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Role of species interactions in the ecology of *Erwinia amylovora*, the causal agent of apple fire blight

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I dedicate this work to my family that allowed me to continue my study and has never stopped me from following my dreams.

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abdomen (indicated by red arrows); B) Under a transilluminator, the abdomen fluoresces, confirming the presence of GFP-labelled *Erwinia amylovora*.

LIST OF SYMBOLS AND ABBREVIATIONS

AGA	D-alanylgriseoluteic acid
AHL	N-Acyl Homoserine Lactone
AI	Autoinducer
BCA	Biocontrol agent
CFU	Colony Forming Units
Ea21	*Erwinia amylovora* Ea21
EFR	EF-TU RECEPTOR
EPPO	European and Mediterranean Plant Protection Organization
EPS	Exopolysaccharides
ETI	Effector-Triggered Immunity
GFP	Green Fluorescent Protein
HR	Hypersensitive Reaction
LB	Lysogeny Broth
NaClO	Sodium hypochlorite
NA	Nutrient Agar
PAMPS	Pathogen-Associated Molecular Patterns
PBSA	Phosphate-Buffered Saline
PM	Biolog Phenotype MicroArray Plates™
PPO	PolyPhenol Oxidases
PSBMT	Partial Stigma-Based Medium
PTI	Pattern-Triggered Immunity
PRR	Pattern Recognition Receptors
QS	Quorum Sensing

QTL	Quantitative Trait Loci
R2A	Reasoner's 2A Agar
RH	Relative Humidity
SA	Salicylic Acid
SDW	Sterile Distilled Water
SNA	Sucrose Nutrient Agar
T3SS	Type Three Secretion System
T6SS	Type Six Secretion System
VBNC	Viable But Not Culturable
VOC	Volatile Organic Compound

ABSTRACT

Fire blight represents a significant threat to apple and pear production worldwide. The causal agent, *Erwinia amylovora*, spreads rapidly within host plants, making this devastating disease particularly difficult to manage. Copper and antibiotics remain the most effective means of controlling fire blight; however, their application contributes to environmental pollution and promotes the emergence of antibiotic-resistant *E. amylovora* populations. For these reasons, there is an urgent need to find new alternatives to such plant protection products. Notably, the presence of *E. amylovora* alone does not necessarily result in the disease development. Instead, fire blight results from multiple interactions established between *E. amylovora* cells, flower microbiota, plant host, insect vectors and environmental factors. For instance, specific humidity and temperature create suitable conditions for *E. amylovora* to grow and reach the cell density needed for plant infection. Once the disease develops, insects act as potential vectors of *E. amylovora*, contributing to its spread. The host plant plays a crucial role, as susceptibility varies among different species within the *Rosaceae* family. Recent studies have shown that apple flower microbiota might either promote or hinder the infection, thus representing a possible source of new biocontrol agents effective in controlling *E. amylovora*. Therefore, studying the influence of the abovementioned factors is necessary to develop novel eco-friendly solutions to manage fire blight disease.

The first objective of this thesis was to investigate the interaction between the plant pathogenic bacterium and the apple flower microbiota to select new potential biocontrol agents. Flowers of *Malus domestica* cv. Golden Delicious were collected from Trentino apple orchards at the 'Balloon stage' and surface-sterilized to isolate only endophytic bacteria. According to the *16S rRNA* gene sequencing, the bacterial isolates mainly belonged to the *Enterobacteriaceae*, *Pseudomonadaceae*,

and *Microbacteriaceae* families. All bacterial strains isolated from apple flowers were tested against *E. amylovora* on detached apple flowers and immature pear slices, which were first treated with bacterial suspensions before being inoculated with *E. amylovora*. Among them, *Pantoea agglomerans* AFF2001 showed the highest efficacy in controlling the plant pathogenic bacterium in both assays. We then conducted several *in vitro* tests to investigate the modes of action underlying the antagonistic activity observed. Based on the results of the experiments conducted on flowers and pears, the bacterial strains selected as potential biocontrol agents grown in a specific medium that mimics the apple stigma nutrient conditions, and the cultural filtrates were tested to evaluate their impact on the growth and virulence of *E. amylovora*, with virulence referring to factors such as motility and biofilm production. To achieve this, the bacterial culture filtrates were used as the medium for the experiments. To further explore the molecular mechanisms involved in its biocontrol activity, knockout mutants of *P. agglomerans* AFF2001 were generated. Mutants with reduced ability to inhibit the growth of *E. amylovora* were subsequently characterised *in vitro* and *in vivo*. The results indicated that differences in motility, biofilm production, and siderophore release distinguished the knockouts from the wildtype, suggesting these factors may contribute to the biocontrol ability of *P. agglomerans* AFF2001 to control *E. amylovora* on apple flowers and pear slices. Altogether, this study highlights the potential of *P. agglomerans* AFF2001 as a promising biopesticide for fire blight disease management.

The second objective of this thesis was to explore the potential role of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) as a vector of *E. amylovora*. Pitfall and olfactometer choice assays were conducted to assess whether *D. suzukii* is attracted to bacterial ooze produced by *E. amylovora.* To do that, *E. amylovora* was cultured on Petri dishes with a specific medium that promotes bacterial ooze formation, and *D. suzukii* was given a choice between inoculated and non-

inoculated plates. The main difference between the two experimental setups was the exposure time, which was longer in the pitfall assay, and the application of airflow in the olfactometer choice assay, which was absent in the pitfall assay. The results showed a tendency of *D. suzukii* flies to be attracted to the ooze produced by *E. amylovora*, suggesting this behaviour may support the dispersal of *E. amylovora* in the environment. The attractiveness was particularly evident for female individuals, and this difference observed between the two sexes might be related to biological factors, such as the reproductive role of females, which may influence their choice. Next, we improved the protocol to test the acquisition of *E. amylovora* by *D. suzukii*. Several parameters were evaluated: the fruit type to inoculate with *E. amylovora* to produce the ooze; the time of exposure of the inoculated fruits to *D. suzukii*; the time points of sampling; the molecular detection of *E. amylovora* in *D. suzukii*. Overall, the results suggested that *D. suzukii* is attracted to *E. amylovora* ooze and can acquire plant pathogenic bacteria. Future studies are needed to assess the ability of *D. suzukii* to spread and transmit *E. amylovora* in the environment, with a particular focus on apple orchards.

CHAPTER 1. INTRODUCTION

1.1 Fire blight disease

Fire blight is a devastating bacterial disease affecting many plant species belonging to the Rosaceae family, including apple (*Malus domestica* L.) and pear (*Pyrus communis* L.), the major hosts. Since its first description in New York State at the end of the 18th century, this destructive disease spread to many countries, and it is now present in New Zealand, Europe, northern Africa, Asia, and the Middle East (Van der Zwet et al. 2012; Jock et al. 2013; Park et al. 2017; Gaganidze et al. 2018, 2021; Doolotkeldieva et al. 2021; Sun et al. 2023). Even though fire blight outbreaks are often sporadic, disease development can rapidly lead to the loss of entire apple and/or pear orchards, therefore representing a significant threat to the apple and pear production of many regions worldwide (Doolotkeldieva et al. 2016). The causal agent of fire blight is *Erwinia amylovora*, a Gram-negative bacterium reported in the European and Mediterranean Plant Protection Organization (EPPO) A2 lists the pests recommended for regulation as quarantine pests (EPPO 2022). Although *E. amylovora* can exploit minor wounds in plant tissues caused by insects or strong winds to invade the plant, flowers are the main site of infection. Colonisation of flowers of Rosaceae plants by *E. amylovora* cells allows them to grow on stigma surfaces and subsequently enter the plant through the hypanthium (Cui et al. 2021c). Once *E. amylovora* enters the plant, it moves systemically through the parenchyma, where its accumulation and its production of biofilms break the epidermis, leading to ooze formation that represents a secondary source of inoculum (Schouten 1989a, b; Slack et al. 2017). Ooze attracts insects that can again become potential vectors and further spread the disease (Boucher et al. 2021a). Notably, *E. amylovora* within the flowers does not inevitably result in disease development. Indeed, several

studies report fire blight only occurs when specific environmental conditions facilitate *E. amylovora* cell movement and multiplication (Dagher et al. 2020; Pusey 2000). However, weather conditions are not the only environmental factors to consider. Increasing evidence indicates that the microbial communities living in flowers may play an essential role in the earliest stage of host colonisation when *E. amylovora* multiplies on stigma surfaces (Cui et al. 2021b).

Since the knowledge of the ecology and biology of plant pathogens is essential to finding new eco-sustainable approaches in plant protection (Morris et al. 2017), this introduction will focus on the interactions between *E. amylovora* and all the other factors involved in the development of fire blight, providing an insight in the ecology of this plant pathogenic bacterium (Figure 1) .

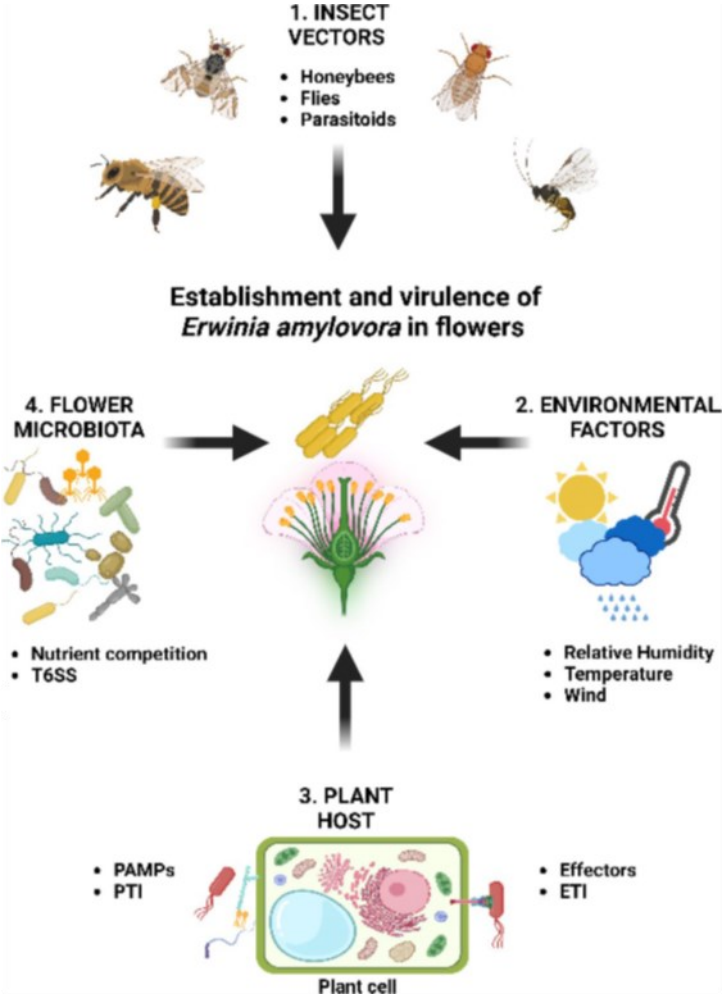

Figure 1. Multiple interactions occur in the environment that may affect the establishment and virulence of *Erwinia amylovora* cells in the flowers of Rosaceous plants. (1) Insects may promote the dissemination of *Erwinia amylovora* cells and allow the plant pathogen to reach the plant hosts; (2) Environmental factors such as temperature and relative humidity may affect *Erwinia amylovora's* ability to colonise flowers and infect the plant hosts; (3) Plant hosts may hinder the success of the infection by *Erwinia amylovora* cells through the perception of Pathogen Associated Molecular Patterns (PAMPs) and effectors leading to the activation of the PAMP Triggered Immunity (PTI) and Effector Triggered Immunity (ETI), respectively; (4) Microbial communities residing in the flowers may compete with *Erwinia amylovora* cells for space and nutrients. Moreover, bacterial communities may interact directly through the Type 6 Secretion System (T6SS), thus affecting *Erwinia amylovora* growth and virulence. (Created with Biorender.com)

1.2 Influence of environmental factors on the establishment and virulence of *Erwinia amylovora*

As Stevens (1960) reported, a favourable environment is one of the three main factors needed for disease development. But what is meant by 'favourable environment' in the case of fire blight?

First, Relative Humidity (RH) has been shown to strongly affect *E. amylovora* population size during flower colonisation, whose density must exceed 10^4 bacterial cells per flower to cause the infection in apple plants (Philion 2014). At a stigmatic level, the epiphytic growth of *E. amylovora* can reach a concentration of more than 10^6 bacterial cells per crab apple flower only when RH is above 55%. In contrast, a higher than 80% RH was required in the floral cup (hypanthium) (Pusey 2000). Rainy weather therefore plays therefore a key role is creating a wet environment suitable for *E. amylovora* cell multiplication. However, flower humidity can also be enhanced by the dew occurring throughout the night, as recently proposed (Slack et al. 2022). According to the same study, light wind would even influence this phenomenon even (Slack et al. 2022). Besides *E. amylovora* cell population size, disease development requires the activation of the Type 3 Secretion System (T3SS), a crucial virulence factor. High RH would contribute to the expression of the genes related to T3SS, thus enabling *E. amylovora* to inject proteins into the host plant to start the infection (Cui et al. 2021a).

Along with RH, the temperature is another essential environmental factor to reach the high cell density required for flower infection (Canada AeA 2006; MAAARO 2011). The optimal temperature for the growth of *E. amylovora* is 28°C (Santander et al. 2017; Van der Zwet et al. 1979), which is consequently the most suitable temperature for fire blight development. However, it was recently shown *E. amylovora* pathogenicity is maintained even at colder temperatures (14°C and 4°C),

proving that the low bacterial cell growth rates slowed down the infection without necessarily preventing it (Santander et al. 2017). Moreover, it is worth noting that pH is also a parameter to consider. Indeed, the optimum pH for the growth of *E. amylovora* is around 7.5 (Shrestha et al. 2005), and it was reported this affects both growth and chemotaxis in *E. amylovora* (Raymundo et al. 1980). In addition, Pester and colleagues (2012) also showed acidic conditions might affect *E. amylovora* pathogenicity. Indeed, the expression of T3SS genes is no longer induced in acidic conditions (pH 4), proving pH has a role in *E. amylovora* virulence (Pester et al. 2012).

The factors described so far are all environmental variables. To a certain extent, the flower itself could be seen as a confined environment in continuous evolution. By their opening, apple and pear flowers undergo several changes that could explain why increasing flower age hinders the colonization ability of *E. amylovora* (Pusey et al. 2008b; Slack et al. 2022). One of these changes is related to the composition of stigmatic oozes, which differs in correlation to the flower opening stage (Pusey et al. 2008a). Moreover, visitors, such as pollinators, can disperse microorganisms that constitute potential competitors for the nutritional sources harboured within the flower, contributing to a shift in the bacterial community (Cui et al. 2020).

All the studies reported above highlighted the environmental conditions that may favour the establishment of *E. amylovora* cells in plant flowers. However, *E. amylovora* cells also have to withstand environmental conditions that may be unfavourable (e.g., dryness). So, it comes naturally to wonder how *E. amylovora* can cope with adverse environmental conditions and what strategies this bacterium may implement to survive and persist in the environment.

It is now widely accepted that biofilm is one of the most important virulence factors required by *E. amylovora* to cause disease (Koczan et al. 2009, 2011; Kharadi and Sundin 2021; Peng et al. 2021). Biofilm consists of *E. amylovora* cells and a polymeric matrix whose main components are exopolysaccharides like amylovoran, levan and cellulose (Castiblanco et al. 2018; Koczan et al. 2009, 2011). In addition, EPS capsules synthesised by *E. amylovora* enhance both dry and cold tolerance since, under these stressful conditions, their production is increased, in particular at low temperatures (Jock et al. 2005; Santander et al. 2017). Moreover, it was reported that levan might protect *E. amylovora* cells from plant defence mechanisms (Geier and Geider, 1993), while amylovoran had a protective effect against desiccation and salinity (Geider, 2000, 2009). Similarly, Ordax and colleagues (2010) showed that amylovoran and levan protected *E. amylovora* cells against the toxic effect of copper ions. Moreover, both amylovoran and levan can be exploited by *E. amylovora* cells as an alternative carbon source when the nutrient availability in the environment is limited (Ordax et al. 2010).

The lack of nutrients is one of the several stresses plant pathogenic microorganisms are exposed to, especially when not in the host plant. It was observed that *E. amylovora* undergoes numerous morphological changes under starvation, altering cell size and shape and producing vesicles whose function has not yet been determined (Santander et al. 2014). Additionally, nutrient deprivation resulted in loss of motility even though flagellar biosynthesis was not reduced, as proved by gene expression analysis (Santander et al. 2014). Strikingly, *E. amylovora* cells kept under starvation conditions were still able to cause symptoms comparable to cells kept under optimal conditions (Santander et al. 2014).

Similarly to other Gram-negative plant pathogenic bacteria (Grey et al. 2001; Kong et al. 2014), *E. amylovora* cells may also enter into a Viable But Not Culturable (VBNC) state to withstand unsuitable environmental conditions. For instance, entry into VBNC is a strategy used by *E. amylovora* cells to counteract the presence of chlorine and copper ions (Ordax et al. 2006, 2009; Santander et al. 2012) and resist starvation. VBNC in *E. amylovora* is also triggered under starvation and is mainly influenced by temperature (Santander et al. 2014), proving once again how much the environment may affect the life of *E. amylovora* cells.

Recent findings showed the expression of virulence genes in *E. amylovora* changes throughout apple flower infection (Schachterle et al. 2022). Specifically, T3SS and flagella are highly induced during the colonization of flower stigmas and at the flower base (Schachterle et al. 2022). The biosynthesis of amylovoran has a similar regulation, while genes involved in sulphur/oxidative stress are expressed during all stages of the infection (Schachterle et al. 2022). These outcomes suggest that *E. amylovora* might modulate its virulence according to the environmental stimuli perceived. Previous studies were carried out to understand how this occurs at a molecular level (Schachterle et al. 2019a, b, 2022; Yang et al. 2020; Kharadi et al. 2022). Yang and colleagues (2020) reported the RelA/SpoT system is activated in *E. amylovora* under adverse conditions, such as starvation and oxidative stress, leading to an increase in the production of the nucleotide second messenger (p)ppGpp that positively regulates T3SS and motility (Yang et al. 2020). Cyclic di-GMP, another nucleotide second messenger, is essential during xylem invasion. Indeed, its accumulation inside *E. amylovora* cells promoted the surface attachment mediated by the Type IV pilus and the production of EPSs, two factors involved in the biofilm formation (Kharadi et al. 2022). However, *E. amylovora* virulence traits and oxidative stress adaptation are also influenced by ArcZ, OmrAB and RmaA, small RNA regulators acting at a

transcriptional and post-transcriptional level (Schachterle et al. 2019a, b). Overall, these observations highlighted the complexity of the regulatory systems activated by *E. amylovora* in response to the environment.

1.3. Influence of the interaction with insects on the dispersal and infection by *Erwinia amylovora*

Due to their essential role in agriculture, honeybees and other pollinators have always been seen as potential carriers of plant pathogenic microorganisms (McArt et al. 2014; Cellini et al. 2019). This is of particular interest in the case of fire blight because pollination occurs in flowers, which are regarded as the primary sites of infection. Honeybees' involvement in *E. amylovora* dispersal was demonstrated in the first half of 1900 (Keitt et al. 1941; Pierstorff et al. 1934). To be transmitted, *E. amylovora* cells must survive inside and/or outside the body of the insects. Early results reported contaminated honeybees can carry *E. amylovora* on the body surface and in the intestine for up to 48 and 36 hours, respectively. However, *E. amylovora* cell longevity strongly depended on temperatures (Alexandrova et al. 2002a, b; Sabatini et al. 2006). In contrast, Choi et al. (2022) recently revealed *E. amylovora* cells can be detected for a longer period inside the honeybees, which can disseminate the plant pathogen in a time span of 10 days after contamination. Moreover, the same authors showed honeybees may acquire *E. amylovora* from diseased apple plants and further spread the pathogen to healthy plant hosts (Choi et al. 2022). This raises questions about how the interaction between *E. amylovora*, pollinators, and host plants is modulated. Honeybees seem to be more attracted by healthy apple flowers rather than infected ones and this phenomenon could be driven by the emission of Volatile Organic Compounds (VOCs) (Cellini et al. 2019). It was hypothesised honeybees may distinguish between diseased and non-diseased apple flowers by recognising their VOC profile. In

particular, methyl salicylate, whose emission is associated with *E. amylovora* infection, would discourage pollinators' visits (Cellini et al. 2019). On one hand, the honeybee's preference for healthy flowers may help prevent the spread of *E. amylovora*, but on the other hand, the attraction of healthy flowers to honeybees infected with *E. amylovora* might facilitate the transmission of the disease (Cellini et al. 2019). The epidemiological implications behind these observations, as highlighted by Cellini (2019), are unclear, and further studies are needed to disentangle the complicated interaction between honeybees, *E. amylovora* and apple flowers.

Even though bees and hoverflies represent the most important pollinators of apple flowers (Delaplane and Mayer, 2000; Klein et al. 2007; Pardo and Borges, 2020), a vast population of non-pollinators visits orchards throughout the seasons. Among them, fruit flies such as *Ceratitis capitata* (the Mediterranean fruit fly or medfly) and the common *Drosophila melanogaster* attracted the attention of fire blight research. Medfly is classified as one of the most dangerous threats to fruit production worldwide due to its invasiveness and ability to quickly settle in new areas, which make it difficult to control this pest (Aluja and Norrbom, 2000). It was observed that this pest can acquire *E. amylovora* by feeding on inoculum drops of infected apples and harbour the plant pathogenic bacterium both in the inner and outer parts of the insect's body for eight and 28 days, respectively, similar to the previous observations conducted on honeybees (Ordax et al. 2015).

Trees infected by fire blight present tiny bacterial ooze droplets on fruits or other plant tissues depending on how the stage of the disease is advanced (Johnson 2000). In contrast to honeybees, flies such as *D. melanogaster* can be contaminated by *E. amylovora* directly from ooze and later transfer the pathogen to a selective medium (Boucher et al. 2019). *E. amylovora* cell acquisition, but not its

abundance, was positively correlated to when *D. melanogaster* individuals were exposed to bacterial ooze (Boucher et al. 2019). Boucher et al. (2019) tested 3, 6, 12 and 24 hours of exposure. Still, it is questionable whether these times represent what happens in the field, where several ooze sources can attract flies and could probably keep flying from one source to another. So, it is difficult to know whether the time they are exposed to ooze is sufficient to acquire enough *E. amylovora* cells to spread further, also because the *E. amylovora* population harboured in ooze could differ.

A recent study on *Delia* (*De.*) *platura*, another fly species, underlined that EPSs contained in ooze would also enhance the attachment of the *E. amylovora* cells to the surface of the insects (Boucher et al. 2020). Surprisingly, insects showed no preference for infected apples with or without ooze droplets (Boucher et al. 2021a), although the attractive effect of ooze on insects is known (Agrios 2008). Investigating the role of odours emitted by infected and uninfected apples, it emerged that *De. platura* prefers healthy fruits to those diseased when the infection is advanced (Boucher et al. 2021a). To a certain extent, this is consistent with what Cellini et al. (2019) reported on the role of VOCs in honeybee transmission. These observations were conducted on controlled experimental conditions, but what happens in open fields? According to recent findings, the role of pollinators in the *E. amylovora* dispersal may be overestimated (Boucher et al. 2021b). However, it is conceivable that data collected in open fields are strongly affected by several environmental factors, such as temperature and humidity. In addition, the outcomes reported by Boucher et al. (2021b) are just preliminary and limited to a specific area. Further studies on other orchards are needed to provide other data to confirm what was observed.

To summarise, both pollinators and non-pollinators showed the ability to acquire *E. amylovora*, which can be maintained in internal parts of the insect body, particularly in the digestive tract. Molecular mechanisms underlying the interaction between *E. amylovora* and insects are still unknown. However, two of the three T3SSs (Inv/Spa-1, Inv/Spa-2) found in the *E. amylovora* genome (Zhao et al. 2009) might be involved, as observed for the establishment of *Sodalis glossinidius* in the tsetse flies (Dale et al. 2001). This hypothesis is supported by Zhao and colleagues (2009), who reported that the two systems are not directly required to interact with plant host, suggesting they may instead function in interactions with insect hosts (Rezzonico et al. 2024). The generation of *E. amylovora* mutants lacking Inv/Spa-1 or Inv/Spa-2 might therefore help determine their role in the colonisation of insect tissues. Notably, *E. amylovora* internalisation might result in its transmission to the progeny. Research carried out on the egg parasitoid *Anaphes nitens* focussed on this aspect. By studying the endosymbionts of this insect, Ribeiro et al. (2022) found *E. amylovora* was inherited by the F1 and F2 generations. Still, it was absent on the eggs of *Gonipterus platensis*, the beetle species parasitised by *A. nitens* (Ribeiro et al. 2022). These outcomes represent the first evidence *E. amylovora* can be vertically transmitted in a parasitoid, raising questions on the possibility of this phenomenon also occurring in other insect species able to disseminate *E. amylovora*. So far, *E. amylovora* has not been detected in the eggs of the medfly (Ordax et al. 2015), but no data are available on honeybees.

1.4. Impact of host plants on the pathogenicity of *Erwinia amylovora*

Plant disease can be seen as a never-ending war in which the invader, the plant pathogenic microorganism, fights to conquer a new niche, represented by the host plant, where it can acquire substances needed for survival (Agrios 2005). It is an arms race where each side tries to attack and

protect itself from the other (Anderson et al. 2010). Due to their short generation time, plant pathogenic bacteria evolve more rapidly than their host plants (Frantzeskakis et al. 2020), giving them an advantage over the host and keeping the interaction with the plant constantly evolving.

Plant immunity is based on two main response mechanisms, namely Pattern-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) (Anderson et al. 2010). In PTI, plant defence reactions are activated after widely conserved microbial molecules (Pathogen-Associated Molecular Patterns, PAMPs) are recognised by specific receptors, namely Pattern Recognition Receptors (PRR) (Chisholm et al. 2006; Zhang and Zhou, 2010). Transcriptional analysis revealed that apple plants activate this basal defence in the first two hours after *E. amylovora* inoculation (Norelli et al. 2009), thus representing the first barrier *E. amylovora* cells must overcome to establish disease. For this reason, improving the earliest immune response of the plant would be a promising strategy to withstand fire blight. As seen for several species belonging to the *Solanaceae* and *Poaceae* families, transgenic plants expressing the *Arabidopsis thaliana* EF-TU RECEPTOR (EFR) have an increased resistance to bacterial plant pathogens (Schoonbeek et al. 2015; Schwessinger et al. 2015; Boschi et al. 2017). Once this PRR was also expressed in the susceptible apple rootstock M.26, the formation of necrotic tissue in the leaves was significantly reduced upon the infection by *E. amylovora* (Piazza et al. 2021). The case reported shows how finding candidate genes in species' genome belonging to families other than *Rosaceae* can be a possible strategy to increase resistance in apple trees. However, the same can be done by focusing on the *Malus* genus. The receptor-like kinase protein (FB_*Mfu10*) found in *Malus fusca* might be involved in the resistance of this wild apple species (Emeriewen et al. 2018). Even though further studies are needed to confirm this hypothesis, this receptor harbours a

domain that might bind the exopolysaccharide amylovoran, thus recognising *E. amylovora* cells and contributing to activating the plant defence (Emeriewen et al. 2018).

Plant pathogens evolved a strategy to silence PTI based on the secretion of specific proteins called effectors. In Gram-negative plant pathogenic bacteria, such as *E. amylovora*, effectors are delivered into host cells through the T3SS encoded by the *hrp* box (Lindgren et al. 1986; Büttner et al. 2009). On the other hand, the plant genome can harbour genes that can specifically suppress the effector's activity, leading to resistance. This gene-for-gene resistance mechanism is called ETI (Effector Triggered Immunity). By acquiring additional effectors or modifying the recognised ones, pathogens can avoid ETI until natural selection leads plants to have new resistance genes. This never-ending evolution in attack-defence response was named 'The zigzag model' (Jones et al. 2006).

It is difficult to apply the zigzag model to *Malus-E. amylovora* pathosystem because several studies showed how complicated it is to understand the gene-for-gene interaction between *E. amylovora* and *Malus* species. However, the possible molecular mechanisms involved in the interaction between *E. amylovora* and *Malus* spp. are summarised below.

In *Erwinia amylovora*, T3SS gene expression is activated in the first 48 hours after flower inoculation (Pester et al. 2012; Schachterle et al. 2022). Particularly, its activation can be strongly induced during the epiphytic colonisation of stigmas and reduced when *E. amylovora* cells reach the hypanthium (Cui et al. 2021a). Several effector genes, namely *hrpN*, encoding for a hairpin protein (Wei et al. 1992; Kim and Beer 1998); *dspE*, involved in plant cell apoptosis (Bogdanove et al. 1998); $hopPtoC_{Ea}$, whose function is not known yet (Zhao et al. 2005); $avrRpt2_{EA}$, homologous to *avrRpt* effector of *Pseudomonas syringae* pv. *tomato* (Zhao et al. 2006); *eop3*, similar to effectors belonging

to the HopX family (Nissinen et al. 2007); *xopX1*$_{Ea}$ (Bocsanczy et al. 2012); *eop1* (Wöhner et al. 2018) were identified in *E. amylovora* sequenced genomes. One of the most widely studied effectors is *avrRpt2*$_{EA}$, which has a single nucleotide polymorphism at position 156 of its amino acidic sequence, where a cysteine residue can be replaced with a serine thus influencing the ability of overcoming plant defences (Vogt et al. 2013). *E. amylovora* strains carrying cysteine, such as *E. amylovora* Ea273, belong to the "C-allele" group. In contrast, those presenting serine, like the Canadian *E. amylovora* strains, belong to the "S-allele" group (Vogt et al. 2013). Recently, Schröpfer and colleagues (2018) observed the effect of *avrRpt2*$_{EA}$ on the susceptible *Malus domestica* cultivar Pinova (Schröpfer et al. 2018). Transgenic lines expressing *avrRpt2*$_{EA}$ activated the salicylic acid-mediated defence response and developed typical fire blight symptoms, suggesting this effector alone is sufficient to cause disease (Schröpfer et al. 2018). Moreover, the interaction between the effector HrpN and the *Malus* spp., a specific protein named HIMP, resulted in the disease development (Wei et al. 1992; Oh and Beer 2008). Consequently, transgenic apple plants with a reduced expression level of *HIMP* were more resistant to *E. amylovora* infection (Campa et al. 2019).

Since most commercial apple tree varieties are susceptible to fire blight, genome mining studies aimed at identifying resistance genes mainly in wild apple genotypes. Resistance quantitative trait loci (QTLs) were found in *Malus floribunda* 821 (Durel et al. 2009), *Malus* × *robusta* 5 (Peil et al. 2007), *Malus fusca* (Emeriewen et al. 2018), *Malus* × *arnoldiana* (Emeriewen et al. 2021), and in the ornamental crab apple *Malus evereste* (Durel et al. 2009). So far, only the *FB_MR5* gene from *Malus* × *robusta* 5 showed a gene-for-gene interaction with effector *avrRpt2*$_{EA}$, but this was strongly influenced by the *E. amylovora* strain inoculated. Indeed, *E. amylovora* strains belonging to the S-allele group could overcome plant defence, unlike strains with the cysteine allele (Vogt et al. 2013).

These results were confirmed by transforming *Malus 9 domestica* cv. 'Gala' with *FB_MR5* (Broggini et al. 2014). Strain-dependent susceptibility was also observed in *M. floribunda* 821 and Evereste, whose resistance mechanism to *E. amylovora* is thought to be related to effector *eop1*, even though it was not proved yet (Wöhner et al. 2018).

In addition to the immunity response triggered by the *FB_MR5* gene, other mechanisms presumably occur during the resistance response. A recent study compared the response of *Malus ×robusta* 5 inoculated with either the wildtype strain *E. amylovora* Ea1189, triggering the ETI and therefore avirulent, and the mutant in the $avrRpt2_{EA}$, which is virulent since the apple plants do not anymore recognise it. Several differentially expressed genes were reported in plants inoculated with the *E. amylovora* Ea1189 virulent or avirulent strain. For instance, *Malus ×robusta* 5 plants induced a higher expression of genes involved in the flavonoids pathway and the biosynthesis of (E)-ß-caryophyllene (Schröpfer et al. 2021), a VOC compound known for its antimicrobial activity (Cellini et al. 2019). These outcomes add information to the resistance reaction in *Malus ×robusta* 5, but further studies are needed to have a more detailed view.

Plant defence may also be triggered indirectly, without physical contact between the plant pathogen and the host. In this regard, the VOC profile emitted by apple (cv. Golden Delicious) plantlets infected with *E. amylovora* Ea ICMP 1540 was characterised (Cellini et al. 2018). Interestingly, Cellini and colleagues (2018) reported the exposure of apple plants to these volatiles enhanced the activation of the signalling pathways related to salicylic acid (SA), a phenolic compound known for its role in plant immunity (Vlot et al. 2009), thus highlighting a possible innovative application of VOCs in the disease management.

Looking at *Erwinia amylovora* during the interaction with apple plants, Puławska and colleagues (2017) inoculated *E. amylovora* strain 650 in a resistant or susceptible apple cultivar. The main difference was seen for *E. amylovora* genes related to stress response, indicating that E. amylovora 650 implemented molecular mechanisms to protect secondary metabolites and cells from toxic compounds. Indeed, genes encoding heat shock protein and those related to multidrug efflux pumps were highly expressed when *E. amylovora* 650 was inoculated in plants of the resistant apple cultivar Free Redstar (Puławska et al. 2017).

1.5. Influence of bacterial communities on *Erwinia amylovora* establishment in flowers

An increasing body of knowledge is highlighting the importance of the plant host microbiota in establishing a pathogenic interaction between a plant pathogenic microorganism and its plant host.

For instance, *Ralstonia solanacearum* caused a drastic shift in the composition of tomato root-associated bacterial taxa that strongly influenced the *R. solanacearum* abundance and the biochemical soil properties (Wei et al. 2018). Similar changes in the rhizosphere microbial communities have been reported also for *Verticillium dahliae*. This plant pathogenic fungus might select collaborative microorganisms within the plant host microbiota through the secretion of a specific effector, attributing these proteins to a new role in plant disease (Snelders et al. 2021). These are just two examples that show manipulation of plant host microbiota might be a strategy exploited by several plant pathogenic microorganisms during plant host colonisation. Does *E. amylovora* also share this ability?

Recently, Cui and colleagues (2021a) tried to understand what happens within the stigmatic bacterial communities upon *E. amylovora* inoculation. Metagenomic analysis revealed that *Pseudomonadaceae* and *Enterobacteriaceae* are the most represented bacterial families on the stigma of healthy flowers (Cui et al. 2021a), consistent with previous observations underlining the conservation of microbial community structure among different apple cultivars (Steven et al. 2018). Moreover, it was seen that the *Pseudomonadaceae* family slowly took over the *Enterobacteriaceae* family in five days, which is the opposite of what was observed in flowers inoculated with *E. amylovora,* where members of the *Enterobacteriaceae* family rapidly became predominant (Cui et al. 2021a). As already mentioned for other plant pathogens, it is conceivable that the changes observed result from a selection promoted by *E. amylovora* aimed at recruiting microbial communities that can support the plant host colonisation and infection. Understanding how *E. amylovora* induces a shift in the microbial communities of apple flowers and how this phenomenon takes place could shed light on the early steps of the infection.

There are several direct and indirect mechanisms *E. amylovora* may exploit to manipulate plant host microbiota. Type Six Secretion System (T6SS) is one of the secretion systems owned by Gram-negative bacteria (Coulthurst 2019). Unlike T3SS, whose function is limited to plant invasion, T6SS can influence the interaction among bacteria, as reported for *Agrobacterium tumefaciens* (Ma et al. 2014). *E. amylovora* harbours three T6SS gene clusters in its genome (Kamber et al. 2017; Tian et al. 2017), and they seem to have a role in antibacterial competition, as reported for *E. amylovora* NCPPB1665 (Tian et al. 2017). Moreover, they can affect both levan and amylovoran production, thus reducing *E. amylovora* NCPPB1665 virulence on immature pears (Tian et al. 2017). To assess whether what was reported by Tian and colleagues (2017) could also apply to other *E. amylovora*

strains, single or double mutants defective in T6SS were created for *E. amylovora* CFBP 1430 by deleting either cluster one, three or both. A slight difference in virulence was reported on apple flowers and shoots, probably due to an alteration in the motility showed by the *E. amylovora* mutants (Kamber et al. 2017). Also, in this case, a possible involvement of T6SSs in antibacterial competition was hypothesised. However, the results of the competition assay performed with *E. coli* revealed *E. coli* survival was not so significantly higher compared with what was observed for the NCPPB1665 strain (Kamber et al. 2017; Tian et al. 2017).

Quorum Sensing (QS) is a regulatory system that controls gene expression according to the bacterial cell population size (Rutherford and Bassler, 2012). QS relies on three main components consisting of a signal molecule released by bacteria cells, namely the autoinducer (AI), an enzyme that synthesises the AI and a transcriptional factor able to perceive the AI (Papenfort and Bassler, 2016). In Gram-negative bacteria, the most common AIs are N-acyl homoserine lactones (AHLs) produced by N-acyl homoserine lactone synthases encoded by *luxI* homologs (Papenfort and Bassler, 2016). However, Gram-negative bacteria may produce another AI called Autoinducer-2 deriving from the cyclisation of 4,5-dihydroxy-2,3-pentanedione synthesised by ribosyl homocysteine-cleavage enzyme encoded by *luxS* homologs (Schauder et al. 2001; Pereira et al. 2013). It is now widely accepted that AIs might be at the basis of interspecies communication within plant pathogenic bacteria and bacterial communities residing on plant host organs (Dulla and Lindow, 2009; Cellini et al. 2020). Homologs of the gene *luxS* were found in *E. amylovora* genomes, but its role is limited to methionine metabolism and does not involve quorum sensing (Rezzonico et al. 2007). Indeed, AI production was not observed for some *E. amylovora* strains isolated in Germany and Switzerland (Mohammadi et al. 2007).

T6SS can be considered as direct mechanisms possibly exploited by *E. amylovora* for microbiota manipulation. However, influence on microbial communities can be exerted indirectly through competition for available nutrient sources (Dubinkina et al. 2019). The major components of stigmatic extracts of pomaceous plants are free sugars and amino acids (Pusey et al. 2008a). Glucose and fructose strongly prevail on sugars such as sorbitol and sucrose, and this pattern is preserved across different apple cultivars (Fuji, Gala, Golden), with an increase in sugar content related to the stage of anther dehiscence (Pusey et al. 2008a). As their amount is normally very low, amino acids are detected in the order of femtograms per pomaceous flowers, with proline, asparagine, glutamine, and glutamic acid being the most abundant ones (Pusey et al. 2008b). Stockwell and collaborators (2010a) reported that *E. amylovora* Ea153 might grow well in a minimal medium amended with one of the principal stigmatic compounds, giving the first evidence that these components support the growth of the plant pathogen. Understanding *E. amylovora*'s nutrient requirements and metabolism is important because they strongly influence its cell multiplication ability and virulence. For instance, apple fruits and shoots inoculated with *E. amylovora* HKN06P1 mutants impaired in the biosynthetic pathways of isoleucine/valine, leucine, methionine, adenine, and tryptophan showed a reduction in the severity of symptoms (Klee et al. 2019). In addition to these amino acids, another study highlighted *E. amylovora* HKN06P1 mutated in the arginine biosynthetic pathway and lost its pathogenicity on immature apples (Ramos et al. 2014). This auxotrophic mutant, but not its dead cells, inhibited the colonisation of the *E. amylovora* HKN06P1 wild-type strain when applied to flowers (Klee et al. 2019). It is not known yet whether this effect is due to nutrient competition alone or other mechanisms (i.e., plant immunity stimulation) are involved. However, Klee and collaborators (2019) proved that

specific amino acids are necessary for the full virulence of *E. amylovora*, but they are not sufficiently available in the host, forcing the plant pathogen to synthesise them.

Iron is another important element for bacterial cell growth because DNA replication, as well as tolerance to oxidative stress, would not be possible without this cofactor (Krewulak et al. 2008; Cornelis et al. 2011). Thus, iron might represent a limiting factor for bacterial cell survival, particularly when several microorganisms are present in the same environmental niche. The latest findings confirmed this hypothesis by comparing the growth of *E. amylovora* CFBP1430 deficient in the iron-uptake receptor gene *foxR* on greenhouse and orchard apple flowers. Greenhouse flowers, which can be considered as semi sterile, well supported the colonisation of the *E. amylovora* CFBP1430 mutant in contrast to those collected from the orchards, whose established microbial communities might be potential iron-competitors (Müller et al. 2022).

1.6. Application of microorganisms for the sustainable control of *Erwinia amylovora*

For many years, antibiotics and copper-based compounds have been the most effective solutions to limit *E. amylovora* spread and damage. However, these plant protection products cause environmental pollution and contribute to the development of resistance that makes their application useless, as reported in the USA, where streptomycin- and copper-resistant *E. amylovora* strains were isolated already in 1991 (Loper et al. 1991). For these two main reasons, research has focused on finding new strategies that can be both effective and eco-friendly.

Besides increasing the knowledge on the fire blight, unveiling how interaction between flower microbiota and *E. amylovora* occurs may also help to improve the sustainable management of the

disease. Indeed, microorganisms, such as yeasts and bacteria, that are able to reduce the ability of plant pathogens to cause disease might be used as biocontrol agents (BCAs) and developed as the main active ingredient of commercial biopesticides (Gupta et al. 2021).

Over the years, microorganisms tested against *E. amylovora* were isolated from different environmental niches (Aktepe et al. 2022; Barbé et al. 2022; Mikiciński et al. 2016; Pourjafari et al. 2022; Pusey et al. 2009). Nevertheless, it is conceivable those isolated from apple flowers can represent the best antagonistic candidates against *E. amylovora* because of their adaptation to living in such an environment. Recent findings showed apple stigma-colonizing bacteria can reduce disease severity when applied on flowers, even though efficacy strongly depends on the bacterial mixtures and is not comparable to streptomycin treatment (Cui et al. 2021b). Among the bacterial isolates tested, treatments including *Pantoea* sp. CT-1039 resulted in the best reduction of disease incidence, indicating that this strain may be responsible for the observed effect (Cui et al. 2021b). Strains belonging to *Pantoea* spp. are indeed the active ingredients of commercial biopesticides used in fire blight management. At the moment, commercial biopesticides developed to control *E. amylovora* are based on Gram-negative bacteria isolated from apple trees, such as *Pantoea* (*Pa.*) *agglomerans* E325 (Bloomtime), *Pa. vagans* C9-1 (BlightBan C9-1) and *Pseudomonas* (*Ps.*) *fluorescens* A506 (BlightBan A506) (Sundin et al. 2009). All these strains can produce antibiotics, such as herbicolin O and pantocin A, although not all strains produce both these antibiotics (Kamber et al. 2012; Stockwell et al. 2010b). Interestingly, *Ps. fluorescens* A506 can release the antibacterial compound only in an iron-rich environment, which is not the case for apple and pear floral surfaces (Temple et al. 2004). The efficacy of *Ps. fluorescens* A506 may be mainly attributed to the nutrient/niche competition (Stockwell et al. 2010a), even though the number of carbon sources utilised by *Ps. fluorescens* A506

was lower than the one of *Pa. vagans* C9-1 and *E. amylovora* Ea135 (Stockwell et al. 2010a). On the opposite, the two BCAs share almost all the nitrogen requirements with *E. amylovora* Ea135 (Stockwell et al. 2010a). In comparison to *E. amylovora* Ea135, both *Pa. vagans* C9-1 and *P. fluorescens* A506 had faster growth when cultivated in the sugar compounds contained in the apple stigmatic oozes (Pusey et al. 2008b; Stockwell et al. 2010a). Unexpectedly, the simultaneous application of both BCAs did not enhance the reduction of fire blight symptoms because *Ps. fluorescens* A506 produces a protease degrading the antibiotic produced by *Pa. vagans* C9-1, thus erasing its effect (Stockwell et al. 2010b). This study highlighted the possible incompatibility among BCAs, which is an important aspect to consider when developing new biopesticides. Considering Gram-positive bacteria, the biopesticide Serenade is based on *Bacillus subtilis* QST713, whose antimicrobial activity is related to the production of lipopeptides (Sundin et al. 2009). The application of Serenade on apple blossoms reduced fire blight symptoms similarly to BlightBan A506, BlightBan C9-1 and Bloomtime (Sundin et al. 2009). Another product used to control fire blight is Blossom Protect, whose active ingredients are the yeast-like fungal strains *Aureobasidium pullulans* CF10 and CF40. Regardless of the incubation temperature, these strains isolated from apple fruits were effective in reducing the fire blight severity both on detached apple flowers and in open fields (Kunz 2004). In a recent study, Temple et al. (2020) compared the efficacy of Blossom Protect to other yeast strains, including *Cystofilobasidium infirmominiatum* YY6 and *Cryptococcus neoformans* C9 and C16. None of the tested yeast strains was as effective as Blossom Protect, whose mode of action is not related to the reduction of *E. amylovora* populations in the flower (Temple et al. 2020).

As the mode of action of *Aureobasidium pullulans* may rely on its ability to compete for space and nutrients, Slack et al. (2019) evaluated the impact of a preventive application of hydrogen and

peroxyacetic acid before the application of Blossom Protect in order to reduce the competition of the microbial populations residing in the apple flowers. However, the removal of the flower microbiota did not result in an increase in the Blossom Protect plant protection efficacy (Slack et al. 2019), an indication that the mode of action of *A. pullulans* might rely on multiple mechanisms. Accordingly, Zeng and colleagues (2023) reported flower treatment with *A. pullulans* would trigger Systemic Acquired Resistance in the apple flowers by increasing the level of salicylic acid and inducing the expression of *PR1* and *PR2* genes. Interestingly, the immune response in the plant host would be active until five days after treatment. As this time period is also the life span of apple flowers, it is conceivable that fewer applications of Blossom Protect would be needed to have an effective plant protection efficacy (Zeng et al. 2023).

Despite the initial promises, years of experiments in the open field showed the application of the above-mentioned BCAs alone was not sufficient in controlling fire blight unless they were used together with streptomycin or other products (Sundin et al. 2009; Johnson et al. 2013, 2022). For instance, a significant increase in fire blight control was achieved by applying Blossom Protect after fruit load thinning treatment with lime sulphur, reaching an efficacy comparable to streptomycin (Johnson et al. 2013). Recently, Johnson and colleagues (2022) suggested that different protection products may be applied at different bloom stages, e.g. Blossom Protect, copper and Serenade at 70% bloom, full bloom and petal fall, respectively. Notably, several trials showed this combination would reduce both disease severity and fruit russeting (Johnson et al. 2022). Besides microbial BCAs, *E. amylovora* might be controlled using bacteriophages, viruses widespread in all habitats where their host, namely bacteria, live (Sieiro et al. 2020). The main characteristic of bacteriophages is they can infect and kill specific bacterial species or strains by lysing their cells, thus representing a valid

alternative to antibiotics in plant disease management (Sieiro et al. 2020). Concerning fire blight, several bacteriophages isolated from apple trees in Germany and North America were characterised and tested against several *E. amylovora* strains (Müller et al. 2011). Müller et al. (2011) showed that bacteriophages belonging to the Myoviridae family significantly reduced the *E. amylovora* population on apple flowers compared to the members of the *Podoviridae* family.

In general, the use of bacteriophages has two important disadvantages, such as the time of application. Since bacteriophage survival is strictly dependent on the presence of their host bacteria, there is reduction of bacteriophage population when flowers do not harbour *E. amylovora* (Ritchie and Klos 1979; Schnabel et al. 1999, 2001). This issue can be solved by using a carrier like *Pa. agglomerans* Eh21-5 to deliver bacteriophages, as developed by Lehman (2007) and employed in later studies (Lehman 2007; Boulé et al. 2011; Geyder et al. 2020). The efficacy of this method might be negatively affected by the sensitivity of the bacterial carrier to the bacteriophages delivered, making the study of the bacteriophage-carrier interactions essential (Geyder et al. 2020). Moreover, *E. amylovora* may rapidly develop resistance to bacteriophages, as seen for other plant pathogenic bacteria (Dong et al. 2018; Fujiwara et al. 2011), thus limiting their efficacy. To overcome this problem, research has focussed on creating mixtures of several bacteriophages that can reduce the emergence of resistance in *E. amylovora* by using different infection strategies (Sieiro et al. 2020). The synergy between several bacteriophages was proved, but in some cases, the validation in planta has not been tested yet (Born et al. 2011, 2015; Geyder et al. 2020; Jo et al. 2023).

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characteristic of bacteriophages is they can infect and kill specific bacterial species or strains by lysing their cells, thus representing a valid alternative to antibiotics in plant disease management (Sieiro et al. 2020). Concerning fire blight, several bacteriophages isolated from apple trees in Germany and North America were characterised and tested against several *E. amylovora* strains (Müller et al. 2011). Müller et al. (2011) showed that bacteriophages belonging to the Myoviridae family significantly reduced the *E. amylovora* population on apple flowers compared to the members of the *Podoviridae* family. In general, the use of bacteriophages has two important disadvantages, the time of application and the development of bacteriophage-resistant *E. amylovora* populations. Since bacteriophage survival is strictly dependent on the presence of their host bacteria, there is reduction of bacteriophage population when flowers do not harbour *E. amylovora* (Ritchie and Klos 1979; Schnabel et al. 1999, 2001). This issue can be solved by using a carrier like *Pa. agglomerans* Eh21-5 to deliver bacteriophages, as developed by Lehman (2007) and employed in later studies (Lehman 2007; Boulé et al. 2011; Geyder et al. 2020). The efficacy of this method might be negatively affected by the sensitivity of the bacterial carrier to the bacteriophages delivered, making the study of the bacteriophage-carrier interactions essential (Geyder et al. 2020). Moreover, *E. amylovora* may rapidly develop resistance to bacteriophages, as seen for other plant pathogenic bacteria (Dong et al. 2018; Fujiwara et al. 2011), thus limiting their efficacy. To overcome this problem, research has focussed on creating mixtures of several bacteriophages that can reduce the emergence of resistance in *E. amylovora* by using different infection strategies (Sieiro et al. 2020). The synergy between several bacteriophages was proved, but in some cases, the validation in planta has not been tested yet (Born et al. 2011, 2015; Geyder et al. 2020; Jo et al. 2023).

1.7. Future perspectives

As discussed so far, fire blight is influenced by many factors that make this disease complex and difficult to study and manage.

For instance, the growth of *E. amylovora* is strongly affected by environmental conditions such as humidity, rain, and temperature, which also influence the efficacy of eco-sustainable strategies such as BCAs. These variables are hard to control, especially nowadays that global warming causes more and more extreme climate events (Seneviratne et al. 2021) and for this reason it is important to test the response of new strategies to adverse environmental phenomena.

In addition to heavy rain and winds, insects might play an important role in fire blight disease. *E. amylovora* can survive on the surface but also inside the body of pollinators and non-pollinators, thus becoming vectors able to spread the plant pathogen to healthy host plants. So far, most of the experiments were carried out in controlled laboratory conditions, and very few data were collected on the impact of insect-mediated transmission in open fields. This is an aspect to be investigated, as well as the transmissibility of *E. amylovora* over generations of insects, which has been recently proved in *A. nitens* (Ribeiro et al. 2022), without testing the viability and the pathogenicity of the bacterial cells. It would also be of interest to dig into the complexity of the system "*E. amylovora* - host plant – vectors" by pursuing the work carried out by Cellini et al. (2019) to understand the attractive/repulsive effect of VOCs, in addition to the stimulation of plant defence.

The interaction between *E. amylovora* and host plants is also an open research field. Since PTI is the earliest immune response activated, it would be conceivable to focus on its implementation.

However, since plant pathogens overcome this barrier by releasing effectors, ETI must not be forgotten. In this view, instead of concentrating only on wild apple varieties, looking at the genomes of other species belonging to the Rosaceae family might lead to identifying QTLs harbouring new resistance candidate genes.

During the earliest phase of infection, when *E. amylovora* cells try to colonize flower stigmas, microbial communities residing in the flowers can influence the infective process, hindering or enhancing the establishment of the *E. amylovora* population. By creating *E. amylovora* knock-out mutants in the genes related to T6SS, it will be possible to assess their involvement in the interaction with the microbiota, expanding the knowledge of the mechanisms that *E. amylovora* might implement to manipulate the flower microbiota. Moreover, studying the bacterial community residing in apple flowers might lead to finding new microbial species to use in the sustainable management of fire blight. So far, commercialized BCAs are not sufficient to achieve complete control of the disease. So, in the future, combining yeasts and bacteria may result in the development of new effective biopesticides.

The combination of different strategies might possibly enhance the chance of reaching a complete fire blight control, avoiding environmental pollution, but further research is required.

*The content presented in this section has been previously published as a review article entitled "**This tree is on fire: a review on the ecology of* **Erwinia amylovora***, the causal agent of fire blight*

***disease**" in the Journal of Plant Pathology (Pedroncelli and Puopolo, 2024). The material has been updated for inclusion in this thesis.*

1.8. Thesis aim

Based on this body of knowledge, this thesis focussed on two of the factors that influence the development of fire blight.

First, we investigated the role of the flower bacterial communities in the interaction with *E. amylovora*. Specifically, we isolated several bacterial strains isolated from apple flowers of Trentino orchards and evaluated them for their ability to control the disease symptoms on flowers. We then characterised the modes of the new potential biocontrol agents by conducting experiments both *in vivo* and *in vitro*. All bacterial strains, including *E. amylovora*, were isolated from the Trentino region.

Second, we assessed the potentiality of *Drosophila suzukii* as a vector of *E. amylovora*.

CHAPTER 2. MATERIALS AND METHODS

2.1. From apple flowers to control fire blight: new potential biocontrol agents of *Erwinia amylovora*?

2.1.1. Isolation of endophytic bacteria from apple flowers

Flowers of *Malus domestica* cv. Golden Delicious from Trentino apple orchards were sampled at the 'Baloon stage'. After determining their weight, flowers were first surface sterilised by putting them in sterile 50 ml tubes and adding ethanol (70%) to completely cover them. Tubes were gently shaken for 1 minute, and ethanol was discarded. Sodium hypochlorite (NaClO) solution (2%) was added to cover all the flowers, and tubes were gently shaken for 90 seconds. After discarding the NaClO solution, flowers were washed with ethanol (70%) for 30 seconds to deactivate the NaClO solution and washed five times with sterile distilled water (SDW). Flowers were then transferred with sterile forceps into sterile Petri dishes and allowed to dry for 30 minutes under the laminar flow. Once dried, flowers were transferred into sterile jars kept on ice and a volume of NaCl (0.85% w/v) + Tween80 (0.1 % v/v) solution was added according to the weight previously determined. All samples were disrupted using a mixer mill (Mixer Mill MM200, Retsch) for 20 seconds at 25 Hz. Jars were put back on the ice; the mince was transferred into 2 ml tubes with cut tips and homogenised by brief vortexing. A brief spin in the centrifuge allowed the separation of flower material from the solution. Finally, serial dilutions were made, and 100 µl of each dilution was plated on Reasoner's 2A Agar (R2A, Oxoid) amended with cycloheximide (50 µg/ml) to avoid the growth of fungi and yeasts.

2.1.2. Maintenance of bacterial strains and preparation of bacterial cell suspensions

All bacterial strains and growth media used in this work are listed in Table 1 and Table 2, respectively. Bacterial strains were stored at length in glycerol (40%) at -80°C and routinely grown for 48 h at 27°C on Reasoner's 2A Agar (R2A, Oxoid) and/or Nutrient Agar (NA, Oxoid). *Erwinia amylovora* Ea21 (Ea21) isolated from a diseased apple plant in Trentino (Albanese et al. 2022) and its spontaneous rifampicin-resistant mutant Ea21Rf^R, selected in this study, were used interchangeably in all the experiments. Ea21Rf^R was routinely grown on NA amended with rifampicin 100 µg/ml. Differences in the bacterial cell growth between Ea21 and Ea21Rf^R were evaluated by estimating the bacterial cell growth in a Partial Stigma-Based Medium (PSBMT; Pusey et al. 2008c) according to the procedure reported below. Moreover, differences in the ability to induce the hypersensitive reaction (HR) in tobacco (*Nicotiana tabacum*) leaves were evaluated according to Klement et al. (1964). After 48 h incubation at 27 C°, bacterial cell suspensions were prepared by adding 3 ml of a sterile saline solution (0.85% NaCl, w/v) into Petri dishes, scraping the bacterial cells with a sterile spatula and collecting them in a sterile 15 ml tube. This procedure was applied in all the experiments, with the exception of the inoculation of detached apple flowers. In this case, bacterial cells were resuspended in sterile distilled water (SDW) to avoid any toxic effect of NaCl on apple flowers. The bacterial cell suspensions were adjusted to a final optical density of 0.1 at 600 nm (OD600), corresponding to ≈ 10^8 colony forming units (cfu)/ml and used in all the experiments, except when otherwise indicated.

Table 1. Bacterial strains used in this study.

Bacterial strain	Relevant characteristics	Reference
Erwinia amylovora strains		
Ea21	Wildtype isolated from *Malus domestica* cv. Fengapi	Albanese et al. 2022
Ea21Rf^{R}	Rifampicin-resistant mutant strain	This study
Ea21GFP	Ea21 carrying pBBRMCS5-GFP (Palluchini et al. 2024)	This study
Bacterial strains isolated from *Malus domestica* cv. Golden Delicious		
Pseudomonas extremaustralis AFC1001		This study
Pseudomonas extremaustralis AFC1003		This study
Subtercola frigoramans AFC2001		This study
Pseudomonas helmanticensis AFF1002		This study
Pseudomonas graminis AFF1005		This study
Pantoea agglomerans AFF2001		This study
Acinetobacter sp. AFF2002		This study
Curtobacterium flaccumfaciens AFF2009		This study
Yersinia similis AFF3001		This study
Agreia sp. AFF4002		This study
Rahnella aquatilis AFF4004		This study
Erwinia billingiae AFF4005		This study
Pseudomonas yamanorum AFF5001		This study
Yersinia rochesterensis AFF5003		This study
Pseudomonas graminis AFF6001		This study
Escherichia coli strains		
DH5α pUT-miniTn5-Km	Donor strain for triparental mating	Alexeyev et al. 1995
HB101 pRK2013	Helper strain for triparental mating	Figurski and Helinski 1979

Table 2. Composition of growth media used in this study.

Medium	Component	Concentration (g/l)
Reasoner's 2A Agar (R2A)	Yeast extract	0.5
	Proteose peptone	0.5
	Casein hydrolysate	0.5
	Glucose	0.5
	Starch	0.5
	Di-potassium phosphate	0.3
	Magnesium sulphate	0.024
	Sodium pyruvate	0.3
	Agar	15
Nutrient Broth/Agar (NB/NA)	Yeast extract	2
	Peptone	5
	Sodium chloride	5
	'Lab-Lemco' powder	1
	Agar (for the solid medium)	15
Partial Stigma-Based Medium (PSBMT)	Glucose	25
	Fructose	25
	Amino acid mix (proline, asparagine, glutamine, serine in ratio of 3:2:2:1)	0.2
	NH_4Cl	0.8
	K_2HPO_4	0.3
	NaH_2PO_4	0.1
	$MgSO_4$	0.4
	Nicotinic acid	0.0012
	Thiamine-HCl	0.0026
	Agar	15
Lysogeny Broth (LB)	Yeast extract	5
	Tryptone	10
	Sodium chloride	10
Sucrose Nutrient Agar (SNA)	Yeast extract	2
	Peptone	5
	Sodium chloride	5
	'Lab-Lemco' powder	1
	Sucrose	50
	Agar	15

2.1.3. Identification of bacterial isolates and comparison of metabolic profiles using Biolog assay

Bacterial isolates recovered from apple flowers were initially selected according to their colony morphology and subsequently identified through *16S rRNA* gene sequencing. The *16S rRNA* coding sequences were amplified with Taq polymerase (GoTaq Green Master Mix, Promega) by using the universal primer pair 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′GGTTACCTTGTTACGACTT-3′) (Lane 1991; Stackebrandt and Liesack1993) according to the manufacturer's and sequenced using BigDye Terminator v 3.1. The resulting DNA sequences were analysed using BLASTN to find homologies with sequences deposited in GenBank. 16S rRNA sequences belonging to wild-type bacterial strains were selected. All the sequences were used for phylogenetic analyses using MEGAX software (Kumar et al. 2018). In detail, Clustal X (Thompson et al. 1997) was used for the alignment, and Kimura's two-parameter model (Kimura 1980) was used to establish the evolutionary distances. The best phylogenetic tree was constructed with the neighbour-joining method (Saitou and Nei 1987), and confidence levels for the branches were assessed through a bootstrap analysis with 1000 replicates (Felsenstein 1985). Since competition for nutrients and space might be one of the factors affecting flower colonization, Biolog Phenotype MicroArray Plates™ (PM) were used to identify and compare the metabolic profiles of all bacteria used in this study. The assay was performed according to the manufacturer's protocol (Biolog Inc., CA, USA). Carbon sources (PM1, PM2A) and nitrogen sources (PM3B) were tested. Plates were inoculated with all the bacteria strains and incubated for 24 hours at 27°C to monitor their growth. The growth curves of the bacterial strains were analysed using Analysis 1.7 software. The signal intensity was measured in Omnilog Units (OU), which correspond to the optical density (OD) derived from the reduction of the tetrazolium dye used in the assay. The results were visualized in a heatmap, where different colours

represent different OD values and thus varying metabolic activity. The colour scale ranges from purple to yellow, with purple indicating zero metabolic activity and yellow representing high metabolic activity.

2.1.4. Evaluation of bacterial strain ability to protect detached apple flower against Erwinia amylovora *Ea21*

Detached apple flower assay was performed according to Pusey (1997) with slight modifications. Briefly, sixty newly open apple flowers of *M. domestica* cv. Golden Delicious were collected and transferred in 5 ml tubes with the peduncle submerged in 4.5 ml of sucrose solution (10% w/v). Apple flower stigmas were inoculated by dropping 2 µl of each bacterial cell suspension (10 µl/flower) and allowed to dry under the laminar flow. Subsequently, apple flowers were enclosed in plastic containers and incubated at 25°C in a growth chamber (80% humidity). After 24 h, 10 µl of an Ea21 suspension (≈10^7 cfu/ml) were deposited on the stigmas as previously described. Apple flowers treated with SDW without inoculation of Ea21 and apple flowers treated with SDW and inoculated with Ea21 were used as controls. Apple flowers were kept at the same incubation conditions reported above. Disease severity was evaluated after four days according to a scale from 0 to 4. In detail, 0: green stigma; 1: necrotic area limited to the tips; 2: half stigma is necrotic; 3: most of the stigma is necrotic; 4: the entire stigma is necrotic. Since each flower has five stigmas, each score represents the average of all stigmas. The experiment was repeated twice, with ten replicates (flowers) for each treatment.

2.1.5. Evaluation of bacterial strain ability to protect immature pear slices against Erwinia amylovora *Ea21*

Immature pears (*Pyrus communis* cv. Williams) were transferred in a plastic container previously sterilised and covered with NaClO solution (2% v/v). The container was left in shaking conditions (60 rpm) for 5 minutes. NaClO solution was discarded, and pears were covered with NaClO solution (0.5% v/v). The container was left in shaking conditions (60 rpm) for 1 minute. The hypochlorite solution was discarded, and the pears were washed three times with SDW. Sterile Petri dishes (90 mm) were filled with three layers of sterile paper moistened with 3 ml of SDW. Pears were cut into small slices with a sterile chopper, and each slice was soaked in one of the bacterial cell suspensions until the entire surface became wet. The dishes were dried under the laminar flow and incubated in a growth chamber at 25°C (80% humidity). After 24 h, 10 µl of an Ea21 cell suspension (≈10^8 cfu/ml) were deposited on each slice and left to dry under the laminar flow. Dishes were kept in the same incubation conditions for five days. Pear slices treated with saline solution without inoculation of Ea21 and pear slices treated with saline solution and inoculated with Ea21 were used as controls. Ea21 virulence on pear slices was evaluated according to a scale from 0 to 6 (Mendes et al. 2021). Symptomless (0); low ooze production with browning in ¼ of the slice (1); production of ooze with browning in half of the slice (2); production of ooze with light browning of the slice (3); production of ooze with dark browning of the slice (4); enhanced production of ooze with intense dark browning of the slice (5); scorched slice (6). Each treatment included three biological replicates (Petri dishes) and three technical replicates (pear slices), and the experiment was repeated twice.

2.1.6. Assessment of potential biocontrol bacteria to prevent hypersensitive reaction in tobacco plants

The test of Klement et al. (1964) was modified as follows. Briefly, bacterial strains were resuspended in sterile saline solution and combined with Ea21Rf^{R} to have a bacterial cell suspension with a final concentration of 10^{8} cfu/ml for each bacterium. The resulting bacterial cell suspension (1 ml) was injected into intercellular spaces (three replicates) per leaf of a healthy tobacco (*N. tabacum*) plant. One tobacco plant was used for each treatment to avoid any crosstalk among injected tobacco leaves. Bacterial strains isolated in this work were injected alone to assess their ability to establish a pathogenic interaction with tobacco plants. Sterile saline solution and Ea21 were injected alone as untreated and positive control, respectively. Tobacco plants were maintained under controlled conditions (25 ± 1 ◦C; 70 ± 10 % RH; 16 h photoperiod) in the greenhouse. The Hypersensitive Response (HR) is one of the plant defence mechanisms against pathogenic bacteria and is characterised by the development of chlorosis on the leaves, followed by localized cell death to restrict the spread of the pathogen (Klement et al. 1964). HR was scored after 48 h by measuring necrotic areas and sectors by using ImageJ software (Schneider et al. 2012). Three replicates were done for each treatment, and the assay was repeated twice. To see whether the bacterial strains were able to reduce the Ea21Rf^{R} population size, the three leaf sectors previously infiltrated were cut into small pieces, transferred into a sterile 15 ml tube containing 3 ml sterile saline solution and briefly vortexed (10 s). The resulting suspension was serially diluted (from 10^{-1} to 10^{-10}) and plated onto NA amended with rifampicin (100 µg/ml) and cycloheximide (100 µg/ml).

2.1.7. Evaluation of contact-dependent and independent antibacterial activity of bacterial strains potential biocontrol agents of Erwinia amylovora *Ea21*

Three bacterial strains were evaluated for their ability to inhibit *E. amylovora* cell growth. The contact-dependent antibacterial activity was evaluated by preparing cell suspensions of Ea21 and potential biocontrol bacteria at 10^7 or 10^8 cfu/ml. In this assay, Ea21Rf^R was used. Volumes of five µl of Ea21 and one of the selected strains were spot co-inoculated onto PSBMT prepared according to Pusey et al. (2008c) with the addition of thiamine-HCl (2.6 mg/l), as illustrated in Table 2. The following combinations were tested: i) Ea21Rf^R (10^8 cfu/ml) + Bacterial strain X (10^8 cfu/ml); ii) Ea21Rf^R (10^8 cfu/ml) + Bacterial strain X (10^7 cfu/ml). Cell suspension at 10^7 and 10^8 cfu/ml of the tested bacterial strains and Ea21Rf^R (10^8 cfu/ml) were spot inoculated individually onto PSBMT as untreated controls. After 48 h incubation at 27°C, each spot was excised from PSBMT with a sterile corkborer (5 mm diameter), transferred in a sterile 1.5 ml microcentrifuge tube containing 1 ml of sterile saline solution and briefly vortexed (10 s). The resulting bacterial cell suspensions were serially diluted (from 10^{-1} to 10^{-10}) and plated onto NA amended with rifampicin (100 µg/ml) to determine the concentration of Ea21Rf^R viable cells. As well, NA was used to determine the concentration of the viable cells of selected bacteria. Three replicates were done for each combination, and the assay was repeated twice.

The contact-independent antibacterial activity of the potential biocontrol bacteria was evaluated by first exposing the bacterial strains to chloroform to kill them, followed by inoculating Ea21. The growth of Ea21 was subsequently monitored to assess whether any secondary metabolites produced by the biocontrol bacteria prior to their death exhibited residual toxicity. This approach

evaluates the potential of biocontrol agents to generate toxic compounds capable of inhibiting pathogen growth even after the bacterial cells are no longer viable. The test was conducted according to the protocol of Puopolo et al. (2016), by spot-inoculating 20 µl of the cell suspensions (10^8 cfu/ml) of potential biocontrol bacteria onto PSBMT and/or R2A contained in Petri dishes (60 mm). After 48 h incubation at 27°C, bacteria cells were killed by using chloroform vapours for one hour under the chemical hood. Subsequently, Petri dishes were aerated under the laminar flow for 30 min and then exposed to the UV rays for another 20 min to avoid any contaminations. Petri dishes containing PSBMT and/or R2A that were not inoculated with any selected bacterial strains were used as a control. A suspension of Ea21 was added to Phosphate-Buffered Saline amended with agar (0.4 g/l; PBSA) to have a final cell concentration of 10^8 cfu/ml. The resulting PBSA containing Ea21 was poured on the treated Petri dishes (3 ml/ Petri dish), and after cooling, Petri dishes were incubated at 27°C. After 48 h, contact-independent antibacterial activity was evaluated by scoring the presence of inhibition haloes surrounding the bacterial cell microcolonies. Three replicates were done for each treatment, and the assay was repeated twice.

2.1.8. Assessment of the mutual influence between Erwinia amylovora *Ea21 and selected bacterial strains on siderophore production*

Siderophore detection assay was performed according to Schwyn and Neiland (1987), and Vasseur-Coronado et al. (2021), with some modifications. PSBMT was poured into Petri dishes (90 mm) and left to dry. Once dried, half part of the growth medium was removed using a sterile scalpel from each Petri dish. CAS Agar was prepared and poured to fill the now empty part of the Petri dishes. Cell suspensions of Ea21 and selected bacterial strains were prepared, and five µl of each cell

suspension of selected bacteria was spot inoculated onto PSBMT at one cm far from the spot of Ea21 cell suspensions (five µl). Both spots were done at 1 cm far from the border with CAS agar. Selected bacterial strains and Ea21 grown alone onto PSBMT at a one cm distance from CAS agar were used as untreated controls. Once the spot inoculated, Petri dishes were incubated at 27°C. After five days, CAS agar discoloration area was calculated with ImageJ software by measuring the side of the halo not in contact with the interacting bacterial strain (when two bacterial strains were tested), thereby enabling the quantification of siderophore production for each strain, or on the corresponding side when one single bacterial strain was tested. Three replicates (Petri dishes) were used for each treatment and the experiment was carried out twice.

2.1.9. Growth curves in Partial Stigma-Based Medium

Selected bacterial strains and Ea21 suspensions were centrifuged at 5,000 rpm for 10 min, washed twice with saline solution, and resuspended in liquid PSBMT to an OD_{600} of 0.1. Each well of a sterile 48-well microplate was filled with 1 ml of each bacterial suspension. The plate was incubated at 27°C in the Synergy 2 (BioTek® Instruments) with horizontal shaking (rpm). The growth of each bacterial culture was estimated by measuring the optical density at 600 nm every hour to build the bacterial growth curves. After 24 h of incubation, the bacterial cell cultures were serially diluted (from 10^{-1} to 10^{-10}) and plated onto NA or R2A to determine the final bacterial cell concentration. The experiment was repeated twice with three replicates for each bacterial strain.

2.1.10. Determination of the effect of bacterial culture filtrates on Erwinia amylovora *Ea21 cell growth and motility*

Selected bacterial strains and Ea21 were inoculated in 12 ml of liquid PSBMT contained in sterile 15 ml tubes and incubated at 27°C on an orbital shaker (180 rpm). Tubes containing PSBMT were used as the untreated controls. After 24h, the bacterial cell cultures were centrifuged at 5,000 rpm for 10 min, and the supernatants were sterilised by filtration (0.2 µm). Once the pH value was determined, half the volume of the supernatant was adjusted to pH 7, while the other half was left unchanged and labelled as *Talis Qualis* (TQ). The supernatants were again filter sterilised, transferred into sterile 15 ml tubes (5 ml each) and inoculated with 100 µl of the Ea21 cell culture. The tubes were incubated overnight at 27°C on an orbital shaker (180 rpm). The resulting Ea21 cell cultures were serially diluted, plated on NA dishes and incubated at 27°C. Ea21 colonies were counted after 48 h. The experiment was repeated three times with three technical replicates for each treatment.

A similar approach was used to test the impact of culture filtrates on Ea21 cell motility. Briefly, the selected bacterial strains were grown in 500 ml bottles containing PSBMT (300 ml) at 27°C on an orbital shaker (180 rpm). After 24h, the bacterial cell cultures were processed as reported above to have half part of the culture filtrate at pH 7, whereas the other half was. A 500 ml flask containing PSBMT (300 ml) was used as the untreated control. Subsequently, 100 ml sterile bottles containing 10 ml of distilled water with two different agar concentrations were autoclaved and mixed with 60 ml of each cultural filtrate. The final agar concentrations used were 0.25% (Zeng et al. 2014) and 0.5% (Rezzonico and Duffy, 2007) for swimming and swarming, respectively. The resulting growth media were poured into Petri dishes (60 mm) and allowed to dry overnight; an aliquot of Ea21 cells grown

on NA dishes (48 h) was placed at the centre of each Petri dish using a one µl loop and incubated at 27°C. The motility area was evaluated with ImageJ software after 24 and 48 hours for swimming and swarming assay, respectively. The experiments were repeated three times with ten technical replicates for each treatment.

2.1.11. Assessment of the effect of bacterial culture filtrates on Erwinia amylovora *Ea21 biofilm production*

The assay was performed according to Peng et al. (2021) with slight modifications. Selected bacterial strains were inoculated in sterile liquid PSBMT (5 ml) contained in sterile 15 ml tubes and grown for 24 h at 27°C on an orbital shaker (180 rpm). Similarly, Ea21 was inoculated in 10 ml of sterile Lysogeny Broth (LB, Fluka) contained in sterile 15 ml tubes and grown for 18 h at 27°C on an orbital shaker (180 rpm). Bacterial cell cultures were centrifuged at 5,000 rpm for 10 min, and the supernatants were handled as reported above. Ea21 cultures were centrifuged at 5,000 rpm for 10 min, washed twice with saline solution and resuspended in LB to an OD_{600} of 2. Wells of a sterile 96-well plate (Costar, 3595) were treated with 200 µl of acetone for 20 s (Davies and Marques, 2009; Peng et al. 2021), dried under the laminar flow hood and filled with 100 µl of the Ea21 suspension. The same volume (100 µl) of either PSBMT, bacterial cell culture filtrate (pH 7 or as it was) or SDW were added to the wells to have a final Ea21 cell OD_{600} of 1. The media resulting from these combinations were PSBMT + LB, culture filtrate + LB, culture filtrate (pH7) + LB, and SDW + LB. The plate was incubated at 27°C, 10 rpm. After 48h, the wells were stained with 10% crystal violet, washed with distilled water and dried. 300 µl of a solution made with methanol acetic acid solution (40% and 10%, respectively) were added to each well. After 1 h of incubation, the biofilm production was assessed

by measuring the absorbance at 594 nm. The experiment was repeated twice with seven replicates for each treatment.

2.1.12. Sequencing and assembly of Pantoea agglomerans *AFF2001 genome*

Genomic DNA was extracted from *Pantoea agglomerans* AFF2001 using DNeasy Blood and Tissue Kit (Qiagen) following the protocol according to the manufacturer's protocol. The whole genome was sequenced through Oxford Nanopore technology at the Edmund Mach Foundation (San Michele all'Adige, Italy). Part of a flow cell version 9.4 was employed. The generated reads were then assembled following the bacterial-genomes pipeline implemented with epi2me flye V.2.9.3-b1797 (Kolmogorov et al. 2019) and polished with medaka V.1.11.3 (medaka: Sequence correction provided by ONT Research). Finally, the genome was annotated with a RAST annotation server (Aziz et al. 2008) and subsequently analysed with antiSMASH 7.0 (Blin et al. 2023) to search for any bioactive secondary metabolites.

2.1.13. Construction and screening of Pantoea agglomerans *AFF2001 knockout mutants*

To investigate the molecular mechanisms involved in the biocontrol activity, knockout mutants of *P. agglomerans* AFF2001 were created via transposon mutagenesis (Alexeyev et al. 1995). Triparental mating was performed according to Williams and Stavrinides (2020), with slight modifications. *E. coli* DH5α pUT-miniTn5-Km was used as donor strain (D), *E. coli* HB101 pRK2013 as helper strain (H), and a spontaneous rifampicin-resistant mutant of *P. agglomerans* AFF2001 as recipient strain (R). Briefly, the three bacterial strains were inoculated in sterile liquid LB amended with either kanamycin 50 µg/ml for donor and helper strains or rifampicin 100 µg/ml in the case of

the recipient strain and grown for 16 hours on an orbital shaker (180 rpm). Overnight bacterial cultures were then centrifuged at 5,000 rpm for 5 min and washed three times with sterile NaCl solution. Bacterial suspensions were mixed in a 20:20:1 (D:H: R) ratio, and 100 µl of the triparental mix was spot inoculated on LBA plates and incubated at 30°C. After 24 hours, each spot was resuspended in 1 ml of sterile saline solution, and 100 µl of the suspensions were plated on NA amended with kanamycin 25 µg/ml and rifampicin 100 µg/ml and incubated at 27°C until single bacterial colonies were visible on the plates.

The screening of the effects of the transformant on the growth of *E. amylovora* Ea21 was done as follows: Ea21 was transformed with plasmid pBBRMCS5-GFP (Pallucchini et al. 2024), which contains the Green Fluorescent Protein (GFP) and named Ea21GFP. Bacterial suspensions of *P. agglomerans* AFF2001 mutants and Ea21GFP suspension were spot co-inoculated onto PSBMT plates and incubated at 27°C. After 48 hours, bacterial spots were exposed to the UV light and the transformants whose co-spot resulted fluorescent or showed a different morphology compared to the positive control (*P. agglomerans* AFF2001 wildtype with Ea21GFP) were selected for further analysis. Fluorescent halos were recorded as an indication of Ea21GFP viability and growth.

To understand the impact of the mutated genes on the biocontrol activity of *P. agglomerans* AFF2001, the plant protection efficacy of all mutants was evaluated both on apple flowers and pear slices to see any differences in the control of fire blight symptoms and *E. amylovora* virulence. To avoid any possible biased results in the assays, the growth rate of the knockouts was determined in Nutrient Broth with an incubation time of 16 hours.

Subsequently, the insertion of the mini-Tn5 transposon in the genome of the selected mutants was determined following what was reported by Ochman et al. (1988), Smith et al. (2016), and Williams and Stavrinides (2020). First, the genomic DNA of each transformant extracted with the Qiagen DNeasy Blood and Tissue Kit was digested with the HincII restriction enzyme. The digestion product was then ligated, purified and used as a template for a PCR reaction with primers npt+772 (5′-TTCGCAGCGCATCGCCTTCTATC-3′) and npt-41 (5′-AGCCGAATAGCCTCTCCACCCAAG-3′). Finally, the transposon insertion sites were identified by BLASTing the amplified sequences against the draft genome of *P. agglomerans* AFF2001 and using the RAST annotation server.

2.1.14. Characterization of Pantoea agglomerans *AFF2001 knockout mutants*

All *Pantoea agglomerans* AFF2001 knockout mutants were characterised *in vitro* by testing motility, siderophore production and bacterial culture filtrates. All these assays were done as previously described. The biofilm formation was instead performed according to Nagórska et al. (2008) and Yaryura et al. (2008) with slight modifications. Briefly, bacterial suspensions of each mutant were prepared (OD_{600}=1). Ten µl of the suspension was inoculate into 1 ml of PSBMT in the wells of a 48-well plate and incubated at 27°C for 48 hours. The medium was removed by inverting the plate and the plate was dried at 37°C for 15 minutes. One ml of methanol was added to the wells for one minute, discarded and washed with sterile distilled water (SDW). After drying again at 37°C for 20 minutes, wells were filled with Cristal Violet (1%) and incubated for 1 minute. Successively, several washes with SDW were done before adding one ml of an ethanol:acetone solution (ratio 4:1).

After 5 minutes of incubation, the biofilm amount was determined at OD_{595}. The experiment was repeated three times with 5 replicates for each bacterial strain.

2.2. *Drosophila suzukii*: a potential vector of *Erwinia amylovora*, the causal agent of apple fire blight?

2.2.1. Maintenance of Drosophila suzukii *colony,* Erwinia amylovora *strains and preparation of bacterial cell suspensions*

The *Drosophila suzukii* colony was started from individuals collected from the Trentino region (Italy) in 2010. Flies were raised according to Rossi-Stacconi et al. (2022). Briefly, all the insects were given a specific diet (Dalton et al. 2016; Rossi-Stacconi et al. 2022) twice a week and kept in flasks at suitable conditions (21°C, 16 L: 8 D photoperiod, 60%-80% relative humidity [RH%]) in an environmental chamber for 3 weeks for fly emergence. According to Boucher and colleagues (2019), food deprived flies were used for all experiments. Three-day-old flies (±1 day) were kept in starvation for 24 hours.

Erwinia amylovora strain Ea21 used in this study was isolated from a diseased apple plant in Trentino (Albanese et al. 2022). Bacterial cells were stored at length in glycerol (40%) at -80°C and routinely grown for 48 h at 27°C on Nutrient Agar (NA, Oxoid). An *E. amylovora* Ea21 strain holding a plasmid containing the Green Fluorescent Protein (GFP) (Pallucchini et al. 2024) was also employed. In this case, NA was amended with gentamycin (25 µg/ml) and chloramphenicol (25 µg/ml). For all the assays, NA amended with 50 g/l of Sucrose (SNA) was inoculated with *E. amylovora* Ea21 and incubated at the same conditions described above. Bacterial cell suspensions

were prepared by adding 3 ml of a sterile saline solution (0.85% NaCl, w/v) into Petri dishes, scraping the bacterial cells with a sterile spatula and collecting them in a sterile 15 ml tube. This procedure was applied in all the experiments. The bacterial cell suspensions of *E. amylovora* Ea21 and *E. amylovora* Ea21GFP were adjusted to a final optical density of 1 at 600 nm (OD_{600}), corresponding to ≈ 10^9 colony forming units cfu/ml and used in all the experiments, except when otherwise indicated.

2.2.2. Assessing the attraction of Drosophila suzukii *to* Erwinia amylovora *Ea21: Pitfall choice test*

The pitfall test was conducted to evaluate the attractiveness of the bacterial ooze produced by *E. amylovora* Ea21 to *D. suzukii*. Individual insects had to choose between a vial inoculated with the *E. amylovora* Ea21 and a non-inoculated vial containing only the growth medium. The experimental setup was designed as follows. Glass vials (20 ml) were filled with 5 ml of SNA, closed with the cap, autoclaved and then left to dry at 45° of inclination. Half of the vials were inoculated with 50 µl of a *E. amylovora* Ea21 suspension, while the other half was not inoculated, representing the treatment and the negative control, respectively. The inoculation was done under the laminar flow hood, and the vials were closed with the cap and incubated for 48 hours at 27°C to allow Ea21 to produce the bacterial ooze. After this time, the vials were opened, and a 5 ml Eppendorf was secured to the opening of the vial with parafilm. To avoid contact with the bacterial medium inside the vial, all Eppendorf tubes were modified by cutting off both ends, one at the tip and the other at the 4 ml mark. This allowed the tubes to function as funnels for introducing *D. suzukii* individuals without allowing them to escape once inside the vial.

Plastic containers (⌀ 12,5 x 12 cm) were used as arenas. The lids were modified by drilling two holes with a diameter of 2.3 cm. One of the holes was sealed using a fine mesh secured with hot

glue. The second hole was plugged with a 50 ml tube previously cut at the 40 ml mark. The tube was fixed to the cap at the hole using hot glue to ensure a tight seal (Figure 2A). Prior to each experimental trial, all the containers and lids were sterilized with ethanol (70%, v/v) and left to dry. One inoculated vial and one non-inoculated vial were placed inside each container (Figure 2B). In addition to the vials, a 1.5 ml Eppendorf sealed with a cotton plug soaked in water was placed in the container to maintain humidity and provide water for the tested *D. suzukii* individuals (Figure 2B).

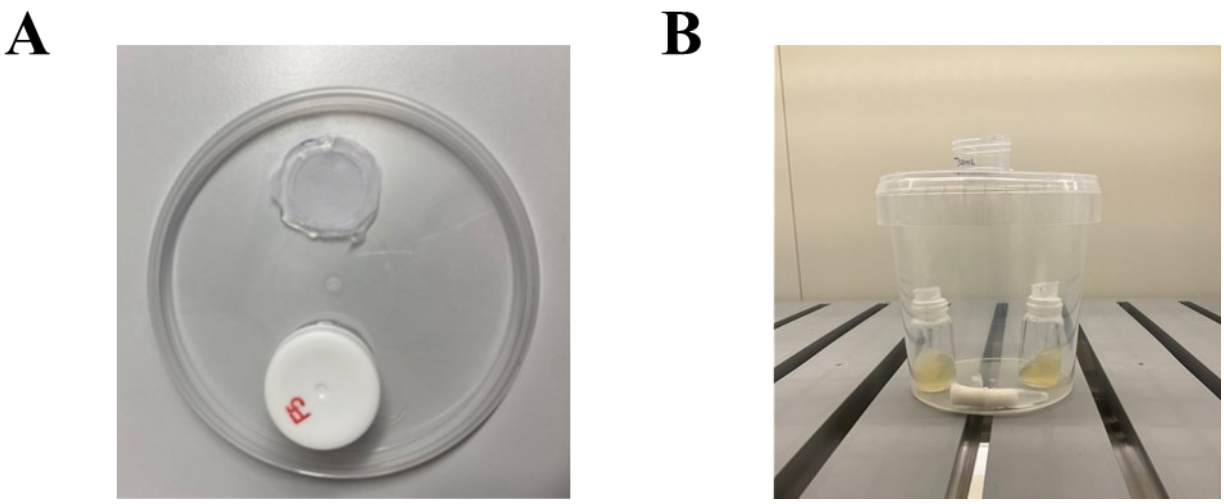

Figure 2. Plastic containers used for the pitfall choice test. A) the cap; B) the vials containing the medium inoculated or non-inoculated with *Erwinia amylovora* Ea21.

Finally, one *D. suzukii* individual was placed into each container subsequently closed with the lid. The distribution of individuals based on sex and the positioning of the vials within the container (inner vs. outer) was randomized as much as possible following the given scheme (Figure 3). All containers were then placed for 48 hours in a climate-controlled chamber at 22°C and 80% relative humidity, with a 16-hour light and 8-hour dark photoperiod. The same shelves were used for all test setups to ensure consistent environmental conditions throughout the trials. A total of 224 replicates

(113 males and 111 females of *D. suzukii*) were conducted using the pitfall choice test across six experimental sessions.

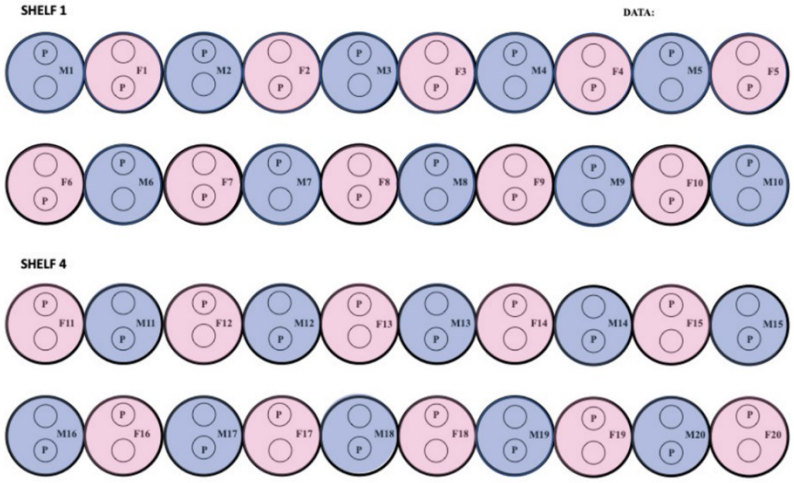

Figure 3. Setup of pitfall choice experiment. Circles represent the plastic container used for the test. Colours indicate whether females (pink) or males (blue) of *Drosophila suzukii* were tested. Letter 'P' (positive control) refers to the vials inoculated with *Erwinia. amylovora* Ea21.

2.2.3. Assessing the attraction of Drosophila suzukii *to* Erwinia amylovora *Ea21: Olfactometer choice test*

The Y-tube olfactometer choice test was performed to assess a possible attraction of *D. suzukii* to any volatile organic compounds (VOCs) released by the bacterial ooze produced by *E. amylovora* Ea21. Compared to the pitfall choice test, the olfactometer experiment presents two main differences. First, the test duration was set to 5 minutes. Second, insect attraction to bacterial ooze was assessed under airflow conditions and not static air, allowing a better analysis of the attractive effect related to volatile compounds emitted by the ooze. The setup used in this study consisted of a Y-tube glass

olfactometer (24 cm; ⌀ 2.5 cm) with two arms connected to sealed plastic containers. In each experiment, one of the two containers presented a SNA petri dish inoculated with 50 µl of a *E. amylovora* Ea21 suspension (namely the treatment), whereas the other contained a SNA plate not inoculated that represented the negative control. An airflow generated by a peristaltic pump (Sera® Precision; Air 275 R Plus) was humidified, purified and split into two equal streams, set at 550 ml min−1 by flow meters (Berk, 2013; Kytola Instruments®, EK-2KR) flowing into the plastic containers and then in the olfactometer's arms (Figure 4).

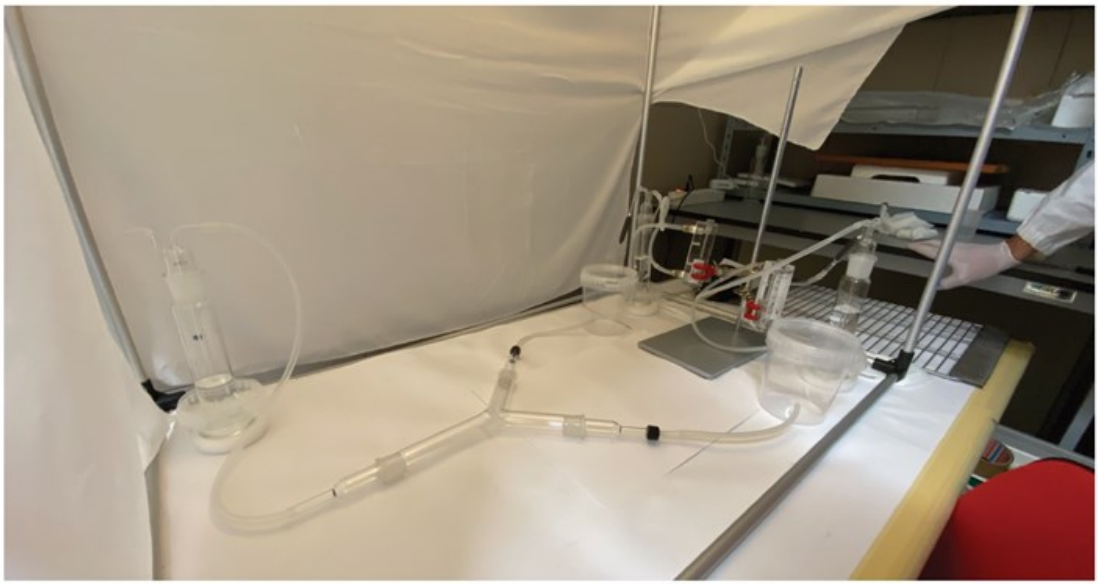

Figure 4. Setup of olfactometer assay. Petri dishes were placed in the two plastic containers at the end of the two arms.

Drosophila suzukii individuals were introduced into the central arm to observe whether they were attracted and moved toward one of the arms where the chemical stimuli were present. *D. suzukii* flies were tested individually in a single examination session consisting of observing one individual at the time. Each session lasted until the individual made a choice, with a maximum duration of 5

minutes. This period was defined as the activation time (*i.e.*, the time allowed for the individual to make a choice). A choice was considered to have occurred when *D. suzukii* flies moved and crossed the midpoint of the two Y-tubes. Individuals that did not move within the allotted 5-minute period were deemed non-responsive and excluded from the analysis. Preliminary experiments tested single individuals; however, due to a very low activation rate, subsequent tests were conducted in groups of 10, with flies separated by sex. A total of 160 individuals (80 females and 80 males) were tested. The experiment was first conducted testing 20 individuals of *D. suzukii* (8 females and 12 males). Subsequently, since no activation was observed, the flies were tested in groups of 10. When tested in groups of 10 individuals, the flies were divided by sex. A total of 16 replicates were conducted, with 8 groups of females and 8 groups of males, for a total of 160 individuals.

2.2.4. Acquisition test: protocol optimization

To assess the acquisition of *E. amylovora* Ea21 by *D. suzukii*, several experiments were performed to test different setups of the acquisition assay reported by Ordax and colleagues (2015).

Inoculation substrate. Several fruits (mature and immature apple and pears) were infected with *E. amylovora* Ea21GFP to evaluate which biological substrate most effectively promoted the production of bacterial ooze. At first, mature apples *Malus domestica* cv. Royal Gala (Biosüdtirol®, VOG) and cv. Golden Delicious (Melinda®) were tested. The fruits were transferred in a plastic container previously sterilised and covered with NaClO solution (2% v/v). The container was left in shaking conditions (60 rpm) for 5 minutes. NaClO solution was discarded, and the apples were covered with NaClO solution (0.5% v/v). The container was left in shaking conditions (60 rpm) for 1 minute. The hypochlorite solution was discarded, and the apples were washed three times with SDW.

The fruits were then left to dry under the laminar flow hood. Eight linear incisions (approximately 0.5 cm deep and 2.5 cm long) were made around the circumference of each fruit with a sterile scalpel and subsequently inoculated with 20 µl of a *E. amylovora* Ea21GFP suspension. Three different bacterial concentrations were inoculated, once for each apple: OD_{600} of 0.1, OD_{600} of 0.5, and OD_{600} of 1. Plastic containers previously sterilised with ethanol (70%, v/v) and UV light were filled with three layers of sterile paper moistened with 1 ml of sterile distilled water to maintain humidity. Apples were then placed inside the containers and incubated at 27 °C for 48 hours. Subsequently, immature pears (*Pyrus communis* cv. Williams) and apples (*Malus domestica* cv. Royal Gala and Golden Delicious) with a diameter of approximately 3 cm, collected in the orchard of the Fondazione Edmund Mach (San Michele all'Adige) were sterilised and inoculated as described above. All infected fruits and *D. suzukii* were placed together inside a cage covered in a plastic film to maintain humidity.

Time of exposure. To determine the experiment duration based on the lifespan of *D. suzukii*, flies were exposed for 7 days to SNA plates exhibiting *E. amylovora* Ea21GFP ooze, as inoculated fruits were not available. Insects were sampled at fours time points (day 0, 3, 5 and 7).

Number of insects. Once the substrate of inoculation and the time exposure were established, the number of *D. suzukii* individuals to be tested was assessed. Thus, flies were exposed to the bacterial ooze developed on SNA plates upon inoculation with *E. amylovora* Ea21GFP. The exposed individuals were pooled together with unexposed flies in ratios 1:1, 1:20 and 1:100 (individuals exposed to *E. amylovora*: individuals not exposed). The pooling was done to evaluate whether the diagnostic method could be applied to large numbers of individuals, as insect captures often yield many specimens, and analysing them individually can be time-consuming. DNA was extracted

according to the method described in the following paragraph and analysed through real-time PCR (qPCR) with the parameters reported below. Four samples (C1, C2, C3, C4) were analysed for each combination and compared to two positive controls (P1, P2), one consisting of a colony of *E. amylovora* Ea21 and the other a previously tested *D. suzukii* harbouring the bacterial plant pathogen. Additionally, two negative controls (N1, N2) were included, one consisting of a *D. suzukii* individual not exposed to the bacterium and the other Milli-Q water. Each sample was tested in duplicate.

DNA extraction. Two DNA extraction methods were compared. In the first method, a single *D. suzukii* fly was put in a 1.5 ml Eppendorf with steel beads, homogenised using a mixer mill (Mixer Mill MM200, Retsch) for 30 seconds at 25 Hz, and filled with 100 µl of 5mm Tris-HCl (pH 8). Eppendorf tubes and steel beads were kept at -20°C prior to homogenisation. Two samples were tested with this 'alternative' method and designated as D1 and D2. In the second one, an individual fly was processed by using a DNA FFPE Tissue Kit (QIAGEN GmbH), eluting the DNA in 100 µl of the suggested buffer and following the manufacturer's protocol. Two samples were tested with the commercial kit and named D3 and D4. A thermal shock (95°C) was subsequently applied to the tubes for 5 minutes, and the sample was stored at -20°C for at least 20 minutes. The effectiveness of both methods was evaluated by performing a qPCR using the primer pairs described by Gottsberger (2010), which target a gene coding for a hypothetical protein. The protocol consisted of 50 cycles of 95°C for 5 sec and 60°C for 30 sec. Before the thermal treatment, dilutions of the samples were plated on SNA plates to assess the viability of *E. amylovora* bacterial cells.

2.2.5. Statistical analysis

Pitfall choice test data were analysed using a G-test with William's correction to evaluate the significance of *D. suzukii*'s choices between the control and the bacterial ooze treatment. This test assessed whether the observed distribution of choices was significantly different from a random distribution.

Concerning the olfactometer trials, a two-tailed exact binomial test was used to assess whether the individuals' attraction to the control or the treatment deviated significantly from a random 50:50 distribution. Additionally, a chi-squared test was performed to determine whether there were significant differences in attraction between males and females in their response to the bacterial ooze. These tests provided insights into both individual preferences and potential sex-based differences in behaviour. All the analyses were conducted with R.

CHAPTER 3. RESULTS

3.1. From apple flowers to control fire blight: new potential biocontrol agents of *Erwinia amylovora*?

3.1.1. Bacterial isolates mainly belong to the Enterobacteriaceae*,* Microbacteriaceae *and* Pseudomonadaceae *bacterial families, and their metabolic profiles revealed differences and similarities with* Erwinia amylovora *Ea21*

Upon a first selection based on the colony morphology, the endophytic bacteria isolated from healthy apple flowers were identified through 16S rRNA gene sequences. The phylogenetic analysis revealed that most of the bacterial strains belonged to *Enterobacteriaceae*, *Microbacteriaceae* and *Pseudomonadaceae* (Figure 5). Among the members of the family *Enterobacteriaceae*, *Erwinia billingiae* AFF4005 belonged to the same genus of *Erwinia amylovora,* and the similarity in the colony morphology was visible on plates. Two bacterial isolates were identified as *Pseudomonas extremaustralis* (AFC1001 and AFC1003) and *Pseudomonas graminis* (AFF1005 and AFF6001). Thus, only one representative isolate per species was tested, namely AFC1001 and AFF1005. *Ps. extremaustralis* AFC1001 had a transparent appearance on R2A plates, while all the other *Pseudomonas* strains were either yellow or white. *Acinetobacter* sp. AFF2002 is the only representative of the *Moraxellaceae* family. Gram-negative bacteria were more abundant since only three out of thirteen endophytes were Gram-positive and belonged to the *Microbacteriaceae* family.

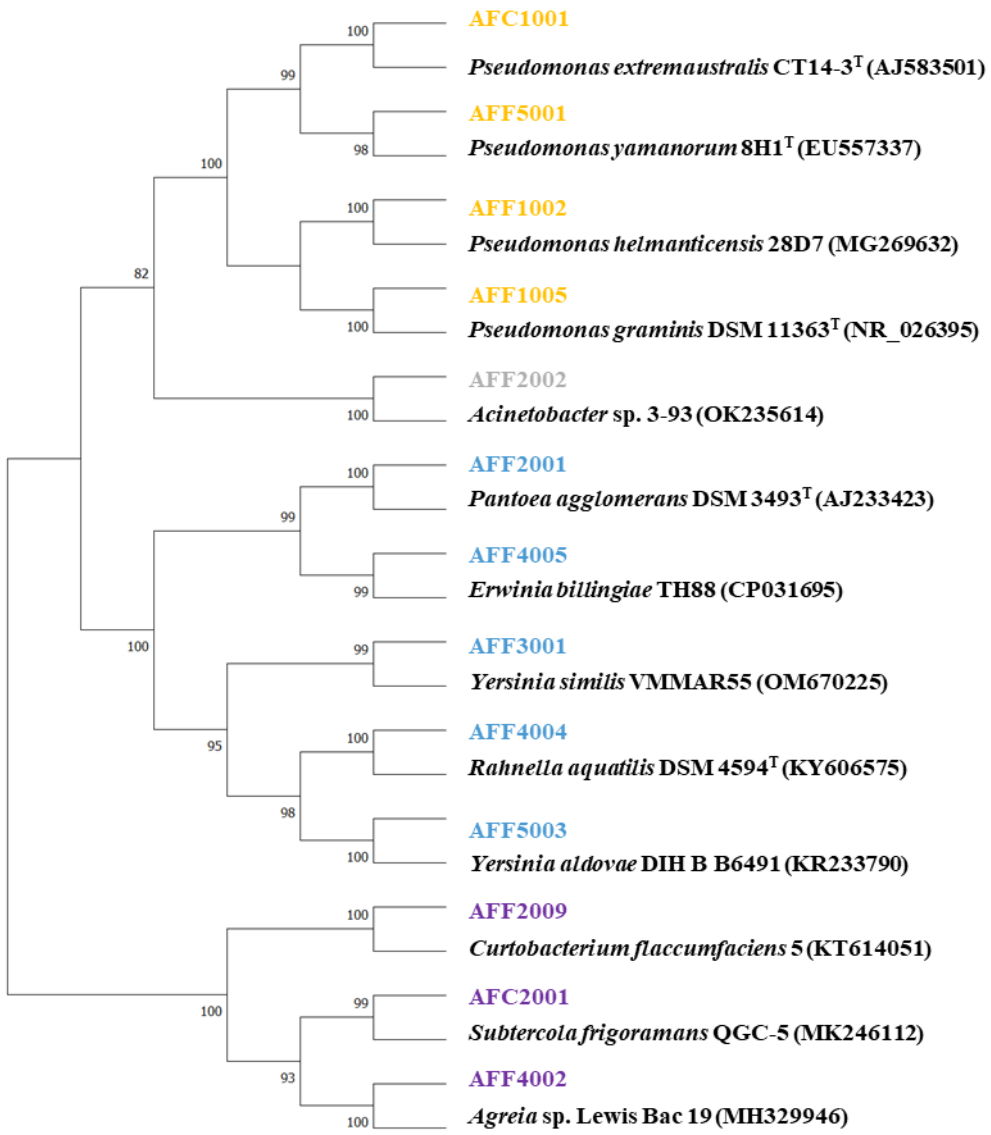

Figure 5. Phylogenetic tree of the endophytic bacteria isolated from apple flowers. Bacterial strains were identified as members of the *Enterobacteriaceae* (blue), *Microbacteriaceae* (violet) and *Pseudomonadaceae* (yellow) families. The analysis was conducted based on *16S rRNA* gene sequences and on the neighbour-joining method (Saitou and Nei 1987). Bootstrap value of 1000 replicates.

The metabolic profiles of apple endophytic bacteria were compared with *E. amylovora* Ea21 to assess a possible overlap in the nutritional requirements, which could lead to competition with the pathogen for floral nutrients, thereby inhibiting its colonization. All bacteria were incubated in the Biolog PM plates to assess whether each compound tested was required for growth. Based on the extent of growth, it was determined whether the compound was metabolized by the bacteria. Bacterial strains belonging to the *Enterobacteriaceae* family (*Pantoea agglomerans* AFF2001, *Erwinia billingiae* AFF4005, *Yersinia similis* AFF3001, *Rahnella aquatilis* AFF4004, *Yersinia aldovae* AFF5003) exhibited a similar carbon source utilisation profile to *E. amylovora* Ea21, with a strong preference for glucose and its derivatives (PM1, Figure 6; PM2A, Figure 7). These substrates include b-methyl-D-glucoside, fructose, galactose, mannitol, N-acetyl-D-glucosamine, ribose, sorbitol, and D-trehalose. High values of Omnilog Units were also measured for gentiobiose, pectin and sucrose. Among the *Enterobacteriaceae* strains, *P. agglomerans* AFF2001 demonstrated the most potential for competition with *E. amylovora* Ea21, particularly for N-acetyl-D-glucosamine, fructose, succinic acid, D-galactose, D-trehalose, and pectin, as indicated by the overlap in the growth curves (data not shown) and by the values in the heatmaps (PM1, Figure 6; PM2A, Figure 7). Interestingly, other bacterial strains from different families, such as *Acinetobacter* sp. AFF2002 and *Subtercola frigoramans* AFC2001, showed very limited assimilation of the carbon sources present in the Biolog plates PM1 and PM2A. The assimilation of nitrogen substrates was more variable among the bacterial strains tested (PM3B, Figure 8). However, it was possible to observe that Ea21 requires specific amino acids, such as asparagine, aspartic acid, cysteine, glutamic acid, glutamine, proline and tryptophan. These were also metabolized by other *Enterobacteriaceae* members (*P. agglomerans* AFF2001, *R. aquatilis* AFF4004, *Y. aldovae* AFF5003), *Pseudomonadaceae* strains (*Pseudomonas extremaustralis*

AFC1001, *Pseudomonas helmanticensis* AFF1002, *Pseudomonas yamanorum* AFF5001) and *Acinetobacter* sp. AFF2002. The endophytic bacteria were generally able to assimilate almost all the amino acids. Only *S. frigoramans* AFC2001, *Curtobacterium flaccumfaciens* AFF2009 and *Acinetobacter* sp. AFF2002 showed low assimilation of the nitrogen sources of the Biolog PM3B plate (Figure 8). Overall, despite their similar metabolic profiles, the main difference among the bacterial strains was the timing of nutrient acquisition, as indicated by the growth curves data recorded by the Omnilog instrument (data not shown).

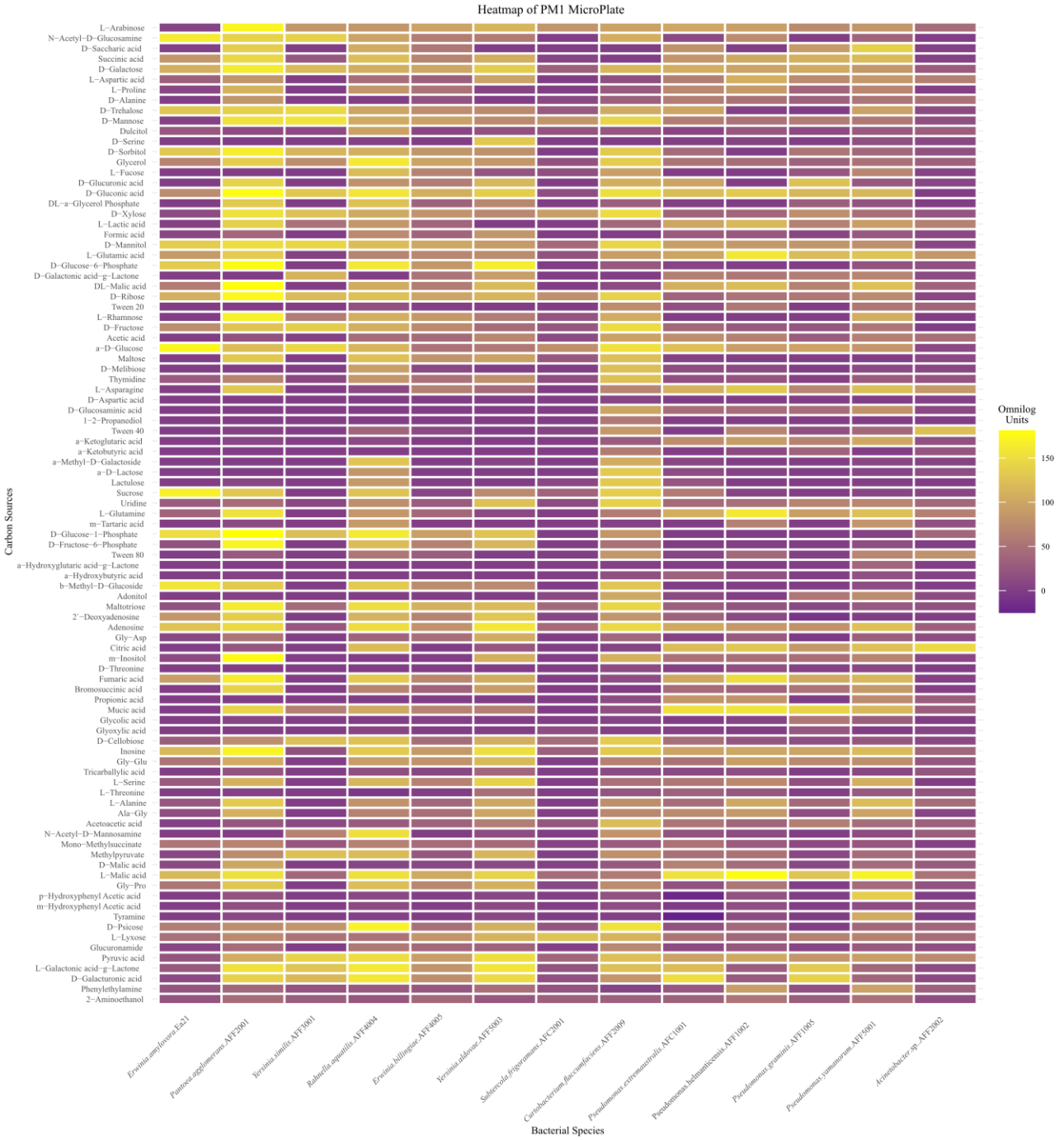

Figure 6. Heat map based on the utilisation patterns of carbon sources from the Biolog PM1 MicroPlate by the strains derived from the microbial communities of apple flowers and *Erwinia amylovora* Ea21. Data were measured in Omnilog Units (OU). The higher is the value, the higher is the assimilation of a certain substrate by the bacterial strain.

Figure 7. Heat map based on the utilisation patterns of carbon sources from the Biolog PM2A MicroPlate by the strains derived from the microbial communities of apple flowers and *Erwinia amylovora* Ea21. Data were measured in Omnilog Units (OU). The higher is the value, the higher is the assimilation of a certain substrate by the bacterial strain.

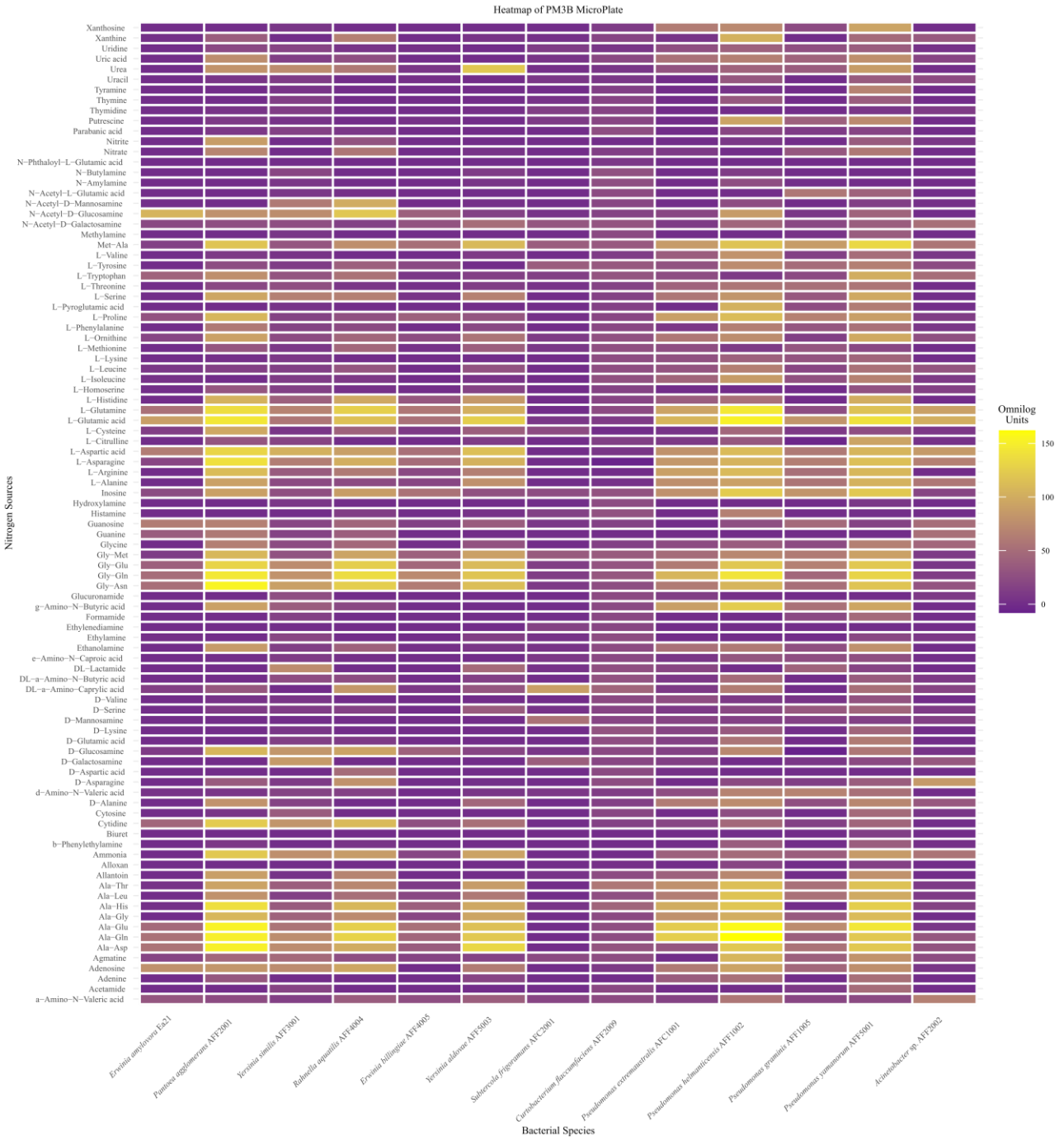

Figure 8. Heat map based on the utilisation patterns of nitrogen sources from the Biolog PM3B MicroPlate by the strains derived from the microbial communities of apple flowers and *Erwinia amylovora* Ea21. Data were measured in Omnilog Units (OU). The higher is the value, the higher is the assimilation of a certain substrate by the bacterial strain.

3.1.2. Acinetobacter sp. *AFF2002,* Curtobacterium flaccumfaciens *AFF2009,* Pantoea agglomerans *AFF2001, and* Pseudomonas extremaustralis *AFC1001 showed the highest efficacy in controlling* Erwinia amylovora *Ea21 on apple flowers and immature pear slices and in preventing hypersensitive reactions in tobacco plants*

To find new potential biocontrol agents, all bacterial strains isolated from healthy apple flowers were tested on newly opened apple flowers to assess their effect on *E. amylovora* Ea21. The stigmas of the flowers were treated by applying 2 µl of each bacterial cell suspension, followed by inoculation with *E. amylovora* Ea21 after 24 h. The symptoms were evaluated on stigmas where *E. amylovora* colonisation takes place at the earliest stage of the infection. Results showed that *Acinetobacter* sp. AFF2002*, C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001, and *Ps. extremaustralis* AFC1001 treatments were effective in controlling fire blight disease development on flowers (Figure 9). The flowers treated with all other bacterial strains did not show any significant reduction in symptoms and were therefore excluded from further analysis. For all these treatments, symptoms of stigmas were significantly reduced compared to the flowers inoculated with Ea21 alone. Except in a few cases, stigmas appeared mildly damaged since the necrotic area was not spread all over the stigmatic surface. This was especially observed in flowers treated with *P. agglomerans* AFF2001, even though the treatment efficacy was not statistically different from that calculated for *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009.

Bacterial strains that were effective on flowers were tested on immature pear slices to assess their effect on the virulence of Ea21. Similarly to what done in the previous assay, pear slices were

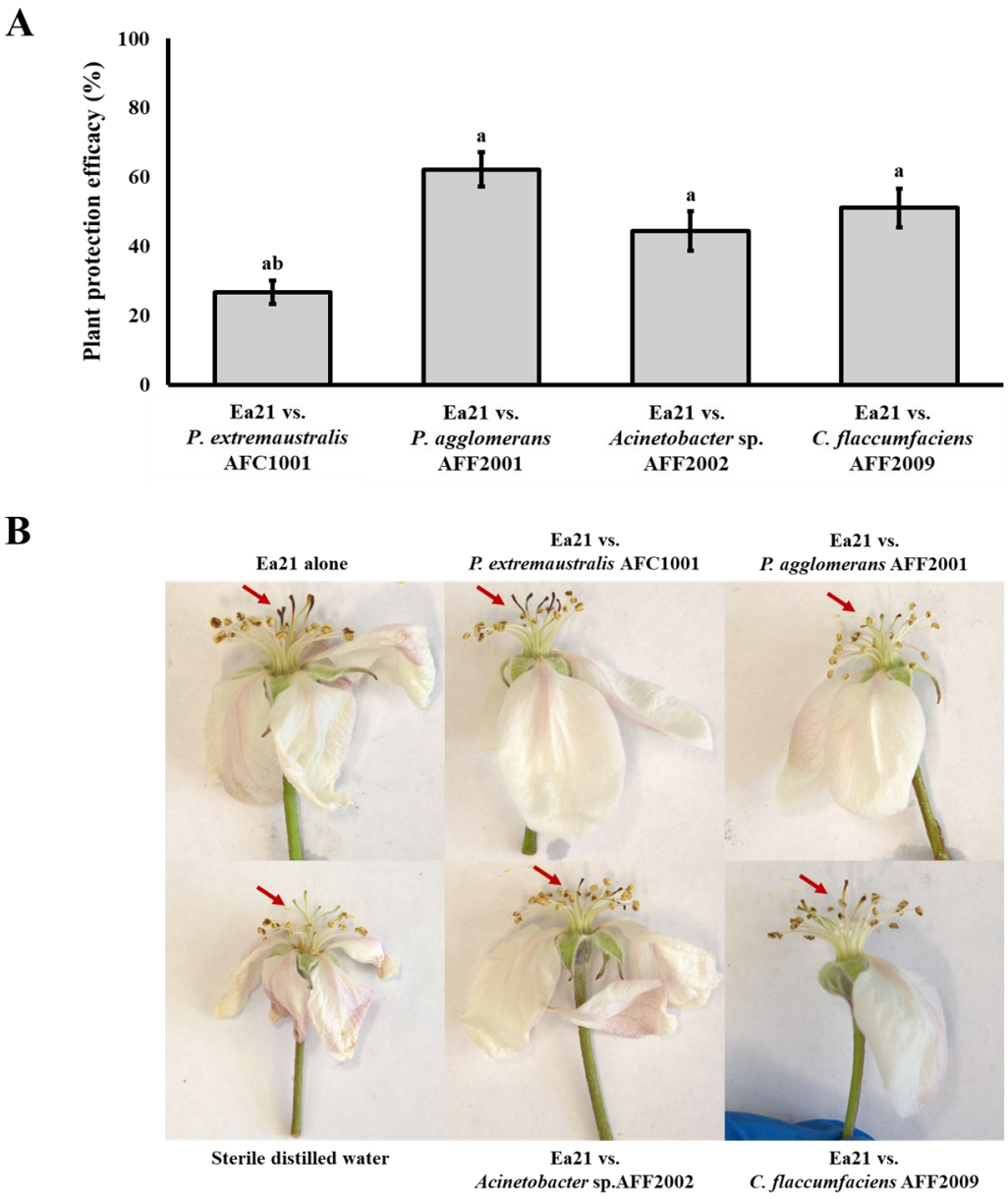

Figure 9. Bacterial strain ability to protect detached apple flowers against *Erwinia amylovora* Ea21. A) Plant protection efficacy. Of all bacterial strains tested, *Acinetobacter* sp. AFF2002, *Curtobacterium flaccumfaciens* AFF2009, *Pantoea agglomerans* AFF2001, and *Pseudomonas extremaustralis* AFC1001 showed the most significant protective effect. Columns represent mean plant protection efficacy ± standard errors of ten replicates. The values indicate the relative percentage change between the flowers treated with the potential biocontrol bacteria prior to the inoculation of Ea21 and those treated with Ea21 alone. Letters indicate values that differ significantly according to Tukey's pairwise comparison test ($\alpha = 0.05$). B) Treated flowers. Red arrows show the necrosis at stigmatic level. Flowers treated with sterile distilled water represent the negative control.

treated with the bacterial strain suspensions 24 h before the inoculation of Ea21. As observed in the detached flower assay, *Acinetobacter* sp. AFF2002, *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 significantly reduced the development of symptoms and their severity (Figure 10A). These include the production of oozes by Ea21 and the occurrence of brown areas on the slices. The efficacy was above 70% in all treatments, but pear slices treated with *P. agglomerans* AFF2001 showed no symptoms at all. When present, the ooze drops were not spread all over the pear slice surface in comparison to the positive control. Moreover, the brownish areas were also strongly reduced. For still unknown reasons, the application of *Ps. extremaustralis* AFC1001 caused side effects. The entire surface of the pear slices appeared brown, including the peel (Figure 10B). However, it was not the typical browning symptoms caused by *E. amylovora.* Thus, it was not possible to evaluate the impact of this bacterial strain on the virulence of Ea21.

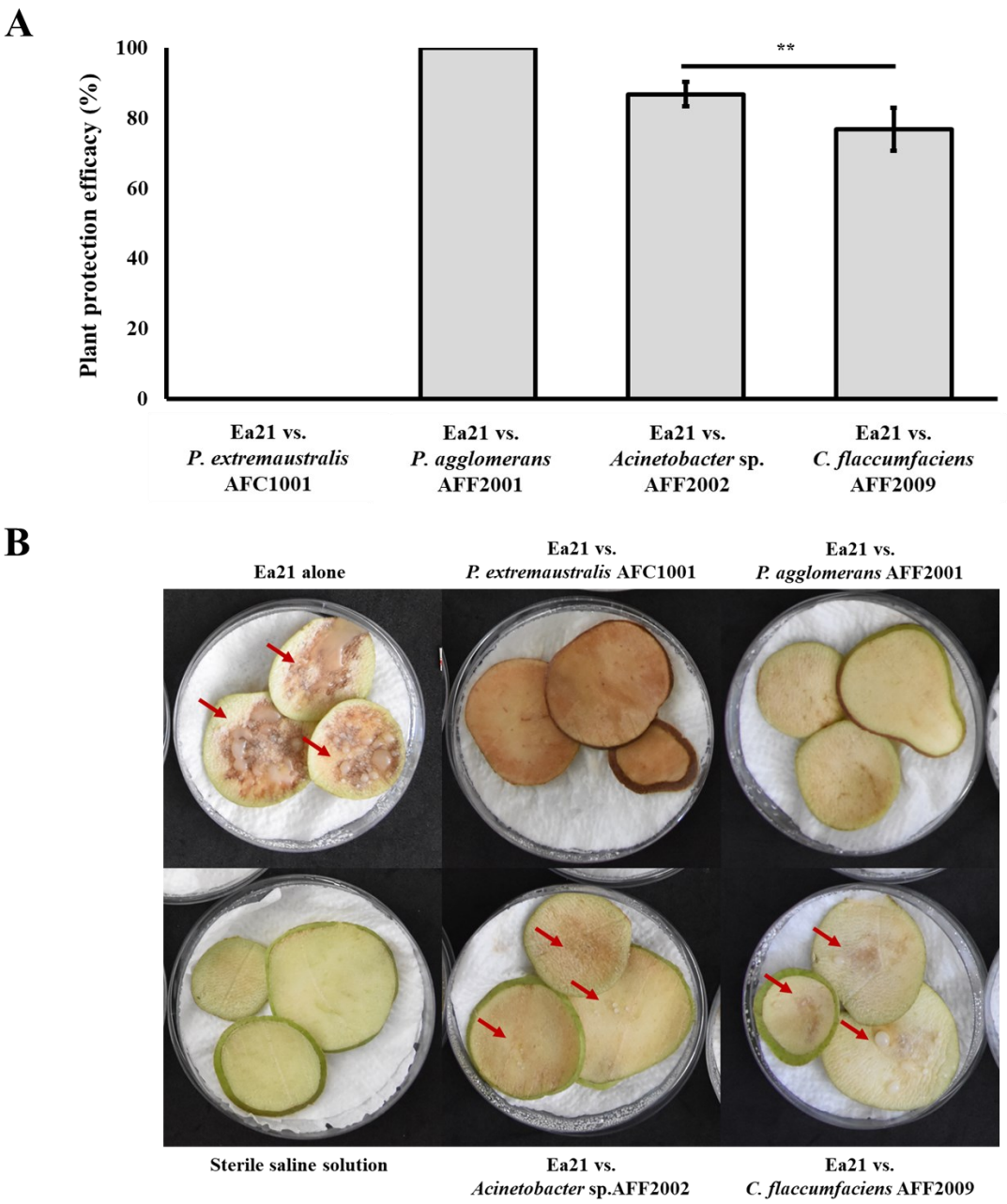

Figure 10. Bacterial strain ability to protect immature pear slices against *Erwinia amylovora* Ea21. A) Plant protection efficacy. *Pantoea agglomerans* AFF2001 showed the highest protective effect of 100% in comparison to the other treatments. However, also *Acinetobacter* sp. AFF2002 and *Curtobacterium flaccumfaciens* AFF2009 were effective. Data related to *Pseudomonas extremaustralis* AFC1001 are not reported since the application of the bacterial suspension had a side effect on the pear slices. Columns represent mean plant protection efficacy ± standard errors of six replicates. The values indicate the relative percentage change between the slices treated with the potential biocontrol bacteria prior to the inoculation of Ea21 and the

slices treated with Ea21 alone. Asterisk indicates values that differ significantly according to the Student's t-test, $p < 0.05$ (* when $p < 0.05$; ** when $p < 0.01$, *** when $p < 0.001$). B) Treated immature pear slices. Red arrows show bacterial ooze produced by Ea21. Pear slices treated with sterile saline solution represent the negative control.

The hypersensitive reaction assay on tobacco was performed to evaluate whether the bacterial strains tested had an impact on the pathogenicity of Ea21 (Figure 11). In contrast to what was observed in the other treatments, *Ps*. *extremaustralis* AFC1001 was not able to prevent HR (Figure 11). The leaf area presenting the symptoms almost corresponded to the leaf area injected. However, the typical collapse of leaf tissue was less severe than in the positive control, meaning there might be a delay in the resistant reaction. Leaf area infiltrated with Ea21Rf^R-*P. agglomerans* AFF2001 had slightly yellow symptoms, and this treatment was the most effective considering the severity of HR. Among the treatments, only *P. agglomerans* AFF2001 significantly reduced the recovery of Ea21Rf^R cells from infected leaf tissue compared to the positive control, with a three-order magnitude difference (10^8 cfu/ml for the positive control and 10^5 cfu/ml for the leaf infiltrated with both *P. agglomerans* AFF2001 and Ea21). Other treatments did not result in a significant reduction of Ea21Rf^R cells.

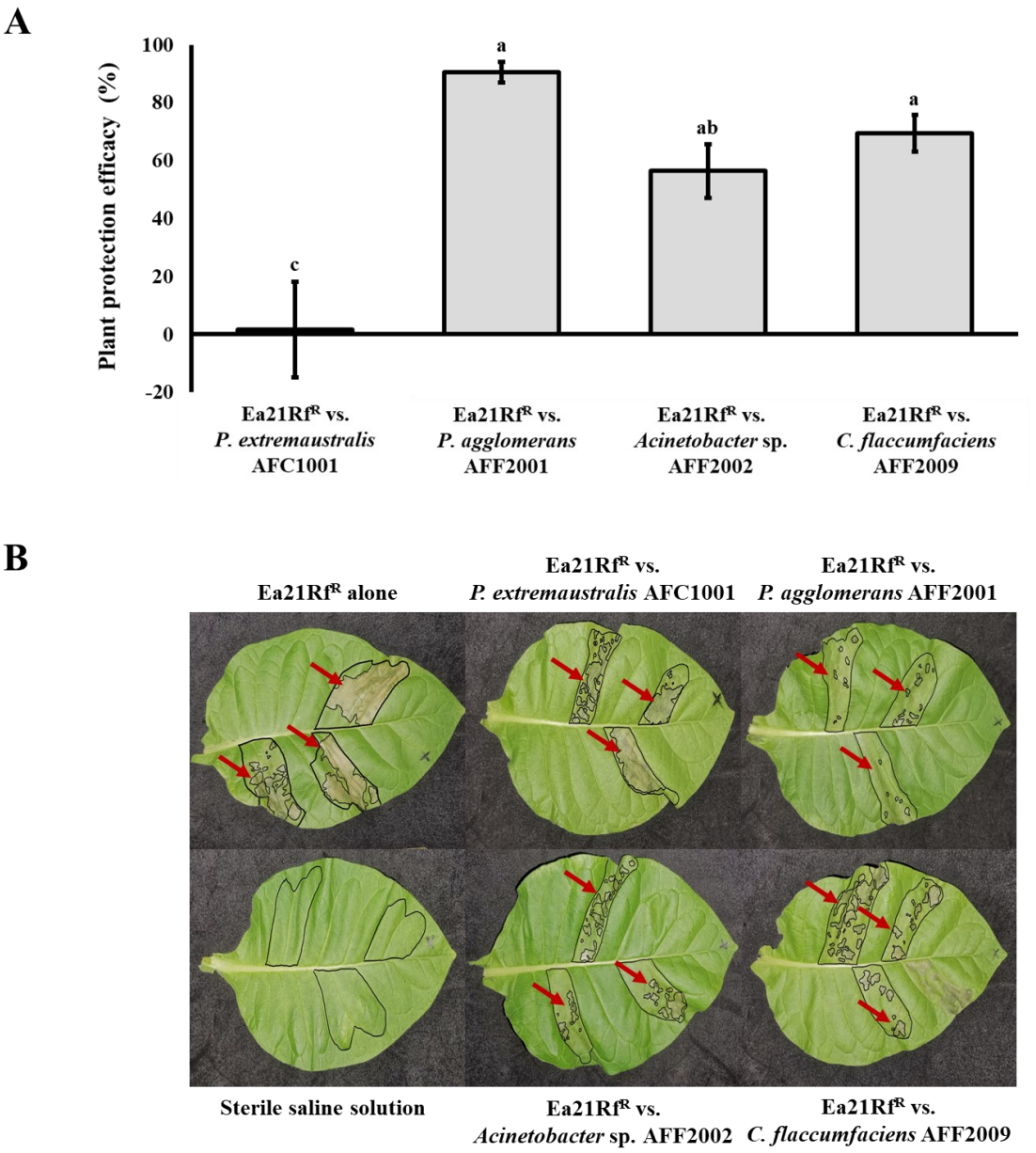

Figure 11. Bacterial strain ability to prevent Hypersensitive Response (HR) in tobacco plants. A) Plant protection efficacy. *Pseudomonas extremaustralis* AFC1001 showed no effect at all since the leaf surface presenting HR symptoms was statistically comparable to the positive control. Columns represent mean plant protection efficacy ± standard errors of three replicates. The values indicate the relative percentage change between the leaves treated with the potential biocontrol bacteria prior to the inoculation of Ea21 and the leaf treated with Ea21 alone. Letters indicate values that differ significantly according to Tukey's pairwise comparison test ($\alpha = 0.05$). B) Infiltrated leaves. The injection of *Erwinia amylovora* Ea21Rf^R suspensions combined with either *Acinetobacter* sp. AFF2002, *Curtobacterium flaccumfaciens* AFF2009, or *Pantoea*

agglomerans AFF2001 produced smaller HR areas in tobacco leaves (red arrows). The infiltration areas are outlined in black. The leaf injected with sterile saline solution represents the negative control.

3.1.3. Pantoea agglomerans *AFF2001 might exert a contact-dependent antibacterial activity against* Erwinia amylovora *Ea21 in the Partial Stigma-Based Medium (PSBMT)*

Acinetobacter sp. AFF2002*, C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 were selected as potential biocontrol agents based on what was observed in apple flowers and in the other plant assays. To investigate their modes of action, the antibacterial activity of the selected bacterial strains on Ea21 cell growth was evaluated.

Ea21Rf^{R} cell suspension was spot co-inoculated with each bacterial strains at different cell concentrations, as reported in Figure 12. All bacterial strains inhibited the growth of Ea21Rf^{R}, even when their cellular concentration was lower than that of the bacterial pathogen. Interestingly, no Ea21Rf^{R} viable cells were recovered in the presence of *P. agglomerans* AFF2001. This bacterial strain showed an inhibition efficacy of 100% and this might indicate a strong contact-dependent antagonism. This antibacterial activity was mild in *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009, whose similar effect was not comparable to that of *P. agglomerans* AFF2001. The biocontrol bacterial cell concentrations ($10^{7}$and 10^{8} cfu/ml) did not influence the observed inhibition effect.

Regarding the assay with chloroform vapours, none of the tested bacterial strains affected Ea21 growth (data not shown), suggesting they likely lack contact-independent antibacterial activity.

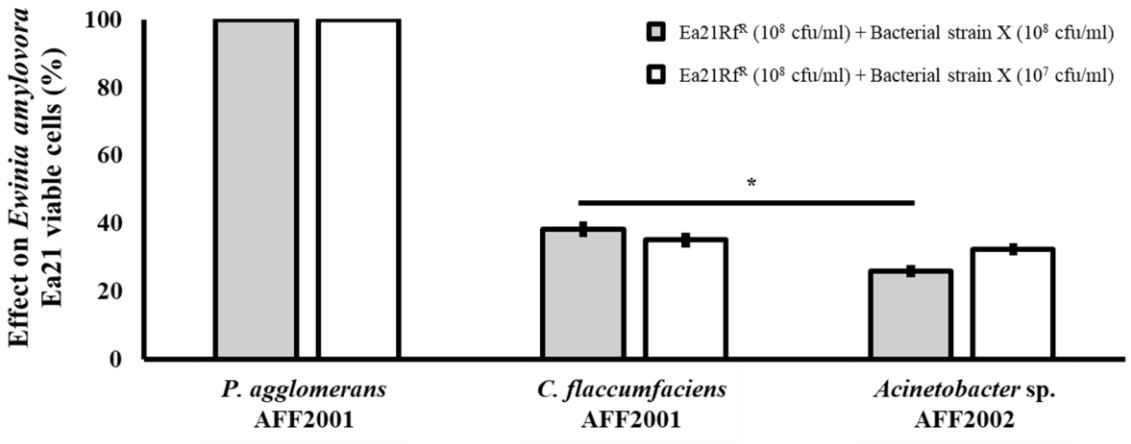

Figure 12. Contact-dependent antibacterial activity of bacterial strains against *Erwinia amylovora* Ea21Rf^R. Ea21Rf^R was spot co-inoculated with each bacterial strain. In all combination tested, a negative effect on the growth of Ea21 was visible. *Pantoea agglomerans* AFF2001 showed the highest efficacy and thus the strongest inhibition ability. Columns represent mean effect ± standard errors of three replicates. For each combination reported, the values indicate the relative percentage change between the growth of Ea21Rf^R in the presence of the potential biocontrol bacteria and the growth of Ea21Rf^R alone. Asterisk indicates values that differ significantly according to the Student's t-test, $p < 0.05$ (* when $p < 0.05$; ** when $p < 0.01$, *** when $p < 0.001$).

3.1.4. The production of siderophores in Pantoea agglomerans *AFF2001 in PSMBT medium is higher in the presence of* Erwinia amylovora *Ea21*

The potential biocontrol agents might compete for nutrients and elements present in the environment. Thus, the iron acquisition ability of each bacterial strain alone and in the presence of Ea21 was evaluated. No CAS agar discolouration area was detected for *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009, underlying these bacterial strains did not release any siderophores under the tested conditions. This was constantly observed even when Ea21 was spot inoculated at 1 cm, meaning there was no competition with bacterial pathogens in PSBMT. However, in the presence of Ea21, an increase in the production of siderophores by *P. agglomerans* AFF2001 was observed, as

shown in Figure 13A, C. Interestingly, *Pantoea agglomerans* AFF2001 seemed to have a similar effect on Ea21, suggesting a possible iron competition between these two bacterial strains. Additionally, the morphology of the microcolonies of Ea21 changed when *P. agglomerans* AFF2001 was spot inoculated at 1 cm. The side of the Ea21 colony facing the other spot appeared less dense (Figure 13C) in comparison to what was observed in the singular spot. Moreover, the Ea21 colony looked more transparent and not fully white as the positive control. Regarding the effect of the selected bacterial strains on Ea21, a higher release of siderophores was observed in Ea21 in the presence of AFF2001 (Figure 13B, C). Interestingly, siderophore production was reduced when Ea21 was grown with AFF2009 and AFF2002 as shown in Figure 13B.

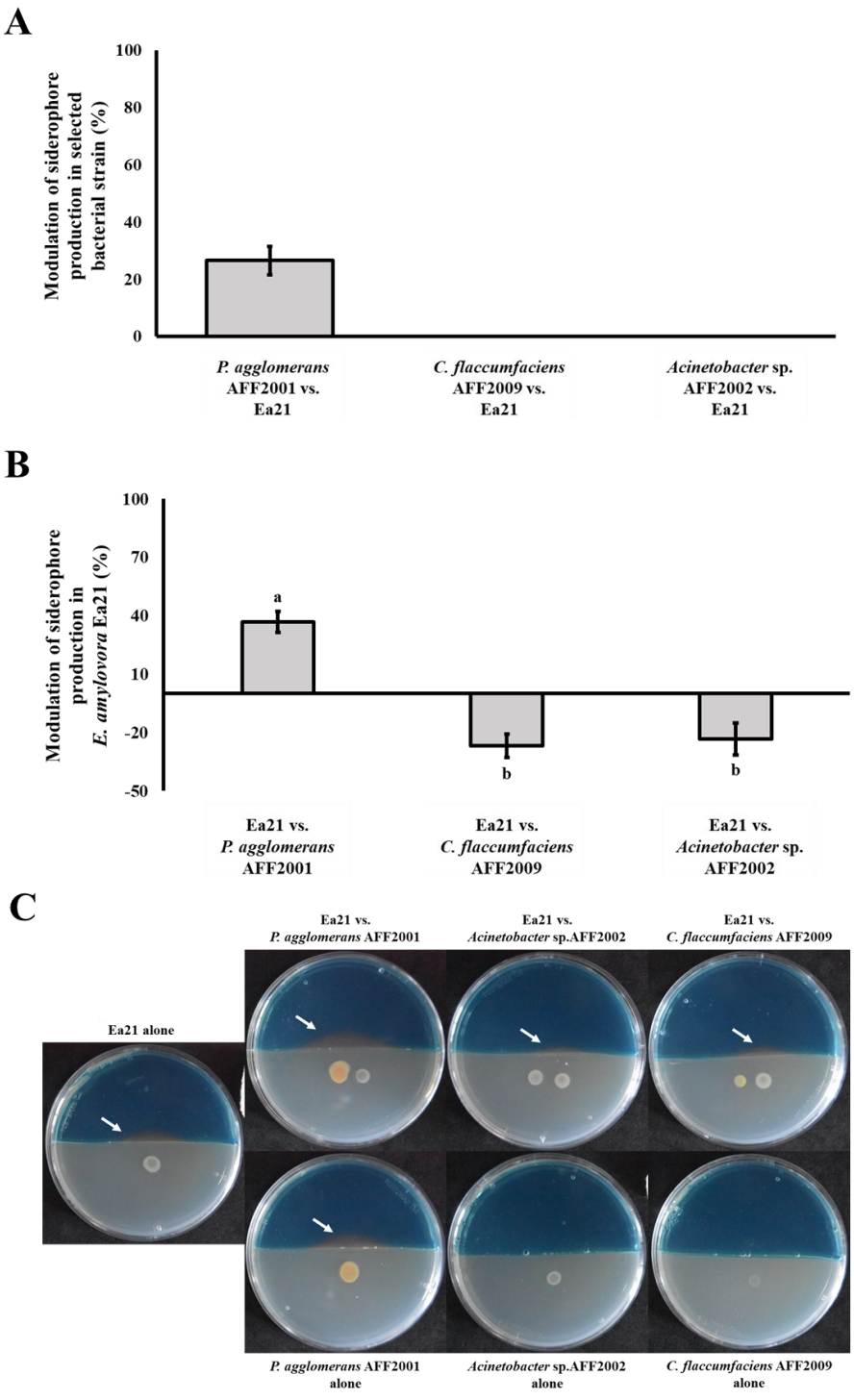

Figure 13. Mutual influence between *Erwinia amylovora* Ea21 and selected bacterial strains on siderophore production. A) *Pantoea agglomerans* AFF2001 increased the release of siderophores in the presence of the

bacterial pathogen. On the other hand, the other two bacterial strains did not seem to need to acquire iron in the tested conditions. Columns represent the mean effect ± standard errors of three replicates. The values indicate the relative percentage change between the area of the discoloration halo produced by each tested bacterial strain in the presence of Ea21 and that observed for each bacterial strain alone. B) During the interaction with *Pantoea agglomerans* AFF2001, Ea21 showed a significant increase in the release of siderophore. On the opposite, the release dropped when Ea21 was grown with *Acinetobacter* sp. AFF2002 and, C*urtobacterium. flaccumfaciens* AFF2009. Columns represent the mean effect ± standard errors of three replicates. The values indicate the relative percentage change between the area of the discoloration halo produced by Ea21 in the presence of the tested bacterial strains and that observed for Ea21 alone. Letters indicate values that differ significantly according to Tukey's pairwise comparison test (α = 0.05). C) Pictures of the plates. White arrows show the discolouration halos that indicate the release of siderophore. n the plates where the two spots are present, EA21 spot is the one on the right.

3.1.5. Bacterial culture filtrates had an impact on Erwinia amylovora *Ea21 cell growth and motility*

To further investigate the modes of action of the bacterial strains selected as potential biocontrol agents, the effect of their bacterial culture filtrates on Ea21 growth and motility was tested. Interestingly, the PSBMT medium, initially at pH 7, became strongly acidic (pH 3.51) upon culturing *P. agglomerans* AFF2001, while the acidification observed in the other treatments was mild (Table 3).

Table 3. pH of *Acinetobacter* sp. AFF2002, *Curtobacterium flaccumfaciens* AFF2009, *Pantoea agglomerans* AFF2001 culture filtrates in Partial Stigma-Based Medium (PSBMT). Values represent the average of three replicates. [1]Letters indicate values that differ significantly according to Tukey's pairwise comparison test (α = 0.05).

Bacterial strain	**pH value**
Pantoea agglomerans AFF2001	3.51b[1]
Acinetobacter sp. AFF2002	6.33a
Curtobacterium flaccumfaciens AFF2009	6.45a

To assess whether the effect observed was mainly influenced by the medium acidification, each bacterial culture filtrate was also tested adjusting the pH to 7. As shown in Figure 14, the bacterial culture filtrates had a negative impact on Ea21 cell viability when their pH was left as originally reported in Table 3 (*Talis quails*, TQ). However, this effect was lower when the pH was raised to 7.

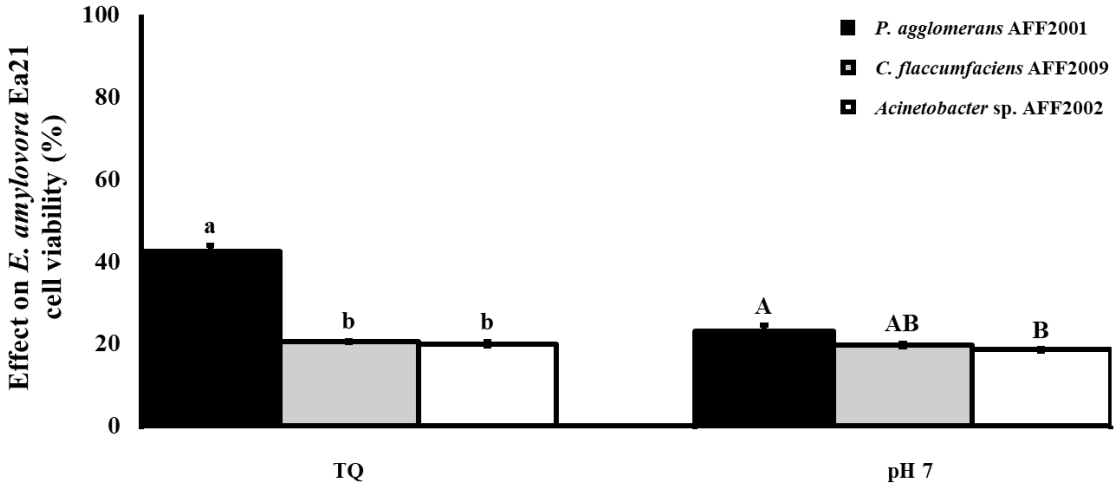

Figure 14. Effect of bacterial culture filtrates on *Erwinia amylovora* Ea21 cell viability. Bacterial culture filtrates were tested without modifying their pH (*Talis Qualis*, TQ) or adjusting it to 7 (pH 7). *Pantoea agglomerans* AFF2001 had the greatest negative impact on Ea21 cell growth in both conditions (TQ and pH 7), but its efficacy was lower than 50%. The columns represent the mean effect ± standard errors of three replicates. The values indicate the relative percentage change between the viability of Ea21 in the bacterial culture filtrates and that observed for Ea21 cultured in PSBMT that had not been previously inoculated. Letters indicate values that differ significantly according to Tukey's pairwise comparison test ($\alpha = 0.05$).

The acidification of *Pantoea agglomerans* AFF2001 culture filtrate significantly inhibited the growth of the bacterial pathogen, which was, however, still able to grow in such acidic conditions. In contrast, the pH 3.51 (*P. agglomerans* AFF2001 TQ) completely inhibited both swimming and swarming motility in Ea21 (Figure 15, Figure 16). Interestingly, inhibition of Ea21 motility was still observed with the *P. agglomerans* AFF2001 culture filtrate at pH 7, showing a 50% and 30%

reduction in swimming and swarming, respectively. This effect was statistically higher than that of the other culture filtrates. According to the results, the *Acinetobacter* sp. AFF2002 culture filtrate (TQ) reduced Ea21 swimming motility similarly to *C. flaccumfaciens* AFF2009 (TQ). However, at pH 7, the *Acinetobacter* filtrate appeared to enhance swimming motility. The opposite trend was observed for swarming motility (Figure 16), where both *C. flaccumfaciens* AFF2009 (pH 7) and *Acinetobacter* sp. AFF2002 (TQ), but not the latter at pH 7, seemed to enhance Ea21 swarming motility.

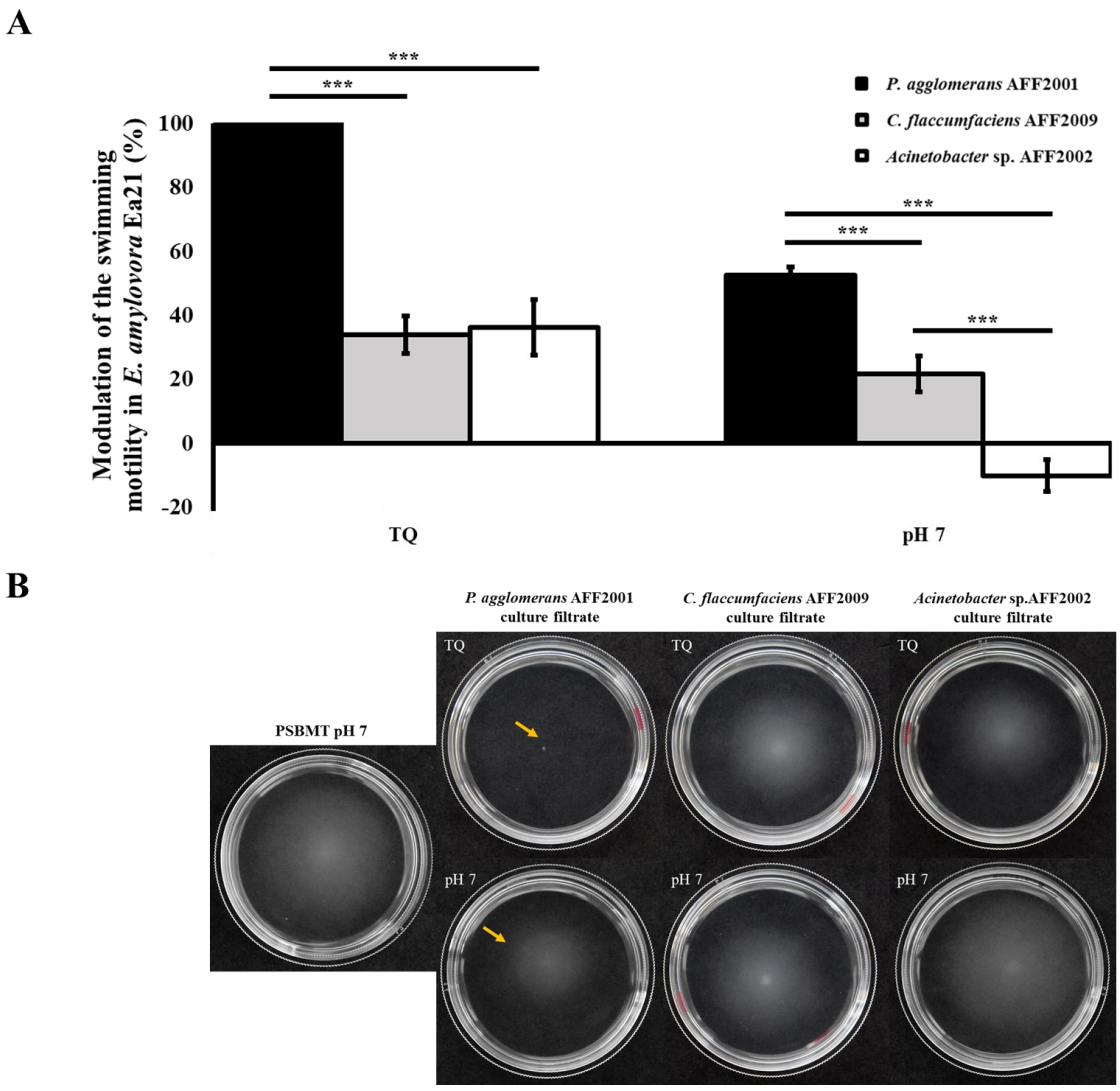

Figure 15. Effect of bacterial culture filtrates on *Erwinia amylovora* Ea21 swimming motility. A) Statistical analysis. Bacterial culture filtrates were tested without modifying their pH (*Talis Qualis,* TQ) or adjusting it to 7 (pH 7). *Pantoea agglomerans* AFF2001 culture filtrate TQ completely inhibited Ea21 swimming motility. The inhibition was lower but still present when the pH was raised to 7. Unexpectedly, *Acinetobacter* sp. AFF2002 culture filtrate pH 7 seemed to enhance the motility of the bacterial pathogen. Columns represent the mean effect ± standard errors of six replicates. The values indicate the relative percentage change between the area of the swimming motility halo produced by Ea21 on the solid bacterial culture filtrates and that observed for Ea21 cultured on solid PSBMT that had not been previously inoculated. Asterisks indicate values that differ significantly according to the Student's t-test, $p < 0.05$ (* when $p < 0.05$; ** when $p < 0.01$, *** when $p < 0.001$). B) Pictures of the plates. Orange arrows indicate the reduced swimming motility halo of Ea21.

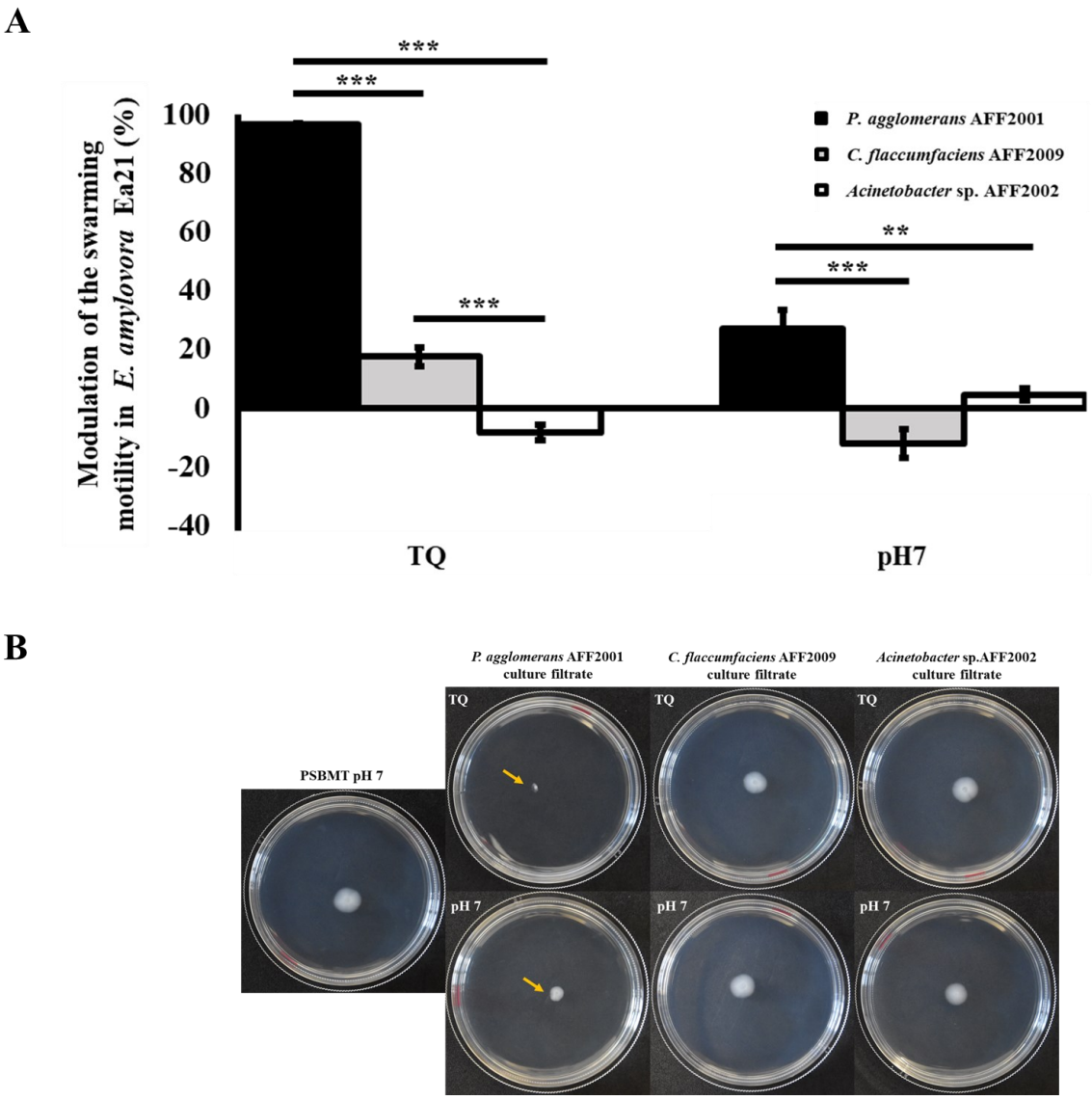

Figure 16. Effect of bacterial culture filtrates on *Erwinia amylovora* Ea21 swarming motility. A) Statistical analysis. Bacterial culture filtrates were tested without modifying their pH (*Talis Qualis*, TQ) or adjusting it to 7 (pH 7). *Pantoea agglomerans* AFF2001 culture filtrate TQ had the most significant inhibitory effect on the swarming motility of Ea21. The inhibition was lower but still present when the pH was raised to 7. Curiously *Acinetobacter* sp. AFF2002 culture filtrate TQ and Curtobacterium *flaccumfaciens* AFF2009 culture filtrate pH 7 did not inhibit the motility. Columns represent the mean effect ± standard errors of six replicates. The values indicate the relative percentage change between the area of the swarming motility halo produced by Ea21 on the solid bacterial culture filtrates and that observed for Ea21 cultured on solid PSBMT that had not been previously inoculated. Asterisks indicate values that differ significantly according to the Student's t-test, $p < 0.05$ (* when $p < 0.05$; ** when $p < 0.01$, *** when $p < 0.001$). B) Pictures of the plates. Orange arrows indicate the reduced swarming motility halo of Ea21.

3.1.6. Uncertain impact of bacterial culture filtrates on biofilm formation in Erwinia amylovora *Ea21of bacterial culture filtrates on biofilm production in* Erwinia amylovora *Ea21*

Since biofilm production is considered a virulence factor of *Erwinia amylovora*, we investigated the effect of bacterial culture filtrates on biofilm production in *Erwinia amylovora* Ea21 (Table 4). Due to the high variability in the data, with both positive and negative values and no clear explanation for this inconsistency, it was not possible to assess the impact or identify consistent trends across treatments.

Table 4. Modulation (%) of *Erwinia amylovora* Ea21 biofilm production by culture filtrates of biocontrol bacteria.

	***P. agglomerans* AFF2001**		***Acinetobacter* sp. AFF2002**		***C. flaccumfaciens* AFF2009**	
	TQ	**pH 7**	**TQ**	**pH 7**	**TQ**	**pH 7**
	36.22	101.79	76.10	-49.07	78.58	74.43
	44.50	19.56	72.39	35.18	-127.63	-4.37
	-75.09	-102.73	-26.75	-3.14	35.60	18.36
	-19.46	41.36	9.88	6.23	29.61	63.40
	26.26	33.37	37.96	12.60	7.12	5.50
	-17.65	-27.42	-43.63	-1.93	9.27	19.29
	-28.07	38.52	20.99	-0.02	43.43	45.77
Average	-4.76	14.92	20.99	-0.02	10.86	32.77
Standard Deviation	42.70	64.29	45.73	25.35	65.58	29.86
Standard Error	16.14	24.30	17.28	9.58	24.79	11.28

3.1.7. Pantoea agglomerans *AFF2001 genome assembly*

The genome of *Pantoea agglomerans* AFF2001 consists of 5,054,504 bp assembled into five contigs (Table 5), with a G+C content of 54.8 %, similar to what was reported for the *P. agglomerans*

type strain FDAARGOS 1447 (55.4%; BioProject PRJNA231221). The *P. agglomerans* AFF2001 genome contains 4932 predicted coding sequences and 98 predicted non-coding RNAs (Table 5).

Table 5. Summary of the genomic characteristics of *Pantoea agglomerans* AFF2001.

	***P. agglomerans* AFF2001**
Contigs	5
Number of bases (bp)	5,054,504
G+C content (%)	54.8
Number of predicted coding sequences	4,932
RNA	98

The longest contig (3,994,567 bp) is predicted to be the chromosome, while BLAST analysis revealed that the other contigs (contig_3, contig_4, and contig_5) contain homologous regions with the plasmids pBH6cv2_D (GenBank accession no. CP134748.1), pASB05p2 (GenBank accession no. CP046724.1), and pASB05p1 (GenBank accession no. CP046723.1), respectively (Table 6).

Table 6. Contigs of *Pantoea agglomerans* AFF2001 with associated features and secondary metabolite predictions.

Contig ID	Length (bp)	Notes	antiSMASH analysis
Contig_1	3,994,567	Predicted chromosome	frederiksenibactin (similarity 84%), aryl polyenes (similarity 94%)
Contig_2	230,479	No homologies	-
Contig_3	54,201	DNA regions homologous to plasmid pBH6cv2_D (identity 97.55%, coverage 57%)	-
Contig_4	196,450	Homologous to plasmid pASB05p2 (identity 99.42%, coverage 100%)	griseoluteic acid (similarity 66%)
Contig_5	578,807	DNA regions homologous to plasmid pASB05p1 (identity 99.09%, coverage 90%)	desferrioxamine E (similarity 100%), carotenoid (similarity 100%)

Plasmids pASB05p1 and pASB05p2 belong to *Pantoea agglomerans* strain ASB05 (Lee et al. 2021), while pBH6cv2_D is associated with *P. agglomerans* strain BH6c (BioProject PRJNA642846). Genome mining with antiSMASH revealed the presence of biosynthetic gene clusters related to frederiksenibactin and aryl polyenes production on Contig_1, while Contig_5 harbours clusters associated with siderophore (desferrioxamine E) and carotenoid (terpene) biosynthesis (Table 6). No known antibiotic-related clusters were identified, except for genes associated with a griseoluteic acid biosynthetic gene cluster located on Contig_4, which shares 66% similarity with reference clusters in the antiSMASH database.

Genes associated with the Type Six Secretion System (T6SS) were also identified with RAST in Contig_1, as shown in Table 7. Moreover, two putative T6SS effectors, homologous to proteins QXB58602.1 and QXB58650.1 from *Pantoea agglomerans* strain FDAARGOS 1447 (BioProject PRJNA231221), were predicted in *P. agglomerans* AFF2001 genome on Contig_1 at positions 306057–306542 and 263367–263846, respectively.

Table 7. Genes associated with the Type Six Secretion System in *Pantoea agglomerans* AFF2001 draft genome.

Gene Function	Location on Contig_1	
	Start	Stop
T6SS component Hcp	263,367	263,846
T6SS component Hcp	306,057	306,542
T6SS associated component FIG00553474	1,373,161	1,373,757
T6SS secretion lipoprotein TssJ (VasD)	1,373,784	1,374,275
T6SS component TssK (ImpJ/VasE)	1,374,275	1,375,624
T6SS outer membrane component TssL	1,375,639	1,376,877
T6SS component TssM (IcmF/VasK)	1,376,881	1,380,510
T6SS associated component TagF (ImpM)	1,380,525	1,381,241
T6SS component TssA (ImpA)	1,381,252	1,382,271
T6SS component TssB (ImpB/VipA)	1,382,353	1,382,886
T6SS component TssC (ImpC/VipB)	1,382,883	1,384,382
T6SS component Hcp	1,384,983	1,385,465
T6SS forkhead associated domain protein ImpI/VasC	1,387,817	1,389,688
T6SS protein serine/threonine phosphatase PppA	1,389,685	1,390,476
T6SS associated component TagJ (ImpE)	1,391,478	1,392,305
T6SS lysozyme-like component TssE	1,392,295	1,392,870
T6SS component TssF (ImpG/VasA)	1,392,873	1,394,744
T6SS component TssG (ImpH/VasB)	1,394,741	1,395,787
T6SS AAA+ chaperone ClpV (TssH)	1,395,842	1,398,451
T6SS Serine/threonine protein kinase (EC 2.7.11.1) PpkA	1,398,479	1,399,984
VgrG protein	1,400,001	1,402,565
T6SS PAAR-repeat protein / RhaS protein	1,404,434	1,408,801
T6SS Serine/threonine protein kinase (EC 2.7.11.1) PpkA	1,436,472	1,435,045
T6SS protein serine/threonine phosphatase PppA	1,438,261	1,437,485
T6SS forkhead associated domain protein ImpI/VasC	1,439,394	1,438,258
T6SS associated component TagF (ImpM)	1,440,129	1,439,431
T6SS component TssM (IcmF/VasK)	1,442,257	1,440,143
T6SS outer membrane component TssL	1,443,474	1,442,257
T6SS associated component FIG00553474	1,444,048	1,443,560
T6SS lysozyme-like component TssE	2,602,338	2,601,886
T6SS secretion lipoprotein TssJ (VasD)	2,602,459	2,602,331
T6SS outer membrane component TssL (ImpK/VasF)	2,602,832	2,602,410
T6SS component TssK (ImpJ/VasE)	2,603,031	2,602,861
T6SS component TssC (ImpC/VipB)	2,603,363	2,603,091
T6SS component TssB (ImpB/VipA)	2,603,903	2,603,403

3.1.8. Pantoea agglomerans *AFF2001 mutants showed a reduced biocontrol activity on apple flowers and immature pear slices*

The efficacy of *Pantoea agglomerans* AFF2001 in controlling *Erwinia amylovora* Ea21 may involve multiple mechanisms. To characterize its biocontrol activity from a molecular perspective, identifying the genes involved, mutants of *P. agglomerans* AFF2001 were generated using the Tn5 transposon system. Over 2,000 knockout mutants were initially screened by observing spot co-inoculation with Ea21GFP on PSBMT. Mutants that failed to inhibit Ea21 growth, displaying a fluorescent co-spot, and/or exhibiting altered spot morphology were selected for further analysis. This screening identified three mutants: A2, A13, and A24. The A2 co-spot showed a fluorescent ring, while the A24 spot appeared transparent. No pigmentation differences were observed in A2 and A13; however, their spots exhibited a lumpy appearance.

Before proceeding with their characterization, the growth of all mutants was tested in NB to rule out any differences that could affect subsequent experiments. The mutants exhibited slightly different growth curves in NB medium (Figure 17A), but their final cell concentrations were comparable (Figure 17B).

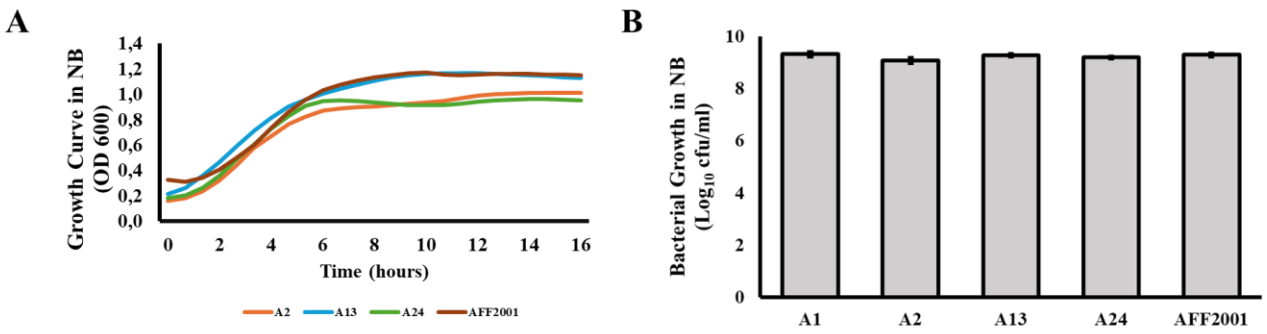

Figure 17. Growth curves of *Pantoea agglomerans* AFF2001 knockout mutants in Nutrient Broth (NB) medium. All mutants showed a growth rate comparable to that of the wild type.

The selected mutants were tested first on flowers and immature pear slices to assess any differences with the wild type in the biocontrol activity against Ea21. All mutants, except for A2, exhibited a significant lower biocontrol activity on flowers compared to the wild type, but none of them completely lost their ability to reduce fire blight symptoms (Figure 18). A24 biocontrol activity was statistically four times lower than *P. agglomerans* AFF2001, with more extensive stigmatic necrosis observed compared to the flowers treated with the other mutants and the wildtype strain. In the immature pear slices assay, only the slices treated with A24 suspension presented Ea21 exudates and slight browning, indicating a lower plant protection efficacy of A24 in comparison to the other mutants (Figure 19A, B). Although the severity of the symptoms was not comparable to that observed in the positive control, the amount of Ea21 cells recovered was equivalent in both treatments (Figure 19C).

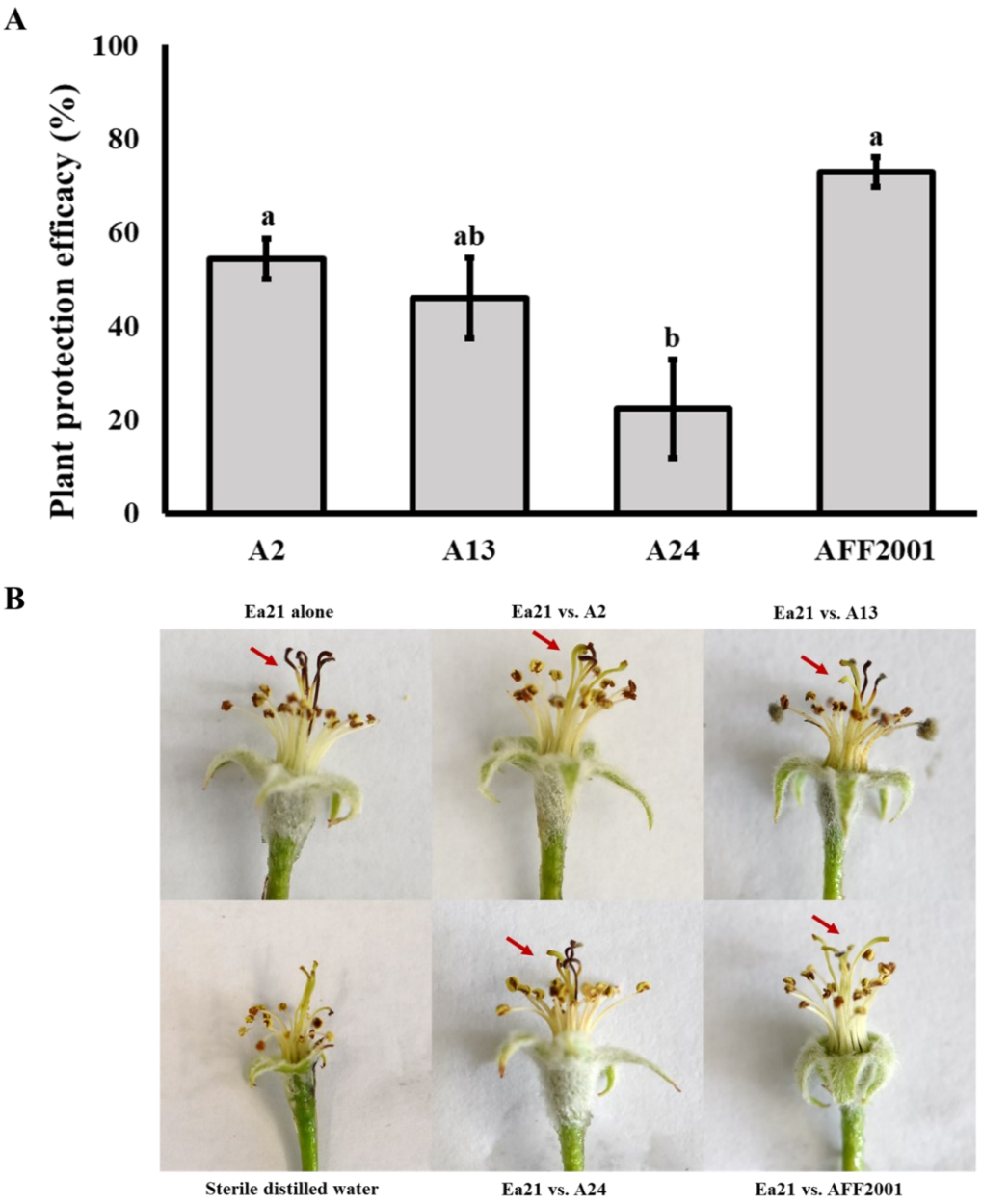

Figure 18. *Pantoea agglomerans* AFF2001 mutants' ability to protect detached apple flowers against *Erwinia amylovora* Ea21. A) Plant protection efficacy. All mutants conserved the biocontrol activity, but only A2 showed a protective effect comparable to the wild type. A24 exhibited the lowest efficacy. Columns represent mean plant protection efficacy ± standard errors of five replicates. The values indicate the relative percentage change between the flowers treated with *Pantoea agglomerans* mutants prior to the inoculation of Ea21 and those treated with Ea21 alone. Letters indicate values that differ significantly according to Tukey's pairwise comparison test ($\alpha = 0.05$). B) Treated flowers. Red arrows show the necrosis at stigmatic level. Flowers treated with sterile distilled water represent the negative control.

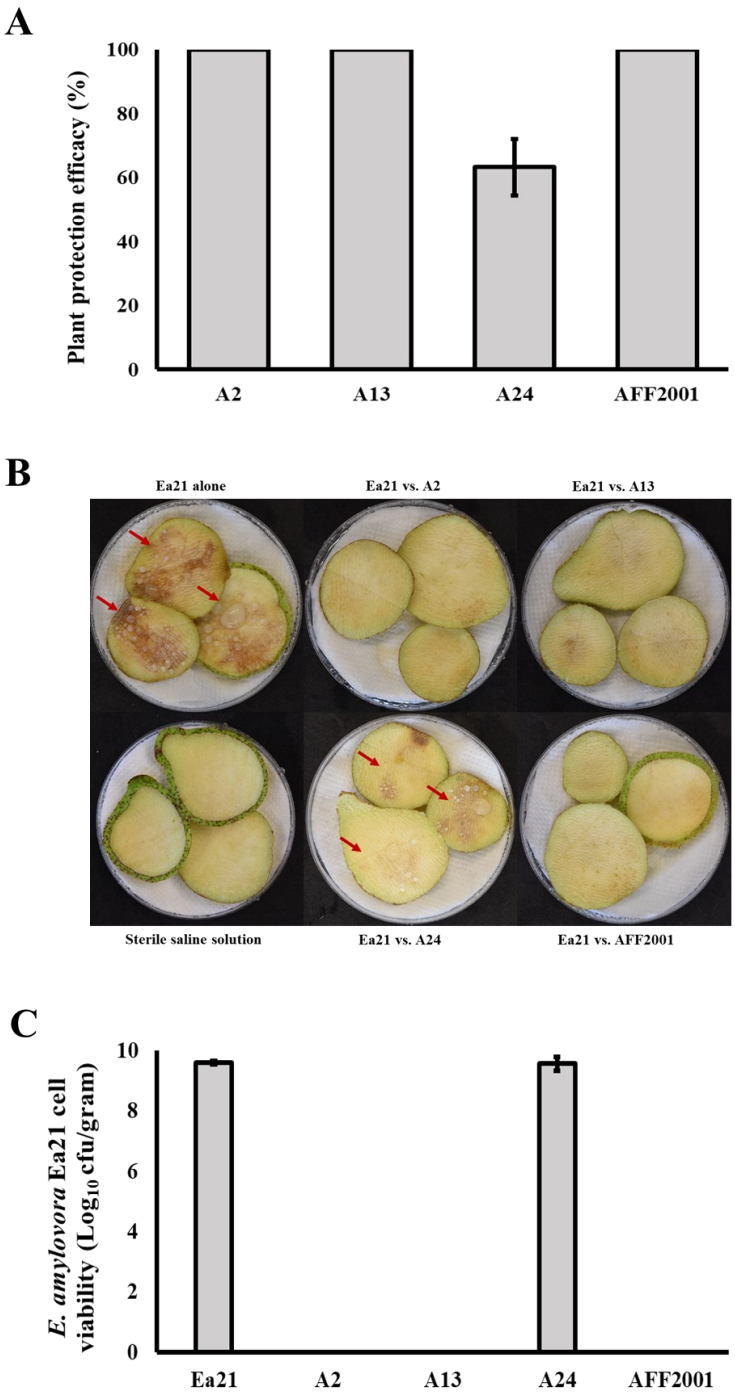

Figure 19. *Pantoea agglomerans* AFF2001 mutants' ability to protect immature pear slices against *Erwinia amylovora* Ea21. A, B) Plant protection efficacy. The reduction in the virulence of Ea21 appeared more evident in A24-treated pear slices, whereas A13 efficacy was almost 100%. Columns represent the mean effect ± standard errors of six replicates. The values indicate the relative percentage change between the flowers treated with *Pantoea agglomerans* mutants prior to the inoculation of Ea21 and those treated with Ea21 alone. Data

related to A24 are typically distributed. Red arrows show bacterial ooze produced by Ea21. Pear slices treated with sterile saline solution represent the negative control. C) Ea21 viable cells recovered from immature pear slices. Columns represent mean plant protection efficacy ± standard errors of three replicates: normal distribution, but no difference according to the Student's t-test, $p < 0.05$.

Since the results observed on the flowers indicated a reduced biocontrol activity in all mutants except A2, the transposon insertion sites in their genomes were identified to determine the disrupted genes. A2 was also included in the analysis, as it could help determine which genes are not involved in the biocontrol activity of *P. agglomerans* AFF2001. The sequences amplified with primers npt+772 and npt-41 were mapped to the *P. agglomerans* AFF2001 draft genome using BLAST. Based on the RAST annotation server, Tn5 insertions were found in the genes *rcsA* encoding the transcriptional regulator RcsA in case of the mutant A2; *pdeR,* encoding the c-di-GMP phosphodiesterase in the mutant A13; and *leuA,* encoding the enzyme responsible for the leucine biosynthesis in the mutant A24 (Table 8).

Table 8. Mini-Tn5 insertion sites in *Pantoea agglomerans* AFF2001.

Mutant	**Genomic region**	**Gene**	**Protein**
P. agglomerans A2	Contig_1: 2,141,131 – 2,141,766	*rcsA*	Colanic acid capsular biosynthesis activation accessory protein RcsA
P. agglomerans A13	Contig_1: 1,860,136 – 1,862,121	*pdeR*	c-di-GMP phosphodiesterase
P. agglomerans A24	Contig_1: 436,542 – 434,980	*leuA*	2-isopropylmalate synthase

3.1.9. Pantoea agglomerans *AFF2001 biocontrol activity might involve genes affecting motility and siderophore production*

To investigate the impact of the knockout genes reported in Table 8 on *Pantoea agglomerans* AFF2001 performance, all mutants were characterised *in vitro* on PSBMT medium for motility, biofilm formation, and siderophore production, which might be potential biocontrol mechanisms.

Except for A2, which exhibited motility comparable to *P. agglomerans* AFF2001, the other mutants displayed different motility patterns. Swimming was impaired in A13 and A24, with the latter showing no movement at all. A13 exhibited reduced motility compared to *P. agglomerans* AFF2001 (Figure 20A). Moreover, the morphology of the A13 halo differed from that of the wild type. The motility area appeared delimited and denser, and the bacterial cells were more concentrated at the centre of the halo as if they were stuck together in a lumpy spot. Differences in the A13 movement area were also seen in the swarming assay (Figure 20B), where there was a visible increase in the spreading ability of this mutant compared to *P. agglomerans* AFF2001. Moreover, in A13, the swarming halo appeared yellow in the centre and white and dense at the edges, while in A2 and AFF2001, the entire halo area was yellow. Similarly to the swimming assay, A24 showed a minimal swarming ability.

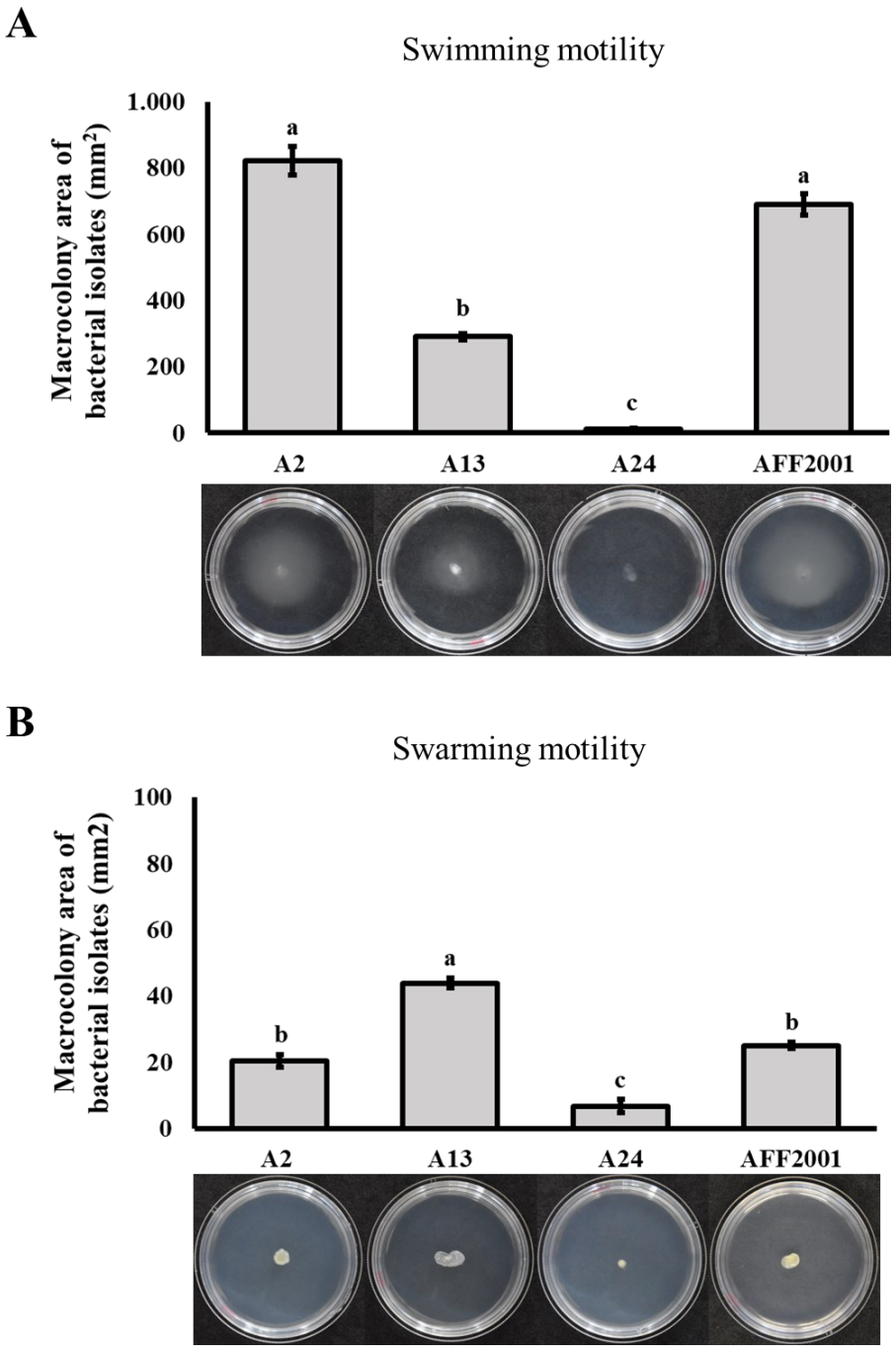

Figure 20**.** Characterisation in vitro of *Pantoea agglomerans* AFF2001 mutants. A) Swimming motility. No differences were observed between A2 and *Pantoea agglomerans* AFF2001 swimming motility. However, there was a significant reduction There was a significant difference in the size of the motility halo for A13, while for A24, it was barely visible. Columns represent mean motility area ± standard errors of six replicates. Letters indicate values that differ significantly according to Tukey's pairwise comparison test (α = 0.05). B)

Swarming motility. Motility was significantly increased in A13 but was nearly absent in A24. Columns represent mean motility area ± standard errors of six replicates. Letters indicate values that differ considerably according to Tukey's pairwise comparison test ($\alpha = 0.05$).

Mutations in *rcsA*, *pdeR* and *leuA* genes affected biofilm formation since in all knockouts showed differences compared to the wildtype (Figure 21A). Interestingly, no biofilm ring was visible in the wells of the 48-well plate inoculated with A24 and no value was measured by the spectrophotometer. On the contrary, in the wells inoculated with A13, the rings were clearly visible and appeared more intense in colour compared to those inoculated with *P. agglomerans* AFF2001. Spectrophotometric analysis confirmed this observation, revealing that the biofilm produced by A13 was nearly twice as abundant as that formed by the wildtype strain. Concerning A2, biofilm production was halved compared to *P. agglomerans* AFF2001, and the rings in the wells were faint.

Since previous results suggested there might be a competition for iron between Ea21 and *P. agglomerans* AFF2001, siderophore production was tested. Mutation in the c-di-GMP phosphodiesterase gene induced a statistically significant reduction in the release of siderophores, as shown in Figure 21B, C. No halo of discolouration was visible on plates inoculated with A24, while data collected for A2 were comparable to the wild type.

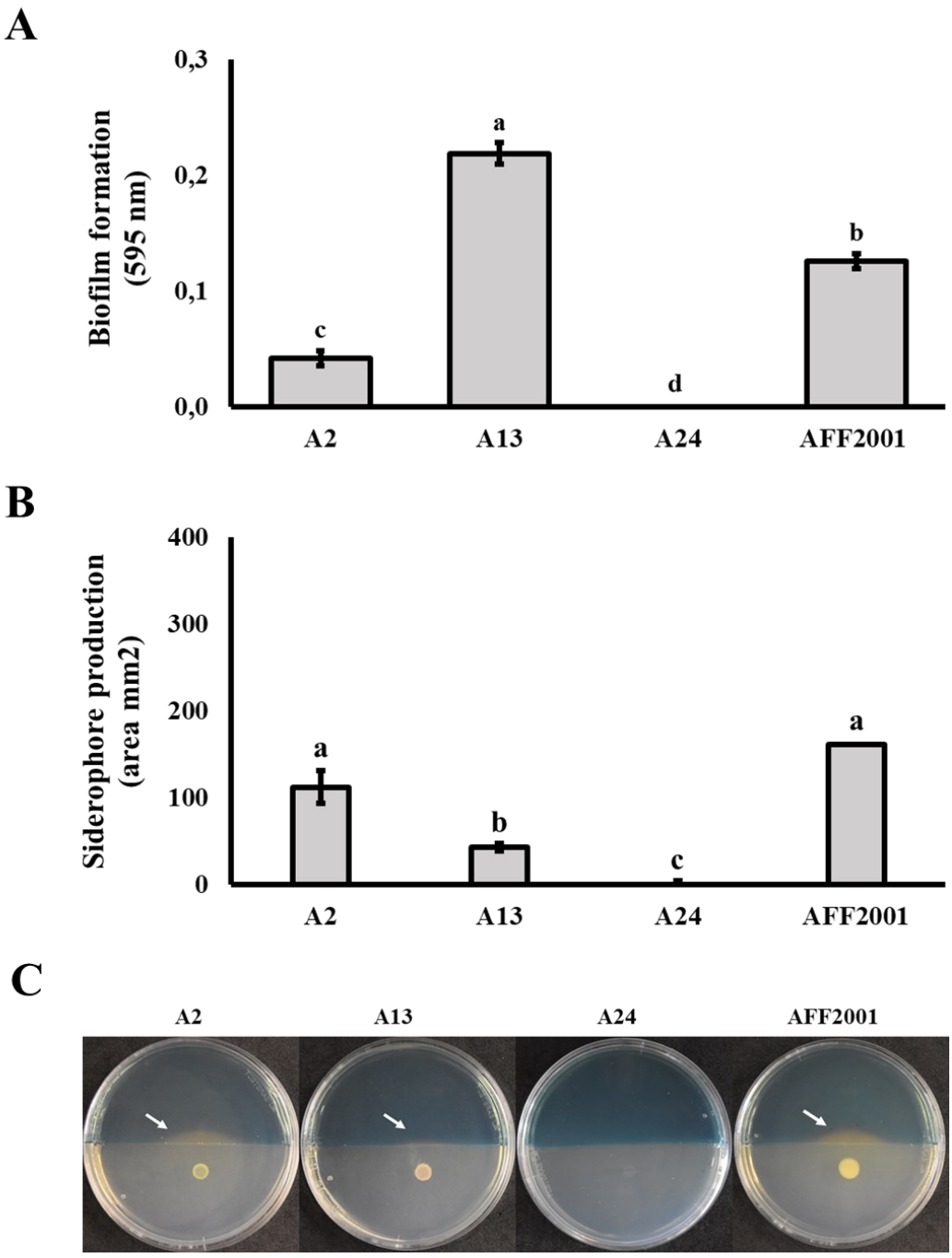

Figure 21. Characterisation in vitro of *Pantoea agglomerans* AFF2001 mutants. A) Biofilm formation. No biofilm was visible or detectable for A24. An increase was measured for A13, while A2 produced less biofilm than wildtype ones. Columns represent mean biofilm production ± standard errors of five replicates. Letters indicate values that differ significantly according to Tukey's pairwise comparison test ($\alpha = 0.05$). B) Siderophore production. A24 appeared incapable of producing siderophores. A2 and *Pantoea agglomerans* AFF2001 produced a statistically equivalent amount of siderophores, whereas a drop was observed for A13.

Columns represent mean siderophore production ± standard errors of three replicates. Letters indicate values that differ significantly according to Tukey's pairwise comparison test (α = 0.05). C) Pictures of the plates. White arrows show the discolouration halos that indicate the release of siderophore.

As *P. agglomerans* AFF2001 culture filtrate showed a strong negative effect on Ea21 cell growth, the same test was conducted using the culture filtrates of A2, A13, and A24 mutants. The pH values of bacterial culture filtrates revealed that A2 and A13 retained the ability to lower the pH of the medium below 4, similar to the wildtype (Figure 22). Acidification was observed also for A24, but the values were closer to 5. Despite the pH values, a reduction in Ea21 cell growth was visible in all treatments (Figure 23). However, the effect was milder when the pH of bacterial culture filtrates was raised to 7. There were no differences between the mutants and the wild type in this case. The impact of A24 bacterial culture filtrates TQ and pH 7 was not statistically equivalent according to the Student's t-test ($p < 0.05$).

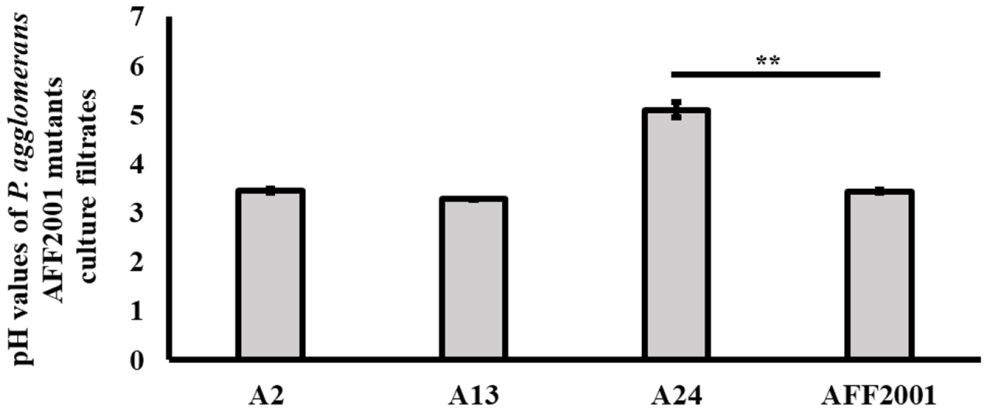

Figure 22. *Pantoea agglomerans* AFF2001 mutants' culture filtrates in Partial Stigma-Based Medium (PSBMT). Values represent the average of three replicates. Asterisk indicates values that differ significantly according to the Student's t-test, $p < 0.05$ (* when $p < 0.05$; ** when $p < 0.01$, *** when $p < 0.001$).

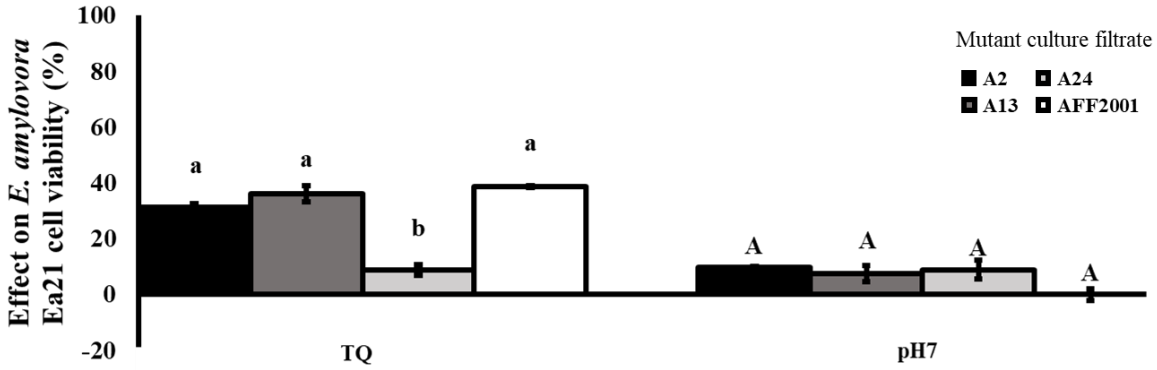

Figure 23. Viability of *Erwinia amylovora* Ea21 cell in *Pantoea agglomerans* AFF2001 mutants culture filtrates. When the pH of bacterial culture filtrates was not modified (*Talis Qualis*, TQ), the negative effect on Ea21 cell growth observed for *Pantoea agglomerans* AFF2001 was still visible in all mutants but A24. When the pH was raised to 7, Ea21 cell viability was still lower than the positive control, but the effect of the treatments was statistically comparable. Columns represent the mean effect ± standard errors of three replicates. Letters indicate values that differ significantly according to Tukey's pairwise comparison test ($\alpha = 0.05$).

The differences observed in the *P. agglomerans* AFF2001 mutants compared to the wild type are summarised in Table 9.

Table 9. Differences in *Pantoea agglomerans* AFF2001 mutants compared to the wild type.

Bacterial strain traits	**A2**	**A13**	**A24**
Plant protection on apple flowers	equal	lower	lower
Plant protection on pear slices	equal	equal	lower
Swimming motility	equal	lower	0
Swarming motility	equal	higher	0
Biofilm formation	lower	higher	0
Siderophore production	equal	lower	0
pH of culture filtrate	equal	equal	higher

3.2. *Drosophila suzukii*: a potential vector of *Erwinia amylovora*, the causal agent of apple fire blight?

3.2.1. Drosophila suzukii *showed a tendency to be attracted to* Erwinia amylovora *Ea21 ooze*

Two assays were conducted to assess a potential attraction of *Drosophila suzukii* to Ea21 ooze, namely pitfall and olfactometer choice tests. Female and male flies were tested separately in both assays. Regarding the pitfall test, the overall activation rate was 64.28%, corresponding to 144 individuals making a choice, with males showing an activation rate of 61.95% (70 individuals) and females displaying a slightly higher rate of 66.67% (74 individuals). Among the activated individuals, 61.54% chose the treatment represented by the SNA medium with *E. amylovora* Ea21 ooze. Analysing the data according to the fly sex (Figure 24), 63.51% (47 individuals) of the activated females and 60.00% (42 individuals) of the activated males were attracted to *E. amylovora* Ea21 ooze. Statistical analysis (Table 10) shows that the overall test result was statistically significant ($p = 0.0045$), indicating a preference for the treatment and a potential attraction to the bacterial ooze produced by *E. amylovora* Ea21. However, when considering the sex, only females demonstrated a significant tendency to be attracted to the positive control ($p = 0.019$), whereas males did not show any significant tendency of attraction ($p = 0.0943$).

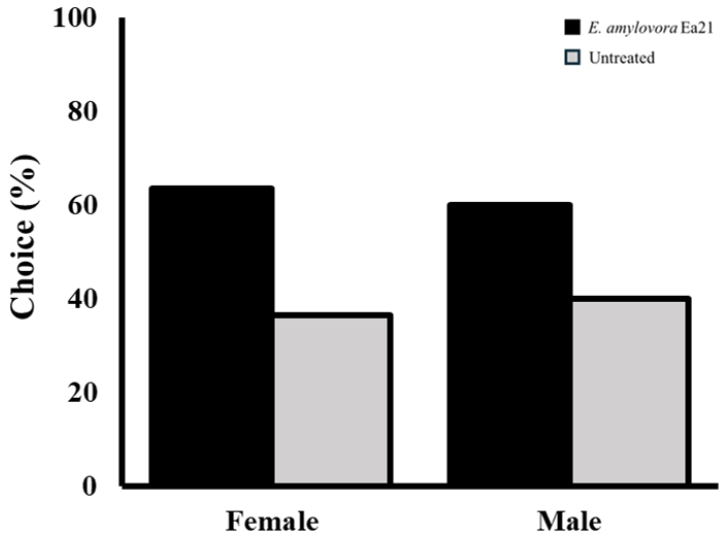

Figure 24. Pitfall test. The chart shows *Drosophila suzukii* individuals' choices according to the treatment and the fly sex.

Table 10. Statistical analysis of pitfall choice test. The logarithm of the likelihood function of the model (L); the likelihood ratio test statistic (G), indicating the difference between the observed and expected frequency distributions (null hypothesis); the adjusted version of G for small sample sizes (Gadj), used when the number of observations in one or more categories is low (<80-100), it corrects the tendency of G to overestimate evidence against the null hypothesis in small samples; the p-value (p) indicates the significance of the result, with values <0.05 (5% of confidence) considered significant.

FEMALES	L	15.435	
	G	5.473	William's correction = 1.003
	Gadj	5.436	$p = 0.0197$
MALES	L	4.094	
	G	2.819	William's correction = 1.003
	Gadj	2.799	$p = 0.0943$
TOTAL	L	57.515	
	G	8.104	William's correction = 1.007
	Gadj	8.076	$p = 0.0045$

To test potential attraction over a shorter time frame compared to the pitfall test, which lasted 48 hours, olfactometer tests were conducted. These tests allow for a more specific assessment of attraction to olfactory stimuli associated with volatile compounds. The experiments were initially conducted on single individuals, but the data showed that only about 35% (n = 7) of *D. suzukii* flies made a choice. Such a low activation rate did not allow for a reliable statistical analysis to make any assumptions. Therefore, the testing approach was adjusted to group experiments. When tested in groups of 10 individuals, the overall activation rate was about 68.13% (n=109), with both males and females showing a significant tendency to prefer the treatment (Binomial test: males, $p<0.001$; females, $p<0.001$). The activation rates were almost identical between sexes, being 67.50% and 68.75% for females and males, respectively. Figure 25 shows that among activated individuals, 88.89% of females (n=48) and 78.18% of males (n=43) chose the treatment.

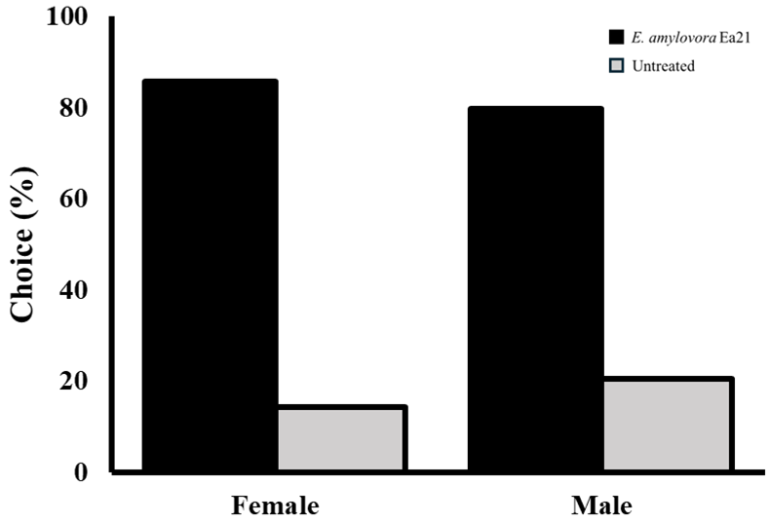

Figure 25. Olfactometer test. The chart shows the choice made by *Drosophila suzukii* flies when tested in groups of ten individuals.

A Chi-square test revealed no significant difference in choices between males and females ($\chi 2 = 1.15$, $p = 0.28$), rejecting the null hypothesis that sex influenced the flies' choice.

3.2.2. Optimized protocol for Erwinia amylovora *Ea21 acquisition by* Drosophila suzukii

Since none of the mature apples inoculated with *E. amylovora* Ea21GFP developed bacterial ooze, even with longer incubation time and higher *E. amylovora* Ea21GFP concentrations, alternative fruits were tested. Mature apples from two cultivars (*Malus domestica* cv. Royal Gala and cv. Golden Delicious) were initially inoculated with Ea21GFP, but this approach was not successful. The focus then shifted to immature apples of the same varieties and immature pears (*Pyrus communis* cv. Williams), and in the latter case, the fruits exhibited visible production of *E. amylovora* Ea21GFP ooze, as shown in Figure 26.

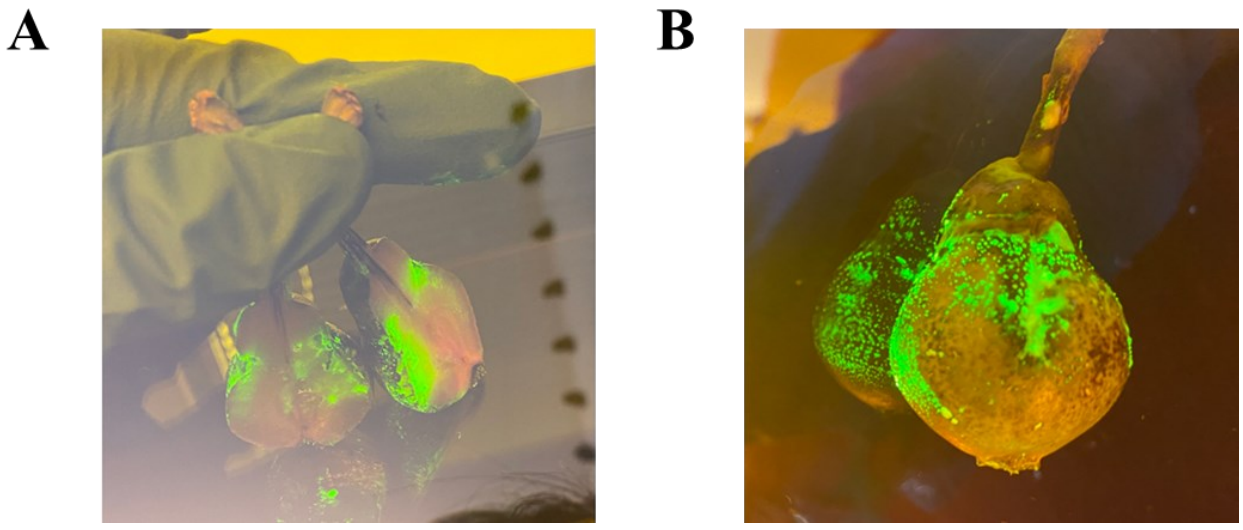

Figure 26. Immature pears (*Pyrus communis* cv. Williams) inoculated with *Erwinia amylovora* Ea21 tagged with the Green Fluorescent Protein (GFP). Fluorescence indicates the presence of bacterial ooze. A) Internal tissue of the pear; B) Fruit surface.

Given that immature pears were not available anymore at the time of the experiments to evaluate the time of exposure and to conduct the molecular analysis through qPCR, *Drosophila suzukii* individuals were exposed to SNA plates containing *Erwinia amylovora* Ea21GFP ooze. Interestingly, *D. suzukii* individuals were able to acquire and potentially ingest Ea21GFP, as indicated by the fluorescence observed in their abdomens following exposure (Figure 27).

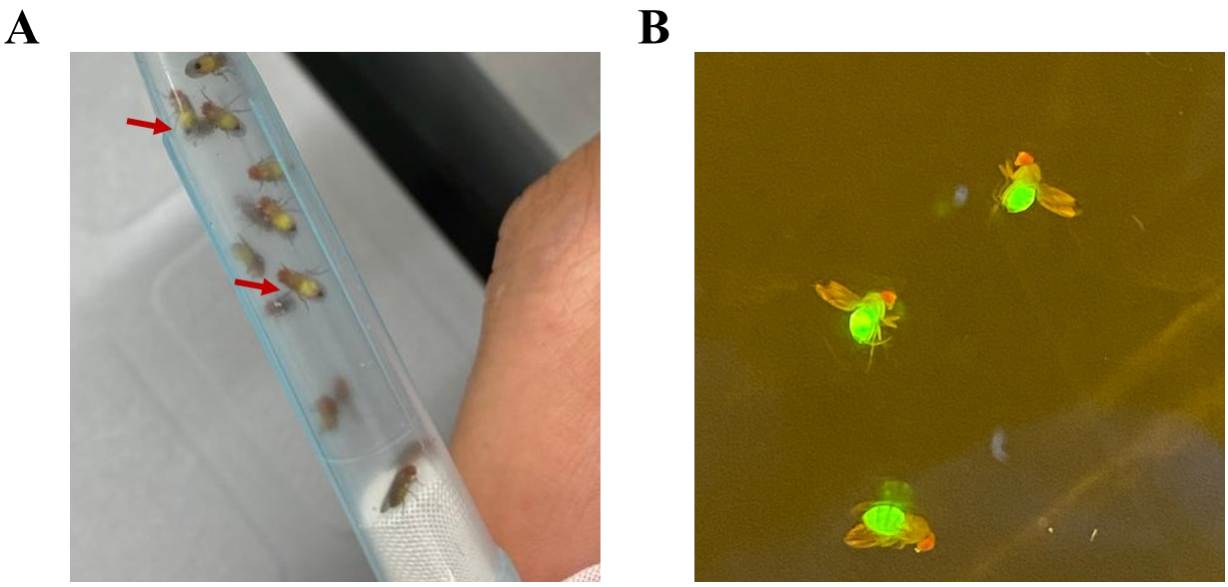

Figure 27. *Drosophila suzukii* after the acquisition of *Erwinia amylovora* Ea21 tagged with the Green Fluorescent Protein (GFP). A) Flies exhibit a yellow colouration in the abdomen (indicated by red arrows); B) Under a transilluminator, the abdomen fluoresces, confirming the presence of GFP-labelled *Erwinia amylovora*.

To assess the persistence of the bacterium on or within the insects, five exposed *D. suzukii* individuals were sampled at multiple time points in a timeframe of 7 days, which was concluded to be the duration of the experiment. Notably, *E. amylovora* cells associated with the surface or internal structures of *D. suzukii* remained viable even after prolonged storage at –20 °C, as evidenced by the growth of fluorescent bacterial colonies upon plating the sample suspension on Petri dishes (data not

shown). Regarding the qPCR analysis, four samples (C1, C2, C3, C4) were tested, each one with different ratios of individuals exposed to *E. amylovora* Ea21GFP to non-exposed individuals, namely 1:1, 1:20, and 1:100. This approach simulates a sampling scenario in which many flies are captured, and analysing multiple insects at once could help expedite the process. Two positive (P1, P2) and negative (N1, N2) controls were included in the analysis. Table 11 highlights the differences in detectability across the tested sample ratios. Since the initial Ea21GFP concentration in the samples was unknown, variability was observed in the mean cycle threshold (Cp). As Cp values are inversely correlated with DNA concentration, lower Cp values indicate higher DNA concentrations, while higher Cp values correspond to lower DNA concentrations. *E. amylovora* was detected in all tested ratios, though its detectability was lower in the 1:100 ratio compared to the others. Since the pathogen was consistently detected, pooling multiple individuals for molecular analysis is a feasible approach for monitoring the presence of *E. amylovora*. The agar plates, where the dilutions of the C1-C4 samples and the P2 control were plated prior to thermal treatment, showed Ea21GFP growth.

Regarding the DNA extraction, two methods were tested: one involving sample homogenization with a mixer mill, as previously described, and the other using a commercial extraction kit. Samples D1 and D2 were processed using the alternative method, while samples D3 and D4 were extracted with the commercial extraction kit. Since the initial bacterial cell concentration on the insects was also unknown in this case, direct comparison of *E. amylovora* Ea21GFP quantification between the methods was not possible. However, these results confirmed that both methods efficiently extract bacterial DNA from the insects (Table 12).

Table 11. Results of the qPCR conducted on *Drosophila suzukii* flies. The mean cycle threshold (Cp) and the standard deviation of the cycles (Std Cp) are reported. C1–C4 represent the tested samples; P1 is the positive control derived from a colony of *E. amylovora* Ea21; P2 is the positive control derived from a *D. suzukii* fly harbouring the bacterial plant pathogen; N1 is the negative control from a *D. suzukii* individual not exposed to Ea21; and N2 is the negative control consisting of sterile Milli-Q water.

Sample	Mean Cp	Std Cp
C1 1:1	21.70	0.22
C1 1:20	25.61	0.10
C1 1:100	27.80	0.01
C2 1:1	23.60	0.06
C2 1:20	27.64	0.08
C2 1:100	29.79	0.06
C3 1:1	22.98	0.06
C3 1:20	26.88	0.16
C3 1:100	29.09	0.03
C4 1:1	24.88	0.03
C4 1:20	29.55	1.25
C4 1:100	29.43	1.80
P1 1:1	17.83	0.16
P1 1:20	21.67	0.02
P1 1:100	23.44	0.19
P2 1:1	21.96	0.01
N1 1:1	35.70	1.22
N1 1:20	37.50	-
N1 1:100	39.32	-
N2 1:1	38.57	-

Table 12. The mean cycles (Cp) and the standard deviation (Std Cp) needed to detect the bacterium assimilated by the four *Drosophila suzukii* individuals.

Sample	Mean Cp	Std Cp
D1	34.57	0.58
D2	33.99	0.12
D3	22.05	0.16
D4	31.57	1.55

Based on these observations, the final protocol for the acquisition test of *Erwinia amylovora* by *Drosophila suzukii* is as follows:

1. Immature pears sterilisation and inoculation.

Immature pears (*Pyrus communis* cv. Williams, ⌀ 3 cm) were sterilised by immersion in a NaClO solution (2% v/v) and shaken at 85 rpm for 5 minutes. Then, the pears were transferred to a 0.5% sodium hypochlorite solution and shaken again at 85 rpm for 1 minute. Afterwards, the pears were rinsed three times with sterile distilled water, shaking at 85 rpm for 3 minutes. The pears were then allowed to dry under a laminar flow hood before eight linear incisions were made around the circumference of each fruit with a sterile scalpel. Each incision was approximately 0.5 cm deep and 2.5 cm long. The cuts were inoculated with 20 µl of an *E. amylovora* suspension at OD_{600} 1. Finally, the inoculated fruits were placed inside plastic containers sterilised with ethanol and UV light. Sterile absorbent paper moistened with 1 mL of sterile distilled water was added to each container to maintain humidity. The pears were incubated for 48 hours at 27°C to promote ooze development.

2. Insect exposure to inoculated fruits.

The inoculated fruits were placed in a 22x22x22 cm cage, sealed with plastic film to retain humidity. Fifty *D. suzukii* individuals (25 females, 25 males) were introduced into the cage for a 7-day exposure period. Samples were collected on days 0, 3, 5, and 7, with five individuals of each sex sampled at each time point. After collection, the exposed insects were stored at -20°C for subsequent molecular analysis.

3. Molecular analysis to detect the presence of E. amylovora.

The insects (5 individuals per Eppendorf tube) were homogenised with steel beads using a mixer mill (Mixer Mill MM200, Retsch) for 30 seconds at 25 Hz, and the homogenisation product was resuspended in 100 µl of 5mm Tris-HCl (pH 8). Eppendorf tubes and steel beads were kept at -20°C before homogenisation. Then, a thermal shock was applied by exposing the samples to 95°C for 5 minutes, followed by storage at -20°C for at least 20 minutes. Before the thermal treatment, dilutions of the homogenate were placed on SNA plates to assess the viability of *E. amylovora* bacterial cells. The plates were incubated for 48 hours at 27°C. The DNA extracts were prepared according to Gottsberger (2010) and the EPPO Standard protocol on Diagnostic "PM 7/20 (3) *Erwinia amylovora*" (2022). A qPCR reaction was run by setting 50 cycles, each consisting of 5 seconds at 95°C and 30 seconds at 60°C.

CHAPTER 4. DISCUSSION

4.1. From apple flowers to control fire blight: new potential biocontrol agents of *Erwinia amylovora*?

It is now widely accepted that microbial communities residing in apple flowers may represent a significant natural barrier to the colonization of *Erwinia amylovora*, thereby hindering the development of fire blight disease. Based on this body of knowledge, in this study, we aimed to investigate the microbiota of apple flowers to select new potential biocontrol agents active against *E. amylovora*. BCAs active against fire blight are already available on the market as biopesticides (Sundin et al. 2009); however, their efficacy remains lower than treatments based on antibiotics and copper-based compounds (Sundin et al. 2009; Johnson et al. 2013, 2022). Therefore, identifying new BCAs that can be combined with existing ones could enhance the effectiveness of fire blight control.

In the present study we isolated bacterial strains from healthy apple flowers (*Malus domestica* cv. Golden delicious) of Trentino orchards. The evaluation of the biocontrol properties of all bacterial strains was done following a specific workflow. We began with *in vivo* testing to assess their efficacy in controlling fire blight development on detached apple flowers, and then we carried out all the *in vitro* experiments to characterize the modes of action. This approach helped avoid biases seen in some studies (Dagher et al. 2020; Aktepe et al. 2022), where the efficacy observed *in vitro* was not replicated *in vivo*. First, we identified the endophytic bacteria we isolated to have a general idea of the bacterial taxa harboured in healthy apple flowers of Trentino orchards. We found bacterial strains mainly belonged to *Enterobacteriaceae*, *Microbacteriaceae* and *Pseudomonadaceae* families. Consistent

with previous studies conducted on different apple varieties, *Enterobacteriaceae* and *Pseudomonadaceae* were the most represented bacterial families (Cui et al. 2021a; Steven et al. 2018). This suggests that the bacterial community's structure is well-conserved among different cultivars. As previously done by Stockwell and colleagues (2010a), we further analysed all bacterial strains from the metabolic point of view through a Biolog assay to spot any differences in nutritional requirements compared to *E. amylovora* Ea21. As expected, most of nutritional overlaps were observed between Ea21 and other members of the *Enterobacteriaceae* family (*Pantoea agglomerans* AFF2001, *Erwinia billingiae* AFF4005, *Yersinia similis* AFF3001, *Rahnella aquatilis* AFF4004, *Yersinia aldovae* AFF5003) to which it belongs, particularly in the assimilation of carbon substrates such as b-methyl-D-glucoside, galactose, mannitol, N-acetyl-D-glucosamine, ribose, sorbitol, and D-trehalose. The data collected on *Pantoea agglomerans* AFF2001 indicate that this bacterial strain has potential as an antagonist to *E. amylovora* Ea21 through nutrient competition, especially for glucose, its derivatives, and fructose. In terms of nitrogen sources, Ea21 demonstrated a marked preference for specific amino acids, such as asparagine, aspartic acid, cysteine, glutamic acid, glutamine, proline, and tryptophan. In contrast, most endophytic strains did not show a strong preference and tended to assimilate a broader or more variable spectrum of amino acids. Bacterial strains such as *P. agglomerans* AFF2001 may compete with Ea21 for specific amino acids, especially glutamine, glutamic acid, and aspartic acid. These data are important for understanding whether endophytic bacteria of apple flowers may hinder (or not) the colonization of *E. amylovora* Ea21 cells by competing for nutrients and space, a common strategy used by biocontrol agents (Pal and Gardener 2006; Stockwell et al. 2010a; Köhl et al. 2019). Notably, the amino acids required by Ea21 are present in flowers at femtogram levels (Pusey et al. 2008a), meaning their scarcity could intensify competition among bacterial species. Detached

apple flowers assay enabled the selection of potential biocontrol agents among the endophytic bacteria isolated. *Acinetobacter* sp. AFF2002*, C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001, and *Ps. extremaustralis* AFC1001 were most effective in controlling disease development. This was not surprising, as some of the biopesticides for fire blight management are based on bacterial strains belonging to the *Pantoea* and *Pseudomonas* genera (Sundin et al. 2009). Moreover, our results were aligning with those reported by Cui and colleagues (2021b), where the most efficient flower treatments were those of *Curtobacterium* sp. 24E2, *Pantoea* sp. 1B4 and *Pseudomonas* sp. 15A4, though in that study isolates were not taxonomically identified at the strain level. We then investigated the effect of the four selected bacteria strains on the virulence of Ea21. Immature pear slices were treated with suspensions of the four bacterial strains, as previously described by Paternoster (2010), and subsequently inoculated with Ea21. While *Acinetobacter sp.* AFF2002, *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 displayed comparable levels of plant protection efficacy in the flower assay, though all below 100%, in this case *P. agglomerans* AFF2001 achieved the highest efficacy reaching full protection (100%). Unexpectedly, *Ps. extremaustralis* AFC1001 caused a side effect on the pear slices that turned brown, which interfered with the assessment parameters and rendered data collection for this strain unfeasible. We hypothesise that an oxidative reaction may have occurred following *Ps. extremaustralis* AFC1001 treatment, as the *Pseudomonas* genus is known to secrete oxidative enzymes, such as polyphenol oxidases (PPOs), which contribute to fruit and vegetable browning by oxidizing polyphenols in plant tissues (Mayer 2006; McMahon 2007; Kumar et al. 2024). Results on immature pear slices underlined that three out of the four bacterial strains evaluated may suppress the virulence of Ea21. To obtain a comprehensive overview of the main characteristics of the tested endophytic bacteria, we finally assessed their impact on the ability of Ea21 to elicit the

hypersensitive response (HR) on tobacco leaves. This test is widely employed to study the pathogenicity of plant pathogens, and it is based on evidence that non-host plants typically show their resistance to plant pathogenic bacteria by inducing a hypersensitive response (Kelman and Sequeira, 1972). Our results showed that only *Ps. extremaustralis* AFC1001 failed to prevent HR formation, suggesting that the other bacterial strains tested may suppress Ea21 pathogenicity. Despite its efficacy in controlling fire blight on apple flowers, *Ps. extremaustralis* AFC1001 neither inhibited the virulence of Ea21 nor suppress the hypersensitive response in tobacco. Consequently, we excluded this bacterial strain from our selection process. Overall, these findings indicated that *Acinetobacter* sp. AFF2002*, C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 may be promising candidates for biocontrol applications against *Erwinia amylovora*.

Next, we investigated the modes of action of these four bacterial strains by performing several *in vitro* tests. In literature, most of the studies related to biocontrol agents against fire blight carried out these assays using general bacterial culture media, such as King's B Medium (Barbé et al. 2022), LB (Cui et al. 2021a), Tryptic Soy Agar (Dagher et al. 2020). The main limitation of using these media is that they significantly differ from the natural conditions found in the flowers, which might affect the observations' reliability. For this reason, we decided to conduct each test on the PSBMT medium developed by Pusey and colleagues (2008c), which aimed to reproduce the nutrient condition found in apple flowers based on the analysis previously done on the stigma oozes of some apple varieties (Pusey et al. 2008a). Gas chromatography and high-performance liquid chromatography revealed that glucose and fructose are the predominant free sugars in the oozes (Pusey et al. 2008a). This finding, together with the Biolog assay, suggests potential competition for these resources between Ea21, *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001. In contrast, sorbitol, inositol, and sucrose

were only detected in trace amounts (Pusey et al. 2008a). Proline, asparagine, glutamine, and serine were the predominant free amino acids, despite their extremely low levels (Pusey et al. 2008a). The scarcity for other amino acids, such as glutamine, glutamic acid, leucine and aspartic acid, may restrict bacterial growth and enhance competitive interactions among microbial communities. For example, *C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001, and *E. amylovora* Ea21 are all able to metabolize tryptophan. Our data suggest that competition for key carbon and nitrogen sources between *E. amylovora* and selected endophytic bacterial strains may contribute to the observed reduction in symptom development.

Nutrient assimilation is one of the factors that strictly influence the growth of *E. amylovora*, consequently affecting its virulence (Ramos et al. 2014). However, in addition to nutrients, competition for iron may also occur. Iron has been identified as a limiting factor for bacterial replication during flower colonization and is therefore essential during the early stages of fire blight infection (Dellagi et al. 1998; Müller et al. 2022). Bacteria can acquire iron from the environment through the release of specific molecules known as siderophores (Lankford 1973), which have a high affinity for the insoluble state of this element (Fe^{3+}) that is converted to its soluble form (Fe^{2+}) once inside the bacterial cells (Bagg and Neilands 1987; Andrews et al. 2003; Müller et al. 2022). To see whether the potential biocontrol bacteria released siderophores, CAS-agar modified test was performed on PSBMT. In contrast to what was observed for *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009, both *P. agglomerans* AFF2001 and Ea21 released siderophores. Supporting this, a genomic analysis of the draft genomes of *E. amylovora* Ea21 (Albanese et al. 2022) and *P. agglomerans* AFF2001 (this study), conducted using antiSMASH, revealed that these bacterial strains harbour the genes responsible for the biosynthesis of the siderophore desferrioxamine E. Specifically,

AFF2001 carries these genes on Contig_5, which shows partial homology to plasmid pASB05p1 from *P. agglomerans* strain ASB05, a plasmid known to encode the aforementioned siderophore (Lee et al., 2021). This siderophore is synthesized by several bacteria, including *P. agglomerans* and *E. amylovora* strains (Berner et al., 1988; Smits and Duffy, 2011). Interestingly, when *P. agglomerans* AFF2001 and *E. amylovora* Ea21 were spot inoculated 1 cm apart, an increase in the discoloration halo, which indicates the release of siderophores, was observed. This observation might support our hypothesis that iron competition might be one of the modes of action behind biocontrol activity.

P. agglomerans AFF2001 showed the strongest potential contact-dependent antibacterial activity, completely preventing the growth of Ea21 when the two bacterial strains were cultured together. All commercially available Gram-negative bacteria biocontrol agents used in fire blight management, namely *P. agglomerans* E325, *P. vagans* C9-1, and *Ps. fluorescens* A506, produce antibiotics active against *E. amylovora* (Pusey et al. 2008c; Sundin 2009; Stockwell et al. 2010b). antiSMASH analysis of the *P. agglomerans* AFF2001 draft genome revealed a D-alanylgriseoluteic acid (AGA) biosynthetic gene cluster on Contig_4, which corresponds to a known phenazine antibiotic cluster previously identified in *Erwinia herbicola* (now *P. agglomerans*) strain Eh1087 (Kearns and Mahanty 1998; Giddens et al. 2002). Since the results of the chloroform assay showed no contact-independent antibacterial activity, we speculate that AGA production may be triggered by the presence of other bacterial strains, such as Ea21. However, further analysis is required to confirm whether the antibiotic predicted *in silico* is produced by *P. agglomerans* AFF2001. In addition to the AGA biosynthetic gene cluster, we also identified genes associated with the Type VI Secretion System (T6SS), which is known to deliver antibacterial effectors capable of killing neighbouring bacterial cells (Ma et al. 2019). The genes encoding the components of the T6SS include those forming the

transmembrane complex (TssA–M), the tubular structure extending outside the cell (Hcp), the tip of the secretion apparatus (PAAR, VgrG), and some accessory protein (TagF, TagJ, TagH, PppA, PpkA) (Cianfanelli et al. 2016; Coulthurst 2019). According to the RAST annotation, all the listed genes are present in the draft genome of *P. agglomerans* AFF2001. In line with previous reports, Hcp and VrgG proteins, which form the outer part of T6SS apparatus, are also known secreted effectors (Pukatzki et al. 2007; De Maayer et al. 2011), and they may play a similar role in *P. agglomerans* AFF2001. Furthermore, two putative T6SS effector genes, homologous to those found in *P. agglomerans* strain FDAARGOS 1447 (QXB58602.1, QXB58650.1), were identified, supporting our hypothesis that T6SS might be involved in the contact-dependent interaction between *P. agglomerans* AFF2001 and Ea21. The function of all these potential effectors should be experimentally confirmed through mutagenesis methods.

It was found that fire blight develops only if the size of the *E. amylovora* population in the flower is bigger than 10^4 bacterial cells (Philion, 2014). The ability of *E. amylovora* to grow and colonise the stigma has, therefore, a key role in the success of the infection, together with all the virulence factors so far identified for this bacterial phytopathogen (Piqué et al. 2015). Thus, we explored the impact of *Acinetobacter* sp. AFF2002*, C. flaccumfaciens* AFF2009, *P. agglomerans* AFF2001 on the growth and virulence of Ea21. We decided to test the effect of the culture filtrates of these bacterial strains as previously done with several *Streptomyces* species (Le et al. 2021; Nguyen et al. 2024). The first detail we noticed was *P. agglomerans* AFF2001 strongly lowered the pH of the PSBMT medium from an initial pH of 7 to 3.51 after 24 hours, whereas *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009 led to an insignificant decrease in the pH values (pH 6.33 and 6.45, respectively). Pusey and colleagues observed a similar behaviour for *P. agglomerans* E325 (2011),

whose cultivation in PSBMT caused a pH drop to 3 after 72 hours. Inoculation of E325 on flowers also caused a decrease in the pH of stigma oozes, although the acidification was less pronounced, ranging from pH 4.8 to 5.1 (Pusey et al. 2008c). Additionally, *P. agglomerans* E325 was shown to produce an antibiotic that remains stable and active under acidic conditions (Pusey et al. 2011). However, acidification was observed independently of antibiotic production, as demonstrated by E325 antibiotic-deficient mutants, suggesting that the pH drop is not driven by antibiotic activity (Pusey et al. 2011). In our study, compared to what observed in PSBMT at pH 7, the acidic conditions of the *P. agglomerans* AFF2001 culture filtrate impaired but did not fully suppress the growth of Ea21. The most considerable reduction in cell viability was observed in Ea21 when grown in *P. agglomerans* AFF2001 culture filtrate. In contrast, the culture filtrates of *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009 had milder effects. We acknowledge that a lower nutrient content availability might limit the growth of Ea21 in a spent medium. However, in the case of *P. agglomerans* AFF2001, we hypothesise that the acidification of PSBMT by *P. agglomerans* AFF2001 might be a primary factor contributing to the observed growth inhibition, as *E. amylovora* grows optimally at around pH 7.5 (Shrestha et al. 2005). We adjusted the pH of each bacterial culture filtrate to 7 to specifically evaluate the effect of pH on *E. amylovora* growth. Even at this adjusted pH, the culture filtrates still exhibited an inhibitory effect, suggesting that, beyond acidification, other mechanisms may also contribute to the inhibition of *E. amylovora*. Chemical analysis is needed to see whether the effect observed was due to the production of antimicrobial compounds or side-products. Notably, Ea21 was still able to grow in the *P. agglomerans* AFF2001 culture filtrate, but it was not in the presence of *P. agglomerans* AFF2001. This indicates that the acidification of pH alone is not sufficient to completely hinder the growth of the bacterial phytopathogen, but other mechanisms including the

competition for nutrients and other resources, as well as the potential contact-dependent antibacterial activity we hypothesised, may be needed.

Several virulence factors have been described for *E. amylovora* (Piqué et al. 2015). Among these, motility is specifically required during the initial phases of infection, when *E. amylovora* cells must reach the nectary of the flowers to get access to the xylem and spread within the plant (Schachterle et al. 2022). Thus, we tested the effect of the bacterial culture filtrates on both the swimming and swarming motility of Ea21. *P. agglomerans* AFF2001 culture filtrate completely inhibited the motility of the bacterial phytopathogen. Acidic conditions have been shown to decrease swimming motility in *E. amylovora* (Raymundo and Ries, 1980), and we thus suppose that low pH may have a similar effect on swarming motility. As observed in the growth assay, adjusting the pH of *P. agglomerans* AFF2001 culture filtrate at 7 still negatively impacted motility. Motility in *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009 culture filtrates was generally reduced, but the effect was milder compared to *P. agglomerans* AFF2001. Interestingly, *Acinetobacter* sp. AFF2002 culture filtrate at pH 7 appeared to enhance the swimming motility of Ea21. As reported by Santander and colleagues (2014), during starvation, *E. amylovora* is unable to move. We might speculate that the reduction in Ea21 motility observed in all bacterial culture filtrates tested may also be related to the lower nutrient content of the spent medium. However, this would not explain the reduced inhibition effect observed when the culture filtrates were adjusted to pH 7. If acidification of the floral environment occurs, it could interfere with *E. amylovora*'s movement from the stigmatic tips toward the base of the flower, potentially hindering colonization and subsequent symptom development.

Finally, we evaluated the effect of the *Acinetobacter* sp. AFF2002 and *C. flaccumfaciens* AFF2009, and *P. agglomerans* AFF2001 culture filtrates on biofilm production. Several studies reported that biofilm formation is essential for *E. amylovora* to survive under stressful conditions, such as low temperature, starvation, and host plant responses, making it one of the most critical virulence factors (Geier and Geider 1993; Jock et al. 2005; Koczan et al. 2009, 2011; Ordax et al. 2010; Santander et al. 2017; Kharadi and Sundin 2021; Peng et al. 2021). We believe the results we obtained are not reliable since we had difficulties in replicating the protocol. No biofilm ring was visible in the control wells inoculated with Ea21 alone, which was unexpected. The dilution of the medium with LB or the plate material might have negatively affected the outcomes of this experiment.

Based on the results described, *P. agglomerans* AFF2001 was the bacterial strain that most effectively controlled the pathogenicity, virulence, growth and motility of *E. amylovora* Ea21, making it the best candidate as a new biocontrol agent among the three bacterial strains tested. For these reasons, we tried to unveil its modes of action by performing a random transposon mutagenesis. We selected three knockout mutants (A2, A13, A24) that were either unable to inhibit the growth of Ea21 and/or exhibited abnormal colony morphology. We then investigated the effects of these mutations, both *in vivo* and *in vitro*, on the PSBMT medium, as previously done for the wild type. Mutants A13 and A24 were less effective than *P. agglomerans* AFF2001 in protecting detached apple flowers, suggesting that the mutations present in their genomes may impact their biocontrol. All mutants except A24 still exhibited a negative effect on Ea21 virulence, as treatment of pear slices with A24 did not prevent the development of Ea21 ooze and slice browning. Although A2 showed plant protection efficacy similar to that of the wild type, we decided to conduct further analysis on this mutant, as it could help identify the gene not involved in the biocontrol activity. The localization of transposon

insertion revealed that A2 was mutated in the *rcsA* gene, encoding the auxiliary regulator protein RcsA. This activator has been previously described in *E. coli* as part of the Regulator of capsule synthesis (Rcs) phosphorelay transduction system, which involves also other genes, namely *rcsB*, *rcsC*, and *rcsD* (Majdalani and Gottesman 2005). RcsA binds to RcsB, forming a heterodimer that activates the transcription of genes involved in EPS and biofilm production (Danese et al. 2000) and represses the transcription of genes responsible for flagellar synthesis (Francez-Charlot et al. 2003). Our results confirmed what was reported in the literature. A2 mutant differed from the wild type in that it exhibited reduced biofilm formation, but motility was comparable to that observed for *P. agglomerans* AFF2001, and siderophore production was reduced. The reason behind a reduction and not a complete inhibition of biofilm production is related to the role of the RcsA protein. In the complex with RcsB, RcsA has the role of a co-regulator that enhances the activity of the activator protein RcsB (Majdalani and Gottesman, 2005; Geider et al. 2006). This suggests the EPS production is still positively regulated by the RcsB protein, whose gene was not mutated. Similarly, *rcsA* mutation may not be relevant in suppressing of flagellar synthesis since RcsB is active. Otherwise, we would have seen an increase in the motility of this mutant in comparison to *P. agglomerans* AFF2001. Concerning A13, the transposon insertion was identified in the *pdeR* gene, encoding a c-di-GMP phosphodiesterase. Swarming activity and biofilm production were enhanced in this mutant while swimming motility and siderophore release were reduced. c-di-GMP is a second messenger that regulates several bacterial functions, including the induction of biofilm formation and the suppression of swimming motility (Römling et al. 2005; Jenal et al. 2006; Cotter et al. 2007; Hengge 2009; Edmunds et al. 2013). This molecule is synthesised by diguanylate cyclases and degraded into 5′-phosphoguanylyl-(3′,5′)-guanosine (pGpG) by phosphodiesterases (Valentini and

Filloux, 2016). A mutation in the gene encoding a phosphodiesterase would likely result in accumulation of intracellular c-di-GMP, as it would no longer be converted into pGpG. This accumulation would lead to increased biofilm production and reduced swimming activity. Our observations perfectly agree with this statement. Moreover, it was found that c-di-GMP is also involved in the suppression of swarming motility and in the induction of siderophores biosynthesis in *Ps. aeruginosa* (Frangipani et al. 2014; Kuchma et al. 2015). These findings suggest that c-di-GMP might have a similar role in *P. agglomerans* AFF2001. The last mutant we selected, A24, was mutated in the *leuA* gene, encoding a 2-isopropylmalate synthase. A24 exhibited distinctly different behaviour in all assays compared to the other knockouts. Neither biofilm nor siderophore production was observed for A24, and this mutant also slightly reduced the pH of PSBMT to approximately 5. As a result, it showed the lowest efficacy in inhibiting Ea21 growth in the bacterial culture filtrate assay. A24 can be defined as an auxotrophic mutant as the mutation in the *2-isopropylmalate synthase* gene led to the loss of its ability to synthesise leucine. Since the Biolog assay revealed that *P. agglomerans* AFF2001 assimilates leucine, even though at a very low level, losing the ability to synthesize it may become essential when the availability of this amino acid is limited or completely absent in the environment, as seen in flower stigmas and in the PSBMT medium, respectively, where only proline, asparagine, glutamine, and serine are present. A24 showed an unusually low growth rate in PSBMT unless leucine was added to the medium (data not shown). Since liquid PSBMT inoculated with A24 looked almost clear after 24 hours of incubation, we initially thought the mutant either did not grow or died in such a medium. However, our hypothesis was rejected since a pellet was visible at the bottom of the tube after it had been centrifuged. We also spot-inoculated a small volume of the bacterial culture in PSBMT on NA plates, and the mutant grew, meaning the bacterial cells were still

alive. Moreover, when we spotted the A24 suspension on PSBMT, the spot was visible, even though the appearance was transparent and not as yellow as the other mutants. Given that numerous studies have shown bacteria enter the VBNC state due to nutrient starvation (Oliver, 2005; Vattakaven et al. 2006; Cook and Bolster, 2007; Oliver, 2010), we hypothesise that A24 may undergo a similar process when grown in PSBMT medium, resulting in reduced metabolic activity while remaining viable. However, unlike typical VBNC bacteria, A24 can still be cultivated in the laboratory, suggesting that its reduced growth is not entirely due to a transition into the VBNC state. Furthermore, A24 'recovered' when subsequently cultured in a leucine-supplemented medium, as observed on NA. The lack of leucine in PSBMT might therefore affect the metabolism and growth of A24, potentially reducing its biocontrol activity. Nonetheless, this hypothesis does not fully explain our observations on immature pear slices, where the treatment with A24 did not completely suppress the virulence of Ea21. This is unexpected, considering pear fruits contain leucine (Rondanelli et al. 2021; USDA); therefore, the acquisition of leucine from the environment should compensate for the mutation, allowing A24 to have the same impact on Ea21 observed in the wild type. It might be that amino acids may not be available in an assimilable form in immature fruits, but this should be confirmed by further research.

The characterization of *P. agglomerans* AFF2001 and its knockout mutants provides a general overview of the mechanisms that may underlie the biocontrol activity of this bacterial strain. *P. agglomerans* AFF2001 might inhibit *E. amylovora* Ea21 flower colonization by acidifying the environment, which becomes unfavourable for Ea21 growth and motility. Additionally, competition for sugars, amino acids, and iron could be another factor limiting Ea21 growth on stigmas. Swimming motility may enable *P. agglomerans* AFF2001 to spread across a larger area of the stigmas and

nectary, enhancing its protective effect. The functionality of T6SS and the production of D-alanylgriseoluteic acid (AGA) have yet to be experimentally confirmed, but they may potentially contribute to the interaction with *E. amylovora*. It also remains unclear whether the limited amount of leucine in the flowers may negatively impact the growth of *P. agglomerans* AFF2001, thus indirectly affecting its biocontrol activity, or whether it might play a different role entirely. Given that several mechanisms might be at play, the mutation of a single gene alone cannot fully account for the loss of biocontrol activity. Therefore, additional studies are required to identify all the genes involved in regulating this activity.

Even though it did not completely inhibit disease development in apple flowers, this study highlights that *Pantoea agglomerans* AFF2001 may represent a promising candidate as a biocontrol agent against fire blight. However, biosafety concerns must be carefully considered. The species *P. agglomerans* is classified as a biosafety level 2 organism under European Union legislation (EU Directive 2000/54/EC). Therefore, before proceeding with further research aimed at developing a formulation for field application, a fluorescent amplified fragment length polymorphism (fAFLP) analysis of the AFF2001 genome should be performed to verify the presence of a specific 474-bp band, which has been shown to differentiate biocontrol *P. agglomerans* strains from clinical isolates that pose a risk to human health (Rezzonico et al. 2009). Although this genomic marker has not yet been formally adopted for biosafety classification, it may still represent a valuable tool for preliminary risk assessment of the strain. Additionally, complementary analyses, such as cytotoxicity testing and antibiotic resistance profiling, may be required by EU Directive 2000/54/EC. Some *P. agglomerans* strains have also been reported as causative agents of disease in various plant species, including shot-hole disease in plum and peaches (Tong et al. 2020), leaf blight on wheat and onion (Sepúlveda et al.

2023; Gutierrez-Castillo et al. 2024), and necrotic disease in *Ziziphus jujuba* (She et al. 2019). For this reason, it is also necessary to conduct an in-depth genomic characterisation of *P. agglomerans* AFF2001 and compare it with known phytopathogenic strains. The infiltration of tobacco leaves with *P. agglomerans* AFF2001 cells did not elicit a hypersensitive response (HR), a standard assay used to assess the pathogenicity of bacterial strains (Klement et al. 1964). However, further studies will be necessary to evaluate the pathogenic potential of *P. agglomerans* AFF2001 across a diverse range of plant species.

Nevertheless, this study underscores the potential of investigating the apple flower microbiota and microbiome as a promising strategy for discovering new solutions to control *E. amylovora*. Along with the other potential biocontrol agents isolated in this study, *P. agglomerans* AFF2001 might be used in the designing of a synthetic microbial community (SynCom) (Grosskopf and Soyer 2014) for the fire blight management. SynComs have recently emerged as a promising approach to enhance plant resilience against pathogens, as they can improve biocontrol efficacy through microbial synergy (Liu et al. 2018; Santhanam et al. 2019; Zhang et al. 2019; Zhou et al. 2022; Qiao et al. 2024). Thus, further research conducted on the bacterial strains isolated in this study may facilitate the development of an effective SynCom.

4.2. *Drosophila suzukii*: a potential vector of *Erwinia amylovora*, the causal agent of apple fire blight?

It is well known that plants infected with *Erwinia amylovora* can develop ooze droplets when the disease reaches an advanced stage (Schouten 1989a, b; Slack et al. 2017). As the ooze consists of sugars and *E. amylovora* cells, it may attract insects, which could then disperse the bacterial pathogen

to healthy plants, contributing to the spread of the disease (Boucher et al. 2021a). In the spring of 2020, a fire blight outbreak occurred in the Valsugana Valley, located in the Trentino region where infection rates exceeded 90% in some areas (Fondazione Edmund Mach & Provincia Autonoma di Trento, 2020). Valsugana is known for the presence of another pest, namely *Drosophila suzukii* (Matsumura) (Tait et al. 2018). Even though *D. suzukii* does not directly affect apple orchards, many orchards in the valley are situated close to fruits commonly targeted by this pest. Interestingly, 29.46% of the fruits collected and monitored in that area during 2020 were infested by *D. suzukii* (Unità Piccoli frutti, FEM). Given these observations and considering dipterans such as *D. melanogaster* might act as potential vectors of *E. amylovora* (Boucher et al. 2019), in this study, we aimed to investigate whether *D. suzukii* might be attracted by bacterial ooze and acquire *E. amylovora*.

To assess this attraction, two tests were conducted: the pitfall choice test and the olfactometer test. In the first test, *D. suzukii* flies were tested individually and exposed to two vials, one containing the bacterial medium with Ea21 ooze and the other containing a non-inoculated medium. Observations were made to determine whether the flies would choose between the two vials. The results of the pitfall choice test showed that more than half of *D. suzukii* individuals actively made a choice and selected the vial containing the bacterial ooze produced by *E. amylovora* Ea21. Food-deprived flies might be attracted by the bacterial ooze since it might be perceived as a food source due to its sugary composition (Dethier et al. 1976; Boucher et al. 2019). However, other factors might have influenced the observed behaviour. Although the overall tendency to be attracted was statistically significant, this attraction was significant only for females, as shown when the results were analysed by sex. The difference between sexes might be explained by several factors. First, the number of male individuals tested may have been insufficient to display a statistical significance. Second, a lower attraction of

males for bacterial ooze might be due to biological reasons that differentiate females from males. Once inseminated, females generate and hold the eggs in their bodies. For this reproduction process, females may need more energy and thus sugars that can be found in the bacterial ooze. The choice might also be affected by the reproductive status of female *D. suzukii*, such as the number of mature eggs, as reported by Wong and colleagues (2018). Females with low numbers of mature eggs seem to have a higher preference for fermented sugars, such as levan and amylovoran, which are components found in *E. amylovora* ooze. On the contrary, females having a higher number of mature eggs would be attracted by fructose and glucose present in ripe fruits, the most suitable place this species chooses for oviposition (Wong et al. 2018; Cavey et al. 2023). The individuals used for the experiments were taken from cages where both sexes were present, thus suggesting that the females tested were mated but that the mature egg loading might have been low. Further studies are required to better understand whether males and females respond differently to *E. amylovora* ooze. In the olfactometer test, the conditions are similar to those of the pitfall test, with the main differences being the longer exposure time of the flies to the medium, which either contains or does not contain Ea21 ooze, and the application of an airflow. This type of test specifically allows for the analysis of the effect of volatile organic compounds (VOCs) produced by *E. amylovora* on *Drosophila suzukii*. The olfactometer is, in fact, a tool commonly used to investigate insects' behaviour in response to chemical stimuli (Barrows, 1907; Wood et al. 1966; Weeks et al. 2011). Interestingly, *D. suzukii* flies exhibited different responses when tested individually compared to in groups of ten. We initially tested single individuals, but the percentage of flies making a choice was too low (35%) to make any reliable assumption. This might suggest that testing single individuals may not provide the best conditions to stimulate a sufficient reaction. Given that the flies were tested in a starved state and likely attracted to

food sources, we first hypothesised that *D. suzukii* might exhibit a behaviour similar to the 'search–aggregation' behaviour observed in *D. melanogaster* (Tinette et al. 2004), where some flies search for food sources and others follow them. However, in the pitfall experiment, single individuals were tested, but the activation rate was higher than 60%. This might highlight that aggregation itself may not be the only significant factor, but environmental conditions or social interactions already described for *D. melanogaster* (Higgins et al. 2005; Kent at al. 2008; Schneider et al. 2014; Rooke et al. 2020), such as social environment and light-dark cycle, may have a crucial role in eliciting the decision-making and response strength to environmental stimuli. To further explore this, we conducted experiments testing groups of ten individuals, either female or male. In this case, the activation rate was higher than that one observed in the pitfall choice test (68.75%), with a slight difference between the sexes and a strong preference for both for *E. amylovora* Ea21 ooze. This experimental setup led to hypothesise that when individuals are exposed to a social context, their behaviour may be influenced by increased competition, social facilitation, or aggregation tendencies as seen for *D. melanogaster* (Rooke et al. 2020). The stronger preference for the inoculated treatment in both sexes during the group trials might reflect heightened sensitivity to signals in a competitive setting or even a learned behaviour where individuals follow others' choices. However, more tests with single individuals need to be performed to develop a better understanding of the dynamics involved since the number of replicates was limited. What is missing in this study is the chemical analysis of the volatile organic compounds (VOCs) likely produced by *E. amylovora* ooze. Identifying the specific volatiles responsible for the observed behaviour would provide a better understanding of *D. suzukii* response to chemical stimuli. Additionally, in this study we used an artificial bacterial growth medium (SNA) that mimics the ooze observed in natural conditions. To gain a more comprehensive

understanding of the interactions between the host plant, *E. amylovora*, and *D. suzukii*, it would be valuable to test and analyse the VOCs emitted by infected fruits and flowers, similarly to the approach taken by Cellini and colleagues (2019) for honeybees.

Even though our results indicate a tendency for *D. suzukii* to be attracted to *E. amylovora* ooze, this attraction alone is not enough to classify *D. suzukii* as a potential vector, as the insect must also be capable of acquiring and transporting the bacterial pathogen. Thus, based on the acquisition protocol reported by Ordax et al. (2015) for *Ceratitis capitata*, we developed a new protocol to assess the acquisition of *E. amylovora* cells by *D. suzukii* from ooze. We replicated natural conditions by inoculating mature and immature fruits to obtain bacterial ooze. Immature pears (*Pyrus communis* cv. Williams) were the only biological substrate tested where ooze was visible after inoculation, thus suggesting the sugar composition of mature fruits may be unfavourable to the ooze development. However, in the future, it might also be interesting to test the acquisition from mature fruits displaying typical symptoms of fire blight. To immediately visualize whether acquisition had occurred, we used *E. amylovora* Ea21GFP, allowing us to observe fluorescence on the insects' bodies by exposing them to a transilluminator. This fluorescence indicates the acquisition of the plant pathogenic bacterium. Similar to what was reported for *Ceratitis capitata* (Ordax et al., 2015), *D. suzukii* was able to acquire the bacterial pathogen on its body surface and potentially also ingest it. However, this last point remains speculative, as we did not dissect the insects and analyse them under the microscope. The qPCR analysis carried out allowed to confirm the acquisition of *E. amylovora* from a molecular point of view. By analysing pools of insects, we also demonstrated this method can be applied to detect *E. amylovora* in pools of up to 100 individuals, making detection easier, especially considering that environmental monitoring may capture multiple insects at once. However, such analysis does not

provide any information about the amount and viability of bacterial cells acquired. This is an important aspect to consider since it is known that fire blight infection requires more than 10^4 bacterial cells per flower to be successful (Philion 2014). So far, we have recovered *E. amylovora* cells from *D. suzukii* individuals previously exposed to Ea21GFP ooze by plating a suspension containing homogenized insects on solid medium, which subsequently showed Ea21GFP cell growth. This suggests that *E. amylovora* cells survive once acquired by the flies; however, no data on the precise bacterial cell titre were collected. Moreover, it is known that *E. amylovora* immediately enters the viable but not culturable state (VBNC) when attached to the body surface of honeybees (Choi et al. 2022), a state in which cells are alive but have low metabolic activity. This may limit the quantification of bacterial cell viability, as VBNC cells are unable to grow on artificial media unless they reacquire cultivability. While qPCR can detect the presence of these bacterial cells, it does not guarantee that they will regain the necessary vitality to cause an infection. Transmission of acquired *E. amylovora* cells to plant and fruit surfaces should also be tested. These are all questions future studies will need to address.

These findings provide a general overview of the potential role of *Drosophila suzukii* as a vector for *Erwinia amylovora*, the causative agent of fire blight. This research proved a significant tendency of *D. suzukii* to be attracted to the bacterial ooze produced by *E. amylovora*, suggesting the ooze may play a key role in pathogen transmission by attracting the insect and eventually spreading it. Direct evidence of *E. amylovora* acquisition from infected fruits was also found by developing a new protocol for testing *E. amylovora* uptake, laying the basis for future studies. This study lays the groundwork for future research aimed at understanding the interaction between *D. suzukii* and *E. amylovora*.

*The sections "Materials and Methods," "Results," and "Discussion," presented under the title "**From apple flowers to control fire blight: new potential biocontrol agents of* **Erwinia amylovora?**" *are part of a manuscript currently in preparation and intended for submission in 2025*.

*The sections "Materials and Methods," "Results," and "Discussion," presented under the title "***Drosophila suzukii:** ***a potential vector of Erwinia amylovora, the causal agent of apple fire blight?***" *have been published in the master's thesis entitled "*Drosophila suzukii*: a possible vector of* Erwinia amylovora*, the causal agent of apple fire blight" by Colmagro Asia (2024)*.

CHAPTER 5. CONCLUSION

This study aimed to deepen the knowledge of fire blight to find sustainable solutions for managing this devastating disease, therefore reducing the negative impact of conventional control strategies involving the use of antibiotics and copper-based compounds.

Several BCAs have been identified in the past years to control *E. amylovora* (Kunz 2004; Sundin et al. 2009), but their application alone is still not sufficient to completely control this plant pathogenic bacterium (Sundin et al. 2009; Johnson et al. 2013, 2022). Thus, it is essential to further study the apple flower microbiota to select a potential BCA to integrate and implement environmentally friendly practices. Of all bacterial strains we isolated from apple flowers*, P. agglomerans* AFF2001 was the one able to effectively control *E. amylovora* Ea21 on apple flowers, significantly reducing the disease development. Moreover, *P. agglomerans* AFF2001 showed to hinder the virulence and pathogenicity of *E. amylovora* Ea21 as demonstrated by the assays conducted on immature pear slices and tobacco leaves, respectively. We found out several mechanisms might be involved in the biocontrol activity observed for this bacterial strain. First, *Pantoea agglomerans* AFF2001 may compete with *Erwinia amylovora* Ea21 for nutrients and space, making key resources such as sugars, amino acids, and iron ions unavailable to the pathogen. This resource competition likely contributes to the inhibition of *E. amylovora* growth at the stigmatic surface of the flower. Additionally, the swimming motility of *P. agglomerans* AFF2001 may play a crucial role in its colonization of floral tissues, further enhancing its competitive advantage over Ea21. Second, the establishment of *Erwinia amylovora* Ea21 cells in the flower may be negatively influenced by acidification of the environment observed *in vitro* in the presence of *Pantoea agglomerans* AFF2001,

although this effect has not yet been confirmed in the flower itself. According to our results, a lower pH impairs both the growth and the swimming motility of *E. amylovora* Ea21, two factors that are essential for successful colonization and disease development. Third, *P. agglomerans* AFF2001 might kill *E. amylovora* Ea21 cells through contact-dependent mechanisms involving both the production of the D-alanylgriseoluteic acid (AGA) antibiotic and the activity of the Type VI Secretion System (T6SS). This hypothesis is based on the identification in the *P. agglomerans* AFF2001 draft genome of the AGA biosynthetic gene cluster and two putative T6SS effector genes. However, these mechanisms have not yet been experimentally validated. No contact-independent antibacterial activity was observed. In conclusion, our findings support that *Pantoea agglomerans* AFF2001 may be a promising new biocontrol agent (BCA) to be developed as a biopesticide. If no concerns regarding its biosafety or phytopathogenicity will be identified, future perspectives may include the development of a synthetic microbial community (SynCom) incorporating *P. agglomerans* AFF2001 together with other bacterial strains isolated in this study, to evaluate whether biocontrol efficacy can be enhanced.

As shown in the fire blight disease cycle, insects may have a role in the dissemination of *E. amylovora*, being attracted by the bacterial ooze produced by the plant pathogenic bacterial cells (Boucher et al. 2021). A few studies have demonstrated that both pollinator and non-pollinator species might acquire *E. amylovora* cells, maintain them outside and/or inside their body and potentially transmit them to other plants (Pierstorff et al. 1934; Keitt et al. 1941; Ordax et al. 2015; Boucher et al. 2019; Ribeiro et al. 2022). Based on the findings reported for *Drosophila melanogaster* (Boucher et al. 2019), we investigated whether the closely related species *Drosophila suzukii* might be regarded as potential vector of *E. amylovora*. We found that *D. suzukii* had a tendency to be attracted to *E. amylovora* Ea21 ooze and could acquire the bacterial cells directly from it. Differences related to the

genre of the insect were seen concerning the attractiveness, likely due to biological reasons. The attraction might be explained as a response to the VOCs released by the bacterial ooze, but this aspect was not analysed in this study. Once observed *D. suzukii* could acquire *E. amylovora* Ea21, we optimized the acquisition protocol described by Ordax and colleagues (2015). To reproduce what happens in environmental conditions, we chose to infect immature pears with *E. amylovora* Ea21, leading to ooze development. We also set up other parameters such as the number of insects and the DNA extraction method, as well as the molecular analysis. In conclusion, this research showed *D. suzukii* showed a tendency to be attracted to the *E. amylovora* Ea21 ooze and can acquire the plant pathogenic bacterium, suggesting its potentiality as vector of *E. amylovora.* The next step would be to assess the ability of this species to transmit *E. amylovora* to healthy tissues. If its vectoring capability is confirmed, strategies should then be developed to manage and control *D. suzukii*.

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