Diversity and biological control of *Sclerotium rolfsii*, causal agent of stem rot of groundnut

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Chapter 1

Introduction and outline of the thesis

Parts of this chapter are integrated in the review: *"Stem rot of groundnut caused by the soil-borne pathogen Sclerotium rolfsii"* (to be submitted)

Introduction and outline of the thesis

Introduction

The central theme in this thesis is biological control of stem rot disease of groundnut in Vietnam. Groundnut (*Arachis hypogaea* L.) is an annual leguminous plant that is cultivated in many countries around the world. In Vietnam, it is the most important oil seed crop with a total area of 249,200 ha and a production of 0.53 million ton in 2009 (FAO 2011). Groundnut cultivation is hampered by a wide range of pests and diseases. One of the most important soil-borne fungal diseases of groundnut is stem rot caused by *Sclerotium rolfsii*. Control of stem rot disease mostly relies on cultural practices and fungicide treatment. However, cultural practices are not always effective due to the wide host range of the pathogen, and fungicides are often too expensive for local groundnut farmers in Vietnam. Biological control has been proposed as a sustainable, affordable and supplementary measure to control *S. rolfsii,* but has not been explored and exploited in detail.

The **overall aim** of the research described in this thesis was to study the feasibility of biological control of stem rot disease on groundnut. The first part of this introduction describes several features of groundnut, in particular its biology, distribution, its symbiosis with nitrogen-fixing bacteria, and the agronomic and economic importance of this crop. Subsequently, the major yield-limiting factors in groundnut cultivation will be presented, followed by a detailed description of stem rot disease on groundnut, the life cycle and characteristics of the pathogen *S. rolfsii*. Then the current state-of-the-art of biological control of stem rot is summarized. The last section of this chapter describes the outline of this thesis.

Groundnut

Groundnut (*Arachis hypogaea* L.), also known as peanut, earthnut, monkey nut, or goobers, is an annual leguminous plant believed to originate in South America in a region encompassing Bolivia, Paraguay, Peru and parts of Western Brazil and Northern Argentina (Hoammons 1994). Groundnut production is important in several countries which are highly populated and where groundnut plays an important role as a food crop (Florkowski 1994). Today, groundnut is widely distributed and is cultivated in more than 80 countries in tropical and sub-tropical regions of the world (Fig. 1). Groundnut requires a

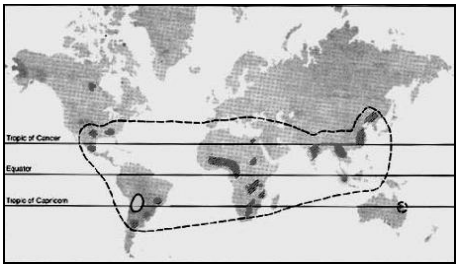

Figure 1. Groundnut growing regions in the world. Solid line: center of origin; dotted line: area of cultivation; black: areas of intensive cultivation. This figure is reproduced from Weiss (2000).

warm, sunny climate with a well-distributed rainfall of at least 500 mm and temperatures ranging from 25 to 30ºC. It thrives best in well-drained sandy loam soils with a pH ranging from 5.5 to 7.0 (Weiss 2000).

Groundnut belongs to the family of Leguminoceae and the genus *Arachis*. *Arachis* is derived from the Greek 'a-rachis', meaning without spine and refers to the absence of erect branches. The species name *hypogaea* is derived from 'hupo-ge' which in Greek means below earth. Cultivated groundnut is an annual, herbaceous plant growing to a height of 30-60 cm with an angular hairy stem with spreading branches (Fig. 2). Leaves occur alternately, one at each node; they are pinnate with two pairs of ovate leaflets (Fig. 2). Flowers are self-pollinating. After pollination, the perianth withers and at the base of the ovary, a meristematic region grows into a stalk-like peg which pushes the ovary into the soil (Fig. 2). Groundnut has a taproot system that is often covered with root nodules resulting from a symbiosis with nitrogen-fixing bacteria (collectively called rhizobia). On groundnut, root nodules develop at sites where lateral roots emerge (Uheda *et al.* 2001). The rhizobia penetrate the root tissue, induce cell division and settle inside root cells where they convert atmospheric nitrogen (N_2) into ammonia, which in turn is used by the plant (Broughton *et al.* 2000). Among the rhizobia identified on groundnut, *Bradyrhizobium* species are the most prominent ones (van Rossum *et al.* 1995; Urtz and Elkan 1996; Zhang *et al.* 1999; Saleena *et al.* 2001). Other bacterial genera associated with groundnut nodules include *Rhizobium*, *Pseudomonas, Enterobacter, Klebsiella*, *Sphingomonas, Bacillus,* and *Paenibacillus* (Zakhia *et al.* 2006). Most of these bacteria were unable to induce nodule formation on groundnut plants, but some did promote plant growth (Ibanez *et al.* 2009).

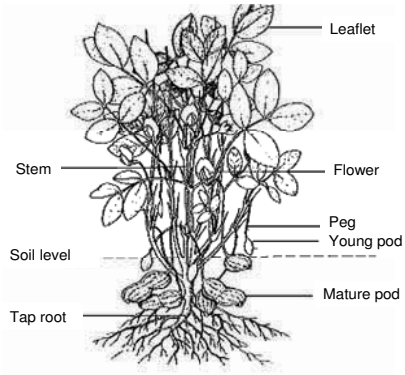

Figure 2. Schematic drawing of the groundnut plant. This figure was reproduced from Rao and Murty (1994).

All parts of the groundnut plant can be used for a variety of purposes. The seeds are rich in protein and vitamins (A, B and some B_2 types) and can be eaten raw, roasted or sweetened. They contain 44-52% edible oil which is also used in making soap, cosmetics and lubricants. The residual oil cake contains considerable amounts of nitrogen, phosphorous and potassium and is used as a fertilizer. The cake is also an important protein source in cattle and poultry feeds and can also be used for manufacturing artificial fiber. The haulms of groundnut can be fed to livestock and the peanut shell is used for manufacturing coarse boards and cork substitutes. In terms of economic importance, groundnut ranks thirteenth on the list of world food crops and tops the list of oil seed crops both in terms of acreage and production. In 2009, it was grown on 23.5 million hectares with an estimated total production of 35.5 million tonnes (FAO 2011). Currently, China is the leading groundnut producer with a share of about 37.5% of

the overall world production, followed by India, the United States of America (USA), Argentina and Vietnam (Fabra *et al.* 2010). India has the largest groundnut cultivation area, but has a low average yield of approximately 1.0 ton ha^{-1}. The USA have the highest average yield of 3.8 ton ha^{-1} (FAO 2011).

Yield limiting factors in groundnut cultivation

Several abiotic and biotic factors affect the growth and development of groundnut leading to qualitative and quantitative yield losses. Temperature, relative humidity, and soil characteristics are the major abiotic factors that can directly or indirectly influence productivity. In addition to the direct impact on growth, these abiotic factors can also have an impact on the proliferation and damage caused by pests and diseases. The major insect pests that attack groundnut are termites, white grubs, thrips, aphids, leaf miners, and caterpillars. The pathogens parasitizing on groundnut include viruses, mycoplasmas, bacteria, oomycetes, fungi, nematodes, and parasitic plants (Middleton *et al.* 1994; Wightman and Ranga Rao 1994). The diseases that are most damaging and cause the largest economic losses are stem rot, damping-off, black collar rot, early and late leaf spot, rust and bacterial wilt (Middleton *et al.* 1994; Podile and Kishore 2002).

Stem rot caused by the fungus *S. rolfsii* will be discussed in detail in the next section. The fungus *Rhizoctonia solani* causes seed decay, pre- and post-emergence damping-off of seedlings, hypocotyl and root necrosis, root rot of young seedlings, peg and pod rot, and foliar blight of mature plants (Porter *et al*. 1984). The fungus *Aspergillus niger* causes black collar rot, an important disease found in all major groundnut-growing areas of the world. Seedlings and young plants are particularly susceptible and infection of juvenile tissue usually results in high mortality rates. Seed rot and pre-emergence damping-off are common symptoms of this disease, but the most obvious symptom is sudden wilting of young plants. Early and late leaf spots caused by the fungi *Cercospora arachidicola* and *Cercosporidium personatum,* respectively, damage the plant by reducing the available photosynthetic area via lesion formation and leaflet abscission. Yield losses can range from 10% to over 50% (McDonald *et al.* 1985). The fungus *Puccinia arachidis* is the causal agent of groundnut rust which*,* in conjunction with leaf spots, is devastating in many South and Central American countries (Porter *et al.* 1984). *Ralstonia solanacearum,* the causal agent of bacterial wilt, is distributed worldwide and is an important yield-limiting factor in groundnut cultivation in Africa and Asia (Porter *et al.* 1984). In Vietnam, stem rot, damping-off, black collar rot, bacterial wilt and leaf spots are the most devastating diseases in many regions (Le 1977; Mehan and Hong 1994; Nguyen *et al.* 1998; Do 2001; Le 2004; Nguyen *et al.* 2004).

Stem rot disease of groundnut caused by *Sclerotium rolfsii*

The first report of stem rot dates back to 1892 with Peter Henry Rolfs' discovery of this fungus in association with tomato blight in Florida (Aycock 1966). The wide host range of *S.*

rolfsii, its prolific growth and ability to produce persistent sclerotia all contribute to the large economic losses associated with this pathogen (Chima Wokocha 1990; Cilliers *et al.* 2003; Singh *et al.* 2003). On groundnut, the disease caused by *S. rolfsii* is reported as stem rot, white mould, or southern blight, but stem rot is most commonly used. The disease occurs in most groundnut production areas in the world and appears to be more serious when the plants also suffer from tomato spotted wilt virus (Branch and Brenneman 2009). Pod yield losses range from 10-25% and sometimes up to 80% (Mehan *et al.* 1994). In North Carolina, groundnut crops sustained higher losses than any other agricultural crop (Aycock 1966). In 1959, the United States Department of Agriculture estimated losses of $10-20 million associated with *S. rolfsii* in the southern groundnut-growing regions, with yield depletions up to 60% in the coastal plains of North Carolina (Garren 1959). In Georgia (USA), economic losses due to stem rot disease and disease management costs were estimated to be approximately $38 million from 2004 to 2007 (Kemerait 2005; 2006; 2007; 2008).

Infection is usually restricted to plant parts that are in direct contact with the soil. On groundnut, *S. rolfsii* attacks stems, roots, leaves, pegs and pods. Initial disease symptoms comprise small, water-soaked lesions on the lower stem or near the soil surface, followed by yellowing and wilting of the lateral branches, main stem, and eventually the entire plant (Fig. 3). Diagnostic signs of the fungus include characteristic white mycelial fans and brown sclerotia extending from infected tissues (Fig. 3). The fungus infects pegs and pods and causes rot (Fig. 3).

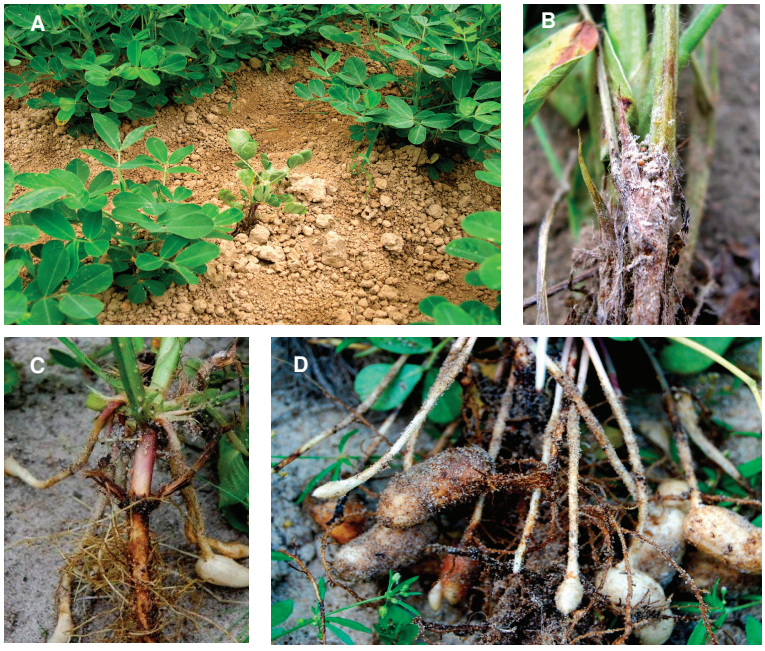

Figure 3. Disease **s**ymptoms on groundnut caused by *Sclerotium rolfsii* on groundnut **A**- yellow leaves and wilting; **B**- mycelium and sclerotia on infected tissue; **C**- stem rot symptoms; **D**- peg and pod rot.

The disease cycle of stem rot on groundnut is shown in Figure 4. It is modeled based on the disease cycle of stem rot on apple (Mullen 2001) and hosta (Upchurch 2000). Sclerotia are the principal overwintering structures and the primary inoculum source for the disease. Under favorable conditions, sclerotia germinate and fungal hyphae grow towards and attack the lower part of the stem base. On diseased tissues, a hyphal mat and sclerotia are produced and, sometimes, also basidiospores are produced. The role of basidiospores in the disease cycle under field conditions has not been investigated in detail.

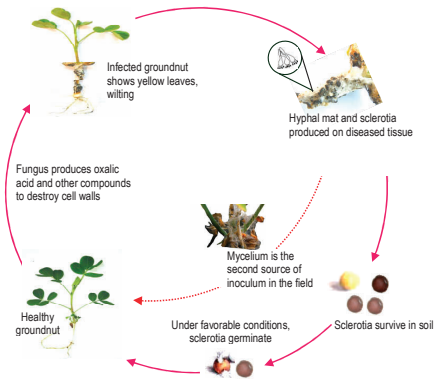

Figure 4. Disease cycle of stem rot on groundnut caused by *Sclerotium rolfsii.*

The soil-borne pathogen *Sclerotium rolfsii*

Sclerotium rolfsii was named by Saccardo in 1911 in recognition of Rolf's pioneer work in Florida referred to in Tu and Kimbrough (1978). Initially, *Sclerotium* was a genus assigned to the artificial class of Fungi Imperfecti that included many diverse species. These species were grouped in one genus because of their shared phenotypic characteristics. They generally form small, tan to dark-brown/black, spherical sclerotia, that function as survival structures. As more became known about these fungi and their sexual states, some were reclassified into other genera, either in the Basidiomycota or the Ascomycota, and sometimes renamed according to their teleomorph. In 1931, Cruzi first described the perfect stage of *S. rolfsii* and *Corticium rolfsii* was the first teleomorph name (referred by Tu and Kimbrough (1978)). In 1978, Tu and Kimbrough proposed to classify the pathogen in the genus *Athelia* and the binominal name, *Athelia rolfsii* (Cruzi) Tu and Kimbrough, has been used since. Some *Sclerotium* species still have no known sexual state, but by exploring molecular identification methods each species can now be assigned to the right genus. For example, based on sequence analysis of the rDNA large subunit (LSU) and internal transcribed spacer (ITS) regions, some *Sclerotium* species were re-named and moved from Ascomycota to Basidiomycota, whereas others, including *Sclerotium denigrans* and *Sclerotium perniciosum,* were moved from the Basidiomycota to the Ascomycota (Xu *et al*. 2010).

S. rolfsii has a wide host range with more than 500 plant species (Aycock 1966). They consist of mono- and di-cotyledons (Farr *et al.* 1989). Until now, no worldwide compilation of host genera has been published, however, more than 270 host genera have been reported in the USA. These include agricultural crops such as sweet potato (*Ipomoea batatas*), pumpkin (*Cucurbita pepo*), corn (*Zea mays*), wheat (*Triticum vulgare*), groundnut (*Arachis hypogeae*), and some horticultural crops such as Narcissus (*Narcissus* spp.), Iris (*Iris* spp.), Lilium (*Lilium*

spp.), Zinnia (*Zinnia* spp.), and Chrysanthemum (*Chrysanthemum* spp.) (Farr *et al.* 1989). In Vietnam, many crops are infected by *S. rolfsii* including groundnut (*Arachis hypogaea*), mungbean (*Vigna radiata*), soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), cabbage (*Brassica oleracea*), cucumber (*Cucumis sativus*) and taro (*Colocasia esculenta*) (Le 1977; Do 2001).

Colonies of *S. rolfsii* can be readily distinguished on infected plant material or artificial media by gross morphological characteristics (Fig. 5). Rapidly growing, silky-white hyphae tend to aggregate into rhizomorphic cords (Aycock 1966; Harlton *et al.* 1995). In culture, agar media are rapidly (2-3 days) covered with mycelium, including aerial hyphae. The optimum temperature for hyphal growth and sclerotial formation is 27-30ºC (Aycock 1966; Mathur and Sinha 1970; Punja 1985; Punja and Rahe 1993). Xu *et al.* (2008) showed that the difference in temperature in the southern and northern parts of the United States of America affects survival of sclerotia. As a result, the severity of *S. rolfsii* in the southern part was higher than that in the northern part. Sclerotia (0.3-3.0 mm diameter) begin to develop after 4-7 days of growth (Punja and Rahe 1993) when hyphae cluster together as a compact mass. After an initial white appearance, the sclerotia quickly become dark brown (Aycock 1966). Sclerotia contain viable hyphae and serve as the primary inoculum source in the disease cycle. Oxalic acid plays an important role in the virulence of *S. rolfsii* (Kritzman *et al.* 1977; Punja 1985). By producing oxalic acid as well as pectinolytic and cellulolytic enzymes, *S. rolfsii* kills and disintegrates host tissues before it penetrates (Prasad and Naik 2008).

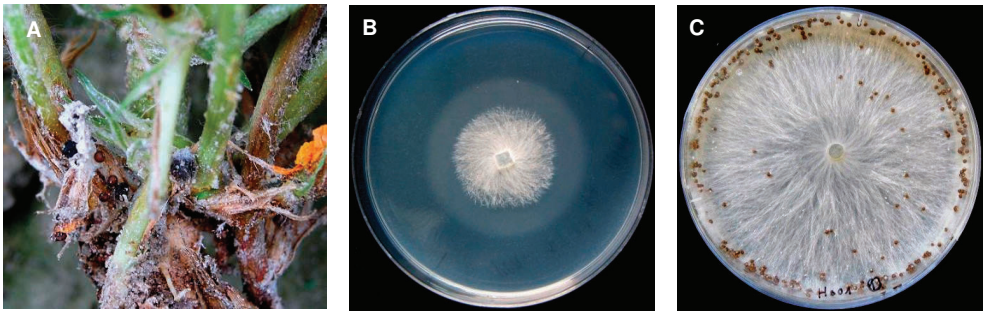

Figure 5. Mycelium and sclerotia of *Sclerotium rolfsii* on diseased tissue (A) and on Potato Dextrose Agar plates (B, C).

The genetic diversity of *S. rolfsii* has been studied by a variety of techniques, including mycelial compatibility (Fig. 6), restriction fragment length polymorphism (RFLP) analysis of ITS-rDNA, and by ITS-rDNA or LSU sequencing (Harlton *et al.* 1995; Okabe *et al.* 2000; Okabe *et al.* 2001; Punja and Sun 2001; Okabe and Matsumoto 2003; Xu *et al.* 2010). Harlton *et al.* (1995) found 49 mycelial compatibility groups (MCGs) and 12 RFLP-ITS groups in a

worldwide collection of isolates, but could not establish correlations between MCGs and pathogenicity. Some RFLP-ITS grouping patterns were correlated with MCGs, but isolates belonging to one MCG sometimes showed different RFLP-ITS patterns and certain patterns were dispersed among different MCGs. Recently, Xu *et al.* (2010) reported a close relationship between *S. rolfsii*, *S. rolfsii* var. *delphinii* and *S. coffeicola* by LSU sequence analysis. The phylogeny of *S. rolfsii*, *S. delphinii* and *S. coffeicola* based on ITS-rDNA shows two clades with most *S. rolfsii* strains. One clade contains most of the *S. delphinii* strains, and one clade contains strains of both *S. rolfsii* and *S. delphinii,* suggesting a close relationship between the latter two species.

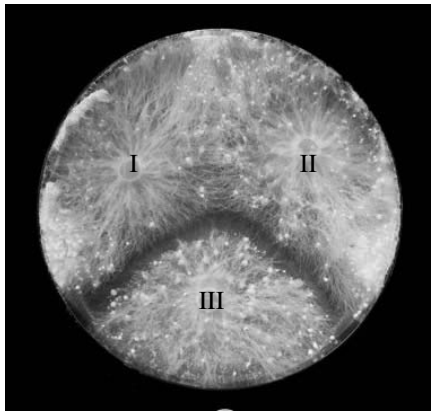

Figure 6. Example of mycelial compatibility analysis with three isolates *of Sclerotium rolfsii*. Hyphae of isolate I and II intermingle and are considered compatible. The barrage zone between isolate III and isolates I and II indicates mycelial incompatibility.

Control methods of stem rot disease

Because of its wide host range, fast growth rate, and the production of large numbers of persistent sclerotia, stem rot disease caused by *S. rolfsii* is very difficult to control (Punja 1985; Lakpale *et al.* 2007). Current control measures comprise the use of resistant cultivars and various physical, chemical, and biological control strategies (Le 1977; Redy and McDonald 1983; Punja 1985; Punja and Rahe 1993; Mehan and Hong 1994; Mehan *et al.* 1995; Le 2004; Nguyen *et al.* 2004; dos Santos *et al.* 2005; Vargas Gil *et al.* 2008).

Physical and cultural control

Cultural and physical methods to control soil-borne *S. rolfsii* are deep ploughing, solar heating, chemical fertilizer and crop rotation. Deep ploughing to bury sclerotia or disease tissues under 6-20 centimetres, was reported to reduce the viability of sclerotia or to kill hyphae of the pathogen in the fields (Elad *et al.* 1980; Porter and Merriman 1983; Mihail and Alcorn 1984). For solar heating, soils mulched with transparent polyethylene for 6 weeks in July-August and then sown with groundnuts the following spring, showed significant decreases in the percentage of diseased plants and rotten pods (Grinstein *et al.* 1979). Ammonium compounds were shown to inhibit germination of sclerotia and promoted colonization of sclerotia by soil microorganisms (Prasad and Naik 2008). However, for control of *S. rolfsii* on groundnut, supplementation with ammonium compounds is not recommended because this reduces the N_2-fixiation in root nodules. Rotation with non-host crops not only improves the

soil nutritional status, but also may adversely affect pathogen inoculum densities. For *S. rolfsii,* however, this strategy is not effective due to its broad host range. Nevertheless, Taylor and Rodriguez Kabana (1999) found that stem rot of groundnut can be suppressed by rotation with cotton. Also paddy rice was recommended as a rotation crop with groundnut in order to reduce stem rot in Vietnam (Le 1977).

Resistant cultivars

Because it is not easy to breed cultivars highly resistant to *S. rolfsii*, disease-tolerant cultivars may be used as a component of an integrated control effort (Punja 1985). In the mid-90s, two cultivars, i.e. Toalson and Southern Runner, were developed which are less susceptible/partially resistant to *S. rolfsii* (Branch and Csinos 1987; Mehan *et al.* 1995; Branch and Brenneman 1999) and more recently, additional partially stem rot resistant groundnut cultivars have been released including C-99R (Gorbet and Shokes 2002a), Florida MDR 98 (Gorbet and Shokes 2002b), Georgia-03L (Branch 2004), Georgia-07W (Branch and Brenneman 2008), and Florida-07 (Gorbet and Tillman 2009). The use of these cultivars is still limited, possibly due to the relatively low level of resistance.

Chemical control

In many cases, fungicides such as tebuconazole, pentachloronitrobenzene (PCNB) and flutolanil are used to control *S. rolfsii*. However, tolerance to tebuconazole and PCNB has been reported for *S. rolfsii* populations in USA (Wadsworth and Melouk 1984; Franke *et al.* 1998; Shim *et al.* 1998). Other fungicides that are used to control stem rot disease include difenoconazole, carbendazim, flusilazole and chlorothalonil (Cilliers *et al*. 2003). When difenoconazole was tested in combination with *Trichoderma harzianum*, a biocontrol agent of *S. rolfsii,* no reduction of the effect of *T. hazianum* was observed (Cilliers *et al.* 2003). Although fungicides can protect groundnut plants from infection by *S. rolfsii*, chemical control should be gradually minimized because of its potential harmful effects to the environment. Therefore integration of several different control measures is proposed to provide sustainable management of *S. rolfsii* and other diseases of groundnut. In this context, biological control can be an alternative or supplement to current management practices for *S. rolfsii* (Singh *et al.* 2003; Dey *et al.* 2004; Tonelli *et al.* 2010).

Biological control

Application of beneficial microorganisms to soil, seeds or planting materials has been proposed as a sustainable and supplementary approach to control plant diseases (Cook and Baker 1989). The most widely studied microorganisms with antagonistic activity against plant pathogens and with beneficial effects on plant growth, belong to the bacterial genera *Bacillus*, *Pseudomonas, Rhizobium,* or the fungal genus *Trichoderma* (Ongena and Jacques 2008; Raaijmakers *et al.* 2009; Lorito *et al.* 2010).

Fungal biocontrol agents may directly or indirectly kill sclerotia or mycelium of *S. rolfsii*. Lectins produced by *S. rolfsii* were proposed to serve as recognition factors for fungal biocontrol agents (Prasad and Naik 2008). Among the fungal biocontrol agents, *Trichoderma* species are the most widely studied (Table 1). In a direct interaction, hyphae of *Trichoderma* penetrate the rind and the cortex of sclerotia and lyse the medullar tissue. Degraded sclerotia become dark, soft and disintegrate under slight pressure (Prasad and Naik 2008) and it was shown that chitinase and β-1,3-glucanase play a role in the interaction between *Trichoderma harzianum* and *S. rolfsii* (Prasad and Naik 2008). Next to *Trichoderma,* several other fungal genera have been tested for their ability to control diseases caused by *S. rolfsii* on bean, carrot, chilli, ginger, wheat, lentil, sesame, soybean, sugar beet, sunflower, tomato, or groundnut. These antagonistic fungi include *Gliocladium virens, Gliocladium roseum, Glomus fascicatum, Penicillium pinophilum, Gigaspora margarita* and also *Sclerotium rolfsii* (Table 1).

For biocontrol of *S. rolfsii,* several bacterial genera and species have been studied. Most of them belong to the genera *Pseudomonas* and *Bacillus* (Table 2). *Pseudomonas* strains can restrict *in vitro* hyphal growth or reduce germination of sclerotia of *S. rolfsii* (Ganesan and Gnanamanickam 1987; Kishore *et al.* 2005; Ganesan *et al.* 2007; de Curtis *et al.* 2010; Pastor *et al.* 2010; Tonelli *et al.* 2010). Although pseudomonads are well-known for the production of a diverse array of antimicrobial compounds, including 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin, rhizoxins, phenazines and lipopeptides (Raaijmakers *et al.* 2002; Haas and Defago 2005; Raaijmakers *et al.* 2009; D'aes *et al.* 2010; Raaijmakers *et al.* 2010) the role of these or other bioactive compounds in biocontrol of *S. rolfsii* has not been studied in detail (Table 2). Also for most of the tested *Pseudomonas* strains, there is a lack of knowledge on the genes involved in their activity against *S. rolfsii*. Next to *Pseudomonas*, several *Bacillus* species and strains have been studied for their efficacy to control stem rot disease of groundnut. Pre-treatment of groundnut seeds with *Bacillus subtilis* protected groundnut seeds against *S. rolfsii* and significantly increased the number of pods (Abd-Allah 2005). Also for the *Bacillus* strains and other bacterial genera tested to date (Table 2), little or no knowledge is available on the fundamental mechanisms involved in their activity against *S. rolfsii*. Moreover, most of these studies were conducted under controlled conditions and only few studies were performed under field conditions.

Table 1. Fungal genera and species tested for biocontrol of *Sclerotium rolfsii* on groundnut and some other crops. Parts of these data were reproduced from Prasad (2008).

Fungal biocontrol agents	Crop	Proposed mechanisms*	References
Gliocladium roseum *Gliocladium virens* *Gliocladium* sp.	carrot, gram, groundnut, sunflower, tomato, mung bean	ISR and parasitism	Prasad and Naik 2008; Yaqub and Shahzad 2008
Glomus caledonium, *Glomus fasciculatum,*	groundnut	parasitism	Ozgonen *et al.* 2010; Prasad and Naik 2008
Gigaspora margarita	groundnut	parasitism	Ozgonen *et al.* 2010
Penicillium pinophilum	sesame	unknown	Prasad and Naik 2008
Sclerotium rolfsii	groundnut	ISR	Nandini *et al.* 2010
Trichoderma aureoviride	tomato, soybean	unknown	Prasad and Naik 2008
Trichoderma harzianum	chilli, groundnut, gram, lentil, mung bean, sesame, soybean, sugar beet, sunflower, tea, tomato, wheat	parasitism ISR	Abada 1994; Bhagat and Pan 2007; Boukaew *et al.* 2011; Elad *et al*. 1982; Ganesan *et al.* 2007; Prasad and Naik 2008; Shaigan *et al.* 2008; Yaqub and Shahzad 2008
Trichoderma koningii	lentil, soybean, tomato	parasitism	Prasad and Naik 2008; Tsahouridou and Thanassoulopoulos 2002
Trichoderma longibrachiatum	groundnut, tea	parasitism	Prasad and Naik 2008; Shaigan *et al.* 2008
Trichoderma pseudokoningii	Chinese cabbage, ginger, sunflower	ISR and parasitism	Cuevas *et al.* 2001; Prasad and Naik 2008; Yaqub and Shahzad 2008
Trichoderma viride	groundnut, gram, lentil, soy bean, sugar beet, tea, tomato	parasitism	Chima Wokocha 1990; Prasad and Naik 2008; Shaigan *et al.* 2008
Trichoderma polysporum,	sunflower	ISR and parasitism	Yaqub and Shahzad 2008
Trichoderma hamatum,	tea	parasitism	Shaigan *et al.* 2008
Trichoderma parceramosum	Chinese cabbage, tea	parasitism	Cuevas *et al.* 2001; Shaigan *et al.* 2008
Trichoderma sp.	bean, groundnut, sunflower, string bean, tomato	parasitism	Liu *et al.* 2008; Prasad and Naik 2008; Sai *et al.* 2010

*ISR = induced systemic resistance

Table 2. Bacterial genera and species tested for biological control of *Sclerotium rolfsii* on groundnut and some other crops.

Bacterial genera/species	Origin	Crop	Test method				Proposed mechanisms*	References
			in vitro	growth chamber	net-house	field		
Pseudomonas								
P. fluorescens	unknown	groundnut	x	x			unknown	Ganesan and Gnanamanickam 1987
P. fluorescens	chickpea rhizosphere	betel vine	x			x	unknown	Singh *et al*. 2003
P. fluorescens	pepper rhizosphere	pepper	x	x			unknown	Abeysinghe 2009
P. fluorescens	culture collection	groundnut	x		x	x	ISR	Senthilraja *et al.* 2010
P. aeruginosa	groundnut seed endophyte	groundnut	x	x			interference with CWDE	Kishore *et al.* 2005
P. aeruginosa	oil palm roots	chili pepper	x	x			ISR	Siddiqui and Meon 2009
P. putida	pepper rhizosphere	pepper	x	x			unknown	Abeysinghe 2009
P. monteilii	unknown	groundnut	x	x			antibiosis	Rakh *et al.* 2011
Pseudomonas sp.	tomato roots	tomato	x	x			ISR and antibiosis	Pastor *et al.* 2010
Pseudomonas sp.	composts	chickpea	x	x			antibiosis	Hameeda *et al.* 2010
Pseudomonas sp.	groundnut	groundnut		x			ISR	Tonelli *et al.* 2011
Bacillus								
B. subtilis	tomato rhizosphere	groundnut	x	x			unknown	Abd-Alla 2003
B. subtilis	tomato rhizosphere	groundnut		x			ISR	Abd-alla 2005; Abd-Allah and El-Didamony 2007
B. subtilis	organic amendments	tomato	x	x		x	unknown	De Curtis *et al.* 2010
B. subtilis	pepper rhizosphere	pepper	x	x			antibiosis	Abeysinghe 2009

'Table 2 continued'

Bacterial genera/species	Origin	Crop	Test method				Proposed mechanisms*	References
			in vitro	growth chamber	net-house	field		
Bacillus spp.	composts	chickpea	x	x			antibiosis	Hameeda *et al.* 2010
Bacillus sp.	groundnut	groundnut		x			ISR	Tonelli *et al.* 2011
Other								
Burkholderia cepacia	organic amendments	tomato	x	x		x	unknown	De Curtis *et al.* 2010
Burkholderia cepacia	oil palm roots	chili pepper	x	x			ISR	Siddiqui and Meon 2009
Steptomyces mycarofaciens	pepper	chili pepper	x	x		x	antibiosis	Boukaew *et al.* 2011
Steptomyces philanthi	pepper	chili pepper	x	x		x	antibiosis	Boukaew *et al.* 2011
Streptomyces spp.	sugar beet soil	sugar beet	x	x			antibiosis	Errakhi *et al.* 2007
Agrobacterium	unknown	tomato	x	x			competition and antibiosis	Pelzer *et al.* 2011
Kluyvera	unknown	tomato	x	x			competition and antibiosis	Pelzer *et al.* 2011
Serratia marcescens	unknown	*S. rolfsii*	x				antibiosis	Ordentlich *et al.* 1988
Methylobacterium sp.	groundnut	groundnut	x		x		ISR	Madhaiyan *et al.* 2006
Rhizobium sp.	unknown	groundnut	x		x		ISR	Madhaiyan *et al.* 2006
Rhizobium sp.	groundnut nodules	groundnut	x	x			antibiosis	Ganesan *et al.* 2007
Rhizobium spp.	tomato rhizosphere	groundnut		x			unknown	Abd-Allah and El-Didamony 2007

* ISR = induced systemic resistance, CWDE = Cell Wall Degrading Enzyme

Outline of this thesis

Groundnut is an important oil seed crop in many countries including Vietnam. Cultivation of groundnut is adversely affected by a wide range of pests and diseases. Stem rot caused by *S. rolfsii* is among the most damaging soil-borne fungal diseases and causes significant economic losses. The **overall aim** of the research described in this thesis was to study the efficacy of biological control of stem rot disease of groundnut in Vietnam. When we started this research project, there was little knowledge on the genetic diversity of *S. rolfsii* populations on groundnut in Vietnam. Moreover, there was hardly any information on the occurrence and distribution of beneficial soil bacteria in groundnut farmer fields and the potential of specific bacterial genera to control stem rot disease under field conditions.

As a first step (**chapter 2**), we made an inventory of the incidence of stem rot disease on groundnut in farmer fields in central Vietnam. Approximately 200 isolates of *S. rolfsii* were successfully isolated from more than 400 diseased samples collected at eight locations in four provinces. We subsequently analyzed the phenotypic diversity of *S. rolfsii* populations based on hyphal growth rate and different sclerotial characteristics. The genetic diversity of *S. rolfsii* populations was assessed by mycelial compatibility assays and ITS-rDNA sequencing. In addition, we tested the pathogenicity of *S. rolfsii* isolates and their sensitivity to tebuconazole, a fungicide commonly used to control stem rot disease.

To date, several bacterial strains have been studied in biocontrol of stem rot of groundnut. However, in most of these studies biocontrol was studied under controlled conditions and the mechanisms involved in the biocontrol activity were not identified. In this thesis (**chapter 3**), the biocontrol efficacy of several well-characterized *Pseudomonas* species and strains was evaluated. These included phenazine-producing strain *Pseudomonas chlororaphis* Phz24 and lipopeptide-producing strains *Pseudomonas* sp. SH-C52, *Pseudomonas fluorescens* SS101, *P. fluorescens* SBW25 and *Pseudomonas putida* 267. To determine the role of phenazines and lipopeptides in inhibition of *S. rolfsii*, mutants deficient in the production of phenazines or lipopeptides were generated and included in the bioassays. Subsequently, strains *P. chlororaphis* Phz24, *Pseudomonas* sp. SH-C52 and *P. fluorescens* SS101 were tested for biocontrol of stem rot of groundnut under nethouse and field conditions in Vietnam. Among these bacterial strains, *Pseudomonas* sp. SH-C52 and *P. fluorescens* SS101 showed promising results in biological control and growth promotion in the field experiments conducted in 2010. Their consistency to control stem rot disease and/or to increase pod yield were again evaluated in field experiments conducted in 2011 (**chapter 5**).

Besides the well-characterized Pseudomonads, we isolated and characterized indigenous bacterial populations from the stem base and roots of groundnut plants grown in farmer fields in central Vietnam (**chapter 4**). Four bacterial isolates, identified by 16S-rDNA sequencing as *Pseudomonas*, *Bacillus* and *Chryseobacterium* species, were tested for growth promotion and biocontrol of stem rot of groundnut. Among the tested bacterial strains, *Pseudomonas* sp. R4D2

was effective in biocontrol of stem rot, whereas *Bacillus* sp. strains S18F11 and S20D12 significantly increased pod yield. All three strains were again tested for biocontrol of stem rot and plant growth promotion in 2011. In these field trials, we also evaluated the effects of these bacterial strains on various other groundnut diseases, including damping-off, black collar rot, bacterial wilt and leaf spots (**chapter 5**).

In **chapter 6**, the diversity of bacteria associated with groundnut nodules and their effects on plant growth were investigated. Two isolates, identified by 16S-rDNA sequencing as *Sphingomonas* and *Rhizobium* species, were tested in field trials for their ability to control stem rot and other groundnut diseases.

The major results obtained in this thesis are summarized and discussed in **chapter 7**. In this final chapter, we also discuss the consistency and future perspectives of biological control of stem rot disease of groundnut.

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Chapter 2

Genetic and phenotypic diversity of *Sclerotium rolfsii* Sacc. in groundnut fields in central Vietnam

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Genetic and phenotypic diversity of *Sclerotium rolfsii* Sacc. in groundnut fields in central Vietnam

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Abstract

Groundnut (*Arachis hypogaea* L.) is an economically important legume crop in Vietnam and many other countries worldwide. Stem and pod rot caused by the soil-borne fungus *Sclerotium rolfsii* Sacc. is a major yield limiting factor in groundnut cultivation. To develop sustainable measures to control this disease, fundamental knowledge of the epidemiology and diversity of *S. rolfsii* populations is essential. In this study, disease incidence was monitored in eight groundnut areas in central Vietnam with a total of 240 plots. The results showed that 5-25% of the field-grown groundnut plants were infected by *S. rolfsii*. Based on ITS-rDNA sequence analyses, three distinct groups were identified among a total of 103 randomly selected *S. rolfsii* field isolates, with the majority of the isolates (*n*=90) in one ITS group. *S. rolfsii* isolates originating from groundnut, tomato and taro were all pathogenic on groundnut and relatively sensitive to the fungicide tebuconazole, but displayed substantial diversity of various genetic and phenotypic traits including mycelial compatibility, growth rate, and sclerotial characteristics.

Keywords: *Athelia rolfsii*, ITS-rDNA, MCG, sclerotia

Introduction

Groundnut (*Arachis hypogaea* L.) is an annual legume crop cultivated in more than 80 countries in the tropics, subtropics, and warm temperate zones (Hammons 1994). It is a major source of edible oil, vitamins, amino acids and used extensively for feed and food (Savage and

Keenan 1994). In terms of economic importance, it ranks thirteenth among the world food crops and tops the list of oil seed crops both in terms of acreage and production. In 2009, groundnut was grown on 23.5 million hectares world wide with an estimated total production of 35.5 million ton (FAO 2010). In Vietnam, groundnut is the most important oil crop with a total area of 256,000 ha and a production of 534,000 ton in 2008 (FAO 2010).

Groundnut cultivation is hampered by a wide range of pests and diseases, including subterranean pests and foliage feeders (Brown 2009), leaf spot, rust, stem rot, seedling diseases, limb and pod rot, nematode and viral diseases (Shew and Waliyar 2005). In Vietnam, black collar root rot caused by *Aspergillus niger* Van Tiegh., damping-off caused by *Rhizoctonia solani* Kühn, and stem and pod rot caused by *Sclerotium rolfsii* Sacc. (teleomorph: *Athelia rolfsii* (Curzi) Tu & Kimbrough) are the most important soil-borne fungal diseases of groundnut (Le 2004; Nguyen *et al.* 2004). The basidiomycete *S. rolfsii* overwinters as mycelium or sclerotia in infected plant tissues and soil. Under favourable conditions, hyphae or germinating sclerotia infect the stembase of the plant and subsequently colonize and invade the root and stem tissue with the typical silky-white mycelium (Brewster 2001). Infected plants become yellow and then wilt, the collar root turns brown and rots; in addition, *S. rolfsii* infects the groundnut pegs and pods leading to yield losses.

S. rolfsii is difficult to control due to its wide host range of over 500 plant species (Aycock 1966; Punja 1985) and persistent sclerotia (Lakpale *et al.* 2007; Punja 1985). Currently, there are only a few resistant cultivars commercially available (Branch and Brenneman 1999; 2009; Woodward *et al.* 2008). In Vietnam, methods to control *S. rolfsii* include rotation with non-host crops or deep coverage of infected crop debris with soil during land preparation. However, these methods are laborious and not effective due to the broad-host range and persistence of *S. rolfsii*. Fungicides currently used to control *S. rolfsii* include pentachloronitrobenzene (PCNB), flutolanil (Scinos 1989) and tebuconazole (Besler *et al.* 2006; Branch and Brenneman 1996; Brenneman and Murphy 1991). All three fungicides are effective in many cases although tolerance to these fungicides was reported for *S. rolfsii* populations from groundnut fields in USA (Franke *et al.* 1998; Shim *et al*. 1998; Wadsworth and Melouk 1984). In Vietnam, these fungicides are not yet used on a regular basis and large scale due to their relatively high costs for subsistence farmers.

To successfully implement management practices (e.g. chemical, biological) to control *S. rolfsii*, knowledge of the distribution and diversity of the pathogen is essential. The diversity of *S. rolfsii* has been assessed for field populations in Georgia (USA) and Ibaraki (Japan) (Franke *et al.* 1998; Okabe and Matsumoto 2000), but for most other groundnut-producing countries, including Vietnam, the information on the distribution, severity and diversity is scarce or not available. Here, we monitored the incidence of Sclerotium stem rot of groundnut in fields in central Vietnam and characterized *S. rolfsii* populations genetically and phenotypically. The implications of our findings for developing sustainable and appropriate strategies to control stem and pod rot in Vietnam are discussed.

Results and Discussion

Disease incidence

Our survey conducted in 2009 at eight locations in central Vietnam, with approximately 240 field plots of 1 m^2, showed that the incidence of stem rot disease of groundnut caused by *S. rolfsii* ranged from approximately 5 to 25% (Fig. 1 and 2). The observed stem rot incidence in

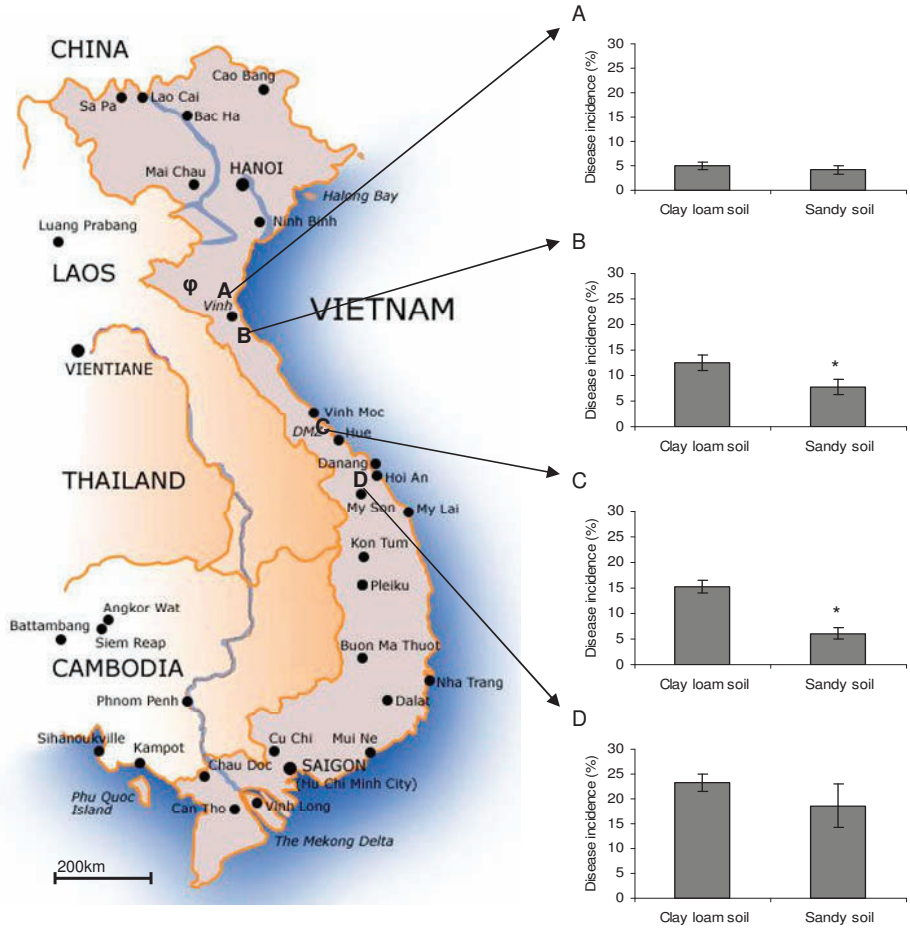

Figure 1. Map of Vietnam showing the provinces where the incidence of stem rot of groundnut was monitored in 2009 and where *Sclerotium rolfsii* isolates were collected. The four provinces surveyed are: A) Nghe An (18º46'N 105º38'E), B) Ha Tinh (18º21'N 105º51'E), C) Thua Thien Hue (16º33'N 107º31'E), and D) Quang Nam (15º47'N 108º21'E). Three more isolates of *S. rolfsii* were collected from groundnut grown in the remote upland area of Nghe An province (φ). For each of the four provinces, stem rot incidence was determined for groundnut plants grown in two soil types (clay loam and sandy soils). For each soil type and each province, the disease was recorded in 30 field plots of 1-m^2. In the graphs on the right, the average disease incidence is presented and the standard error of the mean is indicated. An asterisk indicates a statistically significant difference (p<0.05) between the disease incidence in sandy soil and that in clay loam soil.

central Vietnam is similar to that reported in Georgia (USA) from 1983 to 1985, i.e. approximately 8.5% (Branch and Csinos 1987), but much higher than the disease incidence reported in Texas (USA) in 1992 and 1993 (less than 1%) (Shim *et al.* 1998). On the other hand, it was lower than in Ibaraki (Japan) where disease incidence ranged from 10 to 40% (Okabe and Matsumoto 2000).

Figure 2. A) Overview of the sampling location in Quang Nam province; B) effects of *Sclerotium rolfsii* on emergence and growth of groundnut seedlings in the field, symptoms of stem rot of groundnut (C), tomato (D) and taro (E). For groundnut, tomato and taro, the sclerotia on the infected plant tissues are indicated by an arrow.

Our field survey further indicated a gradient in stem rot disease incidence across central Vietnam with an increase in disease incidence from the northern to the southern field sites (Fig. 1). This might be related to the effect of temperature on disease development (Punja 1985) because along with the lower latitude, the average daily temperature increases from the North to the South of Vietnam. Important to note also is that the field sites surveyed in the two southern provinces (locations C and D, Fig. 1) were sites where tomato and taro were also cultivated. Both crops are hosts of *S. rolfsii* (Fig. 2D and E) and may have contributed to the build-up of pathogen inoculum. For two of the four provinces, i.e. locations B and C, the disease incidence of groundnut plants cultivated in clay loam soil was significantly higher than in sandy soil (Fig. 1). This difference may be related in part to the higher organic content of the clay loam soil, a characteristic that is known to support germination of sclerotia and subsequent hyphal growth toward the host plant (Punja 1985).

Genetic diversity of S. rolfsii

A total of 198 purified *S. rolfsii* isolates, obtained from more than 400 randomly collected samples from different host plants (groundnut, tomato and taro) in the four provinces and in the remote upland region, were subjected to ITS-rDNA sequence analysis. For 103 *S. rolfsii* isolates, high quality forward and reverse sequences were obtained, whereas for the other 95 isolates discrepancies were observed in the assembly of the forward and reverse sequences. Re-sequencing of the ITS-fragments of twelve selected isolates did not resolve this inconsistency, which may suggest polymorphisms in different ITS copies in each of these isolates. ITS polymorphism within a single isolate has been found for several fungal genera and was also reported earlier for *S. rolfsii* by Okabe *et al.* (2001) and Okabe and Matsumoto (2003).

Phylogenetic analysis of the ITS-rDNA sequences of the reference isolates of *S. rolfsii*, i.e. isolates for which ITS-rDNA sequences are present in the NCBI database (Table 1), revealed three main groups designated ITS-groups 1-3 (Fig. 3). Most isolates (*n*=90) collected from central Vietnam belong to ITS-group 1 and were identical to reference isolates of *S. rolfsii* that were previously collected from groundnut, tobacco and sweet pepper (Fig. 3, Table 1). The three isolates in ITS-group 2 were collected from the remote upland region of Nghe An province (Fig. 3) and cluster with an *S. rolfsii* isolate collected from Ascocenda orchids in Florida, USA (Cating *et al.* 2009). Out of the ten isolates in ITS-group 3, eight were collected in Quang Nam province (Fig. 3). Collectively, these results suggest that, based on ITS-rDNA sequences, the *S. rolfsii* population in groundnut fields in central Vietnam appears relatively uniform. However, that ITS-rDNA sequencing does not give detailed insight into the intraspecific diversity and that other molecular markers or traits should be considered.

Table 1. ITS-rDNA sequences of reference strains of *Sclerotium rolfsii, S. delphinii* and *S. coffeicola* used in the phylogenetic analyses.

Accession number	Host	Origin	Year of isolation	Name	Reference
GQ358518	*Ascocentrum* spp., *Ascocenda* spp.	South Florida, USA	2008	*S. rolfsii*	Cating *et al.* 2009
DQ484060	unknown	unknown	unknown	*S. rolfsii*	Matheny *et al.* 2006
DQ484061	unknown	unknown	unknown	*S. rolfsii*	Matheny *et al.* 2006
DQ484062	unknown	unknown	unknown	*S. rolfsii*	Matheny *et al.* 2006
AB075307	*Arachis hypogaea*	Georgia,USA	1991	*S. rolfsii*	Okabe and Matsumoto 2003
GU080230	*Capsicum annuum*	Spain	2009	*S. rolfsii*	Remesal *et al.* 2010
DQ059578	*Nicotiana tabacum*	North Carolina, USA	2007	*S. rolfsii*	Ristaino *et al*. 2007
AB075318	unknown	Washington, USA	1991	*S. delphinii*	Okabe and Matsumoto 2003
AB075314	unknown	Japan	1995	*S. delphinii*	Okabe and Matsumoto 2003
AB075312	unknown	Japan	1992	*S. delphinii*	Okabe and Matsumoto 2003
AB075319	unknown	Surinam	1919	*S. coffeicola*	Okabe and Matsumoto 2003

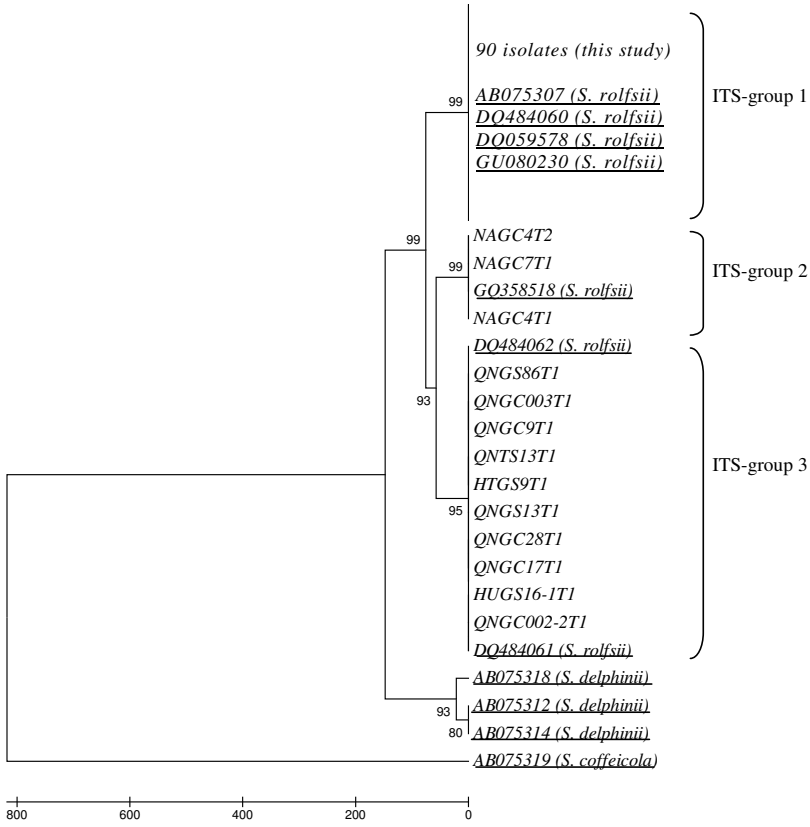

Figure 3. Phylogeny of ITS-rDNA sequences of *Sclerotium rolfsii* isolates from groundnut fields in central Vietnam and of reference strains of *S. rolfsii, S. delphinii* and *S. coffeicola* (underlined, see Table 1) using the Unweighted pair-group method (UPGMA). Bootstrap values with 1,000 replications are indicated at the nodes of the branches. The bar represents the evolutionary distances computed using the Maximum Composite Likelihood method. The sequence codes of the isolates collected in this study refer to the province (first two letters), host crop (third letter), soil type (fourth letter), and the isolate number (digits): NA- Nghe An province, HT- Ha Tinh province, HU- Thua Thien Hue province, QN- Quang Nam province; G- groundnut, T- tomato, M- taro; C- clay loam soil, S- sandy soil (see appendix).

For *S. rolfsii*, several other techniques and molecular markers have been used to assess intraspecific diversity, especially random amplified polymorphic DNA (RAPD) analysis (Punja and Sun 2001). Multiple of these tests aimed at characterizing the intraspecific diversity of the isolates were performed here with an initial seven *S. rolfsii* isolates from the collection, i.e. 3 isolates from Vietnam and 4 reference strains from the culture collection. However, RAPD analysis as well as rep-PCR and ERIC-PCR, performed according to methods previously described for other fungi (McDonald *et al.* 2000), gave inconsistent/irreproducible results and were not considered useful. Therefore, a range of other traits, including pathogenicity, growth rate, sclerotial characteristics, mycelial compatibility and tebuconazole sensitivity, were

analysed to get more insight into the intraspecific diversity of the collection of *S. rolfsii* isolates from central Vietnam.

Pathogenicity

Pathogenicity assays showed that *S. rolfsii* isolates obtained from groundnut (*n*=8), tomato (*n*=5) and taro (*n*=5) from central Vietnam and representing ITS-groups 1-3 were all pathogenic on groundnut (Table 2). The time for the first symptoms to appear ranged from 2-5 days and no statistically significant differences in disease incidence and severity were found between the isolates (Table 2). These results confirmed pathogenicity of i) a subset of *S. rolfsii* isolates collected from groundnut, ii) all *S. rolfsii* isolates collected from other crops (i.e. tomato and taro), and iii) the three isolates collected from groundnut cultivated in the remote upland region in Nghe An province (ITS-group 2).

Table 2. Pathogenicity of *Sclerotium rolfsii* isolates on groundnut. The isolates used for these assays are indicated in the appendix with an asterisk.

Host crop	ITS-group[1]	Incubation period[2] (day)	Disease incidence[3] (%)	Disease severity ± SE[4] (%)
Groundnut (N=5)	1, 3	2-3	100	92.7 ± 2.21
Tomato (N=5)	1, 3	4-5	100	86.3 ± 4.26
Taro (N=5)	1	3-4	100	95.3 ± 1.11
Groundnut (N=3)	2	3-4	100	75.0 ± 2.89

[1] ITS-group as indicated in Fig. 3; [2] Days to first symptom appearance after fungal inoculation; [3]Disease incidence (%) = (diseased plants / total number of plants) × 100%; [4]Disease severity: diseased plants were ranked on a 0-4 scale based on the symptoms with 0: no disease symptoms, 1: infection without visible outgrowth of the fungus, 2: infection with visible outgrowth, 3: partial wilting of the plant, and 4: complete wilting and plant death. Disease severity (%) = [(1 × number of plants classified in scale 1) + (2 × number of plants classified in scale 2) + (3 × number of plants classified in scale 3) + (4 × number of plants classified in scale 4)] × 100% / (4 × total number of plants)].

Growth rate and sclerotial characteristics

S. rolfsii is notorious for its high growth rate *in vitro* (Akram *et al.* 2008; Punja 1985; Punja and Damiani 1996). Indeed, the 103 Vietnamese isolates tested here exhibited a high growth rate ranging from 0.28 to 0.79 mm h^{-1} (Table 3A, appendix). On average, isolates from ITS-groups 1, 2 and 3 showed no significant differences in growth rate (Table 3B). On PDA medium, the *S. rolfsii* isolates produced the typical silky-white mycelium and the brown or dark brown sclerotia (Fig. 4) (Harlton *et al.* 1995). For the 103 isolates tested, considerable variation was observed in the time to form sclerotia, their maturation time, and their number and size (Table 3B, appendix). For example, the number of sclerotia formed per plate after 21 days of incubation ranged from 79 to 1,080 and their size from 0.88 to 2.24 mm (Table 3A). These results confirm and extend the data obtained in previous studies on *S. rolfsii* isolates from other crops and geographic locations (Kokub *et al.* 2007; Prasad and Naik 2008; Punja and Damiani 1996; Xu *et al.* 2010). When comparing the sclerotial traits, the three isolates in ITS-group 2

were different from the isolates in ITS-groups 1 and 3 (Table 3B). ITS-group 2 isolates produced substantially less sclerotia per plate and also the average maturation time and sclerotial diameter were approximately two times higher than for isolates of ITS-groups 1 and 3. These characteristics combined with the light brown colour of the sclerotia of these three ITS-group 2 isolates were also reported for isolates classified morphologically or by LSU-sequencing as *S. rolfsii* var. *delphinii* (Aycock 1966; Punja and Damiani 1996; Xu *et al.* 2010).

Table 3. Growth rate and sclerotial characteristics of *Sclerotium rolfsii* isolates obtained from groundnut fields in central Vietnam. In panel **A**, averages, minimum (Min), and maximum (Max) values of each of these phenotypic characteristics are given for 103 isolates; in panel **B,** averages ± the standard deviation (SD) of these phenotypic characteristics are given for each of the three identified ITS-rDNA groups of *S. rolfsii*.

A	Hyphal growth rate during 48 h (mm h^{-1})	Sclerotia production and size			
		Days to form	Days to mature	Number per plate	Diameter (mm)
Average	0.40	4.8	8.0	589	1.1
Min	0.28	4.0	7.0	79	0.9
Max	0.79	11.0	16.0	1080	2.2
SD	0.06	1.1	1.4	204	0.2

B					
ITS-group					
1 (N=90)	0.41 ± 0.06	4.7 ± 0.6	7.8 ± 0.7	595 ± 181	1.1 ± 0.1
2 (N=3)	0.36 ± 0.04	10.0 ± 1.7	14.3 ± 2.9	98 ± 16	2.0 ± 0.2
3 (N=10)	0.41 ± 0.04	4.2 ± 0.4	7.8 ± 1.2	689 ± 236	1.1 ± 0.2
Average	0.40 ± 0.06	4.8 ± 1.1	8.0 ± 1.4	589 ± 204	1.1 ± 0.2

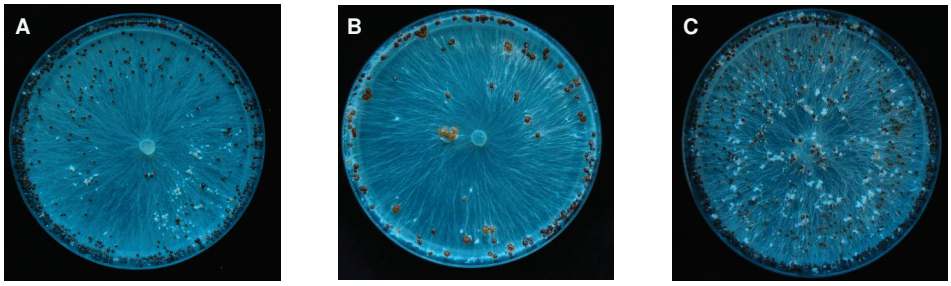

Figure 4. Phenotypic characteristics of mycelium and sclerotia of three *Sclerotium rolfsii* isolates belonging to ITS-rDNA groups 1 (panel A), 2 (panel B) and 3 (panel C). A 5-mm-diameter mycelial agar plug was inoculated in the centre of a 9-cm-diameter PDA agar plate and incubated at 25°C. All isolates have the typical white-silky mycelium with round or irregular, light- to dark-brown sclerotia.

Mycelial Compatibility

For several fungi, mycelial compatibility is used to get insight into the genetic relatedness of isolates or to identify intraspecific variation within field populations of plant pathogens (Kohn *et al.* 1991). When confronted with one another, a demarcation or barrage zone between the fungal colonies places these isolates in a different mycelial compatibility group (MCG). Isolates in the same MCG can be clonal (Kohn *et al.* 1991) or generally exhibit a higher degree of genetic relatedness than isolates from different MCGs (Rayner 1991; Remesal *et al.* 2010). In a study by Punja and Sun (2001) on *S. rolfsii* isolates from 13 countries and 36 different host species, a total of 71 MCGs were found and there was no clear relationship between the original host plant and MCG, except for *S. rolfsii* isolates from turfgrass. Within each of the three ITS-groups identified in our study, a high variation in mycelial compatibility was observed (Table 4). For isolates of ITS-group 1, a total of 91 combinations were tested and only 6.6% of these combinations showed a compatible interaction pointing to at least 9 MCGs. For ITS-group 2 and for ITS-group 3, we found two and four MCGs, respectively (Table 4). In contrast, studies by Adandonon *et al.* (2005) on *S. rolfsii* from cowpea fields in Benin revealed four MCGs among a total of 66 isolates. Similarly, Okabe and Matsumoto (2000) found four MCGs among a total of 132 isolates from groundnut fields in Ibaraki (Japan) and concluded, based on RAPD analysis, that many isolates were clonal. Based on MCG analysis, our results suggest that *S. rolfsii* populations from groundnut fields in central Vietnam are more diverse. This may be partly due to the fact that the fields sampled in our study are far apart, i.e. approximately 400 km from the northern fields sites (location A, Fig. 1) to the southern field sites (location D, Fig. 1) as compared to only 2.5 km in the study by Okabe and Matsumoto (2000).

Table 4. Mycelial compatibility between *Sclerotium rolfsii* isolates for each of the three identified ITS groups.

ITS group	Number of isolates	Number of combinations	Compatible combinations	% compatibility	Number of MCG*
1	14	91	6	6.6	9
2	3	3	1	33.3	2
3	9	36	4	11.1	6

* mycelial compatibility group.

Tebuconazole sensitivity

The results showed that the variation in sensitivity of *S. rolfsii* isolates from groundnut fields in central Vietnam to tebuconazole is substantially less than reported earlier by Franke *et al.* (1998) for the *S. rolfsii* population from Georgia, USA (Table 5, Fig. 5). The three isolates from ITS-group 2, collected from the remote upland region, were significantly more sensitive to tebuconazole than those from ITS-group 1 (Table 5). Although this may be due in part to a sample size difference, it may also be linked to the fact that farmers in this remote area do not use tebuconazole. In the other farmer fields surveyed, however, no records are kept on fungicide use,

so for the fields included in this study no correlation can be established between sensitivity and fungicide use. The study by Franke *et al.* (1998) on sensitivity of *S. rolfsii* to tebuconazole showed a wide variation among a total of 473 field isolates from Georgia, USA; several isolates from their collection showed negative inhibition values which presumably reflects a positive effect of tebuconazole on hyphal growth (Fig. 5). Franke *et al.* (1998) also found a correlation between reduced sensitivity to tebuconazole among isolates from fields with a history of repeated applications of this fungicide. In Vietnam, tebuconazole is used to control *S. rolfsii* but neither on a regular basis nor on a large scale due to its relatively high costs for subsistence farmers.

Table 5. Sensitivity to tebuconazole of *Sclerotium rolfsii* isolates from groundnut fields in central Vietnam. The mean inhibition of radial mycelial growth was determined at the tebuconazole concentration of 0.02 mg liter^{-1} potato dextrose agar. The relative inhibition of mycelial growth was calculated based on the formula: Inhibition (%) = 100 – [(colony diameter on amended medium / colony diameter on control) × 100%].

ITS-group	Growth inhibition (%) (mean ± SD)
1	35.5 ± 8.7
2	45.9 ± 3.1
3	42.7 ± 11.6

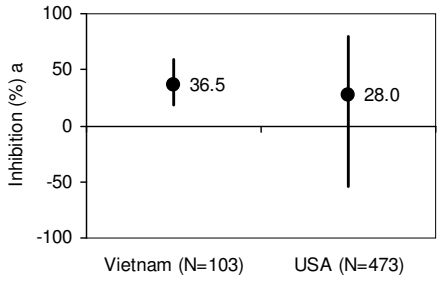

Figure 5. Sensitivity of *Sclerotium rolfsii* isolates collected from groundnut fields in Vietnam to tebuconazole. Sensitivity was tested at 0.02 mg tebuconazole liter^{-1} PDA at 48h and 25°C. For comparison, data reported earlier for *S. rolfsii* isolates from the USA (adapted from Franke et al., 1998) are included. The relative inhibition of radial mycelial growth was calculated by the formula: Inhibition (%) = 100 – [(colony diameter on tebuconazole-amended medium / colony diameter on non-amended plates) × 100%]. The bars represent the minimum and maximum percentage of inhibition observed for single isolates in the collection. Numbers next to the bars indicate the average relative inhibition.

Conclusions

Sclerotium rolfsii Sacc. is a major soil-borne pathogen of groundnut. Systematic knowledge of the diversity and epidemiology of *S. rolfsii* populations in groundnut fields may help to adopt and develop effective and sustainable control measures. The work presented here is the first study on the distribution, incidence and diversity of *S. rolfsii* populations in groundnut fields in central Vietnam. *S. rolfsii* isolates originating from groundnut, tomato and taro were all pathogenic on groundnut and displayed substantial diversity of various genetic and phenotypic traits, including mycelial compatibility, growth rate, and sclerotial characteristics. The observation that the *S. rolfsii* isolates tested were all relatively sensitive to tebuconazole provides opportunities to use this fungicide to control stem rot disease of groundnut in central Vietnam. Combination with other control measures, including biological control, is recommended to prevent resistance development as was observed previously for other *S. rolfsii* populations exposed repeatedly to this fungicide.

Materials and methods

Disease incidence, S. rolfsii isolation and preservation

In 2009, the disease incidence of stem and pod rot was monitored in groundnut fields in four provinces in central Vietnam, i.e. Quang Nam, Thua Thien Hue, Ha Tinh and Nghe An (Fig. 1). In each province, samples were obtained from groundnut plants grown at two locations at least 20 km apart, one with sandy soil and the other with clay loam soil. The disease incidence was assessed at flowering, the developmental stage at which groundnut is infected by *S. rolfsii*. For all eight locations, disease incidence was determined in at least 10 farmer fields for a total of 30 randomly selected plots of 1 m^2. So, in total 240 groundnut plots of 1 m^2 (i.e. 8 locations x 30 plots) were monitored in central Vietnam to determine the disease incidence of stem and pod rot caused by *S. rolfsii*. From each of the eight locations, 50 samples of diseased tissue or sclerotia were collected, stored in plastic bags and brought to the lab for *S. rolfsii* isolation. Samples were collected mainly from groundnut, however, diseased tissues from two other host crops, i.e. tomato (*Solanum lycopersicum*) and taro (*Colocasia esculenta*), grown in the same fields were also collected and included for pathogen isolation. Besides sampling the eight locations, we also sampled groundnut plants from a remote upland location (100 m above sea level) in Nghe An province (Fig. 1).

S. rolfsii was isolated and maintained according to the methods of Punja and Rahe (1993). Diseased tissues and sclerotia were surface sterilized for 2 min in 75% (v/v) ethanol, transferred to wet filter paper in Petri plates and incubated at 28°C for 2-3 days. Outgrowing mycelium was transferred to water agar medium and incubated for 2 days. From each sample, one or two hyphal tips were transferred to potato dextrose agar (PDA) plates to purify the fungal isolates. *S. rolfsii* isolates were kept on PDA slants in duplicate; one sample was covered with mineral oil for long term storage at 20°C. From a total of more than 400 collected samples, 198 *S. rolfsii* isolates were successfully purified, preserved and subjected to ITS-rDNA sequencing (details below).

ITS-rDNA amplification, sequencing and phylogeny

Total DNA of *S. rolfsii* isolates was extracted based on the method of Tendulkar *et al.* (2003). Each *S. rolfsii* isolate was cultured on PDA for 48 h at 25°C, and then approximately 5 mg (fresh weight) of mycelium was transferred to an 1.5-mL tube. Fifty µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA buffer, pH 8) was added and the sample was kept at room temperature (~20ºC) for 10 min, microwaved for 30 s at 650 W and centrifuged at a speed of 10,000 rpm for 10 min. The supernatant containing genomic DNA was used directly for ITS-rDNA amplification. Four µl of genomic DNA template was used in a total reaction mixture volume of 50 µl. The mix contained 2.0 µl of each of the four dNTPs (5 mM stock each); 5.0 µl 10x PCR-buffer; 0.2 µl SuperTaq (5U/µl; SphaeroQ, Netherlands); 2µl of primers ITS1 and ITS4 (10 mM stock each) (White *et al.* 1990); and sterile MQ water. The PCR reaction involved 1 cycle at 94ºC for 5 min, and 35 repetitive cycles with 1 min at 94ºC, 1 min at 55ºC, and 2 min at 72ºC. Two µl of the PCR product was loaded on a 1.5% (w/v) agarose gel to assess the expected size (~700 bp) and quantity of the PCR product. ITS-rDNA fragments were purified and sent for sequencing (Macrogen, Europe). The obtained forward and reverse sequences were assembled and edited in Vector NTI and deposited in GenBank with accession numbers HQ895865 to HQ895967. For the phylogenetic analyses, the edited sequences were trimmed (~550 bp) and aligned to reference sequences available in databases (Table 1); the phylogenetic tree was obtained with MEGA4 software (http://megasoftware.net) using the ITS-rDNA sequence of *Sclerotium coffeicola* as an outgroup.

Pathogenicity assays

Pathogenicity of the *S. rolfsii* isolates was assessed on *Arachis hypogaea* L. cultivar L14, the predominant groundnut cultivar grown in Vietnam. A total of 18 *S. rolfsii* isolates obtained from groundnut, tomato and taro were tested for pathogenicity. A single groundnut seed was sown in a square plastic pot (6 cm width x 8 cm height) containing 250 g of natural field soil obtained from Hue (Vietnam). Pots were placed in a nethouse and watered on

a need-to-need basis. One week after sowing, a mycelial plug (1-cm-diameter) of a 3-day-old *S. rolfsii* PDA plate culture was placed at the stembase. Disease incidence and severity were determined 2 weeks after fungal inoculation. Disease incidence refers to the percentage of infected plants and disease severity was rated on a scale from 0 – 4 (Fig. 6), with 0: no disease symptoms, 1: infection without visible fungal outgrowth, 2: infection with visible fungal outgrowth, 3: partial wilting of the plant, and 4: complete wilting and plant death. For each *S. rolfsii* isolate, three trays of five pots each were used.

Figure 6. Disease scales of groundnut infected by *Sclerotium rolfsii*; 0: no disease symptoms, 1: infection without visible outgrowth of the fungus, 2: infection with visible outgrowth, 3: partial wilting of the plant, and 4: complete wilting and plant death.

Phenotypic characterization

S. rolfsii isolates were cultured on PDA plates at 25°C. Per isolate, two 25-mL PDA agar plate (9-cm-diameter) were inoculated in the centre with a mycelial plug (5-mm-diameter) and radial mycelial growth of the fungal colony was determined 48h after incubation. For each plate, the number of sclerotia was determined 21 days after incubation and the sclerotial diameter was determined for 30 randomly selected sclerotia.

A total of 26 *S. rolfsii* isolates were tested for mycelial compatibility. Multiple combinations of two isolates of *S. rolfsii* were inoculated on PDA plates and incubated at 25°C for 2 weeks. Mycelial compatibility was assessed by the method of Punja and Grogan (1983). If there was a distinct barrage zone at the contact area between two isolates, these isolates were considered to belong to a different mycelial compatibility group (MCG).

The ergosterol biosynthesis inhibitor tebuconazole is a fungicide widely used to control *S. rolfsii*. At a concentration of 0.02 mg liter^{-1} of PDA, tebuconazole reduced hyphal growth rate of *S. rolfsii* by approximately 28% (Franke *et al.* 1998). In our study, *S. rolfsii* isolates were cultured in duplicate on PDA amended with tebuconazole (Sigma-Aldrich) to final concentrations of 0, 0.02 and 0.2 mg liter^{-1}. Hyphal growth was assessed 48 h after incubation at 25°C. Sensitivity of *S. rolfsii* to tebuconazole was calculated with the formula used by Franke *et al.* (1998): Inhibition (%) = 100 – [(colony diameter on amended medium ÷ colony diameter on control medium) × 100].

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Appendix 1. Genetic and phenotypic traits of 103 randomly selected *Sclerotium rolfsii* isolates from groundnut fields in central Vietnam. Isolates used in the pathogenicity assays are indicated with an asterisk; isolates from the remote upland area of Nghe An province are underlined.

Order	Code	Origin			ITS group	Mycelial growth rate during 48 h (mm/h)	Time to produce sclerotia (days)	Time for maturation of the sclerotia (days)	Diameter of sclerotia (mm)				Number of sclerotia per plate	Sensitivity to tebuconazole at 0.02mg/liter^{-1} (%inhibition)
		Province	Soil type	Host plant					Average	min	max	SD		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(15)	(15)
1	HTGS12T1	Ha Tinh	sandy	groundnut	1	0.43	5	8	1.06	0.71	1.32	0.16	1080	19.80
2	HTGS13-2T1	Ha Tinh	sandy	groundnut	1	0.36	5	8	0.89	0.58	1.35	0.19	618	26.26
3	HTGS17T1	Ha Tinh	sandy	groundnut	1	0.32	6	9	1.29	1.02	1.57	0.16	278	22.77
4	HTGS18T1	Ha Tinh	sandy	groundnut	1	0.42	4	7	0.99	0.72	1.26	0.13	616	37.58
5	HTGS20T1	Ha Tinh	sandy	groundnut	1	0.37	5	8	1.14	0.80	1.65	0.20	508	28.19
6	HTGS23T1	Ha Tinh	sandy	groundnut	1	0.36	4	8	0.93	0.67	1.17	0.12	658	47.70
7	HTGS24T1	Ha Tinh	sandy	groundnut	1	0.39	5	8	1.22	0.78	1.43	0.16	326	38.04
8	HTGS6T1	Ha Tinh	sandy	groundnut	1	0.34	5	8	1.00	0.68	1.37	0.14	724	50.07
9	HTGS9T1	Ha Tinh	sandy	groundnut	3	0.35	5	8	1.02	0.72	1.37	0.19	584	45.40
10	HTGC10-2T1	Ha Tinh	clay loam	groundnut	1	0.41	4	7	1.20	0.86	1.53	0.18	568	36.29
11	HTGC10-1T1	Ha Tinh	clay loam	groundnut	1	0.36	5	9	1.07	0.77	1.40	0.19	516	24.25
12	HTGC13-1T1	Ha Tinh	clay loam	groundnut	1	0.41	5	8	1.10	0.85	1.47	0.16	452	42.31
13	HTGC13T1	Ha Tinh	clay loam	groundnut	1	0.33	5	8	1.09	0.67	1.43	0.19	472	28.09
14	HTGC3T1	Ha Tinh	clay loam	groundnut	1	0.40	4	7	1.06	0.81	1.29	0.12	704	38.34
15	HTGC5T1	Ha Tinh	clay loam	groundnut	1	0.38	5	8	1.29	0.90	1.57	0.17	346	19.85
16	HTGC9T1	Ha Tinh	clay loam	groundnut	1	0.45	4	7	1.24	0.90	1.72	0.18	356	53.90
17	HTGC9T2	Ha Tinh	clay loam	groundnut	1	0.44	4	7	1.21	0.99	1.55	0.12	422	42.70
18	NAGS2-1T1	Nghe An	sandy	groundnut	1	0.42	5	8	1.09	0.64	1.50	0.25	576	30.06
19	NAGS1T1 *	Nghe An	sandy	groundnut	1	0.40	4	8	1.04	0.84	1.29	0.13	472	31.97
20	NAGS5T1	Nghe An	sandy	groundnut	1	0.42	4	7	1.09	0.84	1.77	0.18	688	41.64
21	NAGC10T1	Nghe An	clay loam	groundnut	1	0.42	5	8	0.98	0.67	1.35	0.19	574	51.92
22	NAGC12T1	Nghe An	clay loam	groundnut	1	0.35	6	10	1.47	1.00	2.05	0.27	184	25.61
23	NAGC15T1	Nghe An	clay loam	groundnut	1	0.41	5	8	1.14	0.75	1.69	0.22	464	35.90
24	NAGC18T1	Nghe An	clay loam	groundnut	1	0.35	6	10	1.23	0.99	1.61	0.15	282	25.13
25	NAGC1T1	Nghe An	clay loam	groundnut	1	0.39	5	8	1.12	0.79	1.40	0.16	538	34.52
26	NAGC21T1	Nghe An	clay loam	groundnut	1	0.38	4	7	1.09	0.87	1.44	0.14	564	39.42
27	NAGC22T1	Nghe An	clay loam	groundnut	1	0.35	5	8	1.08	0.87	1.40	0.12	542	43.80
28	NAGC2-2T1	Nghe An	clay loam	groundnut	1	0.35	5	7	1.02	0.76	1.45	0.13	605	29.17
29	NAGC27T1	Nghe An	clay loam	groundnut	1	0.41	5	8	1.00	0.75	1.20	0.13	880	38.23
30	NAGC9T1	Nghe An	clay loam	groundnut	1	0.37	6	9	1.23	0.75	1.59	0.20	337	27.75

"Appendix 1 continued"

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(15)	(15)
67	HUGS12T1	Thua Thien Hue	sandy	groundnut	1	0.46	7	11	1.07	0.86	1.41	0.15	520	38.59
68	HUGS11T2	Thua Thien Hue	sandy	groundnut	1	0.42	5	8	1.04	0.73	1.36	0.14	582	27.29
69	HUGS13T1	Thua Thien Hue	sandy	groundnut	1	0.37	5	8	1.09	0.83	1.28	0.11	638	45.72
70	HUGS14T1	Thua Thien Hue	sandy	groundnut	1	0.38	4	8	1.16	0.82	1.36	0.12	616	39.74
71	HUGS16-1T1	Thua Thien Hue	sandy	groundnut	3	0.46	4	7	1.05	0.66	1.44	0.18	652	49.36
72	HUGS1T1	Thua Thien Hue	sandy	groundnut	1	0.41	4	8	1.33	0.87	1.60	0.17	368	19.63
73	HUGS6T1	Thua Thien Hue	sandy	groundnut	1	0.40	4	7	1.03	0.76	1.23	0.12	726	43.08
74	HUGS4T1	Thua Thien Hue	sandy	groundnut	1	0.42	4	7	1.09	0.59	1.33	0.19	488	40.58
75	HUGS5T1	Thua Thien Hue	sandy	groundnut	1	0.39	5	8	1.05	0.64	1.44	0.20	578	37.77
76	HUGS16-2T1	Thua Thien Hue	sandy	groundnut	1	0.45	4	7	1.14	0.80	1.36	0.13	476	27.17
77	HUGS7T1	Thua Thien Hue	sandy	groundnut	1	0.40	4	8	1.08	0.71	1.41	0.17	930	27.78
78	HUGS18T1	Thua Thien Hue	sandy	groundnut	1	0.41	4	7	0.90	0.70	1.25	0.15	932	36.81
79	HUGS18T2	Thua Thien Hue	sandy	groundnut	1	0.42	4	7	1.03	0.83	1.35	0.14	954	55.54
80	RH001	Thua Thien Hue	sandy	groundnut	1	0.42	4	7	1.14	0.89	1.57	0.18	508	38.96
81	H001	Thua Thien Hue	sandy	groundnut	1	0.45	5	8	1.23	0.76	1.55	0.17	338	23.42
82	HUMC22T1 *	Thua Thien Hue	sandy	taro	1	0.40	4	7	1.16	0.85	1.70	0.18	495	22.27
83	HUTS1T2 *	Thua Thien Hue	sandy	tomato	1	0.41	4	7	1.05	0.68	1.41	0.12	562	49.78
84	HUGC10-1T1	Thua Thien Hue	clay loam	groundnut	1	0.42	5	8	1.17	0.74	1.46	0.20	314	38.90
85	HUGC10-2T1	Thua Thien Hue	clay loam	groundnut	1	0.33	5	8	1.09	0.79	1.32	0.12	516	35.37
86	HUGC15T1 *	Thua Thien Hue	clay loam	groundnut	1	0.43	4	7	0.98	0.67	1.28	0.15	752	29.98
87	HUGC16T2	Thua Thien Hue	clay loam	groundnut	1	0.48	4	7	1.02	0.69	1.22	0.11	734	22.73
88	HUGC17T1	Thua Thien Hue	clay loam	groundnut	1	0.44	4	8	0.99	0.69	1.26	0.13	880	23.88
89	HUGC19T1 *	Thua Thien Hue	clay loam	groundnut	1	0.44	4	7	1.02	0.85	1.20	0.10	876	31.16
90	HUGC21T1	Thua Thien Hue	clay loam	groundnut	1	0.37	5	8	1.06	0.72	1.40	0.19	830	25.59
91	HUGC24T1	Thua Thien Hue	clay loam	groundnut	1	0.35	5	8	1.16	0.86	1.48	0.15	342	34.11
92	HUGC26T1	Thua Thien Hue	clay loam	groundnut	1	0.36	5	8	1.06	0.76	1.38	0.16	632	43.57
93	HUGC27T1	Thua Thien Hue	clay loam	groundnut	1	0.40	5	8	1.15	0.77	1.37	0.15	516	18.22
94	HUGC27T2	Thua Thien Hue	clay loam	groundnut	1	0.45	5	8	1.20	0.82	1.46	0.17	576	39.80
95	HUGC29T1	Thua Thien Hue	clay loam	groundnut	1	0.43	4	7	0.88	0.74	1.17	0.10	1042	38.41
96	HUGC30T1	Thua Thien Hue	clay loam	groundnut	1	0.42	5	8	0.95	0.70	1.15	0.10	926	37.67
97	HUGC4T1	Thua Thien Hue	clay loam	groundnut	1	0.47	4	7	0.96	0.80	1.20	0.11	1020	31.73
98	HUGC7T1	Thua Thien Hue	clay loam	groundnut	1	0.33	5	8	0.97	0.63	1.31	0.20	868	30.59
99	HUGC7T2	Thua Thien Hue	clay loam	groundnut	1	0.41	5	8	0.93	0.66	1.39	0.15	724	29.21
100	HUGC7T3	Thua Thien Hue	clay loam	groundnut	1	0.43	5	8	1.01	0.65	1.21	0.15	702	43.07
101	HUGC9T1	Thua Thien Hue	clay loam	groundnut	1	0.41	5	8	1.12	0.78	1.53	0.15	642	41.21
102	HUMC20T1 *	Thua Thien Hue	clay loam	taro	1	0.42	5	8	1.02	0.62	1.46	0.20	635	37.35
103	HUTC1T1 *	Thua Thien Hue	clay loam	tomato	1	0.45	4	7	1.23	0.88	1.60	0.19	492	46.75

"Appendix 1 continued"

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(15)	(15)
67	HUGS12T1	Thua Thien Hue	sandy	groundnut	1	0.46	7	11	1.07	0.86	1.41	0.15	520	38.59
68	HUGS11T2	Thua Thien Hue	sandy	groundnut	1	0.42	5	8	1.04	0.73	1.36	0.14	582	27.29
69	HUGS13T1	Thua Thien Hue	sandy	groundnut	1	0.37	5	8	1.09	0.83	1.28	0.11	638	45.72
70	HUGS14T1	Thua Thien Hue	sandy	groundnut	1	0.38	4	8	1.16	0.82	1.36	0.12	616	39.74
71	HUGS16-1T1	Thua Thien Hue	sandy	groundnut	3	0.46	4	7	1.05	0.66	1.44	0.18	652	49.36
72	HUGS1T1	Thua Thien Hue	sandy	groundnut	1	0.41	4	8	1.33	0.87	1.60	0.17	368	19.63
73	HUGS6T1	Thua Thien Hue	sandy	groundnut	1	0.40	4	7	1.03	0.76	1.23	0.12	726	43.08
74	HUGS4T1	Thua Thien Hue	sandy	groundnut	1	0.42	4	7	1.09	0.59	1.33	0.19	488	40.58
75	HUGS5T1	Thua Thien Hue	sandy	groundnut	1	0.39	5	8	1.05	0.64	1.44	0.20	578	37.77
76	HUGS16-2T1	Thua Thien Hue	sandy	groundnut	1	0.45	4	7	1.14	0.80	1.36	0.13	476	27.17
77	HUGS7T1	Thua Thien Hue	sandy	groundnut	1	0.40	4	8	1.08	0.71	1.41	0.17	930	27.78
78	HUGS18T1	Thua Thien Hue	sandy	groundnut	1	0.41	4	7	0.90	0.70	1.25	0.15	932	36.81
79	HUGS18T2	Thua Thien Hue	sandy	groundnut	1	0.42	4	7	1.03	0.83	1.35	0.14	954	55.54
80	RH001	Thua Thien Hue	sandy	groundnut	1	0.42	4	7	1.14	0.89	1.57	0.18	508	38.96
81	H001	Thua Thien Hue	sandy	groundnut	1	0.45	5	8	1.23	0.76	1.55	0.17	338	23.42
82	HUMC22T1 *	Thua Thien Hue	sandy	taro	1	0.40	4	7	1.16	0.85	1.70	0.18	495	22.27
83	HUTS1T2 *	Thua Thien Hue	sandy	tomato	1	0.41	4	7	1.05	0.68	1.41	0.12	562	49.78
84	HUGC10-1T1	Thua Thien Hue	clay loam	groundnut	1	0.42	5	8	1.17	0.74	1.46	0.20	314	38.90
85	HUGC10-2T1	Thua Thien Hue	clay loam	groundnut	1	0.33	5	8	1.09	0.79	1.32	0.12	516	35.37
86	HUGC15T1 *	Thua Thien Hue	clay loam	groundnut	1	0.43	4	7	0.98	0.67	1.28	0.15	752	29.98
87	HUGC16T2	Thua Thien Hue	clay loam	groundnut	1	0.48	4	7	1.02	0.69	1.22	0.11	734	22.73
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94	HUGC27T2	Thua Thien Hue	clay loam	groundnut	1	0.45	5	8	1.20	0.82	1.46	0.17	576	39.80
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103	HUTC1T1 *	Thua Thien Hue	clay loam	tomato	1	0.45	4	7	1.23	0.88	1.60	0.19	492	46.75

Chapter 3

Involvement of phenazine antibiotics and lipopeptide surfactants in suppression of stem rot disease of groundnut by *Pseudomonas* species

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Involvement of phenazine antibiotics and lipopeptide surfactants in suppression of stem rot disease of groundnut by *Pseudomonas* species

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Abstract

Aims: To determine the efficacy and role of phenazine antibiotics and lipopeptide surfactants produced by different *Pseudomonas* strains in biological control of stem rot disease of groundnut, caused by the fungal pathogen *Sclerotium rolfsii*.

Methods and Results: *Pseudomonas* strains that produce phenazines (PHZ) or lipopeptides (LPs) were tested for antagonism toward *S. rolfsii*. *In vitro* dual culture assays showed that PHZ-producing *Pseudomonas chlororaphis* strain Phz24 did not affect germination of sclerotia but significantly inhibited hyphal growth of *S. rolfsii*. Biosynthetic and regulatory mutants of strain Phz24 deficient in PHZ biosynthesis did not inhibit hyphal growth of *S. rolfsii*. *Pseudomonas fluorescens* strains SS101 and SBW25, producing viscosin-type LPs, and *Pseudomonas putida* strain 267, producing putisolvin-type LPs, only marginally inhibited hyphal growth and did not adversely affect sclerotial germination. In contrast, *Pseudomonas* sp. strain SH-C52, producing the chlorinated LP thanamycin, significantly inhibited hyphal growth of *S. rolfsii* whereas its LP-biosynthesis mutant was not effective. Growth chamber bioassays showed that LP-producing strains SS101, SBW25 and 267 did not suppress stem rot disease of groundnut. PHZ-producing strain Phz24 and LP-producing strain SH-C52 significantly reduced stem rot disease of groundnut, whereas their respective PHZ- or LP-biosynthetic mutants were not effective in disease control. Also in nethouse and field experiments conducted in central Vietnam, strains Phz24 and SH-C52 significantly suppressed stem rot disease of groundnut. LP-producing strain SS101 did not suppress disease but significantly increased groundnut yield.

Conclusion: Phenazine (PHZ) antibiotics and lipopeptides (LPs) play an important role in suppression of stem rot disease of groundnut by specific root-colonizing *Pseudomonas* strains. The results further suggest variation in the

activity of structurally different LPs against the stem rot pathogen *S. rolfsii*, with thanamycin having significant inhibitory effects on hyphal growth and plant infection.

Significance and Impact of Study: PHZ-producing *P. chlororaphis* strain Phz24 and LP-producing *Pseudomonas* strain SH-C52 showed significant control of stem rot disease of groundnut in growth chamber, nethouse and field experiments in Vietnam. Treatment of seeds and/or field soil with these bacterial strains provides a promising supplementary or alternative strategy to control stem rot disease of groundnut.

Keywords: Biological control, *Sclerotium rolfsii, Pseudomonas*, antibiotics.

Introduction

Groundnut (*Arachis hypogaea* L.) is one of the most important oil seed crops worldwide (Florkowski 1994), but its production is hampered by diverse pests and diseases (Brown 2007; Shew and Waliyar 2007). *Sclerotium rolfsii* is among the most important soil-borne fungal pathogens of groundnut and is widely distributed in groundnut-producing countries, including the USA and Vietnam (Branch and Brenneman 2009; Le *et al.* 2011). On groundnut, *S. rolfsii* causes stem, peg and pod rot with yield reductions of up to 80% (Mehan *et al.* 1994). For example, estimated yield losses in Georgia (USA) amounted to approximately 38 mUSD in the period from 2004 to 2007 (Kemerait 2005; 2006; 2007; 2008). Field surveys conducted in central Vietnam in 2009 revealed that 5-25% of the groundnut plants were infected by *S. rolfsii* (Le *et al.* 2011).

Sclerotia are the primary inoculum source for infection and extremely difficult to eradicate (Coleysmi and Cooke 1971; Punja 1985). Current measures to control *S. rolfsii* include crop rotation and, to some extent, the use of disease-tolerant cultivars. In many cases, fungicides such as tebuconazole, pentachloronitrobenzene (PCNB) and flutolanil are used to control *S. rolfsii*. However, none of these control methods alone is effective enough. For example, tolerance to tebuconazole and PCNB has been reported for *S. rolfsii* populations in Georgia and Texas, USA (Wadsworth and Melouk 1984; Franke *et al.* 1998; Shim *et al.* 1998). In Vietnam, these fungicides are not used on a large scale due to their relatively high costs for subsistence farmers. Therefore, integration of several different control measures is proposed to provide sustainable management of *S. rolfsii* and other soil-borne diseases of groundnut. In this context, biological control can be an alternative or supplement to current management practices for *S. rolfsii* (Dey *et al*. 2004; Tonelli *et al.* 2010).

Biocontrol of *S. rolfsii* has not been investigated extensively, but some studies have shown promising results. For example, Ganesan *et al.* (2007) showed that *Rhizobium* in combination with *Trichoderma* improved plant growth and reduced the damage caused by *S.*

rolfsii. Among the beneficial rhizosphere bacteria, *Bacillus* and *Pseudomonas* species are widely studied for their abilities to control soil-borne pathogens. Some *Pseudomonas* species and strains have been tested for their ability to control stem rot disease of groundnut caused by *S. rolfsii* (Ganesan and Gnanamanickam 1987; Kishore *et al.* 2005; Tonelli *et al.* 2011), but the mechanisms and bioactive compounds underlying disease suppression were not identified in these studies. Pseudomonads are well-known for the production of a diverse array of antifungal compounds, including 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin, rhizoxins, PHZ and LPs (Raaijmakers *et al*. 2002; Haas and Defago 2005; Raaijmakers *et al.* 2009; Gross and Loper, 2009; D'aes *et al.* 2010; Raaijmakers *et al.* 2010). Several of these bioactive compounds, in particular the LPs, exhibit substantial structural diversity which may determine the spectrum of their antimicrobial activity (Mavrodi *et al.* 2006; Raaijmakers *et al*. 2010).

Here, we tested the activity of different *Pseudomonas* species and strains against *S. rolfsii* and determined the role of PHZ antibiotics and four structurally different LPs, i.e. massetolide A, viscosin, putisolvin and thanamycin, in disease control. Both *in vitro* and *in vivo* bioassays were conducted to determine the efficacy of these Pseudomonads to: 1) suppress hyphal growth and germination of sclerotia of *S. rolfsii*, 2) colonize the roots and stem base of groundnut plants in the field, and 3) reduce plant infection by *S. rolfsii* under growth chamber, nethouse and field conditions. To determine the role of PHZ and each of the four LPs in the activity of these Pseudomonads against *S. rolfsii*, biosynthetic and regulatory mutants deficient in the production of these bioactive compounds were generated and their activities compared with their parental strains.

Results

In vitro antagonism of phenazine-producing Pseudomonas toward S. rolfsii

PHZ-producing *Ps. chlororaphis* strain Phz24 did not affect sclerotial germination but significantly inhibited hyphal growth of *S. rolfsii* (Table 2). To determine the role of PHZ production in hyphal growth inhibition, random plasposon mutagenesis of strain Phz24 was performed to obtain PHZ-mutants. Out of an initial screening of approximately 2500 random mutants, two mutants, designated M1 and M15, had lost the orange appearance of wild type strain Phz24 (Fig. 1A), a phenotype that is typically associated with the production of 2-hydroxy-phenazine-1-carboxylic acid (Mavrodi *et al.* 2006). Subsequent Y-linker based cloning and sequencing of the regions flanking the plasposon insertion revealed that the plasposon in mutant M1 was integrated in a gene with 98% identity to *phz*C from *Ps. chlororaphis* strain PGS12 (Table 3). The *phz*C gene encodes 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, an enzyme that catalyzes the first step of the shikimate pathway to re-direct intermediates from the primary metabolism toward phenazine biosynthesis (Mavrodi *et al.* 2006). In mutant M15, the plasposon was integrated in a gene with 95% identity to *phz*I, the

autoinducer synthase gene involved in quorum-sensing-based regulation of PHZ biosynthesis (Mavrodi *et al.* 2006). The loss of PHZ production in mutants M1 and M15 was confirmed by RP-HPLC: in cell-free culture extracts of mutants M1 and M15, peaks 1-3 were missing that were present in cell-free extract of wildtype strain Phz24 (Fig. 1B). Peak 3 has the same retention time (RT) (25.3 min) as pure phenazine-1-carboxylic acid (PCA) under the described RP-HPLC conditions and exhibits very similar spectral characteristics as pure PCA (Fig. 1C). Peak 1 has a different RT than PCA and exhibits similar spectral characteristics as 2-hydroxyphenazine (Delaney *et al.* 2001; D. Mavrodi, pers. comm.). Peak 2 with a RT of approximately 22 min was also lacking in mutants M1 and M15 and exhibits spectral characteristics (Fig. 1C) that are somewhat similar to that of 2-OH-PCA (D. Mavrodi, pers. comm.). Subsequent fraction followed by LC/MS-MS will be required to confirm the identity of peak 2.

Table 1. *Pseudomonas* strains and mutants used in this study.

Pseudomonas species, strains and mutants	Origin	Bioactive compound	Target pathogens	Source/ Reference
Ps. chlororaphis strain Phz24	tomato rhizosphere	Phenazines	*Botrytis cinerea, Fusarium oxysporum*	Schoonbeek *et al*. (2002); Mazurier *et al*. (2009)
mutant M1		Phenazine deficient		This study
mutant M15		Phenazine deficient		This study
Pseudomonas sp. strain SH-C52	sugar beet rhizosphere	Thanamycin	*Rhizoctonia solani*	Mendes *et al.* (2011)
mutant O33		Thanamycin deficient		Mendes *et al.* (2011)
Ps. fluorescens strain SS101	wheat rhizosphere	Massetolide A	*Pythium* spp. *Phytophthora infestans* *Phytophthora capsici*	de Souza *et al.* (2003) Tran *et al.* (2007) de Bruijn *et al.* (2008)
mutant MassA		Massetolide A deficient		de Bruijn *et al.* (2008)
Ps. fluorescens strain SBW25	sugar beet phyllosphere	Viscosin	*Phytophthora infestans*	de Bruijn *et al.* (2007)
mutant ViscA		Viscosin deficient		de Bruijn *et al.* (2007)
Ps. putida strain 267	black pepper	Putisolvin	*Phytophthora capsici*	Tran *et al.* (2008)
mutant EP1		Putisolvin deficient		Kruijt *et al.* (2009)

Table 2. Effect of phenazine and lipopeptide-producing *Pseudomonas* strains and their mutants on hyphal growth and germination of sclerotia of *Sclerotium rolfsii*.

Pseudomonas strains/mutants	Hyphal growth inhibition * relative to the control (%)	Germination of sclerotia ** (%)
Control	-	98 a
Ps. chlororaphis Phz24	48 a	100 a
mutant M1	4 cde	100 a
mutant M15	9 b	100 a
Pseudomonas sp. SH-C52	45 a	98 a
mutant O33	2 de	100 a
Ps. fluorescens SS101	6 bc	100 a
mutant *massA*	0 e	100 a
Ps. fluorescens SBW25	1 e	100 a
mutant *viscA*	5 cd	90 a
Ps. putida 267	0 e	100 a
mutant EP1	2 e	100 a

* average of two replicates; ** average of three replicates. The percentages of hyphal growth inhibition and germination of sclerotia was arcsin-transformed prior to statistical analysis. Values within each column followed by the same letter(s) are not significantly different at $P<0.05$ according to Duncan Multiple Range Test.

Table 3. Results of BLASTN analysis of the genes disrupted in two phenazine-deficient mutants of *Pseudomonas chlororaphis* strain Phz24.

Mutant	Number of nucleotides	BLASTN hit	GenBank accession	E-value	Identity (%)
M1	719	*phz*C gene for phenazine biosynthesis (*Ps. chlororaphis* strain PGS12)	FM863699	5e-180	98
M15	509	Autoinducer synthase *phz*I, (*Ps. chlororaphis* strain PCL1391)	AF195615	0.0	95

Thin layer chromatography further revealed that strain Phz24 also produces pyrrolnitrin. This antifungal compound (van Pee and Ligon 2000; Raaijmakers *et al*. 2002; Dwivedi and Johri 2003; Park *et al.* 2011) was detected in higher amounts in culture filtrates of mutants M1 and M15 as compared to wildtype strain Phz24 (Fig. 1D). Hence these mutants enabled us to specifically determine the role of PHZ in growth inhibition of *S. rolfsii.* The dual culture assays showed that both mutants M1 and M15 were not able to inhibit hyphal growth of *S. rolfsii* (Table 2). Collectively, these results indicate that PHZ are the key metabolites involved in *in vitro* antagonism of strain Phz24 toward *S. rolfsii*.

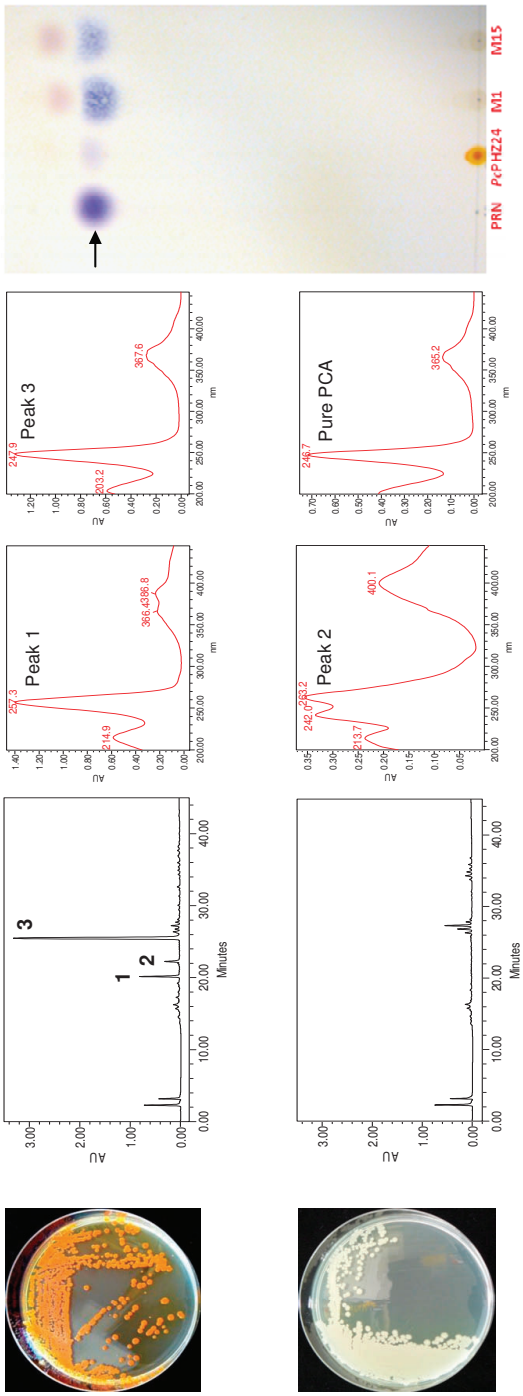

Figure 1. Phenotypic and biochemical analysis of *Pseudomonas chlororaphis* strain Phz24 and mutants M15 and M1. (A) typical phenotype of bacterial colonies on PSA agar plates for wildtype strain Phz24 (top) and for mutant M15 (bottom); the phenotype of mutant M1 is identical to that of M15; (B) RP-HPLC chromatograms (247nm) of cell-free culture extracts of wildtype strain Phz24 (top) and of mutant M15 (bottom); the chromatogram for mutant M1 is identical to that of M15; (C): Spectral characteristics of peaks 1, 2, and 3 shown in panel B for wild type strain Phz24 at retention times (RT) of 20.2, 22.2, and 25.4 min, respectively. As reference, spectral characteristics of pure phenazine-1-carboxylic acid (PCA; RT= 25.3 min) are shown. (D) Thin layer chromatography (TLC) analysis of cell-free culture extracts of wildtype strain Phz24 (*Pc*Phz24) and mutants M1 and M15; pure pyrrolnitrin (PRN; Rf= 0.65) is indicated by an arrow.

In vitro antagonism of lipopeptide-producing Pseudomonas toward S. rolfsii

Four LP-producing *Pseudomonas* strains and their mutants (Table 1) were tested for their ability to inhibit germination of sclerotia and hyphal growth of *S. rolfsii*. None of the bacterial strains and mutants tested inhibited germination of sclerotia of *S. rolfsii* (Table 2). Dual culture assays showed that hyphal growth of *S. rolfsii* was not significantly inhibited by *Pseudomonas* strains 267, SS101 and SBW25 producing putisolvin or viscosin-type LPs. In contrast, *Pseudomonas* sp. strain SH-C52 significantly inhibited hyphal growth of *S. rolfsii*, whereas mutant O33, deficient in the production of the chlorinated LP thanamycin, was not effective. Collectively, these results suggest that inhibition of hyphal growth of *S. rolfsii* is dependent on the type of LP produced by the introduced *Pseudomonas* strain.

Control of stem rot disease of groundnut under growth chamber conditions

Ps. chlororaphis strain Phz24 significantly reduced DI and MR of groundnut plants (Fig. 2). PHZ-mutants M1 and M15 colonized the roots and stem base of groundnut plants to the same extent as wild type strain Phz24, but did not provide significant disease control (Fig. 2). These results suggest that PHZ determines the ability of strain Phz24 to control stem rot disease. The LP-producing strains SS101, SBW25, 267 and their respective LP-biosynthesis mutants did not significantly reduce disease incidence or mortality (Fig. 2). Also strain SH-C52 did not reduce disease incidence but significantly lowered mortality of groundnut plants (Fig. 2). Thanamycin-deficient mutant O33 established a density on the roots of groundnut similar to that of wild type strain SH-C52 (Fig. 2), but was not able to significantly reduce mortality (Fig. 2). Strains SS101, SBW25 and 267 colonized the roots of groundnut significantly less than strains Phz24 and SH-C52: the rhizosphere population densities of strains SBW25, SS101 and 267 were more than 10 to 100-fold (1 to 2 log units) lower than those of Phz24 and SH-C52 (Fig. 2), suggesting a correlation between biocontrol activity and root colonization. However, with respect to the population density of strains Phz24 an SH-C52 on the stem base of groundnut, the correlation with the level of disease control was not evident. For example, strains SS101 and SBW25, which lack biocontrol activity, established similar to higher densities on the stem base than Phz24 and SH-C52, the two strains that did suppress disease (Fig. 2).

Control of stem rot disease under nethouse and field conditions

Under nethouse conditions, only strain SH-C52 significantly reduced disease incidence, but did not reduce disease severity and mortality significantly (Fig. 3). After two weeks of plant growth, strains SS101 and Phz24 established similar densities on roots of groundnut, but neither of the two strains provided disease control under nethouse conditions (Fig. 3). None of the three introduced bacterial strains were detectable on the stem base of groundnut plants after 2 weeks days of plant growth.

In the field experiment in Quang Nam province, disease incidence in the control treatment was relatively low with approximately 10% of the plants naturally infected with *S. rolfsii* at the pod set stage (Fig. 4). Disease incidence increased during the growth season more rapidly in the untreated control plots as compared to field plots with the bacterial strains. At pod set stage, both strains Phz24 and SH-C52 significantly suppressed stem rot disease, whereas strain SS101 did not significantly reduce DI relative to the control treatment. None of the three bacterial strains adversely affected nodulation (Table 4) nor did they adversely affect or increase plant height or the length of the first branch (data not shown). Strains SH-C52 and Phz24 did not increase pod yield, whereas strain SS101 significantly increased yield by on average 12% compared to the control treatment (Table 4).

Table 4. Effects of different *Pseudomonas* strains on nodulation and yield of groundnut plants grown under field conditions in Quang Nam province, Vietnam. Population densities of the introduced *Pseudomonas* strains on the stem base and roots of groundnut plants were determined at two developmental stages (flowering, pod set) of the groundnut plants. Averages of three replicates are given. For each column, different letters indicate a statistically significant difference between the treatments ($P < 0.05$, Duncan Multiple Range Test).

Treatment	Population density introduced *Pseudomonas* strain (log cfu g^{-1})				Nodules per plant	Pod yield (kg ha^{-1})
	Flowering		Pod set			
	Stem base	Roots	Stem base	Roots		
Control					133 a	1220 a
Ps. chlororaphis Phz24	4.9 a	4.6 a	5.1 a	5.6 a	165 a	1260 ab
Pseudomonas sp. SH-C52	5.1 a	4.0 ab	5.9 a	6.0 a	135 a	1190 a
Ps. fluorescens SS101	5.2 a	3.5 b	5.7 a	5.5 a	134 a	1370 b

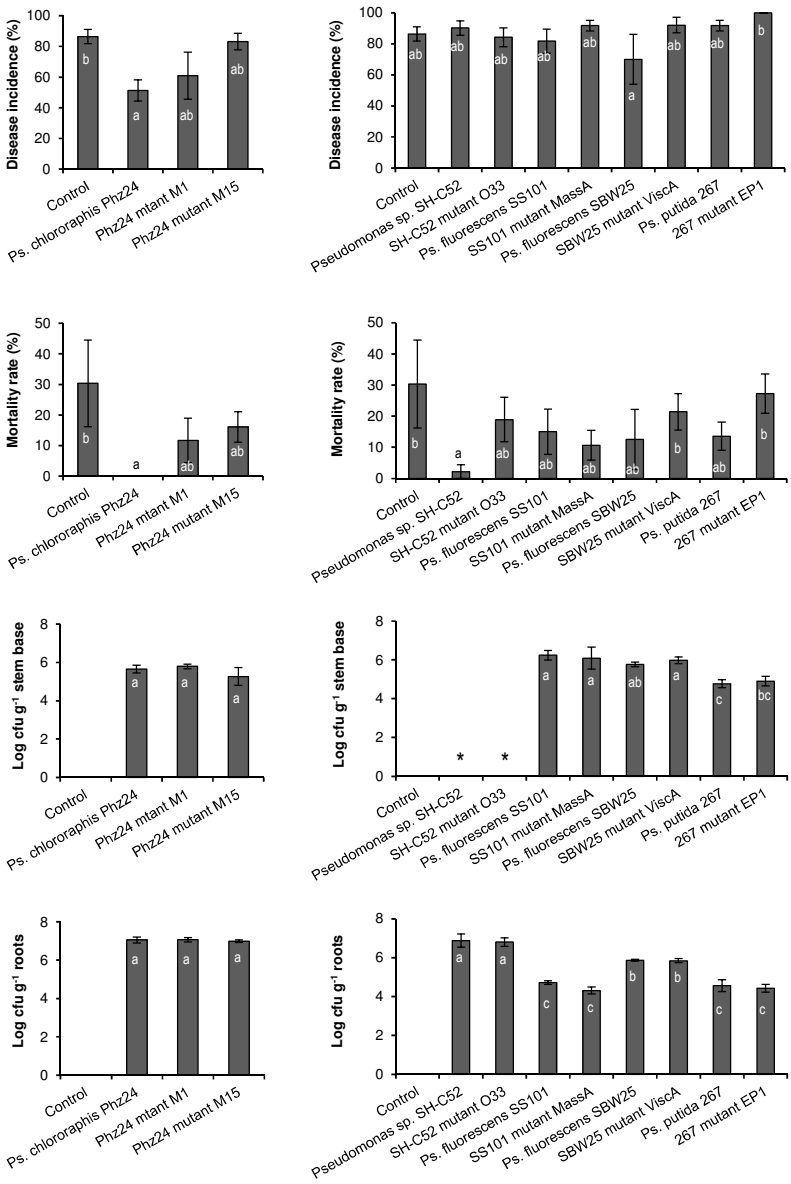

Figure 2. Biocontrol of stem rot disease and colonization of stem base and roots of groundut by different *Pseudomonas* strains under growth chamber conditions. The left panel shows the results obtained with the PHZ-producing *Ps. chlororaphis* Phz24 and its PHZ mutants M1 and M15. The right panel shows the results of the four LP-producing *Pseudomonas* strains and their mutants. Different letters indicate a statistically significant difference between the treatments (*P<0.05,* Duncan Multiple Range Test). For disease incidence (DI) and mortality rate (MR), averages of 5 replicates are given; for bacterial colonization, averages of 3 replicates are given; an asterisk (*) indicates bacterial colonization below the detection limit of 10^4 cfu g^{-1}; error bars represent the standard error of the mean.

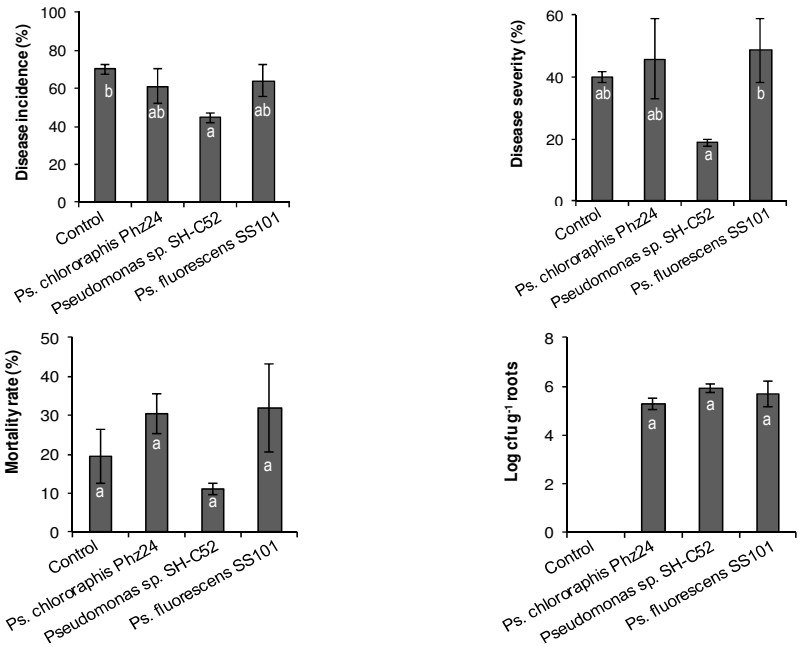

Figure 3. Biocontrol of stem rot disease of groundnut by *Pseudomonas chlororaphis* strain Phz24, *Pseudomonas* sp. strain SH-C52, and *Ps. fluorescens* strain SS101 under nethouse conditions in Vietnam. Averages of 3 replicates are given; error bars represent the standard error of the mean. Different letters indicate a statistically significant difference between the treatments (*P<0.05,* Duncan Multiple Range Test). The lower panel shows the population densities of the applied bacterial strains on roots of groundnut; for bacterial colonization, averages of 3 replicates are given. Error bars represent the standard error of the mean.

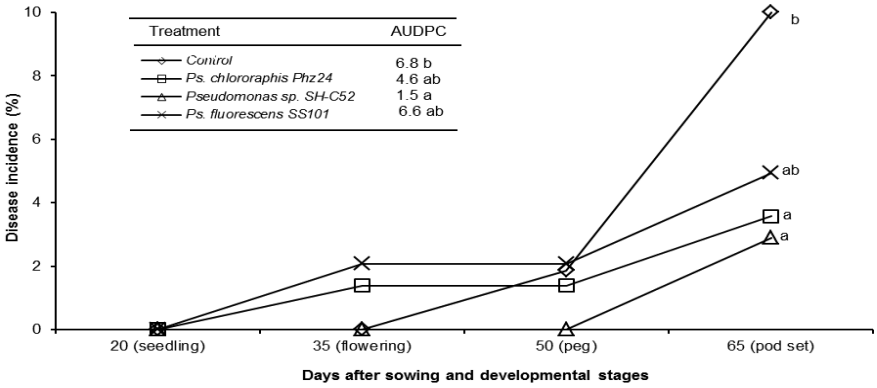

Figure 4. Biocontrol of stem rot of groundnut by *Pseudomonas chlororaphis* strain Phz24, *Pseudomonas* sp. strain SH-C52, and *Ps. fluorescens* strain SS101 under field conditions in Quang Nam province, Vietnam. Disease incidence was monitored at the seedling stage when plants had 3-5 true leaves (20 days after sowing), and subsequently every 15 days. AUDPC represents the Area Under the Disease Progress Curve and was calculated based on the method used by Landa *et al.* (2002) and Kruijt *et al.* (2009). For each developmental stage, averages of 3 replicates are given. Different letters indicate a statistically significant difference between the treatments (*P<0.05,* Duncan Multiple Range Test).

Discussion

Sclerotium rolfsii causes serious diseases on many crops including groundnut. Current methods to control this pathogen are limited or not effective. Hence, development and integration of different control strategies is necessary for sustainable control of this soil-borne pathogen. In a series of experiments conducted in the lab, growth chamber, nethouse and the field, we showed that specific *Pseudomonas* strains, producing either PHZ or LPs, suppressed hyphal growth of *S. rolfsii* and significantly reduced stem rot disease of groundnut. In other studies (Ganesan and Gnanamanickam 1987; Dey *et al.* 2004; Kishore *et al.* 2005; Tonelli *et al*. 2010; Tonelli *et al*. 2011), Pseudomonads were tested mostly under controlled conditions for their efficacy to suppress stem rot disease of groundnut and the mechanisms underlying disease control were not addressed in these studies. By comparing the activities of wildtype *Pseudomonas* strains with that of mutants disrupted in specific biosynthesis and regulatory genes, we demonstrated that PHZ and specific LPs are key bioactive metabolites in biological control of stem rot disease of groundnut.

In vitro and growth chamber bioassays with *Ps. chlororaphis* strain Phz24 showed that PHZ are key metabolites in suppression of hyphal growth of *S. rolfsii* and of stem rot disease. These results confirm and extend results of Rane *et al.* (2007) who showed that *S. rolfsii* could not grow on agar plates amended with phenazine-1-carboxylic acid (PCA) from *Pseudomonas aeruginosa*. Based on RP-HPLC analysis, we showed that strain Phz24 produces at least three PHZ derivatives, i.e. PCA, 2-OH-PHZ and possibly 2-OH-PCA, and that disruption of the *phz*C or *phz*I genes eliminates production of all three PHZ derivatives and concomitantly pathogen inhibition. It is not yet known which of these three PHZ contributes mostly to the activity of strain Phz24 against *S. rolfsii*, but work by Delaney *et al.* (2001) and Smirnov and Kiprianova (1990; reviewed in Delaney *et al*. (2001)) indicated that the hydroxylated PHZ exhibit stronger fungistatic activities. TLC analysis indicated that, next to the phenazines, strain Phz24 also produces pyrrolnitrin, a potent antifungal compound produced by multiple *Pseudomonas* strains and various other bacterial genera (van Pee and Ligon 2000). The observation that pyrrolnitrin production was enhanced in PHZ mutants M1 and M15 suggests that this compound does not play a role in growth inhibition and biocontrol of *S. rolfsii* by strain Phz24.

Sclerotia are the primary inoculum source for infection by *S. rolfsii* and various other soil-borne pathogens, but extremely difficult to eradicate (Coleysmi and Cooke 1971; Punja 1985). Ganesan and Gnanamanickam (1987) reported that some *Ps. fluorescens* strains can reduce germination of sclerotia of *S. rolfsii* by 50-60%. Also culture filtrates of Actinomycetes were shown to inhibit germination of sclerotia of *S. rolfsii* (Errakhi *et al.* 2009). Work by Debode *et al.* (2007) with microsclerotia of *Verticillium dahliae* showed suppressive effects of different *Pseudomonas* strains on germination. By using well-defined mutants, they further demonstrated that PHZ and LPs play an essential role in this suppressive activity. In this study, however, none of the tested phenazine and LP-producing *Pseudomonas* strains significantly reduced

germination of *S. rolfsii* sclerotia. Possible explanations for this discrepancy may be the shorter exposure time of the sclerotia to the bacteria, the potential poor survival of these bacterial strains on the sclerotia, or differences in the sensitivity of *S. rolfsii* to the bacteria and/or bioactive compounds.

In natural habitats, PHZ and LPs can confer a competitive advantage in survival and root colonization (Mazzola *et al.* 1992; Nielsen *et al.* 2005; Tran *et al.* 2007; D'aes *et al.* 2011). For the phenazines, the competitive advantage may relate to their broad-spectrum antimicrobial activities (Mazzola *et al*. 1992; Mavrodi *et al.* 2006) and their role in biofilm formation (Dietrich *et al.* 2008; Maddula *et al.* 2008). Also LPs play a role in biofilm formation and are key metabolites in surface motility of the producing strains and in defense against protozoan predation (Mazzola *et al*. 2009; Raaijmakers *et al.* 2010). The results of our growth chamber experiments, however, showed that the introduced phenazine and LP-producing *Pseudomonas* strains and their biosynthesis or regulatory mutants established similar population densities on the roots and stem base of groundnut. Probably the time point used to determine the population densities, i.e. two weeks after sowing, and the fact that the spatial colonization patterns were not considered in our analysis may account for the discrepancy with results obtained in previous studies.

Among the LPs produced by *Pseudomonas* species, there is considerable structural diversity due to differences in the length and composition of their lipid and peptide moieties (Nybroe and Sorensen 2004; Raaijmakers *et al.* 2006). Based on these structural characteristics, the LPs produced by *Pseudomonas* were initially classified into four major groups, i.e. the viscosin, amphisin, tolaasin and syringomycin groups (Nybroe and Sorensen 2004). In addition, a number of new LPs have been identified for *Pseudomonas* in recent years, including arthrofactin (Morikawa *et al.* 1993; Roongsawang *et al.* 2003), putisolvin (Kuiper *et al.* 2004), orfamide (Paulsen *et al.* 2005; Gross *et al.* 2007), pseudodesmins (Sinnaeve *et al.* 2009), and thanamycin (Mendes *et al*. 2011). In this study, we compared the antifungal activity of *Pseudomonas* strains producing one of four structurally different LPs, i.e. massetolide A by strain SS101, viscosin by strain SBW25, putisolvin by strain 267 and thanamycin by strain SH-C52. Due to the lack of sufficient amounts of pure forms of the four LPs, we were not able to conduct dose-response assays with *S. rolfsii* to determine EC_{50} and minimal inhibitory concentrations (MIC) of these LPs. Instead, we compared the antagonistic activities of the wildtype strains with those of well-defined mutants disrupted in the respective LP-biosynthesis genes.

The results of the *in vitro* dual culture assays showed that strains SS101 and SBW25, producing the viscosin-type LPs, and strain 267, producing putisolvins, did not or only marginally inhibit hyphal growth of *S. rolfsii*. Lack of inhibiting activity of LPs was also observed previously for viscosinamide and putisolvins in hyphal growth assays with oomycete pathogens (Thrane *et al*. 1999; Gross *et al.* 2007; Kruijt *et al.* 2009). In contrast, thanamycin-producing strain SH-C52 significantly inhibited hyphal growth of *S. rolfsii,* whereas its

thanamycin-deficient mutant was not effective. Growth chamber bioassays further showed that strain SH-C52 significantly reduced stem rot disease of groundnut, whereas its thanamycin-deficient mutant and also strains SS101, SBW25 and 267 were not effective in disease control. Collectively, these results suggest differential activity of structurally different LPs against the stem rot pathogen *S. rolfsii*, with thanamycin having significant inhibitory effects on hyphal growth and plant infection. Based on sequence similarities with the syringomycin biosynthesis pathway and bioinformatic analyses, thanamycin is predicted to be a 9-amino acid LP with a C-terminal chlorinated threonine residue (Mendes *et al.* 2011). Due to difficulties to isolate sufficient amounts of pure thanamycin, the predicted structure has not been confirmed by LC-MS/MS and NMR yet. The activity of thanamycin against *S. rolfsii* is most likely related to the C-terminal chlorinated threonine, the residue that is also responsible for most of the antifungal activity of the structurally related LP syringomycin produced by *P. syringae* pv. *syringae* (Grgurina *et al.* 1994). It should be emphasized, however, that the presumed differential activity of the four LPs is based on the assumption that each of the bacterial strains produces similar amounts of the LPs on agar plates and in the groundnut rhizosphere. The culture conditions of the *in vitro* assays are known to induce LP production (X. Cheng, M. van der Voort, J.M. Raaijmakers, unpubl. data), but at this point we have no data on the concentrations of each of the LPs produced on roots of groundnut. To that end, reporter-based studies (Keel *et al.* 1992) or advanced analytical chemical analyses (Ongena *et al.* 2007) should be conducted to confirm that each of the four structurally different LPs is indeed produced *in situ* and to determine the amounts produced.

The proposed mode of action of LPs is pore formation in membranes, leading to an inbalance in transmembrane ion fluxes and cell death (Abada 1994; Bender *et al*. 1999; Baltz 2009). In addition, LPs may induce systemic resistance in plants. For example, massetolide A produced by *Ps. fluorescens* strain SS101 enhanced resistance of tomato against *Phytophthora infestans* (Tran *et al.* 2007). Similarly, fengycins and surfactin produced by *Bacillus* induced systemic protection in bean and tomato leaves against *Botrytis cinerea* (Ongena *et al.* 2007). Whether thanamycin produced by strain SH-C52 directly impacts hyphal growth of *S. rolfsii* in the rhizosphere or on the stem base of groundnut, or limits plant infection through induced systemic resistance is not known. To investigate this, bioassays need to be conducted where the pathogen and the inducers (strain SH-C52, mutant O33, pure thanamycin) are physically separated.

In conclusion, phenazine-producing strain Phz24 and thanamycin-producing strain SH-C52 are promising candidates for biological control of stem rot disease of groundnut caused by *S. rolfsii*. Based on the observed additive and synergistic activity of phenazines and biosurfactant compounds in control of *Pythium* species (Perneel *et al.* 2008) and the fungal pathogen *Rhizctonia solani* (D'aes *et al.* 2011), combination of strains Phz24 and SH-C52 may provide even better or more consistent control of *S. rolfsii*. Alternatively, strain SH-C52 may be transformed with genes for phenazine biosynthesis leading to enhanced antifungal activity, an

approach that was successfully used previously with other *Pseudomonas* strains (Huang *et al.* 2004). Also combining strain SH-C52 with *Ps. fluorescens* strain SS101 should be pursued as the latter strain significantly improved yield in the field experiment. The mechanisms underlying this increase in pod yield by strain SS101 is not yet known and is subject of ongoing investigations.

Materials and methods

Bacterial and fungal strains

The *Pseudomonas* strains used in this study are listed in Table 1. Bacterial strains were grown at 25°C on Pseudomonas Agar (PSA; Difco, France). For all strains, spontaneous rifampicin resistant derivatives were generated in order to monitor their population densities on roots and stem base of groundnut plants. In the *in vitro* and *in vivo* bioassays, *S. rolfsii* strain H001 was used. This strain was collected from groundnut plants in Vietnam in 2008 (Le *et al.* 2011). Sclerotia of strain H001 were obtained as follows: 50 g of dried (110°C overnight) groundnut plants were put in an Erlenmeyer flask, wetted with 100 ml sterile demineralized water, sterilized at 120°C for 30 min and inoculated with three mycelial agar plugs (5-mm-diameter) of *S. rolfsii.* After 3 weeks of incubation at 25ºC, the sclerotia were collected, air-dried and stored at room temperature.

In vitro assays

Inhibition of hyphal growth of *S. rolfsii* by the *Pseudomonas* strains was tested in dual culture assays according to the conditions described previously (Kruijt *et al.* 2009) and determined after 3 days of incubation at 25°C. Inhibition of sclerotial germination by the *Pseudomonas* strains was determined as follows: sclerotia were surface-sterilized with 3% (v/v) sodium hypochlorite for 5 min and rinsed three times with ample sterile distilled water. *Pseudomonas* strains were grown on PSA plates for 24 h at 25ºC, washed three times with sterile distilled water and adjusted to a final density of 10^9 cells ml^{-1} (OD_{600}=1.0). Sclerotia were soaked in sterile distilled water (control) or in bacterial suspension for 30 min and placed on sterile wet filter paper in a Petri dish. For each treatment, three replicate plates were used with fifteen sclerotia per plate. After 48 hours of incubation at 25ºC, germination of sclerotia was examined with a binocular.

Selection and identification of phenazine mutants

Phenazine-producing *Pseudomonas chlororaphis* strain Phz24 was subjected to random transposon mutagenesis using the plasposon Tn*Mod*OTc (Dennis and Zylstra 1998). From approximately 2500 random mutants, two mutants were selected that had lost the typical orange pigment of wildtype strain Phz24 (Fig. 2A) which is linked to the production of 2-hydroxyphenazine-1-carboxylic acid. The loss of PHZ production in these two mutants, designated M1 and M15, was analyzed by RP-HPLC analyses followed by photodiode array spectroscopy according to the methods described by De Souza *et al.* (2003). To identify the genes disrupted in mutants M1 and M15, the Y-linker method described by Kwon and Ricke (2000) was used. Genomic DNA was isolated from each of the mutants, digested with *Sph*I and linked to Y-linkers 1 and 2. The sequence of Y-linker 1 is TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG, and that of Y-linker 2 is TGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT. To amplify the genetic region flanking the transposon insertion, Y-linker primer CTGCTCGAATTCAAGCTTCT and Tn*5* primer GGCCAGATCTGATCAAGAGA were used in the polymerase chain reaction (PCR). DNA amplicons obtained from the two mutants were purified and sent for sequencing (Macrogen Corp. Europe, Amsterdam, The Netherlands). Quality assessment and analysis of the obtained sequences were performed with Vector NTI (Invitrogen, version 8.0). For BLAST searches, databases at the National Center for Biotechnology Information

(http://www.ncbi.nlm.nih.gov) and Computational & Genome Biology Initiative Bioinformatics (CGBI: http://cgbi.cgrb.oregonstate.edu/) were used.

Growth chamber bioassays

The efficacy of the *Pseudomonas* strains and mutants to control stem rot disease of groundnut was investigated in growth chamber assays at Wageningen University (Wageningen, Netherlands). For these bioassays, groundnut cultivar L14 was used, which is the most widely grown cultivar in Vietnam. Growth chamber conditions comprised a continuous temperature of 25ºC during day (12h) and night (12h), a relative air humidity of 80%, and a light intensity of 250 μmol m^{-2} s^{-1}.

Groundnut seeds were surface sterilized in 3% (v/v) sodium hypochlorite solution for 15 min, rinsed with 70% ethanol and rinsed three times with ample sterile distilled water. Sterilized seeds were soaked in sterile water for 4 h at 25°C and then incubated overnight in Petri dishes at 25°C. The pre-germinated seeds were subsequently soaked for 30 min in a bacterial suspension of 10^7 cells ml^{-1}. For the control treatment, germinated seeds were soaked in sterile water for 30 min. Treated seeds were sown in pots containing 250 g of a mix (1:3 (w/w)) of commercial potting soil and river sand. Each treatment consisted of five boxes with eight pots per box. In the growth chamber, the treatments were randomized. Plants with three true leaves (~ two weeks after sowing), were inoculated with the fungal pathogen by placing a mycelial agar plug (5-mm-diameter) of a 3-day-old PDA plate culture of *S. rolfsii* approximately 1 cm below the soil surface close to the base of the stem. The plugs were covered with soil. Two weeks after pathogen inoculation, disease incidence (DI), disease severity (DS) and mortality (MR) were recorded. DI is defined as the number of infected plants divided by the total number of plants tested. For determining the DS, disease symptoms were rated on a scale from 0-4 (Fig. 5) with 0: no disease symptoms, 1: disease symptoms without visible outgrowth of the fungus, 2: disease symptoms with visible outgrowth of the fungus, 3: partial wilting of the plant, and 4: complete wilting and plant death (Le *et al.* 2011). DS was calculated based on the formula: DS= [(1× number of plants rated as scale 1) + (2 × number of plants rated as scale 2) + (3 × number of plants rated as scale 3) + (4 × number of plants rated as scale 4)] × 100 ÷ (4 × total number of plants). MR is the number of wilted and dead plants (scale 4) divided by the total number of plants.

Figure 5. Disease severity index scales of groundnut infected by *Sclerotium rolfsii*. 0: no disease symptoms, 1: disease symptoms without visible outgrowth of the fungus, 2: disease symptoms with visible outgrowth, 3: partial wilting of the plant, and 4: complete wilting and plant death.

Nethouse experiments

For the nethouse experiments conducted at Hue University of Agriculture and Forestry (Hue, Vietnam), the set-up was similar to that of the growth chamber bioassays except that field soil, collected from a groundnut field in Thua Thien Hue province (clay loam soil), was used instead of the potting soil: river sand mixture. Moreover, due to a lack of greenhouses at Hue University for contained used of genetically modified microorganisms, the mutants of the *Pseudomonas* strains could not be tested. Hence, in the nethouse experiments only wildtype strains were tested.

Field experiment

The field experiment for biocontrol of stem rot disease caused by *S. rolfsii* was conducted in 2010 in Quang Nam province (Vietnam), where approximately 20% of the groundnut plants in the field were naturally infected by *S. rolfsii* (Le *et al.* 2011). The experiment consisted of four treatments, i.e. one control treatment and three bacterial treatments. The field experiment was set-up based on a randomized complete block design (RCBD) with three blocks as three replicates and a plot size of 15 m^2 (3 X 5 m). The distance between rows was 30 cm and between plants within a row 10 cm. The bacterial strains tested were *Pseudomonas* Phz24, SH-C52, and SS101. Bacterial strains were grown on PSA plates at 25°C for 48 h, harvested and washed with sterile water. Prior to sowing groundnut seeds, bacterial suspensions were applied to the furrows at a final density of 10^6 cells per cm^2 soil. Bacterial suspensions were applied again at the same density at the flowering stage of the groundnut plants.

The field soil was fertilized with nitrogen (N) at 40 kg/ha, phosphorus (P_2O_5) at 60 kg/ha, potassium (K_2O) at 60 kg/ha, and calcium ($Ca(OH)_2$) at 300 kg/ha. Total phosphorus and calcium was applied at the time of soil preparation. Seeds were sown at a depth of 3-5 cm and covered with soil. When plants had three true leaves (seedling stage), 70% of the nitrogen and 50% of the potassium was applied. The remainder of the N and K fertilization was applied at flowering. Weeds were manually removed at four developmental stages of the groundnut plants, i.e. seedling, flowering, peg and pod set. At these four developmental stages, DI was monitored in 1 m^2 of each plot, i.e. approximately 33 plants per plot. Also other characteristics including plant height, nodulation, and yield were monitored. The number of nodules per plant root system was determined at the pod stage. To that end, three randomly selected plants per plot were harvested; their root systems were washed to remove adhering soil and the number of nodules was counted.

Bacterial colonization of groundnut roots and stem base

The spontaneous rifampicin-resistant derivatives of the *Pseudomonas* strains were re-isolated from roots and stem base of groundnut according to methods described by Tran *et al.* (2008). Briefly, one gram (fresh weight) of groundnut roots or stem base was transferred to a 15-ml tube with 5 ml 10 mM $MgSO_4$-$7H_2O$, followed by vigorous vortexing for 1 min, sonication (Elma D-78224, Singen/HTW, Germany) for 1 min and vortexing again for 15 sec. The suspensions were serially diluted in 10-fold steps and 50 µl of the 100X and 1000X diluted samples was plated onto PSA plates supplemented with chloroamphenicol (12.5 µg ml^{-1}), ampicillin (40 µg ml^{-1}), rifampicin (100 µg ml^{-1}) and delvocid (100 µg ml^{-1}). The number of bacterial colonies was counted after 2- 4 days of incubation at 25°C.

Statistical analysis

All experiments, except the field experiment, were performed at least twice and representative results are shown. DI, DS and MR are expressed in percentages and were arcsin-transformed prior to statistical analysis. Statistical differences ($P < 0.05$) between treatments were analyzed by ANOVA followed by the Dunnet test or the Duncan Multiple Range Test (SPSS Statistics, USA). Normal distribution of the data and homogeneity of variances was tested prior to ANOVA.

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Chapter 4

Diversity and antifungal activity of groundnut-associated bacteria

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To be submitted

Diversity and antifungal activity of groundnut-associated bacteria

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Abstract

Groundnut (*Arachis hypogaea* L.) is an important oil seed crop worldwide and used extensively for feed and food. In Vietnam, groundnut cultivation is hampered by several soil-borne fungal pathogens, in particular *Sclerotium rolfsii.* To develop sustainable measures to control stem rot disease caused by *S. rolfsii*, bacteria were isolated from the stem base and roots of groundnut plants grown in farmer fields in central Vietnam and tested for activity against *S. rolfsii*. Among a total of 3,360 randomly selected bacterial isolates, only thirteen (0.4%) inhibited hyphal growth of *S. rolfsii*. BOX-PCR and 16S-rDNA sequence analyses revealed that these bacterial isolates were genetically diverse and belonged to the γ-Proteobacteria (*Pseudomonas*), Firmicutes (*Bacillus*) and Bacteroidetes (*Chryseobacterium*). Subsequent nethouse and field experiments conducted in central Vietnam showed that treatment of groundnut seeds or soil with strains of each of these three bacterial taxa significantly reduced the incidence of stem rot disease and led to significant yield increases of up to 21%. The level of disease protection provided by the bacterial strains was similar to that achieved by the fungicide tebuconazole. For *Pseudomonas* sp. strain R4D2, two mutants were obtained that did not inhibit hyphal growth of *S. rolfsii* and were not effective in controlling stem rot disease of groundnut under controlled growth chamber conditions. Characterization of these two mutants suggested that *Pseudomonas* sp. strain R4D2 produces (a) biosurfactant compound(s) that might be responsible for biocontrol of *S. rolfsii*.

Keywords: Groundnut, antagonistic bacteria, *Bacillus*, *Pseudomonas, Chryseobacterium, Sclerotium rolfsii*.

Introduction

In Vietnam, groundnut (*Arachis hypogaea* L.) is the second most important annual crop after paddy rice with an area of 249,200 ha and an annual production of approximately 0.53 million tons in 2009 (FAO 2011). Groundnut production can be improved considerably by controlling a number of pests and diseases (Brown 2007; Shew and Waliyar 2007). Among the soil-borne fungal diseases, stem rot caused by *Sclerotium* (*Athelia*) *rolfsii* Sacc. is a destructive disease (Mehan *et al.* 1994). Recent surveys conducted in central Vietnam showed that 5-25% of the groundnut plants in agricultural fields were infected by *S. rolfsii* (Le *et al.* 2011; Chapter 2). This pathogen has a broad-host range and can survive in soil and plant debris for considerable time periods by means of persistent sclerotia (Coleysmi and Cooke 1971; Punja 1985). Sustainable control of this pathogen requires a combination of different strategies including chemical, cultural and biological measures.

To date, studies on biological control of *S. rolfsii* by beneficial microorganisms have shown that bacteria from the genus *Pseudomonas* can restrict *in vitro* hyphal growth of *S. rolfsii* (Ganesan and Gnanamanickam 1987; Kishore *et al.* 2005a; Ganesan *et al.* 2007; de Curtis *et al.* 2010; Pastor *et al.* 2010; Tonelli *et al.* 2010). Germination of sclerotia was reduced by 10-20% and 50-60% after immersion in a bacterial cell suspensions for 1 h and 1 week, respectively (Ganesan and Gnanamanickam 1987). Kishore *et al.* (2005b) further showed that cell-free culture filtrates of *P. aeruginosa* strains GSE18 and GSE19 inhibited the *in vitro* activity of the cell wall degrading enzymes polygalacturonase and cellulase produced by *S. rolfsii*. Strains GSE18 and GSE19 also suppressed the growth of *S. rolfsii* and reduced the incidence of stem rot of groundnut (Kishore *et al.* 2005b). Recent studies conducted in our lab indicated that phenazine-producing *Pseudomonas chlororaphis* strain Phz24 and lipopeptide-producing *Pseudomonas* sp. strain SH-C52 control stem rot disease of groundnut under controlled conditions and in the field in central Vietnam (Chapter 3). Next to pseudomonads, also *Bacillus* species are studied extensively for biocontrol of plant diseases including stem rot disease of groundnut. Pre-treatment of groundnut seeds with *Bacillus subtilis* protected groundnut seeds against *S. rolfsii* and significantly increased the number of pods (Abd-Allah 2005). Other microorganisms tested for control of stem rot disease include *Rhizobium* and *Trichoderma* (Ganesan *et al.* 2007). Collectively, these limited studies indicate that application of antagonistic microorganisms to seeds may provide a promising alternative or supplementary strategy to control stem rot disease of groundnut.

To further develop biocontrol as an integral part of disease management practices to control *S. rolfsii* and other pathogens of groundnut, the biocontrol efficacy of selected beneficial microorganisms needs to be evaluated under field conditions. Most of the microorganisms tested to date for biocontrol of *S. rolfsii,* however, have not been tested under field conditions. Furthermore, most of these microorganisms do not originate from groundnut and may be less adapted to the microenvironment of the groundnut plant and to the (a)biotic conditions

prevailing in groundnut fields. The overall aims of this study were to 1) isolate and characterize bacteria from the stem base and roots of groundnut plants grown in agricultural fields in central Vietnam, 2) test selected bacterial strains under field conditions in Vietnam for their efficacy to control stem rot disease of groundnut and to improve yield, and 3) elucidate the mechanism underlying the biocontrol capacity of one of the selected bacterial strain. The overall set-up and procedures adopted to achieve these goals are included in figure 1.

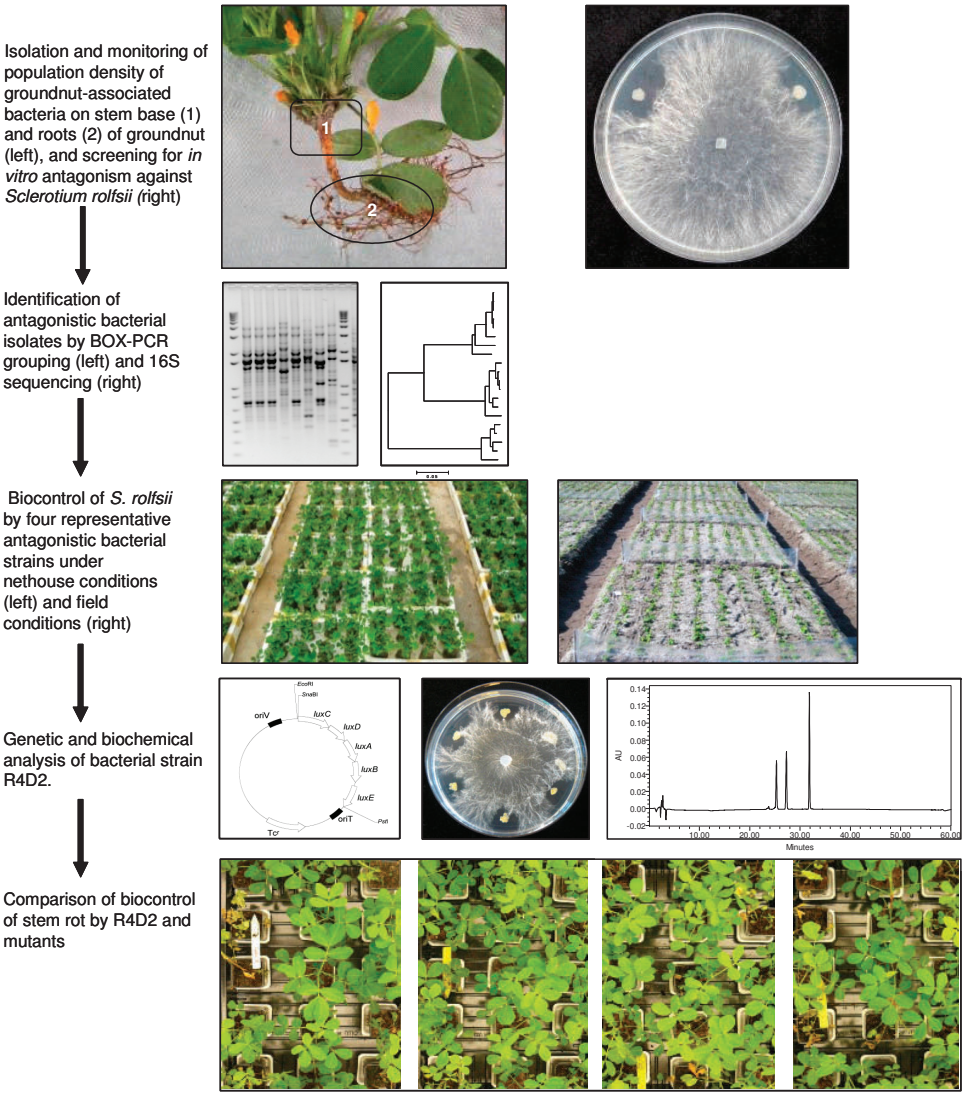

Figure 1. Schematic presentation of approaches and techniques used to isolate, identify, and characterize antagonistic bacteria towards *Sclerotium rolfsii* on stem base and roots of groundnut grown in center on Vietnam.

Results and Discussion

Frequency of antagonistic bacteria on groundnut plants

The number of bacteria isolated on PSA agar plates from the stem base and roots of groundnut plants grown in two farmer fields in central Vietnam, represented densities of approximately 3 X 10^6 CFU per gram (Table 1). Out of a total of 3,360 randomly selected isolates, thirteen (0.4%) inhibited hyphal growth of *S. rolfsii in vitro*, i.e., six isolates from the stem base and seven from roots of groundnut (Table 1). Also Tonelli *et al.* (2010) reported a rather low percentage (1.5%) isolates with inhibitory activity against *S. rolfsii* (only three out of a total of 193 from groundnut plants grown in Córdoba, Argentina). Kishore *et al.* (2005a), who collected bacterial isolates from the rhizosphere of groundnut plants grown in Andhra Pradesh (India), reported that approximately 9% of the isolates (34 out of a total of 393) significantly inhibited hyphal growth of *S. rolfsii.* These differences in frequency of indigenous, groundnut-associated bacteria with *in vitro* activity against *S. rolfsii* can be due to a multitude of factors, including soil type, groundnut cultivar and the developmental stage of the groundnut plants at the time of bacterial isolation. Also the culture conditions, and the *S. rolfsii* isolate used in the inhibition assays may play a role. In hyphal growth inhibition we compared the inhibitory activity of the different bacterial isolates. As shown in Fig. 2, the six bacterial isolates obtained from the stem base of groundnut were substantially more active in inhibition of hyphal growth of *S. rolfsii* than the seven isolates obtained from the roots of groundnut.

Table 1. Frequency and genotypic diversity of antagonistic bacteria isolated from the stem base and roots of groundnut plants grown in agricultural fields in two provinces in central Vietnam.

Province	Plant part	Bacteria*	Antagonism toward *Sclerotium rolfsii***			
			Tested	Inhibitory	(%)	BOX-PCR Group[3]
Quang Nam	Stem base	3.4 x 10^6 ± 0.5 x 10^6	960	2	0.2	1, 3
	Roots	3.5 x 10^6 ± 0.5 x 10^6	960	7	0.7	27, 37
Thua Thien Hue	Stem base	3.0 x 10^6 ± 1.1 x 10^6	720	4	0.6	2, 4
	Roots	3.3 x 10^6 ± 0.8 x 10^6	720	0	0.0	

*Population density of bacteria expressed as CFU g^{-1} stem base or root fresh weight; ± refers to the standard error of the mean. **Number of bacterial isolates tested *in vitro* for hyphal growth inhibition of *Sclerotium rolfsii*. The thirteen antagonistic bacterial isolates and 48 non-antagonistic isolates were subjected to BOX-PCR analysis and grouped in a total of 42 BOX-PCR groups (see also appendix 1).

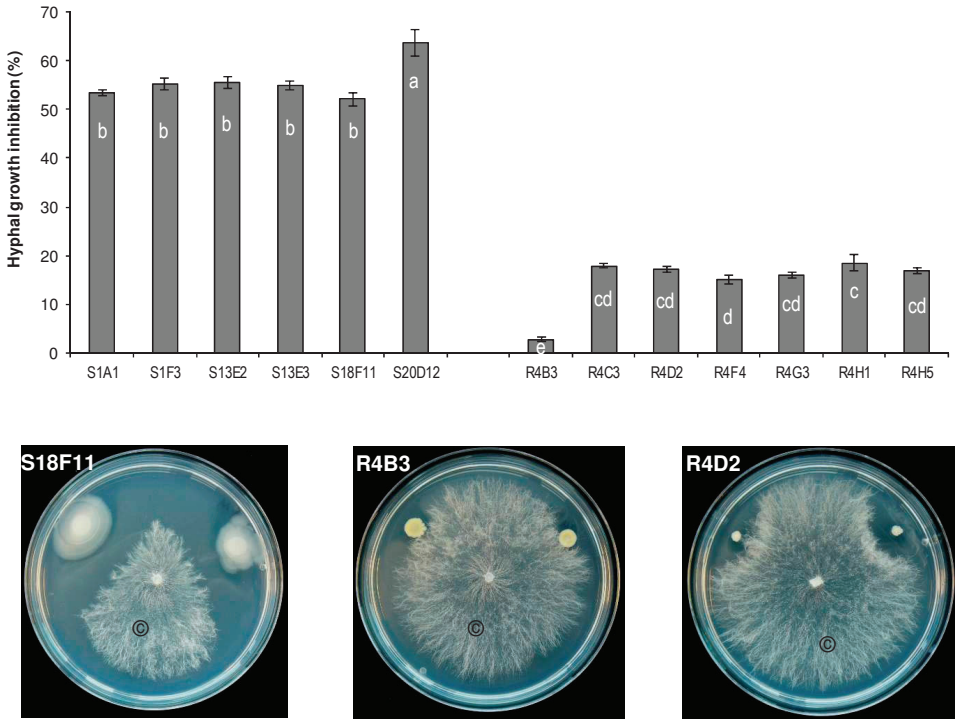

Figure 2. Hyphal growth inhibition (HGI) of *Sclerotium rolfsii* on 1/5th PDA by different bacteria isolated from stem base and roots of groundnut plants in Vietnam. The first letter of the bacterial isolates' code refers to the origin, i.e. stem base (S) or roots (R). The percentage of hyphal growth inhibition (HGI) was arcsin-transformed prior to statistical analysis. The bars show averages of three replicates and error bars represent the standard error of the mean. The pictures at the bottom show examples of the variation in hyphal growth inhibition of *S. rolfsii* among three bacterial isolates on 1/5th PDA plates after 48 h of incubation at 25ºC. The control spot (no bacteria) is indicated by ©

Diversity and classification of the antagonistic bacteria

The genotypic diversity of the 13 antagonistic isolates and of the non-antagonistic isolates obtained from groundnut plants was analyzed by BOX-PCR analysis. The 13 antagonistic isolates were grouped in six BOX groups and were genotypically different from the thirty-six BOX groups found for the non-antagonistic isolates (Table 1, Appendix 1). BOX-groups 37 and 2 harboured most of the antagonistic isolates with six isolates from the roots and three from the stem base of groundnut, respectively (Appendix 1). BOX-groups 1, 3 and 4 harboured one antagonistic isolate each (Appendix 1). The relatively high genotypic diversity of groundnut-associated bacteria that we observed here was also reported by Tonelli *et al.* (2010) for bacterial populations from groundnut plants in Argentina. They showed 20 different genotypic groups for 24 Gram-positive isolates and 8 groups for the 9 Gram-positive bacteria.

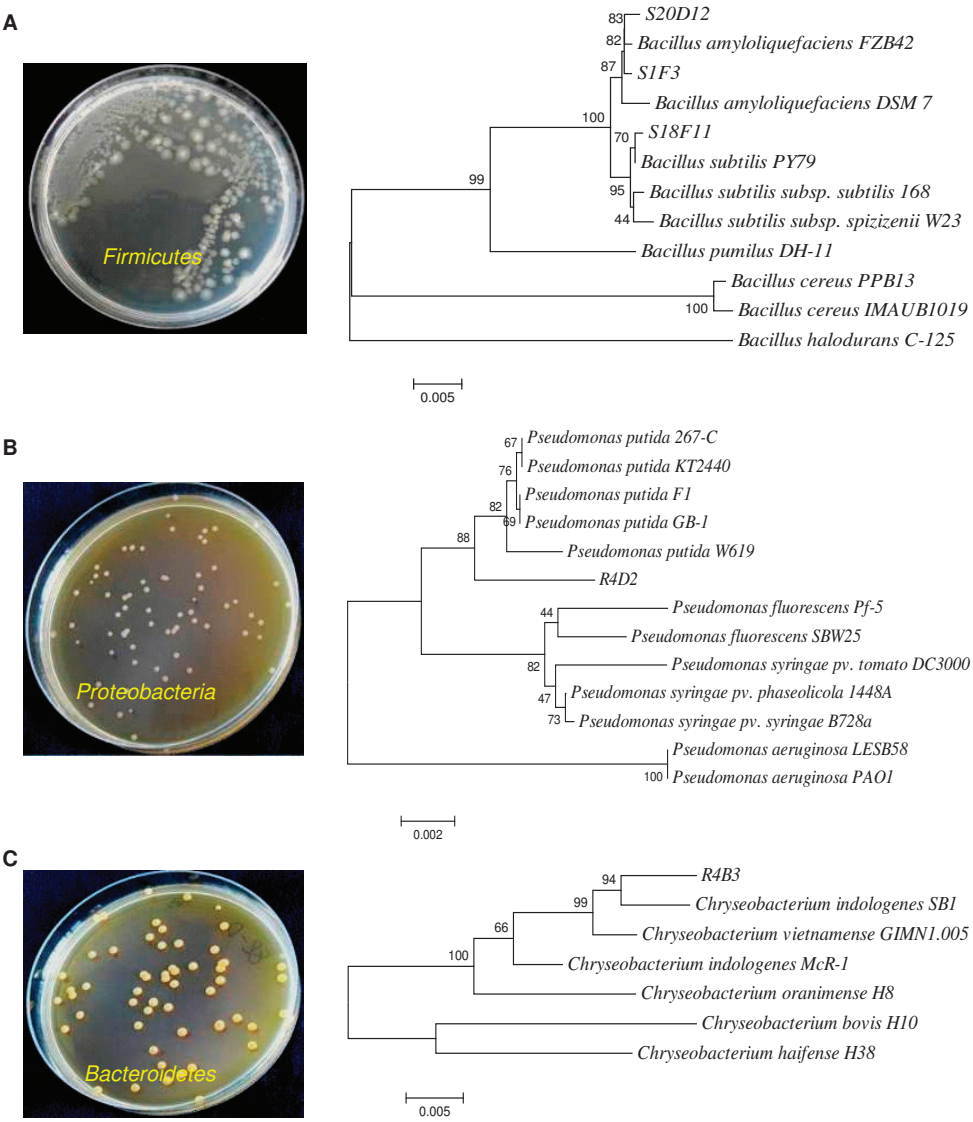

Figure 3. Colony morphology and phylogeny of five selected bacterial isolates (S1F3, S18F11, S20D12, R4D2, and R4B3) that inhibit hyphal growth of *Sclerotium rolfsii*. The branch length indicates the percentage of sequence dissimilarity and numbers at the nodes indicate bootstrap values.

To further identify the antagonistic isolates, one isolate from each of the six BOX-groups was subjected to 16S-rDNA sequencing and phylogenetic analyses. The six isolates, designated S1A1, S1F3, S18F11, S20D12, R4B3 and R4D2 fall in BOX-PCR group 3, 1, 4, 2, 27, and 37, respectively. The obtained 16S-rDNA sequences (~1,300bp) were of high quality except for S1A1 (BOX-group 3), Subsequent re-sequencing did not resolve the poor sequence quality for S1A1. Phylogenetic analyses revealed that bacterial strains S1F3, S18F11, S20D12, R4B3 and

R4D2 belong to three bacterial taxa, i.e. the Firmicutes (*Bacillus*), Proteobacteria (*Pseudomonas*), and Bacteroidetes (*Chryseobacterium*) (Fig. 3). Although the medium used for isolation of these bacterial strains is semi-selective for *Pseudomonas* species, these results indicate that also other bacterial genera can grow on this medium. This is consistent with earlier observations of De Souza *et al.* (2003b) who showed that only 35.8% to 73.5% of the bacteria isolated from wheat roots and plated on this semi-selective medium are *Pseudomonas* species; the other bacteria represented different genera.

The three antagonistic strains classified as *Bacillus* (i.e. S1F3, S18F11, and S20D12) originated from the stem base. At the species level, strains S1F3, and S20D12 were in the same cluster as *Bacillus amyloliquefaciens* reference strains, whereas strain S18F11 clustered with several *Bacillus subtilis* strains (Fig. 3). For the two antagonistic strain originated from the roots of groundnut, strain R4D2 clustered close to the *Pseudomonas putida* group, and strain R4B3 to *Chryseobacterium* species, including *Chryseobacterium vietnamense* strain GIMN1.005 isolated from forest soil in Vietnam (Li and Zhu 2011). Many *Bacillus* and *Pseudomonas* species are well known for their antagonistic activities against plant pathogenic fungi and oomycetes (Ongena and Jacques 2008; Raaijmakers *et al.* 2009; Raaijmakers *et al.* 2010). Several *Bacillus* and *Pseudomonas* strains have been isolated from groundnut and studied as biocontrol agents of *S. rolfsii* on groundnut (Pleban *et al.* 1995; Abd-Alla and Ezzat 2003; Abd-Allah 2005; Abd-Allah and El-Didamony 2007; de Curtis *et al.* 2010; Hameeda *et al.* 2010; Tonelli *et al.* 2010; Tonelli *et al.* 2011). For the genus *Chryseobacterium,* formerly known as *Flavobacterium* (Vandamme *et al*. 1994), relatively little is known about their effects on plant pathogens and plant growth. *Chryseobacterium* was reported to control *Fusarium* and *Rhizoctonia* on tomato and pepper (Domenech *et al.* 2006) and *Phytophthora capsici* on pepper (Kim *et al.* 2008). Recently, *Chryseobacterium* was reported as a potential biocontrol agent of *Pyricularia oryzae* causing rice blast (Gandhi *et al.* 2009; Lucas *et al.* 2009). Interestingly, *Chryseobacterium* was also reported to remove aflatoxin B1 from groundnut milk (Hao and Brackett 1988). Since *Chryseobacterium* is commonly found in the geocarposphere, i.e. the soil surrounding groundnut pods (Kloepper *et al.* 1992), representatives of this bacterial genus may be useful to reduce contamination of groundnut pods with aflatoxin produced by *Aspergillus* species.

Biocontrol of stem rot of groundnut under nethouse and field conditions

Based on the results of the phylogenetic analysis, four antagonistic strains were chosen for the biocontrol assays, i.e. *Bacillus* sp. strains S18F11 and S20D12, *Pseudomonas* sp. strain R4D2 and *Chryseobacterium* sp. strain R4B3. Under nethouse conditions, *Pseudomonas* strain R4D2 significantly reduced stem rot disease incidence and severity (Fig. 4). The other three bacterial isolates did not significantly suppress the disease relative to the control despite the fact that they established similar population densities as strain R4D2 on the stem base and roots of groundnut after two weeks of plant growth (Fig. 4). In the field experiment conducted in Quang

Nam province (Vietnam), all four bacterial strains provided significant disease control at pod set stage to a level similar to that of the fungicide Folicur (Fig. 5). When the disease progress monitored from seedling stage until pod set was taken into account, only strain R4B3 reduced DI significantly. The four applied bacterial strains established population densities on the stem base and roots of groundnut, at flowering and pod set stages, ranging from 4.2-5.5 log cfu g^{-1}. *Bacillus* sp. strain S20D12 established the lowest densities at pod set stage (Table 2). Next to stem rot, damping-off disease, caused by *Rhizoctonia solani,* and black collar rot, caused by *Aspergillus niger,* were monitored. For both diseases, the incidence in the control treatment was not very high (i.e. ~3.7% and 8.3%, respectively) and no significant effects of the bacterial treatments or the chemical Folicur on disease development were observed (data not shown).

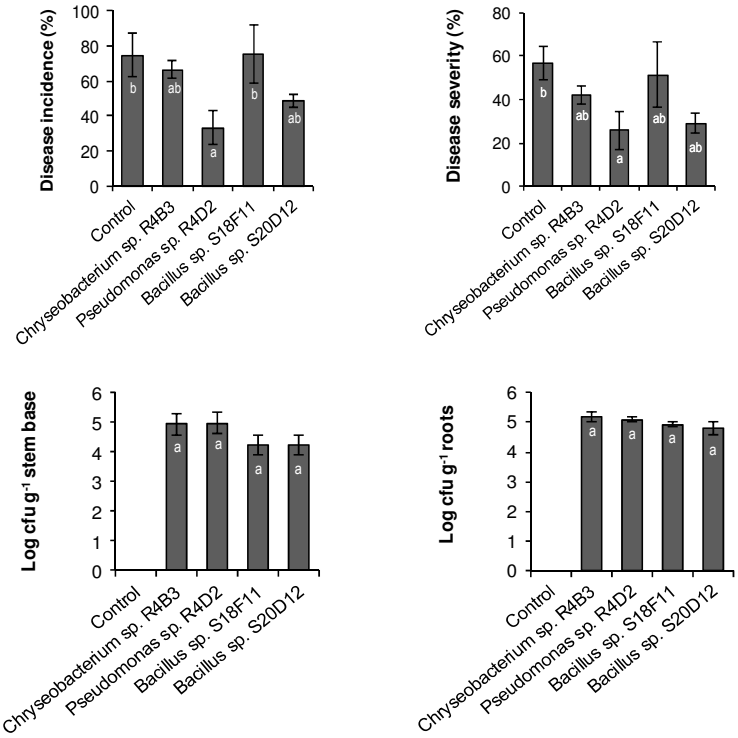

Figure 4. Biocontrol of stem rot of groundnut and colonization of stem base and roots by different bacterial strains under nethouse condition. Averages of three replicates are given. Different letters indicate a statistically significant difference between the treatments (*P=0.05,* Duncan Multiple Range Test). Error bars represent the standard error of the mean.

The bacterial treatments and the chemical Folicur had no effect on plant height, branch length (data not shown), and the number of nodules per plant (Table 2). Only *Bacillus* strain S20D12 significantly increased pod yield by 21% relative to the untreated control, whereas the yield increases observed for most of the other bacterial and chemical treatments were

intermediate between the control and S20D12 treatments (Table 2). Collectively these results suggest that there is no apparent correlation between suppression of stem rot disease, root colonization and yield increase. Indeed, correlation analysis showed that there was no correlation (correlation coefficient = -0.04) between stem rot disease at pod set and pod yield of groundnut.

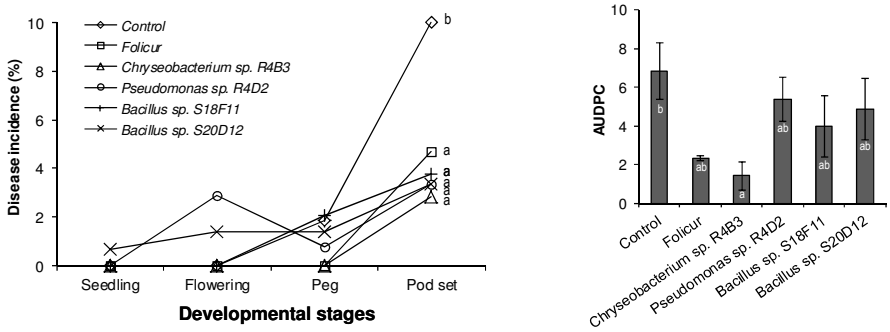

Figure 5. Biocontrol of stem rot of groundnut by chemical fungicide Folicur, and groundnut-associated bacteria *Chryseobacterium* sp. strain R4B3, *Pseudomonas* sp. strain R4D2, *Bacillus* sp. strains S18F11 and S20D12 under field conditions in Quang Nam province, Vietnam. A) Disease incidence was monitored at seedling stage when plants had 3-5 true leaves (20 days after sowing), and subsequently every 15 days. B) AUDPC represents the Area Under the Disease Progress Curve, and was calculated based on the method used by Landa *et al.* (2002) and Kruijt *et al.* (2009). For each developmental stage, averages of three replicates are given. Different letters indicate a statistical significant difference between the treatments (P=0.05, Duncan Multiple Range Test).

Table 2. Effects of four different bacterial strains and the chemical fungicide Folicur on nodulation and pod yield of groundnut plants grown under field conditions in Quang Nam province, Vietnam. Population densities of the introduced bacterial strains on the stem base and roots of groundnut plants were determined at two developmental stages (flowering, pod set). Averages of three replications are given. For each column, different letters indicate a statistically significant difference between the treatments (*P*= 0.05, Duncan Multiple Range Test).

Treatment	Population density introduced bacterial strain (log cfu g^{-1})				Nodules per plant	Dry pod yield (kg ha^{-1})
	Flowering		Pod set			
	Stem base	Roots	Stem base	Roots		
Control					133 a	1220 b
Folicur					126 a	1300 ab
Chryseobacterium sp. R4B3	5.4 a	5.1 a	5.0 ab	5.4 ab	121 a	1190 b
Pseudomonas sp. R4D2	5.3 a	5.1 a	5.2 a	5.5 a	126 a	1320 ab
Bacillus sp. S18F11	5.4 a	5.4 a	5.0 ab	5.0 ab	116 a	1320 ab
Bacillus sp. S20D12	5.5 a	5.2 a	4.4 b	4.2 b	134 a	1480 a

Mechanisms involved in antagonism of Pseudomonas sp. *strain* R4D2 toward *S. rolfsii*

To study the mechanisms underlying disease suppression of the tested bacterial strains, we chose *Pseudomonas* sp. strain R4D2 for an in-depth analysis. This strain represents the largest group of antagonistic bacteria isolated from groundnut based on BOX-PCR analysis (Appendix 1) and gave good consistent results in the biocontrol experiments conducted in the nethouse and field. Furthermore, initial attempts to perform random mutagenesis on the two *Bacillus* and the *Chryseobacterium* strains were not successful.

Pseudomonas species are well-known for the production of a range of antibiotics (Haas and Defago 2005; Gross and Loper 2009). Phenazines, pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol have been shown to play a role in the control of numerous fungal pathogens including *S. rolfsii* (Raaijmakers *et al.* 2002; Haas and Defago 2005; D'aes *et al*. 2011; Chapter 3). Since RP-HPLC and TLC analyses showed that strain R4D2 does not produce any of these four antibiotics in culture (data not shown) it is very unlikely that these compounds contribute to the biocontrol activity of this strain. To identify genes and compounds, random mutagenesis was performed. Among a relatively low number of transformants (154), two mutants, designated MT38 and MT138, were identified that had completely lossed the ability to inhibit hyphal growth of *S. rolfsii in vitro* (Fig. 6A).

Subsequently, a series of phenotypic analyses were performed to begin to identify the nature of the mutations in MT38 and MT138. The drop collapse assay showed that wildtype strain R4D2 produces a surfactant compound which is lacking in mutants MT38 and MT138 (Fig. 6B). To further support the hypothesis that R4D2 produces a surfactant compound, swarming and biofilm assays were conducted. Previous studies with *Pseudomonas* and *Bacillus* strains have shown that surfactants, in particular lipopeptide surfactants, are key metabolites in swarming and biofilm formation (reviewed in Raaijmakers *et al.* 2010). The results showed that mutants MT38 and MT138 formed less biofilm on the walls of plastic multi-well plates (Fig. 6C) and lost the ability to swarm on soft agar plates (Fig. 6D). For many *Pseudomonas* strains, production of lipopeptide surfactants is governed by nonribosomal peptide synthetases (Raaijmakers *et al.* 2010). These enzymes are encoded by large gene clusters of more than 30 kb (Raaijmakers *et al.* 2010), which may explain the high frequency of mutants (i.e. 2 out of 154) in the random mutagenesis experiment described above. To identify the genes disrupted in these two mutants, the Y-linker method was used. Unfortunately, this method yielded no amplicons (for mutant MT38) or amplicons with very poor sequence quality (for mutant MT138). As an alternative, IPCR was used to amplify the genetic regions flanking the transposon insertion, but also this method was so far not successful. Additional experiments are required to support the hypotheses that strain R4D2 produces lipopeptide surfactants and that the transposon insertions in mutants MT38 and MT138 are located in nonribosomal peptide synthetase genes.

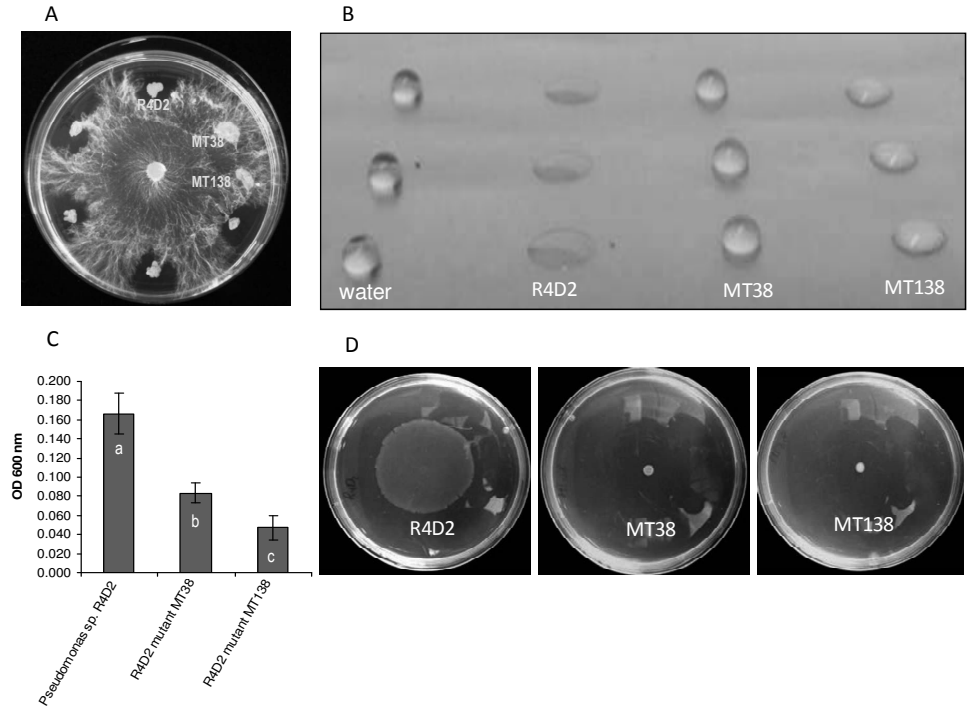

Figure 6. Phenotypic characteristics of *Pseudomonas* sp. strain R4D2, and its mutants MT38 and MT138 that loss of hyphal growth inhibition of *Sclerotium rolfsii*. A) *in vitro* inhibition of hyphal growth of *S. rolfsii* on PDA medium; B) Drop collapse assay. Bacterial cells grown for 2 days at 25°C were resuspended in sterile water (OD600 nm = 1), and 5 µl was dropped on parafilm. Droplets in the same column are replicates from the same isolate; C) Spectrophotometric quantification of the biofilm formed by R4D2 and its mutants; the higher the OD (600 nm) the more biofilm. Different letters indicate statistically significant (*P=0.05*) differences. Error bars represent the standard errors of the means; D) Motility on soft agar medium. Bacterial suspensions were inoculated in the centre of soft agar plates and incubated for 24 h at 25°C. Strain R4D2 swarmed outwards from the point of inoculation, whereas the mutants are impaired in motility.

Comparison of the biocontrol activity of Pseudomonas strain R4D2 and mutants MT38 and MT138

Under growth chamber conditions, strain R4D2 did not reduce the incidence of stem rot disease (Fig. 7A) but did significantly reduce DI and the MR. In contrast, both mutants MT38 and MT138 were not capable to reduce DS and MR. The wildtype strain R4D2 and the two mutants established similar densities on the roots of groundnut, but widtype strain R4D2 colonized the stem base of groundnut significantly better than the two mutants (Fig. 7D). These results suggest that the bioactive compound produced by strain R4D2 plays an important role in biological control of stem rot disease and is involved in stem base colonization. The latter observation again points to a possible role of surfactant compound(s) as they may act as wettability agents of hydrophobic plant surfaces which in turn promotes solubilization and diffusion of substrates for growth (Lindow and Brandl 2003). For several *Pseudomonas* species,

the lipopeptide surfactants have indeed been implicated in plant colonization (Hildebrand *et al.* 1998; Nielsen *et al*. 2005; Tran *et al.* 2007).

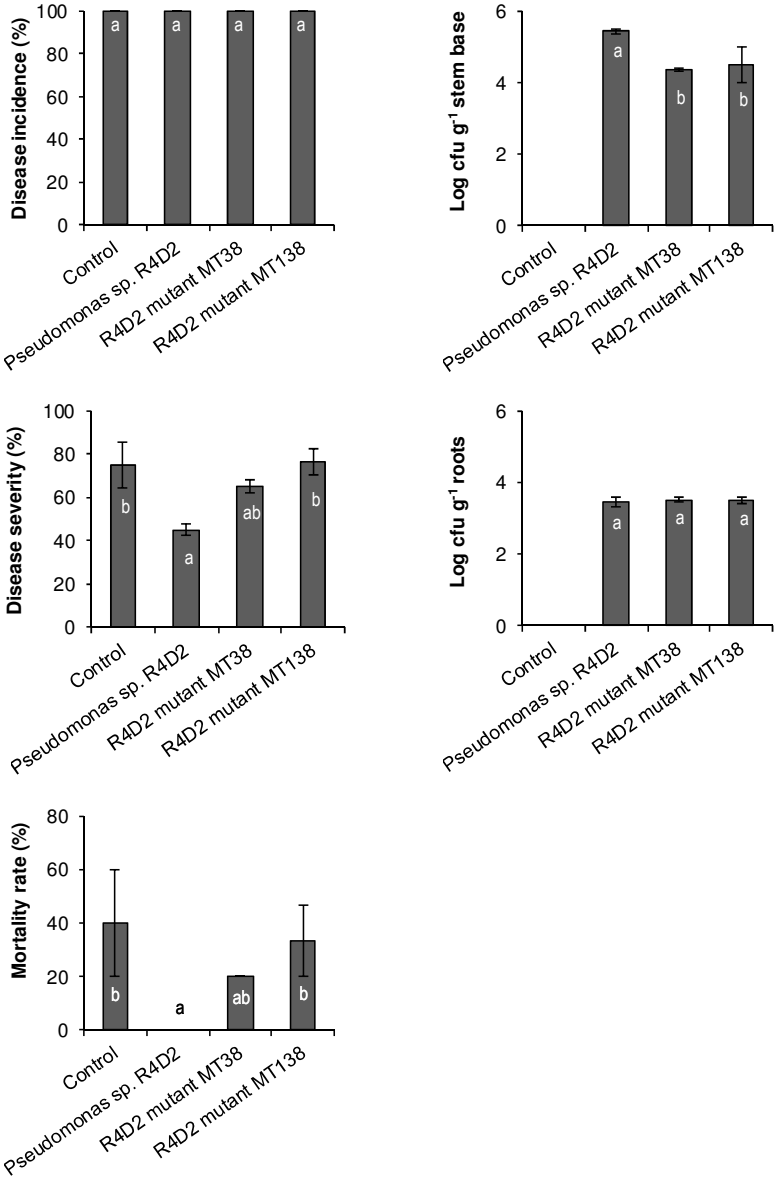

Figure 7. Biocontrol of stem rot of groundnut and colonization of stem base and roots by *Pseudomonas* sp. strain R4D2 and its mutants MT38 and MT138 under growth chamber conditions. Averages of 3 replicates are given. Different letters indicate a statistically significant difference between the treatments (*P=0.05,* Duncan Multiple Range Test). Error bars represent the standard error of the mean.

Material and methods

Bacterial isolation and growth conditions

Healthy groundnut plants were collected from farmer fields in Quang Nam and Thua Thien Hue provinces in Vietnam in 2009. Quang Nam and Thua Thien Hue are located in central Vietnam where groundnut is commonly grown and where stem rot disease caused by *S. rolfsii* is widespread (Le *et al.* 2011). In Quang Nam and Thua Thien Hue, a total of 40 and 30 groundnut plants at flowering stage, respectively, were randomly collected. For each groundnut plant, roots and stem base were separated and kept in plastic bags on ice in an insulated box. Bacterial isolations were performed in the laboratory the next day according to the method of Tran *et al.* (2008). Briefly, approximately 1 gram (fresh weight) of roots with tightly adhering soil or 1 gram of stem base (i.e. 1 cm up and down from the lowest branching point) was transferred to a 15 ml glass tube with 5 ml sterile 10 mM $MgSO_4.7H_2O$, followed by vigorous vortexing for 1 min, sonication (Elma D-78224, Singen/HTW, Germany) for 1 min and vortexing again for 15 sec. The suspensions were then serially diluted in 10-fold steps in sterile 10 mM $MgSO_4.7H_2O$, and 50 µl of the 100X and 1000X diluted samples were plated onto Pseudomonas agar (PSA) plates supplemented with delvocid (100 µg ml^{-1}), ampicilin (40 µg ml^{-1}), and chloroamphenicol (12.5 µg ml^{-1}), and incubated at 25°C for 48-72 h. This medium is semi-selective for fluorescent Pseudomonads (Simon and Ridge 1974), but also allows growth of several other bacterial genera (de Souza *et al.* 2003b). From each replicate sample, forty-eight bacterial colonies were randomly picked and purified on PSA. Those isolates that were inhibitory to the growth of *S. rolfsii* in dual culture inhibition assays (see below) were stored in glycerol (40%, v/v) at -20ºC and -80ºC. For comparison, a set of bacterial isolates that did not show any *in vitro* activity against *S. rolfsii* was also purified and stored.

Hyphal growth inhibition assays

Inhibition of hyphal growth of *S. rolfsii* by bacterial isolates obtained from the stem base and roots of groundnut was tested in dual culture assays according to the method of Kruijt *et al*. (2009). Briefly, bacterial isolates were spot-inoculated at the edge of a 1/5th-strength potato dextrose agar plate (1/5th PDA, pH 6.5). After incubation for 48 h at 25ºC, a 5-mm-diameter agar plug of a 3-day-old culture of *S. rolfsii* strain H001 (Le *et al.* 2011) was placed in the centre of the 1/5th PDA plate and incubated at 25ºC. Inhibition of mycelial growth of *S. rolfsii* by the bacterial isolates was recorded 3-4 days after fungal inoculation. In the initial screen, a total of 3,360 randomly selected bacterial isolates was tested. Isolates that showed *in vitro* inhibition of *S. rolfsii* were tested again and their inhibition of hyphal growth of *S. rolfsii* was quantified. For each bacterial isolate, three plates (replicates) were used. Hyphal growth (in mm) of *S. rolfsii* toward the bacterial colony and the control (no bacterial colony) was measured after 48 h of incubation at 25ºC. Based on these two parameters, hyphal growth inhibition (HGI) of the bacterial isolate relative to the control was calculated.

Bacterial identification

The genotypic diversity of the bacterial isolates with antifungal activity was investigated by BOX-PCR analysis according to methods described by Tran *et al.* (2008). Amplicons ranging from 200 to 5000 bp were scored visually for presence or absence. Bacterial isolates with identical BOX-PCR profiles were considered to be genotypically identical. Representative isolates of several BOX-PCR groups were sent for 16S-rDNA sequencing to Macrogen Inc. (Seoul, South Korea). The obtained forward and reverse sequences were assembled and edited in Vector NTI (Invitrogen, version 8.0) and deposited in GenBank with accession numbers from JN572706 to 572710. For the phylogenetic analyses, the edited sequences were aligned to reference sequences available in databases (http://www.ncbi.nlm.nih.gov/Genomes/ and http://www.pseudomonas.com/overview.jsp). Sequences were trimmed to the same size (~1300 bp) and a phylogenetic tree was obtained with MEGA4 software (http://megasoftware.net).

Biochemical characterization of antagonistic bacterial isolates

After identification by 16S-rDNA sequencing, several of the bacterial strains representative of different BOX-PCR groups and different bacterial genera were tested for the production of well-known antifungal compounds, i.e. phenazines (PHZ), 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT) and pyrrolnitrin (PRN). Thin layer chromatography (TLC) and reverse-phase high performance liquid chromatography (RP-HPLC) were performed according to the methods described by de Souza *et al*. (2003a).

Nethouse experiments

Four selected bacterial strains, designated *Chryseobacterium* sp. R4B3, *Pseudomonas* sp. R4D2, *Bacillus* sp. S18F11, and *Bacillus* sp. S20D12, were tested for biocontrol of stem rot disease of groundnut under nethouse conditions at the Department of Plant Protection, Hue University of Agriculture and Forestry, Vietnam. Groundnut seeds were soaked in water for 4 h at 25°C and then incubated overnight in Petri dishes at 25°C. Bacterial strains were cultured on PSA plates for 48 h at 25ºC, harvested and washed three times with sterile water. The pre-germinated seeds were subsequently soaked for 30 min in bacterial suspension with a density of 10^7 cells ml^{-1}. For the control treatment, germinated seeds were soaked in sterile water for 30 min. One treated seed was sown in a plastic bag containing 250 g of clay loam soil collected from a groundnut field in Thua Thien Hue province. Each treatment consisted of three trays (three replicates) with 12 bags per tray. The trays were randomized. Two weeks after pathogen inoculation at the base of the stem, disease incidence (DI), disease severity (DS) and mortality rate (MR) were recorded. DS was rated on a scale from 0-4 with 0: no disease symptoms, 1: disease symptoms without visible outgrowth of the fungus, 2: disease symptoms with visible outgrowth of the fungus, 3: partial wilting of the plant, and 4: complete wilting and plant death (Le *et al*. 2011). DS is calculated based on the formula: DS= [(1× number of plants rated as scale 1) + (2 × number of plants rated as scale 2) + (3 × number of plants rated as scale 3) + (4 × number of plants rated as scale 4)] × 100 ÷ (4 × total number of plants). MR is the number of dead plants (scale 4) divided by the total number of plants.

Field experiment

The field experiment was conducted in 2010 in Quang Nam province, Vietnam, where approximately 20% of the groundnut plants in the field were naturally infected by *S. rolfsii* (Le *et al.* 2011). The experiment consisted of five treatments, with i) chemical fungicide Folicur, ii) *Chryseobacterium* strain R4B3, iii) *Pseudomonas* strain R4D2, iv) *Bacillus* strain S18F11, and v) *Bacillus* strain S20D12, and one control (no treatment). The field experiment was set-up based on a randomized complete block design (RCBD) with three blocks as three replications and a plot size of 15 m^2 (3 X 5 m). The distance was 30 cm between rows and 10 cm between plants within a row. Bacterial strains were grown on PSA plates at 25°C for 48 h, harvested and washed with sterile water. Prior to sowing groundnut seeds, bacterial suspensions were applied to the furrows at a final density of 10^6 cells per cm^2 soil. Bacterial suspensions were applied again to the soil at the same density at the flowering stage of the groundnut by watering bacterial suspension to the soil.

The field soil was fertilized with nitrogen (N) at 40 kg/ha, phosphorus (P_2O_5) at 60 kg/ha, potassium (K_2O) at 60 kg/ha, and calcium ($Ca(OH)_2$) at 300 kg/ha. Phosphorus and calcium were applied at the time of soil preparation. Seeds were sown at a depth of 3-5 cm and covered with soil. When plants had three true leaves (seedling stage), 70% of the nitrogen and 50% of the potassium was applied. The remainder of the N and K fertilization was applied at flowering. Weeds were manually removed at four developmental stages of the groundnut plants, i.e. seedling, flowering, peg and pod set. At these four developmental stages, DI was monitored in 1 m^2 of each plot, i.e. approximately 33 plants per plot. Also other characteristics such as plant height, nodulation, and pod yield were monitored. The number of nodules per plant root system was determined at the pod stage. To that end, three plants per plot were harvested; their root systems were washed to remove adhering soil and the number of nodules was counted.

Identification of bacterial genes involved in antifungal activity

To identify genes involved in activity of *Pseudomonas* sp. strain R4D2 against *S. rolfsii*, we conducted random transposon mutagenesis by bi-parental mating of a spontaneous rifampicin-resistant derivative of R4D2 with *E. coli S17*-1 (λ*pir*) containing plasmid pUT Lux*CDABE* Tc^R (Winson *et al.* 1998). Mutants were selected on PSA plates supplemented with tetracyclin (100 µg ml^{-1}) and rifampicin (100 µg ml^{-1}) and transferred to microtitre plates containing 100 µl King's medium B per well. After 2 days of growth at 25°C, transformants were tested for *in vitro* activity against *S. rolfsii* as described above. For mutants that had lost the ability to inhibit hyphal growth of *S. rolfsii,* the regions flanking the transposon insertion were amplified using the Y-linker method (Kwon and Ricke 2000). Briefly, genomic DNA was digested with a restriction enzyme (*Nla*III or *Sph*I) and ligated with Y-linkers 1 and 2. The sequence of Y-linker 1 is 5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG-3', and that of Y-linker 2 is 5'-TGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT-3'. To amplify the genetic region flanking the transposon insertion, Y-linker primer 5'-CTGCTCGAATTCAAGCTTCT-3' and Tn*5* primer 5'-GGCCAGATCTGATCAAGAGA-3' were used in PCR. Amplicons were purified and sent for sequencing (Macrogen Corp. Europe, Amsterdam, The Netherlands). Quality assessment and analysis of the obtained sequences were performed with Vector NTI (Invitrogen, version 8.0). For BLAST searches, databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Computational & Genome Biology Initiative Bioinformatics (CGBI: http://cgbi.cgrb.oregonstate.edu/) were used. As a second approach to identify the regions flanking the transposon insertion, we used the inverse PCR method (Lewenza *et al.* 2005). For this, genomic DNA was digested with *Nar*I or *Sst*II. Linear genomic fragments were circularized by ligation with T4 DNA Ligase. The ligation product was used as template for an inverse PCR (IPCR) reaction using outward facing transposon-specific primers. Primer 1 (Tn*5*-*Nar*I) and primer 2 (Tn*5*-out2) for DNA digested by *Nar*I; primer 1 (Tn*5*-*Sst*II), and primer 2 (Tn*5*-out) for DNA digested by *Sst*II.

Mutants of strain R4D2 that showed no or reduced activity against *S. rolfsii* were also characterized phenotypically by analyzing their growth rate, swarming motility, and their capacities to form biofilms and to produce biosurfactant compounds. The methods used for these phenotypic traits were described previously by De Bruijn *et al.* (2007; 2008).

Growth chamber bioassay

The efficacy of *Pseudomonas* strain R4D2 wildtype and its mutants to control *S. rolfsii* infection of groundnut was investigated in growth chamber bioassays at Wageningen University (Wageningen, Netherlands). Growth chamber conditions comprised a continuous temperature of 25ºC during day (12h) and night (12h), a relative air humidity of 80%, and a light intensity of 315 μmol light m^{-2} s^{-1}. Groundnut seeds were surface sterilized in 3% (v/v) sodium hypochlorite solution for 15 min, rinsed with ethanol 70% (v/v) and rinsed three times with ample sterile distilled water. Sterilized seeds were soaked in sterile water for 4 h at 25°C and then incubated in Petri dishes at 25°C overnight. The pre-germinated seeds were subsequently soaked for 30 min in bacterial suspension with a density of 10^7 cells ml^{-1}. For the control treatment, germinated seeds were soaked in sterile water for 30 min. Treated seeds were sown in pots containing 250 g of a mix 1:3 (w/w) of commercial potting soil and river sand. Each treatment consisted of three replications with five plants per replication. In the growth chamber, the treatments were randomized. Infection of seedlings with *S. rolfsii* and disease assessment was monitored as described above for the nethouse experiment.

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Appendix 1. BOX-PCR grouping of bacterial isolates from stem base and roots of groundnut plants grown in farmer fields in 2010 in central Vietnam. *In vitro* antagonism refers to the inhibition of hyphal growth of the fungal pathogen *Sclerotium rolfsii*.

Stem base			Roots		
BOX-PCR Group	No. of isolates	*In vitro* antagonism	BOX-PCR Group	No. of isolates	*In vitro* antagonism
1	1	+	20	4	-
2	3	+	21	1	-
3	1	+	22	2	-
4	1	+	23	3	-
5	1	-	24	1	-
6	1	-	25	1	-
7	1	-	26	1	-
8	1	-	27	1	+
9	1	-	28	2	-
10	1	-	29	4	-
11	1	-	30	1	-
12	1	-	31	1	-
13	1	-	32	1	-
14	1	-	33	2	-
15	1	-	34	1	-
16	1	-	35	1	-
17	1	-	36	2	-
18	1	-	37	6	+
19	1	-	38	1	-
			39	1	-
			40	1	-
			41	1	-
			42	1	-

Chapter 5

Biological control of groundnut diseases and plant growth promotion by beneficial *Pseudomonas* and *Bacillus* species

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To be submitted

Biological control of groundnut diseases and plant growth promotion by beneficial *Pseudomonas* and *Bacillus* species

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Abstract

In Vietnam, groundnut yield is adversely affected by several diseases, including stem rot disease caused by *Sclerotium rolfsii*. In this study, various *Pseudomonas* and *Bacillus* strains were tested in field experiments in Thua Thien Hue province, Vietnam for their abilities to (i) control stem rot and other groundnut diseases, and (ii) promote plant growth and enhance pod yield. *Pseudomonas* sp. strains SH-C52 and R4D2 significantly reduced stem rot disease of groundnut to the same level as the azole fungicide Folicur. Both bacterial strains were not effective against *Rhizoctonia* damping-off disease, black collar rot caused by *Aspergillus niger*, and early and late leaf spot caused by *Cercospora arachidicola* and *Cercosporidium personatum,* respectively. Both strains, however, improved pod yield up to 14% relative to the untreated control. Also *Pseudomonas fluorescens* strain SS101 and *Bacillus* sp. strains S18F11 and S20D12 led to significant increases in pod yield (up to 30%). These three strains were unable to control stem rot, damping-off, black collar rot, and bacterial wilt disease but significantly reduced early and late leaf spot diseases. Collectively, these field experiments in central Vietnam indicate that specific *Pseudomonas* and *Bacillus* strains are promising candidates for integrated management of various groundnut diseases and for improving pod yield.

Keywords: *Bacillus, Pseudomonas, Sclerotium rolfsii*

Introduction

Groundnut (*Arachis hypogaea* L.) is a major source of edible oil, vitamins, and amino acids, and used extensively for food and feed (Woodroof 1983; Florkowski 1994; Savage and Keenan 1994). In terms of economic importance, groundnut ranks thirteenth among the world food crops and tops the list of oil seed crops both in terms of acreage and production (Sharma 2002). It is cultivated in more than 80 countries in the tropics and subtropics (Ramantha Rao and Murty 1994). In 2009, groundnut was grown on 24 million hectares worldwide with an estimated total production of 36.5 million ton (FAO, 2011). In most developing countries, however, groundnut yield is low. For example, yield in India was only around 1,000 kg ha^{-1} in 2009 compared to 3,350 kg ha^{-1} in China and 3,800 kg ha^{-1} in the USA. The average groundnut yield in the world is around 1,500 kg ha^{-1} (FAO, 2011).

In Vietnam, groundnut is the second annual crop after paddy rice with a total area of 249,200 ha and an average yield of 2,100 kg ha^{-1} in 2009 (FAO, 2011). Vietnam ranks fifth among the groundnut-producing countries after China, India, USA and Argentina (Fabra *et al.* 2010). Groundnut cultivation in Vietnam and other countries is hampered by a wide range of pests and diseases (Middleton *et al.* 1994; Wightman and Ranga Rao 1994; Mehan and Hong 1994; Pham 1997; Le 2004; Nguyen *et al.* 2004; Brown 2007; Shew and Waliyar 2007). The most widespread fungal and bacterial diseases of groundnut are stem and pod rot caused by *Sclerotium rolfsii*, black collar rot by *Aspergillus niger,* damping-off by *Rhizoctonia solani,* bacterial wilt by *Ralstonia solanacearum*, rust by *Puccinia arachidis*, early leaf spot by *Cercospora arachidicola,* and late leaf spot by *Cercosporidium personatum* (Porter *et al.* 1984; Shew and Waliyar 2007).

Breeding for new groundnut cultivars, improving fertilization and applying chemical pesticides are measures to improve groundnut growth and yield. However, several of these measures are costly for subsistence farmers in Vietnam and other developing countries. In this context, biological control has been proposed as an environmentally friendly, affordable and sustainable means to control pests and diseases (Fravel 2005; Höfte and Altier 2010). Biological control may also offer a solution for controlling pests and diseases for which no other effective measures are available or when agricultural products must be certified as 'organic' without pesticide residues (Fravel 2005). Biological control based on treating soil, seeds or other planting materials with beneficial microorganisms may not only limit the proliferation of pests and diseases, but may also directly promote plant growth or result in the degradation of toxic soil pollutants (van Loon 2007; Lugtenberg and Kamilova 2009).

In the past decades, various strains of *Pseudomonas* and *Bacillus* species have been screened for their abilities to control specific groundnut diseases (Ganesan and Gnanamanickam 1987; Savithiry and Gnanamanickam 1987; Kokalis-Burelle *et al.* 1992; Shanmugam *et al.* 2002; Abd-Alla and Ezzat 2003; Dey *et al.* 2004; Abd-Allah 2005; Kishore *et al.* 2005a; Kishore *et al.* 2005b; Murugalakshmi *et al.* 2009; Senthilraja *et al.* 2010a; Senthilraja *et al.*

2010b; Taurian *et al.* 2010; Tonelli *et al.* 2010; Tonelli *et al*. 2011). In nethouse and field studies conducted in 2010 in Vietnam, we identified several *Pseudomonas* and *Bacillus* strains that significantly suppressed stem rot disease caused by *S. rolfsii* (Chapters 3 & 4). For two of these strains, i.e. *Pseudomonas* sp. strains SH-C52 and R4D2, the ability to suppress stem rot disease appeared to be linked to the production of biosurfactant compounds (Chapters 3 & 4). Another set of strains, comprising *P. fluorescens* strain SS101 and *Bacillus* sp. strains S18F11 and S20D12, did not significantly or consistently suppress stem rot disease in nethouse and field experiments in 2010, but substantially enhanced pod yield up to 21% relative to the untreated control (Chapters 3 & 4). For successful implementation of one or more of these bacterial strains in groundnut cultivation, their consistency to suppress diseases and/or to improve yield under field conditions should be evaluated as well as their efficacy to control other root and leaf diseases of groundnut. In this study, field experiments were conducted in central Vietnam in 2011 to determine the efficacy of *Pseudomonas* strains SH-C52, R4D2 and SS101, and of *Bacillus* strains S18F11 and S20D12 to (i) suppress stem rot disease, damping-off, black collar rot, leaf spots and bacterial wilt, and (ii) promote plant growth and enhance pod yield.

Results and Discussion

Biocontrol of groundnut diseases by Pseudomonas

As reported previously (Chapters 3 & 4), *Pseudomonas* sp. strains SH-C52 and R4D2 significantly suppressed stem rot disease caused by *S. rolfsii* in nethouse and field experiments conducted in 2010 in Vietnam. Also in the 2011 field experiment, application of *Pseudomonas* sp. strains SH-C52 and R4D2 resulted in a significant suppression of stem rot disease caused by *S. rolfsii* (Fig. 1A, B). At pod set stage, i.e. 10 weeks after sowing, disease incidence was significantly lower in the two bacterial treatments than in the control treatment (Fig. 1A). Both strains were as effective as Folicur (Fig. 1A, B), the azole fungicide commonly used to control *S. rolfsii* (Brenneman and Murphy, 1991). Strains SH-C52 and R4D2 established similar densities on the stem base and roots of groundnut ranging from 4.5- 5 log cfu g^{-1} after 8 weeks of plant growth (Fig. 1C). For strain SH-C25, the chlorinated lipopeptide thanamycin plays a key role in suppression of *S. rolfsii* (Chapter 3). Also for strain R4D2, a biosurfactant appears to be an important factor in growth inhibition and biocontrol of *S. rolfsii* (Chapter 4), but the chemical nature of this biosurfactant and the corresponding biosynthesis genes have not yet been identified.

Given that biosurfactant compounds, and in particular lipopeptides, have a broad-spectrum antimicrobial activity (Raaijmakers 2006; D'aes *et al.* 2010; Raaijmakers *et al.* 2010; Mendes *et al.* 2011), and are known to induce systemic resistance in plants against oomycete and fungal pathogens (Ongena *et al.* 2007; Tran *et al.* 2007), we also monitored the effects of strains SH-C52 and R4D2 on various other diseases of groundnut. The results showed that the incidence of *Rhizoctonia* damping-off disease was low to insignificant and that the bacterial

strains and Folicur did not affect this disease (Table 1). The incidence of black collar rot, caused by *Aspergillus niger*, was higher, but also for this disease no significant effects of the two bacterial treatments and Folicur were observed (Table 1). *Pseudomonas* strain R4D2 significantly reduced bacterial wilt caused by *R. solanacearum* at pod set stage (Table 1). Folicur was not effective and strain SH-C52 reduced wilt disease to a level intermediate between the control and R4D2 treatment (Table 1).

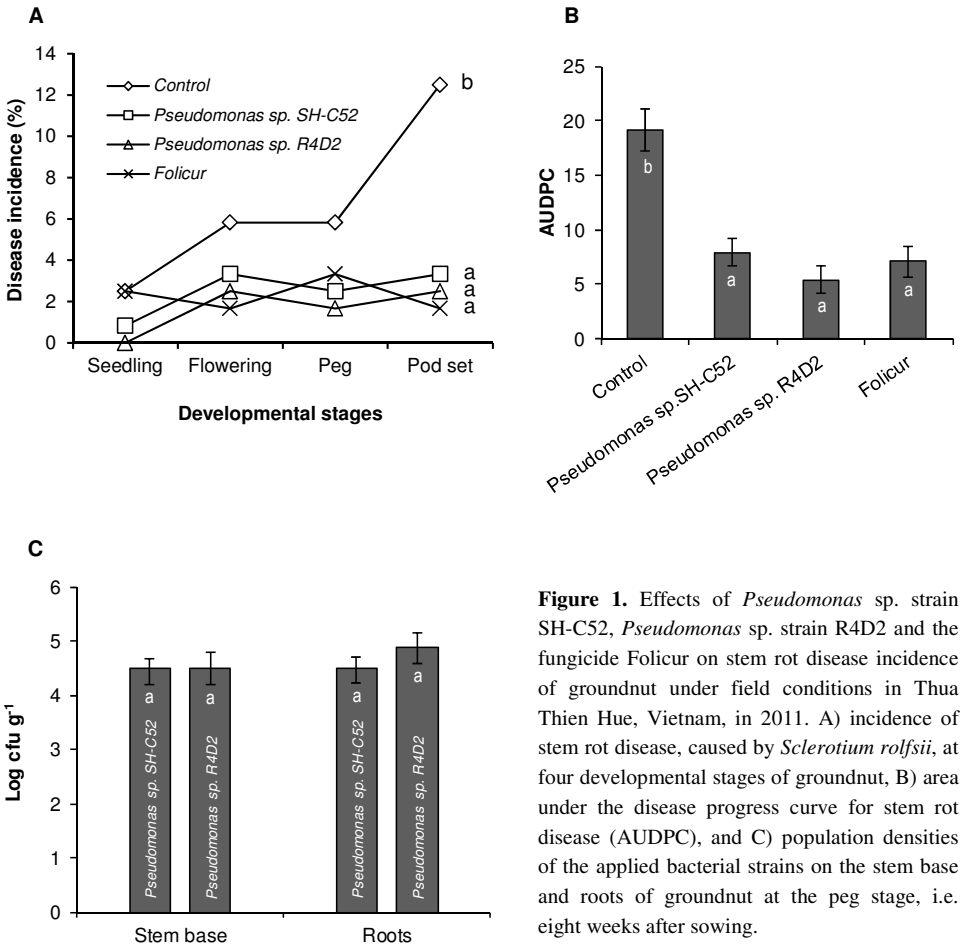

Figure 1. Effects of *Pseudomonas* sp. strain SH-C52, *Pseudomonas* sp. strain R4D2 and the fungicide Folicur on stem rot disease incidence of groundnut under field conditions in Thua Thien Hue, Vietnam, in 2011. A) incidence of stem rot disease, caused by *Sclerotium rolfsii*, at four developmental stages of groundnut, B) area under the disease progress curve for stem rot disease (AUDPC), and C) population densities of the applied bacterial strains on the stem base and roots of groundnut at the peg stage, i.e. eight weeks after sowing.

The incidence and severity of leaf spot diseases caused by *C. arachidicola* and *C. personatum* were not reduced by the two bacterial treatments (Table 1). Also Folicur treatment did not lead to a reduction in leaf spot diseases (Table 1), which is in contrast to results of earlier studies conducted in Georgia and in Texas, USA (Brenneman and Murphy 1991; Besler *et al.* 2006). This may be due to the limited number of fungicide applications in our experiment (i.e. 3 times at a rate of 250 g a.i. ha^{-1}) as compared to other studies, with seven applications in

experiments in Georgia (USA) at a rate of 188 g a.i. ha^{-1} (Brenneman and Murphy 1991), and four applications in Texas (USA) at a rate of 230 g a.i. ha^{-1} (Besler *et al.* 2006). Furthermore, since *S. rolfsii* was the primary target pathogen in our experiment, the fungicide was applied mostly to the stem base of groundnut. As a result, many leaves of groundnut were not exposed to the fungicide.

Table 1. Effects of *Pseudomonas* sp. strain SH-C52, *Pseudomonas* sp. strain R4D2 and the fungicide Folicur on the incidence and severity of different groundnut diseases under field conditions. The experiment was conducted in 2011 in Thua Thien Hue province, Vietnam. For each of the parameters, average values of four replications are given. For each row, different letters indicate a statistically significant difference between the treatments (*P=0.05*, Duncan Multi Range Test).

Disease	Treatment			
	Control	*Pseudomonas* sp. SH-C52	*Pseudomonas* sp. R4D2	Folicur
Damping off (*Rhizoctonia solani*) (%)				
	1.58 a	1.18 a	0.80 a	1.30 a
Black collar rot (*Aspergillus niger*) (AUDPC)*				
	8.75 a	6.25 a	7.92 a	6.25 a
Bacterial wilt (*Ralstonia solanacearum*) (%)				
	2.79 b	1.74 ab	0.94 a	2.04 b
Leaf spot (*Cercospora arachidicola, Cercosporidium personatum*) (%)**				
DI-F	66.5 a	66.5 a	69.0 a	66.5 a
DS-F	26.0 a	25.3 a	26.0 a	24.9 a
DI-Peg	42.0 a	36.0 a	39.5 a	44.0 a
DS-Peg	15.6 a	12.7 a	16.6 a	15.9 a

*AUDPC- area under disease progress curve. **Fifty leaves of each plot were investigated for leaf spot diseases. DI-F: disease incidence at flowering stage, DS-F: disease severity at flowering stage, DI-Peg: DI at peg stage, DS-Peg: DS at peg stage.

Bacterial strains R4D2 and SH-C52 did not promote or adversely affect plant biomass and nodulation (Table 2), and did not significantly alter the nitrogen and carbon contents of groundnut (Appendix 1A). However, both strains did lead to significant pod yield increases of groundnut up to 13.8% for strain R4D2 (Table 2). The pod yield in the R4D2 treatment was also significantly higher than that in the fungicide treatment (Table 2). Given that Folicur reduced stem rot disease to a similar level as the bacterial treatments, there seems to be no apparent correlation between a reduction of stem rot disease incidence and an increase in pod yield.

Table 2. Effects of *Pseudomonas* sp. strain SH-C52, *Pseudomonas* sp. strain R4D2, and the fungicide Folicur on emergence, plant biomass (fresh weight (FW) and dry weight (DW)), number and dry weight of root nodules, number of flowers, and dry pod yield of groundnut. The field experiment was conducted in 2011 in Thua Thien Hue province, Vietnam. For each parameter, averages of four replications are given. For each row, different letters indicate a statistically significant difference between the treatments (*P=0.05*, Duncan Multi Range Test).

Characters	Treatment			
	Control	*Pseudomonas* sp. SH-C52	*Pseudomonas* sp. R4D2	Folicur
Emergence at two weeks after sowing (%)				
	77.8 a	74.3 a	74.8 a	73.1 a
Biomass per plant (gram)				
Shoot FW	50.9 a	49. 9 a	50.2 a	51.4 a
Shoot DW	9.1 a	9.1 a	9.1 a	8.8 a
Roots FW	3.7 ab	3.1 b	4.0 a	3.7 ab
Roots DW	0.6 a	0.5 a	0.7 a	0.6 a
Nodulation				
Nodules plant $^{-1}$	168 a	193 a	202 a	173 a
DW (mg)	109 a	110 a	101 a	107 a
Flowers plant^{-1}				
	39.1 a	37.9 a	36.4 a	36.7 a
Dry pod yield				
kg ha^{-1}	2317 c	2565 ab	2636 a	2408 bc
Average increase (%)		10.7	13.8	3.9

Plant growth promotion by Pseudomonas and Bacillus

As reported in Chapters 3 and 4, the 2010 nethouse and field experiments revealed that *P. fluorescens* strain SS101 and *Bacillus* sp. strains S18F11 and S20D12 can substantially enhance pod yield. In 2011, plant growth promotion by these strains was again evaluated. Application of *P. fluorescens* strain SS101 or *Bacillus* strains S18F11 or S20D12 did not have significant effects on seedling emergence and biomass (Table 3). The three bacterial strains also had no significant adverse or positive effects on nodulation, except that the dry weight of the nodules was significantly higher for groundnut plants treated with strain SS101 (Table 3). All three bacterial strains increased the total number of branches per plant, i.e. the cotyledonary axes (CA), lateral axes and second order branches (Table 3). According to Cattan and Fleury (1998), the number of branches may affect flower production and consequently pod yield. Although there was no statistically significant increase in the number of flowers per plant for each of the three bacterial treatments, both *Bacillus* strains did significantly increase pod weight (P100) and pod yield relative to the control treatment, with yield increases of 25-30% (Table 3). The effect

of strain SS101 on pod weight (P100) and yield was intermediate between the control and the *Bacillus* treatments (Table 3).

Table 3. Effects of *Pseudomonas fluorescens* strain SS101, *Bacillus* sp. strain S18F11 and *Bacillus* sp. strain S20D12 on emergence, number of branches, plant biomass (fresh weight (FW) and dry weight (DW)), number and dry weight of nodules, number of flowers and dry pod yield of groundnut. The field experiment was conducted in 2011 in Thua Thien Hue province, Vietnam. For each parameter, averages of four replications are given. For each row, different letters indicate a statistically significant difference between the treatments (*P=0.05*, Duncan Multi Range Test).

Characters	Treatment			
	Control	*P. fluorescens* SS101	*Bacillus* sp. S18F11	*Bacillus* sp. S20D12
Emergence at two weeks after sowing (%)				
	72 a	78 a	78 a	77 a
No. of branches per plant				
1st	4.1 b	4.1 b	4.6 a	4.2 b
2nd	3.6 a	3.9 a	3.9 a	4.0 a
Total	7.6 c	8.1 b	8.5 a	8.2 ab
Biomass per plant (gram)				
Shoot FW	49.3 a	50.0 a	49.7 a	50.5 a
Shoot DW	8.7 a	8.5 a	8.8 a	9.1 a
Roots FW	3.3 a	3.6 a	3.3 a	3.6 a
Roots DW	0.5 a	0.5 a	0.6 a	0.5 a
Nodulation per plant				
Nodules plant $^{-1}$	144 a	210 a	153 a	182 a
DW (mg)	100 b	130 a	100 b	110 ab
Flowers plant^{-1}				
	42.9 a	47.8 a	46.2 a	47.9 a
Yield components				
No. of pod plant^{-1}	21.3 a	21.8 a	22.0 a	20.5 a
*No. of M-pod plant^{-1}	15.0 a	16.3 a	15.5 a	16.0 a
**P100 (gram)	121 b	126 ab	132 a	133 a
Dry pod yield				
kg ha^{-1}	2244 c	2461 bc	2817 ab	2924 a
Average increase (%)		9.7	25.5	30.3

*Marketable pod, **Dry weight of 100 pods

Suppression of leaf spot by Pseudomonas and Bacillus

Bacillus and *Pseudomonas* species are well-known for their abilities to promote plant growth and yield (Kloepper *et al.* 1980; van Loon 2007; Weller 2007; Lugtenberg and Kamilova 2009). They can directly affect plant growth through, among others, facilitating nutrient acquisition, the production of volatile compounds or plant growth hormones (van Loon 2007; Lugtenberg and Kamilova 2009). Indirect plant growth promotion involves suppression of plant pathogens (van Loon 2007; Lugtenberg and Kamilova 2009). To evaluate the role of pathogen suppression in the observed yield increases by strains SS101, S18F11 and S20D12, the incidence and severity of several groundnut diseases were monitored in this field experiment. These were the same root and leaf diseases as were monitored in the biocontrol experiment described above. The results showed that none of the three bacterial strains significantly reduced stem rot, damping-off, black collar rot and bacterial wilt (Table 4). None of three bacterial strains affected nitrogen and carbon contents of groundnut nor macronutrient concentrations in the soil before and after the experiment (Appendix 1B, Appendix 2). However, all three strains significantly reduced leaf spot diseases, with the two *Bacillus* strains having the most significant effects on disease incidence (Table 4). Both leaf spot pathogens can affect plant growth and yield by reducing the available photosynthetic area via lesion formation and leaflet abscission (Backman and Crawford 1984; McDonald *et al.* 1985; Savary and Zadoks 1992). Early and late leaf spots can cause severe defoliation and pod detachment at harvest (Gremillion *et al.* 2011). The study by Backman and Crawford (1984) indicated that all levels of defoliation resulted in yield losses and that there were no differences in yield loss per unit of disease between *C. arachidicola* and *C. personatum*. Regression analyses by Bourgeois and Boote (1992) further indicated that each 1% increase in necrotic leaf area, caused by *C. personatum,* resulted in a 4% reduction in carbon exchange rate of infected leaflets. Their observation that the affected area is four times larger than the visibly damaged area was attributed to the fact that the infected area extends beyond the visible necrotic area. In addition, other factors such as changes in the physiology of the leaf tissue surrounding the area invaded by the pathogen, or the production of toxins, like cercosporin, may also contribute to the reduced photosynthesis rate (Bourgeois *et al*. 1992; Daub and Ehrenshaft 2000).

Worldwide, yield losses caused by leaf spots range from 10% to more than 50%, but can vary considerably from place to place and between seasons (Backman and Crawford 1984; McDonald *et al.* 1985). In Vietnam, leaf spot diseases of groundnut may cause yield losses up to 20-25% in some provinces in the North (Mehan and Hong 1994). To control leaf spots, several strategies were recommended, including cultural measures, resistant cultivars, fungicides or biological control (McDonald *et al.* 1985; Mehan and Hong 1994; Besler *et al.* 2006; Singh *et al.* 2011). Biological control of early and/or late leaf spot of groundnut has not been studied extensively. Initial work done by Mitchell *et al.* (1987) showed that the mycoparasitic fungus *Dicyma pulvinata* does not primarily act as a protectant against infections by *C. personatum,* but can control the secondary spread of this pathogen. Foliar applications

with *Bacillus cereus* strain 304 (Kokalis-Burelle *et al.* 1992) or *Pseudomonas fluorescens* strain Pf1 (Meena *et al.* 2002) showed promising results in the control of early and late leaf spot, respectively. The results of our study further extend these findings and showed that application of specific *Pseudomonas* and *Bacillus* strains to the seed bed of groundnut can significantly reduce leaf spot diseases and substantially enhance pod yield.

Table 4. Effects of *Pseudomonas fluorescens* strain SS101, *Bacillus* sp. strain S18F11 and *Bacillus* sp. strain S20D12 on the incidence and severity of different diseases of groundnut. The field experiment was conducted in 2011 in Thua Thien Hue province, Vietnam. For each of the parameters, average values of four replications are given. For each row, different letters indicate a statistically significant difference between the treatments (*P=0.05*, Duncan Multi Range Test).

Disease	Treatment			
	Control	*P. fluorescens* SS101	*Bacillus* sp. S18F11	*Bacillus* sp. S20D12
Stem rot (*Sclerotium rolfsii*) (AUDPC) *				
	6.7 a	4.2 a	3.8 a	2.9 a
Damping off (*Rhizoctonia solani*) (%)				
	1.32 a	1.40 a	1.03 a	1.07 a
Black collar rot (*Aspergillus niger*) (AUDPC)				
	6.3 a	2.9 a	2.9 a	4.6 a
Bacterial wilt (*Ralstonia solanacearum*) (%)				
	1.84 a	1.63 a	0.83 a	1.39 a
Leaf spot (*Cercospora arachidicola, Cercosporidium personatum*) (%) **				
DI-F	44.0 a	42.5 a	40.1 a	36.5 a
DS-F	17.3 b	15.8 ab	16.7 ab	13.0 a
DI-Peg	34.5 c	29.5 b	24.5 a	23.5 a
DS-Peg	14.2 b	11.4 a	9.1 a	8.8 a
Root colonization	-	5.7 a	5.1 ab	4.8 b

*AUDPC- area under disease progress curve. **Fifty leaves of each plot were investigated for leaf spot diseases. DI-F: disease incidence at flowering stage, DS-F: disease severity at flowering stage, DI-Peg: DI at peg stage, DS-Peg: DS at peg stage.

The mechanisms underlying suppression of leaf spot diseases by strains SS101, S18F11 and S20D12 are not known yet. Induced systemic resistance (ISR) may be a potential mechanism as was reported earlier by Kloepper *et al*. (2004) for biocontrol of other groundnut diseases, i.e. black collar rot caused by *A. niger.* However, the elaborate study by Zhang *et al*. (2001) with nineteen strains of ISR-bacilli and various chemical elicitors of pathogen resistance indicated that groundnut is not systemically inducible in the same manner as other crops.

Whether induced systemic resistance is one of the mechanisms involved for the three bacterial strains tested here remains to be investigated. In the field experiment, the population densities of the applied bacterial strains were only monitored on the stem base and roots of groundnut (Table 4), but not on the leaflets. Therefore, we cannot rule out direct antagonism between the applied bacterial strains and the leaf spot pathogens.

Conclusions

Each of the five *Pseudomonas* and *Bacillus* strains tested in this study improved pod yield of groundnut plants by 10 to 30%. Similar yield increases were observed in field experiments that we conducted in 2010 in central Vietnam (Chapters 3 & 4). This consistency in yield increases observed in two consecutive years in two independent field experiments at two different locations holds great promise for further development of these bacterial strains as a key component of an integrated strategy to manage multiple diseases of groundnut and/or to improve yield.

Materials and Methods

Bacterial strains and culture conditions

P. fluorescens strain SS101, *Pseudomonas* sp. strains SH-C52 and R4D2, and *Bacillus* sp. strains S18F11 and S20D12 were cultured at 25°C on Pseudomonas Agar (PSA; Difco, France) and maintained in 40% glycerol at -80°C in the Laboratory of Phytopathology, Wageningen University, the Netherlands. Phenotypic and genetic characteristics of these five strains were described previously (Chapters 3 & 4). In the field experiments, spontaneous rifampicin-resistant derivatives of these strains were used to monitor their population densities on roots and stem base of groundnut plants by dilution plating on PSA-medium supplemented with rifampin (100 µg ml^{-1}), chloroamphenicol (12.5 µg ml^{-1}), ampicillin (40 µg ml^{-1}) and delvocid (DSM, 100 µg ml^{-1}) (Chapters 3 & 4).

Biological control of S. rolfsii and other groundnut pathogens

The field experiment was conducted in 2011 in Thua Thien Hue province, Vietnam, where on average 15% of the groundnut plants were naturally infected by *S. rolfsii* (Le *et al.* 2011). The experiment consisted of four treatments, i.e. control, *Pseudomonas* sp. strain SH-C52, *Pseudomonas* sp. strain R4D2, and the chemical fungicide tebuconazole (Folicur 250EC, Bayer Crop Science). The field experiment was laid out in a randomized complete block design (RCBD) with four blocks as four replications and a plot size of 15 m^2 (3 X 5 m) (Fig. 2). The distance between rows was 30 cm and between plants 10 cm. *Pseudomonas* strains SH-C52 and R4D2 were grown on PSA plates at 25°C for 48 h, harvested and washed with sterile water. Prior to sowing groundnut seeds, bacterial suspensions were applied to the furrows at a final density of 10^6 cells per cm^2 soil. After 8 weeks of plant growth, three plants per plot were collected to determine the population densities of the applied bacterial strains on the stem base and roots of groundnut according to the methods described (Chapter 3). Folicur was applied three times during the growth season, i.e. 20, 40 and 60 days after sowing, by spraying 250 g active ingredient (a.i.) ha^{-1}. The field soil was fertilized with nitrogen (N) at 40 kg ha^{-1}, phosphorus (P_2O_5) at 60 kg ha^{-1}, potassium (K_2O) at 60 kg ha^{-1}, and calcium ($Ca(OH)_2$) at 300 kg ha^{-1}. All phosphorus and calcium was applied at seed bed preparation. Seeds were sown at a depth of 3-5 cm and covered with soil. When plants had three true leaves (seedling stage), 70% of the nitrogen and 50% of the potassium were applied. The remainder of the N and K fertilizers was applied at flowering. Emergence was monitored at 7, 10 and 13 days after sowing. Weeds were manually removed at four

developmental stages of the groundnut plants, i.e. seedling, flowering, peg and pod set stages, i.e. 4, 6, 8 and 10 weeks after sowing, respectively (Fig. 2).

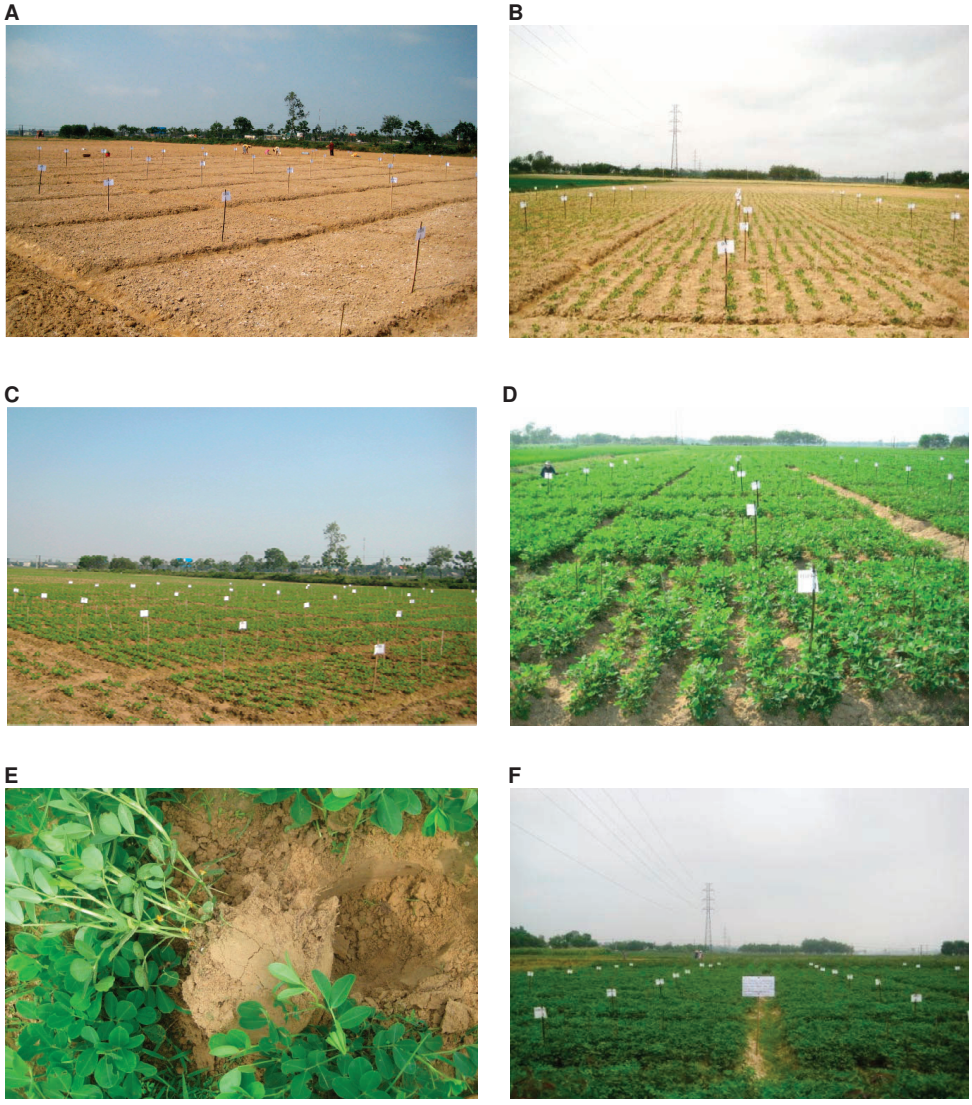

Figure 2. Experimental design of the field experiment conducted in 2011 in Thua Thien Hue province, Vietnam. A) Experimental field three days after sowing; B) Ten plants per plot were labeled at seedling stage (3 true leaves) for measuring plant height, number of branches and branch length; C) Groundnut at seedling stage (5 to 6 true leaves); D) Groundnut at peg stage; E) Sample collection at peg stage; F) Groundnut 10 days before harvesting.

Disease assessments

At each of the four developmental stages (seedling, flowering, peg and pod set), the incidence of stem rot disease by *S. rolfsii* (Fig. 3A) was monitored in 1 m^2 of each plot (~ 33 plants). To further enhance the incidence and

severity of stem rot disease, sclerotia of *S. rolfsii* strain H001 were inoculated into the field soil at a density of approximately 100 sclerotia m^{-2} when the plants had reached the peg stage. Stem rot disease incidence was assessed according to the methods described previously (Chapter 3). Black collar rot caused by *A. niger* was assessed by scoring the number of plants with black collar rot symptoms (Fig. 3B) in 1 m^2 for each plot. Damping-off caused by *R. solani* (Fig. 3C) was monitored at the seedling stage by counting the number of infected plants in 10 m^2 per plot. Bacterial wilt, caused by *R. solanacearum*, was monitored at pod set stage by counting the number of wilted plants (Fig. 3D) in 10 m^2 per plot.

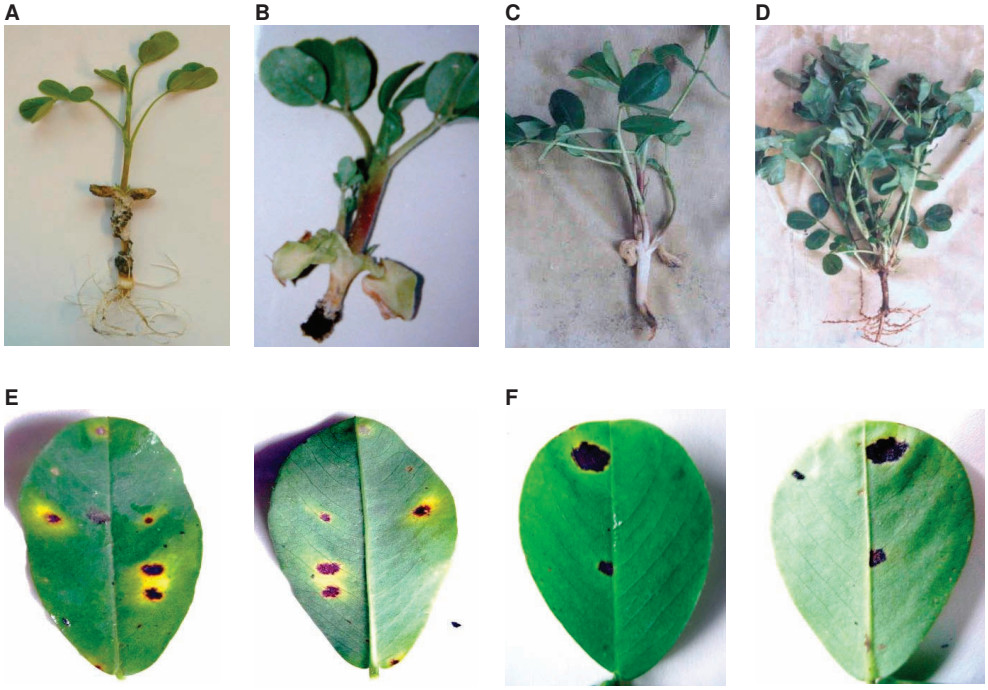

Figure 3. Typical symptoms of major diseases of groundnut monitored in the 2011 field experiment in Thua Thien Hue province, Vietnam. A) Stem rot (*Sclerotium rolfsii*); B) Black collar rot (*Aspergillus niger*); C) damping-off (*Rhizoctonia solani*); D) Bacterial wilt (*Ralstonia solanacearum*); E) Early leaf spot (*Cercospora arachidicola*); F) Late leaf spot (*Cercosporidium personatum*).

Leaf spots caused by *Cercospora arachidicola* and *Cercosporidium personatum* were assessed at flowering and peg stage. Leaf spot incidence (LSI) is defined as the number of infected leaflets divided by the total number of leaflets. Leaf spot severity (LSS) was rated using a scale of 0-5; 0 = no disease symptoms, 1 = <1% of the total leaflet area is affected, 2 = 1- 10%, 3 = 10- 25%, 4 = 25- 50% and 5 = >50% of the total leaflet area is affected. LSS was calculated based on the formula: LSS = [(1× number of leaflets rated as scale 1) + (2 × number of leaflets rated as scale 2) + (3 × number of leaflets rated as scale 3) + (4 × number of leaflet rated as scale 4) +(5 × number of leaflets rated as scale 5)] × 100 ÷ (5 × total number of leaflets). For each of the four plots per treatment, five sites were randomly chosen and for each site ten leaflets were used for LSS assessment.

Plant growth promotion by Pseudomonas and Bacillus

Plant growth promotion by *Pseudomonas fluorescens* strain SS101 and *Bacillus* sp. strains S18F11 and S20D12 was evaluated at the same time and at the same field site in Thua Thien Hue province as the biocontrol experiment described above. Also the experimental set-up and conditions were the same as described above, except

that this experimental field was not inoculated with sclerotia of *S. rolfsii*. Next to the disease assessments described above, plant height, branch length, nodulation, flower production and pod yield were determined. At the seedling stage, ten plants per plot were randomly labelled to determine plant height, branch length, number of pods, number of marketable pods and the weight of 100 pods (P100). During flowering time (from 42 to 64 days after sowing), the number of flowers was monitored daily for five randomly selected plants per plot. Shoots and roots of three randomly selected plants per plot were separated to determine fresh and dry weights. The number and dry weight of root nodules was determined at the peg stage. To that end, three randomly selected plants per plot were harvested; their root systems were washed to remove adhering soil and the number of nodules was counted. Nodules were removed and dried in an oven for 3 days at 105°C to determine dry weight.

Statistical analysis

The Area Under the Disease Progress Curve (AUDPC) of stem rot caused by *S. rolfsii* and black collar rot caused by *A. niger* was calculated using the method of Landa *et al.* (2002) and Kruijt *et al*. (2009). Disease incidence and disease severity values were arcsin-transformed prior to statistical analysis. Statistical differences ($P < 0.05$) between treatments were analyzed by ANOVA followed by the Duncan Multiple Range Test (SPSS Statistics, USA). Normal distribution of the data and homogeneity of variances was tested prior to ANOVA.

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Appendix 1. Carbon (C), nitrogen (N), and C:N ratio in shoots of groundnut plants at peg stage grown in soil untreated (Control) or treated with *Pseudomonas, Bacillus,* or the fungicide Folicur. A) Data from the field plot inoculated with *Sclerotium rolfsii*; B) Data from the field plot without fungal inoculation. Both experiments were conducted in 2011 in Thua Thien Hue province, Vietnam.

A

Treatment	%C	%N	C:N ratio
Control	49.5	3.0	16.6
Pseudomonas sp. SH-C52	49.0	3.2	15.5
Pseudomonas sp. R4D2	48.3	3.3	14.8
Folicur	48.1	3.4	14.1

B

Treatment	%C	%N	C:N ratio
Control	47.2	3.0	15.6
P. fluorescens SS101	47.6	3.5	13.4
Bacillus sp. S18F11	46.7	3.4	13.6
Bacillus sp. S20D12	47.9	3.3	14.6

Appendix 2. Macro-nutrients in the soil prior to and upon completion of the field experiment conducted in 2011 in Thua Thien Hue province, Vietnam.

Sample	pH_{KCl}	OM (%)	Total N (%)	Total K_2O (%)	Total P_2O_5 (%)	Available P_2O_5 (mg 100 g $soil^{-1}$)
Before EX	4.25	1.03	0.05	0.24	0.03	10.75
Control	4.30	1.40	0.04	0.22	0.03	12.65
P. fluorescens SS101	4.31	1.35	0.05	0.22	0.03	11.25
Bacillus sp. S18F11	4.22	1.29	0.05	0.23	0.03	13.68
Bacillus sp. S20D12	4.34	1.45	0.05	0.23	0.05	12.60

Chapter 6

Diversity of endophytic bacteria of groundnut nodules and their effects on plant growth and groundnut diseases

Le, C.N., Thai, T.H. and Raaijmakers, J.M.

Diversity of endophytic bacteria of groundnut nodules and their effects on plant growth and groundnut diseases

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Abstract

Legumes can overcome nitrogen limitation via symbiotic interactions with rhizobia. In this study, bacteria were isolated from surface-sterilized nodules of groundnut plants grown in farmer fields in central Vietnam. Approximately 100 bacterial isolates were grouped by BOX-PCR profiling and identified by 16S-rDNA sequencing. The results showed that the endophytic bacteria of groundnut nodules were genetically diverse. Most of the isolates belonged to the alpha-Proteobacteria, genus *Sphingomonas*. The other bacterial isolates were classified as *Rhizobium, Stenotrophomonas, Ralstonia* and *Burkholderia* species. One group of isolates belonged to the phylum Bacteroidetes, genus *Sphingobacterium*. Most isolates did not induce nodule formation when re-introduced on groundnut plants, except for some *Sphingomonas* isolates. Under field conditions, *Sphingomonas* sp. strain HR32 and *Rhizobium* sp. strain HR9 suppressed leaf spot diseases of groundnut and increased the pod yield. The potential of these endophytic bacteria as inoculants for groundnut cultivation are discussed.

Keywords: nitrogen fixation, *Arachis hypogaea*, *Rhizobium, Sphingomonas*

Introduction

The low availability of nitrogen (N) in agricultural ecosystems often limits plant growth and crop productivity. Legumes can overcome this nutrient limitation via symbiotic interactions with rhizobia (Fabra *et al.* 2010). During this interaction, bacteria invade the plant root and induce the formation of organelle-like structures called nodules. Inside the nodules, rhizobia convert atmospheric di-nitrogen (N_2) into ammonia, which is then used by the plant as a nitrogen source (Broughton *et al.* 2000). N_2 fixation is catalysed by the nitrogenase complex

encoded by the *nifHDK* genes (Dixon and Kahn 2004). Rates of symbiotic N_2 fixation in legumes can vary with host, symbiont and environment, but rates as high as 600 kg N fixed ha^{-1} yr^{-1} have been reported in clover pastures (Unkovich and Pate 2000). Among the legume crops, groundnut is cultivated in more than 80 countries and is the most important oil seed crop worldwide (Rao and Murty 1994). To date, there are only few studies on N_2 fixation in groundnut. The available data suggest that N_2 fixation is highly variable with rates ranging from 32 to 206 kg ha^{-1} (Giller *et al.* 1987; Bell and Wright 1994; Unkovich and Pate 2000).

Before entering the legume root tissue, rhizobia must move towards and colonize the root surface (Fabra *et al.* 2010). In this process of recognition and adhesion to the root, symbiotic signals are exchanged between the host plant and the bacterium to initiate nodule formation and development. Flavonoids are released by plant roots and constitute important signals in interactions between legumes and rhizobia (Broughton *et al.* 2000). Flavonoids belong to a diverse family of aromatic compounds derived from secondary plant metabolism. Groundnut produces some flavonoids such as chrysin, luteolin, apigenin, genistein that were reported to induce expression of *nod* genes in rhizobia (Angelini *et al.* 2003). Depending on the host and bacterium, these compounds activate the bacterial transcriptional regulator NodD, which in turn induces the transcription of other nodulation genes (*nod, nol* and *noe* genes), whose products are involved in the synthesis of the Nod factors (Spaink 2000). Nod factors are lipo-chito-oligosaccharides that cause morphological changes in legume root hairs, leading to infection thread formation, nodule development and symbiotic nitrogen fixation (Fabra *et al.* 2010). Rhizobia enter roots via infection threads but can also enter the roots via wounds or via cavities between undamaged epidermal cells. In groundnut, root nodules develop at the sites of lateral-root emergence (Uheda *et al.* 2001) where the epidermis of the parent roots is broken (Boogerd and van Rossum 1997).

Among the N_2-fixing bacteria on groundnut, *Bradyrhizobium* species were the most identified (van Rossum *et al.* 1995; Urtz and Elkan 1996; Zhang *et al.* 1999; Saleena *et al.* 2001). Morphological, physiological and molecular analyses indicated that groundnut symbionts obtained from different geographical regions are highly diverse (reviewed in Fabra *et al.* (2010)). Based on ITS-RFLP analysis and 16S-rDNA sequencing, Yang and Zhou (2008) reported that isolates from nodules of groundnut in China were phylogenetically related to *Bradyrhizobium liaoningense, Bradyrhizobium japonicum* and *Bradyrhizobium yuanmingense*. Through 16S-rDNA sequencing and RFLP analysis, Taurian *et al.* (2006) and El-Akhal *et al.* (2008) showed that also different *Rhizobium* species were associated with groundnut nodules. In recent studies, various other bacterial genera, including *Pseudomonas, Enterobacter* and *Klebsiella* species, were isolated from surface-sterilized nodules of groundnut (Ibanez *et al.* 2009). These bacteria were unable to induce nodule formation in groundnut plants, but promoted plant growth when re-inoculated on groundnut (Ibanez *et al.* 2009). Also other bacterial genera, including *Inquilinus, Rhodopseudomonas, Phyllobacterium, Ochrobactrum,*

Sphingomonas, Pseudomonas, Microbacterium, Bacillus, and *Paenibacillus,* were reported to be associated with root nodules of legumes in Tunisia, however, they did not form nodules on the wide host spectrum legume species *Macroptilium atropurpureum* (Zakhia *et al.* 2006).

In this study, bacteria were isolated from surface-sterilized nodules of groundnut plants grown in farmer fields in central Vietnam. The genotypic diversity of approximately 100 isolates was assessed by BOX-PCR profiling and representative isolates were subsequently characterized by 16S-rDNA sequencing. The isolates were tested for nodule formation on groundnut and for hyphal growth inhibition of *Sclerotium rolfsii,* an important soil-borne fungal pathogen of groundnut. Two of the nodule-associated bacterial strains, identified as *Sphingomonas* and *Rhizobium* species, were evaluated under field conditions in central Vietnam for their efficacy to promote plant growth and to control stem rot and other diseases of groundnut.

Results and discussion

Isolation and characterization of bacteria associated with groundnut nodules

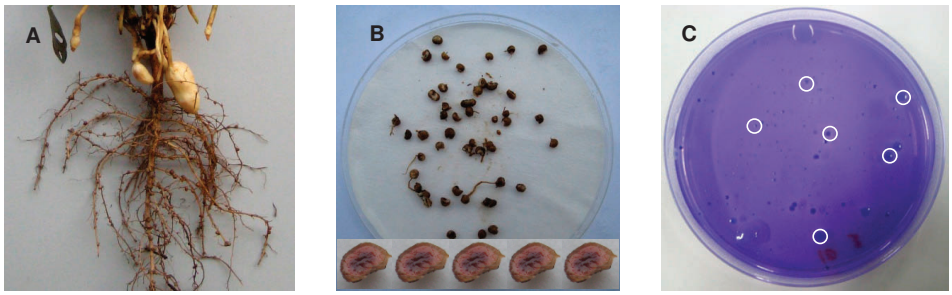

Figure 1. Schematic overview of groundnut nodule selection and isolation of endophytic bacteria. (A) roots of groundnut plants were collected at peg stage, (B) nodules were removed from roots and large nodules (cross sections) were selected, (C) morphology of bacterial colonies isolated from surface-sterilized groundnut nodules on YMA medium supplemented with crystal violet. The watery, translucent colonies that were selected for further analysis are circled.

Bacteria were isolated from surface-sterilized groundnut nodules on YMA medium supplemented with crystal violet (Fig. 1). After incubation at 25°C for three to six days, 99 bacterial colonies with a watery, gummy, translucent phenotype, which is typical for rhizobia (Vincent 1970; Fig. 1), were selected and purified. BOX-PCR analysis showed that these 99 bacterial isolates belonged to 23 groups, designed BG1-BG23 (Table 1). Eleven groups contained only one isolate, whereas several other BOX-PCR groups comprised ten isolates or more (Table 1). These results suggest that bacterial communities living inside groundnut nodules are genotypically diverse, which confirm and extend the results obtained by Angelini *et*

al. (2011), who analyzed, by a similar technique (i.e. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR), the diversity of bacteria from nodules of groundnut plants grown in Argentina.

Table 1. Genetic characterization of endophytic bacteria associated with nodules of groundnut plants grown in farmer fields in central Vietnam. A total of 99 isolates was grouped by BOX-PCR profiling in 23 groups, designated BG-1 through BG-23. One representative isolate of each group was tested for nodule formation on groundnut roots under nethouse conditions. All 99 isolates were tested for their ability to inhibit hyphal growth of *Sclerotium rolfsii in vitro*.

BOX-PCR group*	16S-rDNA sequenced isolate**	Classification based on 16S-rDNA sequence analysis	Nodule formation (No. of nodules plant^{-1})	*S. rolfsii* inhibition *in vitro*
BG-1 (7)	HR101	*Sphingomonas* sp.	0	-
BG-2 (14)	HR32	*Sphingomonas* sp.	10	-
BG-3 (3)	HR118	*Sphingomonas* sp.	3	-
BG-4 (23)	HR71	*Sphingomonas* sp.	5	-
BG-5 (9)	HR67	*Sphingomonas* sp.	4	-
BG-6 (4)	HR64	*Sphingomonas* sp.	1	-
BG-7 (1)	HR7	*Stenotrophomonas* sp.	0	-
BG-8 (1)	HR9	*Rhizobium* sp.	0	-
BG-9 (1)	HR10	*Rhizobium* sp.	1	-
BG-10 (10)	HR42	*Sphingobacterium* sp.	6	-
BG-11 (2)	HR54	*Sphingomonas* sp.	5	-
BG-12 (1)	HR2	*Sphingomonas* sp.	0	-
BG-13 (1)	HR21	*Ralstonia* sp.	0	-
BG-14 (1)	HR27	*Sphingomonas* sp.	3	-
BG-15 (1)	HR49	*Sphingomonas* sp.	0	-
BG-16 (2)	HR51	*Sphingomonas* sp.	0	-
BG-17 (1)	HR55	*Sphingomonas* sp.	0	-
BG-18 (6)	HR75	*Sphingomonas* sp.	10	-
BG-19 (1)	HR77	*Burkholderia* sp.	0	+
BG-20 (2)	HR82	*Sphingomonas* sp.	0	-
BG-21 (1)	HR94	*Sphingomonas* sp.	0	-
BG-22 (1)	HR95	*Sphingomonas* sp.	0	-
BG-23 (6)	HR5	*Burkholderia* sp.	0	+
	Control		0	-

*The numbers of isolates belonging to a certain BOX-PCR group are indicated between brackets; **These isolates were selected for 16S-rDNA sequencing (details see figure 2).

To further identify the bacterial isolates, 16S-rDNA sequencing was performed for one isolate per BOX-PCR group. Ribosomal database comparisons revealed that almost all of the isolates, i.e. 22 out of 23, belonged to the phylum *Proteobacteria* (Fig. 2). One isolate, representative of BOX-PCR group BG-10, was closely related to *Sphingobacterium* species within the phylum Bacteroidetes (Fig. 2). For the 22 isolates that were classified as

Proteobacteria, seventeen belonged to the α-Proteobacteria, two to the β-Proteobacteria and three to the γ-Proteobacteria (Fig. 2). Almost all of the isolates (i.e. 15 out of 17) within the α-Proteobacteria clade were phylogenetically related to *Sphingomonas* species, whereas the two isolates from BOX-PCR groups BG-8 and BG-9 were related to *Rhizobium* (Fig. 2). Zakhia *et al.* (2006) described the isolation of *Sphingomonas* species from nodules of three different legumes (*Astragalus gombiformis, Lotus argenteus,* and *Calycotome villosa*) in Tunisia. These species were, however, not able to form nodules on *Macroptilium atropurpureum*, a legume nodulated by a wide range of bacteria (Zakhia *et al.* 2006). Xie and Yokota (2006) reported that *Sphingomonas azotifigens,* isolated from rice (*Oryza sativa*), was able to fix nitrogen based on the acetylene reduction assay and *nif*H gene detection. Using the same detection assays, Videira *et al.* (2009) also reported N-fixation by *Sphingomonas* isolates from rice in Brazil. They further showed, by sequencing of 16S-rDNA and *nifH* fragments, that most of the nitrogen-fixing isolates clustered apart from *S. azotifigens* (Videira *et al.* 2009). To our knowledge, *Sphingomonas* species have also not been reported to form or associate with nodules on groundnut. When re-introduced into autoclaved field soil, most *Sphingomonas* isolates obtained in our study did not form nodules on groundnut roots (Table 1). However, for some of the identified *Sphingomonas* isolates, in particular isolates H32 (BG-2) and HR75 (BG-18), nodules were found on groundnut roots (Table 1). Whether these isolates colonized and penetrated the root tissue more efficiently than the other isolates, and were actually responsible for nodule formation is not known yet and needs to be investigated. Also the presence of *nif* and *nod* genes as well as the ability of these isolates to fix nitrogen was not determined here and needs to be addressed in future studies.

There are several studies that point to a possible role of *Rhizobium* species in nodule formation on groundnut (Taurian *et al.* 2006; El-Akhal *et al.* 2009). In our study, however, inoculation with isolates HR9 (BG-8) and HR10 (BG-9), which are closely related to *Rhizobium* (Fig. 2), did not or only marginally (i.e. 1 nodule per plant) result in nodule formation on groundnut plants (Table 1). Also isolates HR7, HR21, HR77 and HR5, close relatives of *Stenotrophomonas*, *Ralstonia* or *Burkholderia* (Fig. 2), respectively, did not form nodules on groundnut when re-introduced into autoclaved field soil. *Ralstonia* and *Burkholderia* species have been reported to form nodules on legumes (Chen *et al.* 2003). For example, *Ralstonia taiwanensis* LMG 19424 was reported to form nodules on *Mimosa pudica* (Chen *et al.* 2003). N_2-fixing *Ralstonia* was also isolated from *Dalbergia* species in Madagascar (Rasolomampianina *et al.* 2005).

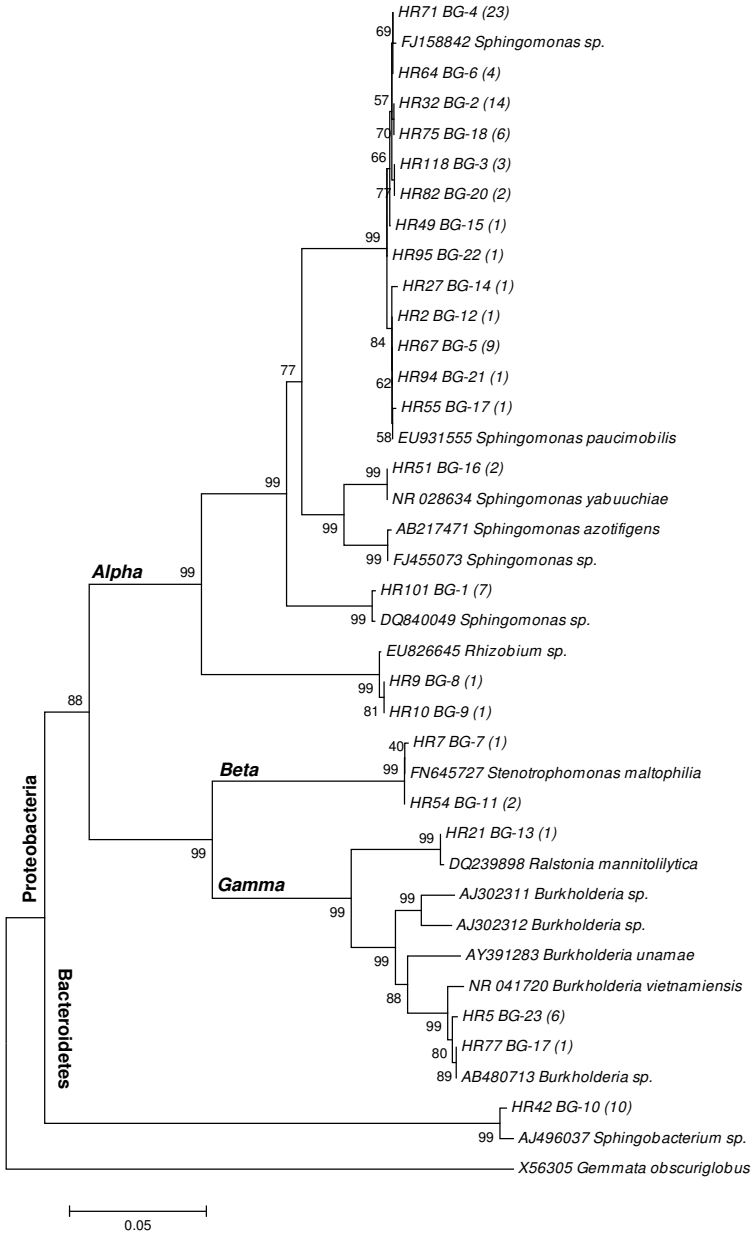

Figure 2. Phylogenetic analysis of 16S-rDNA sequences of 23 bacterial isolates obtained from surface-sterilized groundnut nodules. Each of these 23 isolates is a representative of 23 BOX-PCR groups (designated BG-1 to BG-23) identified among a total of 99 isolates. The branch length in the tree indicates the percentage of sequence dissimilarity and the numbers at the nodes indicate bootstrap values of 1,000 resamplings. In the tree, the name of the isolates is followed by their BOX-PCR group; the number of isolates within each BOX-PCR group is shown between brackets (see Table 1 for details).

Burkholderia species are common inhabitants of the phytosphere and the internal tissues of plants (Compant *et al.* 2008). They are well known as human, animal and plant pathogens, but they can also exert beneficial effects on plants (Compant *et al.* 2008). Nitrogen fixation appears to be common for multiple *Burkholderia* species. The first conclusive report on nodule formation by *Burkholderia* was the study by Moulin *et al.* (2001). In their study, *Burkholderia* sp. strain STM678 contained *nifH* and *nodAB* genes and formed 5 to 20 nodules on roots of a *M. atropurpureum* plant. Other *Burkholderia* species reported to fix nitrogen are *B. vietnamiensis* isolated from rice in Vietnam (Gillis *et al*. 1995), *B. tropica* from sugarcane (Reis *et al.* 2004), *B. unamae* from maize (Caballero-Mellado *et al.* 2004) and *B. silvatlantica* (Perin *et al.* 2006) from teosinte plants. The *Burkholderia* isolates (BG-19, BG-23) identified in our study, however, did not form nodules on groundnut plants (Table 1). Instead, they were the only among the nodule-associated isolates that inhibited hyphal growth of *S. rolfsii* (Table 1).

The *Sphingobacterium* sp. isolate obtained in our study (BG-10) did form nodules per groundnut plant (Table 1). Also here, additional studies will be necessary to confirm these results and to demonstrate N_2-fixation. Previous studies indicated that a *Sphingobacterium* sp. from the rhizosphere of caper plants (*Capparis spinosa*) was able to fix nitrogen (Andrade *et al.* 1997). Members of this genus were also reported to promote plant growth of corn (Marques *et al.* 2010; Mehnaz *et al.* 2010) and to inhibit growth of some *Fusarium* species *in vitro* (Mehnaz *et al.* 2010).

Plant growth promotion and biocontrol of groundnut diseases

For the field experiment conducted in 2011 in central Vietnam, bacterial isolates HR32 and HR9 were selected. Isolate HR32 is closely related to *Sphingomonas* sp., induced nodule formation on groundnut, and represents one of the largest BOX-PCR groups among the endophytic isolates of groundnut nodules (Table 1). Isolate HR9 was chosen because it is closely related to *Rhizobium,* a well-known bacterial genus involved in N_2-fixation and plant growth promotion (Zakhia and de Lajudie 2001). The results of the field experiment showed that both HR9 and HR32 did not significantly increase plant height or the length of the dicotyledonary axes (data not shown). Moreover, no significant effects were observed on seedling emergence, plant biomass, and the number of flowers (Table 2). Although there was a tendency that both HR9 and HR32 increased the number of nodules per plant, these differences were not statistically significant (Table 2). A similar trend was observed for the nitrogen content of the groundnut plants treated with HR9 and HR32 (Appendix 1). Both isolates colonized the roots of groundnut relatively well, reaching densities between 10^5-10^6 CFU g^{-1} of root at the peg stage (Table 3). However, both isolates could not be re-isolated from inside the nodules formed on plants treated with these isolates (data not shown). Despite the fact that no significant effects on plant biomass were found, both bacterial isolates did significantly enhance pod yield, with increases of 15.8 and 18.5% relative to the untreated control (Table 2).

Table 2. Effect of *Rhizobium* sp. isolate HR9 and *Sphingomonas* sp. isolate HR32 on emergence, number of branches, plant biomass (fresh weight (FW) and dry weight (DW)), number and DW of root nodules, number of flowers, yield components and dry pod yield of groundnut. The field experiment was conducted in 2011 in Thua Thien Hue province, Vietnam. For each parameter, averages of four replications are given. For each row, different letters indicate a statistically significant difference between the treatments (*P=0.05*, Duncan Multi Range Test).

	Treatment		
	Control	*Rhizobium* sp. HR9	*Sphingomonas* sp. HR32
Emergence at two weeks after sowing (%)			
	72 a	79 a	73 a
No. of branches per plant			
1st	4.1 b	4.2 a	4.2 a
2nd	3.6 a	3. 8 a	3.9 a
Total	7.6 a	7.9 a	8.1 a
Biomass per plant at peg stage (gram)			
Shoot FW	49.3 a	50.3 a	49.0 a
Shoot DW	8.7 a	9.0 a	8.7 a
Roots FW	3.3 a	3.6 a	3.3 a
Roots DW	0.5 a	0.5 a	0.5 a
Nodulation per plant at peg stage			
No. of nodule plant $^{-1}$	144 a	163 a	177 a
DW (mg)	100 a	120 a	90 a
No. of flowers plant^{-1}			
	42.9 a	42.6 a	42.8 a
Yield components			
No. of pod plant $^{-1}$	21.0 a	21.3 a	23.0 a
* No. M-pod plant^{-1}	15.0 a	14.2 a	15.0 a
** P100 pod (gram)	121 b	123 b	130 a
Dry pod yield			
kg ha^{-1}	2244 b	2598 a	2660 a
Increase (%)		15.8	18.5

*Marketable pods, **Weight of 100 pods.

There was no apparent correlation between these yield increases and the incidence of several major soil-borne diseases: isolates HR9 and HR32 did not provide any control of stem rot, damping-off, black collar rot and bacterial wilt (Table 3). However, both bacterial isolates significantly reduced the incidence and the severity of leaf spots of groundnut, both at flowering and at the peg stage (Table 3). Early and late leaf spots, caused by *C. arachidicola* and *C. personatum,* respectively, seriously damage groundnut with yield reductions up to 50% (McDonald *et al.* 1985). Current control methods mostly rely on the use of fungicides such as penthiopyrad, azoxystrobin, pyraclostrobin and fluoxastrobin (Culbreath *et al.* 2009). To date, there are only few studies on biological control of leaf spot diseases with beneficial

microorganisms (Meena *et al.* 2002; Kishore *et al.* 2005; Meena *et al.* 2006; Galletti *et al*. 2008; Chapter 5). The reduction of leaf spots of groundnut by HR9 and HR32 may have resulted in an increase in photosynthetic leaf area and consequently in the observed yield increases relative to the control. Whether isolates HR9 and HR32 colonize the leaf surfaces of groundnut and interact directly with these fungal leaf pathogens remains to be determined. Another possible mechanism involved in the biocontrol activity of both isolates may be induced systemic resistance. Both mechanisms will be subject of future investigations.

Table 3. Colonization of roots of groundnut by *Rhizobium* sp. HR9 and *Sphingomonas* sp. HR32 and their effects on the incidence and severity of multiple diseases of groundnut. The field experiment was conducted in 2011 in Thua Thien Hue province, Vietnam. For each of the parameters, average values of four replications are given. For each row, different letters indicate a statistically significant difference between the treatments (*P=0.05*, Duncan Multi Range Test).

Characters	Treatment		
	Control	*Rhizobium* sp. HR9	*Sphingomonas* sp. HR32
Colonization (log cfu g^{-1} root)	-	5.8 a	5.5 a
Stem rot (*Sclerotium rolfsii*) (AUDPC)*	6.7 a	4.6 a	5.8 a
Damping off (*Rhizoctonia solani*) (%)	1.33 a	0.83 a	1.28 a
Black collar rot (*Aspergillus niger*) (AUDPC)	6.3 a	5.4 a	4.6 a
Bacterial wilt (*Ralstonia solanacearum*) (%)	1.84 a	1.56 a	1.41 a
Leaf spot (*Cercospora arachidicola, Cercosporidium personatum*) (%)**			
DI-F	44.0 b	36.5 a	34.5 a
DS-F	17.3 b	12.8 a	12.7 a
DI-Peg	34.5 a	29.5 a	29.5 a
DS-Peg	14.2 b	10.7 a	10.8 a

*AUDPC- area under disease progress curve. **Fifty leaves of each plot were investigated for leaf spot diseases. DI-F: disease incidence at flowering stage, DS-F: disease severity at flowering stage, DI-Peg: DI at peg stage, DS-Peg: DS at peg stage.

Materials and methods

Isolation of bacteria from groundnut nodules

Nodules were collected from roots of groundnut plants grown in ten farmer fields in Thua Thien Hue, a province in central Vietnam. In each field, five groundnut plants (peg stage) were collected and ten nodules (on average 2 per plant) were removed from the roots (Fig. 1). From these groundnut nodules, bacteria were isolated based on the method used at the Laboratory of Microorganisms, Faculty of Agronomy, Hue University of Agriculture and Forestry (HUAF), Vietnam. Briefly, the ten collected nodules were washed in 70% ethanol (v/v), surface sterilized in 1% (v/v) sodium hypochlorite (NaOCl) for 3 min and washed three times with sterile distilled

water. Sterilized nodules were crushed with a glass rod in a sterilized Eppendorf tube and 500 µl sterile distilled water was added. Fifty µl of the suspension was plated in duplicate on Yeast Extract Mannitol Agar (YMA) containing crystal violet (10 mg L^{-1}). Plates were incubated at 25°C for three to six days. After incubation, ten to fifteen bacterial colonies with a wet, smooth surface were selected for each sample and purified on YMA plates. In total, 99 purified isolates were purified and stored in 40% (v/v) glycerol stock at -20°C and -80°C for further analysis.

Bacterial identification

The genotypic diversity of the bacterial isolates was investigated by BOX-PCR analysis according to methods described by Tran *et al.* (2008). Amplicons from 200 to 5000 bp were scored visually for presence or absence. Bacterial isolates with identical BOX-PCR profiles were considered to be genotypically identical. Representative isolates of each BOX-PCR group were sent for 16S-rDNA sequencing at Macrogen Inc. (Seoul, South Korea). The obtained forward and reverse sequences were assembled and edited in Vector NTI (Invitrogen, version 8.0) and will be deposited in GenBank. For the phylogenetic analyses, the edited sequences were aligned to sequences available in databases (http://www.ncbi.nlm.nih.gov/Genomes/). Sequences were trimmed to the same size (~1,100 bp) and the phylogenetic tree was obtained with MEGA4 software (http://megasoftware.net).

Nodule formation on groundnut roots

Nodule formation by the bacterial isolates was tested in nethouse. The nethouse experiment was conducted in 2010 at the Plant Protection Department, HUAF, Vietnam. Groundnut seeds were surface sterilized in 3% (v/v) NaOCl for 15 min and rinsed three times with sterile distilled water. Sterilized seeds were soaked in sterile water for 4 h at 25ºC and then incubated overnight in Petri dishes at 25ºC. Bacterial isolates were grown on YMA medium plates at 25ºC, harvested after 3 days of incubation and adjusted to a density of 10^9 cell ml^{-1}. Natural clay loam soil was collected from groundnut fields in Thua Thien Hue province, Vietnam. The soil was autoclaved twice at 120ºC for 30 min with one night in between the two cycles. Autoclaved field soil was transferred to plastic pots (250 g per pot) and two germinated groundnut seeds were sown per pot. The suspension of the bacterial isolate was added to the soil to obtain a final density of 10^6 cells g^{-1} soil. For each bacterial isolate (one treatment), two pots were used. Two weeks after sowing, one plant was kept for every pot. Nodulation was assessed 45 days after sowing. The groundnut plants and soil were taken out of the pots and soil was removed by watering. Then, the nodules were visually observed and counted.

Hyphal growth inhibition of Sclerotium rolfsii

Inhibition of hyphal growth of *S. rolfsii* by the bacterial isolates was tested in dual culture assays according to the method described by Kruijt *et al*. (2009). Briefly, bacterial isolates were spot-inoculated at the edge of a $1/5^{th}$-strength potato dextrose agar plate ($1/5^{th}$ PDA, pH 6.5). After incubation for 48 h at 25ºC, a 5-mm-diameter agar plug of a 3-day-old culture of *S. rolfsii* strain H001 (Le *et al.* 2011) was placed in the centre of the $1/5^{th}$ PDA plate and incubated at 25ºC. Inhibition of mycelial growth of *S. rolfsii* by the bacterial isolates was recorded 3-4 days after fungal inoculation.

Plant growth promotion and biological control

The experiment for plant growth promotion and biocontrol of groundnut diseases was conducted in 2011 in a clay loam field in Thua Thien Hue province, Vietnam. The experiment consisted of three treatments, i.e. one control treatment and two bacterial treatments, i.e. *Rhizobium* sp. strain HR9 and *Sphingomonas* sp. strain HR32. Bacterial strains HR9 and HR32 were grown on YMA plates for 48 h at 25°C, harvested and washed with sterile water. Prior to sowing groundnut seeds, bacterial suspensions were applied to the furrows at a final density of 10^6

cells per cm^2 soil. The field experiment was laid out in a randomized complete block design (RCBD) with four blocks as four replicates with a plot size of 15 m^2 (3 × 5 m).

The field soil was fertilized with nitrogen (N) at 40 kg/ha, phosphorus (P_2O_5) at 60 kg/ha, potassium (K_2O) at 60 kg/ha, and calcium ($Ca(OH)_2$) at 300 kg/ha. Phosphorus and calcium were applied at the time of soil preparation. Seeds were sown at a depth of 3-5 cm and covered with soil. When plants had three true leaves (seedling stage), 70% of the nitrogen and 50% of the potassium was applied. The remainder of the N and K fertilization was applied at flowering. Emergence was monitored at 7, 10 and 13 days after sowing. Weeds were manually removed at four developmental stages of the groundnut plants, i.e. seedling, flowering, peg and pod set stages (4, 6, 8 and 10 weeks after sowing).

To determine the effects of each of the bacterial strains on growth of groundnut, plant height, branch length, nodulation, flower production, and yield were determined. At seedling stage, ten plants per plot were randomly labelled to measure plant height and branch length. Flower production was monitored daily for five random plants per plot during flowering time. The number and dry weight of nodules per plant root system was determined at the peg stage. To that end, three plants per plot were harvested, their root systems were washed to remove adhering soil and the number of nodules was counted. Nodules were removed and dried for at least 3 days at 105°C. Shoots and roots of three plants per plot were separated for measuring fresh weight and dry weight. Three plants per plot were collected to determine the population densities of the introduced bacterial strains HR9 and HR32. For both strains, spontaneous rifampicin-resistant derivatives were generated and their densities on roots of groundnut were determined according to the method described previously (Chapter 3). At harvest, the number of pods, marketable pods and the weight of 100 pods were determined for the ten randomly labeled plants. In addition, some soil properties, including pH_{KCl}, OM (%), total nitrogen (%), potassium (%), total phosphorus (%), and available phosphorus were analyzed prior to and upon completion of the field experiment.

Disease assessments

In the field experiment, the effects of the two bacterial strains HR9 and HR32 on the incidence and severity of several groundnut diseases were also monitored. At each of the four developmental stages (seedling, flowering, peg and pod set), the incidence of stem rot disease by *Sclerotium rolfsii* was monitored in 1 m^2 of each plot (~33 plants).

Stem rot disease incidence was assessed according to the methods described previously (Chapter 5). Black collar rot, caused by *Aspergillus niger,* was assessed by scoring the number of plants with black collar rot symptoms in 1 m^2 for each plot (Chapter 5). Damping-off, caused by *Rhizoctonia solani,* was monitored at the seedling stage by counting the number of infected plants in 10 m^2 per plot according to the methods described previously (Chapter 5). Bacterial wilt, caused by *Ralstonia solanacearum*, was monitored at pod set stage by counting the number of wilted plants in 10 m^2 per plot (Chapter 5).

Leaf spots caused by *Cercospora arachidicola* and *Cercosporidium personatum* was assessed at flowering and at the peg stage. Leaf spot incidence (LSI) is the number of infected leaflets divided by the total number of monitored leaflets. Leaf spot severity (LSS) was rated using the following scale of 0-5, with 0 = no disease symptoms, 1 = diseased area <1% of the leaflet area, 2 = diseased area of 1- 10%, 3 = diseased area of 10- 25%, 4 = diseased area of 25- 50% and 5 = diseased area >50%. LSS was calculated based on the formula: LSS = [(1× number of leaflets rated as scale 1) + (2 × number of leaflets rated as scale 2) + (3 × number of leaflets rated as scale 3) + (4 × number of leaflets rated as scale 4) +(5 × number of leaflets rated as scale 5)] × 100 ÷ (5 × total number of leaflets). For each of the four plots per treatment, five sites were randomly chosen and for each site ten leaflets were used for LSS assessment.

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Appendix 1. Effect of *Rhizobium* sp. HR9 and *Sphingomonas* sp. HR32 on the total carbon carbon (%C) and total nitrogen (%N) contents in shoots of groundnut plants at the peg stage. The groundnut plants were grown in the field in 2011 in Thua Thien Hue, Vietnam. Averages of four replications are shown. For each of the parameters, no statistically significant differences in %C and %N were observed between the treatments [($P_{\%C}$=*0.06,* $P_{\%N}$=*0.30*) except for C:N ($P_{C:N}$=*0.007*) ANOVA statistical test]. An asterisk indicates a significant difference with the untreated control.

Treatment	%C	%N	C:N ratio
Control	47.2	3.0	15.6
Rhizobium sp. HR9	47.6	3.3	14.3 *
Sphingomonas sp. HR32	48.0	3.3	14.2 *

Chapter 7

General discussion

Parts of this chapter are integrated in the review
"Stem rot of groundnut caused by the soil-borne pathogen Sclerotium rolfsii" (to be submitted)

General discussion

Groundnut is one of the most important oil seed crops worldwide, but its production is hampered by several pests and diseases. Among the soil-borne fungal diseases, stem rot caused by *Sclerotium rolfsii* is a major yield-limiting factor in groundnut cultivation in many countries. *S. rolfsii* has a broad host range of more than 500 plant species (Aycock 1966) and causes yield losses ranging from 10-25% and sometimes even up to 80% (Mehan *et al.* 1994). In Vietnam, yield losses due to stem rot disease have not been investigated yet, but it was reported that this disease caused 5-11% plant death in farmer fields in north and central Vietnam (Le 2004; Nguyen *et al.* 2004; Do 2006). Besides stem rot, several other diseases such as *Rhizoctonia* damping-off (*Rhizoctonia solani*), black collar rot (*Aspergillus niger*), leaf spots (*Cercospora arachidicola, Cercosporidium personatum*), and bacterial wilt (*Ralstonia solanacearum*) are also widely distributed and damaging to groundnut plants (Mehan and Hong 1994; Le 2004; Nguyen *et al.* 2004; Do 2006). In order to manage stem rot and other groundnut diseases, fundamental knowledge of the distribution and genetic diversity of the pathogen populations is essential.

Phenotypic and genetic diversity of *S. rolfsii* in central Vietnam

The survey that was conducted showed that the incidence of stem rot disease of groundnut in central Vietnam ranged from approximately 5 to 25% (**chapter 2**). The results further indicated a gradient in stem rot disease incidence across central Vietnam with an increase in disease incidence from the northern to the southern field sites. This might be related to the effect of temperature on disease development (Punja 1985), because along with the lower latitude also the average daily temperature increases from the North to the South of Vietnam. For two of the four provinces, the disease incidence of groundnut plants cultivated in clay loam soil was significantly higher than for plants cultivated in sandy soil. This difference may be related, in part, to the higher organic content of the clay loam soil, a characteristic that is known to support germination of sclerotia and subsequent hyphal growth toward the host plant (Punja 1985).

Based on ITS-rDNA sequencing, *S. rolfsii* populations in groundnut fields in central Vietnam appeared relatively uniform with three ITS-groups (1, 2 and 3). Most isolates were closely related to reference isolates of *S. rolfsii* that were previously collected from groundnut, tobacco and sweet pepper. Unfortunately, RAPD analysis as well as rep-PCR and ERIC-PCR (McDonald *et al.* 2000) gave inconsistent/irreproducible results and were not considered useful to assess the intraspecific diversity of the *S. rolfsii* isolates. Therefore, a range of other traits, including pathogenicity, growth rate, sclerotial characteristics, mycelial compatibility and tebuconazole sensitivity, were analysed. When comparing the sclerotial traits, the three isolates

in ITS-group 2 were clearly different from the isolates in ITS-groups 1 and 3: ITS-group 2 isolates produced substantially less sclerotia per plate and also the average maturation time and sclerotial diameter were approximately two times higher than for isolates of ITS-groups 1 and 3. These characteristics combined with the light brown colour of the sclerotia of these three ITS-group 2 isolates were also reported for isolates classified morphologically or by LSU-sequencing as *S. rolfsii* var. *delphinii* (Aycock 1966; Punja and Damiani 1996; Xu *et al.* 2010). The genetic diversity of *S. rolfsii* isolates from central Vietnam was also investigated by analysis of the mycelial compatibility. Within each of the three ITS-groups identified in our study, a high variation in mycelial compatibility was observed. It should be emphasized, however, that mycelial compatibility described in this thesis is based on macroscopic observations in which hyphae of different isolates readily intermingle without forming a barrage or inhibition zone. Therefore, the mycelial compatibility groups (MCG) found here cannot automatically be considered as vegetative compatibility groups (VCG) or anastomosis groups (AG). Microscopic analysis needs to be performed to confirm heterokaryon formation between isolates.

The results further showed that the variation in sensitivity of *S. rolfsii* isolates from groundnut fields in central Vietnam to the fungicide tebuconazole is substantially less than reported earlier for the *S. rolfsii* population from Georgia, USA (Franke *et al.* 1998). This provides a basis to evaluate the efficacy of this fungicide to control stem rot disease of groundnut in central Vietnam. Combination with other control measures, including biological control, is recommended to prevent resistance development as was observed previously for *S. rolfsii* populations exposed repeatedly to this fungicide (Franke *et al.* 1998).

Biocontrol of stem rot by antagonistic bacteria

Pseudomonas

In a series of experiments conducted in the laboratory, growth chamber, nethouse and field, we showed that specific *Pseudomonas* strains, producing either phenazines (PHZ) or lipopeptides (LPs), suppressed hyphal growth of *S. rolfsii* and significantly reduced stem rot disease of groundnut (**chapter 3**). By comparing the activities of wildtype *Pseudomonas* strains with that of mutants disrupted in specific biosynthesis and regulatory genes, we demonstrated that PHZ and specific LPs are key bioactive metabolites in biological control of stem rot disease of groundnut. The results of the *in vitro* dual culture assays showed that strains SS101 and SBW25, producing the viscosin-type LPs, and strain 267, producing putisolvins, did not or only marginally inhibit hyphal growth of *S. rolfsii*. Lack of inhibitory activity of LPs was also observed previously for viscosinamide and putisolvins in hyphal growth assays with oomycete pathogens (Thrane *et al.* 1999; Gross e*t al.* 2007; Kruijt *et al.* 2009). In contrast, thanamycin-producing strain SH-C52 significantly inhibited hyphal growth of *S. rolfsii,* whereas its

thanamycin-deficient mutant was not effective. These results suggested differential activity of structurally different LPs against the stem rot pathogen *S. rolfsii*, with thanamycin having significant inhibitory effects on hyphal growth and plant infection. This presumed differential activity of the four LPs is based on the assumption that each of the bacterial strains produces similar amounts of the LPs on agar plates and in the groundnut rhizosphere. At this point, however, there are no data available on the concentrations of each of the LPs produced on roots of groundnut. Reporter-based studies (Keel *et al.* 1992) or advanced analytical analyses (Ongena *et al.* 2007) should be conducted to confirm that the LPs are indeed produced *in situ* and to determine the amounts produced.

Next to the well-characterized *Pseudomonas* strains, we also tested novel *Pseudomonas* strains. We describe the isolation, characterization and biocontrol efficacy of *Pseudomonas* sp. R4D2, a strain that was selected from a total of 3,360 indigenous bacterial isolates randomly collected from the stem base and roots of groundnut plants grown in farmer fields in Vietnam (**chapter 4**). Field experiments showed that treatment with strain R4D2 significantly reduced the incidence of stem rot disease. The level of disease protection provided by this bacterial strain was similar to that achieved by the fungicide tebuconazole. For *Pseudomonas* sp. strain R4D2, two mutants were obtained that did not inhibit hyphal growth of *S. rolfsii* and were not effective in controlling stem rot disease of groundnut under growth chamber conditions. Drop collapse assays showed that strain R4D2 produces biosurfactant compound(s) that are not produced by the two mutants. Although these results support the hypothesis that a biosurfactant is responsible for biocontrol of *S. rolfsii,* additional genetic and biochemical analyses are required to prove this and to strengthen the data.

Bacillus

In the past decade, several *Bacillus* species and strains have been studied for their efficacy to control stem rot disease of groundnut (Abd-Alla and Ezzat 2003; Abd-Allah 2005; Abd-Allah and El-Didamony 2007; Tonelli *et al.* 2011). For example, treatment of groundnut seeds with *Bacillus subtilis* protected groundnut against *S. rolfsii* and significantly increased the number of pods (Abd-Allah 2005). In this thesis, we describe the isolation of two promising *Bacillus* strains, designated S20D12 and S18F11, from the stem base of groundnut plants grown in farmer fields in central Vietnam (**chapter 4**). Phylogenetic analyses showed that strain S20D12 belongs to the same phylogenetic cluster as *Bacillus amyloliquefaciens* reference strains, whereas strain S18F11 clustered with several *Bacillus subtilis* reference strains. Both *Bacillus* species have been the subject of numerous studies on biological control of plant pathogens and induced systemic resistance (Kloepper *et al.* 2004; Ongena and Jacques 2008). In the field experiment that we conducted in Quang Nam province in 2010, both *Bacillus* strains provided significant control of stem rot disease to a level similar to that of the fungicide Folicur.

Comparison of the biocontrol efficacy of *Pseudomonas* and *Bacillus*

The two field experiments described in this thesis (**chapters 3, 4, 5**) were conducted in Vietnam in two consecutive years at two different locations: in 2010 in Quang Nam province and in 2011 in Thua Thien Hue province. In both field experiments, several *Pseudomonas* and *Bacillus* strains were tested for their efficacy to control stem rot and other groundnut diseases, and to improve growth and yield. Despite the substantial difference in attainable pod yield between the two locations, several of the applied bacterial strains increased pod yield of groundnut consistently (Table 1). For example, *Pseudomonas* sp. strains SH-C52 and R4D2 significantly suppressed stem rot disease caused by *S. rolfsii* in the field experiments conducted in 2010 (**chapter 3**) and in 2011 (**chapter 5**). Furthermore, *Bacillus* sp. strain S20D12 consistently improved pod yield in both years with increases in pod dry weight of 23-30% (Table 1, **chapters 4, 5**). Despite the consistency in yield increases over two consecutive years, the *Bacillus* strains did not consistently control stem rot disease. For example, in 2010 both *Bacillus* strains significantly suppressed stem rot disease, but in 2011 they did not control this disease nor several other soil-borne diseases such as *Rhizoctonia* damping-off, black collar rot and bacterial wilt. However, both *Bacillus* strains significantly reduced leaf spot diseases (**chapter 5**).

Table 1. Effect of the presence of various bacterial strains on pod yield of groundnut under field conditions in 2010 and 2011 in central Vietnam. The 2010 experiment was conducted on sandy soil in Quang Nam province, the 2011 experiment was conducted on clay loam soil condition in Thua Thien Hue province. Averages of three replicates (2010) or four replicates (2011) are shown.

	2010 Quang Nam province		2011 Thua Thien Hue province	
	Pod yield (kg ha^{-1})	% increase relative to control	Pod yield (kg ha^{-1})	% increase relative to control
Control	1220	-	2317	-
Folicur[#]	1300	8.3	2408	3.9
Pseudomonas sp. SH-C52	1190	-0.8	2565 *	10.7
Pseudomonas sp. R4D2	1320	10.0	2636 *	13.8
Control	1220	-	2244	-
P. fluorescens SS101	1370 *	14.2	2461	9.7
Bacillus sp. S18F11	1320	10.0	2817 *	25.5
Bacillus sp. S20D12	1480 *	23.3	2924 *	30.3

* significant (P<0.05) relative to the control, [#] fungicide treatment

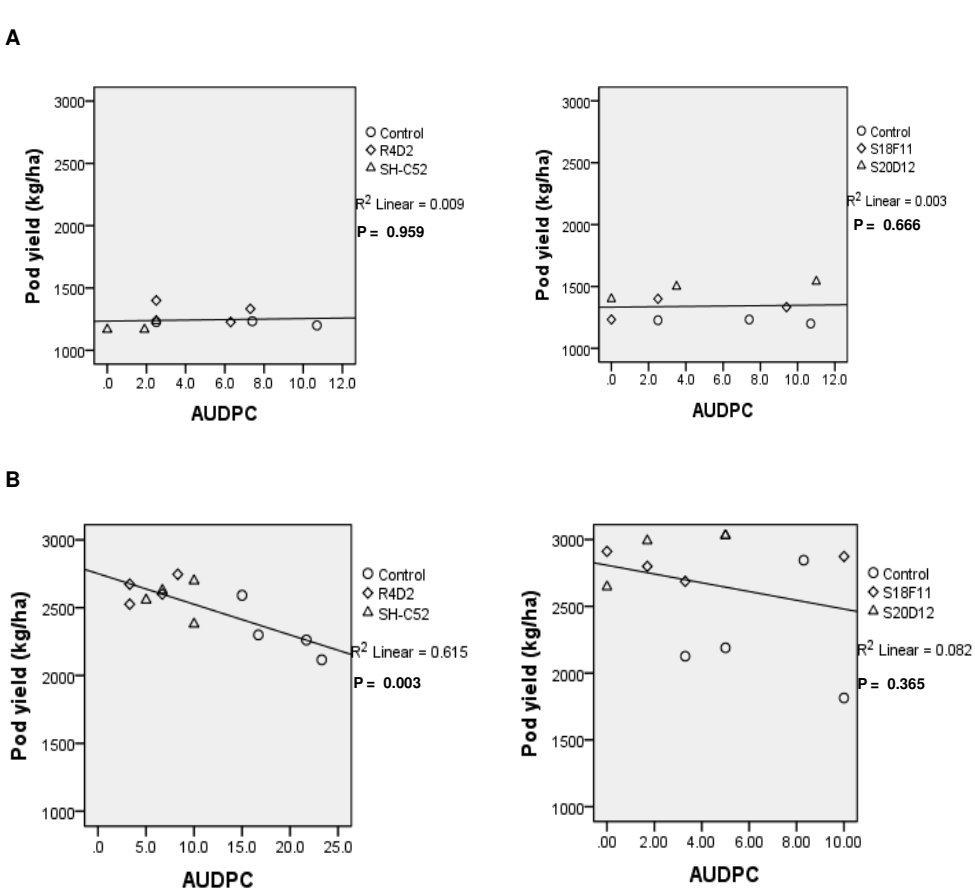

Figure 1. Correlations between stem rot disease incidence and pod yield in field experiments conducted in 2010 in Quang Nam province (panel A) and in 2011 in Thua Thien Hue province (panel B). For both years, the effects of *Pseudomonas* sp. strains R4D2 and SH-C52 (left) and of *Bacillus* sp. strains S18F11 and S20D12 (right) on stem rot disease incidence and pod yield are given. AUDPC refers to the area under the disease progress curve for stem rot disease of groundnut. For each treatment, the data of three or four replicates are given. For details see chapters 3, 4 & 5.

To determine the potential beneficial effects of biocontrol by the tested *Pseudomonas* and *Bacillus* strains on pod yield, regression analyses were performed (Figures 1 and 2). The results of these analyses showed that in the field experiment conducted in 2010, there was no correlation between stem rot disease incidence and pod yield, neither for the two *Pseudomonas* strains nor for the two *Bacillus* strains (Fig. 1A). However, for the field experiment conducted in Hue province in 2011, there was a significant correlation between the reduction of stem rot disease incidence by the two *Pseudomonas* strains and pod yield; for the two *Bacillus* strains, the correlation was not significant (Fig. 1B). When considering other diseases of groundnut that were monitored in the 2011 field experiment (**chapter 5**), a significant relationship was

observed between pod yield and the incidence and severity of leaf spot diseases in the *Bacillus* treatments; however, no significant correlation was found for the two *Pseudomonas* strains (Fig. 2). Collectively, these results suggest that for the two *Pseudomonas* strains, increases in pod yield correlated with suppression of stem rot disease, whereas for the *Bacillus* strains suppression of leaf spot diseases appears to be a more important factor in the observed increases in pod yield.

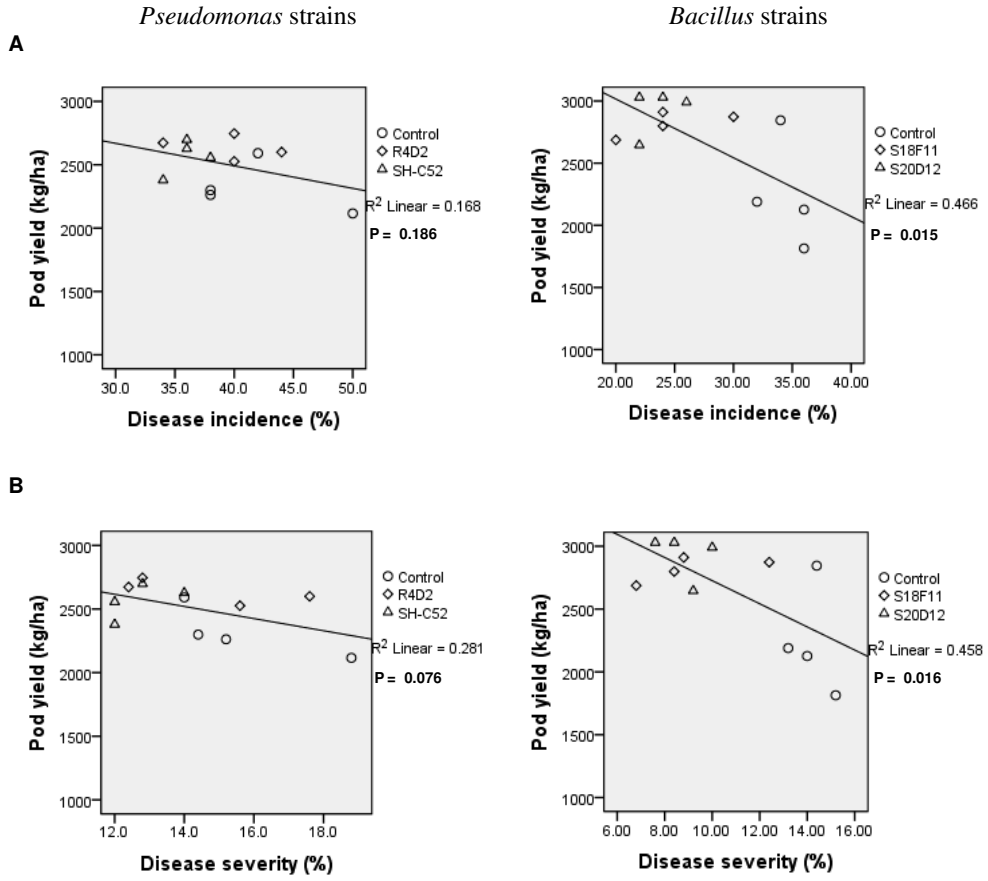

Figure 2. Correlations between pod yield of groundnut and the incidence (panel A) or severity (panel B) of leaf spot diseases caused by *Cercospora* and *Cercosporidium* species in the field experiment conducted in 2011 in Thua Thien Hue province. The effects of *Pseudomonas* sp. strains R4D2 and SH-C52 (left) and of *Bacillus* sp. strains S18F11 and S20D12 (right) on disease incidence/severity and pod yield are given. Disease incidence and severity were assessed at the peg stage of the groundnut plants. For each treatment, the data of three or four replicates are given. For details see chapter 5.

Endophytic bacteria of groundnut nodules as biocontrol agents

To address the question whether endophytic bacteria associated with nitrogen fixing root nodules on groundnut have biocontrol properties, we set out to isolate and characterize these

bacteria. The results (**chapter 6**) showed that bacterial communities living inside groundnut nodules are genotypically diverse, confirming and extending results obtained recently by Angelini *et al.* (2011). Ribosomal database comparisons revealed that these isolates belong to two bacterial phyla, i.e. Proteobacteria and Bacteroidetes. For the isolates that were classified as Proteobacteria, the majority was phylogenetically related to *Sphingomonas* species and the others to *Rhizobium, Stenotrophomonas, Ralstonia,* and *Burkholderia* spp. To our knowledge, *Sphingomonas* species have not been reported to form or associate with nodules on groundnut. When re-introduced into autoclaved field soil, most *Sphingomonas* isolates obtained in our study did not form nodules on groundnut roots. However, in the presence of some of the identified *Sphingomonas* isolates, in particular isolates H32 and HR75, nodules were found on groundnut roots. Inoculation with isolates HR9 and HR10, which are closely related to *Rhizobium,* did not or only marginally result in nodule formation on groundnut plants. Also isolates HR7, HR21, HR77 and HR5, close relatives of *Stenotrophomonas*, *Ralstonia* or *Burkholderia,* did not form nodules on groundnut. Additional studies will be necessary to confirm these results and to determine if the *Sphingomonas* isolates H32 and HR75 are capable of nodule formation and N_2-fixation.

In the 2011 field experiment, both *Rhizobium* sp. strain HR9 and *Sphingomonas* sp. strain HR32 did not significantly increase plant height, branch length, plant biomass or flower production (**chapter 6**). Although there was a tendency that both HR9 and HR32 increased the number of nodules per plant, these differences were not statistically significant. A similar trend was observed for the nitrogen content of the groundnut plants treated with HR9 and HR32. Despite the fact that no significant effects on plant biomass were found, both bacterial isolates did significantly enhance pod yield with increases of 15.8 and 18.5%, respectively. There was also no apparent correlation between these yield increases and the incidence of several major soil-borne diseases: strains HR9 and HR32 did not provide any control of stem rot, damping-off, black collar rot and bacterial wilt. However, both bacterial strains significantly reduced the incidence and the severity of leaf spot diseases of groundnut. As described earlier for the two *Bacillus* strains, the reduction of leaf spot diseases by HR9 and HR32 may have resulted in an increase in photosynthetic leaf area and consequently in the observed pod yield increases. Whether isolates HR9 and HR32 colonize the leaf surfaces of groundnut and interact directly with these fungal leaf pathogens remains to be determined. Another possible mechanism involved in the biocontrol activity of both bacterial strains may be induced systemic resistance. Elucidating the mechanisms involved will be subject of future investigations.

Future perspectives

The observation that *S. rolfsii* populations from groundnut were relatively sensitive to tebuconazole provides opportunities to use this fungicide to control stem rot disease in Vietnam. Combination with other control measures, including biological control, is recommended to

prevent resistance development as was observed previously for *S. rolfsii* populations exposed repeatedly to this fungicide. Several of the *Pseudomonas* strains described in this thesis are particularly effective in controlling stem rot disease, whereas the *Bacillus*, *Sphingomonas* and *Rhizobium* strains can be further exploited to control leaf spot diseases and to improve plant growth and pod yield. Combining chemical pesticides and biocontrol agents for control of stem rot disease has been evaluated before. For example, combining *Trichoderma viride* with pentachloronitrobenzene, captan or aldrex T fungicides controlled stem rot disease on tomato (Chima Wokocha 1990). Similarly, *Trichoderma harzianum, Rhizobium* sp., and carbendazim were combined for management of stem rot of groundnut in India (Muthamilan and Jeyarajan 1996). In both studies, however, the level of disease control achieved by these combinations was not significantly better than that provided by the biological control agents alone. Experiments will be conducted to determine if combination of tebuconazole with *Pseudomonas* or *Bacillus* strains leads to additive or synergistic effects on stem rot and other groundnut diseases. Finally, registration and formulation of the antagonistic bacteria identified in this thesis should be pursued in order to successfully use these microorganisms as an integral part of future management practices to control stem rot and other groundnut diseases.

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Summary

Groundnut (*Arachis hypogaea* L.) is an economically important legume crop in Vietnam and many other countries worldwide. Stem rot disease, caused by the soil-borne fungus *Sclerotium rolfsii* Sacc., is a major yield limiting factor in groundnut cultivation. Current control methods mostly rely on the extensive use of fungicides and on cultural practices. Both methods are not always effective and repeated fungicide use can lead to resistance development in the pathogen population. To further improve disease control and to reduce the use of chemical pesticides in groundnut cultivation, the feasibility of biological control of stem rot was investigated. The project initially focused on evaluating the occurrence and severity of stem rot disease in farmer fields in central Vietnam. It appeared that 5-25% of the groundnut plants in farmer fields in central Vietnam were infected by *S. rolfsii*. From infected fields, *S. rolfsii* isolates were collected and their genetic diversity was investigated as well as the variation in sensitivity to tebuconazole, a fungicide commonly used to control stem rot disease. Based on ITS-rDNA sequence analyses, three distinct groups were identified among a total of 103 randomly selected *S. rolfsii* field isolates, with the majority (n=90) in one ITS group. *S. rolfsii* isolates originating from groundnut, tomato and taro were all pathogenic on groundnut and relatively sensitive to tebuconazole. However, the isolates displayed substantial diversity in various genetic and phenotypic traits, including mycelial compatibility, growth rate, and sclerotial characteristics.

Subsequently, the efficacy of various beneficial bacteria to suppress stem rot disease was investigated. First, the biocontrol activity of well-characterized antagonistic *Pseudomonas* strains was evaluated *in vitro* and in growth chamber, nethouse and field experiments. Secondly, indigenous groundnut-associated bacteria were isolated, their diversity was investigated and their antifungal activity was analysed in nethouse and field experiments in central Vietnam. Finally, endophytic bacteria living inside groundnut nodules were isolated, characterized and tested for biocontrol and plant growth promotion under field conditions. For several of the most promising bacterial strains, the mechanisms involved in biocontrol of stem rot disease were investigated.

The experiments conducted with well-characterized *Pseudomonas* strains showed that only phenazine-producing *Pseudomonas chlororaphis* strain Phz24 and *Pseudomonas* sp. strain SH-C52, producing the chlorinated lipopeptide thanamycin, inhibited hyphal growth of *S. rolfsii* and suppressed stem rot disease. Mutants of these strains that were deficient in phenazine or thanamycin production did not show any activity against *S. rolfsii,* indicating that these metabolites play an important role in suppression of stem rot disease. Other *Pseudomonas* strains producing structurally different lipopeptides did not or only marginally inhibit growth of *S. rolfsii.* These results suggested variation in sensitivity of the stem rot pathogen *S. rolfsii* for structurally different lipopeptides produced by *Pseudomonas*.

Indigenous groundnut-associated bacteria with activity against *S. rolfsii* were classified by 16S-rDNA sequence analysis as γ-Proteobacteria (*Pseudomonas*), Bacteroidetes (*Chryseobacterium*), and Firmicutes (*Bacillus*). Among these indigenous bacterial genera, *Pseudomonas* sp. R4D2 consistently reduced stem rot disease under nethouse and field conditions. The genes and bioactive compounds involved in the biocontrol activity of strain R4D2 have not been identified yet, but phenotypic analyses suggest that biosurfactant production plays a central role. *Chryseobacterium* sp. R4B3 did not reduce stem rot disease in nethouse experiments, but was effective in suppression of stem rot disease under field conditions. Finally, the two indigenous isolates that were identified as *Bacillus* spp. did not reduce stem rot disease in nethouse experiments, but were effective in disease control under field conditions. Furthermore, both *Bacillus* strains significantly increased pod yield with dry weight increases of up to 30% relative to the control.

Endophytic bacteria of groundnut nodules appeared to be genetically diverse. The majority was closely related to *Sphingomonas* while others were classified as *Rhizobium, Burkholderia, Stenotrophomonas*, and *Sphingobacterium* species. In nethouse experiments, occasionally, nodule formation on roots was observed on groundnut plants grown in soil supplemented with some of these endophytic isolates, but further analyses are required to establish that these isolates are indeed responsible for nodule formation and nitrogen fixation. For two of the identified endophytic isolates, i.e. *Rhizobium* sp. HR9 and *Sphingomonas* sp. HR32, field experiments showed that both strains were not effective against stem rot and other soil-borne diseases of groundnut, but significantly reduced leaf spot diseases caused by *Cercospora* and *Cercosporidium* spp. Application of these two endophytic bacterial strains also led to increases in pod yield of groundnut under field conditions.

In conclusion, several of the bacterial strains tested in this study, in particular *Pseudomonas* and *Bacillus* spp., significantly improved pod yield of groundnut by 10 to 30%. The consistency in pathogen control and yield increase observed in two independent field experiments at two different locations in two consecutive years, holds great promise for further development of these bacterial strains as key components of an integrated strategy to manage multiple diseases of groundnut and to improve yield.

Samenvatting

Pinda (*Arachis hypogaea* L.) is een economisch belangrijk gewas in Vietnam en diverse andere landen wereldwijd. Stengelrot, veroorzaakt door de bodemschimmel *Sclerotium rolfsii* Sacc., is een belangrijke opbrengstbeperkende factor in de pindateelt. Momenteel wordt deze ziekte voornamelijk bestreden door middel van fungiciden en teeltmaatregelen. Beide methoden zijn echter niet altijd effectief en veelvuldig gebruik van fungiciden kan leiden tot resistentie-ontwikkeling in de pathogeenpopulatie. Om de bestrijding van stengelrot te verbeteren en het gebruik van fungiciden in de pindateelt te reduceren, hebben wij de haalbaarheid van biologische bestrijding van deze ziekte bestudeerd. In dit onderzoek hebben we in eerste instantie het vóórkomen en de ernst van stengelrot in pindavelden in Vietnam onderzocht. Hieruit bleek dat 5 tot 25% van de pindaplanten in het veld geïnfecteerd was met *S. rolfsii*. Daarnaast werden *S. rolfsii* isolaten verzameld waarvan de genetische diversiteit werd bestudeerd alsmede hun gevoeligheid voor tebuconazole, een fungicide dat veelvuldig gebruikt wordt om stengelrot te bestrijden. Op basis van ITS-rDNA sequenties van 103 willekeurig geselecteerde *S. rolfsii* isolaten werden drie groepen geïdentificeerd; de meerderheid van de isolaten (n=90) behoorde tot één ITS groep. *S. rolfsii* isolaten van pinda, tomaat en taro waren allemaal pathogeen op pinda en relatief gevoelig voor tebuconazole. De *S. rolfsii* isolaten vertoonden echter aanzienlijke diversiteit in verscheidene genetische en fenotypische kenmerken, waaronder vegetatieve compatibiliteit, groeisnelheid en diverse eigenschappen van sclerotiën, de overlevingsstructuren van deze bodemschimmel.

Vervolgens werd onderzocht of stengelrot onderdrukt kan worden door goedaardige bacteriën die de pindaplant koloniseren. Allereerst werd de effectiviteit van bekende *Pseudomonas* stammen geëvalueerd in *in vitro* experimenten en in klimaatcel-, kas- en veldexperimenten. Tevens werden van pindaplanten bacteriën geïsoleerd en gekarakteriseerd; een aantal hiervan werd vervolgens getest op hun schimmelremmende werking in kas- en veldexperimenten in Vietnam. Tot slot werden ook endofytische bacteriën uit de wortelknolletjes van pindaplanten geïsoleerd, gekarakteriseerd en getest op hun vermogen om stengelrot te onderdrukken en plantengroei te bevorderen onder veldomstandigheden. Voor een aantal van de meest veelbelovende isolaten werden de werkingsmechanismen die betrokken zijn bij de onderdrukking van stengelrot nader onderzocht.

De experimenten met de bekende *Pseudomonas* stammen lieten zien dat alleen *Pseudomonas chlororaphis* Phz24, producent van phenazines, en *Pseudomonas* sp. SH-52, producent van het gechloreerde lipopeptide thanamycine, in staat waren de groei van *S. rolfsii* te remmen en stengelrot te onderdrukken. Mutanten van deze stammen, die geen phenazine of thanamycine kunnen produceren, verloren hun activiteit tegen *S. rolfsii* hetgeen een aanwijzing is dat deze twee metabolieten een belangrijke rol spelen in biologische bestrijding van stengelrot. Andere *Pseudomonas* stammen die structureel verschillende lipopeptiden produceren

remden de groei van *S. rolfsii* niet of nauwelijks. Deze resultaten suggereren dat er variatie is in gevoeligheid van *S. rolfsii* voor verschillende lipopeptiden geproduceerd door *Pseudomonas*.

De bacteriën die geïsoleerd waren van pindaplanten en activiteit vertoonden tegen *S. rolfsii* werden op basis van 16S-rRNA analyses geclassificeerd als γ-Proteobacteriën (*Pseudomonas*), Bacteroidetes (*Chryseobacterium*) en Firmicutes (*Bacillus*). Van deze bacteriële genera was *Pseudomonas* sp. stam R4D2 effectief in onderdrukking van stengelrot van pinda zowel in kas- als veldexperimenten. De genen en bioactieve stoffen betrokken bij onderdrukking van stengelrot door R4D2 zijn nog niet opgehelderd, maar diverse analyses suggereren dat de productie van zogenaamde biosurfactants een centrale rol speelt. *Chryseobacterium* sp. R4B3 was niet in staat stengelrot te verminderen in kasexperimenten, maar was wel effectief in de onderdrukking van stengelrot onder veldomstandigheden. De twee isolaten die geïdentificeerd waren als *Bacillus* soorten bleken ook niet in staat om stengelrot te onderdrukken in kasexperimenten, maar waren wel effectief onder veldomstandigheden. Daarnaast zorgden deze *Bacillus* isolaten voor een significante toename in peulopbrengst tot zelfs 30% ten opzichte van de niet-behandelde planten.

De bacteriën die geassocieerd zijn met stikstofbindende wortelknolletjes van pindaplanten bleken genetisch divers te zijn. De meeste isolaten waren nauw verwant aan *Sphingomonas* soorten, terwijl andere isolaten als *Rhizobium*, *Burkholderia*, *Stenotrophomonas* en *Sphingobacterium* soorten geclassificeerd werden. In kasexperimenten werd slechts incidenteel wortelknolvorming gevonden op pindaplanten die geteeld werden op grond waaraan deze bacterie-isolaten waren toegevoegd. Vervolgexperimenten zijn nodig om vast te stellen of deze bacterie-isolaten in staat zijn wortelknolletjes te vormen die stikstof kunnen binden. Twee van deze bacterie-isolaten, te weten *Rhizobium* sp. HR9 en *Sphingomonas* sp. HR32, bleken niet in staat om stengelrot en andere bodemgebonden pindaziekten te onderdrukken, maar gaven wel een significante onderdrukking van bladvlekkenziekten van pinda veroorzaakt door *Cercospora* en *Cercosporidium* soorten. Beide bacterie-isolaten zorgden ook voor een toename in peulopbrengsten van pinda onder veldomstandigheden.

Samenvattend kan geconcludeerd worden dat een aantal van de in deze studie geteste bacteriën, met name *Pseudomonas* en *Bacillus* stammen, de peulopbrengst van pindaplanten aanzienlijk kan verhogen met toenames variërend van 10 tot 30%. De consistentie in ziekte-onderdrukking en opbrengstverhoging in twee onafhankelijke veldexperimenten op twee verschillende locaties in twee opeenvolgende jaren, is veelbelovend voor verdere implementatie van deze bacterie-isolaten in een geïntegreerde strategie om meerdere pindaziekten te onderdrukken en de opbrengst te verhogen.

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Wageningen, 16 December 2011

Cuong N. Le

Curriculum vitae

Cuong N. Le (Lê Như Cương – Vietnamese order) was born on November 15, 1974 in Ha Tinh, Vietnam. He went to university in 1994 and obtained his bachelor degree of Plant Protection in 1998 at the Hue University of Agriculture and Forestry (HUAF), Vietnam. He worked at HUAF as an assistant lecturer from January 1999 to January 2001, after which he continued to work as a lecturer on plant pathology at the Department of Plant Protection of HUAF. From September 2001 to September 2003 he conducted his MSc study on wilt diseases of groundnut and control methods at the same University.

In May 2005, he received a PhD scholarship from the Vietnamese government through project 322 to study abroad. From January 2008 to December 2011, he conducted his PhD study at the Laboratory of Phytopathology, Plant Sciences Group, Wageningen University, the Netherlands. His study on diversity and biological control of *Sclerotium rolfsii*, causal agent of stem rot of groundnut.

After finishing his PhD study in the Netherlands, he will continue his work at the Department of Plant Protection, HUAF, Vietnam as a lecturer on plant pathology.

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Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Le Nhu Cuong**
Date: **16 December 2011**
Group: **Phytopathology, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Diversity and biological control of *Sclerotium rolfsii,* causal agent of stem and pod rot of groundnut in Vietnam	May 27, 2008
► **Writing or rewriting a project proposal**	
Diversity and biological control of *Sclerotium rolfsii*, causal agent of stem and pod rot of groundnut in Vietnam	Jan-May 2008
► **Writing a review or book chapter**	
Review: "Stem rot of groundnut caused by the soil-borne pathogen *Sclerotium rolfsii*"	To be submited
► **MSc courses**	
Molecular and Evolutionary Ecology (GEN-20306)	Mar 03-07, 2008
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*8,1 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student Day, Utrecht University (NL)	Jun 01, 2010
► **EPS theme symposia**	
EPS Symposium Ecology and Experimental Plant Sciences 2	Sep 22, 2009
► **NWO Lunteren days and other National Platforms**	
ALW meeting in Lunteren	Oct 15-16, 2009
CBS Phytopathology joint meeting	Nov 12, 2010
The first Centre for Soil Ecology day	Jun 27, 2011
► **Seminars (series), workshops and symposia**	
Dr. Dagmar Hann: "Early events in Molecular plant-micro interaction"	Aug 08, 2008
Dr. Henkjan Schoonbeek: "Attack and defence mechanisms in the Botrytis cinerea - host interaction: roles of camalexin, ABC transporters and oxalic acid"	Sep 03, 2008
Dr. Daniela Nowara: "Envidence for plant-mediated gene silencing in the obligate biotrophic pathogen *Blumeria gramini*"	Sep 26, 2008
Dr. Rays H.Y. Jian: "Host-pathogen interaction drives genome plasticity in animal and plant pathogens"	Jun 10, 2009
Dr. Hiro Nonogaki: "Seeds, microRNA and Darwin"	Sep 17, 2009
Dr. Ricardo Oliva: "The *Phytophthora infestans* Avrblb2 effector: from one genome to hundreds of genomes"	Sep 17, 2009
Prof. dr. Harro Bouwmeester: "The interaction of plants with their invironments"	Sep 08, 2009
Prof. dr. Ton Bisseling: "From epigenetics to novel organelles and organns"	Sep 08, 2009
Prof. dr. Olaf van Kooten: "Does plant physiology relate to consumer satisfaction?"	Oct 13, 2009
Prof. dr. Jack Leunissen: "The quest for orthologs"	Oct 13, 2009
Prof. dr. Pierre de Wit: "The molecular dialogue between pathogens and plants"	Nov 10, 2009
Prof. dr. Fred van Eeuwijk: "Statistical modeling of genotype to phenotype relations"	Nov 10, 2009
Prof. dr. Naoto Subia : "Chitin perception and signaling in rice and arabidopsis"	Sep 09, 2010
Prof. dr. Regine Kahmann: "Effectors of the plant-pathogen fungus *Ustilago maydis*"	Oct 29, 2010
Prof. dr. Rosie Bradshaw: "The genome of *Dothistroma septosporum* , a close relative of *Cladosporium fulvum*; what have we learnt so far?"	Aug 04, 2011
► **International symposia and congresses**	
Workshop on Biotechnology in the center of Vietnam	Dec 10-11, 2008
International PGPR meeting, Portland, USA	May 18-22, 2009
Plant pathology conference in Hue, Vietnam	Apr 23-24, 2010
International Rhizosphere 3 meeting in Perth, Australia	Sep 25-30, 2011
► **Presentations**	
Poster:On the Evolution of Plant Pathogen interaction: from Principles to Practice	Jun 18-20, 2008
Oral: Workshop on Biotechnology in the center of Vietnam	Dec 10, 2008
Poster: International PGPR meeting, Portlnad, USA	May 18-22, 2009
Oral: Plant pathology meeting in Hue, Vietnam	Apr 23-24, 2010
Poster: Summer school Rhizosphere signaling, Wageningen, NL	Aug 23-25, 2010
Oral: International Rhizosphere 3 meeting, Perth, Autralia	Sep 25-30, 2011
► **IAB interview:** I did not attend due to the field work in Vietnam	
► **Excursions**	
Subtotal Scientific Exposure	*13,8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
On the Evolution of Plant Pathogen interaction: from Principles to Practice	Jun 18-20, 2008
Molecular phylogenies: reconstruction and interpretation	Oct 13-17, 2008
Summer school Rhizosphere Signalling	Aug 23-25, 2010
► **Journal club**	
Literature studygroup Phytopathology	2008-2011
► **Individual research training**	
Field work in Vietnam	2009 - 2011
Subtotal In-Depth Studies	*8,4 credits**

4) Personal development	*date*
► **Skill training courses**	
WGS Course: Information literacy, including Introduction Endnote	May 27-28, 2008
Project- and Time Management	Sep 09,23 & Oct 21, 2008
Techniques for Writing and Presenting a Scientific Paper	Jun 29 -Jul 02, 2010
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33.6**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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Cover front page: Stem rot of groundnut at seedling stage
Cover back page: Groundnut field

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