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The rice sheath rot pathogen *Pseudomonas fuscovaginae*; microbiome and cell-cell signalling studies

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Abstract

Rice sheath rot has been mainly associated with the bacterial pathogen *Pseudomonas fuscovaginae* and in some cases to the fungal pathogen *Sarocladium oryzae*; it is yet unclear if they are part of a complex disease. In this thesis the bacterial and fungal community associated with rice sheath rot symptomatic and asymptomatic rice plants was determined/studied with the main aim to shed light on the pathogen(s) causing rice sheath rot. Three experimental work chapters are presented; the first concerns the pathobiome and microbiome performed on rice plant samples collected from different rice varieties in two locations (highland and lowland) in two rice-growing seasons (wet and dry season) in Burundi. The results have showed that in symptomatic samples the bacterial *Pseudomonas* genus was prevalent in highland in both rice-growing seasons and was not affected by rice plant varieties. *Pseudomonas* sequence reads displayed a significant high similarity to *Pseudomonas fuscovaginae* indicating that it is the causal agent of rice sheath rot as previously reported. The fungal *Sarocladium* genus was on the other hand prevalent in symptomatic samples in lowland only in the wet season; the sequence reads were most significantly similar to *Sarocladium oryzae*. These studies showed that plant microbiome analysis is a very useful approach in determining the microorganisms involved in a plant disease. The second experimental chapter presents the culturable microbiome on rice sheath asymptomatic samples from highland where *P. fuscovaginae* was predominant. This work also includes the purification and characterization of a set of bacterial isolates making up a culture collection. Some phenotypes assays including antibacterial activity against *P. fuscovaginae* have been performed and a bacterial isolate belonging to *Alcaligenes* genus displayed a strong antagonistic activity against the pathogen. The last chapter presents the cell-cell signaling studies of *P. fuscovaginae* since it has been evidenced in the previous chapters that a complex microbial community in the pathobiome is associated with the disease. Previous studies have shown that quorum sensing signalling in *P. fuscovaginae* is inactive *in vitro* but it is active *in planta* and plays a role in virulence. The aim of the final experimental chapter was to shed light on the molecular switching-on system of the quorum sensing cascade. Genetics screening on *P. fuscovaginae* Tn5 mutant bank identified a transcriptional repressor that increases quorum sensing signal production and also regulates an RND efflux pump. This thesis highlights that pathobiome/microbiome studies are instrumental in identifying plant pathogens and that plant microbiome interactions can play a role in the disease process.

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Chapter I. General Introduction

The commonality of all superior living organisms is to live in association with a certain number of microbes constituting the microbiome that contributes to their health. It is possible however that this microbial community plays a role in the diseases process making some diseases more complex. The rice plant (*Sativa oryzae*) is one of the most important cereal crops grown in several parts of the world and also lives in close association with microbes especially bacteria and fungi that play an important role in the plants' health. Microbes inside the microbiome interact among them via intra- and inter-species interactions and with the plant via inter-kingdom signalling. The cooperation established between microbes-microbes or microbes-plant could be beneficial (mutualism), saprophytism, parasitism or via competition/antagonism.

Many microbes (bacteria, fungi and viruses) have been reported to be pathogenic to rice causing several rice diseases. As reported by the *American Phytopathological Society* (APS), many rice microbial diseases have been identified and are currently studied (https://www.apsnet.org/edcenter/resources/commonnames/Pages/Rice.aspx) (Cartwright et al., 2018). Among them there is rice sheath rot which has thus far been associated to different microbial pathogens. It is yet unclear whether these microbes, which cause the same symptoms, are interacting resulting in a complex disease or whether they act independently. In addition, it cannot be excluded that members of the microbiome interact with pathogens playing a role in pathogenicity. Recently the pathogen integrated with the biotic environment of the host has been termed the pathobiome (Vayssier-Taussat et al., 2014).

1.1. Rice Sheath rot disease

Sheath rot is a widespread rice disease reported in several rice growing parts of the world. It has been associated to bacterial and fungal as agent causal of the disease. Below the pathogenic agents and their mode of actions are reviewed. The main toxins involved in rice sheath rot disease are presented in **Table 1.3**.

1.1.1. *Pseudomonas fuscovaginae*; the major pathogenic agent of rice sheath rot; symptoms and distribution

Rice is one of the most important staple crops in several countries and rice sheath rot is responsible for important yield losses. The rice sheath rot disease is associated to the seedborne *Pseudomonas fuscovaginae* (*P. fuscovaginae*) (sheath brown rot) bacterial pathogen and has been identified for the first time in Japan (Miyajima, Tanii, and Akita 1983). *P. fuscovaginae* has also been isolated from other cereal crops like sorghum, maize, wheat, and barley (Duveiller, 1989). The sheath brown rot symptoms appear on rice plants at seedling and at later growth stages; infected seedlings initially show yellow to brown discoloration on the lower leaf sheath (Cottyn; B.; Cerez; M.T.; and Mew; T.W., 1994). The discoloration later turns grey-brown to dark-brown and ultimately the infected seedling may rot and die. Seedling leaves may also display a systemic discoloration of the midrib and veins. The symptoms may be observed on the flag-leaf sheath (booting to heading), other leaf sheaths, and on the panicle of the mature plants. The symptoms of mature-plant and seedlings are older lesions surrounded by an effuse and dark-brown margin. The leaf sheath may also display general water-soaking and necrosis without distinct lesions. Under severe infections, the entire rice leaf sheath may become necrotic and dry out, and the panicle withers (**Figure 1.1**).

The symptoms associated to sheath brown rot have been reported also in Mexico, Guatemala, Panama, Suriname, Colombia, Peru and Brazil (Zeigler, 1987) as well as in South America (G. et al., 1992). In Burundi, *P. fuscovaginae* has been firstly isolated from symptomatic rice in 1988 (Duveiller et al., 1988) (above 1,350 m; in marsh or wetland) and one year later on maize and sorghum (E. Duveller, 1989) (fields between 1,450 and 2,100 meters above sea level). In Madagascar, bacterial sheath brown rot is widespread in irrigated rice grown between 1300 and 2000 metres altitude and the inhibition of panicle emergence increased

with altitude. The local cultivars are less sensitive than the introduced semi-dwarf cultivars from the International Rice Research Institute (https://www.irri.org) or the International Rice Cold Tolerance Nursery. Based on biochemical and serological tests, *P. fuscovaginae* strains isolated from Madagascar, Burundi and Japan displayed a higher similarity and aggressiveness in pathogenicity tests (Duveiller et al., 1990). Recently, sheath brown rot has also been reported to be present is South Korea (Kim et al., 2015) and bacterial strains isolated were classified as *P. fuscovaginae*, with a high probability.

Figure 1. 1 Pictures of rice sheath rot symptoms

(IRRI field- Burundi, 2017)

Table 1. 1 List of symptoms/signs of rice sheath brown rot disease (Plantwise - IRRI)

Part of rice plant	symptoms/signs
Inflorescence	- discoloration panicle
	- lesions on glumes
	- twisting and distortion
Leaves	- abnormal colours
	- necrotic areas
	- rot
	- wilting
Seeds	- discolorations
	- empty grains
	- galls
	- rot
	- shrivelled
Whole plant	- seedling blight

Table 1. 2 List of Host plants / species affected (Plantwise - IRRI)

Species	Family
Agrostis (bent grasses)	
Avena sativa (oats)	
Bromus marginatus (Mountain brome(grass))	
Hordeum vulgare (barley)	
Lolium perenne (perennial ryegrass)	
Oryza sativa (rice)	Poaceae
Poa pratensis (smooth meadow-grass)	
Secale cereale (rye)	
Sorghum bicolor (sorghum)	
Triticale	
Triticum aestivum (wheat)	
Zea mays (maize)	

The plants host of the bacterial pathogen belong all to the same family of Poaceae but the *Oryza sativa* specie is most common plant reported to be associated to the sheath rot symptoms.

1.1.1.1. Classification of *Pseudomonas fuscovaginae*

P. fuscovaginae belongs to the Kingdom of Bacteria, the Phylum of Proteobacteria, the Class of Gamma Proteobacteria, the Order of Pseudomonadales, the Family of Pseudomonadaceae, the Genus of *Pseudomonas*, the Species *Pseudomonas fuscovaginae* and the Binomial name is *Pseudomonas fuscovaginae* (Miyajima et al., 1983; Tanii, Miyajima, & Akita, 1976)

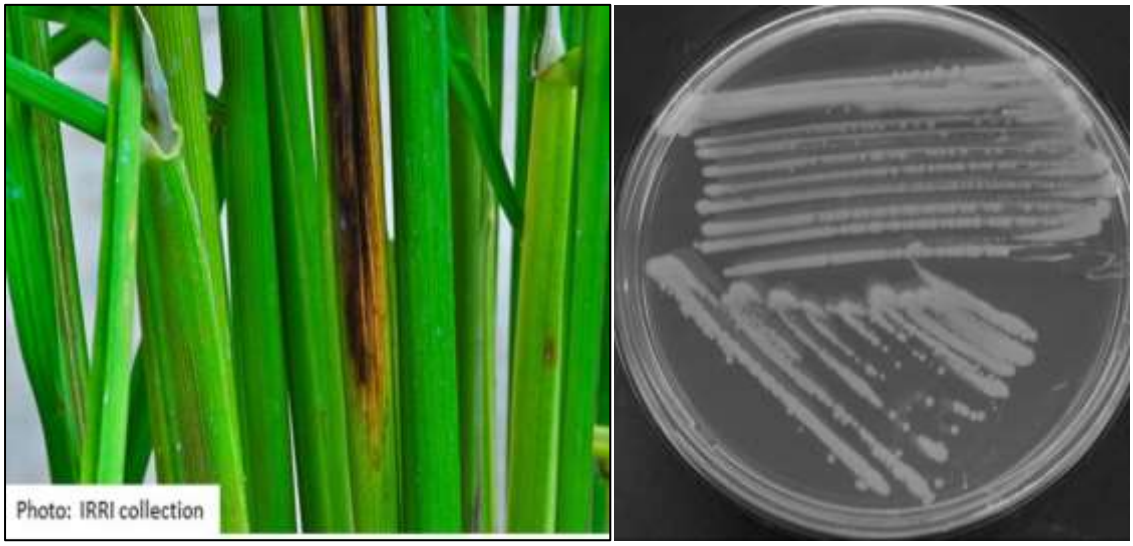


Figure 1. 2 Pictures of sheath brown rot (Image collection IRRI) and *Pseudomonas fuscovaginae* grown on LB agar medium in plate (2019)

1.1.1.2. Description of *Pseudomonas fuscovaginae*

The genus *Pseudomonas* belongs to the subclass Gamma-proteobacteria of the Gram-negative bacteria and currently comprises 144 species. Based on multilocus sequence analysis, *P. fuscovaginae* belongs together with *Pseudomonas asplenii* to the *Pseudomonas asplenii* subgroup as defined by Gomila et al. 2015. These two species are closely related and some authors consider them to be synonymous (Vancanneyt et al., 1996).

The original description of *P. fuscovaginae* in Miyajima, Tanii, and Akita 1983 is the following: the cells are aerobic, gram-negative, non-spore-forming, rod-shaped with round ends, 0.5–0.8 × 2.0–3.5 μm. Cells occur singly or in pairs and are motile by means of one to four polar flagella. They oxidize glucose inoxidation–fermentation medium, and they produce

a green fluorescent pigment, oxidase and arginine dihydrolase. Denitrification, β-glucosidase, pit formation on polypectategel and growth at 37◦C are negative. Characteristics that distinguish this species from other fluorescent pseudomonads which are positive for arginine dihydrolase and oxidase are its inability to utilize 2-ketogluconate or inositol (Miyajima et al., 1983).

P. fuscovaginae belongs to the authentic rRNA group I of pseudomonads, being one of the 18 validly described *Pseudomonas* plant pathogenic species part of the oxidase positive cluster (Anzai et al., 2000; Höfte & De Vos, 2006). A few *P. fuscovaginae* strains genomes have been sequenced (Hitendra Kumar Patel et al., 2012; Xie et al., 2012) and comparative genome analysis of *P. fuscovaginae* strains has revealed that they do not form a single monophyletic group. At least two sub groups have been identified and strains from Madagascar, Japan, China, and Australia clustered separately from *P. fuscovaginae*-like strains from the Philippines (Quibod et al., 2015).

1.1.1.3. Phylogeny of *Pseudomonas* genus and *Pseudomonas fuscovaginae* group

Phylogenetic analysis of the genus *Pseudomonas* was conducted by using the combined *gyrB* and *rpoD* nucleotide sequences of 31 validly described species of *Pseudomonas* (a total of 125 strains) (Yamamoto et al., 2000). *Pseudomonas* strains diverged into two major clusters designated intrageneric cluster I (IGC I) and intrageneric cluster II (IGC II).

According to the four partial sequences of housekeeping genes (16S rRNA, *gyrB*, *rpoB,* and *rpoD*) obtained from 112 complete or draft genomes related to the genus *Pseudomonas* that were available in the database *P. fuscovaginae* has been classified in the *Pseudomonas splenii* by using the multilocus sequence analysis (MLSA) (Gomila et al., 2015).

1.1.1.4. Pathogenicity and virulence of *Pseudomonas fuscovaginae*

The generation of sheath rot symptoms is caused by three different types of phytotoxic metabolites produced by *P. fuscovaginae*, syringotoxin, fuscopeptin A (FP-A) and fuscopeptin B (FP-B) (**Figure 1.3**) (Ballio et al., 1996; Flamand et al., 1996). Syringotoxin belongs to a group of anti-fungal metabolites known as lipodepsipeptides (LDPs) acting at the level of plasma membrane, forming ion channels and consequently increasing membrane permeability (Batoko et al., 1998; Hutchison & Gross, 1997). FP-A and FP-B, equally characterized as LDPs, have the same quantitative amino acid composition differing only for the fatty acid moieties (Ballio et al., 1996). They have similar toxic properties to syringotoxins and are structurally related to syringopeptins produced by plant pathogenic *Pseudomonas syringae* pv. *syringae* strains (Ballio et al., 1991). *P. fuscovaginae* is a seedborne pathogen (Adorada et al., 2015), which can be transmitted by infected seeds or survives as an epiphyte in cereal crops especially in the rice fields, waiting for the favourable conditions for causing the symptoms of the disease.

P. fuscovaginae possesses two acyl-homoserine lactone quorum sensing (QS) systems involved in the regulation of virulence; QS knock-out mutants are less virulent and display less severe symptoms of sheath brown rot (Mattiuzzo et al., 2011). Several other virulence associated loci in *P. fuscovaginae* have been identified by screening a genomic mutant bank (Hitendra Kumar Patel et al., 2014). The genetic loci involved in virulence encode for the following proteins: an arsenic pump, efflux proteins, type IV pilus biogenesis protein PilZ, an N-acetyl-gamma-glutamylphosphate reductase, an acetylglutamate kinase, a phage tail fiber homolog protein, a syringopeptin synthetase C homolog and a bifunctional sulphate adenylyltransferase subunit 1. The genomic functional annotation of *P. fuscovaginae* strain CB98818 (Xie et al., 2012) also revealed pathogenicity-related genes like type VI secretion system, type III and IV secretion system, Hcp- and VgR-like protein, Hrp protein, and

flagellin that are virulence associated genes in many phytopathogenic Gram negative bacteria (Geneious v5.4, 2011). In summary, several genetic loci have been implicated in the pathogenicity of this bacterium even though very few molecular and genetic studies related to the virulence of *P. fuscovaginae* have been performed.

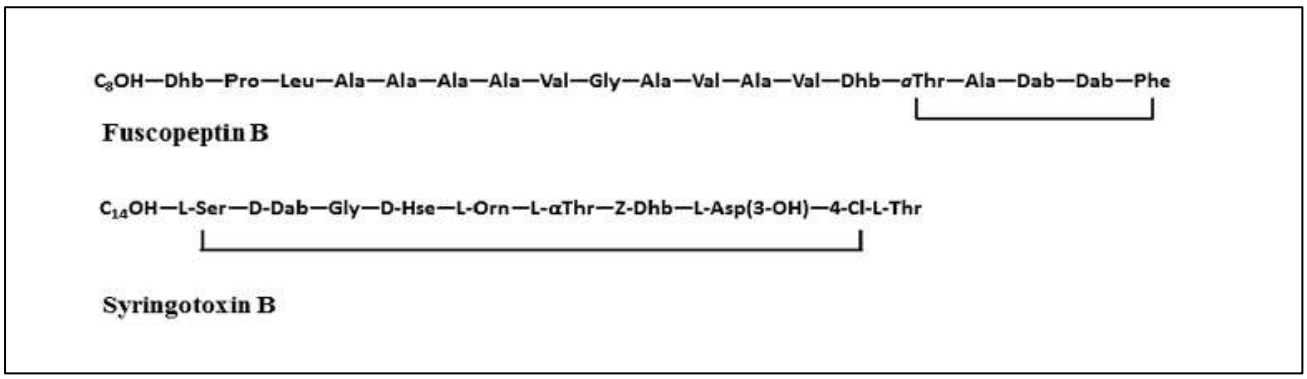


Figure 1. 3 Structures of toxins Fuscopeptin B and syringotoxin B produced by *P. fuscovaginae* (Bigirimana et al. 2015).

1.1.2. *Sarocladium oryzae* and *Fusarium* sp. fungi agent associated to sheath rot

The sheath rot rice disease has bee also associated to other microbial pathogens (Bigirimana et al. 2015). For example, fungi have also been associated with sheath rot symptoms including *Sarocladium oryzae* (*S. oryzae*) (Bills et al., 2004; Giraldo et al., 2015; Purkayastha & Ghosal, 1985; Sreenivasaprasad et al., 2001) and members of the *Fusarium fujikuroi* complex (Abbas et al., 1998; Aoki et al., 2014; Desjardins et al., 1997; Kushiro et al., 2012; Quazi et al., 2013) *S. oryzae* has been originally described as *Acrocylindrium oryzae*, and has been isolated for the first time from rice sheath symptomatic in Taiwan in 1922 (Mew & Gonzales, 2002).

1.1.2.1. Pathogenicity of *Sarocladium oryzae*

The pathogenicity determinants of this pathogen are the secondary metabolites helvolic acid and cerulenin (Ayyadurai et al., 2005; Ghosh et al., 2002; Peeters et al., 2020). *S. oryzae* also produces a cellulolase, a protease, a pectinase, and oxidative enzymes that are thought to play a role in pathogenicity (Joe & Manibhushanrao, 1995; Sreenivasaprasad et al., 2001). Genome sequencing of *S. oryzae* revealed that its genome has evolved with many widespread gene families of proteinases, zinc finger proteins, sugar transporters, dehydrogenases/ reductases, cytochrome P450, WD domain G-beta repeat and FAD-binding proteins (Hittalmani et al., 2016). Gene orthology analysis showed that most of *S. oryzae* genes are orthologous to other Ascomycetes fungi. The orthologous genes are those present in different species and originated of vertical descent from a single gene of the last common ancestor (Fitch, 1970) and they have often, but not always, the same function (Fang et al., 2010). The polyketide synthase dehydratase, ATP-binding cassette (ABC) transporters, amine oxidases, and aldehyde dehydrogenase family proteins are duplicated in larger proportion specifying the adaptive gene duplications to varying environmental conditions. Thirty-nine secondary metabolite gene clusters encode for polyketide synthases and terpene cyclases. Protein homology based analysis indicated that nine putative candidate genes are involved in helvolic acid biosynthesis pathway and they are arranged in cluster and structural organization which is similar to the helvolic acid biosynthesis cluster in *Metarhizium anisophilae.* Other *S. oryzae* genes are identified as putative pathogenicity genes since they have been shown to be involved in virulence in other phytopathogenic fungi and enlisted in pathogen-host interaction database (Hittalmani et al., 2016).

1.1.2.2. Pathogenicity of *Fusarium* sp.

Sheath rot in rice has also been associated with *Fusarium* sp. belonging to the *Fusarium fujikuroi* complex which is largely corresponding to the section Liseola (Wollenweber and Reinking, 1935) that is one of *Fusarium* genus subdivisions (Watanabe et al., 2011). The symptoms caused by *Fusarium proliferatum* are blanked or partially blanked panicle with reddish-brown to off-white florets or kernels that are often covered with a white to pinkish white powder consisting of microconidia and conidiophores. In addition, the enlarging lesion on flag leaf sheath developed rapidly, firstly to dark brown and later off-white to tan with a reddish brown border, which eventually encompasses the entire sheath and may result in the death of the leaf blade. The lower leaf sheaths may eventually develop lesions as well, but rarely more than two leaf sheaths show symptoms; and a dense white to pinkish powder consisting of microconidia and conidiophores of *Fusarium proliferatum* covers the sheath lesions, especially evident during humid periods (Abbas et al., 1998).

Two metabolites involved in plant pathogenicity of *Fusarium* sp. are gibberellins and mycotoxins. According to Wulff et al.(2010), only strains of *Fusarium fujikuroi* were able to produce gibberellin A causing abnormal elongation of rice plants, the so-called bakanae disease. The *Fusarium proliferatum* species is known as specie producing mycotoxins, like fumonisin B and has been associated to rice sheath rot (Abbas et al., 1998). In summary, the two fungal groups (*Sarocladium oryzae* and *Fusarium sp.)* both belong to the Ascomycete phylum and there are very few reports on their pathogenicity on rice plant especially for the *Fusarium sp*.

Table 1. 3 Main toxins involved in rice sheath rot disease

(Bigirimana et al. 2015).

Microbial toxin	**Producing sheath rot pathogen**	**Other producing organisms**	**Class**	**Mode of action**	**Symptom on plants**	**Other activities**
Cerulenin	*Sarocladium oryzae*	Not known	Hexaketide amide	Inhibitor of fatty acid synthetases, interference with flavonoid biosynthesis	Necrosis, growth inhibition	Antibacterial and antifungal activity
Helvolic acid	*Sarocladium oryzae*	*Metarhizium anisopliae, Aspergillus* sp., *Pichia guilliermondii, Alternaria* sp.	Steroid	Interference with chlorophyll biosynthesis	Chlorosis	Antibacterial activity
Fumonisin B	*Fusarium proliferatum, F.verticillioides, F.fujikuroi*	Other *Fusarium* sp., *Aspergillusniger, Tolypocladium* sp., *Alternariaalternata*	Polyketide	Inhibitor of sphingolipid biosynthesis	Necrosis, growth inhibition	Human and animal toxin
Fuscopeptins	*Pseudomonas fuscovaginae*	Not known	Cyclic lipopeptide	Form channels in plasma membranes	Necrosis	Antimicrobial activity
Syringotoxin	*Pseudomonas fuscovaginae*	*Pseudomonas syringae* pv. *syringae*	Cyclic lipopeptide	Interference with ATPase pumps in plasma membrane	Necrosis	Antifungal activity

1.2. Plant microbiome and pathobiome

1.2.1. Definition of plant microbiome

The microbial community which lives in association with the plant is called the plant microbiome or plant microbiota (Schlaeppi & Bulgarelli, 2015) and can be considered as the second plant genome and plays a crucial role in health and nutrient uptake. Plant microbiomes can therefore be a significant ally for the plant in controlling the colonization/infection by plant pathogens. Novel methodologies now allow the analysis of total microbial populations thus opening the avenue on the role of microbiome in plant disease.

The plant microbiome can be sub-divided depending on the location in the plant; (i) the rhizospheric microbiome is the community of microbes most closely associated or attached to the roots, (ii) the endosphere microbiome are the microbes which live inside plants in intercellular spaces and mostly originate from the rhizosphere (Edwards et al., 2015), (iii) the phyllospheric/epiphytic microbiome is located in the surface aerial parts and (iv) the seed microbiome are the vertically transferred microbes. All together these form the plant microbiome as described in **Figure 1.4**.

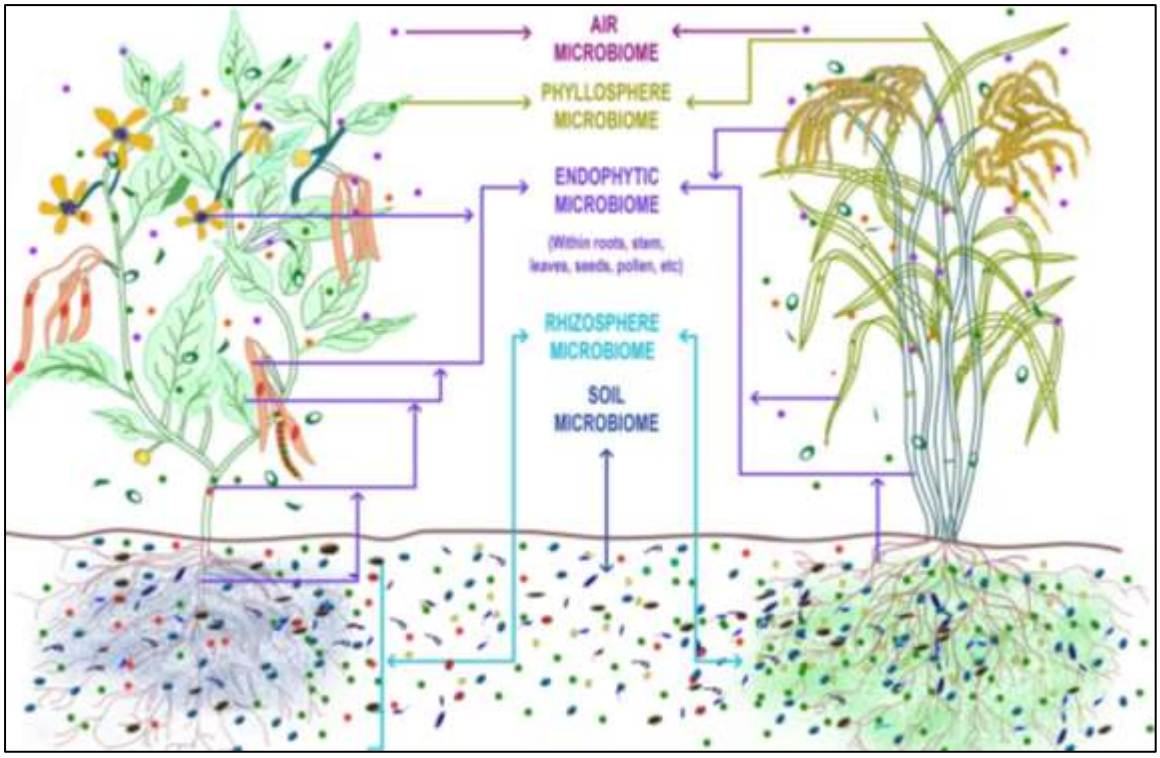


Figure 1. 4 Organization of microbes associated to the plant

(Gopal & Gupta, 2016).

Many microbes of the plant microbiome can offer several benefits to the host plant such as growth promotion by allowing plant to have access to nutrients like phosphate and nitrogen. Alternatively they can stimulate plant growth by producing phytohormones like indole-acetic-acid (IAA) or provide protection from pathogens either via the production of some metabolites that kill/inhibit the pathogens or via nutrient competition (Ali et al., 2012; Coutinho et al., 2015). Plant microbiomes can also play a role in plant resistance to abiotic or biotic stress and agricultural management can have an important impact on the plant microbiome as for example been demonstrated for maize (Wattenburger et al., 2019).

1.2.2. The pathobiome

Pathogenicity has long been believed to be the outcome of interactions between the pathogen, host and the environment. Plant microbiome reports are indicating that plants live in association with a large number of microorganisms that are thought to play important roles in

plant health. Interactions between the pathogen and other microorganisms of the microbiome can affect positively or negatively pathogen establishment and virulence thus adding a fourth dimension to the disease triangle. The perception that the microbiome contributes to disease formation and severity and the discovery of complex diseases involving more than one pathogen, has led to the recent introduction of the term pathobiome, i.e. the pathogen integrated with the biotic environment of the host (Vayssier-Taussat et al., 2014) (**Figure 1.5**). The presence of the pathogens on host induce a shift of the microbiome community as demonstrated recently (Gomes et al., 2019) also the pathogens can cooperate and increase the virulence disease (Jung et al., 2018). In the future, metaomic approaches will most likely shed light in the understanding of the pathogens within the context of microbial communities in the new concept of pathobiome.

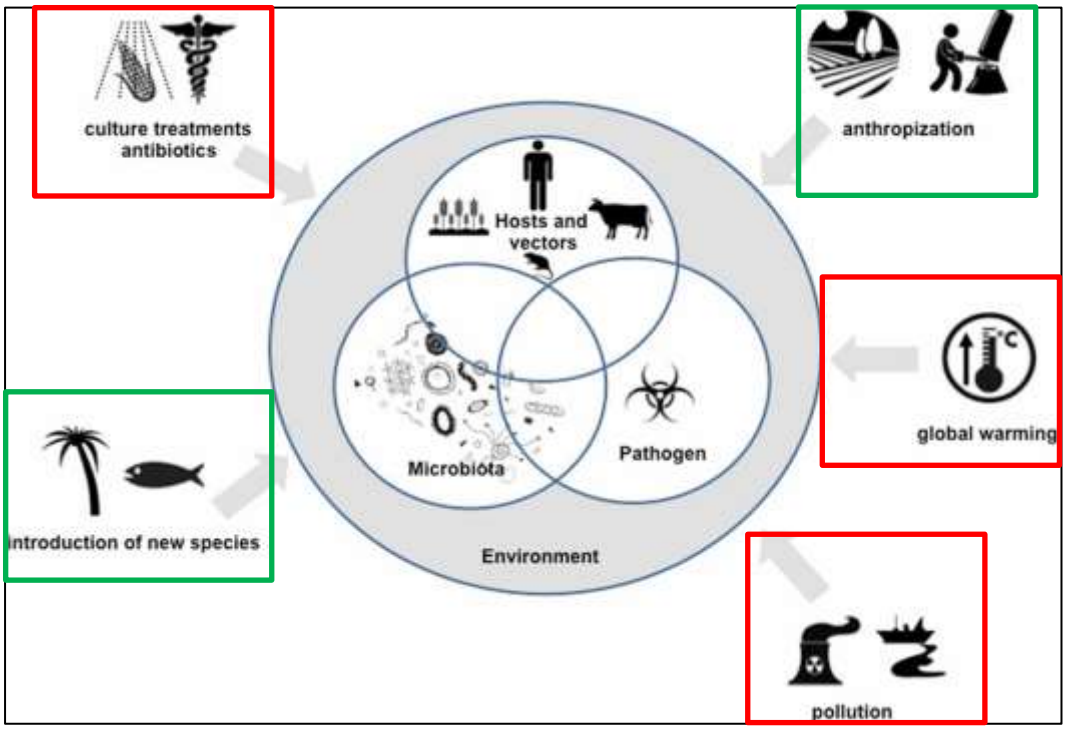


Figure 1. 5 Overview of the pathobiome concept.

Pathogen is influenced by abiotic factors (in red; environmental conditions) and biotic factors (in green ; other microorganisms or organisms including the host) (Vayssier-Taussat et al., 2014).

1.2.3. Pathobiome of sheath rot disease

As mentioned above, rice sheath rot is a devastative disease associated to the bacterial pathogen *P. fuscovaginae*, the fungal pathogen *S. oryzae* and the fungal complex pathogen of *Fusarium spp.* (see above; Bigirimana et al. 2015) and further investigations are necessary for deciphering their possible interkingdom interactions, potential effect on the microbiome and whether sheath rot is a complex disease involving the interaction/cooperation of different pathogens.

Studying the plant microbiome at the site of infection of several rice diseases could reveal potential commensal/resident bacteria or fungi which can cooperate with the pathogen and even the abundance of the pathogens. In human pathology for example, there is a growing awareness that pathogens frequently do not act alone and the study of multispecies synergistic interactions is becoming an important aspect for the understanding of microbial diseases (da Silva et al., 2014). In contrast to mammalian pathology, in plant pathology the concept of monostrain/monospecies infections is deeply rooted. Some initial examples are indicating interactions of different plant pathogens as well as interactions between pathogens and the residential microbiota. Pathobiome studies are likely to considerably increase in the future highlighting possible microbial interspecies interactions in the process of disease.

1.3. Cell-to-cell Signaling and Quorum sensing in *Pseudomonas fuscovaginae*; the causal agent of rice sheath brown rot disease

1.3.1. Definition

Bacteria can undergo cell-cell communication by producing and responding to small diffusible molecules that act as signals; these molecules are often called auto-inducers (AIs). AIs are produced at basal levels and their concentration increases with cell-density and as the signals can diffuse through membranes, their concentration inside cells approximates the

concentration in the environment. Upon reaching a critical concentration, the signal molecules can bind to and activate receptors inside bacterial cells. These receptors can then alter gene expression to activate behaviours that are beneficial under the particular condition encountered. As this phenomenon occurs in a cell-density-dependent manner, it has been termed quorum sensing (W. C. Fuqua et al., 1994).

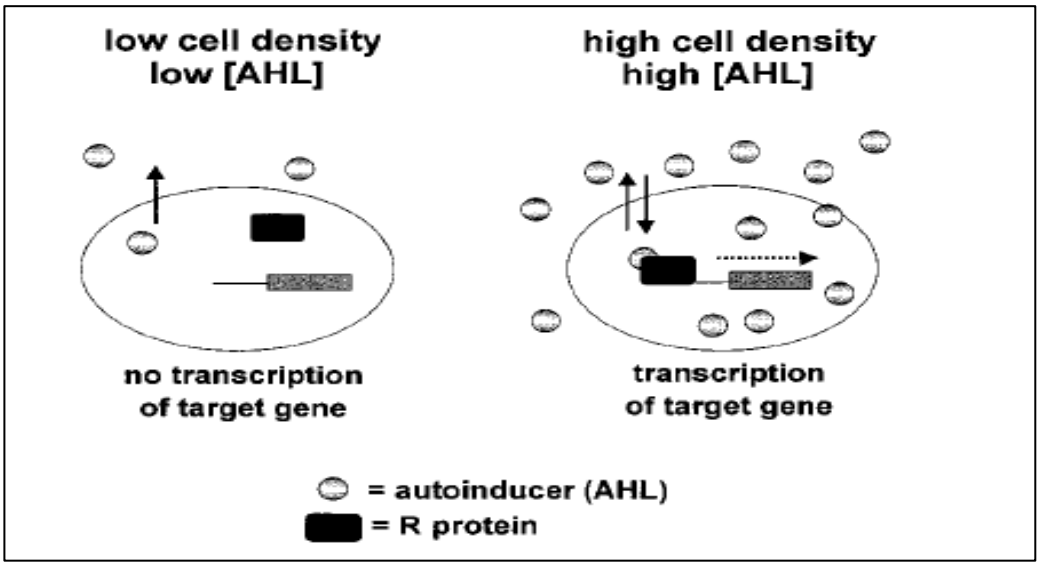


Figure 1. 6 Quorum sensing in gram-negative organisms.

Two regulatory components: the transcriptional activator protein (R protein) and the AI molecule produced by the autoinducer synthase are presented (De Kievit & Iglewski, 2000).

1.3.2. Signals molecules involved in QS and canonical AHL QS system in Gram negative bacteria

Many classes of AIs have been described to date. The most intensely studied AIs are the N-acyl-homoserine lactones (AHLs) of Gram-negative bacteria, the peptides of Gram-positive bacteria and a class of AIs termed AI-2, which is found in both groups of bacteria (Antunes & Ferreira, 2009). AHLs are usually detected through binding to and activation of cytoplasmic receptor proteins, which dimerize upon signal detection and can bind to promoter regions of target genes to activate or repress their transcription (W. C. Fuqua et al., 1994). Peptides are usually detected through binding to membrane sensor proteins of the two-component system family, although some can also be transported to the cytoplasm before interacting with their

receptors (Novick & Geisinger, 2008; Pottathil & Lazazzera, 2003). On the other hand, AI-2 binds a periplasmic protein and then interacts with either a two component system or a transporter depending on the organism (Ng & Bassler, 2009; Taga et al., 2001). Binding to a membrane-associated sensor kinase causes the activation of a phosphorelay cascade, which results in the activation or repression of a response regulator, culminating in altered gene expression (Ng & Bassler, 2009; Novick & Geisinger, 2008)

QS was originally described in the marine luminescent bacterium *Vibrio fischeri*, where it functions as the control mechanism of light production and numerous other traits (Eberhard et al., 1981; J. Engebrecht et al., 1983; J. A. Engebrecht & Silverman, 1984). For years, it was thought that this phenomenon was limited to a few marine organisms but it has later been demonstrated that many bacterial species utilize QS as part of their regulatory machinery (Antunes & Ferreira, 2009; Bassler & Losick, 2006; Lyon & Novick, 2004). Of interest, it is known that bacterial virulence is in many cases controlled by QS (Antunes & Ferreira, 2009). There was then a burst in QS research and it role in the virulence of multiple human and plant pathogens; it has been studied in molecular detail on different bacterial species.

The LuxI and LuxR proteins encoded by *luxI* and *luxR* genes compose the canonical AHL QS system in Gram negative bacteria and they are in most cases genetically adjacent. The LuxI family protein synthetizes AHLs signal molecules. These molecules vary in their structure with different acyl chain lengths (from 4 to 20 carbons). The position C3 of the acyl chain can have the oxidation and can be methylated, ketonated or hydroxylated. The LuxR family, transcriptional regulator, forms a complex with the cognate AHL threshold ("quorum") concentration and the transcriptional status of target genes is affected (C. Fuqua et al., 2001). Acyl-homoserine lactone (HSL) signals are produced by the LuxI enzyme homologues that bind to LuxR homologues to activate expression of target genes. At low cell densities, concentration of the signal is low both inside and outside the cell, with minimal activation of

LuxR. At high cell densities, acyl-HSL activates LuxR through binding and leads to expression of downstream target genes **(Figure 1.7**) (Jayaraman & Wood, 2008).

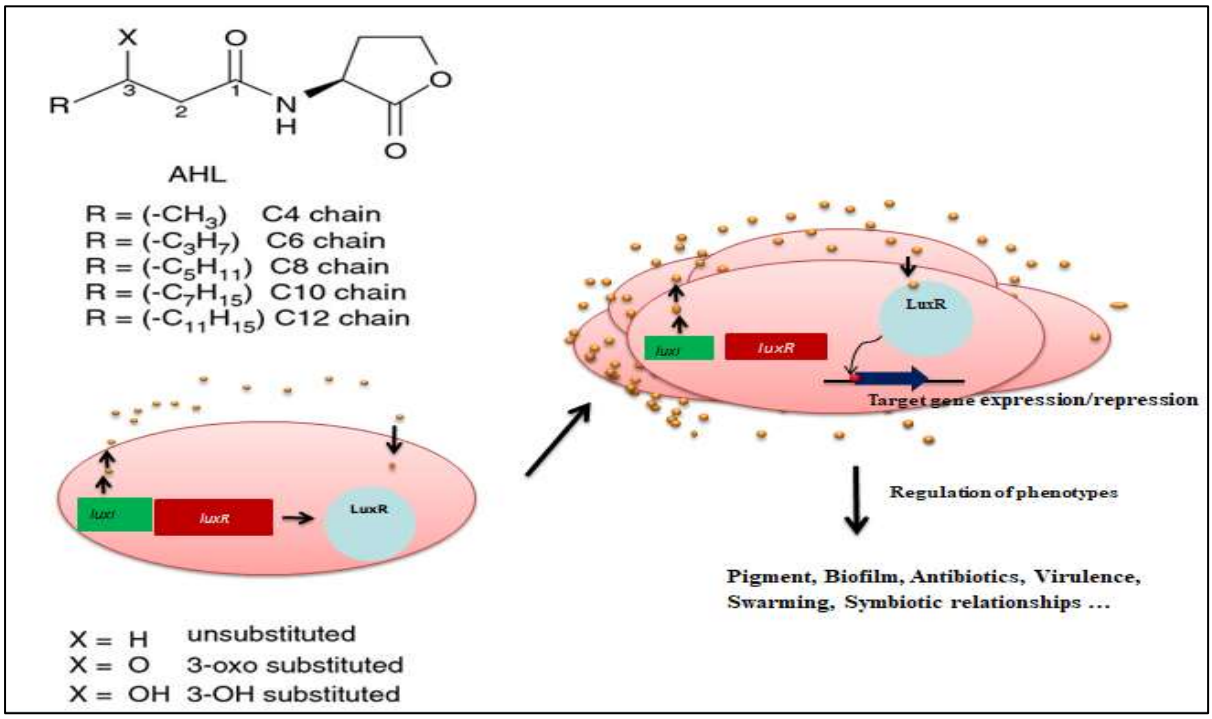
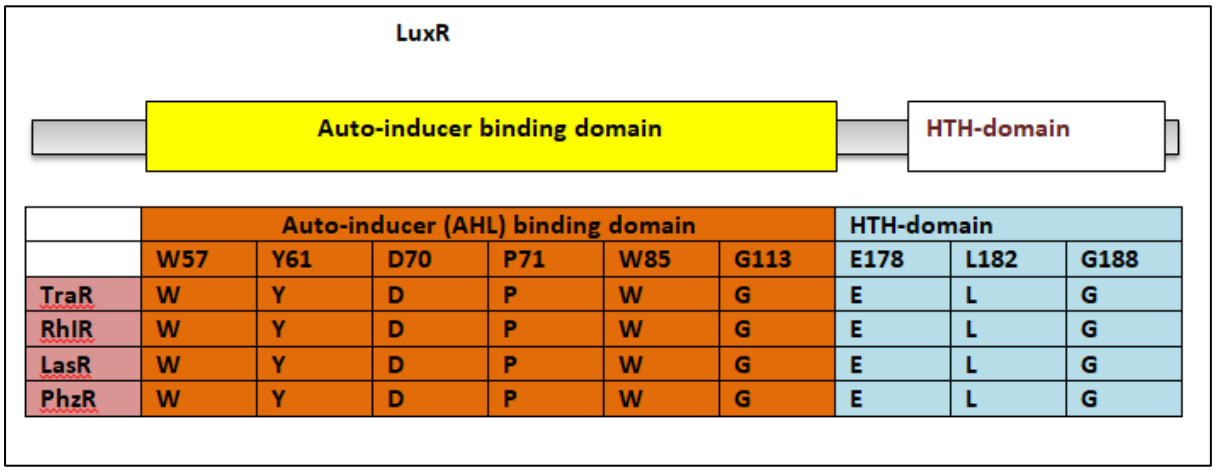


Figure 1. 7 Components of canonical QS system and gene regulation.

The AHL are synthetized and regulated by the LuxI and LuxR transcriptional proteins. The LuxR binds AHL and activate the genes involved in the expression of several phenotypes (Hitendra Kumar Patel et al. 2014 with modifications).

The QS LuxR-family regulatory proteins are modular, composed of approximately 250 amino acids arranged in two domains, which are separated by a short link region an autoinducer-binding domain located in the N-terminal region (Shadel et al., 1990; Slock et al., 1990) and DNA-binding helix-turn-helix (HTH) domain positioned at the C-terminal region (Choi & Greenberg, 1991, 1992; W. C. Fuqua & Winans, 1994). The transcriptional regulation by the LuxR-proteins occurs via DNA-binding in conservative sites of the gene promoter regions called *lux* boxes (Devine et al., 1989; Stevens & Greenberg, 1997). The homologies of QS LuxRs are normally low (18-25%) but nine amino acid residues are highly conserved and are shared at 95% rate (Whitehead et al., 2001; R. guang Zhang et al., 2002). Six of these amino acids are hydrophobic or aromatic and form the cavity of the AHL binding domain while the remaining three are located in the HTH domain (Fuqua C et al., 1996) **(Figure 1.8**). The nine

highly conserved amino acid residues numbers are based on TraR from *Agrobacterium tumefaciens*.



	Auto-inducer (AHL) binding domain						HTH-domain		
	W57	Y61	D70	P71	W85	G113	E178	L182	G188
TraR	W	Y	D	P	W	G	E	L	G
RhlR	W	Y	D	P	W	G	E	L	G
LasR	W	Y	D	P	W	G	E	L	G
PhzR	W	Y	D	P	W	G	E	L	G

Figure 1. 8 LuxR protein domain organization

(Adapted and modified by González and Venturi 2013)

QS-dependent regulation in bacteria is most often involved in the coordinated community action of the bacteria like antibiotic production, biofilm formation, conjugation, bioluminescence, production of extracellular enzymes, virulence factors and pigment formation (Bassler, 2002; C. Fuqua & Greenberg, 2002; Von Bodman et al., 2003; Whitehead et al., 2001) **(Figure 1.7).**

Well-studied examples of QS-dependent regulation of virulence factors include:

- The QS system of *Pseudomonas aeruginosa*, an opportunistic human pathogen in which two different AHL QS circuits (LasI/LasR and RhlI/RhlR) act in synchrony to control the expressions of several virulence factors (Bjarnsholt et al., 2010; Brint & Ohman, 1995; Jones et al., 1993; Passador et al., 1993).
- The AHL QS of *Pseudomonas syringae* pv. *syringae* (AhlI/AhlR) controls several traits including extracellular polysaccharide production, oxidative stress tolerance, swarming motility, promotion of water-soaked lesions in bean plants (Dulla et al., 2005; Quiñones et al., 2005)

- *Pseudomonas aureofaciens,* a plant beneficial bacterium, has two AHL QS system (PhzI/R and CsaI/R) which are required in the production of the phenazine antibiotics and exoproteases that help the bacteria to colonize the wheat rhizosphere and to protect the root against fungal infection (Wood et al., 1997; Wood & Pierson, 1996; Z. Zhang & Pierson, 2001)
- The AHL QS system of plant growth promoting bacteria *Pseudomonas putida* designated as PupI/PupR are involved in the regulation of biofilm formation (Steidle et al., 2002).
- The plant growth promoting bacterium *Pseudomonas fluorescence* NCIMB 10586 possesses the AHL QS system designated by MupI/MupR and it is involved in the regulation of polyketide antibiotic mupirocin production (El-Sayed et al., 2001).
- The plant pathogen *Pseudomonas fuscovaginae* has two AHL QS system (PfsI/PfsR and PfvI/PfvR) which are required in the virulence in rice plant (Mattiuzzo et al., 2011).
- *Hafnia alvei,* an opportunistic pathogen and a dominant psychrophile found in putrid food (Vivas et al., 2008), its QS plays a key role in regulating virulence factors and biofilm production (Hou et al., 2017; Viana et al., 2009).

Additionally to the canonical AHL QS system, in many cases in the genomes of Proteobacteria there are QS *luxR*-type genes which are unpaired to a cognate *luxI* synthase gene. Case, Labbate, and Kjelleberg in 2008, performed an analysis of 265 proteobacterial genomes, showed that 68 had a canonical paired *luxI/R* system and out of these, 45 contained more *luxR* genes than *luxI;* additionally, 45 genomes contained only QS *luxR* genes. These QS LuxR proteins lacking a genetically linked LuxI have been termed “orphans” (C. Fuqua, 2006) or “solos”(Subramoni & Venturi, 2009). A sub-group of LuxR solos has been recently

discovered which are only found in the plant-associated bacteria (PAB) that do not bind AHLs but to plant produced compounds (González & Venturi, 2013) **(Figure 1.9).**

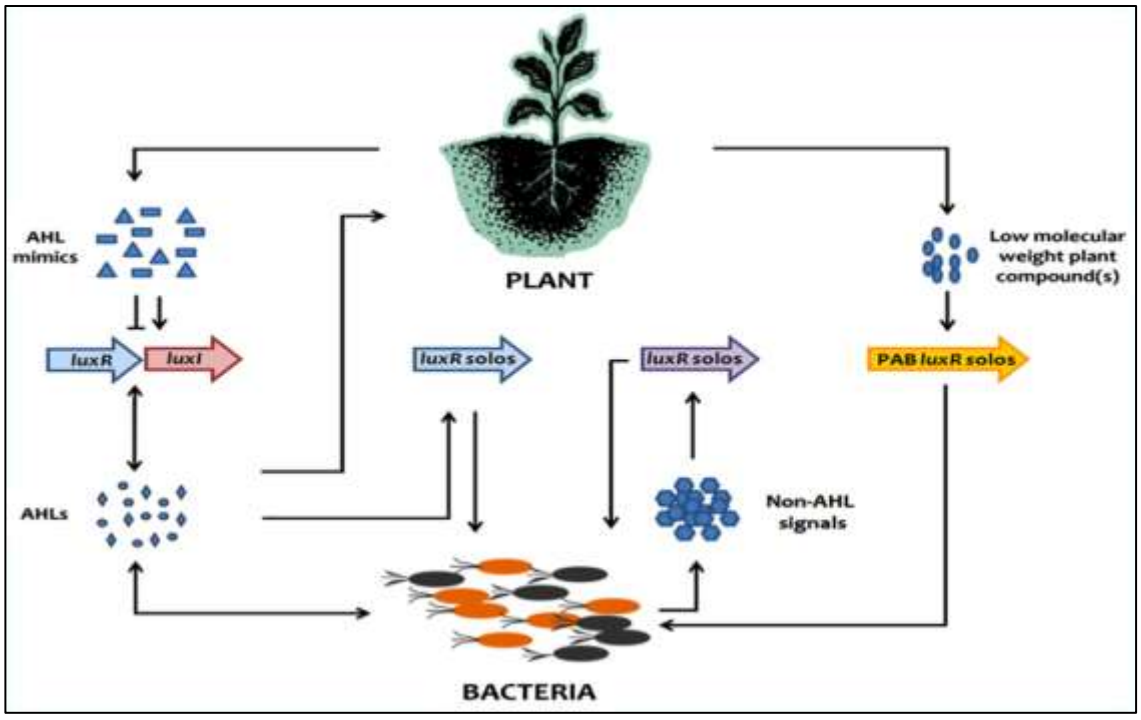


Figure 1. 9 Mode of action of AHL QS and of LuxR solos in signaling between plants and bacteria (Hitendra K. Patel et al., 2013)

1.3.3. Quorum sensing system in *Pseudomonas fuscovaginae*

P. fuscovaginae possesses two N-acyl homoserine lactone (AHL) quorum sensing (QS) systems which are designated PfsI/R and PfvI/R. PfsI synthase is involved in the production of C10- and C12-AHLs signals which are recognized by the PfsR regulator. Instead, the PfvI synthase produces 3-oxo-AHLs such as 3-oxo-C12-, 3-oxo-C10, 3-oxo-C8 and 3-oxo-C6-HSL which are recognized by the cognate PfvR regulator which also in part responds to the AHLs produced by PfsI at high concentration. The two QS systems are not transcriptionally hierarchically organized and both are involved in virulence of rice sheath brown rot. The *psfI/R* and *pfvI/R* systems are stringently negatively regulated by the intergenically located *rsaM* and *rsaL* repressors respectively (Mattiuzzo et al., 2011) **(Figure 1.10)**.

Transcriptomic studies have revealed that the RsaM repressor regulates over 400 genes: 206 are negatively regulated and 260 are positively regulated. More than half of the genes controlled by the PfsI/R system and 65% by the PfvI/R system are also part of the RsaM regulon; this is due to RsaM being involved in the regulation of both systems PfsI/R and PfvI/R (Uzelac et al., 2017). The mode of action of the RsaM repressor remains unknown, it appears not to be a DNA-binding protein and it is believed that it exerts its repressive role on PfsI/R together with other protein(s). The RsaL repressor protein on the other hand, is a DNA-binding protein belonging to the Tetrahelical Superclass of H-T-H Proteins (Rampioni et al., 2007).

P. fuscovaginae also possesses two *luxR* solo genes that lack a cognate *luxI* homolog in the neighbouring genomic region and have been designated as PfvR1 and PfvR2 (Patel HT *et al.,* 2014). PfvR1 most likely belongs to the canonical family of QS LuxR proteins that respond to AHLs whereas the PfvR2 belongs to the sub-family of LuxRs proteins that are found in plant-associated bacteria (PAB) and which bind and respond to yet unknown low molecular weight plant signals (Gonzalez & Venturi, 2013).

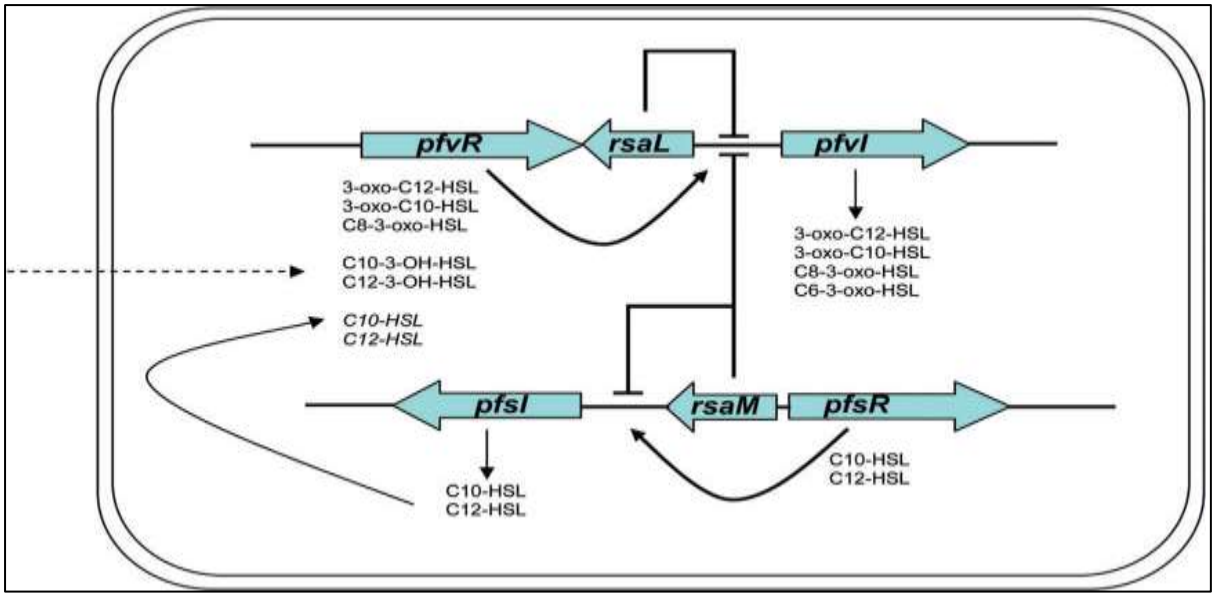


Figure 1. 10 QS system in *Pseudomonas fuscovaginae*

(Mattiuzzo et al., 2011)

1.4. Aims and organization of the thesis

P. fuscovaginae is a seedborne pathogen associated to rice sheath rot. This bacterium has two AHLs QS systems PfvI/R and PfsI/R repressed negatively by RsaL and RsaM (novel repressor); both *P. fuscovaginae* QS systems are involved in the expression of virulence genes. Both of these QS systems are switched off under laboratory conditions and the RsaM repressor involved in the regulation of *P. fuscovaginae* QSs is not a DNA-binding protein and its mechanism of action is currently unknown. With the new concept of pathobiome, it is believed that a pathogen changes the microbiome and cooperates with some of its members; alternatively a biodiverse plant microbiome can play an important role in disease resistance. Microbial interspecies as well as intraspecies signalling is likely to play an important role in these interactions in the pathobiome and microbiome. This could have important implications in devising ways in the control of rice sheath rot. This thesis focuses on studying the rice microbiome and the rice pathobiome (when affected by sheath rot disease) in order identify and compare the microorganisms involved in each plant state (healthy or symptomatic) (i); and in addition, the isolation and characterization of putative microbial (bacteria) probiotics

that could be developed as biocontrol of the disease (ii), and the mechanisms of activation of the PfsI/R and PfvI/R QS systems of the pathogen *P. fuscovaginae* (iii).

The experimental work of this thesis divided into three experimental chapters (II, III and IV); chapter II describes the pathobiome/microbiome studies on sheath rice samples (symptomatic or asymptomatic of sheath rot disease) where the bacterial and fungal communities are described. Chapter III is focused on the culturable microbiome and the isolation and characterization of bacterial isolates isolated from asymptomatic samples in the highland; and the phenotypes assay of these bacterial isolates including the antibacterial activity against *P. fuscovaginae*. Chapter IV presents studies cell-cell signaling of QS *P. fuscovaginae*. In Resume this thesis is focused on the pathobiome/microbiome of rice sheath samples (asymptomatic and symptomatic samples) and the QS system of *P. fuscovaginae*.

Chapter II. Pathobiomes revealed that *Pseudomonas fuscovaginae* and *Sarocladium oryzae* are independently associated with rice sheath rot

2.1. Introduction

Rice sheath rot disease has been identified for the first time in Japan in 1976 (Tanii et al., 1976); it has then been reported in many parts of the world (CABI, 2018) including in Burundi (Duveiller et al., 1988), in Madagascar (Rott, 1989) in Latin America (Zeigler, 1987) in Australia (Cother et al., 2009) and recently in South Korea (Kim et al., 2015). Rice plants can display sheath rot symptoms at all stages of growth; infected seedlings initially show symptoms of yellowish to brown discoloration on the lower leaf sheath and later turn grey-brown to dark-brown and ultimately rot and die (Cottyn; B.; Cerez; M.T.; and Mew; T.W., 1994). The symptoms on mature rice plants are similar to those found on seedlings displaying water-soaking and necrosis without distinct lesions. The causal agent is the bacterium *Pseudomonas fuscovaginae* (Tanii et al., 1976) however several microorganisms have been associated with rice sheath rot symptoms reviewed by (Bigirimana et al., 2015b). *P. fuscovaginae* virulence has been linked with several factors including phytotoxins (Ballio et al., 1996), exopolysaccharides and quorum sensing (Mattiuzzo et al., 2011; Hitendra Kumar Patel et al., 2014). *P. fuscovaginae* is a broad host range pathogen and has also been isolated from other cereal crops like sorghum, maize, wheat, and barley (Duveiller, 1989). Fungi have also been associated with sheath rot symptoms including *Sarocladium oryzae* (Bills et al., 2004; Giraldo et al., 2015; Purkayastha & Ghosal, 1985; Sreenivasaprasad et al., 2001) and members of the *Fusarium fujikuroi* complex (Abbas et al., 1998; Aoki et al., 2014; Desjardins et al., 1997; Kushiro et al., 2012; Quazi et al., 2013). The number and role of the pathogens which are causing sheath rot symptoms is therefore still under study.

Numerous studies on the plant microbiomes have documented that plants are colonized and live in association with a large number of microorganisms which are thought to play important roles in resistance to biotic and abiotic stresses (Dudenhöffer et al., 2016; Grover et al., 2011; Ho et al., 2017; Lata et al., 2018; Rolli et al., 2015; Sziderics et al., 2007; Timm et

al., 2018; Yandigeri et al., 2012; H. Zhang et al., 2008). The plant microbiome is therefore likely to play an important role in the disease development as interactions between the pathogen and other microorganisms of the microbiome could affect positively or negatively virulence. The awareness that the microbiome is involved in disease has led to the introduction of the term pathobiome (Vayssier-Taussat et al., 2014). Plant pathobiome studies are at very early stages, as we know very little on the interactions between plant pathogens and the microbiome. Plant microbiome and pathobiome studies will therefore shed light on the pathogen(s) that are causing the symptoms as well as possible roles of their interactions with the resident microbial community.

In this study the pathobiome and microbiome of sheath rot symptomatic and asymptomatic rice plants were determined in dry and wet growing seasons both in highland and lowland in Burundi where the disease symptoms are strongly present (IRRI.org, **Figure 2.1a, b**). The main aim was therefore to shed light on the pathogen(s) causing rice sheath rot. The rice plants sampled for pathobiome studies presented the typical symptoms of sheath rot that included tissue necrosis. These studies have demonstrated that the *P. fuscovaginae* pathogen is abundantly present in symptomatic samples in the highland in both dry and wet seasons, whereas the fungal rice pathogen *S. oryzae* is significantly present only in diseased samples from the lowland especially during the wet season. These studies have also evidenced members of the pathobiome significantly varies and could therefore play a role in disease establishment/development.

2.2. Materials and Methods

2.2.1. Rice Samplings

Asymptomatic (healthy) or symptomatic (diseased) rice plants of sheath rot (**Figure 2.1a**) were collected in two different rice growing seasons in Burundi. The first sampling was performed during the wet season (April 2017) and the second during the dry season (December 2018). The wet season for rice cultivation is from December to May and it is more rainfall than the dry season that it is from June to November. The sampling sites in Burundi were the highland (Gisha-Ngozi: Latitude S2°55’34,93’’; Longitude E29°56'56,30’’; Altitude 1534,15 m) and the lowland (Gihanga-Bubanza: Latitude S03°10'17"; Longitude E29°21'16"; Altitude 849 m) and samples were then taken to the experimental IRRI Outstation in Burundi (**Figure 2.1b**). For each growing season, 48 samples were collected; 24 from rice plants displaying sheath rot symptoms and 24 from asymptomatic plants. From both growing seasons a total 96 samples was therefore collected. The sampling was performed on asymptomatic or symptomatic rice plant sheaths using cleaned scissors (after each sample the scissors was cleaned by cotton soaked in ethanol 70 %) and wrapped in plastic, and put in cold box (ice bucket) for the transport to the IRRI lab (Bujumbura: Latitude S03°22’41,2’’; Longitude E29°23,18’’; Altitude 855 m) and stored at -20 °C in the fridge.

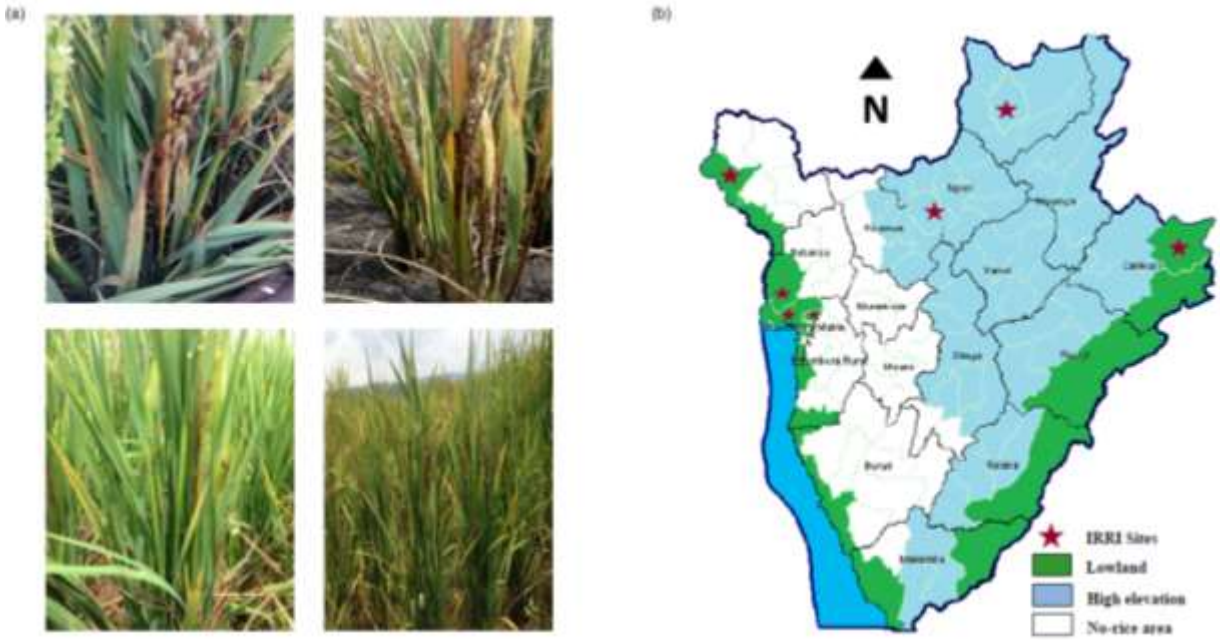


Figure 2. 1 Symptoms of sheath rot in rice field

(a) (necrosis, seeds rotten and sterile); **(b)** Burundi Rice ecology and field sites (lowland and highland) and station of IRRI-Burundi (uploaded by Georges H.), red star designs the IRRI field sites in lowland and highland**.**

The symptomatic (diseased) and asymptomatic (healthy) rice sheath plants were sampled by considering the two locations (lowland land: Gihanga-Bubanza and highland: Gisha-Ngozi) in two seasons; wet season (April 2017) and dry season (December 2018)

2.2.2. DNA extraction from plant material

Rice plant sheath samples (not sterilised) were cut in small pieces and a half of one gram (0.5g) was weighed for each sample and was then used for microbial total DNA extraction. The plant samples were not surface sterilized before. Autoclaved mortar and pestle were used to grind the rice plant material in the presence of liquid nitrogen in order to obtain a powder. DNA extraction was then performed from 0.5g of material according to the DNeasy Power Soil Kit (Qiagen, Hilden, D). DNA samples were lyophilized using a TF-10A Vacuum Freeze Dryer/Lyophilizer in order to facilitate their transport from IRRI laboratory (Bujumbura) to Bacteriology group laboratory (Trieste) and then resuspended in 100µl of sterile water; the quality and the quantity were determined with a UV spectrophotometer (Nanodrop 1000, Thermo Scientific, United States) and dilutions at 5ng/µl were prepared for each DNA sample. The number and samples symptomology are described in Table S1.

2.2.3. 16S rRNA gene and Internal Transcribed Spacer (ITS) amplicon libraries preparation.

The 16S rRNA gene amplicon library was performed by using the following primers: 16S Illumina library FW5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Illumina library RW 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAAATCC. A mix of 2,5 µl (5ng/µl) microbial DNA, 5µL (1µM) of each primers and 12,5 µl of KAPA HiFi HotStart ReadyMix in final volume of 25 µl was used for the first PCR to amplify the V3 and V4 regions of 16S rRNA gene by following this program: initial denaturation of 95°C for 3 min followed by 25 cycles of 95°C for 30 sec, 55°C for 30sec and 72°C for 30 sec; and a final 5min of extension at 72°C and hold at 4°C.

The PCR products were cleaned as described in Illumina protocol, and a second PCR for adding the Illumina index was set. A mix of 5µl (PCR products), 5 µl of each Nextera XT Index Primer (N7xx and S5xx), 25 µl of 2xKAPA HiFi HotStart ReadyMix and 10 µl PCR Grade water in final volume of 50 µl and the following program was used for the second PRC, initial denaturation of 95°C for 3 min, followed by 8 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; and a final 5min of extension at 72°C and hold at 4°C. The second cleaning was done as recommended in the protocol by using AMPure XP beads.

For ITS amplicon library the primers IlluminaITS3-KY02 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGAAGAACGYAGYRAA and IlluminaITS4 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC were used to amplify the ITS-2 region according to (Toju et al., 2012). A mix of 2.5 µl (5ng/µl) microbial DNA, 5µL (1µM) of each primers and 12.5 µl of KAPA HiFi HotStart ReadyMix in final volume of 25 µl was used for the first PCR to ITS2 of ITS rDNA by

following this program: initial denaturation of 95°C for 3 min followed by 25 cycles 98°C for 20 sec, 56°C for 15sec, 72°C for 30 sec; and a final 5min of extension at 72°C and hold at 4°C.

The PCR products were cleaned as described in Illumina protocol, and a second PCR for adding the Illumina index was set. A mix of 15µL (PCR products), 5 µl of each Nextera XT Index Primer (N7xx and S5xx), 25 µl of 2xKAPA HiFi HotStart ReadyMix in final volume of 50 µl; the following program was used for the second PCR: initial denaturation of 95°C for 3 min, followed by 8 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; and a final 5min of extension at 72°C and hold at 4°C. The second cleaning was done as recommended in the protocol by using AMPure XP beads.

For both 16S rRNA gene and ITS amplicon libraries the second amplification products cleaned were quantified using the Qubit Kit (Invitrogen) and the quality (integrity and presence of a unique band) was confirmed by Bioanalyzer equipment (Agilent Inc., Santa Clara, CA, USA). After quantification and normalization all PCR products were diluted to 4nM and aliquots of 5µl of diluted DNA from each library were pooled together and sent to sequence by Illumina Miseq sequencing.

2.2.4. Sequence data processing

FASTQ files were analysed using DADA2 v1.4.0 (Callahan et al., 2016) adapting the methods from the DADA2 Pipeline Tutorial (1.4). R version 3.5.2 was used for all analyses. Briefly, prior to analyses in DADA2, samples were demultiplexed using the QIIME 1.9.1 split_libraries_fastq.py script. The demultiplexed files were then used as the input for DADA2. Cutadapt 1.15 was used for adapter removal and quality filtering. Later quality profiles of the reads were analysed using the DADA2 function; plot Quality Profile, to determine positions at which read quality greatly decrease. Reads were then filtered and trimmed at the identified positions (truncLen=190) using the filterAndTrim function with

standard parameters (maxN=0, truncQ=2, and maxEE=2). Dereplication was performed combining all identical sequencing reads into “unique sequences” with a corresponding “abundance” equal to the number of reads of that unique sequence. DADA2’s error model automatically filters out singletons, removing them before the subsequent sample inference step. Sample inference was performed using the inferred error model and chimeric sequences were removed using the removeBimeraDenovo function.

The RDP reference database (Cole et al., 2014), giving a final OTU table, was used to assign bacterial taxonomy using the assignTaxonomy function with a 97% sequence similarity. For the fungal taxonomy assignment the Greengenes (GG) database was used (McDonald et al., 2012).

2.2.5. Statistical analysis

The sequence table counts and rarefaction curves were determined on sequence count files generated by the analysis pipeline. The number of reads per plant sample ranged from 3556 to 41505 in the first sampling data set and from 5246 to 46059 in the second sampling data set. Both OTU tables were rarefied according to the sample with the lowest number of reads, using the Rarefy function of the GUnifrac library. Low-abundance OTUs were discarded as well as chloroplast and mitochondria presence, resulting in 6217 OTUs in the first sampling dataset and 6429 OTUs in the second sampling dataset. The resulting OTUs were clustered at Genus taxonomic level obtaining a final number of 420 different bacterial taxa for the first sampling and 485 different taxa in the second. For fungal analysis, the total number of taxa was 3422 from 32 samples for the first sampling and 2781 from 48 samples; after removing the unidentified genera the final number at genus level was 182 taxa for the first sampling and 163 taxa for the second sampling.

Statistical analysis were performed using the vegan package version 2.5-4 (Oksanen et al., 2019) and phyloseq package (McMurdie & Holmes, 2013) in R version 3.5.2 (Team, 2014).

Relative abundances of OTUs between samples and the comparative analysis of species richness and diversity indices (Chao1, Shannon, Simpson and ACE) among samples were calculated and Shapiro-Wilk normality test, Krustal- Wallis test or t-test were carried out to determine differences between the two conditions according to the distribution and equality of means of the data. Bray Curtis, Weighted Unifrac and Unweighted Unifrac distance matrices were used to calculate the beta diversity and visualized with Principal Coordinates Analysis (PCoA). Differences in beta diversity between asymptomatic and symptomatic samples were tested with non-parametric analysis of variance based on 999 permutations (PERMANOVA). To test for differential representation of microbial taxa in different samples the Deseq2 package (Love et al., 2014) was used.

2.3. Results

2.3.1. Rice samplings

Two sets of samples were collected in April 2017 and December 2018 during the wet and dry seasons respectively; in both years, asymptomatic and symptomatic samples were collected from the same fields in the highland and lowland locations in Burundi. In the first set during the wet season, samples were collected from six rice varieties from both locations, whereas in the second set during the dry season, samples were taken from two rice varieties in the lowland and four in the highland (**Appendix**). It was decided to sample during the wet and dry seasons and on different rice varieties in order to determine possible differences and commonalities in the pathobiome of rice sheath rot. The symptomatic and asymptomatic samples were cut approximately 2 cm in size covering the sheath rot symptoms (the same zone was cut in asymptomatic plants) and after DNA purification, the quality was determined via 16S rRNA gene and ITS2 region PCR amplification using universal primers (data not shown). The total DNA samples which were then used for bacterial community sequencing over the two years were 24 from highland and 22 from lowland with symptoms and similarly

24 from highland and 22 lowland asymptomatic samples. The total DNA samples over the two years which were of good quality and used for fungal community sequencing were 16 symptomatic and 19 asymptomatic from highland whereas 22 symptomatic and 23 asymptomatic from lowland (**Appendix**).

2.3.2. *Pseudomonas fuscovaginae* is abundant in rice plants displaying sheath rot symptoms in highland

Pathobiome analysis showed that the *Pseudomonas* genus was significantly more abundant in rice plant samples displaying the symptoms of sheath rot in both sampling seasons (wet and dry) from the highland (**Figure 2.2a,b;** p-value <0.01 and p-value < 0.001 respectively). In lowland, the amount of reads taxonomically associated to *Pseudomonas* was significantly different among asymptomatic and symptomatic samples from the dry season (p-value <0.001), while in the wet season there was no significant difference. In samples with sheath rot symptoms, the *Pseudomonas* genus was represented by 21 % and 81% relative abundance in highland in the wet and dry seasons respectively. On the other hand, in lowland samples, the relative abundance of *Pseudomonas* genus was 7% and 10 % in the wet and dry seasons respectively. The *Pseudomonas* genus was therefore predominant in symptomatic samples from highland and not in lowland samples (**Table 2.1**). Among the total number of reads taxonomically assigned to the *Pseudomonas* genus, the percentage of reads belonging to *Pseudomonas fuscovaginae* specifically (homology 99%) was calculated (these reads displayed the highest identity with *P. fuscovaginae*). In order to further confirm the presence of *P. fuscovaginae*, the quorum sensing *rsaM* gene encoding for a repressor, which is unique to *P. fuscovaginae* (Mattiuzzo et al., 2011) was amplified using as template purified DNA from symptomatic and asymptomatic plant material used for the microbiome analysis. The primers used to amplify the *rsaM* gene were RsaM_RV 5'-CGATCGAACATTAAGCCTGC-3' and RsaM_FW 5'ATGCAATCACTCGCCCCA-3'. The *rsaM* locus was only successfully

amplified from symptomatic samples indicating the presence of *P. fuscovaginae* (**Figure 2.4**). In the symptomatic samples from highland during the wet season the abundance of *P. fuscovaginae* was around 46% and 99% during the dry season. On the other hand among the asymptomatic samples from highland the abundance of *P. fuscovaginae* was 18% and 0.2% during wet and dry seasons respectively. In lowland, *P. fuscovaginae* represented 14% and 90% in symptomatic samples and 12% and 10% in asymptomatic samples during the wet and dry season respectively. It was therefore concluded that samples with sheath rot symptoms in the highland of Burundi contained very high abundances of *P. fuscovaginae* bacteria whereas in lowland they did not. This result was regardless of the rice variety since the *Pseudomonas* genus was always predominant in the symptomatic samples especially in the highland (**Figure 2.3a,b**).

Table 2. 1 Bacterial genus abundant (%) in asymptomatic or symptomatic samples from the highland in two rice growing seasons (wet and dry season)

(a)

Genus	First sampling, wet season			
	Highland		Lowland	
	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
Aureimonas	1.76	0.73	2.78	3.74
Bacillus	4.47	7.88	0.49	0.36
Burkholderia	0.17	0.00	9.75	2.38
Chryseobacterium	4.06	6.00	1.03	1.16
Comamonas	5.84	8.89	0.81	0.63
Delftia	2.61	2.97	1.02	0.15
Dickeya	3.23	6.06	0.29	0.39
Enterobacter	3.56	6.30	2.05	1.37
Erwinia	2.78	3.79	0.24	0.32
Herbaspirillum	5.45	10.10	1.17	1.78
Janthinobacterium	1.04	0.00	0.00	0.00
Kosakonia	2.67	3.30	0.24	0.32
Luteibacter	0.16	0.00	1.83	1.91
Methylobacterium	2.12	2.32	13.09	19.64
Microbacterium	0.43	0.40	1.17	1.79
Mycobacterium	0.16	0.00	5.29	2.75
Novosphingobium	2.41	0.10	7.14	5.51
Ochrobactrum	0.80	1.14	1.86	0.00
Pantoea	5.08	4.44	6.14	1.45
Pseudomonas	21.46	13.39	7.43	3.73
Rhizobium	2.09	0.29	7.49	3.60
Sphingobium	0.09	0.02	1.88	2.70
Sphingomonas	2.73	2.11	6.75	8.22
Stenotrophomonas	14.27	7.51	1.09	1.44
Xanthomonas	0.46	0.00	3.76	16.68

(b)

Genus	Second sampling, dry season			
	Highland		Lowland	
	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
Aurantimonas	0.17	4.53	1.24	7.14
Chryseobacterium	0.05	0.99	3.20	13.09
Delftia	2.60	0.87	0.26	0.00
Herbaspirillum	3.46	1.70	2.47	0.38
Hymenobacter	0.11	7.27	0.09	1.78
Janthinobacterium	1.38	0.02	0.00	0.00
Methylobacterium	2.59	40.98	2.35	18.35
Microbacterium	0.01	4.90	0.55	1.11
Mucilaginibacter	0.01	4.09	0.48	4.03
Paenibacillus	0.01	0.05	3.04	0.03
Pantoea	5.48	9.10	41.70	12.73
Pseudomonas	81.37	1.30	10.15	4.53
Rhizobium	0.09	0.51	3.03	3.10
Sphingobacterium	0.01	0.01	3.22	0.00
Sphingobium	0.00	0.08	0.25	0.16
Sphingomonas	0.94	10.13	5.72	22.92
Spirosoma	0.01	2.25	0.12	2.81
Stenotrophomonas	0.20	0.02	10.25	0.01

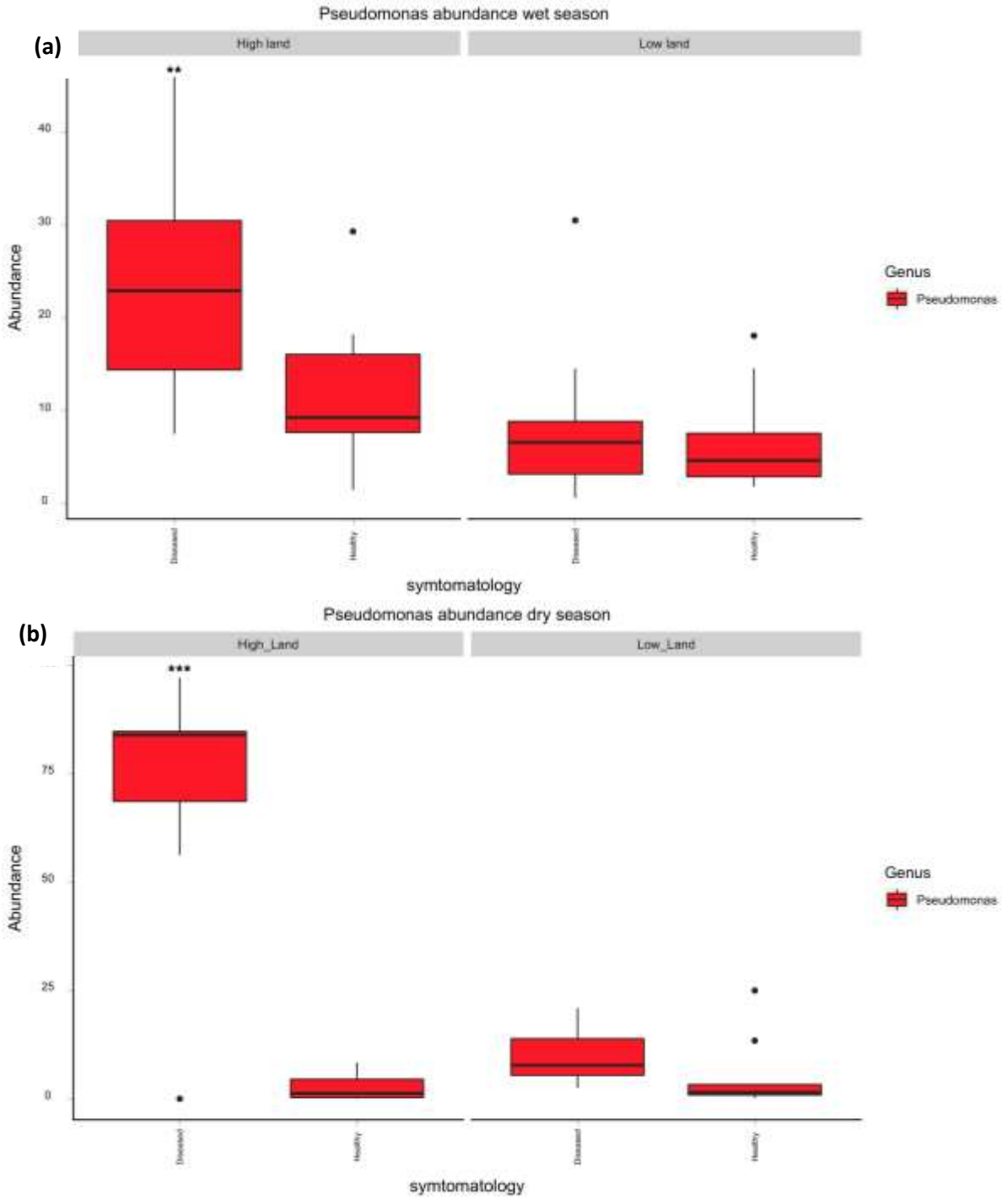
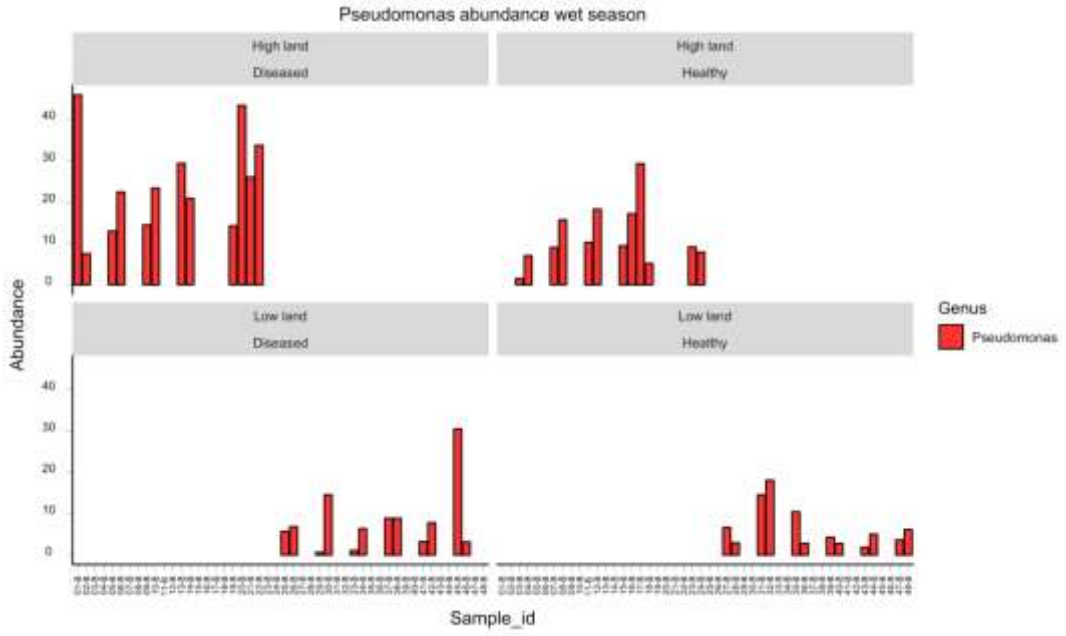


Figure 2. 2 *Pseudomonas* genus abundance according to the symptomology of the samples, the locations (highland and lowland) in wet season (a, 2017) and dry season (b, 2018).

Box plot depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidence intervals (whiskers) and otliers (black dots). Asterisks indicate significant differences between two group of samples (**p-value < 0.01, ***p-value <0.001). Statistical analysis were calculated based on Shapiro- Wilk test and followed by Kruskal-Wallis non-parametric analysis of variance

(a)



(b)

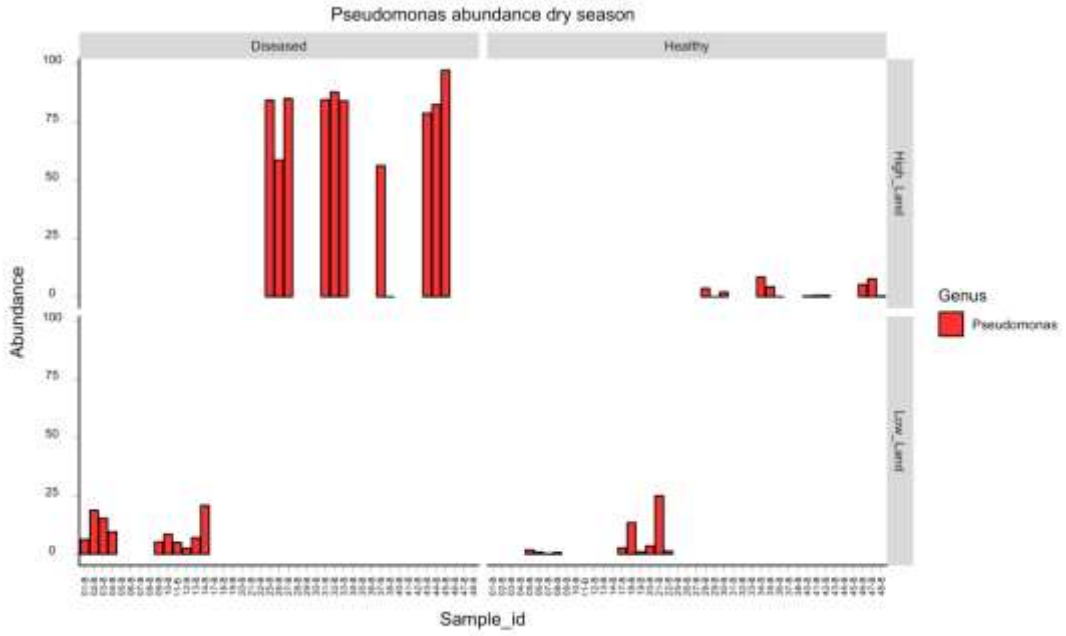


Figure 2. 3 ***Pseudomonas*** **genus abundance level in each sample arranged by symptomatology (Diseased and Healthy) and sampling location (Highland and Lowland).**

(a) Samples from the wet season, (**b**) samples from the dry season.

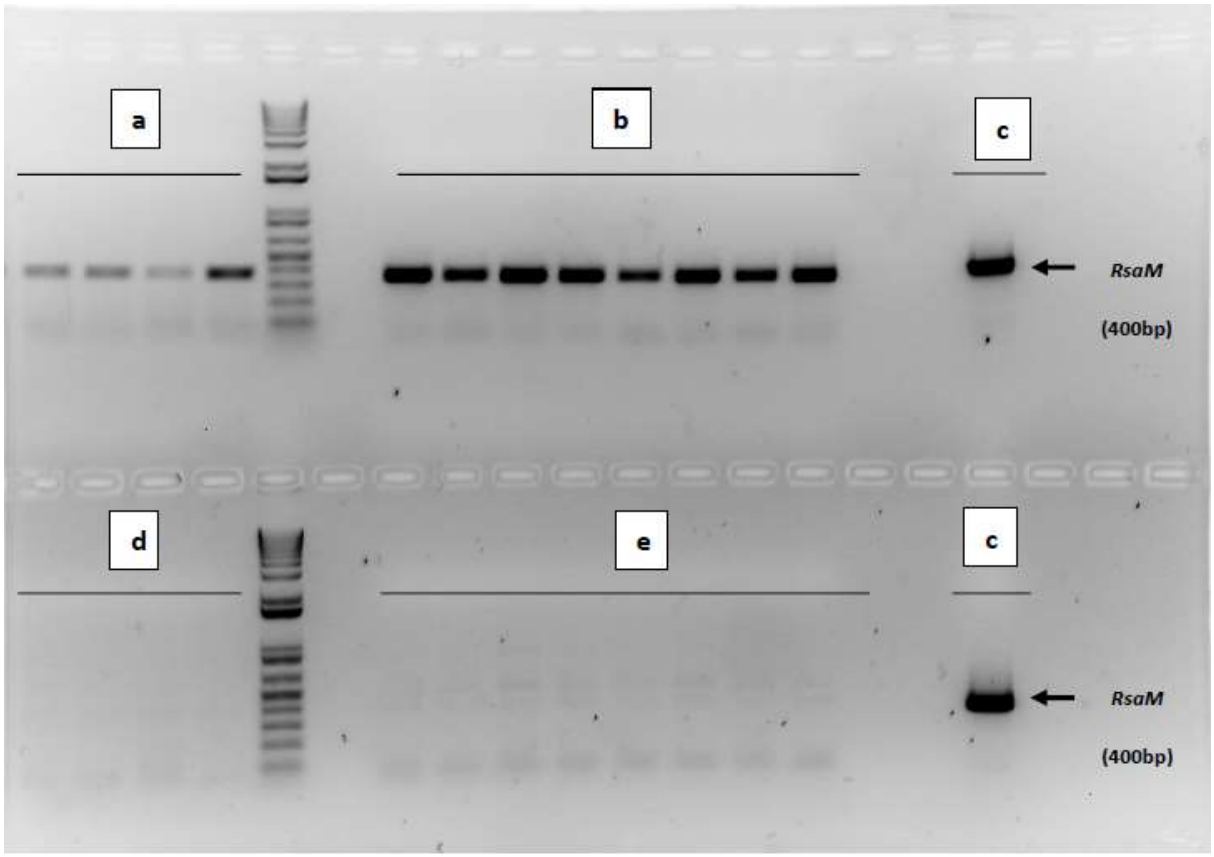


Figure 2. 4 Validation of the presence of *P. fuscovaginae* in the DNA samples extracted from symptomatic and asymptomatic samples in the highland.

The internal sequence of the *rsaM* gene was amplified on symptomatic samples from wet season (**a**), dry season (**b**) and on asymptomatic samples from wet season (**d**) and dry season (**e**). The genomic DNA of *P. fuscovaginae* was used as a control (**c**).

2.3.3. Total bacterial community in asymptomatic and symptomatic rice plants

Having determined that *P. fuscovaginae* was significantly abundant in rice plants displaying sheath rot symptoms in the highland of Burundi, it was then of interest to establish the differences of the total bacterial community among symptomatic and asymptomatic rice plants collected from the same rice fields. The Shannon index was used to determine the alpha diversity (richness) and the observed number of taxa (evenness) to calculate the absolute abundance of species (**Figure 2.5a,b**). Significant differences (p-value <0.001) in Shannon alpha diversity were observed between the symptomatic and asymptomatic samples during the dry season in the highland, while in the other conditions no differences in the

number of different species were detected between symptomatic and asymptomatic samples (**Figure 2.5a**).

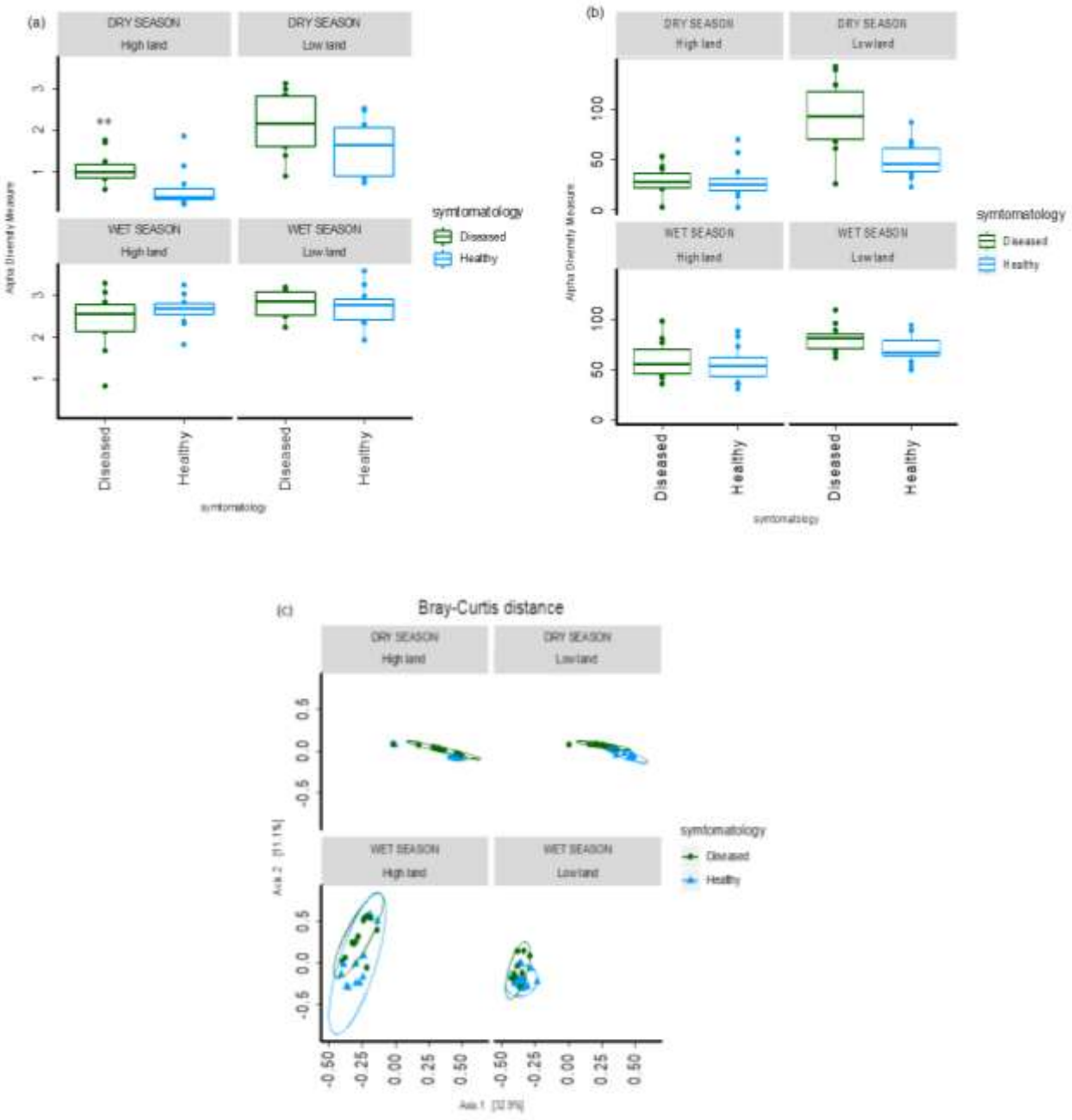


Figure 2. 5 Diversity at community level by determining abundance, richness and correlation analysis according to the symptomology of the samples, the locations (highland and lowland) and the season of the sampling.

(**a**): Alpha diversity by using Shannon index; (**b**): estimation of the observed number of taxa. Box plot depict medians (central horizontal lines), the inter-quartile ranges (boxes), 95% confidance intervals (whiskers) and outliers (black dots). Asterisks indicate significant differences between two group of samples (**p-value< 0.001). Statistical analyses were calculated based on Shapiro -Wilk test and followed by Mann- Whitney-Wilcoxon test **(c):** Principal component analysis (PcoA) of symptomatic (diseased) and asymptomatic (healthy) bacterial communities; green dots represent the symptomatic samples and blue triangle the asymptomatic. This

analysis was performed with preselected bacterial OTUs by random forest analysis using vegan package in R. Statistical results of beta diversity are calculated using adonis function in R

A total of 191 and 241 genera were detected among samples from the wet and dry seasons respectively. The total bacterial community composition according to the relative abundance at bacterial genus level has been determined (**Figure 2.6a,b** and **Figure 2.8a,b**). During the wet season (**Figure 2.6a**), the most predominant genera (>1%) among the symptomatic samples from the highland were *Pseudomonas* 21%, *Stenotrophomonas* 14 %, *Pantoea* 4%, *Comamonas* 5% and *Chryseobacterium* 4 % and among the asymptomatic samples from the same location *Pseudomonas* 13%, *Herbaspirillum* 10%, *Comamonas* 9%, *Bacillus* 8%, *Chryseobacterium* 6%, *and Pantoea* 5%. On the other hand, among the symptomatic samples from the lowland, *Methylobacterium* 13%, *Burkholderia* 10%, *Rhizobium* 8%, *Pseudomonas* 7%, and *Novosphingobium* 7 % were the most abundant whereas among the asymptomatic plants, *Methylobacterium* 20% and *Sphingomonas* 8%, were the two most enriched taxa.

In the dry season (**Figure 2.6b**), the most predominant genera (>1%) across the symptomatic samples from the highland were *Pseudomonas* 81%, *Pantoea* 5%, *Delftia* 3% and *Herbaspirillum* 3% and among the asymptomatic samples from the highland were *Methylobacterium* 41%, *Sphingomonas* 10% and *Pantoea* 9%. In the symptomatic samples from the lowland, *Pantoea* 41%, *Stenotrophomonas* 10% and *Pseudomona*s 10% were the dominant genera and among the asymptomatic plants *Sphingomonas* 22%, *Methylobacterium* 18%, *Pantoea* 13 % and *Chryseobacterium* 13 % and *Rhizobium* 3% were the most relatively abundant ones. In summary, it is clearly evident the very high enrichment of *Pseudomonas* reads in symptomatic samples in highland.

In summary, the genera distribution differs greatly between symptomatic and asymptomatic from the dry season, both in the highland or lowland; it cannot be excluded that the presence at high abundance of the pathogen and the growing season could have an effect on these

differences. No evident differences on the other hand were detected between symptomatic and asymptomatic in the wet season. Interestingly, in asymptomatic samples from the two locations, especially during the dry season, *Methylobacterium* and *Sphingomonas* were found at very high abundance; these two genera are known to be a fundamental part of the phyllospheric microbiome of rice plants (Delmotte et al., 2009; Grady et al., 2019) and could possibly play a role in protecting the plant from biotic stresses.

Beta diversity analysis based on Bray Curtis distance was performed to compare the microbial compositions of different samples (**Figure 2.5c**). There is no defined cluster between asymptomatic and symptomatic samples from wet season in the highland and lowland, as all the samples clustered together, revealing a similar community structure. In the dry season however, the symptomatic and asymptomatic samples are distinctly clustered with a high degree of correlation, showing a different taxa composition. This result is in line with the genus composition described above.

The comparison between symptomatic and asymptomatic samples has been established by differential analysis in order to reveal which were the bacterial taxa significantly enriched in each group of samples (**Figure 2.7**). The comparison was only for the samples from highland as the data showed the prevalence of *P. fuscovaginae* in these samples. Notably, neither in the dry season nor in the wet season there was a taxon that was significantly enriched in the asymptomatic samples compared to symptomatic ones. On the other hand, a few taxa were enriched in the symptomatic samples, among them *Pseudomonas* as already mentioned above (**Figure 2.6a,b**). In particular *Stenotrophomonas* and *Pseudomonas* were more abundant in the symptomatic samples both in the wet and dry season, while *Janthinobacterium*, *Delftia*, *Comamonas*, *Herbaspirillum* and *Pantoea* were more abundant in the symptomatic samples in the wet season. This result confirms again that the *P. fuscovaginae* was significantly more abundant in the symptomatic samples both from the dry and the wet season and that the

microbiome is not excessively modified by the presence of the pathogen. Regardless that this comparison does not show genera that are enriched in asymptomatic samples, the bacterial composition showed that *Methylobacterium*, *Sphingomonas, Sphingobacterium* and *Pantoea* were among the important genera present and relatively abundant in asymptomatic samples (**Table 2.1; Figure 2.6a,b; Figure 2.8a, b**).

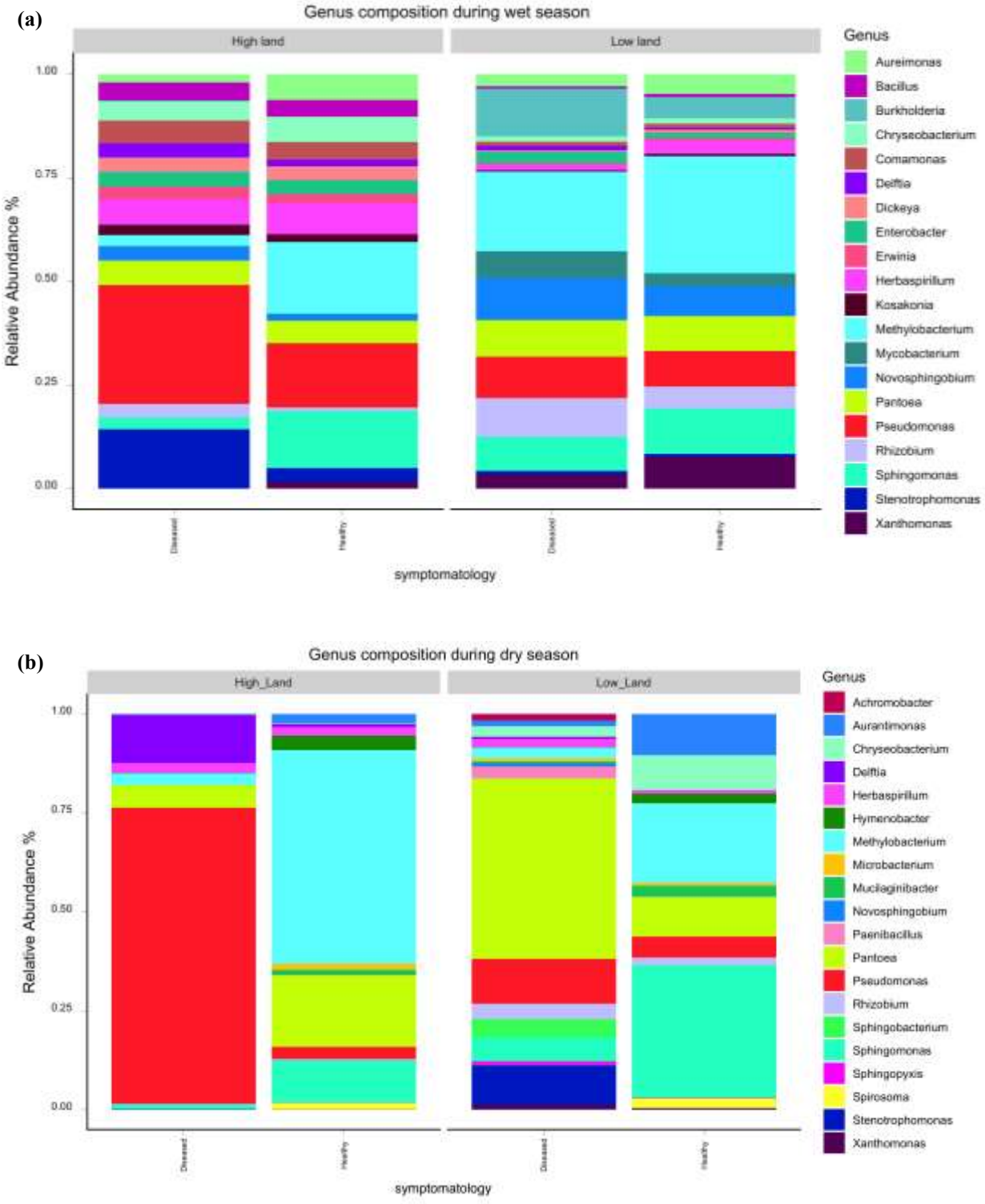


Figure 2. 6 Relative abundance of the most predominant bacterial genera according to symptomatology, sampling location and sampling season.

(**a**) Genus distribution of the predominant OTUs in the wet season; (**b**) Genus distribution of the predominant OTUs in the dry season. Each bar coloured represents a different bacterial genus

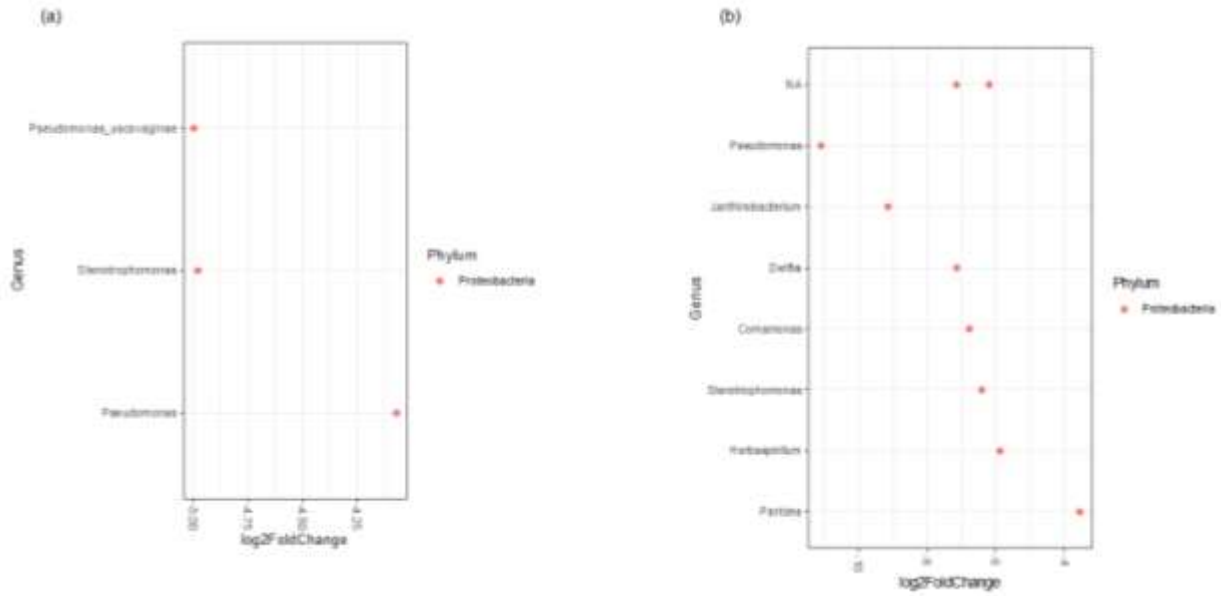
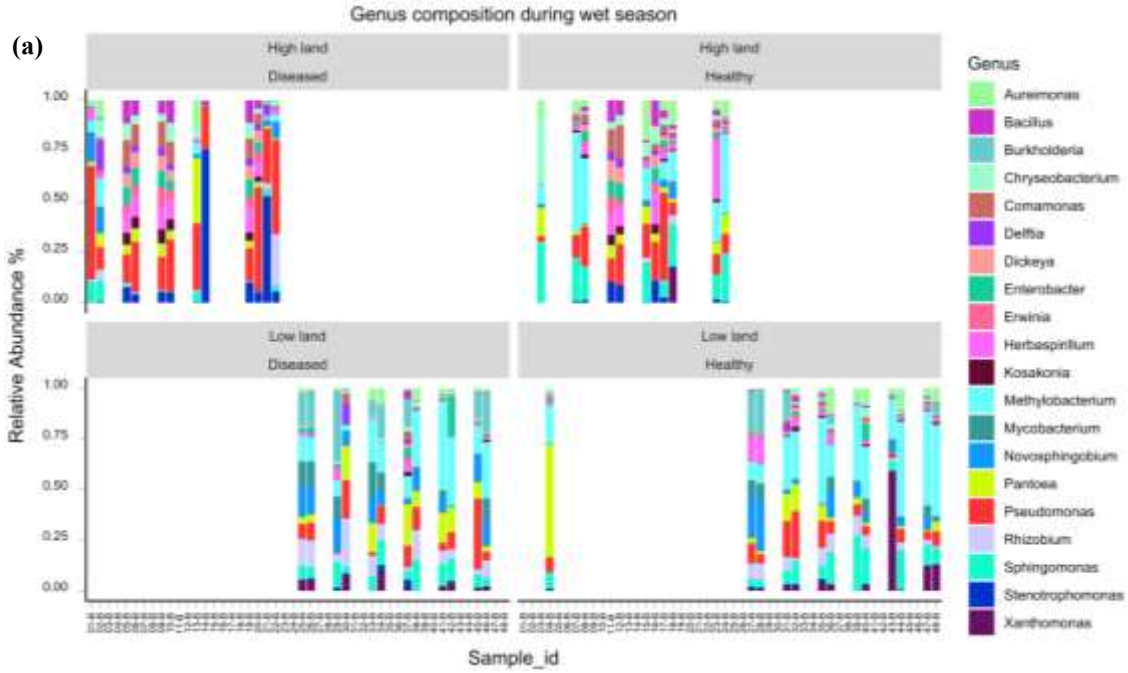


Figure 2. 7 Differential representations of OTUs between asymptomatic and symptomatic samples from both seasons (a) wet and (b) dry in the highland.

Differential abundance of OTUs between the two group of samples tested was assessed by using the R package DESeq2 in conjunction with the Phyloseq package. Taxa are represented as dots in the graph of fold change. Positive values indicate higher representation in symptomatic samples and negative value in asymptomatic samples. Samples with a p-value less than 0.001 and mean representation over all samples higher than 1 are shown.



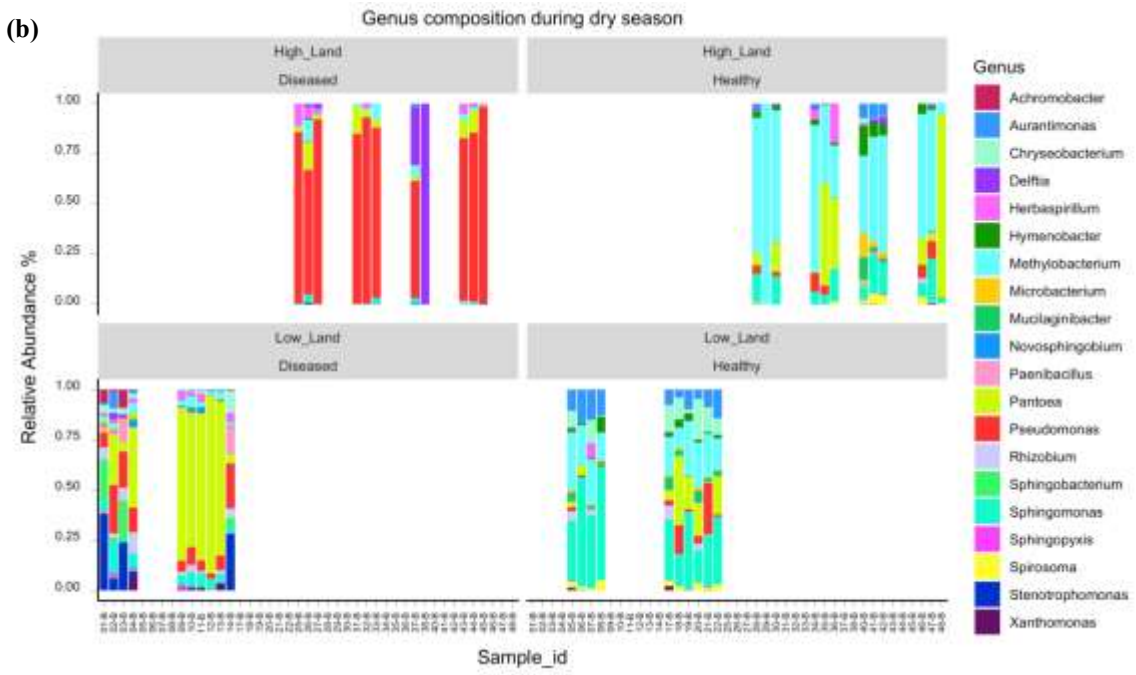


Figure 2. 8 Bacterial genus abundance represented samples by samples from wet season (a) and dry season (b) grouped according to the symptomatology (Diseased and Healthy) and sampling location (Highland and Lowland).

(Cut off >1%; Removed taxa with 0 counts; Removed undefined taxa).

2.3.4. Presence of fungal pathogens in rice sheath rot samples

It was also of interest to determine the fungi present in the microbiome since a few studies have reported the presence of *Sarocladium oryzae* associated to the sheath rot disease. From the same samples used for the bacterial community investigation, the fungal community was also determined. The comparison between symptomatic and asymptomatic samples showed that the *Sarocladium* genus was significantly (p-value <0.001) more present in lowland in symptomatic samples during the wet season (**Figure 2.9a, and Figure 2.10a,b**) where the relative abundance was approximately 18% in symptomatic samples against 2.4% in asymptomatic samples. On the other hand, during the dry season there was no difference in the relative abundance of *Sarocladium* between symptomatic and asymptomatic samples from lowland (1.11% in symptomatic samples against 0.62% in asymptomatic samples). In the samples from highland during the wet season, *Saraclodium* was present at 1.19 % and 0 % in

symptomatic and asymptomatic samples respectively and during the dry season in highland the percentage of *Sarocladium* in symptomatic and asymptomatic samples was at 0.54% and 0.55 %. Interestingly the reads taxonomically associated to *Saraclodium* genus had a very high similarity (>99%) with *Saraclodium oryzae.* It was concluded that samples showing sheath rot symptoms in the lowland of Burundi contained high relative abundance of *S. oryzae* especially in the wet season. In contrast, in highland *Sarocladium* was not significantly present, instead *P. fuscovaginae* was significantly present in symptomatic samples (see above).

2.3.5. Total fungal community in asymptomatic and symptomatic rice sheath rot samples

After determining the presence/abundance of *S. oryzae* in the samples, the total fungal community was determined. A total of 202 and 251 genera were detected among samples from the wet and dry seasons respectively, after the removal of the unidentified reads. The most predominant genera (>1%) across all the asymptomatic and symptomatic samples from the wet (2017) and dry (2018) seasons both in lowland and highland are shown (**Figure 2.9c**,**d)**.

In the wet season, some differences in the fungal community composition were detected comparing asymptomatic and symptomatic plants. In the symptomatic or asymptomatic samples collected in the highland during the wet season the following genera were respectively the most abundant: *Alternaria* (5.20% and 2.98%), *Bipolaris* (25.95% and 0.28%), *Bullera* (1.20% and 4.03%), *and Cladosporium* (25.03% and 36.62 %), *Gibberella* (recently renamed *Fusarium*) (10.69% and 0.15%), *Monographella* (5.92% and 0.27 %) and *Saitozyma* (4.95% and 17.53%). On the other hand in lowland, *Sarocladium* (18% and 2.4%), *Alternaria* (7.57% and 8.73%), *Bipolaris* (25.02% and 3.14%), *Bullera* (16.85% and 22.41%), *Cladosporium* (14.76% and 31.22%), and *Tilletia* (6.87% and 8.60%) were the

predominant ones. In summary, the fungal community composition was not extensively changed by the presence of *S. oryzae* (**Figure 2.11a**). During the dry season, symptomatic and asymptomatic samples from the highland or lowland had no significative differences. The predominant genera were; *Alternaria, Bipolaris, Bullera, Cladosporium, Moesziomyces and Saitozyma*, all of them being present in very similar abundance (**Table 2.2; Figure 2.11b**).

In summary, the most significant difference between symptomatic and asymptomatic samples from highland during the wet season was *Bipolaris* and *Gibberella* (or *Fusarium*), significantly more abundant in the diseased samples. As mentioned above, among the symptomatic and asymptomatic samples from lowland during the wet season the most significative difference was the higher abundance of *Sarocladium* in the samples showing the sheath rot symptoms. Across all samples from the two seasons and symptomatology, the presence of *Cladosporium* was notable. Overall, the fungal community composition during the dry season do not differ greatly neither comparing the samples from highland and lowland or asymptomatic and symptomatic samples from the same location. The low abundance of *S. oryzae* in lowland during the dry season could be due to the resistance of the rice plant varieties or due to the rice growing season/condition.

Table 2. 2 Fungal genus abundance (%) in the symptomatic or asymptomatic samples respectively from the highland and lowland during the dry season that had a high relative abundance

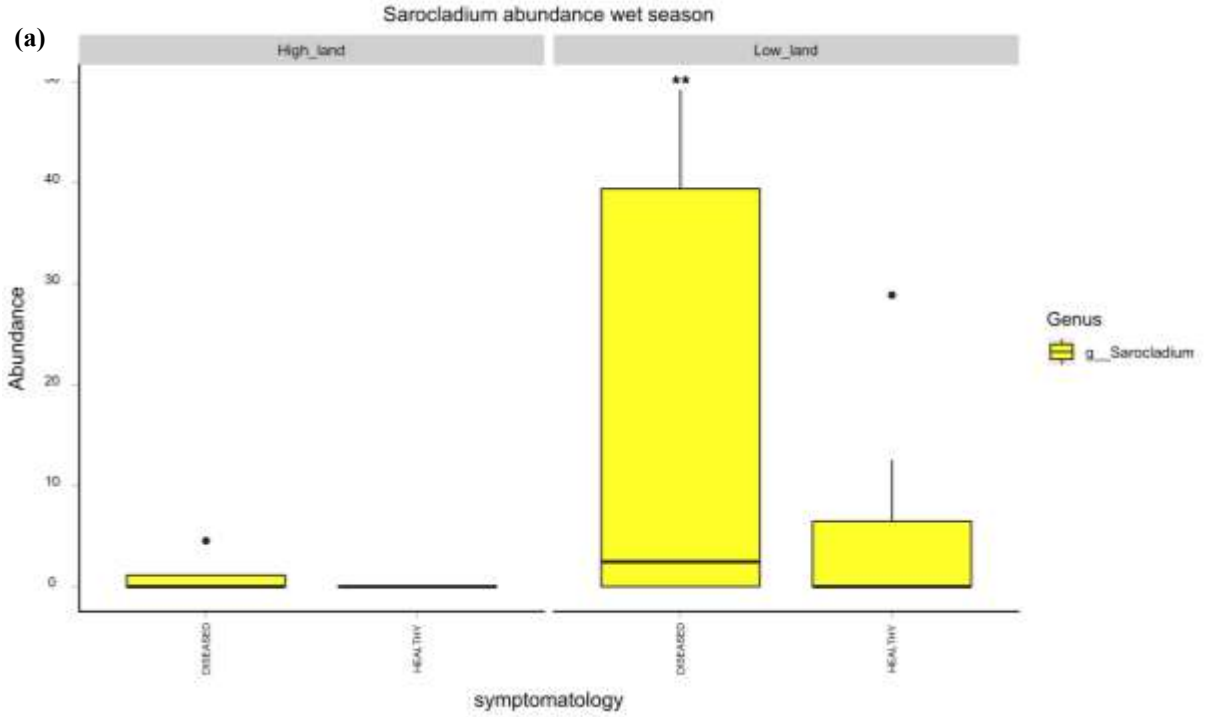
(a)

Genus	First sampling, wet season			
	Highland		Lowland	
	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
Alternaria	5.20	2.98	7.57	8.73
Bipolaris	25.95	0.28	25.02	3.14
Bullera	1.20	4.03	16.85	22.41
Cladosporium	25.03	36.62	14.76	31.22
Gibberella	10.69	0.15	0.03	18.95
Monographella	5.92	0.27	0.00	0.17
Saitozyma	4.95	17.53	0.56	9.28
Sarocladium	1.19	0.00	18.00	2.40
Tilletia	0.00	0.06	6.87	8.60

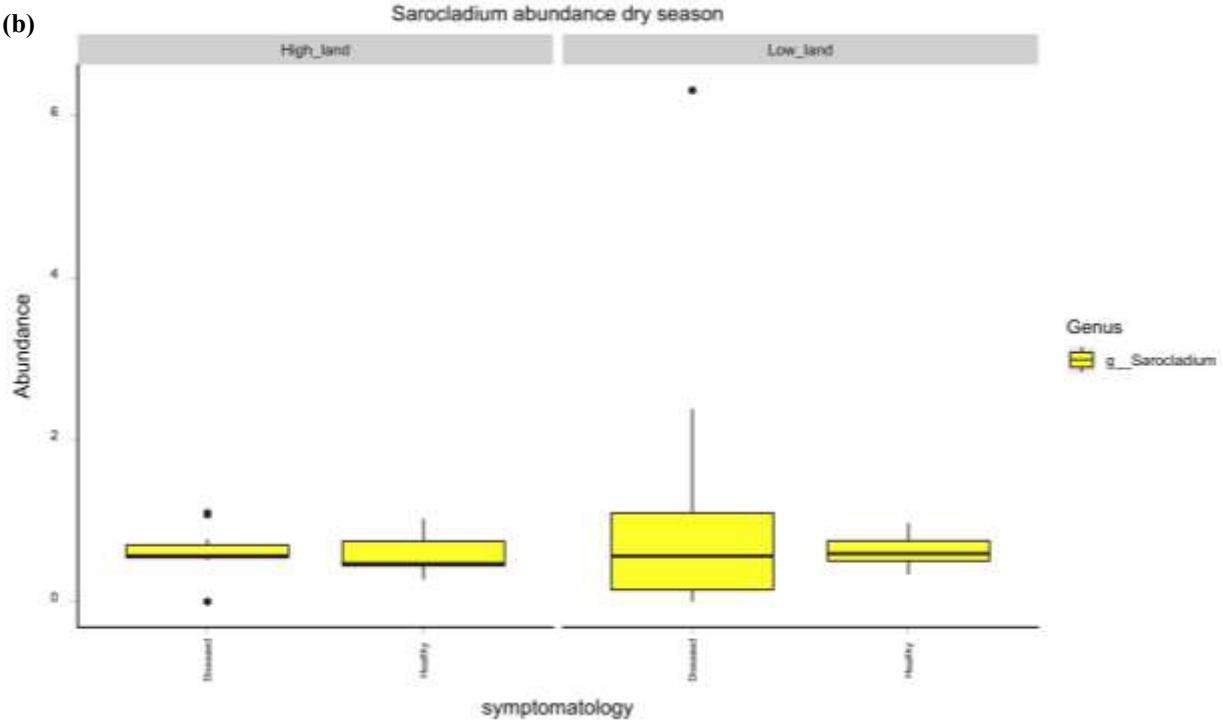
(b)

Genus	Second sampling, dry season			
	Highland		Lowland	
	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
Alternaria	6.12	6.56	5.47	6.48
Bipolaris	18.27	16.59	15.87	19.34
Bullera	2.26	2.38	1.88	2.49
Cladosporium	13.14	14.99	11.28	10.70
Fusarium	0.95	0.88	3.74	0.94
Gibberella	0.26	0.03	3.76	0.17
Moesziomyces	26.32	24.14	27.38	30.91
Saitozyma	4.54	5.52	3.41	3.88
Sarocladium	0.54	0.55	1.11	0.62

(a)


Sarocladium abundance wet season
High_land
Low_land
Abundance
40
30
20
10
0
**
DISEASED
HEALTHY
DISEASED
HEALTHY
symptomatology
Genus
g__Sarocladium

(b)


Sarocladium abundance dry season
High_land
Low_land
Abundance
6
4
2
0
Diseased
Healthy
Diseased
Healthy
symptomatology
Genus
g__Sarocladium

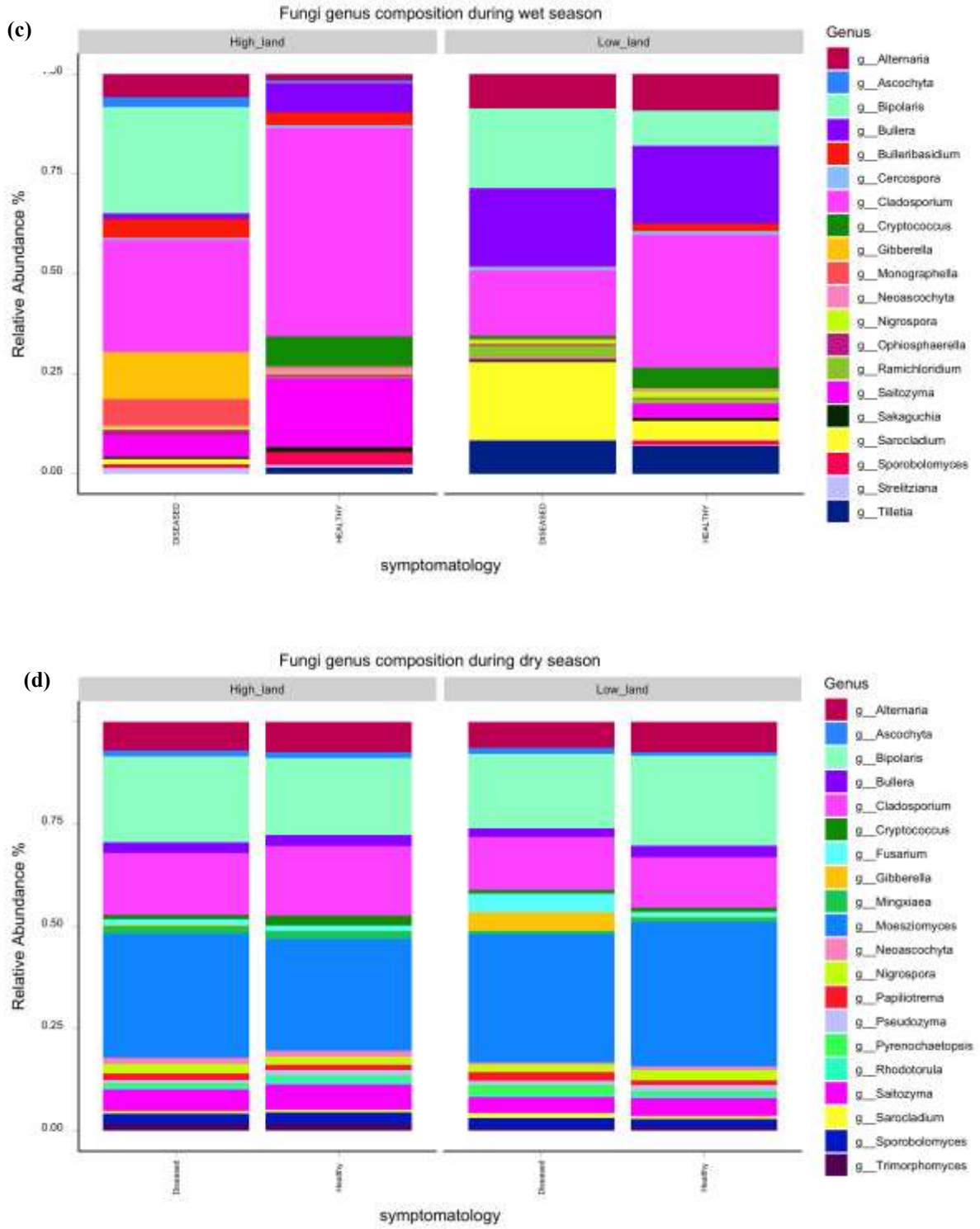


Figure 2. 9 *Sarocladium* genus abundance and comparison of fungal community composition according to the symptomology, the locations (highland and lowland) and the season of rice growing.

Sarocladium abundance in symptomatic and asymptomatic samples from the wet season **(a)** and dry season **(b)**. Asterisks indicate significant differences between two group of samples (**P<0.005). Statistical analyses were calculated based on Shapiro -Wilk test and followed by Mann- Whitney-Wilcoxon test. Comparison of fungal genera composition between symptomatic and asymptomatic samples from the wet season **(c)** and **(d)** dry season. The samples are grouped according to the symptomology (symptomatic= diseased, asymptomatic= healthy) and each bar coloured represents a different fungal genus.

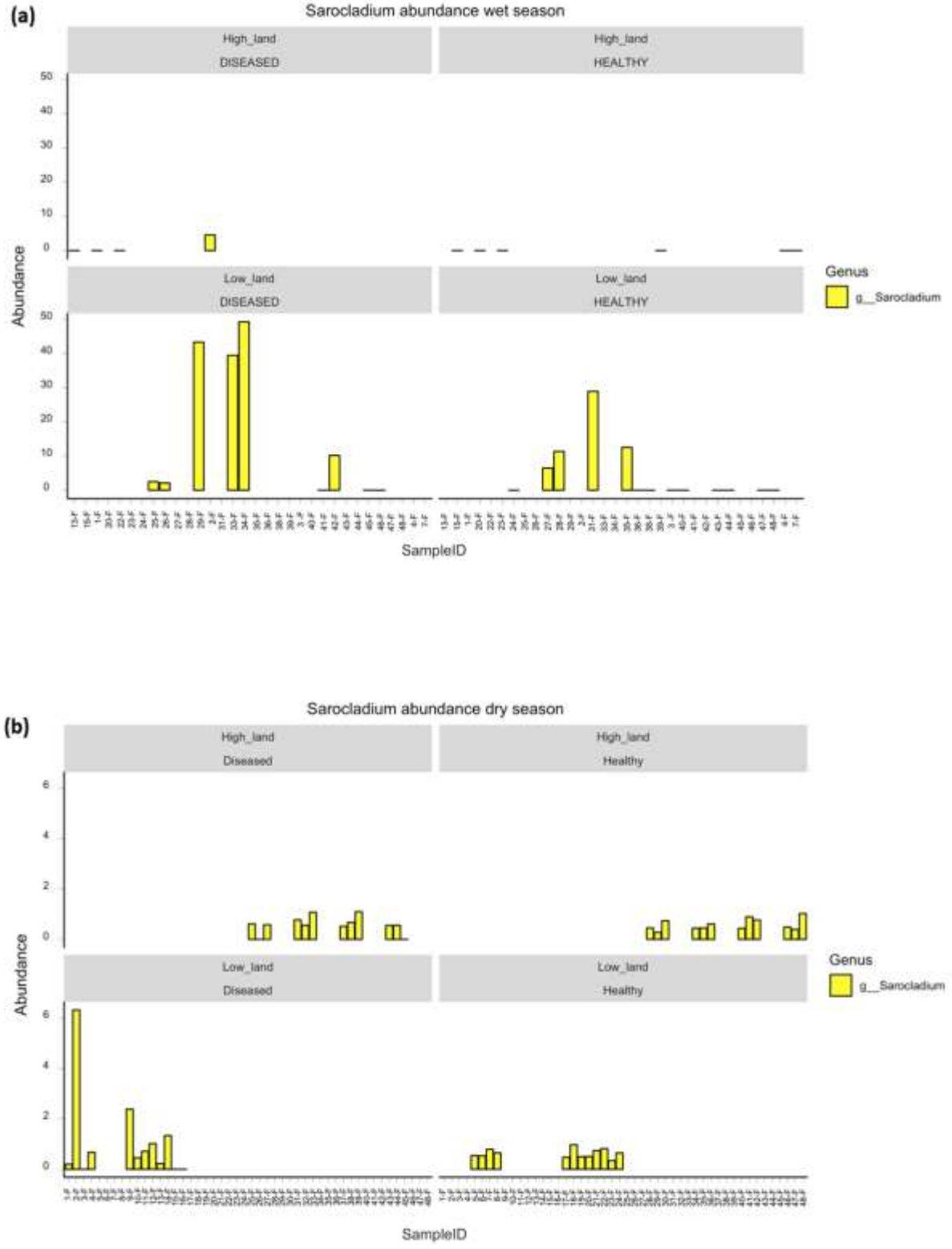


Figure 2. 10 *Sarocladium* genus abundance level in each sample arranged by symptomatology

(Diseased and Healthy) and sampling location (Highland and Lowland). **(a)** Samples from the wet season, **(b)** samples from the dry season.

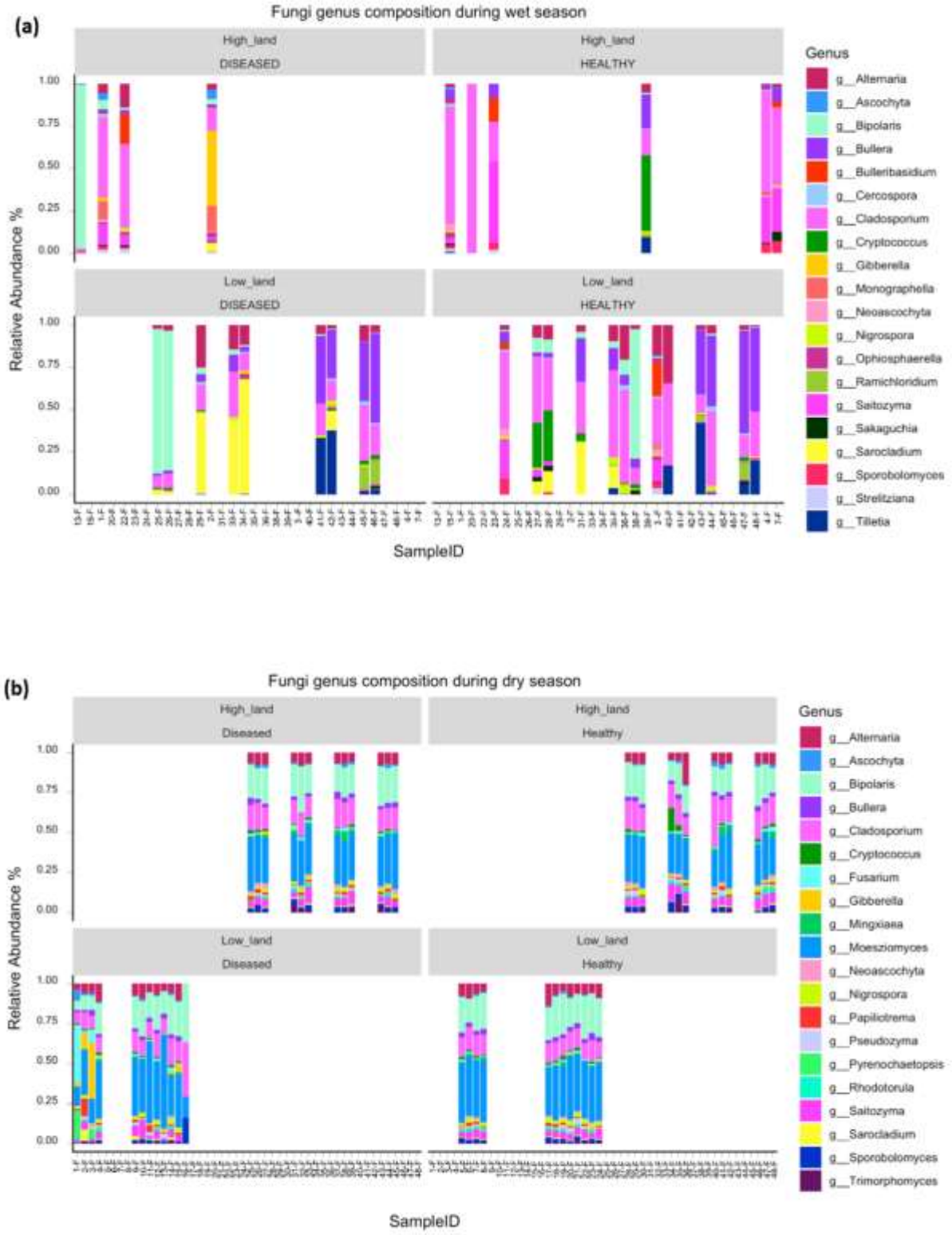


Figure 2. 11 Fungal genus abundance represented by samples.

Wet season **(a)** and dry season **(b)** grouped according to the symptomatology (Diseased and Healthy) and sampling location (Highland and Lowland), Cut off >1%; Removed taxa with 0 counts; Removed undefined taxa

2.4. Discussion

This study is the first report of the microbiology via community sequencing associated with rice sheath rot; the aim was to shed light on the pathogen(s) causing rice sheath rot. Pathobiome studies of rice sheath rot infected plant material from Burundi in two seasons (wet 2017 and dry 2018) revealed that the *Pseudomonas* genus is predominant in all symptomatic samples in highland. The *Pseudomonas* OTUs very likely belong to *P. fuscovaginae* which has been previously identified as a causative agent of rice sheath rot. In lowland however, especially in the wet season, *Pseudomonas* was not significantly present whereas the fungus *S. oryzae* was significantly enriched in the samples displaying the symptoms of sheath rot. Different pathogens therefore independently cause similar sheath rot symptoms depending on the rice growing location and environmental conditions. Pathobiome and microbiome determination is consequently a valid approach to determine the pathogenic agents in plant disease.

P. fuscovaginae has been reported to be the causal agent of sheath rot in many rice growing countries (CABI, 2018) and these studies have clearly associated this pathogen with symptomatic rice samples of sheath rot symptoms. *P. fuscovaginae* has also been reported to be responsible for rice sheath rot in cold and humid tropical highlands in Japan (MIYAJIMA et al., 1983) Burundi (Duveiller et al., 1988) Madagascar (Rott, 1989) and Nepal (Sharma et al., 1997). This study further confirmed that *P. fuscovaginae* is involved with sheath rot at high altitude with high humidity levels. This evidence was strongly supported by *Pseudomonas* reads displaying the highest identity to *P. fuscovaginae* dramatically increasing in symptomatic samples. In addition, a PCR study using primers of specific gene of *P. fuscovaginae* resulted in a clear amplicon only using symptomatic DNA templates. Symptomatic and asymptomatic samples displayed some significant differences in the bacteria present in the microbiome; it is currently unknown whether these differences play a

role in rice sheath rot virulence or in disease resistance. A shift in microbial community can occur when a pathogen arrives or establishes itself to the host (Gomes et al., 2019), some microbes appear or become more abundant while other even disappear in the plant niche. It cannot be excluded that the pathogen can cooperate with members of the microbiome which would result in better growth of the pathogen and this leads to a more aggressive disease (Lamichhane & Venturi, 2015; Rubio-Portillo et al., 2018).

S. oryzae has also been associated to sheath rot in some countries (Hittalmani et al., 2016; Lanoiselet et al., 2012; Naeimi et al., 2003); genome sequencing of *S. oryzae* identified putative loci that could be involved in causing necrosis on host plants (Hittalmani et al., 2016; Sakthivel et al., 2002) and in vivo, *S. oryzae* infected rice plants displayed the symptoms of sheath rot after 30 days (Lanoiselet et al., 2012). The fungal community studies clearly evidenced that *S. oryzae* is abundantly present in sheath rot rice samples only from lowland where *P. fuscovaginae* is not abundantly present. Other fungal genera are also present at high relative abundance, some of them contain known plant pathogenic species like *Bipoloris* (Manamgoda et al., 2014) and *Fusarium* (Roncero et al., 2003). It cannot therefore be excluded that sheath rot symptoms can also be due to a complex disease (Lamichhane & Venturi, 2015) involving more than one type of causal agent.

The fungal genus *Cladosporium* was significantly abundant across all the samples from locations, seasons and symptomatology. This genus is known to be cosmopolitan as reviewed by (Bensch et al., 2012) and has been found associated to the phylloplane (Stohr & Dighton, 2004). It is likely that this presence of *Cladosporium* is associated with the phylloplane and surface sterilization of the samples could have reduced the presence of this genus. However, considering this was a rot disease, it was decided best not to sterilize the samples as this could have compromised the microbiome associated with the disease.

This approach has proved useful in pinpointing possible pathogens and sets the way for the isolation and characterization of the microbes possibly involved in the pathosystem. It can also be used in determining the keystone microbes fundamental in the microbial community network of healthy plants (Poudel et al., 2016)

ChapterIII. A bacterial culture collection from rice sheath in Burundi

3.1. Introduction

Each part of the plant (eg. root, stem, leaf and fruit) is associated with a microbial community that all together forms the plant microbiome. Depending on the plant part, the microbial community can vary considerably consisting of only a few species or being very diverse. Microbiome studies have an important role to bring insight on the composition of these communities that form the plant microbiome. The rhizospheric microbiome is the community of microbes closely associated or attached to the roots whereas the endosphere microbiome are the microbes which live inside plants in intercellular spaces mostly originating from the rhizosphere (Edwards et al., 2015). The phyllospheric/epiphytic microbiome is located on the surface aerial parts and lastly, the seed microbiome which corresponds to the vertically transferred microbes (Gopal and Gupta, 2016). The phyllospheric part of plants represents the largest environmental surface habitat area of microbes on earth (Lindow & Brandl, 2003; Peñuelas & Terradas, 2014; Vorholt, 2012), and much of that surface area is due to agriculture of crops (Foley et al., 2011). Phyllosphere microorganisms or phyllospheric microbiome can be beneficial to plants by (i) increasing stress tolerance (Hamilton et al., 2012; Lindow & Leveau, 2002; Redman et al., 2002), (ii) promoting plant growth (iii) having a role in reproduction (Canto & Herrera, 2012; Doty et al., 2009; Taghavi et al., 2009), (iv) protecting plants against aerial danger like foliar pathogens (Lee et al., 2014), and (v) can be involved in the control of flowering phenology (Wagner et al., 2014). Importantly, these microorganisms also play important roles in Earth's biogeochemical cycles by moderating methanol emissions from plants (Barud et al., 2016; Galbally & Kirstine, 2002) and contributing to global nitrogen fixation (Fürnkranz et al., 2008). Despite this importance, knowledge of phyllosphere microbiomes remains relatively modest, especially for agricultural crops (Hacquard & Schadt, 2015; Vorholt, 2012; Weyens et al., 2009). To leverage plant microbiomes to support productivity and resilience of crops to environmental

stresses both above and below ground (Hassani et al., 2018; Lebeis, 2014; Vandenkoornhuyse et al., 2015), it is important to advance the knowledge on phyllosphere microbiome diversity and dynamics.

The major roles of phyllospheric microbiome in healthy plants has been recently reviewed (Stone et al., 2018). Cultivation-independent studies have revealed that few bacterial phyla predominate in the phyllosphere of different plants and that plant factors are involved in shaping these phyllospheric communities; this is the result of a specific adaptations and multipartite relationships among community members and with the host plant as reviewed by Vorholt (2012). The rice plant (*Oryza sativa*), like other plants, has a microbial community showing differences according to the plant compartment (rhizospheric: root and phyllospheric: stem, leaves, sheath that protect the panicles). In the last decade, several studies reported an emerging rice disease that affect the phyllospheric part of the sheath tissue; the disease is called rice sheath rot. This disease has been mainly associated to *P. fuscovaginae* which is a rice seedborne pathogen. Microbiome and pathobiome studies on rice sheath rot have revealed that *P. fuscovaginae* is much more abundantly present in symptomatic rice plant samples with respect to asymptomatic samples (Chapter II). It is possible that asymptomatic rice samples of the same rice variety in the same area/fields possess a phyllospheric microbiome which promotes plant health and helps the plant fight sheath rot pathogen invasion. It was therefore of interest to perform an analysis of the culturable microbiome and to isolate and characterize bacterial isolates from the asymptomatic sheath rice samples analysed in chapter II. The ultimate aim is the isolation and identification of bacterial isolates that can promote pathogen control.

3.2. Material and Methods

3.2.1. Bacterial strains isolation

A collection of bacterial isolates has been performed from asymptomatic samples of rice sheath which were collected in the wet season of 2017 and dry season of 2018 in Burundi as previously indicated in Chapter II. The no surface sterilized samples that were stored at -80ºC, were resupended in PBS solution and serially diluted. The undiluted and the 10^{-2} dilutions were plated on TSA (Tryptic Soy Agar) and incubated at 28 degrees for 2-3 days. The bacteria grown from the undiluted samples were collected en masse for the genomic DNA extraction for 16S rRNA gene amplicon community sequencing. Single colonies from the 10^{-2} dilutions plates were purified.

3.2.2. Bacterial strains identification

Amplification of the 16S rRNA gene was performed by using Fd1 and Rp2 primers set (Weisburg et al., 1991). Colony PCR was performed after boiling (10' at 95°C) a colony suspension in 50 uL of sterile H_2O. PCR amplification was performed using GoTaq® G2 Enzyme (Promega) according to supplier's instructions and 5 µL of template in a final volume of 50 µL was used for the PCR reaction. Reactions were performed in a T100™ Thermal Cycler (Biorad Laboratories Inc., Hercules, CA, USA) with the following thermal protocol: DNA denaturation for 5 min at 95°C, amplification (30 cycles) at 95°C for 30 s, 54°C for 30 s, and 72°C for 1min30 s, extension 7min at 72°C. Agarose gel electrophoresis was run for the PCR products and DNA from agarose was purified by using EuroGold gel extraction kit (Euroclone SpA, Italy) according to the instructions of the manufacturer. The purified PCR DNA products (16S rRNA gene) were then sequenced with the 907F universal primer by Eurofins Genomics (Germany). Identification of the bacterial isolates was obtained by BLAST analysis at NCBI (http://www.ncbi.nlm.nih.gov).

3.2.3. *In vitro* phenotypic assays

The bacterial isolates were tested for several *in vitro* phenotypes. Assays for antibacterial activity against the rice bacterial plant pathogen *P. fuscovaginae* were performed; isolates were checked for lipolytic activity by streaking the bacterial isolates on 6 times diluted TSA medium amended with 1% tributyrin (Smeltzer, Hart, & Iandolo, 1992); for the proteolytic activity was tested by streaking the bacterial isolates on 6 times diluted TSA medium amended with 2% of powder milk (Huber et al. 2001); for exopolysaccharides (EPS) production was estimated by streaking the bacterial isolates on Yeast Extract Mannitol medium (Zlosnik et al., 2008); the indole acetic acid (IAA) production was tested by streaking the bacterial isolates on nitrocellulose membranes placed on TSA medium plates containing 5mM tryptophan, incubating them for 24h at 28 degree and then removing the nitrocellulose membranes from TSA to place them onto a saturated Whatman paper that was previously treated with the Salkowski reagent (Bric et al. 1991); the IAA production resulted in the formation of a red/purple halo around the streak line growth of the bacterial isolates. Acyl homoserine lactones (AHLs) signal molecules produced by Gram negative bacteria, were analysed by T-streak technique (Steindler & Venturi, 2007) using the biosensor *C. violaceum* CV026 after incubation for 1-2 days. Motility was checked on M8 medium plates with 0.3% (swimming) or 0.5% (swarming) agar (Kohler et al., 2000).

3.2.4. Culturable microbiome analysis

3.2.4.1. Bacterial genomic extraction

Bacterial genomic DNA extraction was performed from the culturable bacteria isolated from rice plant samples. The undiluted suspensions from 10 asymptomatic rice plant samples (see above) were plated on TSA medium and incubated at 28 degrees for 3 days. The bacteria grown were collected in 2 ml of PBS and used for genomic DNA extraction according to the Bacterial Genomic DNA extraction Kit instructions (QIAGEN, Hilden, D). The genomic

DNAs extracted from 10 samples, 6 from the wet season and 4 from the dry season, were used to perform the 16S rRNA gene amplicon library as described below.

3.2.4.2. 16S rRNA gene amplicon library preparation.

The 16S rRNA gene amplicon library was performed by using the following primers: 16S Illumina library FW 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Illumina library RW 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAAA TCC. A mix of 2,5 µl (5ng/µl) microbial DNA, 5µL (1µM) of each primers and 12,5 µl of KAPA HiFi HotStart ReadyMix in final volume of 25 µl was used for the first PCR to amplify the V3 and V4 regions of 16S rRNA gene by following this program: initial denaturation of 95°C for 3 min followed by 25 cycles of 95°C for 30 sec, 55°C for 30sec and 72°C for 30 sec; and a final 5min of extension at 72°C and hold at 4°C.

The PCR products were cleaned as described by Illumina protocol using AMPure XP beads, and a second PCR for adding the Illumina index was set. A mix of 5µl (PCR products), 5 µl of each Nextera XT Index Primer (N7xx and S5xx), 25 µl of 2xKAPA HiFi HotStart ReadyMix and 10 µl PCR Grade water in final volume of 50 µl and the following program was used for the second PCR, initial denaturation of 95°C for 3 min, followed by 8 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; and a final 5min of extension at 72°C and hold at 4°C. The second cleaning was done as recommended in the protocol always using AMPure XP beads.

The second cleaned amplification products of 16S rRNA gene amplicon libraries were quantified using the Qubit Kit (Invitrogen) and the quality (integrity and presence of a unique band) was confirmed by Bioanalyzer equipment (Agilent Inc., Santa Clara, CA, USA). After quantification and normalization all PCR products were diluted to 4nM and aliquots of 5µl of

diluted DNA from each library were pooled together and sent to sequence by Illumina Miseq sequencing platform.

3.2.4.3. Sequence data processing

FASTQ files were analysed using DADA2 v1.4.0 (Callahan et al., 2016) adapting the methods from the DADA2 Pipeline Tutorial (1.4). R version 3.5.2 was used for all analyses. Briefly, prior to analysis in DADA2, samples were demultiplexed using the QIIME 1.9.1 split_libraries_fastq.py script. The demultiplexed files were then used as the input for DADA2. Cutadapt 1.15 was used for adapter removal and quality filtering. Later quality profiles of the reads were analysed using the DADA2 function; plot Quality Profile, to determine positions at which read quality greatly decrease. Reads were then filtered and trimmed at the identified positions (truncLen=190) using the filterAndTrim function with standard parameters (maxN=0, truncQ=2, and maxEE=2). Dereplication was performed combining all identical sequencing reads into "unique sequences" with a corresponding "abundance" equal to the number of reads of that unique sequence. DADA2's error model automatically filters out singletons, removing them before the subsequent sample inference step. Sample inference was performed using the inferred error model and chimeric sequences were removed using the removeBimeraDenovo function. The Greengenes (GG) database (McDonald et al., 2012), giving a final OTU table, was used to assign bacterial taxonomy using the assignTaxonomy function with a 97% sequence similarity.

3.2.4.4. Statistical analysis

The sequence table counts and rarefaction curves were determined on sequence count files generated by the analysis pipeline. The OTU table was rarefied according to the sample with the lowest number of reads, using the Rarefy function of the GUnifrac library. The resulting OTUs were clustered at Genus taxonomic level obtaining a final number of bacterial taxa for the two samplings. Statistical analysis were performed using the vegan package version 2.5-4

(Oksanen et al., 2019) and phyloseq package (McMurdie & Holmes, 2013) in R version 3.5.2 (Team, 2014). Relative abundances of OTUs between samples were calculated.

3.3. Results

3.3.1. Rice samples information

The samples used to perform the analysis of the culturable microbiome and for the isolation of bacteria in order to create a culturable collection of possible *P. fuscovaginae* antagonists, were collected in two different rice growing seasons; the wet season (2017) and the dry season (2018). The collected plant sheath samples were not surface sterilized and were stored in 18% glycerol at -80 °C. Before plating, the samples were then thawed and when necessary diluted in PBS. In total 10 asymptomatic samples were used; 6 samples from the wet season and 4 from the dry season. All samples were from the highland location where the pathogen *P. fuscovaginae* was predominant in symptomatic samples (Chapter II).

3.3.2. Culturable phyllospheric microbiome

It was of interest to compare the total microbiome of asymptomatic samples from highland during the two rice growing seasons (wet 2017 and dry 2018) previously described (Chapter II) with the culturable microbiome detected under laboratory conditions performed on the same samples. In the samples collected in wet season of 2017, 151 taxa were detected in the total microbiome and 108 were detected in the culturable microbiome. In the samples collected during the dry season of 2018, 105 taxa were detected in the total microbiome and 88 taxa were detected in the culturable microbiome. Among the 151 different taxa inferred in the total microbiome from the wet season (Chapter II), 29% of these were found to be culturable under the conditions determined here. Similarly among the 108 taxa identified in the total microbiome from dry season, 31% of these were found to be culturable under laboratory conditions. The number of shared and unique taxa between total and culturable microbiome is shown in the Venn diagram **(Figure 3.1a).** The number of different taxa

observed in each sample and the comparison of the alpha diversity between total and culturable microbiome is shown in **Figure 3.1b**. The difference in the mean value of different taxa observed between the total microbiome and the culturable microbiome is not significant for the asymptomatic samples from the wet season (2017) whereas is significant for the asymptomatic samples from the dry season (2018).

During the wet and dry seasons the most frequent/abundant genera among the asymptomatic samples from the highland were *Herbaspirillum*, *Curtobacterium*, *Enterococcus*, *Methylobacterium*, *Rothia*, *Chryseobacterium, Pantoea*, *Streptococcus*, *Neisseria*, *Microbacterium*, *Sphingomonas* (**Table 3.1**); among them *Microbacterium, Sphingomonas* and *Methylobacterium* have been reported to be part of plant phyllospheric microbiomes (Bertani et al., 2016).

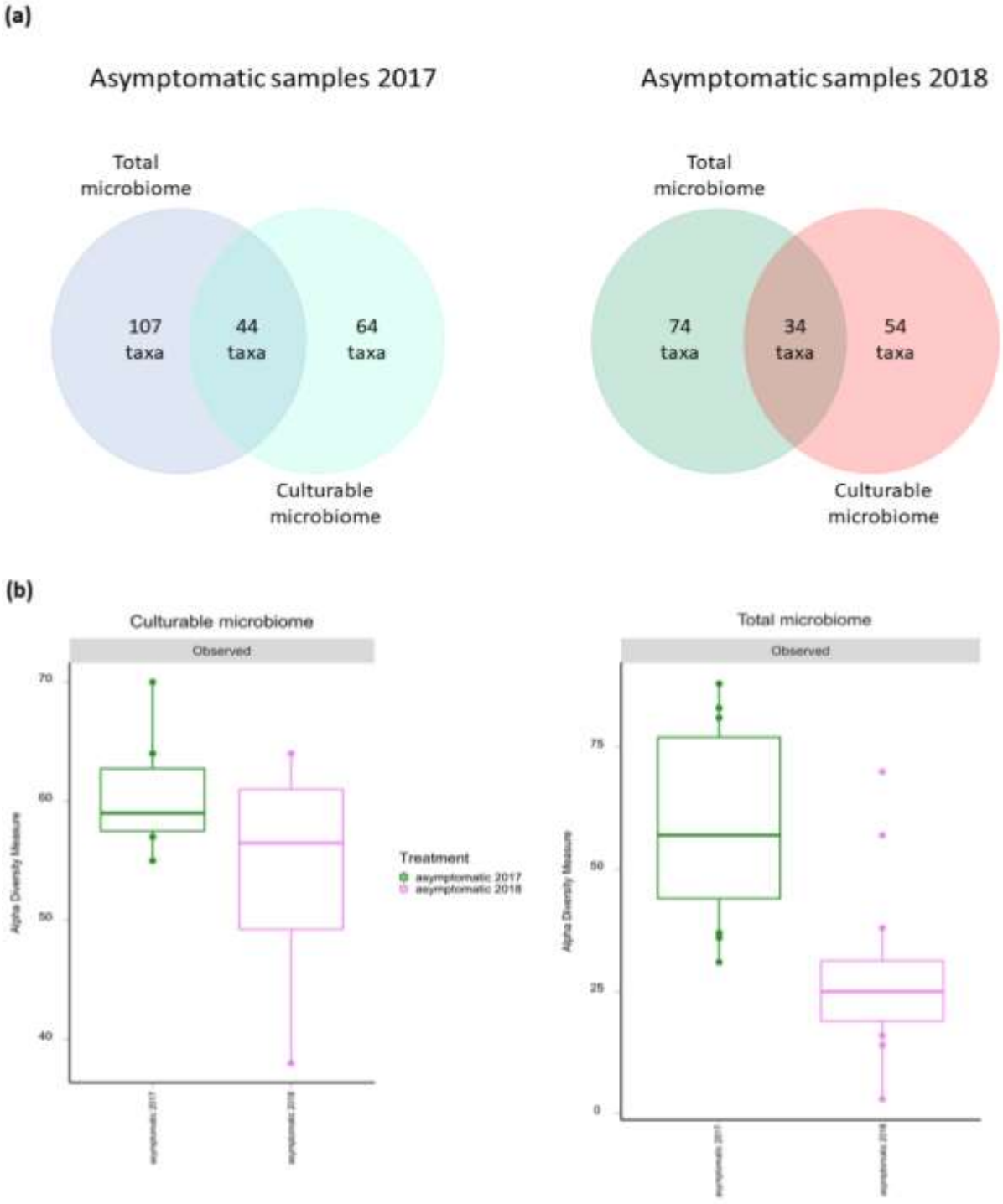


Figure 3. 1 Total microbiome and culturable microbiome.

(a) Venn diagram displaying the number of unique and shared taxa between total and culturable microbiome during the 2017 and 2018 season respectively. **(b)** Alpha diversity of the total microbiome and culturable microbiome for both wet and dry seasons

3.3.3. Bacterial strains isolation and identification from asymptomatic samples

It was also of interest to purify and to isolate the bacteria present in asymptomatic samples since some of these could be involved in pathogen control; 150 pure bacterial colonies were purified and isolated. The 16S rRNA gene amplification and sequencing enabled the classification of 58 bacterial isolates at genus level. The 58 bacterial isolates belonged to 21 genera; among them 16 genera were also identified in the total and/or culturable microbiome study whereas surprisingly 5 were not. This latter result was most likely due to their very low amounts in the samples processed for culturable microbiome study. Among the 58 isolates collected, *Microbacterium*, *Bacillus*, *Sphingomonas* and *Methylobacterium* were the most predominant (**Figure 3.2, Table 3.1).**

Table 3. 1 Genera present in the Total and Culturable microbiome, Bacterial isolates and identification according to 16S rRNA gene

Bacterial isolates present or not in culturable microbiome and total microbiome are listed as genus isolated in the table.

Genus in total microbiome	Genus in culturable microbiome	Genus isolated	Nr of bacterial isolates/genus
-	*g__A17*	-	-
Achromobacter	*g__Achromobacter*	-	-
Acidisoma	-	-	-
Acidovorax	*g__Acidovorax*	*Acidovorax*	1
Acinetobacter	*g__Acinetobacter*	*Acinetobacter*	3
-	*g__Actinomyces*	-	-
Actinomycetospora	-	-	-
Aeromicrobium	-	-	-
Aeromonas	-	-	-
-	*g__Agrobacterium*	-	-
-	*g__Agromyces*	-	-
-	-	*Alcaligenes*	1
-	*g__Alcanivorax*	-	-
-	*g__Alicyclobacillus*	-	-
Alkalibacterium	-	-	-
Alteromonas	-	-	-
-	*g__Ammoniphilus*	-	-
Amnibacterium	-	-	-
Anaerobacillus	-	-	-
-	*g__Anaerovorax*		
Ancylobacter	-	-	-
Aquabacterium	-	-	-
Aquisphaera	-	-	-
Arcicella	-	-	-
Armatimonas	-	-	-
Arthrobacter	*g__Arthrobacter*	-	-
-	*g__Arthrospira*	-	-
-	*g__Asticcacaulis*	-	-
Aurantimonas	-	-	-
Aureimonas	-	*Aureimonas*	1
-	*g__Azohydromonas*	-	-
-	*g__Azorhizobium*	-	-
-	*g__Azospirillum*	-	-
Bacillus	*g__Bacillus*	*Bacillus*	9
Balneimonas	-	-	-
Bdellovibrio	-	-	-
Beijerinckia	-	-	-
Belnapia	-	-	-
Bosea	-	-	-

-	g__Brevibacillus	-	-
-	g__Brevibacterium	-	-
Brevundimonas	g__Brevundimonas	-	-
Burkholderia	-	-	-
-	g__Caldicoprobacter	-	-
-	g__Candidatus Phytoplasma	-	-
Caulobacter	g__Caulobacter	-	-
-	g__Cellvibrio	-	-
Chitinophaga	g__Chitinophaga	-	-
Chryseobacterium	g__Chryseobacterium	Chryseobacterium	2
Citrobacter	-	-	-
Clostridium	g__Clostridium	-	-
-	g__Cohnella	-	-
Comamonas	-	-	-
Conexibacter	g__Conexibacter	-	-
-	g__Coprococcus	-	-
Corynebacterium	g__Corynebacterium	-	-
Croceicoccus	-	-	-
-	g__Cupriavidus	-	-
Curtobacterium	g__Curtobacterium	Curtobacterium	2
Curvibacter	-	-	-
Deinococcus	g__Deinococcus	Deinococcus	1
Delftia	-	-	-
Devosia	g__Devosia	-	
Diaphorobacter	g__Diaphorobacter	-	-
Dickeya	-	-	-
Duganella	-	-	-
Dyadobacter	g__Dyadobacter	-	-
Elizabethkingia	-	-	-
-	g__Emticicia	-	-
Enhydrobacter	-	-	-
Ensifer	-	-	-
Enterobacter	g__Enterobacter	-	-
Enterococcus	g__Enterococcus	-	-
Erwinia	-	-	-
Escherichia/Shigella	-	-	-
Ethanoligenens	-	-	-
Exiguobacterium	-	-	-
Extensimonas	-	-	-
Falsibacillus	-	-	-
Ferruginibacter	-	-	-
Fibrella	-	-	-
Fimbriimonas	g__Fimbriimonas	-	-
-	g__Flavihumibacter	-	-
Flavobacterium	g__Flavobacterium		-
-	g__Fluviicola	-	-
Friedmanniella	-	-	-

Fructobacillus	-	-	-
Gardnerella	-	-	-
Gemella	-	-	-
-	*g__Gemmata*	-	-
Geodermatophilus	-	-	-
-	*g__Gemmatimonas*	-	-
Gibbsiella	-	-	-
-	*g__Glycomyces*	-	-
-	*g__Gracilibacter*	-	-
Haemophilus	-	-	-
Halomonas	-	-	-
Hartmannibacter	-	-	-
Hephaestia	-	-	-
Herbaspirillum	*g__Herbaspirillum*	-	-
Herbiconiux	-	-	-
-	-	*Huakuichenia*	1
-	-	*Humibacter*	1
-	*g__Hydrogenophaga*	-	-
Hymenobacter	-	-	-
	g__Hyphomicrobium	-	-
Janthinobacterium	-	-	-
Jatrophihabitans	-	-	-
-	*g__Kaistobacter*	-	-
-	*g__Kibdelosporangium*	-	-
Kineococcus	-	-	-
Klebsiella	-	-	-
Kocuria	*g__Kocuria*	-	-
Kosakonia	-	-	-
-	*g__Kribbella*	-	-
Labilithrix	-	-	-
Lactobacillus	*g__Lactobacillus*	-	-
Larkinella	*g__Larkinella*	-	-
-	*g__Lautropia*	-	-
-	*g__Leadbetterella*	-	-
Leclercia	-	-	-
Legionella	-	-	-
Leifsonia	-	-	-
-	*g__Lentzea*	-	-
Leucobacter	-	-	-
-	*g__Luteimonas*	-	-
Luteolibacter	*g__Luteolibacter*	-	-
Lysinibacillus	-	-	-
-	*g__Lysobacter*	-	-
-	*g__Magnetospirillum*	-	-
Massilia	*g__Massilia*	*Massilia*	1
Mesorhizobium	*g__Mesorhizobium*	-	-
-	*g__Methylibium*	-	-

Methylobacterium	*g__Methylobacterium*	*Methylobacterium*	5
Methylophilus	-	-	-
-	*g__Methylotenera*	-	-
-	*g__Methyloversatilis*	-	-
Microbacterium	*g__Microbacterium*	*Microbacterium*	12
Micrococcus	*g__Micrococcus*	-	-
Microvirgula	-	-	-
Mitsuaria	-	-	-
Mucilaginibacter	-	-	-
Mumia	-	-	-
Mycetocola	-	-	-
Mycobacterium	-	-	-
Mycoplana	*g__Mycoplana*	-	-
-	-	*Naasia*	1
Nakamurella	-	-	-
Naxibacter	-	-	-
-	*g__Neisseria*	-	-
Neochlamydia	-	-	-
Neorhizobium	-	-	-
-	*g__Niabella*	-	-
-	*g__Niastella*	-	-
Nocardioides	*g__Nocardioides*	-	-
-	*g__Nocardiopsis*	-	-
-	*g__Nonomuraea*	-	-
Novosphingobium	*g__Novosphingobium*	*Novosphingobium*	1
Nubsella	-	-	-
Oceanobacillus	-	-	-
Ochrobactrum	-	-	-
Okibacterium	-	-	-
-	*g__Opitutus*	-	-
Orientia	-	-	-
Paenibacillus	*g__Paenibacillus*	-	-
Pantoea	*g__Pantoea*	*Pantoea*	1
Parachlamydia	-	-	-
Parachlamydia	-	-	-
Paracoccus	*g__Paracoccus*	-	-
Patulibacter	-	-	-
Pedobacter	*g__Pedobacter*	-	-
Pelomonas	-	-	-
Peptoniphilus	-	-	-
Peredibacter	-	-	-
-	*g__Phaeospirillum*	-	-
-	*g__Phenylobacterium*	-	-
-	*g__Phyllobacterium*	-	-
-	*g__Pirellula*	-	-
-	*g__Planctomyces*	-	-
-	*g__planctomycete*	-	-

Pluralibacter	-	-	-
Polaromonas	-	-	-
Propionibacterium	*g__Propionibacterium*	-	-
Prosthecobacter	*g__Prosthecobacter*	-	-
Providencia	-	-	-
Pseudacidovorax	*g__Pseudacidovorax*	-	-
Pseudochrobactrum	-	-	-
Pseudomonas	*g__Pseudomonas*	-	-
Pseudophaeobacter	-	-	-
-	*g__Pseudonocardia*	-	-
-	*g__Pseudonocardia*	-	-
-	*g__Pseudoxanthomonas*	-	-
Quadrisphaera	-	-	-
Ralstonia	-	-	-
Rathayibacter	-	*Rathayibacter*	1
Rhizobacter	-	-	-
Rhizobium	*g__Rhizobium*	-	-
Rhizorhabdus	-	-	-
-	*g__Rhodobacter*	-	-
Rhodanobacter	-	-	-
Rhodococcus	*g__Rhodococcus*	-	-
-	*g__Rhodoplanes*	-	-
Rhodopseudomonas	-	-	-
Rickettsia	-	-	-
Rivibacter	-	-	-
Roseateles	-	-	-
Roseomonas	*g__Roseomonas*	-	-
-	*g__Rothia*	-	-
-	*g__Rubrivivax*	-	-
Rudanella	-	-	-
Salirhabdus	-	-	-
Salmonella	-	-	-
Samsonia	-	-	-
-	*g__Sedimentibacter*	-	-
Segniliparus	-	-	-
Serpens	*g__Serpens*	-	-
Serratia	-	-	-
-	-	*Siccibacter*	1
Shimwellia	-	-	-
-	*g__Shinella*	-	-
Simonsiella	-	-	-
Siphonobacter	-	-	-
-	*g__Solimonas*	-	-
Snodgrassella	-	-	-
Soonwooa	-	-	-
Sphingobacterium	*g__Sphingobacterium*	-	-
Sphingobium	*g__Sphingobium*	-	-

Sphingomonas	g__*Sphingomonas*	*Sphingomonas*	9
Sphingopyxis	-	-	-
Spirosoma	g__*Spirosoma*	-	-
Staphylococcus	g__*Staphylococcus*	-	-
Stenotrophomonas	g__*Stenotrophomonas*	-	-
-	g__*Steroidobacter*	-	-
Streptococcus	g__*Streptococcus*	*Streptococcus*	3
-	g__*Streptomyces*	-	-
-	g__*Symbiobacterium*	-	-
Taibaiella	-	-	-
Tepidisphaera	-	-	-
Terrabacter	-	-	-
-	g__*Terrimonas*	-	-
-	g__*Thermomonas*	-	-
Variovorax	g__*Variovorax*	*Variovorax*	1
-	g__*Verrucomicrobium*	-	-
Williamsia	-	-	-
Xanthomonas	g__*Xanthomonas*	-	-
Yokenella	-	-	-

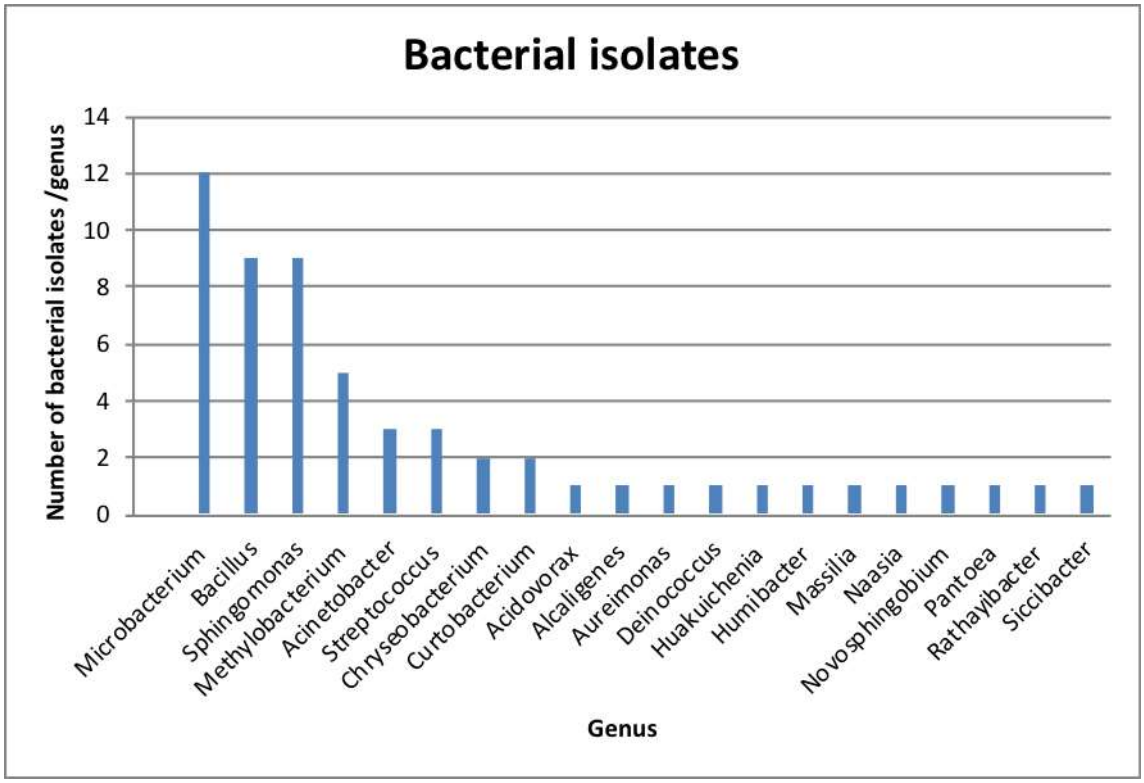


Figure 3. 2 Diagram of bacterial isolates and the numbers of isolates for each genus.

Most of the bacterial isolates belonged *to Microbacterium*, *Sphingomonas*, *Methylobacterium* and *Bacillus* genera

3.3.4. *In vitro* phenotypes characterization of the bacterial isolates

In order to obtain more information on the ability of these bacterial isolates to exert a beneficial direct or indirect effect on the plant, several PGP (plant growth promoting) activities and phenotypes were tested. The 58 isolates were tested for the following activities and phenotypes; proteolytic activity, exopolysaccharides (EPS) production, antibacterial activity against *P. fuscovaginae*, indole acetic acid (IAA) production, acyl homoserine lactones (AHL) signals production, swarming and swimming motility. In summary, 24/58 bacterial isolates displayed proteolytic activity whereas no bacterial isolates displayed lipolytic activity. In addition 17/58 bacterial isolates displayed EPS production, 2/58 produced AHLs, 7/58 displayed swimming motility, 19/58 displayed IAA production activity, 4/58 bacterial showed swarming motility and only 1/58 isolate had anti-*P. fuscovaginae* activity (**Figure 3.3**, **Table 3.2**). This latter bacterial isolate belonged to the *Alcaligenes* genus; interestingly this activity is not due to a protein since it was resistant to the strong protease pronase hence it is most likely a compound without amino acids and peptide bonds.

Table 3. 2 In *vitro* assays on the bacterial isolates from rice asymptomatic samples of sheath rot disease

(+: low activity, ++: medium activity and +++: high activity)

Number	Bacterial isolates	Proteolytic activity	EPS	Antimicrobial activity	Swimming	Swarming	AHLs	IAA
1	*Acidovorax sp.*		+++					
2	*Acinetobacter sp.*	+			++	+		
3	*Acinetobacter sp.*	+			++	+		+
4	*Acinetobacter sp.*		+++					+
5	*Alcaligenes sp.*			+	++	+		++
6	*Aureimonas sp.*		+					+
7	*Bacillus sp.*							
8	*Bacillus sp.*							++
9	*Bacillus sp.*	++						+
10	*Bacillus sp.*	++						+
11	*Bacillus sp.*							+
12	*Bacillus sp.*				+			++
13	*Bacillus sp.*				+			
14	*Bacillus sp.*	++						
15	*Bacillus sp.*	++						+
16	*Chryseobacterium sp.*	+++						
17	*Chryseobacterium sp.*	+++						
18	*Curtobacterium sp.*	+++						
19	*Curtobacterium sp.*	+++						
20	*Deinococcus sp.*	+++	+					
21	*Huakuichenia sp.*	++						
22	*Humibacter sp.*							++
23	*Massilia sp.*	++	+++					
24	*Methylobacterium sp.*							
25	*Methylobacterium sp.*						+	
26	*Methylobacterium sp.*							
27	*Methylobacterium sp.*							
28	*Methylobacterium sp.*							
29	*Microbacterium sp.*	+	+					
30	*Microbacterium sp.*	++	++					
31	*Microbacterium sp.*	++	++					
32	*Microbacterium sp.*		++					
33	*Microbacterium sp.*	++	+					+
34	*Microbacterium sp.*	++	+					
35	*Microbacterium sp.*	++	+++					
36	*Microbacterium sp.*	++	+					
37	*Microbacterium sp.*	+	+					
38	*Microbacterium sp.*	++	++					
39	*Microbacterium sp.*	+++	++					
40	*Microbacterium sp.*	+++	++					
41	*Naasia sp.*							
42	*Novosphingobium sp.*							
43	*Pantoea sp.*							

44	*Rathayibacter sp.*					
45	*Siccibacter sp.*		+++	+	++	++
46	*Sphingomonas sp.*					
47	*Sphingomonas sp.*	+				
48	*Sphingomonas sp.*					++
49	*Sphingomonas sp.*					
50	*Sphingomonas sp.*					
51	*Sphingomonas sp.*					+++
52	*Sphingomonas sp.*					+++
53	*Sphingomonas sp.*					+++
54	*Sphingomonas sp.*					++
55	*Streptococcus sp.*					
56	*Streptococcus sp.*					
57	*Streptococcus sp.*					
58	*Variovorax sp.*		+++			++

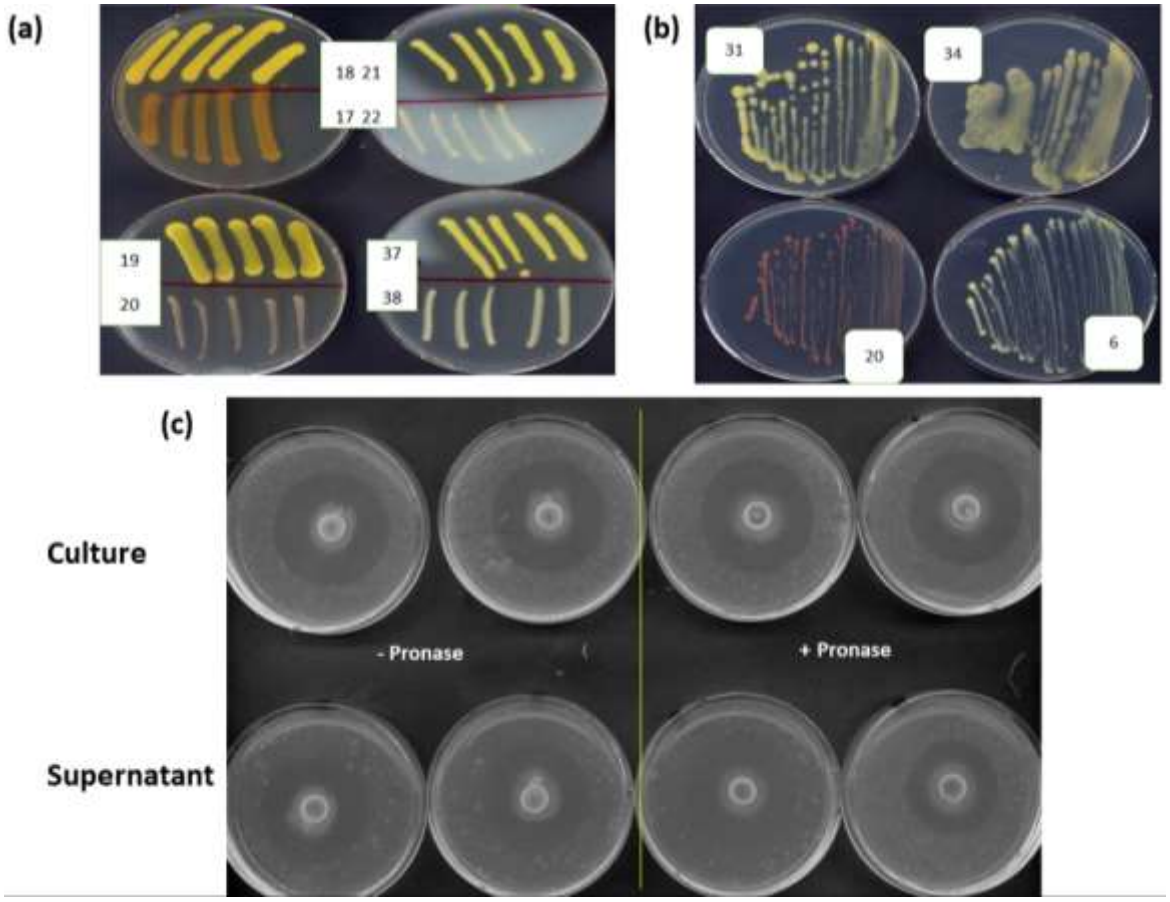


Figure 3. 3 Phenotypes assay of the bacterial isolates.

(a) Proteolytic activity assay; **(b)** Exopolysaccharides production assay; **(c)** *Alcaligenes* antibacterial activity against *P. fuscovaginae* in the presence or not of pronase. Both the *Alcaligenes* bacterial culture alive and its supernatant were used.

3.4. Discussion

The aim of this study was to perform a culturable microbiome and create a bacterial culture collection from healthy/asymptomatic sheath rice plant samples. These asymptomatic samples were collected from rice fields in Burundi, where the rice sheath rot disease is a serious issue. The total microbiome was performed on the same asymptomatic samples as presented in the previous chapter and these had a high number of genera; 151 in 2017 and 105 in 2018. In comparison the culturable microbiome presented in this chapter revealed 108 genera in 2017 and 88 in 2018.

The comparison between the total microbiome and culturable microbiome displayed some differences. Some genera were present in total microbiome and not present in the culturable; most probably some genera in the total microbiome are unculturable or could not grow under the growth conditions used here or were unable to survive as the plant material was frozen in the presence of a cryoprotectant (glycerol). Surprisingly some isolated bacteria of the culturable microbiome belong to genera that were not detected in total microbiome analysis; most likely the growth conditions used are optimal for them and in addition these genera are most probably present in very low abundance thus the total microbiome analysis via 16S rRNA gene amplicon sequencing did not detect them. Many of the genera in the culturable microbiome reported in this chapter were mostly previously reported as being part of the rice phyllospheric microbiome like *Methylobacterium*, *Sphingomonas* and *Microbacterium.* These bacteria that colonize the phyllosphere have the ability for adaptation in a nutrient limiting environment and to survive under high UV radiation (Stone et al., 2018). These members of the phyllospheric microbiome could be involved in providing to the plant resistance to different stress conditions. Interestingly, the bacterial collection reported here, possessed a few genera like *Alcaligenes*, *Massilia*, *Rhayibacter* that have not been reported previously to be associated with the phyllosphere of the rice plant. A possible reason is that the rice sheath

samples used in this work were not surface sterilized meaning that bacterial isolates could contain endophytic and epiphytic bacteria. Many isolates belong to the genus *Microbacterium, Sphingomonas, Bacillus and Methylobacterium*; this could have been caused by enrichment due to the isolation conditions, especially the culture medium (TSA) and the temperature of growth.

The in *vitro* assays performed on the 58 bacterial isolates showed a diversity of phenotypes**;** 24 isolates had proteolytic activity which is an important property involved in the virulence of plant pathogens (Figaj et al., 2019) as well as in biological control of plant disease (Mota et al., 2017)**.** 17 isolates were able to produce EPS, these molecules are known to be produced also by some plant pathogens like *Pseudomonas* and the EPS produced by *Pseudomonas syringae* is involved in biofilm formation, virulence and epiphytic fitness (Laue et al., 2006; Yu et al., 1999). It is possible that bacterial EPS provides some protection to the plant, both from desiccation and from UV damage. Biofilms in the phyllosphere may provide resistance to desiccation unlike those found in water; for example, *Pseudomonas putida* biofilms grown in air retained their morphology better after drying than biofilms grown in liquid medium (Auerbach et al., 2000). *Pseudomonas sp.* are often dominant constituents of the phyllosphere suggesting that naturally occurring biofilms may limit the loss of water and exposure to UV radiation. Plants are exposed to high levels of UV radiation and can suffer developmental and genetic damage (Jansen et al., 1998). Pigmented bacteria are more UV resistant, and the phyllosphere microbiome as a whole becomes more UV tolerant towards the end of the growing season (Jacobs & Sundin, 2001). It is possible that phyllospheric microorganisms may provide some UV protection to the plant through pigmented compounds; interestingly several isolates (many *Microbacterium* sp.) producing EPS were yellow pigmented. It is also known that EPS production is involved in the endophytic colonisation of *Gluconacetobacter*

diazotrophicus since EPS mutants were defective in the colonization of the rice root endosphere (Meneses et al., 2011).

Quorum sensing AHL signals was detected only in 2 of the 58 isolates; more precisely in one *Methylobacterium* and one *Siccibacter* strain. AHL mediated quorum sensing therefore might not play a major role in sheath epiphytic bacteria of rice. IAA was produced mostly by isolates which belonged to *Bacillus* and *Sphingomonas*. The plant hormone (IAA) from phyllospheric microorganisms has an influence on plant growth and the evidence suggests that phyllospheric microorganisms producing it could be involved in increasing plant productivity (Glick, 1995; Romero et al., 2016) and also be involved in the activity of stomata (Tanaka et al., 2006). Swimming and swarming movement was detected on a few isolates; these phenotypes can have an important role in the motility for acquisition of nutrients.

The antimicrobial activity assay against *P. fuscovaginae* showed that only one isolate belonging to the *Alcaligenes* genus displayed a positive test in *vitro*. *Alcaligenes* sp. strains exist in soil, water and environment, as well as in association with humans. The bacteria of this genus are usually non-pathogenic but occasionally can cause opportunistic human infections. Bacterial species belonging to the genus *Alcaligenes* have also demonstrated versatile pollutant bioremediation capability, including phenols (Kumar et al., 2013; Rehfuss & Urban, 2005), phenanthrene (Singleton et al., 2009) as well as having algicidal activity (P. Sun et al., 2015). The *in vitro* antimicrobial assay performed here excludes the isolates that attenuate/block *P. fuscovaginae* pathogenesis/invasion via other mechanisms like competition for nutrients or quorum quenching. It is therefore likely that other bacteria that live in the phyllosphere are involved in promoting plant health by keeping away pathogens like *P. fuscovaginae*.

Chapter IV. Identification of a repressor that regulates quorum sensing and an RND efflux pump in *P. fuscovaginae*

4.1. Introduction

Bacteria can undergo cell-cell communication by producing and responding to small diffusible molecules that act as signals; these are called auto-inducers (AIs). They are produced at basal levels and their concentration increases with cell-density and because of their diffusion through membranes, the concentration inside cells approximates to the concentration in the environment. Upon reaching a critical concentration, the signal molecules can bind to and activate receptors/regulators inside bacterial cells. These regulators can then alter gene expression to activate behaviours that are beneficial under the particular condition encountered. As this phenomenon occurs in a cell-density-dependent manner, it has been termed Quorum Sensing (QS) (W. C. Fuqua et al., 1994). The first QS system was described in the marine luminescent bacterium *Vibrio fischeri*, where it functions as control mechanism for light production (Eberhard et al., 1981; J. Engebrecht et al., 1983; J. A. Engebrecht & Silverman, 1984) and requires an autoinducer synthase protein called LuxI which synthesises an N-acyl homoserine lactone (AHL) and a transcription factor designated as LuxR which responds and binds to AHLs signals. Many Gram negative bacterial species have now been shown to utilize this type of QS system as part of their response to cell density (Antunes & Ferreira, 2009; Bassler & Losick, 2006; Lyon & Novick, 2004) and in many cases it controls virulence (Antunes & Ferreira, 2009). Many classes of AIs have been described and the most intensely studied are the AHLs signals of Gram-negative bacteria and small peptides from Gram positive bacteria (Antunes & Ferreira, 2009). AHLs signals are detected by bacterial cells through binding to cytoplasmic receptor proteins, which, upon signal detection, dimerize and can bind to promoter regions of target genes to activate or repress their transcription (W. C. Fuqua et al., 1994). Peptides on the other hand, are detected through binding to membrane sensor proteins of the two-component system family (Novick & Geisinger, 2008; Pottathil & Lazazzera, 2003)

Pseudomonas fuscovaginae is a bacterial pathogen that causes rice sheath brown rot in several rice (*Oryzae sativa*) growing countries (CABI, 2018). It has been isolated and identified for the first time in Japan (Miyajma, Tanii and Akita 1983; Tanii, Miyajama, and Akita 1976) and belongs to the Gram-negative fluorescent pseudomonads. *P. fuscovaginae* possesses two AHL QS systems designated PfsI/R and PfvI/R; PfsI and PfvI belong to the LuxI family proteins involved in the AHLs signals synthesis whereas PfsR and PfvR belong to the LuxR family involved in AHL detection and transcriptional regulation. The PfsI/R and PfvI/R systems are negatively regulated by repressors which are encoded by genes located intergenically between the AHL synthase and LuxR-family response regulator (Mattiuzzo et al., 2011). The *pfsI/R* system is regulated by a novel repressor designated RsaM while the *pfvI/R* system is regulated by both the characterized DNA-binding RsaL repressor (Venturi et al., 2011) and also by RsaM. The two *P. fuscovaginae* AHL QS systems are not transcriptionally hierarchically organized but share a common AHL signal response and both are required for plant virulence and are involved in rice sheath rot in *P. fuscovaginae* (Mattiuzzo et al., 2011). *P. fuscovaginae* has therefore a unique complex regulatory network composed of at least two different repressors which regulate the AHL QS systems and pathogenicity. *P. fuscovaginae* AHL QS is switched off under laboratory conditions (in *vitro*) but switched on in planta (in *vivo*) (Mattiuzzo et al., 2011; Uzelac et al., 2017) hence the plant niche together with pathobiome microorganisms are likely to play an important role in switching on QS.

The RsaL repressor has a HTH DNA binding domain (Kang et al., 2017; Rampioni et al., 2007) and interacts with the promoter of the *pfvI* gene repressing its transcription (Rampioni et al., 2006).The RsaM is a novel protein and crystallization and DNA-binding studies have evidenced that this repressor does not bind DNA and does not have a DNA binding domain (Michalska et al., 2014). It has been recently also reported in *Burkholderia vietnamiensis* as a

regulator of AHL QS, however its mode of action is not through DNA-binding and currently remains unknown (Le Guillouzer, Groleau, and Déziel 2018; Michalska et al. 2014). RsaM regulates a large number of genes in *P. fuscovaginae* thus being a global regulator; it is involved in the regulation of over 400 genes, 206 are negatively regulated whereas 260 are positively regulated (Uzelac et al., 2017).

RsaM is therefore a pivotal regulator that switches QS on/off in *P. fuscovaginae*. Since both AHL QS systems of *P. fuscovaginae* are switched on *in planta* and are involved in virulence, RsaM could be responding to the pathobiome. As RsaM transcriptionally negatively regulates both AHL QS systems and is not a DNA-binding protein, it needs to influence/interact with another protein(s) in order to exert this indirect negative effect in transcription. In this study, two genetic screens were set up in order to identify possible RsaM protein partners. These screens resulted in the identification of a transcriptional repressor protein that affects *pfsI* transcription, AHLs signals levels and also regulates an efflux pump. The role of this repressor in AHL QS in *P. fuscovaginae* in the pathobiome is discussed.

4.2. Materials and Methods

4.2.1. Bacterial strains, plasmids, media and recombinant DNA techniques

The bacterial strains, plasmids and primers used in this work are listed in **Table 4.1**. *P. fuscovaginae* strains, *Chromobacterium violaceum* reporter strain (CVO26) were grown at 30 °C in Luria-Bertani (LB) /agar medium; DH5α *E. coli* was grown at 37 °C in LB/agar medium (Miller, 1972). When required, antibiotics were added at the following concentrations: ampicillin (Amp) 100 µg /ml (*P. fuscovaginae*, *E. coli*); gentamycin (Gm) 10µg/ml (*E. coli*) kanamycin (Km) 100 µg/ml (*P. fuscovaginae*, *E. coli*); tetracycline (Tc) 10 µg/ml (*E. coli*); 20, 30, 40 and 50µg/ ml (*P. fuscovaginae*); nitrofurantoin (Nf) 100 µg/ ml (*P. fuscovaginae*) and chloramphenicol (Cm), 125, µg/ ml (*P. fuscovaginae*) and 25 µg/ ml (*E. coli*). The 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a final concentration of 80 µg/ ml when it was necessary. Routine DNA manipulation steps like digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase and transformation of *E. coli* were performed as described previously (Sambrook et al., 1989). Plasmids were purified by using EuroGold columns (EuroClone SpA, Milan, Italy); total DNA from *P. fuscovaginae* was isolated by sarkosyl-pronase lysis, as described previously (Better et al., 1983). Digestion with restriction enzymes was conducted according to the supplier's instructions (New England BioLabs, Ipswich, MA, USA). DNA was ligated with T4 DNA ligase (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's recommendations.

PCR amplifications were performed using Expand High Fidelity PCR system (Roche, Basel, CH). Arbitrary PCRs were performed using Vent (exo-) DNA Polymerase (New England BioLabs, Ipswich, MA, USA). Primers were purchased from Sigma. Triparental matings to mobilize DNA from *E. coli* to *P. fuscovaginae* were carried out using the helper strain *E. coli* (pRK2013) (Figurski et al., 1979).

Table 4. 1 Bacterial strains, plasmids vector and primers used in this work

Bacterial strains or mutants	Features	Reference
P. fuscovaginae UPB0736 (Pfv WT)	WT strain isolated from diseased rice in Madagascar	(Maraite & Weyns, 1997)
P. fuscovaginae 0736RSAM	rsaM ::Tn5 of P. fuscovaginae UPB0736; Km^R	(Mattiuzzo et al., 2011)
P. fuscovaginae 0736MexR	mexR:: Tn5 of P. fuscovaginae UPB0736; Km^R	This work
P. fuscovaginae 0736PFSI	*pfsI::*Km of *P. fuscovaginae* UPB0736; KmR	(Mattiuzzo et al., 2011)
C. violaceum CV026	Double transposon mutant of ATCC31532, violacein and AHL negative	(McClean et al., 1997)
E. coli DH5a	l-'80dlacZDM15 D(lacZYA-argF)U169 recA1 endA1 hsdR17(rK – mK -) supE44 thi-1 gyrA relA1	(Hanahan, 1983)
E. coli (pRK2013)	Conjugation helper , Km^R	(Figurski et al., 1979)

Plasmids	Features	Reference
pGEM-T Easy	Cloning vector Amp^R	Promega Corp., Madison, WI, USA;
pBBRmcs5	Broad-host-range vector; Gm^R	(Kovach et al., 1995)
pBBR1TC	PBBRmcs5carrying Tc resistant gene, Gm^RTc^R	This study
pBBBR1pfsITC	PBBR1TC, harbouring pfsI gene promoter and Gm^RTc^R gene	This study
pBluscript II KS	Cloning vector Amp^R	(Alting-Mees & Short, 1989)
pMP77	Promoter probe vector carrying xylie gene reporter; InQ Cm^R	(Spaink, Okker,Wijffelman, Pees, & Lugtenberg, 1987)
pMP77PfsIprom -Tc	pMP77 carrying *pfsI* gene promoter up stream of Tc gene, Cm^R	This study
pMP77MexR	pMP77 carrying *mexR* gene and Cm^R	This study
pMP220	Promoter probe vector, IncP1, Tc^R	(Spaink et al., 1987)
pMP220PFSI	pMP220 carrying *pfsI* gene promoter, Tc^R	This study
pMP220MexR	pMP220carring *mexR* gene promoter, Tc^R	This study
pMP220MexC	pMP220carring *mexC* gene promoter, Tc^R	This study

Primer name	Primer sequence	Reference or source
TcEcoFw	gaattcCGCAGTCAGGCACCGTGTAT	pBBR1MCS-3
TcXbaRev	tctagaTTCCATTCAGGTCGAGGTGG	pBBR1MCS-3
pfsIPROMHindFw	aagcttATGTTCGATCGTGAGAGTTG	This study
pfsIPROMEcoRev	gaattcTTGTCGCGCTGTACCATT	This study
pfsIPROMBamFw	ggatccATGTTCGATCGTGAGAGTTG	This study
pfsIPROMEcoRev	gaattcTTGTCGCGCTGTACCATT	This study
Tn5 Ext	GAACGTTACCATGTTAGGAGGTC	This study
Arb-1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	This study
Tn5 Int	CGGGAAAGGTTCCGTTCAGGACGC	This study
Arb-2	GGCCACGCGTCGACTAGTAC	This study
PROMRepEffluxPUMP KpnFw	ggtaccCTTGAGTTGCATGAAGATCC	This study
PROMRepEffluxPUMP XbaRev	tctagaGGCAGTAAAACCTCGATCAG	This study
PROMEffluxPUMP KpnFw	ggtaccGGCAGTAAAACCTCGATCAG	This study
PROMEffluxPUMP XbaRev	tctagaCTTGAGTTGCATGAAGATCC	This study
RepEffluxPUMP XbaFw	tctagaGAGTTGCATGAAGATCCTCG	This study
RepEffluxPUMP SpeRev	actagtAGACTCACGCATTTTGAC	This study

4.2.2. Screening of *P. fuscovaginae* UPB0736 Tn5 genomic mutants for up-regulation of the *pfsI* promoter

A screening to isolate transposon genomic mutants with up-regulation of the *pfsI* gene promoter via antibiotic resistance acquisition was performed. The screening was conducted on the *P. fuscovaginae* UPB0736 genomic mutant bank obtained by mutagenesis with Tn5 transposon by Mattiuzzo et al. (2011). A construct of *pfsI* gene promoter regulating the transcription of a tetracycline resistance gene has been constructed as follows. The tetracycline resistance gene, deprived of its promoter, was amplified from pBBR1MCS-3 plasmid (Kovach et al., 1995) by using the primers TcEcoFw and TcXbaRev and cloned in pBBR1MCS-5 EcoRI-XbaI generating pBBR1TC. The *pfsI* promoter was amplified from *P. fuscovaginae* genomic DNA using the primers pfsIPROMHindFw and pfsIPROMEcoRev and then cloned upstream the tetracycline resistance gene in pBBR1TC generating pBBBR1pfsITC. From the latter plasmid construct, the *pfsI* promoter fused to the Tc^R gene was then cut with KpnI and XbaI enzymes and transferred to the pMP77 plasmid, generating pMP77PfsIprom-Tc. The fidelity of all these constructs was verified by DNA sequencing (Eurofins Genomics GmbH, Ebersberg, D). The pPfsITc was mobilized from *E. coli* to a *P. fuscovaginae* Tn5 genomic mutant bank by triparental mating using the helper strain *E. coli* (pRK2013) (Figurski et al., 1979). Selection of transconjugants was performed on LB agar plates supplemented with Cm and Km antibiotics.

A mutant selection was then carried on *P. fuscovaginae* Tn5 genomic mutant bank (pPfsITc) on different concentrations of the Tc antibiotic (20, 30, 50 μg/ml) in order to select for mutants where the *pfsI* promoter was up-regulated controlling the transcription of the Tc^R gene; 50000 *P. fuscovaginae* mutants were screened and 67 *P. fuscovaginae* Tc^R resistant mutants were isolated.

4.2.3. Screening of *P. fuscovaginae* UPB0736 Tn5 genomic mutants for overproduction of AHLs

The up-regulation of the *pfsI* gene promoter, via AHLs overproducing mutants, has been screened on the *P. fuscovaginae* UPB0736 genomic mutant bank obtained by mutagenesis with Tn5 transposon by Mattiuzzo et al. (2011).

A further screening was performed in order to isolate transposon genomic mutants overproducing AHLs. 100 µl of a liquid culture of *P. fuscovaginae* UPB0736 Tn5 genomic mutant bank (see above) having 10^3-2.10^3 CFU/ml was plated together with the AHL biosensor *C. violaceum* CV026 using 100 µl of a liquid culture having 10^4-2.10^4 CFU/ml and incubated overnight at 30°C. *P. fuscovaginae* WT produces very low amounts of AHLs and induces very low and almost undetectable levels of pigmentation in proximity of *C. violaceum* CV026. On the other hand CV026 surrounding *P. fuscovaginae* mutants overproducing AHLs turn to a purple colour (**Figure 4.1).** $2{,}5.10^4$ *P. fuscovaginae* mutants were screened and some area zones that turned to purple were identified. These areas were collected and streaked again on LB containing the nitrofurantoin antibiotic (100µg/ml) in order to counter-select CV026 colonies from *P. fuscovaginae* Tn5 genomic mutants; 27 mutants were selected and checked again for AHL production by T-streak technique (Steindler & Venturi, 2007).

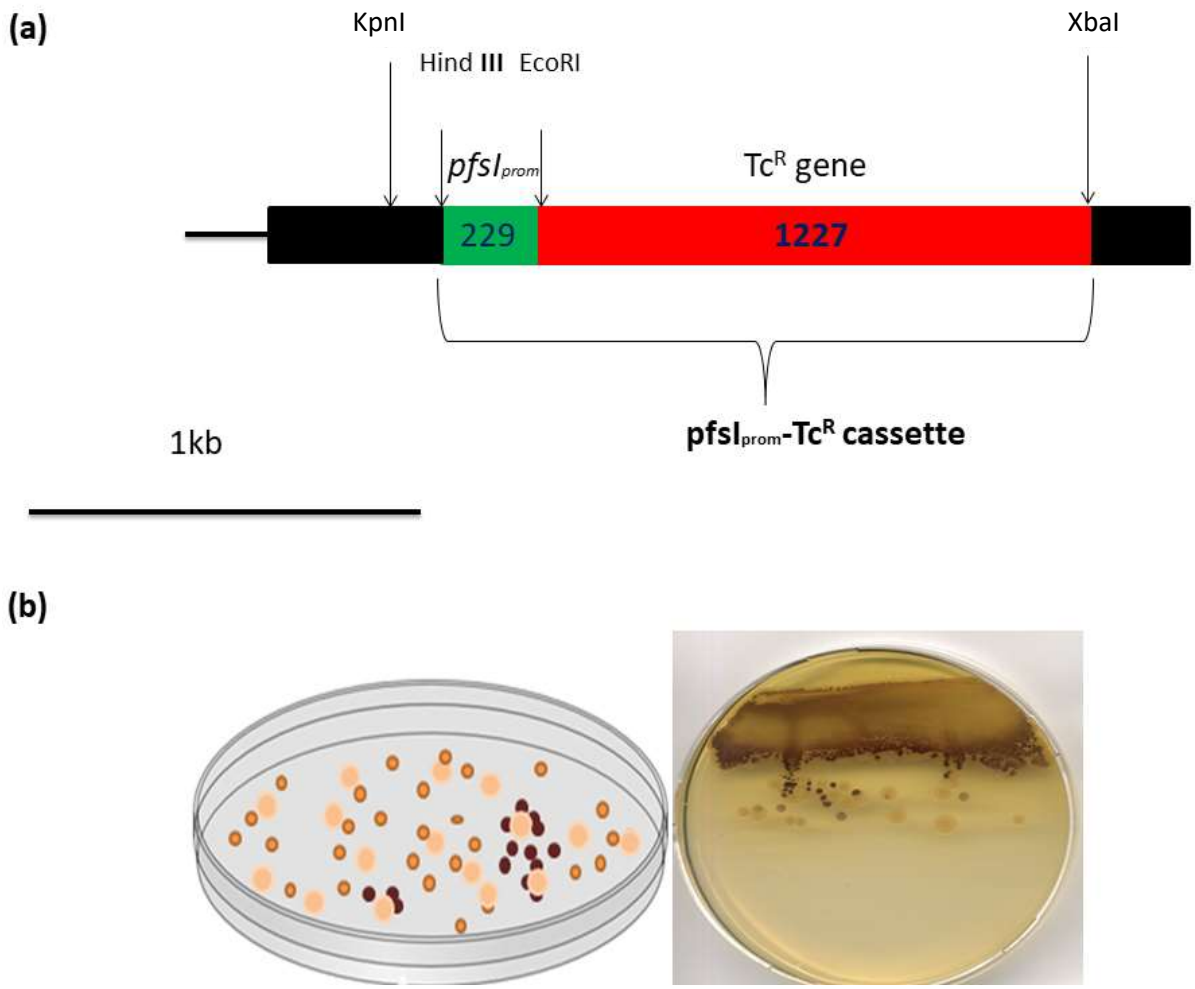


Figure 4. 1 Set-up of the two genetic screens.

(**a**)The construct of the *pfsI* gene promoter (green) controlling the Tc resistance gene (red) inserted in the pMP77 plasmid vector; KpnI, Hind III, EcoRI and XbaI are the restriction enzyme sites as described in Materials and Methods.
(**b**) Screening phases: in the cartoon *P. fuscovaginae* Tn5 mutant bank (bigger light colonies) together with the CVO26 (smaller colonies) biosensor for screening for AHL signals hyper-production.
A *P. fuscovaginae* Tn5 mutant overproducing AHLs signals will make the neighbouring CVO26 colonies becoming purple pigmented as shown in the figure. In the plate it is shown the purification of *P. fuscovaginae* mutant colonies overproducing AHLs signals together with CV026 biosensor colonies.

4.2.4. Mapping of the transposon insertion sites

In order to map the Tn5 transposon insertion sites of the isolated mutants, an arbitrary PCR technique was used as described by O'Toole and Kolter (1998). The arbitrary PCR products were purified, blunted (Quick blunting kit; New England BioLabs, Ipswich, MA, USA) and cloned in the SmaI site of pBluscript II KS (Alting-Mees & Short, 1989). Sequencing revealed the Tn5 transposon insertion sites: insertions occurred independently are

representing different mutants of *P. fuscovaginae*. Among isolated mutants 6 different insertions sites were found inside the same gene encoding for a transcriptional repressor of the multidrug efflux pump *acrAB* and the mutant was named 0736MexR. Transposon mutants in this locus were found using both genetic screens (up-regulation of the *pfsI* gene promoter and increase in AHLs signals production) performed in this study. For this reason mutant 0736MexR was considered for further investigation.

4.2.5. Mutant complementation

To complement the 0736MexR mutant, the transcription repressor together with its promoter was PCR amplified using as template *P. fuscovaginae* genomic DNA using Expand High Fidelity PCR system (Roche, Basel, CH) and the primers RepEffluxPUMP XbaFw and RepEffluxPUMP SpeRev. The PCR product was first cloned in the pGEM plasmid, sequenced, excised with XbaI and SpeI restriction enzymes and further cloned in the pMP77 vector, generating pMP77-PfvMexR.

4.2.6. Gene promoter studies in *P. fuscovaginae* strains

The *pfsI*, *mexR* and *mexC* genes transcriptional fusion plasmid, based on pMP220 promoter probe vector, were constructed. The *pfsI*, *mexR* and *mexC* genes promoter were amplified using the primers respectively pfsIPROMBamFw and pfsIPROMEcoRev, PROMRepEffluxPUMP KpnFw and PROMRepEffluxPUMP XbaRev, RepEffluxPUMP XbaFw and PROMEffluxPUMP XbaRev; and cloned in the BglII-EcoRI sites in pMP220, upstream the promoterless *lacZ* gene, yielding pPfsI220.

β-galactosidase activities were determined, essentially as described by Miller (1972) with the modifications of Stachel et al. (1985), at 4hs, 8hs and overnight time points for *pfsI* gene promoter; and overnight for *mexR* and *mexC* gene promoters. All experiments were performed in biological triplicates.

4.2.7. Extraction and quantification of AHLs

AHLs were extracted from 30 ml overnight cultures of the *P. fuscovaginae* strains. Cultures were centrifuged and the cells free supernatants were filtered (using 0.45u filters, Millipore) and extracted 2 times with an equal volume of ethyl acetate 0.1 % acetic acid. The organic phases were dried in a Speed Vacuum Concentrator (Heto Lab)

The quantification and identification of AHLs has been performed by High Performance Liquid Chromatography (HPLC) and mass spectrometry (MS). For the Chromatography a Shimadzu series 10AD VP LC system was used. The column oven was maintained at 40°C. The HPLC Column used was a Phenomenex Gemini C18 column (3.0 µm, 50 x 3.0 mm) with an appropriate guard column. Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B 0.1% (v/v) formic acid in methanol. The flow rate throughout the chromatographic separation was 450µL/min. The binary gradient initially began at 10% B for 1.0 min, increased linearly to 50% B over 0.5 min, then to 99% B over 4.0 min. The composition remained at 99% B for 1.5 min, decreased to 10% B over 0.1 min, and stayed at this composition for 2.9 min. Total run time per sample was 10 min. The MS system used was an Applied Biosystems Qtrap 4000 hybrid triple-quadrupole linear ion trap mass spectrometer equipped with an electrospray ionisation (ESI) interface. Instrument control, data collection and analysis were conducted using Analyst software. Source parameters were set as: curtain gas: 20.0, ion source potential: 5000 V, temperature: 450 °C, nebulizer gas: 20.0, and auxiliary gas: 15.0.

The Synthetic standards of C4, C6, C8, C10, C12, C14, 3-oxo-C4, 3-oxo-C6, 3-oxo-C8, 3-oxo-C10, 3-oxo-C12, 3-oxo-C14, 3-OH-C4, 3-OH-C6, 3-OH-C8, 3-OH-C10, 3-OH-C12 and 3-OH-C14 AHLs were synthesised and used as AHL standards.

4.2.8. In planta virulence assays

4.2.8.1. Assays on seeds

Virulence tests were performed on rice seeds during germination as described by Weeraratne et al. (2020) with some modifications. The *P. fuscovaginae* strains were grown in LB at 30°C, with shaking at 125 rpm, until they reached an optical density of 1.5 at 600 nm (OD600) and then centrifuged at 4800rpm for 10 min, washed with sterile distilled water (SDW) , resuspended in SDW and diluted to an (OD600) equal to 1. The rice seeds were surface sterilized in 50% hypochloride for 1h, washed five times in SDW, and then put to germinate in the presence of *P. fuscovaginae* strains or SDW as control for 6 days at 30°C. Forty seeds were then allowed to germinate in 20ml of bacterial culture and 20ml of SDW. The seedlings that germinated were then placed to grow in semisolid (0.4% agar) Hoagland solution (Hogland, 1950) in order to compare the impact of bacteria on the growth after germination. Six plantlets were grown for each treatment. Length of shoots and roots after 6-days germination and then after three weeks seedlings growth were determined.

4.2.8.2. Assays on seedlings

Virulence assays were performed as described by Mattiuzzo et al. (2011) and Patel et al. (2014) with some modifications. The infection was performed using 10^9 CFU/ml fresh culture of *P. fuscovaginae* bacterial strains and by needles puncturing two weeks old rice seedling plants grown in semisolid Hoagland solution (Hogland, 1950) . The virulence index was determined after one week from the infection as described by Mattiuzzo et al., 2011. Six plantlets were infected for each case and experiments were performed twice.

After the inoculation, the plants were placed in a growth chamber (28°C, RH = 70%, 16-h photoperiod). Disease severity was evaluated 6 days after inoculation based on the rating scale described by Mattiuzzo et al. (2011) with the following brief amendments: score 0 = no symptoms, only the sign of the injection puncture; 1 =necrosis around the puncture extending

up to 2 cm; 2 = necrosis around the puncture and chlorosis from 2 to 4 cm; 3 = necrosis around the puncture and chlorosis extending up to 5 cm; 4 = necrosis around the puncture and chlorosis throughout two or more leaves or the death of the plant .

4.2.9. Statistical analysis

In seed-soaking assays, measurements of the two parameters (shoot length and root length) were taken from a total of 40 seedlings per treatment. Means of the five treatments were compared and separated by S-test at a 5% confidence interval by Graph Pad Prism 8.3.1.549. Error bars were calculated by determining the standard errors of the means for each treatment.

Disease score data from virulence assays by inoculation of 2-week-old rice plant were averaged and then converted to percent disease index (PDI), taking the score of five as the highest disease incidence (100%). The PDIs of treatments were analyzed using the S-test at a 5% confidence level.

4.3. Results

4.3.1. Identification of transposon genomic mutants involved in the negative regulation of Quorum Sensing in *P. fuscovaginae*

In *P. fuscovaginae*, the AHL QS systems are stringently negatively transcriptionally regulated and switched off *in vitro* (Uzelac et al., 2017). A major player in this negative regulation of the *pfsI/R* and *pfvI/R* systems is the novel RsaM repressor; RsaM is not a DNA-binding protein and its mechanism of action is currently unknown. Both AHL QS systems are involved in virulence (Mattiuzzo et al., 2011) hence they are active *in planta* and this switch-on happens in the pathobiome where the pathogen interacts with many other microorganisms (**see Chapter II**). It is therefore important to determine the RsaM cascade which controls the switch on/off of AHL QS in *P. fuscovaginae*.

In order to identify possible RsaM protein partners involved in the negative regulation of AHL QS, two genetic screens were carried out using a *P. fuscovaginae* Tn5 genomic mutant bank. Firstly, the upregulation of *pfsI* AHL synthase gene was screened by using a construct where its gene promoter controls the transcription of the Tc resistance gene. *P. fuscovaginae* harbouring this construct is not resistant to tetracycline whereas if harboured in a genomic context which results in a significant increase of *pfsI* transcription, it becomes resistant to Tc. This plasmid construct was therefore conjugated *en masse* in a *P. fuscovaginae* Tn5 genomic mutant bank and mutants were assayed for Tc resistance. Approximately 50,000 mutants were screened and 67 mutants displayed Tc resistance and 4 of them had Tn5 insertions in different positions of the same locus (**Figure 4.2**). Curing these 4 *P. fuscovaginae* Tn5 mutants from the plasmid made the strains Tc sensitive confirming that resistance was due to the expression of the Tc resistance gene in the plasmid via the *pfsI* promoter.

The second genetic screen of the Tn5 genomic mutant bank of *P. fuscovaginae* was based on AHL over-production. 25,000 mutants were screened as described in the Materials and

Methods section. 27 AHL over-producing mutants were isolated and 2 of them had a Tn5 insertion in the same locus identified in the previous screen described above (**Figure 4.2**).

In summary, both screens resulted in the isolation of Tn5 transposon mutants which had insertion sites in a *tetR*-family transcriptional repressor gene adjacent to the multidrug efflux RND membrane gene *mexC* (**Figure 4.2**). This locus encoding for a TetR-family repressor was designated as *mexR*.

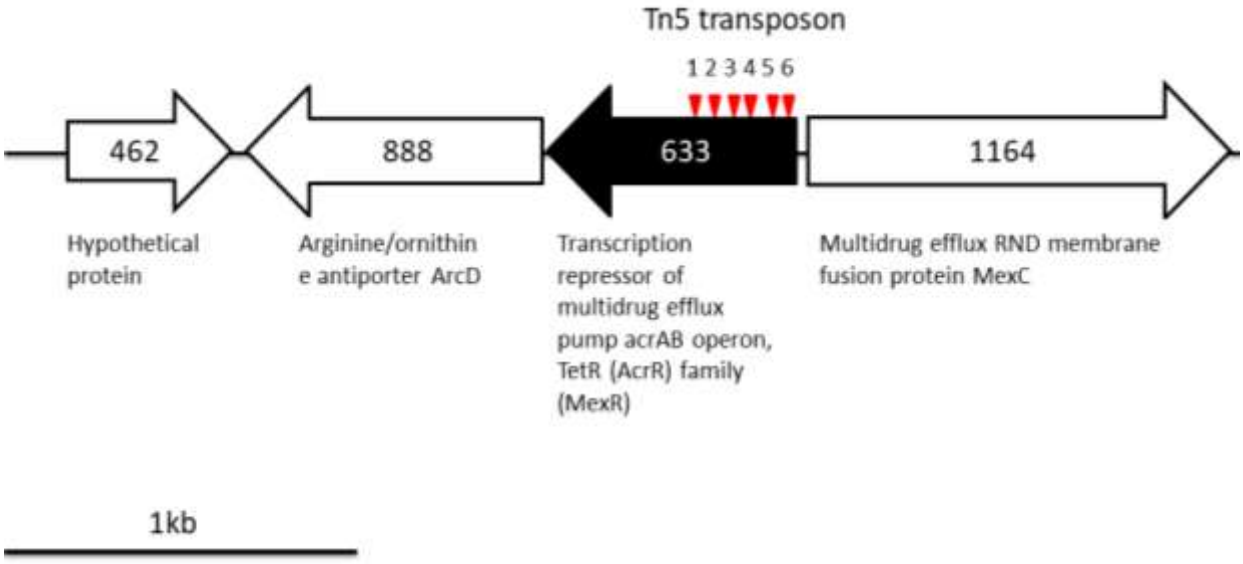


Figure 4. 2 Map of the genetic locus harbouring the Tn5 insertions of the identified *P. fuscovaginae* mutants isolated from the two independent genetic screenings.

The locus harbouring the Tn5 mutants 1, 2, 3, 4, 5 and 6 correspond to the mexR repressor as indicated black-filled. The position of the Tn5 insertion sites are shown by red triangles. The genes and sizes of the adjacent ORFs are shown.

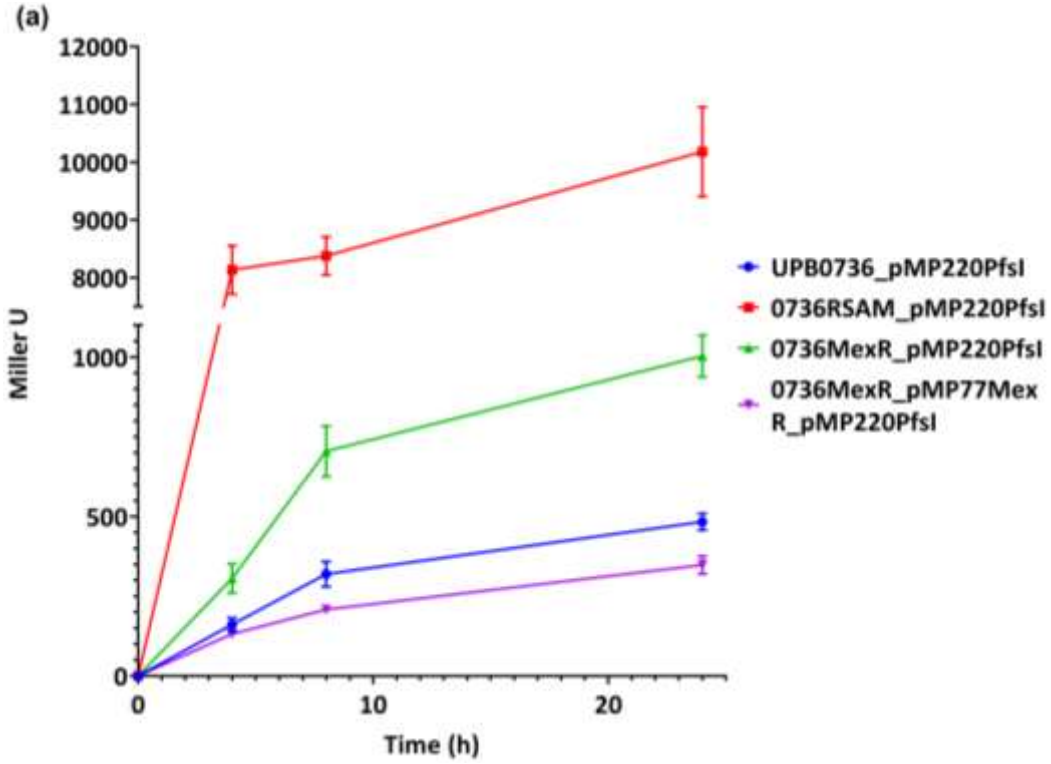
4.3.2. The MexR repressor is involved in the regulation of the *pfsI* AHL synthase

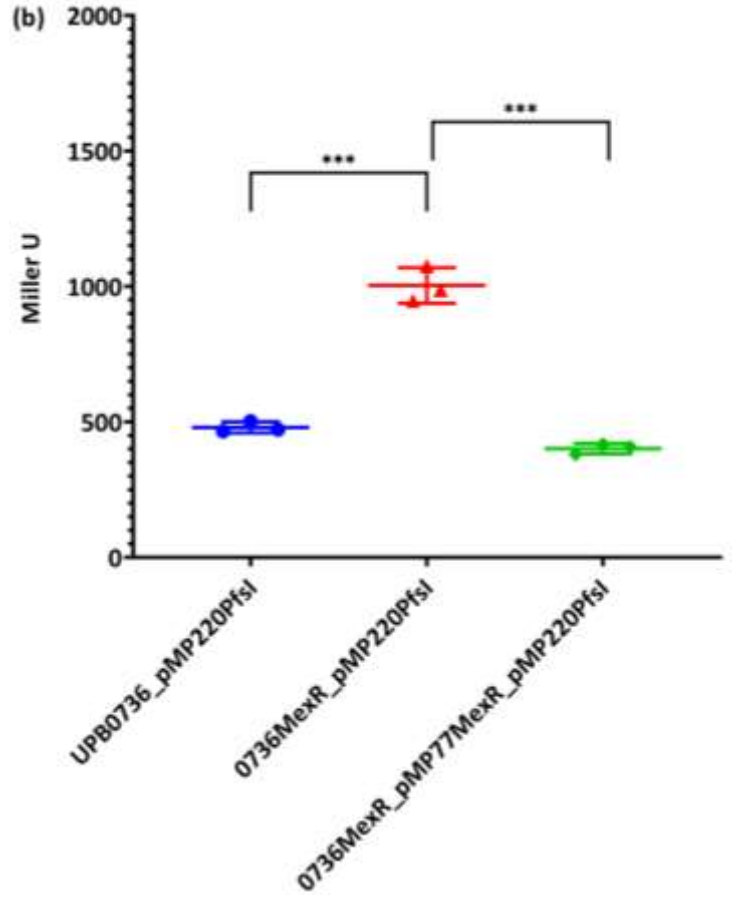
One of the genetic screens which resulted in the isolation of the mutant in the TetR-family repressor gene *mexR* was based on the up-regulation of the *pfsI* promoter. It was therefore of interest to determine the activity of the AHL synthase *pfsI* promoter in the *mexR* Tn5 mutants which were designated as 0736MexR. The upregulation of the *pfsI* gene promoter was determined in *P. fuscovaginae* strains at different liquid growth time points (4H, 8H and 24H) (**Figure 4.3a**) by measuring the activity of the *lacZ* gene product β-galactosidase. It was observed that the *pfsI* transcriptional activity was significantly up-regulated in the *mexR* Tn5 mutant 0736MexR (**Figure 4.3b**). Interestingly, the addition of exogenous AHLs signals (this is a simulation of AHL hyper-production) also resulted in the up-regulation of the *pfsI* gene promoter both in the 0736MexR mutant and in the *P. fuscovaginae* WT (**Figure 4.3b**). The provision of the wild-type *mexR* gene in a plasmid in the 0736MexR mutant resulted in the complementation restoring *pfsI* promoter activity to wild-type levels. In summary, the *mexR* repressor is involved in the AHL QS response in *P. fuscovaginae* by affecting *pfsI* promoter activity.

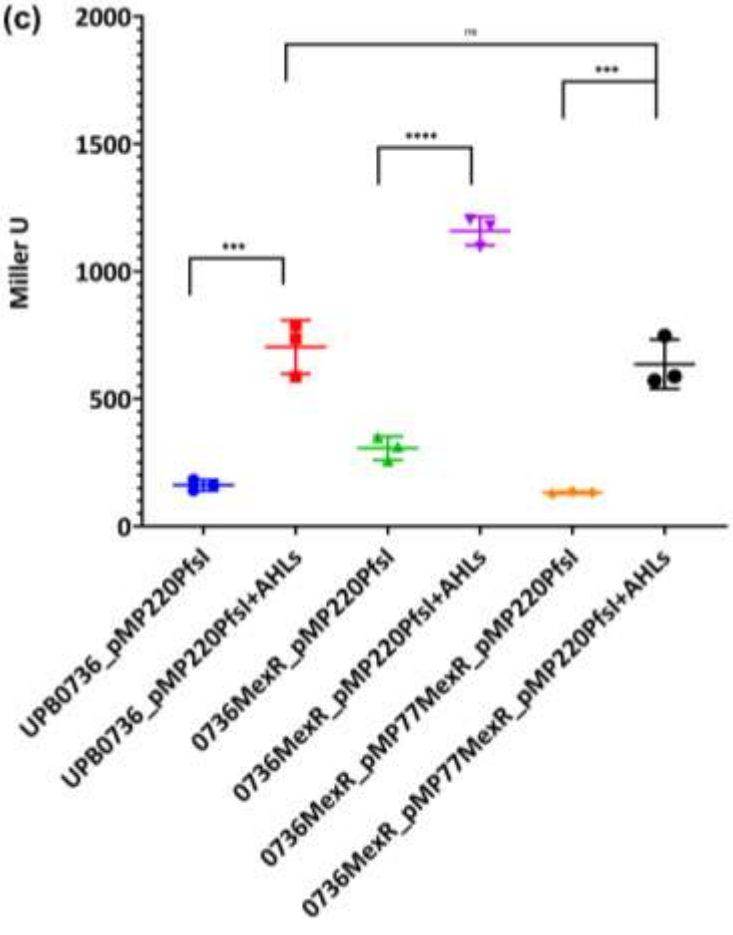
4.3.3. MexR regulates the adjacent *mexC* RND efflux pump

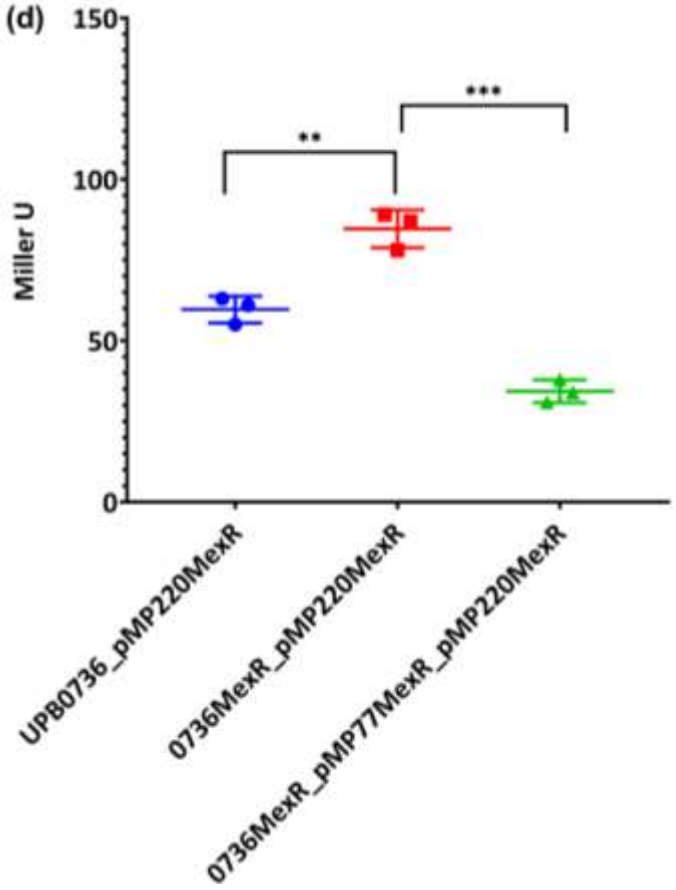
Multidrug resistance (MDR) efflux pumps is one of the major mechanisms used by bacteria to cope with toxic compounds and the resistance nodulation-cell division (RND) family belongs to the larger family of MDR pumps; there are currently five families of MDR pumps in bacteria (Li et al., 2015). In Gram-negative bacteria, the RND family efflux pumps (Murakami et al., 2002, 2006; Nikaido, 1996) consist of an RND protein (the inner membrane component, a membrane fusion protein (MFP: the periplasmic component), and the outer membrane protein (OMP: the outer membrane protein). Next to the *mexR* regulator, the *mexC* gene is located which encodes for the RND protein component (**Figure 4.2, 4.5**); due to this genetic organisation it is likely that MexR regulates *mexC*. It was therefore of interest to

determine whether the transcription of the *mexC* gene was affected by MexR. The gene promoter of *mexC* was cloned in a promoterless probe vector with the *lacZ* gene as reporter and its activity was established in the wild type and 0736MexR mutant. Results clearly indicate that the *mexC* gene promoter was upregulated in the *P. fuscovaginae mexR* mutant (**Figure 4.3e**). This indicates that MexR negatively regulates *mexC* transcription thus regulating the RND efflux pump.




(b)
2000
1500
1000
500
0
Miller U
***
***
UPB0736_pMP220PfsI
0736MexR_pMP220PfsI
0736MexR_pMP77MexR_pMP220PfsI


(c)
2000
1500
1000
500
0
Miller U
ns
***
****
***
UPB0736_pMP220PfsI
UPB0736_pMP220PfsI+AHLs
0736MexR_pMP220PfsI
0736MexR_pMP220PfsI+AHLs
0736MexR_pMP77MexR_pMP220PfsI
0736MexR_pMP77MexR_pMP220PfsI+AHLs


(d)
150
100
50
0
Miller U
**
***
UPB0736_pMP220MexR
0736MexR_pMP220MexR
0736MexR_pMP77MexR_pMP220MexR

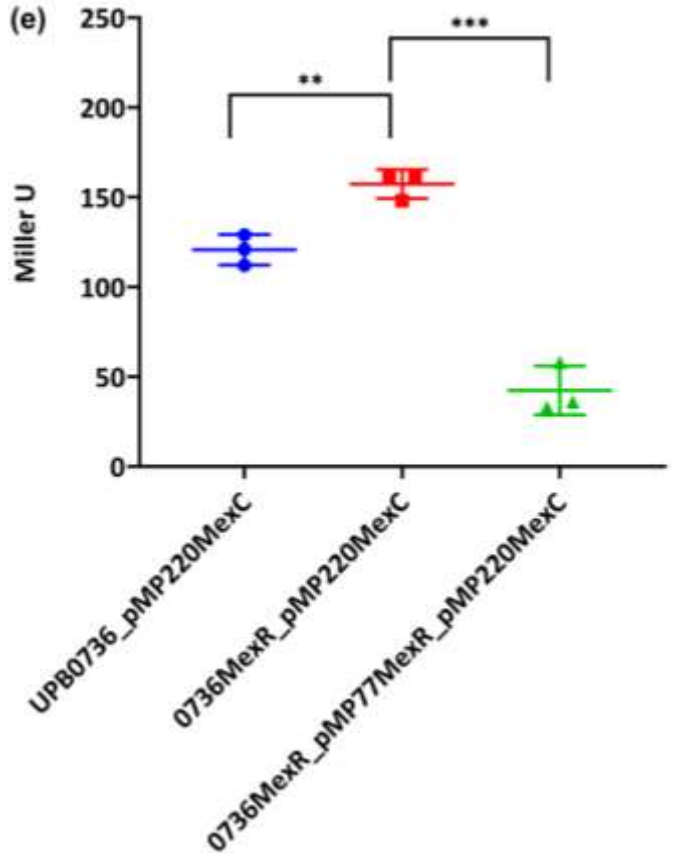

(e)
250
200
150
100
50
0
Miller U
**
***
UPB0736_pMP220MexC
0736MexR_pMP220MexC
0736MexR_pMP77MexR_pMP220MexC

Figure 4. 3 MexR repressor is involved in the regulation of the *pfsI* gene promoter and the *mexC* gene promoter.

(a) Upregulation of *pfsI* gene promoter at three time points (4h, 8h and 24h), 0h represent the starting time. The upregulation of *pfsI* gene promoter was studied in different *P. fuscovaginae* strains; UPB0736 (*P. fuscovaginae* wild type), 0736RSAM (*P. fuscovaginae* mutated in *rsaM* gene), 0736MexR (*P. fuscovaginae* mutated in *mexR* gene) and 0736MexR_pMP77MexR (*P. fuscovaginae* mutated in *mexR* gene and complemented by pMP77 plasmid vector harbouring the *mexR* gene); the activity of *pfsI* gene promoter increased during the time in the 0736RSAM and 0736MexR compared to wild type and the complemented mutant of 0736MexR.

(b) MexR repressor is involved in the regulation of *pfsI* gene promoter;

(c) Exogenous AHLs (C10-AHL and C12-AHL exogenously provided at 1 µM) activated the *pfsI* gene promoter. Measurements were made at 4H of growth. Exogenous AHLs activated pfsI in *P. fuscovaginae* WT, 0736MexR mutant and its complemented 0736MexR_pMP77MexR mutant.

(d) MexR regulates its own transcription. The *mexR* gene promoter is upregulated in the 0736MexR mutant and restored to WT level in its complemented mutant 0736MexR_pMPMexR where the pMP220MexR corresponds to the *mexR*-lacZ transcriptional fusion.

(e) MexR regulates the efflux pump *mexC* gene. The *mexC* gene promoter is upregulated in the 0736MexR mutant and restored to WT level in its complemented mutant 0736MexR_pMP77Mex; the pMP220MexC corresponds to the *mexC*-lacZ transcriptional fusion. (ns: no significative, **: p-value < 0.01, ***: p-value < 0.001, ****: p-value<0.0001)

4.3.4. The MexR repressor is also involved in the regulation of the AHL levels

Since the mexR::Tn5 mutant was isolated in the screening for AHLs signals hyperproduction, it was also of interest to determine the AHLs signals production profile of the *mexR* genomic mutant 0736MexR. LC-MS was used to identify and quantify the AHLs signals produced by several *P. fuscovaginae* strains including the *mexR*, *rsaM* and *pfsI* genomic mutants. Results clearly show that the 0736RSAM mutant produced much larger quantities of AHLs signals as previously reported (Mattiuzzo et al., 2011); interestingly also the 0736MexR mutant produces larger quantities of AHLs signals with respect to the wild-type (**Figure 4.4, Table 4.2**). This increase in AHLs signals production is in line with the screening performed since it was based on increased production of AHLs signals. As determined above; higher AHLs signals concentrations result in stronger *pfsI* promoter activities (see above). In summary, the *mexR* mutant also resulted in higher AHLs signals production in *P. fuscovaginae*.

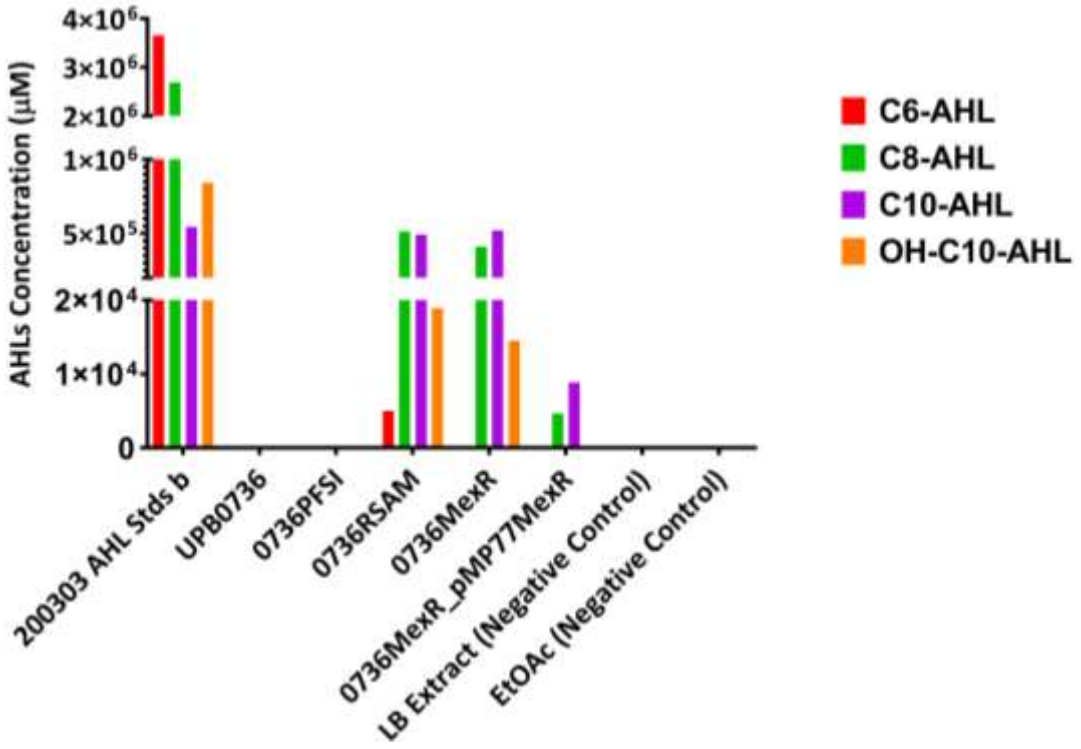


Figure 4. 4 AHLs quantification in *P. fuscovaginae* strains

UPB0736, wild type; 0736PFSI, mutated in AHL synthase *pfsI* gene; 0736RSAM, mutated in rsaM repressor gene; 0736MexR, mutated in mexR repressor gene; 0736MexR_pMP77MexR, *mexR* mutant complemented by the pMP77MexR plasmid vector harbouring the *mexR*; the AHL standards were C4-AHL, C6-AHL, C8-AHL, C10-AHL, C12-AHL, C14-AHL and their derivatives (oxo or hydoxy at position C3). The 0736MexR mutant showed higher production of C8, C10 and OH C10 AHLs like the 0736RSAM mutant, compared to wt. the UPB07360736PFSI and the negative controls (LB; culture medium and EtOAC; extracting solvent) did not show any AHLs signals presence.

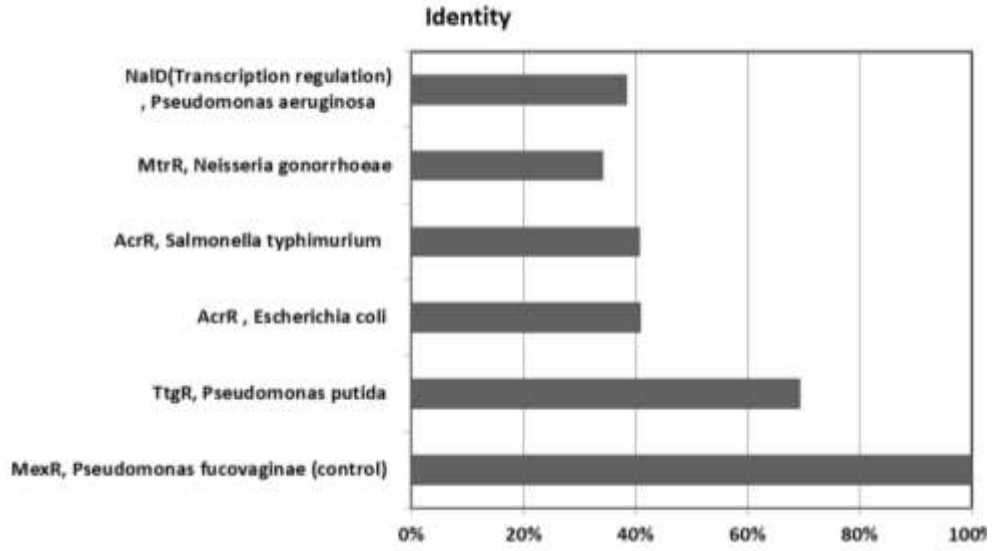
Table 4. 2 Quantification of AHLs signals in *P. fuscovaginae* strains

Sample Name	C6-AHL(µM)	C8-AHL(µM)	C10-AHL(µM)	OH-C10-AHL(µM)
200303 AHL Stds b	3.66E+06	2.69E+06	5.44E+05	8.42E+05
UPB0736	0.00E+00	0.00E+00	0.00E+00	0.00E+00
0736PFSI	0.00E+00	0.00E+00	0.00E+00	0.00E+00
0736RSAM	5.03E+03	5.14E+05	4.92E+05	1.89E+04
0736MexR	0.00E+00	4.10E+05	5.20E+05	1.45E+04
0736MexR_pMP77MexR+Cm	0.00E+00	4.67E+03	8.85E+03	0.00E+00
LB Extract (Negative Control)	0.00E+00	0.00E+00	0.00E+00	0.00E+00
EtOAc (Negative Control)	0.00E+00	0.00E+00	0.00E+00	0.00E+00

4.3.5. MexR repressor in other bacteria

It was of interest to determine if MexR had homologues in other bacteria; an homology search resulted in the identification of five known proteins with 3D structures that displayed homology to MexR (**Figure 4.5a**); all are regulatory proteins; namely TtgR of *P. putida*, AcrR of *E. coli* and *Salmonella typhimurium*, MtrR of *Neisseria gonorrhoeae* and NalD of *P. aeruginosa*. In some cases the homolog of the MexR repressor gene was found next to multidrug efflux system in a similar way adjacent to the *mexC* gene; for example the MexR homolog AcrR of *E. coli* regulates the transcription of the adjacent *acrAB* operon (Dzwokai Ma et al., 1996). Interestingly, the homolog of MexR in *P. aeruginosa*, the NalD repressor, is involved in the transcriptional regulation of the *mex*AB-*oprM* multidrug efflux operon (Sobel et al., 2005) (**Figure 4.5b,c**). The *P. fuscovaginae* multidrug efflux RND membrane protein *mexC* gene does not form an operon with the other two components (*cmeB* and *cmeC*) of the RND efflux system as is the case in *P. aeruginosa* and *E. coli* **(Figure 4.5d)**. The *cmeB* and *cmeC* loci are the likely other components of the RND efflux pump and are organized in an operon and present elsewhere in the chromosome of *P. fuscovaginae* (**Figure 4.5e**). However since *mexR-mexC* and *cmeBC* are located at the border of different contigs of the draft genome of *P. fuscovaginae* UPB0736, it cannot be excluded that they form an operon. It is currently unknown whether *cmeBC* are regulated by MexR.

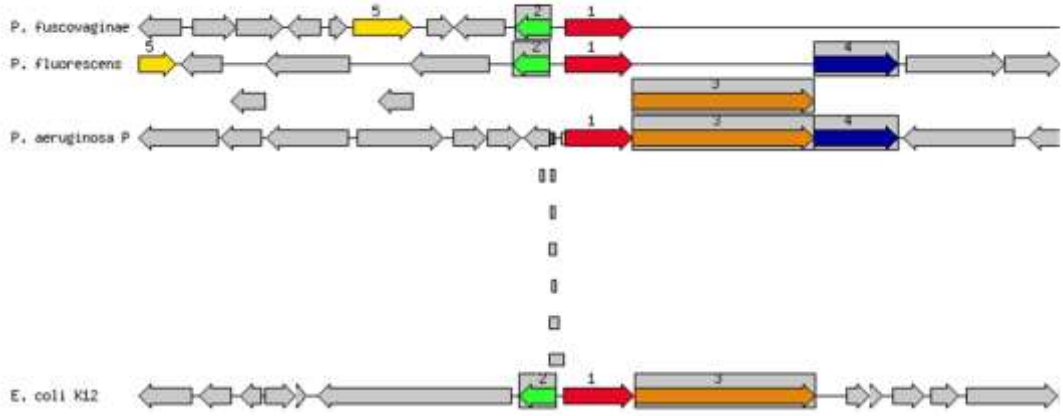
(a)


Identity
NalD(Transcription regulation)
, Pseudomonas aeruginosa
MtrR, Neisseria gonorrhoeae
AcrR, Salmonella typhimurium
AcrR , Escherichia coli
TtgR, Pseudomonas putida
MexR, Pseudomonas fucovaginae (control)
0%
20%
40%
60%
80%
100%

(b)

(c)

(d)


P. fuscovaginae
P. fluorescens
P. aeruginosa P
E. coli K12

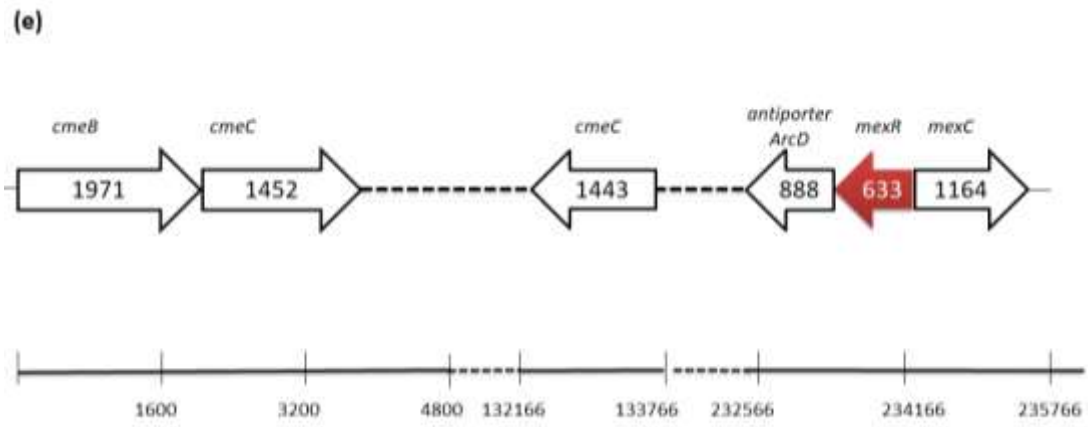


Figure 4. 5 MexR repressor and other bacterial regulators.

(a) The highest identity (>60 %) is with TtgR from *Pseudomonas putida* and the lowest is with MtrR 34.30% from *Neisseria gonorrhoeae*.

(b) Alignment of the fives regulators TtgR, ArcR, NaID and MtrR with MexR of *P. fuscovaginae;* (yellow colour : domain; brown colour: DNA binding domain; symbols *, . and : represent the similarities.

(c) Phylogeny tree of the five proteins (TtgR from *Pseudomonas putida*; ArcR from *Escherichia coli* and *Salmonella typhimurium*, NalD from *Pseudomonas aeruginosa* and MtrR from *Neisseria gonorrhoeae* compared to the MexR sequence from *P. fuscovaginae*.

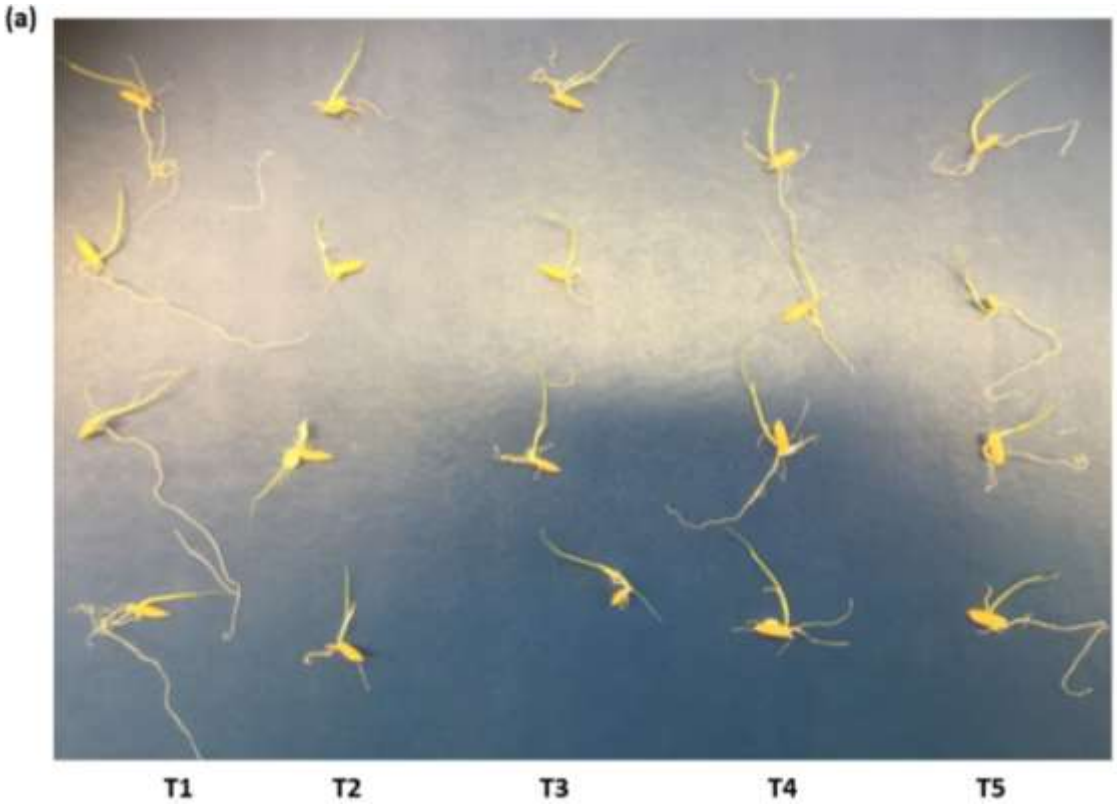
(d) Organization of Efflux Pump genes in three *Pseudomonas sp.* (*P. fuscovaginae*, *P. fluorescens* and *P. aeruginosa*) and *E. coli*; (2) MexR, AcrR; (1) MexC, CmeA, AcrA; (3) CmeB, AcrB; (4) CmeC**.**

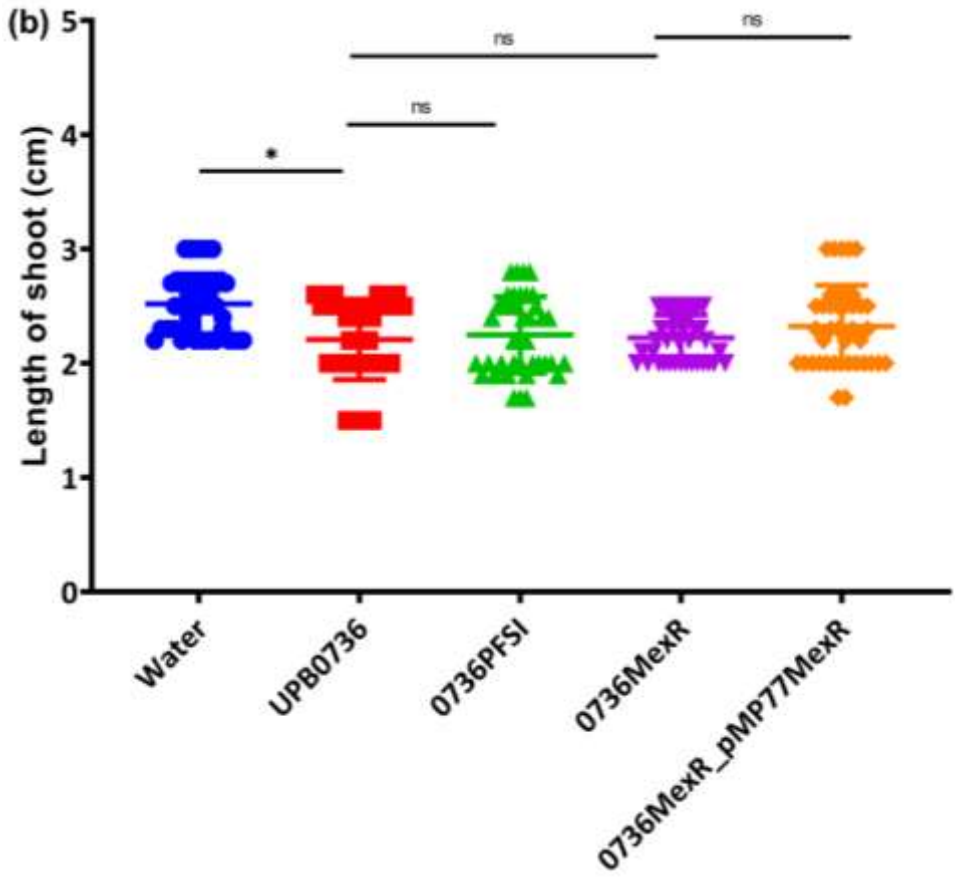
(e) Presence of the *cmeB* and *cmeC* genes two transcriptional components of RNB efflux pump in *P. fuscovaginae* chromosome

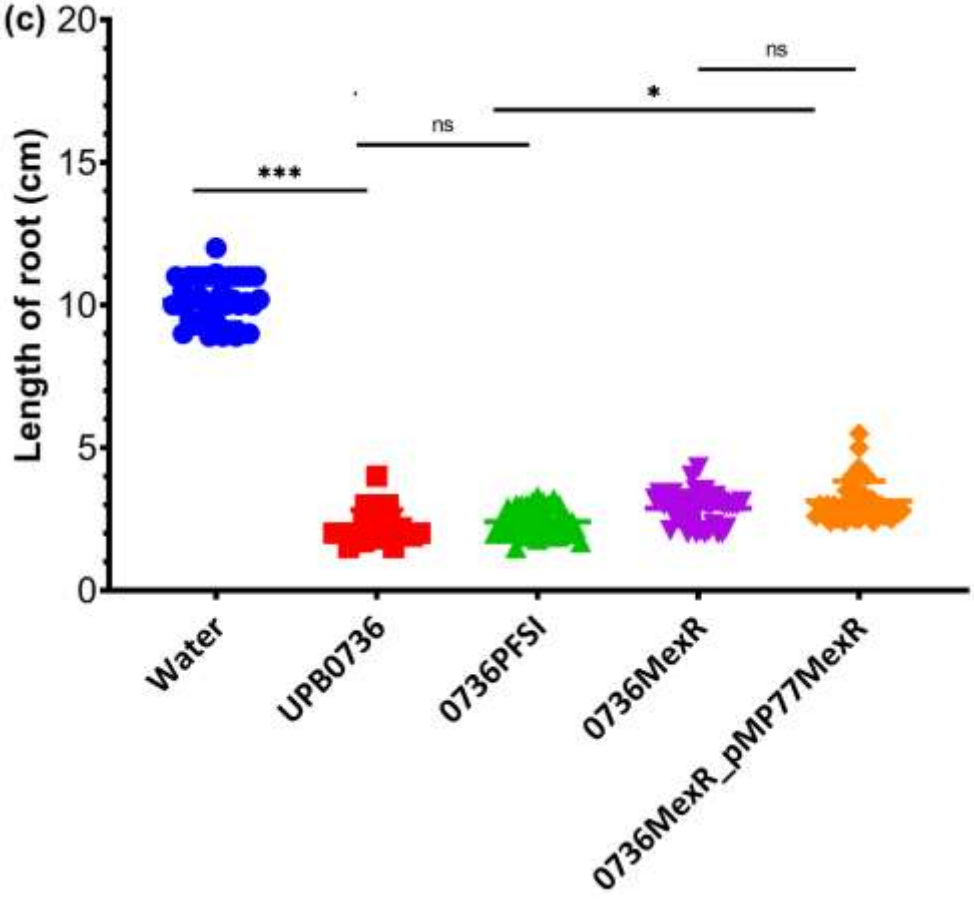
4.3.6. MexR is involved in virulence

It was of interest to determine whether the *mexR* mutant, that had elevated levels of AHLs signals production and higher transcriptional activity of the *pfsI* AHL synthase, was affecting in planta virulence. Two plant virulence assays were performed; one on rice seeds during germination and the other on two weeks old rice seedlings. The assays on rice seeds resulted in germination being 100% for all the strains indicating that 0736MexR mutant had no effect on germination. In addition, the length of the principal root and the shoot (first phases of growth) was measured and no differences were detected of the *mexR* mutant with respect to the wild-type (**Figure 4.3a,b,c**). It is noted that the average length of the principle root from the rice seeds germinated in water controls were five times longer than the ones which were inoculated with the *P. fuscovaginae* strains (**Figure 4.6a,b**). This indicated that the presence of *P. fuscovaginae* affected the initial phase of rice root plant development after germination

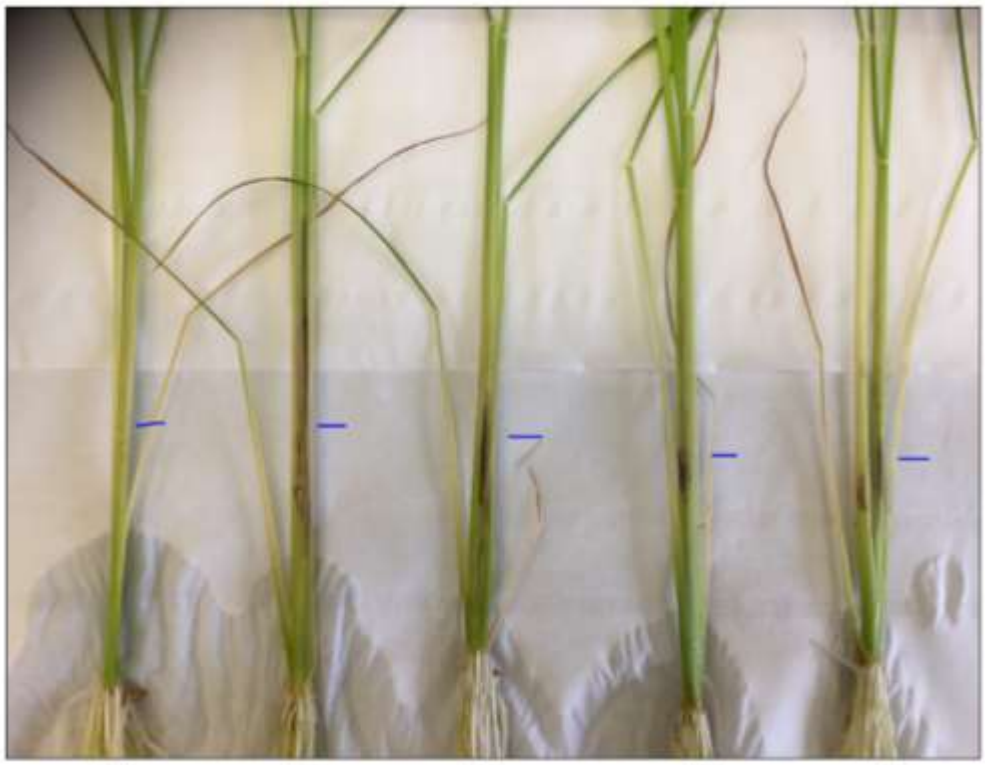
however *mexR* and QS do not play a role in this. The virulence assay on two weeks old rice seedlings on the other hand, resulted in the 0736MexR mutant displaying less virulence than the wild type strain. As previously reported, also QS mutants were less virulent than the wild type *P. fuscovaginae* strain **(Figure 4.6d,e)**. Statistical analysis evidenced that plants infected with 0736MexR were less virulent than the *P. fuscovaginae* wild type (p-value<0.001), however there was no significant difference in the *mexR* complemented mutant. The reason for this lack of complementation is not known, it could be due to plasmid loss since in the complemented strain, *mexR* is harboured in a plasmid and *in planta* no selective pressure can be applied. It was concluded that the MexR is involved in the virulence of *P. fuscovaginae*.




(b)
Length of shoot (cm)
0
1
2
3
4
5
*
ns
ns
ns
Water
UPB0736
0736PFSI
0736MexR
0736MexR_pMP77MexR


(c)
Length of root (cm)
0
5
10
15
20
***
ns
*
ns
Water
UPB0736
0736PFSI
0736MexR
0736MexR_pMP77MexR

(d)


T1
T2
T3
T4
T5

(e)

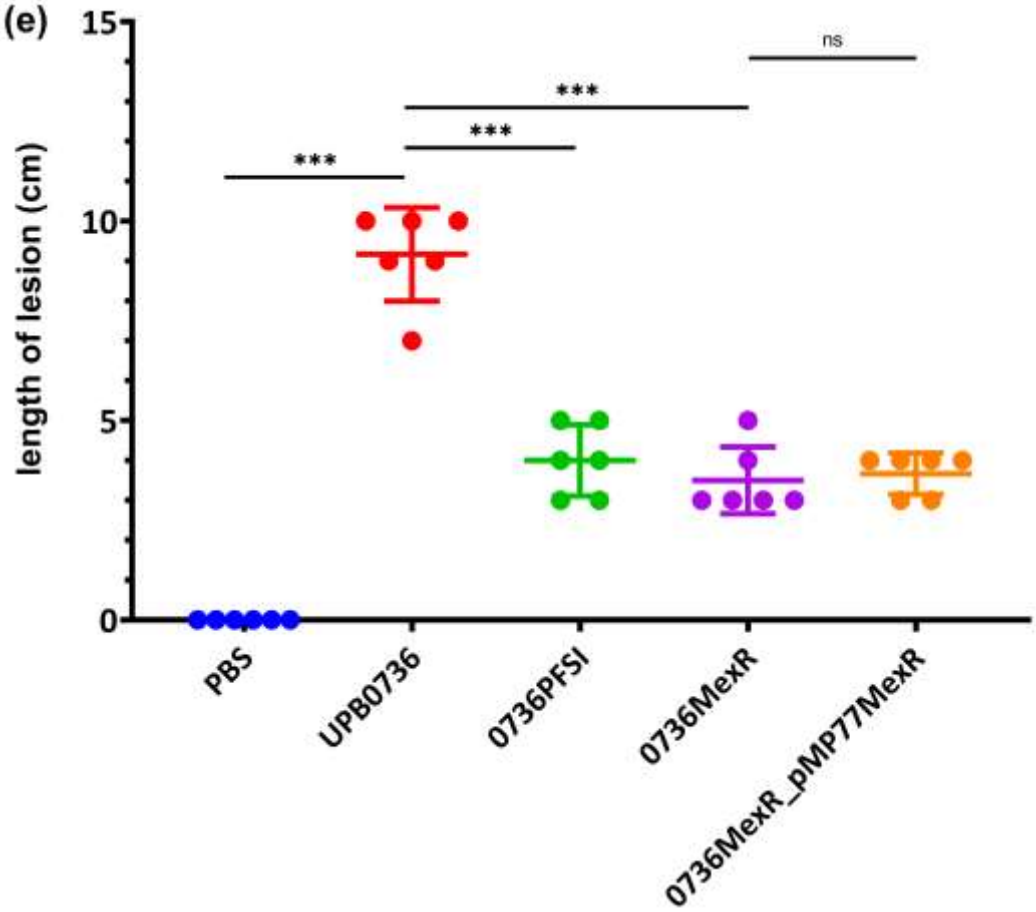

length of lesion (cm)
15
10
5
0
***
***
***
ns
PBS
UPB0736
0736PFSI
0736MexR
0736MexR_pMP77MexR

Figure 4. 6 Virulence assay on rice seeds germination and on two week old rice seedlings.

(a) The presence of the *P. fuscovaginae* strains did not affect the germination of rice seeds, all seeds treated were germinated (T1: treatment with water, controls; T2: UPB0736; T3: 0736PFSI; T4: 0736MexR; T5: 0736MexR_pMP77MexR)

(b) The presence of *P. fuscovaginae* strains did not affect the shoot development, length of shoot average of shoot being the same in treated samples as samples treated by water

(c) The presence of *P. fuscovaginae* affected the root development; length average of root in samples treated by water (control) was high than to those treated by the *P. fuscovaginae* strains

(**d)** The plants infected by *P. fuscovaginae* strains displayed symptoms (T1: treatment with PBS, controls; T2: UPB0736; T3: 0736PFSI; T4: 0736MexR; T5: 0736MexR_pMP77MexR)

(e) *P. fuscovaginae* WT was high virulent, 0736MexR mutant and its complemented mutant did not display any difference in the virulence. (ns: no significative, *: p-value < 0.05, ***: p-value < 0.001)

4.4. Discussion

In this study two genetic screens on *P. fuscovaginae* were performed with the aim to identify negative regulators of AHL QS that might be acting in concert with the novel and non-DNA binding negative regulator RsaM. Both screenings resulted in the isolation of mutants in a transcriptional repressor, designated as MexR, located adjacent to the RND efflux pump gene *mexC*. MexR negatively regulates *pfsI* (affecting AHLs signals production) and *mexC* transcription and is involved in virulence.

MexR belongs to the TetR family of regulators and some of its members are involved in the regulation of the multidrug resistance (MRD) efflux pumps genes in *E. coli* (Dzwokai Ma et al., 1996), in *Acinetobacter nosocomialis* (Subhadra et al., 2018), in *Neisseria gonorrhoeae* (Lucas et al., 1997) and in *P. aeruginosa* (Evans et al., 2001; Saito et al., 1999). The MRD efflux pumps are involved in several bacterial community phenotypes including pathogenicity, QS and biofilm formation (Alvarez-Ortega et al., 2013; J. Sun et al., 2014). The RND is one of the types of MRD efflux pumps and examples include AcrAB-TolC of *E. coli* (D. Ma et al., 1993; Dzwokai Ma et al., 1995) and MexAB-OprM, MexCD-OprJ, MexEF-OprN of *P. aeruginosa* (Gotoh et al., 1995; Köhler et al., 1997; K. Poole et al., 1993; Keith Poole et al., 1996). The MexC of *P. fuscovaginae* reported here is orthologous to the one present in *P. aeruginosa.* MexR regulates transcription of the RND component *mexC*; the other two components of this RND system are present elsewhere as an operon in the *P. fuscovaginae* chromosome (**Figure 4.5e**) and are also likely to be regulated by MexR. It cannot be excluded that the other two components constitute an operon with *mexC* since they are found at the border to two contigs. Future studies will need to determine this possibility.

It has been reported that longer AHLs signals molecules can be trafficked via membrane vesicles (MVs) (Morinaga et al., 2018) and can also be transported by efflux pumps (Black et

al., 1987; Krol & Becker, 2014; Pearson et al., 1999; Van Den Berg et al., 2004). The data presented in this study showed that the *mexC* gene is negatively regulated by MexR thus this RND efflux pump could play a role in AHLs signals transport since a *mexR* mutant produces higher levels of AHLs signals molecules. MexC could play a role in facilitating AHLs signal traffic/transport in *P. fuscovaginae*. It must be noted however that in the *mexR* mutant the transcription of the AHL synthase *pfsI* increases; higher AHLs signals levels in the *mexR* mutants therefore could be due to increased *pfsI* transcription and not to higher AHL transport via increased levels of MexC. Currently it is not known whether MexR is involved directly in the negative transcriptional regulation of *pfsI* or possibly via interaction with RsaM or other proteins; future studies need to determine this. The fact that providing high levels of exogenous AHLs signals also increases *pfsI* gene transcription as reported here argues towards MexR affecting AHL QS via the regulation of the efflux pump.

In *P. aeruginosa,* the efflux pumps MexAB-OprM is involved in the extrusion of the long-chain LasI produced 3-oxo-C12-HSL. Interestingly, overexpressing MexAB results in low accumulation of QS signals making *P. aeruginosa* less virulent (Evans et al., 1998; Minagawa et al., 2012; Sanchez, 2002). In addition, the deletion of MexGHI, another efflux pump in *P. aeruginosa*, reduces the production and secretion of AHLs signals (Aedekerk et al., 2005). Similarly, in the pathogen *Burkholderia pseudomallei*, the secretion of long-chain AHL quorum sensing signals relies in part to the MDR efflux pump BpeAB–OprB (Chan & Chua, 2005; Ying et al., 2007). It is therefore becoming a common feature that longer chain AHLs signals are at least in part transported by efflux pumps.

The *mexR* mutant of *P. fuscovaginae* displayed less virulence towards rice than the wild type. The reason for this is currently unknown; it has been observed that other AHLs signals overproducing mutant strains of *P. fuscovaginae* also displayed reduced virulence (Mattiuzzo et al., 2011) just like the null QS mutants. From these observations it is clear that QS in *P.*

fuscovaginae is involved in plant virulence (Mattiuzzo et al., 2011) and that its activity needs to be strictly modulated in order to infect rice and give rise disease symptoms. Either increasing or decreasing the activity of QS results in a decrease in pathogenicity indicating that QS synchrony and timing are of crucial importance for expressing the virulence factors at the most appropriate time for the pathogen. Finally QS in *P. fuscovaginae* could play an important role in the pathobiome via the interspecies or interkingdom interactions with other microorganisms.

Chapter V. Summarising discussion

5.1.Aim of this thesis

The main aim of this thesis was to study the microbial community of rice sheath rot and to begin to shed light on the biotic factors which participate in the disease process. This disease is a widespread being reported in several rice growing parts of the world including Burundi and it is associated to the bacterial pathogen *P. fuscovaginae* and the fungal pathogen *S. oryzae*. This study included (i) the comparative analysis of the microbial community (bacteria and fungi) from asymptomatic and symptomatic samples of rice sheath rot collected in highland and lowland in Burundi, (ii) analysis of the culturable bacterial microbiome of asymptomatic rice sheath samples from rice grown next to infected rice plants, (iii) generation and characterization of a bacterial culture collection from asymptomatic rice samples ,(iv) investigation on the unique regulation of the *P. fuscovaginae* cell-cell signaling system and its involvement in the sheath rot disease development.

5.2.Microbiome and pathobiome studies revealed that *P. fuscovaginae* and *S. oryzae* are independently associated to rice sheath rot

Microbiome and pathobiome studies of asymptomatic and symptomatic rice sheath samples revealed that *P. fuscovaginae* is more abundant in the symptomatic samples from the highland in Burundi and the *S. oryzae* in lowland samples during the wet season. Plant genotype was not a major driver, whereas altitude was an important factor promoting the colonisation of *Pseudomonas*. In lowland on the other hand, the *Pantoea* bacterial genus was significantly abundant in symptomatic samples and it is currently unknown if it is involved in the sheath rot disease. *Saraclodium* sp. was significantly abundant in symptomatic samples in lowland during the wet season along with the fungus *Bipolaris* resulted to be abundant in all symptomatic samples. It is currently unknown whether *Bipolaris* sp. is involved in the sheath rot disease process. The microbiome/pathobiome analyses revealed that several microbes (bacteria and fungi) were significantly and differentially present/more abundant in the

symptomatic or in the asymptomatic samples, thus it cannot be excluded that interaction among members of the microbial community could have a role in the process/severity of the or in the control of the disease.

5.3. Culturable bacteriome of asymptomatic rice sheath samples

Microbiome and pathobiome studies showed that *P. fuscovaginae* was present at high abundance in highland; it was therefore of interest to analyze the culturable bacterial microbiome of asymptomatic samples from the highland areas in Burundi, where the incidence of rice sheath rot infection is dramatically high. The rationale behind this experiment was that healthy rice plants might contain a microbiome which protects the plant from invasion and colonisation of *P. fuscovaginae*.

From the comparison of the data deriving from the total and culturable microbiomes, a total of 215 taxa were present in the wet season of 2017. Of these, 49.7% resulted from the analysis of the total microbiome, 29.7% of the taxa emerged only from the analysis of the culturable microbiome and 20.5 % were present in both analyses. The same proportions occurred again analyzing the total and culturable communities present in the samples of dry season of 2018 (45.7%, 21% and 33% of the taxa present only in the total analysis, in both of them and only in the culturable one, respectively). These differences are likely to be due to (i) some genera are not culturable, (ii) some genera do not grow in the chosen growth conditions, (iii) some taxa were favoured by the growth conditions chosen and (iv) some taxa were present in very low amounts in the plant samples.

5.4. Generation and characterization of a bacterial culture collection

A collection of approximately 150 bacterial isolates was generated and characterized; several isolates belong to the genera *Microbacterium*, *Methylobacterium*, *Sphingomonas* and *Bacillus*. Interestingly, one bacterial isolate belonging to *Alcaligenes* genus displayed strong

antibacterial activity in vitro against *P. fuscovaginae*; it cannot be excluded that this member of the microbiome could be involved in the control of *P. fuscovaginae,* likely in cooperation with other microbes of the community. It is likely that the microbial community plays an important role in the establishment or control of a biotic disease, thus the interaction and signalling mechanisms between microbes is an important aspect to investigate. It was therefore of interest to perform cell-cell signalling studies of *P. fuscovaginae* and its relation to virulence.

5.5. *P. fuscovaginae* quorum sensing studies

P. fuscovaginae possesses a complex quorum sensing response which is switched on *in planta* condition inducing the transcription of several virulence factors and playing a fundamental role in the development of rice sheath rot disease. Surprisingly, quorum sensing in *P. fuscovaginae* is not working and hence switched off *in vitro*. It has been postulated that the microbial community at the site of infection might play a role in switching on the quorum sensing via cell-cell interspecies and/or interkingdom interactions. The quorum sensing system of *P. fuscovaginae* is stringently controlled by a novel repressor called RsaM which is not a DNA-binding protein and it is currently unknown the cascade that leads to the transcriptional repression of quorum sensing genes. RsaM repressor could be involved in responding to signals coming from other members of the pathobiome, resulting in the regulation of the expression of several quorum sensing genes which are implicated in the development of the sheath rot disease. Molecular studies were aimed to identify possible members of the RsaM cascade which regulate quorum sensing in *P. fuscovaginae*. Two different genetic screens led to the identification of multiple mutants in the same gene, designated *mexR* that encodes for a TetR family transcriptional repressor adjacently located to an operon encoding for an RND efflux pump. It was demonstrated that the MexR repressor has a role in the regulation of AHLs signals production levels and in the regulation of

transcription of the AHL synthase *pfsI*. Importantly it has also been determined that *mexR* negatively regulates the efflux pump hence the increase in AHLs signals levels and in *pfsI* transcription could be indirectly due to the improved transport of AHLs signals.

It is now important to determine whether MexR is part of the cascade involved in the QS regulation via RsaM and/or is affecting quorums sensing via the regulation of the efflux pump involved in the transport of AHLs signals. Interestingly the *P. fuscovaginae mexR* mutant was less virulent in *vivo* and is therefore involved in the regulation of the virulence of *P. fuscovaginae*.

5.6. Future directions

Pathobiome studies could be intensified in order to establish possible microbial partners and co-operators of the pathogens considering the effect/impact of the entire microbial community in the disease process. Similarly, microbiome studies of healthy plants could be fundamental to identify keystone members that are likely to be involved in antagonising and keeping away the pathogens. In addition, a bacterial isolate belonging to *Alcaligenes* genus has been identified showing antibacterial activity against *P. fuscovaginae* in vitro conditions; in vivo assays are needed to establish whether this isolate is involved in the control of disease and whether it can be developed as a biocontrol agent. Furthermore, it has been seen that very likely the entire microbial community is important in the regulation of the *P. fuscovaginae* pathogenesis traits through the involvement of the quorum sensing mechanism and the regulation of a new transcriptional repressor; future works are needed to determine the complete molecular pathway that lead to the control of the quorum sensing in *P. fuscovaginae*.

Chapter VI. References

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Chapter VII. Appendix

List of rice plant samples used to extract total microbial DNA.

The samples are arranged by rice variety, symptomatology, sampling location (ecology) and sampling date (a): wet season, (b): dry season). 48 samples have been collected from each season, divided in 24 symptomatic and 24 asymptomatic. ShRBDI stands for Sheath of rice-Burundi

(a)

Designation	Varieties	Sample symptomology	Ecology	Location	Date
ShRBDI-01	BG90-2	Symptomatic	Highland	Gisha	June 2017
ShRBDI-02	BG90-2	Symptomatic	Highland	Gisha	June 2017
ShRBDI-03	BG90-2	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-04	BG90-2	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-05	IR2793-80-1	Symptomatic	Highland	Gisha	June 2017
ShRBDI-06	IR2793-80-1	Symptomatic	Highland	Gisha	June 2017
ShRBDI-07	IR2793-80-1	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-08	IR2793-80-1	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-09	ITA 304	Symptomatic	Highland	Gisha	June 2017
ShRBDI-10	ITA 304	Symptomatic	Highland	Gisha	June 2017
ShRBDI-11	ITA 304	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-12	ITA 304	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-13	NTNB	Symptomatic	Highland	Gisha	June 2017
ShRBDI-14	NTNB	Symptomatic	Highland	Gisha	June 2017
ShRBDI-15	NTNB	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-16	NTNB	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-17	ZAMBIA	Symptomatic	Highland	Gisha	June 2017
ShRBDI-18	ZAMBIA	Symptomatic	Highland	Gisha	June 2017
ShRBDI-19	ZAMBIA	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-20	ZAMBIA	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-21	KIGEGA	Symptomatic	Highland	Gisha	June 2017
ShRBDI-22	KIGEGA	Symptomatic	Highland	Gisha	June 2017
ShRBDI-23	KIGEGA	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-24	KIGEGA	Asymptomatic	Highland	Gisha	June 2017
ShRBDI-25	BG90-2	Symptomatic	Highland	Gihanga	June 2017
ShRBDI-26	BG90-2	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-27	BG90-2	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-28	BG90-2	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-29	IR2793-80-1	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-30	IR2793-80-1	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-31	IR2793-80-1	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-32	IR2793-80-1	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-33	ITA 304	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-34	ITA 304	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-35	ITA 304	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-36	ITA 304	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-37	NTNB	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-38	NTNB	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-39	NTNB	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-40	NTNB	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-41	ZAMBIA	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-42	ZAMBIA	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-43	ZAMBIA	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-44	ZAMBIA	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-45	KIGEGA	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-46	KIGEGA	Symptomatic	Lowland	Gihanga	June 2017
ShRBDI-47	KIGEGA	Asymptomatic	Lowland	Gihanga	June 2017
ShRBDI-48	KIGEGA	Asymptomatic	Lowland	Gihanga	June 2017

(b)

Designation	Varieties	Sample symptomology	Ecology	Location	Date
ShRBDI-01	Muguiza	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-02	Muguiza	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-03	Muguiza	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-04	Muguiza	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-05	Muguiza	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-06	Muguiza	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-07	Muguiza	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-08	Muguiza	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-09	Kazosi	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-10	Kazosi	Symptomatic	Lowland	Gihanga	Dec-12
ShRBDI-11	Kazosi	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-12	Kazosi	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-13	Kazosi	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-14	Kazosi	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-15	Kazosi	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-16	Kazosi	Symptomatic	Lowland	Gihanga	Dec-18
ShRBDI-17	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-18	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-19	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-20	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-21	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-22	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-23	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-24	Kazosi	Asymptomatic	Lowland	Gihanga	Dec-18
ShRBDI-25	OYT108 RGA 010 234	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-26	OYT108 RGA 010 234	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-27	OYT108 RGA 010 234	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-28	OYT108 RGA 010 234	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-29	OYT108 RGA 010 234	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-30	OYT108 RGA 010 234	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-31	OYT111RGA 011 9	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-32	OYT111RGA 011 9	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-33	OYT111RGA 011 9	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-34	OYT111RGA 011 9	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-35	OYT111RGA 011 9	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-36	OYT111RGA 011 9	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-37	OYT113 RGA 011 17	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-38	OYT113 RGA 011 17	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-39	OYT113 RGA 011 17	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-40	OYT113 RGA 011 17	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-41	OYT113 RGA 011 17	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-42	OYT113 RGA 011 17	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-43	OYT120 RGA 011 51	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-44	OYT120 RGA 011 51	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-45	OYT120 RGA 011 51	Symptomatic	Highland	Gisha	Dec-18
ShRBDI-46	OYT120 RGA 011 51	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-47	OYT120 RGA 011 51	Asymptomatic	Highland	Gisha	Dec-18
ShRBDI-48	OYT120 RGA 011 51	Asymptomatic	Highland	Gisha	Dec-18