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Pseudomonas interactions in the plant microbiome and role in the biocontrol of *Plenodomus tracheiphilus* causal agent of Citrus Mal secco disease

Ph.D. Thesis

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Abstract

Mal secco disease (MSD) is a severe citrus tracheomycosis caused by the mitosporic fungus *Plenodomus tracheiphilus* (*Pt*). In this study we examined the effect of MSD infection on root (rhizosphere and endorhizosphere) and xylem endosphere microbial communities of *Citrus aurantium* (sour orange) seedlings following leaf- and root-inoculation with *Pt* through a metagenomic analysis based on 16S rDNA and ITS amplicons sequencing. The pathogen was detected by real-time PCR in the roots of root-inoculated plants and in the xylem of both leaf- and root-inoculated plants. Our results suggest that leaf and root infection with *Pt* alters the bacterial and fungal community composition of the compartments analyzed through a decrease or increase in the abundance of some key taxa. The genus *Pseudomonas*, which includes strains with known biocontrol activity, was the first taxon represented in the rhizosphere and the second in the endorhizosphere of both inoculated and uninoculated plants. According to differential abundance analysis, bacterial populations of this genus were significantly reduced in the roots of seedlings inoculated with the pathogen. The analysis of co-occurrence networks also suggested, overall, a change in the structure of microbial communities following infection with Pt.

The *P. corrugata* subclade (*P. corrugata*, *P. mediterranea*, *P. brassicacearum*, *P. thivervalensis*, *P. kilonensis*) has plant growth promotion and biocontrol activities against various phytopathogens. A collection of *P. corrugata* and *P. mediterranea* strains from the Phytobacteriology laboratory (Di3A) was screened *in vitro* for biocontrol activity against different isolates of *P. tracheiphilus*. All bacterial strains tested showed *in vitro* antagonistic activity, albeit to varying degrees. The two most effective biocontrol strains *in vitro*, *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C, were selected for further *in vivo* antagonistic assays and whole genome sequencing (WGS). Both strains significantly reduced the incidence and severity of MSD in lemon and sour orange plants especially in the early stages of infection. To investigate genes potentially involved in biocontrol activity, total genomic DNA from both strains was extracted and sequenced using the Illumina NovaSeq PE150 platform. Based on sequencing output, the genomes of *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C harbor numerous biosynthetic gene clusters mainly coding for non-ribosomal peptide synthetases (NRPS) involved in the production of antimicrobial compounds. Collectively, these results indicate that the two selected strains are effective biological control agents against *P. tracheiphilus* both *in vitro* and *in vivo*.

Based on the results obtained from genome analysis, we also studied the biological role of two genes (*crmA*, *solR*), never investigated before, in the production of secondary

metabolites using the model strain CFBP 5454 of *P. corrugata*. *P. corrugata* possesses a canonical quorum sensing (QS) system, PcoI/PcoR, based on the signal molecules acyl-homoserine-lactones (AHL), and in this thesis project we focused on the role of a transcriptional regulator belonging to the LuxR solos family designated SolR. *P. corrugata* produces the cyclic lipopeptides (CLP) cormycin A and corpeptins A and B. The contribution of cormycin A has been investigated here for the first time. The PCOSOLR and PCOCRMA mutant strains were obtained by inactivating, respectively, *solR* and *crmA* genes (the latter coding for a non-ribosomal peptide synthase) of *P. corrugata* CFBP 5454 and the resulting mutational phenotypes were studied. PCOSOLR mutant strain was able to induce hypersensitive response (HR) in the non-host plant tobacco and was more virulent in tomato, probably due to an increased production of CLP with phytotoxic activity. Instead, phenotype analysis of PCOCRMA mutant strain suggested that this strain produces fewer antimicrobial compounds than the wild-type strain. PCOCRMA was in fact unable to cause HR in tobacco and to inhibit the growth of some microorganisms sensitive to CLP, while the symptoms of pith necrosis in tomato were significantly reduced. Based on these results, SolR and CrmA play an important role in the interactions of *P. corrugata* with plants, in virulence, in broad-spectrum antimicrobial activity and, presumably, in the ability of this bacterium to colonize new ecological niches.

Abbreviations

ACC deaminase 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase

AHL Acyl-homoserine-lactones

ANI Average Nucleotide Identity

AUDPC Area Under Disease Progression Curve

BCA Biological Control Agent

BLAST Basic Local Alignment Search Tool

Bot Botrytis cinerea

bp Base pairs

CDS Coding sequence

CFBP Collection Francaise de Bacteries Phytopathogenes, Angers, France

*C*Las *Candidatus* liberibacter asiaticus

CLPs Cyclic Lipopeptides

Ct threshold cycle

CTV Citrus tristeza Virus

CVC Citrus Variegated Chlorosis

DAPG 2,4-diacetylphloroglucinol

DI Disease index

dpi days post inoculation

Forl Fusarium oxysporum f. sp. *radicis-lycopersici*

HCN Hydrogen cyanide

HLB Huanglongbing

HR Hypersensitive response

HTH Helix-turn-helix

HTS High-Throughput Sequencing

IMM Improved Minimal Medium

ITS Internal Transcribed Spacer

Kb Kilobases

LB Luria Broth

LLP Linear lipopeptides

LPs Lipopeptides

M.U. Miller Units

MSD Mal Secco Disease

NDA Nutrient Dextrose Agar

NGS Next Generation Sequencing

NRPS Non-ribosomal peptide synthetase

OTUs Operation taxonomic units

PAB Plant Associated Bacteria

Pco Pseudomonas corrugata

PCoA Principal Coordinates Analysis

PCR Polymerase Chain Reaction

PDA Potato Dextrose Agar

PEP Positive Edge Percentage

Pg picogram

PGP Plant Growth Promoting

PGPR Plant Growth Promoting Rhizobacteria

Pme Pseudomonas mediterranea

Pt Plenodomus tracheiphilus

PVCT Patologia Vegetale, University of Catania, Italy

QS Quorum Sensing

Rpm Revolutions per minute

SD standard deviation

SDW sterile distilled water

SIRPA Sviluppo di Induttori di Resistenza a Patogeni vascolari degli Agrumi

TPN Tomato pith necrosis

WP Work Package

Wt wild-type

X-gal 5-Bromo-4-Chloro-3-Indolyl ß-D-galactopyranoside

1. Introduction

The genus *Pseudomonas* was described at the end of 19th century and is one of the most diverse and ubiquitous bacterial genera and includes more than 190 species; only in the last three years have been described, thanks to the sequencing of the genome, 10 new species a year (Silby *et al*., 2011; Peix *et al*., 2018). The remarkable metabolic versatility and the incredible potential to adapt to changing environmental conditions allow them to live in a wide variety of environments, including soil, water, sediments, air, animals, plants, fungi, algae, compost, human and animal sources. In the last three years some species have been isolated from extreme environments, such as Antarctica or the Atacama desert, and from contaminated water or soil. Some species are pathogenic on plants, animals and humans.

Their importance has increased over time because in addition to the possible implications in plant or human diseases there has been a growing interest in biotechnological applications. The latter concern products of bacterial metabolism with industrial applications or actual applications of bacterial strains or microbial consortia selected in order to improve a particular metabolic capacity of the bioremediation processes, bioaugmentation, biostimulation, biocontrol. Bioremediation, a technology aimed at restoring the original condition of an environment altered by pollutants thanks to the action of microorganisms capable of neutralizing these substances or transforming them into other non-harmful.

Bioaugmentation means the integration of a microbial community with the addition of specific microorganisms (pure strains, selected microbial consortia) in order to improve a particular metabolic capacity of the process.

Biofertilization and Biostimulation (also known as phytostimulation) thanks to microorganisms that facilitate accessibility or increase the supply of nutrients to the plant or that directly promote its growth through the production of phytohormones.

Biocontrol or biological control, relating to the use of biological entities or molecules derived from living organisms to control the growth of pathogens or plant parasites and to contain their harmful action.

1.1 Plant-associated *Pseudomonas*

Microorganisms may establish a contact with plants through soil, air, or water. After this contact, some of them survive in the host plant and start colonization, being sometimes able to be transferred vertically from the the parent plants to their progeny through seeds and to colonize subsequently the root environment (Johnston-Monje and Raizada, 2011; Links *et al*., 2014). Moreover, a microbiome encompassing beneficial microorganisms is shared between generative organs such as anther pockets and moss sporophytes and their host plant (Bragina *et al*., 2012; Fürnkranz *et al*., 2012).

Pseudomonas associated with plants, as other bacterial genera, occupy different niches and vary for their effects on plants and coexisting microorganisms. Specifically, members of this genus show their ecological capabilities through saprophytic, pathogenic or plant growth-promoting behaviours (Silby *et al*., 2011; Palleroni, 2015). *Pseudomonas* contributes to plant-growth promotion both directly, through phosphate solubilization, the synthesis of growth-stimulating phytohormones or the production of volatile growth stimulants, and indirectly, through protection of plants toward phytopathogens (Haas and Défago, 2005; Silby *et al*., 2011; Wu *et al*., 2012; Bulgarelli *et al*., 2013; Schlaeppi and Bulgarelli, 2015). Biocontrol and plant growth promotion seem to be closely related (Agaras *et al*., 2020).

Pseudomonas has been extensively studied as a model beneficial plant-associated bacterium (Emmert and Handelsman, 1999; Weller *et al*., 2002; Raaijmakers *et al*., 2010; Berg *et al*., 2014) and is characterized by a versatile antibiotic production and high rhizocompetence (Bonsall *et al*., 1997; Loper *et al*., 2012). Antibiotics produced by *Pseudomonas* act both in antibiosis against pathogens and in communication with plants (Ryu *et al*., 2003), contributing to different activities, including biofilm formation, signaling, motility and nutrients uptake (Raaijmakers *et al*., 2010; Raaijmakers and Mazzola, 2012). Along with N-acyl homoserine lactones (N-AHLs), signal molecules of Quorum sensing system, antibiotics mediate *Pseudomonas* intra- and interspecies interactions in the plant-soil interface (Steidle *et al*., 2001; DeAngelis *et al*., 2009; Hartmann and Schikora, 2012; Raaijmakers and Mazzola, 2012). The AHLs, for their part, modify the plant transcriptome and root growth and are involved in the induction of systemic resistance to phytopathogens (von Rad *et al*., 2008; Hartmann and Schikora, 2012; Raaijmakers and Mazzola, 2012).

1.1.1 The *Pseudomonas fluorescens* complex

From a taxonomic point of view, the genus *Pseudomonas* has been divided into two main lineages: *P. aeruginosa* and *P. fluorescens* (Mulet *et al*., 2010; Garrido-Sanz *et al*., 2016). Phylogenetic groups have been delineated according to multilocus sequence analysis (MLSA) using the genes 16S rDNA, *gyrB, rpoB* and *rpoD* (Gomila *et al*., 2015; Lalucat *et al*., 2020; Mulet *et al*., 2010). For instance, the *P. fluorescens* group, including many plant-associated species, is composed of three large groups considered complexes of species: *P. fluorescens*, *P. syringae* and *P. putida* (Mulet *et al*., 2010; Marcelletti and Scortichini, 2014; Garrido-Sanz *et al*., 2016). In *P. fluorescens*, the most complex group, these MLSA-based analysis have shown good concordance with whole-genome sequence-based studies, allowing the identification of eight phylogenomic subgroups: *P. fluorescens*, *P. gessardii*, *P. fragi*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. chlororaphis*, *P. protegens* and *P. corrugata* (Garrido-Sanz *et al*., 2016; Hesse *et al*., 2018; Lalucat *et al*., 2020).

According to Vacheron *et al.n*(2016), within the *P. fluorescens* group, *P. chlororaphis*, *P. protegens* and *P. corrugata* subgroups biocontrol properties are more prevalent, while the *P. fluorescens*, *P. mandelii*, *P. jessenii* and *P. koreensis* subgroups, contain PGP strains capable of phytostimulation with fewer biocontrol properties.

1.1.2 Secondary metabolites production in *Pseudomonas* biocontrol strains

Pseudomonas can produce several secondary metabolites, diffusible and/or volatile, with antibiotic properties, and molecules with insecticidal activity. Many reviews are available on this subject dealing with the biosynthesis, chemistry and biological significance and involvement in biocontrol (Haas and Défago, 2005; Gross and Loper, 2009; Raaijmakers and Mazzola, 2012; Olorunleke *et al*., 2015b; Mishra and Arora, 2018).

A clear role of bioactive compounds was demonstrated for a limited number of compounds in biocontrol of plant diseases (Hofte, 2022): hydrogen cyanide (HCN); 2,4-diacetylphloroglucinol (DAPG); phenazines; pyrrolnitrin; pyoluteorin; 2-hexyl-5-propyl-alkylresorcinol; siderophores; and (cyclic) lipopeptides. The production of these compounds is specific of the phylogenetic groups or subgroups within the *Pseudomonas* genus (Hofte, 2022).

Phenazines are typically produced by *Pseudomonas* strains that belong to *P. aeruginosa*, or the *chlororaphis* and *fluorescens* subgroups within the *P. fluorescens* group (Chincholkar and Thomashow, 2013; Hofte, 2022). DAPG is a polyketide antibiotic produced by *Pseudomonas* strains belonging mainly to the *P. protegens* and *P. corrugata* subgroups and a few isolated strains in other taxonomic groups (Almario *et al*., 2017; Hofte, 2022). Pyoluteorin is a hybrid non-ribosomal peptide synthetase/polyketide synthase metabolite reported exclusively in the genus *Pseudomonas* and in strains belonging to the *P. protegens* subgroup and a few *P. aeruginosa* isolates (Hu *et al*., 2005; Ramette *et al*., 2011, Hofte, 2022). Pyrrolnitrin is a chlorinated phenylpyrrol produced by various bacterial genera and first described in *P. chlororaphis* subsp. *aurantiaca* BL915 (Hill *et al*., 1994; Kirner *et al*., 1998) which cluster is found in *P. protegens* and *P. chlororaphis* subgroup (Hill *et al*., 1994; Kirner *et al*., 1998; Hofte, 2022). 2-hexyl-5-propyl-alkylresorcinol (HPR) is similar in structure to DAPG and was described in *P. chlororaphis* subsp. *aurantiaca* BL915 (Nowak-Thompson *et al*., 2003). The biosynthetic gene cluster is present in various biocontrol strains belonging to the *P. chlororaphis* subgroup (Biessy *et al*., 2019; Calderón *et al*., 2013; Hofte, 2022). Hydrogen cyanide (HCN) is a volatile poisonous compound that inhibits cytochrome c oxidase, produced in the *P. aeruginosa* group and in various subgroups of the *P. fluorescens* group. Potentially, all strains capable of producing DAPG in the *P. corrugata* and *P. protegens* and *P. chlororaphis* subgroups also produce HCN (Hofte, 2022).

Pseudomonas strains produce two types of biosurfactants: rhamnolipids and non-ribosomal lipopeptides. Rhamnolipids are glycolipids typically produced by *P. aeruginosa* with a role in biocontrol (Crouzet *et al*., 2020). Lipopeptides are produced by strains in various *Pseudomonas* groups and subgroups and their biosynthesis, structure and biological activity have been reviewed recently (Geudens and Martins, 2018; Götze and Stallforth, 2020).

Among siderophores, most *Pseudomonas* strains produce the fluorescent siderophore pyoverdine and other siderophores, including pyochelin, achromobactin, pseudomonine, corrugatin (Meyer *et al*., 2000; Cornelis, 2010; Schalk *et al*., 2020).

Pseudomonas biocontrol strains belonging to the *P. protegens* and *P. chlororaphis* subgroup produce an insecticidal toxin named Fit (Flury *et al*., 2017; Hofte, 2022).

1.1.3 Cyclic lipopeptides (CLPs)

Among the secondary metabolites produced by *Pseudomonas*, lipopeptides (LPs), consist of a fatty acid tail attached to a peptide (linear, partially, or fully cyclized), mainly described for *P. syringae* complex of species (Berge *et al*., 2014; Dillon *et al*., 2019). Two types of cyclic lipopeptides (CLPs), respectively from the "mycin" and "peptin" families, and a linear LP (LLP) from the "factin" family, are produced (Ballio *et al*., 1988; Ballio *et al*., 1991; Lindeberg *et al*., 2008; Burch *et al*., 2014). Mycins consist of N-acylated peptides of 9 amino acids enclosed in a loop structure between the first and last residues (Takemoto *et al*., 2003). Peptins, larger and more complex than mycins, possess a peptide chain of 18 to 25 amino acids, cyclized in the C-terminal portion to form a lactone ring (5-8 amino acid residues), and N-acylated by the chain of a fatty acid (Dalla Serra *et al*., 2003a). *Pseudomonas* strains mainly produce cyclic lipopeptides (CLPs). CLPs are generally synthesized in a ribosome-independent manner by megaenzymes called non-ribosomal peptide synthetases (NRPSs), encoded by large biosynthetic gene clusters (Marahiel *et al*., 1997; Gross and Loper 2009). CLPs have been classified according to the length and composition of the fatty acid tail and the number, type, and configuration of the amino acids in the peptide moiety into six groups: viscosin, syringomycin, amphisin, putisolvin, tolaasin, and syringopeptin groups (Gross and Loper, 2009). Regarding CLP biosynthesis regulation, multiple global regulatory mechanisms and specific transcriptional regulators located in proximity of the CLP biosynthesis genes are involved in the regulation of production in *Pseudomonas* spp. (Raaijmakers *et al*., 2010).

The GacA-GacS two component regulatory system consists of a membrane-bound sensor kinase protein (GacS) and a cytoplasmic response regulator protein (GacA) and is pivotal for the regulation of syringomycin and syringopeptin toxins in *P. syringae* pv. syringae B301D, controlling hierarchycally their production (Kitten *et al*., 1998 ; Willis and Kinscherf, 2004, Wang *et al*., 2006). Also putisolvins, massetolide A, viscosin and amphisin biosynthesis are regulated by GacA/GacS system (Koch *et al*., 2002; Dubern *et al.*2005; de Bruijn *et al*., 2007, 2008).

Regarding the role of signals triggering CLP biosynthesis, arbutin, a phenolic β-glycosides, and specific sugars found in plant tissues enhanced expression of the *syrB* gene increasing production of syringomycin (Mo and Gross, 1991). Quorum sensing (QS) has also been investigated for a number of CLP-producing *Pseudomonas* strains. N-acylated homoserine lactones (AHLs) are the most common signal molecules of QS system, which synthase is encoded by *luxI*-type genes. At the critical cell density, when the quorum concentration of

AHLs has been reached, AHLs interact directly with the LuxR-type protein; thus the LuxR-AHL complexes can then bind to a specific promoter DNA sequences called lux-boxes of QS-regulated genes affecting their expression (Venturi, 2006). The AHL-QS system is pivotal in viscosin and putisolvin biosynthesis in the plant pathogen *P. fluorescens* strain 5064 and the saprophytic bacterium *P. putida* strain PCL1445, respectively (Cui *et al*., 2005; Dubern *et al*., 2006). Particular attention has also been given to the LuxR-type transcriptional regulators located up- and downstream to the CLP biosynthesis genes. These regulators seem to be involved in the biosynthesis of syringomycin, syringopeptin, syringafactins, putisolvins, viscosin and massetolide (Lu *et al*., 2002; Wang *et al*., 2006; Berti *et al*., 2007; Dubern *et al*., 2008; De Bruijn and Raaijmakers, 2009a). SalA and SyrF belong to a family of transcriptional activators characterized by high sequence similarities to LuxRs; both are critical for the coregulation of syringomycin and syringopeptin production and that together with the sensor kinase GacS mediate transmission of the plant signal molecules to the *syr-syp* biosynthetic cluster (Wang *et al*., 2006). Dubern and collaborators (2008) identified in *P. putida* PCL1445 a luxR-type regulatory gene *psoR*, located upstream to putisolvin biosynthetic gene cluster and required for its expression. Also viscosin production in *P. fluorescens* SBW25 is regulated by two LuxR-type genes exchangeable among different *Pseudomonas* strains, thereby regulating the biosynthesis of structurally different CLPs (De Bruijn and Raaijmakers, 2009a).

Among the other genes regulating CLP production, *P. putida* DnaK and the *P. fluorescens* serine protease ClpP are involved in putisolvin I and II and massetolide production, respectively (Dubern *et al*., 2005, Raaijmakers *et al*., 2006; De Bruijn and Raaijmakers, 2009b; Raaijmakers *et al*., 2010). Channel-forming toxins form mainly two types of pores in cell membranes: the pore "barrel stave" and the “toroidal pore” (Muller *et al*., 2000; Yang *et al*., 2001). At concentrations above the critical micelle concentration, CLPs can directly solubilize plasma membranes, as demonstrated for erythrocytes (Bender *et al*., 1999). Lipids are likely involved in the bilayer process of formation of the pore, promoting the CLP transfer between the inner and the outer membrane layer (Hutchinson, *et al*., 1995; Dalla Serra *et al*., 1999; Menestrina *et al.*, 2003; Scaloni *et al*., 2004; Coraiola *et al.*, 2006). The amphiphilic properties of CLPs are determined by the polar peptide head with the lipophilic fatty acid tail, which can lower surface tension, affecting the integrity of cellular membranes. This latter effect is supposed to determine the interactions with other organisms and in general to be involved in plant pathogenicity and wide-spectrum inhibitory activity (Gross and Loper, 2009). The mechanism of action of the CLPs syringomicin and syringopeptins produced by the plant- pathogen *Pseudomonas syringae*, has been widely explored. The nonapeptide syringomycin targets primarily plasma membrane of host cells thanks to its amphipathic structure, promoting its insertion into the lipid bilayers of

membranes and causing pores; as a consenquence of channel formation, transmembrane fluxes of cytotoxic cations K^+, H^+ and Ca^{2+} increase (Hutchison *et al*., 1995). This cation trasport generates an electrochemical gradient with consequent collapse of the pH gradient of the plasma membrane, resulting in the acidification of the cytoplasm, and the induction of kinase-mediated phosphorylation of membrane proteins (Bidwai and Takemoto, 1987; Bender *et al*., 1999). Moreover, the bacterium benefits from pore formation for the systematic release of nutrients into the intercellular spaces of host tissues (Hutchinson *et al*., 1995) and the alkalization of intercellular fluids, providing a more suitable environment growth (Che *et al*., 1992). A pivotal influence in channel formation by syringomycin is certainly given by lipid bilayer composition; as an example, sterols, particularly ergosterol, promote the binding of syringomycin to cells (Julmanop *et al*., 1993; Taguchi *et al*., 1994). Similarly to syringomycin, syringopeptin appears to induce an H^+-K^+ exchange response in plant cells (Mott and Takemoto, 1989) and to cause electrolyte leakage of plant cells (Iacobellis *et al*., 1992), although many studies reported its increased bioactivity compared to syringomicin (Iacobellis *et al*., 1992; Lavermicocca *et al*., 1997). Analogously with syringomycin, syringopeptins form stable membrane pores modulated by host membrane lipids and particularly sphingolipids and sterol (Malev *et al*., 2002; Takemoto *et al*., 2003; Kaulin *et al*., 2005; Bensaci *et al*., 2011). It is not known precisely how the syringopeptins and syringomycin interact with membrane lipids to form channels (Malev *et al*., 2002), although a role of the electrostatic and hydrophobic interactions between syringopeptins and membrane lipids has been proposed (Scaloni *et al*., 2004; Szabo *et al*., 2004).

1.1.4 The *Pseudomonas corrugata* subgroup

Within the *P. fluorescens* complex, the *P. corrugata* subgroup is composed of six named species: *P. corrugata*, *P. mediterranea*, *P. thivervalensis*, *P. kilonensis*, *P. brassicacearum* (Garrido-Sanz *et al*., 2016; Lalucat *et al*., 2020) and the recently reclassified *P. ogarae* (Garrido-Sanz *et al*., 2021), albeit the number of species of this subgroup is probably underestimated (Garrido-Sanz *et al*., 2016; Garrido-Sanz *et al*., 2017; Nelkner *et al*., 2019; Gislason and de Kievit, 2020). The *P. corrugata* subgroup corresponds to the 'bcm clade', encompassing both pathogenic species and biocontrol agents (Hesse *et al*., 2018; Melnyk *et al*., 2019). *P. corrugata* and *P. mediterranea* are closely related species mainly isolated from the root environment of various host plants and reported as opportunistic pathogens in tomato (Catara, 2007). Some *P. corrugata* strains have been patented for biocontrol and, along with *P.*

mediterranea, elucidated for their activity in many pathosystems (Catara, 2007). *P. corrugata* produces the antimicrobial and phytotoxic CLPs corpeptin A and corpeptin B and cormycin, while in *P. mediterranea* medipeptins have been described (Emanuele *et al*., 1998; Scaloni *et al*., 2004; Strano *et al*., 2015; Zhou *et al*., 2021). Both species produce also volatile poisonous compounds such as HCN (Strano *et al*., 2017).

Among the most characterized beneficial isolates within the *P. corrugata* subgroup, *P. kilonensis* F113, *P. brassicacearum* DF41 and *Pseudomonas* sp. SH-C52 deserve attention.

Pseudomonas kilonensis F113 is a PGPR strain isolated from sugar beet rhizosphere (Shanahan *et al*., 1992; Almario *et al*., 2017). It is known for its biocontrol activity against a wide range of plant pathogenic microorganisms, including *Pythium* sp. (Dunne *et al*., 1998); *P. carotovorum* (Cronin *et al*., 1997a), *F. oxysporum* (Barahona *et al*., 2011). F113 promotes also the growth and development of plants, especially maize (Vacheron *et al.*2016). The production of DAPG is pitovatal in biocontrol activity of this strain (Redondo-Nieto *et al*., 2013). Moreover, the ability of the F113 strain to produce DAPG has been associated with biocontrol of the potato cyst nematode *Globodera rostochiensis*, an important pest of this crop (Cronin *et al*., 1997a).

P. brassicacearum DF41 is an isolate of *Brassica napus* roots capable of suppressing *B. napus* stem rot caused by *Sclerotinia sclerotiorum*, by producing various compounds with antimicrobial activity (hydrocyanic acid, proteases, alginates, lipopeptides, etc..) regulated by a functional GacS/GacA system (Savchuk and Fernando, 2004; Berry *et al*., 2010). In this species, strains that produce lipopeptides do not produce DAPG (Gislason and de Kievit, 2020).

Pseudomonas sp. SH-C52 has been isolated in soils naturally suppressive toward the fungal pathogen *R. solani* (Mendes *et al*., 2011); it produces the cyclic lipopeptides thanapeptin and thanamycin and the 2-amino-acid cyclocarbamate brabantamide, with demonstrated activity against Gram-positive bacteria and Oomycetes (Schmidt *et al*., 2014; Van Der Voort *et al*., 2015; Girard *et al*., 2020).

1.1.5 *Pseudomonas corrugata* as a model bacterium

P. corrugata has been either described as causal agent of tomato pith necrosis however also as biocontrol agent in a number of pathosystems. *P. corrugata* metabolic versatility makes it attractive for the production of commercial biomolecules and bioremediation (Catara, 2007; Trantas *et al*., 2015).

It produces the antimicrobial and phytotoxic cyclic lipopeptides (CLPs) cormycin A, corpeptin A, and corpeptin B (Emanuele *et al*., 1998; Scaloni *et al*., 2004; Licciardello *et al*., 2012). The production of these secondary metabolites seems regulated at high cellular density with a "quorum sensing" mechanism (Licciardello *et al.*, 2007; 2012; Strano *et al*., 2015). For several years, researchers at the University of Catania and the Science and Technology Park of Sicily have been investigating the biomolecules produced by the two bacterial species in the context of several research projects between plant pathology and applied biotechnology. In this framework the team answered to different questions that make *P. corrugata* a useful model to study the LuxR 'solo' in a bacterium that also contain a complete Quorum sensing (QS) system. Different questions have been already answered:

1) *P. corrugata* produces AHLs

Licciardello *et al.* (2007) demonstrated, for the first time, testing a representative collection of strains that *P. corrugata* possess a very well conserved AHL-QS system involved in the regulation of microbiological and phytopathogenic traits of this bacterium. The authors demonstrated that despite their heterogeneous phenotypic and genotypic characteristics, all the strains produce comparable levels of the same AHLs: C6-AHL, C6-3-oxo-AHL and C8-AHL, with C6-AHL apparently being the most abundantly signal molecule produced.

2) The PcoI/R AHL-QS system

The screening of a genomic library of a strain of *P. corrugata* (CFBP 5454) allowed the identification in a cosmid insert of the genetic determinants encoding for an AHL-QS system designated *pcoI* and *pcoR*, encoding for proteins homologous to members of LuxI and LuxR-family proteins, respectively (Licciardello *et al*., 2007). Analysis of the *pcoI* promoter region, revealed a 20 bp imperfect palindrome sequence located 67 bp upstream of the putative translational start site, having high similarity to lux-box-like elements of other *Pseudomonas* spp. and *Vibrio fischeri*. These inverted repeats represent binding sites of LuxR homologs, thus the presence of this putative regulatory element suggested that pcoI is subject to activation by a regulator of the LuxR family. The PcoI putative protein showed the highest similarity to the autoinducer synthase; whereas analysis of the PcoR putative protein revealed that contains an autoinducer binding domain near its amino terminus and a DNA binding domain with a helix–turn–helix motif characteristic of the LuxR-family near its carboxyl terminus.

3) The transcriptional regulator RfiA

In further studies, in order to characterize the DNA region linked to the AHL-QS locus, Licciardello *et al.* (2009) identified located at the right border of the *pcoI* gene, a regulatory

gene *rfiA*. The putative RfiA protein showed high homology with the SalA and SyrF regulators in *P. syringae* pv. syringae B301D (Lu *et al*., 2002) and with the RfiA protein of *P. corrugata* 2140R. The latter is a well known biocontrol strain and the acronym of regulator of fungal inhibition was retained also for CFBP 5454 strain.

RfiA contains a DNA binding domain with a helix-turn-helix (HTH) motif characteristic of the LuxR-family near its carboxy terminus, consisting of four helix bundles in which the central helices form the HTH motif (Fuqua and Greenberg 2002). However, unlike the previous described LuxR homologous, PcoR (Licciardello *et al*., 2007), RfiA does not contain the five highly conserved amino acids (Trp59, Tyr69, Asp79, Pro80, and Gly121) in the N-terminus characteristic of the autoinducer binding subfamily of the QS-LuxR-family proteins. The authors showed that in *P. corrugata*, the newly characterized *rfiA* forms an operon with the *pcoI* AHL synthase gene and it was the first report of a *luxI* homolog cotranscribed with a regulatory gene. As consequence the transposon inserted in the pcoI gene (Licciardello *et al*., 2007) had a polar effect on the cotranscribed *rfiA* gene.

4) Mutational phenotypes of the QS-RfiA system

The inactivation of *pcoI* did not affect pathogenicity when inoculated in tomato, and HR in non-host plant, whereas pcoR mutant determined reduced tomato pith necrosis symptoms, although the population sizes remained similar to that of the wild-type strain. The pivotal importance of RfiA in the development of the disease symptoms in tomato plants was demonstrated by the reduction of lesions and the absence of necrosis within the stem pith tissues inoculated with the *rfiA* mutant compared with those inoculated with the wild-type strain CFBP 5454. Thus, these results together with those one obtained in the previous study the GL1 mutant (the double *pcoI*/*rfiA* mutant) was as virulent in planta as the parent strain (Licciardello *et al*., 2007), allowed to conclude that in *P. corrugata* there are two conditions in which the bacterium is virulent toward tomato: presence of PcoR in the absence of AHL or with RfiA (Licciardello *et al*., 2009).

In conclusion the authors proposed a working model of how the PcoI/PcoR/RfiA system could be involved in virulence, in which either QS regulates the production of RfiA or PcoR regulates virulence independently of AHL. Therefore, it was hypothesized that PcoR acts directly or indirectly in the absence of AHL as an activator or repressor of different virulence associated genes.

5) Genes regulated by QS-RfiA

Different studies addressed this topic two involved gene knock-out and one the RNAseq analysis of the PcoR and RfiA mutant strains.

Downstream of rfiA three ORFs designated *pcoA*, *pcoB* and *pcoC*, constitute an operon (*pcoABC*) that encodes three components of a tripartite resistance nodulation cell division (RND) transporter system (Licciardello *et al*., 2009). The inactivation led only to the partial loss of antimicrobial activity against and none visible influence on virulence. Licciardello *et al.*(2009) demonstrated that the *pcoABC* operon is under positive regulation by RfiA and, indirectly, by the PcoI/R system.

Part of the biosynthetic cluster responsible for corpeptin production, designated as crpCDE including genes transcriptionally joined coding for an NRPS and an ABC efflux system was described by Strano *et al.* (2015). These genes, and hence corpeptins, greatly contributed to *P. corrugata* virulence and plant interaction.

Very interesting also, the results of leaf inoculations of *N. benthamiana*. When strain CFBP 5454 is infiltrated into tobacco mesophyll, it induces the collapse and necrosis of the leaf tissue and, as in an HR, its population declines rapidly. When tested for HR on tobacco the mutant strains that did not produce corpeptins only caused chlorosis, and the population titre of the *crpCDE* mutant (PCONRPS strains) was invariable over a 4-day monitoring period. Wild-type strain CFBP 5454 induces the collapse and necrosis of the leaf tissue and, as in an HR, its population declines rapidly. This result, and the fact that no T3SS was found in the *P. corrugata* CFBP 5454 genome (Licciardello *et al.*, 2014), suggested that corpeptins play a role in the elicitation of HR in *N. benthamiana*. This is in accordance with recent studies, which showed that CLP may induce systemic resistance and which, taken together, suggest that CLPs constitute a novel class of microbial-associated molecular patterns (MAMPs) (reviewed in Raaijmakers *et al*., 2010).

On the regulation point of view Strano *et al.* (2015) also showed that the transcriptional regulators PcoR and RfiA play a pivotal role in the expression of *crpC* and *crpD* genes.

A transcriptional approach was undertaken to identify the specific role of the PcoR and RfiA transcriptional regulators by RNA-seq-based transcriptional analysis of the wild-type (Wt) strain CFBP 5454 in comparison with GL2 (*pcoR* mutant) and GLRFIA (*rfiA* mutant) was performed in cultural conditions favoring CLP production. Genes linked to the biosynthesis of CLPs and alginate were positively controlled by both PcoR and RfiA. Blast homology analysis showed that 19 genes in a large CLP biosynthetic cluster involved in the production of three antimicrobial peptides, which span approximately 3.5% of the genome, are strongly over-expressed in the wild-type strain. Thus, PcoR and RfiA function mainly as activators in the

production of bioactive CLPs, in agreement with phenotype analysis of mutant strains. RNA-seq also revealed that almost all the genes in the structural/biosynthetic cluster of alginate exopolysaccharide (EPS) are under the control of the PcoR–RfiA regulon, as supported by the 10-fold reduction in total EPS yield isolated in both mutant strains in comparison to the parent strain.

6) Genome analysis for other regulatory genes

Recently the draft genome sequences of a few *P. corrugata* strains were obtained opening the opportunity to dissect the mechanisms that *P. corrugata* employs to cause disease, prevent disease caused by other pathogens, and to mine their genomes for genes that encode proteins involved in commercially important chemical pathways (Licciardello *et al*., 2014; Trantas *et al*., 2015). Genome mining of the genome of CFBP 5454 allowed to find a LuxR solo that was also found in the other sequenced strains (unpublished data).

Based on previous knowledge already developed for strain CFBP 5454 at the Di3A (University of Catania), this thesis project aimed at sheding light on the role of this regulator in the biology of the bacterium and if it is involved in modulation of genes regulated by PcoI/R and RfiA.

1.2 Citrus Mal secco disease

Lemon (*Citrus limon*) is the third most important species within the genus *Citrus*, with a global production, together with limes, amounting to 20.5 million tons. The Mediterranean Basin accounts for approximately half of the global lemon production. Turkey is the major producing country (950,000 tons per year), followed by Spain (884,900 tons) and Italy (446,000 tons) (FAO, 2021). Most of the countries of the Mediterranean Basin are affected by Mal secco disease (MSD), a detrimental tracheomycosis caused by the mitosporic fungus *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp & Verkley (formerly: *Phoma tracheiphila* (Petri) Kantschaveli & Gikashvili) (EFSA PLH, 2014). Although several genera in the Rutaceae, such as *Citrus*, *Poncirus*, *Severinia* and their hybrids can serve as hosts, the disease is a major constraint for successful lemon production in the areas where the fungal pathogen is present and many aspects have been reviewed (Migheli *et al.*, 2009; Nigro *et al.*, 2011; Catara and Catara, 2019). Morocco and Portugal, while still free of MSD, have suitable conditions for its

development (Krasnov *et al.*, 2022). Up to the 1950s, MSD destroyed no less than 12,000 hectares of lemon groves in Sicily and 20,000 lemon plants in Mersin Province, Turkey. The average annual yield loss was 12.3% in Turkey and 50–60% in Greece (Nigro *et al.*, 2011). In the last 30 years, the lemon growing area and production in Sicily have decreased by 45% and 41%, respectively (Catara and Catara, 2019).

1.2.1 The causal agent: *Plenodomus tracheiphilus*

P. tracheiphilus is included on the A2 quarantine pest lists of the European and Mediterranean Plant Protection Organization (EPPO), and the A1 lists of several global regional plant protection organizations (EPPO Global Database, https://gd.eppo.int/taxon/DEUTTR). According to the Plant Health panel of EFSA, it has been classified as a regulated non-quarantine pathogen in Europe (EFSA PLH Panel, 2014).

The fungus produces a hyphaenchyma on the outermost cortical elements of infected young branches on which globular black pycnidia differentiate, generally arranged in series or in confluent groups. Initially, pycnidia are astomata, but when mature they have a neck with an ostiolar opening at the end. Inside pycnidia, mother cells give rise to the production of unicellular, hyaline, ellipsoidal in shape, with rounded ends pycnoconidia, which emerge massively from the ostiole in the form of cirri when ripe and are dispersed following laceration of the fruiting body (Catara and Catara, 2019). Other organs of agamic multiplication of hyphal origin are the phialoconidia and thalloconidia. The hyaline and unicellular phialoconidia derive from specialized fertile cells (the phialides), which differentiate on the hyphae, and are abundantly produced on infected plant residues and, in conditions of high humidity, on exposed wood due to wounds or cuts and cracks in the bark and on leaf scars. The thalloconidia, unicellular, irregular in shape and size, are produced by the hyphae in the xylematic vessels and are conveyed by the ascending lymph.

In vitro, the fungus grows at temperature between 5 °C and 30 °C with optimal values of 20-22 °C. Pycnidia prefer temperatures between 12 °C and 24 °C and are more numerous at 15 °C, although they reach a greater size and produce more germinable conidia at 10 °C. The mycelium produces various pigments, including two red anthrachinone derivatives, helmintosporin and cinodontin, and a yellow one, chrysophanol which gives the substrate the characteristic red-orange colour (Quilico *et al.*, 1952; Ballio *et al.*, 1979). The colour of the wood is instead due to the production of gums in the tissues invaded by the fungus.

Goidanich and Ruggeri (1948) considered the fungus as a monotypic entity which, in relation to various factors, could present variations in colour and other morphological characteristics. In particular, the ability to produce red pigment, albeit in variable quantities, would be a constant feature of the fungus. In subsequent studies, the designations "chromogenic" and "non-chromogenic" isolates progressively replaced those of the "DPR" (colonies with dematiaceous mycelium, producing pycnidia and red pigment) and "R" (colonies without dematiaceous mycelium, producing abundant red pigment but no pycnidia) races and the "DP" (colonies with dematiaceous mycelium, producing pycnidia but no red pigment) race. Chromogenic strains, as observed by various authors, occur more frequently than non-chromogenic strains (Magnano di San Lio and Perrotta, 1986). The chromogenic strains of *P. tracheiphilus* also show a high phenotypic instability. When cultured on agar substrates, they frequently form variant sectors (saltations), which differ from the rest of the colony in terms of colouration, speed of development and type of growth of the mycelium. Non-chromogenic strains have shown reduced virulence in various studies (Graniti, 1969; Magnano di San Lio *et al.*, 1986).

1.2.2 Infection process and symptomatology

Infections with *P. tracheiphilus* occur through wounds on leaves and woody organs, and through leaf scars caused by natural or traumatic phylloptosis, but not through stomatal openings (D'Anna *et al.*, 1986). Any injury exposing the vascular bundles of the host constitutes a possible way to entry for the pathogen (Bassi *et al.*, 1980). Small and punctiform leaf injuries remain receptive for 4-5 days and up to 10 days if they are extensive. Wounds caused by traumatic leaf detachment remain receptive for more than 16 days at 15 °C, while those caused by natural phylloptosis are less receptive (D'Anna *et al.*, 1986).

Pycnoconidia that reach the inside of wounds germinate quickly, finding ideal relative humidity conditions, while those that remain outside germinate only at 100% UR, producing abundant mycelial hyphae that penetrate into the host through wounds and initiate the infection process in the presence of suitable humidity conditions (Catara and Catara, 2019). Electron microscope investigations of the infection process taking place in wounded leaves of sour orange showed that *P. tracheiphilus* hyphae develop intercellularly, through the mesophyll, and then localize in the tracheids (Perrotta *et al.*, 1976). Through xylem punctuations or direct attack of the primary wall, the hyphae pass from one vessel to another, which show dark walls covered with an amorphous gummy substance, not necessarily associated with the presence of the fungus (Bassi *et al.*, 1980; Perrotta *et al.*, 1980). Because of such active movement, the fungus colonizes

several xylematic rings (Magnano di San Lio and Perrotta, 1979; Perrotta *et al*., 1980). In xylem vessels, *P. tracheiphilus* remains confined as long as the tissues stay alive and differentiates hyphal-type spores (Catara and Catara, 2019), which are passively transported by the ascendant sap, rapidly spreading the infection in the wood. Therefore, acropetal translocation of the pathogen seems very rapid and occurs prior to symptom expression.

The disease is characterised by a sequence of external and internal symptoms whose distribution and severity depend on xylem colonization and show a seasonal fluctuation (Migheli *et al.*, 2009; Nigro *et al.*, 2011; Catara and Catara, 2019; Batuman *et al.*, 2020).

1.2.3 Host-fungus interactions

Different histo-cytological changes were observed in susceptible and resistant citrus plants. In the xylem of Monachello lemon (resistant), scar tissue was observed around the infection point, which circumscribed the necrotic areas, but gradually the plant had the ability to regenerated new xylem. In susceptible plants, on the other hand, hyperplastic reactions are observed, referable to repair mechanisms, which tend to extend also in a radial sense as the colonisation of the tissues by the pathogen progresses (Perrotta *et al.*, 1979a; Bassi *et al.*, 1980; Perrotta *et al.*, 1981).

An *in planta* and *in vitro* study (Reverberi *et al.*, 2007) on the role of the oxidative stress in the lemon-*P. tracheiphilus* interaction, was conducted using the cvs Monachello, Interdonato, and Femminello, reported as resistant, partially resistant, and susceptible to MSD infections, respectively. The infiltration of *P. tracheiphilus* extracellular proteins into the leaves induced a higher lipoperoxide formation in partially resistant and susceptible citrus varieties, with resistant Monachello reacting earlier. Results from *in vitro* experiments indicated that, when lyophilized twigs and leaves of cv. Femminello were added to the fungal growth medium, the pathogen produced a higher quantity of hydrolytic enzymes, such as polygalacturonase and laccase.

In the leaves of citrus species susceptible to MSD, the extensive clogging of veins, altering water transport inside the xylem vessels (Raimondo *et al.*, 2007), and chloroplast damage, inhibiting the photosynthetic fixation of carbon (Nachmias *et al.*, 1980) have also been reported among the physiological modifications induced by the pathogen. The inhibition of the photosynthetic fixation of carbon has been attributed to the activity of two toxic glycoproteins of 93 KDa and 60 KDa (called Pt60), belonging to the malseccin complex, cabable of inducing chlorosis and necrosis when injected into rough lemon leaves (Fogliano *et al.*, 1998) and to

cause structural changes in protoplasts (Sesto *et al.*, 1990; Gentile *et al.*, 1992), reproducing partially or totally MSD symptoms.

A recent study, carried out with a transcriptomic approach, consolidated previous observations regarding the effect of the pathogen on plant photosynthetic activity; a deep transcriptional reprogramming was in fact observed in rough lemon leaves infected with *P. tracheiphilus*, with a significant down-regulation of genes involved in harvesting and the photosynthetic electron flow (Russo *et al.*, 2021). On the other side, transcriptional analysis of the fungus revealed a multifaceted strategy to infect citrus hosts, involving the destruction of plant defensive metabolites and the optimization of fungus development and pathogenesis (Sicilia *et al.*, 2022).

1.2.4 Resistance to *P. tracheiphilus*

The most effective means to control MSD on a large scale would be to use resistant cultivars or clones and to graft them onto resistant rootstocks (Migheli *et al.*, 2009). In this regard, the search for genetic resistance toward MSD is mainly limited by the long time required to obtain effective and reliable results.

As an example, *in vitro* selection of Femminello lemon calli with *P. tracheiphilus* toxins yielded a cell line named Femminello-S, from which plants low susceptible to the toxins were regenerated from protoplasts, and transferred to the field (Gentile *et al.*, 1992a; 1993). However, despite the promising results, no commercial lemon cultivar were obtained with this brreding method and no information on agronomic and fruit quality traits has been reported (Migheli *et al.*, 2009).

Other approaches, based on innovative biotechnological methods have been pursued over the years to obtain lines resistant to MSD. Somatic hybridization via symmetric and asymmetric protoplast fusion represented a primary strategy for obtaining improved disease-resistant varieties of lemon (Nigro *et al.*, 2011).

A somatic hybrid of Valencia sweet orange with Femminello lemon and two Femminello cybrids was found to be more tolerant than Femminello but less so than the tolerant Monachello variety (Tusa, 1999; Tusa *et al.*, 2000). Interesting results has been achieved also with somatic hybridization of protoplasts of Femminello siracusano lemon (susceptible) with those of Murcott mandarin (less susceptible), that allowed to obtain toxin-tolerant lemon hybrids (Deng *et al.*, 1995).

One more attempt carried out to avoid the limitations of conventional breeding was represented by genetic transformation of commercial varieties, which enabled the obtainment of many varieties and rootstocks with enhanced agronomic traits. As an example, the introduction of the chitinase gene *chit42* from *Trichoderma harzianum* in Femminello siracusano lemon plants through a *Rhizobium*-mediated transformation approach increased the activity of chitinases and glucanases. Lemon transgenic clones showed a reduced susceptibility toward MSD, comparable to that of Monachello and higher to the mother plant (Gentile *et al.*, 2000).

To date, only a few studies were focused on deciphering the genetic basis of resistance/tolerance of lemon lines toward MSD. Reforgiato Recupero *et al.* (1997) analyzed the genetic basis of the resistance to MSD with both field phenotyping and PR proteins production (chitinase) evaluation on *Citrus* species and *Poncirus trifoliata*, identifying three alternative genes (A, B, and C) able to determine the dominant tolerant phenotype and a fourth gene, gene D, that in the condition of dominance was able to determine susceptibility by nullifying allele B action.

Among these promising sequencing approaches, a recent RNA-seq analysis of *Pt*-infected leaves of *C. jambhiri* (rough lemon) led to the identification of promising candidate genes RPM1 and BIR2, that resulted down- and up-regulated respectively in the inoculated sample (Russo *et al.*, 2021).

1.2.5 Control strategies

There are currently no effective methods to control MSD. Where the disease is present, in order to contain infection it is essential to prune (and burn) withered shoots and to remove suckers, which will then reduce the inoculum. Authorised fungicides (including copper-based) applications are recommended in the nursery or orchards, during the rainy season, after hailstorms, frost damage and pruning. Several compounds have been tested in terms of efficacy toward MSD over the last century and the use of many of them are no longer authorized against MSD (Nigro *et al.*, 2011).

Recently, new approaches have been investigated such as the plant defences inducer biocomplex Dentamet® and a super absorbent polymer containing copper (SAP-Cu) have been successfully tested as carriers of copper-based antifungal compounds (Oliveri *et al.*, 2022; El Boumlasy *et al.*, 2022). Both strategies seem to reduce symptoms severity and disease progression, inhibiting the viability of *P. tracheiphilus* in lemon twigs. Studies on the biological control of MSD have focused mainly on resistance induction and microbial antagonism.

First attempts of resistance induction were based on cross-protection with fungi taxonomically different to the pathogen, non-pathogenic toward citrus, taking part in a pathosystem similar to *P. tracheiphilus*-*Citrus* pathosystem. According to results, MSD symptoms were less severe and delayed if sour orange seedlings were pre-inoculated with *Verticillium dahliae* below the site of *P. tracheiphilus* inoculation (Grasso and Tirrò, 1982). Other attempts, based on pre-inoculation of citrus plants with hypovirulent strains of the pathogen, naturally selected or produced by mutation, significantly reduced the severity of symptoms caused by virulent *Pt* isolates, although with variable results depending on temperature (Lima *et al.*, 1994a). In some cases, the effect of cross-inoculation has been lost in the field (Paradies *et al.*, 1985; Ippolito *et al.*, 1988).

The occurrence in the soil and rhizosphere of fungal microorganisms antagonistic to *P. tracheiphilus* was reported by different authors (Nigro *et al.*, 2011); *Gliocladium roseum* and *G. virens*, isolated from soil in Sicilian citrus orchards, showed the greatest *in vitro* antagonistic activity against the pathogen (Leonardi *et al*., 1990). Several bacterial isolates were obtained from citrus plants and tested for their antagonistic activity against *P. tracheiphilus* (Nigro *et al.*, 2011).

In a pioneering study by Lima *et al.* (1994), bacterial isolates obtained from woody tissues of lemon and sour orange plants were tested *in vitro* against the pathogen. The nine most effective isolates were taxonomically identified and inoculated in the stem of sour orange seedlings 15 days before the inoculation with the pathogen. Plants treated with three isolates of *Bacillus subtilis* and one isolate of *Pseudomonas fluorescens* showed a reduction of symptoms severity. MSD symptoms severity has been observed to correlate negatively with the endophytic population titre of these bacterial strains. Subsequent studies focused on the use of strains belonging to *Pseudomonas fluorescens* complex of species.

P. corrugata PVCT 4.3t and *P. mediterranea* PVCT 3C, selected for their efficacy *in vitro* (Coco *et al.*, 2003), were able to colonize sour orange xylem and to reduce fungal spread along the stem, above all when pre-inoculated by stem injection (Coco *et al.*, 2004). The same strains were effective when used to pre-treat lemon plants by leaf-spraying, with results, in the case of *P. mediterranea* PVCT 3C, comparable to those obtained with acibenzolar-S-methyl (Bion 50WG), an antifungal compound stimulating natural defences of plants (Grasso *et al.*, 2008).

More recently, *Bacillus* spp. strains and commercial microbiological products based on *Bacillus* strains were tested *in vitro* toward the pathogen and *in vivo* in susceptible citrus rootstocks artificially inoculated with *Pt*.

Kalai-Grami *et al.*(2014) isolated bacterial endophytes from citrus leaves with potential anti-*Pt* activity. Isolate TEB1, identified as *Bacillus amiloliquefaciens*, showed a reduction of disease

severity and disease incidence when bacterial treatments were carried out by soil drenching near the root zone. Molecular analysis of this isolate revealed the presence of genes responsible of the biosynthesis of the lipopeptides fengycin and iturin, putatively involved in biocontrol activity (Kalai-Grami *et al.*, 2014).

Aiello *et al.* (2022) evaluated the effectiveness *in vitro* and *in vivo* upon artificial inoculation of biological commercial products based on *B. amyloliquefaciens* strains against different isolates of *P. tracheiphilus*. Overall, bacterial cells, filtrates, and volatile organic compounds of all tested strains were able to reduce the growth of the pathogen *in vitro*, with variable results according to the pathogen isolate. Biological control agents were applied by leaf-spraying in *C. volkameriana* seedlings twice before the inoculation through leaves with a virulent isolate of the pathogen and evaluated for their ability to colonize endophytically citrus woody stems. Standard fungicides used on citrus fruits until recently were applied for comparison. According to results, biological products were averagely less effective than standard fungicides, although *Bacillus* strains, re-isolated from treated plants, efficiently colonized citrus plants endosphere.

1.3 Aim of the thesis

This PhD thesis was developed in the framework of the project S.I.R.P.A. (Sviluppo di Induttori di Resistenza a Patogeni vascolari degli Agrumi, Misura 1.1.5 del PO FESR Sicilia 2014/2020, CUP: G68I18000680007). Main aim of the project was to develop defense measure for citrus vascular pathogens, namely *Plenodomus tracheiphilus*, causal agent of Mal secco Disease (MSD) of citrus, and Citrus Tristeza Virus (CTV). In particular, concerning MSD, the project design was based on the induction of resistance and antagonistic activity by biocontrol bacteria.

Di3A (Dipartimento di Agricoltura, Alimentazione e Ambiente) department was engaged with Agrobiotech Soc. coop. in WP2 - Development of a microbial preparation against MSD. The activities, carried out in the laboratory of Phytopathological Bacteriology and Phytosanitary Biotechnology provided for the University partner were OR 2.1 Mass production of bacterial endophyte strains; OR 2.2 Preparation of experimental preparations; OR 2.3 Validation of microbial preparations; OR 2.4 Analysis of mechanisms of action. In particular, the research activities addressed in this doctoral project are:

1) Effect of *P. tracheiphilus* infections on citrus microbiome
2) Selection and evaluation of bacterial strains for MSD control
3) Genome analysis of effective bacterial strains
4) Analysis of the mechanisms of action in the model strain CFBP 5454

1.3.1 The microbiome analysis in S.I.R.P.A. project

Microbial community associated with plants, inside, on the surfaces or in the areas of influence of the plant itself, is very large and extends from prokaryotes to eukaryotes that, together with viruses and viroids, can have beneficial, neutral or adverse effects on the plant (Raaijmakers *et al*., 2009; Vandenkoornhuyse *et al*., 2015).

Recent high-throughput sequencing (HTS) nucleic acid sequencing techniques also known as Next Generation Sequencing (NGS) techniques have changed the vision of the plant as an independent entity in the latest definition of holobiont or "super organism" integrated with the microorganisms that inhabit it (Margulis, 1991; Lundberg *et al*., 2013; Vandenkoornhuyse *et al*., 2015). More generally, the microbiome is then identified as the set of microorganisms, their genomes and environmental interactions that they establish in a given environment (also called 'theater of activity') (Whipps *et al*., 1988).

The microbiome is involved in multiple functions of the plant, from nutrition to resistance to biotic and abiotic factors. So the productivity, vigor and resistance of the plant are a direct consequence of the genetic makeup of the plant itself, but also that of its microbiome (Berg *et al*., 2014; Compant *et al*., 2019; Wagner, 2022). The methods applicable for the study of the microbiome in the agro-environmental field are many and allow to obtain a wide range of information on the micro-organisms that compose it and their functions. In relation to the wide variability and the specificity of the microbial component of the plant, the scholars dividing the microbiome of the plant into several general components, separating the hypogeal portion, that is located below the ground, from the epigeal or aerial portion of the plant, or that in direct contact with the atmosphere, thus allowing us to distinguish: the phyllosphere, the rhizosphere and the endosphere (Compant *et al*., 2010; Liu *et al*., 2020) (Figure 1).

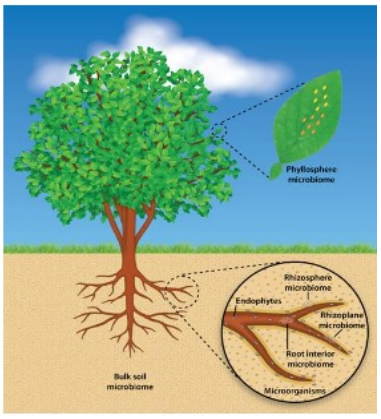

Figure 1: Model of the microbiome. The plant microbiome consists of microorganisms (e.g. bacteria and fungi) in the phylloosphere (portions of the plant outside the soil); the rhizosphere (the area surrounding the plant root), the rhizoplan (the root-soil interface) and the endosphere (internal compartments of the plant). (Source: Wang et al. (2015), *Journal of Citrus Pathology*, *2*, 4–6. doi:10.5070/c421027940.

The microbiome of citrus remains largely unexplored. The long production cycle of citrus leads to fluctuations in the composition of microbial communities, which adapts to environmental change and plant physiology over time. Worldwide, more than 140 countries produce citrus fruits, with most grown in subtropical regions. Several abiotic factors (worldwide distribution of citrus and wide variations in soil, temperature, rainfall, cultivated varieties, cultivation techniques etc...) and biotics (e.g. Huanglongbing disease) play a very important role in the composition of microbial communities. For these reasons, in 2015 the International Consortium for the Citrus Microbiome carried out an extensive study of the structural and functional composition of the citrus rhizosphere microbiome in 23 locations in the main producing countries, including Italy (Wang *et al*., 2015).

The main challenges for industry research:

• defining the profile of the citrus microbiome;

• the study of the effect of age, rootstocks and scions;

• the study of the effect of biotic and abiotic stress;

• the study of the effect of citrus grove management;

• the functional characterisation of the citrus microbiome;

• the engineering of the citrus microbiome for the improvement of productivity and plant health.

Studies on the microbiome of citrus, as with other tree plants, are far fewer in number than those on herbaceous crops or model plants such as *Arabidopsis* (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Schlaeppi *et al*., 2014). For the most part they have been realized on the microbiome of the root system and endosphere, in relation to the health of the plant compared to Huanglonbing infections (HLB)(reviewed in Zhang *et al*., 2021). In the initial stages of

infection of the pathogen *Candidatus* Liberibacter asiaticus, a reduction of certain groups of microorganisms is observed, followed by an increase in the groups of microorganisms with possible beneficial activity (a mechanism exerted by the plant that is called 'cry for help'), while in the advanced stage take over the parasitic and saprophytic microorganisms and wood degraders, responsible for a disbiosis (Rudrappa *et al*., 2008; Lopez-Raez *et al*., 2011; Neal *et al*., 2012; Ginnan *et al*., 2020). Recently, also, a study on foot rot from *Phythophtora* spp. showed a decrease of beneficial bacteria in the infected plants (Yang and Ancona, 2021).

The soil is the reservoir that collects the biomass generated by epigeal infections of MSD and by agronomic operations, specifically plant pruning (Nigro *et al*., 2011; Catara and Catara, 2019). An investigation on the survival of *P. tracheiphilus* in the soil of lemon-growing areas of Syracuse affected by a strong pressure of the disease highlighted that more than 70% of the soil samples analysed in June-July show variable concentrations of fungal DNA, ascertained by means of real-time PCR and numerous viable propagules. The persistence of the propagules also in soils waiting to be replanted with lemon, shows that the soil constitutes an inoculum reservoir of priority importance. In view of the importance of microbial relationships in soil with plant health and the role of soil in the epidemiology of MSD, the S.I.R.P.A. project has studied distinctive elements on the possibilities of use of some beneficial bacteria or their secondary metabolites and which either directly or through modifications at the microbiome level, improve response performance against *P. tracheiphilus*.

1.3.2 The bacteria of the S.I.R.P.A. project

The choice of bacteria to be used for the objectives of the project was made taking into account the importance that the reduction of epigeal and hypogeal infections with sustainable methods passes through the containment of the inoculum both in the natural habitat and in the plant. Therefore, research has been set on some ubiquitous bacteria belonging to the *P. corrugata* subgroup, which includes species described as biological control agents and plant growth promoters. *P. corrugata* and *P. mediterranea* strains have been described in the rhizosphere and endosphere of different plant matrices, in agricultural soil and in natural areas. These species have a marked activity of biocontrol against bacteria, Oomycetes and phytopathogenic fungi and some strains are also subject to patent (Catara, 2007).

Their biological properties are due to the production of several secondary metabolites, some already known as cyclic lipopeptides in the group of 'mycins' and 'peptins' and hydrocyanic acid (HCN), whose molecular mechanisms have been studied in the laboratory of Phytobacteriology/Phytosanitary Biotechnology of Di3A for several years.

The peptins mainly inhibit the growth of the Gram-positive bacteria, the mycins instead have a high antifungal activity and HCN has a role in conidia germination and in the growth of the fungal hyphae following germination (Emanuele *et al*., 1998; Scaloni *et al*., 2004; Strano *et al*., 2017). These aspects were extensively addressed in the introduction. Therefore, at the same time with partner Agrobiotech, the Di3A laboratory evaluated the antagonistic activity of bacterial strains of *P. corrugata* and *P. mediterranea* against isolates of *P. tracheiphilus*, based on previously obtained results (Coco *et al*., 2003; 2004) in relation to the production *in vitro* of metabolites diffusible in the agar medium or volatile substances and to their *in planta* biocontrol activity. Based on the results obtained, the strains with more objective properties were subjected to genome sequence.

1.4 References

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2. Sour orange microbiome is affected by infections of *Plenodomus tracheiphilus* causal agent of Citrus Mal secco disease[1]

Abstract

Mal secco (MSD) is a severe vascular disease of citrus caused by the mitosporic fungus *Plenodomus tracheiphilus* (Pt). The pathogen penetration occurs through wounds on the above- and below-ground part of the tree. Sour orange (*Citrus aurantium*), a susceptible species, is the most popular rootstock for lemon in Italy. In this study, wound-inoculations with Pt of sour orange seedlings were accomplished in roots or leaves. Six months post-inoculation, the microbial communities of rhizosphere, endorhizosphere and xylem samples from inoculated and healthy plants were analyzed by 16S rRNA and ITS amplicons sequencing. The DNA of Pt was quantified by real-time PCR in the three plant compartments. According to results, microbial richness and diversity were reduced in the endorhizosphere of root-inoculated plants, showing the highest concentration of the pathogen DNA. Furthermore, bacterial populations of potentially beneficial taxa (e.g. *Pseudomonas* and *Burkholderia*) were depleted in the rhizosphere of the inoculated plants. The infection though leaves or roots also produced a network-wide rewiring of microbial associations in sour orange roots. Overall, our findings revealed the community-level changes induced by Pt infection in the sour orange root microbiome, providing further insights into the investigation of beneficial multispecies interactions in the *Citrus*-associated microbial communities.

Key words: *Citrus*, Mal secco, root microbiome, real-time PCR, amplicon-based metagenomics.

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2.1 Introduction

A plethora of microorganisms (fungi, oomycetes, bacteria) and smaller biological entities (viruses, viroids) live in tight association with multiple plant microhabitats both as epiphytes or endophytes in different organs of the above below ground organs. Microbial communities associated with plants may have beneficial, neutral or adverse effects on the latter (Raaijmakers *et al.*, 2009; Vandenkoornhuyse *et al.*, 2015). An increasing number of information on plant-microbiome is cumulating since the introduction of the high-throughput sequencing techniques (Liu *et al.*, 2021) As a consequence, a plant can be considered as a "holobiont" that includes the host plant and its microbiome in which selective forces act not only on the genome of the plant itself but also on the microbial community associated with it (Vandenkoornhuyse *et al.*, 2015; Lombardi *et al.*, 2021). This presupposes the need for a more open understanding of evolution that, in this case, acts on the entire holobiont, which becomes the subject or unit of a process of selection and adaptation. Within this theoretical framework, the evolution of the holobiont would lead to variations in both the host genome and its microbiome (Bulgarelli *et al.*, 2013). Genomes and metagenomes in a high-throughput manner has been providing with decreasing processing costs efficient methods to better understand the potential functions of individual microorganisms and of the communities or of entire natural niches (Berg *et al.*, 2020; Liu *et al.*2021). Great attention has been devoted to the plant root environment both the (ecto and endo) rhizosphere. Functions and traits related to plant growth, development and health of the rhizosphere microbiome have been extensively studied in several plant hosts (Berendsen *et al.*, 2012; Mendes *et al.*, 2013). These communities contributes with beneficial properties for the acquisition of nutrient, to greater tolerance to stress, and to protect the plants against soil borne pathogens (Raaijmakers *et al.*, 2009, Compant *et al.*, 2019). A role in host immune regulation has been also linked to the response to the microbiome rhizosphere also to pathogens and pests infecting other plant parts.

Citrus is one of the most important perennial fruit crops in the world (FAO, 2021). Citrus production is facing many challenges including biotic and abiotic stresses and an important role to the microbiome has been attributed in citrus health as for the other plant species (Wang *et al.*, 2017a; 2017b). The study of predominant and core taxa of the global citrus rhizosphere microbiome and their functional traits in field conditions from 23 representative locations in

eight major citrus-producing countries (six continents) has been undertaken by amplicon and deep shotgun metagenomic sequencing of the community members (Xu *et al.*, 2018). A similarity in the microbial community structure of rhizosphere and associated bulk soil samples collected from twelve citrus varieties has been observed, and a host-driven recruitment based on the selection of particular traits postulated (Xu *et al.*, 2018). Citrus core microbial taxa resulted enriched in the rhizosphere compared to the corresponding bulk soil samples and present in more than 75% of the samples from across-the-globe and identified 132 core bacterial genera and seven core fungal genera in the global citrus rhizosphere microbiome (Xu *et al.*, 2018).

Microbiome studies aimed at defining the core citrus microbiome have been made available for wide range of *Citrus* species and several cultivars and genotypes grafted on different rootstocks investigating of microbial communities different compartments (soil, root, leaves, phloem, seeds) (reviewed in Zhang *et al.*, 2021). Only recently a study investigated the effect of rootstocks (Penyalver *et al.*, 2022).

Citrus microbiome has been investigated by metagenomics only in a few pathosistems, although with different aims. The amplicon-based metagenomic approach has been used to explore the etiology of Citrus Decline Disease (CDD) in Iran (Passera *et al.*, 2018), and of the leaf fungal disease Citrus Greasy Spot (CGS) in Italy (Abdelfattah *et al.*, 2017) complex and to date unresolved syndromes. Diseases affect citrus microbiome both when analyzing the same compartment colonized by the pathogen and also at distance. Melanose disease, by *Diaporthe citri*, causes a shift in citrus leaf microbiome, highlighted by the marked reduction of community evenness, the emergence of large numbers of new microbes, and the intense microbial network (Li *et al.*, 2022). A baseline paper on *Phythopthora* spp, causing citrus foot rot, showed a depletion of beneficial bacteria and fungi in the endorhizosphere of diseased symptomatic *C. paradisi* plants grafted on sour orange as compared to the healthy ones (Yang and Ancona, 2021). The authors suggested that the decrease of beneficial microbes could increase susceptibility to environmental stress and colonization of detrimental microbes thus increasing fibrous root decline (Yang and Ancona, 2021).

A large body of information was obtained in the numerous studies on Huanglongbing (HLB). The infections by '*Candidatus* Liberibacter asiaticus' one of the fastidious phloematic bacterium causing HLB affect the structure and composition of the bacterial community associated with the leaves (Blaustein *et al.*, 2017; Munir *et al.*, 2020; Passera *et al.*, 2018) and citrus roots (Blaustein *et al.*, 2017; Ginnan *et al.*, 2020; Li *et al.*, 2021; Padhi *et al.*, 2019; Trivedi *et al.*, 2012; Zhang *et al.*, 2017). In addition, bacterial community composition of either citrus roots or endosphere of all above ground tissues fluctuated due to the application of

biofertilizers (Bai *et al.*, 2019), antibiotic application (Ascunce *et al.*, 2019; Yang *et al.*, 2016; Zhang *et al.*, 2013), and thermotherapy (Yang *et al.*, 2016) for HLB control. A pivotal root for tree decline and death of HLB affected plant was attributed to the changes of roots microbial communities leading to a severe disbiosis status (Ginnan *et al.*, 2020).

To date, no metagenomics study has been carried out on citrus xylem dwellers. Eventhough, however, studies performed by a culturable approach and DDGE on Citrus Variegated Chlorosis (CVC) caused by the xylem-limited bacterium *Xylella fastidiosa* also highlighted that infections induced difference in endophytic culturable bacteria of leaves and branches between symptomatic and asymptomatic *C. sinensis* and *C. reticulata* plants (Lacava *et al.*, 2004).

Mal secco disease (MSD), is a detrimental tracheomycosis of citrus caused by the mitosporic fungus *Plenodomus tracheiphilus*. The disease is a major constraint for lemon production worldwide, although all species of *Citrus*, *Poncirus*, *Severinia* and *Fortunella* can be infected (Migheli *et al.*, 2009; Nigro *et al.*, 2011).

Pathogen penetration occurs *via* wounds in the above- and below-ground part of the tree. The highest incidence of infections is usually registered after meteoric events causing wounds and abscission of leaves or bark and xylem lesions on twigs and stems (Butera *et al.*, 1986; Lanza *et al.*, 1988; Solel, 1976). After penetration of the wounds the fungal hyphae grow towards the xylem (Bassi *et al.*, 1980) and spread progressively in the tree. During this stage the hyphae release in the trachea many phialoconidia which are fastly disseminated to upper parts of the plant through the water flow (Kroitor-Keren *et al.*, 2013). As consequence the vessels are clogged by gums and mycelium accumulation in (Bassi *et al.*, 1980; Perrotta *et al.*, 1979a, Perrotta *et al.*, 1979b), leading to the impairment of water transport (Raimondo *et al.*, 2007; 2010) and associated symptoms of vein yellowing, leaf shading, twig dessication, drying up of the branches and death of the tree. Later, the symptoms become more evident, and the fungus starts to form the pycnidia that will serve as the inoculum for winter dispersal of the new spores.

Root infection is primarily caused by a direct penetration through openings on the root or stem of the spores produced in the infected plant parts (i.e. leaves, twigs, and pruning waste) lying on the ground. Under controlled conditions they may survive about 30 or 120 days (in sandy or clay soil), while in the orchard, they may stay alive for up to 1 year in heavy soil (De Cicco *et al.*, 1987). This kind of infection leads to a sudden wilting of branches or the whole tree, hence named malfulminante (Migheli *et al.*, 2009).

Citrus aurantium (sour orange), the most popular rootstock for lemon (in Italy), has been used in many studies on *P. tracheiphilus*-*Citrus* pathosystem (Nigro *et al.*, 2011; Catara and Catara,

2019). To date, there are no studies examining the influence of MSD caused by *P. tracheiphilus* on the bacterial and fungal communities of citrus roots. Our hypothesis is that Mal secco infection can affect the microbial composition and community structure of these plant compartments. Therefore, we used an amplicon-based (16S rRNA and ITS sequences) metagenomic approach to decipher the root and xylem (including bacterial and fungal) microbiome from healthy sour orange seedlings and seedlings inoculated with *P. tracheiphilus* by wounded leaves or roots, simulating canopy and belowground infections occurring in nature. Microbial multispecies interactions with potential biocontrol applications toward *P. tracheiphilus* were also explored.

2.2 Materials and Methods

2.2.1 Fungal isolates and culture conditions

P. tracheiphilus isolate PVCT Pt57, isolated in 2005 from *Citrus limon* plants affected by Mal secco, was routinely grown at 23°C ± 2 on potato dextrose agar (PDA, Oxoid, Milan, Italy) or carrot-agar (300 g of carrots, 1 L of double distilled water, 1% bacteriological agar).

P. tracheiphilus phialoconidia were obtained in carrot broth (300 g of carrots/l), as described by Salerno and Catara (1967), with minor modifications; two small portions of PDA (Potato Dextrose Agar) along the actively growing area of seven-days-old fungal colonies were transferred to 250 mL-fluted flasks containing 100 mL of carrot broth. The flasks were placed into an orbital shaker under continuous agitation (220 rpm), at 22°C and diffused light up to four days. The culture broth was filtered with four layers of sterile gauze in order to eliminate the mycelial component of the fungus and centrifuged at 6000 x g for 15 minutes. The pellet was washed three times and resuspended in sterile distilled water (SDW). Finally, conidia were counted using a hematocytometer and diluted up to 10^6 mL^{-1} with SDW for the leaf- and root-inoculation assays.

2.2.2 Plant materials

Four-month-old *Citrus aurantium* (sour orange) seedlings in pots (20-cm diameter) filled with a commercial peat-based substrate were leaf-inoculated and root-inoculated (ten seedlings for each inoculation method) with the virulent isolate of *P. tracheiphilus* PVCT Pt57 and grown in unheated greenhouse. Control plants were treated with SDW.

Leaf-inoculations were performed by placing 10 µl of conidial suspension on the secondary veins of young and fully-expanded leaves. Subsequently, leaves were slightly wounded in correspondence of the suspension drops by gently pressing with three entomological needles. For each seedling, four leaves were inoculated at four points. For root-inoculations, after uprooting the plants the root tips were cut and roots immersed in the inoculum suspension for 10-15 minutes. Plants were finally placed into the same peat-filled pot.

Weekly, the leaf and root infection symptoms were monitored and evaluated using the empirical scales proposed by Luisi *et al.*(1979) and Scaramuzzi *et al.*(1964), respectively.

2.2.3 Sample processing

Six months' post-inoculation (leaf-inoculation, root-inoculation and not inoculated control), three compartments were analysed: rhizosphere, endorhizosphere and xylem.

Composite rhizosphere samples were obtained by processing together the rhizospheres of three randomly selected *Citrus aurantium* seedlings. The same sampling method was considered for the endorhizosphere and xylem samples. Three composite samples for each compartment and treatment were considered.

Root samples were processed according to the protocols described by Anzalone *et al.*(2021; 2022) with some modifications. Rhizosphere samples were obtained by shaking gently the roots to remove the non-adhering soil. Roots were then transferred in sterile 50 mL centrifuge tubes containing 25% w/v sterile phosphate saline buffer (PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4) and mixed thoroughly by vortexing for 2 min. Endorhizosphere samples were obtained by surface sterilizing the roots; roots were first submerged in 75% ethanol solution (2 min), followed by 50% sodium hypochlorite solution (2

min), and ethanol 75% (1 min) and rinsed five times in SDW. In order to verify the surface-sterility of the samples, sterilized roots were placed on PDA at 27°C ± 2 for 7 days. The lack of bacterial and fungal growth confirmed the sterility of the root surfaces.

To obtain the xylem samples, after removing the leaves, stems were surface-sterilized by immersion as above described. The bark was peeled off. Endorhizosphere and xylem samples were separately homogenized with a mortar and pestle and suspended in 25% w/v sterile PBS.

The rhizosphere samples and the endorhizosphere and xylem homogenates were finally aliquoted into 2-mL reaction tubes and centrifuged at 13,000 rpm and 4°C for 30 min. Pellets were then stored at -80°C for further processing.

2.2.4 Total DNA extraction and quantitative detection of *P. tracheiphilus*

Total genomic DNA was extracted with DNeasy PowerSoil Pro Kit (Qiagen), according to the manufacturer's instructions. DNA concentration and quality were determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Detection and quantification of *P. trachephilus* DNA in root- and leaf-inoculated seedlings were performed according a species specific real-time PCR protocol (Licciardello *et al.*, 2006; Russo *et al.*, 2011). Amplification was carried out with the primers GR70 and GL1 targeting a 84-pb DNA segment of *P. tracheiphilus* DNA, and the dual-labelled fluorogenic probe PP1 (Licciardello *et al.*, 2006; Russo *et al.*, 2011). Real-time PCR assays were performed using QuantiNova Probe PCR Kit (Qiagen), 400 nM each primer, 200 nM probe and 1 µl of target DNA. All reactions included a blank, replacing DNA with ultrapure water, and positive controls (serial dilutions of *P. tracheiphilus* DNA). The real-time PCR program consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 10 s at 95 °C, 15 s at 62 °C, and 15 s at 72°C. All real-time PCR reactions were performed in Bio-Rad's iCycler® real-time PCR machine (Applied Biosystems).

The standard curve for fungal DNA quantification was constructed using *P. tracheiphilus* PVCT Pt57 isolate DNA (100 ng/µl) serially diluted in sterile distilled water by plotting Ct values versus the logarithm-transformed DNA concentration values of each ten-fold dilution series.

2.2.5 Library preparation and amplicon sequencing

Library preparation and amplicon sequencing were conducted at IGA Technology Services (Udine, Italy). For bacterial community profiling, the V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified with primers 16S-341F and 16S-805R (Klindworth *et al.*, 2013). Peptide nucleic acid (PNA)-clamping was applied during the first 16S rRNA amplification step to block amplification of host chloroplast and mitochondrial 16S rRNA gene sequences. For fungal community profiling, the variable ITS1 region was amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White *et al.*, 1990).

The 16S and ITS libraries were sequenced on a MiSeq instrument (Illumina) in paired end 300-bp mode reads whose ends are overlapped to generate high-quality full-length sequences to ensure an accurate taxonomic classification.

2.2.6 Bioinformatics and statistical analysis

According to Illumina indexing system, 16S and ITS raw Illumina fastq files were demultiplexed and quality-checked by FastQC (Andrews, 2010). Following the QIIME 2 pipelines (Boylen *et al.*, 2019), the USEARCH algorithm (version 8.1.1756) performed chimera filtering, grouping of replicate sequences, sorting sequences per decreasing abundance and OTUs (*Operational Taxonomic Units*) identification and then summarized in feature tables. The most abundant 16S reads in each OTU were selected as representative sequences, extracted, aligned and assigned taxonomically using the RDP classifier classification from the RDP database (Cole *et al.*, 2014) trained to Greengenes 16S rRNA gene database (version 2013_8) (confidence threshold $\geq$ 0.5). UNITE ITS database was performed for fungal ITS region (version 8.1; Kõljalg *et al.*, 2013). Alpha and beta diversity, based on the OTU tables for the bacterial and fungal communities, were evaluated for each treatment (control, leaf inoculation, root inoculation) and compartment (rhizosphere, endorhizosphere, xylem) using the phyloseq package in R (version 4.0.2) (McMurdie and Holmes, 2013, R core team, 2020). The statistical significance was performed using Kruskal-Wallis test for alpha diversity and the two-sided Student's two-sample t-test for beta diversity. Differential abundance analysis was performed with DESeq2 (Love *et al.*, 2014) in order to detect the enriched and depleted bacterial and fungal genera between controls and inoculated samples of the same compartment. Bacterial and

fungal networks were constructed for each treatment (control, leaf inoculation and root inoculation), including in each networks the rhizosphere, endorhizosphere and xylem compartments. The most abundant bacterial and fungal genera were used for the construction of co-occurrence networks. MENAP was used (Deng *et al.*, 2012) following the developer's recommendations, choosing the greedy modularity as separation method, and represented in Cytoscape (Shannon *et al.*, 2003). Differential networks analysis was performed between the networks of the control samples and the networks of the inoculated samples using Diffany framework (Van Landeghem *et al.*, 2016) in Cytoscape.

2.2.7 *Plenodomus* sequence analysis

For sequence analysis, partial ITS1 sequences of the OTUs identified as *Plenodomus* sp. were searched against the National Center for Biotechnology Information (NCBI) nucleotide database using Basic Local Alignment Search Tool BLASTN (https://www.ncbi.nlm.nih.gov/, accessed on 14 November 2022). Only sequences with the highest similarity were considered for the taxonomic assignment. For phylogenetic analysis, sequences of Plenodomus sp. OTUs and partial ITS1 of representative Pleosporales were aligned using MUSCLE in MEGA X (Kumar *et al.*, 2018). A cladogram was constructed by using the maximum likelihood method and Kimura 2-parameter model (Kimura, 1980). The analysis was performed with 1000 bootstrap replications.

2.3 Results

2.3.1 Quantification of *Plenodomus tracheiphilus* in sour orange seedlings

P. tracheiphilus infections in leaf- and root- inoculated sour orange seedlings simulating canopy and belowground infections that occurs *via* wounds were monitored over the time visually and six-month after inoculation by quantitative real-time PCR. Upon leaf inoculation first symptoms

appeared 7 dpi (days post-inoculation) on a small number of infected sites (10%) with and an average disease index of 0.7 (SD ± 0.3). At 14, 21, 28, 35 dpi, average disease indices of 1.3 (SD ± 0.5), 1.9 (SD ± 0.6), 2.4 (SD ± 0.6), 2.8 (SD ± 0.6), respectively were recorded. At the last date (35 dpi) the 90% of sites were symptomatic and the leaf showed typical MSD symptoms consisting in chlorosis extended up to the leaf margin. Two months post-inoculation the majority of leaf-inoculated seedlings showed defoliation. In the remaining leaves chlorosis extended to the whole leaf was observed. Most of the root-inoculated seedlings showed stunted growth as compared to the uninoculated control ones. Mild chlorosis of midribs of new formed leaves was observed six months post-inoculation. The remaining plants were asymptomatic. For both inoculated plants (through leaves and roots), no secondary symptoms were observed (Figure 1).

Figure 1: Symptoms developed upon *Plenodomus tracheiphilus* inoculation in roots (**A**) and leaves (**B**) of sour orange seedlings six and two months post-inoculation, respectively, and non-inoculated control (**C**).

Six months post-inoculation total genomic DNA was extracted from rhizosphere, endorhizosphere and xylem endosphere samples and *P. tracheiphilus* DNA was quantified by a species-specific real-time PCR assay (Table 1). Based on a standard curve for absolute quantification realized with serial dilution of the DNA of *P. tracheiphilus* strain PVCT Pt57 (the same used for the inoculations), the amount of DNA per gram of sample was estimated. Regarding the leaf-inoculated plants the fungus was only detected in the xylem with DNA concentrations from 8.8 to 58.3 pg/g of tissue. No amplification signal was detected from the rhizosphere and endorhizosphere samples of leaf-inoculated seedlings. *P. tracheiphilus* was detected in all samples from root-inoculated seedlings. Variable concentrations of the pathogen DNA, namely 5.3-205.0, 32.6-34862.2, and 5.5-56.2 pg DNA/g tissue were estimated in the rhizosphere, endorhizosphere and xylem samples, respectively.

Table 1: Quantification of *P. tracheiphilus* DNA in composite rhizosphere, endorhizosphere and xylem samples from leaf- and root- inoculated *C. aurantium* seedlings by real-time PCR and *Plenodomus* sp. reads identified by ITS amplicons sequencing.

Treatment/Compartment	Ct[a]	pg DNA g tissue^{-1}	*Plenodomus* sp. (OTU)	Reads[c]
Leaf-inoculation				
Rhizosphere	N.D.	/	N.D.	/
Endorhizosphere	N.D.	/	N.D.	/
Xylem	34.2-36.7	8.8-58.3	OTU[b]	3-10
Root-inoculation				
Rhizosphere	32.7-37.4	5.3-205.0	OTU[b]	4-97
Endorhizosphere	22.6-33.9	32.6-34862.2	OTU[b]	1237-8254
			OTU[c]	0-1
			OTU[d]	0-1
Xylem	34-36.9	5.5-56.2	OTU[b]	6-40

[a]Ct (cycle threshold) values of composite plant samples in real-time PCR.
[b]SH207506.07FU_JF740253_refs; [c]New.ReferenceOTU456. [d]New.CleanUp.ReferenceOTU22126;
[e]Number of *Plenodomus* reads.
N.D.; not detected. Range values are indicated.

2.3.2 Assembly patterns of healthy and diseased sour orange seedlings

2.3.2.1. Alpha and beta diversity

Bacterial and fungal communities were obtained from the rhizosphere, the endorhizosphere and the xylem of *C. aurantium* (sour orange) seedlings colonised by *P. tracheiphilus* upon artificial inoculation either in the leaves or in the roots and from the uninoculated plants. 552,234 and 514,565 reads (for the bacterial and fungal communities respectively) were obtained after quality filtering (length trimming, denoising, chimeric sequences exclusion). For 16S rDNA, a total of 551,069 reads were recovered after the removal of chloroplast and mitochondrial reads. The distribution of the total OTUs were 6651, 5038 and 1556 for rhizosphere, endorhizosphere and xylem samples, respectively. For ITS1, a total of 514,565 reads were recovered after the removal of host sequences reads. The distribution of the total OTUs was 791, 181 and 213 for rhizosphere, endorhizosphere and xylem samples respectively.

The bacterial alpha-diversity, estimated by Chao1 richness and Shannon diversity indices, was significantly different ($P < 0.05$) between the rhizosphere and plant endosphere samples both

endorhizosphere and xylem. The highest values were observed in the rhizosphere and they declined from the endorhizosphere to the xylem (Supplementary Figure S1 A-B). No significant differences in alpha-diversity indices were detected when comparing the control non-inoculated samples versus those ones inoculated with *P. tracheiphilus* although a slight reduction was observed in the rhizosphere of inoculated plants (Supplementary Figure S1). The fungal communities of some of the different sample groups show significant differences either in richness (rhizosphere vs. endorhizosphere and xylem) or diversity (rhizosphere and endorhizosphere vs xylem) (Supplementary Figure S1 C-D). In the endorhizosphere of plants inoculated with the pathogen *via* roots a reduction of both richness and diversity was observed. The high concentration of *P. tracheiphilus*, as ascertained by real- time PCR, caused a reduction other fungi reducing also evenness and therefore diversity. Fungal communities of the rhizosphere of the same samples showed an enriched richness and a reduced diversity.

The PCoA for beta-diversity based on Bray-Curtis dissimilarity distances showed for the bacterial community a significant division between the compartments (P = 0.001) but no effect was observed upon *P. tracheiphilus* inoculation (Supplementary Figure S2 A). Beta diversity analysis of fungal communities highlighted the dispersion of roots-inoculated rhizosphere and endorhizosphere samples, probably due to the already described variation caused by pathogen inoculation (Supplementary Figure S2 B).

2.3.2.2 Relative abundance of bacterial communities

At the phylum level, the most represented bacterial communities of the rhizosphere of control sour orange seedlings were Proteobacteria (26.3%), Firmicutes (14.2%) and Planctomycetes (13.7%), followed by Verrucomicrobia (6.3%) and Bacteroidetes (6.9%); a large group of other bacteria (16.4%) was also observed (Figure 2A). Endophytic bacterial communities both of the root and xylem of control plants were dominated by Firmicutes (46.3 and 49.8%, respectively) and Proteobacteria (29.5 and 25.3%, respectively). Other phyla, more represented in the endorhizosphere than in xylem, were Actinobacteria, Bacteroidetes and Planctomycetes. Only minor changes were observed at phylum level in bacterial communities of the *P. tracheiphilus* inoculated as compared to non-inoculated seedlings. However, at bacterial phylum level, Actinobacteria (8.2%) and Bacteroidetes (4.7%) resulted more abundant in the endorhizosphere of leaf-inoculated plants (4.8 and 2.4 in control plants, respectively); Bacteroidetes were also more abundant in the endorhizosphere of root-inoculated plants (7.7% vs. 2.4% in control plants). Firmicutes decreased in the endorhizosphere of root-inoculated plants (34.5% vs. 46.3%

in control plants). Actinobacteria increased also in the xylem of root-inoculated plants (3.4% vs. 1.9% in control plants, respectively).

The analysis of relative abundance at the family level was investigated to look at the effect of inoculation with *P. tracheiphilus* on sour orange microbiome (Figure 2B). In the rhizosphere samples of control plants, the most relatively abundant bacterial taxa were Planctomycetaceae (5.2%), Pseudomonadaceae (4.7%), Bacillaceae (4.6%), Pirellulaceae (3.6%) and unidientified bacteria (15.8%) (Fig. 2 B). A decrease in Pseudomonadaceae in plants inoculated either *via* roots (1.4%) or leaves (2.1%) than in control plants (4.7%) was observed. Bacterial root endophitic communities of control plants were characterised, at family level, by a higher relative abundances in Paenibacillaceae (15.6%) and Bacillaceae (10.2%), than those observed in the rhizosphere, which together reach up to the 48% in the xylem of control plants. As for the rhizosphere, in the endorhizosphere Pseudomonadaceae were reduced in *P. tracheiphilus* inoculated plants whatever the inoculation site (1.5%, 3.3% and 7.3% for leaf-, root- and uninoculated plants). The families Sphingomonadaceae, Rhizobiaceae and Chitinophagaceae were detected at higher relative abundance in root-inoculated samples (7 vs. 4.4%, 5.3 vs. 2.4% and 5.3 vs. 0.9%, in control plants, respectively), while Paenibacillaceae and Clostridiaceae were less present in the same samples (15.6% vs. 14.3% and 3.6% vs. 2% in control plants, respectively).

The family Staphylococcaceae was detected at higher relative abundance in the xylems of root-inoculated plants, 7% vs. 0.4 and 0.5 % in leaf-inoculated and in control plants, while Bacillaceae and Caulobacteraceae were less represented in the same samples (12.1% vs. 28.8% and 4.6% vs. 10% in control plants, respectively). Other Nostocophycidae and Sphingomonadaceae were more abundant in root-inoculated plants (13.6% vs. 7.7% and 2.3% vs. 3.5% in control samples, respectively).

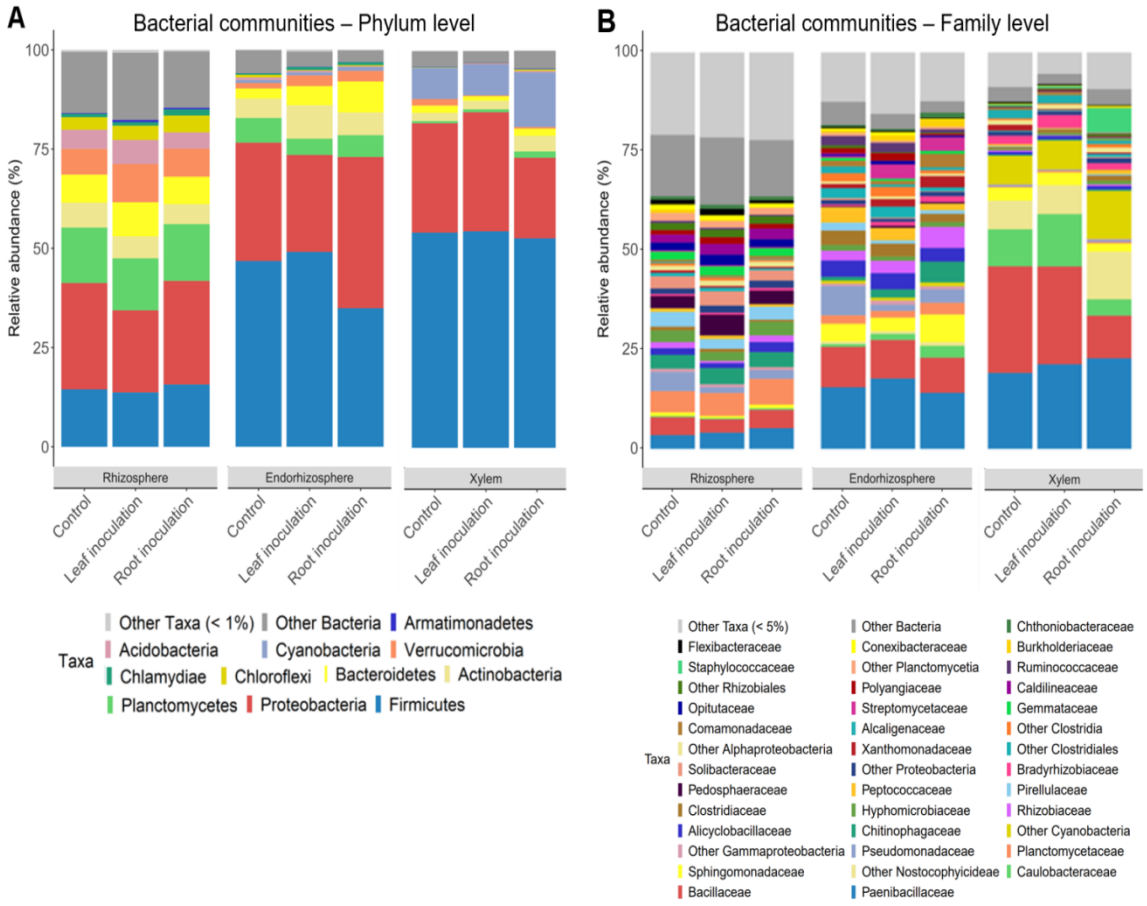

Figure 2: Relative abundances of the bacterial communities at the phylum (**A**) and family (**B**) taxonomic levels in the rhizosphere, endorhizosphere and xylem compartments. Bacterial phyla and family's taxa less abundant than 1% and 5% respectively are reported as "Other Taxa".

2.3.2.3 Relative abundance of fungal communities

At the phylum level, fungal communities of control plants were dominated by Ascomycota in all analysed compartments (81%, 95% and 55% in all analysed sample of the rhizosphere, endorhizosphere and xylem compartments, respectively). Basidiomycota were enriched in the xylem environment. Other represented phyla were Zygomycota and Rozellomycota.

At fungal phylum level, Ascomycota increased in the rhizosphere of root-inoculated plants (97% vs. 81% in control plants, respectively), while Rozellomycota dicreased (1.7% vs. 4.8% in control plants, respectively) (Figure 3A). Basidiomycota and Rozellomycota increased in the endorhizosphere of leaf-inoculated plants compared to control plants (7.2% vs. 3.2% and 1.6% vs. 0.3%, respectively). Zygomycota decreased in the endorhizosphere of root-inoculated plants

(0.03% vs. 0.8% in control plants, respectively) and in the xylem of leaf-inoculated plants (3.4% vs. 6.6% in control plants, respectively).

Fungal community analysis at family level showed that Trichocomaceae which is the most abundant taxon in the rhizosphere was present at higher relative abundance in *P. tracheiphilus* root- and leaf-inoculated samples (71% and 34%, respectively) than in control plants (25%) (Figure 3B). In the rhizosphere, Annulatascaceae were detected at higher relative abundance in root-inoculated plants rhizospheres (2% vs. 0%, in control plants, respectively). On the opposite, Davidiellaceae and Rhizopodiaceae (10% vs. 1%, in control plants, respectively) and Rozellomycota abundances (5% vs. 2% in control plants, respectively) were lower in the rhizosphere of root-inoculated plants than in control plants. In leaf-inoculated plants endorhizosphere, Orbiliaceae (45% vs. 18% in control plants, respectively) were detected at higher relative abundance, whereas Nectriaceae (2% vs. 5%, in control plants, respectively) were less present in both inoculated samples.
In the xylems, the fungal family with the highest realative abundance was Trichocomaceae (10%), in both control and root-inoculated plants. However, Mortierelliaceae (8% vs. 6% and 3% in leaf-inocoulated and control samples, respectively) and Leptosphaeriaceae were more abundant (4% vs. 1% and 0% % in leaf-inocoulated and control samples, respectively).

Leptosphaeriaceae (the family to which *P. tracheiphilus* belongs) resulted the most abundant taxon (58%) in the endorhizosphere of the root-inoculated seedlings. Its presence was also detected, although to a lower extent, in the rhizosphere of root inoculated plants (0.03%) and in the xylem of leaf- (1%) and root-inoculated plants (4%). Three OTUs, namely SH207506.07FU_JF740253_refs (235 bp), New.ReferenceOTU456 (234 bp), and New.CleanUp.ReferenceOTU22126 (251 bp) of the fungal community were identified as *Plenodomus chrysanthemi*, according to UNITE ITS database (Kõljalg *et al.*, 2013). SH207506.07FU_JF740253_refs OTU (total of 9673 reads) was detected both in the rhizosphere, endorhizosphere and xylem of root-inoculated plants and in the xylem of leaf-inoculated plants. BLASTN analysis of these OTUs sequences resulted in 100% identity with *P. tracheiphilus* IS3_15 (MK461058.1), *P. chrysanthemi* ISR2_3 (MK460988.1) and with *P. tracheiphilus* PVCT Pt57 (data not shown). The other two OTUs were instead represented by a total of two reads each in the endorhizosphere of root inoculated samples and showed an identity of 96.5% and 98.3% with the above mentioned GenBank accessions and the *P. tracheiphilus* isolate used in this study.

A phylogenetic tree based on the partial ITS1 gene sequences showed that all the three OTUs cluster within *P. tracheiphilus* phylogroup including both *P. tracheiphilus* and *P. chrysanthemi* isolates (Supplementary Figure S3).

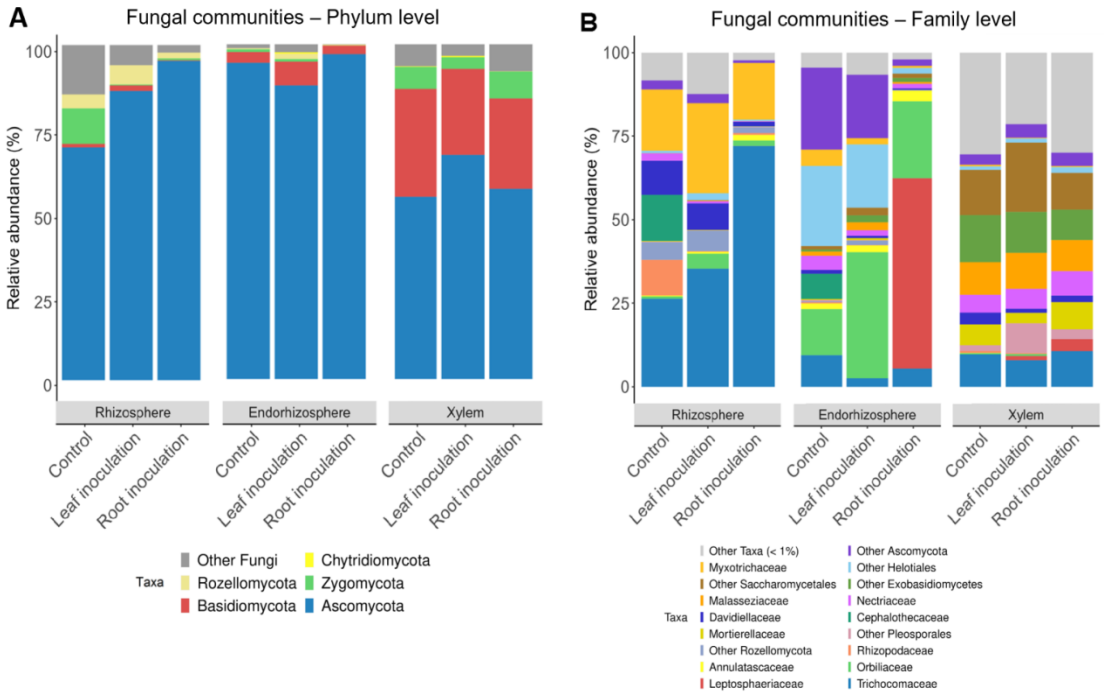

Figure 3: Relative abundances of the fungal communities at the phylum (**A**) and family (**B**) taxonomic levels in the rhizosphere, endorhizosphere and xylem compartments. Fungal families taxa less abundant than 1% are reported as "Other Taxa".

2.3.2.4 Exploring differentially abundant bacterial and fungal genera in inoculated plants

Differentially abundant bacterial and fungal genera were evaluated for each compartment between the sour orange seedlings inoculated with *P. tracheiphilus* by leaf- and root-inoculation and those uninoculated. The differential abundances of the genera that were statistically significant ($p < 0.05$) in at least in one of the pairwise comparison were represented in Figure 4. In the root rhizosphere, 27 and 14 bacterial genera differed significantly when comparing leaf-inoculated (LE) and root-inoculated (RE) to non-inoculated plants, whereas in the endorhizosphere they were 25 and 19, respectively (Figure 4A).

Regarding the rhizosphere, bacterial genera *Longilinea*, *Armatimonas* and *Lewinella* were enriched both in the leaf and root inoculated sour orange seedlings as compared to the non-

inoculated ones (p value < 0.05). Genera enriched following infection through either leaves or roots were *Spirochaeta*, *Fibrobacter*, *Niastella*, *Longilinea* and *Pedosphaera*, and *Sorangium*, *Armatimonas* and *Methylosinus*, respectively (p value < 0.05) (Figure 4A). In contrast, no significant bacterial genera were found depleted in both kind of samples, although the heat map indicate for some families a tendency in that direction. Several genera, namely *Microbacterium*, *Arthrobacter*, *Azohydromonas*, *Pseudomonas*, *Achromobacter*, *Devosia*, *Dyadobacter*, *Oscillochloris*, *Methylobacterium*, *Lutelibacter*, *Rhizobium* and *Hydrogenophaga* were depleted in the leaf-inoculated seedlings, whereas in the case of those inoculated *via* roots only *Rhodococcus*, *Dyella*, *Wautersiella*, *Pedobacter*, *Burkholderia* were depleted (Figure 4A).

Concerning the endorhizosphere, *Streptomyces, Flavilisobacter* and *Spirochaeta* were significantly enriched in both the root- and the leaf-inoculated samples, whereas a depletion of the genus *Lysobacter* was observed. In leaf inoculated samples genera *Fibrobacter, Herbaspirillum, Xanthomonas,* '*Candidatus Methylaciphilum*', *Cystobacter, Methylibium,* '*Candidatus Rhabdochlamydia*' were significantly enriched, whereas significantly less abundant than in the control seedling genera were *Devosia, Achromobacter, Pseudomonas, Microbacterium* and *Demequina*. Considering the root inoculated samples the enriched genera in the endorhizosphere were *Phenylobacterium, Niastella, Chitinophaga, Shinella, Pseudoxanthomoans,* '*Candidatus Xiphinematobacter*' and *Steroidobacter*, whereas only genus *Pimelobacter* was depleted (Figure 4A). In the xylem, no significantly enriched or depleted genera were shared between the treatments. *Hydrogenophaga, Peptoniphilus* and *Finegoldia* were enriched in the leaf-inoculated plants, while *Staphylococcus* was the only bacterial genus enriched after the root-inoculation. *Ochrobactrum* and *Ramlibacter* were depleted in the leaf-inoculated plants (Supplementary Table S1). Differential abundance analysis also highlighted a statistically significant depletion in *Pseudomonas* in the endorhizosphere and rhizosphere of sour orange seedlings inoculated with *P. tracheiphilus* in the leaves. However, from the heatmap it is possible to observe that also the inoculation *via* roots depleted the genus *Pseudomonas* although the data were not statistically significant. Only a few fungal genera were significantly differentially expressed more numerous in the rhizosphere than in the endorhizosphere both in the leaf and root-inoculated sour orange seedlings (Figure 4B). Consistently with previous analysis (relative abundances, real-time PCR), *Plenodomus* was significantly more abundant in the rhizosphere and endorhizosphere samples from root-inoculated seedlings and in the xylem of seedlings inoculated with both methods (Figure 4B, Supplementary Table S1). Accordingly to the increased relative abundance of Trichocomaceae in the rhizosphere of root inoculated seedling, the genus *Penicillium* resulted enriched. Other fungal genera such as *Conlarium* and *Arthrobotrys* were also other enriched whereas *Pilidiella*

was the only enriched genus in the leaf-inoculated ones. Interestingly, *Phialemonium* was depleted in each treatment both in rhizosphere and endorhizosphere. *Sterigmatomyces* in the root-inoculated and *Minimedusa*, *Rhizopus*, *Umbelopsis* in the leaf-inoculated plants were all depleted. In endorizhosphere, *Phialocephala* were enriched in both the treatments. *Conlarium* and *Scytalidium* were enriched and depleted upon the root inoculation, respectively. In xylem, the only differentially abundant fungal genus was *Minimedusa* (Supplementary Table S1).

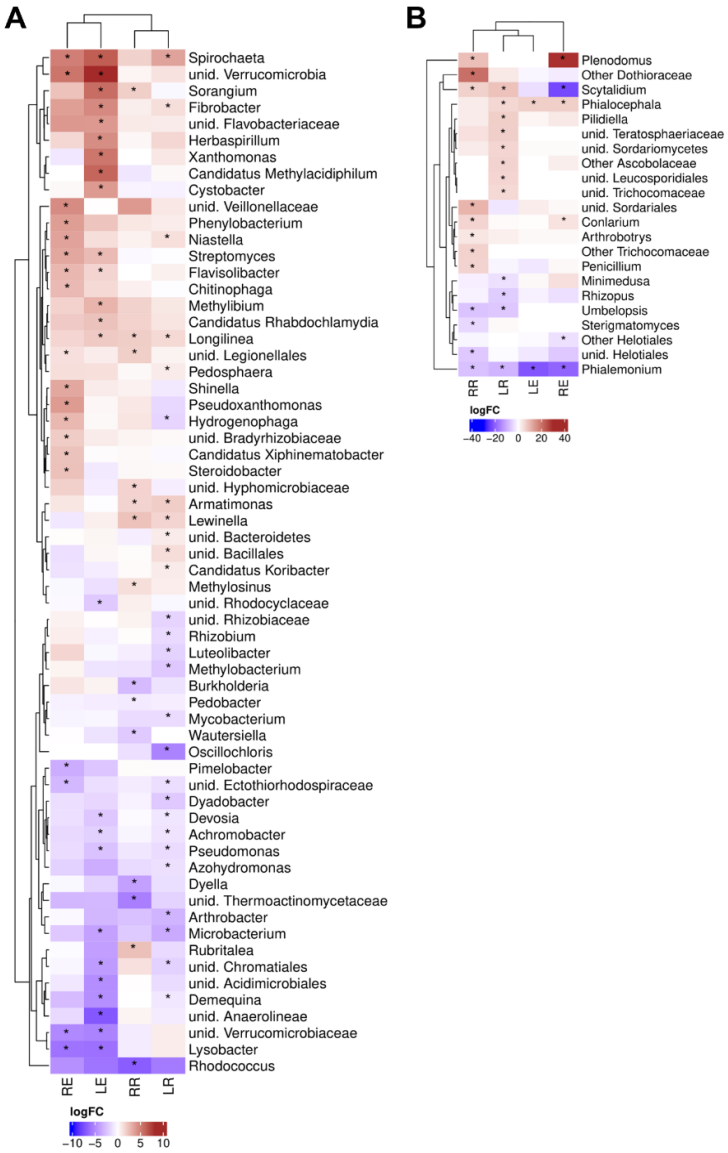

Figure 4: Bacterial (**A**) and fungal (**B**) heatmap of differentially abundant taxa present in the rhizosphere (LR) and endorhizosphere (LE) samples of the leaf-inoculated seedlings and in the rhizosphere (RR) and endorhizosphere (RE) samples of root-inoculated seedlings. Differentially abundant taxa were evaluated among the most significative samples (* indicates $p < 0.05$). Each cell depicts the $\log_2$ fold change (logFC) of each microbial taxon and is colored according to depleted (blue), not differentiated (white), and enriched (red) conditions.

2.3.2.5 Pseudomonas spp. in the microbiome of citrus plants infected with P. tracheiphilus

Pseudomonas was the first represented genus in the rhizosphere and the second in the endorhizosphere of sour orange plants. *Pseudomonas* accounted 131 OTUs; four OTUs (HM344177.1, HM152696.1, New.ReferenceOTU74 and FJ893269.1) represented the 91.8% of the *Pseudomonas* reads in the rhizosphere, endorhizosphere and xylem samples of sour orange inoculated and uninoculated plants. According to species level taxonomic classification, OTUs HM344177.1 and HM152696.1 were identified as *Pseudomonas putida* and *Pseudomonas umsongensis*, respectively, while OTUs New.ReferenceOTU74 and FJ893269.1 were identified only at the genus level (*Pseudomonas* sp.). The effect of *P. tracheiphilus* inoculation (through leaves or roots) on the absolute abundance (total number of reads) of these four *Pseudomonas* OTUs was evaluated in the rhizosphere and endorhizosphere compartment samples. According to results, a reduction of the absolute abundance was observed in the rhizosphere of leaf- and root-inoculated plants for the OTUs HM344177.1, HM152696.1 and New.ReferenceOTU74 compared to uninoculated control plants (Supplementary Figure S4). In the endorhizosphere compartment, a reduction of the absolute abundance of the OTUs HM344177.1, HM152696.1 and New.ReferenceOTU74 was observed in root-inoculated plants and more markedly in the leaf-inoculated plants compared to uninoculated control plants. For FJ893269.1 OTU, the total number of reads resulted reduced only in the endorhizosphere of root-inoculated seedlings, compared to control plants (Supplementary Figure S4).

2.3.2.6 P. tracheiphilus infections altered sour orange microbial co-occurrence network

Infections caused by *P. tracheiphilus* upon wound penetration of the leaves greatly increased the number of nodes and edges in the network, as compared to the network of the non–inoculated plants (89 vs. 141 nodes; 202 vs. 764 edges). In addition, the negative interactions increased in the leaf-inoculated plants, as revealed by a decrease in positive edge percentage (PEP) (Table 2).

Table 2: Topological properties of control, leaf-inoculated and root-inoculated plants' co-occurrence networks.

Network	Number of nodes	Number of edges	Positive edges	Percentage of Positive edges (PEP)	Clustering coefficient	Average Shortest Path	Modularity
Control	89	202	194	90.30%	0.166	3.027	0.415
Leaf-inoculated	141	764	686	81.90%	0.267	2.697	0.375
Root-inoculated	188	1315	1284	93.40%	0.292	2.724	0.286

Similarly, changes in the network of the microbial communities of the root-inoculated sour orange seedlings were characterized by an increase of the total nodes and edges, which respectively were two e five times more in comparison to the nodes and edges in the control network. Moreover, in presence of the pathogen, the networks of the microbial community in the leaf- and root-inoculated samples were more connected and less modularized than those of the control samples (Figure 5 A-C). Comparing the nodes of the microbial communities networks, 65 nodes were shared between the three networks and 50 more nodes were shared between the networks of the inoculated samples, for a total of 115 nodes in common between the inoculated samples (Figure 5D). The differential networks analysis showed that after the leaf- and root-inoculations with *P. tracheiphilus*, the microbial community structure underwent to a rewiring, therefore the correlations in common between the control network and each network of the inoculated samples were few (Figure 5). The networks of the leaf-inoculated and the root-inoculated samples showed 55 nodes shared (which represents the 39% and the 29% of the leaf- and root-inoculated samples' nodes) and 66 edges (Figure 5).

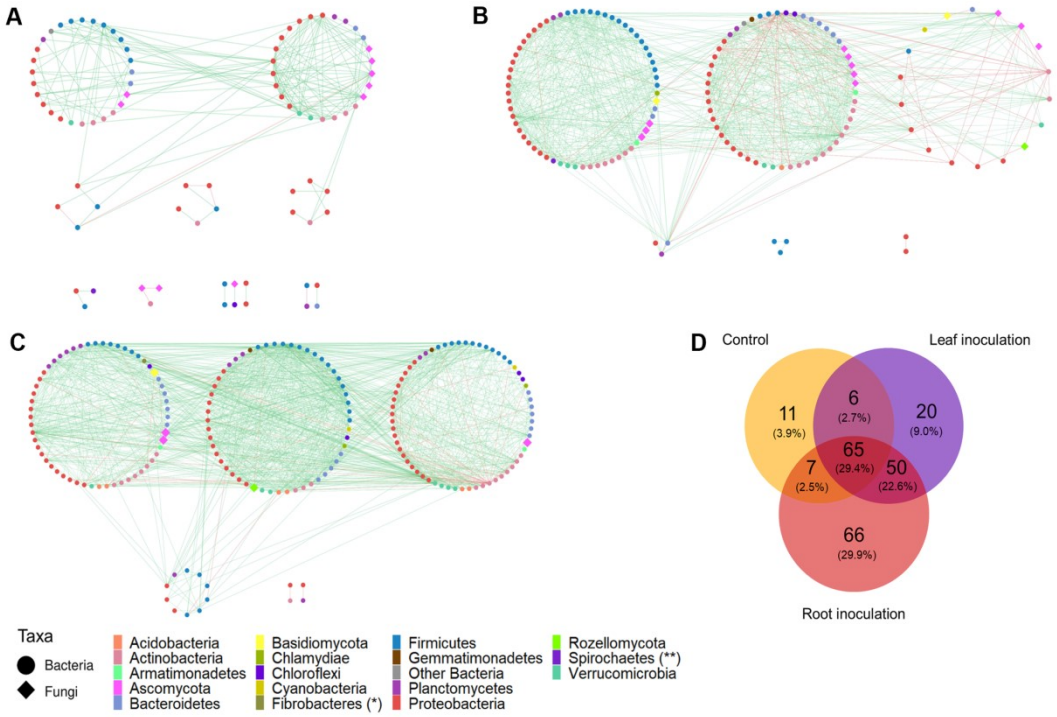

Figure 5: Co-occurrence networks of bacterial and fungal communities in control (**A**), leaf-inoculated (**B**) and root-inoculated (**C**) plants. The nodes and the edges represent the microbial genera and the correlations among them, respectively. Each node is colored according to the phylum taxonomic level. (*) Phylum present only in the network of the root-inoculated plants. (**) Phylum present only in the network of the leaf-inoculated plants. (**D**) Venn-diagrams displaying numbers of bacterial and fungal genera shared between the control samples and the leaf- and root-inoculated samples.

2.4 Discussion

This study was aimed to unravel if and how the infections of *P. tracheiphilus*, causal agent of Mal secco disease (MSD), alter the composition and rewire the interaction of microbial (bacterial and fungal) communities associated to the roots (rhizo-endosphere) and the endosphere of xylem of *Citrus aurantium* (sour orange), the most popular citrus species used as rootstock in Italy.

Since the penetration of the pathogen into the citrus above-ground organs and roots occurs exclusively through wounds (Cutuli, 1972; Zucker and Catara, 1985; D'Anna *et al*., 1986; Perrotta and Graniti, 1988; Migheli *et al*., 2009; Nigro *et al*., 2011; Catara and Catara, 2019) to simulate the natural MSD infections, sour orange seedlings were artificially inoculated through wounded leaves or roots. Through a 16S and ITS amplicon-based metagenomic approach, changes in the microbial community of three sour orange plant compartments (rhizosphere, endorhizosphere and xylem) were investigated. In addition, the colonization of *P. tracheiphilus* was monitored and the pathogen was quantified by real-time PCR (Licciardello *et al*., 2006; Russo *et al*., 2011).

Root-infected seedlings showed leaf symptoms six months post-inoculation, consisting in slight discoloration of midveins. At that time the pathogen was detected by real-time PCR at high concentration in the rhizo-endosphere and in the stem xylem although at very low concentration. According to previous studies, the fungus can reach the leaves before they show disease symptoms by moving inside the vessels through passively transported conidia (Perrotta *et al*., 1979a; 1981). Eventually, it may remain segregated in the inner wood layer for a short time or many years, determining a latent phase of the disease, until it reaches the most external woody rings and the disease progresses very rapidly (Migheli *et al*., 2009; Nigro *et al*., 2011; Catara and Catara 2019).

The effect of infection *via* leaf-inoculations was visible already 14 days post-inoculation with chlorotic halos around the inoculation site, and after a few weeks the chlorosis extended to the leaf margin; about two months post-inoculation many of the infected leaves turned yellow and fell; plants didn't show any basipetal desiccation of the stem over a period of six months. From the leaf-inoculation sites, *P. tracheiphilus* colonized the entire leaf and moved also to stem xylem, where was detected by real-time PCR but at a lower concentration than in root-inoculated seedlings, and was not detected in the roots. The epigeal infections can generate an inoculum source of the pathogen when infected plants organs are left on the soil surface after pruning or fall naturally to the ground (De Cicco *et al*., 1984), as in the case of leaves (Traversa *et al*., 1991; 1992). *Plenodomus sp.* was also detected by amplification and sequencing of ITS1 intergenic region. In particular, three OTUs were identified, within infected samples, as *Plenodomus chrysanthemi*. According to phylogenetic analysis, these OTUs are closely related to both *P. tracheiphilus* and *P. chrysanthemi* isolates.

In this regard, it is worth highlighting that *P. tracheiphilus* and *P. chrysanthemi* could not be differentiated from each other on the basis of their ITS sequences comparison and that the latter is host-specific, being not pathogenic towards sour orange, thus it is reasonable to assume that

the three OTUs identified in this study as *P. chrysanthemi* actually belong to *P. tracheiphilus* species (Baker *et al*., 1985; de Gruyter *et al*., 2013).
Microbial communities of sour orange control seedlings in the overall resemble those already observed in other *Citrus* studies (reviewed in Zhang *et al*., 2021). However, relevant differences were observed in the rhizosphere compartment probably due to our experimental conditions; in this regard, almost all studies investigating the response of citrus microbiome to biotic stresses were performed in the open-field condition with mature citrus plants exposed to natural infections (Trivedi *et al*., 2012; Zhang *et al*., 2017; Passera *et al*., 2018; Ginnan *et al*., 2020; Li *et al*., 2021; Yang and Ancona, 2021). The rhizosphere of sour orange seedlings was characterised by the dominant presence of the bacterial phyla Proteobacteria, Firmicutes and Planctomycetes and amongst fungal communities, Ascomycota. The prevalent prokaryotic phyla found in other citrus rhizosphere studies included Proteobacteria, Actinobacteria, Acidobacteria, and Bacteroidetes, while fungal phyla included Ascomycota, Basidiomycota and to a lesser extent Glomeromycota (Trivedi *et al*., 2010; Zhang *et al*., 2017; Xu *et al*., 2018; Li *et al*., 2021). Sour orange rhizosphere also showed Actinobacteria, Acidobacteria and Bacteroidetes although a higher percentage of Planctomycetes was observed, consistently with previous reports on citrus rhizosphere (Yang *et al*., 2016).

In our study amongst Ascomycota the most represented family in the rhizosphere were Trichocomaceae, Myxotrichaceae, Cephalothecaceae and Rhizopodaceae. Firmicutes and Proteobacteria dominated *C. aurantium* endorhizosphere as also observed in other citrus pathosystems (Trivedi *et al*., 2010; Trivedi *et al*., 2011; Passera *et al*., 2018; Bai *et al*., 2019). Cyanobacteria, the third represented phylum in the endosphere of the xylem, was already observed in citrus endorhizosphere (Yang and Ancona, 2021). At the phylum fungal level, Ascomycota and Basidiomycota were the most representative taxa in the endorhizosphere, consistently with Yang and Ancona (2021).

MSD artificial infections obtained by inoculating either sour orange leaves or roots with *P. tracheiphilus* affected the richness and diversity of both bacterial and fungal microbial communities, markedly in the endorhizosphere of root-inoculated plants, where the highest concentration of *P. tracheiphilus* was detected. Upon root inoculation the endorhizosphere resulted highly colonized by the fungus leading to a shift above all the fungal community and a slight decrease in richness in bacterial communities of the rhizosphere. Variable concentrations of the fungus were detected both by quantification *via* real-time and of absolute counts obtained by ITS targeted metabarcoding as effect of a different degree of colonization of the root endorhizosphere. The highest concentration of the fungus inside the roots the largest the shift of

the other fungal taxa was. *P. tracheiphilus* was detected in the xylem of sour orange seedling as an effect of both inoculations *via* leaves and roots. Only minor differences in beta diversity of root-associated and xylem microbiota were detected between sour orange plants regardless of whether they were inoculated or not with *P. tracheiphilus*; the analysis evidenced only difference between the analyzed plant compartments.

However, the most interesting achievement was that whatever the inoculation was performed relative abundances of bacterial and fungal communities showed some alteration. Notably, Pseudomonadaceae relative abundance upon inoculation was reduced both in the rhizosphere and the endorhizosphere. The differential abundance analysis in addition revealed that, among the other taxa, the genera *Pseudomonas* and *Burkholderia* were depleted in both compartments although more markedly in the rhizosphere of leaf-inoculated plants, while other key taxa (e.g. *Streptomyces*) were increased in the endorhizosphere of plants inoculated both through leaves and roots. The genus *Pseudomonas* belongs to the core member taxa of citrus rhizosphere (Xu *et al*., 2018) and includes many biocontrol agents of plant pathogens (Bonaterra *et al*., 2022). Some *P. mediterranea*, *P. corrugata* and *P. fluorescens* strains have been efficiently used to mitigate MSD infections, as result of inhibition of spore germination and hyphal elongation of *P. tracheiphilus* during leaf and root penetration (Coco *et al*., 2003; 2004). Consistently with our results, the populations of potentially beneficial *Pseudomonas* have decreased in other citrus pathosystems, in particular upon foot rot (Yang and Ancona, 2021), Citrus Decline Disease (Passera *et al*., 2018) and HLB infections (Trivedi *et al*., 2012).

Along with the genus *Pseudomonas*, *Burkholderia* belongs to the core member taxa of citrus rhizosphere, possessing both high rhizocompetence traits (Vancanneyt *et al*., 1996; Lugtenberg *et al*., 2001; Berg *et al*., 2005; Xu *et al*., 2018). The genus is associated with uninfected citrus root samples (Trivedi *et al*., 2010; 2012) and was reported as the dominant rhizosphere-to-rhizoplane enriched genus in HLB-infected citrus plants, encompassing species with plant-beneficial properties, able to contrast the growth of several citrus pathogens by eliciting host defense responses (Riera *et al*., 2017; Zhang *et al*., 2017; Riera *et al.*2018). *Streptomyces* is a member of the core citrus bacterial community across compartments (Blaustein *et al*., 2017; Xu *et al*., 2018; Ginnan *et al*., 2020) and, consistently with our results, was enriched in citrus roots of HLB late-symptomatic citrus trees, thus not alleviating disease symptoms (Ginnan *et al.*, 2020), whereas, by contrast, its root endophytic populations resulted decreased in foot rot-diseased plants (Yang and Ancona, 2021). *Streptomyces* is a natural antagonist of several fungal phytopathogens (LeBlanc, 2021), comprising the xylematic pathogen *Verticillium* (Carlucci *et al*., 2022); thereby a role in competition or antagonism toward *P. tracheiphilus* may be

suggested. In the xylem endosphere, only few bacterial and fungal taxa were either enriched or depleted; among the enriched taxa, *Hydrogenophaga* was significantly but weakly associated with olives with high *Xylella fastidiosa* abundance (Giampietruzzi *et al*., 2020) and the mycoparasitic fungus *Minimedusa* was associated with vascular wilt of poplar (Kwaśna *et al*., 2021). Among the fungal taxa, *Penicillium* was significantly enriched in the rhizosphere of root-inoculated plants. Encompassing species recognized as postharvest pathogens of citrus fruits (Wang *et al*., 2022), *Penicillium* is one of the most abuntant fungal genera associated with citrus roots (Blacutt *et al*., 2020); in HLB-infected plants, the populations of *Penicillium* resulted either increased (Padhi *et al*., 2019) or reduced (Zhang *et al*., 2017).

In order to get insights into the multispecies microbial interactions involving *P. tracheiphilus*, co-occurrence networks were explored in the root environment upon root- or leaf-infections with the pathogen. The study revealed that regardless to the inoculation site (leaves or roots) the root microbiota presented more intense microbial networks than those of uninoculated control plants; moreover, the negative interactions increased in the leaf-inoculated plants and many microbial associations were exclusive of the inoculated plants. Pathogen-induced network-wide changes of microbial interactions have already been observed in different *Citrus* pathosystems and plant compartments. In the HLB pathosystem, the infection determined, in the leaves, negative interactions between *C*Las and few bacterial taxa of citrus core microbiota (Blaustein *et al.*, 2017). Negative interactions among bacterial taxa were also reported under penicillin treatment for HLB control (Ascunce *et al*., 2019). Positive correlations between the abundance of *C*Las and bacterial taxa in the roots and leaves are reported less frequently (Sagaram *et al*., 2009; Trivedi *et al*., 2010; Blaustein *et al*., 2017; Ginnan *et al*., 2020).

With respect to other xylematic pathogens, the soil-borne fungus *Verticillium dalhiae* produced major changes in the topology of bacterial community networks of the rhizosphere and the endorhizosphere of olive trees (Fernández-González *et al*., 2020), while the bacterium *Xylella fastidiosa* revealed negative associations with different bacterial taxa in xylem-infected almond trees (Anguita-Maeso *et al*., 2022). The modification of the microbial communities upon inoculation with the vascular pathogen *P. tracheiphilus,* even when there is not a direct contact as occurred in the leaf-inoculated plants, also suggests it may be mediated by the activation of sour orange host physiological responses to the pathogen attack. **S**imilarly, in citrus plants affected by HLB physiological changes (i.e. diverse photoassimilates distribution) due to the leaf-infection restructured the native microbial community also in the rhizosphere, a compartment lacking of the direct competition with the phloem-limited bacterium *C*Las (Trivedi *et al*., 2012).

Two toxic glycoproteins of 93 KDa and 60 KDa (called Pt60), belonging to the malseccin complex (Nachmias *et al*., 1977; Fogliano *et al*., 1994; Fogliano *et al*., 1998), damage the chloroplasts and inhibit the photosynthetic fixation of carbon in the leaves of rough lemon (Nachmias *et al*., 1980), at times since the first stages of the disease. In many cases, toxins have been shown to reproduce, in part or in whole, the symptoms of the disease (Gentile *et al*., 1992; Fogliano *et al*., 1998). The analysis of *P. tracheiphilus* transcriptome in *C. jambhiri* revealed a multifaceted strategy involving in addition to toxins the destruction of plant defensive secondary metabolites, the optimization of fungus development and pathogenesis to make the host succumb (Sicilia *et al*., 2022). On the other hand, under fungus challenge by leaf-inoculation the rough lemon genes that are involved both in the light harvesting and the photosynthetic electron flow were significantly down-regulated (Russo *et al*., 2021). In addition, in a study on *C. limon* the production of lipoperoxide, hydrolytic enzymes, oxydases and the toxic accumulation of H_2O_2 and NH_4 in the leaves and the differential ability to modulate the lipoperoxidative pathway were pivotal in the infections (Reverberi *et al*., 2007). In the xylem of infected leaves, water transport alteration was determined by the extensive clogging of the veins, due to the progressive degradation of membranes, which increased the hydraulic resistance in the leaves, thus facilitating the spread of the pathogen in the tissue (Raimondo *et al*., 2007).

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Supplementary Material: chapter 2

Sour orange microbiome is affected by infections of Plenodomus tracheiphilus causal agent of Citrus Mal secco disease

Supplementary Figures

Figure S1: Alpha diversity estimations of the bacterial (A-B) and fungal (C-D) communities using Chao1 and Shannon richness and diversity indices, respectively, for the samples (control, leaf-inoculated and root-inoculated plants) grouped by compartment (rhizosphere, endorhizosphere and xylem).

Figure S2: PCoA biplot based on the Bray–Curtis dissimilarity index depicts bacterial (A) and fungal (B) communities beta diversity. Each compartment is represented by a specific symbol (dot for rhizosphere, triangle for endorhizosphere and square for xylem) and associated with specific colours according to the treatment (lightblue: control, orange: leaf inoculation and red: root inoculation).

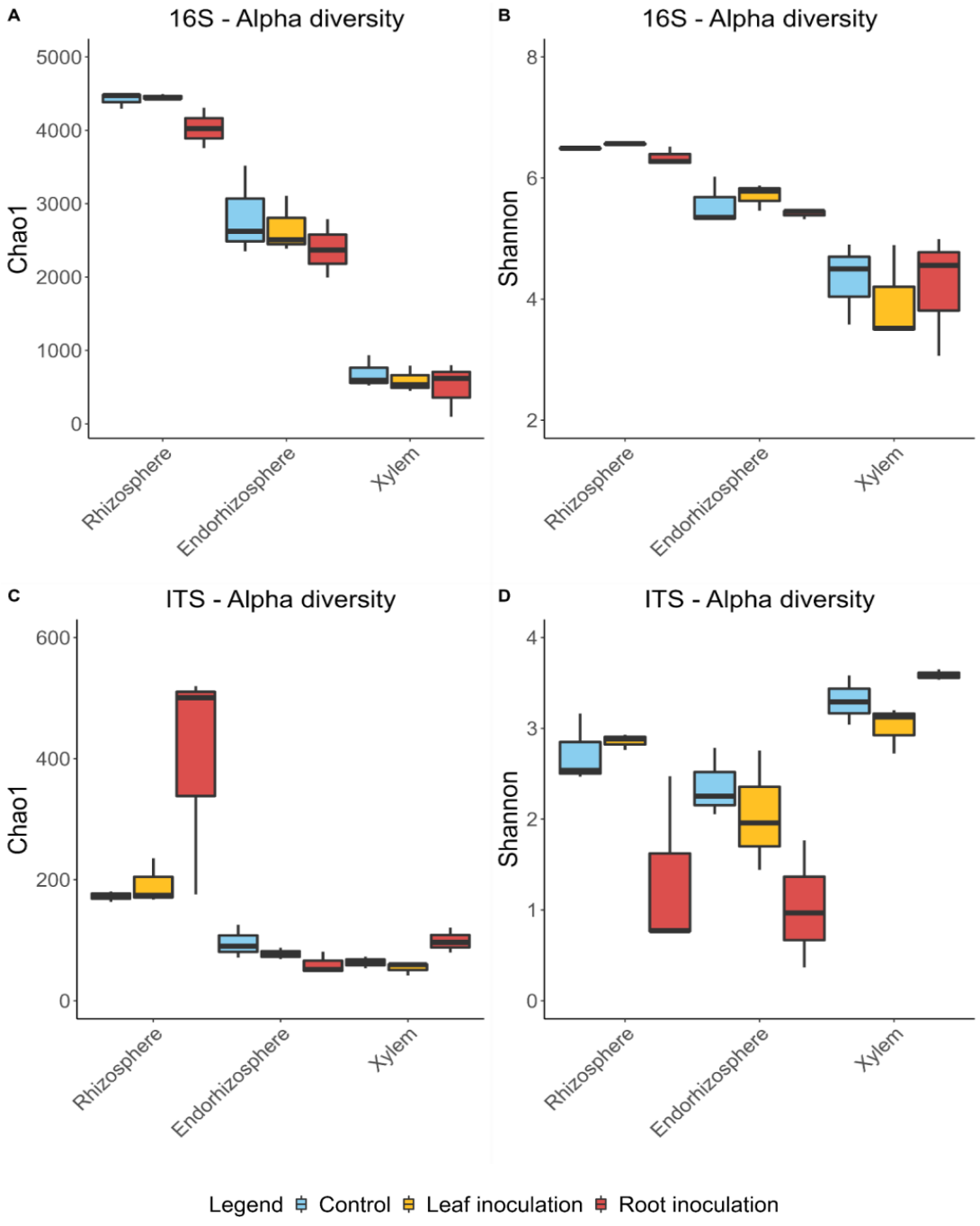
Figure S3: Phylogenetic tree of *Plenodomus tracheiphilus* isolates and representative Pleosporales based on the partial ITS1 gene sequences. The three OTUs identified as *Plenodomus chrysanthemi* in samples from leaf- and root-inoculated plants are reported in bold. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 30.57% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 157 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Figure S4: Absolute abundance of the four most abundant OTUs (HM344177.1, HM152696.1, New.ReferenceOTU74 and FJ893269.1) classified to the genus level as *Pseudomonas*. CR, RR, LR; rhizosphere samples from control (uninoculated), root-inoculated and leaf-inoculated sour orange seedlings, respectively; CE, RE, LE; endorhizosphere samples from control (uninoculated), root-inoculated and leaf-inoculated sour orange seedlings, respectively.

Supplementary Tables

Table S1: Differentially abundant fungal and bacterial taxa in the xylem of leaf- and root-inoculated sour orange plants.

Figure S1: Alpha diversity estimations of the bacterial (A-B) and fungal (C-D) communities using Chao1 and Shannon richness and diversity indices, respectively, for the samples (control, leaf-inoculated and root-inoculated plants) grouped by compartment (rhizosphere, endorhizosphere and xylem).



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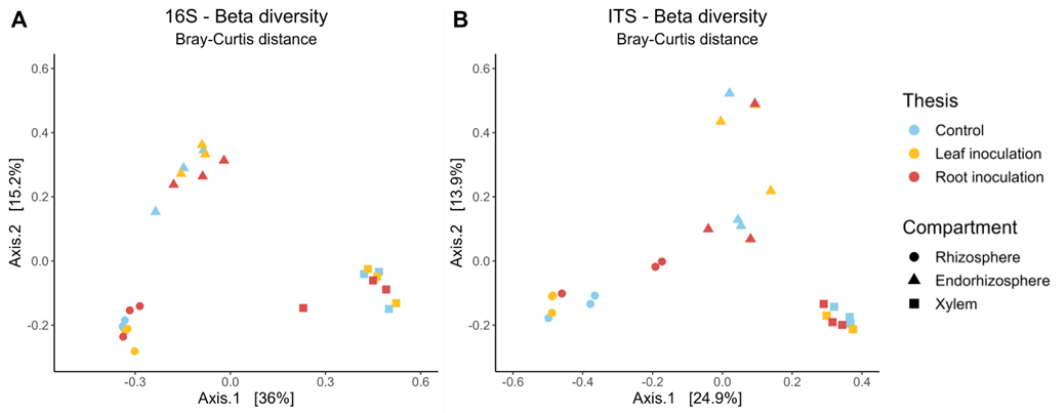
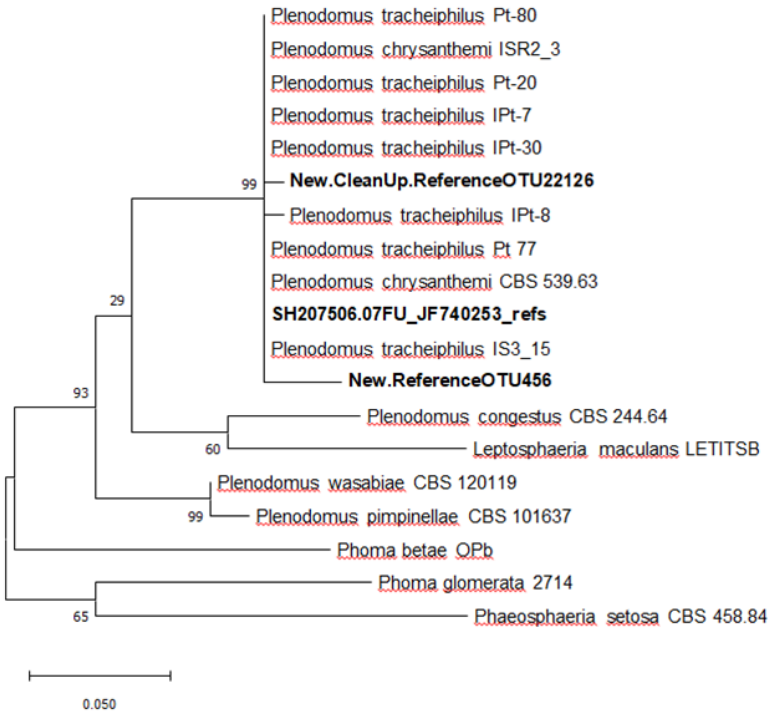


Figure S3: Cladogram of *Plenodomus tracheiphilus* isolates and representative Pleosporales based on the partial ITS1 gene sequences. The three OTUs identified as *Plenodomus chrysanthemi* in samples from leaf- and root-inoculated plants are reported in bold. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 30.57% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 157 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).



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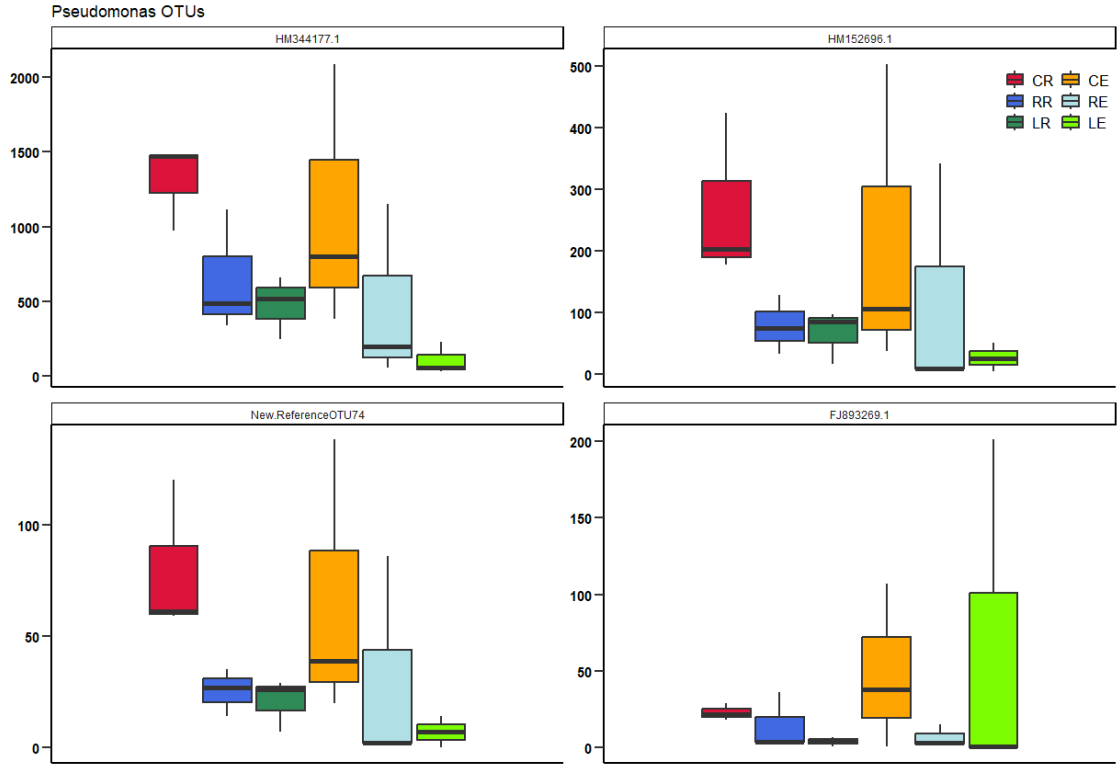

Table S1: Differentially abundant fungal and bacterial taxa in the xylem of leaf- and root-inoculated sour orange plants.

Kingdom	Phylum	Class	Order	Family	Genus	log2FoldChange	*p* value	Adj. *p* value	Treatment
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Other	Other	2.624928566	0.012736436	0.942691007	L.I
	Basidiomycota	Agaricomycetes	Cantharellales	Incertae sedis	Minimedusa	-5.288427445	0.035027791	0.942691007	L.I
	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	Plenodomus	3.597136175	0.022614041	0.988677062	R.I.
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga	20.46820279	4.34366E-15	1.05117E-12	L.I.
	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Peptoniphilus	5.836031827	0.008060717	0.967188348	L.I.
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum	-5.693456822	0.024423643	0.967188348	L.I.
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Other	-8.385115933	0.031927791	0.967188348	L.I.
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ramlibacter	-5.898439856	0.0346811	0.967188348	L.I.
	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Finegoldia	4.35221577	0.049206956	0.967188348	L.I.
	Planctomycetes	Planctomycetia	Other	Other	Other	6.152179426	0.020031571	0.987781033	R.I
	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	2.980635134	0.031195915	0.987781033	R.I

3. Biocontrol activity against *Plenodomus tracheiphilus* and genome analysis of *Pseudomonas corrugata* and *P. mediterranea* strains[2]

Abstract

The *Pseudomonas fluorescens* complex encompasses multiple species recognized for their plant beneficial role, promoting plant growth and competing against phytopathogens.

P. corrugata and *P. mediterranea* are two ubiquitous species associated to the root environment belonging to this complex and, in particular, to the *P. corrugata* phylogenomic subgroup. In this study, a collection of *P. corrugata* and *P. mediterranea* strains was tested *in vitro* in dual-plate assays against different isolates of *Plenodomus tracheiphilus* (*Pt*), causal agent of citrus Mal secco disease. All strains released in the medium compounds with antifungal activity, although to a different extent, with *P. corrugata* being overall slightly more competitive than *P. mediterranea*. Pt isolates PVCT Pt10 and PVCT Pt57 resulted the most virulent. A subset of this *Pseudomonas* collection was further tested toward *P. tracheiphilus* PVCT Pt57 for the activity of their culture filtrates and volatile compounds in terms of inhibition of myceliar growth and conidia germination. Two strains, *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C showed the greatest antifungal activity of culture filtrates and volatiles and were further *in vivo* tested. The leaves of sour orange and lemon plants were pre-treated with a suspension of either PVCT 4.3t or PVCT 3C strain and then inoculated with the fungal pathogen. Pre-treatments of citrus plants significantly reduced disease progression compared to control plants inoculated only with the fungus, although no significant differences were observed between the treatment with the two bacterial strains. Genome sequencing and comparative genomic analysis of these strains revealed that they harbour a repertoire of gene clusters encoding for secondary metabolites, mainly cyclic lipopeptides with wide-spectrum antimicrobial activity.

Key words: *Pseudomonas*, *in vivo* activity, Mal secco, draft genome, metabolites.

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3.1 Introduction

Pseudomonas is a highly diverse and metabolically versatile genus which falls within the gamma subclass of the Proteobacteria ubiquitously found in the environment (Kersters et al., 1996). Multiple *Pseudomonas* species are recognized for their plant beneficial role, known as plant growth-promoting rhizobacteria (PGPR), and are mainly found within isolates and species from the *Pseudomonas fluorescens* complex, including more than fifty named species (Mulet *et al*., 2010; Garrido-Sanz *et al*., 2016; David *et al*., 2018). Species from this complex are described as PGPR useful for biocontrol and biofertilization applications due to their ability to suppress plant diseases (Ellis *et al*., 2000; Raaijmakers *et al*., 2002; Raaijmakers *et al*., 2009) colonizing efficiently plant tissues (Capdevila *et al*., 2004), producing antimicrobial compounds (Weller *et al*., 2007; Pierson and Pierson, 2010; Raaijmakers *et al*., 2010), eliciting plant systemic resistance (Han *et al*., 2006; Bakker *et al*., 2007) and competing with pathogens for iron uptake through siderophores production (Berendsen *et al*., 2015). Other *Pseudomonas* promote host plant growth by solubilizing phosphorus, fixing atmospheric nitrogen and synthesizing phytohormones (Cavalcante *et al*., 2007; Taurian *et al*., 2010; Luo *et al*., 2012; Andrade *et al*., 2014).

Pseudomonas corrugata constitute one of the subgroups within the *Pseudomonas fluorescens* species complex, supported by multilocus sequence analysis (MLSA), phylogenomics (Richter and Rosselló-Móra, 2009) and genome-to-genome blast distance phylogeny (GBDP) approach (Meier-Kolthoff *et al*., 2013). The subgroup is composed of six named species, *P. corrugata*, *P. mediterranea*, *P. thivervalensis*, *P. kilonensis*, *P. brassicacearum*, the recently reclassified *P. ogarae* and *Pseudomonas* sp. SH-C52 (Van der Voort *et al*., 2015; Garrido-Sanz *et al*., 2016; Lalucat *et al*., 2020; Garrido-Sanz *et al*., 2021). Most biocontrol and PGP (Plant Growth Promoting) agents have been described within the *P. corrugata* subgroup; as an example, *P. brassicacearum* strains isolated from *Arabidopsis*, canola, potato, strawberry, tomato, and agricultural soils across a large geographic area demonstrate exceptional biocontrol activity, mainly toward phytopathogenic fungi of cereal crops (Achouak *et al*., 2000; Ortet *et al*., 2011; Zhou *et al*., 2012; Loewen *et al*., 2014; Khayi *et al*., 2015; Novinscak *et al*., 2016; Zachow *et al*., 2017a).

Among the PGPR traits, the production of the antibiotic 2,4-diacetylphloroglucinol (DAPG) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase are probably the most studied within pseudomonads. The biosynthesis of the broad-spectrum antibiotic DAPG (Bangera and

Thomashow, 1999) has also shown to elicit systemic resistance (Weller *et al*., 2012) and stimulate lateral root growth by interacting with the signalling pathway of the phytohormone auxin (Brazelton *et al*., 2008). Biological evidence of DAPG production has been reported in *P*. *ogarae* F113 (Shanahan *et al*., 1992) and in *P*. *brassicacearum* Q8r1-96 (Raaijmakers and Weller, 2001). In *P. brassicacearum*, this compound exerts also a phytotoxic activity, being determinant for pathogenicity on tomato and inhibiting wheat growth and seed germination (Yang *et al.*, 2018; 2020).

Recently, the presence of DAPG biosynthetic genes was identified as a marker for commensalism and negatively correlates with lipopeptides biosynthetic clusters in species within the *P. fluorescens* group (Melnyk *et al*., 2019). Additionally, ACC deaminase promotes root growth (Vacheron *et al*., 2016) and reduces abiotic stresses (Saravanakumar and Samiyappan, 2007; Sapre *et al*., 2019), thus contributing to phytostimulation.

Cyclic lipopeptides (CLPs) and polyketides that are synthesized by non-ribosomal peptide synthetases (NRPS) and by polyketide synthases (PKS), respectively, are major antimicrobial secondary metabolites produced by PGPR *Pseudomonas* (Ghequire and De Mot, 2014; Geudens and Martins, 2018; Stringlis *et al*., 2018) CLPs are active against a wide range of soil-borne phytopathogens (Berry *et al*., 2014; Michelsen *et al*.. 2015; Van Der Voort *et al*., 2015) and variants belonging to the different CLPs families were characterized in *P. corrugata* and *P. mediterranea* (Emanuele *et al*., 1998; Scaloni *et al*., 2004; Licciardello *et al*., 2012). Strains producing three antimicrobial lipopeptides (LPs) within *P. corrugata* subgroup have been recently classified as type I LP-producers, synthetizing compounds with 8 (brabantamide-like), 9 (mycin) and 22 (peptins) amino acids (Oni *et al*., 2022).

Within *P. corrugata* subgroup, *P. corrugata* and *P. mediterranea* are two ubiquitous non-fluorescent species isolated from bulk soil or the root environment of diverse plant species (Catara, 2007) and recognized as causal agents of tomato pith necrosis (Scarlett *et al*., 1978). Although crop losses have occurred due to infection with *P. corrugata* (Catara, 2007; Trantas *et al*., 2015), the bacterium is considered a weak and opportunistic pathogen, infecting tomato under particular growing conditions (CABI, 2006), while acting as PGPR/biocontrol agent in other hosts (Catara *et al*, 2007; Gu *et al*., 2020; Zhou *et al*., 2021). These observations suggest that the genotype and physiological state of the plant, the presence of biotic/abiotic stressors, and the ability of bacteria to manipulate the plant immune system could explain the ambivalent behaviour of this species (Gislason and de Kievit, 2020). Both species do not produce DAPG, probably because this compound can elicit induced systemic resistance (ISR) in plants (Almario *et al*., 2017).

The pathogenicity of *P. corrugata* and *P. mediterranea* is linked to the production of corpeptins and cormycin, phytotoxic cyclic lipopeptides (CLPs) (Strano *et al*., 2015) whose biosynthetic loci are located within a genetic island (named lipopeptide/quorum sensing (LPQ) island) conserved among *P. corrugata* subgroup members (Licciardello *et al*., 2018; Melnyk *et al*., 2019; Girard *et al*., 2020). In *P. corrugata* and *P. mediterranea*, CLPs corpeptins biosynthesis is regulated by the quorum sensing system mediated by the signal molecules acyl-homoserine-lactones (AHLs) (Licciardello *et al*., 2012; 2018).

Many *P. corrugata* strains have been successfully tested as biological control agents in different pathosystems (Catara, 2007) and have been demonstrated to harbour multiple beneficial traits for plants (Trantas *et al*., 2015), which has led to approved field applications of *P. corrugata* with some strains patented for biocontrol (Catara, 2007). *P. corrugata in vitro* antagonistic activity was reported against genera *Bacillus* spp*., Clavibacter michiganensis* subsp. *michiganensis, Erwinia (Brenneria) quercina, Burkholderia cepacia, Pseudomonas syringae* pv. pisi*, P.s.* pv. tomato*, Rhizobium radiobacter* (Bell *et al.,* 1995; Chun and Leary, 1989; Cirvilleri *et al.,* 2001) as well as some phytopathogenic fungi: *Gaeumannomyces graminis* var. *tritici* (Ryder and Rovira, 1993), *Monilinia fructicola* (Smilanick *et al*., 1993); *Gibberella pulicaris* (Schisler and Slilinger, 1994), *Penicillium digitatum*, *Botrytis cinerea* (Cirvilleri *et al.,* 2000), *Sclerotinia sclerotiorum* (Fernando *et al.,* 2005), *Alternaria alternaria* and *Fusarium oxysporium* (Trivedi *et al.,* 2008) and against the chromista *Pythium aphanidermatum* (Zhou and Paulitz, 1993).

P. corrugata strain 2140 was isolated from wheat field soil and its rifampicin resistant derivative strain 2140R colonized wheat roots after application on the seeds (Ryder and Borret, 1991), reducing take all disease of wheat by *G. graminis* var. *tritici* (Barnett *et al.,* 1998; Ryder *et al.,* 1999) and *Pythium* root rot in sugarbeet (Schmidt *et al.,* 2004). A mutant of this strain marked with a Tn7 derived transposon containing *lac*ZY has been authorized for field release in Australia (Ryder *et al.,* 1994) (Choi *et al.,* 2003). A *lux*AB mutant of the same strain (*2140lux*AB) has been employed in a commercial formulation of seed pellet of sugar beet toward *Pythium ultimum* (Schmidt *et al.,* 2004). *P. corrugata* CFBP 2431^{T} significantly reduced fruit dieback from brown rot caused by *Monilinia fructicola* in application up to 12 hours after inoculation of the pathogen and with greater efficacy than the fungicidal compound thiabendazole (Smilanick *et al*., 1993). *P. corrugata* strain 13 reduced the attraction of the zoospores of the oomycete root rot agent *Pythium aphanidermatum* to cucumber root exudates and their germination (Zhou and Paulitz, 1993). In cucumbers grown in greenhouse in rock wool system, the same strain reduced disease severity under high disease levels and increased yields in the absence of the pathogen, suggesting a plant growth promotion (PGPR) effect

(McCullagh *et al.,* 1996). Evidence of the induction of systemic resistance has been observed in the same pathosystem after inoculating the pathogen and the *P. corrugata* biocontrol strain in the spatially separated root system where stem rot was delayed and disease index reduced (Zhou and Paulitz, 1994). Furthermore, a correlation has been observed between resistance induction and salicylic acid production (Chen *et al.,* 1999).

P. corrugata strain NRRLB-30409 a soil bacterium originally isolated from a temperate site of Indian Himalayan Region (IHR) was examined for its antagonistic activities against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum* (Pandey and Palni, 1998; Kumar *et al.,* 2007; Trivedi *et al.,* 2008). The antagonism was affected by growth medium, pH and temperature. Maximum reduction in biomass, of *A. alternata* and *F. oxysporum*, was observed at pH 5.5 and 21°C. The production of siderophore, ammonia, lipase and chitinase in growth medium by *P. corrugata* were considered contributing to the antagonistic activities of the bacterium. *P. corrugata* strain NRRLB-30409 did not produce HCN *in vitro* whereas ammonia production was detected (Trivedi *et al.,* 2008). *Pseudomonas corrugata* strain P94, isolated from agricultural soil, exhibited an important antagonistic activity against *Botrytis cinerea* and other phytopathogenic fungi (*C. fimbriata*, *M. laxa*, *M. grisea*, *P. aphanidermatum*, *P. capsici*, *A. solani*, *R. cerealis*, *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f. sp. *lilii*) and bacteria (*P. corrugata* ICMP 5819, *P. syringae*, *A. avenae*, *R. solanacearum*) (Guo *et al*., 2007). P94 showed also inhibition activity towards tomato grey mildew *in vitro* and produced HCN, protease, phosphatase and indole acetic acid. Sang *et al.* (2008) demonstrated that *P. corrugata* strains CCR04 and CCR80 are suppressive to *Phytophthora* blight of pepper caused by *P. capsici* through an efficient colonization of pepper roots promoted by increased biofilm formation, enhanced motility (swimming and swarming activities) due to the ability to respond to some amino acids, organic acids and sugars present in the root exudates of pepper as well as reduced sensitivity to oxidative species such as H_2O_2 (Sang and Kim, 2014). This phenomenon was reported in previous studies, in which increased microbial activity was associated with suppression of plant diseases, such as damping-off in cucumber caused by *P. ultimum* and *Phytophthora* blight of pepper caused by *P. capsici* (Chen *et al.,* 1988; Kim *et al.,* 2012; Sang and Kim, 2012).

The efficacy of *P. corrugata* as a biological control agent seems linked to its high rhizocompetence. In addition, its *in vitro* wide-spectrum antagonistic activity shows it is able to compete for iron through the siderophore corrugatin and by producing antimicrobial substances (Meyer *et al*., 2002; Risse *et al*., 1998). Antimicrobial activity could be the result of the production CLPs (Emanuele *et al*., 1998; Scaloni *et al*., 2004; Strano *et al*., 2015), hydrogen cyanide (Ramette *et al*., 2003; Strano *et al*., 2017) and, although not confirmed sperimentally, of

other substances, such as inhibitory volatiles (Fernando *et al*., 2005), 2,4-diacetylphluoroglucinol and pyrrolnitrin (Garbeva *et al*., 2001). Both species present also a mangotoxin-like biosynthetic cluster, putatively acting as a regulator of secondary metabolites production (Loper *et al*., 2012; Carrión *et al*., 2014; Van der Voort *et al*., 2015).

In culture, *P. corrugata* produces the cyclic lipopeptides cormycin A and corpeptins (A and B) (Emanuele *et al*., 1998; Scaloni *et al*., 2004; Licciardello *et al*., 2012).

Both CLPs are active against the Gram-positive bacterium *Bacillus megaterium* and cormycin also exhibited activity against the yeast *Rhodotorula pilimanae* (Emanuele *et al*., 1998; Scaloni *et al*., 2004). However, for corpeptins no anti-oomycete activity has been reported (Emanuele *et al*., 1998). A biosynthetic gene cluster highly homologous to the cyclocarbamate brabantamide cluster (*braABC* operon) described in *Pseudomonas* sp. SH-C52 was identified in *P. corrugata* and *P. mediterranea*, although the production of this metabolite has not yet been described in these species (Schmidt *et al*., 2014; Licciardello *et al*., 2018; Girard *et al*., 2020). In *P. corrugata*, genes of the putative brabantamide biosynthesis cluster are, along with corpeptins genes, controlled by the transcriptional regulators PcoR-RfiA (Schmidt *et al*., 2014; Van Der Voort *et al*., 2015; Licciardello *et al*., 2018). Brabantamide showed activity against Gram-positive bacteria (Reder-Christ *et al*., 2011; Schmidt *et al*., 2014) and Oomycetes of the genus *Phytophthora* (Van Der Voort *et al*., 2015).

As PGPR, *P. corrugata* may promote plant health *via* auxin biosynthesis; indole acetic acid (IAA) (Spaepen *et al*., 2007), the main auxin found in plants, plays in fact an important role in growth and development as well as response to abiotic stresses (Benjamins and Scheres, 2008).

P. corrugata and *P. mediterranea* contain indole acetic acid (IAA) biosynthetic genes, while only *P. mediterranea* strains have homologs to the *iacHABICDEFG* cluster involved in IAA catabolism (Leveau and Gerars, 2008). Another way to stimulate plant growth is the solubilization of inorganic phosphate; at this purpose, bacteria produce gluconic acid, which biosynthesis is determined by the glucose dehydrogenases (*gdc*) and redox cofactor pyrroloquinoline quinone (*pqq*) genes (Rodríguez and Fraga, 1999; Rodríguez *et al*., 2001; Arcand and Schneider, 2006; Goldstein, 2007; Choi *et al*., 2008). These genes are conserved in *P. corrugata* and *P. mediterranea* strains (Gislason and de Kievit, 2020).

Among PGP strains, *P. corrugata* SP77 induced a significant increase in the dry weight of the shoots and in the fresh weight of *Medicago* sp. nodules (Zineb *et al*., 2020) due to the high solubilization of the inorganic phosphate; a mutant of the *P. corrugata* NRRLB-30409 is an efficient phosphate solubilizing strain in a wide temperature range (4°C-28°C) (Trivedi *et al*., 2008); *P. corrugata* RM1-1-4 colonizes the rhizosphere of *Brassica napus* and possesses the

biosynthetic clusters for the production of ACC-deaminase, auxin, biofilm, the siderophore rhizobactin and spermidine. There are also genes that predict the synthesis of volatile components: hydrogen cyanide synthase HcnA and orthologs of genes necessary for the biosynthesis of other volatile components such as 2,3-butanediol and its precursor acetoin (Zachow *et al*., 2017b). *P. corrugata* BIPS-11 improves the stem diameter, shoot dry weight and root dry weight of *Sorghum bicolor* plants in hydroponic cultivation and solubilizes phosphate (Amora-Lazcano *et al*., 2022). *P. corrugata* strain 1 (rhizospheric isolate from a subtropical region) and *P. corrugata* strain 7 (rhizoplane isolate from a temperate region) improve growth and yield in *Amaranthus paniculatus* and *Eleusine coracana* (Pandey *et al*., 1999). In comparison to *P. corrugata*, *P. mediterranea* has been used rarely as PGPR and biocontrol agent; the type strain of the species, CFBP 5447, has been well studied to efficiently synthesize polyhydroxyalkanoate (PHA) and extracellular CLPs (Licciardello *et al*., 2012; 2019). *P. mediterranea*, as its closely related species *P. corrugata*, produces under inducing conditions CLPs with antimicrobial activity toward *B. megaterium* and *R. pilimanae* (Emanuele *et al*., 1998; Scaloni *et al*., 2004; Licciardello *et al*., 2012); in particular, *P. mediterranea* strain EDOX, a tomato leaf endophyte, has been demonstrated to produce two novel CLPs designated medipeptins (A and B); medipeptin A exerts a strong antimicrobial activity against Gram-positive bacteria by binding to both lipoteichoic acid (LTA) and lipid II as well as by forming pores in membranes (Zhou *et al*., 2021). The lost of antimicrobial activity toward *B. megaterium* and the plant pathogen *P. syringae* pv. tomato may be associated, for both *P. corrugata* and *P. mediterranea*, to a smooth colony phenotype (Trantas *et al*., 2015). Among biocontrol agents, *P. mediterranea* S58, a tobacco rhizoshere isolate, contains the infections of wheat sharp eyespot (*Rhizoctonia solani*) and tobacco wildfire diseases (*Pseudomonas tabaci*). It showed also PGP activity as it was capable of solubilizing organic phosphate and producing siderophores, proteases, ammonia and 3-indoleacetic acid and, as a consequence, of changing *Arabidopsis thaliana* root system architecture through an increased number of lateral roots. Genome analysis suggested that this strain was a rich producer of cyclic lipopeptides (CLPs) and induced the expression of genes associated with PTI (Pattern-Triggered Immunity) and programmed cell death (Gu *et al*., 2020). Another endophytic *P. mediterranea* strain, HU-9, showed high biocontrol efficacy against crown and root rot caused by *Fusarium* sp., a common wheat disease in arid environments (Ullah *et al*., 2020). *P. mediterranea* and *P. corrugata* strains have been reported to reduce *in vivo* the infections of the fungus *Plenodomus tracheiphilus*, a detrimental vascular pathogen of citrus causing Mal secco disease (MSD) (Coco *et al*., 2003; 2004). No curative approaches have been developed for MSD management. Investigating this pathosystem, we deepened the *in vitro* and *in vivo* biocontrol activity of a

collection of *Pseudomonas* sp. strains belonging to the *P. corrugata* phylogenomic group toward *P. tracheiphilus*, focusing on the potential antifungal activity of different metabolites produced in culture. Genome sequencing and comparative genomic analysis of two promising bacterial strains revealed that both possess a repertoire of secondary metabolites biosynthetic gene clusters, mainly encoding for lipopeptides.

3.2 Materials and Methods

3.2.1 Bacterial and fungal strains and culture conditions

Bacteria and fungi used in this study are listed in Table 1. *P. corrugata* and *P. mediterranea* strains were routinely cultured at 28°C in either nutrient agar (Oxoid, Milan, Italy) supplemented with 1% dextrose (NDA) or Luria-Bertani (LB) broth. *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C cell suspensions were obtained by inoculating 250 mL-Erlenmeyer flasks containing LB broth with a single bacterial colony from a fresh NDA culture. The flasks were placed overnight into an orbital shaker at 28°C under continuous shaking (180 rpm). Bacterial cultures were centrifuged at 9000 x g for 20 minutes and the supernatants were discarded. Bacterial pellets were washed three times, resuspended in sterile distilled water (SDW) and diluted up to 10^8 cfu mL^{-1} for the *in vivo* tests. For bacterial cell-free culture filtrates production, strains were grown in IMM (Improved Minimal Medium), a medium inducing the production of the antimicrobial cyclic lipopeptides (CLPs) (Surico *et al.*, 1988), according to Licciardello *et al.* (2009). 24-h LB cultures of each bacterial strain were transferred into 100 mL-Erlenmeyer flasks up to a final concentration of 10^7 cfu mL^{-1}. The cultures were incubated without shaking at 28°C for four days, centrifuged (9000 x g, 20 min.) and the supernatant was sterilized by filtration using a 0.22-µm Millipore filter (Millipore, Billerica, MA, U.S.A.). The fungal cultures of the citrus pathogen *Plenodomus tracheiphilus*, causal agent of Mal secco disease (MSD), were routinely maintained on Potato Dextrose Agar (PDA, Oxoid) at 23°C or at 25°C (dual-plate antagonistic assays with bacteria). *P. tracheiphilus* phialoconidia were obtained in carrot broth (300 g of carrots/l), as described by Salerno and Catara (1967) with minor modifications; two small portions of PDA along the actively growing area of seven-days-

old fungal colonies were transferred to 250 mL-fluted flasks containing 100 mL of carrot broth. The flasks were placed into an orbital shaker under continuous shaking (220 rpm), at 22°C and diffused light up to four days. The culture broth was filtered with four layers of sterile gauze in order to eliminate the mycelium and centrifuged at 6000 x g for 15 minutes. The pellet was washed three times and resuspended in SDW. Finally, conidia were counted using a hematocytometer and diluted up to 10^6 mL^{-1} with SDW for the *in vitro* and *in vivo* antagonistic tests.

Table 1: Bacterial and fungal strains used in *in vitro* and *in vivo* antagonistic tests.

Strain	Species	Geographic origin	Isolation source	References
PVCT 4.3t	*Pco*	Sicily, Italy	Soil	Catara *et al.*, 1997
PVCT 5.1t	*Pco*	Sicily, Italy	Soil	Catara *et al.*, 1997
CFBP 5404	*Pco*	Tenerife, Spain	Pepper	Sutra *et al.*, 1997
CFBP 5454	*Pco*	Sicily, Italy	Tomato	Catara *et al.*, 1997
NCPPB 2445^T	*Pco*	UK	Tomato	Sutra *et al.*, 1997
PVCT 3C	*Pme*	Sicily, Italy	Tomato	Catara *et al.*, 1997; 2002
PVCT 1.6	*Pme*	Sicily, Italy	Tomato	Catara *et al.*, 1997
PVCT P1	*Pme*	Sicily, Italy	Tomato	Catara *et al.*, 1997
CFBP 5444	*Pme*	Sicily, Italy	Tomato	Catara *et al.*, 1997; 2002
CFBP 5447^T	*Pme*	Sicily, Italy	Tomato	Catara *et al.*, 1997; 2002
PVCT Pt10	*Pt*	Sicily, Italy	Lemon	Grasso and Catara, 2006
PVCT Pt57	*Pt*	Greece	Lemon	Grasso and Catara, 2006
PVCT Pt17	*Pt*	Sicily, Italy	Sour orange	Grasso and Catara, 2006
PVCT Pt67	*Pt*	Greece	Sweet orange	Grasso and Catara, 2006
PVCT Pt52	*Pt*	Greece	Lemon	Grasso and Catara, 2006

CFBP, Collection Francaise des Bacteries Phytopathogenes, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK; PVCT, Patologia Vegetale, University of Catania, Italy. T, type strain. *Pco*, *Pseudomonas corrugata*; *Pme*, *Pseudomonas mediterranea*; *Pt*, *Plenodomus tracheiphilus*.

3.2.2 Biocontrol activity of *Pseudomonas* sp. strains against *P. tracheiphilus*

3.2.2.1 In vitro antagonistic activity

Activity of compounds diffusible in the growth medium

The production of *P. corrugata* and *P. mediterranea* antifungal compounds diffusible in the agar medium was evaluated *in vitro* on PDA. To assess the bacterial antagonistic effect on *P. tracheiphilus*, small masses of bacterial strains from fresh cultures grown on NDA medium were transferred onto two sides of a Petri dish (1 cm from the edge) containing PDA. The following day, four-mm mycelia plugs from well-grown PDA cultures of *P. tracheiphilus* isolates were cut off with a sterile scalpel and placed in the middle of the Petri dishes. For each test, control plates containing only the fungal culture were maintained. Plates were incubated at 25 °C and the radius of the fungal colony was measured for up to two weeks. All bacterial strains were evaluated in three independent replicates. The antifungal activity was expressed as percentage of growth inhibition (PGI) and calculated according to the formula of Vincent (1947) and Zygadlo *et al.* (1994): PGI (%) = 100·(GC-GT)/GC; where GC represents the average value of the mycelium diameter in the control plates (without bacteria) and GT represents the mean value of the fungus diameter on the treated PDA plates contain the antagonistic bacteria.

Activity of culture filtrates

The cell-free bacterial broth of *P. corrugata* and *P. mediterranea* strains was used to assess influence of extracellular metabolites on *P. tracheiphilus* radial growth or phialoconidia germination. Cell-free culture filtrate of each bacterial strain was added to a warm PDA medium (55 °C) at 20% and 50% final concentrations (v/v). PDA plates without culture filtrate were used as controls. Fungal mycelial plugs of 4 mm diameter were placed centrally in the amended medium and incubated at 23°C until negative control growth had covered the whole surface of the plate. The percentage of fungal growth inhibition (PGI) was calculated using the formula of Vincent (1947) and Zygadlo *et al.* (1994) previously described.

The inhibition activity of cell-free culture filtrates against *P. tracheiphilus* phialoconidia germination was assayed in 100 mL-Erlenmeyer flasks containing 70% (v/v) carrot broth and a fresh phialoconidial suspension (10^6 conidia mL^{-1}) of *P. tracheiphilus* PVCT Pt57. Each flask was incorporated aseptically with 20% (v/v) *P. corrugata* PVCT 4.3t or *P. mediterranea* PVCT 3C cell-free culture filtrate and incubated on a rotary shaker (150 rpm) at 25°C with diffused light for 5 days. The culture broth was filtered with four layers of sterile gauze to collect the fungal mycelia and mycelia fresh weights were determined. Then, the fungal mycelia were dried at 68°C to determine dry weights. The inhibition of conidia germination was expressed relative to a control replacing the culture filtrate with IMM medium. For each test, bacterial filtrates were evaluated in three independent replicates.

Activity of volatile compounds

The influence of volatile compounds by *P. corrugata* (strains PVCT 4.3t and CFBP 5454) and *P. mediterranea* (strains PVCT 3C and CFBP 5447^{T}) in antifungal activity against mycelial growth and conidia germination was investigated by a double-dual plate assay (Strano *et al.*, 2017). Petri dishes containing PDA medium were inoculated with four millimetrer mycelium plug or with 30 µl of conidial suspension (1 x 10^3 conidia mL^{-1}) of *P. tracheiphilus* PVCT Pt57. Separate PDA plates were streaked with a 24h-colony of the *Pseudomonas* strain and then placed over the plate with the fungus, avoiding direct contact between the two organisms. The two compartment plates were sealed with three layers of Parafilm® to make a double-dish layer and were incubated at 25°C. The production of volatile compounds was then determined based on inhibition of the PGI of the mycelium radial growth or the number of conidia with visible germ tubes. Control plates were inoculated only with the fungus or the conidial suspension. All bacterial strains were evaluated in three independent replicates.

3.2.2.2 In vivo antagonistic activity

The biocontrol activity of *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C against *P. tracheiphilus*, causal agent of Mal secco disease (MSD) of citrus, was evaluated in the susceptible hosts *Citrus aurantium* (sour orange) and *Citrus limon* (lemon) 'Femminello siracusano 2kr'. Specifically, one-years old plants or seedlings of sour orange and adult plants of lemon were used in different independent trials. Sour orange plants were maintained in unheated greenhouse, whereas trials on lemon were conducted both in unheated greenhouse and outdoor, simulating open filed conditions. All plants were grown in pots filled with agricultural soil. *C. aurantium* and *C. limon* plants were treated with a cell suspension of either *P. corrugata* PVCT 4.3t or *P. mediterranea* PVCT 3C and leaf-inoculated with *P. tracheiphilus* PVCT Pt57.

Specifically, three days before the leaf-inoculation with the fungal pathogen, plants were treated by spraying both the abaxial and adaxial side of the leaves with an aqueous cell suspension (10^8 cfu mL^{-1}) of *P. corrugata* PVCT 4.3t or *P. mediterranea* PVCT 3C. In the greenhouse experiments on *C. limon*, plants were treated only with *P. mediterranea*. Leaf-inoculations with the pathogen were performed by placing 10 µl of aqueous suspension of *P. tracheiphilus* phialoconidia (10^6 conidia mL^{-1}) on the secondary veins of young and fully-expanded leaves of sour orange or lemon plants. Subsequently, leaves were slightly wounded in correspondence of the suspension drops by gently pressing with three entomological needles. For each trial, control plants were treated either only with the bacterial suspensions or with sterile distilled water (SDW) and not inoculated with the fungus (negative controls) or were inoculated only with the

fungus (positive control). In the different trials on sour orange and lemon, for each treatment (*P. corrugata* or *P. mediterranea*), a variable number of plants and leaves was treated and leaf-inoculated with the pathogen (Table 2). Weekly, up to 35 days post-inoculation, the leaf infection symptoms were monitored and evaluated using the empirical scale proposed by Luisi *et al.* (1979), with some modifications: 0, no symptoms; 1, chlorotic halo around the inoculation site; 2, vein chlorosis near the inoculation site; 3, vein chlorosis extended to the leaf margin with a) chlorosis around the leaf veins b) chlorosis of the secondary veins c) yellowing around the leaf veins; 4, diffuse chlorosis and/or necrosis departing from veins. The results were evaluated after processing the disease index (DI) or the Area Under Disease Progression Curve (AUDPC). The global disease index was calculated using the following formula: DI= Σ(Scale value x Number of inoculated points in that rating) /Total number of inoculated points.

Using the weekly values of the disease index of the inoculated leaves, the AUDPC was determined for each trial according to the formula: $AUDPC= \Sigma_i^{n-1}(y_i+y_{i+1})/2(t_{i+1}-t_i)$, where "n" is the total number of observations, "y_i" is the DI at the *i*th observation, and "t_i" is the number of days after *P. tracheiphilus* inoculation at the *i*th observation (Campbel and Madden, 1990).

Table 2: Citrus plants used in the *in vivo* experiments, number of plants per treatment, leaves and points of inoculations for each trial.

Species	Plant age	Treatment[a]	G/F[b]	N. plants	Leaves/ plant	Points/ leaf	Total
C. aurantium	seedlings	*Pme* 3C+*Pt* *Pco* 4.3.t+*Pt*	G	5	6	4	120
C. aurantium	1-year-old	*Pme* 3C+*Pt* *Pco* 4.3.t+*Pt*	G	3	8	4	96
C. limon	adult plants	*Pme* 3C+*Pt*	G	3	5	4	60
C. limon	adult plants	*Pme* 3C+*Pt* *Pco* 4.3.t+*Pt*	F	6	30	6	1260

[a]*Pco*, *Pseudomonas corrugata* (strain PVCT 4.3t); *Pme*, *Pseudomonas mediterranea* (strain PVCT 3C); *Pt*, *Plenodomus tracheiphilus* (isolate PVCT Pt57); G/F[b], after the inoculation plants were maintained in greenhouse (G) or in open field (F).

3.2.2.3 Statistic analysis

For the *in vitro* and *in vivo* tests, data were analyzed using Statgraphics plus Software Version 5.1 (StatPoint Technologies, Inc., Warrenton, VA, USA). Mean values were compared using the Student–Newman–Keuls test. Interaction between the study variables was also investigated. Statistical significance was established as $P \leq 0.001$.

3.2.3 Whole-genome sequencing of bacterial strains

3.2.3.1 DNA extraction

P. corrugata PVCT 4.3t and *P. mediterranea* PVCT 3C were grown under shaking (180 rpm) in Luria-Bertani (LB) broth for 24 h at 28°C and then bacterial cells (1 x 10^9 cfu mL^{-1}) were harvested by centrifugation for DNA extraction using the Gentra Puregene bacterial DNA extraction kit (Qiagen), following the manufacturer's instructions. A NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to check the quantity and quality of the extracted genomic DNA. The high-quality genomic DNA was transferred to Novogene Co. Ltd (Cambridge, UK) to perform the whole-genome sequencing of both strains.

3.2.3.2 Library construction

A total amount of 1µg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, the DNA sample was fragmented by sonication to a size of 350 bp, then DNA fragments were end-polished, A-tailed, and ligated with the NEBNext adapters for Illumina sequencing, and further PCR enriched by P5 and indexed P7 oligos. At last, PCR products were purified (AMPure XP system) and libraries were analysed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR.

3.2.3.3 Sequencing and assembly

The quantified libraries were sequenced after pooling according to its effective concentration and expected data volume using Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd. The raw data were filtered to obtain high quality reads and

assembled using: SOAPdenovo software (version 2.04; Li *et al.*, 2008) with different K-mers (the default were 95, 107, 119), SPAdeS v3.11.1 software (Bankevich *et al.*, 2012) with 2 Different K-mers (the default were 99 and 127) and Abyss v.1.5.2 software (Simpson *et al.*, 2009) with K-mer 64. The assembly results of the three softwares were integrated with CISA v1.3 software (Lin and Liao, 2013) and the assembly result with the least scaffolds was selected. The gapclose software was used to fill the gap of preliminary assembly results. The same lane pollution by filtering the reads with low sequencing depth (less than 0.35 of the average depth) was removed to obtain the final assembly result. Fragments below 500 bp were filtered out and the final result was counted for gene prediction.

3.2.3.4 Genome component prediction

Genome component prediction included the prediction of the coding gene, repetitive sequences, and non-coding RNA. Coding genes were predicted with GeneMarkS program (Besemer *et al.*, 2001). The interspersed repetitive sequences were predicted using the RepeatMasker (http://www.repeatmasker.org/; Saha *et al.*, 2008). The tandem Repeats were analysed by the TRF (Tandem repeats finder; Benson, 1999). Transfer RNA (tRNA) genes were predicted by the tRNAscan-SE (Lowe and Eddy, 1996; Lagesen *et al.*, 2007). Ribosome RNA (rRNA) genes were analysed by the rRNAmmer. Small nuclear RNAs (snRNA) were predicted by BLAST against the Rfam database (Gardner *et al.*, 2009; Nawrocki *et al.*, 2009).

3.2.3.5 Gene function

Six databases were used to predict gene functions: GO (Gene Ontology; Ashburner *et al.*, 2000), KEGG (Kyoto Encyclopedia of Genes and Genomes; Kanehisa *et al.*, 2004), COG (Clusters of Orthologous Groups; Galperin *et al.*, 2015), NR (Non-Redundant Protein Database; (Li *et al.*, 2002), Pfam (Saier *et al.*, 2014) and Swiss-Prot (Bairoch and Apweiler, 2000). A whole genome Blast search (E-value less than 1e-5, minimal alignment length percentage larger than 40%) was performed against above six databases.

3.2.3.6 Genomic comparative analysis

The average nucleotide identity based on BLAST (ANIb) values between the genome sequence of strains PVCT 4.3t and PVCT 3C and the genome sequences of the type strains of the closest related species were estimated by using PYANI (Python module for average nucleotide identity

analyses) software (v0.2.10) (Pritchard *et al.*, 2019). Biosynthetic gene clusters for secondary metabolites were predicted with antiSMASH version 6.1.1 (Blin *et al.*, 2021).

3.3 Results

3.3.1 *In vitro* antagonistic activity against *P. tracheiphilus*

3.3.1.1 Activity of diffusible compounds

A collection of ten *P. corrugata* and *P. mediterranea* strains was screened *in vitro* against five isolates of *P. tracheiphilus* to investigate the antimicrobial potential due to the production of diffusible compounds (Table 1). Strains were tested *in vitro* in dual-culture antagonism assays performed on PDA plates. Ten days post-inoculation, *Pseudomonas* strains significantly inhibited the mycelial growth of *P. tracheiphilus*, showing mean percentages of growth inhibition (PGI) against all fungal isolates ranging from 35 to 65% for *P. corrugata* and from 30 to 60% for *P. mediterranea* as compared to the control plates containing only the target fungus (Table 3).

The fungal isolate PVCT Pt10 resulted significantly less susceptible *in vitro* (33.8% PGI) and grew faster (data not shown) compared to the other isolates, followed by PVCT Pt57 (51.0% PGI).

With regard to the bacterial strains tested, *P. corrugata* strains PVCT 5.1t and PVCT 4.3t showed the greatest inhibitory activity against all challenged *P. tracheiphilus* isolates (Table 3). Moreover, *P. mediterranea* PVCT 3C and *P. corrugata* PVCT 4.3t exhibited a faster growth (data not shown) compared to the other *P. mediterranea* and *P. corrugata* strains respectively.

According to the promising results of this phenotypic screening, *P. corrugata* strains PVCT 4.3t (66.4% PGI) and CFBP 5454 (51.4% PGI) and *P. mediterranea* strains PVCT 3C (53.5% PGI) and CFBP 5447^{T} (57.5% PGI) were selected for further *in vitro* tests.

Table 3: Growth inhibition (%) of *P. tracheiphilus* isolates due to diffusible compounds produced by *Pseudomonas* sp. strains on potato dextrose agar (PDA) medium.

Bacterial strain[a]	*Pt* isolate					Mean[b]
	PVCT Pt10	PVCT Pt57	PVCT Pt17	PVCT Pt67	PVCT Pt52	
Pme CFBP 5444	17.2	26.8	22.6	42.9	40.5	30.0 Aa
Pco NCPPB 2445^T	10.3	41.5	25.8	47.6	50.0	35.0 Aa
Pco CFBP 10148	34.5	46.3	32.3	50.0	59.5	44.5 Bb
Pco CFBP 5454	27.6	51.2	61.3	50.0	66.7	51.4 Cc
Pme PVCT 3C	34.5	53.7	58.1	52.4	69.0	53.5 CDcd
Pme CFBP 5447^T	44.8	51.2	48.4	76.2	66.7	57.5 Decd
Pme PVCT 1.6	31.0	61.0	64.5	66.7	69.0	58.4 Ded
Pme PVCT P1	48.3	46.3	67.7	59.5	78.6	60.1 Ede
Pco PVCT 5.1t	37.9	68.3	77.4	69.0	73.8	65.0 Fe
Pco PVCT 4.3t	51.7	63.4	74.2	64.3	78.6	66.4 Fe
Mean[b]	33.8 Aa	51.0 Bb	53.2 Bb	57.9 Cc	65.2 Dd	52.2

[a]*Pco*, *Pseudomonas corrugata*; *Pme*, *Pseudomonas mediterranea*; *Pt*, *Plenodomus tracheiphilus*; [b]Means with equal letters do not differ from each other for Student's test by p=0.05 (lowercase) and p=0.01 (uppercase).

3.3.1.2 Activity of culture filtrates

The activity of bacterial cell-free culture filtrates was evaluated *in vitro* toward *P. tracheiphilus* PVCT Pt57 one of the most virulent isolates (Oliveri *et al*., 2022). To obtain culture filtrates, *P. corrugata* strains PVCT 4.3t and CFBP 5454 and *P. mediterranea* strains PVCT 3C and CFBP 5447^T were grown in a minimal medium (IMM) inducing the production of antimicrobial cyclic lipopeptides (CLP). Culture supernatants were sterilized by filtration and evaluated in terms of mycelial growth inhibition (PGI%) by amending PDA medium with different concentrations of filtrate (20% or 50%). All tested strains significantly inhibited the growth of the fungus with both doses of filtrate (20% or 50%). Culture filtrates of *P. mediterranea* strains determined a reduction of radial growth of the fungus of about 12-25% at both concentrations (Figure 1A). *P. corrugata* CFBP 5454 induced a growth inhibition of about 30% and 50% respectively at the two concentrations, while strain PVCT 4.3t reduced the growth of the fungal mycelium of about 12-22% at both filtrate concentrations. The addition of bacterial filtrates to the medium, above all at 50% filtrate concentration, induced also a modification of *P. tracheiphilus* colonies morphology and pigmentation (Figure 1A).

The activity of cell-free culture filtrates was also tested for the inhibition activity of conidia germination and the subsequent inhibition of mycelium growth in liquid medium. For this purpose, carrot broth medium containing a suspension of *P. tracheiphilus* phialoconidia was amended with 20% culture filtrates of *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C strains. Mycelial fresh weights and the dry weights were calculated and compared to the control without culture filtrate. Five days post-inoculation, *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C culture filtrates determined a reduction of 70% and 85% respectively of the dry weight of the fungal mycelium and altered also the pigmentation of the mycelium (Figure 1B).

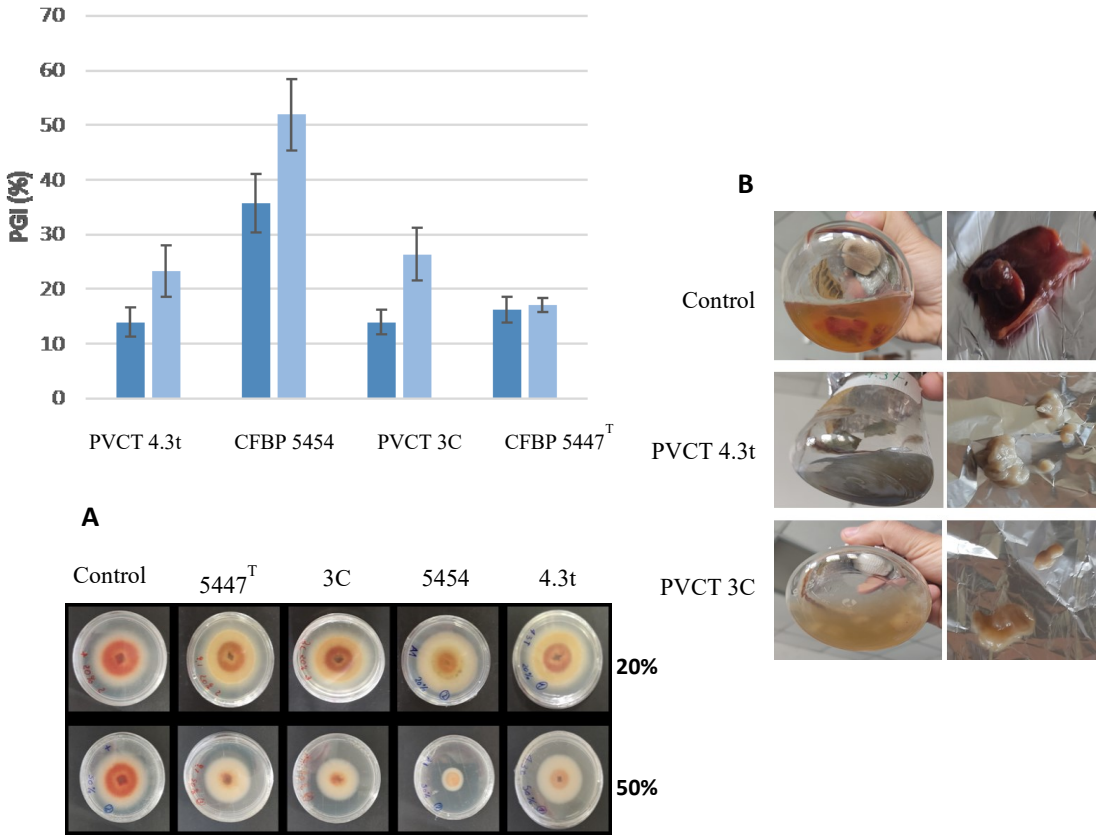

Figure 1: Activity of culture filtrates of *Pseudomonas* sp. on *Plenodomus tracheiphilus* PVCT Pt57. A) Effect on mycelial growth in PDA plates amended with 20% or 50% culture filtrate B) Effect on conidial germination in carrot broth amended with 20% culture filtrate and inoculated with a 10^6 conidial suspension of *P. tracheiphilus*.

3.3.1.3 Activity of volatiles

The potential antifungal activity of volatile compounds produced by *P. corrugata* strains CFBP 5454 and PVCT 4.3t and *P. mediterranea* strains CFBP 5447^{T} and PVCT 3C was tested against *P. tracheiphilus* PVCT Pt57 in terms of inhibition of the growth of the mycelium and conidia germination. For this purpose, *in vitro* tests were conducted providing a spatial separation of the bacteria to be tested from the phytopathogenic fungus through the formation of a sealed double-chamber (Strano *et al*., 2017), made with two combined Petri dishes containing the bacterium in one plate and the fungus in the other. All bacterial strains were able to reduce the mycelial growth of the fungus through the production of poisonous volatile compounds (Figure 2). The percentage of reduction of the diametral growth of the fungus (PGI) compared to the control varied according to the bacterial strain tested and the days after inoculation. Eight days post-inoculation, PGI of 5.5% and 17.5% for *P. corrugata* strains CFBP 5454 and PVCT 4.3t, respectively, and 12% and 22.2% for *P. mediterranea* strains CFBP 5447^{T} and PVCT 3C, respectively, were observed (Figure 2A). A change in mycelium colour was observed in presence of *P. mediterranea* strains (Figure 2A). In terms of reduction of the germination of conidia exposed to bacterial volatiles, a mean percentage reduction of 21.7% and 16.3% was observed for *P. corrugata* strains CFBP 5454 and PVCT 4.3t and of 15.7 and 6.3% for *P. mediterranea* strains CFBP 5447^{T} and PVCT 3C, respectively. After germination, the conidia exposed to *P. mediterranea* PVCT 3C volatiles produced smaller colonies, although less inhibition was observed for this strain (Figure 2B).

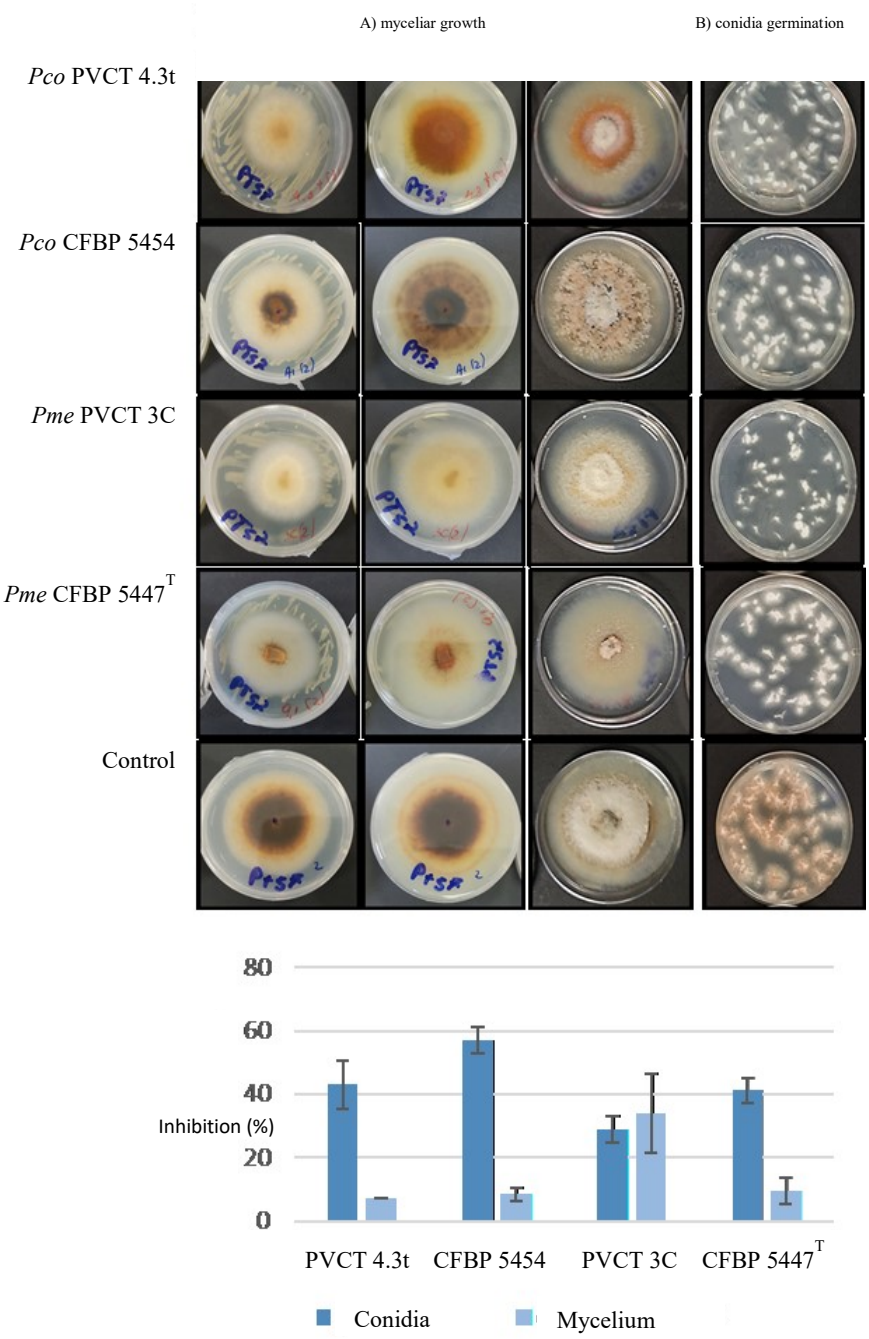

Figure 2: Effect of the production of volatile compounds by *Pseudomonas* spp. on mycelial growth (A) and conidia germination (B) of *P. tracheiphilus* PVCT Pt57. (C) Percentage of inhibition of conidia germination and myceliar diametral growth of *P. tracheiphilus* PVCT Pt57. *Pco*, *P. corrugata*; *Pme*, *P. mediterranea*.

3.3.2 Biological control of Mal secco disease

The *in vivo* activity of *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C against the virulent isolate of *P. tracheiphilus* PVCT Pt57 was tested by treating the leaves of *C. aurantium* (sour orange) or *C. limon* (lemon) plants with a bacterial suspension of the tested strains before the inoculation with the fungal pathogen. Sour orange plants (one-year-old) or seedlings were used for the *in vivo* experiments, while for the experiments with lemon, adult plants were used and maintained, after the pathogen inoculation, in unheated greenhouse or outdoor, simulating open field conditions. Lemon plants grown in unheated greenhouse were treated only with *P. mediterranea* PVCT 3C cells suspension. The evolution of leaf infection symptoms has been evaluated weekly up to 35 days from the inoculation by using the disease index (DI). The progression of the disease measured as the area underlying the curve of the disease (AUDPC) was determined at the end of the experiment, 35 days after inoculation.

In sour orange experiments, the first symptoms appeared from the 7th day after the inoculation only in the assay with one-year plants, where approximately 9.8% of inoculation sites resulted positive in control plants inoculated only with the fungus, with an average disease index of 0.04 (Table 4). Fourteen days post-inoculation in both sour orange experiments (one-year plants or seedlings) pre-treatments of plants with *P. corrugata* or *P. mediterranea* cells suspensions significantly reduced both the percentage of the infected sites and the disease index. Twenty-one, twenty-eight and thirty-five days post-inoculation, the percentage of sites positive to the infection did not differ between treated and not-treated plants (control), although the disease indices were significantly higher in control plants of both experiments at the three time points (Table 4). Treatments with *P. corrugata* PVCT 4.3t or *P. mediterranea* PVCT 3C cells did not differ each other significantly in terms of percentage of infected sites and disease index at each time point (Table 4).

In *C. limon* assays, first symptoms appeared at 21 days post-inoculation in both experiments (greenhouse or open field). Twenty-eight and thirty-five days after inoculation, both percentage of infected sites and disease index did not differ between treated and not-treated plants (Table 4). In order to investigate the different progression of the disease, in the leaves inoculated with the fungal pathogen, according to the treatments (*P. corrugata* or *P. mediterranea*), the AUDPC, an index measuring the area underlying the progression curve of the disease, was determined. The overall value of the disease progression area of each test was examined with two-factor analysis of variance and the means were compared with the Student-Newman-Keuls test (P=0.01). The data were mostly in accordance with those of the DI (Table 4). The

treatments with *P. corrugata* PVCT 4.3t or *P. mediterranea* PVCT 3C significantly reduced disease progression in adult sour orange plants or seedlings in comparison with not-treated control plants (Table 4). Similarly, pre-treatments of lemon plants, maintained in greenhouse or outdoor, with the biological control strains of *P. corrugata* or *P. mediterranea* significantly reduced disease progression compared to plants inoculated only with the fungus and not pre-treated (Table 4). For all experiments, no significant differences were observed for AUDPC between the treatment with *P. corrugata* or *P. mediterranea*, with the exception of the experiment with lemon plants maintained outdoor after the pathogen inoculation.

Table 4: Values of the area under the disease progression curve (AUDPC) of *C. aurantium* and *C. limon* plants leaf-inoculated with *P. tracheiphilus* PVCT Pt57 according to treatments (*P. corrugata* PVCT 4.3t or *P. mediterranea* PVCT 3C).

		Days post-inoculation										
		7		14		21		28		35		
Species/experiment	Treatment	% pos.	DI	% pos.	DI	% pos.	DI	% pos.	DI	% pos.	DI	AUDPC
C. aurantium (seedlings)	3C+*Pt*	0 a	0 a	2.8 a	0.02 a	25.48 a	0.23 a	33.97 a	0.55 ab	33.97a	0.96ab	8.91 a
	4.3t+*Pt*	0 a	0 a	10.0 ab	0.03 a	28.33 a	0.19 a	30.00 a	0.38 a	30.00 a	0.70 a	6.58 a
	Control	0 a	0 a	15.0 b	0.18 b	31.11 a	0.39 b	35.56 a	0.75 b	35.56 a	1.13 b	12.56 b
C. aurantium (1-year-old)	3C+*Pt*	0 a	0 a	5.2 a	0.05 a	95.98 a	0.99 a	100 a	1.27 a	100 a	1.41 a	21.10 a
	4.3t+*Pt*	0 a	0 a	12.2 a	0.12 a	92.44 a	0.96 a	100 a	1.26 a	100 a	1.48 a	21.60 a
	Control	9.84 b	0 b	41.8 b	0.39 b	88.83 a	1.64 b	100 a	2.11 b	100 a	2.43 b	37.65 b
C. limon (greenhouse)	3C+*Pt*	0 a	0 a	3.3 a	0.03 a	50.0 a	0.40 a	60 a	0.72 a	80 a	1.55 a	13.36 a
	Control	0 a	0 a	18.3 b	0.18 b	68.30 a	0.82 b	83 a	1.35 b	91.66 a	2.25 b	23.69 b
C. limon (open field)	3C+*Pt*	0 a	0 a	5.5 a	0.23 a	17.23 a	0.48 a	22.62 a	0.85 a	23.09 a	1.01 a	13.62 a
	4.3t+*Pt*	0 a	0 a	8.6 a	0.15 a	33.72 b	0.60 ab	41.88 b	1.03 a	42.40 b	1.33 a	16.95 ab
	Control	0 a	0 a	21.6 b	0.45 b	32.02 b	0.76 b	39.26 b	1.09 a	41.25 b	1.32 a	19.18 b

Mean values with equal letters do not differ from each other for Student's test by p=0.05. DI, Disease index. % pos, percentage value of infected inoculation sites. Control, plants only leaf-inoculated with *P. tracheiphilus* and not pre-treated.

3.3.3 Draft genome sequence of *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C

3.3.3.1 General features

The draft genome of *Pseudomonas corrugata* strain PVCT 4.3t consists of 65 scaffolds, which are composed of 70 contigs. The N_{50} contig length is 269 kb and the largest contig has a size of approximately 699 kb. Genome coverage was about 99.98% and sequencing depth for scaffolds was 205. The genome is composed of a circular chromosome without any extrachromosomal elements. The genome size was approximately 6,317,970 bp with a G + C content of 61.30 %. A total of 5,595 protein coding genes were predicted, 84 are RNA genes. According to the COG assignment, 4,736 protein coding genes were assigned to a putative function with the 290 remaining annotated as proteins with unknown function (Table 5).

The draft genome of *Pseudomonas mediterranea* strain PVCT 3C consists of 31 scaffolds, which are composed of 34 contigs. The N_{50} contig length is 231 kb and the largest contig has a size of approximately 470 kb. Genome coverage was about 99.99% and sequencing depth for scaffolds was 222. The genome is composed of a circular chromosome without any extrachromosomal elements. The genome size was approximately 6,009,932 bp with a G + C content of 62.05%. A total of 5,305 protein coding genes were predicted and 73 are non coding RNA genes. According to the COG assignment, 4,483 protein coding genes were assigned to a putative function with the 272 remaining annotated as proteins with unknown function (Table 5). The obtained genome sequences were deposited in the NCBI GenBank database with the accession numbers JANIBG000000000 (*P. corrugata* PVCT 4.3t) and JANIBE000000000 (*P. mediterranea* PVCT 3C) .

The size and GC (%) content of the genome of strain PVCT 4.3t was within the expected range based on the genome sequence of *P. corrugata* strains deposited in GenBank at NCBI (ncbi.nlm.nih.gov accessed on 29/11/2022). Conversely, PVCT 3C genome size resulted below (about 300 kb) the expected range based on the genome sequence of *P. mediterranea* strains deposited in GenBank at NCBI. Genome comparison with the type strain of the species (DSM 16733), suggested that PVCT 3C lacks of several prophage-associated gene sequences with unknown function (data not shown).

3.3.3.2 Functional annotation and analysis

Using COG function assignment, the 4,736 protein coding genes of strain PVCT 4.3t could be classified into 24 COG categories. The properties and the statistics of the genome are summarized in Table 6. The most abundant categories of metabolism, information storage and processing, and cellular processes and signaling are related to amino acid transport and metabolism (509, 10.12%), transcription (445, 8.85%), and signal transduction mechanisms (391, 7.77%). Using COG function assignment, the 4,483 protein coding genes strain PVCT 3C could be classified into 24 COG categories. The properties and the statistics of the genome are summarized in Table 6. The most abundant categories of metabolism, information storage and processing, and cellular processes and signaling are related to amino acid transport and metabolism (494, 10,38 %), transcription (440, 9.25 %), and signal transduction mechanisms (373, 7.84 %).

Table 5: Genomic features, gene prediction, and annotation summary.

	P. corrugata PVCT 4.3t	*P. mediterranea* PVCT 3C
Raw Reads (Mb)	1,857	1,906
Clean Reads (Mb)	1,441	1,505
DNA contigs (>500bp)	70	34
DNA, total number of bases in contigs	6,317,920	6,009,902
DNA scaffolds (>500bp)	65	31
DNA, total number of bases in scaffolds	6,317,970	6,009,932
DNA GC%	61.3	62.05
N_{90} contigs length (bp)	64,990	91,695
N_{50} contigs length (bp)	269,418	231,199
N_{90} scaffolds length (bp)	88,459	95,478
N_{50} scaffolds length (bp)	269,418	249,124
Total genes	5,595	5,305
Genes with COG function prediction	4,736	4,483
Protein coding genes assigned to SwissProt	2,550	2,397
RNA genes	84	73
tRNA genes	59	54
Tandem repeats (%genome)	0.61	0.71
Genes assigned to COGs	4,336	4,096
COG's genes with unknown function	290	272
COG's protein coding genes for secondary metabolites biosynthesis, transport and catabolism	151	140
Genes assigned to KEGGs	5,400	5,088
Genes with Pfam domains	3,769	3,606
Genes assigned to GOs	3,769	3,606
GO's protein coding genes with transporter activity	306	304

GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Clusters of Orthologous Groups) and NR (Non-Redundant Protein Database) prediction outputs are reported in supplementary materials (Supplementary Table S1 and Supplementary Figure S1, S2).

Table 6: Number of CDSs associated with COG functional categories.

		P. corrugata PVCT 4.3t		*P. mediterranea* PVCT 3C	
Functional class	Class_description	No. genes	%	No. genes	%
A	RNA processing and modification	1	0.02	2	0.04
B	Chromatin structure and dynamics	4	0.08	3	0.06
C	Energy production and conversion	305	6.07	278	5.85
D	Cell cycle control, cell division, chromosome partitioning	44	0.88	42	0.88
E	Amino acid transport and metabolism	509	10.13	494	10.39
F	Nucleotide transport and metabolism	94	1.87	91	1.91
G	Carbohydrate transport and metabolism	279	5.55	251	5.28
H	Coenzyme transport and metabolism	238	4.74	233	4.90
I	Lipid transport and metabolism	229	4.56	236	4.96
J	Translation, ribosomal structure and biogenesis	259	5.15	247	5.19
K	Transcription	445	8.85	440	9.25
L	Replication, recombination and repair	133	2.65	127	2.67
M	Cell wall/membrane/envelope biogenesis	284	5.65	241	5.07
N	Cell motility	132	2.63	139	2.92
O	Posttranslational modification, protein turnover, chaperones	188	3.74	171	3.60
P	Inorganic ion transport and metabolism	234	4.66	224	4.71
Q	Secondary metabolites biosynthesis, transport and catabolism	151	3.00	140	2.94
R	General function prediction only	489	9.73	461	9.70
S	Function unknown	290	5.77	272	5.72
T	Signal transduction mechanisms	391	7.78	373	7.84
U	Intracellular trafficking, secretion, and vesicular transport	114	2.27	107	2.25
V	Defense mechanisms	121	2.41	113	2.38
W	Extracellular structures	33	0.66	31	0.65
X	Mobilome: prophages, transposons	59	1.17	39	0.82
	TOTAL	5026		4755	

3.3.3.3 Secondary metabolites

Genome mining by antiSMASH 6.1.1 resulted in the prediction of 15 gene clusters in 15 regions (7.85 % of the genome) associated with secondary metabolite biosynthesis in *P. corrugata* strain PVCT 4.3t; 13 gene clusters in 13 regions (7.40 % of the genome) were predicted in *P. mediterranea* strain PVCT 3C (Supplementary Table S2). The majority of the biosynthetic gene clusters of both strains encoded non-ribosomal peptide synthetases (NRPS) and NRPS-like regions in PVCT 4.3t (3.1, 6.2, 12.1, 16.1, 25.1, 28.1, 29.1, 33.1, 45.1) and in PVCT 3C (11.1, 15.2, 16.1, 17.1, 25.1, 26.1) strains. Among the NRPS clusters, in *P. corrugata* PVCT 4.3t genome the gene clusters for corpeptin A and B (region 3.1), brabantamide A (region 16.1), thanamycin (region 29.1) and bicornutin A1 and A2 (region 45.1) showed the highest similarity with known clusters (90-100%), The gene clusters for corpeptin A and B was associated to an homoserine-lactone synthase gene (HSL-NRPS) (Supplementary Table S2).

For *P. mediterranea* PVCT 3C genome 90-100% sequence similarity was found for corpeptin A and B (region 11.1) and nunamycin (region 25.1) gene clusters (Supplementary Table S2).

Moreover, additional gene clusters for a number of secondary metabolite groups were reported in either strains: ribosomally synthesised and post-translationally modified peptide product (RiPP), arylpolyene, siderophores, redox-cofactors, N-acetylglutaminylglutamine amide (NAGGN).

3.3.3.4 Average nucleotide identity

The ANI (ANIb) value above the threshold range (95 to 96%) of species delineation (Richter and Rosselló-Móra, 2009) with the genome of *P. corrugata* type strain BS3649 (98.38% ANI) confirmed that strain PVCT 4.3t belongs to *P. corrugata* species (Figure 3). Strain PVCT 3C showed a ANI value of 99.2 % with the genome of the type strain of the species *P. mediterranea* DSM 16733 (Figure 3).

Comparative genomic analysis showed that both *P. corrugata* and *P. mediterranea* strains share a low intraspecies genetic variability; in particular, *P. mediterranea* strain PVCT 3C shared ANI values ranging from 99.2% (*P. mediterranea* DSM 16733) to 99.9% (*P. mediterranea* strains CFBP 5444 and TEIC1105) with the other *P. mediterranea* genomes available. *P. corrugata* strain PVCT 4.3t shared ANI percentage values ranging from 99.8 (*P. corrugata* CFBP 5403) to 99.5% (*P. corrugata* RM1-1-4) (Figure 3).

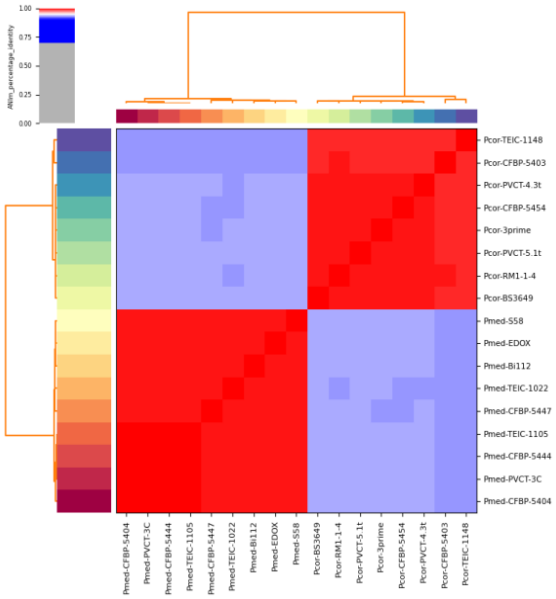

Figure 3: Average Nucleotide Identity (ANIb) of *P. mediterranea* and *P. corrugata* strains deposited in the NCBI database.

3.4 Discussion

P. corrugata is considered an efficient biocontrol agent both *in vitro* and *in vivo* toward several plant pathogenic microorganisms (Coco *et al*., 2003; 2004; Catara, 2007; Sang *et al*., 2008; Trivedi *et al.,* 2008; Sang *et al*., 2012). *P. mediterranea*, a species closely related to *P. corrugata*, have been proposed only in few studies as a biocontrol agent (Coco *et al*., 2003, 2004; Gu *et al*., 2020; Ullah *et al*., 2020; Zhou *et al*., 2021).

In this study, a collection of *Pseudomonas* sp. strains belonging to *P. corrugata* phylogenetic subgroup was investigated to shed some light on its activity *in vitro* and *in vivo* as biological control agents in the Citrus-Mal secco disease (MSD) pathosystem. *In vitro*, the production and bioactivity of compounds diffusibile in the agar medium, extracellular metabolites and volatiles were assessed. Our results showed that all *P. corrugata* and *P. mediterranea* strains, although to a different extent, were able to inhibit the growth *in vitro* of different isolates of *P. tracheiphilus*, the causal agent of MSD. The antimicrobial activity of *P. corrugata* and *P. mediterranea* was supposed to be mainly due to the production of the cyclic lipopeptides (CLPs) corpeptins (Emanuele *et al*., 1998) and cormycin (Scaloni *et al*., 2004) and to the

siderophore corrugatin (Risse *et al*., 1998). CLPs act by forming pores which disrupts the electrical potential across the cell membrane (Bender *et al*., 1999), thus different antifungal activity could depend on the content of sterol in the fungal plasma membrane (Latoud *et al*., 1990).

P. corrugata CFBP strain 5454 and *P. mediterranea* CFBP 5447, type strain of the species, produce in culture both corpeptins and the nonapeptide cormycin A (Licciardello *et al*., 2012). *P. mediterranea* has been recently demonstrated to secrete also two novel CLPs, medipeptin A and B (Zhou *et al*., 2021). Both cormycin and corpeptins have been demonstrated to exert an antagonistic activity *in vitro* against the Gram-positive bacterium *Bacillus megaterium* whereas cormycin resulted also bioactive towards the yeast *Rhodotorula pilimanae* (Emanuele *et al*., 1998; Scaloni *et al*., 2004). Mutant strains for QS-luxR transcriptional regulators genes of *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 and for genes encoding the transcriptional regulator RfiA common to both species were impaired in CLP production. *In vitro*, the mutant strain for the transcriptional regulator *rfiA* was not able to inhibit the growth of different fungal phytopahogens, included *P. tracheiphilus* (Licciardello *et al*., 2012).

In this study, among the tested *Pseudomonas* strains, *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C exerted the strongest antagonistic activity *in vitro* and were selected for further assays *in vivo*; sour orange and lemon plants were treated by leaf-spraying with the two bacterial strains suspensions before the inoculation with *P. tracheiphilus*. *In vivo* trials showed that both PVCT 4.3t and PVCT 3C strains reduced the number of positive infections at the early stages of the disease, slowing down MSD progression on sour orange and lemon. Similar results were obtained with *P. corrugata* CFBP 5454 toward *P. digitatum* on lemon fruits and *B. cinerea* on tomato and zucchini leaves (Strano *et al*., not published). The role of CLPs in biocontrol strictly depends on the pathosystem analysed as in some cases these compounds are not required for disease suppression (Tran *et al*., 2007; Mazzola *et al*., 2007; Berry *et al*., 2010).

Some authors described for *P. corrugata* strains the production of other secondary metabolites or the detection of genes putatively involved in their production and deposited in GenBank such as: inhibitory volatiles (Fernando *et al*., 2005), hydrogen cyanide (Ramette *et al*., 2003), ammonia (Trivedi *et al*., 2008) 2,4-diacetylphluoroglucinol and pyrrolnitrin (Garbeva *et al*., 2001), but none of these aspects were further investigated. *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C genome mining by BLAST research showed don't possess genes involved in the biosynthesis of 2,4-diacetylphloroglucinol (data not shown) but have the *hcnABC* gene cluster for hydrogen cyanide described in *P. corrugata* (Strano *et al*., 2015).

According to our results, *P. corrugata* and *P. mediterranea* produce volatile compounds, which play a relevant role in fungal growth inhibition (Strano *et al*., 2017). The myceliar growth and conidia germination of *P. tracheiphilus* PVCT Pt57 were reduced by the presence of both species, although *P. mediterranea* showed the greatest inhibition activity toward mycelium. Among the volatile compounds, hydrogen cyanide (HCN) is a poisonous secondary metabolites produced by several *Pseudomonas* species, with antagonistic properties (Voisard, *et al*., 1989; Sehrawat *et al*., 2022). *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 produce HCN independently from the AHL-QS system or RfiA (Strano *et al*., 2017). *P. corrugata* and *P. mediterranea* strains are likely to produce volatiles other than HCN; *P. corrugata* strain NRRL B-30409, a biocontrol agent toward phytopathogenic fungi, produce ammonia *in vitro* but not HCN, suggesting that the production of antimicrobial volatile compounds could be strain-dependent (Trivedi *et al.,* 2008).

According to these results, the whole-genome sequence (WGS) of PVCT 4.3t and PVCT 3C strains was obtained to investigate in-depth the molecular mechanisms underlying biocontrol strategies and the annotation was deposited in genbank (at NCBI, http://www.ncbi.nlm.nih.gov/).

The draft genome sequences of *P. corrugata* strain PVCT 4.3t and *P. mediterranea* strain PVCT 3C were subjected to the automated identification of secondary metabolite biosynthesis clusters with the pipeline antiSMASH 6.1 (Blin *et al.,* 2021). Several secondary metabolites gene cluster, in particular NRPS, were found in both genomes, although the draft nature of the genomes made very difficult cluster assignment. The biosynthetic gene clusters of corpeptins and thanamycin/nunamycin were identified with high similarity with known clusters in both species; moreover, in *P. corrugata* PVCT 4.3t gene clusters for the cyclocarbamate brabantamide A (region 16.1) and bicornutin A1 and A2 (region 45.1) were predicted. The analysis of NRPS clusters in the other *P. corrugata* and *P. mediterranea* strains supported the presence of the same CLP biosynthetic clusters for all strains. Consistently, the comparative analysis of the draft genomes of nine *P. corrugata* and *P. mediterranea* strains from different geographical locations revealed the presence of gene clusters for biosynthesis of siderophores, polyketides, non-ribosomal peptides, and hydrogen cyanide and a highly conserved QS system, with differences within the species (Trantas *et al*., 2015).

Brabantamide is a cyclocarbamate described in the biocontrol strain *Pseudomonas* sp. SH-C52 and bioactive against Gram-positive bacteria (Reder-Christ *et al*., 2011; Schmidt *et al*., 2014) and Oomycetes (Van Der Voort *et al*., 2015). An homolog cluster of this metabolite was identified in both *P. corrugata* and *P. mediterranea*, although it was not detected in culture

(Schmidt *et al*., 2014; Licciardello *et al*., 2018; Girard *et al*., 2020). In *P. corrugata*, the expression of the putative biosynthetic gene cluster of a brabantamide-like metabolite is controlled by QS (Licciardello *et al*., 2018). In *P. mediterranea*, the brabantamide biosynthetic cluster was not detected, probably due to the fact that most of the clusters comprising NRPS genes were divided and located in several contigs or due to the low identity percentage with other known brabantamide biosynthetic gene clusters.

The biosynthetic gene cluster responsible for the biosynthesis of the bicornutin isoform A1/A2 was detected with 100% similarity exclusively in *P. brassicacearum* strain DF41, a species belonging to *P. corrugata* subgroup. The bicornutin complex from the entomopathogenic bacteria *Xenorhabdus budapestensis* and *X. szentirmaii* inhibited the growth of *Phytophthora nicotianae* and the fire blight agent *Erwinia amylovora* (Böszörményi *et al*., 2009).

To conclude, we showed that *P. corrugata* and *P. mediterranea* are able to reduce *in vitro* the growth and conidia germination of the fungal pathogen *P. tracheiphilus*, causal agent of Mal secco disease (MSD) of citrus. The mechanism of action involves the production of compounds diffusible in the agar medium and secreted in culture supernatants and volatiles with antifungal activity. Two promising biocontrol strains, *P. corrugata* PVCT 4.3t and *P. mediterranea* PVCT 3C, contrasted also disease progression when used to pre-treat sour orange and lemon plants, two citrus species susceptible to MSD. The search of biosynthetic gene clusters involved in antimicrobial compounds production led to the discovery of several clusters, mainly encoding for non-ribosomal peptide synthase involved in the biosynthesis of antimicrobial cyclic lipopeptides (CLPs).

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Supplementary Material: chapter 3

Genome analysis of Pseudomonas corrugata and P. mediterranea strains with biocontrol activity against Plenodomus tracheiphilus

Supplementary Figures

Figure S1: NR annotation analysis of *Pseudomonas corrugata* strain PVCT 4.3t (A) and *P. mediterranea* strain PVCT 3C (B). X-axis, species ID; Y-axis, number of annotated genes.

Figure S2: Annotation of *Pseudomonas corrugata* strain PVCT 4.3t (A) and *P. mediterranea* strain PVCT 3C (B) genes against KEGG database. X-axis, Kegg pathway type; Y-axis, number of annotated genes.

Supplementary Tables

Table S1: Number of genes assigned to clusters of Orthologous Groups (GO) for *Pseudomonas corrugata* strain PVCT 4.3t and *Pseudomonas mediterranea* strain PVCT 3C.

Table S2: Summary of antiSMASH 6.1.1 results for *Pseudomonas corrugata* strain PVCT 4.3t and *Pseudomonas mediterranea* strain PVCT 3C.

Figure S1: NR annotation analysis of *Pseudomonas corrugata* strain PVCT 4.3t (A) and *Pseudomonas mediterranea* strain PVCT 3C (B). X-axis, species ID; Y-axis, number of annotated genes.

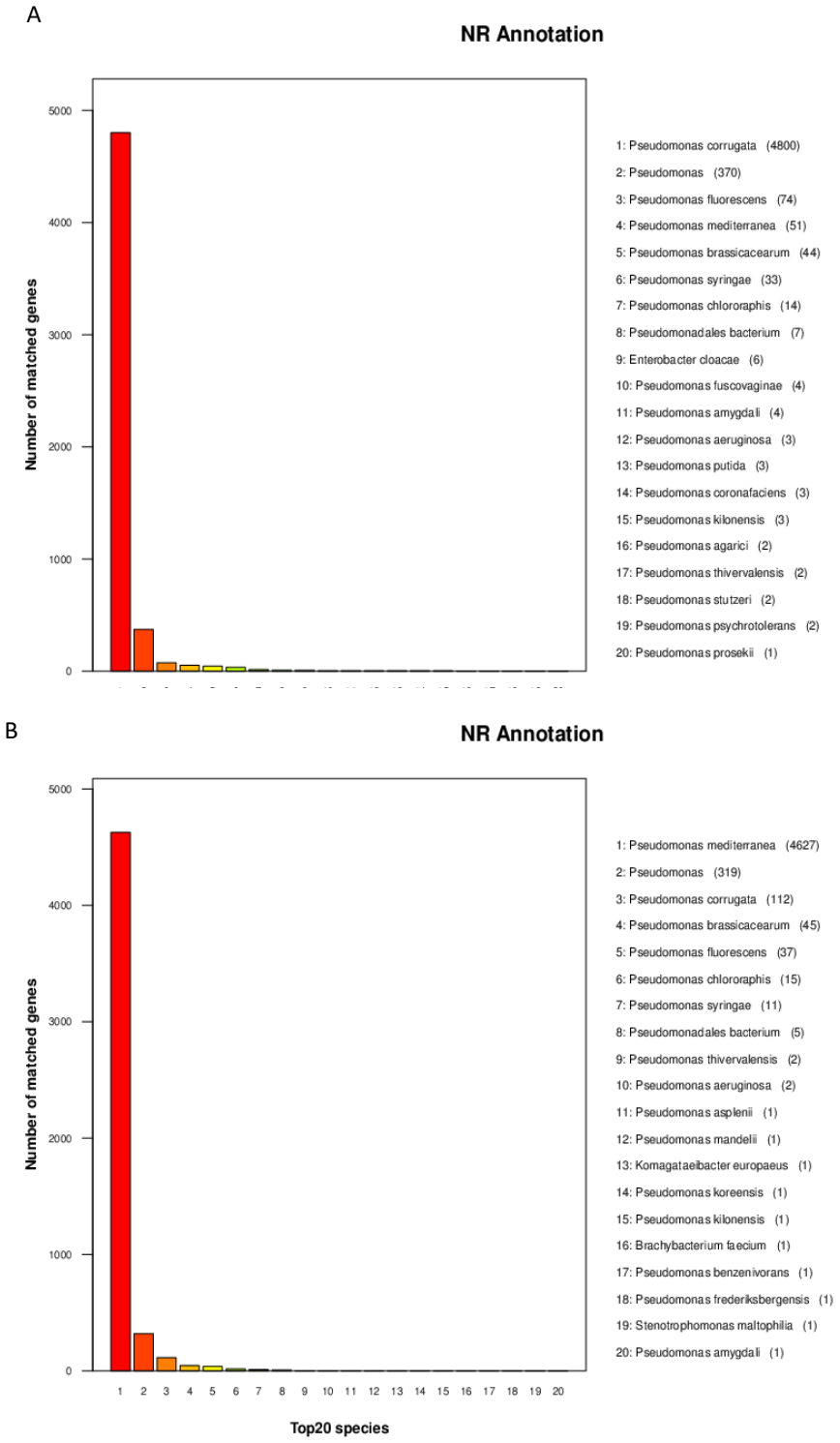
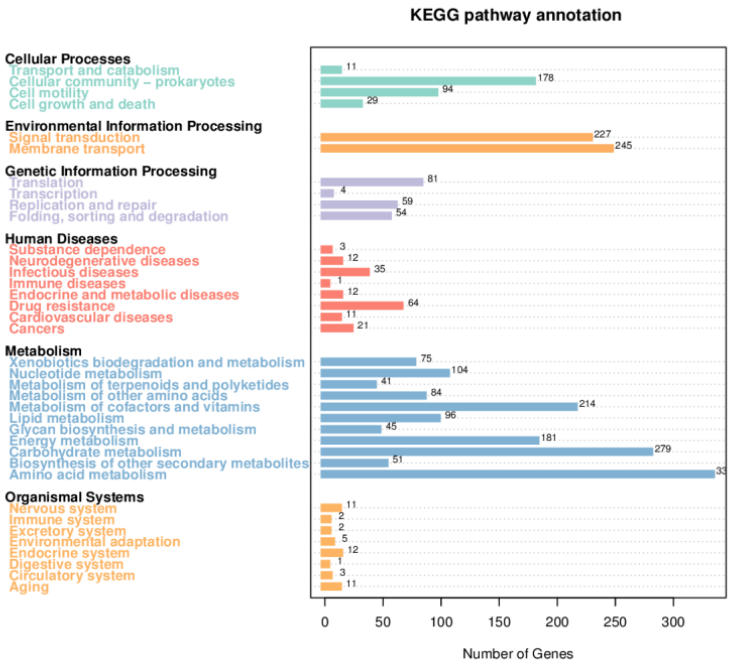


Figure S2: Annotation of *Pseudomonas corrugata* strain PVCT 4.3t (A) and *Pseudomonas mediterranea* strain PVCT 3C (B) genes against KEGG database. X-axis, Kegg pathway type; Y-axis, number of annotated genes.

A



B

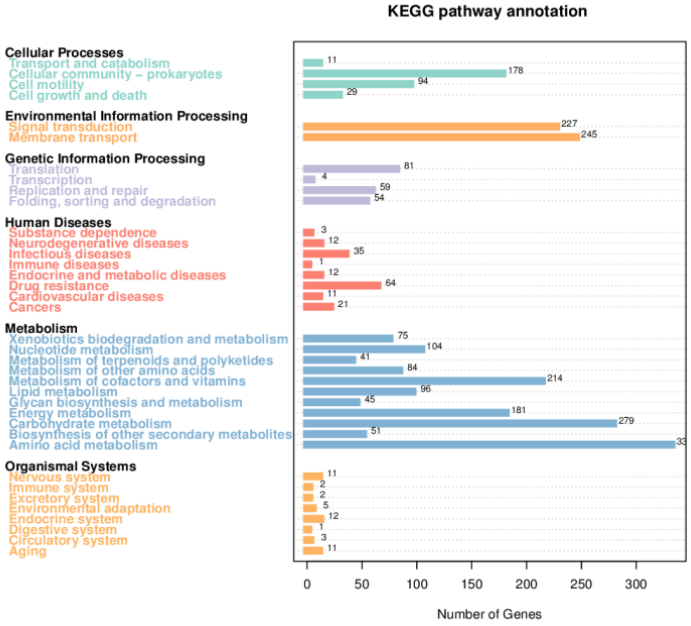


Table S1: Number of genes assigned to clusters of Orthologous Groups (GO) for *Pseudomonas corrugata* strain PVCT 4.3t and *Pseudomonas mediterranea* strain PVCT 3C.

Ontology	Class	*P. corrugata* PVCT 4.3t	*P. mediterranea* PVCT 3C
biological_process	biological adhesion	22	27
	biological regulation	766	760
	cell killing	3	3
	cellular component organization or biogenesis	164	148
	cellular process	2107	2038
	death	3	3
	developmental process	32	25
	establishment of localization	770	771
	growth	1	0
	immune system process	2	5
	localization	790	795
	locomotion	58	60
	metabolic process	2170	2067
	multi-organism process	46	49
	multicellular organismal process	22	20
	negative regulation of biological process	24	23
	nitrogen utilization	2	2
	positive regulation of biological process	19	12
	regulation of biological process	750	746
	reproduction	33	26
	reproductive process	23	18
	response to stimulus	414	409
	signaling	298	291
	viral reproduction	21	15
cellular_component	cell	1327	1283
	cell part	1327	1283
	extracellular region	30	33
	extracellular region part	30	33
	macromolecular complex	200	194
	membrane-enclosed lumen	25	24
	organelle	190	181
	organelle part	87	86
	virion	28	24
	virion part	28	24

molecular_ffunction	antioxidant activity	12	11
	binding	1738	1669
	catalytic activity	2011	1902
	enzyme regulator activity	12	10
	molecular transducer activity	302	295
	nucleic acid binding transcription factor activity	318	322
	protein binding transcription factor activity	65	68
	structural molecule activity	53	49
	transporter activity	306	304

Table S2: Summary of antiSMASH 6.1.1 results for *Pseudomonas corrugata* strain PVCT 4.3t and *Pseudomonas mediterranea* strain PVCT 3C.

Pseudomonas corrugata strain PVCT 4.3t						
Scaffold	Region	Type	From	To	Most similar known cluster	Similarity
1	Region 1.1	NAGGN	146,520	161,313		
	Region 1.2	betalactone	526,373	549,619	fengycin (NRP)	13%
2	Region 2.1	arylpolyene	111,565	155,176	APE Vf	45%
3	Region 3.1	NRPS, hserlactone	1	47,731	corpeptin A / corpeptin B (NRP)	90%
4	Region 4.1	RiPP-like	125,738	136,625		
6	Region 6.1	siderophore	104,588	123,508		
	Region 6.2	T1PKS, NRPS-like	271,848	320,284	entolysin (NRP)	17%
9	Region 9.1	redox-cofactor	60,707	82,854	lankacidin C (NRP + Polyketide)	13%
12	Region 12.1	NRPS-like	157,054	200,590	fragin (NRP)	37%
16	Region 16.1	NRPS	1	48,399	brabantamide A (NRP:Lipopeptide + Saccharide:Hybrid/tailoring)	100%
25	Region 25.1	NRPS	50,475	88,459	crochelin A (NRP + Polyketide)	7%
28	Region 28.1	NRPS	1	36,754		
29	Region 29.1	NRPS-like, NRPS	2	57,853	thanamycin (NRP:Beta-lactam)	100%
33	Region 33.1	NRPS	1	36,548	cichopeptin (NRP)	46%
45	Region 45.1	NRPS	1	5,697	bicornutin A1 / bicornutin A2 (NRP)	100%
Pseudomonas mediterranea strain PVCT 3C						
Scaffold	Region	Type	From	To	Most similar known cluster	Similarity
1	Region 1.1	arylpolyene	258,363	301,974	APE Vf	40%
3	Region 3.1	RiPP-like	146,886	157,773		
4	Region 4.1	T1PKS, NRPS-like	329,482	372,375	entolysin (NRP)	17%
9	Region 9.1	redox-cofactor	147,589	169,736	lankacidin C (NRP + Polyketide)	13%
10	Region 10.1	NAGGN	65,358	80,212		
11	Region 11.1	hserlactone, NRPS	165,719	209,733	corpeptin A / corpeptin B (NRP)	90%
15	Region 15.1	siderophore	13,411	32,317		
	Region 15.2	NRPS	135,263	180,572	anikasin (NRP)	22%
16	Region 16.1	NRPS	134,366	170,582		
17	Region 17.1	NRPS	107,112	143,302	crochelin A (NRP + Polyketide)	7%
25	Region 25.1	NRPS, NRPS-like	3	84,381	nunapeptin / nunamycin (NRP)	100%
26	Region 26.1	NRPS	1	22,167		
27	Region 27.1	Betalactone	19,646	42,881	fengycin (NRP)	13%

APE: arylpolyene
hserlactone: homoserine lactone
NAGGN: N-γ-acetylglutaminyl glutamine 1-amide
NRPS: non-ribosomal peptide synthetase
NRP: non-ribosomal peptide
PKS: Polyketide synthase
RiPP-like: Other unspecified ribosomally synthesised and post-translationally modified peptide product (RiPP) cluster.

4. Genes involved in the production of metabolites with antagonistic activity in *Pseudomonas corrugata*[3]

Abstract

LuxR solos are transcriptional regulators closely related to Quorum Sensing (QS) LuxR-family regulators but devoid of a cognate LuxI-family synthase homologue. They may respond to either endogenous or exogenous AHLs or to plant low-molecular-weight compounds, regulating traits associated to virulence and biocontrol activity. Comparative genome sequence analysis revealed that in addition to a canonical AHL-QS system (PcoI/R), *P. corrugata* harbours another LuxR-family solo transcriptional regulator named SolR. In this species, the cyclic lipopeptides (CLPs) cormycin A and corpeptins A and B are both major virulence factors and antimicrobial compounds, which production is regulated by QS, although little information is available about the specific contribution of cormycin in these activities. The biological role of SolR and a putative cormycin synthase (CrmA) in the model strain *P. corrugata* CFBP 5454 was investigated by inactivating, respectively, the *solR* and *crmA* genes and the resulting mutational phenotypes were investigated. PCOSOLR mutant strain was more virulent in tomato plants than the parent strain and its complementation *in trans* restored a wild-type phenotype. Time-course cell-free culture filtrates activity against a bioindicator microrganism showed that PCOSOLR is a CLP iper-producing strain. In addition, a transcriptional analysis by real-time PCR revealed that genes involved in the biosynthesis and secretion of CLP are probably under SolR regulation. The analysis of the phenotype of PCOCRMA mutant strain suggested it produces compounds diffusible in the agar medium with antifungal activity, although to a lesser extent than the wild-type strain. However, cells and culture filtrates of this strain did not inhibit the growth of the yeast *R. pilimanae* and the Gram-positive bacterium *B. megaterium*, sensitive to CLP. *In vivo*, PCOCRMA is impaired in inducing the hypersensitive response (HR) in the non-host plant *Nicotiana tabacum*, whereas symptoms of pith necrosis in the host plant tomato were significantly reduced.

Key words: *Pseudomonas corrugata*, cormycin, LuxR solo, virulence, mutational phenotype

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4.1 Introduction

First described in the UK in the late 1970s (Scarlett *et al*., 1978), *P. corrugata* was the first of the two species identified as the causal agent of tomato pith necrosis (TPN) (Scarlett *et al*., 1978; Catara, 2007). The species has since been isolated from many other crop plants showing pith necrosis, including pepper, chrysanthemum and geranium (Catara, 2007). Strains have been also isolated from diverse non-diseased plants, as root endophytes, in the rhizosphere or in bulk soil (Catara, 2007). Commensal or phytopathogenic strains show an *in vitro* antimicrobial activity against a wide range of microorganisms (Gram-negative and Gram-positive bacteria, Chromista, yeasts, fungi) (Cirvilleri *et al*., 2001; Catara, 2007) and some of them have been proposed as biological control agents in different pathosystems (Catara, 2007; Strano *et al*., 2017).

P. corrugata has been reported to produce different bioactive compounds; among them, alginate (Fett *et al*., 1996) and a siderophore called corrugatin that could be involved in pathogenicity and colonization (Risse *et al*., 1998). However, the antimicrobial activity is largely attributed to the production of phytotoxic and antimicrobial cyclic lipopeptides (CLPs) (Emanuele *et al*., 1998; Scaloni *et al*., 2004) and other substances such as hydrogen cyanide (Ramette *et al*., 2003), and pyrrolnitrin (Garbeva *et al*., 2001). CLPs act by creating pores in the cell membrane lipids that alter the electrical potential across the membranes, causing cell death (Bender *et al*., 1999).

P. corrugata produces in culture two kinds of CLPs, cormycin A and corpeptins, possessing surfactant, antimicrobial and phytotoxic activities. Corpeptins were isolated from the culture filtrates of the type strain of the species, NCPPB 2445 (Emanuele *et al*., 1998); two isoforms have been described, consisting of 22 amino acid residues: corpeptin A and corpeptin B. Corpeptins induce chlorosis when infiltrated into tobacco leaves and shows antimicrobial activity against the Gram-positive bacterium *Bacillus megaterium* (Emanuele *et al*., 1998). Some *P. corrugata* strains also produce cormycin A, a CLP found in the culture filtrate of strain IPVCT 10.3, whose antimicrobial activity is expressed against *B. megaterium* and *Rhodotorula pilimanae* and which has phytotoxic activity in tobacco, causing chlorosis followed by necrosis (Scaloni *et al*., 2004). In culture, *P. corrugata* CFBP 5454 produce CLPs that are able to diffound through the agar medium and hydrogen cyanide, an antifungal volatile compound (Licciardello *et al*., 2012; Strano *et al*., 2017). In addition, in the genome of *P. corrugata* was

described a biosynthetic gene cluster highly homologous to the cyclocarbamate brabantamide cluster of *Pseudomonas* sp. SH-C52, a biocontrol agent with antagonistic activity against Gram-positive bacteria and Oomycetes (Schmidt *et al*., 2014; Van Der Voort *et al*., 2015; Licciardello *et al*., 2018).

CLPs produced by *Pseudomonas* spp. consist of a fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain (Raaijmakers *et al*., 2006). The structure of corpeptins, as determined by Emanuele *et al.* (1998), is closely related to peptins, characterized by long peptide chains (18-25 amino acids), including fuscopeptins (FPs), produced by the phytopathogenic bacterium *P. fuscovaginae*, syringopeptins, synthesized by phytopathogenic strains of *P. syringae* pv. syringae and tolaasin (ToI-A), produced by the mycopathogenic bacterium *P. tolaasii*. Cormycin belongs to the group of low-molecular-weight nonapeptides (Scaloni *et al*., 2004), together with syringomycin, produced by strains of *P. syringae* pv. syringae (Bender *et al*., 1999; Raaijmakers *et al*., 2006). In *P. corrugata*, the production of nonapeptides is strain-dependent (Scaloni *et al.*, 2004). Cormycin and corpeptins play a fundamental role in the virulence of *P. corrugata*; mutant strains unable to produce one or both compounds retained the colonization ability of tomato stem parenchymal tissues but the symptoms associated with pith necrosis were significantly reduced (Licciardello *et al*., 2012; Strano *et al*., 2015). *P. corrugata* CFBP 5454 produces a set of *N*-hexanoyl-L-homoserine lactones (AHLs), signal molecule of QS system (Licciardello *et al*., 2007).

The AHL-QS system of *P. corrugata* (PcoI/PcoR) consists of an AHL synthase, PcoI, and a transcriptional sensor/regulator belonging to the LuxR-family protein, PcoR, and contributes to virulence in tomato and antimicrobial activity (Licciardello *et al*., 2007). In particular, PcoR is supposed to regulate *P. corrugata* virulence independently of the AHLs (Licciardello *et al*., 2009). Another LuxR-family transcriptional regulator, RfiA, was identified in *P. corrugata*; *rfiA* expression is linked to the AHL-QS system *via* co-transcription with *pcoI* and is necessary for full virulence in tomato and antimicrobial activity (Licciardello *et al*., 2009).
Together, PcoR and RfiA have a role in virulence on tomato and elicite an hypersensitive-like response on non-host plants; *P. corrugata pcoR* and *rfiA* mutant strains were in fact impaired in CLPs production (Licciardello *et al*., 2012), although the the latter retained its *in vitro* inhibition activity against fungal phytopathogens (Strano *et al*., 2017).
Strano *et al.* (2015) identified part of the biosynthetic gene cluster responsible for corpeptins production (*crpCDE*) including genes coding for non-ribosomal peptide synthases (NRPSs) and an ABC efflux system. These genes, and hence corpeptins, are greatly involved in *P. corrugata*-

plants interactions, contributing to virulence in tomato and in the elicitation of HR in non-host plants acting as phytotoxins and systemic resistance inducers, respectively (Strano *et al*., 2015). Expression analysis revealed also that *crpC* transcription occurs at high-cell density and that the transcriptional regulators PcoR and RfiA have a pivotal role in *crpC* expression and thus in corpeptins production. Under CLP-inducing conditions and at high cellular concentrations, genes linked to the biosynthesis of corpeptins and alginate are positively regulated by the PcoR–RfiA regulon in *P. corrugata* CFBP 5454. However, the putative biosynthetic gene cluster for cormycin does not appear to be under PcoR-RfiA control (Licciardello *et al*., 2018).

Regarding the genomic location of CLPs genes, *P. corrugata* strains possess a LPQ (lipopeptide/quorum sensing) genomic island (173 kb), required for phytopathogenic and biocontrol activity; this island is conserved within *P. corrugata* and *P. mandelii* phylogenetic groups (Licciardello *et al*., 2018; Melnyk *et al*., 2019; Girard *et al*., 2020). As mentioned, *P. corrugata* LPQ island harbours the genes for the AHL-QS system and the biosynthetic clusters for the CLPs cormycin and corpeptins and for a brabantamide-like cyclocarbamate metabolite described in the biocontrol agent *Pseudomonas* sp. SH-C52 (Schmidt *et al*., 2014). Although the biosynthetic gene cluster involved in corpeptins production and the biological role of these bioactive molecules have been elucidated (Strano *et al*., 2015), the contribution of cormycin in *P. corrugata* phenotype and the gene cluster organization of this CLP are still unclear.

In Proteobacteria, such as *P. corrugata*, which possess a complete AHL-QS systems as well as in those that apparently do not synthesize AHLs, other QS-LuxR-family regulators have been identified (Subramoni and Venturi, 2009a). This regulators, designed LuxR solos, lack of a cognate LuxI family protein associated with them. LuxR solos have the same modular structure of QS-related LuxR-family proteins, with an AHL-binding domain in the N-terminus and a DNA binding HTH at the C-terminus.

Possibly, LuxR solos present in AHL-producing bacteria could be sensing the endogenously produced AHLs and thus expanding the regulatory repertoire of the typical LuxI/R QS systems to other gene targets whereas in non-AHL-producing bacteria, on the other hand, could allow them to respond to AHLs produced by other bacteria (Patel *et al*., 2013; Venturi and Fuqua, 2013).

A sub-family of these LuxR solos only found in PAB (Plant Associated Bacteria) have lost the capacity to bind AHLs and instead evolved the ability to respond to low-molecular-weight plant compounds, being involved in interkingdom signaling (Patel *et al*., 2013). These LuxRs are very similar to QS LuxRs differing in the conservation of one or two of the six highly conserved amino acids in the AHL-binding domain, which most probably allows them to bind to plant molecules rather than AHLs (Ferluga and Venturi, 2009). In plant pathogens, LuxR solos

contribute to virulence, activating motility genes in some cases or regulating negatively the adhesion in response to plant compounds, thus facilitating the spread of the pathogen in the plant (Ferluga *et al*., 2007; Ferluga and Venturi, 2009; Chatnaparat *et al*., 2012; Gonzalez and Venturi, 2013). In plant-beneficial bacteria, LuxR solos control transcriptional regulation in response to plant compounds of various genes related to antimicrobial activity (Subramoni *et al*., 2011) or are associated with survival under stress and utilization of various carbon sources (Patankar and Gonzalez, 2009). Based on previous knowledge already developed for *P. corrugata* strain CFBP 5454, we have also shed light on the natural role of *solR* and *crmA*, a putative NRPS of cormycin A, in the interactions of *P. corrugata* with plants and other microorganisms through a mutational phenotype analysis and, in the case of SolR, in modulation of genes regulated by the AHL-QS system.

4.2 Materials and Methods

4.2.1 Bacterial and fungal strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas corrugata* strain CFBP 5454 and its derivative mutant strains were routinely cultured at 28°C on either Nutrient Agar (NA, Oxoid, Milan, Italy) supplemented with 1% D-glucose (NDA), Luria-Bertani (LB) agar (Oxoid, Milan, Italy) or in LB broth with shaking (180 rpm). *Escherichia coli* strain DH5α, used as host for the plasmids and for the mutagenesis, and the AHL bacterial biosensor *Chromobacterium violaceum* strain CV026 were respectively grown at 37°C and 30°C on LB agar or LB broth. All the strains were stored at -80°C in Luria-Bertani (LB) broth with the addition of 15% (v/v) of glycerol. The Gram-positive bacterium *Bacillus megaterium* ITM100 and the yeast *Rhodotorula pilimanae* ATCC 26423, used as bioindicators for cyclic lipopeptide (CLP) production, were grown on Potato Dextrose Agar (PDA) (Oxoid, Milan, Italy) respectively at 28°C and 23°C. For the antagonistic tests, the fungal cultures of *Botrytis cinerea* strain PVCTBC1, causal agent of grey mold in pre- and postharvest, *Fusarium oxysporum* f.sp. *radicis-lycopersici* strain Saitama ly1, causal agent of tomato crown and root rot and *Plenodomus tracheiphilus* isolate PVCT Pt57, causal agent of citrus Mal secco disease (MSD), were maintained on PDA at 23°C.

Antibiotics were used at the following final concentrations: ampicillin, 100 µg mL^{-1} (*E. coli*); gentamicin, 15 µg mL^{-1} (*E. coli*) or 40 µg mL^{-1} (*Pseudomonas*); tetracyclin, 15 µg mL^{-1} (*E. coli*) or 40 µg mL^{-1} (*Pseudomonas*); kanamycin, 100 µg mL^{-1} (*Pseudomonas*).

Table 1: Bacterial strains and plasmids used in this study.

Strain, plasmid	Relevant characteristics[a]	Reference or source
Pseudomonas corrugata		
CFBP 5454	Wt, source of *solR* and *crmA*	CFBP[b]
GL1	*pcoI*362::Tn5, Km^r	Licciardello *et al*., 2007
GL2	*pcoR*76::Tn5 of CFBP 5454, Km^r	Licciardello *et al*., 2007
GLRFIA	*rfiA*::pKnock, Km^r	Licciardello *et al*., 2009
PCOCRMA	*crmA*::pEX18Gm, Km^r	This study
PCOSOLR	*solR*::pKnock, Km^r	This study
PCOSOLRC	*P. corrugata* PCOSOLR mutant complemented with plasmid pBBR–SolR, Tc^r	This study
Escherichia coli		
DH5α	F2 f80dlacZDM15 D(lacZYA-argF)U169 endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44	Sambrook *et al*., 1989
Plasmids		
pGEM-T	Cloning vector; Amp^r	Promega
pBluescript KS	Cloning vector, Amp^r	Stratagene
pEX18Gm	Suicide vector for making deletion mutant strains, Gm^r	Hoang *et al*., 1998
pUC4K	pUC7 derivative, Amp^r and Km^r	Addgene, Watertown, MA
pBBR1MCS-3	Broad-host-range vector, Tc^r	Kovach *et al*., 1995
pBBR-SolR	pBBR1MCS-3 containing the full length *P. corrugata* CFBP 5454 *solR* gene	This study
pGEPcoR	*pcoR* promoter cloned in pGEM-T; Amp^r	This study
pGEsolR	*solR* promoter cloned in pGEM-T; Amp^r	This study
pGEsolR2	Full-length *P. corrugata* CFBP 5454 *solR* gene cloned in pGEM-T	This study
pKNOCK-Km^r	Mobilizable suicide vector, Km^r	Alexeyev, 1999
pKMSolR	pKNOCK containing an internal fragment of *P. corrugata* CFBP 5454 *solR* gene	This study
pMP220	Promoter probe vector, IncP Tc^r	Spaink *et al*., 1987
pMPPcoI/RfiA	*pcoI/rfiA* operon promoter cloned in pMP220	Licciardello *et al*., 2009
pMPPcoR	*pcoR* promoter cloned in pMP220	This study
pMPSolR	*solR* promoter cloned in pMP220	This study
pRK2013	Km^r Tra^+ Mob^+ ColE1 replicon	Figurski and Helinski 1979
pEX18Gm-*crmA*	*crmA* sequence depleted of about 1400 bp, cloned in pEX18Gm	This study

[a]Km^r, Tc^r, Gm^r and Amp^r indicate resistance to kanamycin, tetracycline, gentamycin and ampicillin, respectively; [b]CFBP=Collection Francaise de Bacteries Phytopathogenes, Angers, France.

4.2.2 General DNA recombinant techniques

DNA manipulations, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase and transformation of *E. coli* DH5α, were performed as described by Sambrook and associates (1989). Plasmids were purified by using EuroGold plasmid miniprep kit, and agarose gel electrophoresis purification of DNA fragments was performed with EuroGold gel purification kit (EuroClone, Italy). PCR amplifications were made using GoTaq DNA polymerase (Promega) and Expand High FidelityPLUS PCR System (Roche). *P. corrugata* total genomic DNA was isolated with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

4.2.3 Cloning and construction of *P. corrugata* CFBP 5454 mutant strains

4.2.3.1 PCOSOLR and PCOSOLRC mutant strains

P. corrugata CFBP 5454 *solR* gene was insertionally inactivated; the central part of the gene was partially amplified by PCR as a 281-bp fragment using primers solRBamHIfw and solRKpnIrev, cloned into pGEM-T (Promega) according to the manufacturer's instructions and subcloned as a BamHI-KpnI fragment in the corresponding sites in pKNOCK-Km^r suicide vector (Alexeyev, 1999), generating pKMSolR. This latter plasmid was then transferred into *P. corrugata* CFBP 5454 by triparental mating, generating a genomic mutant designated PCOSOLR. Transformants were selected on LB agar plates supplemented with kanamycin (100 μg mL^{-1}) and confirmed by sequencing. PCOSOLR mutant strain was complemented by introducing pBBR1MCS-3 containing the full-length *P. corrugata* CFBP 5454 *solR* gene (709 bp). The sequence was first amplified by PCR using primers SolR_XhoF/SolR_SpeR, cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and removed as a XhoI/SpeI fragment.

The insert was then cloned in the corresponding sites in pBBR1MCS-3 (Kovach *et al.*, 1995), yielding pBBR-SolR. The resulting plasmid was introduced in *P. corrugata* CFBP 5454 derivative mutant strain PCOSOLR by triparental mating, obtaining PCOSOLRC. Primers used are listed in Table 2.

Table 2: Primers used in this study.

Primers	Sequence	Reference or source	Mutant strain
PrsolR-fw	5′-GAGAATTCTCTGCGCTGTCGGTCATG-3′	This study	PCOSOLR
PrsolR-rew	5′-GAGGTACCTTCCACTTGTCCATCGTCG-3′	This study	PCOSOLR
PrgacA-fw	5′-GAGAATTCCTGAGGATGCAGCGAGAC-3′	This study	PCOSOLR
PrgacA-rew	5′-GAGGTACCACTAACACCCTAATCAAGCAG-3′	This study	PCOSOLR
PrpcoR-fw	5′-GAGAATTCACACGGTAGTTGTGGTGG-3′	This study	PCOSOLR
PrpcoR-rew	5′-GAGGTACCAGATGGCTTGAAATGGGCTTG-3′	This study	PCOSOLR
PrpcoI-fw	5′-CGGTACCCACTGTCATCAGTGAAAGC-3′	Licciardello *et al*., 2009	PCOSOLR
PrpcoI-rew	5′-GTCTAGAGGGATTTTCGAGTAAGATG-3′	Licciardello *et al*., 2009	PCOSOLR
SolR_XhoF	5'-ACTCGAGTGAGCGTCTTAACCACC-3'	This study	PCOSOLR
SolR_SpeR	5'-AACTAGTTTTCAAAGTCTGCAAGGCCG-3'	This study	PCOSOLR
solRBamHIfw	5'-AGGATCCTTTGGGATGAGAG-3'	This study	PCOSOLR
solRKpnIrev	5'-AGGTACCATCTCAGTACTTC-3'	This study	PCOSOLR
syrD_2-fw	5'-CAACTGAACACGACACTG-3'	Licciardello *et al*., 2018	PCOSOLR
syrD_2-rew	5'-GTAGGCAATACCGAACAG-3'	Licciardello *et al*., 2018	PCOSOLR
Nrps-fw	5'-ACGGGCCACCCGAAAG-3'	Strano *et al*., 2015	PCOSOLR
Nrps-rev	5'-GAGGCGAAAGCCACGTGAT-3'	Strano *et al*., 2015	PCOSOLR
Pco16s-fw	5'-TGTAGCGGTGAAATGCGTAGAT-3'	Conte *et al*., 2006	PCOSOLR
Pco16s-rew	5'-CCTCAGTGTCAGTATCAGTCCAG-3'	Conte *et al*., 2006	PCOSOLR
SP6	5'-CATACGATTTAGGTGACACTATAG-3'	Eurofins Genomics	PCOSOLR(C)
T7	5′-TAATACGACTCACTATAGGG-3′	Eurofins Genomics	PCOSOLR(C)
800R	5′-TACCAGGGTATCTAATCC-3′	Eurofins Genomics	PCOSOLRC
907R	5′-CCGTCAATTCMTTTRAGTTT-3′	Eurofins Genomics	PCOSOLRC
crm1Kpn_F	5′-GGCTCCACCGGTACCCCGAAAG-3′	This study	PCOCRMA
crm1Hind_R	5′-TTATAAGCTTGCCGTTGGCGGTGAGG-3′	This study	PCOCRMA
crm2Bam_F	5′-TATTAGGATCCCTCAACGACGACGGCGAA-3′	This study	PCOCRMA
crm2Xba_R	5′-TATTTCTAGACCAGCTCGCCTAGCGCATC-3′	This study	PCOCRMA
kanext_F	5′-TGGAATTTAATCGCGGCCTCG-3′	This study	PCOCRMA
kanext_R	5′-CATCTTCCCGACAACGCAGAC-3′	This study	PCOCRMA
crm1ext_F	5′-GCCTTGCTGAACCAGTTGC-3′	This study	PCOCRMA
crm2ext_R	5′-CTTCACCTGGTCGTCGTTGC-3′	This study	PCOCRMA

4 .2.3.2 PCOCRMA mutant strain

A knock-out mutant for *crmA* gene from *P. corrugata* CFBP 5454 was obtained by insertion and deletion using the pEX18Gm plasmid as described previously (Hoang *et al.*, 1998), with some modifications. An internal fragment of about 1400 bp from *crmA* gene was deleted and replaced by a restriction site (PstI). A Km gene cassette, previously extracted from pUC4K plasmid, was cloned inside PstI restriction site. Then, the fragment was excised with KpnI and XbaI restriction enzymes and cloned in the corresponding site in pEX18Gm. The resulting pEX18Gm-derivative plasmid was introduced by triparental conjugation with *E. coli* helper strain pRK2013 in the corresponding *P. corrugata* CFBP 5454 (wild-type strain) genome. Clones with a chromosomal insertion of the pEX18Gm plasmid were selected on LB agar plates supplemented with 40 mg mL^{-1} gentamicin (Gm) and 100 mg mL^{-1} nitrofurantoin (Nf). Plasmid excision from the chromosome was selected on NSLB (No Salt Luria Bertani) agar plates amended with 15% (w/v) sucrose. All mutant strains were verified by PCR using primers specific to the Km cassette and to the genomic DNA sequences upstream and downstream from

the deleted fragment of *crmA* gene. Δ*crmA* mutant strain derived from *P. corrugata* CFBP 5454 was designated PCOCRMA.

4.2.3.3 Bioinformatic analysis

Genome comparative analysis of the nucleotide sequences of homologous *solR* and *crmA* genes present in other strains of *P. corrugata* and gene cluster visualization were carried out using the Integrated Microbial Genomes and Microbiomes (IMG/M) system of the Joint Genome Institute (JGI) portal. The search for restriction sites was carried out using the SerialCloner 2.6.1 software (http://serialbasics.free.fr/Serial_Cloner.html).

4.2.4 β-Galactosidase assay

Transcriptional fusion plasmids for the various gene promoters based on the pMP220 promoter probe vector (Spaink *et al.*, 1987) were constructed as follows. The 567-bp fragment containing the *pcoR* promoter region was amplified by polymerase chain reaction (PCR) by using Expand High FidelityPLUS PCR System (Roche) according to the instructions of the supplier, genomic DNA of *P. corrugata* CFBP 5454 as the template, and oligonucleotides PrpcoR-fw and PrpcoR-rew. The fragment was then cloned in pGEM-T (Promega, Madison, WI, USA), yielding pGEPcoR, and verified by DNA sequencing. The *pcoR* promoter was then removed as a EcoRI-KpnI fragment and cloned in the corresponding sites in pMP220, yielding pMPPcoR. The *solR* promoter was amplified as a 324-bp fragment by using oligonucleotides PrsolR-fw and PrsolR-rew and cloned in pGEM-T, yielding pGESolR. The *solR* promoter was then removed as a EcoRI-KpnI fragment and cloned in pMP220, yielding pMPSolR. Sequencing for verification of cloned DNA inserts was determined with the Eurofins Genomics DNA sequencing service (Tokyo, Japan). Primers used are listed in Table 2. pMP220 derivative constructs were introduced into the wild-type *P. corrugata* CFBP 5454 and its derivative mutant strain by triparental conjugation with the helper strain *E. coli* pRK2013. β-galactosidase activities of bacterial cultures were determined during growth in 20 mL LB medium or IMM medium supplemented with tetracycline (40 μg mL^{-1}) starting with an initial inoculum of 1.0×10^7 cfu mL^{-1}, essentially as described by Miller (1972), with the modifications of Stachel and associates (1985). Average Miller unit values and standard deviations were calculated from three independent experiments and three replicates for each transconjugant.

4.2.5 *In vitro* bioassay for CLP production

P. corrugata CFBP 5454 and its derivative mutant strains were grown in IMM (Improved Minimal Medium: mannitol, 10 g; $MgSO_4$ • 7 H_2O, 0.2 g; L-histidine, 4 g; KH_2PO_4, 0.4 g; K_2HPO_4, 1.114 g; $FeSO_4$ • 7 H_2O, 0.002 g; CaCl, 0.086 g; H_2O, 1 l; pH 5.5 ±2), a medium inducing the production of CLP (Surico *et al.*, 1988). 24-h LB cultures of each strain were transferred into 100 mL-Erlenmeyer flasks up to a final concentration of 10^7 cfu mL^{-1}. The cultures were incubated without shaking at 28°C. Aliquots of filtrates were sampled at the inoculation time (T0) and 1 (T1), 2 (T2), 3 (T3), 6 (T6) days post-inoculation, centrifuged (9000 × g, 20 min) and sterilized by filtration using a 0.22-µm Millipore filters (Millipore, Billerica, MA, U.S.A.). Aliquots of culture filtrate were also sampled at 40h post-inoculation for total RNA extraction and quantitative RT-PCR analysis. Finally, filtrates were lyophilized with the freeze dryer Heto PowerDry LL3000 (Thermo Fisher, USA) and 10X concentrated for LDP bioassay against CLP-indicator microorganisms and phytopathogenic fungi.

4.2.6 RNA sample preparation and RT-Real time PCR

Total RNA from *P. corrugata* CFBP 5454 as well as PCOSOLR mutant strain was extracted from a 40h-culture in IMM ($O.D._{600}$ ~ 3 x 10^8) with the RNeasy Protect Bacteria Mini Kit (Qiagen), according to the manufacturer's instructions. RNA was quantified and estimated for quality using micro-spectrophotometry (NanodropTM 2000C, Thermo ScientificTM, Waltham, MA, United States). Following a DNAse purification step by DNAse I (Invitrogen), first-strand cDNA was synthesized (1 µg of total RNA) using Superscript III (Invitrogen) according to the manufacturer's protocol.

For quantitative RT-PCR, genes annotated as *ppsE_1* and *syrD2*, putatively involved in biosynthesis and secretion of CLPs, were selected. The *16S rRNA* gene was used as an endogenous reference for normalization (Conte *et al.*, 2006). Reactions were conducted with the BioRad iQ Cycler and the SYBR® Select Master Mix for CFX (Applied Biosystem, Life Technologies) according to the manufacturer's protocols. The amplification was performed for both genes in the following thermocycling conditions: 95°C for 10' followed by 40 cycles of 15" at 95°C, 15" s at 56°C and 60 sec at 72°C. Melt-curve analysis was performed to ensure that a single product was amplified. The relative quantitation of *ppsE_1* and *syrD2* expression

among *P. corrugata* CFBP 5454 and its derivative mutant strain PCOSOLR were calculated using the comparative threshold (Ct) method. Three independent triplicates of quantitative PCR experiments were performed for each gene to generate an average Ct. ΔCt was calculated by subtracting the average 16S rRNA Ct from the average Ct of the gene of interest. The comparative expression level of the genes of interest was given by the formula 2-ΔΔCt, where ΔΔCt was calculated by subtracting the ΔCt of PCOSOLR and PCOSOLRC mutant strains from *P. corrugata* CFBP 5454 ΔCt.

4.2.7 Phenotypic analysis of mutant strains

4.2.7.1 Proteolytic activity

P. corrugata CFBP 5454 and its derivative mutant strains were tested for some traits that had revealed intraspecific variability in previous studies. In particular, the presence of proteolytic activity was determined. In accordance Lelliot and Stead (1987), a medium containing casein was used. 111 mL l^{-1} of microfiltered skimmed milk were added to sterile NA medium (50°C). Aliquots of 5 µl of a bacterial suspension of each strain to be tested were then spotted in the center of each Petri dish containing the NA medium amended with milk. The plates were incubated at 28°C for up to approximately 14 days; the test is considered positive when colonies exhibit a clarification halo of protease activity.

4.2.7.2 Motility assays

P. corrugata CFBP 5454 and its derivative mutant strains were assayed in plates for swimming and smarming motility as previously described (Licciardello *et al*., 2007). The swarming motility plates contained 10 g of L^{-1} tryptone, 5 g of L^{-1} NaCl and 0.5% (w/v) or 0.7% (w/v) bacteriological agar. Glucose (10mM) was sterilized by filtration and added just before use to the autoclaved substrate once it had cooled (50°C). The plates for swimming motility contained 10 g of L^{-1} tryptone, 5 g of L^{-1} NaCl and 0.3% (w/v) of bacteriological agar. The plates were inoculated with 1 µl of an overnight bacterial suspension (28°C, 180 rpm) in LB broth of each tested strain and incubated at 28°C for 16-24 hours. The test was carried out twice with three replicates for each bacterial strain.

4.2.7.3 In vitro antagonistic activity

Activity against CLP indicator microorganisms

The culture filtrates of *P. corrugata* CFBP 5454 and its derivative mutant strains were evaluated for the production of cyclic lipopeptides (CLP) by a biological assay based on antimicrobial activity against the yeast *R. pilimanae* and the Gram-positive bacterium *B. megaterium* ITM100, performed as previously described (Licciardello *et al.*, 2009), with some modifications. Aqueous suspensions of the CLP-sensitive bioindicators *B. megaterium* and *R. pilimanae* were prepared from 24-hour PDA cultures and diluted up to a final concentration of 10^7 cfu mL^{-1}. The bioindicators were plated on PDA plates using a sterile swab covering the entire surface of the plate. Wells of 0.5 cm Ø were made using a sterile tip. The wells were sealed with 15 µl of agarose (2%) and 20 µl of 10X concentrated culture filtrate were dispensed onto each well. The plates were incubated at 28 ° C for 48 hours. The antagonistic activity was detected by measuring the inhibition zone between each well and the CLP-indicator microorganisms. For each experiment, the activity of culture filtrates was evaluated in three independent replicates.

Activity against phytopathogenic fungi

In order to determine the effects of *P. corrugata* CFBP 5454 and its derivative mutant strains on fungal mycelia growth, dual-culture antagonism assays were performed on PDA plates. Three plant pathogenic fungi, *Botrytis cinerea*, *Plenodomus tracheiphilus* and *Fusarium oxysporum* f.sp. *radices-lycopersici* were tested. Single colonies from 24h-bacterial cultures grown on NDA medium were transferred onto a Petri dish (1.5 cm from the edge) containing PDA. Four-millimeter mycelia discs from a 7-10 day PDA culture of each fungal pathogen was cut off with a sterile scalpel and placed in the center of the Petri dish. For each test, a control plate containing only the tested fungal pathogen was prepared. Plates were incubated at 25°C and the antifungal activity was expressed as percentage of growth inhibition (PGI) and calculated according to the formula of Vincent (1947) and Zygadlo *et al.* (1994): PGI (%) = 100·(GC-GT)/GC; where GC represent the average diameter of fungal mycelium in PDA (control) and GT represents the average diameter of fungal mycelium on the treated PDA plate contain the antagonistic bacteria. All strains were evaluated in three independent replicates.

4.2.7.4 AHL production assay

AHLs were quantified by adding the culture filtrates of *P. corrugata* CFBP 5454 or PCOSOLR mutant derivative to the biosensor *C. violaceum* strain CV026 liquid cultures and measuring violacein formation, depending on the external addition of medium-chain-length lactones (Licciardello *et al.*, 2009). Experiments were conducted in microtiter-plate-based assay and absorbance values read using the Bioscreen C instrument (Labsystems, Espoo, Finland). *P. corrugata* culture filtrates were added to freshly grown CV026 (1×10^9 cfu mL^{-1}) at a ratio of 1:10 to a final volume of 300 µl (10 replicates for each cultural filtrate) and incubated in the Bioscreen C system for 16 h at 27°C with continuous shaking to allow induction of violacein formation. A 10-replicate lane loaded only with CV026 was also included. Turbidity at 600 nm was used for growth control. After incubation, the plates were dried at 60°C until all medium had evaporated (approximately 6 h or overnight). The violacein was resolubilized by adding 100 µl of dimethyl sulfoxide to each well and incubating the plates in the Bioscreen C system for 2 h with continuous shaking. The amounts of violacein measured at 590 nm by adding the culture filtrates were used as an indirect measure of AHL production. These values were subtracted by those obtained in the wells inoculated only with CV026.

4.2.7.5 Plant inoculations

P. corrugata CFBP 5454 strain and its derivative mutant strains were tested for pathogenicity in tomato seedlings using two different methods. The first to verify the virulence of the strains, and the second to measure the population present in the inoculated tissues of the plant. Greenhouse-grown tomato plants in square pots were used in both assays. During the tests, the plants were kept in a growth chamber with 16/8 h photoperiod and 26°C temperature essentially as described by Licciardello *et al.* (2007; 2009). After the inoculation, the plants were closed in polyethylene bags to maintain 100% relative humidity for one week. Twenty plants per strain were prick-inoculated on the stem at the axil of the first true leaf, using a sterile entomological needle contaminated with bacterial cells from 24-h cultures on NDA. Plants were rated for symptoms 15 days after inoculation by longitudinally dissecting the stems and measuring the length of pith necrosis. Experiments were performed at least twice.

To determine the concentration of the bacterial population within the tissue of the host plant, the inoculated plants were sampled seven days after inoculation. Portions (1 cm) of tomato stems were cut above (1 cm, 2 cm, 3 cm) and below (-1 cm, -2 cm, -3 cm) the inoculation site and ground with a pestle into 1.5 mL eppendorf tubes containing 1 mL in sterile distilled water (SDW). Ten-fold dilutions of these suspensions were plated onto NDA medium supplemented

with the appropriate antibiotics for *P. corrugata* selection and colonies forming units (cfu) were determined for each tomato stem portion.

A hypersensitive response (HR) test was performed by infiltration of *Nicotiana tabacum* leaf mesophyll with a bacterial suspension of (10^8 cfu mL^{-1}) of *P. corrugata* CFBP 5454 parent strain and its mutant derivatives using a blunt syringe. Twenty leaf panels were inoculated per strain. Negative control panels were inoculated only with sterile distilled water (SDW). After inoculation, plants were placed at 25°C in a growth chamber and the collapse/necrosis of the mesophyll was recorded daily.

4.2.8 Statistic analysis

Data were analyzed by one-way ANOVA using GraphPad Prism software (version 8.0.1). Mean values were compared using the Tukey's multiple comparison test and the unpaired t-test for promoter activity analysis. Tukey's multiple comparison test was also used to compare *in planta* virulence and antagonistic activity against CLPs bioindicators. Summary data are reported as mean ± standard deviation (SD). A *P* value of 0.05 was considered significant for all experiments.

4.3 Results

4.3.1 Study on the role of a LuxR solo transcriptional regulator

4.3.1.1 Identification of a LuxR solo transcriptional regulator

The analysis of the genome of *P. corrugata* CFBP 5454 revealed the presence of two *luxR* genes coding for transcriptional regulators with a typical modular structure consisting of an AHL-binding domain and a helix-turn-helix (HTH) DNA-binding domain. One of the two LuxR proteins identified, named PcoR, is the transcriptional regulator of *P. corrugata* QS system PcoR/PcoI (Licciardello *et al.*, 2007). Since the *P. corrugata* CFBP 5454 knock-out of *pcoI*, the

gene encoding the acyl-homoserine lactone synthase of the PcoI/PcoR QS system, is impaired in AHLs production, the second *luxR* identified, lacking of a cognate *luxI* homologue, is a putative *luxR* solo designated *solR*. Interestingly, LuxR solos are widespread among several strains of *P. corrugata* subclade species (Bez *et al*., 2021). Based on its genomic context and primary structure, SolR belongs to the subgroup A of LuxR solos family proteins described by Bez *et al.* (2021)(Figure 1).

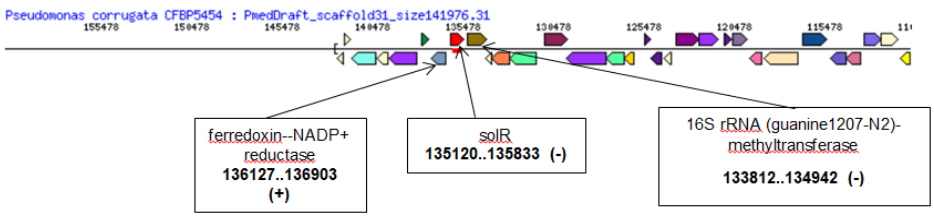

Figure 1: Genomic context of *solR* gene in *P. corrugata* CFBP 5454 provided by the JGI (Joint Genome Institute).

SolR gene genomic context and the conservation of the invariant amino acids of the autoinducer-binding domain were investigated. The analysis revealed that *solR* has the same genomic context of other genes encoding for the already described LuxR solos of *P. putida*, *P. fluorescens*, *P. brassicacearum, P. entomophila*, *P. synxantha*, with a sequence identity of 40-79%.

SolR product did not display changes in the 9 key amino acid residues compared to several characterized AHL-binding QS LuxR protein, suggesting that this protein is likely to bind AHLs. Multiple sequence alignment (BLASTp) showed that SolR of *P. corrugata* retains the same amino acids in the autoinducer-binding domain positions 57 and 61 of previously studied LuxR protein, such as TraR of *Rhizobium radiobacter*, PcoR of *P. corrugata* and LasR of *P. aeruginosa* PA01 (Figure 2). These proteins share the presence of tryptophan (W) in position 57 and tyrosin (Y) in position 61, corroborating the hypothesis that SolR may bind AHLs.

Differently, pairwise sequence alignment by BLASTX of previously described LuxR solos, namely PsoR from *Pseudomonas fluorescens* Pf-5 and OryR from *Xanthomonas oryzae* pv. oryzae, showed substitions for the amino acids in positions 57 and 61, respectively (W), (W) and methionine (M), (W). This most probably reflects the ability of these proteins to bind plant low-molecular-weight compounds, as previously reported (Subramoni and Venturi, 2009a; Patel *et al.*, 2013). In the HTH-domain, amino acids were conserved between all the strain,

specifically glutamic acid (E) in position 178, leucine (L) in position 182 and glycine (G) in position 188.

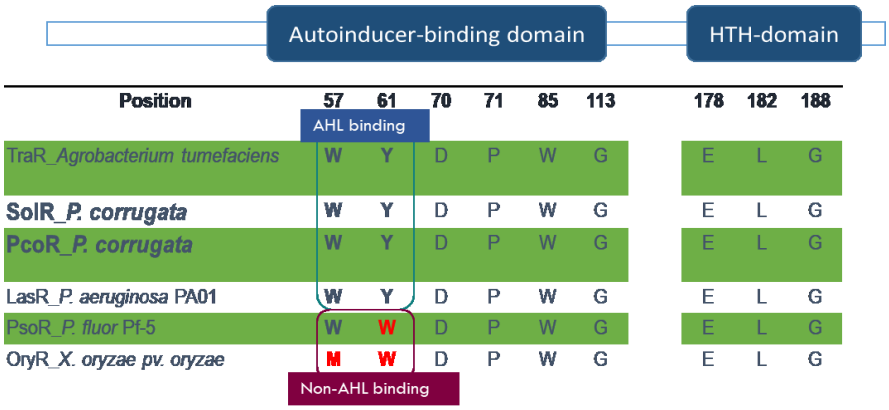

Figure 2: Amino acid multiple sequence alignment of the autoinducer-binding domain and the HTH-domain of LuxR 'solos' from representative bacterial species.

Then, the genomes of the other species taxonomically related to *P. corrugata* were analyzed and we found that the PcoI/PcoR AHL-QS system is common and highly conserved in all *P. corrugata* and *P. mediterranea* strains and in the biocontrol agent *Pseudomonas* sp. SH-C52, but not in the other species of the *P. corrugata* subgroup, whereas SolR resulted conserved in all the strains (identity of 80-95%) (Table 3).

4.3.1.2 Construction of ΔsolR mutant strain

S*olR* gene was independently inactivated by integration of the suicide vector pKNOCK-Km. In the first phase, a region partially inside the genes to be inactivated was amplified by PCR with specific primer pairs provided with an additional sequence containing suitably selected enzyme restriction sites so that they did not cut inside the region to be inactivated and were compatible with the vectors to use in the next steps. The *solR* amplification product was purified and cloned into a broad spectrum host vector (pGEM-T) by transformation into DH5α by heat shock at 42°C. Transformants were selected for resistance to ampicillin and by screening of blue/white colonies. A few white colonies were then taken and confirmed by colony-PCR. In order to purify the inserts, the plasmid DNA was then isolated from the PCR positive clones and subjected to digestion with the restriction enzymes chosen during primer design. The digestions were then purified, quantified and ligated to the pKNOCK-km suicide vector, also previously digested with the same restriction enzymes and quantified in order to have an insert: vector = 10:1 ratio during the ligation phase. The 2.2 kb pKNOCK-Km plasmid contains a marker gene

encoding kanamycin resistance and an R6K (-ori) origin of replication characterizing the suicidal nature of the vector: replication can only occur in *E. coli* strains that synthesize the π protein, such as strain CC118. The presence of the mobilization site (*mob*) also allows its conjugation. CC118 cells were then transformed and selected for kanamycin resistance. Some colonies were then collected and confirmed by colony-PCR. The selected transformants were transferred by triparental conjugation with *E. coli* pRK2013 helper plasmid into the wild-type *P. corrugata* strain CFBP 5454. The pairing of the homologous regions of the genome and vector resulted in a single recombination event leading to the integration of the entire plasmid in the *P. corrugata* genome within the gene to be inactivated. The selection with the appropriate antibiotics has allowed to isolate the mutant strains of a gene that has been called PCOSOLR (Table 1).

PCOSOLR mutant strain was complemented *in trans* by introducing the full-length *solR* gene (709 bp) from *P. corrugata* CFBP 5454. Briefly, a 956 bp fragment including the full-length *solR* gene was amplified by PCR with primers listed in table 2. The amplification product was purified from agarose gel, cloned into a broad spectrum host vector (pGEM-T) and transformed into *E. coli* DH5α by heat shock at 42°C. Transformants were selected for resistance to ampicillin and by screening of blue-white colonies. White colonies were confirmed by sequencing with primers designed on pGEM-T vector (SP6 and T7). The insert was removed as a XhoI/SpeI fragment and cloned in the corresponding sites in pBBR1MCS-3 (Kovach *et al.*, 1995), yielding pBBR-SolR. The pBBR-SolR construct was confirmed by sequencing with primer T7. The positive constructs were then transformed into *E. coli* DH5α by heat shock at 42°C and transformants were selected for resistance to tetracyclin; positive colonies were selected with blue-white screening. The resulting pBBR-derivative plasmid was introduced in *P. corrugata* CFBP 5454 derivative mutant strain PCOSOLR by triparental mating, Transconjugants were confirmed by PCR with primers listed in table 2.

Specifically, the taxonomic identity of each transconjugant was confirmed by sequencing with the universal primers 800R/907R, amplifying partial 16S rDNA gene sequence (not shown).

From positive colonies, partial *solR* gene sequence was amplified with primer pair SolR_XhoF/SolR_SpeR, resulting in a single band of 790 bp for *P. corrugata* CFBP 5454 (wild-type strain) and PCOSOLR complemented mutant strain containing the construct pBBR3-*solR*. No amplification was obtained for PCOSOLR mutant strain (Figure 3), probably due to the size of the amplification product of pKNOCK-derivative contructs (about 3 kb) and the short elongation time (about 1 min) of PCR amplification protocol. PCOSOLR complemented mutant strain was designed PCOSOLRC.

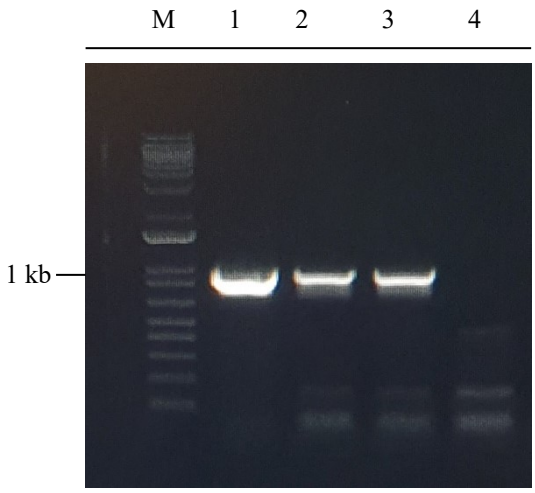

Figure 3: Screening of PCOSOLR complemented mutant strains (PCOSOLRC) containing pBBR3-*solR* construct by *solR* gene amplification. M, molecular weight marker (1 kb). 1: *P. corrugata* CFBP 5454 (wild-type strain); 2: PCOSOLR (pBBR3-*solR*) clone 1; 3: PCOSOLR (pBBR3-*solR*) clone 2; 4: *P. corrugata* CFBP 5454 derivative mutant strain PCOSOLR (Δ*solR*).

4.3.1.3 Phenotypic analysis of PCOSOLR mutant strain

With the aim of sheding light to the biological role of SolR, a LuxR-family solo from *P. corrugata* CFBP 5454, we analyzed the phenotype of PCOSOLR mutant strains, obtained by deletion of *solR* gene. Colony morphology of wild-type strain CFBP 5454 and its derivative mutant strains PCOSOLR and PCOSOLRC was observed on NDA medium. The colonies of the wild-type strain, PCOSOLR (Δ*solR*) and PCOSOLRC complemented mutant strain showed the typical morphology of the species, with a slightly raised and wrinkled surface and wavy margins and produced a yellow pigment diffusible in the substrate (Scarlett *et al.*, 1978).

The production of exoenzyme was investigated in PCOSOLR mutant strain and PCOSOLRC complemented mutant strain by comparison with the wild-type strain CFBP 5454. To determine the production of protease, *P. corrugata* strains were plated on nutrient agar medium containing milk; when positive, bacterial colonies were surrounded by a clarification halo indicating casein hydrolysis. Wild-type strain resulted positive for caseinase, while PCOSOLR mutant strain was impaired in proteolytic activity; the expression of *solR* in trans in PCOSOLR mutant strain (strain PCOSOLRC) restored the proteolytic activity (Figure 4).

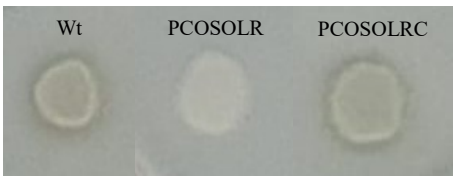

Figure 4: Proteolytic activity of *P. corrugata* CFBP 5454 (Wt) and its derivative mutant strains PCOSOLR and PCOSOLRC (complemented PCOSOLR mutant strain) in Nutrient Agar (NA) medium amended with milk.

The swimming and swarming motility of the wild-type strain and its derivative mutant strains was tested in plates with different concentrations of agar (0.3% for swimming motility, 0.5% and 0.7% for swarming motility). Bacterial motility was evaluated after 24 h.

In 0.3% swim agar plates, PCOSOLR mutant strain did not differ from wild-type strain CFBP 5454, whereas into 0.5 and 0.7% swarm agar plates moved further and more efficiently compared to the parent strain CFBP 5454 (Figure 5). Overall, the complemented mutant strain PCOSOLRC moved as far as the wild-type strain CFBP 5454, with the exception of 0.5% swarm agar plates (Figure 5).

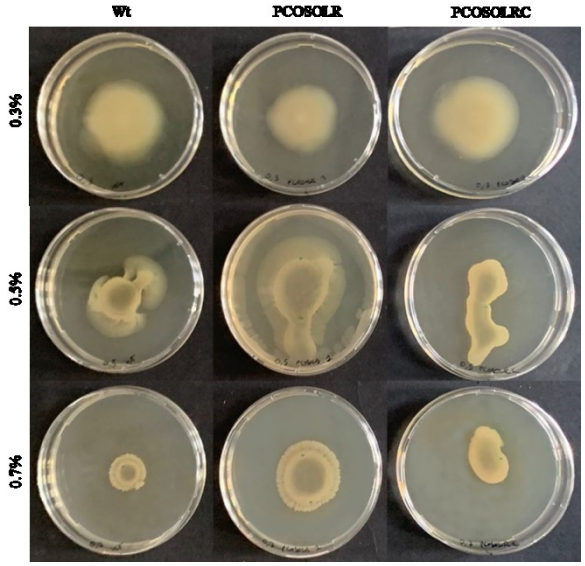

Figure 5: Motility of *P. corrugata* CFBP 5454 (Wt), derivative mutant strain PCOSOLR and complemented mutant strain PCOSOLRC. Motility was assessed in 0.3% (swimming motility) or 0.5% and 0.7% (swarming motility) agar plates.

To investigate the role of *solR* in *P. corrugata*-tomato interaction, *in planta* assays were set up. In the first experiment, the wild-type strain was compared with the mutant strains PCOSOLR and PCOSOLRC on the basis of their ability to induce symptoms in tomato plants prick-inoculated at the first true leaf axil. One-month post inoculation, the inoculated plants were sectioned longitudinally and symptoms were recorded and expressed as length of stems lesion (cm). Plants showed necrosis of the xylem ranging from about 2.0 to 7.0 cm (± 1.34), 3.0 to 13.5 cm (± 3.4), 1.5 to 9.0 (± 2.5) for the wild-type CFBP 5454, PCOSOLR and PCOSOLRC mutant strains, respectively (Figure 6). About 33%, 36% and 10% of tomato plants inoculated

with wild-type strain, PCOSOLR and PCOSOLRC mutant strains respectively showed dark brown lesions extended to the whole pith. The length of the lesions of plants inoculated with PCOSOLR was significantly increased compared to the length of the lesions caused by the parent strain and the PCOSOLRC strain (Figure 6).

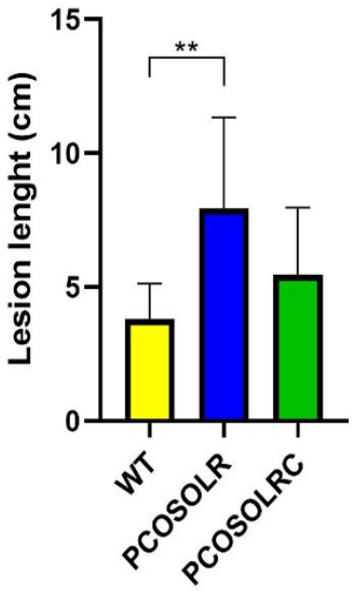

Figure 6: Length of pith necrosis in tomato plants inoculated with the wild-type *P. corrugata* strain CFBP 5454, PCOSOLR mutant derivative and PCOSOLRC complemented mutant strain. Symptoms were recorded one month after inoculation. The mean is obtained from 10 replicates. Vertical bars represent SD of the mean length of pith necrosis. Results were analysed for statistical significance using one-way ANOVA with a Tukey's multiple comparison test ($0.01 \leq *P \leq 0.05$, $0.001 \leq **P \leq 0.01$).

In the second experiment, in order to evaluate if PCOSOLR and PCOSOLRC mutant strains have a differential colonization ability *in planta*, bacterial population concentrations (log cfu/cm) of the three *P. corrugata* strains from inoculated tomato plants were determined seven days post-inoculation at the inoculation site and at different distances below (-1 cm, -2 cm, -3 cm) and above (+1 cm, +2 cm, +3 cm) the inoculation site. Wild-type strain showed a bacterial titer of approximately 10^8 colony forming units (cfu)/cm of stem at the inoculation site and this result was comparable with those of bacterial populations of PCOSOLR and PCOSOLRC mutant strains (Figure 7). No significant differences in terms of bacterial titer of the three tested strains were recorded at 1 and 2 cm above the inoculation site and at 3 cm below (about 10^7 cfu/cm for all three distances). Monitoring at different distances above and below the inoculation site always revealed the presence of all three strains (Figure 7).

The effect of *solR* gene inactivation was evaluated on the ability to cause HR in a non-host species such as *Nicotiana tabacum* by infiltrating the mesophyll of tobacco leaves with a high-inoculum dose of *P. corrugata* CFBP 5454 (wild-type) and the mutant strains PCOSOLR and PCOSOLRC. By 24h after inoculation the leaf panels infiltrated with either the parent strain or its derivative mutant strains PCOSOLR and PCOSOLRC showed the collapse of the mesophyll,

which turned necrotic within the following 24h, with no significant differences between the three strains (Figure 8). Leaf panels inoculated with water (negative control) did not show any symptoms (not shown).

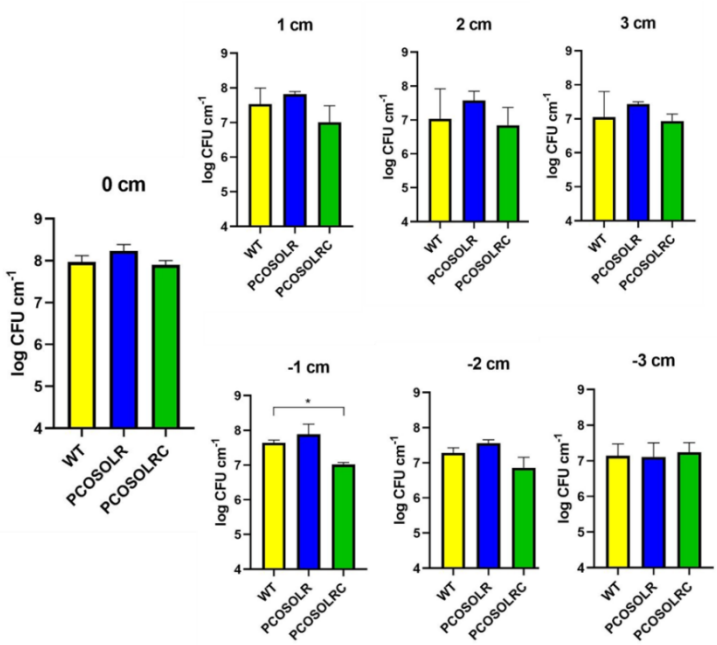

Figure 7: *P. corrugata* strain CFBP 5454, derivative mutant strain PCOSOLR and PCOSOLRC complemented mutant strain population concentrations (log cfu/cm) in tomato stems 7 days after inoculation at the inoculation site and at different distances above (1 cm, 2 cm, 3 cm) and below (-1 cm, -2 cm, -3 cm) the inoculation site.

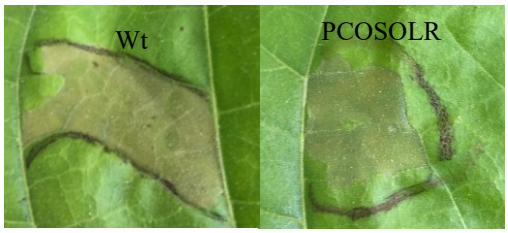

Figure 8: Results of leaf mesophyll inoculations of *N. benthamiana* with bacterial suspensions (1 x 10^{8} cfu mL^{-1}) of *P. corrugata* CFBP 5454 (Wt) and its derivative mutant strain PCOSOLR.

4.3.1.4 CLP production and gene expression

SolR is involved in the production of cyclic lipopeptides and AHLs

CLPs production was indirectly assayed in *P. corrugata* CFBP 5454 and PCOSOLR mutant strain at the inoculation time (T0) and 1 (T1), 2 (T2), 3 (T3), 6 (T6) days after inoculation by inhibition of the growth *in vitro* of the CLP-sensitive bacterium *Bacillus megaterium* (Figure 9). Antimicrobial activity was first detected in culture at T2 for the wild-type strain (1×10^9 cfu mL^{-1}) whereas it was already observed at T1 for PCOSOLR (5×10^7 cfu mL^{-1}), suggesting that in PCOSOLR mutant strain the production of CLPs occurs earlier compared to the parent strain. In addition, inhibition halos measured at the same sampling time (T0, T1, T2, T3, T6) were significantly wider in PCOSOLR compared to the wild-type strain, with a maximum inhibition activity at T6 and T1-T3 (for wild-type and PCOSOLR mutant strain, respectively).

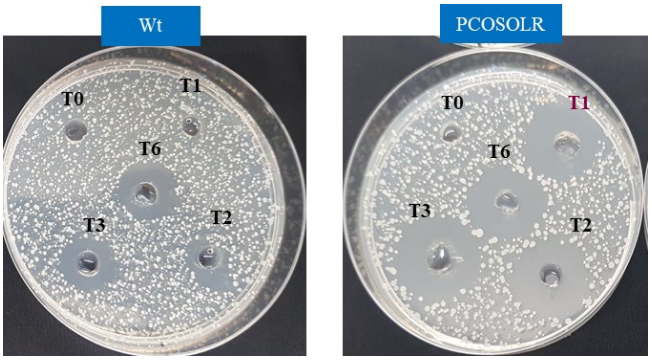

Figure 9: Cyclic lipopeptides (CLPs) production in *P. corrugata* CFBP 5454 (Wt) and PCOSOLR derivative mutant strain during growth phase.

AHLs (acyl-homoserine-lactones) are the signal molecules of the PcoI-PcoR QS system of *P. corrugata*. In the same time-course experiment, AHLs abundance was indirectly quantified by measuring the amount of violacein produced by the biosensor *C. violaceum* CV026 (Figure 10). AHLs production was detected in culture after 24 h of growth for both wild-type strain and PCOSOLR. AHLs concentration increased rapidly at higher bacterial densities; maximum concentration was observed at high-cell densities 2 days after inoculation (T2) and 1 and 3 days after inoculation (T2-T3), while a minimum concentration was detected 6 days after inoculation (T6) and 2 days after inoculation (T2) for wild-type strain and PCOSOLR respectively. For both wild-type strain and PCOSOLR mutant strain minimum and maximum production occurred at the late stationary phase (about 5 x 10^8 cfu mL^{-1}). Overall, at T1, T3 and T6 the

AHL production detected in PCOSOLR mutant strain was significantly increased compared to wild-type strain.

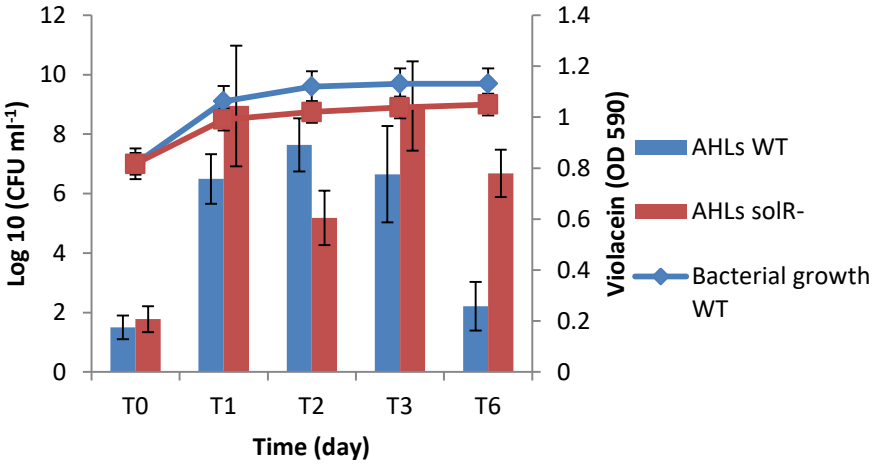

Figure 10: Acyl-homoserine lactones (AHLs) production in *P. corrugata* CFBP 5454 (Wt) and Δ*solR* mutant strain (PCOSOLR) during growth phase at 0 (T0), 1 (T1), 2 (T2), 3 (T3), 6 (T6) days after inoculation. AHL biosynthesis was indirectly measured as violacein produced by a bioassay using the biosensor *Chromobacterium violaceum* CV026. Values are the means of 10 replicates.

SolR influences the transcription of *P. corrugata* AHL-QS system genes

In order to determine whether *solR* regulates the promoter activity of *P. corrugata* AHL-QS genes, the promoter regions of the *pcoI-rfiA* operon and the *luxR* homologue *pcoR* were cloned in the β-galactosidase promoter probe vector pMP220, yielding pMPPcoI-RfiA and pMPPcoR, respectively. Promoter activity, expressed as β-galactosidase activity and reported in Miller Units (M.U.), was determined in a rich medium (Luria Broth, LB) or in a minimal medium inducing CLP production (Improved Minimal Medium, IMM) in the parent strain *P. corrugata* CFBP 5454 and the mutant strain PCOSOLR.

PcoR promoter activity was significantly reduced in *P. corrugata* mutant strain PCOSOLR compared to the parent strain, in both rich or minimal medium. Overall, the promoter activity levels of this gene was higher in minimal medium compared to rich medium (Figure 11). The activity of the promoter of *pcoI-rfiA* operon was significantly reduced in *P. corrugata* mutant strain PCOSOLR compared to the parent strain in minimal medium, although only slight reduction was observed in rich medium. No differences were observed in the promoter activity comparing rich medium to minimal medium (Figure 11). These results suggest that control of

pcoR and *pcoI-rfiA* operon transcriptions are affected by SolR. Moreover, minimal medium provides better conditions for *pcoR* transcription.

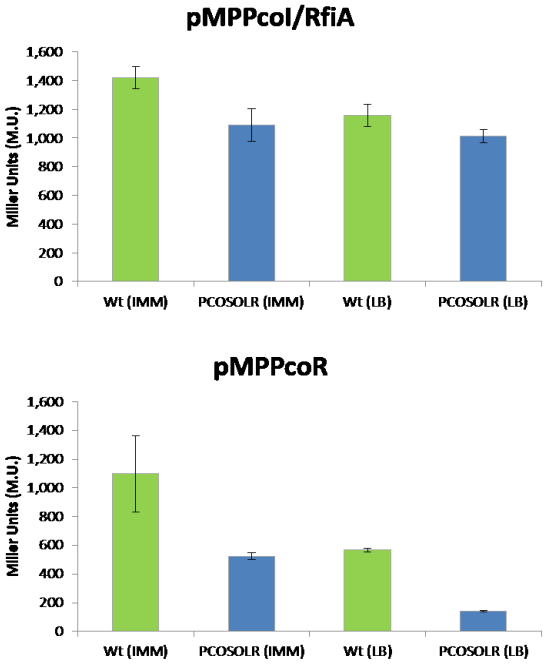

Figure 11: Activity of the promoter of *pcoI-rfiA* operon and *pcoR* gene (*luxR* homolog) in the parent strain *P. corrugata* CFBP 5454 (Wt) and PCOSOLR (Δ*solR*) mutant strain obtained by using the *pcoI/rfiA-lacZ* and *pcoR-lacZ* reporter constructs, respectively. The activity was measured in either rich growth medium (LB, Luria-Bertani broth) or minimal medium (IMM, Improved Minimal Medium). All measures were performed in triplicates. Statistical analyses were performed using one-way ANOVA with a Tukey's multiple comparison test ($P < 0.05$).

In the second experiment, aiming at determing if PcoI, PcoR and RfiA regulate the transcription of *solR* gene and if SolR is under auto-regulation, *solR* promoter region was cloned in pMP220 vector. *SolR* promoter activity, expressed as β-galactosidase activity and reported in Miller Units (M.U.), was determined in a rich medium (Luria Broth, LB) or in a minimal medium inducing CLP production (Improved Minimal Medium, IMM) in *P. corrugata* CFBP 5454 (Wt) and its derivative mutant strains GL1 (*pcoI*$^-$), GL2 (*pcoR*$^-$), GLRFIA (*rfiA*$^-$) and PCOSOLR (Δ*solR*). In IMM, *solR* promoter activity did not differ in GL1, GL2, GLRFIA mutant strains compared to wild-type strain but resulted significantly reduced in PCOSOLR mutant, suggesting that SolR is under partial auto-regulation (Figure 12). In LB, solR promoter activity resulted significantly reduced in all the tested mutant strains, suggesting that PcoI, PcoR and RfiA positively regulate the transcription of *solR* in rich medium (Figure 12).

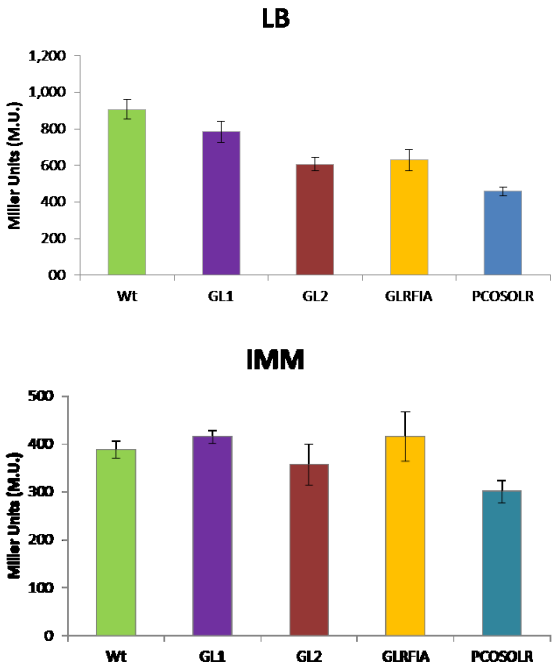

Figure 12: *solR* promoter activity in the parent strain *P. corrugata* CFBP 5454 (Wt) and GL1 (pcoI$^-$), GL2 (pcoR$^-$), GLRFIA (rfiA$^-$) and PCOSOLR (Δ*solR*) mutant strains obtained by using the *solR-lacZ* reporter construct. The activity was measured in either rich growth medium (LB, Luria-Bertani broth) or minimal medium (IMM, Improved Minimal Medium). All measures were performed in triplicates. Statistical analyses were performed using one-way ANOVA with a Tukey's multiple comparison test ($P < 0.05$).

Expression analysis by real-time PCR of genes involved in CLP production/secretion

To determine whether the expression of genes involved in the biosynthesis and secretion of known virulence factors (CLPs) in *P. corrugata* is regulated by SolR, *P. corrugata* CFBP 5454 and derivative mutant PCOSOLR were analysed by quantitative RT real-time PCR. The *P. corrugata* 16S rRNA (Conte *et al.*, 2006) gene was used as housekeeping gene (Figure 13).

The expression of genes annotated as *ppsE_1* and *syrD2* in a previous study (Licciardello *et al.*, 2018) was investigated in 40h-old cultures grown in IMM medium, known to stimulate CLPs production. These genes are involved, in the biosynthesis and secretion of the cyclic lipopeptides corpeptins A e B, rispectively. Results demonstrated that *ppsE_1* and *syrD2* were activated, respectively, sixfold and threefold more in PCOSOLR compared to the parent strain, suggesting that *solR* down-regulates the expression of these target genes (Figure 13).

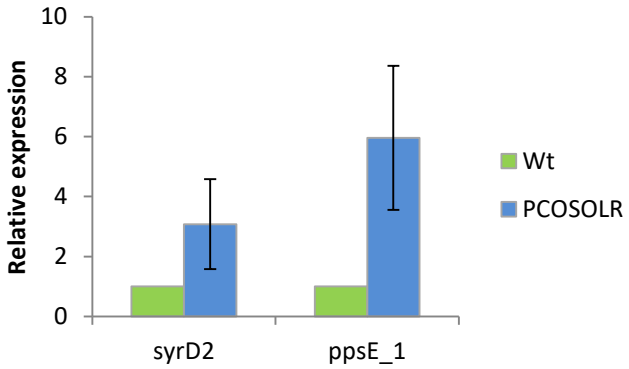

Figure 13: Transcriptional expression of *ppsE_1* and *syrD2* (putatively involved in biosynthesis and secretion of CLPs) by real-time PCR in *P. corrugata* CFBP 5454 (Wt) and PCOSOLR mutant strain (Δ*solR*) grown in IMM for 40 h at the early stationary phase. The results represent the means of three independent experiments.

4.3.2 Study on the role of the gene *crmA*

4.3.2.1 Description of cormycin A biosynthetic gene cluster

P. corrugata produces antimicrobial and phytotoxic CLPs: corpeptins A and B and cormycin A (Emanuele *et al.*, 1998; Scaloni *et al.*, 2004). No information regarding cormycin biosynthesis are available for this species, although the AHL-QS system of *P. corrugata* (PcoI/PcoR) and the transcriptional regulator RfiA have been demonstrated to contribute to virulence via CLP production (Licciardello *et al.*, 2012). Comparison of gene clusters from mycin producer *Pseudomonas* strains with the *P. corrugata* CFBP 5454 draft genome sequence showed that the cormycin gene cluster sequence is incomplete and scattered over contigs, although the syringomycin gene cluster was similar to a fragmented NRPS gene cluster in *P. corrugata* CFBP 5454. For this reason, the *in silico* analysis of *P. corrugata* cormycin A biosynthetic gene cluster was carried out in the annotated complete genome of *P. corrugata* type strain BS3649 (Genbank accession LT629798.1), sharing an average nucleotide identity (ANI) of 99.47% with strain CFBP 5454.

Comparative analysis of gene clusters by BLAST of CLP-producing *Pseudomonas* strains revealed the presence of two coding sequences (CDSs) putatively involved in cormycin biosynthesis in *P. corrugata* BS3649. A first CDS encodes for a putative 5375-amino-acid protein which displays a 88% sequence identity with the non-ribosomal peptide synthetase ThaA (ALG65294.1) of the thanamycin biosynthetic gene cluster from *P. fluorescens*. This CDS was designated *crmA*.

Downstream of *crmA*, separated by only 4 bp, and transcribed in the same direction, another CDS encodes a predicted 4527-amino-acid protein annotated as "amino acid adenylation domain-containing protein". This CDS was designated *crmB*. The *crmA* and *crmB* genes are 16,128 and 13,583 in size, respectively, and composition analysis revealed that the G+C contents were the same (65%).

BLASTp analysis of the translated protein CrmB revealed homologies with a number of non-ribosomal peptide synthetases (NRPS) of CLP-producing *Pseudomonas* species, with a similarity ranging from 75% to 99%. Downstream of *crmB* we identified another CDS (1262 bp), which is separated by 1105 bp and transcribed in the same direction. This CDS codes for a putative rhamnosyltransferase subunit B showing a 97% identity (96% coverage) with RhlB, a TDP-rhamnosyltransferase 1 from *Pseudomonas* sp. SH-C52 (CDF96613.1) involved in the biosynthesis of the cyclocarbamate brabantamide A. Upstream to *crmA*, a further CDS (1,004 bp), divergently oriented to *crmA* was identified separated from it by 363 nucleotides. When translated, this gene potentially encoded a 334-amino-acid protein annotated as cupin-like domain-containing protein sharing a 84% sequence identity with an HlyD family secretion protein (HQ888764.1) from *Pseudomonas* sp. SH-C52. In the phytopathogenic bacterium *P. syringae* pv. syringae, syringomycin biosynthetic gene cluster consists of several genes including *syrD*, *syrP*, *syrC*, *syrB1*, *syrB2*, and *syrE*. No homologues of *syrD*, *syrP* and *syrC* genes were found in the genome of *P. corrugata* B63649, whereas *syrB1 and syrB2* genes from *P. syringae* strain USA0035 (MZ593968.1) showed a 76.2% and 85.7% identity respectively with genes coding for an amino acid adenylation domain-containing protein and an halogenase in *P. corrugata* type strain BS3649. These genes are located about 36 kb upstream to *crmA*. *SyrE* showed a 77.84% identity (79% query coverage) with a genomic region encompassing both *crmA* and *crmB* genes.

4.3.2.2 Construction of ΔcrmA mutant strain

The *crmA* gene was inactivated by insertion and deletion through the insertion of a kanamycin resistance cassette and the deletion of a portion of the gene of about 1400 bp, which involved the use of the suicide vector pEX18Gm. In the first phase, two regions external to the portion of the gene to be inactivated (fragment 1 and fragment 2) were amplified by PCR with specific primer pairs equipped with adapters containing selected enzymatic restriction sites, so that they did not cut within the region to be inactivated and were compatible with the vectors to be used in the following steps (Table 2). The amplification products were purified and cloned in a broad spectrum host vector (pGEM-T, 3000 bp) by transformation into *E. coli* DH5α through thermal shock at 42°C. The transformants were selected for resistance to ampicillin and by blue/white

screening. A few white colonies were then collected and confirmed by sequencing following plasmid extraction. The plasmid DNA of the positive clones was then digested with the restriction enzymes chosen during the primer design (not shown).

The digestions were then purified and subcloned in the pBluescript KS vector (Stratagene), suitably digested and quantified in order to have an insert: vector ratio of 8:1 during the ligation phase.

The constructs in pBluescript were confirmed by digestion and sequencing of plasmids extracted from three *E. coli* clones. Specifically, pBlue-fragment1 was digested with KpnI-HindIII (Figure 14), while pBlue-fragment2 with BamHI-XbaI (Figure 14A). For each digestion, two bands were obtained; the bands with lower molecular weight correspond to fragment 1 (1004 bp) or fragment 2 (1200 bp).

PBlue-fragment1 and pBlue-fragment2 were then sequenced. A kanamycin resistance gene cassette (1256 bp) previously extracted from the pUC4K plasmid was then cloned within the pBlue-fragment2 construct, previously linearized by digestion with PstI (Figure 14B). The obtained construct, named pBlue-fragment2-km, was digested with XbaI-EcoRV; two fragments of 2.9 kb and 2.4 kb, corresponding respectively to the linearized vector and to the construct containing fragment2-km were obtained (Figure 14C). The 2.4 kb fragment was cloned in pBlue-fragment1, digested with XbaI-EcoRV.

pBlue-fragment2-km-fragment1 was digested with XbaI-KpnI, obtaining two fragments (Figure 14D). The fragment with the higher molecular weight, containing the construct fragment2-km-fragment1 construct, was finally cloned in the corresponding site within the suicide vector pEX18Gm. The pEX-based construct was used for transformation into *E. coli* DH5α and confirmed, following plasmid extraction, through the restriction profile obtained by digesting plasmid DNA with the HindIII enzyme; the restriction produced two bands of expected molecular weight: one of about 2000 bp and one of molecular weight compatible with that of the linearized pEX18Gm vector (not shown).

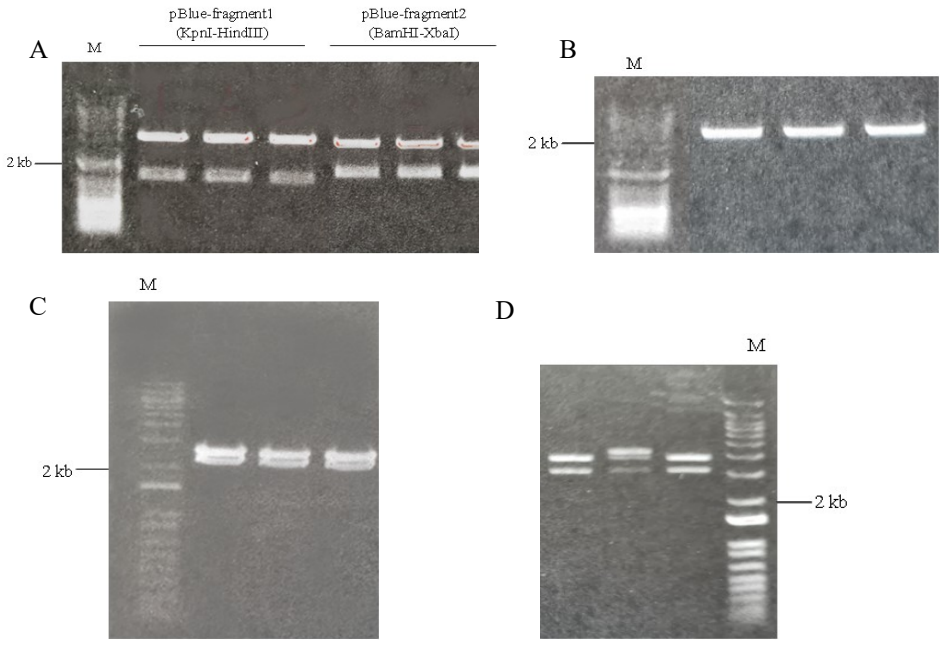

Figure 14: Digestion products of *E. coli* dh5α plasmid containing constructs A) pBlue-fragment1 and pBlue-fragment2; B) pBlue-fragment2; C) pBlue-fragment2-km; D) pBlue-fragment2-km-fragment1. M, molecular weight marker (1 kb).

The pEX18Gm plasmid (5831 bp) contains a marker gene encoding resistance to gentamicin and a *sacB* gene that characterizes the suicidal nature of the vector. The presence of the oriT site also allows for conjugative transfer. *E. coli* DH5α cells were then transformed and screened for resistance to gentamycin. A few colonies were then collected and confirmed by colony-PCR. The pEX18Gm-*crmA*::km construct was transferred by triparental conjugation with the *E. coli* helper strain pRK2013 within the wild-type *P. corrugata* strain CFBP 5454, selecting for Nf (100 mg mL^{-1}; *P. corrugata*), Gm (40 mg mL^{-1}; recombinants that have acquired the vector) and Km (100 mg mL^{-1}; Km resistance cassette). The double cross-over recombinants, which acquired the *crmA*::km construct and lost the pEX18Gm plasmid (therefore did not grow on gentamycin), were subsequently selected on a substrate containing sucrose and tested by colony PCR (Figure 15) using primers designed on the kanamycin resistance gene cassette or on the genomic DNA sequences upstream and downstream of the mutation (Table 2). Amplification with primer pairs crm1ext_F/kanext_R and crm2ext_R/kanext_F primers amplicon produced an amplicon of the expected size (1350 bp and 1660 bp, respectively) (Figure 15).

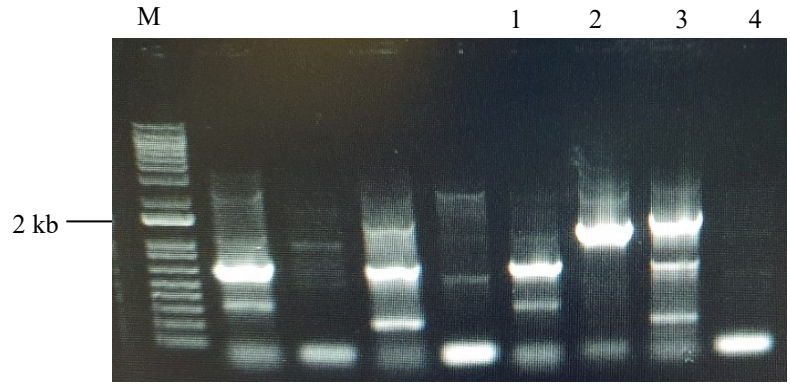

Figure 15: Screening of *P. corrugata* double-crossover mutants containing the *crmA*::Km construct. M, molecular weight marker (1 kb). 1: crm1ext_F- kanext_F primers amplicon; 2: crm1ext_F- kanext_R primers amplicon; 3: crm2ext_R- kanext_F primers amplicon; 4: crm2ext_R- kanext_R primers amplicon.

4.3.2.3 Phenotypic analysis of PCOCRMA mutant strain

In order to investigate the biological role of CLPs in *P. corrugata*, different phenotypic traits of the PCOCRMA (Δ*crmA*) mutant strain (supposingly producing only corpeptins) were compared to the parent strain CFBP 5454 that produces both cormycin and corpeptins and to GLRFIA, a knock-out mutant strain for the transcriptional regulatore rfiA, not producing CLPs.

Colony morphology of wild-type strain CFBP 5454 and its derivative mutant strains PCOCRMA and GLRFIA was observed on NDA medium. The colonies of the wild-type strain showed the typical morphology of the species, with a slightly raised and wrinkled surface and wavy margins and produced a yellow pigment diffusible in the substrate (Scarlett *et al.*, 1978). Conversely, the GLRFIA mutant produced creamy, buttery colonies with entire margins, smooth, slightly raised surfaces, and more mucous, as previously reported (Licciardello *et al.*, 2012). The colonies produced by the PCOCRMA strain, on the other hand, had a very pale creamy-white colour, a buttery appearance, entire margins and a smooth and flat surface. This mutant did not produce any yellow diffusible pigment (Figure 16).

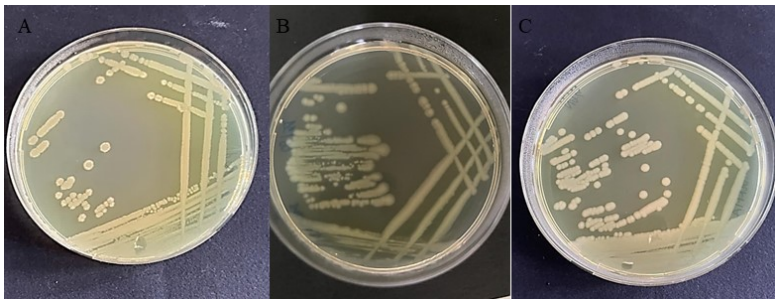

Figure 16: Colony morphology of *P. corrugata* wild-type strain CFBP 5454 (A) and its derivative mutant strains PCOCRMA (B) and GLRFIA (C). The colonies of the wild-type strain show the typical morphology with a slightly raised and wrinkled surface and wavy margins.

The swimming and swarming motility of the wild-type strain and its derivative mutant strains was tested in plates with different concentrations of agar (0.3% for swimming motility, 0.5% and 0.7% for swarming motility). Bacterial motility was evaluated after 24 h. By 24 h, PCOCRMA and GLRFIA mutant strains moved as far as the parent strain CFBP 5454 (Wt) within the agar pores into 0.3% swimming agar plates (Figure 17).

On 0.5% swarm agar plates, 24 h after inoculation the wild-type strain and GLRFIA mutant began to swarm whereas PCOCRMA mutant was unable to swarm over the medium surface and formed a regular colony at the inoculation point. On 0.7% swarm agar plates all the strains started moving 24 hours after inoculation, although the mutant strains swarmed to a lesser extent compared to the wild-type strain. GLRFIA and PCOCRMA mutant strains cells remained mucous at the inoculation point (Figure 17).

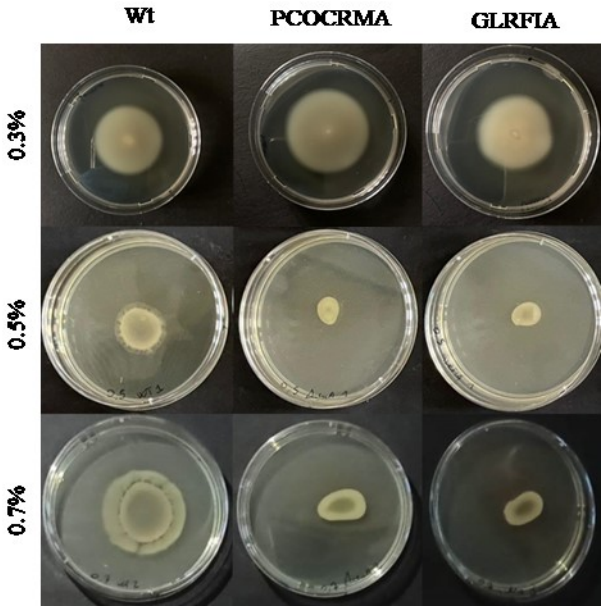

Figure 17: Motility of *P. corrugata* CFBP 5454 (Wt) and derivative strains PCOCRMA (*crmA* mutant) and GLRFIA (*rfiA* mutant). Motility was assessed in 0.3% (swimming motility) or 0.5% and 0.7% (swarming motility) agar plates.

Activity against CLP-indicator microorganisms

The antimicrobial activity of culture filtrates of strain producing both CLPs (wild-type) and of strains putatively able to produce only corpeptins (PCOCRMA, *crmA* mutant) or neither corpeptins and cormycin (GLRFIA, *rfiA* mutant) was tested by a bioassay based on inhibition of in vitro growth of the yeast *Rhodotorula pilimanae* and the Gram-positive bacterium *Bacillus megaterium*. Culture filtrates were obtained by growing the tested *P. corrugata* strains in IMM, a minimal medium inducing CLP production. Culture filtrates of the wild-type strain displayed inhibition activity toward *R. pilimanae* and *B. megaterium* (Figure 18).

However, the antimicrobial activity of culture filtrates was completely abolished in the GLRFIA mutant against *R. pilimanae* and *B. megaterium*, as well as in the PCOCRMA mutant strain, although a slight inihibition was observed toward *B. megaterium* (Figure 18). The same results were observed using bacterial cells of *P. corrugata* CFBP 5454 and its derivative mutant strains PCOCRMA and GLRFIA (not shown).

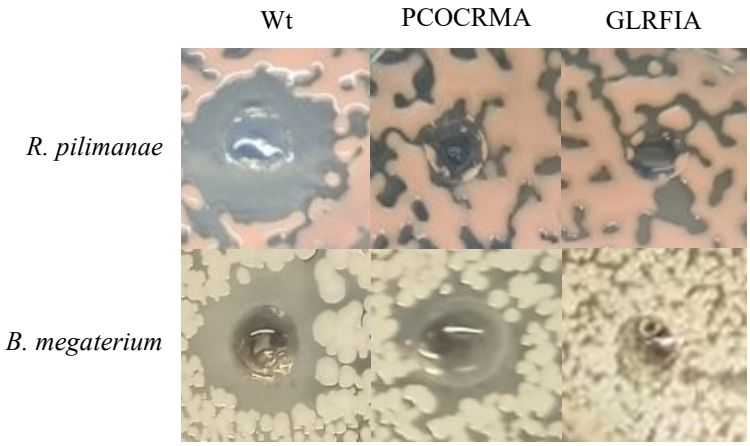

Figure 18: Antagonistic activity of culture filtrates (10X) of the parent strain *P. corrugata* CFBP 5454 (Wt) and PCOCRMA (*crmA* mutant) and GLRFIA (*rfiA* mutant) against the CLP indicator microorganisms *Rhodotorula pilimanae* and *Bacillus megaterium*. GLRFIA mutant was used as non-producing CLP strain control.

Activity against phytopathogenic fungi

In order to determine the effects of *P. corrugata* CFBP 5454 and its derivative mutant strains PCOCRMA and GLRFIA on fungal mycelia growth, dual-culture antagonism assays were performed on PDA plates. Three plant pathogenic fungi, *Botrytis cinerea*, *Plenodomus tracheiphilus* and *Fusarium oxysporum* f.sp. *radicis-lycopersici* were tested. The percentage inhibition (PGI %) of the myceliar growth was calculated at different time points post-inoculation for each fungal pathogen. Eight days after inoculation the wild-type strain reduced the growth of *P. tracheiphilus* mycelium by 60.8% (± 4.4), while the PCOCRMA and GLRFIA mutant strains by 59.7% (± 2. 1) and 61.5% (± 5.6), respectively, compared to the control plates with only the fungus. Four days after inoculation the wild-type strain reduced the growth of *F. oxysporum* fungal mycelium by 55.6% (± 3.1), while the PCOCRMA and GLRFIA mutant strains by 46.5% (± 5.5) and 48.4% (± 1.3), respectively; towards *B. cinerea*, the wild-type strain determined a reduction in the diametral growth of the fungus by 63.8% (± 2.4), while the PCOCRMA and GLRFIA mutant strains by 36.2% (± 2.0) and 41.7% (± 1.7), respectively, compared to control plates containing only the target fungus. Six days after plate inoculation, mycelia of *B. cinerea* and *F. oxysporum* had overgrown the GLRFIA and PCOCRMA bacterial spots (Figure 19). In the overall, PCOCRMA and GLRFIA mutant strains showed a significantly reduced antagonistic activity compared to their parent strain towards *F. oxysporum* ($P < 0.001$) and *B. cinerea* ($P < 0.001$). These results suggest that the inactivation of CrmA affects the wide-spectrum antimicrobial activity of *P. corrugata*.

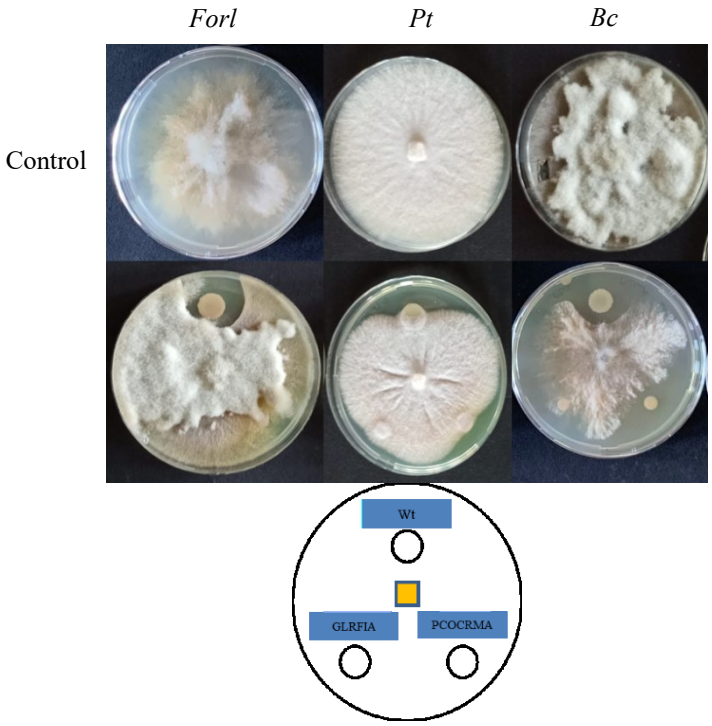

Figure 19: Antifungal activity of *P. corrugata* CFPB 5454 (Wt), and derivative strains PCOCRMA (*solR* mutant) and GLRFIA (*rfiA* mutant) on PDA for diffusible compounds evaluation. *Forl*, *Fusarium oxysporum* f.sp. *radicis-lycopersici*; *Pt*, *Plenodomus tracheiphilus*; *Bc*, *Botrytis cinerea*. The scheme above indicates the disposition of the tested strains in the plates.

Virulence in tomato

Among the other natural functions, CLPs play a key role in virulence of phytopathogenic bacteria due to their phytotoxic activity (Bender *et al.*, 1999; Raaijmakers *et al.*, 2006). *P. corrugata* produces the CLPs cormycin and corpeptins; the latter have a role in virulence in tomato (Strano *et al.*, 2015). The effect of cormycin in the induction of pith necrosis was evaluated in tomato plants inoculated with a mutant strain (PCOCRMA), putatively producing only corpeptins, by comparison with wild-type strain (produces both CLP) or GLRFIA mutant strain (no CLP producing). Tomato seedlings were inoculated with an high-inoculum concentration of cells of *P. corrugata* CFBP 5454 or its derivative mutant strains PCOCRMA and GLRFIA; two weeks after inoculation, the plants were cross-sectioned longitudinally and

the lenght of stem pith necrosis was measured. In plants inoculated with the wild-type strain a dark brown discoloration of the pith ranging from about 2 to 6 cm was observed. The GLRFIA mutant strain did not cause significant lesions in the pith of the inoculated plants, except for a dark discoloration along the pin puncture, while for PCOCRMA mutant strain a drastic reduction of stem pith necrosis length was observed (Figure 20).

Figure 20: Symptoms of tomato pith necrosis on tomato plants inoculated with *P. corrugata* CFBP 5454 (Wt), and its derivative mutant strains PCOCRMA (*crmA* mutant) and GLRFIA (*rfiA* mutant) 15 days post inoculation. GLRFIA mutant strain was used as non-producing CLP strain control.

Induction of HR in tobacco

The effect of *crmA* genes inactivation was evaluated on the ability to cause HR in a non-host species such as *Nicotiana tabacum* by infiltrating the mesophyll of tobacco leaves with a high-inoculum dose of *P. corrugata* CFBP 5454 (wild-type) and the mutant strains PCOCRMA and GLRFIA. By 24h after inoculation the leaf panels infiltrated with the parent strain showed the collapse of the mesophyll, which turned necrotic within the following 24 h. By contrast, leaf panels inoculated with PCOCRMA and GLRFIA mutant strains did not show any response (Figure 21), similarly to those inoculated with water (not shown).

Overall, *in vivo* results clearly demonstrated the importance of CrmA in the development of disease symptoms in the host plant tomato and in the induction of HR in tobacco.

Figure 21: Induction of hypersensitivity response (HR) in *Nicotiana tabacum*. The bacterial suspensions of the strains (wild-type, PCOCRMA, GLRFIA) of *P. corrugata* were infiltrated into the mesophyll of the leaves. GLRFIA mutant was used as non-producing CLP strain control.

4.4 Discussion

LuxR solos are QS-LuxR-family protein regulators occurring without the corresponding LuxI homolog. They are present in bacteria which host a complete AHL-QS system as well as in bacteria that have been proven not to synthesize AHLs (Subramoni and Venturi, 2009a). LuxR solos of AHL-producing bacteria may respond to the endogenously produced AHLs, expanding the regulation to other target genes, while in non-AHL-producing bacteria, on the other hand, could sense AHLs produced exogenously by other bacteria. Some LuxR solos, widespread among plant-associated bacteria (PAB), detect non-AHL compounds produced by plants, being involved in interkingdom signalling (Patel *et al*., 2013). *P. corrugata* is a biocontrol agent from *P. fluorescens* complex of species and constitutes the *P. corrugata* taxonomic subgroup within this complex. *P. corrugata* genome has a conserved AHL-QS system designated PcoI/R and another LuxR-family protein regulator named RfiA (Licciardello *et al*., 2007; 2009).

Recently, the availability of draft genome sequences of *P. corrugata* strains facilitated the study of the mechanisms that *P. corrugata* employs to cause disease or prevent disease caused by other pathogens (Licciardello *et al.*, 2014; Trantas *et al.*, 2015). The inactivation of the *luxI* homologue *pcoI* abolished the production of AHLs therefore Licciardello *et al.* (2007) demonstrated that PcoI is the only acyl-homoserine lactone synthase in *P. corrugata*, suggesting that the new LuxR regulator identified is a LuxR solo. *P. corrugata* LuxR solo was termed SolR. According to its genetic context and primary structure, SolR belongs to the subgroup A of

LuxR solos family proteins (Bez *et al*., 2021) and shared a conserved amino acid structure with other strain belonging to *P. corrugata* subgroup (80-95% identity).

In this work, we analyzed the mutational phenotype of *P. corrugata* CFBP 5454 *solR* mutant strain (PCOSOLR) in order to depict the biological role of SolR. Motility, essentially by swarming migration, is believed to allow the dissemination of bacterial cells when a population is getting too large to inhabit the same niche and is one of the possible phenotypes regulated by QS (Daniels *et al.*, 2004). In *P. corrugata* CFBP 5454, SolR regulates *in vitro* motility, consistently with a previous finding for Ppor, a LuxR solo highly conserved among *P. putida* strains (Fernández-Piñar *et al*., 2011).

Concerning the interaction with plants, SolR contributes to virulence toward tomato, although the *in planta* population concentrations of PCOSOLR mutant strain did not differed. Similarly, the plant-pathogenic bacteria *Xanthomonas oryzae* pv. oryzae and *Xanthomonas campestris* pv. campestris possess two LuxR solos, designated OryR and XccR, which are required for full virulence in rice and cabbage, respectively (Ferluga *et al*., 2007; Zhang *et al*., 2007). PCOSOLR mutant strain was not impaired in HR induction, unlikely the *pcoR* (*luxR* homolog) mutant strain of *P. corrugata* CFBP 5454, that was unable to cause hypersensitive response in tobacco (Licciardello *et al*., 2007). The role of LuxR solos in the interactions of bacteria with non-host plants has beeen explored in few pathosystems; a mutant strain for *xocR*, a *luxR* solo from the rice pathogen *Xanthomonas oryzae* pv. oryzicola, was not altered in the ability to trigger HR on non-host plant tobacco (Xu *et al*., 2015), while the LuxR solos AvhR and AviR from *Agrobacterium vitis*, a species possessing a complete AHL-QS system, are required for induction of hypersensitive response (Zheng *et al*., 2003; Hao *et al*., 2005). *P. corrugata* is known to produce the antimicrobial and phytotoxic cyclic lipopeptides (CLP) cormycin A and corpeptines A and B (Emanuele *et al.*, 1998; Scaloni *et al.*, 2004). In this species, CLPs are major virulence factors and play a role in the induction of systemic resistance in tobacco (Strano *et al*., 2015). In light of this findings, we wondered if the increased virulence of PCOSOLR mutant strain in tomato could be attributed to increased production of these phytotoxic compounds. According to a time-course experiment, PCOSOLR mutant production of CLPs was higher and occurred earlier compared to the parent strain, displaying a stronger antagonistic activity against the CLPs indicator microorganism *Bacillus megaterium*. Previous studies demonstrated that in *P. corrugata* QS modulates the antimicrobial activity and the production of CLPs (Licciardello *et al*., 2007; 2009; 2018). The strong antimicrobial activity of PCOSOLR mutant strain, due to CLPs, further supports the high *in planta* virulence it showed.

Acyl-homoserine-lactones (AHLs) are signal molecules of *P. corrugata* QS system (Licciardello *et al*., 2007). Their abundance, indirectly quantified by measuring the amount of violacein produced by the biosensor *Chromobacterium violaceum* CV026, was higher in PCOSOLR mutant strain during bacterial growth. In presence of AHLs, *P. corrugata* PcoR (LuxR-family protein) activates gene expression of virulence factors via RfiA (Licciardello *et al*., 2009). According to these observations, a higher production of AHLs in PCOSOLR mutant strain may up-regulate the expression of genes involved in the disease expression, thus enhancing virulence in the host plant tomato. PpoR, a LuxR solo from non-AHL-producing strain *Pseudomonas putida* KT2440, regulates iron acquisition and motility independently of AHLs (Fernández-Piñar *et al*., 2011). Moreover, the expression of genes annotated in the CLP biosynthetic gene cluster, *ppsE_1* and *syrD2,* of *P. corrugata*, in the PCOSOLR mutant was upregulated as compared to that of the parent strain under CLP-inducing conditions. As in *P. corrugata* the AHL-QS system regulates CLP production (Strano *et al*., 2015), we investigated if SolR was involved in the transcriptional regulation of the QS system genes *pcoI*/*pcoR* and the luxR-family transcriptional regulator *rfiA* in a rich medium and in a minimal medium inducing CLP production. In turn, this activity was determined for the AHL-QS genes toward *solR*. SolR positively regulated the transcription levels of the AHL-QS genes, markedly of the luxR homolog *pcoR*, with variable results depending on medium used. Unlikely, *ppuI* (*luxI* homolog) promoter of *P. putida* WCS358 exhibited consistently higher expression levels in the luxR solo *ppoR* mutant strain, while *ppuR* (luxR homolog) transcription was not affected (Subramoni and Venturi, 2009b). On the other hand, PcoI, RfiA and PcoR slightly regulated the transcription of *solR* in rich medium. Moreover, SolR was under partial auto-regulation in both media, unlikely reported for the three LuxR solos PsaR1, PsaR2 and PsaR3 of the plant-pathogenic bacterium *P. syringae* pv. actinidiae (Cellini *et al*., 2020). However, the erratic influence of the culture medium on transcription levels and at times the low differential values of transcription made difficult to define the mutual regulation between the endogenous complete AHL-QS system and SolR, suggesting that in *P. corrugata* a more intricate network is most probably taking place. Supposingly, the AHL-QS genes may be indirectly regulated by SolR and *solR* transcription may depend stringently on growth phase, as reported for Ppor, another subgroup A LuxR solo (Subramoni and Venturi, 2009b). *P. corrugata* produces in culture the cyclic lipopeptides cormycin A and corpeptins A and B (Emanuele *et al*., 1998; Scaloni *et al*., 2004). Cyclic lipopeptides (CLPs) are versatile molecules produced by a variety of bacterial genera, including *Pseudomonas* spp. For phytopathogenic *Pseudomonas*, CLPs constitute important virulence factors, while for biocontrol species, CLPs play a key role in antimicrobial activity, motility and biofilm formation (Raaijamakers *et al*., 2006), helping bacteria to colonize plant surfaces richer

in nutrients (Raaijmakers *et al*., 2010). Due to the aforementioned characteristics and the massive presence in the genus *Pseudomonas*, these bioactive compounds have great potential for applications in the biotechnological and biomedical fields (Raaijmakers *et al*., 2006; Sinnaeve *et al*., 2009).

In this study we identified part of the biosynthetic gene cluster responsible for cormycin production, including genes coding for non-ribosomal-type synthetases (NRPS). In the genome of *P. corrugata* CFBP 5454, model strain in our studies, approximately 217 kb coded for putative NRPS biosynthetic clusters (Licciardello *et al*., 2014). Genes encoding for these multimodular enzymes are difficult to assemble and split across several contigs. Thus, our reference strain was BS3649, type strain of *P. corrugata*, for which a complete genome is available. The *P. corrugata* cormycin biosynthetic gene cluster is located within a genomic island of about 173 kb, common to several species belonging to the *P. fluorescens* group, containing genes for the biosynthesis, transport and regulation of compounds with antimicrobial activity (cormycin A, corpeptins A and B, and a brabantamide-like metabolite) and quorum sensing genes (*pcoI*/*pcoR*).

We found out that in this species the biosynthetic cluster of cormycin consists of two NRPS genes named *crmA* and *crmB* and that it resembles the organization observed for other CLP-producing *Pseudomonas*; in particular, *Pseudomonas* sp. SH-C52, a biocontrol strain belonging to *P. corrugata* phylogenomic subgroup known to produce the nonapeptide thanamycin. To get insight into the contribute of cormycin in *P. corrugata* biology, a mutant of the putative NRPS *crmA* gene, PCOCRMA, was obtained and the resulting mutational phenotype was investigated. In terms of biological activity of cormycin, the PCOCRMA mutant strain continued to produce diffusible compounds in the medium exerting antifungal activity, although to a lesser extent than the wild-type strain; this activity can be putatively attributed to the production of corpeptins and a brabantamide-like metabolite described in *Pseudomonas* sp. SH-C52, with known antifungal activity (Schmidt *et al*., 2014; Van der Voort *et al*., 2015). Analogously, a nunamycin mutant strain was impaired in the antifungal activity towards the fungus *Rhizoctonia solani* (Michelsen *et al*., 2015). The thanamycin gene cluster has been shown to be important for the growth inhibition activity of the SH-C52 strain against *R. solani* (Mendes *et al*., 2011); a mutant strain for a *syrE* gene homolog had in fact lost its *in vitro* activity against the fungus *R. solani* (Mendes *et al*., 2011). The culture filtrates of PCOCRMA mutant strain did not inhibit the growth of the yeast *R. pilimanae* and only slightly inhibited the growth of the Gram-positive bacterium *B. megaterium*. Previous studies have shown that *R. pilimanae* is sensitive to cormycin A produced by *P. corrugata*, but not to corpeptins A and B (Scaloni *et al*., 2004), thus

justifying the lack of activity of the PCOCRMA mutant strain against this microorganism. *B. megaterium*, on the other hand, is sensitive to both cormycin A and the two isoforms of corpeptin (Emanuele *et al*., 1998). Other functions of CLPs in nature, above all investigated in biocontrol strains, are their role in motility and in bacteria-plant interactions (Raaijmakers *et al*., 2010). PCOCRMA has been shown to have reduced swarming motility *in vitro*, compared to the parent strain; a mutant strain of *Pseudomonas* sp. 11K1 unable to produce brasmycin showed a marked reduction of swarming motility (Zhao *et al*., 2019). Furthermore, it was demonstrated that a mutant unable to produce the lipopeptide orphamide A exhibited reduced swarming motility compared to the parent strain, overall demonstrating that lipopeptides are physiologically decisive for bacterial motility (Nielsen *et al*., 2005). Concerning pathogenicity toward plants, the wild-type strain induced the hypersensitive response in non-host plant *N. tabacum* and symptoms of pith necrosis in tomato, while the inoculation of the PCOCRMA mutant strain did not produce any symptoms in tobacco. In *P. corrugata* CFBP 5454, mutant strains devoid of corpeptins production only cause chlorosis when inoculated into tobacco (Strano *et al*., 2015). This finding and the lack of a type III secretion system suggest that in *P. corrugata* the production of both compounds is required to trigger HR response, in agreement with studies demonstrating a role of CLPs in the induction of systemic resistance and that CLPs constitute a new class of MAMPs (Raaijmaker *et al*., 2010). *In vivo* trials demonstrated that the mutant strain PCOCRMA, producing putatively only corpeptins, was also clearly less virulent on tomato than the parent strain CFBP 5454. Similarly, a syringomycin mutant strain of *P. syringae* pv. syringae resulted less virulent in cherry fruits (26% reduction) than the parent strain (Scholz-Schroeder *et al*., 2001).

In conclusion, we showed that the LuxR-family solo transcriptional regulator SolR and CrmA synthase (and therefore the nonapeptide cormycin), are determinant for the interactions of *P. corrugata* with other microorganisms and plants, in particular in terms of induction of pith necrosis symptoms and hypersensitive response. Regarding *P. corrugata* LuxR solo, further studies are needed to depict the cross-talk between SolR and the AHL-QS system and to clear what endogenous and/or exogenous signaling compounds this transcriptional regulator is able to respond to.

4.5 References

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5. Concluding remarks

Mal secco disease (MSD), is a detrimental tracheomycosis of citrus caused by the mitosporic fungus *Plenodomus tracheiphilus*. Although partially tolerant lemon clones were selected, these were not always responsive with regard to the qualitative aspects of production. Genetic improvement also gave results that were not entirely responsive. This aspect is common to all tree plant tracheomycosis due to the multiplicity of factors that govern the pathogenic host interactions in the complex process of penetration and colonization of host plants.

The management of the disease was based, from the beginning, on the removal of the symptomatic branches, as reservoirs of the fungal inoculum, and on the repeated epigeal application of fungicides, mainly copper compounds, in order to prevent the germination of the conidia and the penetration into citrus host. Progressive restrictions on the use of fungicides have limited the possibilities of use to copper. Such a strategy is now costly and ineffective, as evidenced by the resurgence of the disease in recent years.

In this consideration, the use of biological control agents (BCAs) to mitigate the symptoms of MSD has been, for over thirty years, research object on the ability of endophyte bacteria to actively penetrate and colonize the same ecological niche invaded by *P. tracheiphilus* and to systemically occupy the host plant with biologically active metabolites without signs of infection or adverse effects on the host.

The engineering of the microbiome of plants passes through the in-depth study of microbial communities and the interactions between them and the host plant. Pathogens have been shown to modify the host microbiome to their advantage in the most understandable example by reducing bacteria that support the plant's health status such as beneficial bacteria.

It is no coincidence that in several pathosystems a reduction of bacteria of the genus *Pseudomonas* in the rhizosphere (*Phytophthora*, *Verticillium*) in infected plants has been observed.

To date, there were no studies examining the influence of MSD caused by *P. tracheiphilus* on the bacterial and fungal communities of citrus roots. Our hypothesis was that Mal secco infection can affect the microbial composition and community structure of these plant compartments. Therefore, we used an amplicon-based (16S rRNA and ITS sequences) metagenomic approach to decipher the root and xylem (including bacterial and fungal) microbiome from healthy sour orange seedlings and seedlings inoculated with *P. tracheiphilus*

by wounded leaves or roots, simulating canopy and belowground infections occurring in nature.

Based on the results obtained, *P. tracheiphilus* manipulates the host plant or rather the 'holobiont' plant-microbiome. This interference occurs both in the compartments of the plant where fungus and microorganisms coexist but also at a distance definitely mediated by the plant's response to stress. The results suggest that there is useful data to think a support through the use of beneficial bacteria can help improve the health of the plant. The choice of beneficial bacteria to be used, mediated by foliar or radical applications, in relation to the microorganism used, must therefore be aimed not only at reducing the fungal pathogen inoculum but also at stimulating the positive response of the plant to stress.

Previous studies highlighted the possibilities to use *Pseudomonas* strains to control MSD. The thesis project was developed in the framework of the project S.I.R.P.A. that aimed to develop a bio- based product to control the disease. Results allowed to focus on two strains belonging to the species *P. corrugata* and *P. mediterranea.* These strains demonstrated antimicrobial activity mediated both by diffusible and volatile compounds against conidia germination and mycelial growth. The results obtained through the *in vitro* and *in planta* biological activity tests of the strains studied for the preparation of a formulation with characteristics different from others currently available are reflected in the genomic profiles emerged from the genome sequencing.

Important are some biological characteristics such as the ability to colonize the rhizosphere and the endosphere of different plants and the ability to produce polyhydroxy-alkanoates in the medium chain that allow bacteria to survive in adverse conditions. The activity of CLP and other biotensioactives, related to their physical-chemical properties, has been studied in particular in various strains of *Pseudomonas* and plays an important role in motility and competition. These metabolites are responsible for the observed antagonistic activity *in vitro* toward conidia germination and mycelial growth of *P. tracheiphilus*.

This analysis also showed that the bacterium putatively produces other secondary metabolites that in other bacteria have a role in the antimicrobial and insecticide activity (e.g. entolysine).

To shed light on the molecular mechanisms that underpin the activity of the two closely related *Pseudomonas* species we worked on the *P. corrugata* strain CFBP 5454. In this model strain by a mutational approach the role of a gene involved in regulation, biosynthesis and export of antimicrobial CLPs was already demonstrated.

In this study we further demonstrated the role of the gene *crmA*, encoding for a non-ribosomal peptide synthase (NRPS) putatively involved in the biosynthesis of the CLP cormycin. The gene was inactivated and the resulting mutational phenotype was studied.

CrmA inactivation reduced motility and antimicrobial activity *in vitro*, virulence in tomato and did not cause HR in the non-host plant tobacco, confirming previous studies demonstrating that lipopeptides are pivotal for bacterial interaction with plants and other microorganisms.

In addition to a canonical AHL-QS system (*pcoI-pcoR*) we identified by genome-wide analysis a *luxR solo*, named *solR*, closely related to Quorum Sensing (QS) *luxR*-family regulators but devoid of a cognate *luxI*-family protein homologue. This regulators may respond to either endogenous or exogenous signal molecules or to plant low-molecular-weight compounds, regulating the interaction of bacteria with plants and other microorganisms. We constructed a knock-out *solR* mutant, PCOSOLR.

In *P. corrugata* CFBP 5454, SolR regulates motility *in vitro* and contributes to virulence toward the host-plant tomato, as symptoms of pith necrosis resulted more severe in PCOSOLR mutant strain. The increased virulence of PCOSOLR mutant strain *in planta* could be attributed to increased production of phytotoxic CLP compounds. Overproduction strategies for antifungal CLPs *in vitro* could be explored.

Publications

Indexed publications

- **Dimaria, G**., Mosca, A., Anzalone, A., Paradiso, G., Nicotra, D., Privitera, G.F., Pulvirenti, A., and Catara, V. (2023). Sour orange microbiome is affected by infections of *Plenodomus tracheiphilus* causal agent of Citrus Mal secco disease. *Agronomy*. *13*, 654. doi:10.3390/agronomy13030654.
- Oliveri, C., Modica, G., Bella, P., **Dimaria, G.**, Cirvilleri, G., Continella, A., and Catara, V. (2022). Preliminary evaluation of a zinc-copper-citric acid biocomplex for the control of *Plenodomus tracheiphilus* causal agent of citrus mal secco disease. *Acta Hortic. 1354*, 231. doi:10.17660/ActaHortic.2022.1354.30.
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- Catara, V., Lo Piero, A.R., Russo, M., Bazzano, M.C., Catalano, C., **Dimaria, G.**, *et al.* (2022). Le biotecnologie sono un supporto fondamentale alla difesa dal Mal secco. Rivista di frutticoltura e di ortofloricoltura, n. 1 gennaio 2022, SPECIALE AGRUMI, pag. 16–22, ISSN 0392-954X.

Informative contributions

- **Dimaria, G.**, Mosca, A., Anzalone, A., Nicotra, D., Russo, M., Catara, V. (2022). Genomica di un batterio multitask. Manualetto divulgativo, progetto S.I.R.P.A. (PO FESR Sicilia 2014 – 2020).
- Mosca, A., **Dimaria, G.**, Paradiso, G., Anzalone, A., Nicotra, D., Lo Cicero, L., Catara, V. (2022). Mal secco e microbioma radicale: effetti sui batteri utili. Manualetto divulgativo, progetto S.I.R.P.A. (PO FESR Sicilia 2014 – 2020).
- Bazzano, M., Catara, A.F., Scuderi, G., Modica, F., Musumeci, S., **Dimaria, G.**, Catara, V., Russo, M. (2022). Verso un nuovo formulato microbico per il contenimento del Mal secco e altre malattie fungine degli agrumi. Manualetto divulgativo, progetto S.I.R.P.A. (PO FESR Sicilia 2014 – 2020).

Contributions at National and International conferences

- **Dimaria, G.**, Mosca, A., Viscardo, O., Russo, Catara, V. (2022). Identification and characterization of *Xanthomonas arboricola* pv. pruni strains isolated from almond in Sicily. Poster presentation. XXVII SIPaV Congress (SIPaV), Palermo (Italy) 21-23 September 2022, *Journal of Plant Pathology*, *104*, 1233.

- Bazzano, M.C., Catara, A.F., Scuderi, G., **Dimaria, G.**, Modica, F., Catara, V., Russo, M. (2022). Dissecting foliar fitness of a *Pseudomonas mediterranea* strain nonpathogenic to citrus to understand the interaction with *Plenodomus tracheiphilus*. XXVII SIPaV Congress (SIPaV), Palermo (Italy) 21-23 September 2022, *Journal of Plant Pathology*, *104*, 1211.

- Catara, V., Nicotra, D., Mosca, A., Anzalone, A., **Dimaria, G.**, Privitera, G. F., Pulvirenti, A. (2022). Microbiome-informed selection of biocontrol bacteria from the tomato endophytome. XXVII SIPaV Congress (SIPaV), Palermo (Italy) 21-23 September 2022, *Journal of Plant Pathology*, *104*, 1224–1225.

- **Dimaria, G.**, Anzalone, A., Bertani, I., Licciardello, G., Paradiso, G., Nicotra, D., Lo Cicero, L., Venturi, V., Catara, V. (2022). SolR, a LuxR-family solo, modulates cyclic lipopeptide production in *Pseudomonas corrugata*. Short oral poster presentation. 14th International Conference on Plant Pathogenic Bacteria (ICPPB), Assisi (Italy) 3-8 July, 2022, Book of abstract, 88.

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- Nicotra, D., Anzalone, A., Mosca, A., **Dimaria, G.**, Modica, F., Cirvilleri, G., Catara, V. (2022). Identification and characterization of tomato bacterial endophytes and preliminary evaluation for consortia-based biocontrol products. 14th International Conference on Plant Pathogenic Bacteria (ICPPB), Assisi (Italy) 3-8 July 2022, Book of abstract, 150.

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- **Dimaria, G.**, Mosca, A., Anzalone, A., Paradiso, G., Pulvirenti, A., Russo, M., Catara, V. (2021). *Plenodomus tracheiphilus* colonization in *Citrus aurantium* and effect on the microbiome. Poster presentation. XXVI Congress of the Italian Phytopathological Society (SIPaV), Verona (virtual form), 15-17 September 2021, Book of abstract, 20.

- **Dimaria, G.**, Musumeci, S., Modica, F., Venticinque, G., Bazzano, M.C., Russo, M., Catara, V. (2021). *In silico* and *in vitro* screening of the potential for biocontrol of bacteria of the *Pseudomonas corrugata* subgroup. Poster presentation. XXVI Congress of the Italian Phytopathological Society (SIPaV), Verona (virtual form), 15-17 September 2021, Book of abstract, 19.

- Russo, M., Scuderi, G., Bazzano, M.C., Leocata, S., Catara, A., **Dimaria, G.**, Mosca, A., Catara, V. (2021). Survey on the persistence of *Plenodomus tracheiphilus* inoculum in lemon groves during summer. XXVI Congress of the Italian Phytopathological Society (SIPaV), Verona (virtual form), 15-17 September 2021, Book of abstract, 49.

- Anzalone, A., **Dimaria, G.**, Mosca, A., Musumeci, S., Privitera, G., Pulvirenti, A., Cirvilleri, G., Catara, V. (2021). Biological control of tomato bacterial diseases by Bacillus sp. and Pseudomonas sp. isolated from tomato endorhizosphere. 4th Annual Conference on Integrating science on Xanthomonadaceae for integrated plant disease management in Europe. 28-30 June 2021

- Anzalone, A., Bergna, A., **Dimaria, G.**, Di Guardo, M., Mosca, A., Leonardi, C., Berg, G., Catara, V. (2021). Comunità batteriche e fungine del pomodoro: dal vivaio alla serra, in suolo e fuori suolo. Congress of XIII Giornate scientifiche SOI, I traguardi di agenda 2030 per l'ortoflorofrutticoltura Italiana, Catania, Italy 22-25 June 2021.

- Urriza, M., **Dimaria, G.**, Añorga, M., Catara, V., Murillo, J. (2021). Genómica comparativa de plásmidos nativos de bacterias fitopatógenas. IX Reunión del grupo Microbiología de Plantas-SEM, Libro de resúmenes, 39.

- Anzalone, A., Bergna, A., Di Guardo, M., **Dimaria, G.**, Leonardi, C., Berg, G., Catara, V. (2020). Beneficial bacteria communities from the tomato seed and root endosphere. Congress of Young Scientists for Plant Health, WEB Workshop (SIGA-SEI-SIBV-SIPAV), 16 December 2020.

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