

Antagonism of walnut endophytic bacteria against six crop pathogenic fungi and its diversity

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ABSTRACT

Plant endophytes are great resources and potential capital in versatile application, including plant diseases biocontrol. We aimed to evaluate the antagonism of English walnut endophytic bacteria against different crop pathogenic fungi, to provide more alternatives for future disease biocontrol agents. The biocontrol capacity of thirty-six walnut endophytic bacterial isolates against the anthracnose fungus, *Gnomonia leptostyla* was evaluated. Fifteen isolates were tested against other five pathogenic fungi such as the cauliflower black spot pathogen, *Alternaria brassicicola*; the tobacco brown spot pathogen, *A. alternata*; the corn leaf spot pathogen *Curvularia lunata*; wheat Fusarium head blight pathogen, *Fusarium graminearum* and the cucumber anthracnose pathogen *Colletotrichum orbiculare*. From our results, it was observed that all the assayed strains suppressed the growth of six fungal pathogens at least 20% in petri dish. Among the endophytes, the highest inhibition rates were observed in endophytes XWR6 and XWL14 against *A. brassicicola* with values of 68.8 and 61.6% respectively; and the endophyte XWS7 against walnut anthracnose pathogen has the inhibition value of 60.5%. Particularly, endophytes XWS7 and XWS4 isolated from walnut stem, showed an inhibition rate above 40% in all the tested pathogens. Biochemical, physiological and phylogenetic analysis based on 16S-rDNA and *gyrB* sequences, allowed the identification of the fifteen endophytes at *Bacillus* spp. However, physiological differences and sequence diversity was observed among the isolates. These findings reveals English walnut endophytic bacteria have strong antagonism activities against crop pathogenic fungi, and show diversity in biochemical, physiological, 16S-rDNA and *gyrB* sequences.

Keywords: Walnut, endophyte, walnut anthracnose, antagonism, *Bacillus* spp.

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INTRODUCTION

In recent years, plant endophyte has demanded extensive interest in research and development. In general endophytes living in plants without causing any symptoms and obvious harm to the plant, in contrast they can benefit their host by protecting them against pathogens, or promoting plant growth in some cases (Alstrom and Van Vurde, 2001; Adame-Ivarez et al., 2014; Ardanov et al., 2012; D'Alessandro et al., 2014). Plant endophytes also could play an important role in reducing pesticides residue and in bioenergy development (Zhang et al., 2014; Afzal et al., 2014).

Several researches in scientific literature have

indicated that plant endophytes show potential ability in control of host and non-host plant diseases. For example, strains of *Bacillus* sp. and *Pseudomonas* sp. isolated from tomato roots have shown promising results as biocontrol agents for *Fusarium oxysporum* (Munif et al., 2012). In addition, plant endophytic bacteria show inhibition of spore germination and growth in filamentous fungi, such as *Rhizoctonia* sp., *Colletotrichum orbiculare* (Andrieu et al., 2000), and the oomycete palm blight *P. palmivora* (Aramsirujiwet et al., 2016).

Other important reports indicate that *Bacillus megaterium* BM1 isolated from wheat grain, inhibits

wheat Fusarium Head Blight (*F. graminearum*) mycelia growth and spore germination (Pan et al., 2015). In the crop field test, the disease incidence and deoxynivalenol (DON) toxin are reduced by 54 and 89.3%, respectively (Pan et al., 2015). *Centella asiatica* endophyte *B. subtilis* BCA31 and *P. fluorescens* BCA08 decreased *C. higginsianum* growth rate by 46 and 82%, respectively (Rakotoniriana et al. 2013). Even more, some crops infected with endophyte attenuated aphids feeding frequently, led to reduce virus infection opportunities indirectly (De Sassi et al., 2006).

Previous research has indicated that walnut endophytic fungi could inhibit several crop pathogens, including cotton blight *Rhizoctonia solani*, apple anthracnose *Gleosporium fructigenum*, pear scab *Venturia pirina*, wheat head blight *F. graminearum* and rape Sclerotinia rot disease *Sclerotinia sclerotiorum* mycelial growth, particularly, 100% inhibition rate on cotton blight (Zhai et al., 2009). The endophytic fungi isolated from walnut root, *Coniothyrium vitivora* G8, its acetyl acetate extract has inhibition against 4 fungi pathogens: apple ring rot *Physalospora piricola*, apple canker *Valsa ceratosperma*, wheat root rot *Cochliobolus sativus* and rice blast *Magnaporthe grisea*, the inhibition rate against these 4 fungi pathogens were all 100% (Xu et al., 2008). It has been observed that walnut endophyte HT-6 fermentation broth against mycelial growth of pepper anthracnose, walnut and apple canker at 80% inhibition rate, suppress spore germination of pear black spot, tomato gray mold and pepper anthracnose pathogens at 100% inhibition rate (Wu et al., 2015).

In this paper, thirty-six endophytic bacteria were isolated and identified from two-year old walnut *Juglans regia* roots, stems and leaves. The biocontrol capacity of the isolates identified against anthracnose fungus, *Gnomonia leptostyla* were evaluated. Another fifteen isolates were tested also against other five pathogenic fungi such as *Alternaria brassicicola*, *A. alternata*, *Curvularia lunata*, *F. graminearum* and *C. orbiculare*. Biochemical, physiological and phylogenetic analysis based on 16S-rDNA and *gyrB* sequences was conducted as well.

MATERIALS AND METHODS

Isolation of walnut endophytes

Samples were obtained from two years old healthy walnut plants. Tissue of roots, stems and leaves were analyzed. In the case of roots and stems, the outer cortex was scratched and cut into 2 cm x 2 cm patches. Samples were disinfected with 0.1% mercury chloride (HgCl) and 75% alcohol and washed with double deionized water (ddH₂O) for five times. The final flushed water and tissue was spread onto Nutrient Agar medium as control to check the surface disinfection effect. Samples were collected in tubes with 1 ml ddH₂O, and crushed with sterilized tweezers and incubated for 20 min and 200 µl were spread onto NA medium with three replicates, which were incubated at 26°C for 2-5 days. Colonies were selected based on different size and color, re-streaked and stored in 30%

glycerol at -80°C.

Targeting fungi pathogens

Isolates of *G. leptostyla* and *A. brassicicola* were collected in our laboratory. Tobacco brown spot *A. alternata*, cucumber anthracnose *C. orbiculare*, corn leaf spot pathogen *C. lunata*, wheat Fusarium head blight *F. graminearum* strains were provided by Hubei Academe of Agricultural Sciences.

Plate confrontation test

The plate confrontation test was conducted on NA medium plates. The target fungi were inoculated as 6 mm diameter plugs in the center of the plates and endophytic bacteria into the plate with a distant of 30 mm. Plates without endophytic bacteria were used as controls. Plates were incubated at 26°C, until the control mycelium grow to the endophyte inoculation point. Inhibitory effects were analyzed by measuring the mycelial growth radius, inhibition radius and minimum Interval Distance (ID, mm) between the pathogen and the endophyte margins. Each experiment was set in five plates, and the experiments were repeated three times. The inhibition rate was calculated based on the following formula:

$$\text{Inhibition rate (\%)} = (\text{control radius} - \text{treated radius}) / (\text{control radius} - 3) \times 100.$$

Clustering based on biochemical and physiological characteristics

The biochemical and physiological characteristics of fifteen strains were tested according to the protocols described in Dong (Dong and Cai, 2001). We conduct the following tests: v-p test, starch hydrolysis, indole produce, urase, acetic acid oxidation, citric acid salt, methyl red, catalase, cellulolase, oxidase, anti-nitrification, pyovendin, milk liquefaction, motility, ethanol oxidation, gelatin liquefaction, salt tolerance. Results were recorded and saved in binary format table (1 represented for positive, 0 represented negative or uncertain). Clustering was conducted using the NTSYSpc2.1 software.

Phylogenetic tree construction based on 16S-rDNA and *gyrB* gene sequences

Bacterial DNA was extracted using TaKaRa DNA Extraction Kit. DNA degradation and quality and quantification were tested by visualizing samples in 1.5% agarose gel electrophoresis and Nanodrop assay. Amplification of 16S rDNA was conducted using primers: 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-GGTTACCTTGTTACGACTT-3'). PCR reaction was prepared according to Pre Mix (Sangon biotech, Shanghai) manual, and cycles with 94°C 5 min, 94°C 50 s, 57°C 1 min, 72°C 90 s, 34 cycles, 72°C 10 min. The amplification of *gyrB* gene was conducted using primers: *gyrB*F 5'-GAAGTCATCATGACCGTTCTGCAYGGN GGNAARTTYGA-3' and *gyrB*R 5'-AGCAGGCTACGGATGTGCGA GCCRTCACRTCNCGKTCNGTCAT-3', PCR reaction compositions and thermal cycles were in accordance to the method described by the company's manual.

The PCR products were detected on 1.5% agarose gel and sequenced in both directions. The identity of the sequences was conducted by comparing nucleotide with the GenBank database. Phylogenetic tree was constructed using MEGA 7.0 software with Neighbor-Joining method using a bootstrap = 1 000 (Saitou and Nei, 1987).

RESULTS

Effect of walnut endophytes against host pathogen *G. leptostyla* HK1

We isolated a total of 36 endophytic bacteria, 8 strains from roots, accounted 22.2%, and code with XWR and number, 12 strains from stems, accounted 33.3%, code with XWS and number, 16 strains from leaves, accounted 44.4%, code with XLW and number.

Our results showed that all of the endophytes have an antagonistic effect on *G. leptostyla* HK1. The minimum inhibition rate was 24.2%. Among which, 4 strains showed an inhibition rate of 20.0-29.9%, 8 strains 30.0 to 39.9%, 21 strains 40.0 to 49.9%. Strains SWS1 and XWS4 showed inhibition rates above 50.0%. The highest inhibition rate was observed in strain XWS4 (60.5%) (Table 1). All the strains that showed inhibition rates higher than 50% were isolated from stems. Strain XWR8 isolated from roots showed an inhibition rate of 49.3% which also has the highest Interval Distance of 3.5 mm.

Based on colony morphology and antagonistic results, fifteen endophytes were chosen for further

investigation on other five crop pathogens.

Antagonistic effect of endophytes against cauliflower black spot pathogen *A. brassicicola*

All the tested fifteen endophytes showed a strong antagonistic effect on *A. brassicicola* mycelial growth. The inhibition rates were higher than 30%. Among of them, 12 endophytes show inhibition rate above 50%. Strains XWL14 and XWR6 showed the highest antagonistic effect. Their inhibition rates were higher than 60%, and the Interval Distance is higher than 4 mm (Table 2). Strain XWR6 showed the strongest antagonistic effect, the rate reached 68.8%. These results suggested that the pathogen was very sensitive to walnut endophytic bacteria.

Antagonistic effect of endophytes against tobacco brown spot pathogen *A. alternata*

All tested fifteen endophytes showed an antagonistic effect on the pathogen *A. alternata* in petri dish. The inhibition rates were between 22.0

to 48.0%, among of them, 10 strains show inhibition rate between 30 and 40%. Three strains XWS13, XWS4 and XWS7 showed the highest antagonistic effect. Their inhibition rates were higher than 40%, and the Interval Distance is higher than 4 mm (Table 3). Strain XWS7 showed the strongest antagonistic effect, the rate reached 48.0%.

Antagonistic effect of endophytes against cucumber anthracnose pathogen *C. lagenarium*

All the tested fifteen endophytes showed antagonistic effect on *C. lagenarium* growth, their values range from 22.4 to 54.8% (Table 4). Among them, 8 endophytes range from 30 to 40%, 5 endophytes above 40%. Strain XWS3 showed the strongest antagonistic effect, the inhibition rate was 54.8%.

Antagonistic effect of endophytes against corn leaf spot pathogen *C. lunata*

All tested fifteen endophytes showed a moderate

Table 1. Inhibition rates of 36 walnut endophytic bacteria on the anthracnose pathogen HK1.

Strains	XWL9	XWL13	XWL15	XWL18	XWL10	XWS8	XWR7	XWR3	XWL5	XWS10	XWS5	XWL8
IR (%)	24.2	27.4	29.4	29.5	32.6	36.1	37.5	37.9	38.6	38.8	39.4	39.7
ID (mm)	0.0	0.0	0.3	1.1	0.3	1.4	0.2	2.3	3.3	0.8	0.5	0.7
Strains	XWS12	XWS9	XWL3	XWS3	XWS11	XWL4	XWR4	XWL11	XWL6	XWS2	XWR2	XWL7
IR (%)	40.9	41.3	41.9	42.4	42.4	44.3	44.8	44.9	45.0	45.0	45.0	45.4
ID (mm)	0.7	0.6	2.8	2.0	0.8	2.2	1.3	1.4	3.3	1.4	2.5	2.1
Strains	XWS13	XWR1	XWL17	XWL14	XWR5	XWR6	XWL12	XWR8	XWL16	XWS1	XWS4	XWS7
IR (%)	45.9	46.0	46.0	46.3	47.7	48.8	48.9	49.3	49.6	50.3	52.1	60.5
ID (mm)	2.2	2.5	2.1	0.8	0.9	1.3	1.1	3.5	1.2	0.6	1.3	0.7

Table 2. Inhibition rates of walnut endophytic bacteria oncauli flower black spot pathogen.

Strains	XWS11	XWS12	XWS10	XWR5	XWS4
IR (%)	31.5	32.0	34.5	50.3	51.6
ID (mm)	0.7	1.0	0.5	5.8	3.3
Strains	XWS7	XWS1	XWS3	XWL17	XWR1
IR (%)	52.8	53.7	54.7	55.8	57.0
ID (mm)	1.6	5.5	7.1	5.0	9.9
Strains	XWS13	XWL12	XWL16	XWL14	XWR6
IR (%)	57.2	57.8	58.9	61.6	68.8
ID (mm)	6.4	6.1	5.7	4.9	4.0

Table 3. Inhibition rates of walnut endophytic bacteria on tobacco brown spot pathogen.

Strains	XWS10	XWS11	XWL17	XWS12	XWR5
IR (%)	22.0	28.4	30.7	32.4	32.7
ID (mm)	0.0	0.1	0.9	0.0	0.9
Strains	XWR6	XWS1	XWL16	XWL12	XWL14
IR (%)	32.9	35.7	37.0	37.1	38.0
ID (mm)	1.2	1.5	0.6	1.0	1.6
Strains	XWR1	XWS3	XWS13	XWS4	XWS7
IR (%)	38.2	38.3	41.5	44.0	48.0
ID (mm)	3.4	3.0	2.3	0.7	1.0

Table 4. Inhibition rates of 15 walnut endophytic bacteria on cucumber anthracnose pathogen.

Strains	XWS10	XWL17	XWS12	XWS1	XWL12
IR (%)	22.4	28.4	30.0	30.6	30.8
ID (mm)	0.0	0.7	0.0	0.8	0.5
Strains	XWL14	XWR6	XWS3	XWS11	XWL16
IR (%)	32.3	33.8	35.9	37.1	37.9
ID (mm)	0.0	0.3	2.9	1.0	0.8
Strains	XWR5	XWS4	XWS7	XWR1	XWS13
IR (%)	40.8	42.9	43.3	48.4	54.8
ID (mm)	0.4	0.1	0.0	0.3	0.0

antagonistic effect on *C. lunata* mycelial growth. The inhibition rates were from 25.8 to 48.7% (Table 5). Among of them, 9 endophytes were between 30 and 40%, 5 endophytes were above 40%.The endophytes effect above 40.0% were all isolated from walnut roots and stems.

Antagonistic effect of endophytes against wheat head blight pathogen *F. graminearum*

All tested fifteen endophytes against *F. graminearum* showed antagonistic effect in some degree, the inhibition rates were higher than 30%. And 6 of them showed

Table 5. Inhibition rates of walnut endophytic bacteria on corn leaf spot pathogen.

Strains	XWS12	XWS11	XWL17	XWS3	XWS10
IR (%)	25.8	30.1	30.4	33.0	33.5
ID (mm)	0	0	2.2	0.6	0
Strains	XWL12	XWS13	XWL14	XWL16	XWR6
IR (%)	34.8	34.9	35.1	36.7	39.0
ID (mm)	2.3	0.7	0.9	2.4	0.8
Strains	XWS4	XWR5	XWR1	XWS1	XWS7
IR (%)	40.6	41.1	42.0	46.7	48.7
ID (mm)	1.1	2.8	0.8	1.2	0.8

Table 6. Inhibition rates of walnut endophytic bacteria on *F. graminearum*.

Strains	XWS12	XWS10	XWS13	XWR5	XWS11
IR (%)	31.8	32.4	35.7	35.8	37.6
ID (mm)	0.0	0.0	0.2	0.9	0.0
Strains	XWS1	XWL12	XWR6	XWL17	XWR1
IR (%)	38.2	39.4	39.8	39.8	40.2
ID (mm)	0.5	2.4	1.0	1.5	0.8
Strains	XWS4	XWS3	XWL16	XWS7	XWL14
IR (%)	41.3	42.3	43.0	44.8	45.1
ID (mm)	0.4	0.0	1.1	0.5	1.9

above 40% (Table 6). Among of them, strain XWL14 isolated from walnut leaf, showed the highest inhibition rate and the biggest Interval Distance with 45.1%, and 1.9 mm respectively.

Integrated evaluation of walnut endophytes antagonist against 6 crop pathogenic fungi

The detected thirty-six walnut endophytes capacity is against its anthracnose pathogen *G. leptostyla* HK1, and fifteen of them are against 5 crop pathogenic fungi. The inhibition rates (total 111 values) were from the lowest, 22.0% to the highest 68.8%, 9 values in 20.0 to 29.9%, accounting for 8.1%; 47 values in 30 to 40%, accounting for 42.3%, 39 values in 40 to 50%, accounting for 35.2%; 13 values in 50 to 60%, accounting for 11.7%. These results indicated that walnut endophytes have abroad antimicrobial spectrum and application potential (Figure 1). Three inhibition rates were higher than 60%, strain XWS7 against HK1, stains SWR6 and SWL14 against tobacco brown spot pathogen *A. alternata*. Amongst the six target fungal pathogens, *A. brassicicola* was the most susceptible, 80% tested endophytes showed inhibition rates above than 50%. The endophytes of walnut against

the host pathogen showed significant role, two-thirds of inhibition rates were above 40%.

Fifteen endophytes antagonistic abilities were evaluated. Inhibition rates 40.0 to 49.9%, marked with Δ , 50.0 to 59.9% marked with \square , higher than 60.0% marked with \blacksquare (Table 7). Based on this evaluation, strains XWS7 and XWS4 showed the highest antagonistic effects, all inhibition rates were higher than 40%. Subsequently strain XWR1 is against 5 target fungi with inhibition rates higher than 40%.

Clustering of walnut endophytes based on biochemical and physiological characteristics

Seventeen biochemical or physiological characters of fifteen walnut endophytic bacteria were assayed. Based on their positive or negative reactions, we convert them into 1 or 0 binary table. We constructed a cladogram using NTSYSpc2.1 software (Figure 2). The highest similarity was 94%, the lowest was 57%. The endophytic bacteria could be divided into six clades based on 76% coefficient. These results implied that the endophytes have abundant diversity. The most effective strains, XWS7, XWS4 and XWR1 belong to 3 different clades,

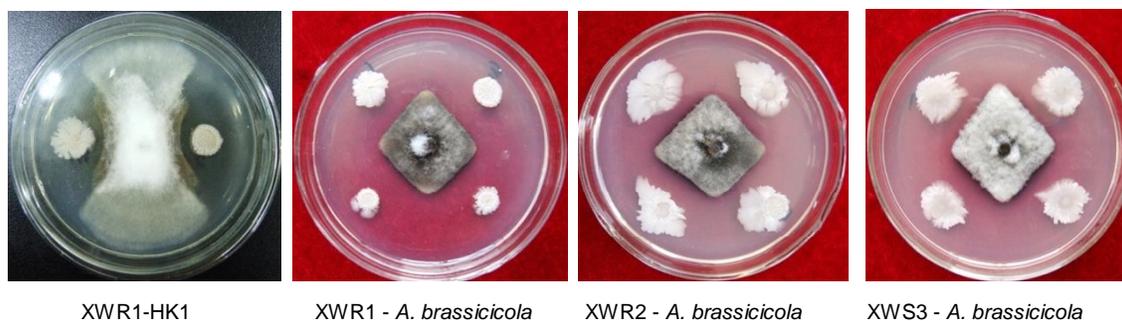


Figure 1. Antagonism of walnut endophytic bacteria against crop fungal pathogens.

Table 7. Inhibitory talent comparison of 15 walnut endophytic bacteria on 6 plant fungal pathogens.

Endophyte	HK1	<i>C. lunata</i>	<i>A. alternata</i>	<i>C. orbiculare</i>	<i>F. graminearum</i>	<i>A. brassicicola</i>
XWS7	■	△	△	△	△	□
XWS4	□	△	△	△	△	□
XWR1	△	△		△	△	□
XWS13	△		△	□		□
XWR5	△	△		△		□
XWS1	□	△				□
XWL14	△				△	■
XWS3	△				△	□
XWL16	△				△	□
XWR6	△					■
XWL12	△					□
XWL17	△					□
XWS11	△					
XWS12	△					
XWS10						

Note: △ means inhibition rates between 40.0 and 49.9%, □ means inhibition rates between 50.0% and 59.9%, ■ means inhibition rates \geq 60.0%, blank means inhibition rates less than 40%.

which indicated that they could not differentiate their antagonistic bacteria efficiency by these biochemical or physiological assayed characteristics.

Phylogenetic tree of based on 16S rDNA and *gyrB* sequences

The sequence analysis of the 16S rDNA and *gyrB* genes indicated that all the 15 endophytes attributed to *Bacillus* spp. which is a big group of endophytic plants (Figure 3). Strain XWL17 shows 99% identity to *B. pumilus*, Strain XWS3, XWS4, XWS12 and XWR5 show 99% identity to *B. subtilis* and *B. tequilensis*. Strains XWL12, XWS7, XWS11, XWL14 and XWR1 were clustered in one clade, sequences analysis showed 99% identity to *B. subtilis* and *B. tequilensis*. However, based on independent *gyrB* gene sequence analysis, all the 15 strains have highest

identity with *B. subtilis*. Besides, these strains could not be clustered into different groups according to isolated tissues or their antagonistic abilities.

DISCUSSION

In this study, the thirty-six endophytes were isolated from the root, stem and leaf of walnut. The strains were tested against walnut anthracnose pathogens, and fifteen of the strains were chosen to test their antagonistic effect against five no-host pathogens. All the assayed results showed inhibition phenomena in some degree. The inhibition rates of strains XWR6 and XWL14 against cauliflower black spot pathogen *A. brassicicola*, the highest 68.8 and 61.6% respectively. The inhibition rate of strain XWS7 against host walnut anthracnose pathogen *G. leptostyla* HK1 was 60.5%. The strains

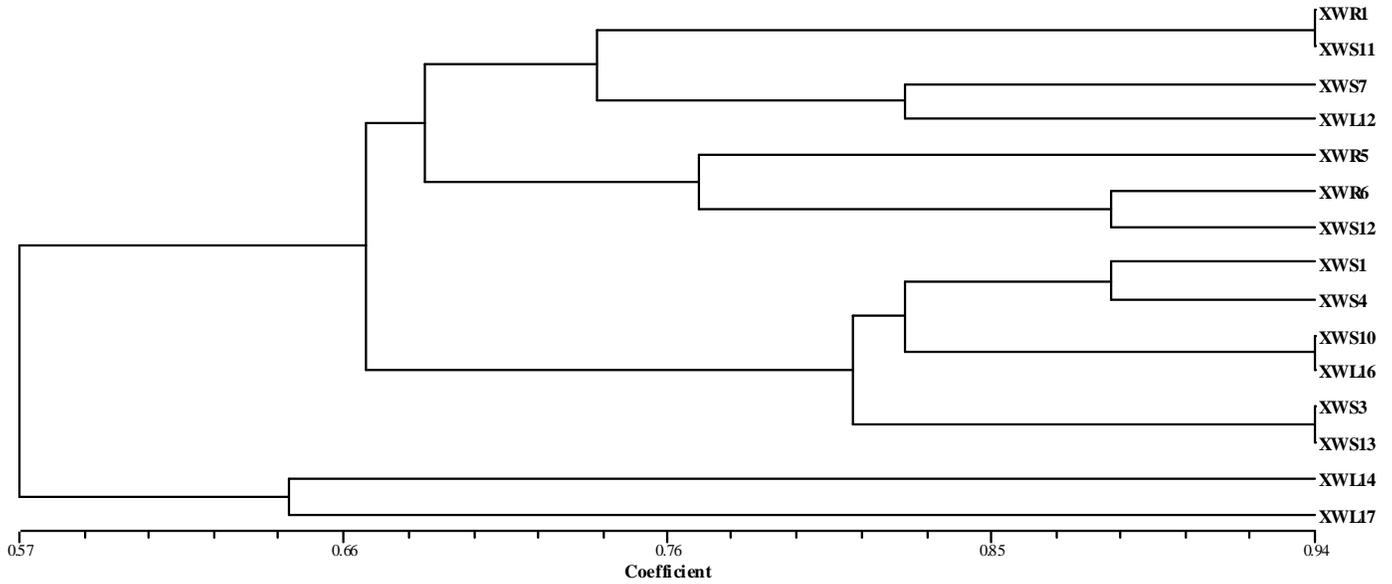


Figure 2. Cluster tree of fifteen walnut endophytic bacteria based on biochemical and physical profiles.

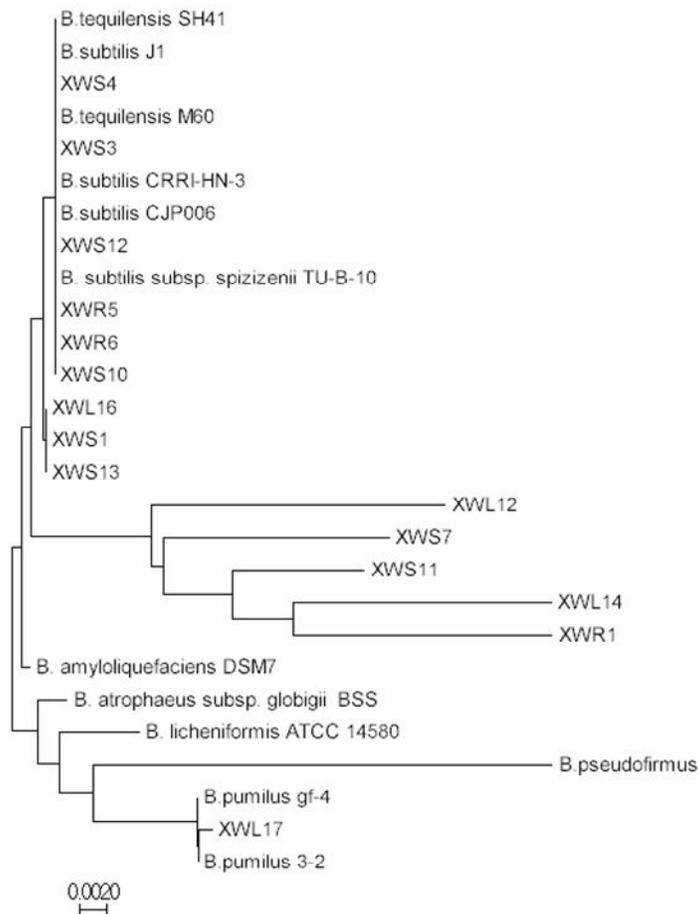


Figure 3. The evolutionary relationships of related species based on 16S rDNA and *gyrB* sequences.

XWS7 and XWS4 isolated from the stem of walnut, showed preeminent and broad antagonistic effect, the inhibition rate to all assayed pathogens were higher than 40%. Besides, the strain XWR1 which was isolated from the root showed an inhibition effect on the five pathogens with values of inhibition higher than 40%.

Amongst these target pathogens, the cauliflower black spot pathogen *A. brassicicola* showed most susceptibility with most of results that are more than 50%. All the endophytes against their host disease pathogen, their effect is higher than 20%. These results indicate that walnut endophyte might play an important role in pathogen inhibition in the plant. The endophytes from the stem with strains (XWS4 and XWS7) showed a stronger effect than the strains from leave, this might be partially explained that the disease that occurred on leaves more serious than that on the stem.

Besides, from the above test, we assayed these endophytes against Chinese cabbage soft rot pathogen *Erwinia carotovora* subsp. *carotovora* and walnut bacterial blight pathogen *Xanthomonas arboricola* pv. *juglandis*, they did not show any diameter of inhibition halo (data not shown), suggesting that there is no antagonistic ability to these bacteria.

Abundant endophytic fungi and bacteria exist *in planta* (Partida et al., 2011). Silva and colleagues isolated 217 bacteria from coffee tissue (Silva et al., 2012), Sid obtained over 500 bacterial isolates from sweet pepper (Sid et al., 2004). However, these endophytes community were with a high diversity in classification and function (Magnani et al., 2010; Vetrivelkai et al., 2010; Rodriguez Estrada et al., 2012; Lugtenberg et al., 2013). Most common endophytic bacteria reported were *P. fluorescens*, *Bacillus*, *Bradyrhizobium*, *Azorhizobium* and *Azospirillum*. The endophyte could affect their host plant physiology and ecology through themselves by communicating or interacting with their host cells (Rodriguez Estrada et al., 2012; Brader et al., 2014; King et al., 2016). The main mechanisms of endophyte as biocontrol agents includes the following (Afzal et al., 2014; Aramsirujwet et al., 2016; Lugtenberg et al., 2013; Verma and Gange, 2014; Zhao et al., 2014), obtaining mineral nutrient directly, such as fixing nitrogen, dissolving inorganic phosphate, producing siderophore to boost iron absorb; promoting plant growth and development; producing plant hormones; inducing plant assistance related genes expression, for example, endophytic bacteria *P. putida* MGY2 can be used to improve papaya resistant to anthracnose *C. gloeosporioides*, due to enhance host plant phenylalanine ammonia lyase, catalase and peroxidase levels; direct antagonistic effect against pathogens, and this effect might produce antibiotics, cellular enzymes, such as chitinase, cellulase, proteinase and β -1,3 glucosylase and its secondary metabolites.

Many research results have showed that plant endophytes have a strong antagonistic role against plant

fungal pathogens. For example, endophytic bacteria isolated from *Nicotiana glauca* suppress tomato Fusarium wilt *F. oxysporum* f. sp. *lycopersici*, and reduce yellowing and wilt symptoms to about 94 and 88%, respectively (Aydi Ben Abdallah et al., 2016). Endophytes, probably *B. subtilis* and *B. licheniformis*, decrease pepper leaf spot disease to 53%, microscopy has observed the target fungi cells cavitation, and mycelial dissolved, cytoplasm leak out (Sid et al., 2004).

In this study, the highest inhibition rate was 68.8%, this probably show that walnut endophyte has an important potential application in biocontrol. However, the actual biocontrol effect should be assayed either by placing potted plants or by using field approach for further studies, as well as the mechanism will be conducted in the future.

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REFERENCES

- Adame-Ivarez RM, Mendiola-Soto J, Heil M, 2014. Order of arrival shifts endophyte-pathogen interactions in bean from resistance induction to disease facilitation. *FEMS Microbiol Lett*, 355: 100–107.
- Afzal M, Khan QM, Sessitsch A, 2014. Endophytic bacteria: Prospects and applications for the phytoremediation of organic pollutants. *Chemosphere*, 117: 232–242.
- Alstrom S, Van Vurde JWL, 2001. Endophytic bacteria and biocontrol of plant diseases. *Plant Pathog Bact*. pp: 60-67.
- Andrieu N, Genet JL, Jaworska G, Bompeix G, 2000. *In vivo* control and in vitro antifungal activity of rhamnolipid B, a glycolipid antibiotic, against *Phytophthora capsici* and *Colletotrichum orbiculare*. *Pest Manag Sci*, 56: 1029–1035.
- Aramsirujwet Y, Gumlangmak C, Kitpreechavanich V, 2016. Studies on antagonistic effect against plant pathogenic fungi from endophytic fungi isolated from *Hottuyntia cordata* Thunb. and screening for siderophore and andole-3-acetic acid production. *KhonKaen Univ Res J*, 21(1): 55-66.
- Ardanov P, Sessitsch A, Häggman H, Kozyrovskaya N, Pirttilä AM. 2012. Methylobacterium-induced endophyte community changes correspond with protection of plants against pathogen attack. *PLoS One*, 7(10): e46802.
- Aydi Ben Abdallah R, Mokni-Tlili S, Nefzi A, Jabnoun-Khiareddine H, Daami-Remadi M, 2016. Biocontrol of Fusarium wilt and growth promotion of tomato plants using endophytic bacteria isolated from *Nicotiana glauca* organs. *Biol Control*, 97:80–88.
- Brader G, Compant S, Mitter B, Trognitz F, Sessitsch A, 2014. Metabolic potential of endophytic bacteria. *Curr Opin Biotechnol*, 27:30–37.
- D'Alessandro M, Erb M, Ton J, Brandenburg A, Karlen D, Zopfi J, Turlings TCJ, 2014. Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic

- interactions. *Plant Cell Environ*, 37: 813–826.
- De Sassi C, Müller CB, Krauss J, 2006.** Fungal plant endosymbionts alter life history and reproductive success of aphid predators. *Proc Royal Soc B: Biol Sci*, 273(1591): 1301-1306.
- Dong XZ, Cai MY, 2001.** Common bacteria systematic identification manual. Beijing: Science Press.
- King KC, Brockhurst MA, Vasieva O, Paterson S, Betts A, Ford S, Frost CL, Horsburgh MJ, Haldenby S, Hurst GDD, 2016.** Rapid evolution of microbe-mediated protection against pathogens in a worm host. *The ISME Journal*, 10: 1915–1924.
- Lugtenberg B, Malfanova N, Kamilova F, Berg G, 2013.** Endophytic bacteria with plant growth promoting and biocontrol abilities. Institute Biology of Leiden Faculty of Science Leiden University. 39. doi: 10.1002/9781118297674.
- Magnani GS, Didonet CM, Cruz LM, Picheth CF, Pedrosa FO, Souza EM, 2010.** Diversity of endophytic bacteria in *Brazilian* sugarcane. *Genet Mol Res*, 9: 250-258.
- Munif A, Hallmann J, Sikora R, 2012.** Isolation of endophytic bacteria from tomato and their biocontrol activities against fungal diseases. *Microbiol Indones*, 6: 148–156.
- Pan D, Mionetto A, Tiscornia S, Bettucci L, 2015.** Endophytic bacteria from wheat grain as biocontrol agents of *Fusarium graminearum* and deoxynivalenol production in wheat. *Mycotoxin Res*, 31: 137–143.
- Partida-Martínez LP, Heil M, 2011.** The microbe-free plant: fact or artifact? *Front Plant Sci*, 2: 100.
- Rakotoniriana EF, Rafamantanana M, Randriamampionona D, Rabemanantsoa C, Urveg-Ratsimamanga S, El Jaziri M, Munaut F, Corbisier AM, Quetin-Leclercq J, Declercq S, 2013.** Study *in vitro* of the impact of endophytic bacteria isolated from *Centella asiatica* on the disease incidence caused by the hemibiotrophic fungus *Colletotrichum higginsianum*. *Antonie van Leeuwenhoek*, 103: 121–133.
- Rodriguez Estrada AE, Jonkers W, Corby Kistler H, May G, 2012.** Interactions between *Fusarium verticillioides*, *Ustilago maydis*, and *Zea mays*: An endophyte, a pathogen, and their shared plant host. *Fungal Genet Biol*, 49:578–587.
- Saitou N, Nei M, 1987.** The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol*, 4: 406-425.
- Sid A, Ezziyyani M, Egea-Gilabert C, Candela ME, 2004.** Selecting bacterial strains for use in the biocontrol of diseases caused by *Phytophthora capsici* and *Alternaria alternata* in sweet pepper plants. *Biologia Plantarum*, 47(4): 569–574.
- Silva HSA, Tozzi JPL, Terrasan CRF, Bettiol W, 2012.** Endophytic microorganisms from coffee tissues as plant growth promoters and biocontrol agents of coffee leaf rust. *Biol Control*, 63: 62–67.
- Verma VC, Gange AC, 2014.** *Advances in Endophytic Research*. Springer-India. pp: 321–333.
- Vetrivelkalai P, Sivakumar M, Jonathan EI, 2010.** Biocontrol potential of endophytic bacteria on *Meloidogyne incognita* and its effect on plant growth in bhendi. *J Biopestic*, 3: 452–457.
- Wu HY, Zheng MY, Min XU, Wang MQ, Hao XJ, 2015.** Antifungal activity of the fermentation liquid of walnut endophytic bacteria HT-6. *J Shanxi Agric Sci*, 43(10): 1315-1328.
- Xu WT, Zhai MZ, Wang W, Gao ZH, Li XM, 2008.** Studies on endophytic fungi from *Juglans regia* L. II. Antimicrobial activity of the fermentation metabolites produced from an endophyte G8 of walnut. *Acta Agriculturae Boreali-occidentalis Sinica*, 17(1): 82-87.
- Zhai MZ, Wen XQ, Liu F, Gao ZH, Zhang YL, 2009.** Isolation of endophytic fungi from walnut and their anti-fungal activities. *J Northwest For Univ*, 24(3): 144-147.
- Zhang Q, Li Y, Xia L, 2014.** An oleaginous endophyte *Bacillus subtilis* HB1310 isolated from thin-shelled walnut and its utilization of cotton stalk hydrolysate for lipid production. *Biotechnol Biofuels*, 7: 152.
- Zhao Y, Selvaraj JN, Xing F, Zhou L, Wang Y, Song H, Tan X, Sun L, Sangare L, Folly YME, Liu Y, 2014.** Antagonistic action of *Bacillus subtilis* strain SG6 on *Fusarium graminearum*. *PLoS ONE*, 9(3): e92486.

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