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Genetic diversity of groundnut rhizosphere antagonistic bacteria and biological control of groundnut wilted diseases in central Vietnam

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ABSTRACT

Stem rot of groundnut caused by *Sclerotium rolfsii*, seriously damages groundnut production in central Vietnam. Biological control is a promising strategy for sustainable groundnut cultivation. In this study, indigenous bacteria were isolated from the rhizosphere of groundnut and tested for fungal inhibition against *S. rolfsii in vitro* and disease control under net house condition. Genetic diversity of isolated bacterial population was evaluated by BOX-PCR and 16S rDNA sequences. Bacterial strains that showed high disease control in net house were evaluated under natural conditions in farmer fields. The antifungal mechanism of the best bacterial strain was identified. Results of the study showed that the antagonistic bacterial population in groundnut rhizosphere is separated in three bacterial genera including *Bacillus*, *Pseudomonas* and *Burkholderia*. One bacterial strain which produces 2,4-DAPG reduced stem rot of groundnut caused by *S. rolfsii* and increased yield from 20.3 to 26.3% compared to the control.

Key words: *Arachis hypogaea*, Bacteria, Groundnut, *Scleortium rolfsii.*

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is the most important oilseed crop in Vietnam with a total of 210,000 ha cultivated in 2014 (FAO, 2017). The yield of groundnut in Vietnam is affected by several soil borne pathogens including *Sclerotium rolfsii*, *Aspergillus niger* and *Ralstonia solanacearum* (Nguyen *et al.*, 2004). In India stem rot caused by *S. rolfsii* inflicts yield loss up to 30% in groundnut (Anonymous, 2012); yield loss may reach 80% in severely infested fields (Mayee and Datar, 1988). In central Vietnam, the disease incidence was reported to be 5-20% at flowering stage (Le *et al.*, 2011). The pathogen survives in the soil as sclerotia for a long period of time making it very difficult to control (Punja, 1985; Smith *et al.*, 1989). To control the disease, there are several recommended methods such as application of fungicides, nutrients and bio control factors. For fungicides, propiconazole, cyproconazole and tebuconazole have been studied (Baird *et al.*, 1991; Culbreath *et al.*, 2009; Franke *et al.*, 1998; Fuller *et al.*, 1990; Grichar, 1995; Yaqub and Shahzad, 2006), however, some *S. rolfsii* strains showed resistance to fungicide (Franke *et al.*, 1998). For cultivation, rotation with non-host crops is recommended; however, it is difficult to select non-host crops of *S. rolfsii* (Punja, 1985). Nutrient supplements reduced disease and increased yield of groundnut (Jadon *et al.*, 2018). Application of nitrogen reduced stem rot disease caused by *S. rolfsii*, however, this method is not recommended for groundnut cultivation being a leguminous crop. Biological control has been studied to control the soil borne pathogens of many crops including *S. rolfsii*, the causal agent of stem rot of groundnut (Ghasemi *et al.*, 2017; Karthikeyan *et al.*, 2006; Le *et al.*, 2018; Le *et al.*, 2012; Rajyaguru *et al.*, 2017)

To control plant diseases, biocontrol factors such as *Bacillus* and *Pseudomonas* have been used for a long time (Abeysinghe, 2009; Fernando *et al.*, 2007; Karthikeyan *et al.*, 2006). Studies on biological control of stem rot of groundnut showed that some antagonistic bacteria inhibited hyphal growth of *S. rolfsii* and suppressed stem rot diseases (Fernando *et al.*, 2007; Ganesan *et al.*, 2007; Karthikeyan *et al.*, 2006; Murugalakshmi *et al.*, 2009). However, there are few studies on antagonistic bacteria in groundnut rhizosphere and evaluation for controlling multiple diseases under natural conditions, where groundnut is infected by several pathogens. The objectives of this study were to isolate and identify the diversity of antagonistic bacteria in the rhizosphere of groundnut in central Vietnam and to control groundnut wilt diseases by antagonistic bacteria.

MATERIALS AND METHODS

Identification of genetic diversity of antagonistic bacteria in groundnut rhizosphere: The antagonistic bacteria were isolated from groundnut rhizosphere in central Vietnam. To

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isolate the antagonistic bacteria, the roots of groundnut, grown in Thua Thien Hue, Quang Nam and Quang Binh provinces were collected. The collected samples were kept in cool condition prior to isolation in the laboratory based on the method of Kruijt *et al.* (2009). Selection of antagonistic bacteria based on hyphal growth inhibition assays was described by Kruijt *et al.* (2009) and Le *et al.* (2018).

The antagonistic bacteria were grouped based on BOX-PCR barcoding as described by Tran *et al.* (2008). One isolate of each BOX-PCR group, which showed high disease suppression under net house condition, was selected for sequencing of 16S rDNA using universal primers 518F (5' CCAGCAGCCGCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3'). The obtained forward and reverse sequences were assembled and edited in Vector NTI (Invitrogen, version 8.0) and deposited in GenBank. For the phylogenetic analyses, the edited sequences were aligned to reference sequences available in databases (http://www.ncbi.nlm.nih.gov). Sequences were trimmed to the same size (~1297 bp) and a phylogenetic tree was obtained using MEGA7 software (http://megasoftware.net).

Biological control of groundnut diseases by antagonistic bacteria: Two bacterial strains that controlled stem rot under net house conditions with artificial *S. rolfsii* inoculation (previous studies) originating from the provinces of Quang Binh (QB), Thua Thien Hue (H) and Quang Nam (QN) were evaluated in natural condition in farmer fields with high stem rot infection in Thu Thien Hue province. The first field experiment was conducted in 2015 and the second in 2016. The field experiment consisted of seven treatments: control (no treatment), *Bacillus* sp. strain QB 5/3, *Pseudomonas* sp. strain H 9/15, *Bacillus* sp. train QN 4/18, *Bacillus* sp. train H 13/8, *Bacillus* sp. strain QN 4/22 and *Bacillus* sp. strain QB 1/1. The field experiment was laid out in a randomized complete block design (RCBD) with 3 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 the row. Groundnut cultivation and bacterial inoculation were conducted according to the methods described by Le *et al.* (2012). Plant mortality was monitored weekly and total number of wilted plant was counted. Mortality rate (MR) was calculated using the formula MR% = 100 × (total wilted plants ÷ total plants per plot) (Le *et al*., 2012).

Mechanism of antifungal activity of antagonistic bacteria: The bacterial strain *Pseudomonas* sp. strain H 9/15 was further studied for bio compounds and gene(s) related to fungal inhibition and disease suppression. The bio compounds in cell free extract of H 9/15 liquid culture were analyzed by HPLC based on the method of Brucker *et al.* (2008). Mutants which lack fungal inhibition were created based on method described by Dennis and Zylstra (1998) and the gene(s) related to fungal inhibition of this strain will be identified.

Statistical analysis: Values of mortality rate expressed in percentages were arcsine-transformed prior to statistical analysis. Normal distribution of the data and homogeneity of variances were tested prior to ANOVA. Statistical differences ($P < 0.05$) between treatments were analyzed by ANOVA followed by the Duncan multiple range test using statistical software SPSS Statistics, Chicago, IL, USA.

RESULTS AND DISCUSSION

Genetic diversity of antagonistic bacteria in groundnut rhizosphere: The genotypic diversity of the 65 isolates from groundnut that inhibited hyphal growth of *S. rolfsii* was analyzed by BOX-PCR analysis. The 65 antagonistic isolates were grouped in 19 BOX groups. The biggest BOX-PCR group harbored eleven isolates while six BOX-PCR groups harbored only one isolate (Table 1). The relatively high

Table 1: Bacterial identification and genetic diversity based on 16S rDNA sequences and BOX-PCR.

Bacterial genera	Number of BOX-PCR	Number of isolates	Diversity of number of isolates per BOX-PCR group
Pseudomonas sp.	10	28	1-6
Burkholderia sp.	1	1	1
Bacillus sp.	8	36	1-11

Table 2: Effect of antagonistic bacteria in groundnut rhizosphere on yield of groundnut in two field experiments conducted in 2015 and 2016.

Treatments	2015 Pod yield (kg ha^{-1})	2015 Increase (%)	2016 Pod yield (kg ha^{-1})	2016 Increase (%)
Control	2596^{cd}	-	3104^{b}	-
Bacillus sp. QB 5/3	3173^{ab}	18.2	3372a^{b}	7.9
Pseudomonas sp. H 9/15	3522^{a}	26.3	3896^{a}	20.3
Bacillus sp. QN 4/18	3077^{abc}	15.6	2849^{b}	-9.0
Bacillus sp. H 13/8	2442^{d}	-6.3	2900^{b}	-7.0
Bacillus sp. QN 4/22	2667^{bcd}	2.7	3270^{ab}	5.1
Bacillus sp. QB 1/1	2719^{bcd}	4.5	3379^{ab}	8.1

QB indicates Quang Binh province, H indicates Thua Thien Hue province and QN indicates Quang Nam province (For each column, data followed by different letters indicate a statistically significant difference between the treatments (*P=0.05*, Duncan Multi Range Test).

genotypic diversity of groundnut-associated bacteria observed here was also reported by Tonelli *et al.* (2010) and Le *et al*. (2018) for bacterial populations from groundnut plants.

Phylogenetic analyses revealed that bacterial strains QB5/3, H14/18, QN18/4, Qb1/1, QN4/18, H13/8, QN22LC1, and QN4/22 belong to the Firmicutes (*Bacillus*) while strains H14/5, QB14/1, H5/5, QN12/22, H9/15, QB9/7, Q2/3, QN7/15-N, QN14/1, QN20/11 and QN3/20 belong to the Proteobacteria (*Pseudomonas, Burkholderia*) (Fig 1). Results of studies showed that antagonistic bacteria belonging to *Bacillus* genera with eight strains represent for 36 isolates in eight BOX-PCR groups; antagonistic bacteria belonging to *Pseudomonas* genera with ten strains represent for 28 isolates in ten BOX-PCR groups. Only one strain belongs to *Burkholderia*. The edited 16S rDNA sequences were deposited in GenBank with accession numbers from MH211366 to MH211384.

Biological control of stem rot by antagonistic bacteria: Results of two year studies on multiple diseases control showed that all tested bacterial strains reduced MR of stem rot compared to the control in 2015 (Fig 2). However, in 2016, only *Pseudomonas* sp. strain H9/15 reduced MR of stem rot compared to the control. The variation in disease control may be due to the changes in natural conditions (Martins *et al.*, 2010) or the pathogenicity of the pathogens

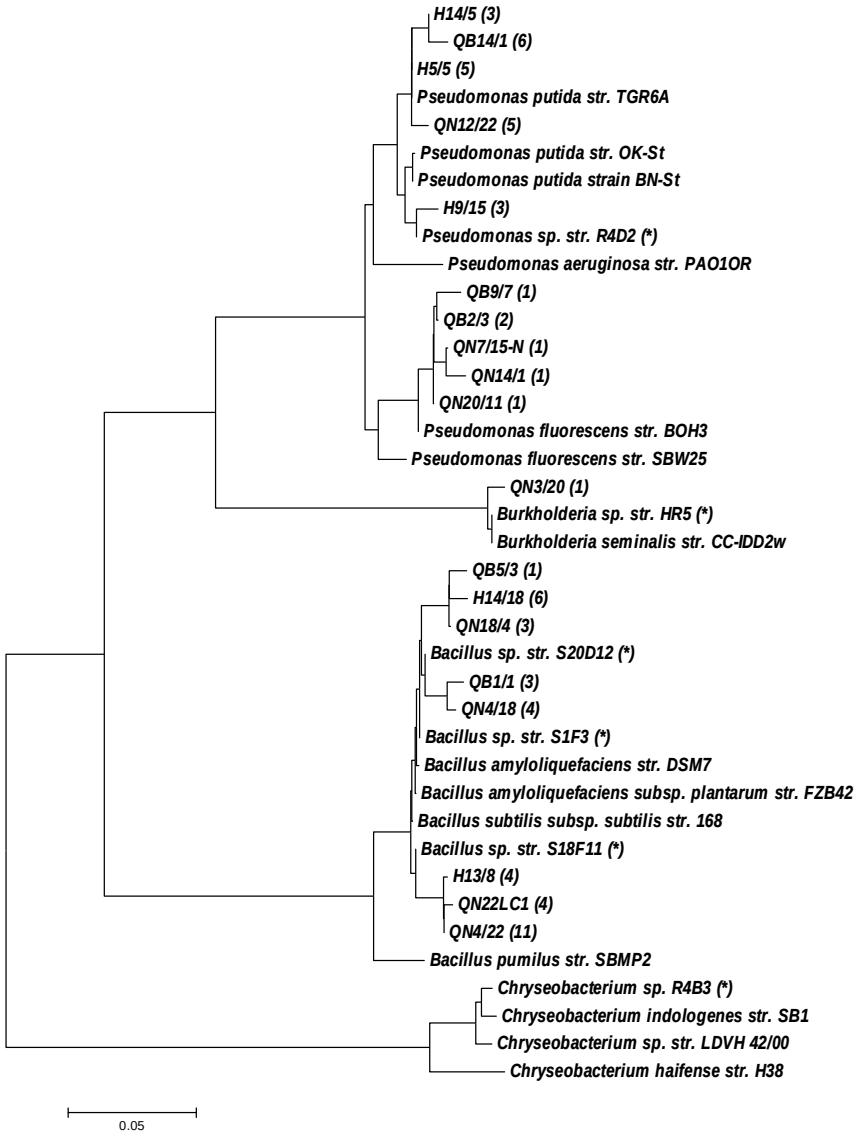

Fig 1: Phylogeny of antagonistic bacterial isolates from groundnut (QN14/1, QN20/11, QN7/15-N, QN12/22, H9/15, H5/5, H14/5, QB14/1, QB2/3, QB9/7, QN3/20, QN4/22, QN4/18, QN18/4, QN22LC1, H14/18, H13/8, QB1/1, QB5/3) that inhibit the hyphal growth of *Sclerotium rolfsii*. The branch length indicates the percentage of sequence dissimilarity; the number in bracket indicates a number of isolates in the same BOX-PCR group; the symbol (*) indicates previous strains which were isolated from groundnut (Le *et al*. 2018).

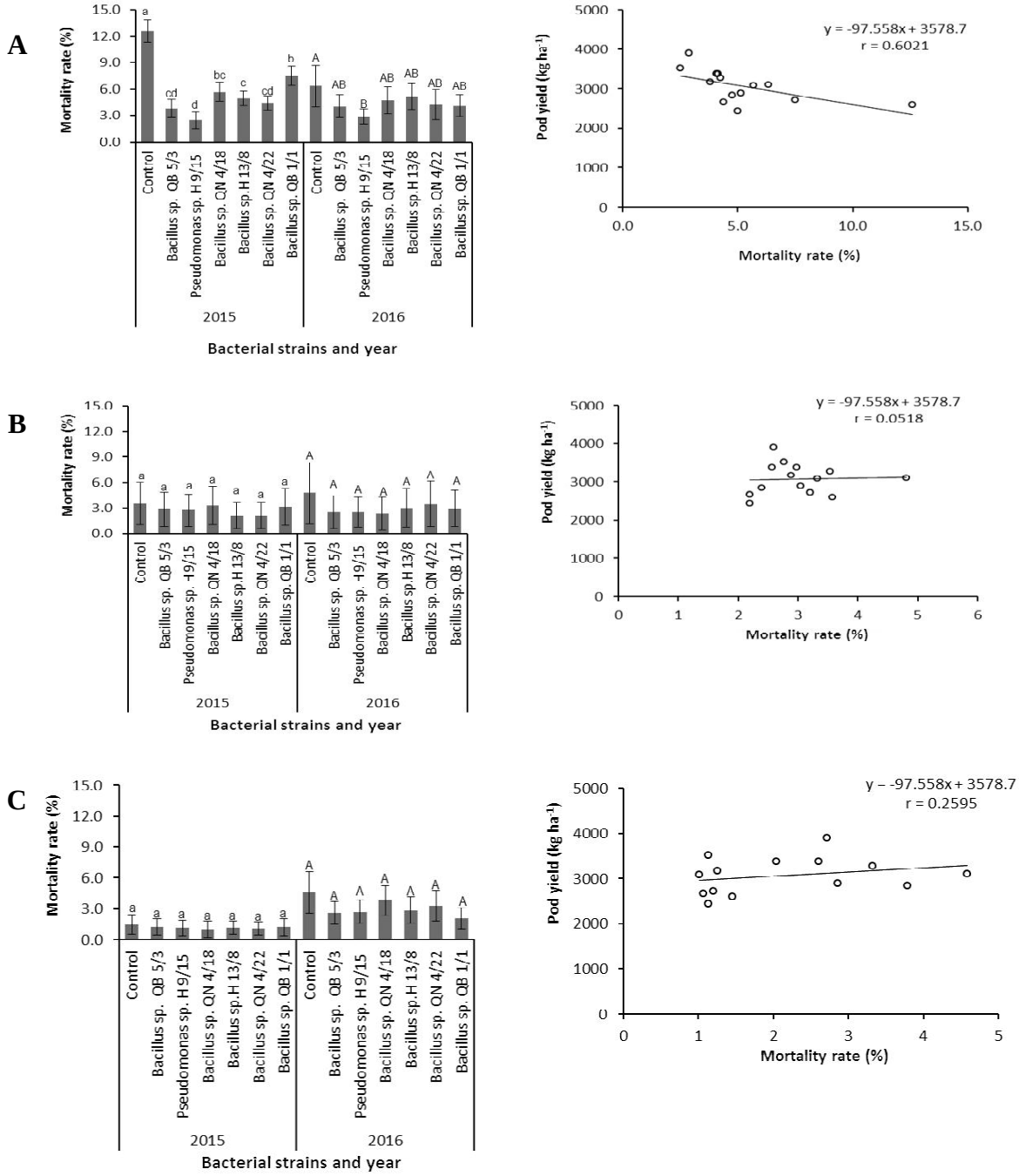

Fig 2: Efficacy of bacterial strains to control multiple wilted diseases of groundnut and correlation between mortality rate and groundnut yield, including (A) stem rot disease (*S. rolfsii*), (B) black collar rot (*A. niger*) and (C) bacterial wilt (*R. solanacearum*). The biocontrol efficacy was tested under field conditions in Thua Thien Hue province, Vietnam, in 2015 and 2016. Different letters indicate a statistically significant difference between the treatments (*p=0.05*, Duncan Multiple Range Test).

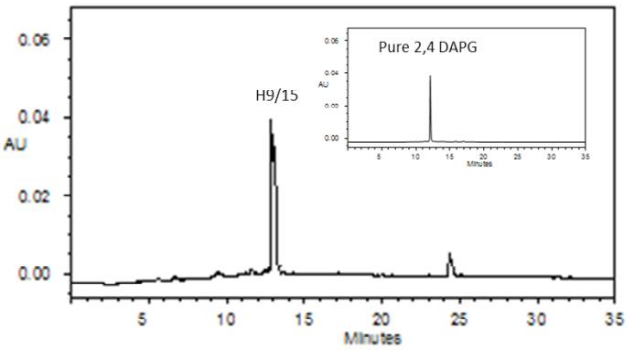

Fig 3: HPLC chromatogram comparing *Pseudomonas* sp. H9/15 crude extract and 2,4-DAPG standard. Both samples had similar retention times on the HPLC chromatograms (~ 13.5 min).

at certain times (Iquebal *et al.*, 2017). Although black collar rot caused by *A. niger* and bacterial wilt caused by *R. solanacearum* are major diseases on groundnut in central of Vietnam, results of the study showed that the tested bacterial strains did not significantly reduced black collar rot and bacterial wilt. This may be due to low MR under natural condition.

The experiment in 2015 showed that *Pseudomonas* sp. strain H 9/15 and *Bacillus* sp. strain QB5/3 significantly improved pod yield compared to the control with an increase of 26.3% and 18.2%, respectively (Table 2). In 2016, only *Pseudomonas* sp. strain H 9/15 significantly improved pod yield by 20.3% compared to the control. The yield increase may be due to disease suppression by these strains. Analysis of correlation between yield and disease showed that stem rot reduced pod yield of groundnut (Fig 2 A) but black collar rot and bacterial wilt did not significantly reduce pod yield of groundnut (Fig 2 B, C).

Mechanisms of antagonism of *Pseudomonas* sp. strain H 9/15: Results of the study revealed that H 9/15 produced 2,4-diacetylphloroglucinol (2,4-DAPG) with the signal similar to pure 2,4-DAPG at a retention time of 13 min (Fig 3). 2,4-DAPG has been shown to play a role in the control of numerous fungal pathogens including *S. rolfsii* (Raaijmakers *et al.*, 2002; Song *et al.*, 2013). The DAPG-producing rhizobacteria were also reported to promote plant growth and inhibit *S. rolfsii*, *Aspergillus flavus* and *A. niger* (Sherathia *et al.* 2016).

CONCLUSION

The antagonistic bacterial population in groundnut rhizosphere showed genetic diversity based on BOX-PCR analysis with the population separated into three bacterial genera (*Bacillus*, *Pseudomonas* and *Burkholdera*). One bacterial strain that produces 2,4-DAPG reduced stem rot of groundnut caused by *S. rolfsii* and increased yield from 20.3 to 26.3% compared to the control under natural conditions.

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