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Assemblages of endophytic bacteria in chili pepper (*Capsicum annuum* L.) and their antifungal activity against phytopathogens *in vitro*

Narayan Chandra Paul[1], Seung Hyun Ji[1], Jian Xin Deng[1,2] and Seung Hun Yu[1*]

[1]Department of Agricultural Biology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Republic of Korea
[2]Department of Plant Protection, College of Agriculture, Yangtze University, Jingzhou 434025, China

***Corresponding author: shunyu@cnu.ac.kr**

Abstract

Endophytic bacteria which show antagonism against phytopathogens were isolated from healthy tissues of leaves, stems and roots of chili pepper plants (*Capsicum annum* L.) in 2010-2011. Antifungal activities of all collected isolates were tested against plant pathogens by dual culture method. Pathogenic fungi used in this study were *Alternaria panax*, *Botrytis cinerea*, *Colletrotichum acutatum*, *Fusarium oxysporum* and *Phytophthora capsici*. A total of 283 bacteria were recovered and grouped into 44 morpho-groups by observing the morphology on nutrient agar media. The isolation rate of endophytic bacteria in leaf, stem and root samples were 4.9%, 44.9% & 50.2%, respectively. 16S rDNA gene sequence analysis detected fourteen distinctive bacterial genotypes at >97% sequence similarity threshold. The most abundant genus was *Pseudomonas* followed by *Bacillus* and *Burkholderia*. A diverse range of other bacterial taxa were isolated and identified- *Actinobacter*, *Arthrobacter*, *Enterobacter*, *Escherichia*, *Kitasatospora*, *Pandoraea*, *Pantoea*, *Rhizobium*, *Ralstonia*, *Paenibacillus*, and *Serratia*. Dual culture antifungal activity indicated that 22 bacterial isolates (12%) inhibited at least one pathogenic fungus tested. *Bacillus tequilensis* (CNU082075), *Burkholderia cepacia* (CNU082111), *Pseudomonas aeruginosa* (CNU082137 and CNU082142) showed antifungal activity against all tested fungi. Crude extracts of selected isolates showed antifungal activity against *Botrytis cinerea* and among others the isolate CNU082111 performed strongest antifungal activity (inhibition zone >55 mm) by paper disk method.

Keywords: Antagonistic activity; Chili pepper; Endophytic bacteria; Molecular taxonomy; 16S rDNA.
Abbreviations: IF_isolation rate; IR_isolation frequency; LB_Luria-Bertani broth; LA_luria agar; NaOCl_sodium hypochlorite; NA_nutrient agar; NB_nutrient broth; PDA_ potato dextrose agar; PDB_potato dextrose broth; TSA_tryptic soy agar; TSB_tryptic soy broth.

Introduction

Endophytes are microorganisms that reside within internal tissues of living plants without visibly harming the host plant (Fisher and Petrini, 1987). Plants constitute vast and diverse niches for endophytic organisms and closer biological associations may have developed between these organisms and their respective hosts than for epiphytes or soil related organisms (Strobel, 2003). Nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel et al., 2004). Only a few of these plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and beneficial endophytic microorganisms among the diversity of plants in different ecosystems is considerable (Ryan et al., 2008). On the other hand, the agriculture and human consumable food sector is moving toward environmental friendly development, while increasing its productivity and simultaneously protecting the natural resources for the future generations and survivals. A renewed interest in the internal colonization of healthy plants by endophytes has arisen as their potential for exploitation in agriculture becomes apparent (Strobel et al., 2004; Sturz et al., 2012).

Bacterial endophytes colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents (Berg et al., 2005). Indeed, numerous reports have shown that endophytic microorganisms can have the capacity to control plant pathogens (Sturz and Matheson, 1996; Krishnamurthy and Gnanamanickam, 1997), insects (Azevedo et al., 2000) and nematodes (Hallmann et al., 1997). The proven advantages of using endophytes for controlling plant diseases or biocontrol agents that they are well adapted to live inside the plants and thus they provide reliable suppression of vascular disease (Lin et al. 2013) and they are not the cause for environmental contamination. Generally, as the endophytic bacteria do not cause visible damage or morphological change on their hosts, so they can benefit the host plants by producing phytohormones, by fixing nitrogen, solubilizar phosphate, by producing antibiotic compounds, or suppression of phytopathogens by competence of invasionsites etc. (Lin et al., 2013; Ryan et al., 2008). However, Endophyte bacteria offer a wide range of benefits to plants. *Capsicum annuum* L. is an economically important cultivated plant for almost all the countries in the world. They are probably the most widely consumed spice in the world (Rozin and Schiller, 1980). Cultivated crop plants like chili pepper may live in association with a variety of mycoflora. Other cultivated plants such as wheat (Coombs and Franco, 2003), rice (Tian at al., 2007), potato (Sessitsch and Berg, 2004), carrots (Surette et al., 2003), tomato & rape (Nejad and Johnson, 2000) and citrus (Araujo et al., 2002) were studied before for their endophytic bacterial association. However, endophytic bacteria in chili pepper

plants have not been studied yet. For that reason, the investigation of endophytic bacteria associated with *Capsicum annuum* L. was carried out. So, the objectives of the present study were (1) to check the occurrence and distribution of endophytic bacteria in different tissues of chili pepper plants (*Capsicum annuum* L.) in Korea and to identify them by 16S rDNA sequence data analysis, (2) to determine whether bacterial endophytes could reduce phytopathogens and to choose the potentially antagonistic bacteria against different plant pathogens.

Results

Assemblages of endophytic bacteria in chili pepper

A total of 283 endophytic bacteria were isolated from leaf, stem and root samples of 45 chili pepper plants. Total 900 tissue segments were plated where 300 tissue segments were plated in every tissue samples. The general isolation frequency was 0.31. However, the IR and IF in leaf, stem and root samples were 4.9%, 44.9% & 50.2% and 0.05, 0.42 & 0.47, respectively (Table 1). Maximum number of isolates was recovered from root samples, whereas leaf samples showed lower endophytic bacterial assemblages. According to the macromorphological characteristics, endophytic bacteria were grouped into 44 morpho-groups and representative isolates were assigned to the genus or species level based on 16S rDNA gene sequence analysis. Fourteen distinctive bacterial genotypes were detected at a >97% sequence similarity threshold (Table 2). A comparison of these sequences with the databases of valid species by using the EzTaxon server showed a very high sequence similarity to the type strains of the corresponding species. The isolate CNU082012 showed 100% sequence similarity with the sequences of bacteria *Bacillus methylotrophicus* CBMB205^T. This GenBank strain is type strain. Many isolates showed 99-100% sequence similariy with the type strain *Pseudomonas aeruginosa* LMG 1242^T and the isolates were CNU082120, CNU082123, CNU082135, CNU082137, CNU082140, CNU082141 and CNU082142 (Fig. 2). Isolates CNU082015, CNU082021, CNU082022, CNU082025 and CNU082026 were 99% identical to the type strain *Bacillus aryabhattai* B8W22^T. The isolate CNU082036 showed 96.1% sequence similarity with its reference strains. In some cases, the sequence similarity below 97% is not acceptable for the identification of bacteria. But the phylogenetic tree showed high bootstrap value (92%) which supported that the isolate would be *Pantoea anthophila* (Fig. 3). Results showed that the most abundant genus was *Pseudomonas* followed by *Bacillus* and *Burkholderia*. A diverse range of other bacterial taxa were isolated and identified, including isolates of the genera *Actinobacter*, *Arthrobacter*, *Enterobacter*, *Escherichia*, *Kitasatospora*, *Pandoraea*, *Pantoea*, *Rhizobium*, *Ralstonia*, *Paenibacillus* and *Serratia*.

Evaluation of antifungal activity

Endophytic bacteria were evaluated for antagonistic activity against five phytopathogenic fungi. Twenty two endophytic bacteria were active against at least one tested fungi. The percentage of endophytic bacteria showed strong pathogenic fungal inhibition were 3.3%, 2.7%, 2.7%, 2.7% and 2.7% against *Colletotrichum acutatum*, *Fusarium oxysporum*, *Phytophthora capsici*, *Alternaria panax* and *Botrytis cinerea*, respectively (Table 3). Species of *Bacillus* (CNU082012, CNU082075), *Paenibacillus* (CNU082099), *Burkholderia* (CNU082110, CNU082111, CNU082112, CNU082114, CNU082115) and *Pseudomonas* (CNU082120, CNU082135, CNU082137, CNU082140, CNU082141, CNU082142) showed strong and broad spectrum antifungal activity against all pathogenic fungi (Table 4). Species of *Rhizobium* (CNU082080) and *Ralstonia* (CNU082081) showed very weak antagonistic activity against one or a few tested fungi.

Table 1. Endophytic bacteria isolated from leaf, stem and root tissues of chili pepper plants in Korea.

Tissue	Segment plated	Isolates recovered	IF[a)]	IR[*] (%)
Leaf	300	14	0.05	4.9
Stem	300	127	0.42	44.9
Root	300	142	0.47	50.2
Total	900	283	0.31	--

[a)] Isolation frequency calculated by the total number of isolates obtained from tissues and total number of segment incubated. [*]Isolation rate calculated by the total number of isolates from tissues and total number of endophytes obtained from chili pepper. The isolation rate is calculated in percentage (%).

Fig 1. Endophytic bacteria isolated from leaf, stem and root samples of chili pepper plants in Korea.

Compound extraction and antifungal activity by paper disk method

Five isolates were selected (minimum one isolate from one antagonistic genus) by dual culture antifungal activity method for chemical extraction and antifungal potentiality check and they were *Bacillus methylotrophicus* CNU082012, *B. tequilensis* CNU082075, *Paenibacillus jamilae* CNU082099, *Burkholderia cepacia* CNU082111 and *Pseudomonas aeruginosa* CNU082142. Compound of CNU082012 and CNU082075 from Hexane, chloroform and ethyl acetate soluble portions did not show any activity against *B. cinerea*. The ethyl acetate soluble portion of CNU082099 and CNU082142 showed weak antagonistic activity (Fig. 4). Two *Bacillus* isolates CNU082012 and CNU082075 showed activity when tested with compound separated by butanol. Extracts from bacterial cells were dissolved in methanol and checked their antagonistic activity against *B. cinerea*. Two bacillus isolates (*B. methylotrophicus* CNU082012 and *B. amyloquifaciens* CNU082075) and the *Burkholderia cepacia* CNU082111 were strongly active as antifungal agent against *B. cinerea*. The antagonistic activity of *B. cepacia* CNU082111 was strongest against *Botrytis cinerea* by paper disk method (Fig. 4). The inhibition zone was >55 mm (data not shown).

Discussion

In this study, the assemblages of culturable endophytic bacteria obtained from chili pepper were investigated. Estimates of the global diversity of bacteria have indicated the existence of millions of species (Blackwell, 2011). However, at present,

Table 2. Sequence similarity (97-100%) between endophytic bacterial isolates and the closest type strains of valid described species based on 16S rDNA gene

Isolate no.	Closest type strains	Tissue	Similarity %	Acc. no. of closest hit
CNU082001	*Pseudomonas taiwanensis* BCRC17751^T	Stem	97.5	EU103629
CNU082008	*Arthrobacter nicotinovorans* DSM420^T	Root/Stem	99.6	X80743
CNU082012	*Bacillus methylotrophicus* CBMB205^T	Root	100	EU194897
CNU082015	*Bacillus aryabhattai* B8W22^T	Root	99.9	EF114313
CNU082017	*Pseudomonas extremorientalis* KMM3447^T	Root	99.8	AF405328
CNU082019	*Bacillus* sp. BSFC10-1	Root	97.0	FJ495144
CNU082020	*Bacillus stratosphericus* 41KF2a^T	Root	97.7	AJ831841
CNU082021	*Bacillus aryabhattai* B8W22^T	Root	99.5	EF114313
CNU082022	*Bacillus aryabhattai* B8W22^T	Root	99.5	EF114313
CNU082025	*Bacillus aryabhattai* B8W22^T	Root	99.5	EF114313
CNU082026	*Bacillus aryabhattai* B8W22^T	Root	99.8	EF114313
CNU082032	*Enterobacter mori* R182^T	Root	98.3	EU721605
CNU082036	*Pantoea anthophila* LMG2558^T	Root	96.1	EF688010
CNU082037	*Pseudomonas vancouverensis* DhA-51^T	Stem	100	AJ011507
CNU082041	*Kitasatospora cineracea* SK-3255^T	Root	99.5	AB022875
CNU082063	*Pseudomonas abietaniphila* ATCC700689^T	Stem	98.4	AJ011504
CNU082075	*Bacillus tequilensis* 10b^T	Root	98.4	HQ223107
CNU082076	*Serratia nematodiphila* DZ0503SBS1^T	Stem	99.9	EU036987
CNU082077	*Pandoraea sputorum* LMG18819^T	Stem	99.8	AF139176
CNU082078	*Pseudomonas rhodesiae* CIP104664^T	Leaf	99.6	AF064459
CNU082080	*Rhizobium miluonense* CCBAU41251^T	Root	99.7	EF061096
CNU082081	*Ralstonia pickettii* ATCC27511^T	Root	100	AY741342
CNU082087	*Enterobacter cowanii* CIP107300^T	Root	99.8	AJ508303
CNU082088	*Paenibacillus cineris* LMG18439^T	Root	100	AJ575658
CNU082098	*Acinetobacter johnsonii* DSM6963^T	Stem	99.3	X81663
CNU082099	*Paenibacillus jamilae* CECT5266^T	Root	99.9	AJ271157
CNU082100	*Rhizobium tibeticum* CCBAU85039^T	Stem	98.8	EU256404
CNU082107	*Pseudomonas fulva* NRIC0180^T	Root	99.9	AB060132
CNU082108	*Enterobacter cancerogenus* LMG2693^T	Stem	99.6	Z96078
CNU082110	*Burkholderia stabilis* LMG14294^T	Root	99.6	AF148554
CNU082111	*Burkholderia stabilis* LMG14294^T	Root	99.6	AF148554
CNU082112	*Burkholderia stabilis* LMG14294^T	Root	99.6	AF148554
CNU082113	*Burkholderia stabilis* LMG14294^T	Root	99.6	AF148554
CNU082115	*Burkholderia stabilis* LMG14294^T	Root	99.6	AF148554
CNU082120	*Pseudomonas aeruginosa* LMG1242^T	Root	99.3	Z76651
CNU082123	*Pseudomonas aeruginosa* LMG 1242^T	Root	99.8	Z76651
CNU082124	*Pseudomonas parafulva* AJ2129^T	Stem/Root	99.9	AB060132
CNU082135	*Pseudomonas aeruginosa* LMG 1242^T	Root	100	Z76651
CNU082137	*Pseudomonas aeruginosa* LMG 1242^T	Root	100	Z76651
CNU082141	*Pseudomonas aeruginosa* LMG 1242^T	Root	99.8	Z76651
CNU082142	*Pseudomonas aeruginosa* LMG 1242^T	Root	100	Z76651
CNU082143	*Escherichia hermannii* GTC347^T	Stem	98.7	AB273738
CNU082148	*Bacillus vallismortis* DSM11031^T	Root	99.3	AB021198
CNU082150	*Bacillus subtilis* subsp. *subtilis* NBRC 13719^T	Root	100	AB271744

only small subsets of potential strains have been isolated from nature and natural resources, there are a great chance to get more active stains from plants as endophytes. A significant opportunity for the discovery of new bacteria exists within plants, a niche found to host a large number of endophytic microorganisms (Bacon and White, 2000). Coupled to these, endophytes are a large and mainly untapped reservoir of genetic and chemical diversity (Strobel, 2003). In this study, 283 bacterial endophytes isolated from chili pepper plants were grouped into 44, belonged to 14 genera by 16S rDNA gene sequence analysis. Sequence based identification of bacteria is common to analyze bacterial diversity, assemblages and distribution. Sequencing of the 16S rDNA genes facilitated the putative taxonomic identification and dereplication of isolates. Recent description (Kim et al., 2012; Li et al., 2012; Miller et al., 2012 and Dourado et al., 2012) also reveled that 16S rDNA gene sequence analysis could give proper identification of bacteria. The endophytic bacterial taxa which have been identified by molecular techniques in this study were *Actinobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Escherichia*, *Kitasatospora*, *Pandoraea*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Ralstonia*, *Paenibacillus* and *Serratia* (Table 2). Previous descriptions and literature also proved the common trend. Phylogenetic trees of the 16S rDNA

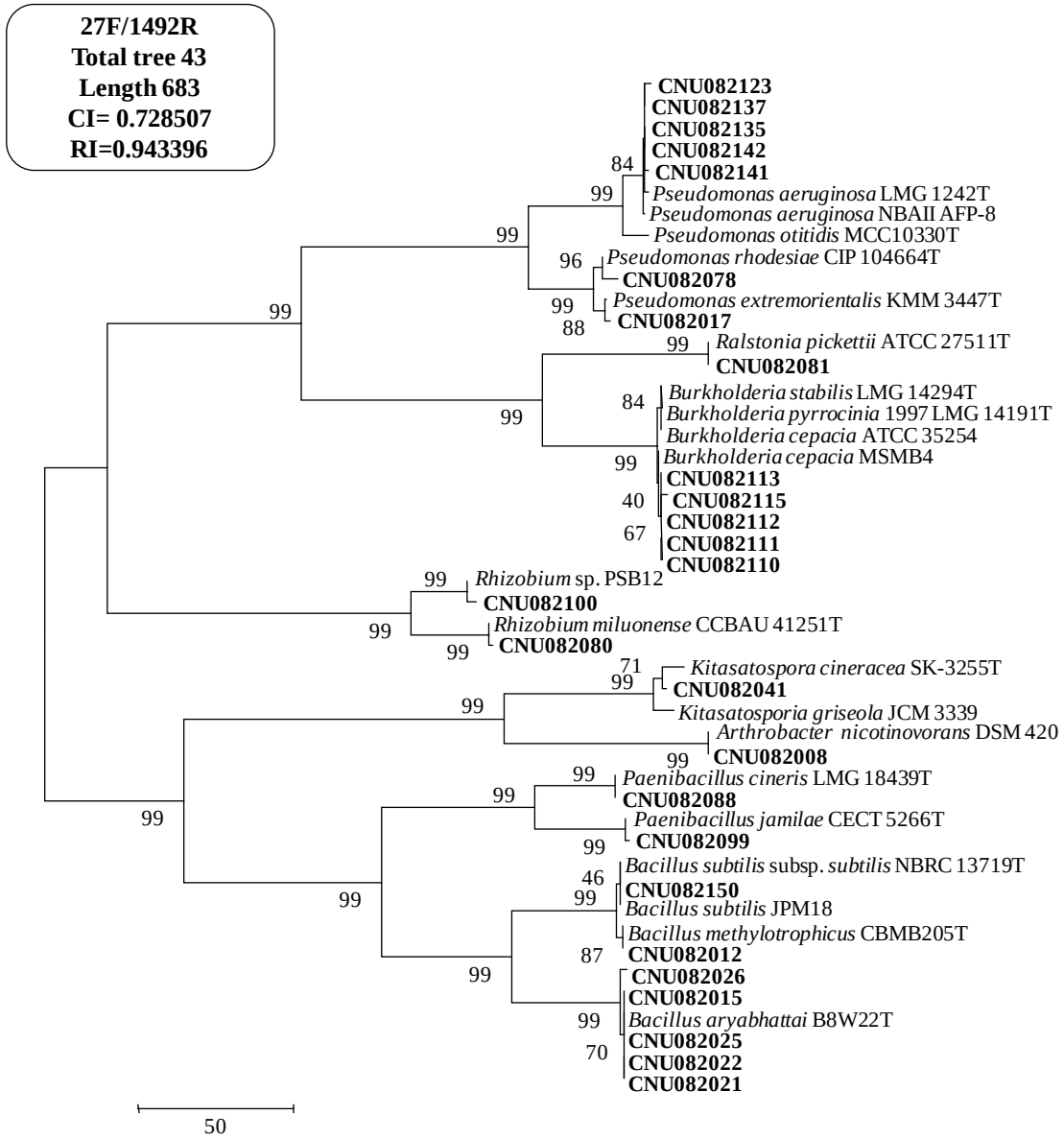

Fig 2. The Maximum Parsimony analysis of the frequently isolated endophytic bacterial sequences and similar sequences from GenBank searched by EZ taxon and BLAST searches. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA program.

gene sequences were largely in agreement with accepted taxonomic divisions and published phylogenies (Araujo et al., 2002; Rosenblueth and Martinez, 2006) (Fig. 2 and Fig. 3). A comparison of these sequences with the databases of valid species by using the EZtaxon server revealed that one strain showed relatively low similarities to the type strain of the corresponding species and therefore, probably represent new taxa. The isolate CNU082036 showed 96.1% sequence similarity with the GenBank data of *Pantoea anthophilla* LMG2558^T which is lower than the recommended. The isolate might be *Pantoea* taxa but the species could be different. Among all endophytic bacteria, most of all showed 99-100% sequence similarity, few of them showed >97 to 99% sequence similarity. More than 97% sequence similarity is accepted as the actual bacterial identification. Endophytic bacteria can act as pathogenic bacteria to plants. So, sometime they are called latent pathogen; however endophytes cause limited, if any, detrimental effects to plants (Carroll, 1988). Considering this, few putative endophytic bacteria *Bacillus*, *Burkholderia* and *Pseudomonas* were isolated from the experimented plants chili pepper. These bacteria contain strains or isolates which are pathogenic to plants or non pathogenic to plants. Although no symptoms of disease were collected or found in chili pepper plants by these bacteria. It is possible that these bacteria were either a pathogen living in latent growth stage or alternatively living mutualistically within the host plant (Miller et al., 2012). Interestingly, *Pseudomonas* spp. was isolated from plants of *Lycium chinense* as endophytic bacteria. It has been previously reported that strains of *Pseudomonas* are successful in controlling pathogenic fungi *Fusarium* sp. (Validov et al., 2007). It is possible that *Pseudomonas* spp. were limiting that plant pathogenic strains of *Fusarium* spp. in *L. chinense* acting as antagonistic bacteria. Among 283 endophytic bacteria 14 (4.9%) were isolated form leaf samples, 127 (44.9%) were from stem and 142 (50.2%) were from root tissues (Table 1). Arvind et al. (2009) showed that as many as 74 strains of

Table 3. Antagonistic activity of endophytic bacteria against different phytopathogenic fungi.

Antifungal activity	Endophytic bacteria against tested fungi				
	Ca[a)]	*Fo*	*Pc*	*Ap*	*Bc*
Strong inhibition	5 (3.3%)	4 (2.7%)	4 (2.7%)	4 (2.7%)	4 (2.7%)
Moderate inhibition	7 (4.7%)	7 (4.7%)	6 (4.0%)	3 (2.0%)	3 (2.0%)
Low inhibition	6 (4.0%)	8 (5.3%)	8 (5.3%)	11 (7.3%)	11 (7.3%)
No activity	132 (88%)	131(87.3%)	132 (88%)	132 (88%)	132 (88%)

[a)] *Ca*, *Colletothichum acutatum*; *Fo*, *Fusarium oxysporum*; *Pc*, *Phytophthora capsici*; *Ap*, *Alternaria panax*; *Bc*, *Botrytis cinerea*. ＞8 mm (+++, strong inhibition), 2-8 mm (++, moderate inhibition) and ＜2 mm (+, weak inhibition).

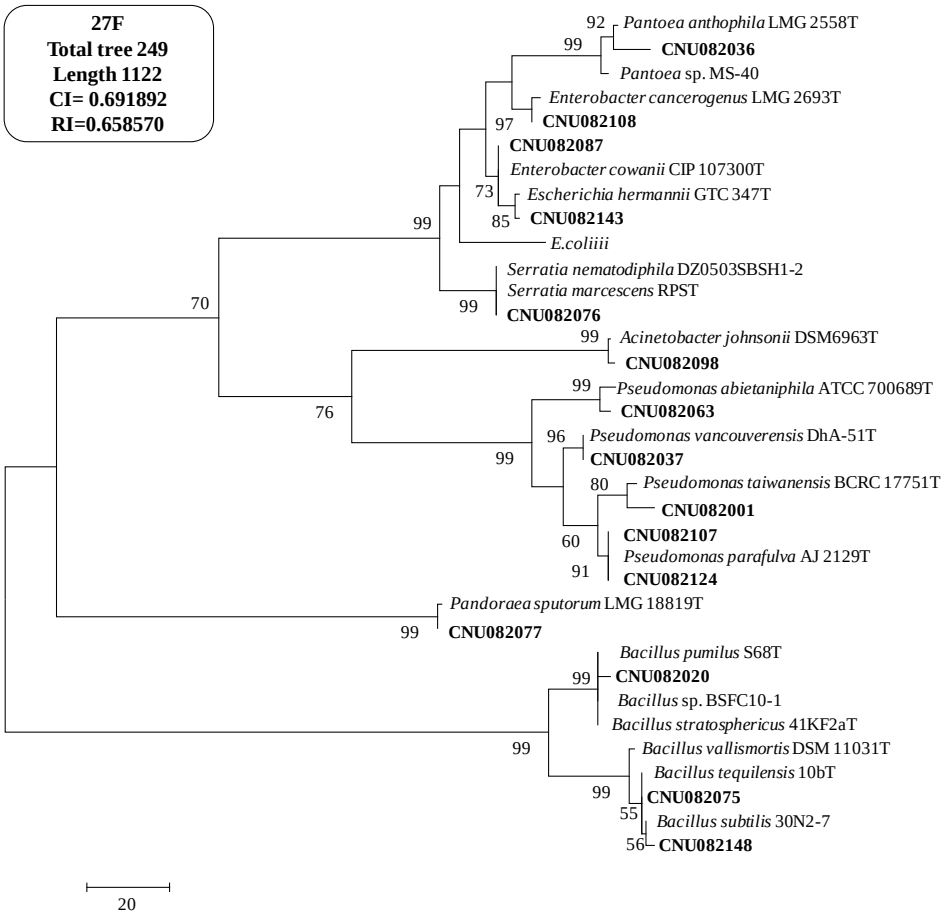

Fig 3. The Maximum Parsimony analysis of the frequently isolated endophytic bacterial sequences and similar sequences from GenBank searched by EZ taxon and BLAST searches. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA program.

bacteria belonging to six genera were isolated from healthy black pepper roots and stems at an average population of 3–4 log and 2–3 log (CFU/g), respectively. The roots harbored more diverse population of endophytic bacteria than stems. The fact that endogenous bacterial population is higher in roots may reflect the fact that the root is the primary site where bacteria gain entry in the plants (Lodewyckx et al., 2002). Close proximity of soil would have contributed to the more diverse population of endophytes in the root tissues than stem tissues.

Most of the endophytic bacteria isolated were Gram positive (80%) and Gram negative constituted only 20%. Among the Gram positives, the dominant one was *Bacillus* spp.. Among the Gram negative *Pseudomonas* spp. dominated followed by *Burkholderia* spp.. Endophytic association in cultivated plants is not common to check assemblages. Scientists follow the woody plants or forest tree species to check the assemblages of endophytic bacteria. In the present study, *Bacillus*, *Pseudomonas*, *Burkholderia* were very common. Endophytic association of *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Micrococcus* and *Curtobacterium* is reported in cultivated potato plants (Sturz et al., 1996) and stem and root tissues of cultivated black pepper plants (*Pepper nigrum* L.) (Arvind et al., 2009). The effectiveness of endophytes as biological control agents (BCAs) is dependent on many factors. These factors include: host specificity, the population dynamics and pattern of host colonization, the ability to move within host

Table 4. *In vitro* antagonistic activity of endophytic bacteria against five different phytopathogenic fungi.

Isolate no.	Host tissue	Endophytic bacteria	Antagonistic activity				
			Ca	*Fo*	*Pc*	*Ap*	*Bc*
CNU082012	Root	*Bacillus methylotrophicus*	++	+	++	++	++
CNU082075	Root	*Bacillus tequilensis*	+++	+++	+++	+++	+++
CNU082078	Leaf	*Pseudomonas rhodesiae*	-	+	-	-	-
CNU082080	Root	*Rhizobium miluonense*	-	+	-	+	-
CNU082081	Root	*Ralstonia pickettii*	+	+	+	+	+
CNU082085	Root	*Pseudomonas aeruginosa*	+	+	-	+	+
CNU082086	Root	*Pseudomonas aeruginosa*	+	-	+	+	+
CNU082088	Root	*Paenibacillus cineris*	+	++	+	+	++
CNU082099	Root	*Paenibacillus jamilae*	++	++	++	++	++
CNU082110	Root	*Burkholderia cepacia*	++	+	++	+	+
CNU082111	Root	*Burkholderia cepacia*	+++	+++	+++	+++	+++
CNU082112	Root	*Burkholderia cepacia*	++	+	+	+	+
CNU082113	Root	*Burkholderia cepacia*	++	++	++	++	+
CNU082114	Stem	*Burkholderia cepacia*	+	-	+	+	+
CNU082115	Root	*Burkholderia cepacia*	+	++	+	+	++
CNU082120	Root	*Pseudomonas aeruginosa*	+++	++	++	+	+
CNU082123	Root	*Pseudomonas aeruginosa*	+	+	+	-	+
CNU082135	Root	*Pseudomonas aeruginosa*	+++	++	++	+	+
CNU082137	Root	*Pseudomonas aeruginosa*	+++	+++	+++	+++	+++
CNU082140	Root	*Pseudomonas aeruginosa*	++	++	+	+	+
CNU082141	Root	*Pseudomonas aeruginosa*	++	++	+	+	+
CNU082142	Root	*Pseudomonas aeruginosa*	+++	+++	+++	+++	+++

Ca, *Colletothichum acutatum*; *Fo*, *Fusarium oxysporum*; *Pc*, *Phytophthora capsici*; *Ap*, *Alternaria panax*; *Bc*, *Botrytis cinerea*. ＞8 mm (+++, strong inhibition), 2-8 mm (++, moderate inhibition) and ＜2 mm (+, weak inhibition).

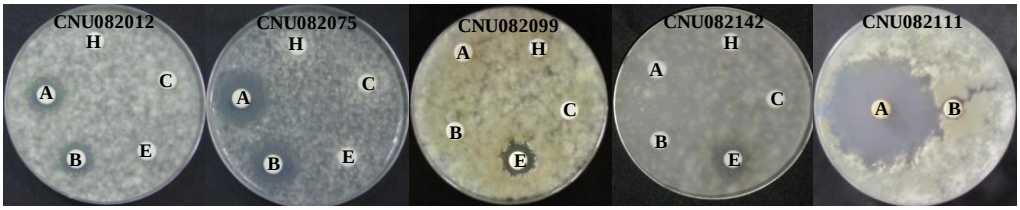

Fig 4. Antagonistic activity of solvent extracts from different bacterial isolates grown on TSB media at 28ºC for 4 days. (H-Hexane, C-Chloroform, E-Ethyl acetate, B-Butanol, A-Acetone).

tissues, and the ability to induce systemic resistance. For example, *Pseudomonas* spp., an onion endophyte, inhibited *Botrytis cinerea* and promoted vine growth in colonized grapevines, demonstrating that divergent hosts could be colonized (Barka et al., 2002). Colonization of multiple hosts has been observed with other species of endophytes and plants. For example: *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166 reduced Cucumber Mosaic Virus in tomatoes and cucumbers (Raupach et al., 1996) as well as anthracnose and Fusarium wilt in cucumber (Liu et al., 1995). Jetiyanon (1994) established that cabbage colonized by endophytes in the greenhouse had season-long reduced black rot in the field due to induction of defense mechanisms. The production of endophytic bioactive compounds was further investigated with isolates. The crude extracts of endophytes (CNU082012, CNU082075, CNU082099, CNU082111 and CNU082142) showed antimicrobial activities against multiple fungi (data not shown). These results support the gene-inferred biosynthetic potential of these isolates. Similar investigations have shown that screening for PKS and NRPS genes identified microbial sponge symbionts which exhibited antimicrobial activities (Zhang et al., 2009). The antifungal activity was produced by the extract of *Pseudomonas aeruginosa* CNU082142 against *Phytophthora capsici* test cultures. Narrow-spectrum activities are consistent with previous reports of *Pseudomonas*-derived compounds in which clinical isolates of *Pseudomonas aeruginosa* produced the low molecular weight compound 2-heptyl-4-hydroxyquinoline N-oxide (Machan et al., 1992), which is active against *Staphylococcus* sp. In the present study, crude extracts of endophytic bacteria showed strong antagonistic activity against fungal plant pathogens and proved that the possibility of strong bioactive compounds produced by endophytic bacteria.

Materials and Methods

Host species and sampling

Sampling sites of this study were Daejeon farmer's field, Chungnam province, middle of Republic of Korea. Forty five plants were selected and leaf, stem and root samples from each plant were randomly excised and brought to the laboratory in separate sterile polyethylene bags (Fig. 1).

Isolation of endophytic bacteria

Samples were cleaned under running tap water to remove debris and then air dried and processed within 5 hrs of collection. From each sample, 10 segments of 1 cm length were separated and treated as replicates. Tissue segments were surface sterilized by immersing in 95% ethanol for 1 min, NaOCl (4% available chlorine) for 4 min and 95% ethanol for 30 sec and the surface sterilized samples were washed in sterile water three times to remove the surface sterilization agents. After the treatment, plant tissues were soaked in 10% $NaHCO_3$ solution in order to inhibit the growth of endophytic fungi. Surface sterilized samples were put in the sterile plates with

filter papers and left to dry in a laminar flow cabinet. To confirm the success of the surface disinfection process, 0.2 ml water used for the final washing step and spread onto the isolation media of PDA and TSA and then incubated at 27℃. Ten segments of chili pepper tissues were placed horizontally on separate Petri dishes containing PDA and TSA media. After incubation at 27℃ for 2, 5 and 7 days, individual bacterial colonies were collected and placed onto NA media and incubated for 2-5 days and confirmed culture purity. Eventually pure cultures were transferred to 25% glycerol stock solution. Strain number were assigned for selected isolates and deposited to the 'Bacterial Culture Collection Center' of the Chungnam National University, Daejeon, Republic of Korea.

Preliminary groupings of the endophytic bacteria

Isolates were tentatively grouped according to their morphological and cultural characteristics, including the properties of colonies on plates, colony color and reverse color and diffusible pigments. These phenotypic properties allowed them to be segregated into distinct groups. Based on the preliminary groupings, representatives of each group were subjected to 16S rDNA gene sequencing analysis.

Genomic DNA extraction and PCR

The selected 44 representative endophytic isolates among 283 were subjected to extraction of genomic DNA for 16S rDNA gene sequence analysis for the identification. Single bacterial colony harvested from NB media was re-suspended in 100 µl sterile distilled water and vortexing for 10 sec. One µl lysate in 50 µl solution was used in polymerase chain reaction (PCR) to amplify 16S rDNA. Primers 27F (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1492R (5`-GGTTACCTTGTTACGACTT-3`) were used for PCR amplifications. PCR amplification was carried out in i-cycler (BIO-RAD, USA) for 30 cycles of 94℃ for 1 min denaturing, 55℃ for 40 sec annealing and 72℃ for 1 min extension. Initial denaturing at 94℃ was extended to 5 min and the final extension was for 10 min at 72℃. The PCR product was purified using Wizard PCR prep. kit (Promega, Madison, WI, USA). Purified double stranded PCR fragments were directly sequenced with BigDye terminator cycle sequencing kits (Applied Bipsystems, Forster City, CA, USA) by following the manufacturer instructions. The gel electrophoresis and data collection were performed on an ABI prism 310 Genetic Analyser (Applied Biosystems, Forster City, CA, USA).

Sequencing and phylogenetic data analysis

The 16S rDNA gene sequences were compared by EZ taxon (http://eztaxon-e.ezbiocloud.net/) and BLAST search (http://blast.ncbi.nlm.nih.gov/) with the sequences available in the GenBank database. Sequences generated from materials in this study and retrieved from GenBank were initially aligned using the CLUSTAL X (Thompson et al., 1997) program and then the alignment was refined manually using the PHYDIT program version 3.2 (Chun, 1995; available at http://plaza.snu.ac.kr/jchun/phydit). Maximum parsimonious trees were constructed using the MEGA5 program (http://www.megasoftware.net/). The bootstrap analysis using 1000 replications were performed to assess the relative stability of the branches.

Test organisms

Dual culture antagonistic activity method was the preliminary screening method of finding antagonistic agents against plant pathogens. Five phytopathogenic fungi were used to evaluate antifungal activity of endophytic bacteria isolated. They were *Alternaria panax* (*Ap*), *Botrytis cinerea* (*Bc*), *Colletrotichum acutatum* (*Co*), *Fusarium oxysporum* (*Fo*) and *Phytophthora capsici* (*Pc*). The phytopathogenic fungi were isolated from the disease affected chili pepper plants and collected from the culture stock of Plant Pathology Laboratory, Chungnam National University, Daejeon, Republic of Korea. The fungal culture had been maintained on PDA slant and 20% glycerol stock solution. Pathogenic fungal inoculums were prepared by growing them for 5-7 days on fresh PDA media.

Evaluation of antifungal activity

One or two days old bacterial colony was placed on 3 points of petri plates containing PDA medium. Test pathogens were inoculated at the center of PDA plates. Plates were incubated at 25℃ for 5-8 days. Antifungal activity was indicative as mycelial growth of test fungus prohibited in the direction of active endophytic bacteria. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony from the fungal growth radius. The width of inhibition zones between the pathogen and the endophytes was evaluated as ＞8 mm (+++, strong inhibition), 2-8 mm (++, moderate inhibition) and ＜2 mm (+, weak inhibition).

Chemical extraction from culture broth and cells

Selected antagonistic bacteria were grown on 50 ml (on 100 ml flask) of NB, PBD, LB and TSB media for 4 days at 28℃ with 150 rpm. After 24 hours, seed cultures were transferred to 100 ml (on 250 ml flask) of same media at 28℃ for 72-96 hours. The culture broths were separated from cells by centrifugation at 1000 rpm for 30 mins. The supernatant were partitioned with equal volume of hexane, chloroform, ethyl acetate and butanol, consecutively. Bacterial cell pellets were washed 3 times with sterilized distilled water. Then added acetone (bacterial cell : acetone = 2 : 8), mixed thoroughly and kept overnight. After 24 hrs, acetone layer were collected and evaporated at 40℃. After each fraction was concentrated and melted with methanol, they were used for antifungal activity through paper disk method.

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