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Isolation, characterization and comparative analysis of plant-associated bacteria for suppression of soil-borne diseases of field-grown groundnut in Vietnam

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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*, plant-associated 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 three bacterial Phyla, i.e. the γ-Proteobacteria (*Pseudomonas*), Firmicutes (*Bacillus*) and Bacteroidetes (*Chryseobacterium*). Nethouse and field experiments conducted in central Vietnam showed that treatment of groundnut seeds or field soil with strains of each of these three bacterial genera significantly reduced the incidence of stem rot disease, led to significant yield increases of up to 21% and did not have adverse effects on nodulation. The level of disease protection provided by the bacterial strains was similar to that achieved by the fungicide tebuconazole. Comparative analysis of the bio-control efficacy of the indigenous *Pseudomonas* strain R4D2 with that of two exogenous, antagonistic *Pseudomonas* strains from the Netherlands showed that in field trials the indigenous strain R4D2 better colonized the roots of groundnut, reduced stem rot (*S. rolfsii*), black collar rot (*Aspergillus niger*), and bacterial wilt (*Ralstonia solanacearum*), and more consistently enhanced groundnut yield.

1. Introduction

In Vietnam, groundnut (*Arachis hypogaea* L.) is the most important oil seed crop with an area of 208,149 ha and an annual production of approximately 0.45 million tons in 2014 (FAO 2017). 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 one of the most destructive diseases (Mehan et al., 1994). Surveys conducted in agricultural fields in central Vietnam showed that 5–25% of the groundnut plants were infected by *S. rolfsii* (Le et al., 2011). 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 hyphal growth of *S. rolfsii in vitro* (Curtis et al., 2010; Ganesan et al., 2007; Ganesan and Gnanamanickam 1987; Kishore et al., 2005a; 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 suspension 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 poly-galacturonase and cellulase produced by *S. rolfsii*. Strains GSE18 and GSE19 also suppressed growth of *S. rolfsii* and reduced the incidence of stem rot of groundnut (Kishore et al., 2005b). Phenazine-producing *Pseudomonas chlororaphis* strain Phz24 and lipopeptide-producing *Pseudomonas* sp. strain SH-C52 suppressed stem rot disease of groundnut under controlled conditions and in field trials in central

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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	$3.4 \times 10^6 \pm 0.5 \times 10^6$	960	2	0.2	1, 3
	Roots	$3.5 \times 10^6 \pm 0.5 \times 10^6$	960	7	0.7	27, 37
Thua Thien Hue	Stem base	$3.0 \times 10^6 \pm 1.1 \times 10^6$	720	4	0.6	2, 4
	Roots	$3.3 \times 10^6 \pm 0.8 \times 10^6$	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 Supplementary Table S1).

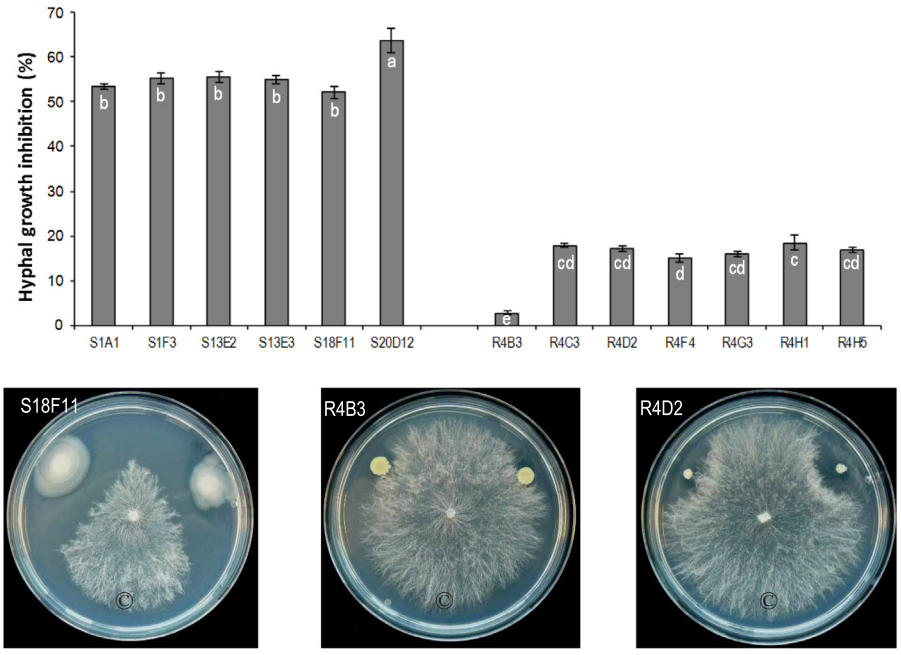

Figure 1. Hyphal growth inhibition (HGI) of *Sclerotium rolfsii* on 1/5th PDA by different bacteria isolated from stem base and roots of groundnut plants in Vietnam. The first letter of the bacterial isolates' code refers to the origin, i.e. stem base (S) or roots (R). The percentage of hyphal growth 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. Different letters indicate a statistically significant difference between the treatments ($P = 0.05$, Duncan Multiple Range Test). The pictures at the bottom show examples of the variation in hyphal growth inhibition of *S. rolfsii* for three bacterial isolates on 1/5th PDA plates after 48 h of incubation at 25 °C. The control spot (no bacteria) is indicated by©.

Vietnam (Le et al., 2012). Next to pseudomonads, also *Bacillus* species are studied extensively for biocontrol of stem rot disease of groundnut. Pre-treatment of groundnut seeds with *Bacillus subtilis* protected 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 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 local groundnut fields. In addition, groundnut is also infected by other pathogens e.g. *Aspergillus niger*, *Rhizoctonia solani*, and *Ralstonia solanacearum*. 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 and other diseases of groundnut and to improve yield, and 3) conduct a comparative analysis of the efficacy of indigenous and exogenous *Pseudomonas* strains to control multiple soil-borne diseases of groundnut.

2. Material and methods

2.1. Bacterial isolation and growth conditions

Healthy groundnut plants were collected from farmer fields in Quang Nam and Thua Thien Hue provinces in Vietnam. 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, 2004, Le et al., 2011). Across the groundnut field, a total of 40 and 30 groundnut plants at flowering stage were randomly collected in farmer fields in Quang Nam and Thua Thien Hue, respectively. 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). From each replicate sample, forty-eight bacterial colonies were randomly picked and purified on Pseudomonas Agar (PSA; Difco, France) medium. Those isolates that were inhibitory to the growth of *S. rolfsii* in dual culture inhibition assays were stored in glycerol (40%, v/v) at −20°C and −80°C.

2.2. 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

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