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ISOLATION AND IDENTIFICATION OF RHIZOBACTERIA HAVING INHIBITORY CAPABILITY ON PATHOGENIC FUNGI, *Pythium* SP.

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ABSTRACT

Hydroponics systems offer an advantage in reducing exposure to soil-borne pathogen; however, root diseases caused by endogenous microorganism especially pathogenic fungi can still occur and make undesirable results. The aim of this study was to isolate and identify rhizobacteria found in hydroponic lettuces, which had inhibitory capability to plant pathogenic fungi, *Pythium* sp. The results showed that a dominant group of rhizobacteria isolated from both infected roots and normal appearance lettuce roots was in the genera *Serratia*. All isolates of rhizobacteria showed inhibitory capability against *Pythium* sp., especially the *Acinetobacter baumannii*, which showed the highest inhibitory capability among all isolates. The lowest inhibitory capability among all of the isolated strain of rhizobacteria was *Serratia entomophila* strain M6. However, this inhibitory ability of rhizobacteria was observed only in the laboratory conditions. Thus, its inhibitory property should be re-tested before the application to real hydroponics farm conditions in order to reduce the infection of pathogenic fungi, which might cause the harmful effects against the vegetable that were grown in the hydroponics system.

Key words: Rhizobacteria, inhibitory capability, root disease, hydroponics system, *Pythium* sp.

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INTRODUCTION

Hydroponics system is a kind of vegetable plantation without soil supports. In this system, vegetable can take essential nutrients in dissolved form directly from circulating water resulting in the reduction of the amount of nutrient in the water solution that required by plants. In Thailand, hydroponics culture of vegetables covers more than 300 rai of surface areas (Tongaram, 2008). The most important and popular vegetable used in hydroponics system is lettuce, *Lactuca sativa* L., (Valenzuela et al., 2002) among wide varieties of vegetable. This system has an advantage in reducing the chance of exposure to soil-borne pathogens, which are generally found in soil support culture systems. However, endogenous root diseases, which are inevitable phenomenon, could still occur and produce more undesirable results.

Pythium is a genus of endogenous parasitic *Oomycetes*, which plays an important role in causing root diseases of vegetables that grow in hydroponics systems, especially in Thailand (Koohakan et al., 2008). *Pythium* spreads very rapidly in plants which grow by using circulating nutrient film cultured system. There are many species of *Pythium* that can cause diseases in the hydroponics system, such as *P. aphanidermatum, P. debaryanum, P. dissotocum, P. intermedium, and P. myriotylum*. But, the most serious pathogens are *P. myriotyrum* and *P. aphanidermatum* (Menzies et al.,1996 ; Sutton et al., 2006). Sutton et al. (2006) reported that *P. aphanidermatum* could grow under temperatures of 24-32 ^{o}C. Some species of *Pythium* can infect plants at temperatures of 20 to 30 ^{o}C. *Pythium* can also cause yield losses in the condition of extremely low nutrient level (Koohakan et al., 2008). The root rot disease that caused by *Pythium* is continually threatened the productivity of various vegetables in hydroponics systems around the world including cucumber, tomato, sweet pepper, spinach, and lettuce (Sutton et al., 2006). It has been shown that the general density of *Pythium* in Thailand is about 2.7-3.8 Log cfu/g in the root of symptomatic *Pythium* infected vegetables, i.e., approximately around 1.9-2.7 Log cfu/g in root of healthy plants, and 0.7-1.3 Log cfu/100 ml in nutrient solution (Koohakan, 2004). Many methods have been performed to identify *Pythium* species, i.e., morphological, biochemical, phenotypic, and molecular methods (Schloter et al., 1995; Reva et al., 2001). In general, scientists identify *Pythium* species by using morphology but it is quite invalid due to the high variability. To date, molecular sequencing and phylogenetic analyses are increasingly being used to identify pathogenic species including *Pythium* (Mchale et al., 2009). In general, the farmer has used fungicides or chemical fertilizers in order to control the problems caused by *Pythium* where it is very costly and may contaminate into not only vegetable crops but also the environments (Pollution Control Department, 2008). In order to avoid these undesirable sequences, an alternative solution or an addition of beneficial microbes has been raised to deal with this problem.

There are many suggestions that rhizobacteria possess an inhibitory capability against pathogenic microorganisms (Kremer and Kennedy, 1996; Compant et al., 2005; Figueiredo et al., 2010). This phenomenon is caused by antagonistic mechanism of beneficial bacteria competing with pathogenic microorganisms. Hultberg et al. (2008) found that *Pseudomonas fluorescens* strain 5.014 and its mutant strain 5-2/4 could be used as a biological controlling tool against *Pythium ultimumon* in the preparation of tomato seedlings. Prakob et al. (2009) found that *Pseudomonas aeruginosa, Bacillus subtilis* and *Paecilomyces lilacinus* were significantly suppressed the infection and growth of root-knot nematodes *Meloidogyne* spp. Loper (1988) found that *Pseudomonas fluorescens* Migula strain 3551 isolated from cotton rhizosphere soil could protect seed colonization and pre-emergence damping-off, which caused by *Pythium ultimum*.

The aim of this study was to isolate and identify the species of rhizobacteria found in the root of lettuces grown in the hydroponics system, which had an inhibitory capability against plant pathogen, *Pythium*.

MATERIALS AND METHODS

Samples collection and preparation

The samples derived from plant tissues, i.e., lettuces, green oak, red oak, cos, and butter head, were collected from the commercially available vegetables grown in hydroponic farms located in several provinces in Eastern Thailand, i.e., Chon Buri, Chachoengsao, and Prachin Buri. The sample was performed in nutrient film technique system (NFT) and dynamics root floating technique system (DRFT). Five individual lettuces were randomly sampled from both vegetables that showed symptomatic infection and non-symptomatic infection of the root. A 50 ml of nutrient solution and water used in the preparation of nutrient solution were also collected and kept in sterilized glass bottles. The lettuces were collected from both of the conventional farms used for commercial and the organic farm (Lung Gai Farm), which were located in Nakhon Ratchasima, Northeastern Thailand. Five individual lettuce plants were randomly sampled from non-symptomatic infection of the root. Nutrient solution, water, and plant samples were collected and separately placed in plastic bags and then kept in iceboxes until used.

Isolation of rhizobacteria from lettuce roots

An isolation of rhizobacteria was performed by spread-plate technique modified from the technique described by Rangjaroen et al. (2008) and Hussen (2003). In brief, a five g of lettuce roots were washed by sterilized water subsequently mixed with 95 ml sterilized water, and mashed with a stomacher. A series of serial ten-fold dilutions were prepared from the root suspension to make 10^{-2} to 10^{-8} dilution. A one ml sample from serial dilutions was spread on nutrient agar (NA) and incubated at 28 ˚C for 24 to 48 h. Each colony with different morphological appearances was counted and subsequently re-streaked onto a new plate using the same media until pure colonies were obtained. The organism from the pure colony was transferred into a nutrient agar slant tube, incubated until full colonies were observed and then kept at 4 ˚C. In the case of nutrient solution and water, a one ml sample was added to a test tube containing nine ml sterilized water. The sample in the tube was vigorously mixed using vortex for one min. Then, a series of serial ten-fold dilutions of the sample were carried out to make 10^{-1} to 10^{-5} dilutions. The sample from the serial dilutions was used as an inoculum in a similar fashion of the previously described isolation procedure.

Identification of the bacterial isolate

Each isolate was grown in 10 ml of nutrient broth placed in multi-stack incubator shaker adjusted at 200 rpm, 28 ºC for 16 h. The bacterial cells were packed by centrifugation at 600 g for two min. Polymerase chain reaction (PCR) was performed to amplify bacterial 16S ribosomal RNA (rRNA) gene by using two primers, 27F (5'GAG-AGT-TTG-ATC-CTG-GCT-CAG'3) and 1492R (5'CTA-CGG-CTA-CCT-TGT-TAC-GA'3). The PCR amplification was carried out under the following conditions, i.e., initial denaturizing at 94 ºC for three min; 30 cycles of denaturizing at 94 ºC for 30 sec, annealing at 58 ºC for 25 sec, elongating at 72 ºC for 50 sec, and final elongating at 72 ºC for seven min. The reaction mixture contained 10 µl of 2X GoTaq, one µl of 10 µM 1492R and 27F primer, one µl of DNA, and seven µl of sterile water.

The amplified products were examined by horizontal electrophoresis in 1% agarose gel stained with ethidium bromide 10 mg/ml, which containing two µl of 100 bp DNA ladder plus and 0.5 µl aliquots of PCR produced at 100 V for 30 min. The PCR products were checked on agarose gel electrophoresis, and it was subsequently purified by the method of Vivantis, Malaysia using DNA extraction kit GF-1 (Tarntip and Sirichom, 2011), and analyzed with first base laboratories SdnBhd Company, Malaysia (Sien et al., 2013). DNA sequences of 16S rRNA gene were analyzed by using software from the blast program provided by the National Center for Biotechnology Information.

Isolation of *Pythium* from lettuce roots

Roots of infected lettuces were selected, cut, and washed with sterile distilled water. A small piece (0.5 cm) was cut and placed on petri dishes, which contained 20 ml of soft agar and incubated at 28 ºC for 24 h. The culture plate was examined and looked for a thallus of fungus. The mycelium of fungi was cut and inoculated onto potato dextrose

agar (PDA) slants and kept at 4 °C.

Evidence of capability to produce infections in plant seed of the fungal isolates

Several seeds were collected from fruits of various plants, i.e., lettuces, green oak, red oak, cos, and butter head in the amount of 30 seeds for each fruit sample. The seed samples were soaked in sterile distilled water for 1 h. Then, the seed sample was transferred and placed on soaked tissue paper in a sterile petri dish, where the isolated organism was placed nearby the seed sample and left in the petri dish for seven days. The seed samples were examined and recorded for the calculation of infection rates.

Identification of the fungal isolates

The fungal isolates were used to extract DNA using DNA extraction kit GF-1 (Vivantis, Malaysia). Each isolate was grown in 10 ml of potato dextrose broth on multi-stack shaking incubator adjusted at 200 rpm under 28 °C conditions for 48 h. The organism in each culture sample was packed by centrifugation at 600 g for 2 min. Ribosomal DNA internal transcribed spacer (ITS) regions were amplified by PCR technique using universal primers. The ITS regions of all isolates were amplified by using the universal primers ITS1 (5'-TCCGTAGGTGAACCT-GCGG-3') and ITS4 (5'-TCCTCCGCTTATTGAGCGG-3') followed the technique described by Al-Saadi et al. (2007). The PCR amplification was performed according to the following conditions, i.e., five min initial denaturation at 94 °C; 30 cycles of denaturation at 94 °C for one min, annealing at 55 °C for one min, and elongation at 72 °C for one min, and final elongation at 72 °C for five min. The sequences were identified by using BLAST with similarity less than 100% to be published sequences in GenBank.

Identifying rhizobacteria with inhibitory capability

The inhibitory capability of rhizobacteria against mycelial growth of the isolates (*Pythium* sp.) was tested using the dual culture technique. The percentage of inhibition of the mycelial growth was calculated from the distance of inhibition zone after it was incubated for 30 h at 28 °C.

Regarding the preparation, each isolate was grown on PDA for 30 h at 28 °C, while rhizobacteria was grown on NA for 24 h at 28 °C. Each isolate of rhizobacteria was streaked on PDA media with a distance about four cm to the *Pythium* isolate and the PDA plate was subsequently incubated at 28 °C in dark conditions. The distance between each inoculated strain of rhizobacteria and each isolate of *Pythium* was recorded. The efficacy of inhibition was calculated using the following formula (1):

$$\text{Percentage inhibition} = \frac{[R_1 - R_2]}{R_1} \times 100 \quad (1)$$

Where, R_1 was the radial distance of the *Pythium* isolate alone without the inhibition of rhizobacteria that served as the control, while R_2 was the distance between the test rhizobacteria and the *Pythium* isolate.

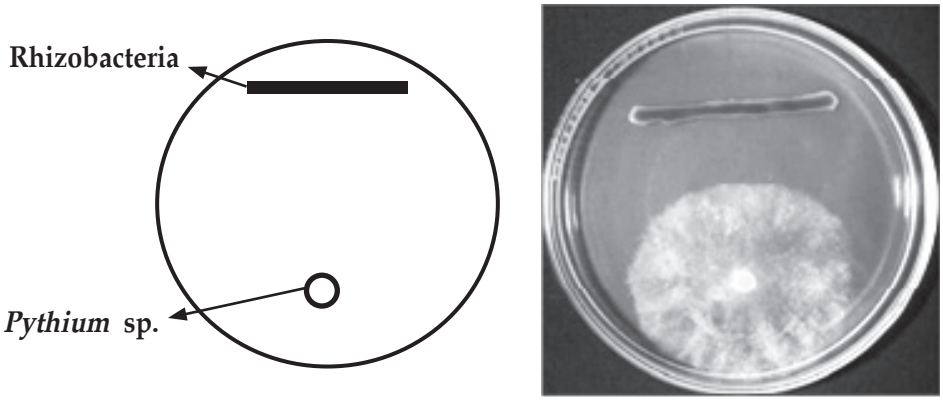

Figure 1. Showing the inhibitory capability tests of rhizobacteria against *Pythium*.

RESULTS

Isolation and identification of Pythium

We could recover 11 isolates of *Pythium* from samples collected from the normal appearance roots of lettuces and the infected ones that showed symptomatic disease appearance. Results are summarized in Table 1. It was found that the Gy-S-4 isolate caused the highest percentage (94.2%) of infection in plants, whereas only 89.9% of infected plants were caused by the isolate Ry-S-4.

Comparing its nucleotide sequences subsequently identified the Gy-S-4, and it was suggested as *P. aphanidermatum* (data not shown).

Table 1. Showing the properties of all isolates that could cause diseases in test plants.

Isolate	Number of dead plant (n=120)*	Percentage of dead plant
Gy - S-4	113.0[a]	94.2[a]
Ry - S-4	108.0[a]	89.9[a]
By - S-4	102.3[b]	85.3[b]
Cy - S-4	98.3[bc]	82.2[bc]
Gn - S-4	94.7[cd]	78.9[cd]
Cn - S-4	92.0[d]	76.7[d]
Rn - S-4	90.0[d]	75.0[d]
Bn - S-4	89.7[d]	74.7[d]
Cn - S-1	38.0[e]	31.7[e]
Rn-C2-1	25.0[f]	20.8[f]
Gy - S-3	6.0[g]	5.0[g]
control	2.7[g]	2.2[g]

Data were analyzed using one-way ANOVA with Duncan's comparison. Different letters indicated a significant difference at the level $p< 0.05$. The symbol, *, represented numbers of dead plants resulted as a mean of four replicates.

Rhizobacteria from lettuce roots and its inhibitory capacity

Fourteen isolates of rhizobacteria were recovered from both symptomatic infected roots and normal appearance lettuce roots, as shown in Table 2. It was found that all isolates of rhizobacteria could inhibit the growth of mycelium of all *Pythium* isolates when compared to the control. There were three isolates of the rhizobacteria, which showed the highest inhibitory capability, i.e., *Acinetobacter baumannii, Serratia marcescens* strain XJtx10.1, and *S. marcescens* strain R9-8A, respectively, whereas the isolate that showed the lowest inhibitory capability was *Serratia entomophila* strain M6. Results of inhibitory capabilities of all isolates are summarized, as shown in Figures 2 and 3.

Results of inhibitory capabilities of each isolate were compared based on the distance of inhibition zone and were further analyzed by one-way ANOVA with Duncan's comparison. Different letters indicated a significant difference at the level $p< 0.05$. Each value represented the value of mean of inhibition zone $\pm$ SD.

Identification of the rhizobacteria isolates recovered from lettuces

All isolates of rhizobacteria that showed inhibitory capabilities were identified by DNA sequence. The amplified DNA was run on 1% agarose gel and the results indicated that its size was 1,600 bp, as shown in Figure 5. The DNA sequence was further analyzed in comparison of the nucleotide sequence with the database stored in the GenBank (FASTA format). Results in Table

4 show the identity percentage of the rhizobacteria isolates. Most of the rhizobacteria isolates fell in the *Serratia* group. Based on 100 % identity of three isolates, i.e., number 1, 4, and 11, were identified as *Acinetobacter baumannii, Staphylococcus hominis* strain RST091219, and *S. nematodiphila* strain XJhbh78.1, respectively.

Table 2. Showing characteristics of all isolates of rhizobacteria that recovered from infected and normal appearance lettuce roots

Isolate No.	Plant species	Type of the isolate	Symptomatic infection
1	Butter head	Root colonizing bacteria	No
2	Green Oak	Root colonizing bacteria	Yes
3	Butter head	Root colonizing bacteria	Yes
4	Green Oak	Rhizoplane	No
5	Green Oak	Root colonizing bacteria	No
6	Cos	Root colonizing bacteria	Yes
7	Green Oak	Root colonizing bacteria	Yes
8	Green Oak	Rhizoplane	No
9	Red Oak	Rhizoplane	No
10	Green Oak	Root colonizing bacteria	Yes
11	Green Oak	Endophytic bacteria	Yes
12	Cos	Root colonizing bacteria	Yes
13	Red Oak	Root colonizing bacteria	No
14	Green Oak	Rhizoplane	No

Table 3. Showing the inhibitory capability of each rhizobacteria isolate.

Isolate No.	Inhibitory zone (cm)	% inhibition
1	2.87	71.67 ± 1.44^{a}
2	2.77	69.17 ± 3.82^{ab}
3	2.67	66.67 ± 1.44^{bc}
4	2.57	64.17 ± 1.44^{c}
5	2.40	60.00 ± 2.50^{d}
6	2.27	56.67 ± 1.44^{e}
7	2.17	54.17 ± 1.44^{ef}
8	2.10	52.50 ± 2.50^{fg}
9	2.07	51.67 ± 1.44^{fg}
10	2.03	50.83 ± 1.44^{fg}
11	2.00	50.00^{g}
12	2.00	50.00 ± 2.50^{g}
13	1.97	49.17 ± 1.44^{g}
14	1.97	49.17 ± 1.44^{g}
Control	4.00	0^{h}

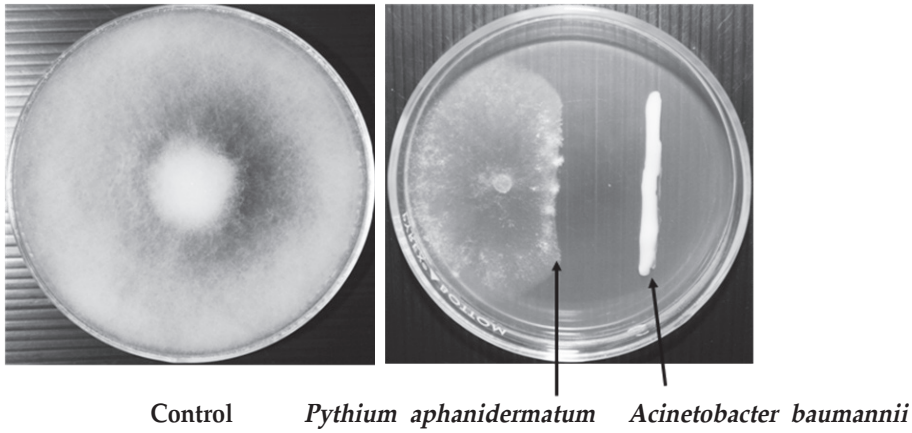

Figure 2. Showing clear zone of the highest inhibitory capability rhizobacteria.

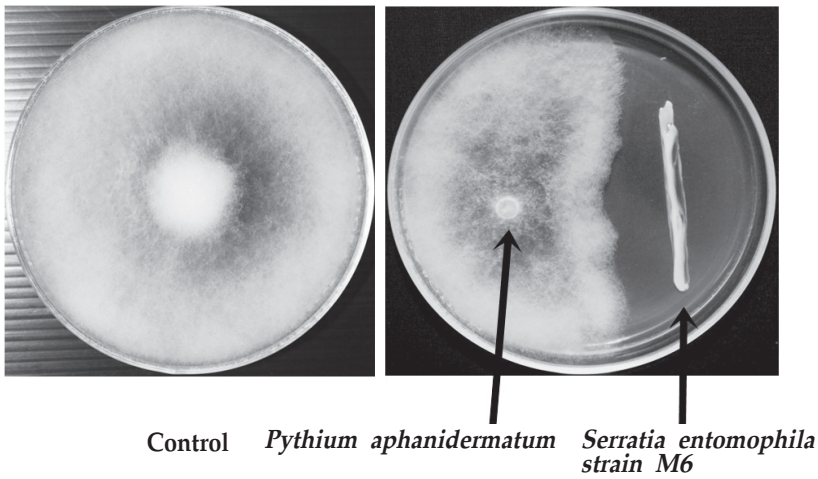

Figure 3. Showing a clear zone of the lowest inhibitory capability rhizobacteria.

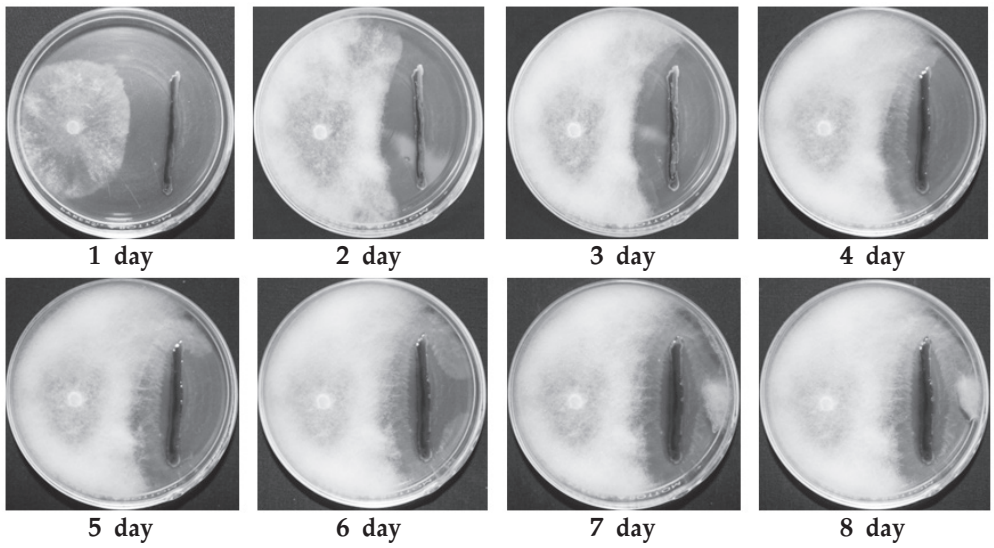

Figure 4. Showing the serial evidences of inhibitory capabilities of *Serratia marcescens* strain YQH50 against *Pythium* sp

Table 4. Showing the percentage of identity of all rhizobacteria isolates.

Isolate (No.) / species of identification	Accession number	% of identities
1 *Acinetobacter baumannii*	FR750378.1	100
2 *Serratia marcescens* strain XJtx10.1	HQ123482.1	99
3 *Serratia marcescens* strain R9-8A	HQ154570.1	99
4 *Staphylococcus hominis* strain RST091219	HM453332.1	100
5 *Serratia marcescens* strain YQH50	HQ143657.1	94
6 *Serratia marcescens* strain d4	HQ166100.1	89
7 *Enterobacter aerogenes* strain PSB28	FJ360760.1	98
8 *Serratia marcescens* strain XJ-01	FJ530951.1	97
9 *Serratia* sp. FS014	HM245061.1	97
10 *Serratia* sp. SS49 (2011)	HQ891979.1	88
11 *Serratia nematodiphila* strain XJhbh78.1	HQ123485.1	100
12 *Serratia marcescens* strain N80	GQ351502.1	99
13 *Serratia entomophila* strain M6	HM240853.1	98
14 *Serratia entomophila* strain M6	HM240853.1	98

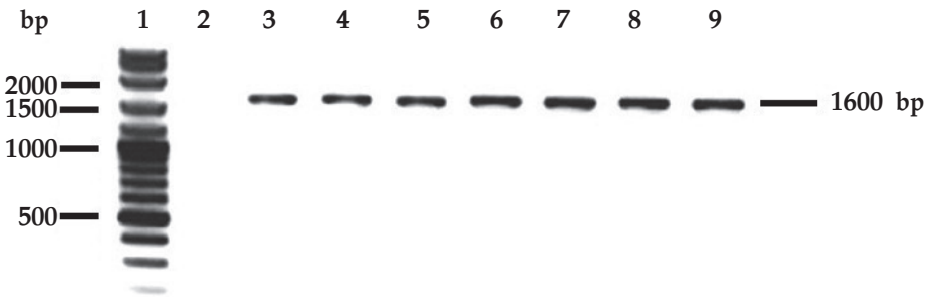

Figure 5. A picture showing PCR amplification of 16 S rRNA on an agarose gel. Lane 1 DNA ladder; Lane 2 negative control; Lane 3-9 sample PCR isolates 1-7 (the amplified band size is 1600 bp)

DISSCUSSIONS

There were 14 rhizobacteria isolates recovered from both asymptomatic and symptomatic infected wide varieties of lettuces, i.e., green oak, red oak, cos, and butter head. When they were identified, it was found that all isolates were rhizobacteria where *Serratia* was the dominant species. This result supported the study of Mohamed et al. (2009), who found that *Serratia* was one of bacterial major group in root-knot diseases.

It was shown that the rhizobacteria isolates that had highest inhibitory capabilities were fallen in the genus *Serratia*. This finding was in agreement with the study of Daayf et al. (2003), who found that bacteria in the genera of *Bacillus*, *Pseudomonas*, *Rahnella*, and *Serratia* showed inhibitory capability in both directly and indirectly antibiosis, which was the induction factor of plant defense systems. Idris et al. (2008) found that *S. marcescens* No.2 caused 80.37% reduction against the growth of *Fusarium*

oxysporum, where Queiroz and Melo (2006) also found that *S. marcescens* R-35 could inhibit root rot diseases caused by *Phytophthora parasitica.* It has been shown that S. *marcescens* has the resistant capability to other pathogenic fungi such as *Alternaria alternate, Aspergillus niger, Fusarium oxysporum, Pyricularia oryzae, Helminthosporium* sp., and *Curvularia* sp. (Jaiganesh et al., 2007; Parani and Saha, 2009).

Robertsa et al. (2007) found that live cells and ethanol extracts from cultured bacterium, *Serratia marcescens*, could significantly suppress the damping-off disease of cucumber that caused by *P. ultimum*. They hypothesized that the enzyme playing an important role in suppression was chitinase and protease, while there were other factors, i.e., antibiotic prodigiosin, the surfactant serrawettin W1, and possibly other unidentified surfactants contributed in the suppressive activity. The findings confirmed the study of Kalbe et al. (1996), who found that rhizobacteria in the genus *Serratia* showed an antifungal activity against different phytopathogenic fungi. They explained that the direct mechanism might be caused by antibiosis (prodigiosin and pyrrolnitrin) and the lytic enzymes (chilinases and β-1,3-glueanases). Cook et al. (1995) also suggested that rhizobacteria could induce suppression mechanism in infected plant responding to the disease caused by soil-borne fungus.

Utkhede and Sholberg (1986) found that *Enterobacter aerogenes* had the capability to control brown rot and alternaria rot, which were post-harvest diseases found in cherry fruit. Utkhede et al. (1997) also found later that *E. aerogenes* B8 could control root rot caused by *Phytophthora lateralis*.

The result of the suppression activity of *Acinetobacter baumannii*, which resulting in controlling root rot caused by *Pythium aphanidermatum* is in agreement with the study of Tan et al. (2006), where they show that this bacterium has the ability in controlling canker disease in citrus plants. Another evidence of the ability to control canker disease by entophytes bacteria, *A. baumannii*, was shown in the study where the isolate from xylem of lemon roots had the same ability (Lima et al., 1994; Araujo et al., 2001).

Results of this study show that the most common rhizobacteria that find in lettuce roots is in the genus of *Serratia*. The rhizobacteria isolates, which recover from both symptomatic and asymptomatic infected vegetables, show the inhibitory capability to pathogenic fungi, *Pythium* sp., especially the *A. baumannii*, where it demonstrates the highest effectiveness. However, this inhibitory capability of rhizobacteria is expressed only under the laboratory condition. Thus, there is a need to evaluate this activity before apply in the real hydroponics farm condition.

It is interesting to note that the control treatment without *Pythium* sp. shows symptomatic infection of lettuces. This observation may be caused by naturally contaminated rhizobacteria in lettuce seed or being introduced by the grower.

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