IMPLEMENTATION of IPM STRATEGIES to CONTROL BACTERIAL DISEASES of STONE FRUITS and NUTS CAUSED by COPPER RESISTANT/ TOLERANT PSEUDOMONADS and XANTHOMONADS

İREM ALTIN

Doctoral Thesis

Phytopathology
Danışman: Prof. Dr. Mustafa Mirik
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T.C.

TEKİRDAĞ NAMIK KEMAL UNIVERSITY

INSTITUTE of NATURAL and APPLIED SCIENCE

DOCTORAL THESIS

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PHYTOPATHOLOGY

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TEKİRDAĞ-2021

I declare that all the information and results presented in this thesis were obtained by me in accordance with academic and ethical rules, and that I have fully cited all the information in the thesis that are not specific to this study.

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On behalf of the Board of Directors of the Graduate School of Natural and Applied Sciences

Doç.Dr. Bahar UYMAZ

Director of The Institution

ASTRATTO

Tesi di Dottorato

IMPLEMENTAZIONE DI STRATEGIE IPM per CONTROLLARE LE MALATTIE BATTERICHE DI DROMI E FRUTTA A GUSCIO CAUSATE DA PSEUDOMONADI E XANTOMONADI RESISTENTI/ TOLLERANTI AL RAME

İrem ALTIN

Tutore: Prof. Dr. Mustafa MİRİK

Il cancro batterico delle drupacee causato da *Pseudomonas syringae* pv. *syringae* e *Pseudomonas syringae* pv. *morsprunorum* e il mal secco del noce causato da *Xanthomonas arboricola* pv. *juglandis*, sono stati studiati per oltre un secolo e hanno portato a significativi progressi nella nostra comprensione dell'epidemiologia delle malattie batteriche fogliari e del ciclo di vita epifita di questi agenti patogeni. Recenti studi sull'ecologia, la caratterizzazione e l'identificazione dei patogeni sono stati stimolati dall'importanza economica delle malattie e dalla mancanza di misure di controllo efficaci. Una collezione di 54 isolati di *P. syringae* spp. da albicocco e 47 isolati di *X. a.* pv. *juglandis* da noce sono stati studiati utilizzando il fingerprinting genomico mediante reazione a catena della polimerasi ripetitiva (PCR), utilizzando i set di primer ERIC, BOX e REP e l'analisi della sequenza multi-locus (MLSA). La tolleranza al rame di tutti i ceppi è stata valutata osservando la crescita batterica su terreni con aggiunta di solfato di rame e i risultati sono stati confrontati con quelli di uno studio di cinque anni nella stessa area. Questa tesi ha mostrato che la frequenza dei batteri resistenti al rame aumenta dopo ripetuti trattamenti con composti a base di rame. Questi risultati suggeriscono che la selezione di ceppi resistenti al rame può essere una delle principali cause di fallimento nel controllo dopo il trattamento con composti rameici. I batteriofagi, in quanto elementi naturalmente presenti nell'ambiente, potrebbero essere un'opzione praticabile per il controllo di queste malattie batteriche. In questa tesi, dieci nuovi fagi che lisano *P. s.* pv. *syringae* e dieci nuovi fagi che lisano *X. a.* pv. *juglandis* sono stati isolati da frutti, foglie, suolo e acqua di irrigazione di albicocche e noci nel nord Italia. La digestione con endonucleasi di restrizione dei fagi ha rivelato che esistono tre gruppi fagici distinti, designati come gruppi 1, 2 e 3. Nonostante il fatto che tutti i fagi isolati fossero specifici per *X. a.* pv. *juglandis*, un'analisi della gamma ospite di fagi che coinvolge 25 diversi *X. a.* pv. *juglandis* ha rivelato che i fagi possono essere suddivisi in quattro classi in base alla loro capacità di lisare i 25 ceppi considerati. Nei test di laboratorio, tutti i fagi hanno avuto un promettente effetto litico su *P. s.* pv. *syringae* e *X. a.* pv. *juglandis*.

Parole Chiave: albicocco, noce, *Pseudomonas syringae* pv. *syringae*, *Xanthomonas arboricola* pv. *juglandis*, batteriofagi.

ABSTRACT

PhD Thesis

IMPLEMENTATION of IPM STRATEGIES to CONTROL BACTERIAL DISEASES of STONE FRUITS and NUTS CAUSED by COPPER RESISTANT/ TOLERANT PSEUDOMONADS and XANTHOMONADS

İrem ALTIN

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Bacterial canker of stone fruits caused by *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *morsprunorum*, and bacterial blight of walnut caused by *Xanthomonas arboricola* pv. *juglandis*, have been studied for over a century and have resulted in significant advances in our understanding of the epidemiology of foliar bacterial diseases and epiphytic life cycle of these pathogens. Recent studies on the ecology, characterization, and identification of pathogens have been prompted by the economic importance of the diseases and the lack of effective control measures. A collection of 54 *P. syringae* spp. on apricot and 47 *X. a.*pv. *juglandis* strains on walnut were studied using repetitive polymerase chain reaction (PCR) genomic fingerprinting using ERIC, BOX, and REP primer sets, and Multi-Locus Sequence Analysis (MLSA). Copper tolerance of all strains were assessed by observing bacterial growth on copper sulphate-added media, and the results were compared to those of a five-year-old study in the same area. This thesis showed that the frequency of copper resistant bacteria increased after repeated treatments with copper-based compounds. These findings suggest that the selection of copper-resistant strains may be a major cause of control failures after copper bactericide treatment. Bacteriophages, as realistic and environmentally friendly microorganisms, could be a viable option for controlling these bacterial diseases. In this thesis, ten novel phages that lyse *P. s.* pv. *syringae* and ten novel phages that lyse *X. a.* pv. *juglandis* were isolated from apricot and walnut fruits, leaves, soil, and irrigation water in Northern Italy. The restriction endonuclease digestion of phages revealed that there are three distinct phage groups, designated as groups 1, 2, and 3. Despite the fact that all isolated phages were specific to *X. a.* pv. *juglandis*, an analysis of the host range of phages involving 25 different *X. a.* pv. *juglandis* strains revealed that the phages can be divided into four classes based on their ability to lyse the 25 strains considered. In laboratory tests, all phages had a promising lytic effect on *P. s.* pv. *syringae* and *X. a.* pv. *juglandis*. *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* showed promising lytic effect in laboratory trials.

Key words: apricot, walnut, *Pseudomonas syringae* pv. *syringae*, *Xanthomonas arboricola* pv. *juglandis*, molecular characterisation, bacteriophages.

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ABBREVIATIONS

µl	: Microliter
Ng	: Nanogram
GYCA	: Glucose yeast calcium agar
NYA	: Nutrient yeast agar
DNA	: Deoxyribonucleic acid
NJ	: Neighbor joining
MLSA	: Multi-locus sequence analysis
NCBI	: National Center of Biotechnology Information
FAO	: Food and Agriculture Organization

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This thesis is dedicated to my mum and my father who raised me to be whatever I wanted to be.

With love and eternal appreciation.

1. INTRODUCTION

Stone fruits, almond (*Prunus* spp.) (Janick, 2005), and walnut (*Juglans* spp.) (Manning, 1978) crops are among the most economically significant trees worldwide, with varying degrees of cultivation on all continents (FAO,2012) (Table 1.1). The number of countries cultivating these crops has increased in the last decade, resulting in an increase in global acreage (FAO, 2012).

The stone fruits are classified in the cosmopolitan genus *Prunus*, which is part of the large and diverse *Rosaceae* family. Peaches, nectarines, apricots, almonds, plums, and cherries are all classified as stone fruits due to their highly lignified seed capsules (Kole and Abbott, 2012). While peaches and nectarines are the most economically significant stone fruits, with a combined global production of 25.7 million tons (FAOSTAT, 2019), the global production of the apricots are shown in Table 1.2. Turkey is the world's largest producer of apricots, while Italy is the fourth largest producer (FAOSTAT, 2019).

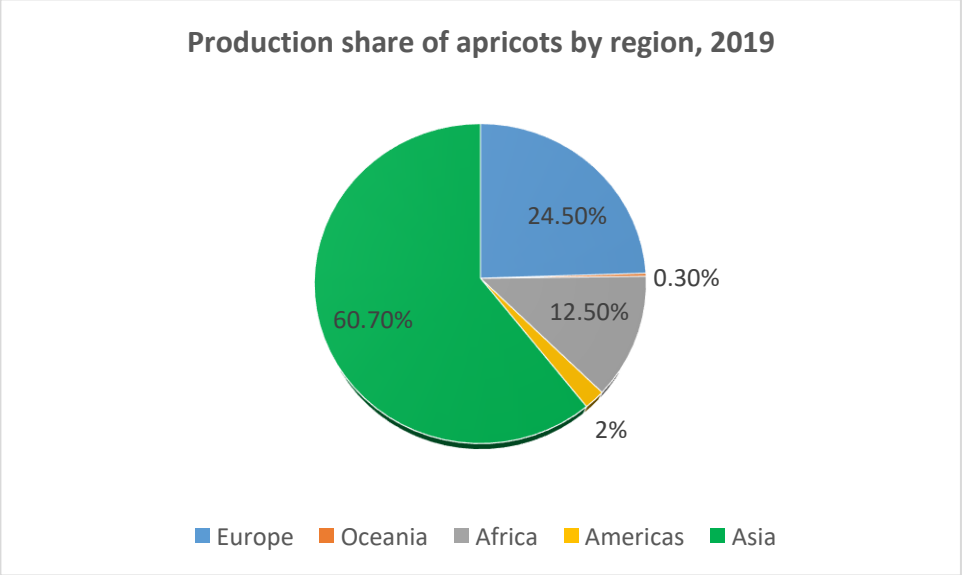

Table 1.1. Worldwide apricot cultivation on all continents

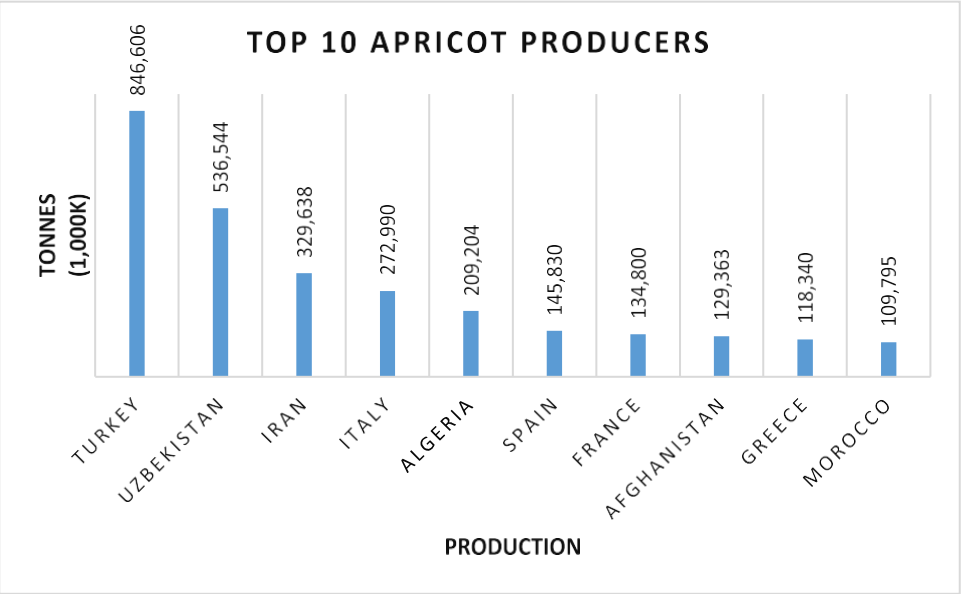

Table 1.2. The global apricot production by countries

The walnut (*J. regia* L.) is the world's most widely distributed nut tree. The Persian walnut, white walnut, English walnut, or common walnut are all common names for the tree. It is a member of the *Juglandaceae* family and is scientifically known as *J. regia*. The global production of walnut is estimated to be around 4,5 million metric tonnes (mMT) (Table 1.3 and 1.4).

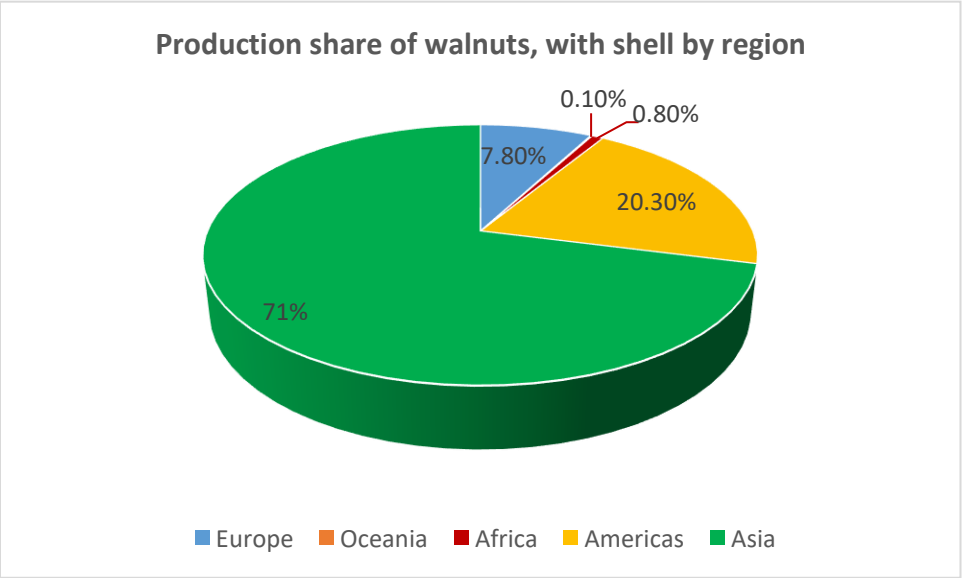

Table 1.3. Worldwide walnut cultivation on all continents

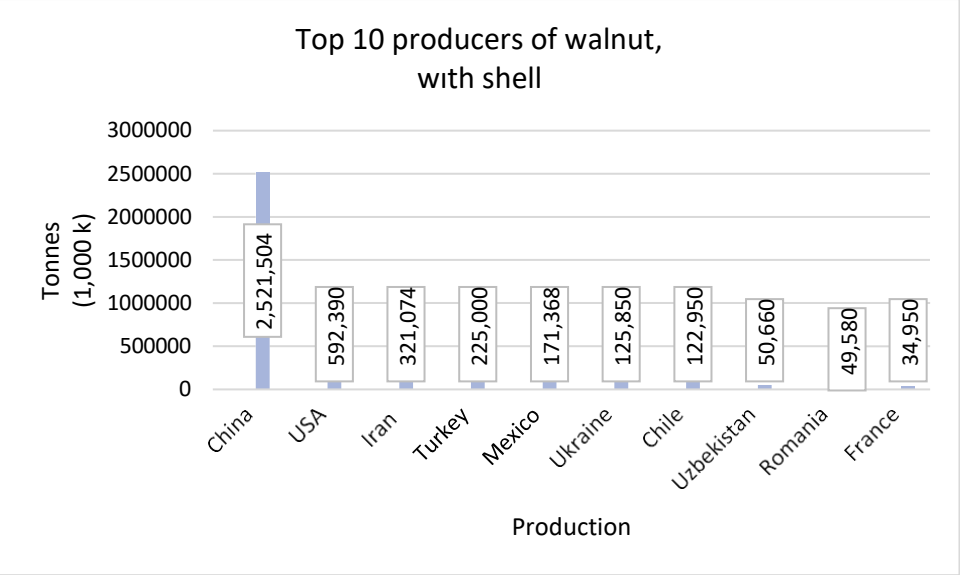

Table 1.4. The global walnut production by countries

Throughout their range of cultivation, *Prunus* and *Juglans* species are frequently attacked by pathogens, resulting in reduced production and significant economic losses. *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum* are the major bacterial pathogens of stone fruit species (Scortichini, 2010). On the other hand, *X. arboricola* has emerged as a significant bacterial pathogen in nuts over the last two decades. Appropriate disease management is critical to minimizing yield losses, which is why numerous recommendations and guidelines for pesticide

rates are included in integrated pest management guides from various countries (Blaauw et al. 2017; DAAM, 2016).

Both bacterial canker and spot on *Prunus* spp. (Smith, 1903) and bacterial blight on *Juglans* spp. (Smith et al. 1912) were described for the first time in the United States during the early twentieth century. Since then, the diseases have spread worldwide (CABI-EPPO. 2001).

The *P. syringae* complex is a collection of plant pathogens found in eight closely related *Pseudomonas* species, including 64 pathovars of *P. syringae* (Young, 2010). These were classified into genomospecies based on DNA: DNA hybridization results. *P. syringae* infecting *Prunus* spp. belong to three pathovars: pv. *syringae*, pv. *morsprunorum* and the more rare pv. *persicae* (Gardan et al. 1999).

Both *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum* are capable of causing bacterial canker in stone fruits. While *P. s.* pv. *syringae* can cause canker on any commercial stone fruit, including pome fruits and herbaceous plants (Gavrilovi, 2009), *P. s.* pv. *morsprunorum* primarily infects sour and sweet cherry, plum (Hattingh and Roos, 1995), and apricot (Hattingh and Roos, 1995; Bultreys and Kaluzna, 2010). Two races of *P. s.* pv. *morsprunorum* have been described based on bacteriophage typing: race 1 (Wormald, 1932), which is pathogenic to cherry, plum, and apricot, and race 2 (Freigoun and Crosse, 1975), which infects cherry (Bultreys and Kaluzna, 2010).

The spring colonization and development of large populations on blossoms initiates the disease cycle of bacterial canker. Blossom populations can grow to be quite large, with 10^4 to 10^6 CFU per blossom, and blast symptoms can appear after extended periods of cool, wet weather or after a frost event. When pathogens attack blossoms, they turn brown and shriveled, and they frequently fall over before fully opening. Frost damage is a known risk factor for bacterial canker infection, and cankers caused by blossom infections are a common symptom. Frost-related damage causes tip dieback symptoms in some cases.

The most common symptoms of bacterial canker on apricot are cankers and necroses on branches and trunks, which are frequently found around spurs, wounds, and branch junctions. The tissue on the branch is sunken, water-soaked, and slightly brown discolored in early infections. Later on, it darkens to a reddish-brown-black color. Orange-brown gummosis is linked to cankers and necroses. When symptoms appear on the leaves, they appear as small

round lesions of varying sizes that are light brown at first and then dark brown; they may be surrounded by a yellowish halo. With time, the necrotized tissue often falls out of the leaf (a condition known as shot-hole symptoms). Sunken brown-black, irregular or regular necroses on immature sweet and sour cherry fruits are well known. Fruit spot symptoms are common on susceptible varieties during the growing season and can appear as water-soaked lesions that turn chocolate-brown. Leaf spot symptoms appear sporadically as well; these spots are surrounded by chlorotic rings early in development, and the spots expand and eventually fall out of the leaves, causing the shothole symptom.

Canker formation is aided by stress events such as freezing temperatures and frost damage, which cause tree weakening and infection susceptibility. Inoculation studies have consistently demonstrated that freeze injury is associated with an increased incidence of infection and canker length (Sobiczewski and Jones, 1992). Canker formation in non-frozen tissue is linked to factors like tissue water-soaking, stem water content, and larger stem diameter (Cao et al. 1999). Cankers that form on scaffold branches and trunks have a sunken appearance and are associated with gummosis. These cankers can grow quite large, girdling branches and trunks and eventually killing entire trees or limbs. Cankers are most commonly caused when a pathogen infiltrates woody tissue after infecting blossoms or dormant buds. *P. syringae* strains can also enter through wounds, and pruning wounds are a particularly problematic entry point for the pathogen in commercial orchards. Dormant buds can be killed by the pathogen as a result of direct infection or systemic spread from a nearby canker, resulting in the "dead-bud" symptom. Trees with a high number of cankers and dead fruit spurs produce fewer flowers, limiting the trees' fruiting potential over time. Furthermore, as with other bacterial fruit tree diseases, like fire blight, young trees are more susceptible to bacterial canker; endophytic infection of these trees is especially important and contributes to rapid decline and death.

There have been numerous reports of bacterial canker in stone fruit orchards worldwide in recent years. *P. s.* pv. *morsprunorum* race 1 and 2 strains were frequently found in cherry and plum orchards in Belgium (Bultreys and Gheysen, 2003; Gilbert et al. 2009). In Turkey, bacterial canker killed nearly 80% of apricot trees in the provinces of Erzurum, Erzincan, and Artvin, and 20% in Malatya (Kotan and Sahin, 2002; Donmez et al. 2010). In addition, *P. syringae* caused leaf-bud and fruit-bud death, as well as branch die-backs, on about 10% of the peach trees grown near Izmir (Ozaktan et al. 2008). *P. s.* pv. *syringae* caused severe twig dieback and plant death in nearly 30% of one-year-old apricot orchards in central Italy

(Scortichini, 2006). In the United States, sweet cherry infections caused by *P. s.* pv. *syringae* have been reported from Oregon (Spotts et al. 2010) and, as previously mentioned, a severe outbreak of bacterial canker caused by *P. s.* pv. *syringae*, *P. s.* pv. *morsprunorum* race 1 and a possible third pathovar has been reported from Michigan (Renick et al. 2008). Bacterial canker has been reported in France, the United Kingdom, Germany, Poland, New Zealand, Lithuania, and other countries for many years, including wild cherry plantations grown for wood (Ménard et al. 2003; Hinrichs-Berger, 2004; Vicente and Roberts, 2007; Janse et al. 2008; Vasinauskiene et al. 2008; Kaluzna et al. 2010a). The disease is observed every year in Poland due to the favorable climate. The most recent severe outbreak occurred in 2007, causing significant economic losses, particularly in sour cherry orchards (Kaluzna et al. 2010b). From infected trees, pathovars and races of *P. s.* pv. *morsprunorum* were isolated.

Pathogenic strains of *X. arboricola* are found in a variety of pathovars (Fischer-Le Saux et al. 2015). Pathovars *pruni*, *corylina*, and *juglandis* are the most economically important in *X. arboricola*. The most important bacterial disease of *Juglans regia* and other *Juglans* species is caused by *X. a.* pv. *juglandis*. *X. a.* pv. *juglandis* is an aerobic, Gram-negative, rod-shaped bacterium (Bradbury, 1986). Bacterial colonies are typically yellow pigmented due to the presence of brominated aryl-polyenes (or xanthomonadins) in the outer-membrane of the bacterial cell walls (Saddler and Bradbury, 2005), which may confer photobiological protection (Poplawsky et al., 2000). Most strains also produce xanthan, an extracellular polysaccharide that gives bacterial colonies a viscous consistency and is important in bacterial survival and plant colonization, and have an optimal growth temperature of 25 °C to 30 °C (Saddler & Bradbury, 2005).

The bacterial walnut blight affects all green tissue on the walnut tree. Surface wetness, particularly due to rainfall, appears to be an important factor in the disease development. On walnut, a high percentage of pistillate flowers and fruits are subjected to infection. The first symptoms are dark green, translucent, or water soaked circular areas. In general, dark brown to black spots appear on new leaves, stems, and the apical portion of fruit. Late-season infections on maturing nuts can darken the hulls, shelles and kernel, rendering them off-grade or unmarketabe. When a developing nut becomes infected, it causes significant economic damage, and in severe infections, many nuts may fall prematurely. Others grow to full size. The kernels of these remaining nuts are frequently blackened, dried, and wrinkled. Pollen from infected catkins may contain *X. a.* pv. *juglandis*. The pathogen overwinters primarily in cankers or

blighted twigs, which produce bacterial exudates in the spring. The bacterium also spends the winter in leaf and catkin buds. Pollen from diseased catkins spread the bacterium to pistillate flowers, resulting in reduced nut set and leaf disease. This perennial source of inoculum explains why walnut blight epidemics can be so severe when only a few cankers are evident. The severity of *X. a.* pv. *juglandis* grows in direct proportion to the timing of flowering.

A new genetic lineage within *X. a.* pv. *juglandis* was identified a few years ago as the causative agent of a new disease known as vertical oozing canker (VOC). Vertical cankers appear on the main trunk, often resulting in trunk deformities. During the summer, infected trees produce black oozing exudates that stain the bark. *X. a.* pv. *juglandis* is also the primary causative agent of a disease complex known as brown apical necrosis. Fungi, especially *Alternaria* and *Fusarium* species, are also associated pathogens. The other bacterial diseases affecting walnut are deep bark canker (*Brenneria rubifaciens*) and shallow bark canker (*Brenneria nigrifluens*). Nonpathogenic *X. a.* pv. *juglandis* strains were also isolated from walnut trees during surveys of French orchards. These strains are unable to cause disease in walnut trees or other plant species. Among the biotic diseases that affect walnut, bacterial blight is regarded as the most serious in all walnut-growing areas.

In the last decade, disease outbreaks of bacterial walnut blight have been reported from Iran (Jami et al. 2005), Spain (Palacio-Bielsa et al 2010), Switzerland (Pothier et al. 2010), Taiwan (Shen et al. 2013), France (Hajri et al. 2010), Iran (Golmohammadi et al. 2002), Lithuania (Burokiene and Pulawska, 2012), and Turkey (Ozaktan et al. 2007). The rate at which these outbreaks are occurring suggests the possibility of future epidemics. To address the consequences of such disease outbreaks, it is critical to first understand the epidemiology of these diseases.

The economic importance of these diseases has prompted recent research into the ecology of pathogens, as well as their characterization and identification. A thorough understanding of the genomic diversity of *P. syringae* and *X. a.* pv. *juglandis* is required for scientists to correctly identify strains, aid in the prevention of future disease outbreaks, and achieve knowledge-informed sustainable disease management in apricot and walnut orchards.

Understanding the origins of infectious disease outbreaks, as well as the evolutionary processes associated with pathogen emergence and spread, are critical areas of research in microbial population biology. Plant diseases have been studied since 750 BC. Nonetheless, it

was not until 1861 that Anton de Bary provided experimental proof beyond any doubt that a causal agent (*Phytophthora infestans*) was to blame for plant losses, *i.e*. potato late blight. The goal of plant pathology at the time was to identify the causal agent, demonstrate pathogenicity, and devise a control strategy. A pathogen lesion on a single leaf may not have a significant economic or ecological impact. However, an epidemic causes significant crop loss. To control the diseases, a plant pathologist must devise methods to control the entire pathogen population. In order to develop rational control strategies, it is critical to understand the population biology of plant pathogens. In general, the focus has not shifted to the present day, despite the fact that our control options and methods have grown more numerous and sophisticated. With the advent of sophisticated molecular methods, large datasets arose, posing analytical challenges but providing a wealth of information that can be used to better understand and control plant diseases.

Because of the phenotypic and genomic heterogeneity of *X. a.* pv. *juglandis* and *P. syringae* pathovars, a multiphasic approach is required to identify these bacteria (López et al. 2010). Since conventional tests are insufficient to distinguish isolates at the pathovar level, similarities and differences between pathovars can be determined using toxin production, genetic techniques (rep-PCR), multilocus sequence analysis (MLSA), and other DNA-based methods.

The study of neutral genetic variation within and among plant pathogen populations, also known as population genetics, is a popular method for investigating plant diseases. Various phenotypic and genotypic markers can be used to study neutral genetic variation, but molecular markers such as microsatellites allozymes and restriction fragment length polymorphisms (RFLP), multiple-locus variable-number tandem-repeat analysis (VNTR/ MLVA), multilocus sequence analysis/typing (MLSA/MLST), virulence gene typing, and DNA fingerprinting patterns are most commonly used. Recent studies characterizing bacterial outbreaks in plants used MLSA/MLST or MLVA (Buhlmann et al. 2014, Newberry et al. 2018, Pruvost et al. 2014, Ravelomanantsoa et al. 2017), aiming to provide a wealth of information both at the population and species levels.

Management of diseases caused by *X. a.* pv. *juglandis* and *P. s.* pv. *syringae* in walnut and apricot, respectively, is currently nearly impossible due to a lack of effective chemical or biological control measures, a lack (and little available knowledge) of host resistance, and the

pathogen's endophytic nature during some stages of the disease cycle. Copper compounds are the most commonly used bactericides for controlling these bacterial diseases.

The first copper-based antimicrobial compound (CBAC) used in agriculture was the Bordeaux mixture (copper sulfate pentahydrate and lime mixture), which was discovered by accident in 1885 by French scientist Pierre-Marie Alexis Millardet. Among the most significant advantages of these compounds there are their relatively high toxicity to plant pathogens, low cost, low mammalian toxicity, and chemical stability, which prevents them from being easily washed from plant surfaces and long residual periods. This has resulted in the widespread use of Cu to control foliar plant pathogens with acceptable levels of disease management. As a result, CBACs have become an important component of integrated pest management (IPM) systems that seek to provide long-term disease management solutions. CBACs are used in conjunction with resistant or tolerant cultivars, cultural, physical, and even biological control methods in the context of IPM. Copper compounds are strictly used as protectants because they have no curative or systemic activity, which means that disease management is improved because they reduce inoculum build-up on susceptible leaf tissues, preventing infection. However, there are several limitations to using copper compounds, including a lack of systemic activity, difficulty in timing, the emergence of resistant strains, and phytotoxicity. Copper is a contact material that does not penetrate to control bacterial populations that are internal, such as those found inside dormant buds, knots, or cankers. Thus, copper applications must be timed to coincide with periods when the host is vulnerable, the pathogen is accessible, and disease conditions are favorable. Such timings may not be possible; for example, copper is highly phytotoxic to walnut and apricot flowers and, during bloom, can only be used at very low levels that are essentially ineffective in lowering pathogen populations on blossoms.

There are concerns that CBACs have negative effects on the environment and biodiversity, such as soil and groundwater contamination, with a significant impact on soil microbiota (Kandeler et al. 1996; Merrington et al. 2002). Furthermore, soil accumulation is likely to have a long-term impact on crop health. Cu availability and toxicity in the soil increase significantly as soil pH decreases. Furthermore, the widespread use of CBACs has resulted in the emergence of copper-resistant (CuR) strains in agriculture, raising concerns about the long-term viability of agricultural production. Many scientists have reported that the widespread use of copper-based bactericides on walnut and apricot for the control of bacterial walnut blight and bacterial canker of stone fruits has resulted in the development and spread of CuR strains of

several plant-damaging bacteria (Adaskaveg and Hine, 1985; Andersen et al. 1991; Sundin et al. 1989; Bender and Cooksey, 1986; Marco and Stall, 1983).

Despite the emergence of CuR bacterial strains, CBACs are still widely used as a standard treatment for foliar diseases. In epidemic years, this compound may need to be applied several times over several seasons and years to minimize crop loss. Several studies have found that this has significantly increased the risk of insufficient disease management (Marco and Stall, 1983; Cazorla et al. 2002). Indeed, once a bacterial strain develops Cu resistance, the continuous selection pressure gradually increases the frequency of the resistant pathogen population and jeopardizes efficacy of copper (Sundin et al. 1989). This is especially true on perennial crops, where the epiphytic and endophytic nature of most plant pathogenic bacteria (Renick et al. 2008) may provide a reservoir of Cu resistance genes from which Cu sensitive strains can acquire resistance (Cazorla et al. 2002; Behlau et al. 2012). Because many bacterial populations thrive on the same host, there is a risk of Cu resistance determinant horizontal gene transfer (Cooksey, 1990; Behlau et al. 2012). Bacteria becoming resistant to Cu through spontaneous mutations is highly unlikely, because Cu resistance is regulated by several genes in bacteria (Cooksey, 1990). Horizontal transfer of Cu resistance plasmids may occur in nature (Bender et al. 1990; Basim et al. 1999) or in laboratory conditions (Stall et al. 1993), indicating that copper resistance plasmids can be shared among different genotypes of bacterial pathogens, as previously demonstrated (Bender and Cooksey, 1986; Cooksey, 1990; Behlau et al. 2012). However, it appears that the frequency of plasmid transfer between strains is greatest when CuR strains thrive in the same environment (Sundin et al. 1989).

Bacterial canker of stone fruits and walnut blight have been primarily controlled by applying copper-containing bactericides as protective sprays at 7-14 days intervals from bud burst. Copper is sometimes sprayed up to ten times per growing season, and this spray program does not always provide adequate disease control. Given the issues raised by the use of copper-based products, it is critical for the agricultural industry to research and develop better management strategies for bacterial walnut blight and bacterial canker of stone fruits.

The use of host-specific bacteriophages is one of the most promising biocontrol strategies for managing plant bacterial diseases (Jones et al. 2007). Phages are bacteria-specific viruses that disrupt the metabolism of their bacterial hosts in order to replicate. Bacteriophages were discovered by Frederick Twort (Twort, 1915) and Felix d'Herelle (d'Herelle, 1917). Prior to Twort and d'Herelle's discovery of antibacterial agents that hinted at the existence of phage,

similar findings of antibacterial agents had been made (Abedon et al. 2011). They were, however, the first to propose that this phenomenon was caused by a virus. The antibacterial potential of phages was quickly recognized, with d'Herelle demonstrating the ability of his phage preparations to treat dysentery patients in 1919 (Wilkinson, 2001). Following this work, numerous early studies and attempts to use phages to treat some human pathogenic bacteria were made (Sulakvelidze et al. 2001). This method of pre-antibiotic control became known as bacteriophage-based control. Phage research was also started with the goal of using phages to control plant diseases. Mallmann and Hemstreet (1924) demonstrated that decomposing cabbage filtrate could be used to inhibit the "cabbage rot organism" *X. campestris* pv. *campestris*. In 1925, Kotila and Coons used bioassays to show that phages could be used to prevent soft rot caused by *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* ssp. *carotovorum* on potato tuber and carrot slices, respectively (Coons and Kotila, 1925; Kotila and Coons, 1925). Thomas (1935) conducted the first field trials, demonstrating that by treating seeds with phage against the phytopathogen *Pantoea stewartii*, he could reduce the incidence of Stewart's wilt disease from 18% (untreated) to 1.5% (treated). However, because understanding of the nature of phages was poor at the time, and data on their efficacy was limited, this type of research became neglected (Okabe and Goto, 1963). For decades, their biology and nature were unknown (Summers, 2005).

In recent years, there has been a surge of interest in using bacteriophages (phages) as phytopathogen-targeting biocontrol agents (BCAs). Their distinct properties make them highly promising, but difficult antimicrobials. Infection of a bacterium by a virulent phage usually results in rapid viral replication, bacterium lysis, and the release of numerous progeny phages. These phages can then go on to infect nearby bacteria. This is a significant advantage over alternative treatments such as antibiotics. Nonetheless, phages are inherently highly specific to bacterial hosts. As a result, the host range of most phages, *i.e*. the species that they are capable of infecting productively, is relatively narrow, typically limited to only a single bacterial genus, species, or, in many cases, a limited number of strains within a given species. This trait has both positive and negative aspects, as it is beneficial in terms of avoiding negative effects on the host microbiota, while also being a hindrance in terms of detection and elimination of the target pathogen.

The primary goal of this study was to estimate the range of copper sensitivity among a large number of *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* strains isolated in Italy between 2018 and 2019, as well as to develop implementation techniques to provide better disease management in apricot and walnut orchards. To accomplish this goal, the phenotype and genotype of each bacterial isolate were used to determine their identity. The identity and genetic variation of bacterial isolates were investigated using rep-PCR, a DNA fingerprinting technique. MLSA was used to determine any correlation between the genetic diversity of the studied strains and the phylogenetic position of *P. s.* pv. *syringae* and *X. a.* pv. *juglandis*. Because we observed that long-term use of copper compounds resulted in copper resistance in *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* strains, we chose phage-based biocontrol as an implementation technique to manage CuR *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* strains. In 2019 and 2020, phages were isolated in northern Italy and characterised using plaque morphology, host range, thermal inactivation, the effect of pH, UV, and copper compounds on phage viability, and restriction fragment length polymorphism (RFLP). Phages, which infect and destroy bacteria, have the potential to significantly reduce the environmental impact of copper use in agriculture while potentially increasing profitability by lowering crop loss in orchards.

2. LITERATURE REVIEW

2.1. The apricot culture

2.1.1. Apricot taxonomy and nomenclature

Apricot (*Prunus armeniaca* Linnaeus, synonyms: *Armeniaca vulgaris* and *Amygdalus armeniaca* (L.) Dumortier) is a species of the *Prunus* L. genus, classified in the *Rosaceae* family's subgenus *Prunophora* Focke. The *Prunus* genus contains approximately 430 species, most of which are economically important fruit crops such as sweet cherry (*P. avium* L.), sour cherry (*P. cerasus*), peach (*P. persica* (L.) Batsch) and plum (*P. domestica* L.). Some *Prunus* species are evergreen and are used as ornamentals (*P. laurocerasus*) (Das et al. 2011).

According to the authors, three to twelve apricot species have been described. The majority of cultivated apricots are of the species *P. armeniaca* L., and six other species are widely recognized as being the most closely linked to *P. armeniaca*: *P. dasycarpa* (black or purple apricot), *P. mume* (Japanese apricot), *P. brigantina* (Apricot from Briançon, Southern French Alps), *P. holosericea* Batal (Tibetan apricot), *P. sibirica* (Siberian apricot) and *P. mandshurica* (from Manchuria and Korea) (Bortiri et al. 2001). In addition, artificial cross-pollination has resulted in the creation of new interspecific hybrids in recent decades. Plumcot is a putative hybrid of diploid apricots and plums, whereas aprium and pluot are complex hybrids thought to be the consequence of interspecific plum and apricot crosses with resulting backcrossing to plum (pluots) or apricot (apriums) (Ahmad et al. 2004).

2.1.2. History of Apricot

The cultivated apricot has 3 potential origins, according to Russian botanist Vavilov (1992). The first, recognized as the "Chinese Center," includes the mountain ranges of Northeastern, Western and Central China; this is the oldest and most likely underexplored group (Zhebentyayeva et al. 2008). The second is the "Central Asian Center," which is found in the mountains of Tien-Shan, Afghanistan, Kyrgyzstan, Tajikistan, Kashmir, west China and northwest India; this is one of the oldest and most diverse group. The majority of apricots in this group have high chilling requirements (Hormaza et al. 2007). The “Near-Eastern Center,” which includes Turkey, Armenia, and parts of Iraq and Iran, is the third center. According to Vavilov (1992), this third center is a secondary center of origin for cultivated apricot.

Apricot pits were discovered during archeological excavations near the Antique Armenian town of Shengavit and the Garni Temple, both near Yerevan, in layers dating as far back over 6,000 years (Morikian, 1982). According to Pliny the Elder, apricots were first cultivated in Italy around 100 B.C. and were common in Roman cuisine by 200 A.D., according to Apicius recipes (Apicius, 1974). Apricot was introduced by the Arabs in Spain in the seventh century and in Southern France before 1000. Apricot was introduced in the 17th century in England and USA, Virginia (Ogawa and Southwick, 1995), and by Spaniards in California in the 18th century (Faust et al. 1998). Schiner is the first person to mention apricot culture in Switzerland (1812).

2.1.3. Botany of Apricot

Apricot is usually diploid (2n = 16), even though tetraploid mutants have been reported (Bailey and Hough, 1975). Arumuganathan and Earle estimated the apricot and peach genome sizes by flow cytometry in 1991 at about 294 and 265 Mbp, respectively, but current sequencing of the peach genome revealed a size of 227 Mbp (The International Peach Genome Initiative, 2013). In either case, this size remains significantly smaller than that of other fruit species such as prune (880 Mbp) or apple (750 Mbp) (Arumuganathan and Earle, 1991), and is barely about twice as large as that of *Arabidopsis thaliana* L. Heynh (115 Mbp, Arabidopsis Genome Initiative, 2000).

Apricot trees are small, varying in height from 4 to 12 meters and with a trunk diameter of up to 40 cm; they produce white to pinkish flowers with 5 sepals and 5 petals and a single pistil. Apricots, like all other *Prunus* species, have only one ovary with two ovules, one of which degenerates shortly after anthesis, and most of the time only one seed is produced (Rodrigo and Herrero, 2002). Apricot trees require a huge amount of chilling to induce flowering, calculated as the number of units (hours) below 7,2°C (Weinberger, 1950). The chilling requirements for flowering vary by apricot variety (Campoy et al. 2012). Since the heat requirement after chilling is so short, apricot trees bloom early in most locations. As a result of the early bloom habit, apricot is susceptible to frost injury and, as a result, the production area is constrained by the risk of spring frost (Mehlenbacher et al. 1990; Layne et al. 1996; Hormaza et al. 2007).

Drupes are fruits that have a single stone that is made up of a hardened lignified endocarp and a seed and is surrounded by a fleshy mesocarp and an exocarp (the fruit skin).

Apricot fruits take 3 to 6 months to develop, depending on cultivar, and their flesh can range from sweet to sour (Jackson and Coombe, 1966). The apricot fruit weight ranges from 30 to 120 grams; it is characterized by total amounts of sugars ranging from 8,700 to 14,200 mg/100g fresh fruit and by good levels of acidity level, with total acids of 1,400 to 3,700 mg/100g fresh fruit (Valentini et al. 2006).

2.1.4. Major pests and diseases

Some viral, fungal, and bacterial diseases also threaten apricot production. Plum pox virus (PPV) is an important apricot pathogen and the causal agent of a disease named "sharka", from the Bulgarian "шарка" (pox). Two strains, PPV-D (Dideron) and PPV-M (Marcus), are virulent for apricot. The Chlorotic Leaf Roll disease, caused by a phytoplasma (*Candidatus* Phytoplasma prunorum), is very common in Europe and causes sap vessel obstruction (Ham, 2009). Despite being a serious threat to apricot production, the genetics of this disease have yet to be studied, and no host resistance has been reported. *Monilia laxa*, *M. fructicola*, and/or *M. fructigena* cause brown rot. *Monilia* spp. are likely the most serious fungal diseases of apricots, causing damage to flowers and shoots (blights) as well as fruits (rots). The disease emergence is linked to weather conditions. In rainy days during flowering is the most critical phase for mycelial growth. Several fungicide applications per year, primarily during bloom and the final stage of fruit ripening, may be required to prevent significant yield losses (Mari et al. 2003). Another fungal pathogen is *Coryneum beijerinckii* (*Stigmina carpophila*), which is common in Eastern Europe and causes shot holes (Balan et al. 1995). Powdery mildew (caused by *Sphaerotheca pannosa* (Wallr.) Lev in the early season or *Podosphaera tridactyla* DeBary, later in the season, are globally more widespread (Corazza and Simeone, 1991). The most devestating bacterial disease of apricot is *P. s.* pv. *syringae* van Hall (1902), which causes significant losses all over the world. Die-back, gummosis, frost injury, and sour sap are all descriptive terms associate with *P. s.* pv. *syringae* activity, which results in severe defoliation and unmarketable fruits (Dingley, 1969; Cunningham, 1925).

2.2. Causal Agent of Bacterial Canker of Stone Fruits: *P. s.* pv. *syringae* and pv. *morsprunorum*

P. s. pv. *syringae* and *P. s.* pv. *morsprunorum* are both capable of causing bacterial canker in stone fruits. While *P. s.* pv. *syringae* infects any stone fruit, including pome fruits and herbaceous plants (Arsenijević, 1997; Gavrilović, 2006; 2009; Gavrilović et al. 2008), *P. s.* pv.

morsprunorum primarily infects plum (Hattingh and Roos, 1995), sweet and sour cherry and apricot (Bultreys and Kaluzna, 2010).

P. s. pv. *morsprunorum* has been classified into two races based on bacteriophage typing: race 1 (Wormald, 1932), which is pathogenic to cherry, plum, and apricot, and race 2 (Freigoun and Crosse, 1975), which infects cherry (Bultreys and Kaluzna, 2010).

Several differences were pointed out between *P. s.* pv. *morsprunorum* race 1 and *P. s.* pv. *syringae* on sweet cherry (Crosse, 1966; Crosse and Garrett, 1966). Garrett et al. (1966) noted that *P. s.* pv. *morsprunorum* race 1 infected leaf scars much more efficiently than *P. s.* pv. *syringae,* yet *P. s.* pv. *syringae* was more efficient via wounds. *P. s.* pv. *syringae* died off in cherry cankers before *P. s.* pv. *morsprunorum* race 1. *P. s.* pv. *syringae* cankers developed in two stages, late autumn and spring, whereas *P. s.* pv. *morsprunorum* race 1 only developed in spring. Eventually, shoot and blossom infections with *P. syringae* were prevalent in spring, but very seldom with *P. s.* pv. *morsprunorum* race 1. Different symptoms on sweet cherry fruits were observed in lesion tests, with a black and sunken lesion for *P. s.* pv. *syringae* and a superficial small brown lesion for *P. s.* pv. *morsprunorum* race 1. (Garrett et al. 1966).

2.2.1. Taxonomy of *P. s.* pv. *syringae*

The taxonomy of the *P. syringae* complex has been widely debated over the last 40 years, but still remains contraversial. The *P. syringae* complex is classified based on host range and symptomatology, consequently *P. syringae* species divided into pathogenic varieties known as pathovars (Dye et al. 1980; Young, 2010).

The *P. syringae* species complex is currently subdivided into more than 60 pathovars described by pathogenic characters based on different host ranges and disease symptoms. Nine genomospecies described by DNA–DNA hybridization, and 13 phylogenetic groups (phylogroups) described by multilocus sequence analysis (Berge et al. 2014; Hwang et al. 2005; Sarkar and Guttman, 2004; Young, 2010; Gomila et al. 2017).

The recent genomospecies classification of *P. syringae* pathovars revealed that *P. s.* pv. *morsprunorum* race 1 and race 2 are totally different organisms because they belong to genomospecies 2 and 3, respectively, while *P. s.* pv. *syringae* belongs to genomospecies 1 (Gardan et al. 1999; Ménard et al. 2003).

P. s. pv. *syringae*, *P. s.* pv. *morsprunorum* race 1 and race 2 should therefore be considered as genetically distant and different pathogens adapted to the same hosts. Since then, genomospecies based on multi-locus sequence analysis (MLSA) have been suggested as the basis for species (Stackebrandt et al. 2002), but official proposals also require comprehensive phenotypic descriptions (Gardan et al. 1999; Young, 2010).

2.2.2. Symptoms and Damage

Diseases caused by pathovars of *P. syringae* van Hall are a major concern in fruit producing areas around the world, are extremely difficult to control, and cause great economic losses. The pathogen is capable of killing both young and old trees. In nurseries, systemic infection and death of young trees is a perennial problem, and canker development resulting death of scaffold limbs and entire trees is a frequent occurrence that can give rise to the rapid downfall of older orchards.

Canker formation can also occur as a result of pathogen colonization with pruning wounds. In a study conducted in Georgia, for example, inoculation of pruning wounds of peach trees with a virulent *P. syringae* isolate ended up in tree mortality ranging from 57% to 100% when trees were pruned in October or December, but no tree mortality was detected when the pruning and wound inoculation was done in April (Chandler and Daniel, 1976). In a study conducted in California, increased *P. syringae* infection was observed on French prune after pruning in December or March as compared to November (Otta and English, 1970). These findings demonstrate that the timing of pruning has a significant impact on disease severity, with autumn/early winter pruning being especially problematic, most probably due to the role of tissue hydration and freezing in *P. syringae* infection of wood (Sobiczewski and Jones, 1992; Vigouroux, 1989; Weaver, 1978).

2.2.3. Localization

P. s. pv. *syringae* and *P. s.* pv. *morsprunorum* grow in or on plants in two interconnected phases: the epiphytic phase, in which bacteria live on the surface of plant tissues (generally the above-ground parts, including leaves, fruits stems, altogether known as the phyllosphere), and the endophytic phase, in which bacteria enter the plant tissue and colonize the intercellular space known as the apoplast. *P. s.* pv. *syringae* are strong epiphytes that have been widely used in microbial ecological studies; however, disease occurs only after the bacteria enter the plant

and multiply in the apoplast (*i.e.*, the endophytic phase). Under favorable environmental conditions, the initial epiphytic populations of some *P. syringae* strains on the plant surface can be good predictors of their later endophytic populations inside the plant tissue and disease outbreaks (Hirano and Upper, 2000; Rouse et al. 1985), demonstrating the importance of dissecting the epiphytic phase for understanding *P. syringae* pathogenesis.

P. s. pv. *syringae* and *P. s.* pv. *morsprunorum* have notable epiphytic and endophytic phases, according to epidemiological studies of bacterial canker. The idea of a plant pathogen sustaining as an epiphyte on healthy tissues was first suggested in studies of *P. s.* pv. *morsprunorum* on sweet cherry (Crosse, 1959, Hirano and Upper, 2000). Crosse (1959) recovered native populations of *P. s.* pv. *morsprunorum* from leaf washings of healthy tissue in his research and stated that these populations were the origin of inoculum for new cankers. The epiphytic phase of *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum* would include growth and survival on seemingly healthy blossoms and leaves for the duration of the growing season (Sundin et al. 1988). The bacterial canker pathogens induce fruit and leaf spot lesions and/or thrive epiphytically on seemingly healthy leaf surfaces during the summer months. During the summer, epiphytic populations often decline to undetectable levels, but they recover in the autumn, when temperature drops and rainfall increases (Crosse, 1957; Crosse, 1966; Sundin et al. 1988).

A study examined and addressed genetic characteristics that are associated with preferable epiphytic or endophytic/pathogenic life styles (Xin and He, 2013; Hirano and Upper, 2000). Tolerance to ultraviolet a dry environment, and light, for example, is widely regarded as essential for a strong epiphytic lifestyle.

For many years, the epidemiology of bacterial canker has been a topic of study, and the adaptability of the *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum* pathogens in colonizing trees both epiphytically and endophytically has played a significant role in restricting effective disease management.

2.2.4. Ice Nucleation Activity

Another characteristic of *P. syringae* bacteria that may be crucial for the epiphytic phase is ice nucleation and the related ability to cause frost injury in plants, which may result in water and nutrient loss from the plant and may generate openings on the plant surface for bacterial

entry. Frost damage in commercial orchards is a rare occurrence that enhances colonization and pathogenesis for these opportunistic diseases. The ability of *P. syringae* to form ice is dependent on the ice nucleation gene INA. INA encodes the ice-nucleating protein, which enables the formation of ice crystals in plants at temperatures above the typical freezing point (Hirano and Upper, 2000; Lindow et al. 1982).

Freezing conditions worsen the severity of *P. syringae* infection of apricot and peach stems and sour cherry leaves (Klement et al. 1984; Stead et al. 2003; Weaver, 1978). The particular temperature required for the production of cankers varies. For example, when shoots infected with *P. s.* pv. *syringae* or *P. s.* pv. *morsprunorum* were subjected to –10°C, necrosis was more serious and pathogen populations increased, but not when shoots inoculated with *P. s.* pv. *syringae* or *P. s.* pv. *morsprunorum* were subjected to –5°C (Sobiczewski and Jones, 1992). Apart from controlled research, cold temperatures in the field have frequently been linked to an increase in the severity of apical necrosis and bacterial canker (Cazorla et al. 1998; Spotts and Cervante, 1994).

The tight relationship of the INA bacteria and ice production with regions of wounded host tissue that is prone to infection appears to be a significant element in the relation between INA and infection. The fact that pathogen cells must be present within 20 minutes after thawing of frost-damaged tissue for successful infection to occur (Süle and Seemüller, 1987) provides correlative evidence for the relevance of INA bacteria, ice, and wounded tissue co-occurrence in infection. Finally, the topic of INA role in pathogen virulence will have to be answered utilizing a specified mutant in inoculation tests on a variety of hosts exposed to a variety of freezing temperatures.

2.2.5. Toxin production

Syringomycin is a cyclic lipodepsinonapeptide phytotoxin generated by *P. s.* pv. *syringae* (Bender et al. 1999). This toxin causes necrosis in host tissues, and plant signal molecules, particularly chemicals found in sweet cherry leaves, stimulate the expression of genes involved in syringomycin production (Mo et al. 1995). In a laboratory sweet cherry fruit infection experiment, syringomycin synthesis reduced pathogenicity, although the relevance of this toxin in canker formation in woody tissues is unknown (Mo et al. 1995).

Coronatine is a phytotoxin generated by *P. s.* pv. *morsprunorum*, the weaker of the two *P. syringae* pathovars that cause bacterial canker (Liang and Jones, 1995). The role of coronatine as a virulence factor in the *P. s.* pv. *morsprunorum*–cherry pathosystem has not been studied.

Persicomycin, a class of 3-(3′-hydroxy) hydroxy fatty acids, was shown to be a necrosis-inducing chemical and virulence factor in *P. s.* pv. *persicae* strains that cause peach disease (Barzic and Guittet, 1996).

2.2.6. Importance of Bacterial canker of Stone Fruits

P. syringae spp. is responsible for economically significant diseases in cultivated plum, cherry, wild cherry, peach and apricot (Scortichini et al. 2003; Vicente and Roberts, 2007; Renick et al. 2008; Gilbert et al. 2009; Kaluzna et al. 2010b).

Bacterial canker of sour cherry trees (*P. cerasus* L.) was already documented in Montenegro (Vučinić et al. 1992): the disease was identified on more than 50% of the trees in young orchards from 1983 to 1987. The disease spread quickly due to the susceptibility of the sour cherry cultivars and suitable climatic conditions. Based on pathogenicity, morphology, and some biochemical–physiological features, the sour cherry isolates were determined to be *P. syringae* (Vučinić et al. 1992).

2.2.7. Identification methods

2.2.7.1. Biochemical Characterization Methods

P. s. pv. *syringae* classification has historically been accomplished using phenotypic methods such as biochemical studies, disease symptoms on the affected host and host range (Lelliott et al. 1966; O'Brien et al. 2011; Studholme, 2011; Young, 2010). The performance of the hypersensitive test and the detection of pyoverdin synthesis using fluorescence techniques are usually the initial steps in identifying plant pathogenic *Pseudomonas* spp. isolated from a variety of hosts (Bultreys and Kaluzna, 2010; Gilbert et al. 2009; Lelliott and Stead, 1987; Whitelaw-Weckert et al. 2011). *P. syringae* can then be distinguished from other *Pseudomonas* species using the levan, oxidase, potato soft rot, arginine dihydrolase, and tobacco leaf hypersensitivity reaction (LOPAT) assays (Hall et al. 2016; Lelliott and Stead, 1987). Gelatin liquefaction, aesculin hydrolase, tyrosinase activity, and tartaric acid utilization (GATTa)

assays are often performed for pathovar differentiation of *P. syringae* strains (Jones, 1971; Lelliott et al. 1966).

2.2.7.2. Molecular Characterization Methods

2.2.7.2.1. DNA-DNA hybridization

The DNA-DNA hybridization (DDH) approach compares genetic similarity between isolates based on how well their genomes hybridize under standard conditions (Johnson, 1973). DDH analysis of several isolates from the same or other species revealed that isolates with similar phenotypic traits contained more than 70% of their genome. Wayne suggested to use the DDH technique to quantitatively designate a bacterial species based on such observations (Wayne, 1988). Following this approach, the DDH method and the 70% hybridization threshold between members of the same species group has become the gold standard for bacterial categorization.

Gardan and colleagues performed DDH studies on strains from the *P. syringae* species complex and categorized them into nine genomospecies groups (Gardan et al. 1999). The bulk of strains and pathovars within the species complex were characterized by four of these genomospecies groups. Although the strains within distinct genomospecies groupings were genetically heterogeneous, carbon source utilization and ribotyping tests comparisons revealed no significant phenotypic differences (Gardan et al. 1999; Young, 2010). As a result, the genomospecies were not given the status of full species.

While the DDH approach is frequently used for categorizing bacterial lineages that lack identifiable morphological or phenotypic traits, it has some serious drawbacks. DDH tests requires a long time and need exceptional precision, making them challenging to use for swiftly identifying novel bacterial isolates. Because of these limits, the DDH technique has gradually been supplanted by other methods for determining the degree of new genetic variation (Martens et al. 2008).

2.2.7.2.2. Ribosomal RNA 16S

With the introduction of low-cost DNA sequencing in the late 1990s, scientists began to look for an alternative to the time-consuming DDH strategy for species categorization. Stackebrandt and Goebel proposed utilizing a comparative sequencing analysis of the 16S ribosomal RNA (rRNA) gene to reflect classification similar to DNA-DNA association

(Stackebrandt and Goebel, 1994). They discovered that bacterial isolates with more than 97% identical 16S rRNA gene sequences are frequently members of the same species (Stackebrandt and Goebel, 1994). The species classifications based on 16S rRNA genes corresponded well with the earlier DDH-based species classification. Furthermore, the availability of the 16S rRNA gene in all lifeforms and the ease of implementation of sequence comparison gave this method a distinct advantage over the time-consuming DDH strategy for efficiently classifying bacterial lineages (Stackebrandt and Goebel, 1994).

Although being the most prevalent strategy for species classification, the 16S rRNA gene has not been frequently employed for *P. syringae* strain classification. A comparison of 16S rRNA sequences from nine strains, including *P. amygdali*, *P. savastanoi*, *P. viridiflava*, *P. avellanae*, *P. caricapapayae*, *P. cichorii*, *P. meliae*, *P. ficuserectae* and *P. syringae* revealed no apparent resolution for distinguishing between uniquely named *Pseudomonas* species (Anzai et al. 2000). This finding suggested that a single gene may not have enough power or resolution to infer relationships between closely related species or strains. As a result, Stackebrandt et al. (2002) proposed using several genetic markers from the entire genome to infer species connections.

2.2.7.2.3. REP-PCR Phylogenetic Analysis

Phylogenetic analysis utilizing polymerase chain reaction (PCR) fingerprinting methods has been crucial in revealing hierarchical clustering of bacterial diseases such as *P. syringae* (Clarke et al. 2010; Gardan et al. 1999; Hwang et al. 2005; Sarkar et al. 2006; Sawada et al. 1999; WhitelawWeckert et al. 2011).

Bacterial chromosomes contain a number of interspersed repetitive sequences that comprise intergenic regions at various locations throughout the genome. Such noncoding, repeating sequence blocks can function as many genetic targets for oligonucleotide probes, allowing the creation of distinct DNA profiles or fingerprints for each bacterial strains. The resolution of different sized DNA fragments obtained from chromosomal or plasmid DNA by restriction endonuclease-mediated digestion and/or DNA amplification to generate a band pattern that acts as a unique identifier is required for DNA fingerprinting. These distinct DNA fingerprints describe each bacterial chromosome without the requirement for gene expression or enzyme function measurements.

Versalovic et al. (1991) developed the repetitive sequence-based polymerase chain reaction (rep-PCR), which produces DNA fingerprints made up of numerous different-sized DNA amplicons. Electrophoresis can be used to separate amplicons of varying sizes, which form the DNA fingerprint patterns unique to individual bacterial strains. The rep-PCR approach has been applied to the DNA fingerprinting of a wide range of prokaryotic and eukaryotic microorganisms (van Belkum, 1994; Versalovic et al. 1994; de Bruijn et al. 1995; Louws et al. 1996). The speed, reliability, absence of radioisotope use, flexibility to intact cell and native tissue preparations, and moderate resource needs of standard equipment available in molecular biology laboratories are key advantages of rep-PCR based chromosomal typing. Rep-PCR genomic fingerprinting has been proven to be most useful at the subspecies/strain level in terms of sensitivity.

2.2.7.2.4. Multi-Locus Sequence Typing (MLST)

In recent years, multi-locus sequence type (MLST) phylogenetic analyzes have become an indispensable instrument for the understanding of bacterial evolution. MLST consists of a number of key sequences of genomes, omnipresents throughout all strains of a bacterial species and important for organism survival (Hwang et al. 2005). Those housekeeping genes are selected because they are less likely to be transferred horizontally and provide an insight into bacteria's developmental history (Hacker and Carniel, 2001). The studies of the developmental history of sixty *P. syringae* isolates using seven housekeeping genes, including aconitate hydratase B (*acn*B), phosphofructokinase (*pkf*), phosphoglucoisomerase (*pgi*), were shown by Sarkar and Guttman (2004). In an effort to get identical results at cheaper expense, Hwang et al. (2005) later refined the number of housekeeping genes to four (citrate synthase, *cts* also called *glt*A; glyzeraldehyde-3-phosphate dehydrogenase, *gap*A; GNA gyrase B, *gyr*B; and sigma factor 70, *rpo*D). The analysis of various genomes can show evolutionary trends indicating changes in lifestyle within a phylogenetic framework (Clarke et al., 2010; Hwang et al., 2005). This also enables genetic modifications to be identified that distinguish between groups that have recently undergone host change (Sarkar et al. 2006).

The *P. syringae* complex was separated into 13 different phylogenetic clusters known as phylogroups in MLST analysis (Berge et al. 2014; Hwang et al. 2005; Sarkar and Guttman, 2004). Seven phylogroups are consistent with the previously recognized genomospecies of Gardan et al (Bull et al. 2011; Gardan et al. 1999). MLST analysis of *P. syringae* benefits from many diagnostic tests by enabling accurate bacterial pathogens to be identified. Dedicated

databases such as PubMLST and PAMDB have been developed for *P. syringae* and many other bacterial pathogens due to extensive applications of MLST techniques for species categorization (Almeida et al. 2010). Through fast comparisons of the individual loci with a collection of known strains, this databasing improved the rate of analysis.

In another study, a pathovar-level investigation employing polymerase chain reaction (PCR) finger printing showed a large overall variance between strains but the clustering of most strains in several groups regardless of host (Scortichini et al. 2003). There is evidence of host specificity between bean strains and grass-infecting *P. s.* pv. *syringae* strains (Cheng et al. 1989, Gross and DeVay 1977), and a genetic diversity analysis indicated that bean strains grouped as a different group from other *P. s.* pv. *syringae* strains (Legard et al. 1993). Also, population-level PCR-fingerprinting studies differ from stone fruit populations in California and ornamental pear in Oklahoma from the strains of *P. s.* pv. *syringae* from other hosts (Little et al. 1998; Sundin et al. 1994). Most *P. s.* pv. *syringae* isolates have only a few common properties, including the transport of genes including *syr*B implicated in toxin syringomycin production and lemon fruit disease (Scortichini et al. 2003). This pathovar is probably a collection of strains, some of which really have a broad spectrum of host, but which most of the pathovars wait to be further classified and delineated by the bigger group. Comparative genomic analyzes could also be used to resolve the problem with the genome sequence of the bean pathogen *P. s.* pv. *syringae* B728a (Feil et al. 2005).

Gasic at al. (2012) evaluated the appropriateness and the differentiating capability of certain common bacteriological and molecular methods with the goal of selecting tests for the rapid and effective differentiation of *P. s.* pv. *syringae*, *P. s.* pv. *morsprunorum* and *P. s.* pv. *persicae*. Strand differentiation utilizing LOPAT, GATTa, ice nucleation tests, sucrose nutrition broth development, and various sources of carbon were performed. The PCR approach has been used for the detection of genes that make toxin: *P. s.* pv. *syringae syr*B and *syr*D and *P. s.* pv. *morsprunorum cfl* gene. In a bioassay with *Geotrichum candidum*, *Saccharomyces cerevisiae* and *Rhodotorula pilimanae* as indicator organisms, syringomycin production was confirmed by *P. s.* pv. *syringae*. Pathogenicity testing of the inoculation material, which may separate *P. s.* pv. *syringae* and the other two pathovars, was carried out both on lemon and inmature nectarine fruit and on string bean pods. Repetitive PCR sequences, REP, ERIC and BOX primers reproduce *P. syringae* pathovars on various genetic profiles.

2.3. Walnut cultivation

Walnut has been consumed in human nourishment from prehistoric times. The walnut tree was cultivated in Europe as early as 1000 BC and is endemic to Central Asia, Uzbekistan, Kyrgyzstan, the western Himalayan chain (Fernandez et al. 2000) Fossil polen deposits explicitly show that *J. regia* was growing in southern Italy, Spain, France, Switzerland (Alps), Greece (Epirus), southwestern Turkey, Bulgaria (Rhodopes Mountains) and Albania during the Upper Pleistocene (126,000–12,000 BP) (Pollegioni et al. 2017). Walnuts were included in the diet of the famed medical school in Salerno, Italy. Many European medical treatises mentioned the claimed medicinal benefits of walnuts. Since then, it has flourished and become well suited to many locations throughout the world with Mediterranean-type ecosystems.

Currently, walnut is commercially grown in southern Europe, eastern Asia, northern Africa, western South America and the United States, China is the world's top producer, followed by the United States, Iran, Turkey, Ukraine, Romania, France, and India, however, production in other countries like as Chile and Argentina has grown fast in recent years.

2.3.1. Botany of Walnuts

Mišic (2002) describes the common walnut as a large-leaved fruit, horticultural, woodland, and park tree with lengthy leaves. A walnut tree can live for hundreds of years if natural conditions are favorable (Ercisli et al. 2012).

The common walnut tree can reach a height of 20–25 m (Kora´c et al. 1997) and a diameter of up to 1 m. The species of *Juglans* genus are all diploid (2n = 32) and interfertile. Flowering takes place between March and May, depending on the cultivar and environmental circumstances. The seed matures in September–October of the same year, thus 19–20 weeks after flowering. The fruits are ovoid drupes 3–4 cm long and 2.5–3 cm wide, with a pulpy epicarp (hull) that is green and rich in tannic acid and detaches when ripe; an indehiscent, woody endocarp (nut) formed of two wrinkly valves, and a cerebrum-shaped seed (kernel) divided into two to four very furrowed lobes, made up of two large and fleshy. Fruits reach full size approximately 10 weeks after blossoming. Between the 12th and 15th week after flowering, the endocarp has finished hardening (Barone et al. 1997).

2.3.2. The significance of the genus *Xanthomonas*

Xanthomonas is a major genus in the *Xanthomonadales* order, belonging to the family *Xanthomonadaceae*. *Xanthomonas* is a genus with 27 species. *Xanthomonas* species cause at least 350 different plant diseases. These pathogenic organisms exhibit a high level of host plant specificity. The genus has been linked to significant crop losses in a number of vital crops such as rice, citrus, cassava, tomato, sugar cane, passionfruit, and brassicas (Hayward, 1993; Brown, 2001; Das, 2003; Quezado-Duval et al. 2004; Strange and Scott, 2005; Wechter et al. 2008; Tripathi et al. 2009). *Xanthomonas* is a difficult impediment for agriculture around the world due to its quick multiplication, handling difficulties, challenges with chemical control, and the severity of the losses of infected plantings (Ryan et al. 2011; Rodriguez et al. 2012). Bacterial plant pathogens of the genus *Xanthomonas* are well adapted to their host plants and are not known to colonize other environments. Each strain host range is often limited to a few host plant species. Bacterial strains that cause the same kinds of symptoms in the same host range form a pathovar.

An examination of published data on *Xanthomonas* hosts (Leyns et al. 1984) revealed that *Xanthomonas* infections occur on at least 124 monocotyledonous and 268 dicotyledonous plant species. The range of hosts among monocotyledons extends over 11 families with at least 70 species, and there are hosts in 57 dicotyledon families with more than 170 genera. There has been no strong relationship of hosts with any of the angiosperm subclasses discovered (Leyns et al. 1984).

Among phytopathogenic Xanthomonads, the *X. arboricola* species contains phytopathogenic bacteria that are responsible for developing diseases all over the world (Boudon et al. 2005; Hajri et al. 2010; Lamichhane and Varvaro, 2014). This species, which is currently split into seven pathovars (Janse et al. 2001; Vauterin et al. 1995), causes diseases in a wide variety of perennial plants, including hazelnut, poinsettia, poplar, *Prunus* spp., and *Juglans* spp. (Janse at al. 2001; Palleroni et al. 1993; Ritchie, 1995, Vauterin et al. 1995). In the EU phytosanitary legislation (EU Directive 2000/29/CE) and the European and Mediterranean Plant Protection Organization lists (EPPO A2 list) (EPPO/OEPP 2004, 2006), *X. arboricola* infecting *Prunus* spp. and hazelnut (the putative *X. a.* pv. *pruni* and *X. a.* pv. *corylina*, respectively) are listed as regulated organisms.

2.4. *X. a.* pv. *juglandis*, the causal agent of walnut bacterial blight.

X. a. pv. *juglandis* is an aerobic, Gram-negative mobile and non-sporing bacterium that measures 0.2-0.8 x 0.8-1.7 m and has a single, polar flagellum (Bradbury, 1986). Colonies are convex, mucous-viscous consistency, translucent with a clear border, and produce a yellow water-insoluble pigment (Hayward and Waterston 1965).

2.4.1. *X. a.* pv. *juglandis* taxonomy

Extensive examination of phenotypic and genotypic traits over the last few decades has enhanced the classification scheme of *Xanthomonas* bacteria to properly reflect their diversity and phylogenetic relationships (Gabriel et al. 1989; Stead, 1989; Van Den Mooter and Swings, 1990; Palleroni et al. 1993; Yang et al. 1993; Vauterin et al. 1995; Rademaker et al. 2005; Saddler and Bradbury, 2005; Young et al. 2008; Parkinson et al. 2009; Rodriguez et al. 2012).

Vauterin et al. (1995) proposed the most recent comprehensive reclassification of the genus based on DNA homology. The genus was split into 20 species in that study, four of which corresponded to previously identified species and 16 of which were composed of one or more former pathovars of *X. campestris*, with few pathovars fitting into more than one species (Vauterin et al. 1995; Vauterin et al. 2000). Following the reclassification proposed by Vauterin et al. (1995), taxonomic modifications were suggested, and a few new species and subspecies were named (Schaad et al. 2000; Vauterin et al. 2000). (Trebaol et al. 2000; Jones et al. 2004; Schaad et al. 2005; Rademaker et al. 2005; Schaad et al. 2006; Young et al. 2010). Currently, the *Xanthomonas* genus contains 28 validly characterized species and 72 pathovars classified among the species *X. axonopodis*, *X. campestris*, *X. oryzae*, *X. dyei*, *X. translucens*, and *X. arboricola*.

The *X. arboricola* species includes seven different pathovars, all of which are plant pathogenic bacteria, including *X. a.* pv. *poinsetticola* on poinsettia, *X. a.* pv. *populi* on poplar, *X. a.* pv. *celebensis* on banana, *X. a.* pv. *fragariae* on strawberry, *X. a.* pv. *corylina* on hazelnut, *X. a.* pv. *pruni* on stone fruits and *X. a.* pv. *juglandis* on walnut (Palleroni et al. 1993; Vauterin et al. 1995; Janse et al. 2001). The last three pathovars, *juglandis*, *corylina*, and *pruni*, are the most closely related with each other and the most serious diseases to fruit production (Scortichini et al. 2003; Young et al. 2008).

Walnut bacterial blight agent was isolated from walnuts for the first time in California by Pierce (1896) and named it *Pseudomonas juglandis* (Pierce, 1901). Further analysis reclassified the pathogen as *Bacterium juglandis* (Smith, 1905), *Phytomonas juglandis* (Bergey et al. 1930), and *X. juglandis* (Bergey et al. 1930) (Dowson, 1939). Later, with the implementation of the International Standards for Naming Pathovars of Phytopathogenic Bacteria, the name was changed to *X. campestris* pv. *juglandis* (Dye et al. 1980). Recent phylogenetic analyses have led to the designation of *X. a.* pv. *juglandis* (Vauterin et al. 1995).

2.4.2. Symptoms of Bacterial Blight on Walnuts

X. a. pv. *juglandis* overwinters largely in walnut buds and catkins (Miller and Bollen, 1946; Mulrean and Schroth, 1982). The centripetal invasion of resident *X. a.* pv. *juglandis* from the outermost area of dormant buds during bud and shoot growth in spring might lead to the infestation of internal bud portions and growing fruits (Mulrean and Schroth, 1982). Since bacteria are easily suspended in rainsplash and then carried onto healthy host tissue, wind-driven rainsplash may play a significant role in the migration of primary inoculum onto developing fruits (Stall et al. 1993). All succulent tissues, including buds, petioles, female flowers, shoots, leaves, rachis, catkins, and nuts have been documented to have symptoms (Miller and Bollen, 1946; Belisario et al. 1999). All parts of the leaf are vulnerable to attack. The early lesions on leaves are usually found on the lamina rather than the veins.

Infections begin as translucent water-soaked spots that progress to brown to blackish greasy necrotic regions. Lesions are initially circular but frequently enlarge into angular patches, which are often encircled by a yellow-green halo. Growing tissues may fold around lesions, causing the leaf to seem twisted (Miller and Bollen, 1946). The bacterium may enter the vascular vessels in later stages. In the spring, the disease emerges on catkins after they have elongated (Rudolph, 1933). Florets that are affected turn black, and catkins twist. Female flowers that have been infected normally shrivel and fall from the tree (Miller and Bollen, 1946). Fruits have black greasy patches that appear on the lateral surface rather than at the blossom end. Necrotic lesions can occur to varying depths and extents, involving many fruit parts including the style, mesocarp, endocarp, and kernel.

Nuts damaged before shell-hardening frequently shrivel, whereas lesions formed after shell-hardening are usually superficial. Many nuts fall prematurely, but the husk, shell, and kernel of those that mature are browned and destroyed. On shoots, black, necrotic patches might

form, and the shoot tip is usually killed back. Lesions can encircle the shoot, be superficial, involve only the bark, or grow into the pith, forming cankers that emit a bacterial slime. The disease usually damages shoot tips in nurseries, producing bud dieback. Bacteria can spread downwards, generating cankers or girdling the stem, causing distal dieback.

The pathogen can live in infected trees in twig lesions, buds, and damaged fruits for years. Since the germs overwinter and live in latent buds, walnut blight is difficult to eradicate. Because they live within the buds, no available nonsystemic substances can reach the internal population.

2.4.3. Genetic variations in *X. a.* pv. *juglandis*

X. a. pv. *juglandis* is commonly isolated from symptomatic walnut leaves and fruits, but also from non-symptomatic walnut plant parts, such as dormant walnut buds and catkins (Lindow et al. 2014), and from non-plant objects, such as orchard machinery (Giovanardi et al. 2015). Genotyping investigations have revealed significant genetic variety in *X. a.* pv. *juglandis*, which is allegedly greater than the diversity identified for other *X. arboricola* pathovars (Hajri et al. 2010). This trait, together with evidence of genetic trade-offs within the species (Barionovi and Scortichini, 2008; Hajri et al. 2012; Merda et al. 2016), indicates an opportunistic pathogen, despite the fact that evidence for *X. a.* pv. *juglandis* environmental reservoirs is still poorly understood (Esterio and Latorre, 1982; Pruvost and Gardan, 1988). These findings are supperted by the isolation of nonpathogenic *X. arboricola* strains, which have been described as phylogenetically diverse, and their separation from the well-defined clusters of pathogenic *X. a.* pv. *juglandis* strains (Essakhi et al. 2015). *X. a.* pv. *juglandis* studied in France (Hajri et al. 2010), Serbia (Ivanovic et al. 2014), and Italy (Giovanardi et al. 2015) revealed high genetic diversity among isolates, implying the existence of many *X. a.* pv. *juglandis* populations. Furthermore, the genetic variations discovered between *X. a.* pv. *juglandis* strains acquired from VOC and WBB symptoms indicate the presence of separate genetic lineages within *X. a.* pv. *juglandis* populations (Hajri et al. 2010).

Numerous hypothesis were proposed to reveal genetic diversity in *X. a.* pv. *juglandis* populations such as geographic location (Loreti et al. 2001; Scortichini et al. 2001), origin of plant propagation material (Hajri et al. 2010), adaptation to specific environmental conditions (Scortichini et al. 2001; Kaluzna et al. 2014), genome flexibility or pathogen virulence (Loreti et al. 2001; Kaluzna et al. 2014), or even selective pressure by the host plant (Marcelletti et al.

2010). Regardless of their valuable contributions, these studies were either based on a small number of bacterial isolates, often obtained without a planned sampling strategy, or on a set of *X. a.* pv. *juglandis* strains from around the world, ignoring important metadata such as date, plant host traits that are required to determine epidemiological patterns (McMahon and Denaxas, 2016; Parkhill and Wren, 2011). In particular, understanding the epidemiological behavior of *X. a.* pv. *juglandis* requires combining extensive genotyping analysis of a coherent set of isolates with meaningful metadata in a single study.

2.4.4. Molecular identification of *X. a.* pv. *juglandis*

Scortichini et al. (2001) examined a global collection of 61 *X. a.* pv. *juglandis* strains isolated from Persian walnut using repetitive polymerase chain reaction (PCR) genomic Fingerprinting with ERIC, BOX, and REP primer sets and polyacrylamide gel electrophoresis. Cluster analysis revealed the existence of three significant strain groups. The first two groups were 85% genetically related, whereas the third was just 78% identical to the first two. Each group might be subdivided into two subgroups based on the geographical origin of the isolates. The ERIC, BOX, and REP primer sets produced reproducible genomic PCR profiles with bands ranging from 100 to 1700 bp. Polyacrylamide electrophoresis distinguished the bands extremely well. A total of 36 repeatable, clearly resolved bands were scored for UPGMA analysis: 14 for primer ERIC, 13 for primer BOX, and 9 for primer REP. In discriminating the *X. a.* pv. *juglandis* strains, ERIC and BOX primers were more discriminative than REP. The similarity matrix creation revealed that no strain from one country had the same profile as any strain from another place. Furthermore, the total genetic similarity of strains from the same nation was consistently greater than that of strains from different nations.

Hajri et al. (2010) conducted research in 79 walnut orchards and nurseries in southeastern and southern France. Based on biochemical testing, bacterial investigation from sick samples generated 36 strains identified as *X. arboricola* and 32 strains identified as *B. nigrifluens*. Pathogenicity testing on walnut revealed that the VOC-causing culprit was *X. arboricola*. F-AFLP was performed on 36 strains of *X. arboricola* collected in this investigation, 24 strains of *X. a.* pv. *juglandis* isolated from walnut blight symptoms, and one strain of *X. a.* pv. *corylina* added as an outgroup. Based on cluster analysis of F-AFLP data, the majority of *X. arboricola* strains responsible for major VOC outbreaks exhibited a significant degree of similarity, producing a cluster clearly distinct from *X. a.* pv. *juglandis* strains isolated from walnut blight symptoms. It is thought that VOC is caused by a unique genetic lineage

within *X. a.* pv. *juglandis*, which is also capable of causing typical bacterial blight symptoms on walnut leaves and fruits.

Burokiene et al. (2012) used genetic diversity analysis of the pathogen with nucleic acid-based techniques to identify and define genetically the causal agent of bacterial walnut blight in Lithuania (rep-PCR and MLST). Five walnut species were examined:*J. cinerea* L., *J. mandshurica* Maxim., *J. ailantifolia* Carr., *J. nigra* L., two hybrids: J. x bixbyi Rehd (J. cinerea x J. ailantifolia) and J. x quadrangulata (Carr.) Rehd. (J. cinerea x J. regia)According to MLST results, all *X. a.* pv. *juglandis* strains were placed in a single monophyletic cluster, with concatenated sequence similarities ranging from 98.2 to 100%. All of the examined genes exhibited identical sequences in the Lithuanian strains. There was a discrepancy with sequences from other nations, but the level of similarity was still high, for example, in the case of genes *fyu*A, *gyr*B, and *rpo*D, it was 98.3-100%, 97.3%, and 100% for Polish strains, respectively.

Kaluzna et al. (2014) collected leaves and fruits from six locations in Poland where walnut trees were showing signs of bacterial blight. Genetic studies (PCR MP, ERIC-, BOX-PCR, and MLSA) revealed similarities between the examined isolates and the French reference strain of *X. a.* pv. *juglandis* CFBP 7179. However, reference strains I-391 from Portugal and LMG 746 from the United Kingdom were not the same. The ERIC-PCR method found to be more suitable for determining the genetic diversity of the *X. a.* pv. *juglandis* isolates than the BOX approach, as 16 distinct patterns were obtained compared to only eight patterns by BOX. A total of 18 and 25 fragments were produced for ERIC and BOX, respectively, with sizes ranging from 100 to 2500 bp. MLSA analysis of partial sequences of the *fyu*A, *gyr*B, and *rpo*D genes from the examined isolates and respective sequences from GenBank of pathotype strains of other *X. arboricola* pathovars revealed that the *X. a.* pv. *juglandis* isolates belonged to different evolutionary lineages. An inconsistency in MLSA gene phylogenies was discovered, as well as evidence of intergenic recombination events.

Fernandes et al. (2017) suggested nine novel unique DNA markers (XAJ1 to XAJ9) that were chosen using dedicated in silico algorithms to identify *X. a.* pv. *juglandis* isolates and detect these bacteria in infected plant material. Dot blot hybridization was performed on a large range of *Xanthomonas* to confirm the efficiency and specificity of these markers. This research enabled the identification of four broad-range markers (XAJ2, XAJ3, XAJ5, XAJ7, and XAJ9), which resulted in the formation of 12 hybridization patterns.

Fernandes et al. (2018) used multilocus sequence analysis (MLSA) and dot blot hybridization patterns obtained with nine *X. a.* pv. *juglandis*-specific DNA markers (XAJ1–XAJ9) to assess genetic diversity. The findings revealed that *Xanthomonas* isolates clustered into ten unique MLSA clusters and hybridization patterns (HP). As demonstrated by MLSA (clusters I to VI), the majority of isolates (112 out of 131) were closely linked to *X. arboricola* strains of pathovar juglandis and hybridized with more than five *X. a.* pv. *juglandis* unique markers. Nineteen isolates were grouped into four MLSA groups (clusters VII–X), none of which contained *X. a.* pv. *juglandis* strains and hybridized to less than five markers. Using this information, it was feasible to distinguish 17 *X. a.* pv. *juglandis* lineages, three *X. arboricola* lineages, and 11 *Xanthomonas* spp. lineages. Some *X. a.* pv. *juglandis* lineages appeared to be widely spread and prevalent across the various bioclimatic zones, seemingly unconstrained by the other factors evaluated. The analysis of type III effector genes and pathogenicity tests revealed that, with the exception of strain CPBF 424, representative lineages of MLSA clusters VII to X were nonpathogenic on walnut, making this bacterium particularly attractive for addressing *Xanthomonas* patho-adaptations to walnut.

Sup Kim et al. (2021) isolated and described *X. a.* pv. *juglandis* in northern Gyeongbuk province of Korea. The pathogens were isolated from walnut buds, and flower buds, shoots, leaves and fruits that had been infected with the disease. Using Biolog GN2 and Vitek 2, nutrient usage profiles of isolated bacteria exhibiting bacterial blight symptoms were described. Additionally, isolates were characterized morphologically, physiologically, and biochemically. The isolates were identified using 16S rDNA sequencing and multi-locus sequence analysis of *atp*D, *dna*K, *efp*, and *rpo*D. They suggested that sequence analysis of the concatenated data from all *X. a.* pv. *juglandis* loci revealed distinct genetic variations between *X. a.* pv. *juglandis* strains and other *Xanthomonas* pathovars found in other parts of the world. The dendrogram was created by concatenating the gene sequences of domestic isolates and comparing them to strains from other countries. The strains (ANU-A35, ANUA36, and ANU-A37) isolated from young walnut trees in the Andong region are most similar to those isolated from *X. a.* pv. *juglandis* in New Zealand and Italy; whereas the strains (ANUY12, ANU-Y13, and ANU-Y15) isolated from old walnut trees in Yeongyang are most similar to those isolated from a Chinese isolate.

2.5. Copper resistance

2.5.1. Copper Resistance Mechanism of action in plant protection

Because copper ions have a multisite mechanism of action, pathogen resistance to copper is unlikely to develop. According to the Fungicide Resistance Action Committee, oomycetes, and fungi have shown no resistance to the numerous copper compounds (FRAC, 2018). Several bacterial pathogens, however, have acquired resistance to the metal. Bacterial resistance was discovered in *X. campestris* pv. *vesicatoria* in 1983. (Marco and Stall,1983). In *Pseudomonas* genus, copper resistance was first discovered in *Pseudomonas syringae* pv. *tomato* in 1986 (Bender and Cooksey, 1986), and then spread to other *Pseudomonads* (Sundin et al. 1989; Cooksey, 1990; Andersen et al. 1991; Goto et al. 1991). Copper tolerance has also been demonstrated in *Erwinia amylovora* populations (Sholberg et al. 2001).

Copper resistance in bacteria is controlled by numerous genes (Cooksey, 1990), which are typically found in mobile genetic elements (plasmids and transposons) (Bondarczuk and Piotrowska-Seget, 2013). According to Yin et al. (2017), the main mechanisms regulating copper resistance in bacteria are as follows: (1) the efflux ATPase pump encoded by *cop*A, which can extrude copper ions from the cytoplasm into the periplasmic space (Rensing and Grass, 2003); (2) the *cus* system, in which the *cus*A gene encodes a resistance nodulation cell protein with an antiport system (Outten et al. 2001; Hasman and Aarestrup, 2002).

2.5.2. Chemical Tolerance and Resistance of *Xanthomonas* spp.

Most bacteria have adapted or evolved mechanisms to tolerate or mitigate active components in common chemical products and antibiotics used in medicine and agriculture. Chemical resistance is common in *Xanthomonas* spp., most notably copper resistance in *Xanthomonas campestris* pv. *viticola* on grapevine, *Xanthomonas citri* on citrus trees, *Xanthomonas* infections on tomato and pepper (Chand et al. 1994; Gardan et al. 1993; Behlau et al. 2011) and *X. a.* pv. *juglandis* on walnut trees. Copper resistance was recently identified in *X. a.* pv. *juglandis* in Italy (Giovanardi et al. 2017). Copper tolerance has been found in *Xanthomonas* spp., such as *X. c.* pv. *vesicatoria* on pepper plants in Australia and *X. citri* on citrus trees in Brazil (Martin et al. 2004; Marin et al. 2019). Resistance and tolerance in *Xanthomonas* spp. could be caused by a variety of mechanisms and processes.

There are numerous mechanisms by which bacteria sequester or overcome copper ions and promote resistance, and *Xanthomonas* spp. are no exception. The *cop*LAB cluster and the *cop*ABCD cluster are two recognized gene clusters in *Xanthomonas* spp. that promote copper resistance (Behlau et al. 2011; Pereira et al. 2015; Richard et al. 2017). *Cop*LAB was found in *Xanthomonas* spp. in 2011 and it was revealed that the gene cluster was required for copper resistance (Behlau et al. 2011; 2012). The *cop*LAB 9 gene cluster regulates how the bacterial cell sequesters copper by providing proteins that bind to copper ions and accumulate copper in the periplasm, preventing the ions from entering the cytoplasm (Cooksey, 1990; Voloudakis et al. 2005). Each of the three genes in the *cop*LAB cluster is essential, and they must all be present for the sequestering mechanism to function (Behlau et al. 2011). The *cop*ABCD cluster was first discovered in *Xanthomonas* spp. in 2015, and later was rediscovered in 2017. (Pereira et al. 2015; Richard et al. 2017). Similar to the *cop*LAB gene cluster, the *cop*ABCD gene cluster encodes copper-binding proteins that sequester ions from the cell (Adaikkalam and Swarup, 2005). Both gene clusters demonstrated that they are up regulated or activated in the presence of a high amount of copper ions, implying that the proteins are not made in significant quantities until the bacterial cell need the copper ions to be sequestered (Adaikkalam and Swarup, 2005; Behlau et al. 2011). When bacterial colonies with copper resistant genes are grown, they turn a blues color due to the buildup of copper ions in the periplasm (Voloudakis et al. 2005).

Some *Xanthomonas* spp. strains show tolerance but not total resistance when compared to resistant strains. Tolerance is described as bacterial strains that can tolerate a specific substance at a lower level than resistant strains but higher level than sensitive strains. There is no known unique genetic marker for tolerant strains, but the current assumption is that tolerance is caused by a set of highly conserved genes (Fan et al. 2018). The *cop*LAB genes, which have been demonstrated to increase in expression in the presence of copper, are the probable gene cluster (Marin et al. 2019). Copper efflux regulator-like proteins have also been linked to copper tolerant strains, as they have been demonstrated to collaborate with DNA and RNA polymerases, increasing the transcription of genes that transport copper ions out of the cell (Ma et al. 2009).

Horizontal gene transfer is a type of bacterial genetic exchange that is important for the bacterial adaptation and survival in their environment. Horizontal gene transfer can occur between bacteria of the same species but in completely distinct taxa. Recent research has shown that distinct xanthomonas pass copper resistant genes to one another (Behlau et al. 2012).

Furthermore, the same study found that even bacteria from unrelated genera, such as *Stenotrophomonas maltophilia,* which is rarely a plant disease, might transfer copper resistance genes to *Xanthomonas* spp. (Behlau et al. 2012).

2.5.3. Copper Resistance in *Pseudomonas syringae* spp.

Copper-based sprays have been used in agriculture since 1882, decades before the first antibiotic was discovered. Psa-3 strains introduced to New Zealand during the global outbreak of kiwifruit canker disease were initially susceptible to copper, but within four years, the first copper-resistant (CuR) Psa-3 strains were detected (Colombi et al. 2017). Sampling the area where CuR strains were initially discovered found that 27% of isolated Psa-3 were CuR. Resistance was obtained by acquiring three unique copper-resistant ICEs and a plasmid containing several resistance loci (*cop*ABCD and *czc*/*cus*ABC systems). Strains isolated from kiwifruit leaves in New Zealand in 1991 and 2010 (*Pseudomonas marginalis* and *P.* s. pv. *actinidifoliorum*, respectively) carried identical CuR encoding ICEs, demonstrating that *P. s.* pv. *actinidiae* had acquired those elements from the co-occurring species that had already evolved resistance to (Colombi et al. 2017).

Cooksey (1990) identified a *P. syringae* strain as the causal agent of a novel impatiens foliar blight. The bacteria were resistant to copper compounds, which are used to prevent bacterial and fungal disease on a number of crops. The bacterium had a single 47-kilobase plasmid (pPSI1) that was homologous to a previously cloned and characterized copper resistance operon from *P. s.* pv. *tomato* plasmid pPT23D. (Bender and Cooksey, 1986). Electroporation was used to change pPSI1 into a copper-sensitive *P. syringae* strain, and the transformants produced were copper resistant. Cooksey developed a physical map of pPSI1 and used Southern hybridizations to evaluate the extent of similarity to pPT23D outside the copper resistance operon. The two plasmids shared around 20 kilobases of homologous DNA, but the remainder of each plasmid lacked detectable homology. While homologous areas hybridized strongly, restriction enzyme recognition sites were rarely conserved.

Jae-Soon and Cooksey (1991) analyzed three of the copper-resistance operon protein (cop) products in order to gain a better understanding of the copper-resistance mechanism and its relation to copper buildup. *Cop*A (72 kDa), *Cop*B (39 kDa), and *Cop*A (12 kDa) proteins were generated only in response to copper induction. *Cop*A and *Cop*C were intracellular proteins, whereas *Cop*B was an extracellular membrane protein. Amino-terminal peptide

sequencing was used to corroborate the *Cop*A, *Cop*B, and *Cop*C leader peptide sequences. *Cop*A, *Cop*B, and *Cop*C were purified and their copper concentration calculated from strain PF23.2. *Cop*A bound 10.9 1.2 atoms of copper while *Cop*C bound 0.6 0.1 atoms of copper. They concluded that the *Cop* proteins appear to act as a copper-resistance mechanism by sequestering copper outside the cytoplasm.

According to Jae-Soon and Cooksey (1993) copper resistance in *P. syringae* harboring the *cop*ABCD operon is related with copper accumulation in the periplasm and outer membrane, likely as a result of the copper-binding activities of the *cop*ABC gene products. *Cop*D, on the other hand, has yet to be assigned a definite role. *P. syringae* cells harboring *cop*CD or *cop*BCD cloned behind the lac promoter were shown to be hypersensitive to copper in their study. Copper buildup was increased in cells having various combinations of cop genes, including *cop*C and *cop*D. Their findings indicated that *Cop*C, a periplasmic copper-binding protein, and *Cop*D, a likely inner membrane copper-binding protein, may cooperate in copper uptake.

Sundin et al. (1994) studied the genetic and plasmid diversity of *P. syringae* wild populations isolated from three ornamental pear nurseries in eastern Oklahoma. Plasmids were classified into two categories based on their homology to the oriV and par sequences from pOSU900, a cryptic plasmid found in *P. s.* pv. *syringae*. The genetic diversity of 100 randomly selected *P. syringae* isolates from nurseries I and III was examined using the arbitrarily primed PCR (AP-PCR) methodology. AP-PCR analysis of the isolates' chromosomal genotypes revealed a significant degree of genetic variety, with the results indicating that the isolates might be classified into two separate groups. Plasmid characteristics were found to be unique to isolates belonging to distinct AP-PCR groups. Among each AP-PCR group, isolates with varying chromosomal genotypes exhibited identical plasmid profiles, showing that plasmid transfer played a significant role in the spread of CuR and Smr within the populations investigated.

Scortichini et al. (2001) discovered that the majority of strains thrived on CYEG at a copper concentration of 7 µg/**ml**, but that seven strains were inhibited. Only three of the four Portuguese strains tested grew at a copper concentration of 20 µg/**ml**, and these likewise grew poorly at 60 µg/**ml**. All 61 *X. a.* pv. *juglandis* strains hydrolized and metabolized the starch in the BS medium.

Cazorla et al. (2002) investigated copper resistance in isolates of *P. syringae* obtained from mango. Copper resistance was determined in 59% of *P. s.* pv. *syringae* isolates. Following repeated treatments with the Bordeaux mixture, a study of a mango orchard found an increase in the frequency of copper-resistant bacteria. These findings imply that selection of copper-resistant bacteria may be a significant factor in control failures following copper bactericide management. While the majority of copper-resistant isolates carried plasmids, the majority of them included a 62-kb plasmid that was also present in copper-sensitive infections. Restriction enzyme analysis and hybridization to *cop*ABCD DNA were used to distinguish the 62-kb plasmids. The most frequently discovered copper-resistant plasmid (62.1) was conjugable. Southern blot hybridizations revealed that determinants largely identical to *cop*ABCD were present in all copper-resistant strains investigated and were frequently associated with plasmids; these determinants were not discovered in copper-sensitive bacteria.

Nakajima et al. (2002) discovered homology between the copper resistance genes of *P. s.* pv. *actinidiae* and *P. s.* pv. *tomato*. Twenty-eight isolates of *P. s.* pv. *actinidiae* were evaluated for resistance to copper sulfate. The strains were obtained in 1984, 1987, and 1988 from kiwifruit plantations in Japan. Copper sensitivity was observed in all isolates identified in 1984, with a minimum inhibitory concentration (MIC) of cupric sulfate of 0.75 mM. Nevertheless, several isolates identified in 1987 and 1988 were resistant, with MIC values ranging from 2.25 to 3.0 mM. All copper-resistant strains carried at least one of the following two plasmids: pPaCul (about 70.5 kb) or pPaCu2 (about 280 kb), or both. DNA sequence analysis of the *cop*A, *cop*B, *cop*R, and cops homologous region genes indicated the presence of four identical open reading frames (ORF A, B, R, and S). ORF A, B, R, and S had an amino acid sequence similarity of 80%, 70%, 97, and 95% with *cop*A, B, R, and S of *P. s.* pv. *tomato*, respectively.

Vanneste and Voyle (2003) investigated the genetic basis of copper resistance in *P. syringae* strains from New Zealand. *P. syringae* strains capable of growing on a minimum medium containing 500 mg/l copper sulphate were obtained in New Zealand from a collection of streptomycin-resistant bacteria. A 1.3 kb DNA was extracted from one of these copper-resistant bacteria using primers designed on genes associated with copper resistance. Sequencing of this fragment revealed that a 572 bp and a 275 bp segment shared 98% and 95% identity with ORF A of *P. s.* pv. *actinidiae*, a copper resistance gene. Smaller segments of this 1.3 kb fragment were similiar (89–100%) to segments of another copper resistance gene (*cop*A)

from *P. s.* pv. *tomato*. Using this 1.3 kb piece of DNA as a probe, similar DNA was discovered in eight other strains of *P. syringae,* all of which were later proven to be copper resistant.

Aiello et al. (2015) used phenotypic, genotypic, and pathogenicity testing to identify *P. s.* pv. syringae isolated from mango. The mangotoxin operon genes *mgo*A and *mgo*B were detected in all strains examined. In some orchards, copper-resistant *P. s.* pv. syringae bacteria with *cus*CBA genes were also detected. This characteristic may account for the ineffectiveness of copper compound-based disease control. No copper-sensitive bacteria were discovered among 71 strains tested in vitro, whereas 44 strains (62%) exhibited high resistance, 16 strains (22.5%) exhibited resistance, and 11 strains (15.5%) exhibited poor resistance to copper sulfate.

Zhang et al. (2017) recovered a total of 35 strains of *P. s.* pv. *phaseolicola* from snap bean samples taken in 2009 and 2010 from commercial orchards in Homestead, Florida. According to the results of this investigation, 80% of *P. s.* pv. *phaseolicola* strains (28 of 35) isolated from commercial snap bean fields were copper resistant, as determined by bacterial populations following 24-hour exposure to liquid NB medium. *P. s.* pv. *phaseolicola* strains grew similarly on CYE agar and liquid NB, both of which were amended with copper, implying that assays on CYE agar amended with copper can be used to rapidly test copper resistance in *P. s.* pv. *phaseolicola* populations. According to greenhouse trials, mancozeb addition increased the efficacy of copper hydroxide in controlling halo blight on snap bean produced by copper-resistant *P. s.* pv. *phaseolicola* strains.

Colombi et al. (2017) hypothesized that horizontal gene transfer could accelerate evolutionary change in *P. s.* pv. *actinidae* populations. In 2010, New Zealand was hit by a global epidemic of kiwifruit canker disease caused by *P. s.* pv. *actinidiae*. While the single clone responsible for the outbreak was susceptible to copper at the time of its introduction, analysis of a set of isolates obtained in 2015 and 2016 revealed that a quarter were copper resistant. Copper resistance, comprised of the *czc*/*cus*ABC and *cop*ABCD systems, as well as resistance to arsenic and cadmium, was acquired by uptake of integrative conjugative elements (ICEs), but also plasmids, as revealed by the genome sequences of seven strains. Comparative investigation revealed that ICEs have a mosaic structure, with one being a tripartite arrangement of two distinct ICEs and a plasmid recovered in 1921 (USA), 1968 (New Zealand), and 1988 (Japan), respectively, from *P. syringae* millet, wheat, and kiwifruit infections. Two *P. s.* pv. *actinidae* ICEs were almost identical to two ICEs isolated from kiwifruit leaf colonists previous to *P. s.* pv. *actinidae* introduction into New Zealand. Additionally, they demonstrated ICE

transfer *in vitro* and *in vivo*, examined the fitness repercussions of ICE carriage, demonstrated the *de novo* synthesis of novel recombinant ICEs, and investigated the ICE host range.

Zhao et al. (2018) found copper-resistant field isolates of *P. s.* pv. *actinidiae* biovar 3, a kiwifruit pathogen, in New Zealand. These isolates invariably contain substantial numbers of unique genetic elements, such as plasmids or integrative conjugative elements (ICEs), which were acquired horizontally from non- *P. s.* pv. *actinidiae* biovar 3 species of bacteria already present in New Zealand. They determined whether these acquired components are both necessary and sufficient to generate the copperresistant phenotype observed. Natural conjugation was used to transfer the 100,000-bp ICEs and big (>100,000 bp) plasmids from copper-resistant isolates to a sensitive recipient strain. The ICEs or plasmids conferred the donor strains' identical copper resistance phenotype, indicating that these components were both essential and sufficient to transmit copper resistance.

2.5.4. Copper Resistance in *X. a.* pv. *juglandis*

Moya-Elizondo et al. (2018) investigated the *in vitro* resistance of *X. a.* pv. *juglandis* bacterial strains to copper at various concentrations. The strains were collected from four orchards in the Biobio Region. Copper resistance was higher in Chile than earlier observed, regardless of the copper bactericides utilized (copper hydroxide, copper sulfate pentahydrate, and copper (I) oxide). Sensible *X. a.* pv. *juglandis* isolates were found in three of the four walnut orchards examined.

Copper tolerance in Australian *Xanthomonas* species associated with bacterial leaf spot was studied by Roach et al (2020). All 44 Australian *Xanthomonas* strains could not develop in media containing more than 1.5 mM CuSO4 5H2O, however they could tolerate at least 0.5 mM CuSO4 5H2O. All strains grew on copper-free media, and the tolerant and sensitive control strains had MICs of 2 mM and 0.2 mM, respectively. Thirteen strains had the same level of copper tolerance as the resistant control (2 mM). Copper tolerance was higher in *X. euvesicatoria, X. perforans,* and *X. vesicatoria* strains (1.5, 1.5, and 2 respectively) than in *X. arboricola* (0.8) and uncharacterized *Xanthomonas* strains (1).

Fu et al. (2021) isolated 60 different *X. a.* pv. *juglandis* strains (59 from Hubei and one from Beijing). When the copper resistant threshold value was set to 125 g/ml, 47 strains were classified as sensitive while 13 strains were resistant to copper. In addition, five strains

demonstrated copper resistance at 270 µg/ml. The *cop*B gene in resistant strains contained a 15-bp insertion and eight dispersed single nucleotide polymorphisms when compared to the *cop*B gene in susceptible strains.

2.6. Bacteriophages

Bacteriophages, sometimes known as phages, are viruses that infect only bacteria. As is the case with all viruses, they are metabolically inert in their extracellular form and must multiply through parasitizing a host. Phages are widespread, inhabiting all niches occupied by bacteria, and have population sizes hundreds of times larger than bacteria. Tailed phages are the most numerous type of phage and are classified in the order *Caudovirales* (Weinbauer, 2004). Virions in this order have an icosahedral symmetric head connected to a hollow helical symmetric tail. The head includes a single linear double stranded DNA genome that is not segmented (dsDNA). Only proteins are encoded by phage genomes. Contractile or non-contractile, straight or flexible, long or short, phage tail-type architectures are possible. Phages are classified into three families based on their tail morphology: *Sipho*-, *Myo*-, and *Podoviridae*. *Siphoviridae* have long non-contractile tails and account for approximately 61% of phages with tails. *Myoviridae* have contractile tails composed of a sheath and a central tube and account for around 25% of tailed phages, whereas *Podoviridae* have short tails and account for around 14% of tailed phages.

2.6.1. Bacteriophages life cycle

In general, the phage's life cycle can be split into stages, beginning with adsorption, nucleic acid transfer, nucleic acid expression, and replication, virion assembly, and finally, virion release and transmission (Lindberg, 1977; Abedon, 2006). Phage adsorption occurs in two stages: a reversible binding of the phage tail fibers to a specific cell surface structure or 'receptor', followed by an irreversible binding. The receptor type varies amongst phages and may consist of sugars, lipopolysaccharide, or pili (Lindberg, 1977). Once the phage has bound to the cell, the phage's base plate may reorganize, generating a hole. The outer sheath contracts, and an internal tube is inserted into the cell through the outer membrane, peptidoglycan, and periplasm (*i.e*. T4). Not all phages possess the structures necessary for physical DNA injection (*i.e*. T7). Rather than that, these phages breakdown the cell wall by the use of enzymes found in the tail or capsid. This results in the formation of holes in the cell wall, allowing phage DNA to enter the cell. Outside of the bacterial cell, the empty phage capsid persists.

Following DNA entry, genetic material can go through a variety of different life cycles (Campbell, 2006; Abedon, 2006; Koch, 2007). In lytic infections, virulent (or lytic) phage nucleic acids lead host metabolism toward the generation of additional virions that are released after cell lysis. Typically, the nucleic acid from temperate phages integrates into the host chromosome during lysogenic infections (Lwoff, 1953; Campbell, 2006; 2007). Lysogeny is the integration of a phage genome, referred to as a prophage, that is not transcribed to generate new virions and hence retains bacterial viability (Stewart and Levin 1984). During host replication, the prophage multiplies. This dormant lysogenic condition can last forever or can be terminated at any time (transition to lytic infection) in response to specific environmental stimuli or pressures (Lwoff, 1953; Campbell, 1961; Stewart and Levin 1984; Campbell, 2006). In pseudolysogenic infections, the phage genome coexists with the host bacterium in an unstable state, where it fails to reproduce (Ripp and Miller, 1997; Weinbauer, 2004; Abedon, 2008). Unlike lysogeny, pseudolysogeny does not involve the integration of the phage genome into the host chromosome.

2.6.2. Interactions between phage and bacteria

On average, roughly 10^{25} phage infections occur per day on Earth, and roughly 10^{15} gene transfer events occur by phage transduction (Brussow et al. 2004, Frost et al. 2005). This continuous arms race between phages and their bacterial hosts has happened over billions of years and has resulted in fundamental modifications to the bacterial genetic architecture. Indeed, a number of pathogenic bacterial strains, including *Clostridium botulinum*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Escherichia coli*, owe their pathogenicity to to temperate phages (Keen, 2012).

To ward off phage predation, bacteria have evolved a variety of mechanisms for blocking phage adsorption or halting phage proliferation within the cell (Hyman and Abedon, 2010). These techniques can be classified into two categories depending on their ability to disrupt specific stages of the phage life cycle:

i) Inhibitors of phage adsorption. These include the inhibition or modification of phage receptors, the creation of biofilms, and the exclusion of superinfection (Sie).

ii) Anti-phage mechanisms. These include restriction modification systems (R-M), Bacteriophage Exclusion Systems (BREX), Abortive Infection Systems (Abi), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR).

In this part, I will further explain only restriction modification systems.

2.6.2.1. Restriction modification systems

Restriction modification (R-M) systems were found in *E. coli* and have now expanded throughout bacteria. R-M systems are mobile genetic elements that can be horizontally transported between bacterial genomes and use restriction enzymes to destroy non-methylated foreign DNA (Kobayashi, 2001). R-M systems are classified into four types (I-IV) and use one of two distinct enzyme types to avoid phage infection. The proteins used in these systems vary greatly, not only in terms of structure, but also in terms of recognition and cleavage sites. R-M systems employ enzymes such as restriction endonucleases, which introduce double-stranded DNA breaks in a sequence-specific way, and methyltransferases, which methylate bacterial DNA to protect it from destruction (Labrie et al. 2010). Although R-M systems defend bacteria from phage invasion, they come at a cost if they are ever lost, as in the instance of *E. coli* K-12, where the loss of a temperature sensitive R-M plasmid decreases bacterial viability (Chinen et al. 2000).

2.6.3. Bacteriophages for the Control of Walnut Bacterial Blight

Romero-Suarez et al. (2012) conducted a study to isolate and characterize bacteriophages from walnut orchards throughout New Zealand. Twenty-six *X. a.* pv. *arboricola* phages were isolated from 326 plant samples representing phyllosphere and rhizosphere populations. Plaque and particle morphology, host range, restriction digest, and phylogenetic analysis, and persistence under various storage conditions were used to describe the phage isolates. The Hind III restriction digestion resulted in phage genome sizes ranging from 38.0 to 52.0 kb, with a 400 kb segment that was similar at the DNA level. Despite having similiar restriction patterns, maximum parsimony bootstrap analysis revealed that the phages belonged to different groups. They hypothesized that these phages could be used in a biocontrol, therefore they examined their storage stability and efficacy. They found that the titres of the phags dropped by more than 50% more than a 12 month storage period. They also observed that deep-freezing temperatures (-34 °C) boosted stability whereas chloroform diminished it.

Dömötör et al. (2016) found that the broad host range phages Xaj2 and Xaj24 had an exceptional ability to destroy *X. a.* pv. *juglandis* in laboratory conditions. After three successive single-plaque purifications, twenty-four phages against *X. a.* pv. *juglandis* were identified from enriched samples. Thirteen phages were isolated from the rhizosphere soil, five from fruits, four from leaves, and two from walnut bark. The host specificity of the chosen phages was tested using 35 strains of *X. a.* pv. *juglandis* and other plant pathogenic bacteria. At least one of the two phages was capable of infecting all *X. a.* pv. *juglandis* strains. The whole genomes of Xaj2 and Xaj24 have been determined. The genomes of Xaj2 and Xaj24 included 49.241 and 44.861 nucleotides, respectively, containing 80 and 53 genes. Comparative genome investigations found that Xaj2 had a distinct genome sequence, whereas Xaj24 was a phiKMV-like phage most closely related to the Prado phage, which is virulent for *Xylella fastidiosa* and *Xanthomonas* spp.

2.6.4. Bacteriophages for the Control of Bacterial Canker of Stone Fruit

Prior et al. (2007) identified and purified bacteriophages from supernatants of the plant pathogenic bacterium *P. s.* pv. *tomato* using density block centrifugation in cesium chloride (CsCl) step gradients. The DNA was extracted from purified phage and digested with the restriction endonucleases *Eco*Rl or *Hind*III. Three distinct DNA fingerprint patterns were discovered, indicating three distinct phage isolates. The phage's genome sizes ranged from 40 to 52 kilobases (kB). Buoyant densities of phage particle buoyant densities in CsCI were from 1.36 to 1.51 g/ml.

Di Lallo et al. (2014) demonstrated the isolation and characterisation of two bacteriophages capable of infecting *P. s.* pv. *actinidae* specifically. ϕPSA1, a member of the *Siphoviridae* family, is a temperate phage with a small host range, a long latency, and a burst size of 178; ϕPSA2, a lytic phage of the *Podoviridae* family, has a wide host range, a short latency, a burst size of 92, and a higher TOD value. ϕPSA1's genomic sequence is 51,090 bp long and has minimal sequence homology with the other siphophages, whereas genome sequence of ϕPSA2 is 40 472 bp long and has 98% homology with *P. putida* bacteriophage gh-1. ϕPSA2 may be a possibility for phage therapy of kiwifruit disease, while ϕPSA1 appears to be unique to the isolates from the recent outbreak and may be relevant for *P. s.*pv. *actinidae* typing.

Frampton et al. (2014) identified 275 phages, with 258 of them active against *P. s.* pv. *actinidiae*. Extensive host range testing on *P. s.* pv. actinidiae, various *Pseudomonads*, and bacteria isolated from kiwifruit plantations revealed that the majority of phages have a restricted host range. Electron microscopy, pulse-field gel electrophoresis, and restriction digestion were used to examine twenty-four samples. Their suitability for biocontrol was evaluated by examining their stability as well as the absence of lysogeny and transduction. A thorough host range analysis was carried out, phage-resistant bacteria were isolated, and resistance to additional phages was investigated. Twenty-one members of the *Myoviridae* family exhibit similiar morphologies and genome sizes but differ in restriction patterns, host range, and resistance, indicating a closely related group. In conclusion, this study describes the identification and characterisation of *P. s.* pv. *actinidiae* phages and provides a foundation for the formulation of phage biocontrol agents against kiwifruit bacterial canker.

Ji-Gang et al. (2016) isolated bacteriophages from soils collected from kiwifruit orchards in Korea and chose seven bacteriophages for further characterisation based on restriction enzyme digestion patterns of genomic DNA. Based on transmission electron microscopy, two of the bacteriophages investigated belong to the *Myoviridae* family and three to the *Podoviridae* family. Host range of the selected bacteriophages was confirmed using 18 strains of *P. s.* pv. *actinidiae*, including the *P. s.* pv. *actinidiae* biovar 2 and *P. s.* pv. *actinidiae* biovar 3 groups, and several were also effective against additional *P. syringae* pathovars. The lytic activity of the selected bacteriophages was sustained *in vitro* for 80 hours, and it was stable at 50oC, pH 11, and UV-B radiation. These findings suggest that the isolated bacteriophages are specific to *P. syringae* species and are resistant to a variety of environmental variables, hinting that they could be used to prevent bacterial canker disease in kiwifruit.

Rombouts et al. (2016) recovered five novel phages from infected fields in Flanders, Belgium, for biocontrol of bacterial blight in leeks caused by the bacterial pathogen *Pseudomonas. syringae* pv. *porri*, and complemented them with one selected host range mutant phage (vB PsyM KIL3b). The phages' genomes ranged in size from 90 to 94 kb, with an average GC content of 44.8%. The stability and lytic capacity of these phages were established *in vitro*. Host range study demonstrated heterogeneity within *P. s.* pv. *porri*, leading to the development of a phage cocktail with a range that includes all 41 strains studied. Specific bio-assays revealed phage effectiveness *in planta*. Furthermore, two parallel field trial tests on three different locations using a phage cocktail of the six phages yielded inconsistent results. Symptom

development was slowed in one trial. These findings show that phage therapy has some potential for suppressing bacterial blight of leeks, pending optimization of formulation and application methods.

Flores et al. (2020) isolated and characterized 13 phages with the potential to suppress *P. s.* pv. *actinidae* infections in kiwifruit plants. The phages were classified based on their host range and the pattern of restriction fragment length polymorphism (RFLP). Four phages were chosen based on their lytic effect on bacteria and tolerance to various environmental circumstances such as temperature (4–37 C), pH (4–7) and UV exposure (30 and 60 min). The sequences of the selected phages (ϕCHF1, ϕCHF7, ϕCHF19, and ϕCHF21) revealed a strong similarity with the podophage of PsaϕphiPSA2. *in vitro* tests on kiwifruit leaf samples revealed that the phage mixture lowered the *P. s.* pv. *actinidae* bacterial load within three hours of treatment and reduced the damage index in 50% of cases. Similarly, experiments with kiwifruit plants grown in greenhouses revealed that these phages were able to lower the *P. s.* pv. *actinidae* bacterial load in more than half of the instances and resulted in a significant decrease in the damage index of treated plants after 30 days. Finally, none of the chosen phages were able to infect the other bacteria found in the kiwifruit plants' native microbiota. These findings indicate that bacteriophages are an appealing alternative for controlling *P. s.* pv. *actinidae* infections in kiwifruit plants.

Jørgensen et al. (2020) isolated three phages from organic waste samples that target *P. syringae* GAW0113: *Pseudomonas* phage Bertil, Strit and Misse. The phages have double-stranded DNA genomes ranging in size from 41342 to 41374 bp, with 50 to 51 open reading frames. The genomes of the three phages are remarkably similar, and genomic comparison analysis suggest that they all belong to the *Autographivirinae* subfamily of the *Podoviridae* family. The phages are only distantly linked to other members of this family and have minimal gene synteny with type-phages from other *Autographivirinae* genera, suggesting that the newly isolated phages may represent a new genus.

Pinheiro et al. (2020) explored the potential use of bacteriophage (or phage) 6 to prevent *P. s.* pv. *actinidae* infections, which is already commercially available. *P. s.* pv. *actinidae* inactivation was tested *in vitro* with liquid culture media and *ex vivo* with intentionally contaminated kiwifruit leaves containing two biovar 3 (a highly aggressive pathogen) strains (*P. s.* pv. *actinidae* CRA-FRU 12.54 and *P. s.* pv. *actinidae* CRA-FRU 14.10). The phage 6 proved efficient against both strains *in vitro* (highest reductions of 2.2 and 1.9 CFU/ml for *P. s.*

pv. *actinidae* CRA-FRU 12.54 and *P. s.* pv. *actinidae* CRA-FRU 14.10, respectively). The decrease was smaller in *ex vivo* experiments (maximum reduction 1.1 log and 1.8 CFU/ml for *P. s.* pv. *actinidae* CRA-FRU 12.54 and *P. s.* pv. *actinidae* CRA-FRU 14.10, respectively). The findings of this study indicate that commercially available phage 6 can be a viable alternative for controlling *P. s.* pv. *actinidae* infections in kiwifruit orchards.

Rabiey et al. (2020) identified seventy phages from cherry leaf, soil and bark to control *P. syringae* infections in the south east of England. Following that, their host range was tested against *P. s.* pv. *syringae*, *P. s.* pv. *morsprunorum* race 1, and *P. s.* pv. *morsprunorum* race 2 strains. While these phages lysed *P. s.* pv. *syringae*, *P. s.* pv. *morsprunorum* and other *P. syringae* pathovar isolates, they did not infect beneficial bacteria such *Pseudomonas fluorescens*. Genome sequencing was used to further describe a group of thirteen phages, finding five separate clades into which the phages could be classified. There were no known toxins or lysogeny-associated genes found. Using bioassays, selected phages were able to effectively limit disease progression *in vivo*, both individually and in cocktails, validating their promise as biocontrol agents.

3. MATERIALS AND METHODS

3.1. PSEUDOMONAS

3.1.1. Establishment of a Bacterial Collection

Symptomatic plant material (twigs, leaves and fruits of apricot) was collected from various apricot cultivars in apricot orchards in the eastern Emilia-Romagna region during 2018 and 2019. Sampling period started in early March, just before bud break, and lasted until late June.

Isolation was done from symptomatic material including leaves, fruits, branch/twig cankers, necrotising buds and wilting flowers. Bacterial isolation and purification were performed on nutrient sucrose agar (NSA) plates (Crosse, 1959). Symptomatic plant samples were surface-sterilized with 70% ethanol for 2 minutes, then washed for 1 minute under tap water. Small sections of surface-sterilized tissue were excised from the margins of diseased areas of buds/fruits/leaves/wood with a sterile scalpel under aseptic conditions and macerated separately for 1 minute in 1 ml sterilised Na PBS buffer. The extracts were then centrifuged at 10,000g for 20 min at 4 °C, and the obtaining pellets were resuspended in 1 ml sterile distilled water to obtain final concentrates. Serial 10-fold dilutions of these concentrates were then produced (i.e., 1:1; 1:10; 1:100 and 1:1000). Twenty microliters per dilution was streaked on NSA and incubated for 48 h at 27 °C.

After incubation, putative *Pseudomonas* colonies (pearl grey and mucoid, with whole margins) were then picked and purified on NSA plates for some further studies. Gram reaction and ability to cause HR of purified Pseudomonas-like isolates were examined in tobacco leaves (Klement and Goodman, 1967).

The detached leaf procedure, as defined by Randhawa and Civerolo (1985), was used to confirm the pathogenicity of all 54 putative *Pseudomonas* spp. strains. Reference strains CFBP 6400 (*P. s.* pv. *syringae*) and CFBP 2351 (*P. s.* pv. *morsprunorum*) as positive controls and sterile distilled water as a negative control were used. Eventually, the putative *Pseudomonas* spp. isolates were preserved at -80°C in Luria-Bertani broth supplemented with 20% sterilised glycerol (Bertani, 1951).

3.1.2. Phenotypic Characterisation

On certain iron-deficient media, such as King's medium B, several strains of *P. syringae* produce yellow, green or blue diffusible pigment, which is particularly useful for demonstrating the development of the siderophore pyoverdin, which is highly fluorescent under UV light (Cody et al. 1987). All putative *P. syringae* isolates were screened for fluorescence on King's B medium. After 24-48 hours of incubation, fluorescence on King's medium B was observed under UV light (Schaad et al. 2001).

3.1.2.1. LOPAT Tests

LOPAT tests is a method which have proven to be very useful in identification of *P. syringae* patovars. These tests include levan production, oxidase reaction, potato rot, production of arginine dehydrolase and hypersensitivity reaction on tobacco (see Table 5.2).

For pathovar identification, LOPAT tests were used according to Lelliott and Stead (1987) as indicated blow:

Levan production (L): 24 h old bacterial culture streaked in Petri dishes containing Nutrient Agar and 5% sucrose (NSA) and then incubated at 26-28°C for 24-48 hours. Levan production is demonstrated by the formation of characteristic mucoid, convex, pearly-white, glossy colonies.

Oxidase production (O): One loopful 24 h old bacteria was taken from King's medium B and transfered on a steril filter paper soaked in 1% (w/v) N,N,N',N'- tetramethyl-p-phenylenediamine dihydrochloride solution. A positive result is shown by the presence of a violet color in less than 10 seconds was considered as a positive result.

Pectinolytic activity (P): Potato slices were sterilized with 1% bleach and washed with sterile water. 24 h old *Pseudomonas syringae* strains were stab-inoculated in the center of potato slices placed in a sterile box containing moist sterile filter paper. Pectolytic ability is verified by the occurrence of softness and soft rot on potato slices after 24-48 hours of incubation at 26-28°C. Sterile water was used as a negative control while a reference strain of *P. carotovorum* was a positive control.

Arginine dihydrolase activity (A): The presence of arginine dihydrolase (A) is determined by transferring 24 h old *P. syringae* strains to tubes containing semi-liquid arginine

(0.01 % peptone, 0.5 % NaCl, K2HPO4, 0.001 % phenol red, L (+) arginine, and 1 %agar). After inoculation, each tube is coated with sterile mineral oil. One tube was left uncoated. After 2-5 days in the anaerobic tube, the color changes from orangered to magenta-amarant, indicated the presence of arginine dihydrolase and evaluated as a positive reaction.

Hypersensitive reaction (HR) (T). A thick suspension (ca. 10^8 cells.ml^{-1}) of 24 h *P. syringae* strains grown on King's medium B was infiltrated into the mesophyll of a fully grown tobacco leaf. Rapid glassy to white necrosis of the infiltrated area 24 h after inoculation indicated the ability to induce a hypersensitivity reaction.

3.1.2.2. GATTa Tests

GATTa tests (Lelliot and Stead, 1987) are widely used to differentiate pathovars within *P. syringae*.

Gelatine hydrolysis (G):

A sterile medium containing 0.3% yeast extract, peptone 0.5%, gelatine 12% in a tube was inoculated with 24 h old *P. syringae* strain. After 7-14 days incubation at 18°C, characteristic liquefaction of gelatine was considered as a positive result (Lelliott and Stead, 1987).

Aesculin hydrolysis (A):

24-48 h old *P. syringae* strains grown on NA medium was introduced into a semi-solid medium containing peptone 1%, esculine 0.1%, 0.05% ferric citrate, agar 2%. After 24-48 h incubation at 26°C, brown colour change in the semi-solid medium was considered positive result and indicated the presence of the *β-glucosidase* enzyme.

Tyrosinase activity (T):

24-48 h old *P. syringae* strains was streaked into a semi-solid medium containing 0.5% sucrose, 1% casamino acid, L-tyrosine 0.1%, 0.05% potassium phosphate, magnesium sulphate heptahydrate 0.0125%, agar 2%, pH 7.2. Red colour change of the media after 7-10 days incubation at 26 °C indicated the activity of tyrosinase.

Tartrate (Ta) utilization:

A liquid medium containing 0.1% ammonium dihydrogen phosphate, potassium chloride 0.02%, magnesium sulfate heptahydrate 0.02%, 1 ml of 4% alcohol bromothymol blue solution at pH 7.0 was inoculated with 24 h old *P. syringae* strains. From green to blue colour change of the medium as evaluated as a positive result (Lelliott and Stead, 1987).

3.1.3. *in vitro* Ice Nucleation Activity of Pseudomonads

All the *P. syringae* strains was checked for their ability to form ice nucleation under *in vitro* conditions. The ice nucleation activity of the strains was assessed at four different temperatures (from -2 °C to -5 °C) with the help of a refrigerated circulating bath (Julabo GmbH F18, Seelbach, Germany). The INA+ *Pseudomonas* spp. strains DLS 58 and DLS 84 described by Tontou et al. (2016) was used as a positive controls in the experiment.

3.1.4. *in vitro* Characterisation of Copper Resistance Level in *P. syringae* strains

The 54 *P. syringae* strains collected from Emilia-Romagna apricot orchards during 2018-2019 was tested to evaluate their copper resistance. The strains were grown on a medium with a low concentration of copper to induce *cus*CBA gene cluster activity (Basim et al. 2005). For this reason, a loopful bacterial culture streaked into NA plates amended with 20 ppm Cu++. Later, copper resistance level of the strains were checked on mannitol glutamate-yeast agar (MGYA) supplemented with increasing concentrations of copper: 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm cupric sulphate (Bender et al. 1990). Bacterial suspensions were adjusted to 10^8 CFU ml^{-1} spectrophotometrically (Ultraspech 1000; Pharmacia Biotech, Boston, Massachusetts, optical density at 600 nm = 0.3; ~1×10^8 CFU ml^{-1}). Ten microlitres of bacterial suspensions into grids were streaked onto copper supplemented MGYA medium. Inoculated plates were incubated at 27 °C for 48 h. Positive controls were two highly copper-resistant *X. a.* pv. *juglandis* strains, Xaj 71 and Xaj 79, previously described by Giovanardi et al. (2015).

3.1.5. Molecular Characterisation

For molecular characterization, isolates were choosen in order to cover all the different cultivars monitored, plant organs, time of sampling, various degree of pathogenicity and

phenotypic traits. Eventually, 28 strains were selected to represent all the obtained *P. syringae* isolates in this thesis.

3.1.5.1. Genomic DNA Extraction

To extract genomic DNA from the *P. syringae* isolates, all the representative strains were grown on NSA medium for 24-48 h and harvested by washing the medium surface with sterile distilled water. The concentration of *P. syringae* cells were adjusted to 1×10^7 CFU ml^{-1} with sterilised water by using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) (optical density at 600 nm = 0.3; ~1×10^8 CFU ml^{-1}). Later, bacterial suspensions were boiled for 10 min on a termal block at 95 °C, placed on ice for 5 minutes and centrifuged for 5 minutes at 10,000 x g. Then, the NanoDrop ND-1000UV-Vis spectrophotometer was used to control the quantification and verification of extracted DNA purity. Eventually, the bacterial DNA suspensions were standardized to a concentration of 50 ng/ µl.

3.1.5.2. Polymerase Chain Reaction for Detection of Toxin-producing Genes

P. syringae pathovars associated with stone fruits contain a number of well-characterized phytotoxic substances that can be used to distinguish pathovars. The PCR method enables to detect genes involved in syringomycin synthesis (*syr*B), syringomycin secretion (*syr*D), and coronatine production (*cfl*).

4.1.5.2.1. *syr*B gene

A set of primers (B1/ B2) (Table 3.1) was used to detect the *syr*B gene (Sorensen et al. 1998) in the representative *P. syringae* strains. The target sequence was amplified by PCR in 25 µl of the following reaction mixture: 1 PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, 0.5 µM of each primer, 0.2 mM deoxynucleoside triphosphate (each), 1.25 U Taq DNA polymerase. 2 µl of bacterial suspension in sterile distilled water was used as a template. The PCR steps were: template denaturation at 94°C for 1.5 min, primer annealing at 60°C for 1.5 min and DNA extension for 3.0 min at 72°C with an additional extension for 10 min at 72°C.

4.1.5.2.2. *syr*D gene

The *syr*D gene was detected using the Bultreys and Gheysen method (1999). The reaction mixtures (25 µl) were prepared using: 1×PCR buffer, 1.25 mM MgCl2, 0.2 mM deoxynucleoside triphosphates (each), 25 pmol of each primer (SyD1/SyD2) (Table 3.1), 0.5 U Taq DNA polymerase and 2 µl of template DNA. Amplification was initiated by incubation at 93°C for 3 min, followed by 35 cycles at 94, 60, and 72°C for 1 min at each temperature and final extension at 72°C for 5 min.

4.1.5.2.3. *cfl* gene

The *cfl* gene, which is involved in coronatine synthesis, was identified using the PCR method described by Bereswill et al. (1994). PCR mixtures were prepared in a final volume of 25 µl, including 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8) and 1.5 mM MgCl2), 0.8 µM of each primer (Primer 1/Primer 2) (Table 3.1), 0.2 mM deoxynucleoside triphosphates (each), 1.5 U Taq DNA polymerase, and 1 µl template DNA. PCR cycling parameters started with an initial denaturation step at 93°C for 2 min and followed by 37 cycles of 2 min at 93°C, 1 min at 67°C and 2 min at 72°C.

Table 3.1. Lists the names of the primers, the oligonucleotide sequences, the expected size of the amplified products, and the literature sources

Primer	**Sequence**	**Tm**	**Reference**
B1	CTTTCCGTGGTCTTGATGAGG	60 °C	Sorensen et al. 1998
B2	TCGATTTTGCCGTGATGAGTC		
*syr*D1	CAGCGGCGTTGCGTCCATTGC	60 °C	Bultreys and Gheysen (1999)
*syrD*2	TGCCGCCGACGATGTAGACCAGC		
cfl-F	GGCGCTCCCTCGCACTT	67°C	Bereswill et al (1994)
cfl-R	GGTATTGGCGGGGGTGC		

The PCR reactions were carried out on a Thermo Cycler GeneAmp 2400 (Applied Biosystem, USA). The amplicons were separated by 1,2% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer, then stained with ethidium bromide (1 g/ml) for 20 min, washed with strerile distilled water and and photographed with a digital imaging camera under UV light (Vilber Lourmat, France).

3.1.5.3. Molecular Fingerprints of *Pseudomonas syringae* Isolates by Combined rep-PCR Analysis

A total of 28 *P. syringae* isolates were subjected to rep-PCR genomic fingerprinting using the primer sets for the BOX, ERIC, and REP elements, as described by Versalovic et al. (1991). The concentration of *P. syringae* cells were adjusted to 1×10^7 CFU ml^{-1} with sterilised water by using a spectrophotometer (optical density at 600 nm = 0.3; ~1×10^8 CFU ml^{-1}). The 25 µl reaction mix consisted of 1x PCR buffer (GoTaq Flexi Buffer, Promega, USA), 1.25 mM deoxyribonucleoside triphosphate, 6.7 mM MgCl2, 60 pmol of each primer (Table 3.2), 2 U Taq DNA polymerase and 1 µl DNA. PCR amplification was carried out in thermocycler under the following conditions: one initial cycle at 95°C for 2 min; 30 cycles of denaturation at 94°C for 3 s and 92°C for 30 s, annealing at 40, 52 or 53°C for 1 min with REP, ERIC and BOX primers respectively extension at 65°C for 8 min; single final extension at 65°C for 8 min and then held at 4°C. The *P. s.* pv. *syringae* reference strains CFBP 6400 and CFBP 4887, as well as the *P. s.* pv. *morsprunorum* reference strain CFBP 2351 and two other *P. syringae* pathovars were included in the study as an outgroup for comparison. Following amplification, the BOX, ERIC, and REP amplicons were run on a %1 agarose gel and stained for 30 minutes with ethidium bromide (0.5 g ml^{-1}); gel images were photographed using BioDoc Analyze (Biometra). The 3 different rep-PCR profiles obtained for each primer set. The profiles then normalized and analyzed with GelCompar 4.1 software (Applied Maths, Kortrijk, Belgium). Using unweighted pair group arithmetic average (UPGMA) clustering analysis, similarity matrices were generated and calculated to create a dendrogram (Sneath and Sokal, 1973). In a combined analysis with the three primer sets mentioned above, the Pearson correlation coefficient was used to investigate the genetic relationships among the *P. syringae* collection.

Table 3.2. Primer list to differentiate bacterial strains by REP-PCR

Primer	Sequence
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC
ERIC 2	AAGTAAGTGACTGGGGTGAGCG
BOX AIR	CTACGGCAAGGCGACGCTGACG
REP 1R	IIIICGICGICATCIGGC
REP 2I	ICGICTTATCIGGCCTAC

3.1.5.4. Multi-locus sequence analysing (MLSA)

This assay was used to differentiate 12 representative *P. syringae* spp. strains and shown to be more specific than 16S rRNA sequence analysis, particularly when analyzing a few housekeeping genes from the bacterial core genome (Maiden et al., 1998; Hwang et al., 2005; Stackebrant et al., 2002). Primers for amplification of four housekeeping genes, *gyrB* (gyrase), *acnB* (aconitate hydratase B), *pgi* (phosphoglucoisomerase), and *pfk* (phosphofructokinase) (Table 3.3) were successfully used following the protocol of Sarkar and Guttman (2004). The 25 µl reaction mix consisted of 1x PCR buffer (GoTaq Flexi Buffer, Promega, USA), 1.25 mM deoxyribonucleoside triphosphate, 5 mM MgCl2, 50 pmol of each primer, 2 U Taq DNA polymerase and 1 µl DNA. PCR protocols were: i.e. annealing temperature of 62ºC, 52°C, 53°C and 56ºC for *gyr*B, *acn*B, *pgi* and *pfk* respectively (Kaluzna et al. 2010a).

Table 3.3. Primer list for MLSA of *Pseduomonas syringae* spp.

Primer	Sequence	Tm	Reference
*gyr*B-F	TCBGCRGCVGARGTSATCATGAC	62,9 °C	Hwang et al. (2005)
*gyr*B-R	TTGTCYTTGGTCTGSGAGCTGAA	60,7 °C	
*acn*B-F	ACATCCCGCTGCACGCYCTGGCC	60°C	Sarkar and Guttman (2004)
*acn*B-R	GTGGTGTCCTGGGAACCGACGGTG	60°C	
pgi-F	TGCAGGACTTCAGCATGCGCGAAGC	60°C	Sarkar and Guttman (2004)
pgi-R	CGAGCCGCCCTGSGCCAGGTACCAG	60°C	
pfk-F	ACCMTGAACCCKGCGCTGGA	63°C	Sarkar and Guttman (2004)
pfk-R	ATRCCGAAVCCGAHCTGGGT	63°C	

3.1.5.5. Copper Resistance

The the whole *P. syringae* collection was investigated using the Aiello et al. (2015) methods for detecting *cus*CBA gene sequences by using primer sets *cus*C-F/*cus*C-R, *cus*B-F/*cus*B-R, and *cus*A-F/*cus*A-R. For the molecular detection of the *cus*CBA gene sequences, the *P. s.* pv. *syringae* strain PSM47 was used as a positive control. Table 3.4 summarizes the primer list. All PCR reactions were carried out in a Bio-Rad thermal cycler under the following cycling conditions: denaturation at 95°C for 5 minutes; 35 cycles of 35 seconds of annealing at 58°C and extension at 72°C for 1 minute; and 5 minutes of final extension at 72°C. PCR products were separated in %1,2 agarose gels and visualized by using BioDoc Analyze (Biometra, Göttingen, Germany).

Table 3.4. Primer list for the molecular detection of the cusCBA gene

Primer	Sequence	Tm	Reference
*cus*C-F	ATGCGCGAGAGGTTCTTTTC	58°C	Aiello et al. (2015)
*cus*C-R	CAGCTCAACCTACAACGCTC	58°C	
*cus*B-F	CGATAGTTTGTACGGCAGCC	58°C	Aiello et al. (2015)
*cus*B-R	CCAAAATACGCCGATGAGCA	58°C	
*cus*A-F	ATCTGCCACCATCGATAGGG	58°C	Aiello et al. (2015)
*cus*A-R	CCTGAAAAGCTGATCGAGGGC	58°C	

3.1.5.6. Detection of the *ina*Z genes

The PCR analysis was carried out to detect the *ina*Z gene using *P. syringae* forward (5'-GCAGACTGCGGGTT ATGAGAGC-3′) and reverse (5'-CGCCGGTC AGTTTGCTTCTATC-3′) primers (Nejad et al. 2006). The *P. s.* pv. *syringae* strain CFBP 1392 was used as a positive control for the detection of the *ina*Z gene sequence at the molecular level. The PCR was carried out in a reaction mixture that included: 2 µl 10X buffer, 2 µl deoxynucleotide mixture (with 2,5 mM of each dGTP, dTTP, dATP, and dCTP), 1.5 µl MgCl2 (25 mM), 2,5 µl primer (10 pM of each), 0.2 µl Taq DNA polymerase, and 2 µl of the dilute template. Milli-Q water was used to dilute the reaction mixture to a final volume of 25 µl. The amplification program was set at 35 cycles with denaturation for 45 sec at 95°C, annealing at 60°C for 45 sec and extension of 1 min at 72°C and with a final extension at 72°C for 5 min.

3.2. X. a. pv. *juglandis*

3.2.1. Bacterial Collection

Field surveys were conducted in Emilia-Romagna between 2018-2019, primarily in orchards where disease severity was constantly observed. Buds, leaves, fruits, twigs, large branch cankers were all sampled. Symptomatic plant samples were surface-sterilized for 2 minutes with 70% ethanol, then washed for 1 minute under tap water. A small section between necrotic area and healthy tissue was taken with a sterile scalpel and subsequently individually macerated in 1ml of sterile distilled water. The macerated homogenate streaked with a sterile loop on GYCA medium (Dye, 1962) (Glucose-Yeast extract-Calcium carbonate Agar). The petri dishes were incubated at 28 ° C for 48 h to obtain putative *X. a.* pv. *juglandis* colonies.

For isolation and purification of *X. a.* pv. *juglandis*, GYCA was regularly used. Agar plates used for isolation and purification were kept at 27 °C for 48–72 hours to obtain putative *X. a.* pv. *juglandis* colonies; these colonies were then picked and used for the hypersensitivity reaction (HR) on bean pods to confirm their pathogenicity potential (Klement and Goodman, 1967). Finally, the putative *X. a.* pv. *juglandis* isolates were preserved at -80°C in Luria-Bertani broth supplemented with 20% sterilised glycerol (Bertani, 1951).

3.2.2. KOH Test

X. a. pv. *juglandis* strains were aseptically removed from the GYCA medium with a sterile toothpick, placed on a glass slide and stirred for 10 seconds in a quick circular motion in a drop of 3% potassium hydoxide (KOH) (Suslow et al. 1982). Gram-negative strains were viscous and formed a mucoid string in 15 seconds.

3.2.3. Patogenicity test of *X. a.* pv. *juglandis*

The pathogenicity of putative *X. a.* pv. *juglandis* isolates was tested on walnut fruitlets according to Aletà et al. (2001). Inoculation was done on a set of 3 immature walnut fruits per isolate to be tested and observing the development of necrotic lesions over a 15-day period was used to estimate virulence.

3.2.4. Molecular Characterization of *X. a.* pv. *juglandis* isolates

3.2.4.1. Bacterial DNA Extraction

To extract genomic DNA from the *X. a.* pv. *juglandis* isolates, all the representative strains were grown on NSA medium for 24-48 h and harvested by washing the medium surface with sterile distilled water. The concentration of *X. a.* pv. *juglandis* cells were adjusted to 1×10^7 CFU ml^{-1} with sterilised water by using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) (optical density at 600 nm = 0.3; ~1×10^8 CFU ml^{-1}). Later, bacterial suspensions were boiled for 10 min on a termal block at 95 °C, placed on ice for 5 minutes and centrifuged for 5 minutes at 10,000 x g. Then, the NanoDrop ND-1000UV-Vis spectrophotometer was used to control the quantification and verification of extracted DNA purity. Eventually, the bacterial DNA suspensions were standardized to a concentration of 50 ng/ µl.

3.2.4.2. Molecular fingerprints of *X. a.* pv. *juglandis* isolates by combined rep-PCR analysis

Molecular fingerprints of the isolates werestudied by rep-PCR according to Versalovic et al. 1991. PCR amplification of the target sequence was performed in 25 µl of the following reaction mixture: 50 pmol each of the primers, 100 ng of template DNA, 1.25 mM of each of 4 dNTPs, 1 U Taq DNA polymerase in 1X PCR buffer (Promega Corp.). PCR amplifications were performed in an automated thermal cycler with an initial denaturation was done at 95°C for 7 min followed by 30 cycles of denaturation at 90°C for 30 sec, annealing [REP, 40°C, 1 min; ERIC, 52°C, 1 min], and extension at 65°C for 8 min with a single final extension at 65°C for 15 min (Table 3.2). The PCR products were then run through a 1% agarose gel, stained with ethidium bromide (0.5 µg ml^{-1}), and analyzed using the BioDoc Analyze (Biometra, Göttingen, Germany). The images were captured, and the BOX profiles were normalized and analyzed using the GelCompar 4.1 software (Applied Maths, Kortrijk, Belgium). Using UPGMA clustering analysis, similarity matrices were created and calculated to generate dendrograms (Sokal and Michener, 1958; Sneath and Sokal, 1973). Pearson and Dice similarity coefficients were used to investigate genetic relationships among the *X. a.* pv. *juglandis* collection.

3.2.4.3. Multi-Locus Sequence Analysis of *X. a.* pv. *juglandis* strains

MLSA was assessed with the four housekeeping genes *dna*K, *fyu*A, *gyr*B, and *rpo*D to investigate intraspecific variability within *X. a.* pv. *juglandis* strains (Table 3.5). For comparison, three other *X. arboricola* pathovars were studied as outgroup: *X. a.* pv. *corylina*, *X. a.* pv. *pruni*, *X. a.* pv. *populi*.

The primer pairs mentioned above were used to amplify gene fragments. Amplifications were performed in a final volume of 25 µl, which contained 1x GoTaq Buffer (Promega), 0.5 M of each primer, 0.50 U GoTaq DNA polymerase (Promega), 200 M of each dNTP (Promega), 1.5 mM MgCl2, and 50 ng template DNA. PCR steps were: denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, extension at 59 °C (*dna*K), 61 °C (*fyu*A), 54 °C (*gyr*B), 54 °C (*rpo*D) for 30 sec and elongation at 72 °C for 30 s, with a final elongation step of 7 min at 72 °C. Electrophoresis was used to separate DNA amplicons in 1% agarose gels. The PCR products were then purified using the mi-PCR Purification Kit (Metabion International AG, Munich, Germany) and sequenced along both strands by BioFab-Research (Rome, Italy) with the same primers used for amplification. The SeqManII software (DNAStar, Madison, USA) program was used to correct and align the sequences. The MEGA 5.0.3 software program (Tamura et al. 2007) was used to build neighbor-joining trees on single and concatenated sequences of the four housekeeping genes, with 1 000 bootstrap replicates to assess node confidence. Furthermore, different trees on single housekeeping genes were constructed in order to highlight the most discriminating gene.

Table 3.5. primer list for MLSA of *X. a.* pv. *juglandis*

Primer	Sequence	Tm	Reference
*dna*K-F	GGTGGAAGACCTGGTCAAGA	59 °C	Curland et al. 2018
*dna*K-R	TCCTTGACYTCGGTGAACTC	59 °C	
*fyu*A-F	AGCTACGAYGTGCGYTACGA	61 °C	Curland et al. 2018
*fyu*A-R	GTTCACGCCRAACTGGTAG	61 °C	
*gyr*B-F	ACGAGTACAACCCGGACAA	54 °C	Parkinson et al. (2007)
*gyr*B-R	CCCATCARGGTGCTGAAGAT	54 °C	
*rpo*D-F	TGGAACAGGGCTATCTGACC	54 °C	Young et al. (2008)
*rpo*D-R	CATTCYAGGTTGGTCTGRTT	54 °C	

4.2.5 Detection of *in vitro* Copper Resistance and CopA/B/L Genes in Copper Resistant *X. a.* pv. *juglandis*

Copper resistance of *X. a.* pv. *juglandis* starins were evaluated according to Basim et al. (2005) and Bender et al. (1990) (see section *Pseudomonas*).

Following DNA extraction and purification, PCR was used to detect specific sequences belonging to the *cop* gene clusters (Cooksey, 1990; Lee et al. 1994) (Table 3.6). A DNA thermal cycler was used to amplify target genes from the strains tested. Each PCR reaction mixture contained 25 µl of total volume, 1X PCR buffer, 25 mM MgCl2, 0.8 mM of each dNTPs (dATP, dTTP, dGTP, and dCTP), 0.5 µl of each primer and 50 µg template DNA. PCR cycles were set up as follows: initial incubation at 95°C for 5 minutes, followed by 30 PCR cycles with the following conditions: denaturation at 95°C for 30 seconds, primer annealing at 60°C for all three cop genes for 30 seconds, and DNA extension at 72°C for 45 seconds. Following the final cycle, the PCR tubes were incubated for 10 minutes at 72°C and then at 4°C. PCR amplicons

were separated using %2 agarose gel. For sizing the PCR product, a 50-bp DNA ladder (Promega Corp.) was used as the standard molecular size marker. After staining and washing the gel with ethidium bromide (0.5 g ml^{-1}) for 20 minutes, the gel images were photographed using a UV transilluminator (Bio-Rad Laboratories).

Table 3.6. Primer list to detect CopABL genes in copper resistant *X. a.* pv. *juglandis*

Primers	**Sequence**	**Tm**	**Reference**
*cop*A-F	CCTCCATGGCACGGACACTTCCATC	60°C	Lee et al. (1994)
*cop*A-R	CCAGACATATCCATCGACCCATGATCCA	60°C	Lee et al. (1994)
*cop*B-F	CTCAGGATCACTCTGCACATCAG	60°C	Lee et al. (1994)
*cop*B-R	GCACGTAGCTCTTAAATCGAGTTGTC	60°C	Lee et al. (1994)
*cop*L-F	CCGTGTCAAGCCTCCTCACTTCTAC	60°C	Lee et al. (1994)
*cop*L-R	CAGCGGCATGACATCCAGGCC	60°C	Lee et al. (1994)

3.3. BACTERIOPHAGES

3.3.1. Bacterial Strains and Media

Bacterial strains used in this study are listed in Table 3.7 and 3.8. These strains were used to test phage host range within *P. s.* pv. *syrinagae* and *X. a.* pv. *juglandis* separately. A further group of strains of *P. s.* pv. *actinidiae, P. s.* pv. *tomato*, *P. s.* pv. *lachrymans* and *X. a.* pv. *pruni*, *B. nigrifluens* and *B. rubrifaciens* were used for testing phage specificity for the strains of *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* population present in Italy. At -80°C, all the bacterial strains were maintained in nutrient broth supplemented with 25% sterile glycerol. The strains were subcultured on nutrient agar (NA) incubated at 27°C during the experiments.

Semisolid nutrient agar yeast extract medium, NYA (0.8 percent Nutrient Broth, 0.2 percent Yeast Extract, 0.6 percent Bacto Agar) was used for bacteriophage isolation and propagation.

Table 3.7. *P. s.* pv. *syringae* strains used in this study to work with phages

Sample Number	Isolation Year	Origin of the Material	Identity
78	2007	Fruits	*P. s.* pv. *syringae*
82	2007	Fruits	*P. s.* pv. *syringae*
361	2008	Fruits	*P. s.* pv. *syringae*
1278	2011	Fruits	*P. s.* pv. *syringae*
1291	2013	Fruits	*P. s.* pv. *syringae*
1293	2014	Leaves	*P. s.* pv. *syringae*
1300	2014	Fruits	*P. s.* pv. *syringae*
1143	2015	Buds	*P. s.* pv. *syringae*
1146	2015	Buds	*P. s.* pv. *syringae*
1148	2015	Buds	*P. s.* pv. *syringae*
1178	2015	Canker	*P. s.* pv. *syringae*
1191	2015	Fruits	*P. s.* pv. *syringae*
1203	2015	Fruits	*P. s.* pv. *syringae*
1333	2016	Fruits	*P. s.* pv. *syringae*
1334	2016	Fruits	*P. s.* pv. *syringae*
1335	2016	Leaves	*P. s.* pv. *syringae*
1337	2016	Fruits	*P. s.* pv. *syringae*
1338	2016	Fruits	*P. s.* pv. *syringae*
1420	2017	Fruits	*P. s.* pv. *syringae*
1421	2017	Branch	*P. s.* pv. *syringae*
1509	2018	Fruits	*P. s.* pv. *syringae*
1514	2018	Canker	*P. s.* pv. *syringae*
1601	2019	Fruits	*P. s.* pv. *syringae*
1619	2019	Fruits	*P. s.* pv. *syringae*
1687	2019	Fruits	*P. s.* pv. *syringae*
IPV-BO *1544*			*P. s.* pv. *tomato*
NCPPB 3739			*P. s.* pv. *actinidae*

Table 3.8. *X. a.* pv. *juglandis* strains used in this study to work with phages

Sample Number	Isolation Year	Origin of the Material	Identity
69	2006	Fruit	*X.a.juglandis*
71	2006	Leave	*X.a.juglandis*
183	2006	Leave	*X.a.juglandis*
320	2007	Fruit	*X.a.juglandis*
355	2007	Canker	*X.a.juglandis*
398	2008	Canker	*X.a.juglandis*
433	2008	Fruit	*X.a.juglandis*
543	2010	Fruit	*X.a.juglandis*
544	2010	Leave	*X.a.juglandis*
605	2010	Leave	*X.a.juglandis*
606	2010	Leave	*X.a.juglandis*
971	2013	Leave	*X.a.juglandis*
972	2013	Fruit	*X.a.juglandis*
1328	2016	Fruit	*X.a.juglandis*
1329	2016	Leave	*X.a.juglandis*
1368	2016	Fruit	*X.a.juglandis*
1371	2016	Fruit	*X.a.juglandis*
1516	2018	Fruit	*X.a.juglandis*
1521	2018	Fruit	*X.a.juglandis*
1529	2018	Fruit	*X.a.juglandis*
1608	2019	Leave	*X.a.juglandis*
1610	2019	Leave	*X.a.juglandis*
1615	2019	Fruit	*X.a.juglandis*
1676	2019	Leave	*X.a.juglandis*
1680	2019	Leave	*X.a.juglandis*
		NCPPB 683	*E.amylovora*
		NCPPB881	*X.gardneri*

3.3.2. Phage Isolation and Purification

Bacteriophage isolation was attempted from the mentioned substrates: above-ground parts (leaves and fruits) of apricot and walnut trees, rhizosphere soil, and irrigation water in apricot and walnut orchards collected from 15 localities in Italy between 2019 and 2020. Substrate samples were incubated with target bacteria (isolates 1508A, 1513A, 1596 for *P. s.* pv. *syringae* and isolates 263, 317, 398 for *X. a.* pv. *juglandis*) in 50 ml NB enriched with 2.5 g $CaCO_3$ to increase the potential for *X. a.* pv. *juglandis*-specific and *P. s.* pv. *syringae*-specific phage isolation. In order to increase phage populations, plant tissue (5 g), irrigation water (50 ml) and soil (10 g) were added to bacterial liquid cultures and incubated on a rotary shaker for 24 hours at 26°C. To remove cells and debris, aliquots (1 ml) of the enrichment culture were centrifuged at 16,000 g for 5 minutes. The supernatant was treated with 10% v/v chloroform for 30 minutes, and the suspensions were stored in microfuge tubes at 4°C (Gasic et al 2012).

All suspensions were examined for the presence of virulent phages by examining lysis of the target bacterium that had initially been used for enrichment. A 100 µl bacterial suspension (10^8 CFU ml^{-1}) from a 24-h-old bacterial culture was prepared in sterile distilled water and pipetted into the center of a sterile Petri dish (90 mm in diameter). Simultaneously, autoclaved NYA medium was poured into plates and mixed with bacterial suspension using a swirling motion (pour-plate procedure). After the medium solidified, plates were spot-inoculated by pipetting 10 µl of the testing phage suspensions onto the medium surface, and positive reactions were scored as either clear or turbid plaques or zones of confluent lysis within the inoculated area after 24-48 h incubation.

Three single plaque isolation steps were used to purify the phage. Phages were transferred from the plaques by stabbing the plaque with a sterile tooth pick and dipping the toothpick into 100 µl of sterile tap water in 1,5 ml eppendorf tubes. This suspension was prepared in two 10-fold dilutions, and 100 µl of each was pipette-mixed with 100 µl of the host bacteria at the bottom of the Petri dish, followed by pour-plate procedure and incubated for 24 hours at 26°C. Phages were extracted after a third purification step by adding 5 ml of sterile distilled water to the plate, washing the plate, and centrifuging the obtained suspension at 8,000 g for 20 minutes. The supernatant was transferred to a microfuge tube and treated with chloroform (10% v/v) for 20 minutes before being stored at 4°C for further testing. The phage titer was determined by plating 100 µl of 10-fold dilutions of purified phage suspensions and 100 µl of the bacterial suspension in NYA medium as previously described. Following a 24 h

incubation period, phage concentration was calculated using the bacterial enumeration formula (Klement et al. 1990) and described as “plaque forming units per ml” (PFU ml^{-1}).

3.3.3. Phage Preservation

100 µl of high-titer phage suspension and 100 µl of bacterial suspension were pipetted and gently mixed and left for 5 minutes to allow phage adsorption before being transferred to NB medium containing 25% glycerol and stored at -80°C for long-term storage.

3.3.4. Optimal Multiplicity of Infection

P. s. pv. *syringae* and *X. a.* pv. *juglandis* (*P. s.* pv. *syringae* 1508A and 398 for *X. a.* pv. *juglandis*) were grown in NB at 26°C until they reached approximately 10^8 CFU ml^{-1} (OD_{600} = 0.3) in order to determine optimal multiplicity of infection (MOI). Bacterial concentrations were also determined by counting the number of CFUs obtained from dilution plating on NA medium (Klement et al. 1990). The bacteria were infected with phage at three different ratios (approx. 0.1, 1, and 10 PFU/CFU). Following 18 h of incubation on a rotary shaker at 120 rpm at 26°C, cultures were treated with chloroform (10% v/v) and caculated to determine phage titer. The tests were done in triplicate. The MOI that resulted in the highest phage titer after 18 hours of incubation was considered optimal and was used in phage propagation.

3.3.5. Host Range Analysis

The host range of the phage isolates was investigated by studying their lytic activity. Infection of 25 strains of *P. s.* pv. *syringae* and 25 strains of *X. a.* pv. *juglandis* isolated from apricot and walnut orchards respectively in Italy. Bacterial lawn was made by suspending 24 h old bacteria in sterile distilled water (10^8 CFU ml^{-1}) and pipetting 100 µl of this suspension into the bottom of an empty Petri dish using the pour-plate method. When the medium had solidified, 4 µl of each phage suspension was spotted onto the surface of the medium in a specific order. Plates were incubated at 26°C for 24 h after being kept in a laminar flow hood for 20 minutes to allow phage suspension to diffuse into the medium. Plaque formation, which indicates host cell lysis, was used to assess phage activity. The plaques were classified as either clear or turbid. The assay was done as three replicate.

3.3.6. One-Step Growth

A one-step growth procedure was used to study the phage life cycle, and the number of particles freed from one cell was represented as "burst size." The protocols of Ellis and Delbrück (1939) and Carlson (2005) were used with small modifications. At a MOI of 0.1, one milliliter of each bacteria (1508A *P. s.* pv. *syringae* and 398 for *X. a.* pv. *juglandis*) in NB at 10^8 CFU ml^{-1} and phage suspension were mixed. The mixture was incubated at 26°C for 5 min to allow phage adsorption. Following that, the mixture was diluted to 10^{-4}, yielding a total of 20 ml of NB in 50 ml flasks. The dilution of the mixture reduces the possibility of unadsorbed or released phages infecting new bacterial cells, and thus an increase in phage concentration represents the number of newly multiplied phage particles. After incubating the diluted mixture in a water bath at 27°C, 100 µl sample was taken at 10 minute intervals. Phage titers were observed on NYA, as previously described. The experiment was carried out three times. The latent period was described as the time between adsorption (excluding the 5 minute pre- incubation period) and the start of the first burst, as stated by the initial rise in phage titer (Adams, 1959; Ellis and Delbrück, 1939). During the latent period, burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells (Adams, 1959).

3.3.7. DNA Extraction

Following the manufacturer's instructions, phage DNA was extracted from a high-titre plate lysate (minimum of 10^8 PFU ml^{-1}) using a Phage DNA Isolation Kit (Norgen Biotek, Thorold, Canada). Extracted DNA was stored at -20°C in a 1.5 ml eppendorf tube until needed. DNA quality and concentration were determined using a NanoDrop ND-1000UV-Vis spectrophotometer.

3.3.8. Restriction Analysis of Phage DNA

According to the manufacturer's instructions, bacteriophage DNA was digested with *Bam*HI, *Hind*III and *Eco*RI restriction enzymes (Promega Corporation). Three microliters of phage DNA suspension were mixed with five µl of Milli-Q water, one µl enzyme buffer, and one µl restriction enzyme (10 units/l) (*Eco*RI, *Hind*III or *Bam*HI). The mixture was incubated at 37°C for 45 min. DNA fragments were separated using 1% agarose gel electrophoresis in

Tris-acetate-EDTA buffer, stained with ethidium bromide (0.5 g ml^{-1}) and photographed using a digital imaging camera using BioDoc Analyze (Biometra).

3.3.9. Thermal Inactivation

The heat stability of phage isolates was investigated by exposing 1 ml of phage suspension (10^7 PFU ml^{-1}) to temperatures ranging from 35 to 75°C in a water bath at 5°C intervals. To determine the thermal inactivation point more precisely, the experiment was repeated three times at 1°C intervals for temperatures ranging from 65 to 75°C. Following a 10-minute incubation, the phage suspensions were rapidly chilled in ice before being spot testing. After 24 hours of incubation at 26°C, plaque formation was observed as an indication of phage activity.

3.3.10. Effect of pH on Phage Viability

To investigate the effect of different pH values on phage viability, phages were suspended in 1 ml SM buffer that had previously been adjusted to pH values of 2, 5, 7, 9, and 11. The concentration of phage suspensions in SM buffer was adjusted to a final concentration of 10^4 PFU/ml. After 24 h incubation at room temperature, 10-fold dilutions of each sample were prepared and tested for phage activity. The assays were performed in three replicate, and the results were reported as the mean number of plaques recorded (PFU/ml).

3.3.11. Effect of UV Light on Phage Survival *in vitro*

Phage suspensions were adjusted to a final concentration of 10^4 PFU/ml in SM buffer and then exposed to UV light 254/366 nm/dark (16 h/8 h) conditions. The phage's titer was determined on the first day and then at various time intervals over the next two months. Three wells were tested per treatment, each with 300 µL of tap water. After 1 minute, rinsates from each well were transferred to microcentrifuge tubes and the titer determined as described previously. The assays were repeated three times and the results expressed as the mean number of plaques counted (PFU/ml).

4. RESULTS

4.1. Biochemical and Molecular Characterization of *P. syringae* spp. from Apricot

4.1.1. Bacterial Stone Fruit Canker Bacterial Collection

Symptoms resembling those caused by pathogenic *P. s.* pv. *syringae* were observed during a 2018–2019 survey of apricot commercial orchards in Northern Italy. Plant materials were collected from symptomatic plant parts to characterize *P. s.* pv. *syringae* strains (Fig. 4.1). On apricot trees, severe fruit and leaf spotting (Fig 4.1) was observed, affecting more than 70% of the canopy. The leaf spots were numerous, round, necrotic, light to dark brown in color, and varied in size, usually surrounded by a halo. Bacterial canker on branches (Fig. 4.1) and gummosis were also symptoms of bacterial stone fruit canker on apricot (Fig. 4.1). Symptomatic samples were collected on a regular basis from commercial apricot orchards in Imola, Forli, Faenza, Ravenna, and Bologna districts. A total of 54 samples were collected during this study (Table 4.1). On NSA medium, all isolates showed the typical *P. syringae* characteristics and morphology: colonies were round, pearl grey in color, mucoid and elevated (levan-type), and they had entire margins. The KOH test revealed that all of the strains were Gram negative. The HR was clearly positive in the majority of cases (Fig 4.2), but not for all isolates tested.

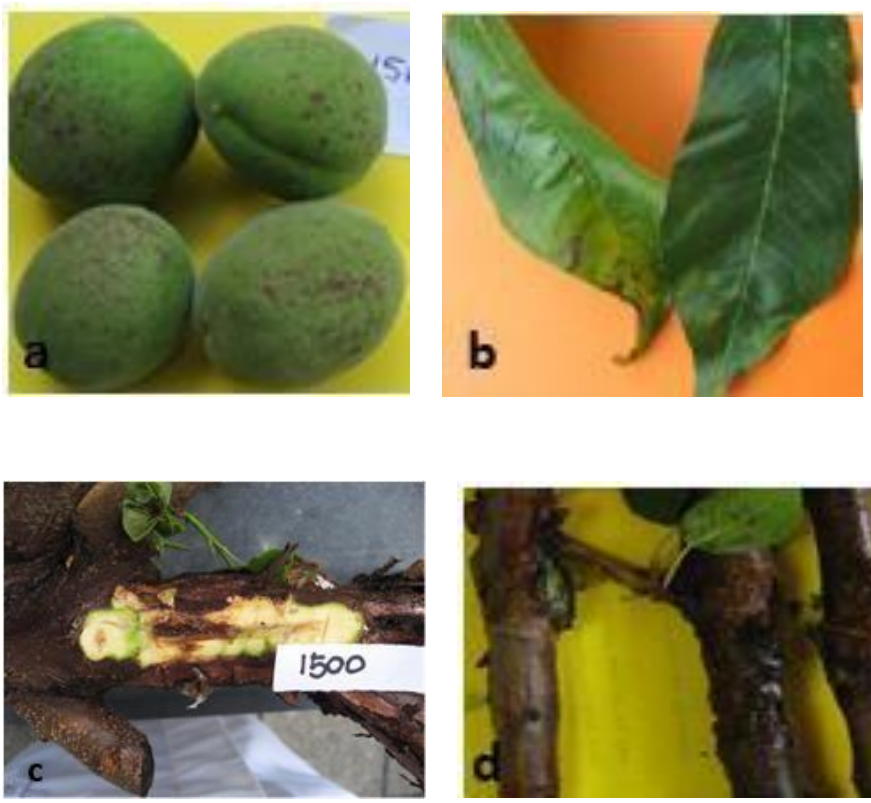

Fig 4.1. Symptomology of bacterial stone fruit canker on apricot: necrotic spots on fruits (a), necrotic spots on leaves (b), bacterial canker on branches (c), gummosis on branches (d)

Fig 4.2. Hypersensitivity reaction of 10^6 cfu/ml P. s. pv. syringae suspension on tobacco leaf. The development of the necrotic area in the infiltrated tissue within 48-72 hours was considered positive

Table 4.1. List of the *P. syringae* strains indicating origin of the strains such as syptomology, locality and variety

Strain nr.	Isolation Date	Symptoms	Source of Strain	
			Variety	Location
1505A	04.05.18	Necrotic spots on fruits	Pink Cot	Sant Agata sul Santerno
1505B	02.05.18	Necrotic spots on fruits	Pink Cot	Sant Agata sul Santerno
1505C	02.05.18	Necrotic spots on fruits	Pink Cot	Sant Agata sul Santerno
1505D	02.05.18	Necrotic spots on fruits	Pink Cot	Sant Agata sul Santerno
1506A	02.05.18	Spots on fruits	Lady Cot	Colle di Faenza
1506B	02.05.18	Spots on fruits	Lady Cot	Colle di Faenza
1507A	02.05.18	Spots on fruits	Lady Cot	Faenza
1507H	02.05.18	Spots on fruits	Lady Cot	Faenza
1508A	02.05.18	Necrotic spots on fruits	Aurora	Lugo
1508B	02.05.18	Necrotic spots on fruits	Aurora	Lugo
1509A	02.05.18	Necrotic spots on fruits	Lady Cot	Bagnoccavallo
1509B	02.05.18	Necrotic spots on fruits	Lady Cot	Bagnoccavallo
1511A	09.05.18	Canker on branches	Farlis	Castel Bolognese
1511B	09.05.18	Canker on branches	Farlis	Castel Bolognese
1511C	09.05.18	Canker on branches	Farlis	Castel Bolognese
1511D	09.05.18	Canker on branches	Farlis	Castel Bolognese
1512A	11.05.18	Necrotic spots on fruits	Lady Cot	Faenza
1512B	11.05.18	Necrotic spots on fruits	Lady Cot	Faenza
1513A	11.05.18	Necrosis on branches	Lady Coot	Faenza
1513C	11.05.18	Necrosis on branches	Lady Cot	Faenza
1514A	11.05.18	Necrotic spots on fruits	Faralia	Faenza
1514B	11.05.18	Necrotic spots on fruits	Faralia	Faenza
1514C	11.05.18	Necrotic spots on fruits	Faralia	Faenza

1525A	25.05.18	Necrotic spots on fruits	Farbele	Ravenna
1525B	25.05.18	Necrotic spots on fruits	Farbele	Ravenna
1525C	25.05.18	Necrotic spots on fruits	Farbele	Ravenna
1525D	25.05.18	Necrotic spots on fruits	Farbele	Ravenna
1526A	25.05.18	Necrotic spots on fruits	Lady Cot	Ravenna
1526B	25.05.18	Necrotic spots on fruits	Lady Cot	Ravenna
1526C	25.05.18	Necrotic spots on fruits	Lady Cot	Ravenna
1527	25.05.18	Necrotic spots on fruits	Farlis	Ravenna
1532A	06.06.18	Necrotic spots on fruits	Lady Cot	Imola
1532B	06.06.18	Necrotic spots on fruits	Lady Cot	Imola
1532C	06.06.18	Necrotic spots on fruits	Lady Cot	Imola
1532D	06.06.18	Necrotic spots on fruits	Lady Cot	Imola
1535A	06.06.18	Necrotic spots on fruits	Lady Cot	Castel Bolognese
1535C	06.06.18	Necrotic spots on fruits	Lady Cot	Castel Bolognese
1535D	06.06.18	Necrotic spots on fruits	Lady Cot	Castel Bolognese
1544E	15.06.18	Necrotic spots on fruits	Lady Cot	Bagnacavallo
1544F	15.06.18	Necrotic spots on fruits	Lady Cot	Bagnacavallo
1544G	15.06.18	Necrotic spots on fruits	Lady Cot	Bagnacavallo
1593	29.03.19	Canker and gummosis on branches	Harval	Faenza
1594	29.03.19	Canker and gummosis on branches	Petra	Faenza
1596	08.04.19	Canker and gummosis on branches	Snee Red	Faenza
1597	08.04.19	Canker on branches	Sunny Cot	Faenza
1600	11.04.19	Necrotic spots on fruits	-	Faenza
1601	11.04.19	Necrotic spots on fruits	-	Faenza

1602	11.04.19	Necrotic spots on fruits	-	Faenza
1605	3.05.19	Necrotic spots on leaves	Bora	Ravenna
1606	3.05.19	Necrotic spots on fruits	-	Ravenna
1618	22.05.19	Necrotic spots on leaves	Delice Cot	Forli
1619	22.05.19	Necrotic spots on fruits	Pricia	Forli
1620	22.05.19	Necrotic spots on fruits	Wonder Cot	Forli
1621	22.05.19	Necrotic spots on leaves	Wonder Cot	Forli

4.1.2. Identification and Phenotypic Characterisation

As a phenotypic characterisation, bacterial strains were characterized using the LOPAT and GATTa tests. On King's B medium, 42 of the 54 isolates tested for fluorescence were positive for fluorescence due to pyoverdin production. All 54 strains tested negative for oxidase and positive for levan. The outcomes of levan production separated isolates into two groups. Forty-six isolates were clearly levan positive and belong to *Pseudomonas* Group Ia, according to the LOPAT scheme. The remaining eight isolates did not clearly produce levan, but the rest of the characteristics were consistent with *Pseudomonas* Group Ia. All strains tested negative for arginine dihydrolase and potato rot. Table 4.2 shows the LOPAT results of all *P. syringae* isolates.

GATTa tests (gelatin liquefaction, aesculin hydrolysis, tyrosinase activity, and tartrate use) are still recommended for distinguishing *P. s.* pv. *syringae* (G + A + T Ta), *P. s.* pv. *mosprunorum* race 1 (G A T + Ta+), and *P. s.* pv. *morsprunorum* race 2 (G + A T Ta)(Latorre and Jones, 1979; Kaluzna et al. 2014). The isolates of *P. syringae* were then further characterized using GATTa tests. The GATTa tests allowed the differentiation of *P. syringae* pathovars and races among the 54 isolates: 39 isolates were classified as *P. s.* pv. *syringae*, ten as *P. s.* pv. *mosprunorum* race 1, and five as *P. s.* pv. *mosprunorum* race 2. (Table 4.2).

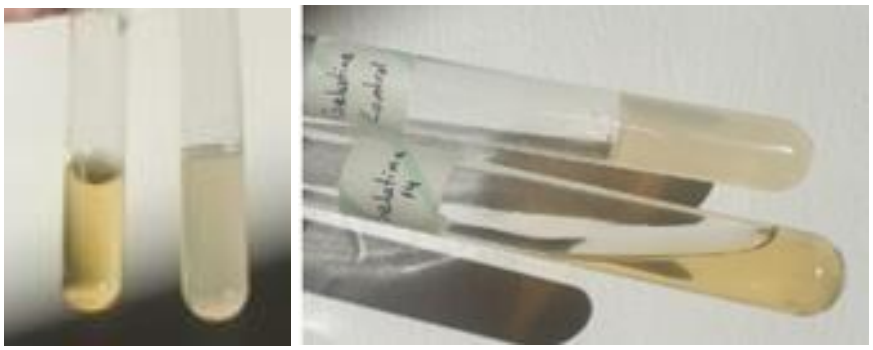

Fig. 4.3.a. Gelatine liquefaction. *P. s.* pv. *syringae* and *P. s.* pv. *morsprunorum* race 2 strains liquefied gelatin after 7- 14 days incubation.

Fig. 4.3.b. Aesculin hydrolysis. Brown colour of the medium after 24-48 h incubation at 26-28°C proved presence of the β-glucosidase enzyme in *P. s.* pv. *syringae* strains.

Fig. 4.3.c. Tyrosinase activity. A colour change to red of the medium after 7-10 days incubation at 26-28°C shows the presence of tyrosinase in *P. s.* pv. *morsprunorum* race 1 strains.

Fig. 4.3.d. Tartrate utilization. A colour change of the medium from green to blue is a positive test result as shown in *P. s.* pv. *morsprunorum* race 1 strains.

Table 4.2. LOPAT and GATTa test results of *P. syringae* pv. *syringae* and *P. s.* pv. *morsprunorum* and differentiation of *P. s.* pv. *morsprunorum* as race 1 and race 2

Strains	F*	L*	O*	P*	A*	T*	G*	A*	T*	Ta*	Identity*
1505A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1505B	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1505C	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1505D	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1506A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1506B	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1507A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1507H	+	+	-	-	-	+	+	-	-	-	*P. s.* pv. *mosprunorum* race 2
1508A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1508B	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1509A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1509B	+	+	-	-	-	+	+	-	-	-	*P. s.* pv. *mosprunorum* race 2
1511A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1511B	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1511C	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1511D	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*

1512A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1512B	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1513A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1513C	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1514A	+	+	-	-	-	+	+	-	-	-	*P. s.* pv. *mosprunorum* race 2
1514B	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1514C	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1525A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1525B	+	+	-	-	-	+	+	-	-	-	*P. s.* pv. *mosprunorum* race 2
1525C	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1525D	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1526A	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1526B	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1526C	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1527	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1532A	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1532B	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1532C	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1532D	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1535A	+	+	-	-	-	+	+	-	-	-	*P. s.* pv. *mosprunorum* race 2
1535C	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1535D	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1544E	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1544F	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *syringae*
1544G	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1593	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1594	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1

1596	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1597	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1600	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1601	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1602	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1605	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1606	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1618	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1619	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*
1620	-	+	-	-	-	+	-	-	+	+	*P. s.* pv. *mosprunorum* race 1
1621	+	+	-	-	-	+	+	+	-	-	*P. s.* pv. *syringae*

*F: Fluoresent strains, *L: Levan, *O: Oxidase reaction, *P: Pectolytic activity, *A: Arginine dihydrolase activity, *T: Hypersensitive reaction on tobacco plant, *G: Gelatine hydrolysis, *A: Aesculin hydrolysis, *T: Tyrosinase activity, *Ta: Tartrate (Ta) utilization, Pathogenicity Test

To demonstrate that *P. syringae* isolates were responsible for disease development on apricot, Koch's postulates were demonstrated by inoculating apricot leaves with *P. syringae* strains and maintaining the leaves in humid conditions. Leaf lesions developed similarly to necrotic spots observed in orchards, with dark necrotic spots surrounded by yellow chlorotic haloes having appeared on the leaves within 7-8 days of inoculation. After inoculation with sterile water, no symptoms developed. After observing the disease's characteristic symptoms on apricot leaves, tissue samples were collected and *P. syringae* was recovered from the leaves as described previously. During pathogenicity tests, characteristic symptoms of the disease were successfully reproduced in inoculated host plant tissues

4.1.3. Identification of *P. s.* pv. *syringae* by PCR

P. s. pv. *syringae* was identified using the PCR method, which enabled the detection of toxin-producing genes: *syr*B and *syr*D in *P. s.* pv. *syringae*, and the *cfl* gene in *P. s.* pv. *morsprunorum* race 1. Regardless of origin, each of the 18 *P. s.* pv. *syringae* isolates demonstrated the presence of a specific *syr*D amplicon by amplifying a DNA fragment (Fig

4.3). The 17 *P. s.* pv. *syringae* strains amplified a fragment using the *syr*B primers (Fig. 4.4). The coronatine coding gene (*cfl*) was identified in five *P. s.* pv. *morsprunorum* strains belonging to race 1 and amplified to 650 bp (Fig 4.5) (Table 4.3).

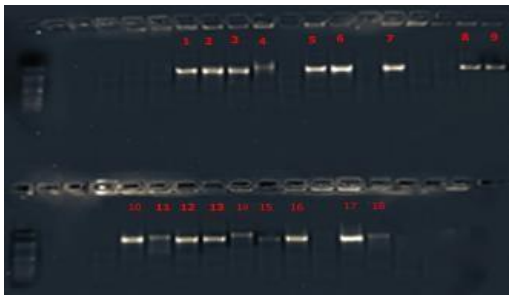

Fig. 4.3. PCR amplification of the 1040 bp fragment of *syr*D. Products were seperated on 1.5% agarose gel.

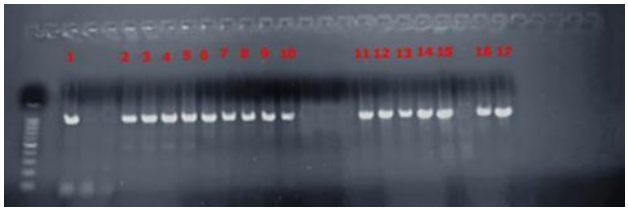

Fig. 4.4. A set of primers (B1/ B2) was used to detect the *syr*B gene in *P. s.* pv. *syringae* strains

Fig. 4.5. cflF/cflR primers were used to verify coronatine production in *P. s.* pv. *morsprunorum* strains.

4.1.4. The Presence of the INA and inaZ Genes

At -4 °C, INA was detected in twenty *P. s.* pv. *syringae* isolates. Among these isolates, ten were extremely active in demonstrating INA: they caused pure water to freeze at a temperature of only -3 °C. None of the *P. s.* pv. *morsprunorum* isolates exhibited INA. Among the 31 isolates tested for the presence of the *ina*Z gene involvedin INA, ten significantly INA-active *P. s.* pv. *syringae* isolates contained the 700 bp specific DNA fragment, confirming the trait's genetic basis (Table 4.3) (Fig 4.6).

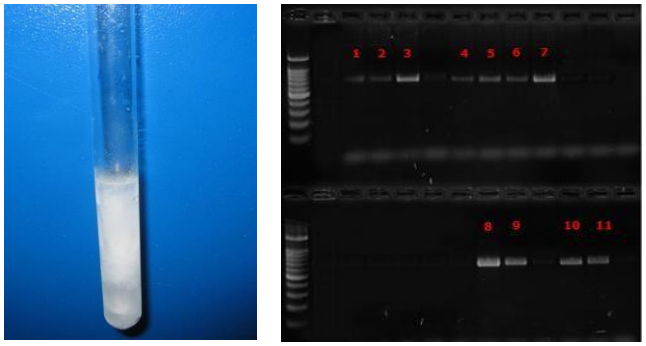

Fig. 4.6. Ice nucleation activity of *P. s.* pv. *syringae* strains demonstrated on the left as an in vitro experiment and agarose gel at 1.2 % illustrated the product of genomic DNA amplification through PCR with an inaZ primer set for *P. s.* pv. *syringae* strains.

Table 4.3. Summary of some molecular features of a collection of *P. syringae* isolates from apricot orchards

No	PCR cfl	PCR syrB	PCR syrD	INA	PCR inaZ	ID based on GATTa results
1505D	-	+	-		+	Pss
1506B	-	+	-		+	Pss
1507H	+	-	-		-	Psm race 1
1508A	-	+	-		-	Pss
1509A	-	+	+		+	Pss
1509B	-	-	-		-	Psm race 2
1511A	-	+	+		-	Pss
1511D	-	+	+		-	Pss
1512B	-	+	+		-	Pss
1513C	+	-	+		-	Psm race 1
1514C	-	-	+		+	Pss
1525A	-	+	+		+	Pss
1525B	-	-	-		-	Psm race 2
1525D	-	+	+		+	Pss
1526C	-	+	+		+	Pss
1527	-	+	+		-	Pss
1532A	+	-	-		-	Psm race 1
1535C	-	-	-		-	Psm race 2
1544E	-	+	+		-	Pss
1544F	+	-	-		-	Psm race 1
1594	+	-	-		-	Psm race 1
1596	-	+	+		+	Pss
1597	-	-	+		+	Pss
1601	-	+	+		+	Pss
1605	-	-	+		-	Pss
1618	-	+	-		-	Pss
1619	-	+	+		+	Pss
1621	-	+	+		-	Pss

4.1.5. Resistance to Copper

Copper-resistant isolates representative of all *P. syringae* species (Table 4.4). All isolates grew on MGYA medium supplemented with copper sulfate at a concentration of 50 ppm. Only 7.4 percent were resistant to copper sulfate at a concentration of 100 ppm, while 11.2 percent were resistant at a concentration of 1000 ppm (Table 4.4). On copper-containing media, some copper-resistant strains of *P. s.* pv. *syringae* accumulated copper and formedblue colonies. The *cus*C gene was detected only in *P. syringae* isolates (10 in total) that were highly resistant to copper while *cus*B was detected in fifteen P. syringae strains and *cus*A gene in nineteen strains.

Table 4.4. List of *P. syringae* strains containing *cus*CBA genes

No	*in vitro* copper resistance	PCR *cus*C	PCR *cus*B	PCR *cus*A	ID based on GATTa results
1505D	500	-	-	-	Pss
1506B	500	-	-	-	Pss
1507H	500	+	-	-	Psm race 1
1508A	400	+	-	+	Pss
1509A	500	-	-	+	Pss
1509B	100	-	-	+	Psm race 2
1511A	500	-	-	+	Pss
1511D	100	-	-	+	Pss
1512B	500	+	+	+	Pss
1513C	500	-	+	+	Psm race 1
1514C	500	-	+	+	Pss
1525A	500	+	+	+	Pss
1525B	100	-	-	-	Psm race 2
1525D	500	+	+	+	Pss
1526C	500	+	+	+	Pss
1527	500	+	+	+	Pss
1532A	500	-	-	+	Psm race 1
1535C	200	-	-	-	Psm race 2
1544E	200	-	+	-	Pss
1544F	500	+	-	+	Psm race 1
1594	300	-	-	+	Psm race 1
1596	300	+	+	+	Pss
1597	200	-	+	-	Pss
1601	200	-	+	-	Pss

1605	200	-	+	-	Pss
1618	300	+	+	+	Pss
1619	500	-	+	+	Pss
1621	500	-	+	+	Pss

4.1.6. Differentiation of *P. syringae* spp. Isolates by REP-PCR

With each fingerprinting technique, distinct banding patterns were generated using the corresponding oligonucleotide primers. The lengths of the amplified bands in BOX ranged from 200 to 3000 bp, while those in ERIC and REP ranged from 150 to 3200 bp and 250 to 300 bp, respectively. After repeating the procedures, reproducible fingerprint profiles were generated with each technique. For the combination of rep-PCR results, a combined dendrogram was calculated using Jaccard's similarity coefficients and unweighted pair group method clustering (UPGMA). Strains were differentiated into two main groups at the 47% similarity level (Fig 4.10). Isolates were classified into four clusters based on the combined dendogram at a similarity level of 64%. Cluster A contains three *P. s.* pv. *morsprunorum* race 1 isolates as well as *P. s.* pv. *morsprunorum* race 1 reference strain (LMG 2222) and seven *P. s.* pv. *syringae* isolates, cluster B contains two *P. s.* pv. *morsprunorum* race 2 isolates as well as *P. s.* pv. *morsprunorum* race 2 (CFBP 3800) and four *P. s.* pv. *syringae* isolates, cluster C contains six *P. s.* pv. *syringae* isolates as well as *P. s.* pv. *syringae* reference strain (CFBP 6400), and cluster E contains six *P. s.* pv. *syringae* isolates. The percentage of polymorphic loci was 57.18% when ERIC primers were used, whereas it was 62.7% and 75.16% when BOX and REP primers were used. These findings revealed a high level of genetic diversity among *P. syringae* pathovars isolated from stone fruits in Northern Italy.

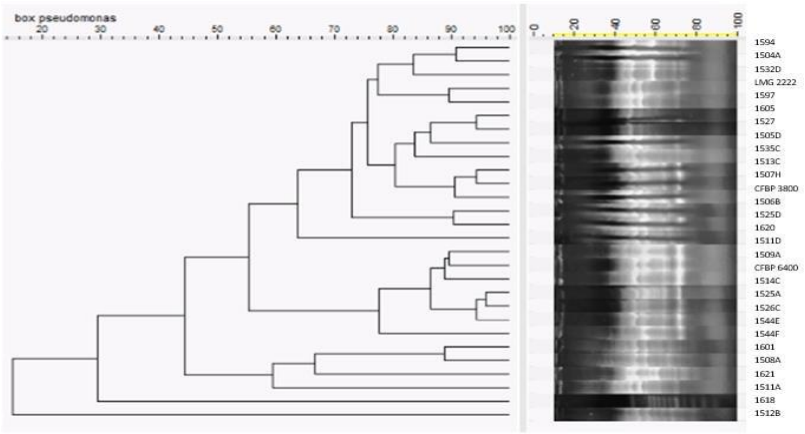

Fig. 4.7. Dendrogram generated after the cluster analysis of digitised combined rep-PCR fingerprints using BOX primers. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400), *P. s.* pv. *morsprunorum* race 1 (LMG 222) and *P. s.* pv. *morsprunorum* race 2 (CFBP 3800).

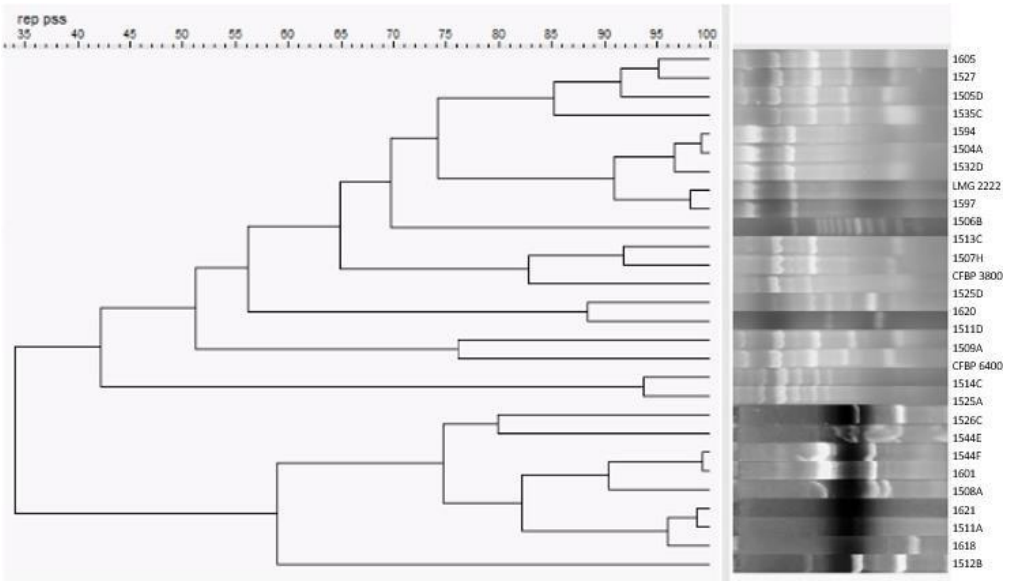

Fig 4.8. Dendrogram generated after the cluster analysis of digitised combined rep-PCR fingerprints using REP primers. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400), *P. s.* pv. *morsprunorum* race 1 (LMG 222) and *P. s.* pv. *morsprunorum* race 2 (CFBP 3800).

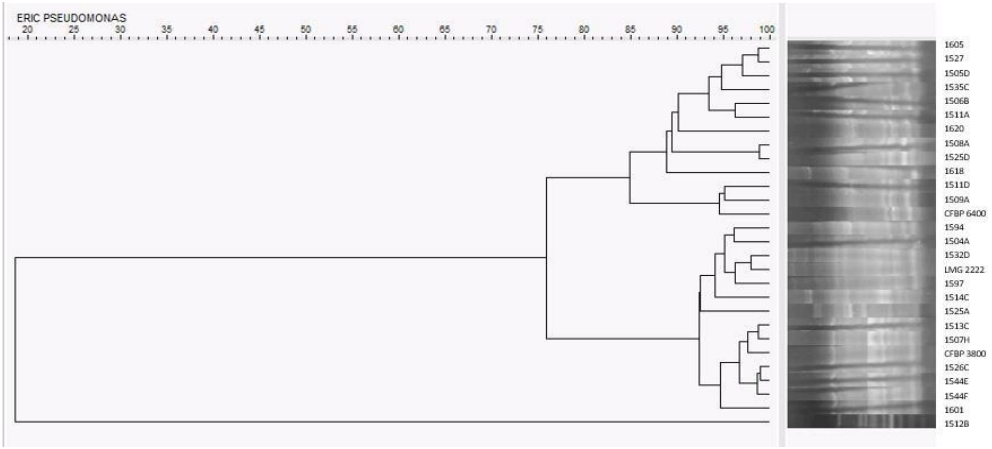

Fig. 4.9. Dendrogram generated after the cluster analysis of digitised combined rep-PCR fingerprints using ERIC primers. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400), *P. s.* pv. *morsprunorum* race 1 (LMG 222) and *P. s.* pv. *morsprunorum* race 2 (CFBP 3800).

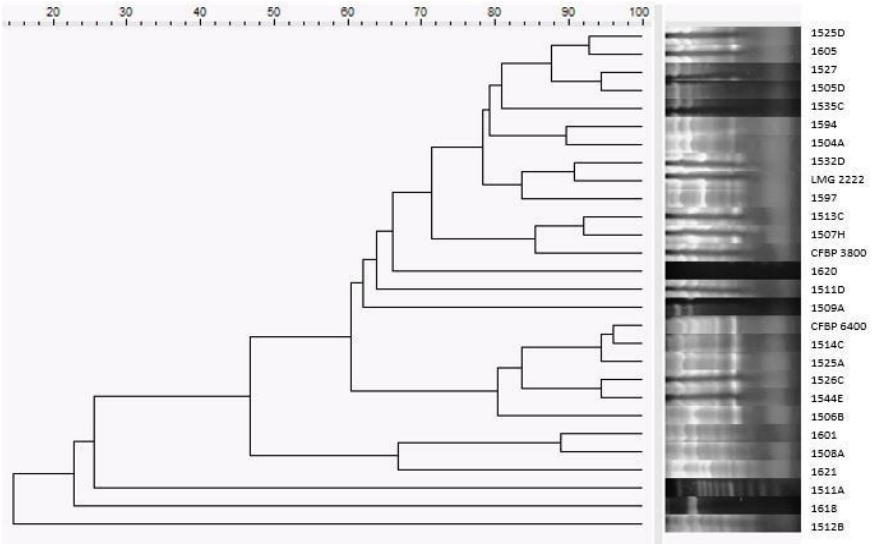

Fig. 4.10. Dendrogram generated after the cluster analysis of concatenated digitised combined rep-PCR fingerprints using BOX, REP and ERIC primer sets. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400), *P. s.* pv. *morsprunorum* race 1 (LMG 222) and *P. s.* pv. *morsprunorum* race 2 (CFBP 3800).

4.1.7. MLSA of Pseudomonas syringae from Apricot

MLST was used to construct a phylogenetic tree from concatenated sequence data for all isolates in this study. The MLST data were used to generate a NJ phylogenetic tree with increased discrimination and bootstrap support for the separation of *P. syringae*. The analysis reveals the presence of two distinct clades with high bootstrap support.

To determine the genetic variability and phylogeny of 12 tested isolates, we used MLSA based on four housekeeping genes (*acn*, cts, *pgi*, and *pfk*)(Fig 4.11, 4.12, 4.13, 4.14). The phylogenetic tree was generated based on concatenated gene sequences consisting of a total 2048 nucleotides in length. The MLSA analysis of 12 representative isolates of *P. syringae* from apricot trees and located in Emilia Romagna revealed genomic differences between the isolates that could not be attributed to geographic origin or plant organs. Nine strains (1532D, 1513C, 1594, 1504A, 1507H, 1527, 1605, 1535C, 1505D) were clustered together on the cumulative dendogram, while strains 1597, 1526B, and 1511A (*P. s.* pv. *morsprunorum* race1) formed another cluster. These two phylogroups were inextricably linked. Strains 1532D, 1513C, and 1594 were distinguished from the rest of the strains and assigned to a distinct subcluster. Second subcluster was formed by strains 1504A, 1507H (*P. s.* pv. *morsprunorum* race 2). Strains 1527, 1605, 1535C, and 1505D were subclustered separately due to their low similarity to the remaining strains in this study (Fig 4.15).

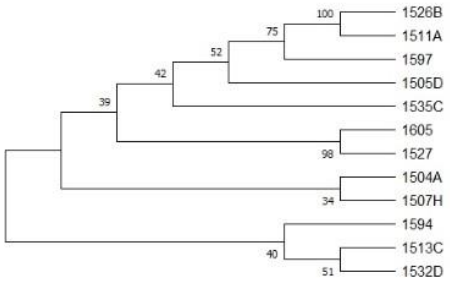

Fig. 4.11. Phylogenetic tree based on the sequence of the *acn* gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400, *P. s.* pv. *morsprunorum* race I (LMG 2222) and *P. s.* pv. *morsprunorum* race II (CFBP 3800)

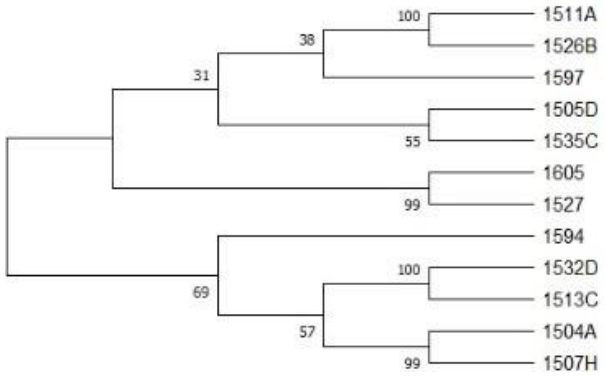

Fig. 4.12. Phylogenetic tree based on the sequence of the *cts* gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400, *P. s.* pv. *morsprunorum* race I (LMG 2222) and *P. s.* pv. *morsprunorum* race II (CFBP 3800)

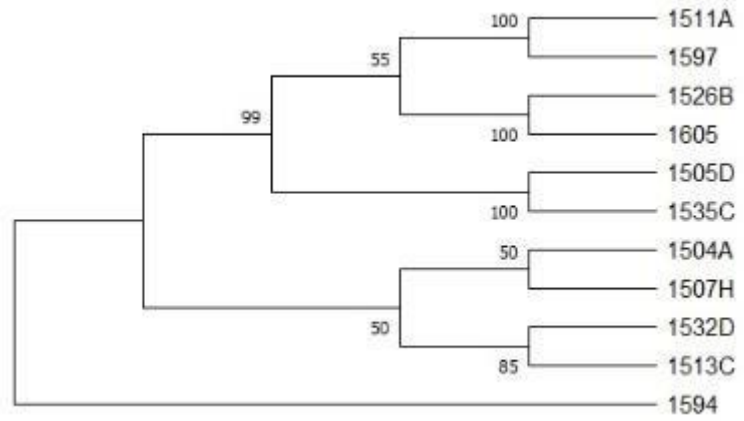

Fig. 4.13. Phylogenetic tree based on the sequence of the *pgi* gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The P. syringae isolates from Italy were compared with the pathotype strains of P. s. pv. syringae (CFBP 6400, P. s. pv. morsprunorum race I (LMG 2222) and P. s. pv. morsprunorum race II (CFBP 3800)

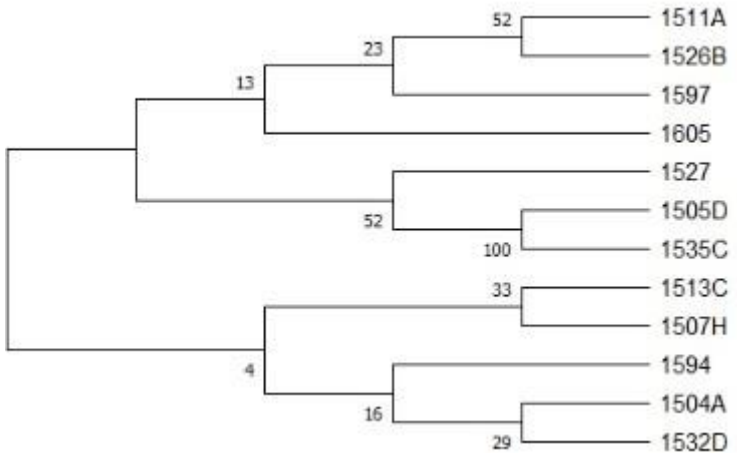

Fig. 4.14. Phylogenetic tree based on the sequence of the *pfk* gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400, *P. s.* pv. *morsprunorum* race I (LMG 2222) and *P. s.* pv. *morsprunorum* race II (CFBP 3800)

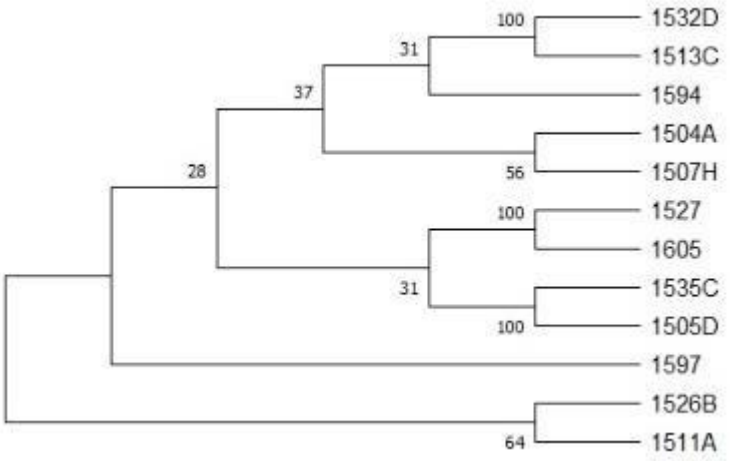

Fig. 4.15. Phylogenetic tree based on the concatenated sequence data of the *acn*, *cts*, *pgi* and *pfk* genes. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *P. syringae* isolates from Italy were compared with the pathotype strains of *P. s.* pv. *syringae* (CFBP 6400, *P. s.* pv. *morsprunorum* race I (LMG 2222) and *P. s.* pv. *morsprunorum* race II (CFBP 3800)

4.2. *X. a.* pv. *juglandis*: Phenotypic And Molecular Characterization

4.2.1. Isolation and Identification of Pathogenic Bacteria from Diseased Walnut Tree Parts

Between May and September, colonies resembling xanthomonads were isolated from diseased walnut trees' leaves, stems, flower buds, and fruits. During the seasons 2018 and 2019, a total of 60 putative Xanthomonad isolates were collected from various wanut cultivars and locations throughout Northern Italy. *X. a.* pv. *juglandis* was identified in 47 isolates (Table 4.5). Following 24-48 hours of incubation at 26°C, all isolates formed yellow mucoid and convex colonies on GYCA medium. When morpho-physiological characteristics were compared to those observed on GYCA, the *X. a.* pv. *juglandis* strains demonstrated distinct visual differences in colony appearance, most notably in terms of color (bright or dark yellow), margin (mucoid or buttery), and surface (smooth and glistening or rough and wrinkled). All strains were Gram-negative, oxidase-negative, and HR-positive on tobacco leaves or bean pods (Table 4.5).

Table 4.5. Summary of origin features of a collection of *X. a.* pv. *juglandis* isolates from walnut orchards

Strains	Isolation Date (m/y)	Symptoms	Location	HR on Tobacco Leaves	HR on Bean Pots	Pathogenicity	Virulence Class
1515A	05/18	Necrotic spots on leaves	Civitella di Romagna	+	+	+	2
1515C	05/18	Necrotic spots on leaves	Civitella di Romagna	+	+	+	1
1515D	05/18	Necrotic spots on leaves	Civitella di Romagna	+	+	+	2
1515E	05/18	Necrotic spots on leaves	Civitella di Romagna	+	+	+	1
1516B	05/18	Necrotic spots on fruitlets	Cotignola	-	+	+	1

1516C	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	3
1517A	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1517B	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	1
1518A	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1518B	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	1
1518C	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	1
1519A	05/18	Necrotic spots on fruitlets	Cotignola	-	+	+	3
1519B	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1519C	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1520A	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	0
1520B	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	3
1520C	05/18	Necrotic spots on fruitlets	Cotignola	-	+	+	2
1521C	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1522A	05/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2

1522B	05/18	Necrotic spots on fruitlets	Cotignola	-	+	+	1
1522C	05/18	Necrotic spots on fruitlets	Cotignola	-	+	+	0
1541A	06/18	Necrotic spots on fruitlets	Cotignola	+	+	+	3
1541B	06/18	Necrotic spots on fruitlets	Cotignola	+	+	+	0
1541C	06/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1541D	06/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1542A	06/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1542B	06/18	Necrotic spots on fruitlets	Cotignola	+	+	+	2
1542C	06/18	Necrotic spots on fruitlets	Cotignola	-	+	+	3
1549B	06/18	Necrotic spots on fruitlets		+	+	+	2
1549C	06/18	Necrotic spots on fruitlets		+	+	+	0
1549D	06/18	Necrotic spots on fruitlets		+	+	+	3
1560	09/18	Very large necrosis on fruits	San Tomè	+	+	+	2
1561	09/18	Necrotic spots on fruitlets	Forli	+	+	+	3

1652	06/19	Necrotic spots on leaves	Cesena	+	+	+	3
1653	06/19	Necrotic spots on leaves	Cesena	-	+	+	2
1654	06/19	Necrotic spots on leaves	Cesena	+	+	+	3
1655	06/19	Necrotic spots on leaves	Cesena	+	+	+	2
1671	06/19	Necrotic spots on leaves	Faenza	-	+	+	3
1672	06/19	Necrotic spots on leaves	Faenza	+	+	+	2
1673	06/19	Necrotic spots on leaves	Faenza	+	+	+	2
1674	06/19	Necrotic spots on leaves	Faenza	+	+	+	0
1675	06/19	Necrotic spots on leaves	Faenza	+	+	+	3
1676	06/19	Necrotic spots on leaves	Faenza	+	+	+	1
1677	06/19	Necrotic spots on leaves	Faenza	+	+	+	3
1678	06/19	Necrotic spots on leaves	Faenza	-	+	+	1
1679	06/19	Necrotic spots on leaves	Faenza	+	+	+	1
1680	06/19	Necrotic spots on leaves	Faenza	+	+	+	1

4.2.2. Test for Pathogenecity

Symptoms developing on walnut fruitlets confirmed the 47 HR positive isolates pathogenicity and demonstrated that they were pathogenic to their host. As a result, these 47 isolates were classified as genuine *X. a.* pv. *juglandis*. The remaining 13 *X. a.* pv. *juglandis*-like isolates that did not induce HR on bean pods or necrosis on walnut fruitlets during the pathogenicity assay were classified as saprophytic, *X. a.* pv. *juglandis*-like bacteria. Fourtheen isolates were highly pathogenic, necrosis and rotting fruitlets up to the cotyledons (virulence class 3); twenty isolates, including the type strain NCPPB 411, necrotized both the mesocarp and endocarp (virulence class 2); and thirteen isolates caused a clear necrosis but limited to the mesocarp (virulence class 1) (Fig 4.16) (Table 4.5).

Taking the origin of the pathogenic strains into account, no correlation between disease severity in the pathogenicity assay and the plant organs and time of isolation of the *X. a.* pv. *juglandis* strains used in this test was observed.

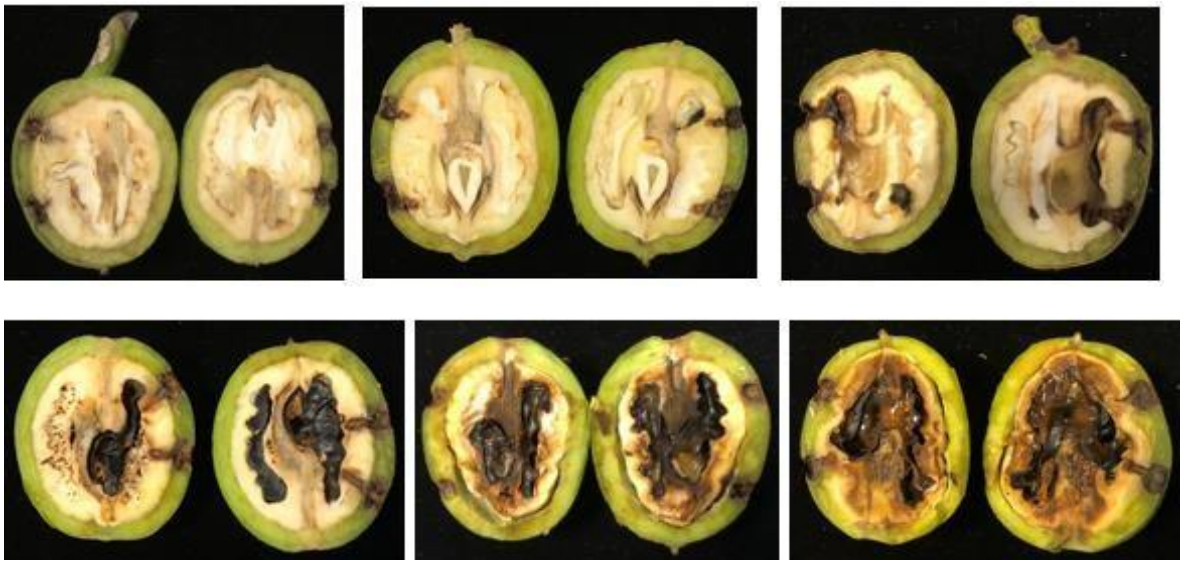

Fig. 4.16. Symptoms that occured during pathogenicity test were categorized on a disease index scale, with 0: indicating no symptom, 1: indicating mesocarp necrosis, 2: indicating mesocarp and endocarp necrosis, and 3: indicating mesocarp and endocarp necrosis. Ultimately, each *X. a.* pv. *juglandis* isolate was allocated to a virulence class based on a phytopathometric evaluation.

4.2.3. The Determination of The Level of Copper Resistance in *Xanthomonas* Strains

Regardless of the species, all CuR *Xanthomonas* strains, including the positive controls, grew on MGYA medium supplemented with 50–1000 ppm of copper (Table 4.6). A few CuR strains grew to confluence at 1.2 mM of copper, whereas the majority reached confluence at 1.6 mM. At 2.4 mM, no growth of CuR strains was observed. CuS negative control strains, on the other hand, did not grow on MGYA medium supplemented with more than 0.2 to 0.3 mM of copper (Table 4.6).

On MGYA medium supplemented with 50 ppm copper sulphate, all *X. a.* pv. *juglandis* isolates, including the type strain NCPPB 411, grew. 88% of *X. a.* pv. *juglandis* isolates grew at a concentration of 100 ppm, 76% at a concentration of 200 ppm, and 68% at a concentration of 300 ppm of Cu++. Surprisingly, 27% of *X. a.* pv. *juglandis* strains grew at increasing concentrations of copper, up to 500 ppm, whereas the majority of them became blue, implying that the bacteria cells accumulated copper.

4.2.4. *cop*LAB Primers and Polymerase Chain Reaction Analysis

*Cop*LF/*cop*LR, *cop*AF/*cop*AR, and *cop*BF/*cop*BR primer sets were effective at discriminating between Cu resistant and Cu sensitive strains of *X. a.* pv. *juglandis*. The *cop*LAB gene cluster was detected in all isolates in our collection using the primer sets *cop*LF/*cop*LR, *cop*AF/*cop*AR, and *cop*BF/*cop*BR. The PCR analysis revealed that the three copper resistance genes are conserved in *X. a.* pv. *juglandis* strains that are resistant to copper. For Cu sensitive strains, no DNA amplifications were observed. Different sizes of *cop*B amplicons were observed between strains tested for the partial region covered by the *cop*BF/*cop*BR primer set. The majority of strains analyzed produced *cop*B with an intermediate size. Of the 37 CuR strains included in this study, one (3%) strain, ten (27%) strains, and twenty-six (70%) strains had a larger, smaller, or intermediate size *cop*B sequence, respectively (Table 4.6). Copper assays on solid medium revealed that the size differences of *cop*B had no effect on copper resistance levels (Table 4.6).

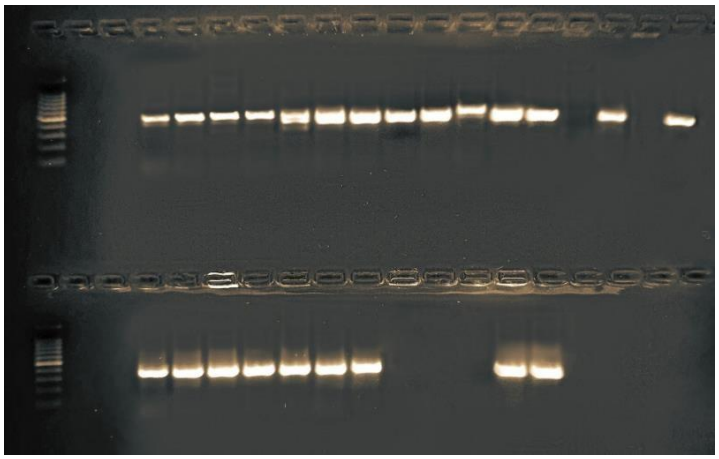

Fig. 4.17. PCR image of *cop*A gene

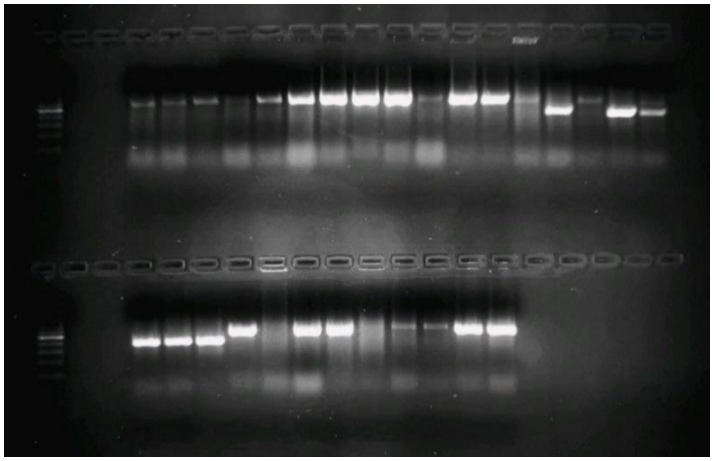

Fig. 4.18. PCR image of *cop*B gene

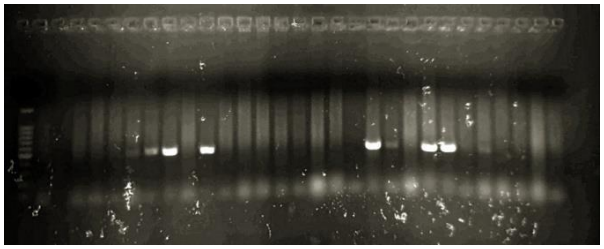

Fig. 4.19. PCR image of *cop*L gene

Table 4.6. List of *X. a.* pv. *juglandis* strains containing *cop*LBA genes

No	*in vitro* copper resistance	PCR *cop*A	PCR *cop*B	PCR *cop*L
1515A	300	+	+	-
1515C	300	+	+	-
1515D	500	+	+	-
1516B	500	+	-	-
1516C	300	+	+	-
1517A	100	+	+	+
1517B	100	+	+	+
1518A	100	-	+	+
1518B	400	+	+	-
1519A	200	+	-	+
1519C	100	+	+	-
1520A	500	+	+	-
1520B	300	+	-	-
1520C	300	+	+	-
1521C	500	+	-	-
1522A	500	+	+	-
1522B	100	+	+	-
1522C	200	+	+	-
1541A	300	+	+	+
1541B	500	+	+	-
1541C	500	+	+	-
1541D	500	+	-	+
1542A	200	-	+	+
1542B	300	-	+	-
1542C	500	+	-	-
1549B	100	-	-	-
1549C	400	+	+	-
1549D	100	-	+	-

4.2.5. REP-PCR of *X. a.* pv. *juglandis* Isolates

Amplicons from a selection of 31 *X. a.* pv. *juglandis* isolates generated using the BOXA1R, REP and ERIC primers (Fig 4.20, 4.21, 4.22). Considering coefficient indices, the UPGMA analysis revealed a greater intra-pathovar heterogeneity. On a cumulative dendogram Pearson's index revealed three distinct clusters (Fig 4.23). The main cluster (A), consisting of thirteen *X. a.* pv. *juglandis* isolates and the type strain NCPPB 411, demonstrated a 51% similarity between the strains; a second cluster (B), consisting of five isolates, demonstrated a 46 % similarity between the strains. Among the strains, a third cluster (C) with thirteen isolates and a similarity coefficient of 40% was observed. Along with the three major clusters, several outlying isolates were identified.

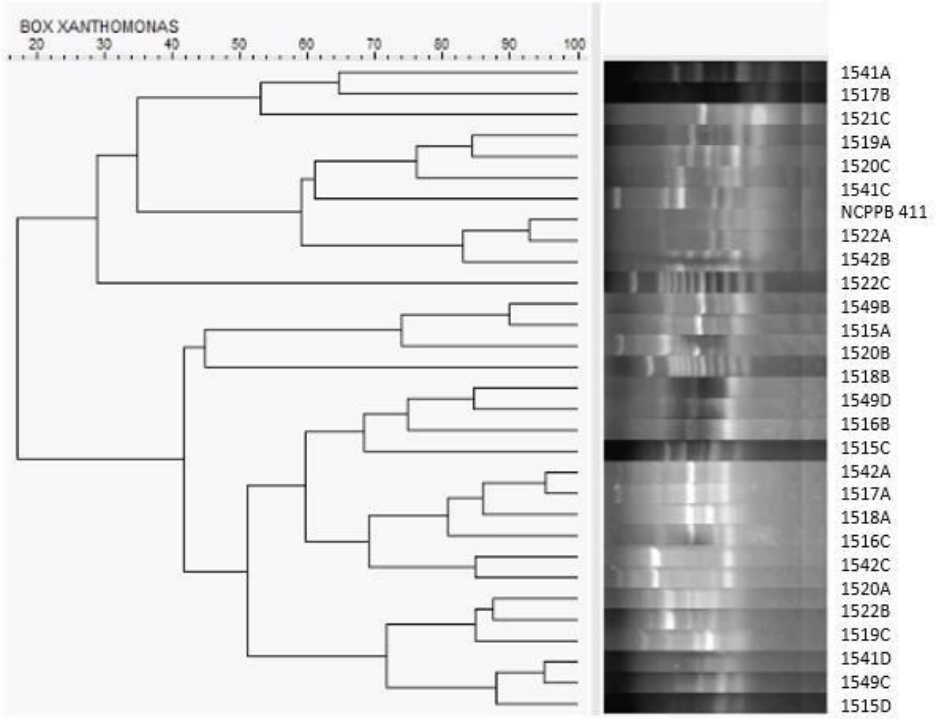

Fig. 4.20. Dendrogram generated after the cluster analysis of digitised combined rep-PCR fingerprints using BOX primer sets. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

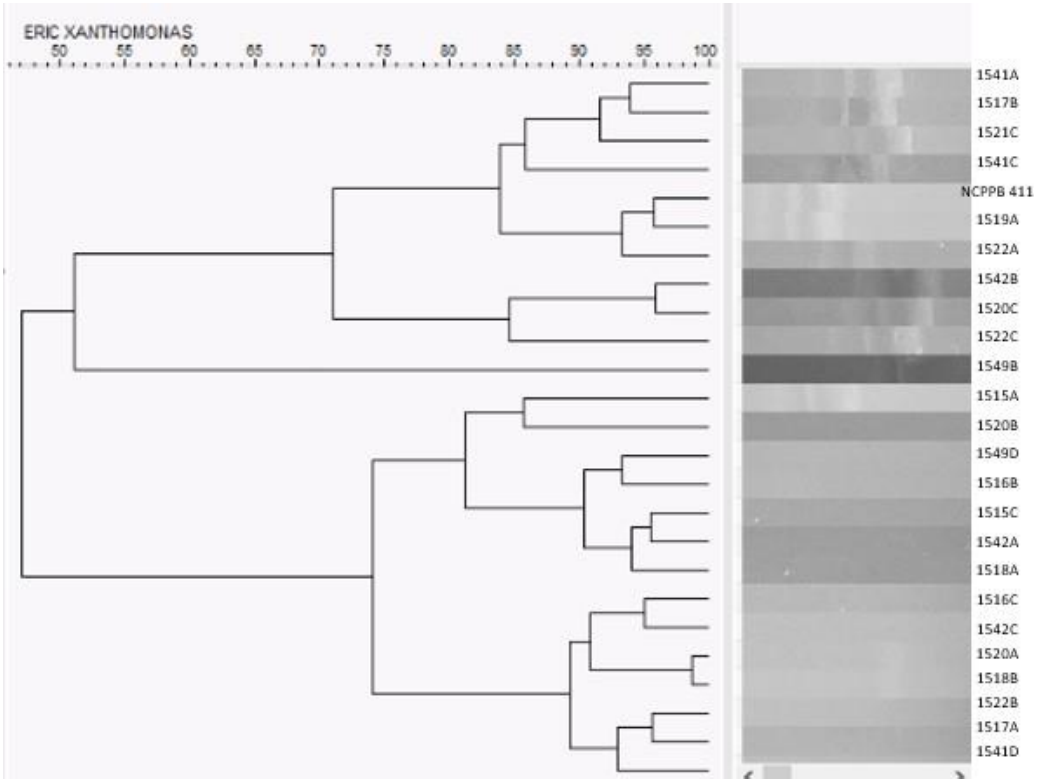

Fig. 4.21. Dendrogram generated after the cluster analysis of digitised combined rep-PCR fingerprints using ERIC primer sets. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

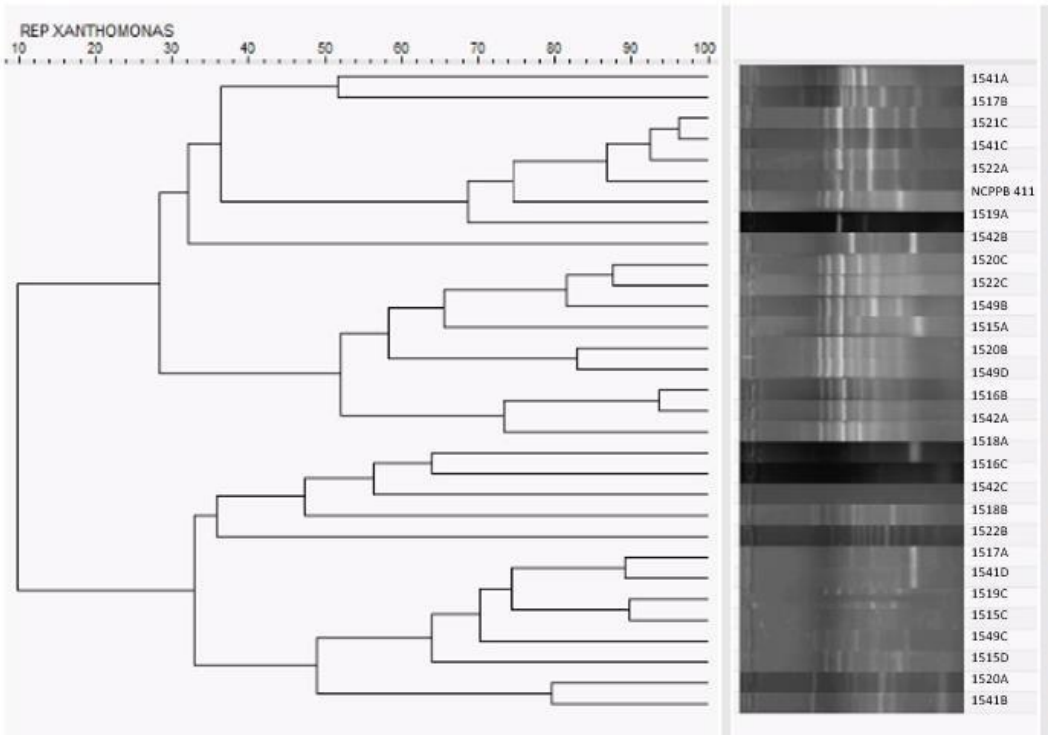

Fig. 4.22. Dendrogram generated after the cluster analysis of digitised combined rep-PCR fingerprints using REP primer sets. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

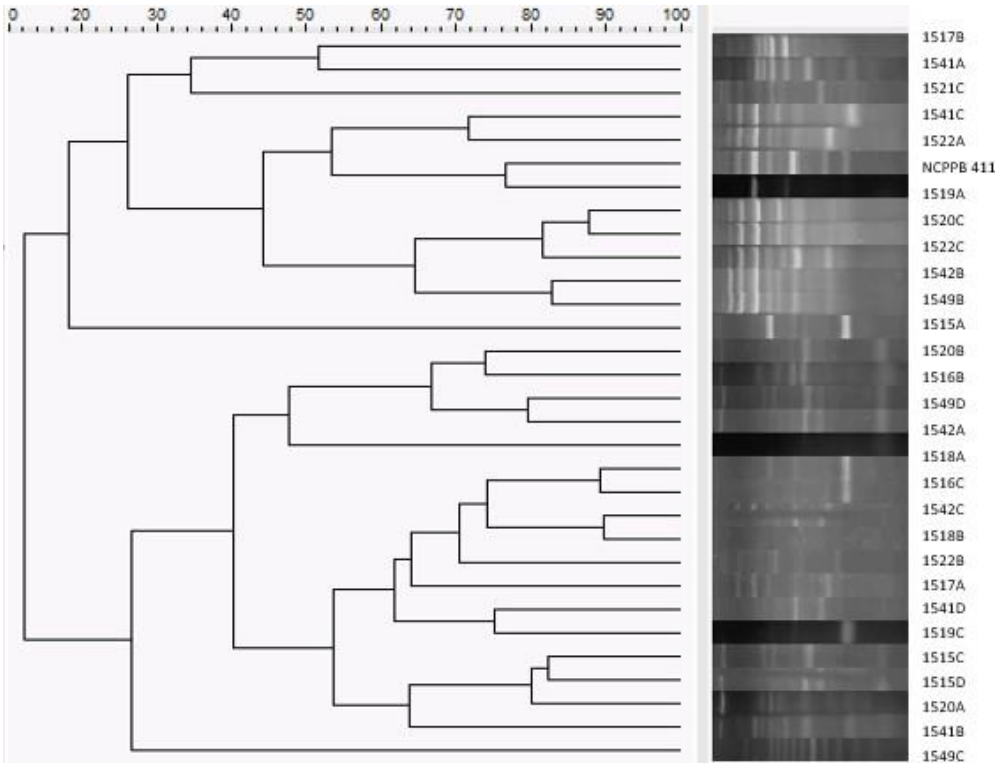

Fig. 4.23. Dendrogram generated after the cluster analysis of concatenated digitised combined rep-PCR fingerprints using BOX, ERIC and REP primer sets. The Dendrogram was obtained using GelCompar 4.1 (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as the percentage values of the Pearson correlation coefficient. The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

4.2.6. MLSA Analysis of *X. a.* pv. *juglandis* Strains

MLSA analysis of representive *X. a.* pv. *juglandis* strains was demonstrated by using four housekeeping genes such as *gyr*B (Fig 4.24), *rpo*D (Fig 4.25), *dna*K (Fig 4.26) and *fyu*A (Fig 4.27). On a phylogenetic tree constructed from concatenated sequences, all *X. a.* pv. *juglandis* isolates are classified into two major clusters, with three minor sub-clusters (Fig 4.28). This phylogenetic analysis confirmed that all the 13 Italian isolates clearly belong to the *X. a.* pv. *juglandis* cluster. The Italian *X. a.* pv. *juglandis* isolates were distributed randomly along the major cluster. The *gyr*B gene exhibited high sequence homology (100 % of bootstrap value) within the tested *X. a.* pv. *juglandis* population (Fig 4.24). The highest degree of discrimination between strains is demonstrated in the *fyu*A sequence (Fig 4.27): seven distinct groups are highlighted. As a result, our collection of *X. a.* pv. *juglandis* isolates demonstrates diversity within the sampled area.

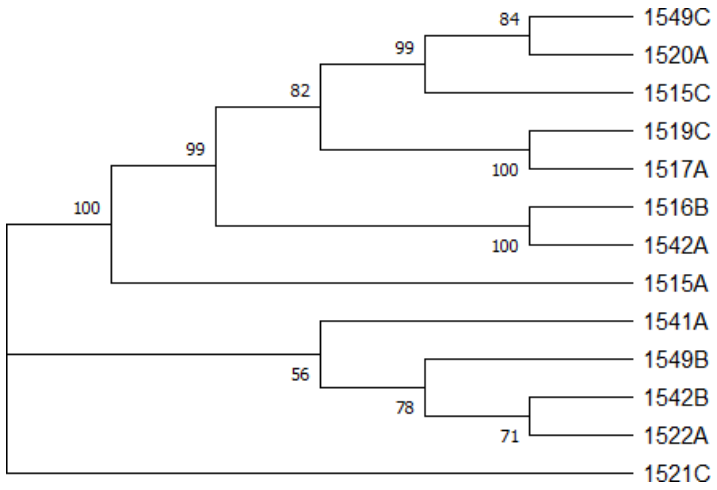

Fig. 4.24. Phylogenetic tree based on the sequence of the *gyr*B gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

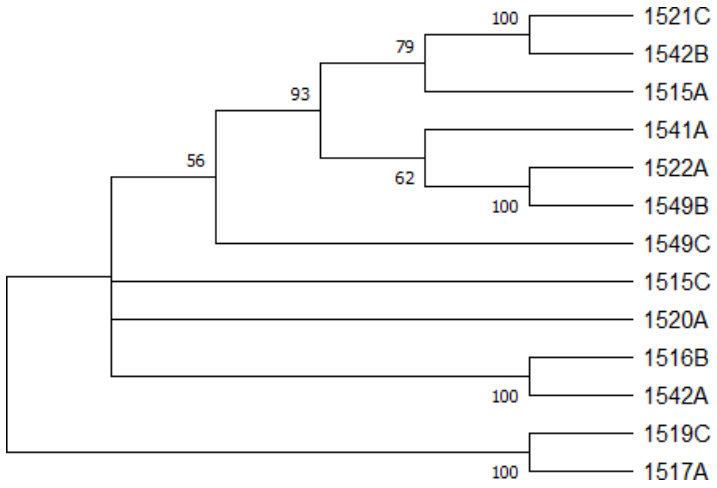

Fig. 4.25. Phylogenetic tree based on the sequence of the *rpo*D gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

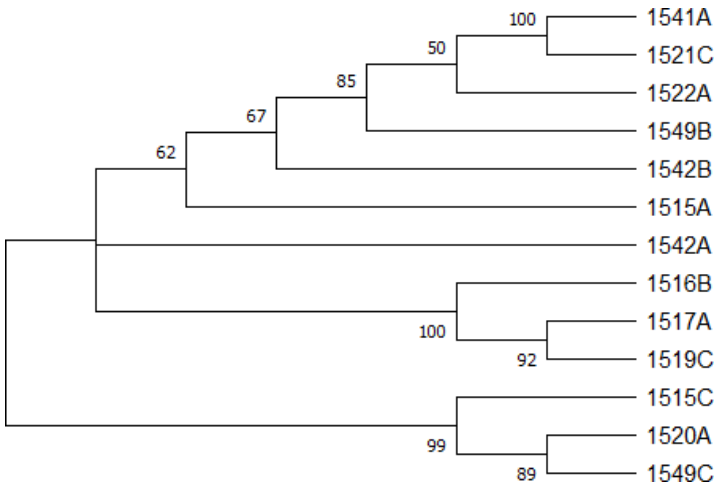

Fig. 4. 26. Phylogenetic tree based on the sequence of the *dna*K gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *X. a.* pv. juglandis isolates from Italy were compared with the pathotype strain NCPPB 411

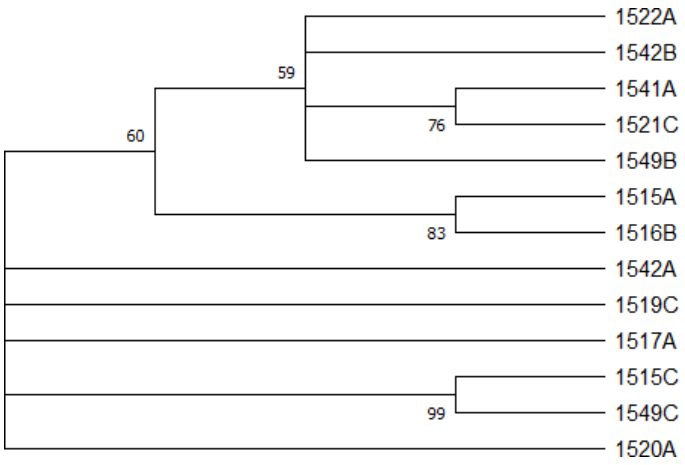

Fig. 4. 27. Phylogenetic tree based on the sequence of the *fyu*A gene. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

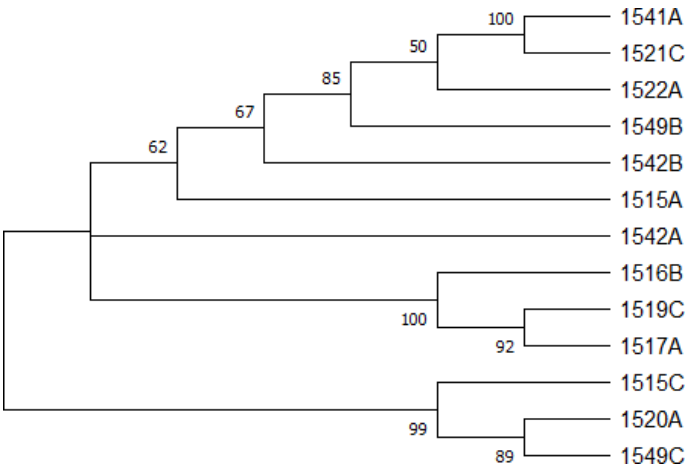

Fig. 4. 28. Phylogenetic tree based on the concatenated sequence of the *gyr*B, rpoD, dnaK and fyuA genes. The tree was constructed using the neighbour-joining method, and the confidence of nodes was determined after 1000 bootstrap simulations using the MEGA 7.0.20 software program (Tamura et al. 2007). The *X. a.* pv. *juglandis* isolates from Italy were compared with the pathotype strain NCPPB 411

4.3. BACTERIOPHAGES

4.3.1. Isolation of Phages

Between 2019 and 2020, a total of twenty bacteriophages were isolated in Italy, ten against *P. s.* pv. *syringae* and ten against *X. a.* pv. *juglandis*, to control populations of the causal agents of stone fruit canker and walnut bacterial blight, respectively.

For *P. s.* pv. *syringae*, four phages were isolated from leaves, four from fruits, and two from soil (Table 4.7). Phage isolation from irrigation water against *P. s.* pv. *syringae* was unsuccessful. Purification and propagation procedures resulted in suspensions of phage with a titer of 10^9 PFU ml-1. Three phage isolates were chosen for further study due to their substrate origin and host specificity (Table 4.7). After 24 hours incubation, the phages formed clear plaques measuring approximately 0.3-0.7 cm in diameter with sharp edges on lawns of *P. s.* pv. *syringae* strain 1508A, 1511A, and 1522. P10 and P11 phage isolates produced identical plaques with an additional translucent halo, whereas the remaining phage isolates produced clear plaques (Fig 4.29).

Table 4.7. List of the phages specific to *P. s.* pv. *syringae* isolated in Northern Italy

Phage	**Locality of isolation**	**Origin of isolation**	**Year**	**Plaque morphology**
Pϕ4	Reggio Emilia	Leaves	2020	0,4 cm clear plaque
Pϕ7	Reggio Emilia	Leaves	2020	0,4 cm clear plaque
Pϕ8	Reggio Emilia	Soil	2020	0,4 cm clear plaque
Pϕ10	Reggio Emilia	Leaves	2020	0,3 cm with halo
Pϕ11	Reggio Emilia	Fruits	2020	0,3 cm with halo
Pϕ12	Reggio Emilia	Fruits	2020	0,7 cm clear plaque
Pϕ12b	Reggio Emilia	Leaves	2020	0,3 cm clear plaque
Pϕ17	Reggio Emilia	Fruits	2020	0,4 cm clear plaque
Pϕ24	Reggio Emilia	Fruits	2020	0,5 cm clear plaque
Pϕ28	Reggio Emilia	Soil	2020	0,6 cm clear plaque

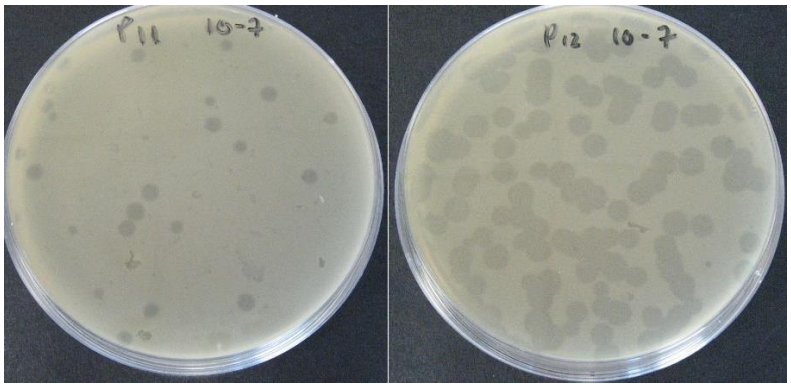

Fig. 4.29. Examples of diversity of phage plaque morphology and plaque sizes. Morphological types of plaques are shown: clear plaques (on the left), plaques with clear centers and turbid plaque (on the right).

After three successive single-plaque purifications, ten phages against *X. a.* pv. *juglandis* were isolated from enriched samples (Table 4.8). We isolated four phages from leaves, three from soil, two from irrigation water, and one from fruits. The strains 1515D, 1519A, and 1549D of *X. a.* pv. *juglandis* were used for enrichment. Purification and propagation procedures yielded phage suspensions with titers ranging from 10^9 to 10^{10} PFU ml-1. Three phage isolates were chosen for further characterization based on their RFLP pattern: Xaj2, Xaj24.

Table 4.8. List of phages specific to *X. a.* pv. *juglandis* isolated in Northern Italy

Phage	Locality of isolation	Origin of isolation	Year	Plaque morphology
Xajϕ1	Faenza	Soil	2019	0,1 cm clear plaque
Xajϕ6	Faenza	Leaves	2019	0,1 cm clear plaque
Xajϕ7	Faenza	Irrigation water	2019	0,1 cm clear plaque
Xajϕ8	Forli	Soil	2019	0,1 cm clear plaque
Xajϕ10	Forli	Leaves	2019	0,1 cm clear plaque
Xajϕ11	Reggio Emilia	Irrigation water	2020	0,2 cm clear plaque with halo
Xajϕ13	Reggio Emilia	Leaves	2020	0,3 cm clear plaque
Xajϕ16	Reggio Emilia	Leaves	2020	0,3 cm clear plaque with halo
Xajϕ21	Reggio Emilia	Soil	2020	0,1 cm clear plaque
Xajϕ22	Reggio Emilia	Fruit	2020	0,2 cm clear plaque

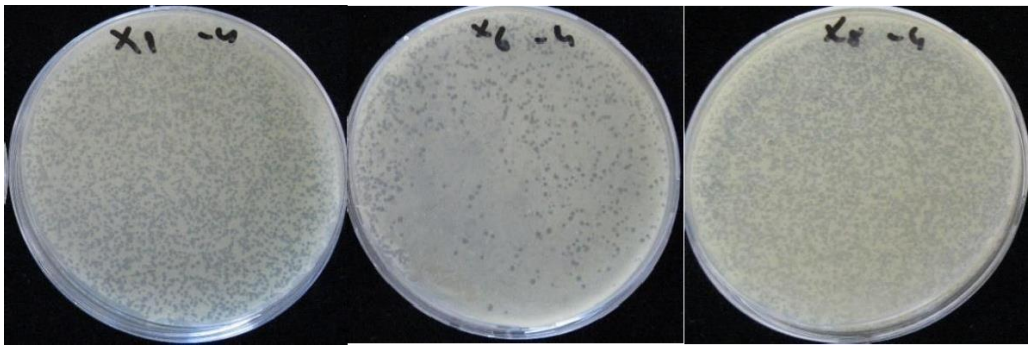

Fig. 4. 30. Example of plaqu morphology of the phages isolated against *X. a.* pv. *juglandis* in this study.

4.3.2. Host Range

All phage isolates tested were only specific for *P. s.* pv. *syringae* or *X. a. pv. juglandis*. The phages for *P. s.* pv. *syringae* did not lyse any of the *P. s.* pv. *tomato* or *P. s.* pv. *actinidae* strains. The spot test results indicated that phages could be classified into three groups according to their specificity for 25 *P. s.* pv. *syringae* strains. At varying degrees, all isolates formed plaques on all 25 *P. s.* pv. *syringae* strains tested (Table 4.9). The presence of clear plaques on the lawn of *P. s.* pv. *syringae* strains indicated complete cell lysis, whereas turbid plaques indicated partial cell lysis. Phages P4, P7, P8, P12, P12b, P17, P24, and P28 formed clear plaques on all *P. s.* pv. *syringae* strains and were classified as group I. Group II phages included P10 and P11, which formed turbid plaques on *P. s.* pv. *syringae* strains.

Table 4.9. Host range analysis of phages against *P. s.* pv. *syringae* isolated in apricot orchards

Bacteria	**Bacteriophages**									
	Pϕ4	Pϕ7	Pϕ8	Pϕ10	Pϕ11	Pϕ12	Pϕ12b	Pϕ17	Pϕ24	Pϕ28
78	-	-	+	-	+	+	+	+	-	+
82	-	+	-	+	+	+	+	+	-	-
361	-	+	-	-	+	+	+	-	-	-
1278	+	+	-	-	+	-	+	-	-	-
1291	+	+	+	-	+	-	+	-	-	+
1293	+	-	+	+	-	+	+	-	+	+
1300	+	-	+	+	+	-	+	+	+	-
1143	+	-	+	+	-	+	+	+	-	-
1146	-	-	+	-	-	+	+	-	-	-
1148	-	+	-	-	-	+	+	-	+	+
1178	+	+	-	+	-	+	+	-	+	-
1191	+	-	+	+	-	+	-	-	+	-
1203	+	-	+	-	+	+	-	+	-	+
1333	+	-	+	-	+	+	-	+	+	+
1334	+	+	+	+	+	-	+	+	+	+
1335	-	+	+	-	+	-	+	-	-	+
1337	-	+	+	-	+	-	-	+	-	+
1338	+	+	+	-	+	-	-	+	-	+

1420	+	+	-	+	-	+	-	+	+	-
1421	+	-	-	+	+	+	+	-	+	+
1509	-	-	-	+	-	+	+	-	+	-
1514	+	-	+	-	-	+	+	-	-	+
1601	-	+	-	+	-	-	+	+	-	+
1619	+	+	-	+	+	-	-	+	+	-
1687	+	-	+	-	+	+	-	+	+	-
IPV-BO *1544*	-	-	-	-	-	-	-	-	-	-
NCPPB 3739	-	-	-	-	-	-	-	-	-	-

Likewise, the phages against *X. a*. pv. *juglandis* did not lyse any of the *X. a.* pv. *pruni* (ICMP51) or *X. a.* pv. *celebensis* (NCPPB1832) strains. The host specificity of 25 X. a. pv. juglandis strains was determined with the selected phages (Table 4.10). At least one of the two phages was susceptible to all *X. a*. pv. *juglandis* strains. Among the 18 *X. a*. pv. *juglandis* strains tested, strains 284, 293 and 294 were each infected with a single bacteriophage (X6, X8, or X21). X1 and X7 were the most effective phages, infecting 15 of 25 strains of *X. a*. pv. *juglandis*.

Table 4.10. Host range analysis of phages against *X. a.* pv. *juglandis* isolated in walnut groves

Strain nr.	**Bacteriophages**									
	Xϕ1	Xϕ6	Xϕ7	Xϕ8	Xϕ10	Xϕ11	Xϕ13	Xϕ16	Xϕ21	Xϕ22
69	+	+	+	+	-	-	-	+	-	-
77	+	+	+	-	-	-	+	+	-	-
97	+	+	-	-	-	-	+	+	-	-
98	+	+	+	-	-	-	+	+	+	-
103	+	-	+	-	+	-	-	+	+	-
105	+	-	+	-	+	-	-	-	+	+
182	+	-	+	+	+	-	-	-	-	+
183	-	+	-	+	+	+	+	+	-	+
265	+	-	+	+	-	+	+	+	-	+
284	-	+	-	-	-	-	-	-	-	-
291	-	-	-	-	-	-	+	-	-	+
293	-	-	-	+	-	-	-	-	-	-
294	-	-	-	-	-	-	-	-	+	-
317	-	+	+	+	-	+	-	+	+	+
357	+	+	-	+	-	+	-	-	+	+
378	+	+	-	+	+	+	+	-	+	+
407	+	-	-	-	+	+	+	-	+	-
423	-	-	-	-	+	-	+	-	+	-
428	-	+	+	-	+	-	-	+	+	-
599	+	+	+	-	-	-	-	+	+	-
604	+	-	+	+	-	+	+	+	-	+
1515	+	-	+	+	+	+	+	+	-	-
1560	-	+	+	+	+	+	+	-	+	-
1652	-	-	+	+	+	+	-	-	+	+
NCPPB41	+	+	+	-	+	-	+	+	-	+
ICMP51	-	-	-	-	-	-	-	-	-	-
NCPPB1832	-	-	-	-	-	-	-	-	-	-

4.3.3. Thermal Inactivation

Phages Xajϕ7, Xajϕ13, Xajϕ16, Xajϕ21 were inactivated after 10 min of exposure to 68°C, while phage isolates Xajϕ1, Xajϕ6, Xajϕ8, Xajϕ10, Xajϕ11, Xajϕ22 were inactivated at 72°C (Table 4.11).

Table 4.11.Thermal inactivation point of the phages specific to *X. a.* pv. *juglandis*

Phages	**Temperature**										
	55°C	65°C	66°C	67°C	68°C	69°C	70°C	71°C	72°C	73°C	75°C
Xajϕ1	+	+	+	+	+	+	+	+	-	-	-
Xajϕ6	+	+	+	+	+	+	+	+	-	-	-
Xajϕ7	+	+	+	+	-	-	-	-	-	-	-
Xajϕ8	+	+	+	+	+	+	+	+	-	-	-
Xajϕ10	+	+	+	+	+	+	+	+	-	-	-
Xajϕ11	+	+	+	+	+	+	+	+	-	-	-
Xajϕ13	+	+	+	+	-	-	-	-	-	-	-
Xajϕ16	+	+	+	+	-	-	-	-	-	-	-
Xajϕ21	+	+	+	+	-	-	-	-	-	-	-
Xajϕ22	+	+	+	+	+	+	+	+	-	-	-

Phages Pϕ4, Pϕ12 and, Pϕ12b were inactivated after 10 min of exposure to 72°C, while phage isolates Pϕ7, Pϕ8, Pϕ10, Pϕ11, Pϕ17, Pϕ24 and Pϕ28 were inactivated at 74°C (Table 4.12).

Table 4.12. Thermal inactivation point of the phages specific to *P. s.* pv. *syringae*

Phages	**Temperature**										
	55°C	65°C	66°C	67°C	68°C	69°C	70°C	71°C	72°C	73°C	74°C
Pϕ4	+	+	+	+	+	+	+	+	-	-	-
Pϕ7	+	+	+	+	+	+	+	+	+	+	-
Pϕ8	+	+	+	+	+	+	+	+	+	+	-
Pϕ10	+	+	+	+	+	+	+	+	+	+	-
Pϕ11	+	+	+	+	+	+	+	+	+	+	-
Pϕ12	+	+	+	+	+	+	+	+	-	-	-
Pϕ12b	+	+	+	+	+	+	+	+	-	-	-
Pϕ17	+	+	+	+	+	+	+	+	+	+	-
Pϕ24	+	+	+	+	+	+	+	+	+	+	-
Pϕ28	+	+	+	+	+	+	+	+	+	+	-

4.3.4. Effect of pH on Phage Viability

All phages of *P. s.* pv. *syringae* survived at range of pH 2-11 during 24 h at room temperature and in dark conditions. Maximum viability was observed at pH 7. Decrease in phage titer was dramatically observed at pH 2 and pH 11 (Fig 4.31).

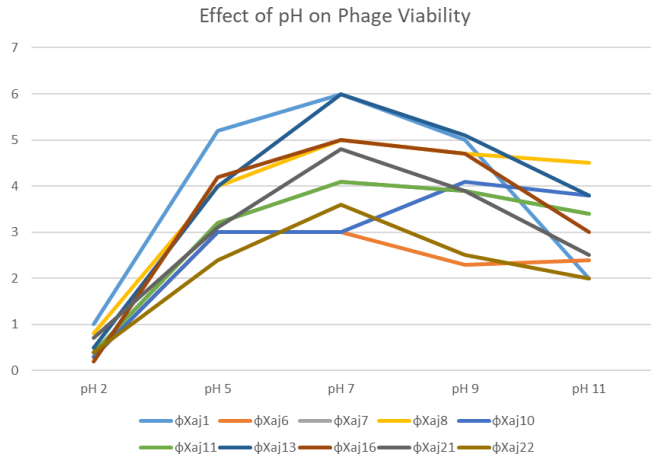

Fig. 4. 31. The effect of different pH values on Xaj sphage during 24 h.

4.3.5. Effect of UV Light on Phage Vitality *in vitro*

When phages were exposed to a 16-hour UV light/8-hour dark photoperiod, their population was significantly reduced compared to the initial concentration. After 24h of incubation, the phage population was undetectable.

4.3.6. DNA Analysis of The Phages

Restriction enzyme digestion and agarose gel electrophoresis were used to analyze the genomic DNA of ten phage isolates. Each phage isolate had a genome that was approximately 26 kb in length. Their DNAs were found to be double-stranded when digested with restriction enzymes specific for double-stranded DNA, such as *Eco*R1.

According to *Eco*RI restriction analysis (Fig 4.32), phages against *P. s.* pv. *syringae* were classified into three RFLP groups (A, B, C). The phages P4, P17, and P28 all had identical restriction patterns during digestion, indicating a high degree of DNA homology, and thus formed group A. Another group with a high degree of DNA homology was designated B and

included the phages P7, P10, P12b, and P24. Phages P8, P11, and P12 formed another group with a high degree of DNA homology and eventually merged into group C.

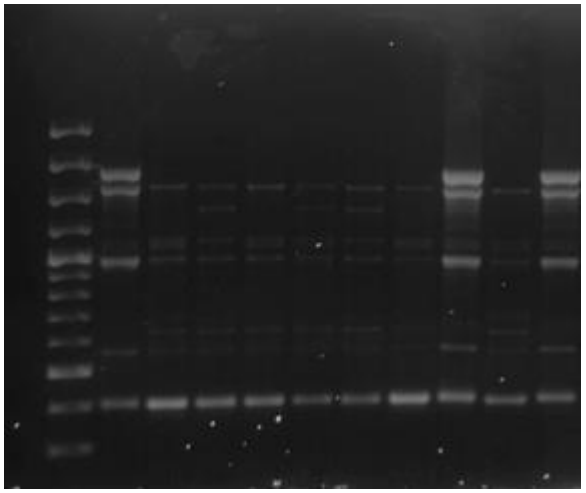

Fig. 4. 32. Agarose gel electrophoresis of DNAs from *P. s.* pv. *syringae* specific bacteriophages. Bacteriophage DNA digested with *Eco*RI enzyme.

Based on the obtained fragment sizes, DNA sizes of phages of *X. a.* pv. *juglandis* were estimated as ranging 15–20 kb. Based on the RFLP patterns by *Eco*R1, the 1o bacteriophage isolates of *X. a.* pv. *juglandis* were apparently separated into two group (Fig 4.33). Xajϕ6, Xajϕ7, Xajϕ8, Xajϕ10, Xajϕ11, Xajϕ13 and, Xajϕ21 had a high DNA homology and grouptogether in group A. Xajϕ21 and Xajϕ22 grouped together in group B. Xajϕ1 showed resistance and did not digested with *Eco*R1.

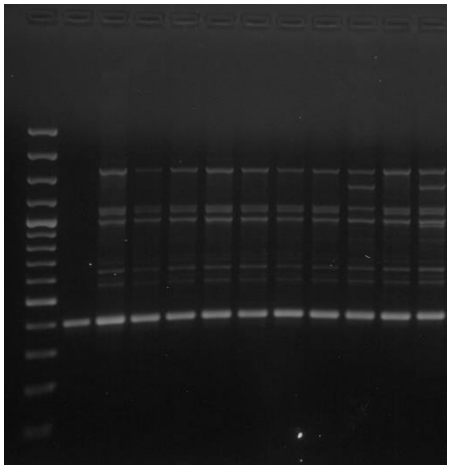

Fig. 4.33. Agarose gel electrophoresis of DNAs from *X. a.* pv. *juglandis* specific bacteriophages. Bacteriophage DNA digested with *Eco*RI enzyme.

5. DISCUSSION AND CONCLUSIONS

A single pathogen lesion on a single leaf has no significant economic or ecological consequences. An epidemic involving thousands or millions of infection events including an entire population of bacteria and their host plants results in significant crop loss. A plant pathologist must develop methods for controlling the entire pathogen population in order to control bacterial diseases. Thus, it is critical to understand plant pathogen population biology in order to develop rational control strategies.

Fruit production generates significant revenue for growers in a number of countries worldwide. Stone fruits are widely cultivated throughout the majority of the European Union, although species distribution varies significantly between Mediterranean and northern European countries (EFSA, 2014). Isolations revealed a predominance of *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* in apricot and walnut samples collected during two growing seasons, respectively. Over 200 symptomatic samples were collected during the two-year survey of orchards, but not all isolates were identified as pathogenic bacteria, indicating a variety of disease etiologies. As a result, symptoms cannot be used as a reliable diagnostic criterion, as similar symptoms can be caused by other biotic or abiotic factors and may be misinterpreted as bacterial infections, resulting in ineffective orchard management. Due to their similar host range, symptomatology, physiological and biochemical characteristics, and physiological and biochemical characteristics, these pathogens are easily misidentified. To develop differential tests for the rapid and reliable identification of *P. s.* pv. *syringae*, *P. s.* pv. *morsprunorum*, and *X. a.* pv. *juglandis*, their pathogenicity was investigated, together with physiological and biochemical characteristics, the presence of toxin-producing genes, and differences in their genetic profile using fingerprinting and MLSA.

Recently, the genetic techniques rep-PCR and MLSA were used to develop effective methods for identifying *P. s.* pv. *morsprunorum* races 1 and 2, demonstrating the genetic homogeneity between these two *P. s.* pv. *morsprunorum* races and the high heterogeneity of *P. s.* pv. *syringae*. Recent studies using these methods have confirmed that each *P. s.* pv. *morsprunorum* race exhibits homogeneity in distinct DNA regions and a high degree of diversity among *P. s.* pv. *syringae* strains (Gilbert et al. 2009; Kaluzna et al. 2010a).

It should be noted that the role of INA in *P. s.* pv. *syringae* virulence is associated with subsequent invasion and tissue infection, not with frost injury to blossoms. In susceptible

varieties, severe frost events are typically accompanied by systemic infection and canker formation. For instance, in 2002, a particularly damaging hard radiational frost occurred in Michigan during bloom (Kennelly et al. 2007). This freeze event severely damaged sweet cherry blossoms and resulted in widespread wood invasion, canker formation, and tree death. Thus, a critical factor in the association between INA and infection appears to be the close association of bacteria and ice formation with sites of injured host tissue that is predisposed to infection. The observation that successful infection requires the presence of pathogen cells within 20 minutes of thawing frost-injured tissue (Süle and Seemüller 1987) provides corroboration for the importance of the co-occurrence of INA bacteria, ice, and injured tissue in infection. Finally, the question of whether INA plays a role in pathogen virulence will need to be addressed using a defined mutant in inoculation studies on a variety of hosts exposed to a variety of different freezing temperatures.

There has been research on secondary metabolites that are important for bacterial ecology and pathogenicity. The two siderophores pyoverdin and yersiniabactine have been investigated for their ecological significance and potential role in virulence (Ahmed and Holmstrom, 2014). Groups with varying degrees of pathogenicity were identified in both pathovars: in *P. s.* pv. *syringae*, between toxic lipodepsipeptide producers and non-producers, as well as between genetic groups of toxic lipodepsipeptide producers; in *P. s.* pv. *morsprunorum* race 1, between coronatine producers and non-producers. In each case, the strains producing these secondary metabolites were identified and detected based on their specificity of production.

To comprehensively characterize the diversity of *X. a.* pv. *juglandis*, casual agent of walnut bacterial blight, and to fully understand their epidemic dynamics, the current research used a two-year sampling plan that included data thought to influence disease epidemiology, namely different walnut cultivars, plant organs (leaves, fruits, branches, buds, and catkins), and plant organs (leaves, fruits, branches, buds, and catkins).

Numerous hypotheses have been advanced to account for the diversity of the *X. a.* pv. *juglandis* population, including geographical location (Loreti et al. 2001; , Scortichini et al. 2001; origin of plant propagation material (Hajri et al. 2010; ,Giovanardi et al. 2015; adaptation to specific environmental conditions (Scortichini et al. 2001; Kaluzna et al. 2014); genome flexibility or pathogen virulence (Ivanovic et al. 2014; Kaluzna et al. 2014; and even selective pressure by the host plant (Hajri et al. 2010; Marcelletti et al. 2010. Regardless of their

significant contributions, these studies relied on a small number of bacterial isolates, frequently obtained without a planned sampling strategy, or on a collection of *X. a.* pv. *juglandis* strains from around the world, omitting critical metadata such as date and plant host traits that are necessary for determining epidemiological patterns (McMahon and Denaxas, 2016; Parkhill and Wren, 2011. Indeed, to fully understand the epidemiological behavior of *X. a.* pv. *juglandis*, it is critical to combine comprehensive genotyping analysis of a coherent set of isolates with insightful metadata in a single study.

In conclusion, our findings reveal previously unknown levels of genetic variability among *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* isolates with multiple co-existing populations. These findings suggest that there are differences in the capacity of various *P. s.* pv. *syringae* and *X. a.* pv. *juglandis* populations to persist/thrive in the plant.

All tested isolates exhibited a high level of resistance to copper, implying that resistance developed in the Italian population of *P. s.* pv. *syringae* and *X. a.* pv. *juglandis*. This could be because copper compounds are frequently used in stone fruit and walnut disease control programs. Copper resistance in plant pathogenic bacteria is not an uncommon occurrence in fruit-growing regions due to intensive chemical protection (Sulikowska and Sobiczewski, 2008; Giovanardi et al. 2015; 2017). However, it is worth noting that the trend of copper resistance increased over time. Indeed, Giovanardi et al. demonstrated that all strains isolated in 2016 were resistant to only 500 ppm Cu++ in the same orchards. After several years, our results confirm that newly isolated strains from the same orchards were copper resistant to more than 500 parts per million (ppm) copper sulphate and up to 1000 parts per million (ppm) copper sulphate.

Despite the development of copper-resistant bacteria, copper-based compounds are routinely used to manage the bacterial plant diseases. However, relying solely on these compounds for disease management poses serious threats to agricultural sustainability. Repeated application of this compound over several seasons and years may be necessary during epidemic years to minimize crop loss. This has significantly increased the risk of insufficient disease management, as several studies have documented (Marco and Stall, 1983; Canteros, 1999; Cazorla et al. 2006). Indeed, once a bacterial strain acquires resistance to copper, continuous selection pressure gradually increases the prevalence of the resistant pathogen population, jeopardizing the efficacy of copper (Sundin et al. 1989). This is especially true for perennial crops, where the epiphytic and partially endophytic nature of the majority of plant

pathogenic bacteria (Renick et al. 2008) may serve as a reservoir of Cu resistance genes that Cu-sensitive strains can acquire (Cazorla et al. 2002; Behlau et al. 2012). Due to the fact that multiple bacterial populations coexist on the same host, the risk of horizontal gene transfer of Cu resistance determinants exists (Cooksey, 1990; Voloudakis et al. 1993; Behlau et al. 2012).

As a result of the current copper resistance problem, bacteriophage-mediated control has the potential to alleviate the growing problem of these diseases, either in place of or in combination with copper treatments.

A thorough characterization of phage characteristics and phage-host interactions is required to assess potential of phages as biocontrol agents. Our results showed that the broad host range phages Xajφ2 and Xajφ24 have an exceptional potential for eradicating *X. a.* pv. *juglandis* in laboratory conditions. This is the first study to characterize *X. a.* pv. *juglandis* phages isolated in Italy, paving the way for future research on bacteriophages that attack *X. a.* pv. *juglandis*.

The detailed characterization of phage characteristics is critical for determining the potential of the phages as biocontrol agents. The phyllosphere is not a safe environment, which results in a dramatic decline in the bacteriophage population (Balogh, 2002; Balogh et al. 2003; Civerolo and Keil, 1969; Iriarte et al. 2007). Numerous studies conducted in the field and in the laboratory have demonstrated that bacteriophages are easily inactivated by exposure to high temperatures, high and low pH values, and sunlight irradiation (particularly the UV-A and UV-B spectrum) (Iriarte et al. 2007). In open field conditions, the population of bacteriophage mixtures decreased rapidly and was almost completely eliminated 36-48 hours after spraying (Tewfike and Desoky, 2015). The short duration of bacteriophage persistence on the leaf surface is the primary constraint on phyllosphere-bacteriophage application.

This thesis demonstrates that phages may be a viable alternative control mechanism for pathogenic *P. s.* pv. syringae and *X. a.* pv. *juglandis* that pose a threat to stone fruits and walnut trees, particularly given the increased risk of copper resistance and regulatory restrictions on antibiotic use in agriculture. Phages developed to control plant pathogenic bacteria take advantage of the numerous and complex host–microbe interactions to significantly reduce disease, economic losses, and environmental and non-target microorganism impact.

In conclusion, while agrochemicals such as copper are still primarily used in the field to control bacterial plant diseases, there is considerable potential for bacteriophage use to reduce

the amount of agrochemicals used or to completely replace those agrochemicals used to control bacterial plant diseases. This will require the collection of additional bacteriophages against a variety of bacterial pathogens, as well as field trials rather than controlled conditions.

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RESUME

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