Integrated Control of *Rhizoctonia* Diseases on Bean and Cabbage

Gia Khuong Hoang Hua

UNIVERSITEIT GENT

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

“Science is not only a disciple of reason but, also, one of romance and passion”

Stephen Hawking

Promotor: Prof. dr. ir. Monica Höfte

Department of Crop Protection, Laboratory of Phytopathology

Ghent University

Dean: Prof. dr. ir. G. Van Huylenbroeck

Rector: Prof. dr. Anne De Paepe

Ghent University
Faculty of Bioscience Engineering
Department of Crop Protection

ir. Gia Khuong Hoang Hua

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Geïntegreerde bestrijding van *Rhizoctonia* ziekten in boon en kool

Cover illustration

Rhizoctonia solani and disease symptoms observed on bean and white cabbage

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Members of the jury

Prof. dr. ir. Monica Höfte (promotor)
Department of Crop Protection
Ghent University

Prof. dr. ir. Geert Haesaert
Department of Applied Biosciences
Ghent University

Prof. dr. ir. Stefaan De Neve
Department of Soil Management
Ghent University

Prof. dr. ir. Marie-Christine Van Labeke
Department of Plant Production
Ghent University

Dr. ir. Jane Debode
Plant Sciences Unit
ILVO

Prof. dr. Jos Raaijmakers
Department of Microbial Ecology
Netherlands Institute of Ecology (NIOO-KNAW)

Dr. ir. Joeke Postma
Plant Research International
Wageningen University

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List of abbreviations

Acyl-HSL	*N*-acyl-L-homoserine lactone
ADIC	2-amino-2-deoxyisochorismic acid
AFLP	Amplified fragment length polymorphism
AG	Anastomosis group
Ap	Ampicillin
BCA	Biological control agent
BNR	Binucleate *Rhizoctonia*
Ca	Calcium
CFU	Colony forming unit
CLP	Cyclic lipopeptide
CLP1	Sessilin
CLP2	Orfamide
C_{org}	Organic carbon
cv	Cultivar
Da	Dalton
DAPG	2,4-diacetylphloroglucinol
DAPI	4,6-diamino-2-phenyl indole
DHHA	*trans*-2,3-dihydro-3-hydroxyanthranillic acid
DHPVD	Dihydropyoverdine
DI	Disease index
dpi	Days post inoculation
DMSO	Dimethyl sulfoxide
Gm	Gentamycin
HAA	3-(3-hydroxyalkanoyloxy)alkanoic acid
HCN	Hydrogen cyanide
hpi	Hours post inoculation
HPLC	High performance liquid chromatography
ISR	Induced systemic resistance
ITS	Internal transcribed spacer region of the rDNA
K	Potassium
KB	King's B
Km	Kanamycin

LB	Luria Bertani medium
LC	Liquid chromatography
LiP	Lignin peroxidase
Mg	Magnesium
MnP	Manganese peroxidase
MS	Mass spectroscopy
Na	Sodium
OD	Optical density
OM	Organic matter content
P	Phosphorus
PCA	Phenazine-1-carboxylic acid
PCN	Phenazine-1-carboxamide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDASM	PDA amended with 100 μg ml^{-1} streptomycin
PDASS	PDA amended with 100 μg ml^{-1} streptomycin and prochloraz (46%)
PDI	Percent disease index
pH	Potential of hydrogen
PIA	*Pseudomonas* isolation agar
PVD	Pyoverdine
PYO	5-*N*-methyl-1-hydroxyphenazine
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
rpm	Rotations per minute
SA	Salicylic acid
Tc	Tetracyclin
TLC	Thin layer chromatography
UV	Ultraviolet
ε	Extinction coefficient
λ	Wave length

Preface

Problem statement

Vegetables, together with grains and fruits, are critically important components of a healthy human diet. Insufficient intake of fruit and vegetables is estimated to cause around 14% of gastrointestinal cancer deaths, 11% of ischaemic heart disease deaths and 9% of stroke deaths globally (WHO 2014). In response to the growing demand for vegetables, world vegetable production has increased continuously in the past decades. A recently published FAO report indicates that the total area used for vegetable cultivation has doubled in the last 30 years (FAOSTAT 2014). With diverse climatic and natural conditions, Vietnam is known as one of the most important vegetable producers in the world. The area planted with vegetables in this country increased from 531 million hectares in 2007 to 680 million hectares in 2012 (FAOSTAT 2014). However, because vegetables are highly sensitive to pathogens, the expansion of the area used for vegetable cultivation is accompanied with an alarming increase of the total quantity of pesticides applied to increase cropping yields and improve quality of the products. In Vietnam, about 5.52 kg of pesticides are used for one hectare of vegetables in each cropping season, almost double the amount needed for one hectare of rice (Anh 2002). In addition to protecting crops, the excessive use of pesticides, especially ones that are banned or restricted, may result in increased pesticide poisoning and ecological damage. Pesticide residues were found on nearly half of leafy vegetables sampled in Ha Noi and Ho Chi Minh. Moreover, the amount of residues in some samples is 50 times higher than the maximum residue levels. As a consequence, Vietnam had more than 7,000 cases of food poisoning from pesticide residues, causing 277 deaths in 37 of the 61 provinces in 2002 (Xuyen 2003). Given these problems, the overall aim of this thesis was to develop an integrated, sustainable strategy that would be able to control diseases caused by *Rhizoctonia solani* on vegetables in Vietnam.

R. solani is one of the most destructive pathogens associated with vegetable production throughout the world. This fungus is a soil inhabitant that infects a wide range of crops, resulting in the development of many serious diseases. Despite the ability to produce basidiospores during the sexual stages (Sneh et al. 1991), most diseases caused by this fungus are initiated by mycelium and/or sclerotia (Keijer 1996).

The model pathosystems employed in our bioassays are *R. solani* (AG 4-HGI and AG 2-2)-bean and *R. solani* (AG 2-1)-cabbage because of the worldwide economic importance of these diseases and the easy cultivation of bean and cabbage in growth chambers. The control strategy that we attempted to develop is based on the use of organic materials to weaken the sclerotia of *R. solani* and the application of antagonistic bacteria to suppress *Rhizoctonia* diseases. More specifically, coir pith is used as soil amendment and *Pseudomonas* sp. CMR12a is used as a biocontrol agent. Coir pith is a lignin-rich organic material widely available in tropical countries. The use of coir pith to control *Rhizoctonia* sclerotia is based on findings of Debode et al. (2005) who reported that the incorporation of Kraft pine lignin and lignin-rich crop residues reduced the viability of *Verticillium* microsclerotia. *Pseudomonas* sp. CMR12a is an antagonistic bacterium isolated from cocoyam roots in Cameroon. This strain can produce phenazines and cyclic lipopeptides (CLPs), antifungal compounds which have been shown to play crucial roles in biocontrol activity (De Jonghe 2006; Debode et al. 2007; Perneel et al. 2007). In parallel with performing experiments with coir pith and CMR12a in the laboratory and growth chambers, a field survey was conducted to isolate and characterize *Rhizoctonia* spp. from diseased *Brassica* vegetables cultivated in Vietnam. The results of our studies are used to address the following research questions:

- Which *Rhizoctonia* AGs are present on *Brassica* vegetables in Vietnam? Is there a link between the AGs found and the agricultural activities in that country?
- Is the biocontrol agent *Pseudomonas* sp. CMR12a active against *R. solani* on bean and cabbage? If yes, do phenazines and CLPs play a role in the antagonism against *R. solani* on these hosts?
- Do growth substrates containing different potting soil and sand ratios affect the biocontrol capacity of *Pseudomonas* sp. CMR12a towards Rhizoctonia root rot of bean?
- Is coir pith incorporation active against the sclerotia of *R. solani*?
- Can biocontrol efficacy be improved by combining coir pith with *Pseudomonas* sp. CMR12a?

Thesis outline

This thesis is divided into seven chapters. In **Chapter 1**, vegetable production in Vietnam and the importance of bean and *Brassica* spp. are introduced. Then, an overview about the genus *Rhizoctonia*, the symptoms of root rot and web blight on bean as well as damping-off and wirestem on *Brassica* vegetables induced by *R. solani* are given. Finally, the use of fluorescent pseudomonads and lignin amendments to control *R. solani* is discussed.

Chapter 2 presents results obtained from a survey conducted on farmer fields in Vietnam. *Brassica* plants with *Rhizoctonia*-like symptoms were collected and the genetic diversity of *Rhizoctonia* populations was assessed using ITS-rDNA sequencing. Then, the virulence of all AGs found towards *Brassica* vegetables, rice and water spinach was evaluated.

The next two chapters are dedicated to the biocontrol activity of *Pseudomonas* sp. CMR12a and the involvement of phenazines and CLP sessilin produced by this strain in biocontrol of Rhizoctonia root rot of bean. In **Chapter 3**, the method used to construct phenazine and sessilin biosynthesis mutants of CMR12a is described. Then, the antagonism of phenazines and sessilin *in vitro* as well as their effect on disease suppression *in vivo* were explored by using these mutants. **Chapter 4** of this thesis focuses on the influence of three potting soil/sand mixtures on the ability of *Pseudomonas* sp. CMR12a to control bean root rot. Moreover, the influence of these growth substrates on the invasion of *Rhizoctonia* hyphae was also investigated.

Chapter 5 marks the switch from *Rhizoctonia*-bean to *Rhizoctonia*-cabbage pathosystem due to the importance of damping-off caused by *R. solani* on *Brassica* vegetables. This chapter starts with pathogenicity tests to determine the virulence of *R. solani* AG 2-1 isolates, which were previously isolated from Belgian cauliflower, on Chinese cabbage seedlings. Then, the effect of phenazines and the two CLPs produced by *Pseudomonas* sp. CMR12a on disease development *in vivo* and viability of *Rhizoctonia* sclerotia *in vitro* was evaluated by comparing the results of treatments with wild type strain CMR12a- with treatments with mutants impaired in phenazine, sessilin and orfamide biosynthesis.

R. solani produces sclerotia and these melanized structures serve as a primary source of inoculum. Thus, reducing the germination of sclerotia in soil is considered an effective strategy to decrease inoculum density of *R. solani* and to control diseases caused by this fungus. In **Chapter 6,** the potential of coir pith, a by-product of coconut industry, as amendment on reducing the viability of *R. solani* AG 2-1 sclerotia was evaluated. Moreover, the combined

effect of coir pith incorporation and *Pseudomonas* sp. CMR12a on sclerotial viability and the suppression of damping-off caused by *R. solani* AG 2-1 on Chinese cabbage were investigated.

In **Chapter 7**, the findings of this study are summarized, a general discussion is given and future research directions are proposed.

Chapter

1

Literature review

Vietnam and its vegetable production

Vietnam is a country situated on the Indochina peninsula bordered by China, Laos and Cambodia. Extending from 8°30' N to 23°22' N, and 102°10' E to 109°30' E, this country has a total area of 330,951 square kilometers. About three-fourth of the country is covered by low mountains and hills. The last one-fourth of the Vietnamese terrain is made up of flat deltas located in the North, South and Central Highlands. The two massive deltas of Vietnam are the Red River delta (approximately 21,050 square kilometers; commonly known as the Northern delta) and the Mekong River delta (approximately 40,553 square kilometers; commonly known as the Southern delta) (GSO 2013; Wikipedia 2014). These deltas serve as major vegetable production areas of Vietnam, following by the Central Highlands and some peri-urban areas (Johnson et al. 2008; Vietnam trade promotion agency 2008). The vegetable growing areas in Vietnam is shown in Table 1.1.

Table 1.1. Area for vegetable production per region in Vietnam (Van Wijk and Everaarts 2007)

Region		Harvested area (1000 hectares)				
		1999	**2000**	**2001**	**2002**	**2003**
North		240.1	245.5	269.7	281.4	296.3
	Red River delta	126.7	125.9	138.5	141.5	148.7
	North East	51	55.2	61.3	67.1	70.9
	North West	9.7	9.7	10.1	11.6	12.5
	North Central Coast	52.7	54.7	59.8	61.2	64.2
South		219	207.4	244.9	279.2	281.5
	South Central Coast	30.9	32.2	34.9	37.6	37.7
	Central Highlands	25.1	31.2	35.4	42.1	41.8
	South East	64.2	48.4	66.2	69	60.1
	Mekong River delta	98.8	95.6	108.4	130.5	141.9

Vietnam has a tropical monsoon type of climate, with average humidity of 84% throughout the year. This country has two major climate regions: (i) Northern Viet Nam (from Hai Van Pass northwards) has a highly humid tropical monsoon climate with four distinguishable seasons (spring, summer, autumn and winter) and is influenced by the Northeast and Southeast monsoon; (ii) Southern Viet Nam (from Hai Van Pass southwards) has a rather moderate tropical climate given the weak influence of monsoon and is characterized by dry and rainy seasons and warm weather all year round (Moinuddin et al. 2012). Annual precipitation of Vietnam varies considerably from place to place, standing between 120 centimeters and 300 centimeters. Nearly 90% of the annual rainfall occurs during the summer. The annual average temperature ranges between 22°C and 27°C. Compared to the South, climatic variation in the mountains and plateaus and in the North is significantly more noticeable, with temperatures ranging from 5°C in winter to 37°C in summer (Ronald 1987).

With a total population of 89 million in 2013, Vietnam is the thirteenth most populous country in the world, and the eighth most populous country in the Asia (Johnson et al. 2008; Wikipedia 2014). Rural population constitutes nearly 70% of the country's population (Moinuddin et al. 2012) and most of rural households grow vegetables (Johnson et al. 2008). Due to the difference in climatic conditions, a wide range of both tropical and temperate vegetables is cultivated (Johnson et al. 2008). About 63% of all vegetables produced is for sale (IFPRI 2002). The general trends in vegetable production of Vietnam from 2002 to 2012 are shown in Figure 1.1.

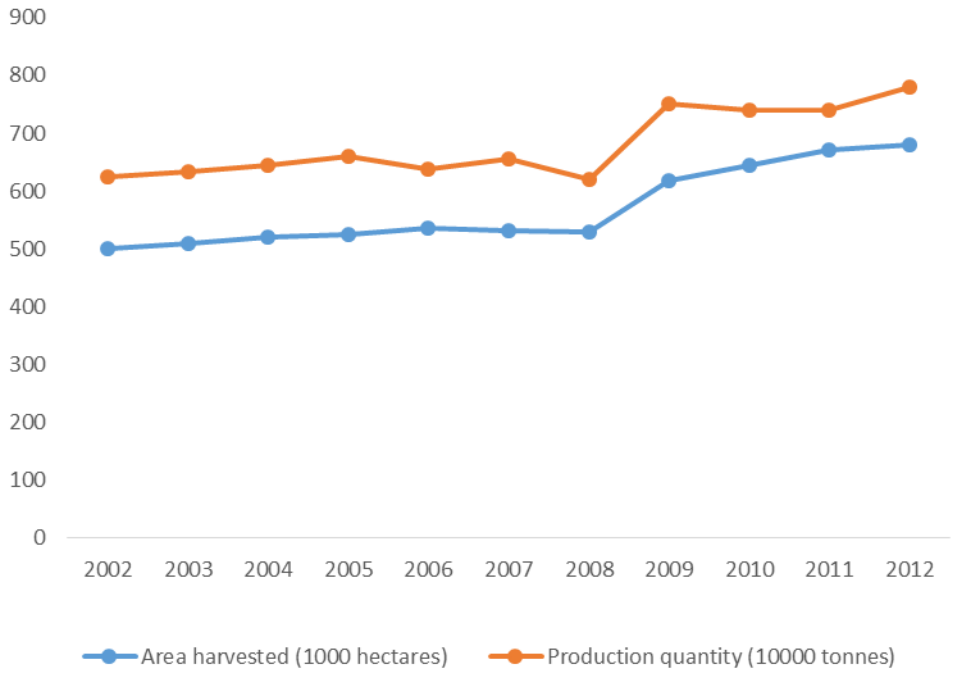


Figure 1.1. Trends in vegetable production and area in Vietnam during the period from 2002 to 2012 (FAOSTAT 2014)

As shown in Figure 1.1, the area planted with vegetables has increased remarkably from 500 thousand hectares in 2002 to 680 thousand hectares in 2012, with the fastest growth observed between 2008 and 2009. The production quantity also increased steadily year by year. According to MARD (2012), Vietnam is strived to obtain 1,200 thousand hectares and 20.6 million tons of vegetables by 2020. Ten most important export markets of Vietnam in 2007 were Taiwan, China, Japan, Russia, USA, the Netherlands, Singapore, Korea, Hongkong and Thailand. The main exported products in that year included tomatoes, carrots, onions, baby corns, cabbages, Chinese cabbage and some other fresh vegetables (Vietnam trade promotion agency 2008).

Importance and utilization of *Brassica* spp.

The genus *Brassica* is a member of the Cruciferae family. This genus includes about 35 different species in which the most important species are *Brassica nigra*, *B. carinata*, *B. oleracea*, *B. napus*, *B. rapa* and *B. juncea*. *Brassica* species seem to have spread from the Mediterranean-Middle Eastern area and they are now widely grown around the world (Rakow 2004; Schmidt and Bancroft 2010). In 2012, about 70 million tons of "cabbages and other Brassicas" were harvested from the area of about 2 million hectares (FAOSTAT 2014). In Vietnam, the area used for the cultivation of "cabbages and other Brassicas" increased from 27 thousand hectares in 2002 to 45 thousand hectares in 2012 in response to the increased demand in vegetable consumption (FAOSTAT 2014). Compared to several other *Brassica* vegetables which are highly restricted to some climatic regions, Chinese cabbage is cultivated throughout Vietnam. In the North, Chinese cabbage is grown from August to October. In the South, this vegetable is cultivated in the period July-April and in Da Lat, Chinese cabbage can be grown year-round (Dan Viet 2013).

Brassica crops are known as an important source of vitamin C, soluble fiber, minerals and antioxidants (Fernández-León et al. 2012). More interestingly, the increased intake of *Brassica* vegetables is associated with reduced cancer risk due to the presence of glucosinolates, sulforaphane, selenium and indole-3-carbinol (Keck and Finley 2004; Finley et al. 2005; Vivar et al. 2009; Banerjee et al. 2012). Aside from providing edible roots, leaves, stems, buds, flowers and seeds, some *Brassica* plants are used as forage, sources of oils and spices, or even ornamentals (Rakow 2004; Schmidt and Bancroft 2010). In 2012, about 494 thousand tons of mustard seed and 65 million tons of rapeseed were harvested worldwide (FAOSTAT 2014).

Importance and utilization of common bean (*Phaseolus vulgaris* L.)

Common bean (*Phaseolsus vulgaris* L.) is the most important herbaceous annual grain legume belonging to the genus *Phaseolus* of the *Fabaceae* family (Graham and Ranalli 1997). Due to the ability to grow under variable climate and soil conditions (Adsule et al. 1998; Omae et al. 2012; Ecocrop 2013), this Latin America-originating crop is now widespread and cultivated in tropical and subtropical areas (Graham and Ranalli 1997; Broughton et al. 2003). Worldwide, about 23 million tons of dry beans and 20 million tons of green beans were harvested from 30

million hectares in 2012. Myanmar and India were the leading producers of dry beans while China was the largest producer of green beans (FAOSTAT 2014).

Beans can be eaten fresh or preserved by freezing or canning. In Latin America and Africa, beans are primarily produced for their edible mature grain whereas in Europe, the United States and other temperate countries beans are considered as vegetable crops grown for the green immature pods. In addition to direct human consumption, bean crop residues and processing by-products from bean production can be used as fodder and organic manure (Purseglove 1974; Wortmann 2006).

Common bean not only supplies high-quality proteins but also contributes a large proportion of dietary fiber, carbohydrates, vitamins and minerals (Beebe et al. 2000; Islam et al. 2002; Winham and Hutchins 2011). It has been proven that common bean consumption may reduce the risk of diabetes, decrease obesity and cardiovascular diseases, lower serum cholesterol concentrations and prevent cancer development (Beninger and Hosfield 2003; Schäfer et al. 2003; Campos-Vega et al. 2013; Chávez-Santoscoy et al. 2014).

The genus *Rhizoctonia*

Genus *Rhizoctonia* is a highly heterogeneous group of filamentous fungi which do not produce asexual spores and share a number of common characteristics in their anamorphic states. Fungi belonging to this genus are widely distributed in both agricultural and forest soils. While some species are pathogenic to a wide range of plants, others are saprophytic on degrading plant tissue or live in symbiosis with orchids (Sneh et al. 1991; García et al. 2006). *Rhizoctonia solani* is by far the most studied among *Rhizoctonia* species. This highly destructive fungus can attack root, shoot and aerial parts to cause seed decay, damping-off of seedlings, wirestem and sore shin, root rot, hypocotyl and stem canker, bottom rot or head rot, crown rot or bud rot, aerial blights, and storage rots on a great variety of crops, including agronomical, ornamental and forestry species worldwide (García et al. 2006; Agarwai 2010).

Taxonomy

The genus concept of *Rhizoctonia* was first described by De Candolle in 1815 and it was reviewed by Parmeter and Whitney (1970). According to these authors, the basic characters of the *Rhizoctonia* genus were the production of sclerotia of uniform texture with hyphal threads

emanating from them and the association of the mycelium with roots of plants (Sneh et al. 1991; García et al. 2006). Because the definition was ambiguous, many unrelated fungi were misidentified as *Rhizoctonia* spp. In 1975, Ogoshi proposed a revised generic concept of *Rhizoctonia* by elevating some characteristics of *R. solani* species to genus level: (i) Branching near the distal septum of cells in young, vegetative hyphae; (ii) Constriction of hyphae and formation of septa a short distance from the point of origin of hyphal branches; (iii) The presence of dolipore septa; and (iv) The absence of clamp connections, conidia, rhizomorphs and sclerotia differentiated into rind and medulla (Sneh et al. 1991). Based on this revised genus concept, species of *Rhizoctonia* can be separated by mycelia color, number of nuclei per young vegetative hyphal cell and the morphology of their teleomorph. Sharon et al. (2008) suggested to divide *Rhizoctonia* isolates into three major groups based on the difference in the number of nuclei per cell including uninucleate *Rhizoctonia* (teleomorph *Ceratobasidium*), binucleate *Rhizoctonia* (teleomorphs *Ceratobasidium* and *Tulasnella*) and multinucleate *Rhizoctonia* (teleomorphs *Thanatephorus* and *Waitea*) (Figure 1.2).

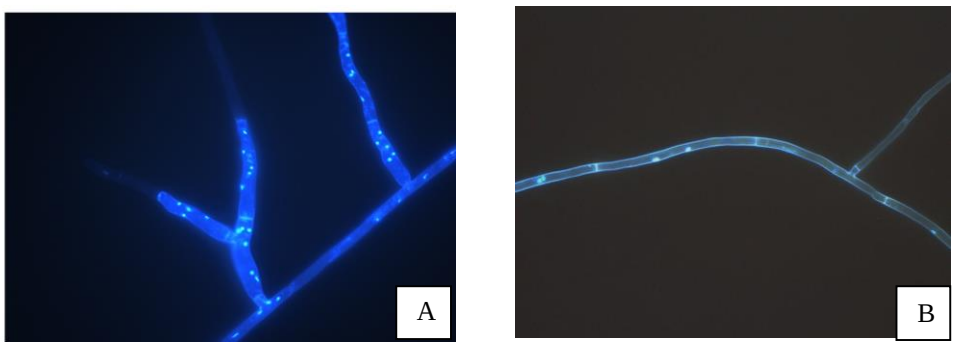


Figure 1.2. Stained nuclei of *Rhizoctonia* species observed under fluorescent microscope
Hyphae of multinucleate *Rhizoctonia* isolate (A) and binucleate *Rhizoctonia* isolate (B) were stained with 4,6-diamino-2-phenyl indole (DAPI).

Rhizoctonia isolates can be further subdivided into anastomosis groups (AGs) based on the ability to anastomose (fuse) hyphae among them. There are four categories (C3 to C0) of hyphal anastomosis reactions in which C3 occurs for the same anastomosis group, same vegetative compatibility population and the same isolate while C0 occurs between different AGs (Carling 1996). Hyphal anastomosis is determined by co-culturing an isolate to be tested with an isolate of known AGs on water agar (Ogoshi 1990). If their cell walls, and probably cytoplasm, connect but no fusion occurs, they belong to the same AG (Ogoshi 1987; García et al. 2006). Currently, thirteen AGs of *R. solani* and sixteen AGs of binucleate *Rhizoctonia* have

been recognized. Among these, *R. solani* AGs 1, 2, 3, 4, 6, 8 and 9 and binucleate *Rhizoctonia* AGs B, D and F have been further divided into subgroups (García et al. 2006; Sharon et al. 2008). Apart from the classical hyphal fusion method, *Rhizoctonia* isolates can also be differentiated into AGs using biochemical and molecular techniques such as isozyme analysis, total cellular fatty acids analysis, electrophoretic karyotyping, DNA-DNA hybridization, DNA fingerprinting based on random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), repetitive probe, AT-rich DNA restriction fragment length polymorphism (RFLP), single-copy nuclear RFLP, rDNA RFLP, and rDNA sequence analysis (Sharon et al. 2006).

Morphology

Due to the lack of the asexual spores, *Rhizoctonia* spp. exist primarily in nature as vegetative hyphae and sclerotia. Members of this genus can produce three sets of hyphae: (i) runner hyphae which are straight and non-infecting; (ii) lobate hyphae which are short, swollen and responsible for the formation of appressoria or dome-shaped infection cushions; and (iii) specialized hyphae consisting of short, broad, varied shaped monilioid cells which involve in the formation of sclerotia (Sneh et al. 1991; Misra et al. 1994; Sharma 2004). Hyphae of *Rhizoctonia* spp. are characterized by the production of branches at right (acute) angles to the main hyphae. The hyphae are broad and hyaline when young, becoming yellow and brown with age because of the accumulation of melanin in the cell walls. Compared to hyphae of multinucleate *R. solani* (diameter = 6-10 µm), hyphae of binucleate *Rhizoctonia* are narrower (diameter = 4-7 µm) (Ogoshi 1987; Sharon et al. 2008).

Sclerotia are survival structures made of compact masses of monilioid cells or undifferentiated hyphae (Sneh et al. 1991). Due to the presence of melanin in the cell walls, sclerotia become resistant to environmental extremes and serve as an important source of *Rhizoctonia* inoculum in soil (Willetts 1971; Misra et al. 1994; Sumner 1996). These resting structure are found primarily in the upper 15 cm of soil in various size and shape (Sumner 1996).

Basidiospores are spores produced during the sexual stages occurring under specific environmental conditions (Sneh et al. 1991). These spores were described by Stalpers and Andersen (1996) as hyaline, thin-walled structures with the shape ranging from round to elliptical or pear-shaped.

Disease cycle

R. solani spreads rapidly through irrigation water, by movement of soil and infected crop residues during land preparation and by using infected planting materials (Abawi 1989). Propagules of this fungus include sclerotia, thick-walled melanized hyphae and basidiospores (Boosalis and Scharen 1959; Parmeter 1970; Naito 1996). A cycle of disease caused by *R. solani* is summarized in Figure 1.3.

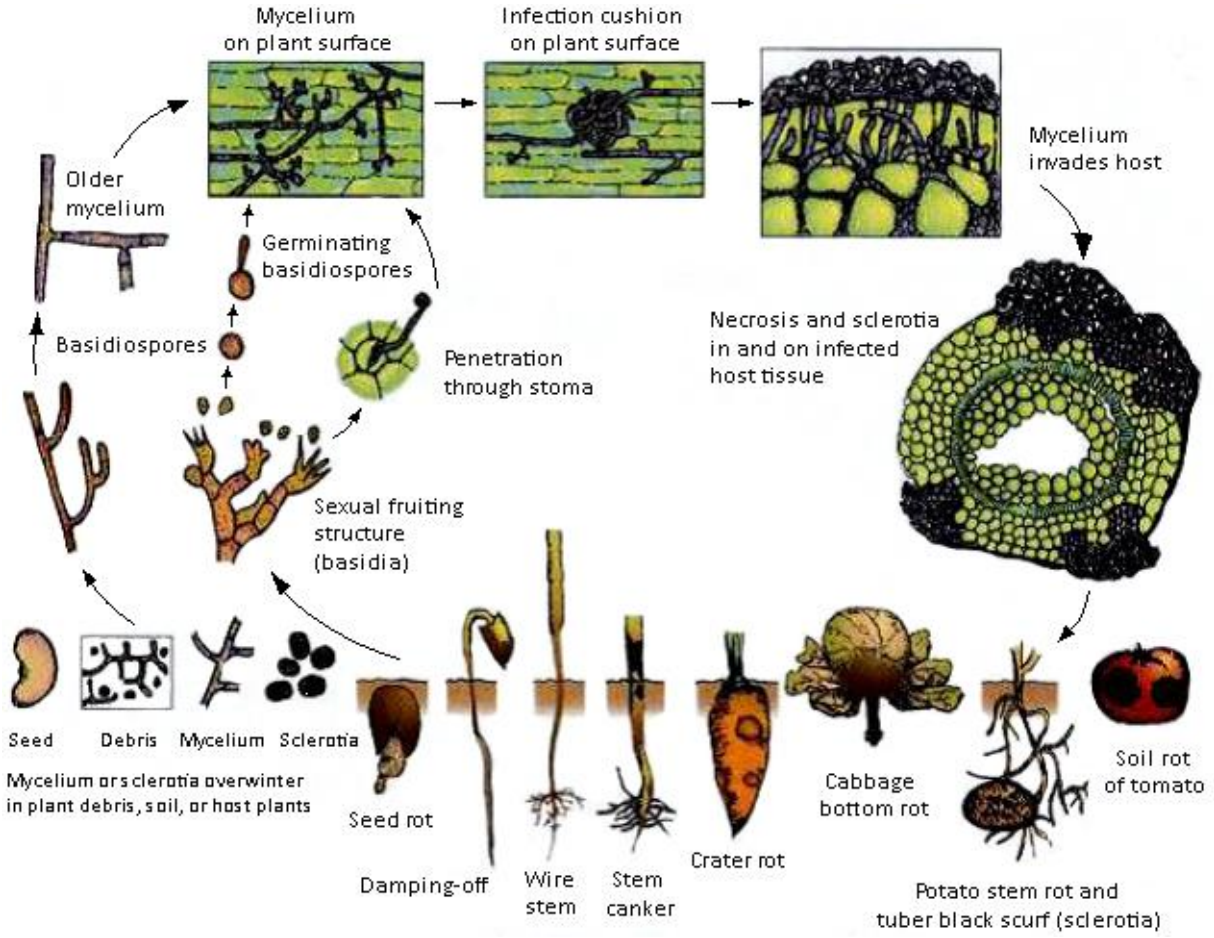


Figure 1.3. Disease cycle of *R. solani* (Agrios 2005)

Compared to infections caused by hyphae or sclerotia, diseases induced by basidiospore infection develop faster. Under favorable field conditions, one complete cycle of disease infection by basidiospores only takes about two weeks. Basidiospores can rapidly spread over a long distance and attack the aerial plant parts (Naito 1996). The infection begins with the germination of basidiospores during periods of high relative humidity (> 99% RH) to form appressoria. These appressoria penetrate directly into the epidermal cells. Then, a stroma-like structure, a special structure which develops only from basidiospore infection, is produced

within the invaded epidermal cells or the upper layer cells of the mesophile. Finally, small, primary lesions are formed on aerial parts of susceptible hosts by hyphae grow out from a stroma-like structure (Naito 1996). Because basidiospores are very fragile and they only survive on host plant parts (Naito 1996), most *Rhizoctonia* diseases result from mycelium and/or sclerotia infections. In contrast to basidiospores, mycelium and sclerotia are able to survive for a long period in plant debris, contaminated seeds or infested soils (Boosalis and Scharen 1959; Sherwood 1970). Under suitable conditions, sclerotia germinate to form hyphae which will grow and become attached to external surface of a compatible host. After attachment, *Rhizoctonia* hyphae start to grow along the anticlinal walls of the epidermal cells and infection cushions are produced when the formation of T-shaped branches is repeated in one or several parental hyphae. Then, some swollen hyphal tips on the infection cushions concurrently form infection pegs (Keijer 1996). The pegs penetrate the cuticle and epidermal cell walls leading to the collapse of these cells (Demirci and Döken 1998). The infection process is promoted by the presence of extracellular enzymes synthesized by the fungi and exudates released by actively growing cells of plant roots. Root exudates, which are very rich in carbohydrates, amino acids, phenols and organic acids, serve as an important source of nutrients for fungal growth and development while enzymatic activity supports the mechanical penetration activity through their involvement in tissue degradation (Keijer 1996; Agarwai 2010).

Rhizoctonia solani as causal agent of root rot and web blight of beans and damping-off and wirestem of *Brassica* vegetables

Symptomology

Root rot of beans

Root rot induced by *R. solani* is a common disease attacking beans grown in Latin America and other bean production regions in the world (Parmeter 1970; Van Bruggen et al. 1986). Isolates of *R. solani* responsible for bean root rot belong to AGs 1, 2-2, 4 and 5 (Galindo et al. 1982; Engelkes and Windels 1996; Karaca et al. 2002; Eken and Demirci 2003; Nerey et al. 2010). Lesions on hypocotyls and roots begin as small, sharp-edged oval to elliptical, reddish-brown, sunken areas. Lesions enlarge with age. Severe infections girdle the stem and cause damping-off of young seedlings (Figure 1.4). On older plants, the fungus can enter and destroy the pith,

produce a brick-red discoloration inside the stem, resulting in plant stunting which eventually kill the infected plant (Stockwell and Hanchey 1984; Abawi 1989; Carisse et al. 2001).

Figure 1.4. Sunken, red-brown lesions developed on beans infected by *R. solani* (Schwartz 2008)

Web blight of beans

Web blight is an important disease in the humid lowland tropics of Latin America and the Caribbean (Gálvez et al. 1989; Godoy-Lutz et al. 2003), Eastern Africa and South America (Masangano and Miles 2004), causing considerable losses in both yield and seed quality (Godoy-Lutz et al. 1998). This disease is known to be induced by *R. solani* AGs 1-IA, 1-IB, 1-IE, 1-IF, 2-2 IV, and 2-2 WB (Godoy-Lutz et al. 2003; Godoy-Lutz et al. 2008). Symptoms of web blight initiated by sclerotia or mycelium fragments are different from those caused by basidiospores. Lesions induced by sclerotia or fungal hyphae first appear on the primary leaves as small necrotic spots (5-10 mm in diameter) with brown centers and olive-green margins. Under warm and humid conditions, these lesions coalesce and affect the entire leaf. On the other hand, lesions produced by basidiospores are distinct, small, necrotic, circular, and measure 2-3 cm in diameter. They are light brown or brick red with a lighter center. These lesions usually do not enlarge much, nor coalesce to form larger lesions, and seldom cause defoliation (Gálvez et al. 1989) (Figure 1.5).

Figure 1.5. Symptoms of web blight disease on beans caused by *R. solani* (Galindo 1982)

Damping-off and wirestem of Brassica vegetables

The infection of *R. solani* AGs 1, 2-1, 2-2, 3, 4, 5, 7, 9 or 10 on *Brassica* crops results in the development of damping-off and wirestem on newly emerged or young seedlings of *Brassica* spp. (Homma et al. 1983; Verma 1996; Yang et al. 1996; Keinath and Farnham 1997; Khangura et al. 1999; Rollins et al. 1999; Sayama 2000; Paulitz et al. 2006; Pannecoucque et al. 2008; Budge et al. 2009). *R. solani* can attack and kill seedlings before or soon after their emergence, causing damping-off disease. On recently emerged seedlings, light brown, water-soaked lesions develop on the stem at the soil level before the plants collapse. For wirestem disease, the stem near the soil line of older seedlings shrivels and brown, cracked lesions develop, resulting in the decay of outer stem tissue. When the outer stem deteriorates, only the fibrous inner xylem is intact and remains as a tough and woody or wiry tissue. Infected seedlings are stunted and severely diseased plants may eventually die (Koike et al. 2006) (Figure 1.6).

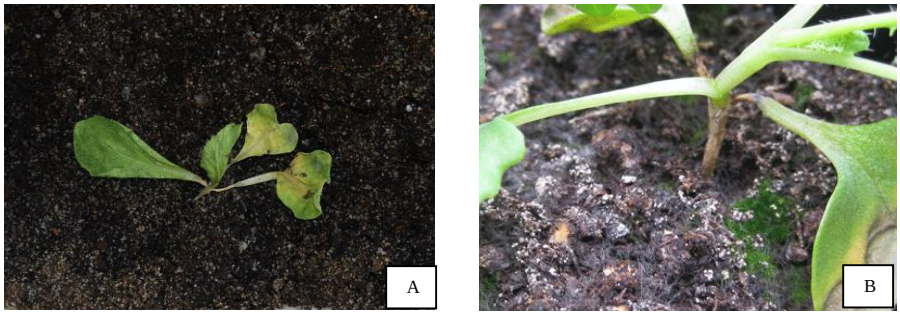


Figure 1.6. Symptoms of Rhizoctonia damping-off (A) and wirestem (B) on Chinese cabbage seedlings

Abiotic factors associated with the development of *Rhizoctonia* diseases

Temperature and moisture levels are two most critical factors for disease development. Bean root rot develops most rapidly in cool (18ºC) soils (Abawi 1989; Harveson 2011) and fungal infection is favored in wet conditions with high to moderate soil moisture levels (Abawi 1989; Harveson 2011). According to Harveson (2011), plants are more susceptible to *Rhizoctonia*-induced diseases because of temperature extremes or over-irrigation.

Incidence of disease is also influenced by nutritional status and density of inoculum (Van Bruggen et al. 1986), soil fertility (Das and Western 1959), potassium availability (Agarwai 2010), and sowing method (Valenciano et al. 2006). The highest proportion of infected plants was observed on beans sown either in raised beds (Valenciano et al. 2006) or in soil containing 250-350 sclerotia per kilogram (Van Bruggen et al. 1986). The effect of soil texture and soil density on the survival of sclerotia, the thick-walled resting structure which determine the quantity and the size of lesions (Van Bruggen et al. 1986), were also reported by Sumner (1996).

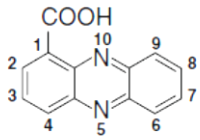
Biological control using fluorescent *Pseudomonas* species

The increasing public concern about the adverse effects of chemical pesticide application in agriculture on environment and human health has heightened the need to search for sustainable control measures. The last decades have seen a drastic expansion in the number and diversity of biocontrol research and among the many potential biocontrol agents studied are fluorescent *Pseudomonas* spp. The mechanisms of biocontrol by these bacteria involve antibiosis (Haas and Défago 2005; Gross and Loper 2009), competition for space and nutrients (Kamilova et al. 2005), competition for iron mediated by siderophores and induction of systemic resistance (De Vleesschauwer and Höfte 2009). Among the number of bioactive metabolites produced by fluorescent *Pseudomonas* spp., phenazines and cyclic lipopeptides (CLPs) are of particular interest. The importance of these two compounds in biological control of plant diseases has recently been reviewed by Olorunleke et al. (in press).

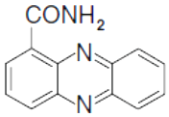
Phenazines

Phenazines are nitrogen-containing tricyclic pigments synthesized by some fluorescent *Pseudomonas* spp. and a few other bacterial genera including *Burkholderia*, *Pectobacterium*, *Brevibacterium*, *Pantoea agglomerans* and *Streptomyces*. Most phenazine producers appear to

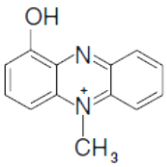
be soil inhabitants and/or plant-associated species (Mavrodi et al. 2006). The three most prominent phenazine producing *Pseudomonas* species are *P. fluorescens*, *P. chlororaphis* and *P. aeruginosa* (Chin-A-Woeng et al. 2003; Gross and Loper 2009). Until recently, about 100 different phenazine-containing compounds of microbial origin have been identified (Mavrodi et al. 2006). The most common phenazine derivatives produced by *Pseudomonas* spp. include phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), pyocyanin (5-*N*-methyl-1-hydroxyphenazine, PYO), 2-hydroxyphenazine, 2-hydroxyphenazine-1-carboxylic acid, and 1-hydroxyphenazine (Turner and Messenger 1986; Mavrodi et al. 2006) (Figure 1.7). Typically, strains of fluorescent pseudomonads are able to produce more than one phenazine derivative which may differ from each other in physical, chemical and antibiotic characteristics. These differences are determined by the functional groups adhered to the aromatic ring in phenazine molecular structure (Mavrodi et al. 2006). The range of the biocontrol ability of antagonistic *Pseudomonas* spp. is partly dependent on the number of antibiotics produced (Chin-A-Woeng et al. 2001).



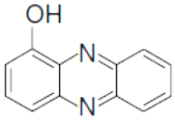
Phenazine-1-carboxylic acid (PCA)



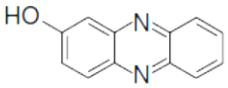
Phenazine-1-carboxamide (PCN)



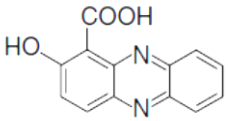
Pyocyanin (PYO)



1-Hydroxyphenazine



2-Hydroxyphenazine



2-Hydroxyphenazine-1-carboxylic acid

Figure 1.7. Structures of common phenazines produced by *Pseudomonas* spp. (Mavrodi et al. 2006)

Phenazines are products of the shikimic acid pathway and phenazine production in fluorescent *Pseudomonas* spp. usually occurs in the early stationary growth phase (Chin-A-Woeng et al. 2000; Laursen and Nielsen 2004). In phenazine biosynthesis, chorismic acid serves as the branch point from which 2-amino-2-deoxyisochorismic acid (ADIC) is synthesized. After that, ADIC is converted to *trans*-2,3-dihydro-3-hydroxyanthranillic acid (DHHA). The nucleus of

the first phenazine derivative PCA is generated by dimerization of two DHHA moieties (Chin-A-Woeng et al. 2003). A cluster of seven genes, *phzABCDEFG*, required for PCA production in *P. aeruginosa* PAO1 (Mavrodi et al. 2001) and *P. fluorescens* 2-79 (Mavrodi et al. 1998). In *P. chlororaphis* 30-84, the PCA biosynthesis is performed with the participation of *phzFABCD* (Pierson et al. 1995). Other phenazine derivatives are obtained by conversion of PCA by specific phenazine-modifying enzymes such as PhzH (converts PCA to PCN) and PhzO (converts PCA to 2-hydroxyphenazine carboxylic acid) (Chin-A-Woeng et al. 2003). More details about biosynthesis and regulation of phenazines were published by Mavrodi et al. (2006) and Chin-A-Woeng et al. (2003) and will not be further discussed in this part.

Because phenazines are broad spectrum antibiotics, phenazine producers are antagonistic to many organisms including bacteria, fungi, and algae (Toohey et al. 1965; Mavrodi et al. 2006). *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 were able to inhibit *Gaeumannomyces graminis* var. *tritici in vitro* and reduced take-all disease of wheat caused by this fungus (Weller and Cook 1983; Thomashow and Weller 1988; Thomashow et al. 1990; Pierson and Thomashow 1992). Similarly, tomato root rot induced by *Fusarium oxysporum* f. sp. *radicis-lycopersici* was successfully controlled by *Pseudomonas chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998). The reduction in severity of rice blast disease caused by *Magnaporthe grisea* by *P. aeruginosa* 7NSK2 (De Vleesschauwer et al. 2006) as well as the suppression of cocoyam root rot caused by *Pythium myriotylum* by *Pseudomonas aeruginosa* PNA1 (Tambong and Höfte 2001; Perneel et al. 2008) were also reported. Recently, phenazines produced by *Pseudomonas* strain CMR12a (D'aes et al. 2011) and *Pseudomonas chlororaphis* Phz24 (Le et al. 2012) were proven to be involved in biocontrol of *R. solani* and *Sclerotium rolfsii* under both *in vitro* and *in vivo* conditions, respectively. In addition to antibiotic activity, phenazines also play a role in pathogenesis, induced systemic resistance, competitiveness, ecological fitness, and biofilm formation of the producers (Mavrodi et al. 2006; Mavrodi et al. 2013).

Cyclic lipopeptides

CLP is one of the two types of biosurfactants commonly produced by biocontrol strains of *Pseudomonas* spp. This compound contains a fatty acyl residue (5 to 16 carbon atoms) and a short (7 to 25 amino acids), cyclic oligopeptide (Raaijmakers et al. 2006; Gross and Loper 2009). Based on the difference in peptide length and amino acid sequence, CLPs of *Pseudomonas* spp. have been assigned into eight different groups including the viscosin, amphisin, syringomycin, syringopeptin, tolaasin, putisolvin, orfamide, and entolysin (Raaijmakers et al. 2006; Li et al.

2013). Chemical structures of some CLPs are illustrated in Figure 1.8. CLPs are reported to play a role in attachment to and colonization of (plant) surfaces, surface motility, antagonism towards other (micro)organisms, induction of systemic resistance and plant pathogenesis (Raaijmakers et al. 2006; D'aes et al. 2010; Raaijmakers et al. 2010).

CLPs are synthesized nonribosomally on large, multifunctional peptide synthetases via a thiotemplate process. CLP production typically takes off in the late exponential or stationary growth phase (Raaijmakers et al. 2006). Factors affect CLP production in *Pseudomonas* spp. include population density of bacteria, nutritional conditions (e.g. the availability of carbon, nitrogen and phosphor), temperature, pH, and oxygen (Nielsen et al. 1999; Raaijmakers et al. 2006). Detailed understanding of biosynthesis and regulation of CLPs produced by *Pseudomonas* spp. is given in the reviews of Raaijmakers et al. (2006; 2010) and Gross and Loper (2009).

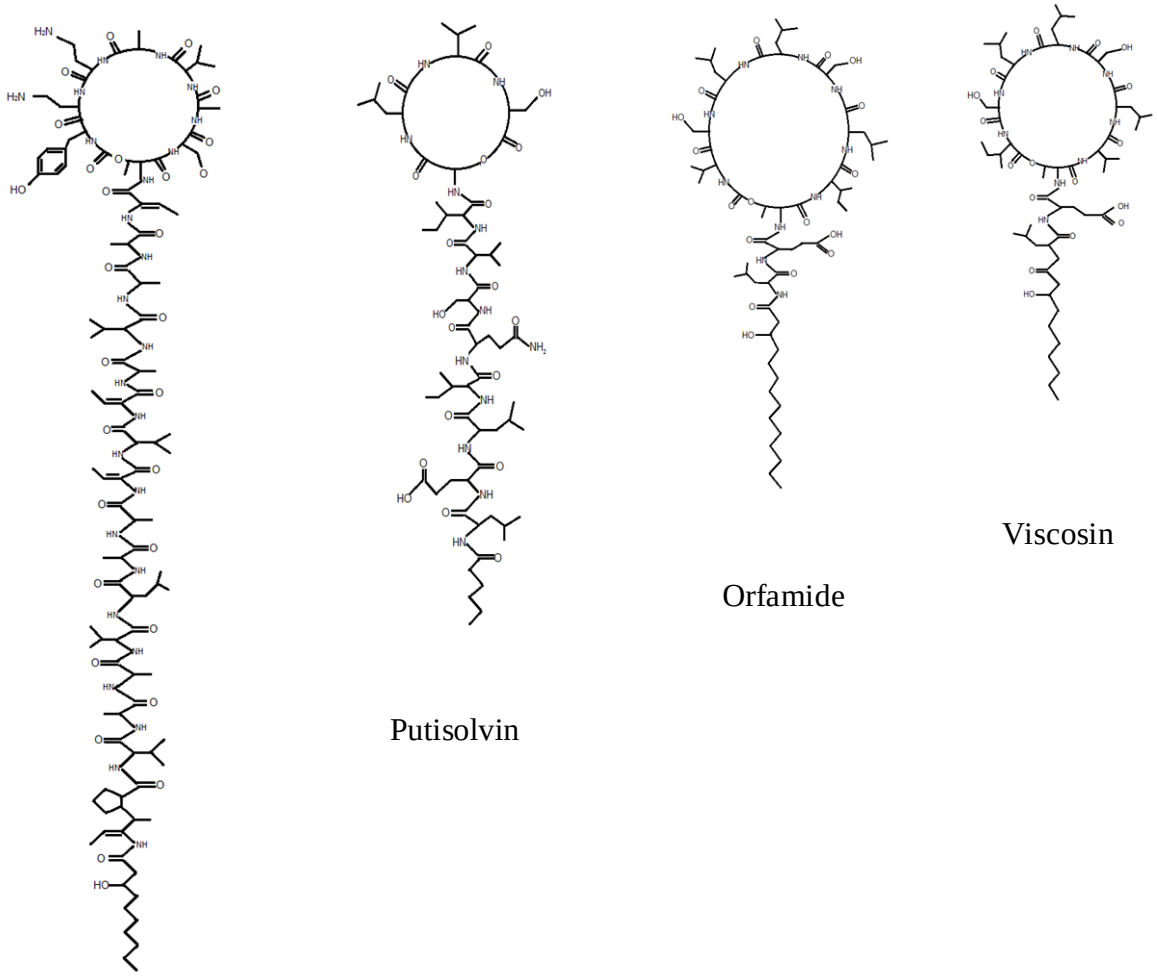


Figure 1.8. Structures of several common cyclic lipopeptides produced by *Pseudomonas* spp. (Raaijmakers et al. 2010)

The first report about the contribution of CLPs in biocontrol dates back to the year 1998, when Nielsen and associates found that viscosinamide produced by *Pseudomonas* strain DR54 successfully suppressed the growth of *Pythium ultimum* and *R. solani in vitro* and reduced damping-off disease caused by *P. ultimum* on sugar beet seedlings (Nielsen et al. 1998). Since then, the crucial role of *Pseudomonas*-originating CLPs in biocontrol of plant pathogens has been intensively studied.

Under *in vitro* conditions, CLPs produced by *Pseudomonas* spp. showed lytic activity towards zoospores of *Phytophthora infestans* (De Bruijn et al. 2007), *Phytophthora ramorum* (Gross et al. 2007) and *Phytophthora capsici* (Kruijt et al. 2009). Additionally, the growth of fungi or other bacteria could be inhibited by CLP-producing *Pseudomonas* strains (Nielsen et al. 1999; Thrane et al. 2000; Andersen et al. 2003; Lo Cantore et al. 2006; Kruijt et al. 2009; Rokni-Zadeh et al. 2012). The presence of CLPs also resulted in the malformation of *R. solani* mycelium (Hansen et al. 2000), the decrease in sclerotia production of *R. solani* (Thrane et al. 2001) and the reduction in oospore production of *P. ultimum* (Thrane et al. 2000). Biocontrol effects of CLPs were also identified *in vivo*. A cell-free crude extract containing the white line inducing principle of *Pseudomonas "reactans"* SPC 8907 was shown to decrease the browning of mushrooms caused by *Pseudomonas tolaasii* (Soler-Rivas et al. 1999). According to De Souza et al. (2003), CLP massetolide A synthesized by *Pseudomonas fluorescens* strain SS101 provided significant protection against root rot caused by *Pythium intermedium* on hyacinth. This compound also effectively suppressed late blight caused by *P. infestans* on tomato (Tran et al. 2007). More recently, Berry et al. (2010) showed that suppression of Sclerotinia stem rot of canola by *Pseudomonas* strain DF41 depended upon CLP sclerosin production, while D'aes et al. (2011) concluded that Rhizoctonia root rot of bean could be partially controlled by CLP sessilin produced by *Pseudomonas* strain CMR12a.

Pseudomonas sp. CMR12a

Pseudomonas strain CMR12a was isolated from the rhizosphere of healthy cocoyam plants (*Xanthosoma sagittifolium*) grown in a *Pythium myriotylum* infested field in Cameroon (Perneel et al. 2007). This strain provided good control of tomato root rot caused by *Phytophthora nicotianae* (De Jonghe 2006), cocoyam root rot caused by *P. myriotylum* (Perneel et al. 2007) and bean root rot caused by *R. solani* (D'aes et al. 2011). In addition, the use of *Pseudomonas* sp. CMR12a could effectively reduce the viability of *Verticillium* microsclerotia *in vitro* (Debode et al. 2007).

Identification of metabolic compounds produced by *Pseudomonas* sp. CMR12a showed that this strain is able to produce phenazines, CLPs, hydrogen cyanide and exoprotease, but not pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol. Two phenazine derivatives synthesized by CMR12a were identified as PCA and PCN (Perneel et al. 2007), whereas two classes of CLPs produced by this strain has recently been classified as sessilins and orfamides (D'aes et al. in press). The main compound of sessilin, namely sessilin A, is made up of a peptide moiety contained 18 amino acid coupled to a β-hydroxyoctanoyl fatty acid. The structure of this compound is highly similar to that of tolaasin, which is a toxin produced by *P. tolaasii* strain Paine 1919, except for the replacement of glutamine in sessilin A with serine in tolaasin at the sixth amino acid. Three major compounds of orfamides are orfamides B, D and E. These orfamides consist of a 10 amino acid peptide moiety and a β-hydroxydodecanoyl or β-hydroxytetradecanoyl fatty acid chain (Table 1.2). Structurally, these compounds are most related to orfamides produced by biocontrol strain *Pseudomonas protegens* Pf-5. In *Pseudomonas* sp. CMR12a, sessilins are required for biofilm formation, whereas orfamides play an important role in swarming motility (D'aes et al. in press).

Table 1.2. Proposed primary structures of sessilin A and orfamides B, D and E produced by *Pseudomonas* sp. CMR12a (D'aes et al. in press)

Name	Molecular weight (Da)	Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sessilin A	2028	C8:0-OH(3)-	dhAbu-	Pro-	Ser-	Leu-	Val-	Gln-	Leu-	Val-	Val-	Gln-	Leu-	Val-	dhAbu-	aThr-	Ile-	Hse-	Dab-	Lys
Orfamide B	1281	C14:0-OH(3)-	Leu-	Glu-	aThr-	Val-	Leu-	Ser-	Leu-	Leu-	Ser-	Val								
Orfamide D	1253	C12:0-OH(3)-	Leu-	Glu-	aThr-	Val-	Leu-	Ser-	Leu-	Leu-	Ser-	Val								
Orfamide E	1279	C14:1-OH(3)-	Leu-	Glu-	aThr-	Val-	Leu-	Ser-	Leu-	Leu-	Ser-	Val								

Lignin-rich organic amendments and their potential in reducing the persistence of *Rhizoctonia* sclerotia

The use of organic amendments has been frequently recommended to improve soil structure and fertility as well as promote saprophytic microbial growth. Amendment of soils with organic materials has also been reported to suppress the incidence of diseases caused by soil-borne pathogens in both conventional and biological agriculture systems (Bailey and Lazarovits 2003;

Bonanomi et al. 2007). Organic matters used as amendments can be divided into four groups: composts, crop residues, peats and organic wastes (Bonanomi et al. 2007). Compost is organic material subjected to aerobic decomposition by the activity of thermophilic and mesophilic microorganisms (Noble and Coventry 2005; Bonanomi et al. 2007). Crop residues include non-degraded materials such as green manures and non-harvestable plant residues (e.g. stems, roots and leaves). Peat is a natural product derived from the progressive accumulation of partially decayed vegetation (mosses and higher plants). Organic wastes consist of all the organic materials which do not fit in the previous three classes, such as undecomposed animal manure and by-products of different industrial processes (Bonanomi et al. 2007). After analyzing 250 articles with 2423 experimental case studies, Bonanomi et al. (2007) found that the incorporation of organic matters into soil significantly suppressed diseases in 45% of the cases in which compost was the most suppressive and peat was the least effective with 50% and 4% of the experiments showing successful disease control, respectively. The disease suppressive effect of organic matters depends on the pathogens. Although *R. solani* was the most studied species, effective control of this fungus was achieved only in 26% of the cases. One of the factors that hinder *R. solani* management is the persistence of its sclerotia. As mentioned above, sclerotia of *R. solani* contain melanin, a dark-pigmented biological phenolic complex polymer which protects the sclerotia against both biotic stress (e.g. attack by lytic enzymes, predatory protozoa and nematodes) and abiotic stress (e.g. UV light and heat) (Hyakumachi et al. 1987; Butler et al. 2005; Plonka and Grabacka 2006). The only report about the effect of soil amendments on viability of *Rhizoctonia* sclerotia is of Van Beneden et al. (2010) who added Kraft pine lignin to a silt loam soil (1%; w/w). Lignin is a heterogeneous aromatic polymer which is very resistant to microbial degradation. Microorganisms which can degrade lignin include bacteria (e.g. *Nocardia* sp. and *Streptomyces* sp.) and fungi (e.g. some members of basidiomycetes and ascomycetes) (Ayyachamy et al. 2013). It is hypothesized that the addition of lignin-rich materials to soil stimulates lignin-degrading microorganisms (Debode et al. 2005). Biodegradation of lignin occurs by the oxidative action of nonspecific extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Due to the ability to produce a wide range of lignin-degrading enzymes, white rot fungi are considered as the major lignin degraders in nature (Ayyachamy et al. 2013). Some lignin degrading enzymes such as MnP produced by white rot fungi are able to lyse fungal melanin (Butler and Day 1998; Butler et al. 2005; De Boer et al. 2005; Van Beneden et al. 2010). Sclerotia with degraded melanin are more susceptible to antagonists and lose their viability (Gómez and Nosanchuk 2003; Butler et al. 2005; Debode et al. 2005; Van Beneden et al. 2010). In addition to the

finding of Van Beneden et al. (2010), the effect of lignin-rich organic materials in reducing the viability of melanin-covered (micro)sclerotia and/or in suppressing diseases caused by sclerotia producing pathogens was also reported by Soltani et al. (2002), Debode et al. (2005) and Montanari and Innocenti (2011), suggesting the potential to use these materials as a control measure of *R. solani* sclerotia.

Chapter

2

Cropping systems and cultural practices determine the *Rhizoctonia* anastomosis groups associated with *Brassica* spp. in Vietnam

Gia Khuong Hoang Hua, Lien Bertier, Saman Soltaninejad and Monica Höfte

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Abstract

Ninety seven *Rhizoctonia* isolates were collected from different *Brassica* species with typical *Rhizoctonia* symptoms in different provinces of Vietnam. The isolates were identified using staining of nuclei and sequencing of the rDNA-ITS barcoding gene. The majority of the isolates were multinucleate *R. solani* and four isolates were binucleate *Rhizoctonia* belonging to anastomosis groups (AGs) AG-A and a new subgroup of AG-F that we introduce here as AG-Fc on the basis of differences in rDNA-ITS sequence. The most prevalent multinucleate AG

was AG 1-IA (45.4% of isolates), followed by AG 1-ID (17.5%), AG 1-IB (13.4%), AG 4-HGI (12.4%), AG 2-2 (5.2%), AG 7 (1.0%) and an unknown AG related to AG 1-IA and AG 1-IE that we introduce here as AG 1-IG (1.0%) on the basis of differences in rDNA-ITS sequence. AG 1-IA and AG 1-ID have not been reported before on *Brassica* spp. Pathogenicity tests revealed that isolates from all AGs, except AG-A, induced symptoms on detached leaves of several cabbage species. In *in vitro* tests on white cabbage and Chinese cabbage, both hosts were severely infected by AG 1-IB, AG 2-2, AG 4-HGI, AG 1-IG and AG-Fc isolates, while under greenhouse conditions, only AG 4-HGI, AG 2-2 and AG-Fc isolates could cause severe disease symptoms. The occurrence of the different AGs seems to be correlated with the dominant crops and the habit to use alternative hosts of *Rhizoctonia* fungus as cover materials in different sampling areas suggesting that agricultural practices determine the AGs associated with *Brassica* plants in Vietnam.

Introduction

Vietnam is a country in Southeast Asia in which the agricultural sector accounts for more than 22% of the GDP, 30% of export and 52% of all employment. Vietnam is not only one of the world leaders in rice and coffee export, but also the third world's largest vegetable producer. *Brassicas* are among the main vegetables produced for both local consumption and export (Vietnam trade promotion agency 2008). Vegetables in Vietnam are mainly produced by poor households living in the Red River and Mekong River delta (see Figure 2.1) in intensive cultivation systems or in rotation with other crops. Due to the lack of knowledge in crop management, limited availability of technology and land fragmentation, farmers are suffering heavy yield losses year after year. Among the limiting factors in vegetable production is the occurrence of *Rhizoctonia* diseases, which has been recognized as one of the most important threats.

Rhizoctonia is a genus of basidiomycete fungi causing many important plant diseases. Based on differences in the number of nuclei per cell, *Rhizoctonia* isolates have been differentiated into uninucleate *Rhizoctonia*, binucleate *Rhizoctonia* (teleomorphs: *Ceratobasidium* spp. and *Tulasnella* spp.) and multinucleate *Rhizoctonia* (teleomorphs: *Thanatephorus* spp. and *Waitea* spp.) (Sharon et al. 2008). *Rhizoctonia* species can also be classified using biochemical and molecular techniques. Among those, rDNA-ITS sequence analysis appears to be the most convenient and reliable method (Sharon et al. 2006). Currently, isolates of *R. solani*, the most

widely recognized species within the multinucleate *Rhizoctonia* group, have been divided into 13 anastomosis groups (AGs), while 16 AGs of binucleate *Rhizoctonia* have been recognized (Carling et al. 1999; Carling et al. 2002a; Carling et al. 2002b; Hyakumachi et al. 2005; Sharon et al. 2006; Sharon et al. 2008). Due to the considerable genetic diversity, several AGs have been further divided into subgroups based on phylogenetic differences. These phylogenetic differences can be associated with differences in morphology, ecology, pathogenicity and biochemical characteristics, although this is not necessarily the case (Sneh et al. 1991).

Compared to binucleate *Rhizoctonia*, multinucleate *R. solani* AGs usually have a wider host range and higher virulence. *R. solani* can survive for a long period in plant debris, contaminated seeds, or infested soils as mycelium or sclerotia (Schwartz 2007; Wharton et al. 2007). Under favorable conditions, sclerotia geminate and form delicate hyphae that will grow toward the host plants (Keijer 1996). *Brassica* vegetables can be attacked by several different AGs of *R. solani* resulting in the development of various diseases such as foliar blight, wirestem and damping-off. In previous studies, AGs 1-IB, 1-IC, 2-1, 2-2 IIIB, 3, 4-HGI, 4-HGII, 4-HGIII, 5, 7, 9 and 10 were shown to be pathogenic on *Brassica* crops grown in Canada (Verma 1996; Yang et al. 1996), Australia (Khangura et al. 1999), Japan (Homma et al. 1983; Sayama 2000), North America (Keinath and Farnham 1997; Rollins et al. 1999; Paulitz et al. 2006), Brazil (Kuramae et al. 2003), China (Yang et al. 2007), Belgium (Pannecoucque et al. 2008), and the UK (Budge et al. 2009). Although *Rhizoctonia* diseases occur severely and frequently on leafy vegetables cultivated in Vietnam, there have been no reports about the AGs and subgroups that attack *Brassica* crops. Therefore, our research aimed at (i) identifying the species and AGs of *Rhizoctonia* present on *Brassica* plants in different vegetable producing regions in Vietnam, and (ii) verifying the susceptibility of *Brassica* vegetables to *Rhizoctonia* isolates collected. Our story revealed that *Rhizoctonia* AGs such as AG 1-IA and AG 1-ID, which have not been reported before on *Brassica* spp., are predominant in Vietnam, which is presumably linked with the cultural practices and cropping systems in the different sampling areas. Moreover, we describe two new AGs that were previously unknown.

Materials and methods

Field sampling and pathogen isolation

Sampling was done on private farms by Gia Khuong Hoang Hua (Vietnamese citizen) with permission from the farmer. In general, permission by the authorities was not required since the sampling studies were carried out on private farms. Only cabbage plants showing symptoms of

the undesired *Rhizoctonia* fungus were sampled, hence the field studies did not involve endangered or protected species. The GPS coordinates of the locations where samples were collected are presented in the Supplementary information (Table A2.1).

The survey was conducted from September to October 2011 on various fields in the Red River delta (Ha Noi; an important vegetable production area of the North), the Mekong River delta (Vinh Long, Can Tho, Hau Giang and Soc Trang; main vegetable production areas of the South), the Central Highlands (Lam Dong; vegetables are mainly produced for export) and the Southeast (Dong Nai; vegetables are mainly produced for domestic consumption) (Figure 2.1). These regions were chosen because of their importance in vegetable production and because they offer a good representation of the different climatic and topographic conditions in Vietnam and, therefore, a good representation of the distribution of *Rhizoctonia* spp. on *Brassica* spp. in Vietnam can be obtained. Total production area, climatic conditions and main agricultural activities of these regions are listed in Table 2.1.

A total of 142 *Brassica* plants with *Rhizoctonia*-like symptoms were sampled. Infected root and leaf tissues were washed in running tap water, surface-disinfected in 1% sodium hypochlorite solution for two min and then rinsed twice in sterile water before placing on 1% water agar medium supplemented with streptomycin (0.05 g L^{-1}). After 24 h of incubation, *Rhizoctonia*-like hyphal tips growing out of these tissues were transferred to fresh potato dextrose agar (PDA; Difco) plates and incubated for two to four days at 28°C.

Ninety seven *Rhizoctonia* isolates were recovered and subjected to nuclei staining and sequencing of the ITS-rDNA region. All isolates are listed in Table 2.2.

Table 2.1. Sampling locations and their relevant characteristics (People's committee of Dong Nai province 2011; Commerce 2014; Department of Culture, Sport and Tourism of Soc Trang 2014; Department of Planning and Investment of Vinh Long 2014; Department of Propaganda and Training of Hau Giang 2014; Integrated water resources management 2014; Viet's life 2014)

Sampling location	Agricultural area (1000 ha)	Average temperature (°C)	Main crop
Red River delta			
Ha Noi	152.24	24	- Cabbage, tomato, cucumber, radish - Rice
Central Highlands region			
Lam Dong	279.00	High land: 14 Low land: 21	- Lettuce, cabbage, carrot, potato - Coffee, tea, cashew-nut tree, cotton
Southeast region			
Dong Nai	289.02	27	- Coffee, cotton, black pepper - Durian, grapefruit, mango
Mekong River delta			
Vinh Long	116.18	27	- Rice - Mango, orange, grapefruit, durian - Cabbage, cucumber, bean
Can Tho	115.00	27	- Rice - Cabbage, cucumber
Hau Giang	139.07	27	- Durian, pineapple, grapefruit - Rice
Soc Trang	278.15	27	- Rice - Grapefruit, mango, durian

Nuclei staining

Rhizoctonia isolates were cultured on sterile glass slides covered by PDA for two days at 28°C. Actively growing fungal hyphae were stained with 10 µg mL^{-1} 4, 6-diamino-2-phenyl indole (DAPI; Sigma-Aldrich) and the number of nuclei per hyphal cell was determined using an Olympus BX51 microscope (Pannecoucque et al. 2008).

DNA extraction, PCR and sequencing of the rDNA-ITS region

The rDNA-ITS region of all collected isolates was sequenced for identification to the AG and subgroup level. The usefulness of the rDNA-ITS region for identification of unknown *Rhizoctonia* spp. has been clearly shown by Sharon et al. (2006; 2008).

Rhizoctonia isolates were grown on potato dextrose broth at 28°C for one week. Mycelial mats were harvested by filtration and ground in liquid nitrogen to produce a fine powder. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). The rDNA-ITS fragment including the 5.8S gene was amplified using primers ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) and ITS5 (5´-GGAAGTAAAAGTCGTAACAAGG-3´) (White et al. 1990). The PCR amplification reactions were performed by adding 2 µL genomic DNA (5-10 ng μL^{-1}) to 23 µL of reaction mixture containing 2.5 µL PCR buffer (10x; Qiagen), 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 11.35 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 at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Cycling ended with a final extension step at 72°C for 10 min. Amplification products were separated in 1% agarose gels in TAE-buffer at 100 V for 30 min and visualized by ethidium bromide staining on a UV transilluminator. The sequences of both strands were determined by LGC Genomics GmbH (Berlin, Germany) using Sanger sequencing.

Identification using BLAST and phylogenetic analysis

Consensus sequences for all 97 isolates were created with BioEdit version 7.1.11. To determine the AG of the isolates, the rDNA-ITS consensus sequences obtained were compared to those in Genbank using the BLASTn tool.

However, since Genbank is an uncurated database, it can contain inaccurately designated *Rhizoctonia* spp., as has been previously shown by Sharon et al. (2006). Therefore, comparison of rDNA-ITS sequences of unknown isolates to a curated database of sequences containing representative rDNA-ITS sequences of all known uninucleate, binucleate and multinucleate *Rhizoctonia* AG and subgroups provides a more reliable identification. Such a database of representative sequences is available from Sharon et al. (2008) and was provided by Michal Sharon to us. However, not all known AGs were present in this database, therefore we added representative isolates of the following AGs: AG 1-1E, AG 1-1F, AG 2-2 WB (Godoy-Lutz et al. 2008) and AG 13 (Carling et al. 2002a). The total number of sequences in the database was 129 and the Genbank accession numbers of all these sequences can be found in Table A2.2.

Multiple alignments for multinucleate and binucleate *Rhizoctonia* isolates were constructed using MUSCLE which is implemented in MEGA 6 (Tamura et al. 2013) and checked manually

afterwards. The resulting alignments had a length of 717 bp (multinucleate *Rhizoctonia* isolates) and 768 bp (binucleate *Rhizoctonia* isolates).

Separate phylogenetic trees were constructed for multinucleate *Rhizoctonia* and binucleate *Rhizoctonia* isolates. For the binucleate tree, one representative isolate for each known binucleate AG was included together with the binucleate *Rhizoctonia* isolates obtained in this work.

For the multinucleate tree, reference isolates from the curated database (Table A2.2) were added for all AGs present in our collection from Vietnam. Another 32 isolates from a characterization study in Vietnam that has not been published (Thuan et al., unpublished; Genbank accession numbers with prefix 'EF' in Table 2.3), and found on a range of crops and belonging to AGs AG 1-IA, AG 1-ID and AG 4-HGI were also added to the multinucleate *Rhizoctonia* alignment for phylogenetic analysis. Phylogenetic trees were built using the neighbour joining algorithm with 1000 bootstrap repeats using MEGA 6 (Tamura et al. 2013). Model testing was done using the software implemented in MEGA 6 and the K2 + G DNA substitution model was chosen.

Aggressiveness of *Rhizoctonia* isolates towards detached leaves of cabbages, rice and water spinach

Nine isolates (representing the nine different *Rhizoctonia* AGs collected) were randomly selected and tested for virulence towards several *Brassica* crops in two independent experiments (Tables 2.7, 2.8 and 2.9). To confirm their pathogenicity, tests were repeated for AGs 1-IA, 1-IB, 1-ID, 2-2, 4-HGI and A since each of these AGs consists of more than one isolate. Results of additional tests are shown in Tables A2.3, A2.4 and A2.5.

Leaves of white cabbage (*Brassica oleracea*), Chinese cabbage (*B. chinensis*), pak choi (*B. chinensis*), mustard cabbage (*B. juncea*) and Chinese flowering cabbage (*B. parachinensis*) were cut into pieces (3 x 3 cm). The growth substrate used in our experiments was a mixture (w/w) of 50% potting soil (Structural; Snebbout, Kaprijke, Belgium) and 50% sand (Cobo garden; Belgium). Sets of six leaf discs were placed in a plastic box (16 x 11 x 6 cm) containing 400 g of substrate. Inoculum of *Rhizoctonia* spp. was produced according to the method described by Scholten et al. (2001). Briefly, water-soaked wheat kernels were autoclaved for 25 min on two successive days and then inoculated with three fungal discs (diameter 5 mm) cut at the edge of a 3-day-old *Rhizoctonia* colony cultured on PDA. Flasks containing the inoculated kernels were incubated for 14 days at 28°C and shaken every 3-4 days to avoid coagulation. Two *Rhizoctonia*-infected kernels which were comparable in size were buried 2 cm below each

leaf disc. Leaf discs inoculated with sterile wheat kernels served as a control. All boxes were incubated in a growth chamber at 22°C.

The detached leaf bio-assay was also conducted to investigate the pathogenicity of the *Rhizoctonia* isolates on rice and water spinach, two important hosts of *Rhizoctonia* spp. in tropical countries. Surface-sterilized seeds of rice (*Oryza sativa* cv. CO39) and water spinach (*Ipomoea aquatic* cv. Trang Nong) were sown in plastic trays (45 x 45 x 10 cm) filled with 4 kg of growth substrate and kept in a growth chamber at 28°C for four weeks before their leaves were detached. Rice leaves were then cut into pieces (8 cm long) and six rice leaf pieces or six water spinach leaves were put in one square Petri dish containing a sterile filter paper moistened with sterile water. Two sterile glass slides were placed in the middle of each Petri dish to keep the leaves away from water. A 5-mm plug harvested from 3-day-old cultures of *Rhizoctonia* spp. on PDA was placed at the center of each leaf or leaf piece and the Petri dishes were incubated at 28°C.

After four days of incubation, disease severity was scored based on the following disease scale: 0 = no symptoms observed; 1 = lesions covered less than 25% of leaf surface; 2 = lesions covered 25-50% of leaf surface; 3 = lesions covered 50-75% of leaf surface; 4 = lesions covered more than 75% of leaf surface or dead leaf. The experiment had a completely randomized design. Each treatment consisted of 12 leaves or leaf pieces equally divided into two boxes or Petri dishes.

In vitro pathogenic potential of _Rhizoctonia_ spp. on seedlings of white cabbage and Chinese cabbage

The same nine *Rhizoctonia* isolates were studied for their *in vitro* pathogenic potential using the method described by Keijer et al. (1997). Six surface-sterilized seeds of white cabbage (*B. oleracea* cv. TN180) or Chinese cabbage (*B. chinensis* cv. Elton) were germinated on Gamborg B5 medium (Gamborg B5 medium including vitamins; Duchefa) in a square Petri dish. Two mycelial disks (5 mm in diameter) from 3-day-old *Rhizoctonia* cultures grown on PDA were placed between seeds. In the control dishes, sterile PDA discs were used for inoculation. The Petri dishes were incubated at 22°C in the dark for two days for seed germination. Then, the second halves of the Petri dishes were covered with aluminum foil to protect the roots from light and placed in an upright position in a growth chamber (22°C, 12 h light). The disease severity was recorded for root and hypocotyl or for leaves after six days of incubation using the following disease scale: 0 = healthy, no symptoms; 1 = lesions covering less than 25% of the

root, hypocotyl or leaf surface; 2 = lesions covering between 25% and 50% of the root, hypocotyl or leaf surface; 3 = wilted plant with lesions covering between 50% and 75% of the root, hypocotyl or leaf surface; 4 = lesions covering more than 75% of root, hypocotyl or leaf surface or dead plant. In this test, a complete randomized design was applied with two Petri dishes (six seedlings each) per treatment and this experiment was done twice.

In vivo pathogenic potential of *Rhizoctonia* spp. on roots and hypocotyls of white cabbage and Chinese cabbage

Surface-sterilized seeds of white cabbage and Chinese cabbage were germinated on wet filter paper in Petri dishes at 22°C one day before sowing into 600 g of growth substrate. Four days after sowing, each perforated plastic box (22 x 15 x 6 cm) with six seedlings was inoculated by placing a row of 12 *Rhizoctonia*-colonized wheat kernels in the middle of the box. The kernels used for inoculation had equivalent sizes and were produced as described previously. Control seedlings were similarly treated with sterile wheat kernels. All plants were incubated at 22°C. Disease severity on root and hypocotyl was evaluated 14 days after inoculation using the same disease scale described for the *in vitro* experiment. A completely randomized design was used with 12 seedlings cultivated in two experimental boxes per treatment and this test was performed twice with the same nine isolates that were used in the previous tests.

Statistical analysis

The severity of *Rhizoctonia* diseases on roots and leaves of *Brassica* seedlings are presented in Tables 2.7-2.9 and Tables A2.3-A2.5 as Disease index (DI). DI was calculated using the following formula:

$$\mathrm{DI} = \frac{\sum(\text{Disease class} \times \text{number of plants within that class})}{\text{Total number of plants within treatment}}$$

Pathogenicity data for the two experiments with nine isolates of nine different AGs were always very similar and no significant interaction was found between the experiments. Therefore, statistical analysis was done on pooled data for the different repeats. The non-parametric Kruskal-Wallis test for k independent samples was used, after which pair-wise comparisons were performed for all treatments using Mann-Whitney tests at a confidence level of $p = 0.05$.

To determine the correlation between the distributions of AGs and sampling locations and between the distributions of AGs and sampled *Brassica* spp., contingency tables were constructed using Excel. Then, potential significant differences between the variables were

revealed with Fisher's Exact Tests. All statistical analyses were conducted in SPSS 22.0 (SPSSinc, Illinois, USA).

Results

Molecular characterization and phylogenetic analysis of *Rhizoctonia* isolates

A total of 142 *Brassica* plants with *Rhizoctonia*-like symptoms were sampled in various important vegetable producing regions in Vietnam (see Figure 2.1). Of the 97 *Rhizoctonia* isolates recovered, only four were binucleate with two nuclei per hyphal cell. The other 93 isolates had multinucleate cells (data not shown).

Analysis of the rDNA-ITS region using the BLASTn tool (against Genbank and against the curated database) revealed that three binucleate *Rhizoctonia* isolates belonged to AG-A while the fourth isolate (LDDL02-1) could not be assigned to any known AG.

Pairwise sequence similarity scores of isolate LDDL02-1 against all isolates in the curated database (representing all known AGs) were determined. Highest pairwise sequence similarities were found with isolates of multinucleate AG 6 (94%) and binucleate AG-Fb (93%) (Table 2.4). When blasting to Genbank, several isolates from taro and ginger from Yunnan Province in China were found to be nearly identical to isolate LDDL02-1.

Figure 2.1 (next page) Location of sites for collection of *Rhizoctonia* isolates from *Brassica* spp. in Vietnam.

The seven provinces sampled are: Ha Noi (districts of Gia Lam, Thanh Tri and Dong Anh), Lam Dong (Da Lat city and Duc Trong district), Dong Nai (Bien Hoa city), Vinh Long (Binh Tan district), Can Tho (Cai Rang district), Hau Giang (Phung Hiep district) and Soc Trang (Soc Trang city and My Xuyen district). In each city or district of one province, one to two wards were surveyed and these wards are marked with a start✦. Different colors are used to highlight the most important AGs found in our survey including AG 1-IA, AG 1-IB, AG 1-ID and AG 4-HGI.

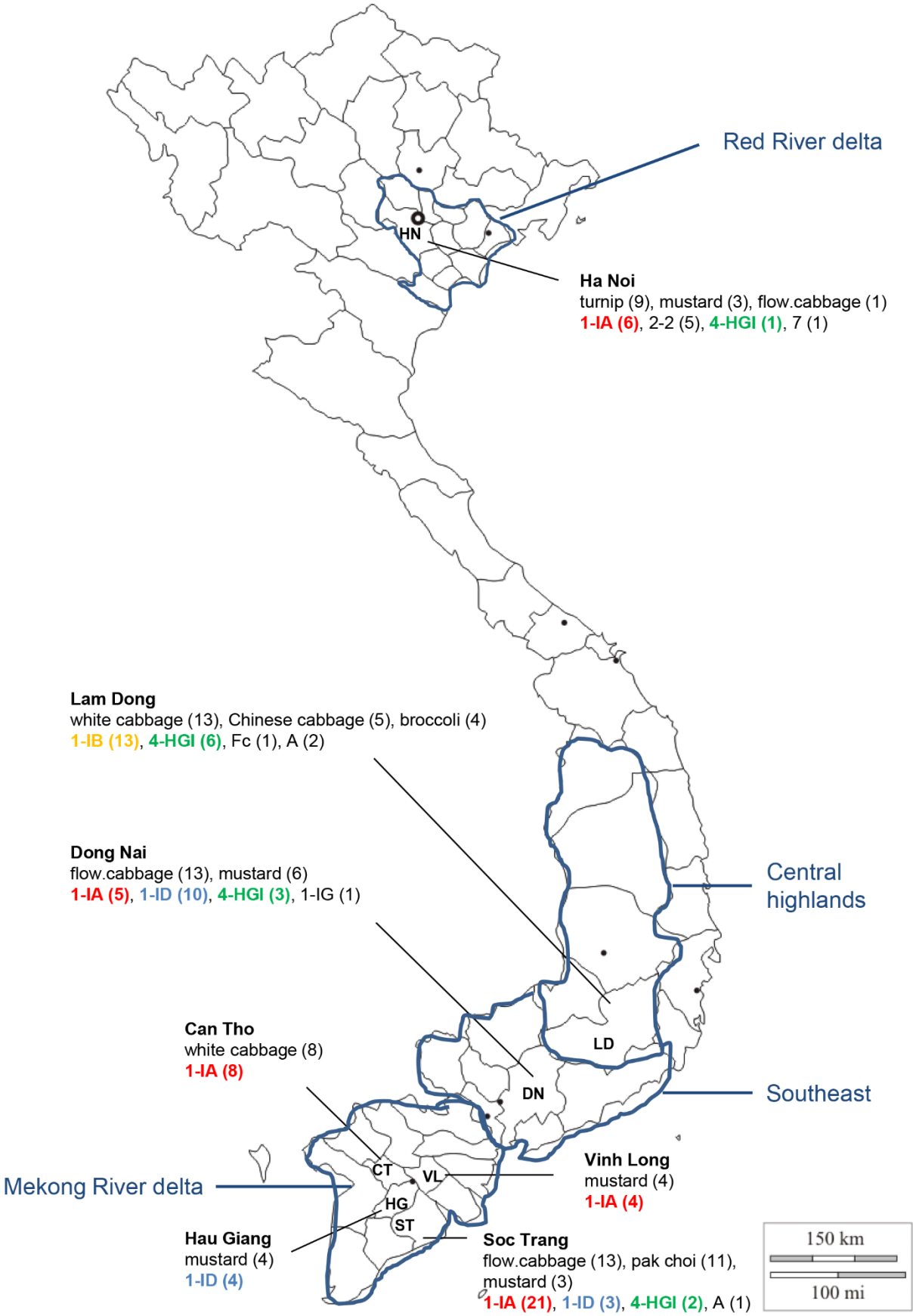

Red River delta
HN
Ha Noi
turnip (9), mustard (3), flow.cabbage (1)
1-IA (6), 2-2 (5), 4-HGI (1), 7 (1)
Lam Dong
white cabbage (13), Chinese cabbage (5), broccoli (4)
1-IB (13), 4-HGI (6), Fc (1), A (2)
Dong Nai
flow.cabbage (13), mustard (6)
1-IA (5), 1-ID (10), 4-HGI (3), 1-IG (1)
Central highlands
LD
Can Tho
white cabbage (8)
1-IA (8)
DN
Southeast
Vinh Long
mustard (4)
1-IA (4)
Mekong River delta
CT
VL
HG
ST
Hau Giang
mustard (4)
1-ID (4)
Soc Trang
flow.cabbage (13), pak choi (11), mustard (3)
1-IA (21), 1-ID (3), 4-HGI (2), A (1)
150 km
100 mi

Table 2.2. Characterization of *Rhizoctonia* isolates collected from diseased *Brassica* crops grown in Vietnam by sequencing the ITS-region

AG /Subgroup	Host plant	Isolate[ab]	Genbank accession numbers
1-IA	*B. parachinensis* (Chinese flowering cabbage)	STST03-1, STST03-3, STST03-4, STST04-2, **STMX04-1**, **STMX04-2**, **STMX04-3**, **STMX04-4**, STMX04-5	KF907702, KF907703, KF907704, KF907705
	B. juncea (Mustard cabbage)	DNBH01-1, DNBH01-2, DNBH01-3, DNBH02-2, DNBH02-3	
		HNGL01-1, HNGL01-2, **HNGL01-3**	KF907706
		STST02-1, STST02-2	
		VLBT01-1, VLBT01-2, VLBT01-3, VLBT01-4	
	B. chinensis (Pak choi)	**STMX01-1**, STMX01-2, **STMX01-4**, STMX01-5, STMX02-1, STMX02-2, **STMX02-3**, **STMX03-1**, **STMX03-2**, STMX03-3	KF907707, KF907708, KF907709, KF907710, KF907711
	B. oleraceae (Turnip cabbage)	HNDD01-1, HNDD01-2, **HNDD01-3**	KF907712
	B. oleraceae (White cabbage)	CTCR01-1, CTCR01-2, **CTCR01-3**, **CTCR02-1**, **CTCR02-2**, CTCR02-3, CTCR03-1, CTCR03-2	KF907713, KF907714, KF907715
1-IB	*B. chinensis* (Chinese cabbage)	LDDT03-1	
	B. oleraceae (Broccoli)	LDDL01-1, **LDDL01-2**, LDDL01-3, LDDL01-4	KF907716
	B. oleraceae (White cabbage)	**LDDL04-1**, LDDL04-2, LDDL04-3, LDDL04-4, LDDL04-5, **LDDL05-1**, LDDL05-2, **LDDL05-3**	KF907717, KF907718, KF907719
1-ID	*B. parachinensis* (Chinese flowering cabbage)	**DNBH03-1**, **DNBH03-2**, **DNBH03-3**, DNBH03-4, DNBH03-5, DNBH05-1-1, DNBH05-1-3, DNBH05-2-2, **DNBH05-3-1**, **DNBH05-4**	KF907720, KF907721, KF907722, KF907723, KF907724
		STST04-1, STST04-3	
	B. juncea (Mustard cabbage)	HGPH01-1, HGPH01-2, **HGPH01-3**, HGPH01-4	KF907725
		STST02-3	
1-IG	*B. parachinensis* (Chinese flowering cabbage)	**DNBH05-1-2**	KF907730
2-2	*B. parachinensis* (Chinese flowering cabbage)	**HNTT01-1**	KF907726
	B. oleraceae (Turnip cabbage)	**HNDA01-1**, HNDA01-2, **HNDA01-3**, **HNDA01-4**	KF907727, KF907728, KF907729
4-HGI	*B. chinensis* (Chinese cabbage)	**LDDT01-1**, LDDT01-2, LDDL02-2	KF907731
	B. parachinensis (Chinese flowering cabbage)	DNBH05-2-1, DNBH05-3-2	
		STST01-1, **STST03-2**	KF907732
	B. juncea (Mustard cabbage)	DNBH02-1	
	B. oleraceae (Turnip cabbage)	**HNDD01-4**	KF907733
	B. oleraceae (White cabbage)	LDDT02-1, LDDT02-2, LDDT02-3	
7	*B. oleraceae* (Turnip cabbage)	**HNDA02-1**	KF907734
A	*B. chinensis* (Pak choi)	STMX01-3	
	B. oleraceae (White cabbage)	**LDDL03-1**, LDDL03-2	KF907735
Fc	*B. chinensis* (Chinese cabbage)	**LDDL02-1**	KF907736

[a] The first two letters represent provinces in which the samples were collected (i.e. CT: Can Tho, VL: Vinh Long, HG: Hau Giang, ST: Soc Trang, DN: Dong Nai, LD: Lam Dong and HN: Ha Noi).

[b] Isolates in bold (unique sequences) are submitted to Genbank.

Table 2.3. Multinucleate and binucleate *Rhizoctonia* isolates derived from Genbank included in the phylogenetic analysis for comparison

AG/Subgroup	Isolate	Host plant	Origin	Genbank accession number	Reference
1-IA	L31-1, L66-1, L73, L59, L38, L52, L62-1	Rice	Vietnam	EF206342, EF429208, EF429211, EF429212, EF429210, EF429209, EF429207	unpublished
	RM61	Water spinach	Vietnam	EF429216	unpublished
	LB71	Water hyacinth	Vietnam	EF429215	unpublished
	DP38	Peanut	Vietnam	EF429214	unpublished
	CLV72-2	Barnyard grass	Vietnam	EF429213	unpublished
	BV71-2, BV61-2, BV50-1	Cotton	Vietnam	EF429206, EF429205, EF206341	unpublished
	CC72	Bermuda grass	Vietnam	EF429204	unpublished
	B34-1	Corn	Vietnam	EF429203	unpublished
1-IG	RMPG28	Chickpea	India	JF701750	Dubey et al. (2012)
1-ID	BV62-1, BV61-6, BV61-5, BV61-4, BV61-1	Cotton	Vietnam	EF197803, EF197804, EF197802, EF197801, EF197800	unpublished
	SR61, SR650	Durian	Vietnam	EF197798, EF197797	Thuan et al. (2008)
	B61-1	Corn	Vietnam	EF197796	unpublished
	CCD61-1	Sugar beet	Vietnam	EF197799	unpublished
4-HGI	XL4	Cauliflower	Vietnam	EF203247	unpublished
	CB63, CB34-2	Cabbage	Vietnam	EF203251, EF203245	unpublished
	CP50-2	Coffee	Vietnam	EF203250	unpublished
	BV68-1, BV68-2	Cotton	Vietnam	EF203249, EF203248	unpublished
	KT63-1	Potato	Vietnam	EF203246	unpublished
Fc	BS-YT-06-5-14, YT, BS-J-06-6-3, DL-jiang-06-2-4, DL-YT-06-4-10, DL-YT-06-4-9, DL-YT-06-3-4	Taro, Ginger	China	HM623619, HM623631, HM623615, HM623622, HM623625, HM623624, HM623623	unpublished

Table 2.4. Pairwise sequence similarities of unknown isolates LDDL02-1 and DNBH05-1-2 to all known AGs from the curated database in Table A2.2. LDDL02-1 shows most similarity to AG 6 and AG-Fb. DNBH05-1-2 shows highest pairwise sequence similarity to AG 1-IA and AG 1-IE.

	LDDL02-1	**DNBH05-1-2**
AG 1-IA	0.88	0.92
AG 1-IB	0.84-0.87	0.84-0.87
AG 1-IC	0.89-0.90	0.89-0.90
AG 1-ID	0.85-0.86	0.85
AG 1-IE	0.89	0.94
AG 1-IF	0.84	0.85
AG 2-1	0.87-0.90	0.82-0.85
AG 2-2	0.86-0.87	0.84-0.85
AG 2-3	0.89-0.90	0.85
AG 3	0.89-0.90	0.84-0.85
AG 4	0.86-0.88	0.85-0.88
AG 5	0.91	0.86-0.87
AG 6	0.89-0.94	0.88-0.90
AG 7	0.91	0.89
AG 8	0.92-0.93	0.89-0.90
AG 9	0.9	0.86
AG 10	0.89-0.90	0.84-0.85
AG 11	0.88-0.89	0.84
AG 12	0.89-0.90	0.87-0.88
AG 13	0.90	0.90
AG 2-BI	0.85-0.86	0.81-0.82
AG-A	0.84	0.84
AG-K	0.84	0.84
AG-Bb	0.79-0.80	0.78-0.79
AG-Q	0.80	0.79
AG-Bo	0.83	0.83
AG-Ba	0.83-0.84	0.83-0.84
AG-C	0.80-0.82	0.82
AG-H	0.81-0.82	0.81
AG-I	0.81	0.80-0.81
AG-D	0.78-0.81	0.77-0.79
AG-G	0.85	0.85
AG-L	0.85	0.85-0.86
AG-O	0.86	0.86

	LDDL02-1	**DNBH05-1-2**
AG-Fb	0.93	0.89
AG-P	0.86-0.89	0.86-0.90
AG-R	0.88	0.85
AG-S	0.88	0.88
AG-Fa	0.91	0.91
AG-E	0.90-0.91	0.89
UNR1	0.82-0.83	0.84
UNR2	0.80	0.82
AG-N	0.67	0.66
W. circinata	0.66-0.67	0.64-0.66

From the binucleate phylogenetic tree (Figure 2.2), it is clear that the unknown isolate LDDL02-1 clusters together with the isolates from Yunnan province, forming a clade with high bootstrap support that is different from any known binucleate AG, with the closest related AG being AG-Fb. So far, nothing has been published about the Chinese isolates. Pairwise sequence similarity within AG-F is 90-100% (Sharon et al. 2008), and the closest related AGs are AG-Fb (93%) and AG-Fa (91%). Therefore, we propose to assign these isolates as a new AG-F subclade, namely AG-Fc.

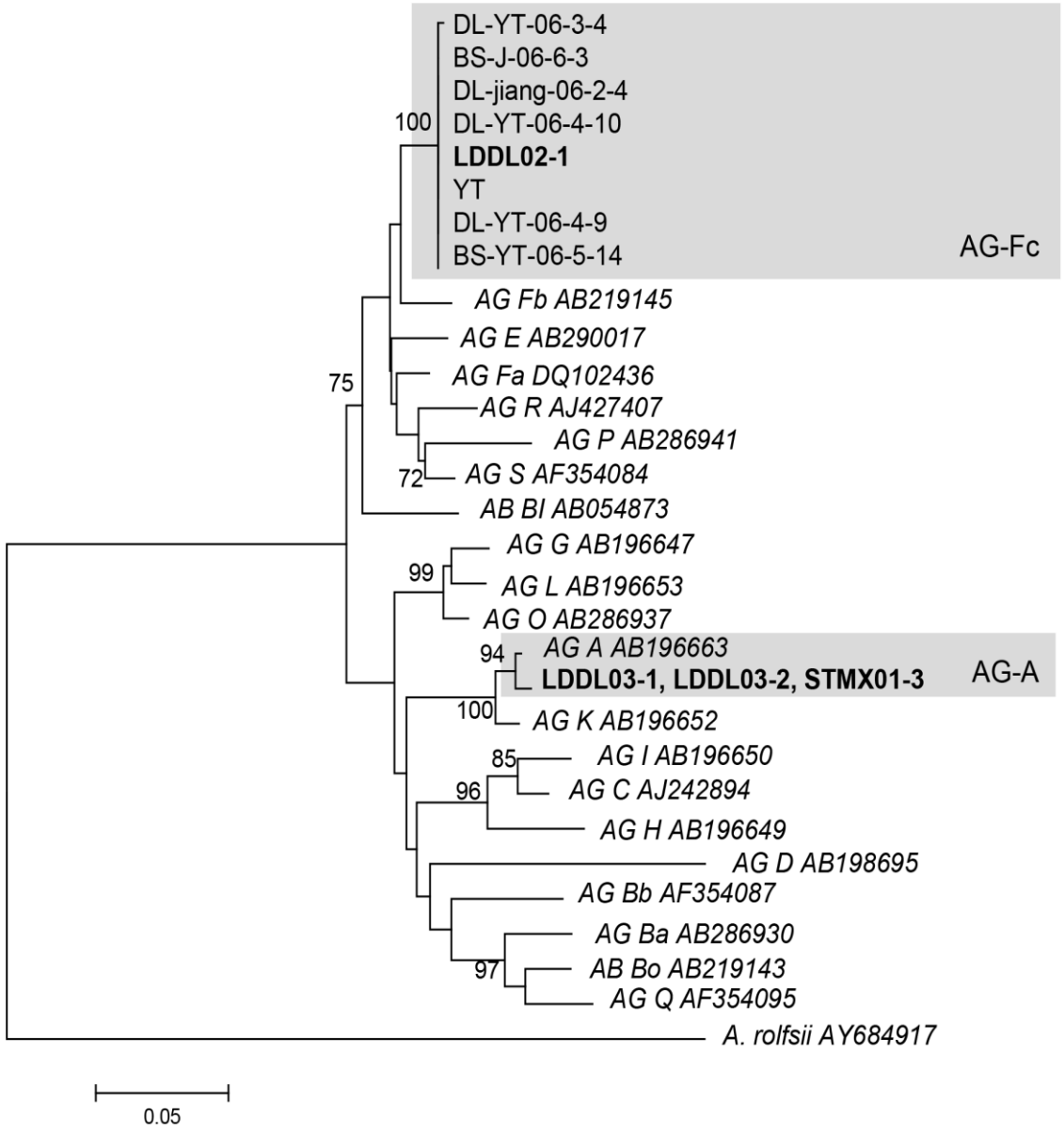


Figure 2.2. rDNA-ITS phylogeny of binucleate *Rhizoctonia* spp. sampled from *Brassica* spp. in Vietnam

Neighbour joining tree derived from the alignment of 31 binucleate *Rhizoctonia* isolates and the outgroup *Athelia rolfsii* (AY684917). Isolates in bold are the 4 isolates derived from *Brassica* spp. in Vietnam during this study. For each known binucleate *Rhizoctonia* AG, a representative isolate (in italics) from the curated database (Table A2.2) is included.

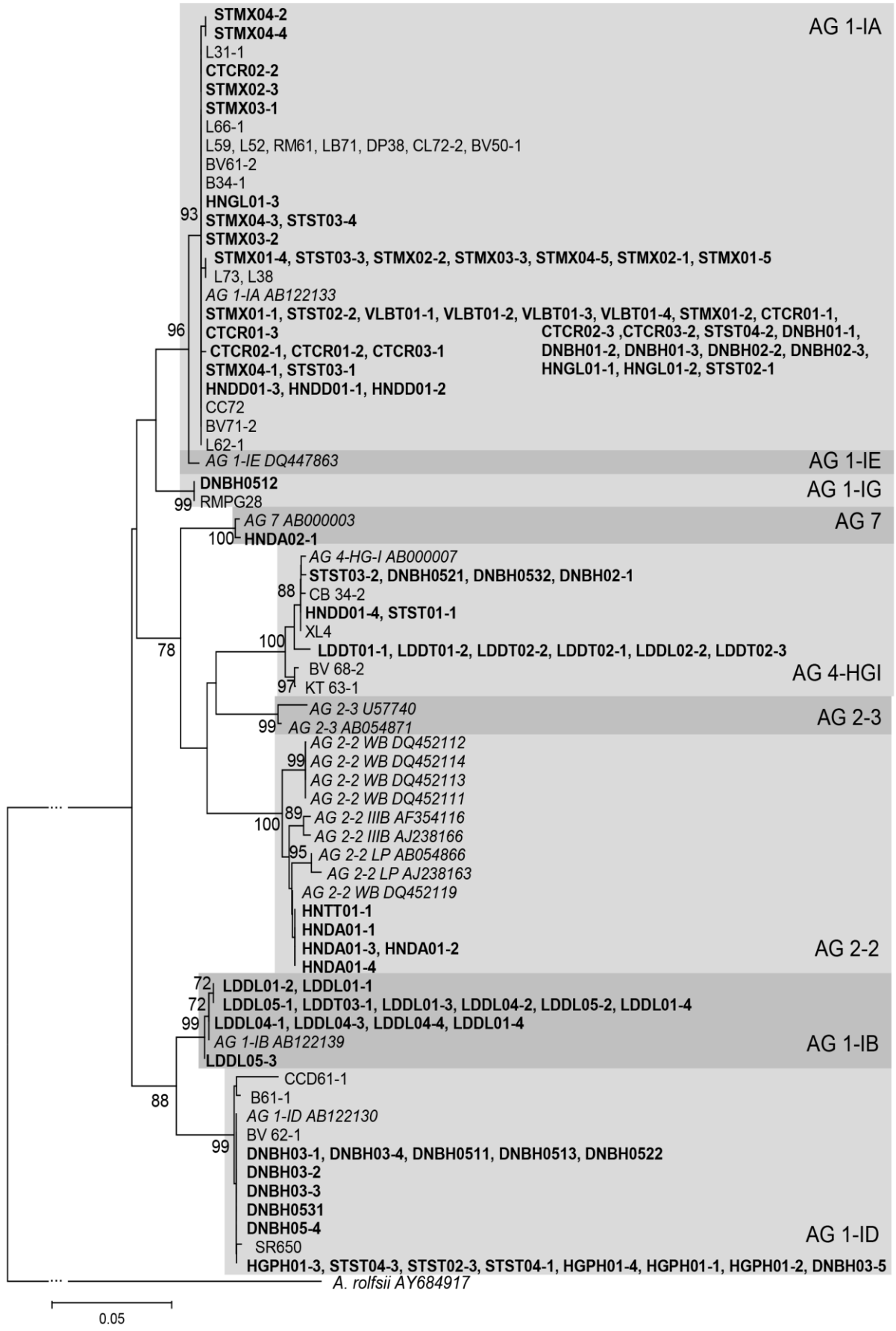
Bootstraps are only given for those branches with bootstrap support higher than 70. The tree was made using only isolates with unique sequences. Isolates with identical sequences were added afterwards on the same line.

In the multinucleate tree, all our Vietnamese isolates form clades with high bootstrap supports together with representative isolates from the curated database (Figure 2.3). The largest group belonged to AG 1-IA (44 isolates), followed by AG 1-ID (17 isolates), AG 1-IB (13 isolates) and AG 4-HGI (12 isolates). Five isolates belonged to AG 2-2. AG 2-2 is further divided into four groups: AG 2-2 IV, AG 2-2 LP, AG 2-2 IIIB and AG 2-2 WB. The similarity scores between our isolates and AG 2-2 IIIB and AG 2-2 LP were comparable (between 97 and 98%). Also, our isolates show 99% similarity with isolate Barranca (DQ452119), which is assigned as AG 2-2 WB by Godoy-Lutz et al. (2008). However, with other isolates from AG 2-2 WB (DQ452111-114), pairwise sequence similarity is lower (between 96 and 97%). Therefore, we decided that it is impossible at this point to assign our isolates to any of the AG 2-2 phylogenetic subgroups. One isolate belonged to AG 7 and the last isolate (DNBH05-1-2) could not be assigned to any of the known multinucleate AG groups in our reference database. The isolate showed the highest pairwise sequence similarity with AG 1-IE (94%, see Table 2.4). A BLASTn search on Genbank identified an isolate from chickpea in India (RMPG28, Dubey et al. 2012) assigned to AG 2-3 to be >99% similar (1 bp substitution) to DNBH05-1-2. However, when comparing these isolates to the AG 2-3 isolates in the curated database, we found low pairwise sequence similarity (85%) (Table 2.4). Also in the phylogenetic tree (see Figure 2.3), isolate DNBH05-1-2 clusters together with isolate RMPG28, but not with the representative AG 2-3 isolates from the curated database, indicating that isolate RMPG28 is wrongly designated as AG 2-3 in Genbank. Hence, these isolates represent a new subgroup of AG 1 and we propose the name AG 1-IG.

Figure 2.3 (next page) rDNA-ITS phylogeny of multinucleate *Rhizoctonia* spp. sampled from *Brassica* spp. in Vietnam

Neighbour joining tree derived from the alignment of 128 multinucleate *Rhizoctonia* isolates and the outgroup *Athelia rolfsii* (AY684917). Isolates in bold are the isolates derived from *Brassica* spp. in Vietnam during this study. For each of the multinucleate *Rhizoctonia* AG subgroups present in our sampling, representative isolates (in italics) from the curated database (Table A2.2) are included.

Bootstraps are only given for those branches with bootstrap support higher than 70. The tree was made using only isolates with unique sequences. Isolates with identical sequences were added afterwards on the same line. Only half of the length of the outgroup branch is shown to increase clarity.


STMX04-2
STMX04-4
L31-1
CTCR02-2
STMX02-3
STMX03-1
L66-1
L59, L52, RM61, LB71, DP38, CL72-2, BV50-1
BV61-2
B34-1
HNGL01-3
STMX04-3, STST03-4
STMX03-2
STMX01-4, STST03-3, STMX02-2, STMX03-3, STMX04-5, STMX02-1, STMX01-5
L73, L38
AG 1-IA AB122133
STMX01-1, STST02-2, VLBT01-1, VLBT01-2, VLBT01-3, VLBT01-4, STMX01-2, CTCR01-1,
CTCR01-3
CTCR02-3 ,CTCR03-2, STST04-2, DNBH01-1,
CTCR02-1, CTCR01-2, CTCR03-1
DNBH01-2, DNBH01-3, DNBH02-2, DNBH02-3,
STMX04-1, STST03-1
HNGL01-1, HNGL01-2, STST02-1
HNDD01-3, HNDD01-1, HNDD01-2
CC72
BV71-2
L62-1
AG 1-IE DQ447863
AG 1-IA
AG 1-IE
DNBH0512
RMPG28
AG 1-IG
AG 7 AB000003
HNDA02-1
AG 7
AG 4-HG-I AB000007
STST03-2, DNBH0521, DNBH0532, DNBH02-1
CB 34-2
HNDD01-4, STST01-1
XL4
LDDT01-1, LDDT01-2, LDDT02-2, LDDT02-1, LDDL02-2, LDDT02-3
BV 68-2
KT 63-1
AG 4-HGI
AG 2-3 U57740
AG 2-3 AB054871
AG 2-3
AG 2-2 WB DQ452112
AG 2-2 WB DQ452114
AG 2-2 WB DQ452113
AG 2-2 WB DQ452111
AG 2-2 IIIB AF354116
AG 2-2 IIIB AJ238166
AG 2-2 LP AB054866
AG 2-2 LP AJ238163
AG 2-2 WB DQ452119
HNTT01-1
HNDA01-1
HNDA01-3, HNDA01-2
HNDA01-4
AG 2-2
LDDL01-2, LDDL01-1
LDDL05-1, LDDT03-1, LDDL01-3, LDDL04-2, LDDL05-2, LDDL01-4
LDDL04-1, LDDL04-3, LDDL04-4, LDDL01-4
AG 1-IB AB122139
LDDL05-3
AG 1-IB
CCD61-1
B61-1
AG 1-ID AB122130
BV 62-1
DNBH03-1, DNBH03-4, DNBH0511, DNBH0513, DNBH0522
DNBH03-2
DNBH03-3
DNBH0531
DNBH05-4
SR650
HGPH01-3, STST04-3, STST02-3, STST04-1, HGPH01-4, HGPH01-1, HGPH01-2, DNBH03-5
AG 1-ID
A. rolfsii AY684917
0.05

For most of the AGs we detected in Vietnam there was very little variation in ITS sequence, except for AG 4-HGI where six variable positions were found between the isolates from Lam Dong and the isolates from the other three provinces where this AG was detected (Soc Trang, Dong Nai and Ha Noi).

The AG 1-IA isolates showed identical or nearly identical (max. 4 SNPs) sequences to AG 1-IA isolates previously isolated from rice, water spinach, water hyacinth and other crops in Vietnam (Table 2.3). The AG 1-ID isolates showed high similarity (identical sequence or one SNP) to isolates detected on coffee, cotton, durian (Thuan et al. 2008), corn and sugar beet (Table 2.3) in Vietnam.

Relationship between the AGs found and sampling locations and between AGs found and *Brassica* species

There seems to be a relationship between the AGs present and the sampling areas (Figure 2.1 and Table 2.5). Thirty three out of 44 isolates belonging to AG 1-IA were recovered from samples collected from Soc Trang, Can Tho and Vinh Long in the Mekong River delta, the main rice production region of Vietnam. On the other hand, no AG 1-IA isolates were detected in Lam Dong although the expected frequency of this group in this province was high (9.98). The observed numbers were also significantly higher than the expected frequencies for AG 1-IB and AG 4-HGI in Lam Dong as well as for AG 1-ID in Hau Giang and Dong Nai.

The relationship between the occurrence of different AGs and the host plants is presented in Table 2.6. Significant AG-host correlations were identified in the following combinations: AG 1-IB and white cabbage, AG 1-IB and broccoli, AG 1-ID and Chinese flowering cabbage, and AG 2-2 and turnip cabbage.

Table 2.5. Contingency table with observed and expected frequencies of anastomosis groups (AGs) of *Rhizoctonia* spp. obtained from *Brassica* fields in different provinces of Vietnam

Province	AG/Subset									Total/ province
	Multinucleate *Rhizoctonia*							Binucleate *Rhizoctonia*		
	1-IA	1-IB	1-ID	1-IG	2-2	4-HGI	7	A	Fc	
Ha Noi	6 (5.90)	0 (1.74)	0 (2.28)	0 (0.13)	5 (0.67)	1 (1.61)	1 (0.13)	0 (0.40)	0 (0.13)	13
Lam Dong	0 (9.98)*	13 (2.95)*	0 (3.86)	0 (0.45)	0 (1.13)	6 (2.72)*	0 (0.23)	2 (0.68)	1 (0.23)	22
Dong Nai	5 (8.62)	0 (2.55)	10 (3.33)*	1 (0.20)	0 (0.98)	3 (2.35)	0 (0.20)	0 (0.59)	0 (0.20)	19
Vinh Long	4 (1.81)	0 (0.54)	0 (0.70)	0 (0.04)	0 (0.21)	0 (0.49)	0 (0.04)	0 (0.12)	0 (0.04)	4
Can Tho	8 (3.63)	0 (1.07)	0 (1.40)	0 (0.08)	0 (0.41)	0 (0.99)	0 (0.08)	0 (0.25)	0 (0.08)	8
Hau Giang	0 (1.81)	0 (0.54)	4 (0.70)*	0 (0.04)	0 (0.21)	0 (0.49)	0 (0.04)	0 (0.12)	0 (0.04)	4
Soc Trang	21 (12.25)*	0 (3.62)	3 (4.73)	0 (0.28)	0 (1.39)	2 (3.34)	0 (0.28)	1 (0.84)	0 (0.28)	27
Total/AG	44	13	17	1	5	12	1	3	1	97

Data show actual numbers of isolates collected among the different provinces. According to Fisher's exact test (p = 0.05), the AGs found are related to the sampling locations. An asterisk* indicates significant differences between observed and expected frequencies. Values in parentheses represent the expected frequencies.

Table 2.6. Contingency table with observed and expected frequencies of anastomosis groups (AGs) of *Rhizoctonia* spp. obtained from field-grown *Brassica* crops in Vietnam

Host plant	AG/Subset[a]									Total/host plant
	Multinucleate *Rhizoctonia*							Binucleate *Rhizoctonia*		
	1-IA	1-IB	1-ID	1-IG	2-2	4-HGI	7	A	Fc	
Mustard cabbage	14 (9.07)	0 (2.68)	5 (3.51)	0 (0.21)	0 (1.03)	1 (2.47)	0 (0.21)	1 (0.62)	0 (0.21)	20
White cabbage	8 (9.53)	8 (2.81)*	0 (3.68)	0 (0.22)	0 (1.08)	3 (2.60)	0 (0.22)	2 (0.65)	0 (0.22)	21
Chinese flowering cabbage	9 (12.25)	0 (3.62)	12 (4.73)*	1 (0.28)	1 (1.39)	4 (3.34)	0 (0.28)	0 (0.84)	0 (0.28)	27
Chinese cabbage	0 (2.27)	1 (0.67)	0 (0.88)	0 (0.05)	0 (0.26)	3 (0.62)	0 (0.05)	0 (0.15)	1 (0.05)	5
Pak choi	10 (4.99)	0 (1.47)	0 (1.93)	0 (0.11)	0 (0.57)	0 (1.36)	0 (0.11)	1 (0.34)	0 (0.11)	11
Broccoli	0 (1.81)	4 (0.54)*	0 (0.70)	0 (0.04)	0 (0.21)	0 (0.49)	0 (0.04)	0 (0.12)	0 (0.04)	4
Turnip cabbage	3 (4.08)	0 (1.21)	0 (1.58)	0 (0.09)	4 (0.46)*	1 (1.11)	1 (0.09)	0 (0.28)	0 (0.09)	9
Total/AG	44	13	17	1	5	12	1	3	1	97

Data show actual numbers of isolates collected among the different *Brassica* crops. According to Fisher's exact test (p = 0.05), the AGs found are related to the crops. An asterisk* indicates significant differences between observed and expected frequencies. Values in parentheses represent the expected frequencies.

Aggressiveness of *Rhizoctonia* isolates towards detached leaves

Nine *Rhizoctonia* isolates, randomly selected from each identified AG, were tested for their pathogenicity on detached leaves. Due to practical reasons, experiments with *Brassica* crops were conducted in a growth chamber specifically built for *Brassica* spp. (22°C, RH = 60%, 12 h photoperiod) and experiments with rice and water spinach were done in a growth chamber specifically built for rice (28°C, RH = 60%, 16 h photoperiod). Although the temperature and humidity in these chambers are slightly lower than those of Vietnam, they are still suitable for the growth of host plants and *Rhizoctonia* isolates involved in our study.

Table 2.7. Aggressiveness of *Rhizoctonia* isolates towards detached leaves of white cabbage, Chinese cabbage, pak choi, mustard cabbage, Chinese flower cabbage, rice and water spinach

AG/ Subgroup	Isolate	Disease index						
		White cabbage	Chinese cabbage	Pak choi	Mustard cabbage	Chinese flowering cabbage	Rice	Water spinach
Control		0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
1-IA	STMX04-2	4.00 d	2.63 d	1.79 d	1.79 c	2.17 e	3.92 d	3.21 f
1-IB	LDDL05-3	3.83 c	0.00 a	4.00 e	3.92 e	3.96 g	1.96 c	2.83 ef
1-ID	DNBH05-4	4.00 d	0.00 a	0.42 b	1.54 c	1.67 d	2.38 c	3.54 f
1-IG	DNBH05-1-2	4.00 d	1.00 b	0.63 bc	1.04 b	1.04 c	1.04 b	2.29 de
2-2	HNDA01-1	3.83 c	0.00 a	0.33 b	0.08 a	0.04 ab	1.29 b	1.75 cd
4-HGI	STST01-1	3.88 cd	1.54 c	2.13 d	3.88 e	3.71 f	2.33 c	2.67 e
7	HNDA02-1	3.13 b	0.00 a	0.00 a	0.13 a	0.17 b	2.29 c	0.46 b
A	DNBH04-1	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
Fc	LDDL02-1	3.38 b	0.71 b	1.17 c	2.58 d	1.17 c	0.00 a	1.38 c

Leaves were scored using a scale ranging from 0 (no disease symptoms) to 4 (lesions covered more than 75% of leaf surface or dead leaf). For rapid visual evaluation of the data, a coloring scale with green ($0 < DI \leq 1$), yellow ($1 < DI \leq 2$), orange ($2 < DI \leq 3$) and red ($3 < DI \leq 4$) was used. The experiment was conducted twice and each treatment consisted of 12 leaves or leaf pieces. The data of the two experiments were pooled before Mann-Whitney comparisons were applied at $p = 0.05$. Within columns, disease severities followed by the same letter are not significantly different.

As shown in Table 2.7, leaves of white cabbage, Chinese cabbage, pak choi, mustard cabbage, Chinese flowering cabbage, rice, and water spinach responded differently to the infection of

Rhizoctonia spp. although each host was affected by at least four AGs. For each plant species, a wide variation in symptom severity induced by different AGs was observed and the only AG that could not cause disease on any of the plants tested was AG-A. White cabbage leaves were most severely infected by all AGs (except AG-A) with a DI varying from 3.13 to 4.00. Compared to other hosts, Chinese cabbage appeared to be most resistant to *Rhizoctonia* isolates. No disease symptoms were observed on Chinese cabbage leaves challenged with AG 1-IB, AG 1-ID, AG 2-2 and AG 7, while other AGs were weak to moderately aggressive (DI varied from 0.71 in AG-Fc to 2.63 in AG 1-IA). Inoculation of pak choi with AG 1-IB resulted in a complete decay of all leaf discs (DI = 4.00). For mustard cabbage and Chinese flowering cabbage, a strong disease pattern was obtained on leaves confronted with AG 1-IB (DI ≥ 3.92) and AG 4-HGI (DI ≥ 3.71). Rice leaves were very susceptible to AG 1-IA and they were severely destroyed within four days of inoculation (DI = 3.92). Disease induced by other *R. solani* isolates on rice was also significantly different from the control although the two binucleate *Rhizoctonia* isolates tested were not pathogenic on rice. For water spinach, a wide variation in aggressiveness of *R. solani* isolates was observed, resulting in DI values ranging from 0.46 to 3.54. Large lesions (DI ≥ 3.21) developed on water spinach leaves inoculated with AG 1-IA and AG 1-ID isolates. The difference in pathogenicity of these AGs towards the different *Brassica* species was confirmed when additional isolates from AGs 1-IA, 1-IB, 1-ID, 2-2, 4-HGI and A were tested in a detached leaf assay on the same series of plants (see Supplementary information, Table A2.3).

In vitro pathogenic potential of *Rhizoctonia* spp. isolates on seedlings of white cabbage and Chinese cabbage

The results of the detached leaf assay revealed that white cabbage leaves were remarkably more susceptible than other cabbage species, while only some AGs could attack the leaves of Chinese cabbage. Therefore, these two plant species were selected as hosts for *in vitro* and *in vivo* pathogenicity tests using the same nine *Rhizoctonia* isolates as mentioned above. Under *in vitro* conditions (Table 2.8), all AGs could cause disease and symptoms were observed on both roots and leaves. Isolates of AG 1-IA, AG 1-IB, AG 2-2, AG 1-IG, AG 4-HGI and AG-Fc were most aggressive towards these hosts (for white cabbage: DI on roots ≥ 3.63 and DI on leaves ≥ 3.42; for Chinese cabbage: DI on roots ≥ 3.08 and DI on leaves ≥ 2.71). Fewer symptoms were recorded on seedlings inoculated with isolates belonging to AG 1-ID, AG 7 and AG-A. The virulence of AG 1-IA, AG 1-IB, AG 2-2 and AG 4-HGI towards white cabbage and Chinese cabbage was confirmed when *in vitro* assays were repeated with additional isolates of these

AGs (Table A2.4). Towards white cabbage, DI on roots varied from 2.17 to 4.00 and DI on leaves fluctuated between 2.33 and 4.00. DI on roots and leaves of Chinese cabbage ranged from 2.08 to 4.00 and from 1.92 to 4.00, respectively.

Table 2.8. Pathogenic potential of *Rhizoctonia* isolates on seedlings of white cabbage and Chinese cabbage in *in vitro* bio-assays

AG/Subgroup	Isolate	Disease Index			
		Root		Leaf	
		White cabbage	Chinese cabbage	White cabbage	Chinese cabbage
Control		0.00 a	0.00 a	0.00 a	0.00 a
1-IA	STMX04-2	3.63 d	3.08 d	3.42 d	2.71 cd
1-IB	LDDL05-3	4.00 e	3.75 ef	4.00 e	3.54 d
1-ID	DNBH05-4	1.88 bc	1.04 b	2.17 c	0.63 b
1-IG	DNBH05-1-2	4.00 e	4.00 g	4.00 e	4.00 e
2-2	HNDA01-1	4.00 e	3.96 fg	4.00 e	3.21 d
4-HGI	STST01-1	4.00 e	4.00 g	4.00 e	4.00 e
7	HNDA02-1	2.50 c	1.83 c	2.29 c	1.58 c
A	DNBH04-1	1.17 b	1.42 bc	0.29 b	0.00 a
Fc	LDDL02-1	3.75 de	3.42 de	3.83 de	3.50 d

Disease severity was assessed on a scale ranging from 0 (no symptoms) to 4 (lesions covering more than 75% of root, hypocotyl or leaf surface or dead plant). For rapid visual evaluation of the data, a coloring scale with green ($0 < DI \leq 1$), yellow ($1 < DI \leq 2$), orange ($2 < DI \leq 3$) and red ($3 < DI \leq 4$) was used. The experiment was done twice with 12 seedlings maintained in two square Petri plates for one treatment. The data of the two experiments were pooled before Mann-Whitney comparisons were applied at $p = 0.05$. Within columns, disease severities followed by the same letter are not significantly different.

In vivo pathogenic potential of *Rhizoctonia* spp. isolates on roots and hypocotyls of white cabbage and Chinese cabbage

The results displayed in Table 2.9 demonstrate that severe *Rhizoctonia*-induced damage on white cabbage roots could only be seen for AG 2-2 (DI = 3.50) and AG 4-HGI (DI = 3.63). Moderate infection (DI = 2.33) was observed in response to AG-Fc. Towards Chinese cabbage, severe disease symptoms were incited by AG 4-HGI (DI = 3.08) and AG-Fc (DI = 2.96). No symptoms were detected on roots and hypocotyls of seedlings challenged with AG-A. In addition, the inoculation of isolates belonging to AG 1-IA, AG 1-IB, AG 1-ID, AG 1-IG and AG 7 did not result in the formation of large lesions on roots of white cabbage and Chinese

cabbage seedlings (DI ≤ 1.57). Data obtained from the tests conducted with additional *Rhizoctonia* isolates belonging to AGs 1-IA, 1-IB, 1-ID, 2-2, 4-HGI and AG-A are presented in Table A2.5. These data confirm the high aggressiveness of AG 2-2 on white cabbage (DI = 4.00) and that of AG 4-HGI on both white cabbage and Chinese cabbage (DI ≥ 3.50).

Table 2.9. Pathogenic potential of *Rhizoctonia* isolates on roots and hypocotyls of white cabbage and Chinese cabbage in *in vivo* experiment

AG/Subgroup	Isolate	Disease Index	
		White cabbage	Chinese cabbage
Control		0.00 a	0.00 a
1-IA	STMX04-2	0.27 b	0.21 ab
1-IB	LDDL05-3	1.57 c	0.29 bc
1-ID	DNBH05-4	0.00 a	0.08 ab
1-IG	DNBH05-1-2	0.00 a	0.13 ab
2-2	HNDA01-1	3.50 e	1.57 d
4-HGI	STST01-1	3.63 e	3.08 e
7	HNDA02-1	0.26 b	0.79 c
A	DNBH04-1	0.00 a	0.00 a
Fc	LDDL02-1	2.33 d	2.96 e

Disease severity on roots was assessed on a scale ranging from 0 (no symptoms) to 4 (seedling dead). For rapid visual evaluation of the data, a coloring scale with green (0 < DI ≤ 1), yellow (1 < DI ≤ 2), orange (2 < DI ≤ 3) and red (3 < DI ≤ 4) was used. The experiment was performed twice with 12 seedlings cultivated in two plastic boxes per treatment. The data of the two experiments were pooled before Mann-Whitney comparisons were applied at p = 0.05. Within columns, disease severities followed by the same letter are not significantly different.

Discussion

Rhizoctonia is an important fungal 'form genus' occurring worldwide and including many important plant pathogenic strains as well as mycorrhizal fungi and hypovirulent or avirulent strains among which there are strains that are capable to protect plants against pathogenic *Rhizoctonia* and other pathogens as well as increase plant growth. Plants can be infected by different *Rhizoctonia* AGs from the time of sowing resulting in the development of both foliar and root diseases. This is the first time *Rhizoctonia* species that attack field-grown *Brassica* crops in Vietnam were isolated and characterized. Ninety seven isolates of *Rhizoctonia* were recovered from symptomatic plant tissues of seven brassicaceous hosts (mustard cabbage, white

cabbage, Chinese flowering cabbage, pak choi, turnip cabbage, Chinese cabbage and broccoli). Of all the isolates collected, 4% were binucleate *Rhizoctonia* and 96% were multinucleate *Rhizoctonia*. Molecular characterization by sequencing of the rDNA-ITS region showed that the binucleate isolates found belonged to AG-A (3 isolates) and an unknown AG introduced here as AG-Fc (1 isolate). Comparison to a curated sequence database of all known *Rhizoctonia* multinucleate, binucleate and uninucleate AGs did not reveal high homology of this isolate to a known AG. Pairwise sequence similarities to all known multinucleate, binucleate and uninucleate *Rhizoctonia* AGs showed highest similarity to AG-Fb, but also to AG 6, which is a multinucleate AG. Close relationships of some binucleate groups with multinucleate groups has been previously noted by Sharon et al. (2008) who did a combined ITS sequence analysis of multinucleate, binucleate and uninucleate groups. These authors stated that the clustering of binucleate and uninucleate groups close to certain multinucleate clusters may indicate a possible evolutionary bridge between multinucleate and binucleate groups and our results support this hypothesis.

We conducted pathogenicity assays on detached leaves, *in vitro* seedlings and plants grown *in vivo*. In general, pathogenicity was highest on roots and leaves under *in vitro* conditions, which is probably due to the high humidity and the young age of the plants in this system. It should be mentioned that we only checked *in vivo* pathogenicity towards roots and hypocotyls, but it should be borne in mind that some of our isolates are mainly leaf pathogens.

Although AG-A isolates were obtained from symptomatic plants, they were unable to induce disease on detached leaves or on seedlings *in vivo*, and were only slightly pathogenic on cabbage seedlings under *in vitro* conditions. This low virulence may be due to the differences in humidity between our experimental conditions and the field situation in Vietnam. Alternatively, AG-A isolates might be avirulent, but since they grow very fast *in vitro*, they may have masked the presence of slower growing, virulent AGs. In contrast to AG-A, AG-Fc appeared to be moderately to highly virulent in all experiments. This also concurs with previous studies that some binucleate *Rhizoctonia* species are highly virulent (Martin 1988; Demirci et al. 2002; Babiker et al. 2013), whereas others are weakly virulent or avirulent (Herr 1995; Poromarto et al. 1998; Ross et al. 1998).

Among the multinucleate AGs, AG 1-IA was the dominant group, followed by AG 1-ID, AG 1-IB, AG 4-HGI, AG 2-2, AG 7 and an unknown AG that we introduced here as AG 1-IG. The occurrence of AG 1-IB and AG 4 on *Brassica* spp. is well known from previous studies. According to Pannecoucque et al. (2008), AG 1-IB is one of the causal agents of wirestem in Belgian cauliflower fields. AG 1-IB isolates are also highly virulent on lettuce in Belgium (Van

Beneden et al. 2009). In our study, AG 1-IB isolates were only recovered from Lam Dong, a province in the cool Central Highlands of Vietnam and the main lettuce production region of Vietnam, suggesting that the presence of this AG is associated with cool climates. The presence of AG 4 has also been reported on *Brassica oleracea* (Yang et al. 2007) and *B. rapa* subsp. *chinensis* (Yang et al. 2004) in China, *B. oleracea* in the UK (Budge et al. 2009), *B. napus* L. and *B. campestris* L. in Canada (Yitbarek et al. 1987), and *B. oleracea* (Rollins et al. 1999) and *B. napus* L. in the US (Baird 1996). AG 4-HGI occurred in most regions sampled. This AG has a wide host range due to its ability to adapt to temperature variation and cropping patterns (Harikrishnan and Yang 2004). AG 4 isolates are able to induce disease on all plant parts and in our pathogenicity trials, the highest disease ratings on cabbage in all bioassays were shown for the AG 4-HGI isolates. For white cabbage, severe disease symptoms were observed on seedlings inoculated with AG 2-2 and AG 4-HGI isolates *in vivo* or on detached leaves challenged with isolates of all AGs. Although *R. solani* AG 2-1 is considered the most dominant and damaging anastomosis group attacking *Brassica* spp. (Pannecoucque et al. 2008; Budge et al. 2009; Ohkura et al. 2009), isolates belonging to this group were not detected in our survey.

The predominance of *R. solani* isolates of AG 1-IA and also the presence of isolates belonging to AG 1-ID in our collection were not anticipated because these AGs have not been described on *Brassica* crops before. The presence of unusual AGs in our sampling is probably due to three aspects: (i) alternative hosts of *Rhizoctonia* present in the sampling locations; (ii) poor cultural practices and (iii) high temperature. AG 1-IA isolates were mainly recovered from samples collected in provinces of the Mekong River delta including Vinh Long, Can Tho and Soc Trang. This is a low-lying coastal region of Vietnam, characterized by high temperature and humidity and prone to flooding every rainy season. Due to water availability and soil type, the agricultural production in this area is dominated by rice (SIHYMETE 2010; IFRC 2013). As previously reported, sheath blight, caused by *R. solani* AG 1-IA, is a major disease of rice cultivated in intensive production systems (Lee and Rush 1983; Ogoshi 1987; Taheri et al. 2007). With the ability to float and to survive in water (Hashiba et al. 1972), sclerotia of AG 1-IA isolates may easily spread from the rice paddy fields to vegetable fields through irrigation or flood water. The recovery of AG 1-IA in the hot region located in the South of Vietnam is consistent with the findings of Harikrishnan and Yang (2004) that temperature can influence growth rate, sclerotia production of *Rhizoctonia* spp. and the distribution of *Rhizoctonia* isolates belonging to different anastomosis groups. AG 1 is a high temperature group (Sneh et al. 1991) and its vegetative growth as well as sclerotia production and survival are inhibited at low

temperatures. The occurrence of AG 1-IA on *Brassica* crops is probably even increased due to farmers' lack of knowledge about crop protection. AG 1 (not specific) has been considered as one of the causal agents of foliar diseases on water hyacinth (*Eichhornia crassipes*) (Freeman et al. 1982), water lettuce (*Pistia stratioites*) and anchoring hyacinth (*E. azurea*) (Zettler and Freeman 1972). In this study, water spinach (*Ipomoea aquatic*) was found to be susceptible to the AG 1-IA isolates that we collected from *Brassica* spp. Vietnamese farmers commonly use these aquatic plants as cover materials in vegetable production and use irrigation water from sources where these plants are present, thus bringing the fungus from these alternative hosts to *Brassica* spp. (Figure 2.4). Isolates of AG 1-IA appeared to be very pathogenic towards leaves of cabbages but they could not induce severe disease on roots, especially under *in vivo* conditions. This finding is in agreement with results reported previously by Yang and Li (2012) that AG 1-IA isolates have a tendency to attack aerial parts of plants. These data also support our hypothesis about the spread of AG 1-IA isolates from rice and water spinach to vegetables. In other words, rice and water spinach that are infected by *R. solani* could be an important source of inoculum that may contribute to the disease caused by AG 1-IA on *Brassica* spp.

In our assays AG 1-ID was mainly pathogenic on leaves of white cabbage. *Rhizoctonia* AG 1-ID was previously found to be pathogenic on durian (Thuan et al. 2008) and coffee (Priyatmojo et al. 2001). Interestingly, ten out of 17 AG 1-ID isolates were collected from Dong Nai, a province where durian, coffee and cotton are widely cultivated (People's committee of Dong Nai province 2011), again suggesting a correlation between cropping patterns and *Rhizoctonia* distribution in Vietnam.

Figure 2.4. *Rhizoctonia*-infected water hyacinth is introduced as cover material to a white cabbage field in Vietnam

Rhizoctonia-infected water hyacinth is taken from a nearby water ditch and used as cover material on the white cabbage field. Via this common practice, Vietnamese farmers unintentionally introduce the *Rhizoctonia* fungus to their crops.

Collectively, it seems that the distribution of *Rhizoctonia* AGs in Vietnam is correlated with the cropping patterns and climatic conditions. However, it is difficult to draw a strong conclusion about the influence of cropping patterns and climatic conditions on the occurrence of *Rhizoctonia* spp. in Vietnam because the sampling regime for *R. solani* isolates comprises two variable parameters, namely the plant species infected by *R. solani* and the sampling site. Due to the variation in soil and climatic conditions, different *Brassica* species are grown in different geographic regions and there is a possibility that particular plant species might be more susceptible to infections by specific *R. solani* AGs than others. Therefore, another field survey with a systematic sampling regime needs to be conducted to confirm our hypothesis.

Our research also points towards the need to have good extension programs to improve the farmers' knowledge about crop protection. Additionally, knowing which AGs are responsible

for *Rhizoctonia* diseases on *Brassica* spp. in Vietnam is an essential prerequisite for developing successful disease management strategies in this country.

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Addendum A2
Supplementary tables

Table A2.1. GPS co-ordinates of the wards in each province of Vietnam where *Rhizoctonia*-infected *Brassica* crops were sampled

Province	District / City	Ward	Latitude	Longitude
Ha Noi	Gia Lam	Dong Du	21° 0'19.04"N	105°55'4.36"E
	Thanh Tri	Ngoc Hoi	20°55'36.59"N	105°50'36.69"E
	Dong Anh	Nam Hong	21°10'4.17"N	105°47'32.86"E
		Van Noi	21° 9'11.05"N	105°48'54.83"E
Lam Dong	Da Lat city	Ward 6	11°58'3.28"N	108°26'1.98"E
		Ward 8	11°59'38.29"N	108°27'30.02"E
	Duc Trong	Ninh Loan	11°34'25.74"N	108°20'42.95"E
		Phu Hoi	11°40'39.69"N	108°21'30.33"E
Dong Nai	Bien Hoa	Tan Bien	10°58'59.62"N	106°53'56.66"E
Vinh Long	Binh Tan	Tan Quoi	10° 6'8.14"N	105°45'36.02"E
Can Tho	Cai Rang	Hung Phu	10° 2'9.99"N	105°47'45.59"E
		Thuong Thanh	9°59'21.92"N	105°45'24.03"E
Hau Giang	Phung Hiep	Long Thanh	9°53'16.55"N	105°44'36.64"E
Soc Trang	Soc Trang	Ward 3	9°35'22.39"N	105°58'36.91"E
	My Xuyen	Dai Tam	9°33'3.54"N	105°55'10.88"E

Table A2.2. Curated database of sequences containing representative rDNA-ITS sequences of all known uninucleate, binucleate and multinucleate *Rhizoctonia* AG and subgroups

AG/Subgroup	Genbank accession numbers
From Sharon et al. (2008)	
W. circinata	AB213577; AB213575; AB213594; AB213597; AB213582; AB213581; AJ000195; AB213589
AG-D	AB198695; AB198694; AB198702; AB198698; AB214367; AB198708
AG-I	AB196650; DQ102443
AG-C	AJ242894
AG-H	AB196649; AB196648
AG-C	AB196642; AB290021; AB290020
AG-Ba	AB286930; AF354088
AG-Bo	AB219143; DQ102430
AG-Q	AF354095
AG-Bb	AF354087; AB122144
AG-K	AB196652; AB122145
AG-A	DQ102407; DQ102421; AB196659; AB196663; U19950
AG-G	AB196647; DQ102401
AG-L	AB196653; AF354093
AG-O	AB286937; AF354094
AG 6	AF354104; AY154304; AF153780; AF153782; AF354102; AB000019; AF153788; AF153790; AF153787; AF153785
AG 4	DQ102449; AY154659; AB000007; AY152704; AY154308; AB000006
AG 5	AF354113; AF153778
AG 2-1	AY154317; AB054852; AB054850
AG 2-4	AB054878; AB054880; AB054879
AG 2-3	U57729; U57740; AB054871
AG 2-2 LP	AB054866; AJ238160; AJ238163
AG 2-2 IIIB	AF354116; AJ238166
AG 2-2 IV	AY270014; AJ238164; AB000014
AG-BI	AB054873; AB054875
AG 3	AB000004; AF153774; AB019023; AB019017
AG 11	AY154313; AF153802
AG 8	AF153797; AF354068; AB000011
AG 9	AF354108; AF354065
AG 10	AF354071; AF153800
AG 12	AF153804; AF153805
AG 7	AF354100; AB000003
AG 1-IA	AB122133; AY270010

AG/Subgroup	Genbank accession numbers
AG 1-IC	AB122142; U19951
AG 1-IB	AB122139; AF308626
AG 1-ID	AB122128; AB122130
AG-Fb	AB219145; AF354081
AG-R	AJ427407; J-04-2
AG-P	AB286941; AB196655; AAB286939; AB286940; AB196664; AB196665
AG-S	AF354084
AG-Fa	DQ102436; AB219144
AG-E	AF354083; AB290017
UNR1	AF472295; AF472278
UNR2	AF200515; AF200517
AG-N	AB286935
A. rolfsii	AY684917
From Carling et al. (2002a)	
AG 13	AB275641
From Godoy-Lutz et al. (2008)	
AG 1-IF	AF308627
AG 1-IE	DQ447863
AG 2-2 WB	DQ452119; DQ452111; DQ452112; DQ452113; DQ452114

Table A2.3. Aggressiveness of *Rhizoctonia* isolates towards white cabbage, Chinese cabbage, pak choi, mustard cabbage, Chinese flower cabbage, rice and water spinach in detached leaf bio-assays

AG/ Subgroup	Isolate	Disease Index						
		White cabbage	Chinese cabbage	Pak choi	Mustard cabbage	Chinese flowering cabbage	Rice	Water spinach
	Control	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
1-IA	VLBT01-4	4.00 c	1.75 b	2.50 de	1.33 c	1.92 cd	4.00 d	3.75 e
	CTCR02-3	3.83 bc	2.17 bc	1.67 cd	1.00 c	2.67 de	3.75 d	3.33 e
	STST03-3	4.00 c	2.75 c	1.67 cd	1.08 c	2.25 d	3.75 d	3.50 e
	STMX02-1	4.00 c	2.67 c	1.50 c	1.17 c	2.08 cd	4.00 d	3.25 de
1-IB	LDDL04-1	4.00 c	0.00 a	3.83 f	4.00 f	3.75 fg	1.08 b	3.00 de
	LDDL05-2	4.00 c	0.50 a	4.00 f	3.75 ef	3.92 g	1.00 b	3.00 de
1-ID	HGPH01-4	4.00 c	0.00 a	0.50 b	1.67 cd	1.25 bc	1.75 c	2.67 cd
	STST02-3	4.00 c	0.00 a	0.58 b	2.00 de	1.75 cd	1.42 bc	2.92 de
	DNBH05-1-1	3.17 b	0.00 a	0.42 b	1.83 d	1.67 cd	1.67 c	2.75 cde
2-2	HNTT01-1	4.00 c	0.00 a	0.50 b	0.00 a	0.00 a	1.08 b	1.92 bc
4-HGI	DNBH05-3-2	3.00 b	2.92 c	2.92 e	3.75 ef	3.17 ef	1.17 b	2.17 c
	LDDT02-3	4.00 c	2.75 c	3.42 ef	3.67 ef	3.33 f	1.42 bc	1.50 b
A	LDDL03-1	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
	LDDL03-2	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a

Leaves were scored using a scale ranging from 0 (no disease symptoms) to 4 (lesions covered more than 75% of leaf surface or dead leaf). For rapid visual evaluation of the data, a coloring scale with green ($0 < DI \leq 1$), yellow ($1 < DI \leq 2$), orange ($2 < DI \leq 3$) and red ($3 < DI \leq 4$) was used. The test was done once with 12 leaves or leaf pieces per treatment. All data were statistically analyzed and within columns, disease severities followed by the same letter are not significantly different.

Table A2.4. Aggressiveness of *Rhizoctonia* isolates towards roots and leaves of white cabbage and Chinese cabbage seedlings in *in vitro* bio-assays

AG/ Subgroup	Isolate	Disease Index			
		Root		Leaf	
		White cabbage	Chinese cabbage	White cabbage	Chinese cabbage
	Control	0.00 a	0.00 a	0.00 a	0.00 a
1-IA	VLBT01-4	3.67 cd	2.67 bc	2.75 def	2.17 cd
	CTCR02-3	2.17 b	2.42 bc	2.33 cde	2.75 d
	STST03-3	2.58 bc	2.08 bc	2.92 def	2.33 cd
	STMX02-1	2.42 bc	3.25 cd	3.08 ef	1.92 bcd
1-IB	LDDL04-1	4.00 d	3.58 cd	4.00 g	2.92 de
	LDDL05-2	3.17 c	3.83 cd	3.33 f	3.17 def
1-ID	HGPH01-4	2.25 bc	1.83 b	1.25 b	1.17 b
	STST02-3	3.33 c	1.92 b	3.33 f	1.00 b
	DNBH05-1-1	1.92 b	1.50 b	2.17 bcde	1.42 bc
2-2	HNTT01-1	4.00 d	3.83 cd	4.00 g	2.42 cd
4-HGI	DNBH05-3-2	4.00 d	4.00 d	4.00 g	4.00 f
	LDDT02-3	4.00 d	3.92 d	4.00 g	3.83 ef
A	LDDL03-1	1.67 b	1.75 b	1.50 bc	1.42 bc
	LDDL03-2	2.08 b	1.75 b	1.67 bcd	1.45 bc

Disease severity on roots or leaves was assessed on a scale ranging from 0 (no symptoms) to 4 (lesions covering more than 75% of root, hypocotyl or leaf surface or dead plant). For rapid visual evaluation of the data, a coloring scale with green ($0 < DI \leq 1$), yellow ($1 < DI \leq 2$), orange ($2 < DI \leq 3$) and red ($3 < DI \leq 4$) was used. Experiment was conducted once with 12 seedlings maintained in two square Petri plates for one treatment. All data were statistically analyzed and within columns, disease severities followed by the same letter are not significantly different.

Table A2.5. Aggressiveness of *Rhizoctonia* isolates towards roots of white cabbage and Chinese cabbage seedlings in *in planta* experiment

AG/Subgroup	Isolate	Disease Index	
		White cabbage	Chinese cabbage
	Control	0. 00 a	0.00 a
1-IA	VLBT01-4	0.25 ab	0.42 b
	CTCR02-3	0.27 ab	0.58 b
	STST03-3	0.58 bc	0.70 b
	STMX02-1	0.30 ab	0.17 ab
1-IB	LDDL04-1	0.92 bc	1.00 bc
	LDDL05-2	1.36 c	0.82 b
1-ID	HGPH01-4	0.56 b	1.00 bc
	STST02-3	0.25 ab	0.42 b
	DNBH05-1-1	0.17 ab	0.25 ab
2-2	HNTT01-1	4.00 d	1.50 c
4-HGI	DNBH05-3-2	4.00 d	3.67 d
	LDDT02-3	3.50 d	3.58 d
A	LDDL03-1	0.00 a	0.00 a
	LDDL03-2	0.00 a	0.00 a

Disease severity on roots was assessed on a scale ranging from 0 (no symptoms) to 4 (seedling dead). For rapid visual evaluation of the data, a coloring scale with green ($0 < DI \leq 1$), yellow ($1 < DI \leq 2$), orange ($2 < DI \leq 3$) and red ($3 < DI \leq 4$) was used. Experiment was performed once. Each treatment consisted of 12 seedlings cultivated in two plastic boxes. Data were statistically analyzed and within columns, disease severities followed by the same letter are not significantly different.

Chapter

3

Biological control of Rhizoctonia root rot on bean by phenazine- and cyclic lipopeptide-producing *Pseudomonas* sp. CMR12a

Jolien D'aes, Gia Khuong Hoang Hua, Katrien De Maeyer, Joke Pannecoucque, Ilse Forrez, Marc Ongena, Lars E. P. Dietrich, Linda S. Thomashow, Dmitri V. Mavrodi, and Monica Höfte

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Abstract

Pseudomonas sp. CMR12a was previously selected as an efficient biocontrol strain producing phenazines and cyclic lipopeptides (CLPs). In this study, biocontrol capacity of *Pseudomonas* sp. CMR12a against Rhizoctonia root rot of bean and the involvement of phenazines and CLPs in this ability were tested. Two different anastomosis groups (AGs) of *R. solani*, the intermediately aggressive AG 2-2 and the highly aggressive AG 4-HGI, were included in growth chamber experiments with bean plants. The wild type strain CMR12a dramatically reduced disease severity caused by both *R. solani* AGs. A CLP-deficient and a phenazine-

deficient mutant of CMR12a still protected bean plants, albeit to a lesser extent compared to the wild type. Two mutants deficient in both phenazine and CLP production completely lost their biocontrol activity. Disease suppressive capacity of CMR12a decreased after washing bacteria before application to growth substrate, and thereby removing metabolites produced during growth on plate. In addition, microscopic observations revealed pronounced branching of hyphal tips of both *R. solani* AGs in the presence of CMR12a. More branched and denser mycelium was also observed for the phenazine-deficient mutant; but neither the CLP-deficient mutant nor the mutants deficient in both CLPs and phenazines influenced hyphal growth. Together, results demonstrate the involvement of phenazines and CLPs during *Pseudomonas* sp. CMR12a-mediated biocontrol of Rhizoctonia root rot of bean.

Introduction

Rhizoctonia solani (teleomorph: *Thanatephorus cucumeris*) is a soil-borne pathogen causing severe damage to a wide range of crops. To date, this fungal species is subdivided into 13 different anastomosis groups (AGs) which may possess similar characteristics, such as host preference, pathogenicity and type of disease symptom caused (Carling et al. 2002a). In the production of common bean (*Phaseolus vulgaris*), root rot caused by *R. solani* is a major constraint leading to severe crop losses, especially under tropical and subtropical climate conditions. Several AGs of *R. solani* have been identified causing root rot on bean, the most frequently reported being AG 2-2 and AG 4 (Muyolo et al. 1993; Balali and Kowsari 2004; Nerey et al. 2010). Knowledge about the prevalence and distribution of different AGs is important, since sensitivity to chemical control treatments and probably also to other control strategies is varying among AGs (Kataria and Gisi 1996; Campion et al. 2003).

Beneficial bacteria have been intensively studied as biocontrol agents against soil-borne diseases (Weller 1988; Handelsman and Stabb 1996). Good results have been obtained with Gram positive *Bacillus* species and Gram negative *Pseudomonas* species in the control of several plant pathogens, including *Gaeumannomyces graminis* var. *tritici*, *Fusarium* spp. and *Pythium* spp. (Kloepper et al. 2004; Haas and Defago 2005; Weller 2007). In case of *R. solani*, studies with different bacterial strains indicated that the biocontrol mechanism can be either induction of systemic resistance or antibiosis (Howell and Stipanovic 1979; Nandakumar et al. 2001). Among the great variety of beneficial bacteria and antibiotic metabolites already discovered our attention has been focused on phenazines and biosurfactants produced by

fluorescent pseudomonads.

Phenazines are broad-spectrum, heterocyclic antibiotics known for their antifungal properties. The most commonly identified phenazine pigments produced by *Pseudomonas* spp. are pyocyanin, phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN) and a number of hydroxy-phenazines (Turner and Messenger 1986). Production of phenazines is essential for the biocontrol capacity of *P. fluorescens* 2-79 (Thomashow and Weller 1988), *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998) and *P. aeruginosa* PNA1 (Tambong and Höfte 2001). In the biological control of *R. solani*, only a few reports suggest the involvement of phenazines. Rosales et al. (1995) observed *in vitro* inhibition of mycelial growth of *R. solani* by *P. aeruginosa* strains which produced PCA and pyocyanin, while disease suppression of Rhizoctonia root rot of wheat *in vivo* was enhanced after transformation of *P. fluorescens* Q8r1-96 with the biosynthesis genes for PCA (Huang et al. 2004). However, conclusive evidence for the importance of phenazines in the biological control of *Rhizoctonia* diseases using phenazine-negative mutants is still lacking.

Biosurfactants are amphiphilic compounds possessing both hydrophilic and lipophilic properties. These surface-active metabolites can be produced by several *Pseudomonas* spp.; the most interesting ones include rhamnolipids and several types of cyclic lipopeptides (CLPs), both of which may possess antifungal properties (Stanghellini and Miller 1997; Ron and Rosenberg 2001; Raaijmakers et al. 2006). Biocontrol related research on biosurfactants has predominantly focused on the control of zoospore producing plant pathogens, such as *Pythium* and *Phytophthora* spp., in which case the mode of action resides on zoospore lysis (Stanghellini and Miller 1997; De Souza et al. 2003; De Jonghe et al. 2005). Only a few studies have demonstrated the involvement of biosurfactants in biological control of non-zoospore producing plant pathogens. A direct effect of biosurfactants on mycelium of *Pythium myriotylum* and *Pythium splendens* was hypothesized by Perneel et al. (2008), while Debode et al. (2007) found a major role for biosurfactants in the reduction of *Verticillium* microsclerotia viability. *In vitro* and in soil microcosm, antifungal properties of biosurfactants against *R. solani* have been described for the CLPs viscosinamide, tensin and amphisin (Nielsen et al. 1999; Nielsen et al. 2000; Thrane et al. 2001; Andersen et al. 2003). However, no literature reports were found which proved a crucial role for CLPs in disease suppression of *R. solani in planta*.

In the present study, we investigate the potential of *Pseudomonas* sp. CMR12a to reduce Rhizoctonia root rot of bean caused by two different anastomosis groups of *R. solani*, AG 2-2 and AG 4-HGI. *Pseudomonas* sp. CMR12a, which produces both cyclic lipopeptide-type

biosurfactants and the phenazines PCN and PCA, is a promising biocontrol strain previously isolated from the cocoyam rhizosphere in Cameroon (Perneel et al. 2007). Analysis of 16S rDNA sequences and phenazine biosynthesis genes indicated that CMR12a probably represents a novel species within the *P. putida* species complex (Perneel et al. 2007; Mavrodi et al. 2010). Mutants of CMR12a, deficient in CLP and/or phenazine production, were used to investigate the role of both compounds in disease suppression of Rhizoctonia root rot on bean.

Materials and methods

Microorganisms and culture conditions

Microorganisms used in this study are listed in Table 3.1. *R. solani* isolates were maintained on Potato dextrose agar (PDA; Difco) plates at 28°C. *Pseudomonas* strains were routinely grown on King's medium B (KB; King et al. 1954) at 28°C. *Escherichia coli* strains were grown on Luria Bertani medium (LB; Sambrook et al. 1989) at 37°C, and *Saccharomyces cerevisiae* on yeast-extract-peptone-dextrose at 30°C (Shanks et al. 2006). When required, gentamycin was added at a concentration of 25 µg ml^{-1} for *E. coli* strains and at 300 µg ml^{-1} for *Pseudomonas* sp. CMR12a and mutant strains.

Table 3.1. Microorganisms, plasmids and primers used in this study

Strains, plasmids, and primers	Relevant characteristics*	Reference
Pseudomonas		
CMR12a	PHZ$^+$, CLP1$^+$, CLP2$^+$, wild type (Cameroon)	Perneel et al. (2007)
CMR12a-GacA	PHZ$^-$, CLP1$^-$, CLP2$^-$; spontaneous *gacA* mutant of CMR12a, formerly designated CMR12a-Reg	Pham et al. (2008)
CMR12a-ΔPhz	PHZ$^-$, CLP1$^+$, CLP2$^+$, mutant with deletion of phenazine biosynthesis operon	This study
CMR12a-Clp1	PHZ^{++}, CLP1$^-$, CLP2$^+$, mutant with insertion in CLP1 biosynthesis genes, Gm^R	This study
CMR12a-ΔPhz-Clp1	PHZ$^-$, CLP1$^-$, CLP2$^+$, phenazine deletion and CLP1 biosynthesis insertion mutant, Gm^R	This study
R. solani		
AG 2-2 CuHav-Rs18	Causal agent of root rot on bean (intermediately aggressive) (Cuba)	Nerey et al. (2010)
AG 4-HGI CuLT-Rs36	Causal agent of root rot on bean (highly aggressive) (Cuba)	Nerey et al. (2010)
E. coli		
DH5α	Host for cloning	Hanahan (1983)
SM10	Donor strain for conjugation; λ pir; Km^R	Herrero et al. (1990)
HB101	Helper strain for conjugation	Boyer and Roulland-Dussoix (1969)
BW29427	Donor strain for conjugation; λ pir	Dietrich et al. (2006)
S. cerevisiae InvSc1	Yeast strain for *in vivo* recombination (*ura3-52/ura3-52* mutation)	Invitrogen
Plasmids		
pMQ30	Gene replacement vector for *Pseudomonas* species; *sacB*, URA3, Gm^R	Shanks et al. (2006)
pEX18Tc	Gene replacement vector with MCS from pUC18; oriT, *sacB*, Tc^R	Hoang et al. (1998)
pRK2013	Conjugation helper plasmid, *ori* ColE1, *tra*$^+$, *mob*$^+$; Km^R	Figurski and Helinski (1979)
pUCGM	Source of Gm^R cassette, Ap^R, Gm^R	Schweizer (1993)
pGV4692 (miniTn*phoA3*)	Mini-TnphoA3 transposon with the Gm cassette on pGV4692, a derivative of pUT (Ap^R)	De Lorenzo et al. (1990); Pattery et al. (1999)
pJET1.2	General cloning vector, Ap^R	Fermentas
Primers (5' → 3')		
PhzA-Up-F	*GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTG*ACA ACTCGTTACCCCACAGG	This study
PhzA-Up-R	*GTCCTTGAGCAACTCTTGGG*TGCCGTCTCAGTGACAGTTC	This study
PhzH-Down-F	*GAACTGTCACTGAGACGGCA*CCCAAGAGTTGCTCAAGGAC	This study
PhzH-Down-R	*CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGAT*AAG GTACGCTGGAGCCAGTA	This study
CLP12a_F	GTGGTCGACATTGATACCTGGCCGCAA	This study
CLP12a_R	GTGGGATCCTGAGGATCGAACTGAACAGC	This study

* PHZ, phenazines; CLP, cyclic lipopeptides; +: positive for trait (similar to wild type), ++: overproduction compared to wild type, -: negative for trait, Gm^r, Tc^r, Ap^r, Km^r, resistant to gentamycin, tetracyclin, ampicillin, kanamycin, respectively. Primer extensions are in italic font.

Construction of vectors and CMR12a mutant strains

Molecular techniques were performed according to standard methods (Sambrook et al. 1989). *Taq* DNA polymerase and Q-solution were purchased from Qiagen (Venlo, the Netherlands) and polymerase chain reaction (PCR) was carried out as recommended by the manufacturer. Plasmids and primers are listed in Table 3.1.

Construction of pMQ30-ΔPhz and mutant CMR12a-ΔPhz

To construct mutant CMR12a-ΔPhz, the complete phenazine biosynthesis operon *phzABCDEFGH* (De Maeyer et al. 2011) was deleted, using allelic replacement vector pMQ30, as described by Shanks et al. (2006) and Hoang et al. (1998). Two regions upstream and downstream of the phenazine biosynthesis operon *phzABCDEFGH* of CMR12a were amplified with primers PhzA-Up-F and PhzA-Up-R (856 bp) and primers PhzH-Down-F and PhzH-Down-R (904 bp), respectively. These PCR products were cloned flanking each other via *in vivo* homologous recombination in the yeast *S. cerevisiae* InvSc1. This procedure yielded deletion plasmid pMQ30-ΔPhz, which was mobilized into CMR12a by conjugation with *E. coli* BW29427 and selection on gentamycin. Transconjugants which had lost the plasmid during a second crossover event were selected on 10 % sucrose, whereupon the deletion was confirmed by PCR.

Cloning of cyclic lipopeptide biosynthesis genes of CMR12a

To track down the genes responsible for biosurfactant synthesis in CMR12a, a transposon mutant library, constructed by mating with *E. coli* SM10 carrying pUT-miniTn5phoA3 (De Chial et al. 2003), was screened for mutants with reduced surface-activity on KB medium by means of a drop collapse test (Jain et al. 1991). In one of these biosurfactant deficient mutants, the transposon interrupted an ORF showing homology with non ribosomal peptide synthetase (NRPS) genes responsible for CLP synthesis in other pseudomonads. This sequence was used as a probe for screening a pRG930 cosmid genomic library of CMR12a (De Maeyer et al. 2011). Primer walking on positive clone 24G3 yielded a 1.9 kb DNA fragment, which was used to create a site-specific CLP mutant in CMR12a.

Construction of pEX18Tc-Clp1 and mutants CMR12a-Clp1 and CMR12a-ΔPhz-Clp1

CLP insertion mutants CMR12a-Clp1 and CMR12a-ΔPhz-Clp1 were constructed using vector pEX18Tc (Hoang et al. 1998). A 1.8 kb fragment of a non-ribosomal peptide synthetase presumably coding for biosurfactant biosynthesis in CMR12a (see above) was amplified with primers CLP12a_F and CLP12a_R. The resulting PCR-product was cloned in pEX18Gm and cut with *Sal*I and *Bam*HI to insert a gentamycin resistance cassette from pUCGM (Schweizer 1993). Following mobilization of the resulting plasmid (pEX18Tc-CLP1) into CMR12a and CMR12a-ΔPhz, transconjugants which lost the suicide plasmid after a second cross-over event were selected on LB with 5 % sucrose. The Gm^R insertion was confirmed by PCR.

Analysis of phenazine and CLP production

Phenazines were extracted and analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) as described before (Perneel et al. 2008; Pham et al. 2008). To obtain quantitative data for phenazine production, spectrophotometric measurements (λ = 369 nm and ε = 11393 M^{-1} cm^{-1}) were performed on supernatant of bacterial cultures grown for 16 h in LB broth. An estimation of the extinction coefficient was derived from a standard curve for unsubstituted phenazine (Sigma-Aldrich), dissolved in water at different concentrations.

The presence of surface-active compounds was tested by the drop collapse technique (Jain et al. 1991). Surface tension was measured as described by Perneel et al. (2007) with minor modifications. Strains were grown on KB medium during 72 h, whereupon cells were scraped from the plates, suspended in sterile distilled water, and cell concentrations were estimated by measuring optical density (OD) at 620 nm. After centrifugation of the suspensions, surface tension of cell-free supernatants was measured with a tensiometer equipped with a Wilhelmy plate. For LC-MS analysis, supernatant samples were collected after 72 h of growth on LB medium and subjected to reverse phase LC-MS (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass spectrometer) on a X-terra MS C18 column (100*4.6 mm, 3.5 µm, Waters). CLPs were eluted with a gradient of acetonitrile in water acidified with 0.1% formic acid at a constant flow rate of 0.5 ml min^{-1} and 40°C. Compounds were detected in the scan mode or SIR mode and in-source settings (electrospray ionization, positive ion) in the SQD were as follows: source temp., 130°C; desolvation temp., 280°C; nitrogen flow, 550 l h^{-1}; cone voltage, 60 or 80 V depending on the compound.

HCN and exoprotease production were determined as described before (Perneel et al. 2007).

Plant material and bacterial application to growth substrate

Plant experiments were carried out using bean seeds of *Phaseolus vulgaris* cv. Prelude (Het Vlaams Zaadhuis, Waarschoot, Belgium). To reduce the variability in emergence, bean seeds were pregerminated before sowing. Seeds were surface sterilized in 1 % sodium hypochlorite solution for 5 min, rinsed twice in sterile distilled water and placed in Petri dishes on sterile moistened filter paper. After 3 days at room temperature, germinated seeds were sown in perforated plastic trays (22 x 15 x 6 cm) filled with 700 g of a growth substrate composed of 50% sand (Cobo garden; Belgium) and 50% non-sterile potting soil (w/w) (Structural; Snebbout, Kaprijke, Belgium). Parameters including pH, organic matter (OM), organic carbon (C_{org}), phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) of the substrate were tested and results are given in Table 3.2.

Table 3.2. Characteristics of a growth substrate used

Parameter	Result
OM (%)	17.6
C_{org} (%)	10.3
pH_{H2O}	5.6
pH_{KCl}	5.2
P (mg/kg)	115.5
Ca (mg/kg)	3238
Mg (mg/kg)	485
K (mg/kg)	407
Na (mg/kg)	41.5

Bacterial inoculum suspensions were prepared by growing CMR12a and its mutants on KB agar plates for 24 to 28 h. Bacterial cells were scraped from the plates and suspended in sterile saline (0.85% NaCl). Concentrations of bacterial suspensions were estimated based on their OD at 620 nm. Washed bacteria were obtained by centrifuging bacterial suspensions twice for 4 min at 13400 rpm and resuspending bacterial pellets in saline prior to determination of their OD_{620}. The amount of suspension to be added to each tray was calculated and diluted to 50 ml with saline. Prior to sowing, the sand/potting soil substrate was thoroughly mixed with the diluted bacterial inoculum to obtain a final concentration of 10^6 colony forming units (CFU) g^{-1} substrate.

Pathogen inoculation and disease rating

Inoculum of *R. solani* AG 2-2 and AG 4-HGI was produced on sterile wheat kernels according to the protocol described by Scholten et al. (2001) with minor modifications. Water soaked wheat kernels were autoclaved twice on two consecutive days and inoculated with three PDA discs (diameter 5 mm) cut at the margin of a 3-day-old *Rhizoctonia* colony. Inoculated wheat kernels were incubated at 28°C during six days for the highly aggressive *R. solani* AG 4-HGI and nine days for the intermediately aggressive *R. solani* AG 2-2. Every 3-4 days wheat kernels were shaken to avoid coagulation. Plastic trays containing bean seedlings were inoculated three days after sowing by placing a row of 40 infected wheat kernels in the middle of the tray at a depth of approximately 2 cm and a distance of 3 cm from the bean seedlings. Six days after inoculation, disease symptoms were evaluated using the following scale: 0 = healthy, absence of symptoms; 1 = small black or brown lesions less than 1 mm in diameter; 2 = lesion covering less than 75% of the stem and/or root surface; 3 = lesion covering more than 75% of the stem and/or root surface; 4 = seedling dead. All plant experiments were carried out in a growth chamber (28°C, RH = 60%, 16 h photoperiod).

Each plant experiment consisted of three replications (trays) per treatment, with 10 bean plants per tray, and in each experiment healthy and infected control treatments were included, together with the bacterial treatments. All experiments were repeated in time.

Evaluation of root colonization by *Pseudomonas* sp. CMR12a and its mutants

At the time of disease rating, bacterial colonization of the bean roots was determined. Roots of five plants per treatment, randomly chosen from the three different trays, were excised and rinsed under tap water to remove most of the growth substrate. After weighing, roots were macerated in sterile saline using mortar and pestle and serial dilutions were plated on KB agar. Bacterial colonies showing the typical morphological characteristics of *Pseudomonas* sp. CMR12a, and which do not appear on control plates, were counted after an incubation period of 36-48 h at 28°C.

Data analysis

Statistical analysis was conducted for the ordinal data of the disease severity and the data of the root colonization experiments using the software package SPSS 15.0 for Windows (SPSSinc, Illinois, USA). Since neither of the data sets met the conditions of normality and

homogeneity of variances, non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($p = 0.05$) were performed.

Microscopic observations

Microscopic experiments were carried out based on a protocol described by Bolwerk et al. (2003) with modifications. Bacterial strains were grown overnight in liquid KB at 28°C on a rotary shaker (150 rpm). Microscopy slides were covered with a thin layer of water agar (1.5%) and placed in Petri dishes containing moistened filter paper. A 5-mm-PDA plug of a 3-day-old *R. solani* colony was placed in the center of glass slide; while a droplet (15 µl) of the overnight culture of *Pseudomonas* sp. CMR12a or mutant strain was pipetted at either side. The Petri plates were sealed with parafilm and incubated for 36 h at 28°C before observation under light microscopy (Olympus BX51 microscope, Aartselaar, Belgium). Digital images were acquired using an Olympus Color View II camera and further processed with Olympus analySIS cell^F software (Olympus Soft Imaging Solutions, Münster, Germany). All experiments were performed twice.

Results

Characterization of *Pseudomonas* sp. CMR12a and its mutants

HPLC analysis and UV absorption measurements of culture supernatant confirmed that both PCA and PCN production were knocked out in mutants CMR12a-ΔPhz, CMR12a-ΔPhz-Clp1. Remarkably, CMR12a-Clp1 produced significantly higher amounts of phenazines than the wild type, an observation which can not be accounted for at this point (Table 3.3).

Various approaches, including reverse phase LC-MS of CMR12a culture extracts and *in silico* analysis of genomic data, provided evidence that CMR12a synthesizes two types of cyclic lipopeptides (results not shown). For each CLP type, several presumably closely related variants seemed to be produced. For the main compound of the first CLP group, which has been designated CLP1, the detected molecular ion had a molecular weight of approximately 2028 Da. Preliminary results indicated that this is a novel compound within the tolaasin group of CLPs (Raaijmakers et al. 2006). Production of CLP1 was completely knocked out in mutants CMR12a-Clp1 and CMR12a-ΔPhz-Clp1. For the second CLP type (designated CLP2) three main compounds with molecular ions of around 1253, 1279 and 1281 Da were detected, which were all still being produced by both CLP mutants at wild type levels. In CMR12a-ΔPhz, the

pattern of CLP production was not altered compared to that of the wild type (results not shown). A more detailed structural characterization of both CLPs is in progress.

Table 3.3. Phenazine and cyclic lipopeptide production by CMR12a and mutants. Measurements of phenazine concentration and surface tension were repeated in time, and representative data from one experiment are shown. Values show means and standard deviations of three independent replicates. <DL: value below detection limit. +: produced at wild type levels, ++: produced at elevated levels compared to wild type, -:compounds is not produced.

Strain	Phenazines			Cyclic lipopeptides		
	HPLC analysis		Phenazine concentration in culture supernatant	HPLC analysis		Surface tension of culture supernatant
	PCA	PCN	(µM/OD_{620})	CLP1	CLP2	(mN/m)
CMR12a	+	+	33 ± 3	+	+	35 ± 2
CMR12a-ΔPhz	-	-	<DL	+	+	36 ± 1
CMR12a-Clp1	+	++	67 ± 6	-	+	47 ± 5
CMR12a-ΔPhz-Clp1	-	-	<DL	-	+	59 ± 6
CMR12a-GacA	-	-	<DL	-	-	54 ± 6

Besides these biosynthesis mutants, a regulatory mutant, CMR12a-GacA (Pham et al. 2008), was also tested for its biocontrol ability against *R. solani* on bean. This mutant has a spontaneous mutation in the GacS/GacA two-component regulatory system (Heeb and Haas 2001). In CMR12a-GacA, not only phenazine production is knocked out, but also synthesis of other secondary metabolites, including exoproteases and HCN (results not shown). In addition, neither CLP1 nor CLP2 production could be detected in supernatant extracts of CMR12a-GacA, indicating that GacA controls production of both lipopeptides.

Biocontrol of Rhizoctonia root rot of bean by *Pseudomonas* sp. CMR12a and its phenazine/CLP mutants

We investigated whether *Pseudomonas* sp. CMR12a could provide protection *in vivo* against Rhizoctonia root rot. In the experiments, four mutants of CMR12a deficient in phenazine and/or CLP production were included (Table 3.3). A significant reduction of disease severity caused by *R. solani* on bean plants was observed for the wild type strain *Pseudomonas* sp. CMR12a (Figure 3.1). The numerical data described further are valid for repetition 1, as shown in Figure 3.1; repetition 2 yielded similar results.

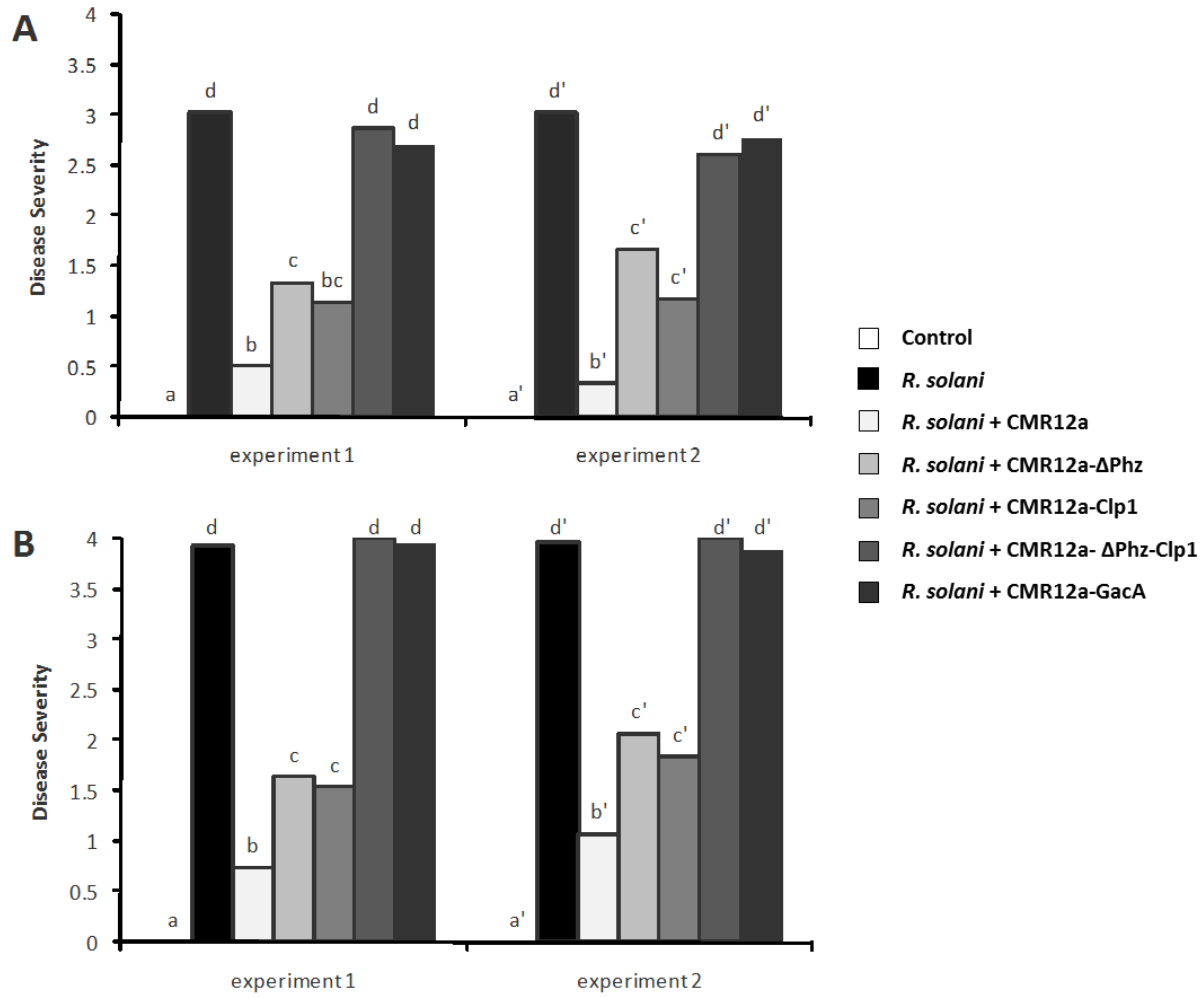

Figure 3.1. Importance of phenazines and cyclic lipopeptides in the biological control of Rhizoctonia root rot of bean

Pseudomonas sp. CMR12a, a phenazine-negative mutant CMR12a-ΔPhz, a CLP-negative mutant CMR12a-Clp1, a double phenazine- and CLP-negative mutant CMR12a-ΔPhz-Clp1 and a spontaneous *gacA* mutant CMR12a-GacA were applied to growth substrate at a concentration of 10^6 CFU g^{-1} substrate three days before inoculation. Bacterial cells were not washed. Bean plants were subsequently inoculated with **A**, *R. solani* AG 2-2 or **B**, *R. solani* AG 4-HGI. Disease symptoms were evaluated after six days using following scale: 0 = healthy, absence of symptoms; 1 = small black or brown lesions less than 1 mm in diameter; 2 = lesion covering less than 75% of the stem and/or root surface; 3 = lesion covering more than 75% of the stem and/or root surface; 4 = seedling dead. Data represent means of disease scale obtained from three replicates with each 10 plants per treatment. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney non-parametric tests (p = 0.05).

For the intermediately aggressive isolate of *R. solani* AG 2-2, a reduction in disease severity from approximately 3.1 ± 0.9 to 0.5 ± 1.0 was recorded ($p < 0.001$); for the very aggressive isolate of *R. solani* AG 4-HGI, which killed almost all plants in the control

treatment, a decrease in disease severity from approximately 3.9 ± 0.3 to 0.7 ± 1.2 was observed ($p < 0.001$). Treatments with the mutant strains performed significantly worse. Mutants CMR12a-ΔPhz and CMR12a-Clp1 were still able to reduce the disease severity caused by both AGs of *R. solani*, although to a lower extent than the wild type. On the other hand, mutants which are deficient in both phenazine and CLP production, biosynthesis mutant CMR12a-ΔPhz-Clp1 and *gacA*-mutant CMR12a-GacA, completely lost their biocontrol capacity and the disease severity for these strains was not statistically different from the diseased control.

Table 3.4. Root colonization data for non-washed bacteria of *Pseudomonas* sp. CMR12a and mutants CMR12a-ΔPhz, CMR12a-Clp1, CMR12a-ΔPhz-Clp1 and CMR12a-GacA in the absence of *R. solani* or after inoculation with *R. solani* AG 2-2 or *R. solani* AG 4-HGI. Data represent means and standard deviations of five replicates per treatment. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney non-parametric tests ($p = 0.05$).

	Treatment	**Bacterial population (in log CFU g^{-1} fresh root)**							
		Repetition 1				**Repetition 2**			
Without *R. solani*	CMR12a	6.14	±	0.37	c	5.61	±	0.46	c
	CMR12a-ΔPhz	5.81	±	0.47	b	6.95	±	0.30	e
	CMR12a-Clp1	5.14	±	0.34	a	6.00	±	0.39	d
	CMR12a-ΔPhz-Clp1	5.22	±	0.35	a	5.27	±	0.39	b
	CMR12a-GacA	5.02	±	0.47	a	4.75	±	0.36	a
R. solani AG 2-2	CMR12a	6.53	±	0.31	c	6.03	±	0.36	d
	CMR12a-ΔPhz	5.86	±	0.71	ab	6.62	±	0.28	e
	CMR12a-Clp1	6.07	±	0.53	b	6.68	±	0.57	e
	CMR12a-ΔPhz-Clp1	5.40	±	0.30	a	5.30	±	0.29	c
	CMR12a-GacA	5.38	±	0.31	a	4.92	±	0.37	b
R. solani AG 4-HGI	CMR12a	6.38	±	0.44	c	5.34	±	0.33	bc
	CMR12a-ΔPhz	6.14	±	0.79	bc	7.39	±	0.24	e
	CMR12a-Clp1	6.55	±	0.45	c	6.84	±	0.37	d
	CMR12a-ΔPhz-Clp1	5.18	±	0.44	a	5.56	±	0.34	c
	CMR12a-GacA	5.68	±	0.32	b	5.16	±	0.38	b

Bacterial counts revealed differences in bacterial populations on the roots (Table 3.4). Bacterial concentrations observed for the parental strain and the mutants CMR12a-ΔPhz and CMR12a-Clp1 were variable among treatments and repetitions in time. However, this did not influence the disease suppressive capacity, suggesting root colonization was at a sufficiently high level for optimal biological control. The two mutants CMR12a-ΔPhz-Clp1 and CMR12a-GacA lacking both phenazine and CLP production predominantly had the lowest bacterial root concentrations and in only one experiment their root concentrations were not significantly lower compared to the wild type. From roots of non-inoculated control plants, CMR12a bacteria were never recovered. Moreover, application of CMR12a or its mutants to the growth substrate never caused visible phytotoxic effects or effects on plant growth, as observed by qualitative inspection of the plants.

Biocontrol capacity of washed CMR12a bacteria and its mutants

To investigate whether metabolites produced during growth of bacterial strains on KB agar plates are involved in the biocontrol capacity of *Pseudomonas* sp. CMR12a, bacteria were washed before application to the sand/potting soil mixture (Figure 3.2). The reduction in disease severity upon treatment with washed bacteria was smaller compared to the non-washed bacteria. For the plants inoculated with the washed wild type strain, disease severity caused by *R. solani* AG 2-2 was reduced from 3.8 ± 0.8 (first repetition) and 2.8 ± 1.0 (second repetition) to approximately 1.5 ± 1.1. Treatment with the washed mutant strains CMR12a-ΔPhz and CMR12a-Clp1 resulted in a disease severity reduction intermediate between the diseased control and the reduction caused by the wild type strain (disease severity = 2.6 ± 1.5 in repetition 1 and disease severity = 2.0 ± 1.2 in repetition 2); whereas mutants CMR12a-ΔPhz-Clp1 and CMR12a-GacA did not influence the disease development and disease severity was not statistically different from the diseased control.

In contrast to the disease reduction of almost 50% observed for *R. solani* AG 2-2, washed bacteria only had a minor effect on the disease caused by the very aggressive isolate of *R. solani* AG 4-HGI. The wild type strain and the mutants CMR12a-ΔPhz and CMR12a-Clp1 still reduced the disease severity significantly from 4.0 ± 0.0 to approximately 3.5 ± 0.6. The two other mutants did not differ from the diseased control and at the time of evaluation all plants were dead. The washing step did not influence root colonization, since bacterial counts after the application of washed bacteria were at comparable level to those for the non-washed bacteria. In the first repetition, CMR12a bacteria were recovered at a concentration of 5.57 ± 0.31 log CFU g^{-1} fresh root after inoculation with *R. solani* AG 2-2 and at 5.58 ± 0.41 log CFU g^{-1} fresh

root after inoculation with *R. solani* AG 4-HGI; in the second repetition bacterial densities were slightly higher with values of 6.51 ± 0.22 and 6.18 ± 0.35 log CFU g^{-1} fresh root, for AG 2-2 and AG 4-HGI, respectively.

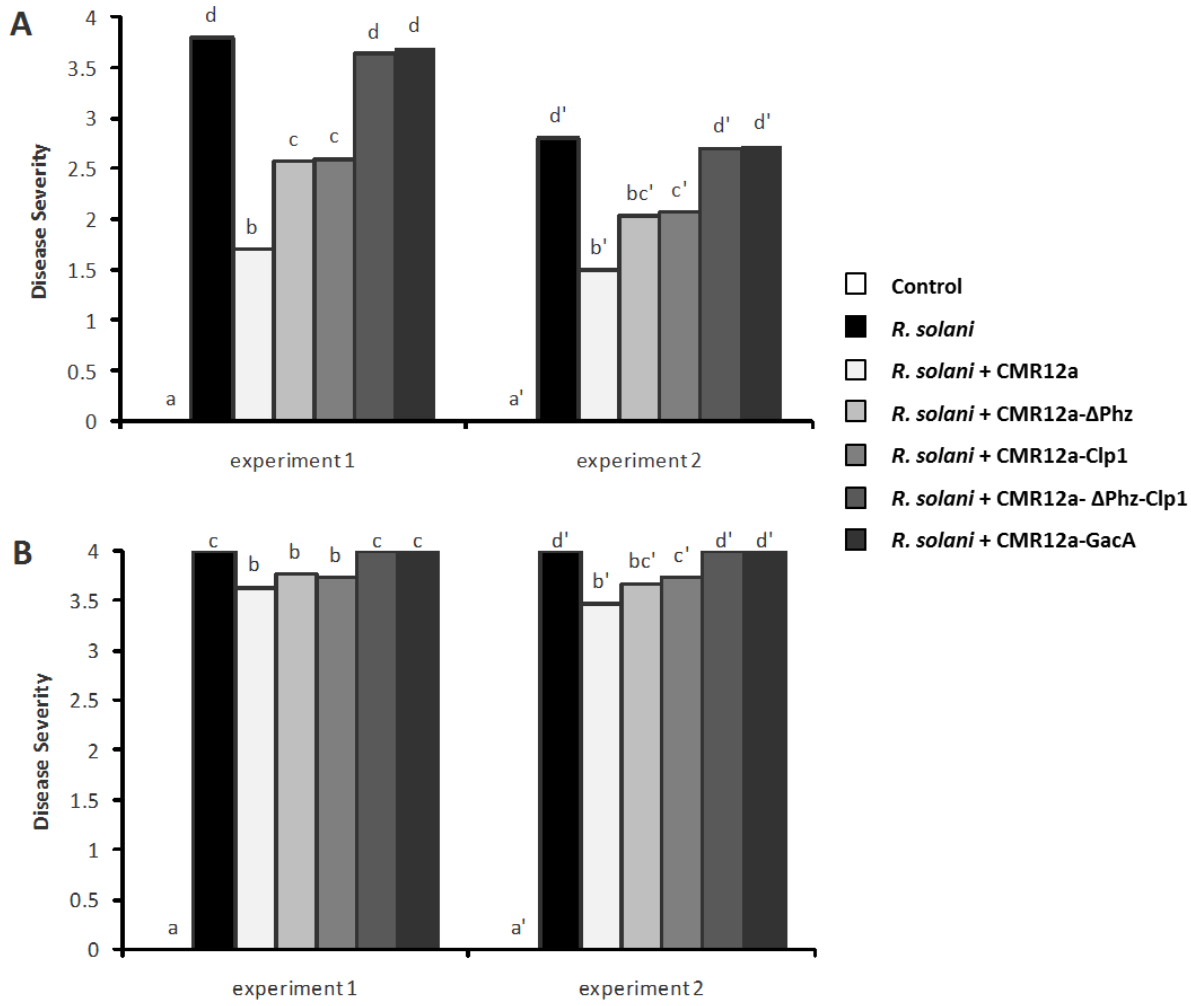

Figure 3.2. Biological control of Rhizoctonia root rot on bean after application of washed *Pseudomonas* sp. CMR12a bacteria and its mutants CMR12a-ΔPhz, CMR12a-Clp1, CMR12a-ΔPhz-Clp1 and CMR12a-GacA

Washed bacteria were applied to potting soil/sand mixture at a concentration of 10^6 CFU g^{-1} mixture three days before inoculation with **A**, *R. solani* AG 2-2 or **B**, *R. solani* AG 4-HGI. Disease symptoms on bean plants were evaluated six days after inoculation using following scale: 0 = healthy, absence of symptoms; 1 = small black or brown lesions less than 1 mm in diameter; 2 = lesion covering less than 75% of the stem and/or root surface; 3 = lesion covering more than 75% of the stem and/or root surface; 4 = seedling dead. Data represent means of disease scale obtained from three replicates with each 10 plants per treatment. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney non-parametric tests (p = 0.05).

Microscopic observations

R. solani hyphae in the presence of bacterial cells were examined with a light microscope to study changes in the growth pattern. At the contact zone between the mycelium of *R. solani* grown on coated microscopic glass slides and the bacterial droplets, morphological differences were observed (Figure 3.3). The mycelium of *R. solani* AG 2-2 and AG 4-HGI was more branched in the presence of CMR12a and CMR12a-ΔPhz bacteria, resulting in a higher density of hyphae. In contrast, the three other mutants, e.g. CMR12a-Clp1, CMR12a-ΔPhz-Clp1 and CMR12a-GacA, did not induce an increase in hyphal branching, and *R. solani* hyphae of both AGs showed similar morphology compared to the control hyphae grown in the absence of bacteria.

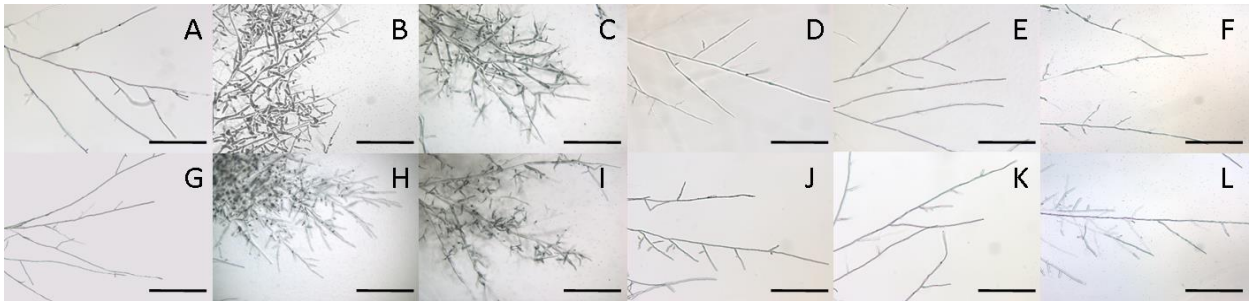

Figure 3.3. Mycelial growth pattern of **A-F,** *R. solani* AG 2-2 and **G-L,** AG 4-HGI microscopically observed after different treatments including **A and G,** control, **B and H,** *Pseudomonas* sp. CMR12a, **C and I,** *Pseudomonas* CMR12a-ΔPhz, **D and J,** *Pseudomonas* CMR12a-Clp1, **E and K,** *Pseudomonas* CMR12a-ΔPhz-Clp1 and **F and L,** *Pseudomonas* CMR12a-GacA. Scale bars = 500 µm.

Discussion

Inhibition zones between hyphae of *R. solani* and *Pseudomonas* sp. CMR12a cells were observed during preliminary *in vitro* dual culture experiments, suggesting a strong antagonistic potential of this bacterial strain towards *R. solani*. In this study, we showed that CMR12a effectively controls Rhizoctonia root rot of bean caused by *R. solani* AG 2-2 or *R. solani* AG 4-HGI. Moreover, the importance of cyclic lipopeptide and phenazine production for the disease suppressive effect of CMR12a was clearly demonstrated. It is unclear whether these effects are due to direct antibiotic activity of CLPs and phenazines. Alternatively, one or both compounds may somehow be involved in the ecological fitness of CMR12a, thus indirectly influencing the biocontrol capacity of CMR12a.

Direct antibiosis of CLPs is likely to be involved, since microscopic experiments clearly showed an increase in hyphal branching of *R. solani* AG 2-2 and AG 4-HGI caused by CMR12a; while a normal growth pattern was observed with the CLP deficient mutant CMR12a-Clp1. In addition, an *in vitro* growth assay with both wild type CMR12a and the CLP1 mutant indicated a strong inhibitory effect of these CLPs on *R. solani* growth (unpublished data). Extensive branching of *R. solani* hyphae was previously observed after treatment with the structurally related CLPs viscosinamide and tensin. The authors assumed that viscosinamide imposes ion channels in the membranes, leading to an increased Ca^{2+} influx and morphological changes of the hyphae (Nielsen et al. 1999). In case of tensin however, an alternative mode of action was suggested possibly involving interference with cell wall synthesis (Nielsen et al. 2000). Phenazines also exert direct antibiotic effects towards several plant pathogens, including *R. solani* (Rosales et al. 1995). However, during our microscopic experiments, we could not observe any effect of phenazines on the growth pattern of *R. solani*. Possibly, the experimental setup did not allow phenazine production to reach concentrations sufficient to inhibit the growth of *R. solani*.

Biosurfactants can interact with other excreted metabolites, thereby enhancing their bioavailability and bioactivity (D'aes et al. 2010). Along these lines, Perneel et al. (2008) showed that simultaneous production of rhamnolipid-type biosurfactants and phenazines is crucial for biological control of *Pythium* species by *Pseudomonas aeruginosa* PNA1. The authors hypothesized that biosurfactants improved physical contact between hyphae and phenazines and increased entry of phenazines into the fungal cell. In this study, both the CLP-negative mutant CMR12a-Clp1 and the phenazine-negative mutant CMR12a-ΔPhz still significantly reduced Rhizoctonia root rot of bean, suggesting an additive effect of CLPs and phenazines. Considering the structural differences between the cyclic lipopeptide-type biosurfactants produced by *Pseudomonas* sp. CMR12a and the rhamnolipid-type biosurfactants produced by *P. aeruginosa* PNA1, it is reasonable to assume that both groups of biosurfactants have different modes of action (Ron and Rosenberg 2001). However, we cannot exclude the possibility that the second CLP, CLP2, which is still being produced by CMR12a-Clp1 (see above), is involved in enhancing phenazine activity.

Numerous studies have acknowledged the importance of CLPs for bacterial motility and root colonization, which are essential features of bacterial biocontrol agents of soil-borne pathogens (Weller 1988; Andersen et al. 2003; Tran et al. 2007; Perneel et al. 2008). An increased rhizosphere competence of biosurfactant producing strains was also demonstrated in this study. Phenazine production can provide an additional advantage during establishment in the

rhizosphere (Mazzola et al. 1992). Consequently, it would not be surprising if phenazines and CLPs produced by CMR12a were involved in the ecological competence of CMR12a. Yet in this study, root counts of CMR12a-ΔPhz and CMR12a-Clp1 were not significantly lower in most cases compared to the wild type. Nevertheless, the two mutants deficient in both phenazines and CLPs yielded significantly lower population levels compared to the parental strain, indicating that CMR12a must produce at least one of both compounds to efficiently defend its ecological niche. It must be noted that the *gacA* mutation in CMR12a-GacA causes pleiotropic effects, which could influence root colonization and biocontrol capacity for reasons not necessarily related to phenazine or CLP production. Notwithstanding the observed differences in root colonization, all bacterial strains were able to reach a concentration of 10^5 CFU g^{-1} root, a crucial colonization level considered necessary for optimal biological control (Raaijmakers et al. 1999; Haas and Defago 2005).

An additional important factor influencing the disease suppressive effect of CMR12a was the experimental setup, more specifically preparation of the bacterial inoculum. Application of washed bacteria to potting soil/sand mixture still had a suppressive effect, although disease severity was only satisfactory reduced after infection with *R. solani* AG 2-2. In case of the highly aggressive isolate of *R. solani* AG 4-HGI, washed CMR12a bacteria did not provide sufficient protection, and the majority of the bean plants was killed. Apparently, metabolites produced on KB plates during growth of the bacteria are important for their biocontrol capacity. These metabolites could provide an advantage to the bacterial cells at the early stages of introduction into growth substrate, preventing a decline in bacterial population generally observed after such an application (Bashan 1998). Nevertheless, bacterial root counts at the evaluation stage did not reveal any significant differences compared to non-washed bacteria. A follow-up of the bacterial population in time should elucidate whether metabolites produced on plate stimulate rapid root colonization. Furthermore, CMR12a produces signal molecules during growth *in vitro*, including homoserine lactones that stimulate the production of phenazines (De Maeyer et al. 2011). Such signal molecules might allow the bacteria to rapidly resume production of phenazines or CLPs once they have colonized the roots.

Together, our results provide evidence for the involvement of phenazines and CLPs in the disease reduction of Rhizoctonia root rot of bean by *Pseudomonas* sp. CMR12a, and demonstrate the negative impact on biocontrol capacity caused by washing bacteria before application. A better insight in the role played by phenazines and biosurfactants in biological control of soil-borne pathogens and the regulation and production of these metabolites *in situ* is imperative for optimization of biological control using *Pseudomonas* strains.

Acknowledgements

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Chapter

4

The role of phenazines and cyclic lipopeptide sessilin in biocontrol of Rhizoctonia root rot on bean (*Phaseolus vulgaris*) by *Pseudomonas* sp. CMR12a is influenced by soil characteristics

Gia Khuong Hoang Hua and Monica Höfte

Submitted to Plant and Soil

Abstract

Aims

Pseudomonas sp. CMR12a is an effective biocontrol agent that produces various antifungal metabolites including phenazine antibiotics and cyclic lipopeptides. In this study, we wanted to investigate the influence of soil characteristics on the role of phenazines and the cyclic lipopeptide (CLP) sessilin in biocontrol of bean root rot caused by *Rhizoctonia solani* AG 2-2.

Methods

Disease severity and spreading rate of *R. solani* was determined in three different potting soil/sand mixtures inoculated with *Pseudomonas* sp. CMR12a or its mutants impaired in phenazine and/or sessilin biosynthesis.

Results

Disease pressure positively correlated with the proportion of sand present in the mixture. In mixtures containing 50% or 75% of potting soil, the presence of either phenazines or sessilin was sufficient to suppress bean root rot. However, in mixtures containing only 25% of potting soil, the involvement of both compounds was required to provide better protection. The ratio between potting soil and sand also determined the spreading rate of *Rhizoctonia* hyphae through the mixtures. The mixture containing 75% of potting soil was the most suppressive against *R. solani* invasion.

Conclusions

The use of various potting soil/sand combinations had a strong influence on the development of bean root rot disease. Due to the difference in disease severity, the production of phenazines or CLPs or both compounds is required for effective biocontrol.

Keywords: biocontrol, cyclic lipopeptides, phenazines, sessilin, fluorescent pseudomonads, *Rhizoctonia solani*

Introduction

Rhizoctonia solani is a soil-borne pathogenic fungus with a wide host range and worldwide distribution. Root and hypocotyl rot induced by this fungus has been considered as one of the most economically important diseases on bean grown in many different regions (Abawi 1989;

Mathew and Gupta 1996; Valenciano et al. 2006) and a yield loss of 5-10% is often recorded (Abawi 1989). The anastomosis groups (AGs) of *R. solani* responsible for hypocotyl and root rot diseases of bean include AGs 1, 2-2, 4 and 5 (Galindo et al. 1982; Engelkes and Windels 1996; Karaca et al. 2002; Eken and Demirci 2003; Nerey et al. 2010). Because the excessive utilization of agrochemicals to control *Rhizoctonia*-induced root rot disease is undesirable and resistance is not available, biocontrol using beneficial microorganisms such as fluorescent pseudomonads may be a valuable alternative.

Fluorescent pseudomonads are gram negative bacteria ubiquitously present in soil, water, plants and sediments. These bacteria are characterized by the ability to produce fluorescent, yellow-green, diffusible pigments on culture media (Meyer 2000) and a wide range of other secondary metabolites including phenazines and cyclic lipopeptides (CLPs) (Raaijmakers et al. 2006; Gross and Loper 2009). Phenazines are a large family of pigmented, heterocyclic nitrogen-containing compounds with an important role in biocontrol of plant diseases (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006; D'aes et al. 2011; Le et al. 2012). CLPs are metabolites composed of a cyclic oligopeptide lactone ring linked to a fatty acid tail (Finking and Marahiel 2004). In *Pseudomonas* spp. these compounds play a crucial role in antagonism towards other microorganisms, pathogenicity, surface motility, and regulation of attachment/detachment to and from surfaces. Various reports about the involvement of CLPs in biocontrol activity of *Pseudomonas* spp. have been published (D'aes et al. 2010; Raaijmakers et al. 2010).

Fluorescent *Pseudomonas* sp. CMR12a (D'aes et al. 2011) was isolated from the rhizosphere of tropical tuber crop cocoyam (*Xanthosoma sagittifolium*) in Cameroon and has excellent *in vivo* biocontrol activity against the cocoyam root rot pathogen *Pythium myriotylum* (Perneel et al. 2007). Taxonomically, *Pseudomonas* sp. CMR12a is positioned in the *Pseudomonas fluorescens* group between *P. protegens* and *P. chlororaphis* and the strain produces phenazines and two different classes of cyclic lipopeptides (D'aes et al. in press). *Pseudomonas* sp. CMR12a was shown to be effective in controlling Rhizoctonia root rot of bean and mutant analysis revealed that in a substrate containing 50% of potting soil and 50% of sand the disease was successfully suppressed by the additive effect of phenazines and CLP sessilin (formerly designated CLP1) (D'aes et al. 2011) (Chapter 3). Interestingly, when performing biocontrol experiments with CMR12a and *P. myriotylum* on cocoyam, D'aes (2012) found that the involvement of phenazines and sessilin in disease suppression was substrate-dependent. In a 75% potting soil/25% sand mixture, the production of phenazines or sessilin could control Pythium root rot. However, in a 50% potting soil/50% sand substrate, no biocontrol effect was observed. On the basis of these observations, we decided to study the effect of different potting

soil/sand mixtures on the role of phenazines and sessilin produced by *Pseudomonas* sp. CMR12a in biocontrol of Rhizoctonia root rot on bean. Because results of the first experiment indicated that there is a significant difference in disease severity among mixtures, another experiment was conducted to determine whether the variation in the infection of seedlings results from the difference in mycelial spread through various potting soil/sand mixtures.

Materials and methods

Microorganisms and culture conditions

Microorganisms used in our experiments and their relevant characteristics are listed in Table 4.1. Bacterial inoculum was prepared by growing *Pseudomonas* sp. CMR12a and its mutants on King´s B plates (KB; King et al. 1954) at 28°C. Bacterial cells from overnight cultures were harvested with sterile saline solution and their concentration was determined spectrophotometrically at 620 nm.

Table 4.1. Microorganisms used in this study with their relevant characteristics

Microorganisms	Relevant characteristics[a]	References
Pseudomonas strains		
CMR12a	PHZ^+, $CLP1^+$, $CLP2^+$; wild type	Perneel et al. (2007)
CMR12a-ΔPhz	PHZ^-, $CLP1^+$, $CLP2^+$; phenazine mutant	D'aes et al. (2011)
CMR12a-Clp1	PHZ^+, $CLP1^-$, $CLP2^+$; sessilin mutant	D'aes et al. (2011)
CMR12a-ΔPhz-Clp1	PHZ^-, $CLP1^-$, $CLP2^+$; phenazine and sessilin mutant	D'aes et al. (2011)
Rhizoctonia solani		
AG 2-2 CuHav-Rs18	Intermediately aggressive isolate of bean root rot in Cuba	Nerey et al. (2010)

[a] PHZ, phenazines; CLP1, sessilin; CLP2, orfamide. A plus$^+$ and a minus$^-$ are used when the production is positive or negative for trait, respectively.

Inoculum of *R. solani* AG 2-2 isolate 18 was produced according to Scholten et al. (2001), with minor modifications. Briefly, three potato dextrose agar (PDA) plugs (diameter = 3 mm) from 3-day-old PDA cultures of *R. solani* were used to inoculate water-soaked wheat kernels, which were autoclaved twice on two consecutive days. Inoculated kernels were incubated at 28°C for nine days before comparable-sized kernels were used for inoculation. To avoid the formation of large, sticky clumps of kernels and fungus, wheat kernels were shaken twice a week.

Physicochemical characteristics of growth substrates

All experiments were conducted in three different non-sterile substrates (S1, S2 and S3), which were produced by adding sand (Cobo garden; Belgium) to potting soil (Structural; Snebbout, Kaprijke, Belgium). Physicochemical characteristics of these substrates are shown in Table 4.2.

Table 4.2. Characteristics of the growth substrates used

	25% potting soil/75% sand (S1)	50% potting soil/50% sand (S2)	75% potting soil/25% sand (S3)
OM (%)	7.3	17.6	20.3
C_{org} (%)	4.3	10.3	11.8
pH_{H2O}	5.9	5.6	5.5
pH_{KCl}	5.3	5.2	5.2
P (mg/kg)	44.6	115.5	197.7
Ca (mg/kg)	1614	3238	4692
Mg (mg/kg)	194	485	756
K (mg/kg)	164	407	706
Na (mg/kg)	7.8	41.5	92.1

Experiment 1. Biological control effect of *Pseudomonas* sp. CMR12a and its mutants towards Rhizoctonia root rot of bean

A factorial experiment was conducted under *in vivo* conditions. Treatments of this experiment included the negative control (with neither pathogen nor antagonist; potting soil/sand mixtures were treated with sterile saline solution and seedlings were inoculated with sterile wheat kernels), the pathogen control (pathogen without antagonist; potting soil/sand mixtures were treated with sterile saline solution), the bacterial control (antagonist only; seedlings were inoculated with sterile wheat kernels), and the biocontrol against *R. solani* (antagonist together with pathogen; potting soil/sand mixtures were treated with bacterial suspensions and seedlings were inoculated with *Rhizoctonia*-colonized wheat kernels). Fifty ml of each bacterial suspension was added to one perforated plastic box (22 x 15 x 6 cm) partially filled with 700 g substrates to give a final concentration of 10^6 CFU g^{-1} of substrate. Bean seeds (*Phaseolus vulgaris* cv. Prelude; Het Vlaams Zaadhuis, Waarschoot, Belgium) were surface sterilized and pre-germinated on moist filter paper three days before sowing. Ten germinated seeds were planted in two rows parallel to the long edge of the box, with 10 cm between rows and about 2

cm between the long edge of the box and the nearer seedling row. Within each row, the seeds were evenly spaced and the seeds nearest to each short edge were 2 cm from the edge.

Rhizoctonia inoculum was used to inoculate 3-day-old seedlings by placing a row of 40 infected wheat kernels in the middle of the box at a depth of about 2 cm from the surface of growth substrates and a distance of 5 cm from each seedling row. All plants were kept in a growth chamber (28°C, RH = 60%, 16 h photoperiod) and were watered three times per week to maintain moisture near field capacity. The development of disease symptoms on roots and hypocotyls was recorded six days after fungal inoculation using the 0-4 rating scale described by D'aes et al. (2011): 0 = healthy, absence of symptoms; 1 = small black or brown lesions <1 mm in diameter; 2 = lesion covering <75% of the stem or root surface; 3 = lesion covering >75% of the stem or root surface; and 4 = seedling dead. A completely randomized design was employed with three replications per treatment, which is equivalent to 30 seedlings in three experimental boxes, and this experiment was performed twice.

In addition to evaluating the disease severity, five plants from three different boxes of each treatment were randomly selected at the time of disease rating to determine the population densities of *Pseudomonas* sp. CMR12a and its mutants colonizing the roots. After rinsing under tap water to remove most of the growth substrate, roots were weighted and macerated in sterile saline solution using mortar and pestle. The suspensions were serially diluted and spread over KB agar plates. These plates were incubated at 28°C for 36-48 h before counting based on the typical morphological characteristics of *Pseudomonas* sp. CMR12a and its mutants.

Experiment 2. Spreading rate of *R. solani* in different potting soil/sand mixtures

Growth substrates, bean seedlings, bacterial suspensions and fungal inoculum used in this test were prepared following the same methods as in the previous experiment. The scheme of this experiment is shown in Figure 4.1.

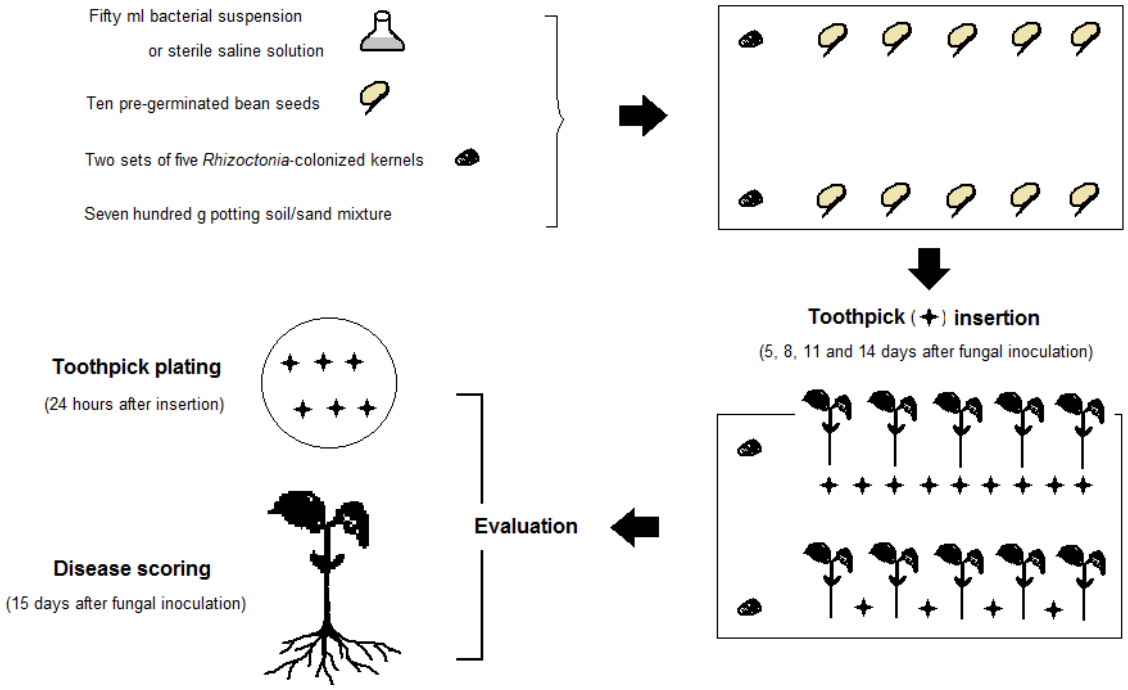

Figure 4.1. Scheme to evaluate the spreading of *R. solani* in different potting soil/sand mixtures

Ten pre-germinated bean seeds were sown in two rows 10 cm apart and each seed was 3 cm apart in each row. The first seed of each row was 8 cm apart from the short border of the box. Sets of five wheat kernels colonized by *Rhizoctonia* hyphae were placed at a distance of 6 cm in front of each seedling row at the time of sowing and all seedlings were maintained in the growth chamber (28°C, RH = 60%, 16 h photoperiod). Then, the toothpick bait technique proposed by Gill et al. (2000) and Paulitz and Schroeder (2005) was employed with some adaptations to determine the spread of *R. solani* through the substrates. Briefly, sterile wooden toothpicks (diameter = 2 mm and length = 3 cm) were inserted into the substrates to a depth of 1.5 cm and 2 cm apart from the seedling rows at 5, 8, 11 and 14 days after fungal inoculation. The distance between toothpicks in the same row was 1.5 cm. After 24 h, these toothpicks were removed from growth substrates and placed on plates containing *Rhizoctonia* selective medium (Ko and Hora 1971). The plates were incubated at room temperature for 24 h and the presence of *R. solani* was checked under a dissecting microscope. To confirm whether the difference in spreading rate of the fungus lead to the variation in disease severity, roots and hypocotyls was scored 15 days after fungal inoculation using the same disease scale described in Experiment 1. This factorial experiment had a completely randomized design with three replications (10 seedlings each) and it was done twice.

Data analysis

Disease severity scores were used to calculate a Percent Disease Index (PDI) using the formula described by Wheeler (1969):

$$\text{PDI (\%)} = \frac{\sum (\text{Disease class x number of plants falling under that class})}{\text{Total number of plants observed x Maximum class}} \text{ x } 100$$

Data collected from root colonization were log10 transformed and percentage of toothpicks colonized by *Rhizoctonia* hyphae were arc-sine transformed before analyses. Data from two repeats of each experiment were pooled and non-parametric Kruskal-Wallis tests followed by Mann-Whitney were performed on pooled data at $p = 0.05$.

Non-parametric factorial data obtained from Experiments 1 and 2 were aligned and ranked using ARTool (Wobbrock et al. 2011). The difference in disease severity (Experiments 1 and 2), root colonization capacity (Experiment 1) and spreading rate (Experiment 2) among treatments and the interaction between bacterial strains and potting soil/sand mixtures on these variables were analyzed based on factorial ANOVA of aligned and ranked data. Results of factorial analysis are shown in the Supplementary information (Tables A4.1-A4.4). All statistic tests were conducted in SPSS 22.0 (SPSSinc, Illinois, USA).

Results

Influence of potting soil/sand mixture on biocontrol capacity of *Pseudomonas* sp. CMR12a and its mutants

Pseudomonas sp. CMR12a, together with its phenazine deletion mutant (CMR12a-ΔPhz), sessilin deletion mutant (CMR12a-Clp1), and a double mutant negative in both phenazine and sessilin production (CMR12a-ΔPhz-Clp1), were tested for their ability to suppress disease induced by *R. solani* in different potting soil/sand combinations. Because the application of bacteria alone did not induce any disease symptoms on bean seedlings, PDI of all bacterial control treatments in Experiment 1 were 0 and they are not shown in Figure 4.2.

Although all seedlings of the pathogen control treatments were heavily infected by *R. solani*, disease symptoms tended to be more severe on seedlings cultivated in the mixture of 25% potting soil/75% sand (S1). In this mixture, the phenazine mutant and the sessilin mutant were significantly less successful in protecting the bean plants against *R. solani* than wild type strain CMR12a. However, in the mixtures with 50% or 75% of potting soil (S2 and S3), less disease

symptoms were recorded and both mutants were equally effective as the wild type strain. The mutant impaired in both phenazine and sessilin production did not show antagonistic ability towards the pathogen in any of the potting soil/sand mixtures tested.

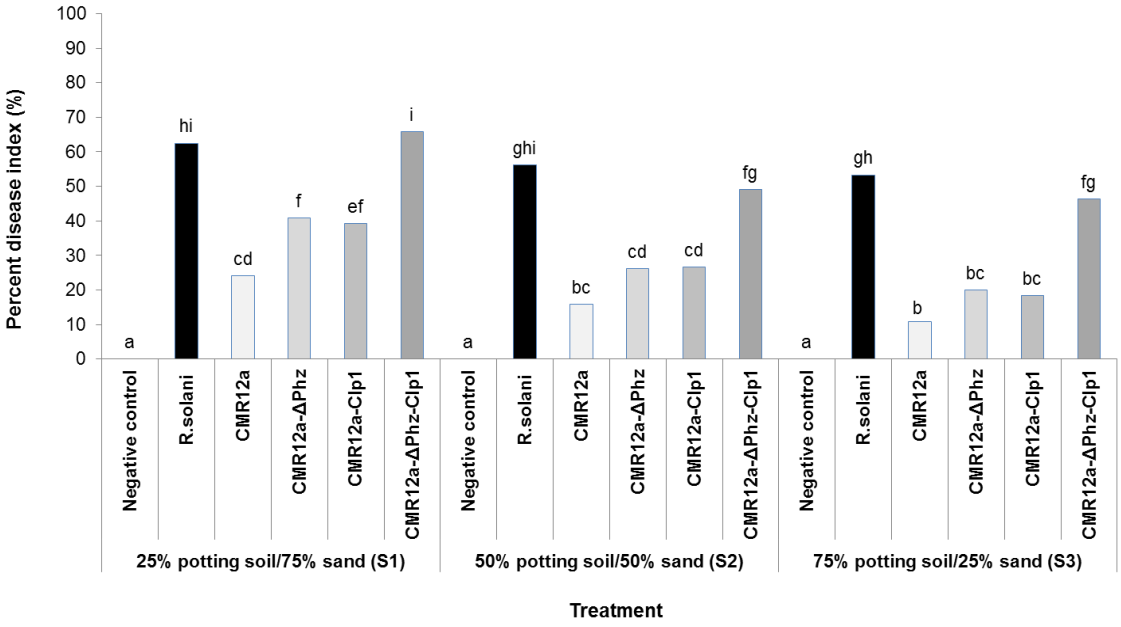

Figure 4.2. Effect of three different potting soil/sand mixtures on biocontrol ability of *Pseudomonas* sp. CMR12a and mutants towards bean root rot when 3-day-old seedlings were inoculated with *R. solani* AG 2-2 inoculum

Pseudomonas strains including wild type (CMR12a), a phenazine-negative mutant (CMR12a-ΔPhz), a sessilin-negative mutant (CMR12a-Clp1) and a double phenazine- and sessilin-negative mutant (CMR12a-ΔPhz-Clp1) were applied to mixture S1 (25% potting soil/75% sand, w/w), S2 (50% potting soil/50% sand, w/w) and S3 (75% potting soil/25% sand, w/w) at a concentration of 10^6 CFU g^{-1} of mixture before sowing. Disease severity on roots and hypocotyls was assessed six days after fungal inoculation using a 0-4 rating scale. Every treatment consisted of 30 seedlings cultivated in three plastic boxes. Experiment was performed twice and data from two repeats were pooled before analysis. Data are expressed as Percent disease index and bars indicated with the same letters are not statistically different based on the Kruskal-Wallis and Mann-Whitney nonparametric tests ($p = 0.05$).

Factorial ANOVA on aligned and ranked PDI data shows that there was a main effect of bacterial strains, with the PDI increasing from seedlings treated with CMR12a, followed by CMR12a-ΔPhz and CMR12a-Clp1, to CMR12a-ΔPhz-Clp1. There was also a main effect of growth substrate, with sand-dominant mixture resulting in more severe disease than that of mixtures containing at least 50% of potting soil. However, there was no interaction between the bacterial strains and growth substrates (Table A4.1).

Table 4.3. Root colonization data of *Pseudomonas* sp. CMR12a and mutants CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1 obtained from roots of bean seedlings six days after inoculation with *R. solani* AG 2-2

Treatment	Potting soil/sand mixture[a]	Population density of *Pseudomonas* sp. CMR12a and its mutants[b] (in log CFU g^{-1} of fresh root)
CMR12a + *R. solani*	S1	5.81 ± 0.37 b
	S2	5.73 ± 0.31 b
	S3	5.39 ± 0.33 a
CMR12a-ΔPhz + *R. solani*	S1	6.67 ± 0.56 d
	S2	6.67 ± 0.39 d
	S3	6.19 ± 0.49 c
CMR12a-Clp1 + *R. solani*	S1	5.32 ± 0.36 a
	S2	5.28 ± 0.25 a
	S3	5.31 ± 0.47 a
CMR12a-ΔPhz-Clp1 + *R. solani*	S1	5.26 ± 0.43 a
	S2	5.30 ± 0.50 a
	S3	5.36 ± 0.33 a

[a] Potting soil/sand mixtures: S1, 25% of potting soil and 75% of sand (w/w); S2: 50% of potting soil and 50% of sand (w/w); S3, 75% of potting soil and 25% of sand (w/w).

[b] Root colonization capacity of *Pseudomonas* strains was determined for five plants randomly selected from three replicate boxes per treatment. Experiment was performed twice. Data from two repeats were log10 transformed and pooled before statistical analysis. Values followed by the same letter are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests (p = 0.05).

Root colonization determined at the time of disease evaluation shows that CMR12a and its mutants were not recovered from roots of bean cultivated in potting soil/sand mixtures treated with sterile saline solution, indicating that these bacterial strains are only present in growth substrates upon inoculation. In other treatments, these bacterial strains possessed the capacity to colonize bean roots, although significant differences were observed among them (Table 4.3). Population densities of *Pseudomonas* sp. CMR12a and its mutants ranged from 5.26 to 6.67 log CFU g^{-1} of root. CMR12a-ΔPhz had the best colonization capacity and the highest bacterial population was observed on roots of seedlings grown in mixtures S1 and S2 treated with this mutant. The second highest bacterial population was found on bean roots treated with CMR12a-ΔPhz in S3, followed by those treated with CMR12a in S1 and S2. Significantly lower bacterial concentrations were detected when potting soil/sand mixtures were inoculated with sessilin-defective mutants or when S3 was inoculated with CMR12a, suggesting the essential role of sessilin in root

colonization capacity of *Pseudomonas* sp. CMR12a in mixtures containing at least 50% of sand (growth substrates S1 and S2). The influence of bacterial strains and potting soil/sand mixtures used on root colonization ability of bacteria as well as the interaction between bacterial strains and potting soil/sand mixtures were confirmed by factorial ANOVA employed on root colonization data (Table A4.2).

Spread of *Rhizoctonia* in different potting soil/sand mixtures

All potting soil/sand mixtures used in our experiments were free from *Rhizoctonia* because no *Rhizoctonia* hyphae were detected on toothpicks inserted in negative control treatments (Figure 4.3). In all treatments inoculated with *R. solani*, the presence of *Rhizoctonia* hyphae on toothpicks steadily increased over time. The fastest invasion was found in mixture S1 while the lowest spread was observed in mixture S3. In control treatments without added bacteria, only 57% of toothpicks inserted into mixture S3 were colonized by *Rhizoctonia* while up to 92% of toothpicks inserted into S1 were covered by fungal hyphae at 15 days after inoculation. There was no significant difference in the percentage of toothpicks covered by *Rhizoctonia* hyphae between control treatments and CMR12a-ΔPhz-Clp1 treatments in the same mixture. In contrast to CMR12a-ΔPhz-Clp1, the application of CMR12a or CMR12a-ΔPhz to potting soil/sand mixtures before sowing significantly inhibited the spread of *R. solani*. CMR12a-Clp1 significantly inhibited the spread of *R. solani* in S2 and S3, but not S1 at 9, 12 or 15 days after inoculation. Strikingly, no *Rhizoctonia* hyphae were detected on toothpicks inserted into any of the potting soil/sand mixtures treated with CMR12a, CMR12a-ΔPhz or CMR12a-Clp1 at six days after inoculation. At 15 days after inoculation, the lowest colonization was observed in mixture S3 treated with CMR12a, CMR12a-ΔPhz or CMR12a-Clp1.

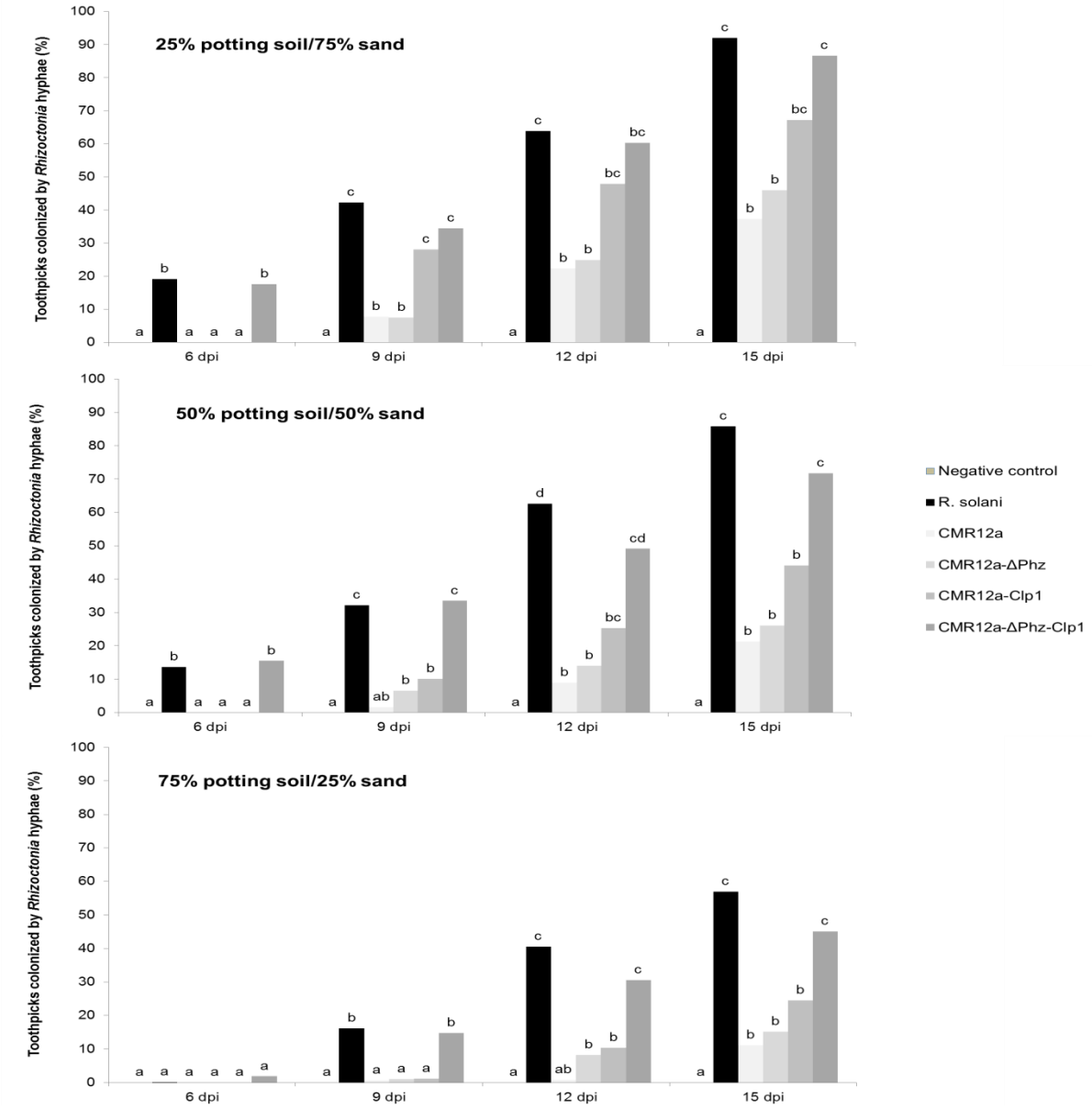

Figure 4.3. Percentage of toothpicks colonized by hyphae of *R. solani* AG 2-2 in three different potting soil/sand mixtures with or without the presence of *Pseudomonas* sp. CMR12a and its mutants at 6, 9, 12 and 15 days after fungal inoculation.

Pseudomonas strains (CMR12a, CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1) were applied to potting soil/sand mixtures to obtain a final concentration of 10^6 CFU g^{-1} of substrate before sowing. Then, sets of five *Rhizoctonia*-colonized wheat kernels were placed 6 cm in front of each seedling row. Sterile toothpicks were inserted at 1.5-cm intervals along the seedling rows at 5, 8, 11 and 14 days post inoculation. After 24 h, these toothpicks were removed from growth substrate and placed on plates containing *Rhizoctonia* selective medium. The colonization of fungal hyphae on toothpicks was observed under the microscope. Experiment was performed twice. Experimental results were arc-sine transformed and data from two repeats were pooled before analysis. In each graph, within each time point, bars indicated with the same letters are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests ($p = 0.05$).

Disease severity data of this experiment are consistent with those of Experiment 1, confirming the influence of growth substrates as well as the involvement of phenazines and sessilin in disease development (Figure 4.4). Factorial analyses show that there were significant effects of both potting soil/sand mixtures and bacterial strains on the spreading rate of *R. solani* at all time points and the severity of disease at 15 days post inoculation. However, the interaction between these two factors was only significant for the spreading rate of *R. solani* at six days and the disease symptoms at 15 days after inoculation (Tables A4.3 and A4.4).

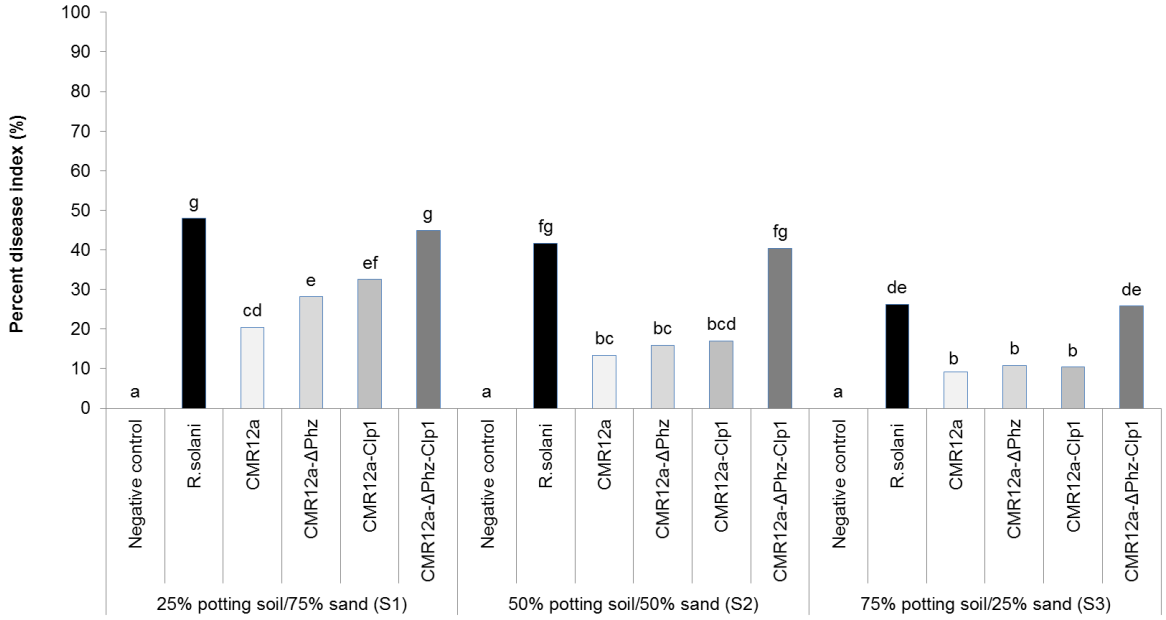

Figure 4.4. Effect of three different potting soil/sand mixtures on disease severity of bean seedlings with or without the presence of *Pseudomonas* sp. CMR12a and its mutants (CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1) when *Rhizoctonia*-colonized wheat kernels were placed 6 cm in front of each seedling row at the time of sowing.

Bacterial strains were applied to substrates S1, S2 and S3 at a concentration of 10^6 CFU g^{-1} of mixture before sowing. The evaluation of disease symptoms on roots and hypocotyls of bean was done 15 days after inoculation using a 0-4 rating scale. Each treatment consisted of 30 seedlings cultivated in three plastic boxes. Experiment was performed twice and data from two repeats were pooled prior to statistical analysis. Data are expressed as Percent disease index and bars indicated with the same letters are not statistically different based on the Kruskal-Wallis and Mann-Whitney nonparametric tests ($p = 0.05$).

Discussion

In this study, we investigated the influence of different potting soil/sand mixtures on the development of *Rhizoctonia* diseases and the biocontrol activity of phenazines and sessilin produced by *Pseudomonas* sp. CMR12a towards bean root rot. Whereas the disease severity data obtained from Experiment 1 only show that disease symptoms increased when more sand was added to the mixtures, those of Experiment 2 also demonstrate a response of disease development to the spread of *Rhizoctonia* through substrates. The fastest fungal invasion, presented in percentage of toothpicks colonized by *Rhizoctonia* hyphae, was recorded in mixture S1 containing 75% of sand. The gradual increase in spreading rate of *R. solani* due to the addition of sand can be explained by differences in C_{org}, which accounts for 4.3% in substrate S1, 10.3% in S2 and 11.8% in S3 (see Table 4.2) since it has been reported that low C content favors *R. solani* (Domsch and Gams, 1970). Physicochemical analyses of three substrates also show the negative correlation between the increase in proportion of sand in the mixtures and the decrease in the content of P, Ca, Mg, K and Na. Whereas there is not enough evidence about the role of Mg and Na in plant diseases, it has been shown that Ca (Huber and Graham 1999), P (Huber and Graham 1999) and K (Baker and Martinson 1970; Amtmann et al. 2008; Christos 2008) may influence the severity of infections caused by fungal pathogens. Ca levels may affect disease incidence by improving cell wall composition, making them more resistant to pathogen penetration (Agrios 2005). Under low disease pressure by *S. rolfsii*, increased Ca levels in peanut tissue may limit disease development (Punja 1985). Ca also may be used in the prevention of pod rot disease induced by *R. solani* (Brenneman 1997). P is most beneficial in reducing fungal diseases of seedlings, where vigorous root development permits plants to escape disease. According to Fageria et al. (2010), the severity of damping-off on pea and root rot on soybean caused by *R. solani* was reduced due to the effect of P. By promoting the development of thicker outer walls in epidermal cells, K may decrease the susceptibility of host plants to disease. Effect of K on reducing the severity of *R. solani*-induced seedling rot in mungbean and cow pea was reported by Tandon and Sekhon (1989). Therefore, based on these findings, we speculate that these mineral nutrients may also play a role in the suppression of Rhizoctonia root rot disease in substrates containing high potting soil ratios. In addition to abiotic factors, the change in total soil microbial population due to the adjustment in the ratio of sand in growth mixtures may associate with the development of disease. When performing a test to observe the spread of *R. solani* in sterile S1, S2 and S3 mixtures using the method described in Experiment 2, we saw that there was no significant difference in spreading rate of *R. solani* among mixtures (Figure A4.1). However, before a strong conclusion can be drawn

about the contribution of mineral elements and soil microorganisms to disease control, more in-depth studies need to be conducted.

Pseudomonas sp. CMR12a is an effective biocontrol agent due to its ability to produce phenazines and CLPs (Debode et al. 2007; Perneel et al. 2007; D'aes et al. 2011; De Maeyer et al. 2013) (Chapter 3). In our experiments, the wild type strain CMR12a, which can simultaneously produce phenazines and sessilin, had excellent biocontrol activity against *R. solani* AG 2-2 in all mixtures tested. The phenazine defective mutant (CMR12a-ΔPhz) and sessilin defective mutant (CMR12a-Clp1) protected seedlings at the same level with the wild type in mixtures containing at least 50% of potting soil (mixtures S2 and S3) but their biocontrol effect was significantly lower than that of the parental strain in the mixture containing only 25% of potting soil (mixture S1). Due to the lack of ability to synthesize phenazines and sessilin, biocontrol capacity of phenazine- and sessilin-negative mutant (CMR12a-ΔPhz-Clp1) was completely lost. These results suggest that both phenazines and sessilin are needed for optimal biocontrol, confirming our previous results about the additive effect of phenazines and sessilin on antagonistic activity against Rhizoctonia root rot of bean (D'aes et al. 2011). In addition, our findings support the results of D'aes (2012) that the biocontrol activity of CMR12a is affected by substrates used. The importance of phenazines and/or CLPs in suppression of various soil-borne diseases has previously been reported (Thomashow et al. 1990; Chin-A-Woeng et al. 1998; Tambong and Höfte 2001; Le et al. 2012). As discussed in our previous work (D'aes et al. 2011) (Chapter 3), the biocontrol activity of *Pseudomonas* sp. CMR12a might be due to direct antifungal effects of sessilin and phenazines, although both compounds could also play a role in the ecological fitness of the strain. Besides their obvious antimicrobial activity, phenazines can act as alternative electron acceptors in microaerobic conditions, can increase iron uptake, and may play a role in biofilm formation and root colonization (Mavrodi et al. 2013). Our root colonization data indicated that wild type and mutant strains involved in our experiments could efficiently colonize bean roots and reached the threshold population level of 10^5 CFU g^{-1} of fresh roots required for successful biocontrol activity (Bull et al. 1991; Raaijmakers et al. 1995; Haas and Défago 2005). Phenazines do not seem to be important for root colonization by *Pseudomonas* sp. CMR12a and the phenazine mutant even was the best root colonizer. However, significantly higher root counts of sessilin producing strains such as wild type CMR12a and mutant CMR12a-ΔPhz were found on seedlings cultivated in the sandy mixtures S1 and S2, compared to those of sessilin mutants CMR12a-Clp1 and CMR12a-ΔPhz-Clp1, suggesting that sessilin is important for rhizosphere competence in substrates containing a high proportion of sand. To explain the increase in

bacterial root counts upon sessilin production in our study, we propose three hypotheses: (i) Sessilin is needed for biofilm formation on the roots. In this context it should be mentioned that D'aes et al. (in press) found that sessilin production by *Pseudomonas* sp. CMR12a is important for biofilm formation on inert substrates; (ii) The presence of CLPs stimulates microbial mineralization (Hernandez et al. 2004) and increases the availability of water-insoluble substrates (Ron and Rosenberg 2001). Products of these biological processes may serve as a source of nutrients for the population growth of *Pseudomonas* spp. in nutrient-poor substrates; (iii) Antimicrobial effect of CLPs provides their producers an advantage in competition for nutrients and ecological niches in the root zone. The contribution of CLPs to root colonization effectiveness has been well-described in previous studies. Amphisin produced by *P. fluorescens* DSS73 was reported to enhance the colonization capacity of this strain on sugar beet seeds and roots (Nielsen et al. 2005) while the synthesis of massetolide A improved the persistence of *P. fluorescens* SS101 on the roots of tomato seedlings (Tran et al. 2007).

Collectively, the severity of Rhizoctonia root rot of bean is determined by the proportion of potting soil to sand in the mixtures. Due to the variation in disease severity, the production of phenazines and/or CLP sessilin by *Pseudomonas* CMR12a is required for disease control. In mixtures having at least 50% of potting soil, disease pressure is low and the presence of either phenazines or sessilin produced by *Pseudomonas* CMR12a is sufficient. However, in the mixture containing only 25% of potting soil, disease pressure is high and the simultaneous production of both compounds is essential for effective control. To our knowledge, most biocontrol studies were conducted on one type of substrate and this is the second study that compare the disease suppressive effect of a biocontrol agent in different substrates. Results obtained from this study give a useful message to researchers concerning the selection of growth substrates for biocontrol experiments of soil-borne pathogens.

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Addendum A4

Supplementary tables

Table A4.1. Analysis of variance of disease severity when *R. solani* AG 2-2 inoculum were placed between two seedling rows maintained in three different potting soil/sand mixtures with the presence of *Pseudomonas* sp. CMR12a and mutants CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1

Factors	df[a]	F	Sig.[b]
Bacterial strains	3	47.79	< 0.001
Potting soil/sand mixtures	2	13.34	< 0.001
Bacterial strains * Potting soil/sand mixtures	6	2.12	0.051

[a] Degree of freedom

[b] Significant level

Table A4.2. Analysis of variance of root colonization capacity of *Pseudomonas* sp. CMR12a and mutants CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1 in three different potting soil/sand mixtures

Factors	df[a]	F	Sig.[b]
Bacterial strains	3	98.12	< 0.001
Potting soil/sand mixtures	2	5.08	0.007
Bacterial strains * Potting soil/sand mixtures	6	3.03	0.007

[a] Degree of freedom

[b] Significant level

Table A4.3. Analysis of variance of disease severity when *R. solani* AG 2-2 inoculum were placed in front of each seedling row maintained in three different potting soil/sand mixtures in the presence of *Pseudomonas* sp. CMR12a and mutants CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1

Factors	df[a]	F	Sig.[b]
Bacterial strains	3	21.97	< 0.001
Potting soil/sand mixtures	2	47.95	< 0.001
Bacterial strains * Potting soil/sand mixtures	6	3.66	0.001

[a] Degree of freedom

[b] Significant level

Table A4.4. Analysis of variance of the spread of *R. solani* AG 2-2 through three different potting soil/sand mixtures with the presence of *Pseudomonas* sp. CMR12a and mutants CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1

	Factors	df[a]	F	Sig.[b]
6 days after sowing				
	Bacterial strains	3	740.31	< 0.001
	Potting soil/sand mixtures	2	20.43	< 0.001
	Bacterial strains * Potting soil/sand mixtures	6	10.76	< 0.001
9 days after sowing				
	Bacterial strains	3	14.51	< 0.001
	Potting soil/sand mixtures	2	13.58	< 0.001
	Bacterial strains * Potting soil/sand mixtures	6	1.15	0.343
12 days after sowing				
	Bacterial strains	3	15.09	< 0.001
	Potting soil/sand mixtures	2	13.52	< 0.001
	Bacterial strains * Potting soil/sand mixtures	6	0.77	0.597
15 days after sowing				
	Bacterial strains	3	16.12	< 0.001
	Potting soil/sand mixtures	2	15.72	< 0.001
	Bacterial strains * Potting soil/sand mixtures	6	0.25	0.958

[a] Degree of freedom

[b] Significant level

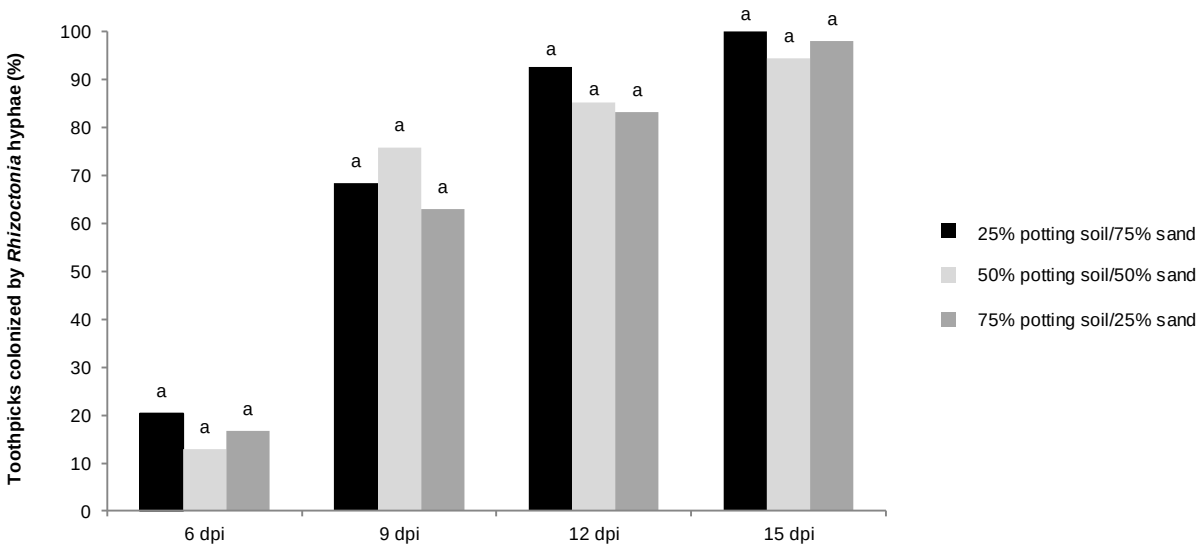

Figure A4.1. Percentage of toothpicks colonized by hyphae of *R. solani* AG 2-2 in three different sterile potting soil/sand mixtures without the presence of *Pseudomonas* sp. CMR12a and its mutants at 6, 9, 12 and 15 days after fungal inoculation

Growth mixtures were sterilized twice on two different days. Then, sets of five *Rhizoctonia*-colonized wheat kernels were placed 6 cm in front of each seedling row. Sterile toothpicks were inserted at 1.5-cm intervals along the seedling rows at 5, 8, 11 and 14 days post inoculation. After 24 h, these toothpicks were removed from growth substrates and placed on plates containing *Rhizoctonia* selective medium. The colonization of fungal hyphae on toothpicks was observed under the microscope. Experiment was performed twice. Experimental results were arc-sine transformed and data from two repeats were pooled before analysis. In each graph, within each time point, bars indicated with the same letters are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests ($p = 0.05$).

Chapter

5

Biocontrol of Rhizoctonia damping-off on Chinese cabbage by *Pseudomonas* sp. CMR12a: Role of phenazines, sessilin and orfamide

Gia Khuong Hoang Hua and Monica Höfte

Abstract

Pseudomonas sp. CMR12a is a promising biocontrol agent due to its ability to produce antimicrobial metabolites phenazines and two distinct types of cyclic lipopeptides (CLPs), sessilin and orfamide. The objectives of the current work were to observe the disease suppressive effect of *Pseudomonas* sp. CMR12a towards damping-off caused by soil-borne pathogenic fungus *Rhizoctonia solani* on Chinese cabbage and to study the capacity of this strain in reducing the viability of *Rhizoctonia* sclerotia. Furthermore, the involvement of phenazines, sessilin and orfamide in biocontrol effect of CMR12a was investigated by comparing the activities of the wild type strain with those of mutants disrupted in phenazines and/or CLPs. Plant experiments and microplate assays demonstrated that phenazines alone or both sessilin and orfamide are needed for successful biocontrol against damping-off of cabbage and for suppressing sclerotial

viability; the presence of individual CLPs is not effective. These results suggest that sessilin and orfamide are acting synergistically in the control of *R. solani*. The synergistic interaction between sessilin and orfamide was confimed by microscopic analyses showing that the combination of a double phenazine- and orfamide-negative mutant and a double phenazine- and sessilin-negative mutant or purified orfamide negatively affected the growth of *Rhizoctonia* hyphae but increased hyphal branching, whereas no inhibition effect was observed when the mutants were inoculated separately. Data obtained also implicate the dose-dependent direct antagonistic effect of orfamide *in vitro* and the essential role of sessilin in root colonization *in planta*.

Introduction

Chinese cabbage (*Brassica chinensis*) is an economically important vegetable universally available in many countries and cultures. Originated from the highlands near the Mediterranean Sea, this leafy vegetable is now grown on a large scale in Asia and present in the daily diet of local inhabitants (Rakow 2004). Unfortunately, the cultivation of this vegetable is hampered by many infectious diseases among which damping-off caused by *Rhizoctonia solani* is the most important (Lancaster 2006; Rimmer et al. 2007). *R. solani* is a widely distributed soil-borne fungus which exists primarily as vegetative mycelium and/or sclerotia (Schwartz 2007; Wharton et al. 2007). Due to the presence of melanin, sclerotia of this fungus are protected from a number of adverse impacts and serve as a source of primary inoculum in soil (Jacobson 2000). For years, the management of *Rhizoctonia* diseases has relied primarily on the application of cultural practices and the use of agrochemicals (Campion et al. 2003). However, whereas agronomic practices alone are not sufficient to control disease, the repeated application of chemical products causes significant harm to humans, wildlife and the environment. Hence, searching for more sustainable approaches which can effectively control diseases caused by *R. solani* as well as reduce the persistence of *Rhizoctonia* sclerotia is an urgent need. The last decades have seen a dramatic expansion in the number and the diversity of microorganisms used to control plant diseases. Among the beneficial rhizospheric bacteria, *Pseudomonas* species are of interest as potential biocontrol agents (BCAs). Pseudomonads are able to produce a wide range of antifungal compounds including pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), hydrocyanide, phenazines and lipopeptides (Leisinger and Margraff 1979; Haas and Keel 2003; Haas and Défago 2005; Reddy 2012). Phenazines constitute a large group of heterocyclic

compounds (Mavrodi et al. 2006) while CLPs are made up of a short, cyclic oligopeptide and a fatty acid tail (Raaijmakers et al. 2006). These compounds have been shown to intimately relate to disease suppression (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006; D'aes et al. 2010; Raaijmakers et al. 2010).

In this study, the biocontrol capacity of *Pseudomonas* sp. CMR12a, an antagonistic bacterium aisolated from the roots of tropical tuber crop cocoyam (*Xanthosoma sagittifolium*) in Cameroon (Perneel et al. 2007), was investigated. *Pseudomonas* sp. CMR12a is able to produce phenazines and two CLPs, sessilin and orfamide (Perneel et al. 2007; D'aes et al. in press). Sessilin is structurally related to tolaasin from *P. tolaasii* whereas the structure of the orfamide produced by CMR12a is very similar to that of orfamides from *P. protegens* Pf-5 (D'aes et al. in press). Given this background, we aimed at determining the effect of phenazines and CLPs on (i) inhibiting hyphal growth and sclerotia germination of *R. solani* under *in vitro* conditions, and (ii) protecting cabbage seedlings from damping-off disease caused by *R. solani* under growth chamber conditions.

Materials and methods

Microorganisms and culture conditions

Pseudomonas strains used in our experiments are listed in Table 5.1. Since the characterization of *Rhizoctonia* spp. associated with *Brassica* crops in Vietnam and the biocontrol experiments of this chapter were conducted in parallel, isolates of *Rhizoctonia* AG 2-1, the most prevalent AG reported on *Brassica* spp. (Pannecoucque et al. 2008; Budge et al. 2009; Ohkura et al. 2009), collected from Belgian cauliflower were used to produce fungal inoculum (see Table 5.1). Bacterial strains were cultured overnight on King´s B (KB; King et al. 1954) agar plates at 28°C. *R. solani* were grown on Potato dextrose agar (PDA; Difco) for three days at 28°C.

Table 5.1. Microorganisms used in this study

Microorganisms	Relevant characteristics[a]	Reference
Pseudomonas strains		
CMR12a	PHZ$^+$, CLP1$^+$, CLP2$^+$; wild type	Perneel et al. (2007)
CMR12a-ΔPhz	PHZ$^-$, CLP1$^+$, CLP2$^+$; phenazine mutant	D'aes et al. (2011)
CMR12a-Clp1	PHZ$^+$, CLP1$^-$, CLP2$^+$; sessilin mutant	D'aes et al. (2011)
CMR12a-ΔClp2	PHZ$^+$, CLP1$^+$, CLP2$^-$; orfamide mutant	D'aes et al. (in press)
CMR12a-ΔClp2-Clp1	PHZ$^+$, CLP1$^-$, CLP2$^-$; sessilin and orfamide mutant	D'aes et al. (in press)
CMR12a-ΔPhz-Clp1	PHZ$^-$, CLP1$^-$, CLP2$^+$; phenazine and sessilin mutant	D'aes et al. (2011)
CMR12a-ΔPhz-ΔClp2	PHZ$^-$, CLP1$^+$, CLP2$^-$; phenazine and orfamide mutant	D'aes et al. (in press)
CMR12a-ΔPhz-ΔClp2-Clp1	PHZ$^-$, CLP1$^-$, CLP2$^-$; phenazine, sessilin and orfamide mutant	D'aes et al. (in press)
Rhizoctonia solani AG 2-1		
BK001-1-1	Highly aggressive isolate of wirestem on cauliflower in Belgium	Pannecoucque et al. (2008)
BK002-1-1	Highly aggressive isolate of wirestem on cauliflower in Belgium	Pannecoucque et al. (2008)
BK004-4-1	Highly aggressive isolate of wirestem on cauliflower in Belgium	Pannecoucque et al. (2008)
BK008-1-1	Highly aggressive isolate of wirestem on cauliflower in Belgium	Pannecoucque et al. (2008)
BK008-2-1	Highly aggressive isolate of wirestem on cauliflower in Belgium	Pannecoucque et al. (2008)
BK012-1-1	Highly aggressive isolate of wirestem on cauliflower in Belgium	Pannecoucque et al. (2008)

[a] PHZ: phenazines; CLP1: cyclic lipopeptide type sessilin; CLP2: cyclic lipopeptide type orfamide; plus$^+$: metabolite is produced; minus$^-$: metabolite is not produced.

Experiment 1. *In vitro* pathogenicity assay

The virulence of *R. solani* AG 2-1 isolates was determined using the method described by Keijer et al. (1997), with some adaptations. Briefly, seeds of Chinese cabbage (*B. chinensis* cv. Elton) were surface disinfected in 1% NaOCl for five min and rinsed twice with sterile water. Sets of six surface-sterilized seeds were evenly spaced on Gamborg B5 medium (Gamborg B5 medium including vitamins; Duchefa) in square Petri dishes (12 x 12 cm; Novolab). Two 5-mm mycelial plugs cut from the edges of the *R. solani* colonies were placed between seeds 2 and 3 and seeds 4 and 5. In the control dishes, sterile PDA plugs were used as inoculum. The Petri dishes were incubated at 22°C in the dark for two days to allow seed germination. Then, the second halves of the Petri dishes were covered with aluminum foil to protect the roots from light and dishes were placed under a 60 degree angle in a growth chamber (22°C, RH = 60%, 16 h photoperiod). Disease severity was scored after eight days of incubation using a 0-to-4 rating scale: 0 = healthy, no symptoms; 1 = lesions covering less than 25% of the root, hypocotyl or leaf surface; 2 = lesions covering between 25% and 50% of the root, hypocotyl or leaf surface; 3 = wilted plant with lesions covering between 50% and 75% of the root, hypocotyl or leaf

surface; 4 = lesions covering more than 75% of root, hypocotyl or leaf surface or dead plant. The experiment was performed in a complete randomized design with two replications per treatment and was done twice.

Experiment 2. *In vivo* pathogenicity assay

Surface-sterilized Chinese cabbage seeds were incubated on wet filter paper for one day at 22°C to allow germination. Each set of six germinated seeds was sown into one perforated plastic tray (22 x 15 x 6 cm) partially filled with 600 g growth substrate composed of 50% potting soil (Structural; Snebbout, Kaprijke, Belgium) and 50% sand (Cobo garden; Belgium) (w/w). The physicochemical characteristics of the 50% potting soil/50% sand mixture is shown in Chapters 3 and 4 of this thesis.

The same *Rhizoctonia* isolates tested in *in vitro* assay were used to produce inoculum for this test according to the method described by Scholten et al. (2001). In short, 5-mm PDA plugs of *R. solani* AG 2-1 were used to inoculate sterile wheat kernels and flasks containing the inoculated kernels were incubated at 22°C for 14 days. For inoculation, comparable-sized kernels fully covered by hyphae of *R. solani* AG 2-1 were placed at 2-cm deep in a row between two 4-day-old seedling rows with a concentration of two infected kernels per seedling. Seedlings inoculated with sterile wheat kernels served as a control. All plants were placed in a growth chamber (22°C, RH = 60%, 16 h photoperiod) and the development of disease symptoms on root and hypocotyl was recorded 14 days after inoculation using the following scale: 0 = healthy, no symptoms; 1 = lesions covering less than 25% of the stem and/or root surface; 2 = lesions covering between 25% and 50% of the stem and/or root surface; 3 = wilted plant with lesions covering between 50% and 75% of the stem and/or root surface; 4 = lesions covering more than 75% of the stem and/or root surface or dead plant. A completely randomized design was employed with four replications per treatment, which is equivalent to 24 seedlings, and this test was done twice. Since *R. solani* AG 2-1 isolate BK008-2-1 was very pathogenic to cabbage seedlings in both conditions tested, it was selected for further studies.

Experiment 3. *In vivo* biocontrol of damping-off disease

Cells of wild type *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production from overnight cultures on KB were harvested with physiological solution and their concentration was determined spectrophotometrically at 620 nm (OD_{620}). Fifty ml of bacterial

suspension was mixed to each tray containing 600 g growth substrate to give a final concentration of 10^7 CFU g^{-1} of substrate before six pre-germinated Chinese cabbage seeds were sown. Fungal inoculum preparation, artificial inoculation and disease evaluation were performed as in Experiment 2. This experiment has a completely randomized design, each treatment was replicated four times (six seedlings each) and the entire experiment was repeated once.

Experiment 4. Competitive colonization capacity of *Pseudomonas* sp. CMR12a and CMR12a-mutants

Since cabbage seedlings in Experiment 3 were uprooted for disease assessment when they were 14 days old, their roots were very delicate and it was not possible to determine densities of root colonizing bacteria. Therefore, to assess colonization ability of *Pseudomonas* sp. CMR12a and its mutants, a root competitive assay was conducted. Bacterial suspensions prepared from overnight cultures were adjusted to OD_{620} = 1.0. One ml of each suspension was pipetted into a sterile eppendorf tube containing five pre-germinated seeds. Tubes were incubated at 28°C for 30 min. Then, seeds were sown into sand, which was autoclaved twice on two successive days (121°C, 60 min). All plants were kept in a growth chamber (22°C, RH = 60%, 16 h photoperiod) and were fertilized every three days with Hoagland solution (Hoagland and Arnon 1950). At 18 days after sowing, five plants per treatment were harvested and their roots were gently swirled a few times in water to wash away the sand particles. Roots were ground with physiological solution and the suspensions were serially diluted to 10^{-3}. Six droplets (10 µl each) of the 10^{-2} and 10^{-3} dilutions were placed on KB plates. The plates were incubated at 28°C for 48 h before counting based on their morphological characteristics.

Experiment 5. Microscopic observations

To study the interaction between sessilin and orfamide, a microscopic experiment was performed according to the protocol of Bolwerk et al. (2003). Orfamide (molecular weight = 1281.8 g mol^{-1}) was purified by the Lab of Phytophathology, Ghent University. The method used for orfamide purification will be published elsewhere (Zongwang Ma, unpublished). Purified orfamide was dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 10 µg ml^{-1}. Sterile microscopic glass slides were covered with a thin, flat layer of water agar (Bacto agar; Difco) and placed in a plastic Petri dish containing a moist filter paper to maintain the humidity suitable for growth of microorganisms. A 3-day-old fungal plug (diameter = 5 mm) taken from full-grown *R. solani* plates was inoculated at the center of the glass slide. Overnight

cultures of CMR12a, CMR12a-ΔPhz-Clp1 and CMR12a-ΔPhz-ΔClp2 were used to prepare bacterial suspensions at a concentration of 10^6 CFU ml^{-1}. Two droplets (15 µl each) of bacterial suspension alone, orfamide alone or a mixture of bacterial suspension and orfamide (50/50; v/v) were placed at two sides of the glass slide (about 2 cm from the fungi). Slides inoculated with KB broth were used as control. The plates were incubated for 36 h at 28°C before evaluating under an Olympus BX51 microscope**.** Images were processed using Photoshop CC (Adobe).

Experiment 6. Microplate assay

Sclerotia of *R. solani* AG 2-1 at relatively uniform size were produced according to the method of Manning et al. (1970). Briefly, a 7-day-old PDA culture was excised into small pieces before it was blended in 50 ml of sterile demineralized water to form a thick suspension. This suspension was used to cover the surface of fresh PDA plates. The plates were incubated at 22°C for five weeks before sclerotia were harvested.

To produce inoculum, bacterial cells harvested from overnight cultures were dissolved in sterile physiological solution. Densities of the bacterial suspensions were measured spectrophotometrically at 620 nm and were adjusted to 10^6 CFU ml^{-1}. Then, one ml of each suspension was used to inoculate 100 ml of KB broth. These cultures were incubated at 28°C for six days while shaking. Cell-free culture supernatants were obtained by centrifugation of cultures at 13,400 rpm for 10 min and filtration twice through 0.22 µm pore size filters (Millipore Corporation; USA). Before setting up the experiment, sclerotia of *R. solani* were surface-disinfested with l% sodium hypochlorite solution for two min and rinsed twice with sterile demineralized water. Surface-disinfested sclerotia were air-dried and sets of ten sclerotia were placed to wells containing 1 ml of culture filtrates (Figure 5.1). At 0, 12, 24, 36, 48 and 60 h after incubation, sclerotia were removed from wells and rinsed twice with sterile water. Each sclerotium was cut into two halves before plating on PDA plates. The plates were incubated at 28°C and the viability of sclerotia was recorded after 36 h of incubation. Each treatment consisted of five replications and the experiment was performed twice.

Figure 5.1. The microplate assay

Each well of a 24-well microplate was filled with ten surface-disinfested sclerotia of *R. solani* and 1 ml of bacteria-free culture filtrates.

Statistical analysis

Disease severity was expressed as Percent disease index (PDI), which was worked out using the formula described by Wheeler (1969):

$$\text{PDI (\%)} = \frac{\sum (\text{Disease class x number of plants falling under that class})}{\text{Total number of plants observed x Maximum class}} \text{ x } 100$$

The disease data collected from two repetitions were pooled in order to obtain a single database. On the other hand, there was a statistically significant interaction between two data sets obtained from two repetitions of the root competive assay; therefore, after the data were log10 transformed, each data set was analyzed independently. Disease severity data and root colonization data were analyzed using non-parametric tests. The differences between groups were detected through a k-sample comparison by the Kruskal-Wallis tests. Finally, a two-sample comparison by the Mann-Whitney test was performed to compare every two treatments ($p = 0.05$).

Viability of the sclerotia was analyzed using logistic regression ($p = 0.05$). All statistic tests were performed with SPSS 22.0 (SPSSinc, Illinois, USA).

Results

Aggressiveness of *R. solani* AG 2-1 isolates towards Chinese cabbage seedlings

Most isolates tested were highly virulent towards cabbage seedlings under *in vitro* conditions (PDI varied from 96 to 100%) and the only isolate that was moderately aggressive *in vitro* was BK002-1-1 (PDI = 52%). Under *in vivo* conditions, the PDI fluctuated from 0 to 76%. The most

aggressive isolates that caused severe damage on seedlings were BK012-1-1 and BK008-2-1. In contrast, the lowest PDI was recorded for BK002-1-1 and no disease symptoms were observed on seedlings inoculated by this isolate (Table 5.2).

Table 5.2. Agressiveness of *R. solani* AG 2-1 isolates towards Chinese cabbage

Treatment	Percent Disease Index (%)	
	***In vitro* experiment**	***In vivo* experiment**
Control	0 c	0 d
BK001-1-1	100 a	52 bc
BK002-1-1	52 b	0 d
BK004-4-1	100 a	61 b
BK008-1-1	100 a	37 c
BK008-2-1	100 a	72 a
BK012-1-1	96 a	76 a

Disease symptoms were scored using a 0-to-4 scale. Each experiment was repeated twice in a complete randomized design. Every treatment consisted of 12 seedlings maintained in two square Petri dishes (*in vitro* experiment) or 24 seedlings cultivated in four perforated plastic boxes (*in vivo* experiment). The results obtained from two repeats of each experiment were pooled before analysis. Values followed by the same letter are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests (p = 0.05).

Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants towards Rhizoctonia damping-off disease of Chinese cabbage under greenhouse conditions

To determine the effect of *Pseudomonas* sp. CMR12a and the importance of phenazines and CLPs in biocontrol of Rhizoctonia damping-off of Chinese cabbage, *Pseudomonas* sp. CMR12a and mutants impaired in the biosynthesis of phenazines and/or CLPs were included in an *in planta* trial with *R. solani* AG 2-1 isolate BK008-2-1. No growth inhibition effects or disease symptoms were observed on seedlings upon bacteria application, indicating that *Pseudomonas* sp. CMR12a and its mutants were not phytotoxic or pathogenic to cabbage seedlings (data not shown).

Damping-off disease was significantly suppressed when the growth substrate was mixed with bacterial strains that can produce phenazines or both CLPs. As shown in Figure 5.2, disease severity was reduced from 78.13% in diseased control to 24.48% in the CMR12a treatment. The same level of protection was detected in treatments where CMR12a-ΔPhz, CMR12a-Clp1, CMR12a-ΔClp2 and CMR12a-ΔClp2-Clp1 were introduced. When CMR12a-ΔPhz-Clp1,

CMR12a-ΔPhz-ΔClp2 or CMR12a-ΔPhz-ΔClp2-Clp1 were applied, no biocontrol effect was observed. These data not only indicate the importance of phenazines and CLPs, but also imply the synergistic effect of the two CLPs in biocontrol activity of *Pseudomonas* sp. CMR12a.

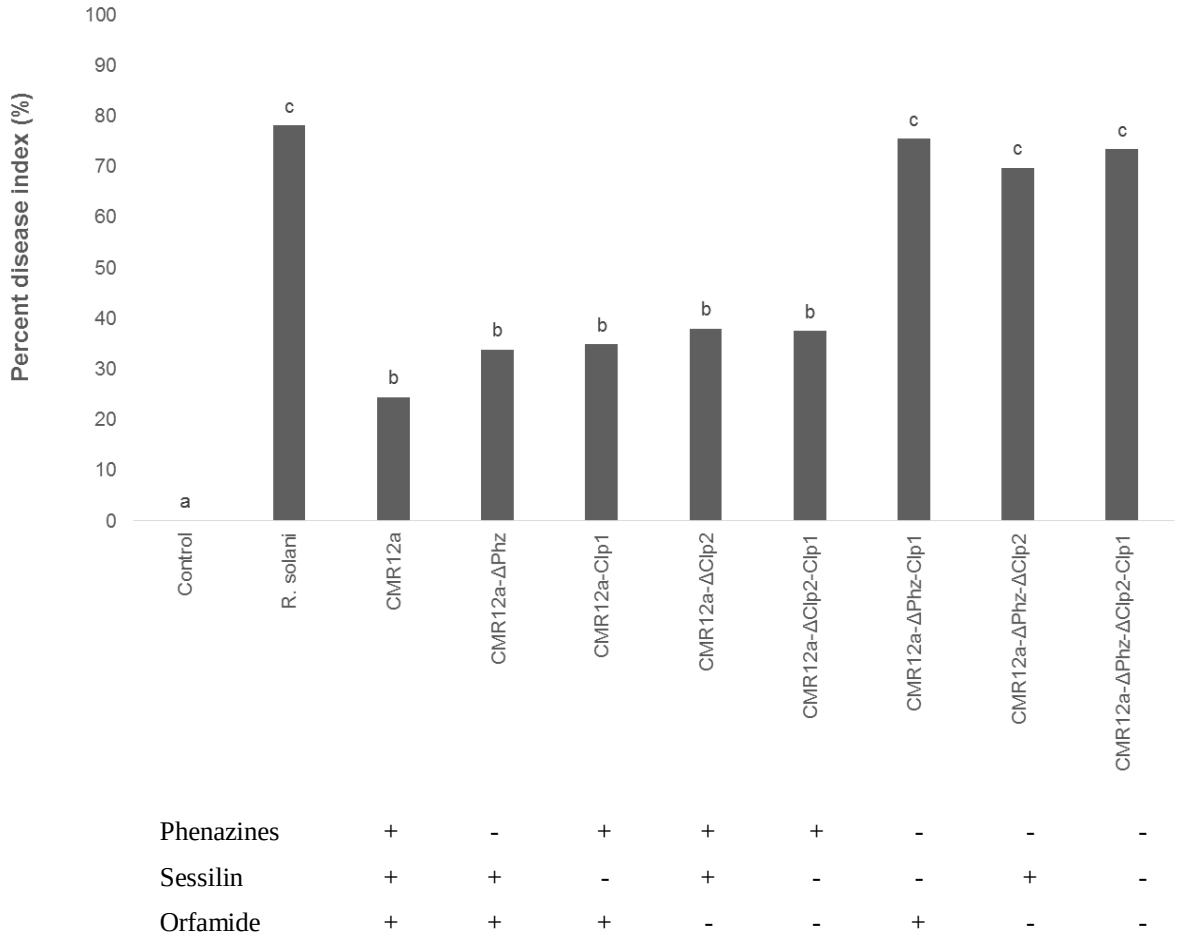

Figure 5.2. Biocontrol effect of *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production towards Rhizoctonia damping-off of Chinese cabbage.

Pseudomonas strains were applied to substrate containing 50% potting soil and 50% sand (w/w) to obtain a final concentration of 10^7 CFU g^{-1} of growth substrate before sowing. Disease severity on roots and hypocotyls was recorded 14 days after inoculation of *R. solani* AG 2-1 isolate BK008-2-1 using the 0-4 rating scale: 0 = healthy, no symptoms; 1 = lesions covering less than 25% of the stem and/or root surface; 2 = lesions covering between 25% and 50% of the stem and/or root surface; 3 = wilted plant with lesions covering between 50% and 75% of the stem and/or root surface; 4 = lesions covering more than 75% of the stem and/or root surface or dead plant. Experiment was performed twice and every treatment consisted of 24 seedlings.

Root colonization capacity of *Pseudomonas* sp. CMR12a and its mutants

The bacterial strains differed in their ability to colonize roots of cabbage (Table 5.3). Bacterial counts varied from 8.25 ± 0.18 to 8.94 ± 0.30 log CFU g^{-1} of fresh root in repetition 1 and from 6.73 ± 0.38 to 7.28 ± 0.15 log CFU g^{-1} of fresh root in repetition 2. The most efficient colonizing strains were CMR12a-ΔClp2, CMR12a-ΔClp2-Clp1 and CMR12a-ΔPhz-ΔClp2 whereas CMR12a, CMR12a-ΔPhz, CMR12a-Clp1 and CMR12a-ΔPhz-Clp1 were the least effective.

Table 5.3. Competitive colonization capacity of *Pseudomonas* sp. CMR12a and mutants impaired in phenazine and/or CLP production on roots of Chinese cabbage grown in sterile sand

Treatment	Phenazines and CLPs produced	Population density of CMR12a and mutants (in log CFU g^{-1} of fresh root)	
		Repetition 1	Repetition 2
Control		0.00 ± 0.00 a	0.00 ± 0.00 a
CMR12a	Phenazines, sessilin and orfamide	8.29 ± 0.11 b	6.82 ± 0.27 b
CMR12a-ΔPhz	Sessilin and orfamide	8.31 ± 0.27 b	6.73 ± 0.38 b
CMR12a-Clp1	Phenazines and orfamide	8.25 ± 0.18 b	6.86 ± 0.16 b
CMR12a-ΔClp2	Phenazines and sessilin	8.94 ± 0.30 d	7.22 ± 0.10 cd
CMR12a-ΔClp2-Clp1	Phenazines	8.84 ± 0.18 d	7.28 ± 0.15 d
CMR12a-ΔPhz-Clp1	Orfamide	8.39 ± 0.25 b	6.78 ± 0.11 b
CMR12a-ΔPhz-ΔClp2	Sessilin	8.92 ± 0.30 d	7.26 ± 0.13 d
CMR12a-ΔPhz-ΔClp2-Clp1		8.64 ± 0.29 c	7.13 ± 0.21 c

Pre-germinated Chinese cabbage seeds were incubated in bacterial suspensions (OD_{620} = 1.0) prepared from overnight cultures of CMR12a and its mutants on KB agar plates. Root colonization data determined 18 days after sowing were log10 transformed before analysis. Experiment was repeated twice in a complete randomized design and each treatment consisted of five seedlings. Within each column, values followed by the same letter are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests (p = 0.05).

Antagonistic activity of sessilin and orfamide produced by *Pseudomonas* sp. CMR12a against *R. solani* AG 2-1

Synergistic interaction between sessilin and orfamide from *Pseudomonas* sp. CMR12a was tested under *in vitro* conditions. In the presence of CMR12a, the growth of *R. solani* was significantly inhibited and the approaching hyphae of this fungus displayed a remarkable increase in branch frequency as compared to that of the negative control. No inhibition zones

were formed between fungus and bacteria when CMR12a-ΔPhz-Clp1 or CMR12a-ΔPhz-ΔClp2 was applied and normal branching appeared in hyphae close to bacterial droplets. However, when the two mutants were concurrently inoculated on the glass slide or when purified orfamide was added to the culture of CMR12a-ΔPhz-ΔClp2, the direct antifungal activity of the wild type strain was completely restored. The antagonistic ability towards *R. solani* of purified orfamide was also observed when *R. solani* was challenged with this compound (Figure 5.3).

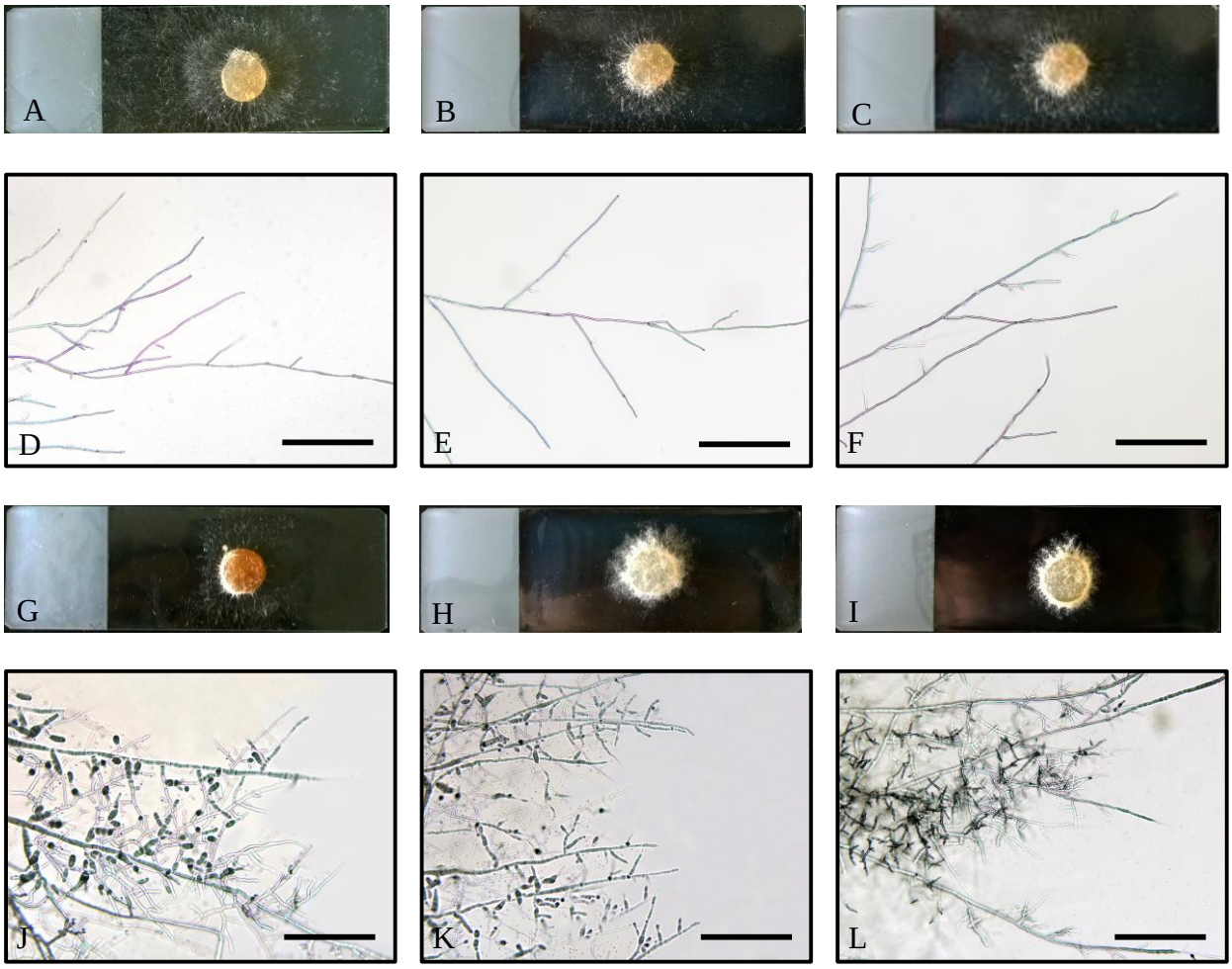

Figure 5.3. Microscopic assays showing inhibited growth and morphological alterations in *R. solani* hyphae caused by bacteria. Interactions were studied on water agar. Treatments include **A and D**, control, **B and E**, *Pseudomonas* CMR12a-ΔPhz-Clp1, **C and F**, *Pseudomonas* CMR12a-ΔPhz-ΔClp2, **G and J**, *Pseudomonas* sp. CMR12a, **H and K**, *Pseudomonas* CMR12a-ΔPhz-ΔClp2 in combination with *Pseudomonas* CMR12a-ΔPhz-Clp1, **I and L**, *Pseudomonas* CMR12a-ΔPhz-ΔClp2 in combination with purified orfamide. The size bar represents 500 µm in all panels.

Influence of phenazines and CLPs on viability of *R. solani* AG 2-1 sclerotia

Soaking sclerotia of *R. solani* in cell-free supernatants of CMR12a, CMR12a-ΔPhz or CMR12a-ΔClp2-Clp1 significantly reduced sclerotial viability (Table 5.4). The reduction in viability of sclerotia was initiated at 24 h after incubation. At 60 h after incubation, all sclerotia were found dead when they were immersed in supernatants collected from the cultures of CMR12a and CMR12a-ΔPhz, whereas the percentage of viable sclerotia in treatment with CMR12a-ΔClp2-Clp1 was 62%. Nearly all (from 96% to 100%) of the sclerotia remained viable when they were treated with KB (control treatment) or supernatants of CMR12a-Clp1, CMR12a-ΔClp2, CMR12a-ΔPhz-Clp1, CMR12a-ΔPhz-ΔClp2 and CMR12a-ΔPhz-ΔClp2-Clp1.

Table 5.4. Effect of phenazines, CLP sessilin and CLP orfamide on the viability of *R. solani* sclerotia

Treatment	Phenazines and CLPs produced	Percentage of viable sclerotia (%)					
		0 hpi	12 hpi	24 hpi	36 hpi	48 hpi	60 hpi
Control		100 a	100 a	100 a	100 a	100 a	100 a
CMR12a	Phenazines, sessilin and orfamide	100 a	99 a	88 b	34 c	1 c	0 c
CMR12a-ΔPhz	Sessilin and orfamide	100 a	99 a	87 b	41 c	2 c	0 c
CMR12a-Clp1	Phenazines and orfamide	100 a	100 a	100 a	100 a	100 a	100 a
CMR12a-ΔClp2	Phenazines and sessilin	100 a	100 a	100 a	100 a	100 a	97 a
CMR12a-ΔClp2-Clp1	Phenazines	100 a	100 a	93 b	76 b	69 b	62 b
CMR12a-ΔPhz-Clp1	Orfamide	100 a	100 a	100 a	100 a	100 a	99 a
CMR12a-ΔPhz-ΔClp2	Sessilin	100 a	100 a	100 a	100 a	100 a	96 a
CMR12a-ΔPhz-ΔClp2-Clp1		100 a	100 a	100 a	100 a	100 a	100 a

Viability of sclerotia was assessed 0, 12, 24, 36, 48 and 60 h after soaking surface-disinfested sclerotia in 1 ml of cell-free culture supernatants of *Pseudomonas* sp. CMR12a and its biosynthesis mutants. Experiment was done twice with five replications (10 sclerotia each) per treatment. Within each column, values followed by the same letter are not significantly different according to logistic regression ($p = 0.05$).

Discussion

Damping-off caused by *R. solani* is one of the most destructive diseases hampering Chinese cabbage production worldwide. Bioassays conducted to study the virulence of *R. solani* AG 2-1, the most prevalent AG found on *Brassica* vegetables (Pannecoucque et al. 2008; Budge et al. 2009; Ohkura et al. 2009), towards Chinese cabbage showed that there was a significant

variation in pathogenicity within this AG, especially under growth chamber conditions. Our pathogenicity data are comparable to those obtained from similar *in vitro* tests carried out by Pannecoucque et al. (2008) on endive. Based on these results, *R. solani* isolate BK008-2-1 was selected for biocontrol experiments.

Pseudomonas sp. CMR12a is known as a potential BCA. In this study, the application of this strain to growth substrate effectively reduced the severity of Rhizoctonia damping-off and PDI of CMR12a-treated treatment was 64% lower than that of the pathogen control treatment. In addition to investigating the biocontrol effect of *Pseudomonas* sp. CMR12a, the relative role of phenazines and CLPs, antagonistic metabolites produced by CMR12a, in the biocontrol of Rhizoctonia damping-off was elucidated via mutant analysis. The results of *in planta* experiments indicated that cabbage seedlings were also successfully protected from disease when the growth substrate was mixed with CMR12a-ΔPhz, CMR12a-Clp1, CMR12a-ΔClp2 or CMR12a-ΔClp2-Clp1 before sowing. Conversely, the addition of CMR12a-ΔPhz-Clp1, CMR12a-ΔPhz-ΔClp2 or CMR12a-ΔPhz-ΔClp2-Clp1 did not provide protection against disease development. These data suggest that phenazines alone are sufficient to suppress Rhizoctonia damping-off whereas the co-production of the sessilin and orfamide is required for successful control of this disease. Our observations not only confirm a previous finding that phenazines and sessilin are important determinants of the biocontrol capacity of CMR12a (D'aes et al. 2011) (Chapters 3 and 4), but also indicate the occurrence of a synergistic interaction between the two types of CLPs in biocontrol of plant diseases.

To determine whether direct antibiosis of CLPs is involved in the biocontrol capacity of CMR12a and to verify the synergistic interaction between sessilin and orfamide, microscopic assays were conducted with a wild type, a double phenazine- and sessilin-negative mutant (CMR12a-ΔPhz-Clp1), a double phenazine- and orfamide-negative mutant (CMR12a-ΔPhz-ΔClp2) and a purified orfamide compound. It was shown that when *R. solani* was challenged with cells of CMR12a-ΔPhz-Clp1 or CMR12a-ΔPhz-ΔClp2, no growth inhibition effect was observed. However, when we combined CMR12a-ΔPhz-ΔClp2 with CMR12a-ΔPhz-Clp1, *R. solani* reduced radial mycelium extension but increased branching, indicating that the disease suppressive ability of CMR12a could be a result of direct antibiotic effect of sessilin and orfamide when these two CLPs were concurrently produced. Additionally, the application of purified orfamide alone or the combination of CMR12a-ΔPhz-ΔClp2 and purified orfamide also showed antifungal effect, suggesting that the ability to suppress fungal growth of orfamide is dose-dependent. Previously, it was also proven that tolaasin and sessilin had direct antimicrobial activity towards various pathogens (unpublished data), including *R. solani*

(Chapter 3). A possible explanation for the synergistic interaction between sessilin and orfamide in this study is that under the conditions tested, these compounds are not synthesized in sufficient amounts and, therefore, the presence of one of the two CLPs is not enough to affect fungal growth or to provide disease control. However, to further elucidate the mechanisms involved in the biological activity of sessilin and orfamide, much more work will be necessary.

The ability to establish population in the root zone is a crucial characteristic of BCAs (De Weert and Bloemberg 2007; Dutta and Podile 2010). Enumeration of the bacterial populations colonizing the plant roots demonstrates that strains which can produced phenazines, sessilin or a combination of phenazines and sessilin appeared to be better root colonizers. The role of sessilin in rhizosphere competence was also indicated in Chapters 3, 4 and 7 of this thesis as mutants impaired in sessilin production were poorer in root colonization capacity compared to the sessilin-producing strains. On the contrary, the competitive colonization abilities of orfamide-producing strains were significantly lower than all other strains included in our tests. These observations point out the crucial role of phenazines and sessilin in rhizosphere competence of the producing strains. Phenazines have been shown to contribute to ecological fitness (Mazzola et al. 1992) and biofilm formation (Maddula et al. 2006; Maddula et al. 2008), whereas the importance of CLPs in surface motility and attachment/detachment to and from surfaces has been reported (D'aes et al. 2010; Raaijmakers et al. 2010). Recent work by D'aes et al. (in press) indicated that sessilin is important for biofilm formation and orfamide appears to be crucial for swarming motility. Together, these data explain why more bacteria were found on roots of seedlings treated with phenazine- and/or sessilin-producing strains.

Although all bacterial strains were able to reach the critical level for good biocontrol capacity of 10^5 CFU g^{-1} of fresh root (Raaijmakers et al. 1999; Haas and Défago 2005), the application of mutants that only produced sessilin or orfamide could not protect seedlings from disease infection. According to Eberl et al. (1999) and Rather (2005), the formation of biofilms can protect bacteria from adverse environmental conditions, whereas swarming motility helps bacteria to reach novel niches. Therefore, co-production of sessilin and orfamide may result in ecological advantages for the producing strains. In contrast, strains that only produce sessilin or orfamide are less competitive, and, as a result, they are less effective in disease suppression.

Another intriguing observation is that phenazines and CLPs produced by *Pseudomonas* sp. CMR12a negatively affected the survival of sclerotia; which is consistent with the findings reported earlier for Pseudomonads and other bacterial genera (Debode et al. 2007; Errakhi et al. 2009). In our bioassays, all sclerotia were killed when they were soaked in the culture extracts of CMR12a and CMR12a-ΔPhz, whereas a significantly lower effect was observed for

CMR12a-ΔClp2-Clp1. These results indicate that sessilin and orfamide, when present together, are more effective in sclerotia viability suppression compared to phenazines. To explain the synergistic effect of sessilin and orfamide, we hypothesize that orfamide allows bacteria to rapidly reach the sclerotia; then, the presence of sessilin allow bacteria to attach and form a thin film on sclerotia (Debode et al. 2007). Since CLPs have the ability to permeabilize cell walls and membranes (Nielsen et al. 1999; Thrane et al. 2000; Perneel et al. 2008), they may attack and kill sclerotia of *R. solani*. Different from CLPs, phenazines represent toxicity towards pathogens through the generation of reactive oxygen intermediates (Hassett et al. 1992; Mahajan-Miklos et al. 1999). According to Hassett et al. (1992), after diffusing across or inserting into the membrane of pathogenic agents, the reduced form of pyocyanin, a type of phenazines, can react with oxygen to generate superoxide (O_2^-), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($^{\bullet}HO$). These toxic compounds can lead to the death of target microorganisms. Since it was hypothesized that biosurfactants can serve as carrier molecules for phenazines, facilitating their access into the target cells (Perneel et al. 2008), the lack of CLP production of sessilin- and orfamide-negative mutants might result in the reduction in their ability to suppress the viability of *Rhizoctonia* sclerotia.

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Chapter

6

The use of coir pith and antagonistic *Pseudomonas* sp. CMR12a for integrated control of damping-off disease caused by *Rhizoctonia solani* AG 2-1 on Chinese cabbage (*Brassica chinensis*)

Gia Khuong Hoang Hua and Monica Höfte

Abstract

Damping-off caused by *Rhizoctonia solani* is one of the major diseases responsible for considerable yield reductions in Chinese cabbage cultivation. With the ability to resist to both biotic and abiotic stress, sclerotia of *R. solani* can survive in soil for years and serve as a source of primary inoculum. In this study, the effect of coir pith incorporation alone or in combination with *Pseudomonas* sp. CMR12a towards the viability of *R. solani* AG 2-1 sclerotia and damping-off disease initiated by these sclerotia was investigated. After four weeks of incubation with 5% coir pith, the viability of *R. solani* sclerotia was significantly decreased from 75% to 20%, while the mean number of dead sclerotia, presumably due to the infection of parasitic fungi, was dramatically raised from 2% to 66%. In contrast to coir pith, the addition of

Pseudomonas sp. CMR12a, an antagonistic bacterium isolated from cocoyam roots in Cameroon, did not affect sclerotial viability. However, this bacterium applied alone partially protected seedlings from the pathogen. An intermediate level of disease protection was also obtained in the 5% coir pith treatment, or in the combined treatments 0.1% coir pith+CMR12a and 1% coir pith+CMR12a. Strikingly, the highest disease reduction of 48.3% was achieved when CMR12a was added to soil amended with 5% coir pith, suggesting the combined effect of coir pith and CMR12a in disease suppression.

Introduction

Rhizoctonia solani is a highly destructive soil-borne fungus that attacks a great diversity of economically important host plants worldwide. Diseases induced by this fungus are difficult to control due to the persistence of its sclerotia. In Chapters 3, 4 and 5 of this thesis, we demonstrated that *Pseudomonas* sp. CMR12a, an antagonistic bacterium isolated from roots of cocoyam, could successfully control root rot of bean and damping-off of Chinese cabbage caused by *R. solani.* Moreover, the incorporation of Kraft pine lignin, a side product of the paper industry, has been shown to effectively suppress *R. solani* basal rot of lettuce (Van Beneden 2009) and reduce the viability of *R. solani* sclerotia (Van Beneden et al. 2010), suggesting the potential to use antagonistic bacteria in combination with lignin-rich organic amendments to sustainably manage this fungus.

Coir pith is non-fibrous, fluffy and light weight croaky material constituting about 50-70% of the husk of coconut palm (*Cocos nucifera*) (Prabhu and Thomas 2002). This by-product consists of lignin 20-40%, cellulose 40-50% and hemicellulose 15-35% (Sjöström 1993). Due to the high lignin content and the availability in great quantity, coir pith could be a good soil amendment to control sclerotia of *R. solani*.

In this study, our objectives were to observe the ability of coconut fiber as soil amendment in reducing the viability of *R. solani* AG 2-1 sclerotia and to investigate whether the application of *Pseudomonas* sp. CMR12a into soil that was previously incubated with coconut fiber would lead to an enhanced suppressive effect towards *Rhizoctonia* damping-off on Chinese cabbage seedlings.

Materials and methods

Microorganisms, culture conditions and inoculum production

Microorganisms used in this study were *Pseudomonas* strain CMR12a and *R. solani* AG 2-1 isolate BK008-2-1.

Pseudomonas sp. CMR12a was isolated from the rhizosphere of cocoyam grown in Cameroon (Perneel et al. 2007). This strain is characterized by the ability to produce phenazines (phenazine-1-carboxylic acid and phenazine-1-carboxamide) and cyclic lipopeptides (CLPs type sessilin and orfamide) (D'aes et al. in press). To produce inoculum, the bacterium was cultured on King´s B (KB; King et al. 1954) agar plates and bacterial cells were harvested after 24 h of incubation at 28°C. The concentration of the bacterial suspension was measured spectrophotometrically at 620 nm and was adjusted to 5 x 10^7 CFU ml^{-1} before using.

R. solani BK008-2-1 was originally isolated from Belgian cauliflower fields (Pannecoucque et al. 2008) and its virulence towards Chinese cabbage was confirmed under *in vitro* and *in vivo* conditions (see Chapter 5). Sclerotia of this fungus were produced as previously described by Manning et al. (1970). In short, a 7-day-old culture on Potato dextrose agar (PDA; Difco) was mixed in 50 ml of sterile demineralized water to form a thick suspension. This suspension was poured over the surface of fresh PDA plates. Sclerotia were harvested after five weeks of incubation at 22°C.

Soil and coir pith preparation

Soil used in this experiment was collected from a commercial field in Bottelare (Belgium) (Table 6.1). Soil taken from the upper 30 cm layer at different locations in the field was sieved (mesh = 5 mm) to remove big particles (e.g. stones and plant residues), homogenized into one sample and stored at 10°C until use. Before amendment with coir pith at the concentrations of 0.1%, 1% and 5% (w/w), moisture content of this soil was measured and adjusted to 16.5% (w/w) by adding sterile water.

Table 6.1. Characteristics of Bottelare soil

Parameter	Result
Soil texture	Loamy sand
OM (%)	1.92
pH_{H2o}	6.84
pH_{KCl}	6.29
P (mg/kg)	444.2
Ca (mg/kg)	1557
Mg (mg/kg)	152
K (mg/kg)	345
Na (mg/kg)	24.8

The absence of *Trichoderma* spp. and fluorescent pseudomonads in coir pith was confirmed by plating before setting up the experiments.

Experiment 1. Pot assay with coir pith and sclerotia of *R. solani* AG 2-1

This experiment was conducted based on the method developed by Van Beneden et al. (2010), with some adaptations. A nylon mesh bag (2.5 x 2.5 cm, mesh = 250 µm) was filled with five sclerotia and five bags were buried in one glass jar containing 200 g soil. Soil without coir pith served as control. Jars were incubated at 22°C for four weeks and sclerotia were removed for surface sterilization in l% sodium hypochlorite followed by rinsing two times with sterile water. Then, each surface-disinfected sclerotium was cut in half. The viability of the sclerotia was tested by plating one half on PDA amended with 100 mg l^{-1} streptomycin and 4 mg l^{-1} prochloraz (as Sporgon WP; BASF Belgium). The other half of the sclerotium was placed on PDA amended with 100 mg l^{-1} streptomycin to observe mycoparasitism. All plates were incubated for two weeks at 22°C and evaluation of sclerotial viability and mycoparasitism started from day six after incubation. Based on morphological characteristics, mycoparasites were classified into three groups including *Trichoderma* spp., *Fusarium* spp. and other fungi.

Changes in *Pseudomonas* and *Trichoderma* population in soil were determined using a soil dilution technique. From each jar described, two replicate soil samples were taken. For *Pseudomonas* counting, serial dilutions of soil samples in sterile physiological solution were spread on *Pseudomonas* isolation agar (PIA; Difco). After 24 h of incubation at 28°C, colonies were counted and transferred to KB agar plates to check for their fluorescence under UV light. To determine *Trichoderma* population, soil samples were mixed with sterile water and soil

suspensions were plated on *Trichoderma* selective medium (Williams et al. 2003). Plates were incubated at 22°C for four days before counting. This experiment was done twice and each treatment consisted of three replicate jars.

Experiment 2. Combined effect of coir pith and *Pseudomonas* sp. CMR12a on sclerotial viability and damping-off disease caused by *R. solani* AG 2-1 on Chinese cabbage

In this experiment, each nylon mesh bag consisting of two sclerotia was buried in a plastic pot (diameter = 15 cm) containing 200 g soil. Pots were covered with plastic bags and aluminum foil before they were incubated at 22°C for four weeks to maintain soil moisture content. A 1% sodium hypochlorite solution was used to sterile Chinese cabbage seeds (*Brassica chinensis* cv. Elton) and surface-sterilized seeds were incubated on moist filter papers for one day at 22°C to allow germination. Then, sets of five germinated seeds were sown in each pot, which had been incubated for four weeks, before two bacterial droplets (5×10^7 CFU ml^{-1}; 500 µl per droplet) were pipetted at two sides of the seeds (Figure 6.1). The severity of damping-off disease was assessed eight days after sowing using the 0-4 scoring system, whereas 0 = healthy, no symptoms observed; 1 = small brown, water-soaked lesions covering less than 25% of stem and/or hypocotyl; 2 = large lesions covering less than 50% of stem and/or hypocotyl; 3 = small wilted plant with large lesions covering less than 75% of stem and/or hypocotyl; 4 = stem and/or hypocotyl is completely decayed and dead plant. The viability of sclerotia and the population of *Pseudomonas* sp. CMR12a were also determined using the same methods described in Experiment 1. Treatments of this test included the negative control (seeds were sown into non-amended soil in the absence of both *R. solani* and CMR12a), the pathogen control (seeds were sown into non-amended soil containing sclerotia of *R. solani* in the absence of CMR12a), the coir pith control (seeds were sown into soil amended with different amount of coir pith in the absence of both *R. solani* and CMR12a) and the treatments containing coir pith alone (seeds were sown into coir pith-amended soil inoculated with sclerotia of *R. solani*; CMR12a was not present) or in combination with CMR12a (seeds were sown into coir pith-amended soil inoculated with sclerotia of *R. solani*; CMR12a was applied to soil at sowing). There were six replicate pots (two sclerotia each) per treatment and the entire experiment was performed twice. Because the number of sclerotia involved in each treatment was relatively small, we only show the percentage of sclerotia which were infected with fungi and killed instead of dividing them into groups according to the mycoparasites as in Experiment 1.

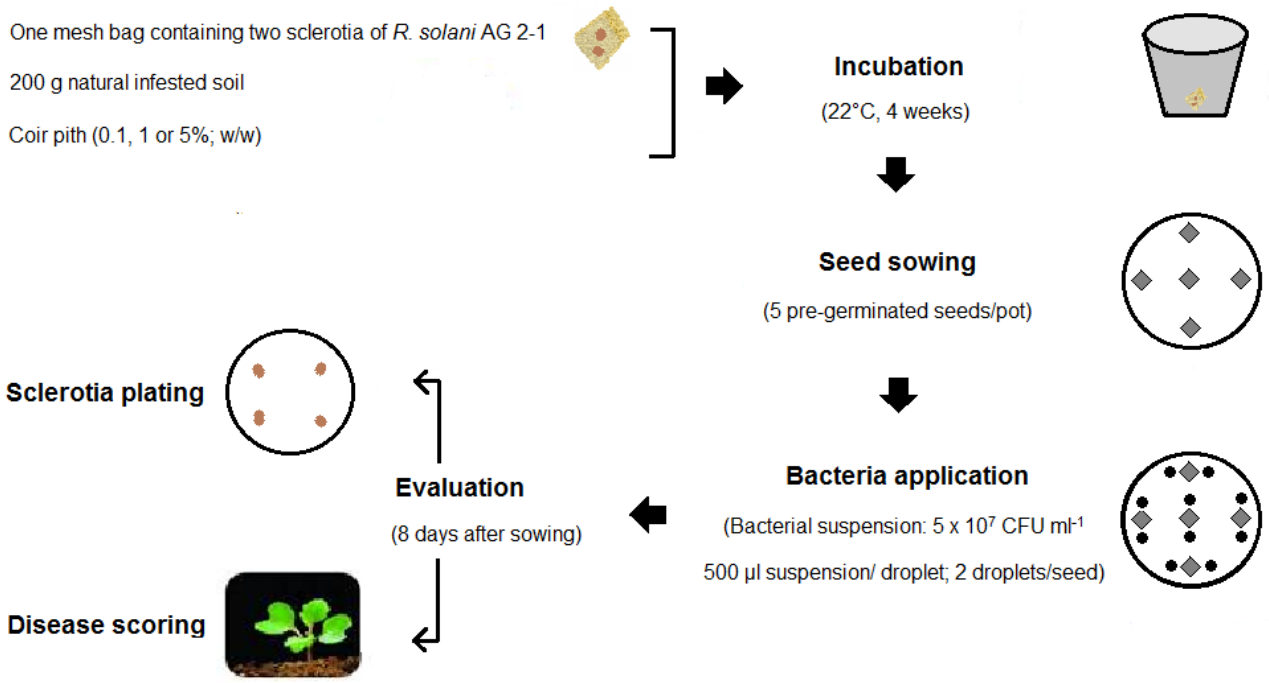

Figure 6.1. Scheme to observe the combined effect of coir pith and *Pseudomonas* sp. CMR12a on sclerotial viability and severity of damping-off disease caused by *R. solani* AG 2-1 on Chinese cabbage

Data analysis

Viability and mycoparasitism of the sclerotia were subjected to logistic regression ($p = 0.05$). Damping-off severity was expressed as Percent disease index (PDI), which was calculated using the formula described by Wheeler (1969):

$$\text{PDI } (\%) = \frac{\sum (\text{Disease class x number of plants falling under that class})}{\text{Total number of plants observed x Maximum class}} \text{ x } 100$$

The population density of *Trichoderma* spp., fluorescent pseudomonads (Experiment 1) and *Pseudomonas* sp. CMR12a (Experiment 2) was log10 transformed prior to statistical analysis. These data were, then, analyzed using non-parametric Kruskal-Wallis tests and Mann-Whitney comparisons ($p = 0.05$).

Results

Influence of coir pith incorporation on the viability of *R. solani* AG 2-1 sclerotia

The difference in sclerotial viability corresponding to the variation in the amount of coir pith added to soil was evaluated after four weeks of incubation (Table 6.2). The viability of *R. solani* sclerotia was reduced to 22% when they were buried in soil amended with 5% of coir pith. Soil containing 1% coir pith also showed a slight decrease in sclerotial viability (59% compared to 79.2% viable sclerotia of unamended soil) but this reduction was not statistically significant. Higher amounts of coir pith added to soil also resulted in a higher percentage of sclerotia infected with fungi. Significantly more infected sclerotia were detected in soils supplemented with 1% or 5% of coconut residue. In soil amended with 5% coir pith, the percentages of dead sclerotia infected with *Trichodema* spp., *Fusarium* spp. and other fungi were 22%, 25% and 13%, respectively. However, in soil amended with 1% coir pith, more sclerotial bodies were infected with fungi other than *Trichoderma* spp. and *Fusarium* spp. Other treatments did not have significantly more infected sclerotia than the control.

Table 6.2. Percentage of viable and dead sclerotia in unamended soil and in soils incorporated with 0.1, 1 and 5% coir pith after four weeks of incubation at 22°C. In each column, values followed by the same letter are not significantly different based on logistic regression (p = 0.05).

Treatment	Viable sclerotia (%)	Dead sclerotia (%)				
		Not infected (%)	Infected (%)			
			Total	*Trichoderma*	*Fusarium*	Other fungi
Control	79.2 b	15.7 a	5.0 a	3.1 a	1.0 a	1.0 a
0.1% coir pith	73.0 b	19.0 a	8.0 a	2.0 a	4.0 a	2.0 ab
1% coir pith	59.0 b	16.0 a	25.0 b	9.0 a	6.0 a	10.0 ab
5% coir pith	22.0 a	18.0 a	60.0 c	22.0 b	25.0 b	13.0 b

Incorporation of 5% coir pith significantly stimulated the growth of fluorescent pseudomonads and *Trichoderma* spp. Populations of fluorescent pseudomonads and *Trichoderma* spp. increased from 2.48 ± 0.17 and 1.69 ± 0.26 log CFU g^{-1} of non-amended soil to 3.26 ± 0.35 and 3.20 ± 0.18 log CFU g^{-1} of soil mixed with 5% coir pith, respectively (Table 6.3).

Table 6.3. Population density of fluorescent pseudomonads and *Trichoderma* spp. present in non-amended control soil and in soils amended with 0.1, 1 and 5% coir pith after four weeks of incubation at 22°C. In each column, values followed by the same letter are not significantly different based on the Kruskal-Wallis and Mann-Whitney nonparametric tests (p = 0.05).

Treatment	Population density (log CFU g^{-1} of soil)	
	Fluorescent pseudomonads	***Trichoderma* spp.**
Control	2.48 ± 0.17 a	1.69 ± 0.26 a
0.1% coir pith	2.54 ± 0.24 a	1.79 ± 0.28 a
1% coir pith	2.67 ± 0.22 a	1.99 ± 0.29 a
5% coir pith	3.26 ± 0.35 b	3.20 ± 0.18 b

Influence of coir pith amendment and *Pseudomonas* sp. CMR12a application on sclerotial viability and mycoparasitism

The effect of coir pith on the survival of *R. solani* AG 2-1 sclerotia with or without the presence of *Pseudomonas* sp. CMR12a was investigated in Experiment 2. Because no *Rhizoctonia* sclerotia were buried in soil of the negative control and the coir pith control, we do not include these treatments in Table 6.4. Obviously, sclerotia incubated in soil amended with 5% of coir pith showed significantly lower viability than the pathogen control. The percentage of viable sclerotia reduced from 91.7% in non-amended control soil to 16.7% in soil supplemented with 5% coir pith. Coir pith amendment also resulted in a steadily increase in sclerotial infection and the highest number of dead sclerotia infected with fungi was observed in treatments contained 5% coir pith.

The results in Table 6.4 also indicate that the application of CMR12a to soil at sowing did not influence sclerotia viability and mycoparasitism. Comparable number of sclerotial bodies was killed in soils amended with the same amount of coir pith regardless of the presence of CMR12a.

Table 6.4. Effect of coir pith incorporation on the viability of *R. solani* AG 2-1 sclerotia with or without the presence of *Pseudomonas* sp. CMR12a. In each column, values followed by the same letter are not significantly different based on logistic regression (p = 0.05).

Treatment		Viable sclerotia (%)[a]	Dead sclerotia (%)[b]
Without CMR12a	*R. solani* (pathogen control)	91.7 b	8.3 a
	R. solani + 0.1% coir pith	91.7 b	8.3 a
	R. solani + 1% coir pith	75.0 b	25.0 a
	R. solani + 5% coir pith	16.7 a	83.3 b
With CMR12a	*R. solani*	91.7 b	8.3 a
	R. solani + 0.1% coir pith	83.3 b	16.7 a
	R. solani + 1% coir pith	75.0 b	25.0 a
	R. solani + 5% coir pith	16.7 a	83.3 b

[a] Percentage of viable sclerotia.

[b] Percentage of dead sclerotia which were infected with fungi.

Disease suppressive effect of coir pith and *Pseudomonas* sp. CMR12a

No phytotoxicity effects or disease symptoms were observed on seedlings of the coir pith control treatments; PDI of these treatments were 0 and they are not shown in Figure 6.2. Although the application of CMR12a to soil did not affect sclerotial viability, a significant reduction in disease severity was detected on Chinese cabbage seedlings cultivated in soils treated with this bacterium. The lowest disease pressure was observed in treatment in which CMR12a was applied to soil previously incubated with 5% coir pith. The use of CMR12a alone or in combination with 0.1 and 1% coir pith still reduced damping-off severity compared to the control, but to a lesser extent compared to the treatment consisting of CMR12a and 5% coir pith. Similar degree of disease supressiveness was found when soil was incorporated with 5% coir pith in the absence of CMR12a. Soil only incubated with 0.1% or 1% coir pith did not show any suppressive effect towards damping-off disease (Figure 6.2).

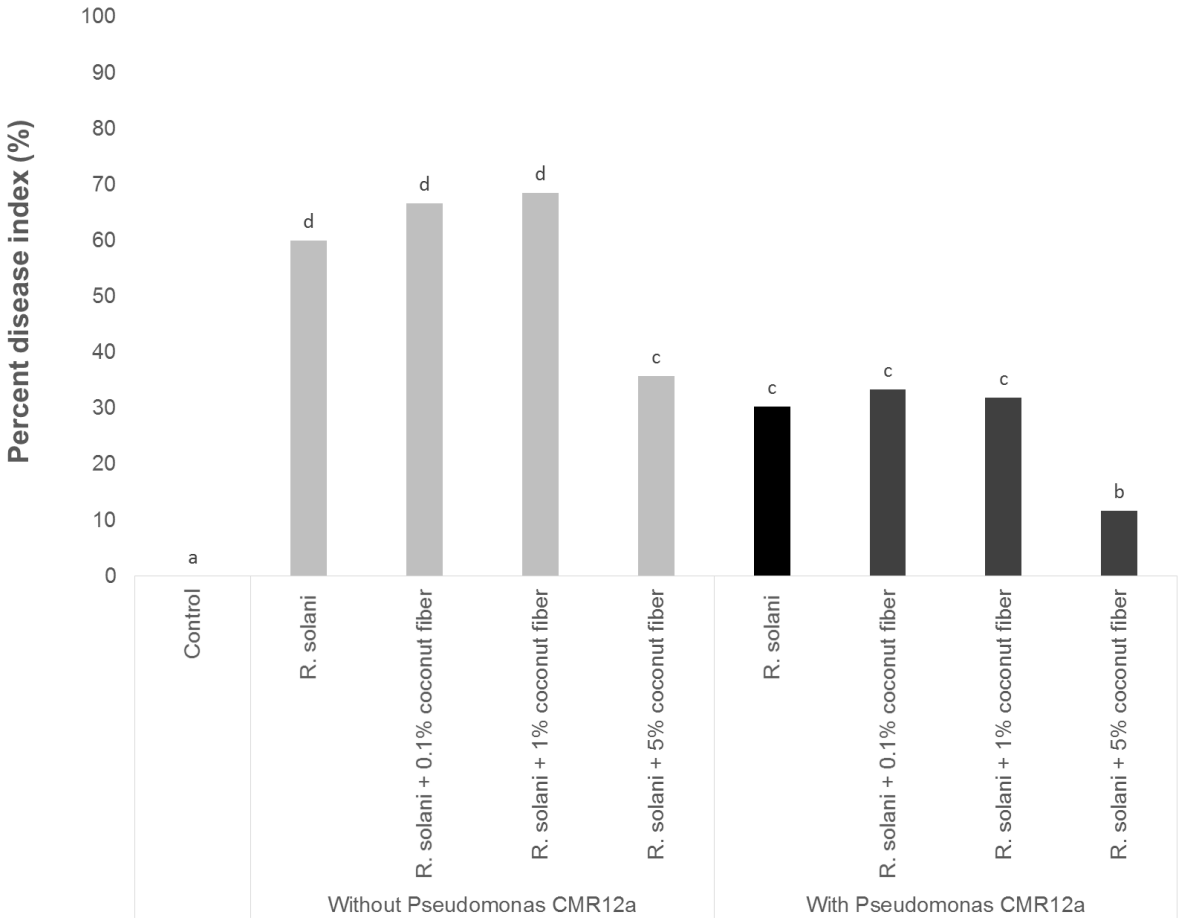

Figure 6.2. Effect of coir pith incorporation on the severity of damping-off disease caused by *R. solani* AG 2-1 on Chinese cabbage seedlings with or without the presence of *Pseudomonas* sp. CMR12a. Bars indicated with the same letters are not statistically different based on the Kruskal-Wallis and Mann-Whitney nonparametric tests (p = 0.05).

Bacterial counts at the end of the experiment show that *Pseudomonas* sp. CMR12a was never recovered from soil of the negative control and coir pith control treatments (data not shown). In soil treated with CMR12a, the population density of this bacterium ranged from 7.20 ± 0.43 to 7.44 ± 0.20 log CFU g^{-1} of soil. The significantly higher bacterial concentrations were found in soil containing 1% or 5% coir pith, indicating that coir pith could stimulate the growth of CMR12a (Table 6.5).

Table 6.5. Density of *Pseudomonas* sp. CMR12a in non-amended control soil and in soils amended with 0.1, 1 and 5% coir pith after four weeks of incubation at 22°C. Values followed by the same letter are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests (p = 0.05).

Treatment	Population of *Pseudomonas* sp. CMR12a (in log CFU g^{-1} of soil)
CMR12a + *R. solani*	7.20 ± 0.43 a
CMR12a + *R. solani* + 0.1% coir pith	7.24 ± 0.45 a
CMR12a + *R. solani* + 1% coir pith	7.47 ± 0.31 b
CMR12a + *R. solani* + 5% coir pith	7.44 ± 0.20 b

Correllation between the viability of sclerotia and the severity of Rhizoctonia damping-off disease

There was a positive correlation between sclerotial viability and disease severity (r = 0.220, p = 0.016) when treatments containing CMR12a were not included in the Spearman'RHO analysis. Treatments having more viable sclerotia would have higher PDI. In other words, those with low percentage of sclerotial viability have less serious disease symptoms than those with high number of viable sclerotia.

Discussion

R. solani is a sclerotia-forming filamentous fungus which attacks and damages agricultural crops worldwide. One of the most effective ways to protect plants from *Rhizoctonia* diseases is to reduce the persistence of its melanized sclerotia. The results of our pot assay clearly demonstrated that the incorporation of 5% coir pith significantly reduced the viability of *Rhizoctonia* sclerotia by 55%. Our observations about the effect of coir pith amendment, which contains 20-40% of lignin (Sjöström 1993), in reducing the survival of melanin-covered sclerotia agree with those of Debode et al. (2005), Van Beneden et al. (2010) and Montanari and Innocenti (2011). Reduction in sclerotial viability was accompanied by a significant increase in the number of sclerotia infected with parasitic fungi in soil amended with 5% coir pith, supporting the lignin-melanin hypothesis proposed by Shetty et al. (2000), Butler et al. (2005) and Debode et al. (2005). According to this hypothesis, the increased susceptibility of sclerotia to antagonists after soil amendments with lignin-rich materials results from the degradation of fungal melanin. Melanin, a component found in the cell walls of *Rhizoctonia*

sclerotia, is a dark-pigmented polymer of phenolic compounds characterized by the ability to confer resistance to biological and chemical attack. Similar to melanin, lignin is an extremely recalcitrant polymer. In nature, lignin can be degraded to various extents by a limited number of microorganisms. With the ability to produce extracellular oxidative enzymes such as laccases, lignin peroxidase and manganese peroxidase, white rot fungi (notably *Phanerochaete chrysosporium*) are known as the most powerful lignin degraders (Bugg et al. 2011). When lignin is added to soil, the growth of lignin degrading microorganisms is stimulated (Shetty et al. 2000; Debode et al. 2005) and their enzyme activity is increased (Van Beneden et al. 2010). Due to the similarity in the structure of lignin and melanin, enzyme activities of lignin degraders can also destroy melanin (Butler and Day 1998). When melanin is broken down, sclerotia become more susceptible to microbial attack (Gómez and Nosanchuk 2003; Butler et al. 2005); which could explain the increase in the number of dead sclerotia colonized by fungi in soil supplemented with coir pith. The identification of the mycoparasites colonizing dead sclerotia in the pot assay showed that the percentage of non-viable sclerotia infected with *Fusarium* spp. and *Trichoderma* spp. in soil amended with 5% coir pith was significantly higher than in un-amended control soil. It has been proven that the addition of coir pith favored the growth and survival of *Trichoderma* spp. (Saju et al. 2002), which are naturally occurring fungi in coir pith (Silveira et al. 2002). *Trichoderma* spp. can directly attack and lyse the cell walls of sclerotia due to the production of chitinase and β-(1,3)-glucanase (Elad et al. 1984; Van Toor et al. 2005). *T. harzianum* was shown to be able to invade and kill sclerotial cells of *Sclerotium rolfsii* (Elad et al. 1984; Upadhyay and Mukhopadhyay 1986). Likewise, the addition of a mixture containing isolates of *Trichoderma* spp. to sawdust led to an increase in mortality of *Ciborinia camelliae* sclerotia (Van Toor et al. 2005). More importantly, the antagonistic effect of *Trichoderma* spp. towards sclerotia of *R. solani* was also reported. According to De Melo and Faull (2000), *T. harzianum* strain Th-9 and *T. koningii* strain Tk-5 not only inhibited the growth of *R. solani*, but also effectively reduced the viability of *Rhizoctonia* sclerotia. Similar effects on sclerotial viability were observed with *T. virens* strain TY009 (Liu et al. 2009) and *Trichoderma* spp. strain TN3, TK3 and TC3 (Prasad and Kumar 2011). Different from *Trichoderma,* several species of *Fusarium* such as *F. oxysporum*, *F. solani* (Falcón et al. 1995; Rodriguez 1996) and *F. proliferatum* (Regalado et al. 1997) possess the ability to engage in lignin breakdown, thereby gaining access to melanin-degraded sclerotia. Therefore, it should be concluded that *Fusarium* spp. and *Trichoderma* spp. have important contributions to both degradation of sclerotial bodies and colonization of melanin-degraded sclerotia.

In addition to fungi, several types of bacteria, including Actinomycetes and *Pseudomonas* spp. (Ahmad et al. 2010), are known to react with lignin (Bugg et al. 2011; Brown and Chang 2014) or to mineralize lignin degradation intermediates (Rüttimann et al. 1991; Vicuña et al. 1993). In our pot assay, population density of fluorescent pseudomonads was positively correlated to the concentration of coir pith added to soil, suggesting that simple substrates released during decomposition of lignin and cellulose in coir pith might be used as a source of nutrient for the growth of bacteria. However, the increased population of these bacteria was not related to the number of dead sclerotia not infected with fungi and this is an evidence that bacterial attack alone was not sufficient to kill the sclerotia.

Because the severity of *Rhizoctonia* diseases is determined by the density and viability of sclerotia (Van Bruggen et al. 1986; Damicone et al. 1993), the reduction in the survival of sclerotia accumulated in soil may lower disease symptoms. In the second experiment, the incorporation of 5% coir pith decreased the viability of sclerotia by 75.0%, confirming the results obtained in Experiment 1 that coir pith amendment could reduce *Rhizoctonia* sclerotial viability. Moreover, the addition of 5% coir pith to soil also decreased the severity of damping-off disease by 24.2%, which is consistent with the findings of Soltani et al. (2002) and Van Beneden (2009) who reported that ammonium lignosulfonate and Kraft pine lignin soil amendment could suppress common scab and verticillium wilt of potato and basal rot of lettuce, respectively.

According to Debode et al. (2007), CLPs produced by *Pseudomonas* sp. CMR12a could reduce the survival of *Verticillium* microsclerotia *in vitro*. Additionally, Chapter 5 of this thesis showed that most sclerotia of *R. solani* were killed after incubating with a supernatant collected from a 6-day-old culture of CMR12a. However, in this study, adding CMR12a to soil did not affect sclerotia viability. The difference in the effect of CMR12a towards the viability of (micro)sclerotia is probably caused by the difference in experimental set up. Whereas in other experiments (micro)sclerotia were soaked in bacterial suspensions or cell-free culture supernatants, in this experiment there was a large distance between sclerotia, which were kept at the bottom of the pot, and the bacterial suspension, which was introduced at the soil surface. Since the viability of sclerotia was evaluated eight days after bacterial application, another explanation could be that the incubation time was not enough for the bacteria to effectively suppress sclerotia germination. Hence, it is interesting to repeat the work and incubate sclerotia in CMR12a-containing soil for a longer period (e.g. four weeks).

Although the application of *Pseudomonas* sp. CMR12a did not reduce sclerotial viability, evaluating disease symptoms at the end of Experiment 2 demonstrated that the combination of

CMR12a with coir pith resulted in better control of Rhizoctonia damping-off. The highest level of disease suppression was achieved when CMR12a was introduced to soil previously incubated with 5% coir pith. *Pseudomonas* sp. CMR12a has been referred to as a biocontrol agent of several plant pathogens, including *R. solani* (De Jonghe 2006; Debode et al. 2007; D'aes et al. 2011) (Chapters 3, 4 and 5). This bacterium can produce several interesting antifungal compounds such as phenazines and CLPs sessilin and orfamide (D'aes et al. in press; Perneel et al. 2007). Both phenazines and CLPs are antifungal compounds and their biocontrol capacity has been well-documented (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006; D'aes et al. 2010; Raaijmakers et al. 2010). Moreover, these metabolites also involved in biofilm formation and root colonization (D'aes et al. in press; Maddula et al. 2006; Maddula et al. 2008). Therefore, it is possible that the significant reduction in the severity of damping-off disease results from the additive effect of coir pith amendment and CMR12a application. Whereas coir pith amendment considerably suppressed the survival of sclerotia, CMR12a effectively colonized roots of cabbage seedlings and protected them from fungal infection. As a consequence, the disease symptoms were significantly less severe when CMR12a was combined with coir pith compared to the two treatments applied separately.

Together, our observations indicate that the incorporation of coir pith at a rate of 5% decreased the viability *R. solani* AG 2-1 sclerotia and increased sclerotial infection, thereby reducing disease symptoms developed on Chinese cabbage seedlings cultivated in this soil. Disease suppressive ability was enhanced when soil was treated with *Pseudomonas* sp. CMR12a at the time of sowing. Significantly less disease symptoms were observed in the combined treatment 5% coir pith+CMR12a. These results suggest the potential to combine lignin-rich materials and antagonistic agents to control sclerotia-forming soil-borne pathogens although more research is needed to fully unravel the mechanism.

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Chapter

7

General discussion and Future prospects

Vietnam is a developing country characterized by the dominance of agricultural production. With a large contribution to GDP, total exports and employment (Ecobichon 2001), agriculture plays an important role for economic growth and social stability in this country (GSO 2008). However, the arable land per capita in Vietnam is quite low because of its large population and a limited amount of cultivable area. Therefore, agricultural production system has been switched from extensive to intensive to meet the increasing domestic and foreign demand for agricultural products. The intensification of agriculture enhances yield and output per acre, but it also results in increased risk of diseases. To protect crops, growers started to use pesticides from the 1950s and pesticide consumption increased from 14 million tons in 1990 to 50 million tons in 2008 (Phung et al. 2012). In addition to harm target pests or pathogens, excessive use and misuse of agrochemicals can pose a great threat to human and environmental health. Therefore, as stated in the first pages of this thesis, the present study is designed to develop an environmentally friendly, safe and cost-effective strategy to manage *Rhizoctonia* diseases on vegetables. To attain this goal, we assessed the genetic diversity of *Rhizoctonia* spp. associated with *Brassica* vegetables in Vietnam. In parallel, we used bean-*Rhizoctonia solani* and cabbage-*R. solani* as models to evaluate the biocontrol effect of antagonistic bacteria and organic amendments. A summary of this study is given in Table 7.1.

Table 7.1 Overview of the thesis

Host plant	Pathogen	Growth substrate	Result	Chapter
Brassica spp.	*Rhizoctonia* spp.	Field soil in Ha Noi, Lam Dong, Dong Nai, Vinh Long, Can Tho, Hau Giang and Soc Trang (Vietnam)	• A collection of 97 *Rhizoctonia* isolates were obtained from different *Brassica* vegetables. Ninety three isolates were multinucleate *R. solani* and the other four were binucleate *Rhizoctonia*. For the first time, multinucleate *R. solani* AG 1-IA and AG 1-ID are found on *Brassica* crops. • There has possibly been a link between the occurrence of the different AGs and the cropping systems and the habit to use alternative hosts of *Rhizoctonia* fungus as cover crops in different sampling areas suggesting that agricultural practices determine the AGs associated with *Brassica* plants in Vietnam.	2
Bean (*Phaseolus vulgaris*)	*R. solani* AG 2-2 *R. solani* AG 4-HGI	50% potting soil and 50% sand	• Phenazines and CLP sessilin show additive effect in the control of bean root rot	3
Bean (*Phaseolus vulgaris*)	*R. solani* AG 2-2	25% potting soil and 75% sand 50% potting soil and 50% sand 75% potting soil and 25% sand	• Both sessilin and phenazines are required to control root rot of bean in a sandy substrate, while either sessilin or phenazines are sufficient to suppress disease in substrates with less sand.	4
Chinese cabbage (*Brassica chinensis*)	*R. solani* AG 2-1	50% potting soil and 50% sand	• Phenazines alone are sufficient to suppress damping-off disease. However, sessilin and orfamide are likely to act synergistically in the biological control of *R. solani* disease on cabbage. • Simultaneous production of sessilin and orfamide is needed to kill sclerotia of *R. solani*	5
Chinese cabbage (*Brassica chinensis*)	*R. solani* AG 2-1	Field soil in Bottelare (Belgium)	• Incorporation of 5% coconut fiber can reduce the viability of sclerotia, thereby decreasing the severity of damping-off disease. • *Pseudomonas* sp. CMR12a did not influence sclerotial viability but damping-off disease is suppressed in response to the application of this bacterium. • Coconut fiber incorporation in combination with *Pseudomonas* sp. CMR12a results in enhanced suppressive effect towards Rhizoctonia damping-off.	6

In the following sections, the most important contributions and limitations of this thesis are discussed from a fundamental and practical point of view. In addition, suggestions are given for future research.

Significance of our study

Variation of *Rhizoctonia* AGs present in *Brassica* fields of Vietnam

One of the most important contributions of this thesis is to provide an overview about the distribution and genetic diversity of *Rhizoctonia* populations associated with *Brassica* diseases in Vietnam. *Rhizoctonia* species are widespread among soils all over the world and some of them have long been recognized as highly destructive pathogens on economic important crops such as cereals, vegetables and potato. Our survey showed that *Brassica* crops cultivated in Vietnam are susceptible to *Rhizoctonia* isolates belonging to nine different AGs. The majority of the isolates were identified as multinucleate *R. solani* isolates. Interestingly, about 45% of isolates was AG 1-IA, the AG responsible for sheath blight on rice. This AG, together with AG 1-ID, has not been reported before on *Brassica* spp. In contrast, there was no AG 2-1 isolate in our collection, although this AG has been reported as the most prevalent pathogen infecting *Brassica* crops grown in other countries (Keinath and Farnham 1997; Khangura et al. 1999; Rollins et al. 1999; Paulitz et al. 2006; Pannecoucque et al. 2008; Budge et al. 2009). Since no survey on the occurrence of *Rhizoctonia spp*. in *Brassica* vegetables was conducted before, this is the first report about the AGs associated with *Rhizoctonia* diseases on these crops in Vietnam. Knowing which AGs of *Rhizoctonia* causing disease is a first and crucial step to develop effective control strategies against these fungi.

In addition to identifying AGs, our results further indicated a notable differences among *Brassica* species in susceptibility to *R. solani*. More interestingly, we found that the occurrence of AGs is correlated with the cropping systems and cultural practices. **From a practical point of view**, the variation in the virulence of *R. solani* AGs on different *Brassica* species may be an important message for growers to better choose the *Brassica* vegetable species to grow. On the other hand, fundamental knowledge about the spreading of *Rhizoctonia* spp. among different plant species in agro-ecosystems may help to reduce the risks of fungal infection in plants and to manage existing outbreaks.

From a fundamental point of view, the introduction of two new AGs namely AG 1-IG and AG-Fc has a significant contribution to the classification of *Rhizoctonia*. Since these AGs have not been described before, the publication of their rDNA-ITS sequences and their deposition in databases facilitate the identification and confirmation of AG affinities of new or existing *Rhizoctonia* spp. isolates without the need to actually use the various AG- representative isolates or the hyphal fusion procedure.

When comparing the rDNA-ITS consensus sequences of our isolates to those of all known multinucleate, binucleate and uninucleate *Rhizoctonia* AGs, we noticed about the presence of wrong DNA sequence information in Genbank. This reinforce the concern of Sharon et al. (2006) about the existence of inaccurate data in the databases available in Genbank, such as mislabeled isolates and other errors introduced during sequence analysis. Therefore, by proving a list of AG-representative isolates, we wish to help reducing misidentification of *Rhizoctonia* AGs due to the use of inaccurate sequence data in Genbank.

Biological control of *Rhizoctonia* diseases by bacterial antagonist

Soil-borne pathogens are notoriously difficult to control because they are mostly associated with the roots of plants (Haas and Défago 2005). Since crop rotation, breeding for resistant plant varieties and the application of pesticides are insufficient to control root diseases (Onokpise et al. 1992; Haas and Défago 2005), the use of antagonistic microorganisms to suppress soil-borne diseases appears to be a promising approach. Therefore, along with investigating genetic diversity of *Rhizoctonia* spp. which infect *Brassica* crops in Vietnam, a large part of this thesis is dedicated to the effect of *Pseudomonas* sp. CMR12a and the putative role of phenazines and CLPs produced by this strain in biocontrol of soil-borne pathogenic fungus *R. solani*.

Soil-plant-microbe interactions involved in biological disease control of Pseudomonas sp. CMR12a

Since soil-borne diseases occur in a dynamic environment at the interface of root and soil, the control of their causal agents is particularly complex and the disease suppressive effect of biocontrol agents (BCAs) is influenced by different factors including the plant, the pathogen, the microbial community on and around the plant and the physical environment (Handelsman and Stabb 1996; Hoitink and Boehm 1999; Whipps 2001). For example, in Chapter 4 of this

thesis, we demonstrated that the biocontrol capacity of *Pseudomonas* sp. CMR12a towards Rhizoctonia root rot of bean is substrate-dependent. In substrates containing a high proportion of potting soil, either sessilin or phenazines are sufficient for biocontrol; however, in sandy substrate, disease development is favored and, therefore, the concurrent production of CLP sessilin and phenazines is required to suppress bean root rot. In Vietnam, bean and *Brassica* vegetables are mainly cultivated on Ferralsols and Fluvisols. The texture of Ferralsols varies from sandy loam to clay whereas the texture of Fluvisols ranges from coarse sand to heavy clay (Chesworth 2008). These suggest that vegetables grown in Vietnam has high risk of *Rhizoctonia* infection. **From a practical point of view**, our research highlights the need to have a good cultural practices (e.g. using transplants instead of direct-seeding, introducing organic materials to soil and preventing the use of *Rhizoctonia*-alternative hosts as cover crops) for better disease management.

The influence of plant-microbe interaction on disease suppressive effect of *Pseudomonas* sp. CMR12a was revealed when comparing biocontrol data on bean and cabbage. Whereas mutants deficient in the production of phenazines or sessilin partly lost their biocontrol activity towards Rhizoctonia root rot of bean (Chapter 3), these mutants could protect cabbage seedlings to a similar extent against Rhizoctonia damping-off in comparison to the wild type (Chapter 5). A plausible explanation for the divergent findings in the two chapters might be that *Pseudomonas* strains interacted differently with bean and cabbage. Through the production of root exudates, plants might favor growth or induce expression of genes in the microorganism involved in disease suppression, attract the BCA to the infection site, or respond to the BCA by inducing a resistance response (ISR). Therefore, different plant species can react differently to BCAs, presumably due to the variation in composition of root exudate among the species (Handelsman and Stabb 1996).

Resulting from the interaction between plant roots and BCA, ISR allows plants to withstand pathogen attack (Harman et al. 2004; Haas and Défago 2005). ISR-eliciting determinants in *Pseudomonas* spp. include siderophores, flagellin, lipopolysaccharides, N-alkylated benzylamine derivative, salicylic acid (SA), 2,3-butanediol, 2,4-diacetylphloroglucinol, pyocyanin and CLPs (Bakker et al. 2007; De Vleesschauwer and Höfte 2009; Jankiewicz and Kołtonowicz 2012). To explore the capacity of *Pseudomonas* sp. CMR12a as well as phenazines and CLPs produced by this bacterium to mediate ISR, an experiment using the bean-web blight as model was conducted. In this experiment, surface-sterilized bean seeds cultivar Prelude (Het Vlaams Zaadhuis, Waarschoot, Belgium) were soaked in suspensions (10^6 CFU ml^{-1}) of CMR12a, CMR12a-ΔPhz, CMR12a-Clp1, CMR12a-ΔClp2, CMR12a-ΔClp2-

Clp1, CMR12a-ΔPhz-Clp1, CMR12a-ΔPhz-ΔClp2 or CMR12a-ΔPhz-ΔClp2-Clp1 for 10 min. Then, seeds were quickly rinsed with sterile water and were incubated on sterile moistened filter paper for three days at 28°C to germinate. Growth substrate made of 50% potting soil (Structural; Snebbout, Kaprijke, Belgium) and 50% sand (Cobo garden; Belgium) (w/w) was sterilized twice on two different days before 50 ml of the bacterial inoculum was applied to obtain a final density of 10^6 CFU g^{-1}. Each set of four germinated seeds was sown in a perforated plastic tray (22 x 15 x 6 cm) filled with 700 g substrate. Ten days after sowing, bacterial application was repeated by introducing bacterial suspesions into the substrate around the root zones of bean seedlings. In negative control and pathogen control treatments, growth substrate was treated with equal volumes of sterile saline in a similar manner. All trays were placed in the growth chamber (28°C, RH = 60%, 16 h photoperiod) and seedlings were watered every two days to maintain moisture of growth substrate near field capacity. A detached leaf assay was performed following the method developed by Takegami et al. (2004), with modifications. In short, fully expanded trifoliate leaves detached from four-week-old bean seedlings were challenged by placing a mycelial plug (diameter = 5 mm), which was collected from a 3-day-old culture of *R. solani* AG 2-2 isolate 18, in the center of each leaflet. Plugs taken from fresh PDA plates were used to inoculate leaflets of the negative control treatment. The leaves were placed in trays containing moistened paper towels. All trays were covered to create a high humidity environment favorable for disease development and they were incubated at 28°C in the dark. Disease severity of each leaflet was evaluated six days after fungal inoculation using the 0-4 scale in which 0 = no visible symptoms of disease; 1 = <30% of the leaf area with symptoms; 2 = 30-60% of the leaf area with symptoms; 3 = 60-80% of the leaf area with symptoms; and 4 = >80% of the leaf area with symptoms. In this experiment, each treatment consisted of three trifoliate leaves (equivalent to 15 leaflets) and the experiment was done once. Bacterial colonization of roots, stems and primary leaves of bean seedlings were determined at the time trifoliolate leaves were detached by plating.

Results of this study (see Figures 7.1 and 7.2) indicates that *Pseudomonas* sp. CMR12a provided a significant induced systemic resistance (ISR)-mediated protective effect on bean plants against *R. solani*. Seedlings colonized with CMR12a-mutants deficient in the production of either sessilin or orfamide yielded similar results. However, CMR12a-mutants deficient in the production of both sessilin and orfamide were not able to trigger ISR and no significant differences in the severity of web blight disease were observed between treatments treated with these mutants and the control, suggesting that sessilin and orfamide are the determinants for induced resistance in wild type *Pseudomonas* sp. CMR12a.

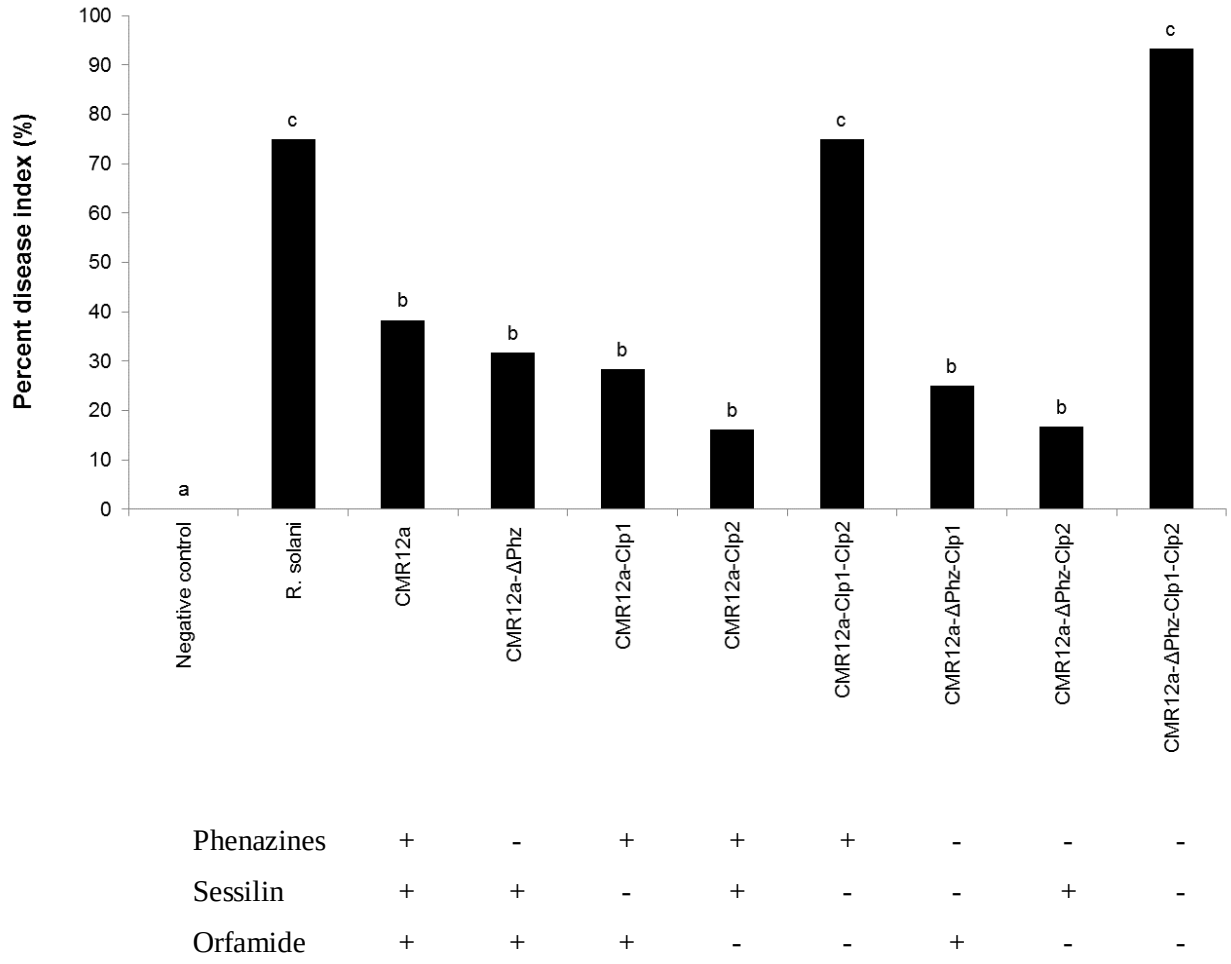

Figure 7.1. Effect of *Pseudomonas* sp. CMR12a and mutants on resistance to *Rhizoctonia solani* in bean. Data are expressed as Percent disease index and different letters indicate statistically significant differences between treatments (Kruskal-Wallis and Mann-Whitney tests, p = 0.05).

Bacterial enumeration (Table 7.2) shows that all strains could establish well on roots of bean seedlings and their population densities varied between 6.21 ± 0.23 and 6.66 ± 0.14 log CFU g^{-1} root. However, the bacteria were not isolated from stems or leaves (data not shown), suggesting that disease suppression effect observed above may result from an increased level of plant resistance to infection by the pathogen upon sessilin and orfamide production since inducing strains and the challenging pathogen were spatially separated.

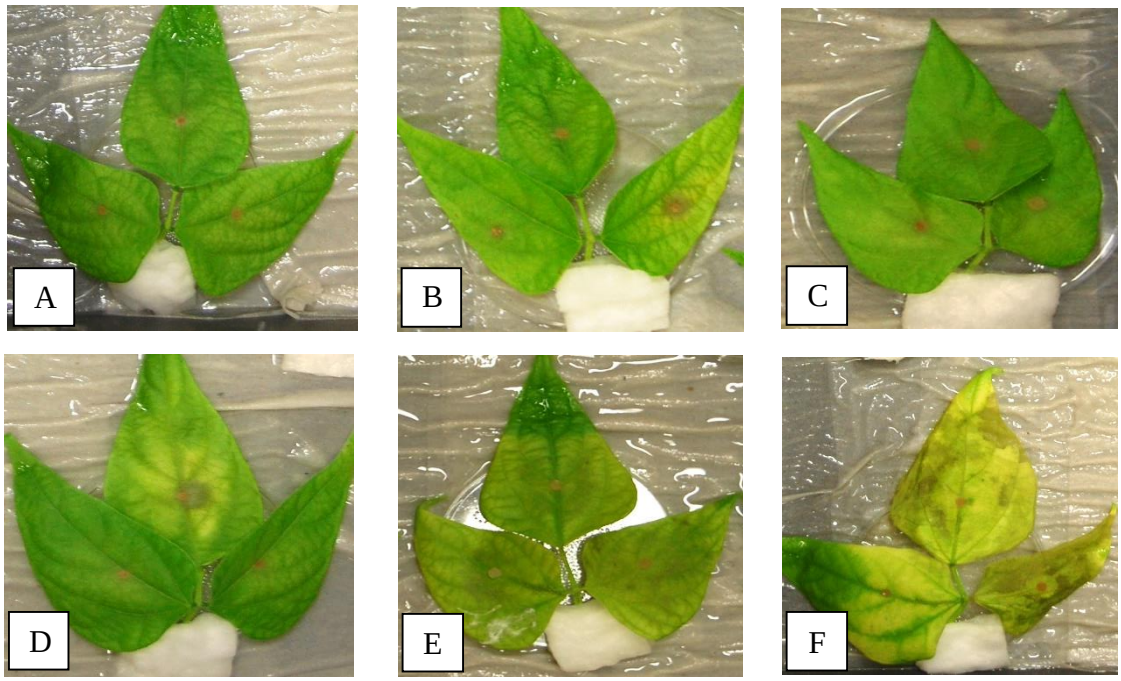

Figure 7.2. Symptoms of web blight caused by *Rhizoctonia solani* AG 2-2 on bean observed after different treatments including **A,** control, **B,** *Pseudomonas* sp. CMR12a, **C,** *Pseudomonas* CMR12a-ΔPhz-Clp1, **D,** *Pseudomonas* CMR12a-ΔPhz-ΔClp2, **E,** *Pseudomonas* CMR12a-ΔClp2-Clp1 and **F,** *Pseudomonas* CMR12a-ΔPhz-ΔClp2-Clp1.

Table 7.2. Densities of *Pseudomonas* sp. CMR12a and its mutants impaired in phenazine and/or CLP production on bean roots. Root colonization was determined for three seedlings per treatment when leaves were harvested for the induction assay. Data were log10 transformed prior to analysis. Values followed by the same letter are not significantly different according to the Kruskal-Wallis and Mann-Whitney nonparametric tests (p = 0.05)

Treatment	Phenazines and CLPs produced	Population density of CMR12a and mutants (in log CFU g^{-1} of fresh root)
CMR12a	Phenazines, sessilin and orfamide	6.30 ± 0.29 a
CMR12a-ΔPhz	Sessilin and orfamide	6.21 ± 0.23 a
CMR12a-Clp1	Phenazines and orfamide	6.39 ± 0.24 a
CMR12a-ΔClp2	Phenazines and sessilin	6.66 ± 0.14 c
CMR12a-ΔClp2-Clp1	Phenazines	6.62 ± 0.35 c
CMR12a-ΔPhz-Clp1	Orfamide	6.31 ± 0.22 a
CMR12a-ΔPhz-ΔClp2	Sessilin	6.58 ± 0.27 c
CMR12a-ΔPhz-ΔClp2-Clp1		6.44 ± 0.26 b

Our results extend the findings of Tran et al. (2007) who stated that the CLP massetolide A of *P. fluorescens* SS101 is efficient at triggering systemic resistance in tomato and the systemic resistance induced by this compound is independent of SA. Another line of evidence that CLPs are important determinants of biocontrol activity was provided by Ongena et al. (2007), who showed that the CLP surfactin from plant-beneficial bacilli has resistance-inducing activities in bean. According to Henry et al. (2011), surfactin may insert and disturb lipid compartmentalization or induce curvature constraints in host cell membranes, leading to the establishment of defensive responses. In our study, although the pathway as well as how sessilin and orfamide from *Pseudomonas* sp. CMR12a induce systemic resistance on bean is yet unknown, the preliminary results might propose an interesting topic for future research.

Phenazines, sessilin and their contribution to competitive root colonization of biocontrol strain Pseudomonas sp. CMR12a

As root colonization is the delivery system of beneficial microbes and their products, effective biocontrol microbes should be rhizosphere competent. It has been shown throughout this thesis that *Pseudomonas* sp. CMR12a is a good rhizosphere colonizer and its population on roots was higher than the population level needed for effective biocontrol. When comparing root colonization ability of wild type strain with that of the mutants, we found that one of the two important traits involved in root colonization capacity of CMR12a is CLP type sessilin (see Chapters 3, 4, 5 and 7). With the ability to form biofilm (D'aes et al. in press), sessilin-producing strains not only successfully colonize plant roots (D'aes et al. 2010) but they are also protected from adverse conditions such as dessication, UV, predation, and bactericides (Costerton et al. 1995; Davey and O'Toole 2000). Additionally, as indicated in Chapters 3 and 5 of this thesis, sessilin may function as an antimicrobial compound; therefore, the production of sessilin confers an advantage for the producers in competition for nutrients and niches. Finally, the abilility to increase nutrient availability of CLP may also contribute to competive colonizaition of the producing strains, especially under nutrient poor environments such as a sandy substrate (see Chapter 4).

Similar to sessilin, the importance of phenazines in root colonization is also demonstrated in Chapters 5 and 7. Based on what we have observed in our experiments, it is hypothesized that the high competitiveness of phenazine-producing strains derives from the contribution of phenazines to bacterial survival and ecological competiveness (Mazzola et al. 1992; Botelho and Mendonça-Hagler 2006; Mavrodi et al. 2006). Redox-active PCA and PCN secreted by

CMR12a are able to reduce ferric iron (De Maeyer 2012), providing an ecological advantage for the producing strains in an iron-limited environment (Hernandez et al. 2004). In addition, as electron acceptors, phenazines can decrease the $NADH/NAD^+$ ratio, leading to an increase in oxygen availability, which is important under oxygen-limited conditions (Price-Whelan et al. 2007; De Maeyer 2012).

Synergistic interaction between sessilin and orfamide

Sessilin and orfamide are two CLPs synthesized by *Pseudomonas* sp. CMR12a. Sessilin can inhibit growth of several pathogens including *Pythium myriotylum*, *P. splendens*, and *Fusarium oxysporum* f. sp. *lycopersici* (unpublished), whereas orfamide A has been shown to lyse zoospores produced by an oomycete plant pathogen (Gross et al. 2007). As indicated in Chapter 3, the presence of sessilin-producing strains at two sides of fungal plugs resulted in reduced fungal growth and increased hyphal branching. However, further *in vitro* assays with orfamide biosynthesis mutants and purified orfamide implicated that the two CLPs have a synergistic effect; mutants deficient in the production of either sessilin or orfamide completely lost their antimicrobial activity, although the use of purified orfamide at a concentration of 10 µM ml^{-1} was shown to suppress fungal growth at the same extent with the wild type (Chapter 5). From these results, it is also reasonable to speculate that the antagonistic activity of sessilin and orfamide is dose-dependent. When these compounds are produced in sufficient quantity, the presence of either sessilin or orfamide can inhibit the growth of *R. solani*; otherwise, sessilin and orfamide need to act synergistically in the control of hyphal growth. The synergistic interaction between sessilin and orfamide is confirmed when we observed that both CLPs are needed to kill sclerotia of *R. solani*, while the individual CLPs are not effective (Chapter 5). Synergisms between antifungal metabolites in biocontrol have been reported before (Dunne et al. 1998; Woo et al. 2002; Fogliano et al. 2007; Perneel et al. 2008); however, this thesis provided for the first time evidence that sessilin acts synergistically with orfamide.

Sessilin is responsible for biofilm formation. In contrast, orfamides appears indispensable for swarming (D'aes et al. in press). With the ability to produce both CLPs, bacteria can attach or detach from surfaces according to need (Verstraeten et al. 2008), leading to better competition in the rhizosphere (Verstraeten et al. 2008; Dutta and Podile 2010). Therefore, we speculate that biocontrol effect of CMR12a is partly attributed to the biological role of sessilin and orfamide.

Role of phenazines in the control of R. solani-mediated diseases

The importance of phenzines in biocontrol activity of *Pseudomonas* sp. CMR12a was demonstrated in different chapters of this thesis. In Chapters 3 and 4, it was shown that phenazine production is essential for the control of root rot of bean, especially under high disease pressure. In Chapter 5, we found that phenazines also play a role in suppression of damping-off disease of cabbage. Although phenazine-producing mutant failed to inhibit the mycelium of *R. solani* (Chapter 3), phenazines appeared to be toxic towards the sclerotia of this fungus (Chapter 5). Phenazine biosynthesis in CMR12a is regulated by PhzI/PhR quorum sensing system (De Maeyer et al. 2011), which is dependent on bacterial population density (Chin-A-Woeng et al. 2001). Additionally, antagonistic activity of phenazines is influenced by a number of physical factors (Georgakopoulos et al. 1994). Hence, it is possible that the conditions employed in the microscopic experiment were not suitable for the multiplication of bacteria and, therefore, bacterial population is lower than the threshold required for phenazine production. As a result, the amount of phenazines present on the slides, presumably produced when bacteria was cultured overnight in KB, is not sufficient to inhibit the growth of *R. solani*. Another explanation could be that direct contact between phenazines and target organisms is needed for this compound to be effective.

Taken together, *Pseudomonas* sp. CMR12a could suppress the growth of *R. solani* and reduce the intensity of several *Rhizoctonia*-caused diseases including root rot (Chapters 3 and 4) and web blight (this chapter) on bean and damping-off on Chinese cabbage (Chapters 5 and 6). **From a biocontrol perspective**, these findings make the first step towards developing an environmentally sound approach to manage diseases induced by *R. solani* on vegetables. Presently, products based on *P. fluorescens* (tradenames: Blight-End, Flick, Fluroissal, Biomonas and Jay-Pseudo) (Exotic Naturals 2014; Khan 2013) and *P. chlororaphis* (tradename: BioAgri) (Khan 2013) are commercially available, presenting an alternative or a supplemental way to chemical pesticides in agriculture. Biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and the ecosystem; it neither eliminates the pathogen nor the disease, but brings them into natural balance (Ramanathan et al. 2002). Together with the application of living microorganisms, the use of purified phenazines as active ingredients of fungicides to suppress the viability of *Rhizoctonia* sclerotia is also an interesting option. Phenazines, which are often synthesized at very high levels (milligrams to grams per liter) when bacteria are grown under *in vitro* conditions (Mavrodi et al. 2006), can be easily extracted from spent growth supernatant of cultures (Perneel et al. 2007). The effectiveness of purified metabolic compounds in biocontrol of several pathogens has been already described by

De Jonghe et al. (2005) and De Vleesschauwer et al. (2006). Using purified compounds also helps to shorten an registration process, which is extremely laborious and complicated (Walsh et al. 2001), applied for antibiotic producing microorganisms.

Coir pith and *Pseudomonas* sp. CMR12a: a promising combination to sustainably control *R. solani*

In Chapter 6, we showed that the incorporation of coir pith to soil reduced the viability of *Rhizoctonia* sclerotia, increased the number of sclerotia infected by parasitic fungi and decreased the severity of damping-off. The results of this chapter also demonstrate the combined effect of coir pith amendment and *Pseudomonas* sp. CMR12a in the control of damping-off disease caused by *R. solani* on cabbage. **From a fundamental point of view**, these findings support the lignin-melanin hypothesis proposed before (Butler and Day 1998; Shetty et al. 2000; Debode et al. 2005). According to this hypothesis, the addition of lignin stimulates the growth of lignin degraders. Ligninolytic enzymes produced by these microorganisms might attack sclerotial melanin, thereby making the sclerotia more susceptible to antagonists. Since the number of viable sclerotia determine the incidence of *Rhizoctonia* diseases (Belmar et al. 1987), the reduction in viability of sclerotia might contribute significantly to disease suppression.

From a practical perspective, our results suggest a possibility to use coir pith as a peat substitute in the nursery industry. Coir pith can function as well as peat in soilless container media for plant growth (Meerow 1997); therefore, there has been an increase in the use of this material in production media (Abad et al. 2005). On one hand, coir pith-based substrates can favor plant growth and protect seedlings from disease, especially when *Pseudomonas* sp. CMR12a was introduced. On the other hand, the use of coir pith to produce planting mix might help to reduce pollution created by the improper management and due to polyphenol leaching (Kumar and Ganesh 2012), especially in leading coconut producer nations such as Vietnam.

Limitations and Future prospects

The contribution of phenazines and CLPs to biocontrol activity of *Pseudomonas* sp. CMR12a towards diseases caused by *R. solani* on bean and cabbage and the reduction of *Rhizoctonia* sclerotial viability were clearly demonstrated in this thesis. However, the mechanism of action

of these metabolites remained unclear. Therefore, it is necessary to purify sessilin from the bacterial cultures and perform more in-depth studies with purified compounds of phenazines, sessilin and orfamide. Presumably, these studies will provide better understanding about the mode of action of phenazines and CLPs in disease suppression as well as the interaction between the two CLPs.

It has been shown that the outcome of biocontrol can be affected by various biotic and abiotic factors such as pathosystem (Åström and Gerhardson 1988; Termorshuizen et al. 2006), soil type (Van Beneden et al. 2010) and soil characteristics (Chapter 4). This points out the need to check the biocontrol effect of *Pseudomonas* sp. CMR12a in *in planta* experiments in Vietnamese soils and on other pathosystems.

The microorganisms introduced as BCAs should be able to adapt and establish in soil before it can play its desirable role (Dutta and Podile 2010). Therefore, it might be necessary to screen for Vietnamese *Pseudomonas* strains that produce both phenazines and CLPs.

In addition to the use of antagonistic *Pseudomonas*, the persistence of *Rhizoctonia* sclerotia as well as the severity of diseases caused by this fungus on cabbage can be suppressed by coir pith incorporation (Chapter 6). To our best knowledge, this is the first report about the use of this side product as soil amendment to control plant diseases. Although similar findings about the disease suppressive ability of lignin-rich substrates have also been described, the mechanism underlying the effect of lignin incorporation in biocontrol of soil-borne pathogens is still poorly understood. Hence, a lot more research is needed to observe the response of soil microbial community structure to lignin-rich amendments and to investigate the involvement of lignin degraders in the degradation of sclerotial melanin. From a practical point of view, our results suggest the use of coir pith and other lignin residues which are available in Vietnam (e.g. straw, sugarcane bagasse, sawdust, paper sludge and compost) as amendments to control *R. solani*. Although some of these materials have shown promise in biocontrol of this fungus (Tuitert et al. 1998; Emran and Md Shamim 2012), it is necessary to check whether they are also effective in Vietnamese soils because the biocontrol ability of lignin-rich organic materials is soil dependent (Van Beneden et al. 2010). Additionally, during the survey, we found that some *Brassica* fields in Lam Dong were heavily infected with *Sclerotinia sclerotiorum*. Similar to *R. solani, S. sclerotiorum* is a sclerotia-forming fungus which can attack hundred species of plants (Saharan and Mehta 2007). It has been documented that *S. sclerotiorum* can be controlled by *Pseudomonas* spp. (Fernando et al. 2007; Li et al. 2011) and the survival of its sclerotia can be reduced by organic amendments (Huang et al. 2002). Hence, it could be fascinating to test the ability to control this pathogen by *Pseudomonas* sp. CMR12a and coir pith amendment.

When performing pathogenicity tests with the Vietnamese isolates of *Rhizoctonia* spp. we found that although three isolates belonging to binucleate *Rhizoctonia* (BNR) AG-A grew very well in 50% potting soil/50% sand mixture and on growing media, they were non-pathogenic to all crops tested (Chapter 2). It has been shown that several BNR isolates can be used as biocontrol agents to suppress *R. solani* diseases on a range of hosts (Cardoso and Echandi 1987; Escande and Echandi 1991; Harris et al. 1994; Villajuan-Abgona et al. 1996; Ross et al. 1998). Disease suppressive effect of BNR isolates may be attributed to one of the following mechanisms: (i) competition for nutrients (Cardoso and Echandi 1987) or infection sites (Sneh et al. 1989) and (ii) induction of systemic resistance in plants (Cardoso and Echandi 1987; Poromarto et al. 1998; Jabaji-Hare et al. 1999). Therefore, it would be interesting to investigate the virulence of these BNR isolates towards a wider range of crops and to explore their potential as antagonistic agent against *R. solani* and other plant pathogens.

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Summary

Vietnam is the third world's largest vegetable producing country in the world. Due to the intensification of cropping systems, vegetable producers confront with the challenges of managing fungal pathogens, such as *Rhizoctonia solani*. This fungus is distributed both in virgin and cultivated soils as mycelium and sclerotia. *R. solani* mainly attacks its hosts when they are in their juvenile stages, causing various diseases on a wide range of economically important crops. Because resistant varieties are not available and cultural control is not sufficient, existing strategies to control *Rhizoctonia* diseases in Vietnam rely heavily on chemical pesticides. However, repeated pesticide applications may result in resistance development in the pathogen population, environmental pollution and negative health impacts. Hence, there is great interest in developing an integrated, sustainable strategy to control *R. solani* on vegetables cultivated in Vietnam.

Before developing a control strategy it is necessary to know about the occurrence and distribution of *Rhizoctonia* spp. in Vietnam. Isolations from infested plants collected from *Brassica* fields showed that *Brassica* vegetables can be attacked by different anastomosis groups (AGs) of *Rhizoctonia* spp. Ninety three out of 97 *Rhizoctonia* isolates collected had multinucleate hyphal cells and were assigned to AG 1-IA, AG 1-ID, AG 1-IB, AG 1-IG, AG 4-HGI, AG 2-2 and AG 7. The other four isolates in our collection belonged to AG-A and AG-Fc of binucleate *Rhizoctonia*. To our best knowledge, this is the first time AG 1-IA and AG 1-ID are isolated from *Brassica* vegetables. Bioassays demonstrated significant divergence in pathogenicity among AGs, in which only isolates of AG 4-HGI, AG 2-2 and AG-Fc could induce severe disease symptoms under all conditions tested. Interestingly, we found that the occurrence of the different AGs are related to the cropping systems and cultural practices, implying that agricultural practices might determine the AGs associated with *Brassica* crops in Vietnam.

Pseudomonas sp. CMR12a is an antagonistic bacterium isolated from the roots of healthy cocoyam in diseased cocoyam fields in Cameroon. This strain excretes phenazines and two types of cyclic lipopeptides (CLPs), designated sessilin and orfamide. In this thesis, we showed that *Pseudomonas* sp. CMR12a successfully protected bean seedlings from disease and the biocontrol activity of this strain is attributed to an additive effect of phenazines and sessilin. Subsequent *in vivo* experiments conducted with the same bacterial strain and *R. solani* AG 2-2 in three different potting soil/sand mixtures revealed that biocontrol activity of phenazines and

CLP sessilin was substrate-dependent. The proportion of sand present in substrates positively correlated with the spreading rate of *Rhizoctonia* hyphae and the disease severity. In substrates containing 50% or 75% of potting soil, the production of phenazines or sessilin was sufficient to suppress bean root rot. However, in sand-dominant substrate, the presence of both phenazines and sessilin was needed to be effective, possibly due to the high disease pressure.

In a further stage of the research, we were interested in exploring the ability to induce systemic resistance against web blight on bean by CMR12a. The application of CMR12a to growth substrate significantly reduced disease caused by *R. solani* AG 2-2 on leaves of seedlings. In order to test whether phenazines, sessilin and orfamide are involved in CMR12a-mediated ISR, mutants deficient in the production of phenazines and/or CLPs production were included in the experiments. Mutants disrupted in sessilin or orfamide biosynthesis were as effective as the parental strain. However, two mutants deficient in the production of both sessilin and orfamide completely lost their disease suppressive effect. These results imply that sessilin and orfamide are the determinants for induced resistance in wild type *Pseudomonas* sp. CMR12a.

In addition to controlling *Rhizoctonia* diseases on bean, *Pseudomonas* sp. CMR12a also showed its biocontrol capacity towards the germination of *Rhizoctonia* sclerotia and the severity of damping-off disease on cabbage. The results of plant experiments and microplate assays indicated that the presence of phenazines or the coexistence of sessilin and orfamide reduced sclerotia viability *in vitro* and suppressed disease development *in vivo*. These observations suggest that there is a synergistic effect of sessilin and orfamide in the control of *R. solani*. The synergistic interaction between these CLPs was confimed by microscopic analyses showing that the combination of a double phenazine- and orfamide-negative mutant and a double phenazine- and sessilin-negative mutant or purified orfamide significantly reduced mycelial growth of *Rhizoctonia* and increased branching of hyphal tips. Data obtained also implicate the dose-dependent direct antagonistic effect of orfamide *in vitro* and the essential role of sessilin in root colonization *in planta*.

In the next part of this work, we focused on the potential of coir pith, a side product abundantly available in Vietnam, to reduce the persistence of *Rhizoctonia* sclerotia and to suppress the severity of damping-off disease. Pot trials showed that sclerotial mortality was 78% when coir pith was added at a rate of 5% (w/w). A reduction in sclerotia viability was observed together with an increase in the number of sclerotia infected with parasitic fungi and the raise in the population density of *Trichoderma* spp. and fluorescent pseudomonads in soil. Based on these results it was hypothesized in accordance to previous studies that the incorporation of lignin favored the growth of lignin-degrading fungi. Some extracellular enzymes such as lignin

peroxidase, manganese peroxidase and laccase produced by lignin degraders can affect melanin. Sclerotia with degraded melanin are more susceptible to antagonists such as *Trichoderma* spp., Actinomycetes and Gram negative bacteria. The decrease in sclerotia viability due to coir pith incorporation was related with a significant decrease in the severity of damping-off disease. Moreover, the disease suppressive ability could be enhanced when coir pith was combined with *Pseudomonas* sp. CMR12a, possibly due to the biocontrol effect of CMR12a.

Samenvatting

Vietnam is de grootste tuinbouwproducent van de derde wereld. De gebruikte teeltsystemen worden steeds intensiever, waardoor producenten van tuinbouwgewassen geconfronteerd worden met de nieuwe uitdaging om schimmelpathogenen zoals *Rhizoctonia solani* onder controle te houden. Deze schimmel komt zowel voor in niet-gecultiveerde als gecultiveerde grond onder de vorm van mycelium en sclerotiën. *R. solani* valt zijn waardplanten voornamelijk aan tijdens de juveniele fase en veroorzaakt zo verschillende ziekten op een waaier van economisch belangrijke gewassen. Doordat resistente rassen niet beschikbaar zijn en cultuurmaatregelen niet volstaan, zijn de huidige strategiën om *Rhizoctonia* ziekten onder controle te houden zeer sterk afhankelijk van chemische pesticiden. Herhaaldelijke pesticide behandelingen kunnen echter resulteren in de ontwikkeling van resistentie binnen de pathogeenpopulatie, milieuverontreiniging en hebben een negatieve impact op de gezondheid. Daarom bestaat er een sterke interesse in de ontwikkeling van een geintegreerde, duurzame strategie om de geteelde gewassen in Vietnam te beschermen tegen *R. solani*.

Vooraleer een gewasbeschermingsstrategie kan ontwikkeld worden, is een beter zicht op het voorkomen en de verspreiding van *Rhizoctonia* spp. in Vietnam noodzakelijk. Isolaten van geinfecteerde planten uit *Brassica* velden toonden dat *Brassica* gewassen aangetast kunnen worden door verschillende anastomosis groepen (AGs) van *Rhizoctonia* spp. Drieënnegentig van de 97 verzamelde *Rhizoctonia* isolaten hadden multinucleate hyfale cellen en werden toegewezen aan AG 1-IA, AG 1-ID, AG 1-IB, AG 1-IG, AG 4-HGI, AG 2-2 en AG 7. De andere 4 isolaten in onze collectie behoren to AG-A en AG-Fc van binucleate *Rhizoctonia*. Voor zover ons bekend is dit de eerste keer dat AG 1-IA en AG 1-ID geisoleerd werden van *Brassica* gewassen. Bioassays toonden een significant verschil in pathogeniteit tussen de AGs, enkel isolaten van AG 4-HGI, AG 2-2 en AG-Fc konden ernstige ziektesymptomen induceren onder alle geteste omstandigheden. Ook noemenswaardig is het feit dat het voorkomen van verschillende AGs gerelateerd was aan de gebruikte teeltsystemen en landbouwpraktijken, wat er op wijst dat landbouwpraktijken kunnen bepalen welke AGs geassocieerd zijn met *Brassica* gewassen in Vietnam.

Pseudomonas sp. CMR12a is een antagonistische bacterie die geïsoleerd werd uit de wortels van gezonde cocoyam planten in zieke cocoyam velden in Kameroen. Deze stam produceert phenazines en 2 types van cyclische lipopetiden (CLPs), genaamd sessilin en orfamide. In dit werk tonen we dat *Pseudomonas* sp. CMR12a in staat is boonzaailingen succesvol te

beschermen tegen ziekte en dat de biocontrole activiteit te wijten is aan een additief effect van fenazines en sessilin. Vervolgens konden we aantonen met *in vivo* experimenten, uitgevoerd met dezelfde bacteriele stam en *R. solani* AG 2-2 in drie verschillende potgrond/zand mengsels, dat de biocontrole activiteit van phenazines en CLP sessilin substraatafhankelijk is. De hoeveelheid zand in het substraat is positief gecorreleerd met de spreidingsgraad van *Rhizoctonia* hyfenn en de ziekte-intensiteit. In substraat dat 50% of 75% potgrond bevatte, was de productie van enkel phenazines of sessilin voldoende om boonwortelrot te onderdrukken, maar in substraat met voornamelijk zand was de aanwezigheid van zowel phenazines als sessilin noodzakelijk voor een effectieve bescherming.

In een volgend stadium van het onderzoek, wilden we onderzoeken of CMR12a systemische resistentie (ISR) tegen boon web blight kan induceren. Toediening van CMR12a aan de grond zorgde voor significant minder ziekte veroorzaakt door *R. solani* AG 2-2 op bladeren van zaailingen. Om na te gaan of phenazines, sessilin en orfamide betrokken zijn in CMR12a-gestuurde ISR, werden mutanten met een deficientie in phenazine en/of CLP productie opgenomen in de experimenten. De mutanten met een uitgeschakelde sessilin of orfamide biosynthese waren net zo effectief als de ouderstam. Twee mutanten die deficient waren in de productie van zowel sessilin als orfamide verloren daarentegen hun ziekte-onderdrukkend effect. Deze resultaten impliceren dat sessilin en orfamide bepalend zijn voor de geinduceerde resistentie door de wild type-stam *Pseudomonas* sp. CMR12a.

Pseusomonas sp. CMR12a bleek niet allen *Rhizoctonia* ziekten op boon te kunnen onderdrukken, maar vertoonde ook biocontrole potentieel op vlak van kieming van sclerotiën en de ernst van de damping-off ziekte op kool. De resultaten van plantenproeven en microplate assays toonde aan dat de aanwezigheid van phenazines en het samen voorkomen van sessilin en orfamide, de levensvatbaarheid van sclerotia *in vitro* verminderde, en de ziekteontwikkeling *in vivo* onderdrukte. Deze observaties suggereren dat er een synergistisch effect is van sessilin en orfamide in de controle tegen *R. solani*. De synergistische interactie tussen deze CLPs kon bevestigd worden via microscopische analyses. Deze toonden dat de combinatie van een dubbele phenazine/orfamide negatieve mutant met een dubbele phenazine/sessilin negatieve mutant of met opgezuiverd orfamide, de myceliumgroei van *Rhizoctonia* onderdrukte en de vertakking van de hyfen versterkte. Onze data wijzen ook op een dosis-afhankelijk direct antagonistisch effect van orfamide *in vitro* en een essentiele rol voor sessilin tijdens wortelkolonizatie *in planta*.

In het volgende deel van het werk, hebben we ons toegespitst of het potentieel van kokosvezels, een bijproduct dat overvloedig beschikbaar is in Vietnam, om de aanwezigheid van *Rhizoctonia*

sclerotiën en de ernst van de damping-off ziekte te verminderen. Experimenten in potten toonden dat sclerotiesterfte 78% bedroeg wanneer 5% (w/w) kokosvezel toegevoegd werd. Samen met een vermindere levensvatbaarheid van de sclerotiën, werd ook vastgesteld dat een stijgend aantal sclerotiën geinfecteerd was met parasitaire schimmels en dat de populatiedensiteit van *Trichoderma* spp. en fluorescente pseudomonaden in de grond tevens toenam. Op basis van deze resultaten en in overeenstemming met eerdere studies, werd de hypothese geopperd dat inmengen van lignine in de bodem de groei van lignine-afbrekende schimmels begunstigt. Sommige extracellulaire enzymes, zoals lignine peroxidase, mangaan peroxidase en laccase, die geproduceerd worden door lignine-afbrekers kunnen melanine aantasten. Sclerotiën met afgebroken melanine zijn gevoeliger voor antagonisten zoals *Trichoderma* spp., actinomyceten en gram negatieve bacteriën. De verminderde levensvatbaarheid van sclerotiën door de toevoeging van kokosvezels was gelinkt aan een significante vermindering van de ernst van de damping-off ziekte. Bovendien kon de ziekte-onderdrukkende werking nog versterkt worden door kokosvezel te combineren met *Pseudomonas* sp. CMR12a, wat te wijten zou kunnen zijn aan het biocontrole effect van CMR12a.

Curriculum vitae

I. Personal information

First name:	Hoang Gia Khuong
Last name:	Hua
Address:	Jubileumlaan 139
	B-9000 Gent, Belgium
Date of birth:	October 23, 1981
Place of birth:	Can Tho
Nationality:	Vietnam
Sex:	Female
Marital status:	Married to Duy Tan Pham
Children:	Hoang Khoi Nguyen Pham; born April 1, 2013
Telephone:	0032486431029
Email:	hoanggiakhuong.hua@ugent.be

II. Education

Main diplomas

2005: Bachelor degree in Agronomy, Can Tho University

2009: Master degree in Bioscience engineering, Ghent University

Dissertation: Biological control of *Rhizoctonia solani* on bean by using coconut fiber and antagonistic *Pseudomonas* bacteria

Additional diplomas

Specialist courses: Plant Breeding and Plant Sexual Reproduction, Ghent University

Bio-Imaging, Ghent University

III. Professional career

November 2005 - May 2007

Research assistant
Department of Plant Protection
College of Agriculture & Applied Biology
Can Tho University, Vietnam.

December 2009 - April 2014

BOF fellowship entitled: ¨Integrated control of soil-borne fungal pathogens in vegetable production in Vietnam¨

IV. Publications

Peer reviewed

D'aes J, Hua GKH, De Maeyer K, Pannecoucque J, Forrez I, Ongena M, Dietrich L, Mavrod DV, Höfte M (2011) Biological control of Rhizoctonia root rot on bean by phenazine and cyclic lipopeptide producing *Pseudomonas* CMR12a. Phytopathology 101(8): 996–1004.

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V. Contribution to Conferences and Symposia

Oral presentation

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pathogens. 14th International Congress on Molecular Plant-Microbe Interactions, July 19-23, 2009, Quebec, Canada.

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