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Characterization *of Pseudomonas syringae* pv. *actinidiae* in Portugal

Master thesis project in Biodiversity and Vegetable Biotechnology, guided by Prof. Drª Joana Cardoso da Costa and Prof. Drº António Manuel Veríssimo Pires, presented at the Department of Life Sciences of Faculty of Science and Technology of the University of Coimbra.

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob orientação científica da Professora Doutora Joana Cardoso da Costa (Universidade de Coimbra) e do Professor Doutor António Manuel Veríssimo Pires (Universidade de Coimbra).

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Abstract

Pseudomonas syringae pv. *actinidiae* (Psa) is a gram-negative bacterium that causes the bacterial canker in both *Actinidia deliciosa* and *A. chinensis*. Psa is a quarantine disease in most countries and since the emergence of an economically devastating outbreak in Japan in the 1980s, it has grown to an international pandemic that is now threatening the sustainability of the kiwi industry in all major kiwi-producing countries, including Portugal (EPPO, 2011/054). Kiwifruit industries worldwide are engaged in coordinated disease control strategies to contain the pandemic and minimize economic loss to growers. Nevertheless, rigorous cultural control measures have not been able to prevent the rapid spread of the pathogen. Portugal is the 10th worldwide Kiwi fruit producer.

Five distinct orchards, known to be colonized with Psa, and representing distinct abiotic conditions (North and Central Portugal), were sampled in consecutive spring and autumn. Leaves were collected from the same four kiwi plants in each orchard and the endophytic and epiphytic Psa diversity was assessed independently. Psa strains were also isolated from natural environments, namely from soil and water samples.

A total of 1673 putative Psa strains were recovered and confirmed using the Gallelli *et al*. (2011) methodology. The fingerprinting analysis, inferred from the BOX-PCR methodology of Psa isolates recovered from each studied orchard, demonstrated that the Psa populations present in Portuguese orchards were heterogeneous. This heterogeneity was found within orchards and between orchards. Additionally, the structure of Psa populations varied over time in the same plant. Higher Psa population's diversity was found among spring Psa populations when compared to those determined in autumn. Furthermore, Psa population diversity was also affected by the location in the leaf and by the geographical location of the orchard.

Psa strains were identified in both soil and water samples, suggesting that these environments provide conditions for Psa persistence and must be considered a probable reservoir for Psa.

In sum, this study evidences the co-existence of several Psa populations in the studied Portuguese orchards. Some of these Psa populations varied with time while other were persistently recovered. This was the first report of a heterogeneous Psa 3 population coexisting in the same plant, at the same time.

Keywords – Bacterial kiwifruit canker, *Pseudomonas syringae* pv. *actinidiae*, Portuguese orchards; population diversity; environmental reservoirs

Resumo

A bactéria Gram negativa *Pseudomonas syringae* pv. *actinidiae* (Psa) é a agente causal do cancro bacteriano da actinídea. Considerada uma doença de quarentena na maioria dos países desde o surto inicial ocorrido no Japão na década de 1980 com efeitos económicos devastadores nas fileiras do kiwi. A área de cultivo afetada tem vindo a crescer sendo atualmente considerada uma pandemia, ameaçando a sustentabilidade da indústria do kiwi nos principais países produtores, incluindo Portugal (EPPO, 2011/054).

A indústria do kiwi tem apoiado o desenvolvimento de novas estratégias de controlo da doença com o objetivo de conter a pandemia e minimizar as perdas económicas. Contudo, a aplicação de medidas rigorosas de controlo não conseguiu evitar a disseminação deste agente patogénico. Atualmente, Portugal é o 10º produtor mundial de kiwi, com registo de um valor de produção de 21 mil toneladas em 2016.

No presente trabalho foram estudados cinco pomares, afetados por Psa, com condições abióticas distintas (Norte e Centro de Portugal). Foram recolhidas amostras na primavera e no outono seguinte. Em cada pomar foram selecionadas 3 plantas e de cada uma foram recolhidas 4 folhas, em cada estação analisada. As populações epífitas e endófitas de Psa foram caraterizadas independentemente.

No total foram isoladas 1673 estirpes putativamente identificadas Psa, e posteriormente confirmadas de acordo com o protocolo descrito por Gallelli *et al.* (2011). A tipagem dos isolados de Psa foi realizada pela metodologia de BOX-PCR.

Os resultados evidenciaram que as populações de Psa presentes nos pomares portugueses são heterogéneas. Tal heterogeneidade foi encontrada em cada um dos pomares e entre os pomares. Além do mais, a estrutura das populações de Psa variou ao longo do tempo na mesma planta. Foi ainda detetada uma maior diversidade entre as populações de Psa isoladas na primavera do que entre os isolados no outono. Para além disso, fatores como a localização na folha e a localização geográfica do pomar influenciaram a diversidade nas populações de Psa.

Foram identificadas estirpes de Psa isoladas de amostras de solo e água recolhidas nos pomares, o que sugere que estes ambientes propiciam as condições necessárias à persistência destas bactérias, pelo que devem ser considerados como prováveis reservatórios ambientais de Psa.

Em suma, este estudo permitiu evidenciar a coexistência de múltiplas populações de Psa presentes nos pomares portugueses. Algumas destas populações variaram nas diferentes amostragens,

enquanto outras foram persistentemente isoladas. Este é o primeiro estudo a reportar a coexistência de populações distintas de Psa3 na mesma planta.

Palavras-chave – cancro bacteriano da actinídia, *Pseudomonas syringae* pv. *actinidiae*, pomares portugueses, diversidade de população, reservatórios ambientais.

Abbreviations

AI – Autumn Isolates

ASM - Acibenzolar-S-methyl

BCAs - Biological control agents

bp – Base pair

DGAV - Direcção Geral de Alimentação e Agricultura

EN – Endophytic isolates/strains

EP – Epiphytic isolates/strains

EPPO - European and Mediterranean Plant Protection Organization

ET - Ethylene

JA - Jasmonic acid

KB - King's B medium

KBc - KB medium modified

MLST - MultiLocus Sequence Typing

NSA - Nutrient Sacarose Agar

PBS - Phosphate Buffered Saline

PCA - Principal Component Analysis

PCR - Polymerase Chain Reaction

Psa - *Pseudomonas syringae* pv. *actinidiae*

Rep-PCR – Repetitive PCR

SA - Salicylic Acid

SI – Spring Isolates

SO – Soil Isolates

TAE - Acetate-EDTA

WA- Water Isolates

Table of contents

List of figures

List of tables

1. Introduction

1.1 The kiwifruit culture

Native to China, the kiwifruit belongs to the *Magnoliophyta* division, *Magnoliopsida* class, *Ericales* order, *Actinidiaceae* family and *Actinidia* Lindl genus (NCBI, 2017). The genus *Actinidia* is characterized as a woody climbing vine, deciduous (with a few exceptions) and dioecious plant (Ferguson, 2013). The fruit, commonly named kiwi, is described as "berries with many seeds embedded in a juicy pericarp" (Ferguson, 2013). *Actinidia* species prefer humid and sheltered environments, with temperatures ranging between -10°C and 40°C. Exposure to cold temperatures for at least 600 hours is a crucial factor in fruit development, therefore, a frost-free period long enough is necessary to allow fruit maturation (Ferguson, 2013).

The genus *Actinidia* comprehend 55 species and 20 varieties, although only tree species have commercial importance in the world agriculture sector. The two mostly cultivated species of *Actinidia* around the world are *A. chinensis* and *A. deliciosa*. In Europe, New Zealand and United States, *A. arguta* is gaining commercial interest and consequently slowly growing in terms of economic importance (Ferguson, 2013).

Several cultivars have emerged since the beginning of global kiwifruit cultivation. The mostly famous cultivar of *A. deliciosa* is "Hayward". This cultivar became responsible for at least 60% of the kiwifruit production throughout the world (Ferguson, 2013). *A. chinensis* also has some cultivars established in international markets, namely "Hort16A", "Jintao" and "Soreli" (Ferguson, 2013; Scortichini *et al.*, 2012).

1.1.1 Economic importance

Since 1999 the production of kiwifruit has been globally increasing. In 2014, the worldwide production reached 3 447 604 tonnes. Regions of Asia, Europe and Oceania were responsible for 47.2%, 27.6% and 15.3%, respectively of this global production (FAOSTAT, 2017). China, Italy and New Zealand, as shown in Figure 1-1, are the major worldwide kiwifruits producers, with more than 410 746 tonnes per year (FAOSTAT, 2017).

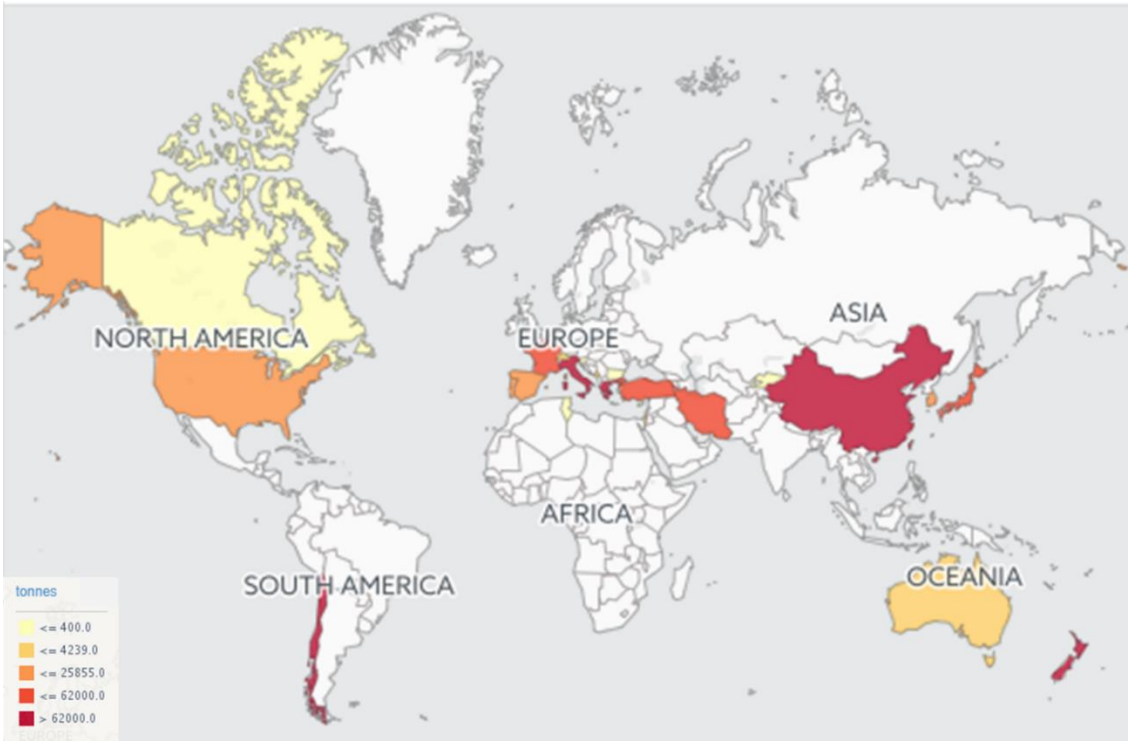


Figure 1-1. World production of kiwifruit. Source: FAOSTAT, 2017

In Portugal, the economic interest in this crop has raised since the 90's. The high nutritional value of kiwifruit - rich in vitamin C, exotic flavour, low cost of production and the few phytosanitary problems were the main reasons for this economical interest (Abelleira *et al.*, 2015; Félix & Cavaco, 2004). Kiwifruit production has become important for local economy especially in the two leading regions: Entre Douro e Minho and Beira Litoral (DGAV, 2014), but climate conditions and the excellent quality of the produced fruit allowed the expansion of this culture to other regions. Nowadays, Portugal is the 10th worldwide kiwifruit producer. In 2016, the production of this fruit reached the highest values ever, with 21 075 tonnes in 2 380 hectares. Entre-Douro e Minho represents the region with major impact in the production with 23 205 tonnes in an area of 1 721 hectares. The export values of kiwi reached 13 167 000€ in 2016 (INE, 2017).

As most cultures, *Actinidia* spp. has been affected by globally distributed diseases caused both by bacteria and fungi. In the last years, this culture has suffered tremendous economic losses caused by the pathogen *Pseudomonas syringae* pv. *actinidiae*, the causal agent of "bacterial canker of kiwifruit" (Donati *et al.*, 2014).

1.1.2 Diseases that affect the kiwifruit culture

Among the diseases that affect the kiwifruit culture the most severe are caused by bacteria and fungi. The common fungi diseases in kiwifruit are caused by *Armillaria* spp. and *Phytophthora* spp., which causes the white rot and the brown rot respectively. Rotting and fluffy degradation of roots are characteristics of the "white rot disease", but the main characteristic is the whitish film that appears covering the stalk, lap and the root of the plant (Sofia, 2003). The "brown rot disease" is known by the wet and reddish rot in the roots. This rot affects the insertion of the main roots causing the yellowing and wilting of leaves. There are no known preventing nor curative measures for these fungi diseases (Chicau & Costa, 2008).

Another disease caused by fungi is named "Esca". In this case a combination of three fungi has been found responsible for the pathogenesis, namely *Phaeoacremonium* spp., *Fomitiporia mediterranea* and *Fusicoccum* spp. The symptoms associated with "Esca" are leave necrosis and wood degradation with consequent weakening of the plant, which affects production and could lead to the death of the plant (Sofia, 2003). Similarly to the other mentioned fungi disease, there are no effective measures to control "Esca".

In terms of the diseases caused by bacteria, the "canker of kiwifruit" is the one that most concerns producers. Caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), this canker is devastating for the most susceptible cultivars, and in some cases, leads to the dead of the plant (Vanneste *et al.*, 2012). *Actinidia* spp. are also affected by other pathovars of *Pseudomonas syringae*, namely *Pseudomonas syringae* pv. *syringae* and pv. v*iridiflava,* which are responsible for the "bacterial wilt". This disease attacks the leaves and the flowers buds, causing brown stains that could get necrotic, which can lower the yield of the production, without causing the dead of plant (Chicau & Costa, 2008; Sofia, 2003).

1.2 "Bacterial canker" of kiwifruit

In the last years, "bacterial canker" of *Actinidiae* spp. has been destroying kiwifruit orchards for all over the world. The causal agent, Psa, can infect both green (*Actinidia deliciosa*) and yellow (*Actinidia chinensis*) kiwifruit (Scortichini *et al.*, 2012) and is considered a quarantine bacterium by the European and Mediterranean Plant Protection Organization - EPPO (EPPO, 2012a). The severity and lack of disease control led to a pandemic infection which caused significant global economic losses, namely in Italy, New Zealand, France, Chile, Spain and Portugal (Vanneste, 2013). It is expected that in Italy and New Zealand the losses will reach over 310 million euros between 2013 and 2018 (Khandan *et al.*, 2013).

1.2.1 Disease history and geographical distribution

First isolation, identification and description of Psa from "Hayward" cultivar, dates to 1984 in Japan (Takikawa, 1989). In 1989 a similar disease was described in China (Butler *et al.*, 2013). In Europe, Italy was the first country to report the presence of this disease in 1992 (Scortichini, 1994). South Korea and Iran also reported the presence of Psa in 1994 (Mazarei & Mostofipour, 1994; Koh *et al.*, 1994). Interestingly, the behaviour of this pathogen was different in Japan and South Korea in comparison to Italy. The disease was much more destructive to kiwifruit orchards in the first two countries, while in Italy there were no considerable losses (Balestra *et al.*, 2010; Ferrante & Scortichini, 2009, 2010). Nevertheless, in 2008 occurred the first destructive epidemics in Italy, devastating the susceptible yellow kiwifruit cultivars "Hort16" and "Jin Tao" and the more resistant green kiwifruit cultivars "Hayward". The disease quickly spread to all the producing regions of Italy in just two years (Scortichini *et al*., 2012).

The disease was rapidly spread between 2011 and 2012 to the rest of the EPPO producing countries, namely Portugal and Spain (Abelleira *et al.*, 2011; Balestra *et al.*, 2010), France (Vanneste *et al.*, 2011c), Switzerland (EPPO, 2011b) and Turkey (Bastas & Karakaya, 2012). New Zealand and Chile also reported the disease in 2011 (EPPO, 2011a; Everett *et al.*, 2011). More recently the presence of Psa in the kiwifruit orchards of Greece was also confirmed (Holeva *et al.*, 2015). The Figure 1-2 shows the current overall distribution map of Psa.

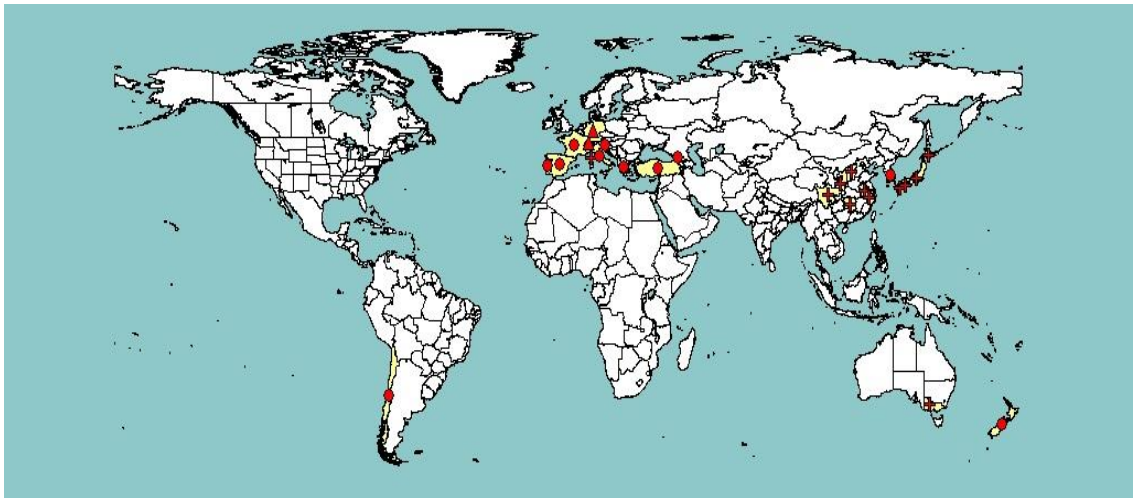


Figure 1-2. Geographical distribution of Psa. Source: EPPO, 2017

Psa was included in EPPO plant quarantine A2 list to prevent further introductions and to limit the spread of this severe disease between Europe countries (EPPO, 2012b). This action limited the movement of plants or pollen between member states, requiring a phytosanitary certificate and if necessary, an inspection with laboratorial tests for bacteria screening. A phytosanitary passport was

created to certify that plants are coming from a Psa-free area, and to guarantee that plants are free of also others quarantine bacteria (Cunty *et al.*, 2015b).

1.2.2 Life cycle, symptomatology and evolution of the disease

"Bacterial canker of kiwifruit" presents a special characteristic: the ease with which Psa spreads within and between orchards (Scortichini *et al.*, 2012). The pathogen entry can occur from natural wounds like stomata, broken trichomes and flowers, leaves and fruit abscission scars (Donati *et al*., 2014; Spinelli *et al*., 2011). Recent studies observed the presence of Psa on the reproductive system of female flowers. Before infection of female flowers, the bacteria can systematically invade the plant (Donati *et al.*, 2014). On male flowers, Psa can survive on pollen grains which constitute a vector for the dispersal of the pathogen (Vanneste *et al.*, 2011a). Psa may also survive and multiply epiphytically or endophytically on flowers and leaves (Stefani & Giovanardi, 2012; Vanneste *et al.*, 2011a).

The Psa life cycle (Figure 1-3) is highly affected by the microclimate of the orchard. Differences between weather conditions, as temperature and relative humidity may determine the severity and dispersal of the disease through the orchard. The infection is favoured by humid weather and mild temperatures, and the dispersal is favoured by strong wind and rain (Donati *et al.*, 2014). Recently, Scortichini and Ferrante (2014) demonstrated that the damage from frost, freeze thawing also promotes the migration of the bacteria within and between orchards. Host plant colonization can occur at any time of the year, although bacteria growth is favoured in certain seasons, like early spring and autumn (Ferrante *et al.*, 2012).

During spring, the pathogen can enter leaves through natural wounds and systemically colonize the host plan through leaf veins and petioles (Donati *et al.*, 2014). In the infected orchards is possible to observe characteristic symptoms, like a white or red-rusty bacterial exudate on trunks (Fig. 1-3f), dormant canes or after bud burst in development twigs. Exudates are the result of bacterial multiplication on the tissues of host plant. The spring weather, with temperatures between 12°C and 18°C, represents the optimum growth temperature for the pathogen, specially in new canes (Serizawa & Ichikawa, 1993). Another characteristic symptoms are dark brown angular leaf spots surrounded by yellow haloes and necrotic flowers (Fig. 1-3b) (Abelleira *et al.*, 2015). Appearance of necrotic zones in the flowers (Fig. 1-3h) could lead to floral abortion, which prevents fruit formation (DGAV, 2014).

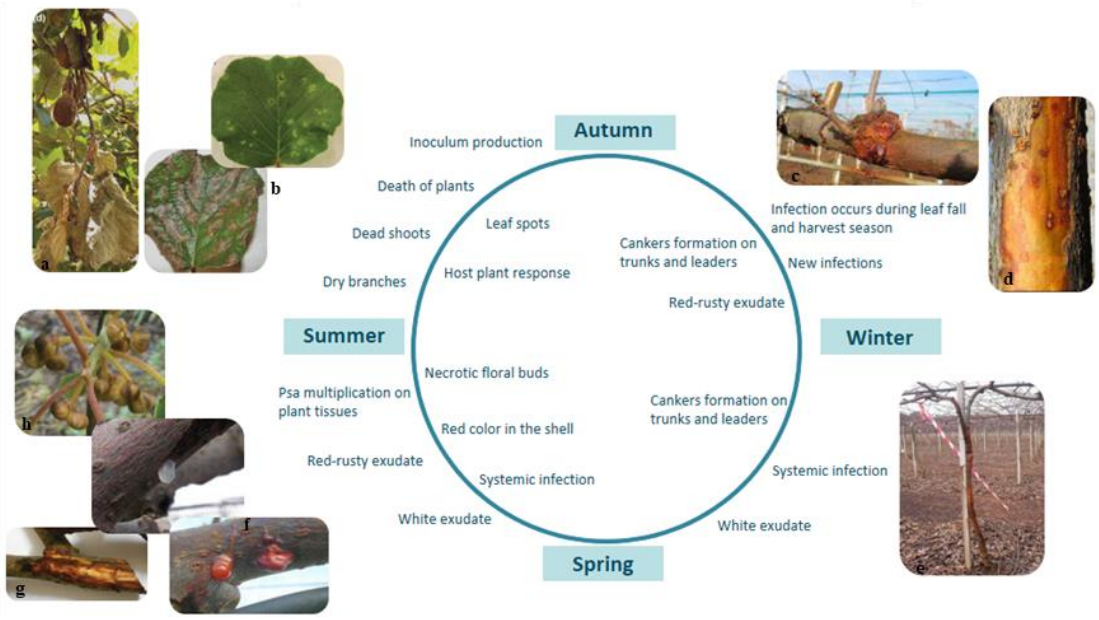


Figure 1-3. Life cycle, symptomatology and evolution of the disease. a: dry branches; b: leaf spots surrounded by yellow haloes; c: bacterial canker; d: red-rusty exudate; e: infected actinidia plant; f: oozing of white and red-rusty exudates; g: red colour in the shell; h: necrotic floral buds. Adapted from: Garcia, 2015; Ferrante *et al.*, 2012.

During summer, the degree of infection is reduced because of the rise in temperature, since Psa growth is inhibited above 25°C. Despite the growth inhibition, bacteria can survive the summer in tissues and continue to colonize the host through stomata and hydathodes, infecting new host plants (Donati *et al.*, 2014; Scortichini *et al.*, 2012). In addition to temperature, the host plant response also contributes to drastically reduce the infection. Host plant response includes the development of wound-healing tissue surrounding the infected area. This defensive process is only effective with temperatures above 22°C. Below 15°C the formation of wound tissue is very low, which weakens the plant physiological status (Donati *et al.*, 2014). Dry branches (Fig. 1-3a), leaf spots (Fig. 1-3b) and death shoots are the main symptoms observed in this season. However, some plants can't resist to infection and it is possible to observe plant death.

In the autumn or early winter, changes in the weather conditions favour the bacterial infection. Beside the decrease of temperature and increase on humidity, autumn is also the kiwi harvest season. The cultural practices facilitates Psa penetration into host plant tissues *(Ferrante et al., 2012; Vanneste et al.*, 2011b). Additionally, wounds left by leaf fall also expose the plant to the entrance of the pathogen. At this stage, Psa colonizes essentially lenticels and buds (Serizawa *et al.*, 1994). These new infections produce a red-rusty bacterial exudate (Fig. 1-1d).

During winter, the pathogen may survive in a latent form in the cortex tissue of infected branches and infect the plant systemically. First frosts promote the oozing of exudates from the wounds, originated by cankers on trunks and leaders (Fig. 1-1c) (Donati *et al.*, 2014).

1.2.3 Dissemination, transmission and environmental reservoir

It is known that Psa can easily spread within and between orchards with several factors conditioning the mode of dissemination. However the knowledge of those factors remains uncertain (Donati *et al.*, 2014; Ferrante *et al.*, 2012).

The dissemination of the disease at small distances, within and between neighbour orchards, it's thought to occur through propagation of epiphytic populations of Psa, through bacterial exudates produced by infected plants or by insects that transport contaminated pollen (Gallelli *et al*., 2011; Vanneste *et al*., 2011a). Both dissemination and transmission can be aided by abiotic or biotic factors and cultural practices. Namely, abiotic factors such as rain and wind, can promote friction between leaves causing wounds and permitting the penetration of the bacterium into a health plant (Garcia, 2015). Rain also may wash exudates becoming a vehicle of transport. In addition, weather conditions such as temperatures between 12° and 18°C and high humidity contributes to pathogen multiplication (Serizawa & Ichikawa, 1993) which increases the success of infection focus. Cultural practices play a significant role in disease transmission, especially if the orchard is infected, by favour the penetration of the bacteria within the plant from wounds caused by some agronomical techniques (Scortichini *et al.*, 2012).

Insects and pollinators are the main biotic factors acting as disseminating vector of infection carrying infected pollen from plant to plant (Tontou *et al.*, 2014; Vanneste, *et al.*, 2013b). Pattermore and his collaborators (2014) detected viable Psa on bees from infected kiwifruit orchards. Another biotic factor that affects transmission is the different susceptibility between young and adult plants, being young plants more susceptible (Vanneste *et al.*, 2011b). Commercial exchange of infected pollen or plants are the principal method of dissemination at long distances: between regions or countries (Vanneste *et al.*, 2011b).

Epidemiology of Psa is now better understood (Ferrante *et al.*, 2012; Scortichini *et al.*, 2012), yet there are still issues that need to be answered. Psa chain of infection comprises both an initial focus of infection on orchard and a host plant (e.g. *Actinidia* spp.) with some entrance portal (such as wounds or stomata) through which bacteria (in this case Psa) will enter and colonize the plant tissues. If the host plant is susceptible to the bacteria and microclimatic conditions on the orchard are appropriate for disease development, the host plant will become infected and bacterium will be considered a

pathogen agent. The chain of infection is resumed with the exit of the inoculum through an exit portal (such as red-rusty bacterial exudate). Thus, the pathogen agent spreads within and between orchards through dissemination modes (e.g wind and leaves friction) and continues the cycle of the disease.

The existence of environmental reservoirs of human bacterial pathogens is well established, and it is known that these reservoirs play an important role in the evolution and disease epidemiology (Monteil *et al.*, 2013). Researches to find the environmental reservoirs for *Pseudomonas syringae* lineages pathogenic to kiwifruit and tomato plants suggested the involvement of some compartments of the water cycle (e.g. freshwater habitats) and other non-agricultural environment reservoirs (e.g. snow zones) (Bartoli *et al.*, 2015, 2016; Monteil *et al.*, 2013). This information supports the hypothesis that irrigation water may be an important environmental reservoir for Psa. If strains were isolated from the water used to irrigate the kiwifruit orchards similar to pathogenic populations, we would be in the presence of a relevant and previously undescribed transmission mode of Psa within the orchard.

Besides water habitats, orchard soil might represent another environmental reservoir of Psa. Indeed, agricultural soil and plants were suggested as reservoir for *Pseudomonas aeruginosa*, detected in 24% of soil samples (Green *et al*., 1974).

Plants commonly act as reservoir for pathogen agent and *Actinidia* spp. are considered environmental reservoir of Psa. After colonization, Psa could survive as epiphytic or endophytic populations on host plant. It seems that epiphytic populations survive on asymptomatic flowers and leaves (Vanneste *et al*., 2011d). According with seasons and also with climatic conditions, Psa is capable to colonize "leaf buds, young and old leaves, shoots, one-year-old twigs, flower buds, open flowers, symptomless lenticels along the twig, trunk and leader, fruit stalk, leaf scars and suckers" (Ferrante *et al*., 2012). After entering plant tissues, endophytic populations can move systemically, probably via the xylem vessels, through the different organs multiplying and originating canker formations (Scortichini *et al.*, 2012). Therefore, host plant contributes to the multiplication and spread of the disease into the host plant itself, being a reservoir promoting dissemination to other susceptible hosts (Vanneste *et al.*, 2011b).

1.2.4 Mitigation methods

Since there are no curative treatments known to control Psa and eradicate the disease from kiwifruit orchards, it was necessary to develop mitigation methods to diminish infection and prevent the dissemination of bacteria within and between orchards (Vanneste *et al.*, 2011b). Indeed cultural, chemical and biological methods have been used with limited success to mitigate the infection and to prevent new focus of infection (Donati *et al.*, 2014). Phytosanitary practices and an appropriate field

management in addition to proper application of the mitigation methods is crucial for containing Psa. Nevertheless, the effectiveness of the mitigation procedures is influenced by abiotic factors, especially microclimatic conditions (Vanneste, 2013).

1.2.4.1 Cultural methods

The main regulated agricultural notices advocate the use of phytosanitary and hygienic practices in orchard maintenance. To avoid introduction of Psa in the orchard the circulation of people should be restrict and all the agricultural materials, like farm gloves and boots, tractor wheels and pruning utensils, must be clean of plant residues and disinfected (Moreira & Coutinho, 2014). Size and number of wounds resulting from usual cultural operations during pruning or harvesting season must be minimized and the wounds should be disinfected and protected with an appropriate covering product (Moreira & Coutinho, 2014). After pruning season, pruning debris should be removed from the kiwifruit orchard and subsequently burned to mitigate new accidental wounds promote by natural phenomena as wind, and to avoid accumulation of potentially infectious plant material on orchard (Cameron & Sarojini, 2014; Vanneste *et al.*, 2011b).

In already infected orchards, plants with symptoms should be carefully managed to minimize spread of the disease. Symptomatic branches, sticks or leaves should be removed and burned, as also dead plants (Vanneste *et al.*, 2011b).

Following Psa inclusion in the EPPO plant quarantine A2 list, Portugal, as member state, started a national action plan for the control of Psa in 2012 (last reviewed and adapted in 2014) elaborated by the Direcção Geral de Alimentação e Agricultura (DGAV). Among other information, this action plan advises pruning of symptomatic plants as a usual method of control in diseased orchards. Depending on the degree of severity of infection observed in the plants, different methods of pruning are suggested: plants that exhibit clear symptoms (e.g. white red-rusty exudate) must be cut below one meter from the infection focus; plants that express symptoms just in branches and/or leaves must be cut below seventy centimetres from the infection focus if symptoms appears just in branches (DGAV, 2014). These pruning measures do not always prevent the propagation of the bacteria throughout the orchard because only symptomatic plants are pruned. In addition, Psa can live epiphytically on plants without causing disease, then according to this pruning methodology, the epiphytic populations are not remove from the orchard constituting a bacterial inoculum. Therefore, it is very difficult to remove the inoculum throughout the orchard's infected area and stop the spread of disease (Vanneste *et al.*, 2011b).

Despite all the cares about pruning, it is also important to control fertilization and watering of orchard. Fertilization should be accomplishing by soil analysis to avoid excessive plant vigour and watering should be improved to keep orchard atmosphere from being excessively moist (Moreira & Coutinho, 2014).

Grafting sticks cannot be collected from sick plants, or from orchards with infected plants or from areas where this pathogen has been detected. To ensure the safety of grafting sticks, samples from the donor orchards must be collected for Psa screening tests (DGAV, 2014). Healthy plants used to plant orchards must be accompanied with a phytosanitary passport which ensures that plants are Psa free.

Nowadays, and since the available cultural control methods are not curative, they are only used in to prevent the entrance and dissemination of the pathogen and must be complemented with chemical methods to successfully contained the spread of the disease (Donati *et al.*, 2014).

The first draft sequence genome of the kiwifruit *A*. *chinensis* (Huang *et al.*, 2013) provided a valuable resource for biological investigation, crop improvement and genomic analysis (Donati *et al.*, 2014). In addition, years of investigations contributed to better understand the life cycle of the disease. Therefore, breeding programs to obtain new cultivars with an increased resistance to Psa have been started in various research institutes (Donati *et al.*, 2014; Vanneste, 2013). Researches based on germplasm screening or induction of mutations have been attempted (Donati *et al.*, 2014). As consequence, a new cultivar of yellow-fleshed kiwifruit varieties, “Gold3” has been released by New Zealand breeding program as an alternative for “Hort16”. This new cultivar apparently is more tolerant to Psa. News cultivars will be certainly developed in the future (Testolin *et al.*, 2016).

1.2.4.2 Chemical methods

Essentially preventive, the mostly often used chemical methods to fight Psa are based in bactericides or bacteriostatic compounds and more recently, resistance inducers (Donati *et al.*, 2014). However, with exception of the later, these products contain heavy metals or antibiotics which limitations their use. In Europe, the application of these compounds is prohibited (Cameron & Sarojini, 2014).

Copper-based compounds are commonly used and play an important role in field control of kiwifruit bacterial diseases, particularly if applied at an early stage of disease development. These compounds are bacteriostatic, capable of inhibit bacterial activity. Pulverizations of cooper-based formulations reduce significantly epiphytic pathogenic populations (Donati *et al.*, 2014). It is recommended that spraying be executed at end of pruning season, postharvest season and at leaf fall to prevent the entering of Psa through wounded tissues, and during bud break (Moreira & Coutinho, 2014; Vanneste

et al., 2011b). These applications must consider both the cultural management and the climatic conditions to which the orchard has been or will be subjected. Another determinant factor to the successful of spray coverage is orchard's canopy density which may affect both cooper rain fastness and longevity on different plant parts (Donati *et al.*, 2014).

Bactericides, such streptomycin are also used for control of Psa (Setsuo Serizawa *et al.*, 1989). New Zealand and Asian countries, in opposite to Europe, allow the application of some antibiotics on agriculture as control methods (Cameron & Sarojini, 2014; Donati *et al.*, 2014). Streptomycin formulations are commonly applied by spraying, such as cooper-based compounds, or by trunk injection. In Korea trunk injection demonstrated efficacy on healing the infected orchard (Koh *et al.*, 1996).

Both copper-based compounds and streptomycin formulations share some common problems such phytotoxicity but also the development of bacterial resistance and accumulation of toxic residues on the fruit (Cameron & Sarojini, 2014; Donati *et al.*, 2014). Studies reported the identification of both streptomycin and cooper resistant genes on Psa genome (Nakajima *et al.*, 1995; Vanneste *et al.*, 2011b), namely *strA* and *strB*, *copR* and *copS* that are respectively responsible by streptomycin (Nakajima *et al.*, 1995) and cooper resistance (Cooksey, 1994).

Agrofood industry in New Zealand has been developing studies in search for new protective compounds that can be applied to control Psa. Some of this new compound are sterilizers (terpene-based compounds, such as geraniol) and polysaccharides, such as chitosan (obtain from shrimp shells) (Cameron & Sarojini, 2014; Donati *et al.*, 2014; Vanneste, 2013). Both products showed some efficacy *in vitro* or in glasshouse trials to control epiphytic populations of Psa (Donati *et al.*, 2014). Phytotoxicity problems have been reported in tomato plants by the use of terpene-based compounds. Chitosan is known for both antimicrobial activity and capability of stimulating plant defences. Moreover, it's biocompatibility and biodegradability make it a promising product (Cameron & Sarojini, 2014; Donati *et al.*, 2014).

Despite all the advances in search for appropriate and effective options to use as control method, these stated above compounds just reduce epiphytic populations having no impact against endophytic bacteria (Donati *et al.*, 2014).

Resistance inducers could provide systemic protection to kiwifruit plant against possible infection risk events. Capable of inducing the natural plant defences system, some known resistance inducers have been subject of studies to ascertain its efficiency on "bacterial canker disease" (Cameron & Sarojini, 2014; Reglinski *et al.*, 2013). Studies were especially linked to phytohormone-mediated signalling pathways, which play a key role on trigger plant response. Kiwifruit plant natural resistance to Psa

seems to be mediated by salicylic acid (SA) signalling pathway mutually antagonist of jasmonic acid (JA)/ethylene (ET) signalling pathways (Donati *et al.*, 2014; Vanneste, 2013). The commercial compound Acibenzolar-S-methyl (ASM) operates as a functional analogue of SA. So, ASM elicit suppression of SA signalling pathway which improve resistance of kiwifruit plant to Psa (Vanneste, 2013). In Italy and New Zealand, glasshouse trials showed effectiveness of ASM to decrease disease incidence in both *A. deliciosa* and *A. chinensis* species (Vanneste *et al.*, 2012). Similar to other chemical methods of control, these compounds also have use limitations due to risk of fruit residues since they have foliar applications (Donati *et al.*, 2014). Resistance inducers must be an integrated option complementing others control methods. The interaction between the compound, pathogen and crop will define the duration of the provided protection (Cameron & Sarojini, 2014).

1.2.4.3 Biological methods

Integrated control of Psa may also include biological strategies mediated by biological control agents (BCAs). There is a great diversity of BCAs based on their mode of action, namely, elicitation of plant response, competition against pathogen or production of pathogen-specific antimicrobial compounds (Donati *et al.*, 2014). Frampton and collaborators developed a biocontrol strategy based on a cocktail of four bacteriophages with proven efficacy on reducing growth of different Psa strains (Frampton *et al.*, 2012, 2014). In Italy were also isolated bacteriophages which infect Psa, to be used as phage therapy on kiwifruit bacterial canker (Lallo *et al.*, 2014). Advances and applications of BCAs can represent an alternative to chemical control methods commonly used. However, their action mechanism, efficacy and impact on orchard environment is limited.

1.3 *Pseudomonas syringae* pv. *actinidiae*

1.3.1 Taxonomy

Pseudomonas syringae pv. *actinidiae* - Psa (Takikawa *et al.*, 1989) is a pathovar of *Pseudomonas syringae*, a member of the family *Pseudomonadaceae*, order *Pseudomonadales* and class *Gammaproteobacteria* of the phylum *Proteobacteria* (EPPO, 2014). *Pseudomonas syringae* species complex is composed by seventy-seven pathovars. From the forty-eight pathovars genetically analysed nine genomospecies were described (Gardan *et al.*, 1999). Later, Psa was placed into genomospecies 8, with *P. syringae* pv. *avellanae* and *P. syringae* pv. *theae* (Scortichini *et al.*, 2012). Currently, Psa is believed to be host-specific, only infecting *Actinidia* spp. (EPPO, 2014).

Recent genetic studies confirm the existence of at least five genetically different populations (also known as biovars) of Psa, all with the ability, at different extents, to infect crops of *Actinidia* spp. worldwide (Chapman *et al.*, 2012; Fujikawa & Sawada, 2016). Psa biovars have been geographically spread and named according to the chronological order of identification as Psa1, Psa2, Psa3, Psa4 and Psa5 (Fujikawa & Sawada, 2016).

The first Psa biovar was isolated from *A. deliciosa* cv. "Hayward" and was associated with the initial epidemic outbreak of the disease in Japan (1984-1989) and Italy (1992). The isolated strains in different countries in different years were genetically similar (Marcelletti *et al.*, 2011). However, the impact of the disease was more intense in Japannase orchards, causing severe economic losses. In Italy, the disease was mantained for 20 years with only sporadic and minor damages. The differences in virulence of the same bacterial pathogen may be explained by climatic conditions and/or agronomical tecnhiques (Scortichini *et al.*, 2012). A characteristic of this biovar is that all strains contains the phaseolotoxin gene cluster, *argK-tox* (Marcelletti *et al.*, 2011), involved in the formation of chlorotic halo lesions (Tamura *et al.*, 2002) which was supposely adquired by horizontal gene transfer (Sawada *et al.*, 1999).

Associated to South Korea disease outbreak, the Psa2 biovar infected both *A. deliciosa* cv. "Hayward" and *A. chinensis* cv. "Hort16A" causing important economic losses. This population was only detected in this country. Unlike Psa1, the operon *argK-tox* was not present in the Psa2 strains. The strains of this population contains a plasmid with genes for the biosynthesis of coronatine, *corR* genes, a non-host-specific phytotoxin (Han *et al.*, 2003). Multilocus sequence typing (MLST) analysis confirms that Psa2 is genetically closer to Psa1 (Chapman *et al.*, 2012).

The pandemic Psa biovar Psa3 was responsible for economic losses worldwide, specially on the EPPO region (EPPO, 2014). This biovar, also known as Psa virulent (Psa-V), is capable to infect both *A. chinensis* and *A. deliciosa* and since 2008 was the predominant population isolated in Italy and in the rest of Europe orchards (Vanneste, 2013). The Psa3 strains are characterized by the lack of both genes for phaseolotoxin and coronatine production (Scortichini *et al.*, 2012). Interestly, Psa3 did not evolve from strains of Psa1 but from a rather unknown common ancestor (Chapman *et al.*, 2012).

Psa strains virulence was related with the presence of effectors and virulence-related genes (Donati *et al.*, 2014). All strains have genes encoding several secretion systems (TSS). A core set of thirty-tree *hop* and six *avr* putative effector genes are conserved on the effector repertoire of Psa strains 1,2 and 3. (Marcelletti *et al.*, 2011). These populations also possess the *repA* gene, which has an important role in replication of a plasmid with genes encoding virulence factors, such TSSIII effectors, phytotoxins, plant hormones and determinants as well as genes for conjugation and insertion sequences elements

(Sundin, 2007). However, four putative effector genes *hopA1*, *hopAA1-2*, *hopH1* and *HopZ2*-like are exclusively present on Psa3 strains (Marcelletti *et al.*, 2011). This suggests that the effector repertoires of the three biovars able to infect *Actinidiae* spp. is variable and may justify the difference between a severe or a milder infection.

A fourth biovar was ideintified -Psa4, comprising strains less virulent (Psa-LV) since they were not able to cause systemic infections or plant death, causeing only leaf spots (Vanneste, 2013). Psa4 has been isolated in Australia, New Zealand, France and more recently in Spain orchards (Abelleira *et al.*, 2015; Chapman *et al.*, 2012; Cunty *et al.*, 2015b). In 2015, Cunty and collegues proposed that Biovar 4 should be considered a new *P. syringae* pathovar, and was renamed as *Pseudomonas syringae* pv. *actinidifoliorum* (Cunty *et al.*, 2015b).

Recently, a new population of Psa was reported in Japan, and named Psa5. Phylogeneticaly, Psa5 is closer to biovar 2 but did not had coronatine biosynthetic genes. Another characteristic of this new population is the absence of phaseolotoxin biosynthetic genes conserved in biovar 1 (Fujikawa & Sawada, 2016). The detection of this biovar is performed using specific PCR primers designed for targeting conserved regions in the strains of this specific population. Until now, biovar 5 is considered an endemic population of Japan (Fujikawa & Sawada, 2016).

1.3.2 Morphological and cultural characteristics

Pseudomonas syringae pv. *actinidiae* according to phenotypic characteristics observed by Takikawa and collaborators is a "gram negative rod-shaped bacterium, aerobic, non-spore forming, and motile with one to three polar flagella" (Takikawa *et al.*, 1989). Metabolically, this bacterium is oxidase-negative and arginine dihydrolase-negative (Donati *et al.*, 2014) and tobacco hypersensitive positive (Everett *et al.*, 2011). Primarily carbon source are glucose, galactose, fructose (Takikawa *et al.*, 1989).

Colonies of Psa grown in Nutrient Sacarose Agar (NSA) are visible after two days and reach to up 2mm of diameter after four days. Morphologically, they are smooth, elevated or convex, round and with the entire margin, pearly whitish in as showed on Figure 1-4a. (EPPO, 2014). The colony morphology changes a little on modified King's B medium (KB) (Fig. 1-4b), and colonies can reach 4-5mm of diameter after only 4-5 days. The smooth characteristic remains, however they became flat, with entire or slightly lobed margin which can be whitish-yellowish (EPPO, 2014).

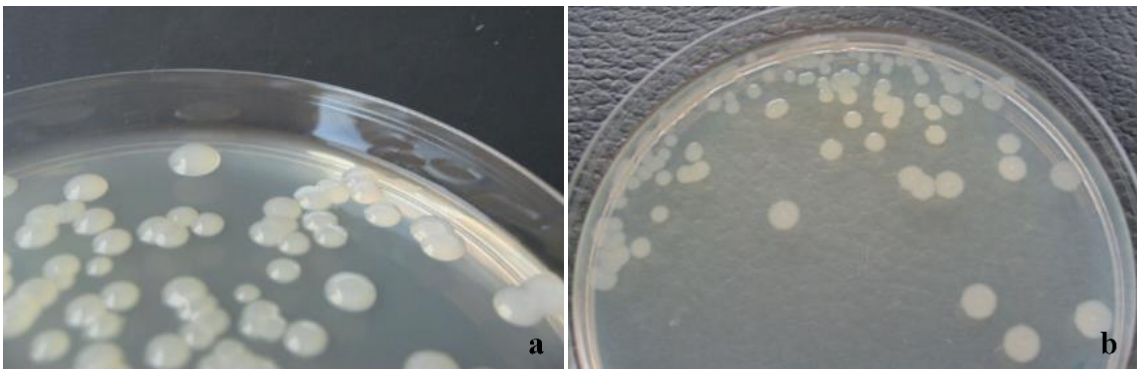


Figure 1-4. Morphology of Psa colonies grown for 5 days in different mediums supplemented with antibiotics. a: 5 days growth on NSA medium; b: 5 days growth on King's B medium. Source: E. Stefani, Dept. of Life Sciences, Reggio Emilia, Italy (EPPO, 2014)

1.3.3 Isolation, identification and fingerprinting

Diagnostic methods for Psa are performed in accordance with EPPO standard regulation (EPPO, 2014). The pathogen can be detected on both symptomatic and asymptomatic aerial parts of infected plants. In symptomatic plants, leave parts with necrotic spots or necrotic lesions and other parts showing the characteristic bacterial canker symptoms can be used as sample for detection of Psa (EPPO, 2014).

Bacteria isolation is attempted on modified King's B medium (Mohan & Shaad, 1987). The addition of boric acid and two antibiotics, cycloheximide and cephalexin, confers selectivity to medium and avoids the growth of undesirable bacteria and fungi which can interfered with the recovery of Psa (EPPO, 2014). The putative colonies of Psa are selected according to their colony morphology for purification and later DNA extraction.

Identification of Psa is based on molecular procedures. Preliminary screening tests suggested by EPPO are performed according to the duplex polymerase chain reaction (PCR), described by Gallelli *et al*. (2011). After the preliminary screening, DNA fingerprinting methods can be used to allow strain identification based on genotyping techniques described for *Pseudomonas* sp. and other bacteria at species and infraspecies level (EPPO, 2014). This genotyping technique consists in a repetitive PCR (rep-PCR) that uses one or more primers which amplifies interspersed repetitive highly conserved DNA sequences present in bacterial genomes (Louws *et al.*, 1994). The BOX-PCR referred on EPPO standard regulation (EPPO, 2014), amplifies the repetitive sequence of BOX element and has been efficiently used to characterized and compare the genetic variability of Psa strains *(Ferrante et al.*, 2012; Ferrante & Scortichini, 2010; Abelleira *et al.*, 2014; Marcelletti *et al.*, 2011).

Another genotyping tool is the Multilocus Sequence Typing (MLST) scheme, applied for an in-depth study of both phylogeny and structure of bacterial populations (Sarkar & Guttman, 2004). MLST

consists in a DNA sequence-based method that relies on PCR amplification and sequencing of internal fragments of four or more housekeeping genes (Elberse *et al.*, 2011). Four housekeeping genes, *gapA*, *gltA*, *gyrB* and *rpoD* are frequently used for Psa typing and for phylogenetic analyses (Abelleira *et al.*, 2014; Cunty *et al.*, 2015a, 2015b).

1.3.3.1 Molecular identification by Duplex-PCR

Gallelli and his collaborators (2011) developed a simple method to identify Psa based in a PCR-method, described in EPPO standard: a duplex-PCR. The duplex-PCR uses two pairs of primers, in this case: KN-F/R and AvrDdpx-F/R. The first pair of primers was designed by Koh and Nou (2002), and it is not specific for Psa, since it also amplifies the same fragment in *P. syringae* pv. *syringae*, pv. *theae* (Rees-George *et al.*, 2010) and pv. *tomato* (Biondi *et al.*, 2013). The second pair of primers was obtained from the sequence of TSSIII effector D1 (*AvrD1)* gene that proved to be specific for Psa strains (Gallelli *et al*., 2011). The duplex-PCR can distinguish Psa strains from those of *P. syringae* pv. *theae*, *P. syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *P. avellanae*, *P. viridiflava* and other bacteria also isolated from infected kiwifruits. A positive test result for Psa detection originates two amplicons with a size of 492 bp and 230bp for KN-F/R primers and AvrDdpx-F/R primers respectively (Gallelli *et al.*, 2011).

1.3.3.2 DNA fingerprinting by BOX-PCR

DNA fingerprinting methods, such rep-PCR, provide indirect access to DNA sequence polymorphism (Versalovic *et al.*, 1994). Rep-PCR, as referred above, means amplification of interspersed repetitive highly conserved DNA sequences present in bacterial genomes by one or more pairs of primers (Louws *et al.*, 1994). Well-defined interspersed repetitive sequences families in prokaryotic genomes such REP (repetitive extragenic palindrome), ERIC (enterobacterial repetitive intergenic consensus) and BOX element sequences are used to design primers which produce amplicons that revels a specific pattern or genomic DNA fingerprinting (Gillings & Holley, 1997; Lupski & Weinstock, 1992).

Some of these methods are proposed as diagnostic tools, more specifically in delineation of species, subspecies or pathovars (Marques *et al.*, 2008). The BOX-PCR applied to Psa uses only one primer: the BOX A1R. (Louws *et al.*, 1994). Amplicon profiles produced in this PCR reaction are unique which allows the application of this molecular tool to differentiate Psa strains (Ferrante & Scortichini, 2010; Marques *et al.*, 2008). Several authors have distinguished *P.s* pathovars and Psa strains worldwide using BOX-PCR (Abelleira *et al.*, 2015; Cunty *et al.*, 2015b; Ferrante & Scortichini, 2010; Mazzaglia *et al.*, 2011).

1.3.3.3 Phylogenetic analysis by Multilocus Sequence Typing (MLST)

MultiLocus Sequence Typing (MLST) analysis is based on nucleotide sequence determinations of internal fragments from multiple housekeeping genes which allows the direct assignment of alleles (Sarkar & Guttman, 2004). The technique is simple and only requires the amplification of DNA fragments by PCR and sequencing of the fragments which can be used to determine bacterial population structures. In addition, phylogenetic relationships between isolates can be inferred (Maiden *et al.*, 1998). The great advantage of MLST is the unambiguity and portability of the sequence data, which allows comparison of results between different laboratories without exchanging strains. A central World-Wide-Web site containing the MLST database permits sharing results between laboratories by reporting and submitting the sequence from housekeeping gene fragments which are related to local isolates found globally (Maiden *et al.*, 1998).

Recent studies have been using MLST as described by Sarkar and Guttman (2004) to establish phylogenetic relationships between Psa and other pathovars or species of *P. syringae* complex (Cunty *et al.*, 2015b; Marcelletti *et al.*, 2011). As referred above, four housekeeping genes are frequently used for typing Psa (Abelleira *et al.*, 2015; Cunty *et al.*, 2015b). The concatenated sequences are analysed in comparison with the complete or partial sequence of corresponding housekeeping genes from several strains of each genomo-species assessed in for example, NCBI databank (Marcelletti *et al.*, 2011). Informatics tools allows construction of several dendrograms or phylogenetic trees based on various algorithms using the total housekeeping gene sequences (Abelleira *et al.*, 2015; Cunty *et al.*, 2015a; Marcelletti *et al.*, 2011).

1.3.4 Presence and prevalence in Portugal

The first report of "bacterial canker of *Actinidia*" in Portugal dates to 2010. The disease was observed on two-years-old plants of *A. deliciosa* cv. "Summer", in kiwifruit orchards in the Entre Douro and Minho region, more specifically in Santa Maria da Feira and Valença (Balestra *et al.*, 2010). In 2011, the disease was detected in North region of Portugal, in infected plants brought from Italy and during that year new focus of the disease were reported in others orchards (Renzi *et al.*, 2012). The disease continued to spread through north and centre regions, and in 2013 a total of 15 regions from Entre Douro and Minho, and 10 in centre region were officially declared affected by the disease (DGAV, 2014).

The characterization of the Psa populations present in Portugal is rather limited. Few studies on the subject were published and all related to northern part of the country (Renzi *et al*., 2012) more specifically to the Entre Douro and Minho region (Moura *et al.*, 2015). Both studies revealed that Psa

isolates analysed from these regions are identical to the most virulent Psa strains identified in Europe and New Zealand, belonging to the biovar 3. However, as in the Italian and New Zealand populations, the Portuguese population from Entre Douro and Minho region presents some degree of genetic variability (Moura *et al.*, 2015). However, more in depth studies are need in order to define the structure of the Portuguese Psa population since this information is vital for the implementation of more efficient control and preventative measures.

1.4 Objectives

Psa has been infecting kiwifruit orchards worldwide, leaving a track of destruction. At least, four populations of Psa have been described: Psa1, Psa2, Psa3 and more recently Psa5. Psa3 is globally distributed and is responsible for important economic losses. In Portugal, the characterization of the disease remains incomplete and the lack of knowledge prevents the design of new strategies or new methods of control. To develop new strategies, there are some questions about the disease that need to be answered: How homogeneous is the Psa population within Portuguese orchards? And between orchards? The population structure varies over time in the same plant? Those the location in the leaf affects the diversity of Psa strains?

In order to answer the abovementioned questions and to characterize the genetic diversity of Psa isolated from distinct Portuguese orchards, the present study has the following objectives:

1. Isolate Psa strains from kiwifruit orchards located in distinct regions over time;
2. Perform molecular characterization of the Psa isolates;
3. Determine the diversity of endophytic and epiphytic Psa strains in the same plant, over time;
4. Determine the diversity of Psa populations in each studied orchard over time;
5. Compare the Psa diversity between orchards;
6. Identify and characterize potential environmental reservoirs of Psa.

2. Materials and Methods

2.1 Field Surveys

The *P. syringae* pv. *actinidiae* collection was obtained from five orchards from different areas of continental Portugal during the year 2016. Two of the orchards were located in the North region, A (Viana do Castelo) and B (Guimarães), and three others in the Centre region, C (Aveiro), D (Coimbra) and E (Coimbra) are shown in Figure 2-1. Orchards A and B were separated by about 70km, while orchards D and E by 4km. The most central orchard, C, was separated by rather than 80km from the northern orchards, and about 50km from the two others.

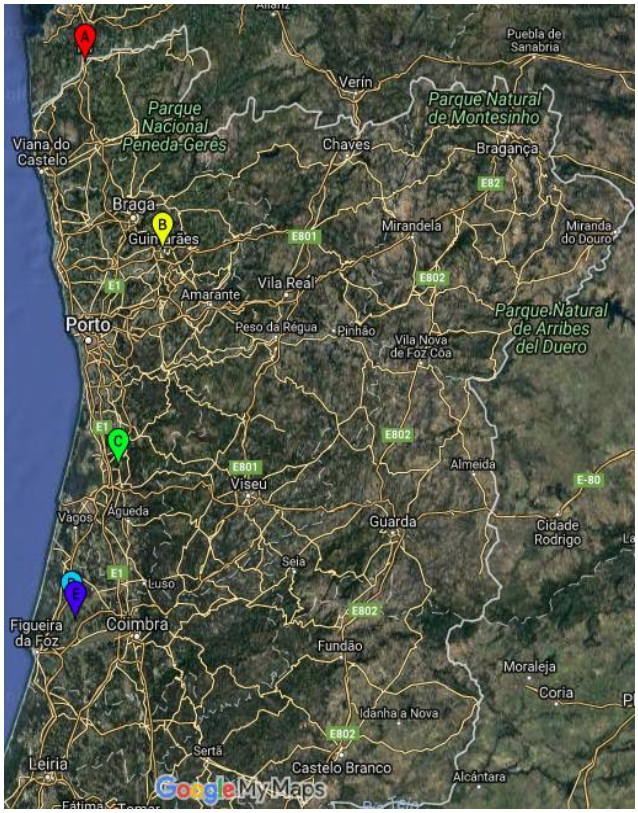


Figure 2-1. Geographical localization of the five sampling orchards in Portugal continental. A: Viana do Castelo; B: Guimarães; C: Aveiro; D: Coimbra; E: Coimbra. Source: https://www.google.pt/maps

Four symptomatic leaves (Fig. 2-2b) from tree individual plants from each orchard were collected, saved in separate sterile bags and processed independently. Plants were marked and their GPS position was recorded (Fig. 2-2a). In the same occasion, soil (Fig. 2-2c) and water (Fig. 2-2d) samples were collected from the orchards. The collected samples were labelled and transported at 4°C. Samples

were processed in the day they were collected. The sampling procedure was realized in two distinct occasions, in late spring (June) and in the following autumn (October) in the same marked plants.

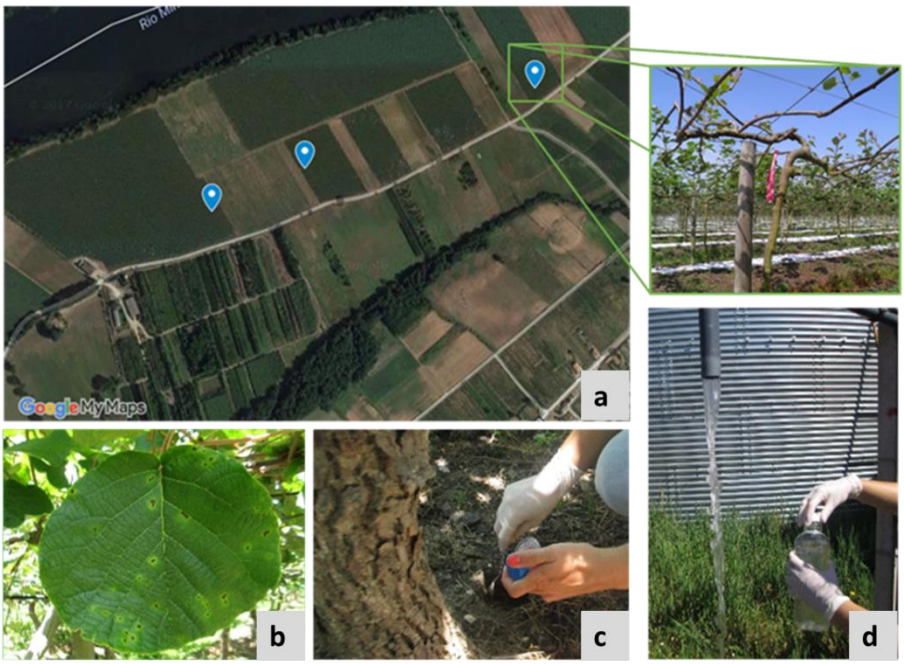


Figure 2-2. Sampling strategy. a: localization and distribution of sampling plants of orchard A; b: example of one of four symptomatic leaves collected; c: soil sampling; d: water sampling. Source: https://www.google.pt/maps

2.1.1 Orchards Description

The description of characteristics observed on each sampling orchard is presented in Table 2.1.1-I. The severity degree of the disease in each orchard, based on the observation of symptoms, was attributed according to the scale shown in Table 2.1.1-II. The studied orchards of *A. deliciosa* cv. "Hayward" (except orchard B) had different ages and presented different degrees of "bacterial canker" severity. Plants in orchard B were from *A. deliciosa* cv. "Erika".

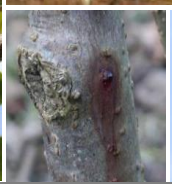
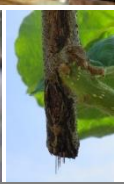
All orchards used micro sprinkler as irrigation system. Orchards A and B were situated near a river and the water used for irrigation was filtered directly from the river. Orchard E was irrigated with filtered water from an artificial lake and orchard C and D used non-filtered water from a well.

Table 2.1.1-I. Description of studied orchards

Orchard	Localization	Cultivar	Age (years)	First detection of Psa	Psa disease severity degree
A	Viana do Castelo	*A. deliciosa* cv. "Hayward"	7	2010	1
B	Guimarães	*A. deliciosa* cv. "Erika"	5	2015	2
C	Aveiro	*A. deliciosa* cv. "Hayward"	16	2016*	1
D	Coimbra	*A. deliciosa* cv. "Hayward"	4	2015	3
E	Coimbra	*A. deliciosa* cv. "Hayward"	30	2016	2

*this study.

Table 2.1.1-II. Severity degree scale of the incidence of "bacterial canker" kiwifruit disease in an orchard, according with the observed symptoms. *

Degree	Symptoms	
0	Asymptomatic plants.	
1	Dark brown angular leaf spots surrounded (or not) by yellow haloes; necrotic floral buds.	 
2	Dark brown angular leaf spots surrounded (or not) by yellow haloes, with exudates in underside of the leaf; necrotic flower buds; initial bacterial cankers; red colour in the shell.	  
3	Dry leaves; dry branches; bacterial cankers on trunks and leaders; Red-rusty/white exudates.	   
4	Completely dry plants.	

*Adapted from a symptomology scale used in pathogenicity assays by Cunty *et al.*, 2015b.

2.1.1.1 Abiotic Conditions

According with the geographical location, the orchards were influenced by distinct abiotic conditions throughout the year. Table 2.1.1-III presents the summary of climatic conditions affecting the orchards during the year of 2016.

Table 2.1.1-III. Summary of some climatic conditions verified in the studied orchards during the year of 2016

Orchard	Average annual temperature (T°)	Annual cumulative rainfall (mm)	Number of cold hours (h)
A	13.5	1800	478
B	12.5	1800	1031
C	14.5	1500	541
D	14.5	1100	541
E	14.5	1100	440

Average annual temperature, annual cumulative rainfall: normal of 1961/90; number of cold hours: total number of hours of TºC below 7.2ºC between 01/10/2015 to 30/04/2016. Accessed online: www.ipma.pt - Portuguese Institute for Sea and Atmosphere, I.P. (IPMA, I.P)

2.2 Culture media and Solutions

2.2.1 Phosphate Buffered Saline (10mM) buffer

The Phosphate Buffered Saline (PBS), described in Table 2.2.1-I, was used as a buffer solution to prepare the bacterial suspensions from epiphytic, endophytic, soil and water samples. The reagents were weight and dissolved in 800mL. To obtain a 7.2 pH solution, the pH was adjusted with an HCl solution. The volume of the solution was adjusted to 1000ml with distilled water. Lastly, the solution was sterilized by autoclaving at 121°C for 20 minutes and stored at room temperature.

Table 2.2.1-I. PBS 10 mM composition.

Reagent	Quantity
Sodium chloride (NaCl)	8.0g
Sodium phosphate (KCl)	0.2g
Sodium Dihydrogen Phosphate Dodecahydrate (Na_2HPO_4. $12H_2O$)	2.9g
Potassium phosphate (KH_2PO_4)	0.2g
Distilled water	Up to 1000 mL

2.2.2 King's medium B (King *et al*., 1954)

King's medium B (KB) is selective for gram negative bacteria and was used as growth medium for Psa isolates. The composition of KB is described in Table 2.2.2-I. All the reagents were weight and dissolved, except the bacteriological agar which was separately added. The pH was adjusted to 7,2 with KOH tablets and the solution volume was adjusted to 1000mL. The medium was sterilized by autoclaving at 121°C for 20 minutes and dispensed in sterile Petri dishes and stored at room temperature.

Table 2.2.2-I. King's B (King et al., 1954) composition.

Reagent	Quantity
Proteose peptone N.3	20.0g
Glycerol	10.0 mL
Dipotassium phosphate (K_2HPO_4)	1.5g
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	1.5g
Bacteriological Agar	15.0g
Distilled water	Up to 1000mL

2.2.3 King's medium B (King *et al*., 1954) modified according to Mohan and Schaad (1987)

The KB medium modified (KBc) was prepared according to Mohan and Schaad (1987) by supplementing with boric acid and two antibiotics. The KBc medium is highly selective and recommended for Psa isolation to avoid the growth of contaminants that can affects the Psa recover.

The composition and procedure of KBc is identical to KB medium, referred on **2.2.2.** The medium (Table 2.2.2-I) was prepared up to 900mL of distilled water, pH solution adjusted to 7.2, sterilized in autoclave and cooled at about 50°C to stabilized. Solutions of acid boric, cycloheximide and cephalexin were previously prepared (Table 2.2.3-I). The antibiotic solutions were sterilized by filtration through a 0.2µm pore size sterile filter (Whatman ® Puradisc 30). After autoclaving and when the medium reached 50°C, 100mL of sterile boric aqueous acid solution, 8mL of cycloheximide and 8mL of cephalexin were added to the medium in aseptic conditions. The medium was dispensed in sterile Petri dishes and stored up to one month in the dark and under refrigerated conditions.

Table 2.2.3-I. Acid boric and both cycloheximide and cephalexin antibiotics solutions composition.

Reagent	Quantity
1.5% boric acid aqueous solution	100mL
25 mg ml^{-1} solution of cycloheximide in 70% ethanol	8mL
25 mg ml^{-1} aqueous solution of cephalexin	8mL

2.2.4 Lysis buffer

The lysis buffer was used for DNA extraction to lyse bacterial cells. To prepare this buffer to a final volume of 3mL, 1500µl of a 2% solution of Tween20 and 300µl of a 10% solution of NZYtech PCR reaction buffer were added to 1200µl of sterile distillate water. After carefully homogenate, the final solution was sterilized by filtration through a 0.2µm pore size sterile filter (Whatman ® Puradisc 30). The final sterile solution was store at -20°C.

2.2.5 King's B freezing medium

The bacterial isolates were cryopreserve at -80°C, in KB medium (**2.2.2.**) with 15% (w/v) glycerol.

2.2.6 Tris acetate-EDTA (TAE) buffer

Acetate-EDTA (TAE) buffer was used for agarose gel electrophoresis in the analyses of DNA fragments resulting from PCR amplification. A stock solution of TAE (50x) was previously prepared with composition described in Table 2.2.6-I.

Table 2.2.6-I. Composition of TAE (x50) stock solution.

Reagent	Quantity
Tris/base	121g
Glacial acetic acid	28.55mL
Aqueous solution EDTA (0.5M)	50mL
H_2O Mili-Q sterile	Up to 500mL

The working solution of TAE (x1) was obtained by diluting the stock solution of TAE (x50) with distilled water.

2.3 Bacterial isolation

2.3.1 From plant samples

Collected plant samples (four leaves per each plant) were processed in order to separately recover epiphytic and endophytic bacterial strains. Epiphytic bacterial communities were obtained by swabbing leaves surface (Fig. 2-3a). The swabs used were shredded, added to 9mL of sterile 10mM PBS (2.2.1.) and vortexed to prepare the epiphytic bacterial suspensions. After recovering the epiphytic bacteria, leaves were processed to collect the endophytic bacterial communities, by following a leaf surface sterilization procedure, described by Eevers *et al.*, 2016. Briefly, leaves were washed 3 min in sterile MiliQ water, 1.5 min in ethanol 70%, 3min NaOCl 1%, 1.5 min ethanol 70%, and finally rinsed 5 times with sterile MiliQ water (Fig. 2-3b); the last rinsing water was inoculated in KB medium and incubated, in order to confirm the absence of bacteria. The sterilized leaves were shredded in a blender (~30ml of sterile 10mM PBS were used per 30g of plant samples) (Fig. 2-3c) and the macerate obtained

passed-thought a sieve (sterile gauze cloth) to remove plant debris to obtain the suspension for Psa endophytic bacterial isolation. All the samples were processed independently, in aseptic conditions.

Several dilutions 1:10 (up 10^{-6}) were prepared from homogenate suspensions obtained in both EP and EN isolation to obtained bacterial suspensions in several concentrations. Serially dilutions were prepared in aseptic condition in 9mL of sterile PBS buffer (10mM) and plated (100µl of each dilution) on the selective KBc medium (composition described in **2.2.3.**). Petri dishes were incubated at 25°C for 72h (EPPO, 2014) and checked at each 24h. Visible colonies were observed and 10 putative Psa CFUs were selected from each 10^{-1} dilution plate sample and re-streaking in KB medium (48h at 25°C) to obtain pure cultures. From remaining dilutions only colonies with different morphology were re-streaking. Re-streaking step was performed as often as necessary until pure cultures were obtained.

Figure 2-3. Sample preparation. a: collection of epiphytic populations by swab technique; b: Sterilization procedure; c: collection of endophytic population by shredded the leaves in a blender, a macerate is obtained.

2.3.2 From soil and water samples

Soil and water samples were processed in order to recover Psa bacterial communities present in these possible reservoirs. Soil suspensions were prepared from each sample, by added 1g of each collected soil to 9mL of sterile PBS buffer (10mM) and vigorously mixed. Serial dilutions 1:10 (up 10-6) in sterile PBS buffer (10mM) were prepared from the homogenate suspensions and, 100µl of each dilution were inoculated by spreading on the selective KBc medium (composition described in 2.2.3.). The cultures were incubated at 25°C for 72h (EPPO, 2014) and checked for colonies every 24h. The isolates were obtained as described before (**2.3.1**).

Water samples (~3L) were filtered through 0.2 µm sterile membrane filters (Whatman® membrane filters mixed cellulose with 47 mm diameter WHA10401770) in a vacuum filtration system, in aseptic conditions. The membrane filters were recovered to sterile plastic bags with 9mL of sterile PBS buffer (10mM). The bags were gently rubbed until a homogeneous bacterial suspension were obtained.

The suspensions diluted were used to isolate Psa bacterial communities as described above.

2.4 Cryopreservation of the isolates and DNA extraction

Isolates were cryopreserved in King's B freezing medium (**2.2.5**). The pure culture, was removed from the KB medium with a 10µl sterile loop and mixed into 800µl of KB freezing medium in cryogenic-tube. The suspension was homogenized and stored at -80ºC for future use.

DNA from each bacterial isolate was extracted. A single colony of each isolate was removed with a sterile loop and mixed to 50µl of lysis buffer (**2.2.4**) in a sterile microtube. The microtube was transferred to a thermoblock (dry bloc heater, FALC) at 97°C for 8 minutes to than placed at -20°C for 5 min and finally centrifuged at 15 000 x g for 5 minutes in a benchtop centrifuge (Eppendorf 5415D). The supernatant containing the DNA was recovered and transferred to a new sterile microtube. The pellet was discarded and DNA stored at -20°C for future use.

2.5 Bacterial identification

Bacterial identification was performed according to Gallelli et al. (2011) duplex-PCR protocol for Psa identification, described in EPPO standard regulation. Amplification reaction was performed using two pairs of primers: KN-F/R, designed by Koh & Nou (2002), and AvrDdpx-F/R (Table 2.7.3-I), which amplified fragments with a size of 492 bp and 230bp, respectively.

Amplification reaction was prepared in a total volume of 25 µl, containing 12,5µl of NZYtaq 2x Colourless Master Mix (nzytech), 1µl of each KN-F and KN-R primer, 0.2µl of each AvrDdpx-F and AvrDdpx-R primer, 2µl of DNA template and 6.9µl of sterile MiliQ water (Table 2.7.3-II). As positive control, Psa reference strain CFBP 7286 (Balestra *et al.*, 2009), were included in each PCR reaction. To verify the absence of contaminations by exogenous DNA, a "blank" control (no template DNA) was included in each amplification reaction. PCR reaction was carried out in a My cyclerTM Thermal cycler (Bio-rad) using following amplification program: initial denaturation step (95°C for 3 min), 30 repeated cycles composed by a denaturation step (94°C for 30 sec), an annealing step (63°C for 45 seconds) and an extension step (72°C for 50 seconds), finally the PCR reactions ends with a single final extension step (72°C for 5 min) (Table 2.7.3-III).

2.5.1 Analysis of duplex-PCR amplicons

Duplex-PCR amplicons were separated by electrophoresis by loading 7µl of the reaction in a 2% agarose gel (w/v) in TAE (1x) buffer (2.2.6) and staining with ethidium bromide. The electrophoresis was performed at 80V for 40 minutes. A molecular weight marker (NZYDNA Ladder III, 200 to 10000

bp, nzytech) were included. The amplifications patterns were visualized and photographed in UV illumination (Image analyser Doc Tm RX +, Bio-rad).

2.6 Bacterial fingerprinting: BOX-PCR

The isolates previously identified as Psa according with duplex-PCR (Gallelli *et al*., 2011) were characterized by BOX-PCR, as described by Louws *et al*. (1994). The fingerprinting analysis was performed using the BOXA1R primer (Table 2.7.3-I). The amplification reaction was performed in a total volume of 25 µl, containing 12,5µl of NZYtaq 2x Colourless Master Mix (nzytech), 5µl of BOXA1R (10µM) primer (Table 2.7.3-II), 5µl of DNA template and 2.5µl of sterile Milli-Q water. To verify the absence of exogenous contamination, a blank (no template DNA) was included in each BOX-PCR reaction. Two Psa3 reference strains were also included in each BOX-PCR reaction for quality of band profile control: CFBP 7286 Italy strain (Balestra *et al*., 2009) and CFBP 7812 New Zealand strain (Vanneste, Yu, Cornish, New, *et al.*, 2013).

BOX-PCR reaction was performed in a My cycler™ Thermal cycler (Bio-rad) according to the following program: initial denaturation step (95°C for 7 min) followed by 30 repeated cycles of denaturation step (94°C for 1 min), an annealing step (53°C for 1 min) and an extension step (65°C for 8 min). A final extension step (65°C for 15 min) was used and samples were maintained at 4ºC until further analysis (Table 2.7.3-III).

2.6.1 Analysis of BOX-PCR amplicons

BOX-PCR amplicons were separated by electrophoresis by loading 25µl of the reaction (3 µl of loading buffer added) in a 2% agarose gel (w/v) in TAE (1x) buffer (**2.2.6**) stained with 8µl of ethidium bromide. The electrophoresis run occurred at 80V for 50 minutes. A molecular weight marker (NZYDNA Ladder III, 200 to 10000 bp, Nzytech) were included in the run for comparison purposes. The amplification patterns were visualized and photographed under UV illumination (Image analyser Doc Tm RX +, Bio-rad).

2.6.2 Analysis of Psa fingerprinting profiles

Fingerprinting profiles obtained from Psa isolates were grouped by visual inspection based on the similarity, namely the number of band and weight of the bands when compared with each other's and with the molecular weight marker. The intensity of the fluorescence of each band was a factor used in the comparison between profiles.

2.7 Multilocus Sequence Typing (MLST) - PCR

Phylogenetic analysis was performed only for representative strains selected from the previously established groups based on the fingerprinting analysis (section **2.6.**). MLST-PCR was performed according to the protocol described by Sarkar and Guttman (2004). Four housekeeping genes, *gapA*, *gltA*, *gyrB* and *rpoD* were amplified from each representative Psa strain with the respective primers: gapA-Fps/Rps, gltA-Fp/Rp, gyrB-Fps/Rps and rpoD-Fp/Rps (Table 2.7.3-I).

Amplification reactions were performed in a total volume of 30µl, containing 3µl of reaction buffer 10x, 1µl of $MgCl_2$ (50mM), 6µl of dNTPs mix (10mM), 1µl of primer (forward and reverse, Table 2.7.3-II), 0.2µl of NZYtaq, 15.8µl of sterile H_2O MiliQ and finally 2µl of DNA template. To verify the absence of contaminations by exogenous DNA, a blank (no template DNA) was included in each MLST-PCR reaction.

MLST-PCR reaction was performed in a My cycler™ Thermal cycler (Bio-rad) according to the following program: initial denaturation step (94°C for 5 min) followed by 30 repeated cycles of denaturation step (94°C for 2 min), an annealing step (temperatures are described on Table 2.6.2-I, for 1 min) and an extension step (72°C for 1 min); finally, MLST-PCR reaction ended with a final extension step (72°C for 10 min) (Table 2.7.3-III). The annealing temperature was lowered in same cases as an attempt to amplify a gene that did not amplify with the standard PCR conditions abovementioned (Table 2.6.2-I).

Table 2.6.2-I. Annealing temperature for amplification of the genes used in the MLST analysis. Standard: temperatures according with Sarkar & Guttman, 2004; Adapted: annealing temperatures used to amplify genes that failed with the standard protocol.

Gene	Primers (Sarkar & Guttman, 2004)	Annealing °C	
		Standard	Adapted
gapA	gapA – Fps gapA – Rps	62°C	58°C
gltA	gltA-Fp gltA-Rp	56°C	53°C
gyrB and ***rpoD***	gyrB-Fps gyrB-Rps rpoD-Fp rpoD-Rps	63°C	58°C

2.7.1 Analysis of MLST amplicons

The purity and yield of each amplicon was verified by electrophoresis by loading 5µl of the reaction (1 µl of loading buffer added) in a 1% agarose gel (w/v) in TAE (1x) buffer (**2.2.6**), stained with 7µl of ethidium bromide. The electrophoresis run occurred at 80V for 30 minutes. A molecular weight marker (NZYDNA Ladder III, 200 to 10000 bp, Nzytech) was included in the run for comparison purposes. Amplicons were visualized and photographed under UV light (Image analyser Doc Tm RX +, Bio-rad).

2.7.2 MLST-PCR amplification products purification

After confirming the amplicon size, PCR products were purified using JETquick PCR products purification kit (GENOMED). Firstly, 25µl of each PCR product was suspended in a binding buffer solution and transferred to a column with a silica gel-based membrane. The column, placed into a 2ml Eppendorf tube, was centrifuged for 1 min to selectively binge the DNA fragments into the membrane. A washing step with washing buffer (containing ethanol) was performed to remove impurities (nucleotides, PCR reagents remains, etc) by centrifuged at maximum speed for 1 min. The flow-through was discarded, followed by an additional centrifugation step (1min) to dry the silica gel-based membrane. Finally, the column was transferred to a sterile Eppendorf 1.5ml and DNA fragments were eluted with 25 µl of sterile warmed MilliQ water (50°C). The column was discarded and the DNA was stored at -20°C.

2.7.3 DNA sequencing of MLST-PCR amplicons

Purified DNA amplicons from each of the housekeeping genes comprising the MLST analysis were sequenced by Sanger's platform, as a contracted service. A multi-well plate was prepared with 5µl of each amplicon and 5µl of the reverse primer used for its amplification Rps or Rs (Table 2.7.3-I), at concentration of 5µM (Turner *et al.*, 1999) and shipped for the contracting service facilities.

2.7.3.1 Phylogenetic analysis

The quality of the received sequences was manually checked with Sequence Scanner program and good quality sequences from the four MLST-based housekeeping genes were edited in BioEdit sequence editor (Hall, 1999) for reverse complement. Alignment of Psa isolates from Portuguese orchards against 10 reference strains of Psa1, 2, 3 and 4 and 7 reference strains of *Pseudomonas syringae* pv. *theae*, *Pseudomonas syringae* pv. *morsprunorum*, *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *phaseolicola*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae*

pv. *tagetis* and *Pseudomonas viridiflava* obtained from the public databases (Table 2.7.3-IV) was performed using the MEGA7 package (Molecular Evolutionary Genetic Analyses) (Kumar *et al.*, 2016). The obtained sequences were concatenated following the alphabetic order of genes as described by (Abelleira *et al.*, 2014). The concatenated data set was 1834 pb long (in the alignment - 1 to 564 bp – *gapA*; 565 to 1009 - *gltA*, 1010 to 1388 - *gyrB* and 1389 to 1834 - *rpoD*). Phylogenetic trees were constructed using neighbour-joining algorithm (Saitou & Nei, 1987) included in the MEGA6 package. The topology of the trees generated from distance matrices calculated with Jukes–Cantor (JC) correction (Jukes *et al.*, 1969) using the neighbour-joining algorithm was evaluated by performing bootstrap analysis (Felsenstein, 1985) of 1000 resampling's of the data set.

Table 2.7.3-I. Primers sequence, amplified gene or target region and amplicon size (bp) used in each PCR based technique.

PCR technique	Primer	Amplified gene/region	Amplicon size (bp)	Sequence (5'-3')	Reference
Duplex-PCR	KN-F KN-R	Genomic DNA fragment	492	CACGATACATGGGCTTATGC CTTTTCATCCACACACTCCG	Koh & Nou, 2002
	AvrDdpx-F AvrDdpx-R	AvrD1	230	TTTCGGTGGTAACGTTGGCA TTCCGCTAGGTGAAAAATGGG	Galleli *et al.*, 2011
BOX-PCR	BOXA1R	BOX element	-	CTACGGCAAGGCGACGCTGACG	Louws *et al.*, 1994
MLST*	gapA-Fps gapA-Rps	*gapA* (glyceraldehyde-3-phosphate dehydrogenase)	675	CGCCATYCGCAACCCG CCCAYTCGTTGTCGTACCA	Sarkar & Guttman, 2004
	gltA-Fp gltA-Rp gltA-Fs gltA-Rs	*gltA* citrate synthase	995	AGTTGATCATCGAGGGCGCWGCC TGATCGGTTTGATCTCGCACGG CCCGTCGAGCTGCCAATWCTGA ATCTCGCACGGSGTRTTGAACATC	
	gyrB-Fps gyrB-Rps	*gyrB* (gyrase B)	674	MGGCGGYAAGTTCGATGACAAYTC TRATBKCAGTCARACCTTCRCGSGC	
	rpoD-Fp rpoD-Fs rpoD-Rps	*rpoD* sigma factor 70	812	AAGGCGARATCGAAATCGCCAAGCG GGAACWKGCGCAGGAAGTCGGCACG AAGCGTATCGAAGAAGGCATYCGTG	

*Primers designed for gene amplification "Fp/Rp; primers designed for gene sequencing "Fs/Rs"; Primers designed for both applications "Fps/Rps". GyrB Fps/ Rps and rpoD Fp/Rps primers were designed by (Sawada *et al.*, 1999).

Table 2.7.3-II. Amplification reactions for each PCR technique in a total volume of 25µl.

PCR technique	Primers	Reagents	Final concentration	Reference
Duplex-PCR	KN–F/R AvrDdpx – F/R	H_2O MiliQ sterile	N.A.	Gallelli *et al*., 2011
		NZYTaq 2x Colourless Master Mix	N.A.	
		KN-F/R (25 µM)	0.5 µM (x2)	
		AvrDdpx-F/R (25 µM)	0.4 µM (x2)	
		Template DNA	20 ng μL^{-1}	
BOX-PCR	BOXA1R	H_2O MiliQ sterile	N.A.	Louws *et al.*, 1994
		NZYTaq 2x Colourless Master Mix	N.A.	
		BOXA1R (10µM)	12.5 µM	
		Template DNA	5	
MLST-PCR*	gapA-Fps/Rps gltA-Fp/Rp gyrB-Fps/Rps rpoD-Fp/Rps	H_2O MiliQ sterile		Sarkar & Guttman, 2004
		Reaction buffer, 10x	1x	
		$MgCl_2$ (50mM)	1	
		dNTPs mix (10mM)	6	
		Cts/*gapA*/*gyrB*/*rpoD* primers (10µM)	1 (x2)	
		NZYTaq (5U/µl)	U	
		Template DNA	0,1-1 ug	

*NZYTaq DNA polymerase (Nzytec) reagents provided, reaction volume of 30µl.

Table 2.7.3-III. Physical conditions of amplification reaction for each PCR technique.

PCR technique	Primers	Temperature (°C)	Time	Cycles	Reference
Duplex-PCR	KN–F/R AvrDdpx – F/R	95	3’		
		94 63 72	30’’ 45’’ 50’’	30x	Gallelli *et al.*, 2011
		72	5’		
BOX-PCR	BOXA1R	95	7’		
		94 53 65	1’ 1’ 8’	30x	Louws *et al.*, 1994
		65	15’		
MLST-PCR	gapA-Fps/Rps gltA-Fp/Rp gyrB-Fps/Rps rpoD-Fp/Rps	94	5’		
		94 **a**/**b**/**c*** 72	2’ 1’ 1’	30x	Sarkar & Guttman, 2004
		72	10’		

’: minutes; **’’**: seconds; *****: Annealing temperature varies according with the primer used: **a** (*gapA*) - 62°C, **b** (*gltA*) - 56°C, **c** (*gyrB* and *rpoD*) - 63°C.

Table 2.7.3-IV. Information on the reference strains used to construct the phylogenetic dendrogram.

Strain	Specie/Pathovar/Biovar	Year of isolation	Country of isolation	GenBank accession number
CFBP4909	Psa1	1984	Japan	*gltA*: KF937505.1
				gapA: KF937408.1
				gyrB: KF937602.1
				rpoD: KF937699.1
T10 04758	Psa3	2010	New Zealand	*gltA*: JN683493.1
				gltA: JN683474.1
				gyrB: JN683455.1
				rpoD: JN683398.1
CFBP8047	Psa3	2010	France	*gltA*: KF937544.1
				gapA: KF937431.1
				gyrB: KF937626.1
				rpoD: KF937738.1
CFBP7811	Psa3	2010	New Zealand	*gltA*: KF937508.1
				gapA: KF937411.1
				gyrB: KF937605.1
				rpoD: KF937702.1
CFBP7287	Psa3	2008	Italy	*gltA*: KF937507.1
				gapA: KF937410.1
				gyrB: KF937604.1
				rpoD: KF937701.1
CFBP2353	*Pseudomonas syringae* pv. *theae*	1970	Japan	*gltA*: KF937503.1
				gapA: KF937406.1
				gyrB: KF937600.1
				rpoD: KF937697.1
ICMP19071*	Psa2	1992	Korea	*gltA*: JN683506.1
				gapA: JN683487.1
				gyrB: JN683468.1
				rpoD: JN683411.1
CFBP2351	*Pseudomonas syringae* pv. *morsprunorum*	1931	USA	*gltA*: KF937599.1
				gapA: KF937405.1
				gyrB: KF937599.1
				rpoD: KF937696.1

Table 2.7.3 -IV (continuation). Information on the reference strains used to construct the phylogenetic dendrogram.

CFBP7951	Psa4	2011	New Zealand	***gltA***: KF937520.1
				gapA: KF937418.1
				gyrB: KF937612.1
				rpoD: KF937714.1
CFBP8041	Psa4	2011	France	*gltA*: KF937538.1
				gapA: KF937448.1
				gyrB: KF937643.1
				rpoD: KF937732.1
CFBP8043	Psa4	2011	France	*gltA*: KF937540.1
				gapA: KF937451.1
				gyrB: KF937646.1
				rpoD: KF937734.1
CFBP8045	Psa4	2010	Australia	*gltA*: KF937542.1
				gapA: KF937421.1
				gyrB: KF937615.1
				rpoD: KF937736.1
CFBP2212	*Pseudomonas syringae* pv. *tomato*	1961	United Kingdom	*gltA*: KF937499.1
				gapA: KF937402.1
				gyrB: JN190421.1
				rpoD: JN185896.1
CFBP1390	*Pseudomonas syringae* pv. *phaseolicola*	1949	Canada	*gltA*: KF937496.1
				gapA: KF937399.1
				gyrB: KF937593.1
				rpoD: KF937690.1
CFBP4702	*Pseudomonas syringae* pv. *syringae*	1950	United Kingdom	*gltA*: KF937504.1
				gapA: KF937407.1
				gyrB: KF937601.1
				rpoD: KF937698.1
CFBP1690	*Pseudomonas syringae* pv. *tagetis*	1920	USA	*gltA*: KF937497.1
				gapA: KF937400.1
				gyrB: KF937594.1
				rpoD: KF937691.1
CFBP2107	*Pseudomonas viridiflava*	1927	Switzerland	*gltA*: KF937498.1
				gapA: KF937401.1
				gyrB: KF937595.1
				rpoD: KF937692.1

Accessed online: Genbank database (https://www.ncbi.nlm.nih.gov/nuccore)

2.8 Statistical analyses

2.8.1 Diversity indexes

The characterization of the Psa population diversity, within and between the Portuguese orchards analysed in this study, was supported by the measurement of two levels of inventory diversity: alpha and beta diversity, as proposed by (Whittaker, 1977).

2.8.1.1 Alpha diversity analysis

The alpha diversity analysis (Hill, 1973) was individually performed for each orchard in order to compare the Psa population diversity between plants and within orchard per sampling condition (considering EP and EN isolates in both seasons). Margalef index (Dmg) was calculated following the formula: $Dmg = \frac{S-1}{\log(N)}$, being S the richness (total number of Psa profiles) and N the total number of Psa isolates found in that plant or orchard. The Shannon index (H') was determined from Psa profiles richness and their relative proportion following the formula: H'= -$\sum pi.\ln(pi)$, being *pi* the specific richness of each Psa profile. This index accounts for both abundance and evenness of the Psa profiles present in the local in study (plant or orchard). Pielou's evenness index (J') measured the ration between the observed diversity (H') and the maximal possible diversity, which accesses how equitable were Psa isolates distributed by the Psa profiles (Pielou, 1966). The Simpson diversity index (1-D) determine the probability of two Psa isolates belong to distinct profiles. It was determined by the complement of Simpson alternative index ($D = \sum(pi)^2$). This index was sensitive to changes in Psa profiles abundance, being an indicative of dominance (Simpson, 1960).

2.8.1.2 Beta diversity

Beta diversity measures the similarity of the Psa population structure between the Portuguese orchards used in this study. A Jaccard index was determined by the quotient between the intersection and the union of the pairwise compared Psa profiles among two orchards. A beta diversity matrix between the five orchards was constructed.

2.8.2 Principal component analysis

A principal component analysis (PCA) – inter-species correlation based on Psa profiles data was performed in order to understand the Psa population distribution over time within and between orchards by using the software package CANOCO (Šmilauer & Lepš, 2014).

3. Results and Discussions

3.1 Bacterial isolation, DNA extraction and cryopreservation

Bacterial isolation was performed on modified King's B medium (Mohan & Shaad, 1987), at 25°C during 72h. According with the colony morphology, which can be observed in Figure 3-1, a total of 1.673 putative *P. syringae* pv. *actinidiae* (Psa) strains were isolated from the five studied orchards (section **2.1.**). After purification, strains were cryopreserved at -80ºC for future use and the corresponding DNA was extracted. Of these strains, 895 were isolated in spring (spring isolates, SI) and 778 in autumn (autumn isolates, AI).

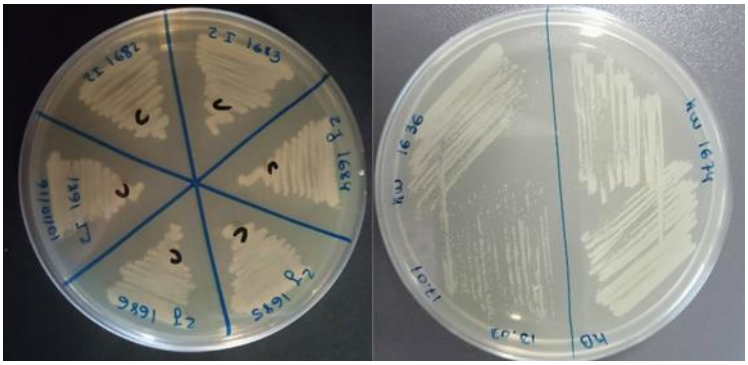


Figure 3-1. Cultures of Psa grown in KBc medium, 72h.

3.2 Characterization of *P. syringae* pv. *actinidiae* (Psa) populations isolated in Portuguese orchards

3.2.1 Orchard A

3.2.1.1 Putative *Pseudomonas* spp. isolation and Psa identification

A total of 337 strains were isolated from plants, soil and water samples collected in orchard A (Table 3.2.1-I); 134 were SI and 203 were AI.

In order to confirm the identity of the isolates as Psa strains a duplex-PCR protocol described by Gallelli *et al*. (2011) was performed using the extracted DNA's. Positive results were considered when two amplicons with 492 bp and 230 bp were simultaneously observed in the electrophoresis gel analyses (Figure 3-2), corresponding to the amplification by KN-F/R and AvrDdpx-F/R primers, respectively.

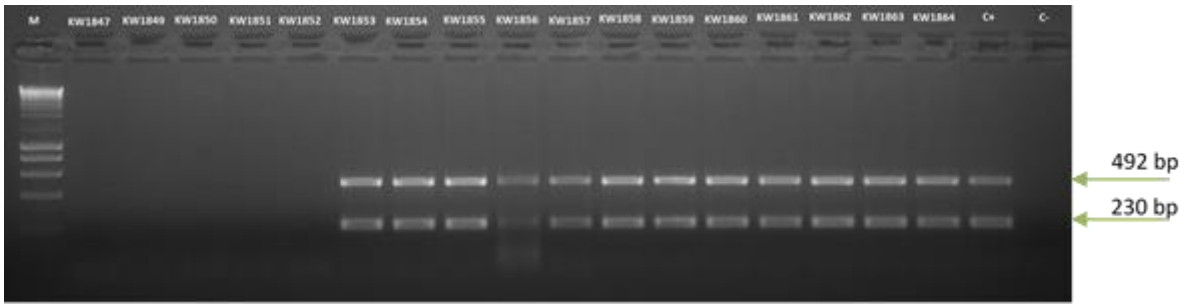


Figure 3-2. Electrophoresis profiles from duplex-PCR amplifications according to Gallelli *et al.*, 2011 M: ladder III (Nzytech). Isolates not identify as Psa: KW1847 to KW1852; Isolates identify as Psa: KW1853 to 1864; C+: positive control, Psa 3 strain - CFBP 7811 (Vanneste *et al.*, 2013). C-: blank control.

Table 3.2.1-I. Total isolates recovered from orchard A in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	SPRING	
	PLANT	LOCALIZATION
KW81; KW82; KW83; KW84; KW85; KW86; KW87; KW88; KW89; KW90; KW91; KW92; KW93; KW94; KW95; KW96; KW97; KW301	P1	EP
KW130; KW131; KW132; KW133; KW134; KW135; KW136; KW137; KW138; KW139; KW168; KW169	P1	EN
KW98; KW99; KW100; KW101; KW102; KW103; KW104; KW105; KW106; KW107; KW108; KW109; KW110; KW111; KW112; KW113; KW341; KW342	P2	EP
KW140; KW141; KW142; KW143; KW144; KW145; KW146; KW147; KW148; KW149; KW150	P2	EN
KW52; KW53; KW54; KW55; KW56; KW57; KW58; KW59; KW60; KW61; KW62; KW63; KW64; KW65; KW66; KW67; KW68; KW69; KW70; KW71; KW114; KW343	P3	EP
KW115; KW170; KW171; KW172; KW173; KW174; KW175; KW176; KW177; KW178; KW179; KW180; KW181; KW182	P3	EN
ISOLATE REFERENCE	**SPRING**	
	RESERVOIR	
KW201; KW202; KW203; KW204; KW205; KW206; KW207; KW208; KW209; KW210; KW211; KW212; KW314; KW315; KW316	SO	
KW196; KW197; KW198; KW199; KW200; KW242; KW243; KW244; KW245; KW246; KW247; KW248; KW249; KW250; KW251; KW252; KW253; KW254; KW255; KW256; KW257	WA	

Table 3.2.1-I (continuation). Total isolates recovered from orchard A in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	AUTUMN	
	PLANT	LOCALIZATION
KW1354; KW1355; KW1586; KW1592; KW1594; KW1600; KW1601; KW1634; KW1635; KW1636; KW1637; KW1638; KW1639; KW1640; KW1641; KW1642; KW1643; KW1644; KW1645; KW1646; KW1647; KW1658; KW1659; KW1660; KW1661; KW1721; KW1722; KW1723; KW1729; KW1737; KW1738; KW1751; KW1752	P1	EP
KW1356; KW1357; KW1358; KW1359; KW1360; KW1361; KW1362; KW1363; KW1364; KW1365; KW1366; KW1451; KW1452; KW1453; KW1551; KW1552; KW1553; KW1554; KW1555; KW1556; KW1557; KW1558; KW1559; KW1560; KW1561; KW1562; KW1563; KW1564; KW1565; KW1598; KW1599; KW1604	P1	EN
KW1426; KW1427; KW1428; KW1429; KW1431; KW1432; KW1433; KW1434; KW1464; KW1465; KW1495; KW1593; KW1619; KW1620; KW1621; KW1622; KW1623; KW1624; KW1632; KW1633; KW1682; KW1688; KW1689	P2	EP
KW1440; KW1441; KW1435; KW1437; KW1438; KW1487; KW1488; KW1489; KW1490; KW1491; KW1574; KW1575; KW1576; KW1577; KW1578; KW1579; KW1580; KW1581; KW1582; KW1680; KW1681	P2	EN
KW1445; KW1446; KW1447; KW1448; KW1449; KW1466; KW1470; KW1471; KW1472; KW1476; KW1477; KW1478; KW1479; KW1480; KW1481; KW1482; KW1483; KW1484; KW1485; KW1486; KW1583; KW1584; KW1585	P3	EP
KW1450; KW1648; KW1649; KW1650; KW1651; KW1652; KW1653; KW1654; KW1655; KW1656; KW1657; KW1662; KW1663; KW1664; KW1665; KW1709	P3	EN
ISOLATE REFERENCE	**AUTUMN**	
	RESERVOIR	
KW1430; KW1459; KW1460; KW1461; KW1462; KW1463; KW1520; KW1521; KW1522; KW1523; KW1524; KW1597; KW1705	SO	
KW1367; KW1368; KW1369; KW1370; KW1371; KW1372; KW1373; KW1374; KW1375; KW1376; KW1377; KW1378; KW1379; KW1380; KW1381; KW1382; KW1383; KW1384; KW1385; KW1386; KW1387; KW1388; KW1389; KW1390; KW1391; KW1392; KW1393; KW1394; KW1395; KW1396; KW1454; KW1455; KW1456; KW1457; KW1458; KW1566; KW1567; KW1568; KW1595; KW1596; KW1690	WA	

The number of total isolates and Psa confirmed strains from the three representative plants, soil and water samples analysed in orchard A is presented in the Table 3.2.1-II. From a total of 337 isolates, 171 (51%) were confirmed as Psa, 77 were SI and 94 were AI (Tab. 3.2.1-II).

The percentage of Psa isolates from plants ranged from 57.8% to 72.4%. No major differences were observed between seasons regarding the persistence of Psa isolates in plants. On average, 57.5% and 46.3% of the total SI and AI were Psa, respectively. From the plant samples, 247 (73.3%) isolates were recovered, 162 were confirmed as Psa, corresponding to 65.6% of total plant isolates. In plant 1, from a total of 97 isolates, 56 (57.7%) were confirmed as Psa, of which 20 (36%) were SI while 36 (64.3%) were AI. In plant 2 and 3 a total of 74 and 76 strains were recovered, of which 51 (69%) and 55 (72.4%) were identified as Psa, respectively. In plant 2, 23 (45%) of the Psa strains were SI and 28 (55%) were AI; while in plant 3, 34 (62%) of the Psa strains were SI and 21 (38.2%) were AI.

A total of 28 (8.3%) strains were isolated from soil samples (Table 3.2.1-II), but only 3 were Psa positive, corresponding to 10.7% of total soil isolates. Curiously, not a single Psa isolate was recovered from soil in spring. Finally, a total of 62 (18.4%) isolates were recovered from water samples of which only 6 were confirmed as Psa, corresponding to 9.7% of total water isolates. Similar to soil samples, not a single Psa isolate was recovered from water in spring (Table 3.2.1-II).

Table 3.2.1-II. Total isolates and strains identified as Psa from plants, soil and water samples in each season from orchard A. SI: spring isolate; AI: autumn isolate.

Plant/ Reservoir	SI	AI	Total	Psa SI	Psa AI	Total Psa
Plant 1	32	65	97	20	36	56
Plant 2	29	45	74	23	28	51
Plant 3	37	39	76	34	21	55
Soil	15	13	28	0	3	3
Water	21	41	62	0	6	6
Total	134	203	337	77	94	171

As abovementioned, the three representative plants from orchard A were sampled in spring and autumn, and each sample was processed in order to separately recover epiphytic and endophytic bacterial strains.

In total, 60 (61.2% of total SI) and 74 (49.7% of total AI) EP strains were isolated in spring and in autumn, respectively; of which, 46 (76.7%) were confirmed as Psa in spring and 23 (31.1%) in autumn. On the

other hand, 38 (38.8% of total SI) and 75 (50.3% of total AI) EN strains were isolated in spring and in autumn, respectively; of which 31 (81.6%) were confirmed as Psa in spring and 62 (82.7%) in autumn (Table 3.2.1-III).

Table 3.2.1-III. Total isolates and strains identified as Psa from plants in each season from orchard A. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

	SI		Total SI	Psa SI		Total Psa SI	AI		Total AI	Psa AI		Total Psa AI
Plant	**EP**	**EN**		**EP**	**EN**		**EP**	**EN**		**EP**	**EN**	
1	18	14	32	12	8	20	33	32	65	7	29	36
2	18	11	29	13	10	23	24	21	45	12	16	28
3	24	13	37	21	13	34	17	22	39	4	17	21
Total	60	38	98	46	31	77	74	75	149	23	62	85

From a total of 97 isolates from plant 1, 32 (33%) were SI, of which 18 (56.2%) were EP SI and 14 (43.8%) were EN SI; while 65 (67%) were AI, of which 33 (50.8%) were EP AI and 32 (49.2%) were EN AI (Table 3.2.1-III). From these, 12 and 8 were confirmed as Psa in EP SI (66.7%) and in EN SI (57.1%), respectively. Additionally, 7 (21.2%) and 29 (90.6%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total of 74 isolates from plant 2, 29 (39%) were SI, of which 18 (62.1%) were EP SI and 11 (37.9%) were EN SI; while 45 (61%) were AI, of which 24 (53.3%) were EP AI and 21 (46.7%) were EN AI (Table 3.2.1-III). From these, 13 (72.2%) and 10 (90.9%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 12 (50%) and 16 (76.2%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total of 76 isolates from plant 3, 37 (48.7%) were SI, of which 24 (64.9%) were EP SI and 13 (35.1%) were EN SI; while 39 (51.3%) were AI, of which 17 (43.9%) were EP AI and 22 (56.4%) were EN AI (Table 3.2.1-III). From these, 21 (87.7%) and 13 (100%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 4 (23.5%) and 17 (77.3%) were confirmed as Psa in EP AI and in EN AI, respectively.

Examining the results for total Psa isolated in orchard A, no obvious relation was observed between EP and EN numbers in SI. On the opposite, in AI there's a clear decrease in the number of EP isolates

when compared to EN. Moreover, comparing the results between the three plants it was possible to observe that the percentage of Psa EN isolates was superior to 50% in all plants (**Figure 3-3**).

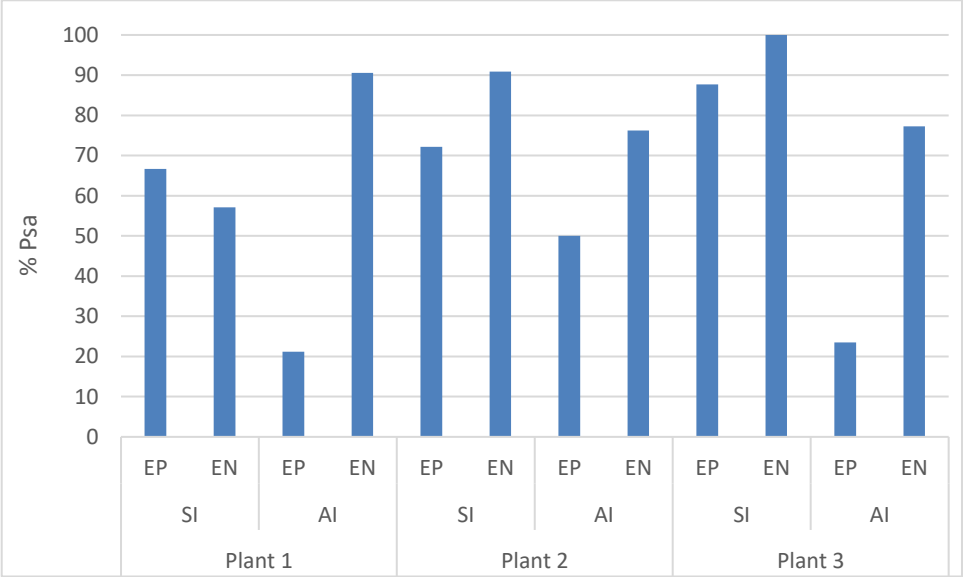


Figure 3-3. Percentage (%) of isolates identified as Psa in each condition from each season from each plant of orchard A. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

3.2.1.2 Characterization of Psa populations in orchard A

The fingerprinting analysis of the Psa isolates was performed by BOX-PCR, as previously described (Louws *et al*., 1994). Fingerprinting profiles (Figure 3-4) obtained from Psa isolates were analysed and grouped as referred in section **2.6.2.**

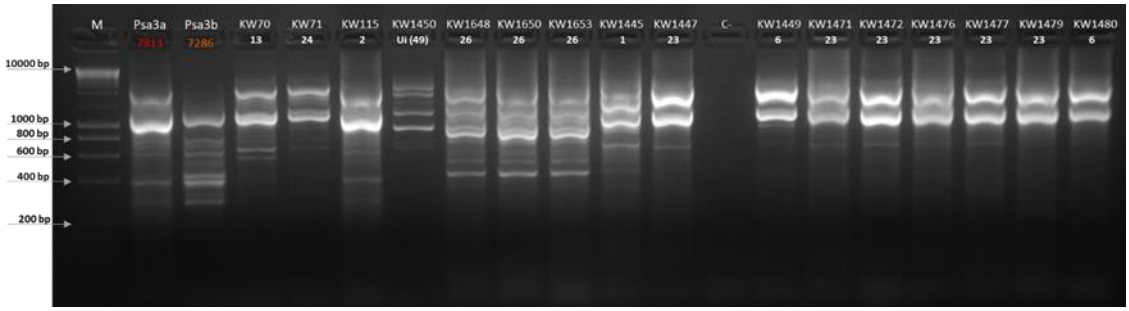


Figure 3-4. BOX-PCR profiles obtained from Psa strains isolated from plant samples. M: ladder III (Nzytech); Psa3a: CFBP 7811 strain (Vanneste *et al*., 2013); Psa3b: CBFP 7286 strain (Balestra *et al*., 2009); KW match strains references; Numbers match Psa profile group; C-: blank.

This methodology allowed clustering isolates into groups and select representative strains to be identified. Figure 4 shows an example of the obtained BOX profiles from several isolates. Isolates KW1447, KW1471, KW1472, KW1476, KW1477 and KW1479 presented an equal BOX profile whereby considered the same strain and were clustered into group 23 (Figure 3-4). From this group, strain KW1447 was selected as a representative and further used for identification purposes.

From the 171 Psa isolates obtain from orchard A, only 146 isolates generated a suitable BOX profile despite several attempts.

Table 3.2.1-IV. Psa isolates and correspondent BOX profile from orchard A. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX PROFILE
	PLANT	LOCALIZATION	
KW81; KW82; KW91	P1	EP	-
KW83; KW84; KW85; KW86; KW89	P1	EP	2
KW97; KW133; KW135	P1	EP	3
KW92; KW93; KW94; KW95	P1	EP	8
KW130	P1	EN	3
KW131; KW132	P1	EN	2
KW137	P1	EN	6
KW168; KW169	P1	EN	13
KW102; KW109; KW110; KW342	P2	EP	-
KW101	P2	EP	5
KW103; KW104; KW105; KW106; KW108; KW111; KW112; KW113	P2	EP	9
KW98; KW99; KW107	P2	EP	36
KW144	P2	EN	-
KW140; KW141; KW142; KW143; KW146	P2	EN	9
KW147; KW148; KW150	P2	EN	10
KW145; KW149	P2	EN	36
KW55; KW56; KW59; KW60; KW62; KW64	P3	EP	-
KW115	P3	EP	2
KW67; KW68	P3	EP	4
KW70	P3	EP	13
KW54; KW57; KW58; KW65; KW69	P3	EP	23
KW61; KW63; KW66; KW71	P3	EP	24
KW343; KW344; KW182	P3	EN	-
KW170; KW171; KW172; KW173; KW174; KW175; KW176; KW177; KW178; KW180; KW181	P3	EN	10

Table 3.2.1-IV (continuation). Psa isolates and correspondent BOX profile from orchard A. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	AUTUMN		BOX PROFILE
	PLANT	LOCALIZATION	
KW1600; KW1646; KW1647; KW1659	P1	EP	-
KW1641	P1	EP	5
KW1729; KW1636; KW1642	P1	EP	19
KW1451; KW1452; KW1453	P1	EN	-
KW1356; KW1357; KW1358; KW1359; KW1360; KW1361; KW1362; KW1363; KW1364; KW1365; KW1365; KW1366; KW1552; KW1553; KW1554; KW1555; KW1556; KW1557; KW1558; KW1559; KW1560; KW1561; KW1562; KW1563; KW1564; KW1565; KW1599	P1	EN	5
KW1427; KW1429	P2	EP	-
KW1431; KW1433; KW1465; KW1619; KW1620; KW1621; KW1622; KW1681; KW1682; KW1688	P2	EP	5
KW1435; KW1438; KW1441; KW1488; KW1574	P2	EN	-
KW1437; KW1440; KW1487; KW1489; KW1490; KW1491; KW1575; KW1576; KW1577; KW1579; KW1580; KW1581; KW1582; KW1680	P2	EN	5
KW1648; KW1650; KW1653	P3	EP	26
KW1450	P3	EP	Ui (49)
KW1448	P3	EN	-
KW1445	P3	EN	1
KW1449; KW1480; KW1482; KW1483; KW1484; KW1485	P3	EN	6
KW1447; KW1471; KW1472; KW1476; KW1477; KW1479; KW1481; KW1486; KW1583; KW1585	P3	EN	23

ISOLATE REFERENCE	AUTUMN	BOX PROFILE
	RESERVOIR	
KW1705	SO	-
KW1523	SO	4
KW1430	SO	Ui (10)
KW1387; KW1388; KW1454	WA	-
KW1458	WA	28
KW1367; KW1566	WA	30
KW1455	WA	Ui (77)
KW1456	WA	Ui (78)

Figure 3-5 shows the distribution of the identified Psa profiles according to the isolation site and season. Psa populations were remarkably distinct between spring and autumn. In spring, a total of 11 Psa profiles were observed, while in the autumn only 6 profiles were detected. Differences in profile diversity between EP/EN strains were also observed in both seasons. Namely, EP SI isolates were split into 10 Psa profiles compared to only 4 profiles detected in EP AI. Similarly, EN SI isolates were split in 7 Psa profiles compared to only 3 profiles detected in EN AI. Furthermore, the total number of EP isolates was lower than the total number of EN isolates, strengthening our observation of a significant difference in the variability among Psa populations between seasons. Indeed, a decrease in Psa population variability was observed in autumn.

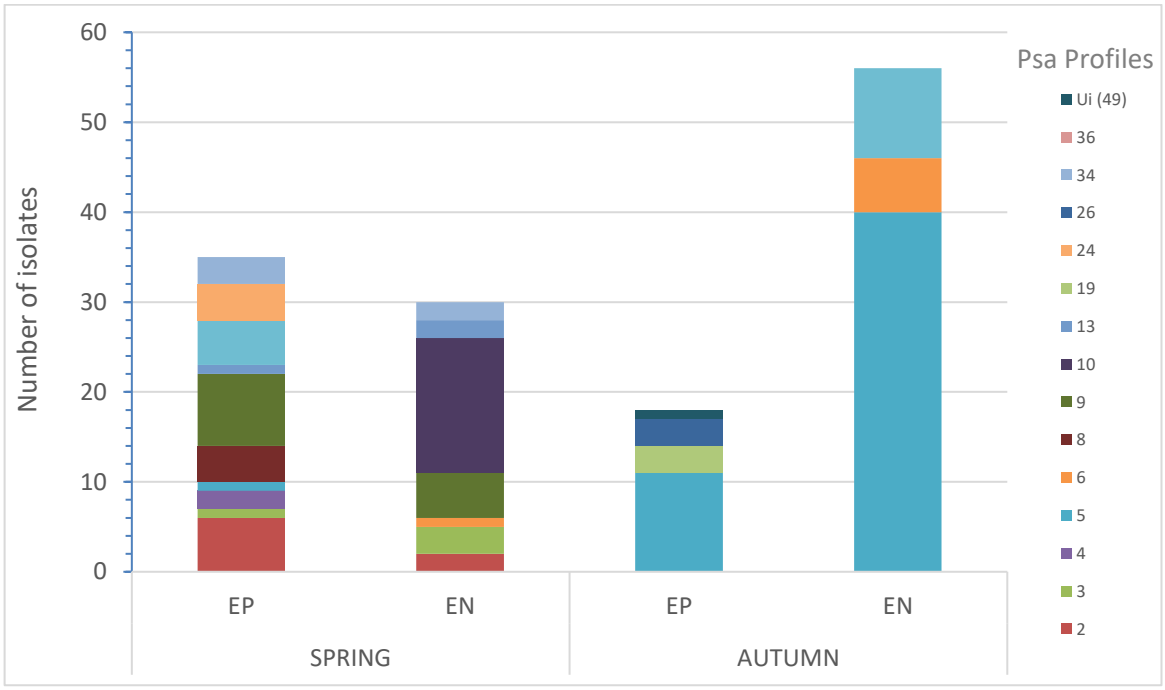


Figure 3-5. Distribution of Psa profiles in plant isolates from orchard A in spring and autumn. EP: epiphytic isolate; EN: endophytic isolate.

In more detail, it was possible to infer a higher diversity in Psa profiles in SI (profiles 2, 3, 4, 5, 6, 9, 10, 13, 23, 24, 34) in relation to AI (5, 6, 19, 23, Ui(49) and 26). Indeed, only 3 Psa profiles - 5, 6 and 23, were common between seasons (Figure 3-5). Among EP isolates from orchard A, the profiles 4, 8 and 24 were only observed in SI. In addition, Psa profiles 19, 26 and Ui (49) were only observed in AI. The only common profile observed between EP SI and AI was profile 5, also present in EN AI. Among the EN isolates, only Psa profile 6 was common in spring and autumn, while Psa profile 5 was found in AI and in EP SI. There was also a unique profile found in Psa EN SI - profile 10. In spring, there were

common Psa profiles between EP and EN, namely profiles 2, 3, 9, 13 and 34 which contrast with only one common profile, Psa profile 5, observed in EP and EN AI.

Our results suggested that there was an obvious higher diversity among Psa populations in spring when compared to autumn. Moreover, there was a clear predominance of Psa profile 5 among EP and EN AI when compared to its restricted distribution in spring. This decrease in Psa variability could be related with the abiotic conditions affecting the orchard between spring and autumn, namely higher temperatures and less humidity (summer conditions), suggesting that the prevalence of Psa profile 5 could be related with its resilient or better adaptation to overcome such conditions.

3.2.1.2.1 Characterization of Psa populations present in each plant

The distribution of Psa profiles among the three sampling plants was depicted in Figure 3-6. It was clear that Psa diversity was quite distinct between plants, and between seasons. In more detail, Psa profiles 3, 8 and 19 were only observed in plant 1. The first one was recovered from both EP and EN SI, while profile 8 and profile 19 were exclusively found in EP SI and EP AI, respectively.

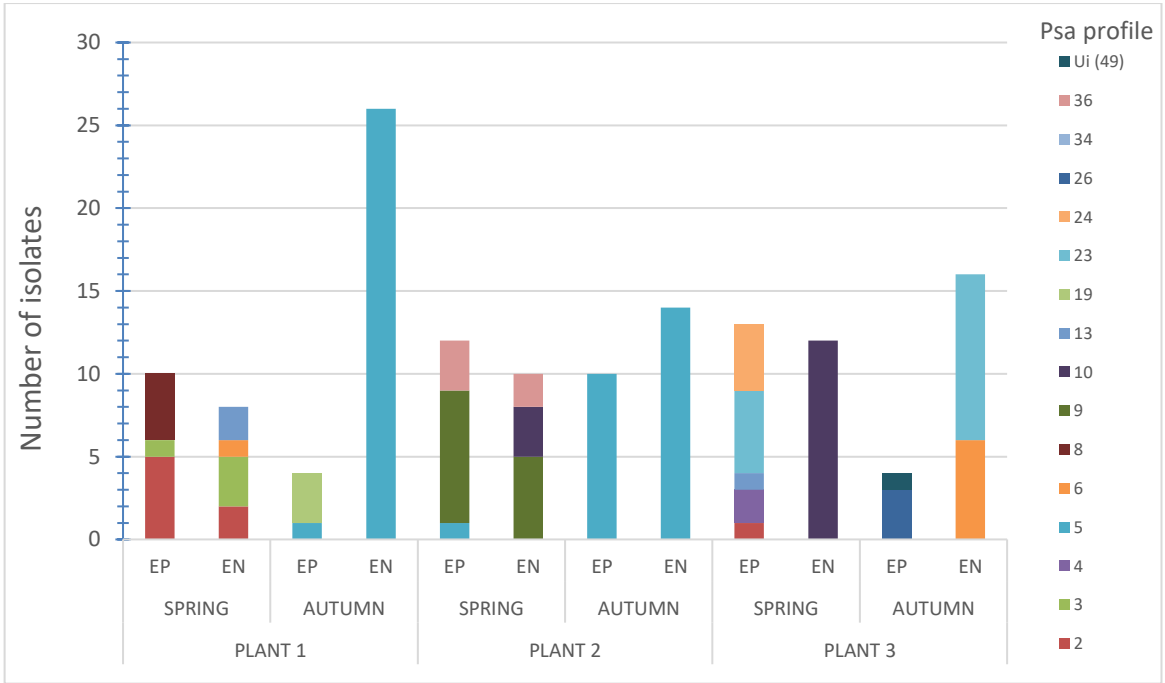


Figure 3-6. Distribution of Psa profiles identified in the three representative plants from orchard A in spring and autumn. EP: Epiphytic isolate; EN: endophytic isolate.

In the plant 2, AI were all characterized as Psa profile 5. In opposition, SI had higher diversity, with two unique profiles (9 and 36) found both EP and EN, and profile 10 isolated from EN.

Plant 3 showed the higher number of profiles when compared with the two other plants, with 5 unique profiles (considering only the isolates of orchard A); namely, profile 4 and 24 from EP SI, profile 26 and UI (49) from EP AI and profile 23 from EP SI and EN AI.

Only Psa profile 5 was common between plant 1 and 2, being the dominant profile in AI. Profile 10 was the only profile common to plants 2 and 3. The isolates with this profile were EN AI in both plants. Plant 1 and 3 shared three common profiles, namely Psa profiles 2, 6 and 13. Considering the number of common profiles, plant 1 and 3 were the most similar. The three mentioned profiles were not related with any specific condition and were isolated either from EP and EN in both seasons.

A higher diversity was observed between Psa profiles isolated in spring than in autumn which suggests that the structure of Psa populations varies over time in the same plant. In addition, there were common profiles between EP and EN in each plant in both seasons, except in the plant 3. These results evidenced the co-existence of several Psa populations; some varied with time while other were persistently recovered.

3.2.1.2.2 Soil and water has potential Psa environmental reservoirs

The total Psa diversity in orchard A was inferred from BOX profiling and is depicted in Figure 3-7. Two and 4 distinct Psa profiles were obtained from soil and water isolates, respectively. All these profiles were obtained from AI and, with one exception, they were restricted to those reservoirs. Indeed, only strains with profile 4 were identified both in soil and in plant (EP SI). These results *per si* argues that both soil and water provide conditions for Psa persistence, although in considerable lower numbers when compared to plant samples. Importantly, and although the isolation season was distinct, soil ought to be considered a reservoir for Psa populations, and included in the management control measures to avoid dispersal of Psa within and between orchards.

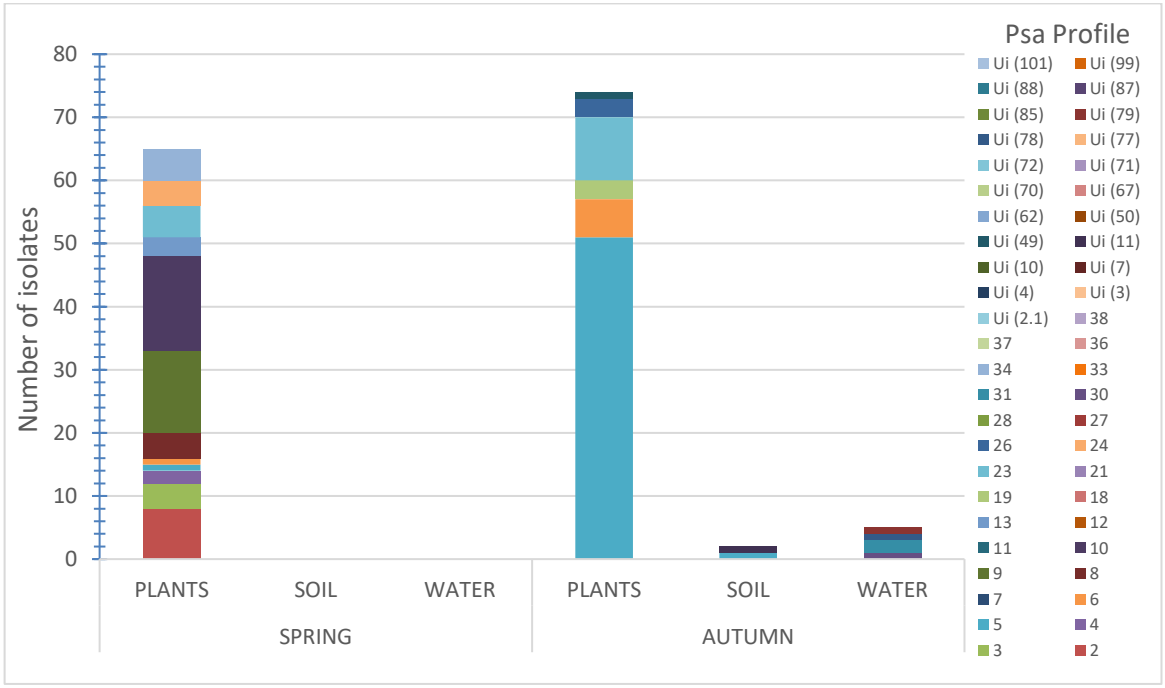


Figure 3-7. Distribution of Psa profiles in plants, soil and water from orchard A.

3.2.1.3 Alpha Diversity

3.2.1.3.1 Between plants

Alpha diversity analysis was performed for each plant in orchard A (Table 3.2.1-V). According to Margalef index (Dmg), which only considers specific richness, plant 2 had the lower value in accordance with the lower number of observed Psa profiles (Figure 3-6).

The Shannon index (H') was calculated from Psa profiles richness and their proportion. Higher values corresponded to a better distribution of Psa isolates through the profiles. In accordance, plant 3 displayed a higher H' meaning that strains were more evenly distributed by the profiles. On the opposite, plant 2 presented a lower H' index supported by the existence of dominate Psa profiles (Figure 3-8.). The Pielou's evenness index (J') measured the ratio between the observed diversity (H') and maximal possible diversity. J' values varied between 0 and 1 (representing a situation in which all Psa profiles were equally abundant), so higher evenness values in a population corresponded to higher J' value. Plant 2 and 3 had both higher J' value with a value of 0.8, meaning that Psa isolates were relatively well distributed by the profiles.

Finally, the Simpson diversity index or dominance index (1-D) was used since it measures the probability of two isolates belong to distinct profiles, varying between 0 (no diversity - dominance) and 1 (high diversity). From this index, we could conclude that plant 3 had higher diversity than plant 1 and 2, were the existence of dominate profiles was evident.

Table 3.2.1-V. Alpha diversity indexes determined for each plant in orchard A. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index.

Index	Orchard A		
	Plant 1	Plant 2	Plant 3
Dmg	3.6	1.8	4.8
H'	1.4	1.1	1.8
J'	0.7	0.8	0.8
1-D	0.6	0.6	0.8

3.2.1.3.2 Within orchard per condition

Considering EP and EN isolates in both spring and autumn seasons, a similar alfa diversity analysis was performed (Table 3.2.1-VI). Observing the values of Dmg, EP SI had the higher value with 5.8. On the opposite, EN AI isolates presented the lower value (1.1), which was expected since it represents the condition with only 3 Psa profiles detected on fingerprinting analysis. According to Shannon index (H') EP SI had the most uniform distribution of Psa profiles with a value of 2.1. On the other hand, EN AI (0.8), presented a lower H' index supported by the existence of dominate Psa profiles, namely profile 5 (Figure 3-5). The J' values were similar between conditions (Table 3.2.1-VI). However, EP SI has the higher evenness (J' value 0.9) which correlates with the Dmg and H' results abovementioned. Finally, 1-D index supported that EP (0.6) and EN (0.4) AI had dominant profiles when compared with EP and EN SI.

Table 3.2.1-VI. Alpha diversity indexes determined for each condition in orchard A. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index; EP SI: epiphytic isolates from spring; EN SI: endophytic isolates from spring. EP AI: epiphytic isolates from autumn; EN AI: endophytic isolates from autumn.

Orchard A	Alpha diversity index			
	Dmg	H'	J'	1-D
EP SI	5,8	2,1	0,9	0,9
EN SI	4,1	1,5	0,8	0,7
EP AI	2,4	1,1	0,8	0,6
EN AI	1,1	0,8	0,7	0,4

In sum, a higher specific richness was found in SI has opposed to AI (Dmg index –Table 3.2.1-VI). Psa strains were more evenly distributed by the profiles in EP and EN SI (H' index – Table 3.2.1-VI) which was in accordance with evenness values (J' index – Table 3.2.1-VI). The autumn populations were characterized by the presence of dominate Psa profiles has evidenced by the Simpson diversity index or dominance index (1-D) (Table 3.2.1-VI).

The data collected from the alfa diversity indexes analysis confirmed our previous evidences that a dramatic changed occurred in Psa populations between spring and autumn supported by a decrease in the variability of Psa profiles accompanied by the raise of dominate ones. This decrease maybe a reflection of alterations in abiotic conditions (temperature and humidity) combined with several implemented orchards cultural practices that varied between spring and autumn.

3.2.1.4 Principal component analyses (PCA)

A inter-species correlation analyses (PCA) of all plants isolates from orchard A is shown in Figure 3-9. In this analysis, it was possible to correlate the weight of a Psa profile with the event, which in this case represents the different conditions: epiphytic isolate or endophytic isolate in both spring and autumn. The PCA analysis allows inferring which Psa profiles differ or cluster samples. AI were clustered together and separated from SI. This configuration was mainly due to profile 5 dominant, in AI. Moreover, several common profiles were found between AI, namely, 6, 19, 26 and Ui (49) reinforcing this clustering. On the contrary, EP and EN SI were separated in the PCA analyses, mainly by profile 10 (EN SI) and profiles 2, 4, 8, 9, 24 and 34 (EP SI). The configuration present in PCA analysis clearly confirms the fingerprinting results, inferring differences in Psa diversity between EP and EN SI.

This analysis reinforced our previous results were the co-existence of distinct Psa populations was reported. Moreover, a succession of Psa populations with seasons was also strength.

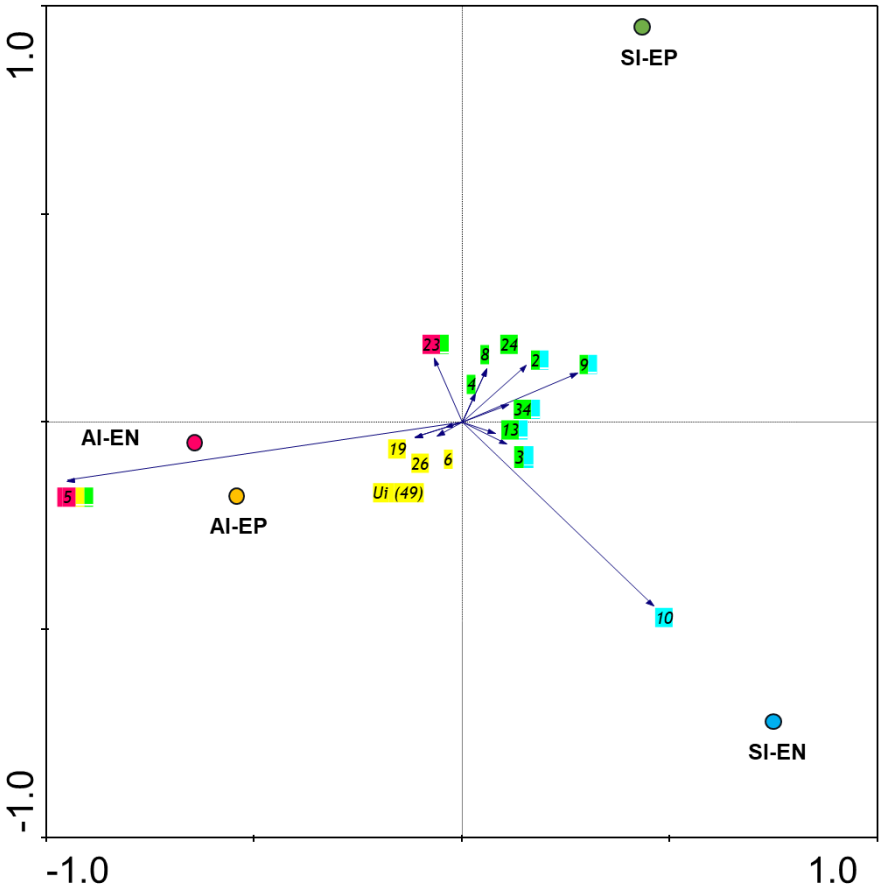


Figure 3-9. Principal component analysis – inter-species correlate – of Psa profiles from orchard A. Green SI-EP: epiphytic isolates, spring; Blue SI-EN: endophytic isolates, spring; Yellow AI-EP: epiphytic isolates, autumn; Pink AI-EN: endophytic isolates, autumn. Numbers correspond to Psa profiles. Colours identify Psa profiles that have more weight in each condition.

3.2.2 Orchard B

3.2.2.1 Putative *Pseudomonas* spp. isolation and Psa identification

A total of 342 strains were obtained from plants, soil and water samples collected in orchard B (Table 3.2.2-I); 176 were SI and 166 were AI.

In order to confirm the identity off the isolates as Psa strains a duplex-PCR protocol described by Gallelli *et al*. (2011) was performed using the extracted DNA's (see section **3.1.1.1.**). The number of total isolates and Psa confirmed strains from the three representative plants, soil and water samples analysed in orchard B is presented in the Table 3.2.1-I. From a total of 342 isolates, 146 (42.7%) were confirmed as Psa, 92 were SI and 54 were AI (Table 3.2.2-III).

Table 3.2.2-I. Total isolates recovered from orchard B in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	SPRING	
	RESERVOIR	
KW213; KW214; KW215; KW216; KW217; KW218; KW219; KW220; KW221; KW222; KW223; KW224; KW225; KW226; KW227; KW228; KW229; KW230; KW231; KW232; KW233; KW234; KW235; KW236; KW237; KW238; KW239; KW240; KW241; KW331	SO	
KW258; KW259; KW260; KW261; KW262; KW263; KW264; KW265; KW266; KW267; KW268; KW269; KW270; KW271; KW272; KW273; KW274; KW275; KW276; KW277; KW278; KW279; KW330; KW332	WA	

ISOLATE REFERENCE	SPRING	
	PLANT	LOCALIZATION
KW1; KW2; KW3; KW4; KW5; KW6; KW7; KW8; KW9; KW10; KW11; KW12; KW13; KW14; KW15; KW16; KW17; KW18; KW116; KW117; KW345	P1	EP
KW19; KW20; KW21; KW22; KW23; KW24; KW25; KW26; KW27; KW28; KW29; KW30; KW31; KW32; KW302; KW303	P1	EN
KW72; KW73; KW74; KW75; KW76; KW77; KW78; KW79; KW80; KW118; KW119; KW120; KW121; KW122; KW123; KW124; KW125; KW126; KW127; KW128; KW346	P2	EP
KW33; KW34; KW35; KW36; KW37; KW38; KW39; KW40; KW41; KW42; KW43; KW44; KW45; KW46; KW47; KW48; KW49; KW50; KW51; KW183; KW304; KW317	P2	EN
KW129; KW151; KW152; KW153; KW154; KW155; KW156; KW157; KW158; KW159; KW160; KW161; KW162; KW163; KW164; KW165; KW166; KW167; KW347; KW348; KW349; KW350	P3	EP
KW184; KW185; KW186; KW187; KW188; KW189; KW190; KW191; KW192; KW193; KW194; KW195	P3	EN

ISOLATE REFERENCE	AUTUMN	
	PLANT	LOCALIZATION
KW1413; KW1414; KW1467; KW1468; KW1469; KW1666; KW1667; KW1668; KW1708; KW1710; KW1711; KW1712; KW1715; KW1725	P1	EP
KW1411; KW1412; KW1415; KW1416; KW1473; KW1474; KW1475; KW1605; KW1606; KW1607; KW1608; KW1609; KW1610; KW1611; KW1612; KW1613; KW1614; KW1615; KW1616	P1	EN
KW1417; KW1418; KW1419; KW1492; KW1493; KW1494; KW1496; KW1497; KW1498; KW1499; KW1500; KW1501; KW1505; KW1506; KW1507	P2	EP

Table 3.2.2-II. (continuation) Total isolates recovered from orchard B in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	AUTUMN	
	PLANT	LOCALIZATION
KW1420; KW1421; KW1422; KW1423; KW1424; KW1502; KW1503; KW1504; KW1508; KW1509; KW1510; KW1511; KW1512; KW1513; KW1514; KW1515; KW1516; KW1517; KW1518; KW1519; KW1570	P2	EN
KW1617	P3	EP
KW1425; KW1618; KW1669; KW1670; KW1671; KW1672; KW1673; KW1674; KW1675; KW1676; KW1677; KW1678; KW1679; KW1694; KW1699; KW1713; KW1714; KW1716; KW1717	P3	EN
ISOLATE REFERENCE	**AUTUMN**	
		RESERVOIR
KW1525; KW1526; KW1527; KW1528; KW1529; KW1530; KW1531; KW1532; KW1533; KW1534; KW1535; KW1536; KW1537; KW1538; KW1539; KW1540; KW1541; KW1571; KW1572; KW1573; KW1574; KW1587; KW1588; KW1589; KW1590; KW1591; KW1602; KW1603; KW1692; KW1696; KW1697; KW1698; KW1700; KW 1701; KW1702; KW1704; KW1720; KW1724; KW1726; KW1727		SO
KW1397; KW1398; KW1399; KW1400; KW1401; KW1402; KW1403; KW1404; KW1405; KW1406; KW1407; KW1408; KW1409; KW1410; KW1443; KW1444; KW1542; KW1543; KW1544; KW1545; KW1546; KW1547; KW1548; KW1549; KW1550; KW1569; KW1684; KW1685; KW1686; KW1687; KW1691; KW1693; KW1695; KW1703; KW1706; KW1707; KW1728		WA

The percentage of Psa isolates from plants sampled ranged from 58.2% to 72.9%. No major differences were observed between seasons regarding the persistence of Psa isolates in plants. On average, 52.3% and 32.5% of the total SI and AI were Psa, respectively. From the plant samples, 203 (59.4%) isolates were recovered, 131 were confirmed as Psa, corresponding to 64.5% of total plant isolates. In plant 1, from a total of 70 isolates, 51 (72.9%) were confirmed as Psa, of which 29 (56.9%) were SI while 22 (43.1%) were AI. In plant 2 and 3 a total of 79 and 54 strains were recovered, of which 46 (58.2%) and 34 (63%) were identified as Psa, respectively. In plant 2, 31 (67.4%) of the Psa strains were SI and 15 (32.6%) were AI; while in plant 3, 26 (76.5%) of the Psa strains were SI and 8 (23.5%) were AI.

A total of 77 (22.5%) strains were isolated from soil samples (Table 3.2.2-III), but only 13 were Psa positive, corresponding to 16.8% of total soil isolates. Finally, a total of 62 (18.1%) isolates were recovered from water samples but only 2 were confirmed as Psa, corresponding to 3.2% of total water isolates. Curiously, not a single Psa isolate was recovered from water in spring.

Table 3.2.2-III. Total isolates and strains identified as Psa strains from plants, soil and water samples in each season from orchard B. SI: spring isolate; AI: autumn isolate.

Plant/ Reservoir	SI	AI	Total	Psa SI	Psa AI	Total Psa
Plant 1	37	33	70	29	22	51
Plant 2	43	36	79	31	15	46
Plant 3	34	20	54	26	8	34
Soil	37	40	77	6	7	13
Water	25	37	62	0	2	2
Total	176	166	342	92	54	146

As abovementioned, the three representative plants from orchard B were sampled in spring and autumn, and each sample was processed in order to separately recover epiphytic and endophytic bacterial strains.

In total, 64 (56.1% of total SI) and 30 (33.7% of total AI) EP strains were isolated in spring and in autumn, respectively; of which, 48 (75%) were confirmed as Psa in spring and 9 (30%) in autumn. On the other hand, 50 (43.9% of total SI) and 59 (66.3% of total AI) EN strains were isolated in spring and in autumn, respectively; of which 38 (76%) were confirmed as Psa in spring and 36 (61%) in autumn (Table 3.2.1-III).

Table 3.2.2-IV. Total isolates and strains identified as Psa from plants in each season from orchard B. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

	SI		Total	Psa SI		Total	AI		Total	Psa AI		Total
Plant	**EP**	**EN**	**SI**	**EP**	**EN**	**Psa SI**	**EP**	**EN**	**AI**	**EP**	**EN**	**Psa AI**
1	21	16	37	17	12	29	14	19	33	5	17	22
2	21	22	43	16	15	31	15	21	36	4	11	15
3	22	12	34	15	11	26	1	19	20	0	8	8
Total	64	50	114	48	38	86	30	59	89	9	36	45

From a total of 70 isolates from plant 1, 37 (53%) were SI, of which 21 (56.8%) were EP SI and 16 (43.2%) were EN SI; while 33 (47%) were AI, of which 14 (42.4%) were EP AI and 19 (57.6%) were EN AI (Table 3.2.1-III). From these, 17 (81%) and 12 (75%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 5 (35.7%) and 17 (89.5%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total 79 of isolates from plant 2, 43 (54%) were SI, of which 21 (48.8%) were EP SI and 16 (51.2%) were EN SI; while 36 (46%) were AI, of which 15 (41.7%) were EP AI and 21 (58.3%) were EN AI (Table 3.2.1-III). From these, 16 (76.2%) and 15 (93.8%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 4 (26.7%) and 11 (52.4%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total of 54 isolates from plant 3, 34 (63%) were SI, of which 22 (64.7%) were EP SI and 12 (35.3%) were EN SI; while 20 (37%) were AI, of which while 1 (5%) was EP AI and 19 (95%) were EN AI (Table 3.2.1-III). From these, 15 (68.2%) and 11 (91.7%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 8 (42.1%) were confirmed as Psa EN AI. The only EP AI recovered from plant 3 was not identified as Psa, so there were no Psa EP AI from plant 3.

Examining the total Psa strains isolated from plants in spring the number of Psa EP was superior to Psa EN. On the opposite, in AI there was a clear decrease in the number of EP isolates when compared to EN (Table 3.2.1-III). In addition, when comparing the results between the three plants was possible to observe that the percentage of Psa EN isolates was superior to 40% in all plants while in EP isolates the percentage varied between 0 and 81% (Figure 3-10).

The increase observed in EN AI was accompanied by an increase in Psa EN AI percent in all the plants. The reduction in EP AI numbers may be related with changes in the edaphoclimatic conditions and associated cultural practises that could restrain the ability of Psa population's to grow or persistency epiphytically on plants.

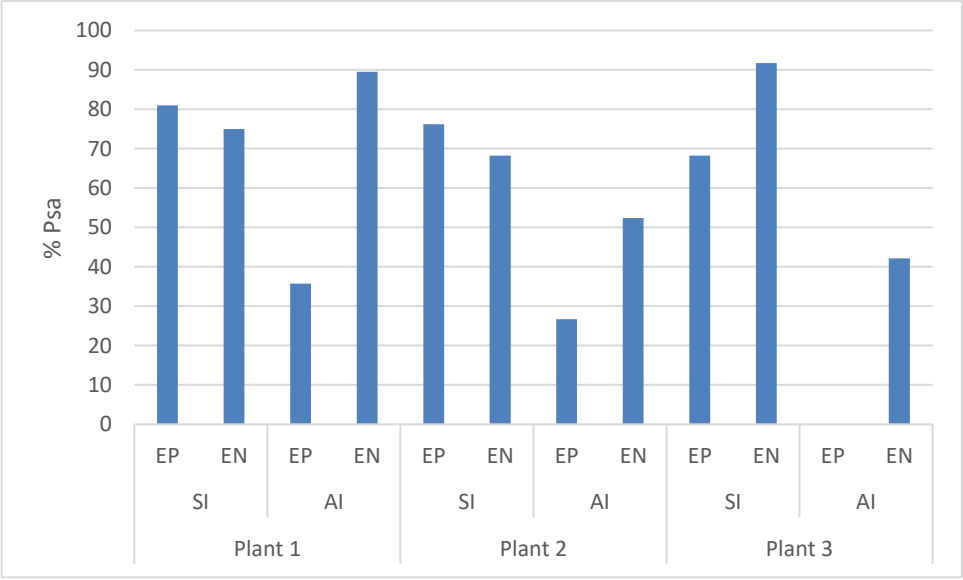


Figure 3-10. Percentage (%) of isolates identified as Psa in each condition from each season from each plant of orchard B. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

3.2.2.2 Characterization of Psa populations in orchard B

The fingerprinting analysis of the Psa isolates was performed by BOX-PCR, as previously described (Louws *et al*., 1994). From the 146 Psa isolates obtain from orchard B, only 130 isolates generated a suitable BOX profile despite several attempts (Table 3.2.2-V).

Table 3.2.2-V. Psa isolates and correspondent BOX profile from orchard B. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX
	PLANT	LOCALIZATION	PROFILE
KW345	P1	EP	-
KW1; KW2; KW5; KW6; KW7; KW18	P1	EP	5
KW3; KW4; KW17	P1	EP	9
KW8; KW9; KW10; KW11; KW12; KW13; KW14	P1	EP	36
KW302	P1	EN	-
KW22; KW23; KW26; KW27; KW28; KW29; KW30; KW31; KW32	P1	EN	5
KW24; KW25	P1	EN	36
KW76; KW80; KW346	P2	EP	-
KW75; KW123; KW124; KW125; KW126; KW127	P2	EP	9
ISOLATE REFERENCE	**SPRING**		**BOX**
	PLANT	**LOCALIZATION**	**PROFILE**
KW74	P2	EP	27
KW73; KW77; KW78; KW79; KW128	P2	EP	36
KW49; KW317	P2	EN	-
KW42; KW43; KW45; KW50	P2	EN	5
KW37; KW46; KW47; KW48; KW304	P2	EN	9
KW38; KW39; KW40; KW44;	P2	EN	36
KW129	P3	EP	-
KW154; KWKW156; KW157; KW158; KW159; KW160; KW161; KW165; KW167	P3	EP	5
KW155; KW162; KW166	P3	EP	7
KW151; KW163	P3	EP	13
KW195	P3	EN	5
KW184; KW185; KW186; KW187; KW189; KW190; KW192; KW193; KW194	P3	EN	7
KW191	P3	EN	33

Table 3.2.2 -VI (continuation). Psa isolates and correspondent BOX profile from orchard B. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX
	RESERVOIR		PROFILE
KW238	SO		-
KW236	SO		28
KW237; KW239; KW240; KW241	SO		38
ISOLATE REFERENCE	**AUTUMN**		**BOX**
	PLANT	**LOCALIZATION**	**PROFILE**
KW1468	P1	EP	-
KW1413; KW1414; KW1667	P1	EP	5
KW1467	P1	EP	Ui (50)
KW1411; KW1609	P1	EN	-
KW1412; KW1415; KW1473; KW1474; KW1475; KW1605; KW1607; KW1610; KW1611; KW1612; KW1613; KW1614; KW1616	P1	EN	5
KW1606; KW1608	P1	EN	37
KW1505	P2	EP	-
KW1500	P2	EP	5
KW1507	P2	EP	13
KW1493	P2	EP	27
KW1503; KW1504; KW1509; KW1510; KW1511; KW1512; KW1513	P2	EN	-
KW1502; KW1514; KW1518; KW1519	P2	EN	5
ISOLATE REFERENCE	**AUTUMN**		**BOX**
	PLANT	**LOCALIZATION**	**PROFILE**
KW1674	P3	EN	1
KW1677; KW1678; KW1679; KW1694; KW1699; KW1716; KW1717	P3	EN	5
KW1618	P3	EN	7
ISOLATE REFERENCE	**AUTUMN**		**BOX**
	RESERVOIR		**PROFILE**
KW1590	SO		-
KW1574	SO		5
KW1724; KW1727	SO		12
KW1540	SO		Ui (11)
KW1726	SO		Ui (67)
KW1692	SO		28
KW1687	WA		-
KW1685	WA		Ui (79)
KW1686	WA		Ui (102)

Figure 3-11 shows the distribution of Psa profiles identified according to the isolation site and season. Psa populations were distinct between spring and autumn. In spring, a total of 7 Psa profiles were observed while in the autumn only 6 profiles were detected. Differences in profile diversity between EP/EN were also observed in both seasons. The EP SI and EP AI were split in 6 and 5 Psa profiles, respectively. However, the EN SI isolates were split in 5 Psa profiles compared to only 2 profiles detected in EN AI. Note that the total number of EP AI isolates was slightly lower than the total number of EN AI isolates which once more, strengthens the observation that a significant difference in diversity exits among EN Psa populations between seasons.

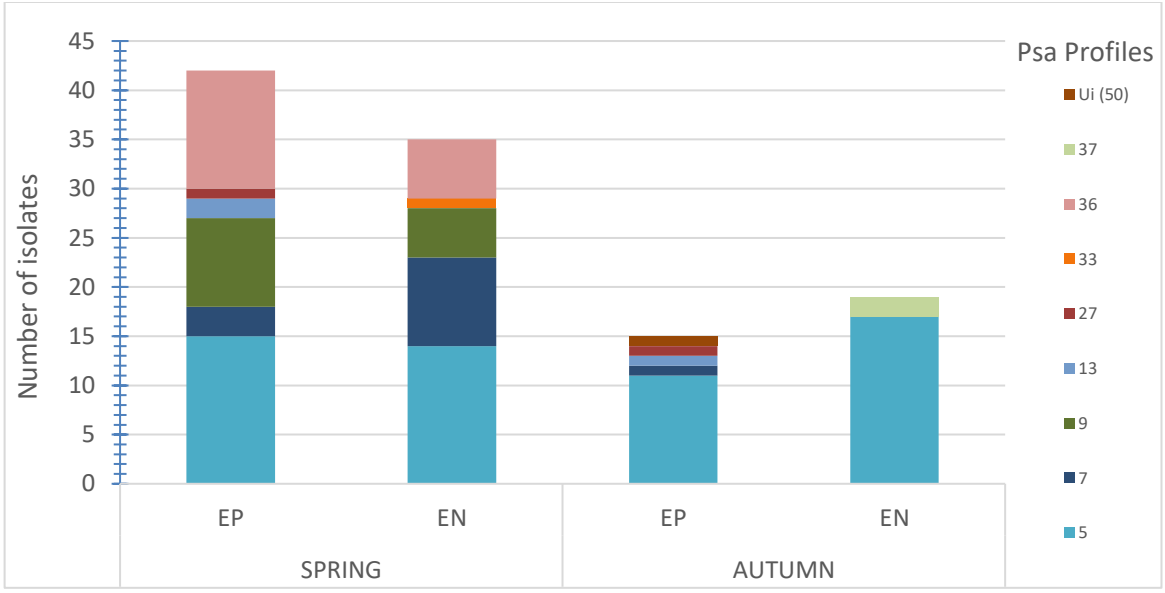


Figure 3-11. Distribution of Psa profiles in plant isolates from orchard B in spring and autumn. EP: epiphytic isolate; EN: endophytic isolate.

In more detail, it was possible to infer a slightly higher diversity in Psa profiles in SI (profiles 5, 7, 9, 13, 27, 33 and 36) in relation to AI (5, 7, 13, 27, Ui (50) and 37). The Psa profiles 5, 7, 13 and 27 were common between seasons (Figure 3-11). The profile 5 was common to all plant isolates. On contrary, profiles 13 and 27 were only common to EP isolates from both seasons. The profile 7 was common between SI and EP AI. Among the SI isolates from orchard B, there were common Psa profiles between EP and EN, namely profiles 9 and 36. There was also a unique profile found only among Psa EN SI - profile 33. In autumn only the profile 5 was common between EP and EN. Unique profiles were observed in both EP and EN - profile Ui (50) and 37, respectively.

Our results suggested that there was a slightly higher diversity among Psa populations in spring when compared to autumn. Moreover, there was a clear predominance of Psa profile 5 among EP and EN in

both seasons. This dominance of Psa profile 5, similar to what was observed in orchard A, could be related with the abiotic conditions affecting the orchard between spring and autumn, namely higher temperatures and less humidity (summer conditions), suggesting that this clone could be more resilient or adapted to overcome such conditions.

3.2.2.2.1 Characterization of Psa populations present in each plant

The distribution of Psa profiles among the three sampling plants is depicted in Figure 3-12. It was clear that Profile 5 was common to all plants, except for EP SI and EN AI from plant 2 and 3, respectively. The diversity observed was similar between plants, and between seasons and it is possible to stablish several common profiles (Figure 3-12). In Plant 1, 5 distinct Psa profiles were identified, of which Psa profile Ui (50) and 37 were exclusive to this plant, recovered from EP and EN AI, respectively. Among SI, three Psa profiles (5, 9, and 36) could be identified. In AI, also three Psa profiles (5, Ui (50) and 37) could be observed.

In the plant 2, 5 Psa profiles (5, 9, 13, 27 and 36) were also observed (Figure 3-12). Differences between SI and AI were due to the detection of profile 9 and 36 which were exclusive from SI (considering only the results of orchard B). These Psa profiles were also identified in plant 1. Observing the EP Psa profiles in both SI and AI it was possible to identify two exclusive profiles – profiles 13 and 27. The last one was also present in EP SI from plant 3. Among EN, the SI had higher diversity than AI, since 3 profiles (5, 9 and 36) were identified while in AI only profile 5 was observed.

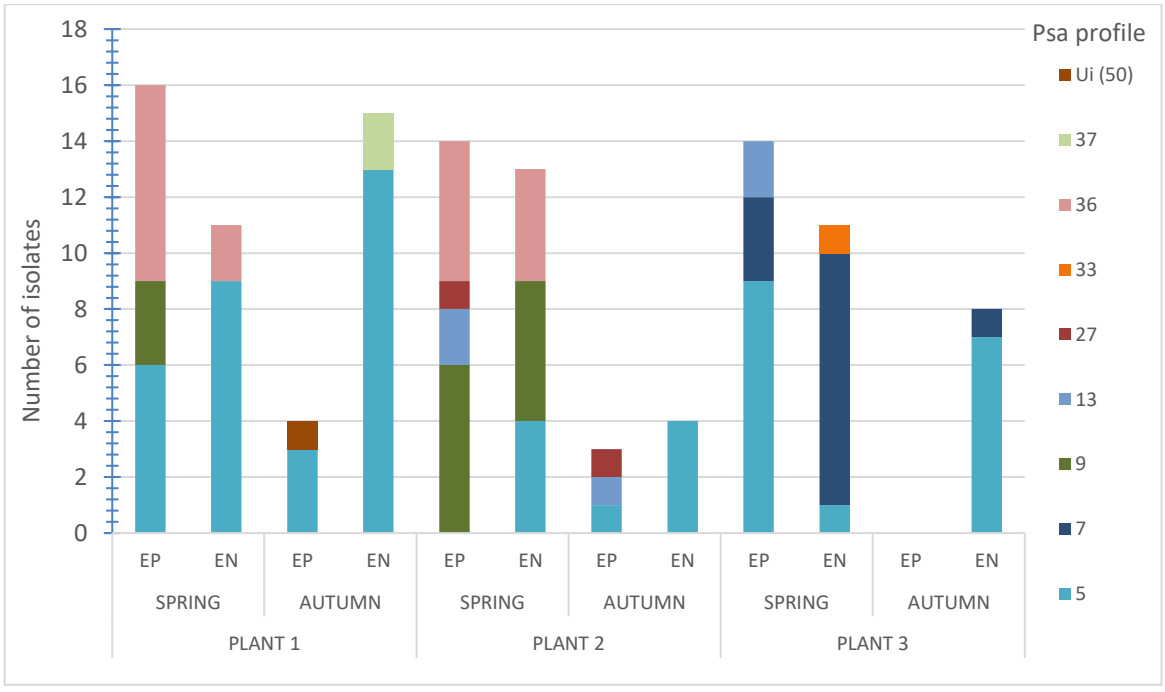


Figure 3-12. Distribution of Psa profiles identified in the three representative plants from orchard B in spring and autumn. EP: Epiphytic isolate; EN: endophytic isolate.

Plant 3 showed a lower diversity when compared with the two other plants, with only 4 identified profiles (5, 7, 13 and 33). However, two of those ones (profiles 7 and 33) were unique of plant 3. The profile 33 was found in all samples, except in EP AI (no Psa isolates was recovered from this samples). Psa profiles were identically distributed between EP and EN isolates, with changes in SI Psa profiles – 13 and 33 isolated in EP and EN, respectively.

As abovementioned, profile 5 was common to the three plants, being dominant in plant 1 and in EN in both SI and AI from plant 2 and 3, respectively. Plant 1 and 2 shared two common profiles, namely Psa profiles 9 and 36. The first profile was related with EP isolates from plant 1 and was present in both EP and EN SI from plant 2. Between plant 2 and plant 3, a common profile - 13 was observed. Psa profile 13 was found in EP SI and AI in both plants. Considering the number of profiles in common, plant 1 and plant 2 were the most similar. The three profiles observed were related to EP and EN isolates from spring season. Overall, there were common profiles between EP and EN in each plant in both seasons. These results evidenced the co-existence of several Psa populations in the same plant and between plants; some varied with time while other were persistently recovered.

No significant differences were observed in diversity between Psa profiles isolated in spring and autumn. However, profiles 9, 33 and 36 were only found in SI such as profile Ui (50) and 37 were only found in AI, which suggests that the structure of Psa population varies over time in the same plant.

3.2.2.2.2 Soil and water has potential Psa environmental reservoirs

The total Psa diversity, inferred from BOX profiling, determined in orchard B is depicted in Figure 3-13. Six and 2 Psa distinct profiles were obtained from soil and water isolates, respectively. The soil profiles were distributed per seasons. A total of 2 Psa profiles (28 and 38) were obtained from SI whereas in AI were obtained 4 Psa profiles (5, 12, ui (67) and 28). From water samples, Psa profiles Ui (79) and Ui (102) were recovered from AI (Figure 3-13).

The Psa profile 5 was commonly identified between soil samples (AI) and both EP and EN from plant samples in both seasons, being widely represent between the orchard B isolates (see Figure 3-10, 3-11 e 3-12). These results *per si* argues that both soil and water provide conditions for Psa persistence, although in considerable lower numbers when compared to plant samples. Importantly, soil ought to be considered a reservoir for Psa populations, and included in the management control measures to avoid dispersal of Psa within and between orchards.

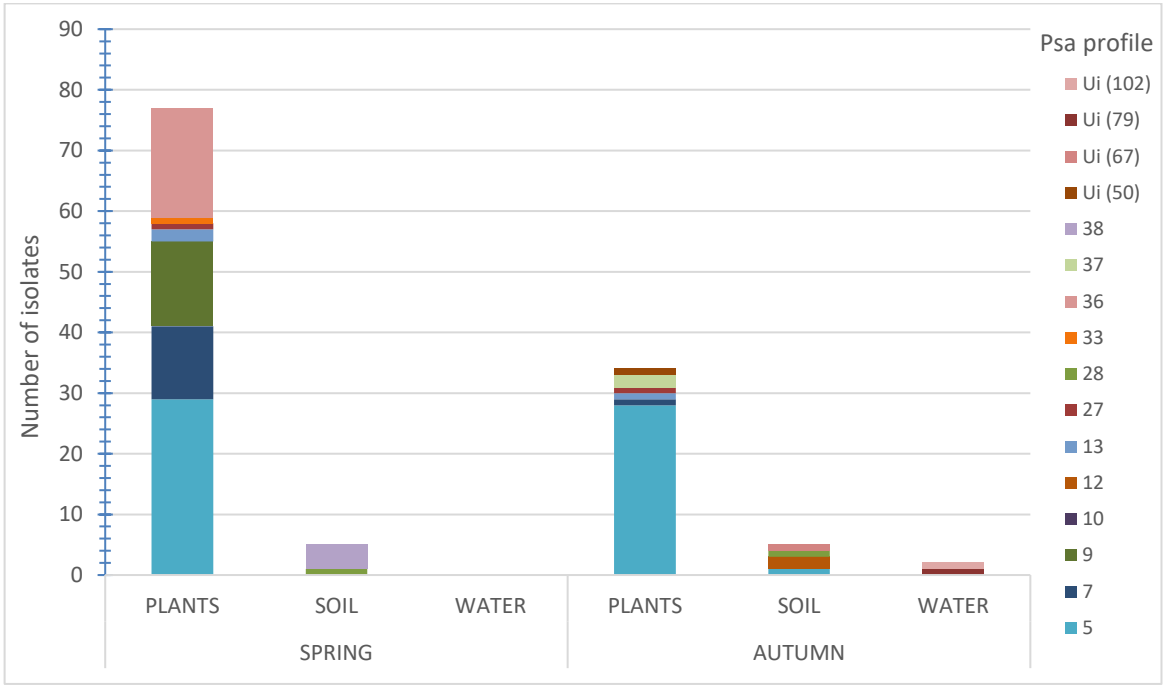


Figure 3-13. Distribution of Psa profiles in plants, soil and water from orchard B.

3.2.2.3 Alfa Diversity

3.2.2.3.1 Between plants

An alfa diversity analysis was performed for each of three plants in orchard B (Table 3.2.2-VII). According to Dmg index, plant 2 had the higher value (Dmg value 2.6) in accordance with the higher number of isolates and Psa profiles (Figure 3.11). On the other hand, plant 3 had the lower value of specific richness (2). The H' index indicates in plant 2 the Psa isolates were better distributes through the profiles, with a value of 1.4, even considering the strong presence of profile 5 (Figure 3-11), which is considered dominant in orchard B. Plant 1 and 3 had the same value of H' (1.0), which indicates that their Psa profile distribution was similar.

In accordance to J' index, in plant 2 and 3 the Psa isolates were more evenly distributed by the Psa profiles with a value of 0.9 and 0.7, respectively (Table 3.2.2-VII). These results mean that the different Psa profiles found in those plants were equally abundant. On the contrary, plant 1 had a lower J' value (0.6) that was associated with the higher relative abundance of profile 5, corresponding to 31 Psa isolates (Figure 3-11). Finally, analysing 1-D values from each plant (Table 3.2.2-VII), no significant diversity differences were noted. The 1-D values in each plant were relatively closer: 0.5, 0.6 and 0.7 to plant 1, 2 and 3, respectively. However, plant 2 had a higher diversity than plant 1 and 3, were the

existence of dominate profiles (such as profile 5 – plant 1, and profiles 5 and 7 – plant 3) was evident (Figure 3-11).

Table 3.2.2-VII. Alpha diversity indexes determined for each plant in orchard B. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index.

Index	Orchard B		
	Plant 1	Plant 2	Plant 3
Dmg	2.4	2.6	2.0
H'	1,0	1,4	1,0
J'	0,6	0,9	0,7
1-D	0,5	0,7	0,6

3.2.2.3.2 Within orchard per conditions

Table 3.2.2-VIII presents a similar alfa diversity analysis performed with EP and EN isolates in spring and autumn. Considering only specific richness, the Dmg index indicates that EP AI had the higher value (3.5) of specific richness. As expected, EN AI had the lower value, with only 1.4. These results were related with the lower number of Psa profiles observed in Psa EN in autumn season (only 2 profiles – see Figure 3-11).

According to H' values, EP and EN SI had the better distribution of Psa profiles with values of 1.5 and 1.4, respectively. On the other hand, EN AI, presented a lower H' index (0.4) supported by the existence of a reduce number of profiles, of which Psa profile 5 was dominant (see Figure 3-11). The J' values were closer between samples, however, the higher J' value was shared by EP and EN SI. These results showed that these isolates had a similar distribution through Psa profiles in this season. The lower J' value was obtained for EN AI, which correlates with both Dmg and H' index and fingerprinting analysis. Finally, 1-D index confirmed that SI had higher diversity than AI (Table 3.2.2-VIII). EP and EN SI had higher 1-D value, with 0.8 and 0.7, respectively.

Table 3.2.2-VIII. Alpha diversity indexes determined for each condition in orchard B. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index; EP SI: epiphytic isolates from spring; EN SI: endophytic isolates from spring. EP AI: epiphytic isolates from autumn; EN AI: endophytic isolates from autumn.

Orchard B	Alpha diversity index			
	Dmg	**H'**	**J'**	**1-D**
EP SI	3.0	1.5	0.9	0.8
EN SI	2.6	1.4	0.9	0.7
EP AI	3.5	1.2	0.8	0.6
EN AI	1.4	0.4	0.4	0.2

Overall, a higher specific richness was found in Psa EP AI (Dmg index – Table 3.2.2-VIII). Psa strains were more evenly distributed in EP and EN SI (H' index – Table 3.2.2-VIII) which was in accordance with the obtained evenness values (J' index – Table 3.2.2-VIII). The Psa populations isolated in autumn were quite different. The EN isolates were characterized by the presence of a dominate Psa profile has evidenced by the Simpson diversity index or dominance index (1-D). On the other hand, the diversity of EP isolates was more similar to EP and EN from SI, according to H', J' and 1-D index (Table 3.2.2-VIII).

The data collected from the alfa diversity index analysis confirmed our previous evidences that a changed occurred in Psa populations between spring and autumns evidenced by a slight decrease in the diversity of Psa profiles (see Figure 3-11) and in the number of Psa isolates. The predominance of Psa profile 5 among EP and ED in both seasons influenced the alfa diversity index values. Most profiles were found in both seasons although an alteration on their relative abundance was observed between seasons. Those results may be related with changes in the ability of some populations to thrive in during summer.

3.2.2.4 PCA analysis

An inter-species correlation analyses (PCA) of all plant isolates from orchard B is shown in Figure 3-14. In this analysis, it was possible to correlate the weight of a Psa profile with the event, which in this case represent the different conditions: epiphytic or endophytic isolates in both spring and autumn. The PCA analyses allowed inferring which Psa profiles differ or cluster samples.

None of the conditions were clustered together. However, AI were closer from each other than did the SI. This proximity was mainly due to profile 5, present in more abundance in AI than in SI. The existence of unique profiles, in both EP and EN AI provided the remoteness of those isolates. Observing SI, the distance between EP and EN was larger than the distance observed for AI, confirming the higher diversity found in SI. The EP and EN SI were separated in the PCA analyses namely by profile 7 and 33 (EN SI) and profiles 9, 13 and 36 (EP SI).

Our results confirmed that EP isolates and EN isolates represented distinct Psa populations both in spring and in autumn, pointing not only for the co-existence of several populations at the same time in the same plant, but mostly interesting for the existence of two distinct niches in the same plant that respond distinctly to the abiotic conditions that differ between spring and autumn.

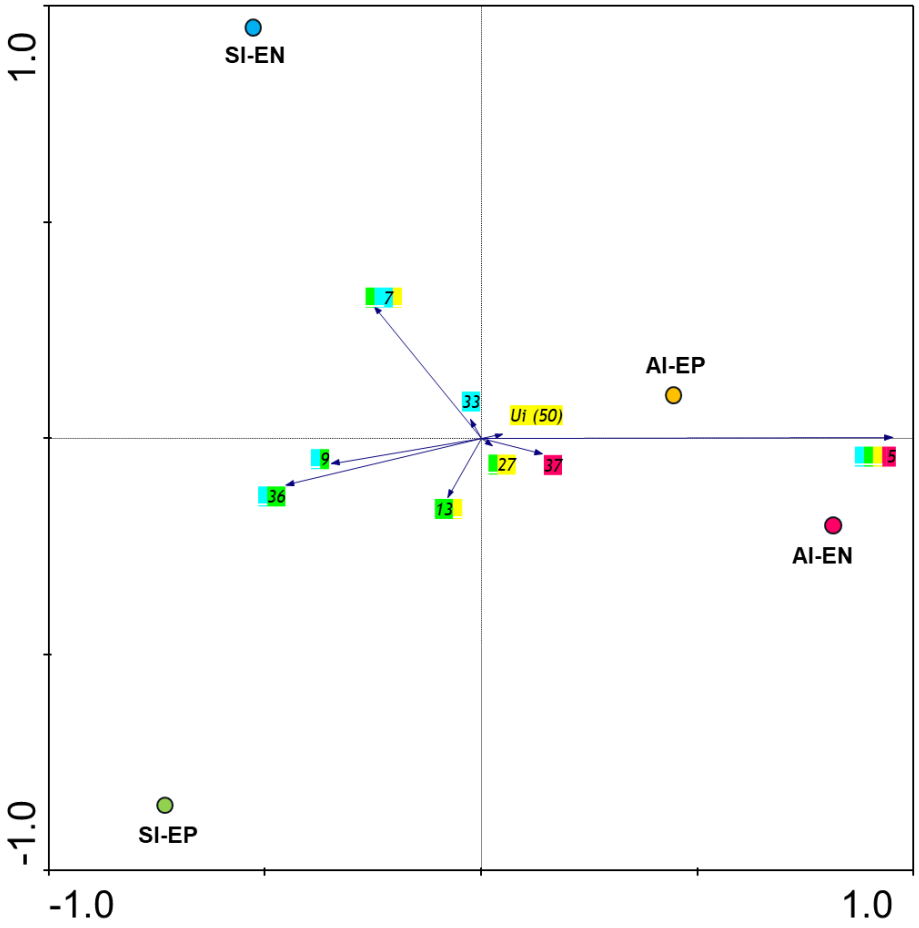


Figure 3-14. Principal component analysis – inter-species correlate – of Psa profiles from orchard B. Green SI-EP: epiphytic isolates, spring; Blue SI-EN: endophytic isolates, spring; Yellow AI-EP: epiphytic isolates, autumn; Pink AI-EN: endophytic isolates, autumn. Numbers correspond to Psa profiles. Colours identify Psa profiles that have more weight in each condition.

3.2.3 Orchard C

3.2.3.1 Putative *Pseudomonas* spp. isolation and Psa identification

A total of 254 strains were obtained from plants, soil and water samples collected in orchard C (Table 3.2.3-I); 198 were SI and 56 were AI.

In order to confirm the identity off the isolates as Psa strains a duplex-PCR protocol described by Gallelli *et al*. (2011) was performed using the extracted DNA´s (see section 3.2.1.).

Table 3.2.3-I. Total isolates recovered from orchard C in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	SPRING	
	PLANT	LOCALIZATION
KW876; KW893; KW994; KW996; KW1177; KW1178; KW1179; KW1180; KW1191; KW1192; KW1193; KW1194; KW1195; KW1196; KW1200; KW1201; KW1202; KW1209; KW1259; KW1283; KW1284; KW1285	P1	EP
KW601; KW602; KW688; KW689; KW690; KW691; KW692; KW693; KW991; KW992; KW993; KW1223; KW1224; KW1258	P1	EN
KW431; KW432; KW433; KW434; KW435; KW436; KW437; KW438; KW439; KW440; KW441; KW442; KW443; KW444; KW445; KW446; KW447; KW448; KW449; KW450; KW451; KW452; KW453; KW454; KW455; KW456; KW457; KW458; KW459; KW468; KW471; KW570; KW610; KW611; KW995; KW997; KW1226; KW1227; KW1228; KW1289; KW1290; KW1291; KW1292; KW1315; KW1316; KW1317; KW1318	P2	EP
KW462; KW463; KW464; KW465; KW466; KW467; KW480; KW481; KW482; KW483; KW484; KW485; KW486; KW487; KW488; KW489; KW490; KW491; KW492; KW493; KW494; KW495; KW496; KW497; KW498; KW612; KW613; KW614; KW615; KW1229; KW1230; KW1231; KW1286	P2	EN
KW571; KW1038; KW1039; KW1040; KW1041; KW1042; KW1043; KW1044; KW1045; KW1111; KW1243; KW1244; KW1246; KW1247; KW1248; KW1249; KW1297; KW1323	P3	EP
KW460; KW461; KW499; KW569; KW604; KW642; KW833; KW834; KW835; KW836; KW837; KW838; KW839; KW922; KW924; KW925; KW926; KW947; KW948; KW949; KW950; KW951; KW952; KW953; KW955; KW956; KW957; KW978; KW1084; KW1085; KW1086; KW1089; KW1090; KW1112; KW1113; KW1114; KW1203; KW1298; KW1299; KW1300; KW1322	P3	EN
ISOLATE REFERENCE	**SPRING**	
	RESERVOIR	
KW567; KW724; KW725; KW726; KW944; KW945; KW1077; KW1220; KW1255; KW1288; KW1312; KW1313; KW1314	SO	
KW568; KW605; KW606; KW607; KW674; KW675; KW676; KW677; KW678; KW875	WA	
ISOLATE REFERENCE	**AUTUMN**	
	PLANT	**LOCALIZATION**
KW1733; KW1735; KW1736; KW1857; KW1873; KW1875; KW1876; KW1877; KW1878; KW1879; KW1881; KW1882; KW1883; KW2107; KW2108	P1	EP
KW1900; KW1966	P1	EN
KW2016; KW2017; KW2018; KW2019; KW2020; KW2021; KW2051	P2	EP
KW1901	P2	EN
KW1902; KW1903; KW1904; KW1965; KW2004; KW2005; KW2006; KW2007; KW2024; KW2050	P3	EP

Table 3.2.3-I (continuation). Total isolates recovered from orchard C in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	AUTUMN	
	PLANT	LOCALIZATION
KW1908; KW1909; KW1921; KW1922; KW1924; KW1925; KW1950; KW1951; KW1952; KW1953; KW1954; KW1955; KW1957;	P3	EN
ISOLATE REFERENCE	**AUTUMN**	
	RESERVOIR	
KW1847; KW1848; KW1923; KW2003; KW2025; KW2026; KW2029; KW2105	SO	

The number of total isolates and Psa confirmed strains from the three representative plants, soil and water samples analysed in orchard C is presented in the Table 3.2.3-II. From a total of 254 isolates, 14 (5.5%) were confirmed as Psa, 9 were SI and 5 were AI (Table 3.2.3-II). This was the first detection of Psa in this orchard.

The percentage of Psa isolates from plants sampled ranged from 4.9% to 5.7%. Differences in persistence of Psa isolates in plants were observed between seasons, namely a reducing in numbers in Psa AI. On average, 4.6% and 8.2% of the total SI and AI were Psa, respectively. From the plant samples, 223 (86.1%) isolates were recovered, 12 were confirmed as Psa, corresponding to 5.4% of total plant isolates. In plant 1, from a total of 53 isolates, only 3 (5.6%) AI were confirmed as Psa. Not a single Psa isolate was recovered from SI. In plant 2 and 3 a total of 88 and 82 strains were recovered, of which 5 (5.7%) and 4 (4.9%) were identified as Psa, respectively. All the Psa isolates identified in plant 2 were SI. In plant 3, 3 (75%) of the Psa strains were SI and 1 (25%) was an AI.

A total of 21 (8.3%) and 10 (3.9%) strains were isolated from soil and water samples, respectively (Table 3.2.3-II). Of these, only one of each was confirmed as Psa. Not a single Psa isolate was recovered from soil in spring or from water in autumn.

Table 3.2.3-II. Total isolates and strains identified as Psa from plants, soil and water samples in each season from orchard C. SI: spring isolate; AI: autumn isolate.

Plant/ Reservoir	SI	AI	Total	Psa SI	Psa AI	Total Psa
Plant 1	36	17	53	0	3	3
Plant 2	80	8	88	5	0	5
Plant 3	59	23	82	3	1	4
Soil	13	8	22	0	1	1
Water	10	0	10	1	0	1
Total	198	56	254	9	5	14

As abovementioned, the three representative plants from orchard C were sampled in spring and autumn, and each sample was processed in order to separately recover epiphytic and endophytic bacterial strains.

In total, 87 (49.7% of total SI) and 32 (66.7% of total AI) EP strains were isolated in spring and in autumn, respectively; of which, 7 (8.0%) were confirmed as Psa in spring and 2 (6.3%) in autumn. On the other hand, 88 (50.3% of total SI) and 16 (33.3% of total AI) EN strains were isolated in spring and in autumn, respectively; of which only 1 (1.1%) was confirmed as Psa in spring and 2 (12.5%) in autumn (Table 3.2.3-III).

Table 3.2.3-III. Total isolates and strains identified as Psa from plants in each season from orchard C. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

Plant	SI		Total SI	Psa SI		Total Psa SI	AI		Total AI	Psa AI		Total Psa AI
	EP	EN		EP	EN		EP	EN		EP	EN	
1	22	14	36	0	0	0	15	2	17	1	2	3
2	47	33	80	4	1	5	7	1	8	0	0	0
3	18	41	59	3	0	3	10	13	23	1	0	1
Total	87	88	175	7	1	8	32	16	48	2	2	4

From a total of 53 isolates from plant 1, 36 (67.9%) were SI, of which 22 (61.1%) were EP SI and 14 (38.9%) were EN SI; while 17 (32.1%) were AI, of which 15 (88.2%) were EP AI and 2 (11.8%) were EN AI (Table 3.2.3-III). From these, not a single SI was confirmed as Psa. Additionally, only 1 (6.7%) and 2 (100%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total of 88 isolates from plant 2, 80 (90.9%) were SI, of which 47 (53.4%) were EP SI and 33 (37.5%) were EN SI; while 8 (9.1%) were AI, of which 7 (87.5%) were EP AI and 1 (12.5%) was EN AI (Table 3.2.3-III). From these, 4 (8.5%) and 1 (3.0%) were confirmed as Psa in EP SI and in EN SI, respectively. Not a single AI was confirmed as Psa.

From a total of 82 isolates from plant 3, 59 (67%) were SI, of which 18 (30.5%) were EP SI and 41 (69.5%) were EN SI; while 23 (28%) were AI, of which 10 (43.5%) were EP AI and 13 were EN AI (56.5%) (Table 3.2.3-III). From these, 3 (16.7%) were confirmed as Psa in EP SI. In autumn, only 1 (10%) EP isolate was confirmed as Psa. Not a single Psa isolate was recovered from both EN SI and AI.

No conclusions could be taken with relation to Psa persistence in orchard C given the low numbers of recovered isolates identified as Psa, 5.5% of total isolates (Figure 3-15). Indeed, the first report of Psa in orchard C was made in this study. Nevertheless, the numbers of Psa isolates in spring were higher

among EP strains than in EN strains (Table 3.2.3-III). Comparing Psa isolates in autumn, no obvious relation was observed between EP and EN numbers.

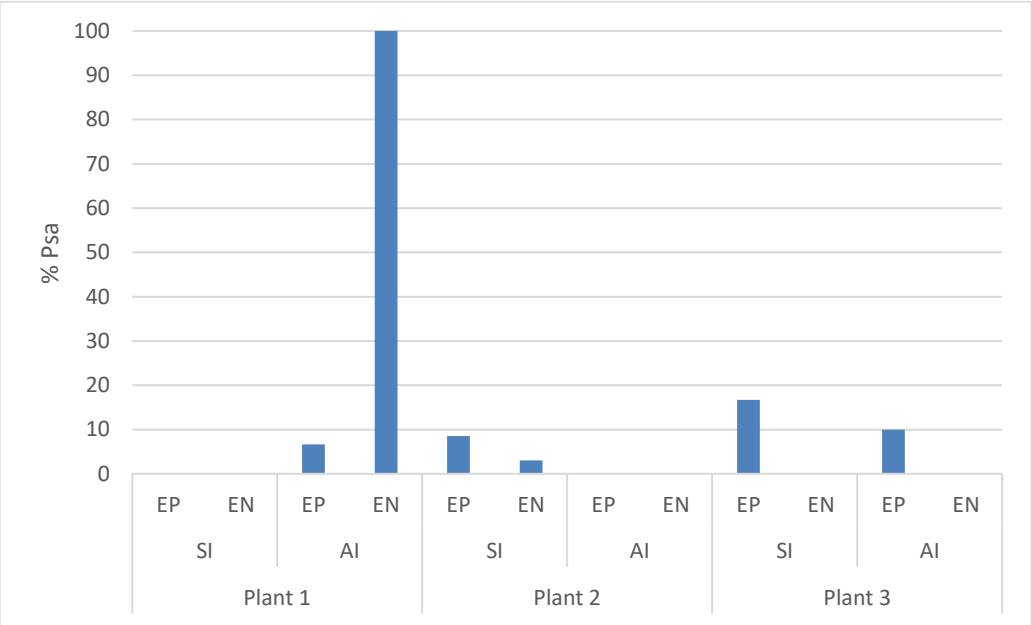


Figure 3-15. Percentage (%) of isolates identified as Psa in each condition from each season from each plant of orchard C. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

3.2.3.2 Characterization of Psa populations in orchard C

The fingerprinting analysis of the Psa isolates was performed by BOX-PCR, as previously described (Louws *et al*., 1994). From the 14 Psa isolates obtain from orchard C, only 9 isolates generated a suitable BOX profile despite several attempts (Table 3.2.3-IV).

Table 3.2.3-IV. Psa isolates and correspondent BOX profile from orchard C. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX
	PLANT	LOCALIZATION	PROFILE
KW434	P2	EP	-
KW436	P2	EP	11
KW435	P2	EP	34
KW432	P2	EP	Ui (2.1)
KW492	P2	EN	Ui (7)
KW571	P3	EP	-
KW1039	P3	EP	Ui (3)
KW1111	P3	EP	Ui (4)

Table 3.2.3-V (continuation). Psa isolates and correspondent BOX profile from orchard C. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX
	RESERVOIR		PROFILE
KW674	WA		-
ISOLATE REFERENCE	**AUTUMN**		**BOX**
	PLANT	**LOCALIZATION**	**PROFILE**
KW1733	P1	EP	34
KW1900; KW1966	P1	EN	34
KW1965	P3	EP	-
ISOLATE REFERENCE	**AUTUMN**		**BOX**
	RESERVOIR		**PROFILE**
KW1923	SO		-

Figure 3-16 shows the distribution of Psa profiles identified according to the isolation site and season. Psa populations were remarkably distinct between spring and autumn. In spring, a total of 6 Psa profiles were observed while in the autumn only 1 profile was detected. Differences in profile diversity between EP were also observed in both seasons. Namely, EP SI were split in 5 Psa profiles compared to only 1 profile detected in EP AI (Figure 3-16). However, the total number of EP SI was higher than the total number of EP AI (Table 3.2.3-III). So, this difference in diversity among EP Psa populations may not be that significant. Psa profiles detected in EN isolates were different between seasons, namely profiles Ui (7) and 34 recovered from SI and AI, respectively. In AI, a predominance of Psa profile 34 among EP and EN was observed (Figure 3-16).

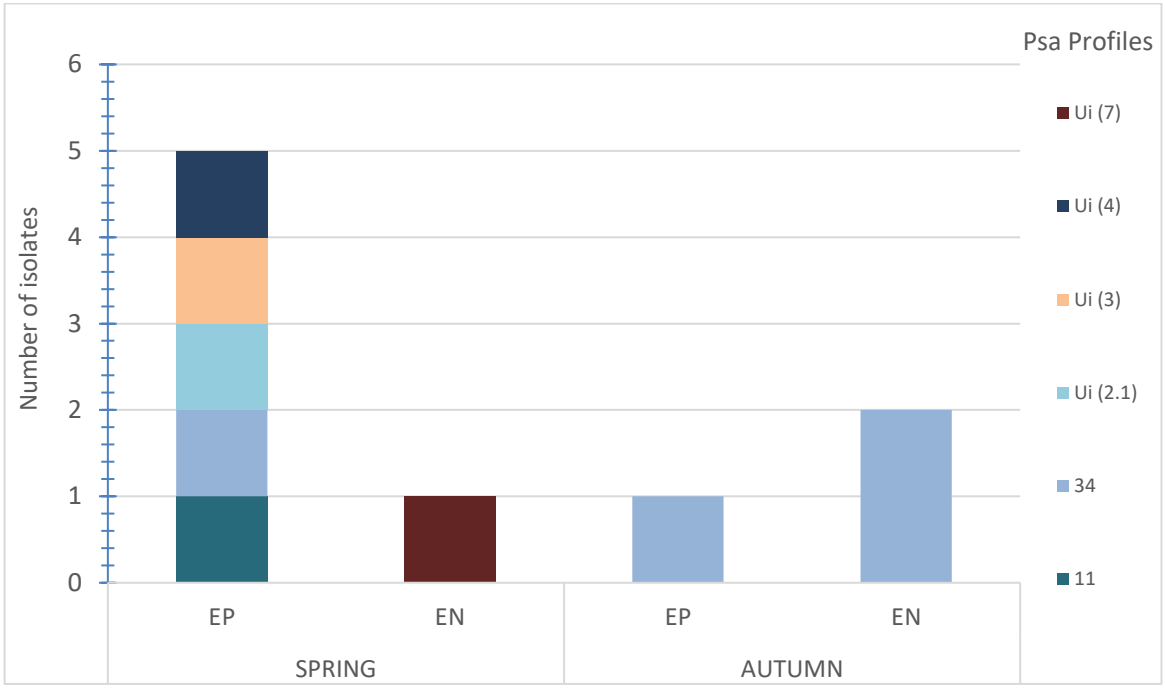


Figure 3-16. Distribution of Psa profiles in plant isolates from orchard C in spring and autumn. EP: epiphytic isolate; EN: endophytic isolate.

In more detail, it was possible to infer a higher diversity in Psa profiles in EP SI, namely profiles Ui (2.1), Ui (3), Ui (4), 11 and 34. The profile 34 was common between seasons since it was recovered from EP SI and both EN and EP AI. The only profile recovered from EN SI was profile Ui (7) corresponding to a single Psa strain (Figure 3-16).

Our results suggested that there was a higher diversity among EP Psa populations in spring. All Psa AI corresponded to profile 34, which was also common to EP SI. However, it was not possible to relate the data from Psa isolates recovered in different sites and seasons given the low numbers of Psa isolates recovered from orchard C. Moreover, this was the first report of Psa detection in this orchard, so the limited presence of EN Psa in EN may be justified by the early colonization of the plants by Psa. In addition, it has been reported that older plants exhibited higher resistance to Psa infection (Vanneste *et al.*, 2011b). Since plants from orchard C have more than 17 years this may have difficult the infection and dissemination of Psa. Considering EP isolates, the reduction on Psa EP AI numbers could be related with abiotic summer conditions, like higher temperatures and less humidity, suggesting that the Psa populations recovered in spring were not able to cope with these events. Only Psa strains from profile 34 were recovered in both seasons indicating that they could be more resilience or better adapted to overcome such conditions.

3.2.3.2.1 Characterization of Psa populations present in each plant

The distribution of Psa profiles among the three sampling plants is depicted in Figure 3-17. It´s clear that Psa diversity was quite distinct between plants and between seasons, even considering the low number of Psa isolates analysed. In more detail, profiles Ui (7), Ui (3) and Ui (4) were only observed in plant 2 and 3, respectively. The first one was recovered from the only EN AI, while profile Ui (3) and Ui (4) were exclusive from EP SI.

In plant 1, not a single Psa SI profile was obtained with BOX-PCR. However, the AI were all characterized as Psa profile 34.

Plant 2 showed a higher diversity of EP Psa profiles, with 2 exclusive Psa profiles and 1 in common with plant 1; namely profile 11, Ui (2.1) and 34, respectively. Only a single Psa isolate were recovered from EN SI, which correspond to profile Ui (7). Not a single Psa isolate were recovered in autumn.

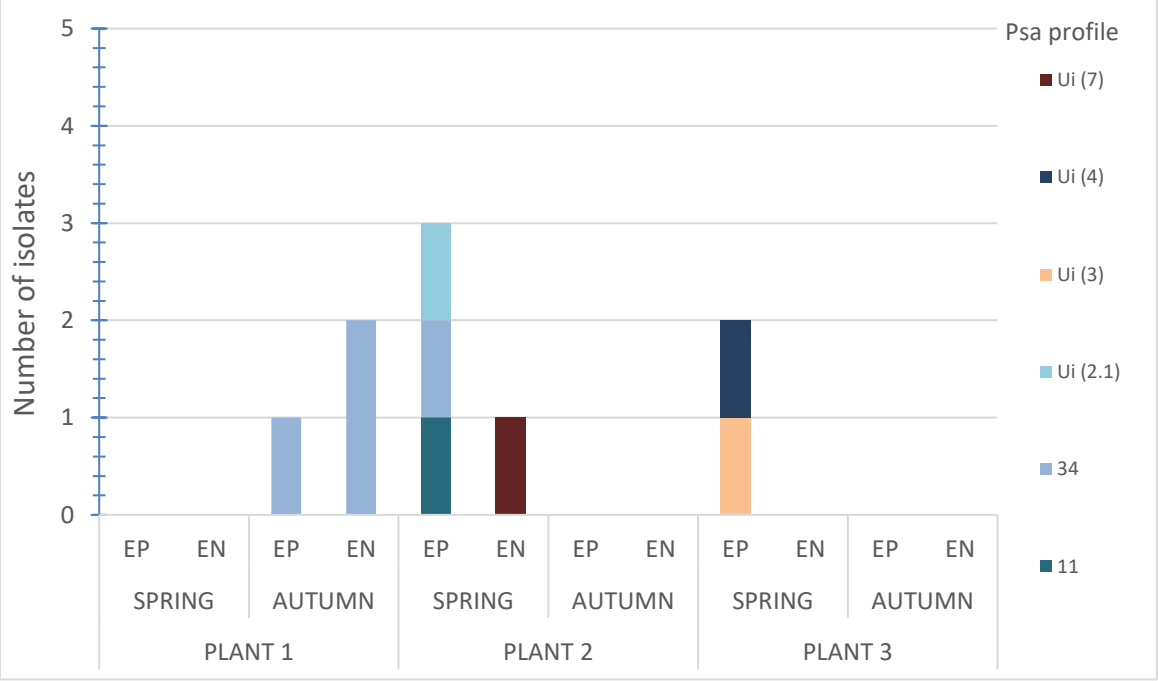


Figure 3-17. Distribution of Psa profiles found in the three representative plants from orchard C in spring and autumn seasons. EP: epiphytic isolate; EN: endophytic isolate.

Observing plant 3, the BOX – PCR analyse was only successful for EP SI. Considering EP SI, two profiles were obtained. Namely, profile Ui (3) and Ui (4), exclusive for plant 3 as abovementioned.

Only profile 34 was common between plant 1 and 2, being the dominant profile in AI. This profile was obtained from plant 1 AI and from EP SI. In addition, this profile was common between EP and EN in

plant 1 which suggests that this Psa population may cause infection both epiphytically and endophytically in the same plant.

Considering the number of Psa isolates analysed, a higher diversity may be observed: 6 Psa profiles in 9 isolates. This diversity was more significant in EP from SI. Moreover, despite the low Psa detection, this result suggests that the structure of Psa population varies over time in the same plant. Even more interesting this early detected infection was due to a heterogeneous Psa population, and not by a single clone.

3.2.3.2.2 Soil and water has potential Psa environmental reservoirs

The fingerprinting analysis of the Psa isolates was performed by BOX-PCR, as abovementioned. Despite several attempts, none of soil or water isolates display a suitable BOX-PCR profile. Therefore, it was not possible to confirm soil and water as potential environmental reservoirs in orchard C.

3.2.3.3 Alfa diversity

Considering the low numbers of Psa isolates and their correspondent Psa profiles, the alfa diversity analysis was not performed.

3.2.3.4 Principal component analyses (PCA)

An inter-species correlation analyses (PCA) of all the plants isolates from orchard C is shown in Figure 3-18. In this analysis, it is possible to correlate the weight of a Psa profile with the event, which in this case represent the different conditions: epiphytic isolate or endophytic isolate in both spring and autumn seasons. The PCA analyses allowed inferring which Psa profiles differ or clusters populations.

Autumn isolates (AI) were clustered together and separated from SI. This configuration was mainly due to profile 34 dominant in AI. On the contrary, EP and EN SI were separated in the PCA analyses, mainly by profile Ui (2.1), Ui (3), Ui(4) and 11 (EP SI) and profile Ui (7) (EN SI). Assessing the spatial distances between samples, it was possible to infer that EN SI were closer to AI than to EP SI. This proximity could be due to profile 34, which was common to each other (Figure 3-16). In sum, PCA analysis inferred that Psa isolates were distinct between seasons, which correlates with the fingerprinting results (Figure 3-16). Moreover, in Psa SI, EP and EN were also distinct. In addition, this analysis reinforced our previous results that there was a co-existence of distinct Psa populations, especially in EP SI.

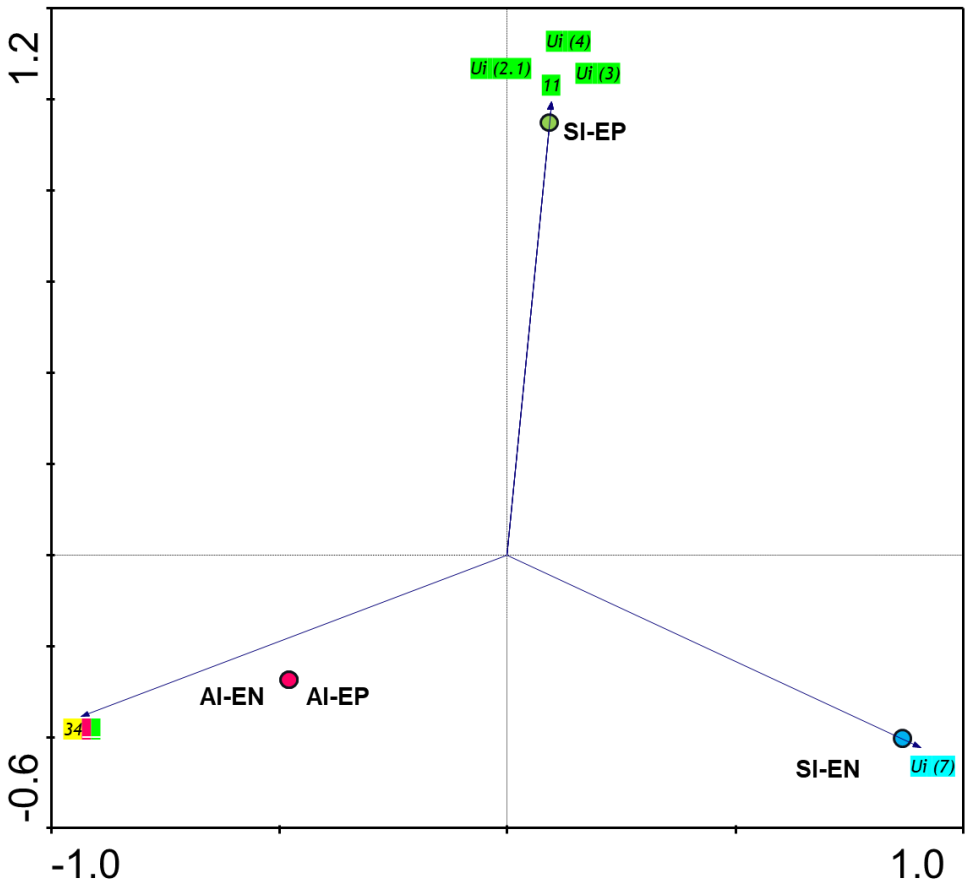


Figure 3-18. Principal component analysis – inter-species correlate – of Psa profiles from orchard C. Green SI-EP: epiphytic isolates, spring; Blue SI-EN: endophytic isolates, spring; Yellow AI-EP: epiphytic isolates, autumn; Pink AI-EN: endophytic isolates, autumn. Numbers correspond to Psa profiles. Colours identify Psa profiles that have more weight in each condition.

3.2.4 Orchard D

3.2.4.1 Putative *Pseudomonas spp*. isolation and Psa identification

A total of 352 isolates were obtained from plants, soil and water samples collected in orchard D (Table 3.2.4-I); 181 were SI and 171 were AI. In order to confirm the identity of the isolates as Psa strains a duplex-PCR protocol described by Gallelli *et al*. (2011) was performed using the extracted DNAs (see **3.2.1.**).

Table 3.2.4-I. Total isolates recovered from orchard D in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	SPRING	
	PLANT	LOCALIZATION
KW522; KW523; KW524; KW525; KW526; KW527; KW528; KW529; KW530; KW531; KW532; KW533; KW534; KW535; KW579; KW940	P1	EP
KW381; KW382; KW383; KW384; KW385; KW386; KW387; KW388; KW389; KW390; KW391; KW469; KW470; KW472; KW473; KW608; KW609; KW863; KW902; KW914; KW937; KW1239; KW1310	P1	EN
KW539; KW540; KW541; KW542; KW543; KW544; KW545; KW546; KW547; KW548; KW549; KW550; KW864; KW870; KW887; KW909; KW910; KW911; KW912; KW913; KW928	P2	EP
KW392; KW393; KW394; KW395; KW396; KW397; KW398; KW399; KW400; KW401; KW402; KW403; KW404; KW405; KW406; KW407; KW408; KW409; KW410; KW474; KW475; KW476; KW477; KW536; KW537; KW538	P2	EN
KW551; KW552; KW553; KW554; KW558; KW599; KW600; KW867; KW868; KW869; KW903; KW904; KW905; KW906; KW907; KW908	P3	EP
KW411; KW412; KW413; KW414; KW415; KW416; KW417; KW418; KW419; KW420; KW421; KW422; KW423; KW424; KW425; KW426; KW427; KW428; KW429; KW430; KW478; KW518; KW866; KW871; KW872; KW873	P3	EN
ISOLATE REFERENCE	**SPRING**	
	RESERVOIR	
KW559; KW560; KW561; KW603; KW707; KW711; KW712; KW716; KW718; KW719; KW720; KW721; KW722; KW723; KW865; KW877; KW891; KW942; KW943; KW960; KW961; KW962; KW979; KW980; KW981; KW982; KW983; KW984; KW985; KW990; KW1072; KW1073; KW1074; KW1075; KW1076; KW1087; KW1088; KW1208; KW1212; KW1213; KW1221; KW1222; KW1238; KW1257; KW1258; KW1301; KW1311	SO	
KW593; KW594; KW595; KW596; KW597; KW598	WA	
ISOLATE REFERENCE	**AUTUMN**	
	PLANT	**LOCALIZATION**
KW1770; KW1771; KW1772; KW1774; KW1775; KW1830; KW1930; KW1938; KW1939; KW1964; KW1972; KW1979; KW1980; KW1981; KW1982; KW1993; KW2052; KW2070; KW2098; KW2114	P1	EP
KW1739; KW1740; KW1741; KW1742; KW1743; KW1744; KW1745; KW1746; KW1747; KW1748; KW1749; KW1750; KW1776; KW1780; KW1781; KW1782; KW1783; KW2053; KW2054; KW2055; KW2056; KW2057; KW2058; KW2075	P1	EN
KW1753; KW1754; KW1755; KW1756; KW1757; KW1763; KW1764; KW1765; KW1766; KW1767; KW1768; KW1769; KW1773; KW1831; KW1832; KW1833; KW1977; KW1978; KW2043; KW2044; KW2111	P2	EP

Table 3.2.4 I (continuation). Total isolates recovered from orchard D in each plant/reservoir, in spring and autumn. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	AUTUMN	
	PLANT	LOCALIZATION
KW1758; KW1759; KW1760; KW1761; KW1762; KW1791; KW1792; KW1793; KW1794; KW1795; KW1802; KW1803; KW1804; KW1805; KW1812; KW1813; KW1858; KW1859; KW1860; KW1863; KW1864; KW2012; KW2040; KW2041; KW2042; KW2079; KW209; KW2092; KW2093; KW2094; KW2095; KW2095	P2	EN
KW1836; KW2001; KW2015; KW2031; KW2032; KW2034; KW2035; KW2099; KW2110; KW2112; KW2121	P3	EP
KW1777; KW1778; KW1779; KW1814; KW1815; KW1816; KW1817; KW1818; KW1819; KW1820; KW1821; KW1822; KW1823; KW1824; KW1825; KW1826; KW1827; KW1828; KW1829; KW2013; KW2071; KW2076; KW2096	P3	EN
ISOLATE REFERENCE	**AUTUMN**	
	RESERVOIR	
KW1834; KW1835; KW1843; KW1844; KW1865; KW1866; KW1867; KW1868; KW1869; KW1870; KW1880; KW1907; KW1919; KW1928; KW1931; KW2036; KW2037; KW2045; KW2046; KW2047; KW2048; KW2049; KW2100; KW2115; KW2116; KW2117; KW2118	SO	
KW1849; KW1850; KW1851; KW1852	WA	

The number of total isolates and Psa confirmed strains from the three representative plants, soil and water samples analysed in orchard D is presented in the Table 3.2.4-II. From a total of 347 isolates, 195 (56.3%) were confirmed as Psa, 100 were SI and 95 were AI (Table 3.2.4-II).

The percentage of Psa isolates from plants sampled ranged from 63.4% to 82.9%. No major differences were observed between seasons regarding the persistence of Psa isolates in plant. On average, 55.3% and 57.2% of the total SI and AI were Psa, respectively. From the plant samples, 263 (75.8%) isolates were recovered, 191 were confirmed as Psa, corresponding to 72.6% of total plant isolates. In plant 1, from a total of 82 isolates, 52 (63.4%) were confirmed as Psa, of which 27 (51.9%) SI while 25 (48.1%) were AI. In plant 2 and 3 a total of 105 and 76 strains were recovered, of which 87 (82.9%) and 52 (68.4%) were identified as Psa, respectively. In plant 2, 41 (47.1%) of the Psa strains were SI and 46 (52.9%) were AI; while in plant 3, 29 (55.8%) of the Psa strains were SI and 23 (44.2%) were AI.

A total of 74 (21%) strains were isolated from soil samples (Table 3.2.4-II), but only 4 were Psa positive, corresponding to 5.4% of total soil isolates. From those, 3 Psa isolates were SI while only 1 was AI. Finally, a total of 10 (2.8%) isolates (Table 3.2.4-II) were recovered from water samples. Not a single Psa isolate was recovered from water in both seasons.

Table 3.2.4-II. Total isolates and strains identified as Psa from plants, soil and water samples in each season from orchard D. SI: spring isolate; AI: autumn isolate.

Plant/ Reservoir	SI	AI	Total	Psa SI	Psa AI	Total Psa
Plant 1	39	43	82	27	25	52
Plant 2	47	58	105	41	46	87
Plant 3	42	34	76	29	23	52
Soil	47	27	74	3	1	4
Water	6	4	10	0	0	0
Total	181	166	347	100	95	195

As abovementioned, the three representative plants from orchard D were sampled in spring and autumn, and each sample was processed in order to separately recover epiphytic and endophytic bacterial strains.

In total, 52 (40.6% of total SI) and 55 (40.7% of total AI) EP strains were isolated in spring and in autumn, respectively; of which, 31 (59.6%) were confirmed as Psa in spring and 28 (50.9%) in autumn. On the other hand, 76 (59.4% of total SI) and 80 (59.3% of total AI) EN strains were isolated in spring and in autumn, respectively; of which 66 (86.8%) were confirmed as Psa in spring and 66 (82.5%) in autumn (Table 3.2.4-III)

Table 3.2.4-III. Total isolates and strains identified as Psa from plants in each season from orchard D. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

	SI		Total SI	Psa SI		Total Psa SI	AI		Total AI	Psa AI		Total Psa AI
Plant	EP	EN		EP	EN		EP	EN		EP	EN	
1	16	23	39	9	18	27	19	24	43	7	18	25
2	21	26	47	15	26	41	25	33	58	21	25	46
3	15	27	42	7	22	29	11	23	34	0	23	23
Total	52	76	128	31	66	97	55	80	135	28	66	94

From a total of 82 isolates from plant 1, 39 (47.6%) were SI, of which 16 (41%) were EP SI and 23 (59%) were EN SI; while 43 (52.4%) were AI, of which 19 (44.2%) were EP AI and 24 (55.8%) were EN AI (Table 3.2.4-III). From these, 9 (56.3%) and 18 (78.3%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 7 (36.8%) and 18 (75%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total of 105 isolates from plant 2, 47 (44.8%) were SI, of which 21 (44.7%) were EP SI and 26 (55.3%) were EN SI; while 58 (55.2%) were AI, of which 25 (43.1%) were EP AI and 33 (56.9%) were EN

AI (Table 3.2.4-III). From these, 15 (71.4%) and 26 (100%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 21 (84%) and 25 (75.8%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total of 76 isolates from plant 3, 42 (55.3%) were SI, of which 15 (35.7%) were EP SI and 27 (64.3%) were EN SI; while 34 (44.7%) were AI, of which 11 (32.4%) were EP AI and 23 (67.6%) were EN AI (Table 3.2.4-III). From these, 7 (46.7%) and 22 (81.5%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 23 (100%) were confirmed as Psa in EN AI. Not a single Psa isolate was recovered from EP AI.

Examining the results for total Psa isolated in orchard D, in general the numbers of EN Psa were superior to EP Psa. These differences were observed in both seasons. Moreover, sampling results were similar between plants. In addition, comparing the results between the three plants it was possible to observe that the percentage of Psa EP and Psa EN isolates was superior to 36.8% and 75%, respectively, in all plants (Figure 3-24). The low isolation of EP Psa comparing to EN Psa maybe related with edaphoclimatic conditions associated to cultural practices that may difficult growth or persistence of epiphytic populations in plants. This was a young orchard, highly susceptible to Psa in which the disease symptoms were widely distributed and severe accounting for the high percentage of endophytic Psa strains corresponding to a systemic infection.

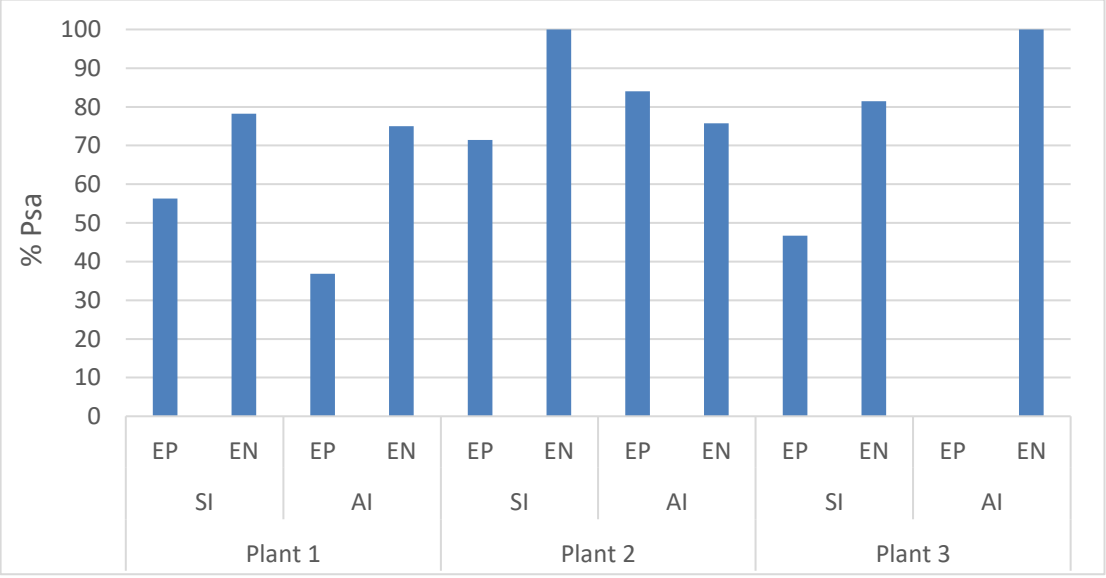


Figure 3-19. Percentage (%) of isolates identified as Psa in each condition from each season from each plant of orchard D. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

3.2.4.2 Characterization of Psa populations in orchard D

The fingerprinting analysis of the Psa isolates was performed by BOX-PCR, as previously described (Louws *et al*., 1994). From the 195 Psa isolates obtain from orchard D, only 182 isolates generated a suitable BOX profile despite several attempts (Table 3.2.4-IV).

Table 3.2.4-IV. Psa isolates and correspondent BOX profile from orchard D. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX
	PLANT	LOCALIZATION	PROFILE
KW528; KW529; KW530	P1	EP	-
KW522; KW524; KW525; KW526; KW527; KW533	P1	EP	5
KW609	P1	EN	-
KW391; KW382; KW383; KW384; KW385; KW386; KW387; KW388; KW389; KW390; KW391; KW472; KW863; KW902; KW914; KW1239	P1	EN	5
KW1239; KW1310	P1	EN	13
KW539; KW540; KW542; KW544; KW545; KW546; KW547; KW548; KW549; KW550; KW870	P2	EP	5
KW543; KW864; KW887	P2	EP	13
KW541	P2	EP	Ui (62)
KW392; KW394; KW395; KW396; KW397; KW398; KW399; KW400; KW401; KW402; KW403; KW404; KW405; KW406; KW407; KW408; KW409	P2	EN	5
KW536; KW537; KW538	P2	EN	6
KW593; KW410	P2	EN	13
KW474; KW475; KW476; KW477	P2	EN	36
KW552	P3	EP	-
KW553; KW600; KW867; KW868; KW869; KW903	P3	EP	5
KW474; KW478	P3	EN	-
KW425; KW427; KW428; KW429; KW430	P3	EN	5
KW415; KW416; KW417; KW418; KW419; KW420; KW421; KW422; KW423; KW424; KW518; KW866; KW871; KW872; KW873	P3	EN	36

ISOLATE REFERENCE	SPRING	BOX
	RESERVOIR	PROFILE
KW1222	SO	-
KW1221	SO	38
KW559	SO	Ui (101)

Table 3.2.4-IV (continuation). Psa isolates and correspondent BOX profile from orchard D. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	AUTUMN		BOX
	PLANT	LOCALIZATION	PROFILE
KW1930; KW1938	P1	EP	-
KW1830	P1	EP	5
KW1771; KW1775; KW1993	P1	EP	7
KW1694	P1	EP	11
KW1741	P1	EN	-
KW1744; KW1745; KW1746; KW1747; KW1780; KW1781; KW1782; KW1783; KW 2055; KW2056; KW2057; KW2058; KW2075	P1	EN	5
KW1776	P2	EP	7
KW1748; KW2053; KW2054	P2	EP	21
KW1753; KW1754; KW1755; KW1756; KW1757; KW1763; KW1764; KW1765; KW1766; KW1767; KW1768; KW1769; KW1773; KW1831; KW1832; KW1833; KW2044; KW2065; KW2066; KW2067; KW2068; KW2069	P2	EP	5
KW1759; KW2042; KW2094	P2	EN	-
KW1760; KW1761; KW1803; KW1804; KW1805; KW1812; KW1813; KW1858; KW1859; KW1860; KW1863; KW1864; KW1977; KW1978; KW2012; KW2040; KW2041; KW2079; KW2092; KW2093; KW2095	P2	EN	5
KW1762	P2	EN	7
KW1777; KW1778; KW1779; KW1814; KW1815; KW1816; KW1817; KW1819; KW1820; KW1821; KW1822; KW1823; KW1825; KW1826; KW1827; KW1828; KW1829; KW2013; KW2071; KW2076; KW2096	P3	EN	5
KW1818; KW1824	P3	EN	6

ISOLATE REFERENCE	AUTUMN	BOX
	RESERVOIR	PROFILE
KW1928	SO	Ui (70)

Figure 3-20 shows the distribution of the identified Psa profiles according to the isolation site and season. Psa populations were different between spring and autumn. A total of 5 Psa profiles were observed in both spring and autumn seasons. A slightly difference in profile diversity between EP/EN were observed in both seasons. Namely, EP SI isolates were split in 4 Psa profiles compared to 3 profiles detected in EP AI. The EN SI and AI were split in 4 Psa profiles.

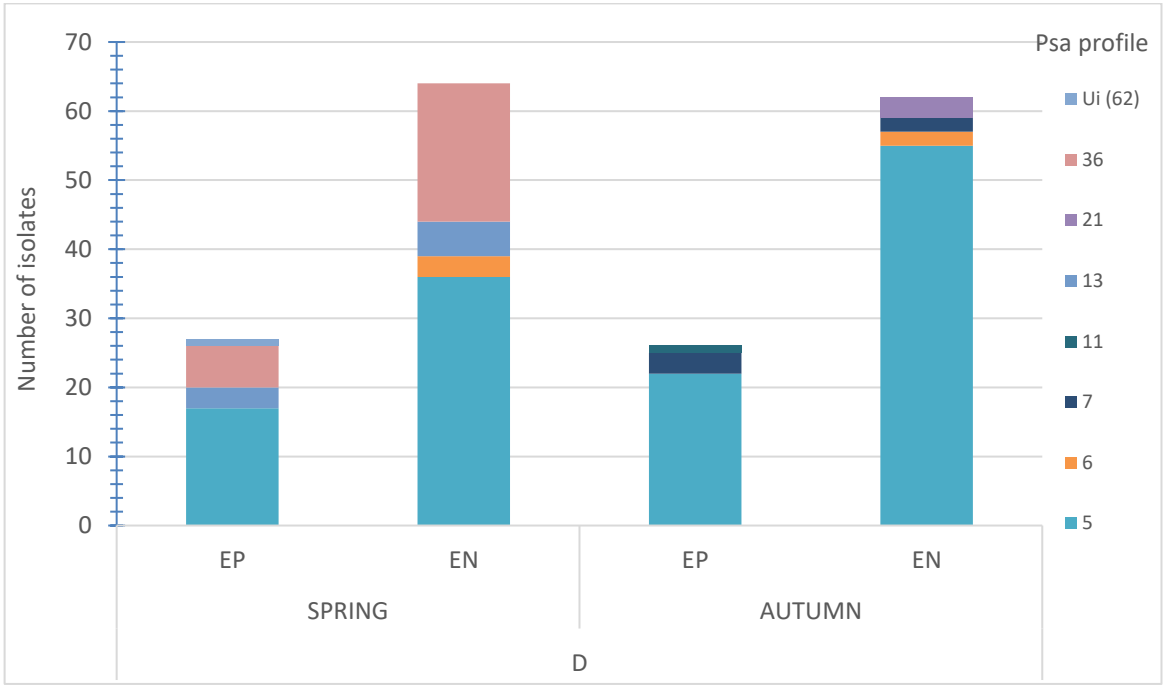


Figure 3-20. Distribution of Psa profiles in plant isolates from orchard D in spring (SI) and autumn (AI) seasons. EP: epiphytic isolate; EN: endophytic isolate.

In more detail, Psa profiles in SI (profiles 5, 6, 13, Ui (62) and 36) were different from AI (5, 6, 7, 11 and 21). Indeed, only 2 Psa profiles – 5 and 6, were common between seasons (Figure 3-20). A clear predominance of Psa profile 5 was observed among EP and EN. The profile 6 was only present in EN. Among the EP isolates from orchard D, the profiles UI (62) and 11 were only observed in SI and AI, respectively. In addition, between EN isolates, an exclusive profile – 11, was observed in AI.

In sum, a low diversity among Psa populations was observed in orchard D. This result was supported by a high number of isolates, recovered from the sampling plants in both seasons (Table 3.2.4-II). The incidence of disease in this young orchard may explain this low diversity among Psa populations since the introduction of the disease was recent (see section **2.1.1.**). Moreover, there was a clear predominance of Psa profile 5 among EP and EN between both seasons. This dominance could be related with the recent introduction of the Psa in this young orchard (Table 2.1.1-I). Psa infection in orchard occurred in the same year of its planting, reason why the plants had not yet developed any resistance to tissue colonization by Psa strains, which could lead to development of other strains. In addition, the ease with which the Psa strains infected the plant may be explained by the poor competition for the colonization of plants by other bacteria.

3.2.4.2.1 Characterization of Psa populations present in each plant

The distribution of Psa profiles of the isolates among the three sampling plants was depicted in Figure 3-21. The Psa diversity was slightly different between plants, and between seasons. In addition, profile 5 was abundantly observed in all plants in both seasons.

In plant 1, SI were all characterized as Psa profile 5. In opposition, AI presented a higher diversity, with two unique profiles - 11 and 23, found in EP and EN.

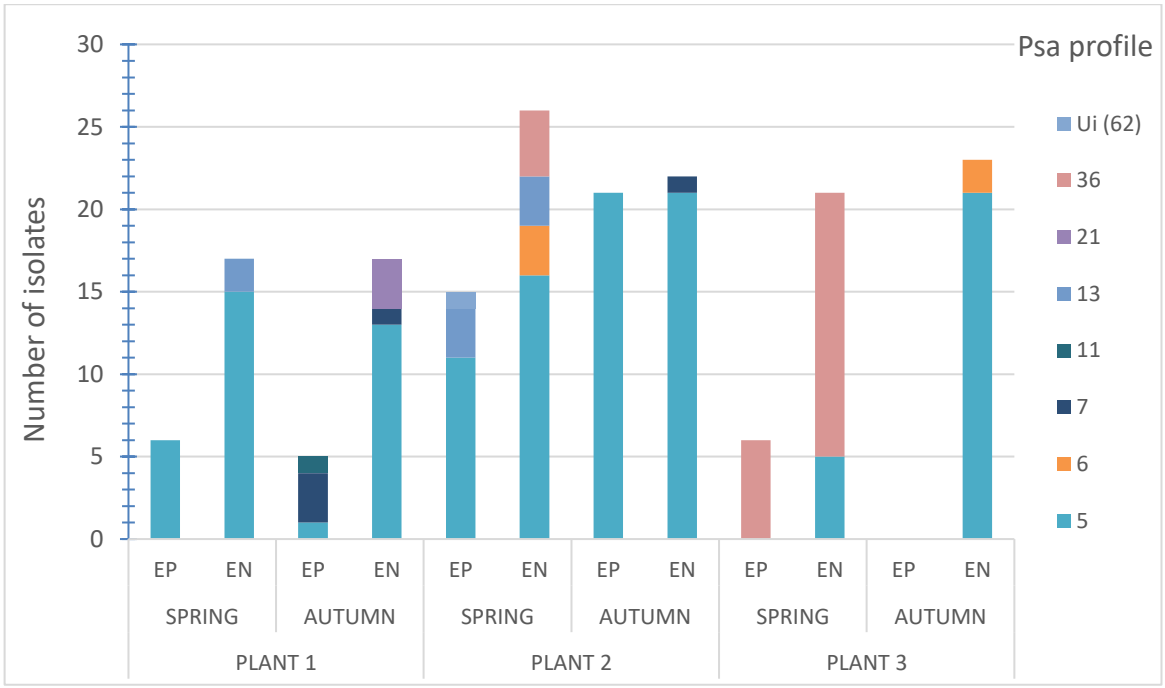


Figure 3-21. Distribution of Psa profiles identified in the three representative plants from orchard D in spring and autumn. EP: Epiphytic isolate; EN: endophytic isolate.

Plant 2 showed a higher number of Psa isolates when compared with the two other plants, split in 6 Psa profiles. Namely, profile 6 and 7 (recovered from EN in SI and AI, respectively), profile 13 (EP and EN SI), Ui (62) recovered from EP SI and profile 5 (recovered from EP and EN in both seasons). There was a higher diversity in SI than in AI. SI was split in 5 Psa profiles while AI was slip in only 2 Psa profiles.

Only 3 Psa profiles were obtained from the 50 Psa isolates analysed by BOX-PCR in plant 3. Namely, profiles 5 (recovered from EN SI and AI), 6 (isolated from EN AI) and 36 (recovered from SI). A clear predominance of Psa profiles 5 and 36 was observed in plant 3 explaining the low diversity of Psa profiles in this plant (Figure 3-21)

Two Psa profiles were shared by plants 1 and 2; Psa profile 7 was recovered from AI in plant 1 and from EN AI in plant 2; Psa profile 13 was isolated from EN SI in plant 1 and recovered from spring in plant 2. Profile 36, was common to plants 2 and 3, and recovered from EN SI and SI, respectively. Profile 6 was also common between plants 2 and 3, found in EN SI and EN AI, respectively. Considering the number of common profiles, and ignoring profile 5, plant 1 and 3 had the most distinct Psa populations in this orchard.

A slightly difference in Psa profiles diversity was observed between both seasons among the plant isolates. There were common profiles between EP and EN in each plant in both seasons. Namely, profiles 5, 13 and 36 in spring – found in plant 2 and plant 3, respectively and profiles 5 and 7 in autumn – found in plant 1. These results evidenced the co-existence of several Psa populations; some varied with time while other were persistently recovered.

Profile 5 was dominant in all the three plants between isolation site and season but in plant 3 this dominance alternates with profile 36 in AI. Other profiles were specific from SI (profiles 13, Ui (62) and 36) and AI (7, 11 and 21). These results support that the structure of Psa populations varies over time in the same plant.

3.2.4.2.2 Potential reservoirs of Psa

The total Psa diversity, inferred from BOX profiling determined in orchard D is depicted in Figure 3-22. Only 3 distinct Psa profiles were obtained from soil isolates, distributed by both seasons. Not a single profile was obtained from water isolates. Even in considerable lower numbers than in plants, soil and water isolates were identified as Psa. In fact, the only Psa profiles obtained from soil isolates were distinct from Psa profiles obtained from plants. However, this result does not exclude these environments has potential Psa reservoirs. Indeed, given the young age of the orchard and the recent infection by Psa is it possible that these environments are not yet colonized with Psa. Further studies are needed to clarify this possibility.

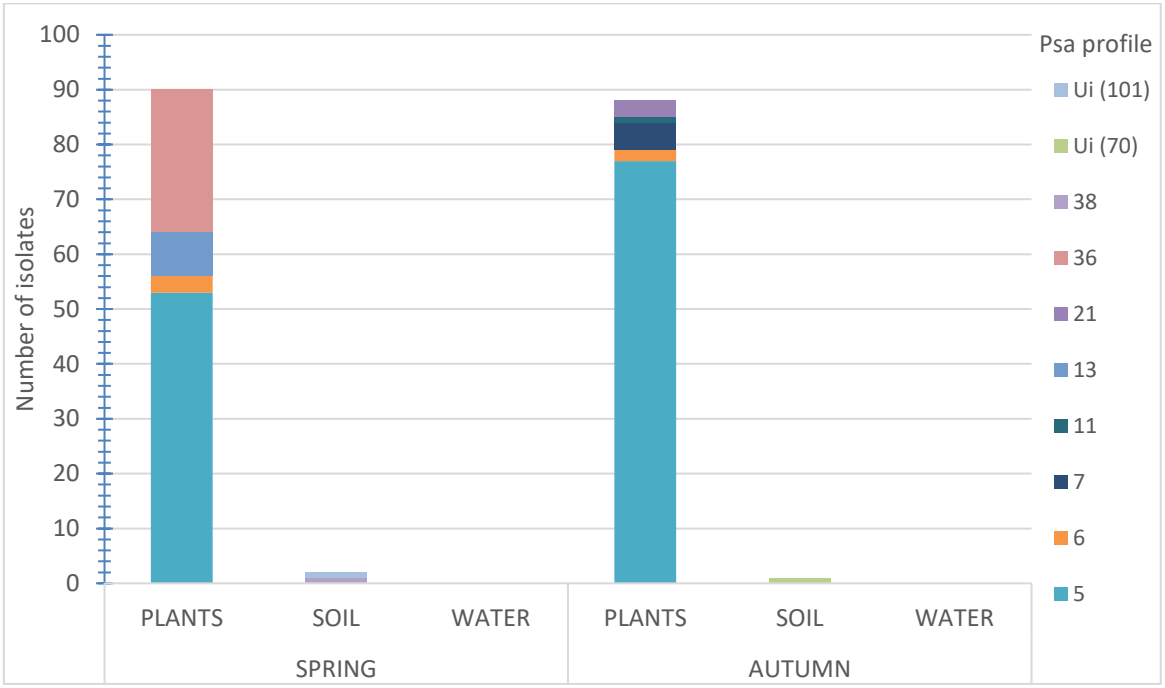


Figure 3-22. Distribution of Psa profiles in plants, soil and water from orchard D.

3.2.4.3 Alfa diversity

3.2.4.3.1 Between plants

Alfa diversity analysis was performed for each of three plants in orchard D (Table 3.2.4-V). According to Dmg values, plant 2 had the higher value in accordance with the higher number of Psa profiles observed (Figure 3-21), not too distant from plant 1 (Table 3.2.4-V). On the other hand, plant 3 had lower value of specific richness, with a value of 1.2.

Observing H' values, it was possible to infer that all the plants share a similar distribution of Psa isolates by the profiles in orchard D, with values between 0.7 and 0.8. Since the values of H' varying between 0 and the H'max (log(S), being S the richness observed in plant), the H' values observed in all plants were considered low since H' max round 1.60 for plant 1 and 2. In fact, the H' max of plant 3 was 1.1, which means that Psa isolates were better distributed by Psa profiles. The J' index values were similar in plants 1 and 2. However, plant 3 had the higher value with 0.8, meaning that Psa profiles were relatively equally abundant in this plant (Table 3.2.4-V).

Finally, the Simpson index was identical for all plants. The higher value was 0.5 in plant 3. Since this index varied between 0 and 1, these values were considered low. This result was in accordance with the results obtained from the fingerprinting analysis where the existence of dominant profiles was evident; namely profile 5 and 36 (Figure 3-21).

Table 3.2.4-V. Alpha diversity indexes determined for each plant in orchard D. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index.

Index	Orchard D		
	Plant 1	Plant 2	Plant 3
Dmg	2.4	2.6	1.2
H'	0.8	0.7	0.8
J'	0.5	0.4	0.8
1-D	0.4	0.3	0.5

3.2.4.3.2 Within orchard per condition

Considering EP and EN isolates in both seasons, a similar alfa diversity analysis was performed (Table 3.2.4-VI). Observing the values of Dmg, EP SI had the higher value with 2.1. On the opposite, EP AI isolates presented a lower value (Dmg value 1.4), which was expected once only 3 Psa profiles were detected, including the dominant profile 5 (Figure 3-21). According to the H' index, both EP and EN SI had a similar distribution of Psa profiles with a value of 1.0. O the other hand, both EP and EN AI, presented an inferior H' value (0.5) supported by the clear existence of dominant Psa profiles, namely profile 5 (Figure 3-21). The J' values were distinct between seasons. Once more, both EP and EN SI had the higher evenness (J' value 0.7) which correlates with the Dmg and H' results abovementioned. Finally, 1-D index shows that EP (0.5) and EN (0.6) SI had a higher diversity than EP and EN AI. The lower values of 1-D index were correlated with the existence of dominant profiles, namely Psa profile 5 and 36, which had influenced all the calculated indexes.

In sum, a higher specific richness was found in EP SI than EN SI and AI (Dmg index – Table 3.2.4-VI). Psa strains were more evenly distributed by the profiles in EP and EN SI (H' index – Table 3.2.4-VI) which was in accordance with evenness values (J' index – Table 3.2.4-VI). Both spring and autumn populations were characterized by the presence of dominant Psa profiles as evidenced by the dominance index (1-D). However, this presence was more evidenced in AI (Table 3.2.4-VI). These results confirms our previous supposition that presence of dominant profiles could be associated with the fact that infection occurred recently in plants with less than a year.

The data collected from the alfa diversity index analysis confirmed our previous evidences that changes occurred in Psa populations between spring and autumns evidenced by a decrease in Psa profiles accompanied by the raise of dominate ones. This alteration maybe a reflection of changes in the abiotic conditions (temperature and humidity) combined with several implemented orchards cultural practices.

Table 3.2.4-VI. Alpha diversity indexes determined for each condition in orchard D. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index; EP SI: epiphytic isolates from spring; EN SI: endophytic isolates from spring. EP AI: epiphytic isolates from autumn; EN AI: endophytic isolates from autumn.

Orchard D	Alpha diversity index			
	Dmg	H'	J'	1-D
EP SI	2.1	1.0	0.7	0.5
EN SI	1.7	1.0	0.7	0.6
EP AI	1.4	0.5	0.5	0.3
EN AI	1.7	0.5	0.3	0.2

3.2.4.4 Principal component analysis (PCA)

A PCA - Inter-species correlation analyses of all the plants isolates from orchard D is shown in Figure 3-23. In this analysis was possible to correlate the weight of a Psa profile with the event, which in this case represent the different conditions: epiphytic isolate (EP) or endophytic isolate (EN) in both spring (SI) and autumn (AI) seasons. The PCA analyses allows inferring which Psa profiles differ or clusters populations. Spring isolates were clustered together and separated from AI. This configuration was mainly due to profiles 36 and 13. Other profiles, namely 6 and Ui (62) reinforced this clustering. On the contrary, EP and EN AI were separated in the PCA analyses, mainly by profiles 7 and 11 (EP AI) and profile 21 (EN AI). The spatial distance among AI was higher, which means that Psa profiles diversity was quite distinct between EP and EN AI.

This analysis reinforced our previous results were a co-existence of distinct Psa populations was reported. Moreover, a succession of Psa populations with seasons was also strength.

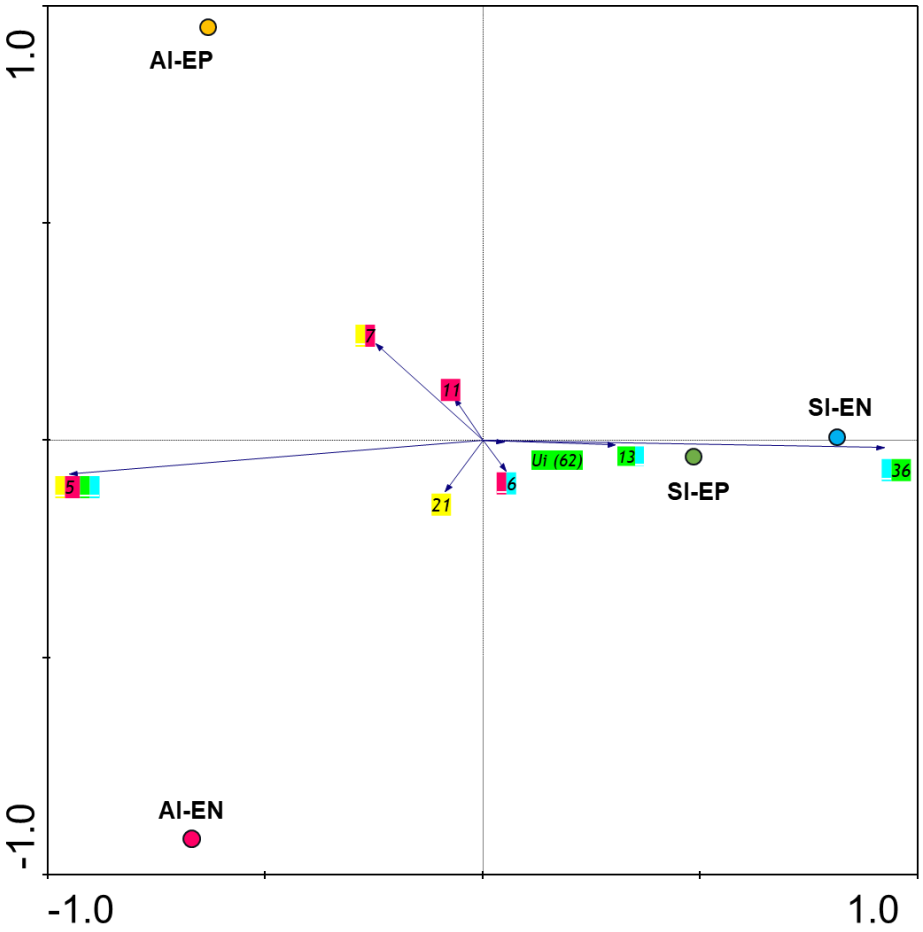


Figure 3-23. Principal component analysis – inter-species correlate – of Psa profiles from orchard D. Green SI-EP: epiphytic isolates, spring; Blue SI-EN: endophytic isolates, spring; Yellow AI-EP: epiphytic isolates, autumn; Pink AI-EN: endophytic isolates, autumn. Numbers correspond to Psa profiles. Colours identify Psa profiles that have more weight in each condition.

3.2.5 Orchard E

3.2.5.1 Putative *Pseudomonas* spp. isolation and Psa identification

A total of 361 isolates were obtained from plants, soil and water samples collected in orchard E (Table 3.2.5-I); 206 were SI and 155 were AI.

In order to confirm the identity of the isolates as Psa strains a duplex-PCR protocol described by Gallelli *et al*. (2011) was performed using the extracted DNAs (see **3.2.1.**).

Table 3.2.5-I. Total isolates recovered from orchard E in each plant/reservoir, in spring and autumn seasons. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	SPRING	
	PLANT	LOCALIZATION
KW517; KW755; KW756; KW757; KW758; KW759; KW760; KW761; KW762; KW763; KW764; KW770; KW820; KW821; KW822; KW889; KW1145; KW1146; KW1147; KW1148; KW1152	P1	EP
KW500; KW501; KW502; KW503; KW504; KW505; KW506; KW507; KW508; KW509; KW510; KW511; KW512; KW513; KW514; KW515; KW516; KW519; KW520; KW521; KW555; KW556; KW616; KW752; KW753; KW754	P1	EN
KW779; KW780; KW784; KW785; KW786; KW787; KW788; KW789; KW790; KW791; KW792; KW793; KW794; KW795; KW796; KW797; KW798; KW799; KW800; KW810; KW850; KW1215	P2	EP
KW775; KW769; KW771; KW772; KW773; KW774; KW776; KW777; KW778; KW781; KW782; KW783; KW785; KW1149; KW1150; KW1151; KW1214; KW1216	P2	EN
KW840; KW841; KW842; KW843; KW844; KW857; KW858; KW859; KW927; KW1014; KW1035; KW1036; KW1037; KW1079; KW1080; KW1081; KW1120	P3	EP
KW801; KW802; KW803; KW804; KW805; KW806; KW807; KW808; KW808; KW809; KW811; KW812; KW813; KW814; KW815; KW816; KW817; KW818; KW819; KW851; KW852; KW853; KW854; KW855; KW856; KW894; KW895; KW1022; KW1023; KW1024; KW1025	P3	EN
ISOLATE REFERENCE	**SPRING**	
	RESERVOIR	
KW562; KW563; KW564; KW565, KW566; KW695; KW696; KW697; KW698; KW699; KW700; KW701; KW702; KW703; KW704; KW705; KW706; KW707; KW708; KW709; KW710; KW713; KW714; KW715; KW727; KW728; KW729; KW730; KW862; KW888; KW890; KW1206; KW1207; KW1211; KW1218; KW1225; KW1287; KW1324	SO	
KW557; KW823; KW824; KW825; KW826; KW827; KW828; KW829; KW845; KW846; KW847; KW848; KW849; KW915; KW923; KW954; KW1015; KW1016; KW1017; KW1127; KW1128; KW1217	WA	
ISOLATE REFERENCE	**AUTUMN**	
	PLANT	**LOCALIZATION**
KW1934; KW1941; KW1973; KW1974; KW1994; KW2073	P1	EP
KW1784; KW1785; KW1786; KW1806; KW1807; KW1808; KW1809; KW1810; KW1837; KW1838; KW1839; KW1840; KW1841; KW1853; KW1854; KW1855; KW1856; KW1861; KW1862; KW1904; KW1927; KW1994; KW2011; KW2064; KW2080; KW2081; KW2082; KW2083; KW2106	P1	EN

Table 3.2.5-I (continuation). Total isolates recovered from orchard E in each plant/reservoir, in spring and autumn seasons. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate.

ISOLATE REFERENCE	AUTUMN	
	PLANT	LOCALIZATION
KW1734; KW1906; KW1961; KW1962; KW1975; KW1976; KW1983; KW2074; KW2101; KW2102; KW2103; KW2104	P2	EP
KW1787; KW1788; KW1789; KW1790; KW1800; KW1801; KW1874; KW1886; KW1887; KW1888; KW1891; KW1894; KW1895; KW1896; KW1897; KW1898; KW1899; KW1920; KW1924; KW1944; KW1945; KW1946; KW2002; KW2010; KW2062; KW2063; KW2084	P2	EN
KW1730; KW1731; KW1732; KW1892; KW1893; KW1932; KW1933; KW1935; KW1936; KW1937; KW1940; KW1970; KW2030	P3	EP
KW1796; KW1797; KW1798; KW1799; KW1871; KW1872; KW1947; KW1948; KW1959; KW1960; KW1963; KW1967; KW1968; KW1969; KW1971; KW2031; KW2059; KW2060; KW2061; KW2077; KW2078; KW2085; KW2086; KW2087; KW2088; KW2089; KW2119; KW2120	P3	EN

ISOLATE REFERENCE	AUTUMN
	RESERVOIR
KW1842; KW1845; KW1884; KW1885; KW1905; KW1942; KW1943; KW2038; KW2113	SO
KW1910; KW1911; KW1912; KW1913; KW1914; KW1915; KW1916; KW1917; KW1918; KW1949; KW1995; KW1996; KW1997; KW1998; KW1999; KW2000; KW2008; KW2009; KW2014; KW2022; KW2023; KW2027; KW2028; KW2033; KW2039; KW2097; KW2122	WA

The numbers of total isolates and Psa confirmed strains from the three representative plants, soil and water samples analysed in orchard E is presented in the Table 3.2.5-II. From a total of 361 isolates, 141 (39%) were confirmed as Psa, 78 were SI and 63 were AI Table 3.2.5-II.

The percentage of Psa isolates from plants sampled ranged from 42.7% to 62.2%. No major differences were observed between seasons regarding the persistence of Psa isolates in plants. On average, 37.9% and 40.7% of the total SI and AI were Psa, respectively. From the plant samples, 257 (71.2%) isolates were recovered, 136 were confirmed as Psa, corresponding to 52.9 % of total plant isolates. In plant 1, from a total of 82 isolates, 51 (62.2%) were confirmed as Psa, of which 25 (49%) were SI while 26 (51%) were AI. In plant 2 and 3 a total of 82 and 93 strains were recovered, of which 35 (42.7%) and 50 (53.8%) were identified as Psa, respectively. In plant 2, 17 (48.6%) of the Psa strains were SI and 18 (51.4%) were AI; while in plant 3, 32 (64%) of the Psa strains were SI and 18 (36%) were AI.

A total of 55 (15.2%) strains were isolated from soil samples Table 3.2.5-II, but only 4 were Psa positive, corresponding to 7.3% of total soil isolates. Finally, a total of 49 (13.6%) isolates were recovered from water samples but only 1 were confirmed as Psa, corresponding to 2% of total water isolates. Not a single Psa isolate were recovered from water in autumn.

Table 3.2.5-II. Total isolates and strains identified as Psa strains from plants, soil and water samples in each season from orchard E. SI: spring isolate; AI: autumn isolate;

Plant/ Reservoir	SI	AI	Total	Psa SI	Psa AI	Total Psa
Plant 1	47	35	82	25	26	51
Plant 2	41	41	82	17	18	35
Plant 3	50	43	93	32	18	50
Soil	46	9	55	3	1	4
Water	22	27	49	1	0	1
Total	206	155	361	78	63	141

As abovementioned, the three representative plants from orchard E were sampled in spring and autumn, and each sample was processed in order to separately recover epiphytic and endophytic bacterial strains.

In total, 60 (43.5% of total SI) and 35 (29.4% of total AI) EP strains were isolated in spring and in autumn, respectively; of which, 16 (26.7%) were confirmed as Psa in spring and 7 (20%) in autumn. On the other hand, 78 (56.5%) and 84 (70.6%) EN strains were isolated in spring and in autumn, respectively; of which 58 (74.4) were confirmed as Psa in spring and 55 (65.5%) in autumn (Table 3.2.5-III).

Table 3.2.5-III. Total isolates and strains identified as Psa from plants in each season from orchard E. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

	SI		Total	Psa SI		Total	AI		Total	Psa AI		Total
Plant	**EP**	**EN**	**SI**	**EP**	**EN**	**Psa SI**	**EP**	**EN**	**AI**	**EP**	**EN**	**Psa AI**
1	21	26	47	3	22	25	5	30	35	2	24	26
2	21	20	41	4	13	17	15	26	41	2	16	18
3	18	32	50	9	23	32	15	28	43	3	15	18
Total	60	78	138	16	58	74	35	84	119	7	55	62

From a total of 82 isolates from plant 1, 47 (57.3%) were SI, of which 21 (44.7%) were EP SI and 26 (55.3%) were EN SI; while 35 (42.7%) were AI, of which 5 (14.3%) were EP AI and 30 (85.7%) were EN AI (Table 3.2.5-III). From these, 3 (14.3%) and 22 (84.6%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 2 (40%) and 24 (80%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total 82 of isolates from plant 2, 41 (50%) were SI, of which 21 (51.2%) were EP SI and 20 (48.8%) were EN SI; while 41 (50%) were AI, of which 15 (36.6%) were EP AI and 26 (63.4%) were EN AI (Table 3.2.5-III). From these, 4 (19.1%) and 13 (65%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 2 (13%) and 16 (61.5%) were confirmed as Psa in EP AI and in EN AI, respectively.

From a total of 93 isolates from plant 3, 50 (53.8%) were SI, of which 18 (36%) were EP SI and 32 (64%) were EN SI; while 43 (46.2%) were AI, of which 15 (34.9%) were EP AI and 28 (65.1%) were EN AI (Table 3.2.5-III). From these, 9 (50%) and 23 (71.9%) were confirmed as Psa in EP SI and in EN SI, respectively. Additionally, 3 (20%) and 15 (53.6%) were confirmed as Psa in EP AI and EN AI, respectively.

Concerning the plant results for total Psa isolated in orchard E, a clear difference was observed among Psa EP and Psa EN numbers in both seasons (Table 3.2.5-III). In general, the number of Psa EN is superior to Psa EP in all plants. Analysing EP, a slightly reduce in EP AI numbers was observed comparing with EP SI numbers. On the opposite, in AI numbers there's a slightly decrease in the number of EN isolates when compared to EN SI. In addition, comparing the results between the three plants is possible to observe that percentage of Psa EN isolates was superior to 50% in all plants (Figure 3-24). The increase observed in EN AI recovering numbers was accompanied by a slightly decrease in Psa EN AI percent in all the plants. Since this orchard has 30 years old, being supposedly more resistant to infection by Psa than young orchards (Vanneste *et al.*, 2011b), this result could suggest that summer conditions promote the competition among other bacteria (which also colonized systemically the plant) and Psa populations, reducing Psa numbers. The reduction in EP AI numbers may be related to edaphoclimatic conditions associated to cultural practises, that may difficult the growth or persistency of epiphytic populations in the plants.

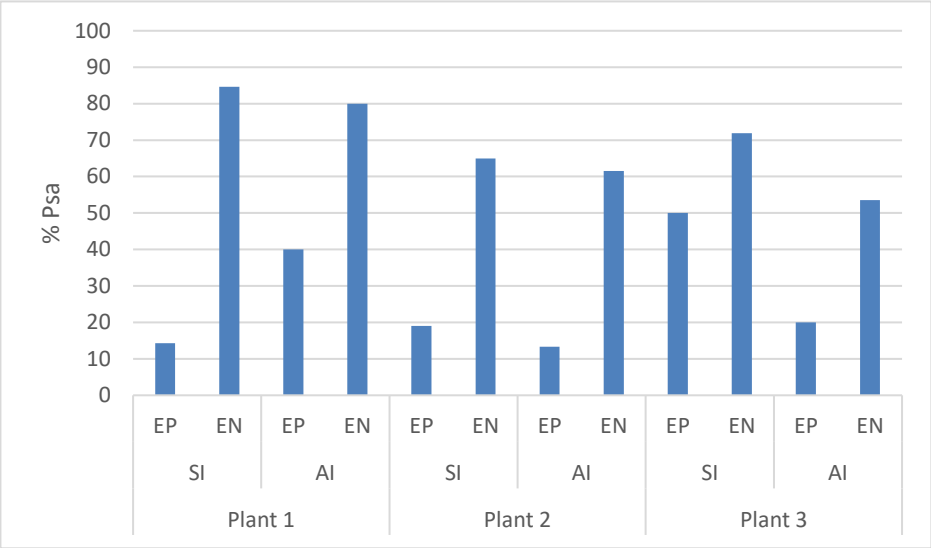


Figure 3-24. Percentage (%) of isolates identified as Psa in each condition from each season from each plant of orchard E. SI: spring isolate; AI: autumn isolate; EP: epiphytic isolate; EN: endophytic isolate.

3.2.5.2 Characterization of Psa populations in orchard E

The fingerprinting analysis of the Psa isolates was performed by BOX-PCR, as previously described (Louws *et al*., 1994). From the 141 Psa isolates obtain from orchard E, only 134 isolates generated a suitable BOX profile (Table 3.2.5-IV) despite several attempts.

Table 3.2.5-IV. Psa isolates and correspondent BOX profile from orchard E. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX
	PLANT	LOCALIZATION	PROFILE
KW1152	P1	EP	13
KW755	P1	EP	31
KW517	P1	EP	33
KW616	P1	EN	-
KW502; KW503; KW505; KW506; KW507; KW508; KW510; KW511; KW512; KW513; KW515; KW516; KW518; KW519; KW520; KW555; KW754	P1	EN	13
KW504; KW514	P1	EN	33
KW850	P2	EP	5
KW779; KW780	P2	EP	36
KW790	P2	EP	Ui (85)
KW769; KW783; KW1013; KW1150	P2	EN	5

Table 3.2.5-IV (continuation). Psa isolates and correspondent BOX profile from orchard E. P1, P2 and P3: plant 1, 2 and 3; EP: epiphytic isolate; EN: endophytic isolate; SO: soil isolate; WA: water isolate; UI: unique profile.

ISOLATE REFERENCE	SPRING		BOX PROFILE
	PLANT	LOCALIZATION	
KW771; KW772; KW773; KW774; KW777; KW778; KW781	P2	EN	36
KW907	P3	EP	-
KW840; KW841; KW842; KW843; KW844; KW1081; KW857; KW858	P3	EP	5
KW1215	P3	EP	13
KW802; KW803; KW804; KW805; KW806; KW807; KW808; KW809; KW811; KW812; KW813; KW815; KW816; KW817; KW853; KW854; KW855; KW856; KW894; KW895; KW1022	P3	EN	5
KW1214; KW1216	P3	EN	13
ISOLATE REFERENCE	**SPRING**		**BOX PROFILE**
	RESERVOIR		
KW1218	SO		Ui (99)
KW1217	WA		Ui (72)
ISOLATE REFERENCE	**AUTUMN**		**BOX**
	PLANT	**LOCALIZATION**	**PROFILE**
KW1973; KW1974	P1	EP	31
KW1927; KW2011	P1	EN	-
KW1784; KW1786; KW1806; KW1807; KW1808; KW1809; KW1810; KW1839; KW1840; KW1841; KW1853; KW1854; KW1855; KW1856; KW1861; KW1862; KW2064; KW2080; KW2081; KW2082; KW2083; KW2106	P1	EN	5
KW1787; KW1788; KW1789; KW1886; KW1887; KW1888; KW1894; KW1895; KW1896; KW1897; KW1898; KW1899; KW1906; KW2062; KW2063; KW2084; KW1983	P2	EN	5
KW1731	P3	EP	Ui (87)
KW1732	P3	EP	Ui (88)
KW1871; KW1872; KW1959; KW1960; KW1967; KW2059; KW2060; KW2061; KW2077; KW2078; KW2085; KW2086; KW2089; KW2119; KW2120	P3	EN	5
ISOLATE REFERENCE	**AUTUMN**		**BOX**
	RESERVOIR		**PROFILE**
KW1885	SO		Ui (71)

Figure 3-25 shows the distribution of Psa profiles identified according to the isolation site and season. Psa populations were remarkably distinct between spring and autumn. In spring, a total of 6 Psa profiles were observed while in the autumn only 4 profiles were detected. Differences in profile

diversity between EP/EN were also observed in both seasons. Namely, EP SI isolates were split in 6 Psa profiles compared to only 3 profiles detected in EP AI. Similarly, EN SI isolates were split in 5 Psa profiles compared to only a single Psa profile detected in EN AI. Furthermore, the total number of EP isolates was lower than the total number of EN isolates, strengthening our observation of a significant difference in the diversity among Psa populations between seasons. Indeed, a decrease in Psa population diversity was observed in autumn.

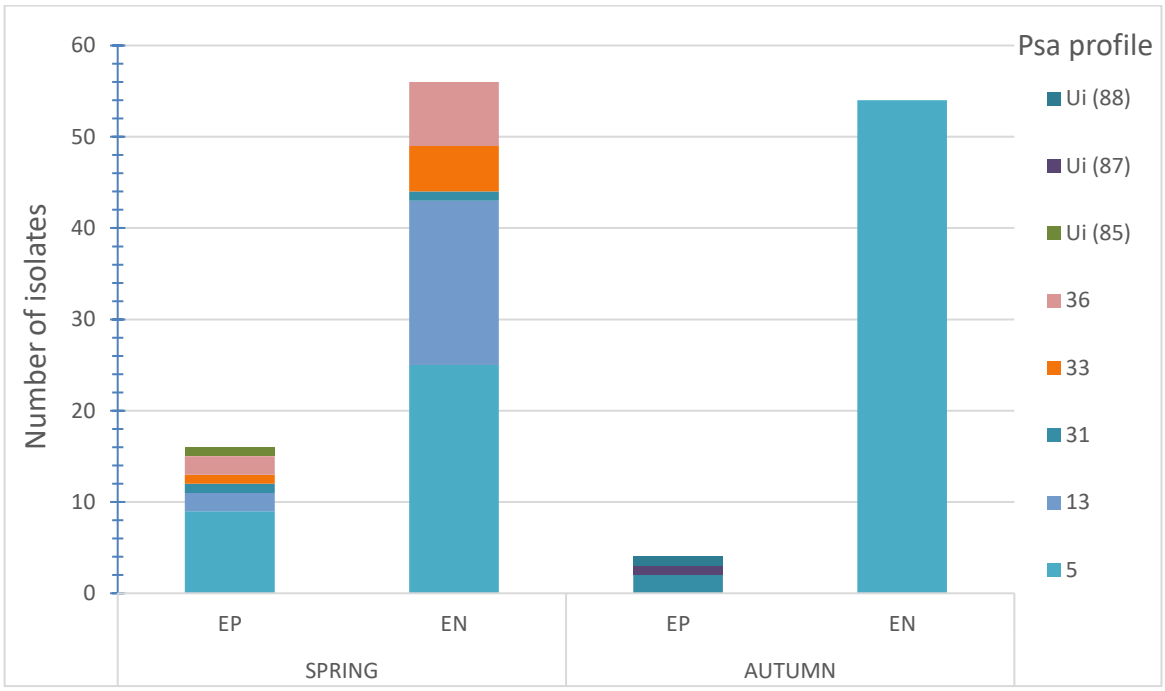


Figure 3-25. Distribution of Psa profiles in plant isolates from orchard E in spring (SI) and autumn (AI). EP: epiphytic isolate; EN: endophytic isolate.

In more detail, it was possible to infer a higher diversity in Psa profiles in SI (profiles 5, 13, 31, 33, Ui (85) and 36) in relation to AI (5, Ui (87), Ui(88) and 31). Indeed, only 2 Psa profiles – 5 and 31, were common between seasons (Figure 3-25). Among the EP isolates from orchard E, the profiles 13, 33, 36 and Ui (85) were only observed in SI. The first three profiles were common between EP and EN, while the last one was EP SI exclusive. In addition, Psa profiles Ui (88) and Ui (89) were only observed in AI. The only common profile observed between EP SI and AI was profile 31, also present in EN SI. Amongst the EN isolates, in SI the Psa profile diversity observed was identical to EP SI. On the opposite, in AI only a single Psa profile – 5 was detected in EN.

Our results suggest that there was a higher diversity among Psa populations in spring when compared to autumn. In addition, the diversity observed in EN SI was lost between seasons, once it was not

detected in more than 50 Psa isolates. Moreover, there was a clear predominance of Psa profile 5 among SI and EN AI. This dominance of Psa profile 5 could be related with abiotic conditions affecting the orchard between spring and autumn, namely higher temperatures and less humidity (summer conditions), suggesting that this clone could be more resilient or adapted to overcome such conditions. Furthermore, as abovementioned, this orchard has 30 years and the disease has been recently introduced, which may suggest that Psa strains detected in SI (such Psa profile 13, 33 and 36) may not able yet to both overcome summer conditions and compete with other colonizer bacteria. These hypotheses may also explain the loss of profile diversity in EN AI.

3.2.5.2.1 Characterization of Psa populations present in each plant

The distribution of Psa profiles among the three sampling plants was depicted in Figure 3-26. The Psa diversity was different between plants, and between seasons. However, it was clear the predominance of profile 5 among all plants, with dominant characteristics.

In more detail, profile 33 was only observed in plant 1 and was recovered from both EP and EN SI. In the plant 2, AI were all characterized as Psa profile 5. In opposition, SI had some diversity, with two exclusives profiles: profile 36 found both EP and EN and profile Ui (85) recovered from EP. Plant 3 had two unique Psa profiles, namely Ui (87) and Ui (88) both detected in EP AI.

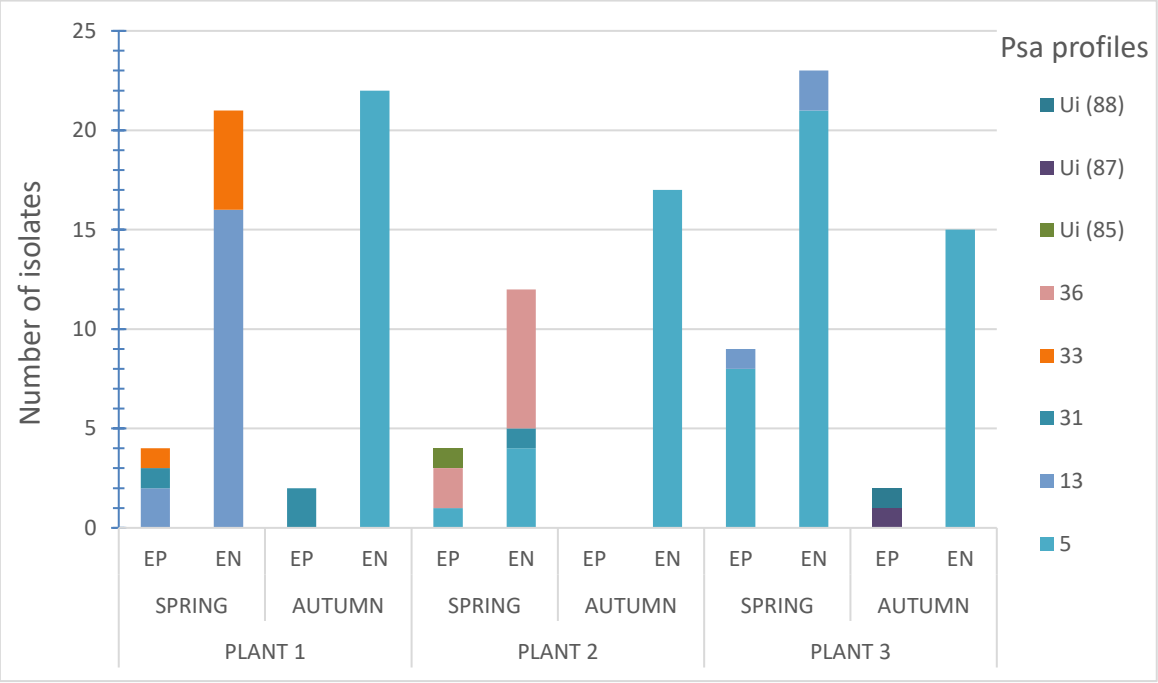


Figure 3-26. Distribution of Psa profiles found in the three representative plants from orchard E in spring and autumn.
EP: epiphytic isolate; EN: endophytic isolate.

Common to all three plants, Psa profile 5 was dominant in EN AI from both plant 1 and 2, and in SI and EN AI from plant 3. The profile 31 was common among plants 1 and 2. The isolates with this profile were from EP and EN SI in plant 1 and EN SI in plant 2. Plant 1 and 3 shared the Psa profile 13, recovered from SI in both plants.

In general, a higher diversity was observed between Psa profiles isolated in spring than in autumn which suggests that structure of Psa population varies over time in the same plant. There were common profiles between EP and EN in each plant in spring. In fact, either plant 1 or 2 had two common Psa profiles between EP and EN. These results suggest that some Psa strains that infects the plant was the same epiphytically and endophytically, at least, in spring. Moreover, these results evidenced the co-existence of several Psa populations; some varied with time (namely, profiles 13, 33 and 36) while other were persistently recovered (such profile 5).

3.2.5.2.2 Soil and water has potential Psa environmental reservoirs

The total Psa diversity, inferred from BOX profiling, determined in orchard E is depicted in Figure 3-27. Two and only one distinct Psa profiles were obtained from soil and water isolates, respectively. The profiles Ui (99) and Ui (71) were recovered from soil in spring and autumn, respectively. The profile Ui (72) was isolated from water in spring. These Psa profiles were restricted to those reservoirs and distinct from Psa profiles obtained from plant isolates. However, even in lesser quantity, soil and water isolates were identified as Psa. Since the infection by Psa was recent, it is possible that these environments are not yet colonized with Psa. Further studies are needed to clarify this possibility.

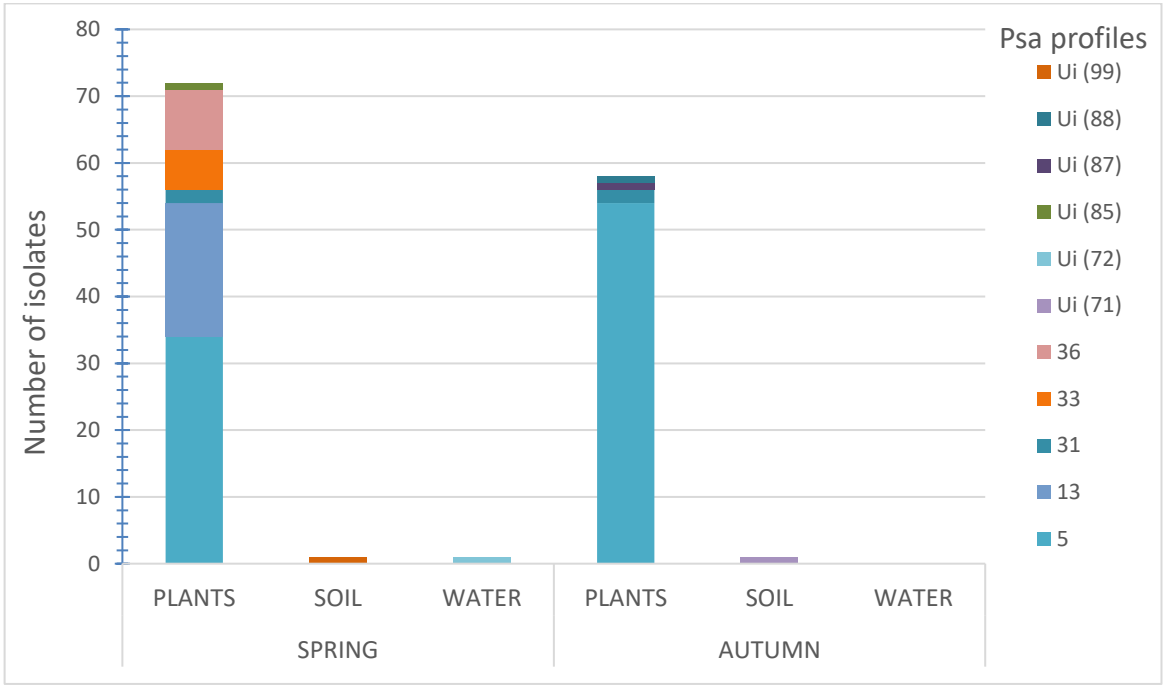


Figure 3-27. Distribution of Psa profiles in plants, soil and water from orchard E.

3.2.5.3 Alpha Diversity

3.2.5.3.1 Between plants

Alpha diversity analysis was performed for each plant in orchard E (Table 3.2.5-V). Observing Dmg index, the values were similar between all plants. However, plant 2 had higher value of specific richness with a value of 2. These results were in accordance with the number of Psa profiles observed (Figure 3-26).

According to H' index, plant 1 displayed a higher H' (H' value 1.2) meaning that strains were more evenly distributed by the Psa profiles. On the opposite, plant 3 presented a lower H' index (H' value 0.4) supported by the existence of dominate Psa profiles, namely profile 5 (Figure 3-26). Observing J' index, Plant 1 had higher J' value with 0.8, meaning that Psa isolates were relatively well distributed by the profiles.

Finally, examining the 1-D index, we could conclude that plant 2 had a higher diversity, not too distant from plant 1 (Table 3.2.5-V). On the opposite, plant 3 had lower 1-D value, which correlated with the evident existence of dominate profiles, as observed in Figure 3-26.

Table 3.2.5-V. Alpha diversity indexes determined for each plant in orchard E. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index.

Index	Orchard E		
	Plant 1	Plant 2	Plant 3
Dmg	1.8	2.0	1.8
H'	1.2	0.8	0.4
J'	0.8	0.6	0.3
1-D	0.6	0.5	0.2

3.2.5.3.2 Within orchard per conditions

Considering EP and EN isolates in both spring and autumn seasons, a similar alfa diversity analysis was performed (Table 3.2.5-VI). Since only one Psa profile was identified in BOX-PCR analysis from EN AI (Figure 3-25), it was not possible to calculate none of the presents indexes for this condition. In accordance with the values of Dmg, EP SI had the higher value with 4.2. On the opposite, EN SI isolates present lower value (Dmg value 2.3). According to H', EP SI has the better distribution of Psa profiles with a value of 1.4, not too distant from EN SI. On the other hand, EP AI (H' value 1.0), presented a lower H' index supported by the lower specific richness (a total of 4 isolates) of Psa profiles, namely profile 31, Ui (87) and Ui (88) (Figure 3-25). J' values were relatively closer each other (Table 3.2.5-VI), however, EP AI has the higher evenness (J' value 0.9). Finally, 1-D index shows that Psa profile diversity was similar among conditions. Concerning the values, EN SI (1-D value 0.7) had a higher diversity than EP SI, and AI.

Overall, a higher specific richness was found in EP has opposed to EN (Dmg index – Table 3.2.5-VI). Psa strains were more evenly distributed by the profiles in SI (H' index – Table 3.2.5-VI) which was in accordance with evenness values (J' index – Table 3.2.5-VI). The 1-D index shows that Psa profile diversity was similar among SI and AI (Table 3.2.5-VI).

Although little evidenced, the data collected from the alfa diversity index analysis confirmed our previous evidences that a changed occurred in Psa populations between spring and autumn. This change was evidenced by the loss of Psa profiles diversity accompanied by the persistence of dominate ones (Figure 3-25 and Table 3.2.5-VI). This alteration maybe a reflection of changes in the abiotic conditions (temperature and humidity) combined with several implemented orchards cultural practices.

Table 3.2.5-VI. Alpha diversity indexes determined for each isolation condition in orchard E. Dmg: Margalef index; H': Shannon index; J': Pielou's evenness index; 1-D: Simpson diversity index; EP SI: epiphytic isolates from spring; EN SI: endophytic isolates from spring. EP AI: epiphytic isolates from autumn; EN AI: endophytic isolates from autumn.

Orchard E	Alpha diversity index			
	Dmg	H'	J'	1-D
EP SI	4.2	1.4	0.8	0.6
EN SI	2.3	1.3	0.8	0.7
EP AI	3.3	1.0	0.9	0.6
EN AI	0.0	0.0	0.0	0.0

3.2.5.4 Principal component analysis (PCA)

A PCA - Inter-species correlation analyses of all the plants isolates from the Orchard E is shown in Figure 3-28. In this analysis, it is possible to correlate the weight of a Psa profile with the event, which in this case represent the different conditions: epiphytic isolate (EP) or endophytic isolate (EN) in both spring (S) and autumn (A) seasons. The PCA analyses allows inferring which Psa profiles differ or clusters populations. Spring isolates were clustered together and separated from AI. This configuration was mainly due to several profiles, namely 13, 33, 36 and Ui (85). The three first ones influenced EN SI and the last one influenced EP SI configuration. On the contrary, EP and EN AI were separated in the PCA analyses mainly by profile 5 (EN AI) and profiles Ui (87), Ui (88) and 31 (EP AI).

This PCA analysis showed the similarity of Psa profiles diversity among SI. In addition, also demonstrate that Psa profiles were distinct between SI and EP AI. Psa profile diversity of EN AI was distinct from the other ones and very influenced by dominant profile 5. Moreover, this analysis reinforced our previous results were a co-existence of distinct Psa populations was reported. In addition, a succession of Psa populations with seasons was also strength.

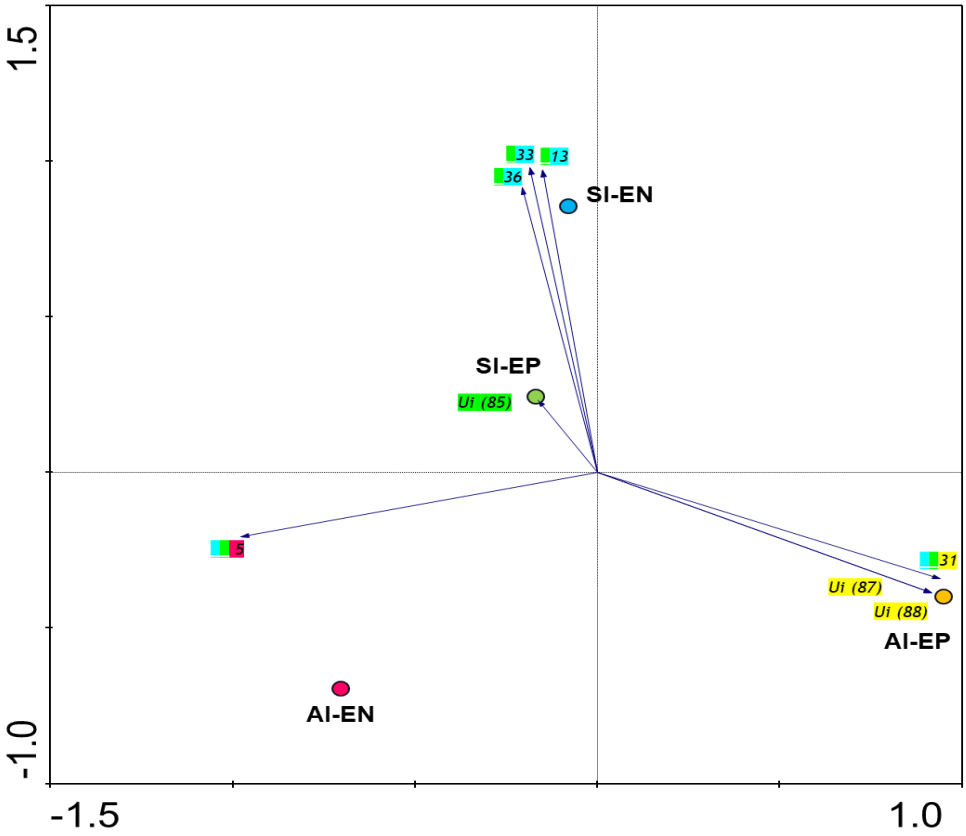


Figure 3-28. Principal component analysis – inter-species correlate – of Psa profiles from orchard E. Green SI-EP: epiphytic isolates, spring; Blue SI-EN: endophytic isolates, spring; Yellow AI-EP: epiphytic isolates, autumn; Pink AI-EN: endophytic isolates, autumn. Numbers correspond to Psa profiles. Colours identify Psa profiles that have more weight in each condition.

3.3 Comparison between the studied orchards

Figure 3-29 shows the distribution of the identified Psa profiles according to the isolation site and season in each of the studied orchards. The overall Psa diversity was remarkable distinct among orchards, namely between the North region (A and B) and the Centre region (D and E). Higher diversity was found in the North region when compared with the Psa diversity observed in the Centre region. Moreover, the structure of the Psa population varied over time. Evident changes in the structure of Psa population occurred between spring and autumn for all the studied orchards. These changes were characterized by a decrease in the diversity of Psa in autumn accompanied by an increase in dominant populations. Beside the differences on the geographical locations (climatic conditions), the location in leaf's affected Psa diversity. Indeed, comparing EP and EN from both seasons in each orchard, EP had higher Psa diversity than EN in all orchards, except in orchard D.

These evidences were based on some relevant results worth notice; namely the recovery of unique Psa profiles in all orchards (Figure 3-29), demonstrating that Psa population's structure varied between the five studied orchards.

In parallel, the presence of widely distributed Psa profiles (5, 13 and 36) was observed (Figure 3-29). Namely, Psa profiles 5 and 13 were found in all orchards, except in orchard C. Besides these populations, orchard A shared some clones with orchards B, C and D; namely Psa profiles 9, 34 and 6, respectively. Psa profile 9 was found in both EP and EN in SI, which suggests that it could represent a population characteristic of spring season in orchards A and B. Psa profile 34 was present in both seasons in orchard C and was detected in SI in orchard A. It seems to be a Psa profile persistent in both seasons, at least in orchard C, where Psa disease was not yet established.

Between orchard A and D, only one Psa profile was common detected in EN in both SI and AI. This Psa profile appeared to be endophytically persistent and resilient to changes in abiotic conditions between seasons. In fact, it was never detected among EP isolates, which may indicate the inability of this Psa clone to survive epiphytically.

Orchard B had only a common Psa profiles with orchard D and E, namely profiles 7 and 33, respectively. Psa profile 7 was recovered from SI and EP AI in orchard B and from AI in orchard D. This Psa profile appears to be able to colonize the plant epiphytic and endophytically in both seasons, even if it was not found in EN AI in orchard B. In addition, Psa profile 33 was found in EN SI and in SI in orchard B and E respectively, which suggests that this could be a Psa clone characteristic of spring in these orchards. Between orchard C and D only one common Psa profile was detected, the Psa profile 11. This profile was recovered from EP SI and EP AI in orchard C and D, respectively. It was not possible to correlate a specific characteristic linked to this Psa profile distribution, however, it was only found in EP in both orchards.

Orchard D and E only shared the Psa profiles better distributed in those regions, namely Psa profile 5, 13 and 36. In general, Psa profile 5 was recovered either in EP or EN in both seasons, being the more representative Psa profile. While Psa profiles 13 and 36 (also detected in orchard B) were only found in both EP and EN from SI, suggesting that they could be spring characteristic Psa strains in these orchards. In comparison with Psa profile 5, those apparently couldn't survive the abiotic conditions that are characteristic of summer.

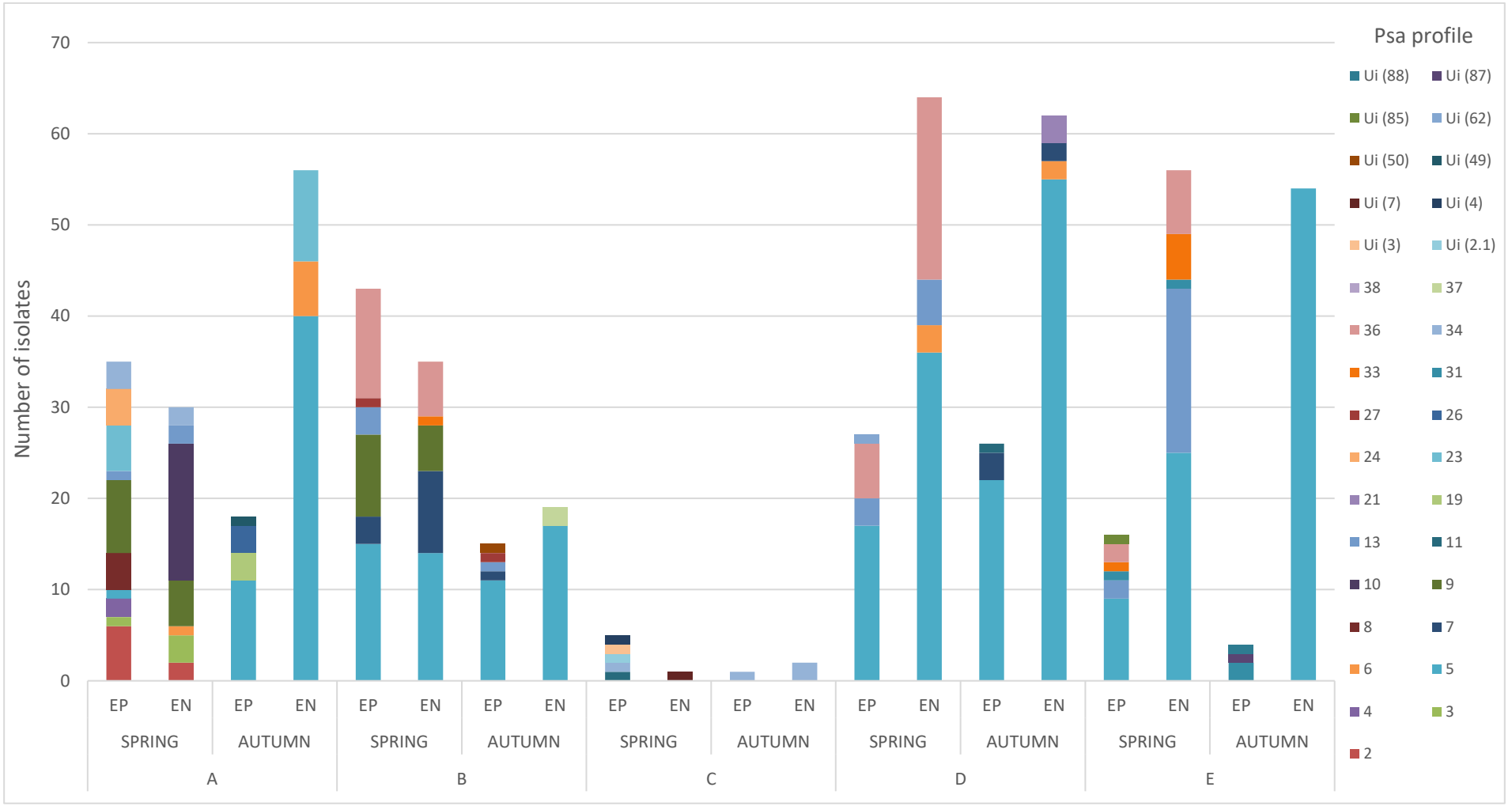


Figure 3-29. Distribution of Psa profiles detected in plant isolates from the studied orchard in spring and autumn. EP: epiphytic isolate; EN: endophytic isolate

From Figure 3-29 it was clear the presence of dominant profiles, namely Psa profile 5 and 36. In fact, Psa profile 5 was detected in more than 300 isolates from all the orchards (except orchard C), being the most representative Psa profile in both regions. Comparing the diversity results obtained from all orchards, this clone could be indeed more resilient or well adapted to overcome the abiotic conditions affecting orchards between spring and autumn (higher temperatures and less humidity). Curiously, observing the Psa profiles obtained from SI it was possible to infer that in the orchards with higher Psa profile diversity, such A and B, the detection of Psa profile 5 was lower than in orchard D and E, characterized by lower Psa profile diversity. This evidence may be related with bacteria competition, since the spring abiotic conditions may favour the growth and develop of other Psa strains (Serizawa & Ichikawa, 1993). In addition, Psa profile 36 was also widely distributed between orchards B, D and E and, recovered from both EP and EN from spring isolates in each of the orchards. Psa profile 36 was not detected in AI, suggesting that contrasting with Psa profile 5, this one couldn't survive the abiotic conditions that are characteristic of summer.

The detection of Psa in Portugal is relatively recent since the first report dates from 2010 (Balestra *et al.*, 2010). The pathogen was detected in EDM region, precisely in orchard A (Valença) and the origin of the infection was related with the plants brought from Italy. This means that the introduced Psa originally infected Italian orchards (since 2008) and was brought to Portugal. The infected plants in EDM orchards had less than a year old and were able to survive Psa infection for the past seven years with low incidence of the disease. This evidence suggests that changes in conditions between Portugal and Italy may have induced a selection between the introduced strains towards the colonization of less virulent strains since the damages in Italy were enormous compared with those in EDM, exacerbated by the high susceptible to Psa infection reported for young plants (Vanneste *et al.*, 2011b).

The incidence and severity of the disease in the orchards of Centre regions, namely D and E, was higher than that observed in Northern orchards (see Table 2.1.1-I). The severity of the disease could be related with the observed decrease in the diversity of Psa strains and the concomitant appearance of dominant strains. This hypothesis was supported by the calculated diversity indexes (namely Simpson index **3.2.1.3**., **3.2.2.3**., **3.2.4.3**. and **3.2.5.3**) and could be connected to abiotic conditions that favoured its persistence, namely the number chilling hours, precipitation and average temperature (see Table 2.1.1-I), in opposition to what was observed for the North region orchards.

Differences in the incidence of disease were observed in central region, namely between orchard D and E. The incidence of the disease was more severe in orchard D in comparison with orchard E. Since Psa was detected at same time and the orchards distance only 4km (same climate conditions), this may explained by differences in plants age, since young plants are more susceptible to disease than oldest

plants (Vanneste *et al.*, 2011b). In fact, plants in orchard D had 4 years old while in orchard E plants were much older - 30 years old.

Finally, from the analysis of orchard C results it was possible to infer that the Psa diversity was quite distinct from the other orchards, despite the low numbers of isolates (Table 3.2.3-II). This orchard had some characteristics that distinguished it from the others and could explain these results. Namely, it was an old orchard and Psa was only detect in 2016 (this study). Moreover, the agricultural practices were very limited, since no application of copper or other chemicals have been implemented. These facts influence the presence of other bacteria that could compete with Psa for the colonization of tissues, reflected in the low number of Psa isolates versus total number of isolates (see **3.2.3.**).

Overall, the fingerprinting analysis supported that Psa populations present in Portuguese orchards were heterogeneous. Indeed, several distinct Psa profiles were obtained from SI and AI, supporting the existence of a highly diverse Psa population. This heterogeneity was found within orchards and between orchards. Some Psa profiles were only found in a specific orchard while others were common to most orchards (such Psa profile 5 and 36). Not a single Psa profile was common to all orchard.

3.3.1 Diversity between orchards

A Beta diversity analysis was performed in order to compare the observed diversity between the five studied orchards (Table 3.3.1-I). This analysis measured changes in the diversity of species (in this case Psa profiles) from one environment (in this case, an orchard) to another. The Jaccard similarity index was applied to compared the Psa profile diversity of each orchard and determine which Psa profiles were shared and which were distinct. The higher the obtained percentage, the more similar two populations are. Analysing the matrix showed in the Table 3.3.1-I, it was possible to infer that the Psa populations were quite distinct in the five orchards, with values of similarity below 20%. These results reinforced our previous conclusions about the heterogeneity of the Psa populations in Portuguese orchards.

According with this analysis, orchards D and E were the most similar (18.75%). Indeed, the location of orchard D and E differs in only 4km. So, this similarity may be explained by the proximity of the two orchards under the influence of the same abiotic conditions. Orchards D and B were equally similar (17.39%), despite located in separate regions. This similarity was mostly explained by the existence of a common profile, Psa profile 7, only detected in these two orchards. On the other hand, orchard C was the most dissimilar, with no similarity with orchards B and E, in accordance with our previous results (Figure 3-29) since not a common profile was identified between them. This dissimilarity was

reinforced by the presence of common Psa profiles - 5, 13 and 36, which well distributed among the remaining orchards.

Table 3.3.1-I. Beta diversity matrix, determined by Jaccard similarity index for the five studied orchards (A, B, C, D and E).

	A	B	C	D	E
A					
B	12.50				
C	3.33	0.00			
D	5.26	17.39	7.14		
E	4.35	12.90	0.00	18.75	

Jacard similarity index, colour scale (%)

20	15	10	5	0

3.3.2 Principal component analysis (PCA)

3.3.2.1 Between Portuguese orchards

A PCA - Inter-species correlation analyses from all plant isolates present in each orchard is shown in Figure 3-30. In this analysis, it was possible to correlate the weight of a Psa profile with the event, which in this case represented the different orchards: A, B, C, D, and E. PCA analyses allowed inferring which Psa profiles differed or clustered each orchard.

Orchard D and E were clustered together and separated from the others (Figure 3-30Figure 3-31). This configuration was mainly due to the presence of Psa profile 5, and to several unique profiles detected in these orchards. Namely profiles 21, Ui (62), 31, 33, Ui (85), Ui(87) and Ui(88). Orchard B was on the centre of the analysis, between orchards D and E, and orchard A, suggesting the existence of common Psa profiles that influenced this configuration. Namely Psa profiles 7, 36 and 33, approaching orchard B with the cluster formed by orchard D and E. On the opposite, Psa profile 9 clustered orchard B with orchard A. Orchard C was completely separated from the other, mainly due to scarcity of common Psa profiles (Figure 3-30). This PCA analysis distributed the different orchards in accordance with the similarity in Psa profile diversity. Orchard D and E clustering correlates well with the Jaccard index, reinforcing the similarity between these orchards. Moreover, Jaccard index also confirmed the disposition of orchard B, more closely related with orchard A, D and E.

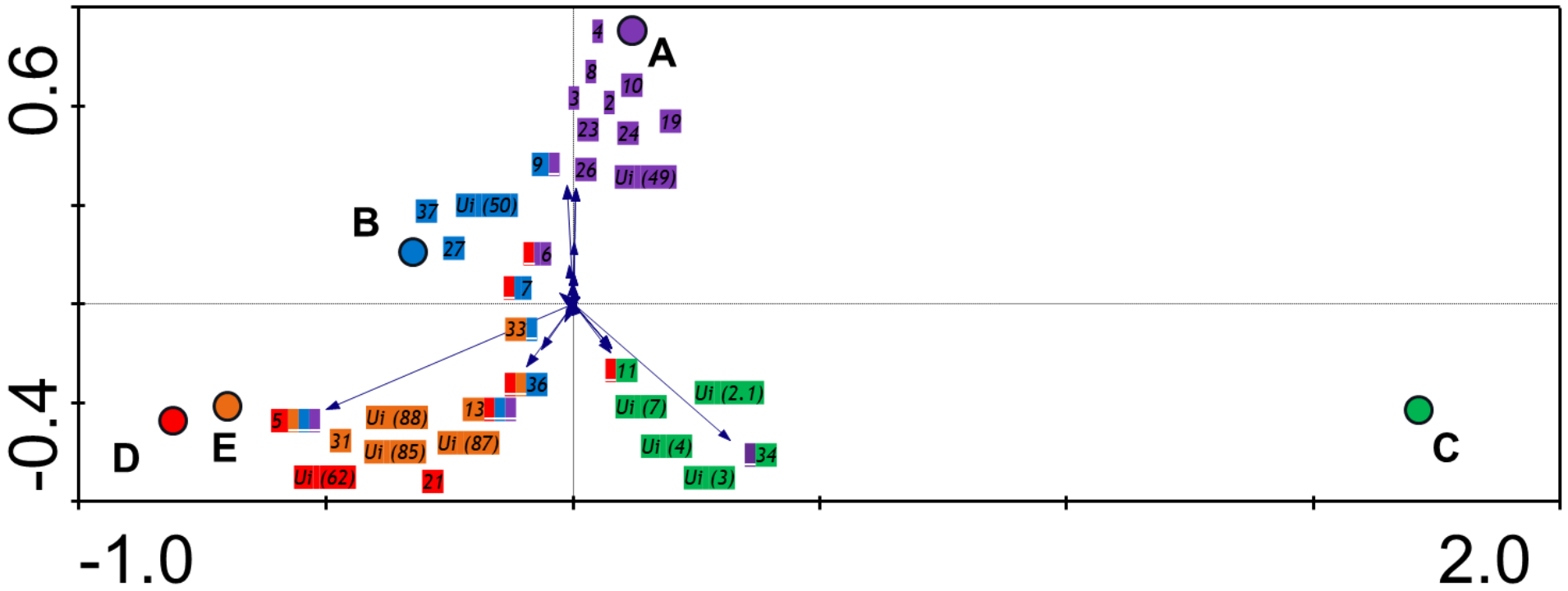


Figure 3-30. Principal component analysis – inter-species correlate – of Psa profiles from each studied orchard. Purple A: total plant isolates from orchard A; Blue B: total plant isolates from orchard B; Green C: total plant isolates from orchard C; Red D: total plant isolates from orchard D; Orange E: total plant isolates from orchard E. Numbers correspond to Psa profiles. Colours identify Psa profiles that have more weight in each condition.

3.3.2.2 Between Portuguese orchards in both seasons

A PCA - Inter-species correlation analyses of all plant isolates from each orchard in both seasons is shown in Figure 3-31. In this analysis, it is possible to correlate the weight of a Psa profile with the event, which in this case represented the different orchards in spring and autumn. From this analysis, it was possible to identify three major clusters; the first grouped autumn samples from all orchards, except orchard C - autumn cluster. This grouping was mainly due to the presence of Psa profile 5, dominant among AI from those orchards.

The second cluster was composed of spring samples from orchards B, D and E - spring cluster. Psa profiles 7, 33, and 36 influence the formation of this cluster (Figure 3-31).

The last identified cluster grouped SI from orchard A and C (Figure 3-31). This cluster comprised the highest Psa profile diversity, since these orchards presented the higher number of unique Psa profiles among SI. This clustering was mainly due to Psa profile 34, which was common between these orchards. This Psa profile also influenced the position of both orchards, separating them from the others, namely from the autumn cluster. Psa profile 34 influenced the cluster formed by SI from orchard A and C, and the AI isolates from orchard C. However, the position of this cluster was closer to spring cluster than to AI from orchard C. This configuration was mainly due to Psa profiles 9 and 11 which were common to A and B SI and to D and C SI, approaching these clusters (Figure 3-31).

This PCA analysis supported our previous assumptions that changes in Psa populations occurred between seasons, which varied over time within and between Portuguese orchards. These results were in accordance with the fingerprinting analysis (Figure 3-29) and with the alfa diversity indexes (sections **3.2.1.3.2.**, **3.2.2.3.2.**, **3.2.4.3.2**. and **3.2.5.3.2.**) determined for each condition.

Moreover, Psa profile diversity was more similar between autumn isolates, except for orchard C. Between spring isolates, Psa profile diversity was also similar between orchards B, D and E. Indeed, the alteration of climatic conditions may be a crucial factor in this differentiation.

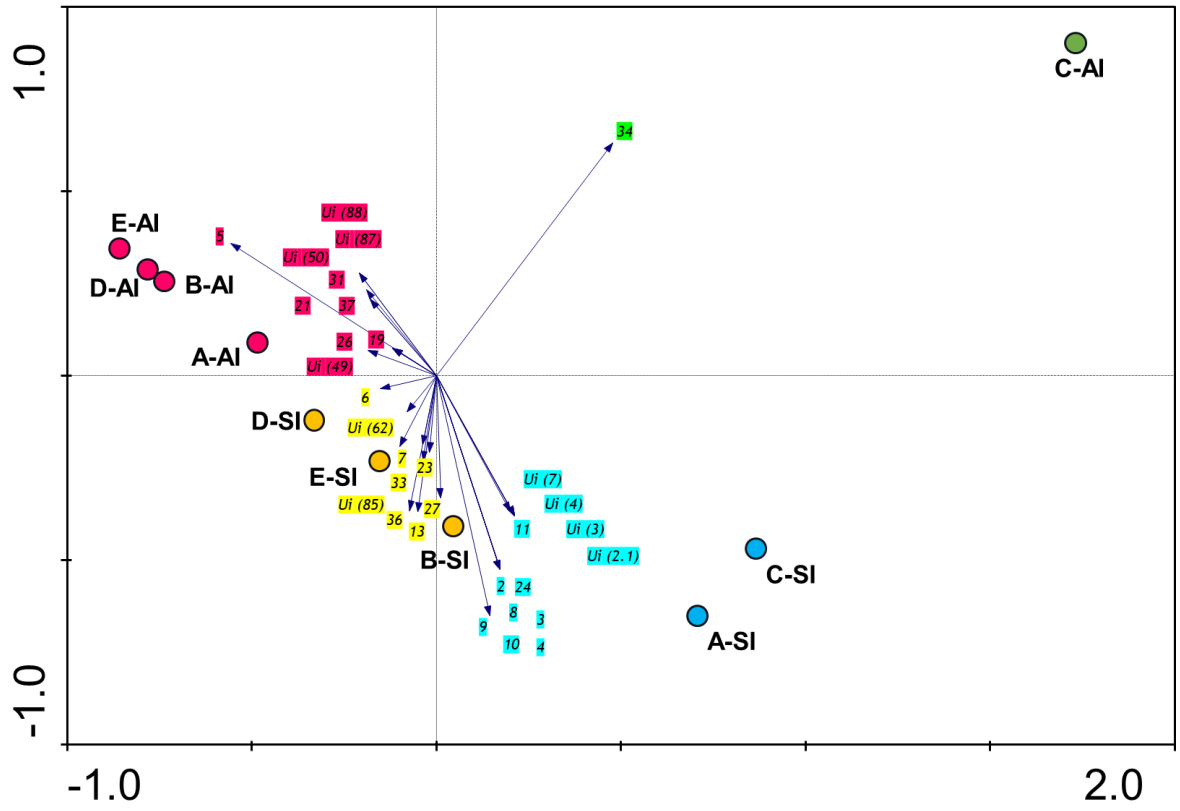


Figure 3-31. Principal component analysis – inter-species correlate – of Psa profiles from each Portuguese orchard in both spring and autumn. Pink AI: autumn isolates from orchard B, D and E; Yellow: spring isolates from orchard B, D and E; Light blue SI: spring isolates from orchard A and C; Green AI: autumn isolates from orchard C. Numbers correspond to Psa profiles. Colours identify Psa profiles that have more weight in each season.

3.3.3 Phylogenetic analysis inferred from the MultiLocus Sequence Typing scheme (MLST)

A phylogenetic tree was constructed from the alignment of the concatenated partial sequences from four housekeeping genes *gapA*, *gltA*, *gyrB* and *rpoD* of 46 strains (Figure 3-32); twenty -nine were Psa isolates from Portuguese orchards (this study) and 17 were reference strains (Table 2.7.3-IV). Due to time constraints, it was only possible to determine the sequence of the four genes in 29 strains. From those, eighteen were isolated from orchard A, six from orchard B, one from orchard C, three from orchard D and one was from orchard E.

Most of the Psa strains isolated in this study were clustered in a discrete group with the reference strains of all Psa biovars, along with the reference strains of *P. syringae* pv. *theae*, *P. syringae* pv. *monosporum* and *P. syringae* pv. *tomate*. Nevertheless, some dissimilarity was found within this previously described cluster that was sub-divided into two groups: the first included the reference strains from Psa biovars 1 and 3 and most of the Psa isolates in this study. This MLST-based analysis was not able to discriminate between Psa1 and Psa3 populations (Figure 3-32). These results were in

accordance with studies that characterized of Psa populations based on MLST scheme (Balestra *et al.*, 2010; Moura *et al.*, 2015; Renzi *et al.*, 2012).

The second cluster included three Psa isolates from this study, KW74, KW146 and KW170, closely related with Psa biovars 2 and 4. Considering that KW74, KW146 and KW170 isolates were identify as Psa through the duplex-PCR analysis (Gallelli *et al.*, 2011), this cluster may represent a new Psa population. Actually, a new population was recently identified in isolates from Japan - Psa biovar 5 (Fujikawa & Sawada, 2016). In order to confirm the existence of this new population, further studies based on the genetic and pathogenicity characterization of the isolates will be performed.

Curiously, strain KW503 (recovered from orchard E) and strain KW541 (recovered from orchard D) were not include in any of the previously identified clusters. The isolate KW503 was positioned closer to *P. syringae* pv. *tomate* (CFBP 2212 strain) and *P. syringae* pv. *phaseolicola* (CFBP 1390 strain) while KW541 was positioned closer to *P. viridiflava* (CFBP 2107 strain). Since these isolates were identified as Psa according to the duplex-PCR analysis (Gallelli *et al.*, 2011), these results come as a surprise. Nevertheless, we propose hypotheses to explain these discrepancies: a) these isolates were not Psa, suggesting that duplex-PCR may present some specificity problems in relation with Psa identification; b) an error occurred in the identification analysis or c) these isolates may be, in fact, Psa and could represent a new population, distinct from the others. Further studies are needed to assess the veracity of these hypotheses.

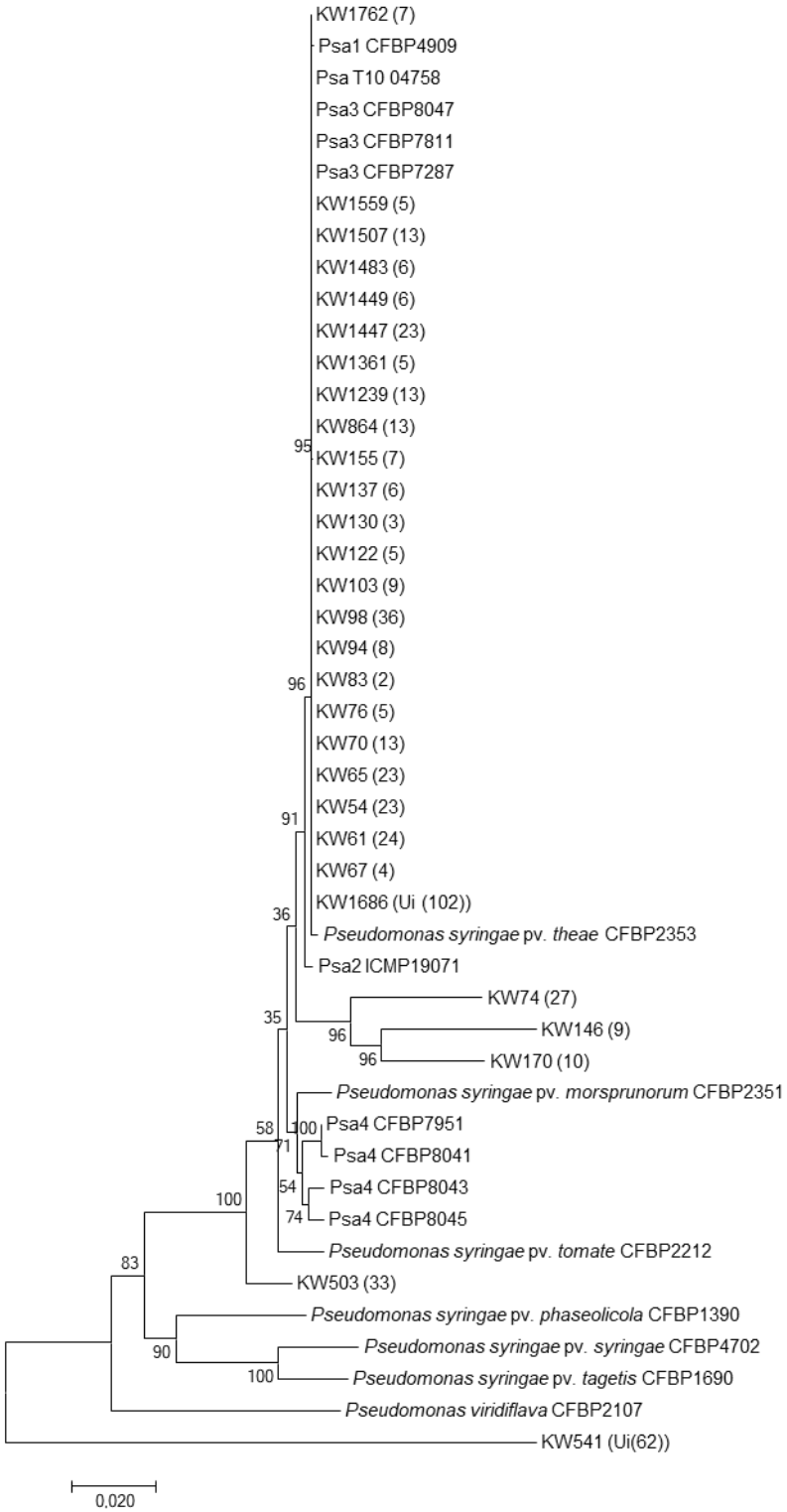


Figure 3-32. Neighbour joining tree constructed with the concatenated partial sequences of four housekeeping genes (*gapA, gltA, gyrB* and *rpoD*). Used strains are described in Table 2.7.3-IV. The scale bar represents the number of nucleotide substitutions per site. Percentage of bootstrap scores obtained for 1000 replicates are indicated at each node.

3.3.3.1 Soil and water has potential environmental reservoirs

Several Psa profiles from soil and water samples recovered from the studied orchards were inferred from BOX profiling. In general, the Psa profiles obtained from these reservoirs were distinct from the Psa profiles found in plant isolates (see Figure 3-7, Figure 3-13, Figure 3-22 and Figure 3-27). However, two Psa profiles were identified in both soil and plant isolates, namely Psa profile 4 – recovered from AI and EP SI, respectively; and Psa profile 5 – recovered from AI and SI/AI, respectively. As referred above, Psa profile 5 was widely distributed in all orchards, being the most abundant Psa profile in both Northern and Centre regions. Nevertheless, this Psa population was not identified in soil isolates from orchard D and E, where it was dominant amongst plant isolates. Since the introduction of the disease was recent in these orchards, it is possible that these environments are not yet colonized by Psa.

No match was found between Psa isolates from water samples, and from plant samples. Nevertheless, a water Psa isolate – KW1682 (representing Psa profile Ui(102)) recovered from orchard B, was clustered together with Psa 1 and Psa 3 biovars. This result confirms the presence of Psa strain in irrigation water that was filtered directly from the river (in this case).

Overall, our results support that both soil and irrigation water should be considered reservoirs for Psa, and included in the management measures to avoid dispersal of Psa within and between orchards.

4. Conclusions

Five distinct orchards, located in North and Centre of Continental Portugal, were studied in order to characterize the genetic diversity of Psa populations over time. Detection of Psa in orchard C was firstly reported in this study.

The fingerprinting analysis inferred from the BOX-PCR methodology of Psa isolates recovered from each studied orchard, demonstrated that the Psa populations present in Portuguese orchards were heterogeneous. This heterogeneity was found within orchards and between orchards, reported by the presence of unique and common Psa profiles. Indeed, not a single Psa profile was common to all orchards.

Furthermore, the structure of Psa populations varied over time in the same plant. The diversity among Psa populations determined in spring was higher when compared to those determined in autumn. The decrease in diversity of Psa in autumn was accompanied by an increase of dominant populations, such Psa profile 5, suggesting that this strain could be more resilient or better adapted to summer conditions. In addition, orchards from the North region presented higher Psa diversity than the orchards from the Centre region, where the presence of dominant profiles was more evident, probably due to a selection influenced by climatic conditions.

Beside differences on the geographical locations (climatic conditions), also the location in leaf's affected Psa diversity. Higher diversity was found among epiphytic Psa populations when compared to endophytic Psa populations.

Importantly, Psa strains were identified in both soil and water isolates, suggesting that these environments provide conditions for Psa persistence and must be considered a probable reservoir for Psa.

In conclusion, this study evidenced the co-existence of several Psa populations in the studied Portuguese orchards. Some of these Psa populations varied with time while other were persistently recovered.

5. Future perspectives

In order to complete the genetic diversity characterization of Psa populations isolated from the studied Portuguese orchards, phylogenetic analysis of representative strains selected from the previously established groups based on the fingerprinting analysis most be concluded. Since MLST-based analysis was not able to discriminate between Psa1 and Psa3 populations, additional analysis need to be performed to confirm the biovar of the Psa isolates recovered from the study orchards.

In addition, further studies based on the genetic and pathogenicity characterization of the Psa strains KW74, KW146 and KW170 (which were grouped in a distinct cluster by the phylogenetic analyses) should be performed to assess the possibility of identifying a new Psa population. Also strains KW503 and KW541, which were not included in any of the clusters determined in the phylogenetic analyses, needs to be the subject to additional studies to assess a more accurate characterization of the strains.

Our results identified soil and water as potential Psa environmental reservoirs. These evidences should be considered when design more efficient strategies of Psa management and control. Furthermore, additional studies on the ability of Psa strains recovered from soil and water samples to survival in those reservoirs and their capability to cause plant infections should be conducted to determine the real impact of those environments as reservoirs in Psa life cycle.

Since this studied reported the presence of dominant Psa strains, apparently well adapted to higher temperatures and less humidity, and also related with the orchards presenting higher incidence of disease, virulence tests should be performed to assess their virulent potential compared with reference strains and less abundant ones. From these results, a correlation between the fingerprinting profile and virulence could be drawn and included in more efficient management strategies adapting cultural practices to the profile of disease evolution and severity.

6. Bibliography

Abelleira, A., Ares, A., Agu, O., Picoaga, A., López, M. M., & Mansilla, P. (2014). Current situation and characterization of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Galicia (northwest, Spain). *Plant Pathology*, *63*, 691–699. https://doi.org/10.1111/ppa.12125

Abelleira, A., Ares, A., Aguin, O., Peñalver, J., Morente, M. C., López, M. M., Sainz, M. J., & Mansilla, J. P. (2015). Detection and characterization of *Pseudomonas syringae* pv. *actinidifoliorum* in kiwifruit in Spain. *Journal of Applied Microbiology*, *119*(6), 1659–1671. https://doi.org/10.1111/jam.12968

Abelleira, A., López, M. M., Peñalver, J., Aguín, O., Mansilla, J. P., Picoaga, A., & García, M. J. (2011). First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Spain. *Plant Disease*, *95*(12), 1583. https://doi.org/10.1094/PDIS-06-11-0537

Balestra, G. M., Mazzaglia, A., Quattrucci, A., Renzi, M., Ricci, L., & Rossetti, A. (2009). Increased spread of bacterial canker of kiwifruit in Italy. *Informatore Agrario*, *65*(24), 58–60. https://doi.org/10.1071/DN09014

Balestra, G. M., Renzi, M., & Mazzaglia, A. (2010). First report of bacterial canker of *Actinidia deliciosa* caused by *Pseudomonas syringae* pv. *actinidiae* in Portugal. *New Disease Reports*, *22*(10), 588–2044.

Bartoli, C., Lamichhane, J. R., Berge, O., Guilbaud, C., Varvaro, L., Balestra, G. M., Vinatzer, B. A., & Morris, C. E. (2015). A framework to gauge the epidemic potential of plant pathogens in environmental reservoirs: The example of kiwifruit canker. *Molecular Plant Pathology*, *16*(2), 137–149. https://doi.org/10.1111/mpp.12167

Bartoli, C., Roux, F., & Lamichhane, J. R. (2016). Molecular mechanisms underlying the emergence of bacterial pathogens : an ecological perspective. *Molecular Plant Pathology*, *17*(2), 303–310. https://doi.org/10.1111/mpp.12284

Bastas, K. K., & Karakaya, A. (2012). First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Turkey. *Plant Disease*, *96*(3), 452.

Biondi, E., Galeone, A., Kuzmanović, N., Ardizzi, S., Lucchese, C., & Bertaccini, A. (2013). *Pseudomonas syringae* pv. *actinidiae* detection in kiwifruit plant tissue and bleeding sap. *Annals of Applied Biology*, *162*(1), 60–70. https://doi.org/10.1111/aab.12001

Butler, M. I., Stockwell, P. A., Black, M. A., Day, R. C., Lamont, I. L., & Poulter, R. T. M. (2013).

Pseudomonas syringae pv. *actinidiae* from recent outbreaks of kiwifruit bacterial canker belong to different clones that originated in China. *Plos One*, *8*(2), e57464.

Cameron, A., & Sarojini, V. (2014). *Pseudomonas syringae* pv . *actinidiae* : chemical control , resistance mechanisms and possible alternatives. *Plant Pathology*, *63*, 1–11. https://doi.org/10.1111/ppa.12066

Chapman, J. R., Taylor, R. K., Weir, B. S., Romberg, M. K., Vanneste, J. L., Luck, J., & Alexander, B. J. R. (2012). Phylogenetic Relationships Among Global Populations of *Pseudomonas syringae* pv . *actinidiae*. *Phytopathology*, *102*, 1034–1044.

Chicau, G., & Costa, J. (2008). Doenças na Actinídea. *DRAPN-N - Divisão de Protecção E Controlo Fitossanitário*.

Cooksey, D. A. (1994). Molecular mechanisms of copper resistance and accumulation in bacteria. *FEMS Microbiology Reviews*, *14*(4), 381–386.

Cunty, A., Cesbron, S., Poliakoff, F., & Jacques, M. (2015). Origin of the Outbreak in France of *Pseudomonas syringae* pv . *actinidiae* Biovar 3, the Causal Agent of Bacterial Canker of Kiwifruit, Revealed by a Multilocus Variable-Number Tandem-Repeat Analysis. *Applied and Environmental Microbiology*, *81*(19), 6773–6789. https://doi.org/10.1128/AEM.01688-15

Cunty, A., Poliakoff, F., Rivoal, C., Cesbron, S., Saux, M. F., Lemaire, C., Jacques, M. A., Manceau, C., & Vanneste, J. L. (2015). Characterization of *Pseudomonas syringae* pv. *actinidiae* (Psa) isolated from France and assignment of Psa biovar 4 to a de novo pathovar: *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov. *Plant Pathology*, *64*, 582–596. https://doi.org/10.1111/ppa.12297

DGAV. (2014). Plano de Ação Nacional para o Controlo da Psa. Lisboa: Ministério da Agricultura e do Mar.

Donati, I., Buriani, G., Cellini, A., Mauri, S., Costa, G., & Spinelli, F. (2014). New insights on the bacterial canker of kiwifruit *(Pseudomonas syringae* pv. *actinidiae*). *Journal of Berry Research*, *4*(2), 53–67.

Eevers, N., Beckers, B., Op de Beeck, M., White, J. C., Vangronsveld, J., & Weyens, N. (2016). Comparison between cultivated and total bacterial communities associated with *Cucurbita pepo* using cultivation-dependent techniques and 454 pyrosequencing. *Systematic and Applied Microbiology*, *39*(1), 58–66. https://doi.org/10.1016/j.syapm.2015.11.001

Elberse, K. E. M., Nunes, S., Sá-Leão, R., van der Heide, H. G. J., & Schouls, L. M. (2011). Multiple-locus variable number tandem repeat analysis for *Streptococcus pneumoniae*: comparison with PFGE and MLST. *PloS One*, *6*(5), e19668.

EPPO. (2011a). First report of *Pseudomonas syringae* pv. *actinidiae* in Chile. Retrieved from http://archives.eppo.int/EPPOReporting/2011/Rse-1103.pdf

EPPO. (2011b). First report of *Pseudomonas syringae* pv. *actinidiae* in Switizerland.

EPPO. (2012a). Pest Risk Analysis for *Pseudomonas syringae* pv. *actinidiae*.

EPPO. (2012b). Revision of EPPO Standard PM 1/2 EPPO A1 and A2 Lists of pests recommended for regulation as quarantine pests. Standards approved by EPPO Council in 2012-09. Retrieved July 20, 2017, from https://www.eppo.int/STANDARDS/council2016.htm

EPPO. (2014). PM 7/120 *Pseudomonas syringae* pv . *actinidiae*. https://doi.org/10.1111/epp.12171

Everett, K. R., Taylor, R. K., Romberg, M. K., Rees-george, J., Fullerton, R. A., Vanneste, J. L., & Manning, M. A. (2011). First report of *Pseudomonas syringae* pv . *actinidiae* causing kiwifruit bacterial canker in New Zealand. *Australasian Plant Disease Notes*, *6*, 67–71. https://doi.org/10.1007/s13314-011-0023-9

FAOSTAT. (2017). Food and agriculture organization of the United Nations - Statistic division. Retrieved from http://www.fao.org/faostat/en/#data/QC/visualize

Félix, A., & Cavaco, M. (2004). Caracterização do ecossistema agrário da cultura da actinídea (*Actinidia deliciosa* A.Chev.) (*Actinidiaceae*). *Direcção-Geral de Protecção Das Culturas*, 1–8.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*(4), 783–791.

Ferguson, A. R. (2013). Kiwifruit: the wild and the cultivated plants. *Advances in Food and Nutrition Research*, *68*, 15–32.

Ferrante, P., Fiorillo, E., Marcelletti, S., Marocchi, F., Mastroleo, M., Simeoni, S., & Scortichini, M. (2012). The importance of the main colonization and penetration sites of *Pseudomonas syringae* pv. *actinidiae* and prevailing weather conditions in the developement of epidemics in yellow kiwifruit, recently observed in central Italy. *Journal of Plant Pathology*, *94*, 455–461.

Ferrante, P., & Scortichini, M. (2009). Identification of *Pseudomonas syringae* pv. *actinidiae* as causal agent of bacterial canker of yellow kiwifruit (*Actinidia chinensis* Planchon) in central Italy. *Journal of Phytopathology*, *157*(11-12), 768–770.

Ferrante, P., & Scortichini, M. (2010). Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. *Plant Pathology*, *59*(5), 954–962.

Ferrante, P., & Scortichini, M. (2014). Frost promotes the pathogenicity of *Pseudomonas syringae* pv.

actinidiae in *Actinidia chinensis* and *A. deliciosa* plants. *Plant Pathology*, *63*(1), 12–19.

Frampton, R. A., Pitman, A. R., & Fineran, P. C. (2012). Advances in bacteriophage-mediated control of plant pathogens. *International Journal of Microbiology*, *2012*, 1–8. https://doi.org/10.1155/2012/326452

Frampton, R. A., Taylor, C., Moreno, A. V. H., Visnovsky, S. B., Petty, N. K., Pitman, A. R., & Fineran, P. C. (2014). Identification of bacteriophages for the biocontrol of the kiwifruit canker phytopathogen *Pseudomonas syringae* pv. *actinidiae*. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.00062-14

Fujikawa, T., & Sawada, H. (2016). Genome analysis of the kiwifruit canker pathogen *Pseudomonas syringae* pv . *actinidiae* biovar 5. *Nature Scientific Reports*, 1–11. https://doi.org/10.1038/srep21399

Gallelli, A., L'aurora, A., & Loreti, S. (2011). Gene sequence analysis for the molecular detection of *Pseudomonas syringae* pv. *actinidiae*: developing diagnostic protocols. *Journal of Plant Pathology*, *93*(2), 425–435.

Garcia, E. (2015). Variabilidade genética e fenotípica de *Pseudomonas syringae* pv . *actinidiae* , agente causal do Cancro da Actinídea , na Região de Entre Douro e Minho.

Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F., & Grimont, P. A. D. (1999). DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov.(ex Sutic and Dowson 1959). *International Journal of Systematic and Evolutionary Microbiology*, *49*(2), 469–478.

Gillings, M., & Holley, M. (1997). Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. *Letters in Applied Microbiology*, 17–21.

Green, S. K., Schroth, M. N., Cho, J. J., Kominos, S. D., & Vitanza-Jack, V. B. (1974). Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa. Applied Microbiology*, *28*(6), 987–991.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. https://doi.org/citeulike-article-id:691774

Han, H. S., Koh, Y. J., Hur, J. S., & Jung, J. S. (2003). Identification and characterization of coronatine-producing *Pseudomonas syringae* pv. *actinidiae*. *Journal of Microbiology and Biotechnology*, *13*(1), 110–118.

Hill, M. O. (1973). Diversity and evenness: a unifying notation and its consequences. *Ecology*, *54*(2), 427–432.

Holeva, M. C., Glynos, P. E., & Karafla, C. D. (2015). First report of bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* in Greece. *Plant Disease*, *99*, 723. https://doi.org/10.1094/PDIS-07-14-0738-PDN

Huang, S., Ding, J., Deng, D., Tang, W., Sun, H., Liu, D., Zhang, L., Niu, X., Zhang, X., & Meng, M. (2013). Draft genome of the kiwifruit *Actinidia chinensis. Nature Communications*, *4*.

INE. (2017). Estatísticas Agrícolas 2016. *Instituto Nacional de Estatística, I.P.* https://doi.org/0079-4139

Jukes, T. H., Cantor, C. R., & Munro, H. N. (1969). Evolution of protein molecules. *Mammalian Protein Metabolism*, *3*(21), 132.

Khandan, H. A. N., Worner, S. P., Jones, E. E., Villjanen-Rollinson, S. L. H., Gallipoli, L., Mazzaglia, A., & Balestra, G. M. (2013). Predicting the potential global distribution of *Pseudomonas syringae* pv. *actinidiae* (Psa). *New Zealand Plant Protection Conference*, 12–15.

Koh, Y. J., Chung, H. J., Cha, B. J., & Lee, D. H. (1994). Outbreak and spread of bacterial canker in kiwifruit. *Korean Journal of Plant Pathology (Korea Republic)*.

Koh, Y. J., & Nou, I. S. (2002). DNA Markers for Identification of *Pseudomonas syringae* pv. *actinidiae*. *Molecules and Cells*, *13*(2), 309–314.

Koh, Y. J., Park, S. Y., & Lee, D. H. (1996). Characteristics of bacterial canker of kiwifruit occurring in Korea and its control by trunk injection. *Korean Journal of Plant Pathology (Korea Republic)*.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874.

Lallo, G. Di, Evangelisti, M., Mancuso, F., Ferrante, P., Marcelletti, S., Tinari, A., Superti, F., Migliore, L., Addabbo, P. D., Frezza, D., Scortichini, M., & Thaller, M. C. (2014). Isolation and partial characterization of bacteriophages infecting *Pseudomonas syringae* pv. *actinidiae* , causal agent of kiwifruit bacterial canker. *Journal of Basic Microbiology*, *54*, 1–12. https://doi.org/10.1002/jobm.201300951

Louws, F. J. ., Fulbright, D. W. ., Stephens, C. T., & De Bruijn, F. J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology*, *60*(7), 2286–2295.

Lupski, J. R., & Weinstock, G. M. (1992). Short , Interspersed Repetitive DNA Sequences in Prokaryotic Genomes. *American Society for Microbiology*, *174*(14), 4525–4529.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., & Caugant, D. A. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences*, *95*(6), 3140–3145.

Marcelletti, S., Ferrante, P., Petriccione, M., Firrao, G., & Scortichini, M. (2011). *Pseudomonas syringae* pv. *actinidia*e draft genomes comparison reveal strain-specific features involved in adaptation and virulence to *Actinidia* species. *PLoS One*, *6*(11), e27297.

Marques, A. S. A., Marchaison, A., Gardan, L., & Samson, R. (2008). BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae* - *P . viridiflava* group. *Genetics and Molecular Biology*, *31*(1), 106–115.

Mazarei, M., & Mostofipour, P. (1994). First report of bacterial canker of kiwifruit in Iran. *Plant Pathology*, *43*(6), 1055–1056.

Mazzaglia, A., Renzi, M., & Balestra, G. M. (2011). Comparison and utilization of different PCR-based approaches for molecular typing of *Pseudomonas syringae* pv. *actinidiae* strains from Italy. *Canadian Journal of Plant Pathology*, *33*(1), 8–18.

Mohan, S. K., & Shaad, N. W. (1987). An improved agar plating assay for detecting *Pseudomonas syringae* pv*. syringae* and *P. s*. pv. *phaseolicola* in contaminated bean seed.

Monteil, C. L., Cai, R., Liu, H., Llontop, M. E. M., Leman, S., Studholme, D. J., Morris, C. E., & Vinatzer, B. A. (2013). Nonagricultural reservoirs contribute to emergence and evolution of *Pseudomonas syringae* crop pathogens. *New Phytologist*, *199*, 800–811. https://doi.org/10.1111/nph.12316

Moreira, J. F., & Coutinho, C. (2014). Avisos Agrícolas. *Circular nº:17/2014*.

Moura, L., Garcia, E., Ares, A., Abelleira, A., & Mansilla, P. (2015). Identificação e caracterização de *Pseudomonas syringae* pv . *actinidiae* (Psa) na Região do Entre Douro e Minho (Portugal). *Sociedade de Ciências Agrárias de Portugal*, *38*(2), 196–205.

Nakajima, M., Yamashita, S., Takikawa, Y., Tsuyumu, S., Hibi, T., & Goto, M. (1995). Similarity of streptomycin resistance gene (s) in *Pseudomonas syringae* pv. *actinidiae* with *strA* and *strB* of plasmid RSF1010. *Japanese Journal of Phytopathology*, *61*(5), 489–492.

NCBI. (2017). Taxonomy browser (*Actinidia*). Retrieved June 15, 2017, from https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=3624&lvl=3&lin=f&keep=1&srchmode=1&unlock

Pattemore, D. E., Goodwin, R. M., McBrydie, H. M., Hoyte, S. M., & Vanneste, J. L. (2014). Evidence of

the role of honey bees (*Apis mellifera*) as vectors of the bacterial plant pathogen *Pseudomonas syringae*. *Australasian Plant Pathology*, *43*(5), 571–575.

Pielou, E. C. (1966). Species-diversity and pattern-diversity in the study of ecological succession. *Journal of Theoretical Biology*, *10*(2), 370–383.

Rees-George, J., Vanneste, J. L., Cornish, D. A., Pushparajah, I. P. S., Yu, J., Templeton, M. D., & Everett, K. R. (2010). Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S–23S rDNA intertranscribed spacer region and comparison with PCR primers based on other gene regions. *Plant Pathology*, *59*(3), 453–464.

Reglinski, T., Vanneste, J. L., Wurms, K., Gould, E., Spinelli, F., & Rikkerink, E. (2013). Using fundamental knowledge of induced resistance to develop control strategies for bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv . *actinidiae*. *Frontiers in Plant Science*, *4*(24), 2009–2012. https://doi.org/10.3389/fpls.2013.00024

Renzi, M., Mazzaglia, A., & Balestra, G. M. (2012). Widespread distribution of kiwifruit bacterial canker caused by the European *Pseudomonas syringae* pv. *actinidiae* genotype in the main production areas of Portugal. *Phytopathologia Mediterranea*, *51*(2), 402–409.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Sarkar, S. F., & Guttman, D. S. (2004). Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology*, *70*(4), 1999–2012.

Sawada, H., Suzuki, F., Matsuda, I., & Saitou, N. (1999). Phylogenetic analysis of *Pseudomonas syringae* pathovars suggests the horizontal gene transfer of *argK* and the evolutionary stability of *hrp* gene cluster. *Journal of Molecular Evolution*, *49*(5), 627–644.

Scortichini, M. (1994). Occurrence of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Italy. *Plant Pathology*, *43*(6), 1035–1038.

Scortichini, M., Marcelletti, S., Ferrante, P., Petriccione, M., & Firrao, G. (2012). Pathogen profile *Pseudomonas syringae* pv . *actinidiae*: a re-emerging, multi-faceted, pandemic pathogen. *Molecular Plant Pathology*, 1–10. https://doi.org/10.1111/J.1364-3703.2012.00788.X

Serizawa, S., & Ichikawa, T. (1993). Epidemiology of bacterial canker of kiwifruit, 3: The seasonal changes of bacterial population in lesions and of its exudation from lesion. *Annals of the Phytopathological Society of Japan (Japan)*, *59*, 469–476.

Serizawa, S., Ichikawa, T., & Suzuki, H. (1994). Epidemiology of bacterial canker of kiwifruit. 5. Effect of

infection in fall to early winter on the disease development in branches and trunk after winter. *Annals of the Phytopathological Society of Japan*, *60*(2), 237–244.

Serizawa, S., Tchikawa, T., Takikawa, Y., Tsuyumu, S., & Goto, M. (1989). Occurrence of Bacterial Canker of Kiwifruit in Japan. *Japanese Journal of Phytopathology*, *55*(4), 427–436.

Simpson, G. G. (1960). Notes on the measurement of faunal resemblance. *American Journal of Science*, *258*(2), 300–311.

Šmilauer, P., & Lepš, J. (2014). Multivariate analysis of ecological data using CANOCO 5. Cambridge university press.

Sofia, J. (2003). Entre Douro e Minho e Beira Litoral: Doenças do Kiwi. *Programa Agro*.

Spinelli, F., Donati, I., Vanneste, J. L., Costa, M., & Costa, G. (2011). Real time monitoring of the interactions between *Pseudomonas syringae* pv. *actinidiae* and *Actinidia species. Acta Horticulturae*, *913*, 461–465.

Stefani, E., & Giovanardi, D. (2012). Dissemination of *Pseudomonas syringae* pv. *actinidiae* through pollen and its epiphytic life on leaves and fruits. *Phytopathologia Mediterranea*, *50*(3), 489–496.

Sundin, G. W. (2007). Genomic insights into the contribution of phytopathogenic bacterial plasmids to the evolutionary history of their hosts. *Annual Review of Phytopathology*, *45*, 129–151.

Takikawa, Y., Serizawa, S., Ichikawa, T., Goto, M., & Takanashi, K. (1989). *Pseudomonas syringae* pv. **actinidiae** pv. nov.: The causal bacterium of canker of Kiwifruit in Japan. *Annals of the Phytopathological Society of Japan*, *55*(4), 437–444.

Tamura, K., Imamura, M., Yoneyama, K., Kohno, Y., Takikawa, Y., Yamaguchi, I., & Takahashi, H. (2002). Role of phaseolotoxin production by *Pseudomonas syringae* pv. *actinidiae* in the formation of halo lesions of kiwifruit canker disease. *Physiological and Molecular Plant Pathology*, *60*(4), 207–214.

Testolin, R., Huang, H.-W., & Ferguson, A. R. (2016). *The Kiwifruit Genome*. Springer.

Tontou, R., Giovanardi, D., & Stefani, E. (2014). Pollen as a possible pathway for the dissemination of *Pseudomonas syringae* pv. *actinidiae* and bacterial canker of kiwifruit. *Phytopathologia Mediterranea*, *53*(2), 333.

Turner, S., Pryer, K. M., Miao, V. P. W., & Palmer, J. D. (1999). Investigating Deep Phylogenetic Relationships among *Cyanobacteria* and plastids by small subunit rRNA Sequence Analysis. *Journal of Eucaryotic Microbiology*, *46*, 327–338.

Vanneste, J. L. (2013). Recent progress on detecting, understanding and controlling *Pseudomonas*

syringae pv. *actinidiae*: a short review. *New Zealand Plant Protection*, *66*, 170–177.

Vanneste, J. L., Giovanardi, D., Yu, J., Cornish, D. A., Kay, C., Spinelli, F., & Stefani, E. (2011). Detection of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit pollen samples. *New Zealand Plant Protection*, *64*, 246–251.

Vanneste, J. L., Kay, C., Onorato, R., Yu, J., Cornish, D. A., Spinelli, F., & Max, S. (2011). Recent Advances in the Characterisation and Control of *Pseudomonas syringae* pv . *actinidiae* , the Causal Agent of Bacterial Canker on Kiwifruit. *Acta Horticulturae*, *913*, 443–456.

Vanneste, J. L., Poliakoff, F., Audusseau, C., Cornish, D. A., Paillard, S., Rivoal, C., & Yu, J. (2011). First report of *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit in France. *Plant Disease*, *95*(10), 1311.

Vanneste, J. L., Spinelli, F., Fiorentini, L., Yu, J., Cellini, A., Cornish, D. A., Donati, I., Costa, G., Moffat, B., & Felman, C. (2012). Reducing susceptibility of kiwifruit plant to *Pseudomonas syringae* pv. *actinidiae* by manipulating the plant metabolism using elicitors and hormones. A snapshot of Psa (*Pseudomonas syringae* pv. *actinidiae*). In *New Zealand Plant Protection Society Symposium*.

Vanneste, J. L., Yu, J., Cornish, D. A., Max, S., & Clark, G. (2011). Presence of *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit, on symptomatic and asymptomatic tissues of kiwifruit. *New Zealand Plant Protection*, *64*, 241–245.

Vanneste, J. L., Yu, J., Cornish, D. A., New, T., & Zealand, N. (2013). Identification , Virulence , and Distribution of Two Biovars of *Pseudomonas syringae* pv . *actinidiae* in New Zealand. *Plant Disease*, *97*(6), 708–719. https://doi.org/10.1094/PDIS-07-12-0700-RE

Vanneste, J. L., Yu, J., Cornish, D. A., Oldham, J. M., Spinelli, F., Pattemore, D. E., Moffat, B., & d'Accolti, A. (2013). Survival of *Pseudomonas syringae* pv. *actinidiae* in the Environment. In *I International Symposium on Bacterial Canker of Kiwifruit 1095* (pp. 105–110).

Versalovic, J., Schneider, M., Bruijn, F. J. de;, & Lupski, J. R. . (1994). Genomic fingerprinting of Bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, *5*, 25–40.

Whittaker, R. H. (1977). Evolution of species diversity in land communities [Birds and vascular plants]. *Evolutionary Biology*.