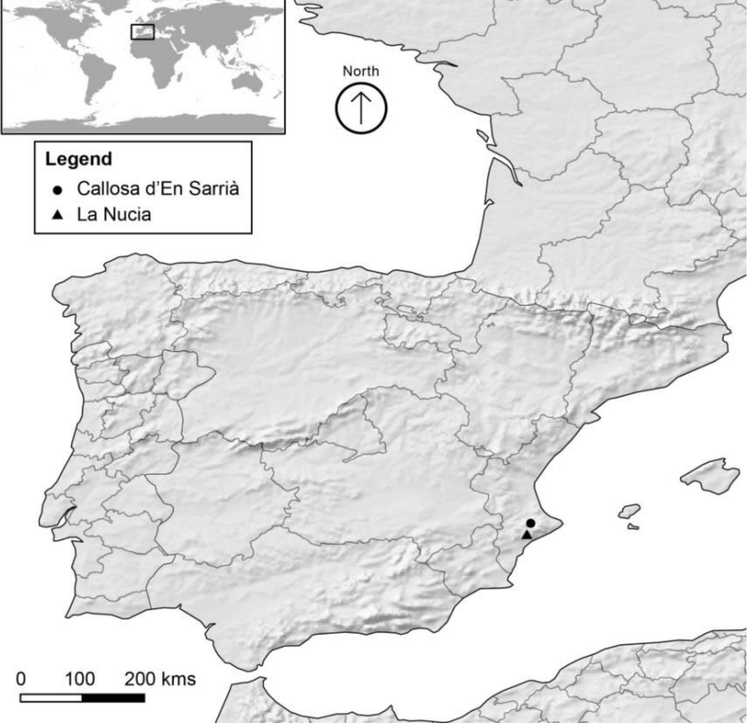

Figure 6 – Sampled locations in the Province of Alicante in November 2015.

An additional set of 30 samples from loquat leaves were added to this study. These were collected from IVIA´s greenhouse and then isolated by another student from IVIA´s research team (María Piñar) (Fig. S1).

1.1 Sample processing and bacterial isolation

Sets of 8 to 11 detached loquat flowers from each loquat tree were kept in plastic bags (Figure 7) and identified with the corresponding tree number. This step was extremely important to identify the loquat tree in case of a positive detection of *E. amylovora* in the surveyed plot.

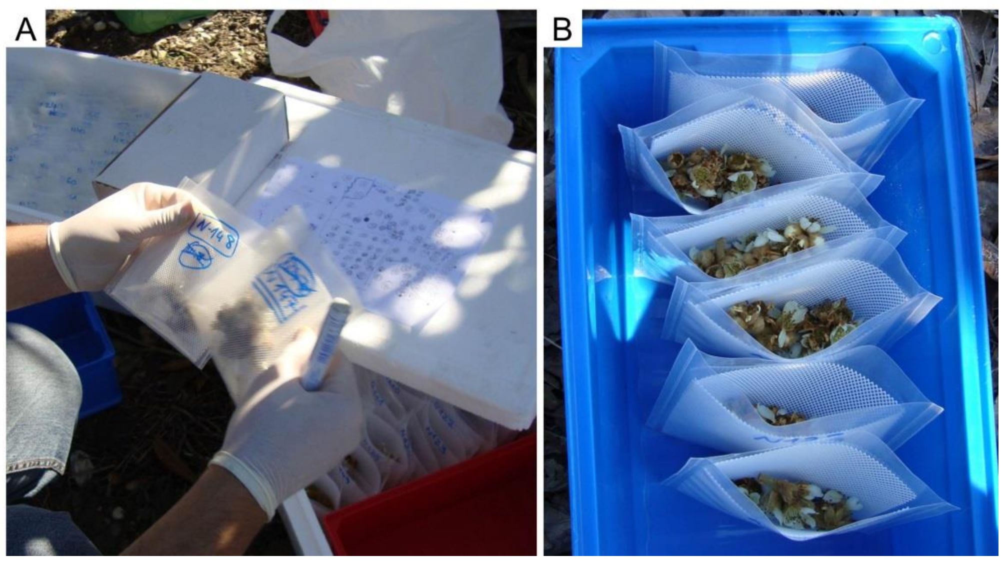

Figure 7 – Sample collection, identification and storage.
(A) Sample identification; and (B) Sample storage.

The pruning shears were disinfected with ethanol (96%) between different trees to avoid cross-contamination. All samples were gathered and maintained in refrigerated conditions until use in the laboratory.

In the day after, the plant material was crushed in an antioxidant maceration buffer (1g /50 ml) (Gorris et al., 1996) (Table S1) and bags were immediately inserted in ice to prevent oxidation. Since the time of inclusion of phosphates can interfere with the number of colonies formed, to media were assayed: King´s medium B (KB) agar (see Table S2 for medium composition) and phosphates autoclaved together, and KB agar and phosphates autoclaved separately and mixed before solidification.

Other aliquots from extracts were enriched in liquid CCT and KB, by mixing 2 ml of each extract with an equal volume of enrichment medium, and incubated without shaking at 25 ºC for 72 hours. This step was necessary to perform the analysis by enrichment DASI-

ELISA using the technique described by Gorris et al. (1994) with a commercial kit (Plant Print Diagnostics S. L., Valencia, Spain).

Before and after the enrichment, aliquots of 50 µl were streaked on King´s medium B + Cycloheximide (KB+C), King´s medium B with phosphates added after autoclaving + Cycloheximide (KB+C+P), CCT (Ishimaru and Klos, 1984) (Table S3), and Levan medium + Cycloheximide (Levan+C), and then incubated at 25ºC. The use of Cycloheximide (50 mg) was to avoid fungal growth. The purpose of using different media was to increase the chances of isolating different types of bacteria.

Another part of extracts was boiled at 100 ºC in a thermoblock during 10 min and maintained at –20 ºC. Finally, glycerol (30%) was added to the remaining extracts and stored at -20ºC for future use. After 24 hours of incubation at 25ºC, colonies representing different morphological types (from the four different media mentioned above) were purified on KB. Then, pure colonies were suspended in 4,5 ml of sterile PBS and streaking again on KB. The process was repeated for 2-3 times, until achieving pure colonies. For long-term preservation, the bacterial isolates were stored in 1 ml of PBS plus glycerol (20%) at –80ºC.

2 Morphological, biochemical and molecular characterization of bacterial strains from loquat microbiota

2.1 Morphology

Morphological descriptions of bacteria colonies were performed, attending their size, shape, colour, texture, elevation and edge (Fig. 8).

Shape referred/ refers to the overall appearance of the colonies: punctiform, circular, irregular, filamentous (individual thin projections), or rhizoid (thin, branching projections). Texture referred/ refers to the characteristics of the colony surface: dry, mucoid (thick, stringy and wet), moist, smooth, rough, wrinkled, or containing centric rings. Margins can be entire, undulate, lobate, filamentous or curled (Fig. 8).

The colony elevation is a description of how the colony grows vertically (looking through the side of the petri dish): flat, raised, convex (sloping up from the edges), pulvinate (sloping steeply from the edges and very high in the center), umbonate (raised center) and crateriform (Fig. 8).

The diameter of the colonies were measured with a ruler and size was reported in millimetres.

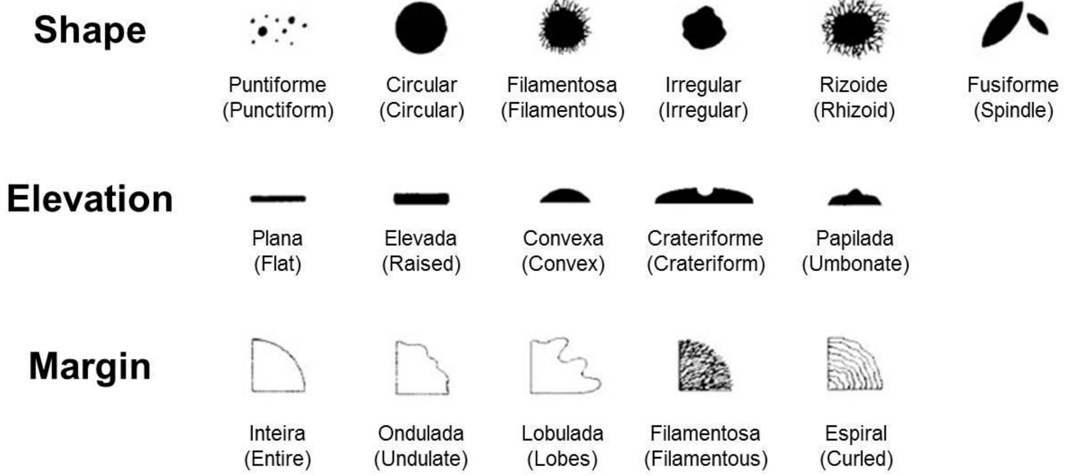

Figure 8 – Morphological characteristics of bacterial colonies.
For each characteristic is the designation used in Table S4 and English translation.
[Adapted from: http://www.biomedicinapadrao.com.br/2013/08/descricao-morfologica-de-colonias-em.html]

2.2 Gram reaction

A solution of 3% potassium hydroxide was used to distinguish Gram-negative and Gram-positive isolates. Thus, one drop of the solution was mixed with a minimal amount of tested colony and then observed the lysis (Gram-negative) or not (Gram-positive) of cells by the viscous or non-viscous consistency of the mix (Gregersen, 1978).

2.3 Oxidase reaction

Oxidase reaction was conducted using the microbiology kit Bactident® Oxidase, which is based on the detection of cytochrome oxidase in microorganisms.

The reaction zone of the test-strips contains N,N-dimethyl-1,4-phenylene diammonium dichloride 0,1 µmol; 1-naphthol 1.0 µmol.

As a positive control it was used a culture of *Vibrio splendidus* (Le Roux et al., 2002).

2.4 API system

Four strains used on the pear blossom assay were characterized using the API system (*Biomerieux*, France) - API 20E, 20NE and 50CH systems. Reactions were evaluated after 24 and 48 hours and reactions were conducted following manufacturer’s instructions (BioMérieux Inc., 2002).

The assimilation tests were inoculated with a minimal medium and it was verified if the bacteria were capable of utilizing the corresponding substrate. Therefore, the strips were used to characterize the isolates, to test some basic biochemical tests and to verify differences between strains, in the use of amino acids and sugars.

2.4.1 API 20E

Preparation of the strip:

- An incubation box (tray and lid) was prepared and about 5 ml of distilled water was distributed into the honeycombed wells of the tray to create a humid atmosphere.
- The reference strains were recorded on the elongated flap of the tray.
- The strip was placed in the incubation box.

Preparation of the inoculum:

- An ampule of API Suspension Medium (5 ml) was opened.
- A single well isolated colony from KB agar with 24h growth was removed using a pipette and carefully emulsify to achieve a homogeneous bacterial suspension. This suspension was used immediately after preparation.

Inoculation of the strip:

- Using the same pipette, both tubes and cupules of the tests CIT, VP and GEL were filled with the bacterial suspensions.
- For the other testes only tube were filled.
- To create anaerobiosis in the tests ADH, LDC, ODC, H_2S and URE mineral oil were overlayed.
- The incubation box was closed with a plastic container to avoid exposure to air and incubated at 24°C for 48 hours.

2.4.2 API 20 NE

The isolates that corresponded to non enterobacteria (*P. rhizosphaerae and C. cancerogenus*) were characterized using API 20 NE system (Biomerieux, 2003) following manufacturer's instructions.

Preparation of the strip:

- An incubation box (tray and lid) was prepared and about 5 ml of distilled water was distributed into the bottom of the tray to create a humid atmosphere.
- The reference strains were recorded on the elongated flap of the tray.

- The strip was placed in the incubation box and closed with a plastic container to avoid exposure to air.

Preparation of the inoculum:

- An ampule of API NaCl 0.85 % Medium (2 ml) was opened.
- 1-4 colonies from KB agar with 24h growth were removed by successive touches, using a pipette.
- Suspensions with a turbidity equivalent to 0.5 McFarland (a weaker inoculum could lead to false negative results) were prepared and used immediately after preparation.

Inoculation of the strip:

- Tests NO_3 to PNPG were inoculated by distributing the suspension into the tubes (and not the cupules), using the same pipette. To avoid the formation of bubbles at the base of the tubes, the strip was tilted slightly forwards and the tip of the pipette was placed against the side of the cupule.
- Approximately 200 µl of the remaining suspension was added to an ampule AUX Medium and homogenize well with the pipette, avoiding the formation of bubbles.
- The tubes and cupules of tests GLU to PAC were filled with the bacterial suspension, with care to leave a flat or slightly convex (but not concave) meniscus, because cupules under or overfilled could give incorrect results.
- Mineral oil was added to the cupules of the 3 underlined tests (GLU, ADH and URE) until a convex meniscus was formed.
- The incubation box was closed with a plastic container to avoid exposure to air and incubated at 24°C for 48 hours.

2.4.3 API 50 CH

Preparation of the strip:

- Each strip is made up of 5 smaller strips each containing 10 numbered tubes.
- An incubation box (tray and lid) was prepared and about 10 ml of distilled water was distributed into the honeycombed wells of the tray to create a humid atmosphere.

Preparation of the inoculum:

- The inoculum was prepared by mixing 1ml of the bacterial suspension in 20 ml of the Ayers medium and used immediately after preparation.

Inoculation of the strips:

- The bacterial suspension was distributed into the 50 tubes by tilting the incubation box slightly forwards, avoiding the formation of bubbles by placing the tip of the pipette against the side of the cupule.
- To maintain anaerobic conditions, the top of the tube was not exceeded (when only the tubes had to be inoculated).
- The formation of a concave or convex meniscus was avoided when tubes and cupules were inoculated.
- The strips were incubated at 30°C for 48 hours.

2.5 Identification by 16S rRNA sequencing

The identification by 16S rRNA partial (about 800 bp fragments) of selected isolates was performed by Genetics PCR solutions (GPS, Orihuela, Alicante, Spain). In some cases, a complete 16S rRNA gene sequencing (about 1400 bp fragments) was required.

2.5.1 Bacillus *screening*

DNA was extracted from Gram-positive isolates by vortexing 1-2 bacteria colonies with 1 ml of 1x AFT and heating the mixture at 100ºC for 10 min. A 163 bp fragment of the 16S rRNA gene was amplified using the primers 16SBACF and 16BACR (Mora *et al.*, 2011) (Table 2). PCR reactions was carried out in a total volume of 25 μl containing 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP (Invitrogen Technologies), 0.2 μM of each primer, 2.0 U of Taq DNA polymerase (Biotools), and 2 μl of genomic DNA. The cycling conditions for the amplification of all targets were: initial denaturation 95 ºC for 4 min, 40 cycles of denaturation at 94 ºC for 1 min, annealing at 59.9 ºC for 1 min, and extension at 70 ºC for 1 min, followed by a final extension step at 70 ºC for 5 min and 4 ºC overnight. Negative controls (distilled water) and positive controls (*Bacillus* spp. strains SF82 and RS6b from the CECT) were run in each reaction.

PCR products were analyzed in a 1,5 % agarose gel in 0,5x Tris-acetate EDTA (TAE buffer), run for 45 min at 100 V, and stained with ethidium bromide/ gel RED for 30 min. Size comparisons were made with a 1-kb Plus Ladder (Invitrogen, California, USA). Gel images were captured with a UV transilluminator.

Table 2 – PCR primers used for *Bacillus* screening.

Primer	Expression product	Sequence (5'-3´)	Gene	Melting T (ºC)	Product size (bp)
16SBACF 16SBACR	16S rRNA	GCTTGCTCCCTGATGTTAGC CGGGTCCATCTGTAAGTGGT	16S rDNA	59,9	163

3 Screening and selection of potential biocontrol agents of *Erwinia amylovora*

3.1 *Ex vivo E. amylovora* inhibition assay

Immature loquat fruits (*Algerie* cultivar) and detached pear blossoms (*Blanquilla* cultivar), obtained from IVIA´s greenhouses, were used to screen for potential biocontrol agents of fire blight.

In the first tests, bacterial strains to be tested were allowed to grow in KB agar at 24 ° C for 24 hours, and 48h in the following assays. Colonies were suspended in PBS and the OD_{600} adjusted at 0,20±0,02 (an approximated concentration of 10^8 CFU/ml), for putative antagonist isolates. The pathogen was the strain *E. amylovora* CFBP 1430 (isolated from infected *Crataegus oxyacantha* in the north of France in 1973), at a concentration of 10^7 CFU/ml.

Experiments were done under controlled environmental conditions. Non-treated controls inoculated with water (negative control) and with the pathogen (infection control) were included in all the assays. Two independent experiments were performed for each kind of plant material.

The experimental design consisted of one replicate per treatment with three immature fruits, or two (hypanthium trial) to three (complete flower trial) flowers, per replicate. Pathogen inoculations and disease assessment were always done under biosafety conditions within a Class II biological safety cabinet (Nuaire Class II UN-426-400E, Nuaire Inc., USA).

3.2 *Ex vivo* immature loquat fruit assay

The loquat fruits used in these experiments were collected from the same tree in batches, in order to be similar and be at the same stage of maturation.

Fruits were collected and kept in the dark at 0ºC to 4 ºC with high relative humidity for three days. Fruits were first washed with sterile water, and then surface-disinfected by immersion in bleach (10%) for 30 minutes, with a gentle move every 5-10 minutes. They were washed three more times by immersion in sterile distilled water. Excess water was removed under air flow in a sterile cabinet. Once dried, they were individually involved in tin foil and placed in the dark, at 0-4ºC. Each fruit was wounded per triplicate in the equatorial zone with a tip of a micropipette (wounds of approximately 2mm diameter and 5 mm depth). In the first experiments, colonies of tested bacterial isolates were allowed to

grow in KB agar at 24ºC for 24 hours under controlled environmental conditions. However, a few trials later, the strategy was modified slightly, allowing the growth of colonies until 48 hours to promote the production of secondary metabolites that could have an inhibitory effect on *E. amylovora*.

Figure 9 – Immature loquat fruit with three wounds for fruit *E. amylovora* inhibition assay.

Immature loquat fruits were sprayed with suspensions of the bacteria to be tested at 1×10^8 CFU/ml (0.4–1ml per fruit) using a microsprayer, and placed in plastic boxes (adapted for the trials) in a controlled-environment chamber at 25±1 °C) and high relative humidity (43%). 50 ml of water were previously added to the boxes, to ensure moisture, an essential condition for *E. amylovora* development.

After 24 hours of incubation the fruit wounds were inoculated with 10 µl of a suspension of *E. amylovora* CFBP 1430 at $1x10^7$ CFU/ml (a tenfold dilution from an initial $1x10^8$ CFU/ml (Cabrefiga et al., 2007; Pusey, 1997; Roselló et al., 2013).The inoculated immature loquats were again incubated under the above mentioned conditions for 4 to 10 days.

According to the literature, two strains were shown to present biocontrol properties (Mora et al., 2011; Nagórska et al., 2007; Schnider et al., 1995). *Bacillus subtilis* QST713, isolated from a commercial product used for fire blight control (Serenade Max, BASF, Ludwigshafen, Germany) and *Pseudomonas fluorescens* CHA0, both at 10^8 CFU/ml, were used as biological control reference strains in the first assays. To minimize cross-contamination, negative controls were sprayed first, then infection controls, followed by the isolates to be tested and finally the biological control reference strains.

Efficacy of infection inhibition by the bacterial strains, disease incidence and disease severity of *E. amylovora* on loquat fruit were evaluated after 4-5, 7-8, and 9-10 days after pathogen inoculation and calculated using the following formulas:

$$E = \left(\frac{I_c - I_t}{T} \right) \times 100$$

where *E* is the efficacy of the infection inhibition for each treatment, *Ic* is the number of infected wounds in the non-treated pathogen inoculated control, *It* is the number of infected wounds in the treatment, and *T* is the number of total wounds (9) for the immature fruits corresponding to each repetition. Additionally, the values of efficacy percentage for bacterial strains were categorized based on their degree of antagonistic activity as: not active (NA, 0-19%), soft active (SA, 20-39%), moderately active (MA, 40-69%), active (A, 70-89%) and very active (VA, 90-100%).

$$I = \left(\frac{F_i}{F_t} \right) \times 100$$

where I is the incidence of infection, Fi the number of infected wounds, and Ft the number of total wounds inoculated (9), for the immature fruits corresponding to each repetition.

$$S = \sum_{i \to 1}^{i} \times \left(\frac{SI_{\,i}}{\mathrm{n} \times 3} \right) \times 100$$

where *S* is the disease severity, *SI* is the severity index, *i* is the number of infected wounds, *n* is the total number of wounds and *3* is the maximum severity index. Wounds were considered infected when drops of bacterial exudates and/or necrosis were detected in and around them. The severity of the infection of each inoculated wound was rated at 4-5, 7-8 and 9-10 days after pathogen inoculation, according to the state of the necrosis in a scale from 0 to 3: (0, no symptoms; 1, presence of a slight necrosis around the wound and/or presence of ooze; 2, presence of an intense necrosis around the wound and/or presence of ooze; 3, necrosis progression through the fruit and/or presence of ooze) (Roselló et al., 2013) (Table 5).

Strains were selected for the detached pear blossom assay based on the results of effectiveness in the inhibition of infections from *E. amylovora* CFBP 1430 in immature loquat fruits, for having negative results in hypersensitive response (HR) on tobacco test

(Danhorn and Fuqua, 2007; Lenaerts et al., 2014; Lugtenberg et al., 2001; Paternoster et al., 2010; Pozo et al., 2014).

3.3 *Ex vivo* detached pear blossoms assay

This experiment tested the inhibition of *E. amylovora* infections by the mentioned strains as potential biocontrol agents on blossom blight.

These assays were performed in pear blossoms from *Blanquilla* cultivar (Fig. 10). Pear blossoms with similar degree of ripeness were collected from one loquat tree from an IVIA´s greenhouse (Fig. 10). In the laboratory, individual flowers were maintained with the cut peduncle submerged in 900 µl of a 10% sucrose solution in a single vial. Two methods were used in this assay: with complete blossoms, and other only with the hypanthium and peduncle.

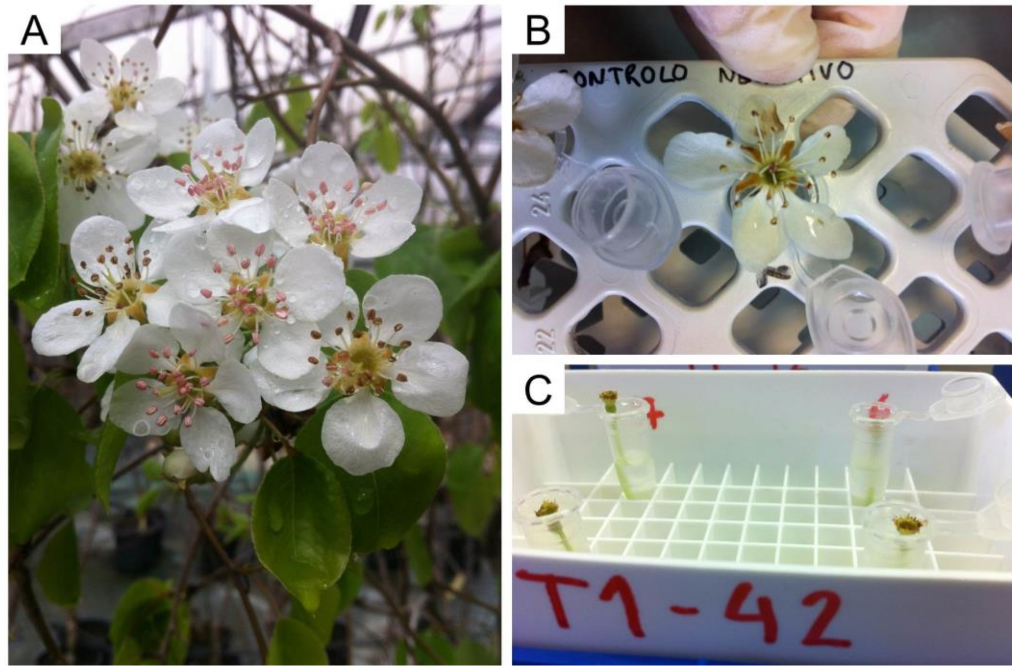

Figure 10 – Pear blossoms sample manipulation.
(A) Pear blossoms from *Blanquilla* cultivar; (B) Tube racks with complete blossoms; and (C) Plastic box with blossoms only with the hypanthium and peduncle.

For the complete blossoms method, flowers were sprayed with suspensions of four selected strains at 1×10^{8} CFU ml^{-1} (0.4–1ml per blossom) by using a microsprayer. For the hypanthium trials, the incomplete flowers were directly inoculated with 10µl of a suspension of two selected strains at $1x10^{8}$ CFU/ml. Non-treated controls inoculated with water (negative control) and with the pathogen (infection/ positive control) were included.

The vials were placed in tube racks (complete blossoms) or plastic boxes (blossoms only with the hypanthium and peduncle) (Fig 10) (both adapted for the trials and previously UV surfaced-disinfected). Each box was placed on a single transparent plastic box, with a thin layer of water and slightly sealed to allow air to flow (Fig. 10).

The plant material was incubated in a controlled environment chamber at 23 °C, high relative humidity (43%) and 16 h of fluorescent light-8 h dark photoperiod. After 24 hours of incubation, the hypanthia of flowers from all possible BCA isolates were inoculated with 10 µl of *E. amylovora* 1430 suspension at 10^7 to 10^3 CFU/ml. The inoculated plant material was again incubated under the above mentioned conditions for 5 days. Flowers were considered infected when necrosis was detected in the hypanthium of each flower.

Efficacy of infection inhibition by the bacterial strains, disease incidence and disease severity of *E. amylovora* on pear blossoms were evaluated after 3 and 5 days after pathogen inoculation and calculated using the following formulas:

$$E = 100 - I$$

where *E* is the efficacy of the infection inhibition for each treatment and *I* is the incidence of infection presented above.

$$I = \left(\frac{F_i}{F_t}\right) \times 100$$

where I is the incidence of infection, Fi is the number of infected wounds, and Ft is the number of total wounds inoculated (9), for the immature fruits corresponding to each repetition.

$$S = \sum_{i \to 1}^{i} \times \left(\frac{SI_{\ i}}{\mathrm{n} \ \times \ 3}\right) \times 100$$

where *S* is the disease severity, *SI* is the severity index, *i* is the number of infected flowers, *n* is the total number of flowers and *3* is the maximum severity index. Severity indexes (*SI*) were determined for each flower according to a state of the necrosis in a scale from 0 to 3: 0, no symptoms; 1, presence of a slight necrosis on the hypanthium; 2, presence of a darker necrosis on the hypanthium; 3, necrosis extending below ovary/ through peduncle (Table 10).

3.4 Hypersensitive response

Bacterial suspensions of the BCA candidates were prepared in PBS from pure cultures grown during 24h on KB, and adjusted turbidimetrically to 10^9 CFU/ml. Then, tobacco leaves (*Nicotiana tabacum* L. cv Xanthi) were injected with each bacterial suspension into leaf veins using a hypodermic needle (Fig. 11), so that the suspension could expand by the leaf mesophyll.

The inoculated area was then demarcated with a permanent marker and identified with the name of the tested strain (Fig. 11). The plants were incubated at room temperature and symptoms were evaluated 72h after infiltrations. *Pseudomonas syringae* pv. *tomato* DC3000 was used as a positive control (a bacterial strain whose positive response is known) and PBS as negative control (Charkowski et al., 1998).

Figure 11 – Tobacco leaf injected with each bacterial suspension into leaf veins.

3.5 Spectrum of antagonistic activity on agar media

Loquat strains that showed the best results in delaying the onset of symptoms of *E. amylovora* on immature pear fruits assay were characterized according to their antagonism on agar medium against *E. amylovora* CFBP1430.

The antagonist activity was assayed with a double layer agar (Iacobellis et al., 2005). The lower layer consisted of KB agar at a concentration of 1.5%; and the upper layer

consisted of 4,5 ml of melted KB agar 0,8%, which was mixed with 0.5 ml of a suspension of *E. amylovora* at 10^8 CFU/ml to obtain a final concentration of 10^7 CFU/ml (1:10 dilution). Other procedure to test the antagonism of the isolates was to prepare a lower layer with a poorer nutrient medium, KB 1:10 dilution, with agar at 1.5%.

Suspensions of antagonistic bacteria, from 48 h grown cultures, were turbidimetrically adjusted at 0,2±0,02 (10^8 CFU/ml), and a drop of 10 µl of each antagonist candidate suspension was transferred to the surface of the overlay agar plates. An infection control (without the antagonistic isolate) was included. *Pseudomonas fluorescens* CHA0 was used as a reference strain. Plates were incubated at 25 ºC and growth inhibition was assessed after 24 and 72 h measuring the diameter of the inhibition zone (Montesinos et al., 1996).

4 Molecular quantification with real-time PCR of selected strains

4.1 Sample isolation and DNA extraction

After the pear blossom assay, each flower was macerated inside filter bags with 800 µl of AFT. The resulting extracts were transferred to labelled eppendorfs, each corresponding to a blossom treated with a specific antagonist (and 24 hours later with *E. amylovora* at different concentrations), the negative control (AFT) and a known concentration of *E. amylovora*.

Bacterial DNA was extracted following the isopropanol extraction procedure (Llop et al., 1999):

1. A volume of 200 µl of AFT was added to the 800 µl extracts in order to dilute them. Eppendorfs with 1000 µl of each samples were centrifuged at 11 000 *rpm* for 10 min. Supernatant was discarded.
2. The pellet was resuspended in 500 µl of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP), vortexed at 750 rpm and left for 1 h at room temperature with continuous shaking.
3. Then it was centrifuged at 5000 g/rpm for 5 min to eliminate extract impurities.
4. A volume of 450 µl of supernatant was carefully transferred to a new labelled eppendorf without pellet carry-over.
5. To each eppendorf, 450 µl of ice-cold isopropanol was added, gently mixed and left for 30 minutes at room temperature.

6. The mixture was centrifuged at 13 000 *rpm* for 5 min, the supernatant discarded and the pellet left to dry overnight.
7. The pellet was hydrated with 200 µl of AFT

4.2 Real time PCR assay

DNA was amplified by real time PCR for *E. amylovora* (Gottsberger, 2010). A total of 145 samples (from flowers at 5 days after pathogen inoculation) were analysed with real time PCR using a LightCycler 480 (Roche). PCR conditions were as follows: 50º C for 3 min, 95º C for 10 min; 45 cycles of 95º C 15 s and 60º C for 1 min (Gottsberger, 2010). Reactions were conducted in 20 µl volumes with PCR mix, and 2 µl sample DNA.

5 Growth curves of selected bacterial strains

The growth rates of the four strains used in the pear blossom assay, in comparison with that of were determined with *E. amylovora* CFBP 1430 strain, were determined with the *Bioscreen C* system.

The growth rate test in Bioscreen was performed for each strain on two plates with 180 µl liquid KB (one plate at 1:1 concentration and the other at 1:10) and 20 µl of bacterial suspension at 10^4 CFU/ml. The different media concentrations simulated different growth environments for bacterial growth. AFT was used as negative control in all experiments. The plates run in the *Bioscreen C* for 91 h at 26ºC. The optical densities (OD) of test wells were read every 30 min at a wavelength of 600 nm, gently shaking the plate for 10 minutes before each reading.

Growth curves produced by the Bioscreen are presented as OD plotted against time. A detection time (determined from a point where a rapid change in OD is verified) can be related to cell number. The detection times can be converted to cell numbers, using calibration graphs.

6 Statistical analyses

Differences between days after *E. amylovora* inoculation for incidence of infection, severity of infection and efficacy of antagonistic activity by bacterial strains were tested using ANOVA with significance at $P<0.05$. HSD Tukey Posthoc test (with 95% confidence intervals) was used to examine differences between overall incidence, severity and efficacy between days after pathogen inoculation and efficacy scales. Number of bacterial

strains in each efficacy category for the three time periods after pathogen inoculation on loquat fruits was compared using a Chi-Square 2 x 2 contingency table test (http://www.socscistatistics.com/tests/chisquare/Default2.aspx). Statistical analyses were conducted in R 3.3.1. and SPSS v15.

CHAPTER III

RESULTS AND DISCUSSION

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1 Sample processing and bacterial isolation

From the samples of loquat blossoms collected from the two localities in the province of Alicante, a total of 223 isolates were counted on the different media assayed. Visually, 193 distinct isolate morphotypes were registered (Table 3). From these, 143 isolates were cryopreserved for further assays, as well as 30 isolates from loquat tree leaves from IVIA greenhouse.

Table 3 – Number of different morphotypes obtained on different culture media from loquat blossoms collected from the two sampled localities.
(KB+ C) King´s medium B + Cycloheximide; (KB+C+P) King´s medium B with phosphates added after autoclaving + Cycloheximide; (CCT) *Erwinia* standard selective medium; and (Levan+C) Levan medium + Cycloheximide.
Numbers in bold refer to the cryopreserved isolates.

Isolation Medium	La Nucia	Callosa d´En Sarrià	Total
KB+C	**32** / 56	**21**[1] / 26	**53** / 82
KB+C+P	**25** / 29	**1** / 1	**26** / 30
CCT	**15** / 17	**8** / 8	**23** / 25
Levan+C	**20** / 28	**21** / 28	**41** / 56
	92 / 130	**51** / 63	**143** / 193

A higher number of different bacterial morphotypes was recovered from La Nucia orchard in all the media assayed, except for Levan+C, where the number was approximately the same from the two orchards. The different numbers are probably due to different environmental and/or agronomic conditions of the two localities.

As a whole, the medium with higher recovery percentage was KB+C followed by Levan+C. As expected, the medium with the lowest yield was CCT, that is semi-selective and, therefore, hamper the growth of some of the bacteria that can grow in routine growth media. Nevertheless, the yield of CCT was, surprisingly, the same that that of KB+C+P. Both KB media, that with phosphates autoclaved together (KB+C) and that with phosphates autoclaved separately (KB+C+P), allowed obtaining a variable number of isolates, depending on the orchard (Table 3). In one case, KB+C+P from Callosa d'En Sarrià, only one isolate was recovered. Both media can be used to grow isolates with distinct morphotypes. Conversely to that observed by Tanaka et al. (2014), who showed that when phosphates are autoclaved together with agar to prepare solid growth media, the number of bacterial colonies becomes remarkably lower than on medium prepared with phosphates autoclaved separately, we observed a higher number of colonies in the KB+C. In our conditions (number and isolates tested in both media), the use of the KB with

[1] Strain C19-A.1 was cryopreserved in duplicate

phosphates autoclaved separately does not seem to confer an advantage for recovering a higher and a more diverse number of bacterial microbiota from loquat plant material.

2 Morphological, biochemical and molecular characterization of bacterial strains from loquat microbiota

An important and necessary step in the biocontrol agent selection process is the characterization of the isolates. This characterization had an important role to select strains with desired traits, and to discard others with deleterious or undesired traits, such as being pathogenic. The selection process is possibly the most difficult step in a biological control program development.

2.1 Morphology

The 143 cryopreserved isolates from loquat blossoms were characterized morphologically based on their size, shape, colour, apparent texture, elevation and edge. Some of the most common characteristics found were yellow colour (70%) and circular shape (54%) (Fig. 12, for more details see Tables S4 and S5).

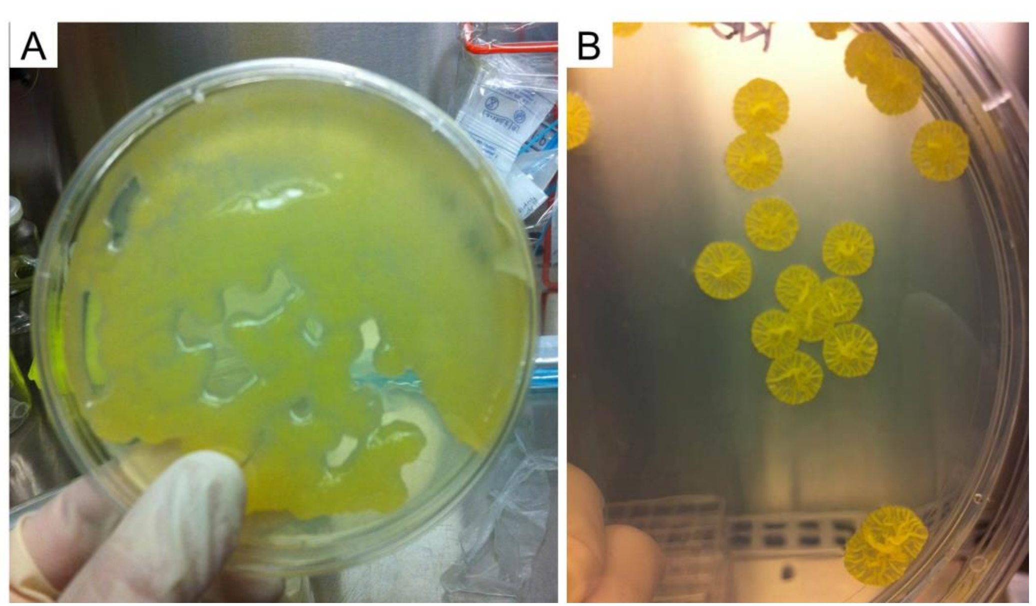

Figure 12 – Morphology of two distinct bacterial colonies after purification on KB medium.
(A) Mucoid yellow bacterial colonies, and (B) Filamentous, umbonate and curled bacterial colonies.

2.2 Gram and oxidase reactions

From the 173 cryopreserved bacterial isolates obtained from loquat blossoms (n=143) and leaves (n=30), 135 isolates (78%) were identified as Gram negative and 38 isolates

(22%) as Gram positive (Tables 4 and S6). Eight Gram negative isolates (6%) were oxidase positive (4 from blossoms and 4 from leaves) (Fig. 13 and Table 4). Therefore, whereas most of the isolates from blossoms were Gram-negative, in leaves a similar percentage of Gram-positive and Gram-negative bacteria were found. This shows that the microbiota found in different plant surfaces can vary greatly.

Table 4 – Results of Gram and oxidase reaction from bacterial isolates obtained from loquat blossoms and leaves.

	Gram -		**Gram +**
	Oxidase -	**Oxidase +**	
Blossoms	113	4	26
Leaves	14	4	12
	127	8	38

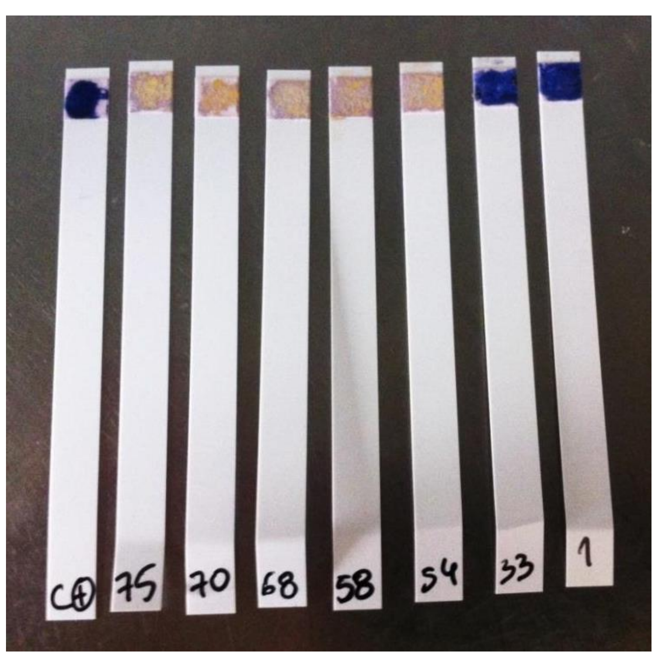

Figure 13 – Cytochrome oxidase test showing negative (non-coloured) and positive (purple) results from different isolates.

2.3 *Bacillus* screening

Since *Bacillus* spp. can display interesting traits related to biocontrol, the presence of *Bacillus* spp. was screened by testing 16 Gram-positive isolates from blossom extract samples (from T1-1 to T1-15 and T1-26) using a PCR protocol with specific primers targeting this bacterial genus (Table 2). As can be seen in Fig. 14, only strain T1-15 exhibited the band of 163 bp, characteristic of the genus *Bacillus*.

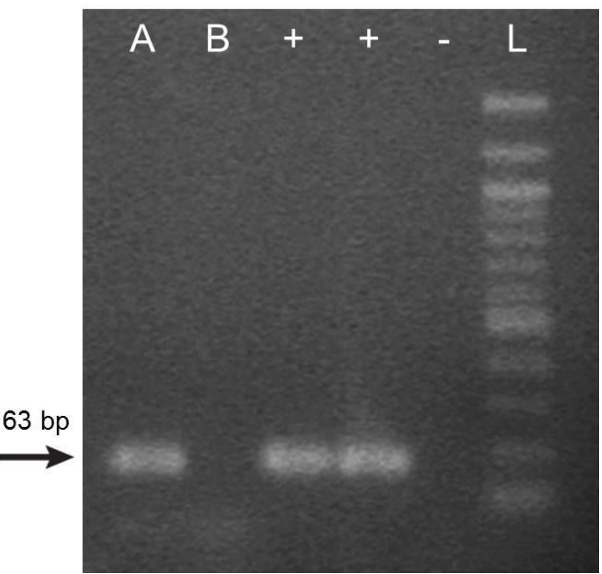

Figure 14 – PCR amplification results with specific *Bacillus* spp. primers of some isolates from loquat tree blossom. (A) Strain T1-15; (B) Strain T1-26; (+) Positive controls, *Bacillus* spp. RS6B and SF82, respectively from left to right; (-) Negative control (ultrapure water); and (L) 1Kb DNA plus ladder (Invitrogen, California, USA).

Although the primers used in this PCR reaction (16SBACF and 16BACR) also can amplify some *P. fluorescens* strains (Mora et al., 2011), the amplification result of strain T1-15 indicates that this strain, positive for Gram reaction test, belongs to the genus *Bacillus*. According to these results, the percentage of *Bacillus* spp. isolates in the analysed samples was very low. It is not surprising, since usually the *Bacillus* population levels in plant samples are not detected or varied strongly with sample type and origin, with isolates being more frequently obtained from soil or rhizosphere than from aerial plant parts (Mora et al., 2011), just those sampled this thesis (blossoms and leaves).

3 Screening and selection of potential biocontrol agents: Screening of *E. amylovora* antagonists

In studies dealing with the development of putative biocontrol agents, a crucial step is the screening of microorganisms to select strains with the ability to inhibit the growth of the pathogen. These tests are proposed as a pre-screening step to discard most of unsuitable isolates and only select candidates with the biocontrol potential. Thus, the selection of a possible biological control agent was based on the criteria of efficiency and consistency in the suppression of necrosis in immature loquat fruits and in pear blossoms, under controlled conditions.

3.1 *Ex vivo E. amylovora* inhibition assay: Immature loquat fruit assay

In this assay, immature fruits are chosen because it is well known that *E. amylovora* develops better in unripe fruits (Doolotkeldieva and Bobusheva, 2016).

3.1.1 Incidence and severity of E. amylovora *infection*

A selection of 160 isolates was tested in immature loquat fruit for its biocontrol potential against *E. amylovora*. The severity of infection of each inoculated wound was rated according to a symptom scale from 0 (no necrosis in the three wounds and ooze) (Table 5) at 4-5, 7-8 and 9-10 days after pathogen inoculation. All negative controls were negative for the times analysed.

Table 5 – Severity levels of *E. amylovora* infection in immature loquat fruit.

	SI=0	SI=1	SI=2	SI=3
Immature loquat fruit assay				

Overall incidence and severity of infection differed significantly between the different times after *E. amylovora* inoculation (ANOVA, df=2, F=53.719, P<0.001 for incidence and df=2, F=84.417, P<0.001). As expected, the percentages of incidence and severity of infection were significantly higher at 9-10 days than at 7-8 days (Tukey HSD, P<0.001) and 4-5 days (Tukey HSD, P<0.01) after inoculation (Fig 15). As can be seen in the plot box of the right part of Figure 15A, at 9-10 days post-infection an incidence of 80-100% was observed in the presence of most of the isolates, whereas an incidence lower than 40% was observed in the presence of only 5 isolates. At the same time, the severity of infection was higher than 60% with most of the strains. In conclusion, only a few bacterial strains successfully delayed disease caused by *E. amylovora*.

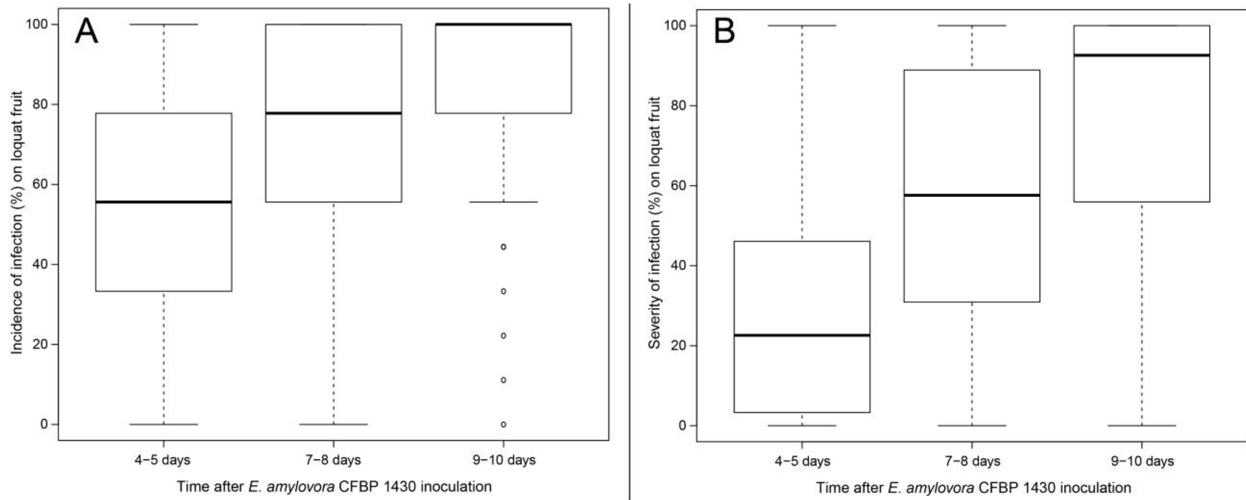

Figure 15 – Overall incidence (A) and severity (B) of infection of 160 bacterial strains on loquat fruit at different days after pathogen inoculation.
Middle line represents the median, box represents the upper and lower quartile, the dashed lines represent the greatest and lowest values excluding the outliers, and circles represent the outliers.

3.1.2 Efficacy of antagonistic activity of bacterial strains against E. amylovora *disease*

The efficacy of the tested bacterial strains on delaying *E. amylovora* necrosis was evaluated at different times after pathogen inoculation. Isolates were grouped by the efficacy in the inhibition of infections in immature loquat fruits, as very active (100-90 %), active (89-70 %), moderately active (69-40 %), soft active (39-20 %) and not active (19-0 %), according to extension of symptoms (Tables S7, S8 and S9).

The results in Fig. 16 show that at 4-5 days after inoculation, 128 strains presented different levels of active inhibition of *E. amylovora* (CFBP 1430) infection: 7 strains were very active, 24 active, 63 moderately active and 34 soft active (Table S7). At 7-8 days after inoculation, 84 strains showed different levels of active inhibition of *E. amylovora* infection: 1 was very active, 7 active, 30 moderately active and 46 soft active (Table S8). At 9-10 days after inoculation, 31 strains were considered to have different levels of active inhibition of infection: 1 was very active, 2 active, 9 moderately active and 19 soft active (Table S9). As shown in Fig. 16, the number of active strains at 9-10 days after *E. amylovora* inoculation (considering VA, A, MA and SA) is significantly lower than at 7-8 days (X^2=20.756, P<0.001) and 4-5 days (X^2=65.273, P<0.001).

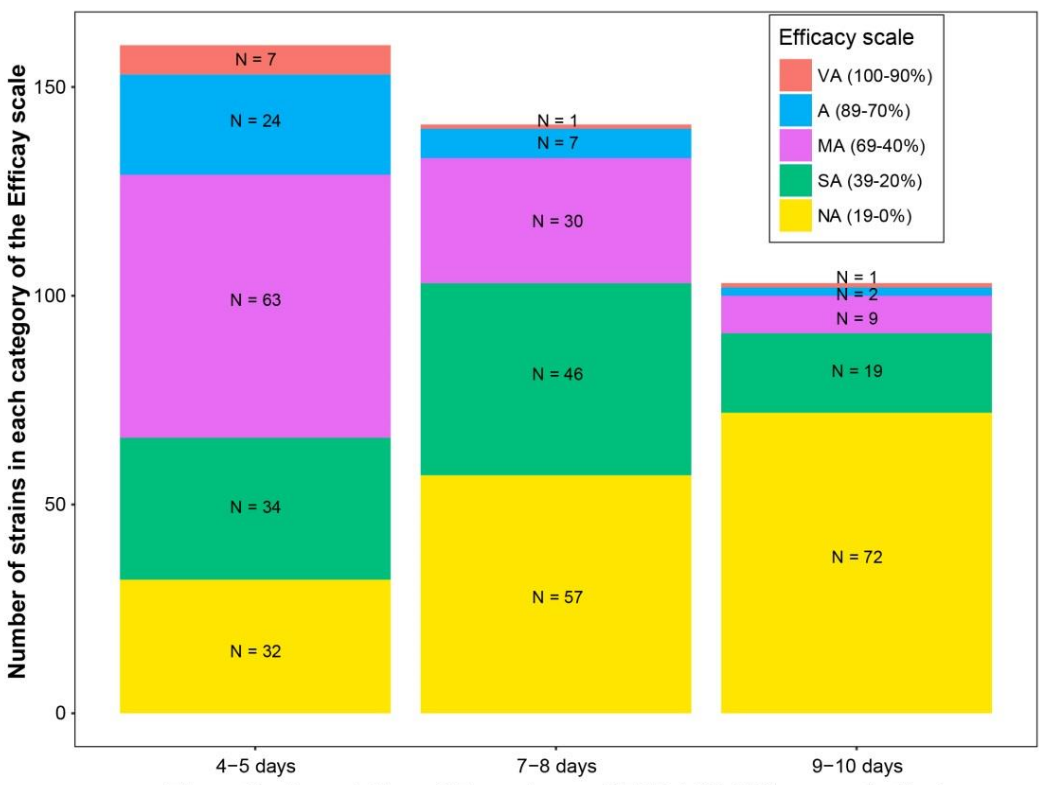

Figure 16 – Efficacy scale of 160 strains in immature loquat fruit at different days of infection by *E. amylovora*.
(VA) Very Active; (A) Active; (MA) Moderately Active; (SA) Soft Active; and (NA) Not Active.
Strains with 0% efficacy in previous days were not considered in following days.
For more details see Tables S7, S8 and S9.

Depending on the antagonistic capabilities of the strains, either by competition in surface colonization or by producing antagonistic substances against *E. amylovora*, signs of necrosis would be visible at different stages (Cabrefiga et al., 2007; Danhorn and Fuqua, 2007; Paternoster et al., 2010; Roselló et al., 2013). As shown in Fig. 17, the percentage of incidence and severity of infection is significantly different between the 5 efficacy scales defined in this study, with the more active strains displaying the lower infection percentages (ANOVA, df=4, F=1569.5, P<0.001 for incidence, and df=4, F=471.97, P<0.001 for severity). In fact, by 9-10 days, only loquat fruits previously inoculated with bacterial strain T3-27 displayed no signs of infection by 9-10 days after pathogen inoculation (Tables 9 and S9).

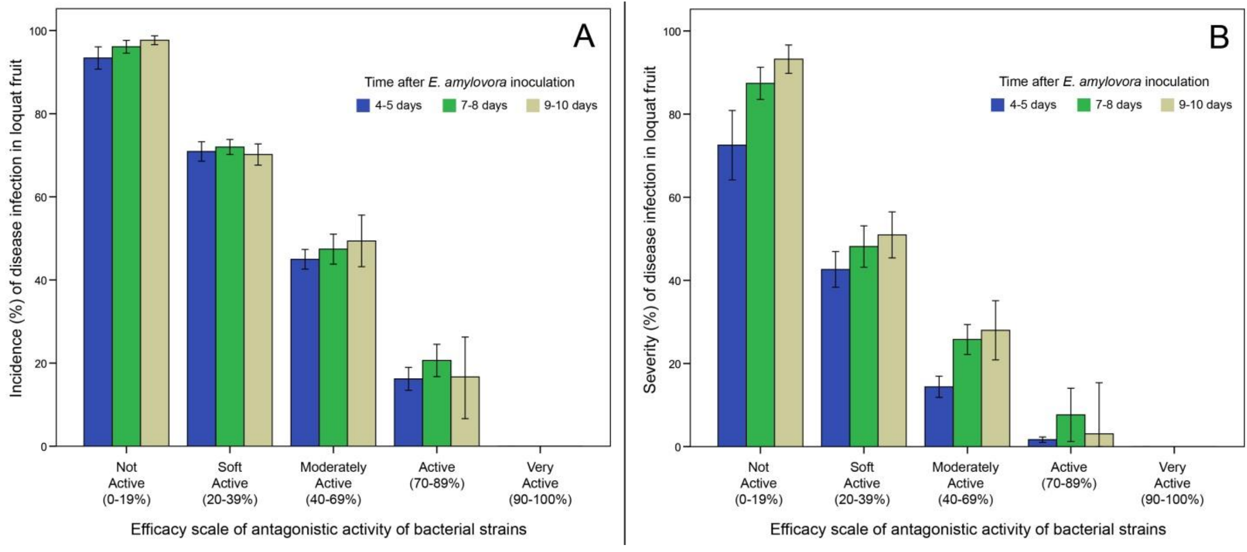

Figure 17 – Incidence and severity of infection of bacterial strains with different antagonistic activity over time. Error bars represent 95% confidence intervals.

An important aspect of these assays is the timing between the application of the potential antagonists and the pathogen inoculation. This is because antagonists can lose their efficacy when co-inoculated or inoculated after the pathogen (Lindow et al., 1996), which can affect the control of disease. The fruit wounds were sprayed with the antagonistic strains 24 hours prior to the *E. amylovora* CFBP 1430 inoculation, to allow wound colonization of the bacterial strains in study (Cabrefiga et al., 2007; Pusey, 1997; Roselló et al., 2013). This also applies to the detached pear blossom assay in section 4.1.

Bacillus subtilis QST713, isolated from a commercial product used for fire blight control (Serenade Max, BASF, Ludwigshafen, Germany) and *P. fluorescens* CHA0, both at 10^8CFU/ml, were used as biological control reference strains in the first fruit assays. To our knowledge this was the first time these were tested in loquat fruits but since these strains did not delay the appearance of necrosis symptoms, were excluded as control reference strains in the course of the experiments.

3.2 Spectrum of antagonistic activity on agar media

The 36 loquat strains that were more effective in fruit bioassays were tested *in vitro* to check their antagonistic activity on agar media under controlled conditions. Antagonism activity was detected by the presence of a halo of *E. amylovora* growth inhibition, around the colony of the bacterial strains in study, indicating the potential of BCA candidates to produce metabolites or substances against *E. amylovora* (Ishimaru et al., 1988).

The results obtained (Fig. 17 and Table 6) showed that only the strains T1-9, T1-40 and *P. fluorescens* CHA0 (used as positive control) had an antagonistic activity against *E. amylovora*, produced a halo of inhibition in the diluted KB medium (1:10) (Fig. 18 and Table 6). This halo was larger in *P. fluorescens* CHA0, followed by T1-40 and T1-9 (Table 6). This medium, which is not a rich nutrient medium, was used to force tested bacterial strains to compete for nutrients and, consequently, produce metabolites against *E. amylovora* (antibiotic properties (Keel et al., 1989)).

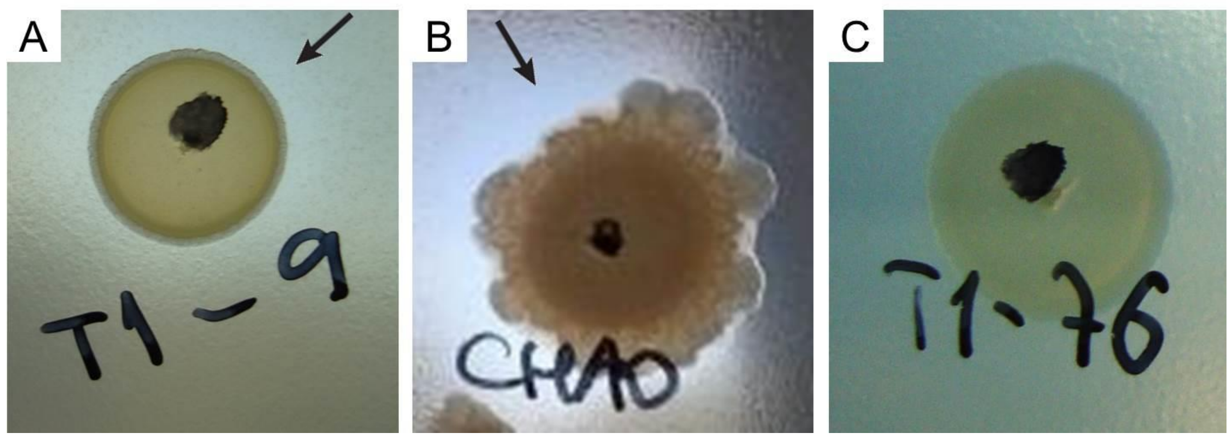

Figure 18 – Growth inhibition by antagonistic activity of some tested strains against *E. amylovora* infection in a double layer agar assay. (A and B) Bacterial strains displaying a halo of inhibition (arrows); and (C) An example of a negative result.

Table 6 – Diameter of the inhibition zone of specific strains against *E. amylovora* infection.

Strain	Diameter of the inhibition zone (mm)
T1-9	2
T1-40	3
P. fluorescens CHA0	7

3.3 Hypersensitive response

Hypersensitive response (HR) is considered to be a major element of plant disease resistance and to show the potential of bacterial isolates to be pathogenic, based on the capacity of non-host plants to respond against the phytopathogens by means of an incompatible reaction. It is a complex form of localized programmed cell death associated with the induction of local and systematic defence response that often leads to macroscopically visible localized tissue necrosis (Charkowski et al., 1998; Govrin and Levine, 2000).

Out of 27 tested strains, 7 (26%) did not produce a hypersensitive response (HR) in tobacco plants (i.e. considered negative for having 0% necrosis, Fig. 19 and Table 7). Six strains (22%) were positive (i.e. having ≥90% necrosis), while in the other 14 strains (52%)

only some spots of necrosis were found (Fig. 19 and Table 7). Positives show that these bacterial strains are pathogenic to tobacco plants and potentially to other plant species, which makes them as unsuitable as biocontrol agents. For this reason, bacterial strains with a positive reaction were discarded from the pear blossom assay.

Occasional occurrence of necrosis may also be associated with specific plant defence mechanisms. All negative controls were negative and all positive controls were positive (e.g. Fig. 19A).

Table 7 – Hypersensitive Response (HR) symptoms in tobacco.

Strain	HR symptoms in tobacco leaves
T1-9	HR + (100% necrosis)
T1-10	HR + (100% necrosis)
T1-12	10% necrosis
T1-14	10% necrosis
T1-20	40% necrosis
T1-38	70% necrosis
T1-39	30% necrosis
T1-40	HR - (0% necrosis)
T1-41	50% necrosis
T1-42	HR- (0% necrosis)
T1-49	70% necrosis
T1-50	HR + (90% necrosis)
T1-56	HR- (0% necrosis)
T1-57	10% necrosis
T1-70	40% necrosis
T1-71	10% necrosis
T1-76	HR + (100% necrosis)
T2-17	40% necrosis
T2-18	HR + (100% necrosis)
T2-22	HR - (0% necrosis)
T2-27	HR - (0% necrosis)
T2-34	HR + (100% necrosis)
T2-43	5% necrosis
T2-53	30% necrosis
T3-3	60% necrosis
T3-14	HR - (0% necrosis)
T3-27	HR - (0% necrosis)

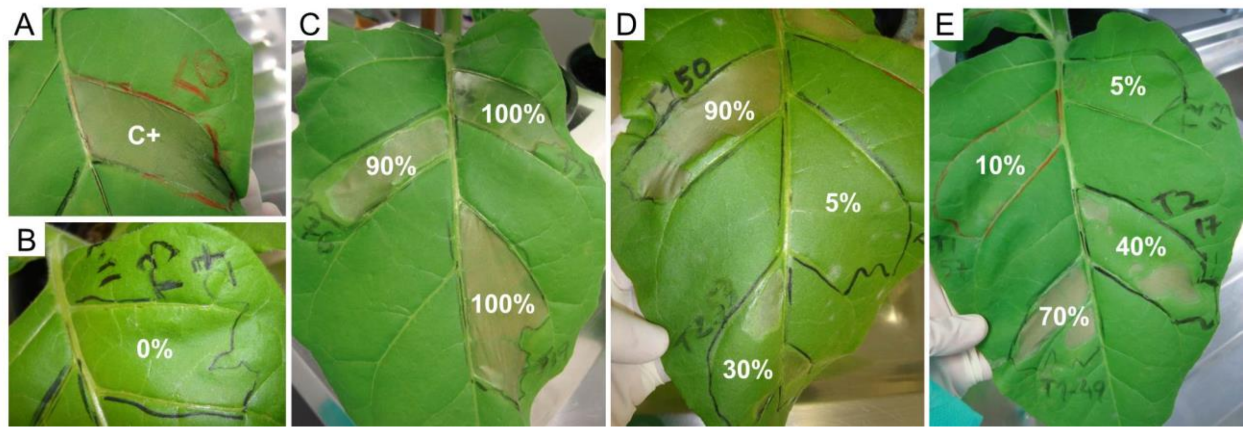

Figure 19 – Example of Hypersensitive Response (HR) test in tobacco plants inoculated with tested strains. Percentages indicate the level of necrosis observed in tobacco leaves. C+) Positive control. Positive response was considered ≥90% necrosis and negative response 0% necrosis (Table 7).

3.4 Molecular identification of bacterial strains by 16S rRNA sequencing

The molecular identification by 16S rRNA sequencing allowed the identification at the species level, of the bacterial strains that showed the best results on delaying the onset of necrosis by *E. amylovora* infection on immature loquat fruit assay (see section 3.2 in the results). *Rosenbergiella epipactidis* species was sequenced in three different loquat flower samples (Table 8), corroborating that they are present on nectar flower, where this species was originally isolated from Lenaerts et al. (2014). Additionally, the identification of T3-27 as a *C. flaccumfaciens* strain could be important since this bacteria species has been suggested to inhibit the growth of *X. fastidiosa*, an important plant pathogen, in *in vitro* experiments (Lacava et al., 2007). Although similarity is equal or similar to 100% for all sequenced bacterial strains, this molecular identification is based only in a single molecular marker and so future studies should further characterize these strains by sequencing other molecular markers. This is especially important because some of these genera are still poorly known, thus with potential diversity yet to be discovered.

Table 8 – Molecular identification of tested bacterial isolates by 16S rRNA sequencing.

Isolates	Species	Similarity (%)	Number of nucleotide differences	Lenght (bp)
T1-12	*Rosenbergiella epipactidis*	99,8	2	790
T1-40	*Erwinia* spp.	99,4	5	830
T1-42	*Pseudomonas rhizosphaerae*	99,6	6	1382
T1-56	*Rosenbergiella epipactidis*	100	0	1362
T1-70	*Erwinia* spp.	99,4	5	772
T1-71	*Pseudomonas azotoformans*	99,9	1	798
T2-27	*Enterobacter cancerogenus*	99,6	5	1393
T2-34	*Rosenbergiella epipactidis*	100	0	792
T3-27	*Curtobacterium flaccumfaciens*	99,7	4	1342

3.5 Selection of active strains for detached pear blossoms assay

From the active strains at 9-10 days, 4 strains were selected for the pear blossom assay based on successful growth and negative results on HR (0% necrosis, see Table 7). These 4 strains were T1-42, T1-56, T2-27 and T3-27. Table 9 summarizes the incidence, severity and efficacy calculations for these selected strains.

Table 9 – Characteristics of the four selected bacterial strains antagonistic activity and *E. amylovora* infection.

Strain	Time	Incidence (%)	Severity (%)	Efficacy (%)	Efficacy scale
T1-42	4-5 days	33.3	7.4	66.7	MA
	7-8 days	33.3	9.9	66.7	MA
	9-10 days	55.6	22.6	44.4	MA
T1-56	4-5 days	22.2	4.9	77.8	A
	7-8 days	77.8	28.8	22.2	SA
	9-10 days	77.8	37.4	22.2	SA
T2-27	4-5 days	0.0	0.0	100.0	VA
	7-8 days	22.2	4.9	77.8	A
	9-10 days	22.2	4.9	77.8	A
T3-27	4-5 days	0.0	0.0	100.0	VA
	7-8 days	0.0	0.0	100.0	VA
	9-10 days	0.0	0.0	100.0	VA

4 Assessment of biocontrol antagonistic activity of four selected bacterial strains against *E. amylovora* infection

4.1 *Ex vivo E. amylovora* inhibition assay: Detached pear blossoms assay with four selected bacterial strains

According to the literature, flowers are more representative models to evaluate the efficiency and consistency of the antagonist effect of selected strains as biocontrol agents of fire blight, since *E. amylovora* can use the aminoacids and sugars present in the flowers to thrive and colonize them (Beer et al., 1984; Lindow et al., 1996).

The blossom assay was selected because some studies indicate that fire blight is controlled when antagonistic bacteria are applied to and become established on the stigmatic surfaces of pear and apple blossoms prior to colonization by *E. amylovora* (Beer et al., 1984; Lindow et al., 1996). The four more effective strains which inhibited *E. amylovora* infections in the immature loquat fruit assay (*P. rhizosphaerae* (T1-42), *R. epipactidis* (T1-56), *E. cancerogenus* (T2-27) and *C. flaccumfaciens* (T3-27)) were tested for their ability to suppress blossom blight.

Two structures of pear flowers were used in this assay: complete blossoms, and incomplete blossoms (hypanthium and peduncle). In order to investigate the influence of

petals and the flower pistil as potential barriers for *E. amylovora* infection petals and pistil were removed from blossoms as described by Paternoster et al. (2010), which can reduce contaminations by fungal infections in blossoms. In addition, the sucrose concentration used (10%) was required for infection to occur (Pusey, 1997).

Table 10 – Severity index levels for *E. amylovora* infection in complete and incomplete loquat flowers.

	SI=0	SI=1	SI=2	SI=3
Complete flower assay				
Incomplete flower assay				

Unexpected results were obtained in both complete and incomplete flower assays with no relationship between inoculation of higher *E. amylovora* concentrations and higher incidence and severity of infection, as well as no apparent relationship between lower *E. amylovora* inoculations and potentially greater antagonistic activity of the tested strains. As shown in Figs. 20 and 21, no relationship between incidence/severity of infection in flowers that were previously treated with a specific bacterial strain, at 3 or 5 days after inoculation with different concentrations of *E. amylovora*. In fact, some of the results seem counter-intuitive because it was expected that the potential antagonistic bacterial strains would increase their antagonistic activity (thus displaying low levels of incidence and severity of infection) for lower *E. amylovora* concentrations.

Nevertheless, the assessment of necrosis in flowers is often difficult because these represent a fragile part of a plant that is easily affected by pathogens or natural oxidation. This is particularly important since some of the negative controls also presented symptoms of necrosis (Table S11). The possibility of pathogen contamination in negative samples was discarded by obtaining negative results in real-time PCR assay to estimate *E. amylovora* concentrations (Table S11). Another difficulty arises from the characterization of severity index levels from hypanthium and peduncle necrosis because it often extends from the ovaries thought the peduncle. Thus, for a better visualization of the necrosis

extension, future studies should incorporate a longitudinal cut from the ovary to the end of the peduncle.

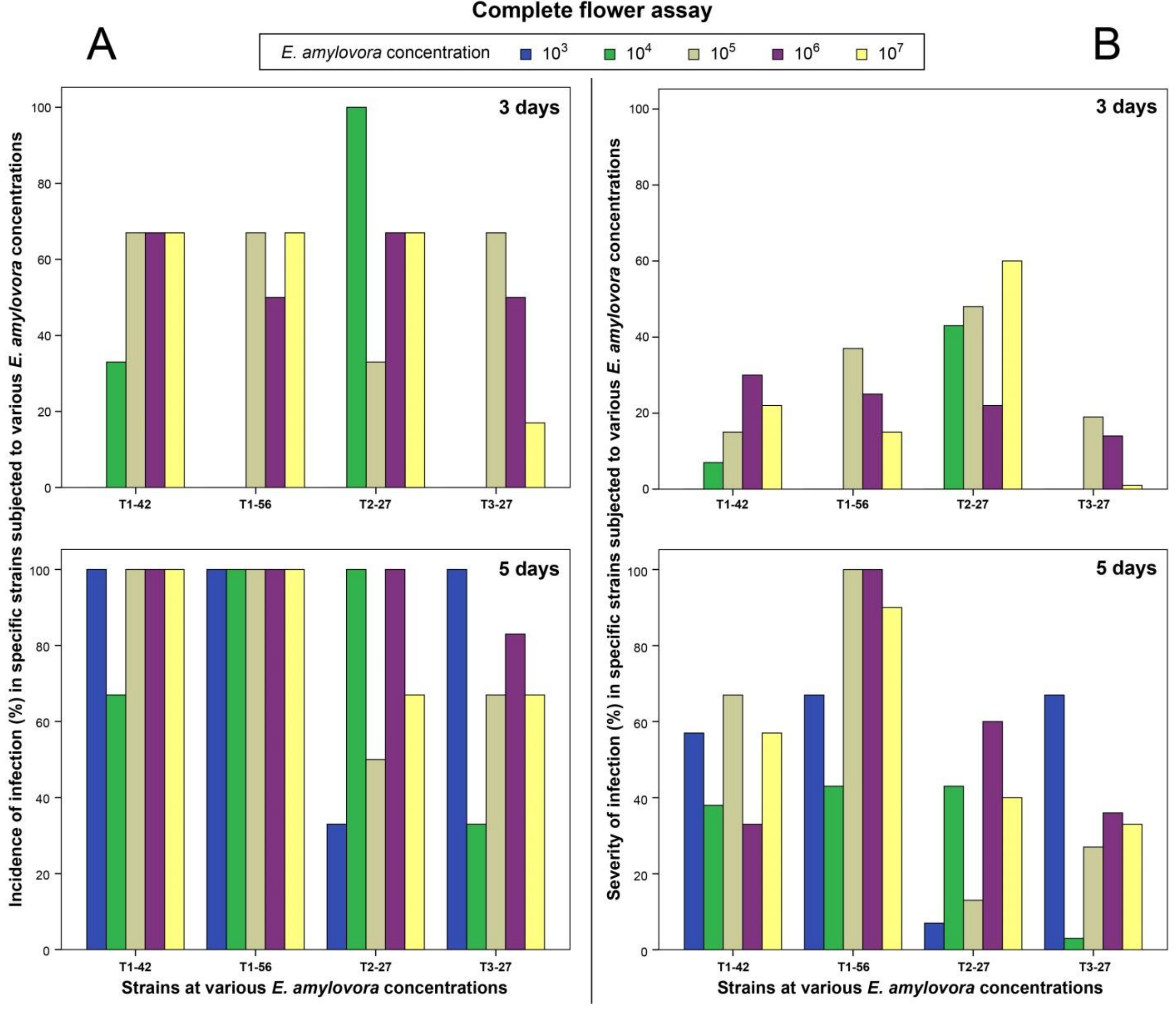

Figure 20 – Incidence and severity of infection by several *E. amylovora* concentrations on complete flowers previously treated with selected bacterial strains.

For the incomplete flower assay, the two strains that may potential as biocontrol agents because of a higher amount of information available on the literature (i.e. T1-42 *P. rhizosphaerae* and T1-56 *R. epipactidis*).

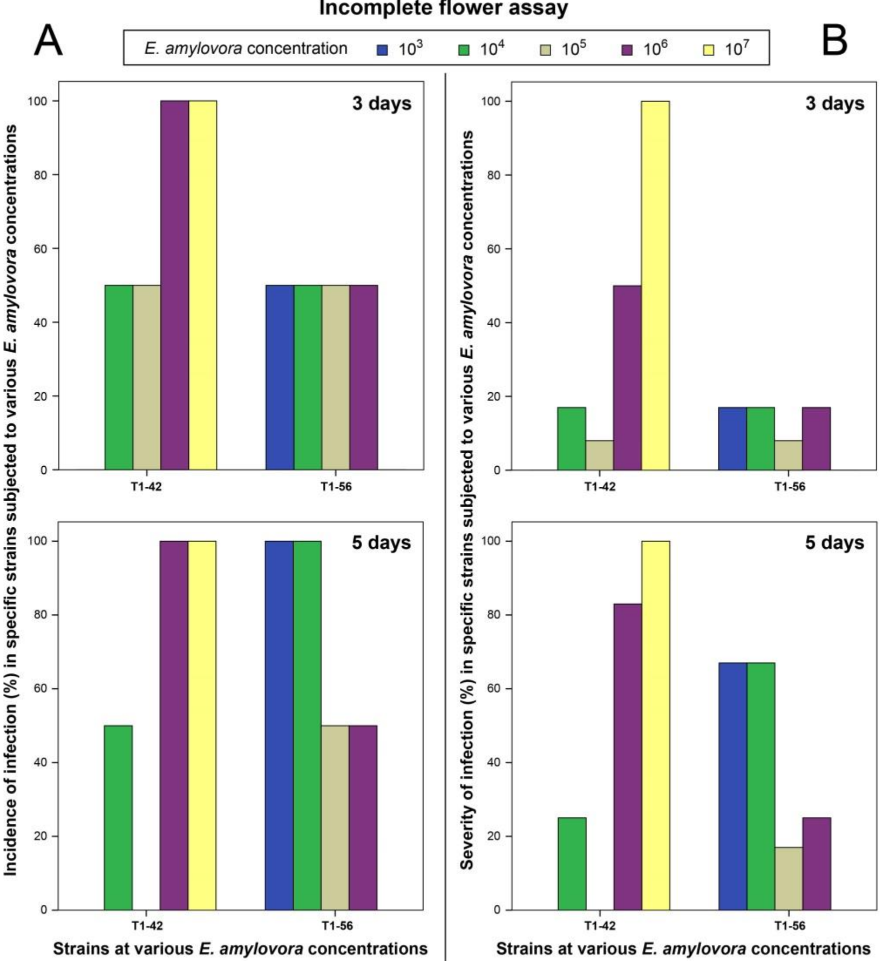

Figure 21 – Incidence and severity of infection by several *E. amylovora* concentrations on incomplete flowers previously treated with selected bacterial strains.

4.2 Molecular quantification with real-time PCR

Real-time PCR allows to estimate the amount of *E. amylovora* in each sample. The lower the cross point cycle (Cp), the higher the amount of *E. amylovora* DNA, thus it was conducted in order to compare the antagonistic activity of bacterial strains against different concentrations of *E. amylovora*. Additionally, it allowed confirming if the occurrence of necrosis in the negative controls was not due to the presence of *E. amylovora*. The fact that no *E. amylovora* was amplified in negative controls shows that the occasional occurrence of necrosis in the negative controls throughout the experiments probably occurred due to natural oxidation of the flowers.

Another unexpected result was the lack of relationship between CPs, flower replicates and inoculated *E. amylovora* concentrations (Table S11 and Fig. S2). These could due to: 1) the inoculation of either the pathogen or antagonistic strain was not successful, and 2) the amplification of targeted fragment was not successful. In fact, the reproduction of real-

time PCR Cp values is often subjected to small variations between plates and machines due to several factors, such as pipetting errors. For this reason, it is recommended to use at least duplicates or triplicates per DNA sample analyzed, unlike the single replicates used per sample in this study. Therefore, future studies should follow these norms to reduce disparities between replicates.

In addition, due to its high sensitivity, real-time PCR is subjected to occasional amplification of false positives (particularly above cycle 30) due to background no-template control (Pujol et al., 2006). Thus, it is possible that some of the late positives in the real-time PCR assay (see Table S11) may in fact be false positives. Once again, the use of triplicates per each flower replicate would allow the exclusion of a possible outlier, which would increase the robustness of the results obtained.

4.3 Biochemical characterization with API system of the four selected bacterial strains

API 20 E system were used to further characterize biochemically the isolates that represent *R. epipactidis* and *E. cancerogenus* species (T1-56 and T2-27, respectively) because these strips are recommended for the Enterobacteriae family and other non-fastidious gram negative bacteria. The results of the 20 biochemical standardized tests including β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H2S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose fermentation or oxidation are presented in Fig. 22.

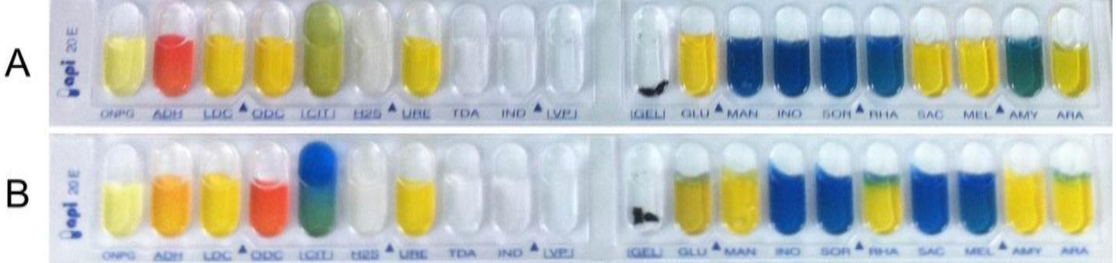

Figure 22 – API 20 E test of isolates that corresponded to *R. epipactidis* and *E. cancerogenus* species. (A) Strain T1-56 (*R. epipactidis*); and (B) Strain T2-27 (*E. cancerogenus*).

The isolates that corresponded to *P. rhizosphaerae* and *C. cancerogenus* were characterized using API 20 NE system, used for gram-negative non enterobacteria. The results of the 21 biochemical standardized test including the reduction of nitrates to nitrites,

reduction of nitrates to nitrogen, indole production, glucose acidification, arginine dihydrolase, urease, β-glucosidases hydrolysis, protease hydrolysis, β-galactosidase, glucose, arabinose, mannose, mannitol, M-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate, phenyl-acetate assimilation, cytochrome oxidase are presented in Fig. 23.

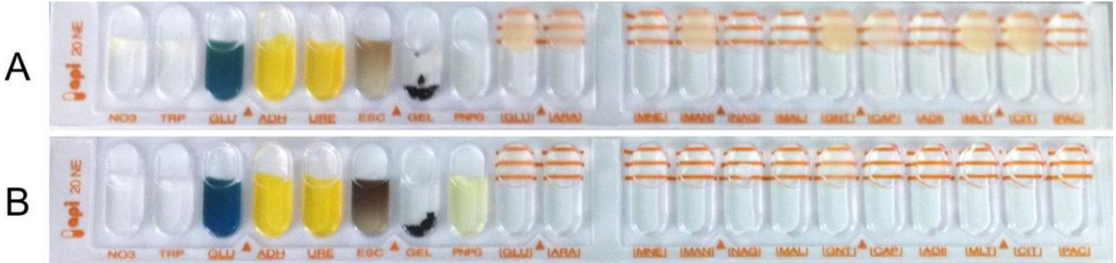

Figure 23 – API 20 NE of isolates that corresponded to *P. rhizosphaerae* and *C. cancerogenus* species. (A) Strain T1-42; and (B) Strain T3-27.

Finally, the four strains (*P. rhizosphaerae* (T1-42), *R. epipactidis* (T1-56), *E. cancerogenus* (T2-27) and *C. flaccumfaciens* (T3-27)) were characterized using API 50 CH, for carbohydrate metabolism characterization (Table S10). Some tests were not conclusive after 24 hours, for this reason results for the three API systems are given at 24h and 48h after inoculation (Table S10).

4.4 Growth curves

Growth curves for each strain used in the detached flower assay (*P. rhizosphaerae* (T1-42), *R. epipactidis* (T1-56), *E. cancerogenus* (T2-27) and *C. flaccumfaciens* (T3-27)) and *E. amylovora* were produced using the Bioscreen C. Growth curves were plotted using Microsoft Excel version 14.0. The software converted kinetically measured OD data into growth curves and, by constructing appropriate calibration graphs, the data was interpreted as bacterial counts.

The purpose of these tests was to evaluate the growth rate of the four strains used in the pear blossom assay and to compare it with the growth rate of *E. amylovora* CFBP 1430. As shown in Figs. 24 and 25, the faster the change in OD, which corresponds to higher cell number, the faster the detection time. In the richest media (KB 1:1), strains T1-42 and T1-56 showed to highest growths (Fig. 24). On the other hand, in a poorer medium the other two bacterial strain had higher growths (KB 1:10) (Fig. 25).

Bioscreen C system was used to directly promote (through incubation and agitation) and measure microorganism growth in treated and in control broths. As microorganisms

grow, they increase the turbidity of their growth medium. Therefore, an optical density (OD) curve was generated by measuring the turbidity of this medium over time, which reflected the growth (increased concentration) of the organism. A higher growth rate of the strains in study may limit *E. amylovora* growth because fewer resources will be available for the pathogen to develop.

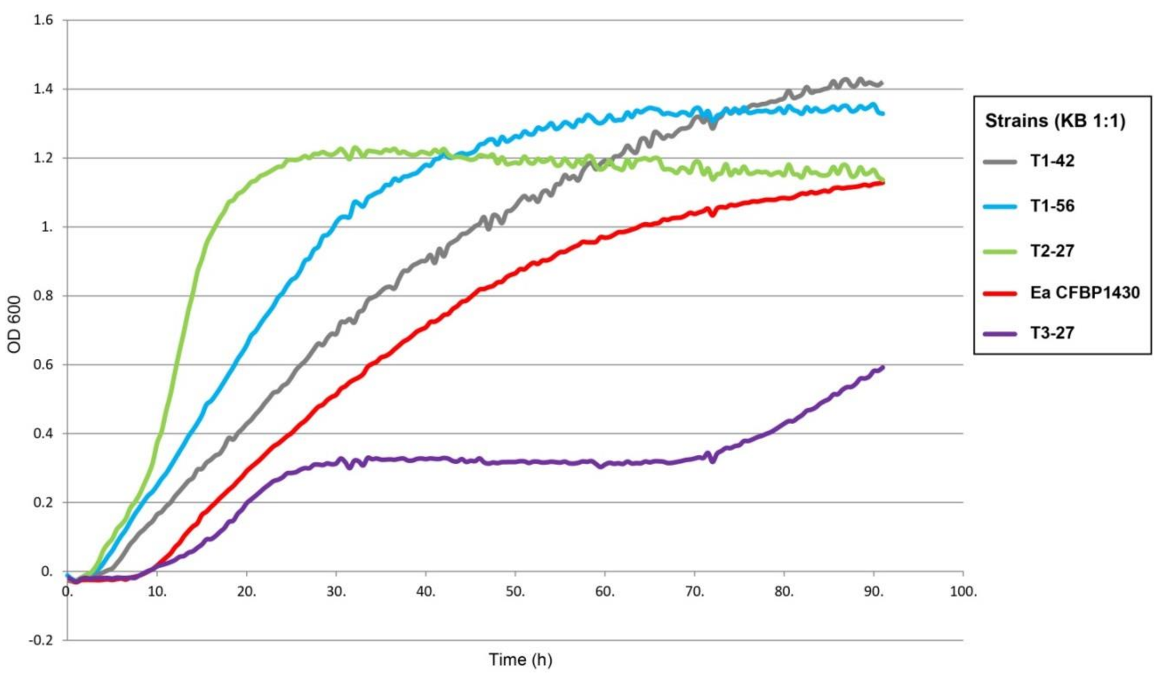

Figure 24 – Growth rates of the four strains assayed in the pear blossom assay compared to *E. amylovora* CFBP 1430 strain in KB medium (1:1).

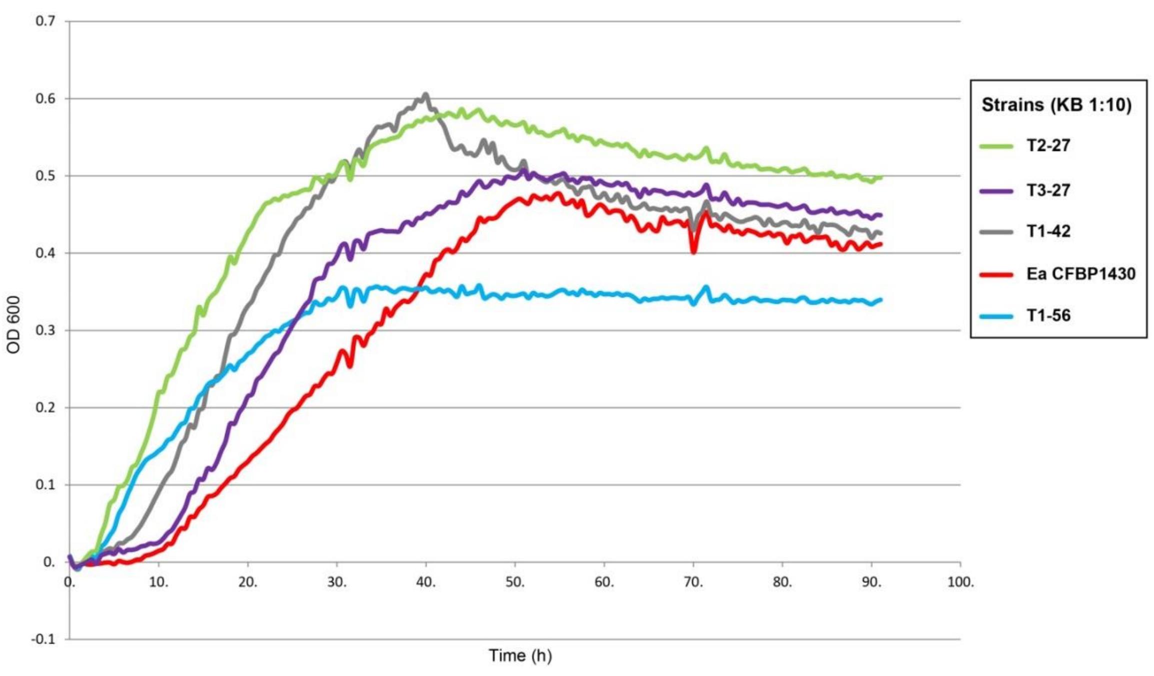

Figure 25 – Growth rates of the four strains assayed in the pear blossom assay compared to *E. amylovora* CFBP 1430 strain in diluted KB medium (1:10).

Biocontrol agents against *E. amylovora* should be efficient colonizers of plant organs and tissues and have to colonize plant surfaces in advance of the pathogen (Glare et al., 2012; Pusey, 2002). For this reason, future studies should evaluate the ability of the antagonistic strains in study to initiate colonization in fruit and stigma, by determining bacterial population levels, and comparing with those of *E. amylovora.*

A study conducted by Pusey (1997), showed that antagonist mixtures have resulted in a greater proportion on blossoms being colonized by at least one antagonist, providing more consistent disease control over a wide range of conditions. Therefore, in future studies, the effect of individual versus mixed antagonists on fruit and stigmatic populations against *E. amylovora* should be tested. An example of a blossom assay methodology would be the combination of 1:1 mixture of strains in study at 10^8 CFU/ml (diluting each strain by one half) to stigmas by placing a droplet of the suspension to each stigma to form a thin film of moisture (0.1 to 0.2 µl per flower).

In certain cases the inhibition of *E. amylovora* infection is not the result of the intrinsic characteristics of the antagonist, but due to uncontrolled factors that favour antagonists or affect the pathogen (Pusey, 1997). Thus, more exhaustive empirical selection procedures, such as tests involving *in vivo* plants under controlled environment and in field conditions should be subjected, to verify their efficacy.

Regarding the study of competition for certain available nutrients (e.g. carbon sources such as fructose or glucose) between the pathogen and antagonistic strains, it is important to compare the nutritional similarity between these bacteria. For instance, the strain T1-56 (*R. epipactidis*) is able to use a broad range of carbohydrates and organic acids which are secreted in the base of the nectaries of flowers (Fridman et al., 2012) , resulting on its capacity to colonize stigma blossoms.

For this, in addition to the system used in this study (API system, which is more often used for clinical and food microbiology), the Biolog system can be used. This system allows the identification of the bacterium at the species level and was designed to identify a wide range of environmental bacteria (SIPH, 2016).

For a more precise quantification of *E. amylovora* with real-time PCR, future studies should estimate unknown sample concentrations with a standard curve method, constructed by using known serial dilutions (Gottsberger, 2010; Salm and Geider, 2004).

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CHAPTER IV
CONCLUSIONS

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The main conclusions of this work were:

1. The medium for bacterial isolation from loquat blossoms with the highest recovery percentage was KB+C, followed by Levan+C, KB+C+P and CCT. Additionally, the use of the KB with phosphates autoclaved separately did not seem to confer an advantage for recovering a higher and a more diverse number of bacterial microbiota from loquat plant material.
2. Most of the bacterial isolates from blossoms were identified as Gram-negative, while in leaves a similar percentage of Gram-positive and Gram-negative bacteria were found. This shows that the microbiota found in different plant surfaces can vary greatly.
3. Incidence and severity of infection in the immature loquat fruit assay increased significantly over time after *E. amylovora* inoculation. This shows that the antagonistic activity of the majority of the tested bacterial strains on *E. amylovora* infection reduces with time, presumably as bacterial strains cannot totally inhibit pathogen growth and/or out-compete it for resources.
4. Additionally, the number of bacterial strains with an active antagonistic activity (with high efficacy of inhibition of *E. amylovora* infection) significantly decreases with time. This highlights that only the more active strains can inhibit *E. amylovora* infection, showing an inverse relationship between incidence/severity of pathogen infection and efficacy of antagonistic activity of bacterial strains.
5. Of all tested strains, only the bacterial strain T3-27 was able to maintain no signs of pathogen infection 9-10 days after pathogen inoculation in the loquat fruit assay, thus having the maximum score of antagonistic efficacy. This shows the difficulties in detecting potential biocontrol agents in natural loquat microbiota against this pathogen based on the rarity of very efficient bacterial strain over longer periods of time.
6. Some bacterial strains with potential as biocontrol agents based on their efficacy of antagonistic inhibition against *E. amylovora*, were discarded because they had a positive response in the hypersensitive test. This shows the importance of analyzing isolated microbiota for their potential pathogenicity.

7. Molecular characterization of the 16S rRNA gene allowed the characterization of bacterial strains to the species level. The four bacterial strains that displayed the best results in the loquat fruit assay were *Curtobacterium flaccumfaciens*, *Enterobacter cancerogenus*, *Rosenbergiella epipactidis*, and *Pseudomonas rhizosphaerae*.
8. Unexpected results were obtained in both complete and incomplete flower assays with no relationship between inoculation of higher *E. amylovora* concentrations and higher incidence and severity of infection, as well as no apparent relationship between lower *E. amylovora* inoculations and potentially greater antagonistic activity of the tested bacterial strains.
9. Similarly, real-time PCR estimations of *E. amylovora* concentration on flowers previously treated with the four selected strains, showed no lack relationship between CPs, flower replicates and inoculated *E. amylovora* concentrations.
10. Comparison of bacterial growth curves with *E. amylovora* showed that three of the four bacterial strains can outgrow *E. amylovora* for a given level of nutrients in the growth media. This emphasizes the potential inhibitory activity of these bacterial strains and their ability to utilize better the available nutrients in the medium in comparison with *E. amylovora*. These were *E. cancerogenus* (T2-27) and *C. flaccumfaciens* (T3-27).

REFERENCES

Amorós, A., Zapata, P., Pretel, M.T., Botella, M.A., Serrano, M., 2003. Physico-Chemical and Physiological Changes during Fruit Development and Ripening of Five Loquat (*Eriobotrya Japonica* Lindl.) Cultivars. Food Sci. Technol. Int. 9, 43–51. doi:10.1177/1082013203009001007

Andrews, J.H., 1992. Biological control in the phyllosphere. Annu. Rev. Phytopathol. 30, 603–635.

Arrebola, E., Sivakumar, D., Bacigalupo, R., Korsten, L., 2010. Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control peach postharvest diseases. Crop Prot. 29, 369–377. doi:10.1016/j.cropro.2009.08.001

Badosa, E., Montesinos, L., Montesinos, E., Feliu, L., Planas, M., Bardají, E., 2014. Prospects and limitations of synthetic antimicrobial peptides for fire blight control. Acta Hortic. 1056, 111–116.

Baker, K.F., 1971. Fire blight of pome fruits: The genesis of the concept that bacteria can be pathogenic to plants. Hilgardia 40, 603–633. doi:10.3733/hilg.v40n18p603

Bastas, K.K., Maden, S., 2007. Evaluation of host resistance inducers and conventional products for fire blight management in loquat and quince. Phytoprotection 88, 93–101. doi:10.7202/018954ar

Beer, S. V., Rundle, J.R., Wodzinski, R.S., 1984. Interaction between *Erwinia amylovora* and *Erwinia herbicola* in vitro, in immature pear fruits and in apple blossoms. Acta Hortic 151, 203–205.

Biomerieux, 2003. api 20 NE. English 1–4. doi:REF 20 050

BioMérieux Inc., 2002. API 20 E Instruction 1–5.

Bonaterra, A., Badosa, E., Cabrefiga, J., Francés, J., Montesinos, E., 2012. Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. Trees 26, 215–226. doi:10.1007/s00468-011-0626-y

Bonaterra, A., Cabrefiga, J., Camps, J., Montesinos, E., 2007. Increasing survival and efficacy of a bacterial biocontrol agent of blight of rosaceous plants by means of osmoadaptation. FEMS Microbiol. Ecol. 61, 185–195. doi:10.1111/j.1574-6941.2007.00313.x

Bonaterra, A., Camps, J., Montesinos, E., 2005. Osmotically induced trehalose and glycine betaine accumulation improves tolerance to desiccation , survival and efficacy of the postharvest biocontrol agent *Pantoea agglomerans* EPS125. FEMS Microbiol. Lett. 250, 1–8. doi:10.1016/j.femsle.2005.06.028

Bonaterra, A., Mari, M., Casalini, L., Montesinos, E., 2003. Biological control of *Monilinia laxa* and *Rhizopus stolonifer* in postharvest of stone fruit by *Pantoea agglomerans* EPS125 and putative mechanisms of antagonism. Int. J. Food Microbiol. 84, 93–104. doi:10.1016/S0168-1605(02)00403-8

CABI, 2016. *Erwinia amylovora* (fireblight) [WWW Document]. Invasive Species Compend. URL http://www.cabi.org/isc/datasheet/21908 (accessed 9.10.16).

Cabrefiga, J., Bonaterra, A., Montesinos, E., 2007. Mechanisms of antagonism of *Pseudomonas fluorescens* EPS62e against *Erwinia amylovora*, the causal agent of fire blight. Int. Microbiol. 10, 123–132. doi:10.2436/20.1501.01.18

Cabrefiga, J., Francés, J., Montesinos, E., A., B., 2014. Improvement of a dry formulation of *Pseudomonas fluorescens* EPS62e for fire blight disease biocontrol by combination of culture osmoadaptation with a freeze-drying lyoprotectant. J. Appl. Microbiol. 117, 1122–1131. doi:10.1111/jam.12582

Cabrefiga, J., Francés, J., Montesinos, E., Bonaterra, A., 2011. Improvement of fitness and efficacy of a fire blight biocontrol agent via nutritional enhancement combined with osmoadaptation. Appl. Environ. Microbiol. 77, 3174–3181. doi:10.1128/AEM.02760-10

Charkowski, A.O., Alfano, J.R., Preston, G., Yuan, J., He, S.Y., Collmer, A., 1998. The *Pseudomonas syringae* pv. tomato HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. J. Bacteriol. 180, 5211–5217.

Cuevas, J., Pinillos, V., Cañete, M.L., González, M., Alonso, F., Fernandéz, M.D., Hueso, J.., 2009. Optimal levels of postharvest deficit irrigation for promoting early flowering and harvest dates in loquat (*Eriobotrya japonica* Lindl). Agric. Water Manag. 96, 831–838. doi:10.1016/j.agwat.2008.11.002

Danhorn, T., Fuqua, C., 2007. Biofilm Formation by Plant-Associated Bacteria. Annu. Rev. Microbiol. 61, 401–422. doi:10.1146/annurev.micro.61.080706.093316

DGADR, 2011. Manual de Boas Práticas para o controlo do Fogo Bacteriano. Lisboa.

Doolotkeldieva, T., Bobusheva, S., 2016. Fire Blight Disease Caused by *Erwinia amylovora* on Rosaceae Plants in Kyrgyzstan and Biological Agents to Control This Disease. Adv. Microbiol. 6, 831–851. doi:10.4236/aim.2016.611080

Eastgate, J.A., 2000. *Erwinia amylovora*: the molecular basis of fireblight disease. Mol. Plant Pathol. 1, 325–329. doi:10.1046/j.1364-3703.2000.00044.x

EFSA, 2014. Scientific Opinion on the pest categorisation of *Erwinia amylovora* (Burr.) Winsl. et al. EFSA J. 12, 1–28. doi:10.2903/j.efsa.2014.xxxx

EPPO, 2013. PM 7/20 (2) *Erwinia amylovora*. EPPO Bull. 43, 21–45. doi:10.1111/epp.12019

Fravel, D.R., 2005. Commercialization and Implementation of Biocontrol. Annu. Rev. Phytopathol. 43, 337–59. doi:10.1146/annurev.phyto.43.032904.092924

Fridman, S., Izhaki, I., Gerchman, Y., Halpern, M., 2012. Bacterial communities in floral nectar. Environ. Microbiol. Rep. 4, 97–104. doi:10.1111/j.1758-2229.2011.00309.x

Gardener, B.B.M., 2004. Ecology of *Bacillus* and *Paenibacillus* spp. in Agricultural Systems. Phytopathology 94, 1252–8. doi:10.1094/PHYTO.2004.94.11.1252

Garrette, S.D., 1965. Towards the biological control of soil- borne plant pathogens, in: Baker, K.F., Snyder, W.C. (Eds.), Ecology of Soil-Borne Plant Pathogens. Univ. Calif. Press, Los Angeles, pp. 4–17.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Köhl, J., Marrone, P.,

Morin, L., Stewart, A., 2012. Have biopesticides come of age? Trends Biotechnol. 30, 250–258. doi:10.1016/j.tibtech.2012.01.003

Gottsberger, R.A., 2010. Development and evaluation of a real-time PCR assay targeting chromosomal DNA of *Erwinia amylovora*. Lett. Appl. Microbiol. 51, 285–292. doi:10.1111/j.1472-765X.2010.02892.x

Govrin, E.M., Levine, A., 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr. Biol. 10, 751–757. doi:10.1016/S0960-9822(00)00560-1

Gregersen, T., 1978. Rapid method for distinction of gram-negative from gram-positive bacteria. Eur. J. Appl. Microbiol. Biotechnol. 5, 123–127. doi:10.1007/BF00498806

Handelsman, J., Stabb, E. V., 1996. Biocontrol of Soilborne Plant Pathogens. The Plant cell. 8, 1855–1869.

Iacobellis, N.S., Lo Cantore, P., Capasso, F., Senatore, F., 2005. Antibacterial activity of *Cuminum cyminum* L. and *Carum carvi* L. essential oils. J. Agric. Food Chem. 53, 57–61. doi:10.1021/jf0487351

Iavicoli, A., Boutet, E., Buchala, A., Métraux, J.-P., 2003. Induced Systemic Resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol. Plant. Microbe. Interact. 16, 851–8. doi:10.1094/MPMI.2003.16.10.851

Ishimaru, C., Klos, E., Brubaker, R., 1988. Multiple antibiotic production by *Erwinia herbicola*. Phytopathology. doi:10.1094/Phyto-78-746

Ishimaru, C., Klos, E.J., 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. Phytopathology 74, 1342–1345.

Janisiewicz, W.J., Usall, J., Bors, B., 1992. Nutritional Enhancement of Biocontrol of Blue Mold on Apples. Phytopathology. 82, 1364–1370.

Jha, C.K., Patel, B., Saraf, M., 2012. Stimulation of the growth of *Jatropha curcas* by the plant growth promoting bacterium *Enterobacter cancerogenus* MSA2. World J. Microbiol. Biotechnol. 28, 891–899. doi:10.1007/s11274-011-0886-0

Johnson, K.B., 2000. Fire blight of apple and pear. Plant Heal. Instr. doi:10.1094/PHI-I-2000-0726-01

Johnson, K.B., Stockwell, V.O., 1998. Management of fire blight: A case study in microbial ecology. Annu. Rev. Phytopathol. 36, 227–248.

Junker, R.R., Loewel, C., Gross, R., Dotterl, S., Keller, A., Bluthgen, N., 2011. Composition of epiphytic bacterial communities differs on petals and leaves. Plant Biol. 13, 918–924. doi:10.1111/j.1438-8677.2011.00454.x

Keck, M., Chartier, R., Zislavsky, W., Lecomte, P., Paulin, J.P., 1995. Heat treatment of plant propagation material for the control of fire blight. Plant Pathol. 44, 124–129. doi:10.1111/j.1365-3059.1995.tb02724.x

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., Defago, G., 1992. Suppression of Root Disease by *Pseudomonas fluorescens* CHA0: Importance of the Bacterial Secondary Metabolite 2,4-Diacetylphloroglucinol. Mol. Plant-Microbe Interact. doi:10.1094/MPMI-5-004

Keel, C., Voisard, C., Berling, C.H., Kahr, G., Defago, G., 1989. Iron Sufficiency , a Prerequisite for the Suppression of Tobacco Black Root Rot by *Pseudomonas fluorescens* Strain CHAO under Gnotobiotic Conditions. Phytopathology 79, 584–589.

Khan, M.A., Zhao, Y., Korban, S.S., 2012. Molecular Mechanisms of Pathogenesis and Resistance to the Bacterial Pathogen *Erwinia amylovora*, Causal Agent of Fire Blight Disease in Rosaceae. Plant Mol. Biol. Report. 30, 247–260. doi:10.1007/s11105-011-0334-1

Koczan, J.M., McGrath, M.J., Zhao, Y., Sundin, G.W., 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. Phytopathology 99, 1237–1244. doi:10.1094/PHYTO-99-11-1237

Krimm, U., Abanda-Nkpwatt, D., Schwab, W., Schreiber, L., 2005. Epiphytic microorganisms on strawberry plants (*Fragaria ananassa* cv . Elsanta): identification of bacterial isolates and analysis of their interaction with leaf surfaces. Fed. Eur. Microbiol. Soc. 53, 483–492. doi:10.1016/j.femsec.2005.02.004

Lacava, P.T., Li, W., Araújo, W.L., Azevedo, J.L., Hartung, J.S., 2007. The Endophyte *Curtobacterium flaccumfaciens* Reduces Symptoms caused by *Xylella fastidiosa* in *Catharanthus roseus*. J. Microbiol. 45, 388–393.

Le Roux, F., Gay, M., Lambert, C., Waechter, M., Poubalanne, S., Chollet, B., Nicolas, J.L., Berthe, F., 2002. Comparative analysis of *Vibrio splendidus*-related strains isolated during *Crassostrea gigas* mortality events. Aquat. Living Resour. 15, 251–258. doi:10.1016/S0990-7440(02)01176-2

Lenaerts, M., Álvarez-Pérez, S., de Vega, C., Van Assche, A., Johnson, S.D., Willems, K.A., Herrera, C.M., Jacquemyn, H., Lievens, B., 2014. *Rosenbergiella australoborealis* sp. nov., *Rosenbergiella collisarenosi* sp. nov. and *Rosenbergiella epipactidis* sp. nov., three novel bacterial species isolated from floral nectar. Syst. Appl. Microbiol. 37, 402–411. doi:10.1016/j.syapm.2014.03.002

Lindow, S., McGourty, G., Elkins, R., 1996. Interactions of Antibiotics with *Pseudomonas fluorescens* Strain A506 in the control of fire blight and frost injury to pear 86, 841–848.

Llop, P., Caruso, P., Cubero, J., Morente, C., L¢pez, M.M., 1999. A simple extractin procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. J. Microbiol. Methods 37, 23–31.

López, M.M., Gorris, M.T., Llop, P., Cambra, M., Roselló, M., Berra, D., 1999. Fire blight in Spain: Situation and Monitoring. Acta Hortic. 489, 187–192.

López, M.M., Ordax, M., Peñalver, J., Roselló, M., Gorris, M.T., Cambra, M., Marco-Noales, E., Biosca., E.G., Palacio-Bielsa, A., Llop, P., 2009. *Erwinia amylovora*: características generales. Métodos de diagnóstico de la enfermedad e identificación de *E. amylovora*., in: El Fuego Bacteriano de Las Rosáceas. pp. 53–74.

Lugtenberg, B.J., Dekkers, L., Bloemberg, G. V, 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu. Rev. Phytopathol. 39, 461–90. doi:10.1146/annurev.phyto.39.1.461

MAGRAMA, 2016. Fuego Bacteriano [WWW Document]. Minist. Agric. Aliment. y Medio

Ambient. URL http://www.mapama.gob.es/ca/agricultura/temas/sanidad-vegetal/fuego-bacteriano/ (accessed 3.27.16).

Malnoy, M., Martens, S., Norelli, J.L., Barny, M.-A., Sundin, G.W., Smits, T.H.M., Duffy, B., 2012. Fire Blight: Applied Genomic Insights of the Pathogen and Host. Annu. Rev. Phytopathol. 50, 475–494. doi:10.1146/annurev-phyto-081211-172931

Mascher, F., Hase, C., Moënne-Loccoz, Y., Défago, G., 2000. The viable-but-nonculturable state induced by abiotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0 does not promote strain persistence in soil. Appl. Environ. Microbiol. 66, 1662–1667. doi:10.1128/AEM.66.4.1662-1667.2000

McManus, P.S., Jones, A.L., 1995. Detection of *Erwinia amylovora* by Nested PCR and PCR-Dot-Blot and Reverse-Blot Hybridisations. Phytopathology 85, 618–623.

McManus, P.S., Stockwell, V.O., Sundin, G.W., Jones, A.L., 2002. Antibiotic use in plant agriculture. Annu. Rev. Phytopathol. 40, 443–465. doi:10.1146/annurev.phyto.40.120301.093927

Mercier, J., Wilson, C.L., 1994. Colonization of Apple Wounds by Naturally Occuring Microflora and Introduced *Candida oleophila* and Their Effect on Infection by *Botrytis cinerea* during Storage. Biol. Control. 4, 138–144.

Montesinos, E., 2003. Development , registration and commercialization of microbial pesticides for plant protection. Int. Microbiol. 6, 245–252. doi:10.1007/s10123-003-0144-x

Montesinos, E., 2007. Antimicrobial peptides and plant disease control. FEMS Microbiol. Lett. 270, 1–11. doi:10.1111/j.1574-6968.2007.00683.x

Montesinos, E., Bonaterra, A., Ophir, Y., Beer, S. V., 1996. Antagonism of Selected Bacterial Strains to *Stemphylium vesicarium* and Biological Control of Brown Spot of Pear Under Controlled Environment Conditions. Phytopathology.

Mora, I., Cabrefiga, J., Montesinos, E., 2011. Antimicrobial peptide genes in *Bacillus* strains from plant environments. Int. Microbiol. 14, 213–223. doi:10.2436/20.1501.01.151

Nagórska, K., Bikowski, M., Obuchowski, M., 2007. Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochim. Pol. 54, 495–508. doi:20071510 [pii]

Norelli, J.L., Jones, A.L., Aldwinckle, H.S., 2003. Fire blight management in the twenty first century using new technologies. Pl. Dis. 87, 756–765.

Pal, K.K., Gardener, B.M., 2006. Biological Control of Plant Pathogens. Plant Heal. Instr. 1–25. doi:10.1094/PHI-A-2006-1117-02.Biological

Paternoster, T., Défago, G., Duffy, B., Gessler, C., Pertot, I., 2010. Selection of a biocontrol agent based on a potential mechanism of action: Degradation of nicotinic acid, a growth factor essential for *Erwinia amylovora*. Int. Microbiol. 13, 195–206. doi:10.2436/20.1501.01.126

Paulin, J.P., 2000. *Erwinia amylovora*: general characteristics, biochemistry and serology, Fire Blight. The Disease and its Causative Agent Erwinia Amylovora. CABI, Wallingford.

Pirc, M., Ravnikar, M., Tomlinson, J., Dreo, T., 2009. Improved fireblight diagnostics using quantitative real-time PCR detection of *Erwinia amylovora* chromosomal DNA. Plant Pathol. 58, 872–881. doi:10.1111/j.1365-3059.2009.02083.x

Pozo, M., Lievens, B., Jacquemyn, H., 2014. Impact of Microorganisms on nectar chemistry, pollinator attraction and plant fitness. Nectar Prod. Chem. Compos. Benefits to Anim. Plants. 41.

Prados, G.R., 2015. Characterization and improvement of plant associated *Lactobacillus plantarum*. Novel biocontrol agents for fire blight disease.

Pujol, M., Badosa, E., Manceau, C., Montesinos, E., 2006. Assessment of the Environmental Fate of the Biological Control Agent of Fire Blight,. Society 72, 2421–2427. doi:10.1128/AEM.72.4.2421

Pusey, P.L., 1997. Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology.pdf 1096–1102.

Pusey, P.L., 2002. Biological Control Agents for Fire Blight of Apple Compared Under Conditions Limiting Natural Dispersal. Plant Dis. 86, 639–644. doi:10.1094/PDIS.2002.86.6.639

Raupach, G.S., Kloepper, J.W., 1998. Mixtures of Plant Growth-Promoting Rhizobacteria Enhance Biological Control of Multiple Cucumber Pathogens. Phytopathology. 88, 1158–1164.

Raymundo, A., Ries, S., 1981. Motility of *Erwinia amylovora*. Phytopathology 71, 45–49.

Raymundo, A.K., Ries, S.M., 1980. Chemotaxis of *Erwinia amylovora*. Phytopathology 70, 1066–1069.

Roselló, G., Bonaterra, A., Francés, J., Montesinos, L., Badosa, E., Montesinos, E., 2013. Biological control of fire blight of apple and pear with antagonistic *Lactobacillus plantarum*. Eur. J. Plant Pathol. 137, 621–633. doi:10.1007/s10658-013-0275-7

Ruz, L., Moragrega, C., Montesinos, E., 2008. Evaluation of four whole-plant inoculation methods to analyze the pathogenicity of *Erwinia amylovora* under quarantine conditions. Int. Microbiol. 11, 111–119. doi:10.2436/20.1501.01.50

Sacherer, P., Défago, G., Haas, D., Defago, G., Haas, D., 1994. Extracellular protease and phospholipase-C are controlled by the global regulatory gene gacA in the biocontrol strain *Pseudomonas fluorescens* CHA0. FEMS Microbiol. Lett. 116, 155–160. doi:10.1111/j.1574-6968.1994.tb06694.x

Salm, H., Geider, K., 2004. Real-time PCR for detection and quantification of *Erwinia amylovora*, the causal agent of fireblight. Plant Pathol. 53, 602–610. doi:10.1111/j.1365-3059.2004.01066.x

Sasu, M.A., Wall, K.L., Stephenson, A.G., 2010. Antimicrobial Nectar inhibits a Florally Transmitted Pathogen of a Wild *Cucurbita pepo* (Cucurbitaceae). Am. J. Bot. 97, 1025–1030. doi:10.3732/ajb.0900381

Schnider, U., Keel, C., Blumer, C., Troxler, J., Defago, G., Haas, D., 1995. Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J. Bacteriol. 177, 5387–5392.

SIPH, 2016. API/Biolog [WWW Document]. ISPWIV. URL http://bacterio.iph.fgov.be/other-activities/molecular-epidemiology/api-biolog (accessed 11.1.16).

Smith, T.J., 1996. A risk assessment model for fire blight of apple and pear. Acta Hortic. 411, 97–104.

Spadaro, D., Gullino, M.L., 2005. Improving the efficacy of biocontrol agents against soilborne pathogens. Crop Prot. 24, 601–613. doi:10.1016/j.cropro.2004.11.003

Stockwell, V.O., Johnson, K.B., Loper, J.E., 1998. Establishment of Bacterial Antagonists of *Erwinia amylovora* on Pear and Apple Blossoms as Influenced by Inoculum Preparation. Phytopathology 88, 506–513. doi:10.1094/PHYTO.1998.88.6.506

Tanaka, T., Kawasaki, K., Daimon, S., Kitagawa, W., Yamamoto, K., Tamaki, H., Tanaka, M., Nakatsu, C.H., Kamagata, Y., 2014. A hidden pitfall in the preparation of agar media undermines microorganism cultivability. Appl. Environ. Microbiol. 80, 7659–7666. doi:10.1128/AEM.02741-14

Thomashow, L.S., Weller, D.M., 1996. Current concepts in the use of introduced bacteria for biological disease control., in: Stacey, G., Keen, N.T. (Eds.), Plant Microbe Interactions. Champman Hall, New York, USA, pp. 187–235.

Thomson, S.V., 2000. Epidemiology of Fire Blight., in: Vanneste, J.L. (Ed.), Fire Blight. The Disease and Its Causative Agent, *Erwinia Amylovora*. CABI Publishing, Wallingford, UK., pp. 9–36. doi:10.1079/9780851992945.0000

Toole, G.O., Kaplan, H.B., Kolter, R., 2000. Biofilm Formation as Microbial Development. Annu. Rev. Microbiol. 54, 49–79.

UC-IPM, 2014. Models: Fire Blight of Pear and Apple--UC-IPM [WWW Document]. Agric. Nat. Resour. URL http://ipm.ucanr.edu/DISEASE/DATABASE/fireblight.html (accessed 8.1.16).

van der Zwet, T., Keil, H.L., 1979. Fire Blight a bacterial disease of Rosaceous plants, Agriculture handbook. Beltsville Agricultural Research Center Beltsville, Beltsville, Maryland.

Van der Zwet, T., Zoller, B.G., Thomson, S.V., 1988. Controlling fire blight of pear and apple by accurate prediction of the blossom blight phase. Plant Dis. 72, 464–472.

Van Loon, L.C., 1997. Induced resistance in plants and the role of pathogenesis-related proteins. Eur. J. Plant Pathol. 753–765.

Van Teylingen, M., 2002. Ornamental hosts of *Erwinia amylovora* and the effect of the fire blight control policy in the Netherlands. Acta Hortic. 590, 81–87.

Vilanova, S., Badenes, M.L., Martínez-Calvo, J., Llácer, G., 2001. Analysis of loquat germplasm (*Eriobotrya japonica* Lindl) by RAPD molecular markers. Euphytica 121, 25–29.

Vrancken, K., Holtappels, M., Schoofs, H., Deckers, T., Valcke, R., 2013. Pathogenicity and infection strategies of the fire blight pathogen *Erwinia amylovora* in Rosaceae: State of the art. Microbiology 159, 823–832. doi:10.1099/mic.0.064881-0

Whipps, J.M., 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52, 487–511.

Wilson, C.L., Wisniewski, M.E., 1994. Biological control of postharvest diseases: Theory and Practice. CRC Press, Boca Raton, USA.

Yang, R., Fan, X., Cai, X., Hu, F., 2014. The inhibitory mechanisms by mixtures of two endophytic bacterial strains isolated from *Ginkgo biloba* against pepper phytophthora blight. Biol. Control. 85, 59–67. doi:10.1016/j.biocontrol.2014.09.013

Yu, T., Wu, P.G., Qi, J.J., Zheng, X.D., Jiang, F., Zhan, X., 2006. Improved control of postharvest blue mold rot in pear fruit by a combination of *Cryptococcus laurentii* and gibberellic acid. Biol. Control. 39, 128–134. doi:10.1016/j.biocontrol.2006.05.008

Zhang, L.H., Dong, Y.H., 2004. Quorum sensing and signal interference : diverse implications. Mol. Microbiol. 53, 1563–1571. doi:10.1111/j.1365-2958.2004.04234.x

Zhang, Y., Geider, K., 1997. Differentiation of *Erwinia amylovora* strains by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 63, 4421–4426.

APPENDICES

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Table S1 – Antioxidant maceration buffer composition.

Antioxidant maceration buffer (Gorris et al., 1996)	
Polyvinylpyrrolidone (PVP-10)	20,0 g
Mannitol	10,0 g
Ascorbic acid	1,76 g
Reduced glutathion	3,0 g
PBS 10 mm pH 7,2	1 L

Table S2 – King´s medium B agar composition.

King´s medium B agar	
Protease peptone nr 3	20 g
Glicerol	10 ml
K_2HPO_4	1,5 g
$MgSO_4 . 7H_2O$	1,5 g
Agar	15 g
Distilled water	1 L

Table S3 – CCT medium composition.

CCT medium (Ishimaru and Klos, 1984)	
Sucrose	100 g
Sorbitol	10 g
1% aqueous solution of tergitol anionic 7	30 ml
0.1% solution of crystal violet in absolute ethanol	2 ml
Nutrient agar	23 g
Distilled water	970 ml

Table S4 – Morphological description of bacterial colonies obtained from loquat blossoms. Strains in bold represent those used for the pear blossom assay.

Bacterial colony	Strain	Medium	Morphological description
N1-A.1	T2-45	KB+C+P	amarela; circular; convexa; inteira; 3mm
N1-B	T1-81	KB+C+P	amarela; irregular; mucosa; convexa; lobulada; 5mm
N1-C	T1-55	KB+C+P	amarela; punctiforme; plana; espiral; 2mm
N1-D.1	T1-7	KB+C+P	2,5 mm; branca; transparente; plana
N1-D.1.1	T1-65	KB+C+P	amarela; filamentosa; papilada; espiral; 3mm
N1-D.1.2.1	T2-46	KB+C+P	amarela; circular; convexa; inteira; 3mm
N1-D.1.2.2	T2-49	KB+C+P	amarela clara; rizóide, crateriforme; filamentosa; 2mm
N1-D.2.1.1	T2-47	KB+C+P	amarela, circular; convexa; inteira; 2mm
N1-D.2.1.2	T2-48	KB+C+P	amarela; rizóide; crateriforme; filamentosa
N1-D.2.2	T1-61	KB+C+P	amarela; irregular; plana; lobulada; 2mm
N12-A	T2-34	KB+C+P	amarela; circular; convexa; inteira; 4mm
N12-B.1	T2-41	KB+C+P	branca; circular; plana; 4mm
N12-B.2	T2-44	KB+C+P	amarela-clara; irregular; elevada; lobulada; 4mm
N16-A	T1-53	KB+C+P	amarela; circular; convexa; inteira; 2mm
N23-A	T2-24	KB+C+P	amarela; muito mucosa; odor intenso
N30-A	T2-1	KB+C+P	amarela; muito mucosa
N36-A	T2-28	KB+C+P	1,5 mm; transparente
N36-B	T2-8	KB+C+P	amarela; mucosa
N36-C	**T1-56**	KB+C+P	amarela; circular; convexa; 3,5mm
N36-D	T2-36	KB+C+P	branca; puntiforme; elevada; inteira
N38-A.1	T2-40	KB+C+P	amarela; mucosa; convexa; irregular; ondulada; 4mm
N88-A.1	T1-28	KB+C+P	amarela clara; circular; plana; inteira; 4mm
N88-A.2	T1-22	KB+C+P	amarela; circular; plana; ondulada; 3,5 mm
N90-A.1	T1-11	KB+C+P	branca; filamentosa; convexa; ondulada; 3mm
N90-A.2	T1-8	KB+C+P	branca; circular; plana; inteira; 3mm
N7-A	T2-7	Levan+C	amarela com pontos no centro; mucosa
N10-A.1	T1-36	Levan+C	amarelo; circular; convexa; inteira; mucosa; 3mm
N10-A.2	T1-67	Levan+C	amarela; muito mucosa
N11-A	T1-40	Levan+C	amarelo; circular; convexa; inteira; mucosa; 2mm
N14-A	T2-20	Levan+C	amarela; muito mucosa, tipo pantoea spp.
N16-A	T2-50	Levan+C	branca; puntiforme; plana; inteira
N16-B.1	T1-13	Levan+C	branca; circular; plana; inteira; 3mm
N16-B.2	T1-2	Levan+C	amarela; circular; plana; inteira; mucosa; 2mm
N16-C	T1-37	Levan+C	amarela; circular; convexa; inteira; 3,5 mm
N30-A	T2-39	Levan+C	branca; puntiforme; plana; inteira
N43-A.1	T1-1	Levan+C	amarela; circular; plana; inteira; 2mm
N51-A.1	T1-71	Levan+C	amarela; puntiforme; plana; ondulada; 2mm
N51-A.2.1	T2-59	Levan+C	2,5 mm; branca; circular; convexa; inteira
N51-A.2.2	T2-60	Levan+C	amarela; circular; convexa; ondolada; 1,5 mm
N51-A.2.3	T2-61	Levan+C	amarela; circular; crateriforme; 2 mm
N56-A	T1-54	Levan+C	amarela; circular; convexa; inteira; 2,5 mm
N76-A	T2-13	Levan+C	esbranquiçada; muito mucosa
N102-A.1.1	T1-73	Levan+C	amarela; rizóide; crateriforme; ondulada; 10mm
N102-A.2	T1-17	Levan+C	branca; puntiforme; plana; inteira; 1,5mm
N147-A	T2-51	Levan+C	branca-amarelada; puntiforme; plana; inteira
N14-A	**T1-42**	KB+C	amarelo forte; circular; convexa; inteira; 5mm
N22-A.1.1	T1-60	KB+C	amarela; circular; papilada; ondulada; 2,5mm
N22-A.1.2	T2-53	KB+C	amarela; rizóide; elevada; filamentosa; 4mm
N22-A.1.3	T1-43	KB+C	amarela; filamentosa; plana; 3mm
N22-A.1.4.1	T1-49	KB+C	amarela; circular; convexa; inteira; 2,5mm
N22-A.1.4.2	T1-66	KB+C	amarela; circular; plana; inteira; 2,5mm
N22-A.2	T1-3	KB+C	amarela; filamentosa; plana; espiral; 3mm
N38-A.1	T1-39	KB+C	amarela; circular; plana; inteira; 4mm
N38-A.2	T1-26	KB+C	esbranquiçada; circular; plana; inteira; 2,5mm

N43-A	T2-29	KB+C	amarela; puntiforme; plana; inteira
N43-B	T2-31	KB+C	amarela; mucosa; circular; convexa; inteira; 4mm
N45-A	T2-25	KB+C	amarela; muito mucosa, tipo pantoea spp.
N49-A.1	T2-11	KB+C	branca; puntiforme; convexa; inteira
N49-A.2	T2-12	KB+C	amarela; irregular; convexa; ondulada; 3mm
N52-A	T1-19	KB+C	amarelo-claro; circular; convexa; inteira; 3mm
N54-A.1	T2-35	KB+C	amarela; circular; crateriforme; inteira; 3mm
N59-A.1	T1-78	KB+C	branca; circular; convexa; ondulada; 3mm
N59-A.2	T1-5	KB+C	amarela; circular; inteira; 2mm
N59-B.1	T2-37	KB+C	amarela; irregular; crateriforme; ondulada; 10mm
N61-A	T2-43	KB+C	amarela; muito mucosa
N66-A.1	T1-34	KB+C	irregular; dispersa por toda a placa; lobulada
N69-A	T2-32	KB+C	amarela; mucosa; circular; convexa; inteira; 4mm
N84-A	T2-22	KB+C	amarela; circular; elevada; inteira; 2,5mm
N88-A.1	T2-33	KB+C	verde fluorescente; filamentosa; convexa; espiral; 3mm
N88-A.2	T2-52	KB+C	rosa avermelhado; circular; elevada; inteira; 2mm
N96-A.1.1.1	T1-44	KB+C	branca; circular; plana; inteira; 2,5mm
N96-A.1.1.2	T1-48	KB+C	branca; circular; convexa; inteira; 1,5mm
N96-A.1.2.1	T1-46	KB+C	branca; circular; convexa; inteira; mucosa; 3mm
N96-A.1.2.2	T1-47	KB+C	branca; circular; crateriforme; inteira; 4mm
N96-A.1.3	T1-79	KB+C	branca; circular; convexa; inteira, 2,5mm
N96-A.2	T1-6	KB+C	branca; circular; plana; espiral; 2,5mm
N109-A	T2-10	KB+C	branca; mucosa; circular; convexa; inteira; 3mm
N7-A.1	T9	CCT	amarela; filamentosa; plana; inteira; 1,5mm
N7-A.2	T10	CCT	amarela; circular; convexa; inteira; 1,5mm
N7-B	T1-30	CCT	amarela esbranquiçada; circular; convexa; inteira; 2mm
N8-A.1	T1-64	CCT	amarela; filamentosa; espiral; mucosa; 2,5mm
N8-A.2	T1-16	CCT	amarela; filamentosa; plana; espiral; 2,5mm
N8-A.3	T1-18	CCT	amarela; irregular; plana; ondulada; mucosa; 3mm
N11-A.1	T2-38	CCT	branca, quase transparente; circular; convexa; inteira; 3mm
N11-A.2	T2-42	CCT	amarela-clara; circular; convexa; inteira; 2mm
N17-A	**T2-27**	CCT	branca; mucosa, tipo pantoea spp.
N19-A.1	T2-54	CCT	amarela; filamentosa; plana; inteira; 3mm
N19-A.2	T2-57	CCT	amarela; irregular; elevada; inteira; 2mm
N19-A.2.1	T2-58	CCT	amarela; fusiforme; plana; inteira; 2mm
N19-B	T1-27	CCT	amarela; circular; plana; inteira; 3mm
N57-A	T1-33	CCT	amarela; circular; convexa; inteira; 2mm
N98-A	T1-69	CCT	branca; circular; plana; inteira; 2,5mm
C33-A	T2-17	KB+C+P	amarela, extremamente mucosa
C1-A	T1-51	Levan+C	amarela; circular; convexa; inteira; 2mm
C1-B.1	T1-70	Levan+C	2mm; transparente; convexa
C1-B.2	T2-55	Levan+C	branca; circular; plana; inteira; 1,5mm
C1-C	T2-5	Levan+C	amarela; circular; convexa; inteira; 2mm
C2-A.1	T1-12	Levan+C	amarela; circular; plana; inteira; 2,5mm
C2-A.2	T1-14	Levan+C	branca; circular; plana; inteira; 2mm
C4-A	T2-19	Levan+C	amarela; irregular; convexa; ondulada; 2,5mm
C5-A.1	T1-21	Levan+C	amarela; circular; convexa; inteira; 2,5mm
C5-A.2.1	T1-72	Levan+C	amarela; puntiforme; plana; inteira; 1,5mm
C5-A.2.2	T2-56	Levan+C	amarela; circular; crateriforme; inteira; 1,5mm
C5-A.3	T1-23	Levan+C	amarela; irregular; plana; lobulada; 2,5mm
C12-A.1.1	T1-76	Levan+C	amarela clara; circular; convexa; inteira; 2mm
C12-A.1.2	T1-74	Levan+C	branca; puntiforme; plana; inteira; 1mm
C12-A.2	T1-24	Levan+C	amarela; circular; plana; inteira; 3mm
C12-A.3	T1-80	Levan+C	branca; circular; plana; inteira; 2mm
C65-A	T2-16	Levan+C	amarela clara; irregular; convexa; 2,5mm
C75-A	T1-41	Levan+C	amarela; circular; convexa; inteira; 2,5mm
C85-A	T1-62	Levan+C	branca; rizóide; plana; espiral; 2,5mm
C86-A	T1-77	Levan+C	branca; puntiforme; plana; inteira; 1mm

C86-B	T1-59	Levan+C	amarela; circular; convexa; inteira; 5mm
C95-A.1	T1-15	Levan+C	branca; circular; plana; inteira; 3mm
Bacillus subtilis	T2-62	KB+C	
C2-A	T2-2	KB+C	amarela; muito mucosa, tipo pantoea spp.
C2-B	T1-52	KB+C	amarela, circular; convexa; inteira; 2mm
C2-C	T2-3	KB+C	amarela clara; circular; elevada; inteira; 2mm
C3-A	T2-6	KB+C	amarela-clara; mucosa; odor intenso
C4-A	T1-38	KB+C	amarela; circular; convexa; ondulada; mucosa; 4,5mm
C4-B	T1-25	KB+C	amarela; circular; convexa; inteira; 4mm
C9-A	T2-18	KB+C	amarela; muito mucosa, tipo pantoea spp.
C19-A.1	T1-29	KB+C	branca; filamentosa; convexa; ondulada; 3mm
C19-A.2	T1-4	KB+C	branca; filamentosa; conxeva; inteira; 2,5mm
C29-A	T2-26	KB+C	amarela, tipo mucosa, tipo pantoea spp.
C39-A.1.1	T1-31	KB+C	amarela; muito mucosa
C39-A.1.2	T1-32	KB+C	amarela; circular; convexa; ondulada; 4 mm
C39-A.2.1	T1-75	KB+C	amarela; circular; convexa; inteira; 3mm
C39-A.2.2.1	T1-45	KB+C	amarela; rizóide; crateriforme; filamentosa; muitíssimo mucosa; 5mm
C39-A.2.2.2	T1-50	KB+C	circular; crateriforme; inteira; muito mucosa; 3,5mm
C55-A.1	T1-68	KB+C	amarela clara; circular; convexa; inteira; 3,5mm
C55-A.2	T1-63	KB+C	amarela; circular; plana; inteira; 1,5mm
C65-A	T2-15	KB+C	amarela, quase transparente; circular; convexa; inteira; 2,5mm
C96-A	T2-14	KB+C	amarela; circular; convexa; inteira; 3mm
C1-A	T1-58	CCT	amarela; circular; convexa; inteira; 3,5mm
C1-B	T2-4	CCT	amarela clara; circular; convexa; inteira; 2mm
C5-A	T2-21	CCT	branca; mucosa; circular; convexa; inteira
C12-A	T1-57	CCT	amarela; filamentosa; convexa; espiral; 6mm
C15-A	T2-30	CCT	amarela; mucosa, tipo pantoea spp.
C33-A	T2-23	CCT	branca-amarelada; muito mucosa.
C87-A	T1-20	CCT	amarela; circular; convexa; inteira; 1,5mm
C93-A	T2-9	CCT	amarela; muito mucosa, tipo pantoea spp.

Figure S1 – Cryotubes containing bacterial isolates from loquat leaves to be preserved at -80ºC.
Samples were isolated from trees in IVIA's greenhouse by María Piñar.
Positions 1 to 30 correspond to strains T3-1 to T3-30, respectively.

Table S5 – Morphological characteristics of some of cryopreserved bacteria isolates obtained from loquat leaves.

Colony	Morphological description
NK-1	Colonia con forma circular, plana y borde entero. Translúcida, de color amarillo y el pigmento es celular.
NK-2	Colonia circular, plana y borde entero. Translúcida, de color amarillo y el pigmento es celular.
NK-3	Colonia con forma irregular, umbonada y borde ondulado. Translúcida, de color amarillo-anaranjado y el pigmento es celular.
NK-4	Colonia circular, convexa y borde entero. Opaca, de color amarillo y el pigmento es celular. Colonia mucosa y brillante.
NK-5	Colonia circular, convexa y borde entero. Opaca, de color blanco y el pigmento es celular. Colonia mucosa y brillante.
NK-6	Colonia circular, plana y borde entero. Opaca, de color blanco y el pigmento es celular. Colonia mucosa y brillante.
NK-7	Colonia circular, plana y borde entero. Translúcida, de color rosado claro y el pigmento es celular. Colonia mucosa y brillante.
NK-8	Colonia circular, convexa y borde entero. Opaca, de color amarillo y el pigmento es celular. Colonia mucosa y brillante.
NK-9	Colonia circular, plana y borde entero. Translúcida, de color amarillo claro y el pigmento es celular. Colina mucosa y brillante.
NK-10	Colonia circular, plana y borde entero. Translúcida, de color blanco y el pigmento es celular. Colonia mucosa y brillante.
NK-11	Colonia circular, plana y borde entero. Opaca, de color blanco y el pigmento es celular.
NK-12	Colonia circular, plana y borde entero. Opaca, de color blanco y el pigmento es celular. Colonia mucosa y brillante.
NK-13	Colonia circular, plana y borde entero. Opaca, de color blanco y el pigmento es celular.
NC-14	Cultivo masivo sin forma de colonia.

Table S6 – Results for Gram and Oxidase tests for the 173 isolates obtained from loquat microbiota.

Strain	From	Gram	Oxidase
T1-1	Flowers	-	-
T1-2	Flowers	-	-
T1-3	Flowers	-	-
T1-4	Flowers	-	-
T1-5	Flowers	-	-
T1-6	Flowers	-	-
T1-7	Flowers	-	-
T1-8	Flowers	+	
T1-9	Flowers	-	-
T1-10	Flowers	-	-
T1-11	Flowers	-	-
T1-12	Flowers	-	-
T1-13	Flowers	-	-
T1-14	Flowers	-	-
T1-15	Flowers	+	
T1-16	Flowers	-	-
T1-17	Flowers	+	
T1-18	Flowers	-	-
T1-19	Flowers	-	-
T1-20	Flowers	-	-
T1-21	Flowers	-	-
T1-22	Flowers	-	-
T1-23	Flowers	-	-
T1-24	Flowers	-	-
T1-25	Flowers	-	-
T1-26	Flowers	-	-
T1-27	Flowers	-	-
T1-28	Flowers	-	-
T1-29	Flowers	-	-
T1-30	Flowers	-	-
T1-31	Flowers	-	-
T1-32	Flowers	-	-
T1-33	Flowers	-	-
T1-34	Flowers	+	
T1-35	Flowers	-	-
T1-36	Flowers	-	-
T1-37	Flowers	-	-
T1-38	Flowers	-	-
T1-39	Flowers	-	-
T1-40	Flowers	+	
T1-41	Flowers	-	-
T1-42	Flowers	-	-
T1-43	Flowers	-	-
T1-44	Flowers	+	
T1-45	Flowers	-	-
T1-46	Flowers	+	
T1-47	Flowers	+	
T1-48	Flowers	-	-
T1-49	Flowers	-	-
T1-50	Flowers	-	-
T1-51	Flowers	-	-
T1-52	Flowers	-	-

Strain	From	Gram	Oxidase
T1-53	Flowers	-	-
T1-54	Flowers	-	-
T1-55	Flowers	-	-
T1-56	Flowers	-	-
T1-57	Flowers	-	-
T1-58	Flowers	-	-
T1-59	Flowers	-	-
T1-60	Flowers	-	-
T1-61	Flowers	-	-
T1-62	Flowers	+	
T1-63	Flowers	-	-
T1-64	Flowers	-	-
T1-65	Flowers	-	-
T1-66	Flowers	+	
T1-67	Flowers	+	
T1-68	Flowers	-	-
T1-69	Flowers	-	-
T1-70	Flowers	-	-
T1-71	Flowers	-	-
T1-72	Flowers	-	-
T1-73	Flowers	+	
T1-74	Flowers	+	
T1-75	Flowers	-	-
T1-76	Flowers	-	-
T1-77	Flowers	-	-
T1-78	Flowers	-	+
T1-79	Flowers	+	
T1-80	Flowers	+	
T1-81	Flowers	-	-
T2-1	Flowers	-	+
T2-2	Flowers	-	-
T2-3	Flowers	-	-
T2-4	Flowers	-	-
T2-5	Flowers	-	-
T2-6	Flowers	-	+
T2-7	Flowers	-	-
T2-8	Flowers	-	-
T2-9	Flowers	-	-
T2-10	Flowers	-	-
T2-11	Flowers	-	-
T2-12	Flowers	-	-
T2-13	Flowers	-	-
T2-14	Flowers	-	-
T2-15	Flowers	-	-
T2-16	Flowers	-	-
T2-17	Flowers	-	-
T2-18	Flowers	-	-
T2-19	Flowers	-	-
T2-20	Flowers	-	-
T2-21	Flowers	-	-
T2-22	Flowers	-	-
T2-23	Flowers	-	-

(Table S6 continued)

Strain	From	Gram	Oxidase
T2-24	Flowers	-	-
T2-25	Flowers	-	-
T2-26	Flowers	-	-
T2-27	Flowers	-	-
T2-28	Flowers	-	-
T2-29	Flowers	-	-
T2-30	Flowers	-	-
T2-31	Flowers	-	-
T2-32	Flowers	-	-
T2-33	Flowers	-	+
T2-34	Flowers	-	-
T2-35	Flowers	-	-
T2-36	Flowers	+	
T2-37	Flowers	+	
T2-38	Flowers	-	-
T2-39	Flowers	+	
T2-40	Flowers	-	-
T2-41	Flowers	-	-
T2-42	Flowers	-	-
T2-43	Flowers	-	-
T2-44	Flowers	-	-
T2-45	Flowers	-	-
T2-46	Flowers	-	-
T2-47	Flowers	-	-
T2-48	Flowers	-	-
T2-49	Flowers	-	-
T2-50	Flowers	+	-
T2-51	Flowers	-	-
T2-52	Flowers	+	
T2-53	Flowers	-	-
T2-54	Flowers	-	-
T2-55	Flowers	+	
T2-56	Flowers	+	
T2-57	Flowers	-	-
T2-58	Flowers	-	-

Strain	From	Gram	Oxidase
T2-59	Flowers	+	
T2-60	Flowers	+	
T2-61	Flowers	+	
T3-1	Leaves	-	-
T3-2	Leaves	-	-
T3-3	Leaves	-	-
T3-4	Leaves	-	-
T3-5	Leaves	+	
T3-6	Leaves	+	
T3-7	Leaves	-	-
T3-8	Leaves	+	
T3-9	Leaves	-	-
T3-10	Leaves	-	-
T3-11	Leaves	+	
T3-12	Leaves	-	+
T3-13	Leaves	-	-
T3-14	Leaves	+	
T3-15	Leaves	+	
T3-16	Leaves	-	-
T3-17	Leaves	+	
T3-18	Leaves	+	
T3-19	Leaves	+	
T3-20	Leaves	-	-
T3-21	Leaves	-	-
T3-22	Leaves	-	+
T3-23	Leaves	-	+
T3-24	Leaves	-	+
T3-25	Leaves	+	
T3-26	Leaves	-	-
T3-27	Leaves	-	-
T3-28	Leaves	+	
T3-29	Leaves	+	
T3-30	Leaves	-	-
Bacillus spp.	Leaves	+	

Table S7 – Efficacy (E) scale of selected bacterial strains at 4-5 days after *E. amylovora* inoculation in immature loquat fruit assay.
(VA) Very Active (100-90 %); (A) Active (89-70 %); (MA) Moderately Active (69-40 %); (SA) Soft Active (39-20 %); and (NA) Not Active (19-0 %).
Strains with 0% efficacy in previous days were not considered in following days.

E scale	**Strain (%) at 4-5 days**									
VA	T1-9 (100%)	T2-26 (100%)	T2-27 (100%)	T2-43 (100%)	T2-56 (100%)	T2-7 (100%)	T3-27 (100%)				
A	T1-10 (89%)	T1-40 (89%)	T1-71 (89%)	T2-14 (89%)	T2-17 (89%)	T2-2 (89%)	T2-28 (89%)	T2-30 (89%)	T2-32 (89%)	T2-37 (89%)	T1-16 (78%)
	T1-26 (78%)	T1-38 (78%)	T1-41 (78%)	T1-50 (78%)	T1-56 (78%)	T1-57 (78%)	T1-69 (78%)	T1-73 (78%)	T1-8 (78%)	T2-25 (78%)	T2-40 (78%)
	T2-6 (78%)	T3-2 (78%)									
MA	T1-25 (67%)	T1-32 (67%)	T1-39 (67%)	T1-42 (67%)	T1-52 (67%)	T2-13 (67%)	T2-18 (67%)	T2-22 (67%)	T2-23 (67%)	T2-31 (67%)	T2-34 (67%)
	T2-4 (67%)	T2-41 (67%)	T2-52 (67%)	T2-53 (67%)	T3-14 (67%)	T3-30 (67%)	T1-27 (56%)	T1-29 (56%)	T1-33 (56%)	T1-35 (56%)	T1-36 (56%)
	T1-43 (56%)	T1-45 (56%)	T1-51 (56%)	T1-61 (56%)	T1-66 (56%)	T1-76 (56%)	T2-19 (56%)	T2-24 (56%)	T2-29 (56%)	T2-3 (56%)	T2-38 (56%)
	T2-47 (56%)	T2-49 (56%)	T2-51 (56%)	T2-55 (56%)	T1-12 (44%)	T1-14 (44%)	T1-20 (44%)	T1-28 (44%)	T1-37 (44%)	T1-58 (44%)	T1-59 (44%)
	T1-60 (44%)	T1-64 (44%)	T1-65 (44%)	T1-70 (44%)	T1-77 (44%)	T1-78 (44%)	T2-10 (44%)	T2-12 (44%)	T2-20 (44%)	T2-33 (44%)	T2-44 (44%)
	T2-46 (44%)	T2-5 (44%)	T2-57 (44%)	T2-8 (44%)	T2-9 (44%)	T3-10 (44%)	T3-11 (44%)	T3-28 (44%)			
SA	T1-15 (33%)	T1-21 (33%)	T1-23 (33%)	T1-44 (33%)	T1-49 (33%)	T1-54 (33%)	T1-55 (33%)	T1-72 (33%)	T2-35 (33%)	T2-58 (33%)	T3-22 (33%)
	T3-26 (33%)	T3-29 (33%)	T3-3 (33%)	T3-5 (33%)	T3-9 (33%)	T1-22 (22%)	T1-24 (22%)	T1-34 (22%)	T1-46 (22%)	T1-47 (22%)	T1-6 (22%)
	T1-63 (22%)	T1-81 (22%)	T2-11 (22%)	T2-21 (22%)	T2-48 (22%)	T2-54 (22%)	T2-59 (22%)	T3-15 (22%)	T3-20 (22%)	T3-21 (22%)	T3-23 (22%)
	T3-7 (22%)										
NA	T1-11 (11%)	T1-19 (11%)	T1-3 (11%)	T1-53 (11%)	T1-68 (11%)	T1-79 (11%)	T1-80 (11%)	T2-16 (11%)	T2-36 (11%)	T2-50 (11%)	T2-61 (11%)
	T3-13 (11%)	T3-24 (11%)	T1-1 (0%)	T1-13 (0%)	T1-2 (0%)	T1-30 (0%)	T1-31 (0%)	T1-4 (0%)	T1-5 (0%)	T1-7 (0%)	T2-1 (0%)
	T2-15 (0%)	T2-39 (0%)	T2-42 (0%)	T2-45 (0%)	T3-1 (0%)	T3-16 (0%)	T3-17 (0%)	T3-18 (0%)	T3-19 (0%)	T3-25 (0%)	

Table S8 – Efficacy (E) scale of selected bacterial strains at 7-8 days after *E. amylovora* inoculation in immature loquat fruit assay.
(VA) Very Active (100-90 %); (A) Active (89-70 %); (MA) Moderately Active (69-40 %); (SA) Soft Active (39-20 %); and (NA) Not Active (19-0 %).

E scale	**Strain (%) at 7-8 days**									
VA	**T3-27 (100%)**									
A	T1-10 (89%)	T1-26 (78%)	T2-26 (78%)	**T2-27 (78%)**	T2-43 (78%)	T2-56 (78%)	T3-28 (78%)			
MA	**T1-42 (67%)**	T2-17 (67%)	T2-18 (67%)	T2-31 (67%)	T2-32 (67%)	T2-34 (67%)	T2-37 (67%)	T1-33 (56%)	T1-50 (56%)	T1-57 (56%)	T1-73 (56%)
	T2-14 (56%)	T2-2 (56%)	T2-22 (56%)	T1-32 (44%)	T1-35 (44%)	T1-36 (44%)	T1-40 (44%)	T1-52 (44%)	T1-58 (44%)	T1-60 (44%)	T1-9 (44%)
	T2-23 (44%)	T2-38 (44%)	T2-41 (44%)	T2-52 (44%)	T2-53 (44%)	T2-57 (44%)	T2-7 (44%)	T3-14 (44%)			
SA	T1-12 (33%)	T1-14 (33%)	T1-20 (33%)	T1-27 (33%)	T1-38 (33%)	T1-41 (33%)	T1-49 (33%)	T1-70 (33%)	T1-71 (33%)	T1-76 (33%)	T1-78 (33%)
	T1-8 (33%)	T2-25 (33%)	T2-28 (33%)	T2-29 (33%)	T2-4 (33%)	T2-40 (33%)	T2-47 (33%)	T2-49 (33%)	T2-51 (33%)	T3-11 (33%)	T3-3 (33%)
	T1-24 (22%)	T1-25 (22%)	T1-39 (22%)	T1-56 (22%)	T1-64 (22%)	T1-65 (22%)	T1-66 (22%)	T1-69 (22%)	T1-72 (22%)	T1-77 (22%)	T1-81 (22%)
	T2-10 (22%)	T2-3 (22%)	T2-30 (22%)	T2-33 (22%)	T2-35 (22%)	T2-44 (22%)	T2-55 (22%)	T2-6 (22%)	T2-8 (22%)	T2-9 (22%)	T3-15 (22%)
	T3-2 (22%)	T3-30 (22%)									
NA	T1-15 (11%)	T1-23 (11%)	T1-34 (11%)	T1-37 (11%)	T1-43 (11%)	T1-51 (11%)	T1-59 (11%)	T1-61 (11%)	T2-12 (11%)	T2-13 (11%)	T2-16 (11%)
	T2-19 (11%)	T2-24 (11%)	T2-36 (11%)	T2-5 (11%)	T3-26 (11%)	T3-29 (11%)	T3-5 (11%)	T3-7 (11%)	T1-11 (0%)	T1-16 (0%)	T1-19 (0%)
	T1-21 (0%)	T1-22 (0%)	T1-28 (0%)	T1-29 (0%)	T1-3 (0%)	T1-44 (0%)	T1-45 (0%)	T1-46 (0%)	T1-47 (0%)	T1-53 (0%)	T1-54 (0%)
	T1-55 (0%)	T1-6 (0%)	T1-63 (0%)	T1-68 (0%)	T1-79 (0%)	T1-80 (0%)	T2-11 (0%)	T2-20 (0%)	T2-21 (0%)	T2-46 (0%)	T2-48 (0%)
	T2-50 (0%)	T2-54 (0%)	T2-58 (0%)	T2-59 (0%)	T2-61 (0%)	T3-10 (0%)	T3-13 (0%)	T3-20 (0%)	T3-21 (0%)	T3-22 (0%)	T3-23 (0%)
	T3-24 (0%)	T3-9 (0%)									

Table S9 – Efficacy (E) scale of selected bacterial strains at 9-10 days after *E. amylovora* inoculation in immature loquat fruit assay.
(VA) Very Active (100-90 %); (A) Active (89-70 %); (MA) Moderately Active (69-40 %); (SA) Soft Active (39-20 %); and (NA) Not Active (19-0 %).

E scale	**Strain (%) at 9-10 days**										
VA	**T3-27 (100%)**										
A	T1-10 (89%)	**T2-27 (78%)**									
MA	T2-56 (67%)	T2-26 (56%)	T2-37 (56%)	T2-43 (56%)	**T1-42 (44%)**	T2-14 (44%)	T2-17 (44%)	T2-32 (44%)	T2-34 (44%)		
SA	T1-14 (33%)	T1-33 (33%)	T1-40 (33%)	T1-49 (33%)	T1-57 (33%)	T1-60 (33%)	T1-70 (33%)	T1-71 (33%)	T1-76 (33%)	T1-78 (33%)	T2-18 (33%)
	T2-2 (33%)	T2-25 (33%)	T1-12 (22%)	T1-36 (22%)	T1-50 (22%)	**T1-56 (22%)**	T1-9 (22%)	T2-23 (22%)			
NA	T1-20 (11%)	T1-26 (11%)	T1-38 (11%)	T1-41 (11%)	T1-58 (11%)	T1-65 (11%)	T2-10 (11%)	T2-22 (11%)	T2-28 (11%)	T2-30 (11%)	T2-41 (11%)
	T2-53 (11%)	T2-7 (11%)	T2-8 (11%)	T2-9 (11%)	T1-15 (0%)	T1-23 (0%)	T1-24 (0%)	T1-25 (0%)	T1-27 (0%)	T1-32 (0%)	T1-34 (0%)
	T1-35 (0%)	T1-37 (0%)	T1-39 (0%)	T1-43 (0%)	T1-51 (0%)	T1-52 (0%)	T1-59 (0%)	T1-61 (0%)	T1-64 (0%)	T1-66 (0%)	T1-69 (0%)
	T1-72 (0%)	T1-73 (0%)	T1-77 (0%)	T1-8 (0%)	T1-81 (0%)	T2-12 (0%)	T2-13 (0%)	T2-16 (0%)	T2-19 (0%)	T2-24 (0%)	T2-29 (0%)
	T2-3 (0%)	T2-31 (0%)	T2-33 (0%)	T2-35 (0%)	T2-36 (0%)	T2-38 (0%)	T2-4 (0%)	T2-40 (0%)	T2-44 (0%)	T2-47 (0%)	T2-49 (0%)
	T2-5 (0%)	T2-51 (0%)	T2-52 (0%)	T2-55 (0%)	T2-57 (0%)	T2-6 (0%)	T3-11 (0%)	T3-14 (0%)	T3-15 (0%)	T3-2 (0%)	T3-26 (0%)
	T3-28 (0%)	T3-29 (0%)	T3-3 (0%)	T3-30 (0%)	T3-5 (0%)	T3-7 (0%)					

Table S10 – API system positive results from the four strains selected for the detached pear blossom assay. ε indicates a weak positive reaction.

Strain	System	Test	Time (h)	Result	Active ingredient	Reactions/enzymes
T1-42	API 20 NE	ESC	24 48	ε + +	esculin ferric citrate	hydrolysis (β-glucosidase) (ESCulin)
T1-42	API 20 NE	GEL	48	ε	gelatin (bovine origin)	hydrolysis (protease) (GELatin)
T1-42	API 20 NE	PNPG	48	+	4-nitrophenyl-βDgalactopyranoside	β-galactosidase (Para-NitroPhenyl-ßDGalactopyranosidase)
T1-42	API 20 NE	GLU	24 48	+ +	D-glucose	assimilation (GLUcose)
T1-42	API 20 NE	ARA	24 48	+ +	L-arabinose	assimilation (ARAbinose)
T1-42	API 20 NE	MNE	24 48	ε + +	D-mannose	assimilation (ManNosE)
T1-42	API 20 NE	MAN	24 48	+ +	D-mannitol	assimilation (MANnitol)
T1-42	API 20 NE	GNT	24 48	+ +	potassium gluconate	assimilation (potassium GlucoNate)
T1-42	API 20 NE	CAP	24 48	+ +	capric acid	assimilation (CAPric acid)
T1-42	API 20 NE	MLT	24 48	+ +	malic acid	assimilation (MaLaTe)
T1-42	API 20 NE	CIT	24 48	+ +	trisodium citrate	assimilation (trisodium CITrate)
T1-42	API 50 CH	4	24 48	εε+ +	L-ARAbinose	
T1-42	API 50 CH	10	48	+	D-GALactose	
T1-42	API 50 CH	11	24 48	ε+ +	D-GLUcose	
T1-42	API 50 CH	13	48	+	D-MaNnosE	
T1-42	API 50 CH	18	48	εε	D-MANnitol	
T1-42	API 50 CH	25	48	+	ESCulin ferric citrate	
T1-42	API 50 CH	36	24 48	εε blue εε blue	AmiDon (starch)	
T1-42	API 50 CH	39	48	εε	GENtiobiose	
T1-42	API 50 CH	43	48	εε	D-FUCose	
T1-42	API 50 CH	45	48	ε	D-ARabitoL	
T1-42	API 50 CH	48	24 48	ε blue + blue	potassium 2-ketogluconate	
T1-56	API 20 E	ONPG	24 48	+ +	2-nitrophenyl-ßD-galactopyranoside	ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase)
T1-56	API 20 E	ADH	24 48	+ +	L-arginine	Arginine DiHydrolase
T1-56	API 20 E	GLU	24 48	+ +	D-glucose	fermentation / oxidation
T1-56	API 20 E	SAC	24 48	+ +	D-sucrose	fermentation / oxidation
T1-56	API 20 E	MEL	24 48	+ +	D-melibiose	fermentation / oxidation
T1-56	API 20 E	AMY	48	εεε	amygdalin	fermentation / oxidation
T1-56	API 20 E	ARA	24 48	+ +	L-arabinose	fermentation / oxidation

T1-56	API 50 CH	1	24 48	εε+ εε	GLYcerol	
T1-56	API 50 CH	4	24 48	εε+ +	L-ARAbinose	
T1-56	API 50 CH	5	48	+	D-RIBose	
T1-56	API 50 CH	6	24 48	εε+ +	D-XYLose	
T1-56	API 50 CH	10	24 48	+ +	D-GALactose	
T1-56	API 50 CH	11	24 48	+ +	D-GLUcose	
T1-56	API 50 CH	12	24 48	εε+ +	D-FRUctose	
T1-56	API 50 CH	13	24 48	ε+ +	D-MaNnosE	
T1-56	API 50 CH	18	48	εε	D-MANnitol	
T1-56	API 50 CH	25	24 48	+ +	ESCulin ferric citrate	
T1-56	API 50 CH	30	24 48	εε+ ε	D-MELibiose	
T1-56	API 50 CH	31	24 48	εε+ ε	D-SACcharose (sucrose)	
T1-56	API 50 CH	39	48	εε	GENtiobiose	
T1-56	API 50 CH	43	48	ε	D-FUCose	
T1-56	API 50 CH	45	48	εε	D-ARabitoL	
T1-56	API 50 CH	48	24 48	ε blue ε blue	potassium 2-ketogluconate	
T1-56	API 50 CH	49	48	ε blue	potassium 5-ketogluconate	
T2-27	API 20 E	ONPG	48	+	2-nitrophenyl-ßD-galactopyranoside	
T2-27	API 20 E	ADH	24 48	ε + +	L-arginine	Arginine DiHydrolase
T2-27	API 20 E	ODC	24 48	+ +	L-ornithine	Ornithine DeCarboxylase
T2-27	API 20 E	CIT	24 48	+ +	trisodium citrate sodium	CITrate utilization
T2-27	API 20 E	VP	48	+	sodium pyruvate	acetoin production (Voges Proskauer)
T2-27	API 20 E	GLU	24 48	+ +	D-glucose	fermentation / oxidation
T2-27	API 20 E	MAN	24 48	+ +	D-mannitol	fermentation / oxidation
T2-27	API 20 E	RHA	24 48	+ +	L-rhamnose	fermentation / oxidation
T2-27	API 20 E	AMY	24 48	+ +	amygdalin	fermentation / oxidation
T2-27	API 20 E	ARA	24 48	+ +	L-arabinose	fermentation / oxidation
T2-27	API 50 CH	1	24 48	+ +	GLYcerol	
T2-27	API 50 CH	3	48	+	D-ARAbinose	
T2-27	API 50 CH	4	24	+	L-ARAbinose	

			48	+		
T2-27	API 50 CH	5	24	+	D-RIBose	
			48	+		
T2-27	API 50 CH	6	24	+	D-XYLose	
			48	+		
T2-27	API 50 CH	10	48	+	D-GALactose	
T2-27	API 50 CH	11	24	+	D-GLUcose	
			48	+		
T2-27	API 50 CH	12	24	+	D-FRUctose	
			48	+		
T2-27	API 50 CH	13	24	+	D-MaNnosE	
			48	+		
T2-27	API 50 CH	15	24	εε+	L-RHAmnose	
			48	εε+		
T2-27	API 50 CH	18	24	+	D-MANnitol	
			48	+		
T2-27	API 50 CH	22	24	+	N-AcetylGlucosamine	
			48	+		
T2-27	API 50 CH	24	24	+	ARButin	
			48	+		
T2-27	API 50 CH	25	24	+	ESCulin ferric citrate	
			48	+		
T2-27	API 50 CH	26	24	+	SAlicin	
			48	+		
T2-27	API 50 CH	27	24	+	D-CELliobiose	
			48	+		
T2-27	API 50 CH	28	24	+	D-MALtose	
			48	+		
T2-27	API 50 CH	32	24	+	D-TREhalose	
			48	+		
T2-27	API 50 CH	39	24	+	GENtiobiose	
T2-27	API 50 CH	44	24	ε+	L-FUCose	
			48	ε		
T2-27	API 50 CH	47	24	ε+	potassium glucoNaTe	
			48	+		
T2-27	API 50 CH	48	48	ε	potassium 2-ketogluconate	
T3-27	API 20 NE	ESC	24	+	esculin ferric citrate	hydrolysis (β-glucosidase) (ESCulin)
			48	+		
T3-27	API 20 NE	GEL	48	εεε	gelatin (bovine origin)	hydrolysis (protease) (GELatin)
T3-27	API 20 NE	PNPG	24	+	4-nitrophenyl-βDgalactopyranoside	β-galactosidase (Para-NitroPhenyl-ßDGalactopyranosidase)
			48	+		
T3-27	API 20 NE	GLU	24	ε+	D-glucose	assimilation (GLUcose)
			48	+		
T3-27	API 20 NE	ARA	48	+	L-arabinose	assimilation (ARAbinose)
T3-27	API 20 NE	MNE	48	+	D-mannose	assimilation (ManNosE)
T3-27	API 20 NE	MAN	48	+	D-mannitol	assimilation (MANnitol)
T3-27	API 20 NE	NAG	48	+	N-acetyl-glucosamine	assimilation (N-Acetyl-Glucosamine)
T3-27	API 20 NE	MAL	24	εε+	D-maltose	assimilation (MALtose)
			48	ε		
T3-27	API 20 NE	GNT	24	ε+	potassium gluconate	assimilation (potassium GlucoNate)
			48	+		

T3-27	API 50 CH	25	24	εεε	ESCulin ferric citrate
T3-27	API 50 CH	48	24 48	ε blue ε blue	potassium 2-ketogluconate

Table S11 – Results from the quantification of *E. amylovora* by real-time PCR on flowers inoculated with four selected bacterial strains.

Strain/Sample	Flower	Ea concentration	Replicate	Symptoms	Cp
Negative	Complete			0	0.00
Negative	Complete			3	0.00
Negative	Complete			0	0.00
Negative	Complete			0	0.00
Negative	Complete			2	0.00
Negative	Complete			2	0.00
Negative	Complete			2	0.00
Negative	Complete			0	0.00
Negative	Complete			0	0.00
Negative	Complete			2	0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Cabine control	Complete				0.00
Positive Ea	Complete				21.91
Infection control I3.1	Complete	10^3	1	3	28.60
Infection control I3.2	Complete	10^3	2	2	0.00
Infection control I3.3	Complete	10^3	3	3	37.82
Infection control I4.1	Complete	10^4	1	3	25.81
Infection control I4.2	Complete	10^4	2	2	29.95
Infection control I4.3	Complete	10^4	3	1	0.00
Infection control I5.1	Complete	10^5	1	2	23.28
Infection control I5.2	Complete	10^5	2	0	22.92
Infection control I5.3	Complete	10^5	3	1	26.50
Infection control I6.1	Complete	10^6	1	2	22.42
Infection control I6.2	Complete	10^6	2	2	28.15
Infection control I6.3	Complete	10^6	3	2	24.20
Infection control I7.1	Complete	10^7	1	3	17.58
Infection control I7.2	Complete	10^7	2	3	19.84
Infection control I7.3	Complete	10^7	3	3	22.02
T1-42	Complete	10^3	1	2	0.00
T1-42	Complete	10^3	2	2	0.00
T1-42	Complete	10^3	3	1	0.00
T1-42	Complete	10^4	3	0	31.48
T1-42	Complete	10^4	1	2	0.00
T1-42	Complete	10^4	2	3	0.00
T1-42	Complete	10^5	1	3	27.51
T1-42	Complete	10^5	2	1	29.79
T1-42	Complete	10^5	3	2	24.83
T1-42	Complete	10^6	1	1	0.00
T1-42	Complete	10^6	2	1	23.41

T1-42	Complete	10^6	3	1	23.34
T1-42	Complete	10^7	1	1	23.99
T1-42	Complete	10^7	2	3	23.62
T1-42	Complete	10^7	3	1	21.20
T1-56	Complete	10^3	1	2	0.00
T1-56	Complete	10^3	2	2	0.00
T1-56	Complete	10^4	1	2	0.00
T1-56	Complete	10^4	2	3	0.00
T1-56	Complete	10^4	3	3	0.00
T1-56	Complete	10^5	1	3	34.27
T1-56	Complete	10^5	2	3	0.00
T1-56	Complete	10^5	3	3	33.11
T1-56	Complete	10^6	1	3	34.80
T1-56	Complete	10^6	2	3	32.58
T1-56	Complete	10^7	1	3	30.28
T1-56	Complete	10^7	2	2	29.96
T1-56	Complete	10^7	3	3	29.07
T2-27	Complete	10^3	1	1	0.00
T2-27	Complete	10^3	2	0	0.00
T2-27	Complete	10^3	3	0	0.00
T2-27	Complete	10^4	1	1	34.99
T2-27	Complete	10^4	2	1	0.00
T2-27	Complete	10^4	3	2	0.00
T2-27	Complete	10^5	2	0	33.76
T2-27	Complete	10^5	4	0	34.89
T2-27	Complete	10^5	5	1	0.00
T2-27	Complete	10^5	6	0	24.69
T2-27	Complete	10^5	1	3	33.60
T2-27	Complete	10^5	3	1	24.35
T2-27	Complete	10^6	1	1	23.66
T2-27	Complete	10^6	4	2	23.21
T2-27	Complete	10^6	5	3	0.00
T2-27	Complete	10^6	6	3	32.68
T2-27	Complete	10^6	2	1	24.85
T2-27	Complete	10^6	3	1	30.39
T2-27	Complete	10^7	1	3	24.92
T2-27	Complete	10^7	5	2	20.15
T2-27	Complete	10^7	4	3	30.35
T2-27	Complete	10^7	5	0	30.31
T2-27	Complete	10^7	6	0	28.08
T2-27	Complete	10^7	4	3	0.00
T3-27	Complete	10^3	1	2	0.00
T3-27	Complete	10^3	2	3	0.00
T3-27	Complete	10^3	3	1	0.00
T3-27	Complete	10^4	1	1	0.00
T3-27	Complete	10^4	2	0	0.00
T3-27	Complete	10^4	3	0	33.96
T3-27	Complete	10^5	3	1	31.24
T3-27	Complete	10^5	4	2	32.91
T3-27	Complete	10^5	5	1	34.20
T3-27	Complete	10^5	6	3	0.00
T3-27	Complete	10^5	1	0	0.00
T3-27	Complete	10^5	2	0	27.78
T3-27	Complete	10^6	1	1	25.58
T3-27	Complete	10^6	2	0	22.17
T3-27	Complete	10^6	4	2	31.04
T3-27	Complete	10^6	5	3	32.13
T3-27	Complete	10^6	6	1	33.38

T3-27	Complete	10^6	3	1	26.09
T3-27	Complete	10^7	3	3	17.56
T3-27	Complete	10^7	2	2	21.36
T3-27	Complete	10^7	4	2	28.98
T3-27	Complete	10^7	5	0	27.77
T3-27	Complete	10^7	6	0	30.46
T3-27	Complete	10^7	1	2	0.00
Negative	Incomplete		1.2	1	0.00
Negative	Incomplete		2.2	2	0.00
Negative	Incomplete		3.1	1	0.00
Negative	Incomplete		4.1	1	0.00
Negative	Incomplete		5.1	1	0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Cabine control	Incomplete				0.00
Positive Ea	Incomplete				18.24
Infection control I3.1	Incomplete	10^3	1	1	0.00
Infection control I3.2	Incomplete	10^3	2	2	0.00
Infection control I4.1	Incomplete	10^4	1	2	0.00
Infection control I4.2	Incomplete	10^4	2	2	0.00
Infection control I5.1	Incomplete	10^5	1	2	0.00
Infection control I5.2	Incomplete	10^5	2	2	32.45
Infection control I6.1	Incomplete	10^6	1	0	31.94
Infection control I6.2	Incomplete	10^6	2	2	0.00
Infection control I7.1	Incomplete	10^7	1	3	27.96
Infection control I7.2	Incomplete	10^7	2	0	28.65
T1-42	Incomplete	10^3	1	0	0.00
T1-42	Incomplete	10^3	2	0	29.87
T1-42	Incomplete	10^4	1	0	34.69
T1-42	Incomplete	10^4	2	3	34.92
T1-42	Incomplete	10^5	1	0	27.39
T1-42	Incomplete	10^5	2	0	21.97
T1-42	Incomplete	10^6	1	3	28.32
T1-42	Incomplete	10^6	2	2	26.84
T1-42	Incomplete	10^7	1	3	24.27
T1-42	Incomplete	10^7	2	3	26.15
T1-56	Incomplete	10^3	1	3	31.55
T1-56	Incomplete	10^3	2	1	32.16
T1-56	Incomplete	10^4	1	2	31.58
T1-56	Incomplete	10^4	2	2	33.61
T1-56	Incomplete	10^5	1	2	33.31
T1-56	Incomplete	10^5	2	0	20.35
T1-56	Incomplete	10^6	1	0	24.21
T1-56	Incomplete	10^6	2	3	27.41
T1-56	Incomplete	10^7	1	0	27.64
T1-56	Incomplete	10^7	2	0	26.55

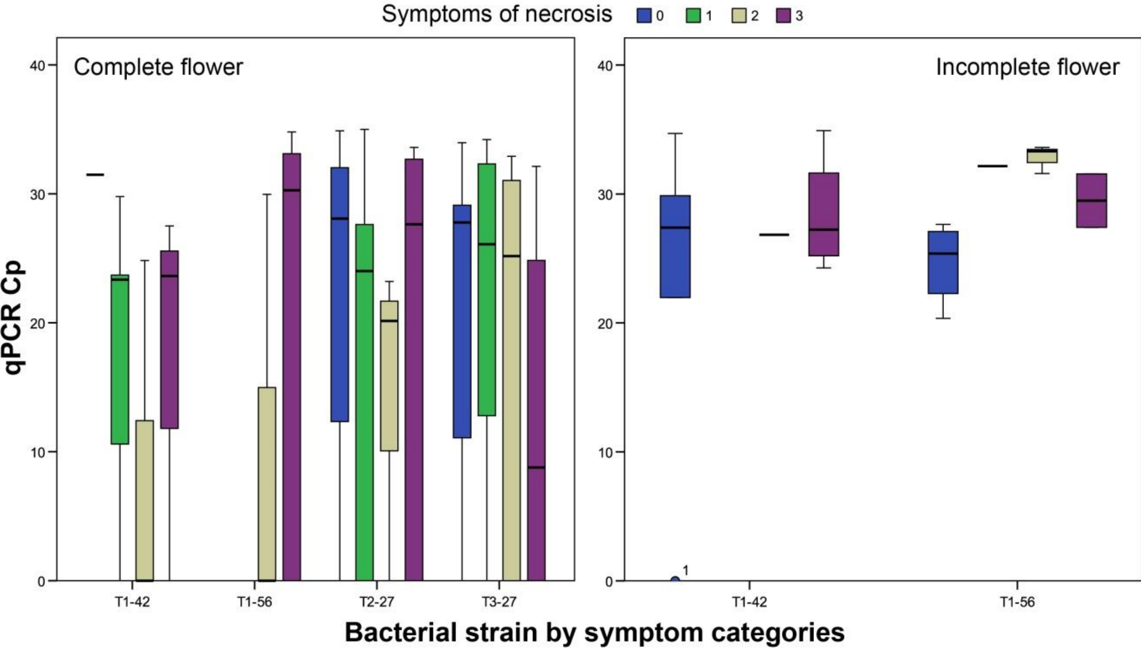

Figure S2 – Relationship between real-time PCR Cp for *E. amylovora* in flowers inoculated with selected bacterial strains and symptoms observed in the complete and incomplete forms.

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