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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 biocontrol 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.

Keywords:

Antagonistic bacteria, *Bacillus, Chryseobacterium, Pseudomonas, Sclerotium rolfsii*.

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 polygalacturonase 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 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.

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. 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 -20ºC and -80°C.

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). In the initial screen, a total of 3,360 randomly selected bacterial isolates was tested. 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. 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 (measured 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) by each of the bacterial isolates was calculated relative to the control with the formula: Hyphal growth inhibition (%) = [(Radial hyphal growth in control – Radial hyphal growth toward bacterial colony) × 100] ÷ (Radial hyphal growth in control)].

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).

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 based on the method of Le et al. (2012). Briefly, 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 of 10^7 cells ml^{-1}. For the control treatment, pre-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. Each treatment consisted of three trays (three replicates) with 12 bags per tray. The trays were randomized. Two weeks after inoculation of the pathogen at the base of the groundnut stem, disease incidence (DI) and disease severity (DS) 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 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).

Field experiments

The first 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 six treatments, i) chemical fungicide Folicur, ii) *Chryseobacterium* strain R4B3, iii) *Pseudomonas* strain R4D2, iv) *Bacillus* strain S18F11, and v) *Bacillus* strain S20D12, and vi) control (no treatment). Follow-up field experiments were conducted in 2014 and 2015 in Thua Thien Hue province, consisting of five treatments: i) control (no treatment), ii) chemical fungicide Folicur, iii) indigenous *Pseudomonas* strain R4D2, iv) exogenous *Pseudomonas corrugata* strain SH-C52 isolated from sugar beet rhizosphere in the Netherlands (Mendes et al. 2011; van der Voort et al. 2015), and v) exogenous *Pseudomonas fluorescens* strain SS101 isolated from wheat rhizosphere in the Netherlands (Souza et al. 2003). The field experiment was set-up in a randomized complete block design (RCBD) with three blocks as three replications and a plot size of 15 m^2 (3 × 5 m). The distance was 30 cm between rows and 10 cm between plants within a row. Groundnut cultivation and bacterial inoculation were conducted according to the methods described by Le et al. (2012).

Statistical analysis

Data are expressed in percentages were arcsin-transformed prior to statistical analysis. Normal distribution of the data and homogeneity of variances was tested prior to ANOVA. Statistical differences ($P < 0.05$) between treatments were analysed by ANOVA followed by the Dunnet test or the Duncan multiple range test using statistical software SPSS Statistics, Chicago, IL, USA.

Results and Discussion

Frequency of bacteria with antagonistic activity against S. rolfsii

The number of culturable 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 3×10^6 CFU per gram of plant tissue (Table 1). Out of a total of 3,360 randomly selected isolates, only 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). 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 (Fig. 1). Also Tonelli et al. (2010) reported a relatively low percentage (1.5%) of bacterial 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 condition (1/5th PDA) and the *S. rolfsii* strain (H001) used in the inhibition assays may affect the outcome of these *in vitro* inhibition assays.

Diversity and taxonomy of the antagonistic bacteria

The genotypic diversity of the 13 isolates from groundnut that inhibited hyphal growth of *S. rolfsii* was analyzed by BOX-PCR analysis. The 13 antagonistic isolates were grouped in six BOX groups and were genotypically different from thirty-six BOX groups found for non-antagonistic isolates (Table 1, Supplementary Table S1). BOX-groups 37 and 2 harbored most of the

antagonistic isolates with six isolates from the roots and three from the stem base of groundnut, respectively (Table S1). BOX-groups 1, 3 and 4 harbored one antagonistic isolate each (Table S1). 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.

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 groups 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 Phyla (genera), i.e. the Firmicutes (*Bacillus*), Proteobacteria (*Pseudomonas*), and Bacteroidetes (*Chryseobacterium*) (Fig. 2). 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 Souza et al. (2003) who showed that only 35.8% - 73.5% of the bacteria isolated from wheat roots on this semi-selective medium were *Pseudomonas* species, whereas the other isolated bacteria represented different genera.

The three antagonistic strains classified as *Bacillus* (i.e. S1F3, S18F11, S20D12) originated from the stem base. At the species level, strains S1F3 and S20D12 were in the same clade as *Bacillus amyloliquefaciens* reference strains, whereas strain S18F11 clustered with several *Bacillus subtilis* strains (Fig. 2). For the two antagonistic strains isolated 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 (Abd-Allah and El-Didamony 2007; Abd-Allah 2005; Abd-Alla and Ezzat 2003; Curtis et al. 2010; Hameeda et al. 2010; Pleban et al. 1995; Tonelli et al. 2011; Tonelli et al. 2010). 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). *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. 3). The other three bacterial strains 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. 3). 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. 4). At seedling, flowering and peg-making stages, the disease incidence was too low to detect suppressive effects of the different treatments (Fig. 4). This

is consistent with previous studies that showed that biological control of the soil-borne fungal pathogen *Fusarium oxysporum* is more pronounced at medium to high disease incidence levels (Raaijmakers et al. 1999). In the field experiment, 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). The bacterial treatments and the chemical Folicur had no effect on plant height, branch length (data not shown), or the number of root 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).

Comparative analysis of the biocontrol efficacy of indigenous and exogenous Pseudomonas strains

It is generally assumed that microorganisms isolated from the host plant and the prospected area of application should be more effective in biological control than microorganisms isolated from other hosts and/or other geographic regions. To start investigating this assumption, we set-up a new set of field trials in 2014 and 2015 in Vietnam comparing the biocontrol efficacy of indigenous *Pseudomonas* strain R4D2, isolated in this study from groundnut in Vietnam, with that of the two exogenous *Pseudomonas* strains SS101 (isolated from wheat roots in the Netherlands; Souza et al. 2003) and SH-C52 (isolated from sugar beet roots in the Netherlands; Mendes et al. 2011; Van der Voort et al. 2015). We not only monitored the effects of these strains on stem rot caused by *S. rolfsii* but also looked into their effects on other groundnut diseases occurring naturally in the field. The results showed that for stem rot (*S. rolfsii*), indigenous strain R4D2 significantly reduced disease development (expressed as area under the disease progress curve, AUDPC) in 2014 and 2015; also exogenous strain SH-C52 showed similar suppressive effects; exogenous strain SS101 also showed significant suppressive effects but less than strain R4D2 (Fig. 5A). For damping-off disease caused by *R. solani*, only strain SH-C52 reduced plant mortality significantly in 2014 and 2015 (Fig. 5B).

For black collar rot (*A. niger*), significant effects on disease development were observed in 2015 for strains R4D2 and SH-C52 (Fig. 5C). Also for bacterial wilt disease (*R. solanacearum*), significant effects were only observed in 2015: all three bacterial strains reduced disease significantly, with strain R4D2 showing the largest reduction (Fig. 5D). Results of these two field trials also showed that the introduced bacterial strains colonized the root and stem base at densities of approximately 5 to 6 log cfu g^{-1} (Table 3). Indigenous strain R4D2 established, in both field trials, higher densities on roots of groundnut than exogenous strains SS101 and SH-C52 (Table 3). Similar to our previous field trials, none of these three strains adversely affected the number of root nodules, whereas the chemical treatment did significantly reduce the number of nodules in the 2014 field trial. Both strains SS101 and R4D2 significantly increased pod yield of groundnut in 2014. In 2015, strain R4D2 was the only bacterial strain that significantly increased pod yield relative to the control. In the field, groundnut is not only infected by *S. rolfsii* but also other pathogens. Therefore, beneficial bacteria targeted for biocontrol in the field should preferably be antagonistic to multiple pathogens. Based on the results of our two-year field trials, we conclude that introduction of different rhizobacterial strains can control a number of soil-borne diseases of groundnut to similar or higher levels than the fungicide Folicur and enhance pod yield significantly. Indigenous *Pseudomonas* strain R4D2 colonized the roots of groundnut to a higher density and provided a more consistent increase in pod yield than the two exogenous *Pseudomonas* strains.

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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

*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).

Table 2. Effects of four different bacterial strains and the 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)

Table 3. Comparative analysis of the biocontrol efficacy of three different *Pseudomonas* strains and the fungicide Folicur on nodulation and pod yield of groundnut plants grown under field conditions in Thua Thien Hue province, Vietnam, in 2014 and 2015. Indigenous strain R4D2 was isolated from groundnut in Vietnam, whereas exogenous strains SS101 and SH-C52 were isolated from wheat rhizosphere and sugar beet rhizosphere in the Netherlands. 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, within a year, different letters indicate a statistically significant difference between the treatments (P= 0.05, Duncan Multiple Range Test)

Supplementary Table S1. 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*

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 25ºC. The control spot (no bacteria) is indicated by ©.

Figure 2. Colony morphology and phylogeny of five selected bacterial isolates from groundnut (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.

Figure 3. Biocontrol of stem rot disease of groundnut (panel A) and colonization of the stem base and roots of groundnut plants (B) by four different bacterial strains under nethouse conditions. 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.

Figure 4. Control of stem rot disease of groundnut by the fungicide Folicur, and groundnut-associated bacteria *Chryseobacterium* sp. strain R4B3, *Pseudomonas* sp. strain R4D2, and *Bacillus* sp. strains S18F11 and S20D12 under field conditions in Quang Nam province, Vietnam. Plant mortality was monitored at seedling stage, when plants had 3-5 true leaves (20 days after sowing), flowering stage, peg-making stage and pod set stage. For each developmental stage, averages of three replicates are given. Error bars represent the standard error of the mean. The asterisk indicates a statistically significant difference between the control and the treatments (*P=0.05*, Dunnett).

Figure 5. Comparative analysis of the efficacy of three *Pseudomonas* strains to control multiple diseases of groundnut, including (A) stem rot disease (*S. rolfsii*), (B) damping-off (*Rhizoctonia solani*), (C) black collar rot (*A. niger*) and (D) bacterial wilt (*Ralstonia solanacearum*). Strain R4D2 represents the indigenous strain from Vietnam, whereas strains SS101 and SH-C52 are the exogenous strains from the Netherlands. The biocontrol efficacy was tested under field conditions in Thua Thien Hue province, Vietnam, in 2014 and 2015. The fungicide Folicur was included as the chemical control. For stem rot disease (A), black collar rot (C) and bacterial wilt (D), 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 plot, the numbers of diseased or wilted plants were counted weekly. For *Rhizoctonia* damping-off (B), plant mortality was monitored at seedling stage only. Different letters indicate a statistical significant difference between the treatments (*P=0.05*, Duncan Multiple Range Test).

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).

Table 2. Effects of four different bacterial strains and the 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}
R4B3	5.4^{a}	5.1^{a}	5.0^{ab}	5.4^{a}	121^{a}	1190^{b}
R4D2	5.3^{a}	5.1^{a}	5.2^{a}	5.5^{a}	126^{a}	1320^{ab}
S18F11	5.4^{a}	5.4^{a}	5.0^{ab}	5.0^{ab}	116^{a}	1320^{ab}
S20D12	5.5^{a}	5.2^{a}	4.4^{b}	4.2^{b}	134^{a}	1480^{a}

Table 3. Comparative analysis of the biocontrol efficacy of three different *Pseudomonas* strains and the fungicide Folicur on nodulation and pod yield of groundnut plants grown under field conditions in Thua Thien Hue province, Vietnam, in 2014 and 2015. Indigenous strain R4D2 was isolated from groundnut in Vietnam, whereas exogenous strains SS101 and SH-C52 were isolated from wheat rhizosphere and sugar beet rhizosphere in the Netherlands. 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, within a year, 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		
2014						
Control	-	-	-	-	149^{a}	1942^{c}
Folicur	-	-	-	-	137^{a}	2249^{bc}
SS101	4.9^{b}	5.1^{b}	5.0^{b}	5.0^{c}	174^{a}	2549^{ab}
SH-C52	5.0^{b}	5.5^{ab}	5.3^{b}	5.4^{b}	157^{a}	2115^{c}
R4D2	5.9^{a}	5.8^{a}	5.8^{a}	5.7^{a}	165^{a}	2633^{a}
2015						
Control	-	-	-	-	167^{ab}	2450^{b}
Folicur	-	-	-	-	141^{b}	2513^{ab}
SS101	5.0^{a}	5.4^{b}	5.0^{a}	5.7^{ab}	192^{a}	2662^{ab}
SH-C52	5.6^{a}	5.6^{ab}	5.1^{a}	5.0^{b}	186^{ab}	2357^{b}
R4D2	5.8^{a}	6.2^{a}	5.6^{a}	6.0^{a}	178^{ab}	2814^{a}

Supplementary Table S1. 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	-

Fig. 1

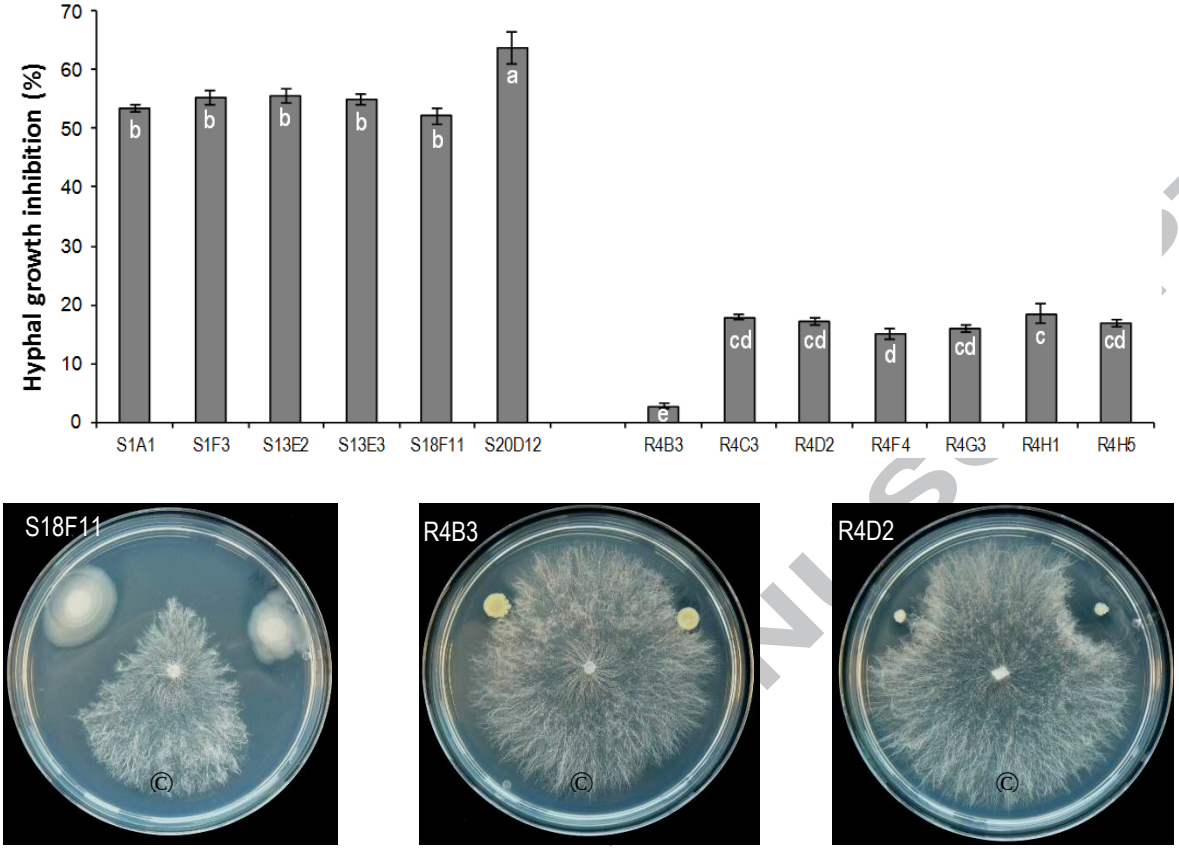


Fig. 2

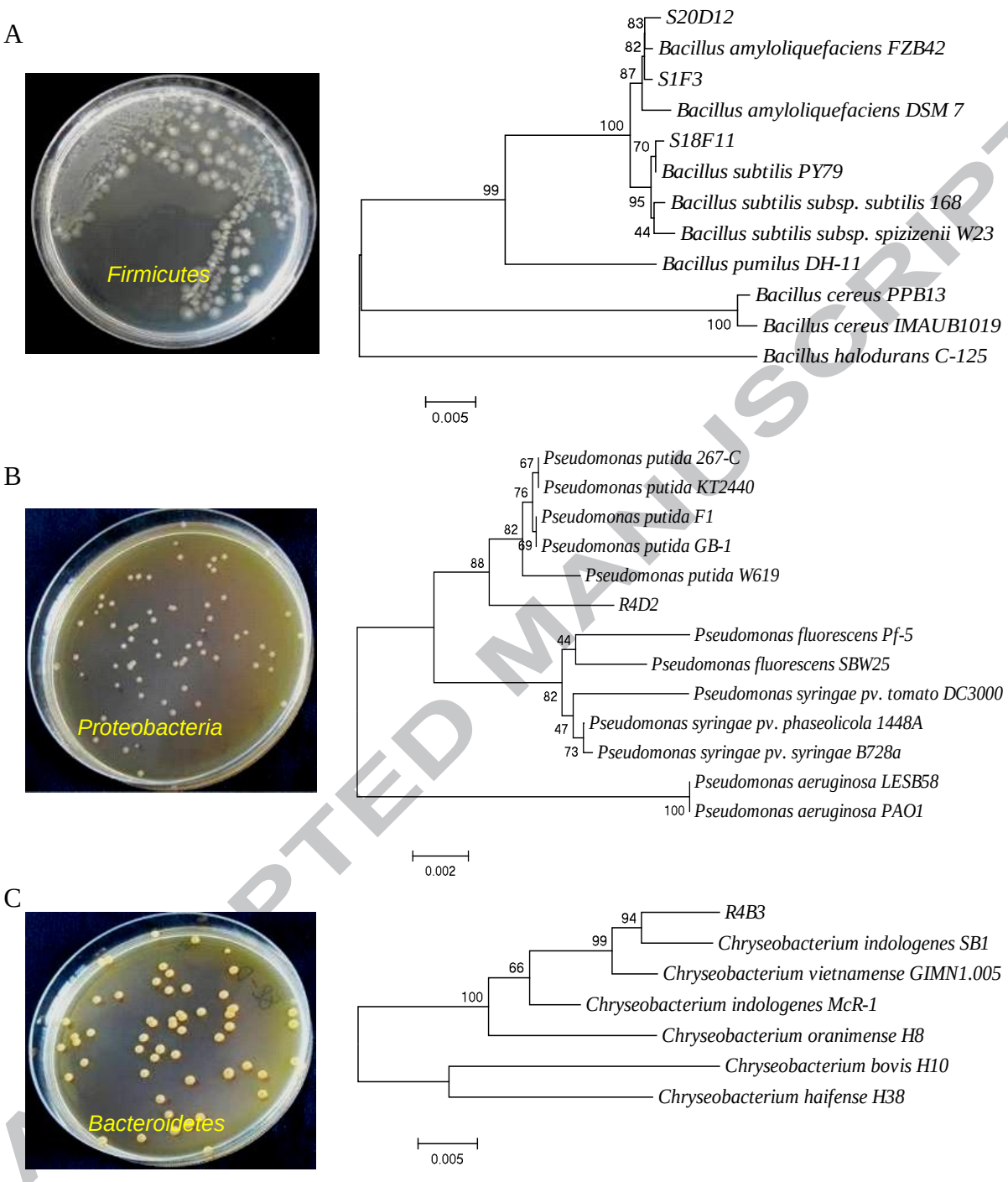


Fig. 3

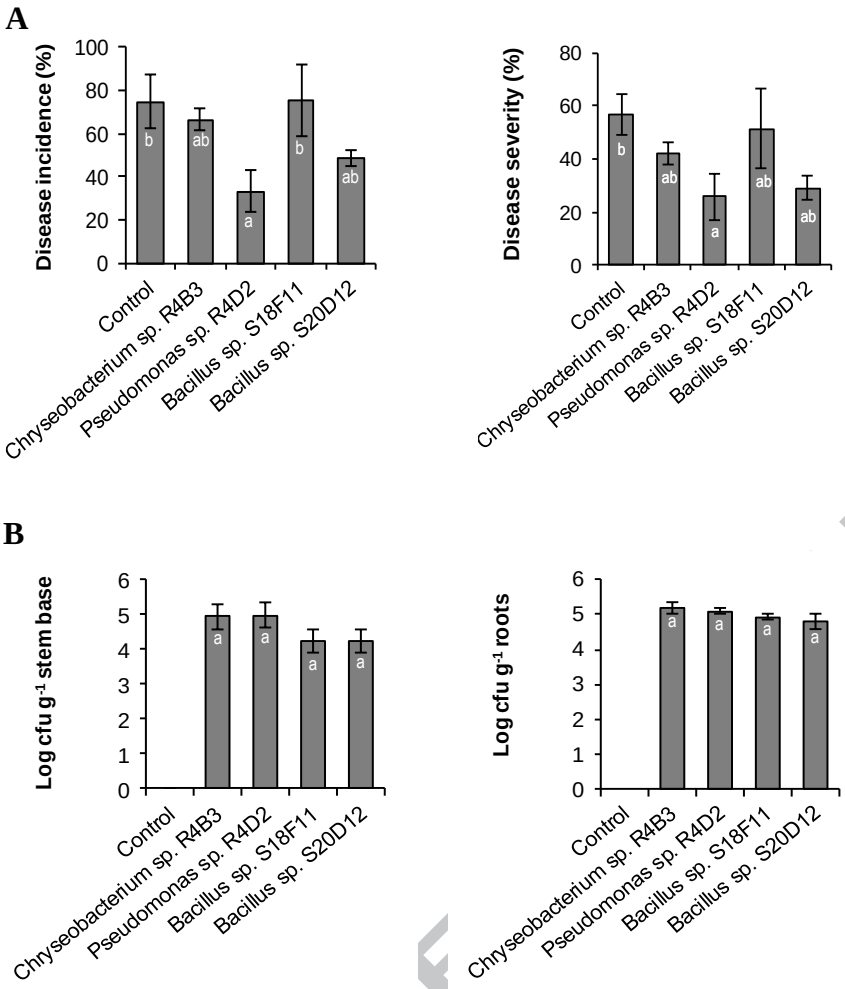


Fig. 4

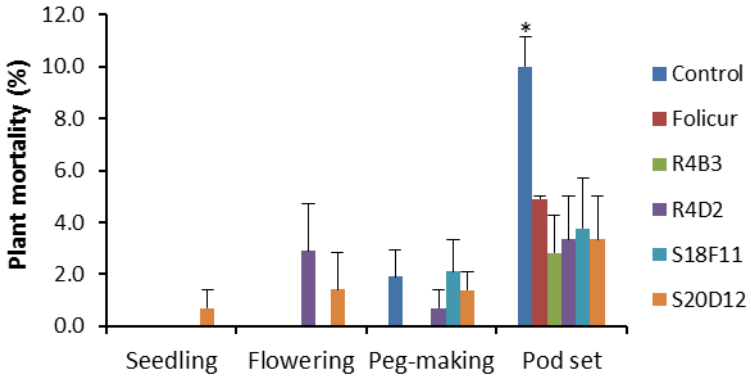
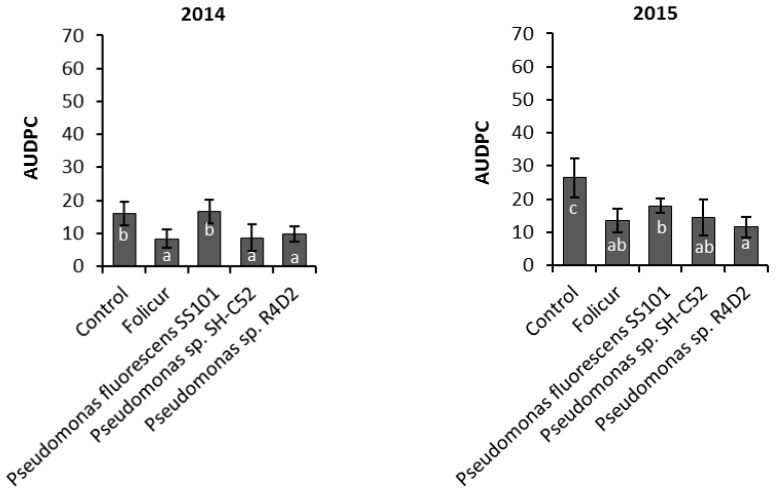


Fig. 5

A



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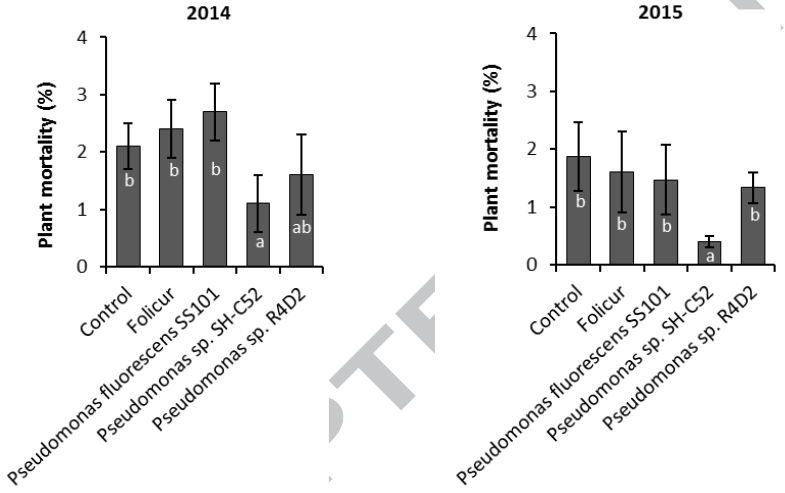

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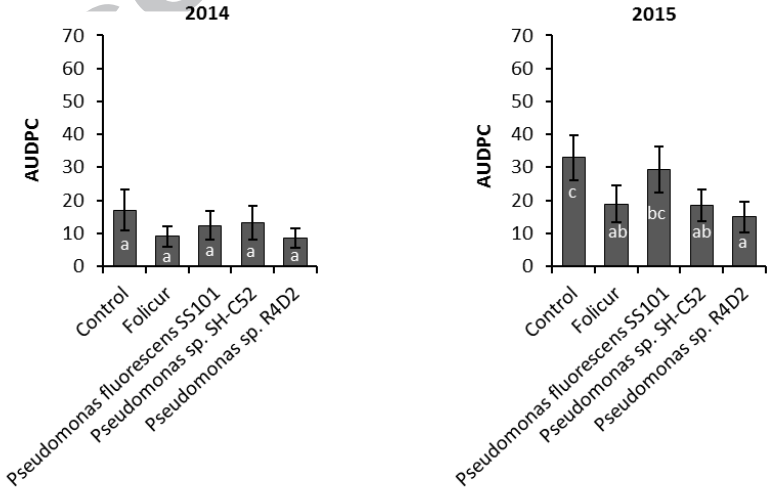

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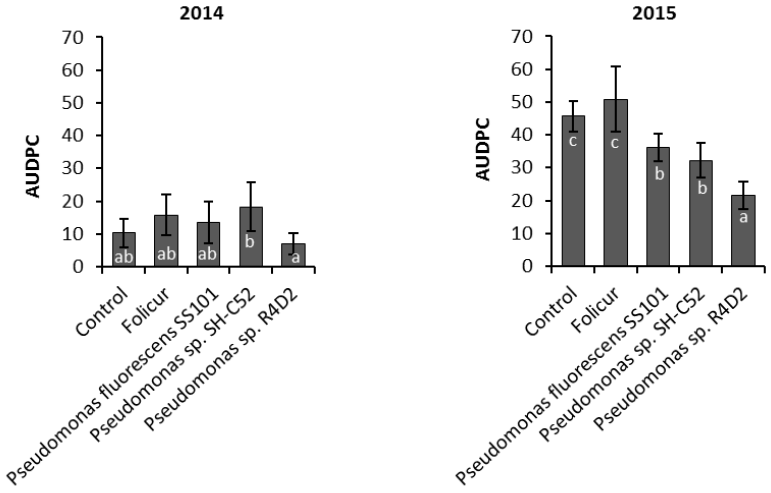

Highlights

Several indigenous bacterial genera are effective in controlling stem rot disease.

Indigenous *Pseudomonas* establishes higher population densities than exogenous.

Indigenous *Pseudomonas* strain reduces multiple soil borne diseases of groundnut.

Indigenous *Pseudomonas* strain consistently increases groundnut yield.