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Characterization of sheath rot pathogens from major rice-growing areas in Rwanda

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title: Karakterisatie van pathogenen die "sheath rot" veroorzaken in de belangrijkste rijstgebieden in Rwanda

Cover illustration: Some sheath rot disease features:

- Left upper side: microscopic picture of the reverse side of *Fusarium andiyazi* isolate RFNG10 on PDA medium;
- Left lower side: microscopic picture of the front side of Fusarium andiyazi isolate RFNG10 isolate on PDA medium;
- Center: illustration of rice sheath rot symptoms on a rice plant;
- Right side: illustration of a phylogenetic tree of *Pseudomonas* isolates associated with rice sheath rot symptoms in Rwanda and the Philippines.

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LIST OF ABBREVIATIONS

ACP	Acyl carrier protein
ANOVA	Analysis of variance
API	Analytical profile index
APS	Adenosine-5'-phosphosulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BTC	Belgian Technical Cooperation
CABI	Centre for Agriculture and Biosciences International
CAVM	College of Agriculture, Animal Science and Veterinary Medicine
CBS	Centraalbureau voor Schimmelcultures/ *KNAW Fungal Biodiversity Centre*
CMC	Carboxymethyl cellulose
CoA	Coenzyme A
DNH	Dihydroxynaphtalene
EDGAR	Efficient Database framework for comparative Genome Analyses using BLAST score Ratios
efe	ethylene forming enzyme
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPS	Extracellular polymeric substances
FAO	Food and Agriculture Organisation
FB	Fumonisin B
FP-A	Fuscopeptin-A
FP-B	Fuscopeptin-B
FSNWG/MAS	Food Security &Nutrition Working Group/Market Analysis Subgroup
GDP	Gross Domestic Product
GRiSP	Global Rice Science Partnership
ICGEB	International Centre for Genetic Engineering and Biotechnology
IPM	Integrated Pest Management
IPPC	International Plant Protection Convention
IRRI	International Rice Research Institute
ISPMs	International Standards for Phytosanitary Measures
ITS	Internal Transcribed Spacer
KB Medium	King's B Medium
LB Medium	Luria Bertani Medium
MEGA	Molecular Evolutionary Genetics Analysis
MINAGRI	Rwanda Ministry of Agriculture and Animal Resources
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NBY	Nutrient broth yeast extract agar
NISR	National Institute of Statistics of Rwanda
ORF	Open reading frame
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDI	Percent Disease Index
Pfv	*Pseudomonas fuscovaginae*
PRA	Pest Risk Analysis

PRB *Périmètre Rizicole de Butare* (Butare Rice-growing Area)
PTE Phosphotriesterase
QS Quorum Sensing
RAB Rwanda Agriculture Board
RAPD Random Amplified Polymorphic DNA
RAST Radioallergosorbent Test
RDB Rwanda Development Board
ROS Reactive Oxygen Species
rpm Revolutions per minute
SAP Shrimp Alkaline Phosphatase
SES Standard Evaluation System
SPS Measures Sanitary and Phytosanitary Measures
T3SS Type Three Secretion System
T6SS Type 6 Secretion System
TAE Tris-Acetate-EDTA
TEF Translational Elongation Factor
USAID United States Agency for International Development
VFDB Virulence Factor Database
WARDA West Africa Rice Development Association
WTO World Trade Organisation
YDC Yeast extract dextrose-$CaCO_3$

rpm Revolutions per minute

RSSP Rural Sector Support Project

SAP Shrimp Alkaline Phosphatase
SES Standard Evaluation System
SPS Measures Sanitary and Phytosanitary Measures
T3SS Type Three Secretion System
T6SS Type 6 Secretion System
TAE Tris-Acetate-EDTA
TEF Translational Elongation Factor

UCORIRWA *Union des Coopératives Rizicoles du Rwanda*
UN-WFP United Nations World Food Program

USAID United States Agency for International Development
VFDB Virulence Factor Database
WARDA West Africa Rice Development Association
WTO World Trade Organisation
YDC Yeast extract dextrose-$CaCO_3$

PROBLEM STATEMENT AND OBJECTIVES

Problem statement

Rwanda is a high-altitude tropical country, characterized by a mountainous landscape and is therefore called "land of the thousand hills". Rwanda has a surface of only 26 338 km^2 and has one of the highest population densities (415 people/km^2) and growth rates (2.9%) in Africa. Its economy is agriculture-based and in 2009, the sector contributed 34% to the Gross Domestic Product (GDP), employed 80% of the active population and generated 70% of foreign earnings (NISR, 2010). Agriculture is perceived as the leading sector for economic transformation (USAID, 2010; Bizimana et al., 2012) and the country considers that its agriculture must be highly productive, value-adding, market-oriented and integrated into the economy. In the part of Rwanda's GDP due to agriculture, 84% comes from food crops (World Bank, 2011; Gapusi et al., 2013) and rice is one of the most commercialized crops, as 47% of its produce is sold on markets (NISR, 2012).

Rice constitutes the primary staple food for more than half of the world population and Asia represents the largest producing and consuming region. In 2013, 740,902.53 metric tons of rice (paddy) were harvested worldwide (IRRI, 2015a). The largest rice-producing countries are China and India plus Indonesia, Bangladesh, Vietnam, Thailand, and Myanmar. The Asian top seven make up 80% of the world's production (GRiSP, 2013). In Latin America, rice is principally produced in Brazil, Peru, Colombia and Ecuador. In the United States rice production is dominant in California and the southern states near the Mississippi river. The leading European producers are Italy, Spain and Russia. Rice is also grown in Australia although its production is currently being threatened by recurring drought (GRiSP, 2013). In Africa, rice is a sought-after staple in many countries, the largest producers being Egypt, Madagascar and Nigeria.

In Rwanda, rice is one of the crops promoted by the agricultural policy. Other important food crops are maize, sorghum, banana, cassava, common bean, soyabean, and wheat. Major cash crops are coffee, tea, pyrethrum (Kathiresan, 2011; Bizimana et al., 2012) and sugar cane, an important industrial crop, grown and processed in the country. Since Rwanda aims to diversify its high-value and export products, efforts are being invested in horticultural products: pineapples, mangoes, avocadoes, passion fruit, macadamia nuts, French beans, dessert banana, courgettes, etc. (MINAGRI, 2009a). In 2012, rice was grown on approximately 12,000 ha and the production estimates were of 80,000 tonnes, with an average yield of 5.5 t/ha (RAB, 2013), which is far higher than the average in the Eastern and

Southern African countries which is below 2t/ha (Singh et al., 2010). The interest in rice production in Rwanda stems from many factors: (1) rice can be grown in marshland areas and as such relieve the increasing pressure on hillside land for food production, currently rice is produced on 12000 ha out of 66094 ha that can be used; (2) Rwanda has many rivers for irrigation and the annual rainfall is about 1500 mm/year (MINAGRI, 2013a); (3) rice is easy to handle and store, has a shorter growing season compared to other tropical crops like cassava and its by-products can be used to feed animals, as a substrate in mushroom production and for energy supply; (4) rice is a profitable crop in Rwanda thanks to relatively low production costs and two rice harvests per year are possible. For all these reasons, rice production has a high potential in Rwanda. It is currently grown in the Western, Southern and Eastern provinces of the country.

However, the national rice production lags far behind the demand and the country imports continuously increasing quantities of rice. Those imports are estimated at 41.13% of the national demand in 2012, from 28.57% in 2008 (RDB, 2014a). Studies have shown that the imports are of long grain rice *indica* type, which is preferred by the urban population, while the majority of the national production is of short grain *japonica* types, which are more adapted to the Rwanda rice-growing agroecology with a tropical climate tempered by the altitude (Promar, 2012). The locally produced rice is considered of lower quality and thus sold at a low price compared to the imported rice (Manneh and El Namaky, 2010; Stryker, 2013). Many attempts have been made to introduce long grain *indica* varieties in Rwanda, but they suffer from abiotic and biotic stress and usually fail in 2-3 growing seasons (RAB, 2014). Initiatives for developing rice varieties specifically adapted to the Rwandan environment, started in the early 1990s and were spearheaded by Japan and China. Unfortunately, these activities were interrupted by war and the genocide in 1994 (Promar, 2012).

Despite a high promise, there are various constraints to rice production in Rwanda, including poor quality of the available seed and grain and lack of knowledge about rice pests and diseases. One of the diseases typically associated with grain discolouration and the reduction of rice grain quality is rice sheath (brown) rot (Zeigler and Alvarez, 1987; Gopalakrishnan et al., 2010). This disease used to be prevalent on rice grown at high altitudes in countries such as Nepal, Japan, Madagascar and Burundi, although its intensity increased with the intensification of the agricultural systems, triggered by the necessity of feeding a rapidly growing human population (Mew et al., 2004b). Based on disease symptoms, there are indications that rice sheath rot may be present in Rwanda, causing poor grain quality. In other

parts of the world, rice sheath rot is caused by fungi like *Sarocladium oryzae* (Lakshmanan, 1993b; Gopalakrishnan et al., 2010), while the name rice sheath brown rot is used when the causal agent is *Pseudomonas fuscovaginae* (Zeigler and Alvarez, 1987), although both organisms cause very similar symptoms. Nothing is known about the causal agents in Rwanda, but since especially *Pseudomonas fuscovaginae* has been associated with sheath rot symptoms at high altitudes, it is to be expected that this pathogen is also present in Rwanda.

Since rice sheath rot disease can be a major cause of rice grain quality reduction, this study aims to understand more about this disease in Rwanda. Knowledge about the causal agents and their pathogenicity mechanisms is essential to develop strategies and practices for control and prevention, and also paves the way for breeding for resistance against sheath rot-causing pathogens.

Research objectives

This study has the following scientific questions:

1. What is the importance and impact of rice sheath rot in Rwanda?
2. Which are the pathogens causing rice sheath rot in Rwanda? Are *Pseudomonas fuscovaginae* and *Sarocladium oryzae*, the most important sheath rot pathogens, present in Rwanda?
3. What is the origin of the sheath rot pathogens found in Rwanda? Are they native or introduced with the plant material?
4. Is there an interaction between these organisms and the prevailing environmental conditions in Rwanda?
5. Which control options can be taken to face rice sheath rot?

At the start of the study we formulated the following hypotheses:

- Lack of adaptation to prevailing abiotic conditions in Rwanda makes introduced rice varieties more susceptible to pests and diseases such as sheath rot;
- Quick rice development in Rwanda, with limited capacity of control and research on pests and diseases, has resulted in the build-up of large populations of pests and diseases, including sheath rot causing pathogens, which also negatively affect the rice grain quality.

Thesis outline

In **Chapter 1** we give information on rice production in Rwanda, looking at its potential and constraints.

In **Chapter 2** we review the current knowledge about the rice sheath rot disease, focusing on the three major pathogens that have been associated with this disease: *Sarocladium oryzae*, *Fusarium* spp., and *Pseudomonas fuscovaginae*.

In **Chapter 3** we have tried to estimate the importance of rice sheath rot in Rwanda and presents data about disease incidence, disease severity and yield loss.

In **Chapter 4**, we isolated and characterized fungi associated with rice sheath rot symptoms in Rwanda. The main genera that were found are *Sarocladium* and *Fusarium* and isolates were identified by morphological and molecular techniques, tested for pathogenicity and toxin production (*Fusarium* spp.).

In **Chapter 5** we isolated and characterized bacteria associated with rice sheath rot in Rwanda and compared them with those found in comparable studies conducted in other parts of the world, especially in the Philippines. Unexpectedly, *Pseudomonas fuscovaginae* was not found, but many other Pseudomonas spp. appear to be associated with rice sheath rot.

In **Chapter 6** we provide a general discussion and develop perspectives for future research. .

The thesis also contains two annexes. **Annex 1** presents a study about virulence associated loci in *Pseudomonas fuscovaginae* that was carried out by researchers in Italy and Australia and to which we contributed by carrying out pathogenicity tests on rice. **Annex A2** contains a list of organisms that qualify as pests of phytosanitary importance in Rwanda useful for routine field inspections and to conduct Pest Risk Analysis (PRA) when doing trade and when receiving or importing seeds.

1 LITERATURE REVIEW ON RICE PRODUCTION IN RWANDA

Rice is one of the important food crops worldwide. It is a priority crop and a very popular food in Rwanda. Rice has been introduced in Rwanda in the 1950s (Stryker, 2013), experimental trials for production were conducted in the 1970s (Baker, 1970) and the country is promoting its development. It is the first most market-oriented crop in the country with 47% of the production being sold (NISR, 2012). It is consumed in all areas in Rwanda, be it urban or rural. Rice is grown in many parts of Rwanda. Potential zones for rice production are distributed throughout the country in the low and middle altitudes. The major rice growing areas in 2004 are presented in Table 1-1 (MINAGRI, 2005) and Figure 1-1. The potential for rice development is high in Rwanda: two production seasons can be organised per year, many marshlands can be developed for rice production and studies show that there are around 66,000 hectares (ha) that are potentially suitable for rice production while now only 12,000 ha are being used (MINAGRI, 2005).

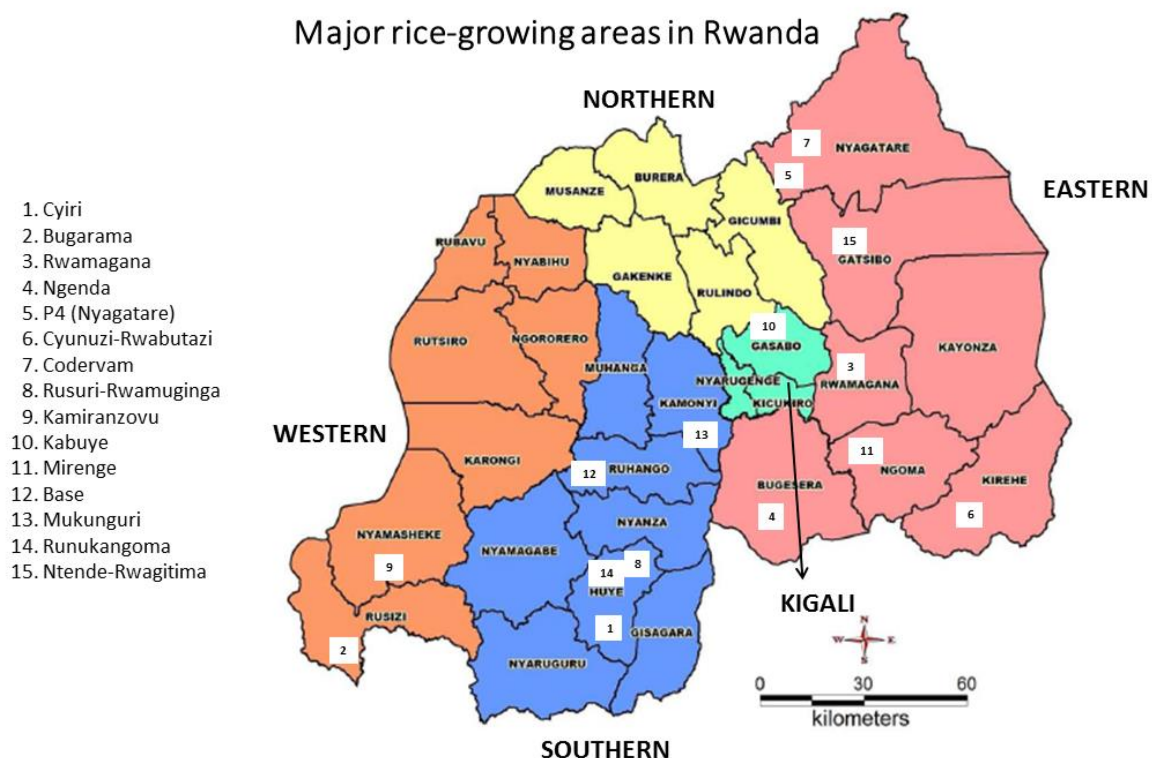

Figure 1-1: Major rice-producing areas in Rwanda

Table 1-1: Major rice-producing areas in Rwanda

Province	District	Sector*	Rice growing-area	Cultivable area/year (2 seasons)	Cultivated area/year (2 seasons)	Yield (t/ha)	Production (tons)**
Eastern	Rwamagana	Kigabiro	Rwamagana (3)	1343	713	7.4	5306
	Nyagatare	Nyagatare	P4 (5)	460	458	5.4	2483
			Codervam (7)	460	430	6.0	2582
	Kirehe	Gatore	Cyunuzi-Rwabutazi (6)***	400	433	4.5	1968
		Nasho	Nasho	160	70	3.0	210
		Mushikiri	Mushikiri	160	25	4.0	100
	Ngoma	Mugesera	Mirenge (11)	600	185	4.0	740
		Rurenge	Gisaya	300	72	7.5	516
		Rukira	Kibaya	240	43	7.0	301
	Gatsibo	Kigabiro	Ntende-Rwagitima (15)	120	86	5.7	490
	Kayonza	Ruramira	Ruramira	90	80	5.0	404
	Bugesera	Mareba	Mareba	200	20	3.0	60
		Mayange	Ngenda (4)	756	643	4.1	2673
	Touches districts of Gasabo, Rwamagana, Gatsibo and Kayonza	Touches many sectors	Muhazi	96	67	5.5	347
Kigali City	Gasabo	Jabana	Kabuye (10)	344	296	2.3	696
Southern	Huye	Rusatira	PRB (8 perimeters including Cyiri) (1)	4358	2621	3.5	9036
		Ruhashya	Rusuri-Rwamuginga-Cyarubare (8)	600	350	1.8	637
			Runukangoma (14)	170	140	3.2	454
	Ruhango	Bweramana	Base (12)	170	170	7.0	1198
	Kamonyi	Mugina	Mukunguri (13)	440	153	2.0	320
Western	Rusizi	Bugarama	Bugarama (2)	2984	2322	6.6	15324
	Nyamasheke	Kagano	Kamiranzovu (9)	460	332	3.3	1098

Adapted from: MINAGRI (2005b). Numbers between bracket refer to Figure 1-1.

*: Some marshlands are scattered in various sectors. The one mentioned here is the one in which most of the marshland is located
**: Paddy rice meaning “rice which has retained its husk after threshing”(FAO and WHO, 1996)
***Caution is needed on the figures presented here as the cultivated area for Cyanuzi-Rwabutazi rice-growing area is higher than the cultivable area. The data have been reproduced as they are presented in the original source. But this might be true, figures of rice-grown areas increasing because of the spontaneous initiatives of the farmers who convert their land to rice production (UCORIRWA and AC Team, 2004).

Rice is produced by small scale farmers, numbering 49907 in 2009 (MINAGRI, 2013a) in an irrigated system, with an average farm area of 0.2 ha per household (Ngango, 2013). Rice is produced in marshlands near major rivers and in inland valleys. Most of the farmers are organized in associations or cooperatives, which provide extension services and collectively purchase inputs. Initial rice marshland development was done in various waves by the Government of Rwanda with the support from Taiwan (1964-1972), China (1972-1982), France through the CCCE (1988-1994) and Canada through CIDA (1977-1981)

(UCORIRWA and AC Team, 2004). In rice development, there are also spontaneous rice development initiatives, in which farmers commit to convert their land to rice production and carry out the initial works, especially the land levelling, installation of water irrigation and drainage systems and find the initial inputs (seeds, fertilizers and pesticides). In the start, rice development initiators taught farmers about rice production techniques. Nowadays, farmers learn about rice farming from their neighbours and the acquisition of new techniques is facilitated by the government and farmers groupings. There are currently many stakeholders in the rice value chain including the following: key ministries and public institutions (MINAGRI, MINICOM, MINEAC, RAB, RBS, PSF), rural development projects and programs (RSSP, PADAB, PAIRB, KWAMP, GAA, CIP, PHHS), international organizations (FAO, IFDC, UN-WFP), bilateral and multilateral agencies (BTC, USAID, DFID, JICA), rice federation (FUCORIRWA), unions, rice farmers' cooperatives, and individual farmers, rice millers/processors, rice traders/importers, input suppliers, etc. (MINAGRI, 2013a).

In Rwanda, the demand for rice is high and outpaces production (Figure 1-2). This is due to the fact that the population of Rwanda has increased exponentially after the genocide period and is now estimated at 12 million and this increased the rice market. In addition to home consumption, rice is an important ceremonial food (Adekunle, 2007). In the rice development policy, Rwanda intends to reach a yield of 7 t/ha and become self-sufficient in rice by 2017 (MINAGRI, 2011a). However, this may be very difficult because there are rampant quality concerns about the rice seed and grain in Rwanda, that need to be addressed before this goal can be reached (Stryker, 2013). Moreover, the planned continuous reduction in rice imports has not been achieved (FSNWG/MAS, 2013, 2014).

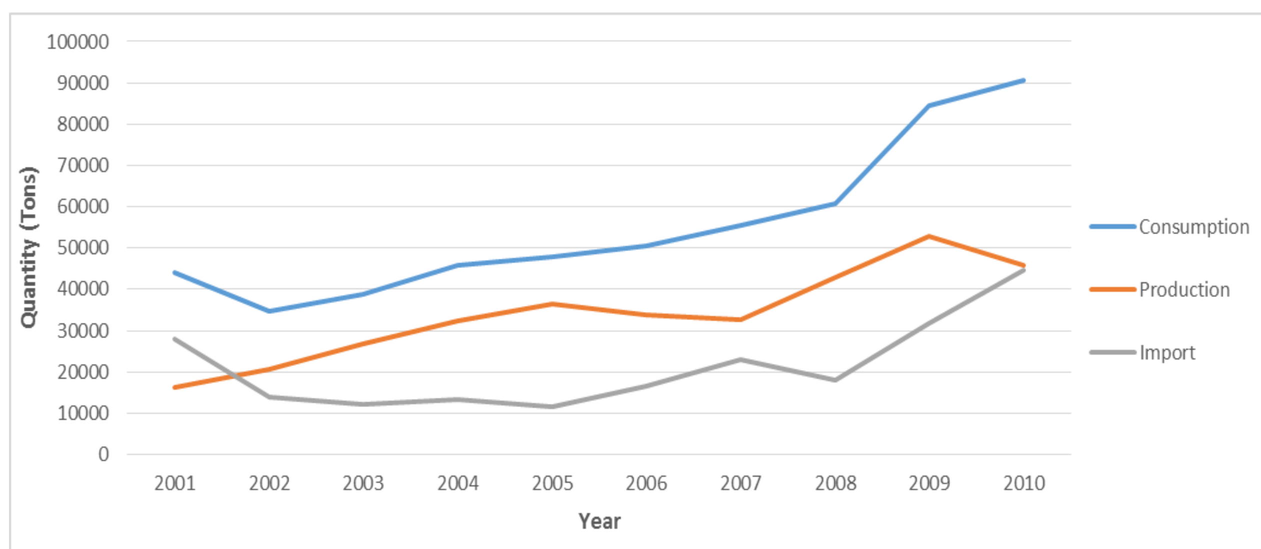

Figure 1-2: Quantity (tons) of rice consumption, production and imports in Rwanda in 2001-2010

Rwanda has a particular climate and topography, as it is mountainous with low temperatures to which most selected rice varieties are not adapted. The average temperature in Rwanda is 19.84°C (NISR, 2013) which is below the best temperature interval for growing rice estimated in the range of 20-38°C (CABI, 2007). This is a problem in most rice production areas in Rwanda except in Bugarama (Western Province) where the average temperature is around 24°C. The effects of the poor adaptation to the climate are long growing cycles and grain sterility (Manneh and El Namaky, 2010). All these conditions warrant a breeding program that would screen available cultivars, create varieties adapted to the Rwandan agro-ecology and contribute in maintaining them. The most successful varieties grown in Rwanda were introduced by Chinese and Japanese breeders before the genocide and have a *Japonica* profile, though precise information is not available (Promar, 2012). A list of varieties that were grown in Rwanda in 2003 is given in Table 1-2 (Jagwe et al., 2003).

Table 1-2: Varieties of rice in Rwanda

Variety	Rice growing area where the variety is grown
Zhong geng (Local name *Kigori*)	Rwamagana, Cyili, Kabuye, Nyagatare, Bugesera, Mukunguri
Yun Keng 136	Rwamagana, Cyili, Kabuye, Nyagatare, Bugesera, Mukunguri
Yun yine 4	Rwamagana
Yunertian 01	Rwamagana, Cyili, Kabuye, Nyagatare, Bugesera, Mukunguri
Xinum 175	Nyagatare, Rwamagana
Fac V046	Cyili
Basmati 370	Bugarama
IRON 280	Bugarama
BG 400-1	Bugarama
IRAT	Bugarama

The short grain, *Kigori* rice, produced in Rwanda, is mostly milled in artisanal structures and is consumed mainly in production and rural areas (Stryker, 2013; Ntirenganya, 2014). In urban areas people prefer long grain imported rice of the *Indica* type, which is considered to be of higher quality (World Bank, 2011; Ntirenganya, 2014). When the locally produced Kigori rice is processed in modern mills, it is not competitive vis-à-vis imported *Indica* rice, as it is sold with a discount of 10-20% (Stryker, 2013).

In 2010, many new rice varieties, mainly of the *Indica* type, were released. Those are: Kigega (Pedigree-P-: VANDANA/IR 64), Nzahaha (P: IR 81431-B-B-162), Terimbere (P: WAB

01291/4*IR64), Rumbuka (P: WAB 56-50/CG 14), Nemeyubutaka (P: WAB 56-50/CG 14), Kimaranzara (P: WAB 56-104/CG 14), Ndamirabahinzi (P: Unknown, Breeding line: WAB 569-35-1-1-1-HB), Muhinzi (P: WAB 56-104/CG 14), Kanyabukungu (P: IRAT 104/PALAWAN), Imberabyombi (P: Unknown, Breeding line: WAB 788-19-1-1-2-HB), Ndengera (P: IR 833-6-2-1-1//IR 1561-149-1/IR 1737), Nsizebashonje (P: PETA/TANGKAI ROTAN), Garukuhinge (P: IR 19660-73-4/IR 2415-90-4-3-2//IR 54), Mbakungahaze (P: SIAM 29/DEE-GEO-WOO-GEN), Ndamirabana (P: SIAM 29/KAOHSIUNG 68), Kungahara (P: WAB450-24-2-3-P3-HB/IG10), Jyambere (P: 11975/IR 13146-45-2-3), Mpembuke (P: Unknown, Breeding line: WAB923-B-6-AL1), Mbangukira (P: Unknown, Breeding line: IR05N499), Cyicaro (P: WAB 56-104/CG 14) (Ndikumana and Gasore, 2010).

There are also the varieties of: Intsinzi, Gakire, Muturage, Tebuka (WAT 1276-22-2) and Intsindagirabigega (B24-2) (MINAGRI, 2009b). Mutware et al. (2014) cite varieties of Nerica, Muturage (WAT 54-TGR-1-5) and Facagro 56.

Some New Rice for Africa (NERICA) varieties have been tested in Rwanda but not many of them are grown. Rwanda practices lowland irrigated rice while most of the NERICA varieties are rainfed upland or lowland varieties, the agroecology of Rwanda, with low temperature may not be suitable to most NERICA varieties (WARDA, 2005; Somado et al., 2008).

The seed is the first input in agriculture, on which depends the performance of other inputs. Currently, the rice varieties in use are degenerated and some of them are a mix of different varieties (Jagwe et al., 2003) as for many years there has not been a rice breeding programme for Rwanda (Promar, 2012). In general, considering all the crops, only 2% of the farmers in Rwanda use improved seeds and Rwanda is a large seed importer (RDB, 2014b).

The existing seed production and distribution system in the country could not keep up with the rapid increase in marshland development and many farmers do not have access to sufficient quantities of quality seed. It is important to be cautious about the quality, as no organized seed evaluation system exists (MINAGRI, 2011a). Progressive farmers feel the need for broadening varietal options as the easily available varieties are short-grain ones, which have a low market value. In recent years, Rwanda has been using seeds imported from international and regional networks. At their arrival in the country, they undergo quick adaptability studies, after which the best performing ones are released to farmers for cultivation. There is not much information about the efficacy after release in Rwanda (Kathiresan, 2010; Mutware and Burger, 2014), but it has been reported that most long grain

Indica varieties fail after 2-3 years of cultivation (Promar, 2012) while short-grain varieties perform better.

Next to the poor quality of the available seeds, Jagwe et al. (2003) defined the following major constraints to rice production in Rwanda: pests and diseases, deterioration and destruction of the drainage and irrigation infrastructure, insufficient research, insufficient use of farm inputs, and lack of adequate drying and processing facilities. Since 2003, there have been improvements on most of these constraints, especially because of a supportive rice development policy but some constraints persist. For infrastructures for drainage and irrigation, the surface of marshlands developed or maintained for rice production increased from 5.500 ha in 2003 to 12.000 ha in 2010 (Promar, 2012). Many farmers unions and cooperatives have their own rice drying facilities (Promar, 2012) and also there are improvements in the processing facilities (Stryker, 2013). The proportion of farmers who use fertilizers increased from 11 to 29% in 2005-2006 to 2010-2011 and the pesticide use rate increased from 24 to 31% in the same period (NISR, 2012).

The problems in rice production in Rwanda that have not been solved up to date deal with poor quality of the available seed and grain, and lack of research about pest and diseases and their management.

Kathiresan (2010) attests that there is poor progress in crop protection practices. Because of this, the pressure of pests and diseases is high as the intensive monocropping of rice has gradually built up pest and disease populations. There is also a lack of knowledge on appropriate control measures and limited access to pest control advice (RAB, 2014).

The locally produced rice grain is of average quality (Adekunle, 2007).The poor physical appearance of rice produced in Rwanda may have different causes: rudimentary milling equipment, varietal appearance, lack of homogeneity in the planting material, physiological stress in development, but can also be due to diseases and pests. Most of the factors cited here have been studied, but less is known about the impact of pest and diseases in reducing the quality of the grain, a problem faced by Rwanda.

The major pests and diseases reported on rice in Rwanda are: *Diopsis thoracica* (stalk-eyed fly) and *Magnaporthe oryzae* (Rice blast) (MINAGRI, 2009b). Given that no research is conducted on pests and diseases, most studies recognize that a large population of pests and diseases has built up throughout the time (MINAGRI, 2011a). In fact, most of these reports are based on surveys where the important informants are farmers, with less connection to

diagnosis systems. *Rice yellow mottle virus* was recently reported (Ndikumana et al., 2011). Rice can potentially be attacked in Rwanda by many more pests and diseases (see Annex 2).

Rice sheath rot disease is associated with grain discolouration and the reduction of grain quality. Until the current study was conducted, the presence of rice sheath rot was reported but no further studies about this disease had been conducted and the causal agents of this disease in Rwanda are unknown. Rice sheath rot disease will be further discussed in the next chapter.

In conclusion it can be stated that the potential of rice as both a food and cash crop in Rwanda is high. However, there are factors that limit the full expression of the potential of rice. It is important to address seed and grain quality issues in rice production in Rwanda as the supply of national production lags behind the demand in quantity and in quality. These two aspects have much in common: the quantity of the produce depends on the used planting material, which is still of low quality, the quality of the produce depends on the production conditions on field, which depend on crop husbandry practices including plant protection against pests and diseases. This last point is difficult to certify as there is not sufficient information on pests and diseases in the country.

Though a lasting solution to these problems can be found in the long run, in the short term it is possible to reinforce the pests and disease control aspects through the evaluation of seeds by organising on-field inspections and laboratory diagnosis and analyses, and collecting information about the planting materials in use in the country and their performance. Research data on the rice performance in the Rwandan environment are also needed. From the information gathered in these initiatives, and by maintaining the good rice policy development that prevailed until now, breeding initiatives can be initiated, having in mind the current market segmentation in which highly demanded rice is the long grain in urban centres while the short grain varieties are more adapted to the Rwandan agroecology and play a capital role in food security in rural areas.

2 REVIEW ON RICE SHEATH ROT: AN EMERGING UBIQUITOUS DESTRUCTIVE DISEASE COMPLEX

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Abstract

Around one century ago, a rice disease characterized mainly by rotting of sheaths was reported in Taiwan. The causal agent was identified as *Acrocylindrium oryzae*, later known as *Sarocladium oryzae*. Since then it has become clear that various other organisms can cause similar disease symptoms, including *Fusarium* spp. and fluorescent pseudomonads. These organisms have in common that they produce a range of phytotoxins that induce necrosis in plants. The same agents also cause grain discoloration, chaffiness and sterility and are all seed-transmitted. Rice sheath rot disease symptoms are found in all rice-growing areas of the world. The disease is now getting momentum and is considered as an important emerging rice production threat. The disease can lead to variable yield losses, which can be as high as 85%. This review aims at improving our understanding of the disease etiology of rice sheath rot and mainly deals with the three most reported rice sheath rot pathogens: *Sarocladium oryzae*, the *Fusarium fujikuroi* complex and *Pseudomonas fuscovaginae*. Causal agents, pathogenicity determinants, interactions among the various pathogens, epidemiology, geographical distribution and control options will be discussed.

2.1 Introduction

Rice is an important crop worldwide, serving as the staple food for half of the world population and additionally being used in industry and for animal feed. Rice is grown in

various agro-ecological zones in tropical and subtropical areas, especially in Asia, the continent accounting for 90% of the world production (IRRI, 2015b). It faces many production constraints, including pests and diseases.

The major feature of rice sheath rot disease is rotting and discoloration of the sheath, leading to chaffiness and sterility of resulting grains. For many years, rice sheath rot was considered as a minor and geographically limited disease. It is only recently that it gained momentum and became widespread. Since the green revolution in Asia in the 1960s, there have been substantial changes in rice farming systems: use of high yielding varieties, increased use of fertilizers, efficient systems of water use, seeding methods, etc. Crop intensification practices such as increased plant density, a high rate of nitrogen fertilizers and the use of semi-dwarf and photoperiod-insensitive cultivars, favor the susceptibility of rice to some diseases and the sheath rot complex is one of them. It is hypothesized that the new photoperiod-insensitive cultivars have lost the capacity of avoiding flowering under conditions of high humidity and high temperature, conditions that are conducive to effective disease attacks (Mew *et al.*, 2004b). Additionally, the last decades saw the boosting of international exchange of planting materials which may have contributed to the spread of the disease.

Rice sheath rot is a disease complex that can be caused by various fungal and bacterial pathogens. Major pathogens associated with rice sheath rot are fungi such as *Sarocladium oryzae* and *Fusarium* spp. belonging to the *Fusarium fujikuroi* complex and the bacterial pathogen *Pseudomonas fuscovaginae*. A variety of other pathogens have been associated with rice sheath rot. An overview is given in Table 2-1.

Table 2-1: Organisms associated with rice sheath rot

Causal agent*	Taxonomic position	Synonyms or other used names	Occurrence	Geographic distribution	References
Fungi**					
Sarocladium oryzae	Ascomycota, Hypocreales	*Acrocylindrium oryzae, Cephalosporium caerulans, Sarocladium attenuatum*	Lowland (< 1250m)	32 countries	(Purkayastha and Ghosal, 1985; Sakthivel, 2001; Bills *et al.*, 2004; Giraldo *et al.*, 2015)
***Fusarium fujikuroi* species complex**	Ascomytota, Hypocreales	*Fusarium fujikuroi, F. proliferatum, F. verticillioides, F. moniliforme*	Ubiquitous	Everywhere	(Desjardins and Plattner, 1997; Abbas *et al.*, 1998; Kushiro *et al.*, 2012; Quazi *et al.*, 2013; Aoki *et al.*, 2014)
***Fusarium graminearum* species complex**	Ascomycota, Hypocreales	*F. zeae*	5-30°C (optimum around 15°C), high relative humidity	Everywhere where temperatures are low and humidity is high	(Singh and Devi, 1990; Naeimi *et al.*, 2003; Goswami and Kistler, 2004; Leplat *et al.*, 2012; Backhouse, 2014; Aoki *et al.*, 2014)
***Fusarium incarnatum-equiseti* species complex**	Ascomycota, Hypocreales	*F. equiseti*	Found in regions with cool through to hot and arid climates	Mainly in wheat-growing areas	(Fisher and Petrini, 1992; Wheeler *et al.*, 1999; Marín *et al.*, 2012)
***Fusarium oxysporum* species complex**	Ascomycota, Hypocreales	-	Ubiquitous	Nepal, Italy	(Fisher and Petrini, 1992; Abbas *et al.*, 1995; Desjardins *et al.*, 2000; Ruiz-Roldán *et al.*, 2015)
Cochliobolus lunatus	Ascomycota, Pleosporales	*Curvularia lunata*	Wide host range and common in paddy fields	India, Bangladesh, China	(Lakshmanan, 1992, 1993a; Shamsi *et al.*, 2003; Liu *et al.*, 2009; Gao *et al.*, 2015)
Gaeumannomyces graminis	Asomycota, Incertae sedis	*Ophiobolus oryzinus*	Wind is an important dissemination factor; found in tropical, subtropical and southern temperate climates	South and North America, Australia	(Walker, 1972; Gnanamanickam and Mew, 1991; Frederick *et al.*, 1999; Elliott, 2005; Peixoto *et al.*, 2013)
Sclerotium hydrophilum	Basidiomycota,	*Ceratorhiza* sp.	Infection on aquatic or	Australia	(Lanoiselet *et al.*, 2002;

Causal agent*	Taxonomic position	Synonyms or other used names	Occurrence	Geographic distribution	References
	Cantharellales		semi-aquatic plants of wet meadows and marshes		Yang *et al.*, 2007; Hu *et al.*, 2008; Xu *et al.*, 2010)
Sclerotium oryzae	Basidiomycota, Agaricales	*Ceratobasidium oryzae-sativae*	Overwintering through stubbles, plant debris and paddy soil	USA, Japan	(Oster, 1992; Lanoiselet *et al.*, 2002; Kimiharu *et al.*, 2004; Hu *et al.*, 2008)
Rhizoctonia oryzae, Rhizoctonia oryzae-sativae	Basidiomycota, Corticiales	*Waitea circinata, Ceratobasidium oryzae-sativae*	Overwintering through stubbles, plant debris and paddy soil	Brazil, Japan	(Prabhu *et al.*, 2002; Kimiharu *et al.*, 2004; Lanoiselet *et al.*, 2007; Chaijuckam and Davis, 2010)
Bacteria					
Pseudomonas fuscovaginae	Gamma proteobacteria	-	Highlands	31 countries	(Miyajima *et al.*, 1983; Zeigler and Alvarez, 1987; Flamand *et al.*, 1996; Batoko *et al.*, 1997)
Pseudomonas syringae	Gamma proteobacteria	-	Ubiquitous epiphytic plant pathogen originally linked to aquatic systems	Hungary, Australia	(Zeigler and Alvarez, 1990; Morris *et al.*, 2013)
Pseudomonas palleroniana	Gamma proteobacteria	-	-	La Réunion (France), Cameroon and Madagascar	(Gardan *et al.*, 2002)
***Pseudomonas* spp.**	Gamma proteobacteria	-	Ubiquitous	Cambodia, Philippines	(Cottyn *et al.*, 1996a; b; Cother *et al.*, 2009; Patel *et al.*, 2014)
Pantoea ananatis	Gamma proteobacteria	*Erwinia herbicola, Enterobacter agglomerans*	Facultative pathogen	Australia, the Philippines, South Korea	(Cottyn *et al.*, 2001; Cother *et al.*, 2004; Sinn *et al.*, 2011; Choi *et al.*, 2012b; Cray *et al.*, 2013)
Burkholderia glumae	Beta proteobacteria	*Pseudomonas glumae*	Adaptability to various habitats	USA, South Korea, Ecuador, South Africa, the Philippines	(Urakami et al., 1994; Cottyn et al., 1996a, 2001, 2009; Sayler et al., 2006; Nandakumar et al., 2009; Kim et al., 2010, 2014; Seo et al., 2011;

Causal agent*	Taxonomic position	Synonyms or other used names	Occurrence	Geographic distribution	References
					Paganin et al., 2011; Riera-Ruiz et al., 2014a)
Burkholderia gladioli	Beta proteobacteria	*Pseudomonas gladioli*	Adaptability to various habitats	USA, Ecuador, Japan, Panama, South Korea, the Philippines	(Urakami et al., 1994; Cottyn et al., 1996a, 2001, 2009; Ura et al., 2006; Nandakumar et al., 2007b, 2009; Riera-Ruiz et al., 2014b)
Acidovorax oryzae	Beta proteobacteria	*Pseudomonas avenae, Acidovorax avenae subsp. avenae*	Transmission by rain, wind and seeds	Philippines	(Cottyn et al., 1996a; Schaad et al., 2008; Liu et al., 2012b)

* : "Causal agents" means here the genus, species or species complex of the agent associated with rice sheath rot

**:For fungi of the *Fusarium* genus, species complex are based on Aoki *et al.* (2014)

The various described sheath rot agents all cause very similar disease symptoms (Cottyn et al., 1996b). This explains why there are practically no comprehensive studies mentioning the link between the presence and quantity of disease inoculum and yield loss (Mew and Gonzales, 2002). The unpredictable nature of the factors acting in the pathosystem explains why losses attributed to *Sarocladium oryzae* can be as variable as in the range of 20-85% (Sakthivel, 2001).

The context of an increasing world population with shrinking natural resources imposes to adopt sustainable production methods, responding to the food demand but also using efficiently and sustainably key resources (Savary *et al.*, 2000; Mew *et al.*, 2004b). The development of sound control practices against rice sheath rot is hampered by the fact that this disease is poorly understood. This review would like to contribute in filling the rice sheath rot missing information gap. It explores the available information on the following aspects: causal agents and symptoms, host range, physiological and biochemical impact, virulence factors, synergism and interactions among causal factors, ecology of causal agents, epidemiology and impact, geographical distribution and relationships with farming systems and control methods. In this review, more emphasis will be put on rice sheath rot symptoms caused by *S. oryzae, Fusarium* spp., and *P. fuscovaginae*, since they are considered to be the most important rice sheath rot pathogens (Table 2-1).

2.2 *Sarocladium oryzae*: the major fungal rice sheath rot pathogen

2.2.1 Pathogen description and symptoms

S. oryzae was originally described as *Acrocylindrium oryzae*, the first organism to be associated with rice sheath rot symptoms isolated in Taiwan in 1922 (Mew and Gonzales, 2002). The genus *Sarocladium* was established in 1975 (Gams and Hawksworth, 1975) and currently encompasses 16 species including plant pathogens, saprobes, mycoparasites, endophytes and potential human pathogens (Giraldo *et al.*, 2015). The genus belongs to the order of the Hypocreales in the Phylum *Ascomycota*. *S. attenuatum* was originally described as a distinct species causing rice sheath rot, but is nowadays considered as a synonym of *S.*

Table 2-2: Main characteristics of the major rice sheath rot pathogens

Pathogen	Survival	Host range	Most susceptible plant stage	Dissemination	Reproduction	Relevant metabolites*	References
Sarocladium oryzae	seeds, plant residues, soil, water	weeds, bamboo, sedge	after booting stage	wind, rain, insects, mites	Aseptate conidia	Helvolic acid, cerulenin	(Pearce *et al.*, 2001; Ghosh *et al.*, 2002; Ayyadurai *et al.*, 2005)
Fusarium fujikuroi	seeds, plant residues, soil	Rice, corn	all stages	wind, rain	macro- and microconidia, no chlamydospores	Fumonisins (low levels in some strains), gibberellins, moniliformin	(Abbas *et al.*, 1999; Lee *et al.*, 2012; Wiemann *et al.*, 2013)
Fusarium proliferatum	seeds, plant residues, soil	wide host range	all stages	wind, rain	macro- and microconidia, no chlamydospores	Fumonisins (high levels), moniliformin	(Abbas *et al.*, 1999)
Fusarium verticillioides	seeds, plant residues, soil	wide host range	all stages	wind, rain	macro- and microconidia, no chlamydospores	Fumonisins (high levels)	(Wulff *et al.*, 2010; Wiemann *et al.*, 2013)
Pseudomonas fuscovaginae	seeds, epiphytically and endophytically on rice	wild and cultivated Graminae	seedling and booting stages	wind, rain	bacterial cells	Fuscopeptin, syringopeptin	(Flamand et al., 1996; Ballio et al., 1996; Batoko et al., 1998)

*'Relevant metabolites' means here metabolites that have been associated with rice sheath rot disease

oryzae (Bridge *et al.*, 1989). Bills *et al.* (2004) showed that also the cerulenin producing fungus *Cephalosporium caerulans* is conspecific with *S. oryzae*.

S. oryzae grows slowly (about 2.5 mm/day on potato dextrose agar at 28°C) and produces a sparsely branched white mycelium. The colony reverse of isolates obtained from rice is generally orange (see Figure 2-1). Conidiophores can be simple or branched. Conidia are cylindrical, aseptate and hyaline, 4-7 x 1-2 µm in size, and arranged in slimy heads (Figure 2-2).

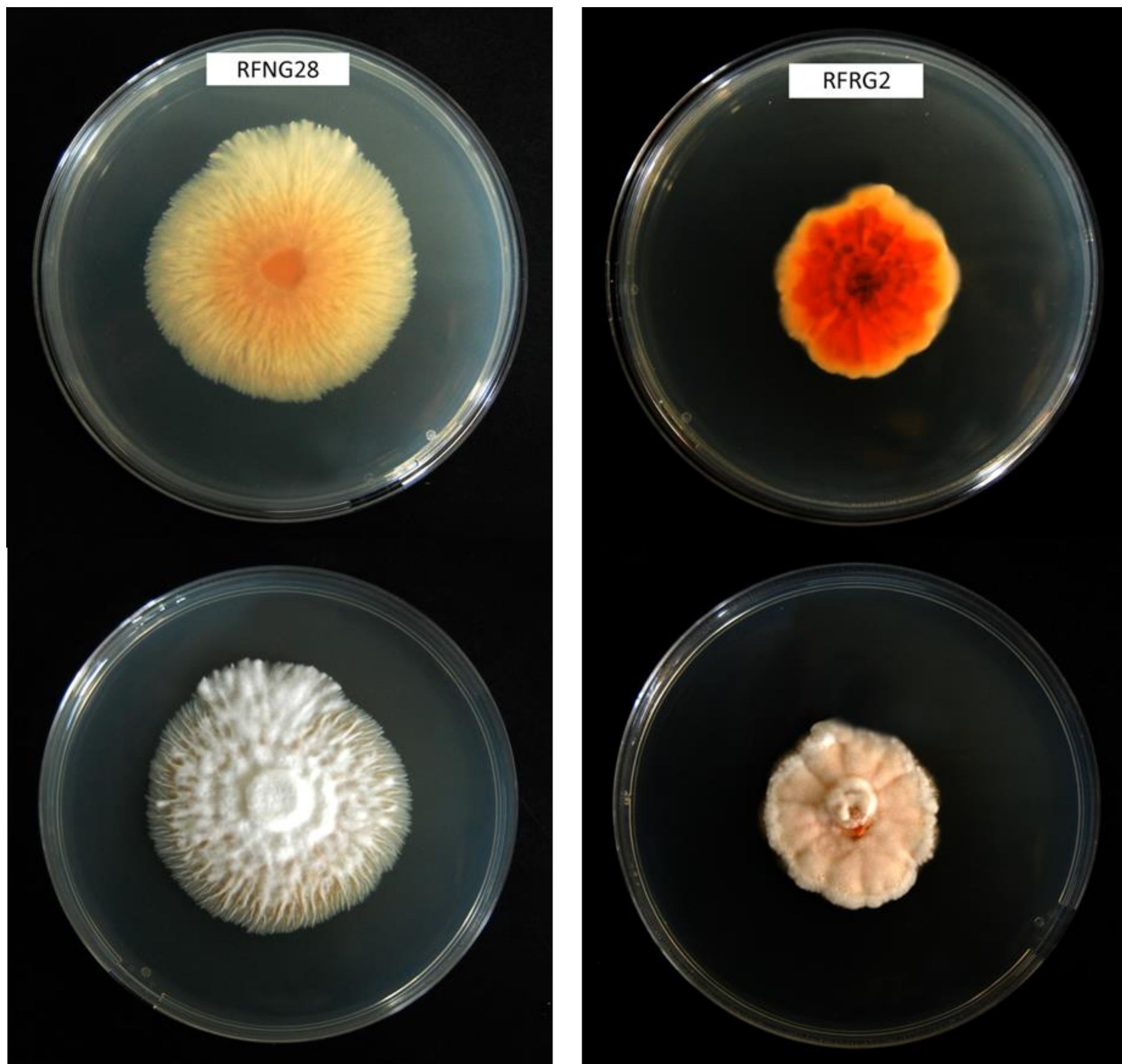

Figure 2-1: Morphology of two different *Sarocladium oryzae* isolates from Rwanda on PDA medium after 14 days of growth at 28°C.
Top is reverse view, bottom is front view.

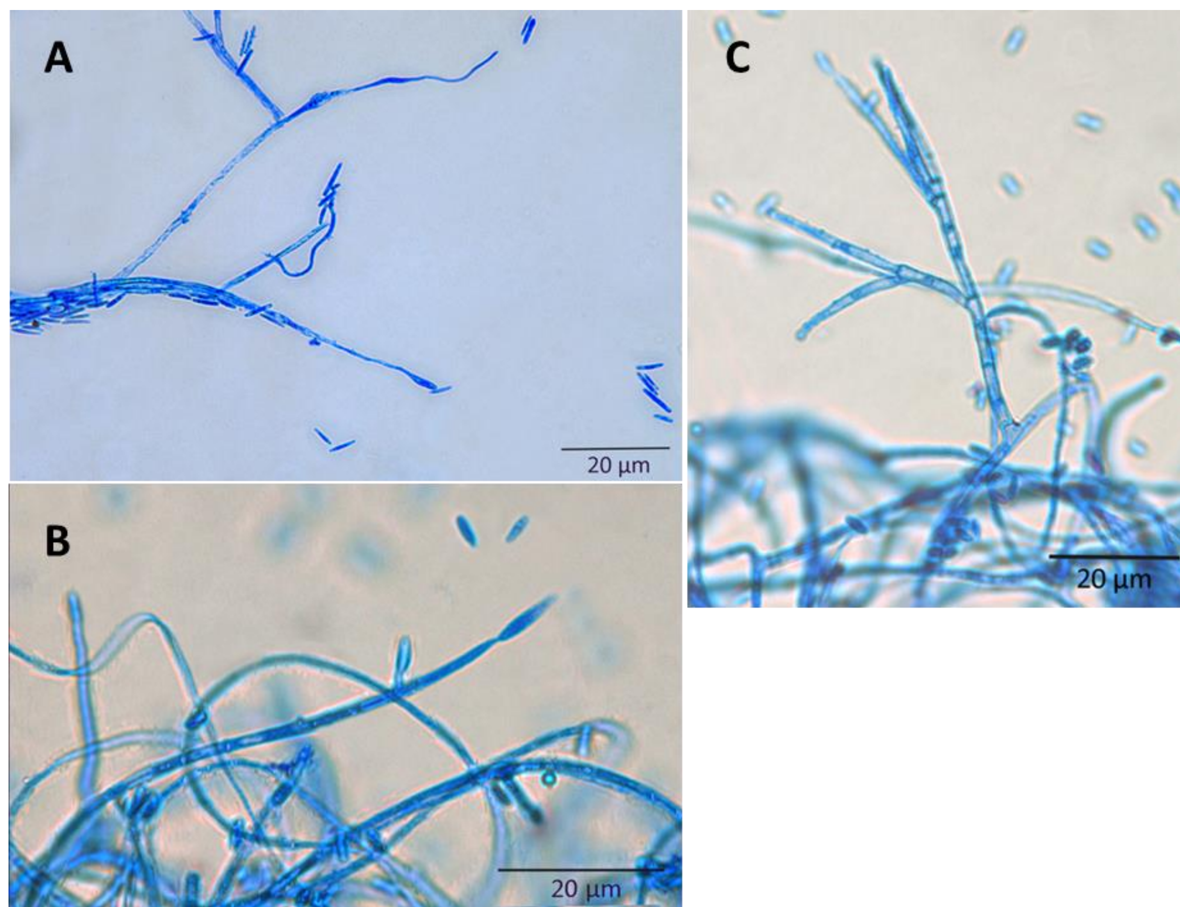

Figure 2-2: Microscopy of *Sarocladium oryzae* grown on PDA medium.
All structures were stained with lactophenol blue. A: Conidia; B: Conidiogenous cell; C: Aerial conidiophores

The major symptoms describing rice sheath rot caused by *S. oryzae* are the following, according to Ou (1985): the rot occurs on the uppermost leaf sheaths enclosing the young panicles; the lesions start as oblong or somewhat irregular spots, 0.5-1.5 cm long, with brown margins and grey centers, or they may be grayish brown throughout; they enlarge and often coalesce and may cover most of the leaf sheath; the young panicles remain within the sheath or only partially emerge; an abundant whitish powdery growth may be found inside affected sheaths and young panicles are rotted. *S. oryzae* infection results in chaffy, discolored grains and affects the viability and nutritional value of seeds (Sakthivel, 2001; Gopalakrishnan *et al.*, 2010). The major symptoms of rice sheath rot incited by *S. oryzae* are presented in Figure 2-3.

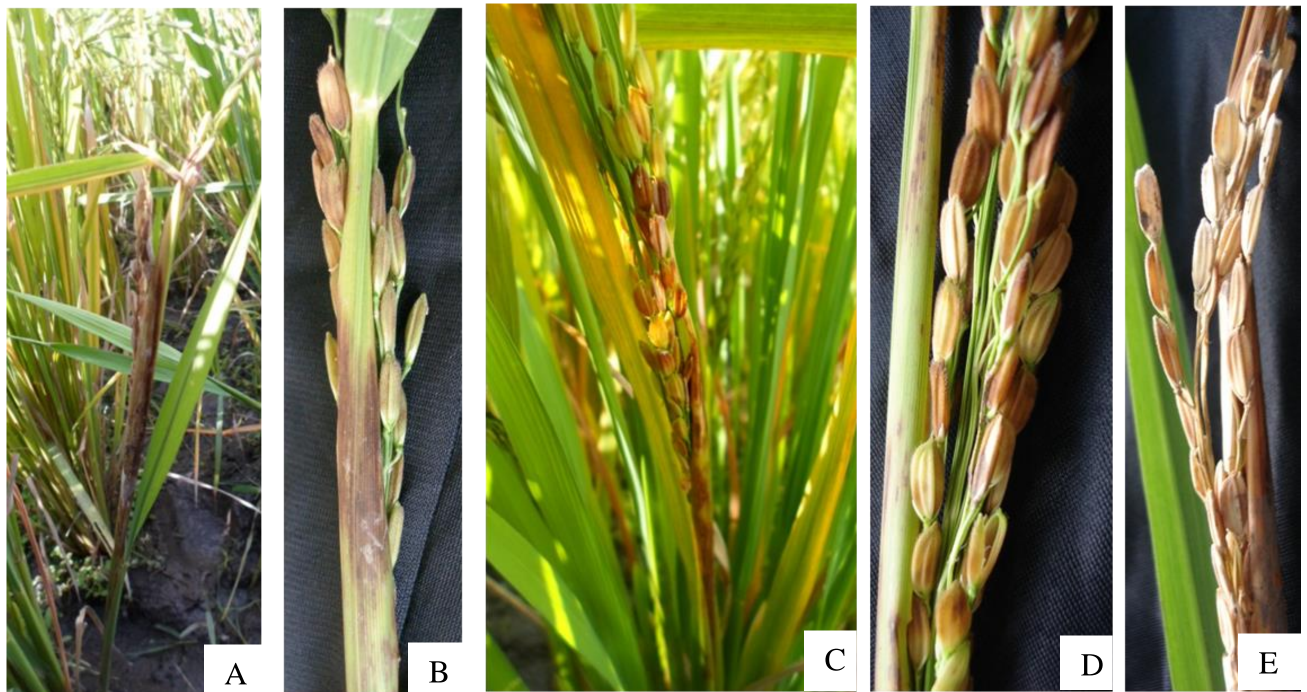

Figure 2-3: Rice sheath rot symptoms caused by *Sarocladium oryzae*. A-B: Diseased sheaths on which white fungal structures can be seen; C-D: Discoloured grains in a diseased panicle; E: Non-filled grains from a diseased plant.

2.2.2 Epidemiology

In general, *S. oryzae* is present in all rice-growing countries worldwide, being very common in rainy seasons (Mew and Gonzales, 2002). It has so far been reported in the following countries (CABI, 2007): Bangladesh, Brunei Darussalam, China, India, Indonesia, Japan, Malaysia, Nepal, Pakistan, Philippines, Saudi Arabia, Sri Lanka, Tajikistan, Thailand, Uzbekistan, Vietnam, Burundi, Cameroon, Côte d'Ivoire, Gambia, Kenya, Madagascar, Niger, Nigeria, Senegal, Tanzania, Mexico, USA, Argentina, Brazil, Venezuela, and Australia. *S. oryzae* is mostly found in lowland environments (Pearce *et al.*, 2001), and hot and humid weather favors the disease (Sakthivel, 2001). Sharma *et al.* (1997) stated that *S. oryzae* infections in Nepal were found below 1250 m. Temperatures of 20-30°C and relative humidity in the range of 65 to 85% favor sheath rot development (Sakthivel, 2001).

The pathogen survives in infected seeds, plant residues (straw, stubble), but also in soil, water or weeds when environmental conditions are favorable. Plants at various growth stages can be affected; the fungus enters through stomata or wounds, and is most destructive after booting stage but also infects other growth stages (Pearce *et al.*, 2001). The entry of *S. oryzae* in the plant is facilitated mostly by insect and mite damage or the weakening of the plant by other pathogens (Pearce *et al.*, 2001). Secondary infections may be wind-borne through injured tissues. Less is known about the seed-borne disease transmission.

Caused yield losses are variable from 20-85%, depending on the pathosystem conditions (Sakthivel, 2001) (Figure 2-4).

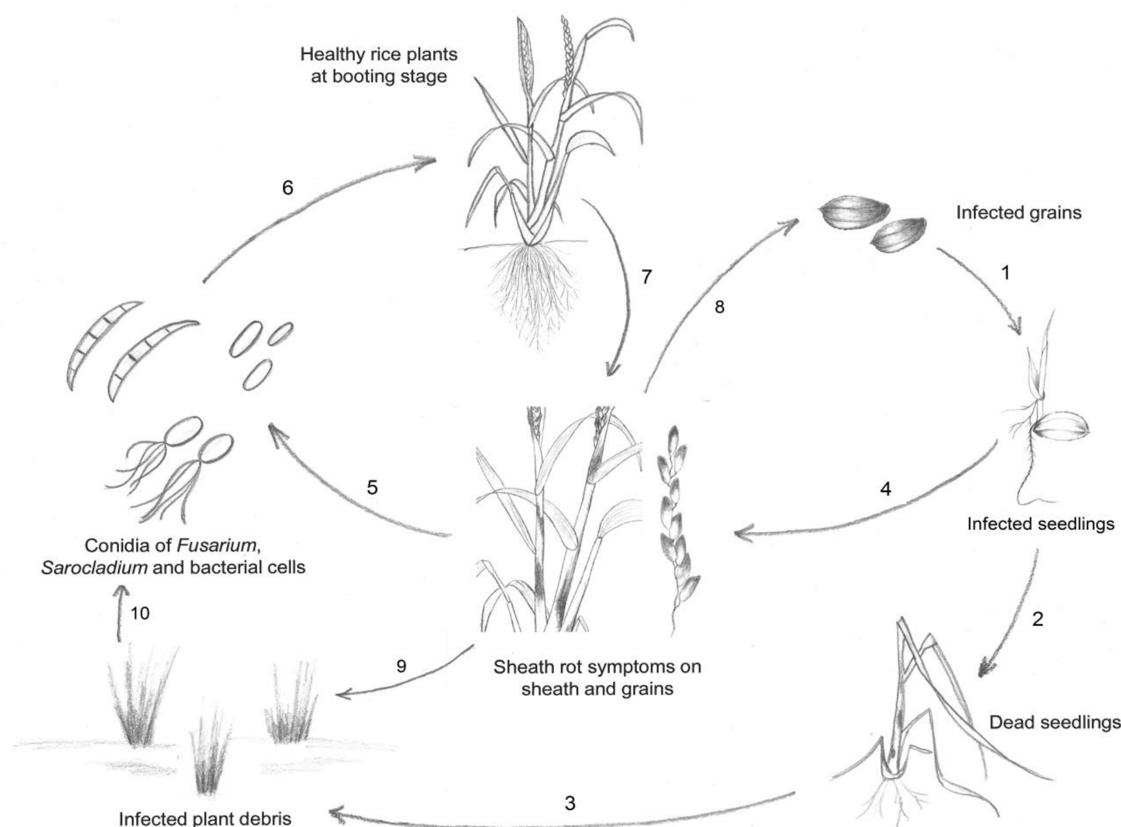

Figure 2-4: Disease cycle of sheath rot caused by *S. oryzae*, *Fusarium sp.* or *Pseudomonas fuscovaginae*. Sheath rot pathogens are seed-transmitted, resulting in infected seedlings (1). Infected seedlings can die (2) resulting in infected plant debris (3) or survive. *P fuscovaginae* can colonize the whole plant as an endophyte or survive epiphytically and infect the inflorescences at booting stage. The seedling transmission of the fungal pathogens is less well understood (4). Secondary infections result from conidia or bacterial cells released from infected plants (5). Conidia or bacterial cells are spread by wind or rain to healthy plants. Plants at booting stage are especially susceptible to infection. In the case of *S. oryzae*, insects and mites can also spread conidia and facilitate infection by creating wounds (6). Rot occurs on the sheath enclosing the young panicles; grains on affected tillers become chaffy and discolored. Grains infected with *Fusarium* sp. can become contaminated with mycotoxins (7). Pathogens can spread to new field via contaminated grains (8). After harvest, infected plant debris will remain in the field (9) serving as inoculum for the next growth cycle (10).

The main host of *S. oryzae* is rice but the pathogen has also been reported as the causal agent of bamboo blight in Bangladesh and India. Bamboo isolates, however, show less infra-population variation than rice isolates (Pearce *et al.*, 2001). *S. oryzae* has also been isolated from grasses and sedges growing in association with rice.

2.2.3 Pathogenicity determinants

Helvolic acid and cerulenin are described as the major secondary metabolites of *S. oryzae* (Ghosh *et al.*, 2002; Ayyadurai *et al.*, 2005) (Table 2-2, Figure 2-5).

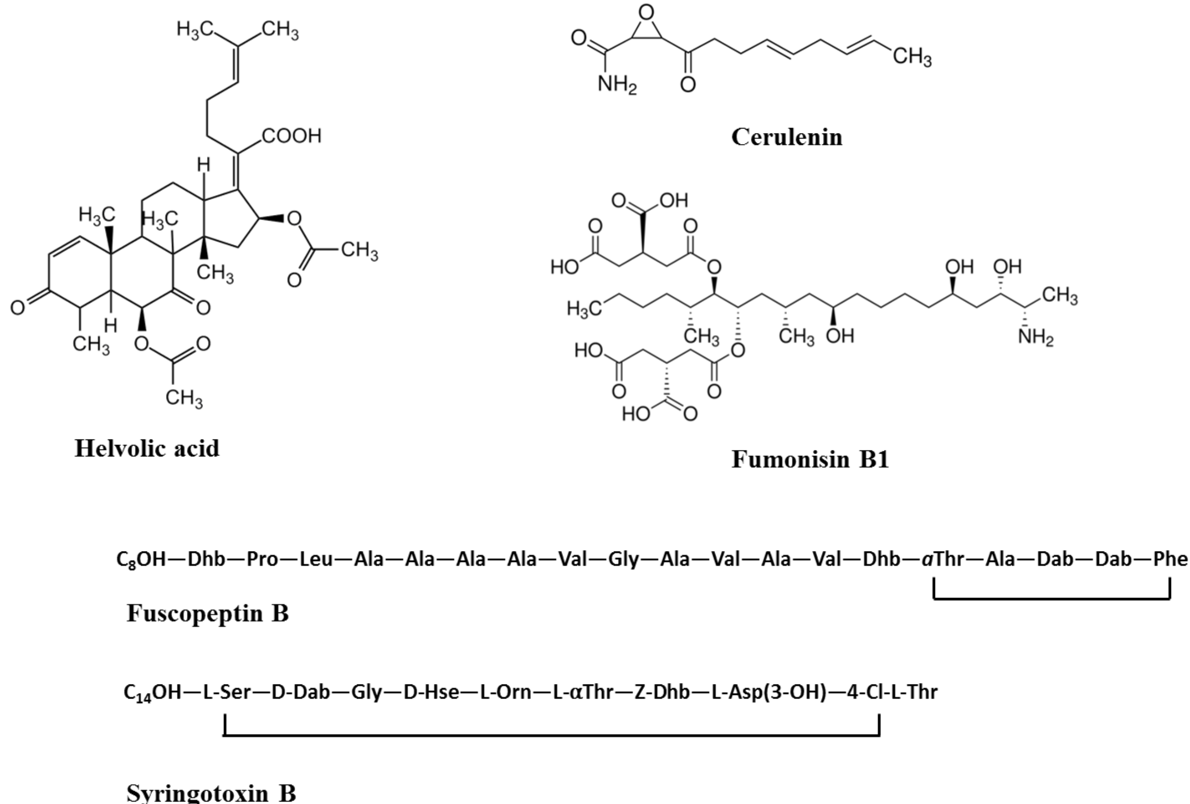

Figure 2-5: Structures of toxins produced by rice sheath rot causing agents. Helvolic acid and cerulenin are produced by *Sarocladium oryzae*; Fumonisin B1 is produced by *Fusarium spp*., Fuscopeptin B and syringotoxin B are produced by *Pseudomonas fuscovaginae.*

Artificial inoculation of those metabolites to host plants reproduced the sheath rot symptoms. Infiltration of rice tissues with cerulenin and helvolic acid leads to electrolyte leakage proportional to the susceptibility to rice sheath rot (Sakthivel *et al.*, 2002). Tschen *et al.* (1997) reproduced *S. oryzae* symptoms on rice seeds, growth retardation and chlorosis, by dipping them in a solution of helvolic acid. Helvolic acid is a tetracyclic triterpenoid that interferes with chlorophyll biosynthesis (Ayyadurai *et al.*, 2005). This compound is also produced by various other fungi including the opportunistic human pathogen *Aspergillus fumigatus*, the entomopathogenic fungus *Metarhizium anisopliae* and by endophytic fungi. Cerulenin is a hexaketide amide that inhibits polyketide synthesis by inhibiting the malonyl-ACP:acyl-ACP condensation step as well as fatty acid synthesis (Omura, 1976) (Table 2-2).

Though the most described virulence factors of *S. oryzae* are helvolic acid and cerulenin, the fungus also produces cellulolytic, proteolytic, pectinolytic and oxidative enzymes that play a role in pathogenicity (Joe and Manibhushanrao, 1995; Pearce *et al.*, 2001). Gopalakrishnan *et al.* (2010) observed a pronounced decrease in sugar, starch and protein and an increase in phenol content in rice seeds infected with *S. oryzae.* This probably explains why infected grains are chaffy and germinate poorly.

2.2.4 Interactions with other diseases and pests

Experimental tests have shown that *S. oryzae*, by the production of toxins, like cerulenin, limits the development of other fungi and emerges as the major pathogen (Gnanamanickam and Mew, 1991; Silva-Lobo *et al.*, 2011). Gnanamanickam and Mew (1991) observed that the antibiotic properties of cerulenin extracted from *S. oryzae* block the development of many rice stem-attacking fungi, like *Sclerotium oryzae, Gaeumannomyces graminis* var. *graminis*, *Magnaporthe oryzae* and *Rhizoctonia solani*. In this context it is interesting to notice that cerulenin has been reported to inhibit melanin biosynthesis in *Colletotrichum lagenarium* (Kubo *et al.*, 1986). DHN (=1,8 dihydroxynapthalene)-melanin in fungi is synthesized by a polyketide pathway which starts from malonyl-CoA which is converted to the first detectable intermediate of the melanin pathway 1,3,6,8-tetrahydroxynapthalene via a polyketide synthase. DHN-melanin is an important virulence factor in several pathogenic fungi including *M. oryzae* and *G. graminis* var. *graminis* (Henson *et al.*, 1999). In addition, helvolic acid has strong antibacterial activities mainly against Gram-positive bacteria (Tschen *et al.*, 1997). This could explain why in many situations *S. oryzae* emerges as the major pathogen.

Table 2-3: Main toxins involved in rice sheath rot disease

Microbial toxin	Producing sheath rot pathogen	Other producing organisms	Class	Mode of action	Symptom on plants	Other activities
Helvolic acid	*Sarocladium oryzae*	*Methahizium anisopliae, Aspergillus spp., Pichia guilliermondii, Alternaria sp.*	steroid	interference with chlorophyll biosynthesis	chlorosis	antibacterial activity
Cerulenin	*Sarocladium oryzae*	not known	hexaketide amide	inhibitor of fatty acid synthetases, interference with flavonoid biosynthesis	necrosis, growth inhibition	antibacterial and antifungal activity
Fumonisin B	*Fusarium proliferatum, F. verticilloides, F. fujikuroi*	other *Fusarium spp., Aspergillus niger, Tolypocladium spp.*	polyketide	inhibitor of sphingolipid biosynthesis	necrosis, growth inhibition	human and animal toxin
Syringotoxin	*Pseudomonas fuscovaginae*	*Pseudomonas syringae pv. syringae*	cyclic lipopeptide	interference with ATPase pumps in plasma membrane	necrosis	antifungal activity
Fuscopeptins	*Pseudomonas fuscovaginae*	not known	cyclic lipopeptide	form channels in plasma membranes	necrosis	antimicrobial activity

Initial work on sheath rot was done in India, and Amin *et al.* (1974) already realized the disease complexity, as the causal agent was already thought to be associated with stem borers. A study on four groups of insects: green leaf hopper, brown plant hopper, mealy bugs and earhead bugs showed that brown plant hoppers and mealy bugs fed on rice infected with *S. oryzae* carry the fungus on their body and can transmit it to healthy plants (Gopalakrishnan *et al.*, 2009). Some of the *S. oryzae* effects like sterility result from its synergism with a mite *Steneotarsonemus spinki* (Ou, 1985; Karmakar, 2008; Hummel *et al.*, 2009). It was observed that wounding of plants facilitated their infection by *S. oryzae* and most of the infected plants proved also to be attacked by stem borers and from time to time by yellow dwarf virus (Ou, 1985). The fact that spraying a spore suspension of *S. oryzae* on earhead bug (*Leptocorisa acuta*)-infected rice tillers results in the development of rice sheath rot disease symptoms in 12 days (Lakshmanan *et al.*, 1992) shows that the entry of *S. oryzae* in rice plants might be facilitated. Sakthivel (2001) realized that the infection occurs after the plant has been weakened by other diseases and stem borer infestation.

Bacterial sheath brown rot, caused by *P. fuscovaginae*, may occur together with sheath rot caused by *S. oryzae*. Other factors that have been associated with *S. oryzae* include rice tungro virus (Venkataraman *et al.*, 1987) and *Fusarium* spp. (Sakthivel, 2001).

2.2.5 Control methods

S. oryzae is controlled by sanitary, chemical and biological measures.

Sanitary control methods involve the following practices (Sakthivel, 2001): using healthy seeds since the disease is referred to as being seed-borne; limiting insect population in rice fields as they are involved in disease transmission; avoiding densely planting as this predisposes plants to fungal attacks; avoiding heavy doses of nitrogen fertilizers; increasing potassium content in the fertilizer formula for reducing the disease impact, as more potassium causes more phenol production; adopting different cultural practices for limiting the disease attack impact: field sanitation, crop residue management, control of weeds, etc.

Various fungicides have been used to control sheath rot but as they cannot kill the fungus inside the glumes, their efficacy is limited (Sakthivel, 2001). Other control tests combined fungicides with insecticides and gave better results (Lakshmanan, 1992). Foliar spray of micronutrients is also said to reduce disease incidence and increase grain yield (Sakthivel, 2001). Some plant extracts are reported to be effective against the disease: neem, pungam oil and rubber cakes (Narasimhan *et al.*, 1998; Sakthivel, 2001).

The use of biological control agents may have potential (Sakthivel and Gnanamanickam, 1987; Mew *et al.*, 2004a). Many pseudomonads can act efficiently for controlling *S. oryzae*, by favoring antagonism, for example through the inhibition of fungal development as do some *P. fluorescens* strains, or by inducing systemic resistance (Saravanakumar *et al.*, 2009).

Breeding for resistance to sheath rot seems the best option, but it is limited by its multiple causal agents. High-yielding nitrogen-responsive rice cultivars are highly susceptible to sheath rot. Resistance to *S. oryzae* has been identified in tall rice varieties (Amin, 1976). Hemalatha *et al.* (1999) developed a method of screening for resistance against *S. oryzae* based on a crude toxin preparation and Lakshmanan (1993) went further by screening for resistance against *S. oryzae* and one of its vectors, the rice mealy bug. The screening of resistance against *S. oryzae* that was developed by Amin (1976) does not seem to have been continued. Ayyadurai *et al.* (2005) analyzed *S. oryzae* isolates from North East and South India and found a high variability in pathogenicity, phytotoxic metabolite production and RAPD band patterns. This variability should be taken into account in breeding efforts.

2.3 *Fusarium fujikuroi*, a species complex associated with rice sheath rot

2.3.1 Pathogen description and symptoms

Sheath rot in rice has also been associated with *Fusarium* spp. belonging to the *Fusarium fujikuroi* complex. The *F. fujikuroi* complex largely corresponds to the Section *Liseola*, established by Wollenweber and Reinking (1935), in which Nelson *et al.* (1983) recognized four species (including *F. moniliforme* and *F. proliferatum)*, but also accommodates certain species originally classified in other *Fusarium* sections. Progress in molecular taxonomy has shown that there are around 50 species in the *F. fujikuroi* complex and the number keeps increasing (Reviewed in Kvas *et al.*, 2009). The complex is currently divided in three large clades, the African clade, the Asian clade and the American clade. The main organisms associated with rice are *F. verticillioides* from the African clade and the closely related species *F. proliferatum* and *F. fujikuroi* from the Asian clade.

Abbas *et al.* (1998) described rice sheath rot symptoms caused by *F. proliferatum* as follows: blanked or partially blanked panicle with reddish-brown to off-white florets or kernels are often covered with a white to pinkish white powder consisting of microconidia and conidiophores of *F. proliferatum*; the flag leaf sheath develops a rapidly enlarging lesion, first dull to dark brown and later off-white to tan with a reddish brown border, that eventually encompasses the entire sheath and may result in the death of the leaf blade; lower leaf sheaths

may eventually develop lesions as well, but rarely more than two leaf sheaths show symptoms; and a dense white to pinkish white powder consisting of microconidia and conidiophores of *F. proliferatum* covers the sheath lesions, especially evident during humid periods.

2.3.2 Epidemiology

Rice-pathogenic *Fusarium* species, because of their high diversity, are ubiquitous in nature (Park *et al.*, 2005). Symptoms of rice sheath rot caused by any of the members of the *Fusarium fujikuroi* species complex are widespread due to their large variability and at least one of their members is found in any part of the rice-growing world. The different species of *Fusarium* forming the *F. fujikuroi* complex (mainly *Fusarium fujikuroi*, *F. verticillioides* and *F. proliferatum*) cause various symptoms on different plant parts and are responsible of yield losses of 40% in Nepal (Desjardins *et al.*, 2000) and even up to 60% in Korea (Park *et al.*, 2005).

Fusarium proliferatum, which is pathogenic to rice, also attacks some other plants of the *Poaceae* family. *F. proliferatum* is widespread and its hosts vary from maize to mango (Leslie *et al.*, 2007), including chestnut (Kushiro *et al.*, 2012), and banana (Li *et al.*, 2012). As the organisms causing rice sheath rot have many hosts, they can easily find alternate hosts in the environment, especially weeds.

Fusarium spp. are seed-transmitted and at maturity, infected grains contain mycotoxins (Wulff *et al.*, 2010). *F. fujikuroi* was one of a number of microbes isolated from the surface of rice seeds; highest levels of microbes were recorded at harvesting. *F. fujikuroi* survived for up to 26 months in infected grains and 28 months in dried stubble of certain rice cultivars. The fungus was detected for up to 10 and 13 months, respectively, in unsterilized and sterilized soils that were infected with fungal propagules (Sunder and Satyavir, 1998). *F. proliferatum* can survive in infected grains even when they are preserved in stressing conditions. In fact, Kushiro *et al.* (2012) could recover *F. proliferatum* in grains preserved at 4-5°C for 6 months. In normal conditions, the survival is longer.

2.3.3 Pathogenicity determinants

Two categories of metabolites are involved in pathogenicity and interaction with plants, gibberellins and mycotoxins. According to Wulff *et al.* (2010), only strains of *F. fujikuroi* were able to produce gibberellin A and these strains cause abnormal elongation of rice plants, the so-called bakanae disease. Main species producing mycotoxins, like fumonisin B (FB)

(Table 2-2, Figure 2-5), have been reported to cause rice sheath rot (Wulff *et al.*, 2010). Fumonisins are linear, polyketide-derived molecules that are also known as major mycotoxins that pose health risks to humans and animals. *F. proliferatum* is among the largest producers of fumonisins and is often associated with rice sheath rot (Abbas *et al.*, 1999; Kushiro *et al.*, 2012; Quazi *et al.*, 2013). In addition, *F. verticillioides* strains are notorious fumonisin producers (Wulff *et al.*, 2010). Isolates belonging to various other related *Fusarium* species have been shown to produce fumonisins (Table 2-2). Fumonisin biosynthetic genes have also been found in more distantly related fungi such as *Aspergillus niger* and *Tolypocladium* spp. The evolution of the fumonisin gene cluster in *Fusarium* is complex and not adequately represented by the species phylogeny. It is hypothesized that a combination of multiple horizontal gene transfer, cluster duplication and loss has shaped the current distribution of the fumonisin gene cluster (Proctor *et al.*, 2013). The role of fumonisins in the ecology and pathology of *Fusarium* is poorly understood. Abbas *et al.* (1998) observed that the concentration of fumonisins coincides with the intensity of sheath and panicle symptoms in rice plants showing sheath rot under *Fusarium* attacks. Toxins are apparently concentrated in the external grain part since their concentration in the grain reduced 75-80% after hulling. One of the major fumonisins, FB1, is conceived as a virulence factor in *Fusarium*-induced diseases in plants (Glenn *et al.*, 2008). FB1 inhibits ceramide synthase (Williams *et al.*, 2007), an enzyme involved in sphingolipid biosynthesis in both animals and plants. This has numerous consequences on the cell physiology and results in increased cell death and inhibition of plasma membrane ATPases (Gutiérrez-Nájera *et al.*, 2005). Members of the *F. fujikuroi* complex also produce a variety of other mycotoxins, including moniliformin. It has been shown that *F. proliferatum* isolates from field samples of rice with Fusarium sheath rot disease are capable of producing both fumonisins and moniliformin in culture. Both mycotoxins were also detected in naturally contaminated rice samples (Abbas *et al.*, 1999). The phytotoxicity of moniliformin is well documented (Abbas *et al.*, 1995). Moniliformin was shown to arrest mitosis of maize root meristematic cells at the metaphase stage (Styer and Cutler, 1984). The factors triggering the infection of *F. proliferatum* to rice plants still need to be further investigated (Kushiro *et al.*, 2012). Genome sequencing revealed the presence of a wide variety of secondary metabolite gene clusters in *F. fujikuroi* and *F. verticillioides*, including clusters for bikaverin, fusarubins, fusarins, fumonisins, and fusaric acid. Beauvericin and gibberellin gene clusters, however, were only present in *F. fujikuroi* (Wiemann *et al.*, 2013).

2.3.4 Interactions with other diseases and pests

There are reports of association of *Fusarium* spp. with *Sarocladium oryzae* in the rice sheath rot disease (Sakthivel, 2001). Islam *et al.* (2000) realized that in many seeds, numerous organisms are detected at the same time as *Fusarium*, including *Alternaria padwickii*, *Curvularia* spp., *S. oryzae*, *Magnaporthe oryzae*, *Bipolaris oryzae* and *Michrodochium oryzae*.

2.3.5 Control methods

Cultural and sanitary methods to control rice sheath rot caused by *Fusarium* spp. include the use of clean seeds and water to separate light weight seeds (IRRI, 2015a). In chemical control, some fungicides are very effective against the fungus: thiophanate-methyl, benomyl, difenoconazole, penconazole (Ilyas and Iftikhar, 1997) and seed treatment is also advised. Seed dressing with antagonistic yeasts in combination with thermotherapy appears to be a promising strategy to control *F. fujikuroi* on rice seeds (Matić *et al.*, 2014). Soil inoculation with the fungus *Talaromyces* sp. isolate KNB422 controlled seed-borne diseases on rice seedlings including *F. fujikuroi* as effectively as chemical pesticides (Miyake *et al.*, 2012).

2.3.6 Other *Fusarium* spp. associated with rice sheath rot

Fusarium graminearum is grouped in the *Fusarium graminearum sambucinum* complex (Aoki *et al.*, 2014) and is pathogenic to many plants, mainly causing wheat head blight (Goswami and Kistler, 2004; Leplat *et al.*, 2012). It has also been reported to cause sheath rot on rice (Singh and Devi, 1990; Naeimi *et al.*, 2003).

Fusarium equiseti belongs to the *Fusarium incarnatum-equiseti* species complex (Aoki *et al.*, 2014) and has been mainly reported as a pathogen for barley (Marín *et al.*, 2012) and wheat (Castellá and Cabañes, 2014). It was also isolated from rice stem tissues (Fisher and Petrini, 1992).

Fusarium oxysporum forms its own group according to the phylogenetic relationships of key *Fusarium* species (Aoki *et al.*, 2014). Though most of the time it has been associated only with vascular diseases and not with *Poaceae* plants (Agrios, 2005), it has been isolated from rice plant tissues (Fisher and Petrini, 1992; Abbas *et al.*, 1995; Desjardins *et al.*, 2000) and is pathogenic on young rice plants (Prabhu and Bedendo, 1983; Fisher and Petrini, 1992). Some *F. oxysporum* isolates are known to produce fumonisins (Proctor *et al.*, 2008), but whether isolates associated with rice sheath rot symptoms produce these mycotoxins has not been tested.

2.3.7 Other reported rice sheath rot associated fungi

Cochliobolus lunatus causes black kernel disease on rice and has been identified as the causal agent of rice sheath rot in India and Bangladesh (Lakshmanan, 1992, 1993a; Shamsi *et al.*, 2003). There are no extensive studies on its pathogenesis on rice, but its virulence is attributed to the methyl 5-(hydroxymethyl) furan-2-carboxylate (M5HF2C) toxin (Liu *et al.*, 2009; Gao *et al.*, 2015).

Gaeumannomyces graminis var. *graminis* (Syn.: *Ophiobolus oryzinus*) causes crown sheath rot or black sheath rot on rice (Walker, 1972; Frederick *et al.*, 1999; Peixoto *et al.*, 2013) and its virulence is linked to the production of DHN-melanin (Frederick *et al.*, 1999).

Sclerotium hydrophilum was recognized as an agent of sheath leaf necrosis by Lanoiselet *et al.* (2002). The fungus was isolated from infected rice sheaths and was shown to cause rice leaf sheath disease. But *S. hydrophilum* is not the only sclerotial disease of rice. *Rhizoctonia fumigata, R. oryzae-sativae, R. oryzae* and *R. solani* are reported to induce the same symptoms as *S. hydrophilum* leaf sheath disease (Kimiharu *et al.*, 2004). The damage caused by all these diseases is high when they reach the top leaf sheath of the plant. The symptoms of all these diseases are pronounced at the heading stage and increase as the plant matures. Most of the time, the rice sclerotial diseases cause overlapping symptoms in places where sheath blight caused by *R. oryzae* frequently occurs, although their pathogenesis is different (Prabhu *et al.*, 2002). These diseases have in common with *Sarocladium oryzae*, the most reported rice sheath rot pathogen, and other sheath rot agents that their symptoms are more pronounced in the reproductive stage and around physiological maturity (Oster, 1992). Also, in the description of the symptoms of *R. oryzae-sativae* (Syn: *Ceratobasidium oryzae-sativae*), (Lanoiselet *et al.*, 2007) mentioned classical sheath rot disease associated symptoms like the rotting of the culm and grain sterility.

The diseases caused by *Cochliobolus lunatus, Gaeumannomyces graminis, Sclerotium hydrophilum, Rhizoctonia fumigata, R. oryzae-sativae, R. oryzae, R. solani*, though they are closer to rice sheath rot agents in terms of symptomatology, will not be extensively covered in this review, considering that they have been primarily described based on plant parts different from the rice sheath.

2.4 *Pseudomonas fuscovaginae*: the most important bacterial pathogen associated with rice sheath rot

2.4.1 Pathogen description and symptoms

Since its isolation in association with rice sheath rot in Japan (Tanii *et al.*, 1976; Miyajima *et al.*, 1983) and its identification as the causal agent of discoloration of rice sheaths, leaves and grains in Latin America (Zeigler and Alvarez, 1987), *Pseudomonas fuscovaginae* is considered as the main bacterium causing rice sheath brown rot. It has been found on both the sheath and the glume (Cother *et al.*, 2009). Zeigler and Alvarez (1987) stated that rice sheath brown rot, caused by *P. fuscovaginae* in Latin America, is characterized by the following features: longitudinal brown to reddish brown necrosis 2-5 mm wide extending the entire length of the leaf sheath and blade; affected sheaths enclosing the panicle may show extensive water-soaking and necrosis with poorly defined margins; glumes discolor before emerging from such panicles; grains on affected tillers may be completely discolored and sterile to nearly symptomless with only small brown spots. To these symptoms, the description by Cottyn *et al.* (1996a) adds the following features: a wide range of sheath and/or grain symptoms, varying from translucent to brown dots to brown blotches to brown streaks to a completely brown sheath, and/or clear to brown spots to brown blotches to completely dark discolored seeds. An illustration of bacteria-induced rice sheath rot is presented in Figure 2-6.

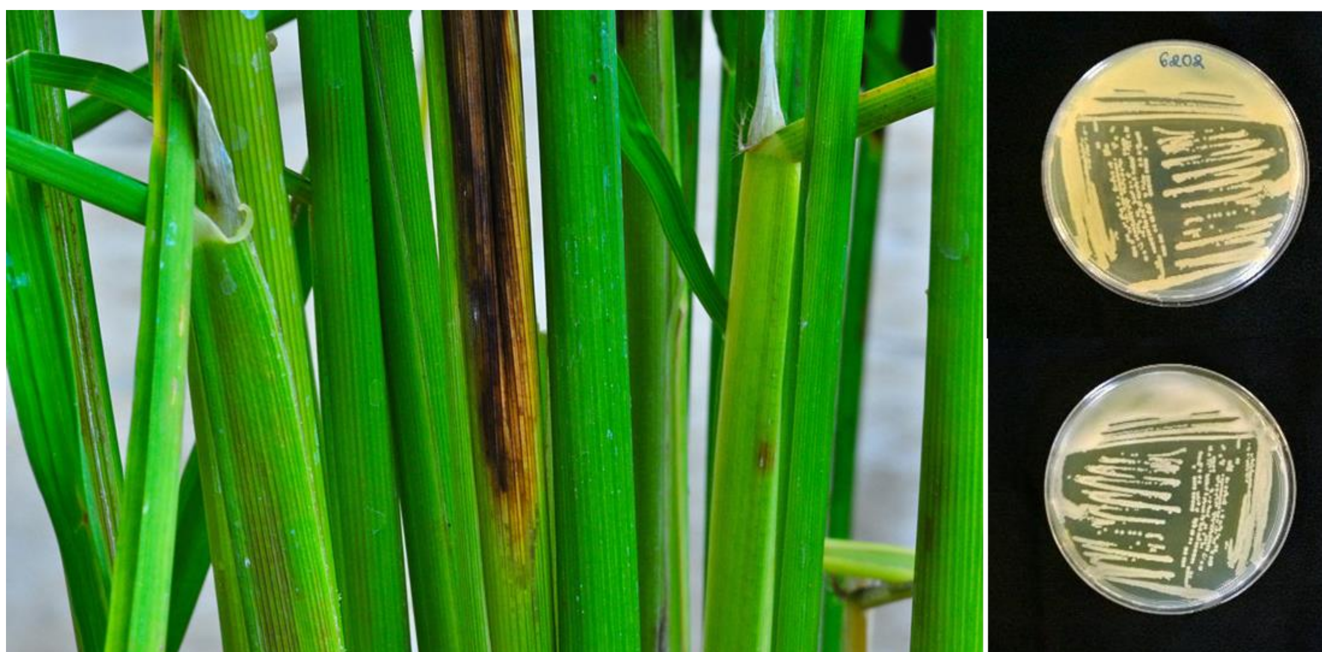

Figure 2-6: Symptoms caused by *Pseudomonas fuscovaginae* and morphology on King's Medium B plates after 48 h of growth at 28°C. Top is reverse side, bottom is front side.

The genus *Pseudomonas* belongs to the subclass *Gammaproteobacteria* of the Gram-negative bacteria and currently comprises 144 species. Based on multilocus sequence analysis, *P. fuscovaginae* belongs together with *P. asplenii* to the *P. 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 *et al.* (1983) is the following: the cells are aerobic, Gram negative, non-spore-forming, rod-shaped with round ends, 0.5 -0.8 x 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 in oxidation-fermentation medium, and they produce a green fluorescent pigment, oxidase and arginine dihydrolase. Denitrification, β-glucosidase, pit formation on polypectate gel 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.

Whole genome sequence analysis of various *P. fuscovaginae* strains has revealed that these pathogens do not form a single monophyletic group. At least two subgroups 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).

2.4.2 Epidemiology

P. fuscovaginae was first reported in literature as the pathogen responsible for rice sheath rot disease in cold and humid tropical highlands in Japan (Miyajima *et al.*, 1983), Burundi (Duveiller *et al.*, 1988), Madagascar (Rott *et al.*, 1989) and Nepal (Sharma *et al.*, 1997), but it was later found even in lowlands (Cottyn et al., 1996b). *P. fuscovaginae* is also associated with high rainfall (Sharma *et al.*, 1997). The bacterium causes quantitative and qualitative losses (Zeigler and Alvarez, 1987). For losses in quality, symptomatic grains cannot be accepted in seed certification chains, mills accept them with a discount and they have a poor marketing value.

CABI (2007) reports the presence of *P. fuscovaginae* in 31 countries: Former Yugoslavia, Russian Federation, China, Indonesia, Japan, Nepal, Philippines, Burundi, Democratic Republic of Congo, Madagascar, Rwanda, Tanzania, Costa Rica, Cuba, Dominican Republic, El Salvador, Guatemala, Jamaica, Nicaragua, Panama, Trinidad and Tobago, Mexico, Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, Peru, Suriname, and Uruguay. Recently, the disease has been diagnosed in Australia (Cother *et al.*, 2009).

The host range of *P. fuscovaginae* seems to be restricted to wild and cultivated *Graminae* (Tanii *et al.*, 1976; Miyajima *et al.*, 1983).

P. fuscovaginae is seed-transmitted and infected seedlings often die. When infection occurs at a later stage, the lower part of the sheath becomes brown and later on, the whole sheath becomes necrotic. The pathogenicity of *P. fuscovaginae* is expressed at grain, seedling and booting stage levels. *P. fuscovaginae* is able to colonize the whole plant as an endophyte (Adorada *et al.*, 2015). If the seedling survives, *P. fuscovaginae* has an epiphytic life until the booting stage when it infects inflorescences, resulting in the formation of infected grains or the panicle abortion. The population of the bacterium is maintained at a low level from early growth stages up to the booting stage. The bacterium can survive epiphytically on the host plant with low pathogen population in the tissue and this explains how the disease can be seed-borne, but only express symptoms at the booting stage (Batoko *et al.*, 1997). This can also be linked to the fact that the booting stage and the reproductive phase in general, is the most stress-sensitive stage in the rice plant development (Fageria, 2007).

2.4.3 Pathogenicity determinants

Different phytotoxins are involved in the disease development. Batoko *et al.* (1997) could reproduce sheath brown rot symptoms by inoculating seedlings with toxins from bacteria. They concluded that toxins are an integral part of the plant-pathogen interactions in rice bacterial sheath rot. Flamand *et al.* (1996) found that a cell-free extract from *P. fuscovaginae* that could induce the same symptoms as *P. fuscovaginae* contained five peptidic compounds (A, B, C, D and E) and two others (fuscopeptins A and B). Peptidic compound D is identical to syringotoxin, a lipodepsinonapeptide containing nine amino acids acylated by 3-hydroxytetradecanoic acid (Table 2-2, Figure 2-5) that is also produced by *P. syringae* pv. *syringae* pathogenic on citrus (Ballio *et al.*, 1990). The structure of fuscopeptins was elucidated by Ballio *et al.* (1996). Fuscopeptins are lipodepsipeptides containing 19 amino acids. Fuscopeptin A is acylated by 3-hydroxyoctanoate while fuscopeptin B is acylated by 3-hydroxydecanoate (Table 2-2, Figure 2-5). Both compounds target the plasma membrane and inhibit H^+-ATPase and act synergistically with syringotoxin (Batoko *et al.*, 1998).

Toxins from *P. fuscovaginae* are non-host specific, the pathogen inducing disease symptoms on many plants of the *Poaceae* family in addition to rice (Miyajima *et al.*, 1983), and have more detrimental effect on culm elongation (Batoko *et al.*, 1997). The non-host specificity may also be justified by the symptoms induction by *P. fuscovaginae* on *Chenopodium quinoa* (Mattiuzzo *et al.*, 2011), a plant belonging to the *Amaranthaceae* family. Toxins are

immediately dissolved in the plant and thus become difficult to recover (Batoko *et al.*, 1997). Phytotoxin concentration increases at the booting stage of rice, which stimulate their large production by the bacterium. The capacity of the plant to detoxify the toxins plays a pivotal role and could constitute a starting point in breeding for resistance against *P. fuscovaginae.*

Patel *et al.* (2014) (see Annex 1) were able to isolate nine mutants of *P. fuscovaginae* via random Tn5 mutagenesis which showed altered virulence on rice. Besides mutants affected in phytotoxin production, also mutants in type IV pili biosynthesis, type VI secretion, arginine biosynthesis and sulfur metabolism were obtained indicating that these processes are also involved in pathogenicity on rice.

2.4.4 Interactions with other diseases and pests

Most of the time *P. fuscovaginae* was found together with *Sarocladium oryzae* in sheath rot diseased plants (Zeigler and Alvarez, 1987; Cottyn et al., 1996b).

2.4.5 Control methods

Some cultural and sanitation practices against *P. fuscovaginae* are indicated like burning farm remains: stubbles, ratoons; treatment of seeds by dipping them in water at 65°C before sowing (Zeigler and Alvarez, 1987); introducing rotation; checking the quality of seeds and as it is a seed-borne disease, using healthy seeds. Host plant resistance is also considered as an option. There are limited sources of resistance to rice sheath rot (Adorada *et al.*, 2013), while this is a must in developing a control strategy against the disease. There are various methods that can be used for screening resistance and Adorada *et al.* (2013) suggested using the pin-prick method. About the chemical control, streptomycin, alone or in combination with oxytetracycline, can effectively control rice sheath rot (CABI, 2007).

2.4.6 Other *Pseudomonas* spp. associated with rice sheath rot

Besides *P. fuscovaginae*, a variety of other poorly characterized fluorescent pseudomonads have been associated with rice sheath rot since the 1950s. The first characterized sheath rot associated *Pseudomonas* was *P. oryzicola* (Klement, 1955). Later on it was decided that this pathogen is equivalent to *P. syringae* pv. *syringae* (Young *et al.*, 1978). Besides *P. syringae* and *P. fuscovaginae*, various other pseudomonads have been consistently found in rice sheath rot related studies (Zeigler and Alvarez, 1987; Cottyn *et al.*, 1996a; b; Cother *et al.*, 2010; Saberi *et al.*, 2013). Only a few of those other pseudomonads have been fully identified except by biochemical tests.

Zeigler and Alvarez (1987) attempted to put rice sheath rot-associated pseudomonads into groups, which were continued and named, based on BIOLOG features, by Cottyn *et al.* (1996a). In their work, they defined, based on the guidelines for the taxonomy of Proteobacteria, originally called purple bacteria (Woese, 1987), four main groups of *Gammaproteobacteria* associated with rice sheath rot and grain discoloration named after the representative species: *P. putida, P. aeruginosa, P. fuscovaginae,* and a composite group related to *P. marginalis, P. corrugata, P. fluorescens, P. aureofaciens* and *P. syringae*. Also Saberi *et al.* (2013) concluded, based on biochemical tests, that sheath rot and grain discoloration caused by *Pseudomonas* spp. in Iran are related to *P. marginalis*, *P. putida*, and *P. syringae*.

The question whether these associated *Pseudomonas* spp. are really pathogenic on rice remains posed for many years. From the start, few species emerged as the most pathogenic compared to others which were causing some low levels of the disease. Zeigler and Alvarez (Zeigler and Alvarez, 1987) already mentioning minor sheath and grain disorders caused by fluorescent pseudomonads, *P. fuscovaginae* being the principal causal agent. Gardan *et al.* (2002) isolated *P. palleroniana* from La Réunion (France), Cameroon and Madagascar from healthy or necrotic rice seeds and from diseased tissue of leaf sheaths. The *P. palleroniana* isolates inoculated to rice seedlings were either non-pathogenic or weakly pathogenic. On the contrary, typical symptoms of bacterial sheath brown rot were induced by *P. fuscovaginae* strain CFBP3078, introduced in the experiment for comparison. This shows that among the pseudomonads found with rice sheath rot, there are differences in virulence and *P. palleroniana* is among the weakly pathogenic organisms.

However, caution is needed in the interpretation of the pathogenicity level for the different species of the pseudomonads associated with rice sheath rot. Cother *et al.*(2010) isolated a pseudomonad causing a widespread disease similar to sheath brown rot in Cambodia. This bacterium was related to *P. parafulva* and *P. fulva*, which belong to the *P. putida* group as defined by Gomila *et al.* (2015), and was clearly pathogenic on rice.

In the meantime, the taxonomy of pseudomonads has made important progress especially thanks to molecular identification method development. In a recently published classification of *Pseudomonas* genus, based on the Multilocus Sequence Analysis Technique (MLSA), Gomila *et al.* (2015) defined 19 groups and subgroups. Most of the sheath rot associated pseudomonads probably belong to the *P. chlororaphis*, *P. fluorescens*, *P. asplenii* (= *P.*

fusovaginae) subgroup or the *P. putida* group, though the groupings are difficult to define currently as many isolates have not yet been fully analyzed.

2.4.7 Other reported sheath rot associated bacteria

Pantoea ananatis, considered globally as a facultative pathogen (Cray *et al.*, 2013), was demonstrated as a sheath rot pathogen with typical symptoms of necrotic spots and discoloration on glumes and stems, indistinct chlorosis but with no water-soaking and its pathogenicity testing satisfied Koch's postulates (Choi *et al.*, 2012a). The disease had previously been reported in the Philippines (Cottyn *et al.*, 2001) and in Australia (Cother *et al.*, 2004), but its importance, though it is reported to reduce the grain quality when it infects the glumes, was never assessed. It was only presumed to be low. Furthermore, in pathogenicity tests, Cother *et al.* (2004) recovered the pathogen from the plants that had not been inoculated, which prompted the hypothesis that the organism lives as an epiphyte and triggers disease symptoms when the plant is under physiological stress. Also Choi *et al.* (2012) linked the disease appearance to favorable environmental conditions.

Burkholderia glumae and *B. gladioli* are becoming important rice pathogens (Nandakumar et al., 2007a). *B. glumae* (formerly *Pseudomonas glumae*) was reported as the agent of rice grain discoloration in Latin America (Zeigler and Alvarez, 1989) after it had been reported as a grain rotter in Asia. It was later detected in North America, in association with *B. gladioli*, causing bacterial panicle blight (Nandakumar *et al.*, 2009). The two pathogens, in addition to being seed-borne, can also be soil-borne (Nandakumar *et al.*, 2008). Disease symptoms are observed at the sheath and grain levels. Though the disease is seed-borne, the presence of the bacteria in the sheath plays a capital role in the infection of the emerging panicle. Toxoflavin, a toxin produced by both species, is considered to be the main pathogenicity determining factor (Suzuki *et al.*, 2004; Ura *et al.*, 2006), while a lipase produced by *Burkholderia glumae* (Pauwels *et al.*, 2012) and tropolone produced by *B. gladioli* (Wang *et al.*, 2013) have also been implicated in pathogenicity.

Acidovorax oryzae (Schaad *et al.*, 2008), formerly called *Pseudomonas avenae* and *Acidovorax avenae* subsp. *avenae* (Willems *et al.*, 1992), causes bacterial brown stripe on rice (Shakya *et al.*, 1985; Kadota *et al.*, 1991; Song *et al.*, 2004). Symptoms start as brown stripes at the bottom of the stems and frequently extend into the sheaths (Liu *et al.*, 2012a). This bacterium has consistently been detected in rice sheath rot related studies (Cottyn *et al.*, 1996a; b; Cortesi *et al.*, 2005; Cother *et al.*, 2010). Recently the type IV pili assembly protein PilP has been implicated in the pathogenicity of *A. oryzae* on rice (Liu *et al.*, 2012a).

2.5 Conclusions and perspectives

Since rice sheath rot symptoms were first described in Taiwan in 1922 and attributed to *Sarocladium oryzae*, various reports of similar or related disease symptoms have been produced in different parts of the world. Rice sheath rot is now getting momentum as an emerging destructive rice disease but on which the scientific understanding is still limited.

There are three main species or complexes of organisms that can cause rice sheath rot: *Sarocladium oryzae,* the *Fusarium fujikuroi* complex and *Pseudomonas fuscovaginae,* but there are many others that are reported to induce symptoms close to those of rice sheath rot. Interestingly, all three groups of major sheath rot causing pathogens produce phytotoxins that cause necrosis and can mimic the disease symptoms, which is probably the reason why they all cause similar looking disease symptoms. The principle that "everything is everywhere, but, the environment selects" (de Wit and Bouvier, 2006) applies to rice sheath rot; organisms that can potentially cause rice sheath rot are many and can be found everywhere nowadays, but the environment probably selects the ones that can adapt to the prevailing environmental conditions in a given area. This situation results in the overlapping of symptoms in the rice sheath rot disease complex (Johanson *et al.*, 1998; Hu *et al.*, 2008) especially at the rice reproductive stage, the most stress-sensitive phase in rice development (Fageria, 2007). There can be even synergism among the rice sheath rot-associated organisms or with arthropods or other groups of organisms. Due to changes in agriculture and in the society in general, like the developments in the farming systems and increased mobility in general, there are also changes in plant health problems, some diseases becoming more important than before, like rice sheath rot, which is now becoming a serious threat to rice production in many parts of the world.

It is proven that most sheath rot associated pathogens have an endophytic (latent) phase in their lifecycle, waiting for the plant to become stressed so that they can attack it (Fisher and Petrini, 1992). This phenomenon is not recent, it was observed since many years. Hsieh *et al.* (1977) attested the presence of *F. moniliforme* (now known as the *F. fujikuroi* complex) on plants without causing visible disease symptoms. New empirical data are needed about most of the organisms thought to be endophytic as some of them have pathogenic potential and are waiting for conducive conditions to attack the plant. Factors governing the expression of the virulence are not yet clearly understood (Andrews and Harris, 2000). There is an urgent need of associating molecular, genetic and pathogenicity data for elucidating the role and

interactions with endophytes given that at the plant level, the answer to pathogens and endophytes is the same (Andrews and Harris, 2000).

The large variability observed in rice sheath rot associated *Pseudomonas* and *Fusarium* genera is intriguing. It would be interesting to investigate whether the isolates in these two groups that can cause sheath rot have obtained phytotoxin-encoding gene clusters by horizontal gene transfer. At least in the case of fumonisins, it has been shown that the fumonisin gene cluster has spread among *Fusarium* spp. and related genera by a combination of horizontal gene transfer, cluster duplication and loss (Proctor *et al.*, 2013). It should be tested whether the sheath rot causing *Fusarium* isolates all contain the fumonisin gene cluster or other phytotoxin encoding gene clusters. Horizontal gene transfer is also a widespread phenomenon in fluorescent pseudomonads (Silby *et al.*, 2011) and it is known that many gene clusters for secondary metabolites, including cyclic lipopeptides, are located on genomic islands. Again, this could be systematically tested for *Pseudomonas* isolates associated with rice sheath rot.

Rice sheath rot has become a highly destructive rice disease with a high variability in yield loss levels varying from 20 to 85%. It is caused by many pathogenic agents varying depending on the area, grown varieties, prevailing environmental conditions, the farming system, other pests, etc. Not much progress has been achieved in the control of the disease, partly because the etiology of the disease is difficult to establish. For facing the disease, a better understanding about it is needed and this review is contributing in that aim. As rice sheath rot disease is complex by nature, its control strategy must be inspired by the Integrated Pest Management (IPM) approach. The solution remains site-specific. Limiting the number of potential pathogens harbored by the plant, making the plant environment less conducive to pathogen development, etc. should be the central elements in the control approach, which can be complemented by other methods, indicated according to the context. The IPM approach is particularly relevant now that there is a need for feeding and responding to the other needs of a constantly increasing population while the production must be conducted in a sustainable way, meaning that the overreliance on pesticide must leave the room to scientifically-proven environmentally-friendly crop protection practices.

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3 ESTIMATION OF THE IMPORTANCE OF RICE SHEATH ROT IN RWANDA BASED ON DIAGNOSIS AND DISEASE INTENSITY EVALUATION

Vincent de Paul Bigirimana, Obedi Ishibwela Nyamangyoku, Monica Höfte

Abstract

Rice is a staple food for half of the world population, and is produced worldwide in tropical and subtropical areas. Rice is one of the priority crops in Rwanda. The demand for rice is high and the country imports, as of 2011, approximately 30% of the rice consumed. Pests and diseases are recognized major limiting factors preventing rice from expressing its full production potential. Rice sheath rot is one of the emerging and destructive rice diseases, yet poorly studied. Its major symptoms are found on the terminal leaf sheath which turns brown, grayish and ends up dying. Grains from diseased plants are discolored, chaffy and germinate poorly. The disease symptoms are more pronounced at the booting stage. A study was undertaken to evaluate the importance of rice sheath rot in Rwanda. Data for rice sheath rot intensity, *id est* incidence and severity, were taken in the agricultural season of September 2013 to January 2014, from the major rice-growing areas of the country: Bugarama, Cyili, Rwamagana, Nyagatare and Rwagitima. Data for yield loss were taken in Rwagitima and Nyagatare. The average disease incidence was 13.5% and the average measure for disease severity (lesion length on a diseased tiller) was 157 mm. In the considered period, the disease was more pronounced in Rwamagana than in other areas. The yield loss is estimated at 57% yield loss based on the weight of diseased grains in comparison to healthy ones. Rice sheath rot symptoms are found in all rice-growing areas of Rwanda and may be caused by many different pathogens like *Sarocladium oryzae, Fusarium* spp., *Pseudomonas fuscovaginae*, *Burkholderia glumae, Acidovorax avenae*, *etc*. and it is impossible to tell the difference between these agents based on induced symptoms; all the sheath rot causing pathogens are seed-transmitted and as the seed system is centrally organized, it is possible for a disease to be transmitted from one place to another through seed movement. *Sarocladium oryzae* and *Fusarium* spp. have recently been diagnosed in Rwanda. Considering the destructive nature of the disease and its widespread distribution, efforts are needed for limiting its impact. As curative options are limited, preventive measures, ensuring that the seed grain is healthy, should be encouraged as most of these pathogens are seed-transmitted and also given that many currently grown rice cultivars are susceptible to sheath rot pathogens.

3.1 Introduction

Rice is the first staple food worldwide, produced all over the world in tropical and subtropical areas. Rice is popular for many reasons among which we can mention the following: it responds well to farm inputs, can be processed and preserved, has an increasing market, gives a good yield compared to many other tropical crops and is adapted to many agroecological zones. Rice production is promoted in many countries including Rwanda. The situation and importance of rice in Rwanda are presented in the section 2.1.

Rice production faces a series of constraints among which pests and diseases are important. Sheath rot disease (see Chapter 2) is one of frequently found rice diseases, but which is not scientifically clearly understood yet. It occurs in many parts of the world.

Many organisms have been associated with rice sheath rot symptoms including fungal pathogens (see Chapter 2 and Bigirimana et al., 2015). It is difficult to tell the causal agent based on the induced symptoms, as the symptoms are highly similar. Losses caused by rice sheath rot vary from 3 to 85% (Ou, 1985).

In the aim of contributing to the generation of information on rice sheath rot, a study was planned to evaluate the importance of sheath rot in rice fields in Rwanda. For this purpose, data about disease incidence, disease severity and yield loss were collected.

3.2 Materials and methods

Data were collected during the agricultural season of September 2013 to January 2014 in the major rice-growing areas of Rwanda (see Figure 1-1 in Chapter 1), which are Bugarama (Bugarama sector, Western Province), Cyili (Rusatira Sector, Southern Province), Rwamagana (Kigabiro Sector, Eastern Province), and Nyagatare and Rwagitima (Nyagatare and Rugarama Sectors, Eastern Province). Altitudes, rainfall data and average temperatures for these areas are given in Table 3-1.

Table 3-1: Altitude, rainfall and average temperature for the major rice-growing areas in Rwanda*

Location	Closest meteorological station	Altitude (m)	Rainfall (mm)	Average temperature (°C)
Rwamagana	Kibungo	1680	979	18.9
Bugarama	Bugarama	900	1098	24.0
Nyagatare and Rwagitima	Gabiro	1470	783	20.4
Cyili	Rubona	1706	1154	19.2

* Source: MINAGRI (2002)

Data were taken in farmers' fields with rice plants at the ripening stage. At the marshland level, a representative random sampling was followed based on the diseased plants availability and the cardinal directions: north, south, west, east and center. In each direction, samples and measurements were taken in the rice plot following a zigzag pattern.

For measuring disease incidence, at each location 3 to 7 fields were sampled and 100 rice plants per plot were counted except in Cyili marshland where the number of counted plants was less than 100 (see Table 3-2 for details). These plants were carefully visually analyzed, searching for any symptom of sheath rot. The total number of plants on which any symptom of sheath rot was detected were noted. Disease incidence is defined as the proportion, expressed in percentage, of the diseased plants out of the total number of plants considered.

To determine the disease severity, only plants showing sheath rot symptoms were considered. One of the obvious symptoms of sheath rot is the disease lesion development. At each location 8 to 20 samples of diseased plants was taken and the length of the disease lesion was measured and expressed in mm.

A method for measuring the yield loss by estimating the impact of the disease on the grain weight was developed. It is presumed that the other plant development factors are the same for diseased and healthy plants in the same rice plot. In one field at Nyagatare and Rwagitima five panicles of healthy plants and panicles of diseased plants were taken and their grains harvested and dried. For each group, grains were weighed. The difference between the weight of healthy and diseased grains was made. The difference was expressed as a percentage over the weight for healthy grains and it is considered as the percentage of the loss.

The collected data were statistically analyzed using *ad hoc* tests and with SPSS 21 Software (IBM, 2012). The assumption of homogeneity of variances was tested through Levene's test (Kutner et al., 2005) and if this condition was not met, data were square-root transformed to meet the normal distribution conditions and the homogeneity of variances. They were then analyzed following a one-way Analysis of Variance (ANOVA). The significance level was fixed at 0.05% and homogenous groups were generated based on the Duncan Multiple-Range Test.

3.3 Results

3.3.1 Disease incidence

Data on disease incidence were taken in the major rice-growing areas. In all the tested rice-growing areas, plants with sheath rot disease symptoms could be found. There were

differences between the rice-growing areas as illustrated in Table 3-2, with the lowest incidence observed in Nyagatare and Rwagitima, the highest being observed in Rwamagana, while Bugarama and Cyili are in an intermediate situation. The average disease incidence was 13.47%.

Table 3-2: Sheath rot disease incidence in various rice producing areas in Rwanda

Location	Field number	Number of plants/tillers	Disease incidence (%)	Average disease incidence (%)	Square root transformed data	Standard deviation	Homogenous group*
Cyili	1.00	50.00	8.00	14.04	2.83	0.65	ab
	2.00	27.00	18.52		4.30		
	3.00	29.00	13.79		3.71		
	4.00	59.00	13.56		3.68		
	5.00	38.00	23.68		4.87		
	6.00	52.00	11.54		3.40		
	7.00	30.00	13.33		3.65		
Bugarama	1.00	100.00	16.00	14.33	4.00	0.66	ab
	2.00	100.00	9.00		3.00		
	3.00	100.00	18.00		4.24		
Nyagatare and Rwagitima	1.00	100.00	1.00	7.40	1.00	1.04	a
	2.00	100.00	10.00		3.16		
	3.00	100.00	12.00		3.46		
	4.00	100.00	10.00		3.16		
	5.00	100.00	4.00		2.00		
Rwamagana	1.00	100.00	30.00	20.00	5.48	1.04	b
	2.00	100.00	15.00		3.87		
	3.00	100.00	15.00		3.87		
Average disease incidence (%)				13.47			

* Data were square-root transformed to meet the normal distribution conditions and the homogeneity of variances. They were then analyzed following a one-way ANOVA. Values followed by the same letter are not significantly different (P=0.05).

3.3.2 Disease severity

Disease severity was assessed by measuring the lesion length on 8 to 20 plants per location. The disease was found to be significantly more severe in Rwamagana, which is different from the three other areas, these being in comparable homogeneity groups (Figure 3-1).

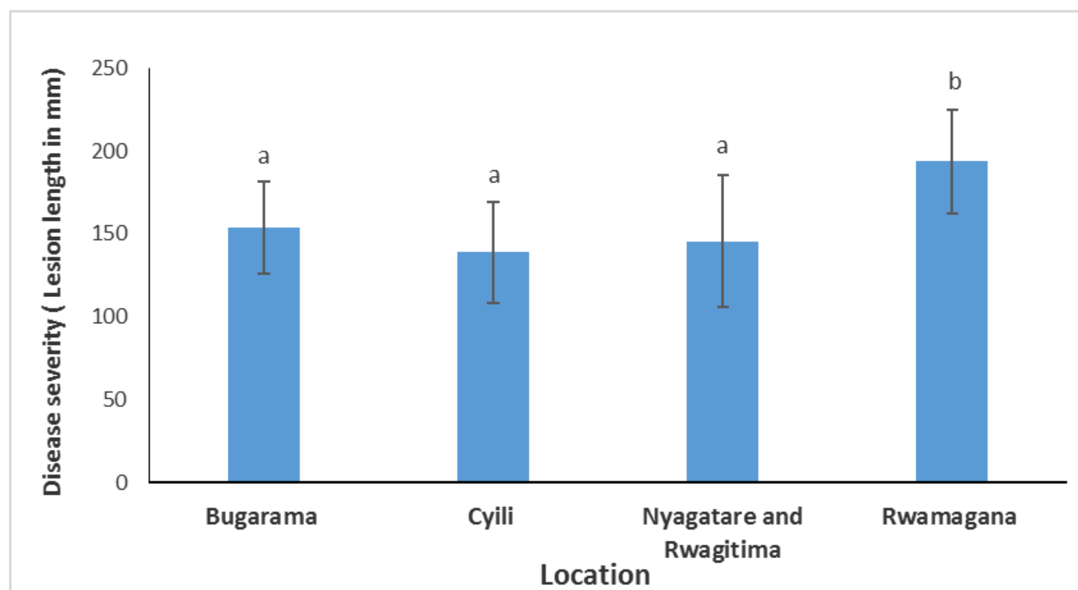

Figure 3-1: Rice sheath rot severity per rice-growing area
Data were analyzed by a one-way ANOVA test. Bars represent mean lesion lengths and standard deviations based on 8 to 20 plants in each location. Bars with the same letters are not significantly different (P=0.05).

3.3.3 Yield loss

Values used for estimating the yield loss were generated on dried grain samples taken in Nyagatare and Rwagitima. The weight of grains from five healthy plants and diseased plants were compared. The average weight of one grain harvested from a healthy plant was 21.22 mg (± 4.51 mg), the average weight of one grain harvested from a diseased plant was 9.14 mg (± 3.19 mg). The calculated yield loss is estimated at 56.93%. A Student test (t-test) revealed that these means are significantly different from each other at P =0.05.

3.4 Discussion

Rice sheath rot symptoms were found in all rice-growing areas of Rwanda considered in this study: Bugarama, Cyili, Rwamagana and Nyagatare and Rwagitima. It is likely that the inoculum is present in nearly all the rice planting materials available in the country. As the seed production system is centrally organized at the national level, the disease development potential is minimized through seed inspection and production but still the planting material released has some level of rice sheath rot pathogens inoculum. In another study, a diagnosis has been conducted on sheath rot associated organisms in Rwanda and it was observed that the major fungal agents of rice sheath rot, *Sarocladium oryzae* and *Fusarium* spp. are present in the country (See Chapter 4). The fact that nearly all the available rice varieties are attacked can be partly explained by the realization that many current rice cultivars are susceptible to attacks of *Sarocladium oryzae* (Mew et al., 2004b) and *Fusarium* disease complexes (Savary

et al., 2012). And it has been proven that some species included in those *Fusarium* complexes cause rice sheath rot (Abbas et al., 1998). It is already established since many years that *Sarocladium oryzae* causes rice sheath rot. Rwamagana showed the highest disease incidence and disease severity. Disease incidence was the lowest at Nyagatare and Rwagitima although data were not significantly different from Cyili and Bugarama due to high variability among fields in Nyagatare and Rwagitima. Disease is influenced by many factors that enter into the disease tetrahedron (Savary et al., 2012): the plant, the pathogen, the environment and the human factors. There are obviously variations in the combination of these factors in the different rice-growing areas, resulting in different disease expressions in those areas. One of the sources of variability may be the rainfall and/or altitude. Rainfall is the lowest in Nyagatare and Rwagitima which may partly explain the lower disease incidence in this region.

The yield loss in terms of grain weight, comparing grains from healthy and diseased plants, is more than the half; it is estimated at 56.93%. This loss may justify the chaffiness that is observed on grains from sheath rot diseased plants (Gopalakrishnan et al., 2010). But it is important to realize that the losses due to the disease are higher than those counted on grains especially in terms of quality. It has been reported that the sheath rot associated pathogens are seed-transmitted, meaning that when the seed is infected, in a system where the seed is most of the time taken from the harvest, the following generation is also exposed to the disease, unless strong preventive measures are taken. This pathogen seed-transmission is reported for both *Sarocladium oryzae* (Ou, 1985) and *Fusarium* spp. (Wulff et al., 2010) that cause sheath rot symptoms. In addition, the yield loss also occurs by the reduction of the marketing quality for the produce when coming from diseased plants as it receives a reduced price. This is particularly true for Rwanda where local rice production is not competitive on the market, being considered of low quality (Stryker, 2013).

The data were taken and analyzed per rice-growing areas and symptoms were found in all the surveyed areas, but the study shows also a high level of variability within the same rice-growing area. The grown rice variety is one of the important factors that can be responsible for the variability in the disease severity due to various disease susceptibility levels for different varieties. Though farmers in the same area in Rwanda use a limited number of varieties per season, there is still a variability in their variety choice, motivated, among others by the disease susceptibility of the planting material. This aspect should be further explored in subsequent studies.

When rice sheath rot is caused by *Fusarium* spp. agents, there is a risk of infection by toxins when consuming the harvested grains as most species of the *Fusarium* spp. complex can produce them (Wulff et al., 2010). This is important for the community in general as food intoxications must be prevented. There is currently no information about toxins infection in Rwanda, but we can presume that it is not negligible, as cereals, *id est* maize, sorghum and wheat, in addition to rice, are among the major crops in the country and that it is not uncommon to see symptoms of attacks by *Fusarium* spp.

Rice is becoming an important food crop in Rwanda. Pests and diseases are among the factors that are still hampering its development. Rice sheath rot disease, one of the important and yet not well known scientifically rice disease is present in Rwanda, in all major rice-growing areas. The disease causes important yield losses, in addition to reducing the grain quality for consumption or use as seed. An action plan for knowing more about the disease and tackling it is highly needed.

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4 *SAROCLADIUM ORYZAE* AND *FUSARIUM SPP.* ARE ASSOCIATED WITH RICE SHEATH ROT IN RWANDA

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The major part of this section has been submitted to *Plant Pathology* and is under revision

Abstract

Sheath rot disease occurs in most rice-growing regions of the world and varying yield losses have been reported. In this study, rice plants showing sheath rot symptoms were sampled from various marshlands in Rwanda and fungal organisms associated with the disease were isolated, purified, and identified by both morphological and molecular methods. *Sarocladium oryzae* and *Fusarium* spp. were recovered from the diseased fields in Rwanda. *Sarocladium oryzae* isolates were genetically very similar, while the majority of the *Fusarium* isolates were closely related to *F. andiyazi*, a species belonging to the *F. fujikuroi* complex. Other *Fusarium* isolates retrieved from sheath rot lesions belonged to the *F. oxysporum*, *F. incarnatum/equiseti* and the *F. graminearum* complex. Phylogenetic analysis reveals a strong relationship between the *S. oryzae* and *F. andiyazi* isolates found in Rwanda and those reported in other rice growing areas. *Sarocladium oryzae* and the *Fusarium* isolates caused very similar symptoms on rice plants although the *indica* variety CO39 appeared to be more resistant to both fungal genera than the *japonica* variety Nipponbare. Fumonisin production could be detected in rice plants inoculated with *Fusarium* isolates from the *fujikuroi* complex and the *oxysporum* complex, but was not correlated with disease severity.

4.1 Introduction

Rwanda is a densely populated high altitude country in Central/East-Africa. There is a lot of interest in rice (*Oryza sativa*) production in Rwanda because the crop can be grown in marshland areas and as such relieve the increasing pressure on hillside land for food production. Rice has been introduced in Rwanda in the 1950s (Stryker, 2013) and is cultivated in irrigated systems at low and middle altitude (900 to 2000 m). The presence of rice sheath rot symptoms has been reported in Rwanda but has not further been investigated (Bigirimana et al., 2013). Rice sheath rot is an emerging disease worldwide and can cause varying yield losses. Rice sheath rot can be caused by toxin-producing fungal pathogens (see Chapter 2 and Bigirimana et al., 2015 for a review). The main fungal species reported is *Sarocladium oryzae* (Sakthivel, 2001; Bills et al., 2004; Ayyadurai et al., 2005), but also *Fusarium* spp. belonging to the *F. fujikuroi* complex such as *F. proliferatum, F. verticillioides,* and *F. fujikuroi* (Abbas et al., 1998; Wulff et al., 2010) have been associated with rice sheath rot. *Sarocladium oryzae* is a seed-borne pathogen and its mycelium can survive in plant residues, weed hosts and soil (Sakthivel, 2001). Pathogenicity of *S. oryzae* is related to its capacity to produce the phytotoxins helvolic acid and cerulenin (Sakthivel et al., 2002). Artificial inoculation of these toxins on rice plants can reproduce sheath rot symptoms (Ghosh et al., 2002; Ayyadurai et al., 2005). The main toxins produced by species belonging to the *F. fujikuroi* complex are the fumonisins (Ma et al., 2013). The role of fumonisins in *Fusarium*-induced pathogenicity is controversial: Abbas et al. (1998) attested that *in planta* fumonisin concentrations were positively correlated with the levels of the disease caused by *Fusarium* spp. but Dastjerdi and Karlovsky (2015) showed that fumonisin-producing and non-producing *Fusarium verticillioides* isolates could both infect seedlings at the same level and thus concluding that fumonisins are not involved in the infection.

Despite the fact that the occurrence of rice sheath rot can cause considerable yield losses, the disease etiology is poorly understood. Thus, the current study aimed at identifying the fungal organisms associated with rice sheath rot in Rwanda. By building the baseline on sheath rot disease in Rwanda, this study paves the way for breeding for resistance and development of management strategies against rice sheath rot.

4.2 Materials and methods

4.2.1 Field sampling

In 2011 and 2013 samples were taken from the traditional rice-growing areas of Bugarama (Bugarama Sector, Western Province), Rwamagana (Kigabiro Sector, Eastern Province), Nyagatare and Rwagitima (respectively Nyagatare and Rugarama Sectors, Eastern Province) in Rwanda. To reflect the change in rice production in Rwanda, in 2013 additional diseased rice plant samples were taken in the new rice growing areas Rugeramigozi (Nyamabuye Sector, Southern Province) and Kabuye (Jabana Sector, Kigali City). All locations where the sampling was conducted are illustrated in Figure 1-1, whereas the major agro-bioclimatic features of the sampling areas are listed in Table 4-1.

Table 4-1: Agro-bioclimatic characteristics of the sampling regions in Rwanda (Gasana, 2002; MINAGRI, 2005)

Province	Sampling region	Altitude (m)	Annual Rice cultivation area (ha)	Average temperature (°C)	Annual rainfall (mm)
Southern Province	Rugeramigozi	1706	160	19.2	1154
Western Province	Bugarama	900	2321	24.0	1098
Eastern Province	Nyagatare and Rwagitima	1470	973	20.4	783
	Rwamagana	1680	713	18.9	979
Kigali City	Kabuye	1270	296	21.7	951

Samples of diseased plants were taken in Agricultural Season B (from March to August) of 2011, and in Agricultural Season A (from September to February) of 2013. In each rice-growing area, sheath rot diseased plant samples were taken in a representative way, following the four cardinal directions (north, south, east and west) and the centre of the marshland. In each direction, there was a careful search for plants exhibiting sheath rot symptoms. Whenever these were found, samples of diseased (rotted) sheaths were collected, following a zigzag pattern.

4.2.2 Pathogen isolation and purification

Plant tissues and un-emerged panicles showing typical sheath rot symptoms were cut in small pieces and surface-disinfected in 70% ethanol for 2 min, followed by rinsing four times with sterile water. Surface-sterilized samples were placed on potato dextrose agar (PDA; Difco) plates and incubated at 28°C for seven to ten days. Fungal hyphae initially developing from diseased plant tissues were transferred to fresh PDA plates and pure cultures were stored on PDA slants at room temperature.

4.2.3 Morphological characterization

Cultures of purified isolates were grown on PDA plates at 28°C for at least two weeks and their morphological characteristics were identified microscopically and macroscopically. For microscopic analysis, thirty randomly selected macroconidia were measured. The presence/absence of chlamydospores was also recorded. For macroscopic analysis, pigmentation on PDA and the growth rate of fungal colony were observed.

4.2.4 DNA extraction, amplification and ITS-sequencing

Fungal isolates were grown on potato dextrose broth (PDB; Difco) at 28°C for one week. The fungal biomass was harvested and ground in liquid nitrogen to produce a fine powder. Total genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) or the DNeasy Plant Mini Kit (Qiagen). The Internal Transcribed Spacer (ITS) region was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCGTAGGTGAACCTGCGG -3') (White et al., 1990). *Fusarium* isolates were further identified by amplifying the Translation Elongation Factor (TEF) using primer pair TEF-1-F (5'-ATGGGTAAGGAAGACAAGAC-3') and TEF-2-R (5'-GGAAGTACCAGTGATCATGTT-3') (O'Donnell et al., 1998). PCR reactions were done in 25 µL of a solution consisting of 2 µL genomic DNA (100 ng $µL^{-1}$), 5 µL PCR buffer (5x; Promega), 5 µL Q-solution (Qiagen), 0.5 µL dNTPs (10 mM; Fermentas GmbH), 1.75 µL of each primer (10 µM), 0.15 µL Taq DNA polymerase (5 units $µL^{-1}$; Fermentas GmbH) and 8.85 µL ultrapure sterile water. Amplification was performed using a Flexcycler PCR Thermal Cycler (Analytik Jena) programmed for an initial denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min. Cycling ended with a final extension step at 72°C for 10 min. The amplicons were separated by horizontal electrophoresis using 2% agarose gels in Tris-Acetate-EDTA (TAE)-buffer at 100 V for 25 min and visualized by ethidium bromide staining on a UV transilluminator. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and the sequences of both strands were determined by LGC Genomics GmbH (Berlin, Germany) using Sanger sequencing. Before use in blasting or building phylogenetic trees, sequences were manually edited.

4.2.5 Identification using BLAST and phylogenetic analysis

Consensus sequences of all isolates generated with BioEdit version 7.1.11 were blasted against GenBank using the BLASTn tool. Isolates belonging to fungal species most related to those present in our collection were selected as reference for phylogenetic tree analysis. Most

Sarocladium sequences were taken from Giraldo et al. (2015). *Fusarium* TEF sequences were retrieved from the Fusarium MLST database or GenBank. Reference isolates belonging to the *F. fujikuroi* group were taken from Wulff et al. (Wulff et al., 2010). Sequences of *Acremonium variecolor* and *Cylindrocarpon* sp. were taken as outgroups.

The accession numbers of all sequences are shown in Table 4-2.

Table 4-2: Sequences of sheath rot-associated isolates and standard strains obtained from GenBank and Fusarium MLST Database used for building phylogenetic trees

Genus	Species	Isolate	Origin	Accession number	Reference
Fusarium spp.	*F. andiyazi*	30ALH	Rice, China	FN252387	(Wulff et al., 2010)
		1ALH	Rice, Burkina Faso	FN252388	(Wulff et al., 2010)
		38ALH	Rice, India	FN252389	(Wulff et al., 2010)
	Fusarium sp.	NRRL 46612	Tomato, Sardinia	-	Fusarium MLST
	Fusarium sp.	NRRL 31654	*Hadrodemas warscewiczianum,* Guatemala	-	Fusarium MLST
	F. fujikuroi	21ALH	Rice, Vietnam	FN252404	(Wulff et al., 2010)
		7ALH	Rice, Nepal	FN252407	(Wulff et al., 2010)
		20ALH	Rice, Vietnam	FN252398	(Wulff et al., 2010)
	F. verticillioides	52 ALH	Rice, Vietnam	FN179342	(Wulff et al., 2010)
		32 ALH	Rice, China	FN179344	(Wulff et al., 2010)
		56ALH	Rice, Bangladesh	FN252385	(Wulff et al., 2010)
	F. proliferatum	24ALH	Rice, Cote d'Ivoire	FN252391	(Wulff et al., 2010)
		34ALH	Rice, China	FN252396	(Wulff et al., 2010)
		51ALH	Rice, Vietnam	FN252397	(Wulff et al., 2010)
	F. boothii	NRRL 29011	South Africa	AF212445	(O'Donnell et al., 2000)
	F. graminearum	NRRL 31084	Wheat, USA	HM744693	(Yli-Mattila et al., 2011)
	F. vorosii	NRRL 38208	Wheat, Japan	DQ459746	(Starkey et al., 2007)
	F. asiaticum	NRRL 13818	Barley, Japan	AF212451	(O'Donnell et al., 2000)
	F. incarnatum-equiseti species complex	NRRL 25080	Brown planthopper, China	JF740711	(O'Donnell et al., 2012)
		F714	Wheat, Spain	KF962952	(Castellá and Cabañes, 2014)
	F. oxysporum species complex	NRRL 38271	Lentil, Colombia	FJ985361	(Donnell et al., 2009)
		NRRL 22543	*Elaeidis*, Suriname	FJ985270	(Donnell et al., 2009)
		NRRL 22554	*Chrysanthemum* sp., Nigeria	FJ985274	(Donnell et al., 2009)
Sarocladium spp.	*S. zeae*	CBS 800.69	*Zea mays* stalk, USA	FN691451	(Giraldo et al., 2015)
	S. bacillisporum	CBS 119.79	Smoked slided meat, Sweden	HG965001	(Giraldo et al., 2015)
		CBS 212.79	Insect, Romania	HG965002	(Giraldo et al., 2015)
		CBS 388.67	Soil, The Netherlands	HG965003	(Giraldo et al., 2015)
		CBS 425.67	Soil, Canada	HE608639	(Giraldo et al., 2012)
		CBS 485.67	Unknown	HG965004	(Giraldo et al., 2015)
		CBS 787.69	Teleutosorus of *Puccinia graminis* on *Lolium temulentum*, Italy	HG965005	(Giraldo et al., 2015)
	S. bactrocephalum	CBS 749.69	*Ustilago* sp., Canada	HG965006	(Giraldo et al., 2015)
		UTHSC 09-384	Eye, USA	HG965007	(Giraldo et al., 2015)

Genus	Species	Isolate	Origin	Accession number	Reference
	S. bifurcatum	CBS 383.73	Bamboo, India	HG965008	(Giraldo et al., 2015)
		UTHSC 05-3311	Bronchoalveolar lavage fluid, USA	HG965009	(Giraldo et al., 2015)
		UTHSC 07-3446	Bronchial wash fluid, USA	HG965010	(Giraldo et al., 2015)
	S. gamsii	CBS 425.73	*Pandanus lerum*, Sri Lanka	HG965014	(Giraldo et al., 2015)
		CBS 707.73	*Pandanus lerum*, Sri Lanka	HG965015	(Giraldo et al., 2015)
	S. glaucum	CBS 191.80	Bamboo, Cambodia	HG965016	(Giraldo et al., 2015)
		CBS 309.74	Air, above sugarcane field, India	HG965017	(Giraldo et al., 2015)
		CBS 382.73	Bamboo, India	HG965018	(Giraldo et al., 2015)
		CBS 456.74	Sugar, Mauritius	HG965019	(Giraldo et al., 2015)
		CBS 100350	Bamboo, Japan	HG965020	(Giraldo et al., 2015)
	S. hominis	UTHSC 02-2564	Leg, USA	HG965011	(Giraldo et al., 2015)
		UTHSC 04-1034	Calf, USA	HG965012	(Giraldo et al., 2015)
		UTHSC 04-3464	Sputum, USA	HG965013	(Giraldo et al., 2015)
	S. implicatum	CBS 397.70A	*Saccharum officinarum*, Jamaica	HG965021	(Giraldo et al., 2015)
		CBS 825.73	*Saccharum officinarum*, India	HG965022	(Giraldo et al., 2015)
		CBS 959.72	Desert soil, Egypt	HG965023	(Giraldo et al., 2015)
	S. kiliense	CBS 122.29	Skin, Germany	FN691446	(Perdomo et al., 2011)
	S. ochraceum	CBS 428.67	*Zea mays*, Kenya	HG965025	(Giraldo et al., 2015)
	S. oryzae	CBS 180.74	Rice, India	HG965026	(Giraldo et al., 2015)
		CBS 399.73	Rice, India	HG965027	(Giraldo et al., 2015)
		LS449-1	Rice, India	KP999971	Genbank
		CBS 414.81	Rice, Nigeria	HG965028	(Giraldo et al., 2015)
		CBS 361.75	Rice, Kenya	AY567005	(Bills et al., 2004)
		CBS 120.817	Rice, Panama		This study
		WA13481	Rice, Australia	JQ965668	(Lanoiselet et al., 2012)
	S. pseudostrictum	UTHSC 02-1892	Sputum, USA	HG965029	(Giraldo et al., 2015)
	S. strictum	CBS 640.75	Decaying wood, The Netherlands	HG965030	(Giraldo et al., 2015)
	S. subulatum	MUCL 9939	Soil, Egypt	HG965031	(Giraldo et al., 2015)
		UTHCS 07-110	Bone, USA	HG965032	(Giraldo et al., 2015)
	S. summerbellii	CBS 430.70	Soil, The Netherlands	HG965034	(Giraldo et al., 2015)
		CBS 797.69	*Canna indica*, The Netherlands	HG965035	(Giraldo et al., 2015)
		CBS891.73	Dead leaf, Sri Lanka	HG965036	(Giraldo et al., 2015)
		CBS951.72	Soil, The Netherlands	HG965037	(Giraldo et al., 2015)
	S. terricola	MUCL 42865	Palm grove, Morocco	HG965040	(Giraldo et al., 2015)
		UTHSC 03-2933	Bronchial wash fluid, USA	HG965041	(Giraldo et al., 2015)
		UTHSC 04-956	Sinus, USA	HG965042	(Giraldo et al., 2015)
		UTHSC 07-3260	Bone, USA	HG965043	(Giraldo et al., 2015)
		UTHSC 08-844	Bronchoalveolar lavage fluid, USA	HG965045	(Giraldo et al., 2015)

For multiple alignments, the MUSCLE algorithm was applied. Phylogenetic trees and distance matrix were constructed using the maximum-likelihood algorithm with 1000 replicates using MEGA6 (Tamura et al., 2013).

4.2.6 Pathogenicity assays

Twelve isolates representing the different fungal species isolated from rice with sheath rot symptoms in Rwanda were selected for pathogenicity tests on rice plants. Inoculum was prepared according to the standard grain inoculum technique (Sakthivel and Gnanamanickam, 1987). Briefly, rice grains were soaked in water for 1 h, excess water was removed and the grains were autoclaved twice on two different days. Ten fungal discs (diameter = 5 mm) taken from the edge of 7-day-old fungal colonies grown on PDA were used to inoculate sterile grains and 0.5 mL of sterile distilled water was added before inoculated grains were incubated at 28°C for 14 days. Every two days, grains were shaken to prevent the formation of clumps.

Rice seeds of *indica* cultivar CO39 and *japonica* cultivar Nipponbare were surface sterilized in 2% sodium hypochlorite solution for 20 min, rinsed five times in sterile distilled water, and placed in Petri dishes containing sterile moistened filter papers. After one week of incubation at 28°C, sets of 12 germinated seeds were sown in a perforated plastic tray (22 x 15 x 6 cm) filled with 900 g potting soil (Structural; Snebbout, Kaprijke, Belgium). Rice seedlings were maintained in a growth chamber (28°C, 60% relative humidity) and 10-week-old plants were used for inoculation. One grain fully colonized by fungal hyphae was introduced in the junction point between the sheath of the second youngest plant leaf and the stem. Inoculation points were covered with moist cotton pad and wrapped with Parafilm to maintain humidity. Plants were evaluated 10 days post inoculation based on the Standard Evaluation System (SES) scale developed by IRRI (IRRI, 2002): 0 = no incidence on flag leaf sheath; 1 = less than 1% of flag leaf sheath area showing symptoms; 3 = 1-5% of flag leaf sheath area showing symptoms; 5 = 6-25% of flag leaf sheath area showing symptoms; 7 = 26-50% of flag leaf sheath area showing symptoms; and 9 = 51-100% of flag leaf sheath area showing symptoms. Each treatment consisted of 12 plants, which were cultivated in the same plastic tray, and the entire experiment was repeated once.

Disease severity was presented as Percent disease index (PDI) according to the following formula:

Disease severity data were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney tests since the conditions of normality and homogeneity of variances were not met. All statistic tests were performed with SPSS 22.0 (SPSS Inc., Illinois, USA) and the significance level was fixed at 0.05.

4.2.7 Fumonisin analysis

Plants infected with *Fusarium* spp. were collected for the detection of fumonisins, toxins that have been known to be responsible for sheath rot disease on rice. Approximately 10 g of leaf sample was ground in liquid nitrogen into a fine powder. Five grams of ground sample was extracted with 25 ml of 70% methanol. Samples were shaken for 3 min and, then, allowed to settle for 2 min. An aliquot of 100 µL of supernatants collected from the top layer of each extract was diluted in 900 µL of distilled water. An aliquot of 50 µl of the solution was analyzed for its total fumonisins using an ELISA kit (AgraQuant Fumonisin Kit; Romer Labs, Singapore).

4.3 Results

4.3.1 Morphological characteristics

After isolation and purification, one hundred and ninety-four forty eight fungal isolates were obtained. Fourty-eight isolates were identified by DNA sequencing. Based on morphological analysis, the isolates were divided into three major subgroups including *Sarocladium*-like, *Fusarium*-like and others. The main characteristics of the *Sarocladium*-like and *Fusarium*-like isolates are presented in Table 4-3 and Figure 4-1. The distribution of identified isolates per location is presented in Figure 4-2. The third subgroup is represented by two identified isolates, one of *Chaetomium* sp. and one of *Epicoccum* sp. Although these fungi are described as rice seed pathogens, they were not considered further since they were only isolated once.

Table 4-3: Morphological characteristics of fungi isolated from Rwanda

Species	Ch*	Pigmentation on PDA	Number of septa		Size of (macro)conidia (µm)	Colony diameter at 3 days after inoculation (mm)
			Macroconidia	Microconidia*		
Fusarium fujikuroi complex	-	Grayish orange to violet grey, dark violet or dark magenta	3-5	+	25-60x2.5-4.0	46
Fusarium graminearum complex	+	Dark violet or dark magenta	5-7	-	38-71x4.0-6.5	67
Fusarium incarnatum-equiseti complex	+	Beige	5-7	-	42-80x3.2-5.6	54
Fusarium oxysporum complex	+	Violet grey to dark violet	3-5	-	21-41x5.0-6.7	43
Sarocladium oryzae	/	Pale orange	/	/	3.5-7.0x1.0-1.5	17

*: Ch: Chlamydospores

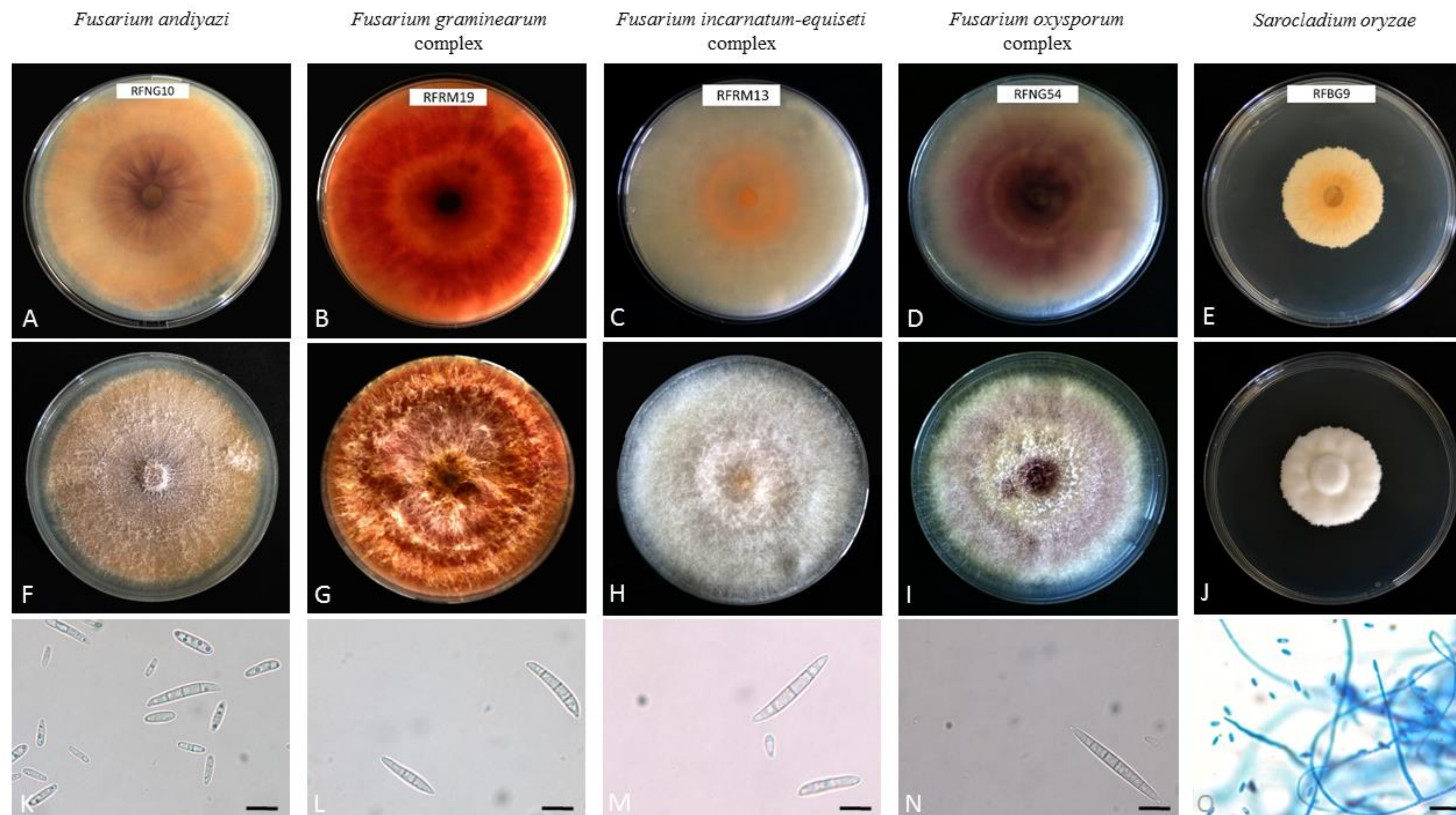

Figure 4-1: Morphological features of *Fusarium spp.* and *Sarocladium oryzae* isolated from rice plants showing sheath rot symptoms in Rwanda. Colony shape: reverse (A-E) and front (F-J) sides of PDA plates. Conidia shapes and sizes (K-O) were observed under the microscope. *Sarocladium oryzae* was stained with 1% methylene blue before examining. Scale bars represent 20 µm.

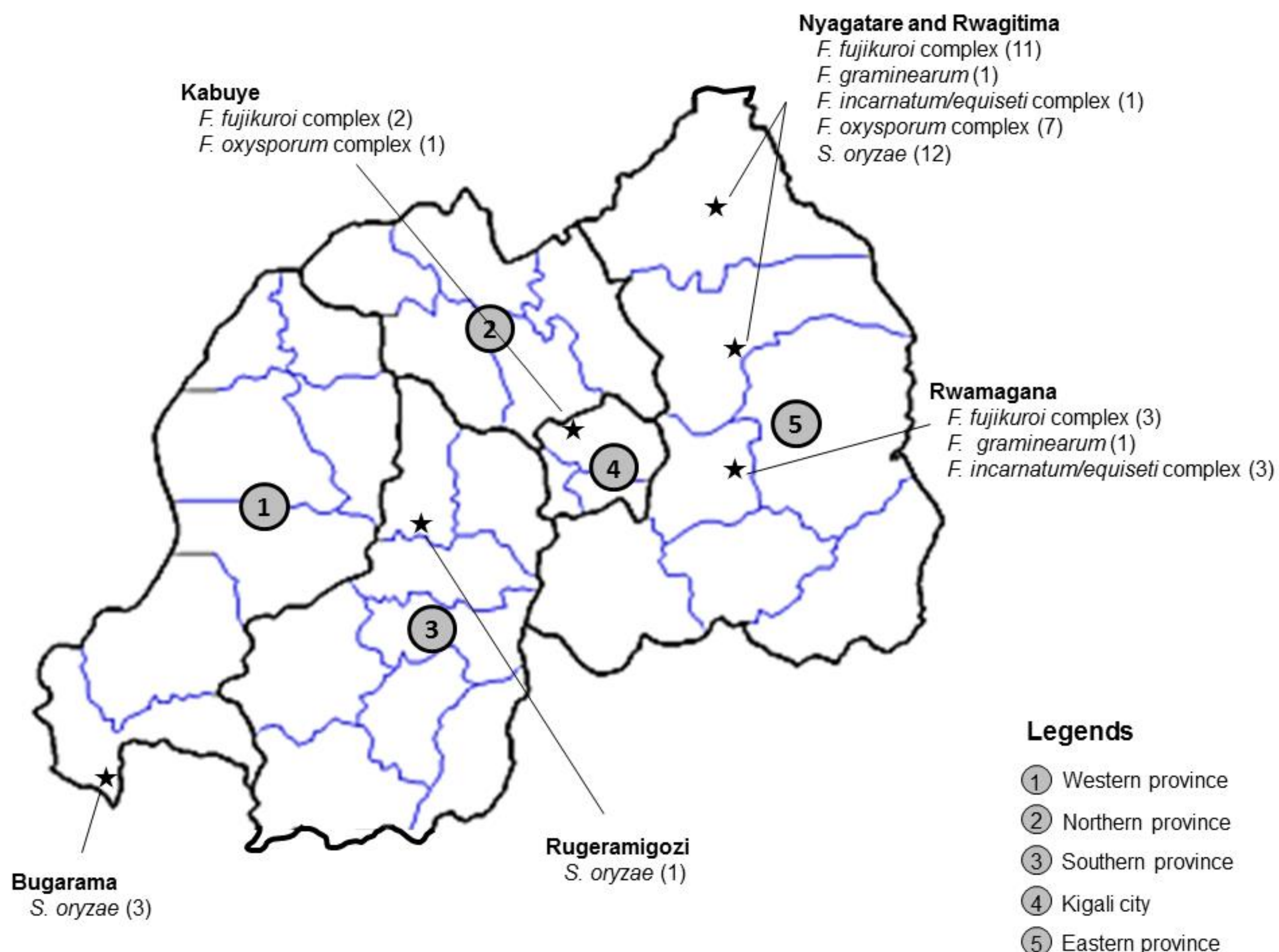

Figure 4-2: Sampling sites for rice sheath rot diseased plants in Rwanda and isolated fungi

Most *Fusarium* species found in this study were able to form chlamydospores and their microconidia were usually one celled. Macroconidia of these *Fusarium* species had three to seven septa and the dimensions of macroconidia ranged from 21.0-80.0 µmx2.5-6.7 µm. Colony colour varied from beige, grayish orange to violet grey, dark violet or dark magenta. For *Sarocladium* isolates, the only colony colour observed was pale orange. Conidia of *Sarocladium* were small with size ranging from 3.5-7.0 µmx1.0-1.5 µm. *Sarocladium* isolates grew significantly slower (17 mm in three days) compared to *Fusarium* spp. isolates (43-67 mm in three days).

4.3.2 Molecular identification and phylogenetic analysis

Forty-six fungal isolates associated with rice sheath rot disease were identified by sequencing the ITS-rDNA region and/or the TEF factor. BLAST results indicated that 16 isolates were closely related to *S. oryzae*. Their identification was based on the ITS-rDNA region

sequencing. These isolates were obtained from Bugarama, Nyagatare and Rwagitima, and Rugeramigozi. The remaining 30 isolates were assigned to different species of the *Fusarium* genus. Most of them were identified by sequencing the TEF factor though both TEF factor and ITS-rDNA region were sequenced for some of them. The majority of the *Fusarium* isolates belonged to the *F. fujikuroi* complex (50% of *Fusarium* isolates) and originated from Kabuye, Nyagatare and Rwagitima, and Rwamagana. The second most dominant group was the *F. oxysporum* complex (30%) found in Kabuye and Nyagatare and Rwagitima, followed by the *F. incarnatum/equiseti* complex (13.3%), and the *F. graminearum* complex (6.7%) (Figure 4-2 and Table 4-4). These last two complexes were found in Nyagatare and Rwagitima, and Rwamagana. For more precise identification, the TEF gene of several *Fusarium* isolates was also sequenced.

Table 4-4: Characterization of fungal isolates collected in sheath rot-affected rice fields in Rwanda

Identification	Isolate	Location	Year of isolation
***Fusarium fujikuroi* complex**	RFKB6	Kabuye	2013
	RFKB1	Kabuye	2013
	RFNG10	Nyagatare and Rwagitima	2011
	RFNG13	Nyagatare and Rwagitima	2011
	RFNG16	Nyagatare and Rwagitima	2011
	RFNG20	Nyagatare and Rwagitima	2011
	RFNG32	Nyagatare and Rwagitima	2011
	RFNG57	Nyagatare and Rwagitima	2011
	RFNG72	Nyagatare and Rwagitima	2011
	RFNG110	Nyagatare and Rwagitima	2011
	RFNG113	Nyagatare and Rwagitima	2011
	RFNG114	Nyagatare and Rwagitima	2011
	RFNG115	Nyagatare and Rwagitima	2011
	RFRM18	Rwamagana	2013
	RFRM35	Rwamagana	2013
	RFRM36	Rwamagana	2013
***Fusarium graminearum* complex**	RFNG127	Nyagatare and Rwagitima	2013
	RFRM19	Rwamagana	2013
***Fusarium incarnatum/equiseti* complex**	RFNG61	Nyagatare and Rwagitima	2011
	RFRM13	Rwamagana	2013
	RFRM14	Rwamagana	2013
	RFRM17	Rwamagana	2013
***Fusarium oxysporum* complex**	RFKB4	Kabuye	2013
	RFNG50	Nyagatare and Rwagitima	2011
	RFNG54	Nyagatare and Rwagitima	2011
	RFNG59	Nyagatare and Rwagitima	2011
	RFNG60	Nyagatare and Rwagitima	2011
	RFNG95	Nyagatare and Rwagitima	2011
	RFNG106	Nyagatare and Rwagitima	2011
	RFNG112	Nyagatare and Rwagitima	2011
Sarocladium oryzae	RFBG3	Bugarama	2011
	RFBG9	Bugarama	2011
	RFBG10	Bugarama	2011
	RFNG23	Nyagatare and Rwagitima	2011
	RFNG27	Nyagatare and Rwagitima	2011
	RFNG28	Nyagatare and Rwagitima	2011
	RFNG30	Nyagatare and Rwagitima	2011
	RFNG33	Nyagatare and Rwagitima	2011
	RFNG41	Nyagatare and Rwagitima	2011
	RFNG51	Nyagatare and Rwagitima	2011
	RFNG94	Nyagatare and Rwagitima	2011
	RFNG97	Nyagatare and Rwagitima	2011
	RFNG100	Nyagatare and Rwagitima	2011
	RFNG122	Nyagatare and Rwagitima	2013
	RFNG124	Nyagatare and Rwagitima	2013
	RFRG2	Rugeramigozi	2013

To check for genetic variability within species, phylogenetic trees of *Sarocladium* isolates and *Fusarium* isolates were constructed based on the analysis of sequenced ITS-rDNA and TEF genes, respectively. Species of sheath rot-associated isolates and standard strains obtained from GenBank and Fusarium MLST Database for which the sequences were used for building

phylogenetic trees are presented in Table 4-2. Only one *S. oryzae* isolate, RFRG2, clustered with CBS strain 180.74 from India, which is the epitype of *S. oryzae*, and strains from Kenya (CBS 361.75), Panama (CBS 120.817) and Australia (WA13481). All other *S. oryzae* isolates clustered with CBS strain 399.73 obtained from India (Figure 4-3).

Sarocladium oryzae strain CBS 414.81 from Nigeria seems more distantly related to the other *S. oryzae* isolates. It should be noted that strains CBS 399.73 and CBS 414.81 were originally classified as *S. attenuatum,* a species that is considered to be conspecific with *S. oryzae* (Bills et al., 2004; Giraldo et al., 2015). The ITS sequence of strain CBS 414.81 is clearly different from strain CBS 399.73 (Result of the comparative blasting in NCBI are: Identity: 98%; Query coverage: 100%).

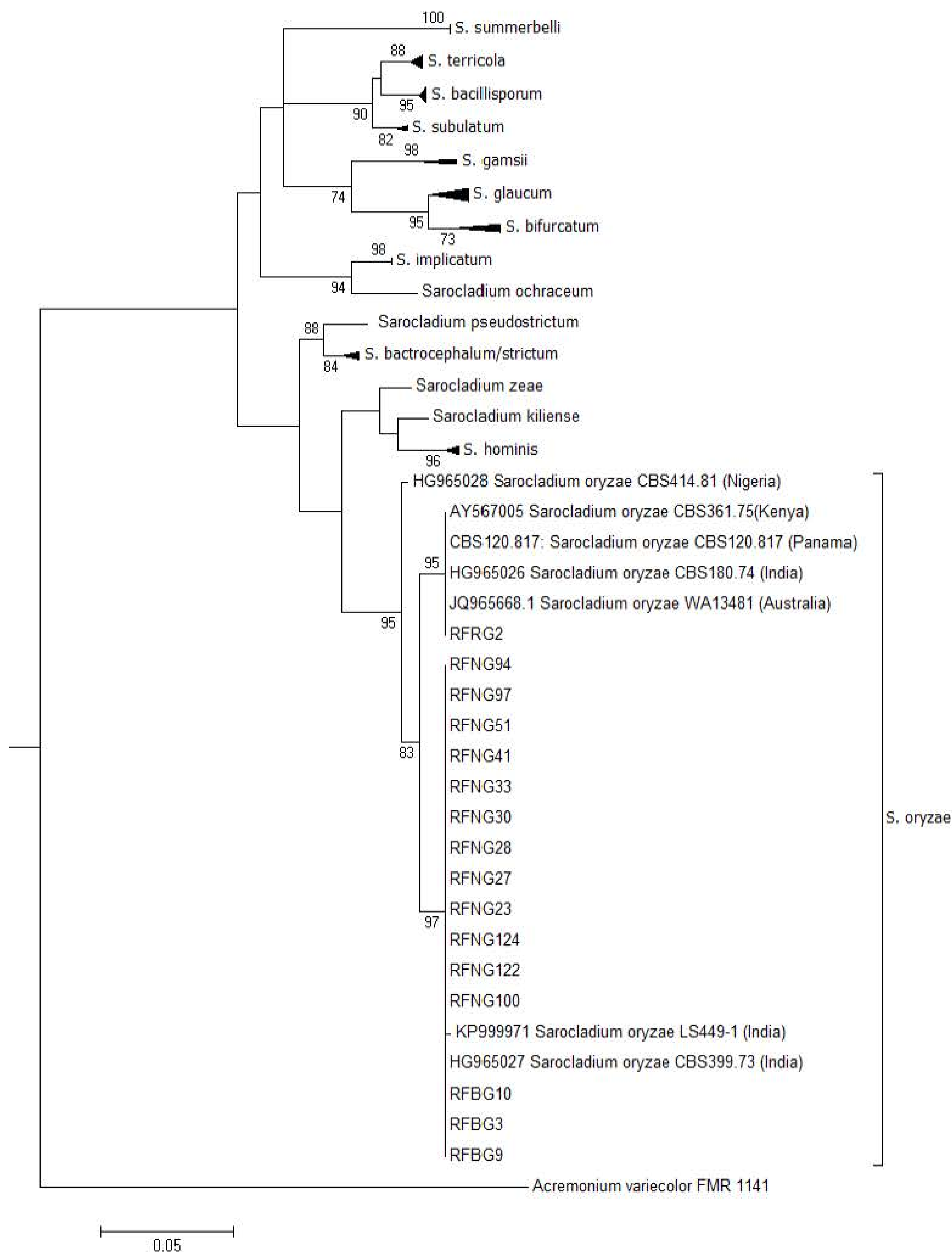

Figure 4-3: Phylogenetic tree for *Sarocladium*-related sequences identified by the ITS region. Sequences were aligned using MUSCLE and the tree was generated by the maximum-likelihood algorithm. The numbers above branches represent bootstrap percentiles from 1,000 replicates. See Table 4-2 for a full list of *Sarocladium* reference isolates used to build the tree.

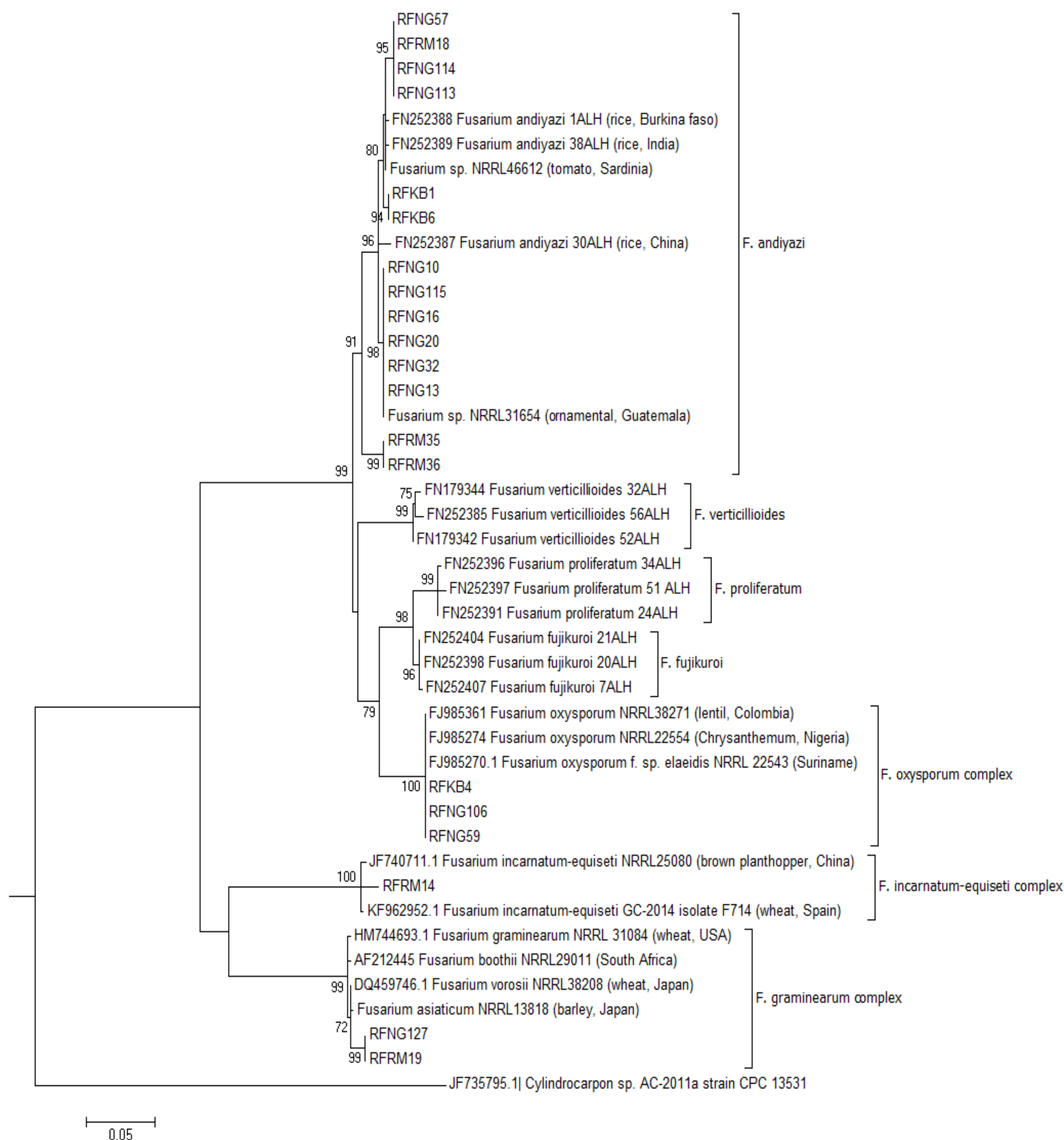

Figure 4-4: **Phylogenetic tree for *Fusarium*-related sequences identified by the TEF factor. Sequences were aligned using MUSCLE and the tree was generated by the maximum-likelihood method. The numbers above branches represent bootstrap percentiles from 1,000 replicates. See Table 4-2 for a full list of *Fusarium* reference isolates used to build the tree.**

In contrast to the homogeneity observed among *Sarocladium* isolates from Rwanda, isolates of the *Fusarium* genus showed more variability (Figure 4-4).

The 14 sampled isolates belonging to the *F. fujikuroi* complex clustered with *F. andiyazi* isolates from rice from India, Burkina Faso and China. Isolates RFNG127 and RFRM19 grouped with isolates belonging to the *F. graminearum* complex and were most closely related to a *F. asiaticum* isolate from barley in Japan (NRRL 13818). The *F. oxysporum* isolates sampled in Rwanda were highly similar and grouped together with *F. oxysporum* isolates from Nigeria and Colombia. The TEF sequence of isolate RFRM14 showed the highest sequence similarity to *F. incarnatum/equiseti* isolates obtained from wheat in Spain (isolate F714) and from a brown planthopper in China (NRRL 25080).

4.3.3 Pathogenicity results

In planta pathogenicity tests showed that *S. oryzae* and all *Fusarium* isolates tested are able to induce sheath rot symptoms on rice (Table 4-5). Interestingly, inoculation of *Fusarium* isolates and *S. oryzae* isolates resulted in a similar pattern of infection and symptomatology on two different rice cultivars: Nipponbare (*Japonica*) (Figure 4-5a) and CO39 (*Indica*) (Figure 4-5b).

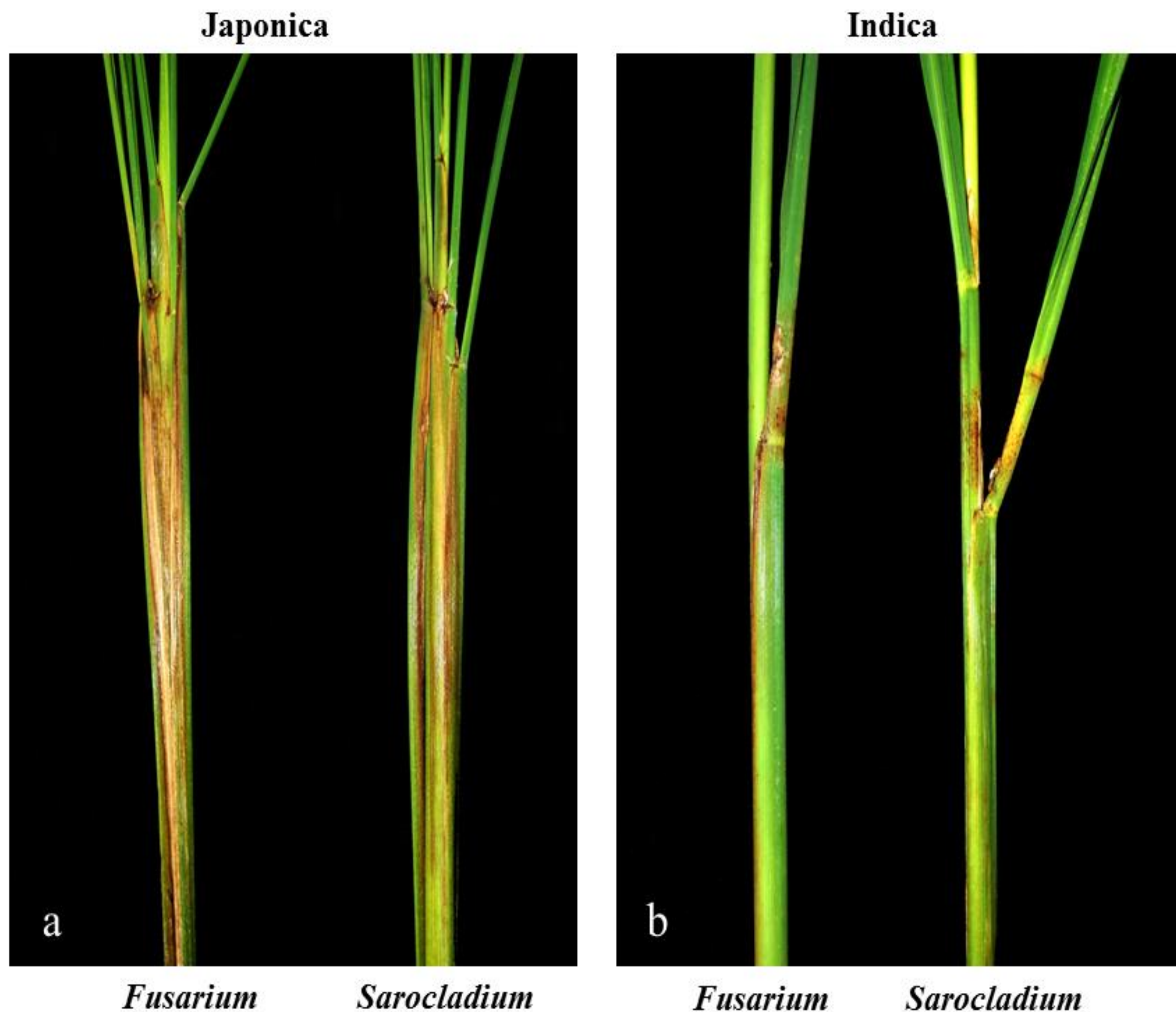

Figure 4-5: Figure 4-6: Similarity in rice sheath rot symptoms caused by *Fusarium* spp. RFNG16 (left) and *Sarocladium oryzae* RFRG2 (right) on japonica cultivar Nipponbare (a) and indica cultivar CO39 (b).

Compared to CO39, the Japonica variety Nipponbare is clearly more susceptible to the infection. The percent disease index (PDI) recorded 10 days after inoculation varied from 33.3% to 58.3% in CO39 and from 60.0% to 77.8% in Nipponbare (Table 4-5).

Table 4-5: Determination of pathogenicity and *in planta* fumonisin production of fungal isolates collected in Rwanda

Identification	Isolate	Rice cultivar and characteristics			
		Nipponbare		CO39	
		PDI*	Fumonisin concentration** (mg/kg)	PDI	Fumonisin concentration (mg/kg)
Control		0.0 a	0.000	0.0 a	0.000
Fusarium fujikuroi species complex	RFNG13	66.7 b	0.000	42.6 c	0.000
	RFNG16	69.7 bc	0.206	37.0 b	0.117
	RFNG113	63.0 b	0.384	37.0 b	0.282
	RFNG114	60.5 b	0.576	40.7 c	0.509
	RFNG115	77.8 c	0.536	35.2 b	0.084
Fusarium graminearum species complex	RFRM19	77.8 c	0.000	40.7 c	0.000
Fusarium incarnatum/equiseti species complex	RFNG61	60.0 b	0.000	35.2 b	0.000
Fusarium oxysporum species complex	RFKB4	66.7 b	0.941	46.3 c	0.547
	RFNG59	63.6 b	0.576	41.4 c	0.293
Sarocladium oryzae	RFBG3	72.2 c	-	44.4 c	-
	RFNG41	72.2 c	-	58.3 c	-
	RFRG2	68.5 bc	-	33.3 b	-

*: Treatments followed by the same letter are not significantly different (P: 0.05)
**: Conversion from ppm to SI (mg/kg) following R-Biopharm (2015)

No association was found between *Fusarium* species and aggressiveness since little variation in pathogenic potential was observed in isolates among and within the species tested. The PDI observed on Nipponbare plants ranged from 60.5% to 77.8% for isolates of *F. fujikuroi* complex, from 63.6% to 66.7% for isolates of *F. oxysporum* complex, was 77.8% for the isolate of the *F. graminearum* complex, and was 60.0% for the isolate from the *F. incarnatum/equiseti* complex.

The levels of disease severity induced by *S. oryzae* on Nipponbare plants were comparable to those of *Fusarium* spp. with a PDI ranging from 68.5 to 72.2%.

4.3.4 Fumonisin production

Fumonisins could be detected in Nipponbare and CO39 rice plants inoculated with *Fusarium* isolates from the *fujikuroi* complex and the *oxysporum* complex. No fumonisins could be detected in rice plants inoculated with isolates belonging to the *graminearum* or *incarnatum/equiseti* complex. Fumonisin production was not correlated with disease severity since non-fumonisin producers were equally aggressive on rice plants as fumonisin producers.

4.4 Discussion

In this study we have shown that rice sheath rot in Rwanda can be caused by both *S. oryzae* and *Fusarium* spp. Both pathogens were found in areas such as Bugarama, Rwamagana, and Nyagatare and Rwagitima where rice is grown since 1960 and in areas where rice production is recent such as Kabuye and Rugeramigozi. No clear link with the occurrence of *S. oryzae* or *Fusarium* spp. and location, altitude or sampling year could be detected. To our knowledge, it is for the first time that these organisms, *Sarocladium sp*. and *Fusarium* sp. associated with rice sheath rot symptoms, are reported in Rwanda.

S. oryzae has been associated with rice sheath rot in 36 countries (CABI, 2015). Most of the Rwandan isolates are clonal with identical ITS sequences and cluster with an Indian isolate of *S. oryzae*. The isolate of *S. oryzae* RFRG2 found in Rugeramigozi in 2013, an area where rice production is very recent, was more closely related to reference strains from India and Kenya than to the other isolates from Rwanda.

Compared to *S. oryzae*, the *Fusarium* population associated with rice sheath rot in Rwanda is more variable although the majority of the isolates are closely related to *F. andiyazi* or belong to the *F. oxysporum* complex. *Fusarium andiyazi* was originally identified in sorghum in Africa (Marasas et al., 2001) but was also isolated from rice seed samples from Burkina Faso, Tanzania, India and Vietnam by Wulff et al. (2010) and from Italian rice seeds by Dal Prà et al. (2010). These rice isolates caused chlorotic and slender leaves (Wulff et al., 2010) or seedling wilt (Dal Prà et al., 2010) but were hardly able to cause crown or stem rot in seed inoculation assays. *Fusarium* species from the *F. fujikuroi* complex that are more typically associated with sheath rot such as *F. verticillioides*, *F. proliferatum* or *F. fujikuroi* (Abbas et al., 1998; Desjardins et al., 2000; Park et al., 2005) were not found in Rwanda. It remains to be investigated whether the widespread presence of *F. andiyazi* on rice in Rwanda is due to specific climatological conditions, the presence of sorghum in the neighbourhood, or other unknown factors. Sorghum is an important crop in Rwanda with an annual production of 145000 tonnes in 2014 (FAO, 2016). *Fusarium oxysporum* is usually associated with vascular diseases, but has been isolated from rice plant tissue before (Abbas et al., 1995; Desjardins et al., 2000) and can be pathogenic on young rice plants (Prabhu and Bedendo, 1983; Fisher and Petrini, 1992). Four *Fusarium* isolates grouped in the *Fusarium incarnatum/equiseti* complex and two isolates grouped in the *Fusarium graminearum* complex. *Fusarium equiseti* has been reported on rice seed samples from Nepal and is considered a weak pathogen, common to subtropical areas (Desjardins et al., 2000). However, at least one of the *F. equiseti* isolates

from Rwanda is as pathogenic on rice sheaths as the other *Fusarium* isolates tested. It is not clear to which species the two isolates from the *graminearum* complex belong but they appear to be most closely related to *F. asiaticum*. *F. asiaticum* (O'Donnell et al., 2004) is causing *Fusarium* head blight on wheat and rice and is predominant in Asia. It has recently also been reported on rice in Brazil (Gomes et al., 2015).

Based on the clonal nature of the *S. oryzae* isolates found in Rwanda it can be presumed that this pathogen was introduced in Rwanda on seeds. The large diversity in *Fusarium* species, however, may indicate that these pathogens are endemic to the country, but it does not exclude that there can also be introductions of *Fusarium* spp. from other parts of the world. Rice seeds used in Rwanda can come from West Africa through the Africa Rice Center (formerly West African Rice Development Association-WARDA-), from Asia through the International Rice Research Institute (IRRI), or from neighbouring countries through regional cooperation. There is no official list of quarantine or regulated non-quarantine pests for rice in Rwanda, which would help in diagnosing plant material entering the country.

Interestingly, *S. oryzae* and the *Fusarium* spp. tested caused very similar symptoms on rice plants and both pathogens were more pathogenic on the *japonica* variety Nipponbare than on the *Indica* variety CO39. Precise information about the rice cultivars grown in Rwanda is hard to find. Because the average temperature in Rwanda is low (see Table 4-1), most successful rice varieties grown in the country seem to have a *japonica* profile, having come from China but really originating from Japan (Promar, 2012). It should be noted that plants were grown at 28°C in our pathogenicity tests. It remains to be investigated whether the difference in susceptibility between *japonica* and *indica* is also observed at lower temperature. Symptoms associated with rice sheath rot are most probably caused by toxin production. Since fumonisins are the predominant mycotoxins produced by the *F. fujikuroi* group (O'Donnell et al., 2013), we did a first test for fumonisin production on our rice plants artificially inoculated with *Fusarium* spp. Fumonisins could be detected in both Nipponbare and CO39 upon inoculation with all *F. andiyazi* strains tested (except isolate RFNG13) and the two *F. oxysporum* isolates tested. But it is necessary to be cautious in the interpretation of these data, given that there were no replications in the preliminary conducted experiment. According to Wulff et al. (2010), *F. andiyazi* only produces trace amounts of fumonisins, but this characteristic is probably strain dependent. Fumonisin production in *F. oxysporum* has been reported before in *F. oxysporum* strain O-1890 (Proctor et al., 2008) and based on fumonisin cluster sequence comparison it appears that this strain has obtained the fumonisin cluster by

horizontal gene transfer from *Fusarium bulbicola* or a closely related strain. It would be interesting to have a closer look at the fumonisin gene cluster in the *F. oxysporum* strains from Rwanda to see whether they are similar to the one described for *F. oxysporum* strain O-1980. Fumonisin production, however, does not explain the symptoms observed upon inoculation with *Fusarium* spp. since non-fumonisin producers such as *F. andiyazi* isolate RFNG13 and the isolates belonging to the *graminearum* and *incarnatum/equiseti* complex were as pathogenic as fumonisin producers. A more extensive analysis of mycotoxins produced *in vitro* and *in planta* by the various *Fusarium* strains from Rwanda is needed and will be the subject of future studies. It should be noted that members of the *F. fujikuroi* complex are also known to produce moniliformin. Moniliformin is phytotoxic and it has been shown that *F. proliferatum* isolates from field samples of rice with sheath rot symptoms are capable of producing both fumonisins and moniliformin in culture. Both mycotoxins were also detected in naturally contaminated rice samples (Abbas et al., 1999). Recently moniliformin was detected on rice samples from Nigeria (Rofiat et al., 2015). *Fusarium oxysporum* and *F. equiseti* are also known to produce moniliformin (Desjardins, 2006). Since moniliformin has not been directly implicated in any outbreak of toxicosis in animals and humans and detection in biological matrices is not easy (Desjardins, 2006), this toxin is not as widely studied as other *Fusarium* mycotoxins.

More information is needed to understand the epidemiology of *S. oryzae* and the *Fusarium* spp. causing sheath rot on in Rwanda. We do not know whether these organisms can co-occur in the same lesion or on the same plant and how they interact with each other or with the plant. Moreover, the influence of cold stress on the occurrence of sheath rot should be studied. Any strategy to control sheath rot in Rwanda should take into account that this disease can be caused by various pathogens.

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5 *PSEUDOMONAS* ISOLATES ASSOCIATED WITH RICE SHEATH ROT DISEASE COMPLEX IN RWANDA AND THE PHILIPPINES

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Abstract

Rice is an important food crop worldwide in tropical and subtropical areas. Rice plants are attacked by many pests and diseases and some of them cause sheath rot symptoms. Less is known about the causal agents of rice sheath rot. In this study, aiming to know more about sheath rot associated bacterial organisms, diseased plants were collected and bacteria were isolated from plant tissues exhibiting sheath rot symptoms. Samples were collected for analysis from the major rice-growing areas of Rwanda. Previously collected isolates from the Philippines were also studied. Bacterial isolates were characterized by morphological, biochemical and molecular features. The molecular diagnosis was based on the sequencing of 16S rDNA, *rpoB* and genes. An initial quick screening based on 16S rDNA revealed the presence of three dominant genera*: Stenotrophomonas, Delftia* and *Pseudomonas*. The distribution of the major isolates was relatively the same in all the sampled areas. More efforts were put in the characterization of samples of *Pseudomonas* genus, and they were put in 4 different subgroups defined after molecular identification, depending on the closely related *Pseudomonas* species: *P. chlororaphis, P. fluorescens, P. asplenii/fuscovaginae* and *P. putida*. Within subgroups, subsets were created according to the similarities among the isolates. There is not a big difference between isolates from Rwanda and those from the Philippines, except that isolates related to *P. saponiphila* are the most present in Rwanda while they are absent in the Philippines. Also, isolates closely related to two pathogenic isolates *P. palleroniana* (*P. fluorescens* subgroup) and *P. fuscovaginae* (*P. asplenii/fuscovaginae* subgroup) were found in the Philippines but not in Rwanda. A subset was tested for pathogenicity on rice plants. Isolates appeared mildly pathogenic, compared to reference related pathogenic strains. The role of identified pseudomonad isolates, as most of them qualify as plant-inhabiting endophytes and/or potential biological control agents, while some others qualify as isolates with low pathogenic potential, is tentatively explained, but still has to be investigated. Considering the complexity of the rice sheath rot disease causing factors, developing an integrated pest management strategy is likely to be the most effective

control option especially as the lifestyle and pathogenic status of identified organisms may have links with the rice plant, its growing environment and other organisms harbored by the plant.

5.1 Introduction

Rice production is threatened by some constraints of which the susceptibility to attacks by pests and diseases is important. Rice sheath rot is one of the diseases attacking rice. Rice sheath rot is characterized by the following symptoms (Zeigler and Alvarez, 1987): a brown stripe on the sheath that can extend along the midrib of the leaf lamina for nearly its entire length, failure of the panicles to emerge properly from the boot when the flag leaf sheath is severely affected, panicles from diseased plants produce discolored and poorly filled grains. The disease can cause significant yield losses, with the possibility of also causing seed discoloration (thus reducing its value), degeneration and even sterility. Some bacteria are known as rice sheath rot causal agents, like *Pseudomonas fuscovagine* (Zeigler and Alvarez, 1987, 1990; Jaunet et al., 1996; CABI, 2007), *Burkholderia glumae* (Fory et al., 2014) and *Acidovorax oryzae* (Cottyn et al., 1996b; Schaad et al., 2008). A review on rice sheath rot associated organisms is presented in Chapter 2 and Bigirimana et al. (2015).

In addition to *Pseudomonas fuscovaginae*, all the studies on rice sheath rot find many other *Pseudomonas* isolates associated with rice sheath rot and grain discoloration symptoms (Zeigler and Alvarez, 1987, 1990; Cottyn et al., 1996a; b; Gardan et al., 2002; Cother et al., 2010), which have not been, until now, completely identified by molecular methods, except for *P. palleroniana* (Gardan et al., 2002). Those pseudomonads have been identified by biochemical/physiological tests.

Cottyn et al. (1996a; b) worked on rice bacterial diseases in the Philippines and conducted a survey to determine which bacterial pathogens were associated with sheath rot and grain discoloration symptoms. Samples were collected from 16 provinces and the disease symptoms were found in samples from 12 provinces. Suspected pathogenic bacteria were tested on seedling sheaths and produced symptoms characteristic of infections caused by *Burkholderia glumae* and *Pseudomonas fuscovaginae*, which are not distinctive according to the causal bacteria. A connection with the isolates from these works has been established by introducing in the current study isolates from the Philippines, taking advantage of the current advances in the molecular identification tools, methods that were not available when the study was first conducted.

Rice is one of the priority crops in Rwanda, a country for which agriculture constitutes the backbone of the economy, given that around 80% of the population depend on it for their livelihoods (MINAGRI, 2011b). In this study, we want to see whether *P. fuscovaginae* is present in Rwanda and isolate any other bacteria associated with rice sheath rot symptoms. The isolated organisms are identified and characterized for pathogenicity. Samples were collected from the major rice-growing areas of Rwanda.

The goal of this study is to generate information that can improve the understanding of the rice sheath rot disease, an emerging rice disease and its causal bacterial agents, especially the less studies pseudomonads, so that a sound management strategy against it can be developed.

5.2 Materials and methods

5.2.1 Research area

Isolation of bacteria associated with sheath rot symptoms on rice has been realized on samples collected from valleys in the major rice-growing areas of Rwanda. The valleys where samples were taken were the following: Bugarama (Bugarama Sector, Western Province), Cyili (Rusatira Sector, Southern Province), Rwamagana (Kigabiro Sector, Eastern Province), Nyagatare and Rwagitima (Nyagatare and Rugarama Sectors, Eastern Province) and Mukunguri (Mugina Sector, Southern Province) (Figure 1-1). Samples were collected in July-August 2011 and 2012, while rice plants were at fruit development according to the growth-stages description (Lancashire et al., 1991).

5.2.2 Sampling methods

A representative sampling of sheath rot diseased rice plants in Rwanda was followed so that the samples taken represent the situation on the whole rice-growing marshland. Samples were taken from plants showing sheath rot symptoms following all the cardinal directions. In each plot or direction, at least three diseased plant samples were taken and labeled. Field samples were preserved at 4 °C before analysis in the laboratory.

5.2.3 Samples preparation for isolation

Sample preparation consisted of 8 steps: (1) Localizing the tissue area where the disease symptoms are well expressed and cutting it from the remaining parts of the plant; (2) Separating the tissues where the disease started and which are at an advanced degradation stage, which are discarded, from the freshly infected tissues, which are retained for the further isolation work, proceeding by dividing that chosen part into small parts of approximately 5 mm of length; (3) Doing surface sterilization by dipping samples of diseased plants tissues in

ethanol 70% for 2 minutes; (4) Cleaning the sterilized diseased plant parts 4 times in sterile distilled water; (5) Measuring 1 ml of the physiological solution 0.85% and pouring it in a sterile mortar where also a small quantity of autoclaved small grains of sand are poured; grinding diseased plant samples with a sterile pestle in the physiological solution in the mortar; (6) Gradual dilution (10^{-1}, 10^{-2}, 10^{-3}) of the liquid from the diseased plants by adding 20 µl of it to 180 µl of the physiological solution; (7) Taking 50 µl of the diluted sample, spreading them on King's B agar medium (KB), tightly closing the Petri dish using parafilm paper and incubating at 28°C; (8) The growth of colonies was evaluated after 48 hours and individual, single and pure colonies constituting isolates were obtained after continuous subcultures. For long term preservation, isolates were maintained in cryotubes in a mixture of 50% of Luria-Bertani (LB) liquid medium and 50% of glycerol 40% at -80°C.

5.2.4 Morphological and biochemical characterization of samples

The bacterial colonies that developed on culture media were first characterized using morphological and biochemical features. The tests were performed on the isolates following the principle that a small number of tests can help predicting the genera of major plant pathogenic bacteria (Schaad et al., 2001) (Table 5-1). The tests conducted are the following: colour observation, Gram coloration, development speed based on the size acquired after 48 hours, observation of the colour of the colonies on YDC (Yeast extract-dextrose-$CaCO_3$) or NBY (Nutrient-Broth yeast extractagar) media, appearance of mucoid colonies at 30°C on YDC medium, fluorescence of pigments on KB medium, diffusible non fluorescent pigments on KB, and growth at 40°C. These biochemical tests were performed searching for three bacterial genera, *Pseudomonas, Burkholderia* and *Acidovorax* recognized as sheath rot pathogens (Cottyn et al., 1996a; b; Cother et al., 2010).

Though there are limited exceptions, four tests are the most important for predicting the genera (Table 5-1): *Pseudomonas* genus can be predicted by the emission of fluorescent pigments on KB medium, *Acidovorax* genus can be predicted by mucoid colonies on YDC at 30°C and growth at 40°C, and *Burkholderia* genus can be predicted by the growth at 40°C and the emission of diffusible non-fluorescent pigments on KB.

Table 5-1: Characters used to differentiate some genera of plant pathogenic prokaryotes that grow on standard media (Schaad et al., 2001)

	Acidovorax	Pseudomonas	Burkholderia
Mucoid colonies on YDC at 30 °C	+	–	–
Fluorescent pigment on KB	–	+	–
Diffusible non-fluorescent pigment on KB	–	–	+
Growth at 40 °C	+	–	+

5.2.5 Molecular identification of samples

A subset of isolated samples was identified by molecular methods. This identification proceeded by DNA extraction or the preparation of the samples so that the DNA is available for reaction (preparation of colonies for PCR), the PCR reaction, and the sequencing of amplified DNA bands evidenced by the presence of bands on the PCR picture.

For the DNA extraction, the protocol described by Louws and Cuppels (2001) was followed. On some isolates, the colony PCR approach was applied through the following steps: (1) growing bacterial colonies on LB medium overnight and diluting them by 1:500 with sterile distilled water; (2) incubation at 96°C for 10 minutes for lysing the cells before the PCR.

The composition of the PCR mixture was the following for 25µl: 5 µl of 5x PCR buffer, 0.5 µl of dNTPs mix 10 mM, 1.25µl of 10 µM of the forward primer, 1.25µl of 10 µM of the reverse primer, 0.125 µl of Taq polymerase 5 µl, 14.375 µl of milli-Q sterile water and 2.5 µl of the bacterial DNA sample. The PCR was run by the following steps: (1) 94°C for 10 min, (2) 94°C for 1 min, (3) 55°C for 1 min, (4) 72°C for 1 min, (5) 72°C for 10 min, (6) maintenance of PCR samples at 4°C; steps (2) to (4) were repeated 39 times.

Three types of primers were used. Initially, samples were analysed by primers based on 16S Ribosomal DNA (Weisburg et al., 1991; Jaunet et al., 1995) (primer sequences in the orientation 5' to 3': fDl- AGAGTTTGATCCTGGCTCAG- and rDl- TAAGGAGGTGATCCAGCC-). When a sample was known or thought to belong to the *Pseudomonas* group and that more precise information about that species was needed, the primers were based on *rpoB* (Frapolli et al., 2007) (primer sequences in the orientation 5' to 3': *rpoB*f1- CAGTTCATGGACCAGAACAACCCGCT- and *rpoB*r1- CCCATCAACGCACGGTTGGCGTC-) and genes (Frapolli et al., 2007; Mulet et al., 2009) (with primer sequences of, respectively, in the orientation of 5’ to 3’: f-

ACTTCCCTGGCACGGTTGACCA- and r-TCGACATGCGACGGTTGATGTC-, PsEG30F-ATYGAAATCGCCAARCG- and PsEG790R- CGGTTGATKTCCTTGA-).

The amplicons were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide. PCR products were purified as instructed in the QIAquick PCR Purification Kit (Qiagen, Helden, Germany) or by the Exo-SAP-IT (Affymetrix, Santa Clara, California, USA). The DNA concentration was measured with the Thermo Nanodrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and when it was higher than 20 ng/µl, the sample was prepared for sequencing, using the same primers as those used in the sequencing. Sequencing was done by LGC Genomics (Berlin, Germany). Sequences were cleaned with BioEdit software. They were then aligned by Multiple Sequence Comparison by Log-Expectation (MUSCLE). Phylogenetic trees were constructed using the maximum likelihood algorithm with 1000 replicates using MEGA 6 Software (Tamura et al., 2013).

5.2.6 Pathogenicity testing

5.2.6.1 Plant material

The rice Indica cultivar Co39 was used for pathogenicity testing. Plantlets used were 5 weeks old. Rice plants were grown in potting soil. They were regularly watered with a nutrient solution composed, per 1 liter, of 2 g of $FeSO_4.7H_2O$ and 0.9 g of NH_4SO_4. The plants were grown in a growth chamber at 28°C, 70% relative humidity and at the day photoperiod of 12/12 hours.

5.2.6.2 The isolates and inoculum preparation

From the collection, bacteria were grown on KB solid medium for 48 hours. Individual colonies were then grown on LB liquid medium for 24 hours. The medium and the bacteria in suspension were centrifuged at 13000 rpm for 2 minutes, after which the liquid supernatant was discarded. The centrifugate was 10 times diluted with 0.85% saline buffer, as indicated (Schaad et al., 2001), taking reference from the used volume of bacteria in suspension in the medium. The bacterial suspension was thoroughly mixed with a vortex. Before inoculation, the bacterial suspension was preserved at 4°C. Inoculation of the bacteria in the plants was performed by injection (Rott et al., 1989) with a 1 ml syringe (Becton Dickinson, Spain) and a 25GX1" needle (Becton Dickinson, Ireland) pumping 100 µl into the plant. After injection, inoculated plantlets were kept in a saturated humid chamber at 28°C for 24 hours.

Thirty-four isolates from Rwanda were tested for pathogenicity. The *Pseudomonas fuscovaginae* isolate LMG2158 was used as the positive control while sterile distilled water served as the negative control. Two other isolates, *Pseudomonas protegens* CHA0 and *P. protegens* Pf-5, taxonomically closely related to the isolates from Rwanda, but known as biological control agents, were also added to the experiment.

5.2.6.3 Experimental design and statistical analysis.

Data for disease development were taken 10 days after inoculation. They were collected based on a scoring scale with 6 levels, adapted after previous works (Mattiuzzo et al., 2011), and following a gradual comparison of symptoms, where 0 represents a healthy plant and 5 a highly diseased plant (Table 5-2). Recorded data were analysed as qualitative data through non-parametric tests. Disease indexes were calculated for the different isolates. The attribution of ranks and equality of means for the different treatments were tested in SPSS 22 software (IBM Corporation, 2013) and the values for pairwise comparison were generated in PAST software Version 2.17C (Hammer et al., 2001). Based on pairwise comparison results, isolates were assigned to homogenous subsets.

Table 5-2: Severity rating scale for *P. fuscovaginae* on rice (Mattiuzzo et al., 2011)

Severity value	Description
0	No symptoms only the sign of the injection puncture
1	Necrosis around the puncture till 1 cm
2	Necrosis around the puncture and chlorosis from 1 to 3 cm on the stem
3	Necrosis around the puncture till 5 cm on the stem
4	Necrosis around the puncture for the two-thirds of the new leaf
5	Necrosis around the puncture throughout the new leaf

5.3 Results

5.3.1 Sampling and quick initial screening for identification

Diseased plants, showing typical sheath rot symptoms were collected from the chosen sampling areas. Efforts were put in capturing, as much as possible, samples coming from the different parts of each rice growing-area, so as to have a representative picture of bacteria associated with sheath rot diseased rice plants. In total, 368 isolates were obtained and the number of isolates recovered in 2011 and 2012 per rice growing area is given in Table 5-3.

Table 5-3: Origin and number of bacterial isolates obtained

Rice- growing area/Year of collection	*2011*	*2012*	*Total*	*% per location*
Bugarama (BG)	24	19	43	11.7
Cyili (CY)	57	31	88	23.9
Mukunguri (MK)	0	43	43	11.7
Nyagatare and Rwagitima (NG)	65	48	113	30.7
Rwamagana (RM)	45	33	78	21.2
Unspecified location in Rwanda (XX)	0	3	3	0.8
Total	**191**	**177**	**368**	

The isolates were screened initially using the morphological tests described in Table 5-1 searching for *Pseudomonas, Burkholderia* and *Acidovorax* genera. Since these tests were not conclusive it was decided to identify a subset of 53 isolates by sequencing based on 16S rDNA. Isolate choice was based on morphological and biochemical features. The molecular identification revealed that no *Burkholderia* or *Acidovorax* isolates could be found. The sequenced isolates belonged to three major genera: *Stenotrophomonas*, *Delftia* and *Pseudomonas* (Table 5-4) and they were found in all rice-growing areas. In addition, 1 isolate of *Enterobacter* and 1 isolate of *Bacillus* were found. A phylogenetic tree based on the partial 16S rDNA sequences of some of these isolates is presented in Figure 5-1.

Table 5-4: Bacterial genera detected in the first screening for the identification of sheath rot associated isolates from Rwanda (based on 16SrDNA)

Rice- growing area/Genus	***Stenotrophomonas***	***Pseudomonas***	***Delftia***	***Total***
Bugarama	4	0	1	5
Cyili	6	3	0	9
Mukunguri	8	2	4	14
Nyagatare and Rwagitima	6	6	4	16
Rwamagana	2	3	1	6
Unspecified location in Rwanda	0	1	0	1
Total	26	15	10	51

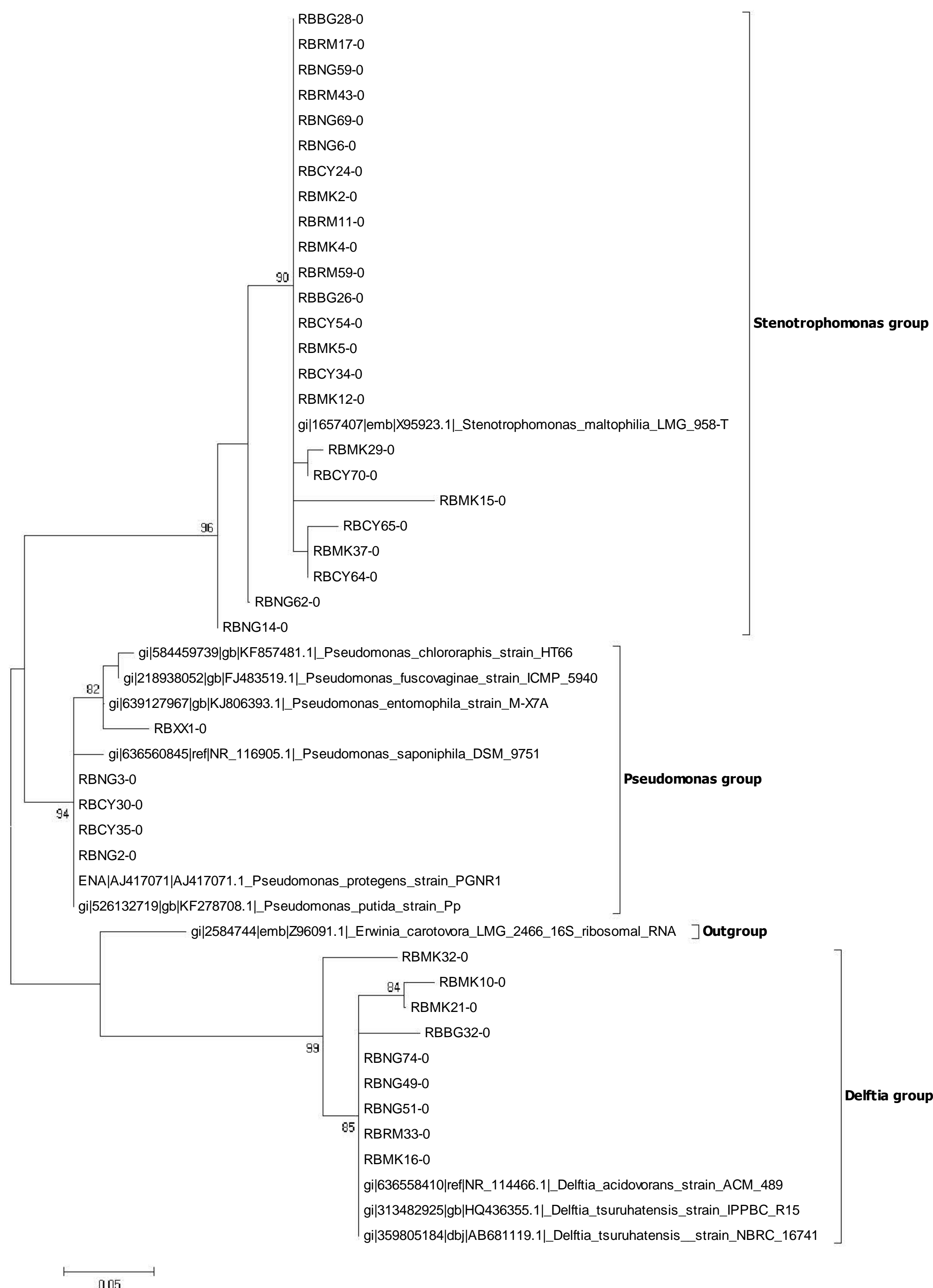

Figure 5-1: Phylogenetic tree for bacterial sequences identified by 16S rDNA. Sequences were aligned using MUSCLE and the tree was generated using the maximum likelihood algorithm. The numbers above branches represent bootstrap percentiles from 1,000 replicates

A first pathogenicity test using a subset of these isolates revealed that *Stenotrophomonas, Delftia* and *Pseudomonas* isolates from Rwanda were not, or only very mildly pathogenic on rice (Figure 5-2): all the tested isolates were clearly less pathogenic than the positive control, consisting of *P. fuscovaginae* strain LMG2158. Since, according to the literature (see Chapter 2), rice sheath rot is most of the time associated with *Pseudomonas* spp. we decided to analyze the *Pseudomonas* population from Rwanda in more detail.

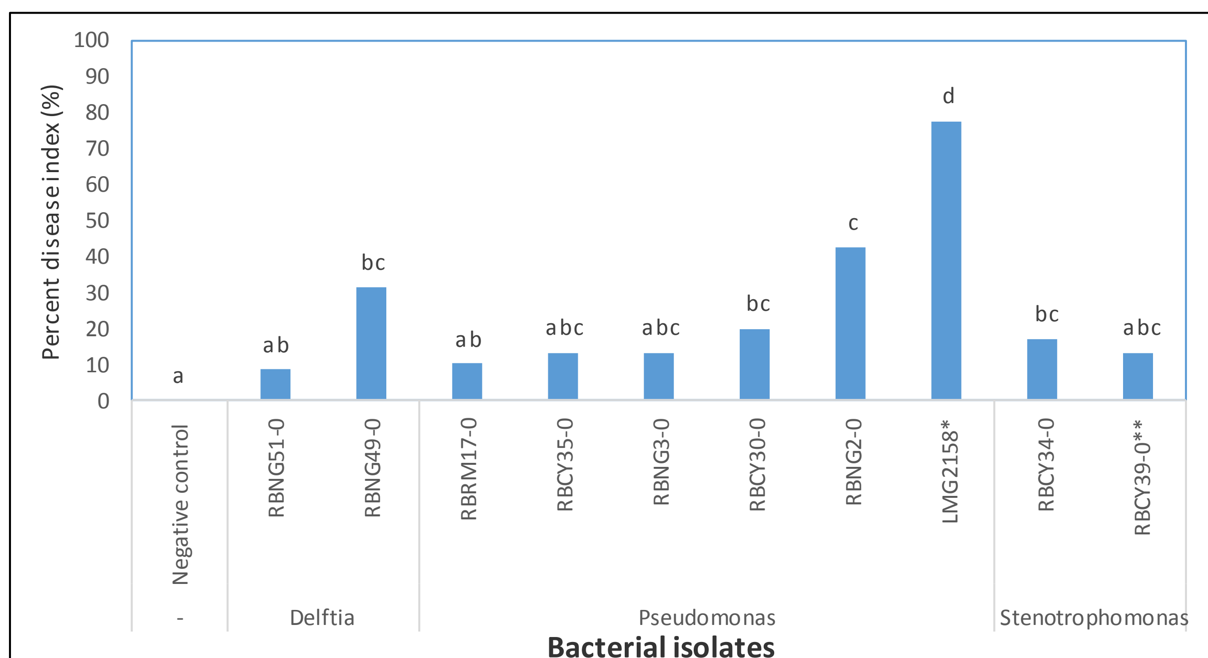

Figure 5-2: Pathogenicity testing for sheath rot associated isolates identified by sequencing the 16S rDNA region (*: *P. fuscovaginae*, used as positive control, **: also identified by *rpoB* and , the latter result retained as considered more accurate).

5.3.2 Molecular identification of *Pseudomonas*-like isolates

After further purification and DNA extraction, we were able to reliably identify 69 bacterial isolates from Rwanda as *Pseudomonas* spp. based on sequencing part of the *rpoB* and gene after PCR amplification. These isolates are listed in Table 5-5. Subgroups were made based on a recent work about *Pseudomonas* taxonomy (Gomila et al., 2015). The species to which these isolates are most closely related are also mentioned. Table 5-5 clearly shows that a diverse group of *Pseumonas* isolates is associated with rice sheath rot symptoms in Rwanda. This is reminiscent of previous studies by Cottyn et al. (1996a; b) in the Philippines, who also found a diversity of *Pseudomonas* isolates associated with rice sheath and seed rot. Since some of these isolates, which at the time had been grouped based on biolog data, were available in the collection of Bart Cottyn, we decided to include them in our analysis and sequenced part of their *rpoB* and genes. These isolates together with the taxomonic group to

which they belong are listed in Table 5-6. We studied the phylogenetic relatedness between the isolates from Rwanda and the Philippines by generating a phylogenetic tree based on the concatenated *rpoB* and sequences. In this tree, also sequences of reference strains (type strains or published species strains closely related to those found in this study) were introduced. The reference strains and some of their features are presented in Table 5-7. The phylogenetic tree is given in Figure 5-3.

Table 5-5: Rwandan *Pseudomonas*-like isolates grouping based on molecular identification

Pseudomonas group (Gomila et al. 2015)	Group in phylogenetic tree	Closest related species	ID Code	Year of collection	Origin
P. chlororaphis subgroup	Pc1	*P. saponiphila*	RBBG8-0	2011	Bugarama
			RBBG9-3(III)	2011	Bugarama
			RBCY29-0(II)	2011	Cyili
			RBCY35-0	2011	Cyili
			RBCY35-1	2011	Cyili
			RBCY39-0(II)	2011	Cyili
			RBCY39-1	2011	Cyili
			RBCY40-0(II)	2011	Cyili
			RBCY29-2(II), RBCY35-2, RBCY-35-3, RBCY35-4, RBCY40-2(III)	2011	Cyili
			RBMK13-1(I)	2012	Mukunguri
			RBMK25-1	2012	Mukunguri
			RBMK13-2(I), RBMK25-2(I)	2012	Mukunguri
			RBNG2-0(IV)	2011	Nyagatare and Rwagitima
			RBNG3-2	2011	Nyagatare and Rwagitima
			RBNG5-0(IV)	2011	Nyagatare and Rwagitima
			RBNG5-3	2011	Nyagatare and Rwagitima
			RBNG81-1	2012	Nyagatare and Rwagitima
			RBNG84-1	2012	Nyagatare and Rwagitima
			RBNG92-1(V)	2012	Nyagatare and Rwagitima
			RBNG3-0	2011	Nyagatare and Rwagitima
			RBNG2-1(IV), RBNG2-2(IV), RBNG3-1, RBNG5-1(IV), RBNG5-2(IV), RBNG5-4(IV), RBNG81-0, RBNG81-2(V), RBNG84-2, RBNG92-0, RBNG92-2	2011	Nyagatare and Rwagitima
	Pc2	*P. protegens*	RBCY29-1	2011	Cyili
			RBCY39-2	2011	Cyili
			RBRM41-1(I)	2012	Rwamagana
			RBRM55-1(II)	2012	Rwamagana
			RBRM55-2(I)	2012	Rwamagana
			RBRM57-2	2012	Rwamagana
			RBRM58-1(II)	2012	Rwamagana
			RBRM58-2(II)	2012	Rwamagana
			RBRM58-3	2012	Rwamagana
			RBRM58-5	2012	Rwamagana
			RBRM40-0, RBRM40-2(II), RBRM 41-0(II), RBRM57-0(II)	2012	Rwamagana
	Pc3	*Pseudomonas* sp. CMR12a	RBCY40-1	2011	Cyili
			RBCY45-1(I)	2012	Cyili
			RBRM57-1(I)	2012	Rwamagana
P. fluorescens subgroup	Pf1	*P. simiae*	RBBG9-1	2011	Bugarama
P. aeruginosa subgroup	Pae	*P. alcaligenes*	RBXX1-1	2012	Rwanda*
P. putida subgroup	Pp3	*P. monteilii*	RBCY30-0(I)	2011	Cyili
			RBCY30-1(I)	2011	Cyili
	Pp1	*P. mosselii*	RBRM17-0(I)	2011	Rwamagana
			RBRM17-4(I)	2011	Rwamagana
			RBRM17-1(II), RBRM17-2, RBRM17-3(II), RBRM17-5(II), RBRM17-6(II)	2011	Rwamagana
	Pp4	*P. fulva*	RBNG90-1	2012	Nyagatare and Rwagitima
			RBNG91-1(I)	2012	Nyagatare and Rwagitima
			RBNG90-0(I), RBNG90-2, RBNG91-2(I)	2012	Nyagatare and Rwagitima

**Isolate from Rwanda for which the information on the exact place of origin is not known.*

Isolates within a phylogenetic group that are followed by the same roman letter are identical in their *rpoB/* sequence.

Table 5-6: Grouping of isolates from the Philippines based on molecular identification

***Pseudomonas* group**	**Group in phylogenetic tree**	**Biolog group (Cottyn et al. 1996a)**	**Closest related species**	**ID Code**	**Tissue**
P. chlororaphis subgroup	**Pc2**	A2	***P. protegens***	5405	sheath
		A2		5440	seed
	Pc3	A3	***Pseudomonas CMR5c***	6244	seed
P. fluorescens subgroup	**Pf2**	B2	***P. palleroniana***	6366	ND
		B2		5067	sheath
		B2		6287*	sheath
		B2		4834	sheath
		B2		5270	sheath
		B2		4846	sheath
		B2		5247	sheath
		B2		5068	sheath
P. asplenii subgroup	**Pas**	B1	***P. fuscovaginae***	6202	seed
		B1		6609	sheath
		B1		7007	sheath
P. putida subgroup	**Pp3**	?	***P. montelii***	7390	ND
	Pp2	A4	***Pseudomonas* sp. CRS01-1**	7174	seed
		A4		7161*	seed
		A4		6594*	sheath
		A4		6593*	sheath
		A4		7407*	seed
		A4		4736*	sheath
		A4		7308	seed
		A4		7327	ND
		A4		7389*	ND
		A4		7313	ND

*Identification based on *rpoB* sequences, no amplification with primers

ND: not defined

Table 5-7: Table of reference strains used to create the *Pseudomonas* phylogenetic tree

***Pseudomonas* subgroup**	**Species**	**ID code**	**Origin of strains closely related to Rwanda/Philippines isolates**	**Genbank accession number *rpoB***	**Genbank accession number**	**Reference**
P. chlororaphis	*P. saponiphila*	DSM 9751^T		HE800515	HE800499	(Lang et al., 2010)
	Pseudomonas sp.	Os17	Biocontrol strain, rhizosphere of rice	POS17_5536	POS_5595	(Takeuchi et al., 2015)
	P. protegens	PGNL1	Biocontrol strain, rhizosphere of tobacco	DQ458655	DQ458687	(Frapolli et al., 2007)
	P. protegens	Pf-5	Biocontrol strain, rhizosphere of cotton	DQ458648	DQ458678	(Ramette et al., 2011)
	P. protegens	CAB57	Biocontrol strain, rhizosphere of shepherd's purse	PPC_5542	PPC_5609	(Takeuchi et al., 2014)
	Pseudomonas sp.	CMR12a	Biocontrol strain, rhizosphere of cocoyam	FJ652703	not in Genbank	(Perneel et al., 2007; Mavrodi et al., 2010)
	Pseudomonas sp.	CMR5c	Biocontrol strain, rhizosphere of cocoyam	not in Genbank	not in Genbank	
	P. chlororaphis	LMG 1245^T		AJ717426	FN554453	(Ramette et al., 2011)
	P. chlororaphis	LMG5004^T		AJ717478	D86036	(Yamamoto and Harayama, 1998; Tayeb et al., 2005)
P. corrugata	*P. brassicacearum*	CIP 107059^T		AJ717436	AM084334	(Ramette et al., 2011)
	P. corrugata	LMG 2172^T		AJ717487	AB039566	(Ramette et al., 2011)
P. fluorescens	*P. fluorescens*	ATCC13535^T		AJ717451	AB039545	(Yamamoto et al., 2000)
	P. marginalis	LMG 2210^T		AJ717425	AB039575	(Ramette et al., 2011)
	P. simiae	CCUG50988^T	Clinical samples	FN554757	FN554513	(Mulet et al., 2010)
	P.simiae	WCS417	Biocontrol strain, rhizosphere of wheat			Berendsen et al., 2015
	P. azotoformans	CIP 106744^T		AJ717458	AB039547	(Ramette et al., 2011)
	P. tolaasii	LMG 23076^T		AJ717467	AB039561	(Ramette et al., 2011)
	P. palleroniana	LMG 23076^T	Weak pathogen, rice	FN554747	FN554497	(Mulet et al., 2010)
P. syringae	*P. cichorii*	LMG 2162T		AJ717418	AB039526	(Ramette et al., 2011)
	P. tremae	LMG 22121^T		FN554761	FN554463	(Ramette et al., 2011)
	P. syringae	LMG 1247^T		AB039516	AJ717484	(Ramette et al., 2011)
P. asplenii	*P. fuscovaginae*	LMG 2158^T		AJ717433	not in genbank?	(Tayeb et al., 2005)
	P. fuscovaginae	S-E1	Pathogen, sheath of rice	not in genbank?	not in genbank?	(Cottyn et al., 2002)
	P. asplenii	LMG 2137^T		AJ717432	AB039593	(Ramette et al., 2011)
P. aeruginosa	*P. alcaligenes*	LMG 1224^T		AJ717475	AB039606	(Ramette et al., 2011)
	P. pseudoalcaligenes	LMG 1225^T		AJ717430	AB039602	(Ramette et al., 2011)
	P. aeruginosa	LMG 1242^T		AJ717442	AJ633568	(Ramette et al., 2011)
P. putida	*P. mosselii*	ATCC BAA-99^T	Clinical samples	FN554744	FN554491	(Mulet et al., 2010)
	P. plecoglossicida	CIP106493T		AJ717456	FN554503	

***Pseudomonas* subgroup**	**Species**	**ID code**	**Origin of strains closely related to Rwanda/Philippines isolates**	**Genbank accession number *rpoB***	**Genbank accession number**	**Reference**
	Pseudomonas sp. (parafulva)	CRS01-1	Biocontrol strain, rhizosphere of rice	CP009747	CP009747	(Liu et al., 2015)
	P. monteilii	CIP104883^T	Clinical samples	AJ717455	FN554488	(Tayeb et al., 2005; Mulet et al., 2010)
	P. putida	LMG 2257^T		AJ717474	AB039581	(Ramette et al., 2011)
	P. parafulva	DSM17004^T		AJ717471	FN554500	(Mulet et al., 2010)
	P. fulva	CIP 106765^T		AJ717419	AB039586	(Tayeb et al., 2005)
	P. fulva	YMC09/4/B4619	Clinical samples	FN599524	FN599525	(Seok et al., 2010)
Outgroup	*E. coli*	K-12 substr. MG1655		U00096.3	U00096.3	(Blattner et al., 1997)

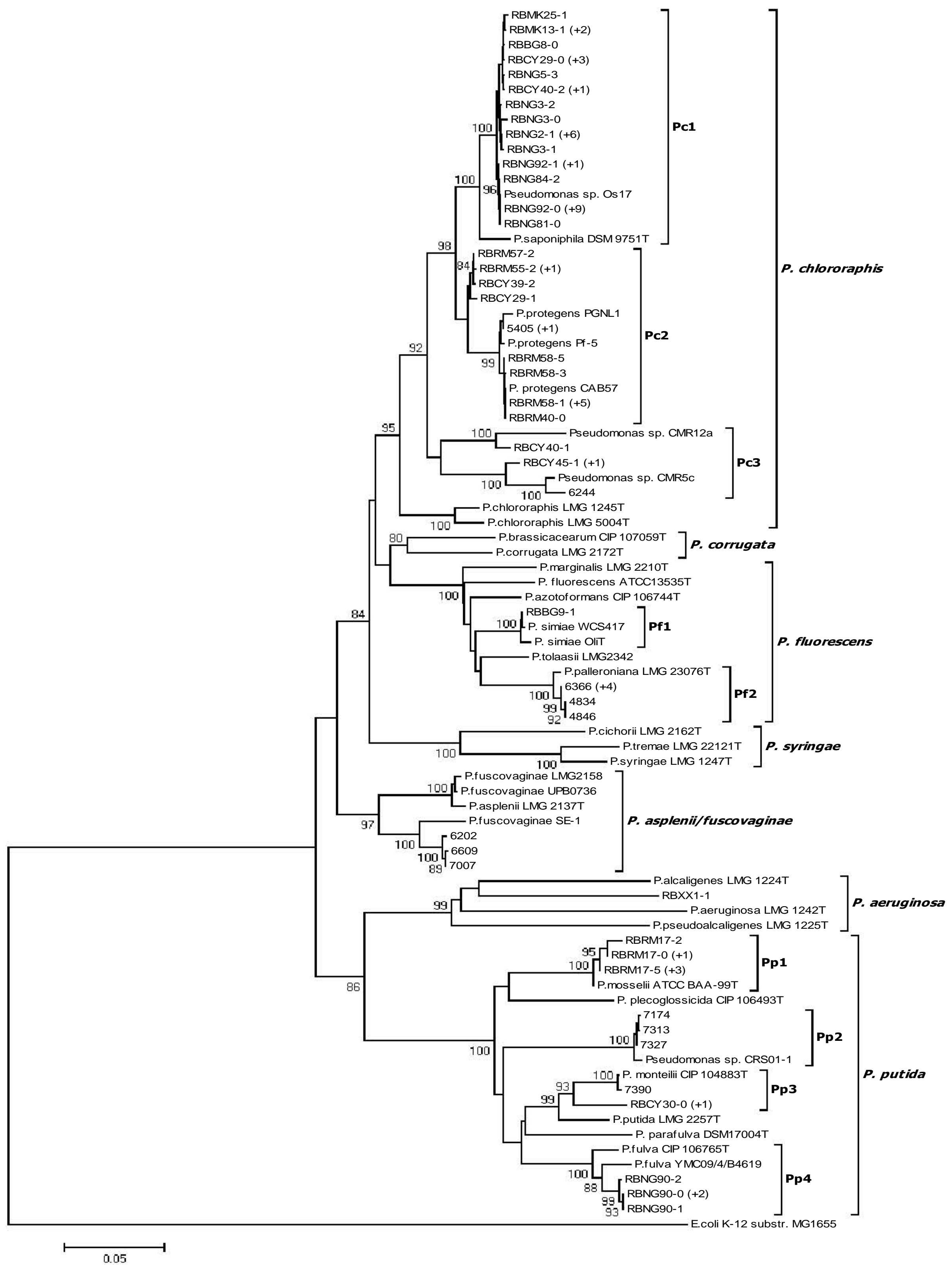

Figure 5-3: Phylogenetic tree for *Pseudomonas* isolates from Rwanda and the Philippines based on the combination of *rpoB* and sequences. Sequences were aligned using MUSCLE and the tree was generated using the maximum-likelyhood method. The numbers at branches represent bootstrap percentiles from 1000 replicates. Only bootstrap values above 70 are shown.

The identified pseudomonad isolates from Rwanda, based on the *Pseudomonas* species to which they are closely related, could be grouped into 4 subgroups (*P. chlororaphis, P. fluorescens, P. putida, P. aeruginosa*) and 8 clusters within these subgroups (written according to the *Pseudomonas* subgroups: Pc1, Pc2, Pc3, Pf1, Pp1, Pp3, Pp4 and *P. aeruginosa*) (Table 5-5). Isolates from the Philippines could be grouped in 4 subgroups (*P. chlororaphis, P. fluorescens, P. asplenii/fuscovaginae, P. putida*) and 6 clusters (Pc2, Pc3, Pf1, Pf2, *P. asplenii/fuscovaginae*, Pp2, Pp3). Cluster Pc2 corresponds with Biolog group A2, cluster Pc3 corresponds with Biolog group A3, cluster Pf2 with Biolog group B2 and cluster Pp2 with Biolog group A4. Biolog groups are illustrated in Cottyn et al. (1996b). Globally, the isolates from Rwanda are found in the same subgroups as those from the Philippines, but the clusters are different. In Rwanda, the majority of the isolates are similar to *Pseudomonas* sp. Os17, a strain with biological control potential obtained from the rhizosphere of rice in Japan and related to *P. saponiphila* (Pc1 cluster) while these were not found among the isolates from the Philippines. The Pf2 cluster, containing isolates related to *P. palleroniana*, and the subgroup of *P. asplenii/fuscovaginae*, the one containing described pathogens, are not represented in Rwanda. The *P. putida* subgroup in the Philippines is dominated by isolates related to *Pseudomonas* sp. CRS01-1, a biocontrol strain obtained from the rhizosphere of rice in China, while in Rwanda this subgroup is dominated by isolates related to *P. mosselii* and *P. fulva*.

5.3.3 Pathogenicity tests

Based on the results of the identification, isolates representing the different *Pseudomonas* subgroups were chosen for undergoing pathogenicity testing. Some isolates induced disease lesion development after inoculation in the plant. The typical lesions induced by *Pseudomonas fuscovaginae*, which served as a reference for scoring on the scale, are presented in Figure 5-4.

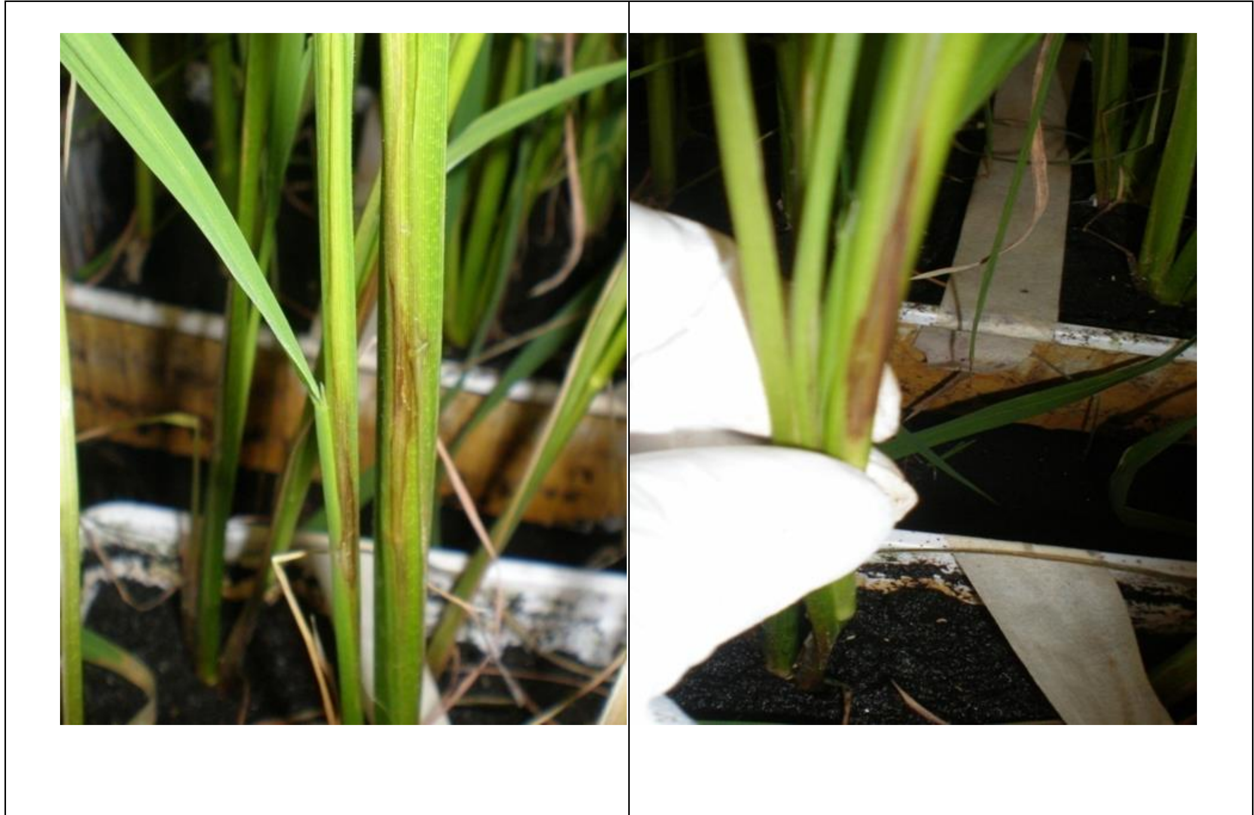

Figure 5-4: Disease lesion caused by *Pseudomonas fuscovaginae* inoculated to rice plantlet

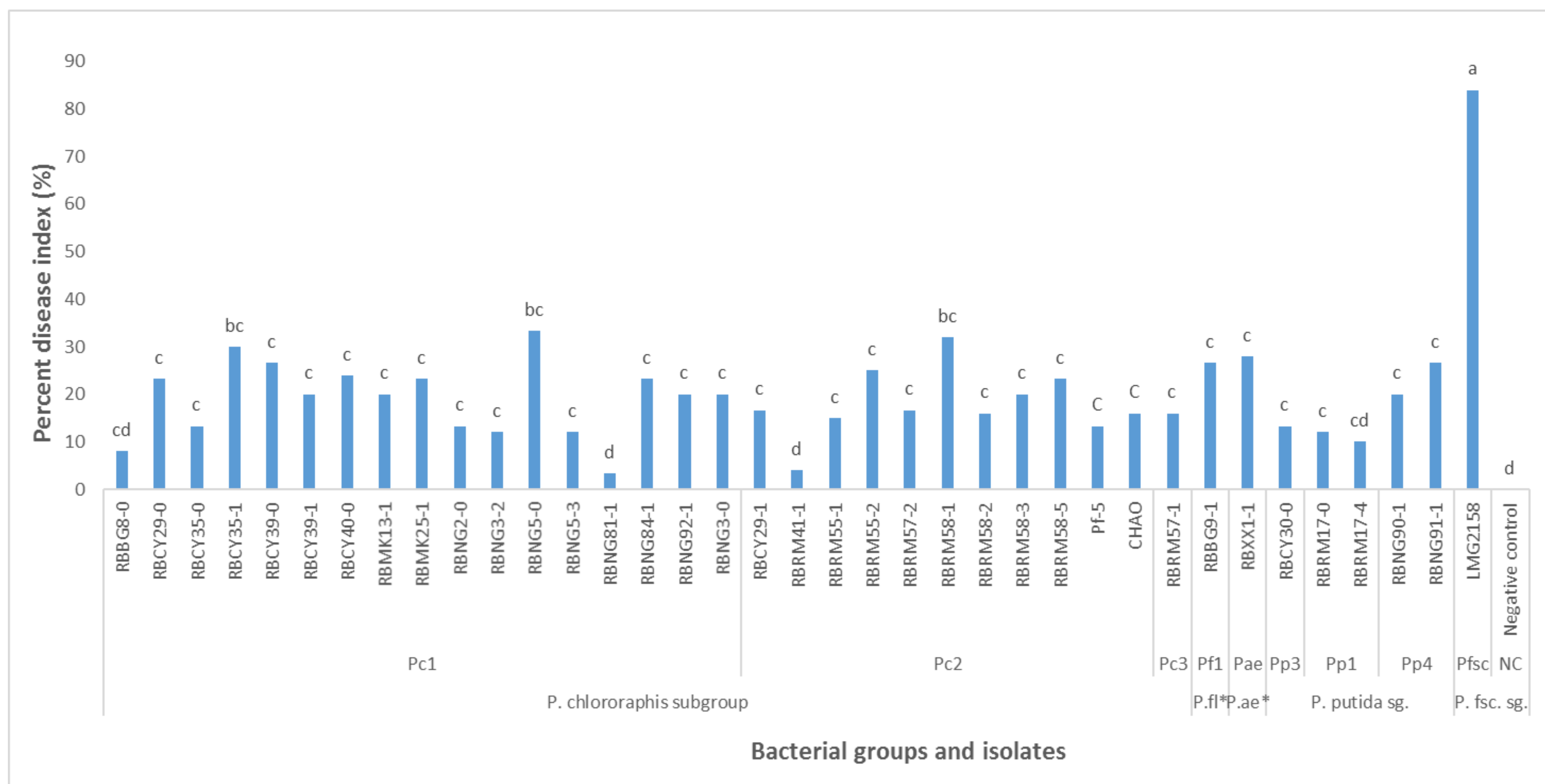

Figure 5-5: Pathogenicity results for bacterial isolates belonging to different subgroups and clusters. Bars indicated with the same letter are not significantly different from each other based on Krsuskal-Wallis non-parametric tests with the significance level of 0.05%.*P. fl: *Pseudomonas fluorescens* subgroup, P.ae: *Pseudomonas aeruginosa* subgroup, NC: Negative Control, P. fsc. sg.: *Pseudomonas fuscovaginae* subgroup .

Pathogenicity data presented in Figure 5-5 show that all these isolates are mildly pathogenic at best and the disease severity they cause is low compared to the disease severity induced by *P. fuscovaginae*, strain LMG2158.

5.4 Discussion

We observed rice with sheath rot symptoms in all the rice-growing areas of Rwanda sampled: Bugarama, Cyili, Mukunguri, Nyagatare and Rwagitima, and Rwamagana. This is in agreement with the general observation that many recent studies point on the emerging importance of rice sheath rot disease in various rice production systems (Mew et al., 2004b; Razak et al., 2009). In this chapter, we specifically focused on bacteria and found three major bacterial genera associated with rice sheath rot symptoms in Rwanda: *Stenotrophomons, Delftia*, and *Pseudomonas.* We emphasized on the further characterization of the *Pseudomonas* spp. since *Pseudomonas fuscovaginae* and other pseudomonads have been associated with rice sheath rot symptoms in other parts of the world. Other reported sheath rot associated bacteria such as *Burkholderia glumae* (Fory et al., 2014), *Burkholderia gladioli* (Nandakumar et al., 2009) and *Acidovorax oryzae* (Cottyn et al., 1996b; Schaad et al., 2008) were not found in Rwanda. The genera *Stenotrophomonas* and *Delftia* were also found to be associated to rice in previous studies but are not considered to be pathogenic on plants (Cottyn et al., 1996b; Mano et al., 2007; Ghasemi et al., 2012).

Stenotrophomonas maltophilia was first described as *Pseudomonas maltophilia* by Hugh et al. (1961). It later became known as *Xanthomonas maltophilia* (Swings et al., 1983) and is now included in the genus *Stenotrophomonas* (Palleroni and Bradbury, 1993). *Stenotrophomonas maltophilia* is an endophyte which is commonly isolated from rice leaves (Mano and Morisaki, 2008) and seeds (Hardoim et al., 2012). Though *S. maltophilia* is known to grow on a limited number of media in the laboratory, it is found in various environments. There is probably a lot of variability within *Stenotrophomonas*: the work of Alavi et al. (2014) has shown that there is a difference in human pathogenic and drug-resistant *S. maltophilia* and the plant-associated *S. maltophilia*. Those differences are seen in virulence factors and heat shock proteins and should be studied in more detail.

Delftia, described as a new genus by Wen et al. (Wen et al., 1999), is considered as a Plant Growth Promoting Bacterium (PGPB), a diazotroph organism and a potential biocontrol agent (Han et al., 2005) and was found in various substrates in the environment. The type species is

Delftia acidovorans (Wen et al., 1999). Another important described species is *Delftia tsuruhatensis*, which was first isolated from activated sludge in Japan (Shigematsu et al., 2003).

Pseudomonas isolates are regularly associated with rice sheath rot and grain discoloration symptoms (Zeigler and Alvarez, 1987, 1990; Cottyn et al., 1996a; b; Gardan et al., 2002; Cother et al., 2010). In the past, those pseudomonads were usually only identified by biochemical/physiological tests, and except for *P. fuscovaginae* and *P. palleroniana,* their taxonomic status had not been unraveled (Gardan et al., 2002). We further identified the *Pseudomonas* isolates by sequencing part of the *rpoB* and genes since taxonomic studies have shown that intragenic diversity among *Pseudomonas* spp. can be specifically evidenced by the *rpoB* and the *rpoD* based sequences (Frapolli et al., 2007; Mulet et al., 2009). Molecular identification techniques have replaced those based on serology, enzymology and metabolism (Höfte and De Vos, 2006) and it is clear that molecular methods are needed to avoid taxonomic inconsistencies (Gomila et al., 2015). Most isolates characterized in this work are related to *Pseudomonas* species that were only recently described, for example *P. saponiphila* (Lang et al., 2010), *P. protegens* (Ramette et al., 2011), *P. fulva* (Uchino et al., 2001), *P. mosselii* (Naik et al., 2008; Mehri et al., 2011) and *P. simiae* (Berendsen et al., 2015), but their similarity with described species is rarely total (100%). Intriguingly, various reference strains closely related to isolates from Rwanda and the Philippines appear to be associated with the rhizosphere and are known as biocontrol agents, such as *Pseudomonas* sp. Os17 (Pc1 in the *P. chlororaphis* group) and *Pseudomonas* sp. CRS01-1 (Pp2 in the P. putida group) isolated from the rice rhizosphere (Takeuchi et al., 2015), *P. protegens* strains Pf-5 from the cotton rhizosphere (Ramette et al., 2011), *P. protegens* Cab57 from shepherd's purse rhizosphere, *Pseudomonas* sp. CMR12a and CMR5c from the cocoyam rhizosphere (Perneel et al., 2007), and *P. simiae* WCS417 from the rhizosphere or wheat (Berendsen et al., 2015). No isolate from Rwanda proved to be a major pathogen and the major rice sheath rot pathogen *P. fuscovaginae*, was not found. The conducted pathogenicity tests have shown that the *Pseudomonas* isolates from Rwanda are weakly pathogenic at best. Isolates from the Philippines included *P. fuscovaginae* and *P. palleroniana*. *P. palleroniana* was isolated from rice sheaths of diseased plants before (Gardan et al., 2002) though it proved to be weakly pathogenic in our pathogenicity tests.

The role played by the identified *Pseudomonas* isolates still has to be further investigated because currently it is unclear whether they are pathogens or may have potential for being used biological

control agents. The possibility of synergism between the organisms and/or favorable agroecological factors in disease induction should not be ruled out. When interpreting pathogenicity data, it is also important to take into account the difficulty of artificially reproducing natural field conditions, and also the short duration of experimentations. As the rice sheath rot is complex by nature, research for improving the understanding on it should continue and check if there are interactions between the detected agents and how they work, if it is by additive effects or by synergism.

The differentiation between pathogenic and beneficial organism is never strict. In fact, studies have shown that some mechanisms found in pathogenic bacteria like the Type Three Secretion System (T3SS) (Höfte and De Vos, 2006) or other mechanisms for host colonization are also found in bacteria thought to be endophytes (Schmidt et al., 2012). This situation is well documented for *Herbaspirillum rubrisubalbicans* (Monteiro et al., 2012) and it is important to check if it is the same situation with the numerous pseudomonads observed with rice sheath rot. Moreover, it has recently been shown that *P. protegens* Pf-5, a well studied biological control bacterium, produces toxoflavin (Philmus et al., 2015), a yellow pigmented toxin. This product is a known virulence factor of *Burkholderia glumae*, causing bacterial wilt on many field crops including rice (Jeong et al., 2003; Koh et al., 2011). This discovery offers new insights about the necessity of further research on the role played by many pseudomonads found in sheath rot diseased plants, potentially producing toxoflavin, which can be partly responsible for sheath rot disease symptoms. On the other hand, *Pseudomonas* bacteria from the *P. chlororaphis* and *P. fluorescens* group are known to produce many secondary metabolites that play a capital role in biocontrol such as phenazines (Mavrodi et al., 2010), 2,4-diacetylphloroglucinol, pyoluteorin (Ramette et al., 2011) and cyclic lipopeptides (Olorunleke et al., 2015), which may have activity against sheath rot fungi such as *Sarocladium oryzae* and *Fusarium* spp., but this remains to be investigated.

The question remains how bacteria that are usually associated with the rhizosphere can end up in the sheath of rice plants. Various hypotheses can be formulated: (i) attraction by plant exudates in the reproduction phase, as it was observed with *P. fuscovaginae* on rice (Batoko et al., 1997); (ii) rice in Rwanda and the Philippines is produced in an irrigated system, in which the root system is most of the time covered with water, limiting oxygen availability which may incite bacteria to move to tissues with more access to oxygen; (iii) they are pathogenic but their

pathogenesis mechanism is not known yet, for example by the production of unknown toxins/polysaccharides; (iv) they have coevolved with other sheath rot pathogens like the fungus *Sarocladium oryzae* and they became resistant to the antibiotics produced by this fungus. Few studies have been conducted on the diversity of organisms in the phyllosphere, compared to the rhizosphere, but it has been documented that the bacterial population in the rhizosphere is not much different from that in the phyllosphere (Sørensen and Sessitsch, 2007; Schlaeppi and Bulgarelli, 2015). Bacteria that have a plant-associated lifestyle without causing any symptoms are generally called endophytes and can be found in the rhizosphere, but also in the phyllosphere, in seeds or in vegetative plant materials.

In the meantime, as it is now established that rice sheath rot is caused by many agents, using healthy planting material can be of utmost importance, considering the possibility of basically breaking the disease cycle. Rice remains an important crop in Rwanda, the Philippines and elsewhere in the world, in tropical and subtropical areas. The planting material exchange keeps increasing, as high quality cultivars are developed in international research centers and sent to countries for production, multiplication and distribution. It is of utmost importance to guaranty the planting material health quality as it has been proven with all the sheath rot associated pathogens that the disease is seed-transmitted. The report that we are presenting is based on a representative sample of the bacteria isolated from sheath rot diseased plants in Rwanda and the Philippines. Conducting more isolates identification is a good option but the fact that most plants in production are infected by various isolates is established. It is urgent to start simple seed treatment practices like the heat therapy mentioned by Zeigler and Alvarez (1987). Putting this in an integrated pest management approach could be more promising, as many factors, probably varying in each agroecosystem, are associated with rice sheath rot disease, to which Rwanda and many other rice-growing countries are exposed.

6 GENERAL DISCUSSION AND PERSPECTIVES

6.1 Introduction

This research was undertaken with the main goal of understanding the situation of rice sheath rot disease in Rwanda, its causal agents, compared to those found elsewhere in the world, and contributing in developing strategies for facing it. This study was motivated by many factors among which we can cite:

- importance gained by rice in Rwanda, where it is one of the priority crops (Bizimana et al., 2012);
- threat posed by rice sheath rot disease in the rice-producing countries;
- improvements in scientific information and tools for studying pathogens,
- suspicion that bacteria or fungi are associated with rice sheath rot in Rwanda,
  necessity of comparing the situation of rice sheath rot disease in Rwanda, as for the importance and the causal agents with the situation in other parts of the world.

Rice is an important crop worldwide. Though Asia contributes in the production at around 90% (IRRI, 2015b), rice is becoming important in other continents also, for example rice production has been increasing on average by 3.5% in Africa since 1961 (Timmer, 2010). Rice provides 21% of energy and 15% of protein to humans (Zibaee, 2013).

Rice has emerged as a promising crop in Rwanda. It is a young crop in the country, having been introduced in the 1950s (Kathiresan, 2010) but the experiments exploring the potential for production at large scale having been conducted only in the 1970s (Baker, 1970). Having been growing rice recently, Rwanda is cooperating with other countries and international research centers, most of which have a long rice growing tradition (UCORIRWA and AC Team, 2004; MINAGRI, 2013a). Rice is produced in an irrigated system in marshlands, performs with a yield of 5.34 tons/ha (IRRI, 2015b), which is high considering the situation in the region and in Africa. The pro-rice promotion policy, followed in the last decades, has also undeniably helped rice development in Rwanda. Still, the rice sector in the country is experiencing some problems in cultivar development and crop protection aspects. In fact, though the production has been increasing, the demand has been much more increasing and the country has to import a large part of the rice consumed. There are problems of presence of less adapted varieties, poor seed quality, low national production quantity, low market value, as the locally produced rice is less

competitive on the market (Stryker, 2013), and poor knowledge on rice pests and diseases (Promar, 2012; MINAGRI, 2013a). Improvements in rice production in Rwanda seem a must for harnessing the potential and respond to the demand and this requires efforts in addressing the limitations including the curbing of pests and diseases, of which many are not yet known, such as rice sheath rot.

There are not many publications about rice production in Rwanda. Like in other areas, there are not many scholarly works about Rwanda. In fact, the country was one of last African countries to be known in the Western World, the first European, Oscar Baumann having entered there only in 1892 (Meyer, 1902). He would be followed by Gustav Adolf von Götzen. When the Berlin conference was held in 1884-1885, the country was vaguely known as part of the African Great Lakes region. Its status would be really defined later with the Brussels conference declaration in 1890.

Rice sheath rot disease has been detected in other parts of the world where it is considered to be caused by fungi like *S. oryzae* and *Fusarium* spp. and bacteria, especially those of the *Pseudomonas* genus, particularly *P. fuscovaginae* (Bigirimana et al., 2015), this study wanted to compare the findings from previous studies and those from Rwanda so as to effectively contribute in rice sheath rot control.

6.2 Research findings in relation with the objectives

Considering the objectives of the study, the following answers can be formulated.

What is the importance and impact of rice sheath rot in Rwanda?

Rice sheath rot is an important disease in Rwanda. It is found in all the rice-growing areas in the country which were surveyed in the study and its intensity (incidence and severity) is high. The comparison between grains from a diseased and a healthy plant has shown that there is a yield loss estimated at 56.93% in diseased plants. To this quantitative loss, must be added a qualitative loss, as most of the pathogens isolated on sheath rot diseased rice plants have the capacity to produce mycotoxins, which are harmful to humans and animals consuming diseased rice (Jestoi, 2008; Wulff et al., 2010; Nesic et al., 2014). Our results gave the indication that toxins are produced by *Fusarium* isolates from Rwanda, but the connection to the disease prevalence needs more extended studies. There are no reference studies on the epidemiology from which a threshold can be established, and this may justify why there is large variability in reported yield

losses (Ou, 1985; CABI, 2007) and also most studies were conducted on a particular development stage, like seedling (Prabhu and Bedendo, 1983; Dastjerdi and Karlovsky, 2015) or harvested kernels (Wulff et al., 2010; Chowdhury et al., 2015).

Which are the pathogens causing rice sheath rot in Rwanda? Are *Pseudomonas fuscovaginae* and *Sarocladium oryzae* the most important sheath rot pathogens present in Rwanda?

Based on the findings from this study, rice sheath rot in Rwanda is probably caused by fungi. *Sarocladium oryzae*, fungi belonging to the *Fusarium fujikuroi*, *F. oxysporum* and *F. incarnatum/equiseti* species complex have been isolated in this study. Not much is known about pathogenesis inducing factors, but given that the isolated species produce toxins, cerulenin and helvolic acid for *Sarocladium oryzae* (Sakthivel et al., 2002; Bills et al., 2004; Hittalmani et al., 2016), and especially fumonisin for *Fusarium* spp.(Abbas et al., 1998; Wulff et al., 2010), it can be hypothesized that sheath rot symptoms are partially due to the effects of toxins produced by fungi.

Unexpectedly, this study did not detect *Pseudomonas fuscovaginae* in Rwanda. *P. fuscovaginae* is the most reported sheath rot causing bacterium (Miyajima et al., 1983; Zeigler and Alvarez, 1987; Cottyn et al., 2002; Cother et al., 2009). But the study has detected many pseudomonads, found in other related studies (Zeigler and Alvarez, 1990; Cottyn et al., 1996a; b; Cother et al., 2010), for which the role, pathogenic or having a beneficial role for the plant, still has to be investigated. One of the merits of this research consists in having given insights in identifying those poorly identified bacteria as these isolates proved, through pathogenicity tests poorly pathogenic.

Sustainable crop protection practices are needed for facing pests and diseases that hamper rice production. In that aim, the starting point is the correct identification of the pests and diseases. In the past, the classical crop protection diagnosis practices were based on morphological or biochemical methods. In this study, *Sarocladium oryzae* identification was based on the sequencing of the ITS region. There are not many new developments in *Sarocladium* identification. *Fusarium* identification took into account the sequencing of the ITS region and the TEF factor and the grouping of isolates was made after Aoki et al. (2014). The general information on the bacteria was obtained through the sequencing based on 16S rDNA and the primers based on *rpoB* and genes were used for *Pseudomonas* isolates, to further put into

evidence the intragenic diversity. The molecular methods of identification, with their high level of precision and the relatively short time that they require, have revolutionised the identification approaches and are incessantly being improved (Atkins and Clark, 2004; López et al., 2009).

Concerning the bacteria, none of the reported major bacterial pathogens causing rice sheath rot elsewhere, such as *Pseudomonas fuscovaginae*, *P. syringae*, *Acidovorax oryzae* (formerly *A. avenae*), *Burkholderia glumae* and *Burkholderia gladioli* has been detected in Rwanda.

One of the striking findings about bacteria is that species traditionally found in the plant rhizosphere, are this time found in the phyllosphere. Although this has not been largely reported, it is probably because there have not been many researches on the organisms living in the phyllosphere; another study confirms that there is not a big difference between rhizosphere and phyllosphere organisms (Sørensen and Sessitsch, 2007). An illustration comes from studies on *Herbaspirillum rubrisubalbicans*, which show that it can move from the rhizosphere to the phyllosphere (Monteiro et al., 2012).

What is the origin of the sheath rot pathogens found in Rwanda? Are they native or introduced with the plant material?

Though Rwanda is a landlocked country, the pathogens that are found there are not far too different from those found in other parts of the world. In the *Sarocladium oryzae* strains, most of them are different from those found elsewhere in the world but few are clustering with reference strains from other parts of the world. *Sarocaldium oryzae* may have been introduced in Rwanda through seed movement. Various types of *Fusarium* spp. isolates were found. The major groups associated with rice sheath rot belonging to the *F. fujikuroi* species complex, *F. incarnatum/equiseti* species complex, and *F. graminearum* species complex are found in Rwanda. These organisms have regularly been found on rice seeds (Mew and Gonzales, 2002). This can be understood in the general observation that *Fusarium* organisms are ubiquitous. There are probably both native and introduced *Fusarium* strains. The pathogenic pseudomonads (*P. fuscovaginae*, *P. syringae*) were not found, it is the poorly studied pseudomonads that were found. Comparing isolates from Rwanda and those from the Philippines, the major groups were the same, except that *P. fuscovaginae* and the mildly pathogenic *P. palleroniana* were found in the Philippines while missing in Rwanda, where the *P. saponiphila*-like isolates are dominating.

Is there an interaction between these organisms and the prevailing environmental conditions in Rwanda?

We can hypothesize that all rice cultivars suffer from the low temperatures prevailing in Rwanda (Promar, 2012) and are susceptible to rice sheath rot. The tolerance found in Japonica varieties, while Indica varieties fail after 2 to 3 seasons of cultivation can be explained by the capacity of the Japonicas to tolerate low temperatures while the Indicas do not have it. On sheath rot disease, however, both types are susceptible, as even our pathogenicity experimentations have shown.

Which control options can be taken to face rice sheath rot?

This study gives the base for studying rice sheath rot in Rwanda. However, the information generated does not lead yet to precise control options. As some of the sheath rot pathogens are known, and taking reference from the review of published information, it is estimated that an action plan adapted to the Rwandan context can be developed. Considering the numerous factors involved in sheath rot disease development, among which there are many causal agents, a large host range and the possibility of disease development in almost all the rice growing areas and in most production systems, while not much research is available, an integrated disease control approach is advisable, the time that more information become available and indicate to give preference to one method or another. Integrated disease management practices, centered on the potential rice pests and diseases presented in Annex 2 and the actions improving and preserving the quality of the seed (Zeigler and Alvarez, 1987, 1989) can be followed, waiting for more durable solutions like the creation of rice lines adapted to the Rwandan environment.

6.3 Connecting findings to hypotheses

This study is based on two hypothesis: (1) lack of adaptation to prevailing abiotic conditions in Rwanda makes introduced rice varieties more susceptible to pests and diseases such as sheath rot; (2) quick rice development in Rwanda, with limited capacity of control and research on pests and diseases, has resulted in the build-up of large populations of pests and diseases, including sheath rot causing pathogens, which also negatively affect the rice grain quality. The data already available tend to support them.

On the first hypothesis, the environment of Rwanda favours rice cultivars of Japonica type, as they are the ones most adapted to low temperatures, but both of the variety types Japonica and Indica are susceptible to rice sheath rot. In the preliminary data on our study, fungal isolates of

Sarocladium oryzae and *Fusarium* spp. induced sheath rot disease symptoms on both *Nipponbare* cultivar of the Japonica type and CO39 cultivar of the Indica type, though symptoms were more pronounced on the former than on the latter. Though this experiment was not replicated many times, it gives preliminary facts but needs to be extended for giving more accurate information about the susceptibility levels. This experiment tends to confirm the susceptibility of the two rice subspecies, confirming that most rice materials currently in use are susceptible to sheath rot attacks (Mew et al., 2004b). Obtaining more information on the genetic features of rice varieties in use in Rwanda can give more insights in understanding the susceptibility. It is not known how comparable Nipponbare is in relation to Rwandan Japonica cultivars. Seeds grown in the 1980s came from Yunnan Province, China and when they became severely attacked by rice blast, Xinan 175 variety, of Japonica type, developed by Japanese in the 1950 in Taiwan, was introduced (Promar, 2012). It is a parent material for many varieties currently used in Rwanda. Besides, the pathogenicity experiment was conducted at 28°C, a temperature far higher than 19.84°C, the average for Rwanda (NISR, 2013), while Japonica varieties are highly affected by a temperature increase (Yoshida, 1981; Shah et al., 2014). It can be presumed that at a high temperature, Japonica type become more stressed and thus more susceptible to the diseases. Putting our findings in relation with the rice situation in Rwanda, as the adapted Japonica cultivars do not have a good market value, most of the last introductions consist of cultivars of Indica type, the ones appreciated on the market. Bugarama, a warm low altitude plain, is the only place where long grain varieties of Indica type are grown satisfactorily. In other places, those Indica varieties become stressed by low temperatures prevailing in Rwanda, and when to this pressure there is also the stress caused by rice sheath rot diseases and other diseases, fail to adapt, and the farmers came back to their "Japonica" varieties, which constitute their "safe haven" varieties. Moreover, the Japonica varieties respond well to the use of farm inputs like fertilizers and the improved production conditions might be predisposing plants to conditions conducive to attack and proliferation of rice sheath rot.

For the second hypothesis, rice is developing very fast in Rwanda, the crop protection practices have not been developing at the same pace. Also, the planting material introduced in the country is not fully checked for its health status. Most sheath rot pathogens are seed-transmitted (Bigirimana et al., 2015; Chowdhury et al., 2015). This situation resulted in the buildup of large stocks of pests and diseases including rice sheath rot.

There are many pathogenic fungal isolates that are found in Rwanda: *Sarocladium oryzae*, *Fusarium* spp. *Sarocladium oryzae* strains present less variability, prompting us to think that it is a recent introduction through seeds and grains. *Fusarium* spp. have more variability, indicating that some of its isolates may be native to the country while others may have been introduced through the seed movement, *Fusarium* spp. being regularly found in rice seeds (Mew and Gonzales, 2002). The disease is associated with many bacterial strains, continuously reported in diagnostic studies (Zeigler and Alvarez, 1990; Cottyn et al., 1996a; b) but their role in the pathogenicity is unknown. The marginal research and practices on rice crop protection that exist in Rwanda, while the country regularly imports seeds and rice for consumption, are unable to realise that a large population of pathogens has built up on rice throughout the time. These pathogens could also be partly responsible of the discolouration and poor quality of the locally-produced rice in Rwanda as this has been observed elsewhere (Zeigler and Alvarez, 1989).

The aim of this research is to contribute in addressing the rice sheath rot problem. If in the long run many actions can be envisaged, there are few options in the short run. Rice can be attacked by many pests and diseases in Rwanda (see Annex A2). In fact, as the country meets tropical and subtropical conditions, most pathogens attacking rice can get established, once they are introduced in Rwanda. It is important to reinforce the disease diagnosis system and develop regulatory tools for preventing the introduction of new pests and diseases. It is likely that the international exchange of planting materials will keep intensifying. A list of pathogens that can attack rice in Rwanda, based on the available information and the PRA methodology is presented in Annex A2. Besides this, there is also an urgency in starting to adopt simple practices, protecting the seed so that it is cleaned before planting, and this must be done on field during production and in post harvest. Some of such practices are displayed by Zeigler and Alvarez (1987, 1989). In the crop husbandry, efforts to promote the good balance in nitrogen fertilization should be reinforced at the same time as maintaining health quality in seed production, multiplication and distribution systems. In the long run, when there is enough information about the pathogens attacking rice and the genetic information on the planting material in use in the country, a breeding program, adapted to the special tropical semi-temperate climate of Rwanda, but also taking lessons from other rice-growing countries like the Philippines which have experienced the same situation, should be mounted. These actions could significantly contribute in addressing the question of the lack of adapted high yielding varieties , resistant to rice sheath

rot and other important pests and diseases and also avail rice with a high marketing value, as the imported Indica long grain varieties are preferred to the adapted short grain varieties.

6.4 Perspectives

There are many perspectives for further studies and field action on rice sheath rot in Rwanda. An action plan can be developed with the available information. This action plan must take into account the following elements: type of pests and diseases present, crop husbandry practices, production objectives, agroecological factors and must also explore the potential for breeding for resistance. In that aim, some of the actions that can be taken fall in the following areas:

- It is important to develop an efficient link between the field practices and the research so that field people can have access to advanced tools like scientific information and diagnosis guidance. Illustrated and annotated manuals of sheath rot pathogens, regularly updated, can help in addressing this issue on the practical side. It is clear that there are pathogen introductions in Rwanda, for example for *Sarocladium oryzae,* and practices must be adapted to prevent such introductions.
- Efforts must be put in understanding the pathogenicity factors of the species identified as associated with rice sheath rot disease. The knowledge on mycotoxins and their role in pathogenicity is of particular importance. This can help in overcoming the controversy existing now in which some studies confirm that mycotoxins constitute a pathogenicity factor (Abbas et al., 1995) while others attest that there is no correlation between mycotoxin production and pathogenicity (Dastjerdi and Karlovsky, 2015). Conducting a study on the infection on the whole growth cycle can provide conclusive information on this issue as the results of these studies come from one plant development stage. The work on the pathogenesis induced by *Sarocladium oryzae* and *Pseudomonas fuscovaginae* will be in the future facilitated by the availability of the information on their genome which has been sequenced (Patel et al., 2012; Hittalmani et al., 2016). Some *Fusarium* species have also been sequenced (Waalwijk et al., 2004).
- The role played by the pseudomonads needs further studies to be clearly elucidated. Their large variability warrants more studies on their importance and their ecology in the phyllosphere, given that they were mainly reported in the rhizosphere. Moreover, their relationships with fungi of the *Sarocladium oryzae* and *Fusarium* genera, both associated with rice sheath rot symptoms, justifies the necessity for conducting those studies. In this

regard, investigations on biofilm formation and the study of the type of secondary metabolism products that are released associated with the coinoculation of *Pseudomonas* and *Sarocladium/Fusarium* on rice plants can lead to more insights about the understanding of the interactions and guide for the biological control exploration. However, it can not be ruled out that those bacteria, or some of them, may be pathogens with an unknown pathogenicity mechanism. Their ecology throughout different development stages deserves also to be studied. It is for example confirmed that *P. fuscovaginae* passes by epiphytic phases before attacking rice and inducing sheath brown rot, taking advantage of the increased susceptibility of the plant at the booting stage (Batoko et al., 1997). This situation should be explored for the other pseudomonads also.

- Research is needed on practices that can reduce the pathogen inoculum in rice seeds, as most sheath rot pathogens are seed-transmitted, building on what is already known.
- The information on the rice genetic material in use in Rwanda is needed. It can give more insights in understanding why rice sheath rot is an important disease in Rwanda.

  Efforts must be put in breeding rice varieties adapted to the Rwanda context: low temperature, good response to input use, good culinary properties. On this last point, there is a need of breeding rice varieties adapted to the unique conditions of a tropical climate tempered by the altitude and this is possible as there are elsewhere many breeding initiatives for taking advantage of the different rice genotypes (Yang et al., 2012). The results presented were obtained on Nipponbare and CO39 varieties. The information on the relationship between those varieties and the ones used in Rwanda can improve in understanding the failure observed with Indica varieties while the Japonica varieties become tolerant. Efforts must be put in enquiring about the situation of the Japan-China initiative in 1990-1994 to develop rice varieties adapted to Rwandan environment (Promar, 2012) that was interrupted so as build on its findings and design anew such kind of breeding activity.
- A comprehensive study on the epidemiology of disease induced by the major rice sheath rot pathogens (*S. oryzae*, *Fusarium* spp., *P. fuscovaginae*) is needed. This study should take into account among others: pathogen variability, seasonal variability, temperature, relative humidity, location, yield losses, rice cultivars and the transmission rate. As the aim of this study would be to advise on sheath rot disease control, trials must be

conducted in both controlled and semi-controlled environments, so as to nearly reproduce the real rice-growing conditions.

ANNEX 1: IDENTIFICATION OF VIRULENCE ASSOCIATED LOCI IN THE EMERGING BROAD HOST RANGE PLANT PATHOGEN *PSEUDOMONAS FUSCOVAGINAE*

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Identification of virulence associated loci in the emerging broad host range plant pathogen *Pseudomonas fuscovaginae*

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Abstract

Background: *Pseudomonas fuscovaginae* (*Pfv*) is an emerging plant pathogen of rice and also of other gramineae plants. It causes sheath brown rot disease in rice with symptoms that are characterized by brown lesions on the flag leaf sheath, grain discoloration and sterility. It was first isolated as a high altitude pathogen in Japan and has since been reported in several countries throughout the world. *Pfv* is a broad host range pathogen and very little is known about its virulence mechanisms.

Results: An *in planta* screen of 1000 random independent Tn*5* genomic mutants resulted in the isolation of nine mutants which showed altered virulence. Some of these isolates are mutated for functions which are known to be virulence associated factors in other phytopathogenic bacteria (eg. *pil* gene, phytotoxins and T6SS) and others might represent novel virulence loci.

Conclusions: Being an emerging pathogen worldwide, the broad host range pathogen *Pfv* has not yet been studied for its virulence functions. The roles of the nine loci identified in the *in planta* screen are discussed in relation to pathogenicity of *Pfv*. In summary, this article reports a first study on the virulence of this pathogen involving *in planta* screening studies and suggests the presence of several virulence features with known and novel functions in the *Pseudomonas* group of bacteria.

Background

Pseudomonas fuscovaginae (*Pfv*) is a Gram-negative, fluorescent pseudomonad and a member of Gamma proteobacteria [1,2]. *Pfv* is one of the 18 validly described *Pseudomonas* plant pathogenic species, which are part of the oxidase positive cluster [3,4]. This bacterium was first identified and reported as a pathogen of rice (*Oryza sativa*) in the temperate region of Japan in 1976 [2]. It has now been described in several other regions of the world where rice and other gramineae food crops are cultivated including Burundi [5], Madagascar [6], Mexico [7], the Philippines [8], Nepal [9], Brazil [10], China [11], Iran [12] and more recently in Malaysia [13] and Australia [14]. *Pfv* causes brown sheath rot disease in rice and also in other gramineae food crops including maize (*Zea mays*), sorghum (*Sorghum bicolour*) and wheat (*Triticum aestivum*) [5,7]. Brown sheath rot symptoms on rice plants appear at all growing stages. At the seedling stage symptoms start with yellow to brown discoloration on the lower leaf sheath which later turns into grey-brown to dark-brown and ultimately, the infected seedlings rot and die. In mature rice plants *Pfv* symptoms can be observed on flag leaf sheaths, other leaf sheaths and also on the panicles. Under severe infection conditions, the entire leaf sheath becomes necrotic and dries out. Spikelets of emerging panicles may be discoloured, sterile or symptomless, except for small brown spots [1,15].

A successful infection by a phytopathogenic bacterium is not a single step process and is coordinated by several functions including bacterial adherence, movement, colonization, invasion, and suppression of host immunity. Type IV pili are one of the best characterized adhesins in *Pseudomonas* pathogens and it has been shown to be involved in several functions including adhesion in

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P. syringae pv. *phaseolicola* [16], epiphytic fitness and survival in *P. syringae* pv. *syringae* [17] and *P. syringae* pv. *tomato* [18] and also in surface motility and virulence in *P. syringae* pv. *tabaci* [19,20]. The colonization process in *Pseudomonas* plant pathogens has been associated with exopolysaccharides (EPSs) shown to be involved in biofilm formation, epiphytic fitness and virulence [21,22]. The invasion process of plant pathogens has also been linked with secretion of several cell wall degrading enzymes including pectinolytic enzymes, cellulases and lipase through protein secretion systems [23]. Plant pathogenic bacteria also have strategies to suppress the host defense responses induced during the infection process by secretion of effector proteins directly into the host cell through a type III secretion system (T3SS) [24]. In addition to these functions, phytopathogenic pseudomonads produce several phytotoxins including coronatine, syringomycin, syringopeptin, phaseolotoxin, tabtoxin, and mangotoxin [25]. Quorum sensing (QS) signaling and its role in virulence has also been studied in several *Pseudomonas* species including *P. aeruginosa* [26-29], *P. syringae* pv. *syringae* [30] and *P. fuscovaginae* [31].

To our knowledge no genetic and molecular studies or screening for virulence-associated systems/functions in *Pfv* have been reported. Only a few biochemical studies have shown the production of three phytotoxins; namely syringotoxin, fuscopeptin A (FP-A) and fuscopeptin B (FP-B) [32,33] which have been shown to be able to generate the disease symptoms. We reported the role of the two QS systems in causing brown sheath rot by *Pfv* in rice [31] and have also determined the first draft genome sequence of a highly virulent *Pfv* strain [34]. In this study an *in planta* screening of 1000 genomic Tn5 mutants has provided some insight into the virulence associated functions in *Pfv*.

Results and discussion

Screening of *P. fuscovaginae* Tn5 mutants for altered virulence *in planta*

As there are no major reports regarding virulence functions of this emerging phytopathogen, we performed an *in planta* screen of 1000 Tn5 genomic mutants to identify genes and/or pathways that might influence *Pfv* virulence potential. A Tn5 mutant bank of *Pfv* was generated as described in the Materials and Methods section and 1000 insertion mutants randomly selected and numbered from 1 to 1000 were tested for virulence on plants of *Chenopodium quinoa*. In this screen *C. quinoa* was chosen as a plant model over rice because the infection protocol for *Pfv* is simpler to perform in *C. quinoa* compared to rice and therefore more suitable for a high-throughput screen involving many mutants [31]. Plant inoculations were performed as described in the Materials and Methods section and virulence was assessed using the virulence score from 0 to 5 as previously described [31] and presented here in Additional file 1. In the first round of screening we obtained 83 mutants that were altered for virulence compared to wild type. In order to verify the results, the 83 mutants were re-assessed for virulence in three biological replicates using two independent plants (total of six replicates). We then obtained a total of 9 mutants (Table 1) that displayed consistent and a significantly reduced virulence compared to wild type *Pfv* (Table 2). None of the mutants were affected in their growth pattern when grown in liquid rich media (data not shown).

In order to verify the virulence deficiency of identified mutants, infection assays were carried out using both *C. quinoa* and rice plants. It was of interest to test all the identified mutants in both models as the strain *Pfv* UPB0736 was first isolated as a rice pathogen. Rice infection was performed by syringe inoculation (100 μl of 10^8 cfu/ml) as described in the Materials and Methods section and virulence was assessed using the virulence score from 0 to 5 and also by measuring the lesion length (as presented in Additional file 2). In rice, 5 out of the 9 selected mutants had similar behaviour as in *C. quinoa*, being reduced in virulence when inoculated with a higher dose of bacteria (100 μl of 10^8 cfu/ml). Three other mutants were also found with reduced virulence, although the virulence level was not significantly different compared to wild type. Surprisingly, *Pfv* 188 on the other hand showed a similar level of virulence compared to wild type in rice (Figure 1).

We localized the Tn5 insertion site in these selected nine Tn5 mutants and mapped their insertion position in the *Pfv* UPB0736 draft genome (Figure 2). The nine Tn5 mutants were localized in genes coding for the following features: an arsenic pump efflux (*Pfv* 80), two hypothetical proteins (*Pfv* 90; *Pfv* 188), the type IV pilus biogenesis protein, PilZ (*Pfv* 102), an N-acetyl-gamma-glutamyl-phosphate reductase (*Pfv* 169), an acetylglutamate kinase (*Pfv* 270), a phage tail fiber homolog protein (*Pfv* 420), a syringopeptin synthatase C homolog (*Pfv* 445) and a bi-functional sulphate adenylyltransferase subunit 1 (*Pfv* 480) (Figure 2).

Validation of the genetic screening by re-generation of knock-out mutants in the same loci and their genetic complementation

In order to further verify the virulence phenotype of selected Tn5 mutants, all mutants in the nine loci were independently re-generated via homologous recombination as described in the Materials and Methods section. In addition we also complemented three Tn5 mutants (*Pfv* 90, *Pfv* 420 and *Pfv* 445) by identifying the genomic region harbouring each of the three loci from a cosmid library. We re-assessed the virulence phenotype of the nine Tn5 mutants, their corresponding re-generated mutants and 3

Table 1 Bacterial strains used in this study

Strains	Relevant characteristics[a]	Reference/source
E. coli.		
DH5α	Cloning strain, Nal^r	[35]
PRK2013	Helper strain for tri-parental conjugation. Km^r	[36]
Pseudomonas fuscovaginae (*Pfv*)		
Pfv UPB0736 (NCPPB 3871) (WT)	Wild-type strain isolated from diseased rice in Madagascar; Nf^r, Amp^r	[34]
Pfv 80	80:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 90	90:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 102	102:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 169	169:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 188	188:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 270	270:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 420	420:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 445	445:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 480	480:: Tn*5* of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 80-pKNOCK	80:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 90 pKNOCK	90:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 102-pKNOCK	102:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 169-pKNOCK	169:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 188-pKNOCK	188:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 270 pKNOCK	270:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 420 pKNOCK	420:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 445 pKNOCK	445:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 480-pKNOCK	480:: pKNOCK mutant of *Pfv* UPB0736; Nf^r, Km^r	This study
Pfv 90 + pCos90	pCos90:: *Pfv* 90; Nf^r, Km^r,Tc^r	This study
Pfv 420 + pCos420	pCos420:: *Pfv* 420; Nf^r, Km^r,Tc^r	This study
Pfv 445 + pCos445	pCos445:: *Pfv* 445, Nf^r, Km^r,Tc^r	This study

[a];Nal^r, Nf^r Km^r, Tc^r and Amp^r indicates nalidixic acid, nitrofurantoin, kanamycin, tetracycline and ampicillin respectively.

Table 2 Virulence screening of Tn*5* transposon mutants of *P. fuscovaginae* in *C. quinoa* plants

Pseudomonas fuscovaginae (*Pfv*) strains	Lesion scores in screen I (Average ± S.D.)	Lesion scores in screen II (Average ± S.D.)
Pfv UPB0736 (WT)	5, 5, 5 (5 ± 0)	5, 5, 5, 5, 5, 5 (5 ± 0)
Pfv 80	0, 1, 1 (0.66 ± 0.58)[a]	2, 2, 2, 2, 2, 3 (2.16 ± 0.40)[a]
Pfv 90	1, 1, 1 (1 ± 0)[a]	0, 0, 0, 0, 0, 1 (0.16 ± 0.40)[a]
Pfv 102	0, 0, 0 (0 ± 0)[a]	0, 1, 1, 2, 2, 3 (1.50 ± 1.05)[a]
Pfv 169	0, 0, 0 (0 ± 0)[a]	0, 0, 0, 0, 0, 0 (0 ± 0)[a]
Pfv 188	0, 0, 0 (0 ± 0)[a]	0, 0, 0, 0, 0, 0 (0 ± 0)[a]
Pfv 270	1, 1, 1 (1 ± 0)[a]	1, 1, 1, 1, 1, 1 (1 ± 0)[a]
Pfv 420	2, 2, 3 (2.33 ± 0.58)[a]	2, 2, 3, 3, 3, 3 (2.66 ± 0.52)[a]
Pfv 445	1, 1, 1 (1 ± 0)[a]	2, 2, 2, 2, 2, 2 (2 ± 0)[a]
Pfv 480	1, 1, 1 (1 ± 0)[a]	1, 1, 1, 1, 1, 1 (1 ± 0)[a]

Table showing disease severity for wild type and selected Tn*5* mutants of *Pfv* based on their rating score in *C. quinoa* plants. A two-tailed, paired '*t*' test with 95% of confidence intervals for independent means was performed between the wild type and each of Tn*5* mutants. [a]; significant difference to WT at $P < 0.05$.

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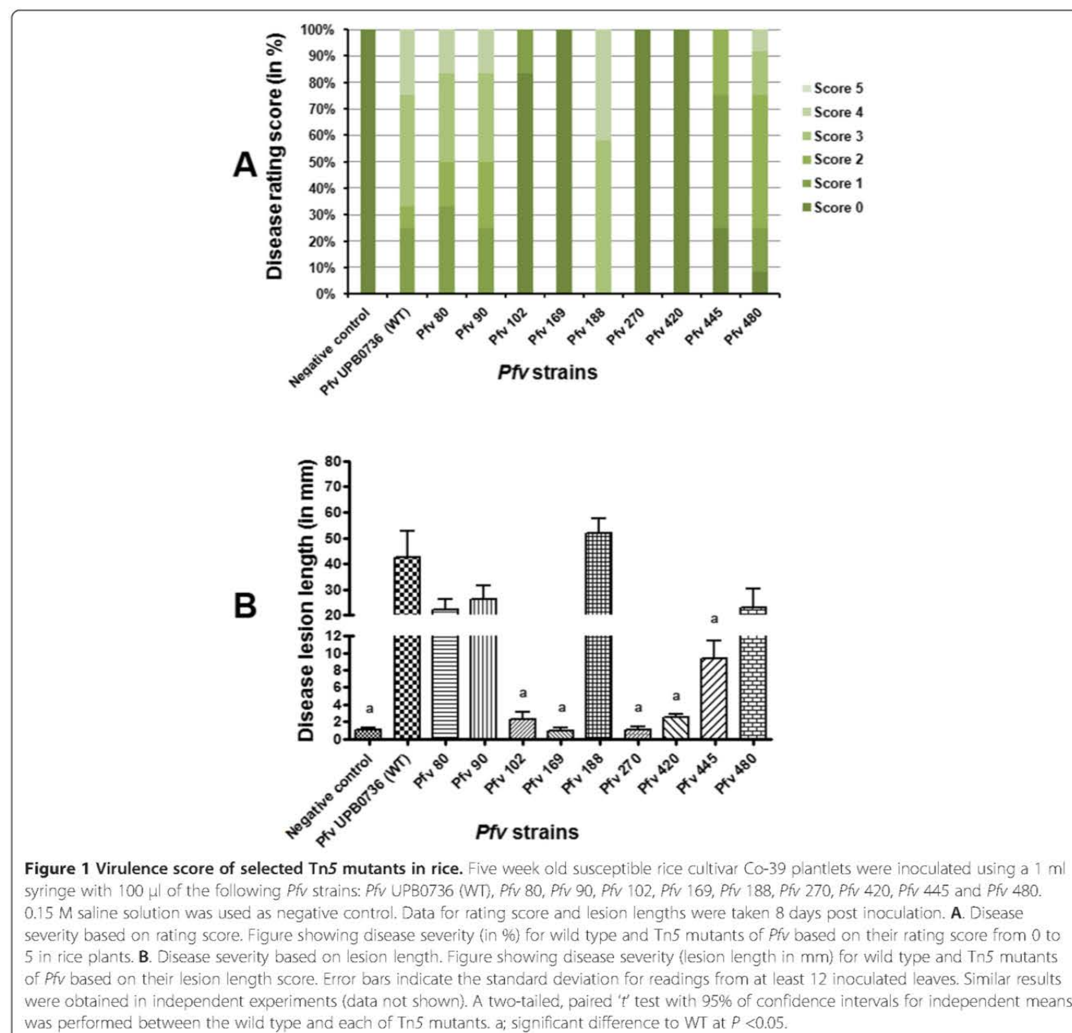

Figure 1 Virulence score of selected Tn*5* mutants in rice. Five week old susceptible rice cultivar Co-39 plantlets were inoculated using a 1 ml syringe with 100 μl of the following *Pfv* strains: *Pfv* UPB0736 (WT), *Pfv* 80, *Pfv* 90, *Pfv* 102, *Pfv* 169, *Pfv* 188, *Pfv* 270, *Pfv* 420, *Pfv* 445 and *Pfv* 480. 0.15 M saline solution was used as negative control. Data for rating score and lesion lengths were taken 8 days post inoculation. **A.** Disease severity based on rating score. Figure showing disease severity (in %) for wild type and Tn*5* mutants of *Pfv* based on their rating score from 0 to 5 in rice plants. **B.** Disease severity based on lesion length. Figure showing disease severity (lesion length in mm) for wild type and Tn*5* mutants of *Pfv* based on their lesion length score. Error bars indicate the standard deviation for readings from at least 12 inoculated leaves. Similar results were obtained in independent experiments (data not shown). A two-tailed, paired '*t*' test with 95% of confidence intervals for independent means was performed between the wild type and each of Tn*5* mutants. a; significant difference to WT at $P < 0.05$.

complemented strains in rice plants by pin prick inoculation (this type of infection results in the inoculation of a lower dose of bacteria as the sterile pin was dipped in a suspension of 10^6 cfu/ml). Eight of the nine Tn*5* mutants and their respective re-generated knock-outs showed similar results displaying virulence deficiency ($P < 0.05$) as observed when inoculated with higher doses of bacteria in rice. Whereas *Pfv* 188 again displayed virulence symptoms similar to wild type strain as obtained with syringe inoculations (Figures 3 and 4). With respect to complementation in these experiments, the mutant *Pfv* 90, harbouring the cosmid clone carrying the corresponding wild-type locus, regained virulence completely. On the other hand the virulence assays with mutants *Pfv* 420 and 445 harbouring cosmid clones isolated from the gene bank did not result in complementation (Figures 3 and 4). Mutant *Pfv* 445 had the Tn*5* inserted in a gene homologous to the one coding for the syringopeptin C of *P. syringae* pv. *syringae* (*Pss*) B728a, thus this gene is most probably involved in the biosynthesis of one of the fuscopeptins produced by *Pfv*. Peptide synthetases are very large ORFs; for example in *Pss* B728a the three syringopeptin genes *sypA* (16119 bp), *sypB* (16410 bp) and *sypC* (40614 bp) are at least 16 kb in size (Additional file 3). The Tn*5* insertion region in mutant *Pfv* 445 was found in a gene homologous to syringopeptin C of *Pss* B728a. This homologous gene in

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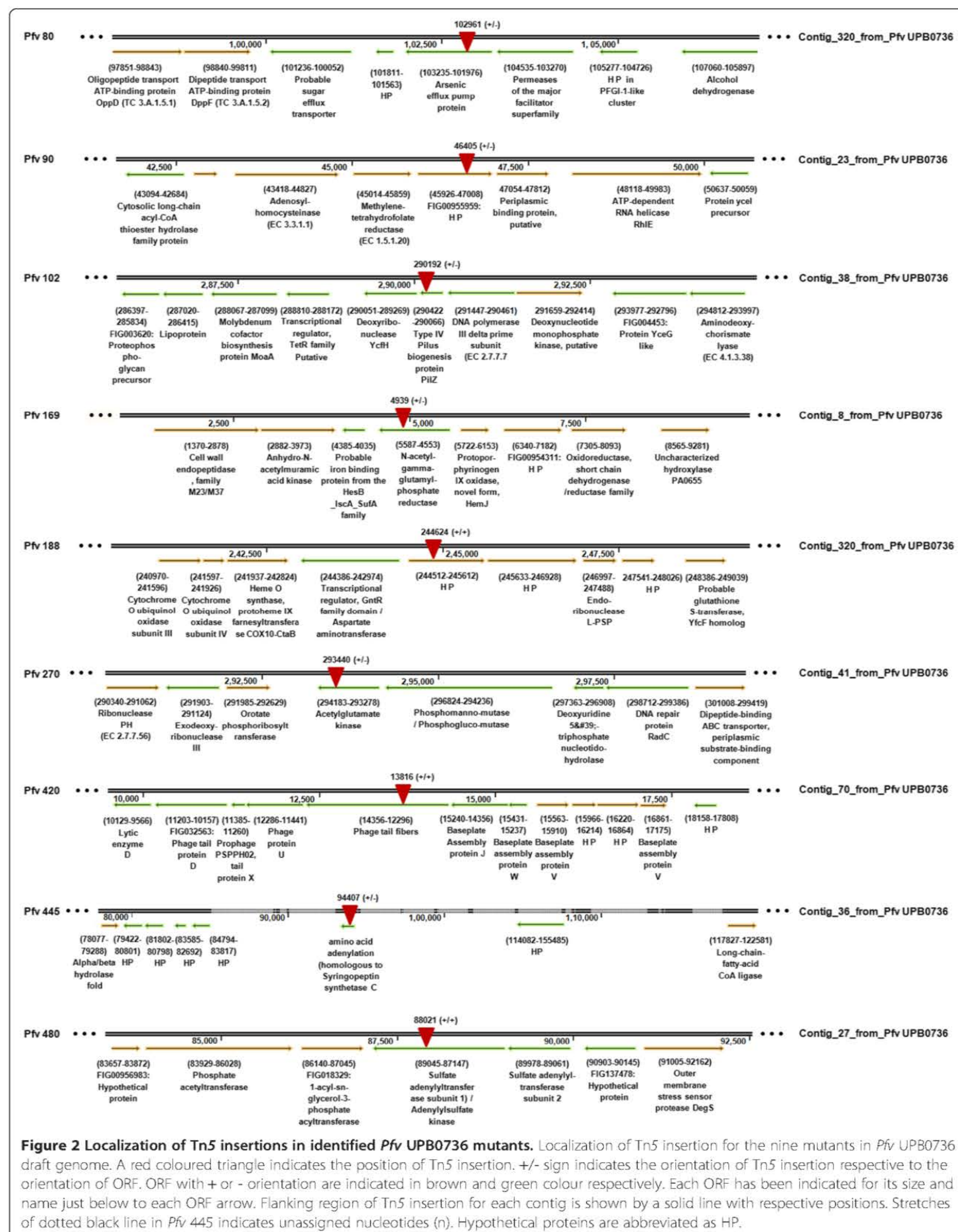

Figure 2 Localization of Tn*5* insertions in identified *Pfv* UPB0736 mutants. Localization of Tn*5* insertion for the nine mutants in *Pfv* UPB0736 draft genome. A red coloured triangle indicates the position of Tn*5* insertion. +/- sign indicates the orientation of Tn*5* insertion respective to the orientation of ORF. ORF with + or - orientation are indicated in brown and green colour respectively. Each ORF has been indicated for its size and name just below to each ORF arrow. Flanking region of Tn*5* insertion for each contig is shown by a solid line with respective positions. Stretches of dotted black line in *Pfv* 445 indicates unassigned nucleotides (n). Hypothetical proteins are abbreviated as HP.

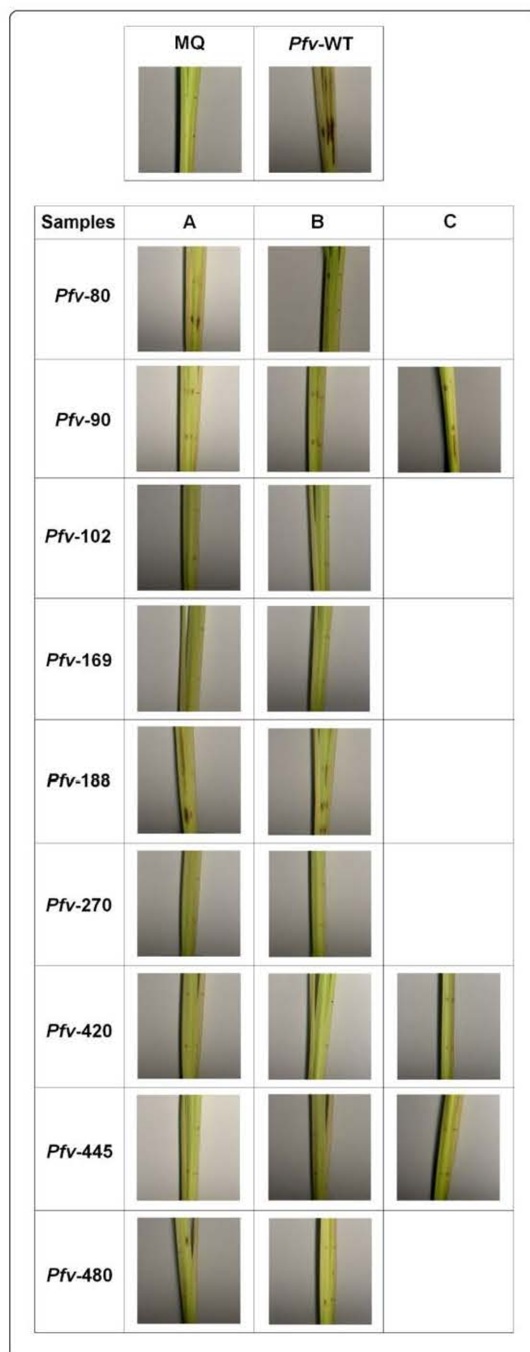

Figure 3 Virulence phenotype of selected *Pfv* strains in rice. Five week old susceptible rice cultivar Baldo were pin prick inoculated by dipping into 10^6 cfu/ml inoculums of *Pfv* strains. **A**: Tn*5* mutants *Pfv* 80, *Pfv* 90, *Pfv* 102, *Pfv* 169, *Pfv* 188, *Pfv* 270, *Pfv* 420, *Pfv* 445 and *Pfv* 480. **B**: knock-out mutants of *Pfv* 80, *Pfv* 90, *Pfv* 102, *Pfv* 169, *Pfv* 188, *Pfv* 270, *Pfv* 420, *Pfv* 445 and *Pfv* 480. **C**: complemented strains *Pfv* 90 + pCos 90, *Pfv* 420 + pCos 420 and *Pfv* 445 + pCos 445. *Pfv* UPB0736 (WT) and MQ water were used as positive and negative control respectively. Figure showing the disease symptoms were taken 10 days post inoculation.

the *Pfv* UPB0736 draft genome sequence contained stretches of unassigned nucleotides and it is likely to be an unusually large ORF. It is therefore possible that the cosmid clone used did not harbour the complete gene hence it could not complement the mutant *Pfv* 445. Mutant *Pfv* 420 had the Tn5 transposon insertion in a gene encoding for a protein with significant homology to a phage tail fiber. In *Pfv* this gene is located in a cluster of genes with phage related functions that are probably part of an operon. Again, lack of complementation could be due to the cosmid clone not containing all the genetic material necessary for the complementation. Another possibility for not having complemented the virulence phenotype of *Pfv* 420 and *Pfv* 445 could be due to multicopy allele effects of these genes which may cause instability or fitness cost. In summary, 8/9 mutants identified using *C. quinoa* as infection model were also found affected for virulence in a similar manner in rice (except *Pfv* 188) when inoculated with low doses of bacteria. The same profile of virulence in rice was also obtained with independently generated mutants in the same loci as the identified virulence defective mutants; all these data further confirm the results of the genetic screen and indicate that the inactivated functions in the identified mutants are directly or indirectly associated with *Pfv* virulence.

The nine genetic loci identified in the screen and their inference in virulence

Here below we describe the nine loci in which the Tn*5* insertions were located in relation to their potential role in virulence.

Virulence deficient mutants Pfv 80, 169, 270 and 480 have transposon insertions in genes involved in various metabolic functions

Pfv 80: the Tn*5* was localized in a gene that displays significant identity to an arsenic efflux pump protein (Figure 2). Arsenic is a toxic metalloid and resistance to this metal has already been described in Gram-positive and also in Gram-negative bacteria [37]. We do not know the exact mechanism of involvement in virulence for arsenic pump efflux protein in *Pfv*. However, being a toxic

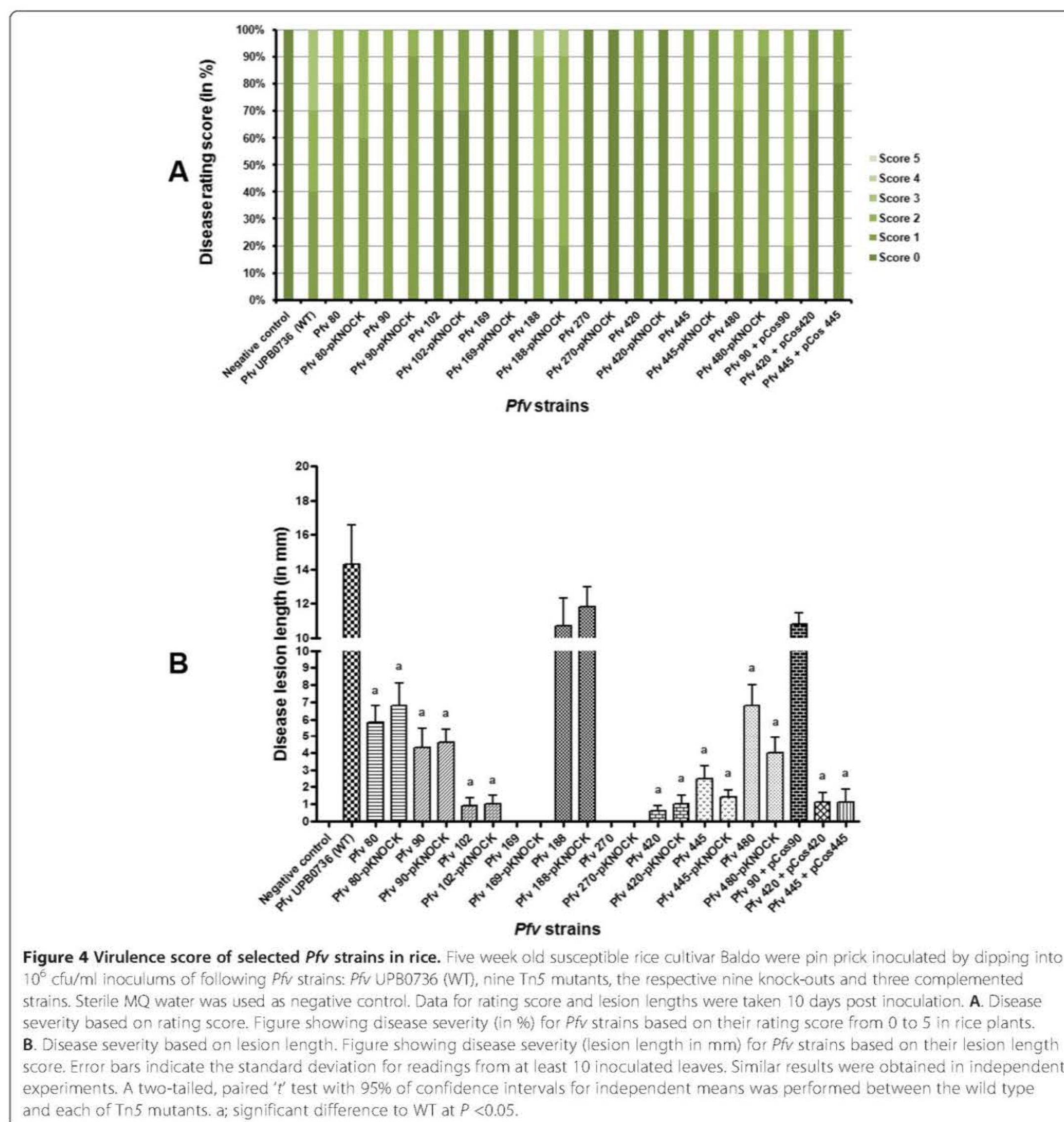

Figure 4 Virulence score of selected *Pfv* strains in rice. Five week old susceptible rice cultivar Baldo were pin prick inoculated by dipping into 10^6 cfu/ml inoculums of following *Pfv* strains: *Pfv* UPB0736 (WT), nine Tn*5* mutants, the respective nine knock-outs and three complemented strains. Sterile MQ water was used as negative control. Data for rating score and lesion lengths were taken 10 days post inoculation. **A.** Disease severity based on rating score. Figure showing disease severity (in %) for *Pfv* strains based on their rating score from 0 to 5 in rice plants. **B.** Disease severity based on lesion length. Figure showing disease severity (lesion length in mm) for *Pfv* strains based on their lesion length score. Error bars indicate the standard deviation for readings from at least 10 inoculated leaves. Similar results were obtained in independent experiments. A two-tailed, paired '*t*' test with 95% of confidence intervals for independent means was performed between the wild type and each of Tn*5* mutants. a; significant difference to WT at $P < 0.05$.

metal, export of arsenic through this efflux protein could be essential for a proper metabolic function and survival of the bacterial cell. It is possible that the inability of the *Pfv* mutant bacterium to expel this or a related toxic metal ion from the cytoplasm diminishes its viability *in planta* and thus makes it less virulent or less fit for growth in this environment compared to the wild type *Pfv*. Virulence studies in the grapevine pathogen, *Xylella fastidiosa* and in the fire blight pathogen, *Erwinia amylovora*, *tolC* mutant affected for efflux pump functions have shown their involvement in virulence and *in planta* fitness [38,39]. It is possible that these efflux pumps are involved in exporting heavy metals, antimicrobials or harmful plant phenolic compounds which are released as part of the plant defense response.

Pfv 169 and *Pfv* 270: In both mutants *Pfv* 169 and *Pfv* 270, the Tn*5* was located in genes involved in the biosynthesis of the amino acid arginine [38]. *Pfv* 169 and

Pfv 270 were found mutated in an N-acetyl-γ-glutamyl-phosphate reductase gene (*argC*) and N-acetylglutamate kinase gene (*argB*) respectively. The two enzymes catalyze the conversion of N-acetylglutamate in N-acetylglutamate semialdehyde, via N-acetylglutamyl phosphate. Specifically, N-acetylglutamate kinase encodes the key enzyme for the biosynthesis of arginine and is inhibited by the reaction product [39,40]. In order to further verify that the two isolated mutants were affected for arginine biosynthesis, an arginine auxotrophy assay was performed as described in the Materials and Methods section. *Pfv* 169 and *Pfv* 270 mutants and their respective re-generated mutants were found affected for growth on M9 agar plates lacking the amino acid arginine. Supplementation of arginine in M9 agar restored the growth defect of *Pfv* 169 and *Pfv* 270 mutants and their respective re-generated mutants (Additional file 4). The chemical complementation by arginine supplementation further confirmed that the two mutants affect the biosynthesis are involved in arginine biosynthesis pathway. Besides having a crucial role in metabolism and growth, arginine was also shown to have a role in virulence. Arginine is one of the components of ethylene biosynthesis and together with oxoglutarate, is used by the ethylene forming enzyme (EFE) to produce ethylene. Mutants in *efe* no longer produce ethylene and were found virulence deficient in *P. syringae* pvs. *glycinea and phaseolicola* [41]. Interestingly, a homolog of *efe* is present in *Pfv* UPB0736 draft genome (data not shown). Arginine is also a fundamental part of the signal peptide that directs the protein to the transport system called twin-arginine translocation system. The consensus sequence of the proteins harbouring the double arginine motif, contains two arginine repeated Ser/Thr-Arg-Arg-X-Phe-Leu-Lys. Furthermore, mutants for the twin-arginine translocation system in *P. syringae* spp. showed reduced viability and virulence *in planta* [42,43]. It is therefore possible that the reduced virulence of mutants *Pfv* 169 and 270 is caused not only by a deficiency in the metabolism of arginine but also due to a role directly related to pathogenesis via ethylene and protein transport.

Pfv 480: Mutant *Pfv* 480 had a mutation in a gene that encodes a bi-functional protein with two enzymatic activities: sulfate adenylyltransferase and adenylylsulfate kinase. Both of these activities are important in the metabolism of sulfur. The sulfate adenylyltransferase catalyzes the first intracellular reaction for the assimilation of sulfur with the use of a molecule of ATP and leading to the formation of adenosine-5-phosphosulfate (APS). This compound is pivotal for the biosynthesis pathway of amino acids that contain sulfur, namely cysteine and methionine. The adenylylsulfate kinase utilized the same substrate as APS catalyzing its conversion into 3'-phosphoadenosine 5'-phosphosulfate (PAPS) using ATP molecules. PAPS is a universal donor of the sulfate group and is used as the substrate for important enzymes such as sulfotransferase [44]. It is currently unknown how these enzymes crucial for the metabolism of sulfur are involved in virulence.

Virulence deficient mutant Pfv 102 is affected in type IV pili biosynthesis

The Tn5 transposon localized in the *Pfv* 102 mutant was found in Type IV pilus biogenesis gene *pilZ* encoding a protein that displayed significant identity (82 to 92%) with PilZ of other pseudomonads. The PilZ protein is one of the several Pil proteins that are associated with type IV pili biosynthesis. PilZ is the only protein that is not part of the pili biosynthesis operon and is located as a single transcriptional unit in the genome of pseudomonads. Type IV pili genes are found not only in pseudomonads but also in other Gram-negative bacteria including xanthomonads and are implicated in a wide spectrum of roles including adhesion, motility, secretion and virulence. The role of PilZ in the formation of Type IV pili has not yet been well established. In some cases, knock-out mutants are incapable of secreting a protein which constitutes the pilus whereas in other cases PilZ does not seem to be essential for the formation of the pilus and for bacterial movement [45-47]. In recent years, sequencing of several bacterial genomes has revealed the presence of a PilZ domain in many proteins and has associated the function of this domain with the binding of the second-messenger cyclic guanosine monophosphate [46]. The c-di-GMP regulates many functions including aggregation, biofilm formation, EPS production, adhesion, movement and virulence [48]. It is possible that PilZ in *Pfv* can influence the signalling cascade mediated by c-di-GMP that could be involved in the pathogenic process.

Virulence deficient mutant Pfv 420 is most likely affected in type VI secretion machinery

Tn5 transposon localized in *Pfv* 420 mutant was found in a gene encoding a protein annotated as a phage tail fiber in *Pfv* UPB0736. This gene was found adjacent to other loci encoding phage related proteins; namely the phage protein U and baseplate assembly protein J (Figure 2). Phage related functions are reported to be present in 25% of the Gram-negative bacterial genomes [49]. These genes encode for protein components that are structurally similar to tailed bacteriophages and are possibly involved in synthesizing a specialized contractile injection system that is known as Type VI secretion system (T6SS). Based on structural similarities, T6SS appeared as an inverted phage tail on the surface of the bacterial cell and it secretes effector proteins into the extracellular milieu or injects them directly into host cells by a puncturing mechanism [50-53]. A common evolutionary history has been

proposed for the two injection machines present in bacteria and bacteriophages [52,54]. In plant associated bacteria, T6SS has been implicated in several functions including tumorigenesis in *Agrobacterium* [55], programmed cell death in the filamentous fungus *Neurospora crassa* by *Pss* B728a [56] and virulence in *Pectobacterium* [57]. Interestingly, *P. fuscovaginae* UPB0736 possesses a T6SS (data not shown). Consequently the Tn5 mutation in phage tail fiber-like protein most likely results in a non functional T6SS secretion machinery for delivery of effector proteins in *Pfv* UPB0736 thus affecting virulence.

Virulence deficient mutant Pfv 445 is most likely affected in phytotoxin production

In this mutant the sequence of the gene inactivated by Tn5 displays 96% identity with the gene of *P. syringae* that encodes an enzyme called syringopeptin synthetase C (*sypC*). In *P. syringae* this gene is 40614 bp long and is part of a gene cluster of 73800 bp that also includes syringopeptin synthetase A (*sypA*) and syringopeptin synthetase B (*sypB*) [58] (Additional file 3). The genetic organization of these loci reveals that several genes flanking this locus are conserved among other *Pseudomonas* cyclic lipopeptides (CLP) biosynthesis clusters, including two genes encoding a putative macrolide transporter MacA and MacB. Genes encoding MacA and MacB have been reported in syringopeptin, syringomycin [59,60] and entolycin biosynthesis gene clusters [61] and they were also found in *Pfv* UPB0736 (data not shown). The three peptide synthetases are responsible for the biosynthesis of non-ribosomal syringopeptin, which represents one of the major virulence factors in *P. syringae* [62]. *Pfv* produces phytotoxins which are similar to syringopeptin and are called fuscopeptins A and B [63]. These lipodepsipeptides show numerous structural and functional characteristics common to syringopeptin isolated from *P. syringae*. The distinguishing feature of the mechanism of action of these lipodepsipeptides is their ability to interact with biological membranes. The amphipathic nature of these molecules allows their insertion into the lipid bilayer, with the consequent formation of ion channels that cause the alteration of the membrane potential and in turn the loss of intracellular material [64]. The leakage from the host cells enriches the intercellular fluid with sucrose, amino acids, inorganic ions and other supplements that could be supporting the bacterial multiplication [63]. These phytotoxins are also able to play a role on disease symptoms by inducing injuries in the host plant. Syringotoxin has also properties of surfactant, fungicidal action and alteration in proton gradient. The antagonistic activity is likely to increase the competitive ability of *Pfv* against other colonizers of leaf surfaces. Although in earlier studies three *Pfv* phytotoxins were characterized biochemically, here we report that a mutant in a vital gene for the biosynthesis of at least one of them results in *Pfv* being significantly less virulent.

Virulence deficient mutant affected in hypothetical proteins (Pfv 90 and Pfv 188)

Pfv 90 mutant had a Tn5 insertion localised in a gene encoding for a hypothetical protein; adjacent to this ORF there is a methylene tetrahydrofolate reductase gene and a gene encoding a periplasmic binding protein (Figure 2). The spacing between the gene encoding the hypothetical protein and the next ORF (periplasmic binding protein) is only 46 bp thus we cannot exclude that the *Pfv* 90 mutant phenotype that could be associated with periplasmic binding gene located downstream in an operonic organization. Mutant *Pfv* 188 on the other hand, had the Tn5 insertion localized in a gene coding for a hypothetical protein that is flanked by a gene coding for a transcriptional regulator possessing a GntR family domain on one side and by a gene encoding a hypothetical protein on the other side. The gene encoding the neighbouring hypothetical protein is 21 bp away from the gene in which Tn5 insertion is localised hence they could be organized in operonic structure. Complementation using cosmid clones resulted in the restoration of virulence for *Pfv* 90 indicating that *Pfv* 90 locus was responsible for causing a virulence deficiency. We did not find any information related to virulence functions for *Pfv* 90 in the literature suggesting that this is a novel gene and is implicated in virulence in *Pfv*.

Conclusions

Despite the importance of *Pfv* as an emerging pathogen worldwide, to date no major studies have been performed to understand the mechanisms of *Pfv* pathogenicity. In 2012 we reported the first genome sequence of a highly virulent strain UPB0736 [34] and since then the genome sequence of another strain has been published [65] and many more genomes will most probably be sequenced in the future. In this study, we sought to identify and characterize some of the genes involved in *Pfv* virulence through an *in planta* screening of 1000 Tn5 mutants; nine mutants that showed virulence deficiency compared to the wild type were identified. The inactivated loci in these mutants include some metabolic functions and also some known virulence associated functions such as type IV pilus biogenesis protein PilZ, T6SS machinery and syringopeptin synthetase. The results of this study highlight the fact that *Pfv* might share features of some of its virulence mechanisms with other phytopathogens. In addition, new loci never reported as being involved in virulence and encoding for hypothetical proteins have been found. Genome mining with future virulence studies will further

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highlight the mechanisms of virulence of this broad host range emerging phytopathogen.

Methods

Bacterial strains, plasmids, and culture media

The bacterial strains used in this work are listed in Table 1. The plasmids used and generated in this study are listed in Additional file 5. *Pseudomonas fuscovaginae* (*Pfv*) strains were grown at 28°C in Luria Bertani (LB)/ King's B (KB) medium, and *E. coli* strains were grown at 37°C in LB medium, as described previously [31]. The concentrations of antibiotics used in this study were as follows: Nitrofurantoin (Nf); 100-150 μg/ml, Ampicillin (Amp); 100 μg/ml, Kanamycin (Km); 50 μg/ml, Tetracyclin (Tc) 30 μg/ml for *Pfv* and Amp; 75 μg/ml, Km; 50 μg/ml, Tc; 15 μg/ml for *E. coli*.

Recombinant DNA techniques

Routine DNA manipulation steps such as digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligations with T4 ligase, radioactive labelling by random priming and transformation of *E. coli* etc. were performed as described previously [66]. Colony hybridizations were performed using N + Hybond membrane (Amersham Biosciences); plasmids were purified using the EuroGold plasmid columns (Euro Clone) or with the alkaline lysis method [67]; total DNA from *Pfv* strains were isolated by Sarkosyl/Pronase lysis as described previously [68]. PCR amplifications were performed using Go-Taq DNA polymerase or pfu DNA polymerase (Promega). The oligonucleotide primers used in this study are listed in Additional file 6. Automated sequencing was performed by Macrogen sequence service (Europe). Triparental matings between *E. coli* and *Pfv* were carried out with the helper strain *E. coli* DH5α (pRK2013) [36].

Generation of Tn*5* mutant library of *Pfv* UPB0736

Tn*5* mutagenesis was performed by using triparental matings between donor *E. coli* (pSUP2021) containing the transposon Tn*5* (Km resistance), a helper *E. coli* strain (pRK2013) and recipient *Pfv* UPB0736 strain. Briefly, *Pfv* UPB0736, donor and helper *E. coli* strains were grown overnight in 20 ml of LB media supplemented with appropriate antibiotics. Cells were pelleted, washed and re-suspended in 10 ml of sterile LB media. Absorbance of all three strains were measured and cells were mixed in the following ratio: recipient *Pfv* UPB0736, 2×10^8 colony forming units (cfu/ml); helper *E. coli*, 4×10^9 cfu/ml; donor *E. coli*, 4×10^9 cfu/ml. The mixture of cells were pelleted out, re-suspended in small volume of LB media and spotted onto Hybond N Plus nylon membrane (Amersham Pharmacia Biotech) that was overlaid on LB agar. Overnight incubated cells grown at 28°C were scraped from the membrane and re-suspended in 1 mL of sterile LB media. The cell suspension (50 μl each) was plated on LB agar plates containing Nf and Km. The plates were incubated at 28°C for 2-3 days to allow the growth of transconjugants (Tn*5* mutants). The *Pfv* Tn*5* mutants were then patched onto LB agar plates with Nf and Km, grown in liquid media and a glycerol stock was prepared and stored at -80°C.

Screening of *Pfv* UPB0736 Tn*5* mutants for virulence deficiency in *Chenopodium quinoa* (*C. quinoa*) and rice plants

One thousand independent Tn*5* mutants of *Pfv* UPB0736 were grown on fresh LB agar plates and inoculated individually in greenhouse-grown 2 week old *C. quinoa* plants. Inoculation was performed using a needle (size; 21 G × 1 ½") by touching the strains on plates and pricking onto twigs of *C. quinoa* plants. After 5 days of inoculations, brown sheath rot disease symptoms were observed and scored on a scale of 0 to 5 as previously reported [31] and shown again here in Additional file 1. *Pfv* Tn*5* mutants with deficiency in virulence compared to wild type were subjected to a second round of screening using two independent plants.

In order to verify the virulence phenotype of selected Tn*5* mutants from the *C. quinoa* screen, mutants were re-inoculated on rice plants which is a real host for this bacterium. Rice plants (cultivar Co-39) were grown in the growth chamber at 28 ± 4°C and approximately 70% relative humidity. Along with wild type *Pfv* UPB0736, the selected Tn*5* mutants were grown for 48 hrs in KB medium. Bacterial cultures were diluted to 10^8 cfu/ml using 0.15 M saline solution and inoculated onto five week-old rice plants using a 1 ml syringe. Inoculation was performed by injecting 100 μl of bacterial culture in the rice plantlet at 5 cm above ground. After inoculation, plantlets were kept in a humid chamber at 20-30°C for disease development. Each bacterial strain was inoculated in rice plantlets in at least 12 replicates. Eight days after inoculation, data for disease severity in lesion lengths (mm) and disease rating score (on a scale from 0 to 5) were collected and analysed for statistical significance using two-tailed, paired '*t*' test with 95% of confidence intervals.

Localization of Tn*5* insertion

Genomic DNA was isolated from selected *Pfv* Tn*5* mutants and double digested either with BamHI + EcoRI, BamHI + SacI or BamHI + ClaI. These double digested products were ligated in pBluescript (double digested with the corresponding set of enzymes), transformed into DH5a *E. coli* cells and selected on LB agar plates with ampicillin (75 μg/ml) + kanamycin (50 μg/ml). These pBluescript clones having the insertion of Tn*5* flanking regions were sequenced using Tn*5* specific Tn*5*-Ext primers

(Additional file 6). Sequences obtained were subjected to homology searches using NCBI Blast and also with the draft genome sequence of *Pfv* UPB0736 using a local blast algorithm. The exact positions of Tn5 insertion were mapped in the *Pfv* UPB0736 draft genome. We also performed arbitrary PCR using pairs of primers mentioned in Additional file 6 as previously described [69]. Arbitrary PCR products cloned in pGEM-T easy were also sequenced using pGEM-T easy vector specific primers.

Confirmation of Tn*5* mutations

The selected Tn*5* mutants were reconstructed by deletion of the wild-type genes via homologous recombination with the use of the pKNOCK-Km suicide delivery system. Briefly, an internal fragment of each Tn*5* insertion locus was amplified by PCR using primers listed in Additional file 6 and cloned in pGEMT-easy vector. EcoRI digested internal fragments were ligated to EcoRI digested pKNOCK-Km and transformed into C118 cells yielding pKNOCK plasmids having internal fragments from selected Tn5 loci. These pKNOCK plasmids were further used as a suicide delivery system and the nine Tn*5* mutants from *Pfv* 80 to *Pfv* 480 mutants were regenerated in the wild-type as previously described [70]. *Pfv* mutant strains were verified by PCR analysis and sequencing.

In order to complement *Pfv* UPB0736 Tn5 mutants, a cosmid library was constructed from the *Pfv* UPB0736 strain by using the cosmid pLAFR3 [71] as a vector. Insert DNA was prepared by partial EcoRI digestion of the genomic DNA and then ligated into the corresponding site in pLAFR3. The ligated DNA was then packaged into λ phage heads using Gigapack III Gold packaging extract (Stratagene) and the phage particles were transduced to *E. coli* HB101 as recommended by the supplier. In order to identify the cosmids containing the genes of interest (90, 420 and 445), the cosmid library was screened using radio-labelled probes for Tn5 insert regions from 90, 420 and 445 in colony hybridization. We obtained respective cosmid clones pCos 90, pCos 420 and pCos 445 in this screen. It is not known whether the cosmids contain the full length genes for 90, 420 and 445 (Additional file 5) and they were introduced in *Pfv* 90, *Pfv* 420 and *Pfv* 445 Tn5 mutants respectively by conjugation. Positive complemented clones were selected on LBA plates supplemented with Nf, Km and Tc.

Arginine auxotrophy assay

Stock solution of arginine-HCl (Sigma Chemicals) was prepared at a concentration of 100 mg/ml in sterile MQ water and filter sterilized. M9 agar plates with 0.2% glucose and with and without arginine (100 μg/ml) were prepared. The *Pfv* wild type strain, the two arginine biosynthesis defective Tn5 mutants *Pfv* 169 and *Pfv* 270, their respective re-created knock-out mutants and one other Tn5 mutant *Pfv* 102 as a control were streaked onto each of these plates and incubated at 30°C for 24-48 hrs.

Virulence assay in rice by pin prick inoculation

Pfv in planta virulence assay was performed on rice plants by pin pricking method with some modifications as described previously [31]. Briefly, the seeds of a susceptible rice variety (Baldo) were surface sterilized using washes with hypochloride and sterile MQ water. Surface sterilized rice seeds were germinated on a sterile filter paper in a petriplate in the dark at 30°C. Rice seedlings of 5 to 6 cm growth stages were planted in 50 ml falcon tubes containing Hoaglands solutions with 0.5% agar. The transplanted rice plants were maintained in a climate controlled room with conditions set to 70% humidity, 16/8 hours light/dark and temperature at 28°C and watered regularly. For inoculation, *Pfv* strains were grown for 24 hrs on LBA medium supplemented with appropriate antibiotics, at 30°C. Bacterial cultures were pelleted down washed with sterile MQ water and adjusted to approximately 10^6 cfu/ml in sterile MQ water. Four to five week old rice plants were pin prick inoculated using a sterile board pin by dipping in the bacterial inoculum. For each strain, 10 plants were inoculated in two different sites each and control plants were inoculated in the similar manner using sterile MQ water. In order to determine *in planta* virulence of *Pfv* strains, disease severity was assessed on the 10th day post inoculation by measuring the browning lesion length neighboring to the zone of inoculation and also by assessing their disease rating score (scale of 0 to 5). Virulence score with average and standard deviations are presented. The statistical significance was performed using two-tailed, paired '*t*' test with 95% of confidence intervals.

Additional files

Additional file 1: Virulence score of *Pfv* strains in *Chenopodium quinoa*. Severity scale used to evaluate disease caused by *Pfv* infection on *Chenopodium quinoa*: severity score 0, No symptoms, severity score 1 Necrosis on less than 2 mm around the puncture, severity score 2, Necrosis on 2 to 10 mm around the puncture, severity score 3, Necrosis on 2 to 10 mm around the puncture and bending of petiole, severity score 4; Collapse of the petiole and severity score 5; Wilting of the leaf.

Additional file 2: Virulence score of *Pfv* strains in rice. Illustration of the rating scores used in evaluating the severity of sheath rot lesions on rice inoculated with bacterial strains. A: severity score 0; No symptoms only the sign of the injection puncture, B: severity score 1; Necrosis around the puncture till 1 cm, C: severity score 2; Necrosis around the puncture and chlorosis from 1 to 2 cm, D: severity score 3; Necrosis around the puncture from 2 to 3 cm, E: severity score 4; Necrosis around the puncture from 3 to 5 cm and F: severity score 5; Necrosis around the puncture from 5 cm and above.

Additional file 3: Syringopeptine operon in *Pss* B728a.

Additional file 4: Arginine auxotrophy. The wild type, two arginine biosynthesis defective Tn5 mutants *Pfv* 169 and *Pfv* 270, their respective

knock-out mutants and one other Tn*5* mutant *Pfv* 100 as a control were streaked onto A: LB agar plate, B: M9 plate with 2% CAS amino acid, C: M9 plate with 2% glucose and D. M9 plate with 2% glucose and 25 μg/ml of arginine-HCl. (+) and (-) indicates growth and no growth respectively.

Additional file 5: List of plasmids used in this study.

Additional file 6: List of primers used in this study.

Competing interests

The authors declare that they have no competing interest.

Author's contributions

HKP, MH and VV designed the experiments. HKP, MM, IB, VB and GJA performed the experiments. HKP, MH and VV drafted the manuscript. All authors read and approved the final manuscript.

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ANNEX 2: CONTRIBUTION TO THE ESTABLISHMENT OF THE LIST OF PESTS OF PHYTOSANITARY IMPORTANCE ON RICE IN RWANDA BY PRA METHODOLOGY AND FIELD SURVEY

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Abstract

Rice (*Oryza sativa*) is one of the priority crops in Rwanda. Efforts are being put in improving rice production and productivity. Also the Rwandan agriculture sector is in the transition from subsistence farming to market-oriented agriculture. Its mission is to transform and modernise agriculture and livestock to ensure food security and contribute to the national economy. One of the requirements to achieve this objective is efficiently organising the plant protection aspects in production and after harvest. Continuous inspection and surveillance by rice professionals are of utmost importance for meeting this requirement. Rice is attacked by many pests and diseases in the many agroecological conditions where it is grown in the world. Rwanda has in general a tropical climate tempered by average high altitude. There is not sufficient information on pests and diseases that can attack rice in conditions of high altitude and low temperature, like those found in Rwanda. This initiative aims at developing a list of pests of phytosanitary importance in Rwanda which can guide rice professionals dealing with plant protection and phytosanitary issues especially in developing the phytosanitary regulation in compliance with the requirements of international trade in accordance with the World Trade Organisation in the agreement on the application of Sanitary and Phytosanitary Measures (WTO-SPS Agreement).

The list of rice pests and diseases is based on published information available in professional and scientific databases, particularly from the Crop Protection Compendium of CABI and also from field surveys. The status of each individual pest or disease, considering the agro ecology of Rwanda, is established based on the Pest Risk Analysis (PRA) methodology. For each pest, the following aspects are covered: taxonomic group, presence or absence in Rwanda, caused losses, likelihood of entry, proliferation and establishment in Rwanda, appreciation of the risk represented expressed in whether it must be included in the list of pests of phytosanitary importance or not.

A final list of pests of phytosanitary importance for rice in Rwanda is suggested. It is composed of 188 organisms from all the groups of pests (fungi, bacteria, viruses, nematodes, arthropods, birds, weeds and rodents) that have been found to meet the phytosanitary importance criteria as specified in the International Standards for Phytosanitary Measures (ISPMs) developed for implementing the International Plant Protection Convention (IPPC) to which Rwanda is signatory.

Introduction

The economy of Rwanda is based on agriculture. As of 2011 this sector employs 80% of the total active population and contributes to the GDP at 31%. The agricultural policy aims to increase the productivity so that the sector can ensure food security and release excess for the market. Agriculture is in transition from subsistence farming to market-oriented agriculture. It is estimated that the agricultural sector will have the greatest impact on the economy in terms of poverty reduction and wealth creation, as it is the largest employer (Bizimana et al., 2012).

Rice is one of the important crops in Rwanda. Although its introduction is recent (Baker, 1970), in 2012, the crop is grown on 12000 ha, the total national production is estimated at 80000 tons and the yield per hectare is 5.5 tons (RAB, 2013), which is high in a developing country context and compared to some countries with a long tradition of rice growing (Singh et al., 2010). Rice is the most marketed crop in Rwanda, with 47% of its production share being sold on markets (NISR, 2012). In the same time, the popularity of rice has continuously been increasing, and the national production cannot meet the demand. The country is now importing more than 40% of the consumed rice (RAB, 2013).

There are many initiatives aiming at increasing rice production in the country by finding solutions to the major constraints. Rice production faces some constraints among which the pressure of pests and diseases is one of the most important (RAB, 2014). The climate of Rwanda, characterized by low temperatures, compared to other tropical and equatorial countries (Sikkens and Steenhuis, 1988) and an elevated topography, is conducive to the development of many pests, as those of tropical and of subtropical areas can be established. Given that the breeding and seed sector in Rwanda is in its development, considering large quantities of rice are imported in the country for consumption or as seed, but also as Rwanda is intending to become a rice exporter in the coming years (MINAGRI, 2011a), legal plant protection measures are perceived as an efficient way that can help crop protection and production professionals in dealing with rice health aspects. The International Plant Protection Convention (IPPC) is the international legal instrument for plant health related issues (Baker et al., 2005). In fact, the IPPC and its related International Standards for Phytosanitary Measures (ISPMs) aim to protect plant health at international level through trade but also at regional and national levels in guiding in the development of diagnostic and surveillance methods meeting international standards (Petter et al., 2008). Thus it is capital to define the phytosanitary importance that must be given to each pest

known to attack rice. A precautionary approach, technically justified based on scientific principles and evidence as explained by Griffin (2000), helps to decide whether the considered pest qualify as a pest of phytosanitary importance or not.

A list of pests of phytosanitary importance on rice in Rwanda has been prepared, which can help Rwanda to conduct PRA and manage rice protection issues according to the principles of the IPPC and the WTO-SPS agreement, Rwanda being signatory of both of these conventions.

Methodology

In the proposed list of pests of phytosanitary importance for Rwanda, a pest is defined as, according to the International Plant Protection Convention (FAO, 1997), "any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products". The pest risk analysis (PRA) is followed for determining the importance of each individual pest and is defined as "the process of evaluating biological and other scientific and economic evidence to determine whether a pest should be regulated and the strength of any phytosanitary measures to be taken against it" (FAO, 1997).

A list of the pests reported on rice has been produced, based on the rice pest in the Commonwealth Agricultural Bureaux database (CABI, 2007) and also from the list of pests in the surveillance report of the Rwanda Ministry of Agriculture and Animal Resources (MINAGRI, 2013b). The MINAGRI conducts routine pest surveillance activities. For rice, the survey covered the areas of Rwamagana, Nyagatare, Cyili, Bugarama and Runda. It consisted in looking at diseased plants and conducting a diagnosis based on pest attack symptom pictures and information in technical literature and manuals.

The Pest Risk Analysis (PRA) was conducted on the pests present on the list. Pests were analysed on the criteria: taxonomic group, presence or absence in Rwanda, level caused losses, likelihood of entry, proliferation and establishment in Rwanda, appreciation of the risk represented expressed in whether it must be included in the list of pests of phytosanitary importance or not. Are included in the list the pests considered as important and which are absent in Rwanda and exceptionally those that are present in Rwanda but which would qualify as regulated non-quarantine pests, the latter expression meaning "a non-quarantine pest whose presence in plants for planting affects the intended use of those plants with an economically unacceptable impact and which is therefore regulated within the territory of the importing contracting party" (FAO, 1997).

Results: Establishing the rice pest list for Rwanda

From the 455 pests analysed, 188 from all the groups (fungi, bacteria, viruses, nematodes, arthropods, birds, weeds and rodents) qualify as pests of phytosanitary importance and are included in the list (Table A2-1).

Table A2-1: List of pests of phytosanitary importance on rice in Rwanda

Pest group	***Pests of phytosanitary importance***
Bacteria	*Acidovorax avenae subsp.* avenae (bacterial leaf blight), *Xanthomonas oryzae pv. oryzae (rice leaf blight), Pseudomonas fuscovaginae* (sheath brown rot), *Pectobacterium carotovorum subsp. carotovorum)* (bacterial root rot of sweet potato)*, Xanthomonas oryzae pv. oryzicola* (bacterial leaf streak of rice), *Pseudomonas syringae pv. syringae* (bacterial canker or blast (stone and pome fruits)), *Burkholderia glumae* (bacterial grain rot).
Birds	*Ploceus cucullatus* (rice weaver bird).
Fungi	*Polymyxa graminis* (vector of streak mosaic: wheat), *Athelia rolfsii* (sclerotium rot), *Cochliobolus miyabeanus* (brown leaf spot of rice), *Sarocladium oryzae* (rice sheath rot), *Cochliobolus sativus* (root and foot rot), *Magnaporthe salvinii (stem rot), Sclerophthora macrospora* (downy mildew), *Cochliobolus lunatus* (head mould of grasses, rice and sorghum)*, Pythium arrhenomanes* (cereals root rot), *Magnaporthe oryzae* (rice blast disease)*, Gibberella fujikuroi var. fujikuroi* (bakanae disease or rice), *Trichoconiella padwickii* (stackburn disease), *Macrophomina phaseolina* (charcoal rot of bean/tobacco*), Sphaerulina oryzina* (narrow brown leaf spot), *Pythium graminicola* (seedling blight of grasses).
Insects	*Trogoderma granarium* (khapra beetle)*, Chlorops oryzae* (rice stem maggot), *Laodelphax striatellus* (small brown planthopper), *Rhyzopertha dominica* (lesser grain borer), *Sitotroga cerealella (grain moth), Oxya chinensis* (rice grasshopper), *Chilo auricilius* (gold-fringed rice borer)*, Nezara viridula* (green stink bug)*, Brevennia rehi* (rice mealybug), *Atta* (leaf-cutter ant), *Dicladispa armigera* (rice hispa*), Nilaparvata lugens* (brown planthopper), *Oebalus pugnax* (rice stinkbug), *Thrips palmi* (melon thrips), *Orseolia oryzae* (rice stem gall midge), *Orseolia oryzivora* (African rice gall midge)*, Lissorhoptrus oryzophilus* (rice water weevil), *Mythimna separata* (paddy armyworm), *Holotrichia serrata* (white grub*), Spodoptera frugiperda* (fall armyworm), *Dimorphopterus, Spodoptera exempta* (black armyworm), *Macrosteles quadrilineatus* (aster leafhopper)*, Earias insulana* (Egyptian stem borer*), Atherigona oryzae* (rice shoot fly), *Sitophilus oryzae* (lesser grain weevil),

Pest group	***Pests of phytosanitary importance***
	Haplothrips culeatus (grass thrips), *Leptocorisa acuta* (rice seed bug), *Sesamia cretica* (greater sugarcane *borer), Parapoynx stagnalis* (rice case bearer), *Agrotis segetum* (turnip moth), *Chilo sacchariphagus* (spotted borer), *Tagosodes orizicolus* (rice delphacid), *Hypomeces squamosus* (green weevil), *Carpophilus dimidiatus* (corn-sap beetle), *Nephotettix cincticeps* (rice green leafhopper), *Spodoptera littoralis* (cotton leafworm), *Liposcelis entomophila* (grain psocid), *Liposcelis paeta* (grain psocid), *Trogoderma variabile* (grain dermestid), *Locusta migratoria* (migratory locust*), Nephotettix virescens* (green paddy leafhopper), *Tetraneura nigriabdominalis* (rice root aphid), *Sesamia inferens* (purple stem borer), *Chondracris rosea* (citrus locust), *Blissus leucopterus* (chinch bug), *Cadra cautella* (dried currant moth), *Conocephalus* cinereus (longhorned green pasture grasshopper), *Pelopidas mathias* (rice skipper), *Sesamia calamistis* (African pink stem borer), *Sesamia nonagrioides* (Mediterranean corn stalk borer), *Anomala pallida, Marasmia trapezalis* (maize webworm), *Mythimna loreyi* (maize caterpillar), *Nomadacris septemfasciata* (red locust), *Plodia interpunctella* (Indian meal moth), *Cryptolestes pusillus* (flat grain beetle), *Atta sexdens* (leaf cutting ant*), Diopsis longicornis* (stalk-eyed fly), *Stegobium paniceum* (drugstore beetle), *Schizaphis graminum* (spring green aphid), *Sogatella furcifera* (white-backed planthopper), *Recilia dorsalis (z*igzag leafhopper).
Mites	*Petrobia latens* (brown wheat mite).
Mollusks	*Pomacea canaliculata* (golden apple snail).
Nematodes	*Pratylenchus brachyurus* (root-lesion nematode), *Aphelenchoides besseyi* (rice leaf nematode), *Pratylenchus penetrans* (nematode, northern root lesion*), Criconemella (ring nematode), Xiphinema americanum* (dagger nematode), *Heterodera sacchari* (sugarcane cyst nematode), *Heterodera oryzicola* (rice cyst nematode), *Helicotylenchus dihystera* (common spiral nematode), *Ditylenchus angustus* (rice stem nematode), *Meloidogyne exigua* (coffee root-knot nematode*), Pratylenchus zeae* (root lesion nematode), *Hirschmanniella oryzae* (rice root nematode*), Hoplolaimus seinhorsti* (lance nematode), *Meloidogyne graminicola* (rice root knot nematode), *Meloidogyne arenaria* (peanut root-knot nematode), *Meloidogyne incognita* (root-knot nematode).
Rodents	*Rattus argentiventer* (rice field rat).

Pest group	***Pests of phytosanitary importance***
Virus	Rice yellow mottle virus, Rice grassy stunt virus, Rice transitory yellowing virus (rice yellow stunt virus), Rice dwarf virus (dwarf disease of rice), Rice tungro bacilliform virus (rice tungro), Rice gall dwarf virus (rice gall dwarf), Rice hoja blanca virus (white leaf disease of rice), Rice ragged stunt virus (rice ragged stunt), Rice stripe virus (rice stripe tenuivirus), Rice tungro spherical virus.
Weeds	*Cynodon dactylon* (Bermuda grass)*, Galinsoga parviflora* (gallant soldier), *Cyperus rotundus* (purple nutsedge), *Echinochloa colona* (junglerice*), Scirpus maritimus* (saltmarsh bulrush), *Paspalum distichum* (knotgrass), *Oryza rufipogon* (wild rice), *Marsilea minuta* (pepperwort), *Eichhornia crassipes* (water hyacinth), *Leersia hexandra* (southern cut grass), *Paspalum conjugatum* (sour paspalum)*, Fimbristylis littoralis* (lesser fimbristylis), *Urochloa plantaginea* (marmeladegrass), *Acanthospermum hispidum* (bristly starbur), *Avena fatua* (wild oat), *Chenopodium album* (fat hen), *Oryza longistaminata* (perennial wild rice), *Polygonum lapathifolium* (pale persicaria), *Monochoria vaginalis* (pickerel weed), *Mimosa pudica* (sensitive plant), *Melochia corchorifolia* (redweed), *Polygonum barbatum* (knot grass), *Conyza canadensis* (Canadian fleabane), *Oxalis latifolia* (sorrel), *Panicum repens* (torpedo grass), *Trianthema portulacastrum* (horse purslane), *Alternanthera philoxeroides (alligator weed), Leptochloa chinensis* (Chinese sprangletop), *Dactyloctenium aegyptium* (crowfoot grass), *Eleusine indica* (goose grass), *Commelina benghalensis* (wandering jew), *Polygonum aviculare* (prostrate knotweed), *Chloris barbata* (purpletop chloris), *Echinochloa crus-galli* (barnyard grass), *Amaranthus retroflexus* (redroot pigweed), *Myriophyllum spicatum* (spiked *watermilfoil), Ceratophyllum demersum* (coontail)*, Commelina diffusa* (spreading dayflower)*, Heliotropium indicum* (Indian heliotrope), *Synedrella nodiflora* (synedrella), *Cirsium vulgare* (spear thistle), *Cenchrus echinatus* (southern sandbur), *Cyperus compressus* (annual *sedge), Polygonum hydropiper* (marsh pepper), *Richardia brasiliensis* (white-eye (Australia)), *Celosia argentea* (celosia)*, Setaria verticillata* (bristly foxtail), *Striga angustifolia* (witchweed), *Corchorus aestuans* (east Indian jew's-mallow (USA)), *Phragmites australis (common reed), Rubus ellipticus* (yellow Himalayan raspberry), *Fimbristylis dichotoma* (tall fringe rush)*, Striga aspera* (witchweed), *Ipomoea aquatica* (swamp morning-glory), *Paspalum urvillei* (Vasey grass), *Hydrilla verticillata* (hydrilla), *Mimosa pigra* (catclaw mimosa), *Cyperus esculentus* (yellow nutsedge), *Digitaria ciliaris* (southern crabgrass), *Aeschynomene indica*

Pest group	*Pests of phytosanitary importance*
	(Indian jointvetch), *Lantana camara (lantana), Ludwigia adscendens* (water primrose), *Ludwigia hyssopifolia* (water primrose), *Ludwigia octovalvis* (primrose willow), *Oxalis corniculata* (creeping woodsorrel (USA)), *Murdannia nudiflora* (doveweed), *Myriophyllum aquaticum* (parrot-feather), *Pistia stratiotes* (water lettuce), *Lemna perpusilla (*duckweed), *Sida acuta* (sida), *Amaranthus spinosus* (spiny amaranth).

Discussion and perspectives

Movements of people and goods keep increasing. This increases the risk of endangering plants and the environment by introducing new pests. In a country with an agriculture-based economy like Rwanda, the introduction of new pests can have disastrous consequences. The global community has established principles for international cooperation in strengthening trade but avoiding the negative impact that would result from the proliferation of pests in regions that were free of them. These principles are expressed in the IPPC and the ISPMs (Petter et al., 2008).

Rwanda aims to apply the principles of IPPC, which is the international legal tool for plant health matters. A list of important pests is a basic tool, from which decisions will be made, based on a PRA approach, whenever there is a need for analyzing seeds, plant consignments, etc. (FAO, 2011). There is a shortage of information about pest prevalence in Rwanda. This work suggests a list of pests of phytosanitary importance for Rwanda. This pest list is intended to serve as a tool for guiding different actors managing rice protection aspects. It is based on the information that is available in the references databases and from field data. It is subject to regular updates when new and more accurate information is found through research, inspection and surveillance. From this list it is possible to strengthen pest-related information reporting. This aspect is critical in a market-oriented agriculture, especially now that there are reports of many cases of rejection of consignments for non-compliance with standards, a reality frequently faced by agriculture in many developing countries (Otieno and Kigamwa, 2011).

Based on this list other more functional lists could be made, for example when defining the list of quarantine pests, areas of low pest prevalence, list of regulated pests, etc. This list will also guide in the daily PRA analysis and will provide the guidance about pests to be checked when issuing or receiving a phytosanitary certificate.

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SUMMARY

Rwanda is a developing country located in Eastern/Central Africa whose economy is based on agriculture. Rice is one of the priority crops in Rwanda. Rice is becoming a popular crop, is supported by the agricultural policy and it has good performance. It was tested in Rwanda in the 1950s but its production at large scale is recent. The current rice demand is significantly exceeding the national production and so a large part of the rice consumed in the country, estimated at around 40%, is imported. One of the factors contributing in limiting rice production is the impact of pests and diseases. There are not many studies on pests and diseases attacking rice in Rwanda. Some of the symptoms of rice sheath rot, an emerging rice disease, like the appearance of extending gray lesions and the non-emergence of infected sheaths, were found in Rwanda and there is a problem of quality in the locally produced rice, expressed for instance by the browning of the grains. The aim of this study was to study the situation of rice sheath rot in Rwanda on the aspects of importance and the causal agents. Bacteria and fungi, were isolated from the major rice-growing areas, identified, tested for pathogenicity and some management options are discussed.

Rice sheath rot as a disease attacking rice is known for more than a century, is ubiquitous and highly destructive. Rice sheath rot was first described in Taiwan. Its symptoms consist of the rotting of the sheath, associated with browning of the grains, which become largely sterile when harvested from a diseased plant. In addition, the germinated seeds can transmit the disease. Second, the disease was reported in most other rice-producing countries all over the world. Third, the disease is destructive, with yield losses varying from 20-85%, depending on the causal agent, used rice cultivars, production system, agro-climatic conditions, etc. Most studies cited *Sarocladium oryzae*, *Fusarium spp*. and *Pseudomonas fuscovaginae* as causal agents, but many more organisms induce sheath rot disease symptoms in various parts of the world where rice is grown. A review on the sheath rot reported agents is presented in this work in the literature chapter. The relationships between the various sheath rot causal agents are not yet known.

The symptoms of rice sheath rot were detected in Rwanda, a country where rice is promoted as a food security and cash crop. The importance of rice sheath rot as a disease was assessed for incidence and severity. Data were taken in 2013-2014, from the major rice-growing areas: Bugarama, Cyili, Rwamagana, Nyagatare and Rwagitima. The disease incidence was 13.5% and

the average measure for disease severity (lesion length on a diseased tiller) was 157 mm. There are variations on the importance of the disease per area, Rwamagana being more affected by the disease in the considered period. As for the yield loss, calculated by comparing weight loss in diseased and healthy grains, it was estimated at 57%. There is an additional loss caused by the poor quality of grains harvested on diseased plants. Rice sheath rot is causing sizeable losses to rice production in Rwanda.

Fungi were isolated from sheath rot diseased rice plants from Rwanda, identified and characterised for pathogenicity on rice plants. The isolated fungi were purified and identified through morphological and molecular methods, by sequencing the ITS and the TEF genomic regions. The species identified are *Sarocladium oryzae* and *Fusarium* spp. *Sarocladium* isolates were similar while there was a large variability in the *Fusarium* isolates, most of them being comparable to *Fusarium andiyazi* and some others being related to species complexes like *F. oxysporum*, *F. incarnatum/equiseti* and *F. graminearum*. The identified isolates were tested for pathogenicity, induced sheath rot symptoms and the caused symptoms were comparable for the two groups. There were differences in susceptibility to the disease between two tested rice cultivars, Nipponbare cultivar of the *Japonica* type being more attacked than CO39 cultivar of the *Indica* type. In Rwanda, it is mainly the cultivars of the japonica types that are grown and which perform well, probably because Rwanda, although the country is located in the tropics, its climate is tempered by the altitude, making it difficult for varieties of the *Indica* type to adapt to low temperatures. The pathogenicity of *Sarocladium* is thought to be linked to toxins, cerulenin and helvolic acid while the pathogenicity of *Fusarium* is attributed to mycotoxins, among which there are fumonisins. Fumonisins were found and measured in most *Fusarium* isolates from Rwanda. Their concentration was not correlated with the disease intensity, though this has to be confirmed by further extensive studies.

Three major genera of bacterial strains were isolated from sheath rot diseased rice plants, *Pseudomonas*, *Stenotrophomonas* and *Delftia*. *Stenotrophomonas* and *Delftia* are commonly associated with the rice plants as endophytes. More emphasis was put on further identification of the *Pseudomonas* isolates by sequencing the *rpoB* and genes. *P. fuscovaginae*, the most reported rice sheath brown rot agent, was not found in the identified Rwandan isolates. Intriguingly, many of the isolates were related to well-known rhizosphere potential biocontrol agents that are not commonly associated with the phyllosphere. These isolates proved to be mildly pathogenic in

artificial inoculations. Isolates from Rwanda were found to be related to the sheath rot associated isolates from the Philippines. But the lack of a 100 % similarity leads to the thinking that most of the isolates from Rwanda may be new species.

An integrated control seems the option that can work for preventing rice sheath rot. In fact the disease is caused by many agents, some of them being unknown. Moreover, even for the known pathogens, pathogenicity mechanisms are not clearly understood. In addition, the interactions between the various pathogenic agents are not understood. It proves then difficult to apply specific control measures; thus it is necessary to develop an integrated control strategy. In the case of Rwanda, a list of potential rice pests, considering the Rwandan agroecology is suggested in Annex A2. It can be updated as more scientific information become available, and can guide in designing integrated control options, adapted to the farming system in use in Rwanda.

This work, based on the knowledge on rice sheath rot in the world and specific data from Rwanda, has paved the way for further studies on rice sheath rot, one of the poorly studied yet important rice pathogen worldwide. Now that rice has become the first staple food worldwide, producing one-fifth of calorie consumption and that its production is expanding, rice sheath rot must be an important element to consider in rice development initiatives, for avoiding important and unnecessary yield losses, which can reach up to 85% of the production, in the world where it is required to increase the production for feeding an ever-increasing world population with limited and degrading natural resources, particularly in tropical and developing countries where most of rice production is conducted. The efforts on rice sheath rot should concentrate on furthering the knowledge of causal agents and the interactions among themselves and with the plant, and understanding the pathogenicity mechanisms, so us to guide the breeding for resistance and the development of sound integrated control options.

SAMENVATTING

Rwanda is een ontwikkelingsland in Oost/Centraal Afrika. De economie is er hoofdzakelijk gebaseerd op landbouw met rijst als een van de belangrijkste gewassen. De rijstproductie kent een stijgende trend, en hoewel het gewas reeds vijftig jaar geleden voor het eerst geteeld werd in Rwanda, is de grootschalige productie ervan slechts een recent verschijnsel. Het landbouwbeleid van Rwanda promoot rijst als een markt gewas ter garantie van de voedselveiligheid. Dit heeft als gevolg dat rijst een sterke stijging in populariteit kent. De vraag naar rijst neemt echter sterker toe dan de productie zelf, met als gevolg dat tot 40% van de geconsumeerde rijst geïmporteerd wordt. De belangrijkste limiterende factoren van de rijstproductie zijn ziekten en plagen, waaronder *Sheath rot*. Een opkomende, maar weinig bestudeerde ziekte in rijst is het sheath rot complex. Sheath rot, oftewel bladschederot, wordt onder meer gekenmerkt door grijsbruine laesies op de bladschede en bruine rijstkorrels. Deze typische symptomen werden geobserveerd in verscheidene rijstvelden in Rwanda. Bovendien is de kwaliteit van lokaal geproduceerde rijst laag ten gevolge van onder meer verkleuring van de granen. Deze studie beoogde bijgevolg het belang van sheath rot in Rwanda te onderzoeken en na te gaan welke pathogenen aan de basis van deze ziekte liggen. Hiertoe werden stalen verzameld uit de belangrijkste rijst producerende regio's in Rwanda waarna de schimmels en bacteriën werden geïsoleerd uit de laesies. Daarnaast werden ook mogelijke controlemethoden behandeld.

Reeds een eeuw geleden werd sheath rot voor het eerst beschreven in Taiwan. Sindsdien heeft de ziekte zich wereldwijd verspreid en zorgt voor productieverliezen van 20-85%. Deze laatste variëren afhankelijk van de ziekteverwekker, de rijstcultivar, het productiesysteem, de agro-klimatologische condities, etc. In de literatuur wordt deze ziekte geassocieerd met voornamelijk *Sarocladium oryzae*, *Fusarium* spp. en *Pseudomonas fuscovaginae*. Al deze pathogenen veroorzaken gelijkaardige symptomen namelijk necrotische laesies op de bladschede waarbij de ontluikende bloeiwijze verkleurt of helemaal wegrot. De geproduceerde zaden zijn steriel, verhard en bruin en bovendien is de ziekte zaad overdraagbaar. Naast deze drie voornaamste pathogenen, kunnen echter nog verscheidene andere pathogenen sheath rot veroorzaken in rijst producerende regio's over heel de wereld. In het literatuur hoofdstuk wordt een overzicht gegeven van de verscheidene sheath rot pathogenen. Het is echter niet duidelijk in welke mate deze pathogenen samen kunnen voorkomen en onderling interageren.

Om het belang van sheath rot in Rwanda te onderzoeken, werden de incidentie en de ziektegraad bepaald. De data werden verzameld in 2013-2014 in de belangrijkste rijst producerende regio's van Rwanda, namelijk Bugarama, Cyili, Rwamagana, Nyagatare en Rwagitima. De incidentie was 13.5% en de gemiddelde ziektegraad (laesielengte) was 157mm. Het belang van de ziekte varieerde naargelang de regio waarbij Rwamagana de sterkst getroffen regio bleek te zijn. De productieverliezen werden bepaald door het gewichtsverschil tussen zieke en gezonde granen. Deze werden geschat op 57%. Daarnaast zijn er echter nog additionele productieverliezen door de daling in de kwaliteit van de rijst met als gevolg dat sheath rot voor aanzienlijke productieverliezen zorgt in Rwanda.

De schimmels die geïsoleerd werden uit planten met sheath rot, werden geïdentificeerd en getest op pathogeniciteit op rijst planten. Met zowel morfologische als moleculaire methoden, namelijk door het sequeneren van de genetische regio's ITS en TEF, werden de schimmels geïdentificeerd als *Sarocladium oryzae* en *Fusarium* spp. De *Sarocladium* isolaten waren genetisch sterk verwant, terwijl de *Fusarium* isolaten een grotere variatie vertoonden. Het merendeel van de *Fusarium* isolaten was nauw verwant met *Fusarium andiyazi*. Daarnaast werden isolaten verwant met *Fusarium* complexen zoals *F. oxysporum*, *F. incarnatum/equiseti* en *F. graminearum* geïsoleerd. De isolaten werden vervolgens getest op pathogeniciteit bij twee rijstcultivars, namelijk de Nipponbare cultivar van het Japonica type en de CO39 cultivar van het indica type. Hieruit bleek dat beide groepen isolaten gelijkaardige symptomen induceerden en dat Nipponbare een grotere gevoeligheid vertoonde dan CO39. Het zijn echter vooral cultivars van het Japonica type die in Rwanda geteeld worden daar deze beter aangepast zijn aan het het gematigde klimaat dat in Rwanda heerst. Hoewel het land zich in de tropen bevindt, heerst een gematigd klimaat ten gevolge van de hoogteligging. De lage temperaturen maken Rwanda ongeschikt voor het kweken van cultivars van het Indica type. De pathogeniteit van *Sarocladium* is te wijten aan de toxines cerulenin en helvolic acid. *Fusarium* spp. daarentegen produceren verscheidenen mycotoxines waaronder fumonisines. Voor merendeel van de *Fusarium* isolaten konden fumonisines gedetecteerd worden. Uit kwantificatie van de geproduceerde fumonisine gehaltes *in planta* bleek dat de concentratie fumonisines niet gecorreleerd was met de ziektegraad. Om dit te bevestigen dienen verdere studies plaats te vinden.

De bacterie isolaten die geïsoleerd werden uit sheath rot laesies behoorden tot de genera *Pseudomonas*, *Stenotrophomonas* en *Delftia*. *Stenotrophomonas* en *Delftia* zijn typische endofyten van rijstplanten. Daarom werd in deze studie de nadruk gelegd op de identificatie van

de Pseudomonas isolaten door middel van sequenering van de en *rpoB* genen. Hieruit bleek dat *P. fuscovaginae*, de meest beschreven sheath brown rot pathogeen, niet geïsoleerd werd uit de sheath rot planten. Het merendeel van de *Pseudomonas* isolaten bleek daarentegen nauw verwant te zijn met bekende biocontrole stammen uit de rhizosfeer. Opmerkelijk is dat, hoewel deze isolaten werden opgezuiverd uit de bladschede, deze zelden geassocieerd zijn met de fyllosfeer. Ook deze isolaten werden getest voor pathogeniciteit door middel van artificiële inoculaties en bleken slechts mild pathogeen te zijn. De isolaten uit Rwanda bleken verwant te zijn met *Pseudomonas* isolaten uit sheath rot planten in de Filippijnen. Omdat er echter geen 100% similariteit is met gekende isolaten, zijn de isolaten uit Rwanda vermoedelijk nieuwe soorten.

Omdat de sheath rot veroorzaakt wordt door verscheidene pathogenen waarvan het mechanisme van pathogeniteit niet gekend is en bovendien niet alle sheath rot pathogenen al gekend zijn, is het zeer moeilijk om specifieke controlemethoden toe te passen. Daarom lijkt een geïntegreerde controlemethode de enige optie om sheath rot te voorkomen. Voor Rwanda werd een lijst opgesteld met de potentiële ziekten en plagen waarbij de agro-ecologie van Rwanda in rekening wordt gebracht. Deze lijst kan dan aangevuld worden wanneer nieuwe informatie beschikbaar wordt. Tevens kan deze lijst als leidraad dienen bij de ontwikkeling van nieuwe geïntegreerde controlemethoden, aangepast aan de gebruikte landbouwmethoden in Rwanda.

Dit doctoraatsproefschrift, dat de reeds gekende informatie over sheath rot wereldwijd combineert met specifieke verworven data uit Rwanda, maakt een synthese van de destructieve doch weinig onderzochte ziekte sheath rot. Deze synthese vormt de basis voor verdere studies over deze ziekte. Het belang van sheath rot wordt voorspeld nog toe te nemen daar, hoewel rijst reeds de belangrijkste voedselbron ter wereld is en 20% van de calorieconsumptie voorziet, de rijstconsumptie nog steeds een stijgende trend kent. Om productieverliezen tot 85% te voorkomen, is het daarom belangrijk om deze destructieve spreidende ziekte in rekening te brengen bij ontwikkelingsinitiatieven in de rijstteelt. Het merendeel van de rijstproductie vindt immers plaats in ontwikkelingslanden, waar een stijging in de rijstproductie vereist is. Opdat gescreend kan worden naar resistente cultivars en doeltreffende geïntegreerde controlemethoden kunnen ontwikkeld worden, is het van groot belang dat verdere studies zich concentreren op de identificatie en karakterisatie van de sheath rot pathogenen. Daarnaast dienen hun pathogeniteitsmechanismen, hun onderlinge interactie en deze met de gastheerplant onderzocht te worden

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CURRICULUM VITAE

I. IDENTIFICATION

Names:	**Vincent de Paul BIGIRIMANA**
Birth date:	01 July, 1979
Birth place:	Nemba-Gakenke, RWANDA
Nationality:	Rwandese
Address:	Ghent University, Faculty of Bioscience Engineering, Department of Crop Protection, Laboratory of Phytopathology, Coupure Links 653, B-9000 GHENT, BELGIUM ***Or*** C/O University of Rwanda, CAVM-SARDAE, Crop Science Department, P.O. Box 210 MUSANZE, RWANDA
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II. EDUCATION

- *November 2010-Now*: Doctoral (PhD) studies in Applied Biological Sciences in the Laboratory of Phytopathology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Belgium.
- *November 2010-Now*: Preparation of the *Doctoral Training Certificate*/Ghent University. Attended courses and activities organised by the Doctoral Schools of Ghent University: Statistics/Statistical Inference, Statistics/Analysis of Variance, Writer Development Course, Opportunity Recognition Workshop, *From PhD to job market* workshop, Advanced Academic English/English for presentation, Quality research skills, Transversal skills (Communication and presentation techniques, Contracts, Intellectual property, Project management), Arctic Microbial Ecology (Summer Erasmus course held in Akureyri, Iceland).
- *January 2010-October 2010*: Training in Pesticide science, University of Liège/Gembloux Agro-Bio Tech. Activities: course work and research.
- *September 2006-September 2007*: M.Sc. Agricultural Sciences and Biological Engineering/Plant Protection, UC Louvain and FUSA Gembloux, Belgium and ENSA-Montpellier, France. Mention: Great Distinction.
- *1999-2005*: Studies at the National University of Rwanda in Butare (now Huye), Rwanda: Agricultural engineering degree (“*Grade d’Ingénieur Agronome”)*/B.Sc. (Hons.) Agr., Option: Crop Production and Horticulture; Internships at Gihindamuyaga Agricultural Station and CARE-Rwanda*;* Mention: Distinction; 2 years of study in Biology (1999-2001).
- *1992-1998*: Secondary studies, Junior Seminaries Saint Jean in Nkumba and Saint Léon in Kabgayi. Rwanda. Option: *Latin-Sciences*.
- 1986-1992: Primary studies at Nemba, Gakenke District, RWANDA.

III. TRAINING

- *11-13 December 2013*: Training on Applied Research: Research Proposal Developing and Writing. Co-organised by UR-CAVM and NUFFIC, Musanze, Rwanda.
- *17-21 June 2013*: "*Laboratory management and equipment operations workshop"*. Co-organised by BECA-ILRI Hub and Holetta Agricultural Research Center, Addis Ababa, Ethiopia.
- *02-22 June 2012*: Training on "*Integrated Pest Management and Food safety*". Organised by Wageningen UR CDI, The Netherlands.
- *06-10 July 2009*: *Fruit fly taxonomy and surveillance* training course. Co-organised by ICIPE, USDA-APHIS, USDA-FAS and RMCA, Nairobi, Kenya.
- *06-09 October 2008*: *Agroecology and Integrated Pest Management* (Short Course). Co-organised by RHODA/MINAGRI/RWANDA and Michigan State University, Kigali, Rwanda
- *10-14 September 2008*: Participatory training on *Good agricultural practices.* Co-organised by WTO, MSU/USA and RHODA/MINAGRI, Kigali, Rwanda.
- *22 January 2008-01 February 2008: Participatory training on IPPC: Pest risk analysis, pests/disease monitoring and surveillance.* Co-organised by RHODA/MINAGRI, WTO and MSU, Butare, Rwanda.
- *28 November 2007-05 December 2007*: *Workshop on entrepreneurship*. Co-organised by Larenstein University, The Netherlands, and ISAE, Musanze, Rwanda.
- *17-26 April 2006*: Training on "*Competency-based learning: Didactic skills I and II*", organised by Larenstein University of Professional Education/The Netherlands.

IV. WORK EXPERIENCE

- *3rd January 2006-Now*: Lecturer at the University of Rwanda (UR), College of Agriculture, Animal Science and Veterinary Medicine (CAVM). Responsibilities in teaching, research and community service (member of the Rwanda NPPO in 2007-2009 -in creation-).
- *1998-1999*: Teacher at Rwaza Secondary School, Rwanda Ministry of Primary and Secondary Education.

V. OTHER QUALIFICATION

- ***PHYTOLICENCE P3* Certificate** (Certificate for distribution, advice or professional use of pesticides, issued by the Belgian Federal Agency for the Safety of the Food Chain AFSCA/FAVV)

VI. PUBLICATIONS

A. PUBLICATIONS IN PEER-REVIEWED JOURNALS

- **BIGIRIMANA V.P.**, HUA G.K.H., NYAMANGYOKU I.O., HOFTE M., 2015. Rice Sheath Rot: An Emerging Ubiquitous Destructive Disease Complex. Frontiers in Plant Science 6: 1066. doi: 10.3389/fpls.2015.01066 (16 pages).

- PATEL H.K., MATTIUZZO M., BERTANI I., **BIGIRIMANA V. P.**, ASH J.G., HOFTE M., VENTURI V., 2014. Identification of virulence associated loci in the emerging broad host range plant pathogen *Pseudomonas fuscovaginae.* BMC Microbiology 14: 274. doi: 10.1186/s12866-014-0274-7 (13 pages).
- **BIGIRIMANA V.P.**, BIZIMANA J.P., 2012. *Evaluation of the efficacy of herbicides in tea (*Camelia sinensis*) production in Rwanda*. International Journal of Applied Agricultural Research, Vol 7, 3:197-202.

B. MANUSCRIPTS SUBMITTED FOR PUBLICATION

- **BIGIRIMANA V. P.,** HUA G. K. H., BERTIER L., NYAMANGYOKU I. O., HÖFTE M. *Sarocladium oryzae* and *Fusarium* spp. are associated with rice sheath rot in Rwanda (Submitted to *Plant Pathology* and currently under revision).

C. PRESENTATIONS AT CONFERENCES

- **BIGIRIMANA V.P.**, NYAMANGYOKU I.O., HOFTE M., 2014. Estimation of the importance of rice sheath rot in Rwanda based on diagnosis and disease intensity evaluation. Poster presentation in the 'Young Researchers Overseas' Day (KAOW-ARSOM), held on December 4, Brussels, BELGIUM.
- **BIGIRIMANA V.P.**, UWUMUKIZA B., HOFTE M., NYAMANGYOKU I.O., Contribution to the establishment of the list of pests of phytosanitary importance on rice in Rwanda by PRA methodology and field survey. Poster presentation in the 'Young Researchers Overseas' Day (KAOW-ARSOM), held on December 4, Brussels, BELGIUM.
- **BIGIRIMANA V.P.,** NYAMANGYOKU I.O., HÖFTE M., 2014. *Rice production in Rwanda: Concerns about seed and grain quality in a dynamic sector.* Poster presentation at the Ghent Africa Platform, Ghent, Belgium, November 27.
- **BIGIRIMANA V.P.,** HOFTE M., NYAMANGYOKU O., 2013. *Isolation and characterisation of fungi associated with rice sheath rot in Rwanda*. Poster presentation, 65th International Symposium on Crop Protection, Ghent, May 21. Book of Abstracts, p. 224.
- **BIGIRIMANA V.P.**, HOFTE M., NYAMANGYOKU O., 2012. *Isolation and characterisation of fungal pathogens causing rice sheath rot in Rwanda*. Poster presentation, Ghent Africa Platform, Ghent, Belgium, December 7.

D. DEGREE RESEARCH WORKS

- **BIGIRIMANA V.P.**, 2007. *Study on the impact of chemotherapy as a way of eradicating Begomoviruses responsible for Cassava mosaic disease*. Thesis for MSc in Agricultural Sciences and Biological Engineering. FUSA Gembloux and UC Louvain, BELGIUM.
- **BIGIRIMANA V.P.,** 2005. *Analysis of factors limiting adoption at large scale of high commercial value potato varieties in Rwanda, Case study of Ruhengeri Province, Rwanda*. Research work for the award of the Agricultural Engineering degree. National University of Rwanda, Butare, RWANDA.

VII. LANGUAGE PRACTICE

- *Kinyarwanda*: Mother tongue.
- *English*: Advanced user for reading, writing, speaking and listening (Advanced Practical English/CEFR C1, Ghent University/UCT; certified for CAE, Cambridge University; Certified for Grammar Skills, UK Writers' College).
- *French*: Proficient user for reading, writing, speaking and listening (Grade A in A level examination).
- *Dutch*: Basic user (CEFR A1, Ghent University/UCT).
- *Swahili*: Basic understanding.
- *Latin*: Passive understanding (Grade A in A level examination).