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Study of the growth parameters of *Polymyxa graminis* f. sp. *colombiana* on rice roots and identification of the first candidate effectors through bioinformatics

Author : Julien van den Eynde de Rivieren
Advisors : Prof. Anne Legrève
Ir. Margaux Genard
Readers : Prof. Claude Bragard
Prof. Michel Ghislain

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Thesis presented in partial fulfillment of the requirements for the degree of Master in agricultural bioengineering.

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Abstract

Polymyxa graminis f. sp. *colombiana* is the vector of the *Rice stripe necrosis virus* (RSNV) and infects rice (*Oryza sativa*). Rice in a major staple food for global consumption, particularly in developing countries, and the RSNV threatens its production by reducing its yield to up to 40%. As of today, the only efficient control against the virus is the use of resistant cultivars and the adoption of culture rotation practices.

A key parameter involved in the infection of host plants by parasites and pathogens is the expression of effectors. Effectors are defined as proteins and small molecules that alter host cell structures and functions, thereby facilitating the colonization of pathogens. As some of these effectors are essential for infection of the host, disturbing their function could lead to novels way of disease control. Therefore, this study aims at identifying candidate effectors responsible for host infection through comparison of genomic data with other relatives of the Plasmodiophorid family.

Identification of candidate effectors is a two steps process. The first aims at identifying effectors through bioinformatics, and the second aims at confirming their presence in a pathosystem involving Pgcol and rice. For that reason, Pgcol first had to be produced on rice.

In this study, Pgcol was grown on rice without much success. Consequently, four bioassays were conducted to study the ecological parameters involved in the development of Pgcol in rice. The influence of the nutritive solution pH, the type of watering system (collective or individual), the life form serving as inoculum and the dynamic of infection were studied. Additionally, the extraction of sporosores and the disinfection of rice seeds were studied.

At the end of this study, no bioassay successfully produced satisfactory amounts of Pgcol. Our results indicate that using individual system over collective is more appropriate for Pgcol production. Several other parameters are hypothesized to potentially improve its production, but more study should be addressed to confirm them.

Predicting candidate effectors was made by using BLAST software by comparing genomic data of Pgcol with three other plasmodiophorids: *Pollymyxa betae*, *Plasmodiophora brassicae*, and *Spongospora subterranea*.

One hundred and nine candidate effectors were identified for the first time for Pgcol. Eleven of them correspond to candidate effectors of Pbras and are described in the literature. Interestingly, one is involved with the disruption of the chitin-triggered immunity of the host plant. Further study of the candidate effectors should be made to confirm their presence and function *in planta*.

List of abbreviations

ABA	Abscisic Acid
Anks	Ankyrin-repeat domains
BaMMV	*Barley Mild Mosaic Virus*
BaYMV	*Barley Yellow Mosaic Virus*
BET	Ethidium bromide
BLAST	Basic Local Alignment Search Tools
Bleach	Sodium hypochlorite
BNYVV	*Beet Necrotic Yellow Vein Virus*
bp	base pair
BSA	Bovine Serum Albumen
CBM18	Chitin-Binding domains carbohydrate-Binding Module family 18
ChBD	Chitin-Binding Effectors
CKs	Cytokinin
CWDEs	Cell Wall Degrading Enzymes
CWMV	*Chinese Wheat Mosaic Virus*
DNA	Desoxyribonucleic Acid
dsRNA	double-stranded Ribonucleic Acid
ERF	Ethylene Response Factor
ET	Ethylene hormone
ETI	Effector-Triggered Immunity
FAO	Food and Agricultural Organisation
FAOSTAT	Food and Agricultural Organisation Statistical Database
GAs	Gibberellins
GPI	Glycosylphophatidylinositol
HR	Hypersensitive response
IPCV	*Indian Peanut Clump Virus*
JA	Jasmonic Acid
MAMPs	Microbe-Associated Molecular Patterns
MAPK	Mitogen-Activated Protein Kinases
mRNA	messenger Ribonucleic Acid
MYC	Myelocytomatosis
NB-LRR	Nucleotide Binding – Leucine Riche Repeat
NGS	Next Generation Sequencing
NOI	Nitrogen Oxide
OGSV	*Oat Golden Stripe Virus*
OMV	*Oat Mosaic Virus*
PAMPs	Pathogen-Associated Molecular Patterns
Pb	*Polymyxa betae*
Pbras	*Plasmodiophora brassicae*

PCR	Polymerase Chain Reaction
PCV	*Peanut Clump Virus*
Pg	*Polymyxa graminis*
Pgcol	*Polymyxa graminis* f. sp. *colombiana*
PLCPs	Papain-Like Cysteine Proteases
PME18	Pectine Methylesterase
PMTV	*Potato Mop-Top Virus*
PRRs	Pattern Recognition Receptors
PRs	Pathogenesis-Related Proteins
PTI	Pattern-Triggered Immunity
R	Resistance
rDNA	Ribosomal DNA
RGAs	Resistance Genes Analogues
RLKs	Receptor-Like Kinases
RNA	Ribonucleic Acid
RNA-seq	RNA-sequencig
RNMV	*Rice Necrosis Mosaic Virus*
ROS	Reactive Oxygen Species
RSNV	*Rice Stripe Necrosis Virus*
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SA	Salicylic Acid
SBCMV	*Soil-Borne Cereal Mosaic Virus*
SBWMV	*Soil-Borne Wheat Mosaic Virus*
SP	Signal Peptide
SrCSV	*Sorghum Chlorotic Spot Virus*
SSPbPs	Small Secreted *Plasmodiophora brassicae* Proteins
Ssub	*Spongospora subterranea*
STAND	Signal Transduction ATPase with Numerous Domains
T3SEs	T3 Secreted Effectors
T3SS	T3 Secretion System
TBE	Tris Borate EDTA
VI	Vigor Index
WSSMW	*Wheat Spindle Streak Mosaic Virus*
WYMW	*Wheat Yellow Mosaic Virus*

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A. Introduction

Rice (*Oryza sativa*) is a major staple food for global consumption, particularly in developing countries, as it plays a crucial role in food security (Muthayya et al. 2014). In 2021, approximately 780 million metric tons of rice had been produced over the world (FAOSTAT, 2023). Given its high consumption, estimated to up to 85 percent of global production (Muthayya et al. 2014), there is a strategic need to maintain this crop in developing countries. Therefore, preserving a high production by minimalizing disease-related pathogens must be addressed.

Rice is affected by several bacterial diseases such as the *sheath brown rot* (*Pseudomonas fuscovaginae*), the *bacteria stripe* (*Acidovorax avenae* subsp. *avenae*), *the bacterial blight* (*Xanthomonas oryzae* pv. *oryzae*), or the *bacterial leaf streak* (*Xanthomonas oryzae* pv. *oryzicola*) (IRRI, 2023). Another important disease in rice is caused by a virus known as the *Rice stripe necrosis virus* (RSNV). It was first detected in Ivory Coast (Fauquet et Thouvenel 1977), then in Colombia, Ecuador, Panama (Morales et al. 1999), but also in Burkina Faso (Sereme et al. 2014), Benin (Oludare et al. 2015), Mali (Decroës et al. 2017), Sierra Leone (Tucker et al. 2020), and Brasil (De Souza et al. 2021), and causes significant damages ranging from 37 to 80% in West African countries and up to 40% yield losses in South and Central America (Bagayoko et al. 2021). Its presence was always associated with *Polymyxa graminis* (Pg) (Fauquet et Thouvenel 1983).

Pg is an obligate endoparasite of roots of many grasses including rice (Ledingham 1939) that causes no known harmful impacts on plants it affects. However, it is the vector of several viruses belonging to the *Benyvirus*, *Bymovirus*, *Furovirus* and *Pecluvirus* genera. These viruses are known to causes severe threats on their hosts (Kanyuka et al. 2003; Sereme et al. 2014). *Polymyxa spp*. are protists belonging to the family of Plasmodiophorids, alongside with *Plasmodiophora sp.* and *Spongospora sp*. (Webster et Weber 2007).

Plasmodiophorids share the same life cycle and have been studied for a long time. However, little is known about the nature of the relationship between hosts and these parasites. In particular, the exact parameters for growth and infection have yet to be all discovered. Additionally, the development of molecular biology techniques at the end of the 20th century has brought new insights on the molecular relationship between hosts and pathogens. It is not until the late 2000s, that plant metabolic pathways were unraveled thanks to the development of transcriptomics and proteomics technologies. It allowed the assembly of the first *Polymyxa species* genome in 2019 and offered new opportunities for studying interactions between *Polymyxa* and his host.

Successful infection of host plant depends on the ability to sequester the necessary nutrients required for growth and reproduction. Secreted proteins, known as “effectors” are believed to be the key governing factors determining host infection and colonization. Effectors are capable of suppressing plant defense responses and later plant physiology to accommodate the pathogenic invaders (Selin et al. 2016). But as

no genomic data were available so far for *Polymyxa graminis* f. sp. *colombiana* (Pgcol), none have yet been identified, hence this present study on identification of candidate effectors.

Currently, control of RNSV disease is difficult as no curative measures are available for its management. Moreover, the virus and its vector can remain in the soil for numerous years, making it even harder for management. Furthermore, new transcriptomics data have shown that *Polymyxa* could manipulate his host immune system and hide from it. One hypothesis would be that effectors play a role in it. Identification of essential effectors protein for infection could therefore help to develop novel methods of control before the occurrence of vector infection.

B. Objectives

This study has been made as part of the thesis of Margaux Genard aiming at assembling the first genome of *Polymyxa graminis* f. sp. *colombiana* and identify the diversity of *Polymyxa* spp. This would allow to review their phylogeny and better understand the interactions between the several actors of the growth system and the parasite. It includes host-*Polymyxa* interactions via the role played by effectors in the infection process and how they intervene in host-*Polymyxa* compatibility.

The final objective of this study is the identification of candidate effectors involved in infection of rice plant by Pgcol. The identification process starts with the comparison of secreted proteins from Pgcol with other secreted and candidate effectors of plasmodiophorids. Then an experimental confirmation would be required. Because of the obligate endoparasitic nature of Pgcol, its growth and development cannot be achieved in axenic conditions. Therefore, a well mastered system must first be developed. Rice plants were grown under controlled conditions in a greenhouse and contaminated with several isolates of Pgcol using several methods of watering. Ecological parameters and parameters outside the system were also studied. Mastering the ecological parameters required for the proper development of Pgcol must be achieved before studying the dynamic of infection. This would allow to properly pinpoint the moment when effectors are released by Pgcol to counter the defense system of its vegetal host.

CHAPTER 1: LITERATURE REVIEW

This review is divided in two sections. A first one discussing the vector, its taxonomy, life cycle, morphology and development, ecological conditions, host range and the virus it transmits; and a second discussing the effectors, their implication in the plant immune system, their target and mechanism of identification, and finally the effectors of other plasmodiophorids. Among the many hosts and viruses of Pg, an accent will be made on Asian rice (*Oryza sativa*) and RSNV.

1.1 *POLYMYXA GRAMINIS* AND OTHER PLASMODIOPHORIDS

Pg was first identified by Ledingham (1939) as an obligate parasite of roots of many grasses (groundnut, barley, rye, oats, wheat, rice) (Barr 1979; Ledingham 1939) that causes no known harmful impacts on plants it affects. However, it must be well understood that Pg is a vector of several cereal viruses causing major yield losses around the world (Kanyuka et al. 2003), listed in at least four major genera or *Benyvirus, Bymovirus, Furovirus* and *Pecluvirus* (Kanyuka et al. 2003; Sereme et al. 2014). Among its many hosts are found: maize, peanut, sorghum, sugarcane, and small grain cereals such as barley, wheat, and rice (Adams et al. 1988; Dieryck et al. 2011; Rao et Brakke 1969; Ratna et al. 1991; Slykhuis et Barr 1977; Thouvenel et Fauquet 1981).

1.1.1 Taxonomy

Plasmodiophorids have been first classified as belonging to the Kingdom of Protozoa by Barr (Barr 1992) and can be considered as a monophyletic group. Several genera (Figure 1) are found in the Plasmodiophoridae family (de Vienne 2016), but only three will be discussed and studied in this work: *Plasmodiophora*, *Spongospora*, and *Polymyxa spp*. These three genera have been chosen because of their available genomic data, enabling further comparison of candidate effectors. The common structural features shared by Plasmodiophorids are the following: **a**. cruciform nuclear division, **b**. zoospores with two, anterior, whiplash flagella of unequal lengths, **c**. multinucleated protoplast (or plasmodia), **d**. environmentally resistant resting spores, and **e**. obligate, intracellular parasitism (Braselton 1995). They differ, however, when it comes to the damages they cause. On one hand, *Plasmodiophora brassicae* (Pbras) and *Spongospora subterraneae* (Ssub) are gall makers. On the other hand, *Polymyxa* species dot not cause direct harm to their vegetal hosts (Dixon 2006; Keskin 1964; Kirk 2008; Ledingham 1939) but are known vectors for several viruses. Ssub is also a virus vector as it transmits the *potato mop top virus* (PMTV).

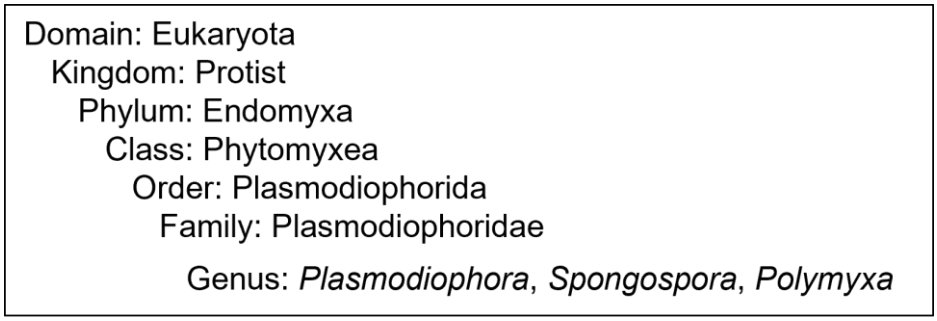

Figure 1: Taxonomy of Plasmodiophorids and several genera studied in this work.

Polymyxa graminis

Up to date, two *Polymyxa* species have been identified, Pg Ledingham (Ledingham 1939) and *Polymyxa betae* (Pb) Keskin (Keskin 1964) based on host range. It has long been debated whether to identify them as fungi or protists. However, it was proposed to classify them as protist until more data was gathered (Barr 1992). More recently (2018), Cavalier-Smith proposed a new classification where *Polymyxa* species are categorized as belonging to the kingdom of Chromista (Cavalier-Smith et al. 2018). Nonetheless, since the theory of Cavalier-smith is still strongly debated, Plasmodiophorids will be classified as protist in this study.

Within Pg, five *formae speciales* (Figure 2) were described based on ecotypes, host range, geographical distribution, and rybotypes: *P. graminis* f. sp. *colombiana* on rice in Africa and South America; *P. graminis* f. sp. *subtropicalis* on barley, maize, sorghum, pearl millet, or wheat in the Indian subcontinent and West Africa; *P. graminis* f. sp. *temperata* on barley or wheat in Belgium, Canada, China, France, Germany, and the United Kingdom; *P. graminis* f. sp. *tepida* on barley, oat, or wheat in Belgium, Canada, and the United Kingdom; and *P. graminis* f. sp. *tropicalis* on maize, sorghum, or pearl millet in India and West Africa (Legrève et al. 2000, 2002).

Polymyxa betae

Pb Keskin was first identified in 1964 (Keskin 1964) on beets. It does not cause any known damages on beets but is, however, the vector of several viruses: the *Beet necrotic yellow vein virus* (BNYVV), *Beet soil-borne virus* (BSBV), *Beet soil-borne mosaic virus* (BSBMV) and *Beet virus Q* (BVQ) (Biancardi et Lewellen 2016; Rysanek et al. 2006). The BNYVV is the most damaging Pb-transmitted virus, as it is the causal agent of rhizomania on sugar beets. It causes foliar symptoms consisting of wilting and general chlorosis of foliage (Wauters et al. 1997). Roots symptoms begin as a light brown discoloration of the vascular rings within the taproot (Biancardi et al. 2002). Classical root symptoms following early infection include small, severely stunted taproots with masses of fine, hairy secondary rootlets giving the roots a “beard”, hence the name “rhizomania” (meaning: root madness) (Harveson 2021).

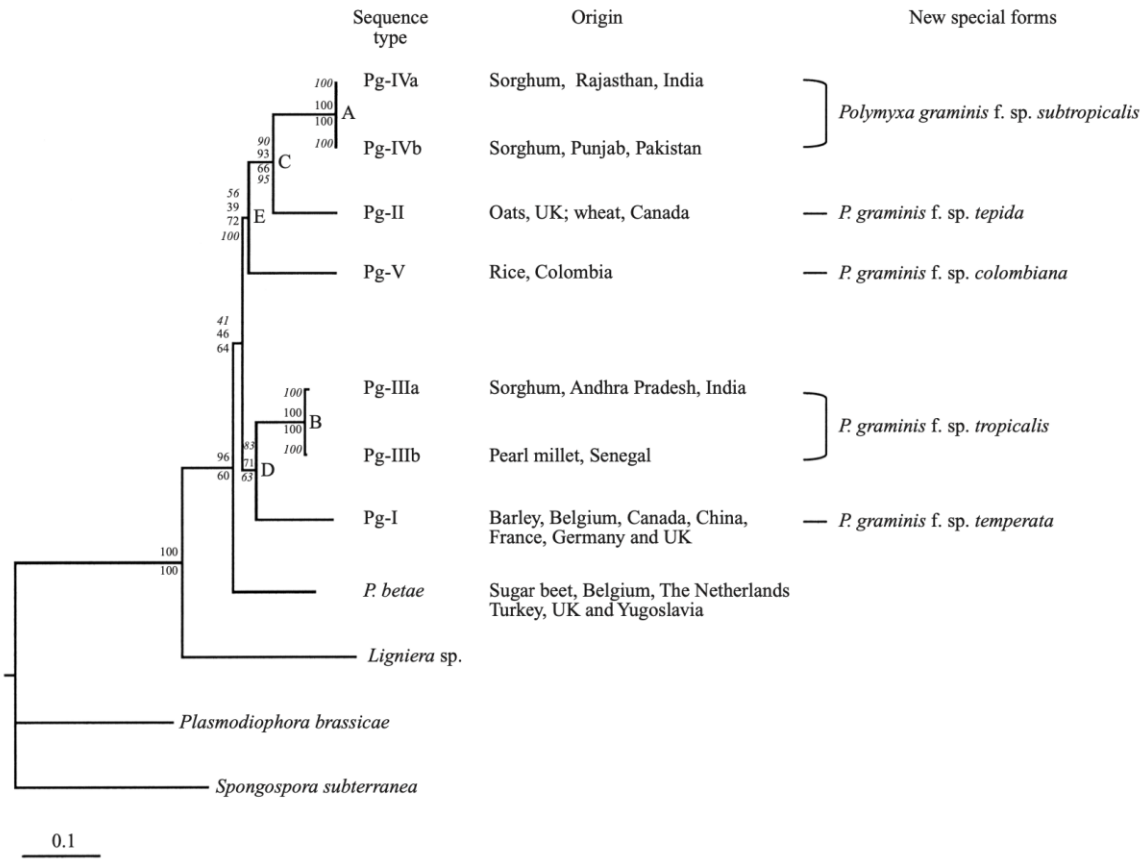

Figure 2: Phylogenetic analysis of Plasmodiophoromycetes on the basis of the rDNA ITS1-5.8S gene-ITS2 sequences. For *Polymyxa graminis*, the origin of associated isolates and the proposed new special forms are given. Tree built by NEIGHBOR analysis using *Spongospora subterranea* as outgroup. The numbers above and below the internodes indicate bootstrap frequencies obtained using the neighbour-joining method and the parsimony method, respectively. In the absence of a number below the internodes, the bootstrap tree was distinct from the one illustrated. The numbers in *italic* indicate the bootstrap frequencies when *Polymyxa* sequences were rooted only with *Ligniera sp*. (Legrève et al. 2002)

Plasmodiophora brassicae

Pbras is the most extensively studied species of the Plasmodiophorids up to date. It is the causal agent of the clubroot disease (Siemens et al. 2009) characterized by the development of galls on infected roots. This disrupts water and nutrient uptake in affected plants, resulting in wilting, stunting and premature ripening of the above-ground organs (Dixon 2006). Pbras preferentially affects the Brassicae family, especially *Brassicae* and associated genera (including *Arabidopsis* and Raphanus) (Dixon 2009; Howard et al. 2010). Clubroot is known to occur in more than 80 countries and results in a 10 to 15% reduction in yields on a global scale (HWANG et al. 2012; Javed et al. 2022).

Spongospora subterranea

Ssub, which is responsible for the development of powdery scab on potatoes, also acts as a vector for the PMTV (Kirk 2008), a *Furovirus*. Powdery scab leads to the formation of lesions on the surface of potato tubers, characterized by scab-like appearances and filled with a predominantly sporosori-based brown powder. Initially, the symptoms manifest as purple or brown pimple-like swellings visible to the naked eye. With the progression of maturation, these pustules enlarge and eventually rupture the periderm. As the lesion continues to mature, it transforms into a shallow cavity containing a powdery mass of resting spores from the pathogen, appropriately known as a scab. This disease can significantly reduce potato yields, occasionally up to 50% (Harrison et al. 1997).

1.1.2 Life cycle and morphology

Although the life cycle of Plasmodiophorids (Figure 3.a) is not yet completely understood, it is well known that they present two stages: the sporangial stage and the sporogenic stage (Barr 1988). The sporangial stage can be seen as the first step of development that produces mobile structures called zoospores that initiate the infection. The sporogenic stage can be seen as the second step occurring once the infection is more advanced and that leads to the production of resting spores, the survival form of Plasmodiophorids (Kanyuka et al. 2003).

It all starts with a single haploid zoospore, called primary zoospores, that germinates from a resting spore (Figure 3a) (Kanyuka et al. 2003). The germination is believed to be mediated by several secreted proteins, called effectors (Balotf et al. 2021), but the exact mechanism has yet to be discovered. It is also thought that zoospores are attracted by several sugars and amino acids exuded by their host root system, but the exact molecules attracting zoospores are not known (Gleason et Lilje 2009). The zoospore then encysts itself in the root of the host. It involves the extension of its structure to form an adhesorium that contains a tubular structure, the Rohr, housing a projectile-like structure called the Stachel required for mechanic penetration of the host's cell wall (Figure 3.b) (Braselton 1995; Kanyuka et al. 2003; Keskin 1964). Almost instantaneously the content of the zoospores, along with the Stachel, is being injected in the host cytoplasm thanks to the swelling of the vacuole (Kanyuka et al. 2003).

Once inside the cell, the zoospores content starts to grow with several synchronous mitotic cruciform nuclear divisions until forming a multinucleate plasmodium (Braselton 1995). Thanks to transmission electron microscopy, cruciform nuclear division was characterized as a mitotic division where the nucleus is elongated perpendicularly to the metaphase plate of chromatin (Braselton et al. 1975; Dylewski et al. 1978). Eventually, a multinucleate sporangial plasmodium, or primary plasmodium is being formed. This process happens through the separation of the nuclei produced in a non-

cruciform manner, and its separation from another by septation (Kanyuka et al. 2003; Webster et Weber 2007).

Sporangial plasmodia then produce thin-walled zoosporangia containing zoospores, called secondary zoospores. Once cruciform divisions have stopped, non-cruciform nuclear division start in zoosporangia as they are being cleaved from the plasmodia, eventually forming secondary zoospores (Braselton 1995). Non-cruciform divisions in sporangial plasmodia are not meiotic as are those in sporogenic plasmodia. These

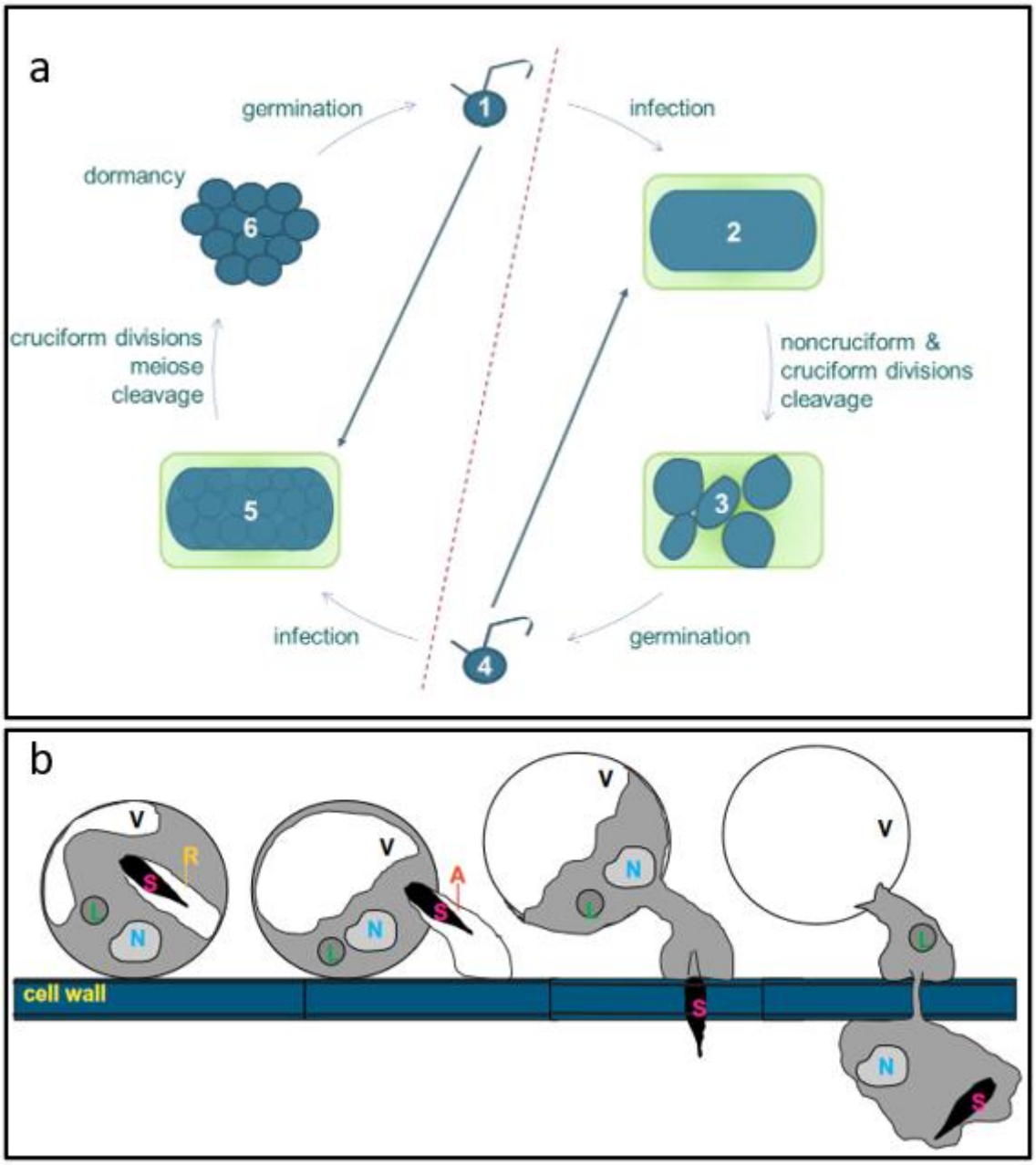

Figure 3: Schematic representation of Pg life cycle and schematic representation of zoospore encystment and penetration of root cells. a-1. Primary zoospore. **a-2.** Plasmodia. **a-3.** Zoosporangia. **a-4.** Secondary zoospores. **a-5.** Sporogenic plasmodia. **a-6.** Resting spores. The red doted line separates the primary infection (on the right) from the secondary infection

(on the left). **b.** Diagram of zoospore encystment and penetration of root cells (S: Stachel; R: Rohr, A: adhesorium, N: nucleus, V: vacuole, L: lipid droplet). Adapted from Decroës (2022) and Kanyuka et al. (2003).

zoospores are called secondary zoospores since they originate from a sporangium and not from a resting spore. Secondary zoospores are then released in the environment or can further infect the host, increasing the number of zoosporangia (Barr 1988).

Eventually, they can lead to the formation of secondary plasmodium or sporogenic plasmodium that are gradually being compartmentalized by septa into multiple lobes which then undergo meiotic nuclear divisions and produces resting spores (Braselton 1995; Decroës 2021; Garber et Aist 1979).

The zoospores are being released into the environment or adjacent cells thanks to exit tubes that were previously formed from zoosporangia (Littlefield et al. 1998). An agglomeration or resting spores, also known as sporosores, are released in the environment after the host tissues break down and serve as the survival structure of the organisms. As time pass, new and older resting spores and older one germinate at different times, thus leading to the overlapping of both sporangial and sporogenic phases (Kanyuka et al. 2003).

1.1.2.1 Pre infectious structure

Resting spores

A resting spore is a structure that can remain dormant for a long period of time. And a sporosore (Figure 4.6) indicated a cluster of resting spores.

Mature resting spores size is commonly about 4 x 5 µm in diameter and appears to have a convex form at their apical end while presenting conical projections at their upper end (Littlefield et al. 1998). However, since its spread is uncontrolled, it will take the shape of the cell it is growing in (Ledingham 1939). Mature sporosores appear to be more discoid or planar than spherical. They are commonly only two to four resting spores thick (Littlefield et al. 1998).

Mature resting spores are uninucleate and contain abundant mitochondria, strands of rough endoplasmic reticulum, Golgi bodies, well-distributed vacuoles, vesicles containing a granular matrix and numerous free ribosomes. Nuclei' size is around 1.6 µm in diameter and each contains a spherical acentric nucleolus (around 0.4 µm in diameter) (Chen JianPing et al. 1996).

Polymyxa sporosores are restricted to cortical and epidermal cells (Littlefield et al. 1997). They are extended throughout much of the length of most root segments, and occur mostly in the proximal half branch roots and into adjoining areas of the root from which the branch arose. The overall size of a sporosore and the number of individual

resting spores per sporosore varies greatly. The cross-section dimension of a sporosore is quite uniform, typically 25 to 35 µm since it is being restricted by the diameter of the host cell it occupies. Sometimes a large sporosore can reach the size of 70 to 90 µm when occupying the entirety of the host cell. The number of resting spores by sporosore varies greatly because of this large variety of size, it ranges from 20 to several hundred (Littlefield et al. 1997).

1.1.2.2 Infectious structures

Primary and secondary zoospores

As mentioned above, two types of zoospores appear during the development of *Polymyxa* species. The primary type is produced by resting spores while the secondary (Figure 4.4 and 4.4bis) is produced upon germination of zoosporangia, and both forms appear similar. Zoospores have a globular/ovate shape of 3.8 up to 7 µm in diameter (Barr et Allan 1982; Ledingham 1939). Secondary zoospores may assume a more flatten shape while being inside their zoosporangia due to pressure exerted from the neighboring cells. Zoospores possess two whiplash flagella of unequal length. The longer emerges from the zoospore posteriorly, while the shorter one (about one quarter or the long one) emerges in an anterior to lateral position but bends back around the zoospores to be positioned laterally or almost posteriorly (Barr et Allan 1982; Ledingham 1939). Zoospores have been found to present multiple vesicles such as conspicuous Golgi apparatus, vesicles and vacuoles of various size, mitochondria and flagellar apparatus (Barr et Allan 1982).

Secondary zoospores are cleaved apart within the cytoplasmic contents of a zoosporangium. As the number of immature zoospores increase, they assume distinctly angular shapes, tightly packed within the zoosporangium. Eventually, each zoospore leaches out of the zoosporangia and appears at first as a small bubble on the surface of the host cell. Zoospores become more rounded as they mature, with flagella being common in intercellular areas that develop progressively between zoospores during those stages. Mature zoosporangia typically become single units or have only a few compartments and contain hundreds of secondary zoospores. Zoospores are release to the outside of the root, into root intercellular spaces, into adjacent host cells or into the same host cell as the zoosporangia. Following release of their zoospores, zoosporangia remain intact as essentially empty structures within their host cells (Littlefield et al. 1998). As it contains no vesicles, zoospores are mature as soon as they leave the discharge tube (Ledingham 1939).

Both types of zoospores move similarly, in a rolling/turning motion thanks to their flagella that lashes around. Depending on the speed at which the zoospore is swimming, its shape might change from spherical at rest to more oval while moving. In most cases, the flagella propel the zoospores and thus are found at the rear. The period during which a zoospore is actively moving ranges from two to three hours or

even more under particularly favorable conditions (Ledingham 1939). As time passes, the activeness of the zoospores decreases up to being motionless and assume once again a more spherical shape, at such point they often lose their infective properties (Keskin 1964; Ledingham 1939).

During encystment, the zoospores enter the root cell through a tubular structure (Rohr) containing a dagger-like body (Stachel) (Figure 3.b). The zoospores cytoplasm is then transferred by the adhesorium to the host cytoplasm. Following the injection, the nucleus of the zoospore rapidly undergoes synchronous nuclear division, resulting in the formation of multinucleate plasmodium (Rochon et al. 2004).

1.1.2.3 Post penetration structures

Sporangial plasmodia

Sporangial plasmodia (Figure 4.2 and 4.2bis) are the first vegetative stage following primary infection. At first, plasmodia are small, multinucleate, rather amorphous masses, lacking a clearly distinguishable limiting membrane. Soon, they appear as numerous clearly defined structures within the host cells commonly located along host cell walls. As plasmodia increase in size, they remain multinucleate (Littlefield et al. 1998).

Sporangial plasmodia produce thin-walled zoosporangia, which contain secondary zoospores. After cruciform divisions have ceased, non-cruciform nuclear divisions occur in secondary plasmodia during the cleavage of the plasmodia into sporangial lobes and subsequently into incipient secondary zoospores. Non-cruciform divisions in sporangial plasmodia are not meiotic as are those in sporogenic plasmodia. Secondary zoospores produced by sporangial plasmodia are released into the environment. After infection of host cells, they may develop into either sporangial or sporogenic plasmodia (Braselton 1995).

Zoosporangia

During early stages of growth, zoosporangia (Figure 4.3) first appear very vacuolate. Later they become larger, more linear structures, as the vacuole gradually decrease in size. Several septa form within each zoosporangium, dividing the latter into segments. Those segments later expand in volume, and many form small exit tubes prior to zoospore formation. Exit tube apices are typically enlarging where they abutted host cell wall. Eventually, zoosporangia become large lobed structures with numerous exit tubes extending from the zoosporangiuma to the host cell wall in which the zoosporangium is contained. The growth is then followed by the appearance of lines of cleavage around each nucleus. At maturity, they are each made of an inner hyaline layer and an outer, smooth, brownish-yellow wall, which is fused with the neighboring cells. The zoosporangial wall is generally uniform in thickness, with the thin, outer

portion being more electron opaque than the thicker, less electron opaque material toward the inside (Ledingham 1939; Littlefield et al. 1997).

The zoosporangium begins its development in the cortical cells of the root as a uninucleate spherical thallus, about 3 to 4 µm in diameter. The next step in the process if the formation of a thallus which will allow the discharge of zoospores in the future. This thallus is created by the successive growth of several nuclei that form lobes one after the other. The thallus growth may either end outside the root or inside an adjacent cell. Should the zoosporangia lie deeper in the cortex of the root, the discharge tubes may then have to pass through adjacent cells to reach the exterior (Ledingham 1939). Usually, there is only one discharging tube per zoosporangia, but in some cases there may be more.

The infection, and thus the development of the zoosporangia, can only be seen once the content of the zoospore has been released inside the cell. It first appears as an immobile, indefinite, difficult to see spot. These spots are first close to the site of infection and later spreading out more widely. Until the stage of maturity, the contents of the zoosporangia appear weakly structured. The homogeneous appearance of the zoosporangia in only marked by its outline. During the last stages of growth, zoospores appear (Keskin 1964).

During the later stages of the development of the zoosporangia, several papillae may appear on a single zoosporangium. Their development is synchronized with the production and maturation process of zoospores. At maturity, the contained zoospores will germinate outside the cell through the zoosporangia (Keskin 1964).

Sporogenic plasmodia

Secondary infection leads to the formation of sporogenic plasmodia (Figure 4.5). Sporogenic plasmodia typically assume large, variable shapes. Such shapes are reflected in the configuration of resulting sporosores formed by cleavage of the sporogenic plasmodia. Only a membrane defines the perimeter of the sporogenic plasmodia (Littlefield et al. 1998).

As the maturation occurs, indistinct cleavages within the plasmodia appear, but no clearly identifiable membranous structures can be observed. As this process evolves, immature resting spores appear. Later, a more distinct electron opaque material is typically appearing between adjacent, immature resting spores, with distinct plasma membrane present on either side of the material. Individual resting spores expand from their initially packed, angular configuration into a more loosely aggregated mass of spherical to elliptical shapes spores. After cruciform divisions have ceased, non-cruciform divisions occur immediately prior to or during cleavage of mature sporogenic plasmodia into resting spores (Braselton 1995). Intercellular spaces formed initially in the interstices grow to make room for the resting spores to grow. Mature sporosores consists of aggregates or resting spores partially fused by remnants of the interstitial matrix. By then, the sporosore is clearly defined. During germination, each resting

spore releases one primary zoospore, which, in turn will initiate the primary infection (Braselton 1995; Littlefield et al. 1998).

In Pb, the first discernible stage of sporosore cleavage is a membranous bilayer that partitions the plasmodial thallus. Progressively thickened walls are built up between the lamina of the bilayer, eventually resulting in numerous thick-walled uninucleate resting spores within mature sporosori (Barr et Asher 1996).

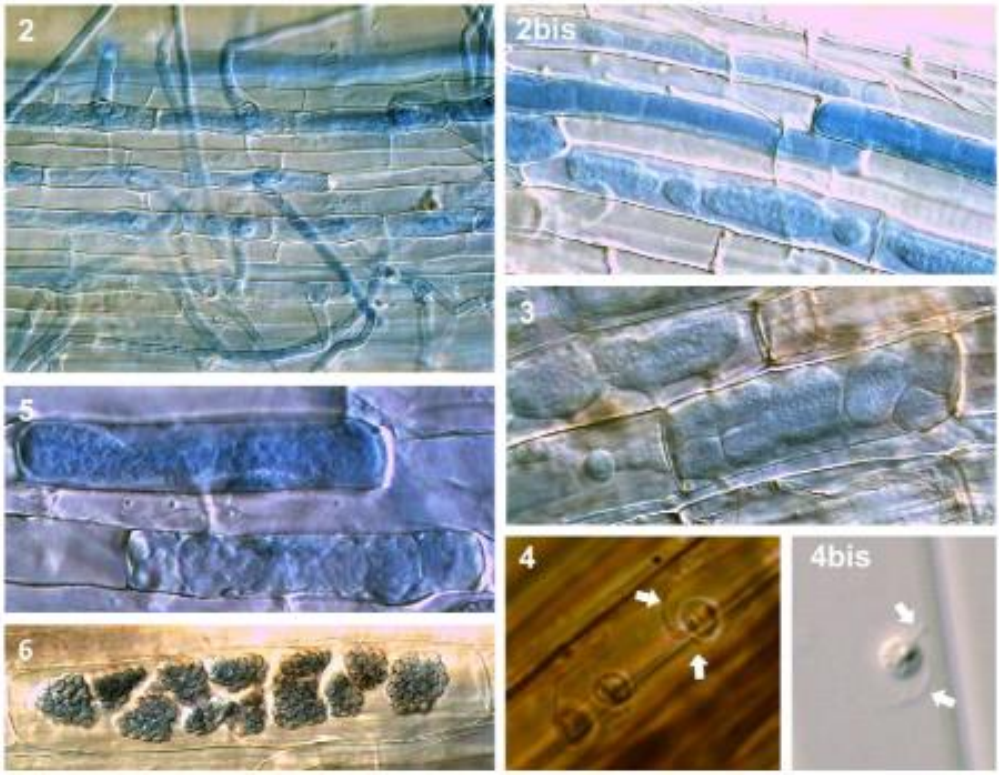

Figure 4: Pictures of each life stage observed in transmitted Nomarski microscopy. Numbers reported in the pictures correspond to those of Figure 3.a. **2**. Rows of cells infected by plasmodia. **2bis.** Zoosporangial plasmodia. **3**. Zoosporangia. **4** and **4bis**. Secondary zoospores inside an outside the roots, respectively. **5**. Sporogenic plasmodia. **6**. Sporosores. White arrows point flagella. Adapted from Decroës (2022).

1.1.3 Ecological requirements

The life cycle of *Plasmodiophorids* is not well understood, as such not all required parameters for efficient growth have been identified. Here are presented some known conditions having an impact on the development of *Polymyxa* genus and its average timeline of infection.

Temperature requirement

Abiotic conditions such as the temperature play an important role in the growth of *Polymyxa graminis formae speciales*.

Polymyxa graminis f. sp. *temperata* grows on Poaceae species including barley and wheat. Infection is favored at temperatures ranging from 15 to 20 °C in temperate regions (Belgium, Canada, China, France, Germany, United Kingdom) (Legrève et al. 1999, 2002)

Polymyxa graminis f. sp. *tepida* also grows on Poaceae species including barley and wheat. Infection is favored at temperatures ranging from 15 to 20 °C (Canada, UK) (Legrève et al. 2002)

Polymyxa graminis f. sp. *tropicalis* infects Poaceae species from tropical regions (India, Senegal), especially sorghum, millet, maize but also barley and wheat. Infection is favored by temperatures ranging from 27 to 30 °C (Legrève et al. 1998, 2002).

Polymyxa graminis f. sp. *subtropicallis* infects Poaceae species such as sorghum and millet, but also barley, peanut, and sugar beet. Infection is favored by temperatures above 23 °C in subtropical regions (India, Pakistan) (Legrève et al. 2002).

Polymyxa graminis f. sp. *colombiana* infects rice. Infection is favored by temperatures above 30 °C (Bagayoko 2022).

Soil pH

The effect of pH of hydroponic solution added to quartz sand on the infection rate of Pg was investigated by Sayama (2010). Three levels of pH ranging from 5 to 7 were tested. Results showed that the infection rate of Pg was 2-2.5% at pH 6 and 7 after eight weeks of germination and 1.8% after ten weeks. Infection rate was 2.3% after eight weeks of germination at pH 5 and 0.5% after ten weeks. This indicated that Pg growth is most favorable at pH 6 and 7 (Sayama 2010). However, Decroës et al. (2017) performed experiments on Pgcol for RSNV detection and determined that a nutritive solution of pH 5.5 was optimal.

Water requirement

As explained before, the infective stage of the life cycle of Plasmodiophorids is the release of zoospores which move in water thanks to their flagella. As such, for the infection to occur, a high content of free liquid water in the soil is needed (Adams et Swaby 1988). It has been suggested that the optimum water content for infection should be slightly less than saturation. A decrease in infection in saturated soil was observed, suggesting that oxygen limitations may override the increase potential for zoospores motility in wetter soils (i.e., at saturation) (Himmel et al. 1992).

Root exudates

The impact of root exudates has been studied on Pbras and its presence increases the germination rate of Pbras (Rashid et al. 2013) compared to resting spore. It can be hypothesized that roots exudates also play a role in the development of Pg as for Pbras.

Zoospores motility

Ledingham (1939) first reported that Pg zoospores could swim for different amount of time depending on the temperature. Adams et Swaby (1988) then studied the effects of several chemical on the motility of zoospores and determined that adding bovine serum albumen (BSA) could increase the swimming time of zoospores and with it their infective properties.

Timeline of infection

In general, the first infection from sporosores is visible two weeks after inoculation. Then depending on the *formae speciales* (i.e. Pg f. sp. *tepida* and Pg f. sp. *temperata* develop more slowly than Pgcol) and hosts, the other specific structures of the life cycle of *Polymyxa* appear. Zoosporangia may appear as early as two weeks after first infection, but for massive development it can be said that they appear within six to twelve weeks and after 14 weeks sporosores starts to appear. Finally, after 19 weeks, sporosores can be considered as matured (Adams et Swaby 1988; Chen JianPing et al. 1996; Legrève et al. 1996, 1998; Littlefield et al. 1998).

1.1.4 Host ranges

1.1.4.1 General host ranges

Since Pg and Pb are morphologically indistinguishable, their distinction is solely made based on their host ranges (Legrève et al. 2003). Pb infects sugar beets while Pg different *formae speciales* infects several cereals (Table 1).

Table 1: Host ranges of *Polymyxa graminis formae specialis*.

Forma specialis	Host	Distribution	Reference
temperata	Barley, wheat, *Poa* sp.	Belgium, Canada, China, France, Germany, United Kingdom	(Legrève et al. 2002; Ward et Adams 1998)
tepida	Barley, wheat, oat, rye	Canada, United Kingdom	(Legrève et al. 2002; Ward et Adams 1998)
tropicallis	Barley, maize, millet, sorghum, wheat	India, Senegal	(Legrève et al. 1998)
substropicallis	Barley, peanut, millet, sorghum, wheat	India, Pakistan	Legrève et al. 2002)
colombiana	Rice	Burkina Faso, Colombia, Mali	(Bagayoko 2022)

1.1.4.2 Rice

As the rice is the specific host of Pgcol, a special emphasis is made on this cereal.

Rice is a monocotyledonous annual herbaceous plant of the family Poaceae and of the genus *Oryza* which is divided into several species, being *O. sativa* (Asian rice), *O. glaberrima* (African rice), *O. rufipogon* (wild rice or brownbeard rice) and *O. barthii* (wild rice). Amongst them *O. sativa* and *O. glaberrima* are cultivated (Barry et al. 2007; Joshi et al. 2000; Vaughan, Morishima, et Kadowaki 2003).

The domestication of rice is thought to have occurred about 10000 years ago in one of the following regions, being either India, South China, the Yangtze River area in China, the so-called “belt-region” (having a great diversity of *Oryza* species) along the southern slope of the Himalayas and the coastal swamp habitats in Southeast Asia (Gross et Zhao 2014).

Rice (*Oryza sativa*) is a major food crop for people around the world, especially in Asia but also in Africa. It serves as a staple food for nearly half of the world population (FAO, 2020). In 2022-2023 about 500 million metric tons of rice have been eaten (Statista, 2023) and about 90% of the rice production in directly consumed as food by humans (FAO, 1998). Rice is also the most important cereal crop in the world. In terms of production, the global rice is estimated at 787.3 million tons in 2021 (FAOSTAT, 2021).

1.1.4.3 Interaction between host and *Polymyxa*

The outcome of the relationship between a microorganism and its host ranges from mutualism to parasitism (Kogel et al. 2006). Commensalism is defined as a relationship between two species in which one species obtains benefits from the other without harming the latter. Mutualism is defined as an association between organisms of two different species in which each benefit. Parasitism is defined as a relationship between two species in which one benefits at the expense of the other (Britannica, 2023).

Among the three plasmodiophorids genera described here, two (Pbras and Ssub) are known to cause direct harm to their hosts and are thus categorized as pathogenic (Decroës 2022). Pb and Pgcol however do not cause direct damage to their host and, in some cases, Pb may even have beneficial effects (Desoignies 2012). Desoignies (2012) reported an enhanced protection of *Beta vulgaris* against *Cercospora* leaf sport after root infection by Pb. Therefore, *Polymyxa spp*. can either be considered as commensalists or even mutualists. However, ultimately the relation between the host and the parasite is of a producer of nutrients and a sink for nutrients. Such interplay makes *Polymyxa* more of a commensalist organism than a mutualist (Decroës 2022).

Since 2015, a few studies have focused on transcriptomic analysis to study the plant-plasmodiophorids interaction. Since the beginning of the 21st century, some have

identified proteins playing a role in plant infection. In the early 2000s, two xyloglucan endo-transglycosylases were found in sugar beet roots infected with Pb and could be involved in root damage repair (Mutasa-Gottgens et al. 2000; Dimmer et al. 2004) Later, it was found that Pb and Pg induce the same basal response in barley, a host for Pg and a non-host for Pb, with less than twenty genes showing increased expression (McGrann et al. 2009). In 2014, Desoignies et al. reported that a fine interplay occurs during the successive stage of Pb life cycle in its host, enabling Pb to hide from the plant immune system. These results were later confirmed by Decroës et al. (2022) who suggested that the hide skill of Pb was provided by small, secreted protein called effectors.

The study of molecular interaction between Pb and the sugar beet (Decroës et al. 2022) showed that Pb induced limited gene expression changes in sugar beet. Most differentially expressed plant genes were down regulated and included resistance gene analogs and cell wall peroxidases. Sugar beet roots infested with Pb showed that cell wall synthesis was mainly repressed. More precisely, genes associated with cellulose synthase complex, hemicellulose synthesis, and pectin synthesis were significantly down regulated. Genes expression related to pathogen detection and plant defense signaling was also found to be modulated by the presence of Pb. They investigated the regulation of genes potentially involved in pathogen recognition, focusing on resistance genes analogues (RGAs) and transcription factors. RGAs are globally repressed in the presence of Pb alone, suggesting that the protist might manipulate his host cell metabolism through the suppression of defense signalling pathways. In previous work with Pb, some proteins potentially involved in the interference of plant defense, such as ankyrin-containing proteins (Chen et al. 2019; Pérez-López et al. 2020; Schwelm et al. 2015), PR-4 homologs (Decroës et al. 2022; Schwelm et al. 2015), and isochorismatases were predicted (Decroës et al. 2022). Ankyrin-repeat domains are believed to have played an important role during the co-evolution of plasmodiophorids with heir hosts as they are involved in various functions including interactions with proteins, lipids and sugars (Decroës et al. 2022; Islam et al. 2018; Li et 2006; Voronin et Kiseleva 2008). PR genes are generally induced in response to an infection or an attack and are part of the plant defense response (Decroës et al. 2022; Schwelm et al. 2015). Isochorismatases secreted effectors of *Verticilum dahlidae* and *Phytophtora sojae* have been experimentally shown to suppress salicylate pathway. Therefore, it is suspected that they may play a similar role for plasmodiophorids (Decroës et al. 2022; Liu et al. 2014; X. Zhu et al. 2017). Finally, the expression of oxidative stress-related genes induced by Pg were studied. Glutathione-S-transferase enzymes, expressed under stress conditions, were up-regulated by the presence of Pb. Similar up-regulation of glutathione-S-transferase has been observed in *Arabidopsis thaliana* (Irani et al. 2018) upon infection by Pbras, indicating their importance in plant-plasmodiophorids interactions. Moreover, two of them were among the 20 most expressed Pb genes, further indicating their crucial role for the protist. This suggest that redox homeostasis plays a crucial role in the interaction between Pb and his host, and this regulation could prove itself to be

beneficial for the protist (Decroës et al. 2022). However, the plant-*Polymyxa* interactions remain poorly understood.

1.1.5 Viruses transmitted by *Polymyxa*

1.1.5.1 Diversity

As previously mentioned, Pg is the vector of several viruses belonging to the genera *Benyvirus*, *Bymovirus*, *Furovirus* and *Pecluvirus* (Table 2).

Two *Benyvirus are* transmitted by *Polymyxa spp*.: the BNYVV and the RSNV. The RSNV is transmitted by Pgcol (Table 3) while the BNYVV is transmitted by Pb. The rhizomania occurs around the globe, in every are cultivating sugar beets. In susceptible varieties, rhizomania can lead to up to 50% yield loss and sometimes even more (Koenig 2008).

Bymovirus transmitted by Pg are the *Barley mild mosaic virus* (BaMMV), *Barley yellow mosaic virus* (BaYMV), *Oat mosaic virus* (OMV), *Rice necrosis mosaic virus* (RNMV), *Wheat spindle streak mosaic virus* (WSSMW), and *Wheat yellow mosaic virus* (WYMV) (Table 2). BaMMV together with BaYMV are the causal agents of the economically important yellow mosaic disease of winter barley in Europe and East Asia. Yield loss associated with these viruses account from 10 to 90% (Lapierre and Hariri 2008; Li et al. 2016). OMV induces a growth spurt in the spring and stunting in the late season in the USA and the UK. Yield losses may range from 25 to 50% in tolerant cultivars and very susceptible cultivars may not produce any grain (Herbert et Panizo 1975). RNMV causes a reduction of growth, apparition of chlorotic streaks and speckles on the leaves, necrotic spots on the leaves, leaf sheath and culms. It is present in Japan and India and yield losses range from 50 to 60% (Inouye s. d.). Soilborne wheat mosaic virus (WSSMW and WYMW) causes mild green to yellow mosaic and parallel streaks on younger leaf, mild stunting of roots and shoots and reduce tillering may occur. Yield losses range from 3 to 87% (Cowger et Weisz s. d.).

Furovirus transmitted by Pg are the *Chinese wheat mosaic virus* (CWMV), *Oat golden stripe virus* (OGSV), *Soil-borne cereal mosaic virus* (SBCMV), *Soil-borne whet mosaic virus* (SBWMV), and *Sorghum chlorotic spot virus* (SrCSV) (Koenig 2008b). The CWMV causes wheat mosaic and typically causes light chlorotic streak on the young leaves and bright yellow chlorotic streaking or even purple chlorotic stripes on old leaves. Yield losses are generally ranging from 10 to 30% but may reach 70% in severe cases (Guo et al. 2019). OGSV causes the same damages as the OMV and causes yield losses ranging from 40 to 60% (Walker et al. 1998). SBCMV causes yield losses of up to 70% and share the same symptoms of other cereal mosaic viruses (Marra et al. 2022). SBWMV causes yield losses of to up to 50% while causing chlorotic patches in field by stunting plants and developing chlorotic mosaic or irregular mottling and streaking on leaves (Cadle-Davidson and Gray 2006). SrCSV causes

symptoms including elongated chlorotic spots and ring spots as well as yellowing on systemically infected leaves of sorghum (Kendall et al. 1988).

Pecluvirus transmitted by Pg are the *Indian peanut clump virus* (IPCV) and *Peanut clump virus* (PCV). Both viruses cause indistinguishable symptoms of stunting, chlorotic streaks, darken leaves, and even early senescence in early infected plants. Yield losses may reach up to 60% (Delfosse 2000; Delfosse et al. 1999)

1.1.5.2 Control

Polymyxa transmitted viruses can survive in soil in the resting spores for a long period of time. As chemical control for soil borne diseases is neither efficient nor acceptable for economic and ecological reasons other methods are required for controlling the diseases. Up to date, culture rotation and use of resistance cultivars are the most efficient ways to control the disease (Koenig 2008).

1.1.5.3 Mechanisms of acquisition and transmission of viruses by *Polymyxa* in the host plants

When discussing the mechanisms of acquisition and transmission of viruses by its vector, two mechanisms need to be differentiated, the *in vitro* transmission and *the in vivo* transmission. The *in vitro* transmission is characterized by an acquisition stage of viral particles outside of plant roots by zoospores (Adams 1991; Campbell 1996) and a release stage in which the virus gains access to the root cell cytoplasm (Rochon et al. 2004). The in vivo transmission is characterized by an acquisition prior to the development of the zoospores and a development inside the host (Rochon et al. 2004).

Viruses transmitted by *Polymyxa spp*. are acquired in the *in vivo* manner, transmitted by their respective viruliferous vector to the plant, and finally developed inside their hosts (Dieryck et al. 2011). The exact acquisition method of viruses by Pg is unknown (Kanyuka et al. 2003). However, it is likely that the process takes place when Pg is under the form of zoospores when either penetrating the host cells or at the sporogenic plasmodia stage (Kanyuka et al. 2003). Moreover, transmembrane domains have been recognized as having a significant importance in transmission of beny-, bymo, furo, and pomoviruses by plasmodiophorids (Dieryck et al. 2013). Studies have characterized the regions of the virus genome involved in the transmission of the virus by the vector. The coat protein readthrough domain of the BNYVV transmitted by Pb and the PMTV transmitted by Ssub was found to be involved in the transmission of the virus by the vector (Dieryck et al. 2011; Reavy et al. 1998).

For viruses transmitted in the *in vivo* manner, the virus is thought to have been acquired by the vector prior to the development of the zoospores (for instance, during *in vitro* acquisition) (Rochon et al. 2004). The BaMMV was confirmed to be present in zoospores and sporangia on several occasions, supporting this hypothesis. However, it hasn't been made possible to detect it in the sporosori phase. The mechanism of injection into the host cells is also unknown, such as for *in vitro* transmission (Adams et al. 2001; Chen J. P. et al. 1991).

1.1.5.4 Symptoms of RSNV

The RSNV) was first identified in 1977 in the Ivory Coast (Louvel et Bidaux 1977). It belongs to the genus *Benyvirus* (Table 2) and family Benyviridae and has recently been reported in other West African countries, such as Burkina Faso, Benin, Mali and Sierra Leone (Decroës et al. 2017; Oludare et al. 2015; Sereme et al. 2014). In South and Central America, the virus has been reported in Colombia, Brazil and Argentina (Lozano et Morales 2009; Maciel et al. 2006; Maurino et al. 2018; Morales et al. 1999; Paz Carrasco et al. 2009).

This virus causes characteristic symptoms of crinkling yellow and deformation of rice leaves (Figure 5). It can result in the death of young seedlings and lead to severe deformity in plants. Affected rice crops typically exhibit prominent yellowish stripes and widespread tissue death on their leaves. They experience stunted growth, and in fully grown plants, the panicles display abnormalities and produce a low seeds quantity (Lozano et Morales 2009). The incidence of the disease ranges from 37% to 80% in West African countries and up to 40% yield loss in South and Central America (Bagayoko et al. 2021).

The RSNV conveyed by Pgcol can cause the death of very young seedlings. For early infected seedling, death may not occur all all time, but infection may result in production of fewer grains (Morales et al. 1999). Yield loss associated with susceptible rice varieties infected by the RSNV can be as high as 40% in West Africa (Gutiérrez et al. 2010) and range from 20 to 40 % in Colombia (Morales et al. 1999). Since *O. globerrima* has shown to be highly resistant to RSNV (Correa et al. 2002), only cultivated *O. sativa* presents a high risk of infection by RSNV.

Table 2: Viruses transmitted by Pg belonging to the genera *Benyvirus, Bymovirus, Furovirus* and *Pecluvirus*. Adapted from Tamada et Kondo (2013) and Dieryck et al. (2011).

Family Genus	Virus species	Acronym	Vector	Virus natural host	Distribution
Unassigned family					
Benyvirus	*Rice stripe necrosis virus*	RSNV	*P. graminis* f. sp. *colombiana*	Rice	West Africa, South and Central America
Potyviridae					
Bymovirus	*Barley mild mosaic virus*	BaMMV	*P. graminis*	Barley	Europe, Japan, China, Korea
	Barley yellow mosaic virus	BaYMV	*P. graminis* f. sp. *temperata, P. graminis* f. sp. *tepida*	Barley	Europe, Japan, China, Korea
	Oat mosaic virus	OMV	*P. graminis*	Oat	Europe, US
	Rice necrosis mosaic virus	RNMV	*P. graminis*	Rice	Japan, India
	Wheat spindle streak mosaic virus	WSSMV	*P. graminis*	Wheat, rye, triticale	North America, Europe, Africa
	Wheat yellow mosaic virus	WYMV	*P. graminis*	Wheat	Japan, China
Virgaviridae					
Furovirus	*Chinese wheat mosaic virus*	CWMV	*P. graminis*	Wheat	China
	Oat golden stripe virus	OGSV	*P. graminis*	Oat	Europe, US
	Soil-borne cereal mosaic virus	SBCMV	*P. graminis* f. sp. *temperata,* *P. graminis* f. sp. *tepida*	Wheat, rye, triticale	Europe
	Soil-borne wheat mosaic virus	SBWMV	*P. graminis*	Wheat, barley, rue, triticale	US, Germany, Brazil, Africa, Japan
	Sorghum chlorotic spot virus	SrCSV	*P. graminis*	Sorghum	US
Pecluvirus	*Indian peanut clump virus*	IPCV	*P. graminis* f. sp. *subtropicalis, P. graminis* f. sp. *tropicalis*	Peanut, sorghum	India, Pakistan
	Peanut clump virus	PCV	*P. graminis* f. sp. *subtropicalis, P. graminis* f. sp. *tropicalis*	Peanut, sorghum	West Africa

Figure 5 : *Rice stripe necrosis virus* symptoms on rice (Lozano et Morales 2009).

1.2 EFFECTORS OF PLASMODIOPHORIDS INVOLVED IN THE INFECTION MECHANISMS OF HOST PLANTS

1.2.1 General information on effectors

The first named effectors were characterized in *Shigella flexneri*, the causal agent of dysentery, and named IpaB, C, and D proteins. They proved to be essential for bacterial entry into epithelial cells (Ménard et al. 1993). Since then, many new effector proteins have been identified. Lately (21st century), our understanding of effector diversity and functions has improved, notably thanks to functional genomic studies (Louet et al. 2022) and the ever-increasing number of available fungal, bacterial and plant genomes (Collemare et al. 2019).

Effectors can be generally defined as proteins and small molecules that alter host cell structures and functions, thereby facilitating the colonization of pathogens (Hogenhout et al. 2009). They are of different natures such as proteins, small RNA, and toxic metabolites (Collemare et al. 2019).

Although pathogens adopt distinct strategies to attack host plants, they all use the delivery of virulence effectors into host cells (De Jonge et al. 2011). Biotrophic pathogens tend to secret effectors to suppress host immunity while minimizing damage to the host cells (Lo Presti et al. 2015) whereas necrotrophic pathogens

secrete effectors to induce host cell necrosis as they proliferate on dead tissues (Lowe et Howlett 2012). Finally, hemibiotrophic pathogens deploy a combination of diverse effectors to suppress innate immunity during the biotrophic phase and elicitation of non-specific cell death during the necrotrophic phase (Horbach et al. 2011; Tariqjaveed et al. 2021).

There are two main areas of action for effectors. Most effectors are delivered into host cells (known as cytoplasmic effectors) and directly manipulate host cellular processes. Other effectors function in the extracellular space (known as apoplastic effectors) (Lovelace et al. 2023). Apoplastic effectors are secreted in the extracellular space between the pathogen and plant cells (Doehlemann et Hemetsberger 2013; Harris et al. 2023). These effectors focus on neutralizing the environmental parameter hindering the safety of the pathogen and helping it avoid detection. They include protease inhibitors, detoxification of secondary metabolites, chitinase binding effectors, and peroxidases inhibitors (van den Burg et al. 2006; Hemetsberger et al. 2012; Ökmen et al. 2013; Rooney et al. 2005; Westrick et al. 2021).

Cytoplasmic effectors modulate various processes involved in host intracellular defence mechanisms, including the inhibition of PAMP-triggered signalling, interruption of the metabolic pathways, deregulation of vesicle trafficking and cytoskeletal networks, host organelle remodeling, and cell death regulation (Djamei et al. 2011; Irieda et al. 2019; Jelenska et al. 2007; Kang et al. 2014; S. Li et al. 2019; Liu et al. 2014; Manning et Ciuffetti 2005; Nomura et al. 2006; Porter et al. 2012). Intracellular effectors in contrast with extracellular effectors are further divided by the compartment they specialize to, for instance, the cytoplasmic effectors or nuclear effectors. Nuclear effectors pass through the nuclear envelope and interfere with different machinery and target either proteins or directly DNA (Harris et al. 2023).

1.2.2 Plant defense system

Plants and pathogens have evolved together for a long period of time during which the pathogens tried to infect plants and plants tried to defend themselves. Such evolution led to the plant immune system and a variety of ways to prevent infection. The plant immune system is divided into two branches. The first uses transmembrane pattern recognition receptors (PRRs) that respond to pathogen-associated molecular patterns (MAMPs & PAMPs). The second acts inside the plant cells, using specific proteins coded by resistance (R) genes (Jones et Dangl 2006). In 2006, Jones and Dangl proposed a simple coevolutionary model of plant-pathogen interactions, called the “zigzag” model having for purpose to describe the interactions between plant and pathogen (Jones et Dangl 2006). Unlike animals, plants lack mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals coming out from infection sites (Ausubel 2005; Chisholm et al. 2006; Dangl et Jones 2001).

The ”zigzag” model encompasses two branches of the plant immune system. In the first branch, conserved molecules shared by many classes of microbe (PAMPs or MAMPs) are recognized, this first branch is now called pattern-triggered immunity (PTI). In the second branch, virulence factors are recognized, and a response is triggered to suppress PTI. This second branch is called effector-triggered immunity (ETI) (Pritchard et Birch 2014).

The current view of the immune plant defense system describes in the zigzag model (Figure 6) by Jones and Dangl (2006):

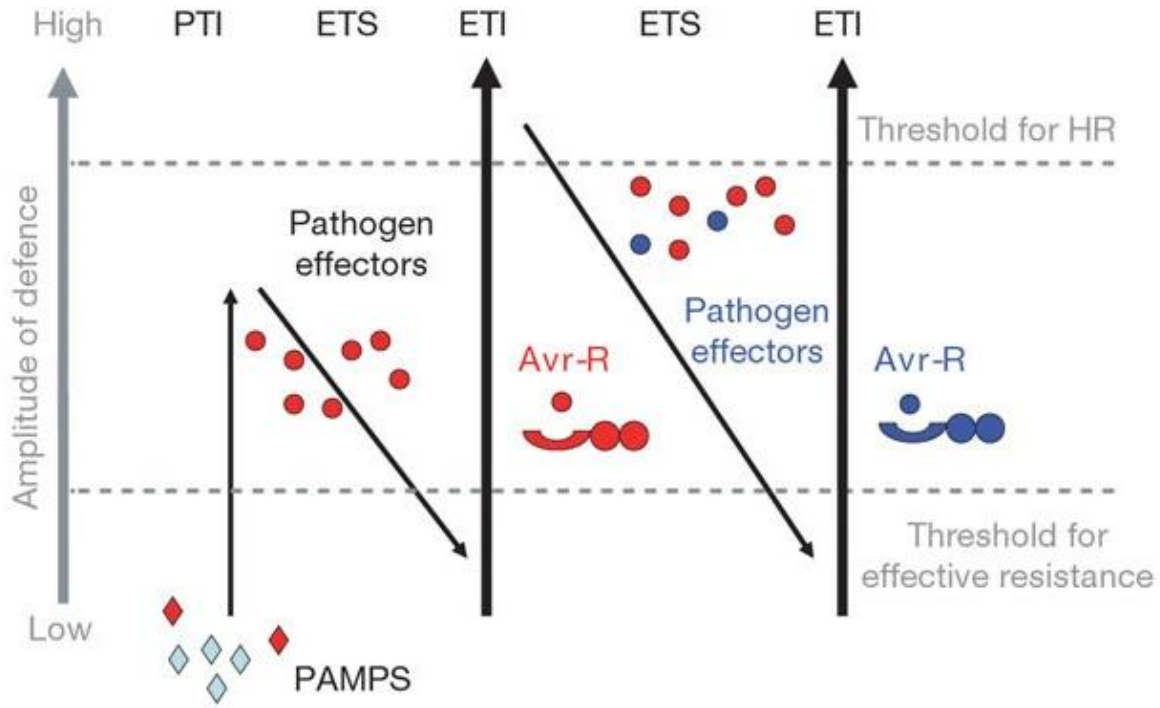

Figure 6: Quantitative output of the plant immune system in the zigzag model proposed by Jones and Dangl (2006). In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI]. In phase 1, plants detect MAMPs or PAMPs via pattern recognition receptors (PRRs) to trigger PTI. In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by a nucleotide binding (NB) and leucine-rich repeat (LRR) protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive response (HR) inducing cell death. In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Natural selection results in new resistance (R) specificities so that ETI can be triggered again.

Plant resistance (R) proteins play a crucial role in plant defense by recognizing pathogen effectors and activating signalling networks (Hogenhout et al. 2009). This type of resistance, known as race-specific resistance or gene-for-gene resistance,

provides complete and specific protection against pathogens (De Jonge et al. 2011). The genes encoding R proteins belong to the NB-LRR gene family (Collier et Moffett 2009). Most R proteins belong to the signal transduction ATPase with numerous domains (STAND) family of proteins, specifically the NBS-LRR subgroup (nucleotide-binding site; leucine-rich repeats) (Ávila Méndez et Romero 2017).

Although the zigzag model is still up-to-date, it presents some limitations when it comes to quantitative or predictive framework to directly study plant-microbe interactions (Pritchard et Birch 2014). As such, other models have been thought to update the zigzag model if required to, such as the invasion model (Cook et al. 2015) or the multi-component model (Andolfo et al. 2016). This shows that plant defenses mechanic is a complicate process involving many molecules.

1.2.3 Host/non-host relationship

Depending on the type of interaction occurring between a plant and a pathogen, plant responses can be divided into two types: non-host response and host response (Ávila Méndez et Romero 2017). Non-host resistance is defined as the event where a plant species is resistant to different pathogens, but these pathogens can infect other plant species (Bellincampi et al. 2014). Host resistance can be seen as a part of plant immunity occurring inside the cells and originates once the host successfully recognizes the effectors secreted by the pathogen (Ávila Méndez et Romero 2017).

The non-host response is induced when MAMPs are recognized by PRR-type membrane receptor proteins. PRR structures are types of receptor-like kinases (RLKs) that have functional modular domains (Monaghan et Zipfel 2012). The PTI is triggered by the recognition of MAMPs. In it are associated with some molecular mechanisms with the production of reactive oxygen species (ROS), Ca^{2+} cascades, and activation of mitogen-activated protein kinases (MAPK) leading to transcriptional reprogramming (Bernoux et al. 2011; Trapet et al. 2015).

Host induced resistance occurs when ETI processes overlap with PTI. Plant resistance (R) proteins can perceive pathogenic secreted effectors and initiate a defense response, including oxidative burst, accumulation of hormones such as salicylic acid (SA) and nitrogen oxide (NOI), MAPK cascades, changes in Ca^{2+} levels, transcriptional reprogramming, synthesis of antimicrobial compounds, and expression of pathogenetic related genes (Ávila Méndez et Romero 2017; Coll et al. 2011; De Bruyne et al. 2014; Hein et al. 2009).

1.2.4 Effectors' target

Parasitic effectors modulate plant immunity through multiple pathways having three distinctive targets: the apoplastic region, the reactive oxygen species generation machinery and the intracellular host defense (Tariqjaveed et al. 2021). In the following

paragraphs, a non-exhaustive list of mechanisms and produced molecules and effectors is presented.

Target: The apoplastic immunity

The apoplast is the defined as the intercellular space filled with gas and water. It is contained between cell membranes, the interfibrillar and intermicellar space of the cell walls, and the xylem extending to the rhizoplane and cuticle of the outer plant surface. The apoplast is involved in a vast number of functions, such as molecules synthesis like those involved in plant defense against pathogens (Farvardin et al. 2020). Upon recognition of elicitors[1] created by a pathogen thanks to PRRs, plant immunity is induced. As countermeasures, apoplastic effectors are created by a pathogen to disrupt the physical and chemical barriers of plants, thus directly interfering with the plant immune system (Tariqjaveed et al. 2021).

Cell wall degrading effectors

Plant parasites have mainly two options when penetrating inside the host: either developing a method to mechanically penetrate the physical defense of the host or produce chemicals enabling them to bypass the plant physical barriers. The secretion of cell wall degrading enzymes (CWDEs), such as glucoside hydrolases, glycosyltransferases and pectin lyases allows them to hydrolyse cell wall constituents (Kubicek et al. 2014).

Some CWDEs secreted by hemibiotrophic and necrotrophic pathogens induce cell death, thus promoting infection. Cell death-inducing CWDEs can generally be divided into two categories: one where effectors act as PAMPs to induce plant cell death, which is independent of their enzymatic activity (for instance. *Fusarium oxysporum* FoEG1 (GH12), *Botrytis cinerea* xyloglucanase BcXYG1 (GH12) and xylanase BcXyl1) (Yang et al. 2018; Zhang et al. 2021; W. Zhu et al. 2017) ; and another that includes a putative endo-β-1,3 glucanases CfGH17-1 and CfGH17-5 in the biotrophic pathogen *C. fuluvm* induce cell death, suggesting that the cell wall products released by these CWDEs are recognized as danger-associated DAMPs (Ökmen et al. 2019; Tariqjaveed et al. 2021).

Chitin-triggered immunity degrading effectors

Chitin is a linear polysaccharide of the amino sugar N-acetyl glucosamine present in most invertebrate animals like arthropods, molluscs, nematodes, but also in fungi (Moussian 2019). Degraded fragments of chitin can be recognized as PAMPs or DAMPs by the plant PRRs and thus inducing immunity (Sánchez-Vallet et al. 2015).

[1] Elicitors are the chemicals or biofactors from various sources that can trigger physiological and morphological responses in the target living organisms (Gowthami s. d.). Elicitors have a broader range than effectors.

Fungi must therefore secrete diverse effectors to inhibit the activation of the chitin-triggered immunity.

Pathogenic plant organisms have developed several mechanisms of action to defend against the glucanases and chitinases present in the apoplastic environment. They can modify the structure of chitin to chitosan but can also produce effectors to disrupt the recognition of chitin by the chitin-binding domain (ChBD) (Sánchez-Vallet et al. 2015). For instance, the tomato leaf fungus *Cladosporium fulvum* secretes, during infection, the Avr4 effector that binds to the chitin-binding domain and reduces the access to host chitinase. This prevents cell wall hydrolysis (Van Den Burg et al. 2006). Another broadly distributed group of chitin-binding effector proteins in the fungal kingdom contains LysM domains and are termed LysM effectors (Sánchez-Vallet et al. 2015). LysM effectors are highly expressed during infection and were demonstrated to be essential for pathogen virulence through suppression of chitin-triggered immunity (Kombrink et Thomma 2013). In addition to chitin-binding effectors LysM and Avr4, the CBM18-containing effectors can avert recognition and digestion of chitin by plant chitinases (Volk et al. 2019).

Papain-like cysteine proteases degrading effectors

Papain-like cysteine proteases (PLCPs) are a versatile group of enzymes present in nearly all living organisms. In plants, PLCPs are involved in various biological processes including plant growth, development, stress responses, senescence, and immune defense (Ozhelvaci et Steczkiewicz 2023). Certain PLCPs have been shown to have essential functions in controlling programmed cell death and enhancing plant immunity (Tariqjaveed et al. 2021).

Considering the importance of PLCPs, fungal pathogens have evolved to produce several effectors to counter their activity (Tariqjaveed et al. 2021). For instance, *C. fulvum* produces the Avr2 effector to promote virulence and inhibits several Cys proteases required for plant defenses (van Esse et al. 2008). *Ustilago maydis* produces Pit2 effector which is involved in the maintenance of biotrophy and tumor induction by inhibiting proteolytic activity of several proteases (Mueller et al. 2013). *Ustilaginoidea virens* produces SCRE1 effector that is involved with immunosuppression (Zhang et al. 2020).

Apoplastic pathogenesis-related degrading effectors

Pathogenesis-related proteins (PRs) are means of defense for plants against biotic and abiotic stress. They possess a wide range of functions, such as chitinases, peroxidases, anti-microbial chemicals, and many others (Zribi et al. 2021).

Fungal pathogens produce several effectors to target plant PRs and regulate their host defenses (Tariqjaveed et al. 2021). Tariqjaveed et al. (2021) reviewed some of the effectors produced by several pathogenic fungi and involved in the disruption of PRs. It includes amongst other the CSEP0055 effector of the biotrophic *Blumeria graminis* f. sp. *hordei* that interacts with PR17c protein to suppress plant defense and the PevD1

effector of *Verticillium dahliae* that interacts with osmotin-like protein GhPR-5 to inhibits its antifungal activity to overcome host defenses.

Target: The reactive oxygen species (ROS) machinery

The ROS describes a group of molecules derived from molecular oxygen (O_2). ROS cause oxidation of lipids, proteins, DNA, RNA, and small molecules in cells. The high reactivity of ROS towards theses cellular components is due to their altered chemistry that allows them to donate an electron or transfer an excited energy state to an acceptor molecule (Halliwell et Gutteridge 2015; Mittler et al. 2022). Response to pathogen infection following pathogen recognition is often initiated by a transient oxidative burst. This burst is followed by an increase in the reduced state of the cytoplasm, the accumulation of the plant hormone salicylic acid and the deposition of callose at the cell wall and plasmodesmata, which prevents pathogen spread (Daudi et al. 2012; Fu and Dong 2013; Torres et al. 2002).

To prevent damages caused by ROS, parasitic plant fungi had to come up with a way to neutralize or inhibit ROS generating machinery. Several fungal effectors have been identified to inhibit ROS generating machinery. *Ustilago maydis* effector Pep1 targets the maize peroxidase POX12, thus inhibiting ROS generating capabilities and early defense responses (Hemetsberger et al. 2012). In *Fusarium oxysporum*, the apoplastic Six1 & Foa1 and cytoplasmic Avr2, Foa2, Foa3 effectors have been identified to promote host colonization by suppressing flg22-induced or chitin-induced ROS (Tintor et al. 2020).

Target: The intracellular host defense

Apart from the apoplastic effectors, intracellular effectors interact with the content of the host cell, being either the organelles or the production of different metabolic pathways such as RNA (Tariqjaveed et al. 2021).

<u>Plasma membrane-associated immunity degrading effectors</u>

Once the cell wall has been crossed, the plasma membrane is the first layer of defense of plant against external pathogens. Recognition is made thanks to receptor-like kinases perceiving the molecules produced by the pathogens, thus initiating an immune response (El Kasmi et al. 2017).

Fungal pathogens produce effectors that disrupt the phosphorylation activity of the plasma membrane-associated proteins. For instance, *Colletotrichum* spp. produce NIS1 effectors (Irieda et al. 2019). Furthermore, the plasma membrane houses several ion channels such as those reserved for K^+ (Tariqjaveed et al. 2021). By disrupting the uptake of K^+, AvrPiz-t effector of *Magnaporthe oryzae* can interfere with the immune system of their host (Shi et al. 2018).

<u>Hormone signaling disruptive effectors</u>

PTI and ETI activation require multiple plant hormones acting as the trigger for plant immune signalling network. Salicylic acid (SA) (Zhang et Li 2019) and jasmonic acid

(JA) (Wasternack et Song 2017) act as the two main hormones, but many others also function as modulators of the plant immune signalling network : ethylene hormones (ET), abscisic acid (ABA), gibberellins (GAs), auxins, and cytokinins (CKs) (C. M. J. Pieterse et al. 2012).

The JA pathway can be further divided into two branches (Pieterse et al. 2012). The ethylene response factor (ERF) branch, regulated by ET, is activated when plants are infected by pathogens with a necrotrophic lifestyle. On the other hand, the myelocytomatosis (MYC) branch, co-regulated by ABA, mainly provides defense against chewing insects. MYC transcription factors regulate JA-related genes involved plant defense and plant growth (Grunewald et al. 2009). Transcription factor's function is transducing external cues into intracellular signals, thereby eliciting specific hormones signalling pathways and gene expression cascades for activation of defense-related targets (Ng et al. 2018). The SA pathway primarily targets pathogens with a biotrophic lifestyle. Therefore, the triggered immunity in the host is determined based upon the infection pathway chosen by the pathogen (Aerts et al. 2020). Additionally, the overall hormonal balance is influenced by various internal factors, such as plant age, and external factors like exposure to other stresses. Ultimately, the final hormone equilibrium and responsiveness are a result of the interplay between plant immunity activation and the factors within the plant (Aerts et al. 2020).

To counter the different hormonal pathways, fungal pathogens can either interfere directly with the biosynthesis of hormones or interact indirectly with the various modulators of hormones regulation pathways (Tariqjaveed et al. 2021).

RNA interfering effectors

Ribonucleic acid (RNA) is a structure from inside the nucleus and present in all living cells. Its principal role is to act as the messenger carrying instruction from DNA for controlling the synthesis of proteins. Fungal effectors are involved in two types of interactions with RNA, the first one as a mean to stop the degradation of RNA and the other to disrupt the host RNA interference machinery.

For biotrophic pathogens the purpose of infection is the survival in the host. However, the plant may induce cell death as a mean of defense. In such context, disrupting the degradation mechanism of host RNA by fungal effectors may ensure a constant income of nutrient as the cell will no longer be perceived as infected. RNase-like effector such as CSEP0064 and BEC1054 produced by *B. graminis* increase its susceptibility in its host, the wheat. In *Fusarium graminearum*, and RNase effector Fg12 is produced to induces cell death and contributes to pathogen virulence (Pennington et al. 2019).

RNA interference (RNAi) is a conserved biological response to double-stranded RNA (dsRNA) that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids and regulates the expression of protein-coding genes (NCBI, 2017). The purpose of the RNAi machinery is the destruction of messenger RNA (mRNA) (Hammond 2005). Incapacitating exogenous RNA silencing is also an

efficient way to promote infection. For instance, the wheat stem rust pathogen *Puccinia graminis* f. sp. *tritici* produces an effector protein PgtSR1 that suppresses RNA silencing.

Nucleus interfering effectors

As explained above, effectors are organized depending on the localization of their target, which ultimately brings the question of the role of the nucleus and its susceptibility to exogenous pathogenic effectors. Nuclear effectors play a disruptive role in various nuclear processes within the host, such as the regulation of host chromatin through epigenetic mechanisms, hindering the trans-kingdom antifungal RNAi machinery, and impacting different types of immunity-associated proteins. These pathways targeted by effectors are highly conserved across different hosts and have a significant influence on a wide range of cellular processes in plants. As a result, these nuclear sites serve as important targets for effectors to undermine the plant defense system and acquire the necessary resources for successful pathogenic growth and spread (Harris et al. 2023; Tariqjaveed et al. 2021).

For instance, the *Phakopsora pachyrhizi* effector candidate 23 with its host transcription factor GmSPL12 suppress his host immunity (Qi et al. 2018). For *Magnaporthe oryzae*, a biotrophic fungi, nuclear effector MoHTR1 and MoHTR2 regulate the expression of immunity-associated genes by binding to the target gene promoters (Kim et al. 2020; Tariqjaveed et al. 2021).

1.2.5 Effector prediction

In the late 50s, the concept of molecular computing was introduced by Feynman (Adleman 1994). At that time, however, the technologies were lacking, and it was not until 1994 that Adleman was able to achieve a molecular computation of DNA for the first time (Adleman 1994). With the development of new generation sequencing technologies, the scale of DNA, RNA, and protein biological database has increased dramatically, leading to a new era of big data sets (Mardis 2008). To analyse such massive information, bioinformatics was introduced. Bioinformatics combines the tools of mathematics, computer science, and biology to elucidate and understand the biological implications and significance of a variety of sequence and structure data as well as other biological data more efficiently (Y. Li et al. 2019; Liang et al. 2019).

Over the years, numerous molecular tools have been developed to explore the role of individual effectors in causing disease, leading to a comprehensive understanding of infection mechanisms. However, the main challenge in effector research lies in predicting these effectors on a genome-wide scale, encompassing the entire repertoire of a pathogen. This difficulty arises from the diverse delivery mechanisms employed by effectors and limited knowledge of these mechanisms in most pathogens (Lovelace et al. 2023). Candidate effectors are generally identified through the recognition of conserved amino-acid motifs that have been previously identified as being responsible for pathogenicity (Jones et al. 2018). However, fungi tend to lack those specific

conserved sequences thus rendering their effectors identification more arduous. Prediction of effectors can be performed based on several pathways, such as protein sequence, structural features, genomic analysis, and gene expression patterns (Lovelace et al. 2023). In general, the discovery of candidate effector is made by using a combination of genomics, transcriptomics, and proteomics (Jones et al. 2021).

Prediction of effectors based on protein sequence

Parasites and pathogens use different effector secretion and/or delivery mechanisms. But a commonly shared feature is the presence of the N-terminal signal peptide (SP), which is a secretion motif (Lovelace et al. 2023). The N-terminal SP is required for protein secretion through the general secretion system (Natale et al. 2008). In eukaryotes, the secretion system is found in the endoplasmic reticulum and function in the secretion of proteins and insertion of membrane proteins that are further directed to their final destination via the vesicle sorting route (Muñiz et al. 2001). In bacteria, the best-studied delivery machinery is the T3 secretion system (T3SS) of Gram-negative bacterial pathogens responsible for transporting T3 secreted effectors (T3SEs). However, for pathogens that lack such delivery machinery, detection of the N-terminal SP is a key criterion for effector prediction (Lovelace et al. 2023). Since 1996, several programs have been created to predict SP-based secreted proteins, such as SignalP. Lovelace et al. (2023) have compiled a number of existing software (Annex D.1) for protein prediction (Lovelace et al. 2023).

Another hint indicating that a parasitic protein may be an effector is the presence of an eukaryotic-specific functional domain (Lovelace et al. 2023). Such domain is thought to produce proteins similar to the hosts to manipulate it by mimicking its proteins. For instance, in *Ralstonia solanacearum* the T3SEs contains F-box motif-like sequence related to protein ubiquitination-based proteosome degradation (Angot et al. 2006). Sequence homology based methods can be used to identify eukaryotic domains within bacterial proteins (Lovelace et al. 2023), such as the Basic Local Alignment Search Tools (BLAST). Another feature used to predict bacterial effector is to target specific compartmentalization signals emitted by pathogens to penetrate a specific cellular compartment such as the nucleus, mitochondria, and chloroplast (Lovelace et al. 2023).

BLAST is a bioinformatic software that finds regions of local similarity between sequences. The program compares nucleotides or proteins sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of the gene families (NCBI BLAST 2023). Several types of action can be performed with BLAST software. Nucleotide BLAST (BLASTn) is used to compare one or more nucleotide query sequences to a subject nucleotide database. BLASTn is useful to determine the evolutionary relationship between different organisms. Translated nucleotide sequence searched against protein sequences (BLASTx) is used to compare a nucleotide sequence query translated in protein sequences and then compared to a protein sequence dataset. BLASTx is often used

to perform the identification of a new nucleotide sequence. Protein sequence searched against translated nucleotide sequences (tBLASTn) is used to compare a protein query sequence against a database of nucleotide sequences. tBLASTn is useful for finding homologous protein coding regions in unannotated nucleotide sequences. Protein BLAST (BLASTp) is used to compare one or more protein query sequences to a subject protein database. BLASTp is useful for identifying a protein (for instance, an effector) (NCBI BLAST 2023).

For the prediction of eukaryotic pathogens (mainly fungi and oomycetes) effectors, bioinformatic tools such as SignalP and PexFinder are used by targeting the N-terminal SP (Lovelace et al. 2023). In fungi, effectors are mostly small (shorter than 150-200 amino acids) and rich in cysteine residue (>3%) (Sperschneider et al. 2015). For oomycetes, effector prediction is easier due to the discovery of host-targeting motifs in the N-terminal region of cytoplasmic effector proteins following the secretion SP. Most of these effectors carry a RxLR motif involved in translocation of protein (Hiller et al. 2004; Rehmany et al. 2005).

Effector prediction using structural features

Structural features are another useful tool for effector prediction. Effectors are known for not sharing homology sequence with others known proteins and they also lack known sequence motives (Lovelace et al. 2023). Structural modelling is available to help predict effector based on structural features, and many structure prediction programs (Annexe D.1) exist and use homology modelling. It involves identifying known experiment structure with sequence similarity. If the effector of interest is structurally similar to a protein with a known domain, it can be inferred that the effector may also share the known domain. Such technic presents, however, some limitation when no homologous protein can be identified (Lovelace et al. 2023).

Effectors prediction facilitated by genomic analysis

Next generation sequencing technologies have allowed fast determination of genomes sequences, enabling the study of effector repertoires. In particular, long-read sequencing technologies such as Pacific BioSciences (PacBio), single-molecule real-time (SMRT), and Oxford Nanopore (ONT) have allowed the accurate resolution of repeat rich region which may contain effectors (Besser et al. 2018).

Thanks to technological advancement, pangenomes analysis rather than individual strains study is now doable. Pangenome is defined as the entire set of genes within a species. It englobes the core genome made of conserved genes encoding proteins that are essential, and the accessory genome made of genes not present in all members of the species (Tettelin et al. 2005). The study of the pangenome is made to survey the effector diversity in a species while the study of the accessory genome allows the study of effector genes (Jones et al. 2021).

Effector prediction based on gene expression patterns

Induced expression in plant is often considered as a feature of genes encoding effectors. It can help to narrow down the identification of candidate effectors before further validation of functional characterization. However, some effector may hide from host immune system and thus will not be detected by this method (Dong et Ma 2021).

For pathogens or parasites in which the regulatory proteins required for infection are poorly understood (such as *Polymyxa sp*.), RNA-sequencing (RNA-seq) has widely been used to identify effector candidates (Lovelace et al. 2023). RNA-seq is a sequencing technology that uses next-generation sequencing (NGS) to enable the detection and quantification of various RNA populations in a biological sample. RNA-seq uses high-throughput sequencing of nucleic acids to determine the nucleotide sequence of RNA molecules as well as the quantities of specific RNAs within populations of RNA molecules (Deshpande et al. 2023). Several sequencing technologies exist. The illumina sequencing, introduced in 2006, is based on sequencing-by-synthesis chemistry and can be used for high sequencing throughput as more than 10 million cDNA fragments can be simultaneously determined (Morganti et al. 2019; Shen 2019). The Nanopore sequencing, introduced in 2014, produces short or long reads from native DNA and RNA fragments of any length. Unlike the illumina sequencing, nanopore sequencing does not require chemical modification or PCR amplification and is capable of sequencing DNA or RNA without these additional steps (Bharagava et al. 2019). The PacBio sequencing, introduced in 2010, generates full-length cDNA sequences (i.e., long reads) that characterize transcripts of targeted genes or across entire transcriptomes (Eid et al. 2009; Vierra et al. 2021). Machine learning using genomic and protein features is another resourceful technology for effector prediction. One of the first algorithms for identifying effectors within bacterial proteomes using machine learning was EffectiveT3 (Annex D.1) (Arnold et al. 2009).

Lastly, candidate effectors should be validated though experimental research. However, functional validation of every candidate effector may prove itself to be fastidious. As such, several methods can be used to make it easier (Lovelace et al. 2023). Microscopy can be used to visualize effectors but has proven to be extremely challenging (Lovelace et al. 2023).

1.2.6 Genomics of plasmodiophorids

Genomic analysis is only a recent practice with an ever-increasing, yet still limited number of full genomes. Out of the previously described organisms, except Pg, the most recent genome assembly was realized by Decroës (Decroës et al. 2022) on Pb. Ssub genome assembly was also only recently published (Ciaghi et al. 2018) while the oldest from the three is Pbras that was assembled in 2015 (Schwelm et al. 2015). In Table 3 are summarized the main information regarding these newly sequenced organisms. Other genome assemblies have been made with other isolates of Pbras.

However, only isolate e3 information are presented here as only isolate e3 will be used for effectors comparison (see Chapter 3). Moreover, only Pb A26-41 isolate is used for effectors comparison (see Chapter 3), therefore only genomics information related to this isolate are presented in this table.

Table 3: Genomes characteristics of Pbras e3 isolate (Schwelm et al. 2015), Ssub SSUBK13 isolate (Ciaghi et al. 2018) and Pb A26-41 isolates (A. Decroës et al. 2022).

Features	Pbras e3	Ssub SSUBK13	Pb A26-41
Assembled genome size (Mb)	24	28.1	26.5
Number of contigs	165	2,340	1040
N50 (kb)	473	28.7	49
Protein-coding genes	9,730	9,742	10,224
GC content (%)	58.5	45.7	45

Decroës et al. (2022) realized a comparative analysis of the hereabove organisms leading to a better understanding of the parasitic lifestyle of the plasmodiophorids and the similarities between Pbras, Ssub and Pb. Their study indicates that Pb is more phylogenetically related to Ssub than to Pbras based on the amount of shared protein. Their study also points out the difference of lower enrichment of proteins with ankyrin-repeat domains, the detection of CatSper complex proteins, different enrichment of GPCR genes for signaling pathways that could be relevant for development and pathogenicity.

1.2.7 Effectors of plasmodiophorids

Until the 21st century, most research on plasmodiophorids were focused on ultrastructural and karyological observations. It is not until the evolution of sequencing technologies that brought the first plasmodiophorids genomes that research on plasmodiophorids effectors could begin (Decroës et al. 2022). To date, many effectors have been identified for pathogenic fungi, oomycetes, bacteria, nematodes, and parasitic plants. However, little is known about the function and molecular mechanisms of effectors from plasmodiophorids (Chen Wang et al. 2021). Moreover, except for plasmodiophorids, only three rhizaria protists genomes are currently available: the Chlorarachnea *Bigelowiella natans* (Glöckner et al. 2014), the Foraminifera *Reticulomyxa filosa* (Curtis et al. 2012) and the Imbricatea *Paulinella micropora* (accession no.: PRJDB3528). Furthermore, as plasmodiophorids are obligate endoparasites, their genome sequencing, assembly and purification always retains some contaminant from their host (Buhariwalla et al. 1995; Delfosse et al. 2000; Qu et Christ 2006). It is thus vital to increase the number of plasmodiophorids genomes to provide new knowledge on the molecular interplay occurring with their hosts.

As current research on plasmodiophorids effectors is mainly focusing on Pbras effectors, the role of effectors in plasmodiophorids life cycle will be investigated for Pbras. Numerous candidate effectors have been identified for Pbras and are summarised in Annex B.1. Among those effectors, several have been more extensively studied and are classified based on their target site located in either the extracellular space or the intracellular space or for interfering with the ROS machinery.

Several studies have identified numerous secreted effectors of Pbras. Chen et al. (2019) identified 33 Pbras secreted proteins during primary infection. These proteins were involved with the suppression and induction of cell death. Their results showed that two secretory proteins (PBCN_002550 and PBCN_005499) were able to induce cell death. Evidence presented in previous studies indicates that recognition of host infection by Pbras occurs during the primary infection (McDonald et al. 2014). It seems that Pbras encodes many proteins capable of suppressing plant immunity. Suppression of cell death is beneficial for biotrophic pathogens as they feed out of their host and cell death would be detrimental for them. Unlike PCBN_005499, PCBN_002550 was able to induce cell death in the nonhost Chinese cabbage. This reflected the specificities of differential interactions of Pbras with his host and nonhost plants. Among the 28 proteins suppressing the PBCN_002550 induced cell death, 18 contained functional domains. These domains have been reported to be involved in pathogen infection, plant disease, and tolerance to biotic and abiotic stresses. For instance, PBCN_007081 was identified to contain a tyrosinase domain, capable to facilitate the conversion of tyrosine to dihydroxyphenylalanine (Langfelder et al. 2003). And dihydroxyphenylalanine has been reported to reduce a reaction involving oxygen species in soybean roots (Soares et al. 2011). This would suggest that a similar process of interfering with the ROS machinery may occur in *Plasmodiophora*. Another significant discovery involved the presence of a MYND zinc finger domain in PBCN_001987, as well as a Ring-H2 zinc finger domain in PBCN_009214. These domains have been reported to enhance tolerance to abiotic stresses (Ko et al. 2006). Enhancing plant resistance to abiotic stress may therefore be beneficial for plasmodiophorids as they live off their host. Several other proteins were found to encompass ankyrin repeats, which are domains known to facilitate interaction between various proteins across a wide range of families (Sedgwick et Smerdon 1999). Many have been linked to activities related to disease resistance, antioxidant metabolism, production of reactive oxygen species, and responses to biotic and abiotic stresses. Unlike for Pb (Decroës et al. 2022), Pbras appears to contain several ankyrin repeats. As the relationship between the host and Pb or Pbras is different, the first being non-pathogenic while the second is, it can be hypothesised that the presence of ankyrin repeats in related to the pathogenic nature of the protist. Finally, a WD40 domain was identified in PBCN_005456 and is associated with roles promoting plant tolerance to abiotic stress and governing the initial stages of infection by Rhizobia in legumes (Kiss et al. 2009). Again, an increase in tolerance to abiotic stresses by the host is beneficial for the plasmodiophorid to enhance its survivability inside its host.

Another important study that identified numerous effector proteins was made by Pérez-López et al. (2020). In this study, 32 small secreted Pbras proteins (SSPbPs) were identified and seven of them presented a functional domain. These SSPbPs are involved in secondary infection, in contrast with the PBCN secreted proteins that are involved in primary infection. Four SSPbPs were identified as cysteine-rich proteins, one with RxLR motifs, three with Pexel-motif. The RxLR motifs have been identified in oomycetes as necessary for translocation in the host cell (Ellis et Dodds 2011; Yaeno et al. 2011). These motifs are required for an effector-host interaction with the cell plasma membrane, thereby mediating entry into the host cell by the pathogen (Kale et al. 2010). No such motifs were detected in Pb (Decroës et al. 2022). Like for the ankyrin repeats, the presence of RxLR motifs might be associated with the nature of the relationship between the plasmodiophorid and its host, being either pathogenic or non-pathogenic. It appeared that more than 25% of the 32 SSPbPs have a role in the regulation of the cell cycle.

Zhan ZongXian et al. (2022) also identified numerous Pbras candidate effectors that can suppress plant immunity. More specifically, *Pb*4_102097 and *Pb*4_108104 can induce cell death through H_2O_2 accumulation and were strongly expressed at the resting spore stage. This suggests that ROS accumulation could be beneficial for the germination of mature resting spores in the soil. Finally, several candidate effectors contained RXLR motifs responsible for functions occurring inside the cytoplasm and the cytomembrane.

Several Pbras effectors were further characterized, to better understand their role in Pbras life cycle.

Muirhead et Pérez-López (2022) studied the chitin-binding domains carbohydrate-binding module family 18 (CBM18). Its role is unknown, but it was observed to be transcriptionally activated during infection. Through coprecipitation, they found that *Pb*ChiB2 and *Pb*ChiB4 bind to spores and to chitin oligomers. They also showed that both suppress chitin-triggered activation of the map kinase proteins MPK3 and MPK6 in their host, *Brassica napus*. Their finding indicates that CBM18 proteins act as effectors to defend Pbras and suppress the chitin-triggered immunity during infection. As chitin is an important component of spore wall and a well-known PAMP, it supports the claim that it is involved in the early stages of infection.

The immunophilin-like protein *Pb*CYP3 studied by Singh et al. (2018) was shown to increase Pbras virulence on its host. Immunophilins are ubiquitous proteins with properties that allow them to regulate protein structure, activity, and stability. Their results showed that *Pb*CYP3 was produced at all life stages of Pbras. A hypothesis is that *Pb*CYP3 could be involved in the penetration mechanism of spores in the host cell.

Stefanowicz et al. (2021) studied the implication of pectin methylesterase (PME18) in the gall formation caused by Pbras. Their results indicated that PME18 level were increased at the late stage of gall formation. PME18 is involved with the demethylation

of pectin within the extracellular matrix. This allows the pathogen to hijack endogenous host mechanisms involved in cell wall loosening to create an optimal cellular environment for completion of its life cycle and the eventual release of resting spores facilitated by degradation of demethylated pectin polymers.

Ssub and Pb infectious effectors have been less studied so far, however a few studies exist describing the main types of effectors of these two organisms. One of them was realized by Decroës et al. (2022) and provides a first insight on the shared features of these organisms effectors. Ankyrin-repeat domains (Anks) are involved in various interactions, primarily with proteins but also with lipids and sugars (Islam et al. 2018; Li et al. 2006). Anks are considered as virulence genes in bacteria since they hijack their host cellular mechanisms (Al-Khodor et al. 2010). It has been shown that Anks are greatly produced in obligate intracellular bacteria which have a reduced genome (Jernigan et Bordenstein 2014). Effectors have also been discovered to attack PRs of their host defense system. For instance, PR4-LRR1 interaction in pepper resulted in suppression of cell death and defense responses (Hwang et al. 2014). Decroës et al. (2022) uncovered three PR-4 protein in Pb isolates but not in Ssub nor Pbras. They also identified other proteins able to interfere with the plant defense, such as the SOBER1-like immunity suppressor protein and some isochorismatases found in all three plasmodiophorids. They also revealed the presence of sperm-specific voltage gated calcium channel in Pb and Ssub but not in Pbras. This supports their hypothesis that Ssub is closer to Pb than Pbras. In mammals, sperm-specific voltage gated calcium channels play a role in sperm hyperactivation, motility, fertility, and chemotaxis to the egg (Ren et al. 2001; Sun et al. 2017). The authors hypothesized that the calcium singling pathway may have a role in zoospore motility regulation and chemotaxis. That hypothesis could therefore explain previous field observations where the presence of Pb was positively correlated with calcium presence. The isochorismatases have been experimentally shown to suppress salicylate pathways (Liu et al. 2014; X. Zhu et al. 2017). It can be assumed that they play a similar function in Pb.

CHAPTER 2: PRODUCTION AND MULTIPLICATION OF *POLYMYXA GRAMINIS* f. sp. *COLOMBIANA*

2.1 Introduction

Pgcol is an obligate root parasite that causes no known symptoms on its host, the rice. Nonetheless, it is the vector of the RSNV that causes important harm and incur up to 40% yield loss to rice cultivation. To investigate the molecular interactions occurring between the vector and the host, a well-mastered study system first needs to be set. However, although plasmodiophorids have been studied for a long time, thus making their life cycle well understood, the exact ecological parameters required to support an optimal development are still unknown.

The objectives of this chapter are to confirm the adequacy of the previously engineered study system (Decroës et al. 2017) used in the thesis of Bagayoko (2022) and study several parameters that could be involved in the development of Pgcol. The following parameters were studied in this chapter to provide new insights on the development of Pgcol in rice: the pH of the nutritive solution, the type (individual or collective) of watering system, and the source of Pgcol inoculum were tested in the present study. Moreover, other parameters were investigated, such as the efficiency of sporosores extraction, the efficiency of seeds disinfection, and the type of Pgcol life form used for primary infection (sporosores or zoospores).

In total, four bioassays were conducted to study these parameters. The first bioassay focused on the production of Pgcol on rice roots with sporosores using the watering system used by Issiaka Bagayoko (2022). Two Pgcol samples (a strain and an isolate) and several soils containing Pgcol were inoculated to maximize the chances of success as well as observe if an isolate infected rice more efficiently. The strain was virus-free while the isolate is associated with RSNV, and Pgcol contained in soils samples was either virus-free or not; this essay could also provide information regarding the effect of RSNV on Polymyxa development. The second bioassay focused on the multiplication of Pgcol in collective watering systems with zoospores originating from the previously infected plant of the first bioassay. A third bioassay was design to study the impact of the pH of the nutritive solution and the watering system on Pgcol production. Two pH (5.5 and 6.5) and two watering system (individual and collective automatic watering system) were tested. A last bioassay was conducted to study the timeline of infection of rice by Pgcol in individual watering systems. Additional parameters such as the efficiency of sporosores extraction (sporosores extraction experiment) and seeds disinfection (seed disinfection experiment) were studied outside the four bioassays.

2.2 Materials and methods

2.2.1 Biological materials

The variety Kogoni of Asian rice (*Oryza sativa*) and two Pgcol isolates (PGML3 and PGML5) were used in this study (Table 4). Both PGML3 and PGML5 were isolated in Belgium by Dr. Issiaka Bagayoko in 2016 and originate from Mali. PGML3 is a monosporous strain while PGML5 is only an isolate. Both PGML5 and PGML3 isolates were previously multiplied by Bagayoko (2022) in Asian rice (*Oryza sativa* cv. Kogoni). Unlike PGML3, PGML5 contains RSNV.

Naturally infested soils with Pg and RSNV (Decroës et al. 2017) were collected in January 2016 by Bagayoko (Bagayoko 2022) in different regions of Burkina Faso and Mali (Table 4). Pgcol and RSNV detection was tested in all soils, results are summarized in Table 4.

Table 4: Origins of soils samples, strain and isolate from dried root samples containing Pg and eventually RSNV from previous study made by Bagayoko (2022). Sample are organized according to the presence of absence of RSNV.

Type of sample Name of sample	Country	Region	Detection of Pgcol	Detection of RSNV
Isolate				
PGML5			(+)	(+)
Soils				
B.137	Mali	Baguineda	(+)	(+)
B.140	Mali	Baguineda	(+)	(+)
K.115	Burkina Faso	Karfiguela	(+)	(+)
T.127	Mali	Samanko-kati	(+)	(+)
T.131	Mali	Samanko-kati	(+)	(+)
T.134	Mali	Samanko-kati	(+)	(+)
V.10	Burkina Faso	Vallé de Kou	(+)	(+)
V.13	Burkina Faso	Vallé de Kou	(+)	(+)
V.39	Burkina Faso	Vallé de Kou	(+)	(+)
V.46	Burkina Faso	Vallé de Kou	(+)	(+)
H.97	Mali	Icrisat	(+)	(-)
S.83	Mali	Icrisat	(+)	(-)

S.89	Mali	Icrisat	(+)	(-)
T.130	Mali	Samanko-kati	(+)	(-)
T.133	Mali	Samanko-kati	(+)	(-)
V.16	Burkina Faso	Vallé de Kou	(+)	(-)
V.16	Burkina Faso	Vallé de Kou	(+)	(-)
V.25	Burkina Faso	Vallé de Kou	(+)	(-)
V.34	Burkina Faso	Vallé de Kou	(+)	(-)
V.5	Burkina Faso	Vallé de Kou	(+)	(-)
Strain				
PGML3			(+)	(-)

2.2.2 Nutritive solution

The nutritive solution is a Yoshida solution (Table 5) adjusted to a pH of 5.5 as studied by Decroës (2017) or 6.5. The pH was measured using an inoLab pH meter Level 1.

Table 5: Concentration of the adjusted Yoshida solution.

MACRO		MICRO	
Element	Concentration (g/L)	Element	Concentration (g/L)
NH_4NO_3	9.12	$MnSO_4.H_2O$	0.1546
$NaH_2PO_4.2H_2O$	4.03	$Na_2MoO_4.2H_2O$	0.0026
K_2SO_4	7.17	H_3BO_3	0.286
$CaCl_2.2H_2O$	8.86	$ZnSO_4.7H_2O$	0.022
$MhSO_4.7H_2O$	32.4	$CuSO_4.5H_2O$	0.008

2.2.3 Seeds disinfection

Throughout this study, the Kogoni seeds were disinfected during 20 minutes in a solution of sodium hypochlorite (bleach) 1° such as made in the previous study of Bagayoko (2022) and rinsed three times with demineralized water for five minutes each. In this study the bleach solution was adjusted to 1° from a 15° initial concentration using demineralized water.

2.2.4 Seed germination

The seeds were germinated on humidified filter paper for up to seven days at 30 °C in the darkness. Seven days post germination, the germinated seeds were taken out of the incubator and transplanted into PVC tube containing autoclaved quartz.

2.2.5 Growth conditions for bioassays

The plants were grown in a greenhouse at 30 °C during the day, 25 °C at night with 70% humidity, and with 14 hours of photoperiod (Decroës et al. 2017).

2.2.6 Sporosores and zoospores extraction

Sporosores extraction (Annex A.1) was performed with roots samples and soils samples (Table 4). Roots were rehydrated then cleaned by rinsing them with demineralized water for 15 minutes. The roots were disinfected by soaking them in ethanol 70% for two minutes and rinsed gain with demineralized water for 10 minutes. The roots were then cut into small fragments inferior to 5 mm with a scalpel and mixed two times 30 seconds with a Virtis (The Virtis company inc, Gardiner, NY, United States) at 2,300 rpm to extract the sporosores from the cell roots. The sporosores suspension was filtered at 100 µm to remove unwanted roots fragments. Finally, sporosores were counted on a Fuchs-Rosenthal cell (Superior marienfield, Germany) with a LEICA DM 2500 microscope.

Zoospores were extracted from living rice roots plants contaminated with Pgcol (Annex A.2). Fresh plants were taken out of their PVC tube and placed in the dark in the same controlled conditions for 24 hours prior to the zoospores release, to synchronize the production of zoospores from zoosporangia. Plants were then removed from their PVC tube and rinsed in demineralized water to remove the remaining quartz from their roots. They were then placed in a small buncher with the smallest possible volume of 12 °C Yoshida pH 5.5 diluted five times and put back in the darkness under the same controlled conditions for one hour to let zoospores release from the roots. Afterward, the suspension was filtered at 4.5 µm to remove unwanted roots fragments. The zoospores concentration was measured on a Thomas cell (Superior marienfield, Germany) with a LEICA DM 2500 microscope.

2.2.7 Inoculation methods

Three inoculation methods were used in this study: inoculation with Pgcol infested soils, inoculation with a suspension of sporosores, and inoculation with a suspension of zoospores.

Inoculation with Pgcol infested soils was conducted as described by Goffart and Maraite (1991). Germinated seeds were inoculated by being directly transplanted in PVC tube containing a 1:4 (v/v) ratio of infested soils and autoclaved quartz.

Inoculation with a suspension of extracted sporosores and a zoospores suspension was similar. For both inoculations, a given volume of the suspension was taken and

injected at the insertion point of the taproot of germinated seed in the PVC tubes and if possible, at several entry points to cover the root system more efficiently.

2.2.8 Assembly of the growing system

Three watering systems were adapted from the design provided by Legrève (1998) to produce and multiply Pgcol: a collective automatic watering system, an individual automatic watering system and an individual manual watering system. The general structure of the assembly is made by using two plastic boxes (30 cm wide, 40 cm long, 6 or 10 cm high) set on top of each other and covered by a lid pierced by several holes (diameter: 3.5 cm) housing the polyethylene tube containing the substrate and the seeds.

Collective automatic watering system

For the collective automatic watering system (Figure 7), the lower container is filled with the nutritive solution and is linked to the upper box by a pump (Submersible water pump; manufactured in China) through a small hole (diameter: 1.6 cm) pierced at the bottom of the upper box. The Yoshida solution is brought from the inferior box to the upper thanks to a tube linked to the pump. The upper box is pierced at its bottom three times, once for the access pipe linked to the pump, secondly for an overflow pipe allowing it to be filled up and finally for by a drain hole allowing it to be completely emptied. The lid at the top of the assembly was pierced with 30 holes each housing a single PVC tube (10 cm high, interior diameter: 2.7 cm, exterior diameter: 3.2 cm). The upper part of the PVC tube is glued with a larger thin PVC ring to prevent it to fall pass the pierced lid. The bottom part of the PVC tubes contains a small sieve (60 - 120µm, pore size) permeable to nutrient solution and sporosores but impermeable to the substrate. Such assembly was used in two dimensions, a bigger one described here above and a smaller one for eight PVC tubes maximum (each container dimensions are: 11.6 cm high, 15 cm wide and 20 cm long). A smaller pump (COKDEZ^{R} Mini Water Pump DH-500 10W; manufactured in China) was used for the smaller assembly.

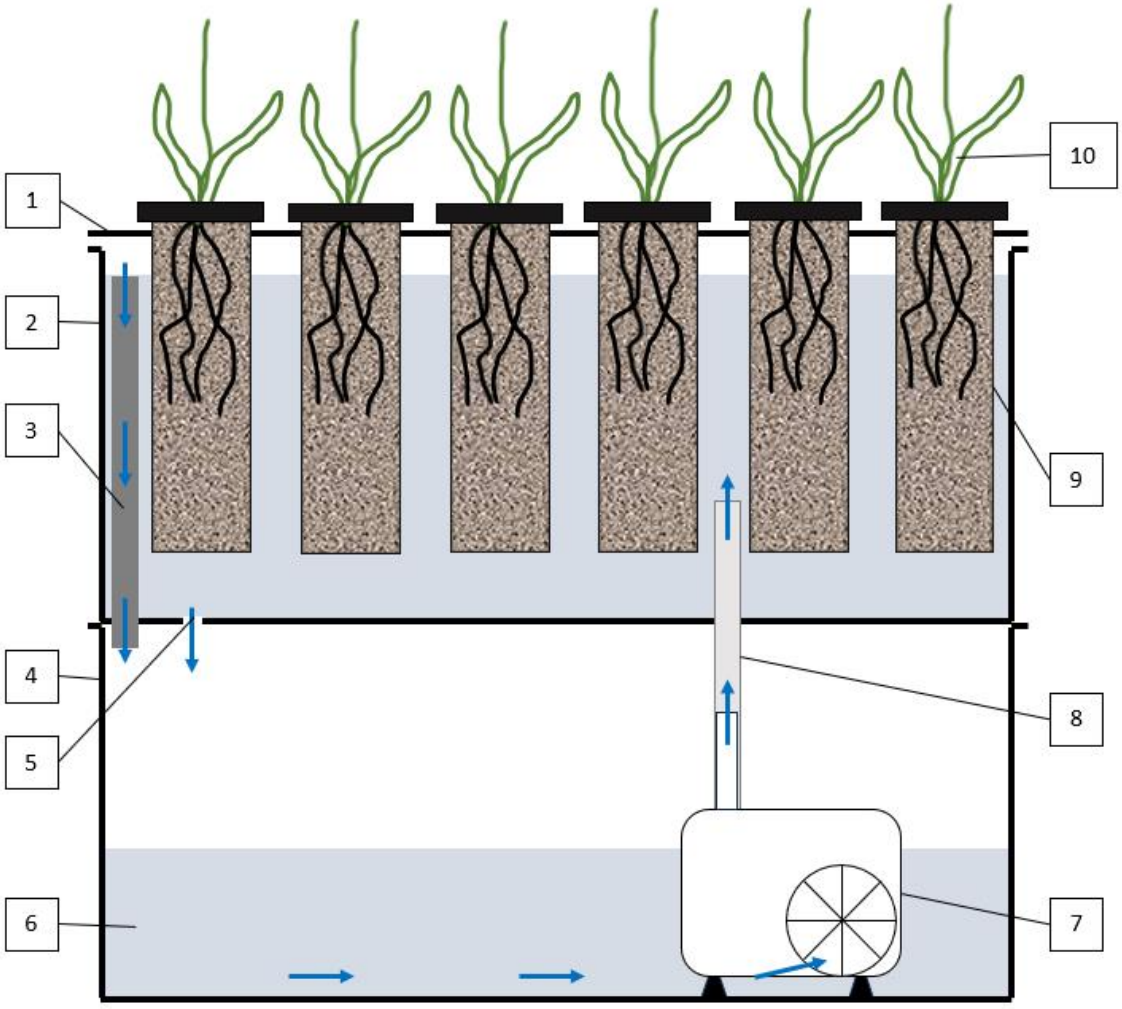

Figure 7: Representation of the collective automatic watering system. 1. Lid; 2. Upper container; 3. Overflow tube; 4. Lower container; 5. Drain hole; 6. Nutritive solution; 7. Pump; 8. Access tube; 9. PVC tubes filled with autoclaved quartz; 10. Rice plant. The blue arrows indicate the movement of the nutritive solution.

Individual automatic watering system

For the individual automatic watering system (Figure 8), the lower box contains demineralized water and is linked to the upper box in the same manner as described above. The upper box contains an aluminum made waterproof container housing individual nutritive solution reservoirs (10 cm high, 5.4 cm wide) meant to feed an individual PVC tube containing a single seed. Like in the previous assembly, the upper box is pierced of three holes, one for the incoming water, another for the overflow tube and the last one acting as a drain. On top of the upper box is found a lid pierced 24 times containing the individual PVC tubes. The individual nutritive solution reservoirs are made of PVC and are slightly bigger than the smaller PVC tube and are filled with nutritive solution up to two third of their maximal capacity.

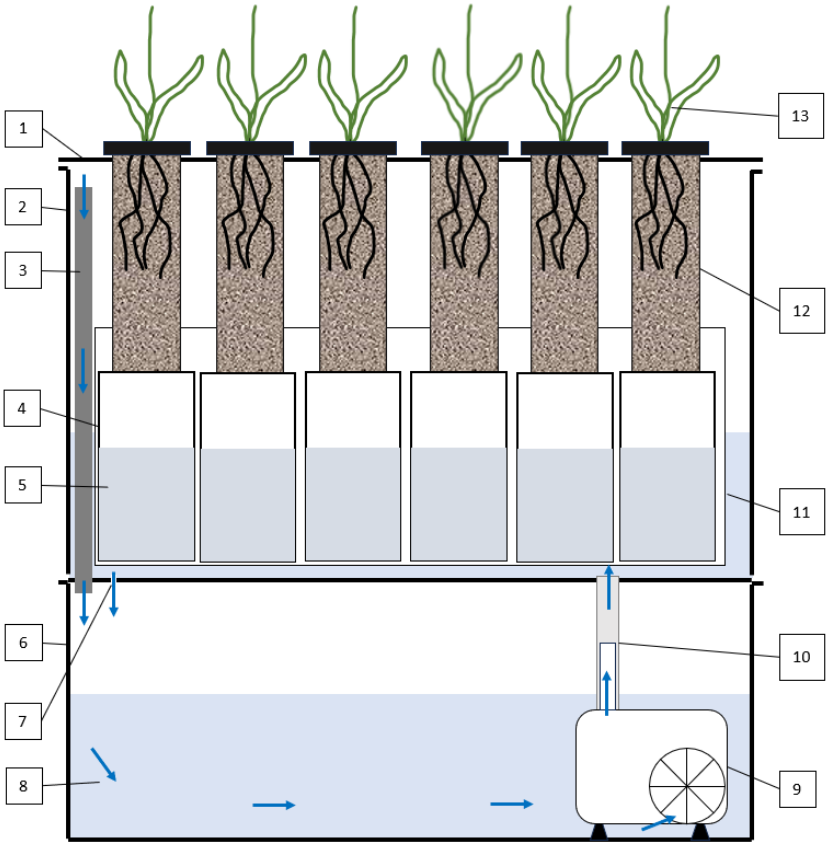

Figure 8: Representation of the individual automatic watering system. 1. Lid; 2. Upper container; 3. Overflow tube; 4. Individual container filled with nutritive solution; 5. Nutritive solution; 6. Lower container; 7. Drain hole; 8. Demineralized water; 9. Pump; 10. Access tube; 11. Waterproof box serving as a lift; 12. PVC tube filled with autoclaved quartz; 13. Rice plant. The blue arrows indicate the movement of the demineralized water.

Individual manual watering system

Lastly, the individual manual watering system (Figure 9) is made of only one box in which are located 24 individual nutritive solution reservoirs housing each a single PVC tube containing a single sample. On top of the box is found a lid pierced with 24 holes housing each a single PVC tube containing one seed.

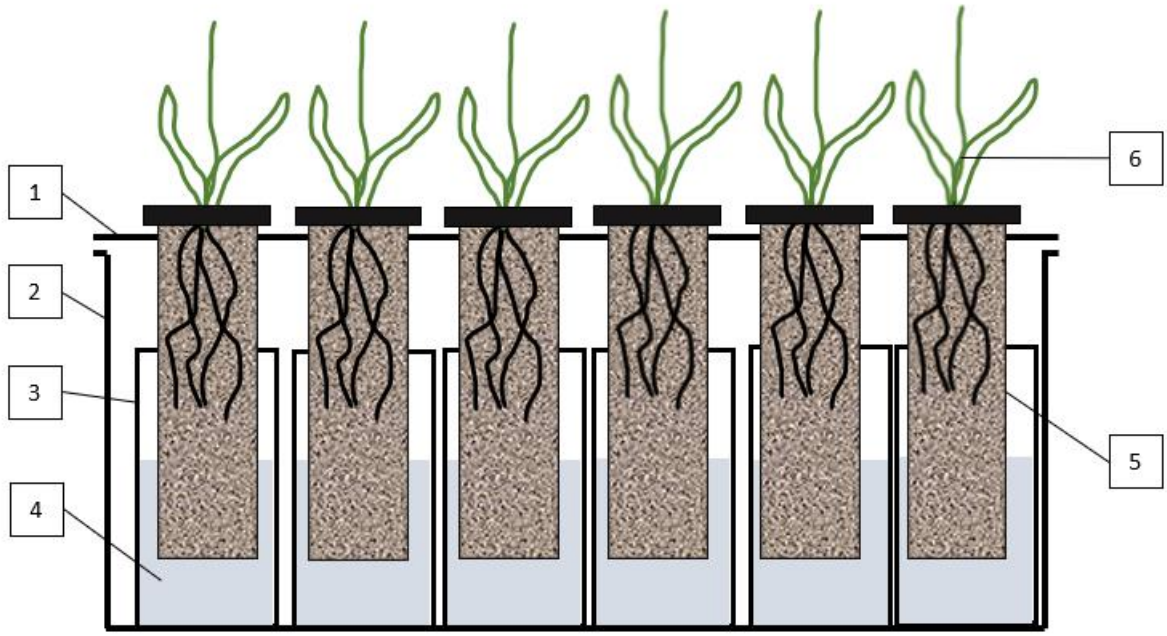

Figure 9: **Representation of the individual manual watering system**. 1. Lid; 2. Container; 3. Individual container filled with nutritive solution; 4. Nutritive solution; 5. PVC tube filled with autoclaved quartz; 6. Rice plant.

Under controlled conditions, young plant having less than two weeks post transplantation were watered every day (excepted during the weekend) by providing nutrient solution by the top of the tube. For plants inoculated with soils samples, this method of watering was kept along the entirety of the experiment. Automatic watering systems were replenished of nutritive and/or demineralized water every two or three days during the entirety of the experiment. Automatic watering system work on a 6-hour shift to ensure that rice cropping was flooded for a certain amount of time during which Pgcol would be actively spreading outside the roots and re-inoculate young cells of the root and also to allow the roots to breath when the pumps were stopped (Legrève et al. 1998).

2.2.9 Harvesting of root samples

Roots were harvested twice to verify successful Pgcol infection of rice roots at two timepoints. The first harvest was made on roots exceeding the PVC tube (Figure 10.a & 10.b), while the second was performed on the full root system (Figure 10.c). Partial root removal was performed in the middle of an experiment, or between three and four weeks, whereas complete removal of the root system was performed at the end of an experiment. All harvested roots were used for PCR detection of Pgcol and microscopic observation. Roots harvested during the ongoing experiment were randomly selected and cut in half for both observation and extraction. For full harvesting, three to four

complete roots were randomly taken for observation and extraction, each. The remaining roots harvested at the end of the experiment were dried and stored.

2.2.10 Observation and analysis

Observation of Pgcol by microscopy

Rice roots were colored by bowling them for two minutes in a solution of blue lactophenol, then rinsed in colorless lactophenol. The colored roots were observed under an Olympus SZ61 macroscope (magnification ranging from 0.75x to 4x) and lighted by a MIC-209 Microscope LED Light in a water environment to detect the presence of Pgcol. Confirmation of the presence was done using a LEICADM 2500 microscope.

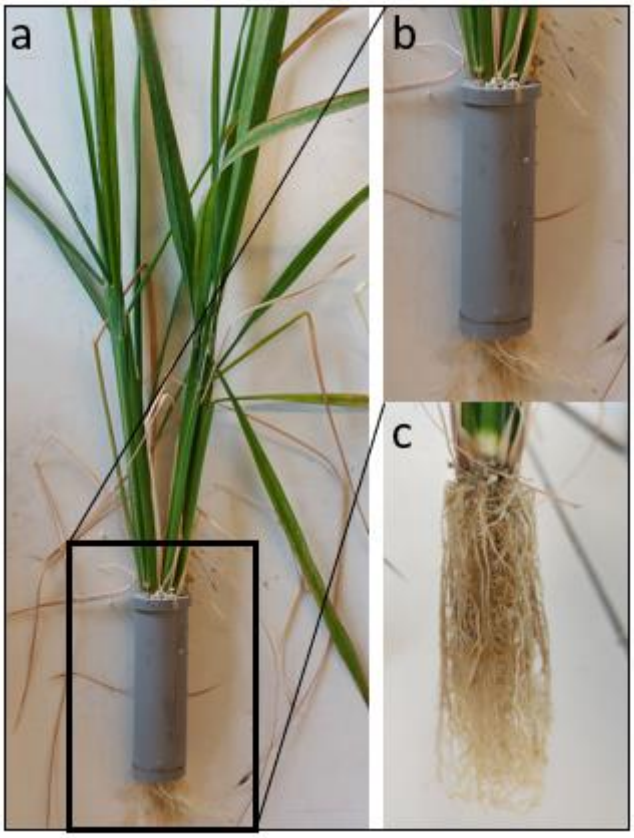

Figure 10: 14-week-old rice plant in PVC tube. **a.** Full view of the plant. **b**. Zoom on the PVC tube and the roots coming out of it. **c.** Full root system of rice plant once taken out of its PVC tube.

Molecular detection of Pgcol and RSNV

RNA extraction (Annex A.3) was performed using 100 mg of fresh root sample grinded 40 sec at speed six in a FastPrep™ FP120 (Bio 101 Thermo electron corporation) with a small ceramic ball in 500µl of Tri-reagent (monophase solution containing 50 to 70% of phenol and 30 to 50% guanidine, thiocyanate (1:1)). The solution was then incubated 10 minutes at room temperature and centrifuged in an Eppendorf centrifuge

5415 R at 4 °C and 13000 rpm for three minutes. The supernatant was then taken out and mixed with 100µl of chloroform, incubated 3 minutes at room temperature and centrifuged at 4 °C and 13000 rpm for 15 minutes. The superior colorless phase was taken out and mixed with 250µl of cooled isopropanol and incubated on ice for 10 minutes. The solution was then centrifuged for 15 minutes at 4 °C and 13000 rpm. The pellet was finally washed with 700 µl of cooled (-20 °C) ethanol 70% after removing the supernatant. The solution was centrifuged one last time at 4 °C, 7500 rpm for 5 minutes before removing the ethanol. Once the pellet was dried, it was suspended in 30µl of DNAse and RNAse-free water. RNA purity and concentration were measured using a Nanodrop™ 1000 spectrophotometer before being stored at -80 °C.

RT-PCR and PCR (Annex A.4) were performed to detect Pg. Two cycles are required to produce complementary DNA from RNA when performing a RT-PCR. The first uses 8.5 µl of biomolecular water, 1 µl the forward primer, and 1µl of RNA for a duration of 10 minutes at 65 °C. In the second, 0.25 µl of reverse transcriptase along with 3.25 µl of biomolecular water, 4 µl of reverse transcriptase buffer, and 2 µl of dNTPs are added to the previous mix and heater at 42 °C for 60 min. The PCR uses 0.125 µl of GoTaq enzyme along with 13.125 µl of biomolecular water, 5 µl of GoTaq buffer, 2.5 µl of $MgCl_2$, 0.75 µl of dNTPs, 0.5 µl of the forward and reverse primers to which are added either 2.5 µl of complementary DNA created through a RT or DNA from an extraction. Several pairs of primers were used (Table 6) to detect Pg and each has a specific PCR cycle (Annex D.2). RT-PCR and PCR were performed in a Sensquest Labcycler.

DNA extraction (Annex A.5) was performed on all samples from bioassays using the CTAB method. One hundred milligrams of fresh roots were taken and grinded twice in a tube containing a small ceramic ball and 500 µl of CTAB (2% cetyl trimethylammonium bromide, 1% polyvinnylpyroolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA) with a Fast Prep for 40 seconds at speed 6 with a 5 minutes break in between. Two incubations at 42 °C and 60 °C followed for 10 minutes each. Then, 500µl of chloroform:isoamyl alcohol in a 24:1 ratio was added, and the mixture was vortexed until complete emulsion before being incubated on ice for 10 minutes and centrifuged for 10 minutes at room temperature and 13000 rpm. The supernatant was taken out and mixed with 194 µl of PEG (30%) and 100 µl of NaCl (5M). After centrifugation for 15 minutes at room temperature and 13000 rpm, the supernatant was removed and 100µl of cooled (-20 °C) ethanol 70% was added to the pellet. Finally, the pellet was diluted in 30µl of DNAse and RNAse-free water. DNA purity and concentration were measured with a Nanodrop™ 1000 spectrophotometer before being stored at -20 °C.

Table 6. Primers used for detection of Pgcol and RSNV.

Organism	Gene	Primers	Sequences	Tm
Polymyxa spp.	*ITS*	Psp 1	TAG-ACG-CAG-GTC-ATC-AAC-CT	60°C
		Psp2rev	AGG-GCT-CTC-GAA-AGC-GCA-A	60°C

	(450-550 bp)			
P. graminis f. sp. *colombiana*	*L10*	Pgcol_L10_f	TGG-TCT-CCG-GTG-AGA-AGG-AG	64°C
		Pgcol_L10_r	AGG-TGT-CCT-TGC-CGG-AAT-AC	62°C
P. graminis f. sp. *colombiana*	*ITS* (195 bp)	Pgrcol_f	ATT-GTC-GGT-TGC-GCG-AAG	62°C
		Pgrcol_r	GAC-TKT-GTG-CTT-CCG-ACC-TG	62°C
RSNV	RNA 1 (927 bp)	RSNV1_f	TGA-ATT-TGG-TGC-TCT-CTT-G	62°C
		RSNV1_r	TGT-GGC-GTT-TCC-AGA-CCT-AAA	62°C

Agarose gel electrophoresis containing 1.2% of agarose were made with either Gel Red or ethidium bromide (BET) (10 µg/ml) (Table 7). DNA migrated for one hour at 130V before being measured with a BIO-RAD Molecular Imager Gel Doc™ XR+ with Image Lab™Software. PCR products from bioassays 1 and 2 were visualised on gels with ethidium bromide whereas the rest were visualised using Gel Red gel.

Table 7. Recipe of agarose electrophoresis gel with Gel Red and Ethidium Bromide colorant.

Gel size	TBE[1] 1x [mL]	Agarose [g]	Gel Red [µL]	Voltage [V]
Small	40	0.48	2	90
Medium	100	1.2	5	90
Large	200	2.4	10	130
Extra large	250	3	12.5	130

Gel size	TBE[1] 1x [mL]	Agarose [g]	BET[2] 10 µg/ml [µL]	Voltage [V]
Small	40	0.48	0.5	90
Medium	100	1.2	1	90
Large	200	2.4	2	130
Extra large	250	3	2.5	130

[1] TBE = Tris Borate EDTA

[2] BET = Ethidium bromide

2.2.11 Production of Pgcol from sporosores (bioassay 1)

The first bioassay that aimed to multiply the strain PGML3 and the isolate PGML5 of Pg was performed using germinating seeds of the variety Kogoni for 36 samples. Seeds were germinated after being disinfected with bleach 1° for 20 minutes and transplanted four days later in PVC tubes in individual automatic watering system with a nutritive solution Yoshida of pH 5.5 (Figure 8). The PGML5 isolate was inoculated at a concentration of 3500 sporosores per plants on 28 plants. The monosporous PGML3 strain was inoculated on eight plants at a concentration of 2000 sporosores per plants. A new batch of seeds was germinated 10 days after the one for PGML3 and PGML5 and 12 plants were transplanted five days post germination in new tubes containing soils of different origins (Table 4) in the individual manual watering system. Additional soils (B.137, B.140, T.127, V.16, S.83, V.5 (Table 4)) were later added to the manual watering system and three others (H.97, V.34, V.13 (Table 4)) were used in small collective automatic watering system for three, three, and two samples, respectively.

Roots exceeding their PVC tube container were harvested seven weeks after inoculation (Figure 10.b). The rest of the plant was left untouched to limit damage, as they will be used for Pgcol multiplication (cfr. bioassay 2). Microscopic observations were made on all harvested samples, but no extraction was performed due to the limited amount of collected roots.

2.2.12 Production of Pgcol from zoospores (bioassay 2)

The second bioassay aimed to multiply Pgcol in rice roots using zoospores as a source of inoculum. Eight PGML3 contaminated plants from the first bioassay were put together with 16 seven-day-old healthy plants in a collective automatic watering system (Figure 7). The PGML5 were divided into three collective automatic watering systems (two big and one small); a large one containing 15 old PGML5 infected plants and 15 seven-day-old healthy plants, another containing 30 healthy seven-day-old healthy plants infected with a zoospore release performed on one confirmed infected sample with PGML5 from bioassay 1, and a small one containing the infected sample with seven seven-day-old healthy plants. Three small collective automatic watering system were used for the soil 5.C isolate: one contained seven seven-day-old plant with the infected 5.C sample, two contained eight seven-day-old healthy plants each infected with a zoospore release performed on soil sample 5.C.

2.2.13 Study of the pH and watering system on Pgcol development (bioassay 3)

A third bioassay named “pH and watering bioassay” was designed to study the effect of the pH and the watering method (Annex D.3) on *Pgcol* infection of rice roots. This parameter was studied because we observed an increase in acidity in the nutritive

solution of some samples in the Bioassay 1 going up to pH 3.5. Therefore, the impact of the pH on the development of Pgcol was investigated by using a less acidic nutritive solution.

Four modalities were established, two collective automatic watering system (Figure 7) at two pH (5.5 and 6.5) and two individual automatic watering system (Figure 8) at two pH (5.5 and 6.5). For each modality, six plants were cultivated. The plants were germinated for seven days after being disinfected with a bleach solution of 1° for 20 minutes and inoculated with 4500 sporosores per plant three weeks post germination. Half of the roots system were harvested for the first three plants at five weeks post inoculation and at six for the three others. After harvesting for observation and extraction, the remaining half root system was put back in the assemblies with new autoclaved quartz. Finally, the complete root system of all plants was harvested at 11 weeks post inoculation and stored.

2.2.14 Study of the dynamic of infection of Pgcol on rice (bioassay 4)

A fourth bioassay, named “Dynamic bioassay” (Annex D.3) aimed at better understanding the dynamics of Pgcol life cycle by studying the timing of optimal zoospore production. This objective was achieved by quantifying zoospore released from Pgcol contaminated rice roots at different time points. For such, 36 seeds were germinated for seven days after disinfection with a bleach solution 1° for 20 minutes and inoculated with 3500 sporosores per plant three weeks post germination. Plants were grown in two individual automatic watering systems until harvest. Three plants were harvested for zoospores release at 10, 12, 14, 17, 19, 21, 24, 26, 28, 31, 33, 35 days post inoculation. After the zoospore release, zoospores were counted using a Thomas cell, and roots of the plant were colored with blue lactophenol for observation of Pgcol structures by microscopy.

2.2.15 Study of the efficiency of sporosores extraction (experiment 1)

A first side experiment, called “sporosore extraction experiment” was performed to study the efficiency of sporosores extraction from roots using three different methods: using a mortar (Annex A.6), using solely scalpels (Annex A.7) or using a Virtis blender (Annex A.1). Sample of *Polymyxa graminis* f. sp. *tropicalis* “Isolate S6” in pearl millet roots was kindly provided by Pr. A. Legrève (Legrève et al. 2000) and used to perform the extractions. Each extraction method was repeated three times for statistical purposes. Extraction using a mortar was performed on dried roots by reducing them into a thin powder by grinding them with a sterile mortar and pestle, then adding demineralized water to form a suspension. The suspension was filtered at 500 µm to remove unwanted roots fragments. Extraction using scalpels was performed on rehydrated roots by cutting them with sterile sharp scalpel until roots fragments were inferior to 5 mm. After adding demineralized water, the suspension was filtered at 500 µm to remove unwanted root fragments. Extraction using the Virtis was performed such as described hereabove and filtered at 500 µm to remove unwanted root

fragments. The most efficient way to extract sporosores was investigated by counting the sporosores on a Fuchs-Rosenthal cell.

2.2.16 Study of the efficiency of several disinfection methods (experiment 2)

A second side experiment, called “disinfection experiment” was conducted to investigate the efficiency of several disinfection methods on the suppression of fungal pathogens present inside the seeds.

Eleven treatments (Annex A.8) were set based on previously realized studies (Chun et al. 1997; Kim et al. 2022; Piernas et Guiraud 1997). Three disinfectants (bleach, ethanol, or hot water) were used. Four combinations of bleach and disinfection timings were tested, bleach 1° for 5 minutes, bleach 1° for 20 minutes, bleach 10° for 5 minutes, bleach 10° for 10 minutes. Two concentrations of ethanol (ethanol 10% and ethanol 50%) were used with a 10 minutes disinfection. Hot water (60 °C) was used as a mean of disinfection, and two disinfection timings (7 minutes and 14 minutes of seeds disinfection). Moreover, two treatments combined two disinfectants together for the same amount of time (7 minutes), a combination of bleach 1° and hot water (60 °C) and a combination of ethanol 10% and hot water (60 °C). Finally, a last batch of seeds were not treated with any disinfectant to serve as a negative control. For each modality, 100 seeds of rice were used.

The number of germinated seeds and the number of infected seeds were observed every day, but the weekend for 14 days. The vigor index (VI) was also calculated to observe the influence of the disinfection method on the development of the rice seeds. The side of the shoot was measured on the 14th day. Each set of eleven modalities was repeated three times for comparison purpose.

2.3 Results

2.3.1 Production of Pgcol from sporosores (bioassay 1)

The first bioassay aimed to achieve Pgcol infection on rice roots using different Pgcol isolates (the monosporous RSNV-free PgML3, the RSNV-transmitting PgML5 isolate and several Pgcol infested soils). The success of Pgcol infection was first verified with microscopic observations (Table 8). Sporosores were observed in the roots of five inoculated plants (Figure 11), proving successful infection of these five plants. Among these five plants, three were inoculated with the isolate PGML5 (ID “D.3”, “F.1”, and “F.4”), one with the strain PGML3 (ID “H.2”), and one with soil samples (ID “5.C”). Structures resembling sporangial plasmodia were also observed in multiple samples but could not exactly be associated with any life form of Pgcol. These structures appeared as regular-coloured vegetal cells and did not present the distinctive zoosporangia present at later stages of sporangial plasmodia development. Often, the colored structures were observed inside the central cortex and so far, Pg has not been proven to infect such regions. Finally, for some samples many contamination were

observed nearby the potential sporangial plasmodia and a question mark was left for further confirmation after extraction. Moreover, the severity of infection was always low.

Table 8: Observations of Pgcol in rice roots at the end of the first experiment with PGML5 and PGML3 strains and naturally infested soils. Blank boxes containing "-" indicate the absence of any life form of Pgcol. Orange boxes indicate an incertitude about the observations and the uncertain structure. Green boxes indicate the presence of Pgcol and the structure observed.

PGML5		PGML3		SOILS	
ID	Observations	ID	Observations	ID	Observations.1
A.1	-	H.1	-	1.A	-
A.2	uncertain plasmodia	H.2	Sporosores	1.B	uncertain plasmodia
A.3	-	H.3		1.C	uncertain plasmodia
A.4	-	H.4		1.D	-
B.1	-	I.1	-	1.E	-
B.2	-	I.2	-	1.F	-
B.3	-	I.3	-	5.A	-
B.4	-	I.4	-	5.B	-
C.1	uncertain plasmodia and zoospores			5.C	Sporosores
C.2	-			5.D	-
C.3	-			5.E	-
C.4	-			5.F	-
D.1	-				
D.2	-				
D.3	Sporosores				
D.4	-				
E.1	-				
E.2	-				
E.3	-				

E.4	-		
F.1	Sporosores		
F.2	-		
F.3			
F.4	Sporosores		
G.1	-		
G.2	-		
G.3	-		
G.4			

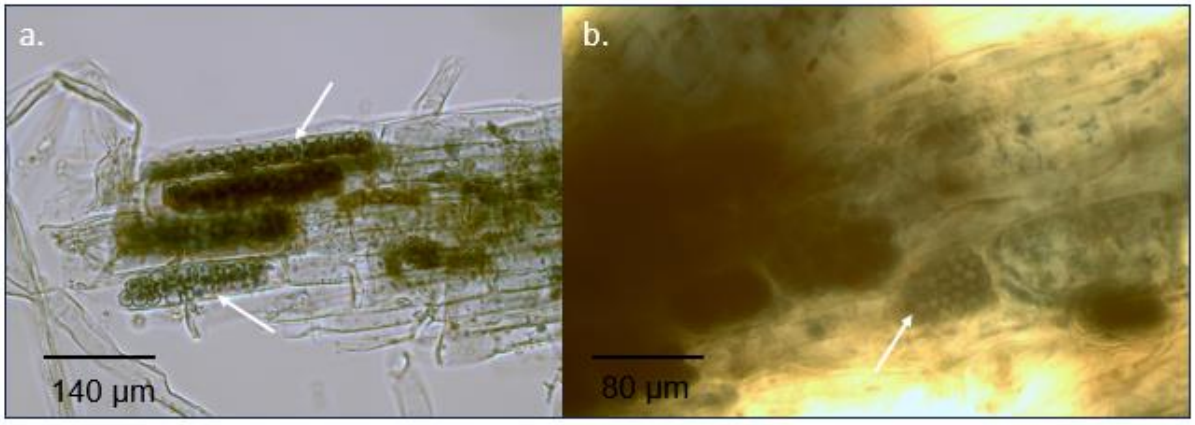

Figure 11: Sporosores observed under the microscope (magnification 400x) on Asian rice (*Oryza sativa* var. Kongoni). **a**. Sporosores present in sample H.2 of the monosporosorous PGML3 strain; **b**. Sporosores present in sample D.3 of the PGML5 isolate. White arrows indicate the position of sporosores.

RNA extraction and RT-PCR detection were not performed on samples infected with PGML3 and PGML5 isolates from the first bioassay. Therefore, Pgcol infection could not be confirmed for root samples containing structures resembling plasmodia.

2.3.2 Multiplication of Pgcol from zoospores (bioassay 2)

The second bioassay aimed to multiply Pgcol from plants inoculated in the first bioassay.

Among the 30 plants multiplied with PGML3 infected plants, the 68 plants multiplied with PgML5 infected plants, and the 24 plants multiplied with soil-inoculated plants, no Pgcol structures were observed by microscopy (Annexe B.2). For the first bioassay, other structures resembling plasmodia were observed.

RNA extraction and RT-PCR (Figure 12) were performed on 17 samples from the PGML3 collective automatic watering system, 45 samples from the PGML5 collective automatic watering systems, and 18 samples from the Soil 5.C collective automatic watering systems. Extraction and amplification were made on a reduced amount of sample as some plants died and others had not enough roots for proper microscopical observation and extraction. Primers Pgcol_L10_f/Pgcol_L10_r were used to specifically detect Pgcol. Although the purity of the extracted RNA samples (Annex C.1) was not great, RNA yields were sufficient for RT-PCR detection. No Pgcol could be detected in plants multiplied either with PGML3, PGML5, or soils infected samples. This is coherent with the lack of Pgcol structures observed by microscopy and confirms that plasmodia-like structures observed in roots are not Pgcol plasmodia.

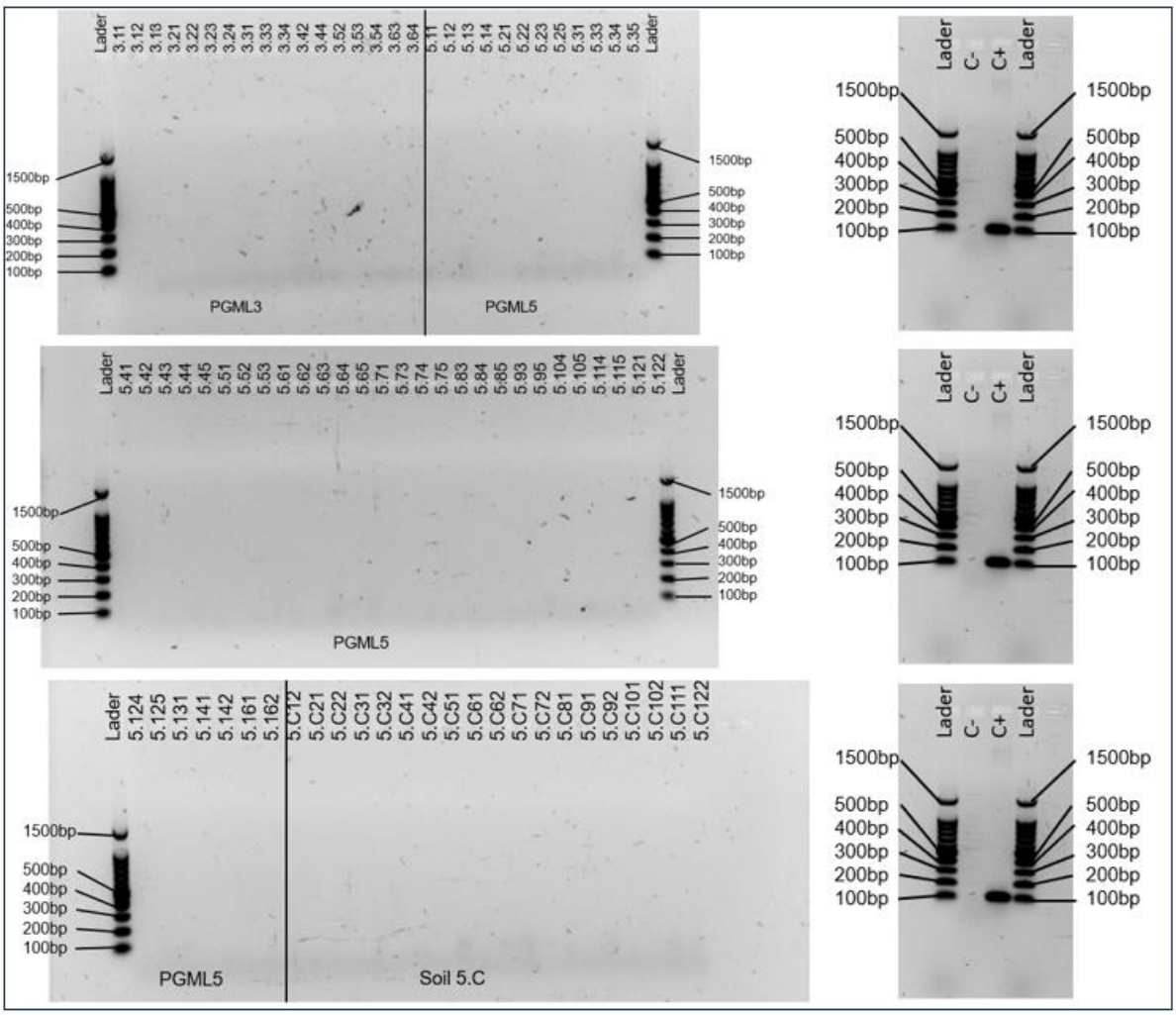

Figure 12: Agarose gel electrophoresis made on the samples harvested in collective automatic watering systems from Experiment 2 using primers Pgcol_L10_f/Pgcol_L10_r. The different inocula are separated from one another by a black line. PGML3, PGML5, Soil 5.C indicate the inoculum. The unit for the ladder is the base pair (bp). The negative and positive controls are represented by C- and C+, respectively.

Samples of plants inoculated with soils (Figure 13a) were also extracted and analysed with RT-PCR using the primers Pgcol_L10Lf/Pgcol_L10_r. However, no Pgcol was detected. Therefore, soil inoculation failed in this second bioassay, with both individual manual watering and collective automatic watering systems.

As some soils samples were inoculated with RSNV-transmitting inoculum, RSNV infection of pants was verified with primers RSNV1_f/RSNV1_r (Figure 13.b). For both positive controls, two amplicons are visible at 4000 bp and 2000 bp. Furthermore, a double line is visible at the bottom of the gel for all samples. They probably correspond to the primer dimers and the advancement of the fragments. The second positive control is less neat than the first indicating concentration likely too high. Strangely the primers used of this detection are supposed to amplify a fragment of 927 bp (Table 6). However, both positive controls do not present any amplicons around 1000 bp and furthermore they both have strong amplicons at 4000 bp. Since both negative controls present an amplicon at 4000 bp it probably means that it originates from the PCR. But no RSNV can be detected on any samples.

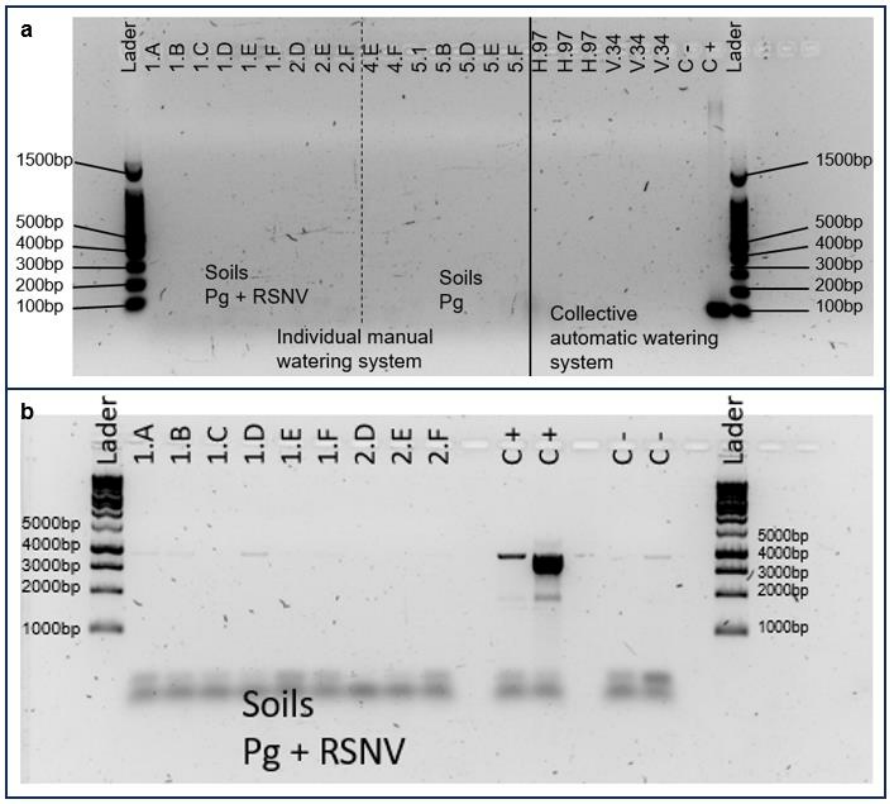

Figure 13: Agarose gel electrophoresis made on samples harvested in individual manual watering system from bioassay 2 for detection of Pgcol and RSNV using Pgcol_L10_f/Pgcol_L10_r and RSNV1_f/RSNV1_r primers, respectively. **a.** Detection of Pgcol on soils samples. The black line separates samples according to their watering system and the dotted black line separates samples containing RSNV from those that do not. **b**. Detection of

RSNV on soils samples containing it. The unit for the ladder is the base pair (bp). The negative and positive controls are represented by C- and C+, respectively.

2.3.3 Study of pH and the watering system (bioassay 3)

As the first two bioassays were not very successful, a third bioassay was conducted to study the impact of pH variation and the watering system on Pgcol production.

Sample of the individual automatic watering system with a pH of 5.5 collected after five weeks (ID “5.5i.1”) and sample of the individual automatic watering system with a pH of 6.5 collected after five weeks (ID “6.5i.1”) both presented a potential presence of sporosores and plasmodia, respectively (Annex B.3). These structures resembled to Pgcol life forms but were seemingly present inside the cortex of the roots. Moreover, in the case of potential sporosores, the size of the resting spores was too big. No other structures were observed for other samples.

The PCR detection realized on 18 DNA extracted samples from the third bioassay using primers Psp1/Psp2rev showed amplification of DNA fragments for most samples (Figure 14). A first gel was made after one purification of the DNA extracts. No amplification can be detected for the second positive control while some can for the first at the desired length of amplification by Psp1/Psp2rev (450-550 bp). This indicates that the problem probably originated from the sample. Although many fragments are visible for both modalities, both individual watering systems present more amplification especially around 450 bp and 150 bp. The amplification at around 150 bp must come from contamination and do not show the presence of Pgcol. A second gel was made after a second purification, but nothing could be concluded from it.

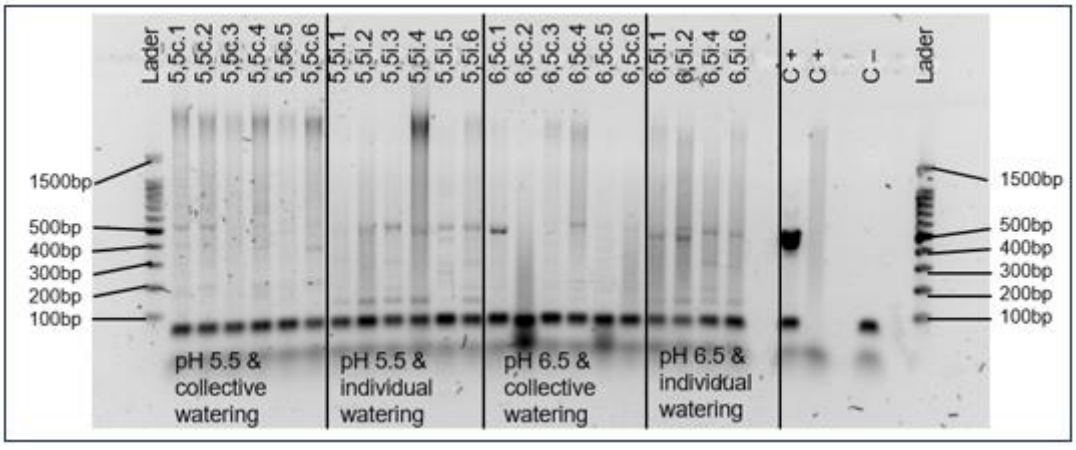

Figure 14: Agarose gel electrophoresis made on rice roots samples infected with Pgcol from the experiment studying the pH and watering system of Experiment 3 using primers Psp1/Psp2rev. The unit for the ladder is the base pair (bp). The negative and positive controls are represented by C- and C+, respectively.

PCR detection using primers Pgcol_L10_f/Pgcol_L10_r was also realized on DNA extracted samples from rice roots of the third bioassay and showed no clear amplification from any samples (Figure 15). However, smears are visible for almost all

samples and much less of the positive control. This indicates either a poor sample quality or that the concentration of the sample was too high. The negative PCR control as well as the positive PCR control show little to no smears indicating that the quality of the control was adequate and thus that the quality of the sample was not.

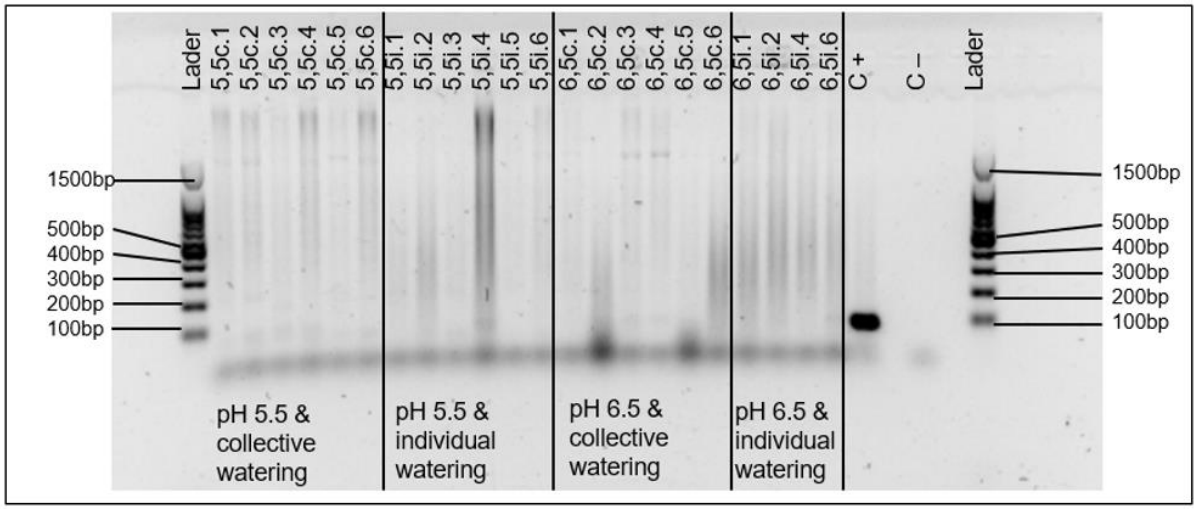

Figure 15: Agarose gel electrophoresis made on rice roots samples infected with Pgcol from the experiment studying the pH and watering system of Experiment 3 using primers Pgcol_L10_f/Pgcol_L10_r. The unit for the ladder is the base pair (bp). The negative and positive controls are represented by C- and C+, respectively.

A third set of primers was used to perform PCR detection on the same samples, Pgrcol_f/Pgrcol_r. This third set a primer was used to confirm the adequacy of the Pgcol_L10_f/Pgcol_L10_r for Pgcol amplification. A first gel was made after one purification, but a lot of smears were present. Therefore, a second gel was made after a second purification (Figure 16). In this gel, the positive control presents smears indicating that the concentration was too high. The size of the amplicon ranges from 200 to 400 bp further indicating that the concentration is too high as the amplification was too spread and should be of 195 bp. No sample show any amplification, but they all present a bottom line corresponding to the primer dimers.

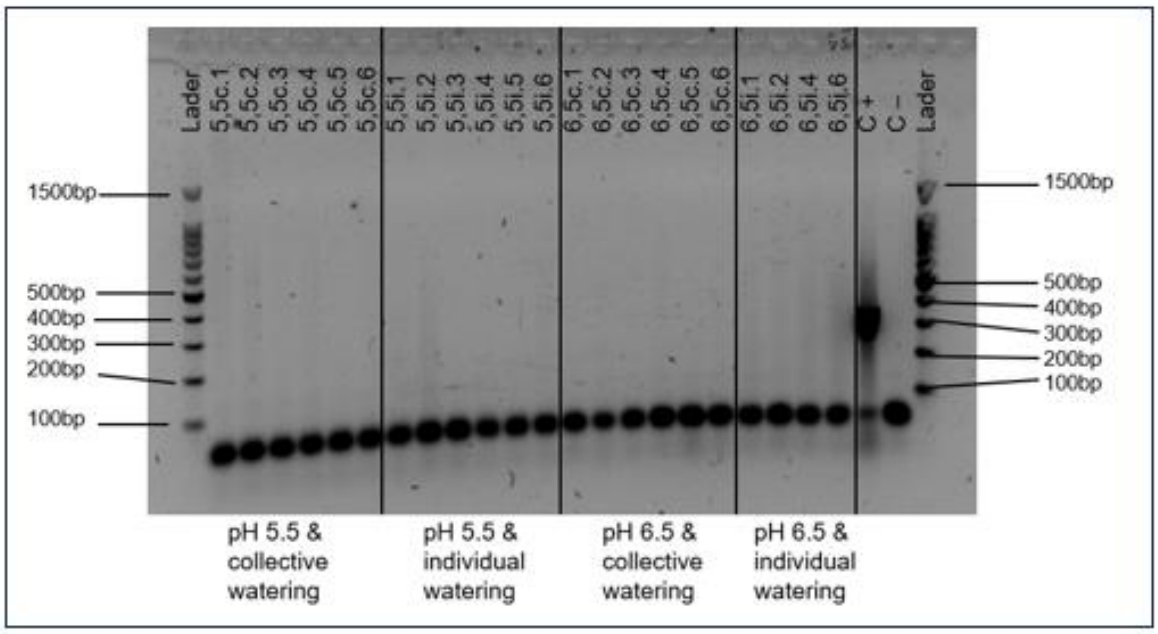

Figure 16: Agarose gel electrophoresis made on rice roots samples infected with Pgcol from the experiment studying the pH and watering system of bioassay 3 using primers Pgrcol_f/Pgrcol_r. The unit for the ladder is the base pair (bp). The negative and positive controls are represented by C- and C+, respectively.

2.3.4 Study of the dynamic of infection of Pgcol in rice (bioassay 4)

The fourth bioassay aimed to better understand Pgcol life cycle by identifying the timing of optimal zoospores production, corresponding to the zoosporangia stage.

Microscopic observations of zoospores released from rice roots contaminated with Pgcol during the study of the dynamic of infection of Experiment 4 did not reveal the presence of any zoospores. However, observation of the roots revealed the presence of Pgcol under the form of sporosores and zoosporangia (Figure 17) in sample “9.1” corresponding to a harvesting time of 28 days post inoculation (Annex B.4).

No PCR detection was performed on these samples and could not confirm the presence or absence of Pgcol in samples.

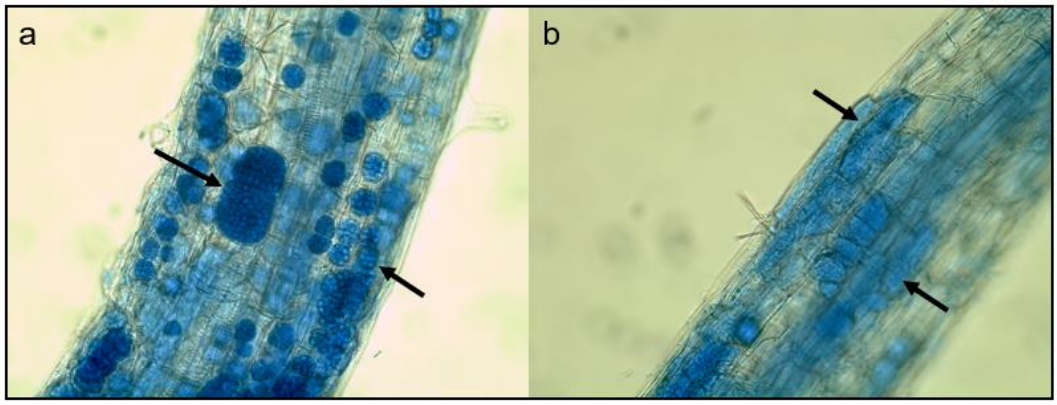

Figure 17: Pgcol structures observed in sample 9.1 of the "dynamic experiment". a. Sporosores. **b**. Zoosporangia. Black arrows indicate the structures.

2.3.5 Improvement of sporosores extraction from dried infected roots

To improve the Pgcol inoculation protocols, sporosores extraction efficiency from dried infected roots was investigated through three methods, by using a mortar, scalpels, and a Virtis blender. In annex B.5 are presented the roots mass used for extraction, the volume of demineralized water used to make the suspension and the amount of sporosores observed per method of extraction. Total sporosores quantity per gram of roots in suspensions of each extraction modality is represented in Figure 18. Extraction using the Virtis blender stands out as the most effective method for sporosores extraction, followed by the extraction method using the scalpels and finally the mortar extraction method. Therefore, it seems that the sporosores extraction method used in the inoculation protocols of Pgcol is optimal.

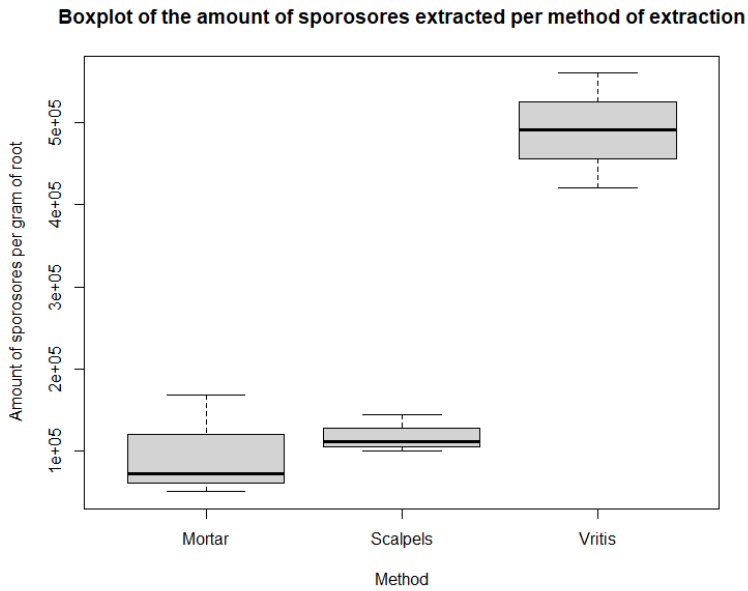

Figure 18: Boxplot of the amount of sporosores of *Polymyxa graminis* f. sp. *tropicalis* extracted with a mortar, scalpels, and a Virtis blender.

2.3.6 Improvement of rice seeds disinfection method

Three parameters were studied to evaluate the impact of the disinfection method on the sanitary status of seeds: the percentage of seed germination (Figure 19), the percentage of contaminated seeds (Figure 20), and the Vigor Index (VI) for all seeds (Figure 22). The VI is calculated by multiplying the seedling length by the percentage of germination ($VI = Seedling\ length * Germination\ [\%]$). In this case, the seedling length was measured in millimeters. Measurements of the percentage of germination and the VI were made to assess how seeds germinated and grew after a certain disinfection method.

The type and concentration of disinfectant appears to have an impact on the germination percentage (Figure 19). In all experiments, the use of bleach disinfectant alone (at 1° or 10°) had a positive impact on germination, as these modalities have higher germination percentages than the untreated seeds. Disinfection with ethanol 10% alone has a similar germination percentage than the untreated seeds at the end of the experiment, but show a slower progression of germination, suggesting a less homogeneous germination. Disinfection with heated water for 7 minutes has a similar germination percentage than the disinfection method using bleach at 1° for 20 minutes. Finally, disinfection methods using either heated water for 14 minutes or ethanol 50% have germination percentages equal to 13% and 0%, respectively. This suggests that higher concentration of ethanol and longer treatment with heated water inhibit the germination of rice seeds.

The disinfection method involving the combined use of bleach 1° with heated water has little to no impact on the germination of seeds. The other combined treatment (ethanol 10% + heated water) significantly impacts the germination of the seeds compared to the untreated seeds. Individually, bleach 1°, ethanol 10%, and heated water for 7 minutes have a better, worse, and worse impact on the germination percentage than the untreated seeds, respectively. The impact of their combination can be seen as the addition of their individual impacts on seed germination and the product of their interaction. This added interaction can be speculated to increase the load on the seeds, thus decreasing their germination percentage. This hypothesis can be confirmed by the increasing impact on seeds germination when two disinfection treatment are combined. Interestingly, all disinfection methods using bleach alone have a better germination percentage than the untreated seeds.

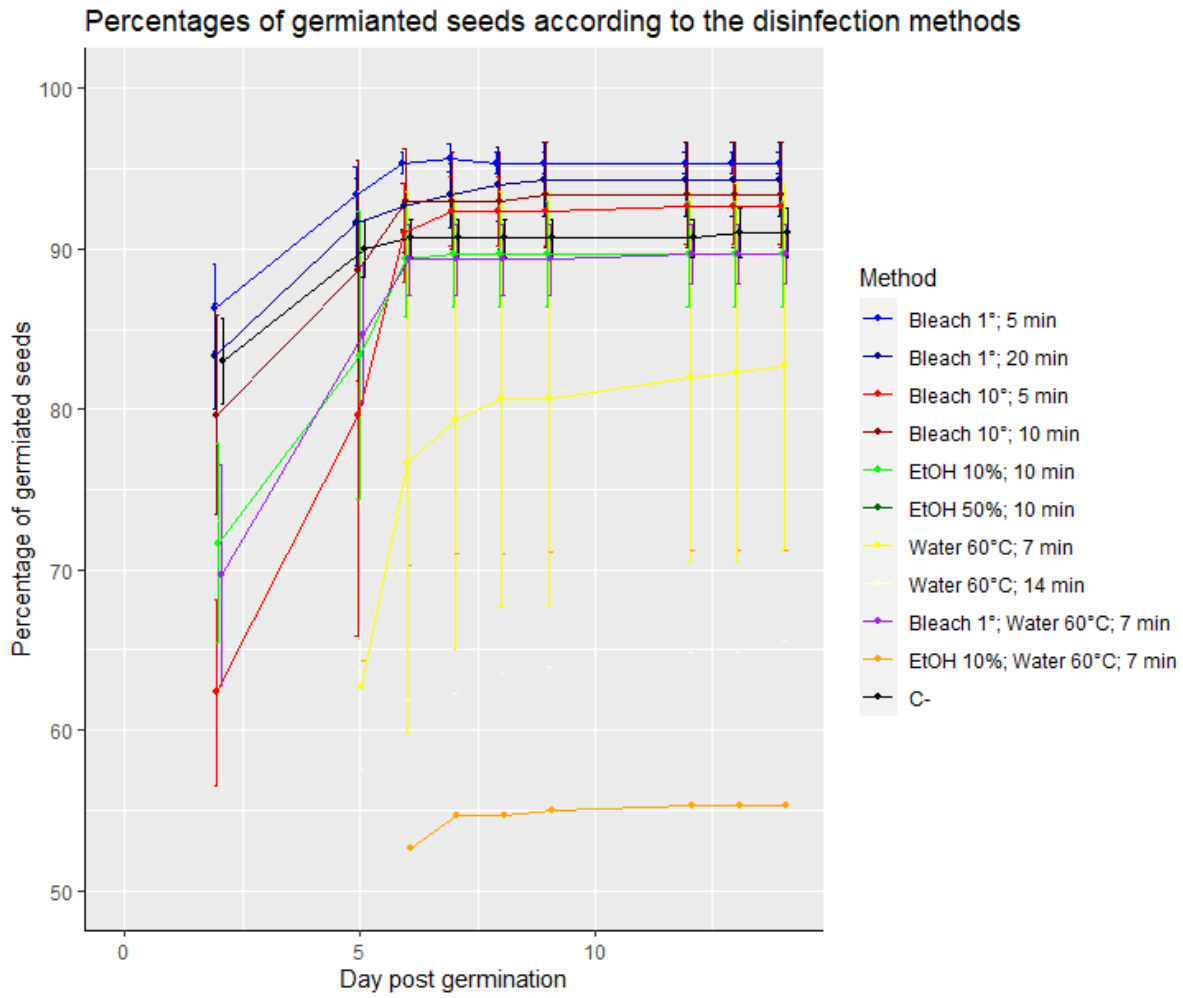

Figure 19: Percentage of germinated seeds according to the disinfection method.

The disinfection methods also had an impact on the level of seed contamination (Figure 20). The highest level of contamination was observed for seeds disinfected with ethanol 10%. The level of seeds contamination of ethanol 10% is similar to the negative control suggesting that disinfecting rice seeds with ethanol 10% for 10 minutes does not prevent the development of endogenous pathogens present in rice seeds. The best disinfection is the method combining the use of ethanol 10% and heated water, followed by the disinfection using bleach 10° for 5 minutes and the other combined method. Two hypotheses can be made on the efficiency of the best disinfection methods (ethanol 10%; 60 °C; 7 minutes, bleach 1°; 60 °C; 7 minutes, bleach 10°; 5 minutes, water 60 °C; 7 minutes) against the development of endogenous rice pathogen present inside the seeds: either the disinfection methods did impact the contamination level originating from seeds or the contaminant were too presents in the seeds and inhibited their germination, thus explaining the lower germination level of the combined methods and for heated water.

Three out of the four disinfection methods using bleach at either 1° or 10° have the same percentage of infection after two weeks. But the method using bleach 10° for 5 minutes is twice more efficient than the others. As heated water is present in four of

the five best disinfection methods, it can be assumed that heated water has a strong impact on the prevention of the observed (see below) endogenous pathogens on rice seeds.

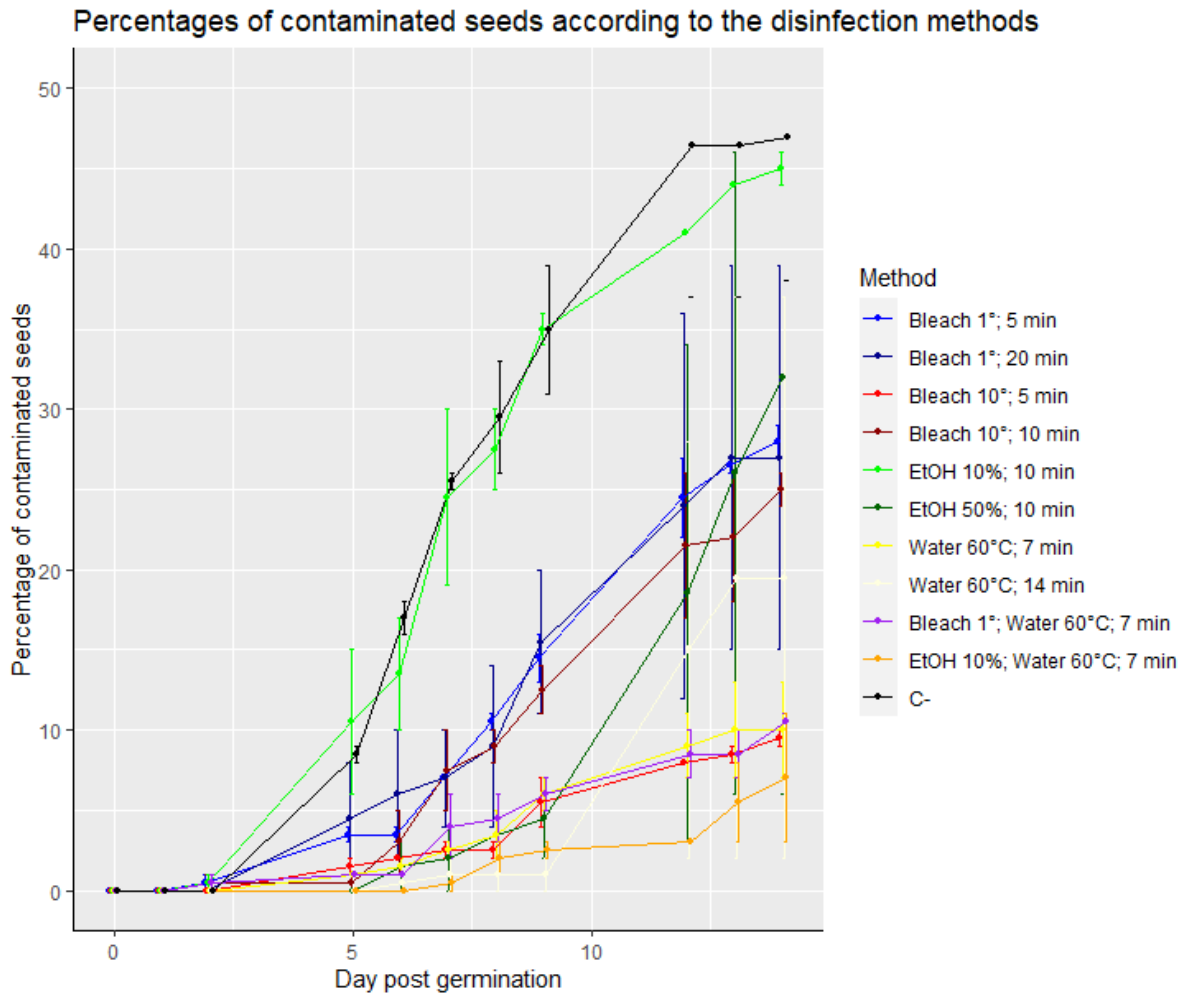

Figure 20: Percentages of contaminated seeds according to the disinfection methods.

Several fungal pathogens were observed during the disinfection tests. Three of them have developed important visual symptoms (Figure 21), *Curvularia lunata*, *Aspergillus niger*, and *Aspergillus oryzae*. Identified fungal pathogens are seed borne since the entirety of the experiment was conducted in sterile conditions.

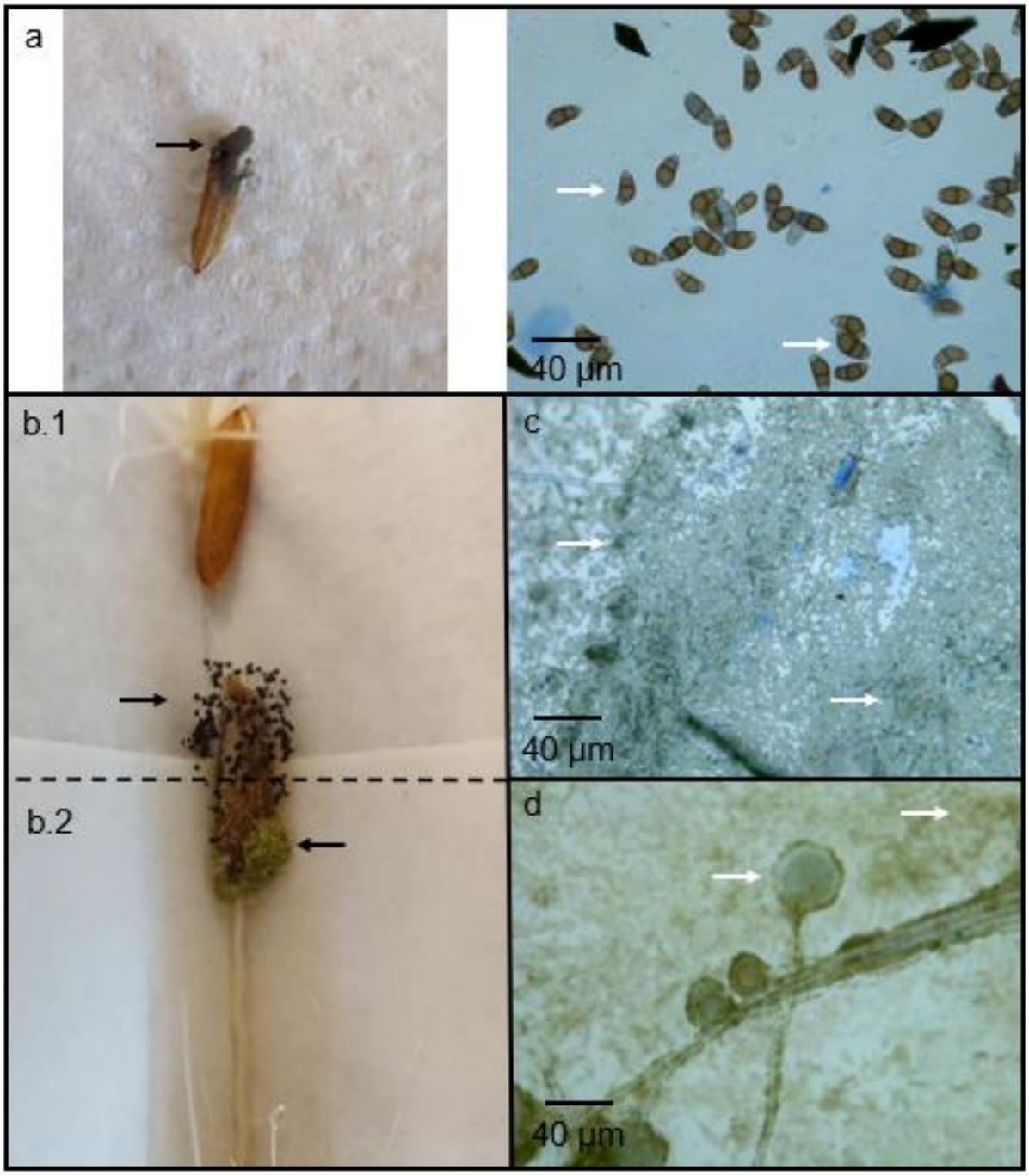

Figure 21: Pictures of fungal pathogens observed during the disinfection tests. a. *Curvularia lunata* on seed (left) or as conidia (right). **b.1** *Aspergillus niger* on seed. **b.2** *Aspergillus oryzae* on seed. **c.** Conidia of *Aspergillus niger*. **d.** Conidia and conidiophores of *Aspergillus oryzae*. The dotted line separates the two Aspergillus development on the same seed. Black arrows point the macroscopic symptoms of the fungal pathogen, and the white arrows point the microscopical structures.

Finally, the vigor index (VI) was also investigated (Figure 22) and showed similar results than germination percentages. Great variations of VI were observed for some disinfection methods (…), indicating a heterogeneous growth of plants.

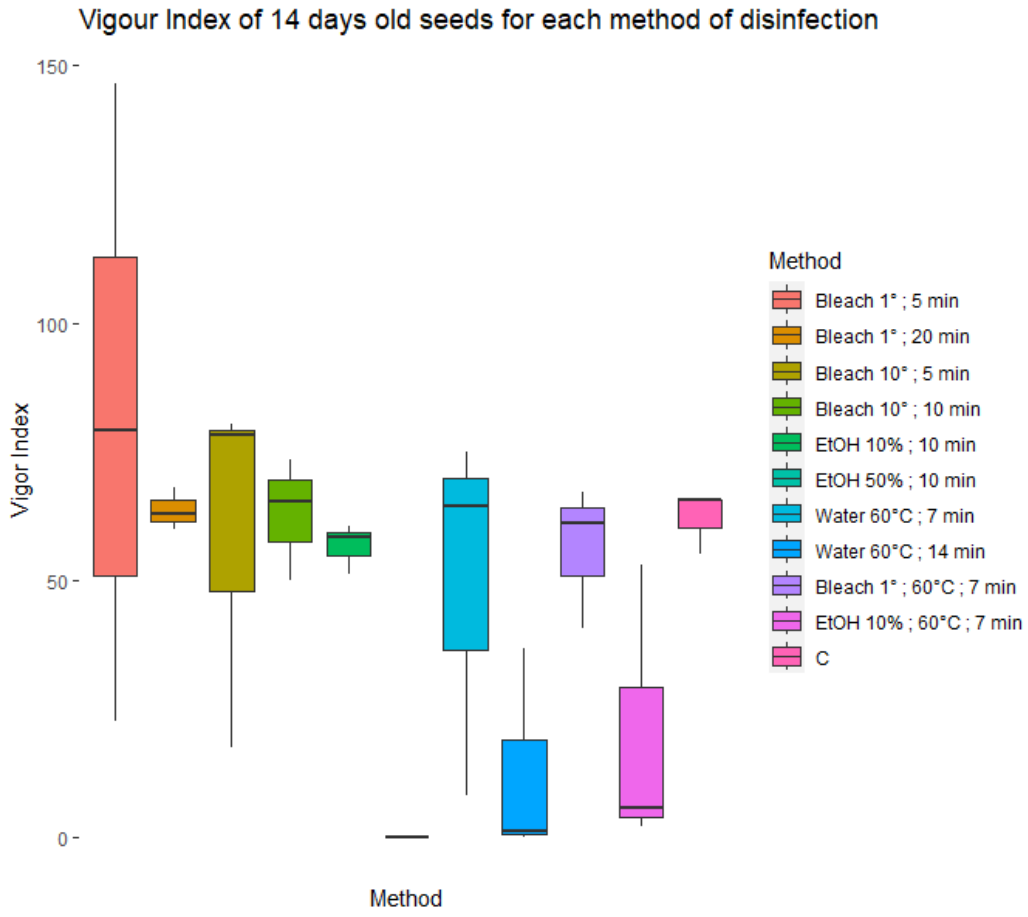

Figure 22: Vigor index for 14 days old seeds disinfected by ten methods.

2.4 Discussion

Four parameters were studied in four bioassays to investigate their impacts on Pgcol production and multiplication: the type of watering system, the pH of the nutritive solution, the origin of Pgcol, and the timeline of Pgcol development. Two additional parameters were also studied in two separate experiments: the efficiency of sporosores extraction, and the efficiency of seeds disinfection.

2.4.1 Influence of the watering system on the multiplication of Pgcol

Multiplication of Pgcol on *O. sativa* cv. Kogoni was made using adapted versions of the immersion systems designed by Legrève et al. (1998). Two types of watering system were developed, collective or individual, and two methods of watering were developed, automatic and manual.

Individual manual and automatic watering systems were used in the first bioassay while collective automatic watering systems were used during the second. However, the influence of the watering system was not specifically studied in these bioassays, as the first bioassay aimed to produce Pgcol from sporosores, whereas the second aimed to promote Pgcol development from zoospores. The lack of Pgcol in the second bioassay, in comparison to the successful infection of Pgcol in the first bioassay demonstrated that one of the changed parameters had an impact on the development of Pgcol. Two parameters were changed: the type of automatic watering system and the infectious structure (from sporosores to zoospores) used for Pgcol propagation. In the third bioassay, the influence of the type of watering systems was specifically studied. Unfortunately, the absence of any stages of Pgcol in the roots harvested from this experiment and the absence of PCR products after PCR amplification for Pgcol revealed that Pgcol did not develop in this bioassay. Therefore, no conclusion cannot be drawn regarding the impact of the watering system on Pgcol development. Finally, a fourth experiment aimed to study the timeline of Pgcol infection in individual automatic watering system by collecting plants after different timepoints. Pgcol infection was only observed in one plant as sporosores (after four weeks post inoculation). Although the third bioassay that specifically studied the influence of the watering system did not yield any results, infection of Pgcol was only achieved in individual watering systems across the four bioassays. It can thus be assumed that individual watering systems might be one of the parameters enabling the proper development of Pgcol. Results were also obtained in the individual manual watering system used in bioassay 1, confirming the hypothesis that individual system offer a better environment for Pgcol development. Moreover, individual watering system were also preferred by Bagayoko (2022). Nevertheless, *Polymyxa* species were successfully grown in collective systems in other studies (Dahm et Buchenauer 1993; Legrève et al. 1998)

Several hypotheses could explain this observation. A first hypothesis is that the dilution of zoospores, responsible for infection, is increased as the volume is far more important in collective systems than for individual systems. For individual systems, the container of the nutritive solution is slightly larger than the tube containing the soil while in a collective system the volume available is far exceeding a single tube. It could be speculated that once zoospores are released in the nutritive solution, they can more easily reach roots than in collective systems. This would explain the increase difficulty for infection between plants but not for reinfection of a same plant root system. Increasing the quantity of zoospores in collective systems may therefore solve the problem of excessive dilution. A second hypothesis would be that the flow of the nutritive solution in collective systems is too important. Zoospores would be taken away and would not be able to contaminate new roots. In contrast, in an individual system, the nutritive solution can be said to be stagnant. Designing a watering system where the tube containing the plant would be dipped into stagnant nutritive solution or watering system with reduced flow of the pumps may solve this problem of flow. A third hypothesis is that the motility of zoospores is not good enough to contaminate new roots. Improving the motility and thereby the infective properties of zoospores can be achieved by adding BSA to the nutritive solution (Adams et Swaby 1988).

2.4.2 Influence of the pH on the multiplication of Pgcol

The influence of pH on the development of Pg has been studied by Sayama (2010) and the development of Pg was determined to be best at pH around 6 to 7. However, in a previous study, Decroës et al. (2017) used a pH of 5.5 while studying the transmission of RSNV by Pgcol. Therefore, the pH was first adjusted to this value to grow and multiply Pgcol in this study. After poor results were obtained in the first two bioassays, the influence of the pH on the development of Pgcol was studied, and the pH was raised from 5.5 to 6.5 to accommodate the observation made by Sayama (2010). A less acidic pH was made based on our observations that an acidification occurred in individual watering systems as time pass and that lowering the starting point of the solution may not be best. However, no conclusion regarding the efficiency of pH on the development of Pgcol can be made because of the absence of infection of Pgcol in rice at this pH.

2.4.3 Influence of the source of inoculum on the multiplication of Pgcol

Several isolates/strains were used to try to achieve Pgcol multiplication. Since PGML3 strain has been sequenced and its genome is being assembled by Margaux Genard, it was preferentially used. However, as the available PGML3 inoculum quantity (sporosori in dried roots) was limited, other isolates were used to enhance the chances of mastery of Pgcol infection. Another isolate (PGML5) stored in dried roots was used as well as several other soils samples known to be infested by Pgcol. In dried roots and soils, the inoculum is present under the form of sporosores. In the second

bioassay, Pgcol was present in the form of zoospores instead of sporosores in previously infected plants.

During the first bioassay, sporosores of Pgcol were successfully observed in several rice plants: one with the PGML3 strain, three with the PGML5 isolate, and one with a soil isolate. During the fourth bioassay, new PGML5 isolate samples were used to infect plants, and sporosores and zoosporangia were observed in one sample. In the second bioassay, no Pgcol could either be observed or identified using the RT-PCR detection method. But in that case, the source of infection originated from zoospores instead of sporosores. Although sporosores were presented at the end of the first experiment, no zoospores release was performed on these samples to confirm their presence. However, it can genuinely be assumed that several life forms except sporosores of Pgcol were present at the same time in the observed roots (Kanyuka et al. 2003; Littlefied et al. 1998).

The results obtained in this study suggest that infection with sporosores is more efficient than with zoospores, contrarily from observed by Bagayoko (2022). However, the concentration of inoculation by sporosores was much greater than the concentration of zoospores. The difference of development of Pgcol in rice root may therefore have been influenced by the concentration of the inoculum. Furthermore, as discussed in section 2.4.1 the second experiment was performed in a collective watering system which appears to be less adequate for Pgcol development. Finally, since sporosores require a dormant phase in their life cycle before germination, it can be assumed that those observed at the end of the first bioassay did not participate in the infection of new plants of the second bioassay.

2.4.4 Dynamic of infection

The following objective after mastering the study system of Pgcol is studying the timeline of infection of the rice by Pgcol. In the fourth bioassay, zoospores release was performed every two days to provide insight on the time required from sporosores germination to zoospores infection to occur. Bagayoko (2022) studied the release period of viruliferous zoospores of Pgcol and observed that zoospores were released from 12 days to 20 days post inoculation and were maximal 16 days after inoculation. However, the study system of Pgcol in this study was not mastered, and no zoospores could be observed in the fourth bioassay. Therefore, nothing can be concluded from it.

2.4.5 Efficiency of sporosores extraction

As discussed above, the amount of dried rice roots containing sporosores of Pgcol used in this study were limited and the concentration of sporosores used as a source of inoculum might play a role in the development of Pgcol. Therefore, three methods of sporosores extraction from dried roots were tested to maximize the amount of

sporosores extracted for a given mass: extraction using a mortar, extraction using scalpels, and extraction using a Virtis blender.

Sporosores extraction experiment showed that the previously used Virtis blender is yet the best tool in comparison with the mortar or the scalpels. Therefore, in the future using the Virtis blender for sporosores extraction would be best.

2.4.6 Efficiency of seeds disinfection and influence on seeds germination and vigor

Another variable studied in this work was the sanitary status of rice seeds. Ten disinfectant treatments were applied to study their impact on the rate of contamination by foreign microorganisms and to evaluate their impact on the germination rate, time, and vigor of rice. Ensuring that a good disinfection method also secures a homogenous growth of rice is important for further designs.

Kogoni rice seeds used in this study were not certified and consequently several fungal pathogens were observed to have grown on the seeds while germinating. The percentage of germination, and the VI were measured to assess the impact of the different method of disinfection on seed germination. The disinfection method used during this study (bleach 1° for 20 minutes) has proven to not impede the germination of the seeds as its germination level is higher than that of the control (Figure 19). The VI of this disinfection method is also relatively close to that of the control (Figure 22). However, when looking at the contamination level (Figure 20), it appears that this method is one of the worst among the ten treatments studied here.

As the amount of rice seeds is not a limiting factor, the percentage of germination should not be regarded as a limiting factor for choosing a more efficient disinfection methods. Among the tested disinfection methods, the disinfection method using bleach at 10° for 5 minutes offered a relatively stable VI after 14 days indicating a good homogeneity of the growth of rice seeds. Furthermore, the percentage of contamination was kept under 10%. Another disinfection treatment offering both a good VI and a good protection again endogenous pathogen is the combined treatment using bleach 1° and heated water (60 °C). Therefore, for future work with rice seeds, it would be best to change the disinfection method from bleach 1° for 20 minutes to either bleach 10° for 5 minutes or the combination of heated water with bleach 1° for 7 minutes.

2.4.7 Other parameters influencing the development of Pgcol

Apart from the studied parameters described above, other parameters may have had an impact on the development of Pgcol.

During the steps preceding the extraction of sporosores from dried roots, a step involving washing the roots for two minutes in ethanol 70% was performed. This step

was made to wash away a maximum of other microorganisms that could have a negative impact on the development of Pgcol. However, it is well known that ethanol containing 10 to 40% water is a powerful disinfectant against bacterial, fungal, and viral organisms. In these proportions, water acts as a catalyst for ethanol allowing it to better permeate through the cell wall and coagulate the protein of the cell, thus killing the organism (Salvage et al. 2014). It can therefore be speculated that sporosores of Pgcol were also killed by the ethanol. But as the disinfectant does not have a 100% efficiency, some sporosores may have survived this treatment, which in turn would explain the few Pgcol infection observed throughout this study.

As discussed above, no infection by Pgcol were observed at the end of the second bioassay. One factor that may have played a role in it is the failure of the automatic watering system. As pumps were getting old, some have failed resulting in the interruption of the alternance of dry and saturated conditions. It resulted in the death of several plants. Dead plants could not be harvested and analysed, therefore creating a loss of potentially infected rice plants.

The death of rice plants could also be explained by the incomplete harvest of their root system during ongoing experiments. As shown in Figure 10.b, roots are coming out of the tube containing the soil substrate. And in Figure 10.c a clear line can be seen once the root system has been taken out of its tube. The loss of important young rootlets may have significantly harmed the plants.

Throughout the entirety of the experiment, levels of Pgcol infection were low. Therefore, it is likely that the observed structures were not representative of the level of infection inside the root system of the observed plant. Such assumption could explain the “disappearance” of Pgcol in the roots of sample “H.2” and “D.3”, contaminated with sporosores of PGML3 strain and PGML5 isolate from the first bioassay to the second, respectively.

CHAPTER 3: IDENTIFICATION OF CANDIDATE EFFECTORS THROUGH THE USE OF BIOINFORMATIC

3.1 Introduction

Plant immune system consists of two branches involving PRRs and R genes to defend against attacks from microorganisms. The first step of the plant defense system involves the recognition of PAMPs via PRRs to trigger PTI. Then virulence factors are recognized, and a response is triggered to suppress PTI. These virulence factors are mediated by secreted proteins called effectors. Effectors are defined as proteins and small molecules that alter host cell structures and functions, thereby facilitating the colonization of pathogens (Hogenhout et al. 2009). They are of different natures such as proteins, small RNA, and toxic metabolites (Collemare et al. 2019).

Lately, our understanding of effectors diversity and function has improved, notably thanks to functional genomic studies (Louet et al. 2022) and the ever-increasing number of available fungal, bacterial, and plant genomes (Collemare et al. 2019). Since 2015, three plasmodiophorids genomes have been sequenced: Pbras (Schwelm et al. 2015), Ssub (Ciaghi et al. 2018), and Pb (Decroës et al. 2022). Several works have focused on identifying candidate effectors involved in pathogenicity for these plasmodiophorids, leading to a better understanding of molecular interactions between plasmodiophorids and their host.

This chapter focuses on the second main objective of this study, which consists of identifying candidate effectors of Pgcol through comparison with the available genomic data of the previously sequenced plasmodiophorids. Indeed, as Pgcol genome as only recently been sequenced (personal communication from Margaux Genard) and started to be assembled, no Pgcol effectors have been identified yet. With this new genomic data, it is possible to identify some Pgcol candidate effectors by homology with other plasmodiophorids candidate and identified effectors.

3.2 Material and method

3.2.1 Material

A list of secreted proteins and candidate effectors of the three plasmodiophorids species that are sequenced so far (Pb, Pbras and SSub) were used in this study for comparison purposes. This lists of predicted secreted proteins and candidate effectors were generated by Decroës (2022) based on the genomes of Pbras isolate e3 (Schwelm et al. 2015), Ssub isolate SSUBK13 (Ciaghi et al. 2018), and two Pb isolates A26-41 and RES F41 (Decroës et al. 2022). They predicted candidate proteins based on a four-step pipeline adapted from Vivek-Ananth et al. (2018) for proteins prediction of Pb isolates, Pbras, and Ssub (Decroës et al., 2022). First, the predicted proteins

were searched for signal peptide with SignalP v5.0b, for Glycosylphosphatidylinositol (GPI) anchor using big-PI with the “Protozoa” learning set and for transmembrane domains using Phobius and TMHMM v2.0. Proteins with a signal peptide and/or a GPI and/or a transmembrane domain were selected through an inclusive rule and retained for the second step. Endoplasmic reticulum retrieval signal was then searched using PS SCAN for the PROSITE pattern PS00014. Proteins with an endoplasmic reticulum signal were discarded. Next, proteins were separated into two groups, one containing proteins with transmembrane domains and/or GPI anchors, and one containing proteins without clues of membrane localization. The last step was the subcellular localization of proteins classified in these two groups using three tools: TargetP v2.0 with the “Nonplant” organism group, WoLF PSORT v0.0.1.1 with the “Animal” organism type, and ProtComp v9.0 (Animal & Fungi). Proteins were considered as secreted or transmembrane if they are predicted as such by at least two tools. Candidate secreted proteins were eventually screened for effector using the EffectorP 2.0 machine learning classifier (Decroës et al. 2022).

The Pgcol reads used in this study were provided by Margaux Genard*. O. sativa* var. Kogoni roots infected with PGML3 strain of Pgcol were sequenced twice on MinION flow cells in 2022. Genomic data obtained from the two sequencing were combined and cleaned with reads from a MinION sequencing of healthy *O. sativa* var. Kogoni roots to remove the genome of the host *O. sativa* and other contamination. Pgcol cleaned reads were not yet assembled when used in this study. Pgcol sequencing is being done as part of the project POGRIVE in collaboration with the IRD.

3.2.2 Method

Pgcol candidate effectors were identified by homology using a BLASTx comparison of Pgcol nucleotide sequences with predicted secreted proteins and candidate effectors of the three plasmodiophorids Pb, Pbras and SSub. This analysis was performed on the Galaxy server using the Blastx v2.10.1 with default parameters. The produced output was displayed in twelve columns (Table 9) among which are found the percentage of identical matches, the length of the sequence, the E-value, and the bit score. These four parameters were used to identify candidate effectors for Pgcol. Selection of candidate effectors and potential secreted proteins used a 30% identical matches threshold (Zvelebil et Baum 2007), a restriction of protein length to 400 amino acids (A. Decroës et al. 2022), a maximal e-value of e-2 (Zvelebil et Baum 2007) and, a minimal bit-score of 50 (Pearson 2013). A list of potential secreted proteins and candidate effectors based on each reference plasmodiophorid (Pb_A26-41, Pb_RES F41, Pbras_e3 and Ssub_SSUBK13) was made for both selection methods.

Lists of candidate secreted proteins and effectors for each plasmodiophorid reference were compared to identify duplicates. This comparison also gives an information regarding the shared effectors between the four references. This comparison was performed with BLASTp. Each amino acid sequence of each candidate effector was

manually blasted again existing NCBI database to find similarities with existing sequenced plasmodiophorids. However, only Pbras_e3 proteins are available on the NCBI database. Proteins of Ssub_SSUBK13 and Pb_A26-41 were downloaded from ENA to perform a BLASTp comparison between candidate effector of Pgcol and Ssub and Pb using Galaxy. Only the sequences sharing the highest homology with the protein sequence used for comparison were retained. For protein sequences compared to Pbras protein in the NCBI database, GenBank accession number were also retained.

Table 9: Description of the output table from BLASTx comparison using Galaxy.

Column	Name	Description
1	Query sequence id (ID of your sequence)	It is the FASTA header of the sequence being searched against the database (the query sequence).
2	Subject sequence id (ID of the database hit)	It is the FASTA header of the sequence in the database that the query sequence has been aligned to (the subject sequence).
3	Percentage of identical matches	It is the extent to which the query and the subject sequences have the same residues at the same positions.
4	Alignment length	It is the length of the alignment.
5	Number of mismatches	It is the number of mismatches.
6	Number of gap openings	It is the number of gap openings in the alignment.
7	Start of alignment in query	It is the start of the alignment in the query sequence.
8	End of alignment in query	It is the end of the alignment in the query sequence.
9	Start of alignment in subject (database hit)	It is the start of the alignment in the subject sequence
10	End of alignment in subject (database hit)	It is the end of the alignment in the subject sequence.
11	Expectation value (E-value)	It is the expect value for the alignment.
12	Bit score	It is the bit-score of the alignment.

3.3 Results

Table 10 summarize the results from the BLASTx comparison that was used to identify Pgcol potential secreted proteins and candidate effectors. For each plasmodiophorid, two files have been analysed: a first one made of the secreted reads of the organism and the second made of the candidate effectors of the same organism. The BLASTx line represents the number of shared reads and associated number of proteins between Pgcol and the mentioned plasmodiophorid. The selection line represents the number of shared reads and associated number of proteins between Pgcol and the mentioned plasmodiophorids after applying the defined threshold for the comparison. The same thresholds were applied on both the secreted proteins and candidate effectors of each plasmodiophorids to visualize the importance of the selection.

Candidate effectors of each plasmodiophorids remaining after the selection were used for further analysis. A BLASTp comparison was performed to identify common effectors across the four plasmodiophorids references (Table 10). Out of the 101 candidate effectors shared with Pbras_e3, 33 remained after the selection. This indicates that 67.33% of the shared candidate effectors between Pgcol and Pbras were removed after the selection. Likewise, these percentages are of 67.78%, 44.44%, and 45.36% for Ssub_SSUBK13, Pb_A26-41, and Pb_RES F41, respectively.

After the BLASTx comparison and application of the threshold levels, Pgcol was found to share 33 candidate effectors with Pbras_e3, 29 with Ssub_SSUBK13, 55 with Pb_A26-41, and 53 with Pb_RES F41. The 170 candidate effectors shared by Pgcol and the four plasmodiophorids isolates were blasted against Pbras_e3 genome to identified if some were shared. Out of the 170, 98 had a corresponding match with Pbras_e3 genome and out of them, eleven have been reviewed in published articles (Table 11). Six originated from the comparison of Pgcol with Pbras only, one from the comparison of Pgcol with Ssub, and one from the comparison of Pgcol with Pb. Furthermore, one candidate effector (GenBank: CEP03452.1) is shared between all plasmodiophorids except Ssub, and two (GenBank: CEP03187.1 and CEO97706.1) are shared by all four plasmodiophorids.

Table 10: Number of matching reads and proteins between Pgcol and Pbras_e3, Ssub_SSUBK13, Pb_A26-41, and Pb_RES F41 after conduction a BLASTx comparison. All reads and corresponding proteins are shared by Pgcol and the organisms specified in the columns. The BLASTx lines summarize the results after the BLASTx comparison, and the selection lines summarize the results after applying the threshold levels.

		Pgcol vs							
		Pbras_e3 secreted protein	Pbras_e3 candidate effectors	Ssub_SSUBK13 secreted proteins	Ssub_SSUBK13 candidate effectors	Pb_A26-41 secreted protein	Pb_A26-41 candidate effectors	Pb_RES F41 secreted proteins	Pb_RES F41 candidate effectors
BLASTx	Number of reads	51417	2932	55505	11038	45084	8352	35992	4900
	Number of corresponding proteins	554	101	332	90	458	99	465	97
Selection	Number of reads	13243	731	22302	2807	19151	2676	15099	1476
	Number of corresponding proteins	364	33	221	29	334	55	346	53

Table 11: Candidate effectors for Pgcol matching with candidate effectors of Pbras, PbA, PbE, and Ssub and described in the literature. "/" indicates that no functional domain has been identified for the candidate effector.

Organism (s)	Name in this study	GenBank or name	Name in the referenced study	Functional domain	Reference
Pb_A26-41	Pbet_jg8668.t1	PBRA_006099	PbFKBP5	FK506-binding proteins	(Singh et al. 2018)
Pbras_e3	Plasmob_jg10145.t1	CEP01301.1	Pb4_111366 or PbChiB2	Chitin recognition protein	(Muirhead et Pérez-López 2022; Zhan ZongXiang et al. 2022a)
Pbras_e3	Plasmob_jg8313.t1	CEO95852.1	Pb4_109192	Pam16	(Zhan ZongXiang et al. 2022)
Pbras_e3	Plasmob_jg459.t1	CEO94501.1	Pb4_111752	RCLR	(Zhan ZongXiang et al. 2022)
Pbras_e3	Plasmob_jg1504.t1	CEO97066.1	Pb4_109724	Reeler domain	(Zhan ZongXiang et al. 2022)
Pbras_e3	Plasmob_jg5293.t1	CEP00143.1	Pb4_106616	Thaumatin family	(Zhan ZongXiang et al. 2022)
Pbras_e3	Plasmob_jg7545.t1	CEO97355.1	Pb4_106058	/	(Zhan ZongXiang et al. 2022)
Pbras_e3	Plasmob_jg2799.t1, Pbet_jg7637.t1, Pbet_jg10022.t1, Pbet_jg6011.t1	CEP03452.1	Pb4_103799	/	(Zhan ZongXiang et al. 2022)
Pbras_e3, Ssub_SSUBK13, Pb_A26-	Plasmob_jg2516.t1, Ssub_jg3743.t1, Pbet_jg7641.t1,	CEP03187.1	Pb4_104106	Sep15/SelM redox domain	(Zhan ZongXiang et al. 2022)

41, Pb_RESF41	Pbet_jg6016.t1				
Pbras_e3, Ssub_SSUBK13, Pb_A26-41, Pb_RESF41	Ssub_jg2768.t1	CEO97706.1	Pb4_105542	/	(Zhan ZongXiang et al. 2022)
Ssub_SSUBK13	Ssub_jg8904.t1	CEO9444.1	Pb4_111688	Kazal-type serine protease inhibitor domain	(Zhan ZongXiang et al. 2022)

However, some overlapping existed in between the three different plasmodiophorids. Therefore, the results of the three comparisons were overlapped to remove repeated candidate effectors appearing in several organisms and to visualize the number of true candidate effectors (Figure 23). From the 170 matches obtained after the selection of the BLASTx comparison, 109 different candidate effectors were deduced to be shared with the three plasmodiophroids genomes used for comparison. Among them, two were shared by all four plasmodiophorids, four between the *Polymyxa species* and Ssub and two between the *Polymyxa species* and Pbras. Naturally, both Pb isolates shared a great number of candidate effectors as there are two isolates of the same organism.

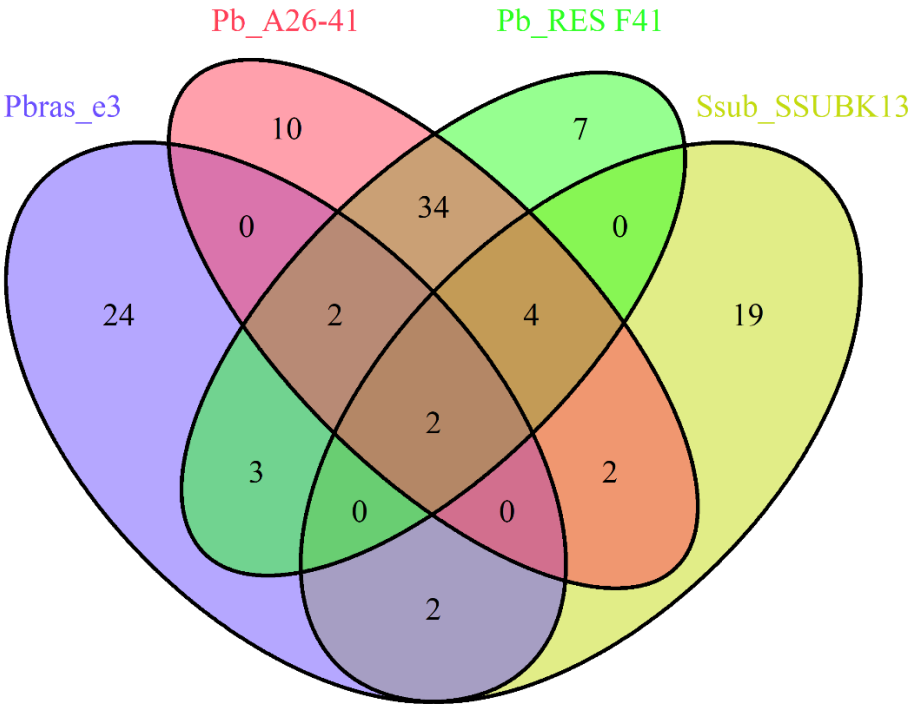

Figure 23: Venn diagram of Pgcol candidate effectors shared with Pbras_e3, Ssub_SSUBK13, Pb_A26-41, and Pb_RES F41 isolates. 170 protein sequences were found to be shared between Pgcol and the other plasmodiophorids. Each sequence was compared via BLASTp against secreted protein of Pbras_e3, Ssub_SSUBK13, and Pb_A26-41 isolates to find similarities. Results were overlapped to visualize the number of shared candidate effectors between each plasmodiophorids.

3.4 Discussion

3.4.1 Homology of Pgcol with other plasmodiophorids

The homology of Pgcol with the compared plasmodiophorids can be investigated through the number of shared candidate effectors (Table 10). Pgcol shares 33 candidate effectors with Pbras_e3, 29 with Ssub_SSUBK13, 55 with Pb_A26-41, and 53 with Pb_RES F41 isolates. As expected Pgcol shares the most candidate effectors with Pb isolates than with the two other plasmodiophorids. It can therefore be concluded that Pgcol is closer to Pb than with the other plasmodiophorids.

Based on the overlapping of the comparisons with the genomic data of each plasmodiophorids, four candidate effectors were revealed to be shared by Pb isolates and Ssub, but only two are shared by Pb isolates and Pbras. This confirms what was previously demonstrated by Decroës et al. (2022) that Pb is closer to Ssub than Pbras. According to shared candidate effectors as well as taxonomy (Legrève et al. 2002), Pgcol and Pb are closer. This suggests that Pgcol may also be closer to Ssub than Pbras. Furthermore, two candidate effectors were shared by all four plasmodiophorids

3.4.2 Host-plasmodiophorids interactions

Candidate effectors targeting the apoplastic immunity are Plasmob_jg10145.t1, Plasmob_jg5293.t1, and Plasmob_jg1504.t1 (Table 11). Plasmob_jg10145.t1 is identical to Pb4_111366 and is involved with the chitin recognition. This candidate effector was confirmed to play a role in suppressing host immunity (Zhan ZongXiang et al. 2022). This candidate effector was also studied by Muirhead et Pérez-López (2022) as *Pb*ChiB2 and was characterized as belonging to the chitin-binding domain carbohydrate-binding module family 18. The *Pb*ChiB2 protein binds to spores and to chitin oligomers and was showed to suppress chitin-triggered activation of the map kinase proteins MPK3 and MPK6 in the host *Brassicae napus*. It would indicate that the *Pb*ChiB2 protein acts as an effector suppressing chitin-triggered immunity and protects the clubroot pathogen. Plasmob_jg5293.t1 is involved with the thaumatin family and Plasmob_jg1504.t1 is involved with the reeler complex (Zhan ZongXiang et al. 2022). Thaumatin-like protein are associated with plant defense responses against biotic and abiotic stresses. When expressed *in vitro*, they inhibit the growth of fungal pathogens by lysing spores, impeding spore germination, and hinder hyphal growth (Rajam et al. 2007). Reeler complex however, have unknown functions. Both candidate effectors were showed to be involved in cell death suppression (Zhan ZongXiang et al. 2022). If these candidate effectors have a similar role in Pgcol, it can be speculated that they would also allow Pgcol to hide from the host immune system.

Two candidate effectors are involved with the interference of the reactive species machinery. The first corresponding to GenBank CEP03187.1 is shared by all four plasmodiophorids studied here and contains a Sep15/SelM redox domain. The

Sep15/SelM redox domain is involved in disulfide bond formation in the endoplasmic reticulum. The Sep15 and SelM can form reversible mixed selenylsulfide bonds during the catalytic cycle of oxidation and reduction (Ferguson et al. 2006). As response to pathogen infection following pathogen recognition is often initiated by an oxidative burst (Daudi et al. 2012), the secretion of Sep15/SelM as a candidate effector may be involved in the suppression of this burst. This candidate effector was confirmed to be involved in the suppression of host immunity (Zhan ZongXiang et al. 2022). The second is only shared with Pbras and corresponds to GenBank CEO94501.1. Unlike the first, it is involved with reactive chlorine species which are powerful antimicrobial antioxidants capable of chlorination and oxidization of a wide range of biomolecules (Parker et al. 2013). However, its mechanisms of action would appear to be similar to the other candidate effector. Disrupting the oxidation balance inside the host cell is a fundamental process for parasitic organisms to allow them to properly develop inside their hosts. Such vital process is emphasized by the fact that all four plasmodiophorids studied here share a candidate effector involved in the ROS machinery.

Finally, the other candidate effectors are involved with the intracellular host defense. Plasmob_jg8313.t1 (GenBank: CEO95852.1) acts in the mitochondrion inner membrane at the Pam16 domain responsible for translocation of transit peptide-containing proteins from the inner membrane into the mitochondrial matrix (D'Silva et al. 2005). Disrupting the Pam16 domain would interfere with the ATPase activity of the cell. This candidate effector was confirmed to be involved in the suppression of host immunity (Zhan ZongXiang et al. 2022). Pbet_jg8668.t1 (name: PBRA_006099) is involve with the FK506 binding proteins, which are known to mediate the immunosuppressive action of drugs. As studied on *Oryza sativa* and *Arabidopsis thaliana*, the FK506 drug could increase the susceptibility of plant to a pathogen (Cheung et al. 2020).

Each function of the hereabove candidate effectors is corresponding to a function described in Pbras and each candidate effector protein sequence corresponds to either Pb_A26-41, Pb_RES F41, Pbras_e3, or Ssub_SSUBK13. This means that the candidate effectors described hereabove are not the exact same candidate effectors of Pgcol. Therefore, the function of these candidate effectors may not be the exact same as real candidate effectors of Pgcol. However, as Pgcol and the other plasmodiophorids used for comparison purposes are relatives, it can be speculated that the candidate effectors of Pgcol may have similar way of action. Further investigation and experiment confirmation should be made to confirm this hypothesis.

CHAPTER 4: CONCLUSION AND PERSPECTIVES

In conclusion, the poor results obtained in this study revealed that the ecological parameters required for proper development of Pgcol in rice are still not well understood. The influence of the pH of the nutritive solution, the life form for inoculation, the influence of the experiment design for watering, and the dynamic of infection were studied here. Additionally, the efficiency of sporosores extraction from dried root samples and the efficiency of seed disinfection were also examined to ptimize future bioassays.

For future bioassays using the same experimental design as described in this study, individual automatic watering systems should be preferred with either certified seeds or better disinfected seeds. In case of seeds disinfection combining bleach with heated water would be advised. However, multiplication of Pgcol in collective automatic watering systems should be further investigated, focusing on the identification of ecological parameters, such as the speed of the nutritive flow, the motility of the zoospores, or the distance between samples that the zoospores must travel. The incidence of disinfecting dried roots containing the sporosores used for inoculation with ethanol 70% should be further investigated to determine whether it played a role in the poor results obtained in this study. Finally, the question of cutting some roots from plant samples during an ongoing experiment or sacrificing an entire plant for observations and analysis should be addressed.

The firsts candidate effectors of Pgcol were identified through homologous comparison of genomic data of three plasmodiophorids: Pb, Pbras, and Ssub. In total, 109 candidate effectors were identified to be shared by Pgcol with the three other plasmodiophorids. Among them two are shared by all four plasmodiophorids. One of the two common candidate effector was identified to possess a Sep15/SelM redox domain involved in the suppression of host immunity by disturbing the oxidative burst responsible for ROS species in Pbras and could have similar functions in all plasmodiophorids life cycles. Another candidate effector shared only by Pgcol and Pbras belongs to the chitin-binding domain carbohydrate-binding module family 18. This type of effector is involved in the suppression of the host immune system targeting chitin structures in parasitic and pathogenic microorganisms, thus preventing the degradation of life forms found outside of the host root system. However, candidate effectors identified in this study were discovered through homology with other plasmodiophorids. Therefore, in this study the functions or the discussed candidate effectors are those of Pb, Pbras, and/or Ssub. In spite of that, it can be assumed that these functions may be similar for Pgcol as these organisms are relatives. A functional validation aiming at demonstrating the virulence function of the candidate effector should be performed either experimentally or thanks to bioinformatics pipelines to confirm the nature of their function.

For further identification of candidate effectors of Pgcol, further progress should be made to obtain a fully annotated genome. Then, as realised in other studies (Decroës et al. 2022; Ciaghi et al. 2018; Schwelm et al. 2015) analyse the Pgcol genome via bioinformatics tools, such as SignalP, to identify candidate effectors from regular secreted proteins. Focussing on different domains, motifs, regions of the genome coupled with the parameters used in this study would allow more accurate prediction of candidate effectors. Finally, conducting homology studies with relative of the same family would allow the identification of shared effectors essential for their development.

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Annexes

Annex A: Protocols

Annex A.1: Protocol for sporosores extraction from dried roots using a Virtis blender.

Protocol for sporosores extraction from dried roots using a Virtis blender

Material:

- 2 scalpels with new blades
- A Virtis blender with its bucket and blades
- 1 pair of tweezers
- 1 large Erlen (3L)
- 1 funnel
- 1 Eppendorf tubes of 50ml
- Sterile demineralized water
- Demineralized water
- 1 dropper of 25ml
- Filters (100µm and 500µm)
- 1 Fuchs-Rosenthal counting chamber
- A microscope
- 1 sterile Petri dish
- A beaker
- Parafilm
- A tile
- Ethanol 70%

Method:

I. Before starting, remove big roots form the sample (optional).
II. Rehydrate roots for 1 to 2 hours by placing them in a sterile petri dish with a few ml of sterile demineralised water and sealing if with parafilm. Place the petri dish in a warm study (~30°C).
III. Rinse the roots under running demineralised water for 15 min an collect the water in the large Erlen flask.
Place the 100µm filter over the funnel, which is itself placed at the opening of the Erlen flask. Place the set-up under a flow od demineralized water and allow the water to tun for 15 min. Carefully collect the wastewater for subsequent sterilisation.
IV. Place the filter and the roots in a beaker containing ethanol 70% for 2 min.

V. Repeat the rinsing process with running demineralised water according to the set-up of step III.
VI. Rince for 5 to 10 min.
VII. Cut the roots on the tile with the scalpels until roots fragments are inferior to 5 mm.
VIII. Place the roots fragments in the Virtis bucket and add 20 ml of sterile demineralised water.
IX. Assemble the Virtis and blend for 30sec at 2300 rpm.
X. Wait for 5 min and blend for 30 sec at 2300 rpm.
XI. Transfer the suspension to an Eppendorf by placing the filter (500µm) over the funnel placed in the Eppendorf.
XII. Observe the contents of the Eppendorf under the microscope using a Fuchs-Rosenthal counting chamber.

Annex A.2: Protocol for zoospores release from fresh root

Zoospores extraction

Protocol:

- Take out a plant from its watering system 24 hours prior zoospores release and place it in a dark environment in the same environment as the rest (i.e. in the same greenhouse).
- Take the plant out of the dark, remove it from its container by rapidly soaking its root system in demineralized water to remove attached quartz.
- Place the quartz-free root system in a small buncher with 12°C nutritive solution diluted 5 times.
- Submerse the roots with the least volume of nutritive solution possible.
- Put the buncher in the dark for 1 hour in the same environment as the rest (i.e. in the same greenhouse).
- Take the plant out of the dark and gather the nutritive solution in a clean Eppendorf tube of 50ml surrounded by aluminium.
  Place a filter (4.5µm) over a funnel, which is itself placed on top of the Eppendorf tube. Hand-squeeze the root system over the filter with clean chirurgical gloves.
- Observe the contents of the Eppendorf tube under the microscope using a Thoma cell.
- Put back the root system of the plant in its container with new autoclaved quartz.

Annex A.3: Protocol for RNA extraction from roots

RNA extraction

Protocol

- Set centrifuge at 4°C.
- Take 10mg of dried root sample or 100 mg of fresh root sample.
- Place in an ice-cooled fast-rep tube with ceramic ball.
- Add 500 µl of Tri-reagent (work under the fume hood).
- Grind in fast prep: 40 sec, speed = 6.
- Incubate 10 min at RT, make sure that the centrifuge is at 4°C.
- Centrifuge 3min, 13 rpm, 40C and transfer the supernatant (500µl) in a new microtube.
- Add 100µl chloroform. Shake vigorously by and for 15 sec.
- Incubate 3 min at RT.
- Centrifuge 15 min, 13000 rpm, 4°C.
- Take carefully the superior colourless phase in anew microtube (+- 150 µl) without touching/disturbing the pellet.
- Add 250 µl of cooled isopropanol. Reverse 5x the tube.
- Incubate 10 min on ice.
- Centrifuge 13000 rpm, 4°C, 15 min.
- Discard the supernatant, add 700µl of cooled 70% ethanol.
- Shake with the fingertip.
- Centrifuge 5min, 4°C, 15 min.
- Discard completely the supernatant by pipetting. Repeat centrifuge if necessary.
- Let the pellet dry for 5 min under the fume-hood to remove residual ethanol.
- Suspend in 30µl of RNase-free water.
- Measure concentration in Nanodrop 1000 spectrophotometer.
- Store at -80°C.

Annex A.4: Protocol for RT-PCR

RT-PCR

Mix RT A:

10 min at 65°C (Vtot = 10,5µl)

- Biomolecular water: 8,5 µl
- Primer forward: 1 µl

Total: 9,5 µl/tube + 1 µl of RNA

Mix RT B:

60 min at 42°C (Vtot = 20 µl)

- Biomolecular water: 3,25 µl
- Buffer RT: 4 µl
- dNTPs: 2 µl
- Reverse transcriptase: 0,25 µl

Total: 9,5 µl added to MIX RT A

Mix PCR:

- Biomolecular water: 13,125 µl
- Buffer GoTaq: 5µl
- $MgCl_2$: 2,5 µl
- dNTPs: 0,75 µl
- Primer froward: 0,5 µl
- Primer reverse: 0,5 µl
- GoTaq: 0,125 µl

Total: 22,5 µl of mix PCR/tube + 2,5 µl pf cDNA

Annex A.5: Protocol for DNA extraction from roots

DNA extraction from roots

Protocol

- Transfer 50-100 mg of fresh roots in a 2ml Fast-prep tube containing a small ceramic ball.
- Add 500 µl of CTAB buffer solution. The CTAB buffer solution must be prepared at the last moment before use by adding 10µm of beta-mercaptoéhtanol and 1µl or proteinase K (20mg/ml) per ml of CTAB buffer.
- Grind 40 sec, speed=6 in the fast prep => 2x with a 5 min incubation on ice in between
- Incubate at 42°C, 10 min. Mix every 3 min by reversing the tubes.
- Incubate at 60°C, 10 min. Mix every 3 min by reversing the tubes.
- Add 500µl of chloroform:isoamyl alcohol (24:1).
- Vortex until everything is well emulsified.
- Incubate on ice 10 min.
- Centrifugate 10 min at 13000 rpm at room temperature (24°C).
- Recover the supernatant (+- 500µl) and transfer it in a new 1,5ml microtube.
- Add 194 µl of PEG 30%.
- Add 100 µl of NaCl 5M.
- Mix by reversing the tube 5 times.
- Centrifugate 15 min at 13000 rpm at room temperature.
- Discard the supernatant.
- Add 100µl of ethanol 70% conserved at -20°C to wash the pellet.
- Retrieve the ethanol.
- Let the pellet dry under the fume-hood.
- Dissolve the pellet in 30µl of RNase-free water, vortex.
- Quantify with a Nanodrop.

Annex A.6: Protocol for sporosores extraction from dried roots using a mortar.

Protocol for sporosores extraction from dried roots using a mortar

Material:

- A sterilized mortar and his masher
- A tweezer
- 1 scalpel with a new blade
- A funnel
- 2 Eppendorf tubes of 50ml
- Sterilized demineralised water
- A 25 ml dropper
- 1 filter (500µm)
- A Fuchs-Rosenthal counting chamber
- A microscope
- Ethanol 70%

Method:

I. Before starting, remove large roots from the sample (optional).
II. Place the dry roots in the mortar.
III. Crush the roots for 10 to 15 minutes, until a root powder is obtained.
IV. Transfer the powder to a clean Eppendorf [1].
V. Add 10ml of sterile demineralised water using the 25 ml dropper.
VI. Shake vigorously for 15 sec.
VII. Leave it rest for 5 to 10 min.
VIII. Filter the suspension using the filter and collect the suspension in a new Eppendorf [2].
IX. Observe the suspension on a Fuchs-Rosenthal counting chamber under the microscope.

Annex A.7: Protocol for sporosores extraction from dried roots using scalpels.

Protocol for sporosores extraction from dried roots using scalpels

Material:

- 2 scalpels with new blades
- 1 large Erlen (3L)
- 1 funnel
- 2 Eppendorf tubes of 50ml
- Sterile demineralized water
- Demineralized water
- 1 dropper of 25ml
- 1 Pastor dropper ant its pear
- Filters (100µm and 500µm)
- 1 Fuchs-Rosenthal counting chamber
- A microscope
- 1 sterile Petri dish
- A beaker
- Parafilm
- A tile
- Ethanol 70%

Method:

I. Before starting, remove big roots form the sample (optional).
II. Rehydrate roots for 1 to 2 hours by placing them in a sterile petri dish with a few ml of sterile demineralised water and sealing if with parafilm. Place the petri dish in a warm study (~30°C).
III. Rinse the roots under running demineralised water for 15 min an collect the water in the large Erlen flask.
Place the 100µm filter over the funnel, which is itself placed at the opening of the Erlen flask. Place the set-up under a flow od demineralized water and allow the water to tun for 15 min. Carefully collect the wastewater for subsequent sterilisation.
IV. Place the filter and the roots in a beaker containing ethanol 70% for 2 min.
V. Repeat the rinsing process with running demineralised water according to the set-up of step III.
VI. Rince for 5 to 10 min.
VII. Place the roots on the tile.
VIII. Cut the roots using the scalpels for 10 to 15 min. Stop once the roots fragments are inferior to 0.5 mm.
IX. Transfer the roots to a sterile Eppendorf [1].

X. Add 10 ml of sterile demineralised water using the 25 ml dropper.
XI. Shake vigorously for 15 sec.
XII. Leave it to rest for 5 to 10 min.
XIII. Filter the contents of the Eppendorf [1] using the 500 µm filter and a funnel to collect the contents in a new Eppendorf [2].
XIV. Observe the contents of Eppendorf [2] under the microscope using a Fuchs-Rosenthal counting chamber.

Annex A.8: Protocol for seeds disinfection using several disinfectants.

Protocol for seeds disinfection using several disinfectants (bleach, ethanol, hot water)

Material:

- Bleach 15° or Etanol 99%
- Demineralised water
- 1 Erlen (250ml)
- Rice seeds
- A tweezer
- 1 graduated dropper
- Tork paper
- A landing net
- A magnetic agitator
- A magnetic bar
- A 50ml Eppendorf tube
- A thermometer

Method for disinfection with bleach or ethanol:

I. Take a sample of 100 seeds.
II. Prepare either:
    i. A 1° bleach solution from bleach 15° and demineralised water
    ii. A 10° bleach solution from bleach 15° and demineralised water
    iii. A 10% ethanol solution from ethanol 99% and demineralised water
    iv. A 50% ethanol solution from ethanol 99% and demineralised water

    Adjust the volume of the final solution to be 5 times greater than the volume occupied by the seeds.

III. Place the seeds in the 250ml Erlen with the magnetic bar and place it in the magnetic agitator for either:
    i. 5 min with the bleach 1° solution
    ii. 5 min with the bleach 10° solution
    iii. 10 min with the bleach 10° solution
    iv. 10 min with the ethanol 10% solution
    v. 10 min with the ethanol 50% solution
IV. Collect the seeds using the landing net and store them in the 50ml Eppendorf after drying them on the tork paper.
    /!\ *Work in a sterile environment provided by a Bunsen burner*.

<u>Method for disinfection using hot water:</u>

I. Take a sample of 100 seeds.
II. Prepare a volume of demineralised water 5 times greater than the volume occupied by the seeds.
III. Place the demineralised water in the Erlen with the magnetic bar and on a heating magnetic agitator.
IV. Measure the temperature by inserting the thermometer and add the seeds when 60°C has been reached.
V. Leave the seeds spinning for 7 or 14 min.
VI. Collect the seeds using a landing net and store them in the Eppendorf after drying them with tork paper.
    /!\ *Work in a sterile environment provided by a Bunsen burner*.

<u>Method for disinfection using hot water and bleach or ethanol:</u>

I. Take a sample of 100 seeds.
II. Prepare a solution of either:
    i. A 1° bleach solution from bleach 15° and demineralised water
    ii. A 10% ethanol solution from ethanol 99% and demineralised water
    Adjust the volume of the final solution to be 5 times greater than the volume occupied by the seeds.
III. Place the solution in the Erlen with the magnetic bar and on a heating magnetic agitator.
IV. Measure the temperature by inserting the thermometer and add the seeds when 60°C has been reached.
V. Leave the seeds spinning for 7 min.
VI. Collect the seeds using a landing net and store them in the Eppendorf after drying them with tork paper.
    /!\ *Work in a sterile environment provided by a Bunsen burner*.

Annex B: Supplementary results

Annex B.1: Candidate effectors of *Plasmodiophora brassicae*

Table 12 : Identified effectors involved with the infection process of *Plasmodiophora brassicae* and action mechanisms.

Name	Target/functional domain	Description	Ref
CBM18 proteins *Pb*ChiB2 & *Pb*ChiB4	Chitin	Transcriptionally activated during infection; bind to spores and to chitin oligomers; suppress chitin-triggered activation of MPK3 and MPK6	(Muirhead et Pérez-López 2022)
Pb4_100008	Chitin	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_100099	Dopa	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_100248	RRLR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_100510	S1/P1 nuclease	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_101162		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_102097		Involved in cell death induction	(Zhan ZongXiang et al. 2022a)
Pb4_102097 & Pb4_108104	ROS	High expression at stage of rest spore maturity; induce cell death, associated with H_2O_2 accumulation	(Zhan ZongXiang et al. 2022b)

Pb4_102521	Anks	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_102877	RHLR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_103257		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_103428	LNAR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_103507	RPLR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_103566		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_103612	Protein kinase domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_103736		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_103799		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_104095	Chitin	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_104106	Sep15/SelM redox domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)

Pb4_104154	IRRFLAK	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_104606		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_104979	Kazal-type serine protease inhibitor domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_105361		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_105542		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_105844	Eukaryotic-type carbonic anhydrase	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_105958	RRLR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_105970	Leucine rich repeat	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_106019		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_106058		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_106352	TLR4 regulator and MIR-interacting MSAP	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)

Pb4_106616	Thaumatin family	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_106798	Polysaccharide deacetylase	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_106909	Thaumatin family	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_107259	Tetrapyrrole methylase	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_107399		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_107465		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_107662		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_108104	Cysteine	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_108186	LNAR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_108188	LFAFLAG	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_108273	MORN repeat	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)

Pb4_108279	MORN repeat	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_108346		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_108437	Leucine rich repeat	Involved in cell death induction	(Zhan ZongXiang et al. 2022a)
Pb4_108519	RQLR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_109005		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_109192	Pam16	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_109599		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_109724	Reeler domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_109755	Phage Tail Collar Domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_110334	Leucine rich repeat	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_110503		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)

Pb4_110819	Anks	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_110950	Cysteine	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111336		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111366	Chitin	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111396	LMAR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111688	Kazal-type serine protease inhibitor domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111712		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111752	RCLR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111854	RWLR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_111952	LYAR	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_112448	Fasciclin domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)

Pb4_112714		Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
Pb4_112853	Ras domain	Involved in cell death suppression	(Zhan ZongXiang et al. 2022a)
PbBSMT	SA	Suppression of immune defenses mediate by SA by conversion of SA into inactive methyl salicylate; methyl transferase present in the cytoplasm	(Baxter et al. 2010; Djavaheri et al. 2019)
*Pb*Chi1	Chitin	Active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*Chi2	Chitin	Active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*Chi3	Chitin	Not active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiB1	Chitin	Contains one module of the CBM18 domain; active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiB3	Chitin	Contains three modules of the CBM18 domain; not active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiBD1	Chitin	Active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiBD2	Chitin	Active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiBD3	Chitin	Active in the apoplastic region	(Muirhead et Pérez-López 2022)

*Pb*ChiBD4	Chitin	Not active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiBD5	Chitin	Active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiBD6	Chitin	Active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiBT1	Chitin	Not active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiD1	Chitin	Not active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiD2	Chitin	Not active in the apoplastic region	(Muirhead et Pérez-López 2022)
*Pb*ChiD3	Chitin	Not active in the apoplastic region	(Muirhead et Pérez-López 2022)
PBCN_000548			(Chen et al. 2019)
PBCN_000619			(Chen et al. 2019)
PBCN_000733	DNA	Contains recA domains that are involved with DNA repair	(Chen et al. 2019)
PBCN_001191			(Chen et al. 2019)
PBCN_001204	Protein-protein interaction	Contains ankyrin repeats that mediates protein-protein interactions in widely diverse families of proteins	(Chen et al. 2019)
PBCN_001442	Virulence factor	Contains HflC domains belonging to the SPFH protein superfamily associated with mitochondrial functions, but also ion transport, signaling, and mechanosensation. In addition,	(Chen et al. 2019)

		SPFH proteins are required for virulence in parasites (Heredia and Rauceo 2021).	
PBCN_001454			(Chen et al. 2019)
PBCN_001856			(Chen et al. 2019)
PBCN_001987	Protein-protein interaction	Contains zf-MYND domain that primarily function as a protein-protein interaction module.	(Chen et al. 2019)
PBCN_002100	Chromosomes	Contains SMC_prok_A domain involved in chromosome condensation, recombination, DNA repair and epigenetic silencing of gene expression.	(Chen et al. 2019)
PBCN_002462			(Chen et al. 2019)
PBCN_002550	ROS	Induce cell death associated with H_2O_2 accumulation and electrolyte leakage	(Chen et al. 2019)
PBCN_002958	Chitin	Contains a ChtBD1 domain involved in recognition or binding of chitin subunits	(Chen et al. 2019)
PBCN_003236	Cell elongation	Contains PRE1 domain involved in regulation of cell elongation	(Chen et al. 2019)
PBCN_003620			(Chen et al. 2019)
PBCN_004344			(Chen et al. 2019)
PBCN_004364			(Chen et al. 2019)
PBCN_004660			(Chen et al. 2019)
PBCN_004708	Cytoplasmic translation	Contains ribosomal_P2 domain involved in cytoplasmic translation	(Chen et al. 2019)

PBCN_004763		Contains PRK14150 domain; PRK govern activation and autoregulation of catalytic activity	(Chen et al. 2019)
PBCN_005031			(Chen et al. 2019)
PBCN_005126	Protein binding	Contains vWFA domain involved in protein binding.	(Chen et al. 2019)
PBCN_005440			(Chen et al. 2019)
PBCN_005456		Contains WD40 domain are associated with plant tolerance to abiotic stresses.	(Chen et al. 2019)
PBCN_005499	ROS	Induce cell death associated with H_2O_2 accumulation and electrolyte leakage	(Chen et al. 2019)
PBCN_005691			(Chen et al. 2019)
PBCN_006649	Protein-protein interaction	Contains ankyrin repeats that mediates protein-protein interactions in widely diverse families of proteins	(Chen et al. 2019)
PBCN_006655	Protein-protein interaction	Contains ankyrin repeats that mediates protein-protein interactions in widely diverse families of proteins	(Chen et al. 2019)
PBCN_006697	Protein-protein interaction	Contains ankyrin repeats that mediates protein-protein interactions in widely diverse families of proteins	(Chen et al. 2019)
PBCN_006785	Protein synthesis	Contains functional domain eIF3_subunit involved in protein synthesis	(Chen et al. 2019)
PBCN_007081	Tyrosine	Contains tyrosinase domain that catalyse tyrosine to DOPA to reduce reaction involving oxygen species	(Chen et al. 2019)

PBCN_007090		Contains CDA1 domain involved in various biological function in human body, like inhibiting cell growth and inhibiting tumor proliferation.	(Chen et al. 2019)
PBCN_007206		Contains pntA domain. PNT domains play a rôle in protein-protein association.	(Chen et al. 2019)
PBCN_007663	Phosphates esters	Contains alkPPc domain. Alkaline phosphatase are non-specific phosphomonoesterases that hydrolyse phosphate esters.	(Chen et al. 2019)
PBCN_007856		Contains a zf-RING-2 domain involved with abiotic stresses tolerance.	(Chen et al. 2019)
PBCN_007868	Protein hydrolysis	Contains Lactamase_B domain. Lactamases catalyse the hydrolysis of S-D-lactoyl-glutathione to form glutathione and D-lactic acid.	(Chen et al. 2019)
PBCN_008279			(Chen et al. 2019)
PBCN_008608			(Chen et al. 2019)
PBCN_009214		Contains a zf-RING-2 domain involved with abiotic stresses tolerance.	(Chen et al. 2019)
PBCN_009249, PBCN_009251	ANK	Contains ankyrin repeats that mediates protein-protein interactions in widely diverse families of proteins	(Chen et al. 2019)
PBCN_009331		Contains CLECT domain involved in cell-cell adhesion, immune response to pathogens and apoptosis.	(Chen et al. 2019)
PBCN_009387		Contains ribosomal_L22 domain involved with the folding and stabilizing the conformation of 23S rRNA.	(Chen et al. 2019)

PBCN_009622			(Chen et al. 2019)
PBCN_009670	C2H2 Zn finger	Contains C2H2 Zn finger domain involved with DNA binding, and sometimes mediate RNA and/or protein interactions	(Chen et al. 2019)
*Pb*CYP3		Increase virulence	(Singh et al. 2018)
$PbGH_3$	Chitin	Auxin and jasmonic acid modification	(Schwelm et al. 2015)
PbRING1	E3 ubiquitin ligases	Not confirmed if it can be considered as an effector	(Yu et al. 2019)
PME18	Pectin	Hijacking of endogenous host mechanisms involved in cell wall loosening	(Stefanowicz et al. 2021)
PRK14510			(Chen et al. 2019; Hossain et al. 2021)
PRO1		Stimulation of resting spore germination through its proteolytic activity	(Feng Jie et al. 2010)
RxLR effector: PBZF1	SnRK1	Inhibition of biological function of SnRK1; highly expressed in resting spores	(Chen Wang et al. 2021)
SSPbP01		Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP02	Glucanase	Glucanase present in the nucleus	(Pérez-López et al. 2020b)
SSPbP03	Serine protease	Serine protease present in the chloroplast and mitochondria	(Pérez-López et al. 2020b)
SSPbP04		Localized in nucleus	(Pérez-López et al. 2020b)

SSPbP05	chromosome	Chromosome segregation proteins present in the nucleus	(Pérez-López et al. 2020b)
SSPbP10		Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP11		Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP18		Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP21	Chromosome	Chromosome segregating proteins present in the nucleus	(Pérez-López et al. 2020b)
SSPbP22 & SSPbP61	Kinase	Kinases present in the cytoplasm and nucleus; interfere with the host cell cycle	(Pérez-López et al. 2020b)
SSPbP31		Localized in the nucleus	(Pérez-López et al. 2020b)
SSPbP32		Localized in the apoplast and chloroplast	(Pérez-López et al. 2020b)
SSPbP41		Localized in the apoplast	(Pérez-López et al. 2020b)
SSPbP42		Localized in the nucleus	(Pérez-López et al. 2020b)
SSPbP43	Anks	Localized in the cytoplasm	(Pérez-López et al. 2020b)

SSPbP44		Localized in the apoplast	(Pérez-López et al. 2020b)
SSPbP51		Localized in the chloroplast, mitochondria, nucleus	(Pérez-López et al. 2020b)
SSPbP52		Localized in the nucleus	(Pérez-López et al. 2020b)
SSPbP53	Papain-like cysteine proteases (PLCPs)	Cysteine protease inhibitor present in the apoplast; interaction with PLCPs *At*XCP1 (for *Arabidopsis taliana*)	(Pérez-López et al. 2020b, 2021)
SSPbP54		Localized in the mitochondria	(Pérez-López et al. 2020b)
SSPbP62		Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP71	Anks	Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP72		Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP73		Localized in the nucleus	(Pérez-López et al. 2020b)
SSPbP81		Cyclin present in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP86		Localized in the cytoplasm	(Pérez-López et al. 2020b)

SSPbP91		Localized in the apoplast	(Pérez-López et al. 2020b)
SSPbP92	Chromosome	Chromosome segregation protein present in the nucleus	(Pérez-López et al. 2020b)
SSPbP93	Anks	Localized in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP94		ADP/ATP translocase present in the cytoplasm	(Pérez-López et al. 2020b)
SSPbP97		Involved in secondary infection and disruption of the host cell wall	(Pérez-López et al. 2020b)

Annex B.2: Observation of bioassay 2 rice roots infected with zoospores.

Table 13: Observations of Pgcol in rice roots from bioassay 2 with PGML5 isolate, PGML3 strains and naturally infested soils. Blank boxes containing "-" indicate the absence of any life form of Pgcol. Orange boxes containing "?" indicate an incertitude about the observations. Green boxes containing "+" indicate the presence of Pgcol, followed by the structure observed. "cont" indicate the presence of contaminations.

PGML5		PGML3		SOILS	
ID	Observations	ID	Observations	ID	Observations
5.11	? ; cont	3.11	-	1.A	-
5.12	-	3.12	-	1.B	-
5.13	?	3.13	-	1.C	-
5.14	? ; cont	3.21	-	1.D	-
5.15	-	3.22	-	1.E	-
5.21	? ; cont	3.53	-	1.F	-
5.23	-	3.54	-	2.D	-
5.24	-	3.61	-	2.E	-
5.25	-	3.63	-	2.F	-
5.31	-	3.63	-	4.E	-
5.33	? ; cont	3.34	?	4.F	-
5.34	?	3.42	-	5.1	-
5.35	? ; cont	3.44	-	5.B	?
5.41	? ; cont	3.51	-	5.D	-
5.42	-	3.52	?	5/E	-
5.43	? ; cont	3.53	-	5.F	-
5.44	-	3.54	-	V.34	-
5.45	-	3.61	-	V.34	-
5.51	-	3.62	-	V.34	-
5.52	? ; cont	3.63	-	H.97	-
5.53	? ; cont	3.64	-	H.97	?

5.61	? ; cont	3.11	-	H.97	?
5.62	-	3.12	-	V.13	-
5.63	? ; cont	3.13	-	5.C11	-
5.64	-	3.21	-	5.C12	-
5.65	? ; cont	3.22	-	5.C21	-
5.71	-	3.53	-	5.C22	-
5.72	-	3.54	-	5.C31	-
5.73	? ; cont	3.61	-	5.C32	-
5.74	? ; cont	3.63	-	5.C41	-
5.75	-	3.63	-	5.C42	?
5.83	-	3.34	?	5.C51	-
5.84	? ; cont	3.42	-	5.C52	-
5.85	-	3.44	-	5.C61	-
5.93	? ; cont	3.51	-	5.C62	-
5.94	-	3.52	?	5.C71	-
5.95	-	3.53	-	5.C72	-
5.102	-	3.54	-	5.C81	-
5.104	? ; cont	3.61	-	5.C91	-
5.105	? ; cont	3.62	-	5.C92	-
5.114	-			5.C101	-
5.115	-			5.C102	-
5.121	-			5.C111	-
5.122	? ; cont			5.C122	-
5.124	- cont				
5.125	? ; cont				
5.131	- ; cont				
5.132	? ; cont				

Annex B.3: Observation of rice roots infected with PGML5 isolate of bioassay 3.

Table 14: Observations of Pgcol PGML5 isolate in rice roots from bioassay 3 studying the impact of the pH and the watering system. Blank boxes containing "-" indicate the absence of any life form of Pgcol. Orange boxes containing "?" indicate an incertitude about the observations. "cont" indicate the presence of contaminations.

Weeks post inoculation	#	Observations
5	5,5i.1	? ; cont
5	5,5i.2	-
5	5,5i.3	-
6	5,5i.4	-
6	5,5i.5	-
6	5,5i.6	-
5	5,5c.1	-
5	5,5c.2	-
5	5,5c.3	-
6	5,5c.4	-
6	5,5c.5	-
6	5,5c.6	-
5	6,5i.1	? ; cont
5	6,5i.2	-
5	6,5i.3	-
6	6,5i.4	-
6	6,5i.5	-
6	6,5i.6	-
5	6,5c.1	-
5	6,5c.2	-
5	6,5c.3	-
6	6,5c.4	-
6	6,5c.5	-
6	6,5c.6	-

Annex B.4: Observation of rice roots infected with PGML5 isolate from bioassay 4.

Table 15: Observations of Pgcol PGML5 isolate in rice roots from bioassay 4 studying the dynamic of infection. Blank boxes containing "-" indicate the absence of any life form of Pgcol. Orange boxes containing "?" indicate an incertitude about the observations. Green boxes containing "+" indicate the presence of Pgcol, followed by the structure observed. "cont" indicate the presence of contaminations.

Days post inoculation	#	Observations
10	1.1	? ; plasmodia
10	1.2	? ; plasmodia
10	1.3	-
12	2.1	-
12	2.2	-
12	2.3	-
14	3.1	? ; plasmodia
14	3.2	-
14	3.3	-
17	4.1	-
17	4.2	? ; plasmodia
17	4.3	-
19	5.1	-
19	5.2	? ; sporosores
19	5.3	-
21	6.1	-
21	6.2	-
21	6.3	? ; plasmodia
24	7.1	? ; plasmodia
24	7.2	-
24	7.3	? ; plasmodia
26	8.1	? ; plasmodia

26	8.2	-
28	9.1	+ ; sporosores
28	9.2	-
31	10.1	- ; cont
31	10.2	-
33	11.1	-
33	11.2	? ; plasmodia
35	12.1	-
35	12.2	? ; plasmodia
35	12.3	? ; plasmodia

Annex B.5: Results of the extraction experiment.

Table 16 : Sporosores extraction values from dry root samples of *Polymyxa graminis* f. sp. *tropicalis* using three methods of extraction.

Repetition	Test 1	Test 2	Test 3
Mortar extraction			
Dry root mass [g]	1.02	0.326	0.324
Volume of demineralized water [ml]	10	10	10
Amount of sporosores observed per cell	16.5	7.5	17.5
Concentration [sporosores/ml]	5156.25	2343.75	5468.75
Amount of sporosores per suspension	51562.5	23437.5	54687.5
Amount of sporosores per suspension per gram or root	50551	71893	168787
Scalpels extraction			
Dry root mass [g]	1.01	0.73	0.826
Volume of demineralized water [ml]	10	10	10
Amount of sporosores observed per cell	32.25	26	38
Concentration [sporosores/ml]	10078.125	8125	11875
Amount of sporosores per suspension	100781.25	81250	118750
Amount of sporosores per suspension per gram or root	99783	111301	143765
Virtis extraction			
Dry root mass [g]	0.98	0.826	0.818
Volume of demineralized water [ml]	20	20	20
Amount of sporosores observed per cell	77	74	55
Concentration [sporosores/ml]	24062.5	23125	17187.5
Amount of sporosores per suspension	481250	462500	343750
Amount of sporosores per suspension per gram or root	491071	559927	420232

Annex C: Nanodrop measurements of RNA and DNA extraction

Annex C.1: Nanodrop values for RNA extraction.

Table 17: Nanodrop values for all RNA extraction on PGML3 strain, PGML5 isolate, and soils samples.

#	cc ([ng/µl]	260/280	260/230
		PGML3	
3.11	217.4	1.72	1.3
3.12	683.3	1.53	0.93
3.13	1016.8	1.58	1.06
3.21	383.2	1.79	1.23
3.22	740.7	1.62	0.93
3.23	1822.9	1.62	1.23
3.24	3023.9	1.43	1.39
3.31	79.1	1.68	1.22
3.33	178.4	1.69	1.05
3.34	223.4	1.74	1.11
3.42	926.4	1.62	0.96
3.44	2155.9	1.77	1.2
3.52	757.4	1.59	0.96
3.53	2741.8	1.93	1.27
3.54	162.2	1.65	1.09
3.63	432	2.02	1.27
3.64	3455.5	1.89	1.42
		PGML5	
5.11	512.7	1.68	0.51
5.12	462.4	1.84	1.09
5.13	358.8	1.85	0.93
5.14	294.7	1.76	0.78
5.21	227.7	1.71	0.6
5.23	79.2	1.64	0.28
5.25	194.5	1.79	0.71
5.31	982.2	1.61	0.5
5.33	420.8	1.8	0.99
5.34	129.3	1.72	0.52
5.35	591.1	1.92	0.97
5.41	314.2	1.83	0.85
5.42	423.5	1.88	1.24
5.43	245.3	1.82	0.74
5.44	245.3	1.83	0.81
5.45	258.2	1.74	0.66
5.51	146.2	1.73	0.53
5.52	86.9	1.7	0.44
5.53	230.5	1.68	0.73
5.61	147.3	1.84	0.47

5.62	64.8	1.85	0.33
5.63	352.7	1.76	1.12
5.64	291.4	1.71	0.51
5.65	399	1.68	0.51
5.71	52.5	1.92	0.35
5.73	67.8	1.75	0.34
5.74	197.5	1.82	1.3
5.75	71.6	1.63	0.49
5.83	188.6	1.8	0.73
5.84	71.6	1.73	0.61
5.85	93	1.39	0.67
5.93	18	1.94	0.18
5.95	113	1.77	0.74
5.104	135	1.79	0.73
5.105	56.7	1.7	0.46
5.114	65.2	1.9	0.32
5.115	97.2	1.73	0.86
5.121	155	1.77	0.77
5.122	113.1	1.7	0.54
5.124	286.6	1.86	1.26
5.125	128.8	1.75	0.86
5.131	76.5	1.55	0.56
5.141	76.5	1.66	0.55
5.142	92.1	1.65	0.61
5.161	215.1	1.78	1.02
5.162	126.5	1.69	0.72
		SOIL 5.C	
5.C11	65.3	1.68	0.78
5.C21	101.1	1.75	0.8
5.C22	109.6	1.64	0.55
5.C31	188.8	1.67	0.59
5.C32	92.8	1.64	0.56
5.C41	160.1	1.72	0.63
5.C42	176.6	1.57	0.58
5.C51	457.3	1.91	1.33
5.C61	190.6	1.82	0.74
5.C62	243.8	1.89	1.07
5.C71	574.5	1.93	1.2
5.C72	174.8	1.84	0.76
5.C81	349.7	1.91	1.05
5.C91	288.9	1.76	1.14
5.C92	509	1.81	1.31
5.C101	91.7	1.8	0.64
5.C102	258.5	1.84	1.26
5.C111	248.4	1.9	1.36
5.C122	366.4	1.85	0.99
		SOILS	

1.A	175.2	1.48	1.61
1.B	126.2	1.65	0.51
1.C	192.2	1.55	0.59
1.D	139.8	1.65	0.56
1.E	203.8	1.711	0.55
1.F	105.7	1.58	0.45
2.D	378.6	1.72	0.93
2.E	150.7	1.61	0.53
2.F	192.1	1.71	0.57
4.E	145.8	1.7	0.55
4.F	167.4	1.56	0.67
5.A	149.2	1.6	0.75
5.B	340.7	1.34	1.06
5.D	82.9	1.38	0.46
5.E	227	1.75	0.72
5.F	109.7	1.61	0.5
H.97	629.3	1.9	1.21
H.97	273.5	1.82	1.11
H.97	607.5	1.94	1.31
V.34	326.4	1.73	0.98
V.34	325.5	1.66	1.07
V.34	291.3	1.33	0.98
V.13	105.1	1.39	0.51

isolat 6	881.3	1.55	0.79
3.34	175.6	1.59	0.51
5.13	195.5	1.64	0.58
5.34	240.5	1.63	0.63
5.132	133.5	1.62	0.44
5.151	169.7	1.69	0.56
5.152	166.9	1.62	0.59
2.D	314.1	1.64	0.86
5.C22	360.7	1.68	0.88
H.97	418.7	1.71	1.05

Annex C.2: Nanodrop values for DNA extraction.

Table 18: Nanodrop values for all DNA extraction performed on PGML3 strain, PGML5 isolate, and soils samples.

#	cc ([ng/µl]	260/280	260/230
isolat 6	226.8	1.61	1.54
3.34	92.6	1?59	1.61
5.13	84.4	1.53	1.43
5.34	72.6	1.63	1.63
5.132	57.4	1.5	1.5
5.151	109.3	1.56	1.52
5.152	229.6	1.41	1.23
2.D	405	1.57	1.86
5.C22	177.5	1.57	1.88
pH and watering system experiment			
5.5c.1	91.4	1.57	1.67
5.5c.2	73.5	1.69	2.04
5.5c.3	93.4	1.52	1.44
5.5c.4	104.9	1.69	1.72
55c.5	56.5	1.61	1.36
5.5c.6	165.2	1.58	1.26
5.5i.1	129.9	1.46	1.47
5.5i.2	104.4	1.46	1.51
5.5i.3	53	1.55	1.31
5.5i.4	70.1	1.57	1.4
5.5i.5	24.4	1.68	1.26
5.5i.6	90.5	1.47	1.39
6.5c.1	47	1.63	1.39
6.5c.2	397.7	1.53	1.69
6.5c.3	52.4	1.53	1.28
6.5c.4	47.6	1.64	1.52
6.5c.5	207.6	1.61	1.62
6.5c.6	117.9	1.37	1.32
6.5i.1	174.8	1.49	1.55
6.5i.2	448.4	1.4	1.35
6.5i.4	129.8	1.53	1.34
6.5i.6	125.7	1.96	1.55
Purification			
5.5c.1	30.9	1.66	2.14
5.5c.2	102	1.51	2.29
5.5c.3	100.6	1.52	2.22
5.5c.4	91.8	1.5	2.27
55c.5	4.5	2.2	1.45
5.5c.6	26.6	1.54	2.03

5.5i.1	144.8	1.51	2.3
5.5i.2	63.3	1.45	2.14
5.5i.3	34.6	1.51	1.98
5.5i.4	14.8	1.68	1.82
5.5i.5	50.9	1.53	2.2
5.5i.6	35.7	1.52	2.02
6.5c.1	9	1.62	1.55
6.5c.2	80.2	1.56	2.19
6.5c.3	419.2	1.84	2.63
6.5c.4	109.4	1?54	2.é7
6.5c.5	52.2	1.73	2.18
6.5c.6	24.6	1.48	1.84
6.5i.1	42.7	1.47	1.98
6.5i.2	12.5	1.63	1.57
6.5i.4	53.3	1.46	2.02
6.5i.6	64.3	1.43	2.2

Annex D: Supplementary information

Annex D.1: Bioinformatics tools review by Lovelace and Jones (2023).

TABLE 1
Bioinformatic tools/resources used for effector prediction

Tool[a]	Description	Availability	Reference
Sequence motif analysis			
MEME-suite	Motif-based sequence analysis	Web API	Bailey et al. (2009) https://meme-suite.org/meme/
HOMER	Promoter motif identification	Stand-alone	Heinz et al. (2010) http://homer.ucsd.edu/homer/motif/
HMMER	HMM profile-based search	Web Stand-alone	Eddy (2009) http://hmmer.org/
MotifScan	Identification of known motifs in sequences	Web	Sun et al. (2018) https://myhits.sib.swiss/cgi-bin/motif_scan
SignalP	Secretion signal prediction	Web Stand-alone	Teufel et al. (2022) https://services.healthtech.dtu.dk/service.php?SignalP
PrediSi	Secretion signal prediction	Web Stand-alone	Hiller et al. (2004) http://www.predisi.de/
Phobius	Secretion signal prediction	Web Stand-alone	Kall et al. (2007) https://phobius.sbc.su.se/
Interproscan	Domain identification	Web Stand-alone	Apweiler et al. (2001) https://www.ebi.ac.uk/interpro/search/sequence/
Protein disorder prediction			
IUPred2A	Prediction of disordered regions in protein sequence	Web Stand-alone	Mészáros et al. (2018) https://iupred2a.elte.hu/
Effector subcellular localization			
LOCALIZER	Intracellular locations of eukaryotic effectors	Web Stand-alone	Sperschneider et al. (2017) https://localizer.csiro.au/
DeepLoc 2.0	Intracellular locations of eukaryotic effectors	Web Stand-alone	Thumuluri et al. (2022) https://services.healthtech.dtu.dk/service.php?DeepLoc-2.0
TargetP 2.0	Intracellular locations of eukaryotic effectors	Web Stand-alone	Almagro Armenteros et al. (2019) https://services.healthtech.dtu.dk/service.php?TargetP-2.0
Genomic feature identification			
ISFinder	Identification of insertion sequences	Web	Siguier et al. (2006) https://isfinder.biotoul.fr/
MGEfinder	Identification of MGE in bacterial genomes	Stand-alone	Durrant et al. (2020) https://github.com/bhattlab/MGEfinder
Islandviewer4	Identification of genomic islands in bacterial genomes	Web API	Bertelli et al. (2017) https://www.pathogenomics.sfu.ca/islandviewer/
PHASTER	Prophage identification	Web API	Arndt et al. (2016) https://phaster.ca/
REPET	Identify TEs in eukaryotic genomes	Stand-alone	Quesneville et al. (2005) https://bio.tools/repet
RepeatMasker	Identification of repetitive regions in genomes	Stand-alone	Smit et al. (2013-2015) https://www.repeatmasker.org/
Protein 3D structure prediction			
MODELLER	Homology modelling	Stand-alone	Webb and Sali (2016) https://salilab.org/modeller/
SWISS-MODEL	Homology modelling	Web	Waterhouse et al. (2018) https://swissmodel.expasy.org/
Phyre2	Homology modelling	Web	Kelley et al. (2015) http://www.sbg.bio.ic.ac.uk/phyre2/
I-TASSER	Homology modelling	Web Stand-alone	Roy et al. (2010) https://zhanggroup.org/I-TASSER/
HHPred	Homology modelling	Web Stand-alone	Söding et al. (2005) https://toolkit.tuebingen.mpg.de/tools/hhpred
RoseTTAFold	Template-free	Web Stand-alone	Baek et al. (2021) https://robetta.bakerlab.org/
AlphaFold2	Template-free	Collabfold (web) Stand-alone	Jumper et al. (2021) https://www.deepmind.com/open-source/alphafold
Machine/deep learning effector prediction			
MacSyfinder	Bacterial secretion systems	Stand-alone	Abby et al. (2016) https://github.com/gem-pasteur/macsyfinder
EffectiveT3	T3, T4 effectors	Web Stand-alone	Arnold et al. (2009) https://effectivedb.org/method/effectivet3
Effectidor	T3 effectors	Web	Wagner et al. (2022) https://effectidor.tau.ac.il/
T3SEpp	T3 effectors	Web Stand-alone	Hui et al. (2020) http://www.szu-bioinf.org/T3SEpp/
Preffector	T1-6 effectors	Web	Dhroso et al. (2018) http://korkinlab.org/preffector
Deepredeff	Bacteria, fungi, oomycetes	Stand-alone	Kristianingsih and MacLean (2021) https://ruthkr.github.io/deepredeff/
EffectorP	Fungal, oomycete	Web Stand-alone	Sperschneider et al. (2016) https://effectorp.csiro.au/
EffectorO	Oomycete	Web Stand-alone	Nur et al. (2021) https://github.com/minur/oomycete-effector-prediction

[a] This table provides a list of representative software for each task but it does not include all possible software available.

Annee D.2: PCR cycles depending on the set of primers used.

Table 19: PCR cycles for the different primers used to detect either Pg, Pgcol or RSNV.

Pgcol_L10_f / Pgcol_L10_r				
Step	Temperature [°C]	Time [mm:ss]	Back to step	# passes
1	94	2:00		1
2	94	30		35
3	60	30		35
4	72	1:00	2	35
5	72	7:00		1
6	4	∝		1
Pgrcol_f / Pgrcol_r				
Step	Temperature [°C]	Time [mm:ss]	Back to step	#passes
1	94	2:00		1
2	94	2:00		35
3	61	30		35
4	72	30	2	35
5	72	7:00		1
6	4	∝		1
Psp1 / Psp2rev				
Step	Temperature [°C]	Time [mm:ss]	Back to step	# passes
1	94	2:00		1
2	94	30		35
3	60	30		35
4	72	35	2	35
5	72	7:00		1
6	4	∝		1
RSNV1_f / RSNV1_r				
Step	Temperature [°C]	Time [mm:ss]	Back to step	# passes
1	94	2:00		1
2	94	30		35
3	56	30		35
4	72	1:10	2	35
5	72	7:00		1
6	4	∝		1

Annex D.3: Picture and schematic representation of bioassay 3 and 4

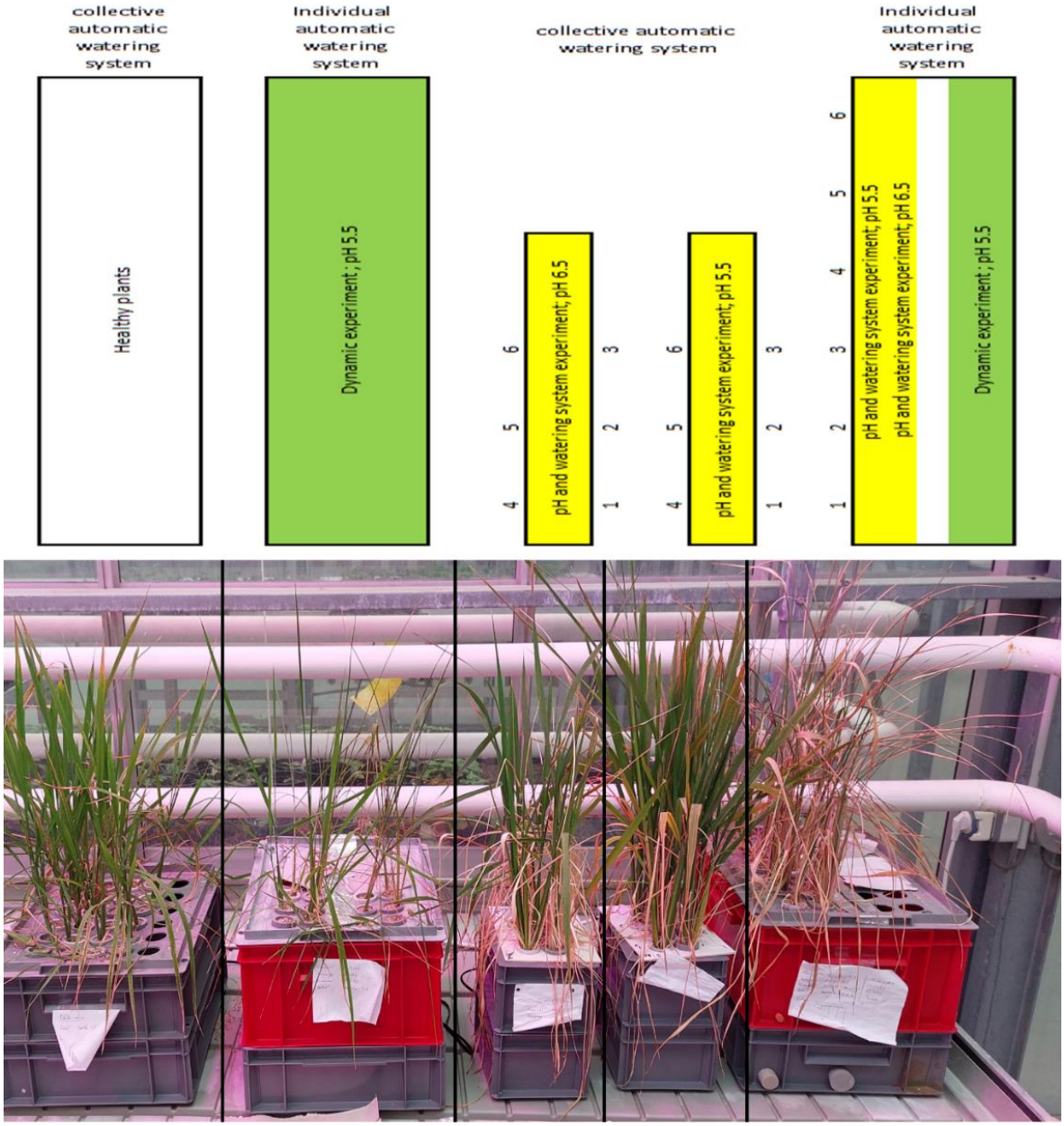

Figure 24: Disposition of the assemblies for the study of the pH, the type of watering system (bioassay 3) and the dynamic of infection (bioassay 4) of rice roots by Pgcol. All plants of the "pH and watering system experiment" are coloured in yellow. All plants of the "Dynamic experiment" are coloured in green. Picture taken when plants of experiment "pH and watering system" were 14 weeks old.

Study of the growth parameters of *Polymyxa graminis* f. sp. *colombiana* on rice roots and identification of the first candidate effectors through bioinformatics

Presented by Julien van den Eynde de Rivieren

Polymyxa graminis f. sp. *colombiana* is the vector of the *Rice stripe necrosis virus* and infects rice (*Oryza sativa*). Rice in a major staple food for global consumption, particularly in developing countries, and the RSNV threatens its production by reducing its yield to up to 40%. As of today, the only efficient control against the virus is the use of resistant cultivars and the adoption of culture rotation practices.

A key parameter involved in the infection of host plants by parasites and pathogens is the expression of effectors. Effectors are defined as proteins and small molecules that alter host cell structures and functions, thereby facilitating the colonization of pathogens. As some of these effectors are essential for infection of the host, disturbing their function could lead to novels way of disease control. Therefore, this study aims at identifying candidate effectors responsible for host infection through comparison of genomic data with other relatives of the Plasmodiophorid family.

Identification of candidate effectors is two step process. The first aims at identifying effectors through bioinformatics and the second aims at confirming their presence in a pathosystem involving Pgcol and rice. For that reason, Pgcol first had to be produced on rice.

In this study Pgcol was grown on rice without much success. Consequently, several bioassays were made to study the ecological parameters involved in the development of Pgcol in rice. The influence of the nutritive solution pH, the type of watering system (collective or individual), the life form serving as inoculum and the dynamic of infection were studied. Additionally, the extraction of sporosores and the disinfection of rice seeds were studied.

At the end of this study, no bioassay successfully produced satisfactory amounts of Pgcol. Our results indicate that using individual system over collective is more appropriate for Pgcol production. Several other parameters are hypothesized to potentially improve its production, but more study should be addressed to confirm them.

Predicting candidate effectors was made by using BLAST software by comparing genomic data of Pgcol with three other plasmodiophorids: *Pollymyxa betae*, *Plasmodiophora brassicae*, and *Spongospora subterranea*.

One hundred and nine candidate effectors were identified for the first time for Pgcol. Eleven of them correspond to candidate effectors of Pbras and are described in the literature. Interestingly, one is involved with the disruption of the chitin-triggered immunity of the host plant. Further study of the candidate effectors should be made to confirm their presence and function *in planta*.

UNIVERSITÉ CATHOLIQUE DE LOUVAIN
Faculté des bioingénieurs
Croix du Sud, 2 bte L7.05.01, 1348 Louvain-la-Neuve, Belgique | **www.uclouvain.be/agro**