FACULTEIT
BIO-INGENIEURSWETENSCHAPPEN

PRIMING FOR INDUCED RESISTANCE IN RICE

Iris Pottie
Stamnummer: 01702454

Promotoren: Prof. dr. Monica Höfte, Prof. dr. Tina Kyndt
Tutoren: Enrico Ferrarini, Willem Desmedt

Masterproef voorgelegd voor het behalen van de graad in Master of Science in de industriële wetenschappen: biochemie

Academiejaar: 2020 - 2021

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Abstract

Induced resistance (IR) refers to a phenotype of enhanced resistance to a biotic stress condition that is induced by an external chemical or biotic stimulus. During this thesis, two novel types of possible IR stimuli were studied in rice, a very important crop in plant science due to its dual role as a model monocot organism and as a staple food. These possible IR stimuli were tested against two different pathogens: *Pseudomonas* strains that produce cyclic lipopeptides (CLPs) against the root-knot nematode *Meloidogyne graminicola* and piperonylic acid (PA), a chemical inhibitor of the CINAMATE-4-HYDROXYLASE enzyme, against the rice blast pathogen *Pyricularia oryzae.* In case of CLP-producing *Pseudomonas* strains, both the direct nematostatic effect and the capacity to induce resistance against *Meloidogyne graminicola* in rice was evaluated, while in case of PA, only the capacity of PA-IR in rice against *Pyricularia oryzae* was investigated.

Here, it was shown that supernatants from several *Pseudomonas* strains have a nematostatic effect, but mutant analysis indicates that (unidentified) secondary metabolites other than CLPs might be the main cause of this effect. Further, it was shown that PA induces resistance in rice against *P. oryzae* through mechanisms that appear to involve phytoalexin production but not lignification.

Key words: induced resistance, rice, pathogens, plant immunity, priming

Samenvatting

Geïnduceerde resistentie (IR) verwijst naar een fenotype gekenmerkt door verhoogde resistentie tegen biotische stress dat wordt geïnduceerd door een externe chemische of biotische stimulus. In deze thesis werden twee nieuwe stimuli die mogelijks IR induceren bestudeerd in rijst, een zeer belangrijk gewas in plantenwetenschap door zijn tweezijdige rol als modelorganisme voor monocotyle planten en als basisvoedsel. Deze mogelijke IR-stimuli werden getest tegen twee verschillende pathogenen: *Pseudomonas*-stammen die cyclische lipopeptiden (CLPs) produceren tegen het wortelknobbelaaltje *Meloidogyne graminocola* en piperonylzuur, een inhibitor van het CINAMAAT-4-HYDROXYLASE enzym, tegen *Pyricularia oryzae*. Voor de CLP-producerende *Pseudomonas* stammen werd zowel hun direct nematostatisch effect als het vermogen om resistentie te induceren in rijst tegen *Meloidogyne graminicola,* geëvalueerd, terwijl bij PA enkel het vermogen om resistentie te induceren in rijst tegen *Pyricularia oryzae* werd nagegaan.

Er werd aangetoond dat supernatants van verschillende *Pseudomonas*-stammen een nematostatisch effect hadden, maar mutantenanalyse wijst erop dat andere, niet-geïdentificeerde, secundaire metabolieten de oorzaak zouden kunnen zijn van dit nematostatisch effect. Verder werd aangetoond dat PA resistentie kan induceren in rijst tegen *P. oryzae,* en dat deze resistentie afhangt van de productie van fytoalexines, maar schijnbaar niet van lignificatie.

Sleutelwoorden: Geïnduceerde resistentie, pathogenen, plant immuniteit, rijst, priming

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List of abbreviations

4-CL	4-COUMARATE:COENZYME A LIGASE
AA	Amino acid
BTH	1,2,3-benzothiadiazole-7-carbothioate
C4H	*trans*-CINNAMATE 4-HYDROXYLASE
CFU	Colony-forming units
CLP	Cyclic lipopeptide
DMSO	Dimethylsulfoxide
Dpi	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
ET	Ethylene
ETI	Effector-triggered immunity
FLS2	FLAGELLIN-SENSING 2
IP	Invasion Pattern
IPM	Integrated pest management
IPR	Invasion Pattern Receptor
IR	Induced resistance
JA	Jasmonic acid
MEP	Methylerythritol 4-phosphate
MPK3	MITOGEN-ACTIVATED PROTEIN KINASE 3
MPK6	MITOGEN-ACTIVATED PROTEIN KINASE 6
PA	Piperonylic acid
PAL	PHENYLALANINE AMMONIA-LYASE
PAMP	Pathogen-associated molecular patterns
Pol	POLYMERASE
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
R gene	Resistance gene
RdDM	RNA-directed DNA methylation
RDRP6	RNA-DEPENDENT RNA POLYMERASE
RKN	Root-knot nematode
ROS	Reactive oxygen species
SA	Salicylic Acid
SD	Soil drench
siRNA	Small interfering RNA
SP 25	Syringopeptin 25
SP22	Syringopeptin 22
ssRNA	Single stranded RNA
WLIP	White line inducing principle

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Preface

Rice (*Oryza sativa* L.) is one of the most important crops worldwide, due to its role as a model organism and its importance as food crop. Because the world population continues to increase, rice demand is expected to grow. However, multiple challenges are threatening rice production. Two of the most damaging of these are *Meloidogyne graminicola,* which causes root-knot disease, and *Pyricularia oryzae,* which causes blast disease. Both pathogens cause severe losses in rice yield, endangering the food security of billions of people. Conventional chemical fungicides and nematicides can be harmful to human health and the environment, and their efficacy can be hampered by issues such as pesticide resistance. Therefore, it is crucial to find new, additional means to combat these pathogens. One of the proposed ways to do so is to use *induced resistance* (IR) to enhance the immune system of the plant, so it can respond more effectively to infection.

IR is used to refer to a phenomenon where plants become more resistant towards certain biotic stressors upon exposure to an exogenous chemical or biotic stimulus. *Priming* refers to a specific subset of the IR response, in which a defense response is not triggered directly by the IR stimulus, but is expressed more rapidly or more intensely in plants treated with the IR stimulus upon later exposure to a biotic stressor.

The efficacy and mechanism of IR is variable and depends on both the specific IR trigger and the pathosystem in which it is tested. During this thesis, two different IR stimuli are studied. First, the role of cyclic lipopeptide (CLP)-producing *Pseudomonas* strains as inducers of resistance against root-knot nematodes in rice is assessed. Second, piperonylic acid (PA), a chemical inhibitor of CINAMATE-4-HYDROXYLASE, is assessed as a possible IR stimulus in rice against rice blast disease, caused by *Pyricularia oryzae.*

The potential of CLP-producing *Pseudomonas* strains was tested in two ways: by testing the *in vitro* nematostatic activity of supernatants from such strains, and of mutants of these strains impaired in CLP production, and by testing whether living *Pseudomonas* cultures could induce resistance in rice to *M. graminicola*.

Second, the capability of PA to induce resistance in rice was assessed. Therefore, infection assays with *P. oryzae* were performed, combined with biochemical assays for guaiacol peroxidase activity, PHENYLALANINE AMMONIA-LYASE activity, lignin content and free and bound phenolic compounds. Furthermore, RT-qPCR was used to investigate the expression pattern of several immunity-related genes in PA-IR in rice against *P. oryzae.*

In the chapter **Literature study**, a more detailed background to this thesis is given. The importance of rice is further explained, as is the importance, life cycle and current management of *Meloidogyne graminicola* and *Pyricularia oryzae*. IR and priming, alongside the broader plant immune system, are then briefly discussed. Finally, current knowledge on PA and CLPs are briefly reviewed.

The chapter **Materials and Methods** provides a description of how experiments were performed. In the chapter **Results,** the results of these experiments are shown, starting with

the experiments involving *Pseudomonas* and then moving on to those involving PA. The chapter **Discussion** summarizes and interprets the results obtained, and compares them to published literature. The discussion ends with providing some future perspectives. Finally, the chapter **General Conclusion** provides a general conclusion to the thesis.

1. Literature study

1.1. Introduction

Rice (*Oryza sativa*) is one of the most important food crops in the world. Especially in tropical and subtropical regions, rice is a staple food of which the annual consumption can surpass 100 kg per capita in some regions (Seck *et al.*, 2012). In 2018, it was estimated that rice production was over 728 million tons worldwide. Asia is responsible for about 90% of this rice production. Some African countries, Brazil and the USA are also important rice producing countries, albeit to a lesser extent (FAO, 2021). Besides of the importance as a staple food, rice plays a crucial role as a model organism for monocotyledons (Izawa & Shimamoto, 1996). For instance, rice is the first cereal crop whose whole genome has been sequenced, partly because its genome is smaller than the genomes of other cereal crops such as sorghum and maize (Jackson, 2016).

Rice production is expected to encounter various challenges during the following decades. First of all, the population is growing, resulting in higher demands for food and thus the need for an increased rice production. Second, climate change and a subsequent competition for water threatens the rice production. Finally, other biotic and abiotic challenges are threats to rice production. The main biotic challenge is the presence of weeds, followed by infection by the rice blast pathogen *Pyricularia oryzae*. Other biotic challenges include nematodes, viruses and bacteria. The main abiotic challenges consist of drought and soils which are nutrient-deficient. Alkalinity and salinity of the soil and low temperatures are other abiotic challenges lowering yield. In order to meet these challenges, research is performed to develop new and better practices in agriculture (Seck *et al*., 2012).

One of the proposed ways to do so is the use of agents inducing plant defense priming as part of integrated pest management (IPM) programs. These agents improve the immune system of plants, without a direct activation of defense genes. This solution can contribute to better and more sustainable agricultural practices (Conrath *et al.*, 2015; Mauch-Mani *et al.*, 2017). This research explores the possible suitability of piperonylic acid and cyclic lipopeptides as a priming agents against *Pyricularia oryzae* (syn: *Magnaporthe oryzae*) and nematodes, respectively.

1.2. Plant parasitic nematodes

Plant parasitic nematodes are nematodes which are parasitic to plants. In general, the life cycle of nematodes consists out of four juveniles and an adult stage, which produces eggs (Bridge & Starr, 2007; Lambert & Bekal, 2002). Plant parasitic nematodes in general molt once into the second juvenile inside the eggs before they hatch, meaning the larvae leaving the eggs are already in the second juvenile stage (Lambert & Bekal, 2002). Approximately half of the economic damage due to plant parasitic nematodes is at the expense of rice and maize, partly because these two crops are cultivated in all parts of the world (Kyndt *et al.*, 2014). However, this is not reflected in the quantity of molecular nematologists executing their research in these crops (Jones *et al.*, 2013; Kyndt *et al.*, 2014). Kyndt *et al.* (2014) proposed that six important

groups of nematodes parasitic on rice can be distinguished. These six groups include root-knot nematodes (RKN, *Meloidogyne* spp.), root lesion nematodes (*Pratylenchus* spp.), cyst nematodes (*Heterodera* spp.), root rot nematodes (*Hirschmanniella* spp.*),* the foliar nematode - *Aphelenchoides besseyi* and the stem nematode *Ditylenchus angustus.* The first difference between these groups is whether the nematodes live in or on the plants. Where root-knot nematodes, root-lesion nematodes, cyst nematodes and root rot nematodes are endoparasitic organisms, *Aphelenchoides besseyi* and *Ditylenchus angustus* are ectoparasitic (Kyndt *et al.*, 2014). Within the endoparasitic nematodes, a further division is made between sedentary and migratory endoparasites. Sedentary endoparasites enter the plant, manufacture a special feeding structure in the plant and lose their ability to move at certain life stages. Root-knot and cyst nematodes belong to the sedentary nematodes. Migratory nematodes on the other hand stay mobile and worm-shaped in each stage of their life. Both rice root rot nematodes and root-lesion nematodes are migratory (Bridge & Starr, 2007).

As mentioned before, RKNs are sedentary (Bridge & Starr, 2007). *Meloidogyne graminicola* and *Meloidogyne incognita* are two notorious species belonging to the group of the RKNs which are able to infect rice plants (Kyndt *et al.*, 2014). The reproduction cycle of RKNs is shown in **Figure 1.** Female RKN lay eggs on the surface of the root or in the root itself. The first molting takes place inside the egg (Jones *et al.*, 2013). When the egg hatches, the infectious juvenile 2 (J2) nematodes are able to penetrate the root by using a stylet. The J2 nematodes move intracellularly until the vascular bundle of the plant, where they grow and make a specific feeding site (Jones *et al.*, 2013; Kyndt *et al.*, 2014). Because of hypertrophy and hyperplasy of adjacent plant cells, galls visible with the naked eye are formed (Kyndt *et al.*, 2014). When the J2 nematodes have finished feeding itself, they molt to become J3 nematodes, which are unable to feed themselves because of the absence of a working stylet. J3 nematodes molt again, becoming J4 nematodes, again unable to feed themselves. Finally, the J4s molt to become adults, which can feed themselves again. Where males are worm-shaped and migrate out of the root, females are pear shaped and stay inside the root, where they lay eggs. Reproduction of plant parasitic nematodes can occur through an array of possible strategies, including parthenogenesis and amphimixis (Jones *et al.*, 2013). In case of *Meloidogyne graminicola,* the sexual ratio is dependent on external factors. Under convenient conditions, almost no males are present and reproduction occurs mostly through parthenogenesis, while under adverse conditions, males become more abundant (Karssen *et al.*, 2013). Yield losses due to infection with root knot nematodes can be as high as 80% (Bui *et al.*, 2020).

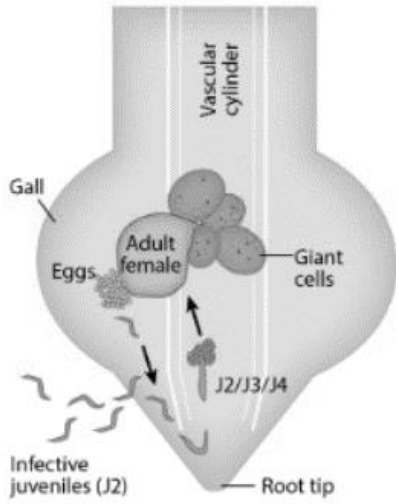

Figure 1: The reproduction cycle of RKN. The infectious juveniles invade the roots and migrate to the vascular bundle, where three moltings take place until female adults form eggs within their bodies. When the eggs hatch, the juveniles have already undergone one molting, so the nematodes are in the second juvenile stage J2. During this reproduction cycle, multiple plant cells merge with the formation of giant cells (Source: Kyndt et al., 2014).

In order to combat the adverse effects of infection with plant parasitic nematodes, different strategies have been developed (Bui *et al.* 2020; Galeng-Lawilao *et al.* 2018). Strategies to control *Meloidogyne graminicola* include applying continuous flooding conditions, rotating crops (Galeng-Lawilao *et al.*, 2018; Thulke & Conrath, 1998) and using chemical nematicides (Galeng-Lawilao *et al.*, 2018; Khan *et al.*, 2012). However, each of these strategies has their own drawbacks. Continuous flooding conditions is difficult due to water scarcity, while crop rotation can lead to increased costs. Chemicals for the control of nematodes in their turn are expensive. Moreover, some of these chemicals have unfavorable effects on the environment and human health. For instance, carbofuran is both an insecticide and a nematicide which is lethal to mammals, fish, birds and wildlife. The adverse effects of these chemicals already led to the banning of some of them from the market, for example the fumigantia 1, 2-di-bromo-3-chloropropane and 1,2-dibromoethane (Galeng-Lawilao *et al.*, 2018). Another unfavorable characteristic of the application of chemicals in rice fields is that under flooded conditions, the chemicals may rapidly dilute and percolate (Khan *et al.*, 2020). In spite of these negative effects, nematicidal chemicals such as oxamyl, fluopyram (Velum), and fluensulfone (Nimitz) are still popular because they are relatively easy to use and the control is reliable (Wram & Zasada, 2019).

1.3. *Pyricularia oryzae*

Pyricularia oryzae (Magnaporthe oryzae) is a plant pathogenic fungus belonging to the phylum Ascomycota and is responsible for the rice blast disease (Boddy, 2016; Dean *et al.*, 2012). *P. oryzae* is a hemi-biotrophic fungus, meaning that the fungus starts the infection process as a biotroph, feeding itself with plant cells without killing them, but subsequently kills the plant cells and feeds on their content (Boddy, 2016). The life cycle of *P. oryzae* starts when a conidium

lands on a rice plant. The spore tip then produces an adhesive in order to adhere strongly to the leaves. Subsequently, several development steps take place. First of all, germination occurs, after which the germ tube grows. A cell specialized for infection, an appressorium, is formed afterwards (Boddy, 2016; Dean *et al.*, 2012). After appressorium formation, the turgor pressure in the appressorium increases through melanization and accumulation of soluble compounds, such as glycerol (Wilson & Talbot, 2009). The increasing pressure leads to the creation of a penetration peg, which can penetrate the underlying plant tissue (Boddy, 2016; Dean *et al.*, 2012; Wilson & Talbot, 2009).The fungus spreads within the plant by branching into neighboring other cells, probable disseminating via the plasmodesmata. The last step of the infection cycle consists of the production of conidia at the conidiophores (Ebbole, 2007). The infection process is shown schematically in **Figure 2**.

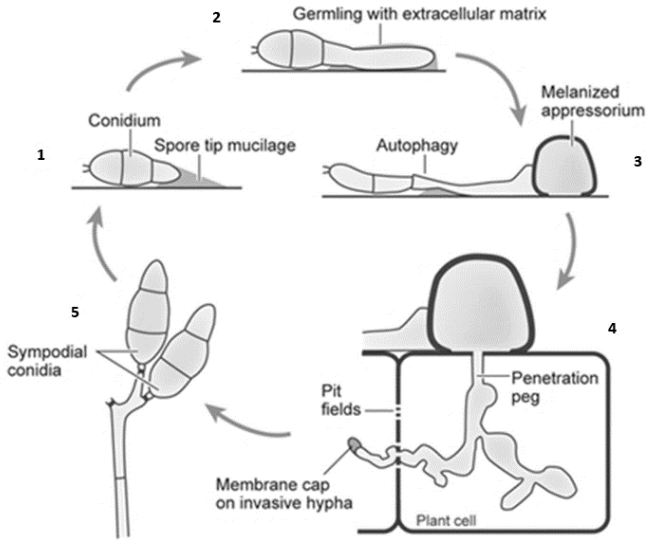

Figure 2: The infection cycle of *Pyricularia oryzae*. In the first step, a spore lands on the plant and adheres firmly on the surface thanks to an adhesive mucilage. Second, germination and elongation of the germ tubes occurs (2). Third, the appressorium is formed, after which the penetration of the host plant happens by a penetration peg. Last, conidia are formed at the conidiophores (Source: adapted from Ebbole, 2007).

Depending on the age of the plants when they are infected, the symptoms vary. In young rice plants, rice blast disease causes tiny necrotic lesions which grow bigger and unite, to form bigger lesions with chlorotic edges. Lesions ultimately cause death of the entire infected leaf. Purple, trigonal lesions appear at the neck nodes. These lesions grow at both sides and impede a proper development of the grains because of the necrotrophic character of the infection. When the neck nodes are entered, the panicles become white. Infections of the neck nodes and the panicles are especially detrimental, causing yield losses up to 80%. Infection at an even later stage of the plant development results in an incomplete filling of the grains (Boddy, 2016). Because of enormous yield losses, rice blast disease is considered as one of the most problematic diseases worldwide. In view of the relevance of *P. oryzae* in global food

supply and because working with *P. oryzae* is relatively easy, *P. oryzae* is used as a model organism to study plant-pathogen interactions (Boddy, 2016; Dean *et al.*, 2012).

In order to combat the great yield losses due to the rice blast disease, different strategies have been developed in order to control it. First of all, rice farmers can use fungicides. For instance, tricyclazole impedes the melanization of the appressorium, making it unable to develop properly and thus prevent the invasion of the plant (Boddy, 2016). Other examples of fungicides which are used are carbendazim, difenoconazole and strobilurines (Yesmin *et al.*, 2020). Fungicides can have harmful effects on the environment and on human health (Boddy, 2016). Moreover, fungicides are relatively costly and the emergence of fungicides-resistant strains are on the rise. Second, the application of six groups of inorganic salts is commonly used in the combat of fungi in general. These include silicates, phosphites, phosphates, bicarbonates, chlorides and sulphates. Where silicate salts and anorganic phosphites already have shown to be useful in the combat against *P. oryzae*, the effectiveness of chlorides, bicarbonates, sulphates and phosphates against the rice blast disease is still unexplored (Yesmin *et al.*, 2020). Third, rice farmers could use rice cultivars which are not susceptible to the rice blast disease, because they have a dominant resistance gene (R gene; Boddy, 2016). These cultivars can be obtained by breeding or by transgenic manipulations (Yesmin *et al.*, 2020). This method was found to be the least harmful method for the environment and to be cost-efficient, which is why it is often applied by rice farmers. However, since the resistance against the disease is usually due to one single gene, the resistance is easily overcome by the fungus, leaving the cultivar susceptible again. Therefore, resistant cultivars can be used only for a few years (Boddy, 2016). In order to circumvent the emergence of resistant strains, cultivars containing multiple resistance genes can be generated (Boddy, 2016; Yesmin *et al.*, 2020). Recent research investigates the possibilities of inducing systemic resistance through both biotic and abiotic stimuli (Yesmin *et al.*, 2020).

1.4. Plant Defense Priming

1.4.1. Terminology

As plants are sessile, they need a very well developed defense system in order to protect themselves against attacks from pathogens or against unfavorable conditions, such as drought. Apart from constitutive chemical and physical protection mechanisms, such as phytoanticipins and a thick cell wall, plants also possess an inducible defense system. This defense system relies on the recognition of specific patterns by a plant recognition receptor, followed by induction of expression of specific defense genes. However, sometimes the expression of defense genes is not induced directly, although the power of the defense capacity increases (Mauch-Mani *et al.*, 2017). This is called *induced resistance* and sometimes *priming*, where priming is a special form of IR, which was originally considered to induce direct defense responses after a pathogen attack exclusively. This definition appeared to be too strict, because research showed that priming agents do not only cause defense responses after a pathogen attack, but also after induction with the agent itself (Mauch-Mani *et al.*, 2017). Therefore, the term induced resistance will be used further on.

In the past, induced resistance has been referred to with numerous overlapping terms, including induced systemic resistance and systemic acquired resistance (Conrath *et al.*, 2015). The distinction between induced systemic resistance and systemic acquired resistance is made based on the agent which induces plant resistance, which are called priming stimuli or IR stimuli. Systemic acquired resistance is by definition caused by pathogens or by chemical molecules, induced systemic resistance is triggered by beneficial micro-organisms (Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017; Pastor *et al.,* 2013). However, it is not always possible to make a clear separation between these types of induced resistance (De Kesel *et al.*, 2021), which is why in this thesis, all kinds of induced resistance are referred to as 'IR'. Interestingly, the state of induced resistance can be passed on to the progeny, this is called *transgenerational IR* (Luna & Ton, 2012).

IR in the field could be effective, as proved by the commercial rollout of several priming agents, such as benzothiadiazole(BTH), commercialized by Syngenta under the product name Bion or Actigard (Thomson *et al.*, 1999). Another example of a commercialized molecule inducing plant defense priming is probenazole. This molecule induces IR in rice plants and helps the combat against the rice blast disease (Iwata *et al.*, 2004). COS-OGA (chitooligosaccharides oligogalacturonides), commercialized as Fytosave®, induces resistance in cucumbers and grapes against powdery mildew (van Aubel *et al.,* 2014).

1.4.2. Plant immune system

The plant immune system has been described by different models. It is important to keep in mind that models are just models, generalizing the reality (Cook *et al.*, 2015). One of the oldest and still most influential model, the zigzag model of Jones & Dangl, divides the plant immune response into four different steps. First, the plant recognizes pathogen-associated molecular patterns (PAMP), which are patterns derived from conserved molecules present in many pests and pathogens, by a pattern recognition receptor (PRR) on the cell-surface. After recognition, the plant will activate its immune system. This leads to PAMP-triggered immunity (PTI). In order to survive and continue symbiosis, the pest or pathogen produces effectors, which are molecules suppressing PTI. This is the second step. Third, the plant evolves intracellular proteins (R proteins) to detect effectors, leading to effector triggered immunity (ETI). In the fourth step, the pest or pathogen evolves in such a way he is able to avoid effector-triggered immunity, for example by not making the effector anymore, by changing the structure of the effector or by producing a new effector (Cook *et al.*, 2015; Jones & Dangl, 2006, Kanyuka & Rudd, 2019; Pritchard & Birch, 2014). Although the zigzag model is able to explain numerous phenomena in plant immunity, it also has some limitations.

These limitations include a flawed usage of the terms "effector" and "PAMP", because they are defined from a different point of view. Where effectors are defined as molecules aiding the symbiosis, attack or infection, PAMPs are patterns recognized by plants. Because of the existence of some overlap between these definitions, the terms are not always applied correctly. In fact, effectors of different pathogens can share similar patterns. Therefore, these patterns can be used as a PAMP by the plant, although their biochemical function consist of

helping the pathogen in the infection process. For example, BcSpl1 is an effector protein produced by virulent *Botrytis cinerea*. This protein has two conserved regions shared by different families of fungi. These conserved regions are recognized by the plant and induce defense responses, including the induction of cell death. Because *Botrytis cinerea* is a necrotrophic fungus, this plant defense response helps in the infection process. However, for the plant, the BcSpl1 protein carries two PAMPs, inducing a defense response. This example clarifies that making a clear distinction between effector and PAMP is difficult, if not impossible. Other limitations of the zigzag model are among others the viewpoint of PAMP and PTI as a static instead of a dynamic phenomenon, the idea that R genes are always host specific, and the separation of broad-spectrum immunity as a distinct form of immunity (Cook *et al.*, 2015). Moreover, it seems impossible to make a distinction between PTI and ETI. After recognition of an attacker, several physiological changes take place in the plant. These changes may include, among others, the deposition of callose, a limited transport of nutrients from cytosol to the apoplast (Ramirez-Prado *et al.*, 2018), production of reactive oxygen species (ROS; Klessig *et al.*, 2018; Ramirez-Prado *et al.*, 2018), closure of the stomata, production of antimicrobial metabolites, nitric oxide (Ramirez-Prado *et al.*, 2018) and the defense hormones salicylic acid, jasmonic acid and ethylene. Comparison between PTI and ETI shows that often the same reactions occur, but ETI usually generates a stronger and longer-lasting response. While it was believed that only ETI was able inducing a hypersensitive response reaction, while PTI is not (Klessig *et al.*, 2018; Ramirez-Prado *et al.*, 2018), other studies showed that both ETI and PTI are able to induce a hypersensitive response, although it occurs more often during ETI (Tsuda & Katagiri, 2010). Also important to point out is that both ETI and PTI were shown to activate defense responses in non-inoculated parts of the plants, causing induced resistance (Klessig *et al.*, 2018).

To address some of these issues, the Invasion Model of plant immunity has been proposed. This model suggests that plants recognize Invasion Patterns (IP) by Invasion Pattern Receptors (IPR). An IP can be any molecule related to an invasion, meaning no division is made between PAMPs, effectors and patterns typical for the infection processes (Cook *et al.*, 2015; Kanyuka & Rudd, 2019). After the detection of the IP, the plant reacts with an IP-triggered reaction. According to this model, two outcomes are possible after an IP-triggered reaction: a cessation or a continuation of the symbiosis or infection. Invaders try to influence the result of the IP-triggered reaction by producing effectors to overcome the immune system and suppress immunity. This, of course, can be unsuccessful, resulting in the end of symbiosis. Besides, invaders can also exploit the IP-triggered response. For instance, necrotrophic organisms use a “pro-death” invasion strategy. For example, *Cohliobolus victoriae* produces an effector called victorin. This effector is recognized by the plant and induces cell death. The dead cells can be used by *Cochliobolus victoriae* (Cook *et al.*, 2015).

In conclusion, different models describing plant immunity exist, of which the two most known are discussed. Both models are based on the recognition of a molecule derived from a pathogen or attacker by a receptor of the plant (Cook *et al.*, 2015).

1.4.3. Changes in naïve plants plant

Different changes occur in naïve plants (plants which are not primed), that lead to the achievement of priming, and thus enhance the defense capacity. These changes can be grouped into physiological, molecular and epigenetic changes.

Physiological changes are mostly transient. The best documented early change after challenging the plant, is the accumulation of the calcium ions in the cytosol of the challenged cells and its neighbors, occurring within seconds or minutes after challenge. The accumulation of calcium in the cells has three major consequences. First of all, the Ca^{2+} present in the cytosol will change ion fluxes, leading to membrane polarization. Membrane polarization in its turn can activate electric signaling from one cell to another in order to transfer signals, enabling to spread the signal to non-challenged parts of the plant. Second, because of the increased level of calcium in the cytosol, enzymes relying on Ca^{2+} present in the cytosol will be more active. Lastly, Ca^{2+} present in the cytosol can induce an accumulation of ROS. This ROS burst is believed to be required for achieving the primed state (Mauch-Mani *et al.*, 2017).

In primed *Arabidopsis* plants, an accumulation of the inactive proteins MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and 6 (MPK6) has been noticed (Conrath *et al.*, 2015; Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017). MPK3/6 has a pivotal role in the cell signaling cascade where it increases cell signals (Conrath *et al.*, 2015; Martinez-Medina *et al.*, 2016). Thus the accumulation of MPK3/6 is a possible technique for a faster phosphorylation and activation of immune responses (Mauch-Mani *et al.*, 2017). However, due to the short life of the inactive MPK3/6 and also some other signaling proteins, it has been argued that long-lasting defense priming cannot be entirely explained by the elevated levels of inactive MPK3/6 (Conrath *et al.*, 2015). Beside of the accumulation of inactive MPK3/6, an accumulation of pattern recognition receptors is also observed as a reaction to the priming stimulus (Conrath *et al.*, 2015; Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017).For example, the level of FLAGELLIN-SENSING 2 (FLS2) receptor, a receptor that recognizes the flg22 epitope at the conserved N-terminal of flagellin, was increased after treatment with benzothiazole in *Arabidopsis.* An increased level of the coreceptor of FLS2, namely BRI1-Associated Kinase 1 (BAK1), was also observed. The FLS2 receptor triggers after a binding event the activation of the MAPK/MPK3/6 signaling cascade, consisting out MAP KINASE/ EXTRACELLULAR SIGNAL-REGULATED KINASE 1 (MEKK1), MAP KINASE KINASE (MKK) 4/5 and MPK 3/6. This discovery corresponds with the observation of the accumulated MPK3/6, providing evidence of the importance of this pathway (Conrath *et al.*, 2015).

These physiological changes are associated with changes on transcriptional level, on both local and systemic level (Mauch-Mani *et al.*, 2017). A recent study aimed to find common transcriptomic changes after induction of plants with priming agents. In order to do so, they compared transcriptional data obtained by publicly accessible RNA-Seq analyses, after inducing resistance in *Arabidopsis thaliana*. They found that genes responsible for fatty acid synthesis and photosynthesis, genes in the methylerythritol 4-phosphate (MEP) pathway, genes for transcription factors for a normal development and genes for transcription factors

inhibiting defense genes were downregulated during the priming phase. Besides of the downregulation of these genes, upregulation of regulators of abscisic acid, MAP kinases, monoterpene synthesis genes, signaling molecules and receptors, transcription factors as *WRKY18* and β-oxidation genes were found. With this findings, a model was built with a "priming fingerprint" of plants, being transcriptional changes similar in all plants (Baccelli *et al.*, 2020). However, it is important to note that besides this common fingerprint, specific transcriptomic changes occur during the priming phase (Baccelli *et al.*, 2020; Mauch-Mani *et al.*, 2017). Moreover, the extent to which this model, based on data obtained with *Arabidopsis*, is applicable on a monocotyledon like rice is unknown.

At the metabolic level, two types of changes have been distinguished. On one hand, increased levels of defense-related hormones, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (Et) have been observed. On the other hand, higher levels of primary metabolites, like xylitol, amino acids (AA), glycerol-3-phosphate and *myo*-inositol appeared (Mauch-Mani *et al.*, 2017). In addition, increased levels of inactive phytoanticipins have been reported after priming with *Pseudomonas syringae* pv. *tomato* in *Arabidopsis* (Mauch-Mani *et al.*, 2017). Phytoanticipins are secondary metabolites which are constitutively expressed or present in order to protect plants (Vanetten *et al.*, 1994). So, in conclusion, both elevated levels of certain primary and secondary metabolites occur after challenge with priming agents (Mauch-Mani *et al.*, 2017; Mhlongo *et al.*, 2016).

The term epigenetics is used to indicate phenotypical modifications or changes in gene expression that can be inherited, but are not caused by a modification in the DNA sequence (Mauch-Mani *et al.*, 2017). One form of gene regulation involves changing the density of chromatin. Two copies of each histone protein H2A, H2B, H3 and H4 are attached to each other, forming an octamer. Around this octamer, 146 base pairs of genomic DNA are encased, making a nucleosome. The nucleosomes, together with histone H1, RNA and nonhistone proteins, form chromatin. Gene regulation can appear on two levels in chromatin. First of all, covalent modifications in histones appear. These modifications encompass acetylation, methylation, poly-UDP-ribosylation and ubiquitination and they take place at the N-terminus of the histones (Conrath *et al.*, 2015). Although the exact function of these modifications in the gene regulation is not fully understood yet, it is generally accepted that acetylation of lysine residues in histones facilitates transcription of genes. Methylation of histones is more complicated than acetylation, probable because both lysine and arginine can be methylated. The second level of controlling gene expression encompasses DNA methylation (Conrath *et al.*, 2015). In plants, methylation of DNA mainly occurs at the cytosine bases (Sanchez, 2016), at both CG and non-CG regions (CGH and CHH respectively, where H is any base but G; Espinas *et al.*, 2016). Small interfering RNAs (siRNA) are responsible for a *de novo* methylation of DNA by both canonical and non-canonical RNA-directed DNA methylation (RdDM) pathways. In the non-canonical PolII-RDR6-dependent pathway, single stranded RNA (ssRNA) is formed by the action of POLYMERASE (Pol) lII. This ssRNA is subsequently converted into dsRNA by RNA-DEPENDENT RNA POLYMERASE (RDRP6), followed by a further processing to a siRNA with a size of 21 to 24 base pairs. This siRNA is loaded into the

ARGONAUTE 6. The resulting complex is guided towards the DNA target sequence, where it causes methylation. In a canonical RdDM pathway, the first step consists of the production of RNA, performed by a specific subunit of PolIV, NUCLEAR RNA POLYMERASE D. Subsequently, different processing steps follow, after which the RNA is loaded into ARGONAUTE 4. Here, it base-pairs to a second RNA strand which is generated by Polymerase V. This complex interacts with DOMAINS REARRANGED METHYLTRANSFERASE, resulting in the methylation of the target DNA sequence (Espinas *et al.*, 2016). Lastly, controlling gene expression is also achieved by non-coding RNA through the RdDM pathway (Kinoshita & Seki, 2014; Luna & Ton, 2012). Different researchers have investigated the importance of DNA methylation for the achievement of a primed state. Luna *et al.* (2012) suggested that the RdDM pathway is an important pathway in the regulation of transgenerational IR. Furthermore, they suggest that IR is transferred to the progeny by a hypomethylation of the CpNpG sites (Luna & Ton, 2012). Another study found that the DNA methylation degree in barley decreased significantly after priming barley with either iso-nicotinic acid methyl ester, N-methyl-nicotinic acid or a culture filtrate of *Bacillus subtilis*. This reduction in methylation degree is associated with an enhanced resistance against the powdery mildew disease (Conrath, 2011).

The importance of epigenetic changes in the regulation of defense genes after priming has been examined by different research groups. This resulted in the hypothesis that initial stimuli eventually alter the chromatin structure in such a way the promotors of defense genes are easier to reach. As the promotor is easier to reach, the activation of the corresponding defense genes is easier, resulting in a faster response upon a next challenge. So, the priming agent does not activate the genes itself, but they make the expression of the genes easier. Because of these epigenetic changes, the long-term character of defense priming, seen in several studies, can be explained. Moreover, changes in DNA methylation are heritable, giving a molecular explanation of a transgenerational primed state. A transgenerational primed state is a phenomenon where the offspring of the plants threated with a priming agent exhibit a primed state. Several studies report such a transgenerational primed state. However, because of the costs related to the activation of defense genes, the primed stated can also be reverted, for instance by demethylation. The duration of the primed state is dependent on different factors, such as the original stress and on how many times this stress has been applied (Mauch-Mani *et al.*, 2017).

1.4.4. Characteristics of defense priming

Martinez-Medina *et al.* (2016) propose six characteristics to distinguish defense priming. These include a low fitness and ecological cost, a broad-spectrum activity, better performance, a better defense in stress conditions and of course the memorization of the stimulus by the plant (Martinez-Medina *et al.*, 2016).

Thanks to the accumulation of receptors on one hand and proteins crucial for downstream phosphorylation processes on the other hand, plants are able to store information on the priming stimulus on the short term (Conrath *et al.*, 2015). Epigenetic changes, including

changes in DNA methylation and histone modifications, provide in their turn a long-term memory (Conrath *et al.*, 2015; Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017). Since the accumulated receptors and proteins are not limited to receptors and proteins against one specific pathogen, the accumulations results in broad spectrum activity (Conrath *et al.*, 2015; Martinez-Medina *et al.*, 2016). Defense priming strengthens the plants' defense capacity against an array of possible invaders, i.e. priming has a broad activity spectrum (Martinez-Medina *et al.*, 2016).

The term *fitness* refers to processes in plants that contribute to its reproductive success. Typically, direct activation of defense genes is associated with high fitness costs in the absence of pests and diseases. As IR in general does not strongly or directly activate defense genes, IR is thought to have a low fitness cost. Nevertheless, there are some fitness costs, called *allocation costs*, being a fitness cost due to the allocation of resources to the immune system instead of to growth (Mauch-Mani *et al.*, 2017). Also *ecological costs*, being costs due to a decreased capacity of the plant to have interactions with their environment may occur (Heil, 2002; Martinez-Medina *et al.*, 2016). Some studies investigated the fitness costs of IR. Most of these studies found that there is either no or a small fitness cost to IR compared to a direct activation of defense-genes expression (Rodríguez *et al.*, 2018; van Hulten *et al.*, 2006). Some studies reported a fitness cost as a result of the IR process (Paudel *et al.,* 2014). These fitness costs were found to be depending on the plant and the dose of the used IR agent, meaning that it is crucial to define the ideal application method for each IR agent (Paudel *et al.*, 2014). In conclusion, IR has a low fitness cost in comparison to direct activation of defense-related gene expression (Conrath *et al.*, 2015; Conrath *et al.*, 2006; Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017).

The ultimate goal of IR in plants is to obtain better performing plants by enhancing their defense capacity. However, although improvement of defense capacity can result in better performance under certain conditions, this is not always the case in other conditions. For example, after induction of plant defense against insects and necrotrophs, a negative hormone crosstalk has been reported for defense responses against biotrophs. These finding suggests that insect and necrotrophic attacks can influence the capacity of the plant to protect itself against biotrophs and vice versa. This example shows the importance of experimental setup in the study of defense priming. Under ideal conditions, defense priming is studied in ecological realistic conditions. Another important factor is that the study preferably determines the survival and reproduction of the plant as the result of the priming step (Martinez-Medina *et al.*, 2016).

1.5. Piperonylic acid

Piperonylic acid is a quasi-irreversible inhibitor of the CINNAMIC 4-HYDROXYLASE (C4H) enzyme (Schoch *et al.*, 2002). This enzyme belongs to the family of the P450 heme thiolate family, consisting out of proteins which catalyze mono-oxygenation reactions in an array of possible substrates in all organisms. C4H in particular catalyzes the mono-oxygenation of *trans*-cinnamic acid to *p*-coumaric acid. This is the second step in the so-called phenylpropanoid pathway. The phenylpropanoid pathway itself consists out of an upstream

part, encompassing three steps which finally results in the production of 4-coumaroyl CoA. These three steps are shown in **Figure 3**. In the first step, phenylalanine is converted into *trans*-cinnamic acid, a reaction catalyzed by PHENYLALANINE AMMONIA-LYASE (PAL). In the second step, *trans*-cinnamic acid is converted to *p*-coumaric acid by C4H. Finally, 4-coumaroyl CoA is formed out of *p*-coumaric acid, catalyzed by 4-COUMARATE:COENZYME A LIGASE. 4-Coumaroyl CoA on its turn can be converted by a sequence of hydroxylation, dehydration and methylation reactions to simple phenylpropanoids, encompassing *p*-coumaric acid, ferulic acid, sinapyl acid, caffeic acid, and simple coumarins (Dixon & Paiva, 1995). These molecules can be converted to a broad range of molecules with a variety of functions in downstream processes, such as the production of lignin, stilbenes, coumarins, flavonoids and isoflavonoids, which have essential roles in protection against UV and microbials or are important signaling molecules (Schalk *et al.*, 1998; Schoch *et al.*, 2002). For instance, *p*-coumaric acid is a precursor for sinapyl, coumaryl and coniferyl alcohol, as it can be converted into these molecules through several hydroxylation, methylation and reduction reactions. Sinapyl, coumaryl and coniferyl alcohols themselves are precursors for lignin, an essential polymer giving plants mechanical strength, with an important role in water transport and protection against pathogen infections by providing a physical blockade (Whetten & Sederoff, 1995; Schoch *et al.*, 2002). Another example yields flavonoids like kaempferol and its derivatives, which are also produced by a series of reactions starting from *p*-coumaric acid, protecting plants against UV light, wounding and microbial infection (Dixon & Paiva, 1995). In other words, these examples show the importance of the phenylpropanoid pathway in plant defense against both biotic and abiotic stress conditions.

In a side branch of the phenylpropanoid pathway, cinnamic acid can be converted to benzoic acid, which in its turn can be converted to SA (Schalk *et al.*, 1998; Schoch *et al.*, 2002), a signaling molecule playing a major role in activating plant defenses and acquiring IR (Chong *et al.*, 2001; Schoch *et al.*, 2002). The synthesis of SA originating from the phenylpropanoid pathway is depending on cinnamic acid. Namely, benzoic acid can be formed out of cinnamic acid, which in its turn can be converted into SA (Schalk *et al.*, 1998; Schoch *et al.*, 2002). However, in the presence of active C4H, cinnamic acid is converted into 4-coumaroyl CoA, resulting in lower levels of cinnamic acid and thus of SA (Schoch *et al.*, 2002). Therefore, inhibitors of C4H were suggested as possible enhancers of plant response upon pathogen attack by redirecting the phenylpropanoid pathway to the synthesis of SA. Piperonylic acid is found to be an inhibitor of C4H (Schalk *et al.*, 1998; Schoch *et al.*, 2002), as it is a substrate analogue of C4H (Schoch *et al.*, 2002). PA inhibits C4H by executing an oxidative attack, resulting in the formation of carben and the emergence of a coordination with the heme iron of C4H (Schalk *et al.*, 1998; Schoch *et al.*, 2002). The structure of both *trans*-cinnamic acid and piperonylic acid is shown in **Figure 4**. Apart from the phenylpropanoid pathway, SA is also produced in a second pathway, the isochorismate pathway. In this pathway, chorismate is converted into isochorismate with the help of an isochorismate synthase homolog. Isochorismate is then bound to glutamate by avrPphB SUSCEPTIBLE 3, forming ischorismate-9-glutamate. Subsequently, SA is formed out of isochorismate-9-glutamate. This can happen spontaneously, or it can be catalyzed by ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1.

It is also suggested isochorismate could be converted directly into SA, but this biosynthesis route is not known yet. In conclusion, SA can be formed either by the isochorismate pathway, starting from chorismate and independent from cinnamic acid, or by the phenylpropanoid pathway. In *Arabidopsis,* the isochorismate pathway is believed to be the main pathway for the production of SA production, while in rice, the phenylpropanoid pathway might be the main pathway for SA production (Lefevere *et al.*, 2020).

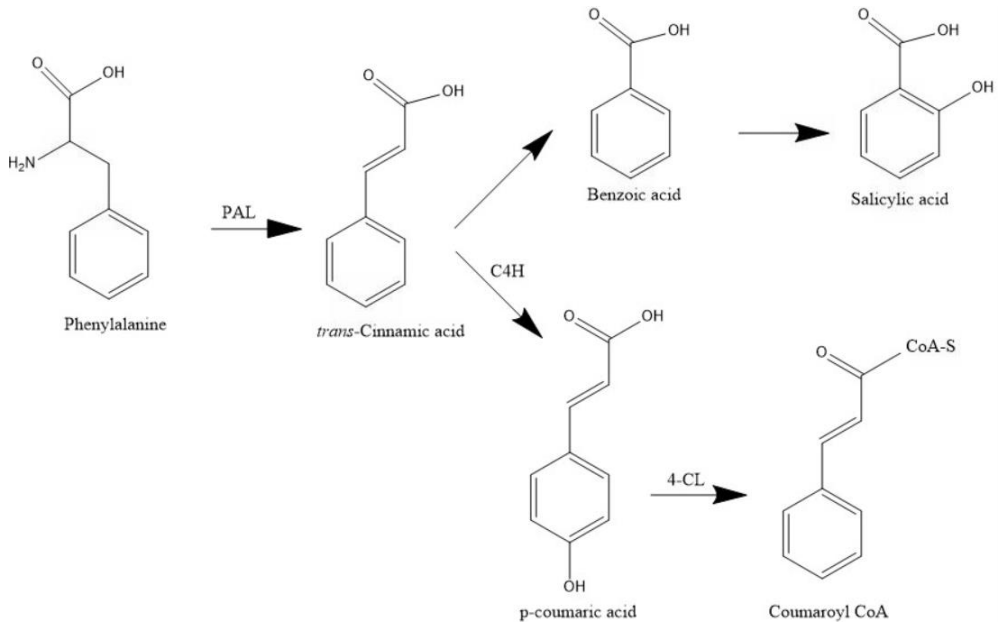

Figure 3: The upstream part of the phenylpropanoid pathway consists out of three steps. First of all, phenylalanine is converted into cinnamic acid by phenylalanine ammonia lyase, after which C4H transforms cinnamic acid into *p*-coumaric acid. Finally, coumaroyl CoA is formed out of p-coumaric acid. Coumaroyl CoA is a precursor for numerous downstream products. The enzymes are PHENYLALANINE AMMONIA-LYASE (PAL), CINNAMIC 4-HYDROXYLASE (C4H) and 4-COUMARATE:COENZYME A LIGASE (4-CL) (Source: adapted from Schalk *et al.*, 1998)

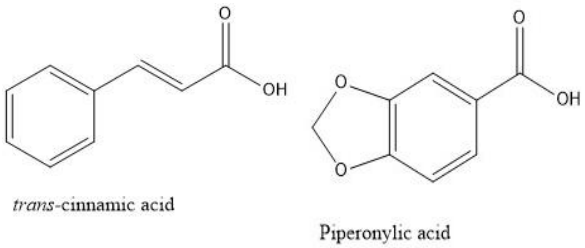

Figure 4: Chemical structure of trans-cinnamic acid (left) and piperonylic acid (right). Both trans-cinnamic acid and piperonylic acid can bind the enzyme CINNAMATE-4-HYDROXYLASE (Source: adapted from Schalk et al., 1998).

1.6. CLPs

1.6.1 Definition and structure

A fatty acid residue and a cyclized oligopeptide, in the form of a lactone ring, forms a cyclic lipopeptide (CLP; Caulier *et al.*, 2019; Ma *et al.*, 2017; Olorunleke *et al.*, 2017; Raaijmakers *et al.*, 2006). Different bacterial genera, including *Bacillus, Pseudomonas* and *Streptomyces* spp. (Geudens & Martins, 2018) produce these CLPs by non-ribosomal peptide synthetases (NRPS; Girard *et al.*, 2020; Oni *et al.*, 2020). Because of their structure, CLPs are amphipathic (Caulier *et al.*, 2019), enabling them to have numerous functions, such as in motility and in the production of biofilms. Some of the CLPs also have an antimicrobial activity (Olorunleke *et al.*, 2017). These natural functions have been noticed by several researchers and have already led to extensive research and ultimately to the development of antibiotic drugs (Baltz *et al.*, 2005). Especially CLPs from the genera *Pseudomonas* and *Bacillus* are already extensively investigated, because of three reasons. First of all, in these genera species with various lifestyle occur. Second, species in these genera live in a wide variety of natural habitats. Lastly, both pathogenic and beneficial organisms are found within these genera (Raaijmakers *et al.*, 2010). Bacterial strains which are natural enemies of one or more plant pests, pathogens or weeds could be used to control their adverse effects and are therefore in this thesis called *biocontrol strains* (Berendsen *et al.*, 2012).

As mentioned before, the production of the CLPs occurs by NRPS. These NRPS are encoded in the genome by large biosynthetic gene clusters. Each gene cluster consists out of different modules, each module incorporates a different AA in the oligonucleotide part of the CLP. One module is composed of three different domains, a C/E domain, an A domain and a T-domain. The A domain has an adenylation activity. This domain recognizes an amino acid, followed by an adenylation of the amino acid. Subsequently, a T-domain with a thiolation activity binds the adenylated AA to the phosphopanteine carrier of the T domain. Lastly, the C-domain (condensation) is responsible for a condensation of the AA, catalyzing the formation of the bound between the new AA and the growing oligopeptide. Besides the condensation activity, this domain can also catalyze the epimerization of the AA, converting the AA from a L configuration to a D configuration. In this specific case, the domain is called a C/E domain, because it is responsible for both a condensation and an adenylation. At the end of the different modules, one or two thioesterase domains are built in. These domains ensure the release and cyclization of the oligopeptide. The synthesis of the CLPs occurs as follows: the fatty acid chain is attached to the first AA by a condensation domain which also shows an N-acylation activity. Subsequently, AAs are added to the growing CLP by either a C domain or a C/E domain. The thioesterase domain(s) are responsible for the final release and cyclization of the CLP(Girard *et al.*, 2020; Oni *et al.*, 2020).

The structure of CLPs is extremely variable due to the diversity of both the fatty acid and oligopeptide components. The fatty acid can vary in its length and substitution pattern, whereas the oligopeptide part has a variable AA composition, chain length and configuration (Raaijmakers *et al.*, 2010). Several classification systems for CLPs have been proposed,

including systems based on the bacterial species that produce the CLPs and systems based on their chemical structure. Based on their structure, different classes are distinguished for both the genera *Bacillus* and *Pseudomonas*. Iturin, fengycin and surfactin are the three main families of CLPs produced by *Bacillus* (Jacques, 2011). Beside these three main classes, other CLPs produced by *Bacillus* have been found, such as kurstakins and polymyxins (Raaijmakers *et al.*, 2010). Also CLPs produced by the genera *Pseudomonas* are further classified into groups. Within one group, the oligopeptide part of all CLPs should have the same length, and both the structure and size of the macrolactone ring should be the same. Furthermore, each group has to contain at least two different CLPs and minimum two distinct strains should produce a CLP of the corresponding group. When all prerequisites for a group of CLPs are met, except the production by distinct strains, the term subgroup instead of group is used. In total, eleven groups and two subgroups are distinguished. These include six groups of short CLPs, having an oligopeptide part between eight and fourteen AAs: xantholysin, viscosin, syringomycin, orfamide, amphisin and pseudofactin. Further, five groups of longer CLPs, with an oligopeptide part between eighteen and 25 AAs, have been characterized: tolaasin, fuscopeptin, corpeptin, syringopeptin 22 (SP22) and syringopeptin 25 (SP25). Last, two subgroups, the putisolvins and the ferrocins and two CLPs which are not-classifiable, entolysin and cocoyamide/gacayamide, are known (Götze and Stallforth, 2020). Furthermore, the recently described bananamide is now considered as a new group (Geudens & Martins, 2018). To date, still a lot of research on *Pseudomonas* CLPs is performed, resulting in discovery of new groups of CLPs, new insights about existing groups, the incorporation of existing groups in other groups, etc. This makes exact classification of *Pseudomonas* CLPs difficult.

In order to examine the potential priming activity of *Pseudomonas* CLPs, different CLP producing strains are examined during this thesis. These include *Pseudomonas* sp. CMR12a, *P. putida* RW10S2, *P. tolaasii* CH36, *P. fuscovaginae* UPB0736, *P. cichorii* SF1-54, *Pseudomonas* sp. COR18, *Pseudomonas* sp. COR33 and their respective mutants. The order of the AAs in the CLPs produced by these strains is shown in **Table 1.** The structure of this or related CLPs, if available, is shown in **Figure 5**.

Pseudomonas sp. CMR12a is a biocontrol strain isolated from the roots of red cocoyam in Cameroon. It produces two cyclic lipopeptides, namely orfamide and sessilin. In addition, it produces an antibiotic: phenazine. Three types of sessilin are produced: sessilin A, sessilin B and sessilin C. Sessilin A is a CLP which shows a high degree of similarity to tolaasin: it consists out of a β-hydroxyoctanoyl fatty acid residue and an 18-AA oligopeptide moiety which is partly cyclized. Thirteen AAs can be found in the linear part; five AAs are part of the cyclic part. The only difference with tolaasin is the sixth AA residue: in the case of tolaasin, this is glutamine, while for sessilin, this is serine. The lactone ring is formed between the hydroxyl group of threonine and the C-terminal of lysine. Sessilin B is probably a non-cyclized form of sessilin A, while in sessilin C, it is believed that the Hse residue is substituted by glycine. Besides sessilins, CMR12a produces orfamides: orfamide D, orfamide B and orfamide E. These orfamides resemble orfamides produced by *Pseudomonas protegens* Pf-5. In the case of orfamides, the oligopeptide moiety consists of 10 AA, which is linked to a β-

hydroxydodecanoyl or a β-hydroxytetradecanoyl fatty acid moiety. Different mutants are available for this strain. *Pseudomonas* sp. CMR12a-ΔClp2 is deficient in the production of orfamide, while *Pseudomonas* sp*.* CMR12a-ΔClp2-Clp1 is deficient in both orfamide and sessilin. Furthermore, *Pseudomonas* sp. CMR12a-ΔPhz- ΔClp2 does not produce orfamide nor phenazine. Finally, *Pseudomonas* sp*.* CMR12a-ΔPhz-ΔClp2-Clp1 is deficient in all of the three metabolites (D'aes *et al.*, 2014). Comparative studies performed on these species showed that sessilin has an important role in biofilm formation, while orfamide has a role in swarming activity of the bacteria. Moreover, sessilin has shown an antagonistic effect against *Rhizoctonia solani,* a pathogen causing root rot on bean (D'aes *et al.*, 2014).

In contrast to *Pseudomonas sp.* CMR12a, *P. cichorii* SF1-54 is a phytopathogenic strain producing CLPs. More specifically, it produces cichofactin and cichopeptin. Cichofactin A and B are two CLPs, both consisting of an oligopeptide part of eight AAs, but while cichofactin A has a C10 fatty acid moiety, cichofactin B has a C12 fatty acid moiety. Cichopeptin belongs to the family of the peptins. It has an oligopeptide moiety of 22 AA, of which five are part of the cyclized part. Besides cichofactin and cichopeptin, a third CLP is produced by *P. cichorii* SF1-54. This CLP belongs to the family of the mycins but is not fully characterized yet. However, data suggests that this CLP resembles pseudomycin (Girard *et al.*, 2020).

P. tolaasii CH36 is responsible for the brown blotch disease on mushrooms. *P. tolaasii* CH36 produces different CLPs, being tolaasin I, tolaasin II and some other tolaasins, tolaasins A-E. Moreover, a recent study revealed that *P. tolaasii* produces pseudodesmin (Hermenau *et al*., 2020). Except for tolaasin A, all tolaasins have a C8 β-hydroxy fatty acid moiety, while tolaasin A has a γ-carboxybutanoyl fatty acid moiety (Bassarello *et al.*, 2004). These tolaasins have shown activity against both Gram-positive and Gram-negative bacteria, fungi and oomycetes (Bassarello *et al.*, 2004; Geudens & Martins, 2018). A mutant deficient in the production of tolaasin is *Pseudomonas tolaasii* Tol-A.

P. fuscovaginae is a plant-pathogenic bacteria causing brown sheath rot disease in rice. The wild type strain *P. fuscovaginae* UPB0736 is the most virulent strain within this species. It produces an array of phytotoxic agents, including the CLPs syringotoxin, which is a member of the mycin family, and fuscopeptin A and B, members of the peptin family. Fuscopeptin resembles syringopeptin of *P. syringae (*Girard *et al.*, 2020*;* Patel *et al.,* 2014*)*. Fuscopeptin A consists of nineteen AA oligopeptide moiety, of which five AA are part of the cyclized part, bound with 3-hydroxyoctanoate or 3-hyroxydecanoate fatty acid moiety for respectively fuscopeptin A and B (Ballio *et al.*, 1996; Girard *et al.*, 2020). Syringotoxin resembles the syringomycin from *P. syringae* (Girard *et al.*, 2020). A mutant of *P. fuscovaginae* UPB0736, *Pseudomonas fuscovaginae* 445, is deficient in fuscopeptin A and B and thus only produces syringotoxin. Studies comparing the wildtype and the mutant showed that fuscopeptins play an essential role in the virulence of *P. fuscovaginae* UPB0736, as the mutant significantly reduced the occurrence of brown sheath rot disease (Patel *et al.*, 2014).

Pseudomonas sp. COR18 is a biocontrol strain belonging to the *Pseudomonas asplenii* group. It produces three different lipopeptides which are not fully chemically characterized

yet: Peptin 19:5, CLP13 and a putative thanamycin. However, there are some characteristics which are already known. Peptin 19:5 is a recently discovered member belonging to the peptin group. This CLP has nineteen AAs, of which five are bound in a lactone ring. This strain is also predicted to produce thanamycin, belonging to the mycin group. For the cyclic lipopeptide CLP13, the number of AAs is determined as thirteen. CLP13 constitutes a novel family of CLPs produced by the *P. asplenii* group like the strains *Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33, the latter one only produces CLP13 and does not produce any peptin or mycin (unpublished).

P. putida RW10S2, a strain isolated from the rhizosphere of rice, produces one CLP, which belongs to the viscosin group: white line inducing principle (WLIP; Rokni-Zadeh et al., 2012). WLIP possesses eleven AA, seven of them are bound in a lactone ring, and a β-hydroxydecanoate fatty acid moiety (Mortishire-Smith *et al.,* 1991*).* WLIP resembles pseudodesmin A, produced by *P. tolaasii.* Only the second amino acid residue is different: for pseudodesmin A, this is glutamine, while for WLIP, this is glutamate (De Vleeschouwer *et al.*, 2020*)*. *P. putida CMPG2120 is a* mutant of *P. putida RW10S2* which is deficient in the production of WLIP (Rokni-Zadeh *et al.,* 2012*).*

Table 1: The order of amino acids in lipopeptides produced by *Pseudomonas* sp. CMR12a (D'aes et al., 2014) *P. cichorii* SF54-1, *P. fuscovaginae* UPB 0736 (Girard et al., 2020), *P. tolaasii* CH36 (Bassarello et al., 2004; Hermenau *et al.*, 2020; De Vleeschouwer *et al.,* 2020) and *P. putida* RW10S2 (Mortishire-Smith et al., 1991).The shaded AAs are the outer parts of the cyclic part. When the stereospecificity of the AA is determined, this is indicated in the table. For the fatty acid moiety, OH or diOH indicate 3-substitutions. For the oligopeptide part, Dab is an abbreviation of 2,4-diaminobutyric acid, Dhb for 2,3-dihydro aminobutyric acid, Dha for dehydroalanine and Dhp for dehydro-2-aminopropanoic acid. Xle stands for Leucine or Isoleucine; αThr for allothreonine, Cl-Thr for 4-chlorothreonine. Hse means homoserine, Asp* is 3-hydroxy-aspartic-acid. Other AAs are indicated by their universal three-letter notation (Girard et al., 2020).

	Fatty acid	1	2	3	4	5	6	7	8	9	10	12	13	14	15	16	17	18	19	20	21	22	23
Orfamide B **Orfamide D** **Orfamide E**	C14:0 -OH(3) C12:0 -OH(3)- C14:1 -OH(3)-	L-Leu	D-Glu	D-αThr	D-Val	L-Leu	D-Ser	L-Leu	L-Leu	D-Ser	L-Val												
Sessilin A	C8:0 -OH(3)-	Dhb	D-Pro	D-Ser	D-Leu	D-Val	D-Gln	D-Leu	D-Val	L-Val	D-Gln	L-Leu	D-Val	xDhb	D-αThr	L-Ile	L-Hse	D-Dab	L-Lys				
Tolaasin I	C8:0 -OH(3)-	Dhb	D-Pro	D-Ser	D-Leu	D-Val	D-Ser	D-Leu	D-Val	L-Val	D-Gln	L-Leu	D-Val	x-Dhb	D-αThr	L-Ile	L-Hse	D-Dab	L-Lys				
Pseudodesmin A	C10:0-OH(3)-	L-Leu	D-Gln	D-αThr	D-Val	D-Leu	D-Ser	L-Leu	D-Ser	L-Ile													
Syringotoxin	C14:0-OH(3)	L-Ser	D-Dab	Gly	D-Hse	L-Orn	L-αThr	zDhb	L-Asp*	Cl-L-Thr													
Fuscopeptin	C8:0 -OH(3)- C10:0 –OH(3)	Dhb	D-Pro	L-Leu	D-Ala	D-Ala	D-Ala	D-Ala	D-Val	Gly	D-Ala	D-Val	D-Ala	D-Val	Dhb	D-αThr	L-Ala	L-Dab	D-Dab	L-Phe			
Cichopeptin A **Cichopeptin B**	C10:0-OH(3)- C12:1-OH(3)	Dhb	Pro	Ala	Ala	Ala	Ala	Val	Dhb	Gly	Val	Ile	Gly	Ala	Val	Ala	Val	Dhb	aThr	Ala	Dab	Ser	Xle Val
Cichofactin A **Cichofactin B**	C10:0-OH (3) C12:0-OH (3)	L-His	L-Leu	L-Leu	D-Gln	L-Leu	D-Gln	L-Val	D-Leu	L-Leu													
WLIP	C10:0-OH(3)-	L-Leu	D-Glu	D-αThr	D-Val	D-Leu	D-Ser	L-Leu	D-Ser	L-Ile													

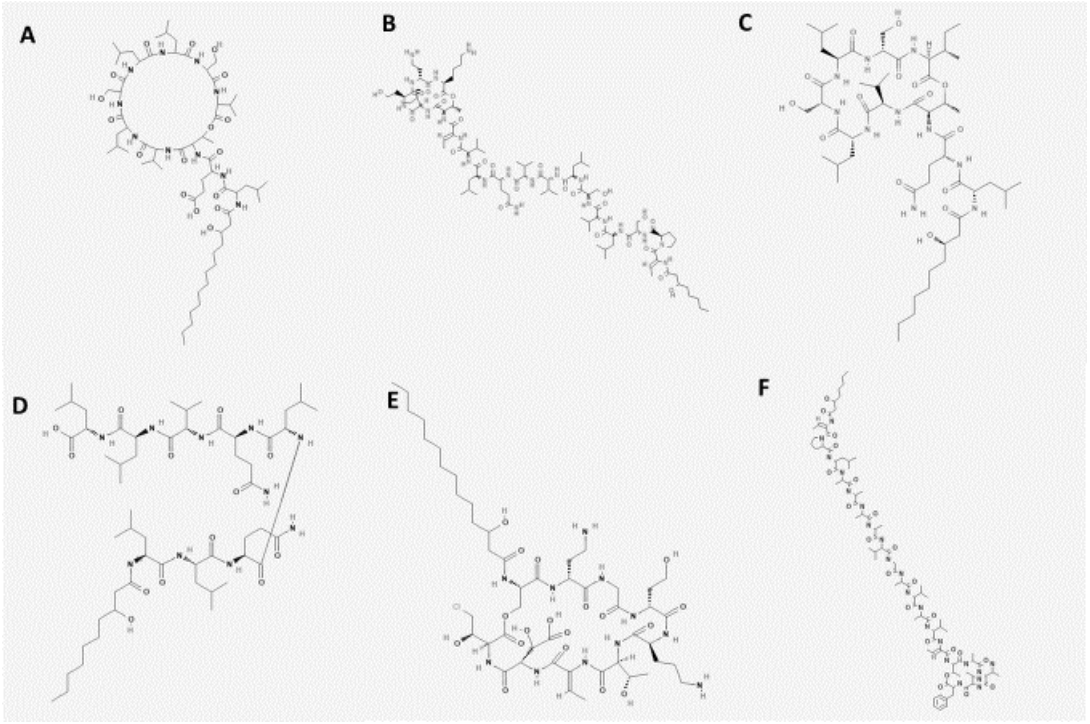

Figure 5: Molecular structure of (A) Orfamide B, (B) Tolaasin I, (C) Pseudodesmin A, (D) Cichofactin A, (E) Syringotoxin and (F) Fuscopeptin (derived from NCBI, 2021b; NCBI, 2021e; NCBI, 2021d; NCBI 2021c; NCBI 2021a; NCBI 2021f).

1.6.2 CLPs and plant protection

Several CLP producing *Pseudomonas* contain plant beneficial strains. Their beneficial effect can have two possible reasons. In the first place, CLPs can have a direct antagonistic effect on the pathogen. Second, it is possible CLPs (or other compounds produced by these genera) cause a state of induced systemic resistance in plants, enabling them to resist infections better (Haas & Défago, 2005). Because of the positive effects of some species in these genera, there have already been investigations on the role of CLPs as an alternative to conventional pesticides in the control of pathogens and pests.

Some investigations have already been done on whether CLPs produced by *Pseudomonas* can induce resistance. For example, massetolide A is a CLP produced by different strains from the genus *Pseudomonas,* with a peptide part of nine AA and a fatty acid moiety of 3-hydroxy decanoid acid (Gerard *et al.*, 1997). A study showed that massetolide A produced by *Pseudomonas fluorescens* SS101 was able to induce IR in tomato plants against the late blight pathogen *Phytophtora infestans*. Furthermore, this study showed that the achievement of this IR was independent of the salicylic acid signaling (Tran *et al.*, 2007). Massetolide A also provides protection against *P. infestans* by a direct antagonism, having a zoosporicidal effect (van de Mortel *et al.*, 2009). However, another study found that massetolide A had no significant effect on the suppression of the oomycete *Pythium* spp. in apple trees, although *Pseudomonas fluorescens* SS101 did suppress infection (Mazzola *et al.,* 2007).

Ma *et al.* (2016) revealed *Pseudomonas* sp. CMR12a is able to protect rice against rice blast by both direct antagonism and IR. In this study, phenazine appeared to have a major role in induction of resistance (Ma *et al.*, 2016). Moreover, a recent study revealed that orfamide produced by *Pseudomonas* sp*.* CMR12a induces plant defense priming in plants against *Cochliobolus miyabeanus,* but not against *Pyricularia oryzae*. Moreover, this study showed orfamide A triggers abscisic acid signaling, as well as increased expression of genes encoding the pathogenesis-related protein PR1b and the transcription factor OsWRKY4. The study suggests that these events might be related to the induction of IR (Ma *et al.*, 2017). Another study executed in the same research group also showed that the orfamides produced by the *Pseudomonas* sp. CMR12a are not able to trigger IR against rice blast. However, this study showed orfamides can suppress *P. oryzae* infection when they are inoculated simultaneously on the leaves of rice, which suggests a direct anti-fungal effect. Moreover, the CLPs xantholysin and N3, did not induce IR in rice against rice blast, although the N3-producing *Pseudomonas* sp*.* COW 3 was found to be a trigger for IR. The *Pseudomonas* sp*.* COR51, which produces the CLP xantholysin, did not significantly decrease disease severity, meaning it does probably not induce IR in rice against *P. oryzae*. Furthermore, the CLPs WLIP, lokisin and endolysin are involved in the induction of IR against rice blast disease in rice plants. This was shown by comparing relative infection of *P. oryzae* in rice plants after priming with WLIP producing strains or WLIP-deficient mutant strains, respectively (Omoboye *et al.*, 2019).

In conclusion, there is growing evidence that CLPs from beneficial microbes play a role in protecting plants through direct toxicity to pests and pathogens, but it is less clear whether they are also able to induce resistance in host plants and to what extent this contributes to the efficacy of such beneficial bacteria.

2. Materials and methods

2.1. Plant material

For assays with *Pyricularia oryzae* VT5M1, rice (*Oryza sativa* L. spp. *indica* 'Co39') was used, while rice (*Oryza sativa* L. subsp. *Japonica* 'Kitaake') was used for infection assays with *Meloidogyne graminicola*. Seeds were first pregerminated by removing the husk, followed by shaking them 25 minutes in a 2% chlorine solution with a droplet of Tween 20. After 5-6 wash steps, the seeds were put on a wet, sterile filter paper in a petri dish and kept in a dark incubator at 25°C for three to five days.

Six to seven seedlings intended for the blast assay were put in a plastic tray with 600 g-700 g non-sterile potting soil (Structural, Type 1) and 250 mL of fertilizer (1 g/L $(NH_4)_2SO_4$ and 2 g/L $FeSO_4$. 7 H_2O). Care was taken to put the root in the soil and the apex above the soil. Rice seedlings intended for *Meloidogyne graminicola* assays were transferred to PVC tubes containing approximately 45.5 g of a 1:1 mixture of sieved potting soil and sand (w/w). Each tube contained one seedling.

Rice plants were kept in a growth chamber with a temperature of 28°C and a light regime of 12 hours day and 12 hours night. The plants were watered twice a week with distilled water and fertilizer (1 g/L $(NH_4)_2SO_4$ and 2 g/L $FeSO_4$. 7 H_2O) was applied once a week.

2.2. Chemical treatments

Two different resistance-inducing chemicals were tested: piperonylic acid (PA; Sigma-Aldrich, catalog nr. P49805) and 1,2,3-benzothiadiazole-7-carbothioate (BTH; Syngenta ActiGard WG50). Both chemicals could be applied as a spray or as a soil drench. BTH was applied as a positive control, as its effectiveness against *P. oryzae* has already been demonstrated (De Vleeschauwer & Höfte, 2009).

A 100 mM stock solution of PA was made in dimethyl sulfoxide (DMSO; Duchefa Biochemie). This solution was diluted with distilled water to a concentration of 300 µM PA, and Tween 20 (Sigma-Aldrich) was added to a concentration of 0.1% (v/v). Approximately 1.5 mL of this solution was sprayed per plant. All other plants were sprayed with a mock solution (1.5 mL distilled water with 0.1% (v/v) DMSO and 0.1% Tween 20). For soil drenching, PA stock solution was diluted with distilled water to 50 µM PA and 13 mL/plant of this solution was applied (resulting in approximately the same total PA dose per plant). The mock soil drench consisted of distilled water with the same concentration of DMSO. PA treatments were executed twice, the first time eight days before infection and the second time one day before infection. BTH was applied once, three days prior to inoculation, as described by, for example, De Vleeschauwer & Höfte (2009).

Three days before infection, treatment with BTH was performed. For soil drench treatment, 80 mL 25 µM BTH was applied per tray. For the treatment with BTH as a spray, 1.5 mL of a spray containing 150 µM active ingredient was sprayed per plant. Because BTH (Syngenta ActiGard WG50) was used as a formulated product, no additional Tween surfactant was added.

2.3. *Pseudomonas* supernatant production

Several CLP-producing *Pseudomonas* strains were selected in order to test the nematocidal activity (see **Table 2**). The *Pseudomonas* strains were grown on King's B medium (King *et al.*, 1954; composition see **Table 3**) in a dark incubation room at 28°C. An individual colony was picked with a pipette tip and put in a glass tube with 5 mL liquid King's B broth. These tubes were put in an incubator at 28°C and 250 rpm for 24 hours. A droplet collapse test was done by pipetting 15 µL on a hydrophobic parafilm surface. Note was taken of the intensity (from complete to none) and the time (from immediate to late) of the droplet collapse. When no difference was seen within media originating from the same strain, the content of this strain tubes was put together in a falcon tube. After centrifugation (15 minutes, 10 000 g), the supernatant was filter-sterilized with a 0.22 µm syringe filter. The resulting cell free supernatant was put in the refrigerator at 4°C until use. Four experiments were performed using this supernatant. All tested strains, their mutants and the CLPs they produce are listed in **Table 2**. Dilutions were made with tap water.

2.4. In vitro nematocidal assay

Each solution was tested by pipetting 1.5 mL in a 6-well plate, followed by adding approximately 50 nematodes, measured by volume. In the second, third and fourth experiment, an additional centrifugation step of the cell free supernatant was performed right before addition of the supernatant to the 6-well plate. Next, the plate was covered with aluminium foil and placed on an orbital shaker. Four, 24 and 48 hours (for the first and second experiment) after adding the nematodes, the number of living and immobilized nematodes was counted under a stereo microscope. For the third and fourth experiment, the nematodes were counted only once, after 24 hours. As a negative control, pure tap water was used, and 5 mL/L of the commercial nematicide Cedroz (Eastman) was used as a positive control.

In the first experiment, the supernatant of the strains *P. tolaasii* CH36, *Pseudomonas* sp. CMR12a, *Pseudomonas putida* RW10S2 and their respective mutants *P. tolaasii* Tol-A, *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2, *Pseudomonas putida* CMPG2120 were tested in a 1:10 and a 1:3 dilution, each treatment was tested in triplicate.

In the second experiment, 1:1 and 1:3-diluted supernatant from the strains *P. cichorii* SF1-54, *P. fuscovaginae* UPB 0736 and its mutant *P. fuscovaginae* 445, *Pseudomonas* sp. COR18, *Pseudomonas* sp. COR33, *Pseudomonas* sp. RHF 3.3-22 and *Pseudomonas* sp. RHF 3.3- 3 were tested, in a 1:1 and a 1:3 dilution in tap water, each dilution was tested in triplicate.

In the third and fourth experiment, 1:3-diluted sterile supernatant originating from *Pseudomonas* sp. CMR12a, *P. tolaasii* CH36, *Pseudomonas* sp. COR18, *Pseudomonas* sp. COR33 and their respective mutants *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2 and *P. tolaasii* Tol-A were tested on their nematocidal activity in six-fold.

2.5. Biological treatments

For this assay, six *Pseudomonas* strains were selected, being *Pseudomonas* sp. CMR12a and its mutant *Pseudomonas* sp. CMR12a- ΔPhz-CLP1-ΔCLP2, *P. tolaasii* CH36 and its mutant *P. tolaasii* Tol-A, *Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33. The *Pseudomonas* strains used for inoculation were grown at 28°C on King's B medium, starting from a stock culture in a -80°C freezer. After 48 hours, the bacteria were suspended in 10 mM $MgSO_4$.

Plants were treated two times with the bacterial suspension. On the day of the transfer, the rootles of the seedlings were dipped in the cell suspension with a cell concentration of approximately 10^7 cells/mL and the bacterial suspension was mixed with the soil to a final concentration of 10^7 cells per gram soil. The second treatment was performed three days before nematode inoculation, 10 mL bacterial suspensions was performed as a soil drench treatment with a final concentration of 10^7 cells/g soil. The positive control was additionally treated with PA as a spray treatment as described earlier. Each treatment was performed in seven-fold for infection with *Meloidogyne graminicola,* three additionally treatments were implemented for assessment of root colonization.

2.6. Extraction of *Meloidogyne graminicola*

Nematodes of the species *Meloidogyne graminicola* (originally isolated from the Philippines and kindly provided by Prof. Dirk De Waele of the Catholic University Leuven) were cultured in *Echinochloa crus-galli* (barnyard grass). Nematodes were extracted using a modified Baerman funnel (Luc *et al.,* 2005). Roots were washed, cut into small pieces and put in a coarse sieve covered with tissue paper. The sieve was kept in a tray, which was filled with tap water without submerging the plant material completely. The tray was covered with aluminium foil. After 48 hours, nematodes were assembled by collection of the residue of a filtration with a filter with a mesh of 20 µm. The number of nematodes per volume unit was counted using a stereo microscope. If the number of nematodes appeared to be too low, the suspension was concentrated by centrifugation for ten minutes at 3 000 rpm, after which the supernatant was discarded.

Table 2: List of the tested CLPs during nematocidal assays, their producing bacteria and their mutants.

Strain	Produced CLPs	Origin	Reference
Pseudomonas sp. CMR12a	Orfamide and sessilin. Additional production of phenazine, an antibiotic	Isolated from the red root cocoyam rhizosphere	(D'aes *et al.,* 2012)
Pseudomonas CMR12a ΔPhz-CLP1-ΔCLP2	None	Triple mutant of *Pseudomonas* sp. CMR12a	(D'aes *et al.,* 2012)
P. putida RW10S2	WLIP	Isolated from the rice rhizosphere from Sri Lanka	(Rokni-Zadeh *et al.*, 2012)
P. putida CMPG2120	None	*wlpC* (NRPS3) mutant of *P. putida* RW10S2 No production of WLIP	(Rokni-Zadeh *et al.*, 2012)
P. fuscovaginae UPB0736	fuscopeptin, CLP13, syringotoxin	Isolated from rice in Madagascar	(Patel *et al.*, 2012)
P. fuscovaginae 445	syringomycin, CLP13	445:pKNOCK mutant of *P. fuscovaginae* UPB0736	(Patel *et al.*, 2014)
Pseudomonas sp. RHF 3.3-3	Peptin 19:5, CLP13 and a putative thanamycin	Isolated from rice in Vietnam	(unpublished)
Pseudomonas sp. RHF 3.1-22	None, produces the antibiotic diacetyl phloroglucinol	Isolated from rice in Vietnam	(unpublished)
Pseudomonas sp. COR18	Peptin 19:5, CLP13, putative thanamycin	Isolated from red cocoyam roots in Cameroon	(Oni *et al.*, 2019; unpublished)
Pseudomonas sp. COR33	CLP13	Isolated from red cocoyam roots in Cameroon	(Oni *et al.*, 2019; unpublished)
P. tolaasii CH36	Tolaasin and pseudodesmin	Causes brown blotch disease on mushrooms	(Rokni-Zadeh *et al.,* 2011; unpublished)
P. tolaasii Tol-A	Pseudodesmin	Tolaasin mutant of *P. tolaasii* CH36	(unpublished)
P. cichorii SF1-54	Cichopeptin A and B	Isolated from green lettuce in Belgium	(Huang *et al.*, 2015 ; Cottyn *et al.*, 2011)

Table 3: Composition of different media

Medium	Composition per L		Reference
King's B (KB) Medium (1 L)	Proteose pepton nr. 3 (Difco)	20 g	(King *et al.*, 1954)
	K_2HPO_4	1.5 g	
	$MgSO_4$	1.5 g	
	Agar	15 g	
	Glycerol	10 mL	
	Distilled water	1000 mL	
Complete medium (CM) (1 L)	$NaNO_3$	6 g	(Talbot *et al.*, 1993)
	KCl	0.52 g	
	$MgSO_4 . 7 H_2O$	0.52 g	
	KH_2PO_4	1.52 g	
	Glucose	10 g	
	Pepton	2 g	
	Yeast extract	1 g	
	Casamino acids	1 g	
	Agar	15 g	
	Trace elements*	1 mL	
	Vitamin solution*	1 mL	
	pH 6.5 with NaOH		
*Trace elements	$ZnSO_4 . 7 H_2O$	22 g	
	H_3BO_3	11 g	
	$MnCl_2 . 4 H_2O$	5 g	
	$FeSO_4 . 7 H_2O$	5 g	
	$CoCl_2 . 6 H_2O$	1.7 g	
	$CuSO_4 . 2 H_2O$	1.6 g	
	$Na_2MoO_4 . 2 H_2O$	1.5 g	
	Na_4EDTA	50 g	
	pH 6.5 with KOH		
*Vitamin solution	Biotin	0.1 g	
	Pyridoxin	0.1 g	
	Thiamine	0.1 g	
	Riboflavin	0.1 g	
	p-aminobenzoic acid	0.1 g	
	Nicotinic acid	0.1 g	

2.7. *Meloidogyne graminicola* infection assay

Two weeks after the transfer of the plants to the PVC tubes, plants were inoculated with approximately 250 nematodes. Fourteen days after inoculation, roots were harvested by taking them carefully out of the PVC tubes, roots were washed with tap water and cooked for one minute in 1:8-diluted Alcoferm raspberry red dye. The roots were destained by shaking them

in acid glycerol (1 mL 37% HCl per L glycerol) for at least two weeks. The number of galls were counted, the roots were washed, dried and weighed and the number of galls was normalized to dry root weight.

2.8. Assessment root colonization

Root colonization by *Pseudomonas* strains was assessed. Roots were harvested by carefully removing them from the PVC tubes, washed with tap water, dried and weighed. They were ground with sterile sand and 10 mL of 10 mM $MgSO_4$ with pestle and mortar. A dilution series was made, the 10^{-3}, 10^{-4} and 10^{-5} dilutions were plated on King's B medium by pipetting 100 µL of the respective dilution on a petri dish, followed by the addition of glass beads and extensive shaking during one minute. The beads were removed, the plates dried and transferred to an incubator at 28°C. Approximately 24 hours later, the number of colonies with a similar morphology compared to a reference were counted on plates containing 15-300 colonies. The number of colony forming units was calculated using following formula:

$$\text{N° CFU/g root} = \frac{V \,.10^{|D|}.CFU}{g \,.P}$$

With N° CFU/g root the number of colony forming units per gram of roots, V the volume of 10 mM $MgSO_4$ used for crushing, $10^{|D|}$ the dilution of the plate, CFU the number of colonies counted on the plate, g the root weight in gram and P the volume of the suspension applied on the plate.

2.9. Rice bioassay with *Pyricularia oryzae*

For the rice blast infection, *Pyricularia oryzae* VT5M1 was used (Thuan *et al.*, 2006). Infection was performed as described by De Vleeschauwer *et al.* (2006). The fungus was grown for two days at 28°C on complete medium (Talbot *et al.*, 1993; composition see **Table 3**) in the dark, after which they were kept under UV light with a light regime of twelve hours light and twelve hours dark for eight days. Spores were suspended in sterile distilled water and mycelia were removed with a 250 µm filter. The spore suspension was adjusted to a final concentration of $5x10^4$ spores mL^{-1} and 0.5% gelatine (Sigma-Aldrich). Approximately 1.5 mL of spore suspension was sprayed on the plants with an airbrush-gun (Badger Airbrush model 150). The infected plants were then transferred to a dark infection room at 26°C and a relative humidity of approximately 100%. After twenty hours, the plants were transferred to the growth room with a temperature of 28°C and a light regime of twelve hours light and twelve hours dark. For evaluation, lesions were scored based on a 0-6 disease index. (see **Figure 6**). Sporulating lesions, with a score greater than 3, were counted on the second youngest developed leaf.

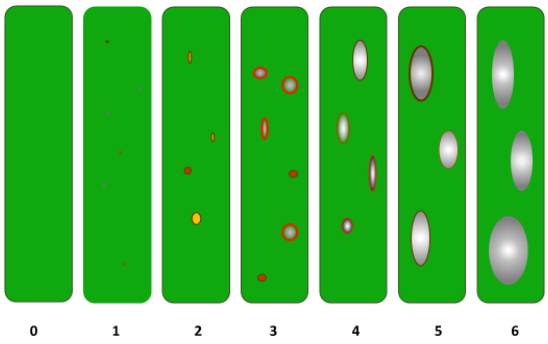

Figure 6: Lesions caused by infection with *Pyricularia oryzae* VT5M1 are scored on a scale from one to six.

This experiment was executed three times. In the first experiment, three treatments were included: PA applied as a spray, BTH as a soil drench, and a non-treated control. BTH was applied as a positive control, as its effectiveness against *P. oryzae* had already been demonstrated (De Vleeschauwer & Höfte, 2009). Each treatment consisted of 6 trays with 6-7 plants. The infection was scored four, five and six days post inoculation (dpi).

In the second experiment, five treatments were assessed four and five dpi: PA and BTH, each applied as both a spray and a soil drench, and a non-treated control. Five trays, each containing 6-7 plants, were used per treatment (except for PA spray, for which six trays were available). In the experiment, the most severely infected leaf from each plant was harvested and frozen after the second scoring at five dpi. Four different assays were executed on each sample: the Folin-Ciocalteu assay for total phenolic content, enzyme assays for PEROXIDASE and PAL activity and the acetyl bromide assay for lignin content.

In the third experiment, only two treatments were performed: PA applied as a spray and a non-treated control. Each treatment consisted of seven trays with six plants, the plants got inoculated as described before. Four trays were used for harvesting, the other three trays were used for scoring the infection. Besides, each treatment contained four trays of six plants which were sprayed with approximately 1.5 mL of a mocking spore solution (0.5% gelatine). Twenty-four hours and three days post infection, the youngest developed and second youngest developed leaves were cut off and frozen in liquid nitrogen. Two leaves were pooled in one sample and used for analysis of gene expression. Four and five dpi, the infection was assessed as described earlier, by counting the number of lesions with a score greater than three.

2.10. Biochemical assays for phenylpropanoid pathway

2.10.1. Analysis of free and cell-wall bound phenolic compounds

Analysis and quantification of free and cell-wall bound phenolic compounds was executed through the Folin-Ciocalteu assay, as described by Ainsworth & Gillespie (2007). After grinding, approximately 70 mg of the sample was weighed and 20 µL/mg cold methanol was added and mixed vigorously through vortexing, after with they were shaken at room

temperature in the dark for thirty minutes. Next, the samples were centrifuged for four minutes at 12 000 rpm and 125 µL of the supernatant was transferred to a new Eppendorf tube, to which 675 µL distilled water, 37.5 µL Folin-Ciocalteu reagent (Sigma-Aldrich) and 375 µL 20 % (v/v) Na_2CO_3 was added. The sample mixture was incubated for thirty minutes in the dark at room temperature, after which it was centrifuged for one minute at 12 000 rpm. Eventually, the absorbance of the sample at a wavelength of 760 nm was measured with a UV-1600 PC spectrophotometer. If the absorbance exceeded a value of 1, the sample was diluted with distilled water. The absorbance was translated to a free phenolics concentration by using a standard curve of gallic acid in methanol in a concentration range of 0 to 1 mg/mL. The concentration of phenolic compounds is therefore expressed in gallic acid equivalents. After removal of the residual supernatant, the remaining pellet was used to measure the cell-wall bound phenolics. Any residual supernatant was removed and the pellet was washed with methanol. Subsequently, 20 µL 1 M NaOH was added per mg sample, this mixture was incubated overnight on a shaker at room temperature. After this, the samples were centrifuged for ten minutes at 12 000 rpm. While the pellet was discarded, 125 µL of the supernatant was mixed with 1 mL distilled water and 37.5 µL Folin-Ciocalteu reagent. Next, the sample was incubated at room temperature for thirty minutes. Finally, the absorbance at a wavelength of 760 nm was measured, which was converted to a gallic acid equivalent.

2.10.2. Analysis of lignin

For analysis of the lignin content of the leaves, the acetyl bromide method was used. The cell wall material was prepared as described by Van Acker *et al*. (2013). Briefly, approximately 100 mg crushed sample was submitted to a sequential extraction process. First, 1 mL water was added, followed by thirty minutes incubation at 98°C and a centrifugation (3 minutes, 14 000 rpm). The supernatant was discarded. Second, 1 mL ethanol was added, followed by thirty minutes incubation at 75°C and centrifugation (3 minutes, 14 000 rpm), the supernatant was discarded and 1 mL chloroform was added to the pellet. This mixture was incubated half an hour at 59°C, after which it was centrifuged for five minutes at 14 000 rpm. The resulting pellet was extracted a last time by addition of 1 mL acetone, followed by incubation for thirty minutes at 54°C and centrifugation (5 minutes, 14 000 rpm). The supernatant was discarded and the pellet was dried overnight in the fume hood, resulting in dry samples. Samples were weighed and suspended in 200 µL acetyl bromide solution (25% acetyl bromide (Sigma-Aldrich) in fresh glacial acetic acid). The samples were then incubated for two hours at 50°C, after which they were put on ice and 1 mL acetic acid was added. The samples where then centrifuged (ten minutes, 14 000 rpm) and 300 µL supernatant was mixed with 300 µL 2.0 M NaOH and 300 µL 0.5 M hydroxylamine (Sigma-Aldrich). After mixing and incubation for ten minutes at room temperature, the absorbance of the samples at a wavelength of 280 nm was measured using a plate reader (Tecan Infinite F200). Finally, the lignin content was calculated according to Barnes & Anderson (2017) with following formula:

$$\text{Lignin content (\% of dry residue)} = \frac{A_{280}}{\varepsilon . L} . \frac{D}{m} . 100\%$$

With A_{280} the absorbance of the sample at a wavelength of 280 nm, corrected with the blank, ε the extinction coefficient of lignin, L the path length, D the dilution factor, and m the mass of the dried sample. An extinction coefficient of 17.75 was used, being the extinction coefficient for maize as reported by Fukushima & Hatfield (2004). For the dilution factor, the ratio of the total sample volume after all dilutions to the volume of acetyl bromide was used. In total, five biological repeats, each consisting of 6 to 7 pooled leaves, were analyzed once for each treatment, except for the treatment PA spray, where 6 biological repeats were analyzed.

2.10.3. Analysis of PAL-activity

PAL was extracted from approximately 70 mg of the grinded sample by adding 10 µL mg^{-1} sample extraction buffer (20 g/L insoluble polyvinylpyrrolidone, 2 mM Ethylenediaminetetraacetic acid (EDTA) and 14 mM β-mercaptoethanol in 100 mM Tris-HCl buffer at pH 8.8), followed by vortexing and centrifugation at 13 000 g during 10 minutes. After centrifugation, 150 µL of the extract was added to 1.2 mL 100 mM Tris-HCl at pH 8.8. Subsequently, 150 µL 60 mM phenylalanine 200 mM Tris-HCl was added to the sample. A blank of each sample was made by adding 150 µL of the extract to 1.2 mL 100 mM Tris-HCl, followed by 150 µL 100 mM Tris-HCl. Next, 500 µL of both the blank and the sample was taken and 50 µL 5 M HCl was added. The remaining volume of each sample was then incubated during 45 minutes at 37°C, after which the reaction was stopped by addition of 100 µL 5 M HCl. The absorbance at a wavelength of 290 nm was measured for each sample and its blank, both before and after incubation. Finally, the difference between the absorbance at a wavelength of 290 nm before incubation compared with the absorbance at a wavelength of 290 nm after incubation was made and taken as a measure for the PAL activity.

2.11. Analysis of peroxidase activity

Peroxidase activity was analyzed using the guaiacol peroxidase assay (MacAdam *et al.*, 1992). Per milligram sample, 8 µL mg^{-1} cold extraction buffer (0.8 M potassium chloride, 80 mg/mL insoluble polyvinylpyrrolidone, 50 mM potassium phosphate buffer pH 6.0) was added to a sample of approximately 50 mg. This mixture was mixed vigorously and centrifuged at 14 000 rpm during ten minutes at 4°C. Thirty µL of the resulting supernatant was transferred to 3 mL assay buffer (0.4 mM H_2O_2, 3.3 mM guaiacol, 100 mM potassium phosphate). At the moment of addition, the absorbance was set on zero. Subsequently, the absorbance increase of the sample at a wavelength of 436 nm was measured each 15 seconds during three minutes. Finally, the absorbance of the sample was plotted as a function of the time and the slope of this curve was used as a measure for the peroxidase activity. Except for the treatment PA spray, having six biological repeats, five biological repeats were measured for each treatment, each consisting of six to seven pooled leaves. The experiment itself was performed once.

2.12. Analysis of gene expression

Leaves were first ground to a fine powder using a TissueLyser (Qiagen). Then, RNA was extracted using Qiagen Quick-start RNeasy® Plant Mini Kit (cat. nos. 74903). Quantity and purity of RNA was evaluated using Nanodrop. RNA-extract was further subjected to DNase I

treatment, using 2 µg RNA (Thermo scientific). Eventually, RNA was converted to cDNA with Tetro cDNA Synthesis Kit (Bioline). Quality of cDNA was evaluated by a normal RT-PCR using expressed protein (EXP) as reference gene. Following program was executed: 5 minutes at 95°C, 40 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 30 seconds at 72°C and a final elongation step at 72°C for five minutes. The resulting PCR mixture was put on a 2.5% (w/v) agarose gel (0.5 x TAE). Gels were stained in an ethidium bromide bath and imaged under trans UV light (Biorad).

RT-qPCR conditions were used as described by De Kesel *et al.* (2020). Gene expression of six genes was evaluated: *OsPR1a, OsWRKY45, OsJAMYB, OsCPS2*, *OsCPS4* and *OsNOMT*. In addition, two reference genes were used, *OsEXP* and *OsEXPNarsai*. Primer sequences are listed in **Table 4.** Four biological replicates per treatment were analyzed, each time in two technical replicates. For analysis of gene expression, a permutation test already implemented in Rest 2009 was used (Pfaffl *et al*, 2002).

Table 4: Primer sequences used in RT-qPCR analysis.

Gene	Forward primer	Reverse primer	Reference
OsNOMT	AAGGTGTTCATGGAGAACTGGTA	CTGGTTGAAGAGCGTGTTGGA	(De Kesel *et al.*, 2020)
OsCPS2	CATGACAGAGAGGCTCATCA	TGAGCTCATCAAGTGCGT	(Li *et al.*, 2012)
OsCPS4	CGGAACGTCTTGGATGGGCTC	GCTCTTCAAGATTGCTGGTCG	(Li *et al.*, 2012)
OsPR1a	ACCTCGGCGTCTTCATCAC	GTCCATACATGCATAAACACGTAGC	(Nahar *et al.*, 2011)
OsWRKY45	AATTCGGTGGTCGTCAAGAA	AAGTAGGCCTTTGGGTGCTT	(Nahar *et al.*, 2012)
OsJAMYB	GAGGACCAGAGTGCAAAAGC	CATGGCATCCTTGAACCTCT	(Nahar *et al.*, 2012)
OsEXP	TGTGAGCAGCTTCTCGTTTG	TGTTGTTGCCTGTGAGATCG	(De Kesel *et al.*, 2020)
OsEXPNarsai	AGGAACATGGAGAAGAACAAGG	CAGAGGTGGTGCAGATGAAA	(De Kesel *et al.*, 2020)

2.13. Data analysis

Data was statistically analyzed in R (v. 3.6.3). Unless otherwise mentioned, all data was analyzed using a non-parametric Kruskal-Wallis test, followed by a *post-hoc* analysis by a non-parametric Conover's test using Bonferroni-Holm correction for multiplicity. A 5% significance level was used ($P \leq 0.05$). In case of experiments where multiple time points were involved, each time point was assessed separately.

3. Results

3.1. Nematocidal assay

In light of the known ability of various *Pseudomonas* biocontrol strains to produce fungicidal and bactericidal compounds (Haas & Défago, 2005), an investigation on whether these organisms also produced compounds with activity against the plant parasitic nematode *Meloidogyne graminicola* was performed.

Supernatants from several *Pseudomonas* strains were investigated. The *Pseudomonas* strains in the first experiments were *P. putida* RW10S2, which produces WLIP, *P. tolaasii* CH36, which produces tolaasin, and *Pseudomonas* sp. CMR12a, which produces orfamide, sessilin and phenazine. Their respective mutants are *P. putida* CMPG2120, lacking WLIP production, *P. tolaasii* Tol-A, lacking the production of tolaasin, and *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2, lacking production of sessilin, orfamide and phenazine. The supernatant of the fermented medium was tested at a 1:3 and 1:10 dilution. After 4, 24 and 48 hours, the number of immobilized and living nematodes were counted. The percentages of the immobilized nematodes relative to the sum of the living and the immobilized nematodes for each treatment after four, 24 and 48 hours is shown in **Figure 7**.

As can be seen in **Figure 7**, the positive control Cedroz (a terpene-based commercial nematicide marketed by Eastman) had a higher immobilization rate than the negative control at all time points. However, the effect was not yet significant after four hours ($P = 0.067$), whereas it was significant after 24 and 48 hours ($P = 0.0340$, $P < 0.0001$, respectively). This shows that the experimental setup worked appropriately. The three-fold dilutions of both CH36 and Tol-A differed from the negative control after four hours, suggesting a nematostatic compound might be excreted in the medium by both *P. tolaasii* CH36 and its mutant *P. tolaasi* Tol-A. However, the difference was not significant ($P = 0.247$ and $P = 0.795$ for *P. tolaasi* CH36 and *P. tolaasi* Tol-A, respectively). Moreover, as the percentage of immobilized nematodes was comparable between these two bacterial strains, tolaasin probably does not contribute to the nematostatic activity. Interestingly, 24 hours after inoculation, the number of immobilized nematodes for both treatments decreased in comparison with the former counting. This may indicate the active compounds paralyzed the nematodes only temporarily, after which the compound was metabolized by the nematode or degraded by micro-organisms. Another possibility is that due to increasing turbidity, the dead nematodes were not seen accurately. A t-test performed on the total number of nematodes counted in each well showed a significantly lower number of counted nematodes after 24 hours of exposure, compared with four hours after incubation ($P = 0.0179$). After 48 hours, the CH36 supernatant had become too turbid to count, while Tol-A supernatant showed a comparable immobilization rate as the control.

The three-fold dilution originating from the sterile supernatant of *Pseudomonas* sp. CMR12a caused a higher immobilization rate compared with the control, although only significant after 48 hours of incubation ($P = 0.287$, $P = 0.124$ and $P = 0.0005$, after four, 24 and 48 hours respectively).

Although the supernatant of *Pseudomonas* sp. CMR12a caused a higher immobilization rate compared with the three-fold dilution of its mutant *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2 at all time points, the difference was not significant (P = 1.000 for each time point). The three-fold diluted sterile supernatant of both *P. putida* RW10S2 and its mutant *P. putida* CMPG2120 seemed to lead to higher immobilization rates compared to the control. After 48 hours both the wildtype *P. putida* RW10S2 and the mutant *P. putida* CMPG2120 differed significantly from the control (P = 0.0291 and P = 0.0194, respectively). However, the two treatments did not differ significantly from each other after 48 hours (P = 1.000). Last, **Figure 7** shows ten times dilutions caused comparable immobilization rates compared with the control, probably because the supernatant was too diluted. Therefore, the next experiments with sterile supernatant were executed without the ten times diluted supernatant.

Also interesting to remark is that, although the Kruskal-Wallis test revealed a significant difference in immobilization rate between different treatments after four hours (P = 0.0369), this was not reflected in the *post-hoc* test.

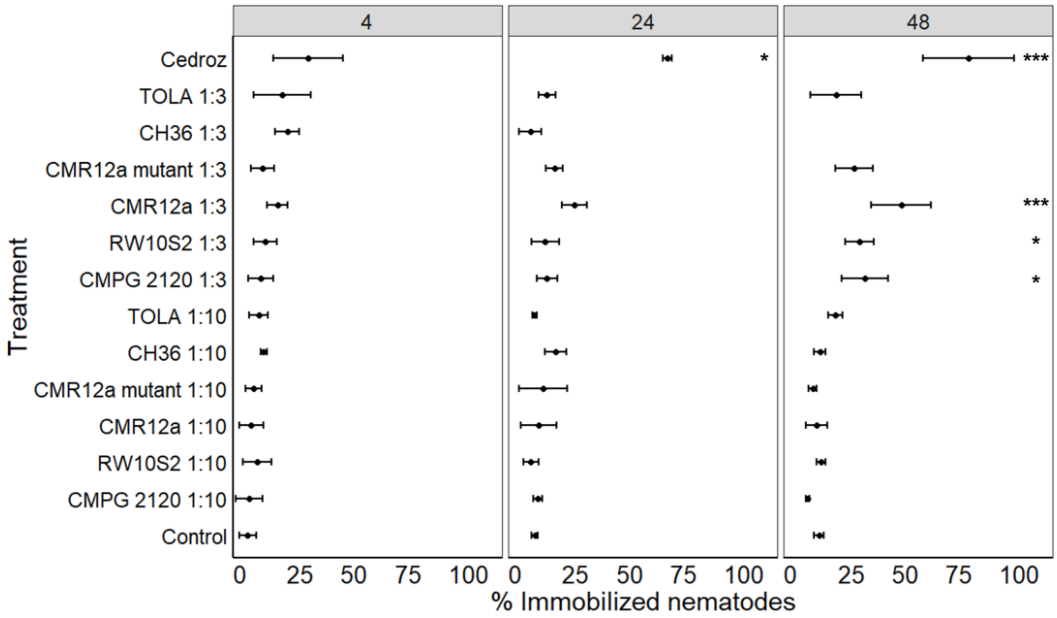

Figure 7: The percentage of immobilized nematodes after four (4), 24 hours (24) and 48 hours (48) incubation in the medium. For each treatment, the dilution is indicated as 1:3 for a three times dilution and 1:10 for a ten times dilution. Control is the negative control, Cedroz the positive control, CH36 and TOLA stands for the supernatant from *P. tolaasii* CH36 (producing the CLP tolaasin and pseudodesmin) and its mutant *P. tolaasii* Tol-A (deficient in tolaasin production), respectively. RW10S2 and CMPG 2120 stands for respectively *P. putida* RW10S2, producing the CLP WLIP, and its mutant *P. putida* CMPG2120, lacking the production of WLIP. CMR12a and CMR12a mutant stands respectively for *Pseudomonas* sp. CMR12a, producing the CLPs sessilin and orfamide and the antibiotic phenazine, and its mutant, *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2, lacking the production of both CLPs and the antibiotic phenazine. Dots represent the mean immobilization rate of three biological replicates (N = 3), error bars indicate the standard deviation and asterisks are an indication of significant differences, compared to the negative control: * indicates a p-value lower than 0.05 (P < 0.05), ** indicates a p-value lower than 0.01 (P < 0.01) and *** indicates a p-value lower than 0.001 (P < 0.001; for data analysis, see chapter Materials and Methods paragraph 2.13 Data Analysis).

In the second experiment, the nematostatic effect of secondary metabolites produced by *P. fuscovaginae* UPB 0736, *P. fuscovaginae* 445, *Pseudomonas cichorii* SF1 -54, *Pseudomonas* sp. COR18, *Pseudomonas* sp. COR33, *Pseudomonas* sp. RHF3.1-22 and *Pseudomonas* sp. RHF3.3-3 was assessed. Cedroz was used as reference treatment. Immobilization rates after four, 24 and 48 hours are shown in **Figure 8.**

Four hours after inoculation, only Cedroz caused an increased immobilization rate compared to the control, albeit not significant (P = 0.1448). All the other treatments showed an immobilization rate comparable to the control, however a slight increase in immobilization rate can be noticed for the three times dilution of the supernatant from *Pseudomonas* sp. COR18 and the pure supernatant of *Pseudomonas* sp. COR18, *Pseudomonas* sp. RHF3.1-22 and *Pseudomonas* sp. RHF3.3-3. Yet, no noteworthy differences within these treatments were observed.

Twenty-four hours after inoculation, a significant higher immobilization rate was observed in the reference treatment Cedroz (P = <0.0001). Moreover, in the undiluted supernatant of both COR18, COR33, RHF 3.3-3 and RHF 3.1-22, a significantly higher immobilization rate was noticed (P = 0.0001, P = 0.0026, P = 0.0045 and P = 0.0179, respectively). The three times diluted supernatant of these strains had no significant effect on motility (P = 1.000 for all treatments). This means there was no evidence that Peptin 19:5 had nematistatic properties, as there was no significant difference in immobilization rate between COR18 and COR33 (P = 1.000), nor between COR33 and RHF 3.3-3 (P = 1.000). Finally, none of the three times dilutions, and the undiluted supernatant from *P. fuscovaginae* UPB 0736, *P. fuscovaginae* 445 and *P. cichorii* SF1-54 seemed to have a notably nematostatic effect.

Forty-eight hours after inoculation, again a significantly increased immobilization rate was observed for the reference treatment Cedroz (P = 0.0454). Immobilization rates comparable to the control were observed in the three times dilution of the supernatants originating from *P. cichorii* SF1 -54, *Pseudomonas* sp. COR33, *Pseudomonas* sp. RHF 3.1-22 and in the undiluted supernatant from *P. fuscovaginae* UPB 0736. A non-significantly lower immobilization rate was observed for both the three times dilution of the supernatant from *P. fuscovaginae* UPB 0736, its mutant *P. fuscovaginae* 445 and the undiluted supernatant from *P. fuscovaginae* 445, compared with the control (P = 0.2427, P = 1.000 and P = 0.0800, respectively). This reduction was most pronounced for the undiluted supernatant from *P. fuscovaginae* 445, followed by the three times dilution of the supernatant from the wild type *P. fuscovaginae* UPB0736, yet the difference with the three times dilution of *P. fuscovaginae* 445 is not significant (P = 1.0000). A higher, but not significant, immobilization rate is observed for three times diluted supernatant from COR18 and RHF3.3-3 (P = 1.000 and P = 1.000, respectively). Pure supernatant of *Pseudomonas* sp. COR33, *Pseudomonas* sp. RHF3.3-3 and *Pseudomonas* sp. RHF3.1-22 was too turbid to count after 48 hours.

Taken together, immobilization rates observed for the nematodes treated with undiluted supernatant from *Pseudomonas* sp. COR18, *Pseudomonas* sp. COR33, *Pseudomonas* sp. RHF 3.3-3, *Pseudomonas* sp. RHF3.1-22 were higher compared with the untreated control,

but lower compared with the reference treatment Cedroz, suggesting one or more nematocidal compounds are secreted by these strains. However, none of three times dilutions of the supernatant originating from these strains showed a notable different immobilization rate compared to the control. Neither the nematodes incubated in the diluted and the undiluted supernatant from *P. cichorii* SF1-54 showed a notable nematostatic effect. After 24 hours, a slightly higher immobilization rate was observed for the undiluted supernatant from *P. fuscovaginae* 445, while treatment with diluted supernatant from *P. fuscovaginae* 445 and both diluted and undiluted supernatant from *P. fuscovaginae* UPB0736 resulted in lower immobilization rates, compared with the untreated control. However, none of these differences was significant (P = 1.000 for all treatments).

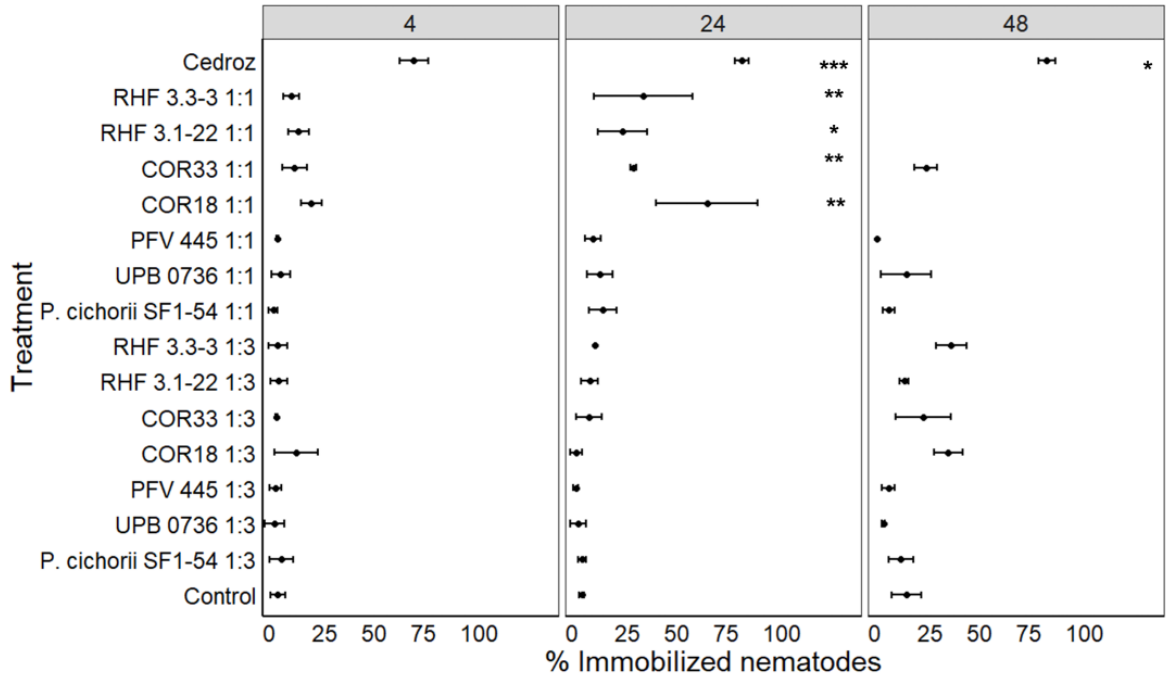

Figure 8: The percentage of immobilized nematodes in function of the supernatant where they were submerged in after four (4), 24 and 48 hours of incubation. UPB 0736 and PFV 445 respectively stands for *P. fuscovaginae* UPB 0736, producing fuscopeptin and syringomycin, and *P. fuscovaginae* 445, only producing syringomycin. *P. cichorii* SF1-54 stands for supernatant from *P. cichorii* SF1-54, producing cichopeptin A and B. COR18 and COR33 stands for *Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33, producing respectively both Peptin 19:5, a putatative thanamycin and CLP13 or CLP13 alone. RHF3.1-22 and RHF 3.3-3, standing for *Pseudomonas* sp. RHF3.1-22 and *Pseudomonas* sp. RHF 3.3-3. *Pseudomonas* sp. RHF 3.3-3 produces respectively no CLPs and CLP13, Peptin 19:5 and a putative thanamycin, *Pseudomonas* sp. RHF 3.1-22 does not produce any CLPs. Supernatant from RHF3.3-3, RHF3.1-22 and COR 18 became too turbid to count after 48 hours. Pure supernatant is indicated as 1:1, 1:3 indicates three times diluted supernatant. Cedroz is the positive control, control is the negative control. Each treatment consisted of three repeats (N = 3). Error bars represent standard deviation, dots represent medians, asterisks indicate significant differences, compared to the negative control: * indicates a p-value lower than 0.05 (P < 0.05), ** indicates a p-value lower than 0.01 (P < 0.01) and *** indicates a p-value lower than 0.001 (P < 0.001; for data analysis, see chapter Materials and Methods, paragraph 2.13 Data Analysis).

In the third experiment, the acute toxicity of three CLP producing strains was reassessed, 24 hours after incubation (**Figure 9 A)**. First, the acute toxicity of the CLPs orfamide and sessilin and the antibiotic phenazine was tested using sterile supernatant from *Pseudomonas* sp. CMR12a and its mutant *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2. Besides, the acute toxicity of tolaasin was reassessed using supernatant from *P. tolaasii* CH36 and its mutant *P. tolaasii* Tol-A. Last, the acute toxicity of CLP13 and Peptin 19:5 was reassessed with the supernatant from *Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33. Cedroz was used as reference treatment, tap water as negative control. As the immobilization rate of Cedroz was lower (42%) than expected, care should be taken by drawing conclusions from this repeat. Both Cedroz, *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2, *P. tolaasii* Tol-A and *P. tolaasii* CH36 significantly increased the immobilization of the nematodes ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$ and $P = 0.0007$, respectively) compared to the control. A non-significant motility reduction compared to the control was observed for the supernatant originating from *Pseudomonas* sp. CMR12a, *Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33 ($P = 0.0516$, $P = 0.0932$ and $P = 0.2200$, respectively). Interestingly, both supernatants originating from the mutants *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2 and *P. tolaasii* Tol-A provoked a greater reduction in mobility compared to their wild-type *Pseudomonas* sp. CMR12a and *P. tolaasii* CH36. This difference was significant for *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2 and *Pseudomonas* sp. CMR12a, but not for *P. tolaasii* Tol-A and *P. tolaasii* CH36 ($P = 0.0307$ and $P = 1.0000$, respectively).

Since Cedroz did not have the expected efficacy on the mobility of the nematodes during the third experiment, the experiment was repeated (**Figure 9 B)**. In the fourth experiment, Cedroz had a significant higher immobilization rate (95.9%) compared with the negative control (6.94%) ($P = 0.0000$), indicating the experimental setup worked appropriately. Overall, except for the supernatant from *Pseudomonas* sp. COR33, all supernatants caused a significant higher immobilization rate ($P = 0.0007$, $P = <0.0001$, $P = <0.0001$, $P = <0.0001$, $P = <0.0001$ and $P = <0.0001$, respectively for *P. tolaasii* CH36, *P. tolaasii* Tol-A, *Pseudomonas* sp. CMR12a, *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2 and *Pseudomonas* sp. COR18). As observed in the third experiment, the tolaasin-producing wild-type *P. tolaasii* CH36 caused a lower immobilization rate compared with *P. tolaasii* Tol-A, and in this experiment the difference was significant ($P = <0.0001$). When comparing *Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33, a significant higher immobilization rate was observed for *Pseudomonas* sp. COR18 ($P = <0.0001$). In contrast to the third experiment, a non-significantly lower immobilization rate was observed for *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2, compared with *Pseudomonas* sp. CMR12a ($P = 0.8017$), suggesting none of the produced CLPs orfamide and sessilin, nor the antibiotic phenazine had a significant effect on the nematostatic activity.

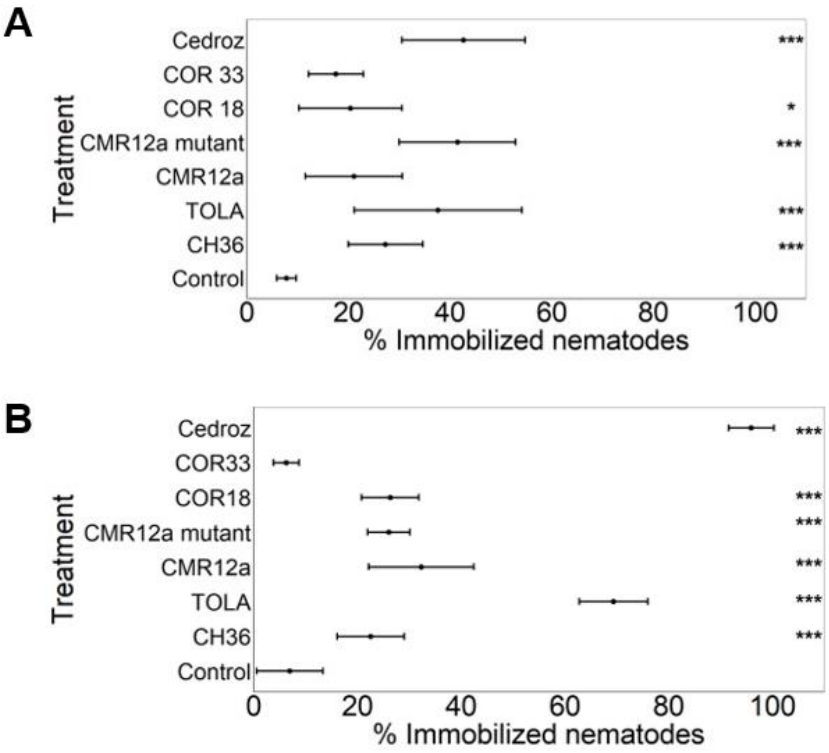

Figure 9: The percentage of immobilized nematodes in function of the supernatant mixture where they were submerged in, counted after 24 hours of incubation. Cedroz is the positive control, Control the negative control, CH36 and TOLA respectively stands for the supernatant from *P. tolaasii* CH36 (producing the CLP tolaasin) and its mutant *P. tolaasii* Tol-A (lacking tolaasin production). CMR12a and CMR12a mutant stands respectively for *Pseudomonas* sp. CMR12a, producing the CLPs sessilin and orfamide and the antibiotic phenazine, and its mutant, *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2, lacking the production of both CLPs and the antibiotic phenazine. COR18 and COR33 are *Pseudomonas* sp. COR18, producing CLP13, Peptin 19:5 and a putative thanamycin, and *Pseudomonas* sp. COR33, only producing CLP13. Exact the same experiment was performed twice on different data. (A) Shows the results of the third experiment (B) shows the results of the fourth experiment. Dots represent the mean immobilization rate of six samples (N= 6), error flags represent the standard deviation, asterisks indicate significant differences, compared to the negative control: * indicates a p-value lower than 0.05 ($P < 0.05$), ** indicates a p-value lower than 0.01 ($P < 0.01$) and *** indicates a p-value lower than 0.001 ($P < 0.001$; for data analysis, see chapter Materials and Methods, paragraph 2.13 Data Analysis).

In all experiments, a higher immobilization rate was observed for the mutant *P. tolaasii* Tol-A, impaired in tolaasin production, compared to the tolaasin-producing wild type *P. tolaasii* CH36. Both wild type and mutant showed a higher immobilization rate compared with an untreated control. These results indicate that *P. tolaasii* produces one or more nematistatic compounds, and that tolaasin is not one of these compounds. The stronger nematostatic properties of the Tol-A mutant could indicate that the metabolic cost of tolaasin production reduces the production of the (unidentified) nematostatic compounds in this species.

Pseudomonas sp. CMR12a and its mutant *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2 provoked higher immobilization rates compared with the untreated control. Whether the sessilin, orfamide and phenazine, whose production is abolished in the CMR12a - ΔPhz-CLP1-ΔCLP2mutant, contribute to nematostatic activity remains unclear, since in two of the three experiments, supernatant from the CLP producing strain *Pseudomonas* sp. CMR12a induced a higher immobilization rate, whereas in the third experiment, the mutant *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2 showed a significantly higher immobilization rate. These results do, however, clearly show that at least some of the nematostatic properties of *Pseudomonas* sp. CMR12a supernatant are caused by metabolites other than sessilin, orfamide or phenazine.

Supernatant from *Pseudomonas* sp. COR33, which contains CLP13, caused a significant lower nematostatic effect than *Pseudomonas* sp. COR18, which produces a putative thanamycin, CLP13 and Peptin 19:5. However, an effect comparable to that of *Pseudomonas* sp. COR33 on the nematode mobility was observed with *Pseudomonas* sp. RHF 3.3-3, also producing Peptin 19:5, indicating that Peptin 19:5 is not nematostatic. Fuscopeptin appeared not to be significantly nematostatic and supernatant from *P. cichorii* SF1-54 also provoked a comparable immobilization rate to the control.

3.2. *In planta* nematocidal test provides inconclusive results

Besides a direct nematostatic effect, *Pseudomonas* strains and the CLPs they produce may also induce resistance in plants (Haas & Défago, 2005). To test whether this is effective against nematodes, a study was set up to investigate whether CLPs are involved in triggering induced resistance in rice plants against *Meloidogyne graminicola.* Plants treated with *Pseudomonas* sp. CMR12a, *P. tolaasii* CH36, *Pseudomonas* sp. COR18 and their respective mutants *Pseudomonas* sp. CMR12a - ΔPhz-CLP1-ΔCLP2, *P. tolaasii* Tol-A and *Pseudomonas* sp. COR33 were infected with nematodes; the resistance inducer PA was used as a positive control. Medium root colonizations of $3.89 . 10^6$, $9.21. 10^6$, $8.26 . 10^6$, $7.76 . 10^6$, $8.56 . 10^6$ and $12.4 . 10^6$ CFU/g root were obtained for plants colonized with *Pseudomonas* sp. CMR12a, *Pseudomonas* sp. CMR12a- ΔPhz-CLP1-ΔCLP2, *Pseudomonas* sp. COR18, *Pseudomonas* sp. COR33, *P. tolaasi* CH36 and *P. tolaasi* Tol-A, respectively (Ferrarini, Verbal communication). The number of galls per root were counted and normalized against the root weight (**Figure 10 A and B**). A low infection pressure was observed (**Figure 10 A**), with gall numbers ranging from 3 to 17 galls per root. Moreover, no significant differences were observed in the number of galls, normalized per gram of root weight (P = 0.1949). Since a comparable number of galls normalized to root weight was observed in both the untreated negative control and the reference treatment (PA), no conclusions can be drawn from this experiment (P = 0.597).

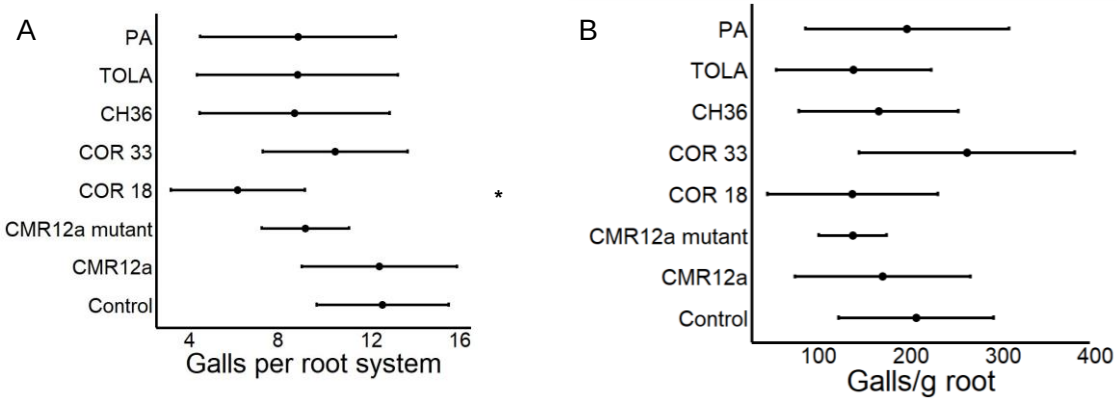

Figure 10: (A) The total number of galls per root system for each treatment. (B) The number of galls, normalized to root weight. Control is the negative control, PA the positive control, CH36 and TOLA stands for bacterial treatment with *P. tolaasi* CH36 (producing the CLP tolaasin) and its mutant *P. tolaasi* Tol-A (deficient in tolaasin production), respectively. Furthermore, COR18 and COR33 stands for respectively *Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33, producing CLP13, a putative thanamycin and Peptin 19:5, or only CLP13, respectively. Last, CMR12a and CMR12a mutant stands respectively for *Pseudomonas* sp. CMR12a, producing the CLPs sessilin and orfamide and the antibiotic phenazine, and its mutant, *Pseudomonas* sp. CMR12a-ΔPhz-CLP1-ΔCLP2, lacking the production of both CLPs and the antibiotic phenazine. Each treatment consists of seven repeats (N = 7), except for PA, where six repeats were included (N = 6). Error bars represent standard deviation, asterisks indicate significant differences compared to the negative control: * indicates a p-value lower than 0.05 (P < 0.05), ** indicates a p-value lower than 0.01 (P < 0.01) and *** indicates a p-value lower than 0.001 (P < 0.001).

3.3. Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*

Two experiments were performed with the intention of investigating whether or not piperonylic acid can induce resistance against *Pyricularia oryzae* in rice plants. In a first experiment, BTH was applied as a soil drench, and PA was applied as a spray. This was done because PA was known from previous research to be highly effective as a spray (Desmedt *et al*., unpublished results), while BTH was usually applied in literature as a soil drench (De Vleeschauwer & Höfte, 2009; Veronico *et al.,* 2018). The results of the first experiment are shown in **Figure 11**. Each plant was considered as one biological replicate. On both the fourth, fifth and sixth day, a significant difference between the treatments was observed (P = <0.0001, P = 0.0090, P = 0.0116, respectively). However, a *post-hoc* analysis shows only BTH differed significantly from the negative control (P = <0.0001, P = 0.0058, P = 0.0092 after four, five and six days, respectively), and not PA (P = 0.0551, P = 0.1762, P = 0.1116, after four, five and six days, respectively). This shows that the data provides insufficient evidence to reject the null hypothesis, being the distribution in the number of lesions greater than or equal to three is equal for plants treated with PA and plants from the control treatment.

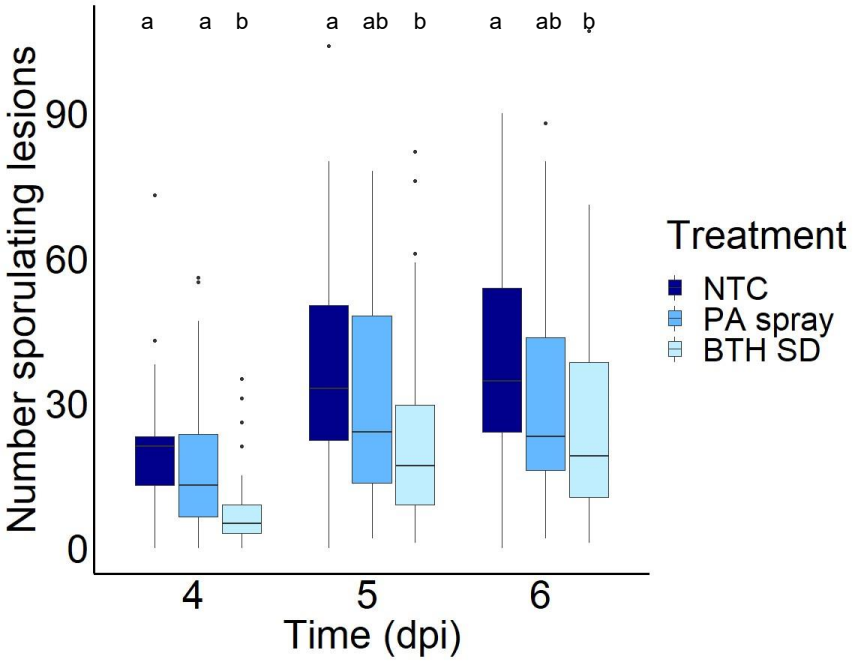

Figure 11: Boxplots showing the number of lesions having a score greater than three in function of time, in days post inoculation (dpi) according to treatment. The experimental conditions consist of piperonylic acid applied as a spray (PA spray), NTC is the non-treated control and benzothiadiazole applied as a soil drench (BTH SD) is the positive control. Each treatment consists of approximately six trays, each with six to seven plants (N = 41, N = 39 and N = 29 four dpi; N= 42, N = 39 and N= 35 five and six dpi for NTC, PA spray and BTH SD, respectively). Treatments with different letters significantly differ from each other (see chapter Materials and Methods paragraph 2.13 Data-analysis).

In a second experiment, five treatments were included: PA and BTH both applied as a spray treatment or as a soil drench, and a control treatment. Four and five days after inoculation, the number of sporulating lesions, having a score equal to or greater than three were counted. The infection was not scored six dpi, because due to the very high level of disease pressure, lesions had begun to merge already at five dpi. Each plant was considered as one replicate, although some plants shared the same tray. As shown in **Figure 12**, both foliar and soil drench treatment with PA significantly enhanced plant resistance against *Pyricularia oryzae* VT5M1 after four days (P = <0.0001 and P = <0.0001, respectively). The positive control treatments BTH spray and soil drench also significantly improved plant resistance against *P. oryzae* VT5M1 after four days (P = <0.0001 and P = 0.0004, respectively). Five days after inoculation, both soil drench and foliar spray treated plants showed a lower number of lesions with a score equal to or greater than 3, compared to the mock-treated control. However, only for the plants treated with BTH and PA as a spray, this difference was significant (P = 0.035 and P 0.046). But since the infection pressure was very high, the number of lesions having a score equal to or greater than 3 was not representative for the real damage to the leaf on the fifth day. Many lesions had coalesced, resulting in fewer, bigger lesions that are not representative of the number of infection sites.

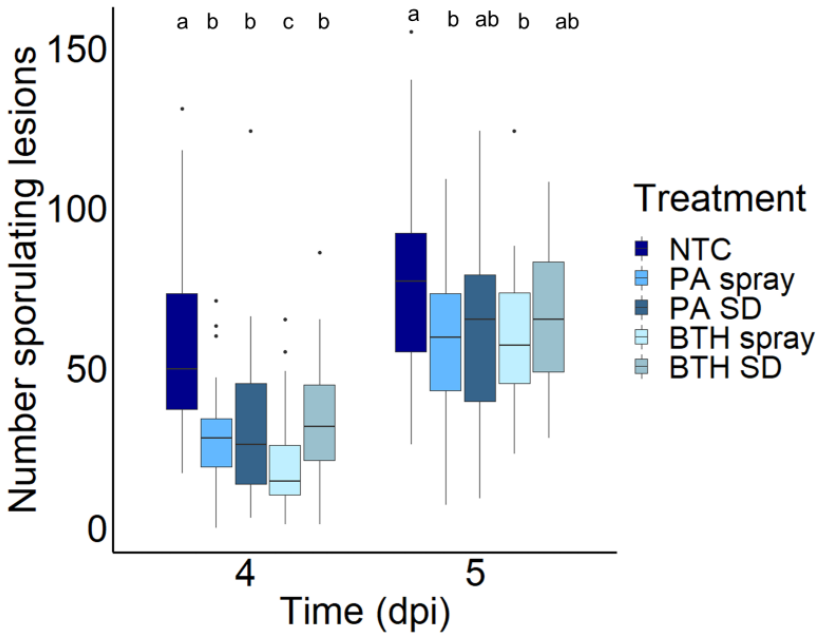

Figure 12: Boxplots showing the number of sporulating lesions in function of time, which is expressed in days post inoculation (dpi), and according to treatment. The experimental treatments consist of plants treated with piperonylic acid, either as a spray (PA spray) or as a soil drench treatment (PA SD). NTC is the non-treated control, benzothiadiazole is the positive control and is also applied as both a spray (BTH spray) and as a soil drench (BTH SD). Each treatment consisted of five to six trays, each with six to seven plants (N = 36 for NTC, N = 39 for PA spray, N = 35 for PA SD and N = 34 for both BTH SD and BTH spray four dpi; N = 37 for NTC, N = 40 for PA spray, N = 35 for both PA SD and BTH spray and N = 34 for both BTH SD five dpi).Letters indicate a significant difference between treatments (see chapter Materials and Methods, paragraph 2.13 Data-analysis).

3.3.1. Treatment with PA might enhance PAL activity

In order to have a deeper insight in which pathways were involved, the activity of PAL, an enzyme upstream of the phenylpropanoid pathway, was determined (**Figure 13 A**). Although no significant differences were observed in PAL activity between different treatments (P = 0.1448), it seemed both plants treated with PA and plants treated with BTH had a higher PAL activity (respectively +178% and +179% for spray treatments and +297% and +467% for soil drench treatments). This suggests both BTH and PA induce resistance in rice against *Pyricularia oryzae* in part by enhancing the PPP pathway.

3.3.2. Treatment with PA seems to enhance guiacol peroxidase activity

Guaiacol peroxidase activity was measured in PA-, BTH- or mock-treated leaves, sampled five days after inoculation with *Pyricularia oryzae* VT5M1 (**Figure 13 B**). Although no significant differences between different treatments were observed (P = 0.2159), a higher guaiacol peroxidase activity can be noted for both foliar and soil drench application of PA (+38% and +49%, respectively). By contrast, guaiacol peroxidase activities comparable to the untreated control were observed for both foliar applied and soil drench applied BTH (+19% and -11%). This suggests PA induces systemic resistance against *P. oryzae* VT5M1 in part by enhancing peroxidase activity, while BTH seems to induce resistance in rice against blast through other mechanisms.

3.3.3. No conclusions can be drawn regarding free and bound phenolic compounds

Free and bound phenolic compounds were measured in leaves sampled five days after inoculation with rice blast to examine the role of the flavonoid metabolism in the induction of resistance by PA and BTH. Two measurements were executed for each biological sample. However, a very high technical variance was observed between the first and the second measurement. Moreover, precipitation occurred in the samples. As such, the results of this experiment are probably not reliable, and an analysis of the results would not be useful (data not shown).

3.3.4. PA seems to not induce systemic resistance through lignification

The lignin content was measured in leaves sampled five days after inoculation with *P. oryzae* VT5M1. No notable differences in lignin content were observed (**Figure 13 C**; P = 0.0574), except BTH soil drench appeared to reduce lignin content in leaves. However, this is not significant. This implies that both BTH and PA probably do not induce resistance in rice against blast by reprogramming the phenylpropanoid pathway towards lignification.

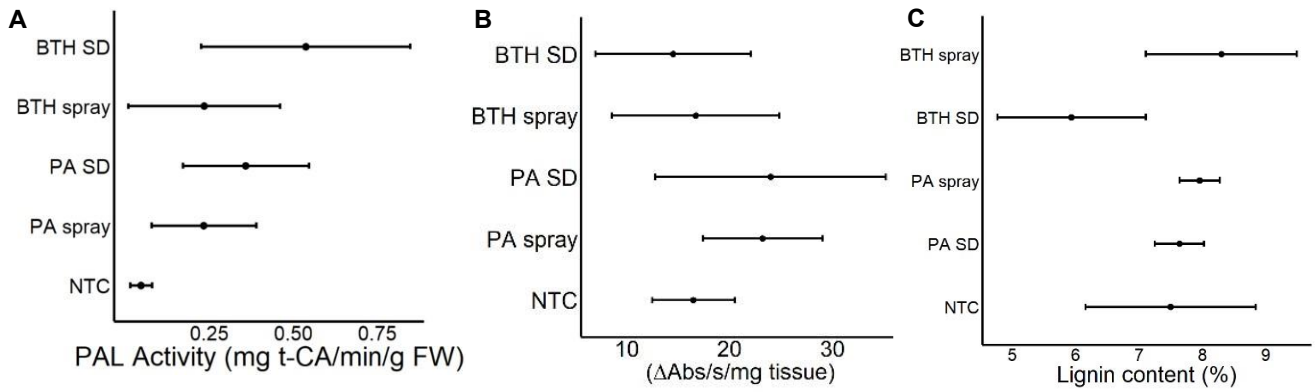

Figure 13: (A) PHENYLALANINE AMMONIA-LYASE (PAL) activity, (B) GUIACOL PEROXIDASE activity and (C) Lignin content of plants infected with *P. oryzae*. The experimental conditions consist of plants treated with piperonylic acid as a spray (PA spray), or soil drench treatment (PA SD). The positive control is benzothiadiazole, applied as soil drench (BTH SD) or as spray (BTH spray) treatment. NTC is the non-treated control. Each treatment consisted of five samples, each a mixture of six leaves from one tray (N = 5). Dots represent the mean, error bars the standard deviation. Asterisks indicate significant difference, compared to the mock-treatment: * indicates a p-value lower than 0.05 (P < 0.05), ** indicates a p-value lower than 0.01 (P < 0.01) and *** indicates a p-value lower than 0.001 (P < 0.001).

3.3.5. PA induces resistance in rice against rice blast by upregulation of *OsNOMT*

In order to reveal which pathways are concerned in the induction of resistance in rice against rice blast, an infection assay was executed, followed by RT-qPCR of several immunity-related genes. In the first place, the infection was assessed by scoring the infection four and five dpi (**Figure 14).** Plants foliarly treated with PA showed a significantly lower number of sporulating lesions compared to the untreated control, both four and five dpi (P = 0.0235 and P = 0.0257). Remarkably, a very low infection pressure was observed, with a number of sporulating lesions per leaf ranging from zero to 68 and a median of six sporulating lesions per plants. The measured change in gene expression as a result of blast infection will therefore probably be representative for a low infection pressure, but not for high infection pressures.

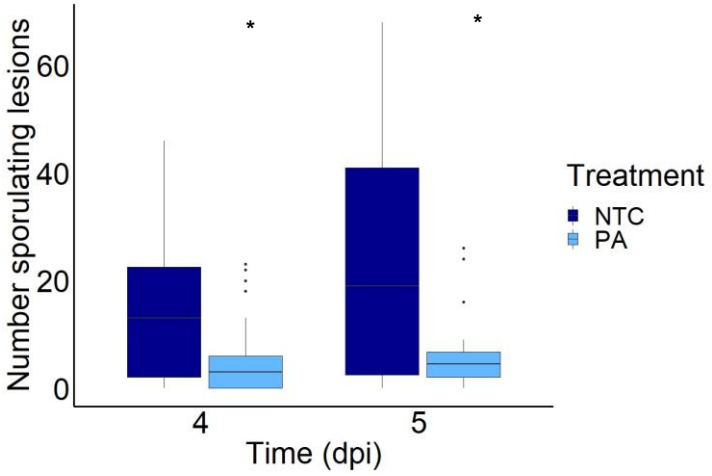

Figure 14: Boxplot of the sporulating number of lesions caused by *P. oryzae* four and five dpi. PA stands for plants treated with piperonylic acid as a spray treatment, NTC is the mock-treated control. Error bars represent the standard deviation, dots represent the mean. Nineteen NTC (N=19) and 24 PA-treated plants were counted. Asterisks indicate significant differences, compared to the mock-treated control: * indicates a p-value lower than 0.05 (P < 0.05), ** indicates a p-value lower than 0.01 (P < 0.01) and *** indicates a p-value lower than 0.001 (P < 0.001).

Five genes were chosen for RT-qPCR analysis: *OsPR1a,* a general marker for immunity (Agrawal *et al.,* 2001), *OsWRKY45* and *OsJAMYB* as markers for a respectively SA or JA response (Lee *et al.,* 2007; Ryu *et al.,* 2006), two genes involved in biosynthesis of diterpenoid phytoalexins (*OsCPS2* and *OsCPS4;* Cho & Lee, 2015) and one gene involved in the biosynthesis of the phenolic phytoalexin sakuranetin *(OsNOMT*; Otomo *et al.,* 2004). Gene expression was measured in four different groups of plants: infected plants treated with PA, uninfected plants treated with PA, mock-treated, infected plants and mock-treated, uninfected plants. Gene expression of plants belonging to the first three groups was compared to gene expression in untreated and uninfected plants (control), the ratio of the relative number of transcripts in the treated sample versus this control is referred to as the fold change (FC). Results are shown in **Figure 15.**

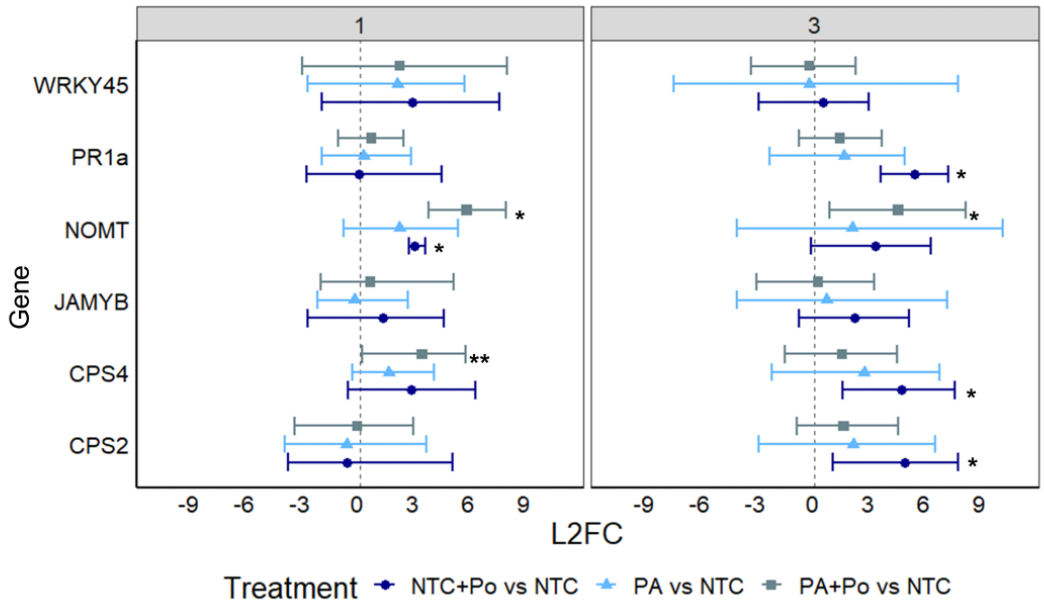

Figure 15: Mean log_2 fold change (L2FC) of gene expression for each treatment one (1) and three (3) days post inoculation, compared to a mock-treated, non-infected control. PA stands for plants treated with piperonylic acid, PA + Po for plants treated with piperonylic acid and infected with *Pyricularia oryzae*, NTC+PO for mock-treated plants infected with *P. oryzae*. Four biological replicates were assessed per treatment (N = 4), each consisting of two technical replicates. Dots represent the mean log_2 fold change, error bars indicate a 95% confidence interval of the log_2 fold change and asterisks indicate a significant different expression level, compared to the mock-treated, uninoculated plants: * indicates a p-value lower than 0.05 (P < 0.05), ** indicates a p-value lower than 0.01 (P < 0.01) and *** indicates a p-value lower than 0.001 (P < 0.001).

One dpi, expression of *OsPR1a* did not differ significantly between any treatment (FC = 1.56; FC = 1.15; FC = 0.95; P = 0.838; P = 0.386; P = 0.92 for respectively PA-treated, uninfected plants, PA treated, infected plants and mock-treated, infected plants). At three dpi, *OsPR1a* was non-significantly induced by PA treatment alone (FC = 2.59, P = 0.13) and by combined PA treatment and *P. oryzae* infection (FC = 2.99, P = 0.25). By contrast, very strong induction was seen in mock-treated, *P. oryzae*-infected plants (FC = 42.5, P = 0.013).

Expression of *OsWRKY45* was non-significantly upregulated in all treatments one dpi. The upregulation was most pronounced in mock-treated, infected plants (FC = 7.15; P = 0.118), followed by PA-treated, infected plants (FC = 4.49; P = 0.325) and finally PA-treated, non-infected plants (FC = 4.07; P = 0.247). Three dpi, there were no major changes in the expression of *WRKY 45* in any treatment (mock-treated, infected: FC = 1.38, P = 0.689; PA-treated, infected: FC = 0.815; P = 0.791; PA-treated, non-infected: FC = 0.816; P = 0.889)

One dpi*, OsJAmyb* appeared non-significantly upregulated in mock-treated, infected plants (FC = 2.40; P = 0.323), and nearly unchanged in PA-treated plants both without infection (FC = 1.50; P = 0.76) and with infection (FC = 0.815; P = 0.741). Three dpi, expression of *OsJAmyb* was non-significantly upregulated in untreated, infected plants (FC = 4.56; P = 0.092), and was comparable to uninfected control plants in both infected (FC = 1.14; P = 0.871) and uninfected (FC = 1.57; P = 0.777) plants treated with PA.

One dpi, two genes involved in biosynthesis of diterpenoid phytoalexins, *OsCPS2* and *OsCPS4,* showed contrasting changes in expression. *OsCPS2* was unaffected, or even slightly downregulated, by all treatments tested at one dpi (FC = 0.917, FC = 0.61, FC = 0.604; P = 0.907, P = 0.386, P = 0.592, for respectively PA treated and infected plants, PA-treated uninfected plants and the mock-treated infected plants). By contrast, *OsCPS4* was significantly upregulated in infected plants treated with PA (FC = 10.32, P = 0.007), and was non-significantly higher in mock-treated, infected rice plants (FC = 6.87; P = 0.105). In uninfected, PA-treated plants, expression of *OsCPS4* was also slightly but non-significantly upregulated (FC = 2.93; P = 0.247). Three dpi, expression of both *OsCPS2* and *OsCPS4* was highest in the untreated, infected control (FC = 28.9 and FC = 25.6; P = 0.028 and P = 0.013). In PA-treated, infected plants, *OsCPS2* and *OsCPS4* were (non-significantly) upregulated (FC = 2.95 and FC = 2.78; P = 0.196 and P = 0.2, respectively), and the same pattern was seen in PA-treated, non-infected plants (FC = 4.25 and FC = 6.41; P = 0.285 and P = 0.201, respectively).

Finally, expression of *OsNOMT* was significantly upregulated one dpi in *P. oryzae*-infected leaves, but much more strongly so in PA-treated than in mock-treated plants (FC = 55.3, P = 0.013 versus FC = 7.92, P = 0.008), which can be an indication of priming. In non-infected, PA-treated plants, a non-significant induction of *OsNOMT* was also seen (FC = 4.47; P = 0.135). Three dpi, expression of *OsNOMT* appeared upregulated in all treatments. The largest change was again seen in PA-treated, infected plants (FC = 22.9, P = 0.041), with smaller and non-significant changes seen in mock-treated, infected plants (FC = 9.60, P = 0.07) and PA-treated, non-infected plants (FC = 4.05, P = 0.471). These results suggest that treatment with

PA results in enhanced induction of *OsNOMT* upon infection with *P. oryzae,* a gene involved in biosynthesis of the phytoalexin sakuranetin.

It should be noted that *OsNOMT, OsCPS2* and *OsCPS4* had very low expression levels in all treatments (especially in the mock-treated plants). More specifically, mean C_q- values of respectively 39.7, 29.8 and 29.9 were observed one dpi, and mean C_q- values of 38.5, 34.0 and 33.6 were observed three dpi for mock-treated, uninfected plants. These low expression levels may reduce the accuracy of quantification for these genes.

4. Discussion

4.1. Nematostatic effect of CLPs on *Meloidogyne graminicola*

In order to determine whether or not secondary metabolites, and in particular CLPs, secreted by *Pseudomonas* strains have a direct nematostatic effect, nematodes were exposed to sterile supernatant obtained from various *Pseudomonas* strains and, if available, from mutants of those strains impaired in the production of one or more CLPs.

Important to notice is that the assay used in this thesis does not distinguish between nematostatic and nematocidal agents: in the experimental setup, nematodes were submerged in supernatant from a *Pseudomonas* strain, and the immobile nematodes were counted after various exposure durations. Immobility can be caused by nematode death or by (reversible) paralysis of the nematode. Distinguishing between these possibilities requires a more complex setup, where nematodes are, after initial immersion in the supernatant, transferred to tap water and their mobility should be reassessed. Dead nematodes do not regain their mobility, whereas reversibly paralyzed nematodes do (Desmedt *et al.,* 2020b). In this experiment, only nematostatic effects were assessed. By consequence, supernatants will be labeled only as nematostatic, not as nematocidal.

The experimental data suggest that supernatants from *Pseudomonas* sp. COR33, *P. cichorii* SF1-54 and *P. fuscovaginae* UPB 0736 (see chapter Results paragraph 3.1 Nematocidal assay **Figure 8** and **Figure 9**) did not significantly affect nematode mobility, which suggests that CLP13, cichopeptin A and B, fuscopeptin and syringomycin do not have a strong nematostatic effect. On the other hand, compounds excreted by *Pseudomonas* sp. CMR12a, *P. tolaasii* CH36, *P. putida* RW10S2 (see chapter Results paragraph 3.1 Nematocidal assay **Figure 7** and **Figure 9**), *Pseudomonas* sp. COR18, *Pseudomonas* sp. RHF 3.3-3 and *Pseudomonas* sp. RHF 3.1-22 appeared to have nematostatic activity (see chapter Results paragraph 3.1 Nematocidal assay **Figure 8** and **Figure 9**).

Interestingly, it seemed that this nematostatic activity might be primarily caused by secondary metabolites other than CLPs. The CLP-producing strains *P. tolaasii, P. putida* and *Pseudomonas* sp. CMR12a showed nematostatic activity, but so did their respective mutants *P. tolaasii* Tol-A, *P. putida* CMPG2120 and *Pseudomonas* sp. CMR12a-ΔPhz-CLP1-ΔCLP2 (see chapter Results paragraph 3.1. Nematocidal assay).

P. tolaasii has been shown to produce several volatile biocidal compounds, such as aminobenzene and dimethyl disulfide (Lo Cantore *et al.*, 2015; Park *et al.*, 1994). Dimethyl sulfide had previously been shown to be nematocidal (Coosemans *et al.,* 2005). *Pseudomonas* sp. CMR12a produces, besides orfamide, sessilin and phenazine, also hydrogen cyanide and exoprotease (D'aes *et al.*, 2011; Perneel *et al.,* 2007). Hydrogen cyanide, extracellular protease and phenazine have all been shown to be nematocidal (Neidig *et al.*, 2011). Finally, *P. putida* can also produce hydrogen cyanide (Guo *et al.,* 2016).

Only for the CLP Peptin19:5, produced by *Pseudomonas* COR18, there was tentative evidence to suggest a nematostatic effect might be present: significantly higher immobilization rates were observed for supernatant from *Pseudomonas* sp. COR18, producing a putative thanamycin, CLP13, Peptin 19:5, compared to the supernatant from *Pseudomonas* sp. COR33, which only produces CLP13. However, supernatant from *Pseudomonas* sp. RHF 3.3-3, also producing CLP13, a putative thanamycin and Peptin 19:5, had a similar nematostatic activity as supernatant from *Pseudomonas* sp. COR33. Possibly, the nematostatic effect was caused by higher CLP production by *Pseudomonas* sp. COR18 compared to *Pseudomonas* RHF 3.3-3. Another possibility is that *Pseudomonas* sp. COR18 excreted, apart from CLP13, Peptin 19: and a putative thanamycin, other nematostatic compounds. Further investigation would be necessary to elucidate the role of these CLPs in nematostatic activity.

Remarkably, the supernatant from the CLP-deficient strain *P. tolaasii* Tol-A caused a significantly higher immobilization rate compared to the supernatant from the tolaasin-producing wild type *P. tolaasii* CH36. Although not significant, the same tendency is observed for the WLIP-producing wild type *P. putida* RW10S2 and its mutant *P. putida* CMPG2120. In the case of *Pseudomonas* sp. CMR12a, producing orfamide, sessilin and phenazine, and its mutant *Pseudomonas* sp. CMR12a-ΔPhz-CLP1-ΔCLP2, this phenomenon was seen in one out of three experiments. A first possible explanation for this phenomenon is that the mutants reproduced at higher rates than the wild type strains, resulting in higher cell densities and thus higher total levels of non-CLP metabolites. Another possibility is that mutant cell lines did not reproduce more rapidly, but instead invested the resources saved by not producing CLPs in increased production of other, possibly nematostatic, secondary metabolites, such as hydrogen cyanide or exoprotease.

To correct for possible differences in reproduction rate between lines in future repetitions of this experiment, it might be desirable to dilute all cultures to a fixed cell concentration before centrifugation, e.g. by diluting to a constant OD_{600}. Another, more laborious, adjustment method would be to extract secondary metabolites from the supernatant (for CLPs, this can be done e.g. through acidic precipitation (De Souza *et al.,* 2003)) and use these extracts instead of supernatants. However, since this experiment is designed as a preliminary screening intended to identify strains that secrete potential nematocidal compounds, such a time-intensive and labor-intensive method is not desired. Based on the data from the screening assays, *Pseudomonas* sp. CMR12a, *P. tolaasii, Pseudomonas* sp. COR18 and *Pseudomonas* sp. COR33 appear to be the most promising strains for further research.

4.2. Role of CLPs to induce resistance in rice against *Meloidogyne graminicola*

Pseudomonas strains are not only able to control certain pathogens and pests by direct antagonism, but also by induction of resistance in plants (Haas & Défago, 2005). Therefore, the potential of some previously tested *Pseudomonas* strains to induce resistance in rice against the root-knot nematode *Meloidogyne graminicola* was further examined. Roots of seedlings were dipped in a cell suspension on the day of transplantation, and a cell suspension was applied twice as a soil drench, once on the day of transfer and once three days before

infection. Plants were inoculated with *Meloidogyne graminicola* and the number of galls normalized to root weight was determined two weeks after inoculation. No significant differences were observed in the number of galls normalized to root weight between treatments. Since this is also the case for the positive control (rice plants sprayed with PA), there might be a technical issue with our experiment and no conclusions can be drawn. It is notable that the mean number of galls per plant is very low, which indicates that something went wrong with the inoculation process (see chapter Results paragraph 3.2 *In planta* nematocidal test provides inconclusive results). This might be due to error in inoculum preparation and application, or it might be a consequence of the choice of substrate (a 1:1 (by weight) sand-potting soil mixture instead of the synthetic-absorbent polymer mixture typically used for nematode infection experiments). The change of substrate was made because it was unknown whether *Pseudomonas* would properly colonize rice in the sand-absorbent polymer substrate. According to Réversat *et al.* (1999), a pure sand substrate is too compacted to allow proper aeration to the roots, and shows very poor water retention. While the mixture of sand and soil that was used in this thesis appeared to retain enough water, it was possible that it was too compacted to allow proper nematode colonization.

In a next experiment, it might be useful to replace the mixture of soil and sand by synthetic-absorbent polymers. However, this substrate appears to be resistant to colonization by bacteria and fungi affecting plant and nematode growth (Réversat *et al*., 1999), making it difficult to work with cell suspensions. Therefore, either the *Pseudomonas* inoculation process must be thoroughly optimized for use in sand-absorbent polymer substrate, or a *Pseudomonas* supernatant should be used instead of living bacterial suspensions.

4.3. Ability of PA to induce resistance in rice against *Pyricularia oryzae*

Piperonylic acid (PA), an inhibitor of cinnamic acid-4-hydroxylase, was tested for its ability to induce resistance in rice against *P. oryzae*. The obtained results show plants treated with PA appeared to have fewer sporulating lesions compared with a mock-treated control, and that PA was effective both as a foliar spray and as a soil drench. Its effectiveness was statistically similar (but generally slightly lower) than that of BTH, even at high infection pressure (see chapter Results paragraph 3.3. Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*).

Nonetheless, this does not necessarily mean PA induces resistance in rice against *P. oryzae,* as it is also possible PA might have a direct fungicidal effect. Experiments examining the fungicidal effect of PA on four different fungal strains, being *Botrytis cinerea, Alternaria solani* and two strains of *Fusarium graminearum* showed PA does not alter germination of fungal spores, and only significantly reduced post-germination mycelium growth in *Fusarium*. However, in this experiment on agar the fungus was exposed to a high PA concentration for up to seven days, which is not necessarily representative of the *in planta* situation (Desmedt *et al.,* 2020a). Furthermore, PA is rapidly conjugated and inactivated after application (Steenackers, 2016), and the observation that soil drench treatment was equally effective as foliar treatment further reduces the likelihood that direct fungicidal effects of PA cause the

observed reduction in disease severity (see chapter Results paragraph 3.3. Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae)*.

Some biochemical assays were performed five days after inoculation, in order to identify pathways that might possibly be involved in PA-IR. These biochemical assays were preferred over RT-qPCR for an initial exploration, because the plant material available was from plants harvested five days after inoculation, a relatively late time point in the infection process. Whereas gene expression changes rapidly, enzymes, secondary metabolites and lignin may remain present for longer once formed.

More specifically, free and bound phenolics, PAL and peroxidase activity and lignin content were measured on plant leaves harvested five days post inoculation. Peroxidase activity was shown to be related to the generation of ROS upon infection, and is used as a marker for immunity (Almagro *et al.,* 2009; Kidway *et al.*, 2020). A recent study revealed GUAIACOL PEROXIDASE activity was higher upon infection in rice varieties resistant to *P. oryzae,* (Kavanashree *et al.,* 2020). Five dpi, a trend towards increased PEROXIDASE was seen in infected plants pre-treated with PA, both as soil drench and as a spray treatment. BTH appeared not to increase peroxidase activity in the rice plants (see chapter Results paragraph 3.3 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). However, it is important to keep in mind that the infection process was already in an advanced stage; Kavanashree *et al.* (2020) showed GUAIACOL PEROXIDASE activity upon infection with *P. oryzae* Cav. peaked at 2 dpi, after which the peroxidase activity declined.

PAL, the first enzyme of the phenylpropanoid pathway (see Literature study paragraph 1.5 Piperonylic acid), is also used as a marker for immunity, as higher PAL activity in general is associated with resistance to pathogen stress (Wang *et al.,* 2019; Yadav *et al.,* 2020). In case of *P. oryzae,* PAL activity was previously reported to be important for resistance in rice (Duan *et al.,* 2014, Giberti *et al.,* 2012). Both PA and BTH appeared to increase PAL activity, although the effect was not statistically significant due to high variability.

Lignin and phenolic compounds are two principal downstream products of the phenylpropanoid pathway, and both play a role in plant defense (Yadav *et al.,* 2020). Due to technical difficulties with the assay, no reliable results were obtained for free and bound phenolics. The assay for lignin was technically successful, and showed no differences were observed in the lignin content of PA- or BTH-treated plants (see chapter Results paragraph 3.3 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*).

The late time point chosen made it impossible to investigate whether a defense response occurred early or late in the infection process; this matters, because in many pathosystems early responses are more effective than late ones (Desmedt *et al.*, 2020b). Therefore, it is possible that PA and BTH did induce resistance through early peroxidase or PAL-dependent activity that we were unable to capture in our assay. Regarding lignin, the highly stable and recalcitrant nature of lignin means that, once formed, it does not disappear and thus would show up in our assay even if formed early in the infection process (Malinovsky *et al.,* 2014). This makes it extremely unlikely that widespread systemic lignification in infected leaves would

be a PA- or BTH-induced immune response against *Pyricularia oryzae.* This is different from PA-IR in tomato against *Meloidogyne incognita,* where a significant higher lignin content was observed in roots of PA-treated tomato plants infected with *M. incognita,* compared to both uninfected PA-treated plants and infected mock-treated plants (Desmedt *et al.,* 2021). No significant difference was seen in lignin content in BTH treated plants as well (see chapter Results paragraph 3.3.4 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*), but BTH-IR has shown to increase lignification in, for example, tomato against *M. incognita* (Veronico *et al.,* 2018) and in Chinese cabbage against soft rot caused by *Pectobacterium carotovorum* ssp. c*arotovorum* (Liu *et al.*, 2019).

Although our data strongly suggested that widespread, systemic lignification is not involved in PA- or BTH-induced resistance against *P. oryzae,* it cannot be entirely ruled out that that minor, highly localized lignification could play a role in resistance against *P. oryzae;* this would have to be addressed through histology, e.g. via the Wiesner stain (Liljegren, 2010; Pomar *et al.,* 2002) rather than through biochemical analysis of bulk tissues. Apart from the relatively late time point, another limitation of this exploratory experiment is that no uninfected controls were present in the experiment, making it impossible to distinguish between primed and directly induced responses (Desmedt *et al.,* 2021).

Both the problems of the late time point and the lack of non-infected controls were addressed in the next experiment, which used RT-qPCR analysis of several immunity-related genes to study PA-IR against rice blast. Six genes involved in different aspects of plant immunity were selected for evaluation: *OsPR1a, OsNOMT, OsCPS2, OsCPS4, OsWRKY45* and *OsJAmyb*. Each gene was examined one and three days post inoculation to capture both early and relatively later defense responses. *PR1a* encodes for a pathogenesis related protein and is used as a general marker for immunity (Agrawal *et al.*, 2001). *OsNOMT, OsCPS2* and *OsCPS* are three genes encoding for enzymes involved in the biosynthesis of phytoalexins (Cho & Lee, 2015; Otomo *et al.,* 2004): *OsCPS2* and *OsCPS4* are involved in the biosynthesis of the principal precursors of diterpenoid phytoalexins, whereas *OsNOMT* is involved in the final biosynthesis step of the phenolic phytoalexin sakuranetin. Last, *OsWRKY45* and *OsJAmyb* are two markers for the key plant defense hormones SA and JA, respectively (Lee *et al.,* 2007; Ryu *et al.,* 2006).

To ensure that the infection was successful, the number of sporulating lesions was visually assessed four and five days post inoculation in a small batch of plants kept aside for this purpose. Although the infection was successful, a markedly lower level of disease pressure was seen than in the previous two infection experiments (see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). As a consequence, the analysis of gene expression changes caused by infection of *P. oryzae* presented in this thesis is representative of a situation with mild infection; expression changes in plants with higher levels of disease pressure might be much more pronounced. Moreover, low expression levels of *OsCPS2, OsCPS4* and *OsNOMT* were observed, making it difficult to draw firm conclusions.

OsPR1a, a gene of which its function is poorly understood, but often used as a marker of plant defense responses (Agrawal *et al.*, 2001; Breen *et al.,* 2017), was not upregulated one dpi in all treatments (FC = 1.15; FC = 1.56; FC = 0.952, respectively for uninfected, treated plants, infected, treated plants and mock-treated, infected plants). However, three dpi, expression of *OsPR1a* was significantly upregulated in mock-treated, infected plants (FC = 42.5), but not in treated, infected (FC = 2.59) or treated uninfected plants (FC = 2.99; see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). The much stronger induction of this general immunity marker gene in mock-treated, infected plants might reflect the much higher level of disease pressure in these plants compared to PA-treated infected plants, which is in accordance with the lower number of sporulating lesions in PA-treated plants, compared to mock-treated plants (**Figure 14,** see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*).

OsWRKY45 is a marker of SA-related defense response in plants, and overexpression has shown to play an important role in resistance against rice blast. Usually, *OsWRKY45* is upregulated upon infection, but induction often comes too late to meaningfully affect the spread of rice blast (Takatsuji, 2014). Early induction of *OsWRKY45* has been shown to be crucial to BTH-IR in rice against blast (Shimono *et al.*, 2007). BTH is a functional analog of SA (Görlach *et al.,* 1996; Lawton *et al.,* 1996), and since expression of *OsWRKY45* is dependent on SA, this might explain why *OsWRKY45* expression is stimulated in BTH-IR (Ryu *et al.*, 2007). In contrast to BTH, it seems expression of *OsWRKY45* is less important in PA-IR. Namely, infected plants treated with PA had a non-significant induction of *OsWRKY45* one dpi, and unchanged *OsWRKY45* expression three dpi (FC = 4.49 and FC = 0.815, respectively; see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). Moreover, expression of *OsWRKY45* both on one and three dpi in PA-treated, infected plants was lower than in mock-treated, infected plants (FC = 7.15, FC = 1.38, respectively; see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). It was previously shown that PA-IR is independent of SA in tomato (Desmedt *et al.*, 2021). Also in rice, PA-IR remains effective against *M. graminicola* in an *OsWRKY45-RNAi* line while PA treatment does not affect SA levels (Desmedt *et al.,* 2020a). These results suggest that PA-IR might similarly be partially or entirely SA-independent in the *P.oryzae*-rice pathosystem.

Besides *OsWRKY45,* the expression of another plant hormone-dependent transcription factor was measured: *OsJAmyb,* dependent on jasmonic acid*. OsJAmyb* is involved in necrosis and cell death. Lee *et al.* (2000) showed expression of *OsJAmyb* was increased after applying an external stress factor, but no alteration in *OsJAmyb* expression was observed due to internal signals. Besides, plants with higher expression of *OsJAmyb* were found to be more susceptible to rice blast disease (Lee *et al.*, 2000). Although according to Lee *et al.* infection with blast significantly induces expression of *OsJAmyb*, in our experiment only a modest, non-significant upregulation was seen one dpi, both for mock-treated plants (FC = 2.40) and plants treated with PA (FC = 1.50; see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces

resistance in rice plants against *Pyricularia oryzae*). This could be explained by the low infection pressure in our experiment, or by the use of another blast pathogen, *P. grisea* IC-17 instead of *P. oryzae* VT5M1, or by the use of another rice variety, *Oryza sativa* spp. *japonica* 'Drew' instead of *Oryza sativa* spp. *indica* 'Co39'. Expression of *OsJAmyb* three dpi appeared to be higher in mock-treated infected plants (FC = 4.56) than in PA-treated infected plants (FC = 1.14, see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*), which could point towards a higher infection pressure and more necrosis and cell death in mock-treated, infected plants. However, no significant differences are observed, so no firm conclusions can be drawn.

OsCPS4 and *OsCPS2* encode two different COPALYL DIPHOSPHATE SYNTHASES, which convert geranylgeranyl pyrophosphate into *syn*-colalyl diphosphate and *ent*-colalyl diphosphate respectively. S*yn*-colalyl diphosphate and *ent*-colalyl diphosphate are the main precursors of rice diterpenoid phytoalexins: *syn*-colalyl diphosphate of momilactones A and B and *ent*-colalyl diphosphate of oryzalexin S, oryzalexins A-F and phytocassanes A-E (Otomo *et al.*, 2004). Hasegawa *et al.* (2010) has shown expression of both *OsCPS4* and *OsCPS2* was upregulated in both susceptible and resistant rice plants upon infection with *Pyricularia oryzae,* but that resistant plants displayed earlier induction of both genes compared to susceptible plants (Hasegawa *et al.*, 2010). In the results obtained in our experiment, expression of *OsCPS4* was significantly increased one day after inoculation with *Pyricularia oryzae* in plants treated with PA (FC = 10.3), while in mock-treated plants a smaller, non-significant upregulation was seen (FC = 6.88). No noteworthy difference was observed in uninfected plants treated with PA (FC = 2.93; see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). Remarkably, expression of *OsCPS2* was not upregulated upon infection one dpi in any of the three treatments. This could be due to a low infection pressure or due to the early stage in the infection process.

However, three dpi, significantly higher expression levels of both *OsCPS2* and *OsCPS4* were observed in mock-treated, infected plants (FC = 28.9, FC = 25.8, respectively). Meanwhile, both genes were much more weakly, and non-significantly, upregulated in PA-treated plants, both in those with and without *P. oryzae* infection (FC = 4.25 and FC = 6.41 respectively in PA-treated, non-infected plants and FC = 2.95 and FC = 2.78 respectively in PA-treated, infected plants; see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). These lack of strong induction of *OsCPS2* and *OsCPS4* at three dpi might again be explained by the much higher infection pressure in mock-treated plants. The early induction of *OsCPS4* in infected, PA-treated plants compared to mock-treated, infected plants at one dpi might contribute to the PA-IR phenotype, a result in accordance with the conclusions of Hasegawa *et al.* (2010) who showed early induction of *OsCPS4* in resistant compared to susceptible rice cultivars.

OsNOMT encodes naringenin 7-*O*-methyltransferase, an enzyme involved in the production of sakuranetin. Being the major flavonoid phytoalexin in rice, sakuranetin plays an important role in the protection of rice against multiple stress factors, including UV radiation and pathogens.

Sakuranetin has been demonstrated to inhibit both germ and mycelial growth of *Pyricularia oryzae* (Cho & Lee, 2015), and it has been observed that higher induced sakuranetin levels in rice plants are correlated with higher blast resistance (Kodama *et al.,* 1992). The results obtained in this thesis show that expression of *OsNOMT* one dpi was upregulated in both mock-treated infected plants (FC = 7.92) and PA-treated infected plants (FC = 55.3), but a much higher upregulation was observed in PA-treated infected plants. This indicates that early accumulation of sakuranetin might contribute to the PA-IR phenotype and that there could be a primed accumulation of sakuranetin upon infection. Remarkably, whereas at three dpi expression of the defense-related gene *OsPR1a* was lower in PA-treated infected plants compared to mock-treated infected plants, *OsNOMT* expression remained much higher in infected plants treated with PA (FC = 22.9) than in mock-treated, infected plants at this time point (FC = 9.60: see chapter Results paragraph 3.3.5 Piperonylic acid treatment induces resistance in rice plants against *Pyricularia oryzae*). This result further supports the hypothesis that sakuranetin production plays a role in PA-IR in rice against *P. oryzae*.

In summary, the data show the potential of alternative, more sustainable plant protection products, being beneficial bacteria and induced resistance in the control of two of the most important diseases in rice. Besides, the data indicates the importance of a phytoalexin of the phenylpropanoid pathway in PA-IR in rice against the pathogen *P. oryzae.*

4.4. Future perspectives

Our results support the hypothesis that several *Pseudomonas* strains produce secondary metabolites with a direct nematostatic effect, although – contrary to our initial hypothesis – these metabolites might not be cyclic lipopeptides. Further research might thus be performed to identify the non-CLP nematostatic compounds that some of the tested *Pseudomonas* strains appear to produce. Given that previous literature suggests that several volatile compounds produced by *Pseudomonas* spp. are nematocidal, gas chromatography analysis of supernatants from the strains used in this thesis might be a useful approach.

Several *Pseudomonas* strains have recently been shown to be capable of inducing resistance against plant-pathogenic nematodes, as recently reviewed by Subedi *et al.* (2020). Moreover, *Pseudomonas* strains also aid in control of other diseases and pathogens, by both a direct antagonistic effect and an induction of defense capacity in plants. Multiple reviews have been published on this subject (Haas & Défago, 2005; Mercado-Blanco, 2015; Oni *et al.*, 2015; Weller, 2007). Given the nematostatic activity observed in this thesis and the encouraging results published to date, *Pseudomonas* strains from the plant rhizosphere warrant further study for use in IPM of plant-parasitic nematodes. Further research into the strains used in this thesis should focus on establishing their efficacy at nematode control *in planta.*

Commercialization of *Pseudomonas* strains remains difficult. In contrast to plant-beneficial *Bacillus* strains, *Pseudomonas* strains are not able to sporulate, resulting in shorter shelf-lives and a more difficult formulation (Tabassum *et al.*, 2017). Moreover, application of microbial strains in general has variable performance, because root colonization is a highly complex process (Tabassum *et al.*, 2017). Yet, commercialization is not impossible, as shown by for

example Cedomon, Cerall and Cedress, which are based on *P. chlororaphis* MA342, commercialized as a fungicide by BioAgri AB (Anderson & Kim, 2018; Mehnaz, 2016). Further experiments into the potential of the strains evaluated in this thesis might involve comparing live *Pseudomonas* bacteria with supernatants (or extracts thereof) as nematode control products *in planta.* The latter would likely be easier to formulate and apply.

The second part of this thesis focused on the ability of PA to induce resistance in rice against the hemibiotrophic rice pathogen *P. oryzae*. During this thesis, PA was shown to induce resistance against *P. oryzae* as a foliar spray at a dose of 300 µM and as a soil drench at a dose of 50 µM (in a six times larger volume of water, so that the total amount of PA per plant was approximately equal). In follow-up experiments aimed at developing PA for use in IPM, it would be interesting to investigate the dose-dependency and importance of moment of application to induce resistance against *P. oryzae*.

P. oryzae is a hemibiotrophic fungus. In earlier experiments, PA-IR was shown to be effective against plant-parasitic root-knot nematodes, the bacterial pathogen *P. syringae* and the necrotrophic fungus *B. cinerea* in dicots (Desmedt *et al.,* 2021). It could be interesting to further investigate whether PA can also induce resistance against necrotrophic fungi in the monocot rice, and even more interesting to investigate whether PA would be effective against biotrophic fungal pathogens, such as *Ustilaginoidea virens* (Sun *et al.,* 2020), to further investigate the activity spectrum of PA-IR.

Another interesting avenue for further research would be to investigate the molecular mechanisms of PA-IR in the rice-*P. oryzae* pathosystem. An obvious starting point for this is the role of phenylpropanoid pathway-derived phytoalexins. The very strong, primed induction of *OsNOMT,* combined with the trend towards higher PAL activity, indicates that phenolic phytoalexins might be important to PA-IR. To this end, it would be interesting to repeat the experiment and to assess the expression of other genes in the same pathway (e.g. *OsCHS* and *OsCHI*; Cho & Lee, 2015*)* and to measure sakuranetin levels directly, e.g. by using mass spectrometry. The importance of sakuranetin to PA-IR could be investigated further by using knock-out mutants in *OsNOMT*.

5. General conclusion

The objective of this thesis was to answer three research questions:

- Do cyclic lipopeptides produced by *Pseudomonas* strains show direct nematostatic activity towards *Meloidogyne graminicola?*
- Do CLP-producing *Pseudomonas* strains induce resistance in rice plants against *Meloidogyne graminicola*?
- Can piperonylic acid induce resistance in rice against *Pyricularia oryzae*?

This thesis has shown that the answer to the first question is complex. Our results show that supernatants from *Pseudomonas* sp. CMR12a, *Pseudomonas* sp. COR18, *P. putida* RW10S2 and *P. tolaasii* CH36 significantly impair nematode mobility. However, it appears that this activity cannot be attributed to CLPs, since mutants deficient in CLP production show equal or even higher immobilization rates. These results suggest that other secondary metabolites, possibly volatile compounds such as cyanide, cause the observed nematostatic activity. Identifying which compounds are involved in the nematostatic activity of the tested *Pseudomonas* strains requires further research.

The second question was not answered in this thesis, as we failed to obtain good colonization by both *Pseumonas* sp. and *M. graminicola.* In order to ensure good infection by *M. graminicola* and good colonization by *Pseudomonas*, further optimization of the used substrate would be needed for future research.

The third research question concerned the ability of PA to induce resistance in rice against the rice blast pathogen, *Pyricularia oryzae.* Our results show PA induces resistance against *P. oryzae,* as the number of sporulating lesions was significantly reduced in plants treated with PA, compared with infected plants. No phytotoxic effects of PA were observed during the experiments, which further supports the potential utility of this molecule at *P. oryzae* control. To further elucidate how PA induces resistance, biochemical assays and RT-qPCR were used. The resulting data suggest that PA-IR in rice against *P. oryzae* is dependent at least partially on phytoalexin biosynthesis, as PAL activity appeared to be higher in plants treated with PA and genes responsible for production of sakuranetin and diterpenoid phytoalexins were upregulated upon infection in plants treated with PA. Conversely, no clear evidence for the involvement of the plant hormones SA and JA or for lignification were found.

6. References

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