Margarida Basaloco Fonseca

Licenciada em Biologia

Identification and Partial Characterization of a Potential Biological Control Agent against Fire Blight

Dissertação para obtenção do Grau de Mestre em Biotecnologia para a Sustentabilidade

Orientador: Dra. Cristina Azevedo, InPP

Co-orientador / Orientador Interno: Prof. Pedro Fevereiro, InPP / ITQB-UNL

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Abstract

Climatic changes combined with the global population growth have contributed to the adoption of new agricultural practices to meet the world's food demand and to fight hunger and food insecurity in a more sustainable way.

Fire blight is a devastating disease that affects several plants from the *Rosaceae* family, including economically important cultivars, such as the case of the Portuguese pear variety Rocha. *Erwinia amylovora* is the causal agent of this disease native from North America and since 2019 is also installed in the most significant Rocha pear production areas in Portugal. Only management practices can be adopted to limit the propagation and minimize economic losses since there is no efficient strategy to eliminate the bacteria.

Therefore, this project aims to identify new candidate Biological Control Agents to be used against this phytopathogen. To select such microorganism and to make sure it is applicable in the Portuguese context, as part of this work, the phytopathogen was isolated from a Portuguese pear orchard greatly impacted by fire blight. Upon pathogen isolation, identification and characterization, a screen of a small library containing 18 bacterial endophytes, coming from pear, apple, almond and olive tree, was performed for its ability to inhibit *E. amylovora* growth *in vitro*.

A *Bacillus* sp. isolate that had been isolated from pear orchards presented great antagonistic activity against the pathogen growth *in vitro*. This candidate Biological Control Agent was then partially characterized genetically using phylogenetic analysis and biochemically tested for its Biological Control and Plant Growth Promoter capacities.

This study could be of great interest for the Portuguese pear industry since identified a pear endophytic bacterium capable of inhibiting *E. amylovora in vitro* at high rates, which could be a promising first step towards developing a new field-validated Biological Control Agent against fire blight.

Keywords: Fire blight, *Erwinia amylovora, Bacillus* sp., Biological Control Agents, pear variety Rocha

Resumo

As alterações climáticas e o rápido aumento da população mundial têm contribuído para a necessidade de adoção de novas práticas agrícolas, de modo a suportar a procura global, combater a fome e a insegurança alimentar de uma forma mais sustentável.

O fogo bacteriano é uma doença que afeta severamente plantas da família das rosáceas, incluindo culturas de grande importância económica, como a variedade de pera portuguesa, Rocha. *Erwinia amylovora* é o agente causal responsável por esta doença nativa da América do Norte e que, desde 2019, já se encontra instalada nas áreas de maior produção de pera Rocha em Portugal. De momento não há uma forma eficiente de eliminar a bactéria, sendo as medidas de controlo a única maneira de limitar a sua propagação e minimizar perdas económicas.

De modo a proporcionar alternativas sustentáveis para o controlo do fogo bacteriano em Portugal, este projeto visa identificar e caracterizar novos microrganismos como possíveis agentes de controlo biológico. Para tal, *Erwinia amylovora* foi isolada de pereiras portuguesas que apresentavam um elevado grau de infeção. Após isolamento, identificação e caracterização do patógeno, uma triagem de 18 bactérias endófitas isoladas de pereira, macieira, amendoeira e oliveira, foi testada na sua capacidade de inibir o crescimento do patógeno *in vitro*. Um *Bacillus* sp. isolado de pereiras, apresentou características antagonistas contra *E. amylovora*, inibindo o seu crescimento *in vitro*. Este potencial agente de controlo de biológico foi parcialmente caracterizado geneticamente através de análise filogenética e também bioquimicamente, para as suas capacidades de controlo tal como para a promoção do crescimento de plantas.

Os dados obtidos neste estudo podem vir a ter grande impacto na indústria portuguesa de produção de peras, tendo em conta que poderá representar um importante avanço no desenvolvimento de novos Agentes de Controlo Biológico contra o fogo bacteriano.

Palavras-chave: Fogo bacteriano, *Erwinia amylovora, Bacillus* sp., agentes de controlo biológico, pera Rocha

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List of abbreviations

ACCd - 1-Aminocyclopropane-1-Carboxylic acid deaminase

BC - Biological Control

BCA - Biological Control Agent

Bp - base pairs

cBCA - candidate Biological Control Agent

CCT - Cycloheximide-Crystal violet - Thallium nitrate media

CM1 - Campo Maior isolate 1 (*E. amylovora*)

DGAV - *Direção-Geral da Alimentação e Veterinária*

DNA - Deoxyribonucleic Acid

DPI - Days Post Inoculation

DRAP - *Direção Regional de Agricultura e Pescas*

EPPO - European and Mediterranean Plant Protection Organization

EU - European Union

GFP - Green Fluorescent Protein

GNR - *Guarda Nacional Republicana*

gyrA - gyrase A

HCN - Hydrogen Cyanide

HR - Highly Resistant

I16 - Isolate 16 (*Bacillus velezensis* QST713)

I21 - Isolate 21 (*Bacillus* sp.)

IAA - Indole-3-acetic acid

KB - King’s B media

LB - Luria-Bertani

MIC - Minimal Inhibitory Concentration

MR - Moderately Resistant

NA - Nutrient Agar media

OD - Optical density

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PGP - Plant Growth Promoter

RNQP - Regulated Non-Quarantine Pest

ROS - Reactive Oxygen Species

rpm - Rotations per minute

rpoB - beta subunit of RNA polymerase

rRNA - ribosomal Ribonucleic Acid

Sm^R - Streptomycin resistant

SOC - Super Optimal broth with Catabolite repression

USA - United States of America

UV - Ultraviolet

π - Nucleotide diversity

1. Introduction

1.1 Challenges of Modern Agriculture

Global population growth has been rising since the middle of the twentieth century, from 2.5 billion in 1950 to 7.8 billion in 2020 (*United Nations Department of Economic and Social Affairs, Population Division (2021). Global Population Growth and Sustainable Development.*). The reduction of mortality along with the decreasing level of fertility due to various social and economic changes led to this demographic transition (Bloom et al., 2003). It's expected that the world's population reaches the 9.7 billion by 2050 (*United Nations Department of Economic and Social Affairs, Population Division (2021). Global Population Growth and Sustainable Development.*, 2022). Nowadays, food and feed production worldwide should meet the planet' needs. However, in 2020, 2.4 billion people were under severe or moderate food insecurity conditions and 786 million suffered from hunger (*FAOSTAT*, 2022). Low-income and lower-middle-income countries are the most affected by hunger and food insecurity. These issues are associated with poverty resulting from economic crisis, environmental changes and armed conflicts. Ending hunger will require an integrated approach in which sustainable agricultural productivity is considered one of the main focus (*United Nations Department of Economic and Social Affairs, Population Division (2021). Global Population Growth and Sustainable Development.*, 2022; Willett et al., 2019).

The ongoing agricultural system has a negative impact on biodiversity, it is responsible for one fifth to one third of the total greenhouse gas emissions and contributes to water and soil pollution, overuses pesticides, herbicides and fertilizers that affect aquatic and soil ecosystems and due to antibiotic administration to control pests and diseases, new antibiotic-resistant pathogens have emerged (*FAOSTAT*, 2022). All these reasons justify why current agriculture practices are considered ineligible for scaling up to meet the needs of a growing population, in a sustainable way (Shukla et al., 2019; Willett et al., 2019). It's predicted that by 2050, the demand for food will increase due to rising incomes, population growth and dietary adjustments (*FAOSTAT*, 2022), and consequently a shift towards a more sustainable production system should be adopted. Moreover, there is an urgent need to combat biodiversity loss, reduce antibiotic and pesticide usage, as well as in reducing excess of fertilization and improving animal welfare.

The *European Commission* has defined a plan, called *The Farm to Fork Strategy*, that addresses the main challenges of food systems and aims to achieve the United Nations' *Sustainable Development Goals* by shifting to a more sustainable agricultural production. European Union (EU) policymaking to protect plant, animal and human health, in addition with the farmers efforts have built up the high-quality standards and safety of European foods. Plant pest and disease management has contributed to an efficient yield increasing in the last 50 years, but climate change has been affecting the way pathogens respond to some of the control measures adopted throughout the years

(Chakraborty & Newton, 2011). Hence, plant protection from emerging pests and diseases requires innovative sustainable approaches. Moreover, the *European Commission* encourage the use of biotechnology and bio-based products on an integrated pest management approach for plant protection, reducing chemical pesticide dependency and the use of more hazardous pesticides by 50% by 2030 (*Farm to Fork Targets - Progress*, 2022).

1.2 Fire Blight Disease

1.2.1 Symptomatology and Epidemiology

Fire blight is a systemic necrotic bacterial disease that affects more than 180 plant species from 39 genera belonging to the *Rosaceae* family. The *Maloideae* subfamily is one of the most susceptible plant groups, including species with high economic importance like pear (*Pyrus* spp.) and apple (*Malus* spp.; Palacio Bielsa & Cambra Alvarez, 2009). Fire blight is one of the most devastating disease affecting pear and apple cultivars worldwide. This disease has been first named in 1817 because of the burnt effect that symptomatic tissues presented, as reported by the author "*turn the leaves suddenly brown, as if they had passed through a hot flame*" (Coxe, 1817). Usually, fire blight symptomatology starts by water soaking, wilting and rapid necrosis, which can be misunderstood by other abiotic or biotic stresses and thus being deceptive to detect (Vanneste, 2000). All aerial host parts including blossoms, leaves, fruits and trunks may present symptoms such as scorched

Figure 1.1 Fire blight symptomatology on pear orchards. (A) necrotic flowers; (B) leaf necrosis; (C) mummified fruits showing ooze exudate; (D) necrotic inner tissues from a freshly removed stem canker. (Source: EPPO Global Database, 2013).

appearance, necrosis and wilting (**Figure 1.1**) but severity may vary depending on environmental conditions and on the host species and variety. Also, the appearance of cankers and exudates containing bacterial cells involved in an exopolysaccharide matrix (bacterial oozes) are typical manifestations of this disease (Vanneste et al., 1995; Zeng et al., 2021).

Fire blight is caused by the phytopathogenic agent *Erwinia amylovora* (*E. amylovora*), which can be spread locally by wind, insects, rain and even by agricultural practices like pruning. For long distance dissemination, the endophytic pathogen is mainly transported by asymptomatic host plants, including ornamentals grown near orchards (Agrios, 2005; Thomson, 2000). Fire blight epidemiologic cycle is still not fully understood due to its complexity and there is evidence that depends on host phenological states and on climatic conditions (Agrios, 2005; Zeng et al., 2021). The disease cycle starts during spring, as temperature rises and endophytic *E. amylovora*, present in the margins of previous years' formed cankers become active and contact with new blossoms (Agrios, 2005; Thomson, 2000, **Figure 1.2**).

The primary infection by *E. amylovora* occurs preferentially during an epiphytic phase in flowers, in which bacteria start to multiply but it can also happen when in contact with wounds or natural openings like stomata or even nectarthodes (Zeng et al., 2021). Secondary sources of bacterial inoculum include the ooze exudates produced throughout the year in high humidity conditions as well as by activation of asymptomatic buds due to environmental changes (Palacio Bielsa & Cambra Alvarez, 2009; Thomson, 2000). Once inside the host, the bacterium quickly starts multiplying and

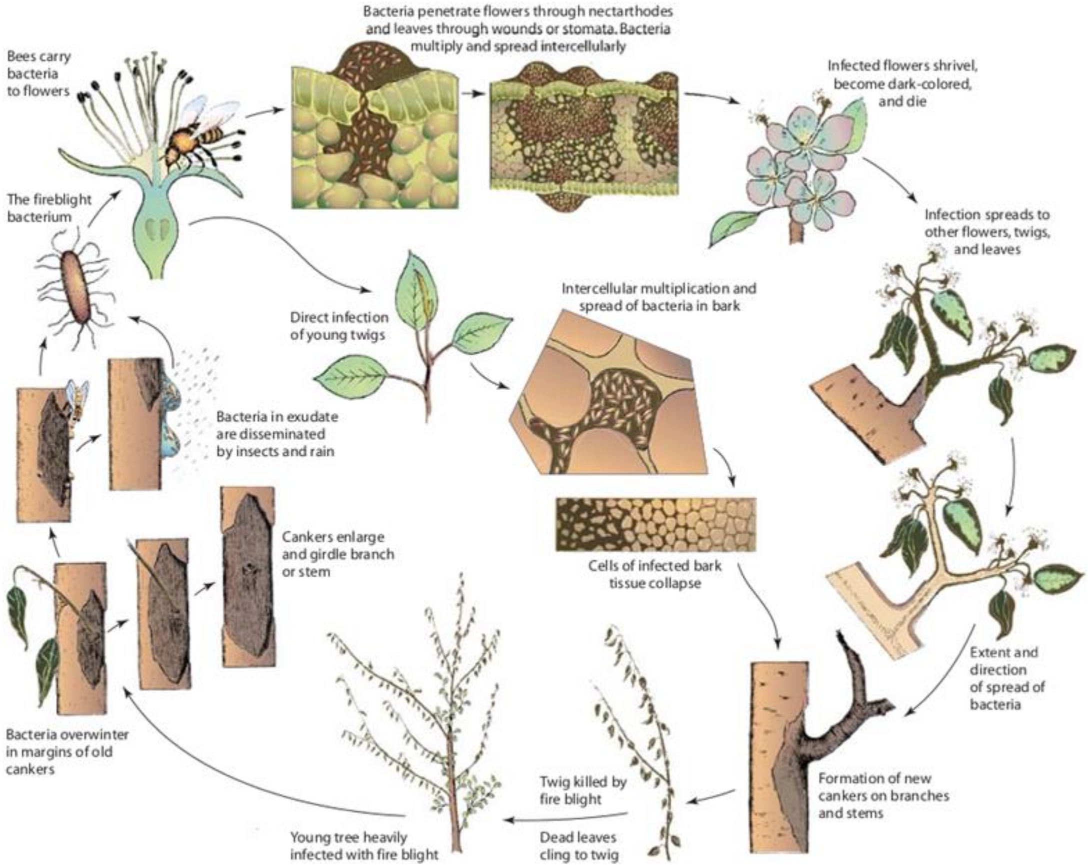


Figure 1.2 Fire blight disease cycle (Source: Agrios, 2005)

migrating to other plant parts, occupying intercellular spaces, and causing adjacent cell collapse (**Figure 1.2**). Consequently, two weeks after infection the first symptoms arise. *E. amylovora* movement inside hosts is variable, taking place into xylem or phloem vessels or even through the cortical parenchyma, depending on the entering site of infection. The growing season ends in late autumn when the environmental conditions turn less favourable and the bacterial proliferation reduces or ceases. By this time, the colonized tissues in trunks or branches evolve to cankers in which bacteria overwinters until a new spring dissemination cycle (Agrios, 2005; Thomson, 2000).

1.2.2 The Causal Agent: *Erwinia amylovora*

The full genome of *E. amylovora* was sequenced in 2010 by Smits (Smits et al., 2010) and Sebaihia (Sebaihia et al., 2010) and was the first bacterium ever described as a causal agent of a plant disease (Burril, 1883). *E. amylovora* is a facultative anaerobe, non-sporulating, rod shaped Gram-negative bacterium that belongs to the *Enteobacteriaceae* family. The *Erwinia* genus comprises pathogenic bacteria associated with a wide range of botanic families, like *E. amylovora, E. piriflorinigrans* and *E. pyrifoliae*, as well as non-phytopathogenic species such as *E. billingiae* and *E. tasmaniensis* (Palacio-Bielsa et al., 2012). The fire blight causal agent can survive from 4º C to 37º C with an optimal growth temperature of 28ºC and retains its pathogenic potential throughout this temperature range, conferring it a highly adaptable behaviour (Santander & G. Biosca, 2017). As fire blight symptomatology can be misinterpreted by other stresses, an integrated approach for diagnosis is recommended, in which pathogen isolation, molecular identification, and pathogenicity should be assessed. *E. amylovora* can be isolated on several nutritional media including King's B (KB), Nutrient Agar (NA), levan medium and Cycloheximide-Crystal violet - Thallium nitrate (CCT) medium. Colonies have different morphologies depending on the culture media in which they are growing. On KB, colonies present a whitish mucoid morphology however, when using CCT, the colour changes to a light pearly blue and a crater like appearance is prevalent (*EPPO Global Database*, 2022; **Figure 1.3**).

Figure 1.3 *E. amylovora* colony morphology on: (A) King's B agar medium; (B) levan medium (NSA) and (C) Cycloheximide-Crystal violet - Thallium nitrate medium. (Source: EPPO Global Database, 2013)

1.2.3 Geographical Distribution and Economic Impact

E. amylovora is native from North America, where it was detected for the first time in 1780 and was restricted to this sub-continent until the 19th century. In 1920 was identified in New Zealand, followed by England in 1957. Soon, Western Europe, Africa and Middle East were promptly colonized. Nowadays fire blight is spread worldwide in more than 50 countries (**Figure 1.4**, *EPPO Global Database*, 2022) and is threatening major producers of pome fruits like China, which was found to be just 200 km apart from the nearest infected field, in the Chinese border with South Korea (Zhao et al., 2019). In 2006, this devastating disease was first reported in Portuguese pear and apple orchards in Fundão, but this outbreak was quickly eradicated. Unfortunately, in 2010 fire blight was again detected in important areas that comprise the majority of pome fruit orchards for national pear and apple production like Oeste and Centre region and Alentejo. Sanitary measures were immediately settled to eradicate *E. amylovora* including total destruction of infected plants. Despite the efforts done towards disease control, in 2017 several other outbreaks were reported in distinct Portuguese areas, from Viana do Castelo, to Mafra and Montemor-o-Novo, Tavira and Monchique. By 2019, Portugal lost its status of last south European country fire blight-free. These outbreaks threaten the Portuguese economy since fruits belong to the 5 most produced commodities in the country and the prevalent pear cultivar Rocha seems to be highly susceptible to fire blight (Cruz et al., 2019; *FAOSTAT*, 2022). Rocha pear dominates the Portuguese pear industry, accounting for 80% to 90% of outputs. Portugal is responsible for 6% of the annual pear production in the EU and in 2020 collected approximately 131,000 tons of pear, representing a decrease of 34% compared with the previous year (*GLOBALAGRIMAR*, *Ficha de internacionalização da pêra rocha; INE_EA_2020*)

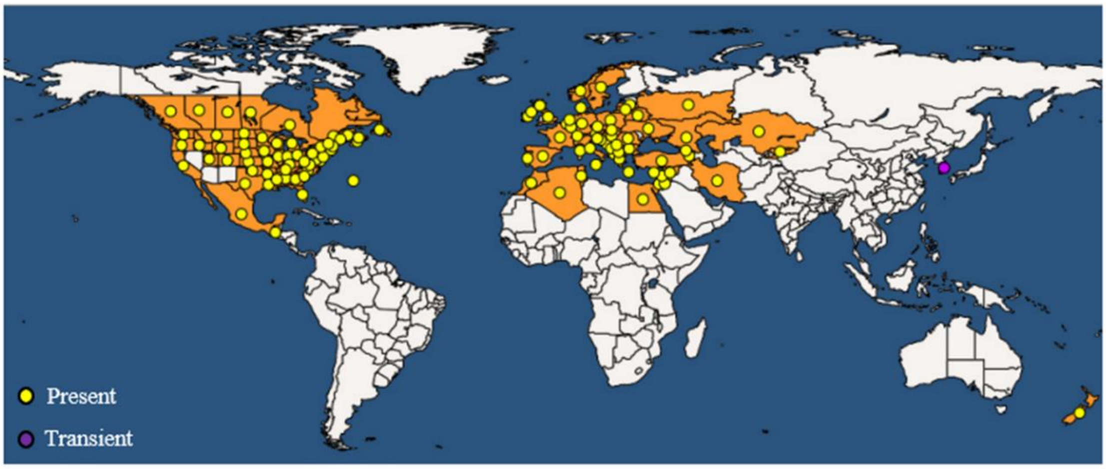


Figure 1.4 Worldwide fire blight distribution (Source: EPPO Global Database, 2022)

This disease is devastating and, in some cases, leads to yield reduction to zero, consequently having a serious impact on economies. Fire blight is a sporadic disease that progresses quickly and affects not only the current production but also the next years' due to the need of fruiting spurs elimination (Norelli et al., 2003). Pear orchards are among the most affected cultivars which is particularly alarming for Europe since it is the second main producer of pears. Europe contributes with 13.9% for the worldwide pear production, after Asia which detains 74.1% (**Figure 1.5**). Economic losses are difficult to predict but, in the United States of America, the financial burden for control measures and crop losses has been predicted to exceed 100 million USD per year (Norelli et al., 2003). In the Hawke's Bay region of New Zealand, an economic impact of about 10 million dollars was estimated for the first outbreaks. Also, during Italy and Lebanon outbreaks approximately 500,000 trees were destroyed by this disease (Calzolari et al., 1998) and in Switzerland the losses reached the 35 million EUR from 1989 to 2003 (Duffy et al., 2005). Therefore, it seems inevitable that the Mediterranean agriculture, including Portugal, will start suffering from more frequent and severe economic losses if the now endemic *E. amylovora* is not controlled.

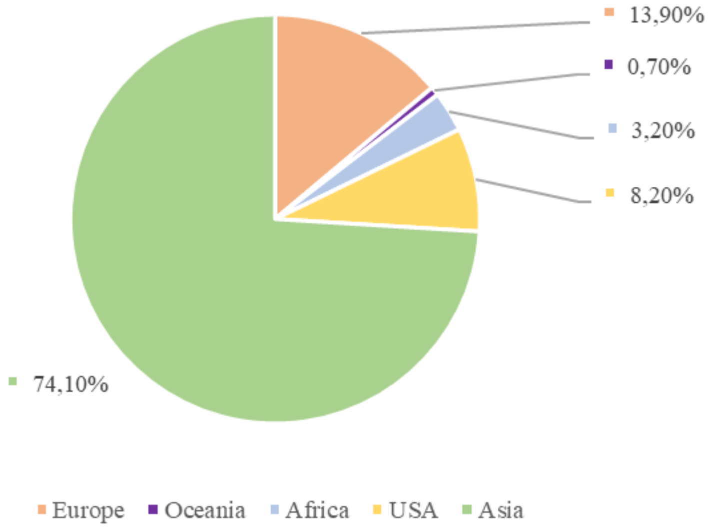


Figure 1.5 World pear production share (Adapted from FAOSTAT Global Database, assessed in April 2022)

1.3 Regulation in Europe and Portugal

Fire blight presents a threat to apple and pear industries and to nursery trades. In the Mediterranean area the risk is increased by the existence of endemic wild hosts as well as favourable climatic conditions. *E. amylovora* is considered a quarantine pathogen in Finland, Latvia, some Italian and Spanish regions and in Corsica Island (France) (EU, 2019). As there is no way to eliminate the pathogen, the European and Mediterranean Plant Protection Organization (EPPO) considered that all plant organs, except seeds, are potential sources of inoculum and thus imposed restrictions on the introduction of some host plants in fire blight-free regions. Portugal was *E. amylovora* free until 2010, apart from the 2006 outbreak. Unfortunately, in 2019 Portugal was removed from the list of the Integral Protected Areas due to rapidly spread of the disease along the territory and *E. amylovora* is now considered a Regulated Non-Quarantine Pest (RNQP) (EU, 2019). A RNQP is defined as "*a non-quarantine pest whose presence in plants for planting affects the intended use of those plants with an* *economically unacceptable impact and which is therefore regulated within the territory of the importing contracting party*" (Fri & Sun, 1997).

1.4 Management and Control

1.4.1 Cultural Practices, Breeding and Transgenic Rootstocks and Varieties

As there is still no cure for fire blight or an effective treatment that eliminates *E. amylovora*, control practices should be adopted. The European legislation comprises a set of phytosanitary measures to prevent and control this plant disease when installing or maintaining orchards (EU, 2019). In RNQP areas, the acquisition of plants for orchards installation must be done at officially controlled nurseries and a plant passport is mandatory for the host species. In Portugal, upon symptoms detection, orchards maintenance must be done and official authorities such *Direção-Geral da Alimentação e Veterinária* (DGAV), *Guarda Nacional Republicana* (GNR) or *Direção Regional de Agricultura e Pescas* (DRAP) need to be notified (EU, 2019). Sanitary pruning should be carried out on less affected trees allowing a longer producing life to the tree (*Direção-Geral de Agricultura e Desenvolvimento*, 2022). During pruning the tools must be disinfected from orchard to orchard and even between cuts. If an affected area is identified, the infected plant material needs to be burnt under the supervision of GNR and DRAP. Other practices such as, elimination of overwintering cankers before spring as well as the reduction of fertilizer application, to reduce plant vitality, should also be followed to help control fire blight (Van Der Zwet & Keil, 1979). These practices contribute to the maintenance and control the disease to a restricted area but do not eradicate the pathogen or treat infection.

Another way to fight fire blight is by breeding for resistance which consequently limits the amount of pesticides needed to control the spread of *E. amylovora* (Lespinasse & Paulin, 1989). Insights on genomics indicate that fire blight resistance is a polygenic trait and most quantitative trait loci (QTL) are strain specific. The majority of plant genes associated with resistance confer a very low level of resistance. To overcome this problem, some breeding programs opted to explore genetic sources coming from areas where both host and pathogen co-evolved (Hokanson et al., 1997). Although being a very sustainable approach, breeding for resistance while maintaining good horticultural characteristics is a laborious and long process and unfortunately an immediate solution is still needed to stop the disease spread (Keller-Przyby\lkowicz et al., 2009). Moreover, in Portugal, the attempt of breeding Rocha variety, the only endemic but fire blight sensitive and most produced pear, which accounts for more than 90% of pear industry, becomes a problem due to the intrinsic cultural importance and challenges in maintaining its organoleptic properties (Silva et al., 2005).

Recently there has been an increasing interest in transgenic pear lines development. There are already well-established procedures for *Agrobacterium tumefaciens* – mediated transformation of apples and pears. However, although some *E. amylovora* resistant apple trees have been obtained, the same procedure failed in pears (Broggini et al., 2014; Vergne et al., 2011). Other methods under development include biolistic transformation, but there are no successful reports of using this methodology. Despite the fact that this strategy seems promising, EU legislation prevents the commercial production and commercialization of genetically modified organisms, thus being hard to introduce as a control measure in countries like Portugal (Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the Deliberate Release into the Environment of Genetically Modified Organisms and Repealing Council Directive 90/220/EEC - Commission Declaration, 2001).

1.4.2 Chemical Control

Chemical control is still the mostly used method to control this and other plant diseases. For fire blight, bactericides can destroy the source when administered in different periods of the host life cycle: in spring when bloom takes place, in summer for secondary blossoms protection, post-bloom and in autumn / spring, before blossom, when the trees are dormant, to inhibit multiplication and reduce inoculum (Psallidas & Tsiantos, 2000).

Two groups of chemical compounds are mostly used to control fire blight: copper compounds and antibiotics (Van Der Zwet & Keil, 1979).

Since 1900, copper compounds which rely on the active copper ion toxicity, have been used as bactericides against fire blight mainly on pome fruit trees. However, copper ions are also phytotoxic and cause necrosis on fruits and leaves. To overcome this problem, mixtures with other compounds have been tested and commercialized, these include the Bordeaux mixture (copper and lime); copper oxychloride, ammoniacal copper sulphate and copper hydroxide. Despite all the efforts to find new bactericide mixtures, all were so far considered phytotoxic at the recommended doses for efficient *E. amylovora* control (Psallidas & Tsiantos, 2000; Van Der Zwet & Keil, 1979). Moreover, other major concerns such as the acquired resistance already detected in other pathogen species (Q.-L. Chen et al., 2019; Cooksey, 1990; Sundin & Wang, 2018), shifts on the structure of soil microbial community (Deng et al., 2012) and water contamination (Fernández-Calviño et al., 2009) have been raised by the administration of this type of chemical compound.

Likewise, antibiotics have been used to control phytopathogens since 1950. After restrictions were imposed for the usage of copper compounds to control fire blight, antibiotics were considered the first management alternatives. Several antibiotics have shown to be effective on inhibiting *E. amylovora* growth *in vitro*, but only few are effective *in planta* (Morgan & Goodman, 1955). Streptomycin has great inhibitory capacity both in laboratory and in the field conditions without high intoxication risk of plant or animals (Ark, 1949). Discovered in 1944 (Schatz et al., 1944), streptomycin is an aminoglycoside, produced by some *Streptomyces griseus*, and when applied into plant it can move systemically into tissues. Streptomycin affects protein synthesis leading to cell malfunction and even death (Gottlieb & Shaw, 1970). In the USA, streptomycin has been deemed safe and efficient for field application to control fire blight since 1950, whereas in Europe its usage has not been approved. Interestingly, streptomycin resistant *E. amylovora* strains have emerged throughout the years in the USA. The first streptomycin resistant strains were detected on pear orchards in 1972 in California and since then more cases have been reported worldwide even where streptomycin had not been used before, like Lebanon (Förster et al., 2015). Genetic and molecular studies have highlighted the possibility of two mechanisms underlying streptomycin resistance such as a mutation on the chromosomal *rpsL* gene or plasmid acquisition containing the *strA*-*strB* gene pair (Förster et al., 2015). These mechanisms result on distinct *E. amylovora* phenotypes, with highly resistant strains that can overcome streptomycin action up to 2,000 µg/mL, and moderately resistant strains which have minimal inhibitory concentrations (MICs) ranging from 500 to 750 µg/mL, respectively (McManus et al., 2002).

An alternative antibiotic tested to surpass the development of resistance was oxytetracycline. However, new emerging resistance has been reported for oxytetracycline (Miller et al., 2022), its bactericidal activity is less effective against fire blight and is considered more toxic (McManus et al., 2002).

Additionally, products that can be used to overcome some of these issues are an oxolinic acid derivate called fosetyl-Al and acibenzolar-S-methyl (ASM) which is an organic compound that enhances the Systemic Acquired Response in plants, limiting the possibility of resistance emergence (Psallidas & Tsiantos, 2000).

In Portugal, chemical treatments against fire blight include, amongst others, copper compounds administration (Cuprital, Cuprital SC, Cuproxi Flo, Cuproxat), organophosphorus-based fosetyl-Al (Aliette Flash, Etylit Premier, Protect Garden Fungicida Sistémico WG, Alfil, Fosletis 80 WG, Maestro 80 WG) and Acibenzolar-S-methyl (BION 50 WG) (*SIFITO*, 2022).

Although *E. amylovora* has been studied for almost two centuries, and most of the developed bactericides were tested against this disease, no sustainable and reliable bactericide can be recommended for field application. The wide host range of the pathogen makes chemical control a harsh task due to compounds features like lack of effectiveness, phytotoxicity and environmental damage.

1.4.3 Biological Control

The emergence of diseases like fire blight that can acquire resistance to chemical treatments coupled with the increased consumer demand for pesticide-free products has led to a need for finding more sustainable and environmentally friendly alternatives (Jaffuel et al., 2019; Sundin & Wang, 2018). Plant microbial diversity plays an important role on plant disease management. Some microorganisms have the capacity of phytopathogenic suppression or inhibition and may also promote plant growth, contribute to nutrients solubilization, or even act on the removal of pollutants. The use of Biological Control Agents (BCAs), which are organisms that can act by inhibiting pathogens activity, is a sustainable control strategy that can overcome the issue caused by antibiotic-resistant strains, and the necessity of chemical usage. These microorganisms can be isolated from many different sources like soil, rain and even been isolated from plants (Köhl et al., 2019; Mechan Llontop et al., 2020).

BCAs may act in distinct ways to decrease the fire blight incidence, including growth inhibition by resource depletion (Wilson & Lindow, 1992) being the efficacy of the control agent dependent on its ecological competencies, production of bactericidal compounds (Saputra et al., 2013) such as secondary metabolites like antibiotics, induction of plant defences (Abo-Elyousr et al., 2019; Fousia et al., 2016) by the recognition of specific microbial compounds that will trigger a cascade of plant metabolic events, higher colonization rates (Wilson & Lindow, 1992) that will ultimately lead to an increased competitive advantage or by a combination of these mechanisms (Neeno-Eckwall et al., 2001).

Despite being environmentally friendly, its efficacy is very dependable on environmental conditions such as rain, UV light and in the case of those applied directly into the soil, on its microbiome (Bonaterra et al., 2012). To improve efficacy, all biocontrol processes involved in the antagonistic interactions must be analysed and defining a precise application schedule is decisive (Xu et al., 2010). When using BCA-based products is best to follow an integrated pest management approach that combine applications with agricultural practices that reduce the level of inoculum, such as the immediate removal and destruction of infected branches and disinfection of pruning material.

The use of BCAs for plant disease management and control offers some advantages over other chemical approaches: has few negative impacts on the ecosystem due to a pathogen-specific action (Alvindia, 2013), allow hosts to allocate energy and resources for other agronomic features by preventing constitutive host immune system response (Wei et al., 2016), and can keep the treatment going for longer time than chemical compounds that need to be re-applied several times (Wei et al., 2016). Despite all this, introduction of new BCAs in the market is covered by a strict regulation and a detailed characterization and identification of the organisms is necessary to ensure health and environmental safety.

Several BCA-based products that act against fire blight in pears and apples are already commercialized on the USA (Edgecomb & Manker, 2006; Wilson & Lindow, 1992). Nonetheless, most of these products are not available in the EU due to regulatory issues (Velivelli et al., 2014).

In Portugal, the *Bacillus velezensis* QST 713 (Serenade; Serenade ASO, Bayer) and *Bacillus amyloliquefaciens* subsp. plantarum, strain D747 (AMYLO-X WG) are the only BCAs approved by DGAV to fight *E. amylovora*. Recently, a Spanish company (Kimitec Agro, Almería, Spain) is developing a phage-based solution to prevent and treat *E. amylovora* infections (PhageFire project). Bacteriophages are viruses that require bacteria to survive and have been observed to positively inhibit the fire blight causal agent (Gill et al., 2003). However, this alternative is still not available on the market.

Overall, a promising approach to control the spread of *E. amylovora* infections is the use of BCAs. However, considering that Portugal has not many available alternatives for a sustainable fire blight control, the biological approach is still not broadly used by the producers (Q.-L. Chen et al., 2021).

1.5 Use of *Bacillus* species as Biological Control Agents

Bacillus is a very heterogenous group of microorganisms that can be found on a large variety of ecological niches like water, soil, surfaces and rhizosphere, gastrointestinal traits and on many other, at times extreme, habitats (Connor et al., 2010). These Gram-positive, rod-shaped and sporulating bacteria belong to the *Firmicutes* phylum and are known for their great ability to produce a wide range of antagonistic compounds. *Bacillus* strains have 5% to 8% of their genome encoding for proteins involved in secondary metabolites biosynthesis (X. H. Chen et al., 2009). Their ability to form biofilms facilitates plant colonization and persistence in unfavourable environments (Pandin et al., 2019; Zeriouh et al., 2014). Also, lipopeptides such as surfactin, produced from *Bacillus* sp. reportedly have a high biological control potential against a wide range of bacterial phytopathogens (Edgecomb & Manker, 2006; Li et al., 2014; Zhao et al., 2014, 2019). Surfactin can act as an antibacterial compound and is capable of inducing pore formation, consequently increasing membrane permeability on Gram-negative bacteria by binding to their lipopolysaccharide cell wall (Hwang et al., 2007). Many other types of lipopeptides can be produced by this group of bacteria, like bacillomycin L and fengycin which are known to have antifungal activity (Li et al., 2014; Zhao et al., 2014). Interestingly, a synergetic approach may be beneficial from a plant protection perspective to improve the biocontrol effect, depending on the lipopeptide mixture (J. Liu et al., 2014).

Bacillus thuringiensis species are one of the most successful cases of biocontrol. These bacteria can kill insects belonging to several orders by disrupting its midgut tissue. This insecticidal effect is just active on larval stages and relies on a mixture of virulence vectors and production of water-soluble insecticidal toxins. The *Bacillus thuringiensis* pore forming toxins have been introduced into a variety of transgenic crops resulting on an effective and more sustainable way to control insect pests in agriculture (Bravo et al., 2011).

The majority of the commercialized biopesticides belong to the *Bacillus thuringiensis* species, followed by the *Bacillus subtilis* group that is mainly used as biological control against phytopathogenic microorganisms (Glare et al., 2012; Rooney et al., 2009). The *B. subtilis* group comprises the important species *B. amyloliquefaciens* amongst others. The identification of some *B. subtilis* members has been a difficult task since phenotypes are very similar and 16S rRNA sequencing does not give enough resolution to distinguish between species (Rooney et al., 2009). As a consequence, *Bacillus* spp. taxonomic misidentifications are frequent and thus detection and characterization can be a confusing process.

The *Bacillus velezensis* QST713, the active ingredient of Serenade ASO (Bayer), has been used for almost ten years as a BCA and is known to form dense biofilms and to swarm on agar plates, suggesting efficiency on plant colonization (Pandin et al., 2018). In Portugal, this biopesticide is available and recommended for fire blight control (*SIFITO*, 2022).

Despite their great potential, *Bacillus*-based biopesticides still do not have market dominance and lag behind chemical usage.

1.6 Project Aim

Fire Blight is a devastating disease for numerous species including the highly sensitive Portuguese pear cultivar Rocha. It's caused by the bacteria E. *amylovora*, which is considered an endemic phytopathogen since 2019. Its control in Europe and in Portugal in particular, is difficult due to the restrictions on the use of antibiotics in agriculture and commercialization of transgenic plants, antibiotic resistance development, and the limited efficacy of alternative inhibitory compounds. The quick dissemination of the phytopathogen is endangering nurseries and orchards throughout the country and there is a great need to find alternative control procedures. The use of BCAs with an antagonistic effect on *E. amylovora* spread, growth or virulence constitutes one of the most promising sustainable and cost-effective approaches to combat fire blight. In Portugal, there are very few biological control alternatives and, for this reason, finding new microorganisms with inhibitory capacity towards *E. amylovora* is crucial. Therefore, this project aims to identify new candidate BCAs to be used against this phytopathogen.

To select such microorganism and to make sure it is applicable in the Portuguese context, as part of this work, the phytopathogen was isolated from a pear orchard in a region greatly impacted by fire blight, Campo Maior. After isolation, molecular identification using Polymerase Chain Reaction (PCR) and Sanger sequencing was carried. To further confirm pathogenicity, tests have been performed using immature pears, as recommended by EPPO, to evaluate its virulence nature. The screen to identify BCAs against *E. amylovora* was performed *in vitro* using a dual-culture solid media approach optimized using a small library of 17 bacterial endophytes coming from Portuguese pear, apple, almond and olive trees and *B. velezensis* QST713 as a positive control for growth inhibition.

A *Bacillus* sp. isolate that had been isolated from pear orchards in the Oeste region presented great inhibitory capacity against *E. amylovora* growth *in vitro* and was then partially genetically and biochemically characterized. This study could be of great interest for the Portuguese pear industry since identifying a pear endophytic bacterium capable of inhibiting *E. amylovora in vitro* at a higher rate than the only commercially available product, *B. velezensis* QST713 (Serenade ASO, Bayer), would be a promising first step towards developing a new field-validated BCA for fire blight.

2. Materials and Methods

2.1 Isolation and Identification of Microorganisms

2.1.1 *Erwinia amylovora* and Endophytic Bacterial Isolation

Secondary shoots were collected from a fire blight symptomatic pear (*Pyrus communis* cultivar Rocha) orchard in Campo Maior as well as from other cultivars (pear, apple, almond and olive) from different Portuguese areas (Campo Maior, Famões, Évora, Gradil, Maxial, Elvas e Redondo) and kept at 4ºC for further use. Plant material, stems and leaves were first disinfected with 70% ethanol to remove epiphytes and two different extraction procedures were tested: stem stamping and macerated leaves (**Figure 2.1**). For stamping which is a more adequate technique for pathogen isolation but can also be used in endophyte isolation, stems were cross-sectioned and stamped in Nutrient Agar (NA, Biolife) and Kings' B Agar media (KB, King et al., 1954). To acquire the endophytic bacterial content from infected and non-infected leaves, they were macerated with 400 µL of 1x PBS (1.37 M NaCl, 2.7 mM KCl; 10 mM Na2HPO$_4$; 1.8 mM KH2PO$_4$; pH=7.4) and 100 µL were spread on nutrient media plates. Morphologically different bacteria were selected and re-streaked several times to purify each culture and obtain single colonies. Stocks for long term storage were made for all isolates using 50% (v/v) glycerol in a 1:1 proportion (v/v) and kept at -80ºC.

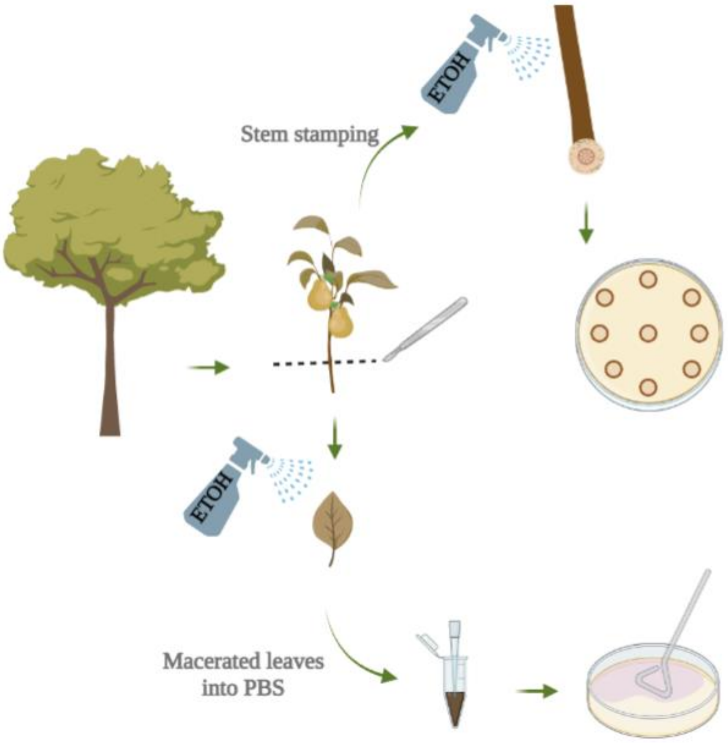


Figure 2.1 Schematic representation of bacterial endophytes isolation from pear trees

2.1.2 Bacterial identification by 16S rRNA amplicon sequencing

Bacterial DNA was obtained using a quick DNA extraction. Individual colonies were eluted in 500 μL of 0.9% (w/v) NaCl, followed by centrifugation at 12,000 xg for 3 min, at room temperature, after which the supernatant was discarded. This step was repeated twice and then the remaining pellet was re-suspended in 100 μL 20 mM NaOH and vortexed for 1 min. This solution was then incubated at 100ºC for 5 min, vortexed vigorously for 2 min and sonicated for 1min. After vortexing again for 2 min the solution was ready to use for PCR. For bacterial identification, a 16S rRNA partial sequence was amplified by PCR using specific primers 16SFw/16SRev, with an expected size of approximately 1,500 bp (**Table 2.1**). The following thermocycler set up was used: 3 min of denaturation at 94ºC, followed by 35 cycles of 30 sec of denaturation at 94ºC, 20 sec annealing at 55ºC and 1:10 min of extension at 72ºC finishing with an extension step of 5 min at 72ºC. An electrophoresis was done to check amplification and size. PCR products were purified using a NZYGelpure kit (NZYTech, Lisbon) and sent to for Sanger sequencing (StabVida, Caparica). The sequences were manually trimmed and searched within NCBI using BLASTn (Altschul et al., 1990). To obtain a more accurate identification, the sequences were also ran against OneTax, which consists of a 3 classifiers' pipeline for taxonomic assignment (InPP developed sequence analysis pipeline, unpublished).

2.1.3 Bacterial growth monitoring

All bacterial growth curves were obtained from cells grown in Luria-Bertani (LB, Liofilchem) liquid media. Growth profiles were estimated by monitoring the optical density at 600 nanometers (OD_{600}) in a MultiSkan Sky Spectrophotometer (Thermofisher). The bacteria were grown overnight at 28ºC, culture OD_{600} were then adjusted to 0.1 with fresh media and then incubated at the same temperature, with linear shaking, using two methodologies. In assay 1, using 5 mL of LB in 15 mL Falcon tubes (corresponding to approximately 3x aeration/volume). The assay was also performed in 96-well plates, assay 2, with 150 μL of LB (corresponding to approximately 1x aeration/volume). In assay 1, absorbance measurements were taken every 60 min for a period of 6 h whereas for assay 2, the data collection was done every 60 min for the first 4 h followed by 30 min intervals.

Table 2.1 Oligonucleotide sequences of primers used in this study

Primer	Sequence (5'-3')	Target	Expected amplicon size (bp)	Reference
16SFw	AGAGTTTGATC(A:C)TGGCTCAG	16S bacterial rRNA	1500	Jensen et al. 2002
16SRev	TACGG(C:T)TACCTTGTTACGACTT	16S bacterial rRNA	1500	Jensen et al. 2002
pEA71Fw	CCTGCATAAATCACCGCTGACAGCTCAATG	*E. amylovora* genomic DNA	187	Taylor et al. 2001
pEA71Rev	GCTACCACTGATCGCTCGAATCAAATCGGC	*E. amylovora* genomic DNA	187	Taylor et al. 2001
M13Fw	CACGACGTTGTAAAACGACGGCCAGTG	pHC60 plasmid	1090	This study
M13Rev	GTGAGCGGATAACAATTTCACACAGG	pHC60 plasmid	1090	This study
rpoBFw	GACGTGGGATGGCTACAACT	*Bacillus* sp. *rpoB* gene	964	Rooney et al. 2009
rpoBRev	ATTGTCGCCTTTAACGATGG	*Bacillus* sp. *rpoB* gene	964	Rooney et al. 2009
gyrAFw	CAGTCAGGAAATGCGTACGTCCTT	*Bacillus* sp. *gyrA* gene	928	Rooney et al. 2009
gyrARev	CAAGGTAATGCTCCAGGCATTGCT	*Bacillus* sp. *gyrA* gene	928	Rooney et al. 2009

2.2 *E. amylovora* identification and characterization

2.2.1 *E. amylovora* identification by pEA71 amplification

A specific genomic partial sequence of this strain (pEA71; Taylor et al., 2001), was amplified by PCR. For that purpose, specific primers for pEA71 (pEA71Fw/pEA71Rev; **Table 2.1**) were used in a PCR program comprising the following steps: 3 min of denaturation at 94ºC, followed by 35 cycles of 30 sec of denaturation at 94ºC, 20 sec annealing at 58ºC and 12 sec of extension at 72ºC finishing with an extension step of 5 min at 72ºC. A fragment with approximately 187 bp was the expected amplicon size. Upon validation, the previously identified *E. amylovora* strain (CM1) DNA was used as template for the amplification control.

2.2.2 Pathogenicity tests

Portuguese Rocha immature pears, with calibre 55/60 mm, from the Oeste region were used as hosts for *E. amylovora* infection. Two approaches were tested: use of whole pears (method A, based on Donat et al., 2005) or using sliced fruits (method B, Duffy et al., 2005). For both methodologies *E. amylovora* isolate CM1 was grown in LB with a linear shaking at 120 rpm for 48 hours. OD_{600} was assessed and 2 mL of the inoculum were centrifuged at 5,000 xg for 5 min. Pellet was suspended in 1x

PBS (pH=7.4) to get a bacterial concentration of 10^9 cfu/mL, which corresponds approximately to an OD_{600} of 1. A surface sterilization of the immature whole pears by submersion with 10% bleach and then with 70% ethanol was followed by a sterile distilled water wash. For method B, the same disinfection steps were performed before cutting the slices. Wounds were done for both experiments: in method A, 3 wounds per fruit using a syringe needle, and for method B, a cross stabbing was done in each slice. For both methods, each wound was inoculated with 10 µL of the bacterial suspension and for the mock control 10 µL of 1x PBS (pH=7.4) were used. Each inoculation sample was constituted by 3 fruits and all were incubated in a humid environment at 25ºC for 7 (method B) or 14 days (method A), after which symptoms were recorded.

2.2.3 Streptomycin antibiograms

E. amylovora isolate CM1 and the reference strain CFBP1430 (CIRM-CFBP, INRAE, France) were grown overnight in LB with 120 rpm linear shaking at 28ºC. The OD_{600} was assessed, adjusted to 0.01 and 100 µL of the inoculum were spread on KB agar plates. An *E. coli* DH5α was used as a sensitive control to streptomycin and plated in KB agar plates at the same OD_{600} as *E. amylovora*. Six antibiotic disks (VWR) with 15 µL each, containing different concentrations of streptomycin (2.5 mg/mL, 1 mg/mL, 500 µg/mL, 100 µg/mL and 50 µg/mL) as well as a mock control with sterile distilled water were then placed on top of the inoculated medium. Plates were incubated at 28ºC for 48h, with halo formation around disks representing susceptibility to the antibiotic, and its absence indicating resistance.

2.3 Introduction of a plasmid containing the green fluorescent protein (GFP) reporter gene into *E. amylovora*

E. amylovora bacterial cells were transformed by electroporation with plasmid pHC60 (Cheng & Walker, 1998) that expresses GFP. To prepare *E. amylovora* competent cells, the bacterium was grown in LB overnight at 28ºC with linear shaking at 120 rpm. The culture was then diluted to an OD_{600} of 0.1 with fresh LB. Bacterial growth was monitored until reaching an OD_{600} of approximately 0.8 and then kept on ice for 20 min. An inoculum volume of 12 mL was centrifuged at 8,000 xg for 5 min at 4ºC and the supernatant discarded. The obtained pellet was washed with ice cold sterile MilliQ water using the same initial volume of inoculum. The resuspended pellet was centrifuged again at 8,000 xg for 5 min and the procedure was repeated decreasing the water volume by half. Another centrifugation was done using the same conditions, the supernatant discarded and the pellet eluted in 250 µL of ice cold sterile MilliQ water. Transformation was performed using 100 µL of the electrocompetent cells. Plasmid DNA was purified with NZYGelpure kit and 550 ng were used for transformation. A control reaction without DNA was also added to the experiment. A 2 mm gap electroporation cuvette was used in a Gene Pulser Xcell™ Electroporation System (BIORAD, Amadora) with the electroporation program described by Spinelli et al., 2005 using a pulse of 800

Ohms, 2.5 kV, 2.5 μFD. Recovery was done by immediate addition of 1mL of SOC medium followed by a 1 h incubation at 28ºC with minimal shaking. Each transformation mixture was then spread on LB agar plates supplemented with 10 μg/mL of tetracycline. No plasmid DNA was added to the negative control of transformation which only contained the competent cells. After 1 day of incubation at 28ºC, transformants were observed under a UV transilluminator to confirm GFP expression. A PCR using the same program set up for 16S rRNA amplification (section 2.1.2) but with primers M13Fw/M13Rev (**Table 2.1**), targeting pHC60 plasmid, was performed to confirm insertion.

2.4 Dual-culture plate assays

To set up a screen for candidate Biological Control Agents (cBCAs) against *E. amylovora* some variables were evaluated, such as: nutrient media, method of inoculation, initial *E. amylovora* inoculum concentration, time-lag between pathogen and cBCAs inoculation. Dual-culture plate assays were tested in LB and KB agar media. Pre-inoculums from CM1 and the 18 cBCAs single colonies were incubated overnight with linear shaking at 28ºC. Bacterial concentration of each isolate was then assessed by OD_{600}. CM1 inoculum was adjusted to two OD_{600} (0.1 and 0.01) to test different starting concentrations. LB and KB agar plates were inoculated with 100 μL of the pathogen's inoculum. Then, two different methodologies were tested to inoculate the cBCAs on top of the already plated pathogen: using wells containing 10 μL of each cBCA liquid culture or inoculate directly 10 μL of each cBCA liquid culture on top of the inoculated pathogen. For the method in which wells were used, after pathogen inoculation into the solid media, wells were made using the top of a sterile 200 μL pipette tip and 10 μL of each cBCA were inoculated inside. The cBCAs inoculation was performed also at two different timings: simultaneous inoculation (SI) in which cBCAs were inoculated 1 h after adding CM1; distinct inoculation (DI) in which cBCAs were inoculated 16h after plating CM1.

The best combination found to perform the BCA screening against *E. amylovora* was using KB plates with an $OD_{600}=0.01$ of the pathogen and a SI of each cBCA. Data collection was also optimized for 2 days post inoculation (2DPI).

2.5 Characterization of the I21 cBCA

2.5.1 I16 and I21 bacterial morphology

I16 (Serenade ASO, Bayer) and I21 morphologies were assessed by re-streaking fresh single colonies of each bacteria onto three different solid media: NA (Biolife), KB Agar (King et al., 1954) and LB Agar (Liofilchem). Plates were incubated at 28ºC and data was collected at 1 DPI.

2.5.2 *rpoB* and *gyrA* amplicon sequencing and phylogeny

cBCA DNA extraction was performed with a quick extraction protocol (see section 2.1). These preparations were used for fragment amplification of *rpoB* and *gyrA* gene sequences with specific primers (rpoBFw/rpoBRev and gyrAFw/gyrARev respectively; **Table 2.1**). A PCR was done for both primer sets with the same set up comprising: 3 min of denaturation at 94ºC, followed by 35 cycles of 30 sec of denaturation at 94ºC, 20 sec annealing at 50ºC and 1 min of extension at 72°C and finally an additional extension step of 5 min at 72ºC. Fragments with approximately 964 bp and 928 bp were, respectively, the expected *rpoB* and *gyrA* amplicon sizes. A control of amplification was done using the 16SFw/16SRev primer pair. DNA products were purified using a NZYGelpure kit and sent to StabVida for Sanger sequencing. Sequences were searched within NCBI using BLASTn. The first 100 accessions with ≥ 80% identity and query coverage, from *rpoB* and *gyrA* genes using *B. velezensis* QST713 sequences as template, were extracted from NCBI along with the *gyrA* and *rpoB* sequences from annotated *Bacillus* spp. strains used as plant pathogen antagonists reported by (Dunlap, 2019). These sequences were used to perform a phylogenetic analysis. Assession numbers for each *Bacillus* sp. used in the phylogenetic analysis are represented on each tree leaf label. For each gene set of sequences, a multiple alignment was done using MAFFT and the sequences were then trimmed using trimAL. Maximum likelihood trees were determined with the online tool IQTree using *E. coli* as outgroup. Bootstrap values were obtained from 1000 pseudreplicates using the default substitution model. Nucleotide diversity (π) was calculated for both *rpoB* and *gyrA* sets of sequences.

2.5.3 Biochemical characterization

1.1.1.1 Catalase activity

Qualitative catalase activity was evaluated by using a fresh LB agar I21 single colony in contact with 3% hydrogen peroxide on a sterile slide (Hansen & Stewart, 1978). Effervescence with oxygen release indicates positive catalase activity, with I16 being used as control.

1.1.1.2 Hydrogen Cyanide production

Qualitative HCN production was assessed by adapting the methodology described by (Castric, 1975). I16 and I21 were streaked onto LB agar supplemented with 4.4 g/L of glycine. After 16h of incubation at 28ºC, a filter paper soaked in a solution containing 0.5% (v/v) of picric acid and 2% (v/v) of sodium carbonate was placed inside the lid's plate and sealed with parafilm. Plates were incubated at 28ºC for 10 days. Colour change from yellow to dark orange represents HCN production, with I16 being used as control for HCN production.

1.1.1.3 Auxins production

Auxins production was evaluated using an adapted colorimetric procedure described by Gilbert et al., 2018. I16 and I21 were incubated in liquid LB at 28ºC with 180 rpm during 16 h. OD_{600} was then adjusted to 0.05 and the inoculum supplemented with L-tryptophan (5 mg/mL). 200 µL of inoculum were added onto a 96-well plate and incubated at 28ºC with 180 rpm for 24 h. Cultures were centrifuged at 3,000 rpm for 30 min and 100 µL of supernatant were pippeted into a new 96-well plate.

Salkowski's reagent (34.3% (v/v) perchloric acid and 10 mM FeCl3, (Salkowski, 1885)) was added in a proportion of 1:1 (v/v). The mixture was incubated for 30 min protected from light at room temperature and colour change was recorded with a MultiSkan microplate reader (Thermofisher). Optical density at 530 nm was recorded. An Indole-3-acetic acid (IAA) standard curve was produced using sequential dilutions (1 mg/mL; 500 µg/mL; 100 µg/mL; 50 µg/mL µg/mL; 10 µg/mL; 5 µg/mL, 1 µg/mL and 0 µg/mL) and also recorded by OD_{530}. Sterile LB supplemented with 5 mg/mL L-tryptophan was used as control and its relative IAA concentration was subtracted from the ones obtained for both bacterial isolates. Data was analysed by comparison with the IAA standard curve. I16 was used as positive control for auxins production.

1.1.1.4 Biofilm formation

Biofilm formation was assessed based on the microtiter dish assay described by O'Toole, 2011. Pre-inoculums of I16 and I21 were incubated in liquid LB at 28ºC with 180 rpm for 16 h. Bacterial concentration was assessed by OD_{600} and adjusted to 0.05. A volume of 100 µL of each inoculum was aliquoted into a 96-well plate and incubated at 28ºC with 180 rpm for 48h. The plates were turned over to remove liquid and washed with tap water. Plates were dried by turning it over into a paper towel and 100 µL of 0.1% crystal violet (w/v) was added to each well. Staining was done for 10 min at room temperature and then plates were washed by submerging on water 3-4 times. After drying for 16 h, 100 µL of 30% acetic acid were added to each well to solubilize the crystal violet. The mixture was incubated by 10 min at room temperature and OD_{550} recorded in a MultiSkan Sky spectrophotometer (Thermofisher). I16 was used as control for biofilm production.

1.1.1.5 1-Aminocyclopropane-1-carboxylic acid deaminase activity

The qualitative assessment of ACCd activity was based on the methodology described by Penrose & Glick, 2003. I16 and I21 were incubated at 28ºC, 180 rpm in liquid LB for 16h. Pre-inoculums were adjusted to an OD_{600} of 0.05 in minimal nutrient medium M9 using ACC as sole nitrogen and carbon source (22 mM KH_2PO_4, 33.7 mM Na_2HPO_4, 9.35 mM NH_4Cl, 8.55 mM NaCl, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 3 mM ACC). A volume of 100 µL of each inoculum were added into a 96-well plate and incubated for 48 h. Bacterial growth indicates ACCd activity and was recorded by OD_{600}. I16 was used as positive control. A blank control was also used and subtracted to the obtained data. Any growth is considered a positive result for ACCd production.

2.6 *Magnaporthe oryzae* inhibition assays

Magnaporthe oryzae strain PR9 was kept growing on Potato Dextrose Agar (PDA, Biolife) plates at 25ºC and 7 mm mycelial plugs were collected from growing edges of 7 days old colonies. I16 and I21 were grown from single colony in 20 mL Luria-Bertani (LB, Liofilchem) and incubated at 28ºC for 2 days at 245 rpm. After incubation OD_{600} was assessed using a MultiSkan Sky

Spectrophotometer (Thermofisher) and the culture was centrifuged at 4,000 rpm for 17 min. The solution was passed through a 0.22 µm filter (Frisenette) and 16 mL of these culture filtrates were added to 24 mL of PDA and 6.8 g/L of bacteriological agar pre-heated at 60ºC, to avoid solidification (60% (v/v) PDA, 40% (v/v) LB and 6.8 g/L of bacteriological agar (VWR)). Small petri dishes (55mm, VWR) were filled with this modified PDA media and inoculated with the 7 mm mycelium agar plugs of the 7 days old PR9. Mock control PDA modified plates containing 40% (v/v) filtrated LB medium were added to the experiment. Growth was assessed by measuring the diameter of the fungal mycelium using Image J, Fiji package (Schindelin et al., 2012) in three different timepoints: 3 DPI, 5 DPI and 7 DPI. Percentage of inhibition was calculated by subtracting the average of growth measurements obtained from media containing the *Bacillus* sp. culture filtrates to the average of growth measurements obtained from the mock control. This result was then divided by the average of mycelium growth presented by PR9 in the mock control plates and finally multiplied by 100.

2.7 Statistical analysis

All data regarding growth inhibition experiments and biochemical characterization were subjected to statistical analysis using Student's t-test in RStudio (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/). Graphical visualization was performed using ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016) and ggbeeswarm (Erik Clarke and Scott Sherrill-Mix (2017). ggbeeswarm: Categorical Scatter (Violin Point) Plots. R package version 0.6.0. https://CRAN.R-project.org/package=ggbeeswarm) R packages. Three biological and technical replicates were used to perform the growth inhibition and growth monitoring experiments, while 8 technical replicates were used to carry the biochemical characterization by evaluation of BC and PGP abilities. Data belonging to bacterial growth monitoring including means and standard deviation was calculated using Microsoft Excel 2016.

3. Results and Discussion

3.1 Erwinia amylovora isolation

To isolate *E. amylovora*, the causal agent of fire blight, infected pear samples were collected from an heavily infected orchard in Campo Maior, Portalegre region (**Figure 3.1**). *E. amylovora* is a culturable bacterium that shows different morphological characteristics depending on the growth media. To increase the probability of successfully isolating this pathogen, two nutritionally rich media were used: Nutrient Agar (NA, Biolife), and King's B (KB, King et al., 1954). Moreover, two different isolation methodologies were used: stamping infected tissue directly onto the media after exogenous sterilization to avoid isolating epiphytes (**Figure 3.2 A, C**) and macerating infected tissue in 1x PBS (**Figure 3.2 B, D**). From each plate, *E. amylovora*-like colonies were selected and re-streaked 2 to 3 times to obtain pure colonies. Once single colonies were obtained genomic DNA was extracted for species identification.

Figure 3.1 Pear trees presenting typical Fire Blight symptoms – fruit wilting and leaf wilting, exhibiting a typical "shepperd's crook" appearance along with some shoot necrosis (Campo Maior Portugal).

E. amylovora has a highly conserved genomic region named pEA71 which is often used as first identification method at molecular level by PCR. (Taylor et al., 2001). Therefore, primers that specifically amplify this DNA fragment (pEA71Fw / pEA71Rev) were used to identify *E. amylovora* strains within the colonies isolated (Taylor et al., 2001).

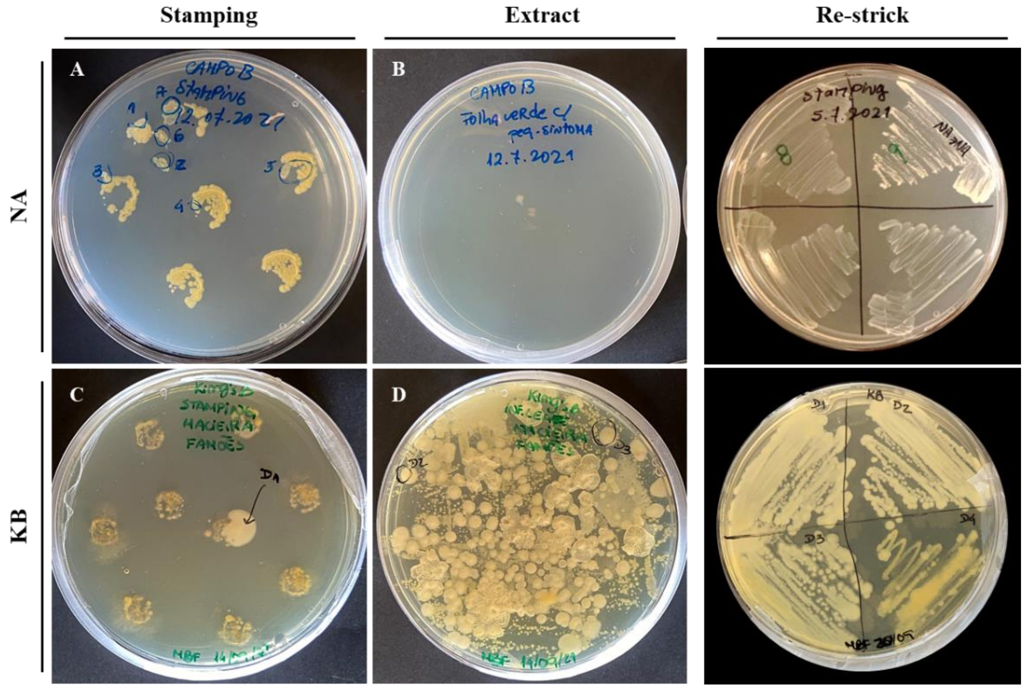


Figure 3.2 Optimization of endophytes' isolation from apple and Rocha pear orchards in Portugal, using stem stamping (A, C) and plant extracts (B, D). Two different media were tested: Nutrient Agar (NA; A, B) and King's B Agar (KB; C, D). Several re-sticks from each bacteria were done to obtain single colonies (re-streak).

E. amylovora pEA71 partial sequences with an expected size of 187 bp were amplified by PCR. As can be observed in Figure 3.3 panel A, isolates A2, A5 and A8 have a band of the expected size (**Figure 3.3A**). To confirm the identity of the isolates, 16S rRNA was partially amplified from colonies and the amplicon sequenced (**Figure 3.3B**). Isolate A8 was successfully confirmed as *E. amylovora* whilst A2 and A5 correspond to a mix of different bacteria, along with the pathogen. Considering that these colonies originally came from the same sample, A8 was chosen to

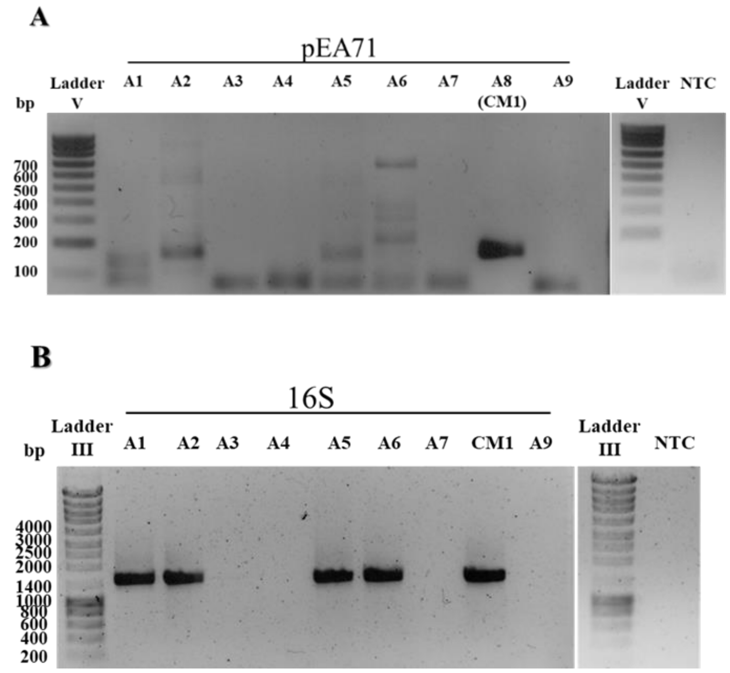


Figure 3.3 PCR amplification of 16S rRNA and pEA71 partial sequences for bacterial identification. Panel A: electrophoresis gel with pEA71 amplicons. Panel B: electrophoresis gel with 16S rRNA amplicons. Expected pEA71 amplicon size of 187 bp. A1-A9: bacterial endophytes isolated from pear trees (Campo Maior, Portugal). Non template control, NTC. Ladder III NZYDNA Ladder III, NZYtech. Ladder V NZYDNA Ladder V, NZYTech.

represent that biological sample hence there was no need to further isolate the mix of bacteria to single colony. A8 was re-named as CM1 - Campo Maior isolate 1.

3.2 Characterization of the *E. amylovora* isolate CM1

3.2.1 Pathogenicity assays on Rocha pears

To confirm that CM1 was the causal agent of the symptomatology observed in Campo Maior's pear orchards, pathogenicity assays were performed on young Rocha pears. This assay was performed using freshly collected pears in mid-summer when harvesting is done. Two types of assays were evaluated: whole pears assays (Method A; Donat et al., 2005; **Figure 3.4**) and fruit slices assays (Method B; Duffy et al., 2005, **Figure 3.5**). As observed in **Figure 3.4**, whole fruits infected with CM1 showed a localized necrosis near the site of inoculation when compared to the controls that were mock inoculated with 1x PBS. Similar results were obtained with Method B, as 8 days post inoculation (DPI), slices inoculated with CM1 showed decay symptoms not observed in the mock control. Both results confirmed that CM1 is pathogenic in young Rocha pears.

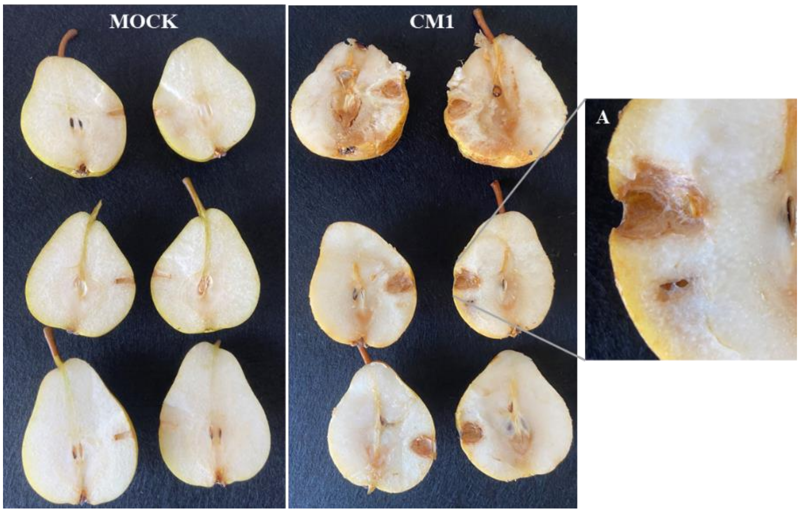


Figure 3.4 Assessing *Erwinia amylovora* CM1 isolate virulence on Rocha pears using whole fruits (method A). Images taken 14 days post inoculation. Mock corresponds to pears inoculated with 1x PBS. CM1 represents pears inoculated with CM1 isolate. (A) represents a close up of a CM1 inoculation site, showing local necrosis.

These data have also demonstrated that by using method A, the symptomatology was more evident and that the assay was easier to perform even if longer time is needed to observe the first infection symptoms (14 DPI, instead of 8 DPI in method B).

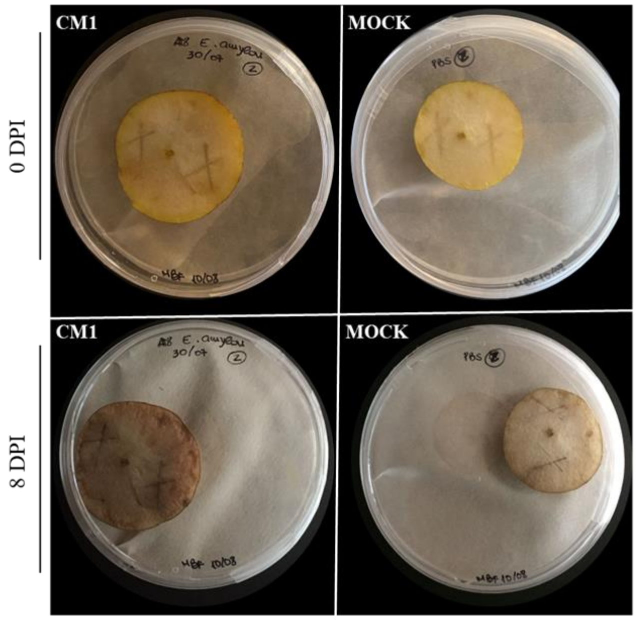


Figure 3.5 Assessing *Erwinia amylovora* CM1 isolate pathogenicity on Rocha pears using sliced fruits (method B). Comparison of the phenotypes between pears slices at 0 (0DPI) and 8 days post inoculation (8DPI). CM1corresponds to pear slice inoculated with isolate CM1. MOCK corresponds to pear slice inoculated with 1x PBS.

3.2.2 Evaluating resistance to streptomycin

Streptomycin is an amyloglycoside antibiotic used in field protection since 1955 and has been used to control fire blight, especially in the US. Over usage of streptomycin has led to the detection of resistant (Sm^R) *E. amylovora* in the US, Canada, New Zealand, Israel and Lebanon (Förster et al., 2015; Tancos et al., 2016; Tancos & Cox, 2016). Fire blight was not occurring in Lebanon until the detection of Sm^R *E. amylovora* strains which may indicate that although no streptomycin was used before in this area, the neighbouring outbreaks may affect the surroundings. Even though no Sm^R isolates have been reported in Europe, CM1 was tested for Sm^R to obtain a more detailed characterization of this isolate. *E. amylovora* strains that show streptomycin minimal inhibitory concentrations (MICs) of 2.5 mg/mL or superior are considered highly resistant (HR). Moderately resistant (MR) strains have MICs of 500-750 µg/mL (Förster et al., 2015). *E. coli* strain DH5α (Thermofisher) was used as negative control on the resistance assay because it does not detain any type of antibiotic resistance. As seen in **Figure 3.6**, no inhibition halo was observed around any of the antibiotic disks corresponding to the different streptomycin concentrations while testing CM1, in contrast to what was observed with *E. coli*. These data indicate that *E. amylovora* strain CM1 isolated from Campo Maior is highly resistant to streptomycin. There are two different known mechanisms underlying streptomycin resistance in *E. amylovora*. One is related to the ribosomal protein S12 on the 30S ribosomal subunit, encoded by the *rpsL* gene, which plays an important role on resistance to streptomycin by this bacterium. A non-silent mutation in codon 43 lead to non-binding of the antibiotic to the ribosome and protein synthesis is maintained, in contrast of what occurs on sensitive strains. This mutation in *rpsL* is reported to confer *E. amylovora* HR to streptomycin, which may be the case of isolate CM1 that did not show an inhibition halo even when in contact with concentrations of 2500 µg/ml. Considering that the usage of streptomycin is forbidden all over Europe, inclusively in Portugal, and that up until now no other *E. amylovora* strains have been reported in Europe, the most likely explanation is that this strain corresponds to a natural introduction of the bacteria from US regions. Another fact that strengthens this possibility is that recent antibiotic resistance acquisition seems to be regulated by other mechanisms, such as plasmid integration, and normally leads to MR instead of HR, which seems not be the case of CM1.

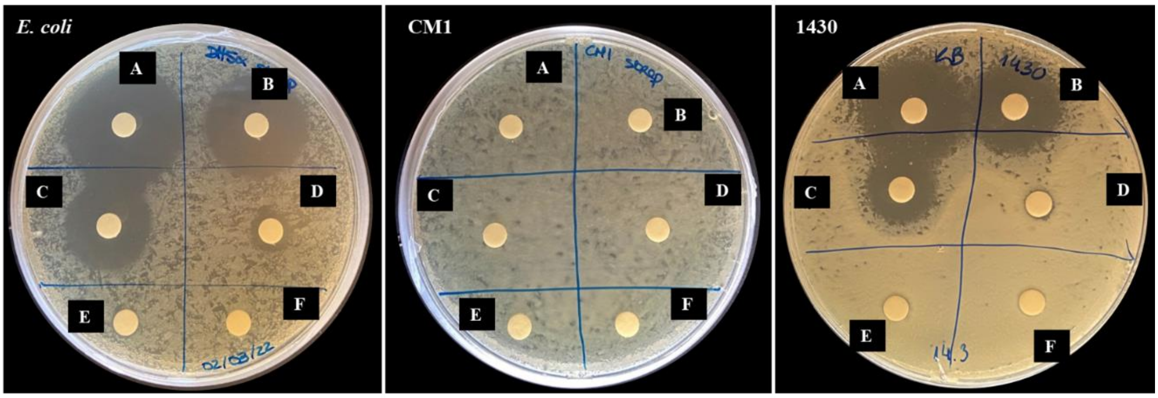


Figure 3.6 Streptomycin antibiogram using antibiotic disks of different concentrations. (A) 2.5 mg/mL; (B) 1 mg/mL; (C) 0.5 mg/mL; (D) 100 µg/mL; (E) 50 µg/mL; (F) sterile miliQ water. *E.coli*: *E. coli DH5α* used as positive bacterial control. CM1: *E. amylovora* CM1 isolate. 1430: *E. amylovora* strain CFBP1430.

3.2.3 CM1 transformation with pHC60 by electroporation

To perform a semi-high throughput fluorescently based screen to identify potentially BCAs and to optimize downstream *in planta* detection assays, *E. amylovora* was transformed with a green fluorescent protein (GFP) marker. CM1 was transformed with a broad host range plasmid that constitutively expresses GFP (pHC60), described as being very stable even in the absence of antibiotic selection (Cheng & Walker, 1998). *E. amylovora* was electroporated using the protocol described by Spinelli *et. al* and transformants were selected in KB agar supplemented with tetracycline (10 µg/ml). A transformation efficiency of 1.18×10^4 cfu/µg was obtained. Considering that many work is done using environmental microorganisms in the laboratory, including sporulating bacteria, and there is a high risk of cross contaminations, a colony PCR with 2 sets of primers was performed to confirm that the transformants were *E. amylovora* and to ensure the presence of the vector. As observed on **Figure 3.7 (Panel A)**, all three colonies tested were confirmed as *E. amylovora* (primers pEA71Fw/pEA71Rev) and contained the plasmid (M13 universal primers), presenting amplicon sizes of 187 bp and 1090bp respectively. These colonies were also observed under UV light where GFP fluorescence can be detected and confirmed that GFP was being expressed (**Figure 3.7, Panel B**). This strain will be used in a semi- high-throughput screening to identify potential BCAs against *E. amylovora* in a systematic way.

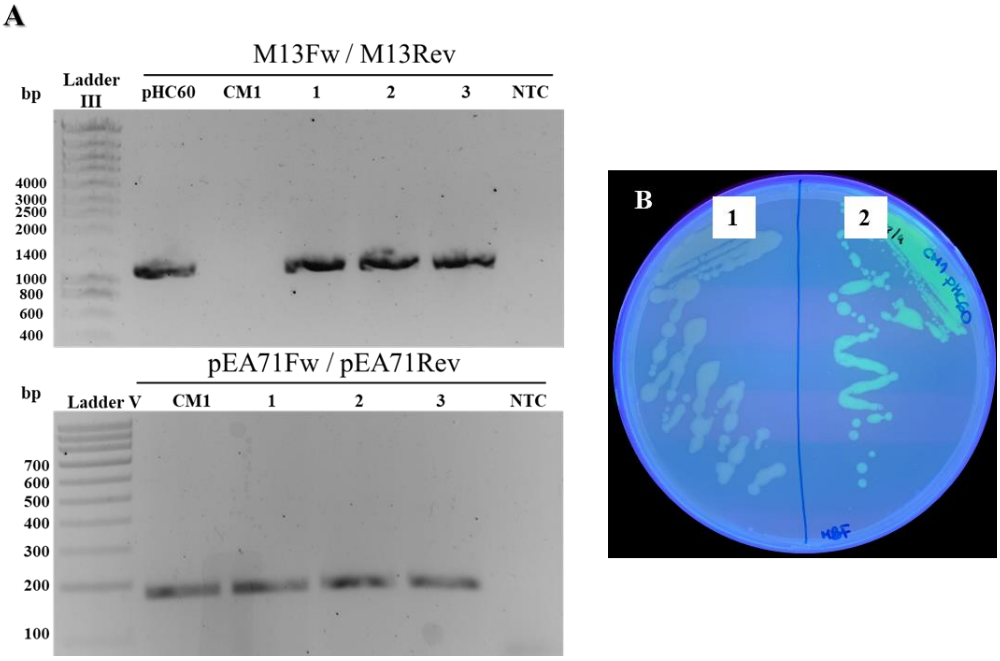


Figure 3.7 Validation of *E. amylovora* CM1 transformation with pHC60 vector. **Panel A**: PCR amplification of pEA71 and pHC60 partial sequences from 3 colonies resulting from *E. amylovora* transformation by electroporation (using pEA71Fw/pEA71Rev and M13Fw/M13Rev primers, respectively). CM1: untransformed *E. amylovora* CM1 control. pEA71 amplicon size of 187 bp. 1-3: independent *E. amylovora* colonies transformed with pHC60. pHC60: plasmid DNA used as positive control for amplification using M13 universal primers. pHC60 amplicon size of 1090 bp. NTC, represents non template control. Ladder III NZYDNA Ladder III, NZYtech. Ladder V NZYDNA Ladder V, NZYTech. **Panel B**: Confirmation of GFP expression under UV light. 1) *E. amylovora* CM1 original isolate growing on KB agar plates without any antibiotic selection, not showing any fluorescence. 2) *E. amylovora* CM1 transformant growing on KB agar plates without any antibiotic selection, presenting fluorescence

3.2.4 Evaluation of bacterial growth using liquid medium

To obtain more information regarding this isolate and evaluate if the BCA screen could be performed in liquid media on a semi-high-throughput system, the growth of CM1 was evaluated in LB. Two alternative methods were tested: 1) the bacteria were grown in a high-volume liquid media (5 mL) in 15 mL falcon tubes; and 2) the bacteria were grown in a small volume (150 µL) in 96 well-plates. The rational being that in a 96-well format the screen could be performed taking advantage of a multi-plate reader, hence speeding up data collection and minimizing the effort.

Growth profiles were investigated in LB media, by measuring the OD_{600}. In the first method (**Figure 3.8**; assay 1) the bacterial culture was grown from an OD_{600} of approximately 0.1, in 15 mL falcons (corresponding to approximately 3x aeration/volume). The assay was replicated in 96-well plates (**Figure 3.8**; assay 2) where CM1 was grown from a starting OD_{600} of approximately 0.1, in 150 µL of LB (corresponding to approximately 1x aeration/volume). Absorbance measurements were taken every 60 min for a period of 6 h in assay 1 whereas the data collection was done every 60 min for the first 4 h followed by 30 min intervals for assay 2.

An exponential growth phase can be observed after 2 h of OD_{600} monitoring in assay 1, using falcon tubes, as seen in **Figure 3.8**. This data was in accordance with the literature reports (Santander & G. Biosca, 2017). Nevertheless, when a comparison between growth profiles was performed, to understand which would be more amenable as for a screen, *g*rowth monitoring in bacteria grown in 96-well plates was not in accordance with the results previously obtained for the growth assay 1 (**Figure 3.8**). One explanation could be the fact that when using 96-well plates, the growth area and volume are much smaller, and aeration is probably deficient when compared with bacteria grown in 15mL falcon tubes. This has probably resulted in an early saturation stage and consequently lead to a premature stationary growth phase. Moreover, some of the wells showed bacterial autoaggregation, which interfere with absorbance measurements. Autoaggregation was observed in bacteria grown in 96-well plates but not when grown with a bigger aeration/volume ratio. This phenomenon has been reported as form of protection against environmental stresses by some bacteria. In this case, the nutrient and oxygen depletion due to culture saturation may led to a switch from a planktonic to an aggregated bacterial growth (Trunk et al., 2018). These results demonstrated that growing bacteria in 96-well plates may not be the best method to perform further large screens targeting the growth of isolate CM1.

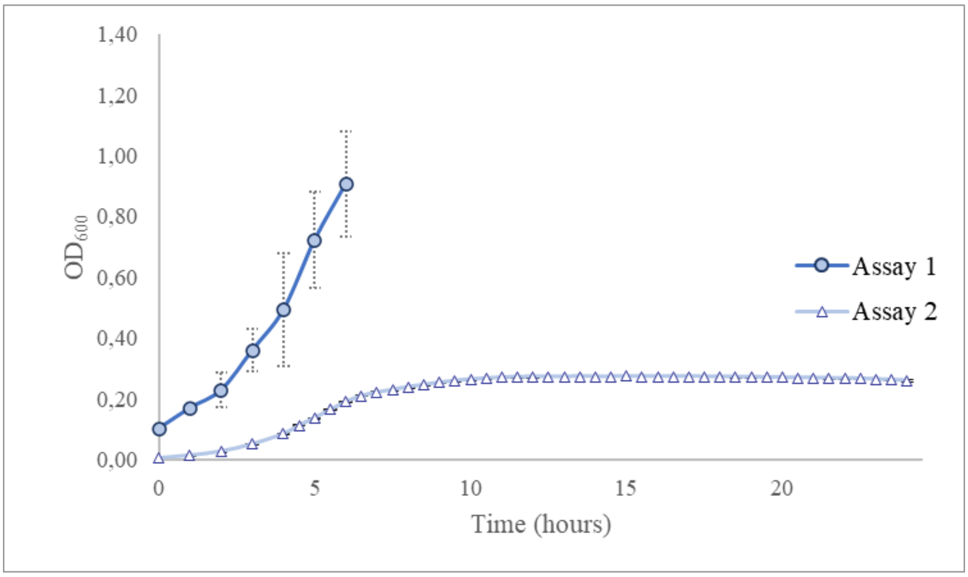


Figure 3.8 CM1 growth profiles in two different growth conditions using LB liquid media. Comparison of two different methods for bacteria growth monitoring. **Assay 1**: growth curve obtained for CM1 isolate using 15 mL of LB in falcon tubes for 6 hours. **Assay 2**: growth profile obtained for CM1 isolate growing in 150 µL of LB in 96-well plates for 24 hours.

3.3 Isolation and selection of candidate Biological Control Agents against *Erwinia amylovora*

In order to optimize a strategy to perform the screening for potential BCAs against *E. amylovora*, 18 test bacteria from InPP bacterial collection and from fire blight infected trees, including a commercialized *B. velezensis* QST713 as a positive control for pathogen's growth inhibition, were used as test. The original screen was idealized to be performed with the GFP expressing *E. amylovora* (section 3.3) however, the fluorescent multiplate reader equipment was not set-up in time and the screen had to be performed with the non-transformed bacteria. Hence, the original idea of a semi-hight throughput screen had to be changed to an alternative methodology. The test bacteria were identified by 16S rRNA amplicon Sanger sequencing (StabVida). The ID of these bacteria was determined by searching NCBI using BLASTn (Altschul et al., 1990) and OneTax (InPP pipeline, **Table 3.1**). OneTax is a pipeline used for taxonomic assignment with 3 classifiers that make use of database Silva for 16S rRNA fragments. Therefore, when ambiguous matches are found the search trusts only on the genus instead of giving potential false species identification. This crosschecking was performed to have a more reliable ID for each bacterial isolate. Moreover, some alignments were done to check if the chosen isolates were different from each other by 16S rRNA amplicon sequences comparison (**Figure 3.9**). Since some pf the bacteria showed polymorphisms within the same genus and others were morphologically different when grown on LB agar, all were tested for antagonist screening against *E. amylovora*.

3.4 Screening for candidate Biological Control Agents against *E. amylovora*

To perform an effective BCA screening the following criteria were considered: sensitivity and specificity, affordability, practicability and reproducibility. Considering the aggregation observed when growing E. amylovora in liquid media using 96-well plates (section 3.4.1), it was decided that the screen would be rather preformed in the more conventional way of dual-culture plate assays to select bacteria microorganisms as potential BCAs. This method was based on inhibition halo measurements when *E. amylovora* (CM1) was grown in the presence of the selected 17 InPP bacterial isolates (**Table 3.1**). Two different solid media were evaluated: LB agar and KB agar. Commercially available *Bacillus velezensis* QST713 (I16), with broad range activity towards different plant pathogenic bacteria and known to be effective in fire blight control (Edgecomb & Manker, 2006) was used as positive control. Several starting bacterial concentrations were evaluated as shown on **Figure 3.10**. Moreover, two different inoculation methods were tested: simultaneous inoculation (SI), in which the pathogen and the test bacteria were placed on the media at the same time, and a differential inoculation (DI), in which the test bacteria were inoculated 16h after the pathogen. The results demonstrated that *E. amylovora* growth was easier to observe when grown on KB agar and the best OD_{600} for CM1 inoculation was 0.01 (**Figure 3.10**). This media and this concentration were therefore chosen to perform the screening. The assay was performed with 100 µL of *E. amylovora* grown in LB media with an OD_{600}=0.01, spread onto KB agar and with concomitant spotting of 10 µL of each cBCA. Each test bacteria were grown in LB media with OD_{600} ranging from 0.8 to 2, with SI as the

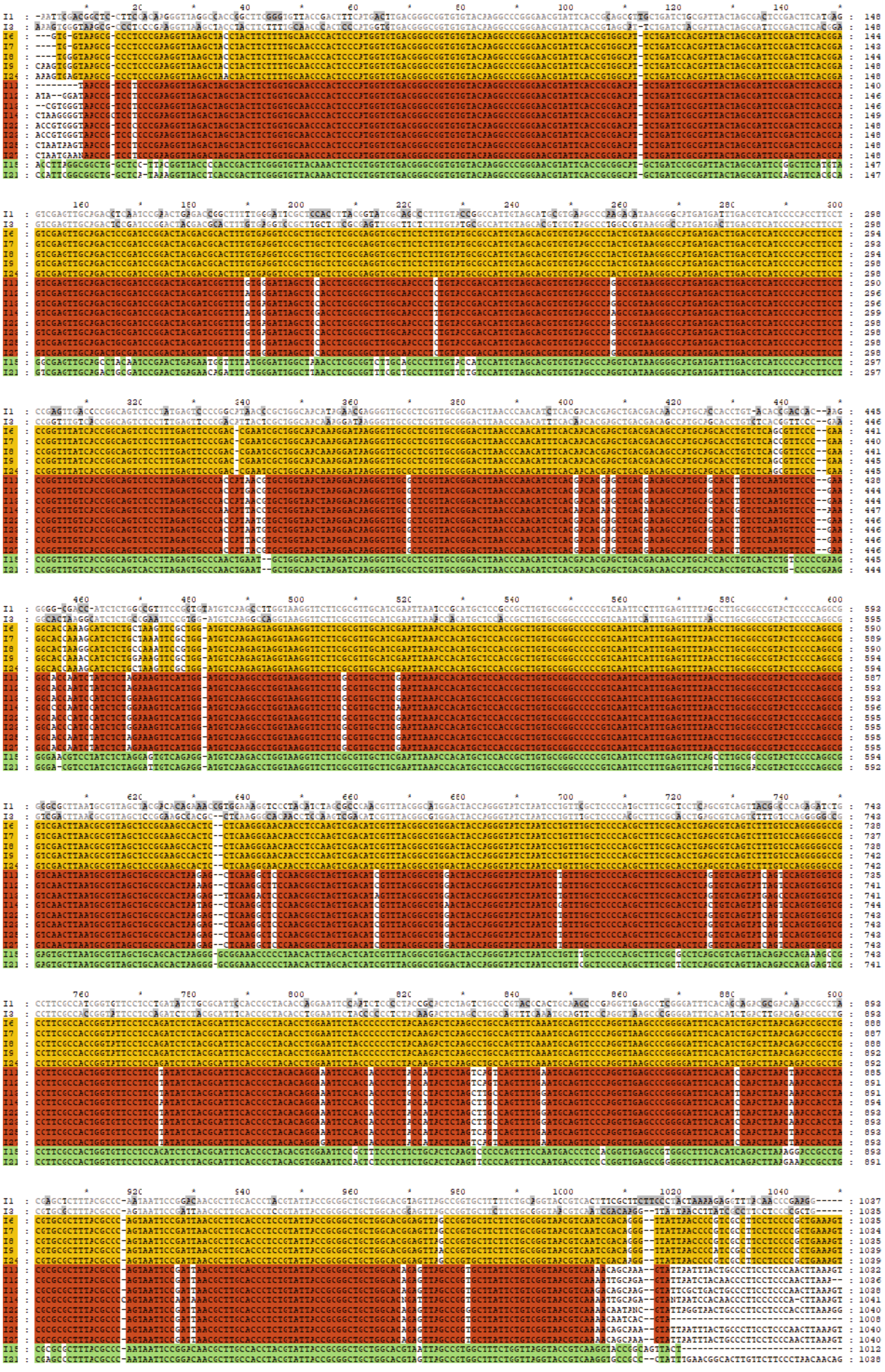


Figure 3.9 Alignment of 16S rRNA partial sequences of the candidate Biological Control Agents used for the screen set up. Bacterial isolates: in green *Bacillus* (I15, I21); in orange *Pseudomonas* (I11, I12, I13, I14, I22, I23, I25, I27), in yellow *Pantoea* (I6, I7, I8, I9, I24); I1 corresponds to a Curtobacterium and I3 is an *Erwinia*. Alignment done using Jalview version 2.11.2.0 and data treatment with GeneDoc.

Table 3.1 Identification of 17 bacterial isolates for inhibition screening of *E. amylovora* using 16S ribosomal RNA partial sequence

Isolate	Host	Geographic origin	Closest identification (NCBI)	% Identity	Accession number	Closest identification (OneTax)
I1	Almond	Évora	*Curtobacterium* sp. strain C-S-S3-9	100	MK398094.1	*Curtobacterium* sp.
I3	Almond	Évora	*Erwinia* sp. strain QL-Z3	99	MH828331.1	*Erwinia billingiae*
I6	Almond	Évora	*Pantoea agglomerans* strain UAEU18	100	CP048033.1	*Pantoea agglomerans*
I7	Almond	Évora	*Pantoea agglomerans* strain KAR13	99	KR054975.1	*Pantoea agglomerans*
I8	Olive	Elvas	*Pantoea agglomerans* strain KABNA4	99	MT605813.1	*Pantoea agglomerans*
I9	Olive	Redondo	*Pantoea agglomerans* strain FDAARGOS	99	CP077366.1	*Pantoea agglomerans*
I11	Almond	Évora	*Pseudomonas* sp. ADAK20 / *Pseudomonas fluorescens* strain Cr4	100	CP052858.1/ MH174651.1	*Pseudomonas* sp.
I12	Olive	Évora	*Pseudomonas azotoformans* strain PgBE29/*Pseudomonas fluorescens* strain FC6846	100	MH144252.1/ MH497588.1	*Pseudomonas* sp.
I13	Almond	Évora	*Pseudomonas rhizosphaerae* strain R2SsM2P1C1	99	KF147109.1	*Pseudomonas* sp.
I14	Olive	Évora	*Pseudomonas reinekei* strain PgBe208	97	MH211298.1	*Pseudomonas* sp.
I15	Pear	Campo Maior	*Bacillus* sp.	99.9	MK954118.1	*Bacillus* sp.
I21	Pear	Famões	*Bacillus* sp.	-	-	*Bacillus* sp.
I22	Pear	Campo Maior	*Pseudomonas* sp. Tigray 2	99.43	KC150861.1	*Pseudomonas* sp.
I23	Pear	Campo Maior	*Pseudomonas syringae*	100	LT629769.1	*Pseudomonas* sp.
I24	Pear	Gradil	*Pantoea agglomerans*	99.99	CP048033.1	*Pantoea agglomerans*
I25	Pear	Gradil	*Pseudomonas* sp. ADAK20	99.9	CP052858.1	*Pseudomonas* sp.
I27	Pear	Maxial	*Pseudomonas* sp.	98.25	MZ642772.1	*Pseudomonas* sp.

screening method. The data collection was also optimized for 2 days after SI, in which inhibition halo sizes stabilized and the growth contrast was more evident.

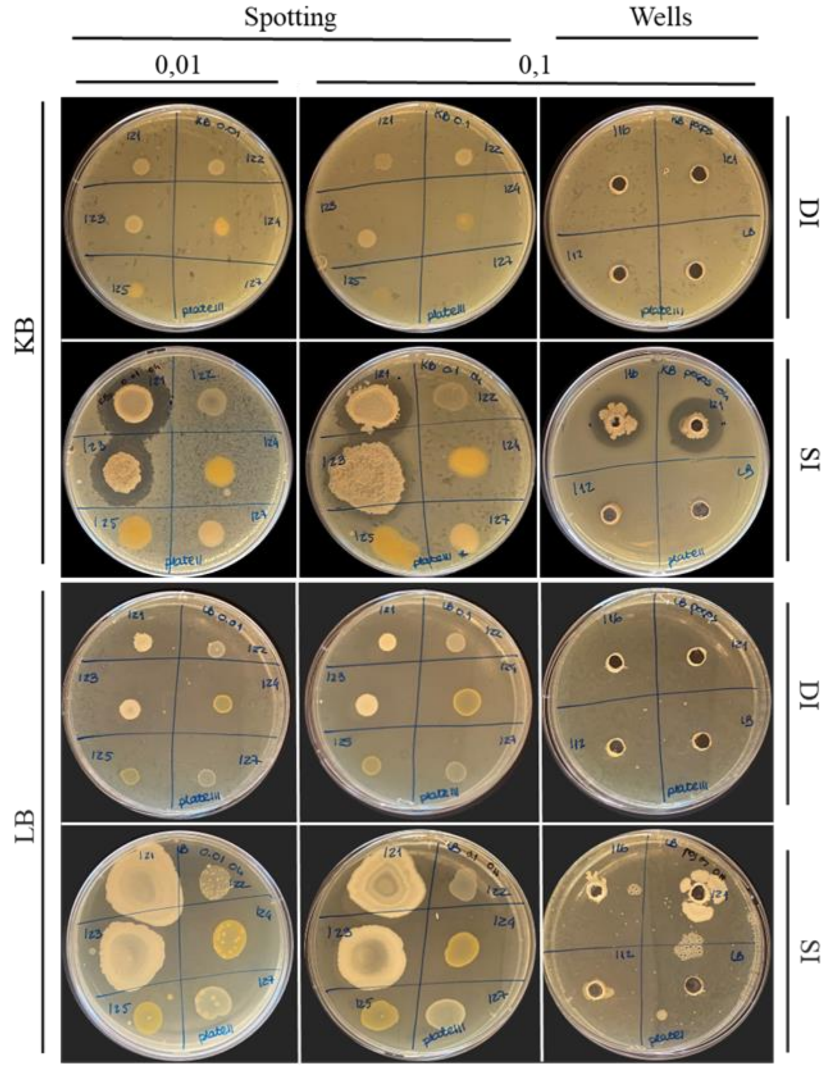


Figure 3.10 Optimization of the screening for *E. amylovora* inhibition in dual-plate assays. Scheme representing variations for the screening set up: different culture media were used (LB and KB); inoculation techniques by using wells or using a small amount of inoculum creating a bacterial spot; time of inoculation after *E. amylovora* platting varying from simultaneous inoculation (SI) to distinct inoculation (DI) done 16h after *E. amylovora* plating and testing different starting OD_{600} (0.01 and 0.1) for the pathogen.

Whilst setting-up the screen, it was observed that one isolate presented interesting inhibitory characteristics when performing the assay on KB agar media (**Figure 3.11**). Isolate 21 (I21), a *Bacillus* sp. isolated from a symptomatic Rocha pear tree from Famões (**Table 3.1**), exhibited great inhibitory capacity against *E. amylovora* as seen by the halo formed (**Figure 3.11**). Several biological and technical replicates confirmed the capability of this isolate to inhibit *E. amylovora* growth. When compared with the positive control, the commercially available *Bacillus velezensis* QST713 (isolate 16, herein called I16), I21 performed better. As observed in **Figure 3.12**, the inhibition halo around

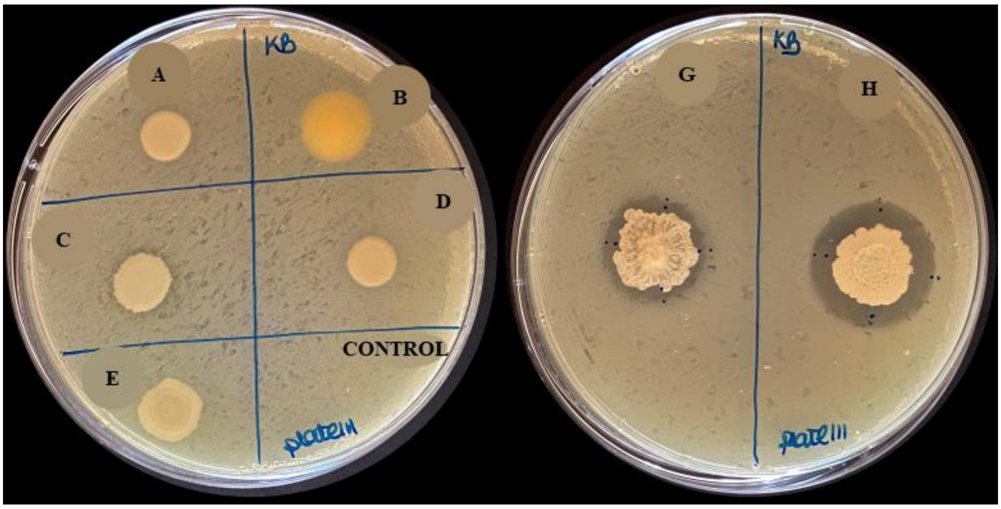


Figure 3.11 On plate screening set up for candidate BCAs (cBCAs) against *E.amylovora*. (A) *P. agglomerans* strain UAEU18*;* (B) *P. agglomerans* strain KABNA4*;* (C) *Pseudomonas* sp.*;* (D) *Pseudomonas* sp.*;* (E) *Pseudomonas* sp.*;* (G) I16 *B. velezensis* QST713*; (H)* I21 *Bacillus sp.;* (CONTROL) LB used as a negative control

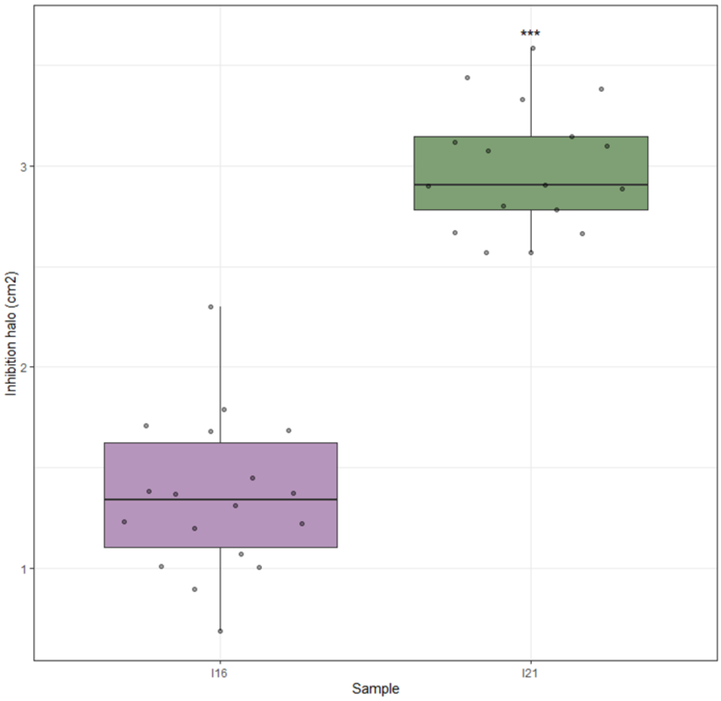


Figure 3.12 *E. amylovora* (CM1) growth inhibition by I16 and I21. Quantification was performed using FiJi image analysis. *** represent significant differences at the 5% level

I21 is bigger than that of the control. The size of the inhibition halos was quantified (**Figure 3.12**) and on average, I21 constrains approximately the double of CM1 growth when compared with the control I16 (**Figure 3.12**). This inhibition capability was also observed against the reference French strain, CFBP1430 (Smits et al., 2010). The growth inhibition of both isolates was observed when grown in KB agar media (**Figure 3.10**).

The carbon source in KB media is glycerol, whereas in LB are catabolized aminoacids instead of sugars. The use of glycerol as a carbon source by some bacteria including *Bacillus* has already been studied and some authors have highlighted its effect on bacterial metabolism (Matuszewska et al., 2021; Soare et al., 2019), generating a metabolic switch that may result on biosurfactants overproduction (Soare et al., 2019). Surfactin lipopeptides are highly produced by *Bacillus velezensis* and are known to be one of the most powerful surfactants (Barale et al., 2022). This compound has a broad spectrum of action including antiviral, antitumoral and antibiotic properties (Fernandes et al., 2007). Besides, it is also known that surfactins have antibiotic activity against multidrug-resistant microorganisms (Fernandes et al., 2007). The production of this type of active compounds will consequently enhance its antimicrobial activity, making *Bacillus* sp. good candidates to explore biological control traits (Soare et al., 2019). Therefore, a deeper characterization of I21 was performed.

3.5 I21 Characterization

3.5.1 Comparison of I16 and I21 morphology and growth profiles

Both I16 and I21 belong to the *Bacillus* genus. *Bacillus* sp. are spore forming bacteria and therefore can easily contaminate other cultures even if working under axenic conditions. To ensure that I16 and I21 were different species, a morphological analysis was performed. For that, the isolates were grown on various media and their morphology compared. As can be observed in **Figure 3.13**, the morphology of the colonies is very distinct specially when grown on KB agar medium.

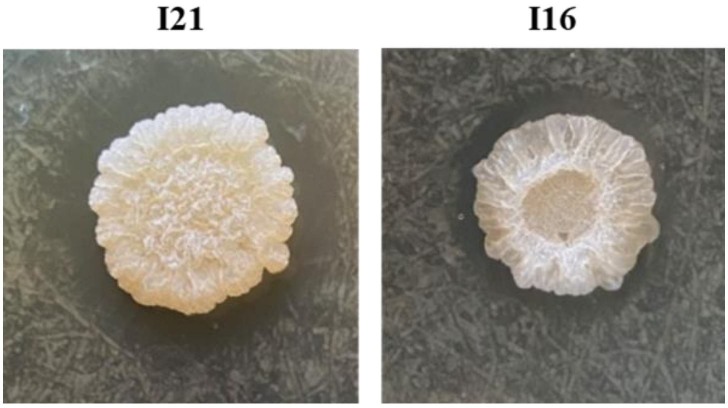


Figure 3.13 Different colony morphologies of I21 (*Bacillus* sp.), **and I16** (*Bacillus velezensis* QST713) on King's B agar plates

Next, their growth profiles on liquid LB media were compared. As can be seen in **Figure 3.14**, the behaviour of I16 and I21 is similar up until 4 h of growth ($OD_{600} \approx 0.6$). However, from this point onwards, isolate I16 it was observed that the OD_{600} starts diminishing whereas I21 keeps on increasing. Although no growth monitoring was done besides 6 h, the cultures were left growing for 2 days and both cultures end with OD_{600} close to 0. Similar results were already reported for I16 (Pandin et al., 2018, 2019) however no explanation was put forward to this phenomena. Events in which negative interactions are displayed by an organism to change the surrounding environment in an auto detrimental way resulting on its own extinction, are called ecological suicide (Ratzke et al., 2018). New insights on this phenomenon showed that the population does not even reach saturation, instead it switches immediately from a growth to a death phase (Ratzke et al., 2018) which seems be occurring with I16 at earlier stages than what happens with I21. Some degree of autoaggregation before the decline in OD_{600} was also observed for both inoculums. This quorum sensing suggests that these bacteria were trying to acquire protection from environmental damage that could be caused by nutrient or oxygen depletion (Trunk et al., 2018), secreted toxins or compounds that may have altered the pH or other primary characteristics of the culture medium. Consequently, high concentrations of these noxious substances may ultimately lead to bacterial death. Is also known that several *Bacillus* sp. secrete various proteases and lipopeptides, with antimicrobial activity, responsible for successful antagonism (Islam et al., 2019; Pandin et al., 2018; Zeriouh et al., 2014). Notwithstanding, other phenomenon can be on the origin of OD_{600} decrease. *Bacillus* sp. are spore forming bacteria, that acquire this type of conformation in response to high cell density and nutritional stresses (Allocati et al., 2014). When endospores are being formed, two distinct cells are created during cell division: a prespore and a mother cell. Upon maturation, programmed cell dead of the mother cell is required for spore release, which involves peptidoglycan hydrolases activity. Some *B. subtilis*, secrete proteins that

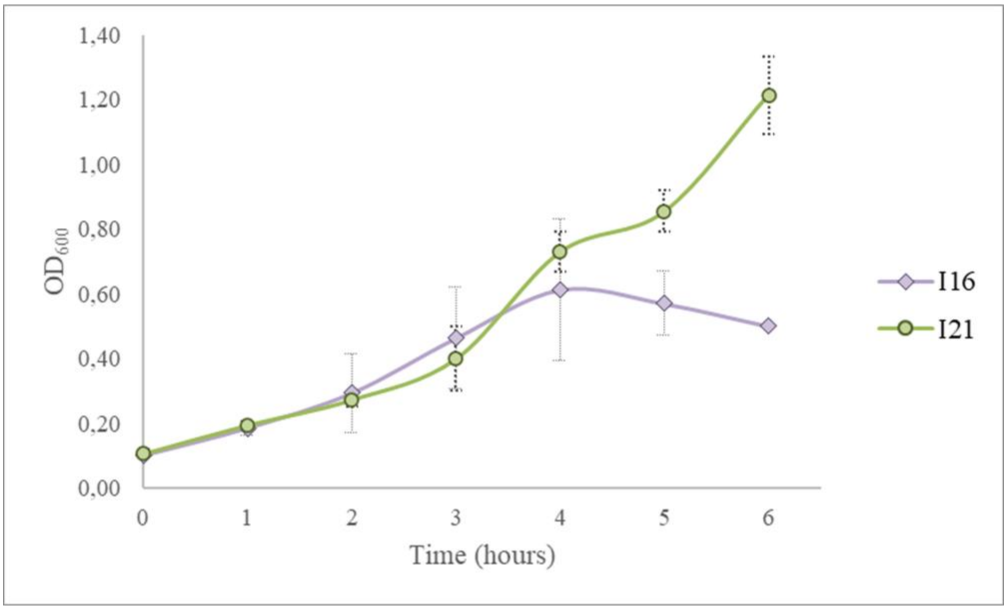


Figure 3.14 Growth profiles of I16 vs I21 in LB media

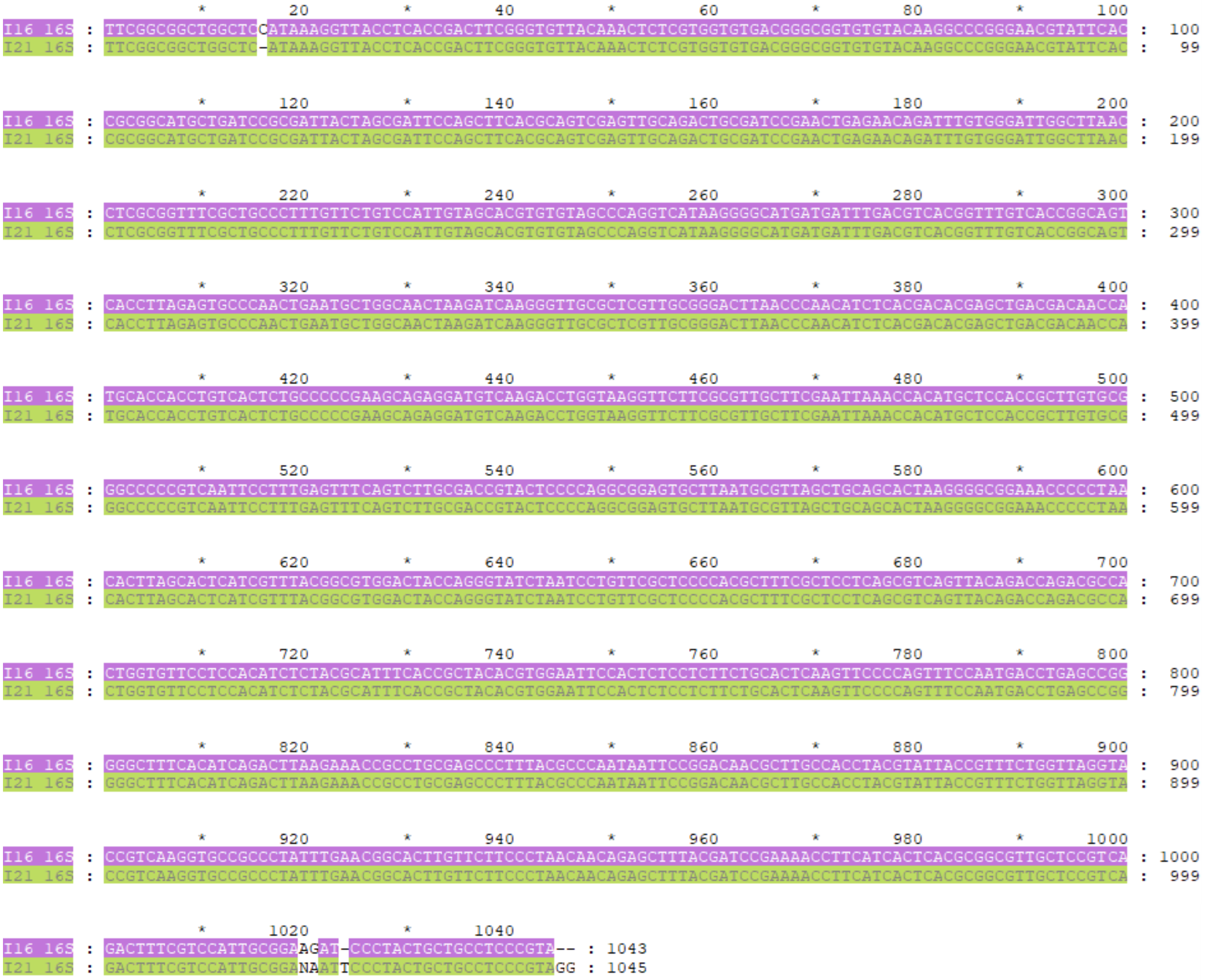
induce cell death by cannibalizing sister cells for the release of nutrients, causing a delay on sporulation. This represents an evolutive advantage when compared with other sporulating bacteria (Allocati et al., 2014). Spores are not as effective as vegetative cells in returning to active growth and when in the absence of cannibalism all bacteria sporulates at the same time, thus making the turn into active growth a slower process. It is also known that the cannibalism molecular mechanism involves several gene clusters that encode for killing factors and peptide toxins, acting also on defence against invading bacteria (Allocati et al., 2014). The *Bacillus* spp. evaluated in this study, presented a decline on OD_{600} that is much probably related with stress caused by nutrient availability or even high cell density. Additionally, it was found some evidence that spores formed by the tested bacteria may have a greater inhibition activity against fungal pathogens. However, in contrast of what was seen, when cannibalism takes place, a higher number of growing cells is maintained during the stationary growth phase (Allocati et al., 2014).

To sum up, the two isolates have different growth patterns and, although in different developmental phases, both I16 and I21 seem to experience ecological suicide.

3.5.2 Phylogenetical analysis

To further characterize I21, a phylogenetic analysis was performed. This approach complemented the 16S rRNA fragment analysis. When comparing the 16S rRNA amplicons there are hardly no differences between I16 and I21 sequences (**Figure 3.15**). The only variations observed are 4 deletions, in which one corresponds to the beginning of the sequence and the remaining are located by the end of the amplicon sequence, most likely corresponding to 5’ and 3’ sequence inaccuracy. When running a BLASTn against NCBI of 16S rRNA sequences from both isolates, several matches were found with a percentage of identity and query coverage superior to 95%, all corresponding to *B. velezensis*, *B. subtilis* or *B. amyloliquefaciens*. Since this region is highly conserved within the *Bacillus* genus, it was not possible to differentiate the two bacteria relying just on this gene information (Chun & Bae, 2000; González et al., 2012; Rooney et al., 2009).

To obtain a more precise taxonomic identification from both isolates, partial sequences of two *Bacillus* housekeeping genes gyrase A (*gyrA*) and the beta subunit of RNA polymerase (*rpoB*), often used to differentiate closely related taxa (Chun & Bae, 2000; González et al., 2012; Rooney et al., 2009) were analysed. These genes were PCR-amplified and the respective amplicons were Sanger sequenced. Sequence alignment of *rpoB* from both I16 and I21, shows that there are 4 sequence polymorphisms on nucleotide positions 16, 82, 247, and being the first one a deletion (**Figure 3.16**). The same is observed when comparing *gyrA* partial nucleotide sequences present on *Figure 3.17*, in which more than 20 polymorphic sites were detected. The *gyrA* partial gene sequence was more divergent when comparing the two *Bacillus* (González et al., 2012; Rooney et al., 2009). When a BLASTn was done at the NCBI database, no perfect matches were found for I21 *rpoB*. The closest ID showed an identity of 99.89% with 3 correspondences, all of them for *B. velezensis* but not for the QST713 strain. These results reinforce our hypothesis that I16 and I21 are different strains. The BLASTn done for I21 *gyrA* partial nucleotide sequence was inconclusive as all matches were related to different *B. velezensis* or *B. amyloliquefaciens* strains. In contrast to what was seen for I16 which



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                   *        20         *        40         *        60         *        80         *       100
I16 16S : TTCGGCGGCTGGCTCCATAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC :  100
I21 16S : TTCGGCGGCTGGCTC-ATAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC :   99

                   *       120         *       140         *       160         *       180         *       200
I16 16S : CGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAAC :  200
I21 16S : CGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAAC :  199

                   *       220         *       240         *       260         *       280         *       300
I16 16S : CTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCACGGTTTGTCACCGGCAGT :  300
I21 16S : CTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCACGGTTTGTCACCGGCAGT :  299

                   *       320         *       340         *       360         *       380         *       400
I16 16S : CACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA :  400
I21 16S : CACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA :  399

                   *       420         *       440         *       460         *       480         *       500
I16 16S : TGCACCACCTGTCACTCTGCCCCCGAAGCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCG :  500
I21 16S : TGCACCACCTGTCACTCTGCCCCCGAAGCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCG :  499

                   *       520         *       540         *       560         *       580         *       600
I16 16S : GGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAA :  600
I21 16S : GGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAA :  599

                   *       620         *       640         *       660         *       680         *       700
I16 16S : CACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGACGCCA :  700
I21 16S : CACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGACGCCA :  699

                   *       720         *       740         *       760         *       780         *       800
I16 16S : CTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCTGAGCCGG :  800
I21 16S : CTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCTGAGCCGG :  799

                   *       820         *       840         *       860         *       880         *       900
I16 16S : GGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGTTTCTGGTTAGGTA :  900
I21 16S : GGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGTTTCTGGTTAGGTA :  899

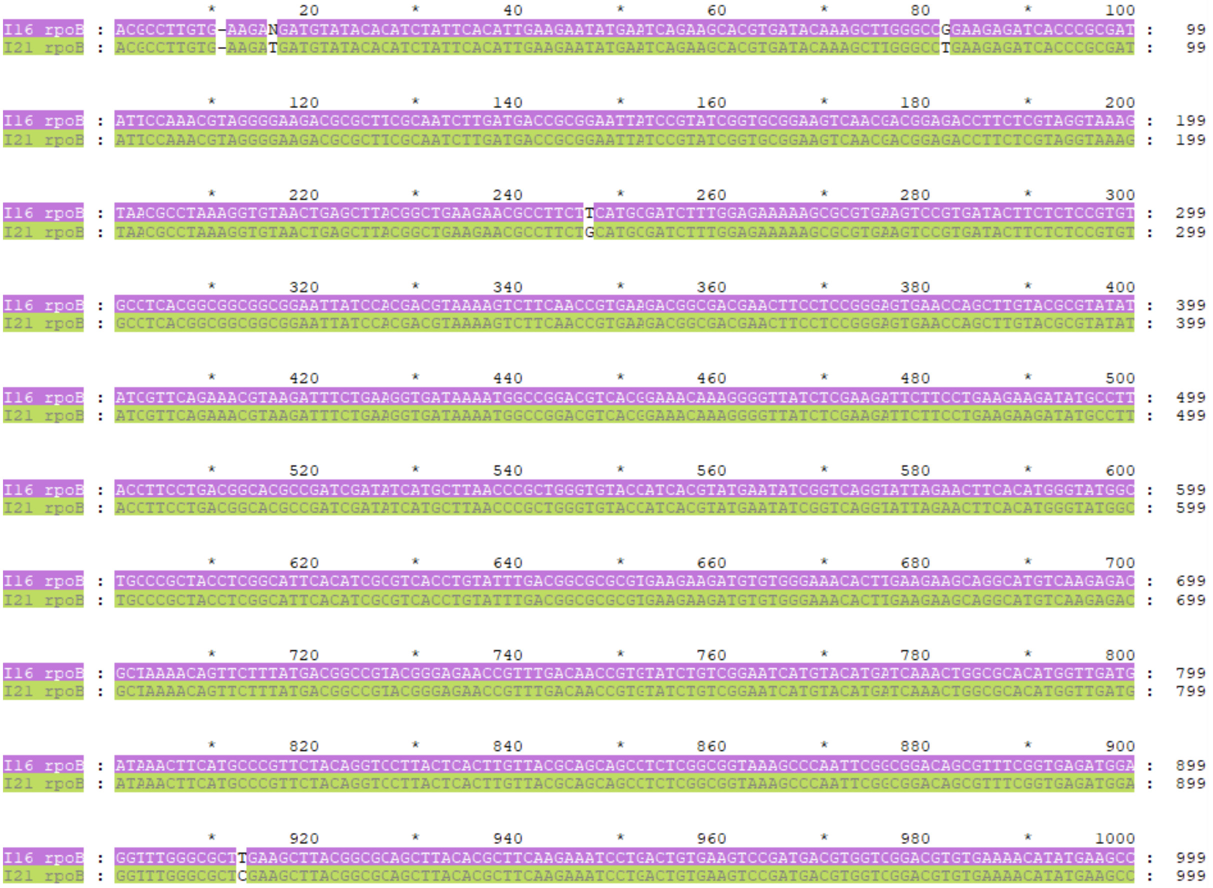
                   *       920         *       940         *       960         *       980         *      1000
I16 16S : CCGTCAAGGTGCCGCCCTATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCA : 1000
I21 16S : CCGTCAAGGTGCCGCCCTATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCA :  999

                   *      1020         *      1040
I16 16S : GACTTTCGTCCATTGCGGAAGAT-CCCTACTGCTGCCTCCCGTA-- : 1043
I21 16S : GACTTTCGTCCATTGCGGANAATTCCCTACTGCTGCCTCCCGTAGG : 1045
```

Figure 3.15 I16 (purple) and I21 (green) **16S rRNA amplicon alignment**. Polymorphisms represented by white coloured nucleotides.

got a similar match for both partial genes sequences with the accession number CP025079.1, a *Bacillus velezensis* strain QST713.

Although 16S rRNA partial sequences are identical between I16 and I21 (**Figure 3.15**), the results retrieved from the analysis of the partial sequences of two other housekeeping genes (**Figures 3.16 and 3.17**) revealed that there are genetic differences between the two isolates. Individual phylogenetic trees were performed using IQTree, with a maximum likelihood analysis of 1,000 resampled data sets, for each gene (**Figures 3.18 and 3.19**) to understand if I16 and I21 are taxonomically distant from each other. Multiple alignment between all bacteria were done using MAFFT and trimmed with trimAL. As shown in **Figure 3.18**, the two isolates seem to fit in the same clade with a bootstrap of 84 for the *rpoB* sequence, I21 appears on a distinct complex with a bootstrap of 95 while I16 presents a bootstrap of 84 for its position. Moreover, a bootstrap of 83 places I21 in a different taxonomic group then the I16 for *gyrA* (**Figure 3.19**). Nucleotide diversity (π) is the average number of pairwise nucleotide variation between sequences and was calculated for both phylogenetic trees. It depends on the number of polymorphic sites along with their frequency (Brown et al., 2004). When π > 0, the data comes from a heterogeneous sample with different species which increases the



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                    *        20         *        40         *        60         *        80         *       100
I16 rpoB : ACGCCTTGTG-AAGANGATGTATACACATCTATTCACATTGAAGAATATGAATCAGAAGCACGTGATACAAAGCTTGGGCCGGAAGAGATCACCCGCGAT :   99
I21 rpoB : ACGCCTTGTG-AAGATGATGTATACACATCTATTCACATTGAAGAATATGAATCAGAAGCACGTGATACAAAGCTTGGGCCTGAAGAGATCACCCGCGAT :   99

                    *       120         *       140         *       160         *       180         *       200
I16 rpoB : ATTCCAAACGTAGGGGAAGACGCGCTTCGCAATCTTGATGACCGCGGAATTATCCGTATCGGTGCGGAAGTCAACGACGGAGACCTTCTCGTAGGTAAAG :  199
I21 rpoB : ATTCCAAACGTAGGGGAAGACGCGCTTCGCAATCTTGATGACCGCGGAATTATCCGTATCGGTGCGGAAGTCAACGACGGAGACCTTCTCGTAGGTAAAG :  199

                    *       220         *       240         *       260         *       280         *       300
I16 rpoB : TAACGCCTAAAGGTGTAACTGAGCTTACGGCTGAAGAACGCCTTCTTCATGCGATCTTTGGAGAAAAAGCGCGTGAAGTCCGTGATACTTCTCTCCGTGT :  299
I21 rpoB : TAACGCCTAAAGGTGTAACTGAGCTTACGGCTGAAGAACGCCTTCTGCATGCGATCTTTGGAGAAAAAGCGCGTGAAGTCCGTGATACTTCTCTCCGTGT :  299

                    *       320         *       340         *       360         *       380         *       400
I16 rpoB : GCCTCACGGCGGCGGCGGAATTATCCACGACGTAAAAGTCTTCAACCGTGAAGACGGCGACGAACTTCCTCCGGGAGTGAACCAGCTTGTACGCGTATAT :  399
I21 rpoB : GCCTCACGGCGGCGGCGGAATTATCCACGACGTAAAAGTCTTCAACCGTGAAGACGGCGACGAACTTCCTCCGGGAGTGAACCAGCTTGTACGCGTATAT :  399

                    *       420         *       440         *       460         *       480         *       500
I16 rpoB : ATCGTTCAGAAACGTAAGATTTCTGAAGGTGATAAAATGGCCGGACGTCACGGAAACAAAGGGGTTATCTCGAAGATTCTTCCTGAAGAAGATATGCCTT :  499
I21 rpoB : ATCGTTCAGAAACGTAAGATTTCTGAAGGTGATAAAATGGCCGGACGTCACGGAAACAAAGGGGTTATCTCGAAGATTCTTCCTGAAGAAGATATGCCTT :  499

                    *       520         *       540         *       560         *       580         *       600
I16 rpoB : ACCTTCCTGACGGCACGCCGATCGATATCATGCTTAACCCGCTGGGTGTACCATCACGTATGAATATCGGTCAGGTATTAGAACTTCACATGGGTATGGC :  599
I21 rpoB : ACCTTCCTGACGGCACGCCGATCGATATCATGCTTAACCCGCTGGGTGTACCATCACGTATGAATATCGGTCAGGTATTAGAACTTCACATGGGTATGGC :  599

                    *       620         *       640         *       660         *       680         *       700
I16 rpoB : TGCCCGCTACCTCGGCATTCACATCGCGTCACCTGTATTTGACGGCGCGCGTGAAGAAGATGTGTGGGAAACACTTGAAGAAGCAGGCATGTCAAGAGAC :  699
I21 rpoB : TGCCCGCTACCTCGGCATTCACATCGCGTCACCTGTATTTGACGGCGCGCGTGAAGAAGATGTGTGGGAAACACTTGAAGAAGCAGGCATGTCAAGAGAC :  699

                    *       720         *       740         *       760         *       780         *       800
I16 rpoB : GCTAAAACAGTTCTTTATGACGGCCGTACGGGAGAACCGTTTGACAACCGTGTATCTGTCGGAATCATGTACATGATCAAACTGGCGCACATGGTTGATG :  799
I21 rpoB : GCTAAAACAGTTCTTTATGACGGCCGTACGGGAGAACCGTTTGACAACCGTGTATCTGTCGGAATCATGTACATGATCAAACTGGCGCACATGGTTGATG :  799

                    *       820         *       840         *       860         *       880         *       900
I16 rpoB : ATAAACTTCATGCCCGTTCTACAGGTCCTTACTCACTTGTTACGCAGCAGCCTCTCGGCGGTAAAGCCCAATTCGGCGGACAGCGTTTCGGTGAGATGGA :  899
I21 rpoB : ATAAACTTCATGCCCGTTCTACAGGTCCTTACTCACTTGTTACGCAGCAGCCTCTCGGCGGTAAAGCCCAATTCGGCGGACAGCGTTTCGGTGAGATGGA :  899

                    *       920         *       940         *       960         *       980         *      1000
I16 rpoB : GGTTTGGGCGCTTGAAGCTTACGGCGCAGCTTACACGCTTCAAGAAATCCTGACTGTGAAGTCCGATGACGTGGTCGGACGTGTGAAAACATATGAAGCC :  999
I21 rpoB : GGTTTGGGCGCTCGAAGCTTACGGCGCAGCTTACACGCTTCAAGAAATCCTGACTGTGAAGTCCGATGACGTGGTCGGACGTGTGAAAACATATGAAGCC :  999
```
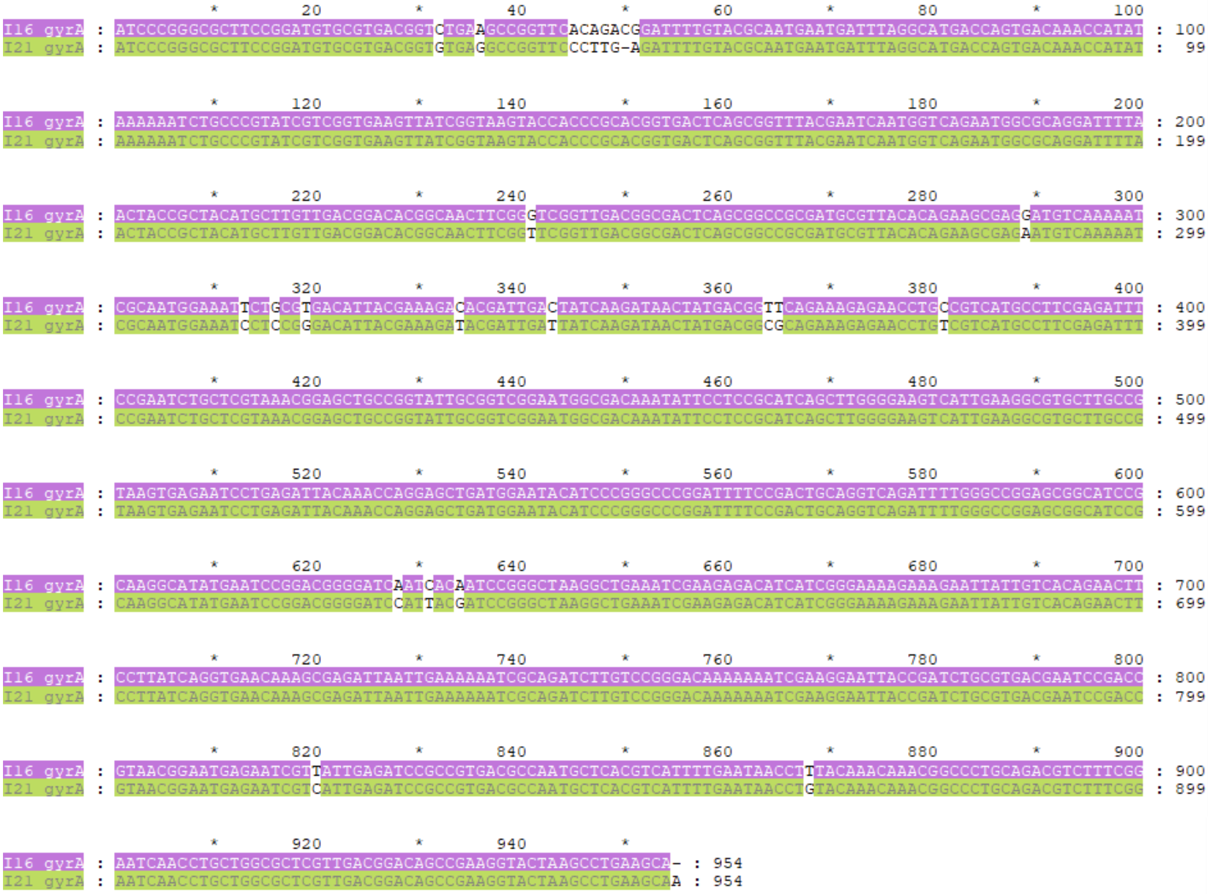

Figure 3.16 I16 (purple) and I21 (green) ***rpoB* amplicon alignment**. Polymorphisms represented by white coloured nucleotides.

phylogenetic tree viability and accuracy. For both *rpoB* and *gyrA* based phylogenetic trees, π values of 0.192 and 0.196 were respectively obtained, confirming the accuracy of the results.

The acquired data proved that although belonging to the same larger clade in the *Bacillus* sp. complex, these two bacteria are phylogenetically distinct. This analysis enabled a more detailed identification of I21, with high homology to *Bacillus velezensis* strain ZeaDK315Endobac16 for these two genes, with the GenBank accession CP043809.1. This identification will be further confirmed by whole genome sequencing.



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                    *        20         *        40         *        60         *        80         *       100
I16 gyrA : ATCCCGGGCGCTTCCGGATGTGCGTGACGGTCTGAAGCCGGTTCACAGACGGATTTTGTACGCAATGAATGATTTAGGCATGACCAGTGACAAACCATAT : 100
I21 gyrA : ATCCCGGGCGCTTCCGGATGTGCGTGACGGTGTGAGGCCGGTTCCCTTG-AGATTTTGTACGCAATGAATGATTTAGGCATGACCAGTGACAAACCATAT :  99

                    *       120         *       140         *       160         *       180         *       200
I16 gyrA : AAAAAATCTGCCCGTATCGTCGGTGAAGTTATCGGTAAGTACCACCCGCACGGTGACTCAGCGGTTTACGAATCAATGGTCAGAATGGCGCAGGATTTTA : 200
I21 gyrA : AAAAAATCTGCCCGTATCGTCGGTGAAGTTATCGGTAAGTACCACCCGCACGGTGACTCAGCGGTTTACGAATCAATGGTCAGAATGGCGCAGGATTTTA : 199

                    *       220         *       240         *       260         *       280         *       300
I16 gyrA : ACTACCGCTACATGCTTGTTGACGGACACGGCAACTTCGGGTCGGTTGACGGCGACTCAGCGGCCGCGATGCGTTACACAGAAGCGAGGATGTCAAAAAT : 300
I21 gyrA : ACTACCGCTACATGCTTGTTGACGGACACGGCAACTTCGGTTCGGTTGACGGCGACTCAGCGGCCGCGATGCGTTACACAGAAGCGAGAATGTCAAAAAT : 299

                    *       320         *       340         *       360         *       380         *       400
I16 gyrA : CGCAATGGAAATTCTGCGTGACATTACGAAAGACACGATTGACTATCAAGATAACTATGACGGTTCAGAAAGAGAACCTGCCGTCATGCCTTCGAGATTT : 400
I21 gyrA : CGCAATGGAAATCCTCCGGGACATTACGAAAGATACGATTGATTATCAAGATAACTATGACGGCGCAGAAAGAGAACCTGTCGTCATGCCTTCGAGATTT : 399

                    *       420         *       440         *       460         *       480         *       500
I16 gyrA : CCGAATCTGCTCGTAAACGGAGCTGCCGGTATTGCGGTCGGAATGGCGACAAATATTCCTCCGCATCAGCTTGGGGAAGTCATTGAAGGCGTGCTTGCCG : 500
I21 gyrA : CCGAATCTGCTCGTAAACGGAGCTGCCGGTATTGCGGTCGGAATGGCGACAAATATTCCTCCGCATCAGCTTGGGGAAGTCATTGAAGGCGTGCTTGCCG : 499

                    *       520         *       540         *       560         *       580         *       600
I16 gyrA : TAAGTGAGAATCCTGAGATTACAAACCAGGAGCTGATGGAATACATCCCGGGCCCGGATTTTCCGACTGCAGGTCAGATTTTGGGCCGGAGCGGCATCCG : 600
I21 gyrA : TAAGTGAGAATCCTGAGATTACAAACCAGGAGCTGATGGAATACATCCCGGGCCCGGATTTTCCGACTGCAGGTCAGATTTTGGGCCGGAGCGGCATCCG : 599

                    *       620         *       640         *       660         *       680         *       700
I16 gyrA : CAAGGCATATGAATCCGGACGGGGATCAATCACAATCCGGGCTAAGGCTGAAATCGAAGAGACATCATCGGGAAAAGAAAGAATTATTGTCACAGAACTT : 700
I21 gyrA : CAAGGCATATGAATCCGGACGGGGATCCATTACGATCCGGGCTAAGGCTGAAATCGAAGAGACATCATCGGGAAAAGAAAGAATTATTGTCACAGAACTT : 699

                    *       720         *       740         *       760         *       780         *       800
I16 gyrA : CCTTATCAGGTGAACAAAGCGAGATTAATTGAAAAAATCGCAGATCTTGTCCGGGACAAAAAAATCGAAGGAATTACCGATCTGCGTGACGAATCCGACC : 800
I21 gyrA : CCTTATCAGGTGAACAAAGCGAGATTAATTGAAAAAATCGCAGATCTTGTCCGGGACAAAAAAATCGAAGGAATTACCGATCTGCGTGACGAATCCGACC : 799

                    *       820         *       840         *       860         *       880         *       900
I16 gyrA : GTAACGGAATGAGAATCGTTATTGAGATCCGCCGTGACGCCAATGCTCACGTCATTTTGAATAACCTTTACAAACAAACGGCCCTGCAGACGTCTTTCGG : 900
I21 gyrA : GTAACGGAATGAGAATCGTCATTGAGATCCGCCGTGACGCCAATGCTCACGTCATTTTGAATAACCTGTACAAACAAACGGCCCTGCAGACGTCTTTCGG : 899

                    *       920         *       940         *
I16 gyrA : AATCAACCTGCTGGCGCTCGTTGACGGACAGCCGAAGGTACTAAGCCTGAAGCA- : 954
I21 gyrA : AATCAACCTGCTGGCGCTCGTTGACGGACAGCCGAAGGTACTAAGCCTGAAGCAA : 954
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Figure 3.17 I16 (purple) and I21 (green) ***gyrA* amplicon alignment**. Polymorphisms represented by white coloured nucleotides

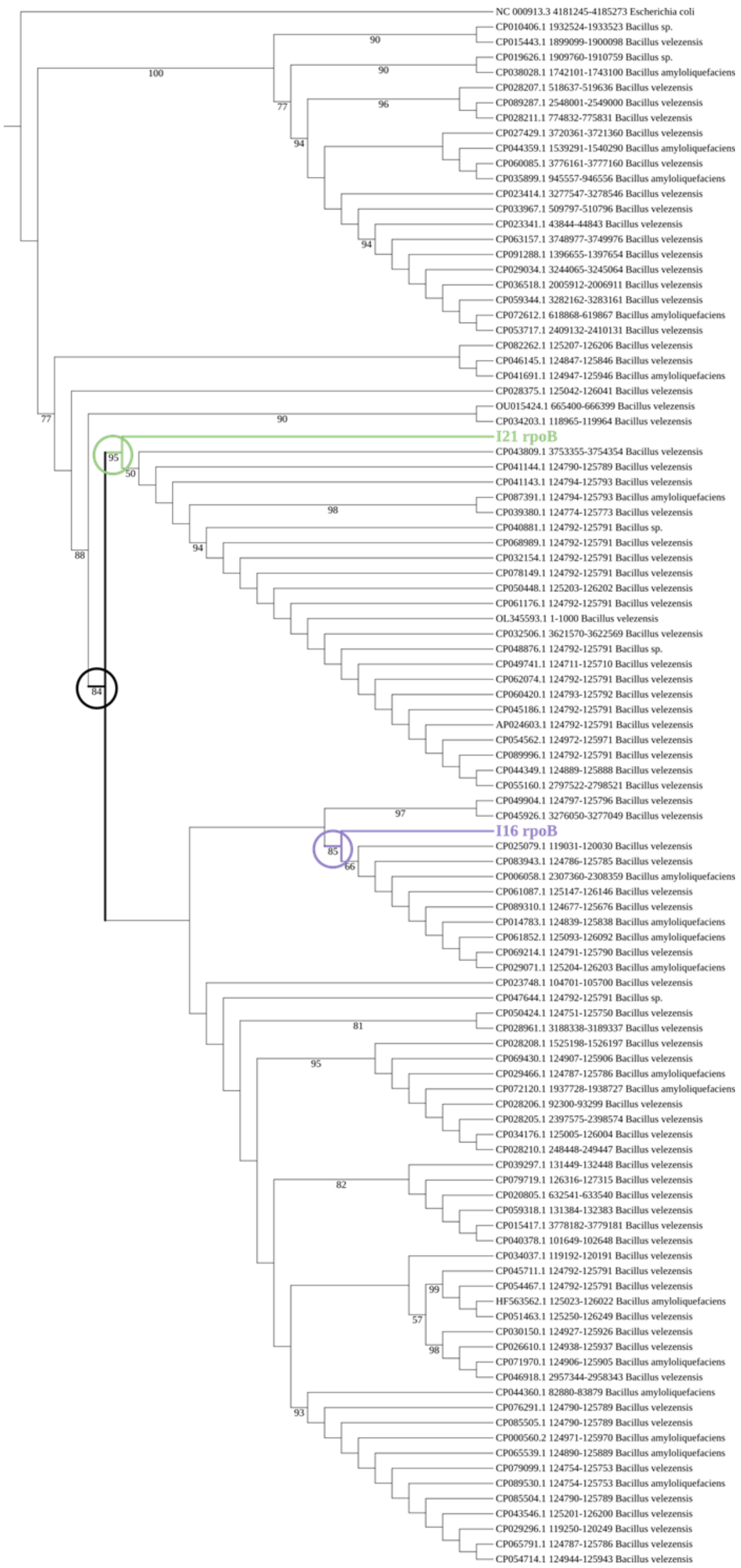

Figure 3.18 Maximum likelihood phylogenetic tree constructed from *rpoB* gene amplicons. Bootstrap values > 50%, based on 1000 pseudoreplicates are indicated on branch points using the substitution model TNe+R3. *Escherichia coli* used as an outgroup. Nucleotide diversity (π) =0,192. Assession numbers for each *Bacillus* sp. are represented on each tree leaf label. In green I21 and in violet I16 *rpoB* sequences

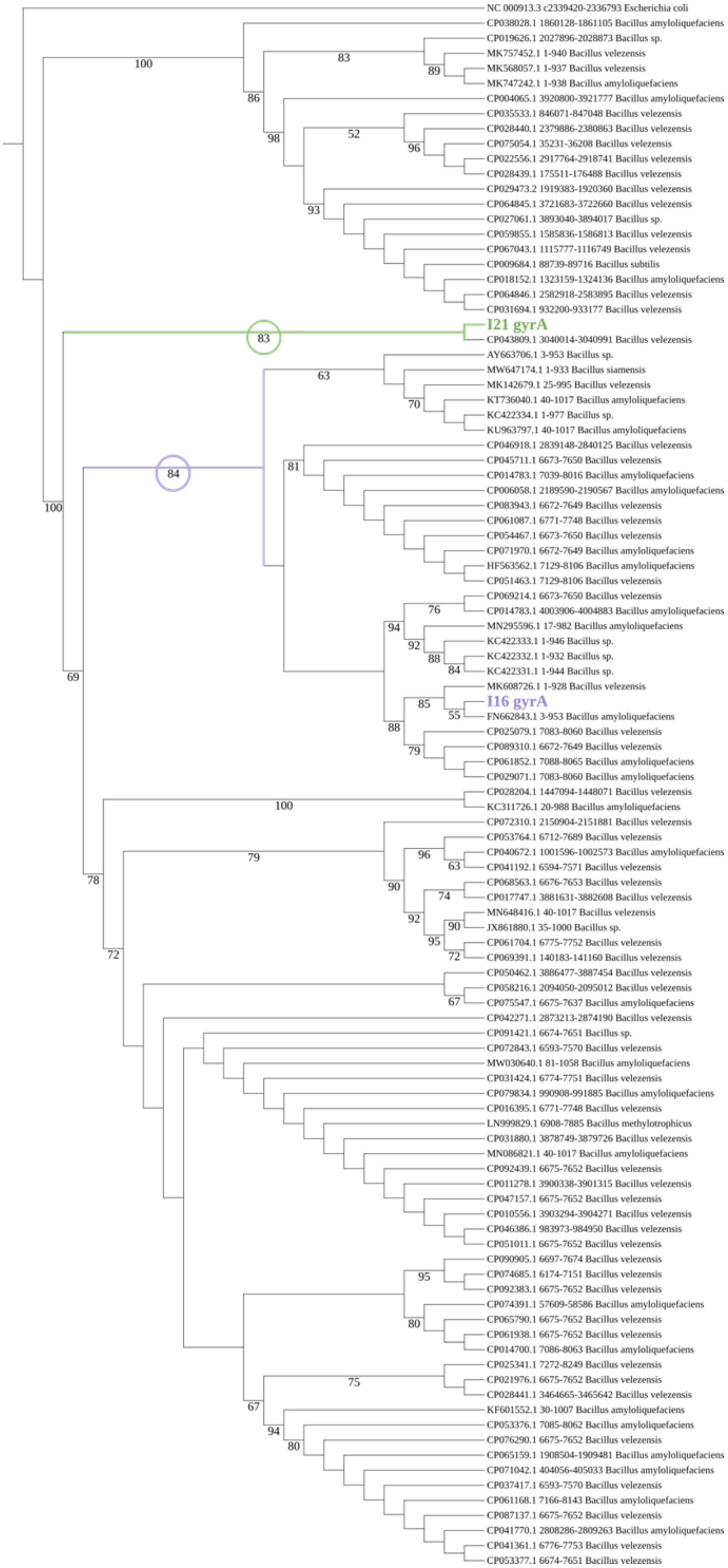

Figure 3.19 Maximum likelihood phylogenetic tree constructed from *gyrA* gene amplicons. Bootstrap values > 50%, based on 1000 pseudoreplicates are indicated on branch points using the substitution model TNe+R3. *Escherichia coli* used as an outgroup. Nucleotide diversity (π) =0,196. Assession numbers for each *Bacillus* sp. are represented on each tree leaf label. In green I21 and in violet I16 *gyrA* sequences

3.6 Characterization of I21 Biological Control and Plant Growth Promoter properties

To complement the characterization of I21 and to understand if these bacteria could have biological control (BC) and plant growth promoter (PGP) capacities, a battery of biochemical tests were performed: catalase activity; hydrogen cyanide (HCN) production; auxins production; biofilm formation; 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd) activity and phosphorous solubilization capacity. For all experiments, I16 was used as a positive control. One of the purposes of doing this type of assays was to differentiate the two *Bacillus* and to complement I21 characterization with biochemical traits. For that reason and because these were just preliminary results that need to be repeated with more biological replicates, all data were interpreted just qualitatively even though we could've obtained quantitative data in some of the experiments.

Bacteria that produce catalase have more chances to survive in environments such as the soil ecosystem, because of the capacity to protect themselves against oxidative stress. This enzyme acts like an antioxidant since it uses non-radical ROS, like hydrogen peroxide, as substrate and converts it into water and oxygen, neutralizing the harmful effect of this noxious compound (Nandi et al., 2019). Therefore, by using this mechanism, PGPs will eventually survive near roots and promote plant growth indirectly by protecting the plant against this harmful substance (Bumunang & Babalola, 2014; Joseph et al., 2007). As seen in **Figure 3.20**, both bacteria were positive for catalase activity. Moreover, I21 showed a slightly different activity since it produced bigger bubbles when compared to I16 performance. Although having different behaviours, this assay does not give enough information regarding the mechanism regulating this reaction and thus, just a qualitative analysis should be considered.

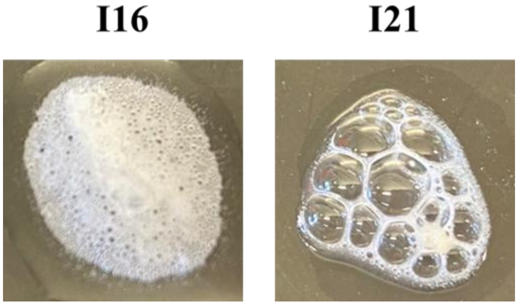

Figure 3.20 Assessing catalase activity for I16 and I21. I16: isolate I16. I21: isolate I21

Hydrogen synthase is an enzyme that catalyses the HCN production using glycine as substrate.(Laville et al., 1998; Lorck, 1948). The production of HCN by rhizobacteria has been described as playing an important role on pathogen's biological control, inducing plant resistance. Beyond its BC action based on toxicity against plant pathogens, HCN is also known to contribute for metal sequestration in soil, facilitating nutrient availability and thus enhance plant growth, indirectly (Gupta & Pandey, 2019, 2019; Kumar et al., 2012; Rijavec & Lapanje, 2016). Thereby, bacteria capable of producing this compound can improve plant growth and protection. **Figure 3.21** shows the ability of both I16 and I21 of producing HCN as seen by a colour change from yellow to a brownish orange tone in the filter paper. No major differences were detected between both isolates.

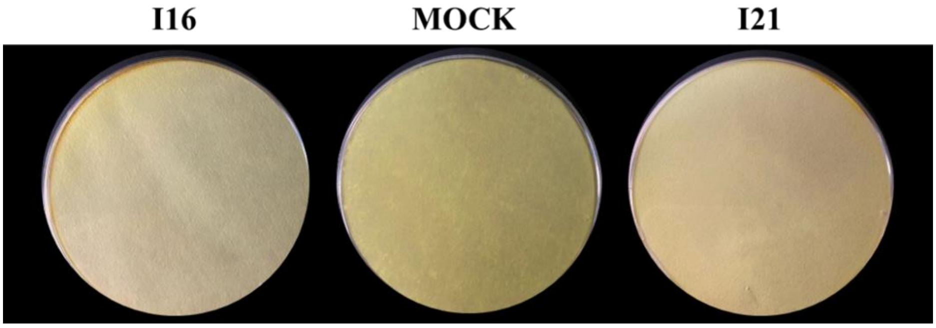

Figure 3.21 Assessing HCN production in I16 and I21. I16: isolate I16. Mock: mock control. I21: isolate I21

Auxins are a group of exhaustively studied phytohormones with growth-inducing activity. These molecules are implicated in a variety of biological systems including signalling and growth regulation. Indole-3-acetic acid (IAA) is a type of auxin frequently found in nature and its production in very common amongst rhizosphere bacteria. Even when in low concentrations, IAA can regulate gene expression promoting root elongation and enhancing plant growth (Gilbert et al., 2018; Gomes & Scortecci, 2021; Gupta & Pandey, 2019). Auxins may also be used as signal molecules between bacteria and plants (P. Liu & Nester, 2006; Spaepen et al., 2007). Hence, under environmental stresses plants will benefit when in contact with auxin producing bacteria. The production of IAA was assessed for each of the isolate using a colorimetric assay. To enhance IAA biosynthesis, the culture medium was supplemented with its precursor amino acid, tryptophan, often found on root exudates. A relative quantification was done against the standard IAA absorbance curve at 530nm. This methodology does not provide information regarding the types of auxins being produced. Results have shown that both I16 and I21 produce auxins, however there was a higher internal variation in I21 (**Figure 3.22**) due to aggregation and this assay needs to be further repeated to validate the results. By visual observation on the plates, both isolates seem to produce similar amounts of auxins.

Biofilms consist of microorganism communities surrounded by a self-produced exopolymer matrix that attach to a surface or to each other. Some of the benefits of bacterial biofilms include protection from abiotic and biotic stresses, increased horizontal gene transfer and antibiotic tolerance (Ahmad & Husain, 2017; Haque et al., 2020; Pandin et al., 2018; Vlamakis et al., 2013). Moreover, it is known that biofilm producing bacteria have a competitive advantage in root colonization and can overproduce secondary metabolites. Thus, PGPs that can form biofilms will easily endure environmental oscillations and consequently will have a fitness advantage (Ahmad & Husain, 2017). *B. velezensis* QST713 (I16) is known to prefer this type of growth under stress conditions. The data obtained for biofilm formation was clearly positive for I16. The parameters used to quantify the biofilm formation were not sensitive enough for the produced quantity by I16 (Pandin et al., 2018; Vlamakis et al., 2013). Because this isolate can form robust and very dense biofilms and the crystal violet in solution was in such high concentrations, the OD_{550} measurements accuracy was compromised. Consequently, the data obtained can just be interpreted qualitatively. **Figure 3.23** shows that both bacteria produced biofilm, although there's evidence of differences regarding the quantity of biofilm produced.

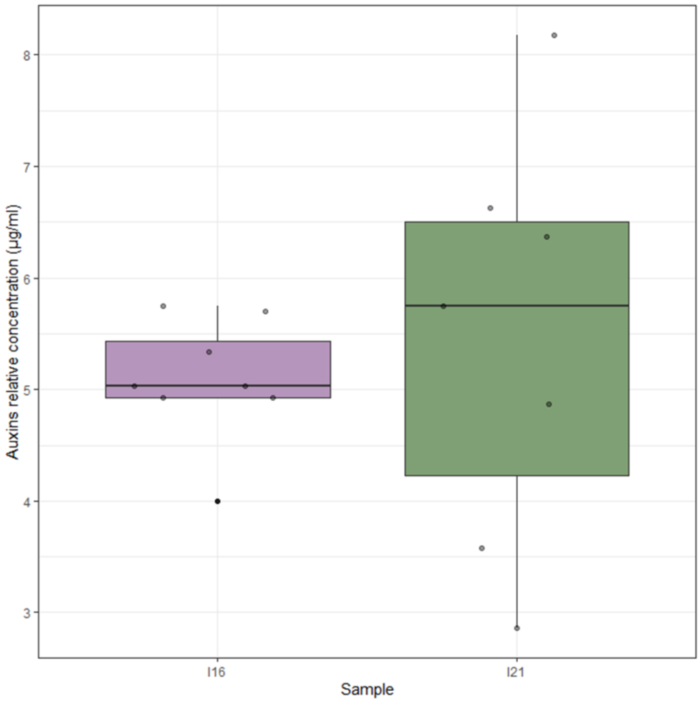

Figure 3.22 Assessing auxins production in I16 and I21. Quantification was performed using FiJi image analysis. I16 represented in violet and I21 in green.

Some PGPs have the ability to hydrolyse aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene biosynthesis. ACC deaminase (ACCd) is the enzyme that catalyses the aforementioned reaction. When in high concentrations, ethylene may become detrimental to shoot and root development affecting plant growth. ACCd reduces the levels of "stress ethylene" present in plants and consequently leads to an increased crop productivity. PGPs with ACCd activity are beneficial to plants in several ways including resistance improvement against some pathogens (Haque et al., 2020; Ojuederie et al., 2019). It has also been described that this enzyme may impact nutrient availability on soils (Gupta & Pandey, 2019). Its presence is assessed by bacterial growth on minimal medium, in which only bacteria that can use ACC as sole nitrogen and carbon souce will grow. **Figure 3.24** represents the bacterial growth of the two isolates monitored by OD_{600} measuring after an overnight incubation at 28ºC. The data shows that both bacteria could grow using ACC as substrate and thus revealing that I16 and I21 most probably possess ACCd activity. Nevertheless, a less efficient growth was exhibited by I21 when compared to I16.

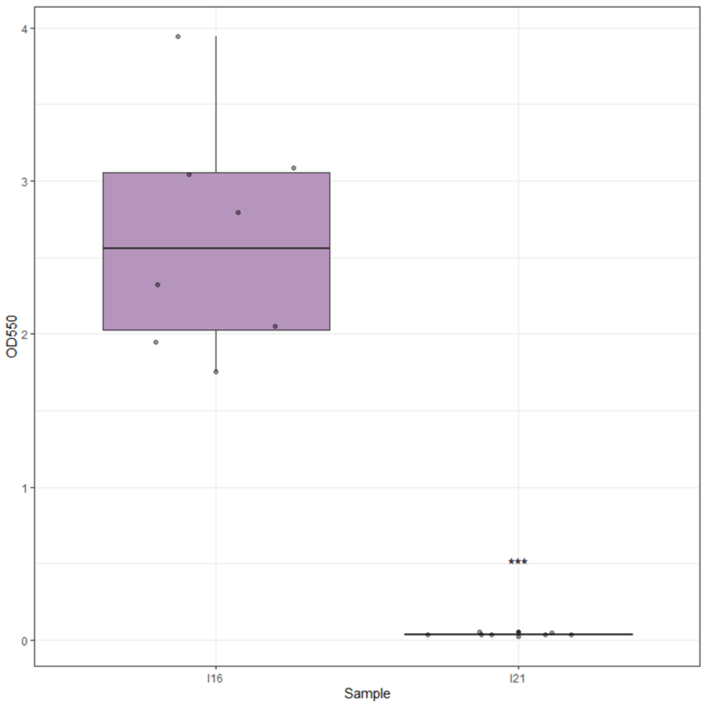

Figure 3.23 Assessing biofilm formation in I16 and I21. Quantification was performed using FiJi image analysis. I16 represented in violet and I21 in green. *** represent significant differences at the 5% level.

All data obtained still need validation through multiple repetitions and biological replicates, but these preliminary results seem to indicate that I21 has both BCA and PGP properties (Chauhan et al., 2017; Gupta & Pandey, 2019; Haque et al., 2020; Ojuederie et al., 2019; Pandin et al., 2018, 2019; Zeriouh et al., 2014).

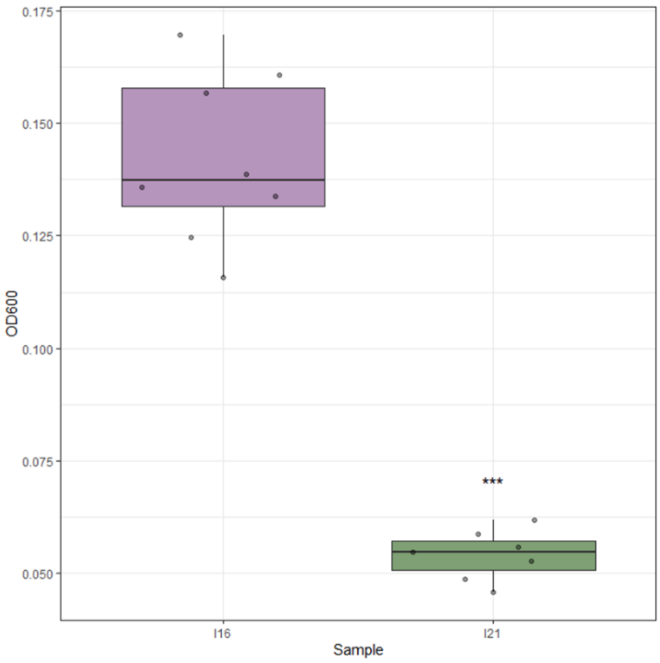

Figure 3.24 Assessing ACCd activity in I16 and I21. Quantification was performed using FiJi image analysis. I16 represented in violet and I21 in green. *** represent significant differences at the 5% level.

3.7 Evaluation of the I21 Antagonistic Specificity

To find out if the growth inhibition showed towards *E. amylovora*, isolate CM1, could be a species-specific trait, an inhibition assay was performed against the fungal pathogen *Magnaporthe oryzae*. This fungus is a phytopathogen that infects rice, causing the widespread and very devastating rice blast disease that annually contributes to major food losses (Fernandez & Orth, 2018). As this fungus was being used in the laboratory, it was decided to test I21 for its inhibitory capacity towards this pathogen. By this time, it was known that on plate assays using active *Bacillus* sp. growing cells could be a difficult task due to its swarming ability and for this reason, this assay was performed using the filtrated LB media in which the bacterial isolates were growing. This decision was done assuming that the LB liquid media would be saturated with compounds responsible for the detected inhibition against *E. amylovora*. For that, a solid PDA media containing a proportion of 40% (v/v) LB filtrates was used to perform the inhibition assays. Mycelium growth was assessed throughout 3 different timepoints (3 DPI, 5 DPI and 7 DPI) and as seen in **Figure 3.25**, the percentage of inhibition was calculated for both isolates. Serenade ASO, is one of the available commercialized products for rice blast control (DGAV, SIFITO, 2022), and, for that reason, I16 was used as a positive control in the inhibition assays. Despite the fact that, at 7 DPI the antagonistic activity seems to slightly decrease, the data confirm that I16 is capable of restrain this fungal pathogen growth by an average 40% of mycelium growth inhibition (**Figure 2.25**). In comparison, I21 shows a much lower inhibitory behaviour of about 18% with not much variance between timepoints (**Figure 2.25**). This data seems to prove that the highly effective antagonistic activity seen in I21 against fire blight might be a species-specific trait, or at least it was not so strong when compared to the assays previously performed using living organisms instead. To facilitate a comparison between the antagonistic effect against the two different phytopathogens, an inhibition assay using filtrates should be performed against *E. amylovora*. Once again, this data highlights the possibility of KB media influence on I21 secretion of toxic compounds.

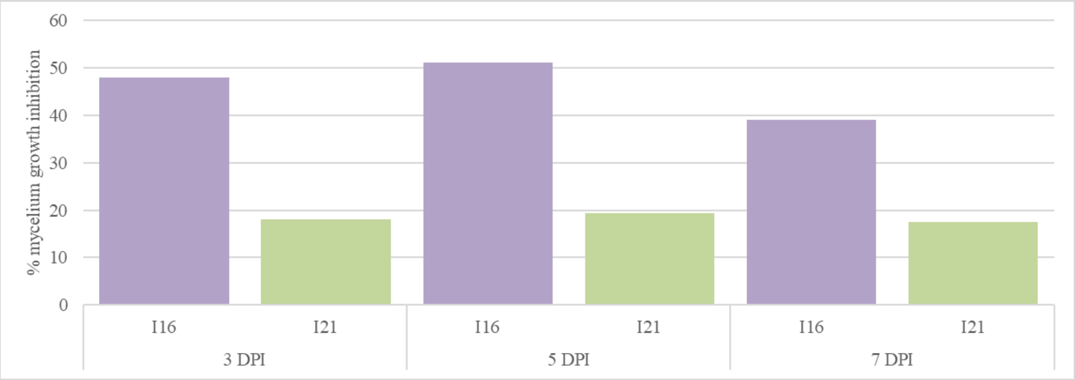

Figure 3.25 Assessing inhibitory capacity of I21 media filtrates against *Magnaporthe oryzae* to test pathogen-specificity. Data collection was done in 3 different timepoints: 3 DPI, 5 DPI and 7 DPI. I16 in violet and I21 represented in green

4. Conclusion and Future Perspectives

In this study, the identification of new cBCAs against *E. amylovora* was proposed as main goal, and consequently some experimental objectives needed to be achieved, such as: i) the isolation, identification and characterization of an *E. amylovora* isolate coming from Portuguese orchards; ii) set up an *in vitro* screening methodology to test cBCAs against the phytopathogen and iii) the identification and characterization of possible antagonistic bacteria capable of inhibiting *E. amylovora* growth *in vitro*. The suggested objectives were totally accomplished and a potential BCA against fire blight was identified and partially characterized. The *Bacillus* sp. identified in this study was isolated from pear orchards in the Oeste region and presented great inhibitory capacity towards *E. amylovora in vitro* at a higher rate than the only commercially available similar product, *B. velezensis* QST713 (Serenade ASO, Bayer).

Although this work uncovers a promising first step towards developing a new field-validated BCA for fire blight, some data need to be validated such as the BC and PGP traits, inhibition against other pathogen species to confirm biological control specificity must be assessed as well as the use of bacterial extract as source of possible bactericidal proteins. Also, the whole genome sequencing of this bacterium should be performed to allow a better taxonomic identification and to understand the metabolic pathways underlying its antagonistic activity. Furthermore, root colonization and inhibition *in planta* should be performed to confirm activity *in vivo*.

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